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Determination of Phenolic Composition of *Tilia Tomentosa* Flowers Using UPLC-ESI-MS/MS

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Abstract: Phenolic compounds, which are secondary metabolites of plants, are one of the main groups of compounds that provide antiallergic, antiinflammatory, antimicrobial, antioxidant, cardioprotective properties of medicinal and aromatic plants. These broad physiological effects that they possess lead researchers to examine the phenolic contents of plants. *Tilia tomentosa* Moench is one of 45 species belonging to *Tiliaceae* family, and the use of flowers in traditional treatment methods is quite common. Although it is well known that *T. tomentosa* flowers are rich in phenolics with various biological functions, there is no recent study on determination of phenolic compounds of *T. tomentosa* flowers using UPLC-ESI-MS/MS. In this study, firstly, *T. tomentosa* flowers were extracted using hexane and volatile oil fractions were separated from the plant. Distilled water:methanol (50:50) mixture was added to the remaining flower part at 40 °C and that subjected to extraction for 15 min. The obtained extract was filtered and dried in a lyophilizer at -70 °C. The residue was redissolved in a mixture of distilled water:methanol (80:20). The sample was analyzed by UPLC-MS/MS (Waters Acquity Ultra Performance LC, Xevo TQ-S MS/MS) by passing through Macherey-Nagel Chromafil Xtra PTFE-20/25 0.20 µm filters. According to the analysis results, 3,4-dihydroxybenzoic acid (66.820 mg/kg), myricetin (29.395 mg/kg), rutin (21.421 mg/kg), ferulic acid (12.334 mg/kg) and 3,4-dihydroxybenzaldehyde (10.383 mg/kg) were detected. *T. tomentosa* flowers have great potential to usage in industries such as food, medicine and cosmetic due to its rich content of phenolics.

Keywords: Phenolics, 3,4-dihydroxybenzoic acid, *Tilia tomentosa*, UPLC-ESI-MS/MS

1. INTRODUCTION

Phenolics or polyphenols are secondary plant metabolites generally found in edible plants and have always been of interest because of their biological functions [1]. Phenolic compounds are important determinants in sensory and nutritional specifications of plants [2, 3]. These compounds presence an aromatic ring with one or more hydroxyl groups and their structures may vary from a simple phenolic molecule to a complex polymer with high-molecular mass [4]. Phenolics have been used for centuries for various medicinal purposes. Phenolic compounds, especially phenolic acids and flavonoids, which are of great interest mostly due to

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their biological functions in human health-related issues, are accepted as main source of pharmacological properties with their presence [5]

Ones of the most widely occurring phytochemicals in plants are phenolic compounds. Phenolic compounds are known to be extremely beneficial in terms of human nutrition, cosmetic and pharmacological [6-9]. As a large group of biologically active chemicals, they have large number of biological functions [10]. As previously reported in literature, phenolic compounds have cardio-protective effect, anti-cancer effect, anti-diabetic effect, anti-aging effect, neuro-protective effect, anti-viral effect, analgesic and anti-inflammatory effects, anti-bacterial effect, anti-parasitic effect and anti-oxidant effects [11, 12]. At the basis of the antioxidant capacities of the phenolic compounds are redox properties which allow them to function as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelating agents [4].

As a result of bioactive compounds found in different parts of plants such as flowers, peel, leaf and increased interest in natural products, medicinal and aromatic plants have found application fields such as pharmaceutical, cosmetic and dye industries. Therefore, they have been subjected to numerous researches [13-15]. Throughout the history of humanity, many diseases (diabetes, jaundice, shortness of breath, etc.) have been studied and tried to be treated using plants. The World Health Organization (WHO) reports that approximately 4 billion people around the world are trying to get rid of health problems with herbal drugs in the first place (80 % of the world population). Furthermore, in developed countries, about 25 % of prescription drugs constitute plant-based active ingredients [16].

Tilia tomentosa Moench is one of 45 species belonging to *Tiliaceae* family [17] and the use of flowers in traditional treatment methods is quite common [18]. Researches on different parts of *T. tomentosa* showed that the plant possesses spasmolytic, diuretic and sedative effects due to its flavonoids, essential oil and mucilage components and has been used to treat disorders such as nervous tension, cough, flu, migraine [19, 20].

T. tomentosa have been studied as novel phenolic compound source and qualitative and quantitative analyzes of phenolic compounds have been carried out using various techniques until today and reported that *T. tomentosa* contains flavonoids, mainly quercetin glycosides (rutin, quercitrin, and isoquercitrin), kaempferol glycosides, tyliroside and phenolic acids (caffeic, *p*-coumaric, and chlorogenic acids) [21, 22]. Additionally, polysaccharides, tannins and terpenoids were identified [22, 23].

Although it is a well-known and frequently used plant, it is seen that the number of studies on *T. tomentosa* is not much. To the best of our knowledge, there is no previous study using UPLC-ESI-MS/MS for the determination of the phenolic composition of *T. tomentosa* flowers.

In this study, the phenolic composition of *T. tomentosa* flowers was determined using UPLS-ESI-MS/MS and the importance of the plant as a medicinal and aromatic plant was evaluated according to the results.

2. MATERIAL and METHODS

2.1. Chemicals and Standards

Phenolic reference standards (pyrogallol, homogentisic acid, 3,4-dihydroxybenzoic acid, gentisicacid, pyrocatechol, galantamine, 4-hydroxy benzoic acid, 3,4-dihydroxybenzaldehyde, catechin hydrate, vanillic acid, caffeic acid, syringic acid, vanillin, epicatechin, catechin gallate, p-coumaric acid, ferulic acid, rutin, trans-2-hydroxy cinnamic acid, myricetin, resveratrol, trans-cinnamic acid, luteolin, quercetin, naringenin, genistein, apigenin, kaempferol, hesperetin, chlorogenic acid, and chrysin) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

All chemicals were analytical grade and purchased from local suppliers. High-Performance Liquid Chromatography (HPLC) grade water (18.2 MΩ) was purified by Millipore Milli-Q system (Molsheim, France) that contains reverse osmosis, ion exchange, and filtration steps.

2.2. Plant Material

T. tomentosa flowers were collected in the time of flowering season (June, 2016) in Muğla, Turkey. The plant was identified from Muğla Sıtkı Koçman University, Faculty of Science, Department of Molecular Biology and Genetics. *T. tomentosa* flowers were collected carefully and studied in fresh form without exposure to extreme temperatures.

2.3. Sample Preparation for the Determination of Phenolic Compounds

Phenolic compounds of *T. tomentosa* flowers were extracted according to the previously reported method [24, 25] with slight modifications. Briefly, flowers of *T. tomentosa* were extracted with hexane at first, and essential oil was removed from the plant. After this extraction, portion of flower was extracted using distilled water:methanol (50:50) at 40 °C for 15 min. This extract was filtered and dried at -70 °C with freeze dryer. Then, the residue was redissolved in water:metanol (80:20) mixture. The mixture was filtered from Macherey-Nagel Chromafil Xtra PTFE-20/25 0.20 µm, and analyzed using UPLC-MS/MS (Waters Acquity Ultra Performance LC, Xevo TQ-S MS-MS) instrument.

2.4. Determination of Phenolic Compounds Using UPLC-ESI-MS/MS

The UPLC-ESI-MS/MS instrument includes Waters (Milford, MA, USA) Acquity Ultra Performance LC with a Waters binary system manager and sample manager coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer with ESI probe. The separation was done with Waters analytical C18 column, Acquity UPLC BEH C18 (1.7 µm 2.1 × 100 mm) at 40°C column oven temperature and 2 µL injection volume with the two mobile phases (mobile phase A, 0.5 % (v/v) acetic acid in ultrapure water and mobile phase B, 0.5 % (v/v) acetic acid in acetonitrile) with a linear gradient mode, 0–1 min 99 % A, 1–10 min 70 % A, 10–12 min 5 % A, 12–13 min 99 % A at 0.650 mL min^{-1} flow rate. The multiple reaction monitoring (MRM) mode executes the transitions of parent ion to daughter ions of m/z, and the optimal instrument parameters of the mass spectrometer were as described in previous methods [25, 26]. MassLynx mass spectrometry software and TargetLynx data processing software (Waters) were used for the identification and evaluation of phenolic compounds by comparing retention time and m/z transitions of commercial standards using established calibration curves.

3. RESULTS and DISCUSSIONS

UPLC analyses of phenolic compounds in flowers of *T. tomentosa* revealed that flowers were highly rich in phenolics. In total, 24 of phenolic compounds were detected among 32 phenolic compounds were scanned.

Genistein, galanthamine, quercetin, pyrocatechol, gentisic acid, trans-2-hydroxy cinnamic acid, homogentisic acid and chlorogenic acid in analyzed sample were not determined.

3,4-dihydroxybenzaldehyde (10.383 mg/kg), 3,4-dihydroxybenzoic acid (66.820 mg/kg), ferulic acid (12.334 mg/kg), myricetin (29.395 mg/kg) and rutin (21.421 mg/kg) were found to be major phenolic compounds while 4-hydroxy benzoic acid (3.542 mg/kg), vanillic acid (5.275 mg/kg), kaempferol (3.683 mg/kg), and catechin hydrate (6.685 mg/kg) were determined as minor compounds. Phenolic compounds which were identified in flowers of *T. tomentosa* are given as ppm (mg/kg) with their method parameters in Table 1.

3,4-dihydroxybenzoic acid was determined as the highest rate of phenolics with the amount of 66.820 mg/kg (39.57% of phenolics), while the apigenin was minimum with the amount of 0.012 mg/kg.

Total phenolic content of *T. tomentosa* flowers was found to be 168.850 mg/kg in this study. Phenolic profile of flowers contained phenolic acids and their derivatives, flavonols, flavanols, hydroxybenzaldehydes and other compounds.

Table 1. Phenolic concentrations (mg/kg ± standard deviation) and method parameters for the analysis of compounds using UPLC-ESI-MS/MS

No	Compounds	Quantification > confirmatory transition (*m*/*z*)	Cone voltage (V)	Collision Energies (V)	Concentration (mg/kg) ± SD
1	Pyrogallol	125.01 > 69.10, 79.04, 81.02	20	17, 17, 14	0.701 ± 0.003
2	Gallic acid	168.95 > 125.02, 107.02, 97.02	20	25, 20, 14	0.232 ± 0.007
3	Homogentisic acid	167.03 > 123.03, 122.08, 108.00	10	20, 20, 10	ND
4	3,4-Dihydroxybenzoic acid	153.06 > 108.00, 81.01, 91.01	10	20, 25, 20	66.820 ± 0.007
5	Gentisic acid	153.05 > 109.04, 108.03, 81.00	10	20, 20, 12	ND
6	Pyrocatechol	153.06 > 81.01, 108.00, 109.04	8	20, 25, 20	ND
7	Galantamin	288.10 > 198.00, 213.09, 230.95	20	32, 23, 17	ND
8	4-Hydroxy benzoic acid	136.98 > 93.03, 65.10	10	25, 14	3.542 ± 0.005
9	3,4-Dihydroxybenzaldehyde	137.00 > 91.93, 107.94, 136.00	8	21, 20, 18	10.383 ± 0.008
10	Catechin hydrate	288.88 > 109.15, 124.99, 245.26	30	25, 20, 15	6.685 ± 0.005
11	Vanillic acid	166.98 > 151.97, 108.03, 123.03	20	18, 12, 14	5.275 ± 0.005
12	Caffeic acid	179.10 > 135.14, 107.10, 133.9	32	23, 23, 24	1.824 ± 0.004
13	Syringic acid	197.20 > 123.00, 167.00, 182.00	15	22, 18, 14	0.320 ± 0.005
14	Vanillin	150.95 > 135.94, 91.90, 107.97	30	20, 20, 14	0.051 ± 0.002
15	*p*-Coumaric acid	163.01 > 119.04, 93.00, 117.01	5	27, 27, 15	0.730 ± 0.005
16	Ferulic acid	193.03 > 134.06, 178.00, 149.02	20	16, 12, 13	12.334 ± 0.011
17	Epicatechin	189.18 > 151.00, 203.00, 205.00	20	20, 20, 20	4.002 ± 0.005
18	Chlorogenic acid	353.02 > 191.01, 179.09, 161.02	30	30, 28, 24	ND
19	Catechin gallate	441.00 > 168.98, 288.97	30	20, 20	0.140 ± 0.005
20	Rutin	609.00 > 254.99, 270.93, 299.90	17	55, 55, 40	21.421 ± 0.005
21	*trans*-2-hydroxycinnamic acid	163.04 > 119.04, 117.01, 93.07	10	25, 22, 13	ND
22	Myricetin	316.90 > 107.07, 137.01, 150.97	30	30, 25, 25	29.395 ± 0.010
23	Resveratrol	227.01 > 143.01, 159.05, 185.03	30	25, 18, 18	0.020 ± 0.004
24	*trans*-Cinnamic acid	146.98 > 103.03, 62.18	30	10, 10	0.922 ± 0.003
25	Luteolin	284.91 > 107.01, 133.05, 151.02	20	30, 33, 30	0.314 ± 0.004
26	Quercetin	303.00 > 137.00, 153.00, 229.00	20	30, 32, 30	ND
27	Naringenin	270.98 > 107.00, 119.04, 150.97	20	25, 25, 20	0.060 ± 0.005
28	Genistein	271.00 > 153.00, 215.00, 243.00	20	27, 25, 24	ND
29	Apigenin	269.10 > 107.00, 117.00, 149.00	20	30, 30, 25	0.012 ± 0.002
30	Kaempferol	284.90 > 158.97, 117.10, 227.14	10	34, 40, 30	3.683 ± 0.006
31	Hesperetin	301.02 > 108.01, 136.00, 163.99	20	36, 30, 24	0.015 ± 0.002
32	Chrysin	252.99 > 63.05, 107.05, 142.99	20	30, 25, 25	0.024 ± 0.005

ND : Not detected

Total ion chromatograms of major phenolic compounds determined in *T. tomentosa* flowers using ultra-performance liquid chromatography with electrospray ionization coupled to tandem mass spectrometry (UPLC-ESI-MS/MS) were given in Figure 1.

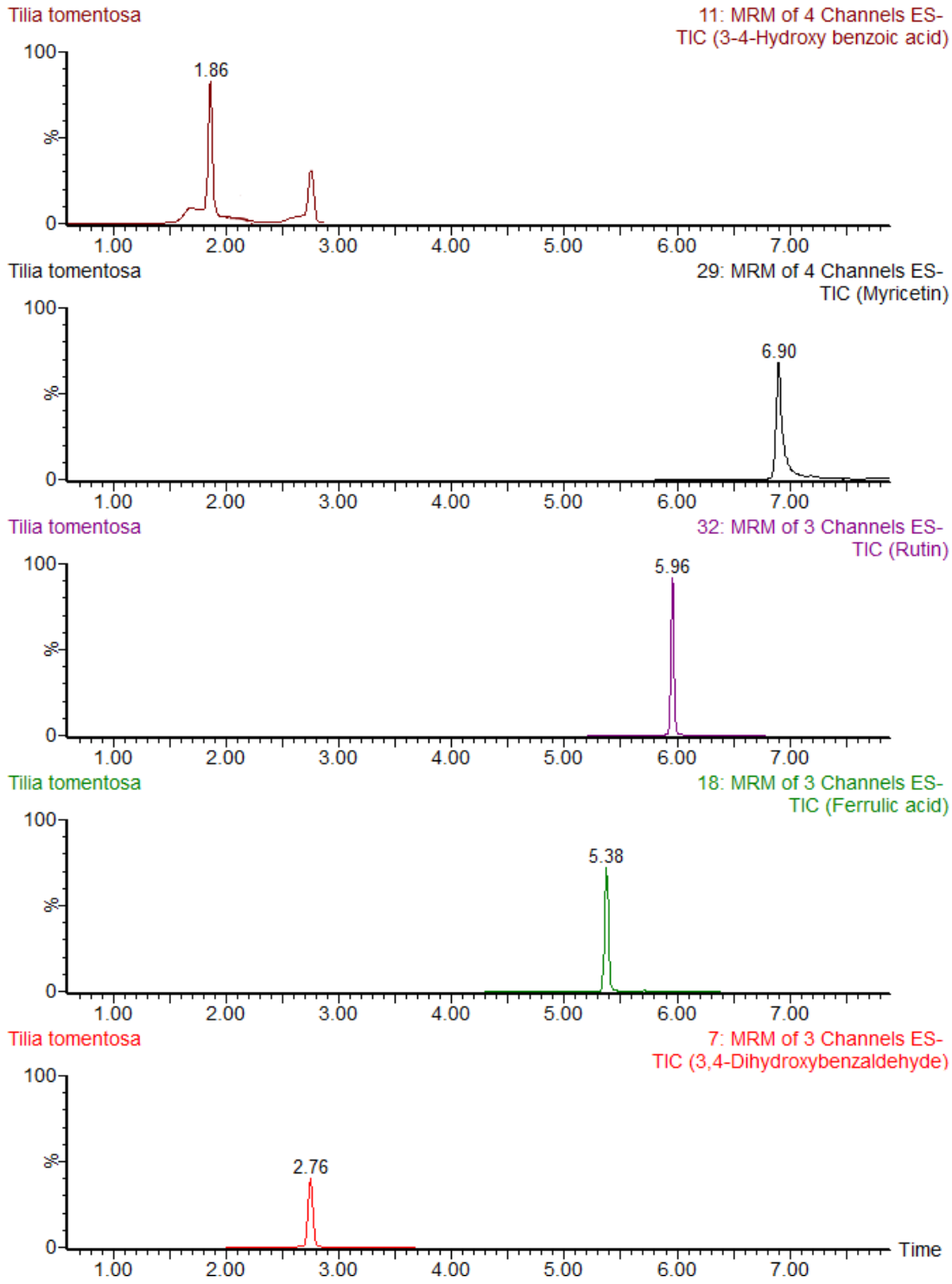

Figure 1. Total ion chromatograms (TIC) of major phenolic compounds analyzed using UPLC-ESI-MS/MS.

Although there is no previous study for the determination of the phenolic composition of *T. tomentosa* flowers, there few literatures related to leaves of *T. tomentosa* (syn. *T. argentea*) Toker et al. [27] reported that 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) in the leaves of *Tilia argentea*. Also Demiray et al. [28] indicated that protocatechuic acid is the major free phenolic compound in acetone and methanol extracts of *T. argentea*. Distilled water showed the highest extraction capacity for catechin, chlorogenic, caffeic and gallic acids.

Aromatic plants are broadly used by food industries but their properties also justify their application by other industries like food packaging, cosmetics, perfumery and pharmaceutical. According to the results of phenolic composition of *T. tomentosa* flowers we can conclude that flowers of *T. tomentosa* are potent natural ingredients for scientists, manufactures and producers to replace their syntetic materials with natural ones.

4. CONCLUSION

Nowadays, it is clear that the escape from artificial substances will further increase the importance of natural phenolic substances. In addition to the possibilities for use food, pharmaceutical and cosmetic industries. It is necessary to understand the mechanisms of action of phenolic substances, which have important effects on human health, and to investigate ways to quantify and use them technologically. For this purpose, phenolic compositions of medicinal and aromatic plants or their different extracts need to be investigated with accuracy and precision using modern instruments.

In this study, the phenolic composition of *T. tomentosa* flowers were identified first time using UPLC-ESI-MS/MS instrument. The lack of information about phenolic composition of *T. tomentosa* flowers using UPLC-ESI-MS/MS makes this study unique and important. The UPLC-ESI-MS/MS demonstrated to be reliable for the unambiguous detection of a large number of compounds, by enabling the determination of phenolic profiles. According to the results, it is understood that *T. tomentosa* flowers are very rich in phenolic acids and flavonols which are the two most important phenolic substance groups. The most abundant ingredients in the samples were 3,4-dihydroxybenzoic acid, myricetin, rutin, ferulic acid and 3,4-dihydroxybenzaldehyde.

The study is a guide for those who want to study the biological activities of *T. tomentosa* flowers include but not limited for antioxidant activity, anticancer activity or antitumor activity.

Conflict of Interests

Authors declare that there is no conflict of interests.

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