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Original Research Article

Phytochemical characterization and anti-cancer properties of extract of *Ephedra foeminea* (Ephedraceae) aerial parts

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Abstract

Purpose: *To evaluate the phytochemical profile of methanol extract of Ephedra foeminea and assess its anti-carcer effect on a large set of normal and cancerous cell lines*

Methods: *Extraction of air-dried powder of aerial parts of E. foeminea was carried out with methanol. The bioactive compounds in the extract were determined using gas chromatography/mass spectrometry (GC-MS). The anti-cancer effect of the extract was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay against various types of normal and cancer cell lines. Serial concentrations of plant extract were used, ranging from 7.812 to1000 µg/mL. Doxorubicin (DOX) served as standard drug. The half-maximal concentration (IC_{50}) values of the extract and DOX for each cell line were determined, and the selectivity index (SI) was computed.*

Results: *Phytochemical analysis showed that the extract contained several bioactive compounds, including alkaloids, flavonoids, sterols and fatty acids. The hazardous ephedra alkaloids (ephedrine and pseudoephedrine) were absent in the plant extract. The extract showed significant anti-proliferative activity against cancer cell lines, when compared with the positive control, doxorubicin ($p < 0.05$). Selective and concentration-dependent cytotoxicity was exhibited in cancer cell lines of breast (MCF-7), lung (A549), colon (Caco-2), liver (HepG-2) and prostate (PC-3). Weak selectivity was produced in other cancer cell lines, i.e., human epithelioma (Hep-2) and cervical carcinoma (Hela). Interestingly, non-cancerous cells showed no or weak cytotoxicity.*

Conclusion: *Ephedra foeminea exerts potential selectivity in anti-proliferative effect against some cancer cell lines. Thus, it is a promising drug source for the production of new and selective anti-cancer medicines.*

Keywords: *Alkaloids, Cytotoxicity, Ephedra foeminea, Medicinal plants, Phytochemical analysis*

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INTRODUCTION

The genus *Ephedra* is a member of the *Ephedraceae* family containing approximately 69 species of non-flowering seed plants. It can be found in different arid and semi-arid areas of the world, especially North Africa, Asia, America and Europe. These *Ephedra* species are characterized by ecological, economic, and recently, medicinal values [1]. The *Ephedra* species are associated with many traditional medicinal uses, including treatment of allergies, cough, oedema, bronchial asthma, headaches, fever, flu, nasal congestion, chills and colds [2]. Moreover, they are the main natural sources of bioactive compounds, including alkaloids such as ephedrine and pseudoephedrine; flavonoids and phenolic compounds such as pro-anthocyanidins, amino acid derivatives, and volatile organic compounds in essential oils such as aromatic and terpenoid compounds [3].

Recently, the idea of using *Ephedra* as an alternative to cancer therapy has become popular. This is particularly true of *Ephedra foeminea*, which many cancer patients in the Middle East region utilise because of the belief that it has cancer-curative properties [4]. Based on these observations, limited studies have investigated the effect of crude extracts of various types of *Ephedra foeminea* on different cancer cell lines [4-7]. In a study using an aqueous decoction of *Ephedra foeminea*, there were no significant effects on the viability of MDA-MB231 and SKBR3 breast cancer cell lines [5]. In contrast, another study showed that extracts and fruit juice of *Ephedra foeminea* significantly decreased the viability of colon cancer cells (HTC116) and breast cancer cells (MDA-MB-213), but no toxic effect was exerted on lung carcinomatous cells (A549) [7]. Likewise, *Ephedra foeminea* extracts exhibited a dose-dependent decrease in viability of human osteosarcoma cells (U2OS) [7]. These contradictory results may be attributed mainly to differences in constituents of various types of *Ephedra foeminea* extracts. There are limited data on the phytochemical constituents of crude extracts of *Ephedra foeminea.* A recent study reported the absence of ephedrine and pseudoephedrine alkaloids from *Ephedra foeminea* [5]. The results obtained from the above studies are contradictory, and further investigations about anti-cancer properties and phytochemical analysis of *Ephedra foeminea* are required.

Since there are limited studies and contradictory results on the anti-cancer properties of *Ephedra foeminea*, this study was aimed at phytochemically analysing a methanol extract of *Ephedra foeminea,* and evaluating its cancer-killing effects on different cancer cell lines.

EXPERIMENTAL

Plant material

The aerial parts of *Ephedra foeminea* were collected from South Jordan, Mutah, Al-Karak, Jordan during the fruiting stage from May to July 2020. They were washed, dried in the shade, and ground to a fine powder. The plant was identified by Professor Al-Gohary, a taxonomist in Department of Plant Ecology and Range, Desert Research Centre, Cairo, Egypt. A voucher specimen (CAIH-0439S) was deposited at the Herbarium of the Centre.

Plant extraction

One hundred grams of air-dried powder of the plant was extracted with methanol (500 ml × 3 times) for 72 h using the cold percolation method. The methanolic extract was filtered using a Buchner funnel. Then, the filtrate was evaporated in a rotary evaporator at a temperature below 40 °C, after which the residue was dried in a dissector to give 25 g/100 g dry weight (DW) of *Ephedra foeminea*. The crude methanol extract was subjected to GC/MS analysis for the determination of bioactive compounds.

Phytochemical screening

The samples of *Ephedra foeminea* were analyzed using GC-MS (Shimadzu GC/MS-QP 5050A) with a DBI column and He carrier gas maintained at a flow rate of 1 ml/min. Injector and ion source temperature was 280 °C, and oven temperature was raised from 40 °C to 280 °C with a ramp of 2 °C/min and a withholding period of 7.5 min. The amount of sample injected was 1 µL. Sample ionization took place in electron impact mode at an ionization voltage of 70 eV and mass span of 50 - 650 m/z. The data interpretation was carried out with Wiley & Nist library databases.

Culture of cell lines

Hepatocellular carcinoma (HepG-2), human epithelioma (Hep-2), breast cancer (MCF-7), as well as Hela, PC-3, Caco-2, A549 and WI-38 cells were sourced from ATTC, USA. The cells were cultured in RPMI 1640 containing 10 % FCS, L-Gln (2 mM) and Na pyruvate (1 mM). In 75-cm^2 cell culture treated flasks (Corning, Amsterdam, Netherlands), the cells were

regularly kept as single-layer cultures (T75 flask) at 37 °C in a 5 % CO_2 incubator, with regular sub-culturing carried out [8].

In vitro cytotoxic effect of *Ephedra foeminea*

The cytotoxic effect of *Ephedra foeminea* was evaluated using the MTT assay. The assay relies on the mitochondrial-dependent reduction of yellow MTT which is converted to purple crystals of formazan through a mitochondrial reduction reaction [9]. Once 70 % confluence was reached, the cells were plated in 96-well plates at a density of $1x10^4$ cells/mL (180 µL/well), followed by overnight incubation at 37 °C in a 5-% CO_2 incubator. After forming a confluent layer of cells, diluted plant extract (0.1 mL) or medium was put in appropriate wells. Serial dilutions of plant extract were obtained by dissolving the extract in growth medium to make final concentrations of 7.812; 15.625; 31.25; 62.5; 125; 250; 500 and 1000 µg/mL, followed by incubation as before, for 96 h in the dark. Thereafter, each well medium was decanted and replaced with 20 µL of 5 mg/mL MTT solution. To allow the development of formazan crystals, the plates were incubated in the dark for 4 h at 37 °C. The supernatant was removed and formazan crystals formed were re-suspended in 150 µL of DMSO. The absorbance of each of the formazan solutions was read at 560 nm in Mindray-96A microplate reader. Doxorubicin was used standard drug, while the medium served as negative control. The assay was carried out in triplicate for each group.

Determination of IC_{50}

The IC_{50} values of the extract and DOX for the cell lines were determined with GraphPad Prism ver. 7 software California, U.S.A. Cell growth inhibition (GI) was calculated using Eq 1.

$$GI\ (\%) = \{100 - (At/Ac)\}100 \quad \ldots\ldots\ldots (1)$$

where *At* and *Ac* are the absorbance values of the test and control samples, respectively.

Selectivity index

The degree of plant extract selectivity towards cancer cells was calculated with selectivity index (SI). This is the ratio of IC_{50} value of plant extract in normal cells (WI-38) to IC_{50} value of plant extract in each cancer cell line. Values >3 suggest that the extract has *in vitro* selective cancer activity, relative to non-cancer cells [10].

Statistical analysis

Statistical analysis was performed using Statistical Packages for Social Sciences (SPSS) software version 19. The results are presented as mean ± standard deviation (SD). Statistical significance of differences was evaluated with *t*-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Phytochemical profile of extract

The GC/MS analysis of the methanol extract revealed that the aerial parts of the *Ephedra foeminea* contained different classes of bioactive metabolites, including alkaloids, flavonoids, sterols and fatty acids (Table 1 and Figure 1). The component with the highest percentage abundance in the methanolic extract of *Ephedra foeminea* was 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl (Relative abundance (RA) = 23.54 %). Eleven alkaloids were identified. These were imidazole alkaloids: imidazole,2-amino-5-[(2-carboxy)vinyl] (R.A. = 0.23 %) and 1H-imidazo[1,2 c]oxazol-5-one (R.A. = 0.10 %); indole alkaloids: 3-(2',6'dichlorobenzylidene)-1,3-dihydroindol-2-one (R.A. = 0.2%) and 2-acetyl-3-(2-benzenesulphonamido)ethyl-7-methoxyindole (R.A. = 0.15 %). Some flavonoids were present: quercetin (R.A. = 3.06 %) and lucenin 2 (R.A. = 2.33 %). Stigmast-5-en-3-ol (R.A. = 1.59 %) sterol compound was also detected. Many fatty acids were identified viz 9-octadecenoic acid (R.A. = 8.03 %), hexadecanoic acid (R.A. = 5.58 %), pentadecanoic acid (R.A. = 0.81%), 13-methyl-methyl ester (R.A. = 0.81 %) and stearic acid, 3-(octadecyloxy) propyl ester (R.A. = 0.82 %).

In vitro cytotoxicity

The cytotoxicity assay (MTT) of the methanol extract was done at different concentrations i.e. 7.812, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL with respect to seven human cancer cell lines. For each cell line, complete concentration response curve and IC_{50} value were developed. The principle employed to classify the cytotoxic effect of the extract was adopted from the U.S. National Cancer Institute (NCI) and Geran protocol [11, 12]. The classification is as follows: $IC_{50} \leq 20$ µg/mL = highly cytotoxic, IC_{50} of 21 - 200 µg/mL = moderately cytotoxic, IC_{50} of 201 - 500 µg/mL = weakly cytotoxic, and $IC_{50} > 501$ µg/mL = not cytotoxic.

Table 1: Chemical constituents of *Ephedra foeminea* identified using GC/MS

S/No.	Compound	MF	MW	R.A.%	Rt
1.	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, trans	C28H44O4	444	8.03	8.72
2.	Benzeneethanamine, 2,5-dimethoxy-α,4-dimethyl	C12H19NO2	209	0.40	11.72
3.	3-(2',6'Dichlorobenzylidene)-1,3-dihydroindol-2-one	C15H9Cl2NO	289	0.20	12.31
4.	2-(dimethylamino)-3-phenylbenzo[b]thiophene	C16H15NS	253	0.1	12.46
5.	Guanosine, 2'-O-methyl	C11H15N5O5	297	0.35	13.82
6.	Imidazole,2-amino-5-[(2-carboxy)vinyl]	C6H7N3O2	153	0.23	14.01
7.	Desulphosinigrin	C10H17NO6S	279	0.51	19.30
8.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	C6H8O4	144	23.54	20.30
9.	Glucosamine,N-acetyl-N-benzoyl	C15H19NO7	325	1.03	22.9
10.	Quercetin	C15H10O7	344	3.06	29.45
11.	1H-Imidazo[1,2 c]oxazol-5-one	C18H30N2O2	306	0.10	31.14
12.	2-Acetyl-3-(2-benzenesulphonamido)ethyl-7-methoxyindole	C19H20N2O4S	372	0.15	31.33
13.	Hexadecanoic acid	C35H68O5	568	5.58	34.64
14.	Dasycarpidan-1-methanol, acetate	C20H26N2O2	326	1.51	34.64
15.	Lucenin 2	C27H30O16	610	2.33	36.15
16.	15-Tetracosenoic acid, methyl ester	C25H48O2	380	0.05	36.45
17.	Pentadecanoic acid, 13-methyl-,methyl ester	C17H34O2	270	0.81	37.18
18.	Stigmast-5-en-3-ol	C29H50O	414	1.59	40.56
19.	3-(4-Chlorophenyl)-4,6-dimethoxyindole-2,7-dicarbaldehyde	C18H14ClNO4	343	0.39	44.40
20.	Stearic acid, 3-(octadecyloxy) propyl ester	C39H78O3	594	0.82	47.92

(MF: Molecular formula: MW: Molecular weight: R.A: Relative abundance: Rt: Retention time)

Table 2: Cytotoxicity of methanol extract of *Ephedra foeminea* on various cell lines

Conc µg/mL	WI-38	HepG2	Hep2	MCF-7	HELA	PC3	Caco-2	A549
7.812	100 ±5	99.6 ±3	99.8 ± 3	100 ± 4	99.9 ± 5	99.8 ± 6	99.1 ± 3	99.4 ± 4
15.625	99.9 ± 4	99.8 ± 5	99.4 ± 6	95.9 ± 5	99 ± 4	99.8 ± 3	99.4 ± 4	99.2 ± 3
31.25	99.3 ± 3	99 ± 4	99 ± 3	52 ± 6	99.7 ± 4	99.5 ± 5	87.9 ± 4	96.6 ± 5
62.5	99.1 ± 4	83.5 ± 5	95.1 ± 4	38.4 ± 5	95.7 ± 4	72 ± 3	45.9 ± 5	53.7± 4
125	99 ± 5	19.7 ± 6	75.7 ± 3	31.4 ± 5	58.6 ± 4	31.9 ± 3	33.1 ± 4	10.4± 6
250	76.7 ± 4	7.4 ± 7	19.5 ± 4	21 ± 5	30.1 ± 7	10.1± 4	14 ± 5	6.7 ± 6
500	42.8 ± 7	5.5 ± 4	5.4 ± 6	9.6 ± 4	16 ± 5	5.3 ± 7	6.5 ± 4	5.8 ± 4
1000	21.7 ± 5	17.1 ± 5	4.8 ± 4	4.9 ± 8	4.9 ± 4	4.4 ± 5	4.2 ± 8	4.1 ± 7

Each value represents mean ± SD from three independent experiments.

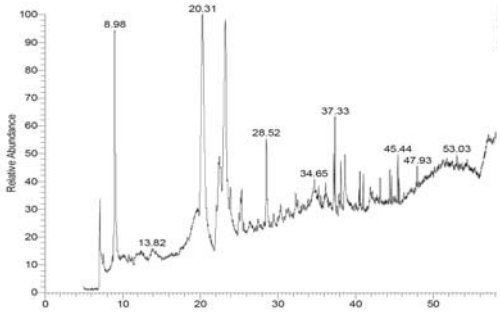

Figure 1: GS/MS spectra of *E. foeminea*

Results showed marked dose-dependent reduction in the viability of all cancer cell lines, relative to DOX (Table 2). Based on the NCI criteria shown above, the *Ephedra foeminea* extract exerted moderate cytotoxic activity against all the cancer cell lines. The highest cytotoxic activity of the plant extract was exhibited against MCF-7, A549 and Caco-2 cell lines, with IC_{50} values of 52 ± 3, 65 ± 4 and 72 ± 2, respectively. This reduction was marked and dose-dependent, relative to DOX ($p < 0.001$). Various potencies of cytotoxic activity were displayed in other cell lines, relative to DOX, with IC_{50} values of 93 ± 8 (HepG-2), 95 ± 5 (PC-3), 168 ± 18 (Hela) and 171 ± 11 (Hep-2). Importantly, no cytotoxic activity was exhibited by the plant extract in healthy foetal (WI 38) cell line (IC_{50} = 464 ± 37 µg/mL; Table 3).

Table 3: Cytotoxic effects of methanol extract of *Ephedra foeminea* on cancer cell lines

	IC_{50} (µg/mL)	
Cell line	***Extract***	***Doxorubicin***
HepG-2	93 ± 8 **	32 ± 4
Hep-2	171 ± 11 *	31 ± 3
MCF-7	52 ± 3 **	23 ± 1
Hela	168 ± 18 **	38 ± 4
PC-3	95 ± 5 **	122 ± 6
Caco-2	72 ± 2**	79 ± 13
A549	65 ± 4 **	85 ± 2
WI-38	464 ± 37 *	79.7±2.5

*Each value represents mean ± SD from three independent experiments. *Significant difference from doxorubicin P<0.05; **High significant difference from doxorubicin P<0.001*

Microscopic analysis of the various cell lines treated for 72 h with *Ephedra foeminea* extract at

a dose of 125 µg/mL was conducted. The examination showed that the plant extract treatment caused the cells of PC-3, MCF-7, Caco-2 and A549 to shrink and become rounded and detached, when compared to untreated control cells. On the other hand, no changes in cell morphology were displayed in Hela, HepG-2 and Hep-2 cells (Figure 2).

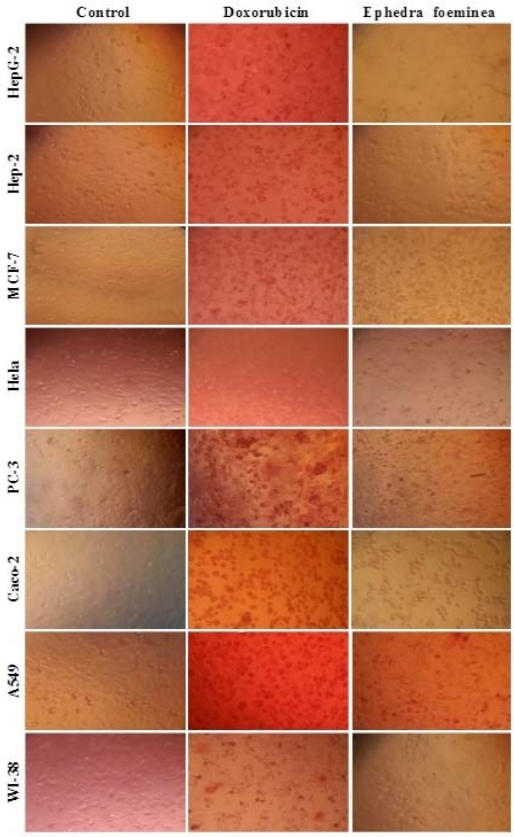

Figure 2: Anticancer impact of *Ephedra foeminea* extract on normal and cancer cell lines. *These cell lines were treated with 125 µg/ml of plants extracts and doxorubicin (positive control) for 72 hours.*

Selective cytotoxic activity

Methanol extract of *Ephedra foeminea* produced highly selective cytotoxicity in MCF-7, A549, Caco-2 cell lines, while weak cytotoxic selectivity was observed against HepG-2 and PC-3 (Table 4). In contrast, no cytotoxic selectivity was exhibited for the plant extract in Hep-2 and Hela cell lines (values < 3).

DISCUSSION

Medicinal herbs are currently playing a leading role in modern drug development. Indeed, many traditional medicines are basically copies or synthetic alterations of natural chemical compounds present in plants. Nowadays, key research investments are dedicated to identifying and characterising new plants with the potential to prevent or hinder development of cancer [13]. Many current techniques may be useful in successful discovery of cancer-suppressing principles from plant sources, as well as elucidation of their modes of action [14]. Although *Ephedra foeminea* has neither a recognised traditional nor an ethno-botanic rationale for curing cancer, the popularity of the herb is on the increase, particularly in the Middle East region [5]. Therefore, this research, explored the phytochemical constituents of methanol crude extract of *Ephedra foeminea*. In addition, we display for the first time, strong evidence of cancer-suppressing effect of *Ephedra foeminea* on a large array of cancer cell lines.

Various plants have been examined for their content of different bioactive compounds and how they affect cell physiological functions, as well as their anti-cancer potential and capacity to prevent cancer cell growth [14]. The phytochemical investigations showed that, unlike other ephedra plants, ephedra alkaloids were absent in *Ephedra foeminea*. This finding is consistent with a previous study which confirmed the absence of potentially hazardous ephedra alkaloids from *Ephedra foeminea* [5].

Interestingly, the analysis found that the major component of the plant methanol extract was 4H-pyran-4 one,2,3-dihydro-3,5-dihydroxy-6-methyl. This compound has been shown to reduce the growth of colon cancer cells by causing apoptotic cell death through inhibition of NF-κB [15]. Furthermore, 9-Octadecenoic acid was one of the main free fatty acids found in the plant methanolic extract. This fatty acid produced potent cytotoxicity against a range of cancer cell lines, particularly colon cancer. This is consistent with the cytotoxicity data which displayed its strong anti-cancer activity against colon cancer cell lines [16,17]. The second major free fatty acid found in the plant extract was hexadecanoic

.

Table 4: Selectivity index values of *Ephedra foeminea* methanol extract and doxorubicin (positive control) for HepG-2, Hep-2, MCF-7, Hela, PC-3, Caco-2 and A549 cancer cells

Extract	SI						
	HepG-2	***Hep-2***	***MCF-7***	***Hela***	***PC3***	***Caco-2***	***A549***
Methanol extract	5	2.7	8.9	2.8	4.9	6.4	7.1

acid. It has been reported that hexadecanoic acid extracted from *Aquilaria malaccensis* leaves exerted antioxidant and anti-cancer effects in various cancer cells [18]. Overall, the major bioactive compounds found in the methanolic extract of *Ephedra foeminea* seem to exert synergetic anti-cancer activity against several cancer cell lines. Further studies are required to fully characterise, isolate and assess the anti-cancer activity of all the bioactive compounds in *Ephedra foeminea.*

Nowadays, naturally-derived compounds are of research interest because they are known to be less toxic than conventional cancer therapy such as chemotherapy. This is particularly true because these compounds may have the potential to target tumour cells without affecting normal cells [14]. The present study reveals, for the first time, a clear selective cytotoxicity in cancer cell lines with no, or minimal effect on normal cells. This selectivity in cytotoxic activity was exhibited only against MCF-7, A549, Caco-2, HepG-2 and PC-3 cell lines. The highest potential cytotoxic activity was reached at certain optimal extract concentrations. The extract showed less selectivity on other cell lines (Hela and Hep-2). These interesting results are consistent with previous studies which demonstrated strong cytotoxic activity of different types of *Ephedra foeminea* crude extracts against certain types of cancer cell lines [4,7]. The ethanolic extract and fruit juice of *Ephedra foeminea* exhibited significant growth-inhibitory activity against colon cancer cells (HTC116) and breast cancer cells (MDA-MB-213) [4]. Moreover, potent cytotoxic activity was displayed in human osteosarcoma cells (U2OS) by treatment with ethyl acetate, ethanol, and water extracts of *Ephedra foeminea* [7]. Importantly, all the IC_{50} values of *Ephedra foeminea* reported here are relatively lower than those obtained in previous studies [4,7]. However, these results contrast with a previous study where water decoction of *Ephedra foeminea* showed no significant cytotoxic effect on MDA-MB231 and SKBR3 breast cancer cell lines [5]. This discrepancy may be attributed to the potency of *Ephedra foeminea,* which probably differs from one cell line to another. In addition, the extraction method used in each study has been shown to influence the final physical, chemical and biological properties of the extract [19]. Alcoholic extracts of *Ephedra foeminea* seem to exert robust cytotoxic activity due to prominent bioactive contents. So far, there is still no mechanistic explanation of the anti-proliferative potential of *Ephedra foeminea.* However, the anti-proliferative potential should be investigated further in future studies to better understand the mechanism involved in the anti-cancer activity of *Ephedra foeminea*.

CONCLUSION

The present study has revealed the presence of several bioactive medicinal components in aerial parts of the *Ephedra foeminea* plant. Importantly, the methanol crude extract of the plant did not contain the hazardous toxic ephedra alkaloids. The extract exerts pronounced cytotoxic activity against most of the various cancer cell lines studied, but not in normal cell line treated with the crude extract. The selectivity in the anti-proliferative activity of *Ephedra foeminea* against certain cancer cell lines holds considerable promise for the isolation of bioactive compounds, thereby providing a basis for developing novel anti-cancer therapies.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

This study was done by the authors named in this manuscript, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Yousef M Al-saraireh, Ahmed M.M. Youssef and Sameeh A Al-Sarayreh. Data were collected and analyzed by Jehad M Al-Shuneigat, Hamzeh M Alrawashdeh and Samir S Mahgoub. Yousef M Al-saraireh wrote the manuscript. All authors read and approved the manuscript for publication.

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