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Exploring the anti-proliferative, pro-apoptotic, and antioxidant properties of *Santolina corsica* Jord. & Fourr. (Asteraceae)

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ABSTRACT

Aims: The bioactivities of *Santolina corsica* Jord. & Fourr. *n*-hexane (EHS) and methanol (EMS) extracts were evaluated in relation to their chemical profile.
Main methods: EHS and EMS were analysed by gas chromatography-mass spectrometry () and high performance liquid chromatography-diode array detection (HPLC-DAD), respectively. Antioxidant activity was determined by β-carotene bleaching, Ferric Reducing Activity Power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) tests. Nitric oxide (NO) production was assessed in LPS-stimulated RAW 264.7 cells. Anti-proliferative activity was evaluated by MTT assay on A549, HeLa, PC3, MCF-7, MDA-MB-231 cancer cells, and non-tumorigenic MCF10 A cells. Cell motility, migration and invasion were assessed by wound-healing scratch, migration and invasion assays, respectively. DNA fragmentation was tested by TUNEL assay. Cells morphology was studied by phase-contrast microscopy. Procaspase-8, -9, poly (ADP-ribose) polymerase and COX-2 expression levels were evaluated by immunoblotting analysis.
Key findings: Kaempferol-3-*O*-glucoside (5878.67 mg/100 g of extract), chlorogenic acid (746.11 mg/100 g), and rosmarinic acid (550.16 mg/100 g) were the dominant EMS constituents. EHS showed myrcene (18.86%) as the main compound, followed by palmitic acid methyl and ethyl esters (9.35 and 9.16%, respectively), β-phellandrene (8.48%), and ar-curcumene (5.63%). Both extracts showed promising anti-proliferative activity on all tested cancer cells, without inducing cytotoxicity in non-tumorigenic cells MCF-10 A. Moreover, extracts inhibited motility, migration, and invasion of MDA-MB-231 cells, inducing apoptosis. EHS decreased NO production, showing anti-inflammatory activity.
Significance: S. corsica extracts might be potentially useful in cancer treatment, since reduce invasive and migratory potential of MDA-MB-231 cells triggering apoptosis.

1. Introduction

During the last years, natural products-based drug discovery is increasing. At present, about 60% of the antitumor drugs are compounds isolated from plants. There are many evidences demonstrating the potential of plant-derived compounds as inhibitors of different stages of tumorigenesis and the associated inflammatory and oxidant processes. Furthermore some works have been published underlying the significance of these products in cancer prevention and therapy [1].

Cancer is a leading cause of death. The burden is expected to grow worldwide due to the growth and aging of the population, mainly in the less developed countries (about 80% of the world's population). The adoption of lifestyle behaviours known to increase cancer risk have risen cancer burden, mainly in the less economically developed countries. Annually, the American Cancer Society estimates the numbers of new cancer cases and deaths in the United States. In 2018, 1,735,350 new cancer cases and 609,640 cancer deaths are estimated. Over the past decade, cancer incidence rate declined by about 2% *per annum* in men, whereas it was constant in women. Death rate caused by cancer declined by about 1.5% in both women and men [2]. The study of natural products resulted in the discovery of several important anticancer agents such as podophyllotoxin derivatives and vinca alkaloids [1].

The genus *Santolina* (Asteraceae, Anthemideae) comprises species

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widely distributed in the Mediterranean area [3]. Phytochemical studies showed the presence of terpenoids, flavonoids, and coumarins as the main classes of compounds of this genus. *Santolina* species showed various biological activities, including antibacterial, antifungal, antiviral, anti-inflammatory, cytotoxic, and hepatoprotective effects [3–6]. *Santolina corsica* Jord. & Fourr. is a polyploid [7] endemic to Corsica (France) and Sardinia (Italy) [8,9], whose chemical composition is yet to be further investigated. In this regard, only three works reported the chemical profile of *S. corsica* essential oil [10–12].

The aim of this study was to test for the first time antioxidant and anti-inflammatory properties of *n*-hexane and methanol *S. corsica* extracts (EHS and EMS, respectively), in relation to their chemical profile. Additionally, their anti-proliferative activity on a wide panel of cancer cell lines, including uterine cervical (HeLa), alveolar (A549), prostate (PC3), luminal and basal breast (MCF7 and MDA-MB-231) cell lines was evaluated.

2. Material and methods

2.1. Chemicals and reagents

Dimethyl sulfoxide (DMSO), ethanol, *n*-hexane, methanol, and SiO_2 60 F254-precoated plates were obtained from VWR (Milan, Italy). All HPLC reagents were of analytical grade and were purchased from Sigma-Aldrich S.p.a. (Milan, Italy). Standard of target phytochemical compounds were supplied by Extrasynthese (Genay-France). Dulbecco's modified essential medium (DMEM), DMEM/F12, horse serum (HS), insulin, hydrocortisone, human epidermal growth factor (hEGF), fetal bovine serum (FBS), cholera toxin, 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium (MTT), doxorubicin, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), β-carotene, ascorbic acid, butylated hydroxytoluene (BHT), propyl gallate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate, linoleic acid, Folin-Ciocalteau reagent, quercetin, chlorogenic acid, diclofenac, Griess reagent, bacterial lipopolysaccharide (LPS), matrigel and Tween 20 were purchased from Sigma-Aldrich (Milan, Italy). L-glutamine and penicillin/streptomycin were obtained from Gibco, Life Technologies.

2.2. Plant materials and extraction procedure

The aerial parts of *S. corsica* were collected in Corsica (France) about two kilometers north of Corte (42.322653 °N, 9.159667 °E) in 8 June 2016. Voucher specimens were deposited in PI (accession numbers 011151 and 011152).

The aerial parts of *S. corsica* (941.97 and 1180.05 g, respectively) were exhaustively extracted by maceration with *n*-hexane and methanol (5 × 2 L) as solvents to give 92.69 and 140.78 g of the respective dried residue (yield of 9.84 and 11.93%, respectively).

2.3. Total phenols content of S. corsica methanol extract

Total phenols content of *S. corsica* methanol extract (EMS) was determined as previously described [13]. Extract was mixed with 0.2 mL of Folin-Ciocalteau reagent, 2 mL of water, and 1 mL of 15% Na_2CO_3. After 2 h of incubation at room temperature, the absorbance was measured at 765 nm (UV Jenway 6003 spectrophotometer). The total phenols content was expressed as milligrams of chlorogenic acid equivalents/g plant materials.

2.4. Total flavonoids content of S. corsica methanol extract

The flavonoids content of *S. corsica* methanol extract was determined as previously described [13]. The absorbance was read at 510 nm. The total flavonoids content was expressed as milligrams of quercetin equivalents/g plant materials.

2.5. RP-HPLC/UV-Vis analysis of bioactive compounds of S. corsica methanol extract

The methanol extract of *S. corsica* was analysed by high performance liquid chromatography-diode array detection (HPLC-DAD) by using a Knauer (Asi Advanced Scientific Instruments, Berlin, Germany) system equipped with two pumps Smartiline Pump 1000, a Rheodyne injection valve (20 μL) and a photodiode array detector UV/VIS equipped with a semi-microcell. Compounds were separated on a TSK gel ODS-100 V (TOSOH Bioscience, Germany) column (250 mm × 3.0 I.D.; 3 μm) at 30 °C and the flow rate used was 0.5 mL/min. The mobile phase consisted of water/formic acid (99.9:0.1, v/v; solvent A) and acetonitrile/formic acid (99.9:0.1, v/v; solvent B), and the gradient profile was as follows: 0.01–20.00 min 5% B isocratic; 20.01–50.00 min, 5–40% B; 40–95% B; 55.01–60.00 min 95% B isocratic. Based on literature data, caffeic acid, chlorogenic acid, (-)-epicatechin, ferulic acid, gallic acid, genistin, kaempferol-3-*O*-glucoside, neochlorogenic acid, protocatechuic acid, quercetin-3-*O*-glucoside, rosmarinic acid, and vanillic acid were chosen as markers and analysed. A calibration straight for each standard was obtained by analysing the standard solution diluted at different concentrations. All solutions were filtered through a 0.45 μm millipore filter (GMF Whatman) and injected into the HPLC system to determine retention times. Identification and quantification were carried out based on recorded retention times in comparison with authentic standards at 280, 254, 330 and 305 nm. Analyses were performed in triplicate. Data processing were carried out using Clarity Software (Chromatography Station for windows). All extracts were dissolved in 10 mL of methanol and filtered through a 0.45 μm Millipore filter (GMF Whatman) before HPLCdetermination. Results were expressed as mg/100 g of extract.

2.6. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) analyses

S. corsica n-hexane extract (EHS) was analysed by GC using GC17 A gas chromatograph (Shimadzu, Milan, Italy) fitted with a HP-5 MS capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) (Agilent, Milan, Italy) and controlled by Borwin Software. Flame ionization detection (FID) was performed at 280 °C. Nitrogen was the carrier gas (1 mL/min). Column temperature was initially kept at 50 °C for 5 min and then increased to 280 °C at 13 °C/min, held for 10 min at 280 °C. analyses were performed on a Hewlett-Packard 6890 gas chromatograph fitted with a fused silica HP-5 capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) (Agilent, Milan, Italy). Ionization energy voltage 70 eV was used. Helium was used as carrier gas. Chromatographic conditions were as given above. Constituents were tentatively identified by comparison of their retention indices either with those of literature or with those of authentic compounds available in our laboratory. Further identification was made by comparison of their mass spectra on both columns with those stored either in Wiley 275 library or with mass spectra from literature and our homemade library [14]. Component relative concentrations were calculated based on GC-FID peak areas without using correction factors.

2.7. Cell cultures

Cell lines used in this study, namely human lung adenocarcinoma cell line (A549), human uterine cervix adenocarcinoma (HeLa), androgen-independent human prostate cancer cell line (PC3), human breast cancer ER^+ cells (MCF-7), triple negative breast adenocarcinoma cell line (MDA-MB-231), murine macrophages (RAW 264.7) and human mammary epithelial cells (MCF 10 A), were purchased from the American Culture Collection (ATCC, Manassas, VA). For maintenance purposes, PC3 and A549 cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin/streptomycin. HeLa, MCF7, MDA-MB-231 and MCF 10 A cells

were cultured as previously reported [15]. RAW 264.7 cell line was cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin. Treatments were performed in appropriate media containing a lower amount of supplemented serum (2%). All cell lines were cultured at 37 °C with 5% CO_2 in a humidified atmosphere.

2.8. Cell viability assay

Cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, as previously reported [16]. A549, HeLa, PC3, MCF7, MDA-MB-231 and MCF 10 A cell lines were seeded in 48-well plates with a density of 2×10^4 cells/well and cultured overnight in complete medium. Then, cells were treated with different concentrations of *S. corsica* extracts for 72 h. DMSO was used as a vehicle control. At the end of treatment period, MTT solution was added to each well (final concentration 0.5 mg/mL), and each plate was incubated at 37 °C for 2 h followed by media removal, DMSO-solubilized formazan in each well was quantified by measuring absorbance at 570 nm using a microplate reader Molecular Devices SpectraMax Plus Plate Reader (Molecular Devices, Celbio, Milan, Italy). Non-linear regression analysis (by GraphPad Prism 7) was used to generate sigmoidal dose-response curves to calculate IC_{50} values for each cell line.

2.9. Wound-healing scratch assay

Cell motility was assessed by wound-healing scratch assay [17,18]. Briefly, MDA-MB-231 cells were seeded into 6-well plate and cultured overnight in complete medium. Upon confluence, a wound was made by scratching across the monolayer cells on the bottom of the wells by using p-200 pipette tip followed by treatment with *S. corsica* extracts. After 24 h, cells were fixed and stained with Coomassie brilliant Blue. Photographs were taken at 4× magnification using phase-contrast microscopy and are representative of three independent experiments. The rate of wound healing was quantified from the picture using Adobe Photoshop software and standard deviations were determined by GraphPad-Prism7 software (GraphPad Inc., San Diego, CA).

2.10. Migration assay

Cell migration was assessed by migration assay [19]. Briefly, 5×10^4 of MDA-MB-231 cells were trypsinized, washed and re-suspended in DMEM/F12 supplemented with 2% FBS and treated with EHS and EMS. Then cells were seeded in the top portion of the chamber (BD, Bioscience). Lower portion of the chamber contained 10% FBS used as a chemoattractant. Chambers were incubated at 37 °C in 5% CO_2 for 24 h, washed three times with PBS and stained with Coomassie brilliant Blue. Photographs were taken at 4× magnification using phase-contrast microscopy and are representative of three independent experiments. Migration rate was quantified from the picture using imageJ software to count cells, standard deviations were determined by GraphPad-Prism7 software (GraphPad Inc., San Diego, CA).

2.11. Invasion assay

Cell invasion was performed by invasion assay [19]. Pre-chilled serum-free DMEM/F12 was mixed with Matrigel (1:5; Sigma-Aldrich). Upper compartments of the chambers were filled with 30 µL of the mixture, and Matrigel was allowed to solidify at room temperature for 1 h. An aliquot of MDA-MB-231 cells (10×10^4) were trypsinized, washed and resuspended in DMEM/F12 supplemented with 2% FBS and treated with EHS or EMS. Then cells were seeded in the top portion of the chamber (BD, Bioscience). Lower portion of the chamber contained 10% FBS, used as a chemoattractant. Chambers were incubated at 37 °C in 5% CO_2 for 24 h, washed three times with PBS and stained with Coomassie brilliant Blue. Photographs were taken at 4× magnification using phase-contrast microscopy, and are representative of three independent experiments. Invasion rate was quantified as migration rate, using GraphPad-Prism7 software.

2.12. Immunoblotting analysis

MDA-MB-231 and RAW 264.7 cells were grown to confluence, treated as before indicated, and lysed as previously described [20]. Briefly, cells were lysed in 500 µL of 50 mM Trisl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS, containing a mixture of protease inhibitors (aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate) for total protein extraction. Amounts of proteins were resolved on 15% SDS-polyacrylamide gel [21], transferred to a nitrocellulose membrane and probed with procaspase-8,-9, cyclooxygenase-2 (COX-2) and Poly (ADP-ribose) polymerase (PARP) specific antibodies (Santa Cruz, Biotechnology, CA, USA and Merck KGaA, DA, DE). Membranes were stripped and incubated with anti-GAPDH antibody (Santa Cruz, Biotechnology, CA, USA), in order to confirm equal loading and transfer. Antigen-antibody complexes were detected by incubation of the membranes with peroxidase-coupled goat anti-mouse or goat anti-rabbit antibodies and revealed using the ECL System (Bio-Rad Laboratories, CA, USA).

2.13. TUNEL assay

DNA fragmentation, a late event of apoptosis, was determined by enzymatic labeling of DNA strand breaks using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL). TUNEL labeling was conducted using TUNEL assay Kit (Promega) and performed as previously described [22]. Briefly, MDA-MB-231 cells treated for 72 h with *S. corsica* extracts were fixed in freshly prepared 4% methanol-free paraformaldehyde solution in PBS (pH 7.4) for 25 min at 4 °C. After fixation, cells were permeabilized in 0.2% Triton® X-100 solution in PBS for 5 min, then, they were washed twice with washing buffer for 5 min, and covered with 100 µL of equilibration buffer at room temperature for The labeling reaction was performed using fluorescin-dUTP mix and rTdT enzyme for each sample and incubated for 1 h at 37 °C. In such reaction rTdT enzyme catalyses fluorescein-dUTP binding to free OH ends in the nicked DNA. After rinsing, cells were washed with 20X SSC solution buffer and subsequently incubated with 100 µL of, 6- diamidino-2-phenylindole (DAPI) in order to stain nuclei, they were protected from light, analysed and photographed by using a fluorescent microscope (10× objective).

2.14. Morphological analysis

MDA-MB-231 cells were seeded into 6-well plate with a density of 8×10^4 cells/well and cultured overnight in complete medium. Then, cells were treated with EHS or EMS for 72 h, using concentrations equal to their IC_{50} values. DMSO was used as a vehicle control. Cells were fixed and stained with May Grünwald-Giemsa (Bio-Optica), as previously described [23]. Photographs were taken at 10× magnification using phase-contrast microscopy, and are representative of three independent experiments.

2.15. Inhibition of NO production in LPS-stimulated RAW 264.7

Nitric oxide (NO) production was measured by performing quantitative evaluation of nitrites (stable oxidized NO compounds) in cell culture media by using Griess reagent, as previously described [24]. RAW 264.7 were seeded into 24-wells plates with a density of 2×10^5 cell/wells and cultured overnight in complete medium. Then, cells were treated with 1 µg/mL bacterial LPS and different concentrations of each *S. corsica* extract for 24 h. At the end of the treatment period, 100 µL of cell culture media were removed and added to 100 µL of Griess reagent into a 96-wells plate followed by spectrophotometric measurement at

550 nm using a microplate reader. Nitrite concentration in the media was determined by comparison with a sodium nitrite standard curve. Cells viability was performed as described in Methods section (par. 2.8) after 24 h of treatment. Diclofenac was used as a positive control.

2.16. In vitro antioxidant tests

2.16.1. ABTS test

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) test was used to investigate the anti-radicals scavenging ability of *S. corsica* extracts [25]. Ascorbic acid was used as positive control. Briefly, ABTS solution was mixed with potassium persulfate and left in the dark for 12 h before use. ABTS solution was diluted with methanol to an absorbance of 0.70 at 734 nm. *S. corsica* extracts μg/mL) were added to the diluted ABTS solution. Absorbance was measured at 734 nm after 6 min.

2.16.2. DPPH test

A methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical at the concentration of 1.0×10^{-4} M was mixed with *S. corsica* extracts (concentrations ranging from 31.3 to 1000 μg/mL) [25]. The reaction mixtures were left in the dark for 30 min. The bleaching of DPPH was determined by measuring the absorbance at 517 nm with-Jenway 6003 spectrophotometer against blank without DPPH. Ascorbic acid was used as positive control.

2.16.3. Ferric Reducing Activity Power (FRAP) test

Antioxidant activity of *S. corsica* extracts was studied by using Ferric Reducing Activity Power (FRAP), as previously reported [25]. FRAP assay is based on a redox reaction involving TPTZ (2,4,6-tripyridyl-striazine)-Fe^{3+} complex. All the samples were tested at a concentration of 2.5 mg/mL. Absorption was measured at 595 nm. Butylated hydroxytoluene (BHT) was the positive control.

2.16.4. β-Carotene bleaching test

In β-carotene bleaching test, a mixture of β-carotene, linoleic acid, and Tween 20 was prepared as previously described [13]. After evaporation of solvent and dilution with water, the obtained emulsion was mixed to *S. corsica* samples at different concentrations μg/mL). Tubes were placed at 45 °C in a water bath for 60 min. Absorbance was read at 470 nm at initial time (t = 0) and after 30 and 60 min of incubation. Propyl gallate was used as a positive control.

2.17. Statistical analysis

The concentration giving 50% of inhibition (IC_{50}) was calculated by nonlinear regression using GraphPad-Prism7 software (GraphPad Inc., San Diego, CA). All results were expressed as means ± SD (standard deviation), taken over ≥ three independent experiments, with ≥ three replicates experiment, unless otherwise stated. Differences among means were tested for statistical significance by using one-way analysis of variance (ANOVA) test. P value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Chemical profile

S. corsica aerial parts were extracted with solvents of increasing polarity to afford *n*-hexane and methanol residues. The *n*-hexane extract was analysed by GC and analyses. As reported in Table 1, 39 compounds (90.21% of total extract) were identified as the main constituents. Myrcene is the dominant compound (18.86%), followed by methyl and ethyl esters of palmitic acid (9.35 and 9.16%, respectively), β-phellandrene (8.48%), ar-curcumene (5.63%), and 1,8-cineole (3.48%).

Table 1
The main constituents of *S. corsica* *n*-hexane extract.

Compound	%	I.M[a]
α-Pinene	0.34 ± 2.5	1,2,3
Camphene	0.28 ± 0.04	1,2,3
Myrcene	18.86 ± 0.7	1,2,3
Sabinene	0.41 ± 0.02	1,2,3
β-Thujene	0.72 ± 1.1	1,2
p-Cymene	0.22 ± 0.01	1,2
α-Terpinene	0.48 ± 0.05	1,2,3
Limonene	1.41 ± 0.03	1,2,3
β-Phellandrene	8.48 ± 1.1	1,2
1,8-Cineole	3.48 ± 1.1	1,2
γ-Terpinene	0.20 ± 0.01	1,2,3
Terpinolene	0.85 ± 0.07	1,2,3
Linalool	0.46 ± 0.02	1,2,3
Camphor	1.31 ± 0.03	1,2
Terpinen-4-ol	0.29 ± 0.02	1,2
α-Terpineol	0.92 ± 0.9	1,2,3
Phellandral	0.21 ± 0.02	1,2
α-Cubebene	0.21 ± 0.01	1,2
α-Copaene	0.17 ± 0.03	1,2
α-Gurjunene	tr	1,2
trans-Caryophyllene	0.26 ± 0.03	1,2,3
γ-Cadinene	0.13 ± 0.01	1,2
δ-Cadinene	0.24 ± 0.02	1,2
(*E*)-β-Farnesene	1.74 ± 0.02	1,2
allo-Aromadendrene	0.52 ± 0.01	1,2
Ar-Curcumene	5.63 ± 0.01	1,2
β-Selinene	tr	1,2
Spathulenol	1.81 ± 0.01	1,2
(*Z*)-Phytol	1.02 ± 0.3	1,2
Palmitic acid methyl ester	9.35 ± 0.7	1,2
Palmitic acid ethyl ester	9.16 ± 0.6	1,2
Heneicosane	0.65 ± 0.03	1,2,3
Tricosane	0.35 ± 0.02	1,2,3
Pentacosane	0.25 ± 0.01	1,2,3
Heptacosane	0.41 ± 0.01	1,2,3
Nonacosane	0.38 ± 0.03	1,2,3
Stigmasta-5,22-dien-3-ol	2.94 ± 0.02	1,2
α-Amyrin	4.24 ± 0.1	1,2
β-Amyrin	3.75 ± 0.3	1,2

Data are expressed as mean ± standard deviation (SD) ($n = 3$). [a]IM: Identification Methods: 1, comparison of retention times; 2, comparison of mass spectra with MS libraries, 3, comparison with authentic compounds. tr: trace (< 0.1%).

Table 2
HPLC-profile of *S. corsica* methanol extract.

Compound	mg/100 g extract
Caffeic acid	170.81 ± 1.2
Chlorogenic acid	746.11 ± 2.9
(-)-Epicatechin	33.86 ± 0.8
Ferulic acid	91.94 ± 1.1
Gallic acid	24.41 ± 0.8
Genistin	253.35 ± 2.3
Kaempferol-3-*O*-glucoside	5878.67 ± 9.6
Neochlorogenic acid	6.86 ± 0.2
Protocatechuic acid	40.77 ± 0.9
Quercetin-3-*O*-glucoside	63.92 ± 0.8
Rosmarinic acid	550.16 ± 2.5
Vanillic acid	58.09 ± 0.4

Data are expressed as mean ± S.D. ($n = 3$).

The methanol extract showed a total phenols content of 611.07 mg of chlorogenic acid equivalents/g of extract and a total flavonoids content of 165.62 mg of quercetin equivalents/g of extract. Twelve compounds, namely caffeic acid, chlorogenic acid, (-)-epicatechin, ferulic acid, gallic acid, genistin, kaempferol-3-*O*-glucoside, neochlorogenic acid, protocatechuic acid, quercetin-3-*O*-glucoside, rosmarinic acid, and vanillic acid, were chosen as markers and analysed by

Table 3
In *vitro* antioxidant activity of *S. corsica* extracts.

	ABTS test IC_{50} (μg/mL)	DPPH test IC_{50} (μg/mL)	FRAP test μM Fe(II)/g[a]	β-Carotene bleaching test IC_{50} (μg/mL)	
				30 min	60 min
S. corsica extract					
n-Hexane	205.22 ± 8.10	43.55 ± 1.84	6.32 ± 0.21	21.47%[b]	24.71%[b]
Methanol	18.26 ± 2.82	6.59 ± 0.42	100.34 ± 7.82	68.90 ± 3.52	78.32 ± 3.83
Positive control					
Ascorbic acid	1.70 ± 0.40	5.01 ± 0.8			
BHT			63.21 ± 4.30		
Propyl gallate				1.01 ± 0.04	1.03 ± 0.06

Data are expressed as mean ± SD ($n = 3$). [a] at concentration of 2.5 mg/mL; [b] at concentration of 100 μg/mL. ABTS test: ANOVA ***$p < 0.0001$ followed by a multicomparison Dunnett's test: ***$p < 0.001$ compared with ascorbic acid. DPPH test: ANOVA ***$p < 0.0001$ followed by a multicomparison Dunnett's test: ***$p < 0.001$ compared with ascorbic acid. FRAP test: ANOVA ***$p < 0.0001$ followed by a multicomparison Dunnett's test: **$p < 0.001$ compared with BHT. β-Carotene bleaching test 30 min: ANOVA ***$p < 0.0001$ followed by a multicomparison Dunnett's test: **$p < 0.001$ compared with propyl gallate. β-Carotene bleaching test 60 min: ANOVA ***$p < 0.0001$ followed by a multicomparison Dunnett's test: **$p < 0.001$ compared with propyl gallate.

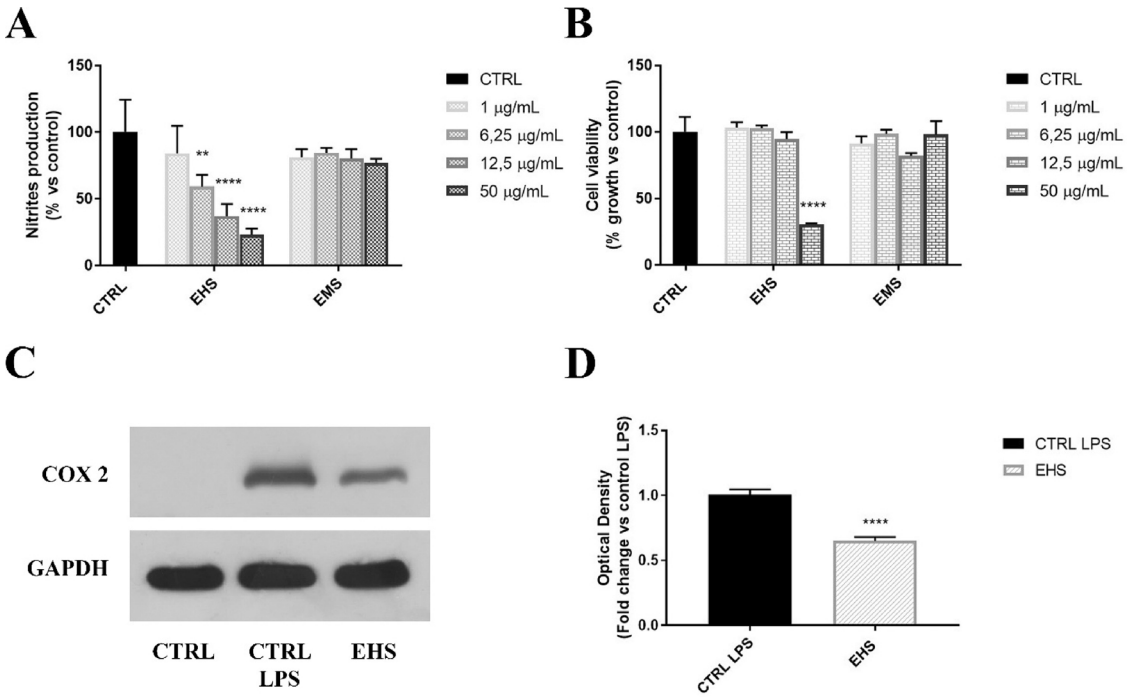

Fig. 1. Anti-inflammatory activity of *n*-hexane (EHS) and methanol (EMS) *Santolina corsica* extracts. (**A**) Nitrites production assessment in LPS stimulated RAW 264.7 cells after treatment for 24 h with different concentrations (from 1 to 50 μg/mL) of EHS or EMS. Results were quantified by Griess assay and expressed as percentage of nitrites production *vs* control (CTRL, cells treated with DMSO). (**B**) Cell growth assessment of RAW 264.7 cells after treatment for 24 h with EHS or EMS (as described above). Assessment was defined by MTT assay. Cell growth was expressed as percentage of treated *vs* control cells. (**C**) Immunoblot of COX 2 from LPS stimulated RAW 264.7 cells treated for 24 h with DMSO (CTRL LPS) or EHS at IC_{50} value. GAPDH was used as a control for equal loading and transfer. **(D)** Histograms represent means ± S.D. of three separate experiments.COX 2 band intensity was evaluated as optical density arbitrary units, and expressed in terms of fold change compared to DMSO-treated samples (CTRL LPS). Data were normalized for GAPDH content. Values represent means ± S.D. of three independent experiments, each performed with triplicate samples (**p < 0.005, ****p < 0.0001, one-way ANOVA calculations).

Table 4
Anti-inflammatory and cytotoxic activity [IC_{50} (μg/mL)] of *S. corsica* extracts.

	Inhibition of NO production	Cytotoxic activity
S. corsica	LPS-stimulated RAW264.7 cells	RAW 264.7 cells
n-Hexane	8.60 ± 1.61	46.22 ± 13.18
Methanol	> 50	> 50
Positive control		
Diclofenac	39.71 ± 2.32	

Data are expressed as mean ± SD ($n = 3$).

RP-HPLC(Table 2). Kaempferol-3-*O*-glucoside (5878.67 mg/100 g of extract), chlorogenic acid (746.11 mg/100 g of extract), rosmarinic acid (550.16 mg/100 g of extract) are the most abundant compounds.

Good amounts of genistin (253.35 mg/100 g of extract) and caffeic acid (170.81 mg/100 g of extract) were found. Other compounds are identified in the range from 6.86 mg/100 g of extract to 91.94 mg/100 g of extract.

3.2. *In vitro* antioxidant activity

Each extract was tested *in vitro* to evaluate its antioxidant activity employing four established *in vitro* models, namely ABTS, DPPH, FRAP, and β-carotene bleaching tests. A concentration-response relationship

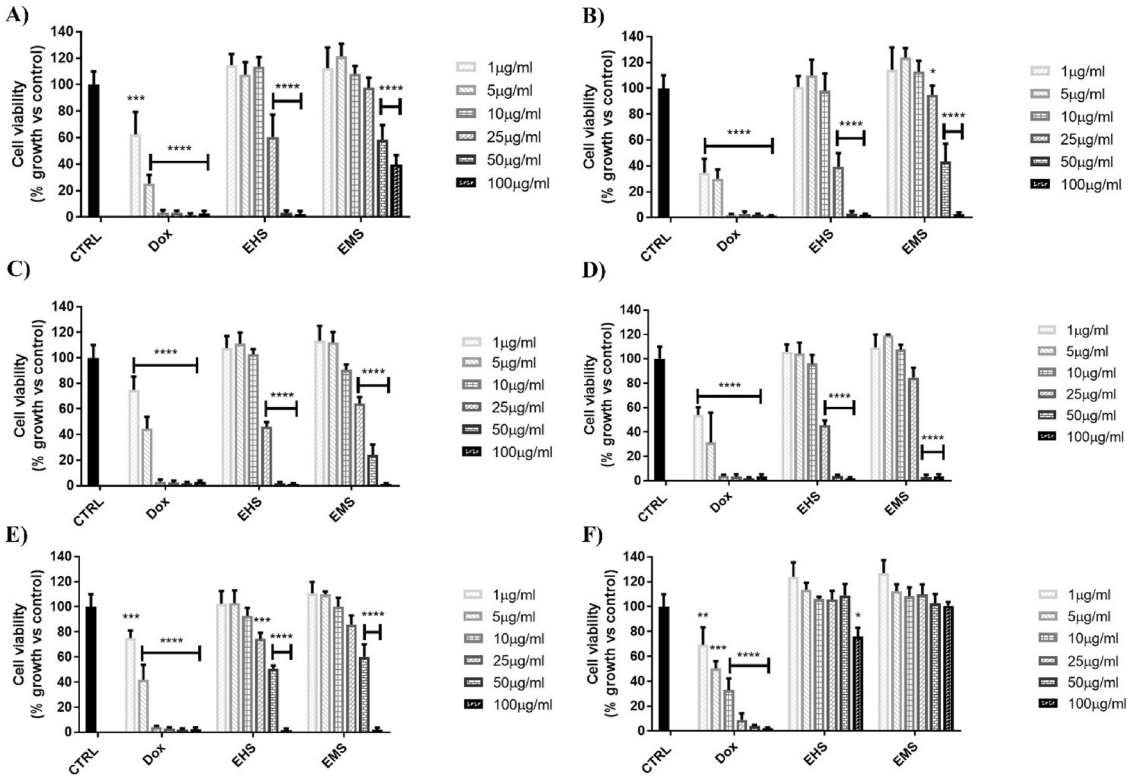

Fig. 2. Effect of EHS or EMS treatment on cell growth. Cell growth assessment after treatment for 72 h of MCF7 **(A)**, MDA-MB-231 **(B)**, Pc3 **(C)**, HeLa **(D)**, A549 **(E)** and MCF-10 A **(F)** cell lines, using different concentrations (from 1 to 100 µg/mL) of EHS or EMS. Doxorubicin was used as a positive control. Results were quantified by MTT assay and expressed as percentage of growth *vs* control (cells treated with DMSO, CTRL). Values represent means ± S.D. of three independent experiments, each performed with triplicate samples. (*p < 0.05, **p < 0.005; ***p < 0.001 ****p < 0.0001, one-way ANOVA calculations).

Table 5
Anti-proliferative activity [IC_{50} (µg/mL)] of *S. corsica* extracts.

	HeLa	MCF-7	MDA-MB-231	A549	PC3	MCF-10 A
S. corsica extracts						
n-Hexane	23.14 ± 6.20	30.10 ± 10.74	24.74 ± 7.77	44.36 ± 9.47	22.56 ± 6.83	> 100
Methanol	37.11 ± 14.13	106.60 ± 30.99	32.39 ± 8.18	58.39 ± 16.19	59.89 ± 22.61	> 100
Positive control						
Doxorubicin	1.34 ± 0.25	1.46 ± 0.20	2.47 ± 0.42	2.39 ± 0.37	0.70 ± 0.14	3.94 ± 0.52

Data are expressed as mean ± SD ($n = 3$). Cancer cell lines: HeLa, human uterine cervix adenocarcinoma; MCF-7, human breast cancer ER^+ cells; MDA-MB-231, triple negative breast adenocarcinoma cell line; A549, human lung adenocarcinoma cell line; PC3, androgen-independent human prostate cancer cell line; MCF 10 A, human mammary epithelial cells.

was observed for tested samples in all assays. Data are reported in Table 3. Generally, the methanol extract was more active than the *n*-hexane extract. An interesting activity was evidenced in FRAP test with a better value (100.34 µM Fe(II)/g) than that observed for the positive control BHT (63.21 µM Fe(II)/g). A good radical scavenging activity in DPPH assay was found, with an IC_{50} value of 6.59 µg/mL. In β-carotene bleaching test, IC_{50} values of 68.90 and 78.32 µg/mL after 30 and 60 min of incubation, respectively, were found for *S. corsica* methanol extract.

3.3. Anti-inflammatory activity of S. corsica n-hexane and methanol extracts

Nowadays, the association between cellular oxidative stress and inflammatory pathways is well known [24]. On this basis, we evaluated NO production, an important mediator of the inflammatory process, in LPS-stimulated RAW 264.7 cells, by Griess assay. According to this test, cells were treated for 24 h with 0, 1, 6.25, 12.5 and 50 µg/mL EHS or EMS, respectively. Positive control was performed by using Diclofenac, an already used drug. Our data (Fig. 1 A and Table 4) showed that EHS concentration-dependently inhibited NO production with an IC_{50} value of 8.6 µg/mL; conversely, EMS was not able to inhibit NO production. Both extracts did not show toxicity on RAW 264.7 cells (Fig. 1B), considering that EHS did not produce cytotoxicity up to a concentration of 50 µg/mL, which was four folds higher than IC_{50} value of anti-inflammatory activity. Based on Griess assay results, we evaluated the expression levels of COX-2, an inducible form of the enzyme related to inflammation pathway. LPS-stimulated RAW 264.7 cells treated with EHS, at IC_{50} concentration (Fig. 1B), displayed COX-2 decreased expression levels, with respect to those found on LPS-stimulated RAW 264.7 cells treated with DMSO (used as an inflammation control), confirming our Griess assay results.

3.4. S. corsica extracts showed a promising anti-proliferative effect on several cancer cell lines

The impact of EHS and EMS on cell viability using a wide panel of cancer cell lines, including uterine cervical (HeLa), alveolar (A549), prostate (PC3), luminal and basal breast (MCF7 and MDA-MB-231) cell lines, was evaluated. Increasing concentrations (1, 5, 10, 25, 50 and

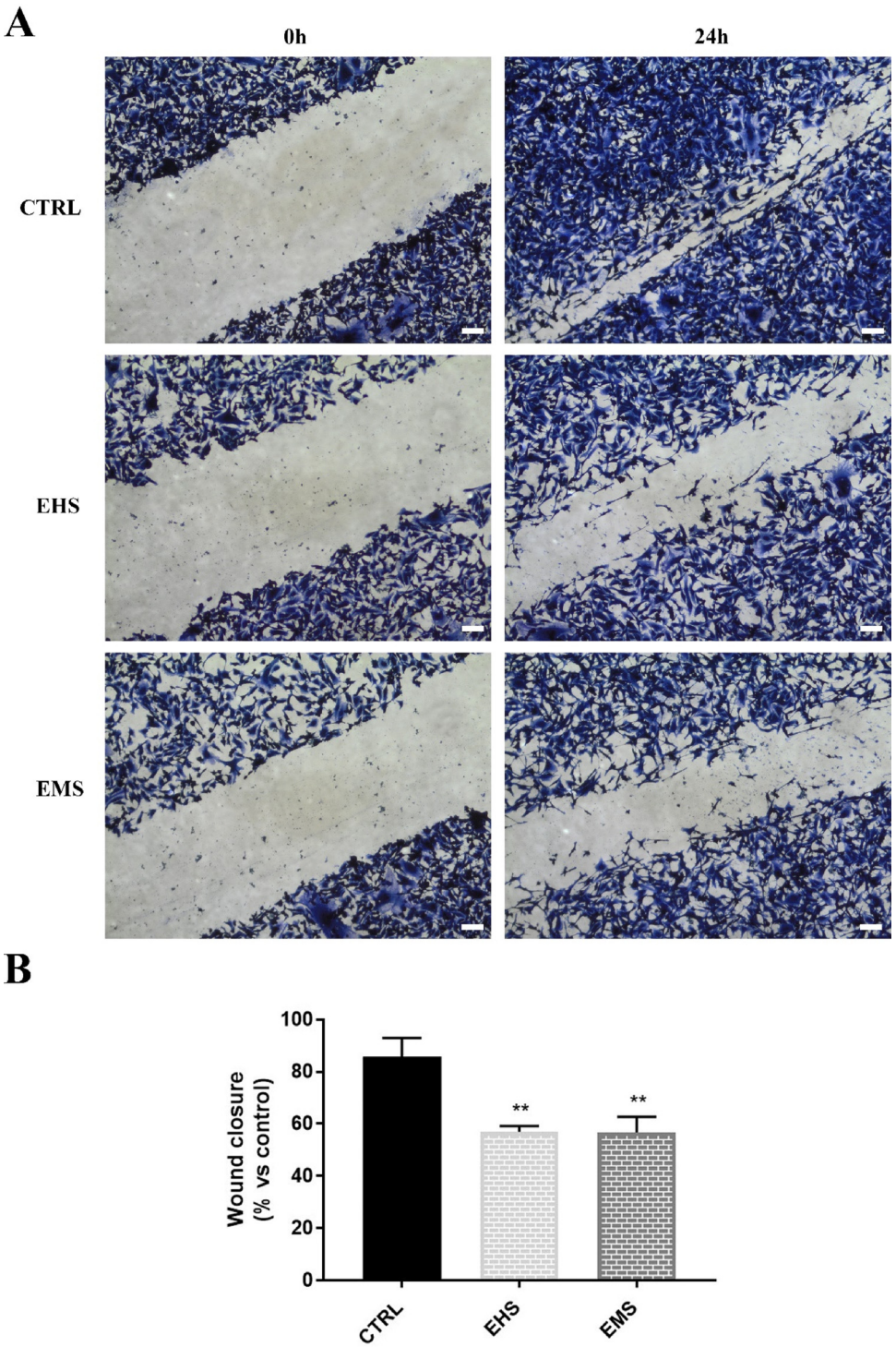

Fig. 3. Effects of EHS or EMS treatment on MDA-MB-231 cell motility. **(A)** Scratch assay on MDA-MB-231 cells treated with DMSO (control, CTRL), EHS and EMS (as indicated) were used at IC_{50} value. Images were taken on Olympus BX41 microscope with CSV1.14 software, using a CAMXC-30 for image acquisition immediately after wounding (time 0) and 24 h later. Scale bars 125 μm. **(B)** Histograms represent the relative percentage of wound closure from three different experiments, each performed with triplicate samples (**$p < 0.005$ *vs* control, one-way ANOVA calculations).

100 μg/mL) of both extracts were used to test cell viability by MTT assay (Fig. 2).

As shown in Table 5, EHS inhibited cell viability on all the tested cancer cell lines, particularly on PC3, HeLa and MDA-MB-231 cells, with IC_{50} values ranging from 22.56 to 24.74 μg/mL. These values fall within the American National Cancer Institute criteria, according to which the IC_{50} limit to consider a crude extract promising for anticancer activity should be lower than 30 μg/mL [26]. EHS anti-proliferative activity on MCF7 cell line was also interesting, showing an IC_{50} value of 30.1 μg/mL. EMS showed weaker anti-proliferative activities than EHS on different cell lines, mainly on A549 (IC_{50} value of 58.39 μg/mL), HeLa (IC_{50} value of 37.1 μg/mL) and MDA-MB-231 (IC_{50} value of 32.4 μg/mL) cell lines. Noticeably, anti-proliferative activities of both extracts were found to be specific for cancer cells, since their IC_{50} values measured on a non-tumorigenic breast epithelial cell line (MCF 10 A) were over 100 μg/mL, values remarkably higher than those

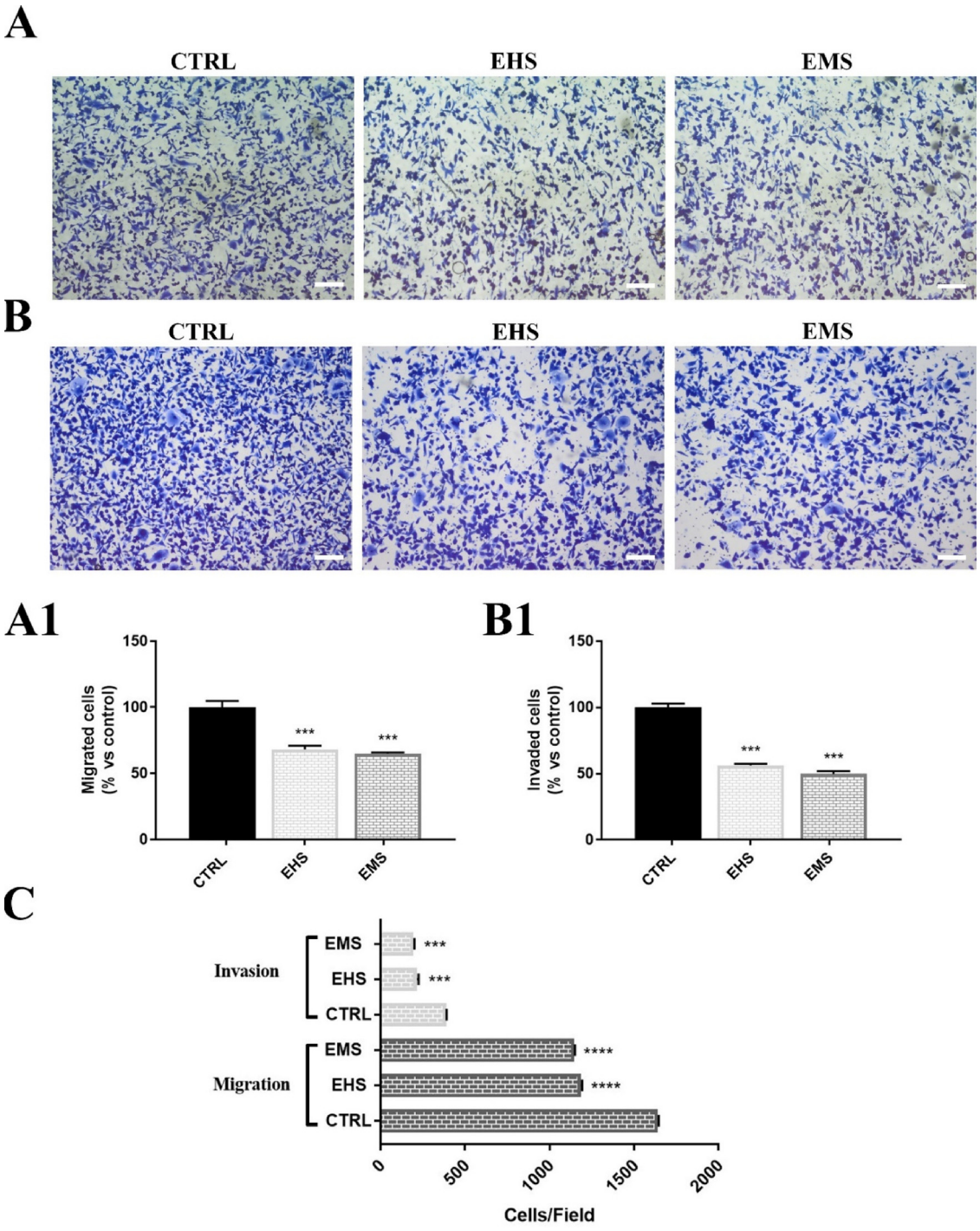

Fig. 4. Effects of EHS or EMS on invasive and migratory potential of MDA-MB-231 cells. **(A)** Migration assay of MDA-MB-231 cell line treated for 24 h with DMSO (control, CTRL) EHS or EMS, used at IC_{50} value. Cells were stained with Coomassie brilliant blue. **(B)** Photographs of MDA-MB-231 cell invasion through the polycarbonate membrane. Cells were treated as indicated and stained as described above. **(A1)** Quantification of migration is expressed as percentage of migrated cells *vs* control cells. **(B1)** Quantification of invasion is expressed as percentage of invaded cells *vs* control cells. **(C)** The number of cells in five random microscopic fields were counted for each group and normalized to cells seeded in the migration assay. Images were taken on Olympus BX41 microscope with CSV1.14 software, using a CAMXC-30 for image acquisition immediately after treatment. Scale bars 125 µm. Histograms values represent means ± S.D. of three independent experiments, each performed with triplicate samples (***$p < 0.001$, ****$p < 0.0001$, one-way ANOVA calculations).

found on all the tested cancer cell lines. Promising results observed on MDA-MB-231 cancer cell line, which is characterized by high invasive and migratory potential, prompted us to further investigate on such cell line, in order to understand how these extracts could contribute to the coordination of cell functions.

3.5. EHS and EMS reduce invasive and migratory potential of MDA-MB-231 cells

We examined the ability *S. corsica* extracts to affect cell movement in wound-healing scratch assays led on MDA-MB-231 cells, which were treated with EHS or EMS, at IC_{50} value. Untreated cells were used as a control. As shown in Fig. 3, after 24 h, cell monolayers were wounded.

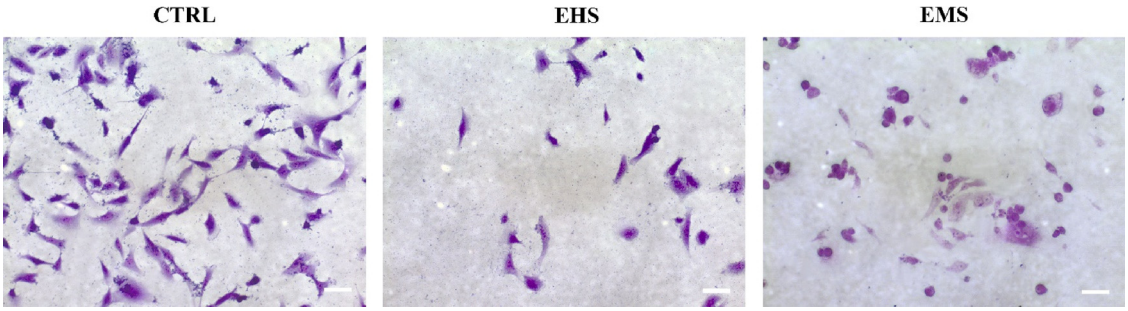

Fig. 5. Effect of EHS or EMS on MDA-MB-231 cell morphology. MDA-MB-231 cells were treated with DMSO (control, CTRL) or each extract (as indicated) at IC_{50} value for 72 h. Images were taken on Olympus BX41 microscope with CSV1.14 software, using a CAMXC-30 for image acquisition. Scale bars 50 μm.

Untreated cells had almost completely invaded the wound area, conversely, movements to close the gap were reduced either in the presence of EHS or EMS treatment. Moreover, the effect of *S. corsica* extracts on MDA-MB-231 cells invasion was tested in Matrigel-coated chambers. Our results displayed that MDA-MB-231 untreated cells migrated to the bottom side of the membrane, whereas EHS or EMS treatment (at IC_{50} value), after 24 h, significantly reduced cell invasion (Fig. 4B). Accordingly, similar results were found by migration assays (Fig. 4A), led as described in Methods section (par. 2.10).

3.6. S. corsica extracts induce morphological changes in MDA-MB-231 cells

The effect of EHS and EMS treatment on MDA-MB-231 cell morphology was studied using a phase-contrast microscope. As shown in Fig. 5, control cells (CTRL) showed a normal cellular morphology, whereas cells treated with EHS or EMS, at IC_{50} value for 72 h, revealed dramatic morphological changes. The majority of cells became small, round-shaped and shrunken, suggesting that EHS or EMS treatment might trigger cell death by apoptosis, since they induced an apoptotic-like morphology.

3.7. S. corsica extracts trigger cell death by apoptosis in MDA-MB-231 cell line

Based on the morphological changes observed on MDA-MB-231 cells after EHS or EMS treatment, we examined if the anti-proliferative activity displayed by these extracts could be due to apoptosis induction. The impact of each extract on apoptosis was performed by using different approaches. Firstly, we evaluated DNA fragmentation, a late event of apoptosis, by TUNEL assay. Our results showed that, after 72 h of EHS or EMS exposure, the percentage of TUNEL-positive MDA-MB-231 cells significantly increased, when compared to the vehicle-treated cells (DMSO), used as a control (Fig. 6A). Next, we examined by immunoblotting analysis the expression levels of different proteins involved in the apoptotic pathway. We evaluated proteolysis of PARP, a facilitator of DNA repair and a key target indicating DNA damage as well as a substrate of effector caspases. After 72 h of treatment with either EHS or EMS, reduced levels of the full-length form of PARP were detected in MDA-MB-231 cells, when compared to the control cells (Fig. 6B). Cell apoptosis is initiated by signals *via* two main pathways, the extrinsic (receptor-mediated) or the intrinsic pathway (mitochondria-mediated) [27]. We assessed expression levels of two proteins involved in these two pathways, procaspase-8 and -9, in order to gain insight into the molecular effector pathway of apoptosis. As shown in Fig. 6B, after treatment with EHS or EMS, procaspase-8 expression levels were significantly decreased, in a time-dependent manner. Such evidence suggested the activation of caspase 8, which is involved in the extrinsic apoptosis pathway. At the same time, Fig. 6B showed that EHS or EMS treatments did not affect expression levels of procaspase-9, excluding a mitochondrial involvement in the apoptotic events triggered by *S. corsica* extracts.

4. Discussion

Natural products are important and fine resources for natural drug development, due to the presence in their extracts of several compounds thought to be responsible for several therapeutic properties such as antimicrobial, anticancer, antioxidant, anti-inflammatory and antifungal [13].

Therefore, natural products play a dominant role in the discovery of effective drugs for the treatment of different human diseases. Herein, for the first time, we demonstrated that *n*-hexane and methanol *S. corsica* extracts have a wide range of biological properties. Antioxidant activity is one of the more intensively studied features, since oxidative damages induced by different sources may arise several diseases [28], including cancer [29,30], Alzheimer's disease [31], Parkinson's disease [32] and others [33,34]. In this work, antioxidant activity of two *S. corsica* extracts was evaluated.

Our findings highlighted an interesting antioxidant activity of both extracts, based on DPPH assay results (IC_{50} value of 6.59 μg/mL for EMS), and FRAP test (IC_{50} value of 6.32 μg/mL for EHS).

During the last decades, different studies showed an interesting correlation between oxidative stress and inflammation [35]. It is known that several inflammatory stimuli, LPS, can induce the NF-κB signaling pathway which, in turn activates and regulates the expression of various genes involved in inflammatory responses, such as cyclooxygenase-2 and the inducible enzyme nitric oxide synthase (iNOS). This latterthe production of NO, which can play a role in host defense as an inflammatory mediator, but its excessive production may promote many chronic inflammatory diseases [36]. Therefore, the inhibition of iNOS pathway represents a notable strategy to control such diseases [37]. In this regard, our experiments performed in LPS-stimulated RAW 264.7 cells, in order to evaluate the ability of EHS and EMS to modulate inflammatory response, highlighted a promising anti-inflammatory activity of EHS. It is noteworthy that this extract was able to significantly reduce NO production, with an IC_{50} value of 8.50 μg/mL, a concentration remarkably lower than that observed for diclofenac (IC_{50} value of 39.7 μg/mL), a commonly used anti-inflammatory drug. In addition, EHS treatment significantly decreased COX-2 expression levels with respect to those of the control (LPS-stimulated RAW 264.7 cells treated with DMSO).

Thisis consistent with the presence in this extract of myrcene, limonene, β-phellandrene and some esters of fatty acids, well-known molecules endowed with anti-inflammatory activity [38,24]. Conversely, EMS was not able to decrease NO production. This could be related to its molecular composition, which is quite different from that of EHS.

Nowadays, the relationships among cellular oxidative stress, inflammation pathways and cancer formation and propagation are well-known [39,40]. Hence, we investigated anti-proliferative activity of EHS and EMS showing that EHS, as well as EMS, inhibited in a dose-dependent manner the proliferation of a wide panel of human cancer cell lines, without inducing cytotoxicity in non-tumorigenic breast

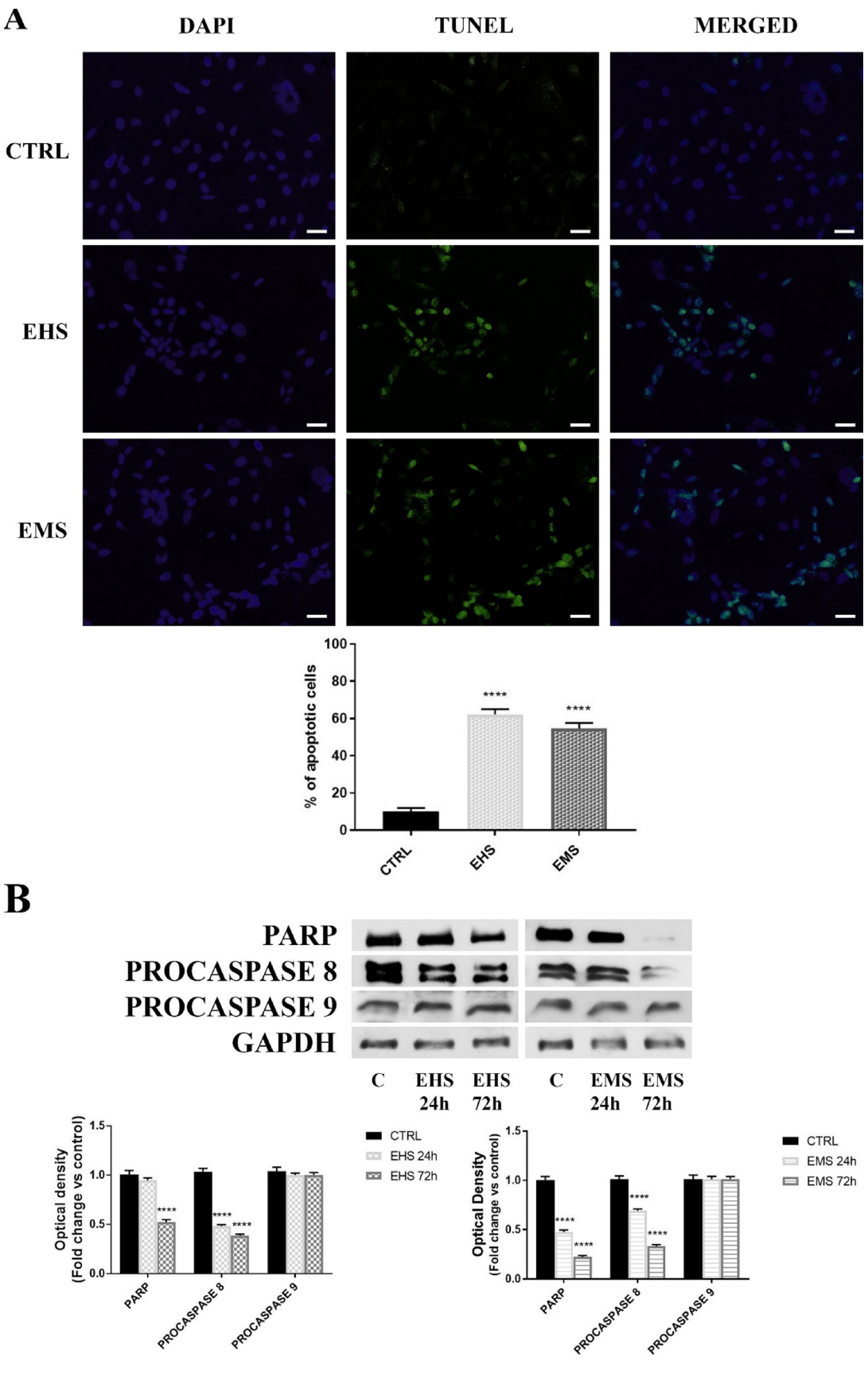

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Fig. 6. EHS and EMS trigger apoptotic cell death in MDA-MB-231 cells. (A) *Upper panel:* Cells were treated for 72 h with DMSO (control, CTRL), EHS or EMS (as indicated), at IC_{50} value, washed with PBS, paraformaldehyde fixed and subjected to TUNEL assay; after removal of enzyme and buffer, fixed cells were DAPI stained to visualize the cell nucleus. Merge (MERGED) was also shown. Images were taken on Olympus BX41 microscope with CSV1.14 software, using a CAMXC-30 for image acquisition; (Scale Bars 50 μm). *Lower panel*: Histograms represent means ± S.D. of apoptotic *vs* control cells from three independent experiments performed in triplicate. **(B)** *Upper panel:* Immunoblot analysis of PARP, procaspase-8 and procaspase-9 expression in MDA-MB-231 cells. Cells were treated as indicated in (A) before lysis. Equal amounts of total cellular extracts werefor PARP, procaspase-8 and procaspase-9 protein expression from total cellular extracts. GAPDH was used as a loading control. *Lower panel*: histograms represent means ± S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units (OD), and expressed as fold change *vs* DMSO-treated samples (****$p < 0.0001$ *vs* control, one-way ANOVA calculations).

epithelial cells, MCF-10 A. This behaviour could be related to their chemical profile, which is characterized by the presence of known molecules endowed with anti-proliferative effects, such as α-pinene [41], myrcene [42], limonene [42], phellandrene and others in EHS, as well as flavonoids [43], quercetin [44] and others in EMS. Moreover, our findings are in complete agreement with previous literature data describing some anti-proliferative effects of different species of *Santolina* genus, highlighting the usefulness of this genus as a source of extracts with potential anticancer activity [6,45].

For the first time, we proved that *S. corsica* extracts exert anti-proliferative activity in triple negative, highly invasive and metastatic MDA-MB-231 cells. Cells invasion represents a negative feature, because it is responsible for tumour dissemination and promotes metastatic processes [46]. Importantly, we have also found that these extracts strongly reduce motility, migration and invasion of MDA-MB-231 cells, highlighting their potential activity in decreasing the metastatic capability of this cancer cell line, which still remains the main responsible for therapy failure.

Additionally, after EHS or EMS treatment, MDA-MB-231 cells became small, circular and shrunk, when compared to control cells. These dramatic morphological changes also entailed shrinkage, suggesting that these two extracts could induce apoptosis, an event usually characterized by cellular morphology changes comparable to those we found [47,48]. On this basis, we investigated if both EHS and EMS were able to induce apoptosis on MDA-MB-231 cell line.

Apoptosis is a mechanism characterized by DNA fragmentation, as a late event in the apoptotic cascade. It is a highly regulated process leading to programmed cell death through the extrinsic (receptor-mediated) or the intrinsic apoptotic pathway (mitochondria-mediated) [27]. It has been reported that the activation of caspase-8 and -9, by cleavage of the corresponding procaspases, occurs in the extrinsic or intrinsic apoptotic pathways, respectively [49]. In this regard, we observed that EHS or EMS treatments were able to induce apoptosis in MDA-MB-231 breast cancer cells, as evidenced by a significant increase of TUNEL-positive cells, as well as by decreased procaspase-8 levels, in a time-dependent manner. Conversely, procaspase-9 levels were not affected. Accordingly, we found a decrease in the levels of the full-length form of PARP, a well-known substrate of effector caspases [50,51]. All these results suggest that the induction of apoptosis, by the extrinsic pathway, may be the mechanism responsible for the anti-proliferative effects exerted by EHS and EMS in MDA-MB-231 breast cancer cells. However, the presence of different classes of compounds detected in both these extracts implies the need to investigate the effects of their individual bioactive constituents.

5. Conclusions

In this study, we demonstrated that *S. corsica* extracts, EHS and EMS, are endowed with antioxidant properties. EHS also shows anti-inflammatory activity, at a concentration considerably lower than IC_{50} value of Diclofenac, a widely used anti-inflammatory drug. Both extracts exert anti-proliferative effects on different cancer cell lines. It's noteworthy that they can inhibit motility, migration, and invasion rate of MDA-MB-231 cells, a highly invasive breast cancer cell line. Such findings emphasize their potential activity in decreasing metastatic capability of breast cancer cells, which still remains the main responsible for therapy failure. Although the underlying mechanism of these effects remains undefined, needing further study, our results highlight that compounds present in both extracts are selectively able to interfere in specific cancer cell mechanisms and to activate the extrinsic apoptosis pathway. Taken together our findings suggest that EHS and EMS might be taken into account for a potential therapeutic application in cancer treatment.

Conflict of interest

The authors declare that there are no conflicts of interest.

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