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Genetic diversity, LCMS based chemical fingerprinting and antioxidant activity of *Epimedium elatum* Morr & Decne

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ABSTRACT

Epimedium elatum Morr & Decne is a perennial herb, endemic to shady coniferous forests of north-western Himalayas, India. It owes its pharmaceutical importance to high concentration of flavonoid glycosides particularly epimedins and Icariin. A lot of medicinal properties are attributed to them like aphrodisiac (PDE-5 inhibition), anti-osteoporosis, anticancer, antioxidant, anti-fatigue and antiviral activities. In the present study, twenty accessions of *E. elatum* were investigated for their genetic diversity and chemo-profiling through molecular markers and fingerprinting, respectively. Further, their phyto-chemical variation and related antioxidant activities are also being reported. Molecular fingerprinting resulted in 277 total loci, out of which 254 were polymorphic, displaying an overall polymorphism of 91.1%. Moreover, fourteen unique bands were amplified, maximum (6) were amplified in GL accession from 3 primers (UBC900, UBC834 & UBC823). The dendrogram topology indicated moderate to high genetic diversity corroborating with diversity index (0.36). Chemo-profiling revealed epimedin B and epimedin C as the major prenylated flavonoids in leaves, while Icariin was found highest in underground parts. However, no correlation could be deduced between molecular and prenylated flavonoid profiling in the present study. Furthermore, ethanolic extracts of rhizomes exhibited stronger antioxidant ability. The study has great implications as the wild resource conservation, germplasm assessment, quality resource explorations have become critical for the sustainability of the species. Efforts are thus needed to conserve the elite accessions of *E. elatum.*

1. Introduction

The genus Epimedium of Berberidaceace family is represented by about 60 species (Sheng et al., 2008; Quan et al., 2011). They are widely distributed in China and only five species of this genus are treated as the official source of Herba Epimedii in Chinese Pharmacopoeia, *E. brevicornum* Maxim, *E. sagittatum* (Sieb. et Zucc) Maxim, *E. pubescens* Maxim, *E. wushanense* T. S. Ying and *E. koreanum* Nakai (Pharmacopoeia Commission of PRC, 2010). They are known by English names like Rowdy Lamb Herb, Barrenwort, Bishop's Hat, Fairy Wings, and Horny Goat Weed (Ma et al., 2011). They have been used in folk medicine as a tonic, aphrodisiac and antirheumatic preparations in China, Japan, and Korea for more than 2000 years (Ma et al., 2011). More than 260 phytochemical compounds have been isolated from different species of *Herba Epimedii* (Ma et al., 2011). Among them, epimedin-A, epimedin-B, epimedin-C and Icariin are the major prenylated flavonoid glycosides (Wu et al., 2003; Pei et al., 2007; Wang et al., 2007, 2010), recommended as quality determining markers for *Herba Epimedii* (Ma et al., 2011). They have been reported to possess aphrodisiac activity (Shin et al., 2015; Kovac et al., 2015) and potential anti-osteoprortic role (Zhang et al., 2014a,b; Xie et al., 2015). Besides, recent studies have also reported *Epimedium* species with wide-reaching pharmacological actions like regulation of NO-cGMP pathway (Zhai et al., 2014; Jin et al., 2014) estrogenic activities (Kang et al., 2012; Ming et al., 2013), improving cardiovascular and cerebrovascular func-

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tions (Ma et al., 2011; Ke et al., 2015), immunological modulation (Ma et al., 2011), anti-oxidant (Zhang et al., 2014a,b; Jang and Kim, 2015), anti-cancer (Zhao et al., 2015; Song et al., 2015; Li et al., 2015a,b; Wang et al., 2015; Zhou et al., 2015), anti-aging (Ma et al., 2011), anti-fatigue (Schluesener and Schluesener, 2014; Wang et al., 2014) and anti-viral actions (Cho et al., 2015; Xiong et al., 2015; Li et al., 2015a,b).

E elatum is a perennial medicinal plant, endemic to high altitude shady forests of India and Pakistan in north-western Himalayas (Nasir and Ali, 2005; Perveen and Qaiser, 2010). Recent reports have indicated its possible ethnomedicinal use in bone related diseases (Arief et al., 2015) and potent PPAR-γ ligand-binding activity (Tantry et al., 2012). In addition, few reports on isolation and simultaneous quantification of its prenylated flavonoids (Tantry et al., 2012; Sofi et al., 2014; Naseer et al., 2015; Arief et al., 2015) have also been published. Chemically, aerial and underground parts of the *E. elatum* have similar concentration of prenylated flavonoids (ABCI) as reported in Chinese pharmacopeia (Naseer et al., 2015). Chemotypic variation of four prenylated flavonoid glycosides, viz epimedin A, epimedin B, epimedin C and Icariin (ABCI) in wild accessions of *E. elatum* has not been broadly characterized. Research on its distributional and altitudinal aspects is also poorly documented. There are no reports on the genetic diversity assessment of this species. So, it was necessary to evaluate the genetic polymorphism of *E. elatum* for future conservation implications. The present study aimed to determine (i) the distribution of this species under natural habitats in Kashmir Himalayas, (ii) genetic diversity using ISSR fingerprinting. (iii) chemo-taxonomic marker variation in different plant parts with respect to four prenylated flavonoids through LCMS fingerprinting (iv) total phenolic (TPC), flavonoid (TFC) and antioxidant potential employing 2,2-diphenyl-1-picryl-hydrazyl-hydrate free radical method (DPPH) and Ferric reducing antioxidant power (FRAP) model systems.

2. Materials and methods

2.1. Plant materials

A total of twenty accessions of *E. elatum* were collected from different eco-geographical regions of Kashmir Himalayas (Table 1). They were identified by taxonomists from two herbarias viz; Centre of biodiversity and plant taxonomy, University of Kashmir and Janaki Amal herbarium, IIIM Jammu, India. Voucher specimens were also deposited at the respective places. The fresh leaves were collected in zip-lock bags for DNA extraction. For phytochemical analysis (LCMS, TPC, TFC, and antioxidant activity), the leaf, stem, rhizome and root were separated, shade dried and chemically extracted.

2.2. DNA isolation and ISSR PCR

Leaf DNA was extracted following CTAB method with some modifications (Doyle and Doyle, 1987; Porebski et al., 1997). The quality and quantity of extracted DNA was estimated by electrophoresis and Nanodrop (ND-2000, Thermo Scientific, USA). The inter-simple sequence repeat (ISSR) primers were selected to study the genetic diversity of *E. elatum*. Forty published primer sequences of the related genus, *Podophyllum* (Xiao et al., 2006; Zong et al., 2008; Naik et al., 2010; Liu et al., 2014) were tried in the present study. PCR optimization showed that 20 μl reaction system was ideal for producing sharp reproducible bands in *E. elatum*. Each 20 μl PCR reaction consisted of 10x PCR buffer (supplied with 15 mM of $MgCl_2$), 2 mM dNTP mix, 10 pmole primer, 50 ng of template DNA, and 1U of Taq DNA polymerase (Bangalore, Genei, India). The PCR was performed using a thermal profile of one cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, specific primers annealed at 48–58 °C for 45 s, and 2 min extension at 72 °C, and a final extension at 72 °C for 5 min. The amplifications were performed in ABI Geneamp 9700 thermal cycler (Thermo Scientific, USA). The amplification products were electrophoresed on 1.4% agarose gels buffered with 1X TAE for 2.5 h at 100 V, detected by ethidium bromide staining, and imaged in the Syngene Bio imaging System (Syngene, UK). Each primer was amplified twice to confirm reliability and reproducibility.

2.3. Genetic statistics and clustering

PCR amplification with each primer was performed thrice and only reproducible and distinct bands were scored. Binary data matrix with presence (1) and absence (0) of bands was prepared for ISSR primers. Allele size was estimated visually by comparing with 100 bp plus ladder (Fermentas) on a gel. All the statistical calculation for resolving power (Rp) (Prevost and Wilkinson, 1999), polymorphic information contents (PIC) (Anderson et al., 1993) and marker indexes (MI) (Milbourne et al., 1997) were done as per the reported methods. Binary matrices were subjected to statistical

Table 1
Sample information of *E. elatum* accessions including habitat characteristics in different regions of Kashmir Himalayas.

Locations	AC[a]	Accession	Altitude	Latitude	Longitude	Habitat characteristics
Gulmarg	GL	22304	2725	34.02°	74.22°	Shade of thick bushes under pine trees.
Babareshi	BR	22306	2694	34.03°	74.23°	Grasses or bushes of shady slopes on hillside under the canopies of Pines.
Drang	DR	22305	2301	33.55°	74.29°	Partial shady slope or open with grasses or on roadside.
Dangarpora	DG	22308	2592	34.05°	74.32°	Sunny slope under small bushes, grown in stone cavities or on roadside.
Boniyar	BY	22307	2148	34.15°	74.21°	Bushes of shady slope on hillside or roadside under canopies of pines.
Yusmarg	YS	22310	2383	33.49°	74.40°	Small bushes in the partial shade of pine trees on hillside or on open slopes.
Dodipathri	DP	22309	2432	33.53°	74.34°	Grasses or Bushes of deep shady slopes on hillside or roadside.
Naranag	NAR	22303	2272	34.21°	74.58°	Bushes of shady slope on streamside and hillside.
Gagangir	GG	22314	2435	34.17°	75.12°	Under partial shade of pine trees on stone cavities near streamside.
Dachigam	DGM	22312	2912	34.08°	75.02°	Bushes of shady slopes on roadside or on stream side.
Pahalgam	PGM	22319	2206	34.00°	75.18°	Bushes of shady slope or under pine trees; facing anthropogenic threats.
Kokernag	KNG	22311	2343	33.34°	75.17°	Bushes of hillside, under the partial shade in stone cavities.
Verinag	VNG	22320	1935	33.32°	75.14°	Bushes of shady slope on hillside or on stone cavities.
Khillanmarg	KMG	22315	3133	34.02°	74.21°	Sunny slope on open bushes or on streamside in stone cliffs.
Chaknala	CNG	22316	2508	34.37°	74.51°	Under partial shade of pine trees or on roadside or hillside.
Sheikhpora	SPG	22317	2646	34.35°	74.59°	Grasses or bushes of shady slopes or on streamside in stone cavities.
Kanzalwan	KZG	22318	2431	34.38°	74.42°	Bushes of shady slope under the canopy of Pinus forests.
Badwan	BDG	22321	2521	33.39°	74.46°	Bushes of shady slope on hillside or on stone cavities.
Hirpora	HP	22322	1818	33.39°	74.57°	Shady slopes on small stony hills or on roadside under the shade of forests.
Aharbal	AB	22313	2425	33.38°	74.74°	Bushes or Grasses or trees of shady slopes on roadside.

[a] Accession codes.

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