SUPPLEMENTARY MATERIAL

Phenolic compounds from the aerial parts of *Clematis viticella* L. and their
*
*in vitro* anti-inflammatory activities

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Abstract

Phytochemical investigations on the EtOH extract of *Clematis viticella* led to the isolation of six flavonoid glycosides, isoorientin (1), isoorientin 3'-O-methyl ether (2), quercetin 7-O-α-L-rhamnopyranoside (3), quercetin 3,7-di-O-α-L-rhamnopyranoside (4), manghaslin (5) and chrysoeriol 7-O-β-D-glucopyranoside (6), one phenylethanol derivative, hydroxytyrosol (7), along with three phenolic acids, caffeic acid (8), (E)-p-coumaric acid (9) and p-hydroxybenzoic acid (10). The structures of the isolates were elucidated on the basis of NMR and HR-MS data. All compounds were isolated from *C. viticella* for the first time.

Compounds 7 and 8 showed significant anti-inflammatory activity at 100 µM by reducing the release of NO in LPS-stimulated macrophages comparable to positive control indomethacin. Compounds 3 and 7 exhibited anti-inflammatory activity through lowering the levels of TNF-α while 1, 3 and 5 decreased the levels of neopterin better than the positive controls.

**Keywords:** *Clematis viticella*; flavonoid glycosides; phenolic acids; hydroxytyrosol; anti-inflammatory activity
1. Experimental

1.1. General
All NMR spectra were recorded on a Varian Mercury-Mx spectrometer (USA) at 600 MHz for $^1$H NMR and at 150 MHz for $^{13}$C NMR, with CD$_3$OD as solvents. HR-MS analyses were performed on APCI-ION TRAP Thermo Deca XP Max. MPLC: Sepacore® Flash Systems X10/X50 (Buchi Labortechnik AG, Flawil, Switzerland). Precoated SiO$_2$ 60F$_{254}$ plates (Merck, Darmstadt, Germany) were utilized for TLC analysis and visualized under UV light and derivatized with 1% Vanillin/ H$_2$SO$_4$ solution, followed by heating at 105°C for 2 min. Column chromatography (CC) was performed with SiO$_2$ 60 (0.063 – 0.200 mm; Merck, Darmstadt), Polyamide (Sigma–Aldrich, St. Louis, MO, USA), and Sephadex LH-20 gel (Sigma–Aldrich, St. Louis, MO, USA). Medium pressure liquid chromatography (MPLC) separation were carried out on Sepacore® Flash Systems X10/X50 (Buchi Labortechnik AG, Flawil, Switzerland) system.

1.2. Plant material
The aerial parts of Clematis viticella Linaceus were collected from Sakarya, in June 2015. The plant material was identified by Prof. Dr. E. Yeşilada. The voucher specimen (YEF 15009) has been deposited at the Herbarium of Faculty of Pharmacy, Yeditepe University, İstanbul.

1.3. Extraction, isolation and structure elucidation
The shade-dried and powdered aerial parts of Clematis viticella (681 g) were macerated overnight with EtOH (3 L) and then extracted twice at room temperature for 4 h. The combined EtOH extracts were concentrated under vacuum to yield a crude extract (46.2 g, yield 6.8 %). The EtOH extract was suspended in 90% MeOH, and partitioned with n-hexane (500 mL x 4). After removing the MeOH, the volume was adjusted to 500 mL with H$_2$O and the partition went on with equal volumes (500 mL x 4) of CH$_2$Cl$_2$, EtOAc and n-BuOH successively. The n-BuOH subextract (2.85 g) was subjected to polyamide column (20 g) eluting with H$_2$O/MeOH (100:0 to 0:100) mixture to give five main fractions (frs. A-E). Fr E (343 mg) was separated by C$_{18}$-Medium Pressure Liquid Chromatography (LiChroprep C$_{18}$-MPLC, 43 g) eluting with H$_2$O/MeOH gradient (85:15 to 50:50) to afford isoorientin (1, 8.3 mg) and subfraction E$_1$ (24.1 mg). Rechromatography of fr. E$_1$ by SiO$_2$ (40 g) column using the gradient mixture of CH$_2$Cl$_2$/MeOH (90:10 to 70:30) yielded isoorientin 3’-O-methyl ether (2, 5 mg). Fr C (249 mg) was subjected to C$_{18}$-MPLC (43 g,
H₂O/MeOH, 85:15 to 50:50) to yield manghaslin (5, 7 mg) and quercetin 3,7-di-O-α-L-rhamnopyranoside (4, 13 mg). EtOAc subextract (2.39 g) was loaded onto a SiO₂ (25 g) column and fractionated by using CH₂Cl₂/MeOH mixtures (90:10 to 50:50) to obtain eight main fractions (frs 1-8). Fr 3 (88 mg) was subjected to SiO₂ (12 g) column (CH₂Cl₂/MeOH, 95:5 to 80:20) to give subfraction fr3a (26 mg) which was further purified by with Sephadex LH-20 CC (6g) column to yield hydroxytyrosol (7, 15 mg). Similarly, fr 4 (161 mg) was separated by SiO₂ (22 g) column eluting with stepwise CH₂Cl₂-MeOH gradient (5 to 15% MeOH) to give the mixture of p-coumaric acid and p-hydroxybenzoic acid (9 + 10, 6 mg). Fr 6 (228 mg) was subjected to SiO₂ (25 g) column (CH₂Cl₂/MeOH, 95:5 to 70:30) to give subfractions fr 6a (32 mg) and 6b (112 mg). Fr 6a was rechromatographed over SiO₂ (6 g) column (CH₂Cl₂/MeOH, 95:5 to 90:10) to give chrysoeriol 7-O-β-D-glucopyranoside (6, 2.5 mg). Purification of Fr 6b by C₁₈-MPLC, (15.5 g, H₂O/MeOH, 80:20 to 40:60) yielded caffeic acid (8, 2 mg) and quercetin 7-O-α-L-rhamnopyranoside (3, 4 mg).

The structures were characterized as isoorientin (1) (Çalış et al. 2006), isoorientin 3′-O-methyl ether (2) (Delezar et al. 2006), quercetin 7-O-α-L-rhamnopyranoside (3), quercetin 3,7-di-O-α-L-rhamnopyranoside (4) (Toker et al. 2004), manghaslin (5) (Afzan et al. 2012), chrysoeriol 7-O-β-D-glucopyranoside (6) (Sarkhail et al. 2006), hydroxytyrosol (7) (Lee et al., 2010), caffeic acid (8), (E)-p-coumaric acid (9) and p-hydroxybenzoic acid (10) by comparing their NMR and MS data with those published previously.

1.4. Antiinflammatory activity assays

Cell Culture and Cell Viability Assay

RAW264.7 murine macrophage cells (ATCC, USA) were maintained in DMEM, supplemented with 10% FBS and 1% streptomycin and penicillin at 37°C in 5% CO₂. Cell viability was examined by using MTT assay. Plated RAW264.7 cells were treated with 100 μM of pure compounds and reference molecules, indomethacin and L-NAME. After 24 hours incubation process, MTT was added to each well at 0.5 mg/mL of concentration and incubated for an additional 2 hours at 37°C. After discarding all medium from plates, 100 μl of isopropanol was added to all wells. Absorbance of the blue formazan was determined at 540 nm by a UV-spectrophotometric plate reader. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells and all measurements were done in triplicates.

Evaluation of Anti-inflammatory Activity by Griess Assay
Anti-inflammatory activities of the isolates were evaluated by measuring the stable nitric oxide (NO) metabolite, nitrite, with Griess reagent (Kiener & Vollmar, 1997). Briefly, RAW264.7 cells were plated in a 48 well-plate and incubated for 24 hours at 37°C in 5% CO₂. Plated cells were pre-treated with the isolated compounds and the reference molecules for 2 hours and then stimulated with 1 µg/mL of LPS for additional 22 hours. The culture supernatant (50 µL) was mixed with Griess reagent and incubated at room temperature for 10 min. The absorbance of the mixture was determined at 570 nm using a microplate reader (Multiskan Ascent, Finland). The amount of nitrite in the samples was calculated by using sodium nitrite standard curve. Indomethacin and L-NAME were used as positive controls at 100 µM.

**Measurement of Neopterin and TNF-α Levels**

Neopterin and TNF-α concentrations were measured in cell culture supernatants by using a commercially available quantitative enzyme-linked immunosorbent assay (ELISA) system (IBL Rat Neopterin ELISA Kit, Germany; Abbkine Rat TNF-α ELISA Kit, China) according to manufacturer's instructions.

1.5. **Statistical analysis**

GraphPad Prism 6 was used for the all statistical analyses. Data related to cell viability, anti-inflammatory activity, neopterin and TNF-α levels were analyzed by using one-way ANOVA following the post-hoc tests by Tukey. Differences were considered as significant at \( p < 0.05 \).
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