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From the aerial parts of *Geranium pyrenaicum* Burm., a new flavonol glycoside, kaempferol 3-*O*-(2'',3''-di-*O*-galloyl)- β -D-glucopyranoside (**7**), along with 6 known related glycosides [kaempferol 3-*O*- β -D-glucopyranoside (**1**), quercetin 3-*O*- β -D-glucopyranoside (**2**), quercetin 3-*O*- β -D-galactopyranoside (**3**), kaempferol 3-*O*-(2''-*O*-galloyl)- β -D-glucopyranoside (**4**), quercetin 3-*O*-(2''-*O*-galloyl)- β -D-glucopyranoside (**5**) and quercetin 3-*O*-(2'',3''-di-*O*-galloyl)- β -D-glucopyranoside (**6**)] and the ellagitannins corilagin (**8**) and tellimagrandin I (**9**) were isolated. Their structures were established from spectroscopic studies (UV, FAB-MS, ¹H and ¹³C-NMR, ¹H-¹H COSY, HETCOR, HMBC). Compounds **1-7** were evaluated for antileishmanial activity against a panel of *Leishmania* species (*L. major*, *L. donovani* and *L. amazonensis*), indicating appreciable in vitro activities (IC₅₀ 4-27 nM) when compared with Pentostam (IC₅₀ 10-11 nM).

Key Words: *Geranium pyrenaicum*, Geraniaceae, Flavonoids, Ellagitannins, Antileishmanial activity.

Introduction

The genus *Geranium* (Geraniaceae), which includes about 400 species distributed throughout the temperate regions, is chemically characterized by the presence of tannins, flavonoids, anthocyanins and essential oils¹. The aerial parts of some *Geranium* species, known as "Turnagagasi", have been employed as a tonic, diuretic, antidiabetic, antidiarrheal and antihemorrhoidal as well as a remedy to treat gastric disorders and to promote the healing of wounds². Despite these medicinal facets, detailed studies on the polyphenolic constituents of *Geranium* species are limited. Among the traditionally used *Geranium* species² *G. pyrenaicum* Burm. is widespread in the central parts of Turkey³. No chemical investigation on this species has so far been

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reported. This paper reports on the isolation and structure elucidation of a series of flavonoids (**1-7**) (Figure 1), including a new (**7**) and a rarely found analogue (**6**), associated with the known ellagitannins corilagin (**8**) and tellimagrandin I (**9**) from this plant source.

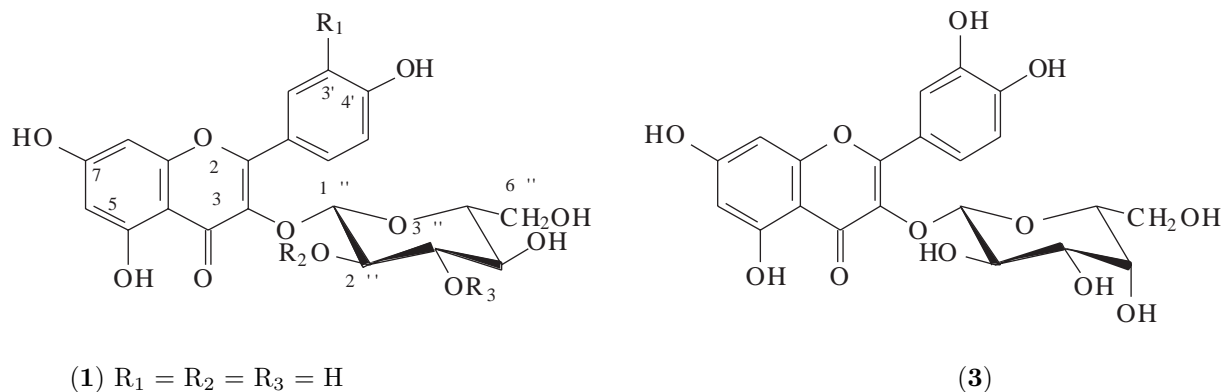


Figure 1. Isolated compounds **1-7**.

Leishmaniasis is a major health problem in most developing countries, but it is beginning to have a major impact on human populations in the developed world as well. In Turkey, cutaneous leishmaniasis and sporadic cases of the visceral form are reported^{4,5}. Continuing our research program to identify new antileishmanial candidates, compounds **1-7** were tested for activity against a panel of *Leishmania* parasites.

Experimental

General Experimental Procedures: 1H and ^{13}C NMR spectra were recorded on a Bruker DPX-400 instrument and chemical shifts are given in δ (ppm) relative to $(Me)_4Si$. HMBC experiments were optimized for $^{2-3}J_{H/C} = 10$ Hz. EI- and FAB-mass spectra were obtained on a Varian MAT CH₇A and Finnigan MAT CH₅DF mass spectrometer, respectively. UV spectra were recorded on a Shimadzu UV160-A model. For prep. TLC, glass supported silica gel plates (0.25 mm layer, 60 F₂₅₄, Merck) were used and Sephadex LH-20 (Fluka) was employed for column chromatography. Compounds were visualized by exposure to UV (254 nm) and then spraying with $FeCl_3$ and Naturstoffreagenz A.

Plant Material: The aerial parts of *Geranium pyrenaicum* were collected from Kızılcahamam, near Ankara, Turkey, in June 2000. A voucher specimen has been deposited in the Herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara (HUEF 00170).

Extraction and Isolation: The dried and powdered aerial parts of *G. pyrenaicum* (800 g) were defatted with petroleum ether (40-60 °C) (2.5 L x 3) and then extracted with 20% aqueous methanol (2 L x 3). The combined extracts were reduced in volume and then successively treated with *n*-hexane (200 mL x 4), EtOAc (200 mL x 4) and 1-BuOH (200 mL x 4).

The ethyl acetate soluble portion (4 g) was initially chromatographed on Sephadex LH-20 (3 x 50 cm) with gradient solvent systems of MeOH-H₂O (1:9→1:0) to afford 26 crude subfractions (E 1-E 26). Following qualitative TLC analysis on silica gel (EtOAc:formic acid:H₂O; 18:1:1) appropriate fractions (20 mL) were combined and further resolved by preparative TLC.

Preparative TLC separation of the subfractions E 11 (10% MeOH; 30 mg), E 14 (15% MeOH; 50 mg), E 21 (40% MeOH; 40 mg) and E 24 (60% MeOH; 110 mg) was performed using EtOAc-MeOH-H₂O-HOAc (17:1:0.5:1.5) as a developing system to yield compounds **2** (6 mg) and **3** (12 mg), **5** (23 mg), **8** (2 mg), and **9** (35 mg), respectively. Preparative TLC purification of E 12 (10% MeOH; 240 mg) with CHCl₃-MeOH-H₂O (65:25:4) afforded compound **1** (15 mg), while E 20 (25% MeOH; 90 mg) using the same mobile system gave compound **4** (28 mg). Similar preparative TLC purification of E 25 (60% MeOH; 300 mg) using CHCl₃-MeOH-H₂O (61:32:7) as the mobile phase afforded compounds **6** (77 mg) and **7** (25 mg).

Quercetin 3-O-(2'', 3''-di-O-galloyl)-β-D-glucopyranoside (6): Amorphous powder. R_f 0.67; CHCl₃-MeOH-H₂O (61:32:7); pos. FAB-MS: *m/z* 791 [M+Na]⁺; 769 [M+H]⁺; 616 [M-Gallic acid+H]⁺; 463 [M-Gallic acid-Gallic acid+H]⁺; 303 [M-Gallic acid-Gallic acid-Glucose+H]⁺. ¹H NMR (CD₃OD): 3.49 (*ddd*, *J* = 2.0, 5.0, 9.4; H-5''), 3.69 (*dd*, *J* = 5.0, 12.0; H_a-6''), 3.80 (*dd*, *J* = 9.0, 9.4; H-4''), 3.84 (*dd*, *J* = 2.0, 12.0; H_b-6''), 5.35 (*dd*, *J* = 7.3, 9.50; H-2''), 5.38 (*dd*, *J* = 9.0, 9.5; H-3''), 5.77 (*d*, *J* = 7.3; H-1''), 6.10 (*d*, *J* = 1.9; H-6), 6.24 (*d*, *J* = 1.9; H-8), 6.77 (*d*, *J* = 8.5; H-5'), 6.96 (*s*, H-2'''), 6.96 (*s*, H-6'''), 7.00 (*s*, H-2'''), 7.00 (*s*, H-6'''), 7.45 (*dd*, *J* = 2.0, 8.5; H-6'), 7.50 (*d*, *J* = 2.0; H-2'). ¹³C NMR: 62.21 (C-6''), 69.64 (C-4''), 74.07 (C-2''), 77.11 (C-3''), 78.75 (C-5''), 95.23 (C-8), 100.65 (C-6), 100.65 (C-1''), 105.20 (C-10), 110.44 (C-2'''), 110.44 (C-6'''), 110.60 (C-2'''), 110.60 (C-6'''), 116.27 (C-5'), 116.94 (C-2'), 120.64 (C-1'''), 120.91 (C-1'''), 122.99 (C-1'), 123.35 (C-6'), 134.92 (C-3), 140.31 (C-4'''), 140.40 (C-4'''), 146.20 (C-3'), 146.33 (C-3'''), 146.33 (C-5'''), 146.42 (C-3'''), 146.42 (C-5'''), 150.03 (C-4'), 158.02 (C-2), 158.56 (C-9), 163.09 (C-5), 167.34 (C-7), 167.96 (C-7'''), 167.96 (C-7'''), 178.86 (C-4).

Kaempferol 3-O-(2'', 3''-di-O-galloyl)-β-D-glucopyranoside (7): Amorphous powder. R_f 0.71; CHCl₃-MeOH-H₂O (61:32:7); UV λ_{max} (MeOH) nm: 269, 292(sh); pos. FAB-MS: *m/z* 775 [M+Na]⁺; 753 [M+H]⁺; 601 [M-Gallic acid+H]⁺; 447 [M-Gallic acid-Gallic acid+H]⁺; 287 [M-Gallic acid-Gallic acid-Glucose+H]⁺. ¹H NMR (CD₃OD): 3.47 (*ddd*, *J* = 2.0, 5.0, 9.5; H-5''), 3.66 (*dd*, *J* = 5.0, 12.0; H_a-6''), 3.77 (*dd*, *J* = 9.0, 9.5; H-4''), 3.81 (*dd*, *J* = 2.0, 12.0; H_b-6''), 5.31 (*dd*, *J* = 7.5, 9.5; H-2''), 5.36 (*dd*, *J* = 9.0, 9.50; H-3''), 5.74 (*d*, *J* = 7.5; H-1''), 6.08 (*d*, *J* = 1.9; H-6), 6.22 (*d*, *J* = 1.9; H-8), 6.80 (*d*, *J* = 8.8; H-3'), 6.80 (*d*, *J* = 8.8; H-5'), 6.96 (*s*, H-2'''), 6.96 (*s*, H-6'''), 7.00 (*s*, H-2'''), 7.00 (*s*, H-6'''), 7.87 (*d*, *J* = 8.8; H-2'), 7.87 (*d*, *J* = 8.8; H-6'). ¹³C NMR: 60.13 (C-6''), 71.86 (C-4''), 74.76 (C-2''), 77.32 (C-3''), 79.06 (C-5''), 94.02 (C-8), 97.80 (C-1''), 99.77 (C-6), 102.36 (C-10), 108.56 (C-2'''), 108.56 (C-6'''), 108.66 (C-2'''), 108.66 (C-6'''), 115.17 (C-3'), 115.17 (C-5'), 117.49 (C-1'''), 118.09 (C-1'''), 120.53 (C-1'), 130.60 (C-2'), 130.60 (C-6'), 131.96 (C-3), 139.51 (C-4'''), 139.91 (C-4'''), 145.39 (C-3'''), 145.39 (C-5'''), 145.39 (C-3'''), 145.39 (C-5'''), 155.25 (C-2), 156.54 (C-9), 160.11 (C-4'), 160.92 (C-5), 164.82 (C-7), 164.82 (C-7'''), 165.11 (C-7'''), 176.19 (C-4).

Assay for leishmanicidal activity: Assays for leishmanicidal activity and cytokine (TNF, IFN-γ and IL-6) inducing potentials were performed as described previously^{6,7}.

For testing the immunomodulatory activities of the compounds, murine macrophage-like RAW246.7 cells were infected with *L. major*, *L. donovani* and *L. amazonensis*. Then the cells were treated with the

compounds. After lysis of the cells, survival of leishmania was detected. In the following step, 5×10^6 cells (infected and non-infected control) were treated with the compounds in their IC_{50} concentration, and after 24 h the supernatants were collected for cytokine measurement using cell lines cytokine-sensitive (L929-TNF, L929-IFN and B9-IL6) and ELISA specific for IFN- α and IFN- γ . Direct toxicity against promastigote leishmania and RAW cells was observed in parallel. Pentostam® was used for comparison as a standard drug of antileishmanial activity^{6,7}.

Results and Discussion

An aqueous methanol extract of the dried aerial parts of *G. pyrenaicum* was successively treated with *n*-hexane, ethyl acetate and 1-butanol. The ethyl acetate soluble portion was chromatographed on Sephadex LH-20 using a water-methanol gradient system. Subsequent preparative TLC separations afforded a series of flavonoid glycosides (**1-7**) along with the ellagitannins corilagin (**8**)⁸ and tellimagrandin I (**9**)⁹. Known compounds amongst the flavonoid metabolites included kaempferol 3-*O*- β -D-glucopyranoside (**1**)¹⁰, quercetin 3-*O*- β -D-glucopyranoside (**2**)¹⁰, quercetin 3-*O*- β -D-galactopyranoside (**3**)¹¹, kaempferol 3-*O*-(2''-*O*-galloyl)- β -D-glucopyranoside (**4**)¹², quercetin 3-*O*-(2''-*O*-galloyl)- β -D-glucopyranoside (**5**)¹³, and quercetin 3-*O*-(2'', 3''-di-*O*-galloyl)- β -D-glucopyranoside (**6**)¹⁴, which were readily identified by comparison of the physical properties with those reported in the literature^{13,14}. Noteworthy is the fact that compound **6** represents a rarely found metabolite, its occurrence being hitherto confined to a single plant source, *Euphorbia maculata* L.¹⁴. Since the NMR spectral data of **6** were previously recorded in acetone- d_4 /D₂O, these are included for comparative purposes (see Experimental).

Compound **7**, a yellow amorphous powder, exhibited a pseudomolecular ion peak at m/z 753 in the positive FAB mass spectrum, consistent with a molecular formula of C₃₅H₂₈O₁₉, and the sodium adduct [M+Na]⁺ peak at m/z 775. The ion peak at m/z 775 yields a product ion at m/z 601 corresponding to cleavage of a single gallic acid bond and an ion peak at m/z 447 corresponding to cleavage of 2 gallic acid moieties. Subsequently an ion peak of m/z 287 corresponds to a loss of 2 gallic acids and glucose moieties. Analysis of the ¹H NMR spectrum of **7** revealed the presence of *meta*-coupling protons (δ 6.08 and 6.22, each d, $J=1.9$ Hz, H-6 and H-8, respectively) and an AA'BB'-spin system for the aromatic A- and B-ring protons (δ 6.80 and 7.87), respectively¹⁰. The ¹H NMR spectrum also displayed an anomeric proton signal at δ 5.74 arising from a β -D-glucopyranosyl moiety ($J_1''2'' = 7.5$ Hz). The anomeric proton of glucose (δ 5.74) was seen further downfield than that of kaempferol 3-*O*- β -D-glucopyranoside (δ 5.22) because of the esterification of glucose moiety¹⁵. Notable differences included the appearance of 2 sharp low-field 2-proton singlets at δ 6.96 and 7.00 for the magnetically equivalent 2- and 6-protons of 2 galloyl groups and the conspicuous deshielding of 2''-H and 3''-H of the glycosyl residue ($\Delta\delta$ 1.86 and 1.95, respectively) relative to their chemical shifts in the non-galloylated analogue of kaempferol 3-*O*- β -D-glucopyranoside (**1**), clearly indicating that the hydroxyl groups at these positions were acylated. Independent support for the location of the galloyl groups at C-2 and C-3 of the carbohydrate moiety was also available from a comparison of the ¹³C NMR data of **7** with those of **1** and its monogalloylated analogue **4**. The position of sugar residue and galloyl moieties were unambiguously determined by the HMBC experiment (Figure 2). Long-range correlations observed in the HMBC spectrum from H-1'' to C-3, and from H-2'' and H-3'' to the carbonyl carbons at δ 164.82 and δ 165.11, respectively, established the points of attachment of both galloyl moieties

to the carbohydrate residue. These spectral features defined compound **7** as a new kaempferol 3-*O*-(2'',3''-di-*O*-galloyl)- β -D-glucopyranoside.

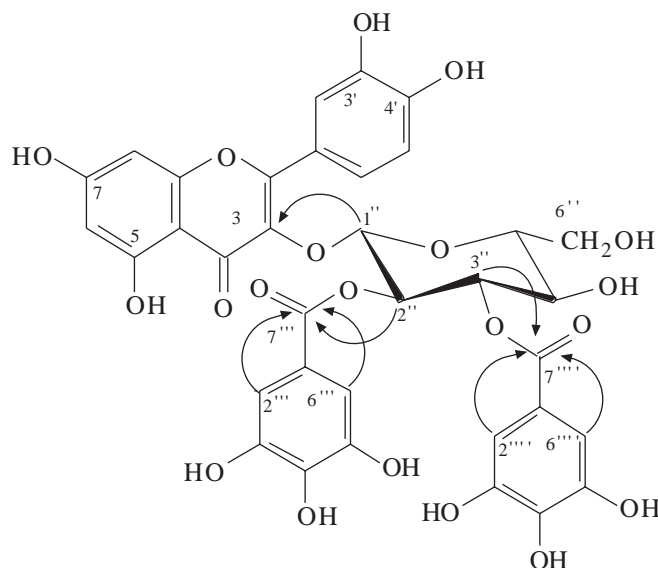


Figure 2. Selected HMBC correlations for compound **7**. Arrows point from H to C.

The characterization of compound **7** not only extends the range of natural *O*-galloylflavonoids but also introduces the first example of a 2'',3''-diacylated kaempferol derivative in this group of secondary products.

Antileishmanial Activity: The antileishmanial activity of compounds **1-7** was assessed against both extracellular promastigotes and intracellular amastigotes of a panel of *Leishmania* species (Table), with *L. major* and *L. donovani* as causative agents of Old World cutaneous and visceral leishmaniasis, respectively. As a parameter for antileishmanial activity, the IC₅₀ value, i.e. the sample concentration causing a 50% reduction in survival/viability of the parasites, was used. None of the samples showed selective toxicity when tested against the promastigote stages of the 3 *Leishmania* species (IC₅₀ > 0.4 μ M). In contrast, all compounds tested reduced the intracellular survival of *Leishmania* amastigotes within RAW 264.7 cells (IC₅₀ 4–27 nM) in our in vitro experiments, when compared with the IC₅₀ values, 10.1–10.6 nM, of the therapeutically used antileishmanial drug Pentostam®. Notably, all samples appeared to be non-toxic against the host cells (IC₅₀ > 0.5 μ M).

Concerning structure-activity relationships, the presence of galloyl groups dramatically decreased the antileishmanial potency, at least in the series of quercetin derivatives (**2**, **3**, **5**, and **6**). For the kaempferol analogues (**1**, **4**, and **7**), no clear correlations were obvious. This phenomenon cannot be satisfactorily explained at present.

Tumor necrosis factor (TNF)- α , produced principally by activated macrophages and monocytes, plays a crucial role in the host defense against various pathogens¹⁶. As shown in the Table, the amounts of TNF induced by the tested flavonoids **1-7** in non-parasitized RAW 264.7 were below the detection limit. In contrast, all samples induced moderate (9-90 U/mL for **2-6**) or significant TNF release (204-270 U/mL for **1**) in *Leishmania*-parasitized RAW 264.7 cells compared to IFN- γ + LPS as a positive control (197-210 U/mL). The considerable TNF-inducing potential of **1** cannot be explained on the basis of structure-activity relationships. Importantly, all test compounds were first subjected to assays for endotoxin contamination

(*Limulus* amoebocyte lysate method), which may stimulate immune cells, of which we found no evidence.

Macrophage functions are intimately related to the IFN system producing not only IFN- α but also, at least under certain conditions, IFN- γ ¹⁷. Accordingly, attention was given to possible IFN-like activities induced by flavonoid glycosides in RAW cells. For this, supernatants of sample-activated RAW cells were analyzed for their capacity to protect L929 cells from the cytopathic effect of encephalomyocarditis virus (EMCV). The relative number of protected, i.e. viable cells, was determined spectrophotometrically using crystal violet as staining reagent. Medium alone served as a negative and a recombinant murine IFN- γ (100 U/mL) as a positive control for cytoprotection. The IFN-like activities found in supernatants of sample-treated infected RAW cells ranged from negligible to moderate (4-42 U/mL) (Table).

Keeping in mind the essential role of IL-6 in the proliferation and differentiation of immune cells¹⁸, this factor was assessed in a functional bioassay employing the selective dependency of murine B9 hybridoma cells on IL-6 for survival and proliferation¹⁹. Appropriate controls were performed using medium alone for minimal and a defined laboratory IL-6 standard (70 U/mL) for maximal cell proliferation. The results listed in the Table show that all compounds stimulated RAW 264.7 cells to some extent for IL-6 release. Of the series of flavonoid glycosides tested, compound **1** activated *Leishmania*-parasitized RAW 264.7 cells to release more IL-6 (25-33 U/mL) than the stimulus IFN- γ / LPS (7-12 U/mL) alone, whereas the remaining compounds exhibited only marginal inducing potentials (2-7 U/mL). As with TNF and IFN, the activation of non-parasitized cells was below detection limits.

Previously structure-activity relationship studies have been performed on some other flavonoids (the chalcones, aurones and flavon-3-ol derivatives). However, the results studies cannot be compared with ours, because their substances are not structurally similar to **1-7**²⁰⁻²².

The present results provide evidence that flavonoid glycosides possess the capability to stimulate defense mechanisms in *Leishmania*-infected RAW 264.7 cells, albeit with moderate potential. Although distinct structural features are difficult to define, the cytokine-inducing potential of **1-7** seemingly decreased with increasing polarity. To investigate this phenomenon, the testing of nanoparticles will be the subject of prospective research.

Table. Antileishmanial activity against a panel of *Leishmania* amastigotes (AM) and TNF, IFN γ - and IL-6 inducing potential of compounds **1-7** on RAW 264.7 cells (IC₅₀ values in nM).

Compound	L. major				L. donovani				L. amazonensis			
	AM	TNF	IFN- γ	IL-6	AM	TNF	IFN- γ	IL-6	AM	TNF	IFN- γ	IL-6
1	6.3	205	42	33	1.5	235	39	26	3.0	270	36	31
2	3.9	72	17	7	3.0	89	15	6	3.6	68	13	6
3	6.4	30	16	6	3.9	31	14	7	3.0	36	12	7
4	2.8	51	12	4	2.5	63	13	3	15.0	59	11	—
5	12.5	34	34	6	12.8	29	26	6	4.8	32	30	3
6	8.7	56	26	3	24.2	36	11	4	20.6	43	21	7
7	3.2	9	4	3	3.0	13	4	4	3.1	11	6	3
Pentostam	11.2	—	—	—	10.6	—	—	10	—	—	—	123
IFN- γ /LPS	—	206	—	10	—	197	—	—	—	210	—	—

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