Grape seed extract suppresses MDA-MB231 breast cancer cell migration and invasion

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Abstract

Background and aim Breast cancer remains a leading cause of mortality among women. In metastasis, cascade migration of cancer cells and invasion of extracellular matrix (ECM) represent critical steps. Urokinase-type plasminogen activator (uPA), as well as metalloproteinases MMP-2 and MMP-9, strongly contribute to ECM remodelling, thus becoming associated with tumour migration and invasion. In addition, the high expression of cytoskeletal (CSK) proteins, as fascin, has been correlated with clinically aggressive metastatic tumours, and CSK proteins are thought to affect the migration of cancer cells. Consumption of fruits and vegetables, characterized by high procyanidin content, has been associated to a reduced mortality for breast cancer. Therefore, we investigated the biological effect of grape seed extract (GSE) on the highly metastatic MDA-MB231 breast cancer cell line, focusing on studying GSE ability in inhibiting two main metastatic processes, i.e., cell migration and invasion.

Methods After MDA-MB231 breast cancer cells stimulated with GSE migration and invasion were evaluated by means of trans-well assays and uPA as well as MMPs activity was detected by gelatin zymography. Fascin, β-catenin and nuclear factor-κB (NF-κB) expression were determined using western blot technique. β-Catenin localization was observed by confocal microscopy.

Results We observed that high concentrations of GSE inhibited cell proliferation and apoptosis. Conversely, low GSE concentration decreased cell migration and invasion, Dinicola Simona and Pasqualato Alessia contributed equally to this work.

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likely by hampering β-catenin expression and localization, fascin and NF-κB expression, as well as by decreasing the activity of uPA, MMP-2 and MMP-9.

Conclusions These results make GSE a powerful candidate for developing preventive agents against cancer metastasis.

Keywords Breast cancer · GSE · Invasion · Metalloproteinases

Introduction

Despite the advances in the understanding mechanisms of cancer progression and in development of different therapeutic strategies, breast tumour is still the leading cause of mortality among women, particularly due to its metastasizing ability [1, 2].

Metastasis is a multistep process, which involves migration, adhesion and invasion of cancer cells into the vessels, extravasation out of the vessel and interaction with the target tissue [3]. Extracellular matrix (ECM) degradation is a critical event in the metastasizing process, and it is well recognized that it is essential in determining tumour prognosis and treatment [4]. The urokinase-type plasminogen activator (uPA) strongly contributes to ECM remodelling, stimulating the conversion of inactive plasminogen to the protease plasin, which mediates cellular invasion both directly by degrading members of the matrix proteins [5, 6] and indirectly by activating matrix metalloproteinases (MMPs) [7].

Among human MMPs, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) represent key enzymes in the degradation of type IV collagen of ECM, thus becoming associated with tumour migration and invasion [8]. Therefore, the inhibition of uPA and MMPs activity is extremely relevant to arrest metastasis, mostly affecting cancer development after carcinogenesis [8, 9]. Moreover, cytoskeleton rearrangement, leading to enhancement of cell motility, is one of the dominant cellular mechanisms that regulates metastasis [10].

It was reported that, in many human carcinomas including breast cancers, fascin, a member of the actin cytoskeletal–bundling proteins contributing to filopodia development, is highly up-regulated [11], correlating with clinically aggressive tumours and metastasis [12]. Also, recent evidence suggested fascin as a novel target gene of β-catenin-TCF signalling [13] that activates the expression of genes involved in invasion and metastasis, such MMPs, at later steps of cancer progression [14, 15]. In addition, fascin facilitates metastasizing process via modulation of metastasis-associated genes, specifically down-regulating the metastasis suppressor BRMS1 and up-regulating the nuclear factor-κB (NF-κB) activity, as well as the induction of uPA, MMP-2 and MMP-9 expression [16]. Therefore, down-regulating β-catenin, fascin, as well as MMPs and their regulatory pathways may represent a useful strategy for anticancer drugs and chemo-preventive agents [17–19].

Frequent consumption of certain fruits and vegetables has been associated with a reduced risk of developing cancer, particularly at breast and colon [20]. Recently, we have observed that grape seed extract (GSE) obtained from different cultivars showed on human colorectal cancer cell lines anticancer properties, in terms of inhibition of proliferation and induction of apoptosis [21–23]. In support of our study, other studies demonstrated that GSE triggers growth arrest and cell death in human breast carcinoma cells [24].

The aim of this study was to determine in a highly metastatic human breast carcinoma cell line, MDA-MB231, the effect of GSE, particularly focusing on migration and invasion processes, associated to metastasis, and to further explain the possible mechanisms.

Materials and methods

Cell lines

The human breast cancer cell lines MDA-MB231 and MCF-7 were obtained from European Collection of Cell Cultures (ECACC). Cells were seeded into 25-cm² flasks (Falcon, Becton–Dickinson Labware, Franklin Lakes, NJ, USA) in DMEM supplemented with 10 % Foetal Calf Serum (FCS) and antibiotics (Penicillin 100 IU/ml, Streptomycin 100 μg/ml, Gentamycin 200 μg/ml). The cultures were kept at 37 °C in an atmosphere of 5 % CO₂ in air, and the medium was changed every third day. At confluence, the cells were sub-cultured after removal with 0.05 % trypsin–0.01 % EDTA.

Grape seed extract preparation

GSE was obtained from Palieri grape cultivar. Fresh grape berry samples were skinned and seeds were separated from pulp, and then, the seeds were gently wiped with filter paper to eliminate pulp residues. Homogeneous and dry material was obtained from seeds, extracted with methanol, and purified and analyzed by ESI–MS according to the previously published method [25]. Palieri GSE contained 6.2 mg/g catechins and 5.6 mg/g procyanidins. GSE was resuspended in 70 % ethanol at a concentration of 30 mg/ml and stored lightless at −20 °C until used. With the purpose of obtaining the concentration of 100 μg/ml (the highest concentration used in our experiments) GSE stock solutions were diluted 1:300.
Cell proliferation assay

MDA-MB231 and MCF-7 human breast cancer cells were seeded in 12-well culture plates (Falcon, Becton-Dickinson Labware, Franklin Lakes, NJ, USA) at densities ranging between of 1 × 10⁴ and 3 × 10⁵ cells/well in a standard medium. After a zero time (T₀) cell count, the cells were stimulated with 25, 50 and 100 μg/ml of Palieri GSE and incubated at 37 °C in an atmosphere of 5 % CO₂ in air. The cells were then detached from wells by trypsinization, and cell count was performed by a cell counter (Beckman Coulter, Inc., Fullerton, CA, USA) and by a Thoma hemocytometer, after staining with the vital stain trypan blue (Sigma Chemical Co., St Louis, MO, USA) after 24, 48, 72 and 96 h. Two replicate wells were used for each data point, and every experiment was performed six times.

Apoptosis analysis

MDA-MB231 and MCF-7 human breast cancer cells were cultured at confluence into 25-cm² flasks (Falcon, Becton–Dickinson Labware, Franklin Lakes, NJ, USA) in a standard medium and stimulated with 25, 50, 100 μg/ml Palieri GSE and incubated at 37 °C in an atmosphere of 5 % CO₂ in air. Control conditions (CTRL) was represented by untreated cells. After 24 h, the cells were trypsinized, washed twice with PBS and stained with FITC-labelled Annexin V/7-AAD (7-aminoactinomycine-D) according to the manufacturer’s instructions (Instrumental Pro3 Laboratory, Cavenago, MI, Italy). The samples were then analyzed by flow cytometry (EPICS Coulter XL, Beckman-Coulter Inc., Fullerton, CA, USA) for the quantification of apoptotic cells. The fluorescence of 20,000 events was measured and an excitation wavelength of 488 nm was used in combination with standard filters to discriminate between the FL1, FL3 channels, forward scatter and side scatter. Apoptosis analysis was performed three times.

Cell migration assay

MDA-MB231 human breast cancer cells (5 × 10³ cells/200 μl medium) were placed in the upper side of 8-μm filters (Falcon, Becton–Dickinson Labware, Franklin Lakes, NJ, USA) (upper chamber), placed in wells of a 24-well plate (Falcon; Becton–Dickinson Labware, Franklin Lakes, NJ, USA) (lower chamber), containing 0.8 ml of medium. After 24 h of starvation, 200 μl of DMEM 1 % FCS was added into the upper chamber, while the lower chamber contained DMEM 10 % FCS (0.8 ml) for untreated cells and DMEM 10 % FCS with 25 μg/ml Palieri GSE for treated cells. Chambers were kept in an incubator for 24 h. After incubation, cells from the upper surface of filters were removed with gentle swabbing, and the migratory cells on the lower surface of membranes were fixed and stained with haematoxylin/eosin. The membranes were examined microscopically, and cellular migration was determined by counting the number of cells on membranes in at least 4–5 randomly selected fields using a Zeiss Axiovert 10 optical microscope. For each data point, three independent experiments in duplicate were performed.

Cell invasion assay

The ability of MDA-MB231 human breast cancer cells for passing through Matrigel-coated 8-μm filters (BD Bio-Coat™ growth factor reduced MATRIGEL™ invasion chamber, BD Biosciences-Discovery Labware, Two Oak Park, Bedford, MA, USA) was measured by the Boyden chamber invasion assay. In brief, cells were treated with 25 μg/ml Palieri GSE. After 24 h, cells were detached by trypsin and resuspended in serum-free medium. Medium containing 10 % FCS for untreated cells and DMEM 10 % FCS with 25 μg/ml Palieri GSE for treated cells was applied to the lower chamber as chemo-attractant, and then, the cells were seeded on the upper chamber at a density of 5 × 10³ cells/well in a 200 μl of serum-free medium. The chamber was incubated for 24 h at 37 °C. At the end of incubation, the cells in the upper surface of the membrane were carefully removed with a cotton swab, and cells invaded across the Matrigel to the lower surface of the membrane were fixed with methanol and stained with haematoxylin and eosin. The invasive cells on the lower surface of the membrane filter were counted with Zeiss Axiovert 10 optical microscope. For each data point, three independent experiments in duplicate were performed.

Urokinase-PA zimography

To test the enzymatic activity of uPA, aliquots of conditioned media of MDA-MB231 human breast cancer untreated control cells and 25 μg/ml Palieri GSE-treated cells were separated by electrophoresis in 10 % polyacrylamide slab gels in the presence of SDS [SDS–polyacrylamide gels (PAGE)] under non-reducing conditions according to the procedure of Laemmli [26]. The uPA was then visualized by placing the Triton-X100-washed gel on a casein–agar–plasminogen underlay as previously described [27]. The lytic zones were plasminogen dependent. Molecular weights were calculated from the position of pre-stained markers subjected to electrophoresis in parallel lines. Densitometric scanning of zymographies was performed to derive a semi-quantitative estimation of protease activities. PA gelatin zimography was performed three times.
MMPs gelatin zymography

The enzymatic activities of MMP-2 and MMP-9 were determined by gelatin zymography. Briefly, conditioned media of MDA-MB231 human breast cancer untreated control cells and 25 μg/ml Palieri GSE-treated cells were prepared with standard SDS-polyacrylamide gel loading buffer containing 0.01% SDS without β-mercaptoethanol and not boiled before loading. Then, prepared samples were subjected to electrophoresis with 12% SDS-PAGE containing 1% gelatin. After electrophoresis, gels were washed twice with distilled water containing 2.5% Triton-X100 for 30 min at room temperature to remove SDS and then incubated in collagenase buffer (0.5 M Tris-HCl pH 7.5, 50 mM CaCl₂ and 2 M NaCl) overnight at 37°C, stained with Coomassie brilliant blue R-250 and destained with destaining solution (30% methanol, 10% acetic acid, and 60% water). MMP gelatin zymography was performed three times.

Western blot

MDA-MB231 human breast cancer cells were incubated with 25 μg/ml Palieri GSE, for 24 h. Untreated cells represented the control conditions. Following the treatment, the cells were washed twice with ice-cold PBS and scraped in RIPA buffer (Sigma Chemical Co., St Louis, MO, USA). A mix of protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany) was added just before use. Cellular extracts were then centrifuged at 8,000 rcf for 10 min. The protein content of supernatants was determined by using the Bradford assay. For western blot experiments, cellular extracts were separated on a 12% SDS-polyacrylamide gel. Proteins were blotted onto nitrocellulose membranes (BIO-RAD, Bio-Rad Laboratories, Hercules, CA, USA) and probed with the following antibodies: β-catenin (sc-7963, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), fascin1 (sc-28265, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), NF-κB (sc-109, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Antigens were detected with an enhanced chemo-luminescence (ECL) kit from Amersham (Amersham Biosciences, Little Chalfont, Buckinghamshire, England) according to the manufacturer’s instructions. For each data point, three independent experiments were performed.

Confocal microscopy

Untreated control cells and 25 μg/ml Palieri GSE-treated MDA-MB231 human breast cancer cells were cultured into 8-well μ-slides (ibidi GmbH, Am Klopferspitz 19, D-82152 Martinsried, Germany) for 48 h. Then, the cells were fixed with 4% paraformaldehyde for 10 min at 4°C and washed twice for 10 min with PBS. The cells were permeabilized for 30 min using PBS, 3% BSA, 0.1% Triton X-100, followed by the anti-β-catenin antibody (sc-7963, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-NF-κB antibody (sc-109, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) staining in PBS, 3% BSA at 4°C overnight. The cells were washed with PBS and incubated for 1 h at room temperature with TRITC fluorophore-conjugated secondary donkey anti-mouse antibody (Invitrogen Molecular Probes Eugene, Oregon). TOPRO-3 labeling was used for nuclei. The slides were then washed with PBS and mounted with 0.1 mM Tris-HCl at pH 9.5: glycerol (2:3). Negative controls were processed in the same conditions besides primary antibody staining. Finally, β-catenin and NF-κB immunolocalization was analyzed using a Leica confocal microscope (Laser Scanning TCS SP2) equipped with Ar/ArKr and He/Ne lasers. Laser line was at 543 nm for TRITC excitation.

Statistical analysis

Results from cell proliferation, cell cycle and apoptosis analyses were expressed as mean ± SD, and statistical analysis was performed through the analysis of variance (ANOVA), followed by the Bonferroni post-test. Results from the other experiments were expressed as mean ± SD, and statistical analysis was performed through unpaired, two-tailed Student’s t test. Differences were considered significant at the level of p < 0.05. Statistical analysis was performed using GraphPad Instat software (GraphPad Software, Inc.; San Diego, CA, USA).

Results

Cell proliferation

To study the sensitivity of MDA-MB231 human breast cancer cell line to GSE, the cells were treated with different...
concentrations of the extract (25, 50 and 100 μg/ml), usually used in bibliography. Then, proliferation rate was recorded every 24 h, until 96 h. GSE induced a statistically significant time-dependent growth inhibition, with respect to control, only at 50 and 100 μg/ml, meanwhile the lowest concentration of GSE failed to trigger a significant proliferation decrease (Fig. 1).

Apoptosis

The possible apoptotic effect of GSE on MDA-MB231 human breast cancer cells was observed after 24 h of incubation with 25, 50 and 100 μg/ml of the extract. GSE increased apoptotic cell death at a significant extent with respect to control only at high concentrations, meanwhile at 25 μg/ml, the amount of apoptotic cells was similar to that observed in control condition (Fig. 2).

GSE effects on proliferation and apoptosis on other breast cancer cells

In order to assess whether GSE could exert its effects on other breast cancer cells, we treated MCF-7 cells with GSE, and we studied proliferation and apoptosis, in the same conditions used for MDA-MB231. We observed that 50 and 100 μg/ml GSE induced a statistically significant time-dependent growth inhibition, with respect to control, starting from 72 h incubation. Otherwise, 25 μg/ml GSE failed to trigger a significant proliferation decrease (Fig. 3a). Moreover, the highest GSE concentration (100 μg/ml) was only to

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**Fig. 2** Effect of GSE on apoptosis of untreated (CTRL) and 25, 50 and 100 μg/ml GSE-treated MDA-MB231 cells after 24 h. Values are means of three independent experiments, with standard deviations represented by vertical bars. *p < 0.05; **p < 0.01. Effect of GSE on apoptosis of untreated (CTRL) and 25, 50 and 100 μg/ml GSE-treated MCF-7 cells after 24 h (b). Values are means of three independent experiments, with standard deviations represented by vertical bars.

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**Fig. 3** Effect of GSE on proliferation of untreated (CTRL) and 25, 50 and 100 μg/ml GSE-treated MCF-7 cells after 24, 48, 72 and 96 h (a). Values are means of six independent experiments performed in duplicate, with standard deviations represented by vertical bars. *p < 0.05; **p < 0.01. Effect of GSE on apoptosis of untreated (CTRL) and 25, 50 and 100 μg/ml GSE-treated MCF-7 cells after 24 h (b). Values are means of three independent experiments, with standard deviations represented by vertical bars.
induce a statistically significant increase in the apoptotic rate after 24 h stimulation (Fig. 3b).

Cell migration and invasion

To study the effect of GSE on MDA-MB231 human breast cancer cells migration and invasion, the not cytotoxic concentration 25 µg/ml was used. For cell migration assay, after 24 h of treatment, we counted the cells moved from the upper side of 8-µm filters to the lower one. The treatment with 25 µg/ml of GSE significantly reduced the number of MDA-MB231 cells passed into the lower chamber (Fig. 4a). For invasion studies, similar experiments were performed using Matrigel-coated 8-µm filters. We observed that the number of treated cells invaded the Matrigel layer was significantly reduced, with respect to control condition (Fig. 4b).

Urokinase-PA and MMPs activity

To provide insights into possible mechanisms underlying GSE-mediated invasion suppression, we examined the activity of uPA, a key metastasis-related factor implicated strongly in breast cancer metastasis. MDA-MB231 human breast cancer cells showed a significant decrease in uPA activity, when treated with 25 µg/ml of GSE both for 24 and 48 h in respect to control (Fig. 5a). Moreover, we observed MMP-2 and MMP-9 activity under the same experimental conditions and we reported that both MMPs decreased their activity. In particular, MMP-2 showed a statistically significant decrease in its activity after 24 h of incubation with 25 µg/ml of GSE in respect to control (Fig. 5b); meanwhile, after 48 h, no relevant differences were observed. Conversely, MMP-9 decreased only after 48 h of treatment with respect to control (Fig. 5c).

Protein levels

Since GSE affects migration and invasion, we determined by western blot analysis the expression of molecular parameters involved in these processes. As first, we showed that β-catenin protein levels were significantly decreased after 48 h of treatment with 25 µg/ml of GSE, with respect to control conditions (Fig. 6a). Moreover, the same trend was observed for fascin (Fig. 6b) and for NF-κB (Fig. 6c).

β-Catenin and NF-κB localization

Since fascin expression may be related to β-catenin localization, we investigated, by means of confocal microscopy, β-catenin immune localization. In the control condition, the fluorescent intensity of the protein was recovered mainly in the nuclei. Besides, we observed the β-catenin signal at perinuclear level and rarely in the cortical part of plasma membrane. Conversely, it is well evident that in 25 µg/ml GSE-treated MDA-MB231 human breast cancer cells, β-catenin nuclear localization disappeared. We observed the protein as diffuse cytoplasmic signal or present in small parts of the cortical plasma membrane (Fig. 7). On the other hand, after 48 h, NF-κB localization was not affected by GSE treatment (data not shown).

Discussion

Several studies documented the biological properties of GSE and its principal compounds, such as catechins and procyanidins, particularly associated to their anticancer and chemo-preventive activities [21–24, 28–31]. Indeed, even this study documented that high concentration (50 and 100 µg/ml) of GSE inhibits cell
proliferation and increases apoptosis in MDA-MB231 human breast cancer cell line.

To support the anti-proliferative and pro-apoptotic role of GSE against breast cancer cells, we determined GSE effect on another breast cancer cell line, MCF-7. In both cell lines, proliferation strongly decreased with 50 and 100 μg/ml GSE. Focusing on the highest concentration, we observed that this effect was earlier in MDA-MB231. In apoptotic assay, we showed a significant increase of cell death only at 100 μg/ml GSE. Differently, in MDA-MB231, this effect was obtained with 50 as well.

As previously reported, this behaviour could be associated to the different malignant phenotype of each breast cancer cell line as well as to the sensitivity to the anticancer effects triggered by GSE [21, 22].

Moreover, we explored the possible effects of GSE against breast cancer cell migration and invasion, two fundamental aspects of the metastatic process. Experiments were performed on MDA-MB231 cells, more malignant and invasive than MCF-7 cells. GSE at a sub-lethal concentration (25 μg/ml) strongly inhibited cell migration and invasion. Actually, this inhibition was accompanied by a decrease in uPA, MMP-2 and MMP-9 activities, as well as in a down-regulation of β-catenin, fascin and NF-κB expression.

These findings are in agreement with previous studies which investigated vegetal extract for their anti-invasive properties. Pachymic acid, derived from Poria cocos was shown to impair breast cancer cell invasion by suppressing matrix metalloproteinase-9 expression [32]. Similar results were obtained with piceatannol, a natural analogue of resveratrol, that was reported to inhibit the invasive capability of human breast cancer cells, attenuating MMP-9 activity [33]. An anti-metastatic effect was observed treating MDA-MB-231 cells with ovatodiolide, the major component of the plant Anisomeles indica [9]. Ovatodiolide inhibited metastasis by reducing MMP-9 and NF-κB activity [9]. In addition, it has been recorded that berberine suppresses the MMP-1 and MMP-9 expression [34].

Interesting data are available about anti-metastatic properties shared by several grape components, such as proanthocyanidins and epigallocatechin, both of which inhibit matrix MMPs release in human prostate carcinoma cells, hindering thereafter MAPK and NF-κB pathways [35, 36].

Fig. 5 GSE inhibitory effect on uPA (a), MMP-2 (b) and MMP-9 (c) activity for 24 and 48 h. Values are means of three independent experiments, with standard deviations represented by vertical bars.
Accumulating evidence has suggested that MMP-2 and MMP-9 activities are highly correlated with breast cancer cell invasion [37, 38]. Thus, agents that could affect them and down-regulate their activity are warranted. Indeed, in our study, we demonstrated that GSE at low concentration inhibited MMP-2 and MMP-9 activities, respectively, after 24 and 48 h of treatment, contributing to decrease the invasive power of MDA-MB231 breast cancer cells.

It has been reported that matrix MMPs, like MMP-2 and MMP-9, may be activated by uPA [7], which is also capable of degrading members of the matrix proteins, such as fibronectin, collagen and laminin by itself [5, 6]. We demonstrated that GSE-treated cells showed a significant decrease in uPA activity after a 24-h treatment with respect to control conditions. As a result, MDA-MB231 cells invaded the Matrigel layer significantly less than the control cells after 24 h. Therefore, it seems likely that uPA inhibition could lead to a “matrix-protective” effect.

MMPs and uPA are primarily regulated at transcriptional and post-transcriptional levels and at the protein level through their activators and inhibitors, and at cell surface localization [39, 40]. Transcription of MMPs and the uPA is regulated by upstream sequences, including motifs corresponding to NF-κB, AP-1 or SP-1 binding sites [39–41].

NF-κB is involved in numerous pathological processes, such as inflammation, cancer cell adhesion, invasion, metastasis and angiogenesis [42, 43]. In this study, we found that GSE significantly decreased NF-κB levels after 48 h of treatment, even though its localization was not affected by GSE treatment. Moreover, recent researches demonstrated a strong link among NF-κB, MMPs, uPA and fascin, a new key player in tumour metastasis [16, 44]. Fascin is a highly conserved actin-bundling protein that localizes to microspikes and filopodia and functions in cell adhesion and motility [45]. It was reported that fascin is over-expressed in large numbers of metastatic cancers, including gastric, breast, colon and ovarian [45, 46]. In our experiments, fascin expression resulted heavily down-regulated in GSE-treated samples, indicating that treated cells showed less motility than those of control conditions. This result was confirmed by the migration assay, which evidenced how the incubation with low concentration of GSE induced MDA-MB231 cells to migrate less than untreated cells.

Lately it was reported that fascin expression correlates with the presence of β-catenin in the nuclei of cells [13].
Thus, we determined the β-catenin localization in MDA-MB231 cell line. GSE-treated cells showed a cytoplasmic and cortical localization of β-catenin, with respect to control cells that exhibited this protein mainly in the nuclei and at perinuclear level. We also valued β-catenin expression and we observed that a 48-h treatment with GSE significantly reduced the expression of this protein. β-catenin, central effector of the Wnt signaling pathway, is involved in several developmental and homeostatic processes [47, 48]. Imbalance in the structural and signaling properties of β-catenin often results in an inappropriate nuclear accumulation of this protein and consequently causes disease and deregulated growth connected to cancer and metastasis [47, 48]. Conversely, in the cytoplasm, β-catenin is normally short-lived: it is recognized by the destruction complex and rapidly targeted for degradation. The fact that the extract induces a translocation from the nucleus to the cytoplasm and cortical level of the protein

Fig. 7 Confocal microscopy of β-catenin distribution in untreated (CTRL) (a, c, e) and 25 μg/ml GSE-treated (b, d, f) MDA-MB231 cells after 48 h. In a and b β-catenin distribution is reported in green.

In e and d TOPRO-3 nuclei labelling is shown. In e and f the merge of green and TOPRO-3 signals is reported. A representative part of samples is shown.
represents a further confirmation of the decrease in the metastatic potential faced by the tested cell line following treatment.

In conclusion, this study showed that GSE may differently affect different metastatic pathways, depending on the GSE concentration. High GSE concentration triggered proliferation arrest and apoptosis; on the contrary, low GSE concentration failed to induce growth inhibition or cell death, otherwise influencing migration and invasion processes.

GSE inhibited the cellular migration of MDA-MB231: that inhibition is likely to be mediated by a reduction in fascin expression, a CSK-related protein. Indeed, MDA-MB231 cells, once incubated with GSE, exhibited a less invasivity that should be ascribed, at least in part, to the inhibited activity of uPA, MMP-2 and MMP-9.

Overall, these results suggest that GSE could be thought a powerful candidate for developing preventive agents against cancer metastasis. Indeed, despite the availability of selected treatment options for breast cancer therapies, yet a natural product like GSE may be a promising adjuvant, able to amplify response obtained with conventional pharmacological cares. High GSE concentrations are likely to induce cancer cells apoptosis indeed; meanwhile, sublethal GSE doses might inhibit the metastatic cascade affecting MMPs, uPA and cytoskeletal factors as β-catenin and fascin. Therefore, a clinical target-oriented study to examine the anticancer effect of GSE is urgently warranted.

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Conflict of interest On behalf of all authors, the corresponding author (Mariano Bizzari) states that there is no conflict of interests.

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