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Antimicrobial and antioxidant activities of *Epimedium elatum* (morr. & decne.) and quantification of its marker compounds

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Abstract

The medicinal importance of *Epimedium elatum* growing wild in Kashmir Himalaya is well documented. Antimicrobial and antioxidant activities of this plant were determined. Isolation, characterization and quantification of phenolics from the plant using different chromatographic and spectral techniques have been achieved. The overall results of this study indicates that the plant extracts and isolated markers have interesting antimicrobial and antioxidant properties and represent a potential source of medicine for the treatment of diseases.

Keywords

Epimedium elatum; Antimicrobial activity; Antioxidant activity; Quantification.

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INTRODUCTION

Epimedium elatum is a herb growing wild in Kashmir Himalaya (J&K), India. The plant usually grows in shady and moist places under the canopies of large gymnosperm trees. Epimedium-derived flavonoids exert beneficial intervention effects on both skeletal and non-skeletal factors related to hip fracture risk in late postmenopausal women[1]. Herbal *Epimedium* derived bioactive compound icariin reduces the incidence of steroid-associated osteonecrosis[2]. Icariin one of the main constituent of Epimedium species, various beneficial effects have been attributed to it[3,4]. Taking into consideration the antioxidant and antimicrobial activity of phenolics we have investigated the antioxidant and antimicrobial potential of *Epimedium elatum* and quantification of its markers (phenolics).

EXPERIMENTAL

General

Melting points are uncorrected and were determined

on BUCHI melting point apparatus. UV spectras were recorded in methanol in nm on Specard S 100 and Shimadzu UV-120-01 spectrophotometer. IR was recorded on a Perkin-Elmer Paragon-1000 spectometer with absorption given in cm^{-1}. ^{1}H NMR and ^{13}C NMR run on 500 MHz Bruker Daltonics instrument using TMS as internal standard. Mass spectra were recorded by using Bruker Esquire 3000 with electrospray ionization. HPLC in a Hewlett-Packard 1100 system with autosampler and quaternary pump coupled to a diode array detector. The column used was 250 mm x 4.6 mm, i.d., 5 μm, Prodigy ODS3 reversed-phase silica (Phenomenex Ltd., Torrance, CA). Column was run using silica gel (60-120 mesh; E. Merck), TLC was run on silica gel G and fluorescent aluminium 60 F_{254} TLC plates (0.25 mm) using solvents chloroform and methanol in different proportions. Spots were visualized on TLC under UV light, ferric chloride, cerric ammonium suplhate, exposure to iodine vapour in an iodine chamber and also by heating the chromatoplates at 100 °C in an oven after spraying with 10% sulphuric acid.

Plant material

The whole plant of *Epimedium elatum* (roots and above ground part) was collected from in august 2007 from both high and low altitudes of well known resort Gulmarg (4000-6000 m), Kashmir, (J&K), India and identified. Voucher specimen was deposited in the Centre of Plant Taxonomy (COPT), University of Kashmir. These plant materials were air dried at room temperature and powdered.

Extraction and isolation

Then 200 g of each powder were macerated in different organic solvents (2.5 l each) at room temperature for 90 h. The filtrate was then concentrated under vacuum to give crude extracts from roots (32 g) and from above ground part (54 g). These extracts were stored at room temperature till further use for bioactivity evaluation. The remaining plant material (whole plant; 900 g) was extracted exhaustively with hexane for 45 h. The plant material was then successively extracted with methanol for 48 h. The methanol extract was concentrated under reduced pressure to have a crude extract 120.5 g. The dried methanolic extract was dissolved in minimum amount of methanol and adsorbed on silica gel to form slurry. The air dried slurry was subjected to silica gel (60-120 mesh) column chromatography. The column was eluted with petroleum ether, chloroform and methanol taking together in different ratios. The markers isolated were identified by Co-TLC and HPLC using respective standard markers.

HPLC quantitation

Quantitation of icariin was achieved using analytical reverse phase HPLC in Hewlett-Packard system with autosampler and quaternary pump coupled to an diode array detector. The column used was 250 mm x 4.6 mm, i.d., 5 μm, Prodigy ODS3 reversed phase silica (Phenomenex Ltd., Torrance, CA) and elution solvents were A, water (containing 5% acetic acid): acetonitrile (50:50) and B, water: methanol (80:20). Solvent gradient was same as used in the proportion of 17% B for 2 min increasing to 25% B after 5 min, to 35% B after a further 8 min and to 50% B after 5 min. Calibration was done by injecting the standards three times at five different concentrations. Peak identification was performed by comparison of retention times and diode array spectral characteristics with the standards and the library spectra. Co-cochromatography was used when neccessray. Samples were injected in duplicate. Results were expressed as mg sample/100 g fresh mass (FW).

Sample extraction for antioxidant assays

Samples of *Epimedium elatum* were extracted with methanol thrice (100 ml the first time, 50 ml the next two times). The homogenate was filtered under reduced pressure through filter (Whatman No. 1) and stored at 5 °C prior to analysis. All extractions were done in duplicate, and subsequent assays were run in triplicate.

Assay technique and antioxidant activity

β-carotene/linoleic acid bleaching method

The antioxidant capacity was determined by the bleaching β-carotene method according to Miller[5], with some modifications[6]. For the preparation of reactive solution, aliquots (30 μl) of β-carotene in chloroform (2 mg/ml) were mixed with linoleic acid (50 μl), chloroform (1 ml) and tween 40 (510 μl). The chloroform was then completely evaporated under nitrogen flow and 100 ml of distilled water saturated with oxygen was added to the mixture. The absorbance was adjusted with water to 0.7. For the oxidation reaction, an aliquot of the sample (100 μl) was mixed with 2400 μl of the β-carotene solution in a cuvette. The samples were submitted to auto-oxidation at 50 °C for 2 h. The absorbance at 470 nm was measured with a spectrophotometer (Ultrospec 2000 UV/Visible) using a methanolic solution of BHT (Butylhydroxytoluene) (50 μM) as control. Results were expressed as 1 mol BHT equivalents/g sample FW.

DPPH radical scavenging activity

The antioxidant capacity was determined also by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical savenging method according to Brand-Williams[7], with some modifications[8]. A 50 μl aliquot of the extract previously diluted and 250 μl of DPPH (0.5 mM) were

shaken and, after 25 min, the absorbance was measured at 517 nm using a Microplate Spectrophotometer (Benchmark Plus, Biord, Hercules, CA), using a methanolic solution of BHT, at different concentrations, as control. The antioxidant capacity was expressed as 1 mol BHT equivalents/g sample FW.

Antimicrobial assays

Microbial strains

Six species of microorganisms (including both Gram (+) and Gram (-) bacteria) namely *Staphylococcus aureus* MTCC 737, *Escherichia coli* MTCC 723, *Klebsiella pneumonia* MTCC 109, *Proteus mirabilis* MTCC 1771, *Bacillus subtilis* MTCC 441, *Salmonella typhi* MTCC 537, *Candida albicans* LMP0204 and *Candida gabrata* LMP0413. The cultures were obtained from Institute of Microbial Technology, Chandigarh, India. These strains were sub-cultured on a fresh appropriate agar plate 24 h prior to any antimicrobial test.

Antimicrobial assay on Gram-positive, Gram-negative bacteria and fungi

Sensitivity test

Agar disc diffusion assay. Preparation of discs: Whatmann filter paper (No. 1) discs of 6 mm diameter were impregnated with 10 μl of the solution of crude extract at 20 mg/ml (200 μg/disc) prepared using DMSO. The discs were evaporated at 37 °C for 24 h. The RA discs (chloramphenicol for bacteria and nystatin for fungi) were prepared as described above using the appropriate concentrations to obtain discs containing 200 μg of drug. Two discs were prepared for each sample.

Diffusion test

The antimicrobial diffusion test was carried out as described by Berghe and Vlietinck[9] using a cell suspension of about 1.5×10^6 CFU/ml. The suspension was standardized by adjusting the optical density to 0.1 at 600 nm (Shimadzu UV-120-01 spectrophotometer)[10]. This was used to inoculate by flooding the surface of MHA plates. Excess liquid was air-dried under a sterile hood and the impregnated discs applied at equidistant points on top of the agar medium. A disc prepared with only the corresponding volume of DMSO was used as negative control. The plates were incubated at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the disc. The assay was repeated twice and the results expressed using signs as follows: (-) for no activity and (+) for samples with IZ > 6mm.

MIC determinations

The MICs of test samples and RA were determined as follows: the test sample was first dissolved in DMSO. The solution obtained was added to NBGP to a final concentration of 15 mg/ml for the crude extracts and 0.025-0.05 mg/ml for the RA. 100 μl of each concentration was added in a well (96-well microplate) containing 95 μl of NBGP and 5 μl of inoculum (standardized at 1.5×10^6 CFU/ml by adjusting the optical density to 0.1 at 600 nm Shimadzu UV-120-01 spectrophotometer). The final concentration of DMSO and Tween in the well was less than 1% (preliminary analyses with 1% (v/v) DMSO/NBGP and 10% (v/v) Tween 20/NBGP affected neither the growth of the test organisms nor the change of color due to this growth). The negative control well consisted of 195 μl of NBGP and 5 μl of the standard inoculums[11]. The plates were covered with a sterile plate sealer, then agitated to mix the content of the wells using a plate shaker and incubated at 37 °C for 24 h. The assay was repeated twice. Microbial growth was determined by observing the change of color in the wells. The lowest concentration showing no color change was considered as the MIC.

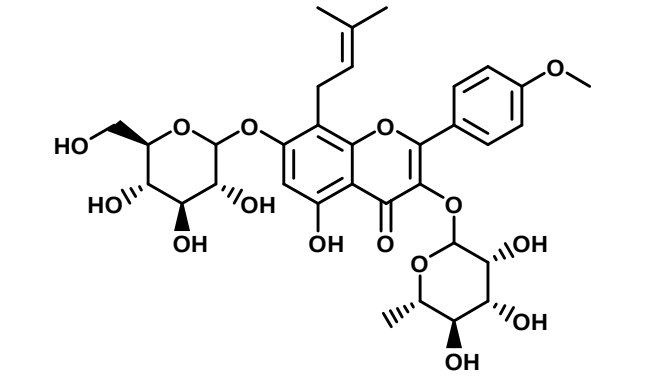
RESULTS AND DISCUSSION

Contents of bioactive compounds

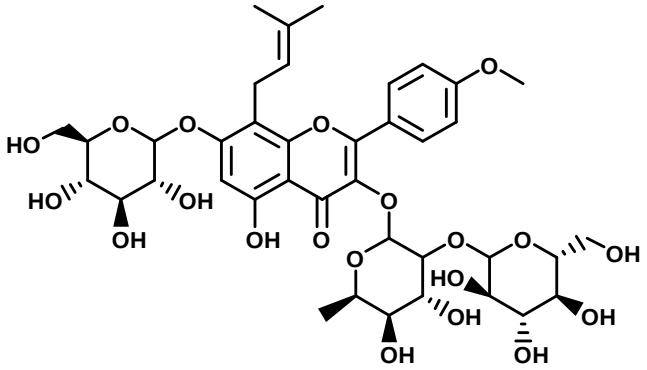
The results showed that *Epimedium* is a good source of bioactive polyphenolics such as flavonoids icariin, to which many beneficial effects have been attributed[12,13]. The main phenolics of *Epimedium elatum* are icariin and other flavonoids present mostly in the aerial parts. Phenolics such as icariin and its analogues are present in more than detectable amounts. The total bioactive content ranged from 0.3 to 1.4 mg/100g FW.

Determination of phenolics

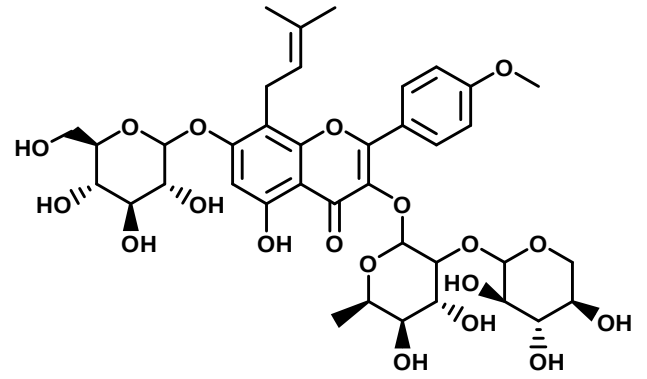
There is a particular interest in the amount of phenolics in *Epimedium elatum* because of increasing evidence of its many biological activities, earlier mentioned. Such type of compounds exists in plant in many derivative forms that differ in solubility, mobility and reactivity, in plant as well as in animal systems[14-19]. Different methods previously reported were tested to choose the best condition for the determination of the total phenol content in *Epimedium elatum*[20]. The results showed that there is significant difference among all methods tested. The best solvent for the extraction of icariin derivatives from *Epimedium* was also determined, among those normally cited. The results showed a huge difference in the total flavonoid content according to the solvent used for extraction. Pure ethylacetate was the least efficient (10.3 mg/100g FW) while as extraction with 100% methanol (12.3 mg/100g FW) was the best. Besides this, other parameters, such as hydrolysis, temperature were tested.



Icariin



Epimedin A



Epimedin B

Structure of three marker compounds

Antioxidant potential

The DPPH• scavenging activity of the methanolic extracts from different parts of *Epimedium elatum* is summarized in TABLE 2. It appeared that at the concentration of 10 mg/ml, the different extract from this plant possess the significantly DPPH• scavenging activity compared to other extracts. Isolated markers possess more activity than extract except methanol extract which showed reverse result. This activity was not significantly ($P < 0.05$) different to that of ascorbic acid used as reference antioxidant compound.

TABLE 1 : Phenolic composition and contents of bioactive compounds (mass; mg/100 g of FW) of *Epimedium elatum*

Constituent	Methanol extract	Acetone extract
Icariin	1.31±0.04	0.61±0.03
Epimedin A	1.10±0.21	0.34±0.07
Epimedin B	1.42±0.03	1.01±0.05

Values are expressed as mean ± SD for triplicate

TABLE 2 : Antioxidant activity of markers and different extracts from *Epimedium elatum*

Constituent	DPPH
Icariin	14.023±1.223
Epimedin A	12.051±0.112
Epimedin B	13.051±0.002
Extract	
Dichloromethane	10.043±1.223
Ethylacetate	11.011±0.112
Methanol	16.003±0.214

DPPH (mg DW/ml)
*Data are referred to DW (dry mass) and the values are the average of five determinations (± S.D.)

Antimicrobial potential

The inhibition activity more than 50% at 1000 μg/ml was also observed with the crude extracts from the whole plant of *Epimedium elatum*. The results of the antimicrobial activity of isolated extracts and four different extracts from *Epimedium elatum* are presented in TABLE 3. The diffusion test showed that all the tested extracts were active on all tested microorganisms, including Gram-positive and Gram-negative bacteria, as well as on the two *Candida* species. The results of the MIC determination indicated MIC values ranging from 1.5 to 12.5 mg/ml for those from the isolated markers and extracts of *Epimedium elatum*.

The lowest MIC value of ranging from 1.5-3g/ml was obtained by Icariin and methanolic extract. While as dichloromethane and ethylacetate extract have shown MIC from 8-12.5 mg/ml.

The overall results of the antimicrobial indicates that all the four extracts can be useful in the development of antimicrobial drugs. This could be confirmed by the results of the MIC determination (TABLE 3). The presence of antimicrobially active metabolites classes such as flavonoids, phenols might explain the wide spectrum of activity of the tested extracts. However, the isolation of the active principles will confirm this hypothesis and provide more explanation on mechanism of action of these extracts. The overall results of this study indicate that the extracts from *Epimedium elatum* represent a potential source of plant drugs. At this stage of our study, we can deduce that the icariin and the methanol extract appeared to be most promising part to be considered as antimicrobial medicines. Also, all parts of this plant could be used as antimicrobials, as they were not toxic following acute toxicity study.

TABLE 3 : Antimicrobial activity of markers and different crude extracts from different of *Epimedium elatum*[a] and reference antibiotics[b,c]

Tested material	Minimal inhibitory concentration (mass : mg/ml)							
	S.aureus	E.coli	K. pneumoniae	P. vulgaris	B. subtilis	S. typhi	C. albicans	C. gabrata
Icariin	2	3	2	2	1.5	2	3	4
Epimedin A	1.5	1.4	3	5.5	6	3	5	3
Epimedin B	1.0	1.1	2	3.5	2.4	4.2	2.5	3.5
Dichloromethane	12	10	10	8	9	11	11	10
Ethylacetate extract	11	14	12	12.5	12.5	13	11	10
Methanolic extract	6	1.5	2.5	7	5.5	6	5	7
Chloramphenicol[b]	0.025	0.05	0.05	0.05	0.05	0.025		
Nystatin[c]							0.05	0.05

[a]All determinations were done in triplicate; [b]Reference antibiotics (RA) for bacteria; [c]Reference antibiotics (RA) for fungi

ABBREVIATIONS

BHT, butylhydroxytoluene; CFU, colony forming units; DMSO, dimethylsulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; GI, growth inhibition; IZ, inhibition zone; MIC, minimal inhibition concentration; MTCC, microbial type culture collection; NA, nutrient agar; NBGP, nutrient broth containing 0.05% phenol red and supplemented with 10% glucose; RA, reference antibiotics; YEM, yeast extract media

CONCLUSION

The overall results of this study indicate that the chemical markers and extracts from the *Epimedium elatum* represent a potential source of plant drugs. At this stage of our study, we can deduce that the plant appeared to be most promising part to be considered as antioxidant and antimicrobial medicines. Also, all parts of this plant could be used as antimicrobials, as they were not toxic following acute toxicity study. However, further investigations will be necessary to confirm our hypothesis.

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REFERENCES

[1] G.Zhang, L.Qin, Y.Shi; Bone, **43**, 64 (2008).
[2] H.Sheng, G.Zhang, L.Qin, Y.Wang, X.Wang, K.Lee, X.Yao, K.Leung; Bone, **43**, 101 (2008).
[3] Y.Pan, L.Kong, X.Xia, W.Zhang, Z.Xia, F.Jiang; Pharmacology Biochemistry and Behavior, **82**, 686 (2005).
[4] X.Zhang, Y.Li, X.Yang, K.Wang, J.Ni, X.Qu; Life Sciences, **78**, 180 (2005).
[5] H.E.Miller; Journal of American Oil Chemists Society, **91**, 48 (1971).
[6] N.M.A.Hassimoto, M.I.Genovese, F.M.Ljolo; Journal of Agricultural and Food Chemistry, **53**, 2958 (2005).
[7] W.Bran-Williams, M.E.Cuvelier, C.Berset; Lebensmittel-Wissenschaft Und-Technologie, **28**, 25 (1995).
[8] J.M.Duarte-Almeid, R.J.Santos, M.I.Geovese, F.M.Lajola; Ciencia e Tecnologia de Alimentos, **2**, 446 (2006).
[9] V.A.Berghe, A.J.Vlietinck; Method in Plant Biochemistry, **6**, 46 (1991).
[10] M.L.Tereschuk, M.V.Q.Riera, G.R.Castro, L.R.Abdala; Journal of Ethnopharmacology, **562**, 27 (1997).
[11] J.R.Zgoda, J.R.Porter; Pharmaceutical Biology, **39**, 221 (2001).
[12] B.H.Chung, J.D.Kim, C.K.Kim, J.H.Kim, M.H.Won, H.S.Lee, M.S.Dong, K.S.Ha, Y.G.Kwon, Y.M.Kim; Biochemical and Biophysical Research Communications, **376**, 404 (2008).
[13] Y.K.Wang, Z.Q.Hang; Pharmacological Research, **52**, 174 (2005).
[14] R.L.Prior, G.Cao, A.Martin, E.Sofi, J.McEwen, C.O'Brien, N.Lischner, M.Ehlenfeldt, W.Kalt, G.Krewer, C.M.Mainland; J.of Agric.Food Chem., **46**, 2686 (1998).
[15] H.Wang, G.Cao, R.L.Prior; J.Agric.Food Chem., **44**, 892 (1996).
[16] M.E.Cuvelier, H.Richard, C.Berset; Biosci.Biotech. Biochem., **56**, 324 (1992).
[17] J.S.Hogg, D.H.Lohmann, K.E.Russell; Can.J.Chem., **39**, 1588 (1961).
[18] G.Cao, S.Sofi, R.L.Prior; J.Agric.Food Chem., **44**, 3426 (1966).
[19] H.Wang, G.Cao, R.L.Prior; J.Agric.Food Chem., **44**, 701 (1996).
[20] H.Y.Zhao, J.H.Sun, M.X.Fan, L.Fan, L.Zhou, Z.Li, J.Han, B.R.Wang, D.A.Guo; Journal of Chromatography A, **1190**, 157 (2008).