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Quantitative Determination by HPLC-DAD of Icariin, Epimedin A, Epimedin B, and Epimedin C in *Epimedium* (Berberidaceae) Species Growing in Turkey

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The genus *Epimedium* is rich in terms of flavonoids, of which icariin, epimedin A, epimedin B and epimedin C are known especially to be biologically active. Therefore, it is important to quantify these compounds. In this study, a HPLC method coupled with DAD detection was developed and validated for the determination of icariin, epimedin A, epimedin B and epimedin C in *Epimedium* species growing in Turkey. The chromatographic separation was performed using a gradient system with a mobile phase of 0.1% formic acid (A) and acetonitrile (B) applied at a flow rate of 1 mL/min using a diode array detector. The highest values were, respectively, icariin 0.65%, epimedin A 0.13%, epimedin B 0.11%, epimedin C 0.06%. The highest values were obtained from the materials collected in Uzungol (Trabzon-Turkey).

Keywords: *Epimedium*, HPLC, Icariin, Epimedin A, Epimedin B, Epimedin C.

Epimedium L. (Berberidaceae**)** is a genus of approximately 52 taxa. In Traditional Chinese Medicine (TCM), more than 15 species of this genus are in use [1]. Two of them, *E. pubigerum* (DC.) Moren & Decaisne and *E. pinnatum* Fischer in DC subsp. *colchicum* (Boiss.) Busch [2] grow in Turkey.

Epimedium species are known as "Keşişkülahı" and "Tekeotu" in Turkey [3]. The genus is rich in flavonoids, and, especially, icariin, epimedin A, epimedin B and epimedin C are known to be biologically active [1a, 1b, 4]. Icariin has various effects on bone, sexual dysfunction, diabetes, and the cardiovascular, nervous and immune systems [5]. Epimedin A, epimedin B and epimedin C also have various biological properties, such as anti-tumor, an immunoenhancing effect, and improvement in the function of the cardiovascular system [5c, 6].

In the present study, aerial and underground parts of *E. pubigerum,* collected from different localities in northeastern Turkey, were quantitatively analyzed for icariin, epimedin A, epimedin B and epimedin C by using high performance liquid chromatography (HPLC). Therefore, in the context of validation procedures, the linearity, precision, limits of detection and specificity of the method were displayed. Specimens of *E. pubigerum* were collected from two different localities in Northeastern Turkey, during and after the flowering period (Table 1).

Table 1: *E. pubigerum* specimens employed in the study.

Voucher Samples No:	Date:	Place:
AEF* 26332!	28.04.2013	Uzungol (Trabzon-Turkey)
AEF* 26335!	03.10.2013	Uzungol (Trabzon-Turkey)
AEF* 26336!	29.04.2013	Macka (Trabzon- Turkey)
AEF* 26337!	03.10.2013	Macka (Trabzon-Turkey)

*AEF: Ankara Üniversitesi Eczacılık Fakultesi Herbaryumu .

The extraction procedure was realized with some modifications of a previously used method [7]. The icariin, epimedin A, epimedin B, and epimedin C contents of each plant extract are shown in Table 2. The highest amounts of icariin and epimedin A were found in aerial parts of *E. pubigerum* during the flowering period, but the amount of epimedin B was at a maximum in aerial parts, and epimedin C in underground parts during and after the flowering period. The highest values were obtained from materials collected from Uzungol. However, the results were lower than in other studies [1a].

Table 2: Contents of icariin, epimedin A, epimedin B, epimedin C in *E. pubigerum* (n=3).

	Standards	*E.pubigerum* MACKA (flw*)	*E.pubigerum* MACKA (aflw**)	*E.pubigerum* UZUNGOL (flw*)	*E.pubigerum* UZUNGOL (aflw**)
Aerial parts	Icariin	0.49%	0.18%	**0.65%**	0.17%
	Epimedin A	**0.13%**	0.07%	0.13%	0.08%
	Epimedin B	0.09%	0.05%	0.08%	0.06%
	Epimedin C	0.05%	0.04%	0.04%	**0.06%**
Underground parts	Icariin	0.10%	0.12%	0.19%	0.22%
	Epimedin A	0.06%	0.08%	0.04%	0.06%
	Epimedin B	0.05%	0.06%	0.06%	**0.11%**
	Epimedin C	0.03%	0.03%	0.04%	0.04%

*flw: flowering period, **aflw: after flowering period

In conclusion, the method used in this study is simple, rapid, efficient and reliable for the HPLC determination of icariin, epimedin A, epimedin B and epimedin C in the extracts of *Epimedium* species.

Experimental

Plant materials: *E. pubigerum* was collected from Macka and Uzungol (Trabzon-Turkey) during flowering and after the flowering period (Table 1).

Preparation of sample and standard solution: Aerial and underground parts of *E. pubigerum* were air-dried, powdered, and samples of each were extracted with 70% ethanol in a Soxhlet for 3 h (80°C). After 3 h, the extracts were concentrated *in vacuo* at 40°C and lyophilized. The lyophilized extract (10 mg) was dissolved in methanol (5 mL). Before the sample was injected (10 µL) into the HPLC column, it was filtered through a 0.45 µm filter.

Icariin, epimedin A, epimedin B and epimedin C were provided by Chromadex. Stock standard solutions of each compound (500 µL) were prepared by dissolving 2 mg in 4 mL methanol.

HPLC: HPLC analysis was carried out using a liquid chromatographic system equipped with a quaternary pump, vacuum

degasser, a thermostated column compartment, an automatic injector and a DAD [7]. Chromatographic separation of compounds was achieved using a Discovery HS C18 column (15 cm x 4.6 mm, 5 µm) maintaining at 40ºC. Elution was performed at a flow rate of 1mL/min in a gradient. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B). Chromatograms were acquired at 270 nm by UV detector. Method validation was performed [8].

Table 3: Intra-day and inter-day precision of the method.

Standards	Amount (µg/mL)	Intra-day precision (RSD*%)	Inter-day precision (RSD*%)
Icariin	12.5	0.692	0.338
	25	0.076	0.099
	50	0.130	0.075
	150	0.326	0.208
	300	0,132	0.079
Epimedin A	2	1.227	1.071
	5	0.469	0.485
	10	1.129	0.833
	20	0.834	0.539
	50	0.736	0.520
Epimedin B	5	0.569	0.449
	10	1.074	0.864
	35	0.135	0.067
	75	0.692	0.057
	150	1.081	0.629
Epimedin C	5	0.401	0.061
	25	0.174	0.177
	50	1.686	0.320
	100	1.088	0.614
	200	0.183	0.185

*RSD: Relative Standard Deviation

The calibration functions for each of the 4 standards were investigated by injecting 5 different concentrations of reference solutions, in triplicate. The calibration plots for icariin, epimedin A, epimedin B and epimedin C were linear within the ranges of 12.5 to 300 µg/mL, 2 to 50 µg/mL, 5 to 150 µg/mL and 5 to 200 µg/mL, respectively. For icariin, epimedin A, epimedin B and epimedin C, the LOD (signal/noise ratio of 3:1) values were determined as 0.084 µg/mL, 0.06 µg/mL, 0.10 µg/mL and 0.096 µg/mL; LOQ (signal/noise ratio of 10:1) values found were 0.281 µg/mL, 0.227 µg/mL, 0.358 µg/mL and 0.322 µg/mL, respectively. The precision of the method was determined by studying intra-day and inter-day variation. Intra-day precision was evaluated by triple injections of standard solution of icariin, epimedin A, epimedin B and epimedin C with different concentrations on the same day. In order to check the inter-day precision, the procedures used for intra-day precision were repeated and performed on different days, and variations were expressed by relative standard deviation (RSD); the results showed that RSD values were always less than 3% (Table 3). A recovery assay was carried out by spiking 3 different known concentrations of standards into sample solutions. Aerial parts of *E. pubigerum* (Uzungol), collected during the flowering period, was used for recovery. The mean extraction recovery of icariin, epimedin A, epimedin B and epimedin C were in the range of 96.5-102.3%, 97.6-104.4%, 98.1-104.5% and 96.6-102.4%, respectively (Table 4).

Table 4: Statistical data showing recovery studies of HPLC (n=3).

Standards	Concentration in sample µg/mL)	Amount spiked (µg/mL)	Mean amount found in mixture (µg/mL)	Mean recovery (%)	RSD* (%)
Icariin	0.078	0.040	0.060	102.3	0.888
		0.080	0.079	101.1	0.963
		0.160	0.114	96.5	0.083
Epimedin A	0.015	0.0075	0.011	102.8	0.837
		0.015	0.015	104.4	0.837
		0.030	0.022	97.6	0.719
Epimedin B	0.009	0.005	0.006	98.1	0.825
		0.010	0.009	99.7	0.718
		0.020	0.015	104.5	0.031
Epimedin C	0.118	0.060	0.086	96.6	0.634
		0.120	0.119	101.0	0.165
		0.240	0.182	102.4	0.611

*RSD: Relative Standard Deviation

The robustness of the method was evaluated by changing the mobile phase composition, column temperature, flow rate, and detector wavelength. These changes had no significant effect on chromatographic resolution.

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