

# Evaluation of medicinal value of *Epimedium elatum* on the basis of pharmacologically active constituents, Icarin and Icariside-II

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**Abstract:** *Epimedium* L. is well known medicinal genus of Chinese pharmacopoeia. Various species are ethnobotanically used against diseases of eye and kidney, impotence, asthma, arthritis and hypertension; besides being used as analeptic, expectorant, antibacterial, hypoglycemic, vasodilator and refrigerant. Recent studies have attributed most of these medicinal properties to its flavonoid glycosides, especially Icarin which is the major pharmacologically active constituent. Icarin has been found to possess effective aphrodisiac, antioxidant, immunomodulatory, hepatoprotective, cardioprotective, vasodilatory, antidepressant and anti-osteoporosis activities. Icariside-II, another active constituent, has cytotoxic and cytostatic effects on 6 cancer cell-lines, and immunosuppressive effects on allograft rejection. In this present study, *Epimedium elatum* Morr. and Decne., the only species of this genus growing in Indian subcontinent, has been investigated for its medicinal value by determining the content of pharmacologically active constituents, Icarin and Icariside-II, by HPLC method. HPLC analysis of alcohol extract of its shade dried parts was performed with reverse phase C-18 column. The mobile phase for Icarin was acetonitrile-water in gradient mode; while for Icariside-II, it was methanol-water. The effluent was monitored at 270nm. The results have revealed an appreciable content of Icarin and Icariside-II in its aerial and underground parts; the content being higher in populations growing at higher altitudes. The substantial presence of pharmacologically active constituents, Icarin and Icariside-II, in this species of *Epimedium*, signifies its value as a medicinal plant.

**Keywords:** *Epimedium elatum*, Flavonoid glycoside, HPLC, Icarin, Icariside-II, medicinal.

## INTRODUCTION

*Epimedium* L. (Berberidaceae), commonly called Horny Goat Weed, is a well-known medicinal genus of Chinese pharmacopoeia (China Pharmacopoeia Committee, 1999). The genus is represented by about fifty-four species of perennial herbs, which disperse from Japan to Algeria and mainly occur in the East Asia and Mediterranean region (China 43 spp; Japan, Korea, Manchuria, Far Eastern Russia 6 spp) (Stearn, 2002; Sun *et al.*, 2005). About 80% of the total species are found in central-southeastern China (Ying, 2001). Species such as *Epimedium grandiflorum* and *E. brevicornum* are grown as ornamental herbs (Naeyer *et al.* 2005); while various other species such as *E. sagittatum*, *E. brevicornum*, *E. wushanense*, *E. koreanum*, *E. pubescens*, *E. alpinum*, *E. grandiflorum* and *E. rugosum* are ethno-botanically used for medicinal purposes against impotence, sterility, cold, numbness, rheumatism, arthritis, hypertension, asthma, chronic bronchitis and diseases of eye and kidney; besides being used as analeptic, expectorant, antitussive, antibacterial, hypoglycemic, vasodilator, aphrodisiac, anodyne, carminative, refrigerant and tonic (Yeung, 1985; Tang and Eisenbrand, 1992; Zhu, 1998). Recent pharmacological and clinical studies on various species have confirmed various medicinal properties such as immunomodulatory (Liang *et al.*, 1997), hepatoprotective (Lee *et al.*, 1995), aphrodisiac and libido-promoting

(Chen and Chiu, 2006), energy-promoting (Cai *et al.*, 1998), memory-enhancing (Liu *et al.*, 1993), anti-oxidant (Xu *et al.*, 2003), angiogenetic (Wang *et al.*, 2004), estrogenic (Naeyer *et al.*, 2005), anti-osteoporosis (Ma *et al.*, 2002), hypoglycemic, expectorant, anti-tussive, anti-hypertension, anti-asthmatic and anti-stress activities (Cai *et al.*, 1998; Zhu, 1998; Lai, 2001).

Most of these medicinal properties have been attributed to its flavonoids (Liang *et al.*, 1996; Liang *et al.*, 1997). Over 141 different flavonoids have been isolated in the genus by most modern techniques (Ma *et al.*, 2011). The active constituents have been found to be flavonoid glycosides, especially Icarin which is the major pharmacologically active constituent (Wang and Huang, 2000; Shen *et al.*, 2007). Icarin (5-hydroxy-4'-methoxyl-8-prenylflavone-3-O- $\alpha$ -L-rhamnopyranoside-7-O- $\beta$ -D-glucopyranoside) has been found to possess effective aphrodisiac (Qiao *et al.*, 2002; Xin *et al.*, 2003; Tian *et al.*, 2004), immunomodulatory (Liang *et al.*, 1997), hepatoprotective (Lee *et al.*, 1995), cardio-protective (Zhang *et al.*, 2000), vasodilatory (Guan *et al.*, 1996), anti-depressant (Pan *et al.*, 2005), anti-osteoporotic (Meng *et al.*, 2005), antioxidant and anti-apoptotic activities (Wang and Huang, 2005). Because of its well-established medicinal properties, Icarin is the quality control standard in many Traditional Chinese Medicine at present (Wang and Lou, 2004).

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Besides Icariin, other important pharmacologically active constituents are Icariside-II, Epimedin A- C, Sagittoside etc. Icariside-II (also called baohuoside-1 or 5,7-dihydroxy-4'-methoxyl-8-prenylflavone-3-O- $\alpha$ -L-rhamnopyranoside), has a great value in the treatment of chronic inflammatory and autoimmune diseases, because of its selectively immunosuppressive effects (on T-cell and B-cell activation) *in-vitro* and effective prevention of heart allograft rejection in rats (Li *et al.*, 1994; Ma *et al.*, 2004). Besides, it has been shown to have cytotoxic and cytostatic effects on 6 cancer cell-lines (Li *et al.*, 1990).

In the present study, *Epimedium elatum* Morr. and Decne., the only species of this genus growing in Indian subcontinent, has been investigated for its medicinal value by discovering its ethnobotanical uses and determining the content of pharmacologically active constituents, Icariin and Icariside-II.

## MATERIALS AND METHODS

### Plant material

*Epimedium elatum* Morr. and Decne., a very little known species of this well known genus, is endemic only to Kashmir Himalaya. It is the only representative of this genus in Indian subcontinent, growing wild under the canopy of coniferous forests. The plants are erect, shade-loving, perennial herbs, 50-100 cm tall, with glabrous stem and branches, sub-serrately toothed leaflets and pale yellowish or yellowish white flowers. Mostly taxonomic study, with relatively little phytochemistry (Tantry *et al.*, 2012), has so far been conducted on this species.

The plant material (aerial and underground parts) was collected from two different populations (I and II), located at two different sites (districts) of Kashmir valley differing in altitude (3000m and 2500m asl). Plants were identified on the basis of the relevant literature (Jafri, 1975) and the specimens of Herbarium KASH, Department of Botany, University of Kashmir. A voucher sample was also deposited in the Herbarium KASH.

### Apparatus and reagents

HPLC machine of Thermo Finnigan make (Thermo Finnigan, Co. Ltd., San Jose, CA USA), consisting of quaternary P4000 pump, AS3000 autosampler, UV6000LP photo diode array detector and a reverse phase C-18 column (5 $\mu$ m, Merck, 250x4mm), was used. The machine is fully computer controlled and data is acquired using Chromquest software (Version 4.0).

Pure standards of Icariin and Icariside-II were a gift from Professor Bao Lin Guo of Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, China. The purity of compounds was more than 99% as determined by HPLC.

All organic solvents (ethanol, chloroform, methanol) used for preparation of crude extract and TLC separation were of analytical grade (E. Merck, Mumbai, India), while methanol and acetonitrile used for HPLC was of HPLC grade (E. Merck, Mumbai, India). The water used was fresh ultra-pure distilled water, purified by a Millipore water purification system (Milford, MA, USA).

### Ethnobotanical survey

Ethnobotanical data were gathered through repeated semi-structured interviews of 60 non-specialist elderly people, comprising of nomads and shepherds (mean age 55; 50% men, 50% women) who were working and staying close to plants in the forests of Pir Panjal range of Jammu and Kashmir. The interviews were conducted according to the well framed questionnaire, the focus being on *E. elatum*. The specimen plant was shown to them & they mentioned its uses. Later, they themselves collected it from the natural populations for its proper identification. The plants were identified and the voucher specimen deposited in the Herbarium KASH. The data were statistically analyzed.

### Drying and extraction

Four different samples of plant material were taken for extraction, two (aerial and underground parts separately) from each of the populations. The plant material was shade-dried, chopped and grinded at room temperature. 100g of the powdered material from each sample was extracted thrice with 1 litre of 95% ethanol for 24 hours at room temperature. The three extracts (washings) were mixed and then dried by evaporating the solvent in a thin film rotavapour (Heidolph Rotavapour). All extractions were done in duplicate and the subsequent assays were run in triplicate.

### Chromatography

The dried extract was run on TLC using solvents chloroform and methanol in 8:2 ratio and co-TLCed with marker compounds, Icariin and Icariside-II. Dried extract was dissolved in HPLC grade methanol at 12.50 mg ml<sup>-1</sup>. The solution was filtered and the filtrate taken for HPLC analysis. The marker compounds, Icariin and Icariside-II, were also dissolved in HPLC grade methanol at 0.11 mg ml<sup>-1</sup> and 0.10 mg ml<sup>-1</sup> respectively and calibrated on HPLC.

High Performance Liquid Chromatographical (HPLC) analysis was performed according to protocols given by Liu *et al.* (2005) & Chen *et al.* (2007), with some modifications. HPLC analysis was performed at an oven temperature of 30°C. The mobile phase was acetonitrile-water in gradient mode as follows: 0-12 min, 28% acetonitrile; 12-20 min, 28-35 % acetonitrile; 20-30 min, 35% acetonitrile; 30-35 min, 35-28% acetonitrile; 35-40 min, 28% acetonitrile. The effluent was monitored at 270nm and the flow rate was 1.0 ml min<sup>-1</sup> constantly.

Injection volume was 4 $\mu$ l for marker and 10 $\mu$ l for samples. Linear calibration curve was obtained for Icariin for concentration ranges of 0.22-1.1 $\mu$ g. However, the mobile phase for Icariside-II was methanol-water (80:20) and the flow rate was 0.6 ml min<sup>-1</sup> constantly. Linear calibration curve was obtained for Icariside-II for concentration ranges of 0.12-0.60 $\mu$ g. Samples were injected in triplicate.

The chromatogram was prepared to show both the qualitative and quantitative data of Icariin and Icariside-II. Peak identification was performed by comparison of retention times with the standards. Co-chromatography was also used to confirm peaks. The results were compared with the calibration curves to compute the quantity of Icariin and Icariside-II as percentage of extract and dry mass of plant material. The data were statistically analyzed by computing standard deviation and LSD at P=0.05.

## RESULTS

The ethnobotanical survey revealed that *Epimedium elatum* Morr. and Decne. is being ethnobotanically used to treat cold, cough, running nose, toothache, tooth-decay, and diseases of bones (joint pain, back aches, stiff necks, etc.). Its extract is applied to heal wounds, besides being sprayed as a rodent and mosquito repellent.

*Epimedium elatum* collected from two different populations (I and II), growing at different altitudinal sites, showed more or less similar morphological characters. However, the plants of population II were taller than population I. The biomass of aerial parts of populations decreased, while the dry mass of underground parts increased, with an increase in altitude of habitat; resulting in higher biomass of whole plants of population II than population I.

TLC and co-TLC of the alcohol extract and marker compounds for the first time showed the presence of Icariin and Icariside-II in both the aerial and underground parts of *Epimedium elatum*. HPLC analysis of alcohol extract confirmed the presence of Icariin as well as Icariside-II. The marker compound, Icariin, was found to elute at a retention time of 8.3 $\pm$ 0.2 minutes, while Icariside-II eluted at 9.8 $\pm$ 0.2 minutes. In population-I, the percentage of Icariin was found to be 2.66% and 3.54% of the dry mass of extract of aerial and underground parts respectively, while the percentage was found to be 0.35% and 0.23% of the total dry mass of aerial and underground parts respectively (table 1). Extractive value of aerial and underground parts was 13.29% and 6.38% respectively. Thus, Icariin percentage (w/w) was higher in dry extract of underground parts than that of aerial parts. But the percentage was lower in underground parts than aerial parts when calculated on plant dry mass basis. Similar

trend was observed in population-II. However, Icariin content, calculated either as percentage of dry mass of extracts or total dry mass of plant parts, was lower in population-II than that of population-I; though the extractive value of aerial parts as well as underground parts of plants of population-II was higher than that of population-I (table 1).

The content of Icariside-II followed the same trend as that of Icariin. Icariside-II content was also higher in underground parts than aerial parts when calculated as percentage of dry mass of extracts. But, it was lower in underground parts than aerial parts when calculated as percentage of total dry mass of plant parts. Icariside-II content of plants of population-I, growing at higher altitude, was also higher than that of population-II (table 1). It was evident from HPLC that the impurities in underground parts are less than that in aerial parts.

## DISCUSSION

Extracts of various species of *Epimedium* have been proven by most modern methods/techniques to possess various medicinal properties (Liang *et al.*, 1997; Cai *et al.*, 1998; Xu *et al.*, 2003). Chemical analysis of the genus has already led to the isolation of over 141 different flavonoids, especially flavonoid glycosides. Evaluation of the bioactivities of these flavonoids has attributed most of these medicinal properties to its flavonoid glycosides, especially Icariin (Liang *et al.*, 1996). Icariin is considered to be the major pharmacologically active constituent of *Epimedium* Herba (Wang and Huang, 2005; Shen *et al.*, 2007) and possess effective medicinal properties such as aphrodisiac, immunomodulatory, hepatoprotective, cardio-protective, vasodilatory, antioxidant, anti-osteoporosis and anti-depressant activities (Lee *et al.*, 1995; Liang *et al.*, 1997; Wang and Huang, 2005; Chen and Chiu, 2006). It is now a recognized biomolecule that is being commercially exploited as a drug in different formulas. Because of its well-established medicinal properties, Icariin is the quality control standard in many Traditional Chinese Medicine at present (Wang and Lou, 2004). The Chinese Pharmacopoeia requires a qualitative determination of this main flavone constituent, Icariin, for this official herbal medicine by TLC comparison with an authentic sample after extraction of the powdered herb with ethanol (Tang and Eisenbrand, 1992). HPLC analysis and TLC comparison of our samples with authentic marker of Icariin have shown that aerial and underground parts of *Epimedium elatum* also contain an appreciable amount of Icariin which can be extracted and harnessed for medicinal purposes (table 1). Our results are, therefore, in compliance with the Quality Control Standard of Chinese Pharmacopoeia, thus signifying its value as a medicinal plant. The substantial content of Icariin in *Epimedium elatum* may be the basis for its ethnobotanical uses

**Table 1:** Extractive value, and Icariin and Icariside-II content of aerial and underground parts of plants of two different populations of *Epimedium elatum* Morr. and Decne. growing at different altitudes\*.

Population	Plant Part Analyzed	Extractive Value (%)	Icariin Content		Icariside-II Content	
			% of dry mass of extract	% of dry mass of plant	% of dry mass of extract	% of dry mass of plant
I	Aerial Part	13.290±0.30	2.661±0.09	0.354±0.04	0.049±0.01	0.007±0.00
	Underground Part	6.378±0.18	3.542±0.11	0.226±0.02	0.081±0.01	0.005±0.00
II	Aerial Part	13.932±0.27	1.809±0.08	0.252±0.03	0.008±0.00	0.001±0.00
	Underground Part	7.796±0.19	3.230±0.09	0.251±0.02	0.048±0.01	0.004±0.00
LSD at P=0.05		1.200	0.250	0.020	0.030	0.001

\* Values are means of three replicates ± SD.

against the diseases of bones (joint pain, back aches, stiff necks, etc.) and tooth-decay.

Icariside-II is another pharmacologically active constituent of the genus that possesses cytotoxic and cytostatic effects on 6 cancer cell-lines (Li *et al.*, 1990) and selective immunosuppressive effects (on T-cell and B-cell activation) effectively preventing heart allograft rejection in rats (Li *et al.*, 1994; Ma *et al.*, 2004). Icariside-II has also been determined in this species (table 1), further establishing its medicinal importance. The ethnobotanical uses against cold, cough, wounds, and toothache may be based on its Icariside-II content.

At higher altitudes, the growth period is shorter producing plants with smaller height and lower herbage/biomass, but with higher content of Icariin and Icariside-II (table 1). This is because of more stress conditions at higher altitudes. Stress conditions are well known to give rise to the production of more secondary metabolites in plants (Pavarini *et al.*, 2012). This is also true in case of our results as higher Icariin and Icariside-II content was found in plants growing at higher altitudes. Thus, *Epimedium* plants growing at higher altitudes are more productive in terms of Icariin and Icariside-II content than plants growing at lower altitudes.

## CONCLUSION

Both the aerial and underground parts of *Epimedium elatum* have an appreciable content of Icariin and Icariside-II which can be extracted and harnessed for medicinal purposes. The present study indicates that the substantial presence of Icariin and Icariside-II in *Epimedium elatum* may be the basis for its ethnobotanical uses, thus, signifying its value as a medicinal plant.

Icariin is usually present in (and extracted from) aerial parts of other species; however, higher Icariin and Icariside-II content (as %age of dry mass of extract) was found in underground parts than aerial parts of this species. The impurities of underground parts being less than the aerial parts, it would be quite efficient and easier to harness this species for these desired compounds. It is,

therefore, suggested that *Epimedium elatum* can prove a promising raw material (industrial crop) for pharmaceutical industry of Indian subcontinent.

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