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DNA barcoding to trace Medicinal and Aromatic Plants from the field to the food supplement

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Summary

The global market of food supplements is growing, along with consumers demand for high-quality herbal products. Nevertheless, substitution fraud, and adulteration cases remain a common safety problem of global concern. In the last years, the DNA barcoding approach has been proposed as a valid identification method and it is now commonly used in the authentication of herbal and food products. The objective of this study was to evaluate whether DNA barcoding can be applied to trace the plant species from the starting raw material to the finished commercial products. We selected a panel of 28 phytoextracts obtained through three different extraction methods (i.e., maceration, percolation and sonication) with different solvents (i.e., ethanol, deionized water and glycerol). Furthermore, we chose six plant species for which we collected and analysed all the intermediates of the industrial production. We sequenced and analyzed the sequence variability at DNA barcoding (*psbA-trnH, ITS*) and minibarcoding (*rbcL* 1-B) marker regions. Phytoextracts obtained through hydroalcoholic treatment, with the lower percentage of ethanol (<40%), and aqueous processing, at the lowest temperature, had major rate of sequencing and identification success. This study proves that DNA barcoding is a useful tool for Medicinal and Aromatic Plants (MAPs) traceability, which would provide consumers with safe and high-quality herbal products.

Key Words: Herbal products, ITS, MAPs, Minibarcode, psbA-trnH, Phytoextracts, *rbcL*

Introduction

Medicinal and Aromatic Plants (MAPs) and their preparations are products used in medicine, cosmetics and food industry, belonging to plants, fungi, algae or lichens (EFSA, 2009). Such products are prepared using plants or their parts to exploit their therapeutic and healthy properties (e.g., antioxidant, anti-inflammatory), as well as their flavor or scent (WHO, 1999). According to a report published by the Persistence Market Research, the global market of herbal supplements had a value of USD 40 billion in 2017 and is expected to reach a market valuation in excess of USD 65 billion by 2025 (PERSISTENCE MARKET RESEARCH, 2017). In the last years, the increasing consumption of natural food supplements and the growing awareness of consumers concerning the healthy benefits of these products have been progressively enhancing the market of MAPs (EFSA, 2009). Although most of the herbal products used as food ingredients have been available to consumers since decades, the regulation of these products differs greatly among jurisdictions. While some countries consider MAPs as reliable ingredients for food production, others regulate them as healthy products or medicines. For example, in the European Union (EU), most products containing Medicinal and Aromatic Plants are sold as food supplements and regulated under the food law (SILANO et al., 2011); in Australia dietary supplements are considered medicinal products and in Canada they are subject to the complex regulation of the Natural Health Products Directorate (NNHPD) of Health Canada (HEALTH CAN., 2015) as medical products (LOW et al., 2017). The lack of a clear and shared global regulation and the large market demand of high-quality plant-based items led to safety problems with the increase of substitution, fraud and adulteration cases. Anyway, more attention is required to guarantee a high quality level of MAPs which necessitates a stable raw material and its assurance. As a matter of fact, frequently, valuable plants are substituted with cheaper raw materials, such as the case of saffron substituted with safflower (BOSMALI et al., 2017). However, adulteration is not necessarily intentional, and herbal products may be altered due to inadvertent substitution, misidentification or confusion resulting from the use of different vernacular names in the countries of production.

According to the World Health Organization (WHO), the adulteration of herbal products is a potential threat to consumers' safety. This condition opens two key issues which refer to the definition of suitable toxicological evaluations to estimate the risks for human health and the setup of an efficient identification system to trace the herbal products from the field to the traded MAPs items. Usually, macroscopic and microscopic examinations are the classic strategies adopted to verify the identity of fresh plants or the origin of plant portions. These tools can be used to trace the herbal products when the plants are processed immediately after being harvested. However, when the herbs undergo drying, fragmentation and pulverization processes the morphological traits cannot be longer used to reliably assess the botanic source. Moreover, many herbal ingredients are obtained by infusion, maceration, distillation or pressing. In these cases, only dedicated chemical analyses of the complex mixtures could permit to achieve a reliable plant identification.

In the last years the DNA barcoding approach was proposed as a valid molecular identification method to provide species-level resolution and it is now more and more used in the authentication of taxonomic provenance of herbal and food products (NEWSMASTER et al., 2013; GALIMBERTI et al., 2013; MOHAMMED et al., 2017). However, the most important limit of this molecular tool is that it can preferentially be adopted on unprocessed material (e.g., dry, fragmented and shredded plant portions) and several difficulties are encountered when dealing with extracts or with any other process that results in the degradation of the DNA. Recently, some manuscripts described the efficacy of minibarcode regions (i.e., the analysis of smaller genome portions – 100-150 bp – usually associated to the largest DNA barcodes) for the identification of processed plant extracts (RACLARIU et al., 2017; LITTLE, 2014). To date, any study addressed the efficacy of a DNA barcoding-based approach to trace herbal products along the entire production chain. In this work, we selected Medicinal and Aromatic Plants in the form of phytoextracts obtained by several industrial companies and subjected to different kind of industrial processes and phytoextraction strategies. The objective of this survey was to evaluate whether or not the DNA barcoding approach (using standard barcodes or minibarcode regions) could be applied to trace the plant species from

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the field to the finished commercial product in the case of food supplements. Therefore, we evaluated which are the industrial processes mostly affecting the efficacy of DNA analysis such as sample pretreatment methods, solvents used for extraction and which are the most suitable DNA markers to achieve a reliable MAPs traceability.

Material and methods

Study design

To test the efficacy of DNA traceability at different steps of the industrial production chain of MAPs, we selected a panel of 28 commercial phytoextracts (Tab. 1) sold by three main European companies. The selected items were obtained starting from 17 plant species (in the initial form of dried raw material) and were processed by the same companies adopting three main extraction procedures, namely maceration, sonication and percolation. Maceration consists in the solubilization of the plant material in different solvents like water and alcohol (e.g., ethanol), while percolation involves the slow descent of a solvent through the plant raw material until it absorbs the molecules of interest. Both methods rely on liquid filtration and concentration. Differently, the sonication provokes cellular cavitation and the release of the phytocomplexes in the solvent used. Three different extraction solvents were considered in this study and specifically, ethanol (i.e., alcoholic), deionized water (i.e., aqueous) and glycerol. During maceration the temperature was maintained under the threshold of 55 °C while in percolation higher temperatures (i.e., > 80 °C) were maintained, and sonication was mainly performed at 30-40 °C. After the percolation process, some phytoextracts are dried at very high temperatures (about 200 °C). Processing details for each tested phytoextract are shown in Tab. 1.

To evaluate the efficacy of DNA barcoding to trace the intermediates of industrial production after different steps of phytoextraction, we selected a panel of six commercial products obtained only by alcoholic and aqueous extraction procedures (Tab. 2). For these samples, we collected and molecularly analysed through DNA barcoding, any intermediate of production (Fig. 1).

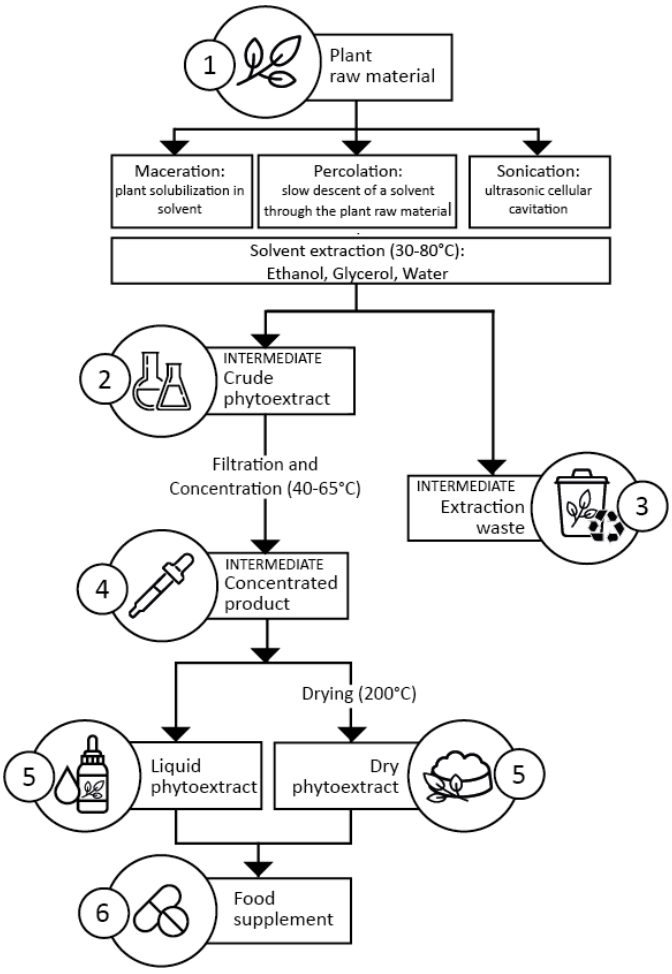

Fig. 1: The industrial flowchart of MAPs production. The numbers indicate the intermediate steps of the industrial process for which the DNA barcoding efficacy has been verified (Tab. 2).

DNA Extraction and DNA barcoding analysis

All commercial products from Tab. 1 were tested for authenticity by sequencing three candidate markers, namely the standard DNA barcoding plastidial intergenic spacer *psbA-trnH* (Steven and Subramanyam, 2009), the nuclear ITS region (primers ITS p5-u4, Cheng et al., 2016) and the minibarcode region *rbcL* 1-B (Little, 2014). Primer details and size of amplified fragments are provided in Tab. A.1.

A total of 50 mg of dried plant raw material, 150 µL of phytoextract (and intermediate products of phytoextraction, see Tab. 2) were treated for DNA extraction by using the EuroGOLD Plant DNA Mini Kit (Euroclone, Pero, Italy). Each commercial phytoextract product was subjected to DNA extraction in three replicates. Purified DNA concentration of each sample was estimated fluorometrically by using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific™).

A PCR amplification for each candidate marker was performed using puReTaq Ready-To-Go PCR beads (GE Healthcare Life Sciences, Italy) in a 25 µL reaction volume according to the manufacturer's instructions containing 1 µL 10mM of each primer and up to 3 µL of DNA template. PCR cycles consisted of an initial denaturation step for 7 min at 94 °C, followed by 35 cycles of denaturation (45 s at 94 °C), annealing (30 s at different temperatures; see Tab. A.1) and extension (1 min at 72 °C), and, hence, a final extension at 72 °C for 7 min.

In the case of the intermediates of production listed in Tab. 2, we amplified and sequenced only the minibarcode locus *rbc*L 1B.

Amplicons occurrence was assessed by electrophoresis on agarose gel using 1.5% agarose TAE gel stained with ethidium bromide and amplicon length was measured by comparison against 100 bp ladder. When a sample did not produce any band or showed multiple or non-specific amplicons, the reaction was repeated increasing the amount of template DNA up to 10 µL.

Purified amplicons were bidirectionally sequenced using an ABI 3730XL automated sequencing machine at Eurofins Genomics (Ebersberg, Germany). The 3' and 5' terminal portions of each sequence were clipped to generate consensus sequences for each sample. After manual editing, primer removal and pairwise alignment, the obtained sequences for dried raw material were submitted to the international GenBank through the EMBL platform (see Tab. A.2 for accession numbers).

For all the tested samples (Tab. 1 and Tab. 2), the reliability of DNA barcoding identification was assessed by adopting a standard comparison approach against a GenBank database with BLASTn. Each barcode sequence was taxonomically assigned to the plant species with the nearest matches (maximum identity >99% and query coverage of 100%) according to Bruni et al. (2015). We performed the identification separately for the three markers.

Results and discussion

Good DNA quality (i.e., A260/A230 and A260/A280 absorbance ratios within the range 1.8 - 2.2) and extraction yield (20-40 ng/µl) were obtained from all the 17 raw material samples. The three can-

Tab. 1: List of the analysed MAPs samples with details concerning their industrial processing to obtain the final phytoextracts. Average yield of DNA extraction (with standard deviation) and assessment of positive sequencing of DNA barcoding markers (×) are also reported.

SAMPLES	Industrial processing		DNA Extraction yield		DNA BARCODING MARKERS		
	Phytoextraction Process	Solvent	Value ng/µl	Standard Deviation	*psbA - trnH*	*ITS*	*rbcL* 1 - B
Achillea millefolium L.	Sonication	EtOH < 40%	12.26	1.55			×
Echinacea pallida (Nutt.) Nutt.	Sonication		16.29	0.97			×
Harpagophytum procumbens (Burch.) DC. ex Meisn.	Sonication		12.94	1.58	×	×	×
Melissa officinalis L.	Percolation		44.63	1.32			×
Mentha × *piperita* L.	Sonication		12.3	0.83	×	×	×
Tilia platyphyllos Scop.	Sonication		9.78	1.28	×	×	×
Zingiber officinale Roscoe	Sonication		13.16	2.13	×	×	×
Arctium lappa L.	Maceration	EtOH > 40%	1.23	0.2			
Echinacea angustifolia DC.	Maceration		2.83	0.5			
Melissa officinalis L.	Percolation		1.4	0.44			
Passiflora incarnata L.	Maceration		1.74	0.17			
Taraxacum officinale Weber ex F.H. Wigg.	Maceration		2.47	0.54			
Thymus vulgaris L.	Maceration		1.78	0.38			
Arctostaphylos uva-ursi (L.) Spreng.	Percolation	WATER	3.1	0.33			
Cetraria islandica (L.) Ach.	Percolation		2.27	0.94			
Echinacea purpurea (L.) Moench	Percolation		3.47	0.71		×	
Epilobium angustifolium L.	Percolation		1.88	0.63			
Malva sylvestris L.	Percolation		2.34	0.69			
Arctostaphylos uva-ursi (L.) Spreng.	Sonication		13.36	1.05			×
Echinacea purpurea (L.) Moench	Sonication		46.41	0.81	×	×	×
Epilobium angustifolium L.	Sonication		14.78	0.97			×
Melissa officinalis L.	Sonication		12,73	0,76	×	×	×
Arctium lappa L.	Maceration	GLYCEROL	2.69	0.64			
Echinacea angustifolia DC.	Maceration		4.73	0.85			×
Melissa officinalis L.	Maceration		2.55	0.76			
Passiflora incarnata L.	Maceration		2.09	0.6			
Taraxacum officinale Weber ex F.H. Wigg.	Maceration		3.12	0.3			
Thymus vulgaris L.	Maceration		2.71	0.8			

Tab. 2: List of six commercial MAPs (phytoextracts) traced along their entire production chain. Each sample (intermediates of industrial production) was treated for DNA extraction and DNA barcoding analysis using the minibarcode region *rbcL* 1-B. Numbers indicate the industrial processing step as described in Fig. 1. Y= correct plant identification by DNA barcoding at *rbcL* 1-B locus, N= DNA extraction or amplification failure, - = Sample was not collected and analysed.

Plant species	Solvent	Steps of the industrial production process					
		1	2	3	4	5	6
Achillea millefolium L.	20% Ethanol	Y	Y	N	Y	Y	Y
Zingiber officinale Roscoe	30% Ethanol	Y	Y	N	Y	Y	Y
Thymus vulgaris L.	60% Ethanol	Y	N	Y	N	N	-
Melissa officinalis L.	70% Ethanol	Y	N	Y	N	N	-
Echinacea purpurea (L.) Moench	Water	Y	Y	N	Y	Y	-
Melissa officinalis L.	Water	Y	Y	N	Y	Y	Y

didate genetic markers exhibited high PCR success and the obtained PCR products were successfully sequenced with high-quality bi-directional sequences. The BLASTn analysis suggested that all the obtained sequences corresponded with 100% maximum identity to the species declared by each company.

We are aware that multiple cases of 100% maximum identity within the same plant genus could occur, especially concerning the DNA minibarcoding *rbcL* 1B region. For example, this plastid region failed in discriminating *Echinacea purpurea (L.)* Moench from congenerics like *Echinacea angustifolia* DC. or *Echinacea pallida* (Nutt.). Such events suggest that in some conditions, the main limit of DNA minibarcoding relies on the reduced discrimination power among congenerics but it allows to detect plant contaminations when the adulterant/s belong to genera different from the target one. Nevertheless, it should be considered that when DNA content is expected to be low (or of low quality), the use of shorter DNA barcoding regions offers the best compromise between amplification universality, sequence quality and taxonomic discrimination (Little, 2014).

Concerning the 17 dry raw material samples, our results agree with the assumptions of Newmaster and co-workers (2013) who suggested that a DNA barcoding approach could be successfully applied to verify the identity of commercial herbal products and to reveal cases of contamination or substitution. Therefore, when herbal products are directly used as ingredients of complex food, medicine or cosmetics items, they are subjected to "soft" processing actions such as cleaning, drying and cutting and the DNA barcoding (achieved using long barcode fragments > 300 bp) represents a useful tool to trace plant species during the processing (De Mattia et al., 2011).

As expected, the efficacy of DNA extraction and amplification decreased when we analyzed the 28 commercial phytoextracts and their intermediates of industrial processing. Overall, the DNA amount obtained after extraction processes ranged from 1.5 to more than 40 ng/μL (Tab. 1). The extracts obtained through hydroalcoholic treatment, with the lower percentage of ethanol (< 40%), and aqueous processing, at the lowest temperature, contained more DNA than the other samples (Tab. 1 and Tab. 2).

In the samples where the DNA barcoding analysis worked well, no contamination or adulteration (i.e., the occurrence of DNA bacodes of other plant species) were observed. Unfortunately, in some groups of extracts, the molecular analysis did not provide reliable DNA extraction or high-quality sequences. At technical level, we hypothesize that in general, the high concentration of ethanol used in the industrial processing steps lead to DNA precipitation. This was confirmed by the data reported in Tab. 2, where samples of *Thymus vulgaris* L. and *Melissa officinalis* L. processed with ethanol at high concentration, showed residual DNA in the extraction waste rather than in the phytoextract. For this reason, both the DNA extraction and DNA barcoding authentication failed when applied to the successive intermediate products of industrial processing and DNA was no longer available in the final herbal supplements. In this case, we conclude that for this kind of industrial production, a DNA-based approach is not suitable to achieve a reliable traceability of the initial plant raw material. Similarly, high temperatures of water during aqueous extraction, followed by a drying step (about 200 °C) probably lead to DNA fragmentation and degradation (Karni et al., 2013) as observed in five of the samples processed with a percolation procedure (Tab. 1). Conversely, the use of more lukewarm water (i.e., < 55° C) allows to achieve a successful DNA extraction, amplification and sequencing of DNA barcoding markers (Tab. 1). Moreover, such conditions also allow the traceability of the intermediate pro-ducts of industrial processing as observed for *Echinacea purpurea* (L.) Moench and *Melissa officinalis* L. extracted using water as solvent.

Concerning glycerol extracts, although this solvent does not act directly on DNA molecules, it usually contains ethylhexylglycerin and phenoxyethanol, which are typically used as additives. According to Langsrud and co-workers (2016) these antibacterial agents could be responsible for DNA loss. For this reason, also the analysis of the DNA minibarcode region did not produce amplicons in glycerine extracts (Tab. 1).

Concerning the industrial treatments, the sonication seems to keep the DNA of raw materials more intact than the other processes (i.e., maceration and percolation). Our results also show that sonicated samples contained higher amounts of DNA (i.e. from 9.73 to 44 ng/μl, Tab. 1) compared to the other categories, thus allowing a successful amplification and sequencing of the DNA minibarcode marker.

Concerning the quality of extracted genetic material, the purity of DNA is more important than the extraction yield to achieve a good amplification and then a reliable identification (Song et al., 2017). It should also be considered that secondary metabolites, like polyphenols and polysaccharides, which are normally extracted along with DNA, may interfere with PCR amplification (Sahu, Thangaraj, and Kathiresan, 2012). These molecules could bind DNA covalently and make the extraction products impure, with several problems for the successive molecular analysis. For example, tannic acids could bind and inactivate Taq polymerase (Opel, Chung, and McCord, 2010). However, in our analysis we hypothesize that the main amplification problem for the phytoextracts is the fragmentation of DNA. In all the tested cases, the DNA minibarcode locus *rbcL* 1-B was most easily amplified and sequenced (Tab. 1) than the other two DNA barcoding markers. This suggests that the DNA obtained from phytoextracts are richer in small DNA fragments (80-200 bp). Such condition is in line with the data reported in recent review articles (Mohammed et al., 2017) suggesting that DNA barcoding is a reliable and suitable technique only for the herbal product that preserve a good quality DNA and with poor fragmentation. In the other cases DNA minibarcoding is the most efficient and reliable tool for traceability purposes (Song et al., 2017).

Nowadays, analytical chemistry methods (TLC, HPLC) represent the most used tools to verify the quality of MAPs, however, these approaches are usually directed to define the concentration of specific bioactive molecules or to estimate chemical contaminants (e.g., heavy metals) rather than to identify the occurrence of plant contaminants (Sgamma et al., 2017). Conversely, the DNA barcoding approach is globally recognized as one of the most reliable DNA-based approaches to identify species if a well populated reference dataset of DNA barcode sequences for the target taxa is available (Galimberti et al., 2013). Moreover, in the case of contamination (or substitution), DNA analyses also allow to simultaneously identify any species (i.e., DNA metabarcoding) using High Throughput Sequencing (HTS) sequencing systems (Galimberti et al., 2015; Mezzasalma et al., 2017). For these reasons, the Pharmacopoeia guidelines of some countries such as that of UK (British Pharmacopoeia Commission, 2017) indicate the DNA barcoding as one of the official traceability systems in the sector of herbal products. Our data support this proposal and the ability of DNA minibarcode makers to provide a reliable tracing of the intermediate products of industrial production. However, it is important to underline that some industrial processes demanding high temperatures and the use of solvents, such as a high concentration of ethanol, can induce DNA degradation and make this molecular tool less effective.

In conclusion, this study leads to two main considerations about the future application of DNA barcoding as a quality control tool in the sectors where the Medicinal and Aromatic Plants constitute relevant ingredients (e.g., food, cosmetics and pharmacology). First of all, the current industrial trends promote the adoption of extraction processes from plant raw material, which rely on the reduction of energy consumption (i.e., low temperatures), and on the use of more 'green' solvents (e.g., water) to obtain exhausted waste products that can be used in other supply chains (e.g., fertilizers). The adoption and

spread of this trend should lead to an increased integrity and quality of DNA in MAPs (and related intermediate products) and therefore enhance the success of DNA barcoding as a universal traceability system.

Secondly, the continuous advances in High Throughput Sequencing and the resulting possibility of exploring multiple short genetic regions simultaneously (i.e. 150-200 bp), could increase the sensitivity of a DNA-based identification.An HTS-DNA metabarcoding approach would allow to check the presence of several plant contaminants in the same sample, even if occurring at low concentrations (Newmaster et al., 2013). Sgamma and co-workers (2017) proposed the introduction of DNA metabarcoding to evaluate the quality and authenticate herbal drug material in the industrial context. The authors proposed a dedicated DNA barcoding flowchart for industrial traceability purposes. Our results could be taken into account to improve this flowchart and to also adapt it to the traceability of intermediates of industrial production. Interestingly, Valentini and co-workers (2017) recently proposed an innovative nanoparticle-DNA barcoding hybrid system called NanoTracer that could potentially revolutionize the world of traceability as it allows for rapid and naked-eye molecular traceability of any food and requires limited instrumentation and cost-effective reagents.

This and other similar applications (Aartse et al., 2017) open the opportunity to really boost the issue of herbal supplements traceability, not only with the industrial actors as the main stakeholders, but also involving a wider cicle of specialists.

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Supplementary material

Tab. A.1: List of primer pairs used for DNA barcoding analysis.

Locus name	Name	5' - 3' Sequence	Tm °C	Amplified samples size	Reference
rbcL	rbcL 1	TTGGCAGCATTYCGAGTAACTCC	50	80-200 bp	PALMIERI (2009)
	rbcL B	AACCYTCTTCAAAAAGGTC	50		
psbA-trnH	psbA	GTTATGCATGAACGTAATGCTC	53	300-600 bp	STEVEN & SUBRAMANYAM (2009)
	trnH	CGCGCATGGTGGATTCACAATCC	53		
ITS	ITS p5	CCTTATCAYTTAGAGGAAGGAG	55	300-750 bp	CHENG (2016)
	ITS u4	RGTTTCTTTTCCTCCGCTTA	55		

Tab. A.2: List of all the analysed plant species. The table include the voucher specimens and GenBank accession numbers of the raw material samples used to authenticate the phytoextracts and their intermediates of industrial production treated in this study.

Species	Type of sample	Specimen voucher	Company	GenBank Accession Number (*rbcL* 1-B; *psbA-trnH*; ITS)
Achillea millefolium L.	Dried raw material	FEM_DBE_001	Company 1	LS999840; LS999856; LS999873
Arctium lappa L.	Dried raw material	FEM_DBE_007	Company 3	LS999841; LS999857; LS999874
Arctostaphylos uva-ursi (L.) Spreng.	Dried raw material	FEM_DBE_010	Company 2	LS999842; LS999858; LS999875
Cetraria islandica (L.) Ach.	Dried raw material	FEM_DBE_013	Company 2	ND; ND; LS999876
Echinacea angustifolia DC.	Dried raw material	FEM_DBE_015	Company 3	LS999843; LS999859; LS999877
Echinacea pallida (Nutt.) Nutt.	Dried raw material	FEM_DBE_018	Company 1	LS999844; LS999860; LS999878
Echinacea purpurea (L.) Moench	Dried raw material	FEM_DBE_020	Company 2	LS999845; LS999861; LS999879
Epilobium angustifolium L.	Dried raw material	FEM_DBE_026	Company 2	LS999846; LS999862; LS999880
Harpagophytum procumbens (Burch.)	Dried raw material	FEM_DBE_029	Company 1	LS999847; LS999863; LS999881
Malva sylvestris L.	Dried raw material	FEM_DBE_031	Company 2	LS999848; LS999864, LS999882
Melissa officinalis L.	Dried raw material	FEM_DBE_033	Company 2	LS999849; LS999865; LS999883
Mentha x piperita L.	Dried raw material	FEM_DBE_045	Company 1	LS999850; LS999866; LS99984
Passiflora incarnata L.	Dried raw material	FEM_DBE_047	Company 3	LS999851; LS999867; LS999885
Taraxacum officinale Weber ex F.H. Wigg.	Dried raw material	FEM_DBE_050	Company 3	LS999852; LS999868; LS999886
Thymus vulgaris L.	Dried raw material	FEM_DBE_053	Company 3	LS999853; LS999869; LS999887
Tilia platyphyllos Scop.	Dried raw material	FEM_DBE_059	Company 1	LS999854; LS999870; LS999888
Zingiber officinale Roscoe	Dried raw material	FEM_DBE_061	Company 1	LS999855; LS999871; LS999889