

EXPERIMENTAL PAPER

Antimicrobial and antioxidant activity of *Epimedium pinnatum*

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S u m m a r y

Epimedium pinnatum (Berberidaceae family) is used as an aphrodisiac in traditional Chinese medicine (TCM). The aim of this study was to evaluate the antimicrobial and antioxidant activity of *E. pinnatum* extracts (ethanol, methanol and aqueous extracts). Total phenolic (TPC) and flavonoid contents (TFC) of each extract were assessed by spectrophotometric methods. It was exhibited that methanol extract had better antimicrobial activity than those of ethanolic extract or aqueous extract. The TPC and TFC of *E. pinnatum* extracts was higher in methanol extract (149 and 36.6 mg/g) than that of ethanolic extract (137.2 and 19.5 mg/g) and aqueous extract (86.2 and 8.4 mg/g). The methanol extract had lower IC₅₀ value (200 µg/ml) than ethanolic (250 µg/ml) and aqueous extract (400 µg/ml). There was a positive correlation between TPC, TFC in *E. pinnatum* extract and their antioxidant and antimicrobial activity.

Key words: *Epimedium pinnatum*, extract, total phenolics, total flavonoids, antimicrobial activity, antioxidant activity

INTRODUCTION

Epimedium belongs to *Berberidaceae* family, also known as horny goat weed. It has a long history of use in traditional Chinese medicine (TCM) as “nourishing the kidney and reinforcing the Yang”. There are famous botanical supplements that they have been widely used as an aphrodisiac remedy for sexual dysfunction. More than 260 chemically active components have been identified in *Epimedium* species and prenyl flavonoids are their main components. There are many scientific researches on their pharmacological activities such as sexual enhancing, improvement of sperm counts and vitality [1-4], anti-osteoporosis [5,6], immune enhancing [7-9], cardiovascular protective [10], anti-hypertensive activity [11,12], anti-arrhythmic effect [13], anti-tumor [14,15], anti-aging and anti-oxidative effect [16-18], anti-hypoxic and anti-fatigue [19,20], and finally anti-inflammatory effect [21]. The survey on the antibacterial and antiviral studies has exhibited that flavonoids content in *Epimedium* genus significantly inhibited *poliovirus* and *enterovirus*. They also showed the inhibitory effect against *Micrococcus pyogenes* var. *albus*, *Staphylococcus aureus*, *Diplococcus pharyngis communis*, *Micrococcus catarrhalis* and *Haemophilus influenzae* [22]. Icarrin, a major component of *Epimedium*, notably inhibited *Aspergillus* sp., *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Penicillium* sp. and *Hansenula* sp. [23].

Epimedium genus has only one species in Iran, namely *E. pinnatum* in wild localities of Iran [24]. *Epimedium pinnatum* is a clump-forming perennial plant and spreads outward by rhizomes. The leaves are hairy-edged, heart-shaped with pointed ends. Young leaves shaded with red/brown and older leaves with mid-green. Flowers are born in sprays in early spring, bright yellow, cup-shaped; five sepals which are bent back from flower center with five small inner petals. The surveys in literature exhibited that there are no studies on antimicrobial and antioxidant activity of *E. pinnatum* extracts in *in vitro* conditions.

MATERIALS AND METHODS

Plant material and extraction

The dried aerial parts of plant was collected from Geno Mountain (Dargir village, Bandar Abbas, Iran) in April 2010 and identified by Agriculture Department of Barij Essence Pharmaceutical Company, Kashan, Iran and authenticated under number 193-1.

The aerial sample was grinded and subjected to hydrodistillation by Clevenger type apparatus for 3 h. The oil was not separated. Extraction was performed with water, methanol and ethanol-water (70:30, v/v). The powdered *E. pinnatum* aerial parts were mixed with solvent at the ratio of 1:10 (w/v) for 24 h at ambient temperature. Then, the mixture was filtered through Whatman filter paper No. 2, the residue rinsed with the same solvent and the extract dried under vacuum.

Total phenolic content (TPC)

Total phenolic contents of crude extracts were determined by a spectrophotometer using the Folin-Ciocalteu's reagent according to the method described by International Organization for Standardization (ISO) 14502-1 [25]. Each dry extract (10 mg) was dissolved in 10 ml of itself solvent (1 mg/ml). 0.1 ml of extract was transferred into a 5 ml volumetric flask. 0.5 ml of Folin-Ciocalteu's reagent (10%) was added and swirled. After 3–8 min, 0.4 ml of 7.5% (w/v) sodium carbonate solution was added and mixed. The solution was mixed thoroughly and allowed to stand at ambient temperature for 1 h until the characteristic blue color was developed. The absorbance of reaction mixture was measured at 765 nm. Quantification of TPC was based on a standard curve generated with gallic acid (GAC) at 765 nm using the following equation:

$$W = ((\text{Abs} - 0.0089) / 0.0647) \times 100,$$

where Abs is absorbance and w is the weight (μg). All tests were conducted in triplicate and averaged. The results were expressed as mg of TPC per g of dry extract as GAC.

Total flavonoid content (TFC)

The aluminum chloride colorimetric method was modified from the procedure reported by Woisky and Salatino [26]. Quercetin was used to make the calibration curve. 10 mg of quercetin was dissolved in ethanol and then diluted to 25, 50 and 100 $\mu\text{g}/\text{ml}$. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at a room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Similarly, 0.5 ml of each diluted extract was reacted with aluminum chloride for the determination of flavonoid content as described above. The results were expressed as mg of TFC per g of dry extract as quercetin (QE).

Antioxidant activity

Free radical-scavenging activity by DPPH method

The hydrogen atom or electron donation ability of extracts was measured from the bleaching of purple coloured methanol solution of DPPH. Radical-scavenging activity of extracts was determined using slightly modified DPPH radical scavenging activity assay [27]. A stock solution of each extract (63 mg/1.6 ml) was

prepared in itself solvent and diluted with methanol in the range of concentrations 7.8 to 2000 $\mu\text{g/ml}$. The diluted extracts (0.1 ml) were added to 2 ml of freshly prepared DPPH methanol solution and mixed. The solution was mixed thoroughly and allowed to stand at ambient temperature for 30 min and the absorbance of reaction mixture was measured at 490 nm by spectrophotometer. The inhibition of free radical DPPH in percent (I%) was calculated as follows:

$$I\% = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and a sample is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as means. The BHT as a reference antioxidant was prepared in ethanol.

β -carotene/linoleic acid bleaching test (BCBT)

The β -carotene bleaching activity of extracts was performed as given elsewhere [28]. A 0.5 mg of β -carotene was dissolved in 1 ml chloroform and this solution was pipetted into a 100 ml round bottom flask. Chloroform was removed using a rotary evaporator under vacuum at 50°C for 10 min, and then 25 μl of linoleic acid, 200 mg of Tween 80 and 100 ml aerated distilled water were added to the flask with vigorous shaking. The emulsion (2.5 ml) was added to a tube containing 0.35 ml of the extract (2 mg/ml) and the absorbance was immediately measured at 490 nm against a blank as zero time. Blank sample, devoid of β -carotene, was prepared for background subtraction. The tubes were placed in a water bath at 50°C and the oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 490 nm over a 120 min period. The antioxidant property (inhibition percentage, I%) of samples was determined using the following equation:

$$I\% = (A_{\beta\text{-carotene after 2 h assay}}/A_{\text{initial } \beta\text{-carotene}}) \times 100,$$

where $A_{\beta\text{-carotene after 2 h assay}}$ is the absorbance of β -carotene after 2 h assay remaining in the samples and $A_{\text{initial } \beta\text{-carotene}}$ is the absorbance of β -carotene at the beginning of the experiments. BHT was used as positive control.

Microbial strains and evaluation of antimicrobial activity by micro-broth dilution assay

Staphylococcus aureus ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Staphylococcus epidermidis* ATCC 14490, *Enterococcus faecium* ATCC 25778, clinical isolate of *Streptococcus agalactiae*, *Streptococcus pneumoniae* ATCC 33400, *Streptococcus*

mutans ATCC 35668, *Streptococcus sobrinus* ATCC 27607, *Bacillus cereus* ATCC 1247, *Bacillus subtilis* ATCC 6051, *Staphylococcus saprophyticus* ATCC 15305, *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 8739, *Salmonella typhimurium* ATCC 14028, *Shigella dysenteriae* PTCC 1188, *Shigella flexeneri* NCTC 8516, *Streptococcus sanguis* ATCC 10556, *Streptococcus salivarius* ATCC 9222, *Enterobacter aerogenes* NCTC 10009, *Pseudomonas aeruginosa* ATCC 9027, and fungi *Candida albicans* ATCC 10231, *Candida glabrata* ATCC 90030, *Aspergillus flavus*, *Aspergillus parasiticus* ATCC 15517, were used. Bacterial suspensions were prepared in Brain Heart Infusion (BHI) broth to a concentration of approximately 10^8 CFU/ml using standard routine spectrophotometric methods. Suspensions of fungi were made in Sabouraud dextrose broth (10^6 CFU/ml). Subsequent dilutions were made from the above mentioned suspensions which were then used in the tests.

The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values of extracts were determined by micro broth dilution assay. The extracts were two fold serially diluted with 10% DMSO containing 25.6–0.2 mg/ml of each extract. These dilutions were prepared in a 96-well micro-titre plate. The MOPS-buffered RPMI 1640 (fungi) [29], cation adjusted Muller Hinton broth (non-fastidious bacteria) [30] and Todd Hewitt broth (fastidious bacteria) [31] were used as broth media. After shaking, 100 μ l of extract dilutions was added to each well. The above microbial suspensions were diluted (1×10^6 CFU/ml for bacteria; 10^4 CFU/ml for fungi) and then 100 μ l was added to each well and incubated at 35°C. MICs were defined as the lowest concentration of extract dilutions that inhibits bacteria and fungi after 24, 48 h, respectively. The MLC values were the first well that showing no growth on solid media.

RESULTS

Total phenolic and total flavonoid content of *E. pinnatum* extract

Total phenolics (TPC) and flavonoid content (TFC) of *E. pinnatum* extracts was evaluated (tab. 1). TPCs of aqueous, methanol and ethanolic extracts were 86.2, 149.1 and 137.2 mg of GAC/g dry extracts. TFC were 8.4, 36.6 and 19.5 mg QE/g dry extracts, respectively. The TPC and TFC were higher in methanol extract than that of ethanolic extract and this amount was lower in water extract.

Table 1.

Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacity of the extracts tested in this study

Solvent	TPC [mg/g]	TFC [mg/g]	IC ₅₀ [μ g/ml]	%IP BCBT
Water	86.2	8.4	400	51.39
Methanol	149.1	36.6	200	63.96
Ethanol 70%	137.2	19.5	250	51.4
BHT	–	–	20	74.6

Antioxidant activity

Evaluation of the antioxidant activity by DPPH radical scavenging assay exhibited that the IC_{50} of methanol extract was lower than that of ethanolic extract and aqueous extract but higher than that of BHT (tab. 1). The IC_{50} value of *E. pinnatum* methanol extract was ten folds higher than that of BHT. The IC_{50} for BHT and methanol extract was 20 and 200 $\mu\text{g/ml}$, respectively. So, the methanol extract had the best radical scavenging activity among different *E. pinnatum* extracts.

Evaluation of the antioxidant activity by β -carotene linoleic acid test (BCBT) showed that methanol extract had higher antioxidant activity than ethanolic and aqueous extracts. The antioxidant activity of *E. pinnatum* ethanolic extract was same as aqueous extract in this system.

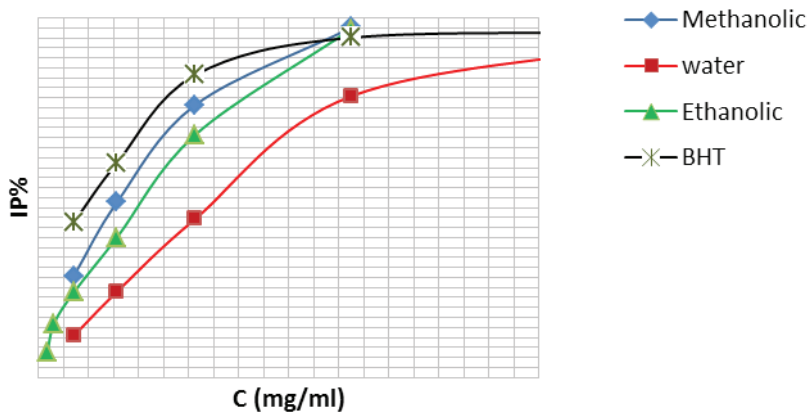


Figure 1.

Antioxidant activity of *E. pinnatum* extracts by DPPH test

Antimicrobial activity of *E. pinnatum* extract

The results of antimicrobial activity of *E. pinnatum* extracts are summarized in table 2. The results showed that the antimicrobial activity of *E. pinnatum* methanol extract against *Staphylococcus* sp. was higher than that of *E. pinnatum* ethanolic or aqueous extract. *S. saprophyticus* had more sensitivity to *E. pinnatum* extracts than that of *S. epidermidis* and *S. aureus*. The antimicrobial activity of methanol extract against *S. pneumoniae*, *E. faecalis*, *E. faecium* and *S. agalactiae* was higher than water extract and the *E. pinnatum* ethanolic extract was less sensitive to these microorganisms. The antimicrobial activity of methanol extract against oral bacteria (*S. mutans*, *S. sobrinus*, *S. sanguis* and *S. mutans*) was weaker than that of aqueous or ethanolic extracts. The antimicrobial effect of aqueous or ethanolic extract against oral *Streptococcus* sp. was the same. Gram-negative bacteria, yeast and

fungi were more sensitive than *Streptococcus* and *Staphylococcus* sp. to *E. pinnatum* extracts. *S. flexeneri*, *S. typhimurium*, *K. pneumoniae*, *C. albicans*, *C. glabrata* and *A. parasiticus* had the same sensitivity to aqueous, ethanolic or methanol extracts. *B. cereus* was more sensitive microorganism to ethanolic extract (MIC, MLC=0.4, 0.8 mg/ml) and aqueous extract (MIC, MLC=0.8, 1.6 mg/ml). *B. subtilis* and *S. dysantheriae* was more sensitive to methanol extract than that of other microorganisms (MIC, MLC=0.4, 0.8 mg/ml). Among the gram-negative bacteria, *S. dysantheriae* was more sensitive to aqueous extract (MIC, MLC=1.6, 1.6 mg/ml), ethanolic extract (MIC, MLC=0.8, 0.8 mg/ml) and methanol extract (MIC, MLC=0.4, 0.8 mg/ml).

Table 2.

Antimicrobial activity of *E. pinnatum* extracts by micro-broth dilution assay

	Water		Ethanolic		Methanol		Antibiotic	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>S. aureus</i>	12.8	25.6	3.2	6.4	0.8	1.6	0.25 ¹	0.5 ¹
<i>S. saprophyticus</i>	6.4	12.8	1.6	3.2	0.8	1.6	2 ¹	4 ¹
<i>S. epidermidis</i>	12.8	25.6	12.8	25.6	6.4	12.8	0.5 ¹	1 ¹
<i>S. pneumoniae</i>	12.8	25.6	25.6	25.6	3.2	6.4	0.25 ¹	0.5 ¹
<i>E. faecium</i>	12.8	25.6	25.6	25.6	3.2	6.4	1 ¹	2 ¹
<i>E. faecalis</i>	12.8	25.6	25.6	25.6	3.2	6.4	1 ¹	2 ¹
<i>S. agalactiae</i>	12.8	25.6	12.8	25.6	12.8	12.8	0.5 ¹	1 ¹
<i>St. mutans</i>	12.8	12.8	12.8	12.8	12.8	25.6	2 ¹	4 ¹
<i>St. sobrinus</i>	12.8	12.8	12.8	12.8	25.6	25.6	0.5 ¹	1 ¹
<i>St. sanguis</i>	12.8	12.8	12.8	12.8	25.6	25.6	0.25 ¹	0.5 ¹
<i>St. salivarius</i>	12.8	25.6	12.8	12.8	25.6	25.6	1 ¹	2 ¹
<i>Sh. flexeneri</i>	6.4	6.4	6.4	6.4	6.4	6.4	0.5 ²	0.5 ²
<i>E. coli</i>	6.4	12.8	6.4	6.4	6.4	6.4	0.5 ²	1 ²
<i>S. typhimurium</i>	6.4	6.4	6.4	6.4	6.4	6.4	1 ²	1 ²
<i>K. pneumoniae</i>	6.4	6.4	6.4	6.4	6.4	6.4	0.25 ²	0.25 ²
<i>Sh. dysantheriae</i>	1.6	1.6	0.8	0.8	0.4	0.8	0.125 ²	0.125 ²
<i>P. aeruginosa</i>	6.4	6.4	3.2	6.4	3.2	3.2	1 ²	1 ²
<i>E. aerogenes</i>	6.4	6.4	6.4	6.4	6.4	6.4	0.25 ²	0.5 ²
<i>B. subtilis</i>	6.4	25.6	6.4	6.4	0.4	0.8	0.125 ¹	0.125 ¹
<i>B. cereus</i>	0.8	1.6	0.4	0.8	3.2	6.4	0.25 ¹	0.5 ¹
<i>C. albicans</i>	6.4	6.4	6.4	6.4	6.4	6.4	0.25 ³	0.5 ³
<i>C. glabrata</i>	6.4	6.4	6.4	6.4	6.4	6.4	0.06 ³	0.06 ³
<i>A. flavus</i>	6.4	6.4	6.4	6.4	6.4	12.8	0.25 ³	0.25 ³
<i>A. parasiticus</i>	6.4	6.4	6.4	6.4	6.4	6.4	0.125 ³	0.125 ³
<i>A. brasiliensis</i>	6.4	12.8	6.4	6.4	6.4	12.8	0.125 ³	0.25 ³

1 - vancomycin, 2 - gentamycin, 3 - amphotericin B

DISCUSSION

The pharmacological activities of medicinal plants are of great importance. *Epimedium* contains 60 genera or more than flowering grassy plant. Among different species of *Epimedium* sp., *E. pinnatum* is native to Iran and grows in northern forests of this country. This research is the first study on antioxidant and antimicrobial activity of *E. pinnatum* aqueous, ethanolic and methanol extracts.

The investigation on antimicrobial and antioxidant activity of *E. pinnatum* extracts showed that among different extracts, methanol extract was found to possess higher antimicrobial activity than that of other extracts (aqueous and ethanolic extracts). Inspection on flavonoid and phenolic compounds in differently prepared extracts from *E. pinnatum* showed that methanol extract contains higher phenolic and flavonoid content. Research on other studies showed that *Epimedium* genus is rich in flavonoids and to date, 141 flavonoids, 9 glycoside flavonoid were identified in this genus.

Researches exhibited that flavonoids inhibit the growth of poliovirus, enterovirus. Regardless from their antiviral activities, flavonoids have inhibitory effect against *Micrococcus pyogenes* var. *albus*, *Staphylococcus aureus*, *Diplococcus pharyngis communis*, *Micrococcus catarrhalis*, *Haemophilus influenza*. Clinical data exhibit that a preparation from *Morindae officinalis* and *Epimedium* sp. (dose of 0.5 ml/kg) decrease the viremia and have positive effect on viral infection induced asthma in children [22]. So, *E. pinnatum*, such as other *Epimedium* sp., had antibacterial activity and this effect may be related to its flavonoid content.

Icarrin is an 8-iso amylene flavonoid glycoside in *Epimedium* genus. It inhibits the food spoilage microorganisms with MIC 0.23% for *Aspergillus* sp., 0.12% for *Escherichia coli* and 0.15% for *Bacillus* sp., *Penicillium* sp. and *Hansenula* sp. [23].

The results of this research showed the direct correlation between higher content of total flavonoids and phenolic contents of *E. pinnatum* methanol extract as well as its antioxidant and antimicrobial activity.

E. pinnatum methanol extract similarly to other species of *Epimedium*, may have other pharmacological activity such as aphrodisiac and anti-inflammatory effects because of high TPC and TFC content. Some other studies are needed to identify icarrin in *E. pinnatum* and to separate the antimicrobial and antioxidant flavonoids agents.

CONCLUSION

The results of this research exhibited that higher content of total flavonoids and phenolic contents of methanol *E. pinnatum* and the direct correlation between antioxidant and antimicrobial activity.

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PRZECIWBAKTERYJNE I PRZECIWUTLENIAJĄCE DZIAŁANIE *EPIMEDIUM PINNATUM*

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Streszczenie

W tradycyjnej medycynie chińskiej *Epimedium pinnatum* (rodzina *Berberidaceae*) używa się jako afrodyzjaku. Celem niniejszej pracy była ocena działania przeciwbakteryjnego i prze-

ciwutleniającego wyciągów: etanolowego, metanolowego i wodnego z *Epimedium pinnatum*. Całkowita zawartość fenoli i całkowita zawartość flawonoidów w każdym z wyciągów została oznaczona metodami spektrofotometrycznymi. Wykazano, że wyciąg metanolowy miał lepsze działanie przeciwbakteryjne niż wyciąg etanolowy i wodny. Całkowita zawartość fenoli i całkowita zawartość flawonoidów była wyższa w wyciągu metanolowym (149 i 36,6 mg/g) niż etanolowym (137,2 i 19,5 mg/g) i wodnym (86,2 i 8,4 mg/g). Wyciąg metanolowy miał niższą wartość IC_{50} (200 μ g/ml) niż wyciąg etanolowy (250 μ g/ml) i wodny (400 μ g/ml). Zauważono pozytywną korelację między całkowitym stężeniem fenoli i całkowitym stężeniem flawonoidów w wyciągach z *E. pinnatum*, a ich działaniem przeciwutleniającym i przeciwbakteryjnym.

Słowa kluczowe: *Epimedium pinnatum*, wyciąg, całkowita zawartość fenoli, całkowita zawartość flawonoidów, działanie przeciwbakteryjne, działanie przeciwutleniające