Pak. J. Bot., 44(2): 595-597, 2012.

FLAVONOIDS IN THE ENDEMIC SPECIES OF *ALCHEMILLA* L., (SECTION *ALCHEMILLA* L. SUBSECTION *CALYCANTHUM* ROTHM. SER. *ELATAE* ROTHM.) FROM NORTH-EAST BLACK SEA REGION IN TURKEY.

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Abstract

This study was undertaken to determine the flavonoids in the species of genus *Alchemilla* L. section *Alchemilla* L. subsection *Calycanthum* Rothm. Ser. *Elatae* Rothm., using the identification techniques such as high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). Eight endemic Turkish species were studied for their flavonoid profiles. They are *A. armeniaca, A. erzincanensis, A. cimilensis, A. orduensis, A. ikizdereensis, A. oriturcica, A. bursensis* and *A. hirsutiflora.* It is the first time that flavonoid profiles of these species have been identified. Flavonol *O*-glycosides and flavone *C*- glycosides were identified in *Alchemilla* endemic species. The flavonoid components determined in the species are rutin (quercetin-3-*O*-rutinoside), hyperoside (quercetin-3-*O*-galactoside), isoquercetin (quercetin-3-*O*-glucopyranoside), quercitrin (quercetin-3-*O*-rhamnoside) as flavonol *O*-glycosides, and orientin (luteolin-8-*C*-glucoside), vitexin (apigenin-8-*C*-glucoside) as the flavone *C*-glycosides.

Introduction

The genus *Alchemilla* L., belongs to the family Rosaceae and it is represented by nearly 80 species in Turkey (Menemen & Hamzaoglu, 2002 a,b; Hayırlıoğlu-Ayaz & İnceer, 2009). There have been some chemical studies on species of the family Rosaceae, but very few on the genus *Alchemilla* L. which currently includes at least 250 (–1000) species (Fröhner, 1995; Gehrke *et al*., 2008). *Alchemilla* has been used as medicinal plant by local people in north-east black sea region of Turkey (Baytop, 1997). Williams & Grayer (2004) indicated that flavonoids captured the interest of scientists from many different disciplines because of their structural diversity, biological and ecological significance in systematic, and health-promoting and anti-cancer properties. It was reported that flavonoid compounds are useful for deliminating the taxa in taxonomy (Harborne 1973, Menemen *et al*., 2002c).

The aim of this study was basically to identify the flavonoid components of the genus *Alchemilla*, which might contribute to scientists working in various disciplines. This study includes the endemic species belonging to the section *Alchemilla* L., subsection *Calycanthum* Rothm. Ser. *Elatae* Rothm., from north-east Black Sea Region in Turkey. The species studied are *A. armeniaca, A. erzincanensis, A. cimilensis, A. orduensis, A. ikizdereensis, A. oriturcica, A. bursensis* and *A. hirsutiflora.*

Materials and Methods

Alchemilla species were collected from the field near Trabzon province. The specimens were identified and are being kept in Kırıkkale University, Anadolu herbarium (ADO) (Table 1 for voucher specimens). Leaf materials used in chemical study were air dried, crushed and extracted according to Mingbo *et al*., (2005) with slight modification.

Table 1. Voucher specimens.

Species	Collection number	Herbarium
A.armeniaca	Yusuf 1439	ADO
A.erzincanensis	Yusuf 1449	ADO
A.cimilensis	Yusuf 1456	ADO
A.orduensis (1)	Yusuf 1453	ADO
A.orduensis (2)	Yusuf 1458	ADO
A.ikizderesis	Yusuf 1461	ADO
A.oriturcica (1)	Yusuf 1437	ADO
A.oriturcica (2)	Yusuf 1467	ADO
A.bursensis	Yusuf 1468	ADO
A.hirsutiflora	Yusuf 1490	ADO

Extraction of flavonoid compounds: Extraction method used in this study was a modification of the method exercised by Mingbo *et al*., (2005). Dried and powdered leaf material (10gr) was extracted with 50% aqueous ethanol in a flask. The extract was subjected to maceration process in incubator for four hours at 150 rpm, at 50°C. It was left to maceration following 20 hours. Then, extract was filtered using a blue band filter paper and Buchner funnel. The hydro alcohol solution was evaporated to dryness under reduced pressure. Hydrolic extract was treated with petroleum ether (40°–60°) in separation funnel. The aqueous phase at the bottom of separation funnel was transferred to another separation funnel. After ethyl acetate was added and the funnel was mixed gently. The part with ethyl acetate was evaporated to dryness under reduced pressure. The soluble material was used for TLC and HPLC chromatographic analysis.

Standard and solvents: The standards used in this study, quercetin, rutin, isorhamnetin, kaempferol, apigenin, luteolin were obtained from Sigma-Aldrich (St. Louis, MO, USA) and the remaining standards hyperoside, quercitrin, isoquercitrin, vitexin, orientin, myricetin, from Extrasyntese (Genay/France).

All solvents were obtained from Riedel-de Haen Company (Selze, Germany). Thin layer chromatography silica gel 60 F254 aluminum plates which are 20 x 20cm with 0.25mm thickness were obtained from Merck (Darmstadt/Germany) company.

Analysis of flavonoid glycoside by TLC: TLC was performed using silica plate according to Wagner & Bladt, (1984). Each flavonoid standard was dissolved in 10 ml methanol. TLC was performed using silica plates. The extracts redissolved in methanol were directly applied with standard flavonoid solutions using a capillary tube on the silica plates and run in ethyl acetate: acetic acid: formic acid: water (100:11:11:27 v/v) as running (mobil phase) solvent. After running, the plates were examined in UV light before and after spraying with NP/PEG (Natural Product Reagent (1% methanolic diphenylboryloxyethylamine / Poly Ethylen Glycol 4000 (5% ethanolic polyethylene glycol 4000)).

Analysis of flavonoid glycosides by HPLC: Qualitative analysis of flavonoids glycosides was achieved by high performance liquid chromatography with direct injection by autosampler. Qualification was carried out with a HPLC Dionex (U.S.A). The system consisted of a ASI-10 autosampler, a P580 pump, STH 585 column heater and UVD 170S UV Visible detector. The data were collected and analyzed with the Chromelon Chromatography Software. Separation and identification was carried out using C18 silica column. This column was 250 x 4.6mm i.d. 5µm particle size (Dionex Corp., USA). Column was placed in the oven set to 27 degrees Celsius. The flow rate was adjusted to 1ml/min isocratic elution. Mobile phase used included acetic acid-methanol-water (5:36:59). Before loading sample mobile phase was put into the column. The injection volume was adjusted to 10µl. The monitoring peaks were detected at 254, 280 and 360nm by automatically Chromelon Chromatography software (Fang *et al*., 2007). *Alchemilla* L. species samples, standard solutions and mobile phases were filtered by a 0.45-µm pour size membrane filter. The filtered standard and ethyl acetate extract were injected under these conditions. The identity of HPLC peaks was confirmed by injection of authentic standards.

Results and Discussion

Flavonoids were determined in the species of genus *Alchemilla* L., section *Alchemilla* L., subsection *Calycanthum* Rothm. Ser. *Elatae* Rothm., using the identification techniques of high performance liquid chromatography and thin layer chromatography. Flavonoid compounds of the endemic species studied are *A. armeniaca, A. erzincanensis, A. cimilensis, A. orduensis* (two samples)*, A. ikizdereensis, A. oriturcica* (two samples)*, A. bursensis* and *A. hirsutiflora* (Table 1).

Analysis of crude ethyl acetate fraction showed the presence of flavonoids. Flavonoid glycosides were determined. Four flavonol *O*-glycosides and 2 flavone *C*-glycosides were identified. The flavonol *O*-glycoside components determined in the species were rutin (quercetin-3-*O*-rutinoside), hyperoside (quercetin-3-*O*-galactoside), isoquercetin (quercetin-3-*O*-glycopyranoside), quercitrin (quercetin-3-*O*-rhamnoside), and flavone *C*-glycosides were identified as orientin (luteolin-8-*C*-glucoside) and vitexin (apigenin-8-*C*-glucoside) (Table 2). However, 3 flavonoid compounds separated on TLC could not be determined. These are shown as Rf_1, Rf_2 and Rf_3. Rf values of flavonoid compounds identified and unidentified their colors under UV light are given in Table 3.

Table 2. Flavonoid compounds of *Alchemilla* species identified on TLC.

Species	Ori	Vit	Rut	Hyp	Iso	Qci	*Rf*1	*Rf*2	*Rf*3
A. armeniaca	+	+	-	+	-	-	-	+	+
A. erzincanensis	-	+	-	-	+	-	-	+	+
A. cimilensis	+	-	+	+	+	-	+	+	+
A. orduensis (1)	+	-	-	+	-	-	+	-	+
A. orduensis (2)	+	+	+	-	+	+	-	+	+
A. ikizdereensis	+	+	+	+	-	-	+	+	+
A. oriturcica (1)	-	-	+	+	+	-	-	-	-
A. oriturcica (2)	-	-	+	+	+	-	+	+	+
A. bursensis	-	-	+	+	+	-	+	+	+
A. hirsutiflora	+	-	+	+	-	+	+	+	+

Table 3. *Rf* values of flavonoid compounds identified and unidentified on TLC and their colours under UV light.

Flavonoids	Rf values	Colours Under UV 365 nm
Orientin	0,70	Yellow
Vitexin	0,77	Green
Rutin	0,44	Orange
Hyperoside	0,65	Orange
Isoquercetin	0,72	Orange
Quercitrin	0,84	Orange
Rf_1	0,36	Orange
Rf_2	0,54	Orange
Rf_3	0,68	Orange

HPLC studies were performed according to the definition of retention time. The results of HPLC studies mostly supported the results obtained from TLC studies. The bibliographical data, the UV/VIS spectra and the retention times permitted the identification of the flavonoids, rutin (quercetin-3-*O*-rutinoside), hyperoside (quercetin-3-*O*-galactoside), isoquercetin (quercetin-3-*O*-glucopyranoside), quercitrin (quercetin-3-*O*-rhamnoside), orientin (luteolin-8-*C*-glucoside), vitexin (apigenin-8-*C*-glucoside), but some unidentified peaks were observed on chromatograms.

A few studies have been found on flavonoids of the genus *Alchemilla*. Fraisse *et al*., (2000) reported quercetin 3-*O*-arabinopyranoside as the main flavonoid compounds from *A. xanthochlora*. Olafsdottir *et al*., (2001) investigated flavonoids in three *Alchemilla* species (*A. faeroensis, A. alpina* and *A. vulgaris*) from Iceland, but they stated that they did not find any flavonoid compounds in those species. In a work carried out by D'Agostino *et al*. (1998) quercetin-3-*O*-*β*-D-glucopyranoside, quercetin-3-*O*-*β*-D-rutinoside, quercetin-3-*O*-*α*-D-arabinofuranoside, 3-*O*-kaempferol-6''-*O*-(p-coumaroyl)-*β*-*D*-glucopyranoside were identified from *A. vulgaris*.

It is the first time that flavonoid profiles of these endemic *Alchemilla* species have been identified in this study. It is hoped that the results obtained in this study will be useful for various scientific disciplines.

Acknowledgements

This research was carried out Kırıkkale University Scientific Research Projects Unit (BAP) No. 2007/44 with the financial support. We thank them for their support.

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(Received for publication 12 February 2011)