SUPPLEMENTARY MATERIAL

Secondary metabolites of *Tilia tomentosa* Moench inflorescences collected in Central Italy: chemotaxonomy relevance and phytochemical rationale of traditional use

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Abstract

In this paper, the phytochemical analysis on the inflorescences (flowers and bracts) of a sample of *Tilia tomentosa* collected from an isolated population in Central Italy, was reported

for the first time. Thirteen compounds (oleanolic acid, maslinic acid, apigenin, luteolin, kaempferol, quercetin, kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucoside, rutin, tiliroside, ellagic acid, shikimic acid and quinic acid), belonging to several different classes of natural products, were identified on the basis of spectroscopic and spectrometric analysis. This sample was found to be mainly composed by flavonoids, followed by organic acids and pentacyclic triterpenes. The chemosystematic aspects of the identified components were also discussed, together with their pharmacologic relevance with respect to the traditional medicinal uses of this plant material.

Keywords: *Tilia tomentosa* Moench, phytochemistry, chemotaxonomy, flavonoids, traditional medicine.

3. Experimental

3.1. Plant material

A quantity of 50.0 g of inflorescences (flowers and bracts) of *Tilia tomentosa* Moench were collected in May 2015 from an isolated population located in the area included between the towns of Collepardo and Vico nel Lazio in the Latium region (Central Italy), (geographical coordinates 41°46'16" N, 13°21'39" E).

The botanical identification was carried out by one of us (M.S.) through comparison with both descriptions reported in literature (Mitchell, 1974; Conti et al., 2005) and with other samples of the same species stored in his industry laboratories (Sarandrea Marco & Co. S.r.l.).

A representative sample of this collection is also stored in our laboratory for further references under the voucher name TT04052015.

3.2. Chemicals

During this study, the following chemicals were employed: ethanol 96% for the extraction procedure; *n*-butanol, distilled water, methanol, dichloromethane as pure solvents or in mixture among them all at several concentrations to be used as eluting systems for the chromatographic separation procedure by Column Chromatography together with silica gel (40-63 μm particle size) as stationary phase; deuterated solvents such as $CDCl_3$, CD_3OD and

D_2O for the identification of compounds by NMR Spectroscopy; HPLC methanol for the identification of compounds by Mass Spectrometry.
All the solvents having RPE purity grade if not differently specified, were purchased from Sigma Aldrich (Saint Louis, Missouri, USA) as well as the deuterated solvents and the HPLC methanol whereas silica gel was purchased from Fluka Analytical (Munich, Germany).

3.3. Instruments

NMR spectra [mono- (^{1}H-, ^{13}C-, DEPT, APT) and bidimensional, homo- (COSY, TOCSY) and heteronuclear (HSQC, HMBC)] were recorded on a Bruker Avance III 400 MHz instrument (Billerica, Massachusetts, USA) operating at 9.4 T at 298 K, with the chemical shifts expressed in ppm. The chemical shifts were expressed from TMS (s, 0 ppm) as internal reference standard for spectra in $CDCl_3$, the internal solvent signal of CD_2HOD (m5, δ_H 3.31 ppm; m7 δ_C 49.00 ppm) was the reference for spectra in CD_3OD while the HDO signal (s, δ_H 4.79 ppm) was set as reference for spectra in D_2O.
MS spectra were performed on a Q-TOF MICRO spectrometer (Micromass, now Waters, Manchester, UK) equipped with an ESI source operating in the negative and/or positive ion mode. The flow rate of sample infusion was 20 μL/min with 50 acquisitions per spectrum. Data were analyzed by using the MassLynx software developed by Waters.

3.4. Extraction of the phytochemicals

The dried plant material consisting of the flowers and bracts for the weight of 30.0 g was extracted with 96% ethanol until complete immersion of it (about 250 mL x 48 h). The procedure was repeated three times for an exhaustive extraction. The ethanolic solutions were collected altogether in a same flask and ethanol was evaporated under reduced pressure by the Rotavapor. Throughout the first concentration of the extract, pH of the solution was checked on litmus paper in order to verify that pH was between the range of 5.5-8.5 because one too acid or too basic solution might cause unwanted reactions in the extract such as the hydrolysis of ester and glycosidic bonds. Once ethanol was completely eliminated, a water suspension was obtained and this was frozen and then lyophilized in order to preserve also the temperature-sensitive compounds. The final dried crude extract, with a dark green coloration, weighed 2.0 g and was stored at 4°C in the dark until further analysis.

3.5. Separation and identification of the phytochemicals

An aliquot of the total dried crude extract (1.5 g) was subjected to a first chromatographic separation using a corresponding amount of silica gel of 45.2 g (ratio 1:30 *w*/*w*). The eluting system was a solution of *n*-butanol and distilled water at the concentration ratio 82:18 (*v*/*v*). During the chromatographic run, the polarity of the eluting system was raised in order to let the elution of the most polar compounds and so this became a solution of *n*-butanol, methanol and distilled water at concentration 70:10:30 (*v*/*v*/*v*).

From this first chromatographic separation, six compounds were identified through comparison with data reported in literature and/or with pure standards available in our laboratory: a mixture (6:2:2:1) of luteolin (**4**) (Venditti et al., 2017a), kaempferol (**5**) (Frezza et al., 2018), quercetin (**6**) (compared with original standard) and apigenin (**3**) (Venditti et al, 2018a) from fraction 11 (25.5 mg); shikimic acid (**12**) and quinic acid (**13**) (Caprioli et al., 2016; Venditti et al., 2017a; Venditti et al., 2018a) as a mixture in ratio 2:1 from the assembly of fractions 80-81 for the total weight of 96.8 mg

Since not all compounds could be clearly detected from this chromatographic step, another chromatographic separation was performed on the assembly of fractions 2-71 deriving from the first one for the total weight of 235.5 mg. The correspective amount of silica gel was 7.0 g (ratio 1:35 *w*/*w*) and the eluting system was a solution of dichloromethane and methanol at different concentrations. The starting eluent concentration was 98:2 (*v*/*v*) but during the chromatographic run this was changed in order to let the elution of the most polar compounds passing to 95:5 (*v*/*v*), 9:1 (*v*/*v*), 8:2 (*v*/*v*), 7:3 (*v*/*v*) and 6:4 (*v*/*v*). From this chromatographic separation, additional components were identified according to the methodology described before: oleanolic acid (**1**) and maslinic acid (**2**) (Venditti et al., 2016a; Seebacher et al., 2003; Hou et al., 2009) from the assembly of fractions 13-17 (11.4 mg) in mixture 3:1; kaempferol-3-*O*-glucoside (astragalin) (**7**) (Wei et al., 2011), quercetin-3-*O*-glucoside (isoquercitrin) (**8**) (Maggi et al., 2015), tiliroside (**10**) (Mekhelfi et al., 2014) and ellagic acid (**11**) (Goriparti et al., 2013) in mixture (2:3:1:1) from the assembly of fractions 30-34 (55.7 mg); rutin (**2**) (Venditti et al., 2016b) from the assembly of fractions 48-56 for the total weight of 8.5 mg. The unequivocal identification of substances in mixture has been conducted on the basis of chemical shift values, multiplicity and the hetero- and homonuclear correlations observed in the 2D-NMR spectra, following a method already applied by our research group in similar

cases and also to describe not previously identified compounds when isolated in mixture (Sciubba et al., 2014; Frezza et al., 2018; Venditti et al., 2016c; 2017b; 2017c; 2018b).

3.6. NMR and MS data of the identified phytochemicals

Oleanolic acid (**1**): ^{1}H-NMR (400 MHz, $CDCl_3$) : 5.25 (1H, t, *J* = 3.4 Hz, H-12), 3.22 (1H, dd, *J* = 10.8, 5.0 Hz, H-3), 1.09 (3H, s, H-27), 0.99 (3H, s, H-25), 0.95 (3H, s, H-30), 0.92 (3H, s, H-29), 0.88 (3H, s, H-26), 0.78 (3H, s, H-24).
ESI-MS: *m/z* 479.60 $[M+Na]^+$; *m/z* 455.35 $[M-H]^-$.

Maslinic acid (**2**): ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 5.25 (1H, t, *J* = 3.4 Hz, H-12), 3.88-3.68 (1H, m, H-2), 1.11 (3H, s, H-27), 1.01 (3H, s, H-23), 0.95 (3H, s, H-25), 0.87 (3H, s, H-30), 0.85 (3H, s, H-29), 0.79 (3H, s, H-24).
ESI-MS: *m/z* 495.72 $[M+Na]^+$; *m/z* 471.32 $[M-H]^-$.

Apigenin (**3**): ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.95 (2H, d, *J* = 8.8 Hz, H-2' and H-6'), 7.09 (2H, d, *J* = 8.8 Hz, H-3' and H-5'), 6.78 (1H, s, H-3), 6.49 (1H, d, *J* = 1.9 Hz, H-8), 6.22 (1H, d, *J* = 1.9 Hz, H-6)
ESI-MS: *m/z* 269.13 $[M-H]^-$.

Luteolin (**4**): ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.43 (1H, d, *J* = 2.0 Hz, H-2'), 7.40 (1H, dd, *J* = 8.2, 2.0 Hz, H-6'), 6.77 (1H, d, *J* = 8.2 Hz, H-5'), 6.47 (1H, s, H-3), 6.40 (1H d, *J* = 1.8 Hz, H-8), 6.21 (1H, d, *J* = 1.8 Hz, H-6).
ESI-MS: *m/z* 287.23 $[M+H]^+$; *m/z* 309.33 $[M+Na]^+$.

Kaempferol (**5**): ^{1}H-NMR (400 MHz, CD_3OD) δ: 8.00 (2H, d, *J* = 8.9 Hz, H-2', H-6'), 6.92 (2H, d, *J* = 8.9 Hz, H-3', H-5'), 6.40 (1H, d, *J* = 2.2 Hz, H-8), 6.21 (1H, d, *J* = 2.2 Hz, H-6).
ESI-MS: *m/z* 285.23 $[M-H]^-$; *m/z* 309.33 $[M+Na]^+$.

Quercetin (**6**): ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.72 (1H, d, *J* = 2.2 Hz, H-2'), 7.62 (1H, dd, *J* = 8.6, 2.1 Hz, H-6'), 6.88 (1H, d, *J* = 8.6 Hz, H-5'), 6.40 (1H, d, *J* = 2.1 Hz, H-8), 6.21 (1H, d, *J* = 2.1 Hz, H-6).
ESI-MS: *m/z* 301.23 $[M-H]^-$; *m/z* 325.22 $[M+Na]^+$.

Kaempferol-3-*O*-glucoside (astragalin) (**7**): ^{1}H-NMR (400 MHz, CD_3OD) δ: 8.02 (2H, d, *J* = 8.5 Hz, H-2', H-6'), 6.79 (2H, d, *J* = 8.5 Hz, H-3', H-5'), 6.39 (1H, *J* = 2.2 Hz, H-8), 6.24 (1H, *J* = 2.2 Hz, H-6), 5.38 (1H, d, *J* = 7.6 Hz, H-1''), other proton signals overlapped.
ESI-MS: *m/z* 471.26 $[M+Na]^+$.

Quercetin-3-*O*-glucoside (isoquercitrin) (**8**): ^{1}H NMR (400 MHz, CD_3OD) δ: 7.78 (1H, d, *J* = 2.0 Hz, H-2'), 7.52 (1H, dd, *J* = 8.6, 2.0 Hz, H-6'), 6.87 (1H, d, *J* = 8.5 Hz, H-5'), 6.39 (1H, d, *J* = 2.1 Hz, H-8), 6.21 (1H, d, *J* = 2.1 Hz, H-6), 4.94 (1H, d, *J* = 7.9 Hz, H-1''), other sugar protons overlapped.
ESI-MS: *m/z* 487.33 $[M+Na]^+$.

Rutin (**9**): ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.72 (1H, d, *J* = 1.6 Hz, H-2'), 7.63 (1H, br. d, *J* = 8.4 Hz, H-6'), 6.89 (1H, d, *J* = 8.4 Hz, H-5'), 6.49 (1H, br. s, H-8), 6.22 (1H, d, *J* = 1.9 Hz, H-6), 5.08 (1H, d, *J* = 7.6 Hz, H-1''), 4.52 (1H, d, *J* = 1.9 Hz, H-1'''), 3.87-3.35 (overlapped signals of saccharides), 1.11 (3H, d, *J* = 6.2 Hz, H-6''').
ESI-MS: *m/z* 609.18 $[M-H]^-$.

Tiliroside (**10**): ^{1}H NMR (400 MHz, CD_3OD) δ 7.98 (2H, d, *J* = 8.9 Hz, H2', H-6'), 7.52 (1H, d, *J* = 15.1 Hz, H-β), 7.32 (2H, d, *J* = 8.6 Hz, H-2''', H-6'''), 6.90 (2H, d, *J* = 8.6 Hz, H-3''', H-5'''), 6.88 (2H, d, *J* = 9.0 Hz, H-3', H-5'), 6.40 (1H, d, *J* = 2.2 Hz, H-8), 6.24 (1H, d, *J* = 2.1 Hz, H-6), 6.07 (1H, d, *J* = 15.1 Hz, H-α), 5.32 (1H, d, *J* = 7.7 Hz, H-1''), other proton signals overplapped.
ESI-MS: *m/z* 617.35 $[M+Na]^+$; *m/z* 593.13 $[M-H]^-$.

Ellagic acid (**11**): ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.44 (2H, s, Ar-H).
ESI-MS: *m/z* 325.21 $[M+Na]^+$, *m/z* 301.13 $[M-H]^-$.

Shikimic acid (**12**): ^{1}H-NMR (400 MHz, D_2O) δ: 6.45 (1H, m, H-2), 4.40 (1H, br. t, *J* = 4.4 Hz, H-3), 3.98-3.94 (1H, m, H-5), 3.58-3.54 (1H, m, H-4), 2.77 (1H, dd, *J* = 18.0/5.4 Hz, H_a-6), 2.20 (1H, dd, *J* = 18.0/7.7 Hz, H_b-6).
ESI-MS: *m/z* 173.16 $[M-H]^-$.

Quinic acid (**13**): ^{1}H-NMR (400 MHz, D_2O) δ: 4.17 (1H, m, H-4), 3.98-3.94 (overlapped signal, H-5), 3.53 (1H, m, H-3), 2.12-2.08 (4H, m, H_a-2, H_b-2, H_a-6, H_b-6).

ESI-MS: *m/z* 191.32 $[M-H]^-$.

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