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Quantification of total bioactive contents and evaluation of the antioxidant and antibacterial activities of crude extracts from *Ephedra altissima* Desf.

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ABSTRACT. The present work describes the *in vitro* biological activities of the crude extracts (petroleum ether, ethyl acetate and *n*-butanol) prepared from the species *Ephedra altissima* Desf. The estimation of total phenolic, flavonoid and tannin contents were carried out using the Folin-Ciocalteu, trichloroaluminum and acidified vanillin methods, respectively. The evaluation of the *in vitro* antioxidant activities were performed by three different methods namely: scavenging of the free radical ABTS, permanganate reducing antioxidant capacity, and potentiometric assay. In addition, the antibacterial activity was assessed by the agar disk diffusion assay against seven bacterial strains. The results of the phytochemical screening revealed the presence of several types of secondary metabolites. The EtOAc extract exhibited the highest content of phenols (125.62 ± 1.51 µg EGA mg^{-1} of extract). The greatest flavonoid and tannin contents were observed for *n*-BuOH extract (19.18 ± 0.39 µg EQ mg^{-1} of extract and 8.95 ± 1.70 µg EC mg^{-1} of extract, respectively). Moreover, the EtOAc extract revealed potent antioxidant activity in all the tested methods. Furthermore, the aqueous extract from the species *E. altissima* showed a good ability to reduce iron III to iron II with a value of 0.68 ± 0.3 moL eq L^{-1} in potentiometric assay. All the crude extracts (PE, EtOAc and *n*-BuOH) displayed inhibition of bacterial growth against at least three strains with values of MIC ranging from 3.125 to 50 µg mL^{-1}. Therefore, these results suggest that *Ephedra altissima* could be used as an important source of natural bioactive compounds with antioxidant and antibacterial properties.

Keywords: medicinal plants; Ephedraceae; biological properties; phenols; potentiometry.

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Introduction

Oxidative stress is a complex process, characterized by an imbalance between the production of free radicals and the ability of the body to eliminate these reactive species through the use of endogenous and exogenous antioxidants (Santos-Sánchez, Salas-Coronado, Villanueva-Cañongo, & Hernández-Carlos, 2019). The overproduction of the reactive oxygen species (ROS) could induce different pathologies including cancers, cardiovascular disorders, inflammation, and neurodegenerative diseases (Morales-Gonzalez, 2013). Indeed, many natural and synthetic compounds showed the ability to reduce oxidative stress by the elimination of free radicals using several mechanisms of actions (scavenging, reducing and chelating). The human body is also exposed to a multitude of microorganisms that can invade its tissues under certain conditions, causing serious infectious diseases. The therapy of bacterial infections is mainly based on the use of antibiotics which selectively inhibit certain metabolic pathways of bacteria, without exerting toxic effects on the human body (Leekha, Terrell, & Edson, 2011). In fact, the inappropriate use of these agents can generate multi-resistant strains. For this reason, several research studies are focused on the discovery of new herbal medicines that can effectively combat the phenomenon of multi-resistance and widely help in the prevention of oxidative stress-related diseases (Gupta & Birdi, 2017).

The genus *Ephedra* (Ephedraceae) including approximately 68 species is characterized by perennials plants and shrubs (Christenhusz & Byng, 2016). The species *Ephedra altissima* is a green dioecious shrub with climbing stems and bud leaves, growing in rocky calcareous slopes and in a well-drained loamy soil. This plant is distributed in the Western Sahara of Algeria (Quezel & Santa, 1963) and used in folk medicine for the treatment of various diseases such as vascular hypertension and respiratory diseases.

The aim of this study is the quantification of total phenolic, flavonoid and tannin contents and the evaluation of *in vitro* antioxidant and antibacterial activities of petroleum ether, ethyl acetate and *n*-butanolic extracts obtained from the species *E. altissima*. To the best of our knowledge, there are no previous reports on the antibacterial activity and the antioxidant properties of this species using potentiometric and potassium permanganate methods.

Material and methods

Apparatus

The measurements of potentiometric method were carried out using a pH meter model: Inolab series WTW720 with a potential measurement function. Measurements were performed by a redox potentiometric cell (Platinum/silver-silver chloride electrode; Pt/Ag/AgCl; 3 M; KCl) (Gomel'SGH-185). For spectrophotometric assays, UV-Vis spectrophotometer SHIMADZU UV-1700 was used.

Chemicals

Gallic acid, quercetin, catechin, Folin-Ciocalteu reagent, trichloroaluminum ($AlCl_3$), ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate ($K_2S_2O_8$), sodium carbonate (Na_2CO_3), butylate hydroxyanisole (BHA) ($C_{11}H_{16}O_2$), ascorbic acid ($C_6H_8O_6$), potassium ferricyanide $K_3[Fe(CN)_6]$, potassium ferrocyanide $[K_4[Fe(CN)_6]\times 3H_2O$, vanillin ($C_8H_8O_3$), hydrochloric acid (HCl), potassium permanganate ($KMnO_4$), potassium hydroxide (KOH), potassium dihydrogen orthophosphate (KH_2PO_4), monosodium phosphate ($NaH_2PO_4\times 2H_2O$), sodium dihydrogen phosphate dodecahydrate ($Na_2HPO_4\times 12H_2O$), dimethyl sulfoxide C_2H_6SO, ethanol, *n*-butanol, petroleum ether, ethyl acetate and chloroform (analytical grade) were purchased from Sigma Aldrich.

Microorganisms

The bacterial strains *Staphylococcus aureus* ATCC 25923, *Streptococcus pneumoniae* ATCC 49619, *Streptococcus mutans* ATCC 25175, *Escherichia coli* CIP 7624, *Escherichia coli* DSM 1103, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* DMS 1117 were supplied by the Institute of Pasteur Algiers, Algeria.

Plant material

The plant material *Ephedra altissima* Desf. was collected in November 2016 in Bouhmama Mountain of vicinity of Khenchla (Aures region) and was identified by Professor Bachir Oudjehih, Agronomic Institute of the University of Batna-1. A voucher specimen was kept under the number 937/LCCE.

Extraction procedure

500 g of the species *Ephedra altissima* (aerial parts) were kept in a dry shelter place. After drying for one month, the plant material was finely grounded using an electric grinder to obtain a powder. This latter was macerated twice (5 L × 2) for 72 hours with solvent mixture ethanol/water (70/30) at room temperature. After filtration, the resulting filtrates were evaporated at 40°C to give 400 mL of the aqueous extract which was fractionated using different solvents with increasing polarities (petroleum ether, ethyl acetate and *n*-butanol). The organic fractions were dried with anhydrous sodium sulfate, filtered and evaporated to provide 3.24, 5.7 and 10.12 g of petroleum ether, ethyl acetate and *n*-butanol extracts, respectively.

The aqueous extract was obtained by infusion of 5 g of the aerial parts of *E. altissima* in 100 mL of boiling distilled water for 15 min. After filtration, the resulting solution was diluted with double distilled water to 100 mL.

Phytochemical screening

The presence of different classes of secondary metabolites contained in the species *Ephedra altissima* extracts (PE, EtOAc and *n*-BuOH) were qualitatively tested using the methods described by Farnsworth (1966).

Detection of flavonoids

500 µL of the tested extract were added to 5 drops of acetic acid, 500 µL of distilled water, 500 µL of concentrated hydrochloric acid, 5 drops of isoamilic acid and a piece of magnesium (Mg). Then, the obtained mixture was stirred for 1 min. The appearance of a pink-orange or red-violet coloration in the supernatant layer reveals the presence of flavonoids.

Detection of tannins

1 mL of the diluted sample was added to 500 µL of ferric chloride solution ($FeCl_3$, 1%). After agitation, the observation of a greenish or bluish color indicates the presence of tannins.

Detection of steroids and terpenoids

2 mL of diluted extract were added to 2 mL of chloroform, 500 µL of acetic anhydride and three drops of concentrated sulfuric acid. The appearance of a blue color suggests the presence of steroids and the observation of red color reveals the existence of terpenoids.

Detection of coumarins

1 mL of the diluted sample was treated with 500 µL of hydroxyl ammonium solution (25%; NH_4OH). After agitation, the observation of intense fluorescence under UV light at 365 nm indicates the presence of coumarins.

Detection of carotenoids

1 mL of the prepared extract was mixed with 1 mL of concentrated hydrochloric acid (HCl) and 1 mL of sulfuric acid (H_2SO_4). The appearance of a blue-green color suggests the presence of carotenoids.

Detection of saponins

5 mL of the decoction obtained from *Ephedra altissima* were added to 20 mL of distilled water. The mixture was then stirred in a graduated cylinder for 15 min. The observation of foam reveals the presence of saponins.

Detection of quinones

1 mL of the tested extract was added to 500 µL of sodium hydroxide (NaOH, 1%). After agitation, the appearance of a red color indicates the existence of quinones.

Detection of anthraquinones

50 µg of each crude extract were stirred with 1 mL of chloroform. The mixture was filtered and then 500 µL of an ammonia solution (10%) were added to the filtrate. The resulting solution was stirred and after standing, two layers were formed. The presence of anthraquinones is supported by the appearance of pink or red coloration in the lower alkaline layer.

Detection of alkaloids

3 drops of Dragendorff reagent were added to 1 mL of each sample. The appearance of orange-red precipitate revealed the presence of alkaloids in the tested extract.

Total phenolic content

The total phenolic content of crude extracts from the plant *E. altissima* was determined by the Folin-Ciocalteu method (Mouffouk et al., 2018). 200 µL of different samples were added to 1000 µL of diluted Folin-Ciocalteu reagent (10%). After 4 min, 800 µL of saturated sodium carbonate (75 g L^{-1}) were added. After 2 hours of incubation at room temperature, the absorbance was measured at 765 nm using UV-Vis spectrophotometer. Gallic acid (0-200 µg mL^{-1}) was used for the standard calibration curve. The results were expressed as microgram equivalents of gallic acid per milligram of dry extract (µg EGA mg^{-1} of extract) and calculated as mean value ± SD (n = 3).

Total flavonoid content

The total flavonoid content was estimated using the trichloroaluminum method (Mouffouk et al., 2018). 1 mL of trichloroaluminum solution prepared in water ($AlCl_3$, 2%) was added to 1 mL of the diluted samples. The mixture was stirred vigorously and after 10 minutes of incubation at room temperature, the absorbance was read at 430 nm. Quercetin (2.5-40 µg mL^{-1}) was used to establish the calibration curve. The results were expressed in microgram equivalents of quercetin per milligram of dry extract (µg EQ mg^{-1} of extract).

Total tannin content

The levels of tannins in crude extracts were quantified by acidified vanillin assay (Mouffouk et al., 2018). 400 µL of samples were added to 3 mL of vanillin solution (4% in methanol) and 1.5 mL of concentrated hydrochloric acid. After 15 min of incubation, the absorbance was read at 500 nm. Catechin (10-400 µg mL^{-1}) was used to establish the calibration curve. The results were expressed as microgram equivalents of catechin per milligram of dry extract (µg EC mg^{-1} extract).

Antioxidant activity

ABTS radical scavenging assay

The antioxidant capacity of the crude extracts to reduce the free radical ABTS was estimated (Sadeer, Montesano, Albrizio, Zengin, & Mahomoodally, 2020). ABTS radical cation ($ABTS^{\bullet+}$) was produced by mixing an equal volume of ABTS solution (7 mm) with potassium persulphate (2.45 mm). The prepared mixture was allowed in the dark place at room temperature for 12-16 hours before use. The obtained solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. 13 µL of the sample at different concentrations were added to 187 µL of $ABTS^{\bullet+}$ solution and the absorbance was read at 734 nm after 6 min. The $ABTS^{\bullet+}$ radical scavenging activity of the tested extracts was calculated according to the calibration curve established by Trolox and the results were expressed as µmoL equivalents of Trolox per gram of dry extract (µmoL ET g^{-1} of extract). All the measurements were performed in triplicate for each sample.

Permanganate reducing antioxidant capacity (PRAC)

The ability of crude extracts to reduce MnO_4^- to MnO_4^{-2} in alkaline medium was determined using the method described by Popović, Štajner, Slavko, and Sandra (2012). 1 mL of each sample at different concentrations was added to 3 mL of $KMnO_4$ solution (80.0 µg L^{-1}; pH = 9). The obtained solutions were incubated at 25°C for 30 min and the absorbance was taken at 525 nm using a spectrophotometer. The blank solution was prepared by replacing the sample with phosphate buffer solution. Results were expressed as percentages of scavenging activity using the following Equation 1:

$$\text{Activity (\%)} = [(A_{Blank} - A_{Sample})/ A_{Blank}] \times 100 \quad (1)$$

where:

A_{Blank} is the absorbance of blank solution and A_{Sample} is the absorbance of sample. The experiments were performed in triplicate and the results were transmitted as the mean (values ± SD).

Antioxidant activity by potentiometric method

The antioxidant activity by the potentiometric method is based on the chemical interactions between antioxidants present in the aqueous extract and the mediator system $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$, which induces electron transfer due to a change in the concentration ratio of oxidized and reduced forms, according Equation 2:

$$n\ Fe(III) + AO \rightarrow n\ Fe(II) + AO_{Ox} \quad (2)$$

where:

AO: antioxidants present in aqueous extract; AO_{Ox}: oxidized form of antioxidants; n: number of electrons involved in the reaction. The antioxidant activity (AOA) was calculated as molar equivalents of antioxidant per liter of aqueous extract (moL eq L^{-1}) as Equation 3:

$$AOA = \frac{C_{ox} - \alpha\, C_{red}}{1 + \alpha};\ \alpha = (C_{ox}/C_{red})\ 10^{(E_1 - E_0)nF/2.3RT} \quad (3)$$

where:

E_0 is potential measured in the system before the addition of sample solution; E_1: potential measured in the system after the addition of sample solution; C_{ox}: concentration of the oxidized form of the mediator system in the cell; C_{red}: concentration of the reduced form of the mediator system in the cell; *n* is the degree of dilution; F: Faraday constant; R: Universal gas constant (J moL^{-1} K^{-1}); T: temperature (K).

To prepare the mediator system 9.8 mL of phosphate buffer solution (pH 7.4) were added to 100 µL of $K_3[Fe(CN)_6]$ (1.0 M) and 100 µL of $K_4[Fe(CN)_6]$ (0.01 M). The obtained solution was placed into electrochemical

cell and the initial potential of the platinum electrode was measured (E_0). Then, 200 µL of diluted aqueous extract were introduced into the electrochemical cell. The mixture was stirred and the potential change was measured. The obtained value was recorded as E_1 (Brainina, Stozhko, Bukharinova, Khamzina, & Vidrevich, 2019). Each experiment was done in triplicate and the results were transmitted as mean (values ± SD).

Antibacterial activity

The antibacterial activity of the crude extracts (PE, EtOAc and *n*-BuOH) from the plant *E. altissima* was assessed by agar disk diffusion assay (Mouffouk, Gómez-Ruiz, Benkhaled, Carralero, & Haba, 2019) against seven bacterial strains, including three Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Streptococus pneumoniae* ATCC 49619 and *Streptococus mutans* ATCC 25175) and four Gram-negative bacteria (*Escherichia coli* CIP 7624, *Escherichia coli* DSM 1103, *Escherichia coli* ATTC 25922 and *Pseudomonas aeruginosa* DMS 1117). The tested bacterial strains were initially isolated by the method of streaking the four quadrants in sterile conditions and at optimum temperatures according to the strain concerned for 24 hours. One or several colonies from each pure culture were transferred into five milliliters of nutrient broth. The bacterial suspension was homogenized and incubated at 37°C for 10-24 hours. After incubation, the absorbance of 1 mL of inoculum was read at 625 nm. Opacity must be equivalent to 0.5 McFarland. Petri dishes containing Mueller Hinton were inoculated by the obtained inoculums using swabbing technic. Wathman paper discs (6 mm) were impregnated with 10 µL of the tested extract at different concentrations (100, 50, 25, 12.5 and 6.25 µg mL^{-1}) and filed carefully on the surface of the inoculated agar with sterile forceps in order to deduce the minimum inhibition concentration (MIC) for each extract. The disc of the negative control was impregnated with DMSO and the discs of positive controls contained the reference antibiotic. The Petri dishes were incubated at 37°C for 24 hours. The tests were performed in triplicate and the results were expressed by the diameters of zones of inhibition around the discs.

Statistical analysis

Results are expressed as mean ± standard deviation from three measurements. Mean comparisons were determined by one way ANOVA followed by the Duncan test. Differences were considered significant at $p < 0.05$ and all the statistics were carried out using Graph Pad prism 5.04.

Results and discussion

Phytochemical screening

Phytochemical screening of the crude extracts (PE, EtOAc and *n*-BuOH) obtained from the plant *E. altissima* revealed the presence of tannins, steroids, flavonoids, coumarins, terpenoids, carotenoids and saponins. While the alkaloids, quinones and anthraquinones were totally absent in all the tested extracts (Table 1).

Table 1. Phytochemical constituents of crude extracts from *Ephedra altissima*.

Phytochemical constituents	Extracts		
	PE	EtOAc	*n*-BuOH
Flavonoids	-	+	+
Tannins	-	-	+
Terpenoids	-	+	-
Steroids	+	-	-
Coumarins	-	-	+
Carotenoids	+	-	-
Saponins	-	-	+
Quinones	-	-	-
Anthraquinones	-	-	-
Alkaloids	-	-	-

(+) presence of phytochemicals, (–) absence of phytochemicals.

Total phenolic, flavonoid and tannin contents

Total bioactive compounds of the crude extracts (PE, EtOAc and *n*-BuOH) from *E. altissima* were estimated and the results are shown in Table 2. The EtOAc extract exhibited the highest content of phenols (125.62 ± 1.51 µg EGA mg^{-1} extract), followed by *n*-BuOH and PE extracts respectively. The greatest flavonoid and

tannin contents were observed in *n*-BuOH extract with values of 19.18 ± 0.39 µg EQ mg^{-1} of extract and 8.95 ± 1.70 µg EC mg^{-1} of extract, respectively.

Table 2. Total phenolic, flavonoid and tannin contents of *Ephedra altissima* extracts.

Extracts	Total phenolic content* (µg EGA mg^{-1})	Total flavonoid content** (µg EQ mg^{-1})	Total tannin content*** (µg EC mg^{-1})
PE	19.55 ± 1.69[a]	7.02 ± 0.98[a]	1.24 ± 0.32[a]
EtOAc	125.62 ± 1.51[c]	16.45 ± 0.87[b]	3.06 ± 0.32[b]
n-BuOH	77.46 ± 0.38[b]	19.18 ± 0.39[c]	8.95 ± 1.70[c]

Results are expressed as mean ± standard deviation of three replicates. Different letters within the same column mean that there are significant differences ($p < 0.05$). *Total phenolic content was expressed as µg equivalents of gallic acid per mg of dry extract. **Total flavonoid content was expressed as µg equivalents of quercetin per mg of dry extract. ***Total tannin content was expressed as µg of catechin equivalents per mg of dry extract.

Antioxidant activity

The results of the antioxidant activity showed that all the tested extracts possessed an ability to scavenge free radicals and to reduce Iron III to Iron II in concentration depending manner (Table 3). In ABTS radical scavenging and potassium permanganate tests, the EtOAc extract revealed the highest antioxidant activity, followed by the *n*-BuOH and PE extracts. Moreover, the aqueous extract prepared from the species *E. altissima* displayed the capacity to reduce iron III to iron II with a value of 0.68 ± 0.3 moL eq L^{-1}.

Table 3. Antioxidant activities of the species *Ephedra altissima*.

Extracts	ABTS assay	PRAC assay	Potentiometric assay
	µmoL ET g^{-1} of extract	µg mL^{-1}	moL eq L^{-1}
PE	5.71 ± 0.1[a]	430.08 ± 0.27[e]	NT
EtOAc	11.64 ± 0.27[c]	204.72 ± 0.32[c]	NT
n-BuOH	7.57 ± 0.14[b]	327.47 ± 0.24[d]	NT
Aqueous extract	NT	NT	0.68 ± 0.3
BHA	NT	193.17 ± 0.56[b]	NT
Ascorbic acid	NT	23.44 ± 1.16[a]	NT

Values expressed are means ± SD of three measurements ($p < 0.05$), NT: not tested.

Antibacterial activity

The crude extracts (PE, EtOAc and *n*-BuOH) from *E. altissima* showed antibacterial activity against most of the tested strains (Gram-positive and Gram-negative) in a dose-dependent manner and the results are illustrated in Table 4 and 5. The ethyl acetate extract had the antibacterial activity against all the tested bacterial strains, whereas the strains *Streptococus mutans* and *Pseudomonas aeruginosa* were the most sensitive with value of MIC at 12.5 µg mL^{-1}. In addition, the PE extract did not reveal any antibacterial effect against all the tested Gram-negative strains. While, this extract exhibited an inhibitory effect against all the Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Streptococus pneumoniae* ATCC 49619 and *Streptococus mutans* ATCC 25175). Furthermore, the *n*-BuOH extract had antibacterial activity against all the Gram-negative strains and only against the Gram-positive bacteria *Streptococcus mutans*. Indeed, the bacterial strain *Pseudomonas aeruginosa* DMS 1117 was the most sensitive with a value of MIC at 3.125 µg mL^{-1}.

Phytoconstituent screening revealed the richness in secondary metabolites of the crude extracts (PE, EtOAc and *n*-BuOH) obtained from the plant *E. altissima*, which could be responsible for the observed biological activities. In fact, all the detected phytochemicals have been previously reported in many species of the same genus (Zhang et al., 2018).

In the present study, moderate contents of phenols, flavonoids and tannins have been found in *E. altissima* extracts. Several studies carried out on total phenolic and flavonoid contents from species of the genus *Ephedra* showed variations in their levels. Indeed, many species like *Ephedra major*, *E. distachya*, *E. monosperma*, *E. fragilis*, *E. foeminea*, *E. alata*, *E. foliata*, *E. vulgaris* and *E. sinica* indicated the lowest contents of phenols and flavonoids compared to the current study (Alali et al., 2007; Song et al., 2010; Ibragic & Sofić, 2015). In addition, the plants *Ephedra sarcocarpa*, *E. strobiliacea*, *E. pachyclada* and *E. laristanica* (Parsaeimehr, Sargsyan, & Javidnia, 2010; Rustaiyan, Javidnia, Farjam, Aboee-Mehrizi, & Ezzatzadeh, 2011a; Rustaiyan, Javidnia, Farjam, Mohammadi, & Mohammadi, 2011b) displayed a higher content of phenols than *E. altissima*. Also, species of the genus *Ephedra* namely *E. intermedia*, *E. przewalskii*, *E. alata*, *E. distachya, E. fragilis, E. californica, E. fasciculata*, *E. nevadensis*, *E. torreyana*, *E. trifurca* and *E. viridis* revealed the presence of tannins (Caveney, Charlet, Freitag, Maier-Stolte, & Starratt, 2001). The observed variations in the results of total phenolic, flavonoid and tannin contents may depend on several factors such as soil characteristics, period and area of harvest, plant storage conditions and analytical quantification method (Ibragic & Sofić, 2015).

Table 4. Antibacterial activity of crude extracts of *Ephedra altissima* against Gram-negative bacterial strains.

Extracts/antibiotics	Dilution (µg mL^{-1})	Inhibition zone (mm)			
		E. coli (CIP 7624)	*E. coli* (DSM 1103)	*E. coli* ATCC 25922	*P. aeruginosa* DMS 1117
PE	100	-	-	-	-
	50	-	-	-	-
	25	-	-	-	-
	12.5	-	-	-	-
	6.25	-	-	-	-
	MIC (µg mL^{-1})	-	-	-	-
EtOAc	100	13.33 ± 1.15	10.67 ± 1.15	9.66 ± 2.88	15.33 ± 1.15
	50	9.0 ± 1.0	8.0 ± 1.0	7.0 ± 1.0	11.0 ± 1.0
	25	7.0 ± 1.0	7.0 ± 1.0	-	9.33 ± 0.57
	12.5	-	-	-	8.0 ± 0.57
	6.25	-	-	-	-
	MIC (µg mL^{-1})	25	25	50	12.5
n-BuOH	100	14.33 ± 0.58	14.33 ± 1.53	17 ± 2.65	28.0 ± 2.0
	50	11.0 ± 1.0	12.67 ± 0.58	10 ± 2	24 ± 3.46
	25	9.67 ± 0.58	10.33 ± 0.57	-	17.33 ± 2.3
	12.5	8.0 ± 1.0	7.33 ± 0.58	-	9.66 ± 0.57
	6.25	-	-	-	8.0 ± 0.14
	MIC (µg mL^{-1})	12.5	12.5	50	3.125
Ciproflxacine	5 mg disc^{-1}	29.0 ± 2.0	42.66 ± 0.57	38.0 ± 1.0	40.66 ± 2.52
Amoxicilline	25 mg disc^{-1}	20.33 ± 1.53	14.66 ± 1.52	9.0 ± 1	18.0 ± 2.0
Ceftazidine	30 mg disc^{-1}	8.0 ± 1.0	8.66 ± 1.52	10.66 ± 1.52	18.66 ± 1.52
Cotrimoxasole	25 mg disc^{-1}	23.33 ± 1.53	26.66 ± 1.52	7.33 ± 0.57	35.33 ± 2.51
Cefotaxime	30 mg disc^{-1}	20.0 ± 1.0	35.66 ± 1.52	18.0 ±1.0	29.33 ± 2.51

Values were expressed as means ± SD of three measurements. (-) No zones of inhibition around the discs. MIC: the minimum inhibitory concentration. ND: not determined.

Table 5. Antibacterial activity of crude extracts of *Ephedra altissima* against Gram-positive bacterial strains.

Extracts/antibiotics	Dilution (µg mL^{-1})	Inhibition zone (mm)		
		S. aureus ATCC 25923	*S. pneumoniae* ATCC 49619	*Streptococus mutans* ATCC 25175
PE	100	8.66 ± 0.57	13.0 ± 1.0	13.33 ± 0.57
	50	7.33 ± 0.58	9.33 ± 1.15	10.33 ± 0.58
	25	-	7.0 ± 1.0	9.33 ± 0.57
	12.5	-	-	8.0 ± 1.0
	6.25	-	-	-
	MIC (µg mL^{-1})	50	25	12.25
EtOAc	100	10.33 ± 0.57	14.33 ± 1.52	16 ± 1.0
	50	8.66 ± 0.57	11.66 ± 0.57	10.33 ± 0.57
	25	-	10.0 ± 1.0	9.33 ± 0.57
	12.5	-	8.66 ± 1.15	8.0 ± 1.0
	6.25	-	-	-
	MIC (µg mL^{-1})	50	12.5	12.5
n-BuOH	100	-	-	13.66 ± 0.57
	50	-	-	11.33 ± 0.57
	25	-	-	8.33 ± 0.58
	12.5	-	-	-
	6.25	-	-	-
	MIC (µg mL^{-1})	-	-	25 ± 0.25
Ciproflxacine	5 mg disc^{-1}	ND	37.66 ± 1.52	31 ± 0.12
Amoxicilline	25 mg disc^{-1}	48.0 ± 1.0	26.33 ± 1.52	23 ± 1.2
Cefoxitine	30 mg disc^{-1}	25.66 ± 1.52	21.66 ± 1.52	ND
Cotrimoxasole	25 mg disc^{-1}	37.66 ± 2.51	25.33 ± 0.57	11 ± 1.5
Oxacilline	1 mg disc^{-1}	23.66 ± 2.51	24.66 ± 2.08	21 ± 0.5

Values were expressed as means ± SD of three measurements. (-) No zones of inhibition around the discs. MIC: the minimum inhibitory concentration. ND: not determined.

To properly evaluate the antioxidant activity of plant extracts, it's preferred to use various methods with different principles. For this reason, the antioxidant activity of the plant *E. altissima* has been assessed using spectrophotometric (permanganate reducing antioxidant capacity and ABTS scavenging activity) and potentiometric technics.

The ABTS radical scavenging activity expressed in Trolox equivalent is one of the most employed methods for determining the antioxidant capacity of crude extracts or pure compounds. Trolox was used as a standard molecule for its amphoteric properties, it can be dissolved in aqueous or organic solution (Philippe & Schaumann, 2014). The good antioxidant activity of the EtOAc extract could be associated with its high

content of phenolic compounds compared to the *n*-BuOH and PE extracts in ABTS assay. Previous researches carried out on the antioxidant activity by ABTS assay of species of the genus *Ephedra* revealed differences in their results. In fact, the methanolic extracts of *Ephedra alata* and *E. sinica* indicated the highest activity in comparison with the results of the present investigation (Alali et al., 2007; Song et al., 2010). This finding is probably due in one hand to the nature and the amounts of secondary metabolites in crude extracts, and in another hand to the employed method of extraction.

Potassium permanganate is a redox-active reagent that is widely used in analytical chemistry for redox titrations (Srivastava, Adholeya, Conlan, & Cahill, 2016). This reagent is suggested to evaluate the total antioxidant capacity of crude extracts or synthetic products. The MnO_4^- ion is considered as a pro-oxidant in the alkaline medium, it is relatively non-toxic and could be measured spectrophotometrically. Discoloration in a permanganate solution indicates an increase in the reducing power of the samples (Srivastava et al., 2016). The important antioxidant activity observed in EtOAc extract could be attributed to the presence of phenolic compounds which are well known for their antioxidant properties.

The evaluation of the antioxidant activity of plant extracts by the potentiometric method is based on the interactions between mediator system $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and antioxidants, in which the results are translated in potential between the platinum electrode and silver chloride electrode. In fact, a shift of redox potential is correlated indirectly with the reducing power of the antioxidants in the tested extract (Kovalchuk, Lupak, Klepach, & Polyuzhyn, 2019). This assay possesses several advantages such as rapid, direct, low cost of equipment and reagents, high sensitivity and easy measurement procedures (Brainina et al., 2019). The high antioxidant activity of aqueous extract from the species *E. altissima* could be attributed to the occurrence of polyphenols that can give electrons to reduce iron III to iron II (Zhao, Zhang, & Yang, 2014).

In this study, the influence of crude extracts from the plant *Ephedra altissima* against the tested bacteria is variable between negligible and significant depending on the used extract and the bacterial strain. The EtOAc extract find to be the most active against all the tested strains. Also, the *n*-BuOH extract exhibited antibacterial activity against the most tested bacteria. The antibacterial activity of the EtOAc and *n*-BuOH extracts could be explained by the presence of different phytoconstituents such as flavonoids, tannins, phenolic acids and terpenes which are known for their antibacterial properties (Cueva et al., 2010; Dhayakaran, Neethirajan, Xue, & Shi, 2015; Javed, Nawaz, & Munazir, 2020). Indeed, phenolic compounds can act at two different levels in the cell membrane and the cell wall of microorganisms (Khameneh, Iranshahy, Soheili, & Bazzaz, 2019). These compounds can also interact with the membrane proteins of the bacteria through hydrogen bonds, causing changes in membrane permeability and cell destruction, penetrating bacterial cells and coagulating the cell contents (Bondar & White, 2012). Furthermore, flavonoids are well known for their role in retarding the growth of microorganisms by inhibiting their nucleic acid synthesis, the function of cytoplasmic membrane and energy metabolism (Wu, Zang, He, Pan, & Xu, 2013; Osonga et al., 2019).

The PE extract did not show any inhibitory effect against all the tested Gram-negative strains. Indeed, the resistance of Gram-negative strains could be justified by the nature of its external cell membrane having selective permeability. In fact, the surface of lipopolysaccharides contains negative charges preventing the diffusion of hydrophobic molecules and porins which block the passage of high molecular weight biomolecules (Epand, Walker, Epand, & Magarvey, 2016).

The lack of the antibacterial activity of PE and *n*-BuOH extracts against some bacterial strains could be explained by the choice of the methods, conditions, and the used tools to perform the test. According to Balouiri, Sadiki, & Ibnsouda (2016), the diffusion method from wells on agar is more suitable for studying the antibacterial activity of aqueous and organic extracts than the diffusion method in agar medium. However, it is possible that the addition of dimethyl sulfoxide (DMSO) to plant extracts reduces its antibacterial activity (Kirkwood, Millar, Downey, & Moore, 2018).

Many biological researches conducted on the antibacterial activity of plants from the genus *Ephedra* like *E. sinicia* (Kwon et al., 2001), *E. breana* (Feresin, Tapia, López, & Zacchino, 2001), *E. nebrodensis* (Cottiglia et al., 2005), *E. alata* (Ghanem & El-Magly, 2008), *E. pachyclada* (Motomura, Noshiro, & Mikage, 2007), *E. gerardiana* (Walter, Shinwari, Afzal, & Malik, 2011), *E. americana* (Bussmann et al., 2010), *E. laristanica* (Rustaiyan et al., 2011b) and *E. sarcocarpa* (Rustaiyan et al., 2011a), *E. pachyclada*, *E. procera* and *E. strobilacea* (Parsaeimehr et al., 2010) showed a good antibacterial activity against Gram-positive and Gram-negative strains which is in agreement with the results of our study.

Conclusion

The present study reported the *in vitro* biological activities of *Ephedra altissima* extracts. The phytochemical screening of the extracts (PE, EtOAc and *n*-BuOH) revealed the presence of several classes of secondary metabolites. The highest total phenolic content was found in the EtOAc extract having the greatest antioxidant activity in all the tested assays. While the aqueous extract from *E. altissima* displayed a good antioxidant activity using the potentiometric method. Furthermore, all the extracts showed an antibacterial effect against at least three strains. Therefore, *Ephedra altissima* extracts could be used as an important source of natural bioactive molecules with antioxidant and antibacterial properties.

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References

Alali, F. Q., Tawaha, K., El-Elimat, T., Syouf, M., El-Fayad, M., Abulaila, K., & Oberlies, N. H. (2007). Antioxidant activity and total phenolic content of aqueous and methanolic extracts of Jordanian plants: an ICBG project. *Natural Product Research, 21*(12), 1121-1131. doi: 10.1080/14786410701590285

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: a review. *Journal of Pharmaceutical Analysis, 6*(2), 71-79. doi: 10.1016/j.jpha.2015.11.005

Bondar, A.-N., & White, S. H. (2012). Hydrogen bond dynamics in membrane protein function. *Biochimica et Biophysica Acta, 1818*(4), 942-950. doi: 10.1016/j.bbamem.2011.11.035

Brainina, K., Stozhko, N., Bukharinova, M., Khamzina, E., & Vidrevich, M., (2019). Potentiometric method of plant microsuspensions antioxidant activity determination. *Food Chemistry, 278*, 653-658. doi: 10.1016/j.foodchem.2018.11.098

Bussmann, R. W., Malca-García, G., Glenn, A., Sharon, D., Chait, G., Díaz, D., ... Benito, M. (2010). Minimum inhibitory concentrations of medicinal plants used in Northern Peru as antibacterial remedies. *Journal of Ethnopharmacology, 132*(1), 101-108. doi: 10.1016/j.jep.2010.07.048

Caveney, S., Charlet, D. A., Freitag, H., Maier-Stolte, M., & Starratt, A. N. (2001). New observations on the secondary chemistry of world *Ephedra* (Ephedraceae). *American Journal of Botany, 88*(7), 1199-1208. doi: 10.2307/3558330

Christenhusz, M. J. M., & Byng, J. W. (2016). The number of known plants species in the world and its annual increase. *Phytotaxa, 261*(3), 201-217. doi: 10.11646/phytotaxa.261.3.1

Cottiglia, F., Bonsignore, L., Casu, L., Deidda, D., Pompei, R., Casu, M., & Floris, C. (2005). Phenolic constituents from *Ephedra nebrodensis*. *Natural Product Research, 19*(2), 117-123. doi: 10.1080/14786410410001704714

Cueva, C., Moreno-Arribas, M. V., Martín-Álvarez, P. J., Bills, G., Vicente, M. F., Basilio, A., ... Bartolomé, B. (2010). Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. *Research in Microbiology, 161*(5), 372-382. doi: 10.1016/j.resmic.2010.04.006

Dhayakaran, R. P. A., Neethirajan, S., Xue, J., & Shi, J. (2015). Characterization of antimicrobial efficacy of soy isoflavones against pathogenic biofilms. *LWT-Food Science and Technology, 63*(2), 859-865. doi: 10.1016/j.lwt.2015.04.053

Epand, R. M., Walker, C., Epand, R. F., & Magarvey, N. A. (2016). Molecular mechanisms of membrane targeting antibiotics. *Biochimica et Biophysica Acta, 1858*(5), 980-987. doi: 10.1016/j.bbamem.2015.10.018

Farnsworth, N. R. (1966). Biological and phytochemical screening of plants. *Journal of Pharmaceutical Sciences, 55*(3), 225-276. doi: 10.1002/jps.2600550302

Feresin, G. E., Tapia, A., López, S. N., & Zacchino, S. A. (2001). Antimicrobial activity of plants used in traditional medicine of San Juan province, Argentine. *Journal of Ethnopharmacology, 78*(1), 103-107. doi: 10.1016/s0378-8741(01)00322-1

Ghanem, S., & El-Magly, U. I. A. (2008). Antimicrobial activity and tentative identification of active compounds from the medicinal *Ephedra alata* male plant. *Journal of Taibah University Medical Sciences, 3*(1), 7-15. doi: 10.1016/s1658-3612(08)70039-8

Gupta, P. D., & Birdi, T. J. (2017). Development of botanicals to combat antibiotic resistance. *Journal of Ayurveda and Integrative Medicine, 8*(4), 266-275. doi: 10.1016/j.jaim.2017.05.004

Ibragic, S., & Sofić, E. (2015). Chemical composition of various *Ephedra* species. *Bosnian Journal of Basic Medical Sciences*, *15*(3), 21-27. doi: 10.17305/bjbms.2015.539

Javed, B., Nawaz, K., & Munazir, M. (2020). Phytochemical analysis and antibacterial activity of tannins extracted from *Salix alba* L. against different Gram-positive and Gram-negative bacterial strains. *Iranian Journal of Science and Technology, Transactions A: Science, 44*, 1303-1314. doi: 10.1007/s40995-020-00937-w

Khameneh, B., Iranshahy, M., Soheili, V., & Bazzaz, B. S. F. (2019). Review on plant antimicrobials: a mechanistic viewpoint. *Antimicrobial Resistance and Infection Control, 8*(118), 1-28. doi: 10.1186/s13756-019-0559-6

Kirkwood, Z. I., Millar, B. C., Downey, D. G., & Moore, J. E. (2018). Antimicrobial effect of dimethyl sulfoxide and N, N-Dimethylformamide on *Mycobacterium abscessus*: Implications for antimicrobial susceptibility testing. *International Journal of Mycobacteriology, 7*(2), 134-136. doi: 10.4103/ijmy.ijmy_35_18

Kovalchuk, H., Lupak, O., Klepach, H., & Polyuzhyn, I. (2019). Potentiometric determination of the antioxidant activity of medicinal plants. *Polonia University Scientific Journal, 35*(4), 102-109. doi: 10.23856/3512

Kwon, Y.-B., Lee, J.-D., Lee, H.-J., Han, H.-J., Mar, W.-C., Kang, S.-K., ... Lee, J.-H. (2001). Bee venom injection into an acupuncture point reduces arthritis associated edema and nociceptive responses. *Pain, 90*(3), 271-280. doi: 10.1016/s0304-3959(00)00412-7

Leekha, S., Terrell, C. L., & Edson, R. S. (2011). General principles of antimicrobial therapy. *Mayo Clinic Proceedings, 86*(2), 156-167. doi: 10.4065/mcp.2010.0639

Morales-Gonzalez, J. A. (2013). *Oxidative stress and chronic degenerative diseases - a role for antioxidants*. Rijeka, HR: InTech.

Motomura, H., Noshiro, S., & Mikage, M. (2007). Variable wood formation and adaptation to the alpine environment of *Ephedra pachyclada* (Gnetales: Ephedraceae) in the Mustang district, Western Nepal. *Annals of Botany, 100*(2), 315-324. doi: 10.1093/aob/mcm111

Mouffouk, C., Hambaba, L., Haba, H., Mouffouk, S., Bensouici, C., Mouffouk, S., ... Khadraoui, H. (2018). Acute toxicity and *in vivo* anti-inflammatory effects and *in vitro* antioxidant and anti-arthritic potential of *Scabiosa stellata*. *Oriental Pharmacy and Experimental Medicine, 18*(5), 335-348. doi: 10.1007/s13596-018-0320-3

Mouffouk, S., Gómez-Ruiz, S., Benkhaled, M., Carralero, S., & Haba, H. (2019). Phytochemical composition, antioxidant and antibacterial activities of crude extracts from the species *Euphorbia atlantica* Coss. *Pharmaceutical Chemistry Journal, 53*(9), 831-837. doi: 10.1007/s11094-019-02086-w

Osonga, F. J., Akgul, A., Miller, R. M., Eshun, G. B., Yazgan, I., Akgul, A., & Sadik, O. A. (2019). Antimicrobial activity of a new class of phosphorylated and modified flavonoids. *ACS Omega, 4*(7), 12865-12871. doi: 10.1021/acsomega.9b00077

Parsaeimehr, A., Sargsyan, E., & Javidnia, K. (2010). A comparative study of the antibacterial, antifungal and antioxidant activity and total content of phenolic compounds of cell cultures and wild plants of three endemic species of *Ephedra*. *Molecules, 15*(3), 1668-1678. doi: 10.3390/molecules15031668

Philippe, A., & Schaumann, G. E. (2014). Interactions of dissolved organic matter with natural and engineered inorganic colloids: a review. *Environmental Science & Technology, 48*(16), 8946-8962. doi: 10.1021/es502342r

Popović, B. M., Štajner, D., Slavko, K., & Sandra, B. (2012). Antioxidant capacity of cornelian cherry (*Cornus mas* L.) – Comparison between permanganate reducing antioxidant capacity and other antioxidant methods. *Food Chemistry, 134*(2), 734-741. doi: 10.1016/j.foodchem.2012.02.170

Quézel, P., & Santa, S. (1963). *Nouvelle flore de l'Algérie et des régions désertiques méridionales* (Vol. 1-2). Paris, FR: Centre National de la Recherche Scientifique.

Rustaiyan, A., Javidnia, K., Farjam, M. H., Aboee-Mehrizi, F., & Ezzatzadeh, E. (2011a). Antimicrobial and antioxidant activity of the *Ephedra sarcocarpa* growing in Iran. *Journal of Medicinal Plants Research, 5*(17), 4251-4255. doi: 10.5897/JMPR.9000172

Rustaiyan, A., Javidnia, K., Farjam, M. H., Mohammadi, M. K., & Mohammadi, N. (2011b). Total phenols, antioxidant potential and antimicrobial activity of the methanolic extracts of *Ephedra laristanica*. *Journal of Medicinal Plants Research, 5*(24), 5713-5717.

Sadeer, N. B., Montesano, D., Albrizio, S., Zengin, G., & Mahomoodally, M. F. (2020). The versatility of antioxidant assays in food science and safety-chemistry, applications, strengths, and limitations. *Antioxidants, 9*(8), 709. doi: 10.3390/antiox9080709

Santos-Sánchez, N. F., Salas-Coronado, R., Villanueva-Cañongo, C., & Hernández-Carlos, B. (2019). Antioxidant compounds and their antioxidant mechanism. In E. Shalaby (Ed.)*, Antioxidants* (p. 1-29). London, GB: IntechOpen.

Song, F.-L., Gan, R.-Y., Zhang, Y., Xiao, Q., Kuang, L., & Li, H.-B. (2010). Total phenolic contents and antioxidant capacities of selected Chinese medicinal plants. *International Journal of Molecular Sciences, 11*(6), 2362-2372. doi: 10.3390/ijms11062362

Srivastava, S., Adholeya, A., Conlan, X. A., & Cahill, D. M. (2016). Acidic potassium permanganate chemiluminescence for the determination of antioxidant potential in three cultivars of *Ocimum basilicum*. *Plant Foods for Human Nutrition, 71*(1), 72-80. doi: 10.1007/s11130-016-0527-8

Walter, C., Shinwari, Z. K., Afzal, I., & Malik, R. N. (2011). Antibacterial activity in herbal products used in Pakistan. *Pakistan Journal of Botany, 43*, 155-162.

Wu, T., Zang, X., He, M., Pan, S., & Xu, X. (2013). Structure-activity relationship of flavonoids on their anti-*Escherichia coli* activity and inhibition of DNA gyrase. *Journal of Agricultural and Food Chemistry, 61*(34), 8185-8190. doi: 10.1021/jf402222v

Zhang, B.-M., Wang, Z.-B., Xin, P., Wang, Q.-H., Bu, H., & Kuang, H.-X. (2018). Phytochemistry and pharmacology of genus *Ephedra*. *Chinese Journal of Natural Medicines, 16*(11), 811-828. doi: 10.1016/s1875-5364(18)30123-7

Zhao, H.-X., Zhang, H.-S, & Yang, S.-F. (2014). Phenolic compounds and its antioxidant activities in ethanolic extracts from seven cultivars of Chinese jujube. *Food Science and Human Wellness, 3*(3-4), 183-190. doi: 10.1016/j.fshw.2014.12.005