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Antioxidant galloylated flavonoids from *Geranium tuberosum* L. subsp. *tuberosum*

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Phytochemical investigations on the most active EtOAc extract led to the isolation of 9 flavonoids [quercetin (**1**), quercetin 3-*O*-β-glucopyranoside (**2**), quercetin 3-*O*-β-galactopyranoside (**3**), quercetin 3-*O*-(2″-*O*-galloyl)-β-glucopyranoside (**4**), quercetin 3-*O*-(2″-*O*-galloyl)-β-galactopyranoside (**5**), quercetin 3-*O*-(6″-*O*-galloyl)-β-glucopyranoside (**6**), quercetin 3-*O*-(6″-*O*-galloyl)-β-galactopyranoside (**7**), quercetin-3-*O*-α-arabinofuranoside (**8**), and quercetin-3-*O*-α-rhamnopyranosyl-(1→6)-β-glucopyranoside (**9**)] and a simple phenolic compound (methyl gallate) (**10**). The structures of the compounds were elucidated by 1- and 2-dimensional NMR techniques (^{1}H, ^{13}C, COSY, HMBC, and HMQC) and ESI-TOF-MS spectrometry. H_2O_2-induced lipid peroxidation inhibitor effects in human red blood cells of different extracts of *G. tuberosum* L. subsp. *tuberosum* aerial parts were also investigated. All the compounds tested showed antioxidant activity.

Key Words: *G. tuberosum* L. subsp. *tuberosum*, flavonol gallate, flavonoids, methyl gallate, antioxidant

Introduction

The genus *Geranium* is known as 'Turnagagası'[1] and represented by 35 species in the flora of Turkey.[2,3] Some *Geranium* species are used in traditional medicine as antidiabetics, hemostatics, stomachics, antihemorrhoidals,

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and antidiarrheics as well as for the treatment of tonsillitis, cough, urticaria, internal bleeding, and dysentery.[4–6] Moreover, leaves of some *Geranium* species are consumed as food in the Aegean region and tubers of *G. tuberosum* subsp. *tuberosum* are eaten in Erzurum.[1] Even though simple phenolics,[5,7] tannins,[5,8] flavonoids,[7,8] lignans,[9] and essential oils[10] have been isolated from *Geranium* species, there is no report on the polyphenolic compounds of *G. tuberosum* subsp. *tuberosum*. The current study describes the isolation and structure elucidation of the polyphenolic compounds from the aerial parts of the title plant and their H_2O_2-induced lipid peroxidation inhibitor effects in human red blood cells.

Experimental

General experimental procedures

The UV spectra were recorded in MeOH using an Agilent 8453 spectrometer. 1D- and 2D-NMR measurements were recorded at room temperature on a Bruker DRX 400 (^{1}H 400 and ^{13}C 100 MHz) MHz for **1-7**, **9**, and **10** and a Bruker ARX 500 MHz for **8** (^{1}H 500 and ^{13}C 125 MHz). Compounds **1-7**, **9**, and **10** were measured in MeOH-d_4 while **8** was measured in DMSO. Chemical shifts were given in ppm with tetramethylsilane (TMS) as an internal standard. ESI-TOF-MS was performed on a Micromass Q-TOF Micro instrument. Sephadex LH-20 (Pharmacia), polyamide (ICN), and Kieselgel 60 (Merck, 0.063-0.200 mm) were used for open column chromatography (CC). Medium-pressure liquid chromatographic (MPLC) separations were carried out on a Büchi (3 × 25 cm) glass column packed with Euchrom C_{18} (20-45 μm), using a Büchi B-684 pump. Vacuum-liquid chromatographic (VLC) separations were realized on a (2.5 × 15 cm) glass column packed with LiChroprep C_{18} (40-63 μm). TLC analyses were carried out on pre-coated Kieselgel 60 F_{254} aluminum plates (Merck). Compounds were detected by UV fluorescence and spraying 1% vanillin/H_2SO_4, followed by heating at 100 °C for 1-2 min.

Plant material: *Geranium tuberosum* L. subsp. *tuberosum* (Geraniaceae) was collected on 6 May 2005 from Atatürk Orman Çifliği, Ankara. Voucher specimens (HUEF 05003) have been deposited at the Herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey.

Extraction and isolation: Dried and powdered aerial parts of *Geranium tuberosum* subsp. *tuberosum* (400 g) were extracted with 80% MeOH (4 × 3.5 L) at room temperature and combined MeOH extracts were concentrated under reduced pressure. The resultant extract was then dissolved in H_2O and the water-soluble portion was partitioned with petroleum ether (40-60 °C) (4 × 150 mL), ethyl acetate (6 × 150 mL), and *n*-BuOH (4 × 150 mL), consecutively. A part of the EtOAc extract (10.4 g) was chromatographed over Sephadex LH-20 (3.2 × 55 cm), eluting with H_2O, followed by increasing concentrations of MeOH in H_2O (0%→100% MeOH) to yield 10 main fractions (Frs. A-J). Fr. C (5%→8% MeOH) (500 mg) was subjected to VLC on LiChroprep C_{18} (2.2 × 18 cm) and elution with H_2O, followed by the MeOH-H_2O solvent system (0%→100%), yielded 5 fractions (Frs. C_1-C_5). Fr. C_2 was found to be compound **10** (90 mg, eluted with 5% MeOH) and Fr. C_4 was found to be compound **9** (3 mg, eluted with 45% MeOH) in pure form. Fr. D (20%→35% MeOH) (800 mg) was subjected to MPLC (Euchrom C_{18}) (3.6 × 42 cm) using the H_2O-MeOH (0%→100) solvent system to yield 5 fractions (Frs. D_1-D_5). VLC fractionation of Fr. D_3 (30 mg) on LiChroprep C_{18} (2.2 × 18 cm) using the same solvent system yielded compounds **10** (8 mg, eluted with 5% MeOH) and **2** (8 mg eluted with 20% MeOH). Fr. D_4 (115 mg) was chromatographed over polyamide (2.2 × 16 cm) eluting with $CHCl_3$-

MeOH-acetone-ethyl methyl ketone (3:1:0.05:0.05) to give compound **3** (9 mg). Fr. J (100% MeOH) (698 mg) was subjected to Sephadex LH-20 (2.2 × 18 cm) and eluting with MeOH yielded 5 fractions (Frs. J_{1-5}). Fr. J_1 (163 mg) was first subjected to a silica gel column (1.3 × 15 cm) with a gradient of $CHCl_3$-MeOH (99:1→95:5) to give 4 subfractions (Frs. J_{1A}-J_{1D}). Fr. J_{1B} was found to be compound **1** (1.8 mg, eluted with 5% MeOH) in pure form. VLC fractionation of Fr. J_{1C} (40 mg) on LiChroprep C_{18} (2.2 × 18 cm) using the MeOH-H_2O (20%→40%) system yielded compound **8** (2 mg, eluted with 35% MeOH). Fr. J_5 (103 mg) applied on VLC on LiChroprep C_{18} (2.2 × 18 cm) using the H_2O-MeOH (0%→100%) solvent system yielded 3 fractions (Frs. J_{3A}-J_{3C}). Fr. J_{3C} was found to be a mixture of compounds **4** and **5** (6 mg, eluted with 38% MeOH). J_{3A} (14 mg) was rechromatographed on Sephadex LH-20 (2.2 × 50 cm) using MeOH, to yield compounds **6** (5.1 mg) and **7** (3.8 mg).

Quercetin (1): $C_{15}H_{10}O_7$; UV λ_{max} (MeOH) nm: 254.0, 267.5 (sh), 298.0 (sh), 368.0. ^{1}H (400 MHz, MeOH-d_4) NMR data were identical to those reported in the literature.[11]

Quercetin 3-*O*-β-glucopyranoside (2): $C_{21}H_{20}O_{12}$; UV λ_{max} (MeOH) nm: 259.0, 270.0 (sh), 361.5. ^{1}H (400 MHz, MeOH-d_4) and ^{13}C (100 MHz, MeOH-d_4) NMR data were identical to those reported in the literature.[11]

Quercetin 3-*O*-β-galactopyranoside (3): $C_{21}H_{20}O_{12}$; UV λ_{max} (MeOH) nm: 255.5, 271 (sh), 303 (sh), 362.5. ^{1}H (400 MHz, MeOH-d_4) and ^{13}C (100 MHz, MeOH-d_4) NMR data were identical to those reported in the literature.[11]

Quercetin 3-*O*-(2″-*O*-galloyl)-β-glucopyranoside (4): $C_{28}H_{24}O_{16}$; ^{1}H (400 MHz, MeOH-d_4) and ^{13}C (100 MHz, MeOH-d_4) NMR data were identical to those reported in the literature.[12]

Quercetin 3-*O*-(2″-*O*-galloyl)-β-galactopyranoside (5): $C_{28}H_{24}O_{16}$; ^{1}H (400 MHz, MeOH-d_4) and ^{13}C (100 MHz, MeOH-d_4) NMR data were identical to those reported in the literature.[12]

Quercetin 3-*O*-(6″-*O*-galloyl)-β-glucopyranoside (6): $C_{28}H_{24}O_{16}$; UV λ_{max} (MeOH) nm: 258, 352. ^{1}H (400 MHz, MeOH-d_4) and ^{13}C (100 MHz, MeOH-d_4) NMR data are given in the Table.[12] $[M+H]^+$ 617.1096

Quercetin 3-*O*-(6″-*O*-galloyl)-β-galactopyranoside (7): $C_{28}H_{24}O_{16}$; UV λ_{max} (MeOH) nm: 254, 354 ^{1}H (400 MHz, MeOH-d_4) and ^{13}C (100 MHz, MeOH-d_4) NMR data are given in the Table.[12] $[M+H]^+$ 617.1099.

Quercetin 3-*O*-α-arabinofuranoside (8): $C_{20}H_{18}O_{11}$; UV λ_{max} (MeOH) nm: 255.5, 358.5. ^{1}H (500 MHz, DMSO-d_6) NMR data were identical to those reported in the literature.[11] ^{13}C-NMR (125 MHz, DMSO-d_6): 178.8 (C-4), 162.6 (C-7), 161.6 (C-5), 158.5 (C-9), 156.1 (C-2), 149.1 (C-4′), 145.6 (C-3′), 132.9 (C-3), 122.2 (C-6′), 120.9 (C-1′), 116.5 (C-5′), 115.8 (C-2′), 108.0 (C-1″), 104.5 (C-10), 99.1 (C-6), 94.2 (C-8), 86.2 (C-4″), 82.1 (C-2″), 76.8 (C-3″), 61.1 (C-5″).[11]

Quercetin 3-*O*-α-rhamnopyranosyl-(1→6)-β-glucopyranoside (9): $C_{27}H_{30}O_{16}$; UV λ_{max} (MeOH) nm: 258.0, 359.0. ^{1}H (400 MHz, CD_3OD) and ^{13}C (100 MHz, MeOH-d_4) NMR data were identical to those reported in the literature.[11]

Methyl gallate (10): $C_8H_9O_5$; ^{1}H (400 MHz, CD_3OD): 7.06 (2H, s) (H-2, H-6), 3.84 (3H, s) ($COOC\underline{H}_3$). ^{13}C-NMR (100 MHz, MeOH-d_4): 169.18 ($\underline{C}OOCH_3$), 146.66 (C-5), 146.67 (C-3), 140.01 (C-4), 121.52 (C-1), 110.16 (C-2, C-6), 52.40 ($COO\underline{C}H_3$).

Table. ^{1}H- and ^{13}C-NMR data for compounds **6** and **7** (^{1}H: 400 MHz, ^{13}C: 100 MHz, MeOH-d_4).

C/H		δ_H ppm, J Hz		δ_C ppm		HMBC (H→C)	
		6	**7**	**6**	**7**	**6**	**7**
2	C			159.51	159.09		
3	C			135.44	135.79		
4	C			179.53	179.63		
5	C			163.01	162.99		
6	CH	6.20 d 2.0	6.21 d 1.6	100.07	100.08	5, 7, 8, 10	5, 7, 8
7	C			166.04	166.14		
8	CH	6.36 d 2.0	6.40 d 1.6	94.98	94.97	6, 9, 10	6, 7, 9
9	C			158.51	158.51		
10	C			105.70	105.67		
1′	C			123.18	123.19		
2′	CH	7.56 d 2.0	7.81 d 1.6	116.08	117.89	1′, 3′	2, 1′, 3′, 4′
3′	C			145.93	145.88		
4′	C			149.85	150.08		
5′	CH	6.74 d 8.0	6.84 d 8.4	117.36	116.23	3′, 4′, 6′	3′, 4′
6′	CH	7.59 dd 2.0/8.0	7.58 dd 1.6/8.4	123.18	122.92	2′, 4′, 5′	2′, 4′
1″	CH	5.23 d 7.2	5.14 d 8.0	104.36	105.66	3	3
2″	CH	3.48-3.56 *	3.87 dd 8.0/9.0	75.82	73.21		
3″	CH	3.48-3.56 *	3.62 dd 2.6/9.0	78.18	75.11		
4″	CH	3.49 *	3.91 bd 2.6	71.61	70.20		
5″	CH	3.50 *	3.82 m	76.02	74.64		6″
6″a	CH_2	4.30 dd 1.6/12.0	4.36 dd 6.8/11.2	64.46	63.94	7‴	5″, 7‴
6″b		4.38 dd 4.8/12.0	4.22 dd 6.0/11.2				4″, 5″, 7‴
1‴	C			121.39	121.21		
2‴	CH	6.96 s	6.91 s	110.22	110.38	3‴, 4‴, 5‴	3‴, 4‴, 5‴
3‴	C			146.44	146.45		
4‴	C			139.87	139.90		
5‴	C			146.57	146.45		
6‴	CH	6.96 s	6.91 s	110.22	110.21	3‴, 4‴, 5‴	3‴, 4‴, 5‴
7‴(C=O)				168.37			

*Signal patterns unclear due to overlapping

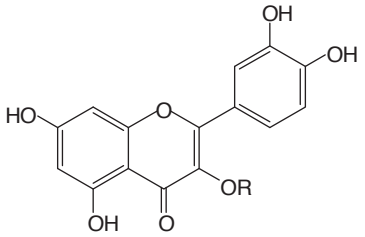
H_2O_2-induced lipid peroxidation inhibitor effect in human red blood cells: Blood samples were obtained from healthy volunteers and collected into heparinized tubes. The whole blood samples were centrifuged for 15 min at 3000 rpm at +4 °C. After removing the plasma and the buffy coat, red blood cells (RBCs) were washed in an equal volume of cold NaCl (0.155 mol/L) 3 times. Following the third saline wash, supernatants were removed and packed RBCs were obtained. When RBCs are treated with H_2O_2 at pH 7.4

in the presence of sodium azide, they undergo lipid peroxidation. This system[13] was used for estimation of antioxidant activity of the samples. First 0.8 mL of buffer (3.4 mM Na_2HPO_4- NaH_2PO_4/0.15 M NaCl/7.8 mM sodium azide (pH 7.4)) was mixed with 0.1 mL of RBCs and with different concentrations of the samples for 30 min. Then 0.8 mL of 10 mM H_2O_2 was added to the tubes and incubated for an additional 120 min. The reaction was stopped by the addition of 1 mL of 28% (w/v) trichloroacetic acid. Then 1 mL of the supernatant was incubated with 1 mL of 1% (w/v) thiobarbituric acid to measure malondialdehyde, an end product of lipid peroxidation, at 532 nm photometrically. Ascorbic acid and trolox were used as positive controls.[13]

Results and discussion

The H_2O_2-induced lipid peroxidation inhibitor effects of different extracts of *G. tuberosum* subsp. *tuberosum* were evaluated. The EtOAc extract was the most active one among the extracts tested. Therefore, further phytochemical studies were carried out on the EtOAc extract. Nine flavonoids (**1-9**) and a simple phenolic compound (**10**) were isolated by means of different chromatographic [(open (Sephadex LH-20, polyamide, silica gel) MPLC (C_{18} silica gel), and VLC (C_{18} silica gel) column] systems.

Quercetin (**1**), quercetin 3-*O*-β-glucopyranoside (**2**), quercetin 3-*O*-β-galactopyranoside (**3**), quercetin 3-*O*- (2″-*O*-galloyl)-β-glucopyranoside (**4**), quercetin 3-*O*-(2″-*O*-galloyl)-β-galactopyranoside (**5**), quercetin-3-*O* − α-arabinofuranoside (**8**), quercetin-3-*O*-rutinoside (**9**), (Figure 1) and methyl gallate (**10**) were identified by comparing their spectroscopic data with those reported in the literature.[11,12]



	R
1	H
2	β-glucopyranose
3	β-galactopyranose
4	(2″-*O*-galloyl)-β-glucopyranose
5	(2″-*O*-galloyl)-β-galactopyranose
6	(6″-*O*-galloyl)-β-glucopyranose
7	(6″-*O*-galloyl)-β-galactopyranose
8	α-arabinofuranose
9	α-rhamnopyranosyl-(1→6)-β-glucopyranose

Figure 1. Flavonoids isolated from *G. tuberosum* subsp. *tuberosum*.

Compounds **6** and **7** were obtained as yellow powders. UV spectra of **6** and **7** in MeOH showed λ_{max} of 258 and 352 nm for **6**, and 254 and 354 nm for **7**, suggesting that both **6** and **7** are flavonols. ESI-TOF-MS of

6 and **7** showed molecular ions at m/z617.1096 $[M+H]^+$ and 617.1099 $[M+H]^+$, respectively, corresponding to the formula $C_{28}H_{24}O_{16}$ (617.1143 calculated) for both. The ion peak at m/z 617 $[M+H]^+$yields product ions at m/z 303 corresponding to $[aglycone+H]^+$ and at m/z 315 corresponding to $[sugar+gallic\ acid+H]^+$ for both **6** and **7**. ^{1}H- and ^{13}C-NMR spectra of compound **6** (Table) showed signals belonging to the aromatic system and sugar moieties. The ^{1}H-NMR spectrum of **6** revealed 3 aromatic protons at δ_H 7.59 (dd J=2.0/8.0), 7.56 (d J=2.0), and 6.74 (d J=8.0), which were observed as an ABX system, suggesting the presence of an *o*-disubstituted B ring. Moreover, 2 *meta* coupled signals in the aromatic region at δ_H 6.20 (1H, d J=2.0, H-6) and 6.36 (1H, d J=2.0, H-8) were consistent with a 5, 7-dihydroxy substituted A ring of flavonoid. A singlet at δ_H 6.96 (2H) was attributed to the H-2‴ and H-6‴ of a gallic acid. The configuration of the anomeric proton of sugar moiety was proposed to be β on the basis of the coupling constant (7.2 Hz). The sugar was found to be a β-glucopyranose according to ^{1}H-NMR data and by comparison with the literature.[14] In the ^{13}C-NMR spectrum of **6** 28 carbon signals were shown. Fifteen carbon signals were assigned to quercetin moiety, 6 carbon signals were assigned to the glucose unit, and the remaining 7 carbon signals were assigned to a gallic acid moiety. The proton signals of H-6″a and H-6″b were shifted downfield approximately 0.70 ppm for H-6″a and 0.65 ppm for H-6″b. Moreover, the C-6″ signal was shifted downfield approximately 1.90 ppm compared to a non-galloylated analogue of quercetin 3-*O*-β-glucopyranoside **(2)**. The observed downfield shifts of H-6″a, H-6″b, and C-6″, proved that the gallic acid moiety was linked to the sugar moiety at C-6″. Moreover, the HMBC spectrum of **6** enabled us to establish the locations to be connected among the quercetin, glucopyranose, and gallic acid moieties of compound **6** on the basis of the cross peaks: one due to the coupling between H-1″ and C-3 and the other due to the coupling between H-6″a and C-7‴. After the complete interpretation of the NMR data based on the COSY, HMQC, and HMBC experiments, and comparing these data with those reported in the literature, compound **6** was determined to be quercetin 3-*O*-β-(6″-*O*-galloyl)-glucopyranoside.[15]

The ^{1}H- and ^{13}C-NMR spectra of compound **7** were similar those of **6**, except for the signals arising from the sugar moiety. The sugar was concluded to be β-galactopyranose based on the ^{1}H- and ^{13}C-NMR signals and the coupling constant of H-1″ (8.0 Hz)[14]. HMBC correlation between H-1″ and C-3 indicated the location of β-galactopyranose, which was attached to C-3 of the aglycone, while correlation between H-6″a and C-7‴ and H-6″b and C-7‴ suggested gallic acid moiety attached to β-galactopyranose from C-6″. From these results, the structure of compound **7** was identified as quercetin 3-*O*-β-(6″-*O*-galloyl)-galactopyranoside, which was confirmed by the comparison of its spectral data with those reported in the literature.[15] This is the first report on the presence of quercetin 3-*O*-(6″-*O*-galloyl)-β-glucopyranoside **(6)** and quercetin 3-*O*-(6″-*O*-galloyl)-β-galactopyranoside **(7)** in the genus *Geranium*.

All extracts showed a lipid peroxidation inhibitory effect and the EtOAc extract was the most active extract (Figures 2 and 3). In addition, the EtOAc extract was found to be more effective than its active components, indicating a possible synergistic interaction of the constituents. The galloyl flavonoids (**6** and **7**) showed strong inhibitor activity and their efficiency was noted to be higher than those of the corresponding non-galloylated flavonol glycosides.[16]

Oxidative stress is thought to play an important role in the etiology and pathogenesis of diabetes, arteriosclerosis, cardiovascular illnesses, cancer, and several neurodegenerative disorders. The chronic hyperglycemia leads to oxidative stress for all tissues because glucose in abnormally high concentrations forms oxygen species. This can lead to oxidative damage in the classical secondary targets of diabetes[17]. There are many studies

about the effect of antioxidants on oxidative stress in diabetes mellitus and it has been shown that antioxidants have a beneficial effect on oxidative stress[18]. The high antioxidant activity of the title plant supported the use of the plant as an antidiabetic in folk medicine.

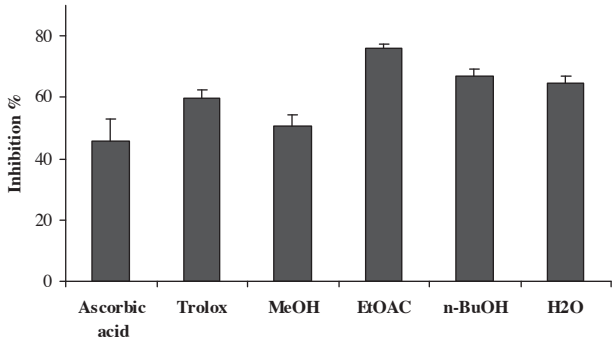


Figure 2. Inhibitory effect of different extracts (50 μg/mL) of *G. tuberosum* subsp. *tuberosum* on H_2O_2-induced lipid peroxidation in human red blood cells.

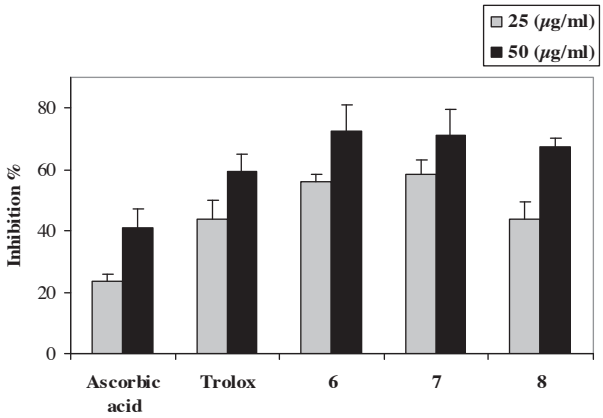


Figure 3. Inhibitory effect of compounds (**6**, **7**, **8**) isolated from *G. tuberosum* subsp. *tuberosum* on H_2O_2-induced lipid peroxidation in human red blood cells.

Acknowledgments

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