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Original article

The role of Algerian *Ephedra alata* ethanolic extract in inhibiting the growth of breast cancer cells by inducing apoptosis in a p53- dependent pathway

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ABSTRACT

Background: *Ephedra alata*, a member of the Ephedraceae family, was used to treat different diseases and it might be shown a strong efficacy to inhibit cancer cell lines.
Methods: Due to the limited research available about this plant, the objective of this research was to evaluate the antioxidant, cytotoxic and apoptotic effects of *Ephedra alata* ethanolic extract (EAEE), against different human cancer cell lines.
Results: EAEE inhibited the growth of the liver (HepG2), breast (MCF-7), and colon cancer cells (Caco-2). MCF-7 cells with an IC_{50} of 153 µg/ml, were the most sensitive to the extract. Furthermore, exploration using flow cytometry using Annexin V-FITC/PI assay demonstrated that EAEE caused death for all human cancer cells mainly through apoptosis. Very interestingly, qRT-PCR analysis using the ΔΔCt method revealed that four genes, Bax, p21, RB1, and TP53 were up-regulated in MCF-7 cells treated either with EAEE or S-FU drug. These findings let us believe that the mechanism by which EAEE kills breast cancer cells seems to be apoptosis via a P53-dependent manner, which involved intrinsic pathways through the induction of Bax, p21, and RB1.
Conclusions: EAEE exhibits good biological properties in contradiction of HepG-2, MCF-7, and Caco-2 cell lines. This study appoints for the first time that EAEE increases the expression in MCF-7 cells of p53 and three more genetic traits that control cellular proliferation and apoptosis. Therefore, this plant could serve as a potential source to find new pro-apoptotic drugs for cancer treatment.

1. Introduction

Due to the increasing number of people dying from cancer, it is becoming more important that the various causes of this disease are identified and studied (Tran et al., 2022). Some of the factors that can be considered include the environment, genetic susceptibility, and improper diet. Around 35% of all cancer cases worldwide are attributed to the wrong diet. Various pharmaceutical and research organizations are working on finding a way to prevent this disease from happening in the future (Lakshmi et al., 2015).

Natural products recurrently have been examined for favorable novel information in pharmaceutical improvement (Yaacob et al., 2010). These compounds and their analogs have been found to be effective anti-cancer composites, and increasingly more plants are also being found to have anti-cancer properties (Prakash et al. 2013). The gymnosperm *Ephedra alata* is a member of the Ephedraceae family and includes a number of well-known pharmacologically potent substances, including phenolic content and alkaloids (Kim et al., 2003). This plant is commonly used in ancient medication to treat various diseases such as respiratory ailments, colds, coughs, and bronchitis (Dousari et al., 2022). In Algeria, *E. alata* is found in the northern and western desert at the level of

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sandy surfaces, regs, and the sandy beds of the valley. It is even present in the sand of tropical understories (Ozenda, 1991). The diverse topography and climate of Algeria make it an ideal location for cultivating a flora that is rich in aromatic and medicinal plants (Miara et al., 2019). It also has over 3,000 species of vascular plants, making it one of the wealthiest countries in the region when it comes to plant diversity (Montes-Moreno et al., 2022). The literature states that various Ephedra species contain alkaloids, such as ephedrine. This substance is known to stimulate the adrenergic receptors and increase peripheral vasoconstriction and cardiac rate (González-Juárez et al., 2020). Besides being a natural source of alkaloids, Ephedra is also a rich source of compounds that have a high antioxidant capacity. These include flavonoids, which have various anticarcinogenic and antimicrobial properties (Elhadef et al., 2020). It is known that antioxidants are capable of scavenging all reactive oxygen species (ROS), thus scientific evidence suggests that the use of diets rich in these compounds reduces the risk of chronic diseases, including cancer (Ogunro et al., 2022). GLOBOCAN 2020 predicts that 19.3 million different tumor cases will be recorded in 2020, while approximately 10.0 million people in the world will life ends from cancer (Sung et al., 2021).

Several recent studies revealed that *E. alata* might have an important anticancer potential *in vivo* and *in vitro* (Danciu et al., 2018; Sioud et al., 2020; Sioud et al., 2022). Many mechanisms were proposed to understand plants' anticancer effects such as cytotoxicity, apoptosis, mitochondrial suppression, cell cycle arrest, vital enzyme inhibition, reduction of DNA damage, and/or angiogenesis prevention (**Azadeh et al., 2022**). Eventually, apoptosis-inducing pathway flaws can result in the proliferation of cancerous cells. A fundamental barrier to effective cancer treatment is the resistance of apoptosis, which promotes abnormal cellular growth and ultimately causes carcinogenesis (Jan and Chaudhr, 2019).

Given the foregoing above, the goal of the current research was to ascertain the antioxidant, the anti-proliferative, and the apoptotic effects of the *E. alata* ethanolic extract against three cancer cell lines. The action mechanism of the *E. alata* Algerian species, has not been studied against breast cancer cells. This study investigated the effect of EAEE on eight genes, namely those implicated in apoptosis and cancer genesis in MCF-7 cells.

2. Materials and methods

2.1. Plant collection and extract preparation

The study focused on the plant's aerial portion (twigs and leaves) *Ephedra alata*, which was obtained from Algerian semi-desert areas. It was identified and authenticated by Dr. Hannane KHANOUF at the University of Jijel, Algeria. The plant samples were dried for 15 days at room temperature to prevent humidity and light from damaging them. The aerial parts were then crushed to obtain a fine powder. The extract was prepared using a combination of 150 ml of 70% ethanol and 50 g of plant powder. The mixture was stirred continuously for 72 h to prevent oxidation. After filtering the extract using Wattman paper, the filtrate obtained was kept in a dry place, and the rest (upper portion) was macerated for the second time following the same preceding steps. Then the two filtrates were recovered and mixed.

The extract was then evaporated at a temperature of 50 °C using a Laborota 400 rotary evaporator. It was weighed and stored in a refrigerator until used. The extraction yield was calculated by the ratio between the obtained weight of the crude extract and the weight of the dry vegetable matter. It is expressed as a percentage according to the following formula:

Yield %= [amount (g) of the dry extract obtained / amount (g) of the dry sample used] × 100.

2.2. In vitro antioxidant activity

In vitro studies were conducted on the plant extract's antioxidant activity to determine its capacity and reducing power. The different methods used included superoxide anion degradation, hydroxyl radical scavenging, and free radical scavenging. The mean values were calculated after all the tests were performed in triplicate.

2.2.1. DPPH• radical scavenging activity

This assessment was carried out with Blois's method (Blois, 1958). The reaction mechanism involves a proton transfer from an antioxidant such as a phenolic compound to the DPPH radical and this leads to the change of color from purple to yellow (Sannigrahi et al., 2009). Various concentrations of crude extract and vitamin C (Vit C), which is used as positive control were incubated with the prepared DPPH• (0.004% in ethanol, w/v) for 30 min. The absorbance of each mixture was measured at 517 nm.

2.2.2. Hydroxyl radical scavenging activity

The scavenging activity of an extract was calculated by the ability to remove hydroxyl radicals from a well plate. This activity was carried out using the concept of the Fe^{2+}–H_2O_2 system, which was described by Smirnoff and Cumbes in 1989. To detect various substances, such as •OH, 50 microletter of 9 mmol/L of salicylic acid, 50 µl of FeSO4, and 50 µl of H2O2 were supplementary to all well. The muddle was then incubated at 37 °C for 60 min. After the mixture was analyzed using a spectrophotometer, at 510 nm, the mixture's absorbance was measured.

2.2.3. Activity for scavenging superoxide anion

Based on the EAEE's capability, superoxide anion (O2•) scavenging activity was measured and inhibit the conversion of nitro blue tetrazolium (NBT) into the formazan (NBT byproduct) via $O2^{\bullet-}$. It is known that these compounds should scavenge the $O2^{\bullet-}$ generated from the auto-oxidation of riboflavin in presence of light (McCord and Fridovich, 1969). In each well of 96 well plates, 200 µl of the reaction mixture was dispensed. This combination comprised riboflavin (0.12 mM), EDTA (0.1 M), 0.0015% NaCN, NBT (1.5 mM), and phosphate buffer, pH 7.8 (67 mM). The mixture contained also various dilutions of EAEE and Vit C. The plate was placed in the sunlight for 15 min after illumination and the optical density at 530 nm was measured.

The ratio of scavenging activity against each free radical was determined as follows: percentage of free radical scavenging = [(AC - AE) /AC] × 100 (Where: AC: the mean of the negative control's absorbances. AE: the mean of each sample's absorbances). The results were expressed as the concentration inhibiting the reaction by 50 % (IC_{50} in µg/ml) and determined using data that were computed using the percentage scavenging activity equation of and GraphPad Instat program.

2.2.4. Power-reducing assay

The Oyaizu method was used to determine the reduction power of the tested extract (Oyaizu, 1986). In this test, antioxidants decrease the Fe^{3+}/ferricyanide combination to the ferrous form, and the process may be observed by observing the development of a blue colour complex at 700 nm. In each well of a 96-well plate, 25 µl of the extract and Vit C at various concentrations were dispensed. Following that, 62.5 µl of pH 6.6, 0.2 M phosphate buffer, and 62.5 µl of potassium ferricyanide ($K_3Fe(CN)_6$) were added per well. The mixtures were incubated at 50 °C for 20 min. After adding 62.5 µl of 10% trichloroacetic acid (TCA) per well, the plate

was centrifuged at 2000 rpm for 20 min. The supernatants were collected, transferred to another plate, and mixed with 100 µl of distilled water and 20 µl of 0.1% ferric chloride ($FeCl_3$). The absorbances were measured at 700 nm. EC50, or the effective concentration that converted 50% of Fe^{3+} into Fe^{2+}, was used to express the results in µg/ml. The data from the equation of the percentage of lowering power activity were used to compute the EC50 using the GraphPadInstat program.

2.2.5. Total amount of antioxidants

The tested extract's total antioxidant capacity (TAC) was quantified colorimetrically in accordance with the specified methodology by Prieto et al (1999) with a minor change. This approach is based on the observation that Mo (V) can be reduced to Mo (IV) by the antioxidants thereby generating eventually a green compound (phosphate/Mo (V)) that have an absorption peak at 695 nm. To prepare the extract, ascorbic acid was added to a mixture of alcohol and 100 l of each of its concentrations. This mixture was then 90 min incubated at 95 °C in an oven. Absorbances were measured at 695 nm after the mixture was thoroughly analyzed. Ethanol is used as a blank instead of an extract. The total antioxidant performance was then calculated by taking into account the microgram equivalents of vit C. The ascorbic acid equivalents were calculated using the standard graph.

2.3. Determination of the cytotoxicity effect of the plant extract on human normal and cancer cell lines

2.3.1. Cell lines and cultures media

Three cancer cell lines, breast cancer cell line (MCF-7), liver cancer cell line (HepG-2), and colon cancer line (Caco-2) along with human normal lung fibroblast cells (Wi-38) ("*ATCC*" *the American Type Culture Collection*", Lonza, USA) were selected to study *in vitro* anticancer efficacy of EAEE. Wi-38 and Caco-2 were grown in DMEM with 10% FBS, while MCF-7 and HepG-2 were cultured in RPMI-1640 improved with 10% fetal bovine serum (FBS). Human normal and cancer cell lines were maintained as adherent cell cultures at 37 °C in an incubator with humidified air and 5% CO2. Cells were seeded in 25 cm^2 culture flasks and incubated in a 5% CO2 incubator. When reaching confluent each cell type was subcultured using trypsinization into a 75 cm^2 culture flask for two weeks before assays.

2.3.2. Cell viability test

Cell viability was assessed employing the dye exclusion technique of Louis and Siegel (2011). It is based on the idea that whereas dead cells lack complete cell walls that keep out certain dyes like trypan blue, live cells have. The hemocytometer was filled with 50 ÂµI of the cell suspension and an equal amount of a 0.5% Trypan blue staining solution. In each of the four corner quadrants, viable (unstained) and nonviable (stained) cells were counted (A, B, C, D). The percentage of cell viability was calculated according to the following formulas: $N/ml = [(A + B + C + D)/4] \times 10^4 \times D$ (N: the amount of live cells, D: diluting the sample (1:1 with the trypan blue). Percentage of cell viability = Number of viable cells/Total number of cells × 100. To utilise the cells for tests, at least 90% of the cells must be alive.

2.3.3. Test for cytotoxicity in vitro

The sensitivity of normal and cancer cells to EAEE was evaluated using an MTT assay, as described by Mosmann (1983) with light modifications. The MTT assay measures cell metabolic activity by detecting the formazan, an insoluble blue product that was produced in viable cells after the reduction of tetrasodium salt (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT)), a yellow colour that is water soluble, via mitochondrial succinate dehydrogenase. The quantity of formazan crystals is inversely correlated with the quantity of active cells since the reduction reaction is only possible in viable cells.

Human cancer and normal cells were placed in a 96-well flat-bottomed plate culture media and permitted to attach for 24 h. After attachment, serial concentrations of EAEE were incubated at 37 °C and 5% CO_2. After 72 h of treatment, all cells were then treated with an MTT solution at pH 7. They were then separated from the solution at 2000 rpm and solubilized with DMSO at 37 °C for 10 min. An ELISA reader for microplates was used to test the mixture's absorbance at 570 nm. The percentage of normal cells and the inhibition of growth rates of treated cancer cells were measured using a triplicate-based method.

Percentage of normal cell proliferation = $[OD_E - OD_B/OD_C - OD_B] - \times 100$ and the inhibition rate (%) where OD_E: is the average of extract absorbances-treated cells, OD_B: the average of blank's absorbances and OD_C: is the mean of absorbances of untreated cells.

The extract's impact on cancer and healthy cells was calculated as IC_{50} (half-maximal inhibitory concentration that causes 50% growth inhibitions of cancer cells) and EC_{50} (effective dose of extract that caused 50% of the normal cells to become cytotoxic) respectively using the GraphPad Instat software. Moreover, the EC_{100} (the safe dose that keeps 100% cell viability) was estimated. The Selectivity index (SI) was calculated using the fraction between the cytotoxicity effect of the extract on normal cells relative to cancer cells (CC_{50}/ IC_{50}).

2.3.4. Cellular morphological changes

IC_{50} of the plant extract was used to examine cellular morphological changes selected before and after 72 h in cancer cell lines of treatment using a digital camera, an image analysis program, and an inverted phase-contrast microscope (Analysis 3.1 Soft Imaging System SIS) (Olympus, Japan).

2.3.5. Annexin V-FITC/PI apoptotic assay and flow cytometry analysis

The plant extract was used at its IC_{50} concentrations to treat the human cancer cell lines MCF-7, HepG-2, and Caco-2 while they were cultured at 37 °C in 5% CO2. Using trypsinization, the cells were collected, cleaned, and resuspended in PBS after 72 h of treatment. By labeling cells with annexin V-biotin (Molecular ProbesTM, USA) and propidium iodide (PI) for 15 min in the dark, the mechanism of cancer cell death was examined. In PBS, cells were suspended after staining with 5 g/ml of streptavidin-fluorescein before being centrifuged and fixed with 4% paraformaldehyde for 10 min (Sigma, USA). A flow cytometer (CyFlow® Space, Germany) was used to measure the cell death rates. Annexin-stained apoptotic cells were recognised using the FITC signal detector (FL1), and necrotic cells were detected using the phycoerythrin emission signal detector (FL2). The investigation was conducted three times, and GraphPad Prism software was used for the flow cytometric analyses.

2.3.6. Effect of the plant extract on GSH and GSSG levels in cancer cells

Glutathione levels were determined by the method of Griffith (1980). The rate of the formation of yellow-colored 2-nitro-5-thiobenzzoic acid was measured using a standard curve and a spectrophotometer. After 72 h of incubation, human cancer cells with plant extract were lysed in a PBS-based inhibitor cocktail (Wei et al., 2022). Then, in a cold solution, a sonicator was used to evaluate the treated and untreated cells. The levels of GSSG and GSH were then measured in the resultant cell lysate. A stock buffer made up of 6 mM of sodium-EDTA and 143 mM of sodium phosphate was prepared to prepare different solutions of 0.1, 0.3, and 6 mg of NADPH, DTNB, and 50 units of GSH reductase, respec-

tively. A reaction mixture consisting of 700 µl of NADPH solution, 100 µl of DTNB, and 100 µl of serial concentrations of GSH or cell lysate was incubated for 10 min at 30 °C, before being transferred to a cuvette containing 10 µl of the GSH reductase, and the rate of absorbance at 412 nm was measured at 0 and 5 min (ΔA). For GSSG, the cell was mixed with L-vinyl-pyridine at room temperature for 2 h, then GSSG was converted to Glutathione by the reductase buffer. The standard curves for GSH and GSG were then made by serially varying the concentrations of 5-sulfonic acid. The cellular GSH and GSSG were determined using the following equations: GSH (nmol/ml) = [(ΔA_S-ΔA_B) - I]/S, GSSG (nmol/ml) = [(ΔA_S- ΔA_B) - I]/S (where: ΔA_S: The mean of change in absorbances per minute of cell lysate. ΔA_B: The mean of change in absorbances per minute of the blank. I: The intercept of the standard curve. S: The slope of the standard curve). The cellular Eh values were calculated using the Nernst equation (in mV) for the respective GSH/GSSG pools: Eh = E_o + R T F log ([GSSG]/[GSH]^2) (Kirlin et al., 1999) (where E_o: The redox potential at standard conditions (−264). R T F: R is the gas constant, T is the temperature in Kelvin and F is the Faraday constant (30).

2.3.7. Gene expression assay

2.3.7.1. RNA extraction and cell culturing. The MCF-7 breast cancer cell line was used to assess the expression patterns of the genes associated with the *E. alata* extract. The extract was selected due to its antiproliferative properties, which were found to be the most potent against breast cancer cells. The cells were then cultured in RPMI-1640 and were maintained as adherent cultures at 37oC and 5 CO2.

After seeding cells in 25 cm2 T-flasks, they were treated with a plant extract and a Fluorouracil (5-FU) drug to improve their efficiency. They were then exposed to growth media at 37 °C for 72 h. untreated cells exhibited negative control and were exposed to growth media. After incubation, the treated and untreated cells were isolated using the PureLinkTMARN Mini Kit, which contains all the necessary equipment for isolating RNA.

2.3.7.2. Reverse transcription-quantitative PCR. RNA samples were reverse-transcribed using MultiScribe™ Reverse Transcriptase (ThermoFisher) with oligo-dT primers. The created complementary DNAs were diluted in distilled water (1:3), and 2 µl of the diluted cDNA samples were added to a real-time PCR reaction mixture that contained 10 µl of 2X SYBR Green PCR Master Mix (ThermoFisher) and 1 µl of particular primer pairs in final volumes of 20 µl. ThermoScientific's PikoRealTM 96 Real-Time PCR System was used to run the samples through 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. All amplifications were conducted in triplicate. The sequences of the primers used in this study were as follows:

BAX (151 bps) (F: CAAACTGGTGCTCAAGGCCC, R: GGGCGTCCCAAAGTAGGAGA); CDKN1 A (151 bps) (F: TACCCTTGTGCCTCGCTCAG, R: GGCGGATTAGGGCTTCCTCT); E2F4 (151 bps) (F: GCATCCAGTGGAAGGGTGTG, R: ACGTTCCGGATGCTCTGCT); RB1 (151 bps) (F: GACCCAGAAGCCATTGAAATCT, R: GGTGTGCTGGAAAAGGGTCC); TP53 (151 bps) (F: GCGTGTTTGTGCCTGTCCTG, R: TGGTTTCTTCTTTGGCTGGG)] **(**Amatori et al., 2017**)**, KRAS1 (157 bps) (F: ACTGAATATAAACTTGTGGTAGTTGGACCT, R: TCAAAGAATGGTCCTGGACC**)(** Roa et al., 2004**).** The housekeeping gene is β-Actin (F: AAGCAGGAGTATGACGAGTCCG; R: GCCTTCATACATCTCAAGTTGG). **(**Jiang et al., 2009**).**

Using the comparative Ct technique, the relative expression of target genes was determined (threshold cycle number at the cross-point between amplification plot and threshold) devised by Livak and Schmittgen (2001). Each target gene's Ct values were adjusted to be equal to those of the housekeeping gene, B-actin, and the ratio of the reference gene to the target gene was determined using the formula: Ct = Ct (target gene) Ct (B-actin). The relative expression levels were determined using the method Ct = Ct (treated sample) Ct (untreated sample). As a result, the number of examined genes was expressed as n-fold changes in comparison to the calibrator sample, as follows: $2^{-\Delta\Delta CT}$.

The data collected during the process were analyzed using Bio-Rad CFX Manager software, which was provided by the instrument manufacturer. The melting curves analysis was performed to verify the amplicon's specificity.

2.4. Statistical analysis

Graph Pad Prism 7 was used to perform a multiple comparisons one-way analysis of variance (ANOVA) and present the results as the mean standard error of the mean (SEM) (GraphPad Software Inc., San Diego, USA). The results of calculating the p-value show that: p 0.05, the difference is not significant (ns); 0.01p 0.05, the difference is significant (*); and 0.001p 0.01, the difference is extremely significant, the difference is very significant (**); 0.0001 < p < 0.001, the difference is extremely significant (***).

3. Results

The ethanolic extraction by maceration of the aerial part of *E. alata* showed a yield of 7.78%. Despite it seems that this yield is low, it is hard to compare these results with those from the literature because the yield is only relative and depends on the procedure and environmental factors used for the extraction.

3.1. In vitro antioxidant activity

3.1.1. Free radical scavenging effect and reducing power activity

The DPPH, hydroxyl radical, and superoxide anion scavenging effects of EAEE were determined by calculating the concentration that inhibits the reaction by 50 % (IC_{50} in µg/ml) (Fig. 1**A**). These results showed that *E. alata* significantly exhibited the strongest capacity to scavenge the three free radicals compared to Vit C ($p < 0.05$) with IC_{50} values correspond to 37.4 ± 1.85, 15.62 ± 1.2 and 10.02 ± 0.05 µg/ml for DPPH, superoxide anion and hydroxyl radical respectively. The reducing effect of the extract (its ability for reduction Fe^{+3} to Fe^{+2}) was determined ording to the method described by Oyaizu, the absorbances were measured at 700 nm and the outcomes were expressed as the effective absorption that reduced 50% of Fe^{3+} into Fe^{2+} (EC_{50} in µg/ml). Fig. 1**B** has shown no significant difference in reducing power activity between EAEE and Vit C ($p > 0.05$). Moreover, the total antioxidant capacity of EAEE was evaluated according to the phosphomolybdenum method in which phosphate/Mo (V) compounds were generated subsequently. TAC of the extract was found to be 14.8 ± 1.3 µg ascorbic acid equivalent/ml of extract.

3.2. In vitro anticancer efficacy

A cytotoxic effect of the studied extract was calculated against human normal lung fibroblast cells (Wi-38) by calculating the extract concentrations (EC_{50} and EC_{100}) at 50% and 100% of cell viability using the colorimetric MTT assay. Fig. 2**A** illustrates the EC_{50} and EC_{100} of the extract (1861.1 ± 6.15 and 922.27 ± 11.7 µg/ml, respectively). This result indicates that the EAEE is considered safe for normal human cells since the low cytotoxicity of the studied extract is indicated by EC_{50}. The EC_{100} concentration will be useful as the maximum concentration in the dose–response curve for the next anticancer experiments. On the other hand, the data obtained from Fig. 2**B** revealed that *E. alata* extract exhibited significant growth inhibition of all cancer cells. A low IC_{50} value refers to a

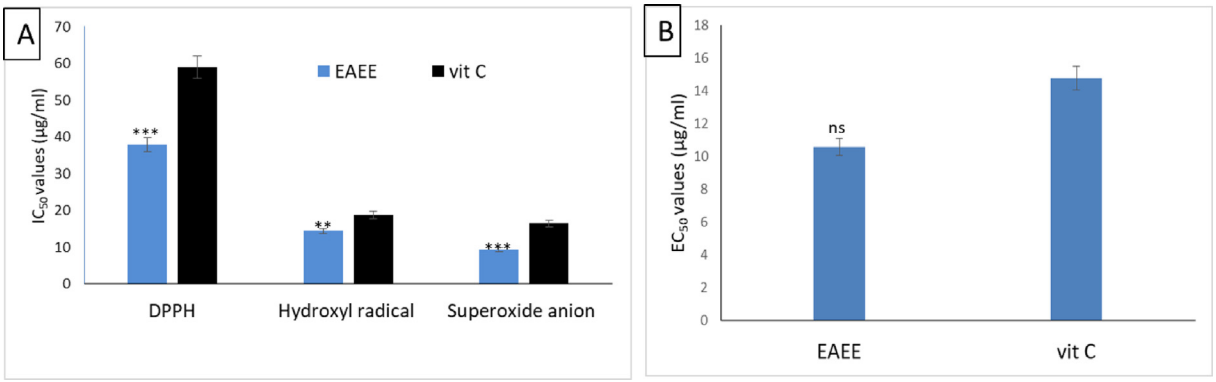

Fig. 1. Free radical scavenging effect and reducing power activity of *E. alata* extract. (A) IC_{50} values of free radicals scavenging activities of EAEE and vit C (µg/ml) and (B) EC_{50} value of reducing power activity of EAEE and vit C (µg/ml). Values are expressed as mean ± SEM. Comparison between EAEE and vitamin C was carried out using two-way ANOVA followed by Sidak's multiple comparisons test for scavenging activity and *t*-test of Student for reducing power. There was no significant (ns) difference between EAEE and Vit C reducing powers (p-value = 0.4192).**p < 0.01, ***p < 0.001.

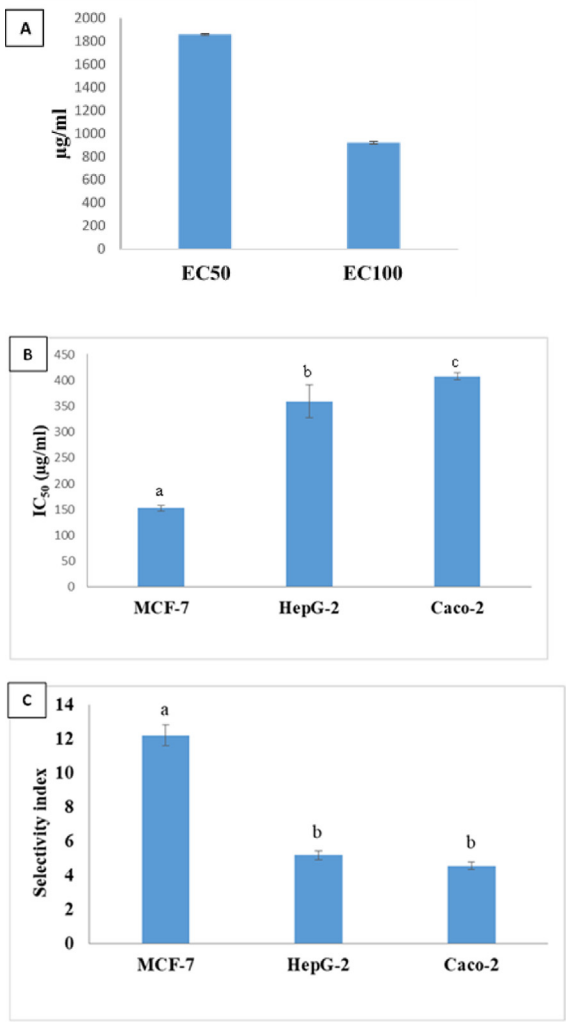

Fig. 2. Cytotoxicity effect of the *E. alata* extract on human normal and cancer cells. (A) EC_{50} and EC_{100} (µg/ml) of plant extract towards the human normal cell line (Wi-38) and (B) their IC_{50} (µg/ml) values against three human cancer cell lines. (C) Selectivity index (SI) of the extract against cancer cells. All values are expressed as mean ± SEM. Comparison between MCF-7, HepG-2 and Caco2 was carried out using one-way ANOVA followed by Tukey's multiple comparisons test. Different letters in the graphic indicate significant differences at p < 0.05.

high anticancer activity of the extract. More importantly, IC_{50} values of extract for human cancer cell lines were lower than its safe dose (EC_{100}) and MCF-7 was the most sensitive cancer cells to the extract, with the lowest IC_{50} < 153 µg/ml, followed by HepG-2 (IC_{50} = 359.43 µg/ml) and Caco-2 (IC_{50} = 407.26 µg/ml) (Fig. 2**B**). Additionally, selectivity indexes (SI) of the *E. alata* extract were evaluated by the ratio of its cytotoxic activity (EC_{50}) on normal cells (Wi-38) concerning the cancer cells (EC_{50}). As presented in Fig. 2**C**, plant extract showed highly selective activity towards the three investigated HepG-2, MCF-7, and Caco-2 cancer cell lines with SI values of 12.20, 5.17, and 4.56 respectively. Noted that the high degree of antiproliferative selectivity of the extract was against breast cancer cell lines.

3.2.1. Cellular morphological changes

The selected cancer cell lines were given IC_{50} doses for 72 h. After the treatment, the examined cells exhibited morphological alterations that were significantly different from the untreated cells. The plant extract had significant impacts on the cell line of breast cancer. This suggests that the extract has a powerful anticancer effect (Fig. 3).

3.2.2. In vitro analysis of apoptotic activity

In the current study, flow cytometry analysis was performed to ascertain the type of cell death induced by *E. alata* extract using a double staining method with annexin V-biotin and propidium iodide. Fig. 4 presents the proportions of living, dying, and dead tissue cells (**A**, **B,** and **C**) and the percentage of total apoptosis in cancer cells (**D**). These results reveal that *E. alata* extract caused death for all human cancer cells mainly through apoptosis. The highest significant apoptosis percentage (77.15%) was noted in MCF-7 followed by HepG-2 and Caco-2 cell lines (73.4% and 51.66%, respectively). This confirmed that the most sensitive cancer cell line for treatment with *Ephedra alata* is breast cancer cells.

3.2.3. Determination of cellular E_h for GSH/GSSG couple in cancer cells

The quantification of Glutathione (GSH) and its disulfide form (GSSG) was performed using one of the most common methods; the enzymatic recycling procedure and the DTNB photometric assay, based on Ellman's reaction. The contents of cellular GSSG and GSH were determined from the GSH and GSSG normal curves presented in Fig. 5**A** and Fig. 5**B** respectively. The obtained concentrations of GSH and GSSG were used to calculate cellular redox potential (E_{hc}) using the Nernst equation as outlined in materials

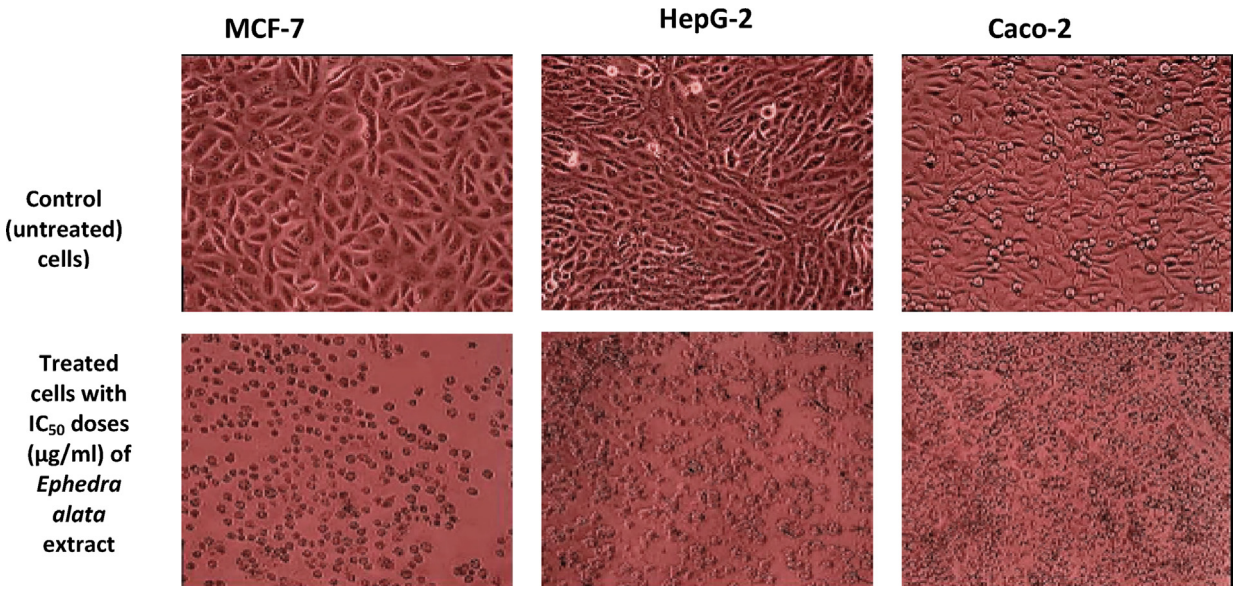

Fig. 3. Morphological alterations of cancer cell lines treated with IC_{50} of the plant extract. Representative photomicrograph shows morphological alterations of selected cancer cells (MCF-7, HepG-2, and Caco-2). Cells were treated with an IC_{50} dose of plant extract for 72 h and imaged by an inverted phase contrast microscope (magnification 200x).

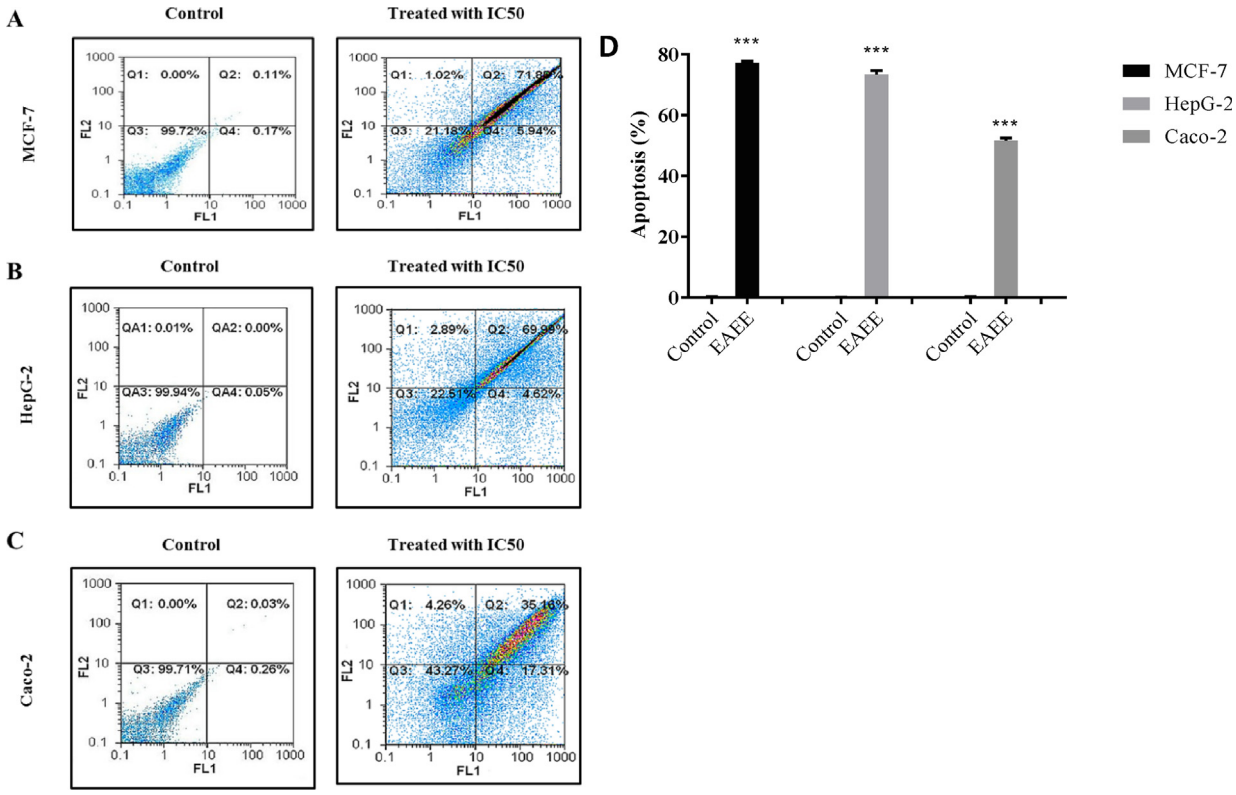

Fig. 4. Apoptotic effect of the plant extract on human cancer cells. (A), (B) and (C): PI/Annexin V double staining of untreated and treated MCF-7 (A), HepG-2 (B), and Caco (C) cells with IC_{50} concentrations for 72 h. The percentage of apoptotic cells was evaluated using flow cytometry. FL1: FITC signal detector for annexin-stained apoptotic cells. FL2: phycoerythrin emission signal detector for PI-stained necrotic cells. For each panel: the upper left quadrant (Q1) represents necrosis (Annexin -/PI +), and the lower left quadrant (Q3) represents viable cells. The lower right quadrant (Q4) shows early apoptosis. The upper right quadrant (Q2) shows late apoptosis (Annexin+/PI +). (**D**) Total percentage of apoptosis in cancer cells. Values are expressed as mean ± SEM. Comparison between the control and treated cells was carried out using two-way ANOVA followed by Sidak's multiple comparisons test. ***$p < 0.001$.

and methods. The outcomes presented in Fig. 5**C** indicated that E_{hc} in cancer cell lines treated with the plant extract has the most negative values compared to those of untreated cells, particularly in breast cancer cell lines. This indicates that the extract caused a substantial alteration ($p < 0.001$) in reduction potential of + 63 mV in MCF-7 cells (e.g. E_{hc} decreased from −259.19 ± 1.5 to −322.79 ± 1.1 mV) followed by a change of + 48 mV in HepG-2 and of + 40 mV in Caco-2 cells.

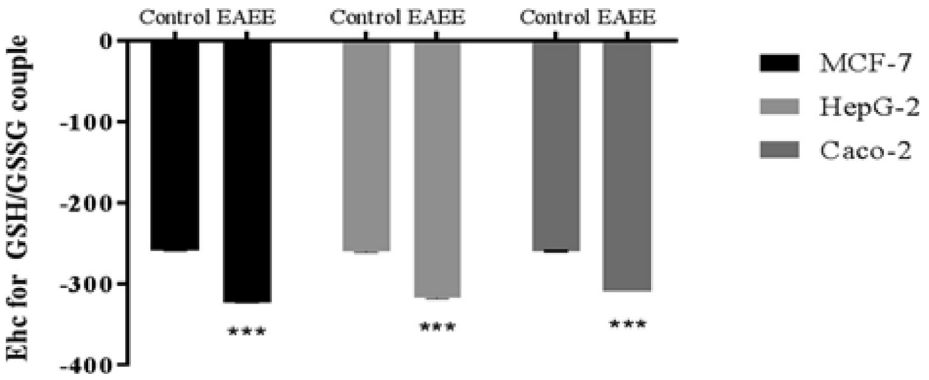

Fig. 5. E_{hc} in cancer cells treated with the plant extract. All values are expressed as mean ± SEM. Comparison between EAEE and the control was carried out using two-way ANOVA followed by Sidak's multiple comparisons test. ***p < 0.001.

3.2.4. Determination of gene expression potential as the mechanism of action

In this study, we have investigated the changes in the IC_{50} concentration-treated MCF-7 cells' gene expression profile of *E. alata* extract and Fluorouracil drug to demonstrate the probable apoptotic mechanism by which the extract kills breast cancer cells. The extracted RNA from all the samples showed high quality and purity (p = 1.80). The gene expression study was done on eight genes, BAX, BCL2, E2F, Cdkn1, RB1, TP53, NF-kB, and KRAS1 using qRT-PCR analysis. The delta-delta Ct technique was used to determine the relative mRNA expression levels of the target genes in terms of fold induction to the untreated cellular population (control). The results were showed in Fig. 6**.** The results exhibited a significant fold rise (p < 0.05) in the expression levels of four genes, Bax, p21, RB1, and TP53 in cells treated either with EAEE or S-FU. Moreover, it was observed that *E. alata* extract induced fold expression changes in these genes better than the S-FU drug. On the other hand, we registered a weak expression of the other genes, BCL2, E2F, and KARS1 in treated MCF-7 cells with either the extract or the drug. As long as the four up-regulated genes are connected to programmed cell death and cell cycle arrest, it may be noted that the plant extract has an important capacity to cause cell death in breast cancer cells especially in a P53- based approach through the mitochondrial pathway and this confirms the results obtained above by the flow cytometry.

4. Discussion

The importance of assessing the antioxidant activity of various raw materials is becoming more apparent due to their functional and health-promoting properties. This process involves identifying the presence of scavengers of free radicals, like as those found in fruits and vegetables (Al-Rimawi et al., 2017). The antioxidant activity in this study of *E. alata* extract was investigated over the subject of *in vitro* assays using DPPH, hydroxyl, and superoxide anion radicals. The test of DPPH is a very common, reliable, and quick measure to assess the *in vitro* antioxidant properties of plant extracts (Mosquera et al., 2007**)**. Results from the DPPH assay and according to Phongpaichit et al. (2007) the IC_{50} value for EAEE varies from 10 to 50 µg/ml (37.4 ± 1.85), indicating that it has significant antioxidant activity. According to statistical analysis, EAEE's IC_{50} value was likewise much higher than that of the positive control. This result is better than that reported by Al-Rimawi et al. (2017) in their study on the same species. The IC_{50} was found to be 78 µg/ml. On the other hand, our result is lower than that mentioned by Noui et al. (2022) in their research on the aqueous-methanolic extract of the same plant, who found that IC_{50} was 12.25 ± 1.09 µg/ml. In a recent study on *E. alata* seeds extract performed by Mufti et al. (2022), their results indicated a remarkable scavenging capacity against DPPH with an IC_{50} of 0.72 ± 0.03 mg/ml.

The reactive oxygen species (ROS) known as hydroxyl radical is capable of causing various types of damage and lipid peroxidation. Its high reactivity allows it to interact with biomolecules in living cells (Mayakrishnan et al., 2013). In this study, researchers looked at the quenching action of each of the Hydroxyl radicals and superoxide anions belonging to *E. alata* extract. The results, expressed as IC_{50} (µg/mL), and presented in Fig. 2**A**, indicated that $OH^{\bullet}$ and O_2 scavenging activities of EAEE (15.62 ± 1.2 and 10.02 ± 0.05 in that order) were noticeably lower (p < 0.05) than those of positive control (18.78 ± 0.13 and 17.4 ± 0.07 respectively). This strongly inhibitory the impact of plant extract on the·OH, and O_2 generation may be explained by phenolic chemicals present in the extract as reported in many studies. Reducing power estimation is considered a very important parameter to evaluate antioxidant activity (Maity et al., 2021). In general, compounds with a reducing effect behave as antioxidants that offer electrons and can turn down the oxidized intermediates of lipid peroxidation (Angelova et al., 2021). In the current research and based on the results displayed in Fig. 2**B**, the *E. alata* extract have a strong reducing capacity (EC_{50} = 10.81 ± 7.7 µg/ml) that presents no significant difference compared to ascorbic acid (p > 0.05). According to many previous studies, this can be explained by the inclusion of flavonoids and polyphenols in the plant extract (Sarfaraz et al., 2021). Phenolic compounds can scavenge free radicals and inhibit lipid peroxidation due to their reducing effect, which is why called antioxidants (Pisoschi et al., 2021). The last experiment to estimate the *in vitro* anti-oxidant performance of the tested extract was the total antioxidant activity. This test was handled in compliance with the known phosphomolybdenum process and TAC was 14.8 ± 1.3 µg ascorbic acid equivalent/ml of extract. Considering all these results, *E. alata* ethanolic extract exhibited strongly *in vitro* antioxidant activity. As regards its composition in phenolics compounds we can point out that these latter most probably act as antioxidants and reducing actors which transform ROS into stable composites. Until now, cancer treatment has been below examination. Only radiation and chemotherapy be able to considered effective and lethal. In traditional medicine, herbs are used for many forms of cancer therapy. Their discovery as anticancer drugs is also progressing (Hani et al., 2021). For that, EAEE was evaluated for antiproliferative activity as a candidate anticancer agent. According to the results obtained above, EAEE was safe toward human normal cells (Wi-38) (EC100 = 922.27 ± 11.7 µg/ml) and exhibited good antiproliferative activity specifically for MCF-7 breast cancer cells, when compared to the three examined cell lines.

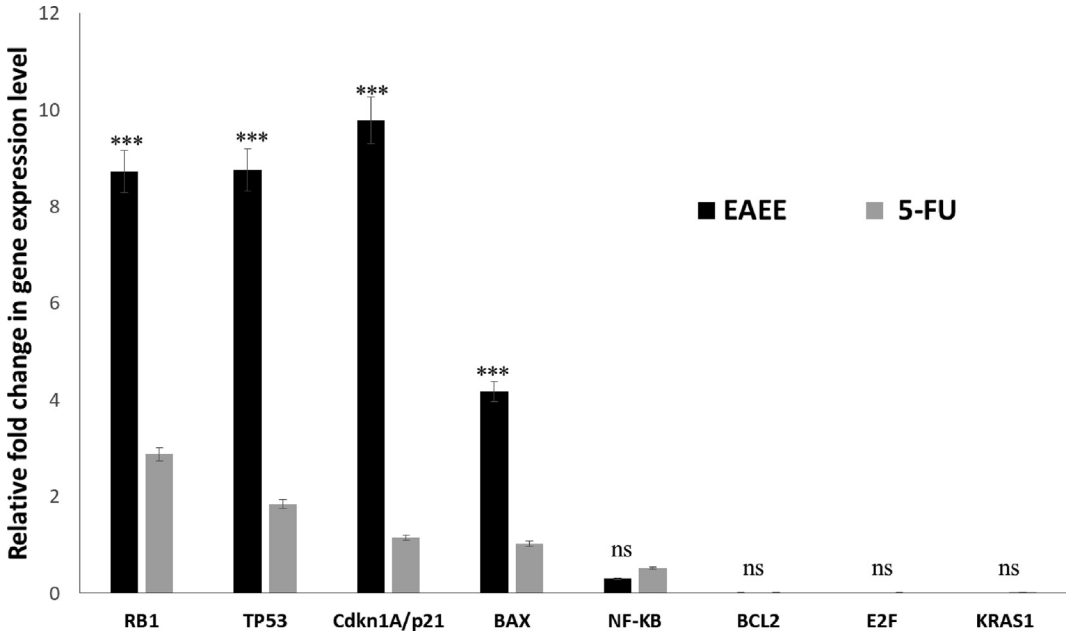

Fig. 6. Fold change in expression of mARN of BAX, E2F, Cdkn1A, KRAS1, RB1, TP53, BCL2, and NF-KB genes in MCF-7 cell lines treated with the extract or the Fluorouracil drug. Two-way ANOVA followed by Sidak's multiple comparisons test was carried out to compare the expression of different genes in MCF-7 cells treated with EAEE and S-FU.

Rare studies have investigated the cytotoxicity effect of *E. alata* from different countries against various cancer cell lines and no one has examined the Algerian species. Kmail et al, (2017) studied the cytotoxic and cytostatic effects (at 24 h and 72 h of cultivation, respectively) of *Ephedra alata* hydroethanolic extract (0–1000 µg/ml) in macrophages derived from human THP-1 in mono- and co-cultures and Hepg2 cells. Cytotoxic activity was observed at containing large amounts than 500 µg/ml. An IC_{50} of 380 µg/ml indicates that the cytostatic impact was stronger in co-cultures. **Corina et al (2018)** examined the antiproliferative activity of the aerial section of *Ephedra alata* Decne's hydroalcoholic extract from the southern part of Djerba (Tunisia) against MCF-7 cells. They found a max growth inhibition with a percentage of 56.45 ± 3.9 for 30 g/ml was the greatest concentration measured. The study directed by Sioud et al (2020) on the use of the *E. alata* methanolic extract revealed that it can induce an anticancer effect against 4 T1 mouse mammary carcinoma cells. It also exhibited a synergic antiproliferative effect when compared to cisplatin. A recent study executed by the same author showed that 4 T1 cell migration was dramatically suppressed at low doses of *E. alata* extract when breast cancer cells were exposed to it. This inhibition was dosage and time-dependent (Sioud et al., 2022). In addition hand, studies on the anticancer properties of specific *Ephedra* species against various cell lines have been conducted. For example, *Ephedra chilensis* K Presl extracts obtained with different solvents have displayed potent antiproliferative activity against MCF-7 breast cancer, HT-29 colon cancer, PC-3, and DU-145 prostate cancer. The *E. chilensis* extracts have shown a higher effect for the PC-3 and MCF-7 cell lines (IC_{50} < 1.0 µg/ml) (Mellado et al., 2019**)**. Another recent study conducted on various extracts of Jordanian *E. aphylla* showed a very weak cytotoxic potential against Vero normal cell line and potent antiproliferative activity against MFC7, and T47D cell lines (Al-Awaida et al., 2018). In another research, the fruit juice and leaf ethanol extracts of *E. foeminea* have shown a reduction in cell viability of MDA-MB-231 breast cancer cells (Mendelovich et al., 2017). The anticancer activity is potentially assigned to the availability of phenolic content in plant extracts since they are well-known as puissant antioxidants and as very much researchers have reported (Thanaset et al., 2014). Selectivity is considered the main problem of compounds in the treatment of cancers. Therefore, the selectivity index (SI) is an important measure to identify compounds with good antiproliferative activity (Iliev et al., 2019). It determines the selective inhibitory activity of the plant extract. High SI values (>2) of a substance present selective toxicity against cancer cells. An SI value of less than two indicates that the substance is toxic even for normal cells (Rodríguez-Juan et al., 2021). According to the literature, EAEE seems to be highly selective towards the three cancer cells and promising for more cancer studies since the SI values were greater than three (especially for MCF-7, SI = 12.02) (Mahavorasirikul et al., 2010). Analyses of EAEE-treated cells by inverted-phase contrast microscopy were the first indication that observed morphological changes characteristic of cell death were induced by plant extract. The results of the double staining method with annexin V-biotin and propidium iodide showed that the mechanism by which the EAEE kills the cancer cells seems to be mainly by apoptosis, especially for breast and liver cancer cells. Nonapoptotic cell deaths could also be involved in the EAEE-induced growth inhibition in these cells. Almost all of the experiments in our study showed that *E.alata* extract might contain bioactive compounds inhibiting increasing numbers of cancer cell lines by the induction of apoptosis. ROS are essential factors for cancer induction and progression, where they are connected to the development of cancer (tumour survival, cell transformation, progression, angiogenesis, and metastasis) (Sarmiento-Salinas et al., 2021). Thus scavenging these radicals may lead to depriving cancer cells of their free radicals are crucially required to continue unchecked proliferation (Waris and Ahsan, 2006). As a result, cancer cells are more vulnerable to being killed by radical scavengers than healthy cells. In short, suppressing the level of ROS than the demand for cancer cells can be halted cancer progression. For this reason, we have checked the effect of *E. alata* extract on GSH/GSSG levels via the determination of cellular redox potential (E_{hc}) in tested cancer cells, using one of the most popular techniques; the enzymatic recycling procedure and the DTNB photometric assay, based on the Ellman's reaction (Pastore et al., 2003). And that's because GSH is considered a very important antioxidant, since it directly detoxifies ROS, and assists other crucial antioxidants, such as Vitamins E and C in their functional ability (Droge et al., 1986**)**. In recent years, the redox environment of a cell is described by the term as it is called redox state, which first

has been used to describe the state of a particular redox pair. The results showed that the E_{hc} in cancer cell lines became more negative, especially for the MCF-7 cell line. These switches of E_{hc} to severely reduced pools may be explained by the effectiveness of radical scavenging of EAEE.

The anticancer mechanism of any compound occurs mainly through either apoptosis or necrosis. For that, the gene expression analysis was done on eight genes, BAX, E2F, cdkn1, KARS1, RB1, TP53, BCL2, and NF-kB to understand the possible molecular mechanism responsible for the programmed cell death in MCF-7 cells treated with EAEE. These genes are engaged in a number of pathways that lead to cancer development, cell cycle progression, cellular senescence, programmed cell death, DNA transcription, and cell survival. We mention that no previous studies have been directed to study the outcome of Algerian *E. alata* on a molecular level. In comparison to the untreated cellular population, changes in gene expression were assessed in terms of fold induction (control) by the $2^{-\Delta\Delta CT}$ method, using RT-qPCR (Tasinov et al., 2022). The purpose of this study was to look at how plant extract affected the expression of various genes in two different cell lines: one that was treated with the extract and the other that was treated with S-FU. The difference between the two groups' fold difference was then analyzed. The results in Fig. 6 indicated that the four genes, namely p21, p53, RB1, and Bax, were up-regulated in the cells that were given the extract treatment. The effects of plant extract on the regulation of certain genes were found to be more potent than those of an anticancer drug. This finding suggests that the use of plant extract could be a more effective strategy for treating cancer. It also showed that p53 expression had risen which suggests that EAEE could potentially affect the regulation of p53, a tumor suppressor gene. It is known that p53 is a gene that plays a role in the cell cycle arrest process and is capable of inducing programmed cell death (Wang, 2021). For example by increasing the expression of genes involved in cell death like Bax and inhibiting anti-apoptotic genes like BCL2, the most crucial proteins of the Bcl-2 family that maintain apoptosis' balance. Bax can reverse the anti-apoptotic activities of BCL2 Thus, the increase in BAX levels ensures the decrease in the Bcl-2/Bax ratio which predisposes cancer cells to apoptosis. This indicates that EAEE induced apoptosis through the mitochondrial pathways (Oren, 2003). In the current study, the plant extract also strongly influenced the expression regulation of the Rb protein. This tumor suppressor gene is known as a central molecule of the signaling pathway that regulates the cell cycle. The link between p53 and RB is presented by the fact that in a cell, p53 is maintained inactive by its association with its inhibitor Mdm2. This later is, in turn negatively regulated by the expression p19ARF induced by the action of E2F. Thus directly connecting the Rb/E2F pathway to p53 expression and cell-programmed death response (Pawge and Khatik, 2021). Furthermore, the family of cyclin-dependent kinase inhibitors (CKI) was affected by the plant extract as long as the p21 expression levels were significantly increased in breast cancer cells after treatment with EAEE. p21 is considered the main important protein of CKI and a negative regulator of the cell cycle maintaining genomic stability (Xiao et al., 2021). Usually, it is found deregulated in cancers that affect humans (Abbas and Dutta, 2009). p21 plays other key roles in DNA repair and apoptosis. It is required for G2/M transition and the progression of mitosis phases (Kuang et al., 2021).

5. Conclusion

The cytotoxic results of EAEE on human cancer cells are interesting. This is the first-ever report illuminating the anticancer activity of 70 % ethanolic extract of aerial parts of the Algerian *E. alata* and apoptosis is induced in the HepG2, Caco-2, and MCF-7 cell lines in a p53-dependent manner through the intrinsic pathway since it up-regulated in MCF-7 cells and the manifestation of four genes related to programmed cell death and cell cycle arrest. Consequently, *E. alata* could provide a potential source of new compounds which have pro-apoptotic effects to treat breast cancer. Due to the limited information about the potential activity of *E. alata*, further studies are needed to determine its molecular mechanism activity and the eventual effect of *E. alata* extract on the cancer cell.

6. Availability of data and material

All data generated or analyzed during this study are included in this published article.

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8. Credit authorship contribution statement

Moufida BENSAM conceived the study. **Safaa M. Ali** and **Moufida BENSAM** performed plant extraction and analyses of the genetic effect of the EAEE. **Marwa M. Abu-Serie** designed and performed an *in vitro* assessment of the EAEE biological activities. **Hocine RECHRECHE** and **Abeer E. Abdelwahab** supervise. All authors were involved in reviewing and editing the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval and consent to participate

No animal or human samples were used in the study.

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