Journal of Functional Foods 116 (2024) 106203

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Anabasis articulata (Forssk.) Moq. food aqueous extract triggers oxidative stress-induced senescence and reduces metastatic power in MDA-MB-231 cells

Valentina Monteleone [a,1], Dounia Ben Menni [b,1], Nabila Belyagoubi-Benhammou [b], Gabriele Di Marco [a], Antonella Canini [a], Angelo Gismondi [a,*]

[a] *Department of Biology, University of Rome "Tor Vergata", Via della Ricerca Scientifica 1, 00133 Rome, Italy*
[b] *Natural Products Laboratory, Department of Biology, Faculty of Natural and Life Sciences, Earth and Universe, University Abou-Bekr Belkaïd, 13000 Tlemcen, Algeria*

ARTICLE INFO

Keywords:
Functional plant food
Antiinvasive properties
Breast cancer
Folk medicine
Algerian plant species
p53/p21$^{WAF1/Cip1}$/p27^{Kip1} pathway
Metalloproteases

ABSTRACT

Ancient ethnobotanical practices handed down through traditional knowledge are still commonly employed to treat various pathologies, although the scientific reasons underlying their biological effects have not been clarified yet. In this contribution, the potential antitumoral activity of the aqueous extract from *A. articulata* (AAE) was investigated to validate the hypothesis of the Algerian folk medicine which would suggest this plant derivative as a functional food for treating breast cancer. *A. articulata* phytocomplex, isolated by maceration following exactly the African recipe, has been already characterized by our research group in previous works. Thus, the antiproliferative function of AAE against MDA-MB-231, a highly aggressive human breast adenocarcinoma cell line, was evaluated. Slowing down of cell growth, absence of cytotoxicity and DNA fragmentation, and cell cycle arrest at the G2/M phase were observed after treatment with AAE at different doses (0.3–6 mg of dried plant material equivalent per mL of culture medium) for 24 and 48 h. Wound and transwell assays proved that AAE possessed both antimigration and antiinvasive capacities, evidence also supported by molecular analyses focused on Metalloproteases (MMP-2 and MMP-9), Vimentin and $\alpha v\beta 3$-Integrin. These results, together with the demonstration of the activation of p53/p21$^{WAF1/Cip1}$/p27^{Kip1} pathway and the increase of oxygen reactive species levels, suggested that AAE triggered a senescence process. The final confirmation was obtained by a specific kit staining senescent cells. All our data would explain the efficacy of the Algerian medicinal remedy based on the intake of the investigated functional plant food and would highlight the basics for developing novel natural pharmacological products based on AAE and showing preventive and therapeutic antineoplastic potentialities against highly aggressive breast cancers.

1. Introduction

The genus *Anabasis* L. belongs to the Chenopodiaceae family and includes 28 halophyte and xerophyte species highly adapted to survive in saline and arid environments. These plants are mainly distributed in central and southwest Asia and around the Mediterranean Basin, colonising deserts, stony areas, and sandy wadies (O'leary and Glenn, 1994; Lauterbach et al., 2019). Among all, *A. articulata* (Forssk.) Moq. is probably the most known for its biological properties. Indeed, for instance, in Algeria, this wild species – commonly named *ajrem* – is widely used in folk medicine. There, ethnobotanical practices have handed down that aqueous decoctions of *A. articulata* leaves can be used to treat diabetes, kidney infections, fever, headache, and skin diseases, such as eczema and itching (Hammiche and Maiza, 2006). In addition, literature has associated to this species other bioactivities, such as larvicidal (Sathiyamoorthy et al., 1997), hepatoprotective (Azza et al., 2014; Mohamed et al., 2014), and anti-inflammatory effect (Abdallah et al., 2014). About the latter, the authors have documented the capacity of *A. articulata* to reduce prostaglandin (i.e., PGE2) and TNF-α levels and to inhibit Cyclooxygenase-2 activity. Recently, Benzineb et al. (2019) have highlighted for this plant a significant anti-microbial power, while El Dine et al. (2018) have demonstrated that the *n*-butanol fraction from

* Corresponding author.
E-mail address: gismondi@scienze.uniroma2.it (A. Gismondi).
[1] These authors equally contributed to the present work.

https://doi.org/10.1016/j.jff.2024.106203
Received 13 September 2023; Received in revised form 12 April 2024; Accepted 22 April 2024
Available online 27 April 2024

A. articulata exhibited a strong agonistic capacity towards the peroxisome proliferator-activated receptor-α in human hepatoma cells (HepG2).

Phytochemical analyses on *A. articulata* extracts have showed the presence of several antioxidant compounds, including coumarins, flavonoids, simple phenols, tannins, and anthraquinones. This evidence has suggested the application of this species as source of antiradical drugs and natural preservatives for food or cosmetic products (Benhammou et al., 2013; Benzineb et al., 2019). In addition, *A. articulata* phytocomplex seems to be characterized also by iridoids, cyanogenic glycosides, cardiac glycosides, alkaloids, and sterols (Eman, 2011; Metwally et al., 2012; Belyagoubi-Benhammou et al., 2019). However, the most abundant components would be the saponins (1.3 %), which have been detected in this halophyte for the first time more than fifty years ago (Segal et al., 1969; Eman, 2011; Mroczek, 2015). In this regard, Kambouche et al. (2009) have reported that these molecules were able to reduce blood glucose levels, providing a scientific proof about the anti-diabetic potential of *A. articulata*, as suggested by the Algerian traditional phytotherapy. According to the same African habits, *ajrem* is an excellent antineoplastic remedy against breast cancer, if mixed with leaves from *Atriplex halimus* L. and *Prunus persica* (L.) Batsch. and dairy products (e.g., yogurt) or honey. Nevertheless, no experimental evidence about the putative antitumoral effect of *A. articulata* has ever been registered in the literature. Therefore, the aim of the present study was to investigate the potential antiproliferative and antimetastatic activity of the *A. articulata* aqueous extract on human breast adenocarcinoma cells (MDA-MB-231), in order to understand the molecular and cellular pathways underlying the biological function related to the phytopreparation adopted by the Algerian folk medicine.

2. Materials and methods

2.1. Plant extract

Anabasis articulata (Forssk.) Moq. plants were collected in March 2017 in the Béchar district (Algeria), after taxonomic identification by Vegetable Ecological Laboratory of Tlemcen University. Aerial portions were washed with ultrapure water, air-dried for a week, grounded, and stored at −20 °C (voucher n. 133AA). The aqueous extract was obtained by dissolving 3 g of dry plant material in 100 mL of distillated water, for 24 h, at room temperature in agitation. After centrifugation at 11,000 g for 15 min, the supernatant was filtered (20 μm, Albet-Jacs filters) and completely desiccated by a vacuum drying system (Concentrator Plus, Eppendorf, Hamburg, Germany). Finally, the residue was resuspended in 3 mL of distillated water and used for cell treatments.

2.2. Cell culture and treatments

The human breast adenocarcinoma cell line (MDA-MB-231) and the human non-tumorigenic mammary epithelial cell line (MCF10A) were cultured under standard conditions in Dulbecco's modified Eagle Medium (DMEM), supplemented with 10 % fetal bovine serum and antibiotics (100 U/mL Penicillin; 100 μg/mL Streptomycin), and grown at 37 °C in humidified atmosphere with 5 % CO_2. For the experiments, cells were treated, for 24 and 48 h, with *Anabasis articulata* aqueous extract at the following concentrations: 0.3, 0.6, 1, 3, and 6 mg (0.3 and 6 mg for MCF10A) of dried plant material equivalent per mL of culture medium. Control cells were treated with pure water, at the highest volume of treatment.

2.3. Cell proliferation and MTT assay

MDA-MB-231 and MCF10A cells were seeded at a density of 2.5×10^5 cells/mL in 6-well plates and treated with the aqueous extract of *A. articulata*, for 24 h and 48 h, at the previously reported doses. Proliferation curves and cytotoxicity were measured using Trypan blue dye (Sigma-Aldrich) and counting alive and dead cells by Neubauer chamber. Then, MDA-MB-231 cell growth was quantified as function of the mitochondrial activity through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Sigma-Aldrich), according to manufacturer's instructions. Results were expressed as absolute number of cells in proliferation graphs, percentage of dead cells, or percentage variation with respect to the control, taken as unit (100 %), in the MTT assay.

2.4. Cell cycle analysis

MDA-MB-231 cells were seeded (4×10^5 cells/mL) and exposed to 3 mg/mL and 6 mg/mL of *A. articulata* extract. After 48 h, cells were washed twice in phosphate-buffered saline (PBS) and fixed, for 30 min at 4 °C, with a cold methanol: acetone (4:1, v/v) solution. Then, cells were treated, at room temperature for 20 min, with RNase A (100 μg/μL) and, for further 20 min, with propidium iodide (1 mg/mL). The analysis was performed by a FACSCalibur instrument (Becton-Dickinson), counting 10,000 events per sample. The percentage of cells in each cell cycle phase, including sub-G0, was measured by CellQuest software.

2.5. Wound healing assay

MDA-MB-231 cells were plated in 6-well plates (1×10^6 cell/well) and cultured to near confluence. Then, wounds were produced on the cell monolayers, using a (20–200 μL) pipette tip, and cellular debris were removed by washing gently with PBS. The wounded monolayers were incubated with the plant extract (0 or control, 3, and 6 mg/mL), for 24 and 48 h, in DMEM. For each sample, photographed under light microscopy, the wound closure was measured by counting the dimension of the scratch in 6 different areas.

2.6. Cell migration and invasion study by transwell system

MDA-MB-231 cells were treated with *A. articulata* (0, 3, and 6 mg/mL) for 24 h. Subsequently, cells were serum-starved for other 24 h, always in presence of plant extract at the respective dose. Cell invasive and migratory property was tested using the principle of the modified Boyden chamber assay (transwell system, Sigma-Aldrich). Briefly, polyvinylpyrrolidone-free polycarbonate filters (8 μM pore size), coated with matrigel (Gibco Geltrex hESC, for the invasion analysis) or not (for migration analysis), were inserted in 24-well plates previously filled with cell culture medium contained 20 % FBS, which acted as a chemoattractant. Then, a total of 8×10^4 (for invasion analysis) and 5×10^4 (for the migration analysis) cells were seeded in the upper compartment and left to migrate and invade for 5 h. After this period, filters were fixed for 15 min in 4 % paraformaldehyde and permeabilize for 10 min in 0.1 % Triton X-100. Membranes were exposed for 20 min to 0.5 % (w/v) Crystal Violet (Sigma-Aldrich) for staining cells and then washed thoroughly with bidistilled water. For each sample, photographed under a light microscopy, the number of cells which migrated to the bottom surface of the insert (and/or invaded the matrigel, if present) was calculated by counting them in 4 different fields of 3 independent filters.

2.7. Zymography

The level of MMPs secreted by tumor cells into the culture medium was monitored by a zymographic analysis. MDA-MB-231 cells were seeded in T-175 flask (20×10^6 cells/flask) and maintained for 24 h. Then, the plant extract was added at a final concentration of 3 and 6 mg/mL for other 48 h. After the incubation, the whole conditioned medium was collected, filtered (20 μm, Albet-Jacs filters), and concentrated, at 4 °C, using Pierce Protein Concentrator PES 30-K MWCO (Thermo Fisher Scientific, Waltham, MA, USA). Secreted proteins were subjected to SDS-PAGE (9 % polyacrylamide gel copolymerized with 1 % gelatin; Sigma-Aldrich). After the run, the gel was incubated for 2 h in 2.5 % Triton-

X100, exposed for 18 h at 37 °C to Collagenase Buffer (0.5 M Tris–HCl pH 7.5, 2 M NaCl, 50 mM dihydrate $CaCl_2$, 0.2 % BRIJ-35), stained for 3 h with Coomassie Brillant Blue R (0.1 % w/v), and destained for 6 h in a methanol:acetic acid:water (3:1:6, v/v/v) solution. Images were captured by the VersaDoc imaging system (Bio-Rad) and spot analyses were performed by the ImageQuant software (GE Healthcare).

2.8. Real-time PCR (qPCR)

After treatment for 48 h, cells were harvested and subjected to total RNA extraction by Pure Link RNA Mini Kit (Invitrogen, Thermo Fisher Scientific). RNA concentration and purity were evaluated through a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham). cDNA synthesis was carried out using FastGene Scriptase II cDNA Kit, starting from 2 μg of RNA. Gene expression was analysed by a StepOne qPCR machine (Applied Biosystems) using SYBR Green reagent (Kapa SYBR Fast qPCR kit; Kapa Biosystems). qPCR amplification parameters were those reported in Nanni et al. (2021), while the primer sequences are reported here: Metalloprotease-9 (MMP-9, F: AGA CCA AGG GTA CAG CCT GTT C, R: GGC ACG CTG GAA TGA TCT AAG); Metalloprotease-2 (MMP2, F: CCC CAT GAA GCC TTG TTT ACC, R: TTG TAG GAG GTG CCC TGG AA); αvβ3-Integrin (F: GAC TGT GTG GAA GAC AAT GTC TGG CAC GTC TT, R: CCA GCT AAG AGT TGA GTT CCA GCC); Vimentin (F: GAC AAT GCG TCT CTG GCA CGT CTT, R: TCC TCC GCC TCC TGC AGG TTC TT); β-Actin (F: ACC ACC ATG TAC CCT GGC ATT, R: CCA CAC GGA GTA CTT GCG CTC A). The number of transcripts was determined using the $2^{-\Delta\Delta Ct}$ method, considering the threshold cycle (Ct) of the sample with respect to the internal reference gene (β-Actin; ΔCt) and to untreated cells (ΔΔCt). Results were reported as percentage compared to the control.

2.9. Western blotting

Cells, exposed or not to the plant extract for 48 h, were resuspended in a lysis buffer containing 350 mM NaCl, 1 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 0.1 mM EGTA, 1 % NP-40, aprotinin 1 mg/mL, phenylmethylsulfonyl fluoride 100 mg/mL and 1 % phosphatase inhibitor cocktail II and III (Sigma-Aldrich). Protein concentration was measured by Bradford reagent (Bio-Rad). Protein samples were resolved by 12 % SDS-PAGE, transferred onto nitrocellulose Protran membrane (Schleicher and Schuell), and incubated with the following primary antibodies and relative antisera: mouse monoclonal anti-β-Actin (used as internal loading control; Santa Cruz Biotechnology, sc-47778); mouse monoclonal anti-p53 (Santa Cruz Biotechnology, sc-126); rabbit polyclonal anti-$p21^{WAF1/Cip1}$ (Santa Cruz Biotechnology, sc-397); mouse monoclonal anti-$p27^{Kip1}$ (Santa Cruz Biotechnology, sc-1641); mouse monoclonal anti-αvβ3-Integrin (Chemicon, MAB1876); mouse monoclonal anti-Vimentin (Merck, V6389). Primary antibodies were revealed using horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Sigma-Aldrich) and a chemiluminescent agent (Luminol, 5-Amino-2,3-dihydro-1,4-ftalazindione, Santa Cruz Biotechnology). Image acquisition and spot analysis were performed by LAS3000 Image System (Fuji) and ImageQuant software (GE Healthcare), respectively. After normalization with β-Actin, the results were shown as percentage variation compared to the control (used as unit, 100 %).

2.10. 2′,7′-dichlorofluorescein diacetate (DCFH-DA) test

DCFH-DA staining was employed to monitor the level of intracellular oxygen reactive species (ROS; particularly H_2O_2). In detail, after the plant treatment for 48 h, cells were incubated, for 15 min at 37 °C, in presence of DCFH-DA 10 μM. Then, they were washed three times with PBS and subjected to flow cytometry analysis. In detail, for each sample (10.000 events), the level of cell fluorescence was detected by a FACSCalibur instrument (Becton Dickinson), using an appropriate filter for fluorescein (FITC, FL1-H), and measured by the CellQuest software. As positive control, cells were exposed to 5 mM H_2O_2 for 4 h. Data were reported as percentage variation of the fluorescence compared to the control (used as unit, 100 %).

2.11. DNA ladder and senescence assays

Tumor cells, treated for 48 h with pure water (control) or *A. articulata* extract (3 and 6 mg/mL), were harvested and subjected to genomic DNA isolation. This procedure was performed by Blood & Cell Culture DNA Kit (QUIAGEN). Contaminating RNA was digested by exposure to RNase A (Roche) for 20 min at 37 °C. Next, DNA samples were separated on 1.5 % agarose gel in Tris-acetic acid-EDTA buffer at 100 V for 40 min, stained with ethidium bromide and visualized under UV-light using a VersaDOC imaging system (Bio-Rad). MW: molecular weight (Hyperladder I, Bioline). Senescence was monitored on MDA-MB-231 and MCF10A cells by phase-contrast microscopy (ZEISS Axio Observer 7, Zeiss, Jena, Germany), after the application of a staining kit (Sigma-Aldrich) which colored in blue/green the senescent cells. Images were captured by Axio Vision Rel 4.6 software (ZEISS, Oberkochen, Germany).

2.12. Statistics

Data were shown as means ± standard error (S.E.) of at least three independent measurements. The significance of the results was evaluated by one-way analysis of variance (ANOVA) and application of the post-hoc lowest standard deviations (LSD) test, through PAST software (p values: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

3. Results

***A. articulata* aqueous extract reduces MDA-MB-231 breast tumor cell proliferation by triggering cell cycle arrest.** The bioactivity of *A. articulata* aqueous extract (AAE) was tested on MDA-MB-231 tumor cells. As shown in Fig. 1A, after 24 h of treatment, significant reductions of cell proliferation were observed in presence of the highest concentrations of AAE (i.e., −20 % at 1 mg/mL; −28 % at 3 mg/mL; −44 % at 6 mg/mL). At 48 h, the cell number decreased by 18 % and 29 %, respectively, with 3 mg/mL and 6 mg/mL of plant extract, with respect to the control. In parallel, the count of dead cells evidenced an increase of cytotoxicity only after 24 h of exposure to 1 (+9.2 %), 3 (+11.6 %), and 6 (+21.2 %) mg/mL AAE, compared to the control (Fig. 1B). To check if the AAE treatment could be safe for normal tissues, the human non-tumorigenic mammary cell line MCF10A was also exposed for 24 and 48 h to the minimum and maximum doses of plant extract previously used on MDA-MB-231 cells. The results, shown in Supplemental Material 1 (panel A and B), revealed that AAE did not exert any significant modulation of the proliferation in MCF10A cells, neither substantial induction of cytotoxicity, as expected considering the common usage and consumption of this plant preparation in the Algerian ethnobotanical tradition. MTT assay was applied to validate the previous data. It indicated that AAE negatively affected the cell growth in a dose-dependent manner, reaching values equal to −45 % and −41 % with respect to the control, in that order, after 24 and 48 h at the concentration of 6 mg/mL (Fig. 1C). As the most significant results were obtained treating cells for 48 h with 3 and 6 mg/mL AAE, we decided to focus our investigations only on these two specific conditions. FACS analysis by propidium iodide demonstrated that the amount of sub-G0 events was comparable to the control (Fig. 1D), confirming the absence of toxicity previously observed at 48 h of treatment (Fig. 1B). Cytofluorimetric profiles clearly evidenced a cell cycle arrest in the G2/M phase (Fig. 1E). Indeed, compared to the control, 3 and 6 mg/mL AAE caused a significant accumulation of G2/M cells by 7.1 % and 9.1 %, respectively, mainly at the expense of the number of G0/G1 events.

Migration and invasive power of MDA-MB-231 cells are inhibited by the plant extract. In order to evaluate the capacity of AAE to

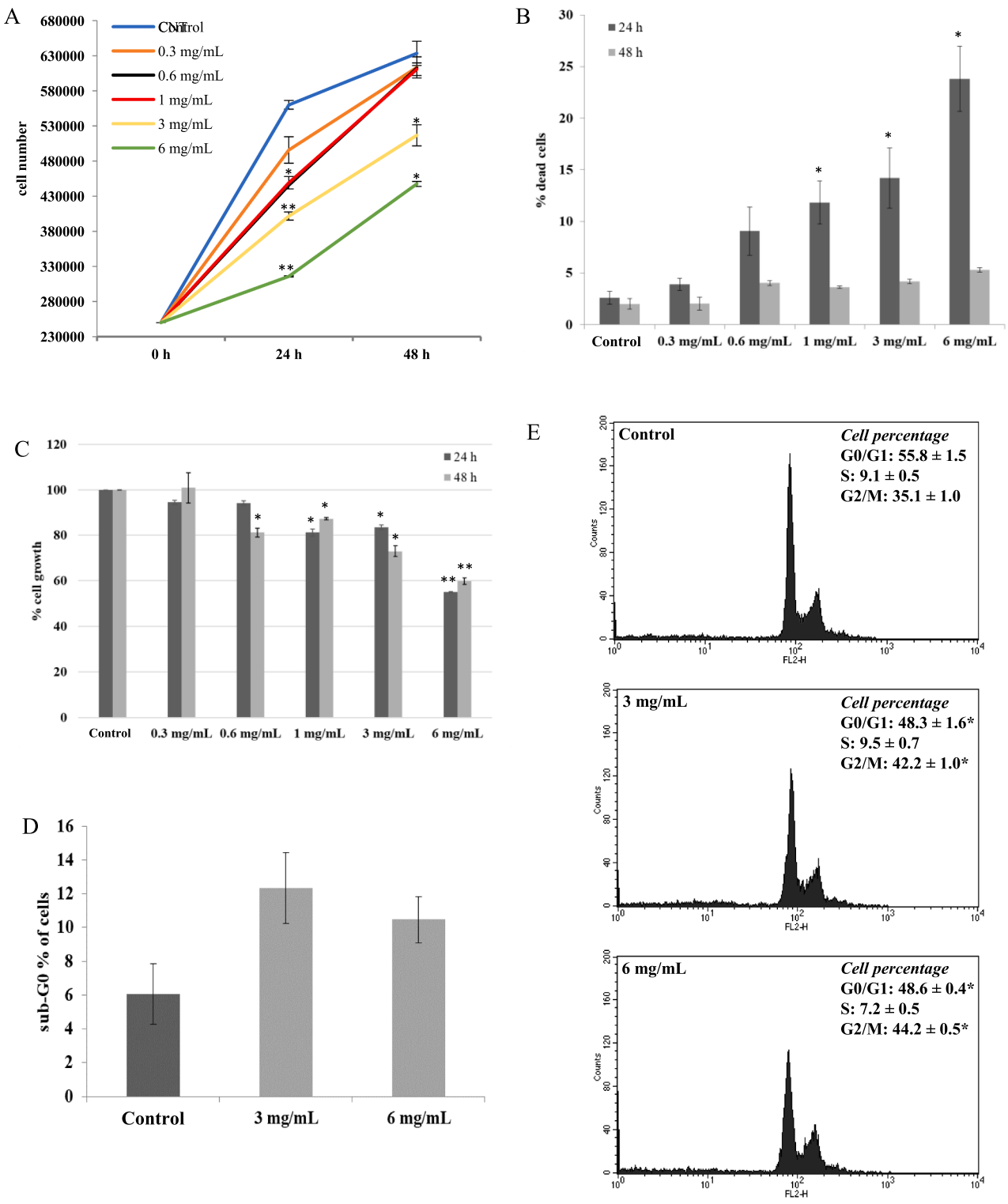


Fig. 1. Effect of *A. articulata* extract (AAE) on the growth of MDA-MB-231 cells. A) Proliferation curves of MDA-MB-231 cells exposed to AAE (from 0.3 to 6 mg of dried plant material equivalent per mL of culture medium) or not (Control), for 24 and 48 h, obtained by counting alive cells (Trypan blue negative cells) using a Neubauer chamber. **B)** Percentage of dead cells (Trypan blue positive cells) after treatment with AAE for 24 and 48 h. **C)** Percentage of cell growth measured by MTT assay after exposure to AAE; untreated cells (Control) were considered as unit (100 %). **D)** Percentage of sub-G0 events detected by FACS analysis after staining with propidium iodide of the tumor cells treated for 48 h with 3 and 6 mg/mL AAE or not (Control). **E)** Cytofluorimetric profiles of MDA-MB-231 exposed to 3 and 6 mg/mL AAE, or not (Control), for 48 h; the number of cells distributed in each cycle phase was indicated as percentage. **A-E)** All results of this figure represent the mean ± s.d. of four independent measurements (p values: *$p < 0.05$; **$p < 0.01$).

contrast the metastatic properties of the tumor cells, the wound assay was carried out. After performing the scratch (0 h), the ability of MDA-MB-231 cells to close the gap in absence (control) or presence of the plant extract, for 24 and 48 h, was assessed. Representative images of the cell test and relative quantitation were shown in Fig. 2A. Results indicated that the wound closure was quite complete at 48 h in control (91 %) and AAE 3 mg/mL (76 %) samples, while the gap remained markedly opened at the highest concentration of AAE (closure rate by 24 %). At the same time, cell migration potential was also investigated using the transwell chamber assay (Fig. 2B). The number of MDA-MB-231 cells able to pass through the pores of the membrane was strongly reduced in presence of AAE for 48 h: −63 % at 3 mg/mL and −74 % at 6 mg/mL, with respect to the control (taken as unit, 100 %). Applying a Matrigel film on the porous membrane, the transwell chamber assay can be also used to simulate the invasive process of tumor cells. The data showed that AAE significantly decreased the invasive capacity of MDA-MB-231 cells by 67 % at the dose of 3 mg/mL and 72 % at 6 mg/mL, compared to the control (Fig. 2C). To understand if this last evidence could be correlated with a possible effect of AAE on the metalloproteases (MMPs), a zymographic approach aimed at investigating the gelatinase activity was applied. Thus, secreted MMPs were recovered from the cell medium, concentrated, and separated, in native form, by electrophoresis on gelatin-enriched acrylamide gel. The capacity of these enzymes to degrade the protein biopolymer present in the gel was put in evidence by Comassie staining (Fig. 2D). The quantitation of the signals revealed that AAE decreased MMP-9 and MMP-2 activity after 48 h of treatment with both the concentrations of plant extract. In detail, MMP-9 proactive and active forms, respectively, were inhibited by 66 % and 83 % at 3 mg/mL AAE and by 73 % and 85 % at 6 mg/mL AAE. In parallel, MMP-2 reduced its biological function by 21 % and 28 %, in that order, after exposure with 3 and 6 mg/mL AAE.

Transcript and protein analysis confirms the potential anti-neoplastic activity of AAE on MDA-MB-231 breast cancer cell line. Total mRNA was extracted from tumor cells exposed to AAE for 48 h and subjected to qPCR assays, for monitoring MMP and cell dynamics-related transcripts (Fig. 3A and 3B). Compared to the control, after the treatments at 3 and 6 mg/mL AAE, respectively, MMP-9 messenger was reduced by 57 % and 85 %, while MMP-2 mRNA by 60 % and 77 %. Similarly, the transcription rate of the VIMENTIN gene appeared inhibited by 3 % and 23 %, in the same conditions. By contrast, αvβ3-INTEGRIN mRNA accumulated in treated cells in a dose dependent manner (+39 % at 3 mg/mL; +46 % at 6 mg/mL AAE). To validate real-time PCR results, western blotting analyses were carried out (Fig. 3C). As expected, protein extracts obtained from MDA-MB-231 cultivated in presence of 6 mg/mL AAE for 48 h showed lower amounts of Vimentin (−47 %) and higher concentrations of αvβ3-Integrin (+1497 %), with respect to the control. In addition, the level of key factors involved in cell cycle progression, that is $p21^{WAF1/Cip1}$, $p27^{Kip1}$ and p53 proteins, were monitored. After AAE treatment at 3 and 6 mg/mL for 48 h, $p21^{WAF1/Cip1}$ increased by 90 % and 97 %, respectively. In parallel, tumor cells raised their p53 levels by 43 % (at 3 mg/mL AAE) and 108 % (at 6 mg/mL AAE), compared to the control. Finally, $p27^{Kip1}$ quantity remained stable at 3 mg/mL AAE, while its signal was intensified (+48 %) at 6 mg/mL AAE.

AAE promotes oxidative stress and senescence in MDA-MB-231 cells. To clarify the molecular mechanism activated by AAE in the tumor cells, the induction of oxygen reactive species, DNA fragmentation, and senescence was investigated. ROS significantly accumulated by 31 % and 37 % in MDA-MB-231 treated for 48 h with 3 and 6 mg/mL, respectively (Fig. 3D). However, after exposure to the same conditions, no pattern of DNA fragmentation was detectable on agarose gel (Fig. 3E). With great surprise, the plant extract triggered senescent phenotypes in the cells. In detail, compared to the control, 48 h of low doses of AAE showed an increase of senescence by + 30.6 %, while high concentrations by + 67 % (Fig. 3F). By contrast, AAE did not determine any senescent effect on non-tumorigenic cells (MCF10A) (Supplemental Material 1, panel C).

4. Discussion

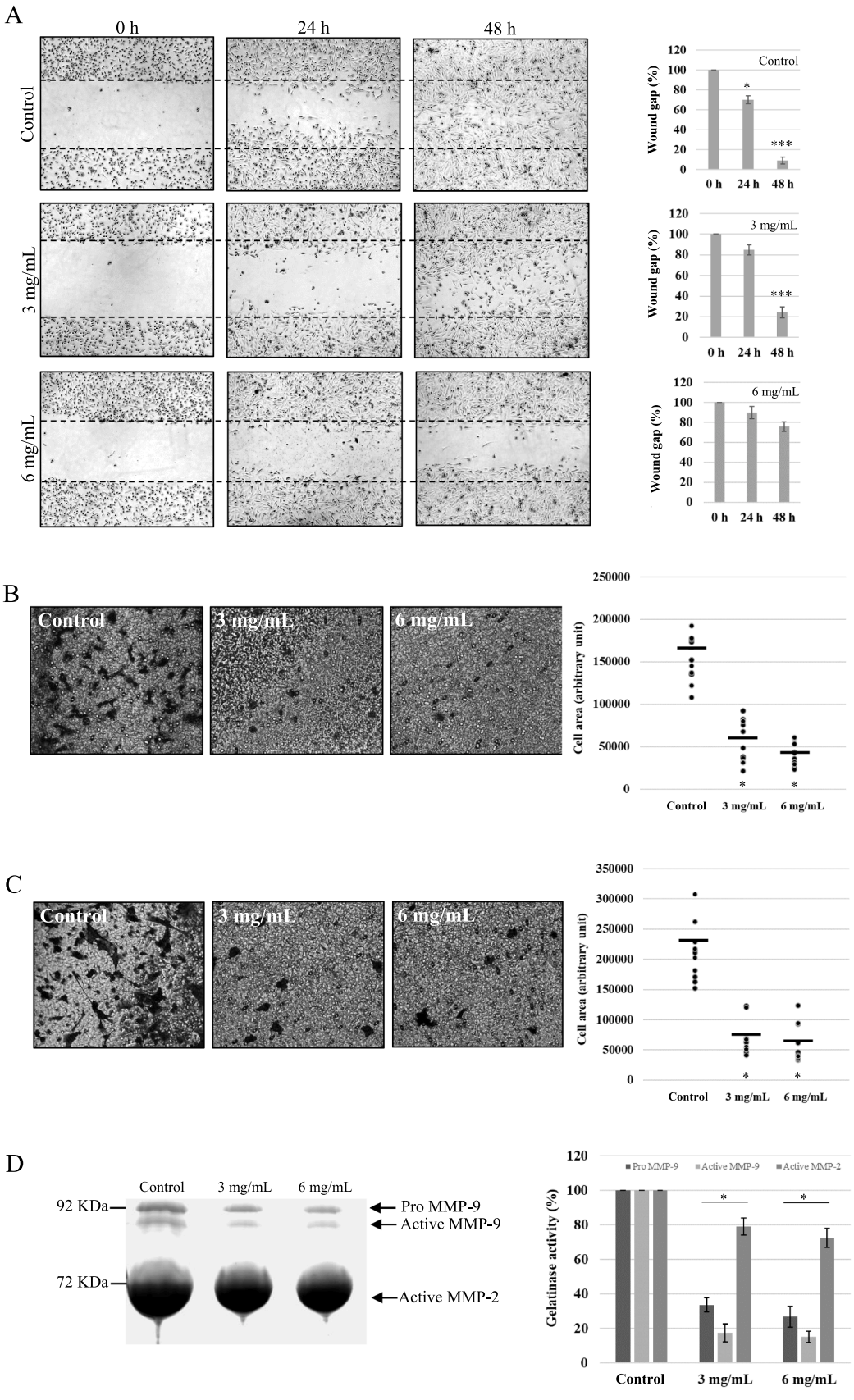
Nowadays, breast cancer represents about 24.5 % of all female tumours and 15.5 % of cancer deaths (Sung et al., 2021).

MDA-MB-231 cells derive from an aggressive, poorly differentiated, and highly metastatic breast cancer. The invasiveness of these cells is mainly due to their elevated extracellular production of proteolytic enzymes, which promote the development of secondary tumoral *foci*. In addition, such type of tumor line cannot be treated with hormonal therapy, usually applied to endocrine cancers, because it lacks oestrogen and progesterone receptor expression (a condition known as triple-negative profile), making the pathology difficult to prevent or eradicate (Liu et al., 2003; Chavez et al., 2010). According to this premise, searching for new therapeutical strategies able to contrast the onset and the development of MDA-MB-231-like tumors appears crucial.

Plant kingdom has always represented an important source of bioactive molecules (e.g., phytochemicals, plant microRNAs). Indeed, since ancient times, human communities have based their primal medicine on herbal practices, reaching today the evidence that about 30% of the modern drugs consists in plant-derived metabolites or compounds synthetized on the chemical structure of the latter and about 60% of the anticancer drugs are phytochemicals (Paterson and Edward, 2005; Gordaliza, 2007; Greenwell and Rahman, 2015; Singh et al., 2016; Newman and Cragg, 2020). In some areas of the world, ethnobotanical recipes handed down through traditional knowledge are still commonly employed to prevent or cure illness and disorders, although the scientific reasons underlying their biological effects have not been clarified yet. Among them, the application of *A. articulata* aqueous extract for treating breast cancer would be suggested by the Algerian folk pharmacognosy. Thus, aim of the current study was the evaluation of the potential antitumoral activity of *A. articulata* against MDA-MB-231 cells, in order to: i) confirm the efficacy of this traditional medicinal remedy; ii) clarify the cellular and molecular mechanisms triggered by the plant treatment; iii) propose a novel pharmacological solution for contrasting aggressive breast cancers.

Profiling plant extracts is fundamental to know the substances which are included into them, to favour their standardization and to predict their potential biological property. It has been proved that the phyto-complex cannot be reproduced synthetically and that it works differently than its single components (Ettorre et al., 2010; Williamson, 2011; Arru et al., 2021). Indeed, as stated by Badolati et al. (2018), phytochemicals would exert their best performance when included into their respective phytocomplexes, that is perfect environments for protecting the molecules from oxidation and degradation. Thus, we decided to perform our investigations by using the whole natural extract of *A. articulata* (AAE) which, to be in line with the African tradition, was obtained by macerating dry plant material in pure water. *A. articulata* phytocomplex has been characterized in the literature (Segal et al., 1969; Eman, 2011; Benhammou et al., 2013; Mroczek, 2015; Belyagoubi-Benhammou et al., 2019; Benzineb et al., 2019; Ben-Menni et al., 2022), highlighting a wide richness in secondary metabolites for this species (e.g., sterols, alkaloids). However, as we have demonstrated in Ben-Menni et al. (2024) by high pressure liquid chromatography analysis, AAE included essentially hydrophilic components (e.g., simple phenols, flavonoids), as expected due to the solvent selected for the extraction. In detail, Epicatechin, Resveratrol, Salicylic acid, Quercetine-3-*O*-glucoside, and Myricetin were the most abundant molecules detected in AAE.

In this contribution, the potential antiproliferative, antimetastatic and antiinvasive capacities of AAE were demonstrated on MDA-MB-231 cell line. In detail, the cell growth measured by counting alive cells in presence of Trypan-blue staining and by MTT assay appeared reduced after exposure to the plant extract, even at low doses, although the most significant results were obtained at 48 h of treatment with the highest



(caption on next page)

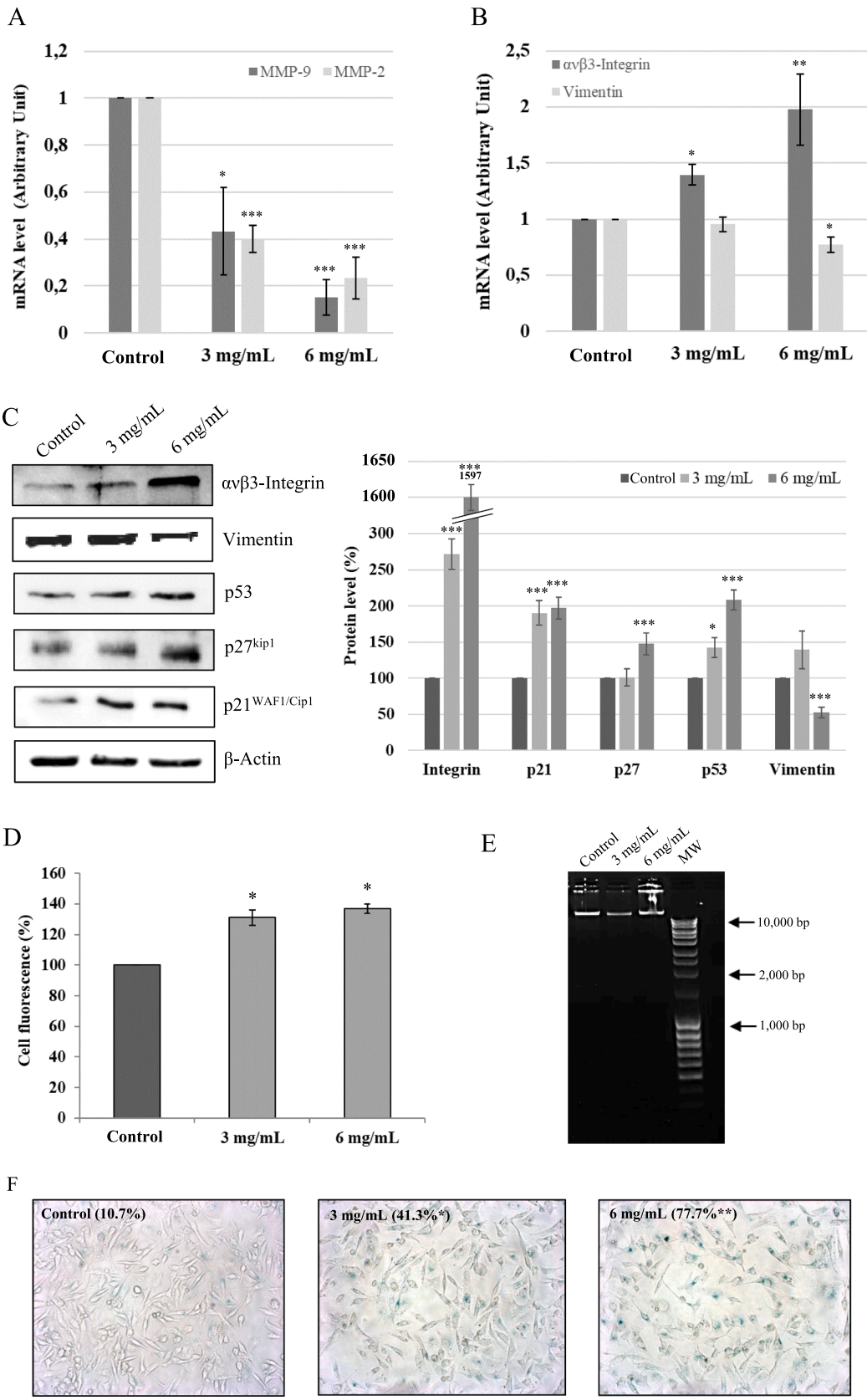
Fig. 2. Antiinvasive and antimigration capacities of AAE on MDA-MB-231 cells. A) Representative images of the wound assay captured by light microscopy at 0, 24 and 48 h after treatment with 3 and 6 mg/mL AAE or not (Control). On the right, graphs reporting the dimension (in percentage with respect to each condition at 0 h) of the respective wound gaps were shown. **B)** Representative images of the filters used in the migration assay performed by the principle of the Boyden chamber assay (transwell system) after exposure of the cells to 3 and 6 mg/mL AAE or not (Control). On the right, a graph of the number of cells (measured in arbitrary units as cell area, or rather black spots) which permeated the respective filter of the transwell system was reported. For each sample, the horizontal bar represents the mean of the measurements (points) carried out on four areas of independent filters (n = 3). **C)** Representative images of the filters used in the invasion assay performed by the principle of the Boyden chamber assay (transwell system plus matrigel) after exposure of the cells to 3 and 6 mg/mL AAE or not (Control). On the right, a graph of the number of cells (measured in arbitrary units as cell area, or rather black spots) which permeated the respective filter of the transwell system was reported. For each sample, the horizontal bar represents the mean of the measurements (points) carried out on four areas of independent filters (n = 3). **D)** A representative gel of the zymography analysis was shown; dark spots indicate the presence of the gelatinase activity at the expense of the active or pro-active isoforms of MMP-2 and MMP-9 secreted by MDA-MB-231 (previously treated with 3 and 6 mg/mL AAE, or not, for 48 h) and purified from the culture medium by specific enzyme concentrators. On the right, a graph indicating the intensity of the bands detected in the gel (measured in percentage compared to the Control used as unit, 100 %) was reported. **A-D)** All results of this figure represent the mean ± s.d. of three independent measurements (p values: *$p < 0.05$; ***$p < 0.001$).

concentrations. Curiously, phytochemicals provoked an initial slight cytotoxicity which then vanished completely, as also confirmed by measuring the amount of sub-G0 events at 48 h. Probably, tumor cells, after a preliminary shock condition, activated a resistance mechanism for avoiding the induction of death processes (Luqmani, 2005; Housman et al., 2014). Diederich and Cerella (2016) have collected literature works describing the capacity of plant compounds to trigger several signal transduction pathways able to lead towards non-canonical programmed cell death patters (e.g., necroptosis, paraptosis, mitotic catastrophe, etc.), changes in cell differentiation status, ultra-structural perturbations, and physiological alterations. All this evidence would suggest that each natural molecule exerts a specific function, and that apoptosis and necrosis should not be considered as the only expected phenomena in human cancer cells exposed to them. Considering that AAE is a phytocomplex, it is important to remind that multiple inputs may have been triggered simultaneously in MDA-MB-231 cells, revealing the complexity in studying the bioactivity of nutraceutical extracts (Riccio et al., 2017).

Cell resistance to AAE toxicity was not concomitant with the reactivation of the mitosis; in fact, at 48 h, the proliferation rate remained slowed down compared to the control. Cytofluorimetric investigations corroborated this trend, showing a cell cycle arrest in G2/M phase. This result was also coherent with the increase of p53, $p21^{WAF1/Cip1}$, and $p27^{Kip1}$ protein levels detected in the cells after treatment with AAE. P53 is one of the most known tumor suppressor genes, whose transcription is activated by several stressing stimuli, such as DNA damage, oncogene signal, hypoxia, mitotic spindle alteration. After the translocation in the nucleus, p53 behaves like a transcription factor for different genes (e.g., $p21^{WAF1/Cip1}$, $p27^{Kip1}$) involved in damage repair or death induction, preventing carcinogenesis (Jin and Levine, 2001; Pitchakarn et al., 2011). Thus, the cell growth arrest observed in MDA-MB-231 exposed to AAE could be linked to the expression of $P21^{WAF1/Cip1}$ and $P27^{Kip1}$ genes, which regulate cell cycle progression, at all phases, by inhibiting cyclin-dependent kinases (Coqueret, 2003). However, the roles of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ are not so univocal; a remarkable increase of $p27^{Kip1}$ has been associated with onset of apoptosis in various cell lines (Wang et al., 2009; Indovina et al., 2012), while the cytoplasmic forms of these factors have been classified as oncoproteins with antiapoptotic properties (Blagosklonny, 2002; Abbastabar et al., 2018). Based on this information, our experiments would suggest for $p27^{Kip1}$ a potential function of evasion to apoptosis, also considering that it increased at non-excessive levels (<50 %) only after 48 h of treatment with AAE, compared to p53 and $p21^{WAF1/Cip1}$. Nevertheless, two main clues would support this hypothesis: i) the lack of significant amounts of dead cells in Trypan blue exclusion test and FACS analysis; ii) the absence of DNA fragmentation, a typical feature of dying cells (e.g., by apoptosis) (Nagata, 2000).

Interestingly, wound test and transwell assays showed that AAE was able to reduce the migration and invasive capacities of MDA-MB-231. In support of this, zymography revealed a decrease of metalloprotease-2 and −9 activity in presence of the plant extract and qPCR approach indicated that even the transcripts of these zinc-dependent proteases were strongly reduced. Since gelatinases are the main enzymes secreted by tumor cells for degrading extracellular matrixes and favoring their spread through blood (Li et al., 2017), our data linked to *A. articulata* a significant antimetastatic effect. Vimentin, a type III intermediate filament, and integrins, transmembrane receptors, are known to be highly accumulated in aggressive cancers during the invasion process, promoting tumour progression and metastasis (Marsico et al., 2018; Berr et al., 2020). Therefore, to understand if AAE acted on these molecular targets, we monitored their protein and mRNA levels by Western Blotting and qPCR, respectively. Coherently with previous results, the expression of Vimentin was inhibited by the phytochemicals but surprisingly αvβ3-Integrin increased. This last outcome seemed to be in contrast with the assumption that AAE possessed antitumoral properties, making difficult its interpretation. However, paying attention to literature, we observed that several scholars have proved that this peculiar integrin was also a marker of cell senescence (Borghesan and O'Loghlen, 2017; Rapisarda et al., 2017). This phenomenon is a generally irreversible process of cell-cycle arrest which is triggered by several stressors (e.g., drug treatment, oncogene stimulation) and driven by the activation of two main pathways, $p53/p21^{WAF1/Cip1}$ and $RB/p16^{INK4A}$. Releasing inflammatory factors, senescent cells recall the immune system to eliminate themselves and to facilitate tissue homeostasis and regeneration (Jun and Lau, 2010; Rapisarda et al., 2017). Since this cell profile perfectly fitted with that obtained in our experimental observations (i.e., proliferation inhibition, $p53/p21^{WAF1/Cip1}$ induction), the final step of the study was focused to investigate the senescence. Using a specific kit, AAE treated cells clearly showed a senescent phenotype, highlighting that the plant extract was able to stimulate the onset of this process. Lastly, as oxidative stress has been mentioned among senescence inducers (Jun and Lau, 2010), the levels of ROS were measured in MDA-MB-231 cells exposed to *A. articulata*. The results allowed to infer that these reactive species might represent the effectors, or the intracellular signals, responsible for the biological activity associated to AAE. Indeed, although phytochemicals are usually considered as antiradical compounds, it has been widely demonstrated that high concentrations of these metabolites may exert a pro-oxidant function in cells (Aquilano et al., 2008; Martin-Cordero et al., 2012; Khan et al., 2014; Nanni et al., 2020), explaining the evidence reported in the current paper and its dependence on the doses of plant extract employed during the treatments.

In conclusion, in the present research, we collected proof about the antiproliferative, antiinvasive, and antimetastatic properties of the aqueous extract of *A. articulata* against a highly aggressive type of human breast cancer. The molecular mechanism triggered by the phytocomplex was defined as oxidative stress-induced senescence, although other investigations are necessary to better elucidate the exact role of ROS in this context. The data reported here would provide a scientific explanation to the empirical observations related to the application of *A. articulata* as an antitumoral drug, confirming the efficacy of this Algerian traditional medicinal remedy. Moreover, they would represent the basics for proposing the development of a novel natural pharmacological product with preventive and therapeutic antineoplastic potentialities against breast cancer.



(caption on next page)

Fig. 3. Molecular investigations and study of the senescence induction by AAE. A) qPCR analysis of MMP-2 and MMP-9 mRNA level in MDA-MB-231 cells exposed to 3 and 6 mg/mL AAE, or not (Control), for 48 h; Control was considered as arbitrary unit (1). B) qPCR quantitation of Vimentin and αvβ3-Integrin transcript level in MDA-MB-231 cells exposed to 3 and 6 mg/mL AAE, or not (Control), for 48 h; Control was considered as arbitrary unit (1). C) Representative images of the immunoblots carried out on the protein extracts purified from the tumor cells treated for 48 h with 3 and 6 mg/mL AAE, or not (Control). Quantitation of each protein signal was reported on the graph in the right part as percentage with respect to the Control (considered as unit, 100 %), after normalization with β-Actin. D) ROS level detected by flow-cytofluorimetry as percentage of fluorescence in MDA-MB-231 cells stained with DCFH-DA after treatment with 3 and 6 mg/mL AAE for 48 h; untreated cells (Control) were considered as unit (100 %). E) DNA ladder assay performed to monitor the fragmentation of the DNA in untreated cells (Control) or exposed to 3 and 6 mg/mL AAE for 48 h; a molecular weight (M.W.) is reported to indicate the efficiency of the run. F) Representative images of MDA-MB-231 treated with AAE for 48 h, or not (Control), and then subjected to a specific kit able to stain in green the senescent cells. The percentage of senescent cells was measured and indicated in each photo. A-F) All results of this figure represent the mean ± s.d. of three independent measurements (p values: *p < 0.05; **p < 0.01; ***p < 0.001).

Compliance with ethics requirement

The authors declare:

- no fundings linked to this research;
- The content of this work was deposed at the Algerian National Institute of Industrial Property (INAPI) as a patent (patent number: 2427; filing number: 220471.
- no involvement of living animals in the research;
- no involvement of living humans in the research.

CRediT authorship contribution statement

Valentina Monteleone: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Dounia Ben Menni:** Writing – review & editing, Investigation, Formal analysis. **Nabila Belyagoubi-Benhammou:** Writing – review & editing, Resources. **Gabriele Di Marco:** Writing – review & editing, Investigation, Formal analysis. **Antonella Canini:** Writing – review & editing, Supervision. **Angelo Gismondi:** Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization.

Declaration of competing interest

The content of this work was deposed at the Algerian National Institute of Industrial Property (INAPI) as a patent (patent number: 2427; filing number: 220471.

Data availability

All data are reported in the MS or in its Supplementary data.

Acknowledgments

The content of this work was deposed at the Algerian National Institute of Industrial Property (INAPI) as a patent (patent number: 12427; filing number: 220471).

Appendix A. Supplementary data

A) Proliferation curves of MCF10A cells exposed to AAE (0.3 or 6 mg of dried plant material equivalent per mL of culture medium) or not (Control), for 24 and 48 h, obtained by counting alive cells (Trypan blue negative cells) using a Neubauer chamber. **B)** Percentage of MCF10A dead cells (Trypan blue positive cells) after treatment with AAE for 24 and 48 h. **C)** Percentage of senescent MCF10A cells measured by the specific kit described in the Materials and Methods section. **A-C)** All results represent the mean ± s.d. of four independent measurements. Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2024.106203.

References

Abbastabar, M., Kheyrollah, M., Azizian, K., Bagherlou, N., Tehrani, S. S., Maniati, M., & Karimian, A. (2018). Multiple functions of p27 in cell cycle, apoptosis, epigenetic modification and transcriptional regulation for the control of cell growth: A double-edged sword protein. *DNA Repair, 69*, 63–72.

Abdallah, H. M., Abdel-Naim, A. B., Ashour, O. M., Shehata, I. A., & Abdel-Sattar, E. A. (2014). Anti-inflammatory activity of selected plants from Saudi Arabia. *Zeitschrift für Naturforschung C, 69*, 1–9.

Aquilano, K., Baldelli, S., Rotilio, G., & Ciriolo, M. R. (2008). Role of nitric oxide synthases in parkinson's disease: A review on the antioxidant and anti-inflammatory activity of polyphenols. *Neurochemical Research, 33*, 2416–2426.

Arru, L., Mussi, F., Forti, L., & Buschini, A. (2021). Biological Effect of Different Spinach Extracts in comparison with the individual components of the phytocomplex. *Foods, 10*(2), 382.

Azza, M., Mohga, A., Maha, R., El-Sayed, M., Abdel-Razik, F., El-Sharabasy, F., Aly, H., & Mohamed, R. M. (2014). Alleviation of dimethylnitrosamine-induced liver injury and fibrosis by supplementation of *Anabasis articulata* extract in rats. *Indian Journal of Clinical Biochemistry, 29*, 418–429.

Badolati, N., Sommella, E., Riccio, G., Salviati, E., Heintz, D., Bottone, S., Di Cicco, E., Dentice, M., Tenore, G., Campiglia, P., Stornaiuolo, M., & Novellino, E. (2018). Annurca apple polyphenols ignite keratin production in hair follicles by inhibiting the pentose phosphate pathway and amino acid oxidation. *Nutrients, 10*(10), 1406.

Belyagoubi-Benhammou, N., Belyagoubi, L., Gismondi, A., Di Marco, G., Canini, A., & Atik-Bekkara, F. (2019). GC/MS analysis, and antioxidant and antimicrobial activities of alkaloids extracted by polar and apolar solvents from the stems of *Anabasis articulata*. *Medicinal Chemistry Research, 28*, 754–767.

Ben Menni, D., Belyagoubi-Benhammou, N., Abdelli, I., Bekkal Brikci, S., Benmahieddine, A., Ben Menni, H., Boutrif, O., Gismondi, A., et al. (2024). Enzyme inhibitory, antioxidant and anti-inflammatory activities of *Anabasis articulata* phenolic-rich extract; *in vitro*, *in vivo*, and *in silico* studies. Plant Biosystems (now under second round of revision).

Ben Menni, D., Belyagoubi-Benhammou, N., Benmahieddine, A., et al. (2022). Identification of Sterols from *Anabasis articulata* (Forssk.) Moq. (Chenopodiaceae) Growing in Algeria and Study of Their Potential Bioactivity. *Waste and Biomass Valorization, 13*, 3283–3295.

Benhammou, N., Ghambaza, N., Benabdelkader, S., Atik-Bekkara, F., & Panovska, F. K. (2013). Phytochemicals and antioxidant properties of extracts from the root and stems of *Anabasis articulata*. *International Food Research Journal, 20*(5), 2057.

Benzineb, E., Kambouche, N., Hamiani, A., Bellahouel, S., Zitouni, H., & Toumi, H. (2019). Phenolics compounds and biological activity of leaves of *Anabasis articulata*, an Algerian medicinal plant. *International Journal of Pharmaceutical Research and Allied Sciences, 8*(4), 1–5.

Berr, A. L., Wiese, K., dos Santos, G., Davis, J. M., Koch, C. M., Anekalla, K. R., Kidd, M., Davis, J. M., Cheng, Y., Hu, Y. S., & Ridge, K. M. (2020). Vimentin is Required for Tumor Progression and Metastasis in a Mouse Model of Non-Small Cell Lung Cancer. *Oncogene, 42*(25), 2074–2087.

Blagosklonny, M. V. (2002). Are p27 and p21 cytoplasmic oncoproteins? *Cell Cycle, 1*(6), 391–393.

Borghesan, M., & O'Loghlen, A. (2017). Integrins in senescence and aging. *Cell Cycle, 16*(10), 909–910.

Chavez, K. J., Garimella, S. V., & Lipkowitz, S. (2010). Triple Negative Breast Cancer Cell Lines: One Tool in the Search for Better Treatment of Triple Negative Breast Cancer. *Breast Disease, 32*(1–2), 35–48.

Coqueret, O. (2003). New roles for p21 and p27 cell-cycle inhibitors: A function for each cell compartment? *Trends in Cell Biology, 13*(2), 65–70.

Diederich, M., & Cerella, C. (2016). Non-canonical programmed cell death mechanisms triggered by natural compounds. In *Seminars in Cancer Biology* (Vol. 40, pp. 4–34). Academic Press.

El Dine, R. S., Abdallah, H. M., Kandil, Z. A., Zaki, A., Khan, S., & Khan, A. (2018). PPARα and γ activation effects of new nor-triterpenoidal saponins from the aerial parts of *Anabasis articulata*. *Planta Medica, 85*, 274–281.

Eman, A. (2011). Phytochemical screening on different plant parts of some succulent plants of Egypt. *New York Science Journal., 4*(2).

Ettorre, A., Frosali, S., Andreassi, M., & Di Stefano, A. (2010). Lycopene phytocomplex, but not pure lycopene, is able to trigger apoptosis and improve the efficacy of photodynamic therapy in HL60 human leukemia cells. *Experimental Biology and Medicine, 235*(9), 1114–1125.

Gordaliza, M. (2007). Natural products as leads to anticancer drugs. *Clinical and Translational Oncology, 9*, 767–776.

Greenwell, M., & Rahman, P. K. (2015). Medicinal plants: Their use in anticancer treatment. *International Journal of Pharmaceutical Sciences and Research, 6*, 4103–4112.

Hammiche, V., & Maiza, K. (2006). Traditional medicine in Central Sahara: Pharmacopoeia of Tassili N'ajjer. *Journal of Ethnopharmacology, 105*, 358–367.

Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N., & Sarkar, S. (2014). Drug resistance in cancer: An overview. *Cancers, 6*(3), 1769–1792.

Indovina, P., Giorgi, F., Rizzo, V., Khadang, B., Schenone, S., Di Marzo, D., Forte, I. M., Tomei, V., Mattioli, E., D'Urso, V., Grilli, B., Botta, M., Giordano, A., & Pentimalli, F. (2012). New pyrazolo-[3, 4-d]-pyrimidine SRC inhibitors induce apoptosis in mesothelioma cell lines through p27 nuclear stabilization. *Oncogene, 31*(7), 929–938.

Jin, S., & Levine, A. J. (2001). The p53 functional circuit. *Journal of cell science, 114*(23), 4139–4140.

Jun, J. I., & Lau, L. F. (2010). The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. *Nature Cell Biology, 12*(7), 676–685.

Kambouche, N., Merah, B., Derdour, A., Bellahouel, S., Bouayed, J., Dicko, A., Younos, C., & Soulimani, R. (2009). Hypoglycemic and antihyperglycemic effects of *Anabasis articulata* (Forssk) Moq (Chenopodiaceae), an Algerian medicinal plant. *African Journal of Biotechnology, 8*, 5589–5594.

Khan, H. Y., Zubair, H., Faisal, M., Ullah, M. F., Farhan, M., Sarkar, F. H., Ahmad, A., & Hadi, S. M. (2014). Plant polyphenol induced cell death in human cancer cells involves mobilization of intracellular copper ions and reactive oxygen species generation: A mechanism for cancer chemopreventive action. *Molecular Nutrition and Food Research, 58*, 437–446.

Lauterbach, M., Veranso-Libalah, M. C., Sukhorukov, A. P., & Kadereit, G. (2019). Biogeography of the xerophytic genus *Anabasis* L. *(Chenopodiaceae). Ecology and Evolution, 9*(6), 3539–3552.

Li, H., Qiu, Z., Li, F., & Wang, C. (2017). The relationship between MMP-2 and MMP-9 expression levels with breast cancer incidence and prognosis. *Oncology Letters, 14*(5), 5865–5870.

Liu, H., Zang, C., Fenner, M. H., Possinger, K., & Elstner, E. (2003). PPARgamma ligands and ATRA inhibit the invasion of human breast cancer cells in vitro. *Breast Cancer Research and Treatment, 79*(1), 63–74.

Luqmani, Y. A. (2005). Mechanisms of drug resistance in cancer chemotherapy. *Medical Principles and Practice, 14*(Suppl. 1), 35–48.

Marsico, G., Russo, L., Quondamatteo, F., & Pandit, A. (2018). Glycosylation and integrin regulation in cancer. *Trends in Cancer, 4*(8), 537–552.

Martin-Cordero, C., Leon-Gonzalez, J. A., Calderon-Montano, M. J., Burgos-Moron, E., & Lopez-Lazaro, M. (2012). Pro-oxidant natural products as anticancer agents. *Current Drug Targets, 13*, 1006–1028.

Metwally, N. S., Mohamed, A. M., & ELSharabasy, F. S. (2012). Chemical constituents of the Egyptian Plant *Anabasis articulata* (Forssk) Moq and its antidiabetic effects on rats with streptozotocin-induced diabetic hepatopathy. *Journal of Applied Pharmaceutical Science, 2*(4), 54.

Mohamed, A. M., Abdalla, M. S., Rizk, M. Z., Mahdy, E. M., Farrag, A. R. H., El-Sharabasy, F. S., Aly, F. F., & Mohamed, M. R. (2014). Alleviation of Dimethylnitrosamine-induced liver injury and fibrosis by supplementation of *Anabasis articulata* extract in rats. *Indian Journal of Clinical Biochemistry, 29*, 418–429.

Mroczek, A. (2015). Phytochemistry and bioactivity of triterpene saponins from Amaranthaceae family. *Phytochemistry Reviews, 14*, 577–605.

Nagata, S. (2000). Apoptotic DNA fragmentation. *Experimental Cell Research, 256*(1), 12–18.

Nanni, V., Di Marco, G., Sacchetti, G., Canini, A., & Gismondi, A. (2020). Oregano phytocomplex induces programmed cell death in melanoma lines via mitochondria and DNA damage. *Foods, 9*(10), 1486.

Newman, D. J., & Cragg, G. M. (2020). Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. *Journal of Natural Products, 83*(3), 770–803.

O'leary, J. W., & Glenn, E. P. (1994). Global distribution and potential for halophytes. In *Halophytes as a resource for livestock and for rehabilitation of degraded lands* (pp. 7–17). Dordrecht: Springer.

Paterson, I., & Edward, A. A. (2005). The renaissance of natural products as drug candidates. *Science, 310*(5747), 451–453.

Pitchakarn, P., Suzuki, S., Ogawa, K., Pompimon, W., Takahashi, S., Asamoto, M., Limtrakul, P., & Shirai, T. (2011). Induction of G1 arrest and apoptosis in androgen-dependent human prostate cancer by Kuguacin J, a triterpenoid from *Momordica charantia* leaf. *Cancer Letters, 306*(2), 142–150.

Rapisarda, V., Borghesan, M., Miguela, V., Encheva, V., Snijders, A. P., Lujambio, A., & O'Loghlen, A. (2017). Integrin beta 3 regulates cellular senescence by activating the TGF-β pathway. *Cell Reports, 18*(10), 2480–2493.

Riccio, G., Maisto, M., Bottone, S., Badolati, N., Rossi, G. B., Tenore, G. C., Stornaiuolo, M., & Novellino, E. (2017). WNT inhibitory activity of *Malus pumila* miller cv annurca and *Malus domestica* cv limoncella apple extracts on human colon-rectal cells carrying familial adenomatous polyposis mutations. *Nutrients, 9*(11), 1262.

Sathiyamoorthy, P., Lugasi-Evgi, H., Van-Damme, P., Abu-Rabia, A., Gopas, J., & Golan-Goldhirsh, A. (1997). Larvicidal activity in desert plants of the Negev and Bedouin market plant products. *International Journal of Pharmacognosy, 35*, 265–273.

Segal, R., Goldzweig-Milo, I., & Zaitschek, D. V. (1969). The sapogenin content of *Anabasis articulata*. *Phytochemistry, 8*, 521.

Singh, S., Sharma, B., Kanwar, S. S., & Kumar, A. (2016). Lead phytochemicals for anticancer drug development. *Frontiers in Plant Science, 7*, 1667.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN esti-mates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A cancer journal for clinicians, 71*(3), 209–249.

Wang, P., Ma, Q., Luo, J., Liu, B., Tan, F., Zhang, Z., & Chen, Z. (2009). Nkx3.1 and p27kip1 cooperate in proliferation inhibition and apoptosis induction in human androgen–independent prostate cancer cells. *Cancer Investigation, 27*(4), 369–375.

Williamson, E. M. (2011). *Phytocomplexes versus single-entity drugs* (p. 519). CRC Press-Taylor & Francis Group.