Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN (E): 2320-3862
ISSN (P): 2394-0530
www.plantsjournal.com
JMPS 2024; 12(4): 333-340

Received: 28-05-2024
Accepted: 03-07-2024

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Cytostatic potential of *Ephedra aphylla* Forssk and *Ephedra foeminea* Forssk. different extract types against HeLa cell line

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DOI: https://doi.org/10.22271/plants.2024.v12.i4d.1726

Abstract
The cytotoxic and cytostatic anticancer potential of *Ephedra aphylla* and *Ephedra foeminae* extracts on the HeLa cell line was assessed via qualitative inverted microscopy morphological screening and quantitative MTT assay using 400, 200, 100, 50, and 25 µg/mL from aqueous, methanol and ethanol extracts. The obtained data showed morphological changes in HeLa cells as they became more rounder, shrunken and detachable than controls after 72 h. Moreover, this was confirmed by MTT assay HeLa cell line viability cytotoxic reduction of 99.7-71.1% and 99.1-72.2% for *E. aphylla* and *E. foeminea* at 25-400 µg/mL, respectively. While, cytostatic reduction was 98.9-10.7% and 98.5-27.9% for *E. aphylla* and *E. foeminea* at 25-400 µg/mL, respectively. Among them, *E. aphylla* ethanol extract followed by *E. foeminea* methanol extract possessed the strongest cytostatic anticancer effect reducing HeLa cell line viability down to 10.7% and 27.9% at 400 µg/mL with nontoxic IC50 of 187.5 and 257.9 µg/mL, respectively.

Keywords: Anticancer, cytostatic, *Ephedra aphylla* Forssk., *Ephedra foeminea* Forssk., HeLa cell line

Introduction
Cancer is a complicated medical disease that primarily affects cells and molecules in a wide variety of ways [1]. So, it is ranked second to cardiovascular diseases as a cause of death [2]. Annually, statistical data indicates that millions of people are unfortunately diagnosed with cancer. Smoking, dietary imbalances, hormones and chronic infections could be considered as major causes of that lethal disease [3]. Radiation, chemotherapy, immunomodulation and surgery are such trials for curing cancer. However, a low success rate is associated with a high mortality rate. Therefore, it seems that developing new plans as chemoprevention including the utilization of natural products including plants [4]. Recent research in synthetic chemistry announced that natural compounds have a good effect that creates better methods for curing and blocking a lot of cancer types [5, 6, 7]. Plant compounds such as alkaloids, phenylpropanoids, and terpenoids were notified to have antitumor activity [8]. This was the reason after the production of several antitumor medications derived from plants, including vinblastine, vincristine, camptothecin, and taxol [9]. This goes along with that over 60% of cancer patients receive therapy from vitamins or herbs as well as diets rich in phytochemicals can minimize cancer danger by 20% [10, 11, 12]. One of the nations with the greatest diversity of floral life in the world is Palestine. In Palestine, The implementation of complementary and alternative therapies is very prevalent [13]. Hence it is interesting to assess if their traditional utilization is only depend on folkloric use or supported by real pharmacological effects [14]. Thus, the finding of new active compounds could be achieved by searching unknown traditional wild medicinal plants. Consequently, the efficiency of new anticancer agents discovery could be improved via the collaboration between informants and scientific institutions in the same field. This should be associated with the scientific exploration of the best extraction, formulation and dosage calculations to obtain maximum benefit from hese herbal remedies [15]. Different investigations in Palestine were carried out considering the antitumor effect of several medicinal plants [16]. Moreover, 115 cancers patient's Palestinian questionnaire and 150 herbalists, traditional healers and rural dwellers ethno-pharmacological

questionnaires were performed regarding the herbal medication used in cancer treatment [15, 17]. One of those plants was *Ephedra*, which is a widespread genus of gymnosperm shrubs that grows on sandy soil or along shores in temperate and subtropical areas [18]. Alanda (*Ephedra foeminea*, or *Ephedra campylopoda*) is the Arabic name for a low stalky Eurasian shrub of the Ephedraceae family. Subsequently, the aerial parts of various *Ephedra* species were proved to hold active alkaloids, such as phenylpropyl amino, ephedrine and pseudoephedrine [19]. During the 20th century, there was an increase interest in the traditional clinical use of *Ephedra*, particularly for the treatment of cancer. World Health Organization (WHO) reported that the fourth most common cancer in women is cervix cancer. In low- and middle-income countries, cervical cancer mortality reaches around 90% among all deaths. A comprehensive strategy that incorporates prevention, early diagnosis, effective screening and treatment programmers could lower the high mortality rate of cervical cancer [20]. Taking into account all the research done in this regard, the ongoing research project issue has emerged. Therefore, this project aimed to investigate the anticancer effect of aqueous, methanol and ethanol extracts of both *Ephedra aphylla* and *Ephedra foeminea* on HeLa cell line in Palestine, as no previous studies on those two plant species against that cancer cell line type were conducted.

Materials and Methods

Plant collection

Ephedra aphylla and *Ephedra foeminea* plants were collected from the West Bank of Palestine. A plant taxonomist from the Biotechnology and Biology Department/Faculty of Science identified them. Representative plant specimens were deposited in the university herbarium and given the voucher numbers 1812 and 1895 after being chemically treated, pressed until dry and mounted on herbarium sheets. Then, whole parts of the two plant species were carefully washed in water and allowed to air dry for about two weeks at room temperature in a shaded area away from the sunlight. After that, the dried parts were ground into a powder and stored at room temperature in a dark place until their use in the plant extracts preparation.

Plant aqueous, methanol and ethanol extracts preparation

One hundred mL of each employed solvent (distilled water, 70% methanol, and 70% ethanol) were added to 10 grams of plant powder, which was then shaken on a rotary shaker at 30 ^{O}C for 72 h. After that, a probe sonicator was used to macerate the soaked plant species for 20 min at 40 ^{O}C (3 seconds of sonication followed by 5 seconds of rest). The mixtures were then centrifuged at 4000 rpm for 10 min. Then, by using pieces of gauze, the resulting extract supernatants were filtered. The filtrates of the ethanol and methanol plant extracts were then transferred to rotary evaporator at 45 ^{O}C. While, the aqueous ones were freeze-dried. The plant extract powders were kept at 4 ^{O}C. Different concentration of methanol and ethanol plant extracts were prepared in 1% fresh dimethyl sulfoxide (DMSO) in Roswell Park Memorial Institute (RPMI 1640) media. Otherwise, aqueous plant extracts were prepared in fresh RPMI 1640 media.

Inverted microscopy visualization screening

Human cervical carcinoma (HeLa) cell line that was obtained from ATCC (American Type Culture Collection) were grown in fresh RPMI 1640 medium with supplements and incubated in a cell culture incubator at 37 ^{O}C, 95% humidity, 5% CO_2 at dark until 90% cell confluence was obtained**.** Later, cells were counted and cultured in a 12 well plate with a total volume of 1000 µl at a density of 20,000 cells per well. They were separately treated with 100 µl of the various extract types concentrations to achieve final concentrations of 25–400 400, 200, 100, 50, and 25 µg/mL. Cells cultured in RPMI media only were used as normal control while those cultured in 1% fresh DMSO in RPMI media were used as negative control. All plates were incubated at cell culture incubator for 72 h. Under the inverted microscope, cell viability, confluence and attachment were detected. At a 10x magnification, microscopic images were captured in the well centers.

MTT assay

This colorimetric assay is based on the reduction of a yellow tetrazolium salt (3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide) (MTT) to a deep purple colored insoluble crystalline formazan by NAD(P)H–dependent oxido–reductase enzymes in the live cells. 20,000 cells were counted for the cytotoxic test and 5,000 cells for the cytostatic test, using the MTT kit (Sigma). Following treatment with 100 µL of varying doses of water, methanol, and ethanol plant extracts of the different research species, each well was incubated for 24 and 72 hours, respectively, for the cytotoxic and cytostatic tests, to reach final concentrations of 25–400 µg/mL. Following incubation, PBS was used to wash the wells and the media was thrown out. Each well was then filled with 100 µL of serum-free RPMI medium, 10 µL of MTT solution (0.5 mg/mL), and incubated for four hours. Cells were treated with 100 µL of acidic isopropanol (0.08N HCl) for 15 minutes after the media was removed and then washed. Using a microplate reader (Labtech, UK), the absorbance of MTT formazan was measured at 570 nm. The following formula was utilized to determine cell viability:

$$\text{Cell Viability (\%)} = \text{A Treatment}/(\text{A Control}) \times 100\%$$

Statistical analysis

The outcomes of the MTT (cell viability data) was assessed with Microsoft Excel using nonlinear regression calculations so as to determine the concentration giving effective 50% inhibition (IC50). By graphing the inhibition percentage (%) against concentration (µg/mL), the dose–response curve was obtained.

Results

Morphological screening

The bioactivity of two *Ephedra* species was investigated by the exposure of HeLa cell line to different concentrations (400, 200, 100, 50, 25 µg/mL) from aqueous, methanol and ethanol extracts for 24 h (cytotoxic) and 72 h (cytostatic). Examination of the treated cancer cells by inverted microscopy revealed an alterations in cancer cells morphology as treated cells became more rounder, shrunken and detachable than controls. The bio-alterations which was noticed in the treated cells could be an indication for cell growth inhibition. However, these effects were more pronounced after 72 h than 24 h. Therefore, this indicates that both plant species under study have a cytostatic effect rather than cytotoxic one under the investigated concentration in a concentration dependent manner. Moreover, the ethanol extract of *E. aphylla* displayed the strongest cytostatic anti-proliferative activity at different concentrations (Figure 2) followed by *E. foeminea* methanol one (Figure 6). None the less, the other examined extract types exhibited cytostatic

effect rather than cytotoxic one (Figures 1, 3, 4, 5). As a result, for further exploration of the observed cytostatic effect in the morphological inverted microscopy secreening, MTT cytotoxic and cytostatic assays were conducted.

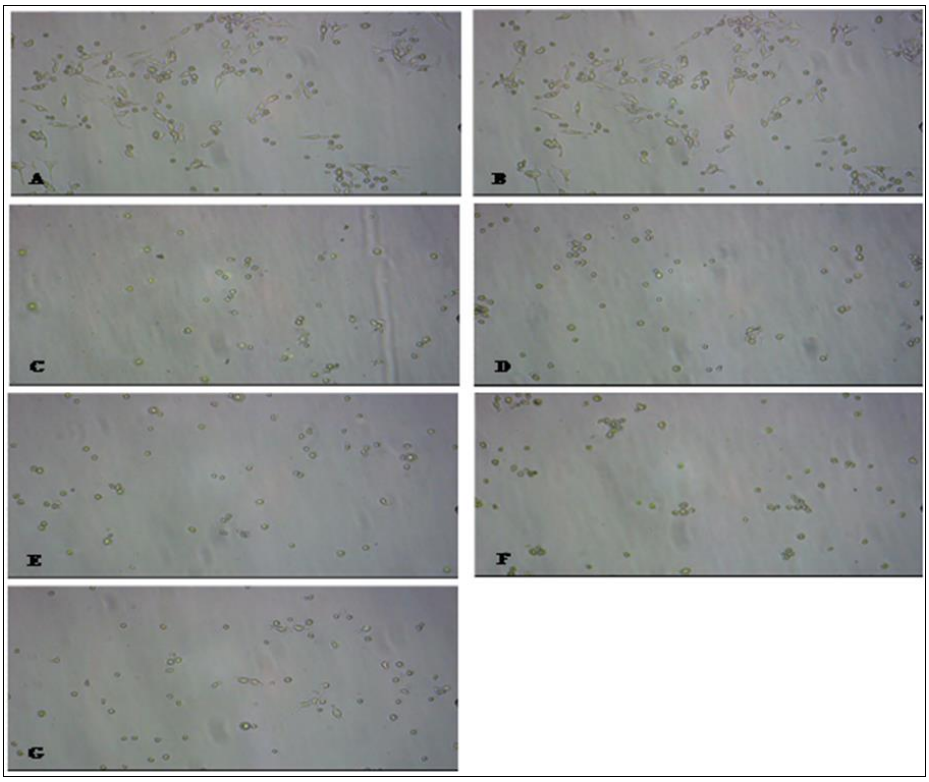

Fig 1: Effect of *Ephedra aphylla* aqueous extract different concentrations on HeLa cells (A: Aqueous control; B: DMSO control; C: 400 µg/mL; D: 200 µg/mL; E: 100 µg/mL; F: 50 µg/mL; G: 25 µg/mL). Images were captured after 24 using inverted microscope at 10x.

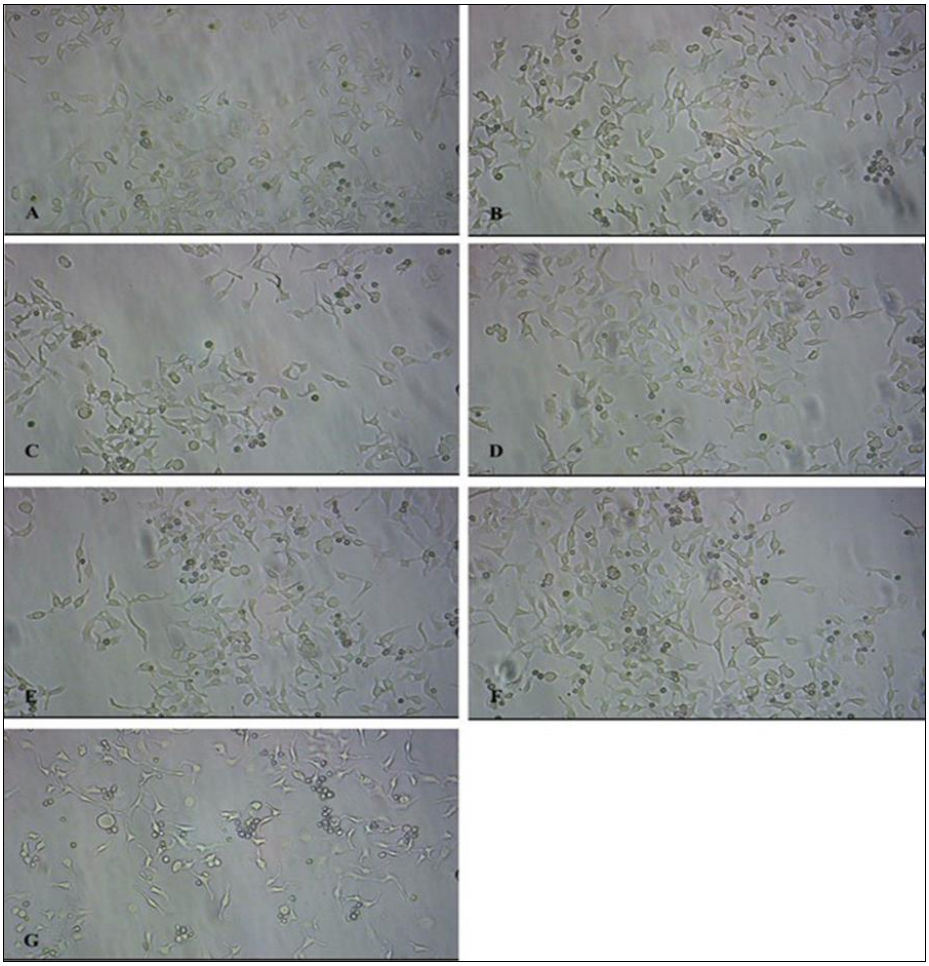

Fig 2: Effect of *Ephedra aphylla* ethanol extract different concentrations on HeLa cells (A: normal control; B: DMSO control; C: 400 µg/mL; D: 200 µg/mL; E: 100 µg/mL; F: 50 µg/mL; G: 25 µg/mL). Images were captured after 24 hours using inverted microscope at 10x.

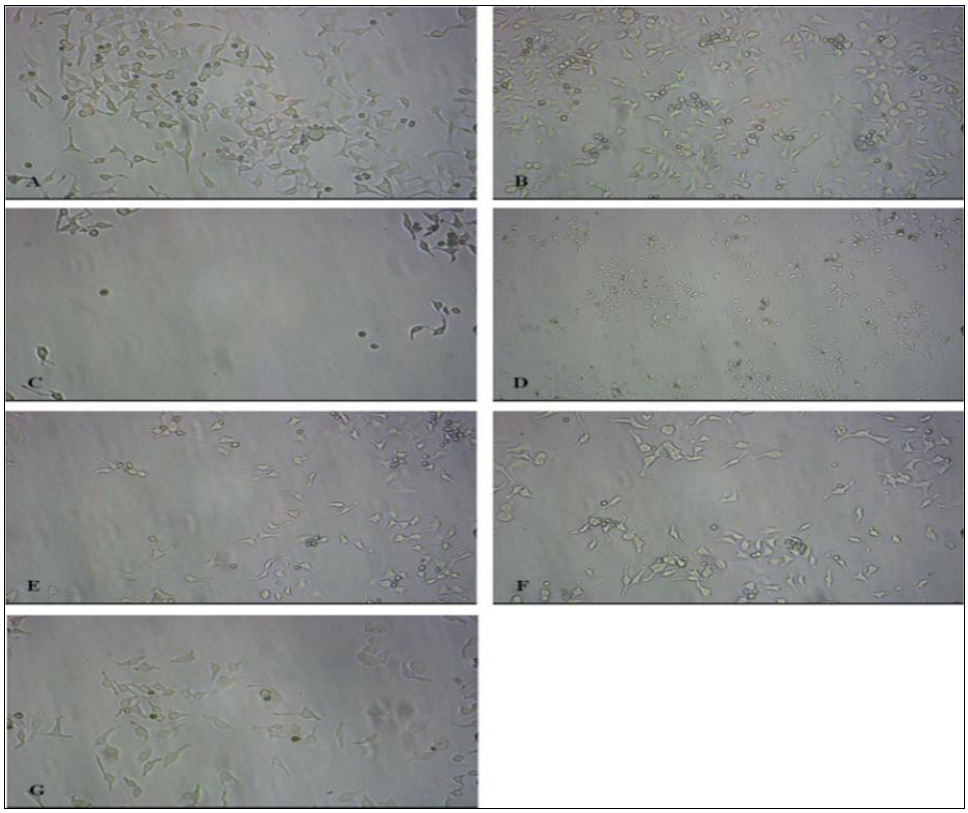

Fig 3: Effect of *Ephedra aphylla* methanol extract different concentrations on HeLa cells (A: normal control; B: DMSO control; C: 400 µg/mL; D: 200 µg/mL; E: 100 µg/mL; F: 50 µg/mL; G: 25 µg/mL). Images were captured after 24 hours using inverted microscope at 10x.

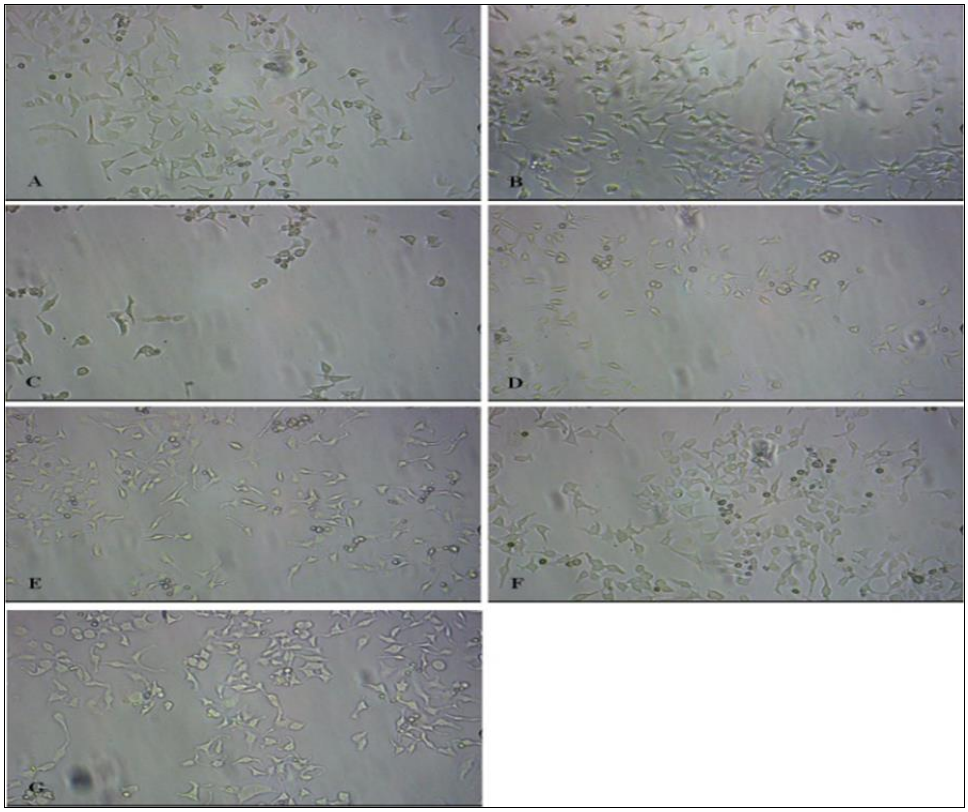

Fig 4: Effect of *Ephedra foeminea* aqueous extract different concentrations on HeLa cells (A: normal control; B: DMSO control; C: 400 µg/mL; D: 200 µg/mL; E: 100 µg/mL; F: 50 µg/mL; G: 25 µg/mL). Images were captured after 72 hours using inverted microscope at 10x.

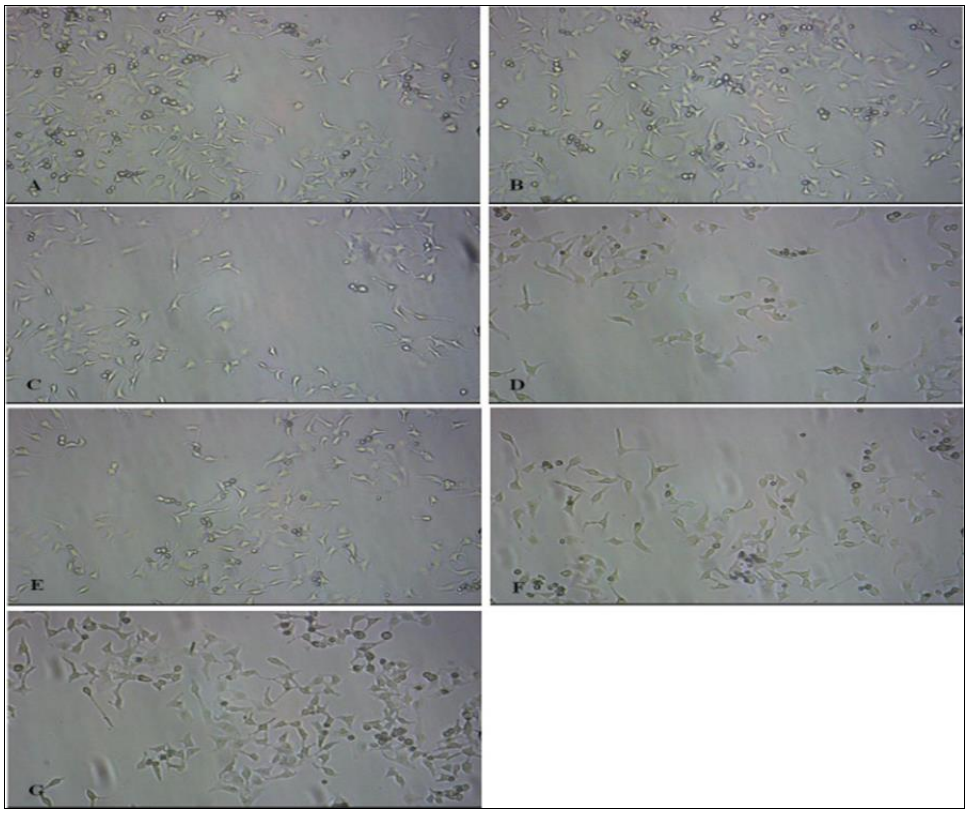

Fig 5: Effect of *Ephedra foeminea* ethanol extract different concentrations on HeLa cells (A: normal control; B: DMSO control; C: 400 µg/mL; D: 200 µg/mL; E: 100 µg/mL; F: 50 µg/mL; G: 25 µg/mL). Images were captured after 72 hours using inverted microscope at 10x.

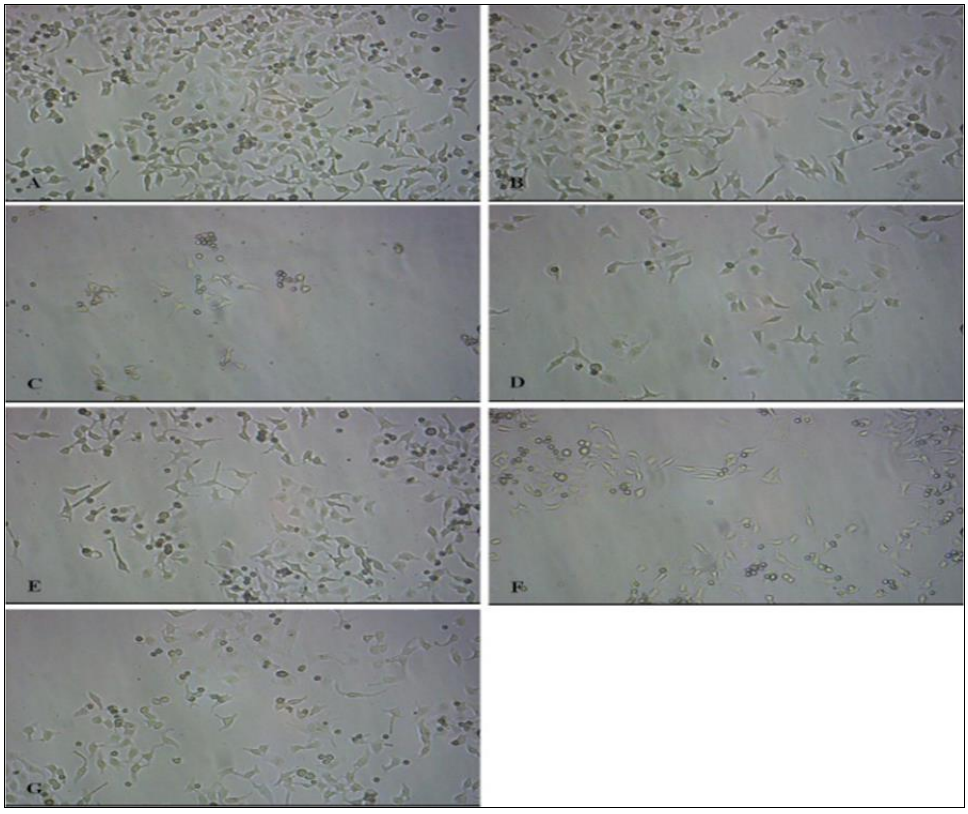

Fig 6: Effect of *Ephedra foeminea* methanol extract different concentrations on HeLa cells (A: normal control; B: DMSO control; C: 400 µg/mL; D: 200 µg/mL; E: 100 µg/mL; F: 50 µg/mL; G: 25 µg/mL). Images were captured after 72 hours using inverted microscope at 10x.

MTT assay

HeLa cell line viability % after 24 h (cytotoxic) showed low reduction in the range of 99.7-71.1% under the effect of aqueous, methanol and ethanol extracts of *E. aphylla* and 99.1-72.2 of *E. foeminea* at 25-400 µg/mL. While, HeLa cell line viability % after 72 h (cytostatic) showed more reduction in the range of 98.9-10.7% under the effect of the three studied extracts of *E. aphylla* and 98.5-27.9% of *E. foeminea* at 25-400 µg/mL. All antitumor cytotoxic and cytostatic bioactivity of all the examined different plant extracts displayed dose dependent manner (Table 1, Table 2).

Table 1. MTT cytotoxic (24 h) and cytostatic (72 h) effect of *Ephedra aphylla* different examined extract types on HeLa cell line viability % under the studied concentrations.

Studied Concentration (µg/mL)	*Ephedra aphylla*					
	Aqueous extract		Methanol extract		Ethanol extract	
	Cytotoxic	Cytostatic	Cytotoxic	Cytostatic	Cytotoxic	Cytostatic
25	99.7	98.9	98.3	95.9	91.7	76.6
50	96.7	93.7	95.1	87.2	90	67.6
100	95.1	88.5	94.4	83.1	88.2	56.6
200	92.4	87.8	92	64.8	83.9	27.2
400	89.9	87.4	87	55.5	71.1	10.7

Table 2: MTT cytotoxic (24 h) and cytostatic (72 h) effect of *Ephedra foeminea* different examined extract types on HeLa cell line viability % under the studied concentrations.

Studied Concentration (µg/mL)	*Ephedra foeminea*					
	Aqueous extract		Methanol extract		Ethanol extract	
	Cytotoxic	Cytostatic	Cytotoxic	Cytostatic	Cytotoxic	Cytostatic
25	99.1	98.5	95.8	93.4	98.6	97.2
50	96.3	94.4	92.7	89.7	92.3	89.7
100	89.4	82.9	84.6	72.4	89.5	82.4
200	88.5	77.4	78.9	54.8	84.6	72
400	79.9	60.4	72.2	27.9	80.1	62.8

The obtained data revealed that both *E. aphylla* (Figure 7) and *E. foeminea* (Figure 8) different examined extract types possessed cytostatic effect rather than cytotoxic one at the studied concentrations (25-400 µg/mL). Moreover, *E.aphylla* ethanol extract reduced the HeLa cell line viability down to 10.7% after 72 h at 400 µg/mL, which could be considered the strongest cytostatic anticancer effect among its other extract types at the different studied concentrations (Figure 7). While, the strongest cytostatic anticancer effect of *E. foeminea* is referred to its methanol extract as showed 27.9% cell viability reduction at 400 µg/mL concentration (Figure 8). Therefore, *E. aphylla* ethanol extract could be considered the most potent anticancer extract against HeLa cell line among all other extract types under study. Furthermore, the two species methanol and ethanol extracts were more effective than the aqueous ones at all examined concentrations. However, both the aqueous and methanol extracts of *E. foeminea* showed more cytostatic anticancer effect than *E. aphylla* ones by the lower recorded cell viability % in HeLa cell line. On the other hand, the ethanol extract of *E. aphylla* had a stronger anticancer effect than *E. foeminea* due the lower recorded cell viability % in HeLa cell line (Table 1 and Table 2).

Hence, due to the pronounced variable cytostatic effect of the examined plant species different extract types at the concentration range under study, the IC50 values were calculated. The recorded IC50 values were in the range 187.5-1187.6 µg/mL, for ethanol and aqueous *E.aphylla* extracts, respectively. The obtained IC50 values confirmed that *E. aphylla* ethanol extract followed by *E. foeminea* methanol one acquired the strongest cytostatic anticancer effect by reducing HeLa cell line viability down to 10.7% and 27.9% at 400 µg/mL with nontoxic IC50 values 187.5 and 257.9 µg/mL, respectively (Table 3 and Figure 9).

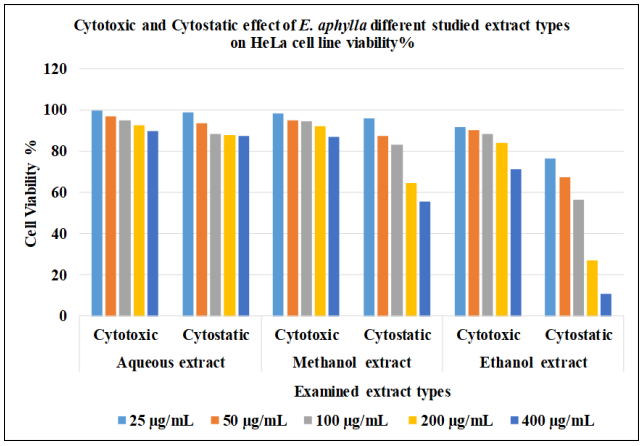

Fig 7: MTT cytotoxic (24 h) vis cytostatic (72 h) effect of *Ephedra aphylla* extracts on HeLa cell line under the examined concentrations.

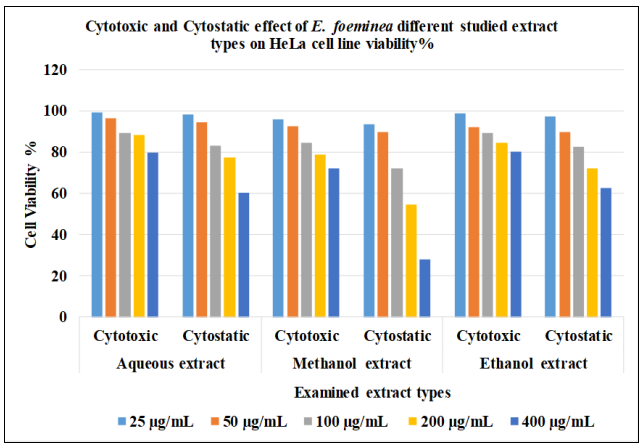

Fig 8: MTT cytotoxic (24 h) vis cytostatic (72 h) effect of *Ephedra foeminea* extracts on HeLa cell line under the examined concentrations.

Table 3: IC_{50} values (µg/mL) of the *E. aphylla* and *E. foeminea* different extract types on HeLa cancer cell line after 72 h at cytostatic experiment using the MTT assay.

Plant species	Extract type		
	Aqueous extract	Methanol extract	Ethanol extract
Ephedra aphylla	1187.6	390.6	187.5
Ephedra foeminea	476.2	257.9	466.9

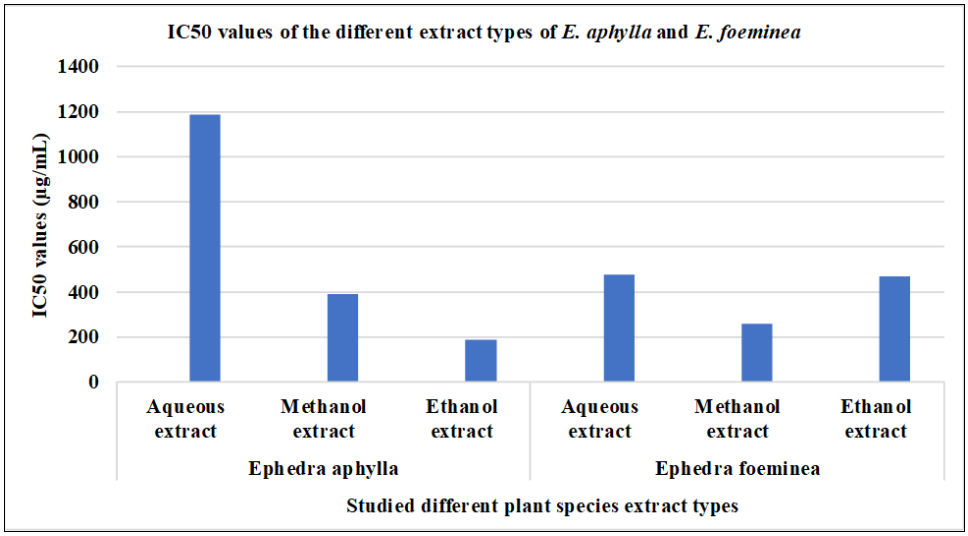

Fig 9: IC_{50} values (µg/mL) of the *E. aphylla* and *E. foeminea* different extract types on HeLa cancer cell line after 72 h at cytostatic experiment using the MTT assay.

Discussion

The ability of medicinal plants to induce apoptosis and/or growth arrest in cancer cells without having negative impacts on normal cells implies that they could be new sources for tumor treatment [21]. Consequently, anticancer medicines derived from plants are efficient in inhibiting tumor cell lines [22]. According to reports, the two Ephedra plant species that are the focus of this study undergo various phytochemical evaluations. Alkaloids, flavonoids, condensed tannins, triterpenes, and cardiac glycosides were found in the phytochemical investigation of *E. aphylla*; these substances have been shown to have therapeutic potential against numerous types of disorders [23]. The presence of flavonoids, saponins, triterpenes and tannins is believed to contribute partially by their anti-proliferative activity through their antioxidant activity [24]. Tannins and polyphenolic compounds have also significant effects on cancer prevention and anticancer activity [25]. Thus, phytochemicals such as alkaloids, phenols and flavonoids may be responsible for the anti-proliferative activity of *E. aphylla*. *Ephedra aphylla* contains different concentrations of phenolic compounds depending on the extract type. It was cited that its methanol extract contains higher phenolic and flavonoids concentration than the aqueous one. Subsequently, the observed strong anti-proliferative potency against the breast cancer cell lines MFC7and T47D could be referred to the presence of these phytochemicals [26]. On that account, the obtained results in the current study agree with the literature cited phytochemical constituents of *E. aphylla*. As its methanol extract showed stronger cytostatic effect than the aqueous one at all examined concentrations. This was confirmed by the lower recorded cytostatic MTT viability % and the inverted microscopy morphological screening for HeLa cell line. None the less, its ethanol extract has a salient cytostatic effect on the examined cell line as caused the lowest HeLa cell line viability % at all concentrations under study among all other its extract types. So, it is highly recommended to subject the ethanol extract of *E. aphylla* for further phytochemical analysis. While, various phytoconstituents were found in the ethanol, methanol, and aqueous extracts of *E. Foeminea*, in which the ethanol extract exhibited higher amounts than the aqueous one. On the other hand, more of those constituents were in the methanol extract, which showed high levels of phenols, sterols/steroids, carbohydrates, flavones and lignin; moderate levels of quinones, tannins, cardiac glycosides, amino acids and phlobatannins; low levels of flavonoids, terpenoids, coumarins, resins, reducing sugars and anthocyanins; and absence of only alkaloids, anthraquinones, saponins, fixed oils and lipids [27]. Those previously notations confirmed the current research out findings as the methanol extract of *E. foeminea* had bid the strongest anti-proliferative effect combined with the highest cytostatic one which is explained by the lowest cell viability among other its extract types in addition to their morphological alterations. This also could be correlated to what has been stated in the literature that the compounds found in *E. foeminea* ethanol extract induced the caspase 3 dependent-cell apoptosis [28]. It is noteworthy that *E. aphylla* ethanol extract is the one that is characterized by the strongest antitumor effect against HeLa cell line among all other extract types under study at all investigated concentrations. This highlights again the importance and necessity of its elaborated phytochemical screening investigation. Additionally, all the identified MTT assay cell viability results and the inverted microscope morphological screening displayed a concentration dependent manner for all studied extract types.

Conclusion

Ephedra aphylla and *E. foeminea* have a cytostatic effect rather than cytotoxic one against HeLa cell line in which cell growth is inhibited as a result of the phenolic chemicals that are present in all extract types. Thus, it is suggested that these plants be employed as antitumor medicines either alone or in combination to treat cancer. Along with that, extensive *in vivo* studies are necessary for more understanding of their medicinal applications against cervical cancer. The selection of an extraction solvent is one of the key steps in the processing of natural products. Together, these findings demonstrated how the plant species, the utilized solvent and the examined concentrations affect the anticancer impact of plant extracts. Therefore, the traditionally used medicinal plants in Palestine can be a source for newly cytostatic anticancer agents. However, further research is required for full characterization of their action in order to either include or exclude the activity of additional compounds in the plant species being investigated in this study. In conclusion, this research validates the effectiveness of the ethnobotanical approach to screening plants for bioactive compounds and supports the traditional usage of medicinal herbs in the treatment of disease.

Acknowledgement
All thanks and appreciation to Biology and Biotechnology Department at An-Najah National University for allowing us to use their laboratory.

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