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Main Flavonoids of *Tilia argentea* DESF. ex DC. Leaves

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Abstract: Kaempferol 3,7-*O*-α-L-dirhamnoside **(I)** and quercetin 3,7-*O*-α-L-dirhamnoside **(II)** were isolated from the leaves of *Tilia argentea* (Tiliaceae). The structure elucidation of the isolated compounds was performed by spectroscopic techniques (^{1}H-NMR, ^{13}C-NMR, HMBC, CHSHF, DEPT and High Resolution FAB-MS). These 2 flavonoids are determined to be the main flavonoids of the leaves and are recommended as indicators for the adulteration of linden inflorescence with leaves known to possess weak healing activity.

Key Words: Flavonol glycosides, Kaempferol 3,7-*O*-α-L-dirhamnoside, Quercetin 3,7-*O*-α-L-dirhamnoside, Lime, Linden, *Tilia argentea*, Tiliaceae.

Introduction

Lime or linden flowers, Tiliae flos, are of prominent importance in phytotherapy. They are stated to possess expectorant, diuretic, diaphoretic, antispasmodic, stomachic and sedative activities and have been used for the treatment of flu, cough, migraine, nervous tension, ingestion problems, various types of spasms and liver and gall bladder disorders[1−4]. The medicinal properties claimed for the drug have been attributed to its flavonoids, volatile oil and mucilage components[5−7]. The use of the leaves as a remedy is not as common as that of the flowers, but they have been suggested to be employed as a diaphoretic; however, the effect has not been evaluated experimentally so far[8].

In European Pharmacopoeia (EP), the inflorescence of *Tilia platyphyllos* Scop., which is rarely found in Turkey, and *T. cordata* Miller, not found, is considered officinal[9]. However, *T. rubra* DC. and *T. argentea* Desf. ex DC. (Syn. *T. tomentosa* auct.) are widespread and used for similar purposes in Turkish folk medicine. In markets, flowers of these species are sold with or without bracts and even sometimes adulterated with leaves.

In our previous research on *Tilia* L. species growing in Turkey, flavonoid compositions of the flowers, bracts and leaves of *T. argentea* were studied using reversed-phase HPLC[10]. The composition of the

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flavonoid fraction of leaves was determined as follows: isoquercitrin+rutin (5.31%), quercitrin (6.00%), astragalin (1.78%) and tiliroside (2.46%). However, 2 unidentified flavonoids were found to be the main components of the leaves; flavonoids **I** and **II** were found in higher concentrations, 16.92% and 67.53%, respectively, in the flavonoid fraction of methanolic extracts. These 2 flavonoids and, in addition, tiliroside, were also found in higher concentrations in the bracts (13.91%, 52.76% and 12.24%, respectively) along with isoquercitrin+rutin (7.62%), quercitrin (8.35%) and astragalin (5.12%) in lesser quantities. However, the flavonoid **II** concentration in flowers was not so high (12.44%), while **I** was absent. Quercitrin (30.03%), isoquercitrin+rutin (28.78%) and astragalin (24.84%) were the main flavonoids of flowers, but the tiliroside concentration was very low (0.16%). Total flavonoid contents were determined to be 0.61%, 0.98% and 0.73% in the flowers, bracts and leaves of *T. argentea*, respectively. Hence, these 2 flavonoids (**I** and **II**) were recommended as indicators for the analyses of linden species, especially for the adulteration of inflorescence with high amounts of leaves or bracts. Recently, these 2 flavonoids were shown to possess significant anti-inflammatory and antinociceptive activities [11].

The aim of this study is to isolate and elucidate the chemical structures of these 2 main flavonoids (**I** and **II**) from the leaves of *T. argentea*.

Experimental

General procedures

TLC was carried out on precoated silica gel 60 F_{254}plastic plates (Merck). Compounds were detected by UV fluorescence before and after spraying with Naturstoff- Polyethylene glycol reagent. For column chromatography silica gel 60 (0.2-0.5 mm and 0.040-0.063 mm, Merck) was used.

UV spectra (λ max) were recorded on a Beckman DU 650 spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were obtained using a JEOL JNM-A500 FT-NMR in CD_3OD 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR). High Resolution FAB-MS spectra were recorded in negative mode on a JEOL JMS HX-110 spectrometer.

Plant materials

T. argentea Desf. ex DC. (Tiliaceae) leaves were collected from the Botanical Garden of the Faculty of Science, Ankara University. The specimens are stored in the Herbarium of Ankara University, Faculty of Pharmacy (AEF.10228).

Extraction and isolation of flavonoids

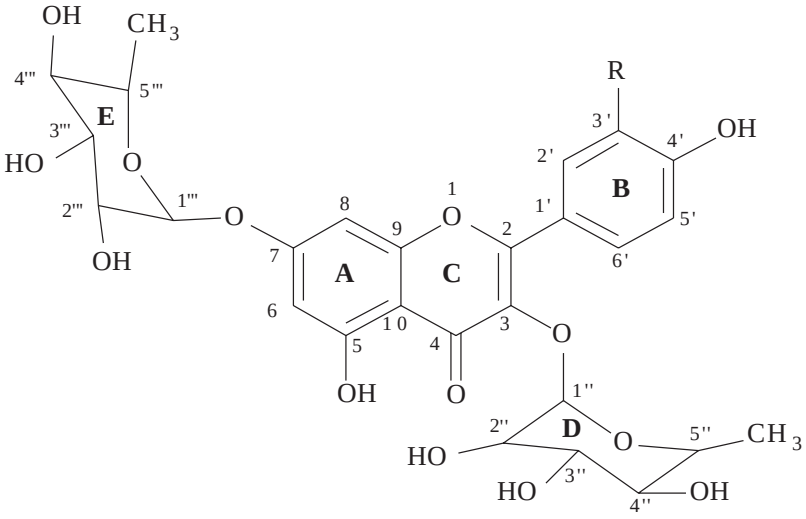
Air dried and powdered leaves of *T. argentea* (640 g) were extracted with 80% ethanol at room temperature several times. The combined ethanolic extract was evaporated in vacuo. The crude extract was dissolved in water and partitioned by successive solvent extraction with chloroform and ethyl acetate. The ethyl acetate extract (7.70 g) was applied to column chromatography on silica gel (0.2-0.5 mm, Merck) and eluted with $CHCl_3$-MeOH-H_2O using gradient elution (8:2:0.1; 8:2:0.2; 6.5:2:0.2; 6.5:2.5:0.4; 6.1:3.2:0.7). The relevant fractions (No. 74-109) (1.14 g) containing flavonoid **I** and **II** were combined and applied to a silica gel column (0.040-0.063 mm, Merck) and eluted with $CHCl_3$-MeOH-H_2O (8:2:0.1 and 8:2:0.2). The combined

fraction (No. 20-22) (0.18 g) was further purified by recrystalization from MeOH to give flavonoid **I** (0.11 g). Flavonoid **II** (0.08 g) was obtained from the combined fraction (No. 30-50)(0.20 g) by preparative TLC using EtOAc-HCO_2H-AcOH-H_2O (100:11:11:27) as the mobile phase. Both compounds were further purified from SEP-PAK C_{18}cartridges (Millipore) with methanol.

Results and Discussion

From the *T. argentea* leaves, 2 known flavonoids were isolated by chromatographic methods. The structures of the compounds were identified by spectroscopic methods (^{1}H-NMR, ^{13}C-NMR, HMBC, CHSHF, DEPT and High Resolution FAB-MS).

Flavonoid **I** was obtained as light yellow crystals. Its UV spectrum (λ max. 263, 317sh, 345) suggested a flavonoid structure. In the ^{1}H-NMR spectrum of **I**, H-6 and H-8 protons appeared separately as doublets at δ 6.46 and 6.71 ppm. The B ring had 4 aromatic protons that split into 2 doublets (δ-7.78 d, H-2′/6′; 6.93d, H-3′/5′). The ^{13}C-NMR signal at δ 116.59 was assigned to an oxygen free aromatic carbon (C-3′). The ^{13}C-NMR signals at δ 159.81 and δ 136.52 were assigned to C-2 and C-3, respectively, of the C-ring of a flavonol structure[12−13]. The methyl groups of sugar moieties showed doublets at δ 0.93(3H) and 1.26 (3H). Two anomeric protons assigned to H-1″ and H-2″ were observed at δ 5.40 and 5.55 as narrow doublets for α configuration of the glycosidic linkage.



R: H Kaempferol 3,7-*O*-α-L-dirhamnoside (I)

R: OH Quercetin 3,7-*O*-α-L-dirhamnoside (II)

Figure. The chemical formula of kaempferol 3,7-*O*-α-L-dirhamnoside (I) and quercetin 3,7-*O*-α-L-dirhamnoside (II).

A CHSHF experiment correlated all proton resonances of **I** with those of corresponding carbons. In the HMBC spectrum, diagnostic long range correlations were observed between C-7 (δ 163.56) and C-3 (δ 136.52) of the aglycone moiety and the anomeric protons H-1‴ (δ 5.55 d, J=1.73 Hz) and H-1″(δ 5.40 d, J= 1.84 Hz) of sugar moiety (Table). Further confirmation of the proposed structure was obtained through C^{13}-NMR DEPT data, which were in good agreement with the reported data[14].

The detected negative ion FAB-MS of compound **I** was 579.1631 (Calcd. for. 578.1636) corresponding to the molecular formula $C_{27}H_{29}O_{14}$.Therefore, flavonoid **I** was identified as kaempferol 3,7-*O*-α-L dirhamnoside.

Table. ^{13}C-NMR and ^{1}H-NMR Data of kaempferol 3,7-*O*-α-L-dirhamnoside (I) and quercetin 3,7-*O*-α-L-dirhamnoside (II).

	Flavonoid I					**Flavonoid II**
C/H	δ_c(ppm)	DEPT	δ_H(ppm)	J (Hz)	HMBC (C to H)	δ_H(ppm)
Aglycone						
2	159.81	C			H-2′, H-6′	
3	136.52	C			H-1″	
4	179.80	C				
5	163.02	C			H-6	
6	99.91	CH	6.46 d	(2.44)	H-8	6.46 d
7	163.56	C			H-1‴, H-6, H-8	
8	95.63	CH	6.71 d	(2.44)	H-6	6.72 d
9	158.10	C			H-8	
10	107.59	C			H-6, H-8	
1′	122.43	C				
2′	131.99	CH	7.78 d	(9.15)		7.37 d
3′	116.59	CH	6.93 d	(9.15)		
4′	161.78	C			H-2′, H-3′	
5′	116.59	CH	6.93 d	(9.15)	H-3′	6.84 d
6′	131.99	CH	7.78 d	(9.15)	H-2′	7.34 dd
3-*O*-Rhamnose						
1″	103.8	CH	5.40 d	(1.84)		5.38 d
2″	71.91	CH	4.22 dd	(1.83/3.34)		4.22 dd
3″	72.18	CH	3.71 dd	(3.05/8.95)	H-4″, H-5″, H-1″	3.78 dd
4″	73.21	CH	3.33-3.34 m		H-4″, H-3″, H-2″, H-6″	3.32-3.33 m
5″	72.10	CH	3.33-3.36 m		H-4″	3.32-3.33 m
6″	17.8	CH_3	0.93 d	(5.48)		0.94 d
7-*O*-Rhamnose						
1‴	100	CH	5.55 d	(1.73)		5.59 d
2‴	71.72	CH	4.02 dd	(1.83/4.05)		4.02 dd
3‴	72.18	CH	3.83 dd	(3.05/9.46)	H-4‴, H-1‴	3.82 dd
4‴	73.62	CH	3.46-3.50 t	(9.15/9.77)	H-5‴	3.46-3.48 m
5‴	71.29	CH	3.59-3.62 m		H-4‴, H-1‴, H-6‴	3.59-3.60 m
6‴	18.00	CH_3	1.26 d	(6.11)		1.28 d

Compound **II** was obtained as yellow amorphous powder. Its UV spectrum (254, 268sh, 298sh,349) suggested a flavonoid structure. In the ^{1}H-NMR spectrum of **II**, 2 meta coupled aromatic protons at δ 6.46 and 6.72 (2 doublets) were clearly attributed to H-6 and H-8 protons. The doublet signal that appeared at δ 7.37 was assigned to H-2′. The signals at δ 6.84 and 7.34 belonged to H-5′ and H-6′. Inspection of ^{1}H-NMR data of compound **II** showed signals very similar to those of compound **I**, except for the C ring. Therefore the structure of the flavonol was clearly proved to be quercetin[12,13]. The secondary methyl groups of sugar moieties showed doublets at δ 0.94 (3H) and 1.28 (3H). H-1″ and H-1‴ rhamnosyl protons were observed at δ 5.38 and 5.59. The sugar parts of compound **II** showed a ^{1}H-NMR spectrum similar to that

of compound **I** (Table). The detected negative ion FAB-MS compound **II** is 594.1581 (Calcd. for. 594.1585) corresponding to the molecular formula $C_{27}H_{29}O_{15}$. Therefore compound **II** was identified as quercetin 3,7-*O*-α-L-dirhamnoside.

In conclusion, the main flavonoid glycosides, showing significant anti-inflammatory and antinociceptive activities in mice, were isolated from the leaves of *T. argentea.* These 2 flavonoids were also reported from *T. platyphyllos* and *T. rubra* bracts and leaves[10]. Kaempferol 3,7-*O*-α-L-dirhamnoside was determined as the second main flavonoid in *T. platyphyllos* leaves (29.46%), and the fourth one in bracts (10.98%), and in lower concentrations in *T. rubra* leaves and bracts. Quercetin 3,7-*O*-α-L-dirhamnoside was the main flavonoid in *T. platyphyllos* bracts (25.96%) and leaves (47.75%), and in *T. rubra* leaves (71.56%), and the second main flavonoid in *T. rubra* bracts (26.25%). However, the concentration of these flavonoids in the flowers of both species was low. Since linden inflorescence (flowers and bracts) is considered the medicinally important part, excess amounts of leaves are regarded as adulteration. Accordingly, these 2 flavonoids were proposed as marker components for analyzing adulteration in linden flowers with excessive amounts of bracts or leaves.

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