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Antioxidant power and anticancer properties of *Ephedra major* Host. alcoholic extract and hydro-distilled essence

Poder antioxidante e propriedades anticâncer do extrato alcoólico e da essência hidrodestilada da *Ephedra major* Host.

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Abstract

In this study, ethanolic and methanolic extracts of *Ephedra major* were extracted by maceration method and essence was prepared by hydro-distilled method. DPPH test was used to assess the antioxidant activity and total phenolic content determined by Folin-Ciocalteu. Also, ethanolic extract and essence were applied by MTT method for cytotoxicity against A549 cancer cells and HDF as normal cells. The percentage of apoptosis was measured by flow-cytometry. Real-time PCR was used to determine the changes of BAX and p53 genes expression. According to results, the antioxidant activity (IC_{50}) of the ethanolic extract was lower while its total phenolic content was higher than methanolic one ($p \leq 0.001$). The lowest IC_{50} for cytotoxicity of ethanolic extract and essence determined as 700 $\mu g.ml^{-1}$ and 280 $\mu g.ml^{-1}$ at 48 hours respectively. The ethanolic extract and essence were not affected HDF proliferation significantly. The apoptosis assay showed that the use of ethanolic extract by IC_{50} concentration led to an increase in early and late apoptosis up to 22% and 29.3% respectively. Also, using the essence by that concentration increased early and late apoptosis up to 14.8% and 25.4%. Based on the results, the ethanolic extract and essence increased the expression of apoptosis-effective genes, p53 ($p \leq 0.001$) and BAX ($p \leq 0.01$) as compared with control. It can be concluded that higher amounts of phenolic compounds in ethanolic extract led to stronger antioxidant power of this extract in comparison with methanolic one. Therefore, the anti-cancer properties of the extract and essence of *Ephedra major* were raised from their antioxidant power.

Keywords: Alcoholic extraction; Antioxidant power; Apoptosis; Cell viability; *Ephedra major*; Essential oils.

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Resumo

Neste estudo, os extratos etanólicos e metanólicos de *Ephedra major* foram extraídos por maceração e a essência foi preparada por hidrodestilação. O teste DPPH foi utilizado para avaliar a atividade antioxidante e o conteúdo fenólico determinado pelo método Folin-Ciocalteu. A citotoxicidade do extrato etanólico e da essência foi avaliada sobre células tumorais A549 e células normais HDF pelo método de MTT. A porcentagem de apoptose foi medida por citometria de fluxo. PCR *real-time* foi utilizado para determinar as mudanças na expressão dos genes BAX e p53. Os resultados mostram que a atividade antioxidante (IC_{50}) do extrato etanólico foi mais baixa, enquanto que o seu conteúdo fenólico total foi mais alto do que no extrato metanólico ($p \leq 0.001$). O IC_{50} mais baixo para a citotoxicidade do extrato etanólico e da essência foi de 700 $\mu g.ml^{-1}$ e 280 $\mu g.ml^{-1}$ em 48 horas, respectivamente. O extrato etanólico e a essência não afetaram significantemente a proliferação das células HDF. O ensaio de apoptose demonstrou que o uso do extrato etanólico (IC_{50}) aumentou a apoptose precoce e a tardia em até 22% e 29,3%, respectivamente. Ainda, a essência (IC_{50}) também aumentou a apoptose precoce e a tardia em até 14,8% e 25,4%. A expressão dos genes p53 ($p \leq 0.001$) e BAX ($p \leq 0.01$) foi aumentada com o extrato etanólico e a essência, quando comparada ao controle. Concluímos que maiores quantidades de compostos fenólicos no extrato etanólico levaram a um maior poder antioxidante em comparação com o extrato metanólico. Desta forma, as propriedades anticâncer do extrato e da essência de *Ephedra major* devem-se ao seu efeito antioxidante.

Palavras-chave: Extração alcoólica; Poder antioxidante; Apoptose; Viabilidade celular; *Ephedra major*; Óleo essencial.

Introduction

Cancer is a condition that results from genetic mutations, which promote the uncontrollable division of cells, either locally or via metastasis.[1] It is considered one of the most important causes of death throughout the world.[2] Among all sorts of cancers, lung cancer is the most common and foremost cause of mortality.[3] Two main types of lung cancer include small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).[4] Worldwide, lung cancer is the most common malignancy in men and the fifth most common cancer in women. Late diagnosis and lack of efficient therapeutic intervention are the chief players that cause limitations in the treatment of this type of cancer.[5] Lung cancer is one of the five most common cancers in Iran. The incidence rate of lung cancer among Iranian men is lower than that of developed countries.[6]

Genome-wide analyses have shown that TP53 is the main risk gene in human cancer.[7] Most importantly, p53 is a transcription factor capable of controlling the expression of numerous coding and non-coding RNAs.[8-9] The p53 is able to protect normal cells from tumor genesis in response to various stimuli, such as oxidative stress, ribosomal dysfunction, nutrient deprivation, oncogene activation, DNA damage and hypoxia.[10-11] The tumor suppressor p53 acts as the main regulator of several major signaling pathways and cell-fate-decision pathways. Following the various stresses, such as DNA damage, oncogene activation, p53 undergoes post-translational modifications that lead to its activation, stabilization, and accumulation in the cell.[12] The tumor-suppressor activity of p53 is mainly related to its transcriptional regulation of genes, which are involved in many cellular processes, such as cell cycle arrest, apoptosis, senescence, DNA repair, and differentiation.[13-14] The p53 promotes apoptosis by inducing pro-apoptotic BCL-2 family members, which facilitates caspase activation and cell death.[15] Apoptosis is a highly regulated program of cell death that plays an important role in embryonic development, tissue homeostasis and the disease progression.[16] In response to various death stimuli, cells often start a mitochondrial-related apoptotic pathway which includes mitochondrial outer membrane permeabilization (MOMP), apoptosome formation, and activation of effector caspases.[17] Generally, the "point

of no return" of MOMP, irreversibly commits cells to apoptosis through the action of two similar effector proteins of the Bcl-2 family, Bax and Bak.[18] Many medicinal plants have been used for pharmacological and clinical applications, including antioxidative, anticarcinogenic, antiatherosclerosis, antimutagenic and antiangiogenesis.[19] These herbs have a wide variety of antioxidants, such as phenolics and nitrogen compounds, vitamins and terpenoids.[20] The Ephedra is a genus belonging to the Ephedra family, which includes totally 35 to 45 species and is found all over the world.[21] This genus was studied due to the high content of ephedrine alkaloids. However, secondary metabolites including alkaloids (amphetamine, imidazole, quinoline, pyrrolidine), flavonoids (flavonols, dihydroflavonol, flavonone, flavanols, flavones, anthocyanins), tannins (dimers, trimers and tetramers of proanthocyanidins), lignans, naphthalenes, esters, terpenides, phenolic acids and quinones, has been reported in different species of Ephedra genus.[22] Despite the previous phytochemical studies of *Ephedra major* Host. There is no study reported on its effect on cancer or on the antioxidant capacity of this species in Iran. Therefore, the aim of this work was to evaluate the antioxidant activity for different ephedra extracts, and to identify the cytotoxicity against cancer cells (A549) line which present the greatest antioxidant activity.

Material and Methods

Plant preparation

Ephedra plant (*Ephedra major*) was collected from Robat Karim in the south of Tehran province, which is located at 51° 10′ 30″ longitude and 35° 24′ 25″ latitude in spring 2022. The plant was air-dried at room temperature and milled to gain powdered material, then stored in fridge until extraction.

Extract preparation

The extraction was carried out by two solvents, including ethanol 85% and methanol 95% (Merck). To 30 grams of the prepared powder, aliquot 300 ml of each alcohol was added and kept in a shaker incubator (Innova 42) at 40°C for 72 hours. Then the extracts filtered by Wattman paper separately. The upper portions macerated again for second time according to the same procedure steps. Then the two obtained extracts of each alcohol were mixed. The extracts were evaporated at 40°C under vacuum in a rotary (model: Heidolph) separately. The crud extracts were weighed and stored in the refrigerator until used.

Preparation of the essential oils

The essential oil (EO) of fresh aerial parts of Ephedra were prepared by hydrodistillation of sterilized aerial parts using a Clevenger-type apparatus. The extraction was carried out for 180 min in 500 ml of water. After dehydrating the EO over anhydrous sodium sulfate, it was weighed and stored at 4°C until use.

HPLC determination of alkaloids

The characterization of alkaloid compounds in *E. major* extracts was performed using a high-performance liquid chromatography system (HPLC) (KNAUER Co., HPLC PUMP K-1001) equipped with reverse-phase C18 column, (Length × ID: 250 × 4.6 mm), (Zorbax Eclipse XDB C18). The diode array detector was set to a scanning range of 200-400 nm. 3 mL of sample was injected into HPLC system under flow rate of 1 $ml.min^{-1}$. The mobile phase is composed of two solvents: Phosphate buffer pH=3 (A), Acetonitrile (B): (94:6 v/v) for 20 min for detecting ephedrine and pseudoephedrine. All chromatographic conditions were performed according to the method described previously by Jdey *et al*.[23] Identification analysis of alkaloid compounds was done by comparison of their retention time with those of pure standards. For the quantitative analysis, a calibration curve was prepared for each identified alkaloid compound using the available standards at 280 nm. The amount of each compound was expressed as microgram per gram of residue (mg. g DW^{-1}).

GC/MS analysis

Identification of essential oils compounds were done by GC-MS analysis. GC-MS analysis is performed on an Agilent 7890A/5975C GC-MS system equipped with a BD-5 fused silica column (30m × 0.25mm i.d. film thickness 0.25 µm). The oven temperature is programmed as follows: the initial temperature of 60°C is immediately increased to 220°C at a rate of 3°C.min^{-1}. Subsequently the temperature is increased to 260°C at 20°C.min^{-1} and held at this temperature for 5 min. The injector and transfer line temperature are 260 and 280°C respectively, carrier gas is helium with a linear velocity of 30.6 cm.s^{-1}, split ratio 1:100, ionization energy 70 eV, scan time 1s, mass range 40-300 a.m.u.

Antioxidant activity

The antioxidant activity of the plant extracts against DPPH (Sigma) was determined using the method proposed by Farhan *et al*.[24] with slight modifications. Various concentrations of crude extract and vitamin C (Neutron) as positive control (10, 25, 50, 100, 200, 250 µg.ml^{-1}), were incubated with the prepared DPPH• for 30 min. One ml of each extract was added to 1 ml of DPPH reagent. The absorbance of each mixture was determined at 517 nm with spectrophotometer (Agilent, cary60). The control sample was prepared by mixing 1 ml DPPH with 1 ml of the selected solvent. The blanks were composed of selected solvents. Antioxidant activity was calculated according to formula presented below, in sequence.

$$\text{Scavenging activity (\%)} = \left(\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}}\right) \times 100$$

The antioxidant power of extracts was reported as IC_{50}.

Total phenolics content

Total phenolics were determined using Folin-Ciocalteau (Merck) reagents according to Singleton and Rossi.[25] Aliquot 100µl of Ephedra plant extracts were mixed with 1.7 ml of Folin-Ciocalteu reagent and allowed to stand at room temperature for 5 min, then 1.2 ml of sodium bicarbonate (Neutron) (7.5%, w/v) was added. The absorbance of the samples was measured at 765 nm after about 60 minutes at room temperature. Aqueous solutions with known concentrations of gallic acid (Sigma Aldrich) in the range of 10 to 500 µg/ml were used for the calibration curve. Results were expressed as mg gallic acid equivalents per gram dry weight (GAE. g DW^{-1}).

Cell culture

The human lung cancer A549 and HDF (human dermal fibroblasts) cell lines were obtained from the genetic resources center of Iran. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Bioidea) supplemented with 10% fetal bovine serum (Bioidea), 1% antibiotics, 1% Non-essential amino acids and 5% CO_2 at 37°C in an incubator (Salvis Lab, Biocenter). In this experiment, HDF cell line was used as control.

MTT assay for cell viability

The effects of Ephedra extract and essence on the A549 and HDF cells viability were determined using an MTT assay. Briefly, in logarithmic growth phase, the cells were digested in 0.1% trypsin and DMEM supplemented with 10% FBS, and the cells were plated in 96-well plates at a final density of 1×10^4 cells.well^{-1}. The cells were treated with a series of diluted concentrations of Ephedra extract (0, 31.25, 62.5, 125, 250, 500, 750, and 1000 µg.ml^{-1}) and essence (0, 5, 25, 50, 100, 200, 300 and 400 µg.ml^{-1}) and incubated at 37°C for 24, 48 and 72 hours. Following incubation, MTT (10 mg.ml^{-1}) solution was added to each well and incubated for 5 hours at 37°C. Then, the supernatant

in each well was separated, and 100 µl dimethylsulfoxide (Neutron) was added. The optical density (OD) at 570 nm was measured using a microplate reader (Biotek, Epoch). Cell viability was calculated as the percentage of viable cells by OD value. The results were reported as IC_{50} concentrations at 24, 48 and 72 hours.

Apoptosis assay

Based on the results obtained from the MTT test, ephedra extract and essence had no significant effects on HDF cells; therefore, this test was not performed on this cell line. The A549 cells were cultured by 10^5 cells.ml^{-1} per well in six well plates at 37°C in a humidified atmosphere containing 5% CO_2. Then, cells were treated with Ephedra extract and essence by IC_{50} doses of cytotoxicity experiment (700 µg.ml^{-1} and 280 µg.ml^{-1} respectively) at 48 hours. The A549 cells were washed twice with ice-cold phosphate-buffered saline (PBS; Bioidea) and re-suspended in 100µl of binding buffer. In order to labeling cells, aliquot 5 µl (5µg.ml^{-1}) of Annexin V-FITC and propidium iodine (PI) were added to the A549 cells and incubated for 15 min in the dark at 25°C. Then, 400µl of binding buffer was added to the mixture samples, and the mechanism of cancer cells death was determined with a flow cytometer.

Total RNA isolation and determining of P53 and BAX expression

A549 cells (5×10^6) were cultured in 24-well plates and treated with 100 µl PBS and 100 µl of *Ephedra major* extract and *Ephedra major* essence by IC_{50} concentration for cytotoxicity experiment at 48 hours (700 µg.ml^{-1} and 280 µg.ml^{-1} respectively based on results obtained from MTT assay). Total RNA was isolated from cell pellets using RiboEx kit (GeneAll, Korea) according to the manufacturer's protocol. The concentration and quantity of total RNA was calculated based upon the OD value 260/280 ratio. The p53 and BAX expressions were examined using the reverse transcriptase-polymerase chain reaction (RT-PCR) method. Total RNA was reversely transcribed into singlestrand cDNA using oligo (dT) as a primer reverse transcriptase (according to easy cDNA synthesis kit protocol, Pars-tous manufacturer's). After cDNA synthesis reaction, the cDNA was used for PCR. After denaturation for 3 min at 94°C, the total amount of reaction products was amplified for 30 cycles for P53 (94°C for 30 sec; 56°C for 30 sec; 72°C for 60 sec) and 35 cycles for BAX (94°C for 45 sec; 64°C for 60 sec; 72°C for 150 sec) on the thermal cycler system (Rotor-Gene6000, Qiagene). The complementary primer pairs used for RT-PCR were listed in Table 1. The GAPDH was used as a quantitative, internal control. Melt curve analysis was performed to confirm the amplification of target genes in the samples. The mRNA fold changes relative to GAPDH as an internal reference gene were calculated with the comparative threshold method. For this, gene expression changes between the untreated and treated cancer cells were calculated by the $2^{-(\Delta\Delta CT)}$ method, where ΔCT=CT (target gene) – CT (GAPDH); ΔΔCT=CT treated – CT untreated; Fold changes = $2^{-(\Delta\Delta CT)}$.

Table 1 - Primer pairs for amplification of P53, BAX and GAPDH.

Gene	Forward	Reverse	Primer length
GAPDH	CTCATTTCCTGGTATGACAACG	CTTCCTCTTGTGCTCTTGCT	122 bp
P53	CCAGCCACCTGAAGTCCAAA	AAACCCAAAATGGCAGGGGA	157 bp
BAX	TGCCTCAGGATGCGTCCACC	CCCCAGTTGAAGTTGCCGTC	175 bp

Source: the authors.

Statistical analysis

The data are presented as means ± SEM of at least 4 independent experiments. The graphs were drawn by Excel 2013 and GraphPad Prism-v10. The means comparison of data carried out by Tukey's test using SPSS-v16 and GraphPad Prism-v10. P values <0.05(*), <0.01(**), <0.001(***) were considered significant differences.

Softwares and companies

Excel 2013 (Microsoft), GraphPad Prism-v10 (Dotmatics), SPSS-v16 (IBM).

Results

GC-MS analysis

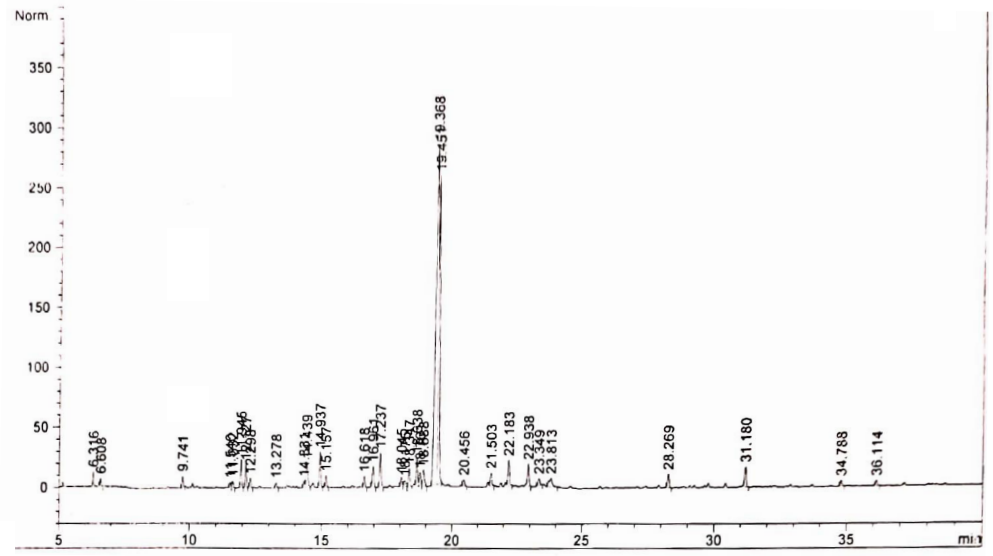
The chromatogram of essential oils composition of ephedra plant is shown in Figure 1. Also, the major compounds in the essential oil with their retention time and relative percentage are presented in Table 2. According to GC-MS results, α-terpineol (43.31%) and myrtenol (21.19%) are the main constituents of essence. Also, *cis*-β-terpineol (2.66%), linalool (2.51%) and terpinene-4-ol (2.02%) are other compounds that presented by high amounts in the essence.

Table 2 - Chemical composition of *Ephedra major* essential oils.

Entry	Retention time (min)	Compound	Area (%)	Kovats index (KI)
1	11.95	*p*-cymene	1.72	1020
2	12.13	Limonene	1.42	1032
3	12.30	1,8 cineol	0.59	1036
4	13.28	γ-terpinene	0.31	1063
5	14.44	*p*-mentha-2,4(8)-diene	2.08	1091
6	14.94	linalool	2.51	1100
7	15.16	nonanal	0.78	1107
8	16.62	1-terpineol	0.78	1135
9	16.96	*trans*-pinocarveol	1.68	1142
10	17.24	*cis*-β-terpineol	2.66	1147
11	18.04	*trans*-β-terpineol	0.71	1161
12	18.35	*cis*-chrysanthenol	1.27	1166
13	18.64	terpinene-4-ol	2.02	1171
14	18.89	*p*-cymene-8-ol	1.50	1175
15	19.37	α-terpineol	43.31	1190
16	19.45	myrtenol	21.19	1201
17	20.46	citronellol	0.52	1230
18	21.50	linallol acetate	0.99	1257
19	22.18	*p*-menth-l-en-7-ol	2.00	1271
20	22.94	thymol	1.71	1287
21	23.35	perilla alcohol	0.61	1296
22	23.81	carvacerol	1.35	1305
23	31.18	ar-curcumene	1.66	1485
24	34.79	3Z-hexenyl benzoate	0.53	1565
25	36.11	caryophyllene oxide	0.45	1585

Source: author's own work.

Figure 1 - Chromatogram of essential oils composition of *Ephedra major*.



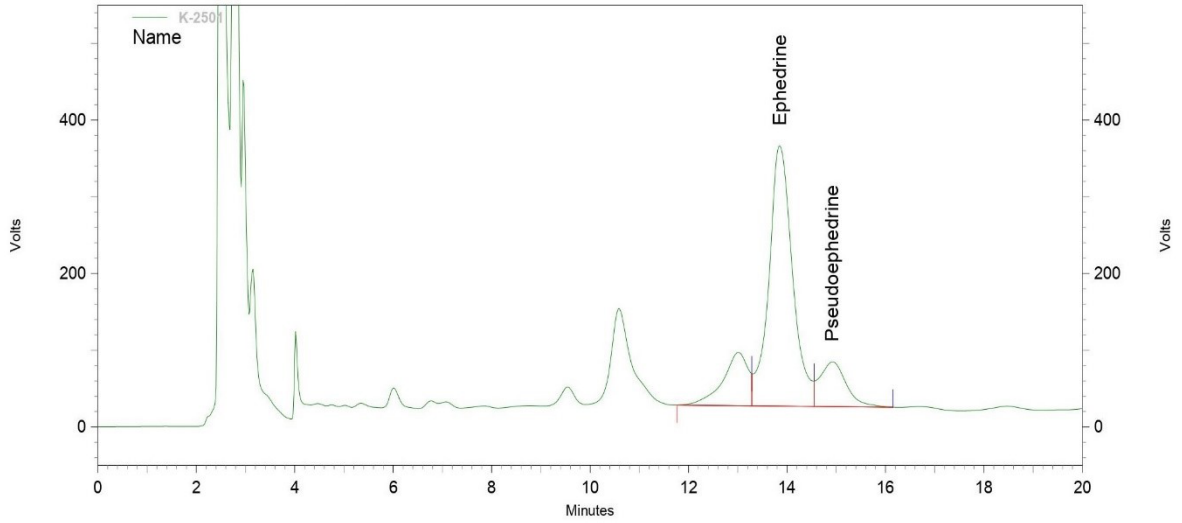
Source: author's own work.

According to Figure 1 the area of each peak and the relative content were calculated. The chemical components of the essence of fresh leaves of *E. major* were identified according to the MS data. The analytical result showed that 25 compounds were listed in Table 2, representing 94.35% of the identified total peak area, from the essence of *E. major*.

HPLC determination of alkaloids

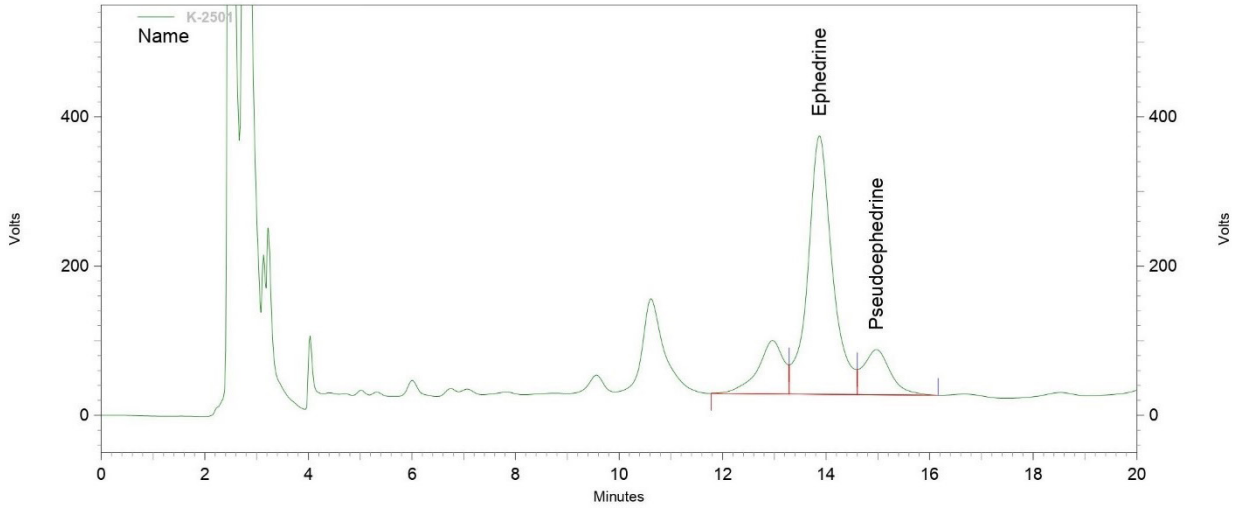
The chromatogram related to measuring the amount of ephedrine and pseudoephedrine in ethanolic and methanolic extracts were shown in the Figure 2 and Figure 3. The results showed that the amounts of ephedrine and pseudoephedrine in both ethanolic and methanolic extracts had no significantly differences. The calculated concentration of ephedrine for ethanolic and methanolic extracts were 276.25 ppm and 278.81 ppm respectively. The amounts of pseudoephedrine were 48.36 ppm and 48.87 ppm for ethanolic and methanolic extracts, respectively.

Figure 2 - Spectra of ephedrine and pseudoephedrine of *Ephedra major* ethanolic extract.



Source: author's own work.

Figure 3 - Spectra of ephedrine and pseudoephedrine of *Ephedra major* methanolic extract.

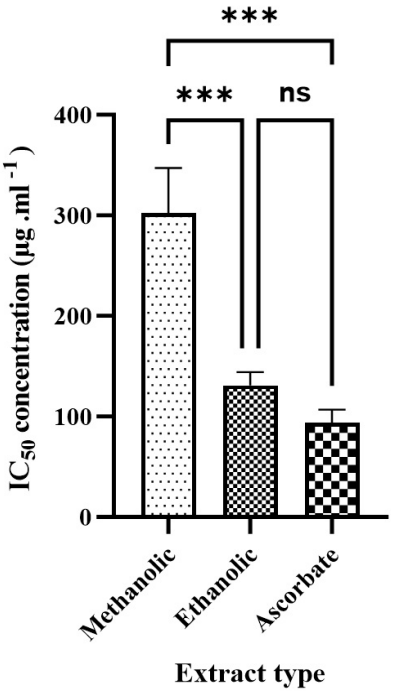


Source: author's own work.

Antioxidant activity

Antioxidant activity of crude extracts of *E. major* against DPPH free radicals, which was reported as IC_{50} is shown in Figure 4. According to results, ethanolic extract could scavenge 50% of DPPH radicals in a lower concentration than the methanolic extract. The comparison of the IC_{50} of ethanolic extract with ascorbic acid did not show any significant difference, which indicates the very high antioxidant power of this extract.

Figure 4 - Means comparison of antioxidant activities of ethanolic and methanolic extracts of *Ephedra major* against DPPH free radicals which reported as IC_{50}. Ascorbate was used as standard. Data are Mean ± SE (n=4). *** differed significantly at $p \leq 0.001$, ns, no significant differences.

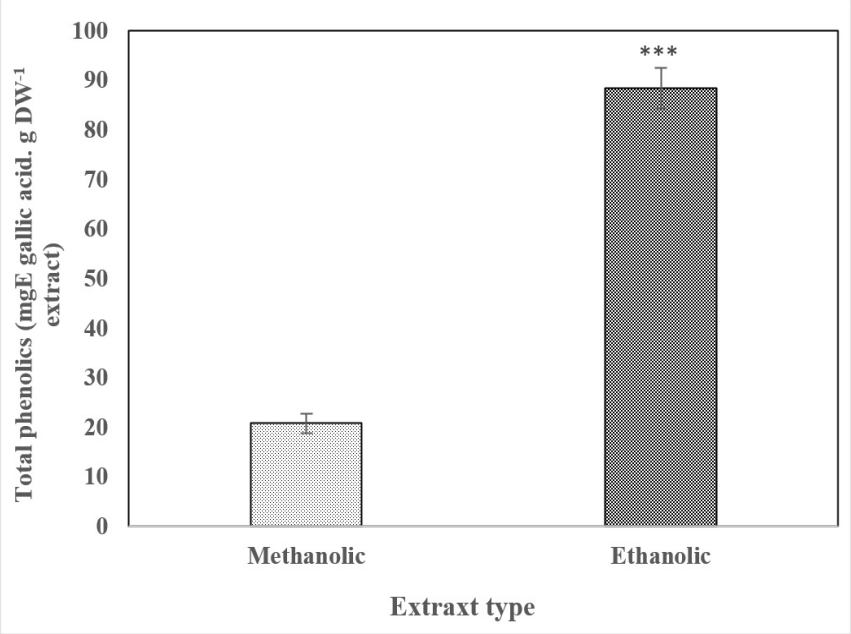


Source: author's own work.

Total phenolics content

The phenolic content as a scale of the antioxidant power of plant extract is shown in Figure 5. Based on the results, the phenolic content of the ethanolic extract was higher than the methanolic extract. The difference in the phenolic content of the extracts was statistically significant at $p \leq 0.001$. Since the ethanolic extract had higher phenolic content and antioxidant power than the methanolic extract, this extract was used in the subsequent experiments.

Figure 5 - Total phenolics content of *Ephedra major* ethanolic and methanolic extracts. Data are Mean ± SE (n=4). *** Show that the data differed significantly at $p \leq 0.001$.



Source: author's own work.

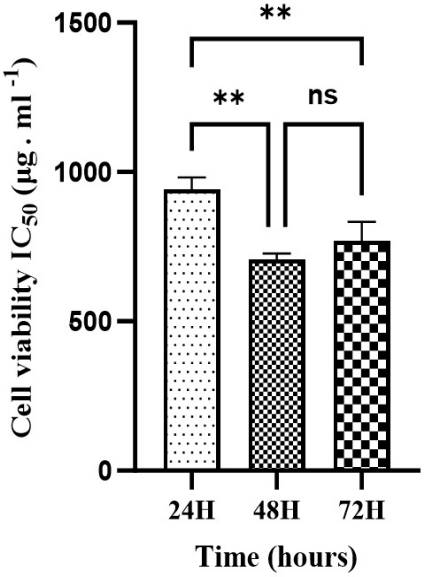
Cell viability

The IC_{50} of different concentrations of ephedra ethanolic extract (0, 31.25, 62.5, 125, 250, 500, 750 and 1000 µg.ml^{-1}) at 24, 48 and 72 hours on the viability rate of A549 cells is shown in the Figure 6. Also, the effects of the noted concentrations of ethanolic extract on HDF cells viability was shown in Figure 7. The results showed that ethanolic extract did not differ the viability of HDF cells significantly in comparison to control. The variance analysis of the data (ANOVA) of effects of ethanolic extract on HDF viability is shown in Table 3. Based on results, the viability of A549 cells decreased by the increase in the concentration of the extract (data were not shown). Accordingly, the effect of the duration of exposure of the cells to the extract was somewhat differed. In this way, the lowest IC_{50} of cell viability was observed at the time of 48 hours, which was determined to be 700 µg.ml^{-1}.

Similarly, the effects of various concentrations of the essence (0, 5, 25, 50, 100, 200, 300, and 400 µl.ml^{-1}) on the cell viability of HDF cells were not significantly differed than the control, Figure 8. The variance analysis of the data (ANOVA) of above noted treatments effects on HDF cells is shown in Table 4. The IC_{50} of different concentrations of essential oil (0, 5, 25, 50, 100, 200, 300, 400 µl.ml^{-1}) on cell viability of A549 cells is shown in Figure 9. In this case, a similar trend by the extract effect on cell viability was observed. The lowest IC_{50} of cell viability was recorded at the time of 48 hours by 280 µg.ml^{-1}. Since the calculated IC_{50} values of HDF cells viability under 24, 48 and

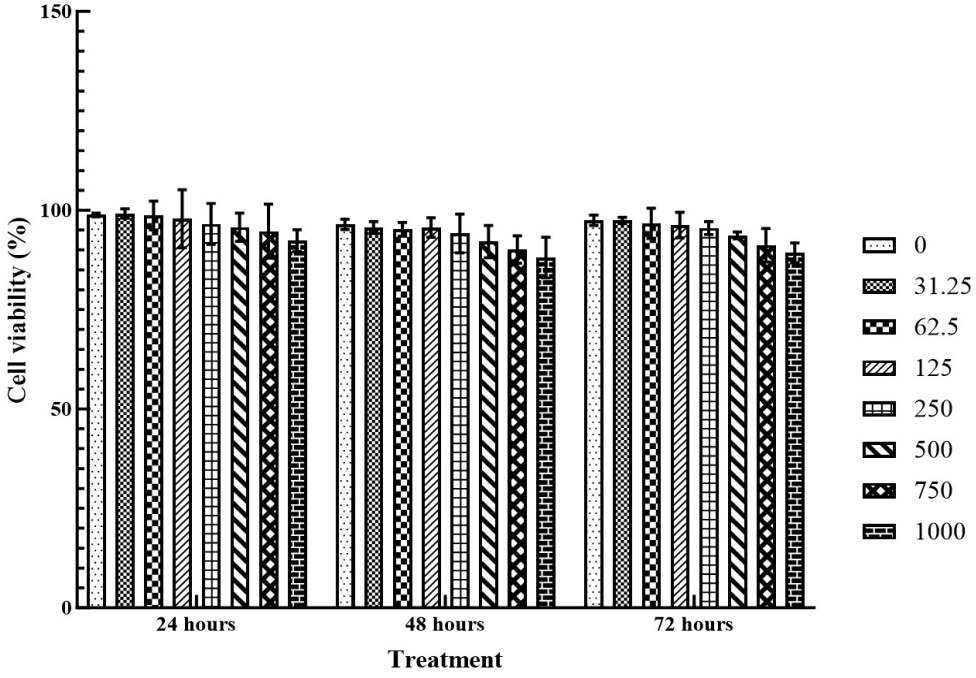
72 hours treatments for ethanolic extract (5179, 4327 and 4859 µg.ml^{-1}) and essence (2372, 1507 and 1751 µl.ml^{-1} respectively) were much higher than the applied concentrations, therefore the IC_{50} graphs for HDF cells under ethanolic extract and essence are not drawn.

Figure 6 - Means comparing of effects of various concentrations of *Ephedra major* ethanolic extracts (0, 31.25, 62.5, 125, 250, 500, 750 and 1000 µg.ml^{-1}) on A549 cells viability. Data are IC_{50} Concentration at 24, 48 and 72 hours. ns: no significant differences, ** differed at p≤0.01.



Source: author's own work.

Figure 7 - Means comparing of effects of various concentrations of *Ephedra major* ethanolic extracts (0, 31.25, 62.5, 125, 250, 500, 750 and 1000 µg.ml^{-1}) on HDF cells viability under 24, 48 and 72 hours treatments.



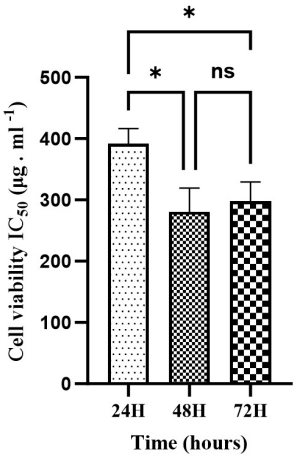
Source: author's own work.

Table 3 - The results variance analysis (ANOVA) of effects of various ethanolic extract concentrations (0, 31.25, 62.5, 125, 250, 500, 750 and 1000 mg.ml^{-1}) under 24, 48 and 72 hours treatments on cell viability of HDF cells.

Source of variation	df	SS	MS	P value	Significant
Time	2	177.4	88.7	0.0017	**
Concentration	7	657.9	93.99	< 0.0001	****
Interaction	14	15.59	1.114	> 0.9999	ns
Total	72	919.5	12.77		

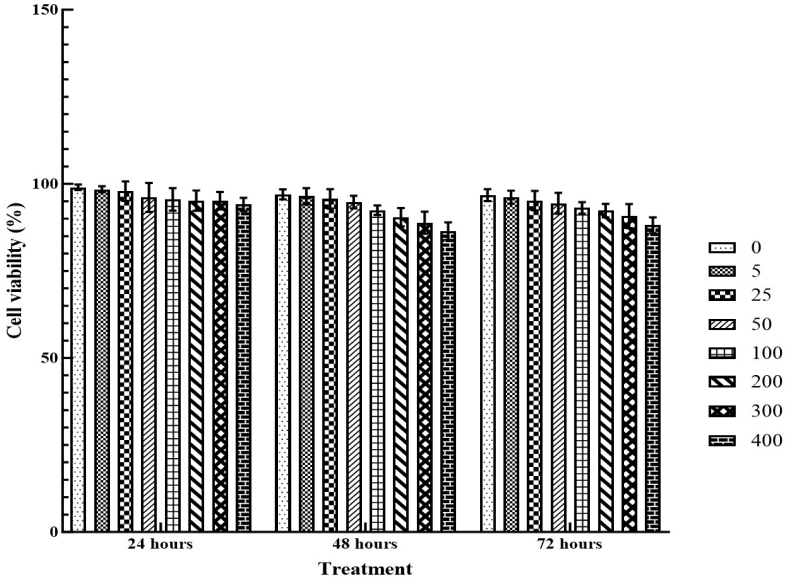
Source: author's own work.

Figure 8 - Means comparing of effects of various concentrations of *Ephedra major* essence (0, 5, 25, 50, 100, 200, 300 and 400 µl.ml^{l-1}) on A549 cells viability at 24, 48 and 72 hours. Data are IC_{50} concentration at 24, 48 and 72 hours. ns: no significant differences, * differed at p≤0.05.



Source: author's own work.

Figure 9 - Means comparing of effects of various concentrations of *Ephedra major* essence (0, 5, 25, 50, 100, 200, 300 and 400 µl.ml^{-1}) on HDF cells viability under 24, 48 and 72 hours treatments.



Source: author's own work.

Table 4 - The results variance analysis (ANOVA) of effects of various concentrations of *Ephedra major* essence (0, 5, 25, 50, 100, 200, 300 and 400 ml.ml^{-1}) under 24, 48 and 72 hours treatments on HDF cells.

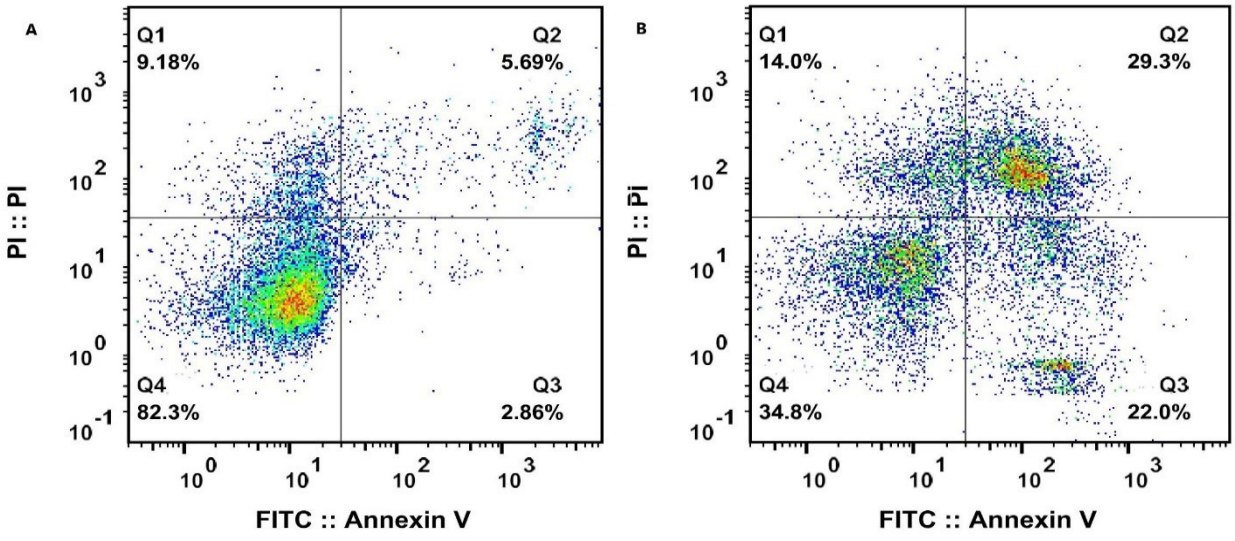
Source of variation	df	SS	MS	P value	Significant
Time	2	248.9	124.5	< 0.0001	****
Concentration	7	665.3	95.05	< 0.0001	****
Interaction	14	81.04	5.788	0.5128	ns
Total	72	439.2	6.1		

Source: author's own work.

Apoptosis assay

According to results obtained from MTT assay, the treatments that led to the lowest cell viability were selected to determine the apoptosis rate in A549 cells. The A549 cells treated with *Ephedra major* extract or essence were double-stained with annexin V and propidium iodide and analyzed by flow cytometry. Each quadrate shown as living [Q_4 (lower left)], necrotic [Q_1 (upper left)], early apoptotic [Q_3 (lower right)] and late apoptotic [Q_2 (upper right)] cells. When A549 cells were treated with Ephedra extract or essence, the apoptotic rates (%) in the early and late stages (Q_3 and Q_2, respectively, in Figure 10 B, Figure 10 C) were evidently increased than in the untreated group (Figure 10 A). It is notable that the proportion of late apoptotic cells was significantly higher under ethanolic extract (29.3%) in comparison with essence (25.4%) or control (5.69%). Moreover, the proportion of early apoptotic cells under Ephedra ethanolic treatment (22%) was significantly higher as compared with essence (14.8%) or untreated group (2.86%) ($P<0.05$).

Figure 10 - Vital cells (Q_4), early apoptosis (Q_3), late apoptosis (Q_2) and necrosis (Q_1) percentage of A549 cells under control (A), ethanolic extract (B) and Essence (C) of *Ephedra major*.



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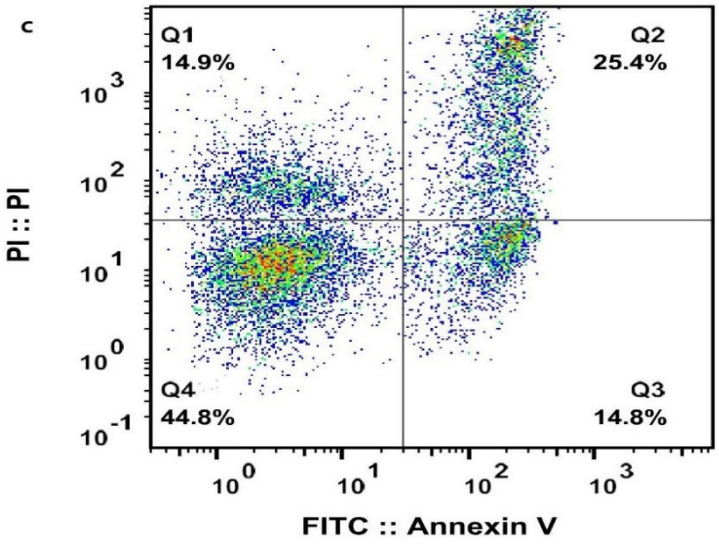
Continuation

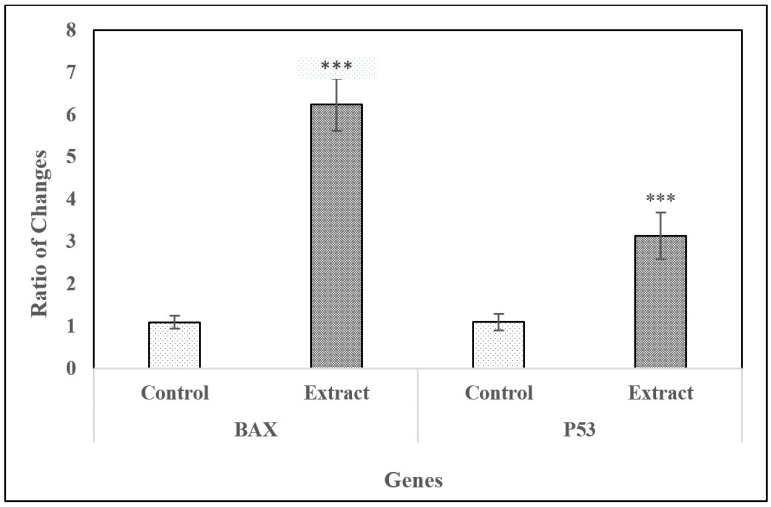


Source: author's own work.

Determining of BAX and p53 expression

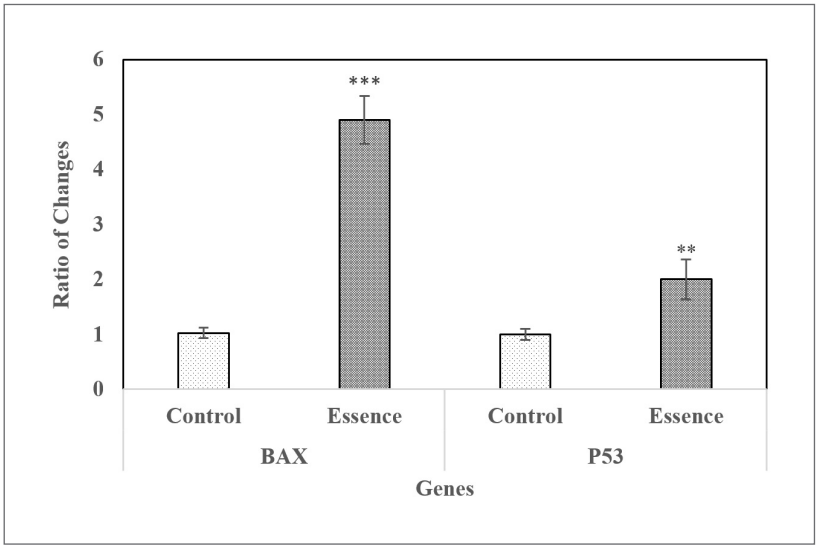
After confirming that *Ephedra major* extract and essence induces A549 cell apoptosis, we attempted to reveal the changes in expression of apoptosis effector genes, BAX and p53. The A549 cells were exposed to *Ephedra major* extract or essence for 48 hours; the mRNA expression levels of Bax and p53 were measured by real-time PCR. Both ethanolic extract and essence of Ephedra, increased the mRNA expression of the pro-apoptotic genes Bax and p53 in A549 cells significantly ($p \leq 0.001$, $p \leq 0.01$). Based on the results, the ethanolic extract increases the expression of the BAX gene by 5.7 times and the p53 gene by 2.84 times as compared with the control (Figure 11). Also, essential oil increases the expression of BAX and p53 genes by 4.7 and 2 times, respectively (Figure 12).

Figure 11 - Effects of *Ephedra major* ethanolic extract on BAX and p53 expression. The relative level of each gene obtained by normalization with GAPDH in the control group, which was set arbitrarily at 1, and the level in other groups was calculated accordingly compared with control (*** $P \leq 0.001$).



Source: author's own work.

Figure 12 - Effects of *Ephedra major* essence on BAX and p53 expression. The relative level of each gene obtained by normalization with GAPDH in the control group, which was set arbitrarily at 1, and the level in other groups was calculated accordingly compared with control (*** P≤0.001, ** P ≤0.01).



Source: author's own work.

Discussion

As shown in Table 2, the main volatile substances in *Ephedra major*, were oxygenated terpenes which followed by other terpene-derivate compounds such as ethers, acids, and esters. Similar to this study, Guo *et al*.[26] reported that alcohols and terpenoids, including α-Terpineol, *p*-Cymene, Ligustrazine, 4-Methoxystyrene, cis-*p*-Menth-2-en-7-ol and Phellandral were the main compounds of *Ephedra herba* essence. Also, Wang *et al*.[27] noted that α-terpineol *p*-vinylanisole, 3-methyl-2-buten-1-ol, terpine-4-ol and α-linalool were the main volatile substances of *Ephedra sinica* essence.

And according to previous studies,[28-29] the ephedrine alkaloids are considered the active constituents of plants belonging to the genus *Ephedra*. The ephedrine is the main and the minor alkaloids include norpseudoephedrine, norephedrine, pseudoephedrine and methylephedrine.[30] In the current study, we determined ephedrine and psedudoephedrin concentrations in ethanolic and methanolic extracts of *E. major*. As demonstrated above, the more abundant alkaloid in both extracts was ephedrine, while pseudoephedrine was present in the extracts by lower amounts than ephedrine. However, the amounts of each of the noted compounds in the both extracts were not significantly differed.

Our results showed that ethanolic extract has higher antioxidant properties as compared with methanolic extract. The antioxidant activity of ethanolic extract at the concentration of 250 $\mu g.ml^{-1}$ was close to that of ascorbate as a standard antioxidant, which indicates a good antioxidant activity. Also, the findings showed that the ethanolic extract has a higher phenolic content compared to the methanolic extract. In accordance with the current study, Ibragic *et al*.[31] By comparing the phenolic content of various extracts of Ephedra, reported that the ethanolic extract has higher phenolic content compared to the methanolic extract. A strong correlation between the TPC and antioxidant activity was reported by Ibragic *et al*.[31] in *Ephedra* spp and by Song *et al*.[32] In *Ephedra sinica*. Saeed *et al*.[33] investigated the antioxidant properties of different fractions of *Torilis leptophylla* extract, their study revealed that TLB and TLE fractions have prominent antioxidant activity; the phenolic compounds are mainly found in these two fractions

and could be attributable to the observed high antiradical properties of these fractions. By measuring the amount of ephedrine and pseudoephedrine flavonoids in ethanolic and methanolic extracts in this study, we found out that the amount of these compounds in the two extracts is not significantly differed, but total phenolic content of the two extracts was significantly differed, and therefore the significant difference in the antioxidant power of the extracts can be attributed to it.

In recent years, the control of cancer has been focused on the search for anticancer agents, which are safer and have higher acceptability for patients. In traditional medicine, herbs are used for many forms of cancer therapy. Their discovery as anticancer drugs is also progressing. For that, Ephedra ethanolic extract and hydrodistiled essence were evaluated for anti-proliferative activity as a candidate anticancer agent. According to the results obtained above, ephedra ethanolic extract and essence were safe toward human normal cells (HDF) and exhibited good anti-proliferative activity, specifically under treatment of 48 hours against A549 cancer cells.

In this regard, various natural agents such as turmeric and polyphenolic, which are generally a part of the human diet or traditional herbal medications, have been given attention.[34] Polyphenolic compounds are reputed as potent chemopreventives and/or chemotherapeutics that prevent formation of tumor and hinder cancer progression by acting on the multiple checkpoints of cancerous cells, and they can induce apoptosis, autophagy, and cell cycle arrest with high specificity.[35] These compounds can interfere with the initiation and progression of cancer by modulating various cellular events, such as cell cycle arrest by decreasing cyclines or apoptosis induction through cytochrome-c release, activation of caspase and down or up-regulation of Bcl-2 family members. The tumor suppressors like p53 and its analogs are key molecular targets of polyphenols and are responsible for their pro-apoptotic effects in cancer models.[36] In the current study, we investigated the effect of *E. major* ethanolic extract in lung cancer cell lines A549 regarding cell viability, apoptosis and its related genes expression. We herein report that *E. major* ethanolic extract, which contains high amounts of phenolic and flavonoid compounds can inhibit cell viability, induced cell apoptosis and increased apoptosis related genes expression in A549 cancer cell line. In this research, we only measured the amounts of ephedrine and pseudoephedrine compounds. But in the studies of other investigators, some other compounds have been identified in ephedra alcoholic extracts that have anti-cancer properties. Wang *et al*., (2019),[37] reported that 1, 4-naphthoquinone derivatives induced apoptosis in gastric cancer cells. Similarly, ferulic acid has been reported to induce cell cycle arrest and autophagy in cervical cancer cells.[38] It has been reported that caffeic acid has anti-cancer effects against breast, cervical, lung, and oral cancers. Its anti-cancer effect is reportedly mediated through the inhibition of cell migration and invasion.[39] 7-Hydroxy-4-methylcoumarin is another phytoconstituent that is reported to possess an anti-cancer effect against skin cancer in mice.[40] Also, etidronic acid is cytotoxic to human breast cancer cells[41] and phytol is reported to be effective against sarcoma and human leukemic cancer cells.[42] L-canavanine is reported to deprive cancer cells and has good anti-cancer action.[43] Scoulerene and formononetin also have anti-cancer actions that are mediated through multiple mechanisms.[44]

A large number of terpenoids show cytotoxic effects against various types of tumor cells, cancer prevention and anticancer efficacy in preclinical animal models. Terpenoids and their derivatives act at different stages of tumor growth, inhibit the beginning and promotion of carcinogenesis, induce tumor cell differentiation and apoptosis, and suppress tumor invasion and metastasis by regulating various growth and transcription factors as well as intracellular signaling mechanisms.[45] A few earlier studies demonstrated anticancer properties of some terpenoids include thymol[46] D-Limonene[47-48] and Carvacerol[49-50] by more details. In the current study, the obtained essence contains anticancer terpenoids such as thymol, D-Limoneneand carvacerol, there-

fore, it can be concluded that the anti-cancer properties of *E. major* essence which determined by measuring the cell viability, apoptosis, and Bax and p53 genes expression in A549 cell line, were due to presence of these compounds.

Conclusion

Finally, as a conclusion, the antioxidant power and the growth inhibitory effects of *Ephedra major* ethanolic extract and hydro-distilled essence against A549 can be attributed to their large amounts of natural medical components, in which alkaloids, polyphenolics and terpenoids are anticancer agents. In order to purify anti-cancer compounds and obtain herbal medicines, it is necessary to carry out additional studies.

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