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Evaluation of antitumoral effect of Hibiscus sabdariffa extract on human breast cancer cells

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ABSTRACT

BackgroundBreast cancer is the most frequent tumor in women. Natural substances represent an important source of innovative therapeutic solutions, eventually integrating or substituting conventional drugs and chemicals. Hibiscus sabdariffa L. is a plant of the Malvaceae family that has raised interest thanks to its anti-inflammatory, antioxidant and anticancer effects. In this work, we evaluated the antitumoral effects of an enriched fraction of Hibiscus sabdariffa L. extract (HsEF) in two human breast cancer cell lines, MCF-7(ERα +) and MDA-MB-231 (triple negative).

Methods and resultsCell viability was assessed by MTT and Trypan blue assays. HsEF reduced both cell lines viability in a dose and time dependent manner and this effect results irreversible. In MCF-7 cells immunofluorescence experiments, demonstrated that HsEF induced ERα *trans*-location from nucleus to perinuclear area and in cytoplasmic compartment. qRT-PCR and western blotting high-lighted that HsEF reduced ERα, BRCA1 and caveolin1 gene and protein expression in MCF-7 cells, but not in MDA-MB-231 cells. Moreover, we demonstrated that HsEF reduced proteasome activity, an increased autophagy, impair migration and invasion in both cell lines.

ConclusionsOur data suggest HsEF has an antitumoral effects on both breast tumor cells examined and that ERα involvement could explain the differences observed between the two cell lines.

1. Introduction

Breast cancer (BC) is the most common tumor in women and the leading cause of cancer-associated mortality worldwide. Furthermore, metastasis increases the malignancy of BC causing extremely high mortality [1]. Multiple risk factors, including genetic changes, biological age, estrogen exposure and obesity are associated with BC [2]. BC can be divided into four subtypes but the most frequent are luminal A (estrogen receptor (ER)-positive, progesterone receptor (PR)-positive and human epidermal growth factor receptor 2 (HER2) negative) and triple-negative breast cancer (TNBC) (ER, PR and HER2 negative). 30–40% of breast cancers are luminal A [3], while TNBC corresponds to 15%–20% of BC cases [4]. Luminal A are classified as low grade cancer and have a better prognosis than other mammary cancers. Differently, TNBC is characterized by aggressive behavior and high ability to metastasize especially at the level of liver, lungs, and central nervous system [4].

Available treatments for BC are surgery, systemic therapies (chemotherapy, hormone therapy and targeted therapy) and immunotherapy [5,6].

Natural products and their metabolites represent an alternative and an interesting therapeutic option, alone or in combination with available antineoplastic agents [4,7–10]. *Hibiscus sabdariffa* L. (HS) is a plant, belonging to Malvaceae family, widely distributed in South Asia and Central Africa. HS extract is characterized by a high content of polyphenol, flavonoids, and anthocyanins, and has been used in folk medicine against liver disease, fever, and hypertension [11]. Thanks to the proven antioxidant and anti-inflammatory properties, the extracts

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obtained from the different parts of HS plants have gained great interest as potential anticancer or chemopreventive compounds [12–15].

In this work we have evaluated *in vitro* the antitumoral effects of the *H. sabdariffa* ethylacetate fraction (HsEF), described in an our previous work [16], against different type of BC cells: MCF-7 and MDA-MB-231, which respectively belong to Luminal A and TNBC type. In Malacrida et al. (2019), this extract has been named HsFC, but in this manuscript the acronym is changed in HsEF [16].

2. Materials and methods

2.1. Cell culture and Hibiscus sabdariffa L

Human breast cancer MCF-7 cells (ATCC, USA) were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 1% glutamine, 1% Penicillin and Streptomycin (Euroclone, Italy). Human breast cancer cells MDA-MB-231 (ATCC, USA) were cultured in DMEM low glucose medium supplemented with 10% FBS, 1% glutamine, 1% Penicillin and Streptomycin (Euroclone, Italy). Cells were incubated at 37 °C and 5% CO_2 in a humidified incubator.

HsEF was obtained from a hydroalcoholic HS calyces extract as previously described [16] and it was dissolved in phosphate-buffered saline (PBS) at 1 g/ml concentration and then diluted directly into culture medium to working concentrations.

2.2. MTT assay

Cells were plated in 96-well plates at $10x10^3$ cells/well density and were treated with increasing concentrations of HsEF (1–5 mg/mL). After 24, 48 and 72 h, MTT assays were performed. Culture medium was removed and cells were washed with PBS and Ca/Mg. A solution of 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) in DMEM without phenol red was added to each well. After 4 h of incubation at 37 °C, MTT solution was removed and formazan crystals were solubilized in 100% ethanol. Absorbance of the solution was read at 560 nm in a microplate reader (BMG-Labtech, Germany).

2.3. Trypan blue vital count assay and reversibility experiments

Cells were plated in 6-well plates at $25x10^4$ cells/well density and were treated with increasing concentrations of HsEF (1–5 mg/ml). After 24, 48 and 72 h, cells were collected after trypsinization and stained with Trypan blue vital dye (Sigma-Aldrich, USA). Viable and dead cells were counted in a Burker hemocytometer under a light microscope (Nikon Eclipse TS100). Doubling time of cells after 48 h of treatment was calculated with the following formula: Doubling time = Duration × ln (2)/ln(Final concentration/Initial concentration).

Trypan blue vital count was also used to assess the reversibility of HsEF. Cells were plated and treated as previously described. After 24 h of treatment, medium containing HsEF was removed and replaced with fresh medium without treatments. Viable cells were counted using Trypan blue after 24, 48, 72 and 96 h.

2.4. qRT-PCR experiments

Cells were plated and treated as described in section 2.3. After 24 h of treatment, cells were washed with PBS and 1 mL of Tripure solution was added to each well. A cell scraper was used to mechanically detach cells from surface. After 5 min in ice, 200 μL of chloroform were added for 15 min. The solution was then centrifuged at 12,000g for 15 min at 4 °C. The supernatant containing the RNA fraction was collected and 500 μL of 2-propanol were added. After 20 min at −20 °C, the solution was centrifuged at 12,000g for 10 min at 4 °C. Obtained pellet was resuspended in 75% ethanol, centrifuged again at 12,000g for 10 min at 4 °C and resuspended in DEPC water.

Extracted RNA was quantified and retrotranscribed adding the retrotranscriptase buffer, dNTP mix and the reverse transcriptase (Thermofisher, USA). The obtained cDNA was mixed with TaqMan gene expression master mix and specific RT primers (Thermofisher, USA, *ESR1* assay ID Hs01046816_m1, *BRCA1* assay ID Hs01556193_m1, *CAV1* assay ID Hs00971716_m1 and *GAPDH*, used as internal control, assay ID Hs02786624_g1 ()). Samples were loaded in 96-well reaction plates and analyzed in a RT-PCR system (7500 Real-Time PCR System, Applied Biosystem, USA).

2.5. Western blotting analysis

Cells were plated and treated as described in section 2.3. After 24 h, culture medium was removed, and cells were washed with PBS. To obtain total protein extracts, cell lysis was performed chemically, with Lysis Buffer (5 mM Hepes pH 7.5, 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1.5 mM EGTA, 4 nM PMSF, 1% Aprotinine, 20 nM sodium pyrophosphate, 10 nM sodium orthovanadate), and mechanically, with a cell scarper.

Total protein samples were then clarified by centrifugation (13,000g, 15 min, 4 °C) and quantified using Bradford method.

10 μg of proteins were loaded in a polyacrylamide gel and after SDS-PAGE electrophoresis, western blotting was performed as described by antibodies manufacturers (anti-ERα, 1:1000, Santa Cruz, USA; anti-BRCA1, 1:50, Cell Signaling, USA; anti-CAV1, 1:1000, Cell Signaling, USA; anti-actin, 1:1000, Santa Cruz, USA; anti-LC3, 1:1000, Cell Signaling, USA; anti-Beclin1, 1:1000, Cell Signaling, USA; anti-rabbit, 1:2000, PerkinElmer, USA; anti-mouse, 1:2000, Chemicon, USA; anti-goat, 1:2000, Chemicon, USA).

2.6. Immunofluorescence experiments

$25x10^4$ cells were plated on glass coverslips and were treated with HsEF 3.5 mg/mL. After 24 h cells were washed with PBS and fixed with paraformaldehyde for 20 min. After washing with PBS, washing with 0.1 M glycine and blocking in 5% BSA and 0.5% Triton X-100, cells were incubated overnight with anti-ERα primary antibody (1:200 in blocking buffer 5% BSA and 0.5% Triton X-100). Then, cells were incubated with secondary antibody (1:200, anti-rabbit, in blocking buffer 5% BSA and 0.5% Triton X-100), DAPI (1:50,000 in PBS) and Phalloidin (1:200 in PBS). After final washing with PBS, glass slides were mounted and observed under a confocal microscope.

2.7. Proteasome activity assay

Cells were seeded, treated and lysed as described in paragraph 2.3, but the lysis buffer was prepared without proteases and phosphatases inhibitors. 40 μg of proteins, 10 μl of 10X proteasome buffer (250 mM Hepes pH 7.5, 5 mM EDTA pH 8.0, 0.5% NP-40, 0.01% SDS) and 10 μl of proteasome substrate (N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin, 7.6 mg/mL) (Sigma-Aldrich, USA) were loaded in each well of a black 96-well plate. After 2 h at 37 °C, fluorescence was quantified in a microplate reader (excitation 380 nm, emission 460 nm) (BMG-Labtech, Germany).

2.8. Acridine orange staining for autophagy evaluation

Cells were seeded over glass coverslips at density of $2.5x10^5$ cells/well in 6-well plates and after 24 h they were treated with HsEF. After 24 h cells were stained with Acridine Orange (AO) (Sigma-Aldrich, USA) at a final concentration of 0.5 μg/mL for 15 min at 37 °C. Cells were then washed 3 times with PBS and observed under a fluorescent microscope (excitation 488 nm, emission 510 nm for green; excitation 460 nm, emission 650 nm for red). Red fluorescence intensity was quantified using ImageJ software.

2.9. Scratch wound healing assay

Cells were plated at high density in 6-well plates. When 90–100% of confluence was reached, culture medium was replaced with serum free medium. After 16 h, a scratch was made on cell monolayer and detached cells were washed away with PBS. Complete medium with or without HsEF was added to each well. Micrographs of the scratch were taken at 0, 24, 48 and 72 h. The area of migration was measured using ImageJ software.

2.10. Boyden chamber assay

Boyden chamber is composed of two compartments. In the lower compartment, culture medium with 10% FBS as chemoattractant was placed. In the upper compartment serum free medium with $5x10^3$ cells was plated. A gelatin coated polycarbonate membrane with 8 μm pores (Biomap, Italy) was placed between the two compartments.

After 24 h of incubation, the membrane was removed and cells on the lower side were fixed and stained with Diff quick staining kit (Biomap, Italy). Cells were then counted under a light microscope.

2.11. Statistical analysis

Data were reported as mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed using GraphPad Prism 3 software. One Way ANOVA analysis of variance followed by Dunnet's multiple comparison test was performed to evaluate the differences between control and treated cells. Statistical significance was set at $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Evaluation of HsEF effects on cell viability of MCF-7 and MDA-MB-231 cells

To evaluate cell viability of MCF-7 and MDA-MB-231, cells were treated with increasing concentrations of HsEF (1–5 mg/mL) and after 24, 48 and 72 h, MTT assay was performed.

Cell viability of both cell lines was reduced in a dose and time dependent manner, but HsEF was more effective in MCF-7 than in MDA-MB-231 cells. IC_{50} at 24 h in MCF-7 was lower than in MDA-MB-231 cells (3.5 ± 0.1 mg/mL and 4.4 ± 0.4 mg/mL respectively). The lowest IC_{50} of MCF-7 was also maintained at subsequent times evaluated (48 and 72 h) (Table 1).

HsEF 3.5 mg/mL was chosen as the concentration to perform the subsequent experiments.

3.2. Evaluation of HsEF effects on cell viability and death of MCF-7 and MDA-MB-231 cells

Trypan blue vital count assay was performed to evaluate the number of viable and dead cells after HsEF 3.5 mg/mL treatment.

In MCF-7 cells, the number of viable cells counted after treatment with HsEF was significantly lower than in untreated controls. This number remained constant for up to 48 h, while it was further reduced after 72 h of treatment (Fig. 1 B). In MDA-MB-231 cells, HsEF treatment significantly reduced the number of viable cells compared to the untreated control, but the number of viable cells remained constant for all the times evaluated (Fig. 1 B).

We also counted the number of dead cells with or without HsEF treatment. Both MCF-7 and MDA-MB-231 untreated cell lines slightly increased the number of dead cells, but this was not significant. After HsEF treatment, the number of MCF-7 dead cells, significantly increased at 48 h and 72 h compared to untreated CTRL, while for MDA-MB-231 cells, the dead cell number remained constant and comparable to untreated CTRL at 24 and 48 h. Only at 72 h, a significant increase in the number of dead cells was observable (Fig. 1 B).

On the basis of the results showed in Fig. 1 B, we have calculated at 48 h MCF-7 and MDA-MB-231 doubling time. Doubling time of MCF-7 untreated cells (CTRL) was 30.4 h, while after HsEF treatment it increased to 126.8 h. For MDA-MB-231, doubling time of untreated cells (CTRL) was 48.8 h and raised to 190.2 h after HsEF treatment (Table 2).

Table 1
IC50 of MCF-7 and MDA-MB-231 treated with HsEF for 24, 48 and 72 h.

IC_{50} (mg/mL)	MCF-7	MDA-MB-231
24 h	3.5 ± 0.1	4.4 ± 0.4
48 h	3.1 ± 0.1	3.9 ± 0.2
72 h	2.8 ± 0.2	3.2 ± 0.1

3.3. Evaluation of HsEF reversibility in MCF-7 and MDA-MB-231 cells

To analyze if HsEF has a reversible effect on MCF-7 and MDA-MB-231 cell viability, we performed Trypan blue experiments in which MCF-7 and MDA-MB-231 cells were treated with HsEF 3.5 mg/mL and after 24 h of treatment, medium was removed and replaced by fresh medium without HsEF. Cells were counted from 0 to 120 h. MCF-7 and MDA-MB-231 untreated cells or subjected to continuous treatment with HsEF represented controls.

As demonstrated in Fig. 1C, the effect of HsEF was not reversible, both in MCF-7 and MDA-MB-231 cells. Cells treated with HsEF for 24 h, did not restart growing after HsEF removal and the number of counted viable cells was comparable to HsEF continuous treatment for all the time points evaluated.

3.4. Evaluation of ESR-1, BRCA1 and CAV1 gene expression modulation in MCF-7 and MDA-MB-231 cells

To evaluate modulation of gene expression in MCF-7 and MDA-MB-231 cells treated with HsEF 3.5 mg/mL for 24 h, RT-PCR analysis was performed on mRNA of ESR1 (gene that code for ERα), BRCA1 and CAV1, that represented genes regulated by ERα.

In MCF-7 cells, mRNA of ESR1, BRCA1 and CAV1 were significantly reduced by HsEF treatment compared to untreated controls, while in MDA-MB-231 cells, mRNA of BRCA1 and CAV1 were not changed by HsEF and are comparable to untreated controls. In MDA-MB-231 cells, mRNA of ESR1 was not quantifiable (Fig. 2 A).

3.5. Evaluation of ERα, BRCA1 and CAV1 protein expression modulation in MCF-7 and MDA-MB-231 cells

To evaluate the modulation of ERα, BRCA1 and CAV1 protein expression levels, MCF-7 and MDA-MB-231 cells were treated with HsEF 3.5 mg/mL for 24 h and western blotting was performed.

ERα was expressed almost exclusively in the nucleus of untreated MCF-7 cells. The treatment with HsEF significantly reduced the expression of the receptor in total extract (Fig. 2 B). Since MDA-MB-231 cells did not express ERα, the Western blot was negative (data not shown).

The treatment with HsEF significantly reduced the expression of BRCA1 and CAV1 proteins in MCF-7 cells (Fig. 2 B). On the contrary, HsEF did not alter the expressione of BRCA1 and CAV1 in MDA-MB-231 cells (Fig. 2C).

3.6. Evaluation of ERα localization in MCF-7 and MDA-MB-231 cells

ERα localization in MCF-7 cells after HsEF treatment (3.5 mg/mL for 24 h) was evaluated by immunofluorescence experiments.

In MCF-7 untreated control cells, ERα was mainly localized in the nucleus (73%) and the signal in the cytoplasm was weak. Treatment of MCF-7 cells with HsEF induced the translocation of ERα from the

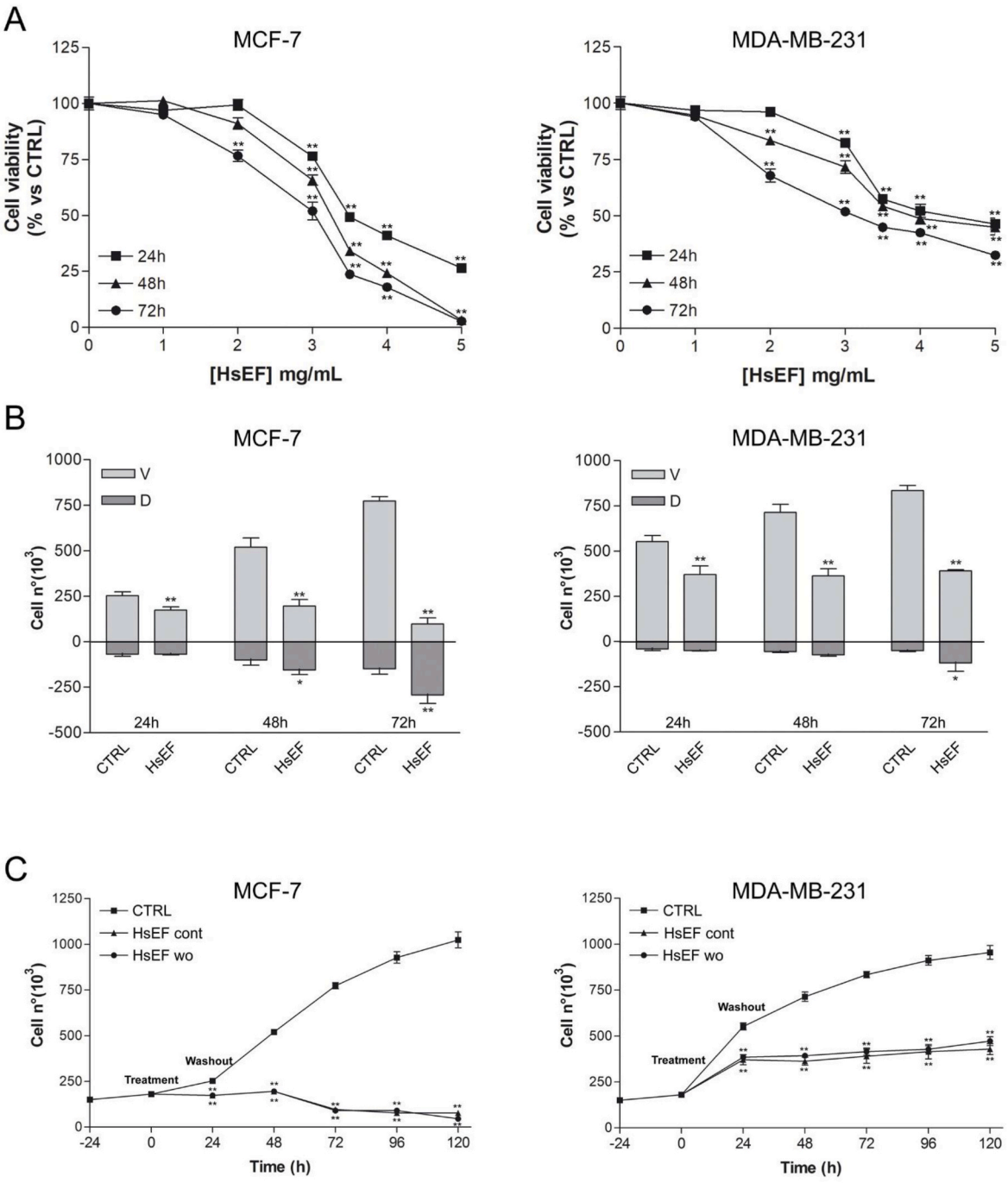

Fig. 1. MCF-7 and MDA-MB-231 cell viability, cell death and reversibility after HsEF treatment. (A) Graphs represent cell viability of MCF-7 and MDA-MB-231 cells treated with increasing concentrations of HsEF (1–5 mg/mL) for 24, 48 and 72 h, and evalueted with MTT assay. (B) Trypan blue vital count of MCF-7 and MDA-MB-231 cells, untreated (CTRL) or treated with HsEF 3.5 mg/mL for 24, 48 and 72 h. Both viable (V) and dead (D) cells are represented in the graphs. (C). Reversibility of HsEF in MCF-7 and in MDA-MB-231 cells. In both figures, graphs represent the number of MCF-7 and MDA-MB-231 counted cells from 0 to 120 h for not treated cells (CTRL), cells treated continuosly with HsEF (HsEF cont), or cells treated with HsEF from 0 to 24 h, than, after washing out, treated only with medium in absence of HsEF (HESC wo). Data are expressed as the mean ± SD of three independent experiments and are compared to untreated controls. *$p<0.05$ and **$p<0.01$ vs CTRL.

Table 2
Doubling time of MCF-7 and MDA-MB-231, calculated after 48 h of treatment with HsEF 3.5 mg/mL or in untreated controls (CTRL).

Doubling Time (hours)	MCF-7	MDA-MB-231
CTRL	30.4	48.8
HsEF	126.8	190.2

nucleus to the cytoplasm, mostly in the perinuclear area, and the percentage of ERα in the nucleus was reduced to 21% (Fig. 3 A and B). On the contrary, ERα was not expressed both in untreated and HsEF treated MDA-MB-231 cells (Fig. 3C).

3.7. Evaluation of MCF-7 and MDA-MB-231 cells proteasome activity

The activity of proteasome was assessed by specific fluorescent activity assay both in untreated MCF-7 and MDA-MB-231 cells, after treatment with HsEF 3.5 mg/mL for 24 h. In both cell lines, proteasome

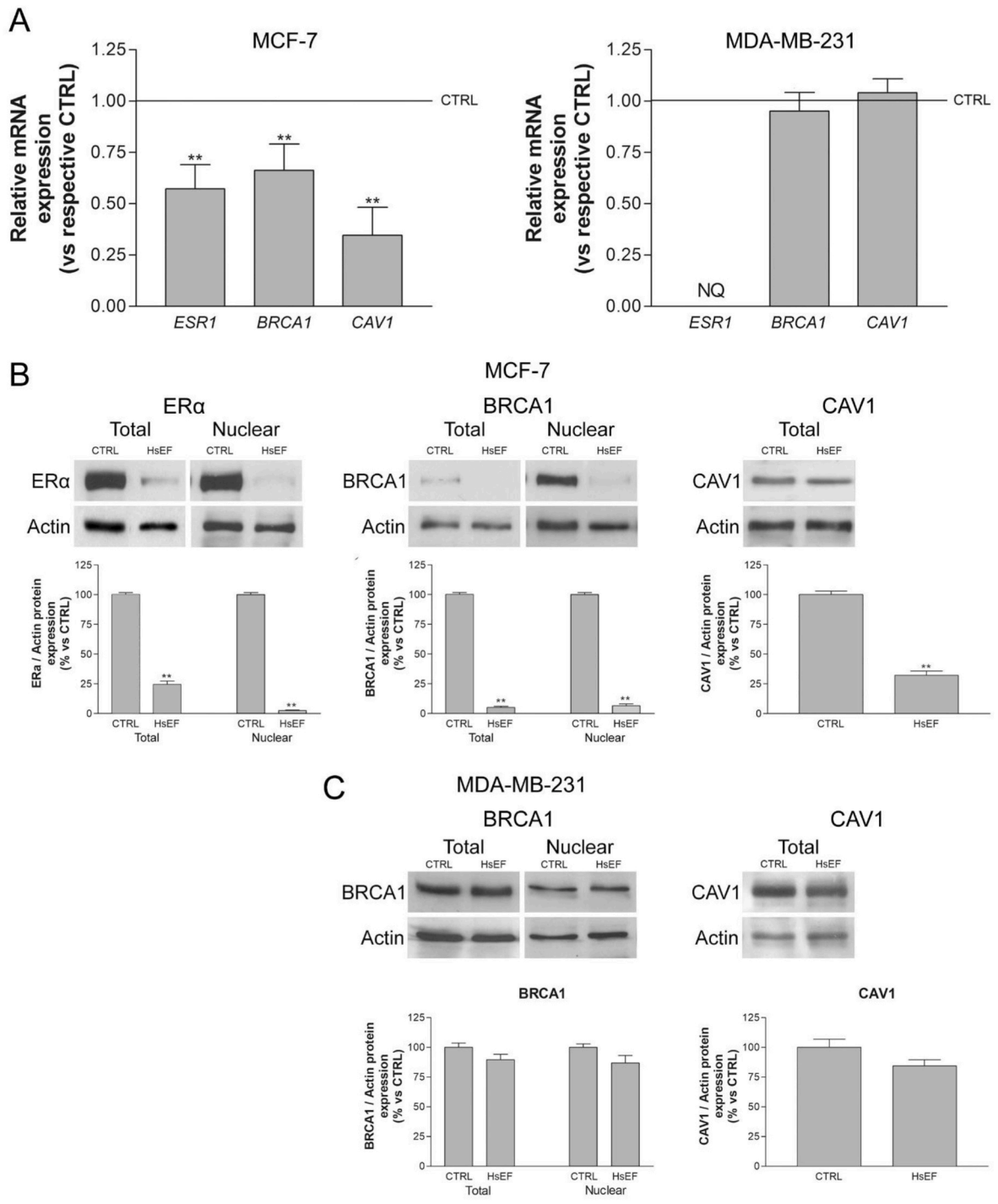

Fig. 2. ESR1, BRCA1 and CAV1 gene expression and immunoblotting analysis in MCF-7 and MDA-MB-231 cells treated with HsEF. (A) RT-PCR analysis of MCF-7 and MDA-MB-231 cells trated with HsEF 3.5 mg/mL or not treated (CTRL) for 24 h. GAPDH represents interal control. **(B)** Representative images and respective graphs of ERα, BRCA1 and CAV1 immunoblotting in MCF-7 cells treated with HsEF 3.5 mg/mL for 24 h. Total protein extracts are represented for ERα, BRCA1 and CAV1. Actin was used as internal control of total protein extracts. **(C)** Representative images and respective graphs of BRCA1 and CAV1 immunoblotting in MDA-MB-231 cells treated with HsEF 3.5 mg/mL for 24 h. Total protein extracts are represented for BRCA1and CAV1. Actin was used as internal control of total protein extracts. Data are expressed as the mean ± SD of three independent experiments and are compared to untreated controls (CTRL, arbitrarily set to 1.00 for RT-PCR and to 100% for immunoblotting). NQ = Not quantifiable. **p<0.01 vs CTRL.

was significantly inhibited by HsEF and its residual activity was lower than 50% (Fig. 4 A). No significant differences have been observed between the two examined cell lines.

3.8. Evaluation of MCF-7 and MDA-MB-231 cells autophagy activity

Autophagy process was assessed by Acridine Orange staining in untreated MCF-7 and MDA-MB-231 cells, after treatment with HsEF 3.5 mg/mL for 24 h.

In both cell lines, autophagy was significantly induced by HsEF. In

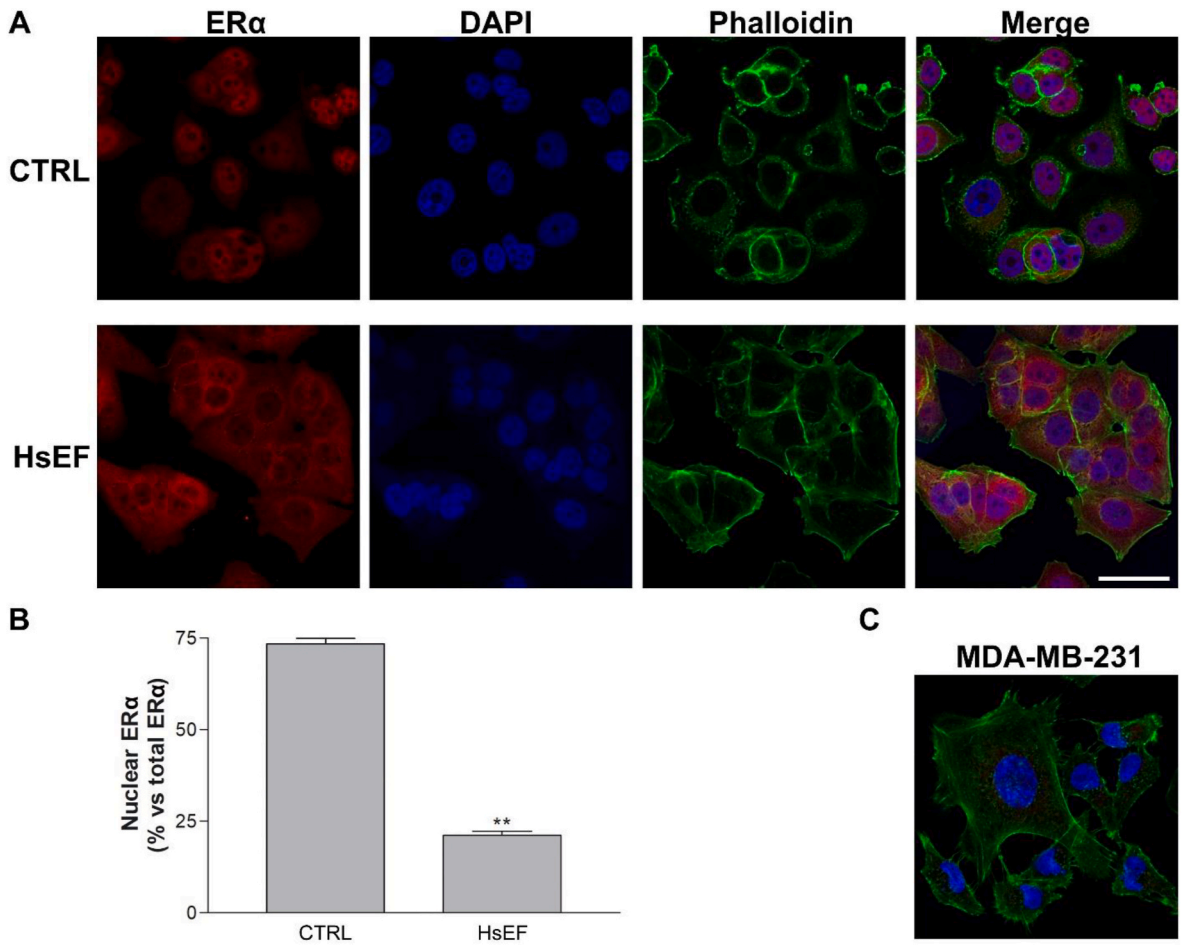

Fig. 3. ERα immunofluorescence in MCF-7 and MDA-MB-231 cells. (A) Representative images of immunofluorescence of ERα in MCF-7 cells, untreated (CTRL) or treated with HsEF 3.5 mg/mL for 24 h. **(B)** Graph represents the percentage of ERα in the nucleus, compared to total ERα in whole cells. **(C)** Representative merge images of immunofluorescence of ERα in untreated and HsEF treated MDA-MB-231 cells. ERα is represented in red, nuclei in blue (DAPI staining) and cytoskeleton in green (phalloidin staining). Data are expressed as the mean ± SD of three independent experiments and are compared to untreated controls. **p<0.01 vs CTRL. Scale bar 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

MCF-7 cells, acidic vesicular organelles (AVOs) intensity was 4 times higher than in untreated controls, while in MDA-MB-231 cells it was 2.7 time higher (Fig. 4 B). Moreover, in both cell lines, the ratio between LC3-II and LC3-I signficantly increased after HsEF treatment, compared to CTRL, indicating an enhanced autophagy activity. Also the expression of Beclin1, a protein involved in the activation of autophagy, was significantly incresaed after HsEF treatment (Fig. 4C).

3.9. Evaluation of MCF-7 and MDA-MB-231 cell migration and invasion

Cell migration and invasion of MCF-7 and MDA-MB-231 after treatment with HsEF 3.5 mg/mL treatment, were analyzed respectively by scratch wound healing assay and Boyden chamber assay.

In scratch wound healing assay, untreated MCF-7 cells migrated and took about 72 h to completely close the scratch. Differently, untreated MDA-MB-231 cells migrated faster and completely closed the scratch already after about 24 h. On the basis of the different times taken by the untreated cells to close the scratch, we evaluated the effect of HsEF on cell migration for MCF-7 cells up to 72 h and for MDA-MB-231 cells up to 24 h. HsEF significatively inhibited MCF-7 cell migration in time dependent manner: at 24, 48 and 72 h the migration was respectively inhibited by 19, 30 and 28% (Fig. 5 A). At 24 h, HsEF reduced cell migration of MDA-MB-231 cells only by 15% (Fig. 5 A).

In Boyden chamber assay, untreated MCF-7 and MDA-MB-231 cells passed through the membrane in presence of 10% FBS as chemoattractant (CTRL pos), while, without serum, cells were not able to pass through the membrane (CTRL neg). HsEF impaired cell passage through the membrane of both cell lines, but MDA-MB-231 were more inhibited than MCF-7 cells (Fig. 5 B).

4. Discussion

Hibiscus is a flowering plant that belong to the family of Malvaceae and include several hundred species. Some Hibiscus species possess interesting biological properties and are used both in natural and traditional medicine. In cancer field, triterpenoids present in *Hibiscus syriacus* L. and an aqueous extract of *Hibiscus rosa-sinesis* L. are able to inhibit triple-negative breast cancer cell viability [7,17]. For the first time, in our manuscript it has been evaluated in breast cancer the antitumoral effect of another species of the Hibiscus family, *Hibiscus sabdariffa* L., using an enriched ethylacetate fraction (HsEF) obtained by ethilacetate extraction [16].

Antitumoral effects of HsEF has been evaluated on two different breast cancer cell lines: MCF-7 and MDA-MB-231 cells. MCF-7 cells are estrogen and progesterone receptor positive and are poorly aggressive. On the contrary, MDA-MB-231 cells are triple-negative and present a high aggressiveness.

Our results demonstrate that HsEF is able to significantly reduce the viability of both MCF-7 and MDA-MB-231 cells and to significantly slow down the duplication rate of both cell lines. Increased duplication time is

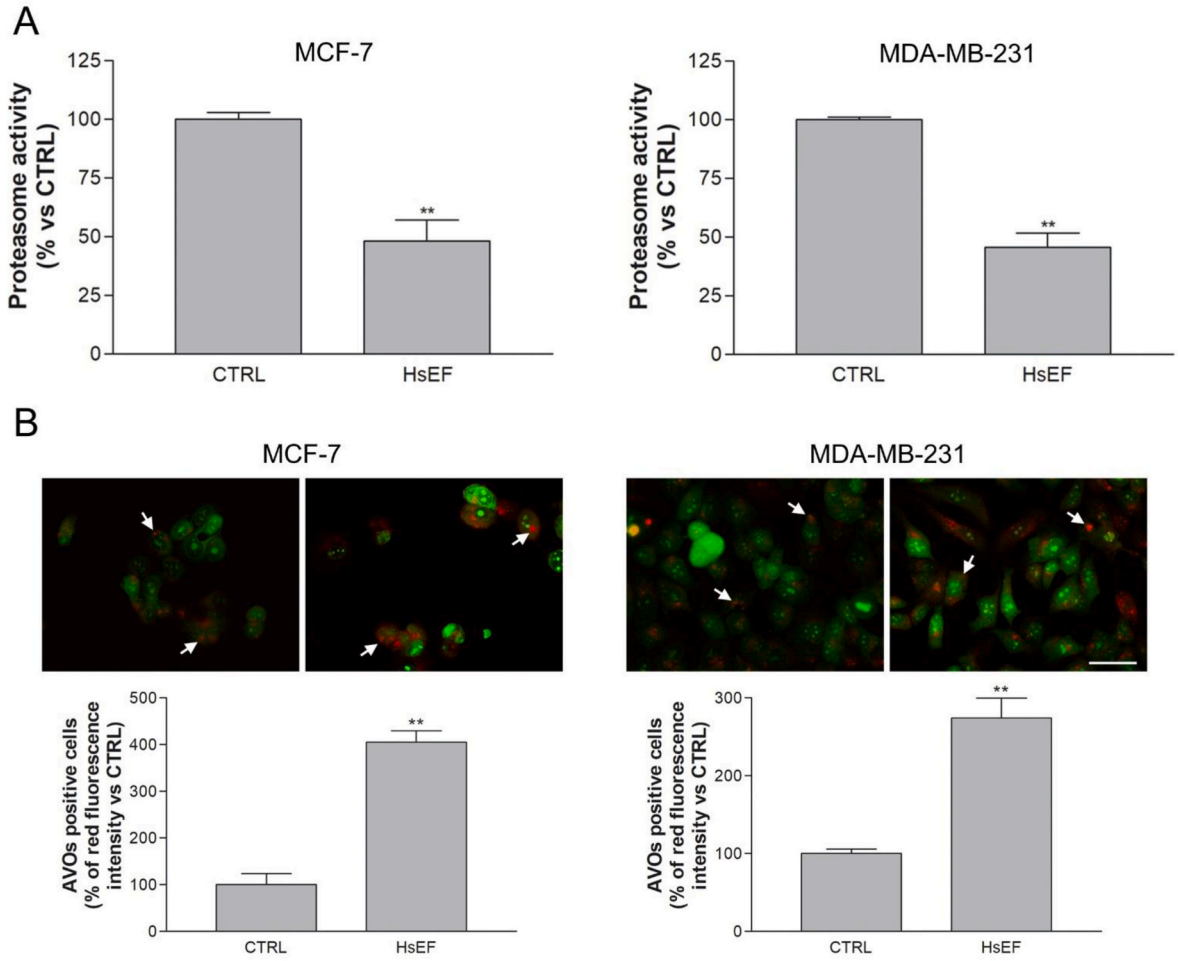

Fig. 4. Proteasome activity and autophagy of MCF-7 and MDA-MB-231 cells after HsEF treatment. (A) MCF-7 and MDA-MB-231 cells proteasome activity after treatment with HsEF 3.5 mg/mL for 24 h. Data are expresses as the mean ± SD of three independent experiments and are compared to untreated controls (CTRL, arbitrarily set to 100%). **(B)** Representative images and respective graphs of acridine orange staining of MCF-7 and MDA-MB-231 cells, untreated or treated with HsEF 3.5 mg/mL for 24 h. Data are expressed as the mean ± SD of red fluorescence intensity of three independent experiments and are compared to untreated controls (CTRL, arbitrarily set to 100%). **p<0.01 vs CTRL. Scale bar 80 μm. (**C**) Representative images and respective graphs of LC3-I, LC3-II and Beclin1 immunoblotting in MCF-7 and MDA-MB-231 cells treated with HsEF 3.5 mg/mL for 24 h. Actin was used as internal control. Data are expressed as the mean ± SD of the ratio between LC3-II and LC3-I or between Beclin1 and Actin. Three independent experiments were performed and results are compared to untreated controls (CTRL, arbitrarily set to 100%). **p<0.01 vs CTRL.

an indication of slowed tumor proliferation and reduced tumor development capacity, a very important aspect in evaluating the efficacy of HsEF. On the basis of the results obtained we can affirm that HsEF is more effective on MCF-7 cells than MDA-MB-231 cells. Furthermore, the number of dead MCF-7 cells after treatment with HsEF already increases at 48 h and further at 72 h, while for MDA-MB-231 cells an increase of dead cells is visible only afterwards 72 h of treatment.

The greater antiproliferative efficacy of HsEF on MCF-7 cells, compared to MDA-MB-231 cells, could be explained by their different characteristics in terms of expression of estrogen receptor. Estrogens play a crucial role in breast cancer progression and ERα is a major player in breast cancer development. Approximately, 10–15% of normal breast epithelial cells express ERα, but this receptor is upregulated in breast cancer and 75% cases are ERα-positive. Most ERα-positive breast cancers depend on estrogen for their growth and progression [18].

For this reason, systemic therapies for breast cancer are based on hormone or endocrine therapy in which drugs block ERα, preventing estrogen interaction, affecting their genomic and nongenomic effects, and decreasing tumoral proliferation [19]. Alternatively, there are drugs that directly decrease the level of estrogen by using an aromatase inhibitor [20].

Our results demonstrate that when MCF-7 cells are treated with HsEF, both mRNA and protein levels of ERα are significantly reduced compared to untreated cells. Moreover, treatment with HsEF induces a change in ERα localization: from nuclear, the receptor becomes mainly perinuclear. Generally perinuclear area localization in breast cells is typical of ERβ [21] and not of ERα. Physiologically, ERα is mainly localized into the nucleus exerting its genomic action. Change of ERα localization has been widely reported. Monje et al. (2001) have demonstrated that in MCF-7 cells, ERα is present not only in the nucleus, but also associated with the plasma membrane [22]. Moreover, in C2C12 murine myoblast cell line, the subcellular localization of native ERα is not only in the nuclear compartment, but also in cytosol and perinuclear area [23]. Localization of ERα is a very important factor so that it can play its role correctly. A change in its localization can greatly affect its action in the cell. Our results demonstrate that, in MCF-7 cells after HsEF treatment, ERα translocate from nucleus to perinuclear area. The observed change of ERα localization could be on the basis of HsEF antitumoral effects inducing the ERα pathway dysregulation. The significant reduction of the expression of ERα and its change of localization imply that estrogen pathway is impaired because the receptor cannot regulate its target genes, that in turn regulates numerous physiological processes (proliferation, DNA repair, differentiation, apoptosis) [24]. These data suggest that, for MCF-7 cells, ERα may be directly or

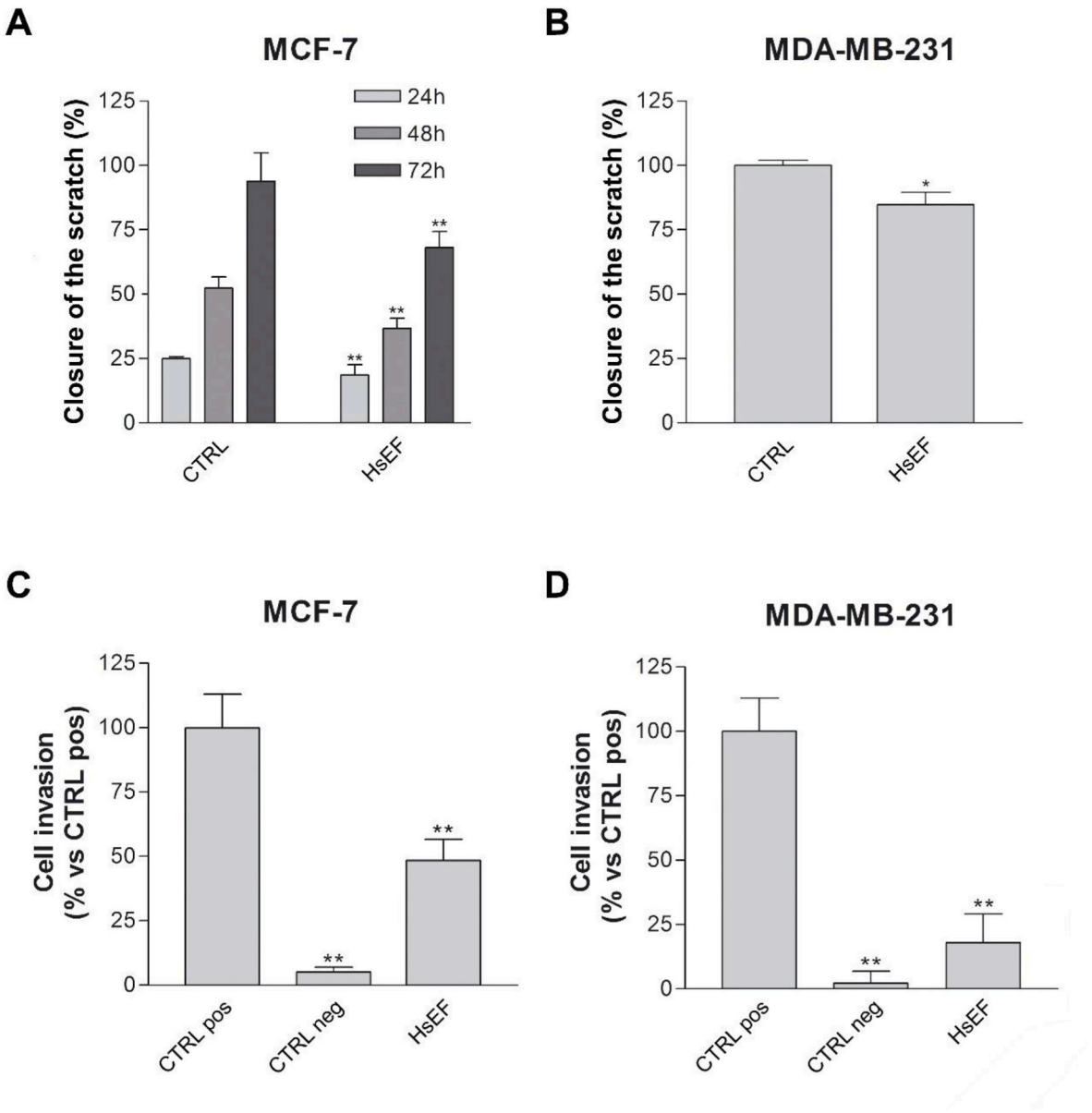

Fig. 5. Effect of HsEF on cell migration and invasion of MCF-7 and MDA-MB-231 cells. (A) Representative images and **(B)** respective graphs representing cell migration of MCF-7 and MDA-MB-231 in scratch wound healing assay. MCF-7 cells were treated with HsEF 3.5 mg/mL for 24, 48 and 72 h, while MDA-MB-231 only for 24 h. Data represent the percentage of scratch closure and are expressed as the mean ± SD of three independent experiments. (C) Representative images and **(D)** respective graphs representing the invasion of MCF-7 and MDA-MB-231 in Boyden chamber assay. Cells were treated with HsEF 3.5 mg/mL for 24 h. Data are expressed as the mean ± SD of three independent experiments and are compared to untreated positive controls at 24 h (CTRL pos, arbitrarily set to 100%), while CTRL neg represent cells that passed through the membrane in absence of chemoattractant. **$p<0.01$ vs CTRL.

indirectly targeted by HsEF antitumoral action. The hypothesis that ER-α dysregulation is on the basis of the decrease of MCF-7 vitality is also demonstrated by Crone et al. (2019) and Salis et al. (2014) after treatment with the herbal remedy Black cohosh and Fluvastatin respectively [25,26].

As a consequence of the dysregulation of ERα, in MCF-7 cells after HsEF treatment, we observed a reduction of mRNA and protein levels of two targets of ERα: BRCA1 and CAV1.

BRCA1 play an important role in DNA damage signaling, DNA repair and in the regulation of cell cycle progression [27]. Estrogens modulate BRCA1 expression in human breast cancer cells *in vitro*, but no estrogen-responsive element has been identified within the promoter of the BRCA1 gene [28]. However, BRCA1 is a regulator of the expression of ERα itself suggesting a functional relationship between this two genes [28]. 40–45% of hereditary breast cancers are caused by inherited mutations of BRCA1. Differently BCRA1 mutations are rarely present in sporadic breast cancers. However, the expression of BRCA1 is often altered in sporadic cancers, suggesting a pivotal role in breast cancer carcinogenesis [29]. Our experiments demonstrate that in MCF-7 treated with HsEF, a significant reduction of mRNA and protein level of BRCA1 is observed. These data are in accord with Crone et al. (2019) and Wang et al. (2014) where BCRA1 decrease is respectively associated to Black cohosh and Doxorubicin effect on MCF-7 cell viability [25,30]. Because BRCA1 play an important role in monitoring genomic integrity and in DNA repair, reduction of BRCA1 induced by HsEF, could be responsible of a decrease of DNA damage repair, allowing MCF-7 cells to be more susceptible to cellular death. In this way BRCA1 decrease could contribute to antiproliferative effect of Hibiscus extract.

CAV1 is a membrane resident protein that regulates breast cancer progression, affecting breast cancer cell invasion and migration. Direct

interaction of CAV1 with ERα, allows its localization on membrane with consequent activation of ER-non-genomic pathways [31]. CAV1 expression is deregulated in breast cancer cells, primary breast cancer and stromal tissues [18]. However, while CAV1 overexpression has been reported to play a role in invasion, metastasis and resistance to antitumoral therapy [32], CAV1 downregulation enhances autophagy process [33]. In our experiments HsEF induces a decrease of mRNA and protein levels of CAV1 in MCF-7 cells, but also a decrease of cell migration and invasion, and an increase of autophagy. These data suggest that in our model CAV1 promote HsEF antitumoral effects and could inhibit the activation of ERα non genomic processes.

Proteasome is a large protein complex responsible for the degradation of intracellular proteins and it is essential for cell proliferation [34]. The use of proteasome inhibitors in the treatment of different types of cancer has been evaluated by different research groups, but to date only Bortezomib, Carfilzomib and Ixazomib are approved for the use in treating multiple myeloma [35].

Autophagy is a physiological cellular process for the degradation and elimination of misfolded proteins and damaged organelles, by forming various membrane structures, including autophagosomes, lysosomes and autolysosomes. The modulation of autophagy plays dual roles in tumor suppression and promotion in many cancers [36].

Proteasome and autophagy, which represent the two main intracellular protein degradation processes within the eukaryotic cells, were originally regarded as rather independent. Instead they seem to be very closely related [37]: the induction of autophagy-dependent cell death was accompanied with the inhibition of proteasome activity [38,39].

Our results demonstrated that both in MCF-7 and MDA-MB-231 cells, treatment with HsEF induces an inhibition of proteasome and an increase of the autophagic process. While proteasome inhibition occurs similarly in the two cell lines, the increase in autophagy is greater in MCF-7 cells than in MDA-MB-231 cells. Recently, autophagy has emerged as a crucial player in the negative regulation of cellular proliferation [30]. It is possible that in cells with faster proliferation rate, such as MCF-7 cells, the increase of autophagy can have a greater effect, thus representing a further modality through which HsEF can perform its antitumor action. In MCF-7 cells, an increase of autophagic process could be an indirect effect of HsEF treatment, mediated by CAV1 reduction [33]. In MDA-MB-231 cells CAV1 is not involved in the increase of autophagy because, after treatment with HsEF, CAV1 downregulation is not observed.

For most of the experiments performed in this manuscript, we evaluated the effect of HsEF at 24 h. This choice is due to the results obtained in reversibility experiments. We have observed that 24 h of HsEF treatment are sufficient to induce an irreversible block of proliferation, both in MCF-7 and MDA-MB-231 cells, although the cells are kept up to 72h in medium without HsEF. This means that the first 24 h are crucial for HsEF action, and it is important to know what happens in this period.

MDA-MB-231 are a cellular model for triple-negative breast cancer (TNBC). The biggest limitation of TNBC treatment is the lack of effective targets [40]. Several therapeutic options are provided for the treatment of TNBC: chemotherapy, PARP inhibitors, immunotherapy and natural products [4,41]. We observed that in MDA-MB-231 cells, HsEF is effective and promote antitumoral effects, but unlike MCF-7 cells, ERα cannot be the target for this action. Furthermore, according to literature [42], we demonstrate that MDA-MB-231 cells express BCRA1 and CAV1, but their reduction after HsEF treatment is not significant, suggesting that BCRA1 and CAV1 do not contribute to the antiproliferative effects of HsEF. Furthermore, considering that CAV1 reduction is not significant, it cannot be responsible for the increase in autophagy observed in MDA-MB-231 cells observed after HsEF treatment.

Except for the proteasome inhibition, which could explain in part the observed effect, we have not yet identified the possible targets of HsEF in MDA-MB-231 cells, so undoubtedly this aspect will have to be investigated in future by further experiments.

National Cancer Institute define metastasis a spread of primary tumor from one part of the body to another. When cancer cells metastasize and form secondary tumors, the cells in the metastatic tumor are like those in the original tumor. Metastases are responsible for about 90% of cancer deaths. Metastasis is a multi-step process that includes migration and invasion of cancer cells. Initially cancer cells must detach from the primary tumor, intravasate into the circulatory and lymphatic systems, evade the immune system, extravasate at distant capillaries, invading and proliferating in distant organs [43].

We have analyzed if HsEF treatment is able to reduce metastatic process both in MCF-7 and MDA-MB-231 cells. These two cell lines represent two type of breast cancer with a different capacity to metastasize. MCF-7 is a poorly-aggressive and noninvasive cell line, normally being considered to have low metastatic potential [44], while MDA-MB-231 cells are highly aggressive and invasive [45].

To assess cell migratory behavior, we have performed two different approaches. The wound healing assay, to analyze migration ability of whole cell masses in terms of area closure, and the Boyden chamber migration/invasion assay, to evaluate the migration or invasiveness cell capacity to directionally respond to chemoattractants [46]. According with their property, MDA-MB-231 cells exhibit migration and invasion greater than MCF-7 cells. HsEF treatment is able to inhibit cell migration and invasion of both cell lines, but HsEF effect is more evident in MDA-MB-231 cells compared to MCF-7.

5. Conclusions

In Malacrida et al., 2016 and 2019 we demonstrated that Hibiscus extracts did not affect cell viability of non-tumor cells, but had antitumoral effects on Myeloma Multiple and Oral Squamous Cell Carcinoma cell lines. Furthermore, HS is not neurotoxic *in vitro* [16,47]) In the present study we demonstrate the antitumoral action of HsEF on breast cancer cells *in vitro*. -We have analyzed the HsEF effects on two breast cancer cell lines, MCF-7 and MDA-MB-231, that have different expression of ERα. HsEF has a greater antiproliferative capacity in MCF-7 than in MDA-MB-231 cells. On the contrary, HsEF contrasts the ability to metastasize more in MDA-MB-231 cells than in MCF-7. Furthermore, our data suggest that for MCF-7 cells, the direct or indirect HsEF target is ERα. For MDA-MB-231 this target has yet to be identified.

HsEF exhibits *in vitro* good antitumoral activity and is efficient both for luminal and triple negative breast cancer. Our results indicate HsEF as possible therapeutic option in treatment of breast cancer. Further studies are necessary to deepen its mechanism of action and for evaluate the possibility that HsEF can be used in combination of antineoplastic agent to improve their effectiveness. **Lastly,** HS will be evaluated in **proper** animal models, to determine its efficacy *in vivo* and **its** anti-inflammatory **properties**, given that inflammation plays a critical role in tumor progression**.**

Author contributions

Conceptualization, A.M., J.E. and M.M.; methodology, A.M., J.E. and V.R.; formal analysis, A.M., J.E. and M.H.; investigation, A.M., J.E., M.H. and V.R.; resources, A.C. and M.M.; data curation, A.M., J.E. and M.M; writing—original draft preparation, A.M. and M.M.; writing—review and editing, A.M. and M.M.; visualization, A.M.; supervision, G.C. and M.M.; project administration, M.M.; funding acquisition, M.M. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial

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Appendix A. Supplementary data

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