ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
26 (2001) 111–121

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Comparative evaluation of the flavonoid content in officinal Tiliae flos and Turkish lime species for quality assessment

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Received 17 December 2000; received in revised form 18 January 2001; accepted 23 January 2001

Abstract

Lime flowers are used for various medicinal purposes in phytotherapy. Flavonoids, volatile oil and mucilage components are known as the active ingredients. In European Pharmacopoeia (EP), a simple thin-layer chromatographic (TLC) technique, which based on the analysis of the flavonoid composition was defined for the qualitative analysis of the drug. In this study, flavonoid composition in the flowers, bracts and leaves of the officinal species, *Tilia platyphyllos* were studied using a reversed-phase high performance liquid chromatographic (HPLC) technique, in order to develop a rapid, reliable and accurate method for quantitative analysis. The results were further compared with in those parts of two common species growing in Turkey, *Tilia rubra* and *Tilia argentea*. Results of the present study revealed that flavonoid composition of each lime species possesses a specific fingerprint HPLC chromatogram depending upon the parts used and evaluation of the data might be helpful in the quality assurance as well as determination of adulteration of the crude drug.

Keywords: Reversed phase HPLC; *Tilia* species; Tiliae flos; Lime; Validation

1. Introduction

Lime flowers, Tiliae flos, have a prominent importance in phytotherapy. It is stated to possess expectorant, diuretic, diaphoretic, antispasmodic, stomachic and sedative activities. It has been used for the treatment of flu, cough, migraine, nervous tension, ingestion, various types of spasms, liver and gall bladder disorders [1,2]. Medicinal properties claimed for the drug have been attributed to its flavonoid, volatile oil and mucilage components.

In European Pharmacopoeia (EP), the inflorescence of *Tilia platyphyllos* Scop., which is rarely found in Turkey and *Tilia cordata* Miller, not found, and an hybrid (*Tilia X vulgaris* Heyne) are accepted as the officinal species. However, *Tilia rubra* DC and *Tilia argentea* Desf. ex DC (Syn. *Tilia tomentosa* auct.) are used for similar purposes in Turkish folk medicine.

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0731-7085/01/$ - see front matter
PII: S0731-7085(01)00351-X

In EP, tests given for the analysis of officinal drug are mainly based on the macroscopic and microscopic features. Moreover, there is also a thin-layer chromatographic (TLC) technique in the monograph, which based on the detection of flavonoid components. Using a mixture of EtOAc–HCO_2H–$C_2H_5OCH_3$–H_2O (50:10:30:10) as mobile phase and caffeic acid, rutin and hyperoside as reference compounds, the plate is migrated under standardized conditions, and the flavonoids are then visualized by diphenylboric acid aminoethyl ester/MeOH reagent. The plate is evaluated according to the characteristic color of spots and relative migration distances compared with those of reference compounds. Utilization of TLC techniques were also described by some other authors, using other mobile systems i.e. EtOAc–HCO_2H–AcH–H_2O (100:11:11:27) [3]. But in all cases, the resolution of this technique seems not sufficient to detect flavonoids with nearly identical R_f-values [4].

Wagner et al. [4] developed a gradient high performance liquid chromatographic (HPLC) technique for official drug (*T. platyphyllos* or *T. cordata*, not defined), but only quercitrin (**6**) (as the main flavonoid), hyperoside (**1**) and tiliroside (**8**) could be detected. HPLC method described by Pietta et al. [5] was based on an isocratic elution technique using reversed-phase column and diode-array ultra voilet (UV) detection system. Quercetin and kaempferol derivatives were identified in the flowers, leaves and herbs (?) of the plant. They also applied micellar electrokinetic chromatography technique to verify the results of HPLC study. The investigators reported that isoquercitrin (**3**), astragalin (**7**) and tiliroside (**8**) were the main components of flowers, as well as quercitrin (**6**) and kaempferol-3-rhamnoside in

Table 1
Results of the HPLC analysis of *T. platyphyllos* flavonoid composition

Peak number	Compound	Mean $X_{Area} \pm$ S.D.	CV%	Flavonoid (%)
T. platyphyllos flowers				
2	Quercetin-3,7-dirhamnoside	115 297.7 ± 4012.3	3.48	5.05
3+4	Isoquercitrin + Rutin	1 265 496.0 ± 54 434	4.30	55.46
6	Quercitrin	138 642.0 ± 4711	3.4	6.08
$?_1$		158 378.0 ± 6560.5	4.14	6.94
7	Astragalin	603 926.7 ± 22647.3	3.75	26.47
T. platyphyllos bracts				
1	Hyperoside	53 602.0 ± 3045	5.68	0.95
2	Quercetin-3,7-dirhamnoside	1 440 601.8 ± 66 935	4.65	25.96
3+4	Isoquercitrin + Rutin	460 545.8 ± 14 415	3.13	8.31
5	Kaempferol-3,7-dirhamnoside	608 681.1 ± 21 121.2	3.47	10.98
6	Quercitrin	1 248 857.1 ± 39 089.2	3.13	22.52
$?_1$		730 951.8 ± 28 716	3.93	13.18
7	Astragalin	232 904.6 ± 6594.6	2.83	4.20
$?_2$		308 564.7 ± 15 776	5.11	5.57
$?_3$		462 073.3 ± 17 096.7	3.70	8.33
T. platyphyllos leaves				
1	Hyperoside	207 707.3 ± 8052.3	3.87	3.95
2	Quercetin-3,7-dirhamnoside	2 509 819.8 ± 96 527	3.85	47.75
3+4	Isoquercitrin + Rutin	29 022.0 ± 1082.5	3.73	0.55
5	Kaempferol-3,7-dirhamnoside	1 548 821.5 ± 58 349	3.77	29.46
6	Quercitrin	513 544.5 ± 18 288	3.56	9.77
$?_1$		237 736.4 ± 10 507.9	4.42	4.52
7	Astragalin	42 683.0 ± 776.8	1.82	0.81
$?_2$		79 323.8 ± 2141.7	2.70	1.51
$?_3$		88 029.3 ± 2359.2	2.68	1.68

Table 2
Results of the HPLC analysis of *T. rubra* flavonoid composition

Peak number	Compound	Mean $X_{Area} \pm$ S.D.	CV%	Flavonoid (%)
T. rubra flowers				
2	Quercetin-3,7-dirhamnoside	29 059.7 ± 999.6	3.44	2.94
3+4	Isoquercitrin+Rutin	520 481.7 ± 6909.9	1.33	52.7
6	Quercitrin	106 757.3 ± 3394.9	3.18	10.81
$?_1$		31 578.3 ± 1563.1	4.95	3.20
7	Astragalin	299 673.3 ± 11 150	3.72	30.35
T. rubra bracts				
2	Quercetin-3,7-dirhamnoside	1 420 332.3 ± 78 701	5.54	26.25
3+4	Isoquercitrin+Rutin	702 711.3 ± 22 274	3.15	12.98
5	Kaempferol-3,7-dirhamnoside	245 062.7 ± 11 287	4.61	4.53
6	Quercitrin	2 174 660.3 ± 127 150	5.85	40.19
$?_1$		362 429.7 ± 19 349	5.34	6.68
7	Astragalin	194 226.7 ± 3489.7	1.95	3.59
$?_2$		42 094.7 ± 1692.2	4.02	0.78
$?_3$		270 520.3 ± 11 729	4.34	5.00
T. rubra leaves				
2	Quercetin-3,7-dirhamnoside	3 731 406.3 ± 226 567	6.07	71.56
3+4	Isoquercitrin+Rutin	191 221.0 ± 8031.3	4.2	3.67
5	Kaempferol-3,7-dirhamnoside	329 970.5 ± 19 319	5.85	6.33
6	Quercitrin	621 181.8 ± 17 890	2.88	11.91
$?_1$		332 025.0 ± 15 770	4.75	6.37
7	Astragalin	8581.7 ± 193.9	2.26	0.16

lesser quantities. In those of leaves, however, quercetin-3-glucoside-7-rhamnoside and 3,7-dirhamnoside (**2**), as well as kaempferol 3-glucoside-7-rhamnoside and 3, 7-dirhamnoside (**5**) were detected. The composition of the plant part defined as 'herb' in this study, was found as a mixture of flavonoids of leaves and flowers. Since this plant is a tree, the definition of 'herb' may actually be 'flowers with bracts' (i.e. inflorescence). Although the flavonoid composition of the plant was studied in detail, the specimen name of the sample was not given since it is supplied from market. In a following methodological study, Pietta et al. [6] used a thermospray liquid chromatography–mass spectrometry (LC–MS) technique to elucidate the flavonoid composition of *T. cordata* leaves and reported an identical result with that given in their previous study.

The aim of this study was to develop a simple, rapid and reliable reversed-phase HPLC method for the qualitative and quantitative analysis of flavonoids in lime samples, in order to employ for the quality assessment of the flower drug (Tiliae flos) as well as determination of adulteration. For this purpose, flavonoid compositions of the flowers, bracts and leaves of the officinal species, *T. platyphyllos* as well as two common species growing in Turkey, *T. rubra* and *T. argentea*, were studied by using HPLC. The results were also compared with those obtained through TLC technique defined in EP.

2. Materials and methods

2.1. Materials

Collection sites of the lime samples used in this study are given below. The specimens are stored in the herbaria of Faculty of Pharmacy, Gazi University and Faculty of Pharmacy, Ankara University (AEF).

T. platyphyllos Scop., Botanical Garden of the Faculty of Science, Ankara University

(AEF.10229), *T. rubra* DC National Park of Kizilcahamam, Ankara (AEF.10227), *T. argentea* Desf. ex DC Botanical Garden of the Faculty of Science, Ankara University (AEF.10228).

2.2. Chemicals

Rutin [quercetin-3-*O*-rutinoside] (**4**) as an authentic sample was purchased from Merck. Hyperoside [quercetin-3-*O*-galactoside] (**1**), isoquercitrin [quercetin-3-*O*-glucoside] (**3**), quercitrin [quercetin-3-*O*-rhamnoside] (**6**), astragalin [kaempferol-3-*O*-glucoside] (**7**), tiliroside [kaempferol-3-*O*-(6-*p*-coumaryl)-glucoside] (**8**) were kindly provided by Professor Ekrem Sezik (Gazi University, Faculty of Pharmacy, Ankara), Professor Nurten Ezer and Associate Professor Zeliha Akdemir (Hacettepe University, Faculty of Pharmacy, Ankara).

Table 3
Results of the HPLC analysis of *T. argentea* flavonoid composition

Peak number	Compound	Mean $X_{Area} \pm$ S.D.	CV%	Flavonoid (%)
T. argentea flowers				
2	Quercetin-3,7-dirhamnoside	222 253.0 ± 12 739	5.73	12.44
3+4	Isoquercitrin+Rutin	512 574.0 ± 23834.7	4.65	28.78
6	Quercitrin	534 860.7 ± 26 690	4.99	30.03
$?_1$		66 025.0 ± 874.5	1.32	3.70
7	Astragalin	442 438.0 ± 19 028	4.30	24.84
8	Tiliroside	2868.0 ± 67.4	2.35	0.16
T. argentea bracts				
2	Quercetin-3,7-dirhamnoside	1 508 881.0 ± 78 603	5.21	52.76
3+4	Isoquercitrin+Rutin	217 895.3 ± 5374.3	2.47	7.62
5	Kaempferol-3,7-dirhamnoside	397 695.7 ± 5230.4	1.32	13.91
6	Quercitrin	238 611.7 ± 2744.6	1.15	8.35
7	Astragalin	146 494.0 ± 2475.2	1.69	5.12
8	Tiliroside	350 056.3 ± 17 354	4.96	12.24
T. argentea leaves				
2	Quercetin-3,7-dirhamnoside	1 439 914.8 ± 37 170	2.58	67.53
3+4	Isoquercitrin+Rutin	113 339.3 ± 4567.6	4.03	5.31
5	Kaempferol-3,7-dirhamnoside	360 718.5 ± 13 738	3.81	16.92
6	Quercitrin	127 841.3 ± 6239.7	4.88	6.00
7	Astragalin	37 904.0 ± 943.8	2.49	1.78
8	Tiliroside	52 508.0 ± 1654	3.15	2.46

Table 4
Within-day analytical precision of the reference flavonoids

Rutin			Hyperoside		
Concentration (μg/ml)	Mean peak-area[a]	CV (%)	Concentration (μg/ml)	Mean peak-area[a]	CV (%)
0.016	498 533.3	2.88	0.016	558 273	3.26
0.024	721 396.3	2.63	0.024	938 457	0.56
0.032	916 613.3	1.08	0.032	1 179 155	1.11
0.040	1 208 541.8	1.61	0.040	1 640 769.7	2.22
0.048	1 406 214	2.39	0.042	1 835 960.3	2.12

[a] Mean, $n = 3$.

Table 5
Variation of the retention time of each flavonoid peak in lime samples by HPLC system employed in this study[a]

Compound	*n*	Mean retention time (min)	±S.D.	CV%
1	11	10.641	±0.4040	3.79
2	35	12.009	±0.4092	3.41
3+4	34	14.550	±0.5177	3.56
5	26	19.865	±0.8990	4.52
6	35	22.143	±0.9482	4.28
7	34	24.152	±0.9766	4.04
8	9	50.520	±1.520	3.01
$?_1$	22	17.614	±0.7154	4.06
$?_2$	15	26.534	±1.344	5.06
$?_3$	10	40.336	±1.534	3.80

[a] *n*, Represents number of data used to estimate retention time. The different numbers in this column originated from the different flavonoid composition of each lime species.

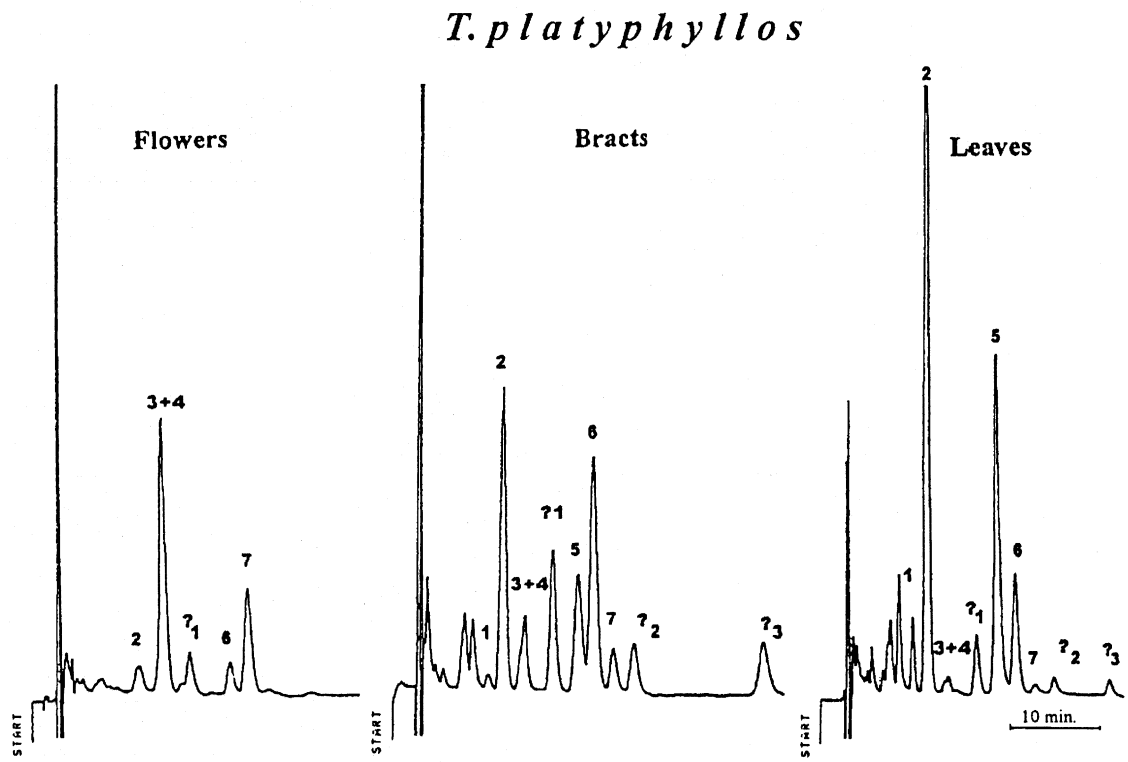

Fig. 1. Representative HPLC chromatograms of the flower, bract and leaf samples of *T. platyphyllos* [absorbency vs. time (min); retention time of each peak was given in Table 2].

2.3. Isolation and structure elucidation of quercetin-3,7-O-dirhamnoside (**2**) and kaempferol-3,7-O-dirhamnoside (**5**)

Eighty percent ethanol extract of *T. argentea* leaves was partitioned between water and ethyl acetate. The EtOAc extract was then applied to open column chromatography on silica gel (Kieselgel 0.2–0.5 mm, Art. No. 7733, Merck) and eluted with EtOAc–MeOH–H_2O in gradient

elution technique (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, v/v/v). The relevant fractions which contained **2** and **5** were combined and applied to a silica gel column (Kieselgel 0.040–0.063 mm, Art. 9385, Merck) and eluted with EtOAc–MeOH–H_2O (8:2:0.1 and 8:2:0.2, v/v).

Table 6
Color intensities of flavonoids on TLC plate by Naturstoff reagent (S_1 and S_2 solvent systems)[a]

Species	1	2	3	4	5	6	7/?$_1$	8	?$_2$	?$_3$
T. platyphyllos										
Flowers		–	****			**	***	–	**	
Bracts	*	***		*	*	****	**	–		*
Leaves	**	****		*	**	***	**	–		*
T. rubra										
Flowers		–	****			**	***			
Bracts		***	**	*	–	****	*			*
Leaves		****	*	***	–	**	*			**
T. argentea										
Flowers		–	***			**	*	**		
Bracts		****		***	*	**		**		
Leaves		****		***	*	**		**		

[a] (–), Not detected by TLC, but was found by HPLC. Color intensity of the spots were expressed by the increasing number of asterisk; i.e. ****, represents the highest and *, represents the weakest color intensity when spraying with reagent.

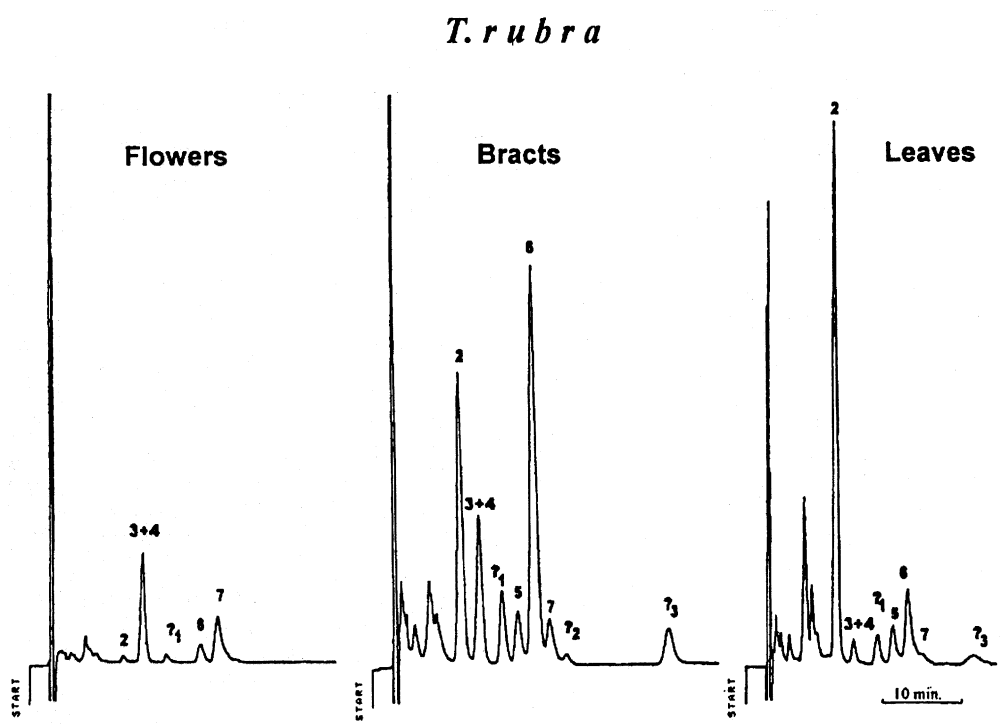

Fig. 2. Representative HPLC chromatograms of the flower, bract and leaf samples of *T. rubra* [absorbency vs. time (min); retention time of each peak was given in Table 2].

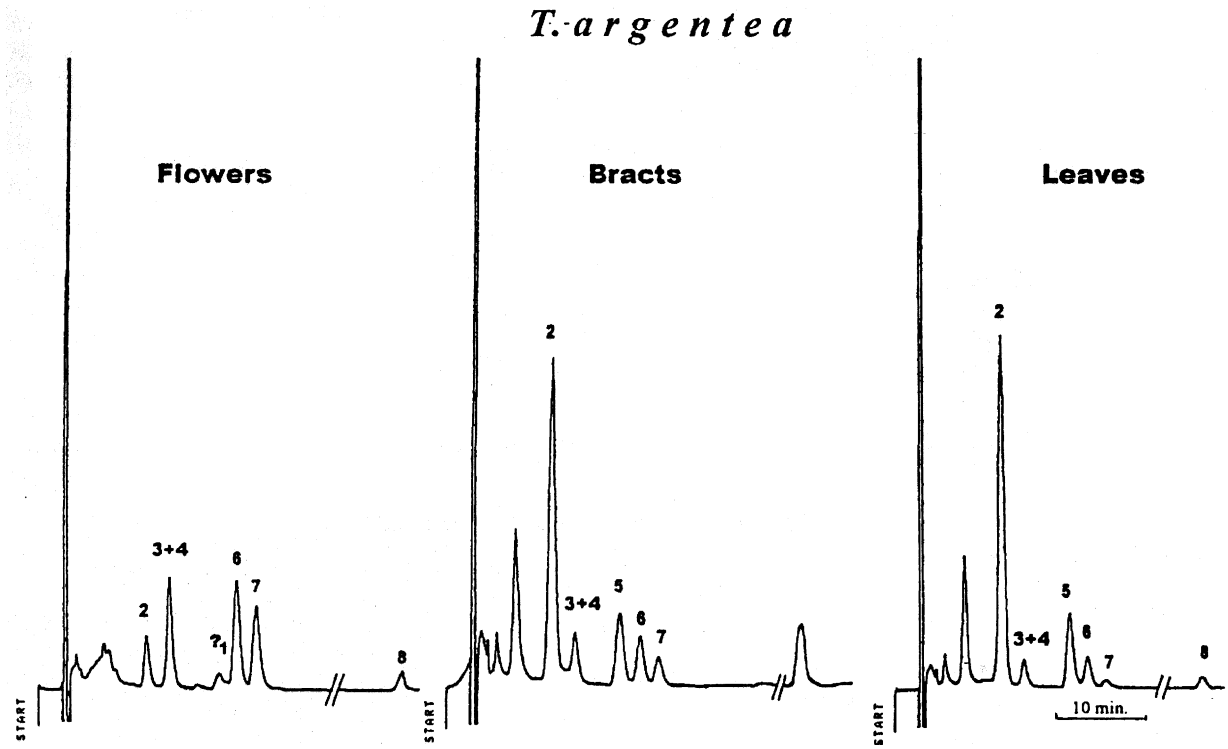

Fig. 3. Representative HPLC chromatograms of the flower, bract and leaf samples of *T. argentea* [absorbency vs. time (min); retention time of each peak was given in Table 2].

Combined fraction (No. 20–22) was further purified by recrystallization from MeOH to give **2**. Flavonoid **5** was obtained from the combined fraction (No. 30–50) by preparative TLC using EtOAc–HCO_2H–AcH–H_2O (100:11:11:25, v/v/v/v) as mobile phase. Both compounds were further purified from SEP-PAK C_{18} cartridges with methanol. The structures were elucidated by using ^1H- and ^{13}C-NMR as well as 2-D-non magnetic resonance (NMR) techniques and high resolution FAB-MS (JEOL HX-110).

Flavonoid **2**, $C_{27}H_{29}O_{15}$ mol wt. 594.15847 (calcd.), 594.1581 (det.), flavonoid **5**, $C_{27}H_{29}O_{14}$ mol wt. 578.163556 (calcd.), 579.1631 (det.).

2.4. Preparation of samples

2.4.1. Plant preparation

Powdered sample (1.0 g) was extracted two times with 50% MeOH (20 ml) overnight at room temperature. Combined extract was evaporated to dryness in vacuo. The crude extract was dissolved in HPLC grade MeOH and suspended particles were removed by filtration through a membrane filter (0.45 μm, Alltech, Germany). Suitable dilutions were prepared with each sample for HPLC analysis.

2.4.2. Standard solutions

Calibration curves were established for rutin and hyperoside by injecting metanolic solutions of 0.4 and 0.3 mg/ml, respectively.

2.5. Apparatus and conditions

TLC was carried out on Kieselgel 60 F_{254} (precoated 0.2 mm thickness plastic plates, Merck) by using the mobile systems; S_1, EtOAc–HCO_2H–AcH–H_2O (100:11:11:27, v/v/v/v) and S_2, EtOAc–HCO_2H–$C_2H_5OCH_3$–H_2O (50:10:30:10, v/v/v/v). Flavonoids were first detected under UV

light (365 nm) and then by spraying the chromatogram with Naturstoff Reagent (diphenylboric acid aminoethyl ester in methanol) and heating.

HPLC system was consisted of Hewlett–Packard HPLC system, model 1050 pump, Rheodyn 7125 injection valve was fitted with 20 μl loop, model 1050 UV detector and 3996 A integrator. Separations were achieved with a reversed-phase column (LiChrospher 100 RP 18e (5 μm particle size; 4×250 mm ID) and H_2O–MeOH–AcH (65:35:5, v/v/v, isocratically) was employed as the mobile system. The flow rate was kept constant at 0.8 ml/min with the column temperature at 40°C and the peak was monitored at 354 nm. HPLC grade solvents and bidistilled water were used for HPLC studies. The mobile phase was degassed in an ultrasonic bath.

2.6. Quantitative determination

For quantification, the external standard method was used. Calibration curves were established by repeated injections ($n = 3$) of reference solutions with concentrations of 6.4, 9.6, 1.28, 1.6, 1.92 μg/ml for rutin and 6.0, 9.0, 12.0, 15.0, 18.0 μg/ml for hyperoside. The R.S.D. of the calculated content was obtained by repeated injections ($n = 3$–7) of the sample solutions.

3. Results

3.1. Results of the HPLC analysis

3.1.1. Linearity

Rutin (**4**) and hyperoside (**1**) were used as reference flavonoids as suggested by EP. The linearity of the detector responses was investigated for each reference substance by plotting peak areas against the injected amounts. The detector response was linearly correlated with concentration, in the ranges of 6.4–19.2 μg/ml for rutin and 6.0–18.0 μg/ml for hyperoside. The regression equations and correlation coefficients determined for the references were [$y = 720889.89\ x + 23211.4$] ($r = 0.9960$) for rutin and [$y = 108035570.0x - 69240.6$] ($r = 0.9819$) for hyperoside. The experimental intercept was not significantly different from theoretical zero value because when we conducted Student's t-test we found $t_{calculated}$ as 1.05 ($n = 3$; $P = 0.05$) for rutin and 2.24 ($n = 3$; $P = 0.05$) for hyperoside, while $t_{tabulated}$ as 4.30. The sample concentrations were deduced by using these equations.

3.1.2. Precision

The precision of the analytical method was determined by assaying at least triplicate applications of each sample and reference. The mean peak area for each flavonoid in the test sample was expressed by $X_{area} \pm$ S.D. and the method precision was calculated as the coefficient of variation (CV%, Tables 1–3). The calculated CV% values were found to vary between 1.15 and 6.07% depending upon the composition of each test material, which was reported within the reasonable limits for crude drugs [7]. Within-day analytical precision of the reference compounds were given in Table 4. In addition, variation of the retention times for each flavonoid peak was studied and a reasonable level of reproducibility was observed (Table 5).

3.1.3. Results of the T. platyphyllos samples (Table 1)

3.1.3.1. Flowers. Main components were isoquercitrin and rutin (**3** + **4**) (55.46% of flavonoid fraction) and astragalin (**7**) (26.47%). Quercetin-3,7-dirhamnoside (**2**) (5.05%), quercitrin (**6**) (6.08%) and unknown compound (**?$_2$**) (6.94%) were also detected in low concentrations.

3.1.3.2. Bracts. Quercetin-3,7-dirhamnoside (**2**) (25.96%) and quercitrin (**6**) (22.52%) were found as the main components. Unknown (**?$_1$**) was found in 13.18% concentration. Kaempferol-3,7-dirhamnoside (**5**) (10.98%) which was not detected in flowers was determined in relatively high ratio. Main components of the flower samples, isoquercitrin + rutin (**3** + **4**) and astragalin (**7**), however, were found in lesser concentrations.

3.1.3.3. Leaves. As the main components quercetin-3,7-dirhamnoside (**2**) (47.75%) and

kaempferol-3,7-dirhamnoside (**5**) (29.46%) were detected. The concentration of quercitrin (**6**) (9.77%) was found lesser as compared with that of bracts. On the other hand, isoquercitrin + rutin (**3** + **4**) and astragalin (**7**) were found almost in trace.

3.1.4. Results of the T. rubra samples (Table 2)

3.1.4.1. Flowers. Main components were observed as isoquercitrin and rutin (**3** + **4**) (52.7%) and astragalin (**7**) (30.35%). Quercitrin (**6**) (10.81%), unknown (**?$_1$**) (3.20%) and quercetin-3,7-dirhamnoside (**2**) (2.94%) were also detected.

3.1.4.2. Bracts. Quercitrin (**6**) (40.19%) and quercetin-3,7-dirhamnoside (**2**) (26.25%) were determined as the main flavonoid components. Isoquercitrin + rutin (**3** + **4**) (12.98%) and unknown (**?$_1$**) (6.68%) were found in lesser concentrations. The concentration of kaempferol-3,7-dirhamnoside (**5**) (4.53%) was found low as compared with that of *T. platyphyllos* bracts.

3.1.4.3. Leaves. Quercetin-3,7-dirhamnoside (**2**) (71.56%) was the main flavonoid component of leaves. Quercitrin (**6**) (11.91%), kaempferol-3,7-dirhamnoside (**5**) (6.33%) and unknown (**?$_1$**) (6.37%) were observed as the other dominant components, while isoquercitrin + rutin (**3** + **4**) and astragalin (**7**) were in minute concentrations.

3.1.5. Results of the T. argentea samples (Table 3)

3.1.5.1. Flowers. As shown in Fig. 1, HPLC pattern of flowers was observed somewhat different than those of *T. platyphyllos* and *T. rubra* flowers. Quercitrin (**6**) (30.03%), isoquercitrin + rutin (**3** + **4**) (28.78%) and astragalin (**7**) (24.84%) were detected as the main flavonoid components, while quercetin-3,7-dirhamnoside (**2**) (12.44%) and unknown (**?$_1$**) (3.70%) were determined in lesser concentrations. The concentration of tiliroside was very low.

3.1.5.2. Bracts. Although quercetin-3,7-dirhamnoside (**2**) (52.76%) was found as the main flavonoid component of bracts as that of official species, other flavonoids were in different concentrations; kaempferol-3,7-dirhamnoside (**5**) (13.91%), tiliroside (**8**) (12.24%), quercitrin (**6**) (8.35%), isoquercitrin + rutin (**3** + **4**) (7.62%) and astragalin (**7**) (5.12%).

3.1.5.3. Leaves. As shown in Fig. 3, the HPLC chromatogram of the leaves was observed as quite similar to that of bracts. As the main components quercetin-3,7-dirhamnoside (**2**) (67.53%), kaempferol-3,7-dirhamnoside (**5**) (16.92%), quercitrin (**6**) (6.00%), isoquercitrin + rutin (**3** + **4**) (5.31%) and tiliroside (**8**) (2.46%) were detected, but the concentration of astragalin (**7**) was found very low as compared with that of bracts.

3.2. Results of the TLC analysis

TLC analysis of the samples were performed using S_1 and S_2 solvent systems. The second system (S_2) was also suggested by EP for the TLC analysis of the flavonoids in lime samples. Almost identical resolution and R_f-values were observed with both solvent systems. TLC plates were evaluated by the color intensities of the spots under UV-365 nm light and after spraying with reagent. Results were summarized in Table 6.

4. Discussion

It has been reported that flavonoid components could be used as a useful indicator for the analysis of lime samples [3,5]. As a simple chromatographic tool, a TLC-technique is described for this purpose in EP. In the present study, however, a simple, rapid and accurate HPLC method was developed. As shown in the representative HPLC chromatograms (Figs. 1–3), flavonoid composition of each lime species possessed a specific fingerprint depending upon the parts used and evaluation of the data might be helpful in the quality assurance as well as determination of adulteration of the crude drug.

Validation of the results confirmed that this method was suitable for the quantitative analysis of lime samples. Moreover, results were further

compared with those of TLC analysis in order to evaluate the reliability of the TLC techniques for the qualitative analysis of lime samples.

It was reported that officinal lime species to contain mainly quercetin and kaempferol derivatives of flavonoids [2], as were also determined in the lime samples employed in this study. The structures of eight were defined (**1**–**8**) by comparison with authentic substances or using spectral techniques (see Tables 3–5), but three of which detected in low concentrations were not to be necessarily to define and symbolized as **?$_1$ -?$_3$**.

HPLC analysis of the flowers of the official species, *T. platyphylos*, revealed that isoquercitrin + rutin (**3** + **4**) and astragalin (**7**) were the main flavonoid components. Due to the low concentration of these compounds in bracts and very low in that of leaves, higher the ratio of these compounds may be the indicator of higher the quality of the drug. On the other hand, the main flavonoids of the leaves, quercetin-3,7-dirhamnoside (**2**) and kaempferol-3,7-dirhamnoside (**5**), may also be employed as an indicator for the adulteration of flowers with leaves. Despite the high ratio of **2** in leaves, the concentration in flowers were found very low, while flowers did not contain **5**. Flavonoids **2** and **5** were also detected in the bracts of the officinal plant. Since EP permits a reasonable ratio of bracts in Tiliae flos, a limit should be set to assess the quality of the drug.

According to the HPLC chromatograms in Figs. 1 and 2, the flavonoid composition of *T. rubra* flowers was found almost similar to that of *T. platyphyllos*. In the flowers of *T. argentea*, however, quercetin-3,7-dirhamnoside (**2**) and quercitrin (**6**) were found in higher concentrations. Thus *T. rubra* flowers may be proposed as an additional officinal species as far as the flavonoid composition in question. The reported volatile oil composition of *T. rubra* flowers was also very similar to that of *T. platyphyllos* which supported this conclusion. On the other hand, volatile oil of *T. argentea* was found rich in esters (34.8–27.0%) [9]. Maybe due to this feature of the *T. argentea* volatile oil, *T. rubra* is esteemed by the people.

Hyperoside (**1**) may be used as another indicator for the quality assessment of the lime samples. This flavonoid was only detected in the leaves and bracts of the officinal species, while were not found in *T. rubra* and *T. argentea* samples.

Although, Hörhammer et al. [8] reported that tiliroside (**8**) was a characteristic flavonoid for all *Tilia* species, it was only detected in *T. argentea* samples. As a matter of fact, this compound was also observed as trace in the all parts of *T. platyphyllos* only by HPLC, but not by TLC. In the HPLC chromatogram given in the study of Wagner et al. [3] for the flowers of officinal species according to DAB8 (*T. platyphyllos* or *T. cordata*, the species' name was not defined), a small concentration of tiliroside was reported same as that observed in the present study for *T. platyphyllos* samples. But in Pietta et al.'s study [6], a high tiliroside content was reported for *T. cordata* leaves. These results suggested that the tiliroside concentration in lime samples could not be evaluated as an index of quality.

Quercetin-3,7-dirhamnoside (**2**) and quercitrin (**6**) were found as the main flavonoids of *T. platyphyllos* and *T. rubra* bracts. Although, **2** was also the main flavonoid of *T. argentea* bracts, kaempferol-3,7-dirhamnoside (**5**) was determined as the second dominant flavonoid component.

The leaves of these three species also contained **2** as the main flavonoid. Although **5** was found as the second highest concentration in the leaves of *T. platyphyllos*, the concentrations in those of *T. rubra* and *T. argentea* were observed relatively low. On the other hand, hyperoside (**1**) was detected only in the leaves of *T. platyphyllos*.

In order to evaluate the reliability of the TLC techniques for the quality assurance of lime samples, the results obtained from HPLC analysis were compared with those of TLC. As was also pointed out by Wagner et al. [4], color intensities of spots in TLC analysis did not reflect the real concentration of each flavonoid in the sample. Despite that the high ratio of **5** in *T. platyphyllos* bracts and leaves, a faint color intensity was observed on TLC-plate. Moreover, flavonoids **1** and **5** as well as **7** and **?$_1$** could not be separated from each other in TLC chromatogram. As shown in Table 6, some flavonoids which detected

in low concentration in HPLC chromatograms could not be seen by TLC; i.e. **2** in the flower samples of all three species and **5** in the bracts and leaves of *T. rubra*.

Although **3** and **4** gave a single peak in HPLC analysis, a significant resolution was achieved in TLC (R_f 0.70 and 0.40, successively). Thus, TLC may serve as a useful tool for the analysis of lime samples from the view point of these two flavonoids. As shown in Table 6, flower samples of these three species contained **3** but not **4**. On the contrary, bracts and leaves of *T. platyphyllos* and *T. argentea* contained **4** but not **3**. Both compounds were detected, however, in the bracts and leaves of *T. rubra*. On the other hand, tiliroside was only determined in *T. argentea* samples by TLC.

As conclusion, results of the present study revealed that flavonoids may be evaluated as a valuable indicator for the quality assurance and determination of adulteration in the lime samples. But reliability of TLC techniques for this purpose, as suggested by EP, seems controversial. For a precise definition and assessment, HPLC techniques should be employed. On the other hand, flavonoid composition of *T. rubra* flowers was found very similar to that of *T. platyphyllos* and was proposed as an additional officinal species.

Acknowledgements

This study is financially supported by Gazi University Research Fund (EF 02-97/4).

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