

Full Length Research Paper

# Total phenols, antioxidant potential and antimicrobial activity of the methanolic extracts of *Ephedra laristanica*

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The antioxidant activities of the methanolic extracts of *Ephedra laristanica* growing in Iran were evaluated using ferric reducing antioxidant power (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assays. FRAP values 2.1 mmol eq quercetin/g extracts, and IC<sub>50</sub> values in the DPPH assay 4.6 mg/mL. This plant showed the high antioxidant activities. FRAP and DPPH assay results showed good correlations with the total phenolic contents of the plants, measured by the Folin-Ciocalteu assay ( $r^2 = 0.926$  and  $0.913$ , respectively,  $p < 0.0001$ ). The antimicrobial capacity was screened against Gram positive and Gram negative bacteria, and fungi. The extract inhibited the growth of Gram negative bacteria, being *Escherichia coli* the most susceptible one with MIC of 32 µg/mL for the extract. The results obtained indicate that *E. laristanica* may become important in the obtainment of a noticeable source of compounds with health protective potential and antimicrobial activity.

**Key words:** Antioxidant(s), ferric reducing antioxidant power (FRAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), total phenol, antimicrobial.

## INTRUCTION

*Ephedra laristanica* (*Ephedraceae*) is a distinct gnetalean genus of ca. 50 to 65 species of shrubs, vines, or rarely small trees (Price, 1996). Over the past 20 years, there has been a lot of interest in the investigation of natural materials as sources of new antibacterial agents. Different extracts from traditional medicinal plants have been tested. Many reports show the effectiveness of traditional herbs against microorganisms, as a result, plants are one of the bedrocks for modern medicine to attain new principles (Evans et al., 2002). The medical use of *Ephedra* dates back to at least 2700 B.C., when the Chinese used Ma Huang (*Ephedra sinica* Stapf.) to

treat asthma, cough, and bronchitis (Groff and Clark, 1928). Almost all commercial applications of *Ephedra* extracts derive from the ephedrine alkaloids found in the stems in many Eurasian species. The best-documented drug made from *Ephedra* is Ma-huang, used in Chinese medicine for 5000 year as a treatment for fever, nasal congestion, and asthma (Zhu et al., 1998). Although, the primary use of *Ephedra* and its products is for weight loss, energy enhancement and respiratory diseases management (Wang, 2006). Antimicrobial activity of some *Ephedra* species including *Ephedra altissima* Desf. (Tricker et al., 1987), *Ephedra transitorai* (Al-Khalil, 1998) and *Ephedra breana* (Feresin et al., 2001) has been noticed in recent years. The plant active compounds such as cyclopropyl amino acids, kynurenates, ephedrine alkaloids, and proanthocyanidins have now been isolated (Gurley et al., 1998). Among *Ephedra* species, 12

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species are known to grow in Iran including *Ephedra major* host as an important species (Ghahreman, 1994).

## MATERIALS AND METHODS

### Reagents and chemicals

Trolox (water soluble equivalent of vitamin E) and quercetin were obtained from Acros Organics (Geel, <http://www.acros.com>). Acetic acid glacial, dimethyl sulphoxide, ferrous sulphate heptahydrate, ferric chloride, Folin–Ciocalteu reagent, hexane, methanol, sodium acetate, sodium carbonate and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were purchased from Merck (Darmstadt, <http://www.merck.de>). Galic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and hydrochloric acid 32% were obtained from Sigma–Aldrich (St. Louis, <http://www.sigma-aldrich.com>).

### Samples

The plant materials were collected in June 2008 from north western Iran. The plants were identified at the Department of Biology, University of Shiraz, Iran and a voucher specimen (no. UZ-18-05) was deposited at the herbarium of the Medicinal and Natural Products Chemistry Research Centre, Shiraz, Iran. Aerial parts of plants were air-dried at room temperature (25°C) in the shade.

### Extraction

Methanolic extracts of the plants were prepared as follows: 7.5 g dry plant, after being defatted with light petroleum, was macerated in 200 mL methanol/water (90/10) for 2 days with one change of solvent after 1 day. The extract was filtered and then concentrated in a rotary vapor in less than 10 min. powders were weighed to calculate the yield, and kept at -20°C until used. Shortly before each experiment, the powder was dissolved in methanol at the desired concentration and tested for antioxidant activity and total phenolic content.

### Assessment of antimicrobial activity

The antimicrobial activity was tested by using the disc-diffusion method (Bauer et al., 1966) and determining the minimal inhibitory concentration (MIC) using the macro dilution broth technique. Each extract and controls were tested in duplicate and the experiments were repeated 4 times.

Briefly, an overnight culture of approximately  $5 \times 10^5$  CFU/ml was inoculated into tubes containing test compound dilutions and incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of test compound able to restrict bacterial growth to a level lower than 0.05 at 650 nm. The extract was screened against 6 bacterial and 3 fungal strains. The bacteria that were used in this study were *Bacillus pumilus* (PTCC 1319), *Escherichia coli* (PTCC 1533), *Kocuria varians* (PTCC 1484), *Pseudomonas aeruginosa* (PTCC 1310), *Salmonella typhi* (PTCC 1609), and *Listeria monocytogenes* (PTCC 1298).

The fungal strains that were used in this study were *Aspergillus niger* (PTCC 5154), *Aspergillus flavus* (PTCC 5006) and *Candida glabrata* (PTCC 5297). All microorganisms were obtained from the Persian type culture collection (PTCC), Tehran, Iran. Microorganisms were cultured for 16 to 24 h at 37°C and prepared to turbidity equivalent to McFarland Standard No. 0.5 (Janssen et al., 1987). The suspensions were then spread on a test plate of Muller–Hinton agar. Sterile discs were impregnated with 2 mg

of the extract and placed on the surface of the test plate. Positive control discs include; gentamicin, ampicillin and ketoconazole for Gram-negative bacteria, Gram-positive bacteria and fungi, respectively.

### Antioxidant activity measured by the ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed as described previously (Benzie and Strain, 1996). Briefly, the FRAP solution was prepared freshly by mixing 10 mL of acetate buffer 300 mM, pH 3.6, 1 mL of ferric chloride hexahydrate, 20 mM, dissolved in distilled water and 1 mL of 2,4,6-tripyridyl-s-triazine (TPTZ), 10 mM, dissolved in HCl 40 mM. Plant extract dissolved in methanol (40 µL) at a concentration of 1 mg/mL was mixed with 4 mL of the FRAP solution. Absorbance was determined at 595 nm after 6 min of incubation at room temperature by a spectrophotometer (Bio-Tek, Model Uvikon XL). Quercetin was tested at the final concentration of 10 µM and used as the reference compound. FRAP values were expressed as µM quercetin equivalents for each g dry weight of plant (DW) and were calculated according to the following formula:

$$\text{FRAP value} = (\Delta\text{AP} / \Delta\text{AQ}) \times Y \times 1000$$

Where  $\Delta\text{AP}$  and  $\Delta\text{AQ}$  are absorbance changes of the FRAP solution in the presence of the plant extract and quercetin, respectively and Y is the extraction yield.

### DPPH radical scavenging activity

Radical scavenging activity of plant extracts against the stable free radical DPPH was measured as described previously (Hwang et al., 2001), with some modifications. Briefly, 4 different concentrations of the plant extract dissolved in methanol were incubated with a methanolic solution of DPPH (100 µM) in 96-well microplates. Concentrations were carefully chosen according to the activity of this plant, in order to produce an appropriate dose-response curve. Plant extract concentrations used in this study ranged from 1.6 to 100 µg/mL. After 30 min of incubation at room temperature in the dark, the absorbance at 490 nm was measured by a microplate reader (Bio-Rad, model 680).

The percentage inhibition (%) for each concentration was calculated by using the absorbance (A) values according to the following formula:

$$\%I = [(ADPPH - AP) / ADPPH] \times 100$$

Where ADPPH and AP were the absorbance of the DPPH solutions containing methanol and plant extract, respectively. The dose-response curve was plotted by using the software SigmaPlot for Windows version 8.0 and  $\text{IC}_{50}$  values for extract was calculated. These values were divided by the extraction yield (Y) to calculate the  $\text{IC}_{50}$  value for the dry plant.

### Total phenolic content

Total phenolic content in plant extracts was determined by the Folin–Ciocalteu colorimetric method, as described previously (Singleton and Rossi, 1965). A methanolic solution of catechin was tested in parallel as a reference compound.

### Statistical analysis

Regression analyses were performed by SigmaPlot 2002 for

**Table 1.** Antimicrobial activity of *Ephedra laristanica*.

Microorganism	MIC of extract	MIC of reference <sup>a</sup>	Zone of inhibition of the extract in mm (Mean $\pm$ SD)	Zone of inhibition of the reference mm (Mean $\pm$ SD) <sup>b</sup>
<i>Escherichia coli</i>	32	16	16.5 $\pm$ 0.7	18 $\pm$ 0.1
<i>Pseudomonas aeruginosa</i>	64	4	13.5 $\pm$ 0.5	15.3 $\pm$ 0.5
<i>Salmonella typhi</i>	64	32	14 $\pm$ 0.9	18.6 $\pm$ 1.1
<i>Bacillus pumilus</i>	256	64	10.5 $\pm$ 0.5	16.1 $\pm$ 0.3
<i>Kocuria varians (Micrococcus varians)</i>	256	16	11.7 $\pm$ 0.1	15.5 $\pm$ 0.5
<i>Listeria monocytogenes</i>	128	16	12.6 $\pm$ 0.5	17.3 $\pm$ 0.1
<i>Aspergillus flavus</i>	512	64	7.8 $\pm$ 0.3	25.5 $\pm$ 0.7
<i>Candida glabrata</i>	256	128	7.5 $\pm$ 0.5	19.1 $\pm$ 0.9
<i>Aspergillus niger</i>	256	64	8.3 $\pm$ 0.2	27 $\pm$ 0.1

<sup>a</sup>Ampicillin, Tetracycline and Fluconazole were used as references for Gram positive, Gram negative bacteria and fungus, respectively. <sup>b</sup>The values represent the mean of four experiments  $\pm$  SD. Ampicillin, gentamicin and ketoconazole (10 lg/disc) were used as references for Gram positive, Gram negative bacteria and fungus, respectively.

Windows version 8.0.

## RESULTS AND DISCUSSION

### Antimicrobial activity

The disc diffusion method for antibacterial activity showed significant reduction in bacterial growth in terms of zone of inhibition around the disc (Table 1). Among bacterial strains tested, *E. coli*, *S. typhi* and *L. monocytogenes* were found to be more sensitive to crude extract. Other bacterial forms were inhibited by the extract. The zone of inhibition increased on increasing the concentration of extract in disc. This showed the concentration dependent activity.

The antibacterial activity of the test compound against the bacterial strains shows high values of MIC (Table 1). Our findings showed that the methanol extract from aerial part of *E. laristanica* had interesting activity against both Gram-negative and Gram-positive bacteria. The extract proved to be active against 4 out of the 6 bacterial strains used and was particularly active against *E. coli*, *P. aeruginosa* and *S. typhi* (MIC values of 32  $\mu$ g/ml for the first and 64  $\mu$ g/ml for the others, respectively). As for *L. monocytogenes* an MIC value of 128  $\mu$ g/ml was found, while *Bacillus pumilus* and *Kocuria varians* was the least affected with an MIC value of 256  $\mu$ g/ml. Due to the continuous emergence of antibiotic-resistant strains there is continual demand for new antibiotics. In many developing countries about 80% of available drugs come from medicinal plants and in industrialized countries plants make up the raw material for processes, which synthesize pure chemical derivatives (Penso, 1980). The methanol extract from aerial part showed an inhibiting activity on disease causing Gram-negative and Gram-positive bacteria, the most inhibited being *E. coli*. This is particularly interesting from a medical point of view because this microbial agent is responsible for severe

opportunistic infections. We also screened the antifungal activity of the extract of *E. laristanica*. The extract showed mildly significant activity against fungal.

### Antioxidant activity

Several methods have been used to determine antioxidant activity of plants. Our present study therefore involved three various established methods to evaluate antioxidative activity of this extract, namely, DPPH radical-scavenging activity, ferric reducing/antioxidant power (FRAP) assay and total antioxidant capacity. Antioxidant activities of the plant extracts are reported in Table 2. A good correlation was found between FRAP and DPPH assays.

Total phenolic contents of the tested plants showed good correlations with the results of FRAP ( $r^2 = 0.926$ ,  $p < 0.0001$ ) and DPPH methods ( $r^2 = 0.913$ ,  $p < 0.0001$ ). This means that phenolic compounds provide the major contribution to the antioxidant activity of the plant extracts measured by these assays. This is in line with the observation of other authors who found similar correlations between total phenolic content and antioxidant activity of various plants (Nencini et al., 2007).

### DPPH radical- scavenging activity

DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples (Sakanaka et al., 2005; Shimoji et al., 2002). It was a stable free radical with a characteristic absorption at 517 nm that was used to study the radical-scavenging effects of extracts. As antioxidants donate protons to this radical, the absorption decreases. To evaluate the scavenging effects of DPPH of methanol extract, DPPH inhibition was investigated. Antioxidants, on interaction with DPPH, either transfer an electron or hydrogen atom

**Table 2.** Antioxidant activity and total phenolic contents of *Ephedra laristanica*.

Plant name	DPPH IC <sub>50</sub> (mg/ml)	FRAP value (mmol eq quercetin/g extracts)	Total phenolic content (μmol eq gallic acid/g extract)
<i>Ephedra laristanica</i>	4.6 ± 0.019	2.1 ± 0.07	513.03 ± 18.11

Values represent the mean of three experiments ± SD. Quercetin was tested as a reference compound in the DPPH and FRAP assay. Values with different letters in the same column are significantly different.

to DPPH, thus neutralizing its free radical character (Naik et al., 2003). The color was changed from purple to yellow and its absorbance at wavelength 517 nm was decreased (Table 2). The growing interest in the substitution of synthetic food antioxidants with natural ones has been fostered research on plant sources and screening of raw materials to identify new antioxidants. Interest in oxidation reactions is not confined to the food industry, as antioxidants are widely needed to prevent deterioration of other perishable goods, such as cosmetics, pharmaceuticals and plastics. In addition, other biological properties such as anticarcinogenicity, antimutagenicity, antiallergicity and antiaging activity have been reported for natural and synthetic antioxidants (Moure et al., 2001).

#### Ferric reducing activity based on FRAP assay

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe<sup>3+</sup>-TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe<sup>2+</sup>-TPTZ) (Benzie and Strain, 1996). Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). According to Benzie and Strain (1996), the reduction of Fe<sup>3+</sup>-TPTZ complex to blue-colored Fe<sup>2+</sup>-TPTZ occurs at low pH.

#### Phenolic compounds analysis

Total phenol content of methanol extract was determined using the Folin-Ciocalteu technique (Singleton and Rossi, 1965). Briefly, a 50 μl aliquot of methanol extracts was assayed with 250 μl Folin reagent and 500 μl sodium carbonate (20%, w/v). The mixture was vortexed and diluted with water to a final volume of 5 ml. After incubation for 30 min at room temperature, the absorbance was read at 765 nm in a cuvette of 1 cm and total phenols in the guarana ethanol extract were expressed as gallic acid equivalents (GAE), using a calibration curve of a freshly prepared gallic acid solution.

Phenolics or polyphenols have received considerable attention because of their physiological function, including antioxidant, antimutagenic and antitumour activities (Othman et al., 2007). Plant phenolics present in fruit and vegetables, because of their potential antioxidant activity, have been received considerable attention (Dziedzic and Hudson, 1983; Lopez-Velez et al., 2003). Phenolic compounds due to their antioxidant activities and free radical-scavenging abilities, are widely distributed in plants (Li et al., 2006), which have gained much attention and potentially have beneficial implications for human health (Govindarajan et al., 2007). Total phenol content (TPC) was determined in comparison with standard gallic acid and the results expressed in terms of μmol eq gallic acid/g extract.

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