

Polyphenolic Constituents and Antioxidant Potential of *Geranium stepporum* Davis

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Abstract: The crude MeOH extract prepared from the aerial parts of *G. stepporum* and its EtOAc, *n*-BuOH, H₂O subextracts were screened for their inhibitory activity on H₂O₂-induced lipid peroxidation in human red blood cells. The EtOAc subextract was found to be the most active one and its chemical composition was further analysed. Six polyphenolic secondary metabolites including quercetin (**1**), rutin (**2**), methyl gallate (**3**), pusilagin (**4**), 1,3,6-tri-*O*-galloyl- β -glucospyranose (**5**) and 1,2,3,4,6-penta-*O*-galloyl- β -glucopyranose (**6**) were isolated from the EtOAc extract by successive chromatographic methods (Sephadex CC, silica gel CC and P-TLC). The structures of the isolates were elucidated by 1D- and 2D-NMR and MS. This is the first report of occurrence of hydrolysable tannins in *G. stepporum*.

Keywords: *Geranium stepporum*; Geraniaceae; galloylglucopyranose; hydrolysable tannin; flavonoid; antioxidant effect.

1. Introduction

The genus *Geranium* L. (Geraniaceae) comprises almost 400 species in temperate areas and tropical mountains throughout the world [1]. It is represented by 35 species in the flora of Turkey [2,3]. Some *Geranium* species are used in traditional medicine as antidiabetic, hemostatic, antihemorrhoidal, antidiarrheic and in treatment of tonsillitis, cough, whooping cough, urticaria, dysentery, kidney pain, and gastrointestinal ailments [4-6]. In previous studies, different types of

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compounds, such as tannins [5,6], flavonoids [7], lignans [8] as well as essential oils [9] have been isolated from *Geranium* species. In our preliminary study, we reported the isolation of some galloylated flavonoid glycosides from the title plant [7]. However, there is no biological activity studies on *Geranium stepporum*. As a continuation of this work, we now report the H₂O₂-induced lipid peroxidation inhibitory effect of different extracts of *G. stepporum* as well as the isolation and structure elucidation of secondary metabolites from the most active EtOAc extract.

2. Materials and Methods

2.1. General Experimental Procedures :

The UV spectra were recorded in MeOH using an Agilent 8453 spectrometer. 1D- and 2D-NMR measurements were recorded in MeOH-*d*₄ at room temperature on a Bruker DRX 400 (¹H 400 and ¹³C 100 MHz) spectrometer. Chemical shifts were given in ppm with tetramethylsilane (TMS) as an internal standard. HR-MS was performed on a Micromass Q-TOF Micro instrument (ESI ionisation). Sephadex LH-20 (Pharmacia) and Kieselgel 60 (Merck, 0.063-0.200 mm) were used for column chromatography (CC). Preparative TLC (P-TLC) was carried out on Kieselgel GF₂₅₄ glass plates (Analtech- Uniplate 81300). Qualitative TLC analyses were carried out on pre-coated Kieselgel 60 F₂₅₄ aluminum plates (Merck). Compounds were detected by UV fluorescence and spraying 1% vanillin/H₂SO₄ or 3% FeCl₃ followed by heating at 100 °C.

2.2. Plant Material

Geranium stepporum DAVIS was collected from Kayseri, Pınarbaşı, Eğrisöğüt, Turkey, in May 2006. A voucher specimen has been deposited at the Herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 06001).

2.3. Extraction and Isolation:

Powdered aerial parts of *Geranium stepporum* (270 g) were extracted with MeOH for four times at 35 °C, and then filtered and combined MeOH extracts were concentrated under reduced pressure. The resultant extract was then dissolved in H₂O and water-soluble portion was partitioned with petroleum ether (40-60 °C) (4x 100 mL), EtOAc (6x 100 mL) and *n*-BuOH (4x 100 mL), respectively. A part of EtOAc extract (20 g) was chromatographed over Sephadex LH-20 (3.2 x 55 cm), eluting with H₂O, followed by increasing concentrations of MeOH in H₂O (0%→100% MeOH) to yield 10 fractions (Fr. A-J). Fr. B (860 mg) was subjected to Sephadex LH 20 using with MeOH to afford 3 fractions (Frs B₁-B₃). Fr. B₂ (120 mg) was first applied to silica gel CC with CH₃Cl₃/MeOH (80:20→60:40) mixture, and yielded Fr. B_{2A}, and compound **2** (4 mg). Fr. B_{2A} (25 mg) was then purified by P-TLC using CH₃Cl₃/MeOH/H₂O (70:30:3) mobile phase, and yielded compound **3** (6 mg). CC fractionation of Fr. C (890 mg) on Sephadex LH 20 CC with MeOH as eluent gave compound **4** (21 mg). Fr H (290 mg) was chromatographed over Sephadex LH 20 CC with MeOH to give compound **5** (11 mg) in pure form. Fr I (240 mg) was applied to Sephadex LH 20 CC, eluting with MeOH, to give Frs. I₁-I₃ and compound **6** (12 mg). Fr I₂ was then purified on a silica gel column, eluting with CH₃Cl₃-MeOH (99:2→92:8) mixture, and yielded compound **1** (3.5 mg).

2.4. H₂O₂-induced lipid peroxidation inhibitor effect in human red blood cells:

Red blood cells (RBCs) obtained from healthy volunteers, were treated with H₂O₂ at pH 7.4 in the presence of sodium azide. Thus, they undergo lipid peroxidation. 0.8mL of buffer (3.4 mM Na₂HPO₄- NaH₂PO₄/0.15 M NaCl/7.8 mM sodium azide (pH 7.4)) was mixed with 0.1 mL of RBCs

and the samples for 30 minutes. 0.8 mL 10 mM H₂O₂ was added to the tubes and incubated for an additional 120 min. Reaction was stopped by addition of 1mL of 28% (w/v) trichloroacetic acid. 1mL of the supernatant was incubated with 1mL of 1% (w/v) thiobarbituric acid to measure malondialdehyde, an end product of lipid peroxidation, at 532 nm photometrically [10]. Ascorbic acid and trolox were used as positive controls.

2.5. Statistical analysis

Student t-test was performed as a nonparametric test to evaluate the significance of the differences between groups, and $p < 0.05$ was accepted as significant.

3. Results and Discussion

Different extracts prepared from the aerial parts of *G. stepporum* were evaluated for their H₂O₂-induced lipid peroxidation inhibitory effect in human red blood cells. Among the tested extracts, the EtOAc extract displayed higher inhibitory effect. Further phytochemical studies on the EtOAc extract, led to the isolation of six polyphenolic compounds (**1-6**) by using different chromatographic methods (Sephadex CC, silica gel CC and P-TLC)

Compound **5** and **6** were obtained as amorphous powders. Initial definition of compounds **5** and **6** as ellagitannins clearly followed from their visualisation on silica gel plates upon treatment with FeCl₃ (dark blue), and vanillin/H₂SO₄ (pink). Diagnostic features in the ¹H NMR spectra of this group of hydrolysable tannins were the presence of a two proton singlet at ca δ_H 7.1 for the magnetically equivalent 2- and 6- protons of a galloyl group in each instance, and aliphatic proton signals attributable to a sugar moiety (Table 1). The molecular formula of **5** was determined as C₂₇H₂₅O₁₈, [M+H]⁺ by positive-ion ESI-TOF-MS ([M+H]⁺ at 637.1045). In the ¹H NMR spectra of **5**, three two-proton singlets due to galloyl groups appeared in the aromatic region, and seven signals arising from a sugar moiety were observed in the aliphatic region (Table). The anomeric proton resonance at δ_H 5.86 and the signals in the region of δ_H 5.32-3.80 together with the corresponding C resonances in the ¹³C-NMR spectrum (Table 1) indicated the presence of glucopyranose unit in the structure of **5** [11]. The configuration of the glucopyranose unit was determined to be β on the basis of coupling constant of anomeric proton ($J=8.0$ Hz). The complete assignments of **5** were made by 2D-NMR experiments (DQF-COSY, HMQC, and HMBC). In the sugar region, ¹³C resonances of C-1, C-3 and C-6 appeared to be significantly shifted to a lower field comparing those of non-substituted glucose. These findings clearly indicated that the acylation sites of the glucose core were C-1, C-3 and C-6. HMBC correlations between glucose protons and the carbonyl group of gallic acids H-1 and C-A7 (δ_C 166.97), H-3 and C-B7 (δ_C 168.26) and H-6 and C-C7 (δ_C 168.36) demonstrated that gallic acid units were attached to C-1, C-3 and C-6 of the glucose unit. Detailed analysis of the 1D- and 2D-NMR data revealed the structure of **5** as 1,3,6-tri-*O*-galloyl- β -glucose [12].

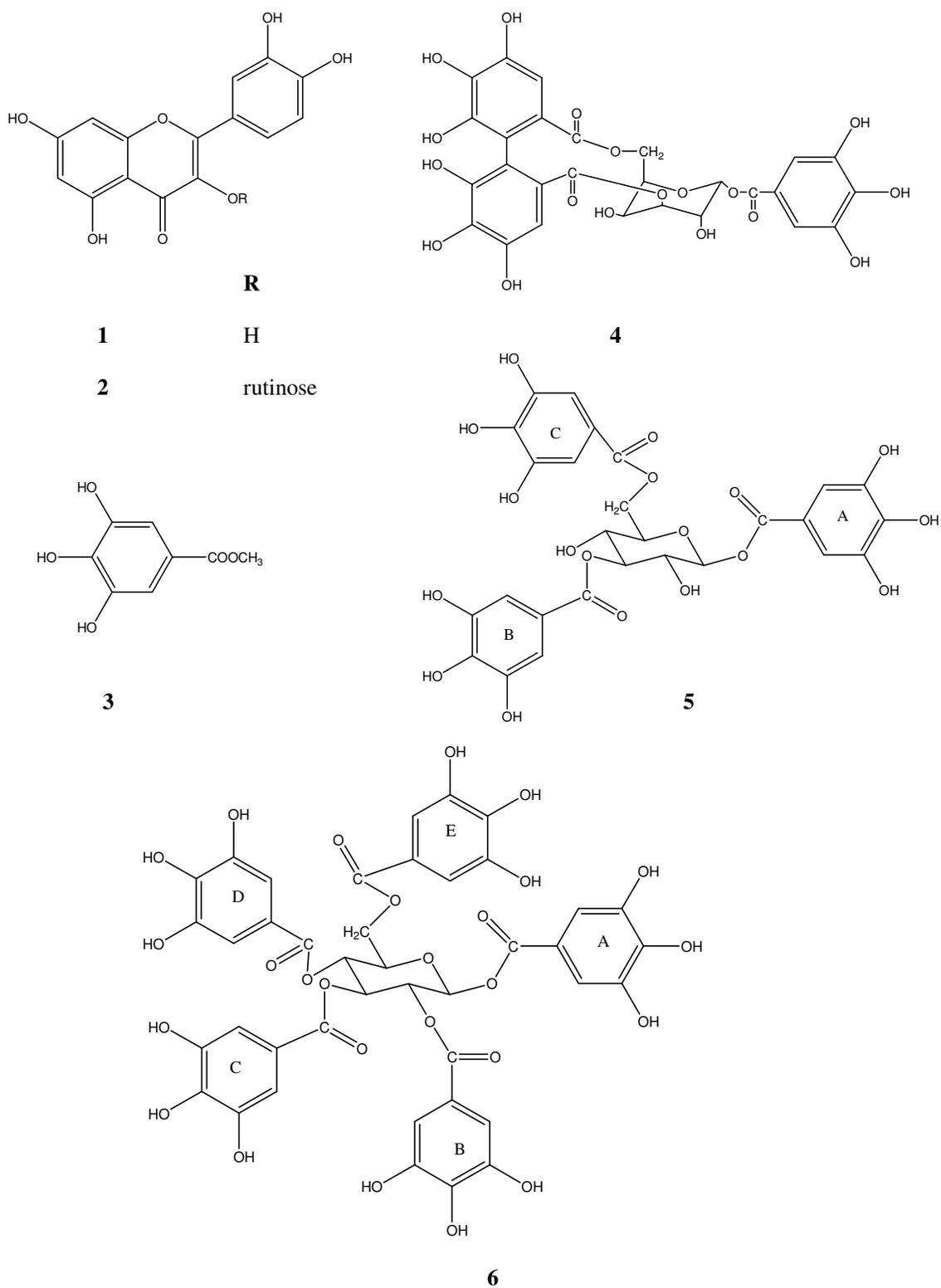


Figure 1. Compounds isolated from *Geranium stepporum*

Table 1. ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data of compound **5** and **6**; (δ in ppm from TMS, J in Hz, in CD_3OD)

Atom	5		6	
glucose	δ H	δ C	δ H	δ C
1	5.86 <i>d</i> (8.0)	96.02	6.23 <i>d</i> (8.0)	93.94
2	3.78-3.82 Φ	72.77	5.62 <i>dd</i> (8.0/9.5)	72.32
3	5.32 <i>t</i> (9.2)	79.07	5.90 <i>t</i> (9.5)	74.23
4	3.80-3.85 Φ	69.85	5.58 <i>t</i> (9.5)	69.93
5	3.91 <i>dd</i> (2.0/4.8)	76.56	4.51 <i>d</i> (10.5)	74.53
6a	4.61 <i>dd</i> (2.0/12.0)	64.36	4.40 <i>m</i>	63.23
6b	4.80 <i>dd</i> (4.8/12.0)		4.55 <i>dd</i> (4.8/10.5)	
Galloyl ring A				
1		121.76		119.84
2,6	7.17 <i>s</i>	110.55	7.99 <i>s</i>	110.76
3,5		146.56		146.66
4		140.60		140.89
7		166.97		166.35
Galloyl ring B				
1		120.63		120.47
2,6	7.12 <i>s</i>	110.35	7.01 <i>s</i>	110.60
3,5		146.66		146.49
4		140.04		140.54
7		168.26		167.15
Galloyl ring C				
1		121.41		120.32
2,6	7.19 <i>s</i>	110.74	6.99 <i>s</i>	110.51
3,5		146.57		146.54
4		139.92		140.48
7		168.36		167.43
Galloyl ring D				
1				120.35
2,6			7.94 <i>s</i>	110.54
3,5				146.39
4				140.25
7				167.05
Galloyl ring E				
1				121.16
2,6			7.14 <i>s</i>	110.47
3,5				146.57
4				140.12
7				168.06

Φ Signal patterns unclear due to overlapping

The ESI-TOF-MS of compound **6** showed the $[\text{M}+\text{H}]^+$ peak at m/z 941.1255, corresponding to the molecular formula $\text{C}_{41}\text{H}_{33}\text{O}_{26}$. Comparison of the ^1H NMR data for compound **6** with those of compound **5** revealed its close structural resemblance to this compound. Notable differences in the ^1H NMR spectra were the presence of two additional two-proton singlets arising from the galloyl units in the aromatic region. The chemical shifts and coupling patterns of the sugar protons and corresponding carbon resonances were assigned by ^1H - ^1H COSY and HMQC were typical of a glucopyranose residue. Noteworthy all proton and carbon resonances of the glucose core were shifted to the low field,

suggesting the acylation of glucose in each OH groups. The complete assignments of **6** were made by 2D-NMR experiments (DQF-COSY, HMQC, and HMBC) and the structure of **6** was identified as 1,2,3,4,6-penta-*O*-galloyl- β -glucose [13].

Quercetin [14] (**1**), rutin [14] (**2**), methyl gallate [13] (**3**), and pusilagin [15] (**4**), were also isolated and identified by comparison of their spectroscopic (UV, TOF-ESI-MS, 1D- and 2D-NMR) data with those published in the literature. Present study is the first example of the occurrence of a hydrolysable tannins in *Geranium stepporum*.

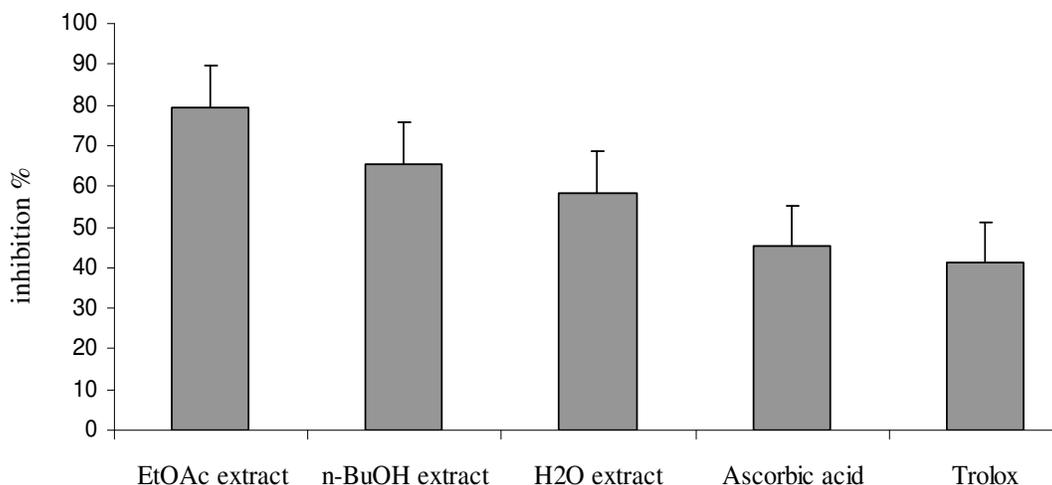


Figure 2. Inhibitory effects of different extracts of *G. stepporum* on H_2O_2 -induced lipid peroxidation in human red blood cells (50 μ g/mL)

Figure 2 shows the inhibitory effects of different extracts of *G. stepporum* on H_2O_2 -induced lipid peroxidation in human red blood cells at 50 μ g/mL. All extracts showed lipid peroxidation inhibitory effect, and the EtOAc extract was the most potent one. All extracts were possessed higher antioxidant effect than those of ascorbic acid and trolox which were used as reference substances.

Lipid peroxidation is a marker of oxidative stress and also one of the prime factors involved in cellular damage caused by free radicals. It has been implicated in a number of deleterious effects such as increased membrane rigidity, osmotic fragility, decreased cellular deformability, reduced erythrocyte survival, lipid fluidity, neurodegenerative disorders, the promotion and progression stages of carcinogenesis and development of atherosclerosis [16, 17]. Moreover, H_2O_2 could traverse membranes and exerts cytotoxic effects on cells in the proximity of those responsible for its production [18]. Polyphenolic compounds have an important role in stabilising lipid peroxidation, as they are potential antioxidants [19]. The results demonstrated in this study shown that the EtOAc, *n*-BuOH and H_2O extracts of *G. stepporum* inhibited the lipid peroxidation. Therefore, it can be concluded that EtOAc, *n*-BuOH and H_2O extracts prepared from *G. stepporum* can be used for antioxidative purposes. The use of naturally occurring phenolic compounds may provide new strategies to protect or halt oxidative stress associated diseases.

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