

University of Milano-Bicocca
Department of Biotechnology and Bioscience
PhD program in Converging Technologies for
Biomolecular Systems - Cycle XXXVIII

**From plant biodiversity to neuroprotection:
identification and validation of natural bioactive
compounds in models of Parkinson's disease**



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Academic Year 2024/2025

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Prologue

This thesis is the result of the research work carried out at the University of Milano-Bicocca (Italy) under the supervision of Prof. Paola Coccetti. It also includes part of the results obtained during my external research activity at the Molecular Genetics of Development and Biomedical Models Laboratory, led by Prof. Nuria Paricio, in the Department of Genetics at the University of Valencia (Spain).

This thesis addresses the investigation of innovative neuroprotective strategies, with particular focus on Parkinson's disease (PD), a rapidly increasing neurodegenerative disorder (NDD) worldwide. The aggregation of α -synuclein (α -syn) protein is a central pathological hallmark of PD, and the identification of compounds able to prevent or attenuate α -syn aggregation represents a significant challenge in biomedical research.

The main project of this thesis was conducted in close collaboration with researchers of the National Biodiversity Future Centre (NBFC) (Adopted by the Italian Ministry of University and Research, CUP, H43C22000530001, Spoke 6, Project title "National Biodiversity Future Center-NBFC", <https://www.nbfc.it/>), which aims to valorize Italy's rich plant biodiversity. Within this framework, a screening of 60 Italian plant species was performed to identify bioactive molecules with the capacity to inhibit α -syn aggregation and exert neuroprotective effects.

Concurrently, within the scope of the “ON FOODS” project (Project code PE0000003 ON FOODS—CUP:H43C22000820001—Spoke 6, Project title “ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON Foods”), focused on the valorization of agro-industrial waste products, the neuroprotective potential of discarded by-products such as cocoa bean shells and leaves of *Glycyrrhiza glabra* were also evaluated.

The efficacy of these compounds was assessed using multiple experimental models: *Saccharomyces cerevisiae* yeast cells and human SH-SY5Y neuroblastoma cells, both genetically engineered to overexpress α -syn, as well as a *Drosophila melanogaster* *DJ-16* model of PD to investigate neuroprotective effects at the whole-organism level.

The findings of this research are presented across three independent chapters, each containing a thorough introduction. The following sections will detail the individual studies, emphasizing the thematic and methodological continuity that underpins this multidisciplinary research effort.

Introduction

1. Aging and neurodegenerative diseases

Aging is a gradual and irreversible biological process characterized by the progressive decline of physiological functions, which affects cells, tissues, and entire organ systems. This multifactorial process involves, at the molecular level, genomic instability, telomere attrition, epigenetic alterations, mitochondrial dysfunction, loss of proteostasis and impaired autophagy, among other key hallmarks. At the cellular level, aging is associated with cellular senescence, stem cell exhaustion, accumulation of damaged proteins and organelles, increased oxidative stress and a progressive decline in tissue regenerative capacity. At the systemic level, these alterations lead to diminished homeostatic resilience and impaired intercellular communication, ultimately increasing the organism's susceptibility to environmental stressors, infections, chronic disorders and age-related neurodegenerative diseases [1].

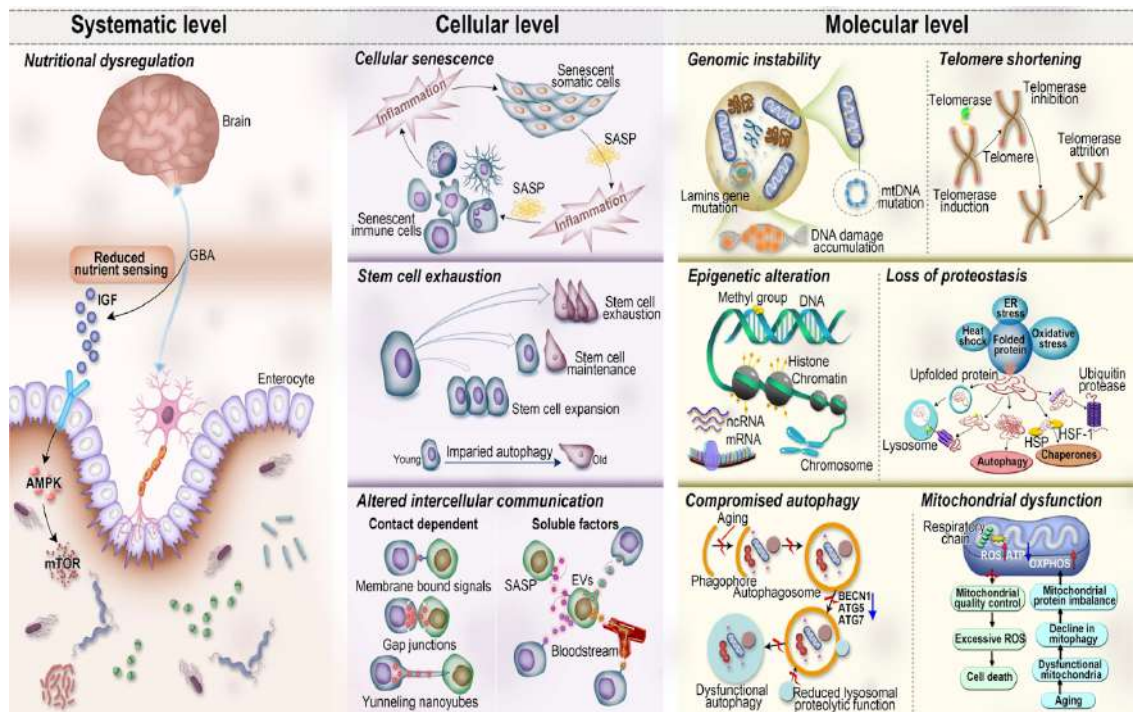


Fig. 1. Multiple molecular signaling networks involved in the aging processes of organisms [1]

Neurodegenerative diseases represent a particularly severe consequence of aging. Disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD) are tightly linked to advanced age, reflecting both cumulative cellular damage and declining protective mechanisms in the nervous system [2].

At the molecular level, neurodegeneration is characterized by the interplay of multiple pathogenic mechanisms. Protein misfolding and aggregation are central features; for example, α -syn in PD forms intracellular inclusions known as Lewy

bodies, while β -amyloid plaques and hyperphosphorylated tau tangles are hallmarks of AD. These aggregates disrupt cellular homeostasis by impairing mitochondrial function, increasing reactive oxygen species (ROS) production, triggering neuroinflammatory pathways and compromising protein degradation systems, including the ubiquitin-proteasome system and autophagic pathways. The resulting cascade of events ultimately leads to synaptic dysfunction, neuronal loss, progressive decline in cognitive and motor abilities [3].

The multifactorial nature of neurodegeneration underscores the need for multi-target strategies aimed at slowing down disease progression rather than merely alleviating symptoms. Traditional pharmacological approaches often focus on single pathways, such as dopamine replacement in PD, that treat symptoms but fail to address upstream triggers of neuronal damage. Consequently, research has increasingly emphasized preventive and neuroprotective interventions, which aim to maintain cellular homeostasis, reduce oxidative stress, modulate inflammatory responses, and enhance intrinsic repair mechanisms. Such strategies are especially relevant in the context of aging, where early intervention may preserve neuronal function and delay the onset of clinical symptoms [4].

2. Pathogenetic mechanisms of Parkinson's disease

PD is the second most common chronic and progressive neurodegenerative disorder (NDD) worldwide [5]. Its incidence increases significantly in individuals over 60 years of age, with prevalence estimates indicating that 1-2% of people in this age group are affected [6]. Overall, the number of PD cases ranges from 5 to 35 per 100,000 individuals and the prevalence is expected to double by 2030, reflecting a substantial rise in the disease's burden, particularly among older populations [7]. It is characterized by the selective degeneration of dopaminergic neurons located in the substantia nigra pars compacta (SNpc), a midbrain structure critically involved in the regulation of motor control through dopaminergic projections to the striatum [8] and widespread intracellular α -syn accumulation [9]. The resulting dopaminergic deficit leads to impaired motor circuit function and to the emergence of hallmark clinical symptoms such as resting tremor, muscle rigidity, bradykinesia and postural instability [10]. In addition to motor disturbances, the disease encompasses a wide spectrum of non-motor symptoms, including cognitive decline, sleep disorders, autonomic dysfunction, neuropsychiatric manifestations and sensory deficits, which often precede motor onset and reflect widespread neural system involvement [11].

In recent years, genome-wide association studies (GWAS) have highlighted the complex polygenic architecture of PD [12]. To date, approximately 100 genes or

genetic loci have been identified in association with this heterogeneous disease [13]. PD can be classified into sporadic and familial forms. Sporadic PD, which represents the majority of cases, approximately 80-90%, occurs without a clear family history or identifiable genetic risk factors and it is thought to result from a complex interaction between environmental and genetic factors, although its precise etiology remains unclear [14]. In addition to sporadic forms, several atypical parkinsonian syndromes are associated with exposure to environmental toxicants capable of inducing dopaminergic cell death, such as neurotoxins 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), or pesticides like rotenone and paraquat, and endotoxins such as lipopolysaccharide (LPS) [13].

Familial PD accounts for only 5-15% of cases and is caused by specific gene mutations [14].

Locus	Gene	Chromosomal Region	Inheritance	Role of the Encoded Protein
PARK1/4	<i>SNCA</i>	4q22.1	AD	-synaptic vesicles mobility
PARK2	<i>PRKN</i>	6q26	AR	-mitophagy -ubiquitin-dependent protein degradation
PARK3	—	2p13	AD	-unknown
PARK5	<i>UCH-L1</i>	4p13	AD	-participate in generation of ubiquitin monomers
PARK6	<i>PINK1</i>	1p36.12	AR	-mitophagy
PARK7	<i>DJ1</i>	1p36.23	AR	-redox-sensitive chaperone (sensor for oxidative stress)
PARK8	<i>LRRK2</i>	12p12	AD	-chaperone mediated autophagy -participation in neuronal cell death -neuronal plasticity -vesicle trafficking
PARK9	<i>ATP13A2</i>	1p36.13	AR	-inorganic cations transport
PARK10	—	1p32	—	-unknown
PARK11	<i>GIGYF2</i>	2q37.1	AD	-repressor of translation initiation
PARK12	—	Xq21-q25	X-linked	-unknown
PARK13	<i>HTRA2</i>	2p13.1	AD	-caspase-dependent apoptosis
PARK14	<i>PLA2G6</i>	22q13.1	AR	-catalyse the release of fatty acids from phospholipids
PARK15	<i>FBXO7</i>	22q12.3	AR	-ubiquitination mediator
PARK16	—	1q32	—	-unknown
PARK17	<i>VPS35</i>	16q11.2	AD	-retrograde transport of proteins from endosomes to the trans-Golgi network
PARK18	<i>EIF4G1</i>	3q27.1	AD	-mRNA binding in translation process
PARK19	<i>DNAJC6</i>	1p31.3	AR	-clarithin-mediated endocytosis
PARK20	<i>SYNJ1</i>	21q22.11	AR	-regulates levels of membrane phosphatidylinositol-4,5-bisphosphate
PARK21	<i>DNAJC13/ TMEM230</i>	3q22.1	AD	-co-chaperone of heat shock protein -stimulation of ATP hydrolysis
PARK22	<i>CHCHD2</i>	7p11.2	AD	-negative regulator of mitochondria-mediated apoptosis -reduction of oxidative stress
PARK23	<i>VPS13C</i>	15q22.2	AR	-proper mitochondrial function (maintenance of mitochondrial transmembrane potential)

AD=Autosomal Dominant; AR= Autosomal recessive

Table 1. Summary of monogenic variants associated with Parkinson's disease [15].

Although monogenic forms are relatively rare, the identification of gene mutations has been instrumental in elucidating molecular pathways implicated in familial PD but also in sporadic PD. The affected pathways involve α -syn homeostasis, mitochondrial efficiency, redox balance, calcium handling, axonal transport and chronic neuroinflammation [9].

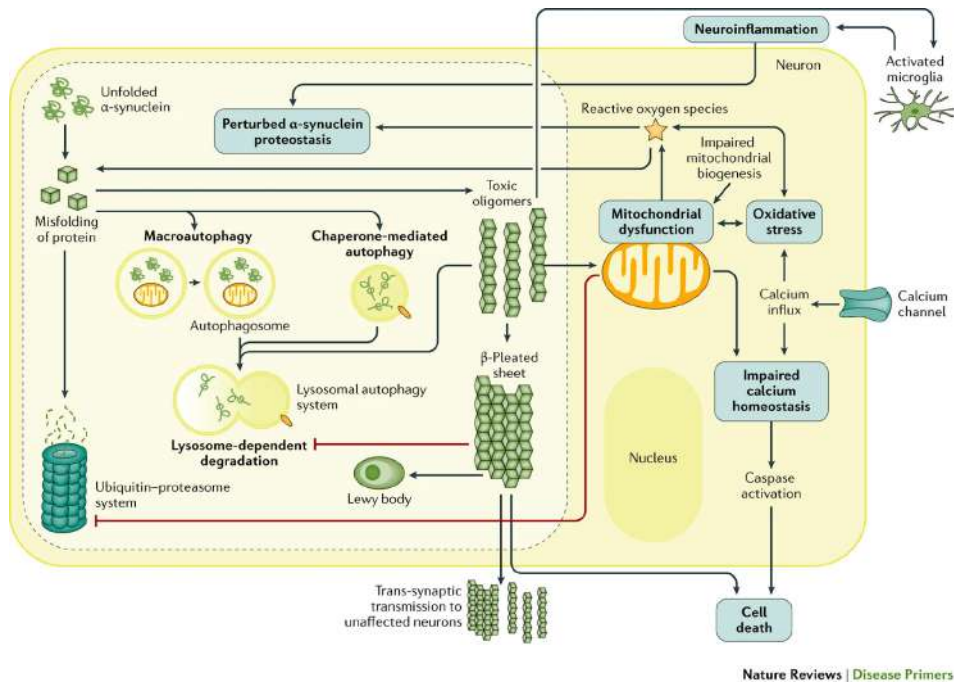


Fig. 2. Molecular mechanisms involved in PD [9].

2.1 α -Synuclein

α -Syn belongs to the synuclein family, a group of soluble proteins highly expressed mainly in the neural tissue [16,17], but also in other tissues, including the olfactory epithelium [18], skeletal muscle [19], heart [20], pancreas [21] and hematopoietic cells [22], in particular in the erythrocytes (> 99%) [23], while the remaining amount is split between plasma (0.1%), leukocytes (0.05%) and platelets (0.2%) [24]. This family is composed of three distinct isoforms, α -, β - and γ -synuclein, composed of 140, 134 and 127 amino acids respectively [25]. All three have 55-62% identical sequences and a comparable domain organization [26].

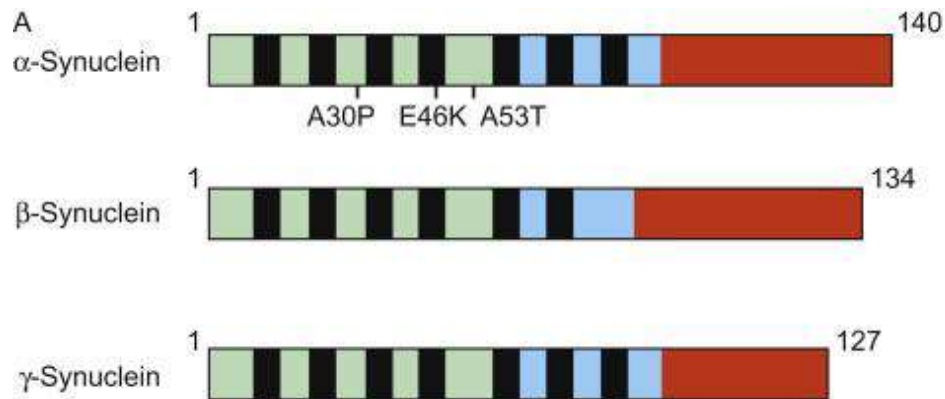


Fig. 3 Diagram of the three human synucleins, which range from 127 to 140 amino acids in length [27].

α -Syn is the most extensively studied thanks to its higher abundance in the brain and its established involvement in the pathogenesis of PD [28].

It was first identified in the electric organ of the ray *Torpedo californica* using an antibody to purified cholinergic vesicles [29].

α -Syn, encoded by the SNCA gene, is a 140-amino-acid protein that lacks a stable, well-defined structure in aqueous solution, which is why it is classified as an intrinsically disordered protein (IDP). However, it can adopt α -helical conformations upon binding to negatively charged lipids, such as phospholipids in cellular membranes and can form β -sheet-rich structures during prolonged incubation *in vitro* [30].

The protein is organized into three main regions [30,31]:

1. N-terminal region (residues 1-60): contains motifs similar to those of apolipoproteins, which favor the formation of amphipathic helices and enable membrane binding.
2. Central hydrophobic region (residues 61-95), known as the NAC (non-A β component): confers β -sheet propensity and aggregation potential.
3. C-terminal region (residues 96-140): highly negatively charged and largely unstructured, involved in protein-protein interactions and binding to small molecules.

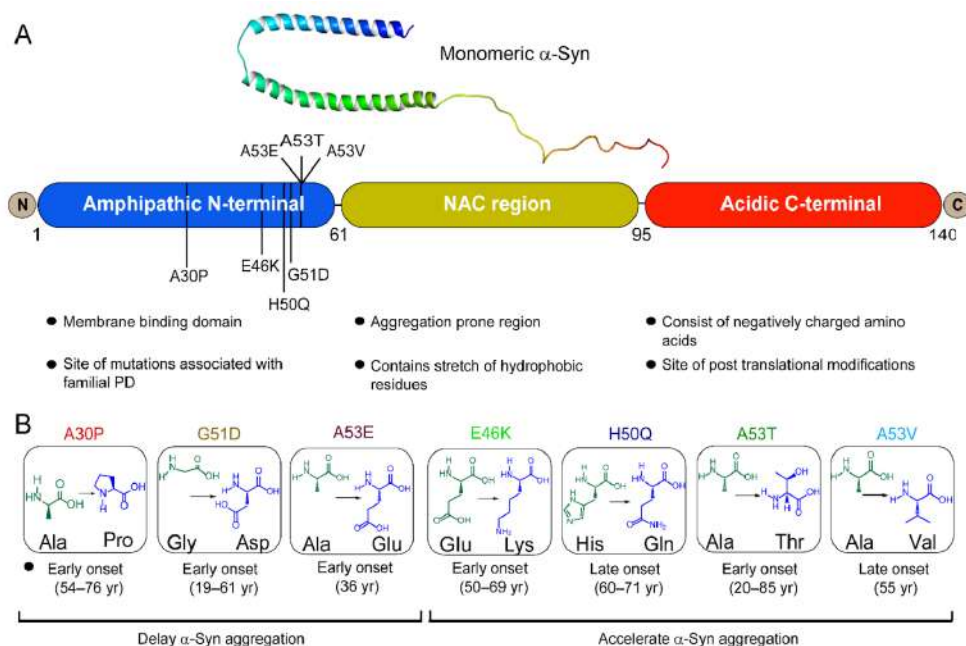


Fig. 4. Domain structure of α -syn and its disease-associated mutations. (A) Structure of micelle-bound human α -syn and schematic representation of the primary sequence of α -syn with three distinct domains, (i) the N-terminus (blue) contains lipid-binding motif and is the site of all familial mutations of α -syn, and (ii) the central NAC domain (yellow) contains the stretch of hydrophobic residues. (iii) the C-terminal (red) is rich in acidic amino acids. (B) Schematic diagram of seven mutational variants of α -syn associated with familial PD along with their age of onset. A30P, G51D, and A53E delay and E46K, H50Q, A53T and A53V accelerate α -syn amyloid formation [31].

The SNCA gene also gives rise to at least two shorter isoforms via alternative splicing, although their precise physiological and pathological roles are not fully understood [30].

α -Syn is enriched at presynaptic terminals. Although its precise roles remain unresolved, α -syn's localization suggests a central role in regulating neurotransmitter release, dopamine metabolism and synaptic vesicle dynamics [32].

Notably, α -syn is a key protein not only in PD but also in other synucleinopathies, including dementia with Lewy bodies (DLB), multiple system atrophy (MSA) and the Lewy body variant of AD [33,34]. Seven missense mutations of SNCA (A30P, E46K,

H50Q, G51D, A53V, A53T, A53E, see fig. 4) are directly linked to autosomal dominant familial PD [16,35]. Variants located within the promoter region or within distal regulatory elements of SNCA can further modulate α -syn expression, thereby increasing susceptibility to PD. Additionally, genomic rearrangements such as SNCA duplications and triplications have been shown to play a key role in determining inheritance patterns as well as influencing the age of onset and clinical severity of the disease [36]. Beyond SNCA, mutations in several other genes contribute to both dominant and recessive hereditary forms of PD. These include LRRK2 (leucine-rich repeat kinase 2), VPS35 (vacuolar protein sorting 35), PINK1 (PTEN-induced kinase 1), PARK2 (Parkin), UCHL1 (ubiquitin carboxyl-terminal hydrolase L1), GBA (glucocerebrosidase) and PARK7 (DJ-1), all of which participate in molecular pathways relevant to PD pathogenesis [37].

LRRK2 is a multi-domain serine/threonine kinase involved in the positive regulation of autophagy through calcium-dependent activation of the CaMKK/AMPK pathway, leading to increased lysosomal pH. Pathogenic LRRK2 variants are associated with autosomal-dominant PD and are mostly gain-of-function mutations that increase kinase activity. Mutant LRRK2 induces neuronal toxicity and has been implicated in α -syn propagation. Increased LRRK2 activity leads to enlarged lysosomes with impaired degradative capacity, thereby altering lysosomal trafficking pathways [38].

VPS35 encodes a core component of the retromer complex, and it is composed of two major subunits, the cargo recognition trimer (CSC, which includes VPS35) and the sorting nexin dimer (SNX). It regulates retrograde vesicular trafficking from early endosomes to the trans-Golgi network or the plasma membrane. VPS35 also influences the dynamic balance of mitochondria and participates in autophagy. VPS35 mutations are inherited in an autosomal-dominant manner and impair α -syn degradation by disrupting endosomal-lysosomal trafficking. VPS35 represents a key hub connecting PD-associated genes such as PINK1 and LRRK2, reinforcing its centrality in PD-related vesicular pathways [39].

PINK1 is a mitochondrial serine/threonine kinase that protects against mitochondrial stress by phosphorylating mitochondrial substrates and recruiting Parkin to initiate mitophagy. Recessive PINK1 mutations lead to severe mitochondrial dysfunction, dopaminergic neurodegeneration, and motor impairment [40].

PARK2 encodes the E3 ubiquitin ligase Parkin, which catalyzes Lys-63-linked polyubiquitination to target misfolded or damaged proteins for degradation. Parkin plays a key role in mitophagy, acting downstream of PINK1 to eliminate dysfunctional mitochondria. Recessive loss-of-function PARK2 mutations cause early-onset PD. Parkin deficiency disrupts protein quality control and exacerbates

mitochondrial dysfunction, including increased susceptibility to mitochondrial DNA deletions [41].

UCHL1 is a neuron-specific deubiquitinating protein and is one of the most abundant proteins in the brain (1-5% of total soluble proteins). It generates ubiquitin monomers and prevents monoubiquitin degradation in lysosomes. UCHL1 is essential for the ubiquitin-proteasome system. Loss of UCHL1 activity impairs protein degradation and enhances α -syn pathology. In mouse models, excess α -syn exacerbates disease severity in the absence of UCHL1 [42].

GBA encodes the lysosomal enzyme β -glucocerebrosidase, which hydrolyzes glucosylceramide into glucose and ceramide, and plays a central role in sphingolipid metabolism. GBA1 mutations are the most common genetic risk factor for PD. Loss-of-function variants reduce lysosomal activity, leading to altered sphingolipid homeostasis, impaired α -syn processing and clearance and subsequent α -syn accumulation and aggregation. GBA dysfunction also affects broader lysosomal trafficking pathways [43].

DJ-1 is a redox-sensitive protein that functions as an oxidative stress sensor and cellular antioxidant. Under conditions of oxidative stress, DJ-1 displays multiple protective activities, including those of a molecular chaperone, protease, glyoxalase, transcriptional regulator, and autophagy regulator, all of which contribute to mitochondrial protection. Through its chaperone function, DJ-1 also

prevents the aggregation of α -synuclein. Recessive mutations in DJ-1 lead to early-onset PD, impairing antioxidant defenses and resulting in elevated oxidative stress and mitochondrial dysfunction, as consistently observed in DJ-1 knockout models [44,45].

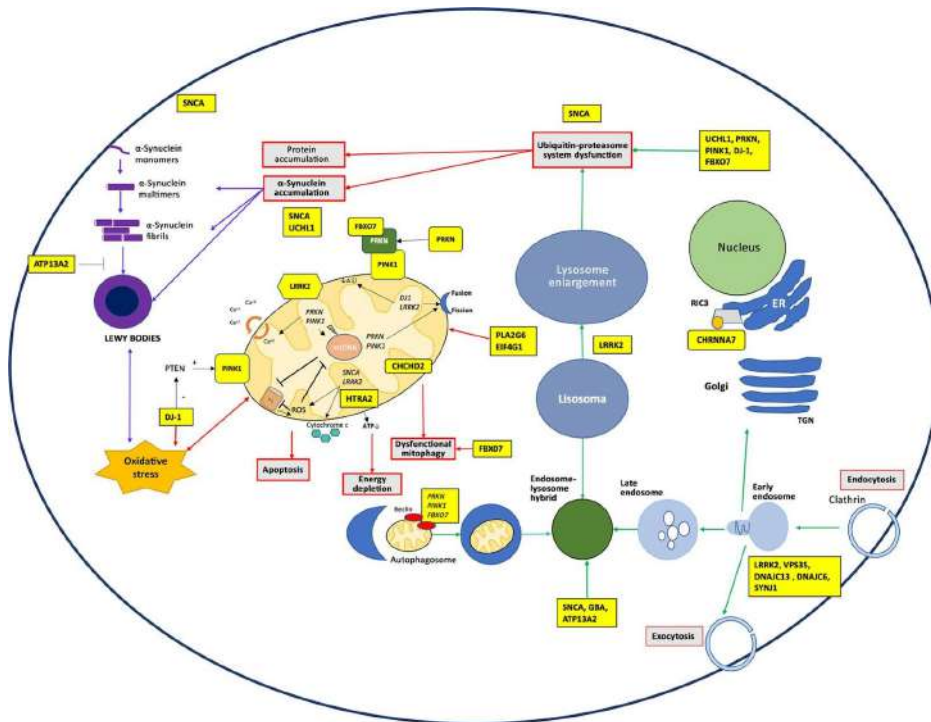


Fig. 5. Relationship between mutated genes and altered molecular pathways in PD pathogenesis [46].

All these mutations lead to elevated expression levels or enhanced aggregation propensity of α -syn [47]. Indeed, aggregated α -syn is the major constituent of Lewy bodies and Lewy neurites, the characteristic protein inclusions composed primarily of insoluble, fibrillar α -syn [48].

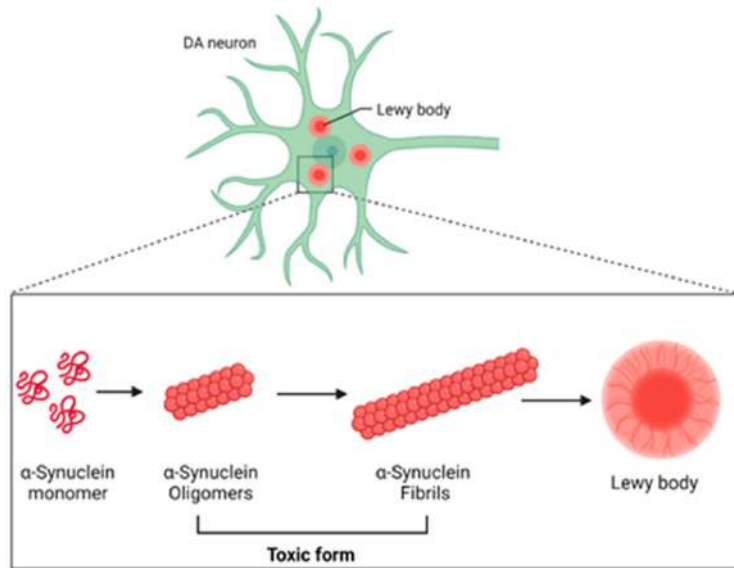


Fig. 6. The aggregation process of α -syn [49].

α -Syn acquires neurotoxic properties, central to the pathogenesis of PD and other synucleinopathies, when it undergoes a pathological transition from its soluble monomeric state to oligomers, protofibrils and ultimately insoluble fibrils. Unfolded monomers interact within the cytosol to generate dimers that are initially unstable. As this process progresses, these dimers give rise to oligomers with diverse structural features, which subsequently develop into protofibrils and ultimately into insoluble fibrils. Oligomers represent the earliest aggregation species and, compared with the more advanced aggregates, they are smaller, soluble, highly mobile and exhibit pronounced cytotoxic effects on neighboring cells [50].

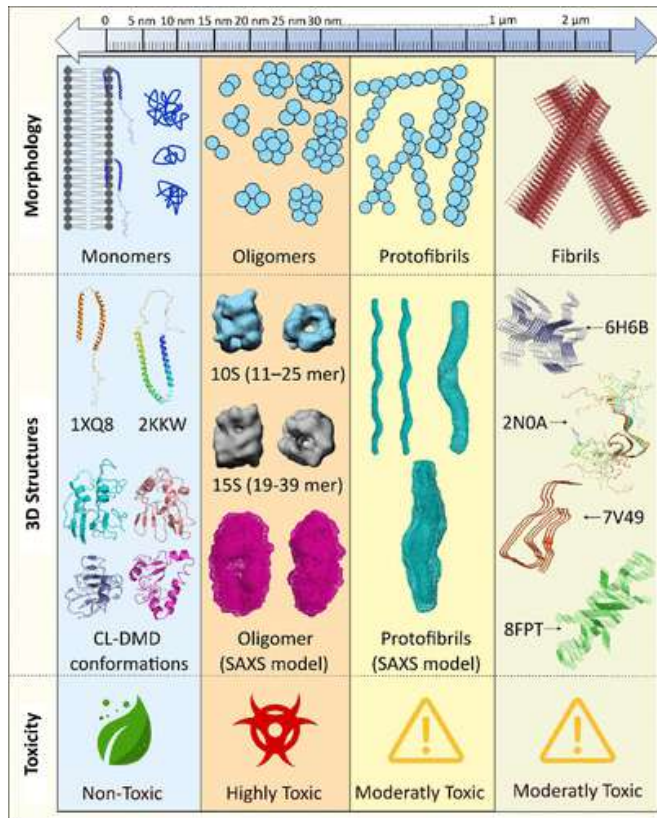


Fig. 7. α -Syn aggregation pathway, structural morphology, and its toxicity [50].

The aggregation can be initiated by several factors, including not only protein overexpression and mutations that increase its misfolding and oligomerization propensity, but also stress conditions and a wide range of post-translational modifications (PTMs). Notably, most α -syn in LBs is phosphorylated at serine 129 [51]. In addition to phosphorylation mechanisms, also ubiquitination, SUMOylation, acetylation, glycation, O-GlcNAcylation, nitration, oxidation, polyamination, arginylation, and truncation further modulate the fibrillization process of α -syn. Indeed, they increase its misfolding, denaturation and

oligomerization and/or cause impairments in the cellular pathways responsible for α -syn clearance [52].

The aggregation process of α -syn follows a characteristic sigmoidal kinetic profile composed of a lag phase, an exponential growth phase and reaches a final plateau, each reflecting a distinct conformational change during fibril assembly (Fig. 8).

During the lag phase, monomers exist predominantly in soluble states and form small, highly unstable, and disordered aggregates that retain elements of the native conformation. These early species progressively reorganize into β -sheet-rich structures, gaining stability and facilitating rapid fibril assembly. α -Syn aggregation proceeds via a nucleation-dependent conformational conversion mechanism, in which misfolded aggregates require a rate-limiting structural transition to become nucleation-competent (primary nucleation). In the exponential (growth) phase, additional monomers are rapidly recruited, driving the elongation of fibrils, through secondary nucleation. Fibril fragmentation generates new ends, providing additional reactive surfaces that enhance fibril propagation and markedly accelerate aggregate formation. In the stationary (plateau) phase, most soluble α -syn has been converted into insoluble amyloid-like fibrils, and the system reaches a dynamic equilibrium between fibrils and monomers. The total fibril mass no longer increases significantly, marking the end of rapid fibril growth. Together,

these three phases depict the transition from soluble monomers to stable, β -sheet-rich fibrillar aggregates, highlighting the nucleation-dependent mechanism underlying α -synuclein aggregation, as illustrated in Figure 8 [53–55].

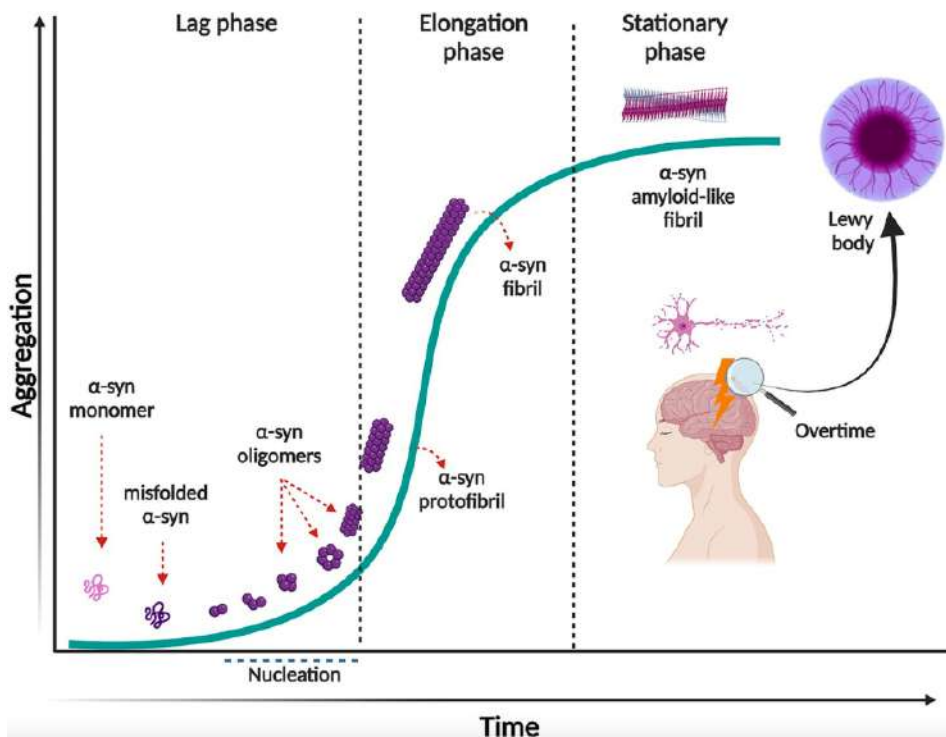


Fig. 8. Nucleation-dependent polymerization model of α -syn aggregation [55].

Among the various α -syn species, β -sheet-rich oligomers are considered the most neurotoxic. These oligomers induce elevated ROS, which in turn promote further oligomerization, creating a self-amplifying toxic cycle. Their partially organized hydrophobic core exposes hydrophobic residues, increasing structural flexibility and enabling aberrant interactions with membranes and other proteins. This leads

to membrane disruption, altered calcium permeability, mitochondrial dysfunction, impairment of ER-Golgi trafficking through soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) inhibition, activation of glial cells and widespread disturbance of cellular proteostasis [37,53].

Altogether, the interplay between α -syn misfolding, oligomer toxicity and compromised cellular homeostasis is thought to drive dopaminergic neuron degeneration and the progression of PD [53].

Intracellular α -syn homeostasis is regulated primarily by the ubiquitin-proteasome system and the autophagy pathway.

2.2 α -Syn homeostasis

Degradation of α -syn via the ubiquitin-proteasome system (UPS) and autophagy pathways is crucial for preventing its pathogenic accumulation [56].

The UPS is responsible for the selective degradation of soluble, misfolded, or damaged proteins. Proteins destined for degradation are tagged with poly-ubiquitin, a small regulatory protein, which signals their recognition and processing by the 26S proteasome, a large proteolytic complex. The proteasome then breaks down these proteins into short peptides, preventing the accumulation of potentially toxic species and maintaining cellular protein quality. The 26S proteasome consists of a catalytic 20S core, where actual proteolysis occurs, and a

19S regulatory particle, which recognizes ubiquitinated substrates and unfolds them for degradation [57].

Substrate specificity is mediated by E3 ubiquitin ligases, such as Parkin and SIAH, and proteasome-associated proteins. Notably, small soluble oligomers of α -syn, including phosphorylated pS129 species, are efficiently degraded by the UPS, whereas larger oligomeric or fibrillar forms are resistant and can even impair proteasomal activity. Monoubiquitination by SIAH can stabilize α -syn monomers but may also promote aggregation and inclusion formation, highlighting the complex regulation of UPS-mediated degradation [58].

Autophagy represents a bulk degradation pathway that targets large protein aggregates, dysfunctional organelles, and other cytoplasmic components. It is modulated through inhibition of mTORC1 and activation of AMPK. Autophagy plays a critical role in the clearance of α -syn aggregates, yet its efficiency declines with aging and under pathological conditions, contributing to the accumulation of neurotoxic species. The process of autophagy includes three main types: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Among these autophagic pathways, both CMA and macroautophagy contribute to α -syn degradation. CMA selectively targets cytosolic proteins containing a KFERQ-like motif via recognition by Hsc70 and translocation through the lysosomal receptor LAMP2A, which acts as a rate-limiting factor. In macroautophagy, the

initiation of autophagy starts with the nucleation of phagophore, which membranes originate from the endoplasmic reticulum (ER). During elongation, the phagophore engulfs large α -syn aggregates. The phagophore closes forming the double-membrane vesicle autophagosome. Then, it fuses with the lysosome, where enzymatic degradation occurs and α -syn aggregates are degraded by the lysosomal hydrolases. The resulting macromolecules are then recycled to the cytoplasm to support cellular metabolism. CMA is primarily responsible for monomeric α -syn, while macroautophagy and other endosomal-lysosomal pathways are essential for the removal of oligomeric and fibrillar forms. Pharmacological or genetic inhibition of these pathways leads to accumulation of high molecular weight α -syn oligomers, whereas upregulation of macroautophagy enhances clearance of all α -syn species [9,59–61].

In PD, this degradative balance becomes disrupted: lysosomal enzyme activity is reduced in nigral neurons, CMA markers such as LAMP2A are diminished, autophagosomes accumulate, and the crosstalk between macroautophagy and CMA is impaired. α -Syn oligomers can directly inhibit the UPS, aggregated α -syn can suppress macroautophagy, and wild-type, mutant, or post-translationally modified forms of α -syn interfere with CMA efficiency [62]. Moreover, α -syn fibrils can resist proteolytic clearance even by macroautophagy, requiring alternative endosomal-lysosomal or exosome-mediated pathways for removal [63]. Collectively, these

findings highlight that UPS and ALP are functionally connected, such that the impairment of one influence the other [61].

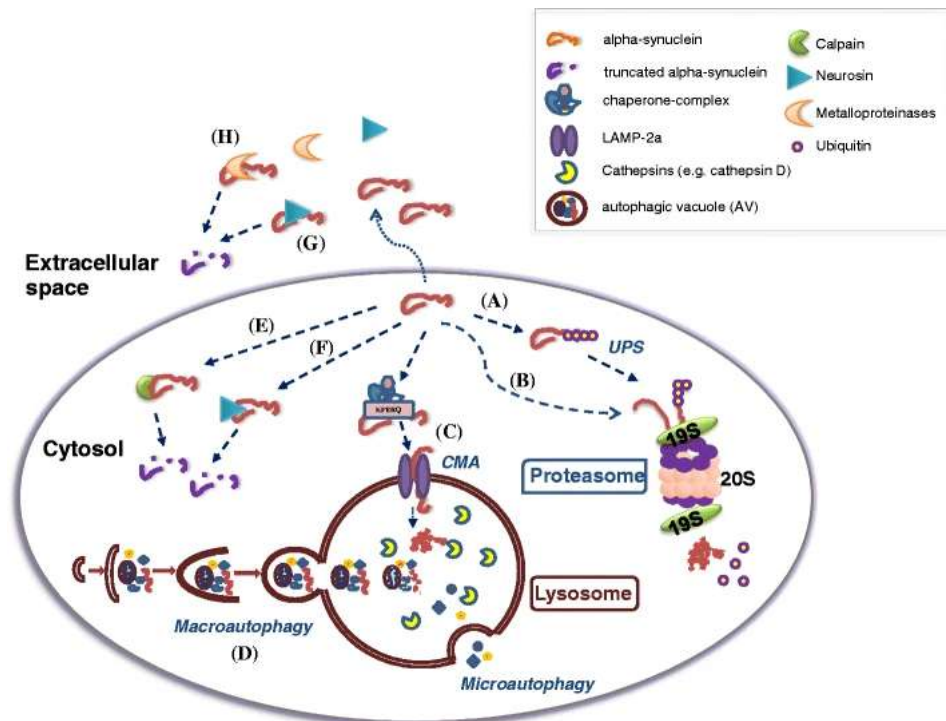


Fig. 9. Intracellular α -syn homeostasis processes: ubiquitin-proteasome system and autophagy [60].

2.3 Mitochondrial dysfunction

Mitochondrial dysfunction has been widely implicated as a central element in PD pathogenesis. Evidence suggests a vicious cycle in which α -syn aggregation and mitochondrial impairment exacerbate each other, explaining why these cellular alterations often coexist in degenerating neurons [64]. Activity of mitochondrial complex I, a key component of the electron transport chain, is reduced in brain tissues from PD patients. Moreover, genes regulated by the transcriptional coactivator PGC-1 α , a master regulator of mitochondrial function, are generally downregulated in PD. Normally, low levels of α -syn are present in mitochondria, but excessive accumulation of the protein can impair complex I activity and increase oxidative stress. PGC-1 α activation reduces α -syn oligomerization and toxicity *in vitro*, while PGC-1 α deficiency increases vulnerability to α -syn oligomers. Conversely, exposure to α -syn oligomers decreases cellular PGC-1 α levels. Animal models in which mitochondrial function is compromised replicate key features of PD neuropathology, including nigrostriatal degeneration, axonal pathology, neuroinflammation and altered autophagy. These findings suggest that axonal degeneration, potentially driven by energy deficits, may represent an early pathogenic event in PD. Finally, mutations in Parkinson's disease-associated genes, such as LRRK2, PARK2, and PINK1, impair mitophagy and the clearance of damaged mitochondria [9,65].

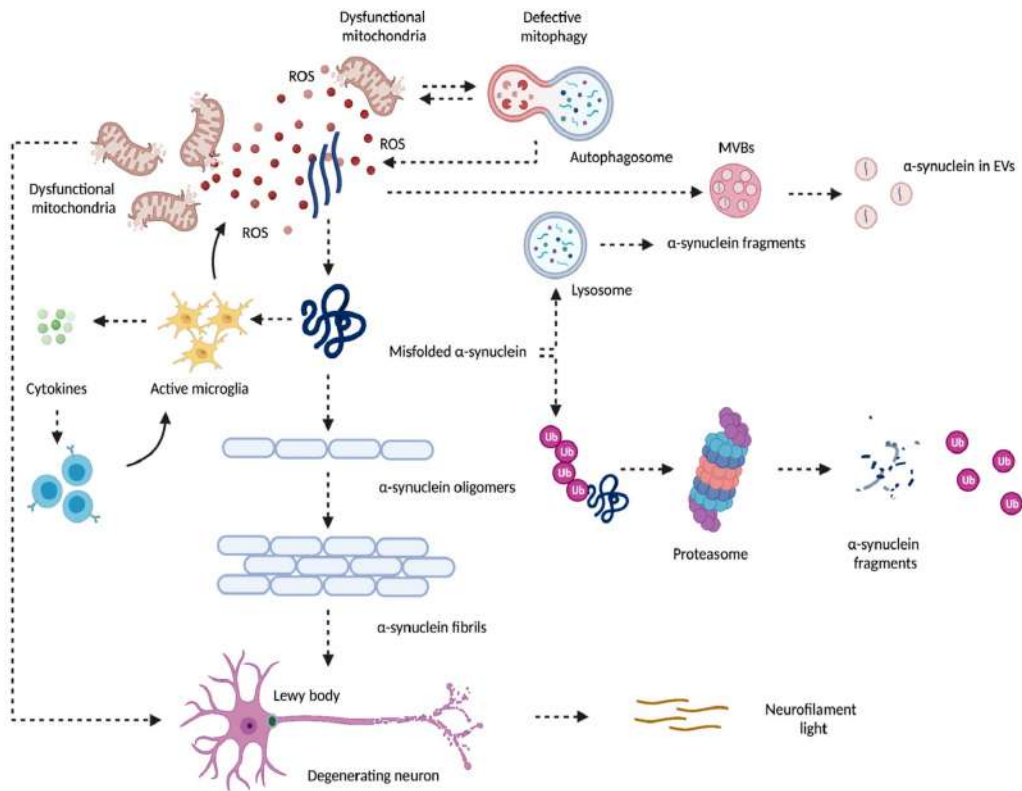


Fig. 10. Mitochondrial dysfunction in PD [65].

2.4 Oxidative stress

Oxidative stress is closely linked to mitochondrial dysfunction and is elevated in the brains of PD patients. Indeed, it is one of the key mechanisms underlying the pathogenesis of PD, directly contributing to the vulnerability and progressive loss of dopaminergic neurons in the nervous system (SN). Their vulnerability is due to their long unmyelinated axons, high synaptic density, autonomous pacemaking activity, and high cytosolic dopamine levels [9,66]. The loss of dopaminergic neurons results from excessive production of ROS generated by multiple cellular

processes. Dopamine metabolism represents a major source of ROS: dopamine auto-oxidation and degradation mediated by monoamine oxidase B (MAO-B) produce free radicals and hydrogen peroxide, which, in the presence of iron, catalyze the formation of highly toxic hydroxyl radicals. The accumulation of iron and calcium in the SN further exacerbates ROS production, as iron promotes Fenton reactions and the opening of L-type calcium channels in dopaminergic neurons increases energy consumption and oxidative stress-effects that are amplified by other cellular alterations characteristic of PD. Mitochondrial dysfunction, particularly impairment of Complex I of the electron transport chain, constitutes another critical source of ROS. Damaged mitochondria generate free radicals that, in turn, exacerbate mitochondrial injury, establishing a vicious oxidative cycle. Neuroinflammation, mediated by activated microglia, also contributes to ROS production and the release of pro-inflammatory cytokines. The release of oxidized lipids, proteins, and DNA from dying neurons further stimulates microglial activation, amplifying neuronal damage. In addition to these metabolic and environmental factors, genetic mutations associated with PD play a significant role in redox stress. Alterations in genes such as PRKN and PINK1, which regulate mitophagy and mitochondrial quality control, increase susceptibility to oxidative stress due to the accumulation of depolarized and dysfunctional mitochondria. Mutations in DJ-1 reduce cellular antioxidant capacity by impairing the activation

of protective genes via NRF2, whereas mutations in LRRK2, FBXO7, and ATP13A2 exacerbate mitochondrial fragmentation and further enhance ROS production. The central role of oxidative stress has also led to the identification of potential biomarkers, including oxidized DJ-1, uric acid, oxidized Coenzyme Q10 (CoQ10), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and lipid peroxidation products (MDA, HNE, F2-isoprostanes), which may be useful for monitoring disease progression and evaluating the efficacy of antioxidant therapies. In fact, antioxidants such as creatine, vitamin E, CoQ10, MitoQ, and pioglitazone represent promising therapeutic strategies to modulate PD progression, although clinical outcomes to date have been limited [67].

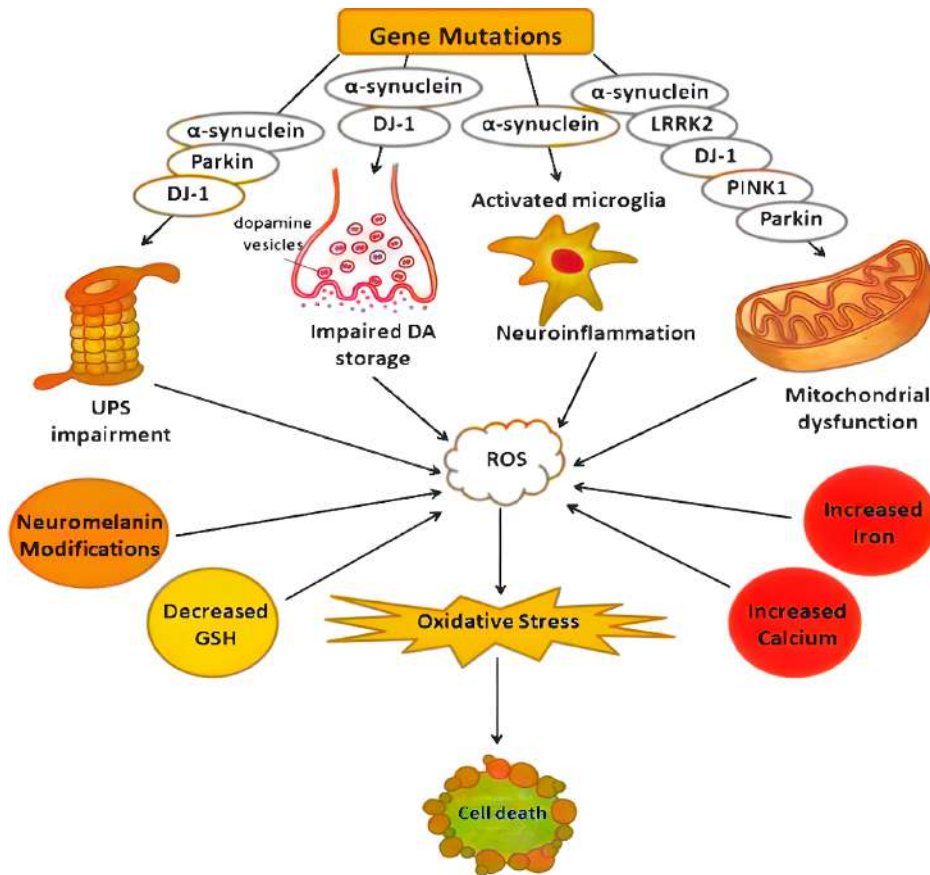


Fig. 11. Mechanisms leading to oxidative stress in PD and the role of PD-related gene products in this process [66].

2.5 Role of DJ-1

DJ-1 (PARK7) is a predominantly cytosolic homodimeric protein, ubiquitously expressed in both brain and peripheral tissues [68].

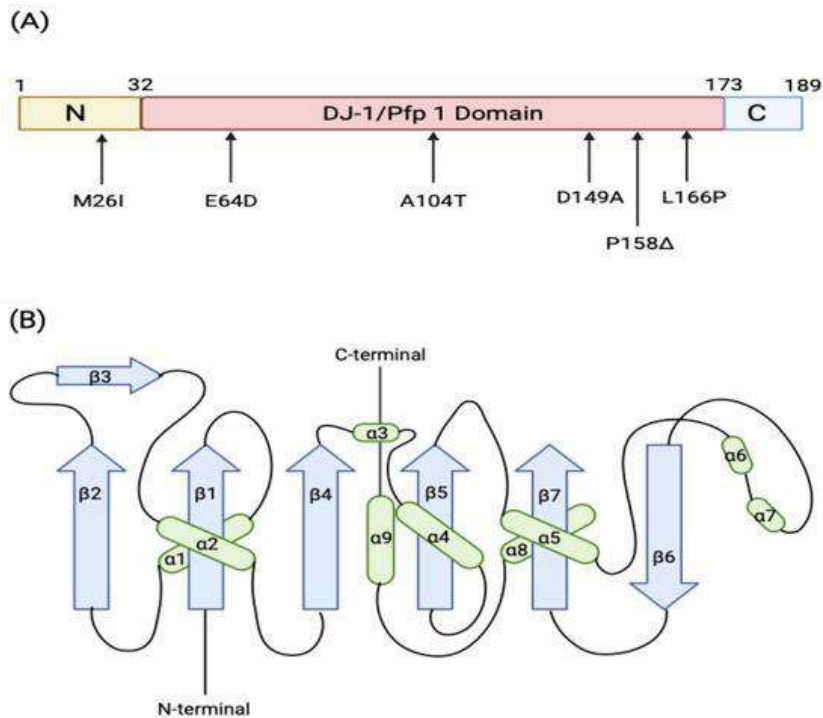


Fig. 12. Domain structure of DJ-1 monomer. (A) Three DJ-1 domains and familial mutations of DJ-1 associated with PD. (B) DJ-1 monomer topology. The α -helices are represented by ovals (green) and the β -strands (blue) by arrows [14].

It is a multifunctional protein with reported roles in neuroprotection, Ras-dependent cell transformation, fertility, regulation of protein-RNA interactions, modulation of androgen receptor signaling and as a protein chaperone and protease. DJ-1 also interacts with other PD-linked proteins, including PINK1 and Parkin. Mutations in DJ-1 cause early-onset autosomal recessive PD and are associated with increased cellular oxidative stress. DJ-1 is responsive to oxidative conditions and protects cells against ROS. Functionally, DJ-1 contributes to

neuronal survival by modulating ROS levels, supporting mitochondrial stability, and activating protective cellular pathways. Loss of DJ-1 function increases oxidative damage in dopaminergic neurons, enhances susceptibility to α -syn toxicity, and impairs mitochondrial integrity. In addition, DJ-1 may influence protein quality control and autophagic processes, indirectly promoting the clearance of toxic proteins such as α -syn [68–71]

2.6 Neuroinflammation

Inflammation is a defense mechanism of the organism against infections, trauma, and other pathological conditions. When this process affects the central nervous system, it is referred to as neuroinflammation, a complex phenomenon involving different glial cell types, particularly microglia and astrocytes, which communicate through cytokines, neurotransmitters, and neurotrophic factors. Under physiological conditions, microglia contribute to the maintenance of brain homeostasis by performing immune surveillance, phagocytosis of cellular debris and abnormal proteins and supporting neuronal repair. In the early stages of AD, for example, microglia may exert a protective role by promoting β -amyloid clearance. However, excessive, or prolonged neuroinflammation, promoted by aging, metabolic disorders, or viral infections, can become detrimental and contribute to neurodegeneration. Activated microglia can acquire a pro-inflammatory M1 phenotype, characterized by NF- κ B activation and the production

of inflammatory cytokines, reactive oxygen species and neurotoxic mediators, or an anti-inflammatory and neuroprotective M2 phenotype, which is associated with enhanced phagocytic activity, tissue repair and support of synaptic plasticity. Similarly, astrocytes can differentiate into a pro-inflammatory and neurotoxic A1 state or a neuroprotective A2 state that promotes neuronal survival and reparative processes. Although the M1/M2 and A1/A2 classifications represent an oversimplification, as glial cells can display intermediate phenotypes, this framework is useful for understanding the role of neuroinflammation in central nervous system pathologies [72].

Neuroinflammation is a key pathogenic factor in neurodegenerative diseases such as PD, AD and amyotrophic lateral sclerosis. In PD, post-mortem, imaging and biomarker studies indicate that neuroinflammation is a central feature of the disease. Aggregation of α -syn can activate innate and adaptive immune responses, while inflammation itself may further promote protein misfolding, creating a vicious cycle. Genetic factors, such as LRRK2 (which regulates autophagic processes in immune cells) and environmental influences, including the gut microbiota, also contribute to microglial activation and disease progression. Despite its pathogenic role, the immune response is not exclusively harmful: microglia can also contribute to the clearance of α -syn aggregates, a mechanism exploited by current immunotherapeutic strategies under clinical development [9].

Collectively, α -syn aggregation, impaired proteostasis, mitochondrial dysfunction, oxidative stress and neuroinflammation, form a complex pathogenic network in PD, highlighting multiple potential therapeutic targets for intervention.

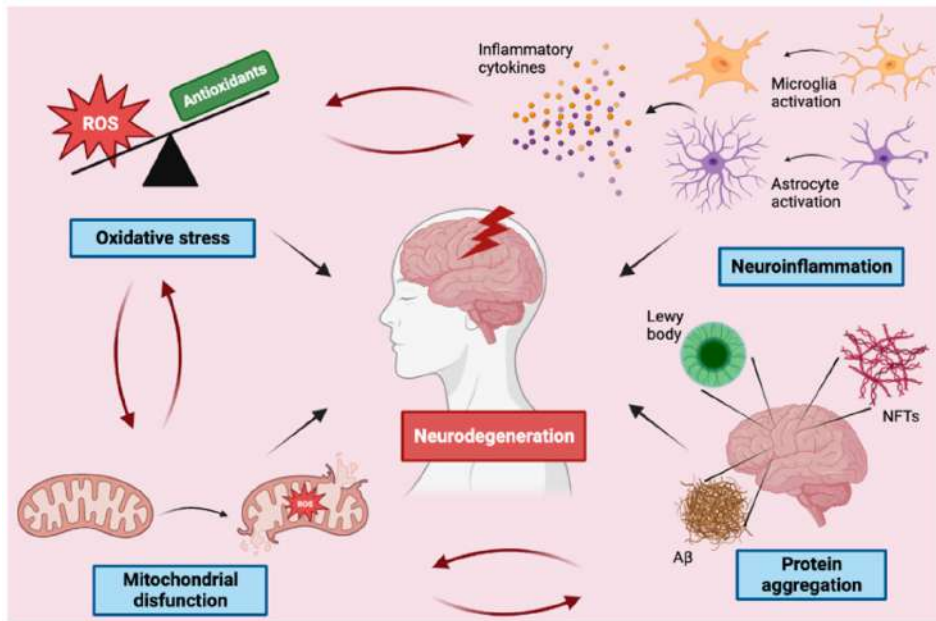


Fig. 13. Main mechanisms involved in neurodegeneration [72].

2.7 Gut-brain axis

The understanding of the gut-brain axis in the pathogenesis of synucleinopathies, has challenged the traditional view of PD as a purely brain-confined disorder. Since 1988, α -syn has been recognized as a neuronal protein located in the nucleus and presynaptic terminals. However, post-mortem studies by Heiko Braak in 2003 revealed that α -syn aggregation in LBs is not restricted to the *substantia nigra*, but

is also consistently found in the vagus nerve, suggesting that the pathology may originate or manifest initially in the gut [73,74].

This concept was further supported by observations in fetal neuronal transplants, where previously healthy tissue developed LB pathology over time, consistent with a prion-like propagation of α -syn from the periphery to the CNS. Subsequent research highlighted the intestinal microbiota as a key modulator of the disease: alterations in microbial composition, reduced levels of short-chain fatty acid (SCFA), lipopolysaccharide-producing bacteria and increased abundance of pro-inflammatory species such as *Lactobacillus*, *Bifidobacterium*, and *Akkermansia* contribute to gut inflammation, epithelial barrier dysfunction, and α -synuclein aggregation, often years before the onset of motor symptoms.

The gut-brain axis represents a bidirectional communication network linking the CNS and the enteric nervous system (ENS), mediated by sympathetic and parasympathetic fibers. Microbiota changes can directly influence neuroinflammatory processes and facilitate α -syn propagation along the vagus nerve to the *substantia nigra*, in line with Braak's hypothesis and the dual-hit model, which proposes that PD may initiate simultaneously in neurons of the gut and of the olfactory bulb.

Additionally, specific microbes, including *Helicobacter pylori* and *Escherichia coli*, appear to promote α -syn aggregation through extracellular mechanisms, accelerating pathology both in the gut and brain. These findings emphasize that the gut microbiota is not only a potential biomarker but also a promising target for preventive and therapeutic strategies in PD [75,76].

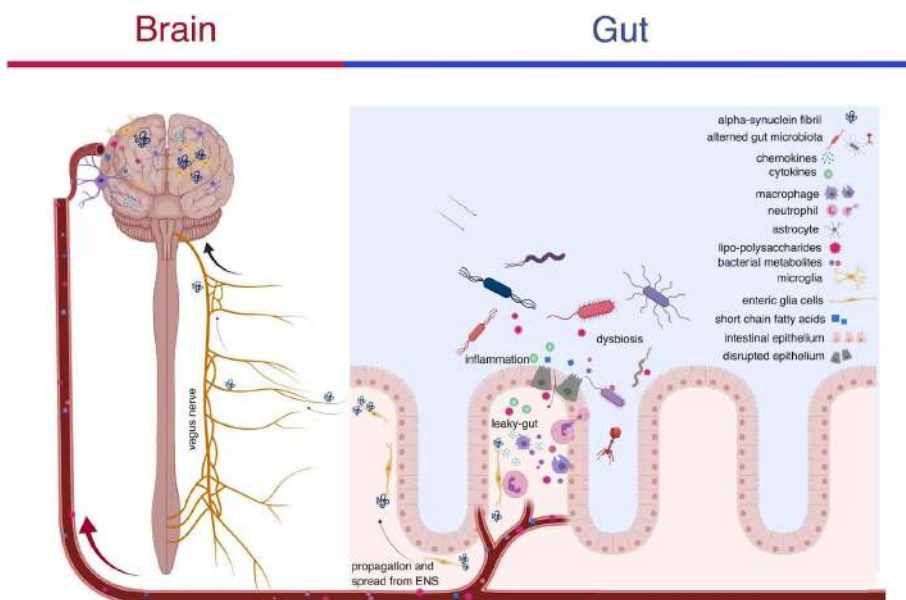


Fig. 14. A schematic representation of the microbiota-gut-brain axis [76].

2.8 Limitations of conventional therapies

Levodopa remains the most widely prescribed and effective first-line therapy for PD [77], which provides symptomatic benefit and does not slow or alter the underlying neurodegenerative process. This agent works by restoring dopamine

signaling or mimicking its physiological effects, yet they are unable to prevent or reverse the progressive degeneration of dopaminergic neurons [78].

Long-term administration of levodopa, especially at higher doses, frequently leads to motor complications, such as dyskinesias and motor fluctuations marked by a premature waning of therapeutic effect or unpredictable transitions between “on” and “off” states. When these issues arise, adjunctive therapies may be introduced to mitigate motor complications and reduce Levodopa requirements. Commonly used add-on treatments include dopamine agonists and drugs that inhibit dopamine metabolism or reuptake, such as catechol-O-methyltransferase (COMT) and monoamine oxidase-B (MAO-B) inhibitors [79].

Given these limitations, there is a growing imperative to explore alternative therapeutic strategies that not only alleviate symptoms but also target the molecular and cellular mechanisms driving neurodegeneration in PD.

3. Plant-derived bioactive compounds as emerging therapeutic strategies in PD

The growing interest in the use of natural products and plant-derived bioactive compounds for managing diseases such as PD is rooted in solid historical and scientific foundations, highlighting the chemical richness and biological diversity of the plant kingdom.

Natural products, characterized by a wide spectrum of secondary metabolites with diverse chemical structures and biological activities, have long been used in traditional medicine, the oldest known medical practice, to treat or alleviate human disorders and diseases. This historical relevance has driven modern research efforts aimed at gaining an in-depth understanding of the effects and mechanisms of action of plant-derived molecules in various model systems, with the goal of leveraging them mainly for disease prevention. Nevertheless, the use of natural extracts or isolated compounds represents a highly interesting challenge in neurological therapies. [80,81].

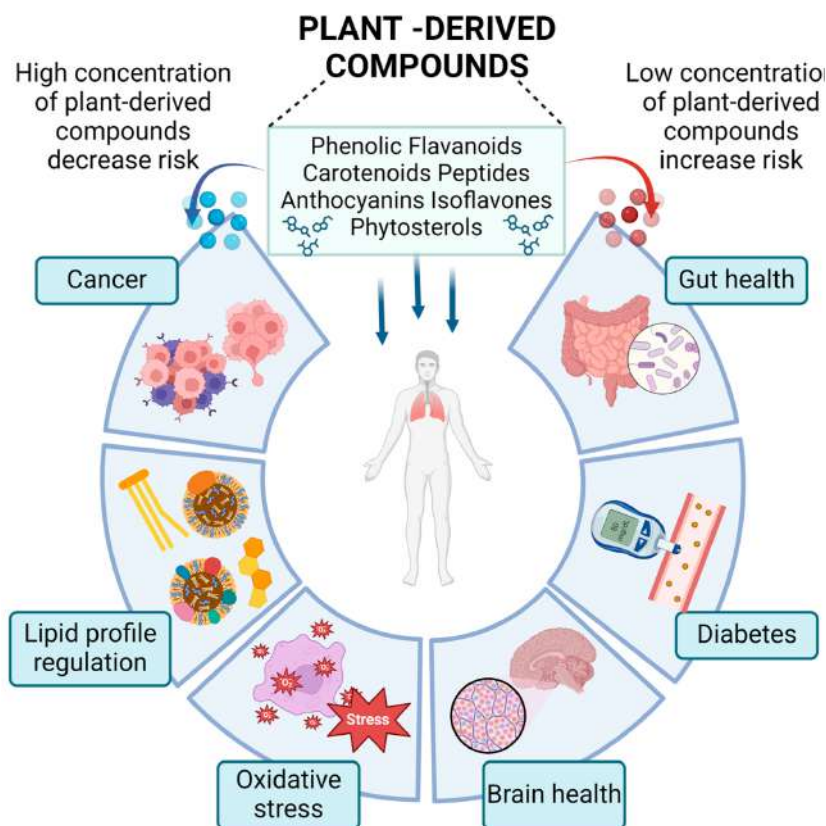


Fig. 15. The multifaceted role of plant-derived bioactive compounds in chronic diseases [82].

Plants synthesize secondary metabolites, distinct from primary metabolites necessary for growth, as essential molecules for interaction and defense within their environment. These compounds play crucial ecological roles, including defense against pathogens (bacteria, fungi, viruses), competition with other plant species and plant-herbivore interactions [83].

Secondary metabolites are classified into several major classes:

- Terpenes: the largest family of natural products, with structures ranging from linear to highly polycyclic, all synthesized from isoprene (C₅) units. Terpenes encompass organic compounds (plant volatiles), cardiac glycosides, carotenoids, and sterols.
- Phenolic compounds: a widely distributed group defined by the presence of a hydroxyl functional group bound to an aromatic ring, encompassing simple phenols as well as high-molecular-weight polymers such as tannins. Phenolics include phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins, and lignin.
- Alkaloids: a broad class characterized by one or more nitrogen-containing heterocyclic rings, typically synthesized from amino acids. Alkaloids are part of nitrogen containing compounds, such as glucosinolates.

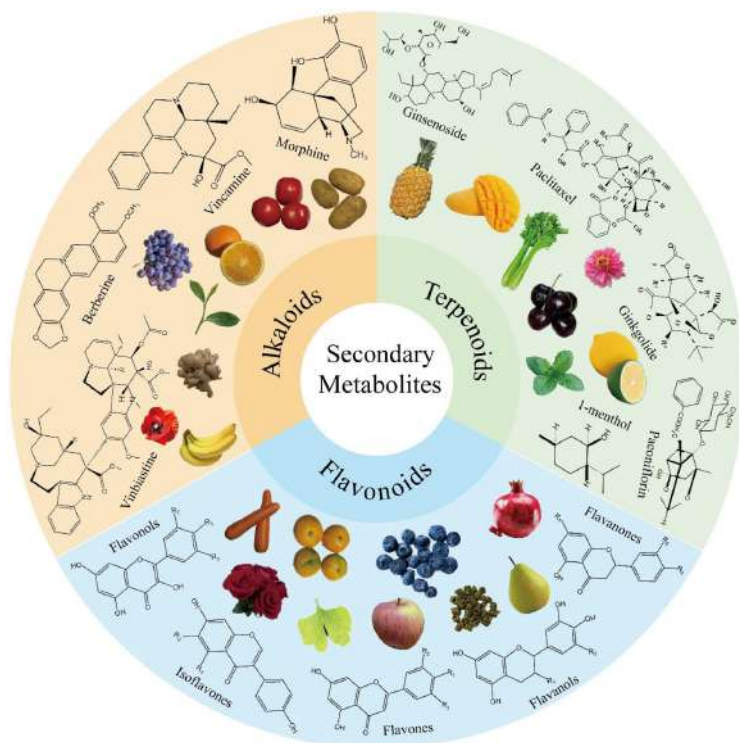


Fig.16. A range of horticultural species and their secondary metabolites [84].

The structural diversity of secondary metabolites enables a broad range of biochemical activities that are highly valuable in pharmaceutical and medical contexts [85].

In recent years, increasing attention has been directed toward the use of medicinal plants and plant-derived bioactive compounds as complementary or alternative approaches in the management of PD with a more favorable safety profile [86]. Natural sources such as mushrooms, plants, microalgae, and cyanobacteria, which are rich in essential micronutrients and bioactive secondary metabolites, represent

a valuable reservoir of molecules with potent neuroprotective properties [87,88]. These compounds are characterized by their multiple phenol rings and include flavonoids, terpenoids, alkaloids, phenols, and polysaccharides. They have been intensively explored and studied and they have shown promising results in preclinical models of neurodegenerative disease, due to their ability to modulate multiple key pathways involved in neurodegeneration. These bioactive molecules exert multifaceted actions, including preservation of cognitive functions, Acetylcholinesterase (AChE) inhibition, anti-neuroinflammation, anti-apoptotic, anti-amyloidogenic and autophagy stimulation [87].

Moreover, advances in delivery technologies, such as nanocarriers and other modern formulation strategies, are helping overcome key obstacles related to stability, bioavailability and tissue targeting of natural products [89].

Therefore, integrating plant-derived bioactive molecules into modern pharmacological research may offer a significant opportunity to identify more effective and sustainable therapeutic solutions for the preventive management of PD.

4. The One Health framework

The One Health approach is based on an integrated vision of health as a unified and interconnected system, aiming to promote a lasting balance between human, animal and environmental well-being [90].



Fig. 17. Schematic representation of the One Health framework [91].

This perspective recognizes that human health is closely linked to that of animals, both domestic and wild, as well as to the health of plants and natural ecosystems, highlighting their mutual interdependence [92].

The profound effects of climate change and the complex relationships between human activities, wildlife, and the environment, increasingly demand an integrated and multidisciplinary model. Cooperation among different scientific fields and

sectors enables a broader and more effective approach to the prevention, control, and management of diseases, thereby reinforcing global health security [93].

The historical roots of this concept date back to the early 20th century, with William Osler and James H. Steele, who highlighted the links between human and animal health. In 1964, veterinary public health expert Calvin Schwabe formally introduced the term “One Medicine” to describe the close interconnections between human and animal health. Schwabe’s concept emphasized collaboration between medical and veterinary professions but remained largely clinically focused. Over the following decades, the idea evolved to incorporate broader environmental and ecological dimensions. The term “One Health” was officially coined in 2004, inspired by the need for a holistic approach to emerging zoonotic diseases and it was subsequently formalized through initiatives such as the Manhattan Principles, which promoted integrated strategies across human, animal, and environmental health. This shift marked the transition from a purely clinical perspective to a systems-oriented understanding of health interdependence [90].

Today, it is widely recognized that biodiversity loss negatively affects human health, both directly and indirectly. The reduction in biological diversity undermines ecosystem stability, making systems less resilient and more vulnerable to

environmental stress, with a consequent decline in essential ecosystem services that support human survival and well-being [94].

The link between health and nature is further emphasized by the fact that many pharmaceutical compounds are directly derived from natural organisms [95].

These considerations have fostered the development of research approaches that are more sustainable and aligned with current environmental changes. In particular, the concept of global health has prompted the establishment of numerous initiatives both internationally and in Italy, notably the National Biodiversity Future Centre (NBFC) and the On Foods project, both funded by the National Recovery and Resilience Plan (PNRR).

The NBFC focuses on the protection of the Mediterranean area, recognized as a biodiversity hotspot, addressing challenges related to the conservation and restoration of marine, coastal, transitional, and terrestrial ecosystems. The project supports research activities aimed at monitoring, conserving, restoring, and enhancing Italian biodiversity, with the goal of mitigating the impacts of human activities and climate change. As part of biodiversity valorization, the study of natural resources enables the identification of nature-based solutions, including the development of innovative pharmaceuticals, nutraceuticals, and functional foods,

thereby increasing the economic, social, and environmental value of natural heritage and promoting ecosystem preservation (<https://www.nbfc.it/>).

Aligned with the 2030 Agenda for Sustainable Development, the “ON FOODS” project seeks to counteract climate change and conserve natural resources by promoting sustainable practices across the entire food supply chain, from production and distribution to consumption. A key objective is the implementation of a circular system to enhance the value of food by-products and reduce waste, thereby improving both the efficiency and sustainability of food products (<https://onfoods.it/>).

Overall, the convergence of One Health principles, the exceptional biodiversity of the Mediterranean basin, the food waste valorization and modern pharmacological screening strategies creates an ideal framework for identifying neuroprotective plant-derived molecules, particularly for preventive applications. Within this integrated perspective, the present doctoral research represents a concrete example of how ecological knowledge and biomedical innovation can be combined to develop sustainable, effective approaches for the management of PD.

5. Valorization of natural waste matrices

In the context of a One Health-oriented and sustainability-driven research framework, the valorization of plant waste matrices represents a promising and environmentally responsible strategy for the discovery of bioactive compounds relevant to neuroprotection. Agro-industrial by-products, often generated in large quantities, mainly consist of inedible plant parts that are traditionally discarded. The accumulation of such by-products contributes to environmental pressure and economic loss, thereby reinforcing the need for sustainable strategies to recover and reuse plant waste matrices as natural sources of functional compounds [96]. These by-products are increasingly recognized as valuable reservoirs of secondary metabolites with antibacterial, anti-inflammatory, antiproliferative, antioxidant and enzymatic inhibitory activities [97]. Their use not only reduces environmental impact and promotes circular bioeconomy principles, but also expands the range of natural sources available for pharmacological exploration [98].

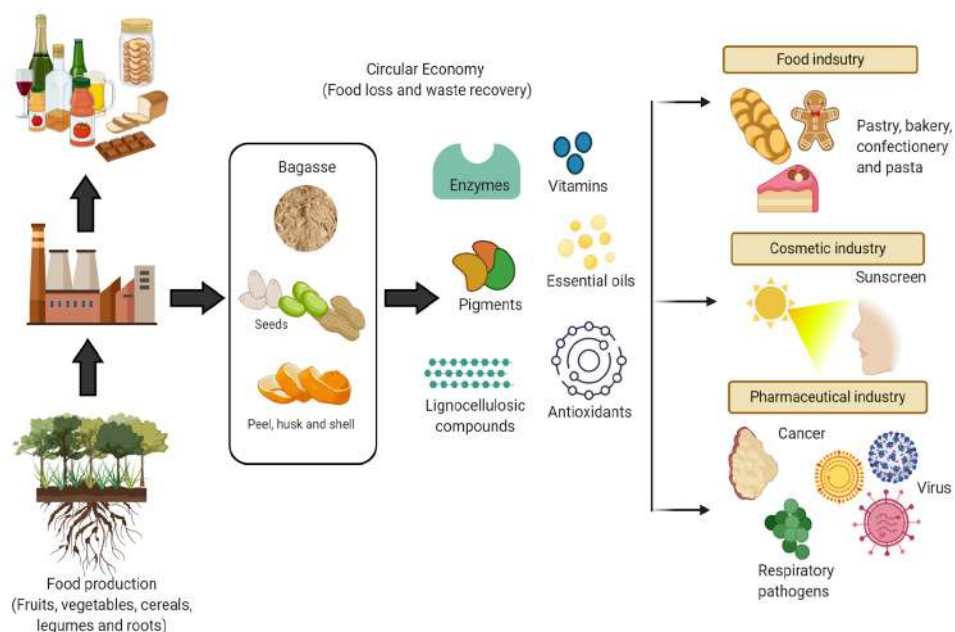


Fig.18. Schematic overview of agri-food waste valorization and its applications in the food, cosmetic and pharmaceutical industries within a circular economy model [99].

Advances in green extraction and biorefinery technologies have enabled the efficient recovery of bioactive compounds from plant residues, turning agro-industrial by-products into valuable resources. This approach supports sustainability, circular economy principles and zero-waste goals, while consumer awareness and regulatory frameworks increasingly favor plant-based bioactives [100]. Integrating these undervalued matrices into neuroprotective research offers cost-effective, high-yield sources of natural compounds, facilitating their

translation into preventive or therapeutic strategies for neurodegenerative diseases [72].

Research focus

The central focus of this PhD thesis is the investigation of innovative neuroprotective strategies to prevent development of PD, a rapidly increasing neurodegenerative disorder. The primary pathological process addressed throughout this research is the aggregation of α -syn, a hallmark mechanism driving PD onset and progression. Identifying natural bioactive compounds capable of preventing or mitigating α -syn aggregation - and the cascade of cellular dysfunction associated with it - constitutes a major challenge in modern biomedical research and represents the main objective of this work.

This project is conceptually framed within the One Health perspective, which emphasizes sustainability and the interconnectedness between human, environmental, and ecosystem health. Within this framework, the research is organized into two complementary experimental lines, both devoted to the valorization of natural matrices - either plant biodiversity or agro-industrial by-products - to identify molecules with neuroprotective properties relevant to PD:

1. Valorization of Plant Biodiversity (NBFC Project).
2. Valorization of Agro-Industrial By-Products (ON FOODS Project).

The thesis is structured into three interconnected research chapters (Chapters 2, 3, and 4), preceded by a conceptual review (Chapter 1), forming a coherent scientific progression.

Chapter 1 provides the theoretical foundation, reviewing natural compounds as multi-target agents for aging-related neurodegenerative diseases and highlighting the relevance of target mechanisms such as protein aggregation, oxidative stress and mitochondrial dysfunction.

Chapter 2 introduces the first experimental research line, developed within the ON FOODS sustainability framework, focused on underexploited waste matrices. Part A demonstrates the neuroprotective activity of a cocoa bean shell extract (CBSE) and Part B explores extracts from Calabrian *Glycyrrhiza glabra* leaves, identifying its potential neuroprotective effect, based on production area and harvest time.

Chapter 3 presents the second research line, conducted in collaboration with the National Biodiversity Future Centre (NBFC), describing the screening pipeline that led to the identification of a neuroprotective compound, Acteoside from *Verbascum thapsus*.

Chapter 4 extends the work from Chapter 2B by providing a detailed investigation of a flavonoid from *Glycyrrhiza glabra* leaves, pinocembrin, evaluating its protective effects.

In summary, this thesis integrates biodiversity-driven discovery, sustainable bioprospecting and biological validation to identify natural compounds capable of modulating key pathogenic mechanisms of PD. Despite arising from two distinct valorization strategies (agro-industrial by-products and plant biodiversity), all experimental chapters converge on a common scientific goal: the identification of multi-target natural molecules capable of reducing α -syn aggregation and oxidative stress. This consistent thematic progression ensures a coherent narrative aligned with the overarching principles of the One Health framework and the long-term goal of developing novel neuroprotective preventive strategies for PD.

Chapter 1

Complementary strategies aimed at preventing aging-associated neurodegenerative diseases can be achieved through compounds derived from natural extracts, which have the potential to delay aging and protect neuronal function. We examined recent research on bioactive molecules from diverse natural sources, including mushrooms, plants, microalgae and cyanobacteria, emphasizing their reported neuroprotective effects.

Given that neurodegeneration involves multiple pathological mechanisms, we discussed compounds capable of modulating several processes simultaneously, thus preventing premature neuronal death and functional decline. Specifically, we analysed molecules derived from secondary metabolism, such as phenols, flavonoids, terpenes, alkaloids and polysaccharides, based on their key bioactivities.

Furthermore, we highlighted four extensively studied natural molecules, curcumin, resveratrol, ergothioneine and phycocyanin, as models for developing multi-target neuroprotective agents:

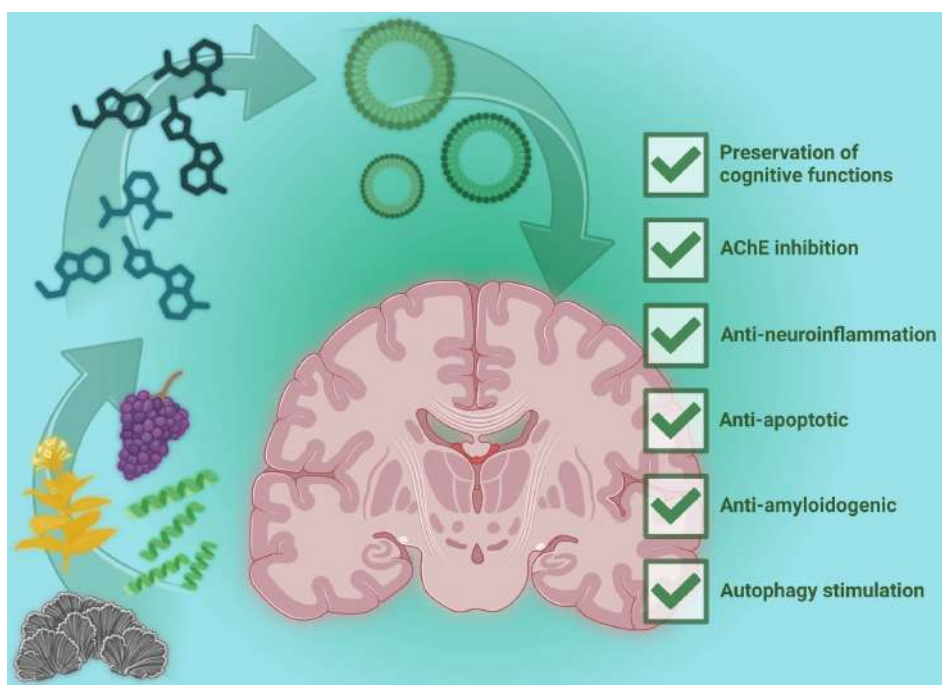
1. Curcumin: a polyphenol from *Curcuma longa* with antioxidant, anti-inflammatory and anti-amyloidogenic properties, also inducing

macroautophagy via Akt/mTOR inhibition. Clinical trials with bioavailable forms have suggested improvements in attention and memory.

2. Resveratrol: a polyphenol from red grapes and wine that exerts antioxidant and anti-inflammatory effects, activates SIRT1 and has slowed cognitive decline in AD patients.
3. Ergothioneine (EGT): a sulfur-containing amino acid abundant in mushrooms, acting as a ROS scavenger and reducing A β plaque accumulation in AD models.
4. Phycocyanin (PC): a pigment-protein complex from cyanobacteria (*Arthrospira platensis*) with antioxidant and anti-inflammatory properties, improving visual learning and working memory in elderly subjects.

Finally, we discussed the challenges of translating these preclinical findings into clinical applications, including low bioavailability, poor solubility and limited blood–brain barrier (BBB) penetration. We reviewed promising strategies such as nanoencapsulation and surface modification of nanocarriers to enhance stability, absorption and BBB crossing, as well as modulation of the gut-brain axis, which represents a novel avenue for mitigating neurodegeneration.

Overall, this review emphasizes the potential of natural compounds as multi-target neuroprotective agents and highlights strategies to bridge the gap between basic research and clinical application, ultimately aiming to improve the quality of life for individuals affected by neurodegenerative diseases.



Graphical abstract

Exploiting natural niches with neuroprotective properties: a comprehensive review

Moukham H. & Lambiase A. et al., 2024.

Abstract

Natural products from mushrooms, plants, microalgae, and cyanobacteria have been intensively explored and studied for their preventive or therapeutic potential. Among age-related pathologies, neurodegenerative diseases (such as Alzheimer's and Parkinson's diseases) represent a worldwide health and social problem. Since several pathological mechanisms are associated with neurodegeneration, promising strategies against neurodegenerative diseases are aimed to target multiple processes. These approaches usually avoid premature cell death and the loss of function of damaged neurons. This review focuses attention on the preventive and therapeutic potential of several compounds derived from natural sources, which could be exploited for their neuroprotective effect. Curcumin, resveratrol, ergothioneine, and phycocyanin are presented as examples of successful approaches, with a special focus on possible strategies to improve their delivery to the brain.

Keywords: neuroprotection; curcumin; resveratrol; ergothioneine; phycocyanin; Alzheimer's disease (AD); Parkinson's disease (PD); delivery strategies

1. Introduction

Aging is a natural condition characterized by the progressive deterioration of physiological functions. It involves several different biological processes, and it is strongly influenced by environmental conditions, affecting lifespan and playing an essential role in the development of age-related diseases. Among these, Alzheimer's disease (AD) and Parkinson's disease (PD), involving cognitive and physical decline, are massive public health and social issues. From a molecular point of view, these diseases are characterized by neuroinflammation and the accumulation of protein aggregates. In detail, β -amyloid plaques and phospho-tau neurofibrillary tangles are found in the brain of AD patients, while α -synuclein fibrils are observed in PD patients. Despite the great efforts in developing new drugs against these pathologies, no effective or definitive treatment is currently available [1]. A complementary approach would consist of the prevention of these aging-associated diseases through the assumption of natural extracts or specific compounds derived from them, which can delay aging and prevent neurodegeneration.

The aim of this review is to present research developments in the use of molecules derived from natural sources (mushrooms, plants, microalgae, and cyanobacteria) with neuroprotective effects (Figure 1). In particular, the bioactivities associated with specific identified compounds are selectively taken into consideration in this manuscript. In fact, although several natural extracts have shown neuroprotective effects, for most of them, the specific mechanism of action is still not known.

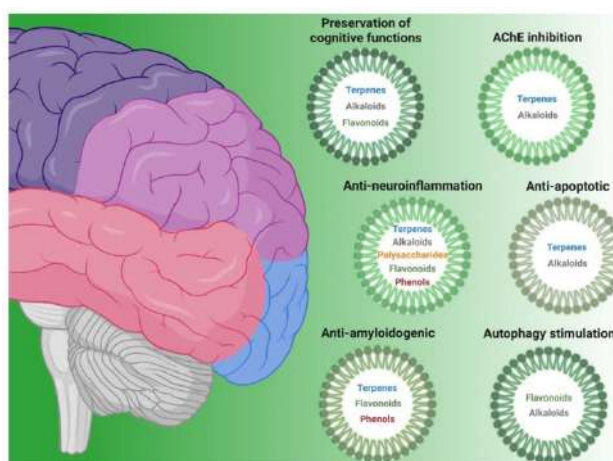


Figure 1. Classes of natural compounds (flavonoids, terpenes, phenols, alkaloids, and polysaccharides) with neuroprotective properties, grouped according to their bioactivity (preservation of cognitive functions, AChE inhibition, anti-neuroinflammation, anti-apoptotic, anti-amyloidogenic, and autophagy stimulation).

Here, we describe compounds derived mainly from the secondary metabolic pathways of shikimate (such as phenols and flavonoids), terpenes, alkaloids, as well

as polysaccharides. Furthermore, we discuss them as a function of their mechanisms of action through a narrative literature methodology.

In addition, four widely studied molecules (curcumin, resveratrol, ergothioneine, and phycocyanin) are presented as model compounds for the development of novel neuroprotective molecules, with a focus on possible delivery strategies to improve bioavailability.

2. Active Natural Compounds and Targeted Bioactivities

A plethora of natural extracts derived from different matrices, such as plants, microalgae, cyanobacteria, or mushrooms, are rich in bioactivities. Searching scientific publications with “natural compounds AND neuroprotection” as keywords in bibliographic databases, 3864 results are shown via PubMed (<https://pubmed.ncbi.nlm.nih.gov/?term=natural+compounds+AND+neuroprotection>, accessed on 23/02/2024) and 11,060 through ScienceDirect (natural compounds AND neuroprotection—Search|ScienceDirect.com, accessed on 23/02/2024). In total, 2841 review articles and 3943 research articles (out of the 8558 manuscripts suggested by the ScienceDirect database) were published over the last 10 years (natural compounds AND neuroprotection—Search|ScienceDirect.com, accessed on 23/02/2024). This indicates a deep interest and prolific research activity in this field in recent years.

Neurodegenerative diseases, such as AD and PD, share some common features at the molecular level (e.g., protein aggregation, oxidative stress, neuroinflammation, and neuronal death) but also present disease-specific characteristics [2]. In the following section, we aim to examine the main processes involved in these pathologies and explore many emerging natural molecules presenting interesting bioactivities. Table 1 reports the classes of compounds and specific metabolites associated with their main bioactivities, which have been selected for this manuscript. Antioxidant properties were not extensively discussed since they have been widely explored in several other recent reviews [3–5]; therefore, they are only briefly mentioned here.

Table 1. Bioactive compounds from natural resources comprehensively classified by the class of bioactivity.

Class of Compound	Compound Name	Natural Source	Ref.
Anti-amyloidogenic			
terpenes	Polyozellin, thelephoric acid, polyozellic acid	<i>Polyozellus multiplex</i>	[6]
	Erinacine A	<i>Hericium erinaceus</i>	[7,8]
	Tanshinone IIA	<i>Salvia miltiorrhiza</i>	[9,10]
phenols	Oleuropein aglycone	Extra virgin olive oil	[11]
	Hydroxytyrosol	Extra virgin olive oil	[11]

	Salvianolic acid B	<i>Salvia miltiorrhiza</i>	[12,13]
flavonoids	Epigallocatechin gallate	<i>Camellia sinensis</i>	[14–16]
AChe inhibitors			
terpenes	Triterpenes	<i>Ganoderma lucidum</i>	[17]
	Polyozellin, thelephoric acid, Polyozellic acid	<i>Polyozellus multiplex</i>	[6]
alkaloids	Nostocarboline	<i>Nostoc 78-12A</i>	[18]
	Anatoxin-a(s)	<i>Anabaena flos-aquae</i> <i>strain NRC 525-17</i>	[19]
	Galanthamine	Amaryllidaceae	[20]
Anti-apoptotic			

terpenes	Triterpenes	<i>Ganoderma lucidum</i>	[21,22]
	Erinacine A	<i>Hericium erinaceus</i>	[8]
	Astragaloside IV	<i>Astragalus membranaceus</i>	[23,24]
alkaloids	Caffeine	Coffee and cocoa beans, tea leaves, guarana berries	[25,26]
flavonoids	Fisetin	Strawberry, apple, persimmon, grape, onion, cucumber	[27–31]
	GardeninA	<i>Gardenia resinifera</i> , <i>Tamarix dioica</i> , <i>Murraya paniculata</i>	[32]

Autophagy stimulation

alkaloids	Caffeine	Coffee and cocoa beans, tea leaves, guarana berries	[33]
flavonoids	Isoquercitrin	<i>Apocynum venetum</i>	[34,35]
	Hesperidin	<i>Citrus</i> fruits	[36]
	Epigallocatechin gallate	<i>Camellia sinensis</i>	[37]
	Fisetin	Strawberry, apple, persimmon, grape, onion, cucumber	[38]
	Morin	Mulberry	[39]
Anti-neuroinflammation			
polysacchar ides	β-glucans	<i>Grifola frondosa</i>	[40]

terpenes	Erinacine A	<i>Hericium erinaceus</i>	[41]
	Astragaloside IV	<i>Astragalus membranaceus</i>	[42,43]
flavonoids	Fisetin	Strawberry, apple, persimmon, grape, onion, cucumber	[44]
	Epigallocatechin gallate	<i>Camellia sinensis</i>	[45–47]
	GardeninA	<i>Gardenia resinifera</i> , <i>Tamarix dioica</i> , <i>Murraya</i> <i>paniculata</i>	[32]
alkaloids	Caffeine	Coffee and cocoa beans, tea leaves, guarana berries	[33,48–52]

Preservation of cognitive functions

polysaccharides	β -glucans	<i>Grifola frondosa</i>	[40]
terpenes	Erinacine A	<i>Hericium erinaceus</i>	[7,53,54]
	Astragaloside IV	<i>Astragalus membranaceus</i>	[42,55]
alkaloids	Caffeine	Coffee and cocoa beans, tea leaves, guarana berries	[56–61]
flavonoids	Epigallocatechin gallate	<i>Camellia sinensis</i>	[62–64]
	GardeninA	<i>Gardenia resinifera</i> , <i>Tamarix dioica</i> , <i>Murraya paniculata</i>	[32]
	Morin	Mulberry	[65,66]
	Hesperidin	Oranges	[67–72]

	Isoquercitrin	<i>Apocynum venetum</i>	[73]
phenols	6-Shogaol	Ginger rhizomes (<i>Zingiber officinale</i>)	[74,75]
	Hydroxytyrosol	Extra virgin olive oil	[76,77]
	Oleuropein aglycone	Extra virgin olive oil	[11]
	Salvianolic acid B	<i>Salvia miltiorrhiza</i>	[78,79]
terpenes	Tanshinone IIA	<i>Salvia miltiorrhiza</i>	[80]

2.1. Anti-Amyloidogenic Compounds

Pathological protein aggregation is one of the hallmarks of neurodegenerative diseases and is associated with clinical outcomes since their presence often correlates with the progression of the symptoms [2]. However, mature aggregates or fibrils are not the major toxic species, while oligomeric assemblies are recognized as mainly responsible for cellular toxicity.

In AD patients, two distinctive aggregates are found that induce oxidative stress and neuronal loss. The first is represented by plaques of amyloid- β peptide ($A\beta$), a short proteolytic fragment derived from the amyloid protein precursor (APP) through the action of β - and γ -secretases. These insoluble deposits are found extracellularly near the synapses and lead to synaptic dysfunction and neuronal death. The second is represented by neurofibrillary tangles of the phosphorylated tau protein (p-tau), a microtubule-associated protein that, when hyperphosphorylated, accumulates intracellularly and leads to reduced neuronal functions and neurotoxicity. PD is instead characterized by the accumulation of aggregates of the presynaptic protein α -synuclein, which induces neurotoxicity and, finally, neuronal loss.

Therefore, compounds acting on protein aggregation have been widely studied in recent years (Table 1).

Different terpenoid molecules have been shown to affect protein aggregation, such as those from the black mushroom *Polyozellus multiplex* used in Asia (especially Korea, China, and Japan). Specifically, different p-terphenyls were purified from this mushroom, named polyozellin, thelephoric acid, and polyozellic acid, which strongly reduce extracellular $A\beta$ 1-42 release from the human Swedish mutant APP-transfected mouse neuroblastoma cell line [6]. Another interesting terpene derives

from *Hericium erinaceus*, which is one of the most used medicinal mushrooms, also known as lion's mane or Yamabushitake. The interest in this mushroom is due to the presence of a large amount of different bioactive metabolites, both in the fruit body and in the mycelia [81]. Among them, Erinacine A, a diterpenoid isolated from the mycelium, can reduce A β plaque formation in a mouse model of AD (APP/PS1 transgenic mouse), both by increasing A β degradation and inhibiting its production [7]. In addition, Erinacine A reduces ROS levels in mouse neuronal cells treated with a neurotoxin that inhibits complex I of the electron transport chain (ETC) and consequently also cell death [8].

Another intriguing bioactive terpenoid is Tanshinone IIA (Tan IIA), a diterpene phenanthrenequinone extracted from the roots and the rhizomes of the plant *Salvia miltiorrhiza*. Tan IIA presents a variety of bioactivities, including anti-apoptotic, anti-inflammatory, blood–brain barrier (BBB) protection, and antioxidant effects [82]. Tan IIA also inhibits amyloid fibril formation and disaggregates the A β 1-42 amyloid peptide via specific binding to a hydrophobic β -sheet groove [10], as well as its reduced A β -induced toxicity in neuronal cells in vitro [9].

Also, phenolic compounds, like Oleuropein aglycone (OleA) and Hydroxytyrosol (HT), found in extra virgin olive oil, are known for their anti-amyloidogenic

properties. These compounds exhibit strong antioxidant effects and are suggested to modulate the aggregation of the A β 1-42 peptide, decreasing the formation of toxic aggregates [83–85]. OleA primarily interacts with the *N*-terminus of A β 1-42, hindering the development of toxic oligomers and their transition into mature fibrils. In contrast, HT accelerates fibril formation, possibly due to increased hydrophilicity. Therefore, treatment with OleA and HT reduces the cytotoxicity of A β 1-42, steering the aggregation process towards harmless outcomes even at low concentrations. Notably, evidence suggests that these polyphenols can penetrate the blood–brain barrier, accumulating in the brain parenchyma with regular intake [11] and supporting their development as an anti-aggregant *in vivo*.

Likewise, Salvianolic acid B (SalB), a dominant water-soluble component from *Salvia miltiorrhiza*, is known for its ability to ameliorate A β -induced neuronal degeneration and neuroinflammation in AD [12]. It has been reported that Salvianolic acid B not only inhibits A β fibril formation but also disaggregates preformed fibrils by converting them to an amorphous structure, thereby providing protection against A β -induced cytotoxicity. In a study *in vitro*, SalB was shown to significantly decrease the levels of A β 1-42 in SH-SY5Y cells overexpressing the human APP (amyloid precursor protein) Swedish mutant [13].

Among the metabolites derived from the shikimic acid pathway, flavonoids represent a class of very interesting ones. Among these, Epigallocatechin gallate (EGCG), present in green tea, has received considerable attention in recent years due to its potential health advantages in preventing numerous chronic diseases, including neurodegenerative diseases. In transgenic mice overexpressing the mutant amyloid precursor protein (Tg APPsw), the administration of EGCG via intraperitoneal injection (20 mg/kg) led to a significant decrease in the brain levels of the A β peptide and a reduction in A β plaques. Additionally, EGCG promoted cleavage in the α -C-terminal fragment of APP, leading to elevated levels of soluble APP- α , indicative of enhanced α -secretase cleavage activity, which correlates with lower A β levels [14]. Strikingly, EGCG showed comparable effectiveness in diminishing A β deposition when administered orally via drinking water (50 mg/kg) [86]. In vitro, it was demonstrated that EGCG impedes the fibrillogenesis process of both α -synuclein and amyloid- β by directly interacting with the intrinsically disordered polypeptides, preventing their conversion into toxic species and promoting the formation of amorphous, non-toxic aggregates [15,16].

2.2. Inhibitors of AChE

Acetylcholine (ACh) is a neurotransmitter that activates cholinergic neurons in the central nervous system and is involved in the processes of memory and learning.

Acetylcholinesterases (AChEs) are the enzymes responsible for the degradation of ACh and are potential targets for the treatment of AD since the dysfunction of cholinergic neurons is considered one of the major causes of cognitive impairment in AD patients. A second enzyme, butyrylcholinesterase (BChE), with a broader substrate specificity, seems to also be involved in ACh hydrolysis in the cholinergic synapse, and its levels are increased in the later stages of AD [87]. Therefore, a reduction in the level or the activity of AChE and BChE could lead to an increase in the level of ACh in the synaptic cleft, thus improving cognitive functions. Donepezil hydrochloride and rivastigmine are prescribed cholinesterase inhibitors that reversibly inhibit synaptic acetylcholinesterases, enhancing cholinergic transmission in AD patients [88,89]. However, these drugs present side peripheral cholinergic effects; therefore, the identification of new natural-based inhibitors of AChE activity is of valuable interest in the field.

Several mushroom extracts have been shown to inhibit AChE activity, although in most cases, the effect was not attributed to specific compounds. *Ganoderma lucidum* is a Basidiomycetes fungus, highly used in traditional medicine in Asia since ancient times for its numerous health-promoting benefits [21]. In *G. lucidum*, several triterpenes were tested in vitro and showed AChE inhibition, with IC₅₀ in the micromolar range [17]. Similarly, the above-mentioned p-terphenyls from the black

mushroom *Polyozellus multiplex* present β -secretase non-competitive inhibitor activity in vitro [6].

Also, some alkaloids have been shown to inhibit acetylcholinesterases. Nostocarboline was isolated from the freshwater cyanobacterium *Nostoc 78-12A* and is a potent butyrylcholinesterase (BChE) inhibitor (IC_{50} of 13.2 μ M) [18]. In cyanobacteria, the function of AChE inhibitors is required for the defense against the invertebrate larval settlement of competitor organisms [90,91]. Of interest is the anatoxin-a(s) compound, isolated from the cyanobacterium *Anabaena flos-aquae* strain NRC 525-17, which showed severe cholinergic overstimulation in rats along with a robust inhibition on both AChE and BChE enzymes in vitro [19].

Other alkaloids inhibiting AChE activity come from the Amaryllidaceae plant family. In fact, over 100 cultivars from the Amaryllidoideae subfamily have been studied for their AChE inhibitory activity [92]. Compounds derived from diverse skeleton types have demonstrated potent AChE inhibitory properties, thereby presenting avenues for structure optimization and the semi-synthesis of potent AChE inhibitors [93]. Among these, Galanthamine, which was approved in 2001 by the FDA for AD treatment, showed a strong AChE inhibitory activity (IC_{50} of 0.3–0.5 μ M) [20], exerted by inhibiting substrate accommodation and hydrolysis in the active site of the enzyme [94].

2.3. Natural Compound with Anti-Apoptotic Effect

The most prominent effect of neurodegeneration is the resulting neuronal loss due to the ultimate activation of the apoptotic pathway. Apoptosis is crucial during the development of the central nervous system, but its improper activation can be detrimental in the adult brain and is involved not only in neurodegeneration but also in several other diseases [95].

Several compounds belonging to different chemical classes have been shown to prevent neuronal apoptosis in AD and PD models (Table 1).

G. lucidum triterpenoids, which also present antioxidant activity, have been shown to decrease apoptosis both in vitro and in vivo in the hippocampus of an APP/PS1 mouse model of AD, with an effect on caspase-3 activation and on the ROCK signaling pathway [22]. This results in improved cognitive functions in the same transgenic AD mouse model, thus supporting the development of these triterpenoids as anti-apoptotic agents in neurons.

Another mushroom-derived terpenoid, the aforementioned Erinacine A from *H. erinaceus*, was shown to inhibit apoptosis induced by MPP (1-methyl-4-phenylpyridinium) treatment in a mouse neuronal cell line in vitro. This effect is associated with the increased phosphorylation of some proteins involved in survival, such as PAK1 and LIMK2 both in vitro and in the striatum of a 1-methyl-4-

phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mouse model of PD [8]. Another terpenoid regulating apoptosis is Astragaloside IV (AS-IV), also known as Astragalus saponin IV, which is a primary bioactive component derived from the plant *Astragalus membranaceus*, which is widely used in treating various diseases in traditionally Chinese medicine. AS-IV can protect primary cerebral cortex neurons exposed to oxygen and glucose deprivation by modulating the PKA/CREB signaling pathway and preserving mitochondrial function [23]. AS-IV has also been shown to decrease the apoptosis rate in primary astrocytes, downregulating p-JNK, modifying the Bax/Bcl-2 ratio, and influencing caspase-3 activity [24].

Among the alkaloids, one of the most studied is caffeine (1,3,7-trimethylxanthine), a natural central nervous system (CNS) stimulant of the methylxanthine class. It is mostly sourced from coffee beans but can also be found in certain types of tea and cacao beans and is one common ingredient of many energy beverages [96]. It was shown that the PI3K/Akt signaling pathway, known for its role in cell survival, is implicated in the anti-apoptotic effects of caffeine by regulating the p-JNK and ERK protein kinase of both in vitro cultures [26] and in a mouse model of AD [25].

Fisetin (3,3',4',7-tetrahydroxyflavone) is another plant-derived compound that has garnered attention for its potential anti-apoptotic effects in the brain. It is a flavonoid abundant in fruit and vegetables, including apples, strawberries,

cucumber, and persimmon [27,97]. In 6-hydroxydopamine-treated cells, which are used as a model of PD or traumatic brain injury (TBI), treatment with Fisetin inhibits the activation of key apoptotic proteins, including caspase-3 and caspase-9, suggesting a potential mechanism for preventing neuronal cell death [28,29]. Moreover, Fisetin has been observed to enhance Bcl-2 expression and reduce Bax expression in the mouse brain, thereby shifting the balance in favor of cell survival [30,31]. Furthermore, Fisetin treatment can reduce the expression of other apoptotic regulators, such as ASK-1, p-JNK, p53, and cytochrome C, in the cortex and hippocampus of mice [31]. The inhibition of these apoptotic molecules by Fisetin is triggered through the activation of the PI3K/Akt signaling pathway [28].

Finally, the flavonoid GardeninA (GA), found in the gum of the medicinal plant *Gardenia resinifera*, has shown a regulatory effect on apoptotic pathways in a *Drosophila* model of PD. Indeed, it was observed that GA pretreatment reduced the levels of active caspase-3 induced by paraquat (PQ), thereby mitigating apoptotic cell death. Moreover, GA pretreatment resulted in the downregulation of *reaper* expression, which was induced by PQ. This is significant as *reaper* can induce apoptosis by inactivating inhibitors of apoptosis proteins (IAPs), consequently allowing caspases to escape proteasomal degradation and trigger cell death [32].

2.4. Autophagy Stimulation

Autophagy is an evolutionarily conserved process that leads to the trafficking and degradation of intracellular components into the lysosome to maintain cellular homeostasis [98]. The targets of autophagy comprise misfolded or aggregated proteins, as well as damaged organelles, such as mitochondria, endoplasmic reticulum, and peroxisomes; in particular, mitophagy is the selective removal of damaged mitochondria via the macroautophagy pathway, which is crucial for monitoring their quality and decreasing cellular oxidative stress [99]. Autophagy is particularly important in neurons since it is involved in neurodevelopment as well as in the maintenance of neuronal homeostasis, and basal beneficial autophagy is necessary to maintain lifespan and prolong longevity [100]. Moreover, autophagy also exerts a key role in degrading aggregation-prone proteins, such as α -synuclein [101], β -amyloid [102], and the mutant protein Huntingtin [103]. For this reason, autophagy is considered a target for the prevention of neurodegenerative diseases, and a variety of natural compounds are reported to modulate autophagy and exert neuroprotective effects. Interestingly, most of the literature describes the efficacy of natural compounds in autophagy induction, and some of them also stimulate beneficial effects in particular conditions. Here, we collected the current evidence indicating their neuroprotective role (Table 1).

The aforementioned caffeine was shown to enhance autophagy by increasing the microtubule-associated protein, LC-3, reducing the receptor protein sequestosome 1 (SQSTM1/p62) and chaperone-mediated autophagy and regulating the expression of LAMP2A (lysosomal associated membrane protein 2). Chronic caffeine treatment re-establishes macroautophagy activity and broadly targets a variety of protein aggregates in the brain. Notably, caffeine treatment was shown to attenuate abnormal α -Syn aggregation and neurotoxicity by re-establishing autophagy activity in animal models of PD [33].

Most known plant-derived compounds regulating autophagy belong to the class of flavonoids (Table 1), such as Isoquercitrin, Hesperidin, Epigallocatechin gallate, Fisetin, and Morin.

Isoquercitrin, a flavonoid from *Apocynum venetum*, also known as quercetin-3-O- β -d-glucopyranoside, is a flavonoid that is also present in a variety of medicinal herbs, fruits, and vegetables as well as plant-based foods and drinks [104]. Isoquercetin can mitigate cellular damage in Schwann cells exposed to high glucose levels by upregulating the expression of Beclin-1 and LC3, thus reversing the inhibition of neural cell proliferation [34]. Likewise, in the model organism *C. elegans*, Isoquercetin counteracts the neurotoxic effects of peptide A β 1-42 through

the activation of both macroautophagy and proteasomal degradation pathways [35].

Hesperidin, identified as 3,5,7-trihydroxy flavanone-7-rhamnoglucoside, is a flavanone glycoside predominantly found in citrus fruits. In a study, Hesperidin treatment was able to counteract the increase in Beclin-1, LC3A, and LC3B induced by sodium fluoride, in this case, indicating its potential role in mitigating autophagy-related neurotoxicity. Furthermore, Hesperidin treatment was shown to stimulate the PI3K/Akt/mTOR signaling pathway, which is essential for cell survival [36].

The aforementioned Epigallocatechin gallate was shown to induce autophagy in SH-SY5Y cells by activating the Sirt1 pathway, specifically targeting the neurotoxicity and mitochondrial damage caused by the prion protein. EGCG induces an increase in LC3-II expression alongside a decrease in p62 levels, indicating enhanced autophagic activity [37]. Additionally, the natural flavonoids Fisetin and Morin have also been recognized for their ability to stimulate autophagy. Morin is highly present in the leaves, stems, branches, and fruits of plants belonging to the Moraceae family, and it possesses significant antioxidant and anti-inflammatory properties that offer neuroprotective benefits. In addition, Morin enhances mitophagy, which is essential for removing malfunctioning mitochondria. Strikingly, it accomplishes this by promoting the relocation of transcription factor EB (TFEB) to

the nucleus and triggering the AMPK-ULK1 pathway. These actions have been proven to confer neuroprotection in a mouse model of PD induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), highlighting Morin's potential [39]. As for Fisetin, it was observed that it can induce autophagy within cortical neurons by activating the Nrf2 transcription factors and TFEB, likely via the inhibition of mTORC1. Such activation is crucial for the disassembly of p-tau in cortical neurons, positioning Fisetin as a promising neuroprotective molecule [38].

2.5. Anti-Neuroinflammatory Compounds

Neuroinflammation is another typical hallmark of neurodegenerative diseases [2], which has been observed as an early event in AD development. Chronic inflammation involving microglia may occur in the presence of misfolded or aggregated proteins, high ROS levels, or damaged synapses. In addition, astrocytes are also activated in neurodegenerative diseases, affecting synaptic functions, neuron metabolism, protein aggregation, and, therefore, taking part in the disease process. For these reasons, the modulation of neuroinflammation is a relevant aspect of the prevention of neurodegeneration, and several compounds have been studied for their ability to modulate neuroinflammation (Table 1).

Grifola frondosa, also known as Maitake, is a well-studied medicinal mushroom used for centuries in China and Japan. It is particularly rich in polysaccharides,

especially β -glucans, which have been shown to possess several health-promoting activities [105]. It was recently reported that treatment with proteo- β -glucans derived from Maitake increases astrocytes and microglia activation in a mouse model of AD and promotes the recruitment of microglia to the A β plaques in the cortex and hippocampal region, thus promoting the clearance of A β and reducing the A β plaque burden [40].

Another mushroom-derived compound, the well-studied terpenoid Erinacine A from the mushroom *H. erinaceus*, exerts an anti-inflammatory effect. Indeed, it reduces TNF- α expression upon lipopolysaccharide (LPS) stimulation in rat astrocytes and reduces NO production and iNOS expression in a mouse microglial cell line following treatments with LPS and IFN- α . Remarkably, Erinacine A treatment shows the same anti-inflammatory effect in vivo, leading to improved motor functions in an animal model of PD [41].

Considering triterpenoids, Astragaloside IV (AS-IV) also exhibits anti-inflammatory effects. In particular, it promotes the activation of nuclear factor-kappa B (NF- κ B), reducing the expression of pro-inflammatory genes in microglial cell cultures and in vivo [42]. In addition, this molecule has been reported to modulate the activity of MAPKs and to inhibit the mRNA expression of various pro-inflammatory cytokines

of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) enzymes, contributing to reducing the inflammation [42,43].

Among flavonoids, Fisetin exhibits a remarkable ability to decrease the expression of the glial fibrillary acidic protein (GFAP) in the cortex, hippocampus, and hypothalamus of mice, which is a hallmark of neuroinflammation [44].

GardeninA, among the class of flavonoids, reduces both iNOS and NO levels in the PD model in *Drosophila*, thus providing protection against NO-mediated inflammatory effects [32].

Finally, several lines of evidence indicate that caffeine can limit neuroinflammation associated with neurodegenerative disorders. Indeed, it was reported that caffeine reduces both microglia activation and astrocyte reactivity [49,51].

2.6. Compounds Improving Cognitive Functions

Neurodegenerative diseases are characterized by a strong and progressive cognitive decline. AD and PD patients have difficulties in learning and memory, as well as in speech, due to the neuroinflammation and progressive degeneration of neurons [106]. Thus, the evaluation of the effects of natural compounds on cognitive functions is often the biggest preclinical readout of the effect of these molecules. In Table 1, we report natural compounds that can improve cognitive

functions; interestingly, some of them are also discussed as anti-neuroinflammation molecules (see the previous section). Indeed, as expected, the prevention of cognitive impairment is strictly connected to neuroinflammation reduction.

Different mushroom-derived compounds have been tested for their effects on cognitive functions. For instance, it was recently reported that treatment with proteo- β -glucans derived from Maitake mushrooms improves learning and memory dysfunctions in a mouse model of AD [40]. Another mushroom-derived compound, Erinacine A from *H. erinaceus* mycelium, was also shown to ameliorate behavior deficits in the same model, in particular, burrowing and nesting behavior, spatial learning, and memory [7]. Strikingly, in humans, two double-blind clinical trials showed that the *H. erinaceus* fruiting body supplement (about 3 g/day) improves cognitive functions in healthy Japanese 50-year-old individuals and individuals with mild cognitive impairment [53,54]. Although in these two studies no specific molecule was used, rather, the total dried fruiting body of *H. erinaceus* was used, and although it was not tested on patients affected by neurodegenerative diseases, these preliminary clinical trials suggest the potential of this mushroom to prevent cognitive impairment.

Among terpenes, Astragaloside IV treatment is able to reverse cognitive impairment, as indicated by improved learning and memory abilities in an AD mouse model [42], as well as in a rat model of vascular dementia [55].

Also, caffeine has been shown to potentially prevent memory decline in animal models of AD and PD. Moderate treatment with caffeine led to an enhanced capacity for memory and a reversal of AD pathology in mice [56], also preventing β -amyloid-induced cognitive impairment [57]. Additionally, caffeine consumption prevented hippocampal neurodegeneration induced by streptozotocin (STZ) in a rat model of sporadic dementia [58]. A study examining risk factors for AD in individuals over 65 years old found that coffee drinkers had a 31% lower risk of developing the disease [59]. Similar results were observed in a further study, which showed a reduced risk of AD/dementia in moderate coffee drinkers of 3–5 cups every day compared to those who consumed 0–2 cups of coffee over a 21-year period [60]. In a cross-sectional study involving 196 untreated patients with early-stage PD, coffee consumption was significantly associated with a reduction in the severity of the mood/cognition domain of the non-motor symptoms scale (NMSS) [61].

Numerous flavonoid compounds, including Morin, EGCG, Hesperidin, Isoquercitrin, and 6-Shogaol, have been documented to support the maintenance of cognitive abilities, as outlined in Table 1. Despite the high use of green tea polyphenols, there

is a lack of satisfactory clinical trials involving EGCG in humans with neurodegenerative diseases, but several studies on animals are available. For instance, it was reported that the long-term oral consumption of EGCG (25 mg/kg) induced significant improvement in memory function in an AD mouse model [62]. In a study on cognitive performance, EGCG was found to enhance neurological effects during brain wave stimulation, resulting in a rise in tranquility and a reduction in stress [64]. Taken together, these data support the notion of EGCG dietary supplementation as a potentially safe and effective neuroprotective agent that should also be evaluated in subjects with neurodegeneration.

As concerns GardeninA, its ability to confer protection against mobility deficits induced by paraquat (PQ) in a *Drosophila* PD model has been examined, and it showed that pretreatment with GA significantly improved climbing performances [32].

Also, Morin was shown to improve learning and memory by attenuating oxidative stress, neuroinflammation, and neuronal death in the hippocampus in a rat model of AD [65]. Moreover, in an MPTP mouse model of PD, Morin (20 to 100 mg/kg) significantly reduced nigrostriatal lesions, dopaminergic neuronal death, striatal dopamine depletion, and long-term behavioral impairments [66]. Based on these

studies, we can hypothesize that Morin could be beneficial in reducing cognitive dysfunction both in PD and AD.

Hesperidin has been demonstrated to facilitate the enhancement of spatial memory indirectly through the increased release of TGF β -1 and the promotion of synaptogenic activity in cortical astrocytes of newborn Swiss mice [68]. Hesperidin addressed both motor and non-motor symptoms, such as anxiety-like behaviors and memory deficiencies, while concurrently mitigating monoaminergic abnormalities [107]. Another study investigated the impact of Hesperidin on learning and memory following prolonged exposure to mild stress. Albino Wistar rats, subjected to chronic mild stress and supplemented with Hesperidin (40 mg/kg for 28 days), demonstrated preserved cognitive functions compared to the control group. Notably, there was a statistically significant enhancement in memory consolidation observed after the learning process [69]. Strikingly, several clinical trials have also been conducted in humans, especially using Hesperidin-rich orange juice. In a clinical investigation involving 37 healthy adults aged 60–81, Hesperidin was tested at two different doses with the use of juice containing a high Hesperidin concentration (549 mg/L) or a lower Hesperidin content (64 mg/L). Following eight weeks, the group with elevated Hesperidin levels exhibited markedly enhanced cognitive function, executive function, and episodic memory compared to the group with lower Hesperidin levels [71]. A separate acute study involving 44 young

adults consuming 500 mL of citrus juice with 42.15 mg of Hesperidin demonstrated a notable enhancement in cerebral blood flow (CBF) and executive function in the right frontal gyrus. However, no direct correlation was identified between behavioral improvement and the increase in CBF [70]. Another investigation centered on beverages rich in Hesperidin in healthy middle-aged men (30–65) revealed significant enhancements in attention, executive cognitive function, and psychomotor speed six hours after consuming orange juice with added orange pomace fiber (containing 220.46 mg Hesperidin) compared to a placebo group [67]. In a cohort study encompassing 13,373 Japanese elderly participants, the consumption of citrus displayed a dose-dependent and inversely correlated association with dementia incidence, indicating the potential safeguarding role of citrus flavonoids in diminishing the risk of dementia [72]. These studies proposed that dietary approaches rich in Hesperidin could potentially forestall cognitive decline in elderly individuals, although the specific mechanism remains somewhat unclear.

Among flavonoids, Isoquercetin has been attracting much attention for the treatment and prevention of AD because it contributes to the enhancement of cognitive performance, attenuating neurochemical and neurobehavioral changes in a rat model of AD-induced by colchicine through the activation of antioxidant and anti-inflammatory pathways [73].

6-Shogaol, Hydroxytyrosol (HT), Oleuropein aglycone (OleA), and Salvianolic acid B (SalB) are all recognized phenolic compounds that offer neuroprotection, particularly through the improvement of cognitive abilities. 6-Shogaol, a principal bioactive constituent of ginger, has demonstrated effectiveness in reducing cognitive impairments in animal models of dementia, highlighting its potential in combating cognitive decline. A study illustrated the influence of 6-Shogaol on memory deficits by affecting astrocyte and microglia activation in a mouse model of AD induced by injecting A β 1-42 oligomers into the hippocampus. The results of the study revealed that 6-Shogaol treatment reduced inflammation and neuronal death, leading to an improvement in learning and mitigation of memory deficits [74]. In another investigation, significant enhancements were noted in behavioral and memory tests in a mouse model of AD after the oral administration of 6-Shogaol for two months. Consequently, the study proposed that 6-Shogaol, acting as an inhibitor of the cysteinyl leukotriene 1 receptor (CysLT1R) and cathepsin B (both implicated in the pathogenesis of AD), could act as a neuroprotective agent, preventing neuronal death and cognitive deficits [75]. Hydroxytyrosol (HT) and Oleuropein aglycone (OleA) are compounds found in extra virgin olive oil, and their dietary intake has been linked to protective effects against AD [108]. In an animal model of AD, the administration of HT (at 5 mg/kg/day for 6 months) slightly improved the cognitive behavior of mice [76]. Likewise, the administration of HT

(50 mg/kg) to mice with AD for a duration of 8 weeks significantly improved their cognitive abilities, significantly reducing amyloid aggregates in the cortex [77]. Similarly, OleA is reported to protect against cognitive deterioration by reducing A β levels and plaque deposits in the animal brain together with increased synaptic function (LTP) [109]. Salvianolic acid B (SalB), extracted from the roots and rhizome of *Salviae miltiorrhiza*, is used in traditional Chinese medicine for its neuroprotective properties, attributed to its anti-inflammatory and antioxidant actions. As a water-soluble polyphenolic compound, SalB is characterized by low toxicity and high activity [110]. Notably, SalB demonstrated the ability to traverse the blood–brain barrier and directly impact the central nervous system. In a study involving 12-month-old male mice with cognitive impairment, treatment with Salvianolic acid B (20 or 40 mg/kg/day) led to improved cognitive function and the inhibition of neuroinflammation [78].

Besides SalB, *Salvia miltiorrhiza* contains Tanshinone IIA (Tan IIA), a lipophilic terpenoid compound with a pharmacological activity that enhances learning and memory in AD mouse models. Tan IIA treatments have exhibited preventive effects on behavioral tests in the early stages of the disease in six-month-old males. In the conducted research, which involved the Novel Object Recognition test, it was noted that Tan IIA was able to mitigate short-term memory deterioration in mice. Additionally, during the Morris Water Maze test, Tan IIA reversed the impairments

and improved the swimming strategy with which the mice tracked down the platform, suggesting an attenuation of spatial memory deficits and an improvement in the learning capability of Tan IIA treatment [80].

3. Four Widely Explored Molecules with Neuroprotective Effects

As highlighted in the previous section, the potentialities of natural compounds are limitless, and an increasing number of manuscripts describing their neuroprotective effects have been published in recent years. In this section, we describe in more detail four examples of natural compounds that have been extensively studied as neuroprotective agents, having shown effects on multiple processes involved in neurodegeneration, and which are currently under investigation in clinical trials for their great potential (Figure 2).

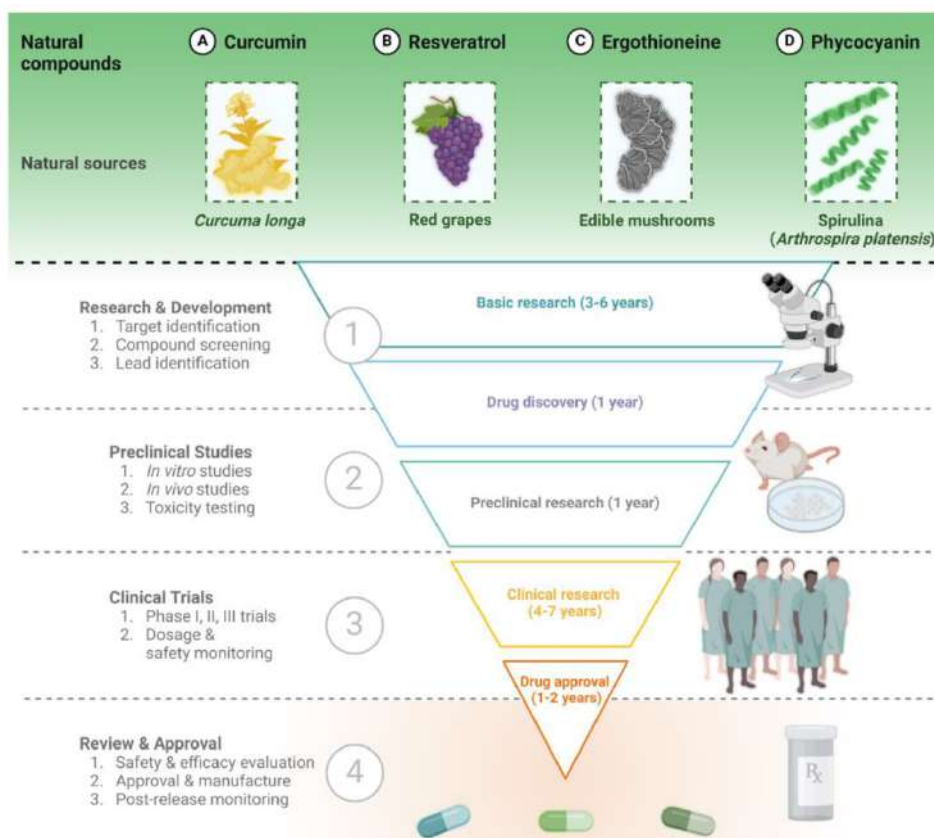


Figure 2. Exemplary natural compounds (curcumin, resveratrol, ergothioneine, and phycocyanin) and the general steps of bioactive substances from natural sources towards clinical approval.

3.1. Curcumin

Curcumin is a natural polyphenol derived from the rhizomes of the turmeric plant *Curcuma longa* [111], also called the “spice of life” for its history deeply interwoven with cultural, medicinal, and culinary traditions [112]. Curcumin is the primary component of turmeric, responsible for the plant’s intense yellow color [113] and

has emerged as a focal point in extensive scientific research for its potential therapeutic application. In particular, scientific evidence from in vitro studies, in vivo experiments, and clinical trials highlights the neuroprotective effect of curcumin, which modulates several pathways associated with neurodegenerative diseases [114].

Like several polyphenol compounds, curcumin acts as a scavenger for free radicals and enhances antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), thus protecting neurons from oxidative stress [115].

Curcumin also has anti-inflammatory effects by inhibiting diverse inflammatory markers, including pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6, thus mitigating the brain's inflammatory response [116]. Additionally, it downregulates the activity of pro-inflammatory enzymes, such as COX-2 and iNOS, which lead to reduced levels of prostaglandins and nitric oxide [117]. It also modulates the signaling pathways of NF- κ B, a key transcription factor involved in the regulation of inflammatory responses, along with Wnt5 (Wnt Family Member 5A) and JNK1 (c-Jun *N*-terminal kinases), which are both crucial for neuronal cell survival, inflammation, and apoptosis [118]. Interestingly, curcumin has been investigated for its ability to interfere with the misfolding and aggregation of proteins linked to

neurodegenerative disorders, including A β 1-42 and α -synuclein [119]. Based on experimental findings, curcumin has been shown to directly bind to misfolded proteins, altering their conformation and hindering their ability to aggregate into toxic forms [120,121]. In addition, it promotes the degradation of misfolding proteins through cellular protein degradation pathways, such as the ubiquitin–proteasome system and autophagy, helping to prevent the accumulation of toxic aggregates [122]. Indeed, many studies have demonstrated curcumin’s important role in inducing macroautophagy. Specifically, in PD models both in vitro and in vivo, curcumin activates autophagy through the inhibition of the Akt/mTOR pathway, contributing to the neuroprotective effects. Interestingly, it was found that curcumin supplementation substantially increases the LC3-II/LC3-I ratio and Beclin-1 and reduces the accumulation of α -synuclein [123,124]. In keeping with this, curcumin downregulates the PI3K/Akt/mTOR pathway and increases the expression of Beclin-1 and the LC3-II/LC3-I ratio, promoting the formation of autophagosomes and reducing the aggregation of A β in a mouse model of AD [125].

Several clinical trials have been performed to address the clinical potential of curcumin as a neuroprotective agent in humans. Initial placebo-controlled trials in subjects with mild-to-moderate AD did not show any evidence of efficacy [126,127], probably because of the limited bioavailability of curcumin used (despite the high dose of up to 4 g/day) or due to the short time of the trial (6 months). However, the

use of a solid lipid formulation of curcumin (Longvida, about 80 mg/day of curcumin) in healthy elderly subjects suggested an increase in attention, as well as in working memory and mood upon acute administration (1 h) and after 4 or 12 weeks of administration [128,129]. Interestingly, the administration of a lipidated form of curcumin (80 mg/day) also increased catalase activities and decreased A β concentration in the plasma after 4 weeks of treatment [130]. In a longer (18 months) double-blind placebo-controlled trial using a bioavailable form of curcumin (Theracurmin, 180 mg/day), curcumin treatment led to significant memory and an improvement in attention in non-demented adults. Strikingly, these results were accompanied by a reduction in FDDNP-PET (2-(1-{6-[(2-[fluorine-18]fluoroethyl)(methyl)amino]-2-naphthyl}-ethylidene)malononitrile positron emission tomography) binding, which can indicate the brain deposition of amyloid plaques and tau tangles, particularly in the amygdala and hypothalamus, which are crucial regions for mood and memory [131]. All these findings support the use of curcumin to preserve cognitive functions in humans, although high attention must be paid to its bioavailability (see also Section 4).

Nowadays, curcumin is widely used not only in the food industry but also in the pharmaceutical and cosmetic industries. The global Curcumin market is expected to undergo substantial growth from 2023 to 2030. The market size was USD 207 million in 2022 but is projected to increase to USD 265 million by 2028. India is at

the forefront of this market, contributing over 70%, followed by China and the USA. Strikingly, pharmaceutical-grade Curcumin represents the largest product segment, accounting for over 65% [132].

3.2. Resveratrol

Resveratrol is a natural polyphenol present in wine, red grapes, berries, chocolate, and peanuts, and it is attracting more and more attention due to its potential neuroprotective properties [133]. In fact, numerous studies suggest that resveratrol may safeguard the brain through various mechanisms. The compound's antioxidant properties are among the most studied activities of resveratrol, which is able to neutralize harmful free radicals that can damage brain cells. Resveratrol showed potent antioxidant activity in hippocampal neuronal cells by scavenging free radicals and shielding against NO toxicity [134]. Furthermore, it reduces quinone reductase 2 activity, which contributes to age-related metabolic stress and cognitive deficit in hippocampal neuronal cells treated with menadione [135], and also upregulated the endogenous antioxidant response in a mouse model exposed to ischemia [136]. Resveratrol also activates protein kinase AMPK and its upstream regulator, LKB1, in neuronal cell lines, primary neurons, and the brain [137]. In addition, it activates the Nrf2 pathway, regulating antioxidant enzyme expression in a number of in vitro models (PC12 cells, astrocytes, cerebellar granule neurons,

and oligodendroglial cells) as well as in in vivo models [138]. In primary microglia cultures exposed to LPS in rats, resveratrol decreases the production of prostaglandins, NO, TNF α [139], and COX-1 [140], showing strong anti-inflammatory properties, also reported by other studies [140,141]. Resveratrol activates SIRT1, leading to a reduction in the NF- κ B signaling pathway in glial cells exposed to A β 1-42-induced toxicity. This effect is particularly relevant since the down-regulation of SIRT1 expression abolishes the protective action of resveratrol in neuroblastoma cell lines exposed to the neurotoxic compound 6-hydroxydopamine (6-OHDA) [142].

Interestingly, resveratrol binds to the peptide A β 1-42 in vitro, and by changing the conformation of oligomers, it probably attenuates their cytotoxicity [143].

The neuroprotective effects of resveratrol have also been tested in human subjects in randomized, double-blind, placebo-controlled clinical trials. In healthy young men, a single oral dose increased cerebral blood flow in a dose-dependent manner, but no effect on cognitive function was observed [144]. However, another trial on young adults receiving resveratrol for 28 days demonstrated cognitive benefits related to working memory tasks [145]. Resveratrol (150–200 mg/day) has also been tested in elderly subjects. Initial evidence suggested improved mood and cognitive performances in post-menopausal women after 14 weeks of

administration [146] and improved retention of words over a 30 min delay after 6 months of administration [147]. However, the following study failed to show any effect of resveratrol on verbal memory performance [148]. In a longer study (52 weeks) on AD patients, a higher dose of resveratrol (up to 2 g/day) decreased the level of metalloproteinase MMP9, which is involved in maintaining the integrity of the BBB, in the cerebrospinal fluid, and slowed down progressive cognitive decline [149], strongly supporting the role of resveratrol as a neuroprotective agent also in AD patients.

Despite challenges such as low bioavailability, resveratrol tolerability, and its safety profile position it as a promising candidate for long-term treatment against cognitive decline in humans [150], and advanced pharmaceutical technologies aim to improve resveratrol's bioavailability [151].

The increasing demand for resveratrol is attributed to its widespread use across various sectors, including cosmetics, dietary supplements, medications, and personal care items. Projections for the global resveratrol market indicate substantial growth between 2023 and 2030. As of 2022, the market is steadily expanding, and with significant strategies adopted by key players, it is expected to surpass the anticipated growth trajectory. As of 2021, the global resveratrol market size was estimated to be USD 165.73 million. Forecasts suggest a Compound Annual

Growth Rate (CAGR) of 8.52%, leading to a market value of USD 270.69 million by 2027. Leading the resveratrol market are North America, Mexico, and South America, Asiatic-Pacific areas, the Middle East, Africa, and Europe [152].

3.3. Ergothioneine

Ergothioneine (EGT) is a non-proteinogenic, sulfur-containing amino acid characterized by good stability and solubility. Its name comes from the ergot fungus *Claviceps purpurea*, from which it was first isolated in 1909. It is an essential amino acid that can be introduced into the diet and is particularly abundant in mushrooms, although it can also be found in certain meat products (such as chicken liver) and grains and at a much lower concentration in other food sources, like eggs, rice, garlic, and beans [153]. It is absorbed in the gut through the transporter OCTN1 (the organic cation transporter novel, type 1) [154] and is also highly distributed in the brain. Importantly, in the brain, OCTN1 is expressed in neurons, dendrites, and microglia but not in astrocytes [155,156]. Strikingly, a decline in EGT has been observed in patients with dementia, including AD [157], suggesting that EGT reduction may be associated with a higher risk of brain diseases. Thus, recently, EGT has gained great attention as an anti-aging compound, and it is often called the “longevity vitamin” [158–160]. The role of EGT as an antioxidant agent has been widely investigated; it acts both as a direct reactive oxygen species (ROS) scavenger

as well as through the activation of the antioxidant cellular processes mediated by Nrf2 [153]. It was also observed that EGT reduces A β plaques in a 5XFAD mouse model of AD [161].

Despite these data, the effect of EGT administration on human subjects was poorly explored. In 2017, EGT uptake and clearance were measured, but very limited effects on biomarkers of oxidative damage were observed, probably due to the short time of administration (7 days) [162]. Some other clinical trials are ongoing (see www.clinicaltrials.gov, accessed on 15/02/2024), but at the moment, no clear results on humans have been reported, and further studies are required to clarify EGT health benefits in humans.

Yet, EGT is widely commercialized as a diet supplement for its antioxidant and anti-aging properties, especially in the United States, China, and Europe. The price of EGT is about USD 700/kg; the global market of EGT was estimated to be USD 15 million in 2020 and is expanding more and more; it is expected to reach USD 125 million in 2027 [163], thus representing a huge developing market. For these reasons, new strategies for EGT extraction from mushrooms, but also for production from engineered strains and through chemical synthesis, are being developed [163].

3.4. Phycocyanin and Spirulina Extract

Phycocyanin (PC) is a pigment–protein complex from the light-harvesting phycobiliprotein family, which also includes Allophycocyanin, Phycoerythrin, and Phycoerythrocyanin [164,165]. Phycobiliproteins aggregate to form clusters that adhere to the membrane, called Phycobilisomes. PCs are found in cyanobacteria as an accessory pigment to chlorophyll and with a molecular weight of around 30 KDa. PCs are composed of two subunits, α and β , combined together [166–168]. These proteins have a light blue color, absorbing orange, and red light near 620 nm and emitting fluorescence at around 650 nm. The light absorption and the fluorescence emission are type-dependent (e.g., Allophycocyanin absorbs and emits at longer wavelengths than C-Phycocyanin or R-Phycocyanin). Approximately 20–45% of the protein fraction from the cyanobacterium *Arthrospira platensis* (Spirulina) is PCs [169,170]. The utilization of an appropriate extraction method is of high importance to obtain a pure and stable PC since the release of this pigment depends on the cell membrane disintegration [171].

Several studies describe PC's beneficial characteristics and antioxidant properties in vitro and in vivo [165,172]. In particular, PCs are able to scavenge reactive oxygen and nitrogen species to counteract lipid peroxidation and to inhibit enzymes, such as NADPH oxidase and COX-2 [173]. In animal models of multiple sclerosis and

ischemic stroke, these compounds induce remyelination, as demonstrated by electron microscopy, also showing a reduction in pro-inflammatory cytokines and induction of immune suppressive genes [173]. Several studies focus on the effects of *Spirulina* in the brain, highlighting its beneficial anti-inflammatory and antioxidant effects acting on glial cell activation and, in the prevention, and/or progression of neurodegenerative diseases (in particular, AD and PD) [174].

Spirulina is not only rich in PC but is a rich source of other bioactive compounds, including proteins, polysaccharides, polyunsaturated fatty acids, vitamins, and carotenoids [175–177]. Different studies have demonstrated the neuroprotective role of *Spirulina* on the development of the neural system, senility, and a number of pathological conditions, including neurological and neurodegenerative diseases [178–181].

Extracts of *Spirulina maxima* (70% ethanol) were studied in a 12-week double-blind placebo-controlled clinical trial in the elderly, showing a significant improvement in visual learning and visual working memory [182], although the exact components of this extract were not specified by the authors.

Nowadays, cyanobacteria- and microalgae-based bioactive compounds are generally commercially available. Their market demand will continue to grow due to the increasing demand for natural products in the food industry [177]. The global

market regarding microalgal-based proteins is foreseen to reach USD 0.84 billion by 2023 [183]. In particular, C-Phycocyanin is expected to reach a market value of USD 409.8 million within the 2030s [183]. Spirulina, as a protein-rich cyanobacterium (60–70% (w/w)) and an important source for C-Phycocyanin, has also gained attention as a promising feedstock for large-scale production. It is cultivated in different countries (e.g., Germany, Portugal, the USA, and China) and mainly commercialized as dried biomass. The exclusive production of high-value proteins and C-Phycocyanin is still emerging.

4. Delivery Strategies

We have described how a plethora of natural compounds exhibit beneficial activities [184]; nevertheless, the translation of the huge number of preclinical studies to clinical trials is, of course, very challenging. One of the biggest issues is represented by the fact that most bioactive substances are environmentally sensitive, with low solubility, chemical instability, and low bioavailability, as well as an inappropriate molecular size [185]. To overcome these problems, diverse strategies have been employed to improve the bioavailability of natural products [186]. Recently, there has been an increasing interest in nanotechnology, which encompasses the generation and utilization of materials within the nanometer scale [187] to deliver natural compounds, leading to numerous advantages.

Nanoparticles are favored for their abundance, biocompatibility, bioavailability, biodegradability, increased chemical and physical stability, as well as for extended shelf-life of nanoparticles [188]. Nanoencapsulation is a new frontier in nanoscience in the food, pharmaceutical, and cosmeceutical industries. This technique consists of enclosing a bioactive compound or a drug in liquid, solid, or gaseous states within a matrix or inert material, commonly using a polymer matrix or 'wall material'. This encapsulation aids in enhancing protection, increasing stability, solubility, and availability through the cells [189]. The choice of the preparation method for an agent's encapsulation depends mostly on its properties, such as the state of aggregation, sensitivity, and especially the size of molecules. In the last few years, numerous methods for the encapsulation of natural products, such as extract or pure natural compounds, have been developed.

Some of the compounds described in the previous section have been nano-formulated to improve their functional properties. Among them, curcumin (described in Section 3.1), due to its low solubility, biochemical degradation, and poor bioavailability, has been encapsulated in nanoemulsion, which has been described as an excellent carrier of lipophilic curcumin [190,191]. In nanoemulsion delivery systems, curcumin molecules are typically located within the hydrophobic interior of oil droplets. In this way, curcumin is protected from active substances within the aqueous phase that would typically promote its chemical degradation.

In addition, this formulation leads to enhanced intestinal absorption of curcumin, which is related to the inhibition of curcumin metabolism by nanoparticles based on hydrogen bonding-driven self-assembly. The enhanced cellular uptake of curcumin by encapsulation also provides pH-triggered intestinal targeted release properties and strong antioxidant capacity [192].

Curcumin's stability is affected by the composition and structure of delivery systems. Numerous studies have demonstrated that the stability of Curcumin is influenced by the nature and concentrations of emulsifiers. Kharat and colleagues examined the impact of emulsifier type (sodium caseinate, Tween20, quillaja saponin, gum arabic) and concentration on the stability of curcumin-loaded nanoemulsions. They found that the degradation of Curcumin in saponin-stabilized nanoemulsions accelerated curcumin degradation (after 15 days) due to its ability to promote peroxidation reactions [193]. Similarly, Artiga-Artigas et al. assessed the impact of three molecularly different surfactants, lecithin, Tween20, and sucrose monopalmitate, and their concentration on the stability of Curcumin-loaded nanoemulsions and evaluated their antioxidant capacity. They found that Curcumin-loaded nanoemulsions with lecithin showed long-term stability and exhibited higher antioxidant capacity compared to those made with the other surfactants. This enhanced performance is attributed to lecithin's phosphate ions, which can form hydrogen bonds with phenolic hydroxyls of curcumin [194].

Many studies have shown that the bioavailability of curcumin within the gastrointestinal tract (GIT) can be greatly increased by emulsion-based systems compared to crystalline curcumin dispersed within water [195,196]. Researchers have subjected curcumin formulations to a simulated GIT model consisting of the mouth, stomach, and small intestine phases. They have shown that the bioaccessibility value of curcumin encapsulated (74–79%) is significantly higher than that of the curcumin solution (10%). In addition, it suggests that encapsulating curcumin within small lipid particles may be advantageous for improving its absorption from the GIT [197].

With a similar approach, polysaccharides from *G. lucidum* were also encapsulated in a nanoparticle to achieve better health-promoting effects. However, *G. lucidum* polysaccharides have some challenging characteristics, such as low solubility and low yield. Accordingly, to surmount these problems, a polysaccharide-based nanoparticulate co-delivery system was developed to obtain excellent functional properties and processing benefits [198]. Correspondingly, research demonstrated that *G. lucidum* polysaccharides in nanoparticles not only perform their original functions but also contribute to preventing the rapid metabolism of other active components. This leads to an improved loading rate and encapsulation efficiency, thereby enhancing the bioactivity and pharmacological effects of polysaccharide-based nano-systems [199].

As mentioned above, *Spirulina* provides a high protein content and a full range of essential amino acids, which is why it has become of special interest as a good protein source. Additionally, it exhibits antioxidant and anti-inflammatory effects on the human body, as presented in Section 3.4. Nevertheless, it brings some challenging characteristics that limit its use in food products, such as the strong, dark, green-blue color, derived from pigments of chlorophyll A, phycocyanin, and carotenes. Therefore, nanotechnology, such as microencapsulation by spray drying and enzymatic treatment, has become of interest to overcome *Spirulina*'s restricted use [200]. Maag et al. applied microencapsulation and additional enzymatic hydrolysis to the *Spirulina* extract in order to reduce the intense green color, optimize thermal stability, increase bioavailability, enhance protein powder solubility, and moreover, improve digestibility. This method consists of entrapping sensitive nutrients within a wall material maltodextrin and gum arabic in order to protect them from physico-chemical influences and external factors, such as heat and oxidative processes caused by air and light. They have shown that enzymatic treatment or encapsulation enhances the protein solubility between 10 and 30% compared with the untreated samples. Furthermore, a considerable reduction in green color intensity was observed in the encapsulated samples [201].

When considering neuroprotective agents, the BBB is a major challenge against obtaining the optimum effect from natural compounds [202]. Also, in this case,

nanotechnologies, by means of various drug delivery systems, propose good strategies owing to their unique ability to target the BBB and to assist in the better delivery of natural compounds to the brain than natural compounds on their own. In addition, with the help of the surface modification of nanocarriers to take advantage of receptors overexpressed at the BBB, nanocarriers have been shown to efficiently overcome the BBB and deliver the selected compound to specific sites within the brain. To exemplify, the surface modification of curcumin nanocarriers incorporates targeting ligands to bind receptors and transporters at the BBB, which enables them to bypass receptor-mediated endocytosis, thus improving central nervous system selectivity and permeability [203]. Another study reported that curcumin-encapsulated poly(lactide-co-glycolide) nanoparticles (cur-NPs) induced the proliferation of neural stem cells and their differentiation to neurons in vitro and in the hippocampus and subventricular zone (SVZ) of adult rats. The nanoparticles increased curcumin levels in the rat brain compared to similar doses of bulk curcumin, and the presence of cur-NPs in the hippocampus and SVZ confirmed the ability of these nanoparticles to cross the BBB and induce neurogenesis [204].

Bidirectional communication between the human gut microbiota and the brain, commonly referred to as the microbiota–gut–brain axis or gut–brain axis, exerts a significant influence on behavior and brain function. The microbiota–gut–brain axis

represents an intricate communication network that connects the gastrointestinal tract with the brain and facilitates bidirectional interactions through neural, endocrine, and immune pathways [205]. The investigation into dietary impact on the gut microbiota demonstrates a major impact on the brain, especially on the development and function of microglia and the BBB [206]. The gut microbiota produces various neurotransmitters, such as GABA, dopamine, and serotonin; amino acids as well as their derivatives, such as tryptophan and tyramine; and microbial metabolites, like short-chain fatty acids and aryl hydrocarbon receptor (AhR) ligands. These compounds can influence the local gut physiology and can circulate through the blood to interact with the host immune system, which directly signals the brain and may affect microglial activation [207]. Notably, early microbiome changes were found in patients with preclinical AD and PD, and compelling evidence shows that the altered gut microbiome can drive neurodegenerative disease pathogenesis [205]. Strikingly, changes to the gut microbiome can cause the misfolding and abnormal aggregation of α synuclein, which can be transported from the periphery to the central nervous system (CNS) via the vagus nerve [208].

This insight into the microbiota–gut–brain axis makes it a promising target for neuroprotective strategies. In fact, adjusting the gut microbiota through dietary changes could potentially influence microglial activity and mitigate the progression

of neurodegenerative diseases [205]. Therefore, the bioavailability of natural compound nanoparticles within the gastrointestinal tract can affect the gut microbiota, thereby impacting brain-related diseases. For instance, Qu and colleagues developed Honokiol nanoparticles, a plant bioactive compound, which demonstrated the improved bioavailability and bioactivity of Honokiol. It has been shown that this nanoparticle suppresses the neuroinflammatory response, regulating the gut microbiota, reducing β -amyloidosis, and, consequently, exhibiting neuroprotective properties in a mouse model of AD [209].

To summarize, the use of nanoparticles is a promising approach to deliver natural compounds due to their abundance, biocompatibility, bioavailability, biodegradability, and increased chemical, physical, and shelf-life. The bioavailability of natural compound nanoparticles affects the gut–brain connection, thereby preventing neurodegenerative diseases. Nevertheless, even if it is successful in various in vitro and in vivo BBB models, further studies are needed to investigate the efficacy of these approaches in humans. In addition, despite the success of this technique, it is imperative to evaluate more types of nanomaterials and identify additional potential health effects.

5. How a Better Understanding of Natural Molecules with Neuroprotective Effects Could Improve Their Utilization in the Next Decades

A better understanding of natural molecules with neuroprotective effects could lead to the development of more effective strategies to prevent neurodegenerative disorders [210]. The free radical theory of aging postulated by Harman in 1956 points out that excessive production and accumulation of ROS causes a subsequently altered cellular integrity that eventually leads to severe and irreversible damage [210,211]. Therefore, oxidative stress plays a crucial role in neuronal damage associated with aging as well as neurodegenerative disorders [210,212,213]. Epidemiological and biochemical studies have identified food components as promising agents for neuroprotection [210]. Neuroprotection includes mechanisms such as the activation of specific receptors, changes in enzymatic neuronal activity, and the synthesis and secretion of different bioactive molecules. All these mechanisms are focused on preventing neuronal damage and alleviating the consequences of massive cell loss. Some neuropathological disorders selectively affect particular neuronal populations, but their neurochemical and anatomical properties in order to design effective therapies should be known. Although the design of such treatments could be specific to neuronal groups sensitive to damage, the effect would have an impact on the whole nervous system [210].

The development of new strategies, like nanocarriers (see Section 4), that help to promote the access of neuroprotective molecules to the brain is needed to provide more effective therapies for disorders of the central nervous system (CNS) [210]. In order to trace the success of these nanoplateforms on the release of the bioactive cargo in the CNS and determine the concentration at trace levels of target biomolecules, the use of analytical chemistry and concrete separation instrumental techniques are essential. Currently, these techniques are used for the determination and identification of natural neuroprotective molecules in complex matrixes at different concentration levels [210].

An alternative, not a mutually exclusive approach, is represented by the chemical modification of natural compounds to mainly increase their stability and/or solubility. Researchers have chemically modified several natural compounds with neuroprotective or anti-aging activity [213–215]. Fucoidan is a class of sulfated polysaccharides rich in fucose, and fucoidans from different species have different structures [216]. As investigated via electron microscopic images and signaling pathway studies, this compound, despite the fact that does not inhibit the aggregation of A β 1-42, can block caspase-3 and caspase-9 and then apoptosis [217]. Naive Bayesian and recursive partitioning algorithms were applied by Fang et al., for example, to construct classifiers to predict active molecules against 25 key targets toward AD using the multitarget-quantitative structure–activity

relationships (mt-QSAR) method [218]. In this context, fucoidan has significant structural diversity: its structure–activity relationship could be investigated to further rational design and modification, achieving efficient clinical development [214].

6. Conclusions

In this comprehensive review, we delve into the fascinating properties of natural products sourced from mushrooms, plants, microalgae, and cyanobacteria. These bioactive compounds hold promise in the prevention and treatment of neurodegenerative diseases. Ergothioneine, curcumin, resveratrol, and phycocyanin emerge as powerful allies in the fight against neurodegeneration. Their origins span various natural niches, potentially sharing a common purpose: safeguarding neuronal health. Rather than targeting a single pathway, these compounds adopt multifaceted strategies, and by addressing multiple processes simultaneously, they can mitigate premature cell death and preserve neuronal function.

While their potential is undeniable, effective delivery to the brain remains a hurdle. Strategies to enhance bioavailability and overcome the blood–brain barrier are critical for translating these discoveries into clinical success. Collaborations between researchers, clinicians, and pharmaceutical industries are essential for the

further development of this scientific field. Innovative drug delivery systems hold promise in optimizing the therapeutic impact of these natural neuroprotective agents. Moreover, the gut–brain axis represents a promising target for the development of new therapies for neurodegenerative and neurodevelopmental disorders. Exploiting natural compounds with neuroprotective properties could provide a novel approach to enhance this axis and promote brain health. More research is needed to fully understand the complex interactions between the gut microbiota, the gut–brain axis, and natural neuroprotective niches.

In summary, this manuscript underscores the need to harness the potential of natural compounds for neuroprotection. By bridging the gap between basic research and practical application, the way can be paved for novel treatments, improving the lives of individuals affected by neurodegenerative diseases.

Author Contributions: Conceptualization, P.C. and F.T.; writing—original draft preparation H.M., A.L., G.D.B., and F.T.; writing—review and editing, P.C. and F.T. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.4—call for tender No. 3138 of 16 December 2021, rectified by Decree No.3175 of 18 December 2021 of the Italian Ministry of University and Research funded by the European Union—

NextGenerationEU; Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022. Adopted by the Italian Ministry of University and Research, CUP, H43C22000530001, Spoke 6, Project title “National Biodiversity Future Center—NBFC”. This work was also supported by the project “ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON Foods” (CUP:H43C22000820001) of the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.3—Research funded by the European Union—NextGenerationEU.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The authors declare no conflicts of interest.

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Chapter 2

To address the critical challenge of neurodegenerative disorders such as PD, here we have adopted an innovative approach grounded in the principles of the circular economy and bioeconomy: the valorization of agro-industrial waste matrices. This strategy not only provides environmental and economic benefits, but also exploits underutilized by-products as sustainable sources of bioactive compounds with potential neuroprotective effects. The two articles presented in this chapter collectively present our integrated research aimed at exploring and characterizing the neuroprotective potential of two distinct agro-industrial waste streams against α -syn pathology using advanced cellular models of PD.

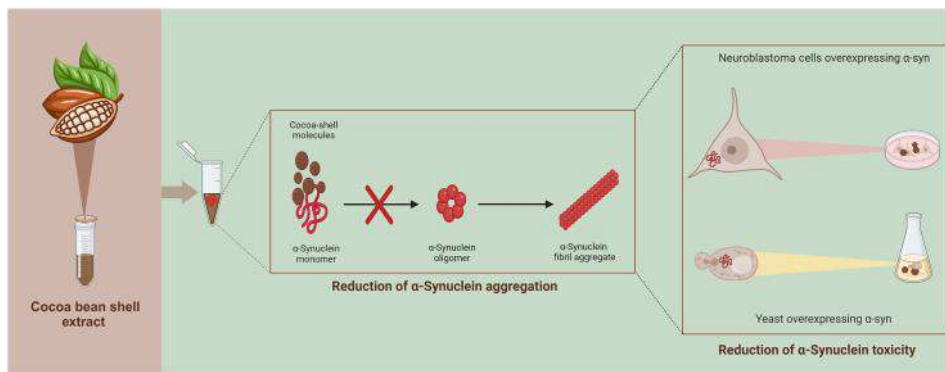
In the first study (chapter 3A), we examined a cocoa bean shell extract (CBSE), a by-product generated in large quantities during cocoa bean roasting. The extract, obtained through a green pressurized liquid extraction (PLE) method, was chemically characterized and found to contain amino acids, organic acids and methylxanthines (caffeine and theobromine). We demonstrated that CBSE supplementation significantly enhanced the longevity of α -syn-expressing yeast cells while reducing ROS levels and intracellular protein aggresomes. Furthermore, in SH-SY5Y neuroblastoma cells expressing α -syn, CBSE reduced the formation of toxic α -syn oligomers and aggresomes. Surface Plasmon Resonance (SPR) analysis

confirmed that CBSE directly binds α -syn in a concentration-dependent manner, inhibiting toxic amyloid aggregation. CBSE also modulated cellular stress responses by stimulating autophagy in yeast and activating the AMPK signaling pathway in neuroblastoma cells, suggesting a multi-mechanistic neuroprotective role.

In the second study (chapter 3B), we investigated the potential of *Glycyrrhiza glabra* leaves (GGL), the vegetative biomass of a highly valued Calabrian licorice variety with Protected Designation of Origin (PDO) status, which is typically discarded during root harvesting. We demonstrated that GGL extracts are rich in specialized metabolites, primarily prenylated flavanones (such as pinocembrin, licoflavanone and glabranin) and prenylated dihydrostilbenes, and that their content and overall antioxidant capacity were strongly influenced by cultivation area and harvest time. Using a *Saccharomyces cerevisiae* yeast model overexpressing human α -syn, we showed that GGL extracts possess significant antioxidant and anti-aging properties, effectively attenuating α -syn toxicity, reducing intracellular reactive oxygen species (ROS) and notably prolonging chronological lifespan (CLS) in yeast cells. These findings identified GGL as a promising, untapped resource for nutraceutical and pharmaceutical applications.

In conclusion, the results presented in these two articles highlight the immense potential of waste matrices, such as licorice leaves and cocoa bean shells, as

sustainable and bio-accessible sources of neuroprotective compounds. We have established that extracts derived from these undervalued resources effectively mitigate key pathological features of synucleinopathies, including α -syn aggregation, oxidative stress and cellular senescence. We believe that these discoveries provide a solid theoretical and technical foundation for the development of novel functional ingredients and nutraceuticals aimed at preventing age-related neurodegenerative disorders.



Graphical abstract

Targeting protein aggregation using a cocoa-bean shell extract to reduce α -synuclein toxicity in models of Parkinson's disease

Tripodi F..et al., 2024.

Abstract

Neurodegenerative diseases are among the major challenges in modern medicine, due to the progressive aging of the world population. Among these, Parkinson's disease (PD) affects 10 million people worldwide and is associated with the aggregation of the presynaptic protein α -synuclein (α -syn). Here we use two different PD models, yeast cells and neuroblastoma cells overexpressing α -syn, to investigate the protective effect of an extract from the cocoa shell, which is a by-product of the roasting process of cocoa beans. The LC-ESI-qTOF-MS and NMR analyses allow the identification of amino acids (including the essential ones), organic acids, lactate and glycerol, confirming also the presence of the two methylxanthines, namely caffeine and theobromine. The present study demonstrates that the supplementation with the cocoa bean shell extract (CBSE) strongly improves the longevity of yeast cells expressing α -syn, reducing the level of reactive oxygen species, activating autophagy and reducing the intracellular

protein aggresomes. These anti-aggregation properties are confirmed also in neuroblastoma cells, where CBSE treatment leads to activation of AMPK kinase and to a significant reduction of toxic α -syn oligomers. Results obtained by surface plasmon resonance (SPR) assay highlights that CBSE binds α -syn protein in a concentration-dependent manner, supporting its inhibitory role on the amyloid aggregation of α -syn. These findings suggest that the supplementation with CBSE in the form of nutraceuticals may represent a promising way to prevent neurodegenerative diseases associated with α -syn aggregation.

Keywords: *Saccharomyces cerevisiae*, human α -synuclein, Parkinson's disease (PD), cocoa, food waste valorization

1. Introduction

The food industry produces a high amount of waste during food processing and production. Under a circular economy perspective, the valorization of these by-products and waste is convenient not only from an economic and environmental point of view, but also because they are rich in active compounds with potentially useful bioactivities [1, 2].

Cocoa beans (*Theobroma cacao* L.) are widely used in food production, but also for pharmaceutical and cosmetic purposes [3]. One of the main by-product of cocoa processing is the cocoa bean shell, which is produced in large quantities during the roasting process [2, 4]. It has been estimated that global cocoa bean production in the 2015/2016 harvest reached approximately 3972 thousand tons, with the shell making up to 20% of the bean [5, 6]. This represents roughly 600 thousand tons, most of which are discarded as waste and remain under-utilised, with limited applications as boiler fuel, animal feed, or fertilizer [5]. However, in recent years, cocoa shells have gained attention as a rich source of phenolic compounds, including caffeine and theobromine [5, 7–10]. These two compounds, belonging to the methylxanthine class, are well-known for their antioxidant and anti-inflammatory properties [11–13], which contribute to mitigating oxidative stress, a key factor in neurodegenerative diseases. While the spotlight is often on cocoa bean, recent studies suggest that cocoa shells, given their abundant availability and cost-effectiveness, can offer a significant potential as a sustainable resource for developing therapeutic agents [14–16]. Besides, new insights into the toxicological safety of two cocoa shell matrices has been also provided, opening opportunities for their use as functional food and nutraceutical products [10]. Recently, a new environmentally friendly and automated pressurised liquid extraction method has

been developed and optimised to selectively recover theobromine and caffeine from cocoa shell by-products [8].

Parkinson's disease (PD), characterised by aberrant aggregates of the presynaptic protein α -synuclein (α -syn), is the second most common neurodegenerative disease [17–19]. Many *in vitro* and *in vivo* models mimicking α -syn pathology have been used over the years [20]; among them, *Saccharomyces cerevisiae* has been extensively employed as a model of synucleinopathies [21–24]. This unicellular eukaryote is a valuable tool in research due to its small size, short generation time, non-pathogenic nature, and ease of genetic manipulation [25]. The deep conservation of cellular mechanisms, such as DNA replication, cell division, and protein folding, from yeast to pluricellular eukaryotes, underscores its relevance for human disease studies. The most commonly used yeast model for Parkinson's disease research involves the heterologous expression of human α -synuclein, which induces toxicity associated with aggregate formation, leading to vesicular trafficking impairment, increased oxidative stress and mitochondrial dysfunction, reduced lifespan, disturbed calcium signaling and altered autophagy [22, 23, 26–28]. As in more complex systems, the autophagic pathway is the major responsible for the clearance of oligomers and toxic aggregates, which cannot be degraded by the proteasome [29].

Although *S. cerevisiae* provides an excellent platform for preliminary studies, pluricellular eukaryotic models such as *Caenorhabditis elegans* [30, 31], *Drosophila melanogaster* [32, 33] and mice [34] are also extensively employed. Interestingly, a SH-SY5Y cell line overexpressing α -synuclein through a doxycycline-inducible promoter, is a valuable model for studying the molecular mechanisms underlying neuronal degeneration [35]. This eukaryotic system provides a robust platform for the *in vitro* screening of neuroprotective compounds to evaluate potential pharmaceutical compounds.

Here we show that an extract from cocoa bean shells increases lifespan and reduces reactive oxygen species (ROS) levels in a yeast model of synucleinopathy. Its anti-aging properties are associated with a stimulation of autophagy during the first two days of the stationary phase and a strong anti-aggregation feature both *in vivo* and *in vitro*. Consistently, our data also highlight a significant reduction of toxic α -syn oligomers in neuroblastoma cells expressing α -syn, supporting the potential use of cocoa bean shell extract as a preventive agent against aggregation and its pathogenic effects.

2. Materials and methods

2.1 Chemical reagents

All chemicals were purchased from Merck unless otherwise stated. MS-grade solvents used for UPLC analysis, acetonitrile, water, and formic acid were provided by Romil. Reagents for SPR experiments were purchased from Cytiva. Cell culture media and supplements were provided by Euroclone, Biolog plates and reagents were provided by Rigel Process and Lab.

2.2 Cocoa bean shell extract (CBSE) preparation

Cocoa bean shell (Trinitario variety) was kindly provided by a cocoa processor after being roasted at 225°C for approximately 20 min. The cocoa bean shell extract (CBSE) was obtained by Pressurised Liquid Extraction (PLE) using Dionex ASE350 (Dionex Sunnyvale, CA) as previously reported [8]. Briefly, 1 g of dry matrix, previously blended and sieved (300-600 µm) to obtain homogeneous samples, was extracted in 5 mL stainless steel cells using 15% EtOH solution, 90°C temperature, 5 cycles and a static time of 6 min at a pressure of 100 bar. After the extraction the ethanol was removed using a rotary evaporator (G3, heiVAP core, Heidolph Germany) with the bath temperature set at 40°C and the extract was freeze-dried (ALPHA 1-2 LSC BASIC, Christ Germany) with a yield of 20.68 ± 1.25 % of dry matrix.

2.3 Chemical characterization and quantitative analysis of caffeine and theobromine

The CBSE was analysed by Synapt G2-Si QToF instrument (equipped with a ZsprayTM ESI-probe) (Waters) coupled with an Acquity UPLC I-Class chromatography system (Waters). The UPLC analyses were carried out by a ACQUITY UPLC HSS T3 column (100×2.1 mm, 1.8 µm, Waters) fitted with a VanGuard cartridge (Waters) maintained at a fixed temperature of 35°C. The products were separated using a linear gradient elution program, which consisted of water (A) and acetonitrile (B) (both with 0.1% formic acid) varying from 5 to 95% B (0-20 min). A flow rate of 0.4 mL/min and an injection volume of 4 µL were adopted. The PDA acquisition wavelength range was 190–400 nm. For mass spectrometry analyses, both negative and positive ionization modes were applied. The ESI-modes were acquired in the range of 50-1200 m/z with a fixed source temperature of 120°C and a desolvation temperature of 150°C. A desolvation gas flow of 600 L h⁻¹ was employed. The capillary voltage was 3 kV (positive ionization mode) and -2 kV (negative ionization mode). The instrument was controlled by a MassLynxTM v4.2 software (Waters). All MS acquisitions were performed the same day, with blank control between injections.

For quantitative analysis, the UPLC system was coupled with a UV detector acquiring data 283 nm. An external standard calibration method was used to quantify theobromine and caffeine in the extract. Standard solutions of theobromine and caffeine, each at a concentration of 1 mg/mL, were properly diluted with H₂O to create six-level calibration curves ranging from 1 to 200 µg/mL. The linearity of each calibration curve was verified using analysis of variance (ANOVA), and the linear model was found appropriate for the concentrations used and each level was acquired in triplicate. The instrument was controlled by a MassLynx™ v4.2 software (Waters). All MS acquisitions were performed the same day, with blank control between injections.

In addition, to detect analytes not visible with mass spectrometry, an NMR analysis was also conducted. Extract aliquots (4.3-4.7 mg) were resuspended in 1 mL deuterated water, containing 50 mM phosphate buffer pH7.4 and 0.4 mM TSP (internal standard). 700 µL of this solution were transferred to NMR tubes and analysed. NMR analyses were performed with a JEOL JNM-ECZ 600R spectrometer (resonance ¹H 600.17 MHz) equipped with a 5 mm FG/RO DIGITAL AUTOTUNE probe. Monodimensional experiments were carried out in the following conditions: 128 scans, 4 dummy scans, presaturation of the residual water signal, impulse at 90°C of 8.3 µs, 64K data points, time of acquisition 7.7 s. For metabolite identification, literature data referred to NMR studies on cocoa samples [36, 37]

were used. To quantify the identified metabolites in the aqueous solution, the integrals of the selected ^1H resonances were measured with respect to TSP as previously described [38]. Three replicates were made, and the results were expressed as $\mu\text{g}/\text{mg}$ of extract $\pm$ SD.

2.4 Proteomic characterization

Proteins present in the CBSE were concentrated and separated from small molecules using molecular cut-off filtration (Amicon 10000 Da MWCO, Merck) with two washes with water. After protein precipitation with 80% Acetonitrile, pellets were resuspended in 25 mmol/L NH_4HCO_3 containing 0.1% RapiGest (Waters Corporation) and sonicated by immersion for 10 sec before digestion, as previously described [39]. After 15 min of incubation at 80 °C, proteins were reduced with 5 mmol/L DTT at 60°C for 15 min, and carbamidomethylated with 10 mmol/L iodoacetamide for 30 min at room temperature in darkness. Digestion was performed with sequencing-grade trypsin (Promega) (1 μg every 25 μg of proteins) overnight at 37°C. After digestion, 2% TFA was added to hydrolyze RapiGest and inactivate trypsin.

Tryptic peptides were used for label-free mass spectrometry analysis, LC-MS^E, which was performed on a hybrid quadrupole time-of-flight mass spectrometer (Xevo G2-XS, Waters Corporation), coupled with a UPLC H-class system and

equipped with an ESI source (Waters Corporation). Samples were injected into an analytical column ACQUITY Premier HSS T3 C18, 100 Å, 1.8 µm, 2.1mm x 150 mm equipped with a vanguard FIT cartridge (Waters Corporation), for elution at a flow rate of 200 µl/min for 3 min at 2% mobile phase B before increasing the organic solvent B concentration from 2 to 50% over 90 min, using 0.1% v/v formic acid in water as reversed phase solvent A, and 0.1% v/v formic acid in acetonitrile as reversed phase solvent B. All of the analyses were performed in duplicate and analysed by LC-MS^E as previously detailed [39]. In particular, in the low-energy MS mode, the data were collected with Masslynx software at a constant collision energy of 6 eV, while in the high-energy mode, fragmentation was achieved by applying a ramp from 15 to 35 eV. Scan time of 0.1 sec, capillary voltage of 1 kV, cone voltage of 40 V, source temperature 120°C, desolvation temperature of 600°C with desolvation gas at 800 L/h were applied to acquire spectra in the range 50-1990 m/z. The time-of-flight analyzer was externally calibrated using Sodium formate from m/z 50 to 1990, and data were post-acquisition lock mass corrected using the monoisotopic mass of the doubly charged precursor of [Glu1]-fibrinopeptide B (m/z 785.8426) delivered to the mass spectrometer at 100 fmol/µL. The reference sprayer was sampled every 30 s. The radio frequency (RF) applied to the quadrupole mass analyzer was adjusted in such a way that ions from m/z 300 to 2,000 were efficiently transmitted, thus ensuring that any ion with a

mass of less than m/z 300 only arose from dissociations in the collision cell. Peak detection and protein identification were performed with PLGS software (v 3.0.3) using a Uniprot *Theobroma cocoa* sequence database (v2024_04, 40947 unreviewed entries, 16 reviewed entries) and NCBI database (167551 entries). The following search criteria were used for protein identification: the default search parameters included the “automatic” setting for mass accuracy (approximately 10 ppm for precursor ions and 25 ppm for product ions); a minimum of one peptide match per protein, a minimum of two consecutive product ion matches per peptide, and a minimum of five total product ion matches per protein; up to one missed cleavage site allowed; carbamidomethyl-cysteine as fixed modification; and methionine oxidation as variable modification.

2.5 Yeast strains, growth analysis and chronological lifespan (CLS) determination

The *Saccharomyces cerevisiae* strains used in this paper are reported in Table 1. Yeast cells were grown at 30°C in minimal medium (Difco Yeast Nitrogen Base without amino acids 6.7 g/L), with 2% w/v glucose and supplements added in excess [41]. Cell growth was monitored by determining cell number using a Coulter Counter-Particle Count and Size Analyser, as described [42]. In parallel, the extracellular concentration of glucose and ethanol were measured in medium samples collected at different time-points using enzymatic assays (K-HKGLU and K-

ETOH Megazyme) [41]. Duplication time (T_d) was obtained by linear regression of the cell number increase over time on a semi-logarithmic plot. CLS of Fig. 1 was measured according to [43] by counting colony-forming units (CFU) starting with 72 h (day 3, first-age point) after diauxic shift (day 0). The number of CFU on day 3 was considered the initial survival (100%). CBSE, dissolved in 20% ethanol by using an ultrasonic bath at 28 khz frequency and 90 W power for 3 min, was added to yeast cultures at the final concentration of 0.2% w/v. A 25X stock solution was prepared to properly dissolve the raw extract and at the same time to limit perturbations in cell culture medium composition after the supplementation. CLS experiments of Fig. 2-3 were performed adding CBSE in the exponential phase, as in [44]. Briefly, cells were pre-grown until mid-late exponential phase and then inoculated at 0.150 OD/mL into flasks containing fresh medium in the presence of CBSE at the final concentration of 0.05%, 0.1% or 0.2% w/v. Then, the medium was filtered through 0.22 μ m filters and 0.1 mM ampicillin was added to preserve sterility throughout the duration of the experiments. Survival was assessed by propidium iodide staining (PI) at different time points with the Cytotflex cytofluorimeter (Beckman Coulter) and analysed with the Cytotflex software.

Table 1.

Yeast strains used in this study.

Yeast strain	Genotype	Source
<i>wt [empty]</i>	<i>BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 [pYX242]</i>	[44]
<i>wt [αSyn]</i>	<i>BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 [pYX242-SNCA]</i>	[44]
<i>wt [αSyn] [ATG8-GFP]</i>	<i>BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 [pYX242-SNCA][pCu-ATG8-GFP]</i>	[44]
<i>atg8Δ [αSyn]</i>	<i>BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 atg8Δ::KanMX [pYX242-SNCA]</i>	This study

2.6 Analysis of Reactive Oxygen Species (ROS) Levels

ROS levels were analysed as previously reported [44]. Briefly, yeast cells were collected after 24 h treatment with the extract and 0.2 OD of cells were resuspended in PBS and stained with 5 µg/mL dihydroethidium (DHE) for 10 min. FACS analyses were performed with a Cytoflex cytofluorimeter (Beckman Coulter) and analysed with the Cytoflex software.

2.7 Protein extraction and immunoblotting from yeast proteins

Equal amounts of cells were collected and quenched using TCA 6% and lysed in lysis buffer (6M UREA, 1% SDS, 50 mM Tris-HCl pH7.5, 5 mM EDTA), as reported in [44]. Western blot analysis was performed using anti-GFP antibody (Roche), anti-α-synuclein antibody (Sigma Aldrich) or anti-Cdc34 antibody [45].

2.8 Analysis of aggresomes in yeast

The intracellular protein aggresomes were analysed using the PROTEOSTAT® Aggresome detection kit (ENZO Life Sciences). Cells were collected following a 24 h treatment with 0.2% CBSE and 0.2 OD were suspended in PBS buffer and stained with the PROTEOSTAT® Aggresome detection reagent at a dilution of 1:1500 [44]. FACS analyses were conducted using a Cytoflex cytofluorimeter (Beckman Coulter) and analysed with Cytoflex software.

2.9 *In vitro* aggregation of α -syn and ThT assay

α -syn was purchased from Merck and dissolved at 70 μ M in PBS. Protein samples (20 μ L) were incubated at 37°C in PBS up to 72 h under constant shaking at 900 rpm with a thermo-mixer in the absence (cnt) or in the presence of the extract at 0.1 and 0.025 mg/mL or in the presence of caffeine and theobromine solution at 0.01 mg/mL. The ThT binding assay was performed according to [46], using a 20 μ M ThT solution in PBS buffer. 180 μ L of ThT solution were added to 20 μ L of the aggregated α -syn samples, transferred into a black 96-well clear bottom multiwell plate and ThT fluorescence was read at the maximum intensity of fluorescence of 485 nm using a Victor X3 plate reader (Perkin Elmer); fluorescence of blank samples was subtracted from the fluorescence values of all samples. In control experiments, no interference of the extract on ThT fluorescence was observed.

2.10 Surface plasmon resonance (SPR) analysis

The BIACORE X100 system (Cytiva-Pall) was utilised to analyse molecular interactions between α -syn and the CBSE via Surface Plasmon Resonance (SPR). α -syn was immobilised onto a carboxymethylated dextran surface of a CM5 sensor chip using amine-coupling chemistry, as recommended by the manufacturer (Biacore Sensor Surface handbook BR100571), with the instrument temperature set at 25°C. The amine coupling procedure was performed using HBS-EP running buffer (0.01 M HEPES, 0.15 M NaCl, 0.003 M EDTA, and 0.005% v/v Surfactant P20, pH 7.4) at a flow rate of 5 μ L/min. The CM5 sensor chip was activated by injecting EDC/NHS (1:1) into both flow cells 1 and 2 for 10 min. α -syn was then injected into flow cell 2 at a concentration of 200 μ g/mL in 10 mM sodium acetate, pH 3.1, and covalently immobilised at level of 1200 Response Units (RU). The remaining activated sites on the chip were subsequently blocked using 1 M ethanolamine (pH 8.5) in both cells. The association capacity of α -syn was determined by injecting two control antibodies, anti- α -syn (Sigma) recognizing the whole α -syn (positive control) and anti- α -syn33 (Sigma) recognizing α -syn oligomers (negative control), at a dilution of 1:2500 in HBS-EP running buffer at a flow rate of 10 μ L/min. The lyophilized CBSE was resuspended in HBS-EP running buffer and injected at multiple concentrations for 5 min at 25°C and a flow rate of 10 μ L/min, with the running buffer injected as a blank under the same conditions. After injection, the analyte solutions were

replaced by the running buffer at a continuous flow rate of 10 $\mu\text{L}/\text{min}$ for 5 min. Surface regeneration was achieved by injecting 50 mM NaOH for a contact time of 1 min. Each sensorgram was corrected for the response observed in the control flow cell 1 (no immobilized protein) and normalized to a baseline of 0 RU. The sensorgram curves were acquired using the BiacoreX100 Control software, version 2.0.2 (Cytiva-Pall), in manual run mode.

2.11 Biolog OmniLog System

The effect of CBSE was evaluated for its impact on metabolic abilities using various chemical agents. This was done using the Biolog OmniLog Phenotype MicroArray chemical sensitivity panels PM21-PM25, which include 120 chemical compounds at four different concentrations. The Biolog OmniLog System was employed to compare the chemical sensitivity for each drug of *wt[αsyn]* yeast cells with and without 0.2% CBSE. All plates were prepared following the manufacturer's instructions as outlined in the OmniLog ID System User Guide (Biolog). Yeast cell cultures were grown on agar plates at 30°C and inoculated into 8 mL of minimal medium containing 2% glucose and YNB in sterile glass tubes. The cell suspension was measured using the BIOLOG Turbidimeter (Biolog) until a transmittance of 62% T was achieved. The suspension was prepared according to the BIOLOG PM protocol for yeast cells, using Dye E. 100 μL of cell suspension was added to each well and

microplates were incubated in the OmniLog™ system at 30°C for 72 h. The resistance and sensitivity profiles were compared using the appropriate OmniLog Biolog database (Biolog), with the y-maximum value of each kinetic growth curve being used for the analysis. Ratio between CBSE-treated and control cells were calculated, and compounds which showed a fold change >5 or <0.2 in at least three concentrations for each compound were considered as compounds towards which CBSE increases or decreases sensitivity.

2.12 Cell cultures

SH-SY5Y pTet-SNCA-FLAG were purchased from Merck. Cells were cultured on geltrex-coated plates at 37 °C in DMEM/F12 medium, containing 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin, in a humidified 5% CO₂ incubator. Doxycycline-inducible α-syn-expressing cells were selected against the antibiotic puromycin with a dose of 2 µg/mL. Induction of α-syn expression was achieved by adding 6 µg/mL doxycycline (doxy, from a 6 mg/mL stock in DMSO) for 48 h or 72 h. The CBSE was resuspended in water, sterile filtered and added to the medium at a final concentration of 150 µg/mL. For immunofluorescence assays, 160.000 cells were seeded on geltrex-coated glass cover slips in wells of a 24 multiwell plate and treated the day after for 48 h.

2.13 Protein extraction and immunoblotting for mammalian proteins

Total cell extracts were prepared using RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS) plus protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail (Merck). Protein concentration was determined using the Bradford protein assay (Bio-Rad). Western blot analysis was performed using anti- α -syn antibody (Merck), anti-phospho-T172-AMPK antibody (Cell Signaling), anti-AMPK α antibody (Cell signalling), anti-p62/SQSTM1 antibody (Merck), anti-phospho-Ser555-ULK1 antibody (Merck), anti-ULK1 antibody (Calbiochem) and anti-vinculin antibody (Sigma).

2.14 Immunofluorescence assay

After treating cells with doxycycline (Doxy) alone or in combination with CBSE (Doxy + Cocoa) for 48h, cells were washed with PBS, fixed with 4% formaldehyde for 15 min, and permeabilized with PBS-0.2% Triton X-100 for 10 min. Then, cells were washed three times in blocking solution (PBS-1% BSA), blocked at room temperature for 60 min, and then incubated overnight at 4°C with the primary antibodies dissolved in blocking solution as follows: anti-oligomer A11 Polyclonal Antibody (1:40, Invitrogen), anti α - syn antibody (1:200, Merck). Then, cells were incubated with anti-rabbit secondary antibody (1:200) conjugated with AlexaFluor488, dissolved in a blocking solution, for 1 h at room temperature shield

from light. Glasses were mounted with a DAPI containing mounting solution. The PROTEOSTAT R Protein aggregation assay (ENZO Life Sciences) was used to measure α -syn aggregates in cells as described by the manufacturer's instructions. Briefly, after treating cells as mentioned above, cells were washed carefully twice with 1X PBS, then fixed with 4% formaldehyde for 30 min at room temperature and permeabilized with Permeabilizing solution for 30 min on ice. Following PBS washes, the slides were dispensed in Proteostat dye and incubated for 30 min at room temperature. All treated slides were washed and mounted with a mounting medium with DAPI for nuclear staining and imaged under a Thunder fluorescence microscope (Leica). Image analysis was performed using the ImageJ software (NIH).

2.15 Statistical Analysis

Experiments were conducted in triplicate. Results are presented as mean values $\pm$ standard deviations (SD). Statistical data analyses were made using the two-tailed Student's t-test with significance set at $p < 0.05$, or by one-way ANOVA test (* $p \leq 0.05$ and ** $p \leq 0.01$).

3. Results

3.1 Characterization of the cocoa bean shell extract

The cocoa bean shell extract (CBSE), which was optimised and partially characterised previously [8], was subjected to further analyses to better define its composition. For this purpose, both UPLC-PDA-MS and NMR analyses were performed, and the compounds identified or tentatively identified are described in Tables 2 and 3. The identity of some compounds was achieved on the basis of the accurate mass and the associated errors, isotopic distribution, m/z values comparison with those reported in the literature and using literature databases.

Table 2. Metabolites identified by NMR analysis in the ^1H spectrum.

Metabolite	^1H chemical shift (ppm)	Multiplicity (J [Hz])	Concentration $\mu\text{g}/\text{mg} \pm \text{SD}$
Leucine	0.96	d [6.2]	3.37 $\pm$ 0.09
Isoleucine	1.01	d [7.1]	1.82 $\pm$ 0.02

Valine	1.05	d [7.1]	3.22±0.01
2,3-Butanediol	1.15	d [6.4]	1.51±0.10
Lactate	1.33	d [6.9]	31.56±1.12
Alanine	1.48	d [7.2]	4.64±0.09
Acetate	1.92	s	7.43±0.22
GABA	2.30	t [6.1]	2.42±0.06
Succinate	2.41	s	3.74±0.06
Citrate	2.55	d [15.3]	13.02±0.12
Aspartate	2.82	dd [17.4, 3.8]	1.69±0.01
Glycerol	3.66	dd [11.7, 4.3]	15.90±0.56
Mannitol	3.87	dd [11.9, 2.9]	4.76±0.04

Pyroglutamate	4.18	dd [9.1, 5.9]	7.16±0.03
Malate	4.30	dd [10.2, 2.9]	3.53±0.15
Fumarate	6.53	s	0.08±0.01
Tyrosine	6.9	d [8.4]	1.26±0.02
Phenylalanine	7.43	m	4.03±0.05
Formate	8.46	s	0.46±0.01

Table 3.

Chemical compounds identified or tentatively identified by UPLC-PDA-MS.

m/z expected	m/z calculated	Ionization Mode	Error (ppm)	Molecular formula	Proposed compound	Reference
181.0720	181.0733	M + H	5.5	C ₇ H ₈ N ₄ O ₂	Theobromine	[8,59]
195.0877	195.0888	M + H	5.9	C ₈ H ₁₀ N ₄ O ₂	Caffeine	[8]
	263.0636	M + H			Unknown	
	279.0407	M + H			Unknown	
	297.0509	M + H			Unknown	
305.0695	305.0699	M-H	-0.57	C ₁₂ H ₁₈ O ₇ S	Hydroxy-jasmonic acid sulfate	[59]
327.0510	327.0517	M-H	-2.06	C ₁₇ H ₁₂ O ₇	Unknown	
399.0838	399.0835	M + H	0.78	C ₂₈ H ₁₄ OS	Unknown	
	563.1732	M + H			Unknown	
579.1497	579.1486	M + H	1.90	C ₃₀ H ₂₆ O ₁₂	Procyanidin B	[8,60]
601.1188	601.1183	M + H	0.83	C ₂₈ H ₂₄ O ₁₅	Cyanidin-3-O(2''galloyl)-galactoside	[60]
	617.0954	M + H			Unknown	
467.1195	467.1214	M-H	-4.07	C ₂₁ H ₂₄ O ₁₂	Unknown	

NMR analysis identified and quantified mainly amino acids, such as Ala, Leu, Ile, Val, Phe, Asp, Tyr, and intermediates of TCA cycle such as fumarate, succinate, malate and citrate. The last one, together with lactate and glycerol, were the most abundant ones (Table 2, Fig. S1A). MS analysis revealed the presence of the two methylxanthines, caffeine and theobromine (theobromine: $52.74 \pm 8.12 \mu\text{g}/\text{mg}$ extract, caffeine: $12.98 \pm 3.96 \mu\text{g}/\text{mg}$ extract), and of other less abundant metabolites, like hydroxy-jasmonic acid sulphate, procyanin B, cyanidin-3-O(2''galloyl)-galactoside, as well as of some unknown compounds that could not be clearly identified (Table 3, Fig. S1B).

According to a Bradford quantification, the extract also contained $6 \mu\text{g}/\text{mg}$ of proteins. Although the amount of protein was very low, a proteomic analysis was performed to achieve a characterization. Mass spectrometry analysis of the proteins present in the extract revealed the presence of 5 proteins from *Theobroma cacao* (Table 4), with Vicilins and a 21 kDa seed protein being the most abundant ones according to peptide intensity, as expected from literature [46]. However, we cannot exclude the presence of other proteins not identified due to low level of annotation in the database of *Theobroma cacao*, although both UniProt and NCBI databases were used.

Entry (Database)	Accession	protein.Description	protein score	MW	protein Matched Products	protein matched Peptides	Cov (%)	Peptide Intensity Sum	Sequence	MH + Error (ppm)	Score
ASP_THECC (Uniprot)	P32765	21 kDa seed protein	615	24438	30	7	30.77	203620	(R)HSDDDGQIR(L)	0.9339	6.6629
									(R)SDLDNGTPVIFSADSKDDVVR(V)	0.4055	5.9953
									(K)DDVVR(V)	-2.412	5.6277
									(R)VSTDVNIEFVPIR(D)	2.7383	5.3816
									(R)RSDDLNGTPVIFSADSK(D)	-2.3835	5.2396
									(R)LDNYDNSAGK(W)	-3.3845	5.1266
									(R)ATGQSCPEIVVQR(R)	1.7676	4.8949
A0A061EM85_THECC (Uniprot)	A0A061EM85	Vicilin-A_putative	200	66198	37	9	17.67	75441	(R)QDRR(E)	-1.8736	6.231
									(K)EQER(G)	-0.3204	5.622
									(K)ELSFVPSK(L)	1.3752	5.6153
									(R)SEEEGQQR(N)	-1.5498	5.3042
									(R)	3.0933	5.2171
									GTVVSPAGSTVYVVSQDNQEK(L)		
									(R)EQEESEETFGFQQVK(A)	1.6377	5.1098
VCL_THECC (Uniprot)	Q43358	Vicilin	167	61483	32	8	17.33	55233	(R)QDRR(E)	-1.8736	6.231
									(K)EQER(G)	-0.3204	5.622
									(R)SEEEGQQR(N)	-1.5498	5.3042
									(R)	3.0933	5.2171
									GTVVSPAGSTVYVVSQDNQEK(L)		
									(R)EQEESEETFGFQQVK(A)	1.6377	5.1098
									(R)QEEELQR(Q)	-3.0807	4.8992
A0A061GTA7_THECC (Uniprot)	A0A061GTA7	Uncharacterized protein	117	10317	4	1	7.53	5242	(R)EKEEILEEQR(G)	-1.8284	4.7921
									(K)LTIAVLALPVNSPGK(Y)	-0.6553	4.6108
									(K)IEEHQSY(-)	-0.1395	5.2605
A0A061GZ27_THECC (Uniprot)	A0A061GZ27	Putative plant transposon protein domain-containing protein	100	12772	6	1	13.76	6904	(M)NQCHFSEVSCICQK(V)	0.6846	4.8622

CAA44494.1 (NCBI)	CAA44494.1	vicilin_partial	1582	54423	81	13	23.7	503177	(R)EQEEEESEETFGF(-)	0.2798	7.8005
									(R)QDRR(E)	-1.8736	7.1311
									(R)REQEEEESEETFGF(-)	-0.781	6.6848
									(R)NNPYYPFK(R)	0.4712	6.3261
									(R)DEEGNFK(I)	1.2679	6.2127
									(R)SEEEGQQR(N)	-1.5498	6.203
									(K)ESYNVQR(G)	2.3721	6.1794
									(R)	3.0933	6.1217
									GTVVSPAGSTVYVVSQDNQEK		
									(L)		
									(K)EQER(G)	-1.8439	5.9501
									(R)QQEELQR(Q)	-3.0807	5.7974
									(R)EKLEIEEQR(G)	-1.8284	5.6899
									(K)LTIAVLALPVNSPGK(Y)	-0.6553	5.5105
									(K)LEEIEEQR(G)	3.2998	5.3213
									(R)EQEEEESEET(T)	1.8693	0
									(R)EQEEEESEET(F)	1.9878	0
									(R)EQEEEESEETFGF(F)	-1.9716	0
									(R)EQEEEESEETFGF(-)	0.1171	0
									(R)EQEEEESEET(E)	-2.1412	0

Table 4.

List of proteins by LC-MSE in cocoa extract with the corresponding peptides.

3.2 Supplementation of CBSE extends CLS of yeast cells expressing human α -syn

Since yeast *S.cerevisiae* has been extensively employed as model system to study the cytotoxic effects of α -syn in PD and other synucleinopathies [23], we wished to test in the context of a standard CLS experiment [47] whether cocoa-shell treatment might have any ameliorating effect on the age-dependent α -syn-mediated cell death [27]. To this end a humanised yeast model of PD overexpressing human α -syn was used. As shown in Fig. 1A, no significant differences were observed in the duplication time (Td) between cells expressing α -syn and wt ones grown on minimal medium in 2% glucose (Fig. 1A). Consistently, during the exponential phase, when growth is sustained by a prevalent fermentation-based metabolism, the glucose decrease was accompanied by ethanol accumulation that in both yeast cultures followed the same kinetics (Fig. 1B). Once defined the growth profile, CBSE was added to both cultures at the onset of chronological aging, namely at the diauxic shift, and CLS was determined by CFU scoring. In line with previous reports [27], α -syn expression reduced CLS (Fig. 1C). Interestingly, CBSE supplementation increased both mean and maximum CLS (Fig. 1C and D), as well as the survival integral (SI, Fig. 1D), defined as the area under the CLS curves [48]. Indeed, the SI increased by about 38% for wt and 89% for α -syn expressing cells, indicative of a pro-longevity effect of the CBSE. Starting from these

results and being specifically interested in α -syn aggregation and its cytotoxic outcome, subsequent analyses were performed only with cells expressing α -syn.

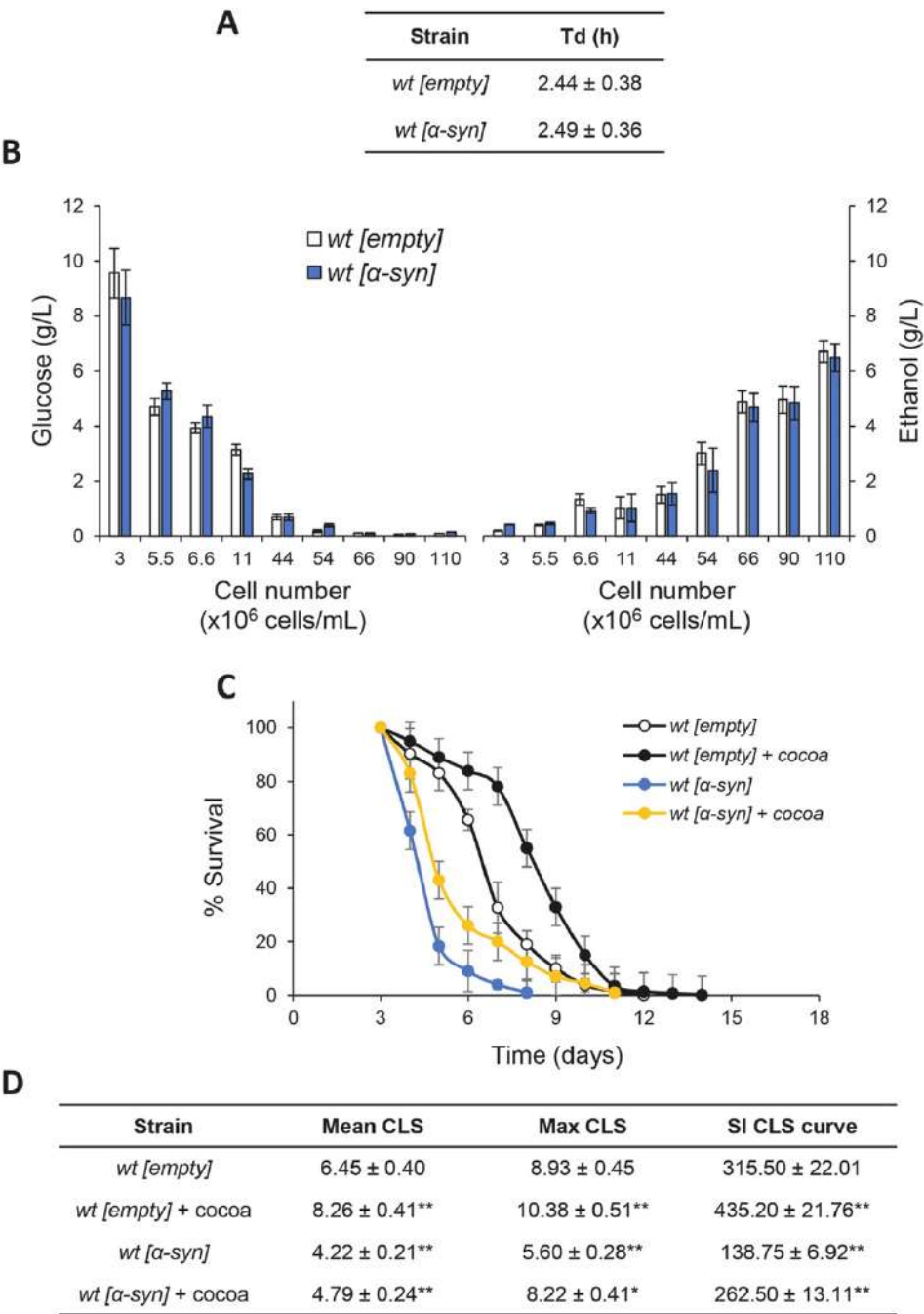


Figure 1. CBSE supplementation at the diauxic shift extends CLS. Wild-type (*wt[empty]*) and α -syn overexpressing (*wt[α -syn]*) cells were grown in minimal medium containing 2% glucose and required supplements in excess. (A) Cell growth was monitored by counting cell number over time and duplication time (Td) of wt and α -syn expressing cells was calculated as $\ln 2/k$, where k is the constant rate of exponential growth. In parallel, (B) extracellular concentration of (left) glucose and (right) ethanol were measured in medium samples collected at different time-points. At the diauxic shift (day 0), CBSE (cocoa) was added and (C) survival over time of the indicated strains was assessed by colony-forming capacity on YEPD plates. 72 h after the diauxic shift (day 3) was considered the first age-point, corresponding to 100% survival. (D) Quantification of chronological survival: data referring to the time-points (days) where chronological aging cultures showed 50% (Mean CLS) and 10% (Max CLS) of survival, as well as, survival integral (SI) measured as reported [49]. All data refer to mean values determined in three independent experiments with three technical replicates each. Standard deviations (SD) are indicated. * $p \leq 0.05$ and ** $p \leq 0.01$.

When CBSE was added to exponentially growing cells, up to 0.2%, no effect on the growth rate was observed (data not shown), while a significant dose-dependent reduction of intracellular ROS was detected 1 day after its addition, supporting an antioxidant effect (Fig. 2A). Nevertheless, both the mean and the maximal CLS, as

well as the SI increased (more than 70%) only at the highest concentration (0.2%) (Fig. 2B), in accordance with the results reported above.

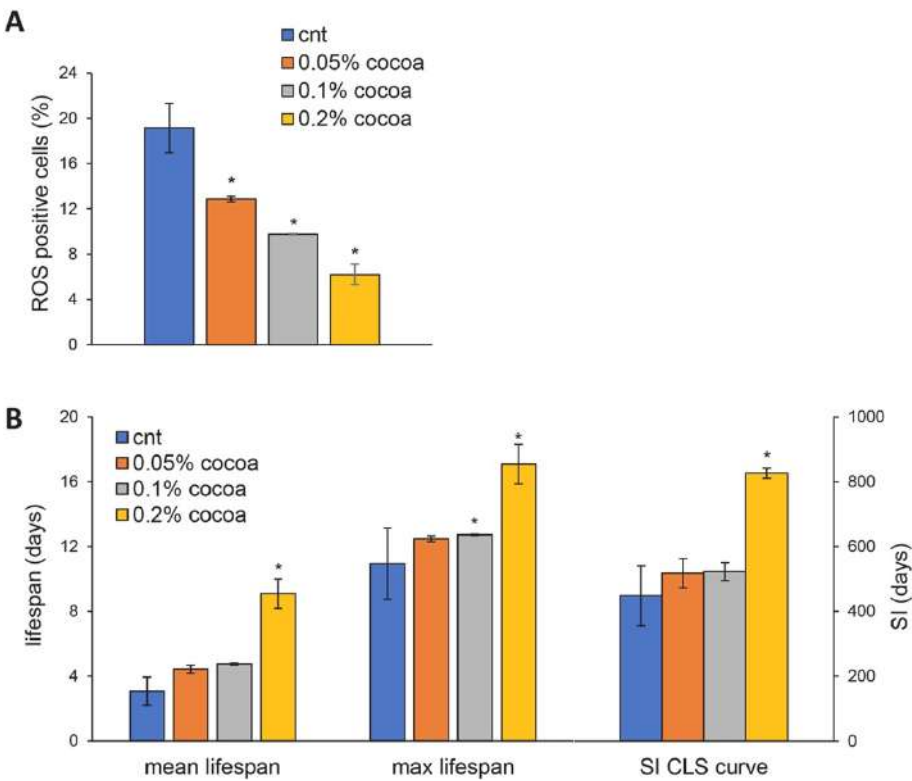


Figure 2. The CBSE extends yeast lifespan and reduces ROS levels. (A) ROS content of yeast *wt[α-syn]* cells grown in medium containing 2% glucose in the absence or presence of 0.05%, 0.1% or 0.2% CBSE, added in the exponential phase of growth. (B) Mean and maximal lifespan and SI of cells in (A). Histograms represent mean ± standard deviation of at least two independent experiments. *p<0.05.

3.3 The anti-aging effect is independent from caffeine and theobromine

Since the CBSE contains both metabolites and proteins (Tables 2-4), we performed a size-exclusion fractionation to separate the protein fraction (molecular weight >10 kDa) from the metabolite fraction and tested them separately on the yeast model. As shown in Fig. 3 A-B, the metabolite fraction increased mean lifespan and reduced ROS levels (albeit to a lesser extent than the total extract), while the protein fraction showed no significant activity, suggesting that metabolites are the main responsible for the antioxidant and anti-aging effect of the CBSE. This is consistent with the very low amount of protein present in the extract.

Among the metabolites identified in the CBSE, caffeine and theobromine represent about 10% of the total extract (see paragraph 3.1). To analyse whether the observed anti-aging effect could be attributed to the presence of these two methylxanthines, α -syn overexpressing cells were treated with caffeine, theobromine or a combination of both, mimicking their abundance in the CBSE. Neither single treatments, nor their combination, showed any anti-aging effect (Fig. 3C) or antioxidant properties (Fig. 3D), suggesting that the pro-longevity function might depend on other metabolites or result from a synergistic/combined action with other molecules of the CBSE.

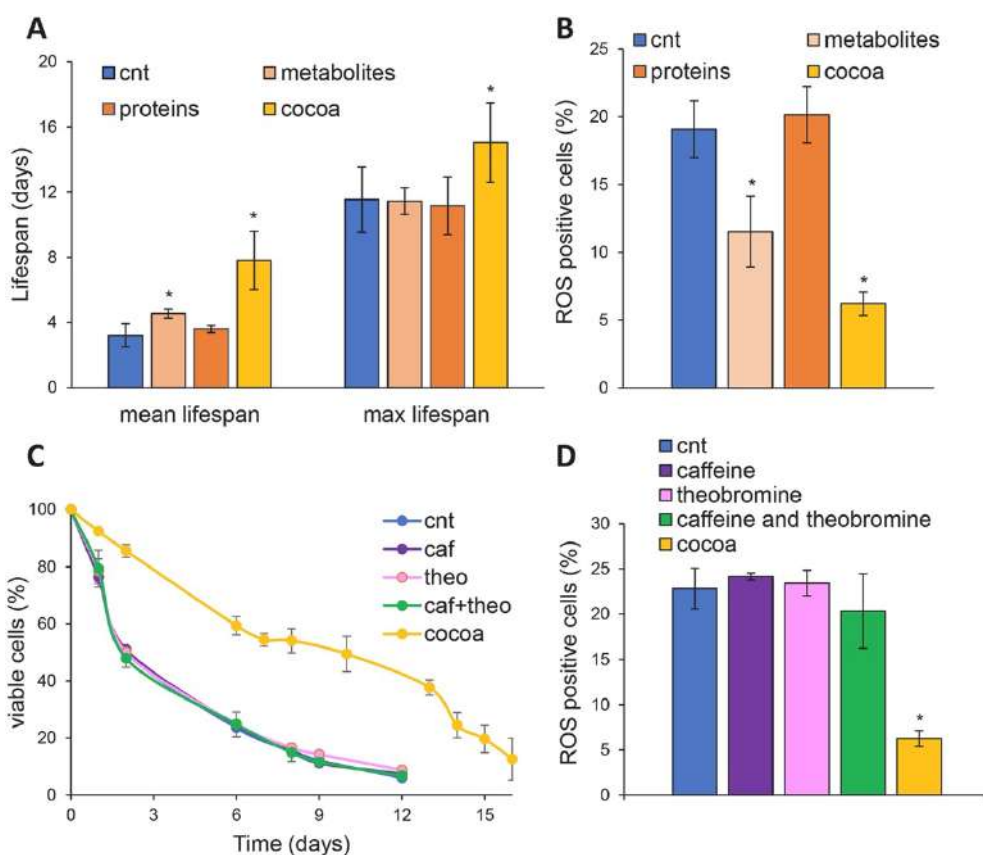


Figure 3. The effect of the CBSE is independent from caffeine and theobromine.

(A) CLS of yeast *wt* [α -*syn*] cells in medium containing 2% glucose in the absence (cnt) or presence of 0.2% CBSE, and its contained metabolites and proteins. (B) ROS content of cells treated for 24 h as in (A). (C) CLS of yeast *wt* [α -*syn*] cells in medium containing 2% glucose in the absence or presence of 0.2% CBSE, caffeine, theobromine or a combination of the two (25.96 μ g/mL caffeine and 105.4 μ g/mL theobromine). (D) ROS content of cells treated for 24 h as in (C). Results are reported as the mean $\pm$ standard deviation of three independent experiments.

* $p < 0.05$.

3.4 The CBSE binds α -syn and reduces its aggregation

In the last years, several data reported that natural extracts could exhibit direct fibrillation-inhibiting effects [49]. Thus, we wondered if the anti-aging effect of the CBSE could be due to a reduction in α -syn aggregation. A very strong decrease of intracellular aggresomes was observed in yeast cells treated with the CBSE for 24 h, with a 5-fold decrease compared to control cells (Fig. 4A), showing its potential in reducing the aggregation of misfolded proteins. Thus, to evaluate a direct effect of the extracts on the aggregation process, α -syn fibrillation experiments were performed *in vitro* and the ThT emission fluorescence signal was used to quantify fibrils formation over time. In the absence of the CBSE, the ThT fluorescence showed the typical sigmoidal shape, indicating the aggregation of the protein; this behaviour completely disappeared in the presence of the extract (at both concentrations, Fig. 4B). Interestingly, caffeine and theobromine together, although had no effect on yeast longevity and ROS content (Fig. 3C,D), showed a partial inhibitory effect on the aggregation of α -syn, Fig. S2A).

A direct effect of the extract on aggregation would imply a direct interaction among the components of the CBSE and α -syn. To explore this hypothesis, α -syn protein was immobilised on a CM5 sensor chip for surface plasmon resonance (SPR) analysis. To validate the suitability and selectivity of the chip, anti α -syn (which

binds free and aggregated α -syn) and anti- α -syn33 (which binds only α -syn aggregates) antibodies were utilised as positive and negative controls, respectively, and were injected into the SPR system. While the anti- α -syn antibody bound the protein on the chip, the one specific for the aggregated α -syn showed no binding at all (Fig. S2B). These results showed that the immobilised α -syn protein on the sensor chip surface was in its non-aggregated form and thus was employed to assess its direct binding with the CBSE. Results obtained from the SPR assay indicate that the CBSE could bind to α -syn protein in a concentration-dependent manner (Fig. 4C). Indeed, five increasing concentrations of the extract were tested (0.64 mg/mL, 1.27 mg/mL, 2.54 mg/mL, 5.08 mg/mL and 8 mg/mL) and the response signal increased as a function of the rising concentration of the sample (Fig. 4D). This indicates that there are compounds in the CBSE that directly bind to α -syn, and can explain the inhibitory effect on the amyloid aggregation of α -syn (Fig. 4B). Finally, we tested caffeine and theobromine, which did not appear to bind to the protein (data not shown). However, considering that the molecular weights of caffeine (194.19 g/mol) and theobromine (180.164 g/mol) are near the detection limit of the instrument (100 Da), we cannot exclude that the binding was not detected due to technical limitations.

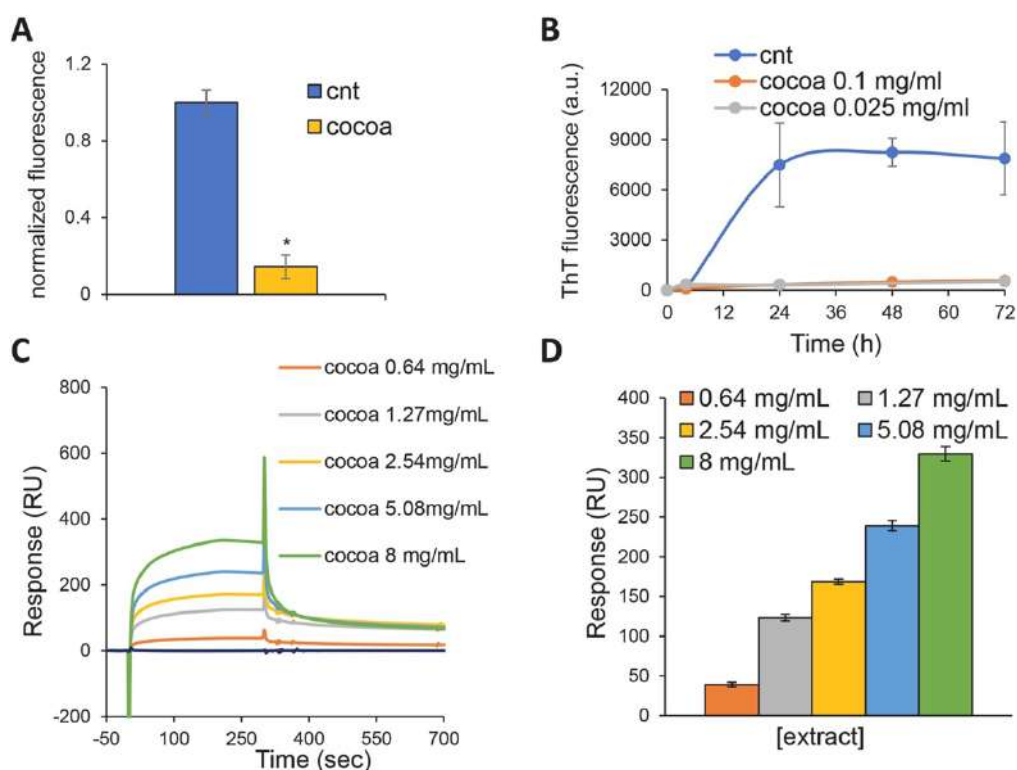


Figure 4. The CBSE inhibits α -syn aggregation. (A) Fluorescence intensity obtained from flow cytometry analysis of aggresomes of yeast *wt* [α -syn] cells in medium containing 2% glucose in the absence (cnt) or presence of 0.2% CBSE. (B) α -syn aggregation process, followed by ThT fluorescence, in the absence (cnt) or presence of the CBSE (0.1 and 0.025 mg/ml). (C) SPR sensorgrams of CBSE at different concentrations (8 mg/mL, 5.08 mg/mL, 2.54 mg/mL, 1.27 mg/mL, 0.64 mg/mL) display binding toward α -syn on CM5 sensor chip surface. (D) The CBSE shows dose-dependent binding activity to α -syn protein. Results are reported as the mean $\pm$ standard deviation of at three independent experiments.

3.5 The CBSE stimulates autophagy in yeast cells

In an attempt to identify cellular changes occurring upon treatment with the CBSE, a high throughput screening for sensitivity against antibiotics, chemicals and osmolytes was performed. The chemical resistance and sensitivity profile due to the CBSE of the yeast strain overexpressing α -syn was measured using the Biolog Phenotype MicroArrays PM21-PM25 chemical sensitivity panel, which contains 120 assays of chemical sensitivity. Each plate contains 24 different chemical agents in 4 different concentrations, that were divided into 6 groups based on their structure and function: ions, cyclic compounds, organic compounds, chelators, antibiotics, and nitrogen compounds (Table S1). In the presence of 0.2% extract, yeast cells showed increased resistance to several compounds; interestingly most of them have been described for their effect on autophagy in different models (Fig. 5, Table S2).

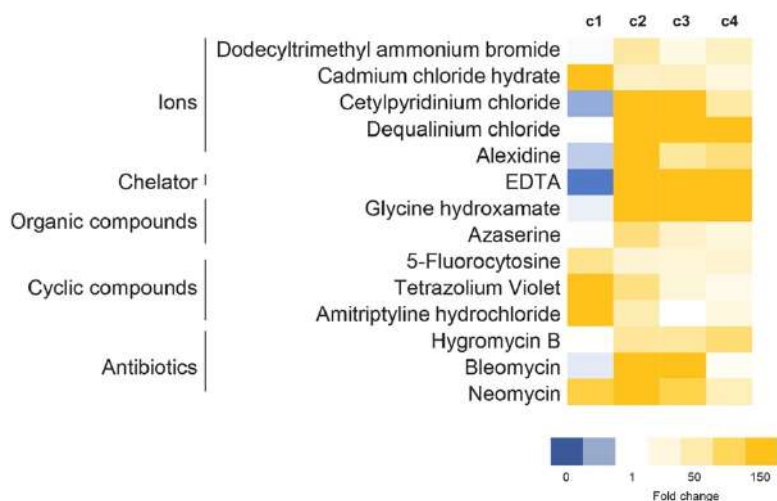


Figure 5. Drug sensitivity upon CBSE treatment. Heatmap of sensitivity of *wt[α-syn]* cells to selected drugs in the presence of CBSE compared to the control condition, measured by Biolog OmniLog Phenotype MicroArray. Fold changes (treated/cnt) in γ-maximum value were calculated and compounds were selected when fold change was >5 or <0.2 in at least three concentrations. Colour scale indicates increased resistance (yellow) or decreased resistance (blue) after 72 h growth.

The autophagic pathway is normally activated in stationary phase cells and is the major process involved in the clearance of α-syn aggregates [22]. Therefore, to evaluate the activation of the autophagic process in cells treated with the CBSE, we monitored the accumulation of free GFP in cells expressing Atg8-GFP fusion protein, whose cleavage is indicative of autophagy activation. Interestingly, a significant increase in the cleavage of Atg8-GFP was observed 1 day, and even more, 2 days after the extract addition, reflecting the activation of the autophagic process in such condition (Fig. 6A,B).

However, in *atg8Δ* cells, CBSE was still able to significantly reduce both intracellular ROS level and aggresomes (Fig. 6 C,D), suggesting that the stimulation of the autophagic process is not the only pathway involved in the pro-longevity function of the CBSE.

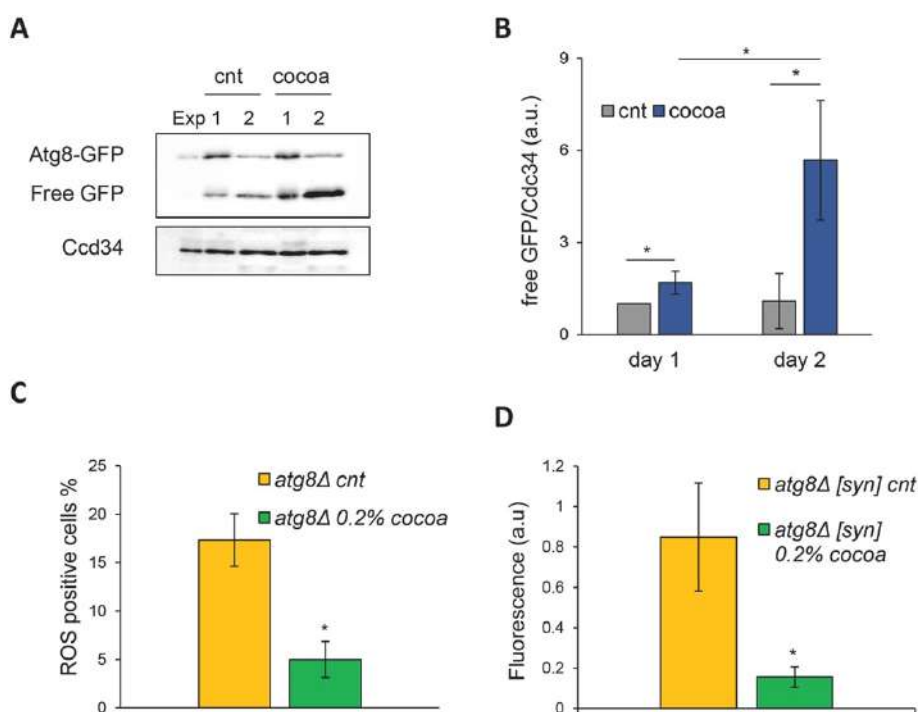


Figure 6. The CBSE activates autophagy in yeast. (A) Western analysis using anti-GFP antibody on total extracts from *wt[α-syn][Atg8-GFP]* cells treated with 0.2% CBSE for 1 and 2 days. Anti-Cdc34 antibody was used as loading control. (B) Quantification of free GFP of three independent experiments performed as in (A). (C) ROS content of *atg8Δ[α-syn]* cells in medium containing 2% glucose in the absence (cnt) or presence of 0.2% CBSE for 24 h. (D) Fluorescence intensity obtained from flow cytometry analysis of aggregates of yeast *atg8Δ[α-syn]* cells in medium containing 2% glucose in the absence (cnt) or presence of 0.2% CBSE for 24 h. Results are reported as the mean ± standard deviation. *p<0.05.

3.6 The CBSE reduces α -syn aggregates in neuroblastoma cells

In order to further investigate the effects of the CBSE, we turned to SH-SY5Y neuroblastoma cells expressing α -syn under a doxycycline-inducible promoter [34]. As expected, doxycycline induced an increase of monomeric α -syn level, which was not affected by treatment with the CBSE both at 48 h and 72 h (Fig. 7A,C). Although the extract induced the phosphorylation of the energy sensor AMPK, no change of either pULK1 or p62 level was observed, suggesting that the CBSE does not activate the autophagic pathway in neuroblastoma cells (Fig. 7A-B).

One of the key processes for the pathogenesis of Parkinson's disease is the assembly of toxic oligomeric species of α -syn. Then, since we have shown that the CBSE is able to bind and inhibit α -syn aggregation (Fig. 4), the level of α -syn oligomers was investigated in neuroblastoma cells treated with the CBSE. Strikingly, a significant reduction of α -syn oligomers, as well as of intracellular aggresomes were observed upon CBSE treatment (Fig. 7D-E). These data suggest that the CBSE prevents the formation of toxic oligomeric species and not their clearance through the autophagic degradation.

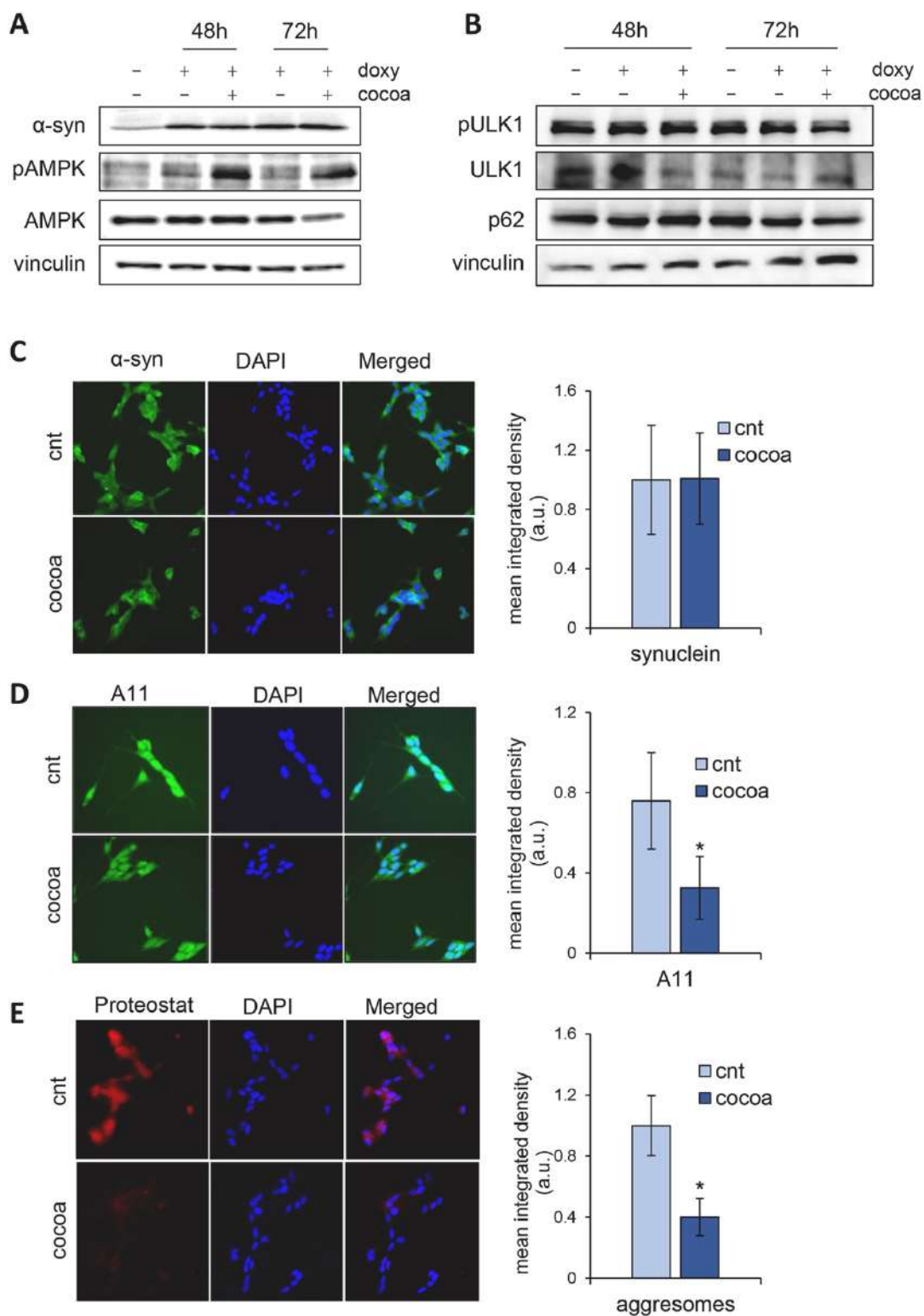


Figure 7. The CBSE reduces α -syn toxicity in neuroblastoma cells. (A-B) Western blot analysis using anti- α -syn, anti-phospho-T172-AMPK, anti-AMPK α , anti-vinculin antibodies (A) and anti-p62/SQSTM1 anti-phospho-Ser555-ULK1, anti-ULK1 and anti-vinculin antibodies (B) on protein extracts from SH-SY5Y pTet-SNCA-FLAG cells untreated, treated with doxycycline or treated with doxycycline and 150 μ g/mL CBSE for 48 and 72 h. (C) Representative immunofluorescence (60x) images of SH-SY5Y cells treated with doxycycline (Doxy) alone and in combination with 150 μ g/ml CBSE (Doxy + Cocoa) for 48 h, immunolabeled with anti α -syn antibody (C), A11 anti-oligomer antibody (D), and Proteostat R dye (E). Nuclei were stained by DAPI (Blue). Histograms represent mean $\pm$ standard deviation of cell fluorescence quantified with the ImageJ software.

4. Discussion

Accumulation of pathological protein aggregates is associated with a wide range of human diseases. Among these, aggregates of β -amyloid, p-tau or α -syn in the brain are found in patients with Alzheimer's and Parkinson's diseases and correlate with the progression of neurodegeneration [50]. Considering the consequent induction of neurotoxicity and neuronal loss, there is an increasing interest in the study of secondary metabolites, such as terpenes, flavonoids and phenols, able to inhibit

protein aggregation and/or stimulate the clearance of these toxic aggregates. In recent years, the protective effects of a number of bioactive compounds have been highlighted on a wide variety of diseases, among which neurodegenerative ones [49, 51].

In the context of the research of still unexplored bioactive molecules, nature is an unlimited reservoir for the discovery of novel therapeutics not only against broad-spectrum diseases, but also for applications in the cosmetic and food industries. In line with this, the valorization of by-products generated by the conventional linear food industry is an emerging strategy to identify new potential useful bioactivities and to reduce food waste.

In the present study we have employed this approach for the utilisation of cocoa bean shells, a by-product typically discarded during the roasting process of cocoa beans [7].

Here we show that CBSE, obtained by a green extraction and rich in amino acids, organic acids and methylxanthines (Tables 2, 3), strongly improves yeast longevity and reduces the toxicity of human α -syn, by decreasing intracellular protein aggregates (Fig. 1, 2, 4, 7). Different eukaryotic models, yeast cells and a neuroblastoma cell line, were used to verify the bioactivity of the CBSE. Although the effects identified are not completely superimposable in the two systems, this

approach highlights the importance of using multiple models to better identify all the biological pathways that contribute to the neuroprotective activity of natural compounds. Indeed, while CBSE stimulates autophagy in the yeast model of PD (Fig. 6), this is not the case in neuroblastoma cells, even if the stress responsive kinase AMP-activated protein kinase (AMPK) is activated (Fig. 7). We consider this result particularly relevant because energy metabolism defects are commonly described in neurodegeneration and several studies reported the implication of AMPK in various signalling pathways that are involved in the progression of neurodegeneration [52]. Thus, the stimulation of both autophagy and AMPK signalling appears to represent as two complementary responses induced by CBSE which together contribute to protect the cell from the toxicity of misfolded proteins.

Results obtained by surface plasmon resonance (SPR) assays indicate also that CBSE binds α -syn protein in a concentration-dependent manner, supporting a direct association of the cacao-bean shell extract with monomers of α -syn by preventing its aggregation into toxic oligomers and amyloid fibrils (Fig. 4B-D). What remains to be elucidated are the specific compounds exerting this role. Although caffeine and theobromine, as well as the very small fraction of proteins contained in the extract, do not have any effect on yeast longevity and ROS content (Fig. 3), the methylxanthines together show a partial inhibitory effect on the aggregation of α -

syn *in vitro* (Fig. S2A). The protective functions of methylxanthines are well documented, since they reduce inflammation and preserve cognitive functions [15, 53–55]. Then, the inactivity of both caffeine and theobromine in our yeast model could suggest a possible synergistic or combined role of different molecules within the extract.

The promising results obtained in this study represents the first step for the development of the CBSE as a neuroprotective agent. In order to address if the reported bioactivity may be relevant also under physiological conditions, CBSE bioavailability, absorption rate and metabolism need to be further investigated both in animal models and in clinical studies. In addition, to reach the brain, the active compounds of CBSE have to pass through the gastrointestinal tract and to cross the blood brain barrier, without losing any efficacy. Remarkably, toxicology studies performed in mice, both in acute and sub-chronic assays, indicate that the oral administration of both cacao shell flour or extracts is safe, without significant histopathological alterations [9]. In line with this, an interesting approach of encapsulation has been reported to enrich chocolate bars with phenolic antioxidant compounds extracted from cocoa bean shells [56].

Altogether, our results along with these studies support the potentiality of this waste matrix upcycling as a safe and neuroprotective ingredient for functional

foods opening the route to using cocoa bean shell extract, in the form of nutraceutical, as a direct anti-aggregant agent against intracellular protein misfolding and toxicity.

5. Conclusions

During industrial food processing, a large amount of waste is produced. The cocoa bean shell is a valuable by-product of the chocolate industry, thus its valorisation may reduce the environmental impact and provide economic benefits. Nowadays, the cocoa bean shells are mainly used for feedstuff, as biofuel and in the agriculture and food industry [2]. Here we propose a new valorization approach for this waste product, since through a sustainable, rapid, and cost-effective procedure, we have developed an extract with anti-aggregant properties. Although our study represents the first step towards the use of CBSE as a protective agent, both preclinical and clinical data are still required in the perspective of the development of novel sustainable treatments to prevent neurodegeneration.

ACKNOWLEDGMENTS

This work was supported by the project “ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON

Foods” (CUP:H43C22000820001) of the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.3—Research funded by the European Union—NextGenerationEU.

This work was also supported by the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.4—call for tender No. 3138 of 16 December 2021, rectified by Decree No. 3175 of 18 December 2021 of the Italian Ministry of University and Research funded by the European Union—NextGenerationEU; Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022. Adopted by the Italian Ministry of University and Research, CUP, H43C22000530001, Spoke 6, Project title “National Biodiversity Future Center—NBFC”.

CONFLICT OF INTEREST

The authors have declared no conflict of interests.

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Data availability. Raw data will be made available on request.

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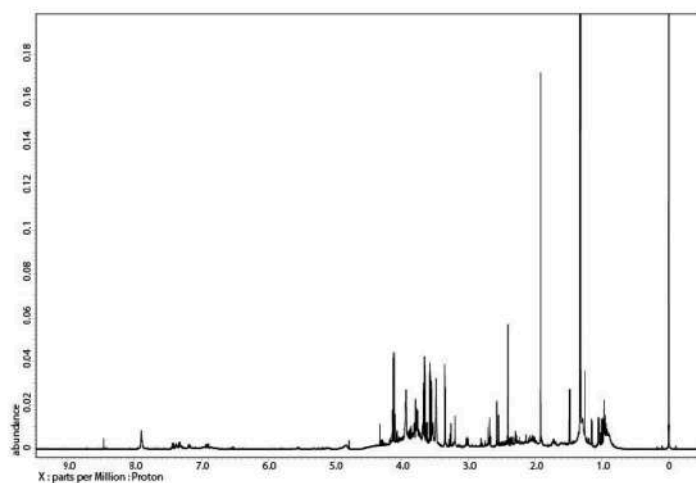
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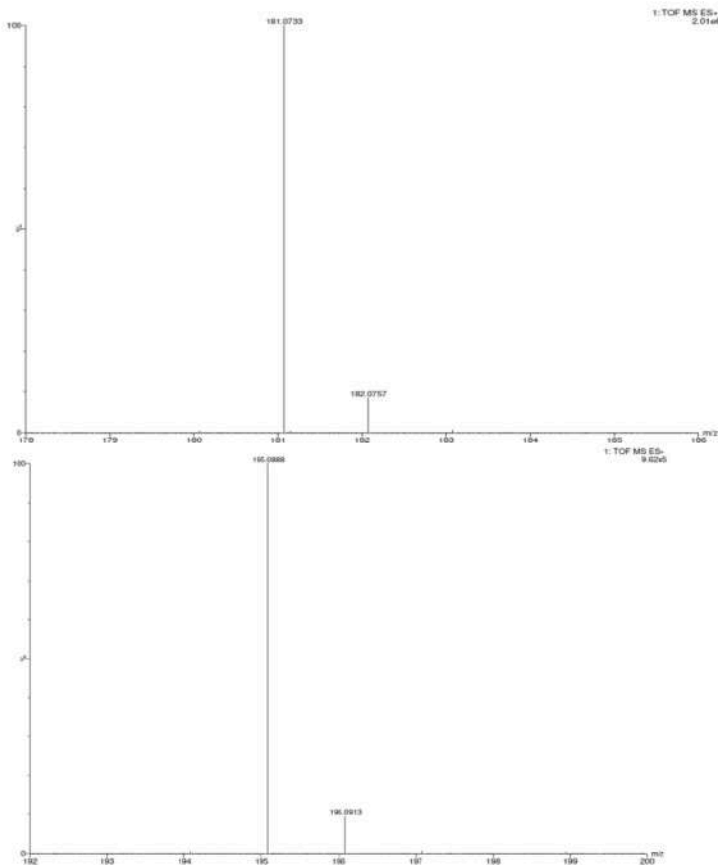
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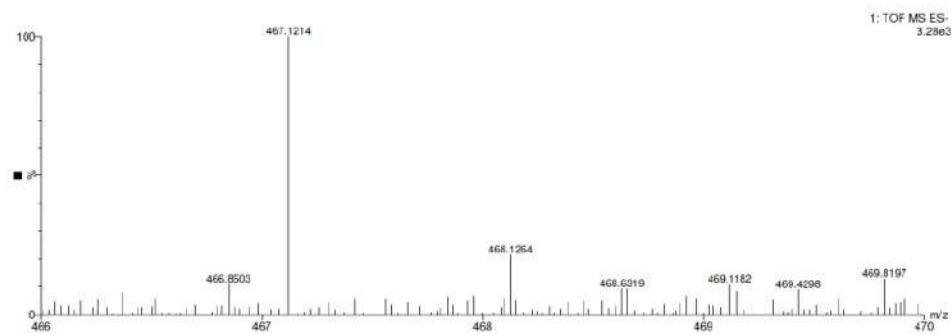
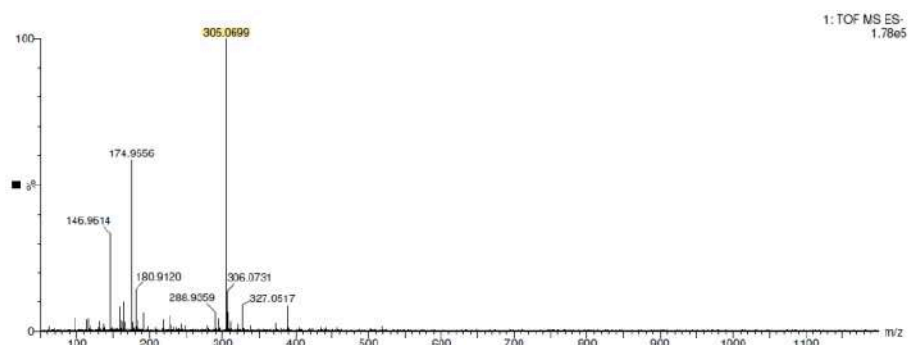
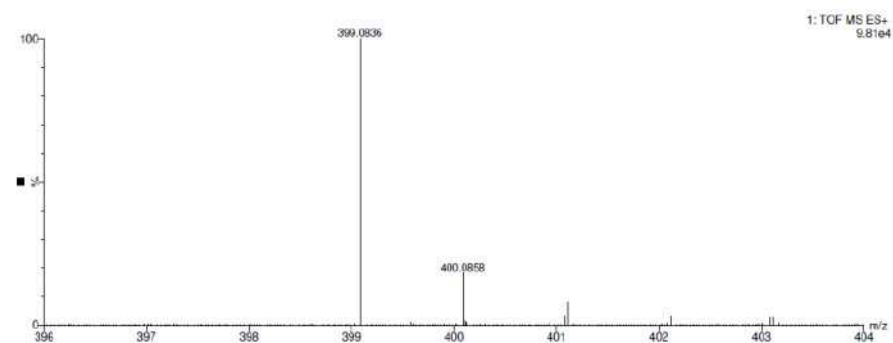
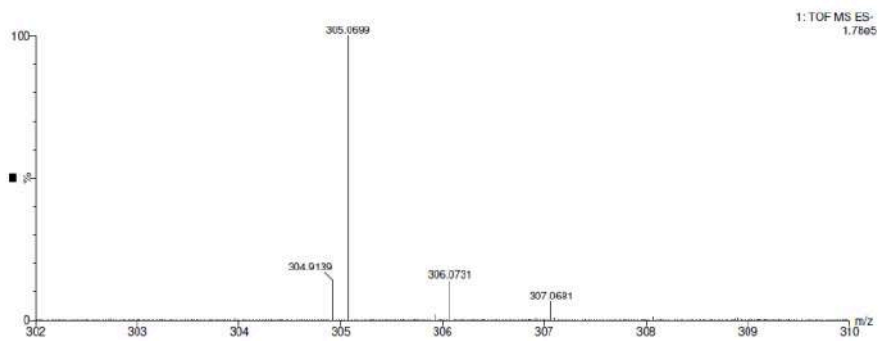
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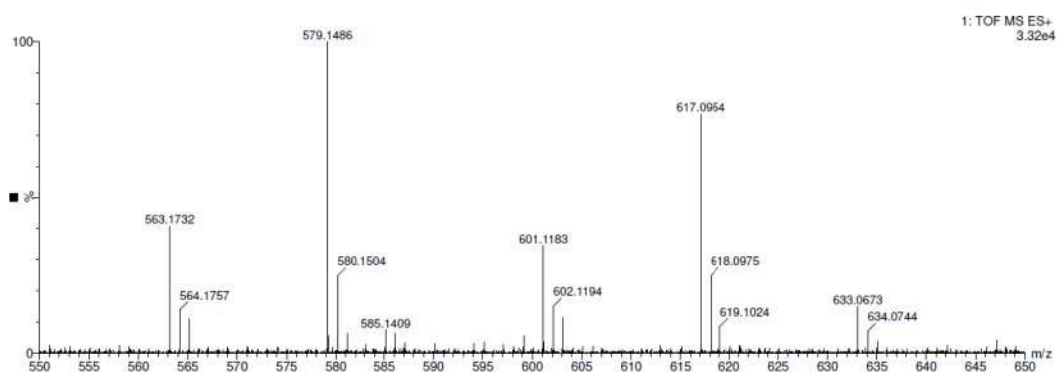
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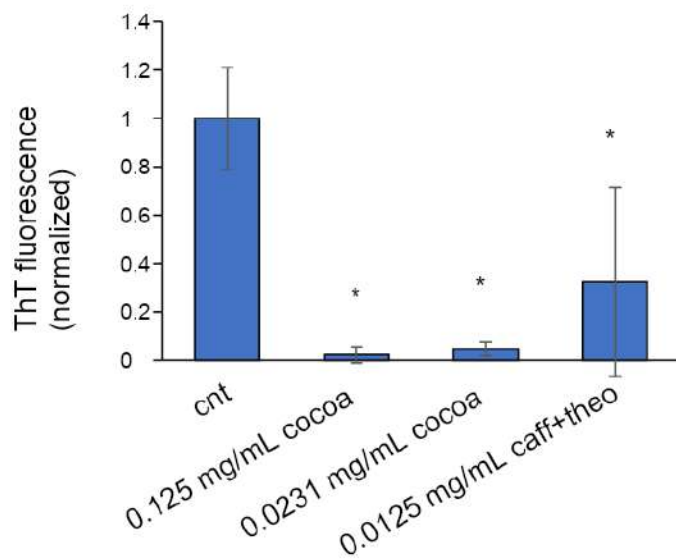
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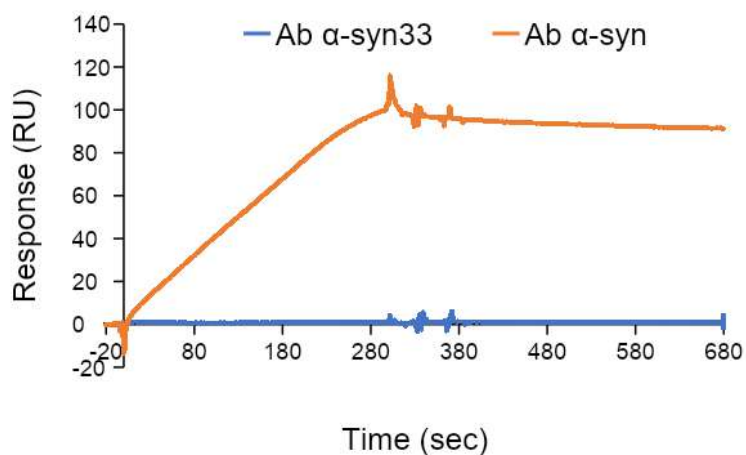






Supplementary Figure S1. (A) 600.17 MHz ^1H NMR spectrum of cocoa bean shell extract solubilized in D_2O containing 50 mM phosphate buffer pH7.4 and 0.4 mM TSP as internal standard. (B) MS spectra of the metabolites reported in Table 3.





Supplementary Figure S2. (A) α -syn aggregation process, followed by ThT fluorescence, in the absence (cnt) or presence of the 0.01 mg/mL of caffeine and theobromine. (B) The bioactivity of the CM5 sensor chip was determined by injecting positive (Ab anti- α -syn) and negative (Ab anti- α -syn33) controls onto immobilised α syn protein.

Groups	Drugs	Concentration 1	Concentration 2	Concentration 3	Concentration 4
Ions	Sodium dichromate	0,56755694	0,623737383	7,381328311	2,483020329
	Sodium arsenate	0,841201818	0,441488076	0,263049085	0,553268749
	Sodium arsenite	0,623167727	0,603344035	3,28058388	0,35487225
	Sodium cyanate	0,210577222	0,416450047	0,846042364	0,712103787
	Sodium orthovanadate	0,62798072	8,259770115	3,681876558	0,995323275
	Sodium selenate	0,53817279	0,738086826	0,636880754	0,561544201
	Sodium selenite	0,655254226	0,654684717	0,698000318	0,989909457
	Sodium cyanide	0,439544254	4,112331961	1,891101462	14,76218067
	Sodium thiosulfate	1,604610244	0,275876794	0,280566619	1,838490702
	Sodium metasilicate	0,883069693	0,891283232	0,394572046	1,333282403
	Sodium metaborate tetrahydrate	0,695186934	0,842256989	4,86350968	24,8077
	Sodium (meta)periodate	0,83390097	0,97915076	0,645144944	0,417492025
	Sodium metavanadate	0,827466657	10,24852308	2,405979901	0,794820439
	Sodium fluoride	0,503614264	0,580450783	0,350336041	0,383772359
	Potassium chromate	0,327640103	0,981842969	28,24144061	84,36847015
	Copper (II) sulfate	0,747458247	20,74520728	5,492579009	4,989526063
	Thallium(I) acetate	0,940828245	2,937511091	0,198247209	0,036576987
	Cadmium chloride hydrate	61,4026	12,1278	13,6688	7,3805
	Chromium(III) chloride	0,571719143	0,445148885	0,483630963	0,006671906
	Cobalt(II) chloride hexahydrate	0,842009238	0,775133174	1,138481402	24,255
	Cupric chloride dihydrate	0,884542825	0,709319263	3,531806223	0,474513442
	Manganese(II) chloride	0,833367317	0,780040293	1,465098904	2,443568654
	Magnesium chloride	1,026656283	0,853738467	0,835873339	0,803963298

	Nickel chloride	0,591160386	0,644647782	0,698002771	0,979207561
	Zinc chloride	15,23019711	0,056186229	0,230945003	0,019225223
	Benzethonium chloride	0,007129952	1,035027628	105,0063	16,826
	Protamine sulfate	0,561902125	0,580162114	0,617216763	1,352058413
	Poly-L-lysine hydrochloride	0,611234594	3,562414167	3,155770409	0,526878808
	Cetylpyridinium chloride	0,425402419	86,4866	135,1999	18,7961
	Domiphen bromide	0,819764134	0,905590688	112,7816	93,6274
	Ammonium sulfate	0,540205287	5,319490917	1,544981682	0,031908716
	Lithium chloride	0,943351534	0,746747686	30,6876	12,906
	Aluminum sulfate	0,779923711	0,386402754	3,144156585	1,803129048
	Dequalinium chloride	1,332086944	100,9274	85,8249	131,6576
	Methyl viologen dichloride hydrate	4,242206939	0,08879181	3,602566226	0,076127254
	Dodecyltrimethyl ammonium bromide	0,974836251	18,9137	6,0426	13,2209
	Alexidine	0,651846331	71,9475	20,1407	26,2381
	Palladium(II) chloride	0,566142722	1,385820421	0,301842675	0,783230504
Cyclic Compounds	Promethazine	0,576656114	6,096486012	1,006633863	0,027519553
	Chlorpromazine hydrochloride	712,8908758	63,1161	51,79611041	1
	Cycloheximide	0,761704193	0,731793407	0,488277445	0,527546316
	Trifluoperazine	6,547707462	3,297627119	1,187339328	1,731815026
	Thioridazine hydrochloride	0,720892195	81,0159	14,5204	1
	Fluconazole	0,726109997	0,481668798	1,385987151	0,24024972
	Tamoxifen	0,680299273	3,987947109	1,103025398	2,973247057
	Miconazole nitrate	2,408180002	3,023601838	0,661160353	0,622347455

	Tetrazolium Violet	67,9221	25,351	8,318149151	5,289687198
	Amitriptyline hydrochloride	94,3484	16,0938	1	7,008987045
	Zaragozic acid A	0,859037153	0,621385014	0,441239083	0,027109013
	Berberine chloride	0,535496557	0,534476374	0,768498603	2,817774606
Organic Compounds	L-Aspartic acid β -hydroxamate	0,527201202	0,272220529	1,9301426	0,192475759
	L-Glutamic acid g-hydroxamate	0,919580258	0,3252395	1,28355	14,9237
	L-Arginine hydroxamate	0,272995571	0,766395991	1,242364894	97,1232
	Glycine hydroxamate	0,90047343	125,8618	130,341	57,4253
	D,L-Serine hydroxamate	0,71005631	4,094940018	0,980196621	0,779312226
	D-Serine	0,921085688	0,788998572	1,846717435	0,423404055
	Thialysine	0,641415402	0,379136651	0,093830636	0,616206436
	5-Fluorocytosine	22,9953	9,5643	8,8397	10,0702
	5-Fluorouracil	0,434224832	0,589562953	0,082166511	0,017349635
	Propiconazole	1,019319459	0,944899564	1,717231504	7,835893028
	Sodium benzoate	0,658086943	0,595137316	0,414627415	0,294794006
	Hydroxyurea	0,927303752	0,973626743	1,4635933	34,6698
	Azaserine	0,993592886	26,1043	11,2406	8,0074
	Urea hydrogen peroxide	0,882311325	0,707142777	0,83524981	0,647146476
	Succinic acid	0,588558759	0,749455819	4,130858919	6,484421809
	Malic acid	0,81200416	0,746892881	116,1171117	11,93
	Tartaric acid	0,715899056	0,863348214	1,349553024	0,472823356
	Fumaric acid	0,555503965	0,729482266	0,391694516	0,029410403
	Cinnamic acid	0,7279324	0,60283866	0,820517866	0,255579915
	Fluorodeoxyuridine	0,738949625	0,88403082	0,64534617	0,025929643

	Sodium caprylate	0,668569995	0,000492457	0,785142149	0,379514342
	Sodium salicylate	0,871446245	1,451566972	1,116425516	1,158721632
	Glycine hydrochloride	0,305464516	0,768195944	6,427656032	1,6664
	Caffeine	0,581068288	0,584763512	-0,027557556	0,407093056
	Chloroalanine hydrochloride	0,639348855	1,207465797	1	24,1644
	2-Deoxy-D-glucose	0,933549672	0,04898887	0,694162056	0,026088681
	Clomiphene citrate	0,743879384	10,84070322	1,25755908	0,523860233
	Ibuprofen	0,575054435	0,526822838	0,009472334	0,009164139
	Chloroquine	0,90438508	1,952014801	95,1792	1,148586166
	Guanidine hydrochloride	0,560514614	0,472036148	16,4684	15,9444
	Myclobutanil	0,881334733	0,671275072	0,910570001	0,120332201
	Niaproof	0,204424672	0,326835204	0,013396314	1,22084938
	Polymyxin B	0,681104213	0,808369196	131,5629	31,2397
	Miltefosine	0,764050047	0,536530472	70,0266	80,4243
	FCCP	0,770966091	0,722195143	0,609809362	0,017221316
	CCCP	0,536238434	0,698859432	1,534041249	2,951303286
	Triclosan	0,535580491	19,74128152	1,5741031	14,31785083
Chelators	1-Hydroxypyridine-2-thione	0,659377411	0,713809015	0,872699075	0,326881858
	EDTA	0,060303352	139,2722	131,1858	59,3468
	BAPTA	0,694033001	0,754568557	108,2045	2,685478603
	EGTA	3,32378184	5,524298841	1,919503343	7,9772
	Sodium pyrophosphate decahydrate	0,898079159	0,004844206	0,038167939	0,5009648
	Benzamidine	0,430321088	14,51694264	13,8748	1,269193523

	2,2'-Dipyridyl	0,935680597	0,806389763	0,614217589	17,77987064
Antibiotics	Nystatin	0,601073449	0,42770244	17,8622	12,70788148
	D-Cycloserine	8,930651007	3,426809899	1,0490449	1,153149434
	Apramycin sulfate	0,321616446	0,680379857	0,97953284	1,703078976
	Blasticidin hydrochloride	0,185428493	0,981976352	0,617247989	0,186421111
	Chlortetracycline hydrochloride	0,570075091	0,962393456	3,964574495	0,877984397
	Kanamycin monosulfate	0,803968911	0,394460886	0,17710836	1,119419547
	Paromomycin	9,09245123	3,369269126	2,545472069	1,473417018
	Isoniazid	0,664297147	2,074950488	10,86864453	1,814650767
	Bleomycin	0,858727239	128,0577	60,0417	3,171
	Doxycycline hyclate	0,747915979	0,129392821	0,921354992	2,945978636
	Tobramycin	0,652001938	34,1163	24,0324	3,491393894
	Hygromycin B	1,574526464	20,5526	20,2186	28,2038
	Neomycin	38,85467451	117,1281	36,5881	14,89980923
	Pentamidine isethionate	0,770121877	1,048934036	0,03021075	2,278929717
Nitrogen Compounds	6-Azauracil	0,785968919	0,629048691	0,935396047	1,873251453
	Diamide	0,419083236	0,622899859	0,000447212	2,04423676
	Thiourea	0,087760744	0,604307191	12,64837595	0,425097891
	4-Aminopyridine	0,131371275	0,352979042	29,8476	37,4864
	Sodium azide	0,477258253	0,853401303	0,277645862	25,055
	Sodium nitrite	0,747728396	0,898391171	1,13225515	1,313812402
	Cisplatin	8,865921185	9,5186	4,3742	11,5504
	Aminacrine	1,192822883	7,286863135	1,707009235	2,750340736
	3-Amino-1,2,4-triazole	0,588343945	0,632295326	143,026	39,5857

4-Nitroquinoline-N-oxide	0,859550858	0,737068282	0,724918708	0,984268379
Hydroxylamine hydrochloride	0,861382083	0,698133389	1,059703744	0,000944028
Compound 48/80	0,386493942	2,525333984	136,429	61,3583

Supplementary Table S1. PM21-PM25 chemical compounds divided into functional groups. Drugs from plates PMs 21–25 divided into 6 groups, based on their structure and function: ions, cyclic compounds, organic compounds, chelators, antibiotics and nitrogen compounds. These 24 different chemical agents are presented in 4 different concentrations. The data presented for each drug concentration represent the ratio between the y-maximum value of cells grown with the cocoa-shell treatment and the untreated contro

Groups	Drugs	Effects	References
Ions	Dodecyltrimethyl ammonium bromide	ability to promote disaggregation of the fibrillar assembly (<i>in vitro</i>)	(Pandey et al., 2013)
	Cadmium chloride hydrate	autophagy inducer (in hepatocytes)	(Niture et al., 2021)
	Cetylpyridinium chloride	antimicrobial effect (drug dose-dependently inhibit mitochondrial complex 1 and potent and dose-dependent AMPK inducer)	(Allen et al., 2020)
	Dequalinium chloride	act as an inducer and/or stabilizer of the protofibrils of α -synuclein (<i>in vitro</i>)	(Lee et al., 2006)
	Alexidine	antimicrobial effect	(Kim et al., 2013)

Chelators	EDTA	autophagy inducer (in <i>A. fumigatus</i>)	(Richie et al., 2007)
organic compounds	Glycine hydroxamate	autophagy inhibitor (in neuron through the AMPK pathway both <i>in vitro</i> and <i>in vivo</i>)	(Cai et al., 2019)
	Azaserine	antioxidant effect	(Rajapakse et al., 2009)
Cyclic Compounds	5-Fluorocytosine	autophagy inducer (in BGC-823 cells)	(X.-X. He et al., 2018)
	Tetrazolium Violet	apoptotic inducer (antitumor effect in Human Lung Cancer A549 cells)	(Zhang et al., 2012)
	Amitriptyline hydrochloride	autophagy inhibitor (not block the beginning of autophagy, block its turnover, via inhibiting autophagosome maturation in neuronal cells)	(Kwon et al., 2020)
antibiotics	Hygromycin B	inhibit growth (in <i>S. cerevisiae</i>)	(Kaster et al., 1984)
	Bleomycin	autophagy inducer (in idiopathic pulmonary fibrosis)	(Yue et al., 2022)
	Neomycin	autophagy inducer (in both cochlear HCs and HEIOC-1 cells after neomycin injury)	(Z. He et al., 2017)

Supplementary Table S2. Compounds towards which cocoa-shell extract alters sensitivity, reported in Fig. 5. Compounds were selected when the fold change (ratio between y-maximum value of CBSE treated vs control cells) was >5 or

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Exploring influence of production area and harvest time on specialized metabolite content of *Glycyrrhiza glabra* leaves and evaluation of antioxidant and anti-ageing properties

Docimo T. et al., 2024

Abstract

Calabrian *Glycyrrhiza glabra* is one of the most appreciated licorice varieties worldwide and its leaves are emerging as a valuable source of bioactive compounds. Nevertheless, this biomass is usually discarded, and its valorization could contribute to boost the economic value of licorice-production chain. In this study the effects of production area and harvest time on specialized metabolite content of *G. glabra* leaves (GGL), antioxidant and anti-ageing properties are evaluated to explore the potential of this untapped resource and to select the most optimal harvesting practices. GGL exhibited high levels of specialized metabolites (4-30 g/100 g of dry leaf) and the most abundant ones are pinocembrin, prenylated flavanones (licoflavanone and glabranin) and prenylated dihydrostilbenes. Their levels and antioxidant capacity in extracts are influenced by both production area and harvest time, showing a decisive role on specialized metabolites accumulation.

Interestingly, GGL extracts strongly attenuate the toxicity of α -synuclein, the intracellular ROS content and cellular senescence on *Saccharomyces cerevisiae* expressing human α -synuclein model, showing great potential to prevent aging and age-related disorders. These results provide insights into the phytochemical dynamics of GGL, identifying the best harvesting site and period to obtain a bioactive-rich sources with potential uses in food, nutraceutical and pharmaceutical sectors.

Keywords: *Glycyrrhiza glabra* leaves; untapped resource; prenylated flavanone; prenylated dihydrostilbene; sustainable agriculture; bioeconomy; *Saccharomyces cerevisiae*; human α -synuclein; neurodegenerative disorders

1. Introduction

Glycyrrhiza species are flowering, herbaceous perennial plants of the bean family Fabaceae, worldwide cultivated but native to Europe, Asia, and most of North America. The genus *Glycyrrhiza* comprises approximately thirty species, among which the European *G. glabra*, the chinese *G. uralensis* and *G. inflata*, and the american *G. lepidota* are the most studied [1]. Roots and rhizomes of *G. glabra* (licorice) stand out for well-documented and widely appreciated distinct characteristics related to botanical, culinary, and pharmacological properties [2].

Notably, licorice has a long history of therapeutic applications, including expectorant, carminative, anti-inflammatory, antiulcerous, antibacterial, antifungal, antiviral, antiallergic, and immunostimulant activities. Besides, it is also used as natural sweetener and flavour additive in foods, beverages, functional foods, and food supplements [2,3]. However, excessive consumption of licorice can lead to some side effects such as hypertension and edema [4]. As regards, a particularly distinctive feature of Calabrian *G. glabra* is its lower content in glycyrrhizin [5], which is the compound responsible for the sweet tasting of roots but also for the hypertensive effects. The amount of this compound tends to vary depending on root harvesting period, but also on factors such as soil conditions, climate, and agricultural practice. Indeed, the unique climatic and environmental conditions of the Calabrian region in Southern Italy have been shown having a deep impact on the distinctive aroma and sweetness of Calabrian licorice [5] which holds the "Protected Designation of Origin" (PDO) status.

Besides roots, this perennial plant has a vigorous vegetative biomass which is emerging as a valuable source of bioactive compounds positively affecting human well-being. Nevertheless, during the blossom season this biomass is an untapped resource, and its valorization could contribute indirectly to boost the economic value of licorice cultivation. Current harvesting practices of PDO licorice occur during the fall foliage season, when any leaves present are often discarded or left

to decompose on the soil, resulting in poor quality and quantity of this biomass. Until now, root harvesting has prioritized yield over chemical composition, and a limited exploration into the influence of harvest times and environmental factors on the chemical and biological activity of root extracts has been reported. A recent study, has highlighted the impact of different harvesting periods on the chemical profile and bioactivity of *G. glabra* roots, envisaging alternative harvest times [6, 7, 8, 9]. As regards leaves, however, no studies have been conducted to investigate the effects of growth stage and environment on the phytochemical content and biological activities of *G. glabra* leaves. Knowledge of the influence of these factors on metabolic leaf content and related potentiality might be crucial for envisaging either the most optimal harvesting practices for whole plant valorization and exploration of the potential uses of this untapped resource. These aspects could strongly improve the economic value of Calabrian licorice-production chain. Indeed, various untapped plant materials are currently being investigated as potential sources of bioactive compounds for the production of nutraceuticals and functional ingredients of food. Recent studies indicate that leaves of well-known crops and wild growing plants are unconventional and valuable source of functional and bioactive compound [10,11].

Glycyrrhiza leaf extracts have been assayed for various biological activities, including antiproliferative, antimicrobial, antioxidant, anti-inflammatory, and α -

glucosidase inhibiting activities [12-16]. Recently our UHPLC-HRMS profiling of aboveground and belowground tissues of Calabrian *G. glabra* revealed different distinctive chemical composition of the leaves as compared to roots [17]. In agreement with [14], flavanones were found to be the most abundant class of secondary metabolites, with pinocembrin, licoflavanone, and glabranin as majoritary compounds. The second distinctive class of phytochemicals of *G. glabra* leaves is represented by prenylated dihydrostilbenes, which together with prenylated flavanones represented distinctive chemical markers of leaves [17]. Among the chemical modifications of specialized metabolites, prenylation is the mostly impacting on structural diversity and on biological activity. As compared to non-prenylated counterparts, prenylation confer higher lipid solubility, higher capability to penetrate the membranes and higher potential to interact with different cellular targets [18]. For this reason, prenylated phenolic compounds are promising molecules as drugs for their wide pharmacological spectrum of activities [19]. As an example, many plants within medicinal and food resources, such as *Morus alba*, *Humulus lupulus*, *Glycine max*, *Ficus carica* and *Cannabis sativa* accumulate prenylated phenols, and their extracts have been exploited in oncotherapy [20], metabolic disorders [21], and cardiovascular activities [22]. Regarding *Glycyrrhiza* metabolites, prenylated dihydrostilbenes and flavonoids from *G. uralensis* leaves extracts were investigated as therapeutic agents in liver

fibrosis [12]. Recently, the potential of prenylated phenolics from Mulberry leaves has been also exploited for neuroprotective activity [20]. In the field of neurodegenerative diseases accounting for a large and *increasing* health burden worldwide, drug discovery and development is urgently needed.

Neurodegenerative diseases, characterized by aberrant aggregates of the presynaptic protein α -synuclein, are collectively referred to as synucleinopathies, the second most common group of neurodegenerative diseases [23]. Despite the advances in the study of these pathologies, the detailed molecular mechanism of neuronal degeneration is still largely unknown. Several studies underline the relevant role of cellular models for a better understanding of the molecular regulation of synucleinopathies [24]. Many different models using yeast strains overexpressing human genes associated with diseases like Alzheimer's disease (AD), Parkinson's disease (PD) or Huntington's disease (HD) have also been exploited in the last decades. Among them, one of the most studied yeast model consists on the expression of α -synuclein (α -syn) [25-27], a presynaptic protein whose alteration is associated with PD [23,28,29].

Being *G. glabra* leaves (GGL) a valuable source of beneficial compounds, to harness their potential, in this study we investigated the effect of geographical area of cultivation and harvesting period on the profile of GGL specialized metabolites and

their biological activities. To do so a quantitative profiling of the main GGL compounds from *G. glabra* cultivated in three typical PDO production areas of Calabria region and harvested during three developmental stages, was performed. The influence of these factors on metabolite content was statistically determined to distinguish the patterns either for cultivation area than for harvesting times. Afterwards, *in vitro* antioxidant capacity of the GGL extracts and their chemical markers were evaluated, as well as their anti-ageing and antioxidant properties on a yeast model of *Saccharomyces cerevisiae* expressing human α -synuclein.

2. Materials and Methods

2.1. Chemical and standards

Analytical grade ethanol (EtOH), MS grade formic acid (HCOOH), 6-hydroxy-2,5,7,8-acid tetramethylcroman-2-carboxylic acid (Trolox), 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and the reference standards of isoquercitrin and rutin were provided by Merck Chemicals (Milan, Italy). MS grade acetonitrile and water were purchased from Romil (Cambridge, UK). Ultrapure water (18 M Ω) was prepared using a Milli-Q purification system (Millipore, Bedford, USA).

Pinocembrin (L29), licoflavanone (L36), glabranin (L46), dihydro-3,3',4'-trihydroxy-5-O-prenylstilbene (L33) and dihydro-3,5,4'-trihydroxy-4,5'-diprenylstilbene (L44) were purified by RP-HPLC preparative from *G. glabra* exudate and characterized by 1D- and 2D-NMR (purity $\geq$ 96% from HPLC). Finally, vicienin-2 was previously isolated from natural sources [30].

2.2. Samples

Leaves of *G. glabra* var. *typica* (GGL) were kindly supplied by Nature Med S.r.l. (Castrovillari (CS), Italy) of the Consortium for Protected Designation of Origin "Liquirizia di Calabria PDO". The cultivation and harvesting were conducted according to the disciplinary of the "Liquirizia of Calabria PDO". Plant materials were collected from 3–4 year old plants in three typical PDO production areas of Calabria region: Villapiana (GGL-A), (Cosenza; coordinates (lat, long): 39.810000, 16.470000, elevation: 16 m), Castrolibero (GGL-B) (Cosenza; coordinates (lat, long): 39.322460, 16.192634, elevation: 290 m) and Scandale (GGL-C) (Crotone; coordinates (lat, long): 39.152599, 17.011761, elevation: 63 m). Leaf samples were harvested during three growing phases of 2019: Harvesting Time 1 (HT1), corresponding to the pre-flowering stage, at early May; HT 2, corresponding to the flowering stage, at mid-June; HT 3, corresponding to the senescence stage (root collection), at late October.

For each geographical area at each HT, three representative samples (about 0.5 kg each replicate) were randomly collected.

Drying of leaf materials (ventilated oven with a temperature of 50 °C) was carried out by Nature Med S.r.l. using standardized procedures in accordance to set rules of PDO disciplinary. Dried leaf samples were ground using a knife mill Grindomix GM 200 (Retsch, Haan, Germany), operating at intervals of brief cycles. The resulting ground materials were sieved through a test sieve with a range of 300 - 600 µm to obtain a sample with a homogeneous particle size distribution.

2.3. Ultrasound-assisted extraction (UAE)

Exhaustive extracts of GGL samples from each biological triplicate were prepared according to the methodology proposed by Celano et al. [17]. Briefly, 500 mg of homogenized samples were extracted in duplicate with 10 mL of aqueous ethanol (70 % v/v), for 30 min at 30 °C in a thermostat-controlled ultrasounds bath (Labsonic LBS2, Treviglio, Italy). The extraction process was repeated three times with fresh solvent. The extracts were pooled and diluted with distilled water to 50 mL, then filtered with 0.45 µm regenerated cellulose filters (Chromafil Xtra RC-20/13, Delchimica, Italy) for the chromatographic analysis.

For ABTS assay and biological assay, triplicate solution of each GGL sample were freeze-dried (freeze dryer Alpha 1–2 LD, Christ, Germany), after the removal of the

organic solvent under vacuum at 40°C in a rotary evaporator (Rotavapor R-200, Buchi Italia s.r.l, Cornaredo, Italy). The dried extracts were properly dissolved before assays according to procedures reported in sections 2.5 and 2.6.

2.4. Quantitative analysis

Quantitative analysis of GGL extracts was performed using a Platin Blue UHPLC system, (Knauer, Labservice Analytica, Bologna, Italy) consisting of two Ultra High-Pressure Pumps, an autosampler, a column temperature manager and a diode array detector. UHPLC separation was performed using a previously developed method [17]. Briefly, the chromatographic separations were performed using a Kinetex C18 column (2.1 × 100 mm, 1.6 µm; Phenomenex, Bologna, Italy) protected by a C18 Guard Cartridge (2.1 mm I.D.) and thermostated at 25 °C. A binary gradient of H₂O and MeCN, both containing 0.1% of formic acid, at a flow rate of 500 µL min⁻¹ was employed. After each injection (5 µL), the column was washed with 98% B for 4 min and re-equilibrated (4 min). UV spectra were acquired in the range of 200-600 nm and three wavelengths were selected for the detection of target analytes based on the maximum absorbance of pure compounds (Figure S1): 220 (prenylated dihydrostilbenes), 290 (flavanones) and 360 (flavones-C-glycosides and flavonols-O-glycosides) nm.

External standards method was employed to determine the levels of main compounds in the GGL extracts. Stock solutions of reference standards (vicenin-2, rutin, isoquercitrin, pinocembrin, licoflavanone, glabranin, dihydro-3,3',4'-trihydroxy-5-O-prenylstilbene (L33) and dihydro-3,5,4'-trihydroxy-4,5'-diprenylstilbene (L44)) were prepared in MeOH at a concentration of 5 mg mL⁻¹ and stored at -20 °C. A mixture of reference standards was prepared at concentration of 1 mg mL⁻¹, and the calibration levels were prepared by appropriate serial dilution with MeOH/H₂O, 1:1 v/v, and analysed in triplicate. The linearity of the calibration curves was evaluated in the concentration range of 6.25 – 100 µg mL⁻¹ for vincenin 2, rutin and Isoquercitrin, and 12.5 - 200 µg mL⁻¹ for the remaining reference compounds. The regression curves were tested with the analysis of variance (ANOVA) and linear model was found appropriate over tested concentration range (R² values > 0.999). The samples were analysed after appropriate dilutions, to fall within the dynamic calibration range. The quantification of compounds of GGL extracts, for which no reference standards were available, was determined using the calibration curves of standards belonging to the same class of secondary metabolites (similar chromophore). The amount of the compounds was expressed as mg per g of dry leaf material (mg/g DM) ± standard deviation (n = 3 biological replicates).

2.5. ABTS assay

The antioxidant activities of the exhaustive extracts and the major GGL compounds, vicianin-2, rutin, isoquercitrin, pinocembrin, licoflavanone, glabranin, dihydro-3,3',4'-trihydroxy-5-O-prenylstilbene (L33) and dihydro-3,5,4'-trihydroxy-4,5'-diprenylstilbene(L44), were evaluated using ABTS assays adapted for use in 96-well plates as reported in [31]. A microplate spectrophotometer reader Multiskan Go (Thermo Scientific) was employed for the assays. GGL dried extracts were dissolved in methanol at the concentration of 1 mg/mL and then diluted with with 5 mM PBS (pH 7.4) to provide approximately 20–80% control absorbance. ABTS results were expressed as Trolox equivalent antioxidant capacity (TEAC) per g of extract (mmol TE/g) or per mmol of pure compound (mmol TE/mmol). In the ABTS assay, the curves of Trolox, pure compounds and extracts were obtained by plotting concentration (mM for Trolox and standards and mg g⁻¹ for leaf extracts) against the average % Inhibition of radical absorbances.

2.6. Yeast Strains and Media

Yeast strain used to evaluate the biological activity of dried GGL extracts is BY4742 MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0 [pYX242- SNCA] overexpressing human α -synuclein. Cells were grown at 30 °C in minimal medium containing 2% glucose as a carbon source and 0.67% yeast nitrogen base without amino acids and supplemented with 50 mg/L of required amino acids and bases for which the strain

was auxotrophic, with a constant shaking (120 rpm). GGL extracts were resuspended in 150 μ L of EtOH 100%, vortexed, incubated in the termomixer at 25 °C for 1 hour, sonicated for 20 minutes and dissolved in the liquid medium at a concentration of 0.2% of the yeast strain and filtered through 0.22 μ m filters, as previously reported [28,29].

2.7. Chronological Lifespan Experiments (CLS)

Yeast cells were grown in liquid medium until the mid-late exponential phase, centrifugated (13000 rpm, 5 min) and then inoculated at 0.150 OD/mL (Optical Density /mL) in flasks containing medium in the absence (negative control) or presence of the extracts (0.2% concentration). Survival was assessed by propidium iodide staining (PI) at the indicated time points with the Cytoflex cytofluorimeter (Beckman Coulter) and analyzed with the Cytoflex software, as previously reported [28,29].

2.8. Analysis of Reactive Oxygen Species (ROS) Levels

Analysis of cellular superoxide was performed by DHE (dihydroethidium) staining. Cells were collected after 24 h treatment with 0.2% GGL extracts and 0.2% were resuspended in PBS and stained with 5 μ g/mL for 10 min. FACS analyses were performed with Cytoflex cytofluorimeter (Beckman Coulter) and analyzed with the Cytoflex software, as previously reported [25,26].

2.9. Statistical analysis

The quantitative data and TEAC values were subjected to analysis of variance (ANOVA), using Statgraphic Centurion 18 software from Statgraphics Technologies (Arezzo, Italy). A two-way ANOVA (3 production areas $\times$ 3 HTs) was applied to each GGL marker, the total of metabolite classes and TEAC values. The means were separated using the Tukey HSD test only when the F-test for factors and their interaction were significant at the P-values < 0.05 probability level.

With regard to the CLS assays and analysis of ROS levels, experiments were performed in duplicate, and results are expressed as mean $\pm$ SD (standard deviation). Results were compared by using the two-sided Student's t-test. Differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Quantitative profile of *Glycyrrhiza glabra* leaves

Our recent study, conducted on *G. glabra* var. *typica* cultivated for the production of PDO certified Calabrian licorice, has revealed that the leaves represent a rich source of unusual bioactive compounds with potential uses in nutraceutical, pharmaceutical and cosmetic sectors [17].

In the current study, the quantitative profiles of *G. glabra* leaves (GGL) from different production areas of Calabria and collected in different harvest times were determined by UHPLC-UV analysis to investigate the influence of cultivation site and phenological stages on the phytochemicals content of this untapped resource. GGL samples were harvested from three typical areas dedicated to the production of PDO Calabrian licorice: Villapiana (GGL-A), located near the Ionian Sea; Castrolibero (GGL-B), located in the hinterland of the Tyrrhenian coast; and Scandale (GGL-C), located further south in the hinterland of the Ionian coast. From each production area the leaf samples were collected in three different harvesting times corresponding to three growing stages HT1, (pre-flowering stage); HT2, (flowering stage); HT3, (senescence stage, corresponding to the root collection) [32].

Untargeted UHPLC-HRMS analysis [17] indicated that the qualitative profiles of investigated GGL extracts were comparable among production areas and harvesting times. The main compounds detected in UHPLC-PDA profiles (Figure 1) were: the flavanones pinocembrin (L29), licoflavanone (L36), 8/6-prenylnaringenin (L35), glabranin (L46) and glabranin isomer (L48); the flavone C-glycosides vincenin 2 (1) and iso/shaftoside (2); the flavonol O-glycosides rutin (L11), isoquercitrin (L12) and astragalin (L17); and the prenylated dihydrostilbenes dihydro-3,5,3',4'-tetrahydroxy-5'-prenylstilbene (L28), dihydro-3,3',4'-trihydroxy-5-O-prenylstilbene

(L33), dihydro-3,5,3',4'-tetrahydroxy-4,5'-diprenylstilbene (L41) and dihydro-3,5,4'-trihydroxy-4,5'-diprenylstilbene (L44). These compounds were assumed as GGL markers and quantified in all GGL samples by UHPLC-UV analysis and external calibration method (Table S1).

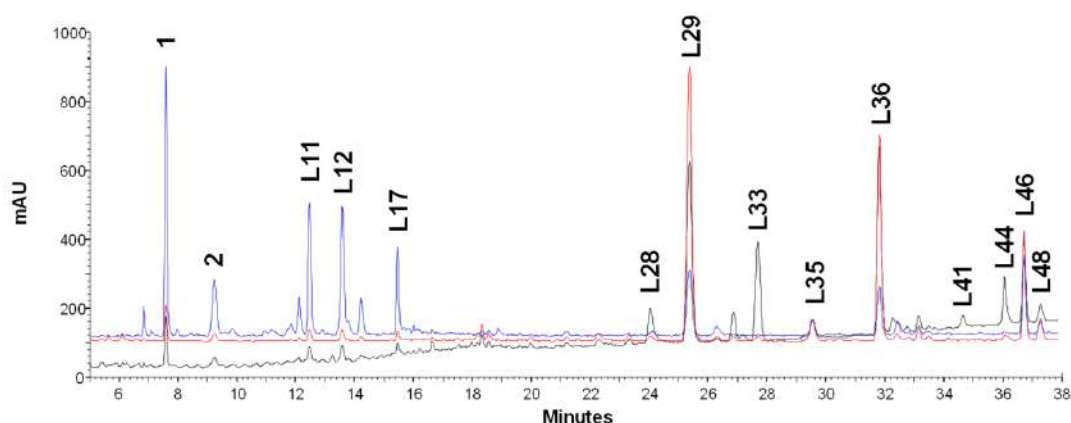


Figure 1. A representative UHPLC-UV profile of *G. glabra* leaves (GGL-B2): black line, 220 nm; red line 290 nm; blue line 360 nm.

As reported in Table S1, the leaves of Calabrian *G. glabra* exhibited high levels of specialized metabolites, with a total content ranging from 4 to 30 g/100 g of dry leaf DM. The changes in the level of abundance of each metabolite class and the most abundant compounds (pinocembrin, licoflavanone, glabranin and stilbene L33) are shown in Figure 2. Overall, the most abundant class of secondary metabolites was represented by flavanones, constituting 33-61% of the total content of quantified metabolites, with pinocembrin (L29) and licoflavanone (L36)

as the major compounds (each accounting for 11-21% of the total content). Following, in order of abundance, prenylated dihydrostilbenes with a content ranging from 10 to 37% of the total content (L33 and L44 as the most abundant compounds), flavone C-glycosides (17-32% of the total content), and flavonol O-glycosides (4-10% of the total content) (Table S1 and Figure 2).

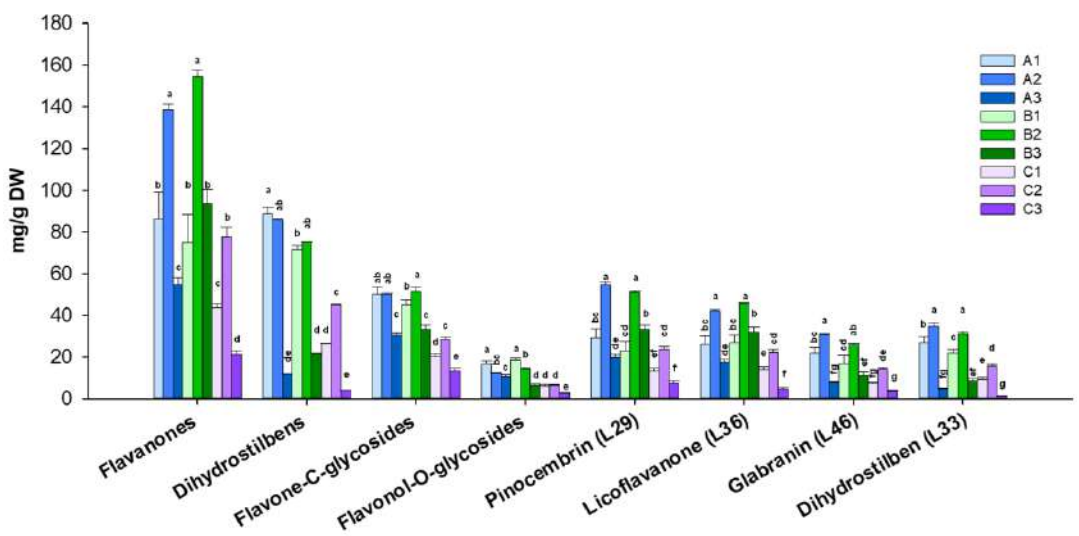


Figure 2. Levels of metabolite classes and the most abundant compounds from three production areas (P) and three harvest times (HT) of *G. glabra* leaves. Different letters within each metabolite class or compound indicate significant differences between extracts ($p \leq 0.05$ by a post-hoc Tukey's HSD test).

Quantitative data of GGL phytochemicals revealed considerable variations of their content both in relation to the production areas (P) and harvesting times (HT) (Table S1 and Figure 2). The levels of each GGL marker and of metabolite classes were

statistically evaluated according to the influence of P and HT. Two-way ANOVA analysis indicated that both P and HT statistically affected the levels of all GGL markers. Also, the interaction between the P and HT proved to be statistically significant for all the analyzed compounds and the metabolite classes (Table S1 and Table 1).

Factor	Flavanones	Dihydrostilbenes	Flavones	Flavonols	L29	L36	L46	L33	TEAC
P (2) ^b	183 *	132 *	417 *	294 *	210 *	211 *	101 *	216 *	40 *
HT (2) ^b	239 *	318 *	202 *	182 *	250 *	171 *	168 *	531 *	114 *
P x HT (4) ^b	15 *	21 *	9 *	27 *	26 *	13 *	11 *	25 *	3.4 *

^a Values are given as F-ratio, * denote significant at $p \leq 0.001$. of Fisher; ^b degrees of freedom in brackets.

Table 1. F ratios of production area (P) and harvest time (HT) and their interaction of specialized metabolites content in *G. glabra* leaves ^a.

Particularly, the variation in the levels of the most abundant compound classes, flavanones and prenylated dihydrostilbenes, is predominantly attributed to the

dynamic influence of development stages (HT), which shaped the overall composition of bioactive compounds, according to the plant's growth cycle. On the other hands, the contribution of P on the variance was greater for flavone C-glycosides and flavonol O-glycosides (Table 1). The effects of P and HT on the content of the different metabolite classes agree with their distinct localization in the leaf [17], suggesting a different biological role. Namely, the phytochemicals flavanones and dihydrostilbenes, accumulated in the leaf exudate are likely responsible for the resistance to pathogens and herbivores attack and drought stress; while glycosylated flavones and flavonols, distributed solely in the inner leaf tissue, might be involved in photoprotection shielding plant cells from photooxidation [17,33].

Considering the harvesting time, the levels of GGL specialized metabolites resulted significantly different among the three time frame of our study (Figure S2). HT3 exhibited the lowest levels of each compound or metabolite class, regardless of the production area (Table S1 and Figure 2). These results can be easily attributed to developmental changes associated with plant growth and, in particular, with leaf senescence and the plant recycling activities of catabolism, which are necessary for nutrient remobilization from senescing leaves to sink tissues [33-35]. Namely, different trends in the content of the various compound classes are observed, especially for the most abundant classes. A significant variation in the flavanone

levels was observed in the three HTs: both the total content and the major flavanones (pinocembrin, licoflavanone and glabranin) more or less doubled between HT1 and HT2, and then decreased in HT 3 by 61, 40 and 73 % in GGL-A, GGL-B and GGL-C, respectively. On the contrary, the levels of prenylated dihydrostilbenes did not show relevant variations between HT1 and HT2, but undergo a drastic reduction, ranging from 71% to 91%, from HT2 to HT3 for all three production areas (Figure S2). Pinocembrin, prenylated flavanones and dihydrostilbenes are accumulated in the sticky exudate that cover the surface of *G. glabra* leaves [17]. It has been reported that *G. glabra* exudate is most likely responsible for the resistance to biotic and abiotic stresses such as pathogens, herbivores attack and drought stress [36]. The well-known antimicrobial and cytotoxic activities of pinocembrin and licoflavanone further support their role in insect deterrence and defence against enemies feeding [12,13,37,38]. This is in agreement to what reported in eucalypt oil glands, where pinocembrin has been identified as main component and its biological properties, such as antibacterial, antifungal and antifeedant activities, clearly related to plant defence [39-41]. Thus, during flowering the high levels of flavanones and prenylated dihydrostilbenes are most likely part of a defensive strategies of licorice plant [42].

A similar variation in the flavone C-glycosides was observed, regardless of the production area. Flavonol-O-glycosides instead showed a significant content

decrease in both A and B production areas from HT1 to HT2, whereas in C their content was not significantly changed during these two harvesting times (Table S1 and Figure 2). This different behaviour between A/B and C production areas during the three HTs can be associated with environmental conditions. HT1 e HT2 (from late Spring to Summer) encompass seasons characterized by high UV light, especially in Southern Italy, then the accumulation of these antioxidant compounds is expected to increase, but our results suggest that possibly plants located in A and B areas might experience a better shading than plants growing in the C are [43].

Our data also highlight relevant differences among the different production areas. For all quantified compounds, GGL-C displayed content means statistically different compared to GGL-A and GGL-B; on the contrary, no statistically significant differences were observed between A and B areas, except for the content of prenylated flavanones (Figure S2). In details GGL-A and GGL-B, in all three studied harvest times showed the highest content of metabolites (10-29 and 15-30 g/100 g DM, respectively), whereas in GGL-C the lowest total content of metabolites (4-16 g/100 g DM) was detected, and a statistically significant lower content of each analyzed compounds was observed in these samples as compared to the other two (Table S1 and Figure 2). In particular, since it is widely known that pedoclimatic conditions play a crucial role on the accumulation of secondary metabolites by influencing the uptake of water and nutrients, these results to point out that the

significantly lower phytochemical amounts in GGL-C samples might reflect the different, and possibly less pleasant, growth condition experienced by *G. glabra* in the hinterland of Ionian coast [44]. On the other hand, GGL-A and GGL-B extracts, exhibited a similar content of phytochemicals, which is more than double compared to GGL-C, and generally no marked differences among the different metabolite classes were observed.

Overall, the quantitative profile of GGL indicates that this untapped resource can provide a bioactive-rich matrix if harvested in A and B area and in the first two harvesting times.

3.2. Antioxidant activity in vitro and in vivo of Glycyrrhiza glabra extracts

In order to evaluate how the differences recorded in GGL phytochemical profiles reflect biological properties, the antioxidant capacity of GGL extracts were evaluated spectrophotometrically by ABTS *in vitro* assay (Figure 3a). As well, to ascertain which specialized metabolites mostly contribute to the antioxidant activity, distinctive GGL compounds were assayed for their antioxidant potential (Table 2).

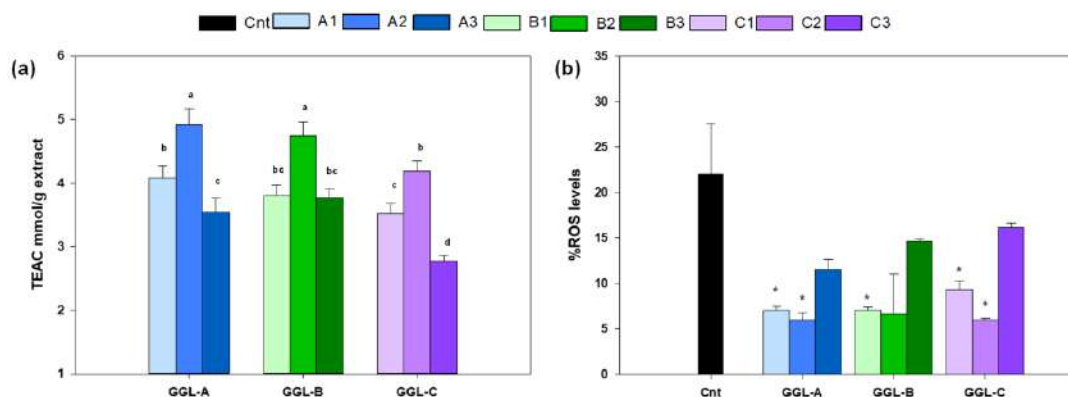


Figure 3. Antioxidant capacity of *G. glabra* leaf extracts from three production areas and three harvest times, measured as (a) TEAC equivalents by ABTS assay and (b) ROS content (assayed by DHE staining) of yeast cells BY4742 bearing pYX242-SNCA plasmid grown in minimal medium containing 2% glucose for 24 hours in the absence (cnt) or presence of 0.2% *G. glabra* leaves extracts. Different letters in (a) indicate significant differences between extracts ($p \leq 0.05$ by Tukey's HSD test). * $p < 0.05$ relative to control cells. Data of (b) represents mean and standard deviation of at least two independent experiments.

Also, for *in vitro* antioxidant capacity, two-way ANOVA analysis revealed a statistically significant variation across the production area (P) and according to the harvesting time, as well as the interaction P x HT (Table 1). Antioxidant activity of GGL extracts (Figure 3a) changed according to the quantitative profiles. As regards to the harvest time, GGL samples collected in HT 2 showed significantly higher antioxidant activities than HT1 and HT3 (Figure S2), consistently with the

metabolite enrichment characteristic of this growth stage. Conversely, no statistically significant differences were observed between A and B areas (Figure S2). These data further support the best harvest conditions identified by the quantitative analysis of GGL markers, suggesting that these compounds are most likely responsible for antioxidant activity. Indeed, as shown in Table 2, the tested compounds showed higher antioxidant powers than Trolox, with the following activity order licoflavanone > isoquercitrin > rutin > stilbene L33> glabranin > pinocembrin > stilbene L44 > vincenin 2. Interestingly, the most abundant compounds (licoflavanone, stilbene L33, glabranin and pinocembrin) showed superior or comparable antioxidant activity to the flavonols rutin and isoquercetrin, the content of which was distinctly lower in the GGL extracts. Thus, the antioxidant capacity of GGL might be attributed to high content of prenylated flavanones and dihydrostilbenes. These results are in agreement with what has been reported in previous studies [15,45,46] on bioactivity of *G. glabra* leaf extracts.

Table 2. Antioxidant activity of GGL main compounds by ABTS assay.

Compound	TEAC (mmol TE/ mmol±SD)
Vincenin 2	1.12±0.01

Rutin	6.00±0.04
Isoquercitrin	6.45±0.03
Pinocembrin	3.21±0.01
Licoflavanone	9.55±0.04
Glabranin	4.08±0.03
Prenylstilbene (L33)	5.09±0.01
Prenylstilbene (L44)	2.50±0.01

^aValues are means of three replicates.

Oxidative stress, resulting from the excessive intracellular accumulation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and other free radical species, contributes to the onset and progression of various diseases, among which Parkinson's disease (PD) [47]. Based on results of *in vitro* antioxidant capacity of *G. glabra* leaves and its main specialized metabolites and in order to estimate the ability of GGL extracts to exert an antioxidant response at the cellular level, not just

their radical scavenging capacity, the antioxidant effect of the GGL extracts was evaluated on a yeast model of *Saccharomyces cerevisiae* expressing the human α -synuclein. As shown in Figure 3b, the treatment with GGL extracts remarkably reduced intracellular ROS level in exponentially growing yeast cells, with significant antioxidant effects for the GGL extracts with the highest phytochemical contents (harvesting times 1 and 2).

3.3. Anti-aging effects of Glycyrrhiza glabra extracts in yeast cells expressing human α -synuclein

α -Synuclein is a presynaptic protein associated with the pathophysiology of synucleinopathies, including Parkinson's disease [23,48] and budding yeast has been extensively employed in models of synucleinopathies [25,26]. Thus, the effect of GGL extracts on the longevity of yeast cells over-expressing the human α -synuclein was evaluated. In order to evaluate whether GGL extracts could exert anti-aging effects, they were added to exponentially growing yeast cells expressing the human α -synuclein at a concentration of 0.2% in a synthetic minimal medium. Results show that all the extracts prolonged chronological lifespan (CLS) of yeast cells (Figure 4a), with a significant marked increase in both mean and maximal lifespan. In fact, mean lifespan resulted 2.5 times longer while maximal lifespan increased up to 3 times relative to the control (Figure 4b).

Overall, the data presented indicate that *G. glabra* extracts delay senescence by reducing intracellular ROS level and α -synuclein toxicity.

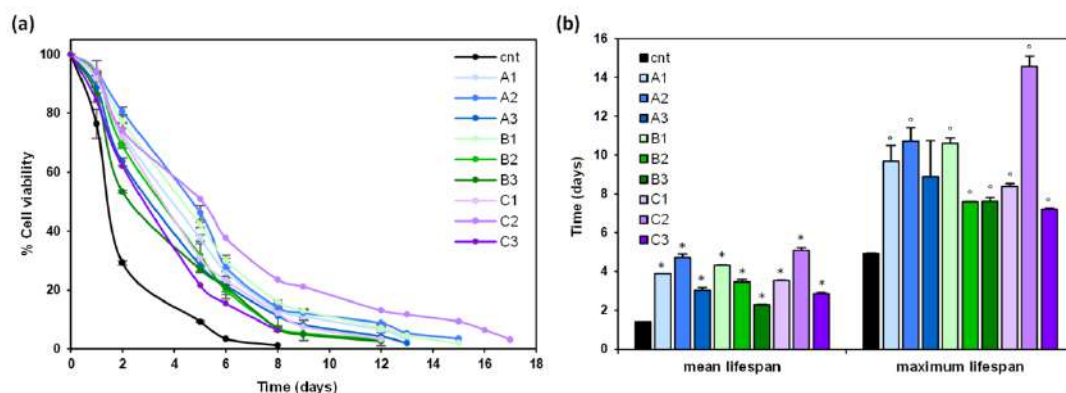


Figure 4. Effect of *G. glabra* leaf extracts on α -synuclein toxicity: (a) Chronological lifespan (CLS) of yeast cells BY4742 bearing pYX242-SNCA plasmid grown in minimal medium containing 2% glucose in the absence (cnt) or presence of 0.2% GGL extracts; (b) mean and maximum lifespan of CLS of yeast cells as in (a). * $p < 0.05$ relative to mean lifespan in control cells, ° $p < 0.05$ relative to maximum lifespan in control cells.

4. Conclusions

In this study, we explored the influence of different harvest times and cultivation areas on *G. glabra* leaves phytochemical content, showing a direct influence of these factors on specialized metabolites production. According to the results, different harvest periods have a strong impact on the concentration of bioactive

compounds especially on flavanones and dehydrostilbenes, thus influencing also GGL extracts bioactivities.

Regarding antioxidant and antiaging power of GGL extracts, our results clearly indicate that harvesting time is essential for obtaining desired bioactive chemical constituents. Aging and age-related neurodegeneration are among the major challenges in modern medicine because of the progressive increase in the number of elderly in the world population. Nutrition, which has evident long-term consequences for health, is an important way to prevent diseases and achieve healthy aging. Our study successfully demonstrates the beneficial effect of GGL extracts on senescence related to α -synuclein toxicity along with their notable antioxidant properties, supporting the role GGL extracts as a functional food in age-related disorders.

Overall, this study provides important theoretical foundations and technical support for selecting best cultivation area in order to optimize *G. glabra* cultivation in Calabrian region, also offering the possibility to envisage the most favorable harvest time for valorization of *G. glabra* leaf resource according to the principles of sustainable development in agricultural processes.

Supplementary Materials

The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox13010093/s1>, Table S1: Level of the main *G. glabra* leaves according to the production area (P) and harvesting time (HT) on metabolite content of *G. glabra* leaves; Figure S1: UV spectra of main GGL compounds; Figure S2: Means plot of metabolite contents and TEAC of *G. glabra* leaves according to the production area (P) and harvesting time (HT).

Author Contributions: Conceptualization, T.D., R.C., M.R. and A.L.P; methodology, T.D., R.C., P.C. and A.L.P; validation, R.C., V.S. and A.L.P; formal analysis, T.D., A.L., R.D.S., S.S. and V.S.; investigation, T.D., R.C., A.L., R.D.S., S.S. and V.S.; resources, P.C., M.R., L.R. and A.L.P; writing—original draft preparation, T.D., R.C., P.C. and A.L.P; supervision, R.C. and A.L.P; project administration, M.R., L.R. and A.L.P; funding acquisition, M.R. and L.R. All authors have read and agreed to the published version of the manuscript.

Funding: Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.4—Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union—NextGenerationEU; Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17

June 2022. Adopted by the Italian Ministry of University and Research, CUP, H43C22000530001, Spoke 6, Project title “National Biodiversity Future Center—NBFC”.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: This work was supported by the project “OroNero” (CUP: J88C17000130006) POR Calabria 204-2020 and by Research Infrastructure Saf@med-Food Safety platform, and the project “ON Foods - Research and innovation network on food and nutrition Sustainability, Safety and Security – Working ON Foods” (CUP:H43C22000820001) of National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.3—Research funded by the European Union—NextGenerationEU. We also thank to Dr. Antonio Massarotto and Dr. Marco Massarotto for the support in the sampling and treatment of plant materials.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 3

An innovative and multidisciplinary approach was adopted to the discovery of novel neuroprotective agents from Mediterranean plants. Rooted in the “One Health” concept, which recognizes the interconnectedness of human, animal, and environmental health, we leveraged bioprospecting within plant biodiversity, focusing on a collection of 60 Italian plant species representing the Mediterranean flora, a significant and exceptionally rich biodiversity hotspot.

We developed an integrated strategy to select the most promising neuroprotective plant extracts. This approach combined an *in silico* phylogenetic analysis, aimed at identifying related medicinal plants, with a high-throughput *in vivo* screening using a *Saccharomyces cerevisiae* yeast model of PD overexpressing human α -syn. This integrated workflow allowed the rapid and cost-effective selection of biologically relevant candidates.

Our analysis led to the identification of four plant extracts with promising neuroprotective properties: *Allium lusitanicum*, *Salvia pratensis*, *Verbascum Thapsus* and *Glaucium flavum*. These extracts demonstrated strong anti-aging and antioxidant activities in the yeast model and robust inhibitory effects on α -syn amyloid aggregation *in vitro* and in neuroblastoma cells overexpressing the protein.

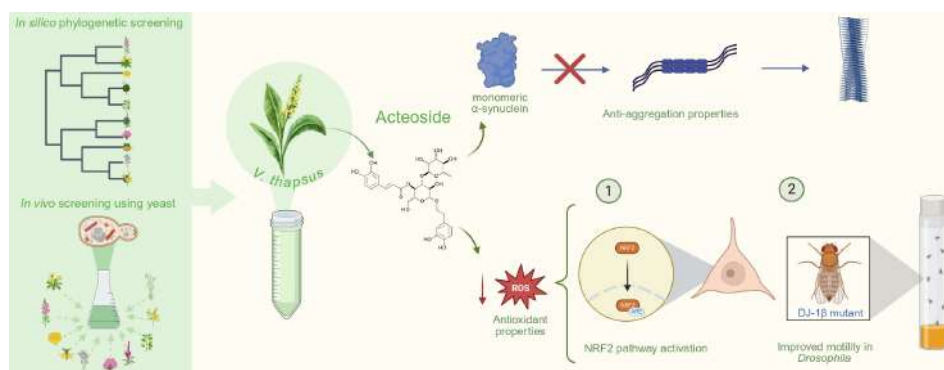
As detailed in this article, we subsequently identified the key metabolite responsible for the anti-aggregant activity. Using a size exclusion chromatography affinity approach coupled with mass spectrometry (SEC AS-MS), we identified the phenylpropanoid glycoside acteoside (also known as verbascoside) from the extract of the edible plant *V. thapsus* as the molecule that directly binds α -syn and effectively inhibits its fibril formation.

We further evaluated the neuroprotective properties of acteoside using biophysical techniques, neuroblastoma cell cultures and a *Drosophila melanogaster* PD model based on inactivation of the *DJ-1 β* gene.

The results presented in this article demonstrate that acteoside exerts a dual action: it efficiently inhibits α -syn aggregation by targeting both primary nucleation and fibril elongation phases, and it significantly reduces oxidative stress. The antioxidant effect of acteoside is mediated through activation of the NRF2 signaling pathway in neuroblastoma cells. Furthermore, in the *Drosophila* PD model, acteoside improved motor performance and reduced oxidative protein damage, even when treatment was initiated in adulthood.

In conclusion, the findings presented in this article provide robust evidence that acteoside possesses both inhibitory effects on α -syn aggregation and strong antioxidant properties, supporting its potential as a neuroprotective candidate for

PD. We believe these discoveries offer a promising pathway for the development of novel strategies targeting PD-related synucleinopathies.



Graphical abstract

Acteoside exerts neuroprotective effects by preventing α -synuclein aggregation and oxidative stress in models of Parkinson's disease

Lambiase A. et al., 2025

Abstract

α -Synuclein is a small presynaptic protein whose aggregation is one of the hallmarks of Parkinson's disease (PD). In our quest to identify novel preventive or therapeutic treatments for PD, we collected 60 Italian plant species, representative of part of the Mediterranean flora, which were screened by a phylogenetic analysis in conjunction with a high-throughput screening in a yeast model of PD expressing human α -syn. The integration of these approaches led to the identification of four plants, *Allium lusitanicum*, *Salvia pratensis*, *Verbascum thapsus* and *Glaucium flavum*, whose extracts, characterized by a metabolomic analysis, exhibit robust inhibitory activity against the amyloid aggregation of α -synuclein *in vitro*, as well as in neuroblastoma cells overexpressing the protein. By employing a size exclusion chromatography affinity approach coupled to mass spectrometry, we identified the phenylpropanoid glycoside acteoside from the extract of the edible plant *V. thapsus* as the metabolite that directly binds α -synuclein and effectively inhibits its fibril

formation. In addition, acteoside reduces oxidative stress in neuroblastoma cells exposed to α -synuclein fibrils and activates the NRF2 pathway. Notably, acteoside improves motor performance in a *Drosophila* model of PD and exhibits a significant reduction of protein carbonyl groups, suggesting that this compound may mitigate oxidative stress-induced protein damage. Our findings could pave the way for the development of new strategies aimed at discovering novel neuroprotective agents targeting PD-related diseases.

Keywords: *Verbascum thapsus*; verbascoside; lifespan; *Saccharomyces cerevisiae*; *Drosophila melanogaster*

Introduction

Aging is a complex physiological condition influenced by various biological pathways that not only affect lifespan but also play a crucial role in the development and progression of age-related diseases. Among these, Parkinson's disease (PD) is a neurodegenerative disorder affecting 2-3 % of the population over 65 years, clinically characterized by movement disorders and cognitive impairment, and by the misfolding and aggregation of α -synuclein at the molecular level [1–3]. α -synuclein (α -syn) is a small presynaptic protein, mainly expressed in neurons of the central and peripheral nervous system, involved in the regulation of vesicle

trafficking and neurotransmitter release [4]. It is an intrinsically disordered protein that can adopt an α -helical conformation when associated with biological membranes. Mutations or overexpression of α -syn can lead to aggregation into toxic oligomers and fibrils, which alter vesicle trafficking, mitochondrial activity and induce cellular toxicity, ultimately leading to the loss of dopaminergic neurons [2].

The onset of age-related diseases is also shaped by environmental factors. According to the One Health concept, the health of humans, animals, plants, and the environment are closely linked and inter-dependent, and by means of a collaborative and *trans*-disciplinary approach it aims to achieve improved health and well-being outcomes. Through the bioprospecting approach, biodiversity enables the identification of a number of compounds (from diverse sources such as plants, marine organisms, and microorganisms) with bioactive properties beneficial for preventing or treating human diseases. Indeed, the potential of natural compounds is huge, and recent years have seen a surge in publications highlighting their numerous neuroprotective effects [5,6]. In this context, selecting plant candidates is a critical step before analyzing their biological activities. As Najmi et al. [7] suggested, various approaches can be employed: selection based on ethnopharmacological knowledge, traditional medicine systems, or random selection. However, it has been suggested that closely related taxa may share biosynthetic pathways of similar phyto-metabolites and that their chemical

molecules are generally conserved with each other [8,9]. Molecular phylogenetic approaches offer an alternative method to identify plant-based natural products reducing the time and cost of subsequent biological analysis [9,10].

The National Biodiversity Future Center in Italy is dedicated to conserving, restoring, monitoring, and enhancing biodiversity within the Mediterranean biodiversity hotspot [11] (<https://www.nbfc.it>). The Mediterranean basin, with its diverse and heterogeneous plant life, boasts a significant number of endemic species, likely due to its varied geography, environmental conditions, and historical influences [12]. Although many Mediterranean plants have been described, they remain a vital resource for research in biochemistry, phytochemistry, and pharmacology, potentially leading to the discovery of new therapeutic molecules. This study was designed to develop an innovative integrated approach to select the most promising neuroprotective plant extracts from a library of Mediterranean plants. We exploited the information present in a broad phylogenetic framework, combined with a high-throughput screening in a yeast model of PD expressing human α -syn. From the selected plant extracts, we identified the phenylpropanoid glycoside acteoside, with strong anti-aggregant activity against α -syn fibrillation. The neuroprotective properties of acteoside were deeply evaluated through biophysical techniques, in neuroblastoma cell cultures and in a *Drosophila*

melanogaster model of PD based on the inactivation of the *DJ-16* gene [13], the fly ortholog of human *DJ-1* (*PARK7*), mutated in early-onset familial PD forms [14].

Materials and methods

Sample collection and extraction

Aerial vegetative organs (leaves and eventually young herbaceous stems) of the 60 plant species investigated were collected in triplicate from plants growing in the botanical garden of Padova (Italy), grown in a nursery or in the open field, as specified in Supplementary Table S1. The samples were immediately frozen in liquid nitrogen, ground into a fine powder using an A11 basic analytical mill (IKA-Werke, Staufen, Germany), and then stored at -80°C. Approximately 1 g of frozen powder was subjected to extraction using 10 volumes (w/v) of 100% LC-MS grade methanol (Honeywell, Seelze, Germany). The mixture was vortexed for 30 s, sonicated on ice for 10 min in a 40-kHz ultrasonic bath (SOLTEC, Milano, Italy), and then centrifuged at 14,000 g for 10 min at 4 °C. The collected supernatants were divided into ten 1.5 mL tubes, with each tube containing 1 mL of extract, and stored at -20 °C. Nine aliquots were then subjected to processing with a vacuum evaporation system (SpeedVac, Heto-Holten, Frederiksborg, Denmark) and allocated for the following *in vitro* bioassays, while one aliquot was kept for LC-MS analysis.

Untargeted Liquid Chromatography-Mass Spectrometry (LC-MS) metabolomics analysis

The untargeted LC-MS metabolomics analyses were performed using the instrumentation and methodology described in previous studies [15,16] with slight modifications. In brief, the extracts were diluted at 1:10, 1:50 and 1:100 with LC-MS grade water (Honeywell) and filtered through 0.22 μ m Minisart filters (Sartorius-Stedim Biotech, Göttingen, Germany). The analyses were performed with Ultra-Performance Liquid Chromatography-High-Resolution Mass Spectrometry (UPLC-HRMS) and 5 and 1 μ L were injected into the instrument for analysis in both negative and positive ionization modes, respectively. The elution was carried out in gradient mode among two mobile phases (A: 0.1% formic acid in water, B: 100% acetonitrile). The elution gradient commenced at 1% B, held for 1 min, then increased to 40% B at 10 min, 70% B at 13.5 min, 90% B at 15 min, and reached 99% B at 16.5 min. The method was then maintained at 99% B for 3.5 min before returning to 1% B at 20.1 min. The method concluded with an isocratic phase (1% B) at 25 min. MS data were acquired in profile and sensitivity modes and by using a scan range of 50-2000 m/z. The scan time was set at 0.3 s and the MS data were acquired with function 1 (no fragmentation) and function 2 (CID fragmentation with argon at 35 eV). Furthermore, a FAST-DDA data-dependent analysis was conducted to improve metabolite identification. This involved

assessing the accurate mass (derived from the m/z ratio), retention time, and fragmentation pattern (MS/MS from the FAST-DDA analysis) for each ion, followed by cross-referencing with a proprietary library of authentic standard compounds, an *in silico* database of plant compounds, and relevant scientific literature and public databases such as Pubchem, MoNA and MassBank. Identification levels were established according to metabolomics standards initiative (MSI): level 1, unambiguous identification with reference standards; level 2, putative assignment based on MS data similarity with literature data or databases; level 3, putative assignment established by spectral similarity to chemical class of compounds and chemotaxonomic data.

Phylogenetic selection approach

The lists of plants with anti-aggregant, neuroprotective and anti-Parkinson's biological activities were downloaded from the Dr. Duke's Phytochemical and Ethnobotanical Databases[17,18] to determine their phylogenetic clumping. Hereinafter, we will refer to these plants as 'Medicinal Plants' (MP). The MP belonging to the Pteridophyta and Ceratophyllales (Supplementary Table S1) were excluded from the phylogenetic analyses due to their low number in the database. The Maximum Likelihood (ML)-based chronogram produced by Zanne et al. [19]

was downloaded and four monophyletic subtrees (i.e., Monocotyledoneae with 7,060 tips, Superrosidae with 10,009 tips, Superasteridae with 11,324 tips, and Basal eudicots with 948 tips as defined in [19]) representing 29,341 species in total, were extracted for use as reference in subsequent analyses.

The functions available in the R package ‘pm4mp’ (<https://github.com/gzecca/pm4mp>) were used as follows. The function “*clean_and_match()*” was applied with the argument “*reftype = z*” and the argument “*match_thr = 0.9*” to standardise the species names and to find the fuzzy matches between the MP names and tree tip names. The resulting fuzzy matches were manually checked for misspelling on the Plants of the World Online (<https://www.plantsoftheworldonline.org>) and on the World Flora Online (<https://www.worldfloraonline.org>) databases and corrected when necessary in the MP lists (Supplementary Table S2). The “*tree4nodesigl()*” and “*sample4nodesigl()*” functions were used to prepare input files for PHYLOCOM v4.2, which was used to identify the “*hot nodes*” in the reference phylogenies [20]. The “*hot nodes*” are the nodes on a phylogenetic tree that contain an overabundance of taxa of a given category compared to a random pattern of distribution [21,22]. The function “*nodesiglr()*” with parameters “*start = 1*” and “*stop = 1500*” was used to run PHYLOCOM from R and to perform 1500 repetitions of the “*nodesigl*” command using 999 randomizations each. Repetitions were necessary because of

the stochastic nature of the null model used by PHYLOCOM to test the statistical significance of hot nodes. The appropriate number of repetitions was determined based on preliminary analyses, and chosen to exceed the number of replicates after which the detected *hot nodes* remained constant (data not shown). The function "*nodesigl_harvesteR()*" with the argument "*fract* = 5" was used to keep the (stable) *hot nodes* present in all 1500 runs that contained at least 5% of MPs and to extract their corresponding "*hot trees*" in Newick format. We define the "*hot trees*" as subtrees of the reference phylogenies containing independent subsets of phylogenetically nested "hot nodes" and therefore containing different sets of species (for details see the "*pm4mp*" package documentation). The 60 plant species selected by the NBFC project (Supplementary Table S1, S2) were searched within the identified *hot trees*. The "*hot_tree_painteR()*" function was used to plot the *hot trees* where at least one plant species selected by the NBFC project was found.

Yeast Strains and Media

The yeast strain utilized to assess the biological activity of plant extracts is BY4742[pYX242-*SNCA*], overexpressing human α -syn. Cell cultures were grown in minimal medium containing 2% glucose as the carbon source and 0.67% yeast nitrogen base lacking amino acids, supplemented with 50 mg/L of essential amino acids and bases necessary for the auxotrophic strain's requirements and with 0.1

mM ampicillin to prevent contaminations. Cells were grown at 30 °C under continuous agitation at 120 rpm. For treatments with the extracts, the dried extracts, obtained by 100 mg of fresh leaves, were resuspended in 75 µL of 100% ethanol, vortexed and sonicated for 20 min at 28 KHz and then added to 5 mL of the medium. In the control condition, 75 µL of ethanol was added in the medium (final ethanol concentration of 1.5%).

Biolog OmniLog System

The Biolog OmniLog System was utilized to compare the cellular phenotypes of BY4742[pYX242-*SNCA*] yeast cells supplemented with 60 different extracts. Yeast cell cultures were grown on agar plates at 30 °C, then inoculated into 8 mL of 2% glucose minimal medium in sterile glass tubes. The cell suspension was measured using the BIOLOG Turbidimeter (Biolog, Hayward, CA) until reaching a cell suspension transmittance of 62% T. The suspension was prepared according to the BIOLOG PM protocol for yeast cells: Dye D 75x (Biolog, Hayward, CA), cells 48x, and medium. This suspension was then pipetted into the 96-well plates (100 µL per well). All dried extracts were resuspended in 75 µL of 100% EtOH and 1.5 µL of extract, corresponding to 20 mg of fresh leaves/mL medium, were added to each well of the plates (1.5% EtOH final concentration) to reach a 100 µL total volume. Microplates were then incubated in the OmniLogTM system at 30 °C for 72 h. The

experiment was conducted in triplicate. Growth profiles were compared using the y-maximum value of each kinetic curve.

Chronological lifespan assay (CLS)

Cells were grown in liquid medium until reaching the mid-late exponential phase, after which they were inoculated at a density of 0.150 OD/mL into flasks containing medium either without extracts (solvent control) or supplemented with the extract (corresponding to 20 mg of fresh leaves/mL medium). Survival (expressed as percentage) was evaluated by propidium iodide (PI) staining at different time points using the Cytoflex cytofluorimeter (Beckman Coulter, Milan, Italy) and analysed with Cytoflex software (version 2.6.0.105), as previously reported [23].

Analysis of reactive oxygen species (ROS) and aggresome levels in yeast

ROS level within the cells was assessed through DHE (dihydroethidium) staining. Following a 24 h treatment with the extracts, cells were harvested, resuspended in phosphate-buffered saline (PBS) solution, and stained with 5 µg/mL of DHE for 10 min, as previously reported [23]. Flow cytometric analyses were conducted using the Cytoflex cytofluorimeter (Beckman Coulter) and data were analysed using Cytoflex software (version 2.6.0.105). The intracellular protein aggresomes were analysed using the PROTEOSTAT® Aggresome detection kit (ENZO Life Sciences). Yeast cells were collected after 24 h treatment with the extracts; 0.2 OD of cells

were collected and resuspended in PBS buffer and stained with the PROTEOSTAT® Aggresome detection reagent at a dilution of 1:1500, as previously reported [24]. Fluorescence-activated cell sorting (FACS) analyses were conducted using a Cytoflex cytofluorimeter (Beckman Coulter) and analysed with Cytoflex software.

Thioflavin T (ThT) assay

α -syn was purchased from Merck-Millipore and dissolved at 70 μ M in PBS. Protein samples (20 μ L, 70 μ M concentration of α -syn) were incubated at 37 °C in PBS up to 72 h under constant shaking at 900 rpm with a thermo-mixer in the absence (cnt) or in the presence of the extracts. Dried extracts, corresponding to 100 mg of fresh leaves resuspended in 75 μ L of 100% ethanol, were diluted 1:10 in PBS and 5 μ L were added to α -syn to test their effect on protein aggregation; in control experiments ethanol was used as solvent control. The ThT binding assay was performed according to [25] using a 20 μ M ThT solution in the PBS buffer. 180 μ L of ThT solution were added to 20 μ L of the aggregated α -syn samples, transferred into a black 96-well clear bottom multiwell plate and ThT fluorescence was read at the maximum intensity of fluorescence of 485 nm using a Victor X3 plate reader (Perkin Elmer). Fluorescence of blank samples was subtracted from the fluorescence values of all samples. In control experiments with each extract, no interference with ThT fluorescence was observed.

Seed fibrils formation

For seed fibrils formation 250 μL of $\alpha\text{-syn}$ at 70 μM was incubated at 37 $^{\circ}\text{C}$ in PBS for 72 h under constant shaking at 900 rpm in a thermo-mixer. Fibrils formation was verified through the Thioflavin T assay by mixing 10 μL of fibril samples with 20 μM ThT in a final volume of 100 μL . Fluorescence emission at 485 nm was recorded using a plate reader (BioTek Synergy H1, Agilent).

Aggregation kinetics

For kinetic experiments, 35 μM $\alpha\text{-syn}$ (100 μL) was incubated at 37 $^{\circ}\text{C}$ in PBS, with 20 μM ThT solution in PBS, up to 72 h under constant shaking at 425 cpm using a plate reader (BioTek Synergy H1, Agilent) in the absence (cnt) or in the presence of *V. thapsus* extract or acteoside (50 μM and 100 μM). The dried extract of *V. thapsus* was resuspended in 75 μL of 100% EtOH and 2.5 μL were added to $\alpha\text{-syn}$ to test the effect on protein aggregation; in control experiments ethanol was used as solvent control. For fibril elongation experiments, 1 μM preformed seed fibrils were added to 35 μM $\alpha\text{-syn}$ in PBS. To probe fibril elongation, protein samples (100 μL) were incubated at 37 $^{\circ}$ C in PBS, with 20 μM ThT solution in PBS, under constant shaking at 425 cpm using a plate reader (BioTek Synergy H1, Agilent) in the absence (cnt) or in the presence of acteoside (1 μM , 25 μM , 50 μM and 100 μM). Acteoside was

purchased from Merck and was resuspended in water and added to α -syn; in control experiments water was used as solvent control.

Transmission electron microscopy (TEM) imaging

For the visualization of α -syn fibrils by TEM, α -syn was used at 70 μ M in PBS. Protein samples (250 μ L) were incubated at 37°C in PBS, with 20 μ M ThT solution in PBS under constant shaking at 900 rpm with a thermo-mixer up to 72h, in absence (cnt) or in the presence of acteoside (50 μ M). Samples were loaded on carbon coated copper TEM grids (5 μ L) and let them air-dried. Then a negative staining (Uranyl Acetate, 1%) was performed to enhance the contrast and to improve the observations. TEM micrographs were acquired with JEOL JEM 2100Plus Transmission Electron Microscope (JEOL, Japan) operating with an acceleration voltage of 200 kV, and equipped with an 8 megapixel Gatan (Gatan, USA) Rio Complementary Metal-Oxide-Superconductur (CMOS) camera.

Cell cultures

SH-SY5Y cell lines harboring *pTet-SNCA-FLAG* were purchased from Merck. Cells were cultured on plates at 37 °C in DMEM/F12 medium, containing 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin and 100 μ g/mL streptomycin, in a humidified 5% CO₂ incubator. Doxycycline-inducible α -syn-expressing cells were selected against the antibiotic puromycin with a dose of 2 μ g/mL. Induction of α -

syn expression was achieved by adding 6 µg/mL doxycycline (from a 6 mg/mL stock in DMSO) for 48 h.

The extracts obtained from 100 mg of fresh leaves were resuspended in 75 µL of 100% ethanol and used as 200x stocks (corresponding to 6.6 mg/mL of medium, final ethanol concentration 0.5%); 0.5% ethanol was used as solvent control. Acteoside was resuspended in water and used at a final concentration of 100 µM. For immunofluorescence assays, 1.6×10^5 cells were seeded on geltrex-coated glass cover slips in wells of a 24 multiwell plate and treated the day after for 48 h.

SH-SY5Y cells were purchased from ATCC. Cells were cultured on plates at 37 °C in DMEM medium, containing 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin, in a humidified 5% CO₂ incubator. For analysis of ROS level 8×10^3 cells were seeded in wells of a 96 multiwell plate and treated with 5 µM of α-syn fibrils for 72 h. To obtain the fibrils, α-syn (70 µM) was incubated at 37 °C in PBS up to 72 h under constant shaking at 900 rpm with a thermo-mixer in the presence or in the absence of acteoside (1 mM).

Analysis of acteoside availability in cells

To evaluate the intracellular/cell-associated level of acteoside, 2×10^6 SH-SY5Y *pTet-SNCA-FLAG* cells were seeded in 60-mm dishes and treated with acteoside at 100 µM up to 24 h. For metabolite extraction, cells were washed twice with PBS before

being lysed with ice cold 80% methanol solution for 5 min. The lysate was transferred into a 1.5 mL tube and sonicated in bath 1 min on and 1 min off for 5 times. Samples were centrifuged at 14000 g for 30 min at 4 °C and the supernatant was filtered using a 3 kDa molecular weight cut-off filter at 14000g for 1h at 4 °C. The eluate was transferred in LC-MS vials and stored at -20 °C.

Samples were injected into a Xevo G2-XS quadrupole time of flight mass spectrometer coupled to a Waters ACQUITY ultra-performance liquid chromatography (UPLC) H-Class system through an ESI source (Waters Corporation, Milford, MA, USA). The instrument was operated in negative ion current in sensitivity mode. The capillary voltage was maintained at 1.5 kV with the source temperature held constant at 140 °C.

An ACQUITY UPLC® BEH C18 1.7µm, 2.1mm x 50mm, (Waters Corporation, Milford, MA, USA) was used for analyte separation. The flow rate was set to 400 µL/min with a column temperature of 40 °C. A gradient of solvent A (99.9% LC-MS-grade water with 0.1% formic acid) and solvent B (LC-MS-grade 99.9% acetonitrile with 0.1% formic acid) was applied with a total run time of 12 min as follows: 0-0.3 min at 2% B; 0.3-6.3 min linear increase from 2 to 98% B; 6.3-8.3 min 98% B; 9-12 min 2% B. Transition selection and optimization of collision energy were performed manually on acteoside and its isomer isoacteoside standard solutions, and data acquisition in

MRM mode was performed with Masslynx (v4.2). The transitions monitored were m/z 623.1 (CE 32V) $\rightarrow$ m/z 161.1 (quantifier) and 461.1 at CE 28V (qualifier) for acteoside and isoacteoside. A standard curve 8-5000 nM of both the analytes was run to test the linearity of the response and to confirm the different retention time of the two isomers. The accuracy of each standard point was calculated with TargetLynx based on the determined linear regression curve and should be between 80% and 120% of the expected value. Analyte quantity (acteoside + isoverbascoside) was extrapolated from the respective standard curves.

Cell viability assay

Cells viability was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. After treating cells for 72 h as described above, the culture medium was removed, and cells were incubated for 2 h at 37 °C in 100 μ L of DMEM containing 0.5 mg/mL MTT. Then, 100 μ L of solubilization buffer (10% Triton X-100, 0.1 N HCl in isopropanol) was added to cells. Absorbance was measured using a microplate reader (BioTek Synergy H1, Agilent) at 570 nm.

Analysis of Reactive Oxygen Species (ROS) level in SH-SY5Y cells

Intracellular ROS were determined using fluorescent probe 2',7'-dichlorofluorescein diacetate, acetyl ester (H₂DCFDA, Merck). After treating cells for 72 h as described above, the culture medium was removed, and cells were washed twice with PBS.

Then, 5 μ M of H₂DCFDA was added to each well for 30 min. After washing cells twice with PBS solution, the fluorescence was measured using a microplate reader (BioTek Synergy H1, Agilent) at excitation wavelength of 490 nm and emission wavelength of 520 nm. Data were normalized on total viability measured with MTT assay.

Western blot and native dot blot analyses

Cells were lysed in RIPA buffer (Thermo Scientific) supplemented with protease inhibitors (Roche) and phosphatase inhibitor cocktails (Merck). The lysates were centrifuged at 4 °C and the supernatants were collected. Protein concentration was determined using the Bradford assay (Bio-Rad). Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. After the transfer, the membranes were blocked with 5% nonfat dry milk and primary antibodies anti-NRF2 (1:1000, Thermofisher), anti- α -syn (Merck, 1:1000) and anti-vinculin (1:5000, Merck) were used. Conjugated secondary antibodies were applied, and the signal was detected using enhanced chemiluminescence (ECL). The resulting signals were captured using the Chemidoc system (Bio-Rad). For native dot blot analysis, samples from *in vitro* α -syn aggregation assays, performed in the absence or in the presence of the plant extracts, were dropped onto nitrocellulose membranes and

incubated with the oligomer specific A11 mouse antibody (1:500, Thermo Fisher) or with the anti- α -syn antibody (1:1000, Merck).

Immunofluorescence assay

Cells grown on cover slips were washed with PBS, fixed with 4% formaldehyde for 15 min, and permeabilized with PBS 0.2% Triton X-100 for 10 min. Then, cells were washed in blocking solution (PBS 1% BSA), blocked at room temperature for 60 min, and then incubated overnight at 4°C with the primary antibody anti- α -Syn33 (1:40, Merck) recognizing α -syn oligomers, or with the primary antibody anti-NRF2 (1:200, Thermo Fisher). After that, cells were incubated with anti-rabbit AlexaFluor488 secondary antibodies (1:200, Thermo Fisher) for 1 h. Coverslips were mounted with a DAPI containing mounting medium for nuclear staining. Image acquisition was carried out on a Thunder fluorescence microscope (Leica Microsystems). For the analysis of α -syn oligomers, fluorescence images were analyzed using ImageJ software (NIH) and the Integrated Density (IntDen) of the α -syn fluorescence signal was quantified. Quantitative analysis of NRF2 nuclear localization was performed using Cell-ACDC, an open source user friendly GUI-based framework for segmentation [26]. Nuclei were segmented by applying an automated thresholding algorithm (Otsu method) on the DAPI channel, generating accurate nuclear masks. Within each segmented nucleus, the concentration parameter, defined as the total

fluorescence intensity normalized to the nuclear area, was calculated. The resulting output data were exported and statistically analyzed to compare NRF2 localization across different treatment conditions.

Real Time PCR

Total RNA was isolated using the Quick-RNA Mini-Prep Kit (Zymo Research), according to manufacturer's instructions. RNA (1 µg) was reverse-transcribed using iScript supermix (Bio-Rad), according to the manufacturer's protocol. For each real-time PCR, cDNA were amplified using the ChamQ mastermix (Vazyme) and specific primers. Each sample was normalized for total RNA content using ACTB and GAPDH as housekeeping genes. The primers used are the following: ACTB-F: CACCATTGGCAATGAGCGGT, ACTB-R: GTCTTTGCGGATGTCCACGT; GAPDH-F: AGAAGGCTGGGGCTCATTTG, GAPDH-R: TGCTAAGCAGTTGGTGGTGC; NQO1-F: TCCGGAGTAAGAAGGCAGTG, NQO1-R: TCCAGGCGTTTCTTCCATCC; HMOX1-F: TTCAAGCAGCTCTACCGCTC, HMOX1-R: TTGCACTTTGTTGCTGGCCC.

Size exclusion chromatography coupled to reversed phase LC-MS (SEC AS-MS)

For the SEC AS-MS analysis, the dry extracts were first diluted in 1 mL of dichloromethane (DCM) and dried under nitrogen to obtain the accurate weight. A 10 mg/mL solution in methanol was prepared and centrifuged for 5 min at 13000 rpm and then diluted in water:methanol (70:30 v/v) to obtain a final concentration

of 2 mg/mL. Incubation solutions (500 μ L) containing 10 μ M α -syn and the selected plant extracts (2 mg/mL) were prepared in 0.1 M potassium dihydrogen phosphate buffer at pH = 7 and incubated for 96 h at 4°C. SEC AS-MS experiments of the incubated samples were carried out with two chromatographic systems (System 1 and System 2) as described in [15]. Briefly, System 1 was used for SEC analyses using a TSK Gel Super SW2000 125 Å column (300 $\times$ 4.6 mm ID, 4 μ m) from Tosoh Bioscience. Analysis was carried out in isocratic elution (KH_2PO_4 0.1 M, 0.05% NaN_3 , pH=7) at a flow rate of 350 μ L/min. α -Syn was detected at λ = 280 nm and the peak was eluted in the window 6.5-8 min. The elution fraction (750 μ L final volume) was focused on an Agilent Zorbax Eclipse Plus C18 analytical trap column (12.5 $\times$ 4.6 mm ID, 5 μ m). The trap column was connected to System 2 on the head of an analytical column, an XTerra MS C18 column (250 $\times$ 2.1 mm ID, 5 μ m) from Waters. Chromatographic separation of the plant extracts fished analytes and the analyses of the fingerprinting of the extracts were carried out through gradient elution where the mobile phase was water 0.1% formic acid (solvent A) and acetonitrile 0.1% formic acid (solvent B). Specifically, after an isocratic step at 1% B for 11.50 min, a linear gradient of B from 1% to 40% was applied in 22 min. Then, three sequential linear gradient steps were performed (from 40% to 70% in 8.5 min, from 70% to 90% in 3.5 min and from 90% to 100% in 3.5 min). Lastly, an isocratic step was carried out at 100% B for 8.5 min. Compound identification was achieved as

reported in [15] through mass spectrometry (MS) using a linear ion trap mass spectrometer (LTQ) equipped with an electrospray ion source (ESI) (Thermo Fisher Scientific, Waltham, MA, USA). Full scan mass range was set up in the 250-1500 Da range.

Native Mass Spectrometry (Native-MS)

Native-MS analysis was performed to assess the interaction of α -syn with acteoside. Commercial α -syn (Merck Millipore) and acteoside (Merck) powders were individually resuspended in 100 mM ammonium acetate pH 7 at 1 mg/mL stock concentration. Titration experiments were performed preparing independent samples at fixed α -syn concentration (7 μ M) and variable acteoside concentrations (0-1400 μ M). Each sample (10 μ L) was directly infused into the mass spectrometer (Orbitrap Fusion, Thermo Fisher Scientific) by a static nano-electrospray source, employing borosilicate, metal-coated capillaries with 1- μ m internal diameter (Thermo Fisher Scientific). Analytes desolvation and charging were obtained applying a 1.1-1.2 kV ion spray voltage. The main instrumental parameters were set as follows: resolving power at m/z 200, 120,000; detection window, 700-2600 m/z ; IRM pressure, 3 mTorr (intact protein mode); ion-transfer tube temperature, 275 °C; in-source fragmentation, 0-5 V; AGC target, 4×10^5 ; maximum injection time, 100 ms. Tandem MS analysis at selected charge states of the protein-ligand complex

was performed by collision-induced dissociation (CID) with helium gas. Final spectra were obtained by averaging the signal over 30 s acquisition. A ligand-binding curve was generated calculating the relative intensity ratio of bound vs. total α -syn peak signals.

Fly culture and acteoside treatment

DJ-16^{ex54} mutant flies (hereafter called *DJ-16*) were used as PD model flies [27]. They were cultured on standard *Drosophila* food at 25 °C. For acteoside treatments, *DJ-16* mutant second instar (L2) larvae were cultured on standard medium containing 0.1% dimethyl sulfoxide (DMSO) (untreated flies) or supplemented with 250 or 400 μ M acteoside (GenoChem World). After eclosion, adult female flies were transferred to new tubes and cultured for five days in vials containing DMSO or acteoside. Subsequently, flies were directly evaluated in climbing assays or frozen in liquid nitrogen and kept at -80 °C for quantification of protein carbonylation level. The effect of acteoside was also evaluated in flies treated only after eclosion: 1-day-old *DJ-16* mutant female flies were transferred to new vials containing either 0.1% DMSO (control) or acteoside-supplemented food (400 μ M) and climbing assay was carried out 5 days and 10 days later.

Climbing assay and determination of carbonylation protein level

Locomotor performance of *DJ-16* mutant flies was analyzed using climbing assay, which exploit flies' innate negative geotaxis, an upward movement response following mechanical stimulation. This simple, robust, and cost-effective behavioral test is widely used to evaluate motor function in fly models of neurodegenerative diseases [28]. In these assays, a total of 80-120 5-day-old female flies were analyzed per condition. Groups of 10-20 female flies were transferred to graduated plastic tubes (1.4 cm diameter), acclimated for 1 min, gently tapped down to the bottom and allowed to climb for 10 s, recording the process. Moreover, climbing assays were also carried out in 5-day-old and 10-day-old female flies treated only after eclosion. The climbing ability of untreated (DMSO) and acteoside-treated flies was determined as the average of the height reached by each fly after this time [29]. Protein carbonyl groups were measured in extracts from *DJ-16* female flies of different ages and subjected to different treatment regimes using 2,4-dinitrophenylhydrazine (DNPH) derivatization in 96-well plates (Greiner 96 well plate, polypropylene) as previously described [30].

Statistical analysis

All experiments were conducted in triplicate. Results are presented as mean $\pm$ standard deviation (SD). Statistical analyses were made using the two-tailed Student's t-test, with significance set at $p < 0.05$. For *Drosophila* experiments, groups

were statistically compared by one-way ANOVA analysis followed by Tukey's post hoc test (* $p < 0.05$).

Results

Plant species selection

Within a collection of plant species belonging to the Italian flora, in the frame of the Italian initiative NBFC (National Biodiversity Future Center, <https://www.nbfc.it>), extracts from 60 plant species rich in specialized metabolites were selected and tested. While the chemical structures of primary metabolites are common across various species, specialized metabolites sharing core structures often exhibit a distribution linked to specific genetic lineages [31]. Thus, to facilitate a broad exploration of specialized metabolites with their bioactivities, species from all major clades of Angiosperms were included in this investigation. The species, listed in Supplementary Table S1, encompass monocots and eudicots, representing a total of 24 orders and 40 families. Additionally, two more primitive vascular species belonging to the Pteridophyta group were also included. Among the 60 analysed species, the leaves of 11 plants (*Althaea officinalis*, *Aloysia citrodora*, *Artemisia abrotanum*, *Beta vulgaris*, *Castanea sativa*, *Diospyros kaki*, *Filipendula ulmaria*, *Lysimachia vulgaris*, *Rosa canina*, *Salvia pratensis*, *Verbascum thapsus*) are included

in the Belfrit list, which defines a common reference for the use of plants in food supplements [32]. The inclusion of these species is particularly relevant, as it allows for a deeper evaluation of their specialized metabolites for public health applications [33].

To screen for the promising neuroprotective properties of this plant extract library, we conducted two complementary analyses: an *in silico* phylogenetic analysis to select potential medicinal plants, and a screening using a yeast model expressing the human protein α -syn, to select protective extracts against misfolded α -syn also in a cellular system.

Phylogenetic selection approach

Phylogenetic analyses identified 34, 45 and 50 hot trees related to antiaggregant, antiparkinsonian, and neuroprotective biological activities, respectively (Supplementary Table S3). The hot trees containing at least a taxa included in the list of 60 plants of NBFC project were plotted (Supplementary Figure S1). All taxa included in hot trees were defined as Potential Medicinal Plants (PMP) if these were not already recognized as Medicinal Plants (MP) in the databases used. The plant species appearing in both the list of 60 plants and the hot trees are listed in Table 1 specifying if these are MP or PMP. In Figures 1A and 1B two examples of hot trees are plotted, wherein the clade encompassing the plant species identified by the

phylogenetic approach applied in this study (Table 1) was enlarged for clarity. Altogether, our analysis identified 7 plants of our library associated with the antiaggregant biological function, 8 plants with the antiparkinsonian effect and 4 plants with the neuroprotective properties (Table 1). Therefore, a total of 11 plants (*Allium angulosum*, *Allium lusitanicum*, *Castanea sativa*, *Medicago marina*, *Salvia pratensis*, *Verbascum thapsus*, *Glaucium flavum*, *Acorus calamus*, *Althea officinalis*, *Malva alcea*, *Artemisia abrotanum*), selected on the basis of the phylogenetic analysis (Table 1), have a high probability to present a medicinal value.

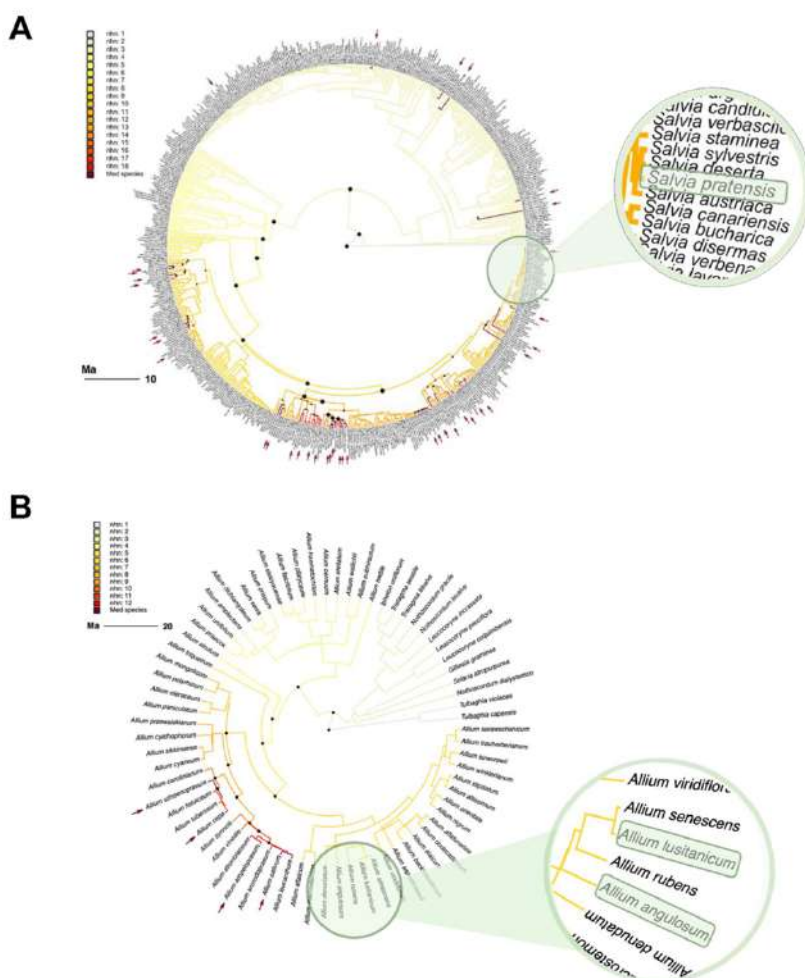


Figure 1. Phylogenetic analysis. Phylogenetic hot tree associated with neuroprotective (A) and antiaggregant (B) biological activity. The branch colours increase from white to red proportionally to the number of nested hot nodes (nhn). Medicinal Plants (MP) are indicated by an arrow and their branches are highlighted in purple. All hot nodes are indicated with a black circle. Horizontal bar giving the scale of the branch lengths in million years (Ma).

Biological Activity	NBFC-project	Category
Antiaggregant	<i>Allium angulosum</i>	PMP
	<i>Allium lusitanicum</i>	PMP
	<i>Castanea sativa</i>	MP
	<i>Medicago marina</i>	PMP
	<i>Salvia pratensis</i>	PMP
	<i>Verbascum thapsus</i>	MP
	<i>Glaucium flavum</i>	MP
Antiparkinsonian	<i>Acorus calamus</i>	MP
	<i>Allium angulosum</i>	PMP
	<i>Allium lusitanicum</i>	PMP
	<i>Althaea officinalis</i>	MP
	<i>Castanea sativa</i>	MP
	<i>Malva alcea</i>	PMP
	<i>Artemisia abrotanum</i>	PMP
	<i>Salvia pratensis</i>	PMP
Neuroprotective	<i>Allium angulosum</i>	PMP
	<i>Allium lusitanicum</i>	PMP
	<i>Artemisia abrotanum</i>	PMP
	<i>Salvia pratensis</i>	PMP

Table 1. Plant species appearing in both the list of 60 plants of NBFC-project and the hot trees subdivided for biological activity. In the category column: MP = Medicinal Plants, PMP = Potential Medicinal Plants.

High-throughput screening of plant extracts with anti-aging and antioxidant effects

In parallel with the *in silico* analysis, a high-throughput screening on the 60 extracts of our library was conducted *in vivo* using a *S. cerevisiae* PD model, in which the

human protein α -syn is overexpressed [34,35]. The Omnilog Biolog instrument was first employed to evaluate the effect of all plant extracts (at a concentration corresponding to 20 mg of fresh leaves/mL medium) on yeast cell growth. This step allowed us to exclude extracts exhibiting cytotoxicity, enabling a focused analysis of biologically relevant candidates to be considered in the following assays. Out of 60 plant extracts tested, only two obtained from *Heuchera sanguinea* and *Empetrum hermaphroditum*, showed relevant cellular toxicity and were thus excluded from further analysis (Fig. 2A). Of the other 58, 10 extracts did not significantly influence cell growth, while the remaining ones (48) stimulated the cell proliferation (expressed as maximal growth obtained after 72 h). On the basis of these data, we proceeded with a detailed analysis of the 58 non-toxic extracts in order to explore their potential anti-aging, antioxidant, and neuroprotective properties.

These 58 extracts were tested in the context of a standard chronological life span (CLS) experiment [36], to evaluate any ameliorating effect on the age-dependent α -syn mediated cell death, as we also recently reported [23]. Most extracts increased both mean and maximal lifespan of yeast cells (as indicated by the yellow color in the heatmap, Fig. 2B), except for those from *Glandularia tenera*, *Pseudosasa japonica* and *Acorus calamus*. Notably, 18 different extracts (*Echinophora spinosa*, *Acalypha virginica*, *Pulsatilla montana*, *Butomus umbellatus*, *Ranunculus lingua*,

Typha laxmannii, *Centranthus ruber*, *Aquilegia atrata*, *Allium lusitanicum*, *Eryngium maritimum*, *Glaucium flavum*, *Verbascum thapsus*, *Succisa pratensis*, *Valeriana dioica*, *Salvia pratensis*, *Akebia quinata*, *Sternbergia lutea* and *Adenophora liliifolia*) exhibited very potent anti-aging activities, increasing mean lifespan more than 3.5 fold compared to the control condition. Coherently, these extracts also extended the maximum lifespan, although at different extents (Fig. 2B).

Alongside a decreased lifespan, oxidative stress is a typical feature of toxic protein aggregation induced by α -syn [37]; therefore the intracellular ROS level after 24 h treatment with the extracts were also quantified. Strikingly, 16 extracts (*Pulsatilla montana*, *Sternbergia lutea*, *Osyris alba*, *Rosa canina*, *Nonea lutea*, *Ranunculus lingua*, *Salvia pratensis*, *Malva alcea*, *Glaucium flavum*, *Succisa pratensis*, *Verbascum thapsus*, *Euonymus europaeus*, *Adenophora liliifolia*, *Allium lusitanicum*, *Inula salicina*, *Valeriana dioica*) decreased intracellular ROS level more than 2.5 fold (Fig. 2B). Therefore, 10 extracts showed the ability to attenuate both senescence and oxidative stress, two key hallmarks of age-related diseases (Fig. 2C), they derived from *Ranunculus lingua*, *Verbascum thapsus*, *Adenophora liliifolia*, *Valeriana dioica*, *Allium lusitanicum*, *Succisa pratensis*, *Salvia pratensis*, *Pulsatilla montana*, *Glaucium flavum* and *Sternbergia lutea*. These plants belong to different plant families but, with the exception of *Allium lusitanicum* that contains only saponins, the other plant extracts share some chemical similarities as eight species

are characterized by the presence of flavonoids. Specifically, *Adenophora lilifolia*, *Ranunculus lingua*, *Succisa pratensis* and *Verbascum thapsus* have compounds belonging to the flavone class (*e.i.* luteolin and derivatives), while *Glaucium flavum*, *Pulsatilla montana*, *Sternbergia lutea* and *Valeriana dioica* have compounds belonging to the flavonol class (*e.i.* quercetin and derivatives). Furthermore, six of these ten plant extracts with antiaging and antioxidant activity (*Glaucium flavum*, *Pulsatilla montana*, *Ranunculus lingua*, *Salvia pratensis*, *Succisa pratensis* and *Valeriana dioica*) are characterized by the presence of compounds from the cinnamyl derivative class, known for their antioxidant properties [38].

From the combination of the three screening methods, *i.e.* phylogenetic identification as a medicinal plant, increase of mean lifespan (>3.5 fold) and decrease of ROS level (>2.5 fold), we identified 4 plant extracts responding to all three criteria (Fig. 2C). In fact, from the phylogenetic analysis, 11 species were identified as potential medicinal plants (PMP) or medicinal plants (MP) (Table 1), but only 4 of them, *A. lusitanicum*, *G. flavum*, *S. pratensis* and *V. thapsus*, were also able to improve cell longevity (>3.5 fold increase) and reduce intracellular ROS (>2.5 fold decrease) in the yeast model (Fig. 2C). Consequently, the combination of these approaches underscores the potential preventive and therapeutic benefits of *A. lusitanicum*, *G. flavum*, *S. pratensis* and *V. thapsus* against neurodegenerative diseases such as Parkinson's disease.

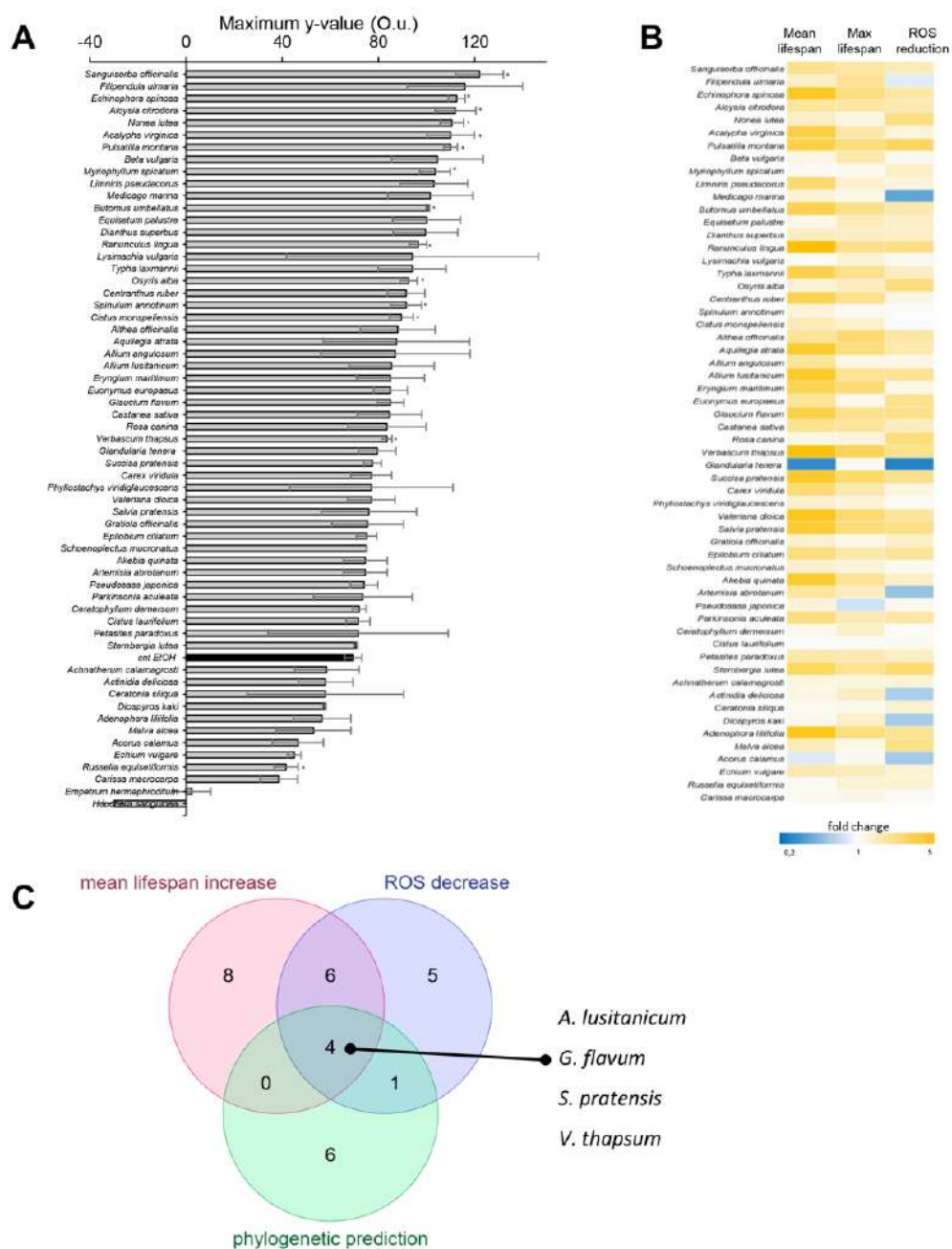


Figure 2 Screening in yeast cells. (A) Effect of the extracts on cell growth: maximum y-value of growth kinetics of yeast cells overexpressing α -syn, grown in minimal

medium containing 2 % glucose for 72 h in the absence (cnt) or presence of the extracts (corresponding to 20 mg fresh leaves/mL medium). The black bar identifies the control (cnt). * $p < 0.05$ relative to maximum y-value in control cells ($n = 3$). (B) Heatmap showing the effect of the plant extracts on mean lifespan, maximal lifespan and ROS reduction on yeast cells overexpressing α -syn. Data are expressed as fold change compared to control condition and are visualized using a color scale, with yellow indicating an increase and blue indicating a decrease of each parameter ($n = 3$). (C) Venn diagram of the plant extracts resulting from the phylogenetic analysis and the screening in yeast cells. The increase of mean lifespan of at least 3.5 fold and the reduction of ROS level of at least 2.5 fold have been considered. The extracts from *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* have been selected by all three screening systems.

Phytochemical profiles of *A. lusitanicum*, *G. flavum*, *S. pratensis* and *V. thapsus* extracts

Building on the promising biological findings, the phytochemical profiles of the above selected species were obtained and shown in Fig. 3 as base peak chromatograms recorded in negative ionization (only for *G. flavum* we reported also the positive ionization chromatogram to highlight the exclusive occurrence of alkaloids). The four species are characterized by the presence of different classes of

secondary metabolites, their identity and LC-MS features are reported in Table 2.

A. lusitanicum accumulates different glycosides of steroidal saponins characterized by furostane, spirostane and cholestane skeletons (Fig. 3A, Table 2). A more exhaustive phytochemical characterization of this species is reported in a previous published work [15]. *G. flavum* is characterized by the accumulation of large amounts of glaucine and other aporphine/isoquinoline alkaloids, while rutin is the most abundant flavonoid among the polyphenols detected (Fig. 3B, Table 2). *S. pratensis* accumulates a wide array of polyphenols, mainly rosmarinic acid and its derivatives as well as other caffeic acid esters such as salvianolic acids (Fig. 3C, Table 2). *V. thapsus* presents a more diversified profile with the occurrence of iridoid glucosides (mainly catalpol) and their derivatives esterified to various hydroxycinnamic acids; moreover, this species presents large amounts of acteoside (Supplementary Fig. S2) and minor amounts of acteoside isomers and other phenylpropanoid/phenylethanoid glycosidic esters (such as leucosceptoside and martynoside) (Fig. 3D, Table 2).

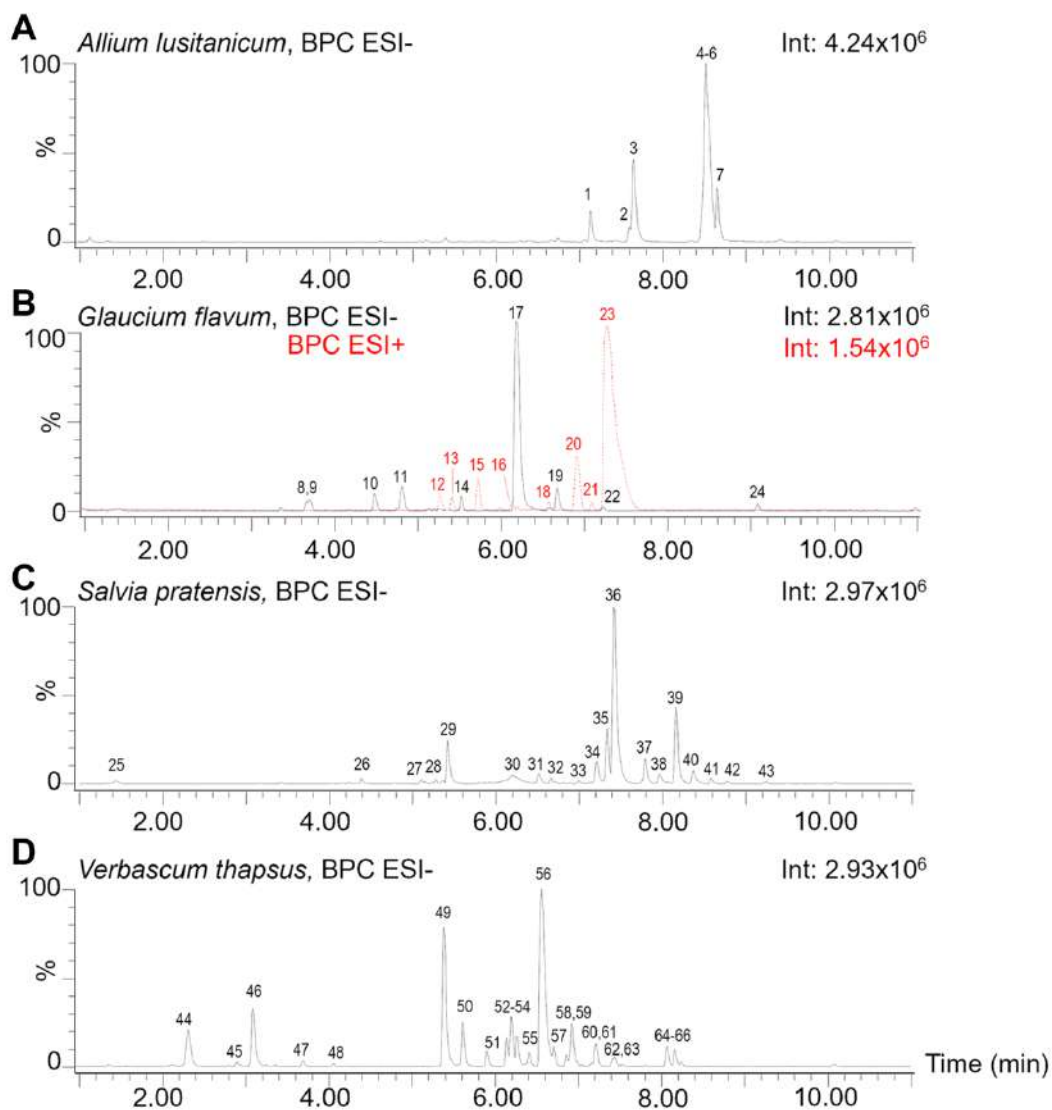


Figure 3. Specialized metabolite-profiling of the selected plant extracts. Base peak chromatograms, in negative ionization mode, of *Allium lusitanicum* (A), *Glaucium flavum* (B), *Salvia pratensis* (C) and *Verbascum thapsus* (D). For *G. flavum* also the positive ionization chromatogram is shown in red. The ID of the peaks are the same as Table 2.

Peak ID	Compound	Rt (min)	Molecular formula	Precursor ion	m/z observed	Error (ppm)	Diagnostic product ions (m/z)	MSI level ^a
<i>Allium lusitanicum</i>								
1	Saponin class IV4 + 2 hexoses + desoxyhexose	7.13	C ₄₅ H ₇₄ O ₁₉	[M+FA-H]	963.481	-1.45	447.312; 609.364; 771.423; 917.480	2
2	Saponin class IV4 + 2 hexoses + desoxyhexose + acetyl group	7.60	C ₄₇ H ₇₆ O ₂₀	[M+FA-H]	1005.492	-1.99	447.312; 609.364; 771.423; 917.480	2
3	Saponin class IV4 + 2 hexoses + desoxyhexose+ acetyl group	7.65	C ₄₇ H ₇₆ O ₂₀	[M+FA-H]	1005.492	-1.99	447.311; 609.364; 771.423; 899.468; 917.477; 959.485	2
4	Saponin class IV3 + 2 hexoses + 2 desoxyhexoses	8.46	C ₅₁ H ₈₄ O ₂₂	[M+FA-H]	1093.545	-1.55	431.319; 593.369; 755.426; 901.482; 1047.541	2

5	Saponin class IV3 + 2 hexoses + 3 desoxyhexoses	8.51	C ₅₇ H ₉₄ O ₂₆	[M-H] ⁻	1193.598	-2.51	431.315; 593.369; 883.470; 1047.541	575.356; 755.420; 901.482;	2
6	Saponin class IV3 + 2 hexoses + desoxyhexose + pentose	8.55	C ₅₀ H ₈₂ O ₂₂	[M+FA-H] ⁻	1079.531	-3.71	431.319; 755.426; 901.482	593.369;	2
7	Saponin class IV3 + 2 hexoses + desoxyhexose	8.65	C ₄₅ H ₇₄ O ₁₈	[M+FA-H] ⁻	947.487	-1.79	431.315; 755.426; 901.482	593.374;	2
<i>Glaucium flavum</i>									
8	Vanillyl alcohol hexoside isomer 1	3.71	C ₁₄ H ₂₀ O ₈	[M-H] ⁻	315.108	-0.04			2
9	Vanillyl alcohol hexoside isomer 2	3.75	C ₁₄ H ₂₀ O ₈	[M-H] ⁻	315.108	-0.04	153.056; 135.046		2
10	Coumaroyl quinic acid	4.52	C ₁₆ H ₁₈ O ₈	[M-H] ⁻	337.0923	0.11	191.057; 163.041; 119.050		2
11	Feruloyl quinic acid	4.87	C ₁₇ H ₂₀ O ₉	[M-H] ⁻	367.1029	0.01	193.051; 149.061; 134.037		2

12	Laurifoline	5.27	$C_{20}H_{24}NO_4^+$	$[M]^+$	342.1705	0.07	297.114; 282.087; 265.087; 237.090; 222.070; 219.080; 207.080; 191.085; 165.072	2
13	Isoboldine/Salutaridine isomer	5.44	$C_{19}H_{21}NO_4$	$[M+H]^+$	328.1549	-0.08	297.110; 285.12; 265.084; 239.069; 237.093; 192.101	2
14	Caffeoyl malic acid	5.57	$C_{13}H_{12}O_8$	$[M-H]^-$	295.0454	-0.03	179.033; 135.046; 133.015; 115.003	2
15	Isoboldine or Salutaridine isomer 3	5.78	$C_{19}H_{21}NO_4$	$[M+H]^+$	328.1549	-0.08	297.114; 265.087; 237.090; 233.060; 222.069; 205.066; 194.071; 177.069	2
16	Corydine / Isocorydine isomer	6.14	$C_{20}H_{23}NO_4$	$[M+H]^+$	342.1689	4.75	236.083; 265.084	2
17	Rutin	6.17	$C_{27}H_{32}O_{16}$	$[M-H]^-$	609.1456	-0.08	300.026; 271.026; 255.031	1
18	Corydine / Isocorydine isomer	6.61	$C_{20}H_{23}NO_4$	$[M+H]^+$	342.1705	0.07	311.129; 279.095; 251.106; 236.083	2

19	Kaempferol hexose-deoxyhexose	6.70	C ₂₇ H ₃₀ O ₁₅	[M-H] ⁻	593.1506	0.06	285.041; 255.031; 227.035	2
20	Protopine	6.86	C ₂₀ H ₁₉ NO ₅	[M+H] ⁺	354.1341	0.12	336.121; 275.068; 247.073; 206.080; 188.071; 149.059	2
21	Norchelidonine	7.09	C ₁₉ H ₁₇ NO ₅	[M+H] ⁺	340.1164	6.15	275.068; 189.069; 247.077; 305.079; 323.091	2
22	UI	7.24	C ₂₀ H ₃₀ O ₁₀	[M-H] ⁻	429.1761	-0.09	249.113; 205.124; 179.052; 161.133; 119.039	
23	Glaucine	7.26	C ₂₁ H ₂₅ NO ₄	[M+H] ⁺	356.1862	-0.07	325.142; 310.119; 294.128; 279.099; 251.106; 236.083	2
24	3β,23-dihydroxy-30-nor-olean-12,20-dien-28-oic acid hexose-hexuronic acid	9.08	C ₄₁ H ₆₂ O ₁₅	[M-H] ⁻	793.401	0.03	631.352; 455.312	2
<i>Salvia pratensis</i>								

25	UI	1.44	C ₁₀ H ₆ O ₈	[M-H] ⁻	252.9984	0.17	136.985; 116.928; 115.002; 71.008	
26	Caffeoyl threonic acid	4.39	C ₁₃ H ₁₄ O ₈	[M-H] ⁻	297.0629	-6.26	179.036; 161.029; 135.030; 117.017; 75.006	2
27	C ₉ H ₁₂ O ₃ -hexose-deoxyhexose	5.11	C ₂₁ H ₃₂ O ₁₂	[M+FA-H] ⁻	521.1863	1.38	475.184; 113.024; 329.124; 161.047; 149.061; 311.113; 167.074	3
28	Tuberonic acid hexoside	5.28	C ₁₈ H ₂₆ O ₉	[M-H] ⁻	387.1664	-2.33	207.103; 163.113	2
29	Rosmarinic acid hydrate	5.42	C ₁₈ H ₁₈ O ₉	[M-H] ⁻	377.0873	-0.12	359.0787; 197.0464; 179.0338; 161.0261; 135.0465; 133.0301	2
30	Salvianolic acid A	6.17	C ₂₆ H ₂₂ O ₁₀	[M-H] ⁻	493.1135	-0.07	313.0726; 295.0627; 185.0256; 109.0307	2
31	Luteolin-O-hexoside	6.51	C ₂₁ H ₂₆ O ₁₁	[M-H] ⁻	447.0943	-3.51	285.038	2

32	Rosmarinic acid hexose	6.70	C ₂₄ H ₂₆ O ₁₃	[M-H] ⁻	521.1287	1.55	359.075; 323.077; 179.034; 161.023; 135.044	341.086; 197.046;	2
33	Sagerinic acid	6.99	C ₂₆ H ₃₂ O ₁₆	[M-H] ⁻	719.1615	-0.41	161.026; 197.044; 341.067; 135.044	359.075; 179.036;	2
34	Luteolin acetylhexoside	7.19	C ₂₃ H ₂₂ O ₁₂	[M-H] ⁻	489.1029	0.81	447.090; 285.042		2
35	Salvianolic acid K	7.34	C ₂₇ H ₂₄ O ₁₃	[M-H] ⁻	555.1139	-0.07	493.115; 295.059; 179.034; 161.026; 135.046	359.079; 197.046;	2
36	Rosmarinic acid	7.41	C ₁₈ H ₁₆ O ₈	[M-H] ⁻	359.0767	-0.03	197.046; 161.026; 133.030; 123.046	179.036; 135.046;	1
37	Lithospermic acid	7.77	C ₂₇ H ₂₂ O ₁₂	[M-H] ⁻	537.1033	-0.01	493.115; 295.059; 197.046; 161.023; 135.044; 133.030	359.079; 221.044; 179.036;	2

38	Chrysoeriol-(3-Hydroxy-3-methylglutaroyl)-O-hexoside	7.96	C ₂₈ H ₃₀ O ₁₅	[M-H] ⁻	605.1477	4.86	503.119; 461.107; 299.056; 284.034; 255.030	2
39	Octene alcohol-hexose-pentose	8.15	C ₁₉ H ₃₄ O ₁₀	[M+FA-H] ⁻	467.2129	-0.13	421.208; 289.169; 161.044; 149.046; 131.033; 113.024; 101.022	2
40	Methyl rosmarinate	8.37	C ₁₉ H ₁₈ O ₈	[M-H] ⁻	373.0923	0.10	197.046; 179.036; 175.039; 160.017; 135.044; 123.046	2
41	UI	8.58	C ₂₈ H ₂₈ O ₁₅	[M-H] ⁻	603.1355	-0.85	317.065; 137.025; 197.044; 87.008; 93.032	
42	UI	8.77	C ₂₁ H ₃₆ O ₁₀	[M+FA-H] ⁻	493.2289	-0.83	447.224; 315.180; 161.047; 359.083	
43	Salvianolic acid L	9.23	C ₂₆ H ₃₆ O ₁₆	[M-H] ⁻	717.1456	-0.06	519.095; 338.052; 179.034; 161.023; 135.044	2

<i>Verbascum thapsus</i>								
44	Catalpol	2.30	C ₂₅ H ₂₂ O ₁₀	[M+FA-H] ⁻	407.1192	-0.62	199.061; 181.052; 163.039; 137.025	169.052; 151.040; 2
45	UI	2.89	C ₂₁ H ₃₂ O ₁₄	[M+FA-H] ⁻	553.1761	1.36	507.176; 181.049; 163.041; 231.088; 327.111	345.121; 315.103; 151.039; 2
46	Aucubin	3.08	C ₂₅ H ₂₂ O ₉	[M+FA-H] ⁻	391.124	1.10	345.121; 183.065; 165.054	179.053; 2
47	Dihydroaucubin	3.69	C ₂₅ H ₂₄ O ₉	[M+FA-H] ⁻	393.142	-5.90	347.131; 179.055; 167.072	185.081; 3
48	Forsythoside	4.05	C ₂₀ H ₃₀ O ₁₂	[M-H] ⁻	461.165	1.94	135.046; 315.110; 297.100; 161.045	113.025; 2

49	Hydroxy-saccatoside (Catalpol-O-caffeoyl-deoxyhexoside) isomer 1	5.39	C ₃₀ H ₃₈ O ₁₇	[M-H] ⁻	669.205	-2.59	161.025; 133.029; 163.039; 179.033; 135.044	3
50	Hydroxy-saccatoside (Catalpol-O-caffeoyl-deoxyhexoside) isomer 2	5.61	C ₃₀ H ₃₈ O ₁₇	[M-H] ⁻	669.205	-2.59	161.025; 133.029; 163.039; 179.033; 135.044	3
51	Saccatoside (Catalpol-O-coumaroyl-deoxyhexoside)	5.9	C ₃₀ H ₃₈ O ₁₆	[M-H] ⁻	653.205	4.21	145.031; 377.124; 163.041; 491.157; 291.087; 117.035	3
52	Methoxy-saccatoside (Catalpol-O-feruloyl-deoxyhexoside)	6.14	C ₃₁ H ₄₀ O ₁₇	[M-H] ⁻	683.219	-0.13	175.041; 160.016; 407.136; 193.051	3
53	Acteoside-pentose	6.19	C ₃₄ H ₄₄ O ₁₉	[M-H] ⁻	755.241	-1.93	593.210; 161.025; 133.029; 447.151; 179.033; 135.046; 125.025	2
54	Apigenin-hexose-hexuronide	6.27	C ₂₇ H ₂₈ O ₁₆	[M-H] ⁻	607.130	0.17	269.046; 431.102; 225.057	2

55	Vermynoside (caffeoyl-catalpol)	6.41	C ₂₄ H ₂₈ O ₁₃	[M-H] ⁻	523.147	-2.95	161.025; 179.033; 135.046; 133.029	2
56	Acteoside	6.56	C ₂₉ H ₃₆ O ₁₅	[M-H] ⁻	623.197	1.58	161.025; 461.165; 133.029; 179.036; 135.046; 113.025	1
57	Leucosceptoside A-pentose	6.70	C ₃₅ H ₄₆ O ₁₉	[M-H] ⁻	769.256	-0.01	593.210; 175.041; 160.016; 193.051; 447.151;	2
58	Acteoside isomer 1	6.86	C ₂₉ H ₃₆ O ₁₅	[M-H] ⁻	623.197	1.42	161.025; 461.165; 133.029; 179.036; 135.046; 113.025	2
59	Acteoside isomer 2	6.92	C ₂₉ H ₃₆ O ₁₅	[M-H] ⁻	623.197	1.42	161.025; 461.165; 133.029; 179.036; 135.046; 113.025	2
60	Scutellarioside II (p-coumaroyl-catalpol)	7.16	C ₂₄ H ₂₈ O ₁₂	[M-H] ⁻	507.153	-4.45	145.028; 231.066; 345.098; 163.041; 119.050	2

61	Leucosceptoside A	7.21	C ₃₀ H ₃₈ O ₁₅	[M-H] ⁻	637.214	-0.42	461.165; 175.041; 160.016; 193.051; 135.046; 113.025	2
62	Martynoside-pentose	7.41	C ₃₆ H ₄₈ O ₁₉	[M-H] ⁻	783.273	-1.73	175.041; 607.226; 193.051; 160.016; 461.169	2
63	Leucosceptoside A isomer	7.43	C ₃₀ H ₃₈ O ₁₅	[M-H] ⁻	637.214	-0.42	461.165; 175.041; 160.016; 193.051; 135.046; 113.025	2
64	Martynoside	8.07	C ₃₁ H ₄₀ O ₁₅	[M+FA-H] ⁻	697.237	-4.21	651.230; 175.041; 160.016; 193.051; 475.182	2
65	Octene alcohol-hexose-pentose	8.15	C ₂₉ H ₃₈ O ₁₀	[M+FA-H] ⁻	467.211	4.15	289.166; 421.206; 161.048; 101.025; 113.025	3
66	Martynoside isomer	8.23	C ₃₁ H ₄₀ O ₁₅	[M-H] ⁻	651.230	-0.95	175.041; 160.016; 193.051; 475.182	2

Table 2. LC-MS features of the main specialized metabolites detected in the extracts of *Allium lusitanicum*, *Glaucium flavum*, *Salvia pratensis* and *Verbascum thapsus*. Peak IDs refer to peak numbers of Fig. 3. Abbreviations: FA, formic acid; Rt, retention time; UI, unidentified.

The extracts of *S. pratensis*, *V. thapsus* and *G. flavum* inhibit α -syn aggregation

Recent studies indicate that a number of natural extracts can display a direct inhibition of amyloid fibril formation [6,39]. These findings have spurred interest in exploring plant-derived compounds as potential therapeutic agents targeting protein misfolding and aggregation.

To investigate these promising features, the anti-aggregant properties of the four selected extracts, *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* were evaluated through a multi-tiered approach, that integrates complementary systems, including yeast cultures and neuroblastoma cells expressing human α -syn, as well as direct protein interaction studies. This multifaceted strategy allowed us to explore their effects at various biological levels, ranging from cellular outputs to direct molecular interactions.

Firstly, we investigated whether the positive effects of the extracts on yeast longevity (Fig. 4A-C), also reported in the heatmap of Fig. 2B, could be associated with their influence on the formation of intracellular aggresomes. It is widely

recognized that these inclusion bodies of aggregated, misfolded proteins can arise in response to different cellular stress. Their abundance increases during the stationary phase and is markedly elevated in cells overexpressing α -synuclein (Supplementary Fig. S3, [24]); indeed they represent a typical hallmark of PD and other neurodegenerative diseases [40]. Notably, treatment with all four extracts resulted in a reduction of intracellular aggregates, albeit to varying extents, ranging from 40% (*G. flavum*) to a stronger reduction of 60% (*A. lusitanicum* and *V. thapsus*) and 80% (*S. pratensis*) (Fig. 4D).

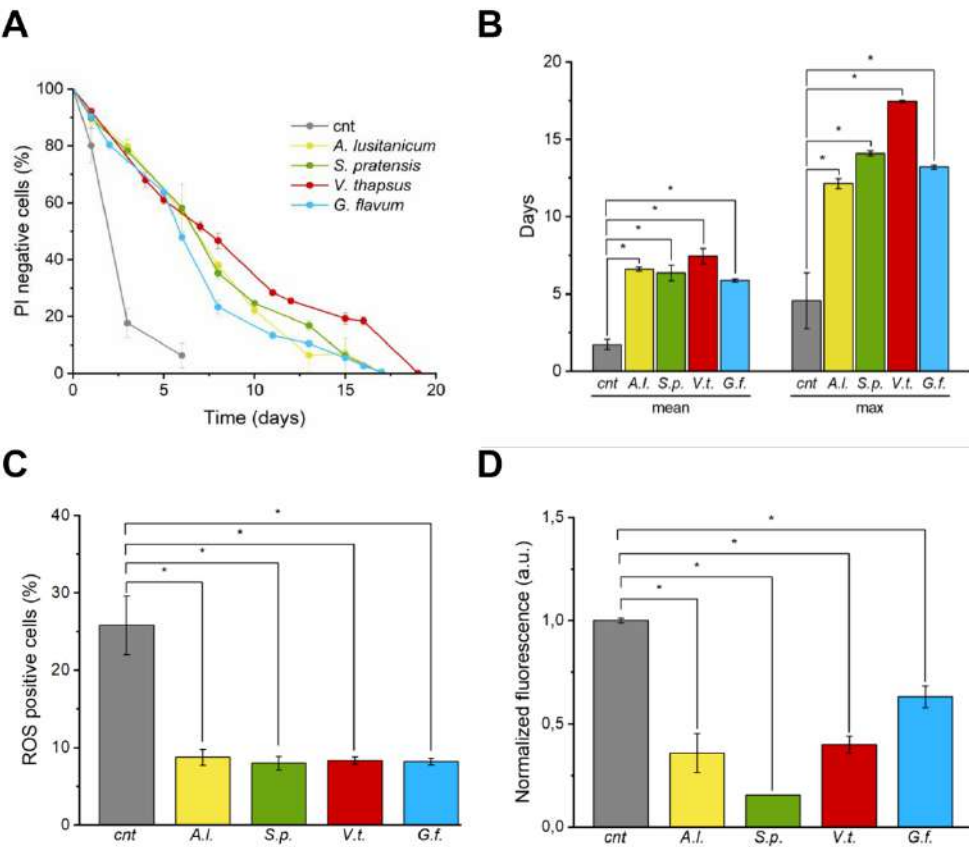


Figure 4. The extracts from *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* extend yeast lifespan. (A) CLS of yeast cells overexpressing α -syn grown in medium containing 2 % glucose in the absence or presence of *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* extracts (at a concentration corresponding to 20 mg of fresh leaves/mL medium), added in exponential phase of growth. (B) Mean and maximal lifespan of cells in (A). (C) ROS content of cells treated for 24 h as in (A). (D) Aggresomes level of cells treated for 24 h as in (A). Histograms represent mean $\pm$ SD of at least three independent experiments. * $p < 0.05$.

To further evaluate the ability of the selected extracts to prevent protein misfolding, SH-SY5Y neuroblastoma cell cultures conditionally expressing α -syn were also used [41]. Cells were treated with doxycycline to induce the overexpression of α -syn in the presence of the extracts, and the toxic oligomeric species of α -syn were detected following 48 h treatment. Notably, a significant decrease in α -syn oligomers was observed with *S. pratensis*, *V. thapsus*, and *G. flavum* extracts (around 40%) (Fig. 5A-B), which also slightly affected cell viability (Supplementary Fig. S4A). Despite the difference in oligomeric forms, the total level of α -syn (both endogenous and overexpressed, which bears a FLAG-tag that enables to discriminate it from the endogenous protein) did not vary significantly with the treatments (Fig. 5C). In contrast, the extract from *A. lusitanicum*, which strongly reduced cell viability (Supplementary Fig. S4A), did not lower α -syn oligomer level

at all (Fig. 5A-B). We speculate that its toxicity could overcome the potential activity of other beneficial compounds within the extract. Alternatively, these active molecules exhibited very low permeability, resulting in no detectable positive effect on cells.

Considering these results, we next investigated whether the extracts could inhibit the aggregation of the purified α -syn protein. To this end, we took advantage of the Thioflavin T (ThT) fibrillation assays to monitor α -syn aggregation over 72 h. In the absence of the extracts, ThT fluorescence exhibited a strong increase, indicating substantial amyloid fibril formation (Fig. 5D), as already known [25,42]. Strikingly, the presence of the extracts completely abolished this signal, with residual ThT fluorescence level of less than 2% for *A. lusitanicum* and *G. flavum*, and 9% and 17% for *V. thapsus* and *S. pratensis*, respectively (Fig. 5D). The inhibition of fibril formation was also confirmed with a dot blot using A11 antibody, which specifically recognizes oligomeric α -syn (Fig. 5E) and by TEM analysis (Fig. 5F). The results obtained strongly highlight that all the four extracts inhibit the aggregation of the purified α -syn protein *in vitro*.

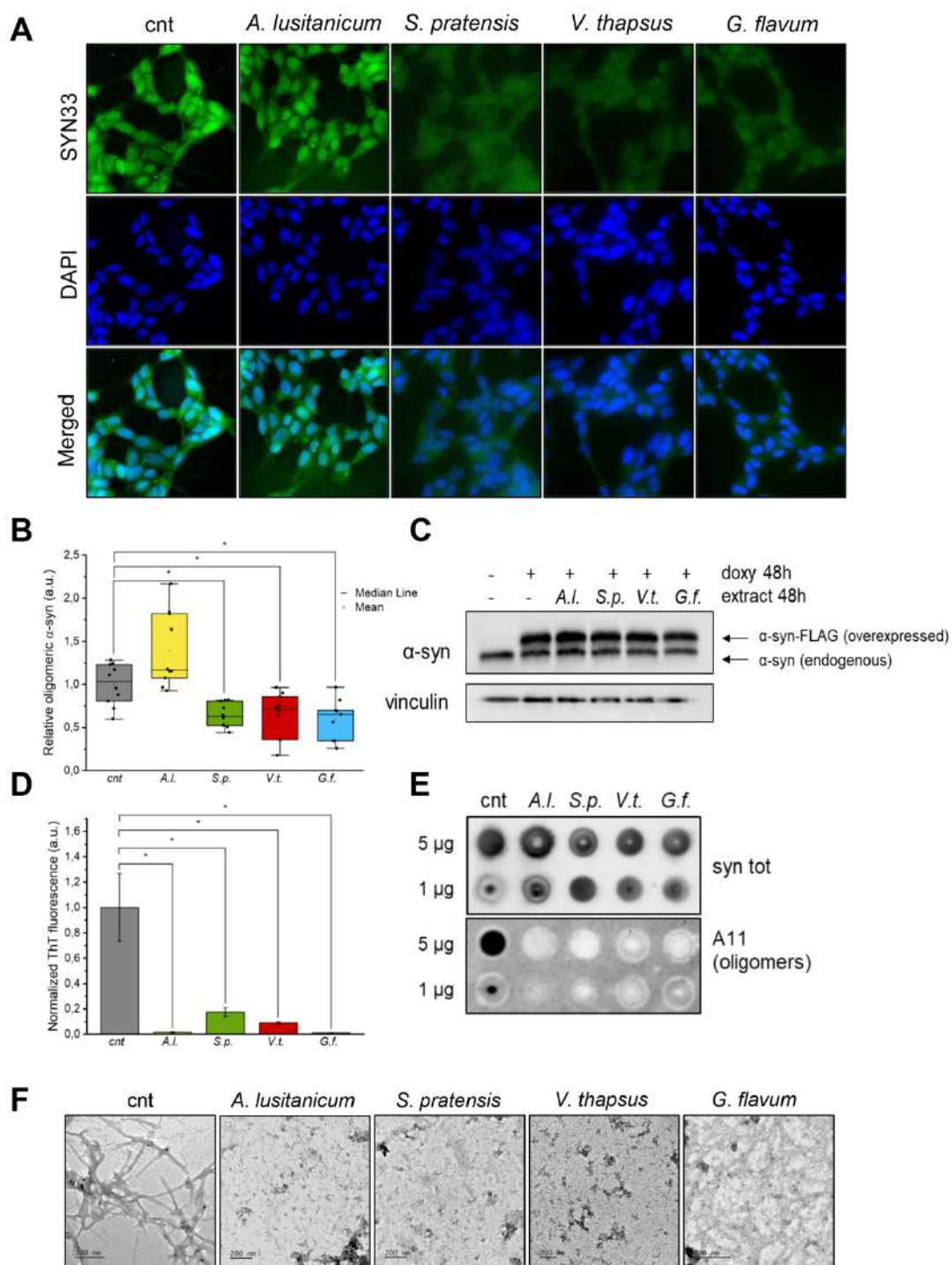


Figure 5. The extracts from *S. pratensis*, *V. thapsus* and *G. flavum* reduce α -syn aggregation. (A–B) SH-SY5Y *pTet-SNCA-FLAG* cells were treated with 6 μ g/mL doxycycline to induce α -syn overexpression, in the absence or presence of the extracts from *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* for 48 h. Representative immunofluorescence images (63x) of fixed cells, immunostained with anti-Syn33 antibody (specific for oligomeric species). Nuclei were stained by DAPI (blue). Graphs in B represent mean $\pm$ SD of oligomeric α -syn fluorescence quantified as Integrated Density (IntDen) using ImageJ software. (C) SH-SY5Y *pTet-SNCA-FLAG* cells were treated with 6 μ g/mL doxycycline to induce α -syn overexpression, in the absence or presence of the extracts from *A. lusitanicum*, *S. pratensis*, *G. flavum* and *V. thapsus* for 48 h. The level of α -syn (both endogenous and FLAG-tagged overexpressed proteins) was evaluated by Western blot. Vinculin was used as loading control. (D) α -syn aggregation process, followed by ThT fluorescence, in the absence (cnt) or presence of *A. lusitanicum*, *S. pratensis*, *V. thapsus* or *G. flavum* extracts for 72 h. (E) Dot-blot analysis of α -syn (1 and 5 μ g) after 72 h of aggregation in the absence (cnt) or presence of *A. lusitanicum*, *S. pratensis*, *V. thapsus* or *G. flavum* extracts using A11 anti-oligomer antibody and anti- α -syn antibody as a control. (F) TEM images of α -syn after 72 h of aggregation in the absence (cnt) or presence of *A. lusitanicum*, *S. pratensis*, *V. thapsus* or *G. flavum* extracts. Data represent mean $\pm$ SD from three independent experiments.

Then, surface plasmon resonance (SPR) analysis was employed to assess direct interactions between the extracts and α -syn protein by immobilizing the protein on a CM5 sensor chip (as previously reported in [23]). The anti- α -syn antibody bound to the protein on the chip, whereas the oligomer-specific anti-Syn33 antibody did not (Supplementary Fig. S5A), confirming that the immobilized α -syn was in its monomeric form. This system was then used to test direct interactions between α -syn and the extracts from *A. lusitanicum*, *S. pratensis*, *V. thapsus*, and *G. flavum*. All extracts showed concentration-dependent binding to α -syn, with increasing extract concentrations producing higher SPR signals (Supplementary Fig. S5B-I). These results, along with previous ones, suggest that the extracts contain compounds that directly bind α -syn and inhibit its amyloid aggregation.

In conclusion, integrating data from yeast, neuroblastoma cells, and SPR analysis, extracts from *S. pratensis*, *V. thapsus*, and *G. flavum* consistently inhibited the formation of toxic α -syn oligomers across all models, indicating the presence of anti-aggregant compounds.

Acteoside from *V. thapsus* binds α -syn

In order to identify which metabolites present in the extracts can directly bind to α -syn protein, the extracts from *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* were analysed by size exclusion chromatography coupled to reversed phase LC-MS

(SEC AS-MS). We decided to make this analysis considering all the extracts because the one from *A. lusitanicum* was able to affect α -syn aggregation *in vitro* (Fig. 5D-F), despite its inconsistent effect on neuroblastoma cells (Fig. 5A-B). An already established ligand fishing assay, based on size exclusion chromatography coupled to reversed phase LC-MS for the analysis of botanical extracts, was modified for the affinity screening of the four extracts towards α -syn [15].

Each extract was incubated with α -syn and injected in the SEC column to isolate and collect α -syn complexes. Bound ligands were desorbed from the trap column and analysed by RPLC-MS. The LC-MS chromatograms of *G. flavum* and *S. pratensis* extracts obtained by the described fishing method did not reveal the presence of any bound ligands. As concerns the *A. lusitanicum* extract, we observed the fishing of 3 different saponins, putatively identified through the classification of the aglycone class and the number and type of sugars attached to it, in accordance with Geng et al. 2021 [43]: saponin class IV3 +2 hexoses +3 desoxyhexoses, saponin class IV3 +2 hexoses +1 desoxyhexose + 1 pentose, saponin class IV3 +2 hexoses +1 desoxyhexoses (Supplementary Fig. S6A-C).

Notably, in the case of *V. thapsus* extract, the most abundant metabolite acteoside was successfully fished as reported in Fig. 6A. In particular, the chromatogram shows two peaks at retention times 27.35 and 28.20 corresponding to both

isoforms of the molecule, able to bind α -syn (Fig. 6A, lower panel). These peaks correspond to acteoside and its isomer and were identified by extracting the ionic current in the mass spectrum. Their presence was confirmed through fingerprinting and by comparing their mass values with those of the main molecules in the extract, as reported in Table 2. In the non-treated extract, they appeared at retention times 27.25 and 28.09 (Fig. 6A, upper panel), while in the SEC-AS-MS analysis of the incubated extract with α -syn, they shift slightly to 27.35 and 28.20. Interestingly, in the *V. thapsus* extract there are different structurally related compounds, such as leucosceptoside (phenylethanoid glycosides) or verminoside (caffeoyl-catalpol), but only acteoside was able to bind α -syn in the SEC AS-MS analysis, supporting the specificity of this binding.

Then, our analysis highlights that saponins from the extract of *A. lusitanicum* and acteoside from *V. thapsus* were able to bind α -syn protein. Interestingly, the abundance of the fished metabolites was always lower in the incubated samples compared to the extracts, as the result of the binding equilibrium.

We decided to focus our attention on the molecule acteoside (Fig. 6B), which is well known for its neuroprotective functions [44], and the interaction between α -syn and acteoside was further investigated by biophysical methods. Native-MS was employed to directly detect the protein-metabolite complex, thanks to the

preservation of non-covalent interactions during the analysis. The nano-ESI-MS spectrum of α -syn under non-denaturing conditions (Supplementary Fig. S7A) showed a multimodal charge state distribution, in agreement with the heterogeneous conformational ensemble populated by the intrinsically disordered polypeptide chain. The titration of α -syn with acteoside highlighted the comparison of new peaks in the spectrum, with a position that corresponds to the mass of the 1:1 protein-ligand complex (Fig. 6C). These new signals increased in intensity at high metabolite concentrations, and mainly involve the low- and medium-charge states of the protein, corresponding to the compact and intermediate conformational components (Fig. 6D). The complex identity of the signals was further confirmed by collision-induced dissociation in gas-phase, with the detection of the unbound partners upon collision with helium atoms (Supplementary Fig. S7B). Despite the semi-quantitative nature of Native-MS experiments, the simultaneous detection of free and bound protein states allowed the determination of a binding curve (Fig. 6E) and a rough estimation of the (apparent) dissociation constant of the complex ($K_d \sim 0.9$ mM).

by α -syn (lower panel). Extracted ion acteoside, 623.19 m/z . (B) Chemical structure of acteoside. (C) Acteoside- α -syn interaction by Native-MS. Enlargement in the region of α -syn 7+ charge state of Native-MS spectra, obtained at distinct acteoside concentrations (0–280 μ M). The peaks corresponding to unbound α -syn (blue circle) and 1:1 α -syn-acteoside complex (red circle) are labeled. (D) Histogram reporting the fraction of unbound α -syn (blue) and α -syn-acteoside complex (red) as a function of the analyte charge state. (E) Fraction of protein in the bound state vs. total ligand concentration. The solid line represents the non-linear fit of the experimental data with a 1:1 binding curve ($K_d \sim 0.9$ mM).

These results indicate a low affinity of α -syn for the metabolite, and are in agreement with the ones obtained by SPR. Indeed, this analysis also showed a direct binding of acteoside to α -syn, with a progressive increase of SPR-signal with ligand concentration. However, the accurate determination of the binding parameters was not possible, due to the lack of saturation caused by the very low affinity of the metabolite for the protein (Supplementary Fig. S8).

To conclude, considering its interaction with α -syn protein, the molecule acteoside from *V. thapsus* was considered for extended investigation.

Acteoside inhibits α -syn aggregation

Building upon the identification of acteoside as a key metabolite binding to α -syn, we further elucidated its potential neuroprotective properties. Acteoside (Fig. 6B), the predominant phenylpropanoid glycoside in *V. thapsus* extract (Fig. 3C, Table 2), is commonly found in a variety of plants, especially of the *Verbascum* species, but also in more than 200 other plant species belonging to 23 different families, and it is well known for its strong antioxidant activity [45]. In order to investigate the direct effect of acteoside on the protein aggregation process, the Thioflavin T (ThT) assay was used. The incubation of α -syn (35 μ M) in the presence of 100 μ M or 50 μ M acteoside revealed that the molecule was exceptionally active, abrogating almost completely the formation of fibrils, with residual ThT fluorescence level of less than 1% at both concentrations tested, similarly to the effect exerted by the whole *V. thapsus* extract (Fig. 7A). To better understand the inhibition of α -syn aggregation, we also investigated the protein aggregation kinetics. Notably, we did not observe the characteristic curve of α -syn aggregation when the protein was incubated with 100 μ M and 50 μ M of acteoside (Fig. 7B). This finding suggests that acteoside inhibits the formation of nucleation sites, which are essential for the initiation of the fibrillation phase and occur approximately 24 h from the beginning of the aggregation assay, as seen in the control sample (Fig. 7B). Remarkably, the inhibition of α -syn primary nucleation was also confirmed by the visualization of α -

syn fibrils by TEM. Indeed, fibril formation was clearly visible when α -syn was incubated alone, while the presence of acteoside completely abolished the formation of the fibrillar species (Fig. 7C).

We next analyzed whether acteoside can influence fibril elongation [46], by studying aggregation of monomeric α -syn in the presence of preformed fibrils (PFF) of α -syn that act as seeds for secondary nucleation. Strikingly, we observed that acteoside inhibited fibril elongation in a dose-dependent manner: 1 μ M acteoside did not affect the fibril elongation phase, whereas at higher concentrations (25 μ M, 50 μ M and 100 μ M) fibril elongation was completely abolished, as highlighted by the absence of the characteristic curve of aggregation (Fig. 7D).

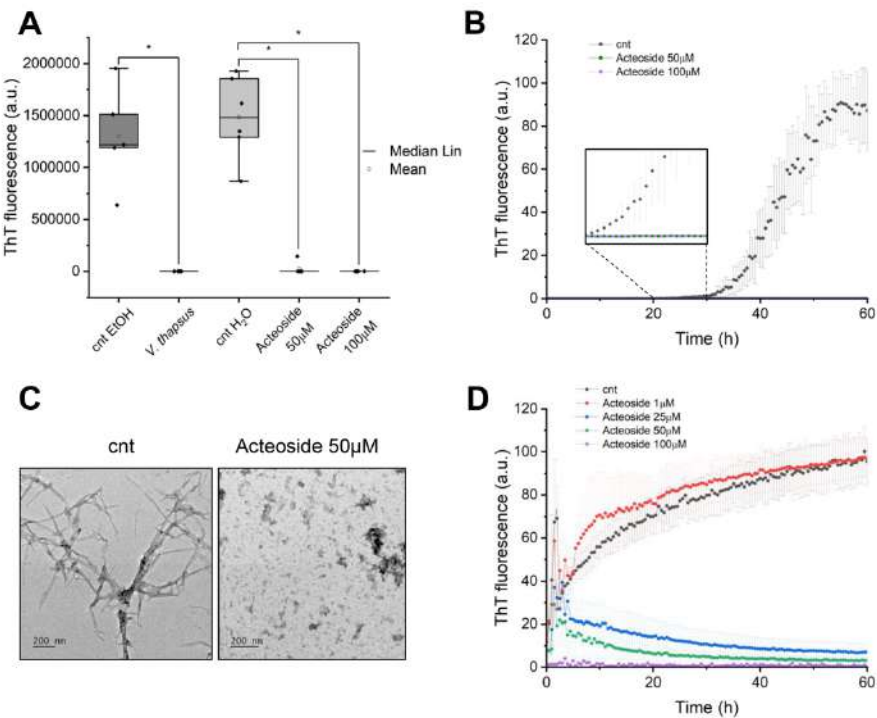


Figure 7. Acteoside inhibits α -syn aggregation. (A) α -syn aggregation process, followed by ThT fluorescence after 72 h, in the absence (cnt 1, solvent control 2.5 % ethanol) or presence of *V. thapsus* (solvent 2.5 % ethanol) and in the absence (cnt 2, solvent control water) or presence of acteoside (100 μ M or 50 μ M in aqueous solution). (B) Kinetic of α -syn aggregation process, detected by ThT fluorescence, in the absence (cnt) or presence of acteoside (100 μ M or 50 μ M), with a zoom of the 20–30 h time interval. (C) TEM images of 70 μ M α -syn after 72 h of aggregation in the absence (cnt) or presence of acteoside (50 μ M in aqueous solution). (D) α -syn fibril elongation: 35 μ M monomeric protein was incubated with 1 μ M of preformed fibrils, detected by ThT fluorescence, in the absence (cnt) or presence of increasing concentration of acteoside (1 μ M, 25 μ M, 50 μ M and 100 μ M). Representative data from $n = 3$ independent experiments.

Altogether, these data indicate that acteoside plays a key role in the anti-aggregant effects of *V. thapsus* extract, influencing both primary nucleation and fibril elongation phases.

Acteoside activates the NRF2 signaling pathway

Before investigating acteoside effects on cell cultures, its cellular availability was assessed in human neuroblastoma cells by measuring intracellular/cell-associated level of acteoside in cells treated with 100 μ M of acteoside, a concentration which was not toxic up to 72 h (Supplementary Fig. S4B). As shown in Fig. 8A, the highest intracellular/cell-associated acteoside levels were observed after 1 h of treatment, and it gradually decreased over time. Then, in order to confirm the protective role of acteoside against oxidative stress, which is strictly connected with the onset of PD [47], SH-SY5Y cells were exposed to 5 μ M extracellular α -syn aggregates, obtained in the absence or presence of 1 mM acteoside. Intracellular ROS level induced by PFF decreased by 54% with acteoside in comparison with the control (Fig. 8B).

Strikingly, in SH-SY5Y neuroblastoma cells conditionally expressing α -syn, treatment with 100 μ M acteoside reduced the level of aggregated α -syn, as measured by immunofluorescence analysis using an antibody specific for the oligomers (Fig. 8C), without affecting the total level of α -syn, both endogenous and overexpressed (which bears a FLAG tag that enables to discriminate it from the endogenous protein, Fig. 8D).

To investigate the molecular mechanism underlying acteoside's antioxidant activity, we focused on NRF2, a well known pathway responsible for mediating antioxidant responses and maintaining redox homeostasis [48]. Indeed, we observed an increase in NRF2 protein level in cells treated with acteoside (Fig. 8E,F). Moreover, a clear nuclear enrichment of NRF2 was observed upon acteoside treatment (Fig. 8G,H). Accordingly, the mRNA level of two NRF2-dependent genes (NQO1 and HMOX1) increased in cells treated with acteoside (Fig. 8I), supporting NRF2 functional activation. In addition, pretreatment with acteoside prevented the increase of ROS level in cells treated with fibrils, but this effect was not observed when acteoside was added together with the NRF2 inhibitor (Fig. 8J).

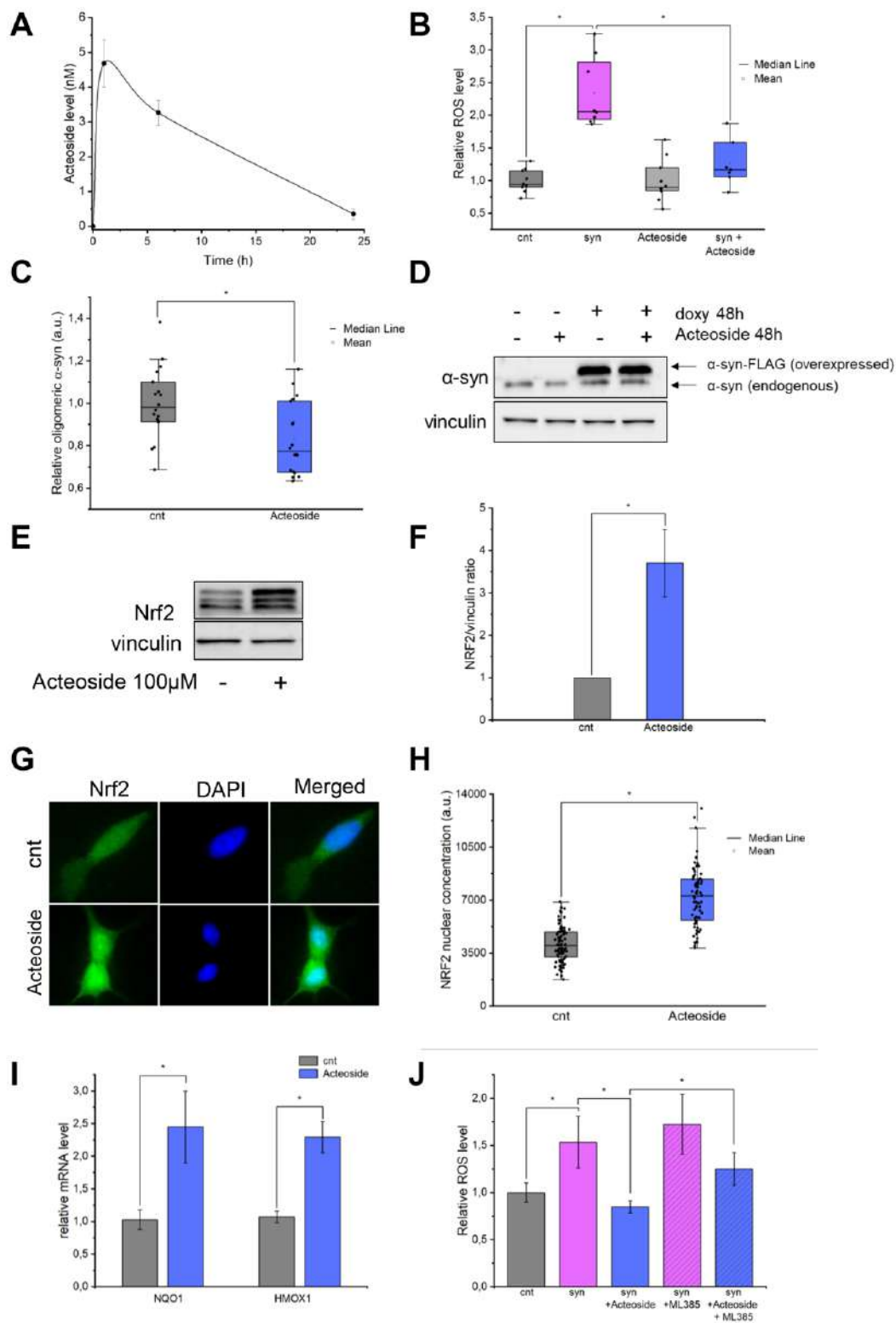


Figure 8. Acetoside activates the NRF2 signaling pathway. (A) Temporal course of intracellular/cell-associated level of acetoside in neuroblastoma cells. Data are expressed as pmol acetoside/ μ g proteins. (B) SH-SY5Y cells were grown for 72 h in the absence or presence of 5 μ M α -syn, obtained after 72 h of aggregation without or with 1 mM acetoside. ROS level was evaluated by H₂DCFDA staining. * $p < 0.05$. (C) SH-SY5Y *pTet-SNCA-FLAG* cells were treated with 6 μ g/mL doxycycline to induce α -syn overexpression, in the absence or presence of 100 μ M acetoside for 48 h. Cells were fixed and immunostained with anti-Syn33 antibody (specific for oligomeric species). Graphs represent mean $\pm$ SD of oligomeric α -syn fluorescence quantified as Integrated Density (IntDen) using ImageJ software. (D) SH-SY5Y *pTet-SNCA-FLAG* cells were treated with 6 μ g/mL doxycycline to induce α -syn overexpression, in the absence or presence of 100 μ M acetoside for 48 h. The level of α -syn (both endogenous and FLAG-tagged overexpressed) was evaluated by Western blot. Vinculin was used as loading control. (E) Western analysis using anti-NRF2 and anti-vinculin antibody on protein extracts from neuroblastoma cells grown for 48 h in the absence (cnt) or in the presence of acetoside (100 μ M). (F) Quantitative analysis of NRF2 protein level in (E). (G) Immunofluorescence images of neuroblastoma cells treated with 100 μ M of acetoside for 48 h. Fixed cells were immunostained with anti-NRF2 antibody. Nuclei were stained by DAPI (blue). (H) Quantitative analysis of nuclear localization in (G) performed using Cell-ACDC

software. (I) Relative mRNA expression levels of NQO1 and HMOX1 genes in neuroblastoma cells grown in the absence (cnt) or in the presence of acteoside (100 μ M) for 48 h. (J) SH-SY5Y cells were pretreated with 100 μ M acteoside and/or 5 μ M ML385 (NRF2 inhibitor) for 2 h, then treated with 5 μ M α -syn fibrils for 72 h. ROS level was evaluated by H₂DCFDA staining. Data represent mean $\pm$ SD from $n = 3$ independent experiments, * $p < 0.05$.

Altogether, these findings suggest that acteoside reduces intracellular ROS level through the activation of the NRF2 signaling pathway in neuroblastoma cells, in line with its antioxidant and neuroprotective effect.

Acteoside improves locomotor performance and reduces oxidative protein damage in a *Drosophila melanogaster* model of PD

Considering the antioxidant efficacy of acteoside in various biological systems [49–52], including rotenone-treated flies (a *Drosophila* model of sporadic PD) [53], we tested acteoside in a *Drosophila* PD model based on *DJ-1 β* inactivation. The DJ-1 protein plays a central role in cellular defense against oxidative stress and is involved in ROS scavenging, transcriptional regulation, redox-dependent chaperone activity, mitochondrial function, and anti-apoptotic signaling [54]. Accordingly, *DJ-1 β* mutant flies exhibit several PD-relevant phenotypes, including locomotor deficits, reduced lifespan, ROS accumulation, and hypersensitivity to

oxidative toxins [29,55,56]. Thanks to its high pharmacological responsiveness, this *Drosophila* PD model has been successfully used to evaluate the therapeutic potential of multiple compounds with different mechanisms of action [29,56–58]. Besides, it should be mentioned that mutations in the DJ-1 protein cause autosomal recessive forms of PD, and oxidatively damaged DJ-1 protein accumulates in the brains of patients with idiopathic PD [59]. Therefore, to assess the potential therapeutic effect of acteoside, *DJ-1 β* mutant flies were cultured on medium supplemented with the compound throughout development, from larval stage to five days post-eclosion. To determine the optimal dose for acteoside supplementation in flies, two concentrations of the compound were used (250 μ M and 400 μ M) and locomotor performance was assessed via climbing assays in 5-day-old flies. Acteoside administration resulted in a significant, dose-dependent improvement in motor function, producing a beneficial effect at both tested concentrations (Fig. 9A).

DJ-1 β mutant flies also exhibit increased ROS production, resulting in oxidative damage of cellular components [57,60]. A notable marker of oxidative damage is protein carbonylation, whose level is elevated in *DJ-1 β* mutant flies [61]. To determine whether acteoside influences oxidative protein damage, we measured protein carbonylation level in extracts from 5-day-old *DJ-1 β* mutant flies treated with vehicle or with the compound at the tested concentrations (250 μ M and 400

μM). Consistent with the climbing assay results, flies treated with 400 μM acteoside exhibited a significant reduction of protein carbonyl groups (Fig. 9B), indicating that this compound can attenuate oxidative stress-induced protein damage, likely contributing to the observed improvements in motor performance. Despite the strong effect on motor function, treatment with 400 μM acteoside had no significant impact on fly longevity (Supplementary Fig. S9), suggesting that it may improve healthspan rather than extend lifespan.

To further evaluate whether acteoside could exert a beneficial effect also when administered in adulthood, more accurately reflecting the timing of PD, flies were treated with acteoside (at a concentration of 400 μM) after eclosion and climbing assays were performed 5 and 10 days following treatment initiation. As shown in Fig. 9C, acteoside significantly improved motor ability at both time points, demonstrating that it retains its efficacy also when administered post-eclosion. In agreement with these results, protein carbonylation level was also significantly decreased after 10 days of treatment (Fig. 9D). Therefore, the beneficial effect of acteoside emerges not only with early supplementation but also when treatment is initiated at advanced stages, reflecting the temporal dynamics and progression of PD pathology.

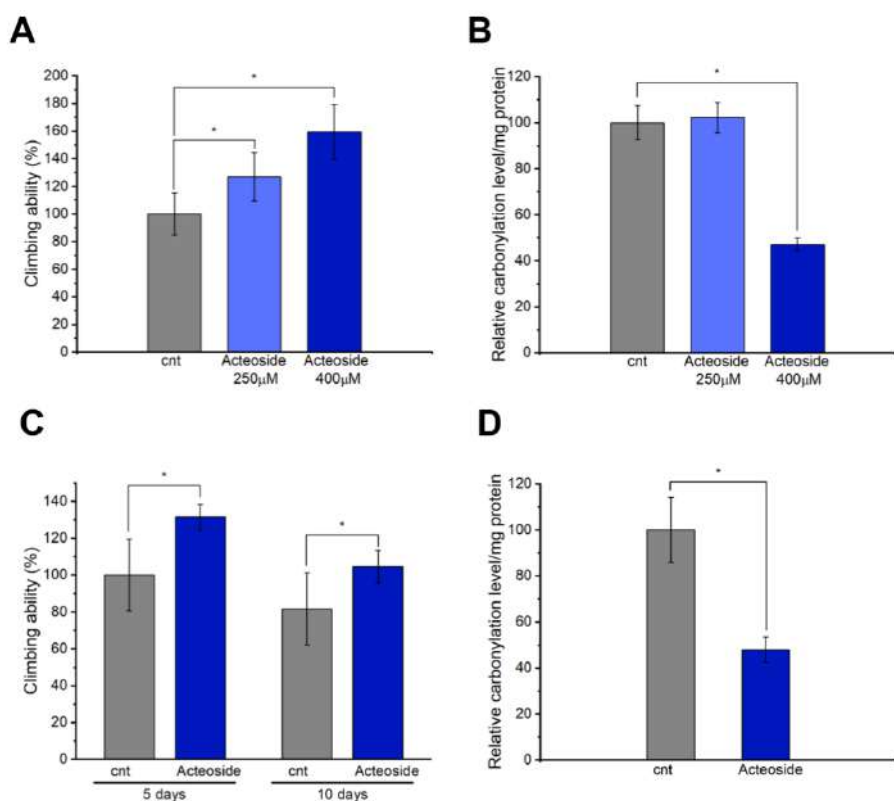


Figure 9. Effect of acteoside on *DJ-1b* mutant flies. (A) Motor performance of *DJ-1b* mutant flies treated with 250 and 400 μ M acteoside was evaluated by performing a climbing assay ($n = 80$ – 120 5-day-old female flies for each condition). (B) Protein carbonylation level in *DJ-1b* mutant flies treated with 250 and 400 μ M acteoside was analyzed using 2,4-dinitrophenylhydrazine (DNPH) derivatization, data are expressed as arbitrary units (a.u.) per mg of protein. Results were normalized to data obtained from flies cultured in vehicle medium (0.1 % DMSO). Values are expressed as mean $\pm$ SD from three independent experiments in which three biological replicates were used. Groups were statistically compared by one-

way ANOVA analysis followed by Tukey's post hoc test ($*p < 0.05$). (C) Motor performance of *DJ-1b* mutant flies treated post-eclosion with 400 μ M acteoside was evaluated by performing a climbing assay 5 and 10 days after treatment initiation ($n = 80$ – 120 female flies per condition). (D) Protein carbonylation level in *DJ-1b* mutant flies treated post-eclosion with 400 μ M acteoside was analyzed 10 days after treatment using 2,4-dinitrophenylhydrazine (DNPH) derivatization, data are expressed as arbitrary units (a.u.) per mg of protein. Results were normalized to data obtained from control flies cultured in vehicle medium (0.1 % DMSO). Values are expressed as mean $\pm$ SD from three independent experiments. $*p < 0.05$.

In conclusion, the observed functional recovery in *DJ-1b* mutants suggests that acteoside can effectively counteract deficits linked to *DJ-1b* dysfunction, thereby modulating PD-related phenotypes and validating its therapeutic potential.

Discussion

Plant biodiversity is an exceptional source of secondary metabolites, nevertheless no more than 15% of higher plants have been systematically investigated for medicinal properties [62]. Consequently, the approach of bioprospecting, which promotes the research in biodiversity for new resources with potential income, remains a valuable opportunity to identify new bioactive compounds, or new

sources of known compounds, increasing the value of biodiversity and supporting its conservation. Natural substances play a central role in creating innovative solutions for disease prevention/treatment and the advancement of analytical techniques, combined with high-throughput screening methods, can significantly improve the process of drug discovery. The biodiversity of the Mediterranean hotspot [11] is an extraordinary resource of bioactive compounds for advancing innovative therapies targeting non-communicable diseases, including neurodegenerative disorders.

A growing number of patients worldwide suffer from PD and the currently available drugs, which regulate dopaminergic and cholinergic transmission, primarily aim to control symptoms and improve patients' quality of life. However, there is still no definitive cure for this disease, making it necessary to develop new drugs, with particular attention to preventive strategies.

In this paper, we present a multidisciplinary approach to examining 60 different Italian plants representative of part of the Mediterranean flora. We combined an *in silico* screening system based on phylogenetic methods with a high-throughput screening using as a model the budding yeast expressing human α -syn.

This approach allowed a rapid and economic high throughput analysis and led us to focus on phytoextracts from four plants, namely *A. lusitanicum*, *S. pratensis*, *V.*

thapsus and *G. flavum*, which are promising sources of neuroprotective compounds for the preventive treatment of PD, being strongly active in reducing the toxic intracellular species of aggregated proteins. Notably, acteoside, one of the major metabolites found in the *V. thapsus* extract, emerges as the key molecule that binds α -syn. Of paramount importance, *V. thapsus* is already included in the Belfrit list [32], supporting its utilization as a public health product [33]. Acteoside, also known as verbascoside, was first isolated in 1963 from *Verbascum sinuatum* L., belonging to the family *Scrophulariaceae* [45]. Chemically, acteoside (Fig. 6B), with a formula of $C_{29}H_{36}O_{15}$ and a molecular weight of 624 Da, is a phenylethanoid glycoside [63]. Acteoside contains a hydroxyl salidroside residue condensed with caffeoyl and rhamnose moieties and is among the most widespread disaccharide caffeoyl esters. While mainly detected in *Verbascum* species, acteoside has also been found in 23 different plant families [45,64]. It is extracted from various traditional Chinese medicine sources including *Rehmannia glutinosa*, *Cistanche deserticola* and *Pedicularis kansuensis* [65] and is considered a crucial secondary metabolite in folk medicine [66,67] with a variety of pharmacological activities [68–72]. Several studies have reported that acteoside exerts strong antioxidant activities, and it is especially able to enhance the endogenous antioxidant defences of the cell (for a review see [45]). The neuroprotective effects of acteoside have

been linked not only to its ability to mitigate oxidative stress, but also to inhibit neuroinflammation and to modulate autophagy [53,67].

Here we also show that acteoside directly binds to α -syn and inhibits its amyloid fibrils formation (Fig. 6 and 7), a key event in the prevention of PD. Specifically, since α -syn aggregation involves both nucleation and growth phases [73], we show under different aggregation conditions that acteoside inhibits primary nucleation, by preventing nuclei formation during the lag phase (Fig. 7B,C), as well as fibril-induced amplification during the growth phase (Fig. 7D), indicating that acteoside functions as a protein aggregation inhibitor at multiple stages of the process. Intriguingly, previous studies have shown that acteoside reduces the aggregation of another prion-like protein, the amyloid β (A β) peptide, thereby decreasing its toxicity in Alzheimer's disease models [74–76].

The discovery that acteoside also inhibits α -synuclein fibrillation paves the way for its potential development as a preventive treatment for PD. Given that PD is a disorder with complex evolution, a broad-spectrum approach to target different pathological pathways could be a promising strategy. In this context, oxidative stress is strongly associated with this disease and it is linked with various cellular processes, which all seem to contribute to neurodegeneration [77]. Notably, in SH-SY5Y cells exposed to toxic α -syn aggregates, acteoside decreases intracellular ROS

level (Fig. 8B). This is associated with the activation of the NRF2 signaling pathway, leading to the stimulation of the antioxidant response (Fig. 8). In agreement with our data, previous reports have highlighted the role of acteoside in modulating the NRF2 pathway *in vitro* and both in mouse and Zebrafish PD models [78,79]. Moreover, docking analyses have also reported possible binding of acteoside to NRF2 protein [80]. Remarkably, acteoside treatment restored locomotor function and reduced protein carbonylation *in vivo* in a *Drosophila* model of PD based on loss of *DJ-1 β* function (Fig. 9). This dual action in a different PD model affecting a gene involved in both familial and sporadic form of the disease underscores its potential as a multifunctional therapeutic candidate for PD. Although preliminary, these results, obtained in a multicellular model organism, provide a solid basis for future studies aimed at dissecting the molecular pathways targeted by acteoside and evaluating its translational relevance in mammalian PD models.

Understanding acteoside's pharmacokinetic properties is crucial to assess its therapeutic potential and translational value. Despite its well documented antioxidant and neuroprotective activities, and its high level of safety [71,81–83], acteoside exhibits poor bioavailability [51]. *In vivo* studies in rats and beagle dogs have shown low systemic availability after oral administration, with bioavailability ranging from 0.1% to 4%. This poor bioavailability is primarily attributed to acteoside's high hydrophilicity, low membrane permeability and active efflux

transport through P-glycoprotein (P-gp) [44]. Our findings are consistent with the previous observations. Intracellular or cell-associated acteoside reaches approximately 0.1% after 1h of treatment (Fig. 8A). This low but measurable uptake confirms acteoside's ability to enter cells or interact with their membranes, supporting its bioactivity. In addition, promising studies in rat models indicate that acteoside is able to reach the brain [84] and its blood-brain barrier permeability can be further enhanced through lipid encapsulation [85], suggesting that proper formulations of this compound are essential to maximize its activity *in vivo*. In conclusion, this study provides new evidence that acteoside possesses an inhibitory effect on α -syn aggregation and supports its antioxidant role, suggesting the relevance of this molecule as a candidate for neuroprotection in PD. Future studies will be essential to investigate the molecular pathways involved in the anti-aggregant properties of acteoside and to develop new strategies to increase its potential value as a preventive agent against synucleinopathies *in vivo*.

ACKNOWLEDGMENTS

Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.4—Call for tender No. 3138 of December 16, 2021, rectified by Decree n.3175 of December 18, 2021 of Italian Ministry of University

and Research funded by the European Union—NextGenerationEU; Award Number: Project code CN_00000033, Concession Decree No. 1034 of June 17, 2022. Adopted by the Italian Ministry of University and Research, CUP, H43C22000530001, Spoke 6, Project title “National Biodiversity Future Center—NBFC”. Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.3—Call for tender No. 3138, 16 December 2021, rectified by Decree n.341 of 15 March 2022 of Italian Ministry of University and Research funded by the European Union—NextGenerationEU; Project code PE0000003 ON FOODS—CUP:H43C22000820001—Spoke 6, Project title “ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON Foods”. Project funded by the University of Valencia to Nuria Paricio, Grantcode UV-INV-AE-3664989.

Abbreviations

α -syn: α -synuclein

CLS: Chronological lifespan assay

MP: Medicinal Plants

PD: Parkinson’s disease

PFF: preformed fibrils

PMP: Potential Medicinal Plants

ROS: reactive oxygen species

RPLC-MS: reverse phase liquid chromatography mass spectrometry

SEC AS-MS: size exclusion chromatography coupled to reversed phase LC-MS

SPR: Surface Plasmon Resonance

TEM: Transmission electron microscopy

ThT: Thioflavin T

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Ethical statement

Not required as no humans or animals are involved in this work.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary Methods

Surface plasmon resonance (SPR) analysis

The BIACORE X100 system (Cytiva-Pall) was utilized to analyse molecular interactions between α -syn and the selected extracts via SPR. α -syn was immobilised onto a carboxymethylated dextran surface of a CM5 sensor chip using amine-coupling chemistry as previously described [1]. The association capacity of α -syn was determined by injecting two control antibodies, anti- α -syn (Merck) recognizing the whole α -syn (positive control) and anti- α -Syn33 (Merck) recognizing α -syn oligomers (negative control) [1].

The extracts were lyophilized to be weighed, and resuspended in 100% EtOH at the appropriate concentrations, then sonicated for 30 min at 40 kHz and centrifuged for 5 min at 13000 rpm. Afterwards, the samples were diluted in HBS-EP running buffer to achieve a 10% EtOH concentration, sonicated for 20 min at 40 kHz and injected at multiple concentrations for 5 min at 25°C and a flow rate of 10 μ L/min, with the same running buffer (HBS-EP) containing 10% EtOH. The blank (HBS-EP containing 10% EtOH) was injected under the same conditions. After injection, the analyte solutions were replaced by the running buffer at a continuous flow rate of 10 μ L/min for 5 min.

For experiments with acteoside, the compound was first dissolved in water, then diluted in HBS-EP running buffer, sonicated 30 min at 28 kHz and injected at different concentrations (5 mM, 2.5 mM, 1.25 mM, 0.625 mM, 0.312 mM). Each concentration was injected over the α -syn surface for 1 min at 25 °C with a constant flow rate of 10 μ L/min using the same HBS-EP as the running buffer.

Surface regeneration was achieved by injecting 50 mM NaOH for a contact time of 1 min. Each sensorgram was double reference subtracted, for the response observed in the control flow cell 1 (no immobilized protein) and, also, for the blank subtraction. The sensorgram curves were acquired using the BiacoreX100 Control software, version 2.0.2 (Cytiva-Pall), in manual run mode.

Lifespan assay

Groups of 75 1-day-old *DJ-1 β* mutant female flies were collected for each experimental condition. Flies were divided into groups of 25 individuals and transferred into separate vials containing either food supplemented with 0.1% DMSO (control) or food supplemented with acteoside (400 μ M). Every two or three days, the number of dead flies in each vial was recorded, and the flies were transferred to fresh vials every seven days. This procedure was continued until all flies had died and the percentage of flies alive at each time point was graphed [2]. Survival data were analyzed using Kaplan–Meier curves, and statistical differences

between groups were evaluated with a semiparametric log-rank test [3]. The analysis was conducted using GraphPad Prism 4 software.

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Supplementary Tables and Figures

Division		Order	Family	Species	Source	Organs collected	Number of plants per replicate
Pteridophyta	L	Lycopodiales	Lycopodiaceae	<i>Spinulum annotinum</i> (L.) A.Haines subsp. <i>annotinum</i>	OF (Plateau of Asiago, Vicenza, Italy)	stems + leaves	1
	P	Equisetales	Equisetaceae	<i>Equisetum palustre</i> L.	BG	stems + leaves	5
Magnoliophyta (Angiosperms)	M	Acorales	Acoraceae	<i>Acorus calamus</i> L.	BG	leaves	3
	M	Alismatales	Butomaceae	<i>Butomus umbellatus</i> L.	BG	leaves	3
	M	Asparagales	Amaryllidaceae	<i>Allium angulosum</i> L.	N	leaves	2
	M	Asparagales	Amaryllidaceae	<i>Allium lusitanicum</i> Lam.	N	leaves	2
	M	Asparagales	Amaryllidaceae	<i>Sternbergia lutea</i> (L.) Ker Gawl. ex Spreng.	BG	leaves	5
	M	Asparagales	Iridaceae	<i>Limniris pseudacorus</i> (L.) Fuss	N	leaves	2
	M	Poales	Cyperaceae	<i>Carex viridula</i> Michx.	N	leaves	2
	M	Poales	Cyperaceae	<i>Schoenoplectus mucronatus</i> (L.) Palla	N	leaves	2
	M	Poales	Poaceae	<i>Achnatherum calamagrostis</i> (L.) P. Beauv.	N	leaves	2
	M	Poales	Poaceae	<i>Phyllostachys viridiglaucescens</i> (Carrière) Rivière & C. Rivière	BG	leaves	5
	M	Poales	Poaceae	<i>Pseudosasa japonica</i> (Siebold & Zucc. ex Steud.) Makino ex Nakai	BG	leaves	5
	M	Poales	Typhaceae	<i>Typha laxmannii</i> Lepech.	N	leaves	2
	EU	Ranunculales	Lardizabalaceae	<i>Akebia quinata</i> (Thunm. ex Houtt.) Decne.	BG	leaves	2

EU	Ranunculales	Papaveraceae	<i>Glaucium flavum</i> Crantz	BG	leaves	2
EU	Ranunculales	Ranunculaceae	<i>Aquilegia atrata</i> W.D.J. Koch	N	leaves	2
EU	Ranunculales	Ranunculaceae	<i>Pulsatilla montana</i> (Hoppe) Rchb.	N	leaves	2
EU	Ranunculales	Ranunculaceae	<i>Ranunculus lingua</i> L.	N	leaves	2
EU	Ceratophyllales	Ceratophyllaceae	<i>Ceratophyllum demersum</i> L.	N	stems + leaves	1
CE	Saxifragales	Haloragaceae	<i>Myriophyllum spicatum</i> L.	N	stems + leaves	1
CE	Saxifragales	Saxifragaceae	<i>Heuchera sanguinea</i> Engelm	BG	leaves	2
R	Celastrales	Celastraceae	<i>Euonymus europaeus</i> L.	BG	leaves	1
R	Fabales	Fabaceae	<i>Ceratonia siliqua</i> L.	BG	leaves	1
R	Fabales	Fabaceae	<i>Medicago marina</i> L.	N	stems + leaves	2
R	Fabales	Fabaceae	<i>Parkinsonia aculeata</i> L.	BG	leaves	1
R	Fagales	Fagaceae	<i>Castanea sativa</i> Mill	BG	leaves	1
R	Malpighiales	Euphorbiaceae	<i>Acalypha virginica</i> L.	OF (Parco dell'Adige, Verona, Italy)	leaves	2
R	Malvales	Cistaceae	<i>Cistus laurifolius</i> L.	BG	leaves	1
R	Malvales	Cistaceae	<i>Cistus monspeliensis</i> L.	BG	leaves	1
R	Malvales	Malvaceae	<i>Althaea officinalis</i> L.	N	leaves	2
R	Malvales	Malvaceae	<i>Malva alcea</i> L.	N	leaves	2
R	Myrtales	Onagraceae	<i>Epilobium ciliatum</i> Raf.	N	stems + leaves	2
R	Rosales	Rosaceae	<i>Filipendula ulmaria</i> (L.) Maxim.	N	leaves	2
R	Rosales	Rosaceae	<i>Rosa canina</i> L.	BG	leaves	1
R	Rosales	Rosaceae	<i>Sanguisorba officinalis</i> L.	BG	leaves	3
A	Caryophyllales	Amaranthaceae	<i>Beta vulgaris</i> L.	N	leaves	6
A	Caryophyllales	Caryophyllaceae	<i>Dianthus superbus</i> L. subsp. <i>superbus</i>	N	stems + leaves	2
A	Santalales	Santalaceae	<i>Osyris alba</i> L.	BG	stems + leaves	3

A	Apiales	Apiaceae	<i>Echinophora spinosa</i> L.	N	stems + leaves	2
A	Apiales	Apiaceae	<i>Eryngium maritimum</i> L.	N	stems + leaves	2
A	Asterales	Asteraceae	<i>Artemisia abrotanum</i> L.	BG	leaves	1
A	Asterales	Asteraceae	<i>Petasites paradoxus</i> (Retz.) Baumg.	N	leaves	2
A	Asterales	Campanulaceae	<i>Adenophora liliifolia</i> (L.) Ledeb.ex A.DC.	N	leaves	2
A	Boraginales	Boraginaceae	<i>Echium vulgare</i> L.	BG	leaves	3
A	Boraginales	Boraginaceae	<i>Nonea lutea</i> (Desr.) DC.	BG	leaves	5
A	Dipsacales	Dipsacaceae	<i>Succisa pratensis</i> Moench	N	leaves	2
A	Dipsacales	Valerianaceae	<i>Centranthus ruber</i> (L.) DC.	BG	leaves	1
A	Dipsacales	Valerianaceae	<i>Valeriana dioica</i> L.	N	leaves	2
A	Ericales	Actinidiaceae	<i>Actinidia deliciosa</i> (A.Chev.) C.F.Liang & A.R.Ferguson	OF (Local producer, Verona, Italy)	leaves	6
A	Ericales	Ebenaceae	<i>Diospyros kaki</i> Thunb.	BG	leaves	1
A	Ericales	Ericaceae	<i>Empetrum hermaphroditum</i> Hagerup	OF (Plateau of Asiago, Vicenza, Italy)	stems + leaves	1
A	Ericales	Primulaceae	<i>Lysimachia vulgaris</i> L.	N	leaves	2
A	Gentianales	Apocynaceae	<i>Carissa macrocarpa</i> (Eckl.) A.DC.	BG	leaves	1
A	Lamiales	Lamiaceae	<i>Salvia pratensis</i> L.	N	leaves	2
A	Lamiales	Plantaginaceae	<i>Gratiola officinalis</i> L.	N	leaves	2
A	Lamiales	Plantaginaceae	<i>Russelia equisetiformis</i> Schltld. & Cham.	BG	stems + leaves	2
A	Lamiales	Scrophulariaceae	<i>Verbascum thapsus</i> L.	BG	leaves	1
A	Lamiales	Verbenaceae	<i>Aloysia citrodora</i> Paláu	BG	leaves	1
A	Lamiales	Verbenaceae	<i>Glandularia tenera</i> (Spreng.) Cabrera	BG	leaves	3

Table S1. Taxonomy and sampling details of the plant species collected.

Abbreviations: Taxonomy – M (Monocots), EU (Eudicots), CE (Core Eudicots), R (Rosids), A (Asterids), L (Lycopodiophyta), P (Polypodiophyta); Source - BG= Botanic Garden of Padova (Italy); N= Nursery (Centre for biodiversity and outside-forest activities; Montecchio Precalcino, Vicenza, Italy); OF= open field. For each species, three biological replicates, each one represented by a pool of more independent individuals (from 2 to 6) have been collected and analyzed. One single individual per replicate was used only when more individuals were not available.

Plants	Reference phylogeny
<i>Acalypha virginica</i>	-
<i>Achnatherum calamagrostis</i>	Monocotyledoneae
<i>Acorus calamus</i>	Monocotyledoneae
<i>Actinidia deliciosa</i>	Superasteridae
<i>Adenophora liliifolia</i>	Superasteridae
<i>Akebia quinata</i>	Basal eudicots
<i>Allium angulosum</i>	Monocotyledoneae
<i>Allium lusitanicum</i>	Monocotyledoneae
<i>Aloysia citrodora</i>	-

<i>Althaea officinalis</i>	Superrosidae
<i>Aquilegia atrata</i>	Basal eudicots
<i>Artemisia abrotanum</i>	Superasteridae
<i>Beta vulgaris</i>	Superasteridae
<i>Butomus umbellatus</i>	Monocotyledoneae
<i>Carex viridula</i>	Monocotyledoneae
<i>Carissa macrocarpa</i>	Superasteridae
<i>Castanea sativa</i>	Superrosidae
<i>Centranthus ruber</i>	Superasteridae
<i>Ceratonia siliqua</i>	Superrosidae
<i>Ceratophyllum demersum</i>	Ceratophyllales

<i>Cistus laurifolius</i>	Superrosidae
<i>Cistus monspeliensis</i>	Superrosidae
<i>Dianthus superbus</i>	Superasteridae
<i>Diospyros kaki</i>	Superasteridae
<i>Echinophora spinosa</i>	-
<i>Echium vulgare</i>	Superasteridae
<i>Empetrum hermaphroditum</i>	Superasteridae
<i>Epilobium ciliatum</i>	Superrosidae
<i>Equisetum palustre</i>	Pteridophyta
<i>Eryngium maritimum</i>	Superasteridae
<i>Euonymus europaeus</i>	Superrosidae

<i>Filipendula ulmaria</i>	Superrosidae
<i>Glaucium flavum</i>	Basal eudicots
<i>Gratiola officinalis</i>	Superasteridae
<i>Heuchera sanguinea</i>	Superrosidae
<i>Limniris pseudoacorus (Iris pseudacorus)</i>	Monocotyledoneae
<i>Spinulum annotinum (Lycopodium annotinum)</i>	Pteridophyta
<i>Lysimachia vulgaris</i>	Superasteridae
<i>Malva alcea</i>	Superrosidae
<i>Medicago marina</i>	Superrosidae

<i>Myriophyllum spicatum</i>	Superrosidae
<i>Nonea lutea</i>	-
<i>Osyris alba</i>	Superasteridae
<i>Parkinsonia aculeata</i>	Superrosidae
<i>Petasites paradoxus</i>	-
<i>Phyllostachys viridiglaucescens</i>	-
<i>Pseudosasa japonica</i>	Monocotyledoneae
<i>Pulsatilla montana</i>	Basal eudicots
<i>Ranunculus lingua</i>	Basal eudicots
<i>Rosa canina</i>	Superrosidae
<i>Russelia equisetiformis</i>	Superasteridae

<i>Salvia pratensis</i>	Superasteridae
<i>Sanguisorba officinalis</i>	Superrosidae
<i>Schoenoplectus mucronatus</i> (<i>Schoenoplectiella mucronata</i>)	Monocotyledoneae
<i>Sternbergia lutea</i>	Monocotyledoneae
<i>Succisa pratensis</i>	Superasteridae
<i>Typha laxmannii</i>	Monocotyledoneae
<i>Valeriana dioica</i>	Superasteridae
<i>Verbascum thapsus</i>	Superasteridae
<i>Glandularia tenera</i> (<i>Verbena tenera</i>)	-

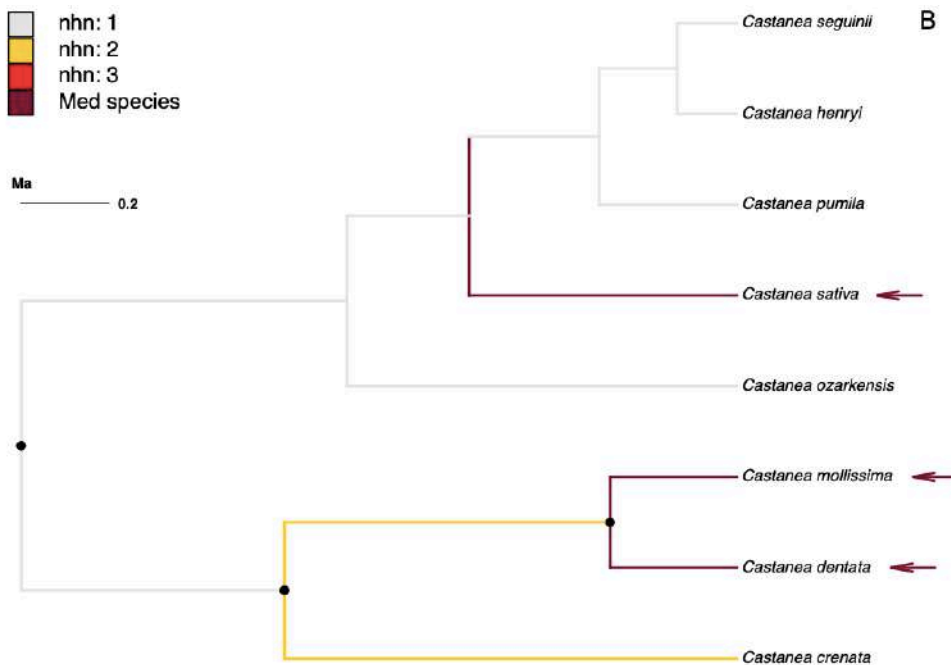
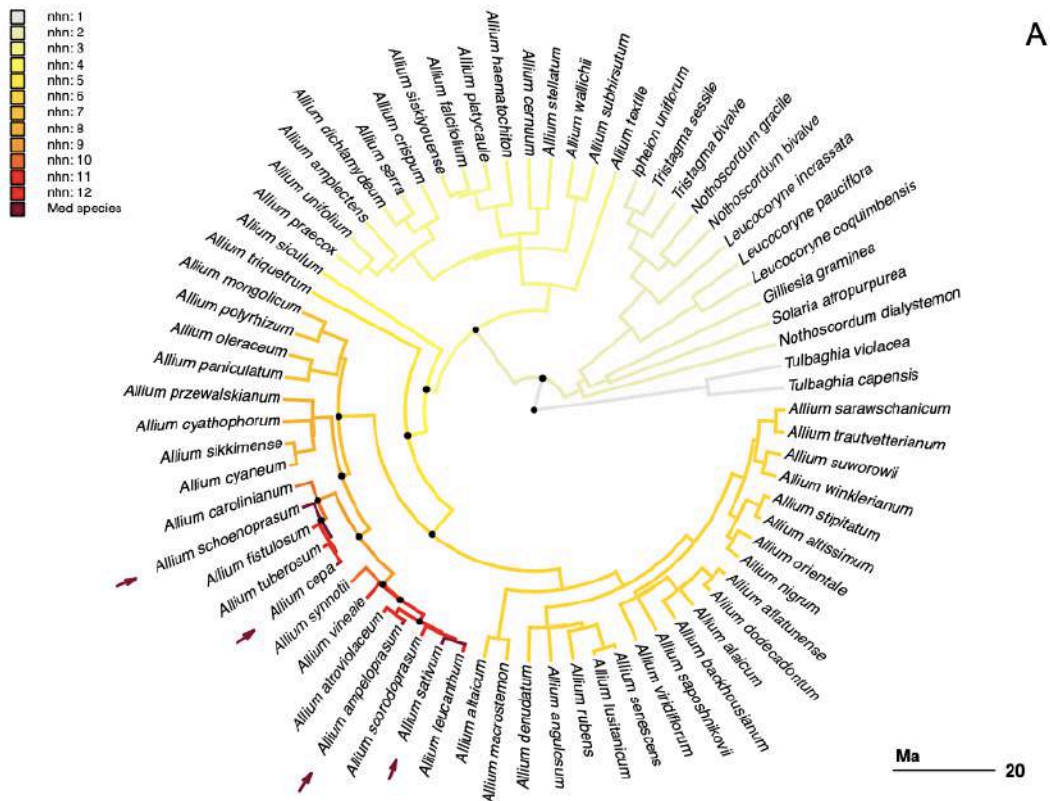
Table S2. List of the 60 plant species selected by the NBFC project. In brackets the name presents in the reference phylogeny when different (synonyms names from World Flora Online, <http://www.worldfloraonline.org/>, 10 of January 2024). Reference phylogeny = tree of the reference phylogeny where the plant is present or a dash if the plant is absent.

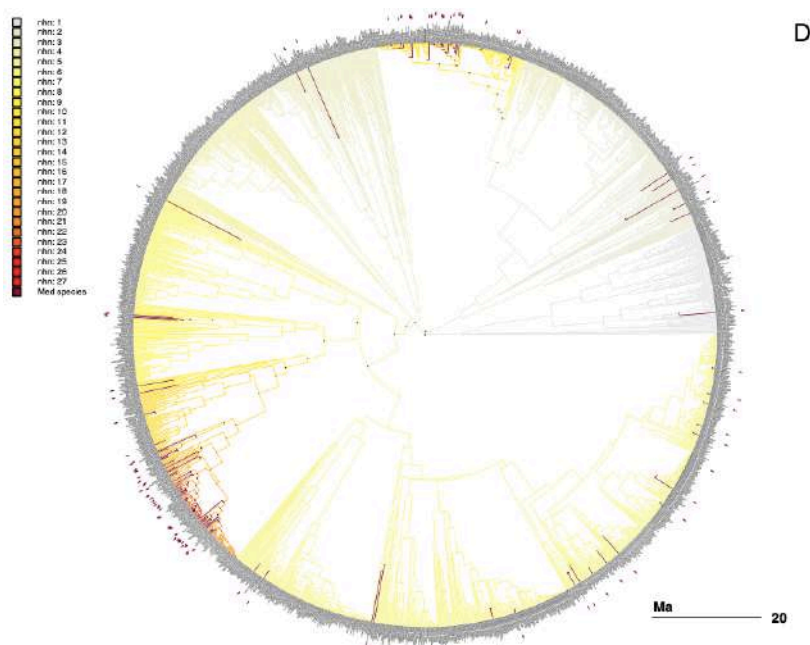
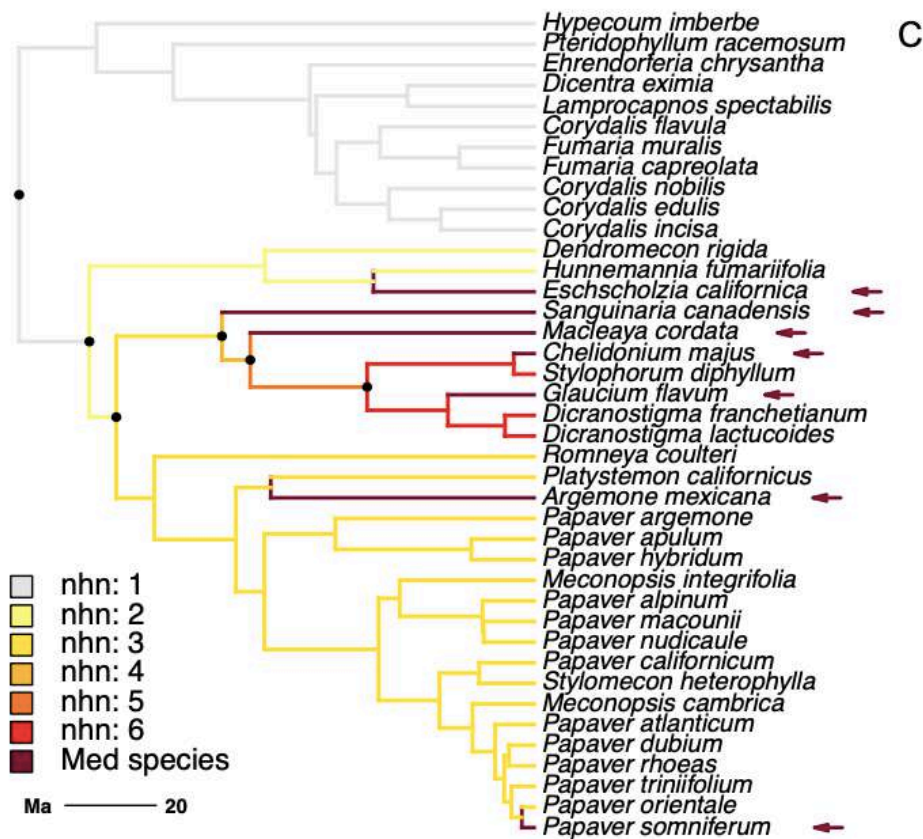
Antiaggregant				
Reference phylogeny	hot nodes	hot trees	taxa	PMP
MO	38	8	289	268
SR	216	22	2687	2488
SA	85	1	1372	1297
BE	10	3	57	45

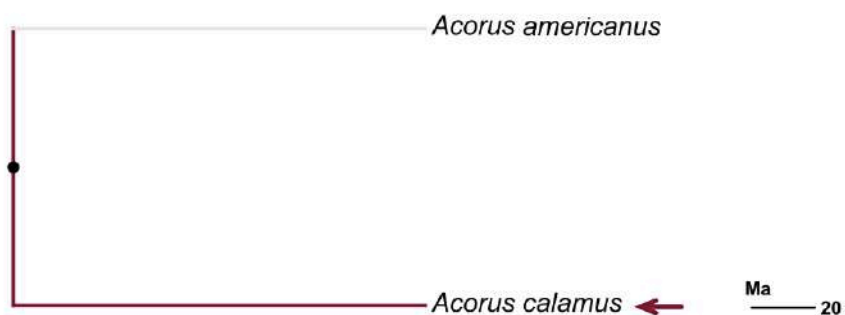
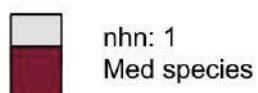
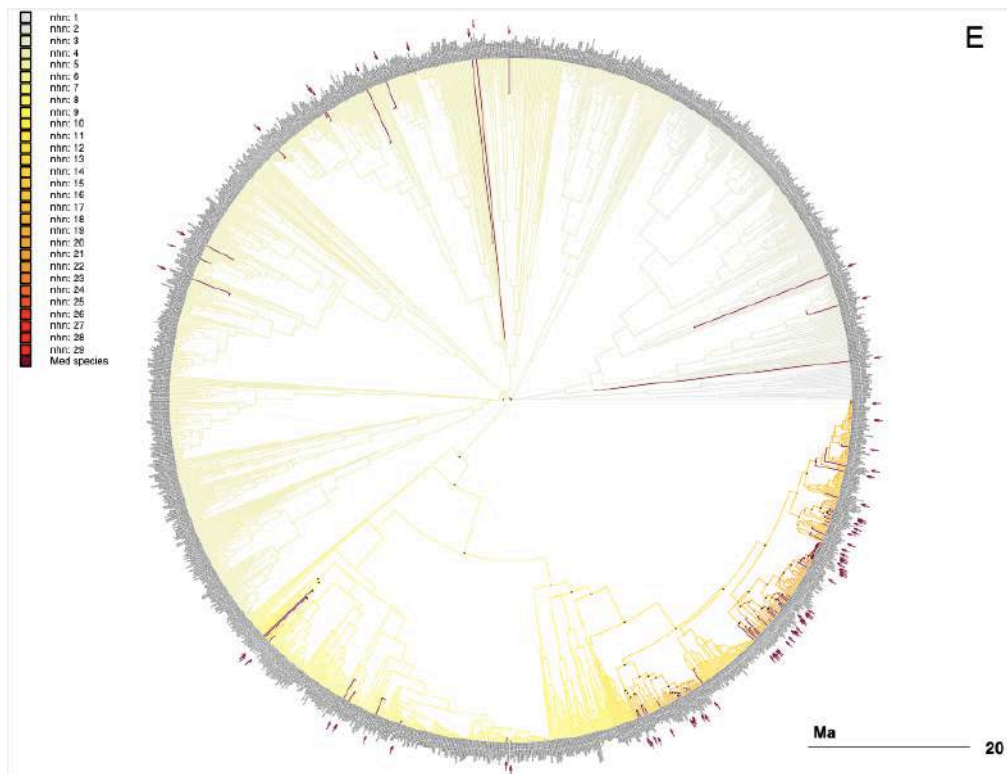
Antiparkinsonian				
Reference phylogeny	hot nodes	hot trees	taxa	PMP
MO	35	14	168	148
SR	150	19	1180	1083
SA	111	11	1267	1191
BE	2	1	35	32
Neuroprotective				
Reference phylogeny	hot nodes	hot trees	taxa	PMP
MO	47	15	227	203

SR	147	19	1332	1228
SA	126	15	1178	1084
BE	1	1	35	32

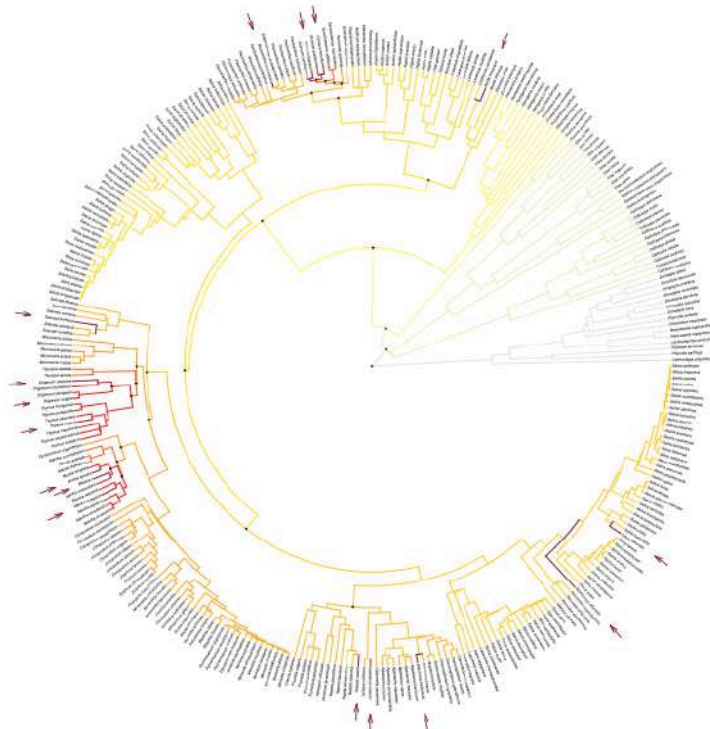
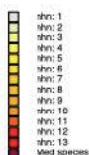
Table S3. Summary of phylogenetic results divided for biological activity and reference phylogeny. MO=Monocotyledoneae, SR=Superrosidae, SA=Superasteridae, BE=Basal eudicots. taxa = total number of plant species present in the *hot tree*. PMP = Potential Medicinal Plants.



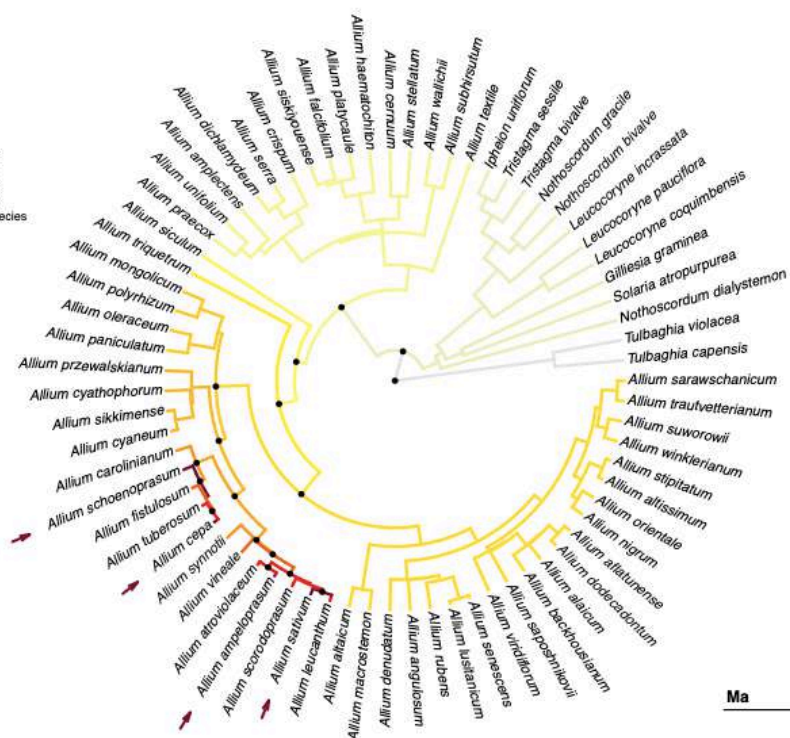
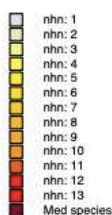








K



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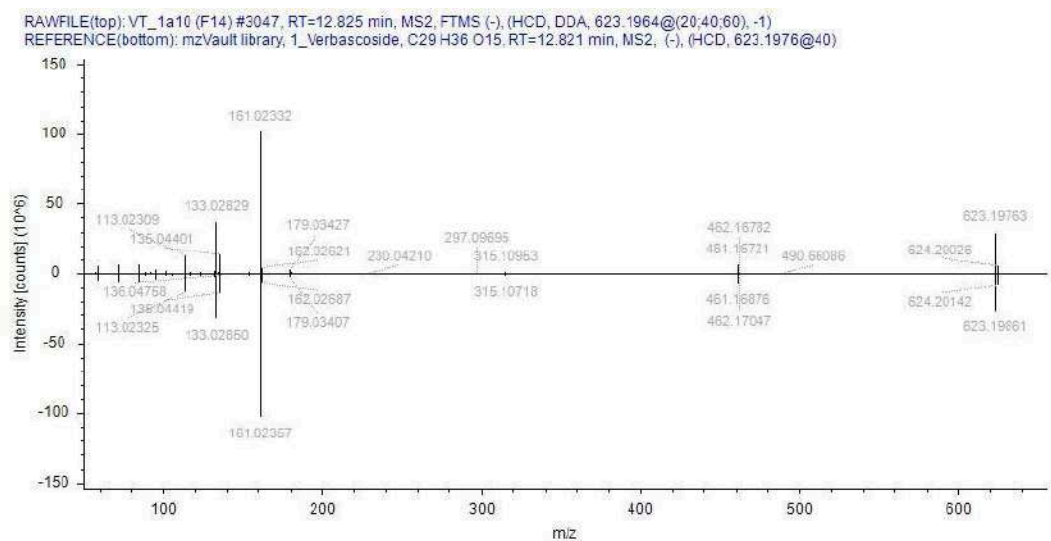


Figure S2. (-)-HRMS/MS spectra of acteoside (at 6.6 min) in *V. thapsus* extract (top) and reference standard (bottom).

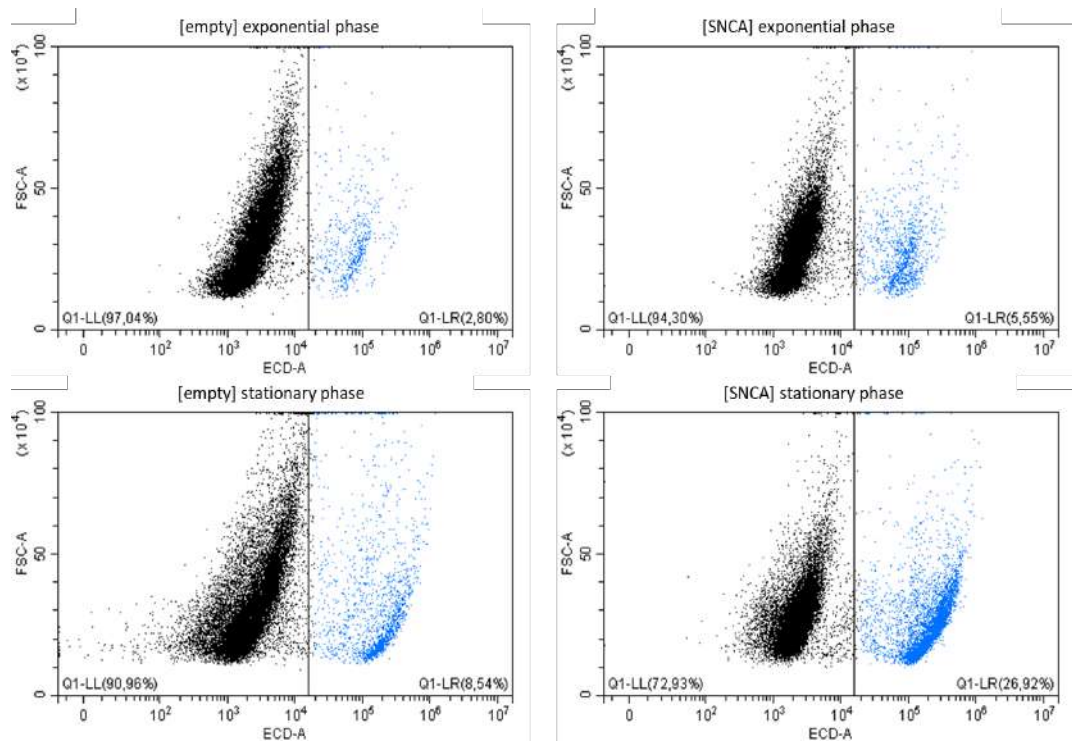


Figure S3. Increased aggresomes formation in cells overexpressing α -syn. Scatter plot of *wt[empty]* and *wt[SNCA]* cells in exponential phase (upper panels) and in early stationary phase (lower panels) stained using the the PROTEOSTAT Aggresome kit. In blue aggresome-positive cells are represented.

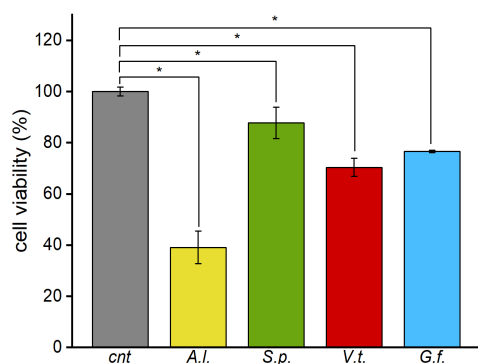
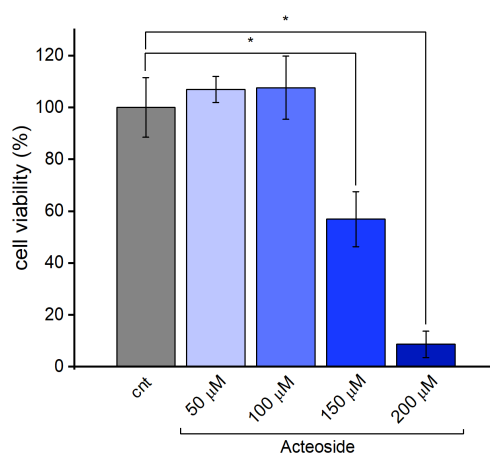
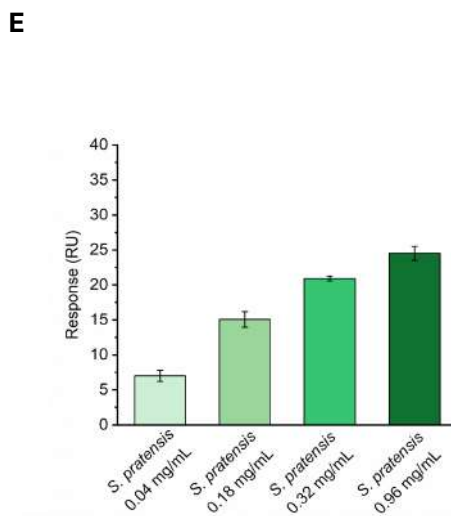
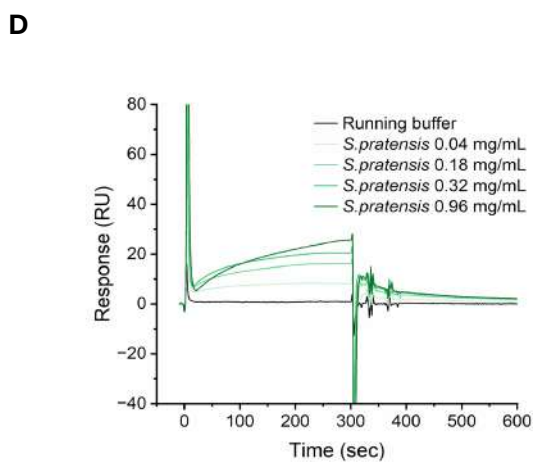
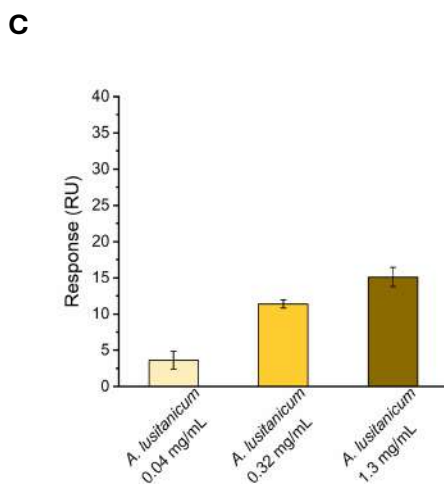
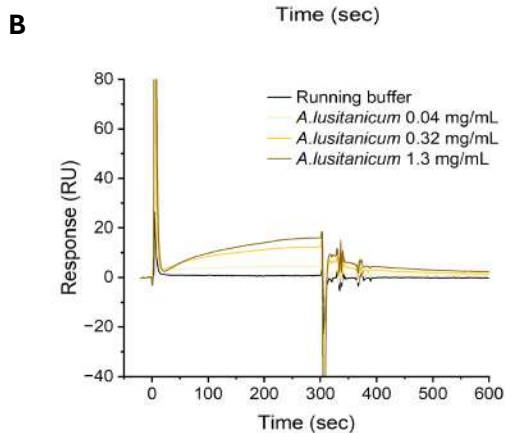
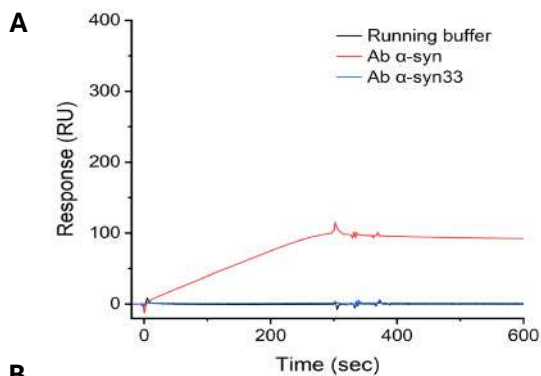
A**B**

Figure S4. Effect of the treatments on cell viability. (A) Cell viability of SH-SY5Y *pTet-SNCA-FLAG* was determined using MTT assay, after treating cells for 72 h with the indicated extracts (corresponding to 6.6 mg of fresh leaves extract/mL of medium). (B) Cell viability of SH-SY5Y was determined using MTT assay, after treatment with acteoside at the indicated concentrations for 72 h.



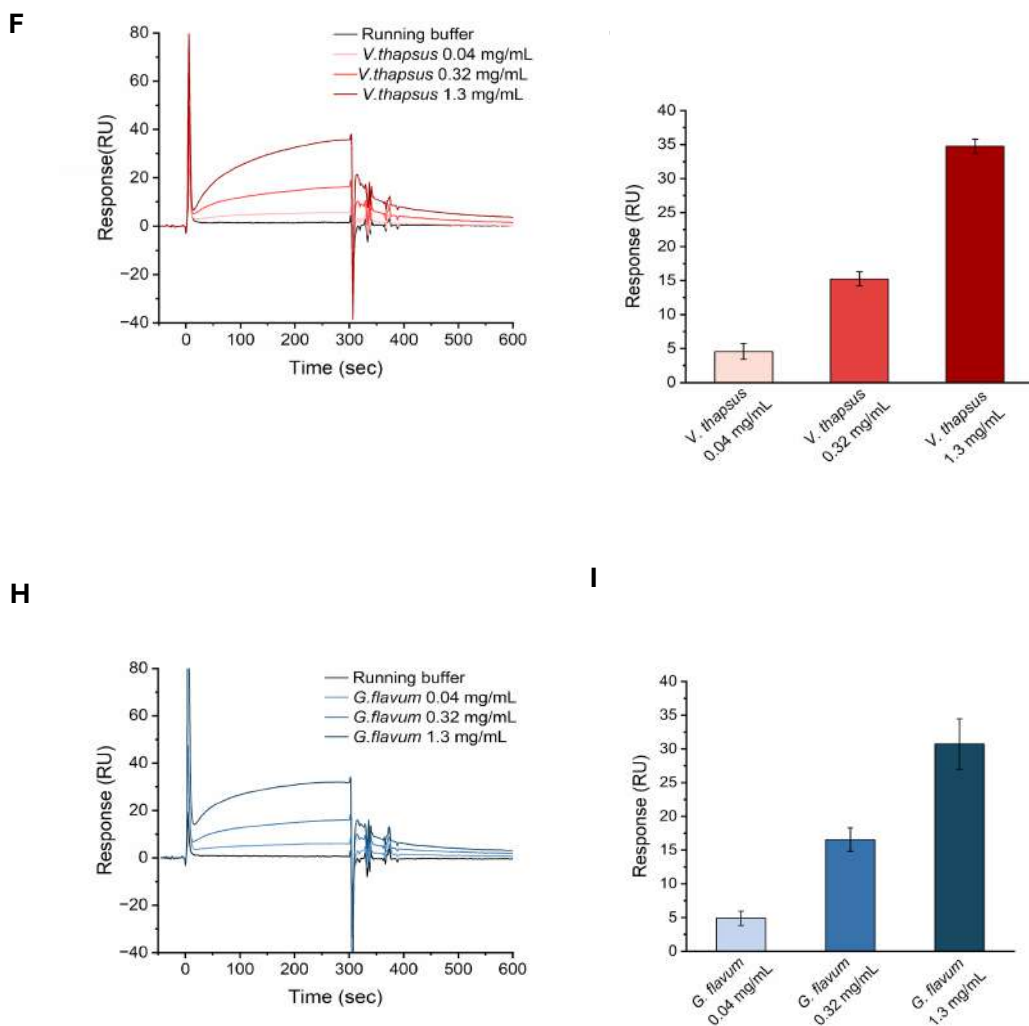
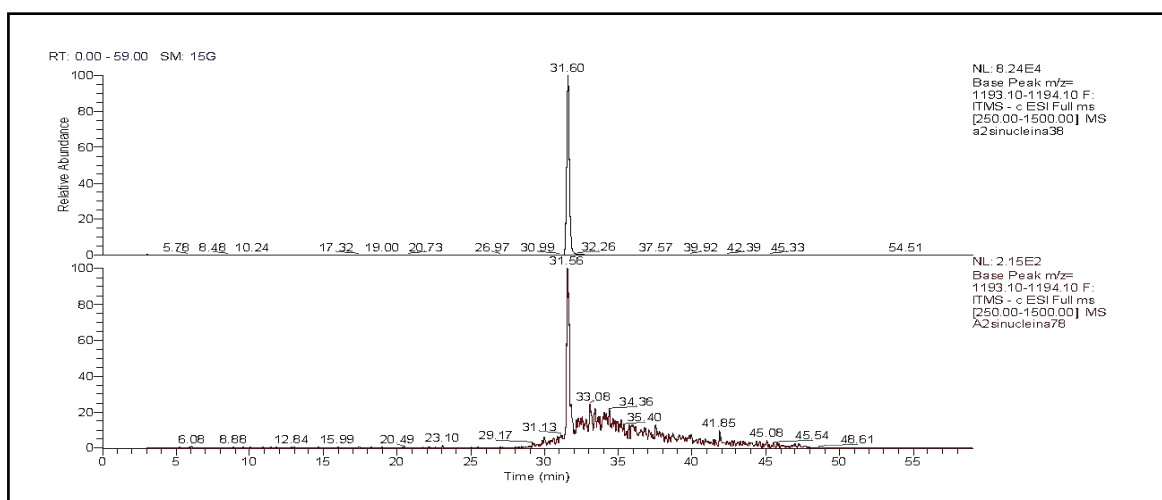


Figure S5. The extracts from *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum*

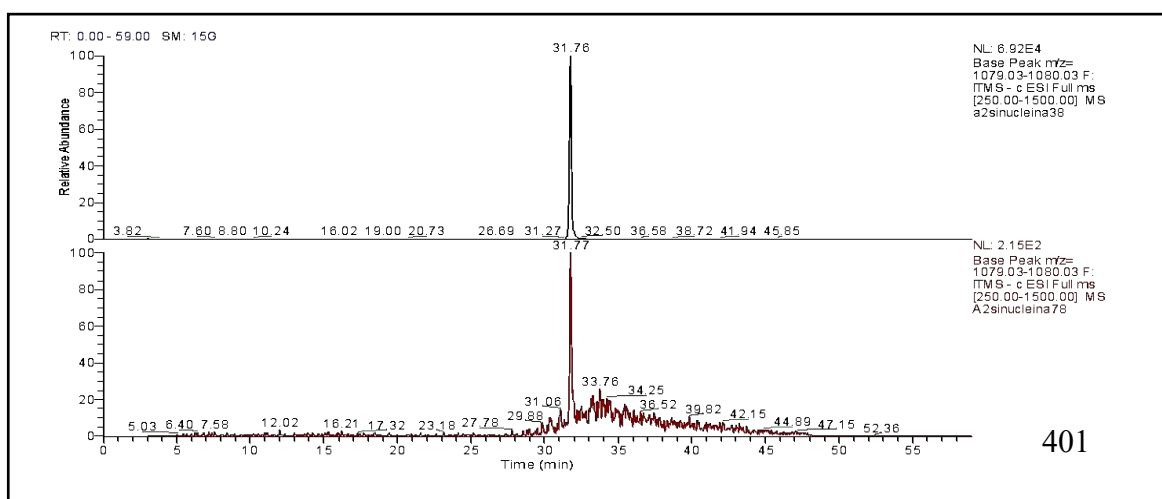
bind α -syn *in vitro*. (A) Assessment of CM5 Sensor Chip: the bioactivity of the CM5 sensor chip was determined by injecting positive (Ab anti- α -syn) and negative (Ab anti- α -Syn33, oligomer specific) controls onto immobilized α -syn protein. (B-I) SPR sensorgrams of *A. lusitanicum* (B-C), *S. pratensis* (D-E), *V. thapsus* (F-G) or *G. flavum*

(H-I) extracts at different concentrations display binding toward α -syn on CM5 sensor chip surface. Results are reported as the mean $\pm$ standard deviation of three independent experiments.

A



B



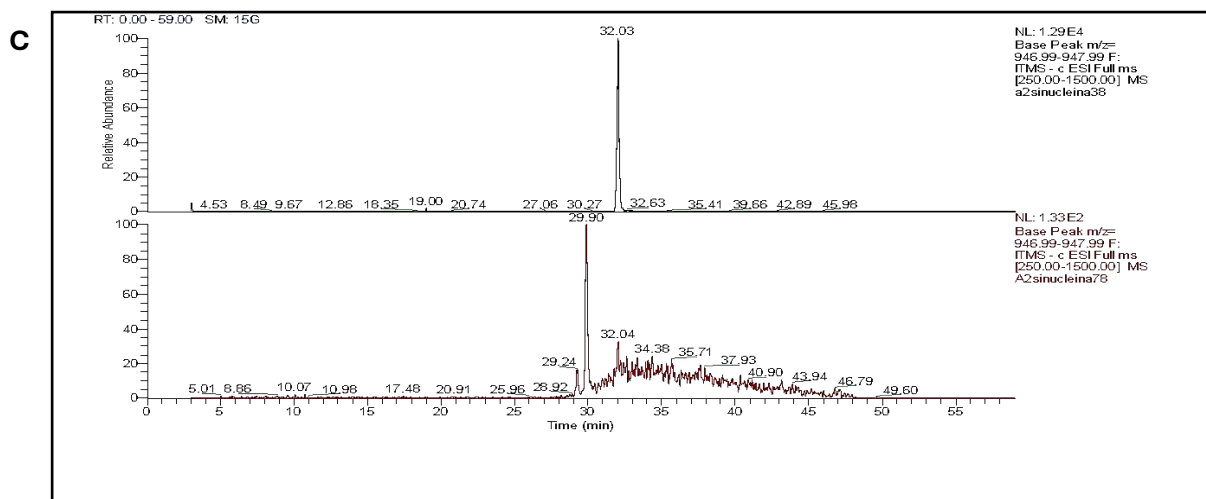


Figure S6. SEC-AS-MS analyses reveal that saponins bind to α -syn. (A) RPLC-MS chromatograms in negative ion mode of *A. lusitanicum* crude extract (upper panel) and *A. lusitanicum* crude extract fished by α -syn (lower panel). Extracted ion saponin class IV3 +2 hexoses +3 desoxyhexoses, 1193.596m/z. (B) RPLC-MS chromatograms in negative ion mode of *Allium lusitanicum* crude extract (upper panel) and *A. lusitanicum* crude extract fished by α -syn (lower panel). Extracted ion saponin class IV3 +2 hexoses +1 desoxyhexose + 1 pentose, 1079.531m/z. (C) RPLC-MS chromatograms in negative ion mode of *A. lusitanicum* crude extract (upper panel) and *A. lusitanicum* crude extract fished by α -syn (lower panel). Extracted ion saponin class IV3 +2 hexoses +1 desoxyhexoses, 947.487m/z.

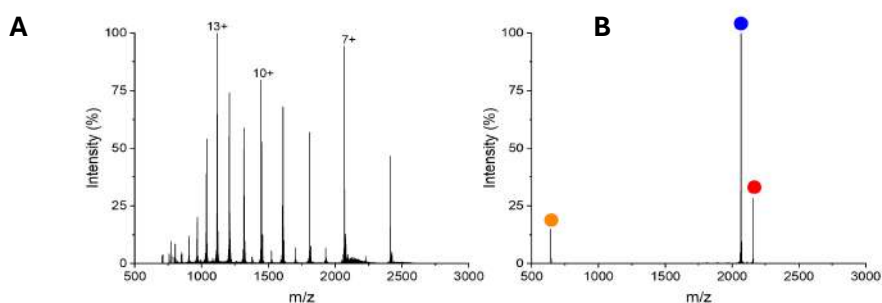


Figure S7. Native-MS analysis. (A) nano-ESI-MS spectrum of α -syn 7 μ M in 100 mM ammonium acetate pH 7. The peaks corresponding to the main distributions are labeled by the corresponding charge states. (B) nano-ESI-MS/MS spectrum of 7 μ M α -syn, 140 μ M acteoside in 100 mM ammonium acetate pH 7. Collision-induced dissociation of the 1:1 α -syn-acteoside complex (parent ion, red circle) at 15 V collision energy gives free α -syn (blue circle) and free acteoside (orange circle) as dissociation products.

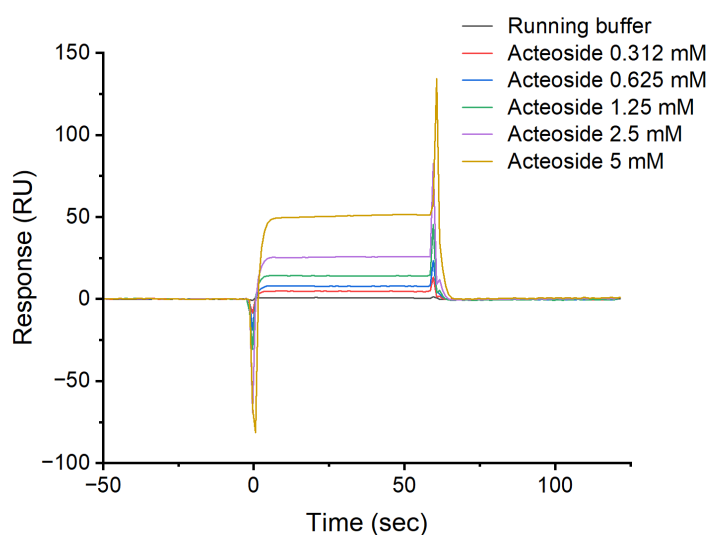


Figure S8. Interaction of acteoside with a-syn by Surface Plasmon Resonance (SPR). SPR sensorgrams of acteoside at different concentrations (5 mM, 2.5 mM, 1.25 mM, 0.625 mM, 0.312 mM) display a dose-dependent binding toward a-syn immobilized on CM5 sensor chip surface.

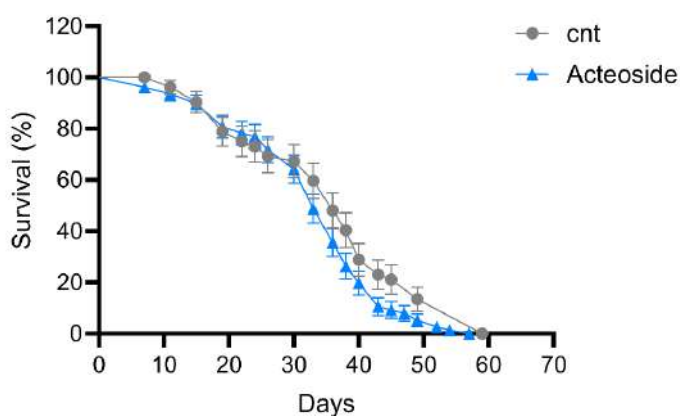


Figure S9. Acteoside does not affect *DJ-1b* mutant flies lifespan. Survival curves of *DJ-1b* mutant flies cultured in vehicle (0.1% DMSO, grey) or treated with 400 μ M acteoside (blue) from the larval stage. Survival was analyzed using the Kaplan–Meier method and compared by log-rank.

Chapter 4

Other results (paper in preparation)

Pinocembrin: a neuroprotective flavonoid against α -synuclein toxicity, oxidative stress and energetic dysfunction in models of Parkinson's disease

Abstract

Glycyrrhiza glabra leaves are an emerging source of specialized metabolites, among which the flavanone pinocembrin represents one of the most abundant constituents. In the context of valorizing this underexploited biomass and exploring its relevance as a neuroprotective agent, we investigated the biological profile of pinocembrin, through complementary experimental approaches. The study employed *in vitro* assays to examine its impact on α -synuclein aggregation, as well as analyses on a yeast Parkinson's disease model to evaluate its effects on oxidative stress and cellular aging. To extend the analysis to a multicellular model, the *Drosophila melanogaster* DJ-1 β mutant strain was used as an established model of Parkinson's disease, to assess the compound's impact on motor function and biochemical markers related to energy homeostasis. This integrated strategy aims

to clarify the molecular features and biological activities of pinocembrin, providing a framework for understanding its potential relevance within the broader context of licorice leaf valorization and the exploration of natural compounds with neuroprotective activity.

Keywords: *Glycyrrhiza glabra*; bioactive compound; liquorice; neuroprotection; lifespan; *Saccharomyces cerevisiae*; *Drosophila melanogaster*

Introduction

Neurodegenerative diseases, such as Parkinson's disease (PD), are complex and multifactorial disorders characterized by pathological hallmarks including oxidative stress, mitochondrial dysfunction, and the accumulation and aggregation of misfolded proteins, most notably α -synuclein (α -syn) [1,2]. The convergence of these processes contributes to neuronal dysfunction and progressive degeneration, emphasizing the need for therapeutic strategies capable of targeting multiple disease-related pathways simultaneously [3]. Natural compounds with pleiotropic activities, such as flavonoids, represent promising candidates for neuroprotective intervention [4,5]. Flavonoids constitute a large and diverse class of naturally occurring polyphenolic compounds widely distributed in edible plants and well

recognized for their beneficial effects on human health [6]. Among them, pinocembrin (5,7-dihydroxyflavanone) is a flavonoid belonging to the flavanone subclass, found in a variety of plants, herbs, fruits, vegetables, nuts, seeds, spices, stems, flowers, tea, red wine and fungi and particularly abundant in hive products such as honey and propolis [7,8]. Pinocembrin is also a major flavonoid constituent of *Glycyrrhiza glabra* leaves, which contain additional bioactive compounds such as glabranin and licoflavanone. Previous studies have shown that *G. glabra* leaf extracts exhibit anti-aging and antioxidant properties in a model of PD, suggesting a potential role in neuroprotection [9] (chapter 2B). These observations have prompted further interest in elucidating the specific contribution of pinocembrin to the biological activities of the extracts and its relevance in neurodegenerative contexts.

The biological activities and therapeutic potential of pinocembrin have been extensively investigated through preclinical *in vitro* and *in vivo* studies, which are essential for elucidating its mechanisms of action and translational relevance. *In vitro* studies have identified molecular and cellular targets involved in oxidative stress regulation, inflammatory signaling, and cytoprotection. Specifically, these effects were associated with modulation of neuroinflammatory responses, activation of cytoprotective pathways, particularly the Nrf2/heme oxygenase-1 (HO-1) axis, suppression of key inflammatory signaling cascades including NF- κ B,

iNOS, COX-2, and proinflammatory cytokines, as well as modulation of microglial activation through mitogen-activated protein kinase (MAPK) pathways [10].

These mechanisms underlie pinocembrin's protective effects in models of neurodegenerative diseases. In Alzheimer's disease (AD) models, pinocembrin prevented A β -induced neurotoxicity, preserved the ultrastructure of nerve fibers, inhibited RAGE-mediated inflammatory signaling, and supported cholinergic function via the ERK-CREB-BDNF pathway. Similarly, in PD models, pinocembrin was shown to mitigate dopaminergic neuronal loss, oxidative stress, apoptosis, and mitochondrial dysfunction, in part through ERK1/2 activation, HO-1 induction, and modulation of the Nrf2/antioxidant response element pathway [11]

Studies in rats have provided critical insights into systemic effects, pharmacokinetics, tissue distribution, and safety, showing that pinocembrin improves neurological outcomes and preserves the blood-brain barrier (BBB) integrity [10,12,13]. Notably, pinocembrin has been shown to efficiently cross the BBB, due to its low molecular weight and favorable lipophilicity, a critical feature for compounds aimed at treating central nervous system disorders [13,14].

Despite encouraging preclinical evidence, the translation of pinocembrin into clinical applications is limited by its low aqueous solubility and reduced oral bioavailability. In fact, preclinical and clinical studies have shown that oral

bioavailability is relatively low. Ongoing research efforts are therefore focused on improving its pharmacokinetic profile through the development of structural analogs and advanced drug delivery systems, including amino acid derivatives, lecithin complexes, and polymeric micelles, which have been shown to enhance bioavailability by more than 2.5-fold. While phase I clinical trials have indicated a favorable safety profile, demonstrating that pinocembrin is well-tolerated at intravenous doses up to 120 mg/day in 58 healthy volunteers without significant adverse effects, additional studies are required to fully define its therapeutic potential [7].

Within this context and aligned with the ON FOODS sustainability framework, the present study aims to explore the neuroprotective potential of pinocembrin in experimental models relevant to PD, with a particular focus on protein aggregation, oxidative stress and the maintenance of cellular energy homeostasis.

Materials and Methods

Yeast strains and culture conditions

The yeast strain used to evaluate the biological effects of pinocembrin was BY4742[pYX242-SNCA], which overexpresses human α -synuclein. Cells were

cultured in minimal medium containing 2% glucose as the carbon source and 0.67% yeast nitrogen base without amino acids, supplemented with 50 mg/L of the amino acids and nitrogenous bases required by the auxotrophic strain, as well as 0.1 mM ampicillin to avoid bacterial contamination. Cultures were maintained at 30°C under constant shaking at 120 rpm. For pinocembrin treatments, the compound was dissolved in 100% ethanol, vortexed, and sonicated for 20 min at 28 kHz before being added to 5 mL of culture medium at a final concentration of 145 μ M. This concentration reflects the amount of pinocembrin present in 0.2% GGL extract, which was previously tested in the same yeast model [9]. Control cultures were treated with 1.5% ethanol (solvent control) .

Chronological lifespan (CLS) assay

Yeast cells were grown in liquid medium until reaching the mid-to-late exponential growth phase and then inoculated at a density of 0.150 OD/mL into fresh medium either lacking pinocembrin (solvent control) or supplemented with the compound (145 μ M). Cell survival was assessed at selected time points by propidium iodide (PI) staining and analyzed by flow cytometry using a CytoFLEX cytofluorimeter (Beckman Coulter, Milan, Italy). Data analysis was performed with CytExpert software (version 2.6.0.105), as previously described [15].

Assessment of intracellular reactive oxygen species (ROS)

Intracellular ROS levels were determined using dihydroethidium (DHE) staining. After 24 h of treatment, cells were collected, resuspended in phosphate-buffered saline (PBS), and incubated with 5 µg/mL DHE for 10 min, following an established protocol [15]. Fluorescence was measured by flow cytometry using the CytoFLEX instrument, and data were processed with CytExpert software (version 2.6.0.105).

Thioflavin T (ThT) aggregation kinetic assay

To monitor protein aggregation over time, 100 µL of 35 µM α-syn (recombinant α-syn, from Merck-Millipore) was incubated at 37 °C in PBS, in the presence of 20 µM ThT solution in PBS, for up to 72 h under constant shaking at 425 cpm using a plate reader (BioTek Synergy H1, Agilent). Pinocembrin, obtained from Merck, was dissolved at 117 mM in 100% ethanol, and 2.5 µL of this solution was added to the α-syn samples to assess its effect on protein aggregation (final concentration of pinocembrin 1 mM); in control reactions, ethanol was used as solvent control.

Drosophila culture and drug administration

DJ-18^{Δex54} mutant flies (hereafter referred to as DJ-1β) were used as a *Drosophila* model of PD [16] and maintained on standard fly food at 25 °C. Pinocembrin was dissolved at 117 mM in 100% dimethyl sulfoxide (DMSO). Second instar (L2) *DJ-18* larvae were reared on food containing either 0.1% DMSO (control) or pinocembrin at concentrations of 100 or 200 µM. After eclosion, adult female flies were

transferred to fresh vials containing the same treatments and maintained for 5 days, 10 days and 15 days. Flies were then subjected to climbing assays or frozen in liquid nitrogen and stored at -80 °C for subsequent biochemical analyses.

Climbing assay

Motor performance was evaluated using a negative geotaxis climbing assay, which measures the innate tendency of flies to climb upward after mechanical stimulation and is commonly employed in neurodegeneration models [17]. For each condition, 80-120 5-day-old *DJ-16* female flies were tested. Groups of 10-20 flies were placed in graduated plastic tubes (1.4 cm diameter), allowed to acclimate for 1 min, gently tapped to the bottom and given 10 seconds to climb. The climbing ability was calculated as the mean height reached by individual flies within the allotted time [18].

Protein carbonylation assay

Protein carbonyl content was quantified in extracts from groups of 10 5-day-old *DJ-16* female flies using derivatization with 2,4-dinitrophenylhydrazine (DNPH) in 96-well plates, following previously described procedures [19].

ATP quantification

ATP levels were determined using the ATP determination kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, groups of 5 5-day-old *DJ-1 β* mutant and control female flies were homogenized in 200 μ L of reaction buffer. Homogenates were boiled for 4 min and centrifuged at 18500 $\times$ g for 10 min at 4 °C to remove debris. Subsequently, 5 μ L of each extract were mixed with 100 μ L of reaction solution in a white 96-well plate, and luminescence was recorded using an Infinite 200 PRO plate reader (Tecan). Results were expressed as relative luminescence units per mg of proteins and normalized to control samples.

Glycogen determination

Glycogen content was measured following a protocol adapted from Foray et al. [20]. Groups of 5 5-day-old *DJ-1 β* mutant and control female flies were homogenized in 200 μ L PBS using a TissueLyser LT (Qiagen) with a steel bead for 2 min at 50 Hz. Samples were centrifuged at 180 $\times$ g for 10 min at 4 °C to remove debris. From the supernatant, 90 μ L were collected and mixed with 10 μ L of 20% (w/v) sodium sulfate and 750 μ L ethanol. After vortexing, samples were centrifuged at 180 $\times$ g for 15 min at 4 °C, resulting in glycogen precipitation. Pellets were washed twice with 80% methanol, resuspended in 1 mL anthrone reagent (1.42 mg/mL in 70% sulfuric acid), and incubated at 90 °C for 15 min. After cooling and

centrifugation, 200 μ L of each sample were transferred to a 96-well plate, and absorbance was measured at 625 nm using an Infinite 200 PRO reader.

Lifespan analysis

For lifespan experiments, groups of 75 1-day-old *DJ-1b* mutant female flies were collected for each condition. Flies were divided into groups of 25 and housed in vials containing either control food (0.1% DMSO) or food supplemented with pinocembrin (200 μ M). Mortality was recorded every two to three days, and flies were transferred to fresh food weekly. This process continued until all individuals had died, and survival curves were generated [21]. Statistical analysis of survival data was performed using Kaplan-Meier plots and log-rank tests [22] with GraphPad Prism 4 software.

Statistical analysis

All experiments were performed at least in triplicate. Data are presented as mean $\pm$ standard deviation (SD). Statistical significance was evaluated using two-tailed Student's *t*-tests, with $p < 0.05$ considered significant. For *Drosophila* experiments, comparisons among groups were conducted using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).

Results

Anti-aging effects of pinocembrin in yeast model of PD

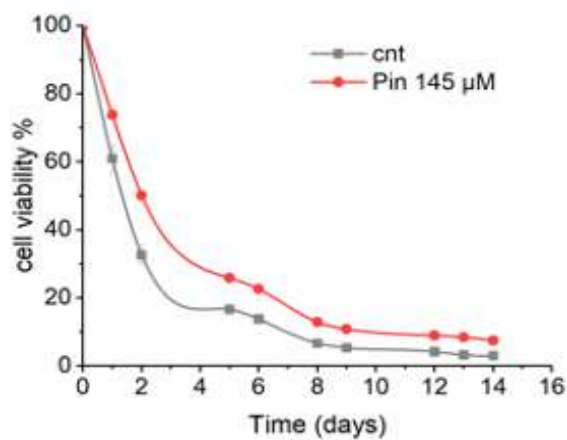
α -Syn is a presynaptic protein critically involved in the pathogenesis of synucleinopathies, including PD [23], and *S. cerevisiae* has been widely used as an experimental model to study α -syn-associated toxicity [24]. To evaluate the potential anti-aging effect of pinocembrin, the impact of pinocembrin on the survival of yeast cells overexpressing human α -syn was investigated. To assess its potential anti-aging properties, pinocembrin was administered to exponentially growing α -syn-expressing yeast cells at a concentration of 145 μ M (which corresponds to the concentration of pinocembrin present in 0.2% GGL extract, see chapter 2B) in synthetic minimal medium. The results indicate that pinocembrin significantly extended the chronological lifespan (CLS) of yeast cells (Fig. 1A), leading to an increase in both mean and maximum lifespan. Specifically, mean lifespan was extended by approximately 1.5-fold, while maximum lifespan increased up to 1.6-fold compared with control conditions (Fig. 1B).

Antioxidant activity of pinocembrin in the yeast model of PD

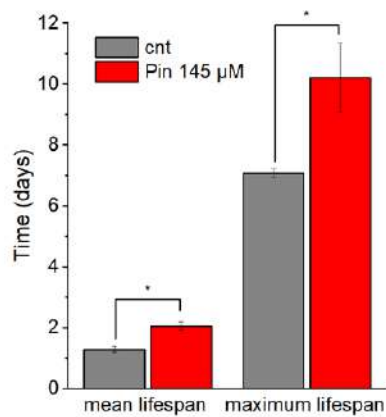
Oxidative stress, driven by the excessive intracellular accumulation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and other radical species, plays a crucial role in the development and progression of PD [25]. To investigate

the antioxidant potential of pinocembrin at the cellular level, its effects were assessed in *S. cerevisiae* cells overexpressing human α -syn. As illustrated in figure 1C, 24 h exposure to pinocembrin led to a significant decrease in intracellular ROS levels by approximately 16%.

A



B



C

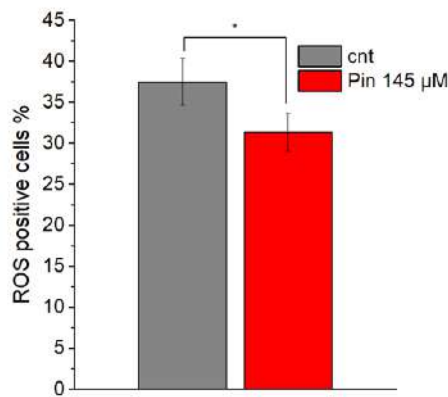


Figure 1. **Effect of pinocembrin on α -synuclein toxicity.** (A) Chronological lifespan (CLS) of yeast cells BY4742 bearing pYX242-SNCA plasmid grown in minimal medium containing 2% glucose in the absence (cnt) or presence of pinocembrin 145 μ M. (B) Mean and maximum lifespan of yeast cells in A. (C) ROS content of cells treated for 24 h as in (A). * $p < 0.05$ relative to control cells.

Pinocembrin inhibits α -syn aggregation *in vitro*

To investigate the direct effect of pinocembrin on α -syn aggregation over time, aggregation kinetic was analyzed in the presence of the compound. Remarkably, the typical sigmoidal aggregation curve of α -syn was not observed when the protein was incubated with 1 mM pinocembrin (Fig. 2). These results suggest that pinocembrin effectively prevents the formation of nucleation sites, which are essential for initiating the fibrillation phase and normally appear around 24 h after the start of the experiment, as observed in control samples (Fig. 2).

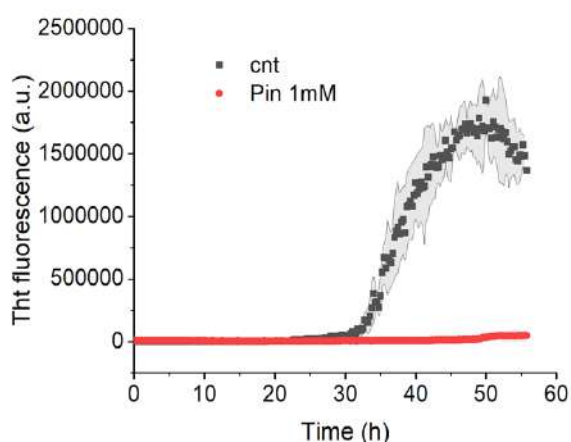


Figure 2. **Pinocembrin inhibits α -syn aggregation.** Kinetic of α -syn aggregation process, detected by ThT fluorescence, in the absence (cnt) or presence of pinocembrin (1 mM).

Pinocembrin improves locomotor performance and reduces oxidative protein damage in a *Drosophila melanogaster* model of PD

Given the well-established antioxidant and neuroprotective effects of pinocembrin in various biological models [7,10] , as well as the observed antioxidant effect on the yeast PD model (Fig. 1C), we assessed its impact in a *Drosophila* model of PD based on *DJ-1 β* deficiency. DJ-1 is a key protein involved in cellular defense against oxidative stress, contributing to ROS scavenging, transcriptional regulation, redox-dependent chaperone activity, mitochondrial maintenance, and anti-apoptotic signaling [26]. *DJ-1 β* mutant flies exhibit several PD-related phenotypes, including impaired locomotion, shortened lifespan, elevated ROS levels, and increased susceptibility to oxidative stress [18,27,28,29]. This *Drosophila* model is highly responsive to pharmacological treatments and has been extensively used to evaluate the efficacy of compounds with different mechanisms of action [18,27,30,31]. Notably, mutations in *DJ-1* cause autosomal recessive forms of PD,

and oxidatively modified DJ-1 accumulates in the brains of patients with sporadic PD [26].

To explore the therapeutic potential of pinocembrin, *DJ-1 β* mutant flies were raised on food supplemented with the compound throughout development, from the larval stage to five days after eclosion. Two concentrations of the compound (100 μ M and 200 μ M) were tested, and locomotor activity was assessed in 5-day-old adults using climbing assays. Pinocembrin treatment produced a significant improvement in motor performance, with positive effects observed at the higher concentration of 200 μ M (Fig. 3A).

DJ-1 β mutant flies also display increased ROS production, leading to oxidative damage of cellular macromolecules [28]. Protein carbonylation, a common marker of oxidative protein damage, is elevated in these mutants [32]. To determine whether pinocembrin could reduce oxidative protein modifications, carbonyl content was measured in extracts from 5-day-old flies treated with vehicle or pinocembrin. In line with the locomotor data, flies treated at both concentrations 100 μ M and 200 μ M pinocembrin exhibited a significant decrease in protein carbonyl levels (Fig. 3B), suggesting that the compound mitigates oxidative stress-induced protein damage and contributes to improved motor function. However, although oxidative protein damage was reduced at both concentrations, the

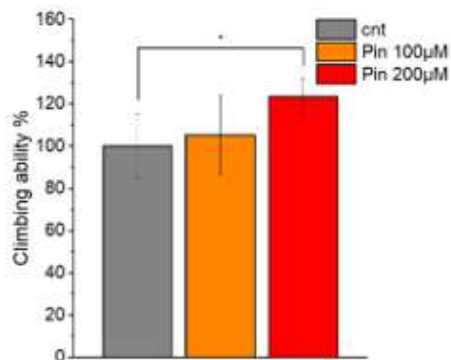
decrease achieved at 100 μ M was likely insufficient to reach the threshold required for functional recovery at the organismal level. This suggests that a more pronounced attenuation of oxidative stress, as achieved at 200 μ M, is necessary to translate molecular protection into measurable improvements in motor performance.

Despite this effect, 200 μ M pinocembrin did not significantly increase fly lifespan (Fig. 3D), indicating a potential enhancement of healthspan rather than longevity.

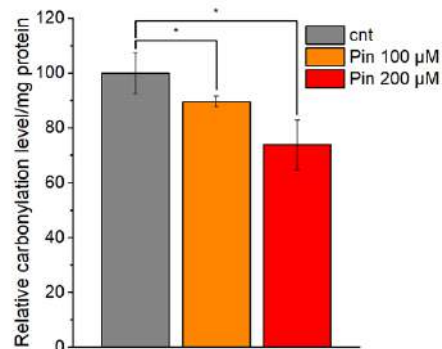
To further assess the efficacy of pinocembrin during adulthood, a stage more relevant to the clinical onset of PD, flies were treated with 200 μ M pinocembrin in larval stage and climbing assays were conducted both at 5 days (as in Fig. 1A) and at 15 days after eclosion. As shown in Fig. 3C, pinocembrin significantly enhanced locomotor performance at 5 and 15 days, demonstrating that the compound can be beneficial both at early and late stages of the disease, consistent with the progressive nature of PD pathology.

In conclusion, the functional improvements observed in *DJ-1 β* mutant flies indicate that pinocembrin can effectively mitigate deficits resulting from *DJ-1 β* dysfunction, ameliorate PD-related phenotypes, and highlight its potential as a therapeutic intervention.

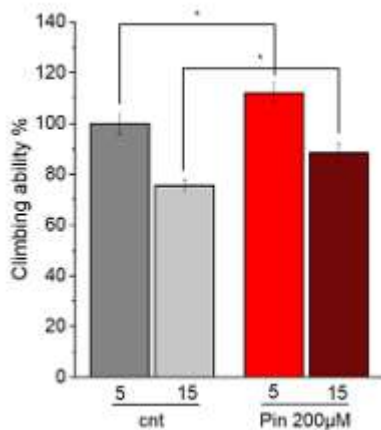
A



B



C



D

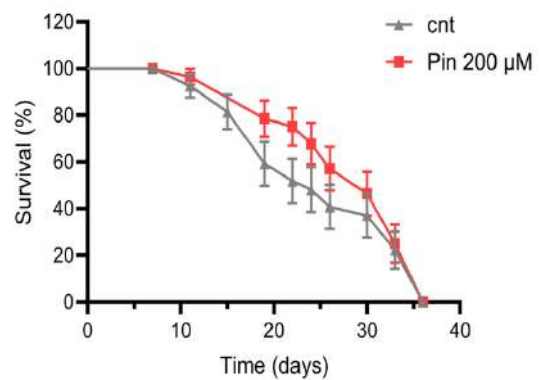


Figure 3. **Effect of pinocembrin on *DJ-1b* mutant flies.** (A) Motor performance of *DJ-1b* mutant flies treated with 100 or 200 µM pinocembrin was evaluated by a climbing assay (n=80-120 5-day-old female flies for each condition). (B) Protein carbonylation level in *DJ-1b* mutant flies treated with 100 or 200 µM pinocembrin

was analyzed using 2,4-dinitrophenylhydrazine (DNPH) derivatization, data are expressed as arbitrary units (a.u.) per mg of protein. Results were normalized to data obtained from control flies cultured in vehicle medium (0.1% DMSO). Values are expressed as mean $\pm$ SD from three independent experiments in which three biological replicates were used. Groups were statistically compared by one-way ANOVA analysis followed by Tukey's post hoc test (* $p < 0.05$). (C) Motor performance of *DJ-1 β* mutant flies with 200 μ M pinocembrin was evaluated by performing a climbing assay 5 and 15 days after eclosion (n=80-120 female flies per condition). Values are expressed as mean $\pm$ SD from three independent experiments. * $p < 0.05$. (D) Survival curves of *DJ-1 β* mutant flies cultured in vehicle (0.1% DMSO, grey) or treated with 200 μ M pinocembrin (red) from the larval stage. Survival was analyzed using the Kaplan-Meier method and compared by log-rank.

Pinocembrin increases ATP levels and glycogen content in *DJ-1 β* mutant flies

Alterations in cellular energy metabolism are increasingly recognized as central features of neurodegenerative diseases, including Parkinson's disease. Mitochondrial dysfunction, oxidative stress, and impaired glucose utilization contribute to reduced ATP production, which compromises neuronal function and survival. In this context, glycogen, traditionally considered an energy reserve in liver and muscle, has emerged as an important regulator of neuronal energy

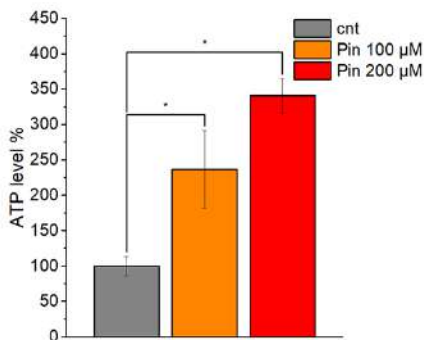
homeostasis. Glycogen stored in astrocytes can be mobilized to maintain ATP levels during metabolic stress, hypoxia, or oxidative challenges, thereby supporting synaptic function, plasticity and overall neuronal resilience [33]. Evidence from *Drosophila* and *in vitro* PD models suggests that disruptions in glycolysis and mitochondrial activity may trigger compensatory mechanisms, including increased glycolytic flux and glycogen mobilization, to sustain cellular energy demands. Accordingly, interventions that enhance ATP availability and glycogen storage, such as treatment with metabolic modulators or compounds, could provide neuroprotective benefits by restoring energy balance, reducing oxidative damage, and supporting neuronal survival. These observations underscore the potential of targeting cellular energy metabolism, particularly ATP generation and glycogen dynamics, as a strategy to counteract neurodegenerative processes [19,33].

To investigate the metabolic effects of pinocembrin, ATP and glycogen levels were measured in 5-day-old female *DJ-1 β* mutant flies treated with 100 μ M and 200 μ M of the compound. Pinocembrin treatment resulted in a dose-dependent increase in ATP levels, with both concentrations significantly enhancing cellular energy content compared to vehicle-treated controls (Fig. 4A). This finding suggests that pinocembrin improves cellular energy metabolism in mutant flies.

Glycogen content, a marker of energy reserves [34], was also assessed under the same conditions. Interestingly, although both concentrations increased ATP, a significant increase in glycogen was observed only at the higher concentration (200 μ M) (Fig. 4B). This indicates that higher doses of pinocembrin promote the accumulation of energy reserves, providing additional metabolic support to the cells.

Overall, these results show that pinocembrin exerts a positive effect on the energy metabolism of *DJ-1B* mutant flies: at both concentrations pinocembrin improves cellular energy availability, while only at the higher concentration it increases glycogen storage, suggesting a modulatory effect on energy metabolism and intracellular energy reserves.

A



B

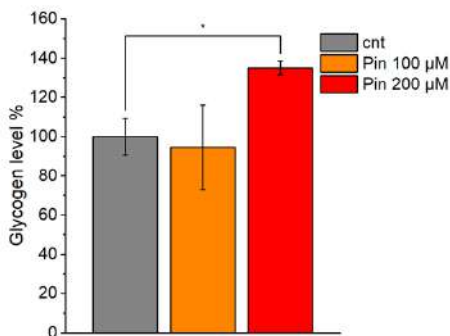


Figure 4. Effect of pinocembrin on energy metabolism and intracellular energy reserves on *DJ-1 β* mutant flies. (A) Relative ATP levels in 5-day-old *DJ-1 β* mutant flies treated with 100 or 200 μ M pinocembrin compared to controls of the same age. (B) Glycogen levels in 5-day-old *DJ-1 β* mutant flies treated with 100 or 200 μ M pinocembrin were analyzed by absorbance using the anthrone method. In all cases, results are expressed as arbitrary units (a.u.) and are normalized to data obtained in control flies. Values are expressed as mean $\pm$ SD from three independent experiments. * $p < 0.05$.

Conclusion

The progressive global increase in the prevalence of PD highlights the urgent need to complement symptomatic therapies with preventive strategies capable of intervening in the early stages of pathogenesis. Given the multifactorial nature of the disease, mainly characterized by the interplay of oxidative stress, mitochondrial dysfunction, and protein aggregation, the identification of compounds able to modulate multiple pathological pathways at an early stage represents a priority objective. Plant secondary metabolites emerge as a particularly promising resource for the development of preventive approaches, although only a limited fraction of higher plants has been systematically explored for neuroprotective properties [35].

Among these compounds, flavonoids have garnered growing interest due to their pleiotropic biological activity and generally favorable safety profile. These characteristics, combined with a suitable bioavailability and a lack of significant adverse effects, make them ideal candidates for long-term preventive strategies. Specifically, flavonoids such as pinocembrin are especially promising for their ability to target multiple pathological pathways simultaneously. In PD, where oxidative stress, mitochondrial dysfunction, and α -syn aggregation converge to drive neurodegeneration, multi-target compounds are especially valuable.

In this study, we provide comprehensive evidence that pinocembrin, a flavanone abundantly present in *G. glabra* leaves, exerts multifaceted neuroprotective effects in different experimental models of PD. In yeast overexpressing human α -syn, pinocembrin inhibited α -syn aggregation, reduced intracellular ROS levels and significantly extended chronological lifespan, highlighting both anti-aggregation, antioxidant and anti-aging properties. In *DJ-1 β* mutant *Drosophila*, pinocembrin produced dose-dependent improvements in locomotor performance, reduced protein carbonylation, increased ATP and elevated glycogen content, supporting enhanced energy metabolism and cellular resilience under PD-related stress.

Importantly, pinocembrin is not only biologically relevant but also particularly attractive from a sustainability perspective. In this study, the compound derives

from the GGL, an agro-industrial by-product that is typically discarded during licorice processing. The agro-food industry generates large amounts of plant waste rich in secondary metabolites, which can be transformed into valuable resources rather than treated as disposable residues. The valorization of GGL aligns with current efforts to promote circular economy models, reduce environmental impact, and recover high-value bioactive compounds from low-cost renewable biomass. Moreover, increasing consumer demand for health-promoting and environmentally sustainable products further supports the development of nutraceutical or pharmaceutical ingredients obtained through green and solvent-reduced extraction techniques.

Overall, our integrated preclinical approach demonstrated that pinocembrin could modulate key pathological features of PD, including α -syn aggregation, oxidative stress, and energy metabolism, validating its potential as a multi-target neuroprotective agent. These findings provide a strong rationale for further preclinical and clinical investigations aimed at optimizing its bioavailability and therapeutic application in PD.

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Discussion and future perspective

This doctoral project successfully achieved its main objective of identifying innovative neuroprotective strategies aimed at counteracting PD pathogenesis by targeting α -syn aggregation and associated cellular dysfunctions. Two complementary experimental pillars were developed: the valorization of agro-industrial waste matrices (ON FOODS Project) and the exploration of Mediterranean plant biodiversity (NBFC Project), both converging on the identification of multi-target natural compounds with neuroprotective potential.

A major strength of this work lies in the use of multiple experimental PD models, including *Saccharomyces cerevisiae*, human neuroblastoma cells, and the *Drosophila melanogaster DJ-1 β* mutant model. This multi-level approach enabled rapid screening and robust validation of bioactivities across increasing levels of biological complexity and enhanced the translational relevance of the findings.

Within the agro-industrial by-product valorization line, the cocoa bean shell extract (CBSE) emerged as a promising neuroprotective phytocomplex. Rich in amino acids, organic acids, and methylxanthines, CBSE significantly extended the chronological lifespan of α -syn-expressing yeast while reducing intracellular protein aggregation and oxidative stress. Surface Plasmon Resonance analysis demonstrated that CBSE components directly bind α -syn in a concentration-dependent manner, supporting

a direct mechanism of aggregation inhibition. Importantly, CBSE also activated cellular stress-response pathways, stimulating autophagy in yeast and activating AMPK signaling in neuroblastoma cells. These findings suggest that the extract exerts a synergistic, multi-mechanistic action rather than relying on single bioactive molecules, as isolated methylxanthines alone were insufficient to fully reproduce the observed protective effects.

Similarly, *Glycyrrhiza glabra* leaf extracts (GGL), derived from a typically discarded agricultural biomass, were shown to possess anti-aging and strong antioxidant properties. The flavanone pinocembrin was identified as a key bioactive component responsible for attenuating α -syn toxicity. Detailed mechanistic analyses demonstrated that pinocembrin significantly extended yeast lifespan, reduced intracellular ROS levels and inhibited α -syn aggregation *in vitro*. Crucially, analyses in the *Drosophila DJ-1 β* PD model revealed dose-dependent improvements in locomotor performance, reduced oxidative protein damage, and a marked increase in ATP levels and glycogen content. These findings highlight pinocembrin as a potent metabolic modulator capable of restoring cellular energy homeostasis, a process increasingly recognized as central to neurodegenerative disease progression.

The second research line, focused on plant biodiversity valorization, employed an integrated phylogenetic and high-throughput screening strategy that led to the identification of *Verbascum thapsus* as a promising neuroprotective species. Subsequent analyses identified acteoside as the principal bioactive metabolite responsible for the observed effects. Acteoside demonstrated a dual mode of action: it efficiently inhibited α -syn aggregation by targeting both nucleation and fibril elongation phases, and it significantly reduced oxidative stress through activation of the NRF2 antioxidant pathway. Functional validation in the *Drosophila* *DJ-1 β* model confirmed its *in vivo* efficacy, as acteoside improved motor behavior and reduced oxidative protein damage even when treatment was initiated during adulthood, mirroring the temporal course and advancement of Parkinson's pathology.

Collectively, these results position acteoside and pinocembrin as strong multi-target neuroprotective candidates capable of simultaneously modulating protein aggregation, oxidative stress, and energy metabolism, three interconnected pathogenic hallmarks of synucleinopathies.

These two compounds, derived from Mediterranean plants, further highlight the Mediterranean flora as an extraordinarily vast and still largely unexplored pool of bioactive molecules with significant potential for the development of novel

therapeutics against neurodegenerative disorders. In this perspective, the preservation of biodiversity emerges as a critical factor, not only for the protection of ecosystems but also for safeguarding unique natural resources that may prove essential for future pharmacological discoveries.

Despite the compelling preclinical evidence presented in this thesis, several challenges must be addressed to enable the successful clinical translation of the identified natural compounds. A primary limitation concerns their pharmacokinetic properties, particularly low aqueous solubility, limited oral bioavailability and restricted penetration of the BBB.

Acteoside, although highly bioactive *in vitro* and *in vivo* models, exhibits low systemic availability due to its hydrophilic nature, low BBB permeability and active efflux mechanisms. Pinocembrin, while more lipophilic and capable of BBB crossing, is still constrained by poor solubility and reduced bioavailability following oral administration. Consequently, future research should focus on the development of advanced drug delivery strategies, such as nanoencapsulation, lipid-based carriers, polymeric micelles or targeted nanocarriers, to improve compound stability, absorption and central nervous system delivery.

In addition, further validation in mammalian PD models, such as mouse models, will be essential to confirm the therapeutic potential observed in *Drosophila* and to fully

elucidate the molecular mechanisms involved, such as NRF2 activation by acteoside and the modulation of energy metabolism by pinocembrin.

Finally, emerging evidence implicates the microbiota-gut-brain axis as a critical contributor to PD pathogenesis, particularly in relation to α -syn misfolding and neuroinflammation. Future studies should therefore investigate whether acteoside, pinocembrin and the complex extracts (CBSE and GGL) can modulate gut microbiota composition and function, potentially offering additional neuroprotective benefits through this bidirectional communication pathway.

By addressing these translational challenges within a One Health framework, the findings of this thesis lay the groundwork for the development of sustainable, natural and multi-target preventive strategies for PD and related synucleinopathies.

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Short CV and “extra” products

Published papers

- Moukham, H., **Lambiase, A.***, Barone, G. D., Tripodi, F., & Coccetti, P. (2024). Exploiting Natural Niches with Neuroprotective Properties: A Comprehensive Review. *Nutrients*, 16(9), 1298.
<https://doi.org/10.3390/nu16091298>. * Co-first author
<https://hdl.handle.net/10281/477920>
- Docimo, T., Celano, R., **Lambiase, A.**, Di Sanzo, R., Serio, S., Santoro, V., Coccetti, P., Russo, M., Rastrelli, L., & Piccinelli, A. L. (2024). Exploring Influence of Production Area and Harvest Time on Specialized Metabolite Content of *Glycyrrhiza glabra* Leaves and Evaluation of Antioxidant and Anti-Aging Properties. *Antioxidants*, 13(1), 93.
<https://doi.org/10.3390/antiox13010093>.
<https://hdl.handle.net/10281/458519>
- Tripodi, F., **Lambiase, A.**, Moukham, H., Spandri, G., Brioschi, M., Falletta, E., D'Urzo, A., Vai, M., Abbiati, F., Pagliari, S., Salvo, A., Spano, M., Campone, L., Labra, M., & Coccetti, P. (2024). Targeting protein aggregation using a cocoa-bean shell extract to reduce α -synuclein toxicity in models of Parkinson's disease. *Current research in food science*, 9, 100888.
<https://doi.org/10.1016/j.crfs.2024.100888>.
<https://hdl.handle.net/10281/523738>
- **Lambiase, A.**, Spandri, G., Moukham, H., Toini, E., D'Urzo, A., Zecca, G., Commisso, M., Guzzo, F., Santoro, V., Piccinelli, A. L., Calleri, E., Salerno, S., Rinaldi, F., Negri, S., Santambrogio, C., Brioschi, M., Solana-Manrique, C.,

Labra, M., Grassi, F., Paricio, N., ... Coccetti, P. (2025). Acteoside exerts neuroprotective effects by preventing α -synuclein aggregation and oxidative stress in models of Parkinson's disease. *Neurotherapeutics: the journal of the American Society for Experimental NeuroTherapeutics*, e00825. Advance online publication. <https://doi.org/10.1016/j.neurot.2025.e00825>.
<https://hdl.handle.net/10281/565881>

Contributes to congresses

- Participation in the congress of National Biodiversity Future Center (NBFC) “Biodiversity PhD day 2023”, at the Botanical Garden of University of Padova, Padova, May 31 and June 1, 2023; <https://hdl.handle.net/10281/507539>
- Participation in the congress PhD Meeting 2023 of Tecsbi PhD course, with Poster and Oral presentation "Characterization of plant bioactive molecules in eukaryotic models of Parkinson’s disease”, at Certosa 1515, Avigliana (TO) and Istituto di ricerche farmacologiche Mario Negri, Milan, September 25-27, 2023; <https://hdl.handle.net/10281/507559>
- Participation in the congress NeuroMI 2023-Annual Meeting of the Milan Center for Neuroscience, with Poster " Characterization of Cocoa extract in eukaryotic models of Parkinson’s disease”, at the University of Milan-Bicocca, Milan, December 5, 2023; <https://hdl.handle.net/10281/507579>
- Participation in the congress BtbsDay 2024 with Poster " Anti-aging effects of plant extracts on eukaryotic models of Parkinson’s disease”, at the University of Milan- Bicocca, Milan, February 8, 2024; <https://hdl.handle.net/10281/507580>

- Participation in the congress of National Biodiversity Future Center (NBFC) “Forum Nazionale della Biodiversità 2024”, with Poster " Neuroprotective effects of plant extracts on eukaryotic models of Parkinson’s disease”, at the University of Palermo, May 20-21, 2024; <https://hdl.handle.net/10281/507599>
- Participation in the congress of National Biodiversity Future Center (NBFC) “Biodiversity PhD day 2024” with Poster " Neuroprotective effects of plant extracts on eukaryotic models of Parkinson’s disease” at the Botanical Garden of University of Padova, Padova, June 3-5, 2024; <https://hdl.handle.net/10281/507619>
- Participation in the 48th FEBS congress, with Poster " Anti-aging effects of plant extracts on eukaryotic models of Parkinson’s disease” at Alianz Mico, Milano, June 29 – July 3, 2024; <https://hdl.handle.net/10281/507659>
- Participation in the Spring meeting 2023 of Tecsbi PhD course, with Oral presentation “Characterization of plant bioactive molecules in eukaryotic models of Parkinson’s disease”, at the University of Milan-Bicocca, Milan, May 24, 2023; <https://hdl.handle.net/10281/507660>
- Participation in the Spring meeting 2024 of Tecsbi PhD course, with Oral presentation “Neuroprotective effects of plant extracts on eukaryotic models of Parkinson’s disease”, at the University of Milan-Bicocca, Milan, May 15, 2024; <https://hdl.handle.net/10281/507679>
- Participation in the congress PhD Meeting 2024 of Tecsbi PhD course, with Oral presentation “Neuroprotective and anti-aging effects of plant extracts on eukaryotic models of Parkinson’s disease”, at the University of Milan-Bicocca, Milan, September 16-18, 2024; <https://hdl.handle.net/10281/565809>

- Participation in the congress NeuroMi 2024-Annual Meeting of the Milan Center for Neuroscience, with Poster “Neuroprotective and anti-aging effects of cocoa-shell extract on eukaryotic models of Parkinson’s disease” at the University of Milan- Bicocca, Milan, October 24-25, 2024; <https://hdl.handle.net/10281/565810>
- Participation in the congress BtbsDay 2025 with Poster “Identification of new sustainable biomolecules with neuroprotective effects: a multifaceted screening of the Mediterranean biodiversity” at the University of Milan- Bicocca, Milan, February 18, 2025; <https://hdl.handle.net/10281/565811>
- Participation in the congress of National Biodiversity Future Center (NBFC) “Forum Nazionale della Biodiversità 2025”, with Poster “Glabranin as a natural modulator of AMPK signaling and lipid metabolism in non-alcoholic fatty liver disease”, at the University of Milan-Bicocca, Milan, May 19-21, 2025; <https://hdl.handle.net/10281/565862>
- Participation in the congress of National Biodiversity Future Center (NBFC) “Biodiversity PhD day 2025” with Oral Presentation “Neuroprotective effects of verbascoside on eukaryotic models of Parkinson’s disease” at Palazzo Bo, Padova, May 26-27, 2025. <https://hdl.handle.net/10281/565812>

Scientific collaborations

Collaborations with TeCSBi PhD students:

- **PhD student Elisa Toini** – Collaborated on the identification and selection of plant extracts with neuroprotective properties. Her expertise in phylogenetic analysis was crucial in selecting candidate species. We co-authored the manuscript *"Acteoside exerts neuroprotective effects by*

preventing α -synuclein aggregation and oxidative stress in models of Parkinson's disease," currently under peer review.

- **PhD student Chiara Giustra** – As a chemistry PhD student, she provided a variety of plant and agri-food matrix extracts, which were tested in my neurodegenerative disease models.
- **PhD student Stefania Pagliari** – As a chemistry PhD student, she supplied the cocoa bean shell extract that I used in my experimental work. This collaboration resulted in the publication of the article: *“Targeting protein aggregation using a cocoa-bean shell extract to reduce α -synuclein toxicity in models of Parkinson's disease.”*
- **PhD student Hind Moukham** – We collaborated on the identification of plant extracts and bioactive compounds with neuroprotective effects. This collaboration led to the publication of one research article: *“Targeting protein aggregation using a cocoa-bean shell extract to reduce α -synuclein toxicity in models of Parkinson's disease,”* one review article: *“Exploiting Natural Niches with Neuroprotective Properties: A Comprehensive Review,”* and one manuscript currently under peer review: *“Acteoside exerts neuroprotective effects by preventing α -synuclein aggregation and oxidative stress in models of Parkinson's disease.”*
- **PhD student Francesco Abbiati** – Contributed to the research leading to the publication of the article: *“Targeting protein aggregation using a cocoa-bean shell extract to reduce α -synuclein toxicity in models of Parkinson's disease.”*

Collaborations with PhD students from other Universities:

- **Phd student Simona Serio (University of Salerno)** – As a chemist, she provided the *Glycyrrhiza glabra* leaf extract. This agri-food waste matrix was used in my studies, and our collaboration led to the publication of: *“Targeting protein aggregation using a cocoa- bean shell extract to reduce α -synuclein toxicity in models of Parkinson's disease.”*
- **PhD student Sofia Salerno (University of Pavia)** – Collaboration on ligand-fishing experiments involving α -synuclein and selected extracts contributed to the manuscript currently under peer review: *“Acteoside exerts neuroprotective effects by preventing α - synuclein aggregation and oxidative stress in models of Parkinson's disease.”*

Membership to scientific societies

- Società Italiana di Biochimica e Biologia Molecolare (SIB), Gruppo “Proteine” e “Nutrizione e Ambiente”.
- Best4Food, centro di scienza e tecnologia sul cibo Bicocca.

Participation to third mission events

- “Hands on RRI” interactive course on “DEVELOPING RESPONSIBLE RESEARCH”, March 4, 2024, 5 hours; [ID IRIS: PEN-5145](#)
- Participation in the Evento divulgativo “Giornata di Ateneo Unimib”, at the University of Milan-Bicocca, Milan, March 20, 2024;
- Participation in the Evento divulgativo FEBS, “Meet the Biochemist in the Secret Garden”, with the Poster " Characterization of plant bioactive

molecules in eukaryotic models of Parkinson's disease", at the Botanical Garden of Milano, Milan, June 29, 2024. ID IRIS: PEN-5144

- Participation in the event "Scienza in Azione: Mercoledì con i Giovani", with the Oral presentation "Neuroprotective effects of verbascoside on eukaryotic models of Parkinson's disease, online, May 21, 2025; ID IRIS: PEN-6510
- Invited speaker at seminar "*Seminarios Biotecmed 2025*" titled "*Neuroprotective effects of plant bioactive compounds on eukaryotic models of Parkinson's disease*", at the University of Valencia, June 27, 2025; ID IRIS: PEN-6511
- Organization of the PhD meeting 2023 for the Tecsbi PhD course, at Certosa 1515, Avigliana (TO), and the Mario Negri Institute for Pharmacological Research, Milan, September 25-27, 2023; ID IRIS: PEN-6514
- Organization of the PhD meeting 2024 at the Mario Negri Institute for Pharmacological Research, Milan, May 28-30, 2024; ID IRIS: PEN-6513
- Organization of the PhD meeting 2024 for the Tecsbi PhD at the University of Milano- Bicocca, Milan, September 16-18, 2024; P ID IRIS: PEN-5390
- Organization of the PhD meeting 2025 for the Tecsbi PhD course at Villa Cagnola in Gazzada Schianno (VA), September 22-24, 2025. ID IRIS: PEN-6515

International mobility

Spain, Valencia, University of Valencia, Department of Genetics, Developmental genetics and biomedical models Lab of Prof. Nuria Paricio, from February 2, 2025, to November 4, 2025, 9 months.

Tutoring activities

- Tutoring “FONDO GIOVANI” - A.A. 2024/25 – 20 hours.
- Students tutoring of 3 bachelor’s stages.
- Students tutoring of 4 master’s theses:
 1. “Study of the anti-aging and anti-cancerogenic action of different Mediterranean plant extracts” – Master thesis of University of Barcellona.
 2. “High-throughput screening analysis of phytoextracts activities exploiting a humanized yeast model expressing Alpha synuclein” – Master thesis of University of Milano-Bicocca.
 3. “Neuroprotective effects of plant extracts on eukaryotic models of Parkinson’s disease” - Master thesis of University of Milano-Bicocca.
 4. “New bioactive natural compounds regulate lipid accumulation and metabolism in non-Alcoholic fatty liver disease” – Master thesis of University of Milano- Bicocca.

Courses

- “Time & Self-management” course, July 7, 2023, Romilde Manzoni, 8 hours.
- “Hands on RRI” interactive course on “DEVELOPING RESPONSIBLE RESEARCH”, 2023, 5 hours.
- “Hands on RRI” interactive course on “DEVELOPING RESPONSIBLE RESEARCH”, March 4, 2024, 5 hours.
- “Surfing the academic job market: how to publish in high impact international journals” course, March 29, 2023, Prof. Marco Brambilla, 8 hours.

- “Literature review 2: managing and evaluating your bibliography” course, November 4, 2024, Prof. Stefania Fraschetta, Prof. Anna Vanzetti, Prof. Margherita Zambotto, 12 hours.
- “Hands on RRI” interactive course on “DEVELOPING RESPONSIBLE RESEARCH”, October 29, 2025, 5 hours.
- “Basic of biobased processes and biorefineries” course, December 2025, Prof. Paola Branduardi, 8 hours.

Seminars

- “Systems Biology of Yeast Metabolism”, at the University of Milan-Bicocca, Milan January 19, 2023, Jens Nielsen, 60 min.
- “Challenges of the study of microbial degradation of plastics and their degradative functions for biotechnological applications”, at the University of Milan-Bicocca, Milan, February 9, 2023, Jessica Zampolli, 60 min.
- “High Mobility Group Box 1 (HMGB1) and sterile inflammation: From tissue damage & regeneration to cancer immunity”, at the University of Milan-Bicocca, Milan, February 16, 2023, Marco E. Bianchi, 60 min.
- “Architecture of a toxic alpha-synuclein oligomer”, at the University of Milan-Bicocca, Milan, February 23, 2023, Salvador Ventura, 60 min.
- “Targeting liquid-liquid phase separation in disease: a new modality in drug development?”, at the University of Milan-Bicocca, February 23, 2023, Peter Tompa, 60 min.
- “Cold-loving enzymes: from fundamentals to biotechnological exploitations”, at the University of Milan-Bicocca, Milan, March 9, 2023, Marco Mangiagalli, 60 min.

- “Biacore X 100, Biosensore a Risonanza Plasmonica di Superficie (SPR, surface plasmon resonance)”, at the University of Milan-Bicocca, Milan, October 5, 2023, Annalisa D’urzo, 60 min.
- “Fostering Autophagy and Endolysosomal homeostasis in neurodegenerative diseases: a new avenue for the development of new therapeutic tools”, at the University of Milan- Bicocca, Milan, October 12, 2023, Luca Muzio, 60 min.
- “Progranulin oncogenic axis in solid tumors: novel targets for therapy”, at the University of Milan-Bicocca, Milan, June 18, 2024, Andrea Morrione, 60 min.
- “Nanoparticle- Based Delivery of Biological Drugs: Applications and Perspectives”, at the University of Milan-Bicocca, Milan, September 25, 2024, Lucia Salvioni, 60 min.
- “Signalling architecture underlying breast cancer cell motility”, at the University of Milan-Bicocca, Milan, September 12, 2025, Chiara Francavilla, 60 min.
- “Integrative Antibody Discovery for Challenging Protein Aggregates”, at the University of Milan-Bicocca, Milan, October 10, 2025, Francesco Aprile, 60 min.
- “Effects of stressful experiences during. early life on the plasticity of inhibitory networks. Implications for psychiatric disorders”, at the University of Valencia, Valencia, February 21, 2025, Julia Alcaide Pozo, 60 min.
- “Functional cell-based assays and live cell imaging in 2D and 3D”, at the University of Valencia, Valencia, March 13, 2025, Dajana Preub, 60 min.

- “Discover innovative perspectives on biology through single-cell and special technique”, at the University of Valencia, Valencia, April 3, 2025, Ana Borges, 60 min.
- “Exploring the Role of Sox9 in Cortical Neural Stem Cell Behavior and Fate”, at the University of Valencia, Valencia, April 3, 2025, Jaime Fabra, 60 min.
- “Nuevos mecanismos de regulación de la expresión del represor transcripcional de Start de levadura Whi7”, at the University of Valencia, Valencia, April 30, 2025, Marcos Leonarte, 60 min.
- “Engineering humanized gene switches for gene therapy”, at the University of Valencia, Valencia, June 2, 2025, Mark Isalan, 60 min.
- “Economía circular en agricultura como fuente de alternativas a fertilizantes de síntesis química”, at the University of Valencia, Valencia, June 2, 2025, Begoña Renau Morata, 60 min.

Review

Exploiting Natural Niches with Neuroprotective Properties: A Comprehensive Review

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and Paola Coccetti ^{1,2} 

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Abstract: Natural products from mushrooms, plants, microalgae, and cyanobacteria have been intensively explored and studied for their preventive or therapeutic potential. Among age-related pathologies, neurodegenerative diseases (such as Alzheimer's and Parkinson's diseases) represent a worldwide health and social problem. Since several pathological mechanisms are associated with neurodegeneration, promising strategies against neurodegenerative diseases are aimed to target multiple processes. These approaches usually avoid premature cell death and the loss of function of damaged neurons. This review focuses attention on the preventive and therapeutic potential of several compounds derived from natural sources, which could be exploited for their neuroprotective effect. Curcumin, resveratrol, ergothioneine, and phycocyanin are presented as examples of successful approaches, with a special focus on possible strategies to improve their delivery to the brain.

Keywords: neuroprotection; curcumin; resveratrol; ergothioneine; phycocyanin; Alzheimer's disease (AD); Parkinson's disease (PD); delivery strategies



Citation: Moukham, H.; Lambiase, A.; Barone, G.D.; Tripodi, F.; Coccetti, P. Exploiting Natural Niches with Neuroprotective Properties: A Comprehensive Review. *Nutrients* **2024**, *16*, 1298. <https://doi.org/10.3390/nu16091298>

Academic Editor: Edgard Delvin

Received: 3 April 2024

Revised: 18 April 2024

Accepted: 24 April 2024

Published: 26 April 2024



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1. Introduction

Aging is a natural condition characterized by the progressive deterioration of physiological functions. It involves several different biological processes, and it is strongly influenced by environmental conditions, affecting lifespan and playing an essential role in the development of age-related diseases. Among these, Alzheimer's disease (AD) and Parkinson's disease (PD), involving cognitive and physical decline, are massive public health and social issues. From a molecular point of view, these diseases are characterized by neuroinflammation and the accumulation of protein aggregates. In detail, β -amyloid plaques and phospho-tau neurofibrillary tangles are found in the brain of AD patients, while α -synuclein fibrils are observed in PD patients. Despite the great efforts in developing new drugs against these pathologies, no effective or definitive treatment is currently available [1]. A complementary approach would consist of the prevention of these aging-associated diseases through the assumption of natural extracts or specific compounds derived from them, which can delay aging and prevent neurodegeneration.

The aim of this review is to present research developments in the use of molecules derived from natural sources (mushrooms, plants, microalgae, and cyanobacteria) with neuroprotective effects (Figure 1). In particular, the bioactivities associated with specific identified compounds are selectively taken into consideration in this manuscript. In fact, although several natural extracts have shown neuroprotective effects, for most of them, the specific mechanism of action is still not known.

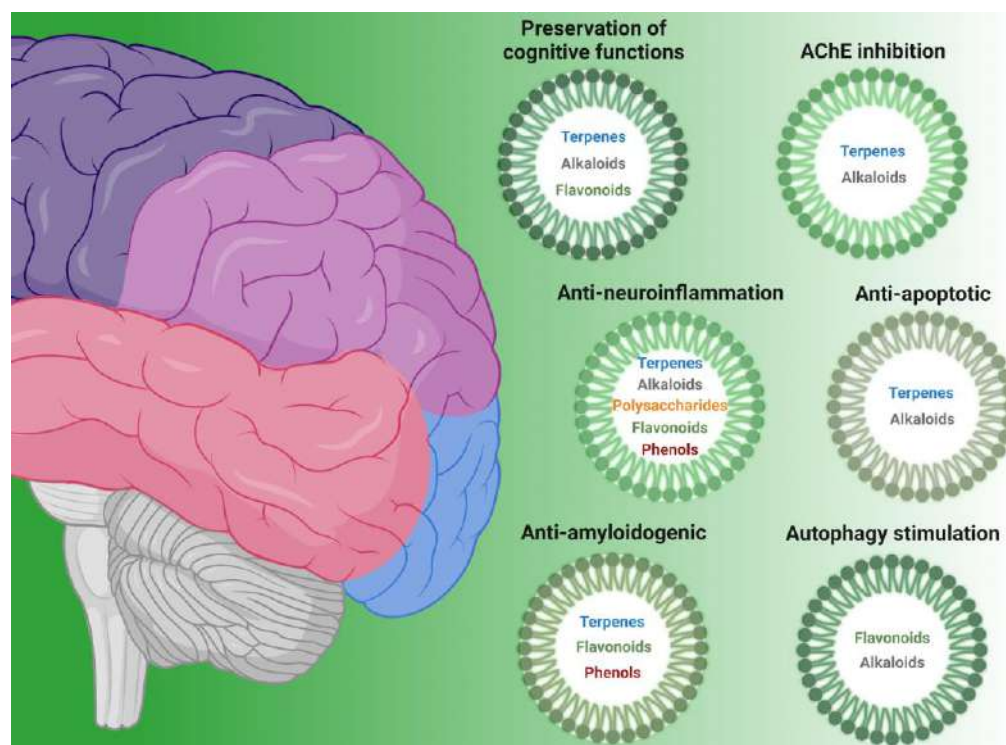


Figure 1. Classes of natural compounds (flavonoids, terpenes, phenols, alkaloids, and polysaccharides) with neuroprotective properties, grouped according to their bioactivity (preservation of cognitive functions, AChE inhibition, anti-neuroinflammation, anti-apoptotic, anti-amyloidogenic, and autophagy stimulation).

Here, we describe compounds derived mainly from the secondary metabolic pathways of shikimate (such as phenols and flavonoids), terpenes, alkaloids, as well as polysaccharides. Furthermore, we discuss them as a function of their mechanisms of action through a narrative literature methodology.

In addition, four widely studied molecules (curcumin, resveratrol, ergothioneine, and phycocyanin) are presented as model compounds for the development of novel neuroprotective molecules, with a focus on possible delivery strategies to improve bioavailability.

2. Active Natural Compounds and Targeted Bioactivities

A plethora of natural extracts derived from different matrices, such as plants, microalgae, cyanobacteria, or mushrooms, are rich in bioactivities. Searching scientific publications with “natural compounds AND neuroprotection” as keywords in bibliographic databases, 3864 results are shown via PubMed (<https://pubmed.ncbi.nlm.nih.gov/?term=natural+compounds+AND+neuroprotection>, accessed on 23 February 2024) and 11,060 through ScienceDirect (natural compounds AND neuroprotection—Search | ScienceDirect.com, accessed on 23 February 2024). In total, 2841 review articles and 3943 research articles (out of the 8558 manuscripts suggested by the ScienceDirect database) were published over the last 10 years (natural compounds AND neuroprotection—Search | ScienceDirect.com, accessed on 23 February 2024). This indicates a deep interest and prolific research activity in this field in recent years.

Neurodegenerative diseases, such as AD and PD, share some common features at the molecular level (e.g., protein aggregation, oxidative stress, neuroinflammation, and neuronal death) but also present disease-specific characteristics [2]. In the following section, we aim to examine the main processes involved in these pathologies and explore many emerging natural molecules presenting interesting bioactivities. Table 1 reports the classes of compounds and specific metabolites associated with their main bioactivities, which have

been selected for this manuscript. Antioxidant properties were not extensively discussed since they have been widely explored in several other recent reviews [3–5]; therefore, they are only briefly mentioned here.

Table 1. Bioactive compounds from natural resources comprehensively classified by the class of bioactivity.

Class of Compound	Compound Name	Natural Source	Ref.
Anti-amyloidogenic			
terpenes	Polyozellin, thelephoric acid, polyozellic acid	<i>Polyozellus multiplex</i>	[6]
	Erinacine A	<i>Hericium erinaceus</i>	[7,8]
	Tanshinone IIA	<i>Salvia miltiorrhiza</i>	[9,10]
phenols	Oleuropein aglycone	Extra virgin olive oil	[11]
	Hydroxytyrosol	Extra virgin olive oil	[11]
	Salvianolic acid B	<i>Salvia miltiorrhiza</i>	[12,13]
flavonoids	Epigallocatechin gallate	<i>Camellia sinensis</i>	[14–16]
AChe inhibitors			
terpenes	Triterpenes	<i>Ganoderma lucidum</i>	[17]
	Polyozellin, thelephoric acid, Polyozellic acid	<i>Polyozellus multiplex</i>	[6]
alkaloids	Nostocarboline	<i>Nostoc</i> 78-12A	[18]
	Anatoxin-a(s)	<i>Anabaena flos-aquae</i> strain NRC 525-17	[19]
	Galanthamine	Amoryllidaceae	[20]
Anti-apoptotic			
terpenes	Triterpenes	<i>Ganoderma lucidum</i>	[21,22]
	Erinacine A	<i>Hericium erinaceus</i>	[8]
	Astragaloside IV	<i>Astragalus membranaceus</i>	[23,24]
alkaloids	Caffeine	Coffee and cocoa beans, tea leaves, guarana berries	[25,26]
flavonoids	Fisetin	Strawberry, apple, persimmon, grape, onion, cucumber	[27–31]
	GardeninA	<i>Gardenia resinifera</i> , <i>Tamarix dioica</i> , <i>Murraya paniculata</i>	[32]
Autophagy stimulation			
alkaloids	Caffeine	Coffee and cocoa beans, tea leaves, guarana berries	[33]
flavonoids	Isoquercitrin	<i>Apocynum venetum</i>	[34,35]
	Hesperidin	Citrus fruits	[36]
	Epigallocatechin gallate	<i>Camellia sinensis</i>	[37]
	Fisetin	Strawberry, apple, persimmon, grape, onion, cucumber	[38]
	Morin	Mulberry	[39]
Anti-neuroinflammation			
polysaccharides	β -glucans	<i>Grifola frondosa</i>	[40]
terpenes	Erinacine A	<i>Hericium erinaceus</i>	[41]
	Astragaloside IV	<i>Astragalus membranaceus</i>	[42,43]
flavonoids	Fisetin	Strawberry, apple, persimmon, grape, onion, cucumber	[44]
	Epigallocatechin gallate	<i>Camellia sinensis</i>	[45–47]
	GardeninA	<i>Gardenia resinifera</i> , <i>Tamarix dioica</i> , <i>Murraya paniculata</i>	[32]
alkaloids	Caffeine	Coffee and cocoa beans, tea leaves, guarana berries	[33,48–52]

Table 1. Cont.

Class of Compound	Compound Name	Natural Source	Ref.
Preservation of cognitive functions			
polysaccharides	β -glucans	<i>Grifola frondosa</i>	[40]
terpenes	Erinacine A	<i>Hericium erinaceus</i>	[7,53,54]
	Astragaloside IV	<i>Astragalus membranaceus</i>	[42,55]
alkaloids	Caffeine	Coffee and cocoa beans, tea leaves, guarana berries	[56–61]
flavonoids	Epigallocatechin gallate	<i>Camellia sinensis</i>	[62–64]
	GardeninA	<i>Gardenia resinifera</i> , <i>Tamarix dioica</i> , <i>Murraya paniculata</i>	[32]
	Morin	Mulberry	[65,66]
	Hesperidin	Oranges	[67–72]
	Isoquercitrin	<i>Apocynum venetum</i>	[73]
phenols	6-Shogaol	Ginger rhizomes (<i>Zingiber officinale</i>)	[74,75]
	Hydroxytyrosol	Extra virgin olive oil	[76,77]
	Oleuropein aglycone	Extra virgin olive oil	[11]
	Salvianolic acid B	<i>Salvia miltiorrhiza</i>	[78,79]
terpenes	Tanshinone IIA	<i>Salvia miltiorrhiza</i>	[80]

2.1. Anti-Amyloidogenic Compounds

Pathological protein aggregation is one of the hallmarks of neurodegenerative diseases and is associated with clinical outcomes since their presence often correlates with the progression of the symptoms [2]. However, mature aggregates or fibrils are not the major toxic species, while oligomeric assemblies are recognized as mainly responsible for cellular toxicity.

In AD patients, two distinctive aggregates are found that induce oxidative stress and neuronal loss. The first is represented by plaques of amyloid- β peptide (A β), a short proteolytic fragment derived from the amyloid protein precursor (APP) through the action of β - and γ -secretases. These insoluble deposits are found extracellularly near the synapses and lead to synaptic dysfunction and neuronal death. The second is represented by neurofibrillary tangles of the phosphorylated tau protein (p-tau), a microtubule-associated protein that, when hyperphosphorylated, accumulates intracellularly and leads to reduced neuronal functions and neurotoxicity. PD is instead characterized by the accumulation of aggregates of the presynaptic protein α -synuclein, which induces neurotoxicity and, finally, neuronal loss.

Therefore, compounds acting on protein aggregation have been widely studied in recent years (Table 1).

Different terpenoid molecules have been shown to affect protein aggregation, such as those from the black mushroom *Polyozellus multiplex* used in Asia (especially Korea, China, and Japan). Specifically, different p-terphenyls were purified from this mushroom, named polyozellin, thelephoric acid, and polyozellic acid, which strongly reduce extracellular A β 1-42 release from the human Swedish mutant APP-transfected mouse neuroblastoma cell line [6]. Another interesting terpene derives from *Hericium erinaceus*, which is one of the most used medicinal mushrooms, also known as lion's mane or Yamabushitake. The interest in this mushroom is due to the presence of a large amount of different bioactive metabolites, both in the fruit body and in the mycelia [81]. Among them, Erinacine A, a diterpenoid isolated from the mycelium, can reduce A β plaque formation in a mouse model of AD (APP/PS1 transgenic mouse), both by increasing A β degradation and inhibiting its production [7]. In addition, Erinacine A reduces ROS levels in mouse neuronal cells treated with a neurotoxin that inhibits complex I of the electron transport chain (ETC) and consequently also cell death [8].

Another intriguing bioactive terpenoid is Tanshinone IIA (Tan IIA), a diterpene phenanthrenequinone extracted from the roots and the rhizomes of the plant *Salvia miltiorrhiza*. Tan IIA presents a variety of bioactivities, including anti-apoptotic, anti-inflammatory, blood–brain barrier (BBB) protection, and antioxidant effects [82]. Tan IIA also inhibits amyloid fibril formation and disaggregates the A β 1–42 amyloid peptide via specific binding to a hydrophobic β -sheet groove [10], as well as its reduced A β -induced toxicity in neuronal cells in vitro [9].

Also, phenolic compounds, like Oleuropein aglycone (OleA) and Hydroxytyrosol (HT), found in extra virgin olive oil, are known for their anti-amyloidogenic properties. These compounds exhibit strong antioxidant effects and are suggested to modulate the aggregation of the A β 1–42 peptide, decreasing the formation of toxic aggregates [83–85]. OleA primarily interacts with the N-terminus of A β 1–42, hindering the development of toxic oligomers and their transition into mature fibrils. In contrast, HT accelerates fibril formation, possibly due to increased hydrophilicity. Therefore, treatment with OleA and HT reduces the cytotoxicity of A β 1–42, steering the aggregation process towards harmless outcomes even at low concentrations. Notably, evidence suggests that these polyphenols can penetrate the blood–brain barrier, accumulating in the brain parenchyma with regular intake [11] and supporting their development as an anti-aggregant in vivo.

Likewise, Salvianolic acid B (SalB), a dominant water-soluble component from *Salvia miltiorrhiza*, is known for its ability to ameliorate A β -induced neuronal degeneration and neuroinflammation in AD [12]. It has been reported that Salvianolic acid B not only inhibits A β fibril formation but also disaggregates preformed fibrils by converting them to an amorphous structure, thereby providing protection against A β -induced cytotoxicity. In a study in vitro, SalB was shown to significantly decrease the levels of A β 1–42 in SH-SY5Y cells overexpressing the human APP (amyloid precursor protein) Swedish mutant [13].

Among the metabolites derived from the shikimic acid pathway, flavonoids represent a class of very interesting ones. Among these, Epigallocatechin gallate (EGCG), present in green tea, has received considerable attention in recent years due to its potential health advantages in preventing numerous chronic diseases, including neurodegenerative diseases. In transgenic mice overexpressing the mutant amyloid precursor protein (Tg APPsw), the administration of EGCG via intraperitoneal injection (20 mg/kg) led to a significant decrease in the brain levels of the A β peptide and a reduction in A β plaques. Additionally, EGCG promoted cleavage in the α -C-terminal fragment of APP, leading to elevated levels of soluble APP- α , indicative of enhanced α -secretase cleavage activity, which correlates with lower A β levels [14]. Strikingly, EGCG showed comparable effectiveness in diminishing A β deposition when administered orally via drinking water (50 mg/kg) [86]. In vitro, it was demonstrated that EGCG impedes the fibrillogenesis process of both α -synuclein and amyloid- β by directly interacting with the intrinsically disordered polypeptides, preventing their conversion into toxic species and promoting the formation of amorphous, non-toxic aggregates [15,16].

2.2. Inhibitors of AChE

Acetylcholine (ACh) is a neurotransmitter that activates cholinergic neurons in the central nervous system and is involved in the processes of memory and learning. Acetylcholinesterases (AChEs) are the enzymes responsible for the degradation of ACh and are potential targets for the treatment of AD since the dysfunction of cholinergic neurons is considered one of the major causes of cognitive impairment in AD patients. A second enzyme, butyrylcholinesterase (BChE), with a broader substrate specificity, seems to also be involved in ACh hydrolysis in the cholinergic synapse, and its levels are increased in the later stages of AD [87]. Therefore, a reduction in the level or the activity of AChE and BChE could lead to an increase in the level of ACh in the synaptic cleft, thus improving cognitive functions. Donepezil hydrochloride and rivastigmine are prescribed cholinesterase inhibitors that reversibly inhibit synaptic acetylcholinesterases, enhancing cholinergic transmission in AD patients [88,89]. However, these drugs present side peripheral cholinergic effects; therefore,

the identification of new natural-based inhibitors of AChE activity is of valuable interest in the field.

Several mushroom extracts have been shown to inhibit AChE activity, although in most cases, the effect was not attributed to specific compounds. *Ganoderma lucidum* is a Basidiomycetes fungus, highly used in traditional medicine in Asia since ancient times for its numerous health-promoting benefits [21]. In *G. lucidum*, several triterpenes were tested in vitro and showed AChE inhibition, with IC₅₀ in the micromolar range [17]. Similarly, the above-mentioned p-terphenyls from the black mushroom *Polyozellus multiplex* present β -secretase non-competitive inhibitor activity in vitro [6].

Also, some alkaloids have been shown to inhibit acetylcholinesterases. Nostocarboline was isolated from the freshwater cyanobacterium *Nostoc 78-12A* and is a potent butyrylcholinesterase (BChE) inhibitor (IC₅₀ of 13.2 μ M) [18]. In cyanobacteria, the function of AChE inhibitors is required for the defense against the invertebrate larval settlement of competitor organisms [90,91]. Of interest is the anatoxin-a(s) compound, isolated from the cyanobacterium *Anabaena flos-aquae* strain NRC 525-17, which showed severe cholinergic overstimulation in rats along with a robust inhibition on both AChE and BChE enzymes in vitro [19].

Other alkaloids inhibiting AChE activity come from the Amaryllidaceae plant family. In fact, over 100 cultivars from the Amaryllidoideae subfamily have been studied for their AChE inhibitory activity [92]. Compounds derived from diverse skeleton types have demonstrated potent AChE inhibitory properties, thereby presenting avenues for structure optimization and the semi-synthesis of potent AChE inhibitors [93]. Among these, Galanthamine, which was approved in 2001 by the FDA for AD treatment, showed a strong AChE inhibitory activity (IC₅₀ of 0.3–0.5 μ M) [20], exerted by inhibiting substrate accommodation and hydrolysis in the active site of the enzyme [94].

2.3. Natural Compound with Anti-Apoptotic Effect

The most prominent effect of neurodegeneration is the resulting neuronal loss due to the ultimate activation of the apoptotic pathway. Apoptosis is crucial during the development of the central nervous system, but its improper activation can be detrimental in the adult brain and is involved not only in neurodegeneration but also in several other diseases [95].

Several compounds belonging to different chemical classes have been shown to prevent neuronal apoptosis in AD and PD models (Table 1).

G. lucidum triterpenoids, which also present antioxidant activity, have been shown to decrease apoptosis both in vitro and in vivo in the hippocampus of an APP/PS1 mouse model of AD, with an effect on caspase-3 activation and on the ROCK signaling pathway [22]. This results in improved cognitive functions in the same transgenic AD mouse model, thus supporting the development of these triterpenoids as anti-apoptotic agents in neurons.

Another mushroom-derived terpenoid, the aforementioned Erinacine A from *H. erinaceus*, was shown to inhibit apoptosis induced by MPP (1-methyl-4-phenylpyridinium) treatment in a mouse neuronal cell line in vitro. This effect is associated with the increased phosphorylation of some proteins involved in survival, such as PAK1 and LIMK2 both in vitro and in the striatum of a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mouse model of PD [8]. Another terpenoid regulating apoptosis is Astragaloside IV (AS-IV), also known as Astragalus saponin IV, which is a primary bioactive component derived from the plant *Astragalus membranaceus*, which is widely used in treating various diseases in traditionally Chinese medicine. AS-IV can protect primary cerebral cortex neurons exposed to oxygen and glucose deprivation by modulating the PKA/CREB signaling pathway and preserving mitochondrial function [23]. AS-IV has also been shown to decrease the apoptosis rate in primary astrocytes, downregulating p-JNK, modifying the Bax/Bcl-2 ratio, and influencing caspase-3 activity [24].

Among the alkaloids, one of the most studied is caffeine (1,3,7-trimethylxanthine), a natural central nervous system (CNS) stimulant of the methylxanthine class. It is mostly sourced from coffee beans but can also be found in certain types of tea and cacao beans and is one common ingredient of many energy beverages [96]. It was shown that the PI3K/Akt signaling pathway, known for its role in cell survival, is implicated in the anti-apoptotic effects of caffeine by regulating the p-JNK and ERK protein kinase of both in vitro cultures [26] and in a mouse model of AD [25].

Fisetin (3,3',4',7-tetrahydroxyflavone) is another plant-derived compound that has garnered attention for its potential anti-apoptotic effects in the brain. It is a flavonoid abundant in fruit and vegetables, including apples, strawberries, cucumber, and persimmon [27,97]. In 6-hydroxydopamine-treated cells, which are used as a model of PD or traumatic brain injury (TBI), treatment with Fisetin inhibits the activation of key apoptotic proteins, including caspase-3 and caspase-9, suggesting a potential mechanism for preventing neuronal cell death [28,29]. Moreover, Fisetin has been observed to enhance Bcl-2 expression and reduce Bax expression in the mouse brain, thereby shifting the balance in favor of cell survival [30,31]. Furthermore, Fisetin treatment can reduce the expression of other apoptotic regulators, such as ASK-1, p-JNK, p53, and cytochrome C, in the cortex and hippocampus of mice [31]. The inhibition of these apoptotic molecules by Fisetin is triggered through the activation of the PI3K/Akt signaling pathway [28].

Finally, the flavonoid GardeninA (GA), found in the gum of the medicinal plant *Gardenia resinifera*, has shown a regulatory effect on apoptotic pathways in a *Drosophila* model of PD. Indeed, it was observed that GA pretreatment reduced the levels of active caspase-3 induced by paraquat (PQ), thereby mitigating apoptotic cell death. Moreover, GA pretreatment resulted in the downregulation of *reaper* expression, which was induced by PQ. This is significant as *reaper* can induce apoptosis by inactivating inhibitors of apoptosis proteins (IAPs), consequently allowing caspases to escape proteasomal degradation and trigger cell death [32].

2.4. Autophagy Stimulation

Autophagy is an evolutionarily conserved process that leads to the trafficking and degradation of intracellular components into the lysosome to maintain cellular homeostasis [98]. The targets of autophagy comprise misfolded or aggregated proteins, as well as damaged organelles, such as mitochondria, endoplasmic reticulum, and peroxisomes; in particular, mitophagy is the selective removal of damaged mitochondria via the macroautophagy pathway, which is crucial for monitoring their quality and decreasing cellular oxidative stress [99]. Autophagy is particularly important in neurons since it is involved in neurodevelopment as well as in the maintenance of neuronal homeostasis, and basal beneficial autophagy is necessary to maintain lifespan and prolong longevity [100]. Moreover, autophagy also exerts a key role in degrading aggregation-prone proteins, such as α -synuclein [101], β -amyloid [102], and the mutant protein Huntingtin [103]. For this reason, autophagy is considered a target for the prevention of neurodegenerative diseases, and a variety of natural compounds are reported to modulate autophagy and exert neuroprotective effects. Interestingly, most of the literature describes the efficacy of natural compounds in autophagy induction, and some of them also stimulate beneficial effects in particular conditions. Here, we collected the current evidence indicating their neuroprotective role (Table 1).

The aforementioned caffeine was shown to enhance autophagy by increasing the microtubule-associated protein, LC-3, reducing the receptor protein sequestosome 1 (SQSTM1/p62) and chaperone-mediated autophagy and regulating the expression of LAMP2A (lysosomal associated membrane protein 2). Chronic caffeine treatment re-establishes macroautophagy activity and broadly targets a variety of protein aggregates in the brain. Notably, caffeine treatment was shown to attenuate abnormal α -Syn aggregation and neurotoxicity by re-establishing autophagy activity in animal models of PD [33].

Most known plant-derived compounds regulating autophagy belong to the class of flavonoids (Table 1), such as Isoquercitrin, Hesperidin, Epigallocatechin gallate, Fisetin, and Morin.

Isoquercitrin, a flavonoid from *Apocynum venetum*, also known as quercetin-3-O- β -D-glucopyranoside, is a flavonoid that is also present in a variety of medicinal herbs, fruits, and vegetables as well as plant-based foods and drinks [104]. Isoquercetin can mitigate cellular damage in Schwann cells exposed to high glucose levels by upregulating the expression of Beclin-1 and LC3, thus reversing the inhibition of neural cell proliferation [34]. Likewise, in the model organism *C. elegans*, Isoquercetin counteracts the neurotoxic effects of peptide A β 1-42 through the activation of both macroautophagy and proteasomal degradation pathways [35].

Hesperidin, identified as 3,5,7-trihydroxy flavanone-7-rhamnoglucoside, is a flavanone glycoside predominantly found in citrus fruits. In a study, Hesperidin treatment was able to counteract the increase in Beclin-1, LC3A, and LC3B induced by sodium fluoride, in this case, indicating its potential role in mitigating autophagy-related neurotoxicity. Furthermore, Hesperidin treatment was shown to stimulate the PI3K/Akt/mTOR signaling pathway, which is essential for cell survival [36].

The aforementioned Epigallocatechin gallate was shown to induce autophagy in SH-SY5Y cells by activating the Sirt1 pathway, specifically targeting the neurotoxicity and mitochondrial damage caused by the prion protein. EGCG induces an increase in LC3-II expression alongside a decrease in p62 levels, indicating enhanced autophagic activity [37]. Additionally, the natural flavonoids Fisetin and Morin have also been recognized for their ability to stimulate autophagy. Morin is highly present in the leaves, stems, branches, and fruits of plants belonging to the Moraceae family, and it possesses significant antioxidant and anti-inflammatory properties that offer neuroprotective benefits. In addition, Morin enhances mitophagy, which is essential for removing malfunctioning mitochondria. Strikingly, it accomplishes this by promoting the relocation of transcription factor EB (TFEB) to the nucleus and triggering the AMPK-ULK1 pathway. These actions have been proven to confer neuroprotection in a mouse model of PD induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), highlighting Morin's potential [39]. As for Fisetin, it was observed that it can induce autophagy within cortical neurons by activating the Nrf2 transcription factors and TFEB, likely via the inhibition of mTORC1. Such activation is crucial for the disassembly of p-tau in cortical neurons, positioning Fisetin as a promising neuroprotective molecule [38].

2.5. Anti-Neuroinflammatory Compounds

Neuroinflammation is another typical hallmark of neurodegenerative diseases [2], which has been observed as an early event in AD development. Chronic inflammation involving microglia may occur in the presence of misfolded or aggregated proteins, high ROS levels, or damaged synapses. In addition, astrocytes are also activated in neurodegenerative diseases, affecting synaptic functions, neuron metabolism, protein aggregation, and, therefore, taking part in the disease process. For these reasons, the modulation of neuroinflammation is a relevant aspect of the prevention of neurodegeneration, and several compounds have been studied for their ability to modulate neuroinflammation (Table 1).

Grifola frondosa, also known as Maitake, is a well-studied medicinal mushroom used for centuries in China and Japan. It is particularly rich in polysaccharides, especially β -glucans, which have been shown to possess several health-promoting activities [105]. It was recently reported that treatment with proteo- β -glucans derived from Maitake increases astrocytes and microglia activation in a mouse model of AD and promotes the recruitment of microglia to the A β plaques in the cortex and hippocampal region, thus promoting the clearance of A β and reducing the A β plaque burden [40].

Another mushroom-derived compound, the well-studied terpenoid Erinacine A from the mushroom *H. erinaceus*, exerts an anti-inflammatory effect. Indeed, it reduces TNF- α expression upon lipopolysaccharide (LPS) stimulation in rat astrocytes and reduces NO

production and iNOS expression in a mouse microglial cell line following treatments with LPS and IFN- α . Remarkably, Erinacine A treatment shows the same anti-inflammatory effect in vivo, leading to improved motor functions in an animal model of PD [41].

Considering triterpenoids, Astragaloside IV (AS-IV) also exhibits anti-inflammatory effects. In particular, it promotes the activation of nuclear factor-kappa B (NF- κ B), reducing the expression of pro-inflammatory genes in microglial cell cultures and in vivo [42]. In addition, this molecule has been reported to modulate the activity of MAPKs and to inhibit the mRNA expression of various pro-inflammatory cytokines of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) enzymes, contributing to reducing the inflammation [42,43].

Among flavonoids, Fisetin exhibits a remarkable ability to decrease the expression of the glial fibrillary acidic protein (GFAP) in the cortex, hippocampus, and hypothalamus of mice, which is a hallmark of neuroinflammation [44].

GardeninA, among the class of flavonoids, reduces both iNOS and NO levels in the PD model in *Drosophila*, thus providing protection against NO-mediated inflammatory effects [32].

Finally, several lines of evidence indicate that caffeine can limit neuroinflammation associated with neurodegenerative disorders. Indeed, it was reported that caffeine reduces both microglia activation and astrocyte reactivity [49,51].

2.6. Compounds Improving Cognitive Functions

Neurodegenerative diseases are characterized by a strong and progressive cognitive decline. AD and PD patients have difficulties in learning and memory, as well as in speech, due to the neuroinflammation and progressive degeneration of neurons [106]. Thus, the evaluation of the effects of natural compounds on cognitive functions is often the biggest preclinical readout of the effect of these molecules. In Table 1, we report natural compounds that can improve cognitive functions; interestingly, some of them are also discussed as anti-neuroinflammation molecules (see the previous section). Indeed, as expected, the prevention of cognitive impairment is strictly connected to neuroinflammation reduction.

Different mushroom-derived compounds have been tested for their effects on cognitive functions. For instance, it was recently reported that treatment with proteo- β -glucans derived from Maitake mushrooms improves learning and memory dysfunctions in a mouse model of AD [40]. Another mushroom-derived compound, Erinacine A from *H. erinaceus* mycelium, was also shown to ameliorate behavior deficits in the same model, in particular, burrowing and nesting behavior, spatial learning, and memory [7]. Strikingly, in humans, two double-blind clinical trials showed that the *H. erinaceus* fruiting body supplement (about 3 g/day) improves cognitive functions in healthy Japanese 50-year-old individuals and individuals with mild cognitive impairment [53,54]. Although in these two studies no specific molecule was used, rather, the total dried fruiting body of *H. erinaceus* was used, and although it was not tested on patients affected by neurodegenerative diseases, these preliminary clinical trials suggest the potential of this mushroom to prevent cognitive impairment.

Among terpenes, Astragaloside IV treatment is able to reverse cognitive impairment, as indicated by improved learning and memory abilities in an AD mouse model [42], as well as in a rat model of vascular dementia [55].

Also, caffeine has been shown to potentially prevent memory decline in animal models of AD and PD. Moderate treatment with caffeine led to an enhanced capacity for memory and a reversal of AD pathology in mice [56], also preventing β -amyloid-induced cognitive impairment [57]. Additionally, caffeine consumption prevented hippocampal neurodegeneration induced by streptozotocin (STZ) in a rat model of sporadic dementia [58]. A study examining risk factors for AD in individuals over 65 years old found that coffee drinkers had a 31% lower risk of developing the disease [59]. Similar results were observed in a further study, which showed a reduced risk of AD/dementia in moderate coffee drinkers of 3–5 cups every day compared to those who consumed 0–2 cups of coffee over a 21-year

period [60]. In a cross-sectional study involving 196 untreated patients with early-stage PD, coffee consumption was significantly associated with a reduction in the severity of the mood/cognition domain of the non-motor symptoms scale (NMSS) [61].

Numerous flavonoid compounds, including Morin, EGCG, Hesperidin, Isoquercitrin, and 6-Shogaol, have been documented to support the maintenance of cognitive abilities, as outlined in Table 1. Despite the high use of green tea polyphenols, there is a lack of satisfactory clinical trials involving EGCG in humans with neurodegenerative diseases, but several studies on animals are available. For instance, it was reported that the long-term oral consumption of EGCG (25 mg/kg) induced significant improvement in memory function in an AD mouse model [62]. In a study on cognitive performance, EGCG was found to enhance neurological effects during brain wave stimulation, resulting in a rise in tranquility and a reduction in stress [64]. Taken together, these data support the notion of EGCG dietary supplementation as a potentially safe and effective neuroprotective agent that should also be evaluated in subjects with neurodegeneration.

As concerns GardeninA, its ability to confer protection against mobility deficits induced by paraquat (PQ) in a *Drosophila* PD model has been examined, and it showed that pretreatment with GA significantly improved climbing performances [32].

Also, Morin was shown to improve learning and memory by attenuating oxidative stress, neuroinflammation, and neuronal death in the hippocampus in a rat model of AD [65]. Moreover, in an MPTP mouse model of PD, Morin (20 to 100 mg/kg) significantly reduced nigrostriatal lesions, dopaminergic neuronal death, striatal dopamine depletion, and long-term behavioral impairments [66]. Based on these studies, we can hypothesize that Morin could be beneficial in reducing cognitive dysfunction both in PD and AD.

Hesperidin has been demonstrated to facilitate the enhancement of spatial memory indirectly through the increased release of TGF β -1 and the promotion of synaptogenic activity in cortical astrocytes of newborn Swiss mice [68]. Hesperidin addressed both motor and non-motor symptoms, such as anxiety-like behaviors and memory deficiencies, while concurrently mitigating monoaminergic abnormalities [107]. Another study investigated the impact of Hesperidin on learning and memory following prolonged exposure to mild stress. Albino Wistar rats, subjected to chronic mild stress and supplemented with Hesperidin (40 mg/kg for 28 days), demonstrated preserved cognitive functions compared to the control group. Notably, there was a statistically significant enhancement in memory consolidation observed after the learning process [69]. Strikingly, several clinical trials have also been conducted in humans, especially using Hesperidin-rich orange juice. In a clinical investigation involving 37 healthy adults aged 60–81, Hesperidin was tested at two different doses with the use of juice containing a high Hesperidin concentration (549 mg/L) or a lower Hesperidin content (64 mg/L). Following eight weeks, the group with elevated Hesperidin levels exhibited markedly enhanced cognitive function, executive function, and episodic memory compared to the group with lower Hesperidin levels [71]. A separate acute study involving 44 young adults consuming 500 mL of citrus juice with 42.15 mg of Hesperidin demonstrated a notable enhancement in cerebral blood flow (CBF) and executive function in the right frontal gyrus. However, no direct correlation was identified between behavioral improvement and the increase in CBF [70]. Another investigation centered on beverages rich in Hesperidin in healthy middle-aged men (30–65) revealed significant enhancements in attention, executive cognitive function, and psychomotor speed six hours after consuming orange juice with added orange pomace fiber (containing 220.46 mg Hesperidin) compared to a placebo group [67]. In a cohort study encompassing 13,373 Japanese elderly participants, the consumption of citrus displayed a dose-dependent and inversely correlated association with dementia incidence, indicating the potential safeguarding role of citrus flavonoids in diminishing the risk of dementia [72]. These studies proposed that dietary approaches rich in Hesperidin could potentially forestall cognitive decline in elderly individuals, although the specific mechanism remains somewhat unclear.

Among flavonoids, Isoquercetin has been attracting much attention for the treatment and prevention of AD because it contributes to the enhancement of cognitive performance,

attenuating neurochemical and neurobehavioral changes in a rat model of AD-induced by colchicine through the activation of antioxidant and anti-inflammatory pathways [73].

6-Shogaol, Hydroxytyrosol (HT), Oleuropein aglycone (OleA), and Salvianolic acid B (SalB) are all recognized phenolic compounds that offer neuroprotection, particularly through the improvement of cognitive abilities. 6-Shogaol, a principal bioactive constituent of ginger, has demonstrated effectiveness in reducing cognitive impairments in animal models of dementia, highlighting its potential in combating cognitive decline. A study illustrated the influence of 6-Shogaol on memory deficits by affecting astrocyte and microglia activation in a mouse model of AD induced by injecting A β 1-42 oligomers into the hippocampus. The results of the study revealed that 6-Shogaol treatment reduced inflammation and neuronal death, leading to an improvement in learning and mitigation of memory deficits [74]. In another investigation, significant enhancements were noted in behavioral and memory tests in a mouse model of AD after the oral administration of 6-Shogaol for two months. Consequently, the study proposed that 6-Shogaol, acting as an inhibitor of the cysteinyl leukotriene 1 receptor (CysLT1R) and cathepsin B (both implicated in the pathogenesis of AD), could act as a neuroprotective agent, preventing neuronal death and cognitive deficits [75]. Hydroxytyrosol (HT) and Oleuropein aglycone (OleA) are compounds found in extra virgin olive oil, and their dietary intake has been linked to protective effects against AD [108]. In an animal model of AD, the administration of HT (at 5 mg/kg/day for 6 months) slightly improved the cognitive behavior of mice [76]. Likewise, the administration of HT (50 mg/kg) to mice with AD for a duration of 8 weeks significantly improved their cognitive abilities, significantly reducing amyloid aggregates in the cortex [77]. Similarly, OleA is reported to protect against cognitive deterioration by reducing A β levels and plaque deposits in the animal brain together with increased synaptic function (LTP) [109]. Salvianolic acid B (SalB), extracted from the roots and rhizome of *Salviae miltiorrhiza*, is used in traditional Chinese medicine for its neuroprotective properties, attributed to its anti-inflammatory and antioxidant actions. As a water-soluble polyphenolic compound, SalB is characterized by low toxicity and high activity [110]. Notably, SalB demonstrated the ability to traverse the blood–brain barrier and directly impact the central nervous system. In a study involving 12-month-old male mice with cognitive impairment, treatment with Salvianolic acid B (20 or 40 mg/kg/day) led to improved cognitive function and the inhibition of neuroinflammation [78].

Besides SalB, *Salvia miltiorrhiza* contains Tanshinone IIA (Tan IIA), a lipophilic terpenoid compound with a pharmacological activity that enhances learning and memory in AD mouse models. Tan IIA treatments have exhibited preventive effects on behavioral tests in the early stages of the disease in six-month-old males. In the conducted research, which involved the Novel Object Recognition test, it was noted that Tan IIA was able to mitigate short-term memory deterioration in mice. Additionally, during the Morris Water Maze test, Tan IIA reversed the impairments and improved the swimming strategy with which the mice tracked down the platform, suggesting an attenuation of spatial memory deficits and an improvement in the learning capability of Tan IIA treatment [80].

3. Four Widely Explored Molecules with Neuroprotective Effects

As highlighted in the previous section, the potentialities of natural compounds are limitless, and an increasing number of manuscripts describing their neuroprotective effects have been published in recent years. In this section, we describe in more detail four examples of natural compounds that have been extensively studied as neuroprotective agents, having shown effects on multiple processes involved in neurodegeneration, and which are currently under investigation in clinical trials for their great potential (Figure 2).

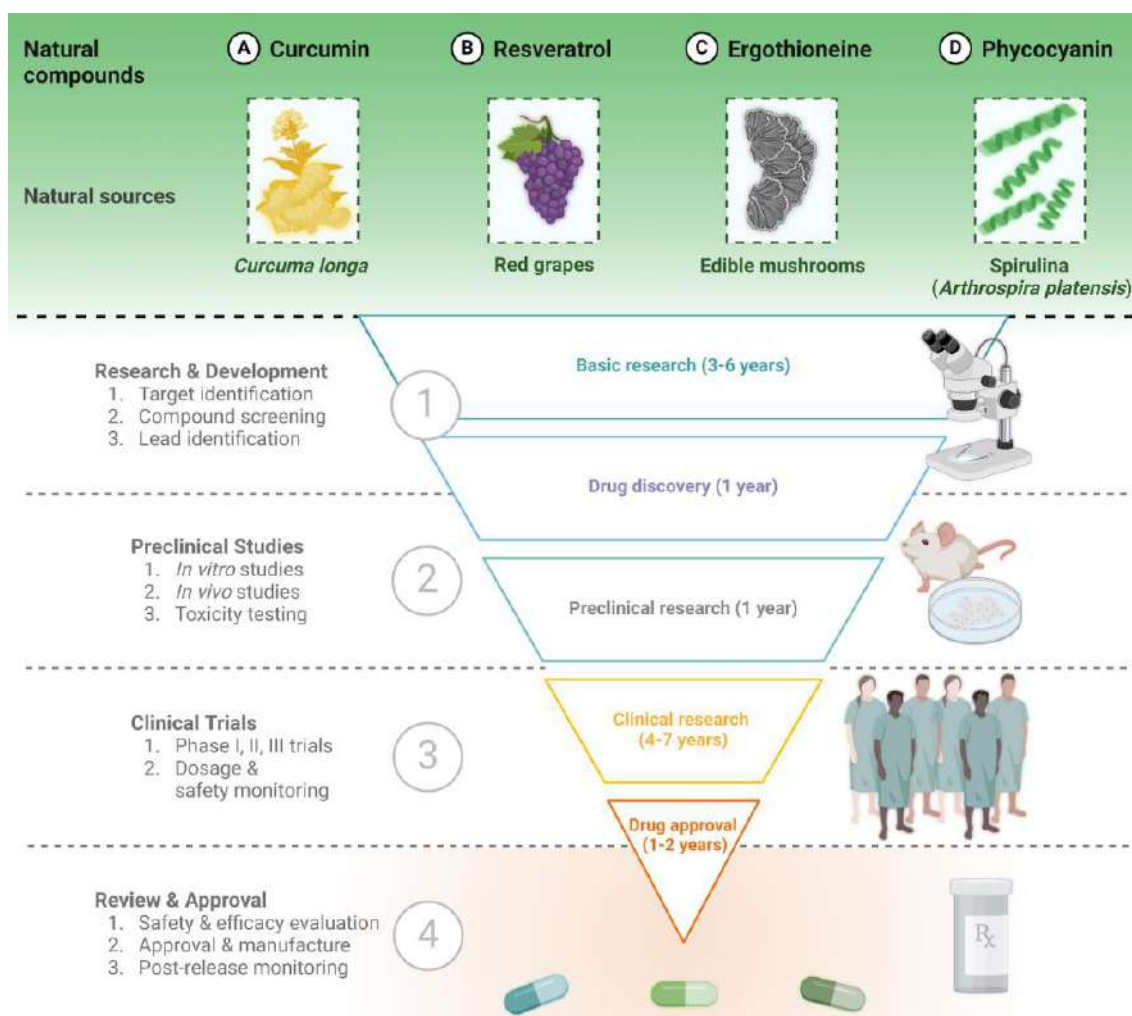


Figure 2. Exemplary natural compounds (curcumin, resveratrol, ergothioneine, and phycocyanin) and the general steps of bioactive substances from natural sources towards clinical approval.

3.1. Curcumin

Curcumin is a natural polyphenol derived from the rhizomes of the turmeric plant *Curcuma longa* [111], also called the “spice of life” for its history deeply interwoven with cultural, medicinal, and culinary traditions [112]. Curcumin is the primary component of turmeric, responsible for the plant’s intense yellow color [113], and has emerged as a focal point in extensive scientific research for its potential therapeutic application. In particular, scientific evidence from *in vitro* studies, *in vivo* experiments, and clinical trials highlights the neuroprotective effect of curcumin, which modulates several pathways associated with neurodegenerative diseases [114].

Like several polyphenol compounds, curcumin acts as a scavenger for free radicals and enhances antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), thus protecting neurons from oxidative stress [115].

Curcumin also has anti-inflammatory effects by inhibiting diverse inflammatory markers, including pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6, thus mitigating the brain’s inflammatory response [116]. Additionally, it downregulates the activity of pro-inflammatory enzymes, such as COX-2 and iNOS, which lead to reduced levels of prostaglandins and nitric oxide [117]. It also modulates the signaling pathways of NF- κ B, a key transcription factor involved in the regulation of inflammatory responses, along with Wnt5 (Wnt Family Member 5A) and JNK1 (c-Jun N-terminal kinases), which are both crucial for neuronal cell survival, inflammation, and apoptosis [118]. Interestingly, curcumin has

been investigated for its ability to interfere with the misfolding and aggregation of proteins linked to neurodegenerative disorders, including A β 1-42 and α -synuclein [119]. Based on experimental findings, curcumin has been shown to directly bind to misfolded proteins, altering their conformation and hindering their ability to aggregate into toxic forms [120,121]. In addition, it promotes the degradation of misfolding proteins through cellular protein degradation pathways, such as the ubiquitin–proteasome system and autophagy, helping to prevent the accumulation of toxic aggregates [122]. Indeed, many studies have demonstrated curcumin's important role in inducing macroautophagy. Specifically, in PD models both in vitro and in vivo, curcumin activates autophagy through the inhibition of the Akt/mTOR pathway, contributing to the neuroprotective effects. Interestingly, it was found that curcumin supplementation substantially increases the LC3-II/LC3-I ratio and Beclin-1 and reduces the accumulation of α -synuclein [123,124]. In keeping with this, curcumin downregulates the PI3K/Akt/mTOR pathway and increases the expression of Beclin-1 and the LC3-II/LC3-I ratio, promoting the formation of autophagosomes and reducing the aggregation of A β in a mouse model of AD [125].

Several clinical trials have been performed to address the clinical potential of curcumin as a neuroprotective agent in humans. Initial placebo-controlled trials in subjects with mild-to-moderate AD did not show any evidence of efficacy [126,127], probably because of the limited bioavailability of curcumin used (despite the high dose of up to 4 g/day) or due to the short time of the trial (6 months). However, the use of a solid lipid formulation of curcumin (Longvida, about 80 mg/day of curcumin) in healthy elderly subjects suggested an increase in attention, as well as in working memory and mood upon acute administration (1 h) and after 4 or 12 weeks of administration [128,129]. Interestingly, the administration of a lipidated form of curcumin (80 mg/day) also increased catalase activities and decreased A β concentration in the plasma after 4 weeks of treatment [130]. In a longer (18 months) double-blind placebo-controlled trial using a bioavailable form of curcumin (Theracurmin, 180 mg/day), curcumin treatment led to significant memory and an improvement in attention in non-demented adults. Strikingly, these results were accompanied by a reduction in FDDNP-PET (2-(1-{6-[(2-[fluorine-18]fluoroethyl)(methyl)amino]-2-naphthyl}ethylidene)malononitrile positron emission tomography) binding, which can indicate the brain deposition of amyloid plaques and tau tangles, particularly in the amygdala and hypothalamus, which are crucial regions for mood and memory [131]. All these findings support the use of curcumin to preserve cognitive functions in humans, although high attention must be paid to its bioavailability (see also Section 4).

Nowadays, curcumin is widely used not only in the food industry but also in the pharmaceutical and cosmetic industries. The global Curcumin market is expected to undergo substantial growth from 2023 to 2030. The market size was USD 207 million in 2022 but is projected to increase to USD 265 million by 2028. India is at the forefront of this market, contributing over 70%, followed by China and the USA. Strikingly, pharmaceutical-grade Curcumin represents the largest product segment, accounting for over 65% [132].

3.2. Resveratrol

Resveratrol is a natural polyphenol present in wine, red grapes, berries, chocolate, and peanuts, and it is attracting more and more attention due to its potential neuroprotective properties [133]. In fact, numerous studies suggest that resveratrol may safeguard the brain through various mechanisms. The compound's antioxidant properties are among the most studied activities of resveratrol, which is able to neutralize harmful free radicals that can damage brain cells. Resveratrol showed potent antioxidant activity in hippocampal neuronal cells by scavenging free radicals and shielding against NO toxicity [134]. Furthermore, it reduces quinone reductase 2 activity, which contributes to age-related metabolic stress and cognitive deficit in hippocampal neuronal cells treated with menadione [135], and also upregulated the endogenous antioxidant response in a mouse model exposed to ischemia [136]. Resveratrol also activates protein kinase AMPK and its upstream regulator, LKB1, in neuronal cell lines, primary neurons, and the brain [137]. In addition, it activates

the Nrf2 pathway, regulating antioxidant enzyme expression in a number of in vitro models (PC12 cells, astrocytes, cerebellar granule neurons, and oligodendroglial cells) as well as in in vivo models [138]. In primary microglia cultures exposed to LPS in rats, resveratrol decreases the production of prostaglandins, NO, TNF α [139], and COX-1 [140], showing strong anti-inflammatory properties, also reported by other studies [140,141]. Resveratrol activates SIRT1, leading to a reduction in the NF- κ B signaling pathway in glial cells exposed to A β 1-42-induced toxicity. This effect is particularly relevant since the down-regulation of SIRT1 expression abolishes the protective action of resveratrol in neuroblastoma cell lines exposed to the neurotoxic compound 6-hydroxydopamine (6-OHDA) [142].

Interestingly, resveratrol binds to the peptide A β 1-42 in vitro, and by changing the conformation of oligomers, it probably attenuates their cytotoxicity [143].

The neuroprotective effects of resveratrol have also been tested in human subjects in randomized, double-blind, placebo-controlled clinical trials. In healthy young men, a single oral dose increased cerebral blood flow in a dose-dependent manner, but no effect on cognitive function was observed [144]. However, another trial on young adults receiving resveratrol for 28 days demonstrated cognitive benefits related to working memory tasks [145]. Resveratrol (150–200 mg/day) has also been tested in elderly subjects. Initial evidence suggested improved mood and cognitive performances in post-menopausal women after 14 weeks of administration [146] and improved retention of words over a 30 min delay after 6 months of administration [147]. However, the following study failed to show any effect of resveratrol on verbal memory performance [148]. In a longer study (52 weeks) on AD patients, a higher dose of resveratrol (up to 2 g/day) decreased the level of metalloproteinase MMP9, which is involved in maintaining the integrity of the BBB, in the cerebrospinal fluid, and slowed down progressive cognitive decline [149], strongly supporting the role of resveratrol as a neuroprotective agent also in AD patients.

Despite challenges such as low bioavailability, resveratrol tolerability, and its safety profile position it as a promising candidate for long-term treatment against cognitive decline in humans [150], and advanced pharmaceutical technologies aim to improve resveratrol's bioavailability [151].

The increasing demand for resveratrol is attributed to its widespread use across various sectors, including cosmetics, dietary supplements, medications, and personal care items. Projections for the global resveratrol market indicate substantial growth between 2023 and 2030. As of 2022, the market is steadily expanding, and with significant strategies adopted by key players, it is expected to surpass the anticipated growth trajectory. As of 2021, the global resveratrol market size was estimated to be USD 165.73 million. Forecasts suggest a Compound Annual Growth Rate (CAGR) of 8.52%, leading to a market value of USD 270.69 million by 2027. Leading the resveratrol market are North America, Mexico, and South America, Asiatic-Pacific areas, the Middle East, Africa, and Europe [152].

3.3. Ergothioneine

Ergothioneine (EGT) is a non-proteinogenic, sulfur-containing amino acid characterized by good stability and solubility. Its name comes from the ergot fungus *Claviceps purpurea*, from which it was first isolated in 1909. It is an essential amino acid that can be introduced into the diet and is particularly abundant in mushrooms, although it can also be found in certain meat products (such as chicken liver) and grains and at a much lower concentration in other food sources, like eggs, rice, garlic, and beans [153]. It is absorbed in the gut through the transporter OCTN1 (the organic cation transporter novel, type 1) [154] and is also highly distributed in the brain. Importantly, in the brain, OCTN1 is expressed in neurons, dendrites, and microglia but not in astrocytes [155,156]. Strikingly, a decline in EGT has been observed in patients with dementia, including AD [157], suggesting that EGT reduction may be associated with a higher risk of brain diseases. Thus, recently, EGT has gained great attention as an anti-aging compound, and it is often called the “longevity vitamin” [158–160]. The role of EGT as an antioxidant agent has been widely investigated; it acts both as a direct reactive oxygen species (ROS) scavenger as well as through the

activation of the antioxidant cellular processes mediated by Nrf2 [153]. It was also observed that EGT reduces A β plaques in a 5XFAD mouse model of AD [161].

Despite these data, the effect of EGT administration on human subjects was poorly explored. In 2017, EGT uptake and clearance were measured, but very limited effects on biomarkers of oxidative damage were observed, probably due to the short time of administration (7 days) [162]. Some other clinical trials are ongoing (see www.clinicaltrials.gov, accessed on 15 February 2024), but at the moment, no clear results on humans have been reported, and further studies are required to clarify EGT health benefits in humans.

Yet, EGT is widely commercialized as a diet supplement for its antioxidant and anti-aging properties, especially in the United States, China, and Europe. The price of EGT is about USD 700/kg; the global market of EGT was estimated to be USD 15 million in 2020 and is expanding more and more; it is expected to reach USD 125 million in 2027 [163], thus representing a huge developing market. For these reasons, new strategies for EGT extraction from mushrooms, but also for production from engineered strains and through chemical synthesis, are being developed [163].

3.4. Phycocyanin and Spirulina Extract

Phycocyanin (PC) is a pigment–protein complex from the light-harvesting phycobiliprotein family, which also includes Allophycocyanin, Phycoerythrin, and Phycoerythrocyanin [164,165]. Phycobiliproteins aggregate to form clusters that adhere to the membrane, called Phycobilisomes. PCs are found in cyanobacteria as an accessory pigment to chlorophyll and with a molecular weight of around 30 KDa. PCs are composed of two subunits, α and β , combined together [166–168]. These proteins have a light blue color, absorbing orange, and red light near 620 nm and emitting fluorescence at around 650 nm. The light absorption and the fluorescence emission are type-dependent (e.g., Allophycocyanin absorbs and emits at longer wavelengths than C-Phycocyanin or R-Phycocyanin). Approximately 20–45% of the protein fraction from the cyanobacterium *Arthrospira platensis* (Spirulina) is PCs [169,170]. The utilization of an appropriate extraction method is of high importance to obtain a pure and stable PC since the release of this pigment depends on the cell membrane disintegration [171].

Several studies describe PC's beneficial characteristics and antioxidant properties in vitro and in vivo [165,172]. In particular, PCs are able to scavenge reactive oxygen and nitrogen species to counteract lipid peroxidation and to inhibit enzymes, such as NADPH oxidase and COX-2 [173]. In animal models of multiple sclerosis and ischemic stroke, these compounds induce remyelination, as demonstrated by electron microscopy, also showing a reduction in pro-inflammatory cytokines and induction of immune suppressive genes [173]. Several studies focus on the effects of Spirulina in the brain, highlighting its beneficial anti-inflammatory and antioxidant effects acting on glial cell activation and in the prevention and/or progression of neurodegenerative diseases (in particular, AD and PD) [174].

Spirulina is not only rich in PC but is a rich source of other bioactive compounds, including proteins, polysaccharides, polyunsaturated fatty acids, vitamins, and carotenoids [175–177]. Different studies have demonstrated the neuroprotective role of Spirulina on the development of the neural system, senility, and a number of pathological conditions, including neurological and neurodegenerative diseases [178–181].

Extracts of *Spirulina maxima* (70% ethanol) were studied in a 12-week double-blind placebo-controlled clinical trial in the elderly, showing a significant improvement in visual learning and visual working memory [182], although the exact components of this extract were not specified by the authors.

Nowadays, cyanobacteria- and microalgae-based bioactive compounds are generally commercially available. Their market demand will continue to grow due to the increasing demand for natural products in the food industry [177]. The global market regarding microalgal-based proteins is foreseen to reach USD 0.84 billion by 2023 [183]. In particular, C-Phycocyanin is expected to reach a market value of USD 409.8 million within the

2030s [183]. Spirulina, as a protein-rich cyanobacterium (60–70% (*w/w*)) and an important source for C-Phycocyanin, has also gained attention as a promising feedstock for large-scale production. It is cultivated in different countries (e.g., Germany, Portugal, the USA, and China) and mainly commercialized as dried biomass. The exclusive production of high-value proteins and C-Phycocyanin is still emerging.

4. Delivery Strategies

We have described how a plethora of natural compounds exhibit beneficial activities [184]; nevertheless, the translation of the huge number of preclinical studies to clinical trials is, of course, very challenging. One of the biggest issues is represented by the fact that most bioactive substances are environmentally sensitive, with low solubility, chemical instability, and low bioavailability, as well as an inappropriate molecular size [185]. To overcome these problems, diverse strategies have been employed to improve the bioavailability of natural products [186]. Recently, there has been an increasing interest in nanotechnology, which encompasses the generation and utilization of materials within the nanometer scale [187] to deliver natural compounds, leading to numerous advantages. Nanoparticles are favored for their abundance, biocompatibility, bioavailability, biodegradability, increased chemical and physical stability, as well as for extended shelf-life of nanoparticles [188]. Nanoencapsulation is a new frontier in nanoscience in the food, pharmaceutical, and cosmeceutical industries. This technique consists of enclosing a bioactive compound or a drug in liquid, solid, or gaseous states within a matrix or inert material, commonly using a polymer matrix or ‘wall material’. This encapsulation aids in enhancing protection, increasing stability, solubility, and availability through the cells [189]. The choice of the preparation method for an agent’s encapsulation depends mostly on its properties, such as the state of aggregation, sensitivity, and especially the size of molecules. In the last few years, numerous methods for the encapsulation of natural products, such as extract or pure natural compounds, have been developed.

Some of the compounds described in the previous section have been nano-formulated to improve their functional properties. Among them, curcumin (described in Section 3.1), due to its low solubility, biochemical degradation, and poor bioavailability, has been encapsulated in nanoemulsion, which has been described as an excellent carrier of lipophilic curcumin [190,191]. In nanoemulsion delivery systems, curcumin molecules are typically located within the hydrophobic interior of oil droplets. In this way, curcumin is protected from active substances within the aqueous phase that would typically promote its chemical degradation. In addition, this formulation leads to enhanced intestinal absorption of curcumin, which is related to the inhibition of curcumin metabolism by nanoparticles based on hydrogen bonding-driven self-assembly. The enhanced cellular uptake of curcumin by encapsulation also provides pH-triggered intestinal targeted release properties and strong antioxidant capacity [192].

Curcumin’s stability is affected by the composition and structure of delivery systems. Numerous studies have demonstrated that the stability of Curcumin is influenced by the nature and concentrations of emulsifiers. Kharat and colleagues examined the impact of emulsifier type (sodium caseinate, Tween20, quillaja saponin, gum arabic) and concentration on the stability of curcumin-loaded nanoemulsions. They found that the degradation of Curcumin in saponin-stabilized nanoemulsions accelerated curcumin degradation (after 15 days) due to its ability to promote peroxidation reactions [193]. Similarly, Artiga-Artigas et al. assessed the impact of three molecularly different surfactants, lecithin, Tween20, and sucrose monopalmitate, and their concentration on the stability of Curcumin-loaded nanoemulsions and evaluated their antioxidant capacity. They found that Curcumin-loaded nanoemulsions with lecithin showed long-term stability and exhibited higher antioxidant capacity compared to those made with the other surfactants. This enhanced performance is attributed to lecithin’s phosphate ions, which can form hydrogen bonds with phenolic hydroxyls of curcumin [194].

Many studies have shown that the bioavailability of curcumin within the gastrointestinal tract (GIT) can be greatly increased by emulsion-based systems compared to crystalline curcumin dispersed within water [195,196]. Researchers have subjected curcumin formulations to a simulated GIT model consisting of the mouth, stomach, and small intestine phases. They have shown that the bioaccessibility value of curcumin encapsulated (74–79%) is significantly higher than that of the curcumin solution (10%). In addition, it suggests that encapsulating curcumin within small lipid particles may be advantageous for improving its absorption from the GIT [197].

With a similar approach, polysaccharides from *G. lucidum* were also encapsulated in a nanoparticle to achieve better health-promoting effects. However, *G. lucidum* polysaccharides have some challenging characteristics, such as low solubility and low yield. Accordingly, to surmount these problems, a polysaccharide-based nanoparticulate co-delivery system was developed to obtain excellent functional properties and processing benefits [198]. Correspondingly, research demonstrated that *G. lucidum* polysaccharides in nanoparticles not only perform their original functions but also contribute to preventing the rapid metabolism of other active components. This leads to an improved loading rate and encapsulation efficiency, thereby enhancing the bioactivity and pharmacological effects of polysaccharide-based nano-systems [199].

As mentioned above, Spirulina provides a high protein content and a full range of essential amino acids, which is why it has become of special interest as a good protein source. Additionally, it exhibits antioxidant and anti-inflammatory effects on the human body, as presented in Section 3.4. Nevertheless, it brings some challenging characteristics that limit its use in food products, such as the strong, dark, green-blue color, derived from pigments of chlorophyll A, phycocyanin, and carotenes. Therefore, nanotechnology, such as microencapsulation by spray drying and enzymatic treatment, has become of interest to overcome Spirulina's restricted use [200]. Maag et al. applied microencapsulation and additional enzymatic hydrolysis to the Spirulina extract in order to reduce the intense green color, optimize thermal stability, increase bioavailability, enhance protein powder solubility, and moreover, improve digestibility. This method consists of entrapping sensitive nutrients within a wall material maltodextrin and gum arabic in order to protect them from physico-chemical influences and external factors, such as heat and oxidative processes caused by air and light. They have shown that enzymatic treatment or encapsulation enhances the protein solubility between 10 and 30% compared with the untreated samples. Furthermore, a considerable reduction in green color intensity was observed in the encapsulated samples [201].

When considering neuroprotective agents, the BBB is a major challenge against obtaining the optimum effect from natural compounds [202]. Also, in this case, nanotechnologies, by means of various drug delivery systems, propose good strategies owing to their unique ability to target the BBB and to assist in the better delivery of natural compounds to the brain than natural compounds on their own. In addition, with the help of the surface modification of nanocarriers to take advantage of receptors overexpressed at the BBB, nanocarriers have been shown to efficiently overcome the BBB and deliver the selected compound to specific sites within the brain. To exemplify, the surface modification of curcumin nanocarriers incorporates targeting ligands to bind receptors and transporters at the BBB, which enables them to bypass receptor-mediated endocytosis, thus improving central nervous system selectivity and permeability [203]. Another study reported that curcumin-encapsulated poly(lactide-co-glycolide) nanoparticles (cur-NPs) induced the proliferation of neural stem cells and their differentiation to neurons in vitro and in the hippocampus and subventricular zone (SVZ) of adult rats. The nanoparticles increased curcumin levels in the rat brain compared to similar doses of bulk curcumin, and the presence of cur-NPs in the hippocampus and SVZ confirmed the ability of these nanoparticles to cross the BBB and induce neurogenesis [204].

Bidirectional communication between the human gut microbiota and the brain, commonly referred to as the microbiota–gut–brain axis or gut–brain axis, exerts a significant

influence on behavior and brain function. The microbiota–gut–brain axis represents an intricate communication network that connects the gastrointestinal tract with the brain and facilitates bidirectional interactions through neural, endocrine, and immune pathways [205]. The investigation into dietary impact on the gut microbiota demonstrates a major impact on the brain, especially on the development and function of microglia and the BBB [206]. The gut microbiota produces various neurotransmitters, such as GABA, dopamine, and serotonin; amino acids as well as their derivatives, such as tryptophan and tyramine; and microbial metabolites, like short-chain fatty acids and aryl hydrocarbon receptor (AhR) ligands. These compounds can influence the local gut physiology and can circulate through the blood to interact with the host immune system, which directly signals the brain and may affect microglial activation [207]. Notably, early microbiome changes were found in patients with preclinical AD and PD, and compelling evidence shows that the altered gut microbiome can drive neurodegenerative disease pathogenesis [205]. Strikingly, changes to the gut microbiome can cause the misfolding and abnormal aggregation of α synuclein, which can be transported from the periphery to the central nervous system (CNS) via the vagus nerve [208].

This insight into the microbiota–gut–brain axis makes it a promising target for neuro-protective strategies. In fact, adjusting the gut microbiota through dietary changes could potentially influence microglial activity and mitigate the progression of neurodegenerative diseases [205]. Therefore, the bioavailability of natural compound nanoparticles within the gastrointestinal tract can affect the gut microbiota, thereby impacting brain-related diseases. For instance, Qu and colleagues developed Honokiol nanoparticles, a plant bioactive compound, which demonstrated the improved bioavailability and bioactivity of Honokiol. It has been shown that this nanoparticle suppresses the neuroinflammatory response, regulating the gut microbiota, reducing β -amyloidosis, and, consequently, exhibiting neuroprotective properties in a mouse model of AD [209].

To summarize, the use of nanoparticles is a promising approach to deliver natural compounds due to their abundance, biocompatibility, bioavailability, biodegradability, and increased chemical, physical, and shelf-life. The bioavailability of natural compound nanoparticles affects the gut–brain connection, thereby preventing neurodegenerative diseases. Nevertheless, even if it is successful in various *in vitro* and *in vivo* BBB models, further studies are needed to investigate the efficacy of these approaches in humans. In addition, despite the success of this technique, it is imperative to evaluate more types of nanomaterials and identify additional potential health effects.

5. How a Better Understanding of Natural Molecules with Neuroprotective Effects Could Improve Their Utilization in the Next Decades

A better understanding of natural molecules with neuroprotective effects could lead to the development of more effective strategies to prevent neurodegenerative disorders [210]. The free radical theory of aging postulated by Harman in 1956 points out that excessive production and accumulation of ROS causes a subsequently altered cellular integrity that eventually leads to severe and irreversible damage [210,211]. Therefore, oxidative stress plays a crucial role in neuronal damage associated with aging as well as neurodegenerative disorders [210,212,213]. Epidemiological and biochemical studies have identified food components as promising agents for neuroprotection [210]. Neuroprotection includes mechanisms such as the activation of specific receptors, changes in enzymatic neuronal activity, and the synthesis and secretion of different bioactive molecules. All these mechanisms are focused on preventing neuronal damage and alleviating the consequences of massive cell loss. Some neuropathological disorders selectively affect particular neuronal populations, but their neurochemical and anatomical properties in order to design effective therapies should be known. Although the design of such treatments could be specific to neuronal groups sensitive to damage, the effect would have an impact on the whole nervous system [210].

The development of new strategies, like nanocarriers (see Section 4), that help to promote the access of neuroprotective molecules to the brain is needed to provide more effective therapies for disorders of the central nervous system (CNS) [210]. In order to trace the success of these nanoplatforms on the release of the bioactive cargo in the CNS and determine the concentration at trace levels of target biomolecules, the use of analytical chemistry and concrete separation instrumental techniques are essential. Currently, these techniques are used for the determination and identification of natural neuroprotective molecules in complex matrixes at different concentration levels [210].

An alternative, not a mutually exclusive approach, is represented by the chemical modification of natural compounds to mainly increase their stability and/or solubility. Researchers have chemically modified several natural compounds with neuroprotective or anti-aging activity [213–215]. Fucoidan is a class of sulfated polysaccharides rich in fucose, and fucoidans from different species have different structures [216]. As investigated via electron microscopic images and signaling pathway studies, this compound, despite the fact that does not inhibit the aggregation of A β 1-42, can block caspase-3 and caspase-9 and then apoptosis [217]. Naive Bayesian and recursive partitioning algorithms were applied by Fang et al., for example, to construct classifiers to predict active molecules against 25 key targets toward AD using the multitarget-quantitative structure–activity relationships (mt-QSAR) method [218]. In this context, fucoidan has significant structural diversity: its structure–activity relationship could be investigated to further rational design and modification, achieving efficient clinical development [214].

6. Conclusions

In this comprehensive review, we delve into the fascinating properties of natural products sourced from mushrooms, plants, microalgae, and cyanobacteria. These bioactive compounds hold promise in the prevention and treatment of neurodegenerative diseases. Ergothioneine, curcumin, resveratrol, and phycocyanin emerge as powerful allies in the fight against neurodegeneration. Their origins span various natural niches, potentially sharing a common purpose: safeguarding neuronal health. Rather than targeting a single pathway, these compounds adopt multifaceted strategies, and by addressing multiple processes simultaneously, they can mitigate premature cell death and preserve neuronal function.

While their potential is undeniable, effective delivery to the brain remains a hurdle. Strategies to enhance bioavailability and overcome the blood–brain barrier are critical for translating these discoveries into clinical success. Collaborations between researchers, clinicians, and pharmaceutical industries are essential for the further development of this scientific field. Innovative drug delivery systems hold promise in optimizing the therapeutic impact of these natural neuroprotective agents. Moreover, the gut–brain axis represents a promising target for the development of new therapies for neurodegenerative and neurodevelopmental disorders. Exploiting natural compounds with neuroprotective properties could provide a novel approach to enhance this axis and promote brain health. More research is needed to fully understand the complex interactions between the gut microbiota, the gut–brain axis, and natural neuroprotective niches.

In summary, this manuscript underscores the need to harness the potential of natural compounds for neuroprotection. By bridging the gap between basic research and practical application, the way can be paved for novel treatments, improving the lives of individuals affected by neurodegenerative diseases.

Author Contributions: Conceptualization, P.C. and F.T.; writing—original draft preparation H.M., A.L., G.D.B. and F.T.; writing—review and editing, P.C. and F.T. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.4—call for tender No. 3138 of 16 December 2021, rectified by Decree No. 3175 of 18 December 2021 of the Italian Ministry of University and Research funded by the European Union—NextGenerationEU; Award Number: Project code CN_00000033, Concession

Decree No. 1034 of 17 June 2022. Adopted by the Italian Ministry of University and Research, CUP, H43C22000530001, Spoke 6, Project title “National Biodiversity Future Center—NBFC”. This work was also supported by the project “ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON Foods” (CUP:H43C22000820001) of the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.3—Research funded by the European Union—NextGenerationEU.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The authors declare no conflicts of interest.

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Targeting protein aggregation using a cocoa-bean shell extract to reduce α -synuclein toxicity in models of Parkinson's disease

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ARTICLE INFO

Handling Editor: Dr. Yeonhwa Park

Keywords:

Saccharomyces cerevisiae

Human α -synuclein

Parkinson's disease (PD)

Cocoa

Food waste valorization

ABSTRACT

Neurodegenerative diseases are among the major challenges in modern medicine, due to the progressive aging of the world population. Among these, Parkinson's disease (PD) affects 10 million people worldwide and is associated with the aggregation of the presynaptic protein α -synuclein (α -syn). Here we use two different PD models, yeast cells and neuroblastoma cells overexpressing α -syn, to investigate the protective effect of an extract from the cocoa shell, which is a by-product of the roasting process of cocoa beans. The LC-ESI-qTOF-MS and NMR analyses allow the identification of amino acids (including the essential ones), organic acids, lactate and glycerol, confirming also the presence of the two methylxanthines, namely caffeine and theobromine. The present study demonstrates that the supplementation with the cocoa bean shell extract (CBSE) strongly improves the longevity of yeast cells expressing α -syn, reducing the level of reactive oxygen species, activating autophagy and reducing the intracellular protein aggregates. These anti-aggregation properties are confirmed also in neuroblastoma cells, where CBSE treatment leads to activation of AMPK kinase and to a significant reduction of toxic α -syn oligomers. Results obtained by surface plasmon resonance (SPR) assay highlights that CBSE binds α -syn protein in a concentration-dependent manner, supporting its inhibitory role on the amyloid aggregation of α -syn. These findings suggest that the supplementation with CBSE in the form of nutraceuticals may represent a promising way to prevent neurodegenerative diseases associated with α -syn aggregation.

1. Introduction

The food industry produces a high amount of waste during food processing and production. Under a circular economy perspective, the valorization of these by-products and waste is convenient not only from an economic and environmental point of view, but also because they are rich in active compounds with potentially useful bioactivities (Oreopoulou and Russ, 2007; Panak Balentić et al., 2018).

Cocoa beans (*Theobroma cacao* L.) are widely used in food production, but also for pharmaceutical and cosmetic purposes (Mazzutti et al., 2018). One of the main by-product of cocoa processing is the cocoa bean shell, which is produced in large quantities during the roasting process (Panak Balentić et al., 2018; Okiyama et al., 2017). It has been estimated that global cocoa bean production in the 2015/2016 harvest reached

approximately 3972 thousand tons, with the shell making up to 20% of the bean (Okiyama et al., 2018; Soares and Oliveira, 2022). This represents roughly 600 thousand tons, most of which are discarded as waste and remain under-utilised, with limited applications as boiler fuel, animal feed, or fertilizer (Okiyama et al., 2018). However, in recent years, cocoa shells have gained attention as a rich source of phenolic compounds, including caffeine and theobromine (Okiyama et al., 2018; Arlorio et al., 2005; Pagliari et al., 2022; Visioli et al., 2012; Gil-Ramírez et al., 2024). These two compounds, belonging to the methylxanthine class, are well-known for their antioxidant and anti-inflammatory properties (Rebollo-Hernanz et al., 2019, 2022; Cadoná et al., 2022), which contribute to mitigating oxidative stress, a key factor in neurodegenerative diseases. While the spotlight is often on cocoa bean, recent studies suggest that cocoa shells, given their abundant availability and

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<https://doi.org/10.1016/j.crfs.2024.100888>

Received 17 August 2024; Received in revised form 9 October 2024; Accepted 16 October 2024

Available online 24 October 2024

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cost-effectiveness, can offer a significant potential as a sustainable resource for developing therapeutic agents (Rojo-Poveda et al., 2020; Cinar et al., 2021; Sánchez et al., 2023). Besides, new insights into the toxicological safety of two cocoa shell matrices has been also provided, opening opportunities for their use as functional food and nutraceutical products (Gil-Ramírez et al., 2024). Recently, a new environmentally friendly and automated pressurised liquid extraction method has been developed and optimised to selectively recover theobromine and caffeine from cocoa shell by-products (Pagliari et al., 2022).

Parkinson's disease (PD), characterised by aberrant aggregates of the presynaptic protein α -synuclein (α -syn), is the second most common neurodegenerative disease (Poewe et al., 2017; Calabresi et al., 2023; Fields et al., 2019). Many *in vitro* and *in vivo* models mimicking α -syn pathology have been used over the years (Delenclos et al., 2019); among them, *Saccharomyces cerevisiae* has been extensively employed as a model of synucleinopathies (Fruhmann et al., 2017; Sampaio-Marques et al., 2018, 2019; Tenreiro et al., 2017). This unicellular eukaryote is a valuable tool in research due to its small size, short generation time, non-pathogenic nature, and ease of genetic manipulation (Longo et al., 2012). The deep conservation of cellular mechanisms, such as DNA replication, cell division, and protein folding, from yeast to pluricellular eukaryotes, underscores its relevance for human disease studies. The most commonly used yeast model for Parkinson's disease research involves the heterologous expression of human α -syn, which induces toxicity associated with aggregate formation, leading to vesicular trafficking impairment, increased oxidative stress and mitochondrial dysfunction, reduced lifespan, disturbed calcium signaling and altered autophagy (Sampaio-Marques et al., 2018, 2019; Tenreiro et al., 2016; Outeiro and Lindquist, 2003; Büttner et al., 2008). As in more complex systems, the autophagic pathway is the major responsible for the clearance of oligomers and toxic aggregates, which cannot be degraded by the proteasome (Popova et al., 2015).

Although *S. cerevisiae* provides an excellent platform for preliminary studies, pluricellular eukaryotic models such as *Caenorhabditis elegans* (da Silva et al., 2024; Hughes et al., 2022), *Drosophila melanogaster* (Sanz et al., 2017; Avallone et al., 2024) and mice (Dovonou et al., 2023) are also extensively employed. Interestingly, a SH-SY5Y cell line over-expressing α -syn through a doxycycline-inducible promoter, is a valuable model for studying the molecular mechanisms underlying neuronal degeneration (Vasquez et al., 2018). This eukaryotic system provides a robust platform for the *in vitro* screening of neuroprotective compounds to evaluate potential pharmaceutical compounds.

Here we show that an extract from cocoa bean shells increases lifespan and reduces reactive oxygen species (ROS) levels in a yeast model of synucleinopathy. Its anti-aging properties are associated with a stimulation of autophagy during the first two days of the stationary phase and a strong anti-aggregation feature both *in vivo* and *in vitro*. Consistently, our data also highlight a significant reduction of toxic α -syn oligomers in neuroblastoma cells expressing α -syn, supporting the potential use of cocoa bean shell extract as a preventive agent against aggregation and its pathogenic effects.

2. Materials and methods

2.1. Chemical reagents

All chemicals were purchased from Merck unless otherwise stated. MS-grade solvents used for UPLC analysis, acetonitrile, water, and formic acid were provided by Romil. Reagents for SPR experiments were purchased from Cytiva. Cell culture media and supplements were provided by Euroclone, Biolog plates and reagents were provided by Rigel Process and Lab.

2.2. Cocoa bean shell extract (CBSE) preparation

Cocoa bean shell (Trinitario variety) was kindly provided by a cocoa

processor after being roasted at 225 °C for approximately 20 min. The cocoa bean shell extract (CBSE) was obtained by Pressurised Liquid Extraction (PLE) using Dionex ASE350 (Dionex Sunnyvale, CA) as previously reported (Pagliari et al., 2022). Briefly, 1 g of dry matrix, previously blended and sieved (300–600 μ m) to obtain homogeneous samples, was extracted in 5 mL stainless steel cells using 15% EtOH solution, 90 °C temperature, 5 cycles and a static time of 6 min at a pressure of 100 bar. After the extraction the ethanol was removed using a rotary evaporator (G3, heiVAP core, Heidolph Germany) with the bath temperature set at 40 °C and the extract was freeze-dried (ALPHA 1–2 LSC BASIC, Christ Germany) with a yield of 20.68 ± 1.25 % of dry matrix.

2.3. Chemical characterization and quantitative analysis of caffeine and theobromine

The CBSE was analysed by Synapt G2-Si QToF instrument (equipped with a ZsprayTM ESI-probe) (Waters) coupled with an Acquity UPLC I-Class chromatography system (Waters). The UPLC analyses were carried out by a ACQUITY UPLC HSS T3 column (100 $\times$ 2.1 mm, 1.8 μ m, Waters) fitted with a VanGuard cartridge (Waters) maintained at a fixed temperature of 35 °C. The products were separated using a linear gradient elution program, which consisted of water (A) and acetonitrile (B) (both with 0.1% formic acid) varying from 5 to 95% B (0–20 min). A flow rate of 0.4 mL/min and an injection volume of 4 μ L were adopted. The PDA acquisition wavelength range was 190–400 nm. For mass spectrometry analyses, both negative and positive ionization modes were applied. The ESI-modes were acquired in the range of 50–1200 m/z with a fixed source temperature of 120 °C and a desolvation temperature of 150 °C. A desolvation gas flow of 600 L/h was employed. The capillary voltage was 3 kV (positive ionization mode) and –2 kV (negative ionization mode). The instrument was controlled by a MassLynxTM v4.2 software (Waters). All MS acquisitions were performed the same day, with blank control between injections.

For quantitative analysis, the UPLC system was coupled with a UV detector acquiring data 283 nm. An external standard calibration method was used to quantify theobromine and caffeine in the extract. Standard solutions of theobromine and caffeine, each at a concentration of 1 mg/mL, were properly diluted with H₂O to create six-level calibration curves ranging from 1 to 200 μ g/mL. The linearity of each calibration curve was verified using analysis of variance (ANOVA), and the linear model was found appropriate for the concentrations used and each level was acquired in triplicate. The instrument was controlled by a MassLynxTM v4.2 software (Waters). All MS acquisitions were performed the same day, with blank control between injections.

In addition, to detect analytes not visible with mass spectrometry, an NMR analysis was also conducted. Extract aliquots (4.3–4.7 mg) were resuspended in 1 mL deuterated water, containing 50 mM phosphate buffer pH7.4 and 0.4 mM TSP (internal standard). 700 μ L of this solution were transferred to NMR tubes and analysed. NMR analyses were performed with a JEOL JNM-ECZ 600R spectrometer (resonance ¹H 600.17 MHz) equipped with a 5 mm FG/RO DIGITAL AUTOTUNE probe. Monodimensional experiments were carried out in the following conditions: 128 scans, 4 dummy scans, presaturation of the residual water signal, impulse at 90 °C of 8.3 μ s, 64K data points, time of acquisition 7.7 s. For metabolite identification, literature data referred to NMR studies on cocoa samples (Caligiani et al., 2010, 2014) were used. To quantify the identified metabolites in the aqueous solution, the integrals of the selected ¹H resonances were measured with respect to TSP as previously described (Spano et al., 2024). Three replicates were made, and the results were expressed as μ g/mg of extract $\pm$ SD.

2.4. Proteomic characterization

Proteins present in the CBSE were concentrated and separated from small molecules using molecular cut-off filtration (Amicon 10000 Da

MWCO, Merck) with two washes with water. After protein precipitation with 80% Acetonitrile, pellets were resuspended in 25 mmol/L NH_4HCO_3 containing 0.1% RapiGest (Waters Corporation) and sonicated by immersion for 10 s before digestion, as previously described (Brioschi et al., 2013). After 15 min of incubation at 80 °C, proteins were reduced with 5 mmol/L DTT at 60 °C for 15 min, and carbamidomethylated with 10 mmol/L iodoacetamide for 30 min at room temperature in darkness. Digestion was performed with sequencing-grade trypsin (Promega) (1 µg every 25 µg of proteins) overnight at 37 °C. After digestion, 2% TFA was added to hydrolyze RapiGest and inactivate trypsin.

Tryptic peptides were used for label-free mass spectrometry analysis, LC-MS^E, which was performed on a hybrid quadrupole time-of-flight mass spectrometer (Xevo G2-XS, Waters Corporation), coupled with a UPLC H-class system and equipped with an ESI source (Waters Corporation). Samples were injected into an analytical column ACQUITY Premier HSS T3 C18, 100 Å, 1.8 µm, 2.1 mm × 150 mm equipped with a vanguard FIT cartridge (Waters Corporation), for elution at a flow rate of 200 µl/min for 3 min at 2% mobile phase B before increasing the organic solvent B concentration from 2 to 50% over 90 min, using 0.1% v/v formic acid in water as reversed phase solvent A, and 0.1% v/v formic acid in acetonitrile as reversed phase solvent B. All of the analyses were performed in duplicate and analysed by LC-MS^E as previously detailed (Brioschi et al., 2014). In particular, in the low-energy MS mode, the data were collected with Masslynx software at a constant collision energy of 6 eV, while in the high-energy mode, fragmentation was achieved by applying a ramp from 15 to 35 eV. Scan time of 0.1 s, capillary voltage of 1 kV, cone voltage of 40 V, source temperature 120 °C, desolvation temperature of 600 °C with desolvation gas at 800 L/h were applied to acquire spectra in the range 50–1990 m/z. The time-of-flight analyzer was externally calibrated using Sodium formate from m/z 50 to 1990, and data were post-acquisition lock mass corrected using the monoisotopic mass of the doubly charged precursor of [Glu1]-fibrinopeptide B (m/z 785.8426) delivered to the mass spectrometer at 100 fmol/µL. The reference sprayer was sampled every 30 s. The radio frequency (RF) applied to the quadrupole mass analyzer was adjusted in such a way that ions from m/z 300 to 2000 were efficiently transmitted, thus ensuring that any ion with a mass of less than m/z 300 only arose from dissociations in the collision cell. Peak detection and protein identification were performed with PLGS software (v 3.0.3) using a Uniprot *Theobroma cocoa* sequence database (v2024_04, 40947 unreviewed entries, 16 reviewed entries) and NCBI database (167551 entries). The following search criteria were used for protein identification: the default search parameters included the “automatic” setting for mass accuracy (approximately 10 ppm for precursor ions and 25 ppm for product ions); a minimum of one peptide match per protein, a minimum of two consecutive product ion matches per peptide, and a minimum of five total product ion matches per protein; up to one missed cleavage site allowed; carbamidomethyl-cysteine as fixed modification; and methionine oxidation as variable modification.

Table 1
Yeast strains used in this study.

Yeast strain	Genotype	Source
wt [empty]	BY4742 MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 [pYX242]	Tripodi et al. (2020)
wt [aSyn]	BY4742 MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 [pYX242-SNCA]	Tripodi et al. (2020)
wt [aSyn] [ATG8-GFP]	BY4742 MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 [pYX242-SNCA][pCu-ATG8-GFP]	Tripodi et al. (2022)
atg8Δ [aSyn]	BY4742 MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 atg8Δ::KanMX [pYX242-SNCA]	This study

2.5. Yeast strains, growth analysis and chronological lifespan (CLS) determination

The *S. cerevisiae* strains used in this paper are reported in Table 1. Yeast cells were grown at 30 °C in minimal medium (Difco Yeast Nitrogen Base without amino acids 6.7 g/L), with 2% w/v glucose and supplements added in excess (Orlandi et al., 2014). Cell growth was monitored by determining cell number using a Coulter Counter-Particle Count and Size Analyser, as described (Vanoni et al., 1983). In parallel, the extracellular concentration of glucose and ethanol were measured in medium samples collected at different time-points using enzymatic assays (K-HKGLU and K-ETOH Megazyme) (Orlandi et al., 2014). Duplication time (Td) was obtained by linear regression of the cell number increase over time on a semi-logarithmic plot. CLS of Fig. 1 was measured according to (Fabrizio et al., 2005) by counting colony-forming units (CFU) starting with 72 h (day 3, first-age point) after diauxic shift (day 0). The number of CFU on day 3 was considered the initial survival (100%). CBSE, dissolved in 20% ethanol by using an ultrasonic bath at 28 khz frequency and 90 W power for 3 min, was added to yeast cultures at the final concentration of 0.2% w/v. A 25X stock solution was prepared to properly dissolve the raw extract and at the same time to limit perturbations in cell culture medium composition after the supplementation. CLS experiments of Figs. 2 and 3 were performed adding CBSE in the exponential phase, as in (Tripodi et al., 2022). Briefly, cells were pre-grown until mid-late exponential phase and then inoculated at 0.150 OD/mL into flasks containing fresh medium in the presence of CBSE at the final concentration of 0.05%, 0.1% or 0.2% w/v. Then, the medium was filtered through 0.22 µm filters and 0.1 mM ampicillin was added to preserve sterility throughout the duration of the experiments. Survival was assessed by propidium iodide staining (PI) at different time points with the Cytoflex cytofluorimeter (Beckman Coulter) and analysed with the Cytoflex software.

2.6. Analysis of reactive oxygen species (ROS) levels

ROS levels were analysed as previously reported (Tripodi et al., 2022). Briefly, yeast cells were collected after 24 h treatment with the extract and 0.2 OD of cells were resuspended in PBS and stained with 5 µg/mL dihydroethidium (DHE) for 10 min. FACS analyses were performed with a Cytoflex cytofluorimeter (Beckman Coulter) and analysed with the Cytoflex software.

2.7. Protein extraction and immunoblotting from yeast proteins

Equal amounts of cells were collected and quenched using TCA 6% and lysed in lysis buffer (6M UREA, 1% SDS, 50 mM Tris-HCl pH7.5, 5 mM EDTA), as reported in (Tripodi et al., 2022). Western blot analysis was performed using anti-GFP antibody (Roche), anti-α-synuclein antibody (Sigma Aldrich) or anti-Cdc34 antibody (Coccetti et al., 2008).

2.8. Analysis of aggresomes in yeast

The intracellular protein aggresomes were analysed using the PROTEOSTAT® Aggresome detection kit (ENZO Life Sciences). Cells were collected following a 24 h treatment with 0.2% CBSE and 0.2 OD were suspended in PBS buffer and stained with the PROTEOSTAT® Aggresome detection reagent at a dilution of 1:1500 (Tripodi et al., 2022). FACS analyses were conducted using a Cytoflex cytofluorimeter (Beckman Coulter) and analysed with Cytoflex software.

2.9. In vitro aggregation of α-syn and ThT assay

α-syn was purchased from Merck and dissolved at 70 µM in PBS. Protein samples (20 µL) were incubated at 37 °C in PBS up to 72 h under constant shaking at 900 rpm with a thermo-mixer in the absence (cnt) or in the presence of the extract at 0.1 and 0.025 mg/mL or in the presence

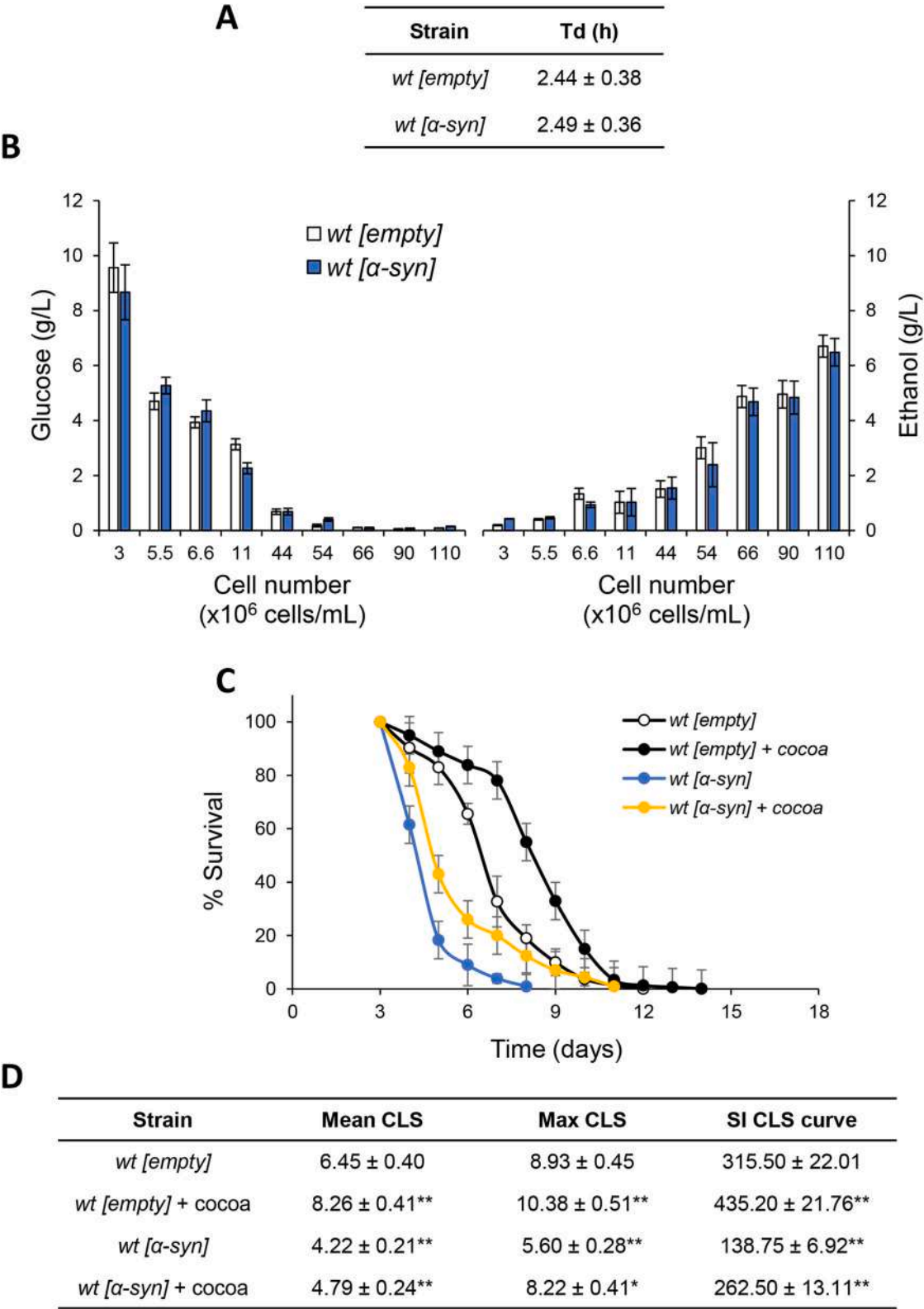


Fig. 1. CBSE supplementation at the diauxic shift extends CLS. Wild-type (*wt[empty]*) and α -syn overexpressing (*wt[α-syn]*) cells were grown in minimal medium containing 2% glucose and required supplements in excess. (A) Cell growth was monitored by counting cell number over time and duplication time (Td) of wt and α -syn expressing cells was calculated as $\ln 2/k$, where k is the constant rate of exponential growth. In parallel, (B) extracellular concentration of (left) glucose and (right) ethanol were measured in medium samples collected at different time-points. At the diauxic shift (day 0), CBSE (cocoa) was added and (C) survival over time of the indicated strains was assessed by colony-forming capacity on YEPD plates. 72 h after the diauxic shift (day 3) was considered the first age-point, corresponding to 100% survival. (D) Quantification of chronological survival: data referring to the time-points (days) where chronological aging cultures showed 50% (Mean CLS) and 10% (Max CLS) of survival, as well as, survival integral (SI) measured as reported (Murakami and Kaeblerlein, 2009). All data refer to mean values determined in three independent experiments with three technical replicates each. Standard deviations (SD) are indicated. * $p \leq 0.05$ and ** $p \leq 0.01$.

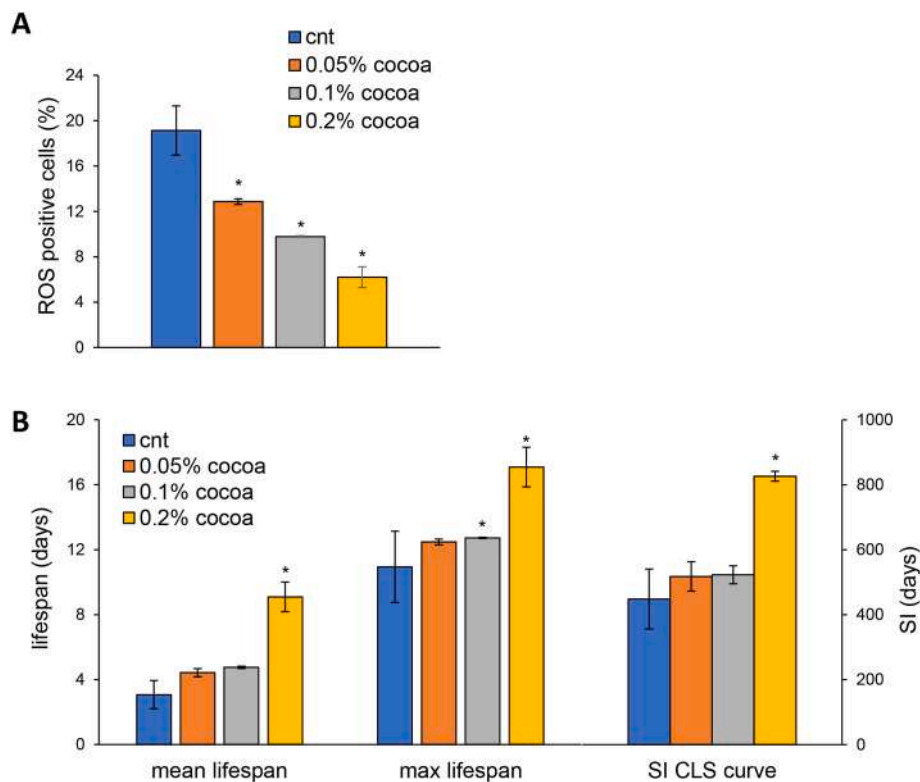


Fig. 2. The CBSE extends yeast lifespan and reduces ROS levels. (A) ROS content of yeast *wt[α-syn]* cells grown in medium containing 2% glucose in the absence or presence of 0.05%, 0.1% or 0.2% CBSE, added in the exponential phase of growth. (B) Mean and maximal lifespan and SI of cells in (A). Histograms represent mean $\pm$ standard deviation of at least two independent experiments. * $p < 0.05$.

of caffeine and theobromine solution at 0.01 mg/mL. The ThT binding assay was performed according to (LeVine, 1999), using a 20 μ M ThT solution in PBS buffer. 180 μ L of ThT solution were added to 20 μ L of the aggregated α -syn samples, transferred into a black 96-well clear bottom multiwell plate and ThT fluorescence was read at the maximum intensity of fluorescence of 485 nm using a Victor X3 plate reader (PerkinElmer); fluorescence of blank samples was subtracted from the fluorescence values of all samples. In control experiments, no interference of the extract on ThT fluorescence was observed.

2.10. Surface plasmon resonance (SPR) analysis

The BIACORE X100 system (Cytiva-Pall) was utilised to analyse molecular interactions between α -syn and the CBSE via Surface Plasmon Resonance (SPR). α -syn was immobilised onto a carboxymethylated dextran surface of a CM5 sensor chip using amine-coupling chemistry, as recommended by the manufacturer (Biacore Sensor Surface handbook BR100571), with the instrument temperature set at 25 $^{\circ}$ C. The amine coupling procedure was performed using HBS-EP running buffer (0.01 M HEPES, 0.15 M NaCl, 0.003 M EDTA, and 0.005% v/v Surfactant P20, pH 7.4) at a flow rate of 5 μ L/min. The CM5 sensor chip was activated by injecting EDC/NHS (1:1) into both flow cells 1 and 2 for 10 min. α -syn was then injected into flow cell 2 at a concentration of 200 μ g/mL in 10 mM sodium acetate, pH 3.1, and covalently immobilised at level of 1200 Response Units (RU). The remaining activated sites on the chip were subsequently blocked using 1 M ethanolamine (pH 8.5) in both cells. The association capacity of α -syn was determined by injecting two control antibodies, anti- α -syn (Sigma) recognizing the whole α -syn (positive control) and anti- α -syn33 (Sigma) recognizing α -syn oligomers (negative control), at a dilution of 1:2500 in HBS-EP running buffer at a flow rate of 10 μ L/min. The lyophilized CBSE was resuspended in HBS-EP running buffer and injected at multiple concentrations for 5 min at 25 $^{\circ}$ C and a flow rate of 10 μ L/min, with the running buffer injected as a

blank under the same conditions. After injection, the analyte solutions were replaced by the running buffer at a continuous flow rate of 10 μ L/min for 5 min. Surface regeneration was achieved by injecting 50 mM NaOH for a contact time of 1 min. Each sensorgram was corrected for the response observed in the control flow cell 1 (no immobilised protein) and normalized to a baseline of 0 RU. The sensorgram curves were acquired using the BiacoreX100 Control software, version 2.0.2 (Cytiva-Pall), in manual run mode.

2.11. Biolog OmniLog system

The effect of CBSE was evaluated for its impact on metabolic abilities using various chemical agents. This was done using the Biolog OmniLog Phenotype MicroArray chemical sensitivity panels PM21-PM25, which include 120 chemical compounds at four different concentrations. The Biolog OmniLog System was employed to compare the chemical sensitivity for each drug of *wt[α-syn]* yeast cells with and without 0.2% CBSE. All plates were prepared following the manufacturer's instructions as outlined in the OmniLog ID System User Guide (Biolog). Yeast cell cultures were grown on agar plates at 30 $^{\circ}$ C and inoculated into 8 mL of minimal medium containing 2% glucose and YNB in sterile glass tubes. The cell suspension was measured using the BIOLOG Turbidimeter (Biolog) until a transmittance of 62% T was achieved. The suspension was prepared according to the BIOLOG PM protocol for yeast cells, using Dye E. 100 μ L of cell suspension was added to each well and microplates were incubated in the OmniLog™ system at 30 $^{\circ}$ C for 72 h. The resistance and sensitivity profiles were compared using the appropriate OmniLog Biolog database (Biolog), with the y-maximum value of each kinetic growth curve being used for the analysis. Ratio between CBSE-treated and control cells were calculated, and compounds which showed a fold change >5 or <0.2 in at least three concentrations for each compound were considered as compounds towards which CBSE increases or decreases sensitivity.

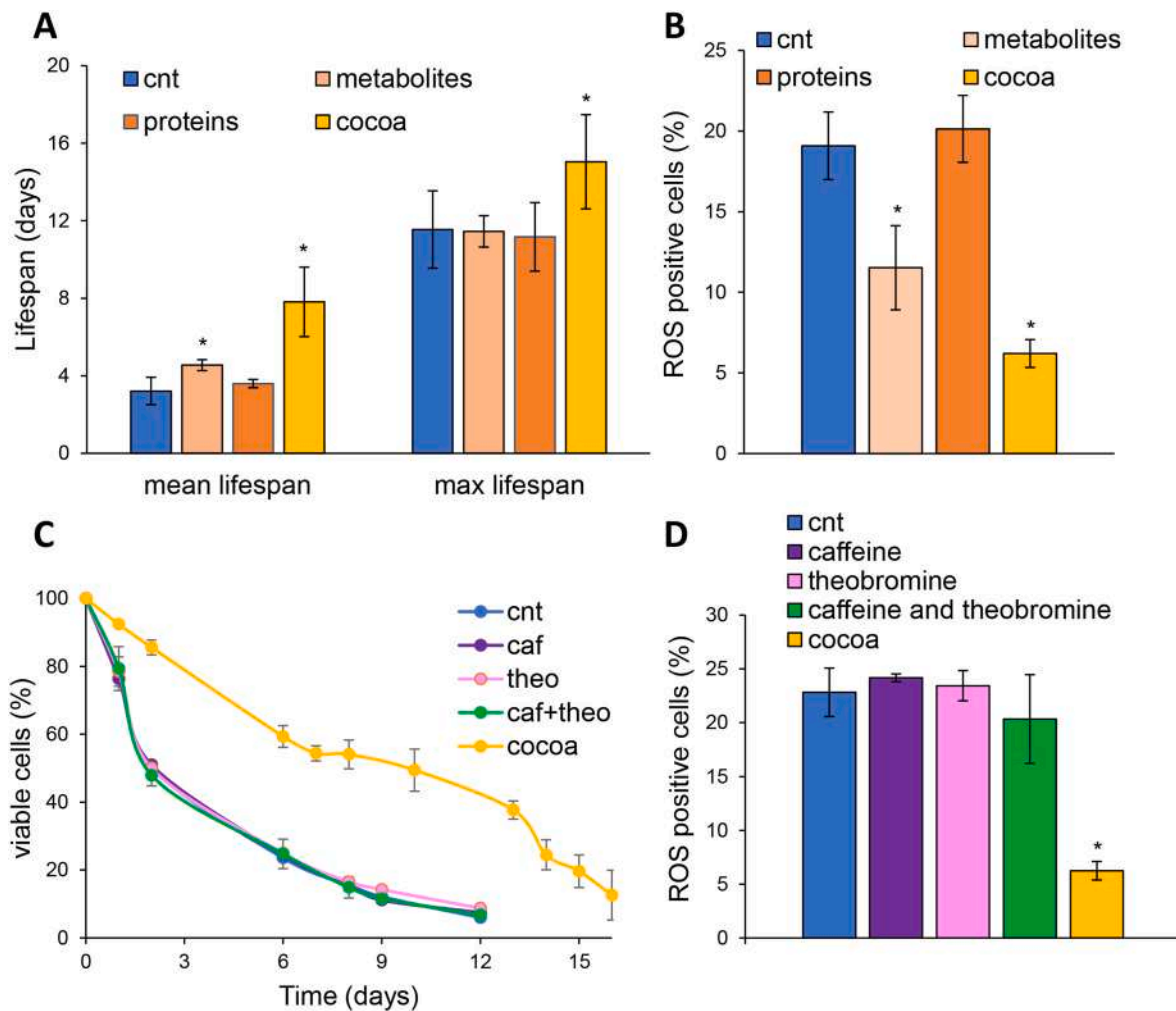


Fig. 3. The effect of the CBSE is independent from caffeine and theobromine. (A) CLS of yeast *wt* [α -syn] cells in medium containing 2% glucose in the absence (cnt) or presence of 0.2% CBSE, and its contained metabolites and proteins. (B) ROS content of cells treated for 24 h as in (A). (C) CLS of yeast *wt* [α -syn] cells in medium containing 2% glucose in the absence or presence of 0.2% CBSE, caffeine, theobromine or a combination of the two (25.96 μ g/mL caffeine and 105.4 μ g/mL theobromine). (D) ROS content of cells treated for 24 h as in (C). Results are reported as the mean $\pm$ standard deviation of three independent experiments. * $p < 0.05$.

2.12. Cell cultures

SH-SY5Y pTet-SNCA-FLAG were purchased from Merck. Cells were cultured on geltrex-coated plates at 37 °C in DMEM/F12 medium, containing 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin and 100 μ g/mL streptomycin, in a humidified 5% CO₂ incubator. Doxycycline-inducible α -syn-expressing cells were selected against the antibiotic puromycin with a dose of 2 μ g/mL. Induction of α -syn expression was achieved by adding 6 μ g/mL doxycycline (doxy, from a 6 mg/mL stock in DMSO) for 48 h or 72 h. The CBSE was resuspended in water, sterile filtered and added to the medium at a final concentration of 150 μ g/mL. For immunofluorescence assays, 160.000 cells were seeded on geltrex-coated glass cover slips in wells of a 24 multiwell plate and treated the day after for 48 h.

2.13. Protein extraction and immunoblotting for mammalian proteins

Total cell extracts were prepared using RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS) plus protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail (Merck). Protein concentration was determined using the Bradford protein assay (Bio-Rad). Western blot analysis was performed using anti- α -syn antibody (Merck), anti-phospho-T172-AMPK antibody (Cell Signaling), anti-AMPK α antibody (Cell signalling), anti-p62/

SQSTM1 antibody (Merck), anti-phospho-Ser555-ULK1 antibody (Merck), anti-ULK1 antibody (Calbiochem) and anti-vinculin antibody (Sigma).

2.14. Immunofluorescence assay

After treating cells with doxycycline (Doxy) alone or in combination with CBSE (Doxy + Cocoa) for 48h, cells were washed with PBS, fixed with 4% formaldehyde for 15 min, and permeabilized with PBS-0.2% Triton X-100 for 10 min. Then, cells were washed three times in blocking solution (PBS-1% BSA), blocked at room temperature for 60 min, and then incubated overnight at 4 °C with the primary antibodies dissolved in blocking solution as follows: anti-oligomer A11 Polyclonal Antibody (1:40, Invitrogen), anti α -syn antibody (1:200, Merck). Then, cells were incubated with anti-rabbit secondary antibody (1:200) conjugated with AlexaFluor488, dissolved in a blocking solution, for 1 h at room temperature shield from light. Glasses were mounted with a DAPI containing mounting solution. The PROTEOSTAT R Protein aggregation assay (ENZO Life Sciences) was used to measure α -syn aggregates in cells as described by the manufacturer's instructions. Briefly, after treating cells as mentioned above, cells were washed carefully twice with 1X PBS, then fixed with 4% formaldehyde for 30 min at room temperature and permeabilized with Permeabilizing solution for 30 min on ice. Following PBS washes, the slides were dispensed in Proteostat dye and incubated

for 30 min at room temperature. All treated slides were washed and mounted with a mounting medium with DAPI for nuclear staining and imaged under a Thunder fluorescence microscope (Leica). Image analysis was performed using the ImageJ software (NIH).

2.15. Statistical Analysis

Experiments were conducted in triplicate. Results are presented as mean values ± standard deviations (SD). Statistical data analyses were made using the two-tailed Student’s t-test with significance set at $p < 0.05$, or by one-way ANOVA test ($*p \leq 0.05$ and $**p \leq 0.01$).

3. Results

3.1. Characterization of the cocoa bean shell extract

The cocoa bean shell extract (CBSE), which was optimised and partially characterised previously (Pagliari et al., 2022), was subjected to further analyses to better define its composition. For this purpose, both UPLC-PDA-MS and NMR analyses were performed, and the compounds identified or tentatively identified are described in Tables 2 and 3. The identity of some compounds was achieved on the basis of the accurate mass and the associated errors, isotopic distribution, m/z values comparison with those reported in the literature and using literature databases.

NMR analysis identified and quantified mainly amino acids, such as Ala, Leu, Ile, Val, Phe, Asp, Tyr, and intermediates of TCA cycle such as fumarate, succinate, malate and citrate. The last one, together with lactate and glycerol, were the most abundant ones (Table 2, Fig. S1A). MS analysis revealed the presence of the two methylxanthines, caffeine and theobromine (theobromine: $52.74 \pm 8.12 \mu\text{g}/\text{mg}$ extract, caffeine: $12.98 \pm 3.96 \mu\text{g}/\text{mg}$ extract), and of other less abundant metabolites, like hydroxy-jasmonic acid sulfate, procyanin B, cyanidin-3-O (2''galloyl)-galactoside, as well as of some unknown compounds that could not be clearly identified (Table 3, Fig. S1B).

According to a Bradford quantification, the extract also contained 6 $\mu\text{g}/\text{mg}$ of proteins. Although the amount of protein was very low, a proteomic analysis was performed to achieve a characterization. Mass spectrometry analysis of the proteins present in the extract revealed the presence of 5 proteins from *Theobroma cacao* (Table 4), with Vicilins and a 21 kDa seed protein being the most abundant ones according to peptide intensity, as expected from literature (Rawel et al., 2019). However, we cannot exclude the presence of other proteins not identified due to

low level of annotation in the database of *T. cacao*, although both UniProt and NCBI databases were used.

3.2. Supplementation of CBSE extends CLS of yeast cells expressing human α -syn

Since yeast *S.cerevisiae* has been extensively employed as model system to study the cytotoxic effects of α -syn in PD and other synucleinopathies (Tenreiro et al., 2017), we wished to test in the context of a standard CLS experiment (Fabrizio and Longo, 2007) whether cocoa-shell treatment might have any ameliorating effect on the age-dependent α -syn-mediated cell death (Büttner et al., 2008). To this end a humanised yeast model of PD overexpressing human α -syn was used. As shown in Fig. 1A, no significant differences were observed in the duplication time (Td) between cells expressing α -syn and wt ones grown on minimal medium in 2% glucose (Fig. 1A). Consistently, during the exponential phase, when growth is sustained by a prevalent fermentation-based metabolism, the glucose decrease was accompanied by ethanol accumulation that in both yeast cultures followed the same kinetics (Fig. 1B). Once defined the growth profile, CBSE was added to both cultures at the onset of chronological aging, namely at the diauxic shift, and CLS was determined by CFU scoring. In line with previous reports (Büttner et al., 2008), α -syn expression reduced CLS (Fig. 1C). Interestingly, CBSE supplementation increased both mean and maximum CLS (Fig. 1C and D), as well as the survival integral (SI, Fig. 1D), defined as the area under the CLS curves (Murakami and Kaeberlein, 2009). Indeed, the SI increased by about 38% for wt and 89% for α -syn expressing cells, indicative of a pro-longevity effect of the CBSE. Starting from these results and being specifically interested in α -syn aggregation and its cytotoxic outcome, subsequent analyses were performed only with cells expressing α -syn.

When CBSE was added to exponentially growing cells, up to 0.2%, no effect on the growth rate was observed (data not shown), while a significant dose-dependent reduction of intracellular ROS was detected 1 day after its addition, supporting an antioxidant effect (Fig. 2A). Nevertheless, both the mean and the maximal CLS, as well as the SI increased (more than 70%) only at the highest concentration (0.2%) (Fig. 2B), in accordance with the results reported above.

3.3. The anti-aging effect is independent from caffeine and theobromine

Since the CBSE contains both metabolites and proteins (Tables 2–4), we performed a size-exclusion fractionation to separate the protein fraction (molecular weight >10 kDa) from the metabolite fraction and tested them separately on the yeast model. As shown in Fig. 4A and B, the metabolite fraction increased mean lifespan and reduced ROS levels (albeit to a lesser extent than the total extract), while the protein fraction showed no significant activity, suggesting that metabolites are the main responsible for the antioxidant and anti-aging effect of the CBSE. This is consistent with the very low amount of protein present in the extract.

Among the metabolites identified in the CBSE, caffeine and theobromine represent about 10% of the total extract (see paragraph 3.1). To analyse whether the observed anti-aging effect could be attributed to the presence of these two methylxanthines, α -syn overexpressing cells were treated with caffeine, theobromine or a combination of both, mimicking their abundance in the CBSE. Neither single treatments, nor their combination, showed any anti-aging effect (Fig. 4C) or antioxidant properties (Fig. 4D), suggesting that the pro-longevity function might depend on other metabolites or result from a synergistic/combined action with other molecules of the CBSE.

3.4. The CBSE binds α -syn and reduces its aggregation

In the last years, several data reported that natural extracts could exhibit direct fibrillation-inhibiting effects (Moukham et al., 2024). Thus, we wondered if the anti-aging effect of the CBSE could be due to a

Table 2
Metabolites identified by NMR analysis in the ¹H spectrum.

Metabolite	¹ H chemical shift (ppm)	Multiplicity (J [Hz])	Concentration $\mu\text{g}/\text{mg} \pm \text{SD}$
Leucine	0.96	d [6.2]	3.37 ± 0.09
Isoleucine	1.01	d [7.1]	1.82 ± 0.02
Valine	1.05	dd [7.1]	3.22 ± 0.01
2,3-Butanediol	1.15	d [6.4]	1.51 ± 0.10
Lactate	1.33	d [6.9]	31.56 ± 1.12
Alanine	1.48	d [7.2]	4.64 ± 0.09
Acetate	1.92	s	7.43 ± 0.22
GABA	2.30	t [6.1]	2.42 ± 0.06
Succinate	2.41	s	3.74 ± 0.06
Citrate	2.55	d [15.3]	13.02 ± 0.12
Aspartate	2.82	dd [17.4, 3.8]	1.69 ± 0.01
Glycerol	3.66	dd [11.7, 4.3]	15.90 ± 0.56
Mannitol	3.87	dd [11.9, 2.9]	4.76 ± 0.04
Pyroglutamate	4.18	dd [9.1, 5.9]	7.16 ± 0.03
Malate	4.30	dd [10.2, 2.9]	3.53 ± 0.15
Fumarate	6.53	s	0.08 ± 0.01
Tyrosine	6.9	d [8.4]	1.26 ± 0.02
Phenylalanine	7.43	m	4.03 ± 0.05
Formate	8.46	s	0.46 ± 0.01

Table 3

Chemical compounds identified or tentatively identified by UPLC-PDA-MS.

m/z expected	m/z calculated	Ionization Mode	Error (ppm)	Molecular formula	Proposed compound	Reference
181.0720	181.0733	M + H	5.5	C ₇ H ₈ N ₄ O ₂	Theobromine	(Pagliari et al., 2022; Greño et al., 2022)
195.0877	195.0888	M + H	5.9	C ₈ H ₁₀ N ₄ O ₂	Caffeine	
	263.0636	M + H			Unknown	
	279.0407	M + H			Unknown	
	297.0509	M + H			Unknown	Greño et al. (2022)
305.0695	305.0699	M-H	-0.57	C ₁₂ H ₁₈ O ₇ S	Hydroxy-jasmonic acid sulfate	
327.0510	327.0517	M-H	-2.06	C ₁₇ H ₁₂ O ₇	Unknown	
399.0838	399.0835	M + H	0.78	C ₂₈ H ₁₄ OS	Unknown	
	563.1732	M + H			Unknown	(Pagliari et al., 2022; Alves et al., 2023)
579.1497	579.1486	M + H	1.90	C ₃₀ H ₂₆ O ₁₂	Procyanidin B	
601.1188	601.1183	M + H	0.83	C ₂₈ H ₂₄ O ₁₅	Cyanidin-3-O(2''galloyl)-galactoside	
	617.0954	M + H			Unknown	
467.1195	467.1214	M-H	-4.07	C ₂₁ H ₂₄ O ₁₂	Unknown	

reduction in α -syn aggregation. A very strong decrease of intracellular aggresomes was observed in yeast cells treated with the CBSE for 24 h, with a 5-fold decrease compared to control cells (Fig. 4A), showing its potential in reducing the aggregation of misfolded proteins. Thus, to evaluate a direct effect of the extract on the aggregation process, α -syn fibrillation experiments were performed *in vitro* and the ThT emission fluorescence signal was used to quantify fibrils formation over time. In the absence of the CBSE, the ThT fluorescence showed the typical sigmoidal shape, indicating the aggregation of the protein; this behaviour completely disappeared in the presence of the extract (at both concentrations, Fig. 4B). Interestingly, caffeine and theobromine together, although had no effect on yeast longevity and ROS content (Fig. 3C and D), showed a partial inhibitory effect on the aggregation of α -syn, Fig. S2A).

A direct effect of the extract on aggregation would imply a direct interaction among the components of the CBSE and α -syn. To explore this hypothesis, α -syn protein was immobilised on a CM5 sensor chip for surface plasmon resonance (SPR) analysis. To validate the suitability and selectivity of the chip, anti α -syn (which binds free and aggregated α -syn) and anti- α -syn33 (which binds only α -syn aggregates) antibodies were utilised as positive and negative controls, respectively, and were injected into the SPR system. While the anti- α -syn antibody bound the protein on the chip, the one specific for the aggregated α -syn showed no binding at all (Fig. S2B). These results showed that the immobilised α -syn protein on the sensor chip surface was in its non-aggregated form and thus was employed to assess its direct binding with the CBSE. Results obtained from the SPR assay indicate that the CBSE could bind to α -syn protein in a concentration-dependent manner (Fig. 4C). Indeed, five increasing concentrations of the extract were tested (0.64 mg/mL, 1.27 mg/mL, 2.54 mg/mL, 5.08 mg/mL and 8 mg/mL) and the response signal increased as a function of the rising concentration of the sample (Fig. 4D). This indicates that there are compounds in the CBSE that directly bind to α -syn, and can explain the inhibitory effect on the amyloid aggregation of α -syn (Fig. 4B). Finally, we tested caffeine and theobromine, which did not appear to bind to the protein (data not shown). However, considering that the molecular weights of caffeine (194.19 g/mol) and theobromine (180.164 g/mol) are near the detection limit of the instrument (100 Da), we cannot exclude that the binding was not detected due to technical limitations.

3.5. The CBSE stimulates autophagy in yeast cells

In an attempt to identify cellular changes occurring upon treatment with the CBSE, a high throughput screening for sensitivity against antibiotics, chemicals and osmolytes was performed. The chemical resistance and sensitivity profile due to the CBSE of the yeast strain overexpressing α -syn was measured using the Biolog Phenotype Micro-Arrays PM21-PM25 chemical sensitivity panel, which contains 120 assays of chemical sensitivity. Each plate contains 24 different chemical

agents in 4 different concentrations, that were divided into 6 groups based on their structure and function: ions, cyclic compounds, organic compounds, chelators, antibiotics, and nitrogen compounds (Table S1). In the presence of 0.2% extract, yeast cells showed increased resistance to several compounds; interestingly most of them have been described for their effect on autophagy in different models (Fig. 5, Table S2).

The autophagic pathway is normally activated in stationary phase cells and is the major process involved in the clearance of α -syn aggregates (Sampaio-Marques et al., 2019). Therefore, to evaluate the activation of the autophagic process in cells treated with the CBSE, we monitored the accumulation of free GFP in cells expressing Atg8-GFP fusion protein, whose cleavage is indicative of autophagy activation. Interestingly, a significant increase in the cleavage of Atg8-GFP was observed 1 day, and even more, 2 days after the extract addition, reflecting the activation of the autophagic process in such condition (Fig. 6A,B).

However, in *atg8Δ* cells, CBSE was still able to significantly reduce both intracellular ROS level and aggresomes (Fig. 6C and D), suggesting that the stimulation of the autophagic process is not the only pathway involved in the pro-longevity function of the CBSE.

3.6. The CBSE reduces α -syn aggregates in neuroblastoma cells

In order to further investigate the effects of the CBSE, we turned to SH-SY5Y neuroblastoma cells expressing α -syn under a doxycycline-inducible promoter (Vasquez et al., 2018). As expected, doxycycline induced an increase of monomeric α -syn level, which was not affected by treatment with the CBSE both at 48 h and 72 h (Fig. 7A–C). Although the extract induced the phosphorylation of the energy sensor AMPK, no change of either pULK1 or p62 level was observed, suggesting that the CBSE does not activate the autophagic pathway in neuroblastoma cells (Fig. 7A and B).

One of the key processes for the pathogenesis of Parkinson's disease is the assembly of toxic oligomeric species of α -syn. Then, since we have shown that the CBSE is able to bind and inhibit α -syn aggregation (Fig. 4), the level of α -syn oligomers was investigated in neuroblastoma cells treated with the CBSE. Strikingly, a significant reduction of α -syn oligomers, as well as of intracellular aggresomes were observed upon CBSE treatment (Fig. 7D and E). These data suggest that the CBSE prevents the formation of toxic oligomeric species and not their clearance through the autophagic degradation.

4. Discussion

Accumulation of pathological protein aggregates is associated with a wide range of human diseases. Among these, aggregates of β -amyloid, p-tau or α -syn in the brain are found in patients with Alzheimer's and Parkinson's diseases and correlate with the progression of neurodegeneration (Wilson et al., 2023). Considering the consequent

Table 4
List of proteins by LC-MS^E in cocoa extract with the corresponding peptides.

Entry (Database)	Accession	protein.Description	protein score	MW	protein Matched Products	protein matched Peptides	Cov (%)	Peptide Intensity Sum	Sequence	MH + Error (ppm)	Score
ASP_THECC (Uniprot)	P32765	21 kDa seed protein	615	24438	30	7	30.77	203620	(R)HSDDDGQIR(L)	0.9339	6.6629
									(R)SDDLNGTPVIFSNADSKDDVVR(V)	0.4055	5.9953
									(K)DDVVR(V)	−2.412	5.6277
									(R)VSTDVNIEFVPIR(D)	2.7383	5.3816
									(R)RSDLDNGTPVIFSNADSK(D)	−2.3835	5.2396
									(R)LDNYDNSAGK(W)	−3.3845	5.1266
									(R)ATGQSCPEIVVQR(R)	1.7676	4.8949
A0A061EM85_THECC (Uniprot)	A0A061EM85	Vicilin-A_ putative	200	66198	37	9	17.67	75441	(R)QDRR(E)	−1.8736	6.231
									(K)EQER(G)	−0.3204	5.622
									(K)ELSFVGVP SK(L)	1.3752	5.6153
									(R)SEEEEGQQR(N)	−1.5498	5.3042
									(R)	3.0933	5.2171
									GTVVSV PAGSTVYVVSQDNQEK(L)		
									(R)EQEESEEEETFG EFQQVK(A)	1.6377	5.1098
									(R)QEEEELQR(Q)	−3.0807	4.8992
									(R)EKLEEELEEQR(G)	−1.8284	4.7921
VCL_THECC (Uniprot)	Q43358	Vicilin	167	61483	32	8	17.33	55233	(R)QDRR(E)	−1.8736	6.231
									(K)EQER(G)	−0.3204	5.622
									(R)SEEEEGQQR(N)	−1.5498	5.3042
									(R)	3.0933	5.2171
									GTVVSV PAGSTVYVVSQDNQEK(L)		
									(R)EQEESEEEETFG EFQQVK(A)	1.6377	5.1098
									(R)QEEEELQR(Q)	−3.0807	4.8992
									(R)EKLEEELEEQR(G)	−1.8284	4.7921
									(K)LTIAVLALPVNSPGK(Y)	−0.6553	4.6108
A0A061GTA7_THECC (Uniprot)	A0A061GTA7	Uncharacterized protein	117	10317	4	1	7.53	5242	(K)IEEHQSY(−)	−0.1395	5.2605
A0A061GZ27_THECC (Uniprot)	A0A061GZ27	Putative plant transposon protein domain-containing protein	100	12772	6	1	13.76	6904	(M)NQCHFSEVSCSICQK(V)	0.6846	4.8622
CAA44494.1 (NCBI)	CAA44494.1	vicilin_ partial	1582	54423	81	13	23.7	503177	(R)EQEESEEEETFG EF(−)	0.2798	7.8005
									(R)QDRR(E)	−1.8736	7.1311
									(R)REQEESEEEETFG EF(−)	−0.781	6.6848
									(R)NNPY YFPK(R)	0.4712	6.3261
									(R)DEEGNFK(I)	−1.2679	6.2127
									(R)SEEEEGQQR(N)	−1.5498	6.203
									(K)ESYNVQR(G)	2.3721	6.1794
									(R)	3.0933	6.1217
									GTVVSV PAGSTVYVVSQDNQEK(L)		
									(K)EQER(G)	−1.8439	5.9501
									(R)QEEEELQR(Q)	−3.0807	5.7974
									(R)EKLEEELEEQR(G)	−1.8284	5.6899
									(K)LTIAVLALPVNSPGK(Y)	−0.6553	5.5105
									(K)LEEILEEQR(G)	3.2998	5.3213
									(R)EQEESEEEET(T)	1.8693	0
									(R)EQEESEEEET(F)	1.9878	0
									(R)EQEESEEEETFG EF(F)	−1.9716	0
									(R)EQEESEEEETFG EF(−)	0.1171	0
									(R)EQEESEEE(E)	−2.1412	0

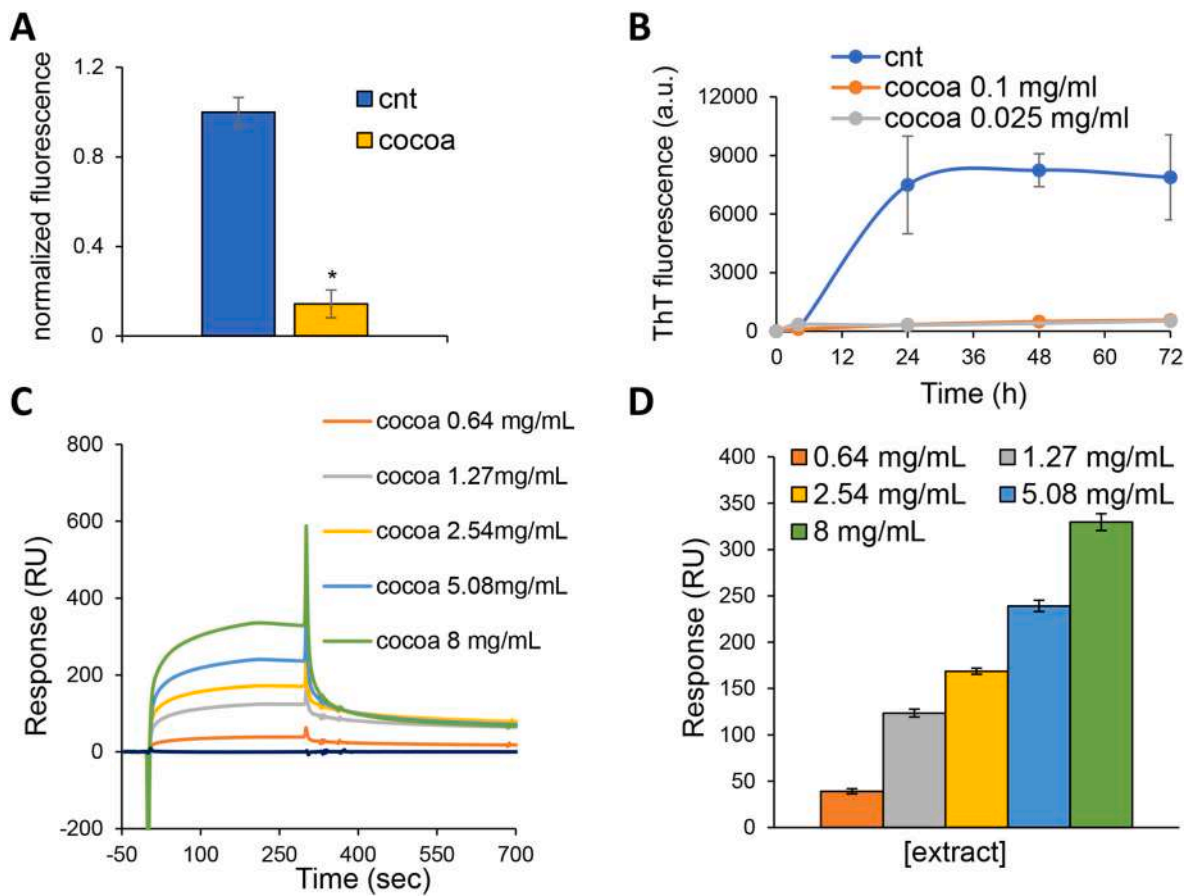


Fig. 4. The CBSE inhibits α -syn aggregation. (A) Fluorescence intensity obtained from flow cytometry analysis of aggregates of yeast wt [α -syn] cells in medium containing 2% glucose in the absence (cnt) or presence of 0.2% CBSE. (B) α -syn aggregation process, followed by ThT fluorescence, in the absence (cnt) or presence of the CBSE (0.1 and 0.025 mg/ml). (C) SPR sensorgrams of CBSE at different concentrations (8 mg/mL, 5.08 mg/mL, 2.54 mg/mL, 1.27 mg/mL, 0.64 mg/mL) display binding toward α -syn on CM5 sensor chip surface. (D) The CBSE shows dose-dependent binding activity to α -syn protein. Results are reported as the mean $\pm$ standard deviation of at three independent experiments.

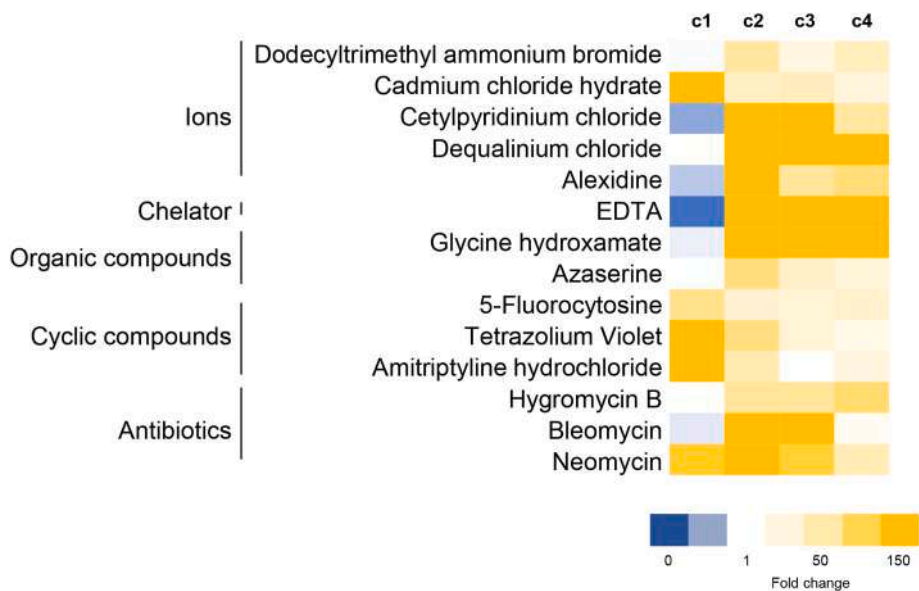


Fig. 5. Drug sensitivity upon CBSE treatment. Heatmap of sensitivity of wt [α -syn] cells to selected drugs in the presence of CBSE compared to the control condition, measured by Biolog OmniLog Phenotype MicroArray. Fold changes (treated/cnt) in y-maximum value were calculated and compounds were selected when fold change was >5 or <0.2 in at least three concentrations. Colour scale indicates increased resistance (yellow) or decreased resistance (blue) after 72 h growth.

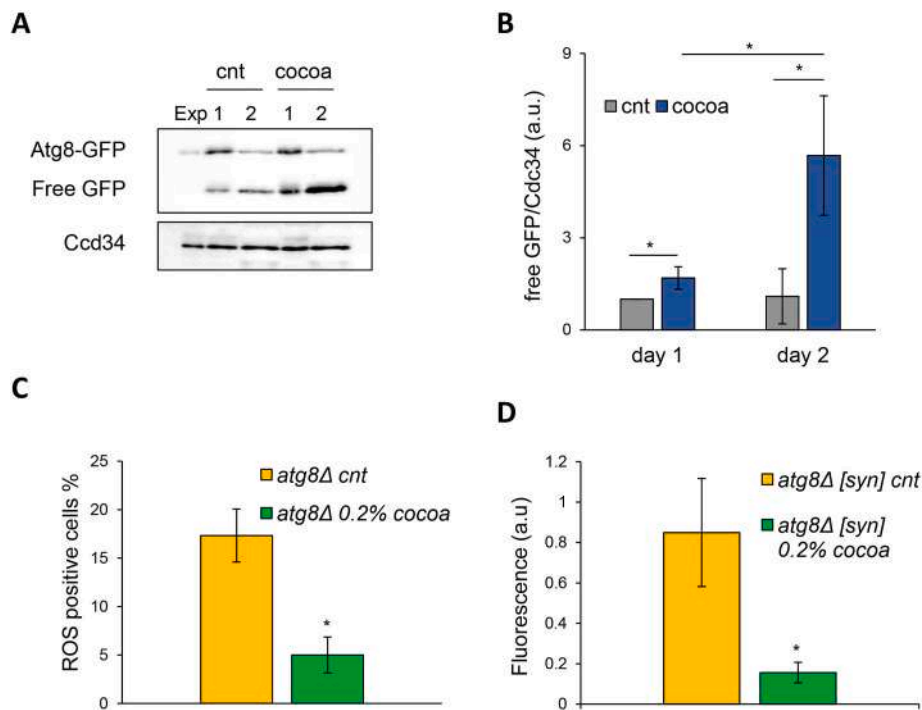


Fig. 6. The CBSE activates autophagy in yeast. (A) Western analysis using anti-GFP antibody on total extracts from *wt[α-syn][Atg8-GFP]* cells treated with 0.2% CBSE for 1 and 2 days. Anti-Cdc34 antibody was used as loading control. (B) Quantification of free GFP of three independent experiments performed as in (A). (C) ROS content of *atg8Δ[α-syn]* cells in medium containing 2% glucose in the absence (cnt) or presence of 0.2% CBSE for 24 h. (D) Fluorescence intensity obtained from flow cytometry analysis of aggregates of yeast *atg8Δ[α-syn]* cells in medium containing 2% glucose in the absence (cnt) or presence of 0.2% CBSE for 24 h. Results are reported as the mean ± standard deviation. **p* < 0.05.

induction of neurotoxicity and neuronal loss, there is an increasing interest in the study of secondary metabolites, such as terpenes, flavonoids and phenols, able to inhibit protein aggregation and/or stimulate the clearance of these toxic aggregates. In recent years, the protective effects of a number of bioactive compounds have been highlighted on a wide variety of diseases, among which neurodegenerative ones (Moukham et al., 2024; Pohl et al., 2018).

In the context of the research of still unexplored bioactive molecules, nature is an unlimited reservoir for the discovery of novel therapeutics not only against broad-spectrum diseases, but also for applications in the cosmetic and food industries. In line with this, the valorization of by-products generated by the conventional linear food industry is an emerging strategy to identify new potential useful bioactivities and to reduce food waste.

In the present study we have employed this approach for the utilisation of cocoa bean shells, a by-product typically discarded during the roasting process of cocoa beans (Pagliari et al., 2022).

Here we show that CBSE, obtained by a green extraction and rich in amino acids, organic acids and methylxanthines (Tables 2 and 3), strongly improves yeast longevity and reduces the toxicity of human α-syn, by decreasing intracellular protein aggregates (Figs. 1, 2, 4 and 7). Different eukaryotic models, yeast cells and a neuroblastoma cell line, were used to verify the bioactivity of the CBSE. Although the effects identified are not completely superimposable in the two systems, this approach highlights the importance of using multiple models to better identify all the biological pathways that contribute to the neuroprotective activity of natural compounds. Indeed, while CBSE stimulates autophagy in the yeast model of PD (Fig. 6), this is not the case in neuroblastoma cells, even if the stress responsive kinase AMP-activated protein kinase (AMPK) is activated (Fig. 7). We consider this result particularly relevant because energy metabolism defects are commonly described in neurodegeneration and several studies reported the implication of AMPK in various signalling pathways that are involved in the progression of neurodegeneration (Domise and Vingtdoux, 2016). Thus,

the stimulation of both autophagy and AMPK signalling appears to represent as two complementary responses induced by CBSE which together contribute to protect the cell from the toxicity of misfolded proteins.

Results obtained by surface plasmon resonance (SPR) assays indicate also that CBSE binds α-syn protein in a concentration-dependent manner, supporting a direct association of the cacao-bean shell extract with monomers of α-syn by preventing its aggregation into toxic oligomers and amyloid fibrils (Fig. 4B–D). What remains to be elucidated are the specific compounds exerting this role. Although caffeine and theobromine, as well as the very small fraction of proteins contained in the extract, do not have any effect on yeast longevity and ROS content (Fig. 3), the methylxanthines together show a partial inhibitory effect on the aggregation of α-syn *in vitro* (Fig. S2A). The protective functions of methylxanthines are well documented, since they reduce inflammation and preserve cognitive functions (Sánchez et al., 2023; Dong et al., 2020; Eskelinen and Kivipelto, 2010; Valada et al., 2022). Then, the inactivity of both caffeine and theobromine in our yeast model could suggest a possible synergistic or combined role of different molecules within the extract.

The promising results obtained in this study represents the first step for the development of the CBSE as a neuroprotective agent. In order to address if the reported bioactivity may be relevant also under physiological conditions, CBSE bioavailability, absorption rate and metabolism need to be further investigated both in animal models and in clinical studies. In addition, to reach the brain, the active compounds of CBSE have to pass through the gastrointestinal tract and to cross the blood brain barrier, without losing any efficacy. Remarkably, toxicology studies performed in mice, both in acute and sub-chronic assays, indicate that the oral administration of both cacao shell flour or extracts is safe, without significant histopathological alterations (Gil-Ramírez et al., 2024). In line with this, an interesting approach of encapsulation has been reported to enrich chocolate bars with phenolic antioxidant compounds extracted from cocoa bean shells (Grassia et al., 2021).

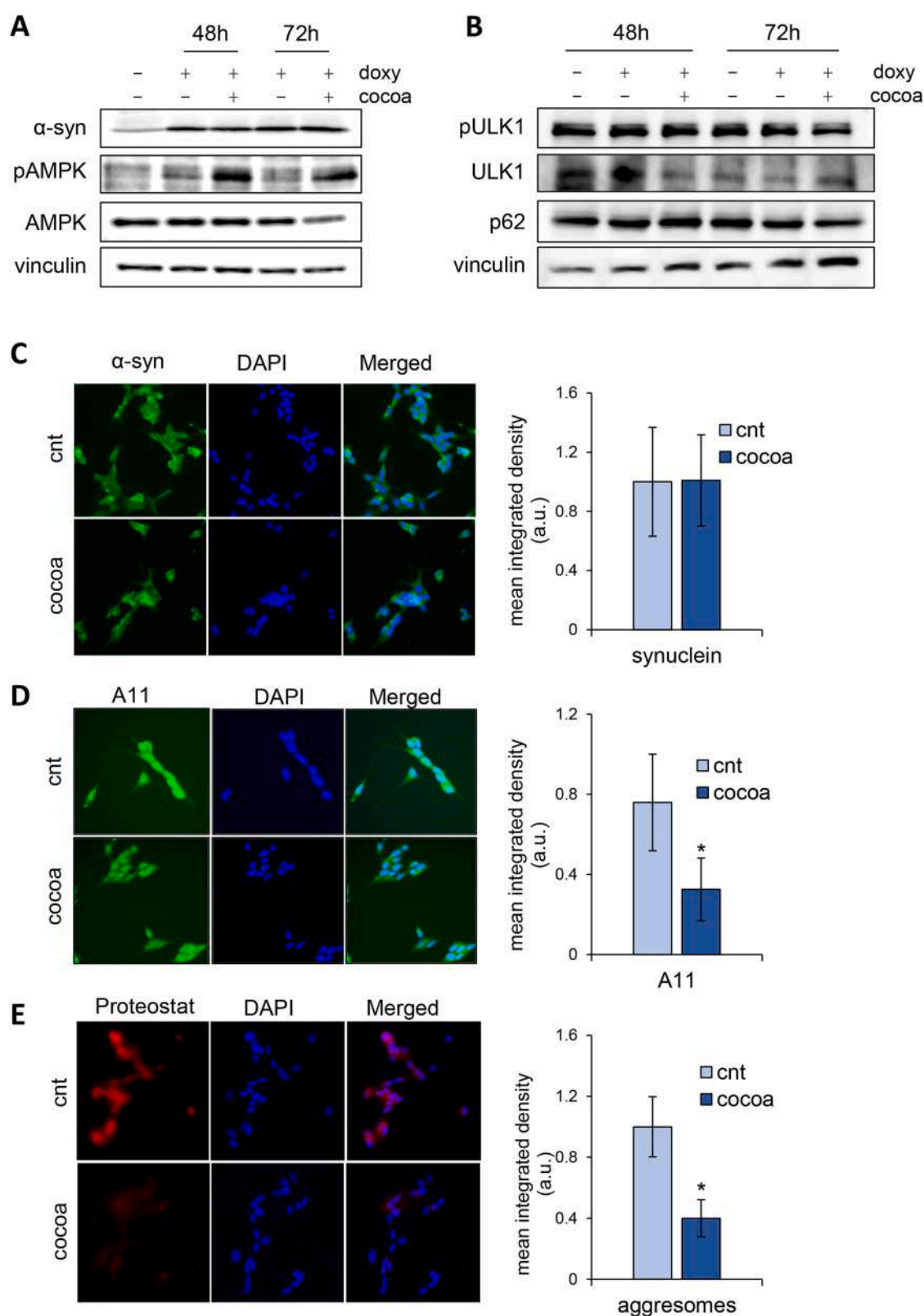


Fig. 7. The CBSE reduces α-syn toxicity in neuroblastoma cells. (A–B) Western blot analysis using anti-α-syn, anti-phospho-T172-AMPK, anti-AMPKα, anti-vinculin antibodies (A) and anti-p62/SQSTM1 anti-phospho-Ser555-ULK1, anti-ULK1 and anti-vinculin antibodies (B) on protein extracts from SH-SY5Y pTet-SNCA-FLAG cells untreated, treated with doxycycline or treated with doxycycline and 150 μg/mL CBSE for 48 and 72 h. (C) Representative immunofluorescence (60x) images of SH-SY5Y cells treated with doxycycline (Doxy) alone and in combination with 150 μg/mL CBSE (Doxy + Cocoa) for 48 h, immunolabeled with anti-α-syn antibody (C), A11 anti-oligomer antibody (D), and Proteostat R dye (E). Nuclei were stained by DAPI (Blue). Histograms represent mean ± standard deviation of cell fluorescence quantified with the ImageJ software.

Altogether, our results along with these studies support the potentiality of this waste matrix upcycling as a safe and neuroprotective ingredient for functional foods opening the route to using cocoa bean shell extract, in the form of nutraceutical, as a direct anti-aggregant agent against intracellular protein misfolding and toxicity.

5. Conclusions

During industrial food processing, a large amount of waste is produced. The cocoa bean shell is a valuable by-product of the chocolate industry, thus its valorization may reduce the environmental impact and provide economic benefits. Nowadays, the cocoa bean shells are mainly used for feedstuff, as biofuel and in the agriculture and food industry (Panak Balentić et al., 2018). Here we propose a new valorization approach for this waste product, since through a sustainable, rapid, and cost-effective procedure, we have developed an extract with anti-aggregant properties. Although our study represents the first step towards the use of CBSE as a protective agent, both preclinical and clinical data are still required in the perspective of the development of novel sustainable treatments to prevent neurodegeneration.

CRediT authorship contribution statement

Farida Tripodi: Writing – original draft, Conceptualization, Writing – review & editing. **Alessia Lambiase:** Methodology, Investigation, Formal analysis, Data curation, Visualization. **Hind Moukham:** Methodology, Investigation, Formal analysis, Data curation, Visualization. **Giorgia Spandri:** Methodology, Investigation, Formal analysis, Data curation, Visualization. **Maura Brioschi:** Methodology, Investigation, Formal analysis, Data curation, Visualization. **Ermelinda Falletta:** Methodology, Investigation, Formal analysis, Data curation, Visualization. **Annalisa D’Urzo:** Methodology, Investigation, Formal analysis, Data curation, Visualization. **Marina Vai:** Conceptualization, Writing – review & editing. **Francesco Abbiati:** Methodology, Investigation, Formal analysis, Data curation, Visualization. **Stefania Pagliari:** Methodology, Investigation, Formal analysis, Data curation, Visualization. **Andrea Salvo:** Methodology, Investigation, Formal analysis, Data curation, Visualization. **Mattia Spano:** Methodology, Investigation, Formal analysis, Data curation, Visualization. **Luca Campone:** Methodology, Investigation, Formal analysis, Data curation, Visualization, Funding acquisition, Conceptualization, Writing – review & editing. **Massimo Labra:** Funding acquisition. **Paola Coccetti:** Writing – original draft, Funding acquisition, Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

The authors have declared no conflict of interests.

Acknowledgments

This work was supported by the project “ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON Foods” (CUP:H43C22000820001) of the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.3—Research funded by the European Union—NextGenerationEU.

This work was also supported by the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.4—call for tender No. 3138 of December 16, 2021, rectified by Decree No. 3175 of December 18, 2021 of the Italian Ministry of University and Research funded by the European Union—NextGenerationEU; Award Number: Project code CN_00000033, Concession Decree No. 1034 of June 17, 2022. Adopted by the Italian Ministry of University and Research, CUP, H43C22000530001, Spoke 6, Project title “National Biodiversity Future Center—NBFC”.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crfs.2024.100888>.

Data availability

Data will be made available on request.

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Article

Exploring Influence of Production Area and Harvest Time on Specialized Metabolite Content of *Glycyrrhiza glabra* Leaves and Evaluation of Antioxidant and Anti-Aging Properties

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Citation: Docimo, T.; Celano, R.; Lambiase, A.; Di Sanzo, R.; Serio, S.; Santoro, V.; Coccetti, P.; Russo, M.; Rastrelli, L.; Piccinelli, A.L. Exploring Influence of Production Area and Harvest Time on Specialized Metabolite Content of *Glycyrrhiza glabra* Leaves and Evaluation of Antioxidant and Anti-Aging Properties. *Antioxidants* **2024**, *13*, 93. <https://doi.org/10.3390/antiox13010093>

Academic Editor: Susana M. Cardoso

Received: 18 December 2023

Revised: 3 January 2024

Accepted: 10 January 2024

Published: 12 January 2024

Correction Statement: This article has been republished with a minor change. The change does not affect the scientific content of the article and further details are available within the backmatter of the website version of this article.

Abstract: Calabrian *Glycyrrhiza glabra* is one of the most appreciated licorice varieties worldwide, and its leaves are emerging as a valuable source of bioactive compounds. Nevertheless, this biomass is usually discarded, and its valorization could contribute to boost the economic value of the licorice production chain. In this study, the effects of production area and harvest time on the specialized metabolite content of *G. glabra* leaves (GGL) and also the antioxidant and anti-aging properties are evaluated to explore the potential of this untapped resource and to select the most optimal harvesting practices. GGL exhibited high levels of specialized metabolites (4–30 g/100 g of dry leaf) and the most abundant ones are pinocembrin, prenylated flavanones (licoflavanone and glabranin), and prenylated dihydrostilbenes. Their levels and antioxidant capacity in extracts are influenced by both production area and harvest time, showing a decisive role on specialized metabolites accumulation. Interestingly, GGL extracts strongly attenuate the toxicity of α -synuclein, the intracellular reactive oxygen species (ROS) content, and cellular senescence on *Saccharomyces cerevisiae* expressing human α -synuclein model, showing great potential to prevent aging and age-related disorders. These results provide insights into the phytochemical dynamics of GGL, identifying the best harvesting site and period to obtain bioactive-rich sources with potential uses in the food, nutraceutical, and pharmaceutical sectors.

Keywords: *Glycyrrhiza glabra* leaves; untapped resource; prenylated flavanone; prenylated dihydrostilbene; sustainable agriculture; bioeconomy; *Saccharomyces cerevisiae*; human α -synuclein; neurodegenerative disorders



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1. Introduction

Glycyrrhiza species are flowering, herbaceous, perennial plants of the bean family Fabaceae, cultivated worldwide but native to Europe, Asia, and most of North America. The genus *Glycyrrhiza* comprises approximately thirty species, among which the European *G. glabra*, the Chinese *G. uralensis* and *G. inflata*, and the American *G. lepidota* are the most studied [1]. Roots and rhizomes of *G. glabra* (licorice) stand out for their well-documented

and widely appreciated distinct characteristics related to botanical, culinary, and pharmacological properties [2]. Notably, licorice has a long history of therapeutic applications, including expectorant, carminative, anti-inflammatory, antiulcerous, antibacterial, antifungal, antiviral, antiallergic, and immunostimulant activities. Besides, it is also used as natural sweetener and flavor additive in foods, beverages, functional foods, and food supplements [2,3]. However, excessive consumption of licorice can lead to some side effects such as hypertension and edema [4]. A particularly distinctive feature of Calabrian *G. glabra* is its lower content of glycyrrhizin [5], which is the compound responsible for the sweet taste of roots but also for the hypertensive effects. The amount of this compound tends to vary depending on the root harvesting period, but also on factors such as soil conditions, climate, and agricultural practice. Indeed, the unique climatic and environmental conditions of the Calabrian region in Southern Italy have been shown having a deep impact on the distinctive aroma and sweetness of Calabrian licorice [5], which holds the “Protected Designation of Origin” (PDO) status.

Besides roots, this perennial plant has a vigorous vegetative biomass, which is emerging as a valuable source of bioactive compounds positively affecting human well-being. Nevertheless, during the blossom season, this biomass is an untapped resource, and its valorization could contribute indirectly to boost the economic value of licorice cultivation. Current harvesting practices of PDO licorice occur during the fall foliage season, when any leaves present are often discarded or left to decompose on the soil, resulting in poor quality and quantity of this biomass. Until now, root harvesting has prioritized yield over chemical composition, and a limited exploration into the influence of harvest times and environmental factors on the chemical and biological activity of root extracts has been reported. A recent study highlighted the impact of different harvesting periods on the chemical profile and bioactivity of *G. glabra* roots, envisaging alternative harvest times [6–9]. Regarding leaves, no studies have been conducted to investigate the effects of growth stage and environment on the phytochemical content and biological activities of *G. glabra* leaves. Knowledge of the influence of these factors on metabolic leaf content and the related potentiality might be crucial for envisaging either the most optimal harvesting practices for whole plant valorization or exploration of the potential uses of this untapped resource. These aspects could strongly improve the economic value of the Calabrian licorice production chain. Indeed, various untapped plant materials are currently being investigated as potential sources of bioactive compounds for the production of nutraceuticals and functional ingredients of food. Recent studies indicate that leaves of well-known crops and wild growing plants are unconventional and valuable source of functional and bioactive compounds [10,11].

Glycyrrhiza leaf extracts have been assayed for various biological activities, including antiproliferative, antimicrobial, antioxidant, anti-inflammatory, and α -glucosidase-inhibiting activities [12–16]. Recently, our UHPLC-HRMS profiling of aboveground and belowground tissues of Calabrian *G. glabra* revealed a different distinctive chemical composition of the leaves compared to the roots [17]. In agreement with [14], flavanones were found to be the most abundant class of secondary metabolites, with pinocembrin, licoflavanone, and glabranin as major compounds. The second distinctive class of phytochemicals of *G. glabra* leaves is represented by prenylated dihydrostilbenes, which together with prenylated flavanones represent distinctive chemical markers of leaves [17]. Among the chemical modifications of specialized metabolites, prenylation has the greatest impact on structural diversity and on biological activity. Compared with their non-prenylated counterparts, prenylation confers higher lipid solubility, higher capability to penetrate the membranes, and higher potential to interact with different cellular targets [18]. For this reason, prenylated phenolic compounds are promising molecules as drugs for their wide pharmacological spectrum of activities [19]. As an example, many plants within medicinal and food resources—such as *Morus alba*, *Humulus lupulus*, *Glycine max*, *Ficus carica*, and *Cannabis sativa*—accumulate prenylated phenols, and their extracts have been exploited in oncotherapy [20], metabolic disorders [21], and cardiovascular activities [22]. Regarding

Glycyrrhiza metabolites, prenylated dihydrostilbenes and flavonoids from *G. uralensis* leaf extracts were investigated as therapeutic agents in liver fibrosis [12]. Recently, the potential of prenylated phenolics from Mulberry leaves has been also exploited for neuroprotective activity [20]. In the field of neurodegenerative diseases, accounting for a large and increasing health burden worldwide, drug discovery and development are urgently needed.

Neurodegenerative diseases, characterized by aberrant aggregates of the presynaptic protein α -synuclein, are collectively referred to as synucleinopathies, the second most common group of neurodegenerative diseases [23]. Despite the advances in the study of these pathologies, the detailed molecular mechanism of neuronal degeneration is still largely unknown. Several studies underline the relevant role of cellular models for a better understanding of the molecular regulation of synucleinopathies [24]. Many different models using yeast strains overexpressing human genes associated with diseases like Alzheimer's disease (AD), Parkinson's disease (PD), or Huntington's disease (HD) have also been exploited in the last decades. Among them, one of the most studied yeast models concerns the expression of α -synuclein (α -syn) [25–27], a presynaptic protein whose alteration is associated with PD [23,28,29].

As *G. glabra* leaves (GGL) are a valuable source of beneficial compounds, to harness their potential, in this study we investigated the effect of the geographical area of cultivation and harvesting period on the profile of GGL specialized metabolites and their biological activities. To do so, a quantitative profiling of the main GGL compounds from *G. glabra* cultivated in three typical PDO production areas of the Calabria region and harvested during three developmental stages was performed. The influence of these factors on metabolite content was statistically determined to distinguish the patterns for cultivation area and for harvesting times. Afterwards, the in vitro antioxidant capacity of the GGL extracts and their chemical markers were evaluated, as well as their anti-aging and antioxidant properties, on a yeast model of *Saccharomyces cerevisiae* expressing human α -synuclein.

2. Materials and Methods

2.1. Chemical and Standards

Analytical grade ethanol (EtOH), MS grade formic acid (HCOOH), 6-hydroxy-2,5,7,8-acid tetramethylcroman-2-carboxylic acid (Trolox), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and the reference standards of isoquercitrin and rutin were provided by Merck Chemicals (Milan, Italy). MS grade acetonitrile and water were purchased from Romil (Cambridge, UK). Ultrapure water (18 M Ω) was prepared using a Milli-Q purification system (Millipore, Bedford, TX, USA).

Pinocembrin (L29), licoflavanone (L36), glabranin (L46), dihydro-3,3',4'-trihydroxy-5-O-prenylstilbene (L33), and dihydro-3,5,4'-trihydroxy-4,5'-diprenylstilbene (L44) were purified by RP-HPLC prepared from *G. glabra* exudate and characterized by 1D- and 2D-NMR (purity $\geq$ 96% from HPLC). Finally, vicianin-2 was previously isolated from natural sources [30].

2.2. Samples

Leaves of *G. glabra* var. *typica* (GGL) were kindly supplied by Nature Med S.r.l. (Castrovillari, CS, Italy) of the Consortium for Protected Designation of Origin "Liquirizia di Calabria PDO". The cultivation and harvesting were conducted according to the disciplinary of the "Liquirizia di Calabria PDO". Plant materials were collected from 3–4 year old plants in three typical PDO production areas of Calabria region: Villapiana (GGL-A), (Cosenza, Italy; coordinates (lat, long): 39.810000, 16.470000; elevation: 16 m), Castrolibero (GGL-B) (Cosenza, Italy; coordinates (lat, long): 39.322460, 16.192634; elevation: 290 m), and Scandale (GGL-C) (Crotone, Italy; coordinates (lat, long): 39.152599, 17.011761; elevation: 63 m). Leaf samples were harvested during three growing phases of 2019: Harvesting Time 1 (HT1), corresponding to the pre-flowering stage, in early May; HT 2, corresponding to the flowering stage, in mid-June; and HT 3, corresponding to the senescence stage (root

collection), in late October. For each geographical area at each HT, three representative samples (about 0.5 kg each replicate) were randomly collected.

Drying of leaf materials (ventilated oven with a temperature of 50 °C) was carried out by Nature Med Seral. using standardized procedures in accordance to set rules of PDO disciplinary. Dried leaf samples were ground using a knife mill Grindomix GM 200 (Retsch, Haan, Germany) operating at intervals of brief cycles. The resulting ground materials were sieved through a test sieve with a range of 300–600 µm to obtain a sample with a homogeneous particle size distribution.

2.3. Ultrasound-Assisted Extraction (UAE)

Exhaustive extracts of GGL samples from each biological triplicate were prepared according to the methodology proposed by Celano et al. [17]. Briefly, 500 mg of homogenized samples was extracted in duplicate with 10 mL of aqueous ethanol (70% *v/v*) for 30 min at 30 °C in a thermostat-controlled ultrasound bath (Labsonic LBS2, Treviglio, Italy). The extraction process was repeated three times with fresh solvent. The extracts were pooled and diluted with distilled water to 50 mL, then filtered with 0.45 µm regenerated cellulose filters (Chromafil Xtra RC-20/13, Delchimica, Italy) for the chromatographic analysis.

For ABTS assay and biological assay, a triplicate solution of each GGL sample was freeze-dried (freeze dryer Alpha 1–2 LD, Christ, Germany) after removal of the organic solvent under vacuum at 40 °C in a rotary evaporator (Rotavapor R-200, Buchi Italia s.r.l., Cornaredo, Italy). The dried extracts were properly dissolved before assays according to procedures reported in Sections 2.5 and 2.6.

2.4. Quantitative Analysis

Quantitative analysis of GGL extracts was performed using a Platin Blue UHPLC system (Knauer, Labservice Analytica, Bologna, Italy) consisting of two ultra-high-pressure pumps, an autosampler, a column temperature manager, and a diode array detector. UHPLC separation was performed using a previously developed method [17]. Briefly, the chromatographic separations were performed using a Kinetex C18 column (2.1 × 100 mm, 1.6 µm; Phenomenex, Bologna, Italy) protected by a C18 Guard Cartridge (2.1 mm I.D.) and thermostated at 25 °C. A binary gradient of H₂O and MeCN, both containing 0.1% of formic acid, at a flow rate of 500 µL min^{−1} was employed. After each injection (5 µL), the column was washed with 98% B for 4 min and re-equilibrated (4 min). UV spectra were acquired in the range of 200–600 nm and three wavelengths were selected for the detection of target analytes based on the maximum absorbance of pure compounds (Figure S1): 220 (prenylated dihydrostilbenes), 290 (flavanones), and 360 (flavones-C-glycosides and flavonols-O-glycosides) nm.

The external standards method was employed to determine the levels of main compounds in the GGL extracts. Stock solutions of reference standards (vicenin-2, rutin, isoquercitrin, pinocembrin, licoflavanone, glabranin, dihydro-3,3',4'-trihydroxy-5-O-prenylstilbene (L33), and dihydro-3,5,4'-trihydroxy-4,5'-diprenylstilbene (L44)) were prepared in MeOH at a concentration of 5 mg mL^{−1} and stored at −20 °C. A mixture of reference standards was prepared at a concentration of 1 mg mL^{−1}, and the calibration levels were prepared by appropriate serial dilution with MeOH/H₂O, 1:1 *v/v*, and analyzed in triplicate. The linearity of the calibration curves was evaluated in the concentration range of 6.25–100 µg mL^{−1} for vicenin 2, rutin, and isoquercitrin, and 12.5–200 µg mL^{−1} for the remaining reference compounds. The regression curves were tested with the analysis of variance (ANOVA), and the linear model was found to be appropriate over the tested concentration range (*R*² values > 0.999). The samples were analyzed after appropriate dilutions to fall within the dynamic calibration range. The quantification of compounds of GGL extracts, for which no reference standards were available, was determined using the calibration curves of standards belonging to the same class of secondary metabolites (similar chromophore). The amount of the compounds was expressed as mg per g of dry leaf material (mg/g DM) ± standard deviation (*n* = 3 biological replicates).

2.5. ABTS Assay

The antioxidant activities of the exhaustive extracts and the major GGL compounds, vicenin-2, rutin, isoquercitrin, pinocembrin, licoflavanone, glabranin, dihydro-3,3',4'-trihydroxy-5-O-prenylstilbene (L33) and dihydro-3,5,4'-trihydroxy-4,5'-diprenylstilbene (L44), were evaluated using ABTS assays adapted for use in 96-well plates, as reported in [31]. A microplate spectrophotometer reader Multiskan Go (Thermo Scientific, Milan, Italy) was employed for the assays. GGL dried extracts were dissolved in methanol at a concentration of 1 mg/mL and then diluted with 5 mM PBS (pH 7.4) to provide approximately 20–80% control absorbance. ABTS results were expressed as Trolox equivalent antioxidant capacity (TEAC) per g of extract (mmol TE/g) or per mmol of pure compound (mmol TE/mmol). In the ABTS assay, the curves of Trolox, pure compounds, and extracts were obtained by plotting concentration (mM for Trolox; standards and mg g⁻¹ for leaf extracts) against the average % inhibition of radical absorbances.

2.6. Yeast Strains and Media

The yeast strain used to evaluate the biological activity of dried GGL extracts is BY4742 MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0 [pYX242-SNCA] overexpressing human α -synuclein. Cells were grown at 30 °C in minimal medium, containing 2% glucose as a carbon source and 0.67% yeast nitrogen base without amino acids, and supplemented with 50 mg/L of required amino acids and bases for which the strain was auxotrophic, with constant shaking (120 rpm). GGL extracts were resuspended in 150 μ L of EtOH 100%, vortexed, incubated in the thermomixer at 25 °C for 1 h, sonicated for 20 min, dissolved in the liquid medium at a concentration of 0.2% of the yeast strain, and filtered through 0.22 μ m filters, as previously reported [28,29].

2.7. Chronological Lifespan Experiments (CLS)

Yeast cells were grown in liquid medium until the mid-late exponential phase, centrifuged (13,000 rpm, 5 min), and then inoculated at 0.150 OD/mL (Optical Density/mL) in flasks containing medium in the absence (negative control) or presence of the extracts (0.2% concentration). Survival was assessed by propidium iodide staining (PI) at the indicated time points with a Cytoflex cytofluorimeter (Beckman Coulter, Milan, Italy) and analyzed with the Cytoflex software (version 2.6.0.105), as previously reported [28,29].

2.8. Analysis of Reactive Oxygen Species (ROS) Levels

Analysis of cellular superoxide was performed by DHE (dihydroethidium) staining. Cells were collected after 24 h treatment with 0.2% GGL extracts, and 0.2% were resuspended in PBS and stained with 5 μ g/mL for 10 min. FACS analyses were performed with Cytoflex cytofluorimeter (Beckman Coulter, Milan, Italy) and analyzed with the Cytoflex software (version 2.6.0.105), as previously reported [28,29].

2.9. Statistical Analysis

The quantitative data and TEAC values were subjected to analysis of variance (ANOVA) using Statgraphic Centurion 18 software from Statgraphics Technologies (Arezzo, Italy). A two-way ANOVA (3 production areas $\times$ 3 HTs) was applied to each GGL marker, the total of metabolite classes, and TEAC values. The means were separated using the Tukey HSD test only when the F-test for factors and their interaction were significant at the p -value < 0.05 probability level.

With regard to the CLS assays and analysis of ROS levels, experiments were performed in duplicate. The results are expressed as mean $\pm$ SD (standard deviation). Results were compared using the two-sided Student's t -test. Differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Quantitative Profile of *Glycyrrhiza glabra* Leaves

Our recent study, conducted on *G. glabra* var. *typica* cultivated for the production of PDO certified Calabrian licorice, has revealed that the leaves represent a rich source of unusual bioactive compounds with potential uses in the nutraceutical, pharmaceutical, and cosmetic sectors [17].

In the current study, the quantitative profiles of *G. glabra* leaves (GGL) from different production areas of Calabria and collected at different harvest times were determined by UHPLC-UV analysis to investigate the influence of cultivation site and phenological stages on the phytochemical content of this untapped resource. GGL samples were harvested from three typical areas dedicated to the production of PDO Calabrian licorice: Villapiana (GGL-A), located near the Ionian Sea; Castrolibero (GGL-B), located in the hinterland of the Tyrrhenian coast; and Scandale (GGL-C), located further south in the hinterland of the Ionian coast. From each production area, the leaf samples were collected at three different harvesting times corresponding to three growing stages: HT1 (pre-flowering stage), HT2 (flowering stage), and HT3 (senescence stage, corresponding to the root collection) [32].

Untargeted UHPLC-HRMS analysis [17] indicated that the qualitative profiles of investigated GGL extracts were comparable among production areas and harvesting times. The main compounds detected in UHPLC-PDA profiles (Figure 1) are as follows: the flavanones pinocembrin (L29), licoflavanone (L36), 8/6-prenylnaringenin (L35), glabranin (L46), and glabranin isomer (L48); the flavone C-glycosides vincenin 2 (1) and iso/shaftoside (2); the flavonol O-glycosides rutin (L11), isoquercitrin (L12), and astragalin (L17); and the prenylated dihydrostilbenes dihydro-3,5,3',4'-tetrahydroxy-5'-prenylstilbene (L28), dihydro-3,3',4'-trihydroxy-5-O-prenylstilbene (L33), dihydro-3,5,3',4'-tetrahydroxy-4,5'-diprenylstilbene (L41), and dihydro-3,5,4'-trihydroxy-4,5'-diprenylstilbene (L44). These compounds were assumed as GGL markers and quantified in all GGL samples by UHPLC-UV analysis and external calibration method (Table S1).

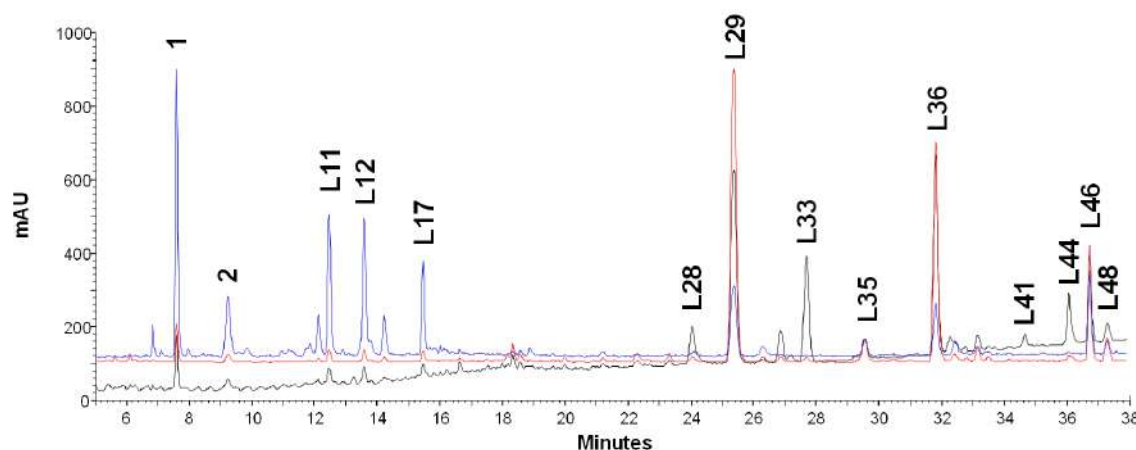


Figure 1. A representative UHPLC-UV profile of *G. glabra* leaves (GGL-B2): black line, 220 nm; red line, 290 nm; blue line, 360 nm.

As reported in Table S1, the leaves of Calabrian *G. glabra* exhibited high levels of specialized metabolites, with a total content ranging from 4 to 30 g/100 g of dry leaf DM. The changes in the level of abundance of each metabolite class and the most abundant compounds (pinocembrin, licoflavanone, glabranin, and stilbene L33) are shown in Figure 2. Overall, the most abundant class of secondary metabolites was represented by flavanones, constituting 33–61% of the total content of quantified metabolites, with pinocembrin (L29) and licoflavanone (L36) being the major compounds (each accounting for 11–21% of the total content) followed by—in order of abundance—prenylated dihydrostilbenes with a content ranging from 10 to 37% of the total content (L33 and L44 as the most abundant

compounds), flavone C-glycosides (17–32% of the total content), and flavonol O-glycosides (4–10% of the total content) (Table S1 and Figure 2).

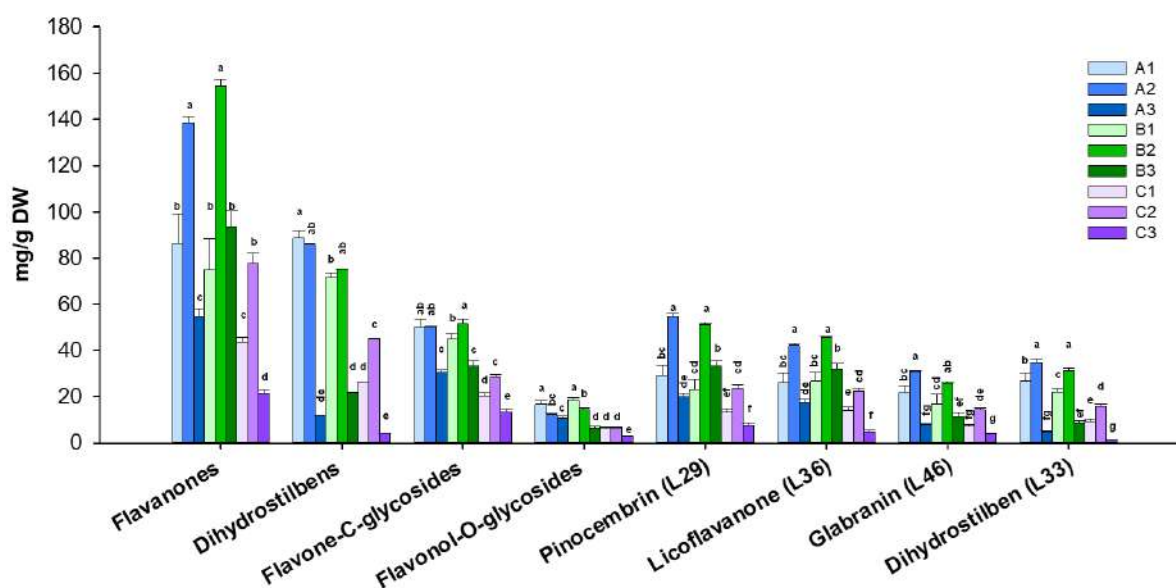


Figure 2. Levels of metabolite classes and the most abundant compounds from three production areas (P) and three harvest times (HT) of *G. glabra* leaves. Different letters within each metabolite class or compound indicate significant differences between extracts ($p \leq 0.05$ by a post-hoc Tukey's HSD test).

The quantitative data of GGL phytochemicals revealed considerable variations in their content in relation to both the production areas (P) and harvesting times (HT) (Table S1 and Figure 2). The levels of each GGL marker and of metabolite classes were statistically evaluated according to the influence of P and HT. The two-way ANOVA analysis indicated that both P and HT statistically affected the levels of all GGL markers. Also, the interaction between the P and HT proved to be statistically significant for all the analyzed compounds and the metabolite classes (Tables S1 and 1).

Table 1. F ratios of production area (P) and harvest time (HT) and their interaction of specialized metabolites content in *G. glabra* leaves ^a.

Factor	Flavanones	Dihydrostilbenes	Flavones	Flavonols	L29	L36	L46	L33	TEAC
P (2) ^b	183 *	132 *	417 *	294 *	210 *	211 *	101 *	216 *	40 *
HT (2) ^b	239 *	318 *	202 *	182 *	250 *	171 *	168 *	531 *	114 *
P × HT (4) ^b	15 *	21 *	9 *	27 *	26 *	13 *	11 *	25 *	3.4 *

^a Values are given as F-ratio; * denote significant at $p \leq 0.001$ of Fisher; ^b degrees of freedom in brackets.

Particularly, the variation in the levels of the most abundant compound classes, flavanones and prenylated dihydrostilbenes, is predominantly attributed to the dynamic influence of development stages (HT), which shapes the overall composition of bioactive compounds according to the plant's growth cycle. On the other hand, the contribution of P on the variance was greater for flavone C-glycosides and flavonol O-glycosides (Table 1). The effects of P and HT on the content of the different metabolite classes agree with their distinct localization in the leaf [17], suggesting a different biological role. Namely, the phytochemicals flavanones and dihydrostilbenes accumulated in the leaf exudate are likely responsible for the resistance to pathogens, herbivore attacks, and drought stress; meanwhile, glycosylated flavones and flavonols, distributed solely in the inner leaf tissue, might be involved in photoprotection, shielding plant cells from photo-oxidation [17,33].

Considering the harvesting time, the levels of GGL specialized metabolites were found to be significantly different among the three timeframes of our study (Figure S2). HT3 exhibited the lowest levels of each compound or metabolite class, regardless of the production area (Table S1 and Figure 2). These results can be easily attributed to developmental changes associated with plant growth and, in particular, with leaf senescence and the plant recycling activities of catabolism, which are necessary for nutrient remobilization from senescing leaves to sink tissues [33–35]. Namely, different trends in the content of the various compound classes are observed, especially for the most abundant classes. A significant variation in the flavanone levels was observed in the three HTs: both the total content and the major flavanones (pinocembrin, licoflavanone, and glabranin) more or less doubled between HT1 and HT2, and then decreased in HT 3 by 61, 40, and 73% in GGL-A, GGL-B, and GGL-C, respectively. On the contrary, the levels of prenylated dihydrostilbenes did not show relevant variations between HT1 and HT2 but underwent a drastic reduction, ranging from 71% to 91%, from HT2 to HT3, for all three production areas (Figure S2). Pinocembrin, prenylated flavanones, and dihydrostilbenes are accumulated in the sticky exudate that cover the surface of *G. glabra* leaves [17]. It has been reported that *G. glabra* exudate is most likely responsible for the resistance to biotic and abiotic stresses such as pathogens, herbivore attacks, and drought stress [36]. The well-known antimicrobial and cytotoxic activities of pinocembrin and licoflavanone further support their role in insect deterrence and defense against enemies feeding [12,13,37,38]. This is in agreement with reports on eucalypt oil glands, where pinocembrin has been identified as a main component and its biological properties, such as antibacterial, antifungal, and antifeedant activities, are clearly related to plant defense [39–41]. Thus, during flowering, the high levels of flavanones and prenylated dihydrostilbenes are most likely part of the defensive strategies of the licorice plant [42].

A similar variation in the flavone C-glycosides was observed, regardless of the production area. Flavonol-O-glycosides instead showed a significant content decrease in both A and B production areas from HT1 to HT2, whereas in C, their content did not significantly change during these two harvesting times (Table S1 and Figure 2). This different behavior between A/B and C production areas during the three HTs can be associated with environmental conditions. HT1 and HT2 (from late Spring to Summer) encompass seasons characterized by high UV light, especially in Southern Italy; thus, the accumulation of these antioxidant compounds is expected to increase, but our results suggest that plants located in A and B areas might experience better shading than plants growing in the C area [43].

Our data also highlight relevant differences among the different production areas. For all quantified compounds, GGL-C displayed content means that were statistically different compared to GGL-A and GGL-B; on the contrary, no statistically significant differences were observed between the A and B areas, except for the content of prenylated flavanones (Figure S2). In detail, GGL-A and GGL-B at all three studied harvest times showed the highest content of metabolites (10–29 and 15–30 g/100 g DM, respectively), whereas in GGL-C, the lowest total content of metabolites (4–16 g/100 g DM) was detected, and a statistically significant lower content of each analyzed compound was observed in these samples compared to the other two (Table S1 and Figure 2). In particular, since it is widely known that pedoclimatic conditions play a crucial role in the accumulation of secondary metabolites by influencing the uptake of water and nutrients, these results point out that the significantly lower phytochemical amounts in GGL-C samples might reflect the different, and possibly less pleasant, growth conditions experienced by *G. glabra* in the hinterland of the Ionian coast [44]. On the other hand, GGL-A and GGL-B extracts exhibited a similar content of phytochemicals, which is more than double compared to GGL-C. Generally, no marked differences among the different metabolite classes were observed.

Overall, the quantitative profile of GGL indicates that this untapped resource can provide a bioactive-rich matrix if harvested in the A and B areas and at the first two harvesting times.

3.2. Antioxidant Activity In Vitro and In Vivo of Glycyrrhiza glabra Extracts

In order to evaluate how the differences recorded in GGL phytochemical profiles reflect biological properties, the antioxidant capacity of GGL extracts was evaluated spectrophotometrically by ABTS in vitro assay (Figure 3a). Furthermore, to ascertain which specialized metabolites mostly contribute to the antioxidant activity, distinctive GGL compounds were assayed for their antioxidant potential (Table 2).

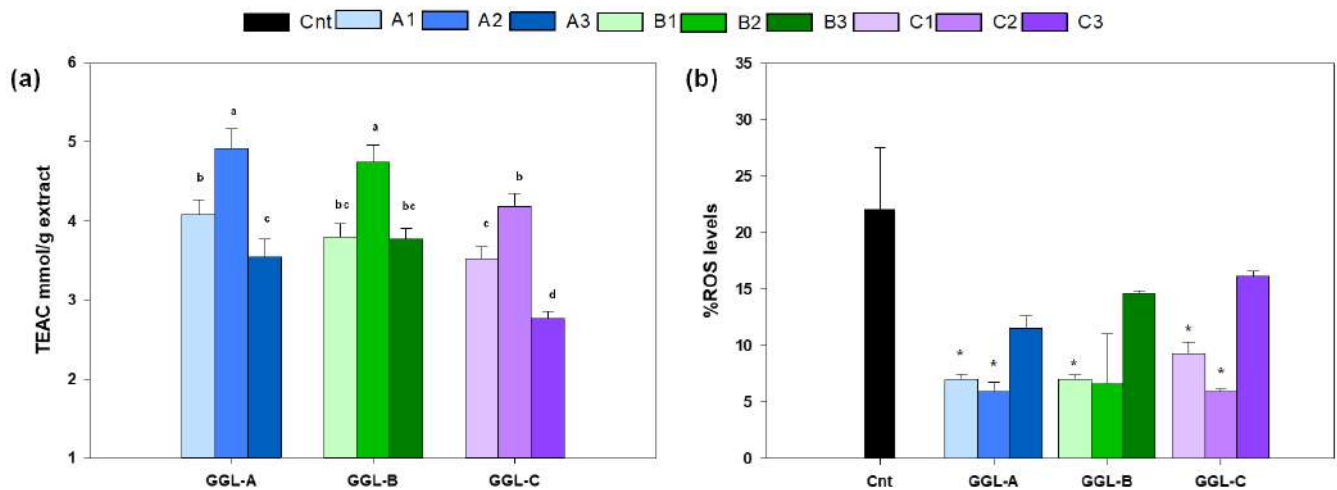


Figure 3. Antioxidant capacity of *G. glabra* leaf extracts from three production areas and three harvest times, measured as (a) TEAC equivalents by ABTS assay and (b) ROS content (assayed by DHE staining) of yeast cells BY4742 bearing pYX242-SNCA plasmid grown in minimal medium containing 2% glucose for 24 h in the absence (cnt) or presence of 0.2% *G. glabra* leaf extracts. Different letters in (a) indicate significant differences between extracts ($p \leq 0.05$ by Tukey's HSD test). * $p < 0.05$ relative to control cells. Data of (b) represent mean and standard deviation of at least two independent experiments.

Table 2. Antioxidant activity of GGL main compounds by ABTS assay ^a.

Compound	TEAC (mmol TE/mmol $\pm$ SD)
Vincenin 2	1.12 $\pm$ 0.01
Rutin	6.00 $\pm$ 0.04
Isoquercitrin	6.45 $\pm$ 0.03
Pinocembrin	3.21 $\pm$ 0.01
Licoflavanone	9.55 $\pm$ 0.04
Glabranin	4.08 $\pm$ 0.03
Prenylstilbene (L33)	5.09 $\pm$ 0.01
Prenylstilbene (L44)	2.50 $\pm$ 0.01

^a Values are means of three replicates.

Also, for in vitro antioxidant capacity, two-way ANOVA analysis revealed a statistically significant variation across the production area (P) and according to the harvesting time, as well as the interaction $P \times HT$ (Table 1). Antioxidant activity of GGL extracts (Figure 3a) changed according to the quantitative profiles. Regarding the harvest time, GGL samples collected in HT 2 showed significantly higher antioxidant activities than HT1 and HT3 (Figure S2), consistent with the metabolite enrichment characteristic of this growth stage. Conversely, no statistically significant differences were observed between the A and B areas (Figure S2). These data further support the best harvest conditions identified by the quantitative analysis of GGL markers, suggesting that these compounds are most likely responsible for antioxidant activity. Indeed, as shown in Table 2, the tested compounds showed higher antioxidant powers than Trolox, with the following activity order: licoflavanone > isoquercitrin > rutin > stilbene L33 > glabranin > pinocembrin >

stilbene L44 > vincenin 2. Interestingly, the most abundant compounds (licoflavone, stilbene L33, glabranin, and pinocembrin) showed superior or comparable antioxidant activity to the flavonols rutin and isoquercetin, the content of which was distinctly lower in the GGL extracts. Thus, the antioxidant capacity of GGL might be attributed to the high content of prenylated flavanones and dihydrostilbenes. These results are in agreement with what has been reported in previous studies [15,45,46] on the bioactivity of *G. glabra* leaf extracts.

Oxidative stress, resulting from the excessive intracellular accumulation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and other free radical species, contributes to the onset and progression of various diseases, among which is Parkinson's disease (PD) [47]. Based on results of the in vitro antioxidant capacity of *G. glabra* leaves and its main specialized metabolites and in order to estimate the ability of GGL extracts to exert an antioxidant response at the cellular level, not just their radical scavenging capacity, the antioxidant effect of the GGL extracts was evaluated on a yeast model of *Saccharomyces cerevisiae* expressing human α -synuclein. As shown in Figure 3b, the treatment with GGL extracts remarkably reduced the intracellular ROS level in exponentially growing yeast cells, with significant antioxidant effects for the GGL extracts with the highest phytochemical contents (harvesting times 1 and 2).

3.3. Anti-Aging Effects of Glycyrrhiza glabra Extracts in Yeast Cells Expressing Human α -Synuclein

α -Synuclein is a presynaptic protein associated with the pathophysiology of synucleinopathies, including Parkinson's disease [23,48], and budding yeast has been extensively employed in models of synucleinopathies [25,26]. Thus, the effect of GGL extracts on the longevity of yeast cells over-expressing the human α -synuclein was evaluated. In order to evaluate whether GGL extracts could exert anti-aging effects, they were added to exponentially growing yeast cells expressing human α -synuclein at a concentration of 0.2% in a synthetic minimal medium. Results show that all the extracts prolonged the chronological lifespan (CLS) of yeast cells (Figure 4a), with a significant marked increase in both mean and maximal lifespan. In fact, mean lifespan resulted 2.5 times longer while maximal lifespan increased up to 3 times relative to the control (Figure 4b).

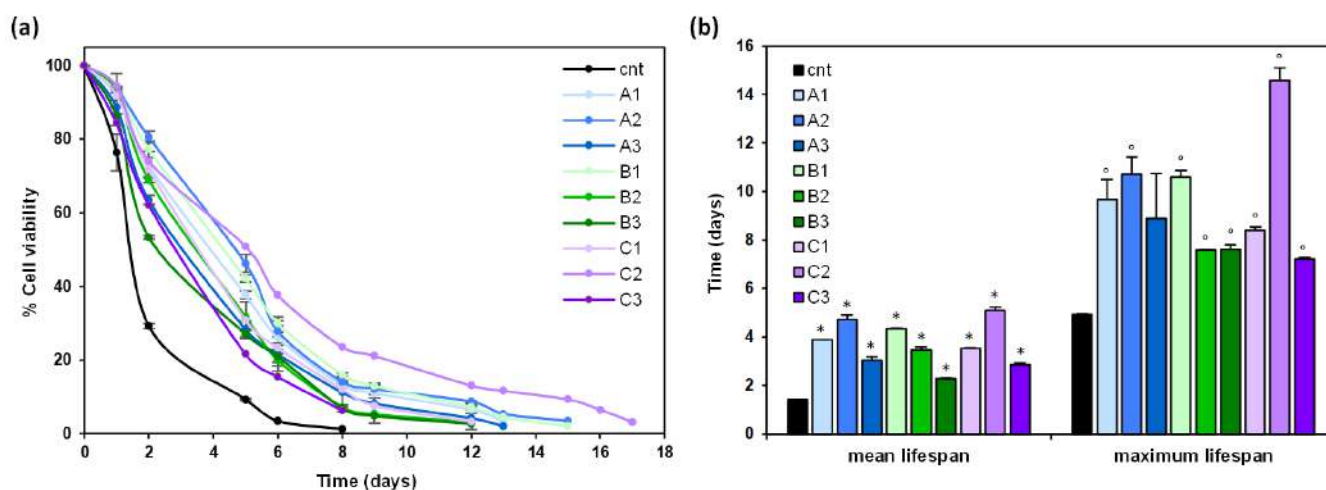


Figure 4. Effect of *G. glabra* leaf extracts on α -synuclein toxicity: (a) chronological lifespan (CLS) of yeast cells BY4742 bearing pYX242-SNCA plasmid grown in minimal medium containing 2% glucose in the absence (cnt) or presence of 0.2% GGL extracts; (b) mean and maximum lifespan of CLS of yeast cells as in (a). * $p < 0.05$ relative to mean lifespan in control cells; ^o $p < 0.05$ relative to maximum lifespan in control cells.

Overall, the data presented indicate that *G. glabra* extracts delay senescence by reducing intracellular ROS level and α -synuclein toxicity.

4. Conclusions

In this study, we explored the influence of different harvest times and cultivation areas on the phytochemical content of *G. glabra* leaves, showing a direct influence of these factors on specialized metabolites production. According to the results, different harvest periods have a strong impact on the concentration of bioactive compounds, especially on flavanones and dehydrostilbenes, thus also influencing GGL extracts' bioactivities.

Regarding the antioxidant and antiaging power of GGL extracts, our results clearly indicate that harvesting time is essential for obtaining desired bioactive chemical constituents. Aging and age-related neurodegeneration are among the major challenges in modern medicine because of the progressive increase in the number of elderly in the world population. Nutrition, which has evident long-term consequences for health, is an important way to prevent diseases and achieve healthy aging. Our study successfully demonstrates the beneficial effect of GGL extracts on senescence related to α -synuclein toxicity along with their notable antioxidant properties, supporting the role of GGL extracts as a functional food in age-related disorders.

Overall, this study provides important theoretical foundations and technical support for selecting the best cultivation area in order to optimize *G. glabra* cultivation in the Calabria region, also offering the possibility to envisage the most favorable harvest time for valorization of *G. glabra* leaf resources according to the principles of sustainable development in agricultural processes.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox13010093/s1>, Table S1: Level of the main *G. glabra* leaves according to the production area (P) and harvesting time (HT) on metabolite content of *G. glabra* leaves; Figure S1: UV spectra of main GGL compounds; Figure S2: Means plot of metabolite contents and TEAC of *G. glabra* leaves according to the production area (P) and harvesting time (HT).

Author Contributions: Conceptualization, T.D., R.C., M.R. and A.L.P.; methodology, T.D., R.C., P.C. and A.L.P.; validation, R.C., V.S. and A.L.P.; formal analysis, T.D., A.L., R.D.S., S.S. and V.S.; investigation, T.D., R.C., A.L., R.D.S., S.S. and V.S.; resources, P.C., M.R., L.R. and A.L.P.; writing—original draft preparation, T.D., R.C., P.C. and A.L.P.; supervision, R.C. and A.L.P.; project administration, M.R., L.R. and A.L.P.; funding acquisition, M.R. and L.R. All authors have read and agreed to the published version of the manuscript.

Funding: Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.4—Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union—NextGenerationEU; Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022. Adopted by the Italian Ministry of University and Research, CUP, D43C22001260001, Spoke 6, Project title “National Biodiversity Future Center—NBFC”.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: This work was supported by the project “OroNero” (CUP: J88C17000130006) POR Calabria 2014-2020 and by Research Infrastructure Saf@med-Food Safety platform, and the project “ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON Foods” (CUP:H43C22000820001) of National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.3—Research funded by the European Union—NextGenerationEU. We also thank to Antonio Massarotto and Marco Massarotto for the support in the sampling and treatment of plant materials.

Conflicts of Interest: The authors declare no conflicts of interest.

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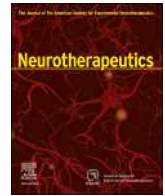
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Original Article

Acteoside exerts neuroprotective effects by preventing α -synuclein aggregation and oxidative stress in models of Parkinson's disease

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ARTICLE INFO

Keywords:

Verbascum thapsus

Verbascoside

Lifaspan

Saccharomyces cerevisiae

Drosophila melanogaster

ABSTRACT

α -Synuclein is a small presynaptic protein whose aggregation is one of the hallmarks of Parkinson's disease (PD). In our quest to identify novel preventive or therapeutic treatments for PD, we collected 60 Italian plant species, representative of part of the Mediterranean flora, which were screened by a phylogenetic analysis in conjunction with a high-throughput screening in a yeast model of PD expressing human α -synuclein. The integration of these approaches led to the identification of four plants, *Allium lusitanicum*, *Salvia pratensis*, *Verbascum thapsus* and *Glaucium flavum*, whose extracts, characterized by a metabolomic analysis, exhibit robust inhibitory activity against the amyloid aggregation of α -synuclein *in vitro*, as well as in neuroblastoma cells overexpressing the protein. By employing a size exclusion chromatography affinity approach coupled to mass spectrometry, we identified the phenylpropanoid glycoside acteoside from the extract of the edible plant *V. thapsus* as the metabolite that directly binds α -synuclein and effectively inhibits its fibril formation. In addition, acteoside reduces oxidative stress in neuroblastoma cells exposed to α -synuclein fibrils and activates the NRF2 pathway. Notably, acteoside improves motor performance in a *Drosophila* model of PD and exhibits a significant reduction of protein carbonyl groups, suggesting that this compound may mitigate oxidative stress-induced protein damage. Our findings could pave the way for the development of new strategies aimed at discovering novel neuroprotective agents targeting PD-related diseases.

Introduction

Aging is a complex physiological condition influenced by various biological pathways that not only affect lifespan but also play a crucial role in the development and progression of age-related diseases. Among these, Parkinson's disease (PD) is a neurodegenerative disorder affecting 2–3 % of the population over 65 years, clinically characterized by movement disorders and cognitive impairment, and by the misfolding and aggregation of α -synuclein at the molecular level [1–3]. α -Synuclein (α -syn) is a small presynaptic protein, mainly expressed in neurons of the

central and peripheral nervous system, involved in the regulation of vesicle trafficking and neurotransmitter release [4]. It is an intrinsically disordered protein that can adopt an α -helical conformation when associated with biological membranes. Mutations or overexpression of α -syn can lead to aggregation into toxic oligomers and fibrils, which alter vesicle trafficking, mitochondrial activity and induce cellular toxicity, ultimately leading to the loss of dopaminergic neurons [2].

The onset of age-related diseases is also shaped by environmental factors. According to the One Health concept, the health of humans, animals, plants, and the environment are closely linked and inter-dependent, and by

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Received 4 September 2025; Received in revised form 19 November 2025; Accepted 8 December 2025

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means of a collaborative and *trans*-disciplinary approach it aims to achieve improved health and well-being outcomes. Through the bioprospecting approach, biodiversity enables the identification of a number of compounds (from diverse sources such as plants, marine organisms, and microorganisms) with bioactive properties beneficial for preventing or treating human diseases. Indeed, the potential of natural compounds is huge, and recent years have seen a surge in publications highlighting their numerous neuroprotective effects [5,6]. In this context, selecting plant candidates is a critical step before analyzing their biological activities. As Najmi et al. [7] suggested, various approaches can be employed: selection based on ethnopharmacological knowledge, traditional medicine systems, or random selection. However, it has been suggested that closely related taxa may share biosynthetic pathways of similar phyto-metabolites and that their chemical molecules are generally conserved with each other [8,9]. Molecular phylogenetic approaches offer an alternative method to identify plant-based natural products reducing the time and cost of subsequent biological analysis [9,10].

The National Biodiversity Future Center in Italy is dedicated to conserving, restoring, monitoring, and enhancing biodiversity within the Mediterranean biodiversity hotspot [11] (<https://www.nbfc.it>). The Mediterranean basin, with its diverse and heterogeneous plant life, boasts a significant number of endemic species, likely due to its varied geography, environmental conditions, and historical influences [12]. Although many Mediterranean plants have been described, they remain a vital resource for research in biochemistry, phytochemistry, and pharmacology, potentially leading to the discovery of new therapeutic molecules.

This study was designed to develop an innovative integrated approach to select the most promising neuroprotective plant extracts from a library of Mediterranean plants. We exploited the information present in a broad phylogenetic framework, combined with a high-throughput screening in a yeast model of PD expressing human α -syn. From the selected plant extracts, we identified the phenylpropanoid glycoside acteoside, with strong anti-aggregant activity against α -syn fibrillation. The neuroprotective properties of acteoside were deeply evaluated through biophysical techniques, in neuroblastoma cell cultures and in a *Drosophila melanogaster* model of PD based on the inactivation of the *DJ-1 β* gene [13], the fly ortholog of human *DJ-1* (*PARK7*), mutated in early-onset familial PD forms [14].

Materials and methods

Sample collection and extraction

Aerial vegetative organs (leaves and eventually young herbaceous stems) of the 60 plant species investigated were collected in triplicate from plants growing in the botanical garden of Padova (Italy), grown in a nursery or in the open field, as specified in [Supplementary Table S1](#). The samples were immediately frozen in liquid nitrogen, ground into a fine powder using an A11 basic analytical mill (IKA-Werke, Staufen, Germany), and then stored at -80°C . Approximately 1 g of frozen powder was subjected to extraction using 10 vol (w/v) of 100 % LC-MS grade methanol (Honeywell, Seelze, Germany). The mixture was vortexed for 30 s, sonicated on ice for 10 min in a 40-kHz ultrasonic bath (SOLTEC, Milano, Italy), and then centrifuged at 14,000 g for 10 min at 4°C . The collected supernatants were divided into ten 1.5 mL tubes, with each tube containing 1 mL of extract, and stored at -20°C . Nine aliquots were then subjected to processing with a vacuum evaporation system (SpeedVac, Heto-Holten, Frederiksberg, Denmark) and allocated for the following *in vitro* bioassays, while one aliquot was kept for LC-MS analysis.

Untargeted liquid chromatography-mass spectrometry (LC-MS) metabolomics analysis

The untargeted LC-MS metabolomics analyses were performed using the instrumentation and methodology described in previous studies [15,16] with slight modifications. In brief, the extracts were diluted at 1:10, 1:50 and 1:100 with LC-MS grade water (Honeywell) and filtered

through $0.22\ \mu\text{m}$ Minisart filters (Sartorius-Stedim Biotech, Göttingen, Germany). The analyses were performed with Ultra-Performance Liquid Chromatography-High-Resolution Mass Spectrometry (UPLC-HRMS) and 5 and $1\ \mu\text{L}$ were injected into the instrument for analysis in both negative and positive ionization modes, respectively. The elution was carried out in gradient mode among two mobile phases (A: 0.1 % formic acid in water, B: 100 % acetonitrile). The elution gradient commenced at 1 % B, held for 1 min, then increased to 40 % B at 10 min, 70 % B at 13.5 min, 90 % B at 15 min, and reached 99 % B at 16.5 min. The method was then maintained at 99 % B for 3.5 min before returning to 1 % B at 20.1 min. The method concluded with an isocratic phase (1 % B) at 25 min. MS data were acquired in profile and sensitivity modes and by using a scan range of 50–2000 m/z . The scan time was set at 0.3 s and the MS data were acquired with function 1 (no fragmentation) and function 2 (CID fragmentation with argon at 35 eV). Furthermore, a FAST-DDA data-dependent analysis was conducted to improve metabolite identification. This involved assessing the accurate mass (derived from the m/z ratio), retention time, and fragmentation pattern (MS/MS from the FAST-DDA analysis) for each ion, followed by cross-referencing with a proprietary library of authentic standard compounds, an *in silico* database of plant compounds, and relevant scientific literature and public databases such as Pubchem, MoNA and MassBank. Identification levels were established according to metabolomics standards initiative (MSI): level 1, unambiguous identification with reference standards; level 2, putative assignment based on MS data similarity with literature data or databases; level 3, putative assignment established by spectral similarity to chemical class of compounds and chemotaxonomic data.

Phylogenetic selection approach

The lists of plants with anti-aggregant, neuroprotective and anti-Parkinson's biological activities were downloaded from the Dr. Duke's Phytochemical and Ethnobotanical Databases [17,18] to determine their phylogenetic clumping. Hereinafter, we will refer to these plants as 'Medicinal Plants' (MP). The MP belonging to the Pteridophyta and Ceratophyllales ([Supplementary Table S1](#)) were excluded from the phylogenetic analyses due to their low number in the database. The Maximum Likelihood (ML)-based chronogram produced by Zanne et al. [19] was downloaded and four monophyletic subtrees (i.e., Monocotyledoneae with 7060 tips, Superrosidae with 10,009 tips, Superasteridae with 11,324 tips, and Basal eudicots with 948 tips as defined in Ref. [19]) representing 29,341 species in total, were extracted for use as reference in subsequent analyses.

The functions available in the R package 'pm4mp' (<https://github.com/gzecca/pm4mp>) were used as follows. The function "`clean_and_match()`" was applied with the argument "`reftype = z`" and the argument "`match_thr = 0.9`" to standardise the species names and to find the fuzzy matches between the MP names and tree tip names. The resulting fuzzy matches were manually checked for misspelling on the Plants of the World Online (<https://www.plantsoftheworldonline.org>) and on the World Flora Online (<https://www.worldfloraonline.org>) databases and corrected when necessary in the MP lists ([Supplementary Table S2](#)). The "`tree4nodesigl()`" and "`sample4nodesigl()`" functions were used to prepare input files for PHYLOCOM v4.2, which was used to identify the "*hot nodes*" in the reference phylogenies [20]. The "*hot nodes*" are the nodes on a phylogenetic tree that contain an overabundance of taxa of a given category compared to a random pattern of distribution [21,22]. The function "`nodesiglR()`" with parameters "`start = 1`" and "`stop = 1500`" was used to run PHYLOCOM from R and to perform 1500 repetitions of the "`nodesigl`" command using 999 randomizations each. Repetitions were necessary because of the stochastic nature of the null model used by PHYLOCOM to test the statistical significance of hot nodes. The appropriate number of repetitions was determined based on preliminary analyses, and chosen to exceed the number of replicates after which the detected *hot nodes* remained constant (data not shown). The function "`nodesigl_harvesteR()`" with the

argument “*fract* = 5” was used to keep the (stable) *hot nodes* present in all 1500 runs that contained at least 5 % of MPs and to extract their corresponding “*hot trees*” in Newick format. We define the “*hot trees*” as subtrees of the reference phylogenies containing independent subsets of phylogenetically nested “*hot nodes*” and therefore containing different sets of species (for details see the “*pm4mp*” package documentation). The 60 plant species selected by the NBFC project (Supplementary Tables S1 and S2) were searched within the identified *hot trees*. The “*hot-tree-painteR()*” function was used to plot the *hot trees* where at least one plant species selected by the NBFC project was found.

Yeast strains and media

The yeast strain utilized to assess the biological activity of plant extracts is BY4742[pYX242-SNCA], overexpressing human α -syn. Cell cultures were grown in minimal medium containing 2 % glucose as the carbon source and 0.67 % yeast nitrogen base lacking amino acids, supplemented with 50 mg/L of essential amino acids and bases necessary for the auxotrophic strain's requirements and with 0.1 mM ampicillin to prevent contaminations. Cells were grown at 30 °C under continuous agitation at 120 rpm. For treatments with the extracts, the dried extracts, obtained by 100 mg of fresh leaves, were resuspended in 75 μ L of 100 % ethanol, vortexed and sonicated for 20 min at 28 KHz and then added to 5 mL of the medium. In the control condition, 75 μ L of ethanol was added in the medium (final ethanol concentration of 1.5 %).

Biolog OmniLog system

The Biolog OmniLog System was utilized to compare the cellular phenotypes of BY4742[pYX242-SNCA] yeast cells supplemented with 60 different extracts. Yeast cell cultures were grown on agar plates at 30 °C, then inoculated into 8 mL of 2 % glucose minimal medium in sterile glass tubes. The cell suspension was measured using the BIOLOG Turbidimeter (Biolog, Hayward, CA) until reaching a cell suspension transmittance of 62 % T. The suspension was prepared according to the BIOLOG PM protocol for yeast cells: Dye D 75x (Biolog, Hayward, CA), cells 48x, and medium. This suspension was then pipetted into the 96-well plates (100 μ L per well). All dried extracts were resuspended in 75 μ L of 100 % EtOH and 1.5 μ L of extract, corresponding to 20 mg of fresh leaves/mL medium, were added to each well of the plates (1.5 % EtOH final concentration) to reach a 100 μ L total volume. Microplates were then incubated in the OmniLogTM system at 30 °C for 72 h. The experiment was conducted in triplicate. Growth profiles were compared using the y-maximum value of each kinetic curve.

Chronological lifespan assay (CLS)

Cells were grown in liquid medium until reaching the mid-late exponential phase, after which they were inoculated at a density of 0.150 OD/mL into flasks containing medium either without extracts (solvent control) or supplemented with the extract (corresponding to 20 mg of fresh leaves/mL medium). Survival (expressed as percentage) was evaluated by propidium iodide (PI) staining at different time points using the Cytoflex cytofluorimeter (Beckman Coulter, Milan, Italy) and analyzed with Cytoflex software (version 2.6.0.105), as previously reported [23].

Analysis of reactive oxygen species (ROS) and aggresome levels in yeast

ROS level within the cells was assessed through DHE (dihydroethidium) staining. Following a 24 h treatment with the extracts, cells were harvested, resuspended in phosphate-buffered saline (PBS) solution, and stained with 5 μ g/mL of DHE for 10 min, as previously reported [23]. Flow cytometric analyses were conducted using the Cytoflex cytofluorimeter (Beckman Coulter) and data were analyzed using Cytoflex software (version 2.6.0.105). The intracellular protein aggresomes were analyzed using the PROTEOSTAT[®] Aggresome detection kit (ENZO Life

Sciences). Yeast cells were collected after 24 h treatment with the extracts; 0.2 OD of cells were collected and resuspended in PBS buffer and stained with the PROTEOSTAT[®] Aggresome detection reagent at a dilution of 1:1500, as previously reported [24]. Fluorescence-activated cell sorting (FACS) analyses were conducted using a Cytoflex cytofluorimeter (Beckman Coulter) and analyzed with Cytoflex software.

Thioflavin T (ThT) assay

α -syn was purchased from Merck-Millipore and dissolved at 70 μ M in PBS. Protein samples (20 μ L, 70 μ M concentration of α -syn) were incubated at 37 °C in PBS up to 72 h under constant shaking at 900 rpm with a thermo-mixer in the absence (cnt) or in the presence of the extracts. Dried extracts, corresponding to 100 mg of fresh leaves resuspended in 75 μ L of 100 % ethanol, were diluted 1:10 in PBS and 5 μ L were added to α -syn to test their effect on protein aggregation; in control experiments ethanol was used as solvent control. The ThT binding assay was performed according to Ref. [25] using a 20 μ M ThT solution in the PBS buffer. 180 μ L of ThT solution were added to 20 μ L of the aggregated α -syn samples, transferred into a black 96-well clear bottom multiwell plate and ThT fluorescence was read at the maximum intensity of fluorescence of 485 nm using a Victor X3 plate reader (PerkinElmer). Fluorescence of blank samples was subtracted from the fluorescence values of all samples. In control experiments with each extract, no interference with ThT fluorescence was observed.

Seed fibrils formation

For seed fibrils formation 250 μ L of α -syn at 70 μ M was incubated at 37 °C in PBS for 72 h under constant shaking at 900 rpm in a thermo-mixer. Fibrils formation was verified through the Thioflavin T assay by mixing 10 μ L of fibril samples with 20 μ M ThT in a final volume of 100 μ L. Fluorescence emission at 485 nm was recorded using a plate reader (BioTek Synergy H1, Agilent).

Aggregation kinetics

For kinetic experiments, 35 μ M α -syn (100 μ L) was incubated at 37 °C in PBS, with 20 μ M ThT solution in PBS, up to 72 h under constant shaking at 425 cpm using a plate reader (BioTek Synergy H1, Agilent) in the absence (cnt) or in the presence of *V. thapsus* extract or acteoside (50 μ M and 100 μ M). The dried extract of *V. thapsus* was resuspended in 75 μ L of 100 % EtOH and 2.5 μ L were added to α -syn to test the effect on protein aggregation; in control experiments ethanol was used as solvent control. For fibril elongation experiments, 1 μ M preformed seed fibrils were added to 35 μ M α -syn in PBS. To probe fibril elongation, protein samples (100 μ L) were incubated at 37 °C in PBS, with 20 μ M ThT solution in PBS, under constant shaking at 425 cpm using a plate reader (BioTek Synergy H1, Agilent) in the absence (cnt) or in the presence of acteoside (1 μ M, 25 μ M, 50 μ M and 100 μ M). Acteoside was purchased from Merck and was resuspended in water and added to α -syn; in control experiments water was used as solvent control.

Transmission electron microscopy (TEM) imaging

For the visualization of α -syn fibrils by TEM, α -syn was used at 70 μ M in PBS. Protein samples (250 μ L) were incubated at 37 °C in PBS, with 20 μ M ThT solution in PBS under constant shaking at 900 rpm with a thermo-mixer up to 72 h, in absence (cnt) or in the presence of acteoside (50 μ M). Samples were loaded on carbon coated copper TEM grids (5 μ L) and let them air-dried. Then a negative staining (Uranyl Acetate, 1 %) was performed to enhance the contrast and to improve the observations. TEM micrographs were acquired with JEOL JEM 2100Plus Transmission Electron Microscope (JEOL, Japan) operating with an acceleration voltage of 200 kV, and equipped with an 8 megapixel Gatan (Gatan, USA) Rio Complementary Metal-Oxide-superconductor (CMOS) camera.

Cell cultures

SH-SY5Y cell lines harboring *pTet-SNCA-FLAG* were purchased from Merck. Cells were cultured on plates at 37 °C in DMEM/F12 medium, containing 10 % fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin, in a humidified 5 % CO₂ incubator. Doxycycline-inducible α -syn-expressing cells were selected against the antibiotic puromycin with a dose of 2 µg/mL. Induction of α -syn expression was achieved by adding 6 µg/mL doxycycline (from a 6 mg/mL stock in DMSO) for 48 h.

The extracts obtained from 100 mg of fresh leaves were resuspended in 75 µL of 100 % ethanol and used as 200x stocks (corresponding to 6.6 mg/mL of medium, final ethanol concentration 0.5 %); 0.5 % ethanol was used as solvent control. Acteoside was resuspended in water and used at a final concentration of 100 µM. For immunofluorescence assays, 1.6×10^5 cells were seeded on geltrex-coated glass cover slips in wells of a 24 multiwell plate and treated the day after for 48 h.

SH-SY5Y cells were purchased from ATCC. Cells were cultured on plates at 37 °C in DMEM medium, containing 10 % fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin, in a humidified 5 % CO₂ incubator. For analysis of ROS level 8×10^3 cells were seeded in wells of a 96 multiwell plate and treated with 5 µM of α -syn fibrils for 72 h. To obtain the fibrils, α -syn (70 µM) was incubated at 37 °C in PBS up to 72 h under constant shaking at 900 rpm with a thermo-mixer in the presence or in the absence of acteoside (1 mM).

Analysis of acteoside availability in cells

To evaluate the intracellular/cell-associated level of acteoside, 2×10^6 SH-SY5Y *pTet-SNCA-FLAG* cells were seeded in 60-mm dishes and treated with acteoside at 100 µM up to 24 h. For metabolite extraction, cells were washed twice with PBS before being lysed with ice cold 80 % methanol solution for 5 min. The lysate was transferred into a 1.5 mL tube and sonicated in bath 1 min on and 1 min off for 5 times. Samples were centrifuged at 14,000 g for 30 min at 4 °C and the supernatant was filtered using a 3 kDa molecular weight cut-off filter at 14000 g for 1 h at 4 °C. The eluate was transferred in LC-MS vials and stored at -20 °C.

Samples were injected into a Xevo G2-XS quadrupole time of flight mass spectrometer coupled to a Waters ACQUITY ultra-performance liquid chromatography (UPLC) H-Class system through an ESI source (Waters Corporation, Milford, MA, USA). The instrument was operated in negative ion current in sensitivity mode. The capillary voltage was maintained at 1.5 kV with the source temperature held constant at 140 °C.

An ACQUITY UPLC® BEH C18 1.7 µm, 2.1 mm × 50 mm, (Waters Corporation, Milford, MA, USA) was used for analyte separation. The flow rate was set to 400 µL/min with a column temperature of 40 °C. A gradient of solvent A (99.9 % LC-MS-grade water with 0.1 % formic acid) and solvent B (LC-MS-grade 99.9 % acetonitrile with 0.1 % formic acid) was applied with a total run time of 12 min as follows: 0–0.3 min at 2 % B; 0.3–6.3 min linear increase from 2 to 98 % B; 6.3–8.3 min 98 % B; 9–12 min 2 % B. Transition selection and optimization of collision energy were performed manually on acteoside and its isomer isoacteoside standard solutions, and data acquisition in MRM mode was performed with Masslynx (v4.2). The transitions monitored were *m/z* 623.1 (CE 32 V) → *m/z* 161.1 (quantifier) and 461.1 at CE 28 V (qualifier) for acteoside and isoacteoside. A standard curve 8–5000 nM of both the analytes was run to test the linearity of the response and to confirm the different retention time of the two isomers. The accuracy of each standard point was calculated with TargetLynx based on the determined linear regression curve and should be between 80 % and 120 % of the expected value. Analyte quantity (acteoside + isoverbasoside) was extrapolated from the respective standard curves.

Cell viability assay

Cells viability was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. After treating cells for 72 h as described above, the culture medium was removed and cells were incubated for 2 h at 37 °C in 100 µL of DMEM containing 0.5 mg/mL MTT. Then, 100 µL of solubilization buffer (10 % Triton X-100, 0.1 N HCl in isopropanol) was added to cells. Absorbance was measured using a microplate reader (BioTek Synergy H1, Agilent) at 570 nm.

Analysis of reactive oxygen species (ROS) level in SH-SY5Y cells

Intracellular ROS were determined using fluorescent probe 2',7'-dichlorofluorescein diacetate, acetyl ester (H₂DCFDA, Merck). After treating cells for 72 h as described above, the culture medium was removed and cells were washed twice with PBS. Then, 5 µM of H₂DCFDA was added to each well for 30 min. After washing cells twice with PBS solution, the fluorescence was measured using a microplate reader (BioTek Synergy H1, Agilent) at excitation wavelength of 490 nm and emission wavelength of 520 nm. Data were normalized on total viability measured with MTT assay.

Western blot and native dot blot analyses

Cells were lysed in RIPA buffer (Thermo Scientific) supplemented with protease inhibitors (Roche) and phosphatase inhibitor cocktails (Merck). The lysates were centrifuged at 4 °C and the supernatants were collected. Protein concentration was determined using the Bradford assay (Bio-Rad). Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. After the transfer, the membranes were blocked with 5 % nonfat dry milk and primary antibodies anti-NRF2 (1:1000, ThermoFisher), anti- α -syn (Merck, 1:1000) and anti-vinculin (1:5000, Merck) were used. Conjugated secondary antibodies were applied, and the signal was detected using enhanced chemiluminescence (ECL). The resulting signals were captured using the Chemidoc system (Bio-Rad). For native dot blot analysis, samples from *in vitro* α -syn aggregation assays, performed in the absence or in the presence of the plant extracts, were dropped onto nitrocellulose membranes and incubated with the oligomer specific A11 mouse antibody (1:500, Thermo Fisher) or with the anti- α -syn antibody (1:1000, Merck).

Immunofluorescence assay

Cells grown on cover slips were washed with PBS, fixed with 4 % formaldehyde for 15 min, and permeabilized with PBS 0.2 % Triton X-100 for 10 min. Then, cells were washed in blocking solution (PBS 1 % BSA), blocked at room temperature for 60 min, and then incubated overnight at 4 °C with the primary antibody anti- α -Syn33 (1:40, Merck) recognizing α -syn oligomers, or with the primary antibody anti-NRF2 (1:200, Thermo Fisher). After that, cells were incubated with anti-rabbit AlexaFluor488 secondary antibodies (1:200, Thermo Fisher) for 1 h. Coverslips were mounted with a DAPI containing mounting medium for nuclear staining. Image acquisition was carried out on a Thunder fluorescence microscope (Leica Microsystems). For the analysis of α -syn oligomers, fluorescence images were analyzed using ImageJ software (NIH) and the Integrated Density (IntDen) of the α -syn fluorescence signal was quantified. Quantitative analysis of NRF2 nuclear localization was performed using Cell-ACDC, an open source user friendly GUI-based framework for segmentation [26]. Nuclei were segmented by applying an automated thresholding algorithm (Otsu method) on the DAPI channel, generating accurate nuclear masks. Within each segmented nucleus, the concentration parameter, defined as the total fluorescence intensity normalized to the nuclear area, was calculated. The resulting output data were exported and statistically analyzed to compare NRF2 localization across different treatment conditions.

Real time PCR

Total RNA was isolated using the Quick-RNA Mini-Prep Kit (Zymo Research), according to manufacturer's instructions. RNA (1 µg) was reverse-transcribed using iScript supermix (Bio-Rad), according to the manufacturer's protocol. For each real-time PCR, cDNA were amplified using the ChamQ mastermix (Vazyme) and specific primers. Each sample was normalized for total RNA content using ACTB and GAPDH as housekeeping genes. The primers used are the following: ACTB-F: CACCATTGGCAATGAGCGGT, ACTB-R: GTCCTTGCGGATGTCCACGT; GAPDH-F: AGAAGGCTGGGGCTCATTTG, GAPDH-R: TGCTAAGCAGTTGGTGGTGC; NQO1-F: TCCGGAGTAAGAAGGCAGTG, NQO1-R: TCCA GCGTTTCTTCCATCC; HMOX1-F: TTCAAGCAGCTCTACCGCTC, HMOX1-R: TTGCACTTTGTTGCTGGCCC.

Size exclusion chromatography coupled to reversed phase LC-MS (SEC AS-MS)

For the SEC AS-MS analysis, the dry extracts were first diluted in 1 mL of dichloromethane (DCM) and dried under nitrogen to obtain the accurate weight. A 10 mg/mL solution in methanol was prepared and centrifuged for 5 min at 13,000 rpm and then diluted in water:methanol (70:30 v/v) to obtain a final concentration of 2 mg/mL. Incubation solutions (500 µL) containing 10 µM α -syn and the selected plant extracts (2 mg/mL) were prepared in 0.1 M potassium dihydrogen phosphate buffer at pH = 7 and incubated for 96 h at 4 °C. SEC AS-MS experiments of the incubated samples were carried out with two chromatographic systems (System 1 and System 2) as described in Ref. [15]. Briefly, System 1 was used for SEC analyses using a TSK Gel Super SW2000 125 Å column (300 × 4.6 mm ID, 4 µm) from Tosoh Bioscience. Analysis was carried out in isocratic elution (KH₂PO₄ 0.1 M, 0.05 % NaN₃, pH = 7) at a flow rate of 350 µL/min α -Syn was detected at λ = 280 nm and the peak was eluted in the window 6.5–8 min. The elution fraction (750 µL final volume) was focused on an Agilent Zorbax Eclipse Plus C18 analytical trap column (12.5 × 4.6 mm ID, 5 µm). The trap column was connected to System 2 on the head of an analytical column, an XTerra MS C18 column (250 × 2.1 mm ID, 5 µm) from Waters. Chromatographic separation of the plant extracts fished analytes and the analyses of the fingerprinting of the extracts were carried out through gradient elution where the mobile phase was water 0.1 % formic acid (solvent A) and acetonitrile 0.1 % formic acid (solvent B). Specifically, after an isocratic step at 1 % B for 11.50 min, a linear gradient of B from 1 % to 40 % was applied in 22 min. Then, three sequential linear gradient steps were performed (from 40 % to 70 % in 8.5 min, from 70 % to 90 % in 3.5 min and from 90 % to 100 % in 3.5 min). Lastly, an isocratic step was carried out at 100 % B for 8.5 min. Compound identification was achieved as reported in Ref. [15] through mass spectrometry (MS) using a linear ion trap mass spectrometer (LTQ) equipped with an electrospray ion source (ESI) (Thermo Fisher Scientific, Waltham, MA, USA). Full scan mass range was set up in the 250–1500 Da range.

Native mass spectrometry (Native-MS)

Native-MS analysis was performed to assess the interaction of α -syn with acteoside. Commercial α -syn (Merck Millipore) and acteoside (Merck) powders were individually resuspended in 100 mM ammonium acetate pH 7 at 1 mg/mL stock concentration. Titration experiments were performed preparing independent samples at fixed α -syn concentration (7 µM) and variable acteoside concentrations (0–1400 µM). Each sample (10 µL) was directly infused into the mass spectrometer (Orbitrap Fusion, Thermo Fisher Scientific) by a static nano-electrospray source, employing borosilicate, metal-coated capillaries with 1-µm internal diameter (Thermo Fisher Scientific). Analytes desolvation and charging were obtained applying a 1.1–1.2 kV ion spray voltage. The main instrumental parameters were set as follows: resolving power at m/z 200, 120,000; detection window, 700–2600 m/z ; IRM pressure, 3

mTorr (intact protein mode); ion-transfer tube temperature, 275 °C; in-source fragmentation, 0–5 V; AGC target, 4×10^5 ; maximum injection time, 100 ms. Tandem MS analysis at selected charge states of the protein-ligand complex was performed by collision-induced dissociation (CID) with helium gas. Final spectra were obtained by averaging the signal over 30 s acquisition. A ligand-binding curve was generated calculating the relative intensity ratio of bound vs. total α -syn peak signals.

Fly culture and acteoside treatment

DJ-1^{ex54} mutant flies (hereafter called *DJ-1 β*) were used as PD model flies [27]. They were cultured on standard *Drosophila* food at 25 °C. For acteoside treatments, *DJ-1 β* mutant second instar (L2) larvae were cultured on standard medium containing 0.1 % dimethyl sulfoxide (DMSO) (untreated flies) or supplemented with 250 or 400 µM acteoside (GenoChem World). After eclosion, adult female flies were transferred to new tubes and cultured for five days in vials containing DMSO or acteoside. Subsequently, flies were directly evaluated in climbing assays or frozen in liquid nitrogen and kept at –80 °C for quantification of protein carbonylation level. The effect of acteoside was also evaluated in flies treated only after eclosion: 1-day-old *DJ-1 β* mutant female flies were transferred to new vials containing either 0.1 % DMSO (control) or acteoside-supplemented food (400 µM) and climbing assay was carried out 5 days and 10 days later.

Climbing assay and determination of carbonylation protein level

Locomotor performance of *DJ-1 β* mutant flies was analyzed using climbing assay, which exploits flies' innate negative geotaxis, an upward movement response following mechanical stimulation. This simple, robust, and cost-effective behavioral test is widely used to evaluate motor function in fly models of neurodegenerative diseases [28]. In these assays, a total of 80–120 5-day-old female flies were analyzed per condition. Groups of 10–20 female flies were transferred to graduated plastic tubes (1.4 cm diameter), acclimated for 1 min, gently tapped down to the bottom and allowed to climb for 10 s, recording the process. Moreover, climbing assays were also carried out in 5-day-old and 10-day-old female flies treated only after eclosion. The climbing ability of untreated (DMSO) and acteoside-treated flies was determined as the average of the height reached by each fly after this time [29]. Protein carbonyl groups were measured in extracts from *DJ-1 β* female flies of different ages and subjected to different treatment regimes using 2, 4-dinitrophenylhydrazine (DNPH) derivatization in 96-well plates (Greiner 96 well plate, polypropylene) as previously described [30].

Statistical analysis

All experiments were conducted in triplicate. Results are presented as mean $\pm$ standard deviation (SD). Statistical analyses were made using the two-tailed Student's t-test, with significance set at $p < 0.05$. For *Drosophila* experiments, groups were statistically compared by one-way ANOVA analysis followed by Tukey's post hoc test ($*p < 0.05$).

Results

Plant species selection

Within a collection of plant species belonging to the Italian flora, in the frame of the Italian initiative NBFC (National Biodiversity Future Center, <https://www.nbfc.it>), extracts from 60 plant species rich in specialized metabolites were selected and tested. While the chemical structures of primary metabolites are common across various species, specialized metabolites sharing core structures often exhibit a distribution linked to specific genetic lineages [31]. Thus, to facilitate a broad exploration of specialized metabolites with their bioactivities, species

from all major clades of Angiosperms were included in this investigation. The species, listed in [Supplementary Table S1](#), encompass monocots and eudicots, representing a total of 24 orders and 40 families. Additionally, two more primitive vascular species belonging to the Pteridophyta group were also included. Among the 60 analyzed species, the leaves of 11 plants (*Althaea officinalis*, *Aloysia citrodora*, *Artemisia abrotanum*, *Beta vulgaris*, *Castanea sativa*, *Diospyros kaki*, *Filipendula ulmaria*, *Lysimachia vulgaris*, *Rosa canina*, *Salvia pratensis*, *Verbascum thapsus*) are included in the Belfrit list, which defines a common reference for the use of plants in food supplements [32]. The inclusion of these species is particularly relevant, as it allows for a deeper evaluation of their specialized metabolites for public health applications [33].

To screen for the promising neuroprotective properties of this plant extract library, we conducted two complementary analyses: an *in silico* phylogenetic analysis to select potential medicinal plants, and a screening using a yeast model expressing the human protein α -syn, to select protective extracts against misfolded α -syn also in a cellular system.

Phylogenetic selection approach

Phylogenetic analyses identified 34, 45 and 50 hot trees related to antiaggregant, antiparkinsonian, and neuroprotective biological activities, respectively ([Supplementary Table S3](#)). The hot trees containing at least a taxa included in the list of 60 plants of NBFC project were plotted ([Supplementary Fig. S1](#)). All taxa included in hot trees were defined as Potential Medicinal Plants (PMP) if these were not already recognized as Medicinal Plants (MP) in the databases used. The plant species appearing in both the list of 60 plants and the hot trees are listed in [Table 1](#) specifying if these are MP or PMP. In [Fig. 1A](#) and [B](#) two examples of hot trees are plotted, wherein the clade encompassing the plant species identified by the phylogenetic approach applied in this study ([Table 1](#)) was enlarged for clarity. Altogether, our analysis identified 7 plants of our library associated with the antiaggregant biological function, 8 plants with the antiparkinsonian effect and 4 plants with the neuroprotective properties ([Table 1](#)). Therefore, a total of 11 plants (*Allium angulosum*, *Allium lusitanicum*, *Castanea sativa*, *Medicago marina*, *Salvia pratensis*, *Verbascum thapsus*, *Glaucium flavum*, *Acorus calamus*, *Althaea officinalis*, *Malva alcea*, *Artemisia abrotanum*), selected on the basis of the phylogenetic analysis ([Table 1](#)), have a high probability to present a medicinal value.

High-throughput screening of plant extracts with anti-aging and antioxidant effects

In parallel with the *in silico* analysis, a high-throughput screening on the 60 extracts of our library was conducted *in vivo* using a *S. cerevisiae* PD model, in which the human protein α -syn is overexpressed [34,35]. The Omnilog Biolog instrument was first employed to evaluate the effect of all plant extracts (at a concentration corresponding to 20 mg of fresh leaves/mL medium) on yeast cell growth. This step allowed us to exclude extracts exhibiting cytotoxicity, enabling a focused analysis of biologically relevant candidates to be considered in the following assays. Out of 60 plant extracts tested, only two obtained from *Heuchera sanguinea* and *Empetrum hermaphroditum*, showed relevant cellular toxicity and were thus excluded from further analysis ([Fig. 2A](#)). Of the other 58, 10 extracts did not significantly influence cell growth, while the remaining ones (48) stimulated the cell proliferation (expressed as maximal growth obtained after 72 h). On the basis of these data, we proceeded with a detailed analysis of the 58 non-toxic extracts in order to explore their potential anti-aging, antioxidant, and neuroprotective properties.

These 58 extracts were tested in the context of a standard chronological life span (CLS) experiment [36], to evaluate any ameliorating effect on the age-dependent α -syn mediated cell death, as we also recently reported [23]. Most extracts increased both mean and maximal

Table 1

Plant species appearing in both the list of 60 plants of NBFC-project and the hot trees subdivided for biological activity. In the category column: MP = Medicinal Plants, PMP = Potential Medicinal Plants.

Biological Activity	NBFC-project	Category
Antiaggregant	<i>Allium angulosum</i>	PMP
	<i>Allium lusitanicum</i>	PMP
	<i>Castanea sativa</i>	MP
	<i>Medicago marina</i>	PMP
	<i>Salvia pratensis</i>	PMP
	<i>Verbascum thapsus</i>	MP
	<i>Glaucium flavum</i>	MP
	<i>Acorus calamus</i>	MP
	<i>Allium angulosum</i>	PMP
Antiparkinsonian	<i>Allium lusitanicum</i>	PMP
	<i>Althaea officinalis</i>	MP
	<i>Castanea sativa</i>	MP
	<i>Malva alcea</i>	PMP
	<i>Artemisia abrotanum</i>	PMP
	<i>Salvia pratensis</i>	PMP
	<i>Allium angulosum</i>	PMP
	<i>Allium lusitanicum</i>	PMP
	<i>Artemisia abrotanum</i>	PMP
Neuroprotective	<i>Salvia pratensis</i>	PMP
	<i>Salvia pratensis</i>	PMP

lifespan of yeast cells (as indicated by the yellow color in the heatmap, [Fig. 2B](#)), except for those from *Glandularia tenera*, *Pseudosasa japonica* and *Acorus calamus*. Notably, 18 different extracts (*Echinophora spinosa*, *Acalypha virginica*, *Pulsatilla montana*, *Butomus umbellatus*, *Ranunculus lingua*, *Typha laxmannii*, *Centranthus ruber*, *Aquilegia atrata*, *Allium lusitanicum*, *Eryngium maritimum*, *Glaucium flavum*, *Verbascum thapsus*, *Succisa pratensis*, *Valeriana dioica*, *Salvia pratensis*, *Akebia quinata*, *Sternbergia lutea* and *Adenophora liliifolia*) exhibited very potent anti-aging activities, increasing mean lifespan more than 3.5 fold compared to the control condition. Coherently, these extracts also extended the maximum lifespan, although at different extents ([Fig. 2B](#)).

Alongside a decreased lifespan, oxidative stress is a typical feature of toxic protein aggregation induced by α -syn [37]; therefore the intracellular ROS level after 24 h treatment with the extracts were also quantified. Strikingly, 16 extracts (*Pulsatilla montana*, *Sternbergia lutea*, *Osyris alba*, *Rosa canina*, *Nonea lutea*, *Ranunculus lingua*, *Salvia pratensis*, *Malva alcea*, *Glaucium flavum*, *Succisa pratensis*, *Verbascum thapsus*, *Euonymus europaeus*, *Adenophora liliifolia*, *Allium lusitanicum*, *Inula salicina*, *Valeriana dioica*) decreased intracellular ROS level more than 2.5 fold ([Fig. 2B](#)). Therefore, 10 extracts showed the ability to attenuate both senescence and oxidative stress, two key hallmarks of age-related diseases ([Fig. 2C](#)), they derived from *Ranunculus lingua*, *Verbascum thapsus*, *Adenophora liliifolia*, *Valeriana dioica*, *Allium lusitanicum*, *Succisa pratensis*, *Salvia pratensis*, *Pulsatilla montana*, *Glaucium flavum* and *Sternbergia lutea*. These plants belong to different plant families but, with the exception of *Allium lusitanicum* that contains only saponins, the other plant extracts share some chemical similarities as eight species are characterized by the presence of flavonoids. Specifically, *Adenophora liliifolia*, *Ranunculus lingua*, *Succisa pratensis* and *Verbascum thapsus* have compounds belonging to the flavone class (e.i. luteolin and derivatives), while *Glaucium flavum*, *Pulsatilla montana*, *Sternbergia lutea* and *Valeriana dioica* have compounds belonging to the flavanol class (e.i. quercetin and derivatives). Furthermore, six of these ten plant extracts with antiaging and antioxidant activity (*Glaucium flavum*, *Pulsatilla montana*, *Ranunculus lingua*, *Salvia pratensis*, *Succisa pratensis* and *Valeriana dioica*) are characterized by the presence of compounds from the cinnamyl derivative class, known for their antioxidant properties [38].

From the combination of the three screening methods, i.e. phylogenetic identification as a medicinal plant, increase of mean lifespan (>3.5 fold) and decrease of ROS level (>2.5 fold), we identified 4 plant extracts responding to all three criteria ([Fig. 2C](#)). In fact, from the phylogenetic analysis, 11 species were identified as potential medicinal plants

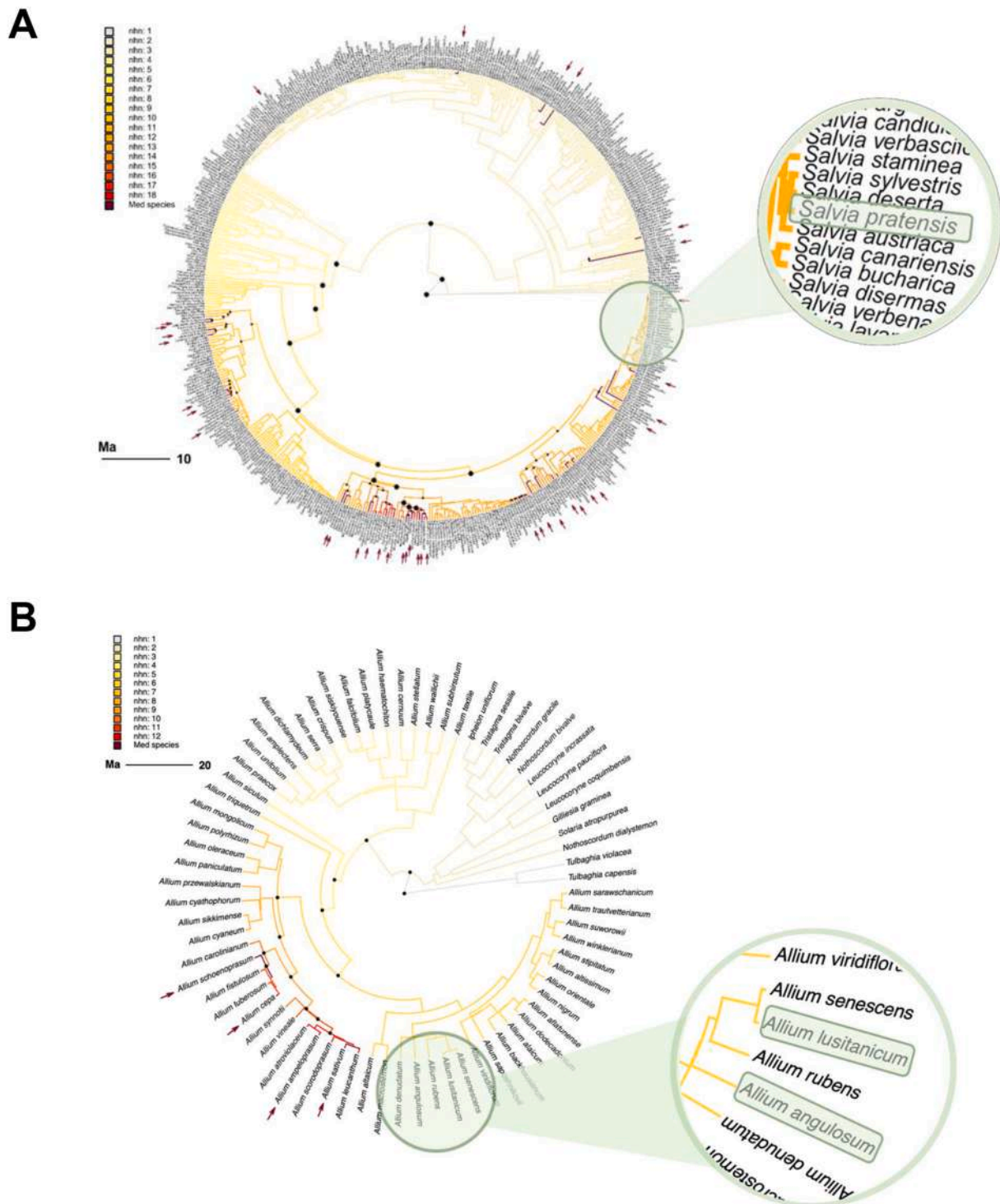
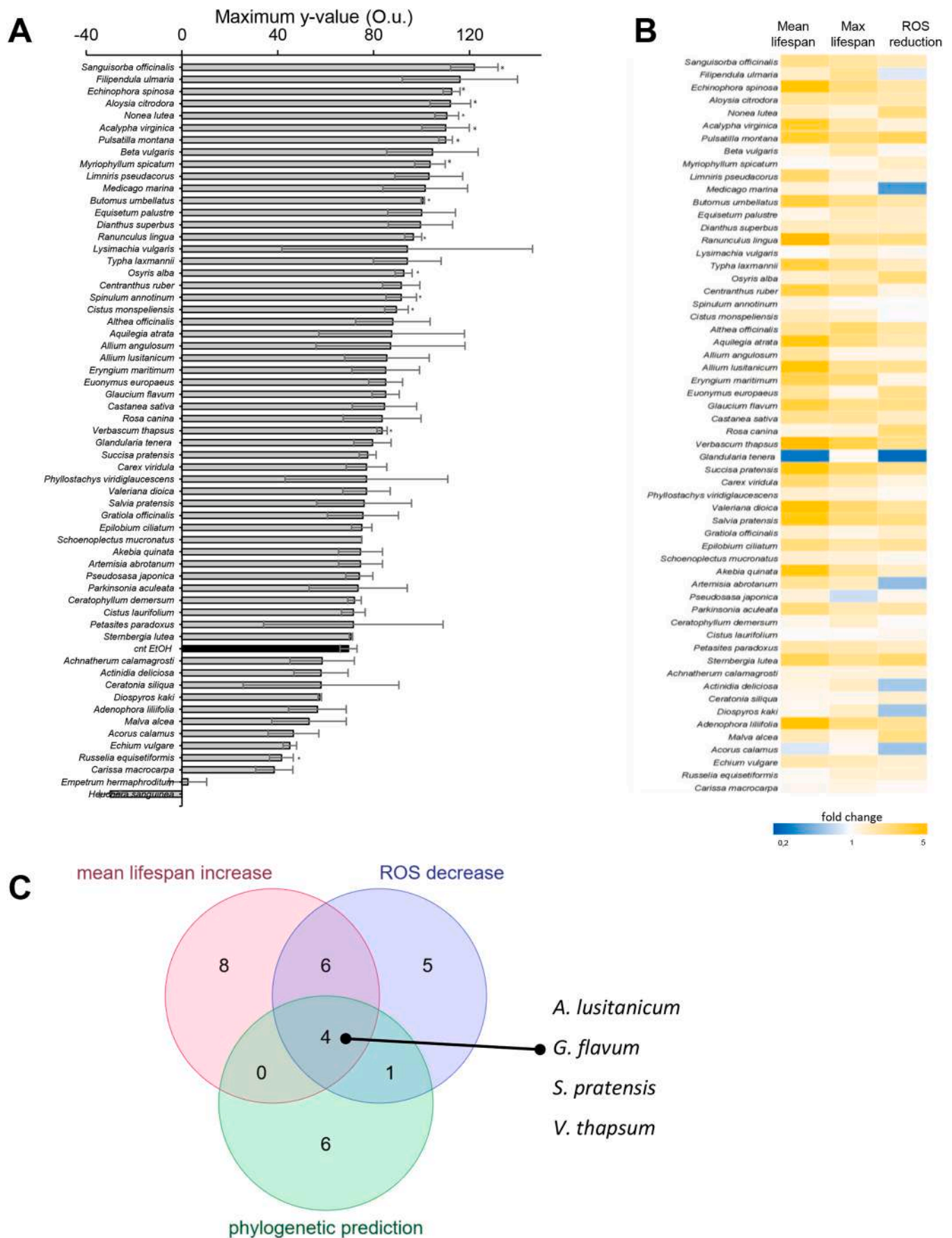


Fig. 1. Phylogenetic analysis. Phylogenetic hot tree associated with neuroprotective (A) and antiaggregant (B) biological activity. The branch colors increase from white to red proportionally to the number of nested hot nodes (nhn). Medicinal Plants (MP) are indicated by an arrow and their branches are highlighted in purple. All hot nodes are indicated with a black circle. Horizontal bar giving the scale of the branch lengths in million years (Ma).

(PMP) or medicinal plants (MP) (Table 1), but only 4 of them, *A. lusitanicum*, *G. flavum*, *S. pratensis* and *V. thapsus*, were also able to improve cell longevity (>3.5 fold increase) and reduce intracellular ROS (>2.5 fold decrease) in the yeast model (Fig. 2C). Consequently, the combination of these approaches underscores the potential preventive and therapeutic benefits of *A. lusitanicum*, *G. flavum*, *S. pratensis* and *V. thapsus* against neurodegenerative diseases such as Parkinson's disease.

Phytochemical profiles of *A. lusitanicum*, *G. flavum*, *S. pratensis* and *V. thapsus* extracts

Building on the promising biological findings, the phytochemical profiles of the above selected species were obtained and shown in Fig. 3 as base peak chromatograms recorded in negative ionization (only for *G. flavum* we reported also the positive ionization chromatogram to highlight the exclusive occurrence of alkaloids). The four species are characterized by



(caption on next page)

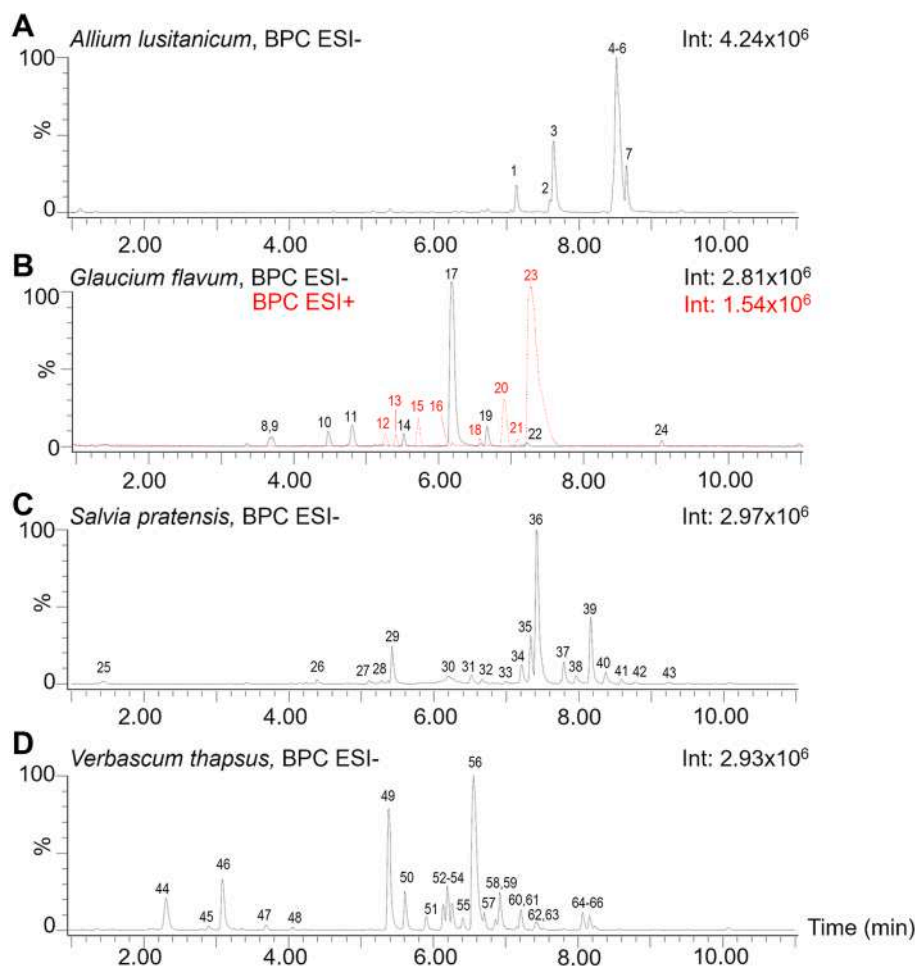


Fig. 3. Specialized metabolite-profiling of the selected plant extracts. Base peak chromatograms, in negative ionization mode, of *Allium lusitanicum* (A), *Glaucium flavum* (B), *Salvia pratensis* (C) and *Verbascum thapsus* (D). For *G. flavum* also the positive ionization chromatogram is shown in red. The ID of the peaks are the same as Table 2.

the presence of different classes of secondary metabolites, their identity and LC-MS features are reported in Table 2. *A. lusitanicum* accumulates different glycosides of steroidal saponins characterized by furostane, spirostane and cholestane skeletons (Fig. 3A, Table 2). A more exhaustive phytochemical characterization of this species is reported in a previous published work [15]. *G. flavum* is characterized by the accumulation of large amounts of glaucine and other aporphine/isoquinoline alkaloids, while rutin is the most abundant flavonoid among the polyphenols detected (Fig. 3B, Table 2). *S. pratensis* accumulates a wide array of polyphenols, mainly rosmarinic acid and its derivatives as well as other caffeic acid esters such as salvianolic acids (Fig. 3C, Table 2). *V. thapsus* presents a more diversified profile with the occurrence of iridoid glucosides (mainly catalpol) and their derivatives esterified to various hydroxycinnamic acids; moreover, this species presents large amounts of acteoside (Supplementary Fig. S2) and minor amounts of acteoside isomers and other phenylpropanoid/phenylethanoid glycosidic esters (such as leucosceptoside and martynoside) (Fig. 3D, Table 2).

The extracts of S. pratensis, V. thapsus and G. flavum inhibit α -syn aggregation

Recent studies indicate that a number of natural extracts can display a direct inhibition of amyloid fibril formation [6,39]. These findings have spurred interest in exploring plant-derived compounds as potential therapeutic agents targeting protein misfolding and aggregation.

To investigate these promising features, the anti-aggregant properties of the four selected extracts, *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* were evaluated through a multi-tiered approach, that integrates complementary systems, including yeast cultures and neuroblastoma cells expressing human α -syn, as well as direct protein interaction studies. This multifaceted strategy allowed us to explore their effects at various biological levels, ranging from cellular outputs to direct molecular interactions.

Firstly, we investigated whether the positive effects of the extracts on yeast longevity (Fig. 4A–C), also reported in the heatmap of Fig. 2B,

Fig. 2. Screening in yeast cells. (A) Effect of the extracts on cell growth: maximum y-value of growth kinetics of yeast cells overexpressing α -syn, grown in minimal medium containing 2 % glucose for 72 h in the absence (cnt) or presence of the extracts (corresponding to 20 mg fresh leaves/mL medium). The black bar identifies the control (cnt). * $p < 0.05$ relative to maximum y-value in control cells ($n = 3$). (B) Heatmap showing the effect of the plant extracts on mean lifespan, maximal lifespan and ROS reduction on yeast cells overexpressing α -syn. Data are expressed as fold change compared to control condition and are visualized using a color scale, with yellow indicating an increase and blue indicating a decrease of each parameter ($n = 3$). (C) Venn diagram of the plant extracts resulting from the phylogenetic analysis and the screening in yeast cells. The increase of mean lifespan of at least 3.5 fold and the reduction of ROS level of at least 2.5 fold have been considered. The extracts from *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* have been selected by all three screening systems.

Table 2

LC-MS features of the main specialized metabolites detected in the extracts of *Allium lusitanicum*, *Glaucium flavum*, *Salvia pratensis* and *Verbascum thapsus*. Peak IDs refer to peak numbers of Fig. 3. Abbreviations: FA, formic acid; Rt, retention time; UI, unidentified.

Peak ID	Compound	Rt (min)	Molecular formula	Precursor ion	m/z observed	Error (ppm)	Diagnostic product ions (m/z)	MSI level ^a
<i>Allium lusitanicum</i>								
1	Saponin class IV4 + 2 hexoses + desoxyhexose	7.13	C ₄₅ H ₇₄ O ₁₉	[M + FA-H] ⁺	963.481	−1.45	447.312; 609.364; 771.423; 917.480	2
2	Saponin class IV4 + 2						hexoses + desoxyhexose + acetyl group	7.60
	C ₄₇ H ₇₆ O ₂₀		[M + FA-H] ⁺	1005.492	−1.99		447.312; 609.364; 771.423; 917.480	2
3	Saponin class IV4 + 2						hexoses + desoxyhexose + acetyl group	7.65
	C ₄₇ H ₇₆ O ₂₀		[M + FA-H] ⁺	1005.492	−1.99		447.311; 609.364; 771.423; 899.468; 917.477; 959.485	2
4	Saponin class IV3 + 2 hexoses + 2 desoxyhexoses	8.46	C ₅₁ H ₈₄ O ₂₂	[M + FA-H] ⁺	1093.545	−1.55	431.319; 593.369; 755.426; 901.482; 1047.541	2
5	Saponin class IV3 + 2 hexoses + 3 desoxyhexoses	8.51	C ₅₇ H ₉₄ O ₂₆	[M − H] ⁺	1193.598	−2.51	431.315; 575.356; 593.369; 755.420; 883.470; 901.482; 1047.541	2
6	Saponin class IV3 + 2						hexoses + desoxyhexose + pentose	8.55
	C ₅₀ H ₈₂ O ₂₂		[M + FA-H] ⁺	1079.531	−3.71		431.319; 593.369; 755.426; 901.482	2
7	Saponin class IV3 + 2 hexoses + desoxyhexose	8.65	C ₄₅ H ₇₄ O ₁₈	[M + FA-H] ⁺	947.487	−1.79	431.315; 593.374; 755.426; 901.482	2
<i>Glaucium flavum</i>								
8	Vanillyl alcohol hexoside isomer 1	3.71	C ₁₄ H ₂₀ O ₈	[M − H] ⁺	315.108	−0.04		2
9	Vanillyl alcohol hexoside isomer 2	3.75	C ₁₄ H ₂₀ O ₈	[M − H] ⁺	315.108	−0.04	153.056; 135.046	2
10	Coumaroyl quinic acid	4.52	C ₁₆ H ₁₈ O ₈	[M − H] ⁺	337.0923	0.11	191.057; 163.041; 119.050	2
11	Feruloyl quinic acid	4.87	C ₁₇ H ₂₀ O ₉	[M − H] ⁺	367.1029	0.01	193.051; 149.061; 134.037	2
12	Laurifoline	5.27	C ₂₀ H ₂₄ NO ₄ ⁺	[M] ⁺	342.1705	0.07	297.114; 282.087; 265.087; 237.090; 222.070; 219.080; 207.080; 191.085; 165.072	2
13	Isoboldine/Salutaridine isomer	5.44	C ₁₉ H ₂₁ NO ₄	[M+H] ⁺	328.1549	−0.08	297.110; 285.12; 265.084; 239.069; 237.093; 192.101	2
14	Caffeoyl malic acid	5.57	C ₁₃ H ₁₂ O ₈	[M − H] ⁺	295.0454	−0.03	179.033; 135.046; 133.015; 115.003	2
15	Isoboldine or Salutaridine isomer 3	5.78	C ₁₉ H ₂₁ NO ₄	[M+H] ⁺	328.1549	−0.08	297.114; 265.087; 237.090; 233.060; 222.069; 205.066; 194.071; 177.069	2
16	Corydine/Isocorydine isomer	6.14	C ₂₀ H ₂₃ NO ₄	[M+H] ⁺	342.1689	4.75	236.083; 265.084	2
17	Rutin	6.17	C ₂₇ H ₃₀ O ₁₆	[M − H] ⁺	609.1456	−0.08	300.026; 271.026; 255.031	1
18	Corydine/Isocorydine isomer	6.61	C ₂₀ H ₂₃ NO ₄	[M+H] ⁺	342.1705	0.07	311.129; 279.095; 251.106; 236.083	2
19	Kaempferol hexose-deoxyhexose	6.70	C ₂₇ H ₃₀ O ₁₅	[M − H] ⁺	593.1506	0.06	285.041; 255.031; 227.035	2
20	Protopine	6.86	C ₂₀ H ₁₉ NO ₅	[M+H] ⁺	354.1341	0.12	336.121; 275.068; 247.073; 206.080; 188.071; 149.059	2
21	Norchelidonine	7.09	C ₁₉ H ₁₇ NO ₅	[M+H] ⁺	340.1164	6.15	275.068; 189.069; 247.077; 305.079; 323.091	2
22	UI	7.24	C ₂₀ H ₃₀ O ₁₀	[M − H] ⁺	429.1761	−0.09	249.113; 205.124; 179.052; 161.133; 119.039	2
23	Glaucine	7.26	C ₂₁ H ₂₅ NO ₄	[M+H] ⁺	356.1862	−0.07	325.142; 310.119; 294.128; 279.099; 251.106; 236.083	2
24	3β,23-dihydroxy-30-nor-olean-12,20-dien-28-oic acid hexose-hexuronic acid	9.08	C ₄₁ H ₆₂ O ₁₅	[M − H] ⁺	793.401	0.03	631.352; 455.312	2
<i>Salvia pratensis</i>								
25	UI	1.44	C ₁₀ H ₆ O ₈	[M − H] ⁺	252.9984	0.17	136.985; 116.928; 115.002; 71.008	
26	Caffeoyl threonic acid	4.39	C ₁₃ H ₁₄ O ₈	[M − H] ⁺	297.0629	−6.26	179.036; 161.029; 135.030; 117.017; 75.006	2
27	C ₉ H ₁₂ O ₃ -hexose-deoxyhexose	5.11	C ₂₁ H ₃₂ O ₁₂	[M + FA-H] ⁺	521.1863	1.38	475.184; 113.024; 329.124; 161.047; 149.061; 311.113; 167.074	3
28	Tuberonic acid hexoside	5.28	C ₁₈ H ₂₈ O ₉	[M − H] ⁺	387.1664	−2.33	207.103; 163.113	2
29	Rosmarinic acid hydrate	5.42	C ₁₈ H ₁₈ O ₉	[M − H] ⁺	377.0873	−0.12	359.0787; 197.0464; 179.0338; 161.0261; 135.0465; 133.0301	2
30	Salvianolic acid A	6.17	C ₂₆ H ₂₂ O ₁₀	[M − H] ⁺	493.1135	−0.07	313.0726; 295.0627; 185.0256; 109.0307	2
31	Luteolin-O-hexoside	6.51	C ₂₁ H ₂₀ O ₁₁	[M − H] ⁺	447.0943	−3.51	285.038	2
32	Rosmarinic acid hexose	6.70	C ₂₄ H ₂₆ O ₁₃	[M − H] ⁺	521.1287	1.55	359.075; 341.086; 323.077; 197.046; 179.034; 161.023; 135.044	2
33	Sagerinic acid	6.99	C ₃₆ H ₃₂ O ₁₆	[M − H] ⁺	719.1615	−0.41	161.026; 359.075; 197.044; 179.036; 341.067; 135.044	2
34	Luteolin acetylhexoside	7.19	C ₂₃ H ₂₂ O ₁₂	[M − H] ⁺	489.1029	0.81	447.090; 285.042	2
35	Salvianolic acid K	7.34	C ₂₇ H ₂₄ O ₁₃	[M − H] ⁺	555.1139	−0.07	493.115; 359.079; 295.059; 197.046; 179.034; 161.026; 135.046	2
36	Rosmarinic acid	7.41	C ₁₈ H ₁₆ O ₈	[M − H] ⁺	359.0767	−0.03	197.046; 179.036; 161.026; 135.046; 133.030; 123.046	1

(continued on next page)

Table 2 (continued)

Peak ID	Compound	Rt (min)	Molecular formula	Precursor ion	m/z observed	Error (ppm)	Diagnostic product ions (m/z)	MSI level ^a
37	Lithospermic acid	7.77	C ₂₇ H ₂₂ O ₁₂	[M – H] [–]	537.1033	–0.01	493.115; 359.079; 295.059; 221.044; 197.046; 179.036; 161.023; 135.044; 133.030	2
38	Chrysoeriol-(3-Hydroxy-3-methylglutaryl)-O-hexoside	7.96	C ₂₈ H ₃₀ O ₁₅	[M – H] [–]	605.1477	4.86	503.119; 461.107; 299.056; 284.034; 255.030	2
39	Octene alcohol-hexose-pentose	8.15	C ₁₉ H ₃₄ O ₁₀	[M + FA-H] ⁺	467.2129	–0.13	421.208; 289.169; 161.044; 149.046; 131.033; 113.024; 101.022	2
40	Methyl rosmarinate	8.37	C ₁₉ H ₁₈ O ₈	[M – H] [–]	373.0923	0.10	197.046; 179.036; 175.039; 160.017; 135.044; 123.046	2
41	UI	8.58	C ₂₈ H ₂₈ O ₁₅	[M – H] [–]	603.1355	–0.85	317.065; 137.025; 197.044; 87.008; 93.032	
42	UI	8.77	C ₂₁ H ₃₆ O ₁₀	[M + FA-H] ⁺	493.2289	–0.83	447.224; 315.180; 161.047; 359.083	
43	Salvianolic acid L	9.23	C ₃₆ H ₃₀ O ₁₆	[M – H] [–]	717.1456	–0.06	519.095; 338.052; 179.034; 161.023; 135.044	2
<i>Verbascum thapsus</i>								
44	Catalpol	2.30	C ₁₅ H ₂₂ O ₁₀	[M + FA-H] ⁺	407.1192	–0.62	199.061; 169.052; 181.052; 151.040; 163.039; 137.025	2
45	UI	2.89	C ₂₁ H ₃₂ O ₁₄	[M + FA-H] ⁺	553.1761	1.36	507.176; 345.121; 181.049; 315.103; 163.041; 151.039; 231.088; 327.111	
46	Aucubin	3.08	C ₁₅ H ₂₂ O ₉	[M + FA-H] ⁺	391.124	1.10	345.121; 179.053; 183.065; 165.054	2
47	Dihydroaucubin	3.69	C ₁₅ H ₂₄ O ₉	[M + FA-H] ⁺	393.142	–5.90	347.131; 185.081; 179.055; 167.072	3
48	Forsythoside	4.05	C ₂₀ H ₃₀ O ₁₂	[M – H] [–]	461.165	1.94	135.046; 113.025; 315.110; 297.100; 161.045	2
49	Hydroxy-saccatoside (Catalpol-O-caffeoyl-deoxyhexoside) isomer 1	5.39	C ₃₀ H ₃₈ O ₁₇	[M – H] [–]	669.205	–2.59	161.025; 133.029; 163.039; 179.033; 135.044	3
50	Hydroxy-saccatoside (Catalpol-O-caffeoyl-deoxyhexoside) isomer 2	5.61	C ₃₀ H ₃₈ O ₁₇	[M – H] [–]	669.205	–2.59	161.025; 133.029; 163.039; 179.033; 135.044	3
51	Saccatoside (Catalpol-O-coumaroyl-deoxyhexoside)	5.9	C ₃₀ H ₃₈ O ₁₆	[M – H] [–]	653.205	4.21	145.031; 377.124; 163.041; 491.157; 291.087; 117.035	3
52	Methoxy-saccatoside (Catalpol-O-feruloyl-deoxyhexoside)	6.14	C ₃₁ H ₄₀ O ₁₇	[M – H] [–]	683.219	–0.13	175.041; 160.016; 407.136; 193.051	3
53	Acteoside-pentose	6.19	C ₃₄ H ₄₄ O ₁₉	[M – H] [–]	755.241	–1.93	593.210; 161.025; 133.029; 447.151; 179.033; 135.046; 125.025	2
54	Apigenin-hexose-hexuronide	6.27	C ₂₇ H ₂₈ O ₁₆	[M – H] [–]	607.130	0.17	269.046; 431.102; 225.057	2
55	Vermiside (caffeoyl-catalpol)	6.41	C ₂₄ H ₂₈ O ₁₃	[M – H] [–]	523.147	–2.95	161.025; 179.033; 135.046; 133.029	2
56	Acteoside	6.56	C ₂₉ H ₃₆ O ₁₅	[M – H] [–]	623.197	1.58	161.025; 461.165; 133.029; 179.036; 135.046; 113.025	1
57	Leucosceptoside A-pentose	6.70	C ₃₅ H ₄₆ O ₁₉	[M – H] [–]	769.256	–0.01	593.210; 175.041; 160.016; 193.051; 447.151;	2
58	Acteoside isomer 1	6.86	C ₂₉ H ₃₆ O ₁₅	[M – H] [–]	623.197	1.42	161.025; 461.165; 133.029; 179.036; 135.046; 113.025	2
59	Acteoside isomer 2	6.92	C ₂₉ H ₃₆ O ₁₅	[M – H] [–]	623.197	1.42	161.025; 461.165; 133.029; 179.036; 135.046; 113.025	2
60	Scutellarioside II (p-coumaroyl-catalpol)	7.16	C ₂₄ H ₂₈ O ₁₂	[M – H] [–]	507.153	–4.45	145.028; 231.066; 345.098; 163.041; 119.050	2
61	Leucosceptoside A	7.21	C ₃₀ H ₃₈ O ₁₅	[M – H] [–]	637.214	–0.42	461.165; 175.041; 160.016; 193.051; 135.046; 113.025	2
62	Martynoside-pentose	7.41	C ₃₆ H ₄₈ O ₁₉	[M – H] [–]	783.273	–1.73	175.041; 607.226; 193.051; 160.016; 461.169	2
63	Leucosceptoside A isomer	7.43	C ₃₀ H ₃₈ O ₁₅	[M – H] [–]	637.214	–0.42	461.165; 175.041; 160.016; 193.051; 135.046; 113.025	2
64	Martynoside	8.07	C ₃₁ H ₄₀ O ₁₅	[M + FA-H] ⁺	697.237	–4.21	651.230; 175.041; 160.016; 193.051; 475.182	2
65	Octene alcohol-hexose-pentose	8.15	C ₁₉ H ₃₄ O ₁₀	[M + FA-H] ⁺	467.211	4.15	289.166; 421.206; 161.048; 101.025; 113.025	3
66	Martynoside isomer	8.23	C ₃₁ H ₄₀ O ₁₅	[M – H] [–]	651.230	–0.95	175.041; 160.016; 193.051; 475.182	2

^a According to metabolomics standards initiative (MSI).

could be associated with their influence on the formation of intracellular aggresomes. It is widely recognized that these inclusion bodies of aggregated, misfolded proteins can arise in response to different cellular stress. Their abundance increases during the stationary phase and is markedly elevated in cells overexpressing α -synuclein (Supplementary Fig. S3, [24]); indeed they represent a typical hallmark of PD and other neurodegenerative diseases [40]. Notably, treatment with all four extracts resulted in a reduction of intracellular aggresomes, albeit to varying extents, ranging from 40 % (*G. flavum*) to a stronger reduction of

60 % (*A. lusitanicum* and *V. thapsus*) and 80 % (*S. pratensis*) (Fig. 4D). To further evaluate the ability of the selected extracts to prevent protein misfolding, SH-SY5Y neuroblastoma cell cultures conditionally expressing α -syn were also used [41]. Cells were treated with doxycycline to induce the overexpression of α -syn in the presence of the extracts, and the toxic oligomeric species of α -syn were detected following 48 h treatment. Notably, a significant decrease in α -syn oligomers was observed with *S. pratensis*, *V. thapsus*, and *G. flavum* extracts (around 40 %) (Fig. 5A–B), which also slightly affected cell viability

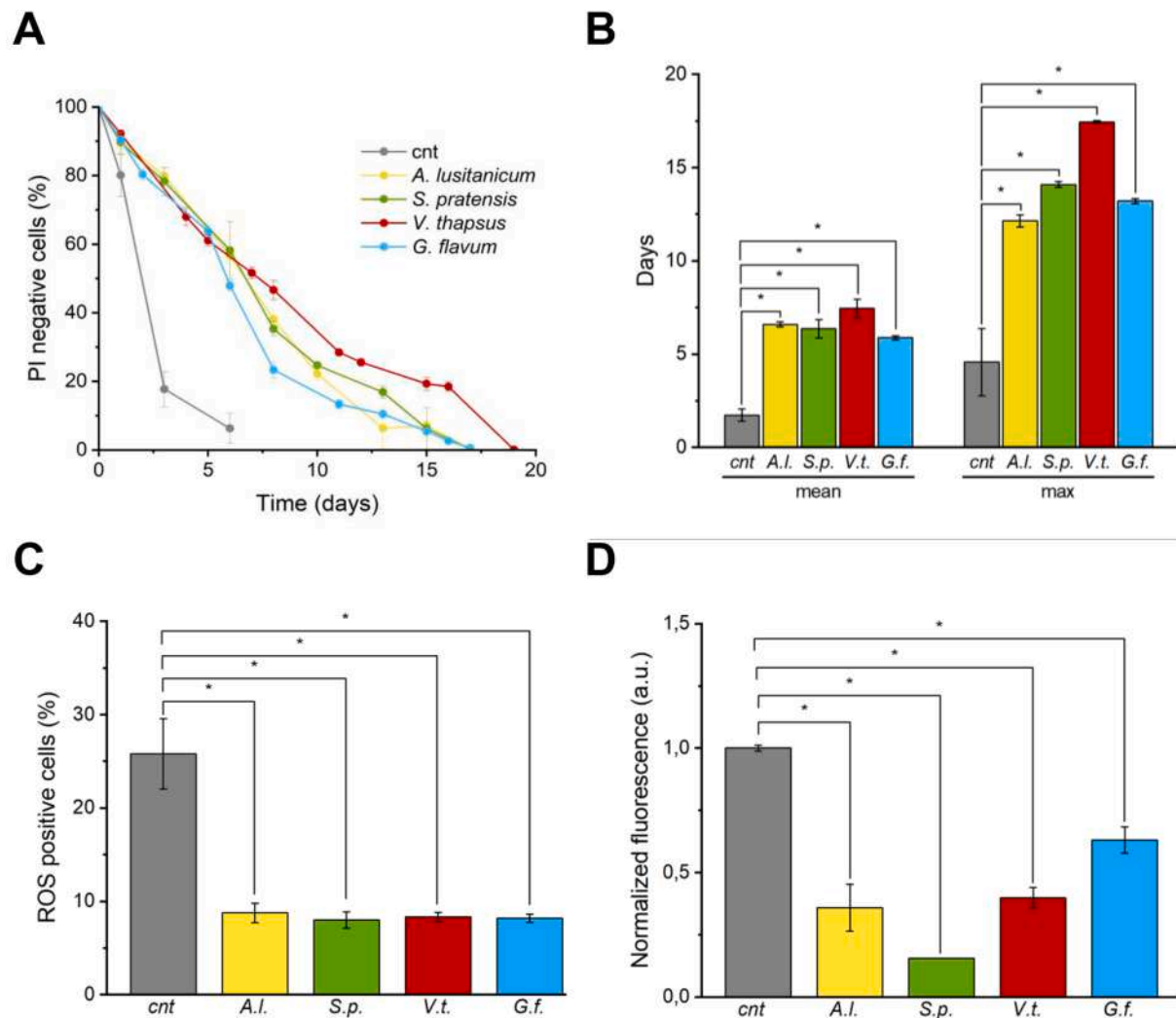


Fig. 4. The extracts from *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* extend yeast lifespan. (A) CLS of yeast cells overexpressing α -syn grown in medium containing 2 % glucose in the absence or presence of *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* extracts (at a concentration corresponding to 20 mg of fresh leaves/mL medium), added in exponential phase of growth. (B) Mean and maximal lifespan of cells in (A). (C) ROS content of cells treated for 24 h as in (A). (D) Aggregates level of cells treated for 24 h as in (A). Histograms represent mean $\pm$ SD of at least three independent experiments. * $p < 0.05$.

(Supplementary Fig. S4A). Despite the difference in oligomeric forms, the total level of α -syn (both endogenous and overexpressed, which bears a FLAG-tag that enables to discriminate it from the endogenous protein) did not vary significantly with the treatments (Fig. 5C). In contrast, the extract from *A. lusitanicum*, which strongly reduced cell viability (Supplementary Fig. S4A), did not lower α -syn oligomer level at all (Fig. 5A–B). We speculate that its toxicity could overcome the potential activity of other beneficial compounds within the extract. Alternatively, these active molecules exhibited very low permeability, resulting in no detectable positive effect on cells.

Considering these results, we next investigated whether the extracts could inhibit the aggregation of the purified α -syn protein. To this end, we took advantage of the Thioflavin T (ThT) fibrillation assays to monitor α -syn aggregation over 72 h. In the absence of the extracts, ThT fluorescence exhibited a strong increase, indicating substantial amyloid fibril formation (Fig. 5D), as already known [25,42]. Strikingly, the presence of the extracts completely abolished this signal, with residual ThT fluorescence level of less than 2 % for *A. lusitanicum* and *G. flavum*, and 9 % and 17 % for *V. thapsus* and *S. pratensis*, respectively (Fig. 5D). The inhibition of fibril formation was also confirmed with a dot blot using A11 antibody, which specifically recognizes oligomeric α -syn

(Fig. 5E) and by TEM analysis (Fig. 5F). The results obtained strongly highlight that all the four extracts inhibit the aggregation of the purified α -syn protein *in vitro*.

Then, surface plasmon resonance (SPR) analysis was employed to assess direct interactions between the extracts and α -syn protein by immobilizing the protein on a CM5 sensor chip (as previously reported in Ref. [23]). The anti- α -syn antibody bound to the protein on the chip, whereas the oligomer-specific anti-Syn33 antibody did not (Supplementary Fig. S5A), confirming that the immobilized α -syn was in its monomeric form. This system was then used to test direct interactions between α -syn and the extracts from *A. lusitanicum*, *S. pratensis*, *V. thapsus*, and *G. flavum*. All extracts showed concentration-dependent binding to α -syn, with increasing extract concentrations producing higher SPR signals (Supplementary Fig. S5B–I). These results, along with previous ones, suggest that the extracts contain compounds that directly bind α -syn and inhibit its amyloid aggregation.

In conclusion, integrating data from yeast, neuroblastoma cells, and SPR analysis, extracts from *S. pratensis*, *V. thapsus*, and *G. flavum* consistently inhibited the formation of toxic α -syn oligomers across all models, indicating the presence of anti-aggregant compounds.

Acteoside from *V. thapsus* binds α -syn

In order to identify which metabolites present in the extracts can directly bind to α -syn protein, the extracts from *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* were analyzed by size exclusion chromatography coupled to reversed phase LC-MS (SEC AS-MS). We decided to make this analysis considering all the extracts because the one from *A. lusitanicum* was able to affect α -syn aggregation *in vitro* (Fig. 5D–F), despite its inconsistent effect on neuroblastoma cells (Fig. 5A–B). An already established ligand fishing assay, based on size exclusion chromatography coupled to reversed phase LC-MS for the analysis of botanical extracts, was modified for the affinity screening of the four extracts toward α -syn [15].

Each extract was incubated with α -syn and injected in the SEC column to isolate and collect α -syn complexes. Bound ligands were desorbed from the trap column and analyzed by RPLC-MS. The LC-MS chromatograms of *G. flavum* and *S. pratensis* extracts obtained by the described fishing method did not reveal the presence of any bound ligands. As concerns the *A. lusitanicum* extract, we observed the fishing of 3 different saponins, putatively identified through the classification of the aglycone class and the number and type of sugars attached to it, in accordance with Geng et al. 2021 [43]: saponin class IV3 +2 hexoses +3 desoxyhexoses, saponin class IV3 +2 hexoses +1 desoxyhexose +1 pentose, saponin class IV3 +2 hexoses +1 desoxyhexoses (Supplementary Fig. S6A–C).

Notably, in the case of *V. thapsus* extract, the most abundant metabolite acteoside was successfully fished as reported in Fig. 6A. In particular, the chromatogram shows two peaks at retention times 27.35 and 28.20 corresponding to both isoforms of the molecule, able to bind α -syn (Fig. 6A, lower panel). These peaks correspond to acteoside and its isomer, and were identified by extracting the ionic current in the mass spectrum. Their presence was confirmed through fingerprinting and by comparing their mass values with those of the main molecules in the extract, as reported in Table 2. In the non-treated extract, they appeared at retention times 27.25 and 28.09 (Fig. 6A, upper panel), while in the SEC-AS-MS analysis of the incubated extract with α -syn, they shift slightly to 27.35 and 28.20. Interestingly, in the *V. thapsus* extract there are different structurally related compounds, such as leucosceptoside (phenylethanoid glycosides) or verminoside (caffeoyl-catalpol), but only acteoside was able to bind α -syn in the SEC AS-MS analysis, supporting the specificity of this binding.

Then, our analysis highlights that saponins from the extract of *A. lusitanicum* and acteoside from *V. thapsus* were able to bind α -syn protein. Interestingly, the abundance of the fished metabolites was always lower in the incubated samples compared to the extracts, as the result of the binding equilibrium.

We decided to focus our attention on the molecule acteoside (Fig. 6B), which is well known for its neuroprotective functions [44], and the interaction between α -syn and acteoside was further investigated by biophysical methods. Native-MS was employed to directly detect the protein-metabolite complex, thanks to the preservation of non-covalent interactions during the analysis. The nano-ESI-MS spectrum of α -syn under non-denaturing conditions (Supplementary Fig. S7A) showed a multimodal charge state distribution, in agreement with the heterogeneous conformational ensemble populated by the intrinsically disordered polypeptide chain. The titration of α -syn with acteoside highlighted the comparison of new peaks in the spectrum, with a position that corresponds to the mass of the 1:1 protein-ligand complex (Fig. 6C). These new signals increased in intensity at high metabolite concentrations, and mainly involve the low- and medium-charge states of the protein, corresponding to the compact and intermediate conformational components (Fig. 6D). The complex identity of the signals was further confirmed by collision-induced dissociation in gas-phase, with the detection of the unbound partners upon collision with helium atoms (Supplementary Fig. S7B). Despite the semi-quantitative nature of Native-MS experiments, the simultaneous

detection of free and bound protein states allowed the determination of a binding curve (Fig. 6E) and a rough estimation of the (apparent) dissociation constant of the complex ($K_d \sim 0.9$ mM). These results indicate a low affinity of α -syn for the metabolite, and are in agreement with the ones obtained by SPR. Indeed, this analysis also showed a direct binding of acteoside to α -syn, with a progressive increase of SPR-signal with ligand concentration. However, the accurate determination of the binding parameters was not possible, due to the lack of saturation caused by the very low affinity of the metabolite for the protein (Supplementary Fig. S8).

To conclude, considering its interaction with α -syn protein, the molecule acteoside from *V. thapsus* was considered for extended investigation.

Acteoside inhibits α -syn aggregation

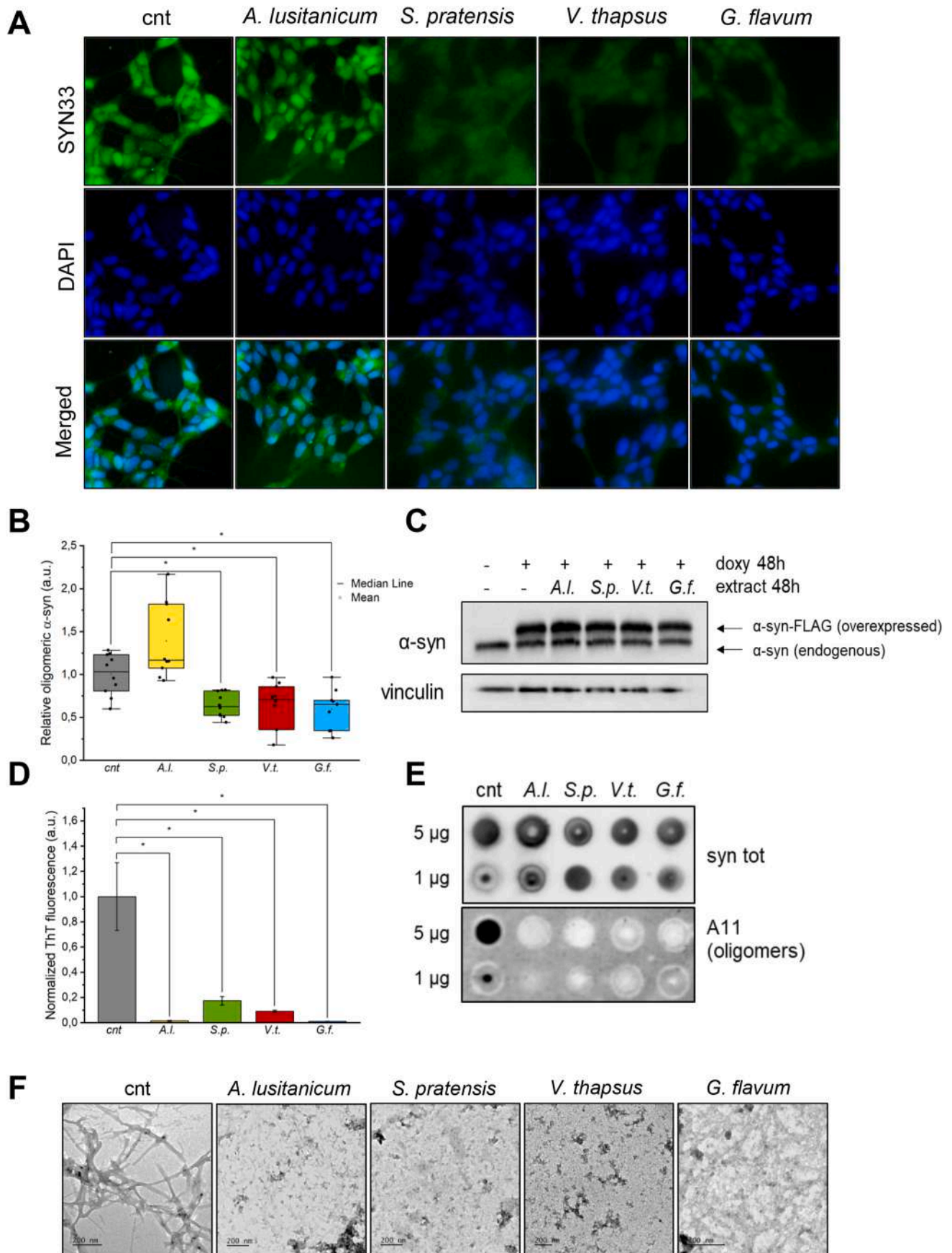
Building upon the identification of acteoside as a key metabolite binding to α -syn, we further elucidated its potential neuroprotective properties. Acteoside (Fig. 6B), the predominant phenylpropanoid glycoside in *V. thapsus* extract (Fig. 3C, Table 2), is commonly found in a variety of plants, especially of the *Verbascum* species, but also in more than 200 other plant species belonging to 23 different families, and it is well known for its strong antioxidant activity [45]. In order to investigate the direct effect of acteoside on the protein aggregation process, the Thioflavin T (ThT) assay was used. The incubation of α -syn (35 μ M) in the presence of 100 μ M or 50 μ M acteoside revealed that the molecule was exceptionally active, abrogating almost completely the formation of fibrils, with residual ThT fluorescence level of less than 1 % at both concentrations tested, similarly to the effect exerted by the whole *V. thapsus* extract (Fig. 7A). To better understand the inhibition of α -syn aggregation, we also investigated the protein aggregation kinetics. Notably, we did not observe the characteristic curve of α -syn aggregation when the protein was incubated with 100 μ M and 50 μ M of acteoside (Fig. 7B). This finding suggests that acteoside inhibits the formation of nucleation sites, which are essential for the initiation of the fibrillation phase and occur approximately 24 h from the beginning of the aggregation assay, as seen in the control sample (Fig. 7B). Remarkably, the inhibition of α -syn primary nucleation was also confirmed by the visualization of α -syn fibrils by TEM. Indeed, fibril formation was clearly visible when α -syn was incubated alone, while the presence of acteoside completely abolished the formation of the fibrillar species (Fig. 7C).

We next analyzed whether acteoside can influence fibril elongation [46], by studying aggregation of monomeric α -syn in the presence of preformed fibrils (PFF) of α -syn that act as seeds for secondary nucleation. Strikingly, we observed that acteoside inhibited fibril elongation in a dose-dependent manner: 1 μ M acteoside did not affect the fibril elongation phase, whereas at higher concentrations (25 μ M, 50 μ M and 100 μ M) fibril elongation was completely abolished, as highlighted by the absence of the characteristic curve of aggregation (Fig. 7D).

Altogether, these data indicate that acteoside plays a key role in the anti-aggregant effects of *V. thapsus* extract, influencing both primary nucleation and fibril elongation phases.

Acteoside activates the NRF2 signaling pathway

Before investigating acteoside effects on cell cultures, its cellular availability was assessed in human neuroblastoma cells by measuring intracellular/cell-associated level of acteoside in cells treated with 100 μ M of acteoside, a concentration which was not toxic up to 72 h (Supplementary Fig. S4B). As shown in Fig. 8A, the highest intracellular/cell-associated acteoside levels were observed after 1 h of treatment, and it gradually decreased over time. Then, in order to confirm the protective role of acteoside against oxidative stress, which is strictly connected with the onset of PD [47], SH-SY5Y cells were exposed to 5 μ M extracellular α -syn aggregates, obtained in the absence or



(caption on next page)

presence of 1 mM acteoside. Intracellular ROS level induced by PFF decreased by 54 % with acteoside in comparison with the control (Fig. 8B).

Strikingly, in SH-SY5Y neuroblastoma cells conditionally expressing α -syn, treatment with 100 μ M acteoside reduced the level of aggregated α -syn, as measured by immunofluorescence analysis using an antibody specific for the oligomers (Fig. 8C), without affecting the total level of α -syn, both endogenous and overexpressed (which bears a FLAG tag that enables to discriminate it from the endogenous protein, Fig. 8D).

To investigate the molecular mechanism underlying acteoside's antioxidant activity, we focused on NRF2, a well known pathway responsible for mediating antioxidant responses and maintaining redox homeostasis [48]. Indeed, we observed an increase in NRF2 protein level in cells treated with acteoside (Fig. 8E and F). Moreover, a clear nuclear enrichment of NRF2 was observed upon acteoside treatment (Fig. 8G and H). Accordingly, the mRNA level of two NRF2-dependent genes (NQO1 and HMOX1) increased in cells treated with acteoside (Fig. 8I), supporting NRF2 functional activation. In addition, pretreatment with acteoside prevented the increase of ROS level in cells treated with fibrils, but this effect was not observed when acteoside was added together with the NRF2 inhibitor (Fig. 8J). Altogether, these findings suggest that acteoside reduces intracellular ROS level through the activation of the NRF2 signaling pathway in neuroblastoma cells, in line with its antioxidant and neuroprotective effect.

Acteoside improves locomotor performance and reduces oxidative protein damage in a *Drosophila melanogaster* model of PD

Considering the antioxidant efficacy of acteoside in various biological systems [49–52], including rotenone-treated flies (a *Drosophila* model of sporadic PD) [53], we tested acteoside in a *Drosophila* PD model based on *DJ-1* inactivation. The *DJ-1* protein plays a central role in cellular defense against oxidative stress and is involved in ROS scavenging, transcriptional regulation, redox-dependent chaperone activity, mitochondrial function, and anti-apoptotic signaling [54]. Accordingly, *DJ-1* mutant flies exhibit several PD-relevant phenotypes, including locomotor deficits, reduced lifespan, ROS accumulation, and hypersensitivity to oxidative toxins [29,55,56]. Thanks to its high pharmacological responsiveness, this *Drosophila* PD model has been successfully used to evaluate the therapeutic potential of multiple compounds with different mechanisms of action [29,56–58]. Besides, it should be mentioned that mutations in the *DJ-1* protein cause autosomal recessive forms of PD, and oxidatively damaged *DJ-1* protein accumulates in the brains of patients with idiopathic PD [59]. Therefore, to assess the potential therapeutic effect of acteoside, *DJ-1* mutant flies were cultured on medium supplemented with the compound throughout development, from larval stage to five days post-eclosion. To determine the optimal dose for acteoside supplementation in flies, two concentrations of the compound were used (250 μ M and 400 μ M) and locomotor performance was assessed via climbing assays in 5-day-old flies. Acteoside administration resulted in a significant, dose-dependent improvement in motor function, producing a beneficial effect at both tested concentrations (Fig. 9A).

DJ-1 mutant flies also exhibit increased ROS production, resulting in oxidative damage of cellular components [57,60]. A notable marker

of oxidative damage is protein carbonylation, whose level is elevated in *DJ-1* mutant flies [61]. To determine whether acteoside influences oxidative protein damage, we measured protein carbonylation level in extracts from 5-day-old *DJ-1* mutant flies treated with vehicle or with the compound at the tested concentrations (250 μ M and 400 μ M). Consistent with the climbing assay results, flies treated with 400 μ M acteoside exhibited a significant reduction of protein carbonyl groups (Fig. 9B), indicating that this compound can attenuate oxidative stress-induced protein damage, likely contributing to the observed improvements in motor performance. Despite the strong effect on motor function, treatment with 400 μ M acteoside had no significant impact on fly longevity (Supplementary Fig. S9), suggesting that it may improve healthspan rather than extend lifespan.

To further evaluate whether acteoside could exert a beneficial effect also when administered in adulthood, more accurately reflecting the timing of PD, flies were treated with acteoside (at a concentration of 400 μ M) after eclosion and climbing assays were performed 5 and 10 days following treatment initiation. As shown in Fig. 9C, acteoside significantly improved motor ability at both time points, demonstrating that it retains its efficacy also when administered post-eclosion. In agreement with these results, protein carbonylation level was also significantly decreased after 10 days of treatment (Fig. 9D). Therefore, the beneficial effect of acteoside emerges not only with early supplementation but also when treatment is initiated at advanced stages, reflecting the temporal dynamics and progression of PD pathology.

In conclusion, the observed functional recovery in *DJ-1* mutants suggests that acteoside can effectively counteract deficits linked to *DJ-1* dysfunction, thereby modulating PD-related phenotypes and validating its therapeutic potential.

Discussion

Plant biodiversity is an exceptional source of secondary metabolites, nevertheless no more than 15 % of higher plants have been systematically investigated for medicinal properties [62]. Consequently, the approach of bioprospecting, which promotes the research in biodiversity for new resources with potential income, remains a valuable opportunity to identify new bioactive compounds, or new sources of known compounds, increasing the value of biodiversity and supporting its conservation. Natural substances play a central role in creating innovative solutions for disease prevention/treatment and the advancement of analytical techniques, combined with high-throughput screening methods, can significantly improve the process of drug discovery. The biodiversity of the Mediterranean hotspot [11] is an extraordinary resource of bioactive compounds for advancing innovative therapies targeting non-communicable diseases, including neurodegenerative disorders.

A growing number of patients worldwide suffer from PD and the currently available drugs, which regulate dopaminergic and cholinergic transmission, primarily aim to control symptoms and improve patients' quality of life. However, there is still no definitive cure for this disease, making it necessary to develop new drugs, with particular attention to preventive strategies.

In this paper, we present a multidisciplinary approach to examining 60 different Italian plants representative of part of the Mediterranean

Fig. 5. The extracts from *S. pratensis*, *V. thapsus* and *G. flavum* reduce α -syn aggregation. (A–B) SH-SY5Y *pTet-SNCA-FLAG* cells were treated with 6 μ g/mL doxycycline to induce α -syn overexpression, in the absence or presence of the extracts from *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum* for 48 h. Representative immunofluorescence images (63x) of fixed cells, immunostained with anti-Syn33 antibody (specific for oligomeric species). Nuclei were stained by DAPI (blue). Graphs in B represent mean $\pm$ SD of oligomeric α -syn fluorescence quantified as Integrated Density (IntDen) using ImageJ software. (C) SH-SY5Y *pTet-SNCA-FLAG* cells were treated with 6 μ g/mL doxycycline to induce α -syn overexpression, in the absence or presence of the extracts from *A. lusitanicum*, *S. pratensis*, *G. flavum* and *V. thapsus* for 48 h. The level of α -syn (both endogenous and FLAG-tagged overexpressed proteins) was evaluated by Western blot. Vinculin was used as loading control. (D) α -syn aggregation process, followed by ThT fluorescence, in the absence (cnt) or presence of *A. lusitanicum*, *S. pratensis*, *V. thapsus* or *G. flavum* extracts for 72 h. (E) Dot-blot analysis of α -syn (1 and 5 μ g) after 72 h of aggregation in the absence (cnt) or presence of *A. lusitanicum*, *S. pratensis*, *V. thapsus* or *G. flavum* extracts using A11 anti-oligomer antibody and anti- α -syn antibody as a control. (F) TEM images of α -syn after 72 h of aggregation in the absence (cnt) or presence of *A. lusitanicum*, *S. pratensis*, *V. thapsus* or *G. flavum* extracts. Data represent mean $\pm$ SD from three independent experiments.

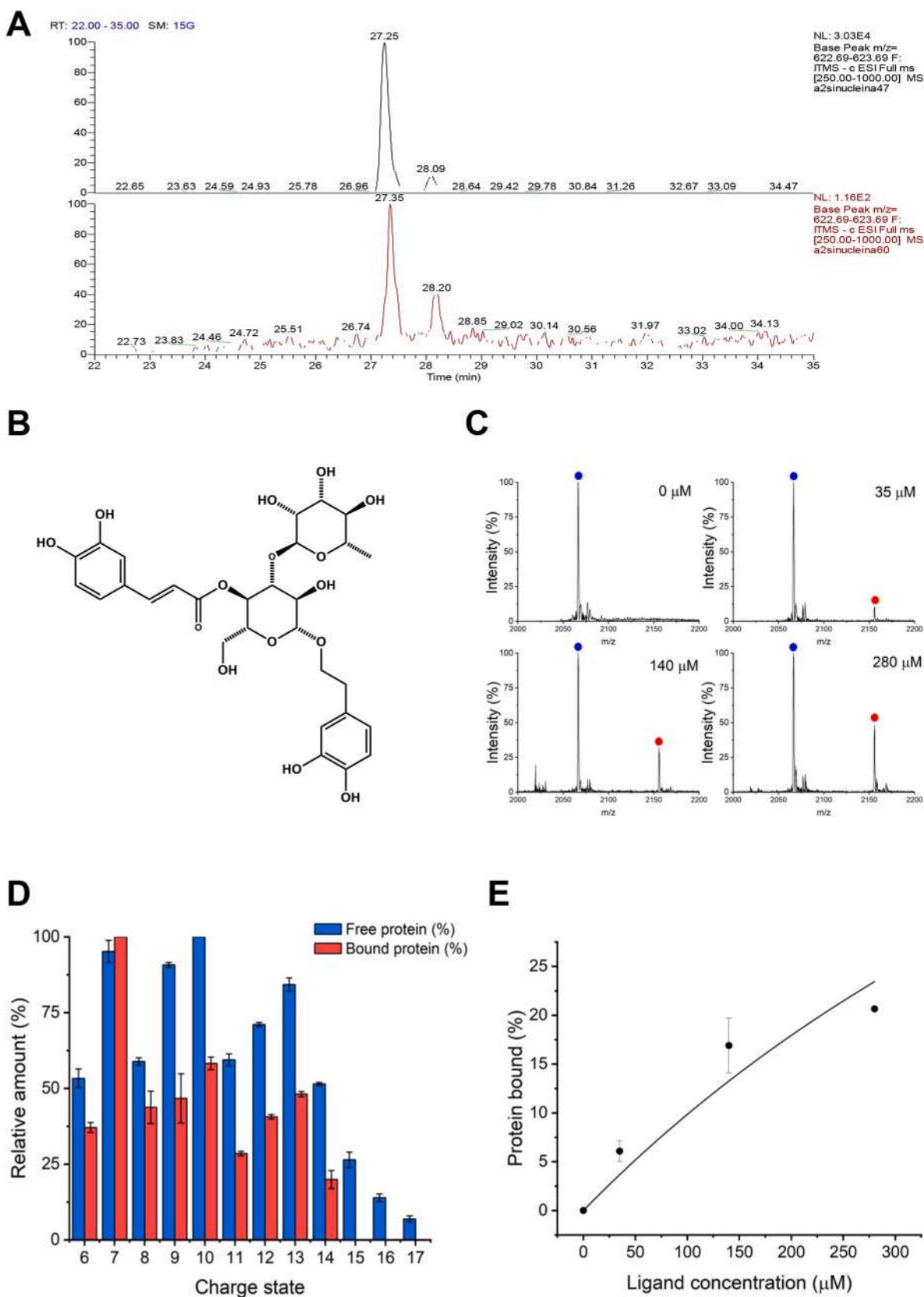


Fig. 6. Acteoside binds to α -syn. (A) RPLC-MS chromatograms in negative ion mode of *V. thapsus* crude extract (upper panel) and *V. thapsus* crude extract fished by α -syn (lower panel). Extracted ion acteoside, 623.19 m/z . (B) Chemical structure of acteoside. (C) Acteoside- α -syn interaction by Native-MS. Enlargement in the region of α -syn 7+ charge state of Native-MS spectra, obtained at distinct acteoside concentrations (0–280 μ M). The peaks corresponding to unbound α -syn (blue circle) and 1:1 α -syn-acteoside complex (red circle) are labeled. (D) Histogram reporting the fraction of unbound α -syn (blue) and α -syn-acteoside complex (red) as a function of the analyte charge state. (E) Fraction of protein in the bound state vs. total ligand concentration. The solid line represents the non-linear fit of the experimental data with a 1:1 binding curve ($K_d \sim 0.9$ mM).

flora. We combined an *in silico* screening system based on phylogenetic methods with a high-throughput screening using as a model the budding yeast expressing human α -syn.

This approach allowed a rapid and economic high-throughput analysis and led us to focus on phytoextracts from four plants, namely *A. lusitanicum*, *S. pratensis*, *V. thapsus* and *G. flavum*, which are promising sources of neuroprotective compounds for the preventive treatment of PD, being strongly active in reducing the toxic intracellular species of aggregated proteins. Notably, acteoside, one of the major metabolites found in the *V. thapsus* extract, emerges as the key molecule that binds α -syn. Of paramount importance, *V. thapsus* is already included in the Belfrit list [32], supporting its utilization as a public health product [33]. Acteoside, also known as verbascoside, was first isolated in 1963 from *Verbascum sinuatum* L., belonging to the family *Scrophulariaceae* [45]. Chemically, acteoside (Fig. 6B), with a formula of $C_{29}H_{36}O_{15}$ and a molecular weight of 624 Da, is a phenylethanoid glycoside [63]. Acteoside contains a hydroxyl salidroside residue condensed with caffeoyl and rhamnose moieties, and is among the most widespread disaccharide caffeoyl esters. While mainly detected in *Verbascum*

species, acteoside has also been found in 23 different plant families [45, 64]. It is extracted from various traditional Chinese medicine sources including *Rehmannia glutinosa*, *Cistanche deserticola* and *Pedicularis kansuensis* [65] and is considered a crucial secondary metabolite in folk medicine [66,67] with a variety of pharmacological activities [68–72]. Several studies have reported that acteoside exerts strong antioxidant activities, and it is especially able to enhance the endogenous antioxidant defences of the cell (for a review see Ref. [45]). The neuroprotective effects of acteoside have been linked not only to its ability to mitigate oxidative stress, but also to inhibit neuroinflammation and to modulate autophagy [53,67].

Here we also show that acteoside directly binds to α -syn and inhibits its amyloid fibrils formation (Figs. 6 and 7), a key event in the prevention of PD. Specifically, since α -syn aggregation involves both nucleation and growth phases [73], we show under different aggregation conditions that acteoside inhibits primary nucleation, by preventing nuclei formation during the lag phase (Fig. 7B and C), as well as fibril-induced amplification during the growth phase (Fig. 7D), indicating that acteoside functions as a protein aggregation inhibitor at multiple stages of the

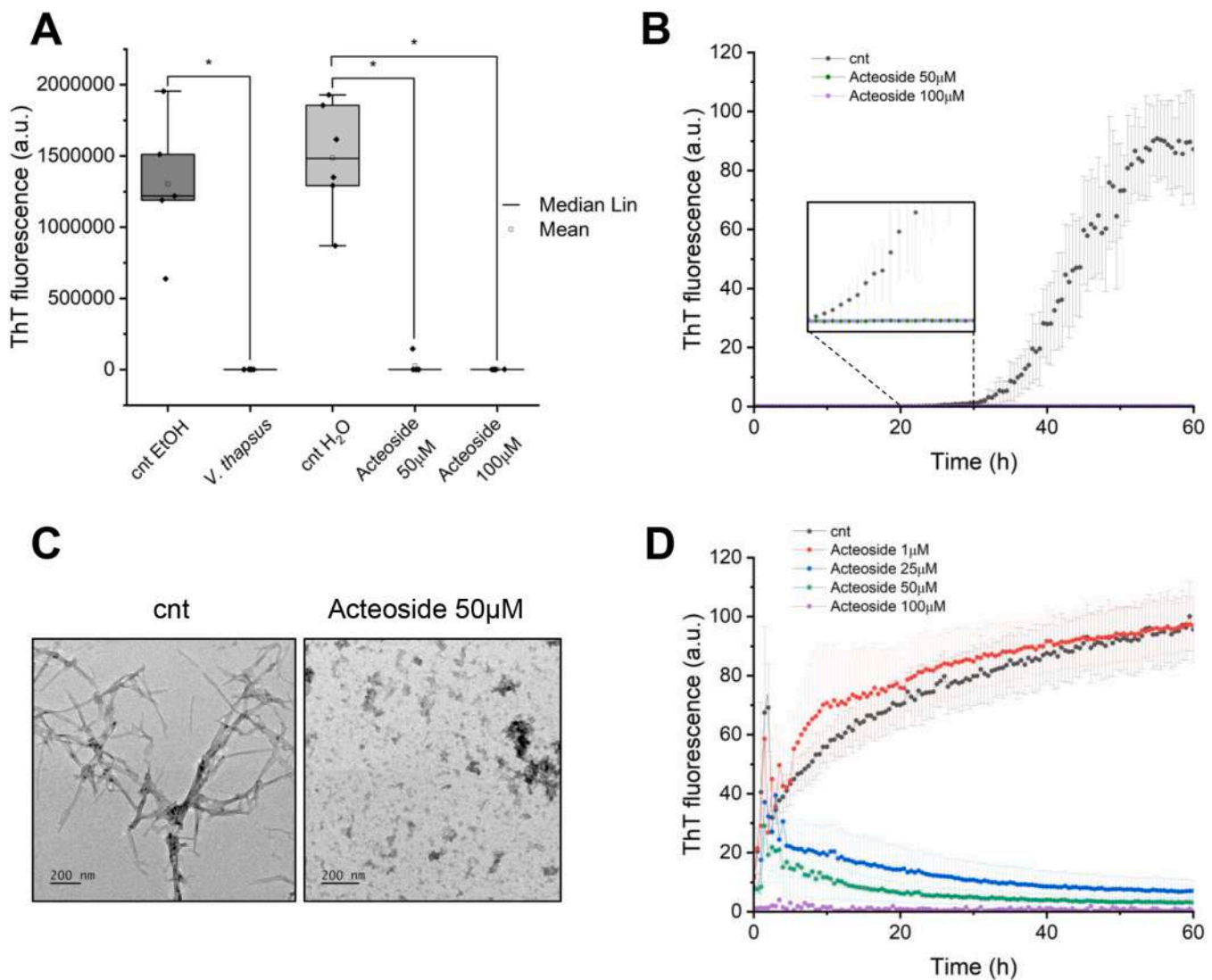
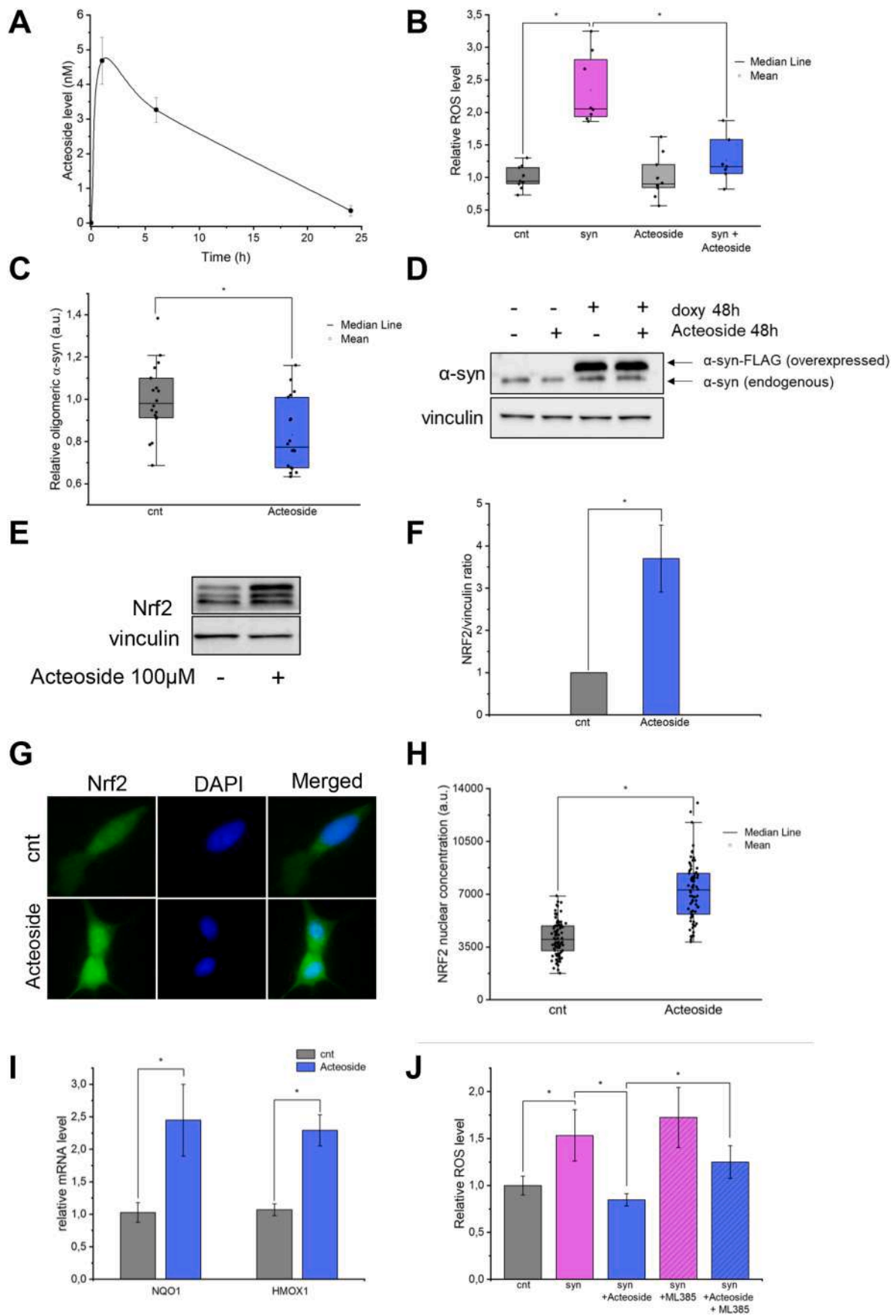


Fig. 7. Acteoside inhibits α -syn aggregation. (A) α -syn aggregation process, followed by ThT fluorescence after 72 h, in the absence (cnt 1, solvent control 2.5 % ethanol) or presence of *V. thapsus* (solvent 2.5 % ethanol) and in the absence (cnt 2, solvent control water) or presence of acteoside (100 μ M or 50 μ M in aqueous solution). (B) Kinetic of α -syn aggregation process, detected by ThT fluorescence, in the absence (cnt) or presence of acteoside (100 μ M or 50 μ M), with a zoom of the 20–30 h time interval. (C) TEM images of 70 μ M α -syn after 72 h of aggregation in the absence (cnt) or presence of acteoside (50 μ M in aqueous solution). (D) α -syn fibril elongation: 35 μ M monomeric protein was incubated with 1 μ M of preformed fibrils, detected by ThT fluorescence, in the absence (cnt) or presence of increasing concentration of acteoside (1 μ M, 25 μ M, 50 μ M and 100 μ M). Representative data from $n = 3$ independent experiments.



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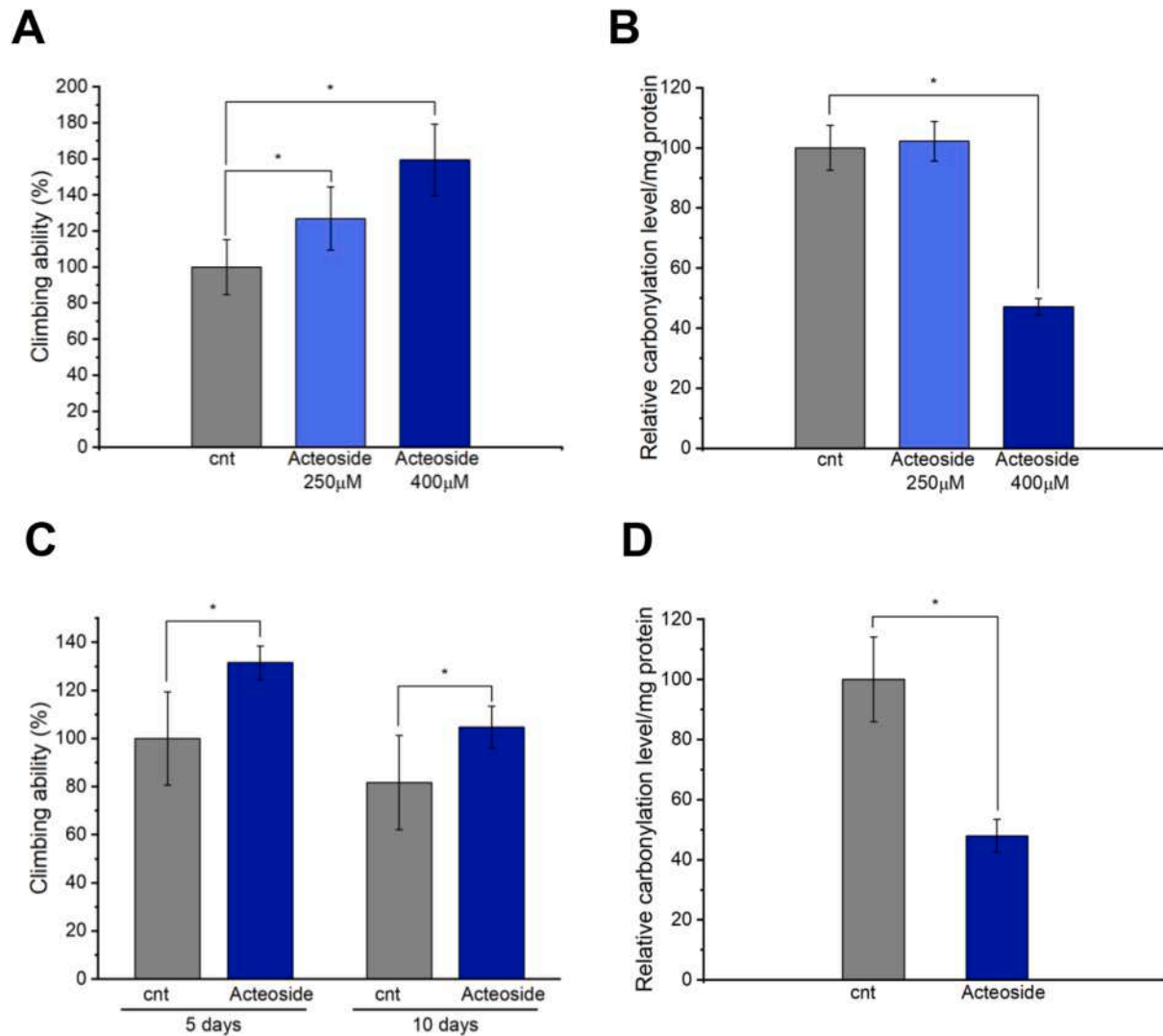


Fig. 9. Effect of acteoside on *DJ-1β* mutant flies. (A) Motor performance of *DJ-1β* mutant flies treated with 250 and 400 μM acteoside was evaluated by performing a climbing assay ($n = 80$ – 120 5-day-old female flies for each condition). (B) Protein carbonylation level in *DJ-1β* mutant flies treated with 250 and 400 μM acteoside was analyzed using 2,4-dinitrophenylhydrazine (DNPH) derivatization, data are expressed as arbitrary units (a.u.) per mg of protein. Results were normalized to data obtained from flies cultured in vehicle medium (0.1 % DMSO). Values are expressed as mean $\pm$ SD from three independent experiments in which three biological replicates were used. Groups were statistically compared by one-way ANOVA analysis followed by Tukey's post hoc test ($*p < 0.05$). (C) Motor performance of *DJ-1β* mutant flies treated post-eclosion with 400 μM acteoside was evaluated by performing a climbing assay 5 and 10 days after treatment initiation ($n = 80$ – 120 female flies per condition). (D) Protein carbonylation level in *DJ-1β* mutant flies treated post-eclosion with 400 μM acteoside was analyzed 10 days after treatment using 2,4-dinitrophenylhydrazine (DNPH) derivatization, data are expressed as arbitrary units (a.u.) per mg of protein. Results were normalized to data obtained from control flies cultured in vehicle medium (0.1 % DMSO). Values are expressed as mean $\pm$ SD from three independent experiments. $*p < 0.05$.

process. Intriguingly, previous studies have shown that acteoside reduces the aggregation of another prion-like protein, the amyloid β ($A\beta$) peptide, thereby decreasing its toxicity in Alzheimer's disease models [74–76].

The discovery that acteoside also inhibits α -synuclein fibrillation paves the way for its potential development as a preventive treatment for PD. Given that PD is a disorder with complex evolution, a broad-

Fig. 8. Acteoside activates the NRF2 signaling pathway. (A) Temporal course of intracellular/cell-associated level of acteoside in neuroblastoma cells. Data are expressed as pmol acteoside/μg proteins. (B) SH-SY5Y cells were grown for 72 h in the absence or presence of 5 μM α -syn, obtained after 72 h of aggregation without or with 1 mM acteoside. ROS level was evaluated by H_2DCFDA staining. $*p < 0.05$. (C) SH-SY5Y *pTet-SNCA-FLAG* cells were treated with 6 μg/mL doxycycline to induce α -syn overexpression, in the absence or presence of 100 μM acteoside for 48 h. Cells were fixed and immunostained with anti-Syn33 antibody (specific for oligomeric species). Graphs represent mean $\pm$ SD of oligomeric α -syn fluorescence quantified as Integrated Density (IntDen) using ImageJ software. (D) SH-SY5Y *pTet-SNCA-FLAG* cells were treated with 6 μg/mL doxycycline to induce α -syn overexpression, in the absence or presence of 100 μM acteoside for 48 h. The level of α -syn (both endogenous and FLAG-tagged overexpressed) was evaluated by Western blot. Vinculin was used as loading control. (E) Western analysis using anti-NRF2 and anti-vinculin antibody on protein extracts from neuroblastoma cells grown for 48 h in the absence (cnt) or in the presence of acteoside (100 μM). (F) Quantitative analysis of NRF2 protein level in (E). (G) Immunofluorescence images of neuroblastoma cells treated with 100 μM of acteoside for 48 h. Fixed cells were immunostained with anti-NRF2 antibody. Nuclei were stained by DAPI (blue). (H) Quantitative analysis of nuclear localization in (G) performed using Cell-ACDC software. (I) Relative mRNA expression levels of NQO1 and HMOX1 genes in neuroblastoma cells grown in the absence (cnt) or in the presence of acteoside (100 μM) for 48 h. (J) SH-SY5Y cells were pretreated with 100 μM acteoside and/or 5 μM ML385 (NRF2 inhibitor) for 2 h, then treated with 5 μM α -syn fibrils for 72 h. ROS level was evaluated by H_2DCFDA staining. Data represent mean $\pm$ SD from $n = 3$ independent experiments, $*p < 0.05$.

spectrum approach to target different pathological pathways could be a promising strategy. In this context, oxidative stress is strongly associated with this disease and it is linked with various cellular processes, which all seem to contribute to neurodegeneration [77]. Notably, in SH-SY5Y cells exposed to toxic α -syn aggregates, acteoside decreases intracellular ROS level (Fig. 8B). This is associated with the activation of the NRF2 signaling pathway, leading to the stimulation of the antioxidant response (Fig. 8). In agreement with our data, previous reports have highlighted the role of acteoside in modulating the NRF2 pathway *in vitro* and both in mouse and Zebrafish PD models [78,79]. Moreover, docking analyses have also reported possible binding of acteoside to NRF2 protein [80]. Remarkably, acteoside treatment restored locomotor function and reduced protein carbonylation *in vivo* in a *Drosophila* model of PD based on loss of *DJ-1* β function (Fig. 9). This dual action in a different PD model affecting a gene involved in both familial and sporadic form of the disease underscores its potential as a multifunctional therapeutic candidate for PD. Although preliminary, these results, obtained in a multicellular model organism, provide a solid basis for future studies aimed at dissecting the molecular pathways targeted by acteoside and evaluating its translational relevance in mammalian PD models.

Understanding acteoside's pharmacokinetic properties is crucial to assess its therapeutic potential and translational value. Despite its well documented antioxidant and neuroprotective activities, and its high level of safety [71,81–83], acteoside exhibits poor bioavailability [51]. *In vivo* studies in rats and beagle dogs have shown low systemic availability after oral administration, with bioavailability ranging from 0.1 % to 4 %. This poor bioavailability is primarily attributed to acteoside's high hydrophilicity, low membrane permeability and active efflux transport through P-glycoprotein (P-gp) [44]. Our findings are consistent with the previous observations. Intracellular or cell-associated acteoside reaches approximately 0.1 % after 1 h of treatment (Fig. 8A). This low but measurable uptake confirms acteoside's ability to enter cells or interact with their membranes, supporting its bioactivity. In addition, promising studies in rat models indicate that acteoside is able to reach the brain [84] and its blood-brain barrier permeability can be further enhanced through lipid encapsulation [85], suggesting that proper formulations of this compound are essential to maximize its activity *in vivo*. In conclusion, this study provides new evidence that acteoside possesses an inhibitory effect on α -syn aggregation and supports its antioxidant role, suggesting the relevance of this molecule as a candidate for neuroprotection in PD. Future studies will be essential to investigate the molecular pathways involved in the anti-aggregant properties of acteoside and to develop new strategies to increase its potential value as a preventive agent against synucleinopathies *in vivo*.

Author contributions

Alessia Lambiase: Investigation; Validation; Visualization; Writing – original draft; Writing – review & editing.

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Hind Moukham: Investigation; Visualization.

Elisa Toini: Investigation; Methodology; Visualization.

Annalisa D'Urzo: Investigation; Methodology.

Giovanni Zecca: Investigation; Methodology; Writing – original draft; Writing – review & editing.

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Ethical statement

Not required as no humans or animals are involved in this work.

Data availability

Data will be made available on request.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.4—Call for tender No. 3138 of December 16, 2021, rectified by Decree n.3175 of December 18, 2021 of Italian Ministry of University and Research funded by the European Union—NextGenerationEU; Award Number: Project code CN_00000033, Concession Decree No. 1034 of June 17, 2022. Adopted by the Italian Ministry of University and Research, CUP, H43C22000530001, Spoke 6, Project title “National Biodiversity Future Center—NBFC”. Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4. Component 2 Investment 1.3—Call for tender No. 3138, 16 December 2021, rectified by Decree n.341 of 15 March 2022 of Italian Ministry of University and Research funded by the European Union—NextGenerationEU; Project code PE00000003 ON FOODS—CUP: H43C22000820001—Spoke 6, Project title “ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON Foods”. Project funded by the University of Valencia to Nuria Paricio, Grantcode UV-INV-AE-3664989.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neurot.2025.e00825>.

Abbreviations

α -syn	α -synuclein
CLS	Chronological lifespan assay
MP	Medicinal Plants
PD	Parkinson's disease
PFF	preformed fibrils
PMP	Potential Medicinal Plants
ROS	reactive oxygen species
RPLC-MS	reverse phase liquid chromatography mass spectrometry
SEC AS-MS	size exclusion chromatography coupled to reversed phase LC-MS
SPR	Surface Plasmon Resonance
TEM	Transmission electron microscopy
ThT	Thioflavin T

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