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Antimicrobial, antioxidant and anticancer activity of *Ephedra alata* growing in East of Libya

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Highlights

- **Methanolic extract of *Ephedra alata* plant contains alkaloids, carbohydrates, flavonoids, steroids, tannins, and phenolic compounds.**
- **The methanolic extract has a significant anti-bacterial activity against representatives of Gram positive and Gram negative as well as has anti-fungal activity.**
- ***Ephedra alata* has a significant antioxidant, and cytotoxic effects.**

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ABSTRACT

Introduction: The plant *Ephedra alata* belongs to the *Ephedriaceae* family. These plants distributed in North Africa, Palestine, Libya, Egypt, Saudi Arabia, and Iraq. *Ephedra alata* has been commonly used in folk medicine in Libya and most of the Arabian countries for treatment of asthma, hay fever, and the common cold.
Aim: This study aimed to investigate the phyto-constituents and to assess its anti-microbial, antioxidant activities, in addition, to study its cytotoxic effect.
Methods: The aerial part dried of the plant was subjected to a phytochemical screening and investigated for their antimicrobial activity, antioxidant effect, and cytotoxic effects**.**
Results: The phytochemical investigation of the methanolic extracts of *Ephedra alata* revealed the presence of alkaloids, carbohydrates, flavonoides, steroids, tannins, and phenolic compounds. The results of anti-microbial activity revealed that; there was a significant antibacterial activity against representatives of Gram positive (*Staphylococcus aureus, Bacillus subtilis*) and Gram-negative (*Escherichia coli, Pseudomonas aeruginosa*), while there was no antifungal effect against *Aspergillus flavus* and *Candida albicans.* In terms of antioxidant activity, the methanolic extract of *Ephedra alata* showed a significant scavenging effect on the DPPH radical, compared to gallic acid. The results showed an honest correlation between antioxidant activity and flavonoid content. The plant extract displayed a significant cytotoxic effect on MCF7with IC_{50} and IC_{90} comparing with doxorubicin as a positive control.
Conclusion: The results indicated that the extract of *Ephedra alata* had significant antioxidant, and cytotoxic effects in addition to their antimicrobial activity.

1. Introduction

It is known that complementary and alternative therapies are increasing throughout the world. Peoples looking for unconventional therapies, such as herbal medicine when conventional medicine fails to cure chronic diseases. Therefore, medicinal herbs are gaining more attention and popularity to replace synthetic medicine in the treatment and prevention of several diseases. WHO; reported that about 80% of the world use traditional therapies, such as plant, for health care (WHO, 2004).

It is reported that several types of plant extracts consist of a large range of phytochemicals, which used for different therapeutic effects and many present drugs were obtained or derived from phytochemicals (Atanasov *et al.*, 2015). Through time, plants have considered as an important source of new drugs with different therapeutic effects such as antimicrobial, antioxidative, anticancer, and other effects.

The antioxidant effects exert by phenolic components, which act as free radical-scavenger, oxygen radical absorbent and metal ions chelator and interfere with the cytochrome P450 mixed-function oxidases (Reyhane Hoshyar *et al.*, 2015). It was reported that phenolic compounds reduce cellular damages of oxidative stress and commonly used for cancer treatment and prevention (Sen and Chakraborty 2011, Ma *et al.*, 2011).

It is known that multiple drug resistance has developed due to misuse of antimicrobial drugs and individuals recognized that the effective life span of antibiotics is limited and microbial resistance developed as a result of misuse of traditional antibiotics (Alam *et al.*, 2009). In addition, using antibiotics is often associated with side effects. Therefore, it was necessary to find new alternative antimicrobial drugs (Agarwal *et al.*, 1996). Accordingly, plants have been considered as an important source of new antimicrobial agents, which used to treat infectious diseases (Cowan, 1999). Furthermore, Plants have a long history of use in the treatment of cancer and continue to be a major source of new drugs (Cragg and Newman 2013).

Ephedra species are widespread in many countries, native to Southwestern North America, Southern Europe and North Africa. *Ephedra alata* is among several medicinal plants, which extensively spread in Libya and used traditionally. Ephedra used in most Arabian countries in common cold, fever, and to cure nasal congestion. It was used also in case of allergies and asthma. In addition, it was

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added as dietary nutritional supplements for their pronounced stimulant effects. Currently, the interest to use ephedra extract in a form of capsules or hydro-alcoholic extract has been increased (White *et al*., 1997; Barnes *et al*., 2007).

Ephedra is a genus of gymnosperm shrubs, It is a small shrub with less than 1 meter height. Today, the main prominence of Ephedra species in the medicine is due to the presence of the alkaloids derived from phenyl-alanine (e.g., ephedrine and other related compounds, such as pseudoephedrine, norpseudoephedrine, norephedrine, methylephedrine, and methyl pseudoephedrine), which has a sympathomimetic effect. Also, Ephedra contains another constituent which considered as Non-alkaloid Parts such as Volatile oils, Sterols and triterpenes, Carbohydrates, and Flavonoids (Ibragic and Sofi, 2015).

There are some studies that described the biological activity of Ephedra species, there is a paucity of studies in the literature on the antimicrobial activity of *Ephedra alata* Decne. Ethanolic extracts of the aerial part of Tunisian *Ephedra alata* Decne contain polyphenolic phytocompounds that provide the antioxidant activity of the plant. The extract has a pronounce antimicrobial activity specifically against the Gram-positive cocci and Candida *Spp.* and cytotoxic effect against the MCF-7 human breast cancer cell line (Danciu *et al*., 2019).

Although, the extract of this herb (decoction, aqueous extract, mixture and another form) is proposed to patients and used as traditional medicine for several decades ago in countries of the Mediterranean basin. There is not any scientific data about Libyan *Ephedra Alata.* For that reason, the present study aimed to identify the phytochemical characterization of the methanolic extract of the aerial part of Libyan *Ephedra alata*. In addition, the present study designed to determine the potential antibacterial, antioxidant activities and the cytotoxic effect of the same extract.

2. Materials and Methods

2.1. Plant collection and preparation

The aerial part of *Ephedra alata* was collected from the village in East mountain of Libya called Jardaas Al`Abid in June 2018. The plant was identified in the department of Pharmacognosy (Faculty of Pharmacy, University of Benghazi) and left to dry at open air for 72 hours. The dried plant powdered finely by using a blender to be used for extraction, phytochemical screening, chromatographic screening, antimicrobial, antioxidant and cytotoxic studies.

2.2. Extraction of the plant

The methanolic extract (70%) obtained through exhaustive cold maceration of the dried plant for 7 days. The solvent was evaporated in a rotary evaporator under reduced pressure (at 40°C). The solid residue then dissolved in 2 ml by the same solvent and stored at 4°C.

2.3. Phytochemical qualitative screening

The phytochemical screening of methanol extract was done to identify the main groups of chemical constituents such as carbohydrate, phenols and tannins, flavonoids, saponins, glycosides**,** steroids and terpenoids using standard qualitative methods as described by Trease and Evans (2002).

2.4. Antimicrobial study

Antibacterial activity was performed using the agar well diffusion method. Strains concentrations on plates were carried out using modified BD quality control procedures (Baver *et al*., 1996). Briefly, fresh colonies from Nutrient ager plates suspended into normal saline (pH 7). Microbial concentrations were adjusted by the turbidity, after 15 minutes the inoculum was collected by dipping a sterile cotton swab into the suspension, swab rotated several times on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swabs then applied on Mueller-Hinton agar (pH 7.4), plates then left for at least 3 min to allow inoculum to be absorbed. The plates were incubated while inverted, at 35-37°C for 24-48 hours in case of bacteria and at 25°C for 48 hours in case of fungi. 10 µl of Dimethyl sulfoxide (DMSO) was used as negative control and tetracycline and amphotericin B were used as positive controls (standard) for bacteria and fungi, respectively.

2.5. The measurement of antioxidant activity

The Antioxidant activity of methanolic extract of *E. alata* was examined on the basis of scavenging effects using stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Sreenivasan *et al*., 2007). Freshly DPPH solution was prepared and kept in the dark at 4°C. 3.2 μl of (DPPH) was dissolved in 25 ml methanol with steering regularly up to 30 minutes. Methanolic solutions of DPPH (90 μl) were added to 10 μl of plants extracts solution with different concentration. The extract was incubated for 30 minutes at 37°C at room temperature; after incubation, the absorbance was measured at 490 nm using multi-plate reader (Bio-Tek Elx800™, Instruments, Inc., USA). Galic acid was used as standard whereas dimethyl sulfoxide (DMSO) was used as a negative control. Every determination was performed in triplicate. Percentage inhibition of the radical scavenging activity of test samples was calculated (Amiš *et al*., 2003). Extracts concentration providing 50% inhibitions (IC_{50}) was calculated from the graph plotted of inhibition percentage against extracts concentration.

2.6. Cytotoxic effect on human cell lines.

The cytotoxic activity of the methanolic extract of the aerial parts of *E. alata* was assessed by the mitochondrial-dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan (Mosmann, 1983). The cells were plated in 96-multi well plate (10^4 cell/well) for 24 hrs before treatment with the tested extracts to allow the attachment of cells to the wall of the plate. Different concentrations of each extract under test (0, 1, 2.5, 5, 10 mg/well) were added to the cell monolayer. Triplicate wells were prepared for each dose. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration was platted to get the survival curve of each tumor cell line after the specified concentration. By fitting the curves to the straight-line equation, IC_{50} and IC_{90} could be calculated. This work was performed on MCF7 [Human Caucasian breast adenocarcinoma]. The IC50 (dose of the extract which reduces survival to 50% by µg/ml) was calculated.

3. Results

The qualitative phytochemical screening of the methanol extract of *Ephedra alata* was carried out in order to assess the presence of bioactive compounds. The presence of Carbohydrates, Sterols and triterpenes, Flavonoids, Tannin & Volatile oils, and alkaloides was investigated in the extract (Table 1).

Table 1

Phytochemical composition of the methanolic extract of *E. alata*

Test	Constituents	Aerial parts
Sudan III	Steam volatile substances	+
Lieberman-burchard's test	Sterols and/or triterpenes	+
Fehling's solutions test Benedict's reagent test	Carbohydrates	+
Shinoda test	Flavonoids (free and combined)	+
Ferric chloride test	Tannins (condensed)	+
Dragendoff's test Mayer's test Wagner's test	Alkaloids	+
"foam formation" Froth Test	Saponins	–
1% HCl	Coumarins	–
Borntrager's test	Anthraquinones	–
Keller-kilani test	Cardiac glycosides	–

(+) found, (–) not found

In the present study, the antimicrobial activity of methanolic extract of *Ephedra alata* was studied against the gram positive bacteria (*Staphylococcus aureus*, and *Bacillus subtilis*) and gram negative bacteria: (*Escherichia coli*, and *Pseudomonas aeruginosa.* While, the antifungal effect of extract was done against *Aspergillus flavus* and *Candida albicans*. Zone of inhibition were measured and diameters less than 5 mm were considered as having an inhibitory effect. Diameter of zone of inhibition (mm) and potency percentage of the tested samples of the studied was recorded in Table 2.

Table 2

The antimicrobial activity of methanolic extract of *E. alata*

		Diameter of zone of inhibition (mm) (% potency)					
		Gram positive bacteria		Gram negative bacteria		Fungi	
		S. Aureus	*B. Subtilis*	*E Coli*	*P. aeruginosa*	*A. flavus*	*C. Albicans*
Standard	Tetracycline	19 mm	29 mm	30 mm	30 mm	–	–
	Amphotericin B	–	–	–	–	17 mm	19 mm
	Methanolic extract	12 mm 63.15%	12 mm 41.38%	14 mm 46.67%	13 mm 43.33%	3 mm 18%	4 mm 21%

To evaluate the effect of extracts on cell proliferation, the cytotoxic effects was investigated on the growth of human breast cancer (MCF-7). The result demonstrated in Table 3 showed a significant cytotoxic effect on MCF7with IC_{50} and IC_{90} (38.7±2.8 and 99.9±0.65 respectively) comparing with doxorubicin as positive control.

Table 3

Cytotoxic effect of methanolic extract on MCF7

Sample Code	IC_{50} (µg/ml)	IC_{90} (µg/ml)	Remarks
E. alata	38.7	99.9	83.2% at 100ppm
DMSO	--------	--------	3% at 100ppm
Negative control	--------	--------	0 %
Positive control	28.3	48.8	

IC_{50}: Lethal Concentration of the sample, which causes the death of 50% of cells in 48 hrs; IC_{90}: Lethal concentration of the sample, which causes the death of 90% of cells in 48 hrs

To determine free radical scavenging ability of plant extract; the DPPH method was used. The results (Table 4) indicated that the methanolic extract of *E. alata* showed significant scavenging effects on the DPPH radical, compared to gallic acid.

Table 4

Antioxidant effect of methanolic extract of *E. alata*

Sample	**DPPH % inhibition** (Mean±SEM)
Methanolic extract of *E. alata*	0.16±0.02
Gallic Acid	0.03±0.002

4. Discussion

4.1. Phytochemical qualitative screening

According to the results illustrated in Table 1, it was found that the aerial parts of the plant were responded almost similar to the applied tests to give a strongly positive response which indicated that; the plant extract was enriched in sterols and/or triterpenes, carbohydrates, flavonoids (free and combined), tannin and alkaloid. Meanwhile, coumarins, anthraquinones, saponin and cardiac glycosides were undetected and considered as absent.

4.2. Antimicrobial activity activities

The antimicrobial activity of methanolic extract of *Ephedra alata* using a disc diffusion method was studied against the gram positive bacteria (*Staphylococcus aureus*, and *Bacillus subtilis*) and gram negative bacteria: (*Escherichia coli*, and *Pseudomonas aeruginosa.* While the antifungal effect of the extract was done against *Aspergillus flavus* and *Candida albicans*. Zone of inhibition was measured and diameters less than 5 mm were considered as having an inhibitory effect. The diameter of the zone of inhibition (mm) and potency percentage of the tested samples of the studied was recorded in Table 2.

The results were showed a powerful antibacterial activity of methanolic extract of the studied plant against gram positive specially *Staphylococcus aureus* with inhibition zone (63.15%) which was the most sensitive strain to the extract. Moderate activity for both *Bacillus subtilis* and Gram-negative bacteria; *Pseudomonus aeruginosa* and *Escherichia coli* were obtained with the same extract, which showed nearly similar effects with zones of inhibition 41.38%, 43.33% and 46.67% respectively. This result supports the previous studies which presented that butanol, ethyl acetate, and dichloromethane extracts from *Ephedra alata* were active against both Gram-positive and G- bacteria (Chebouat *et al*., 2014). Concerning the antifungal activity, the effect of extract against *Aspergillus flavus* represents 18% while the effect against *C. Albicans* was 21%.

4.3. Antioxidant Activity

Evaluation of antioxidant activity is becoming increasingly appropriate within the field of nutrition because it gives beneficial information with relevancy health helping and functional quality of raw materials whether or not they are fruits, vegetables, or medicinal plants. This factor accounts for the presence of efficient oxygen radical scavengers, like phenolic compounds. The antioxidant activity of phenolic compounds is principally due to their redox properties, which make them act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Scalfi *et al.,* 2000).

The methanolic extract of *E. alata* had significant scavenging effects on the DPPH radical, compared to gallic acid. The results showed an honest correlation between antioxidant activity and flavonoid contents. This finding was also supported by earlier reports that showed that the *Ephedra alata* grown in Palestine is rich in antioxidants, phenolic compounds and flavonoids. Their antioxidant activity is comparable or higher to that of Ephedra of other countries. There is a correlation between antioxidant activities and total phenolic content (Al-Rimawi *et al*., 2017).

4.4. Cytotoxic Activity

The extract of *E. alata* showed a significant cytotoxic effect on MCF7with IC_{50} and IC_{90} (38.7±2.8 and 99.9±0.65 respectively) comparing with doxorubicin as a positive control. This study was found a similar profile to study reported that some species of Ephedra have been assigned anticancer potential against various cell lines. For instance, extracts obtained with different solvents from *Ephedra aphylla* have shown antiproliferative activity against the T47D and MCF-7 carcinoma. The identical study showed that the

extracts have weak antiproliferative potential against Vero, a normal kidney cell line (Al-Awaida *et al*., 2018).

5. Conclusion

The phytochemical screening showed that the *Ephedra alata* plant extract contains a mixture of phytochemicals such as alkaloids, reducing sugars, flavonoids and phenolic compounds. The results of the antimicrobial activity of the methanolic extract were revealed that the methanolic extract showed antibacterial activity when used against 4 strains bacteria (2 gram positive) and (2 gram negative) as well as antifungal activities. According to the findings, it could confirm that the plant has potent antioxidant properties. Furthermore, it showed a significant cytotoxic effect on MCF7. Consequently, *Ephedra alata* can be considered a good choice for biological and chemical analysis and can be further subjected to the isolation of the therapeutically active compounds with anticancer potency and also for further pharmacological evaluations. Moreover, other parts of the examined plant are also needed to be assessed for their antimicrobial, antioxidant and cytotoxic activities.

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