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Full Length Research Paper

Antimicrobial and antioxidant activity of the *Ephedra sarcocarpa* growing in Iran

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The antimicrobial capacity of the methanolic extracts of *Ephedra sarcocarpa* growing in Iran is screened against gram-positive and gram-negative bacteria and fungi. The extract inhibited the growth of Gram-negative bacteria, being *Pseudomonas aeroginosa* the most susceptible one with MIC of 16 µg/mL for the extract. The antioxidant activities are 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_{50} values in the DPPH assay 4.6 mg/mL. This plant had shown the high antioxidant activities. FRAP and DPPH assay results were shown good correlations with the total phenolic contents of the plants, measured by the Folin-ciocalteau assay: (r^2 = 0.920 and 0.893, respectively, p < 0.0001).

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

INTRODUCTION

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 was shown 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). *Ephedra L.* (Ephedraceae) is a distinct gnetalean genus of ca. 50 to 65 species of shrubs, vines, or rarely small trees (Price, 1996).

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 that have been found in the stems in many *Eurasian* species. Although, the primary use of Ephedra and its products was for weight loss, energy enhancement and respiratory diseases management (Wang et al., 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. Among *Ephedra* species, 12 species has been known to grow in Iran including Ephedra major Host as an important species (Ghahreman, 1994).

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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–ciocalteau 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).

Sample

The plant materials were collected in June 2010 from north western Iran. The plants were identified at the herbarium of the Medicinal and Natural Products Chemistry Research Centre, Shiraz, Iran (http://mncrc.sums.ac.ir) by Dr. Maryam Gholami and a voucher specimen (no. PC-18-21) representing this collection was deposited at the herbarium of the Medicinal and Natural Products Chemistry Research Centre. 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 evaporator 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 was tested for antioxidant activity and total phenolic content.

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:

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

Where ΔAP and ΔAQ 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 were dissolved in methanol, 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 extracts concentrations that were 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 (%I) 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 IC_{50} values for extract was calculated. These values were divided by the extraction yield (Y) to calculate the IC_{50} value for the dry plant.

Total phenolic content

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

Total phenol content

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 obtain 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 ethanol extract were expressed as gallic acid equivalents (GAE), using a calibration curve of a freshly prepared gallic acid solution.

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 5×10^5 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 were included; gentamicin, ampicillin and ketoconazole for Gram-negative bacteria, Gram-positive bacteria and fungi, respectively.

Statistical analysis

Regression analyses were performed by SigmaPlot 2002 for Windows version 8.0.

Table 1. Antimicrobial activity of the extract of *Ephedra sarcocarpa.*

Microorganism	MIC of extract	MIC of reference[a]	Zone of inhibition of the extract in mm (Mean ± SD)	Zone of inhibition of the reference mm (Mean ± SD)[b]
Escherichia coli	64	16	14.5 ± 0.5	17.5 ± 0.3
Pseudomonas aeroginosa	16	8	16 ± 0.1	14.5 ± 0.5
Salmonella typhi	64	32	17.5 ± 0.5	17.7 ± 0.3
Bacillus pumilus	128	64	7.5 ± 0.7	15 ± 0
Klebsiella pneumonia	128	32	9.5 ± 0.3	16 ± 0.1
Listeria monocytogenes	256	16	15.3 ± 0.2	15 ± 0.3
Aspergillus flavus	512	64	6.4 ± 0	21 ± 0.9
Candida glabrata	512	64	6.4 ± 0	23.5 ± 0.2
Aspergillus niger	512	32	7.5 ± 0.2	25 ± 0

[a]Ampicillin, tetracycline and fluconazole were used as references for Gram-positive, Gram-negative bacteria and fungus, respectively. [b]The values represent the mean of four experiments ± SD. ampicillin, gentamicin and ketoconazole (10 lg/disc) were used as references for Gram-positive, Gram-negative bacteria and fungus, respectively.

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

Plant name	DPPH IC_{50} (mg/ml)	FRAP value (µM/g DW)	Total phenolic content (mg catechin equivalent/g extract)
Ephedra sarcocarpa	5.3 ± 0.027	6.7 ± 0.16	709.18 ± 14.08

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 were significantly different.

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, *S. typhi,* P*. aeroginosa, L. monocytogenes* and *E. coli* were found to be more sensitive to crude extract. Other bacterial forms were inhibited by the extract. The zone of inhibition was added on increasing the concentration of extract in disc. This was showed the concentration dependent activity.

The antibacterial activity of the test compound against the bacterial strains shows high values of minimum inhibitory concentration (MIC) (Table 1). Our findings showed that the methanol extract from aerial part of *E. sarcocarpa* had interesting activity against both Gram-negative and Gram-positive bacteria. The extract proved to be active against 5 out of the 6 bacterial strains used and was particularly active against *E. coli*, *P. aeroginosa*, *E. coli* and *S. typhi* (MIC values of 16 µg/ml for the first and 64 µg/ml for the others, respectively). As for *B. subtilis* and *k. pneumoniae* an MIC value of 128 µg/ml was found, while *L. monocytogenes* was the least affected with an MIC value of 256 µg/ml. Due to the continuous emergence of antibiotic-resistant strains, there was 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).

We also screened the antifungal activity of the extract of *E. sarcocarpa* the extract that show 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 are found between FRAP and DPPH assays. Total phenolic contents of the tested plants are showed good correlations with the results of FRAP (r^2 = 0.920, $p < 0.0001$, data not shown) and DPPH methods (r^2 = 0.893, $p < 0.0001$). 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 last observations 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

$DPPH^{\bullet}$ is a free radical compound and was 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 to $DPPH^{\bullet}$, thus neutralizing its free radical character (Naik et al., 2003).The colour 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 anti carcinogenicity, anti mutagenicity, anti allergenicity and anti aging 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^{3+}–TPTZ) complex and producing a coloured ferrous tripyridyltriazine (Fe^{2+}–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^{3+}–TPTZ complex to blue-coloured Fe^{2+}–TPTZ occurs at low pH.

Mechanism of antioxidant action of phenolic compounds

The presence of free radicals, $R^{\bullet}$, in a biological system containing polyunsaturated fat, RH, and other material susceptible to oxidative destruction is potentially dangerous because the damage associated with the initial production of the radicals can be greatly amplified through a chain mechanism involving peroxyl radicals, $ROO^{\bullet}$. Although the chain reaction eventually becomes self-terminating when two peroxyl radicals combine to give non-radical products, in the meantime there may occur a massive amount of destruction of the unsaturated lipid, depending on the chain length. Chain reactions can be strongly inhibited by the addition of a small amount of a chain-breaking antioxidant. In biological systems, the lipid-soluble antioxidant almost invariably is the hindered phenol, atocopherol (vitamin E) (Burton et al., 1982).

Plant phenolics present in fruit and vegetables, because of their potential antioxidant activity (Dziedzic and Hudson, 1983; Lopez-Velez et al., 2003) have been received considerable attention. 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).

In general, phenols, phenolics or polyphenols, are able to compete very effectively with an oxidizable substrate for peroxyl radicals because the phenolic hydrogen atom is much more readily abstracted. The relatively stable phenoxyl radical that is produced is so unreactive towards either the substrate or O_2, that is, it is unable to continue the chain.

$$ArOH + ROO^{\bullet} \rightarrow ArO^{\bullet} + ROOH$$

Eventually, the phenoxyl radical is destroyed by reaction with a second peroxyl radicalor; it may be reduce back to the original phenol by a water-soluble agent such as vitamin C.

$$ArO^{\bullet} + ROO^{\bullet} \rightarrow \text{inactive products}$$
$$ArO^{\bullet} + AH\ (-) \rightarrow ArOH + A\ (-)^{\bullet}$$

Total phenol content (TPC) was determined in comparison with standard galic acid and the results expressed in terms of µmol eq galic acid/g extract.

Conclusions

The results obtained indicate that *E. sarcocarpa* may become important in the obtainment of a noticeable source of compounds with health protective potential and antimicrobial activity. It shows an inhibiting activity on disease causing Gram-negative and Gram-positive bacteria, the most inhibited being *Pseudomonas aeroginosa*. This is particularly interesting from a medical point of view, because this microbial agent is responsible for severe opportunistic infections.

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