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Antioxidant Activity and Cytotoxicity of Methanol Extracts of *Geranium macrorrhizum* and Chemical Composition of its Essential Oil

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ABSTRACT

Geranium macrorrhizum, an herbaceous medicinal plant in the Balkans, has shown promising biological activity. The phytochemical composition and bioactivities of *G. macrorrhizum* cultivated in Germany were compared with previously published reports of the plant. The essential oil of flowering *G. macrorrhizum* plants, collected from an ornamental garden in Heidelberg, Germany, was obtained by hydrodistillation and analyzed by gas-liquid chromatography-mass spectrometry (GLC-MS). Methanol extracts of roots and leaves were evaluated for total phenolic and flavonoid contents and for antioxidant activity. The major oil component was the sesquiterpene germacrone (60.14%). Methanol extracts from leaves and roots screened for cytotoxic activity using the MTT assay, exhibited a moderate cytotoxicity against two human leukemia cell lines (CCRF-CEM and CEM/ADR 5000). The IC₅₀ values were 22.4 and 98.3 µg/mL for CCRF-CEM and 112.3 and 154.2 µg/mL for CEM/ADR 5000 cells that apparently over expressed ABC transporters, inhibiting the activity of ABC transporters, such as p-gp. The methanol extract from roots contained high concentrations of phenolic compounds and exhibited substantial antioxidant activity. Results were consistent with previous reports of this plant from other geographical locations.

INTRODUCTION

Geranium macrorrhizum L. (family Geraniaceae) is a perennial herb commonly known as “zdravetz” Bigroot geranium, Bulgarian geranium, and Rock

cranesbill. The plant, which is native to the Balkan Peninsula, Carpathian, Southern Alps, and Apennine Mountains (Ivancheva *et al.*, 2006; Öner *et al.*, 2010), has been cultivated and naturalized in eastern and central European countries as a decorative and aromatic plant (Öner *et al.*, 2010).

In traditional medicine, *G. macrorrhizum* is used as an aphrodisiac and as an emblematic plant for the treatment of stomach disorders by Balkan peoples (Radulovic *et al.*, 2010). Extracts of the plant have been evaluated for antimicrobial, hypotensive, spasmolytic, astringent, cardiotonic, antioxidant, capillary, and sedative activities (Radulovic *et al.*, 2010). Essential oil from the plant inhibits *Bacillus subtilis* (Radulovic *et al.*, 2010).

The plant is a natural source of antioxidant compounds, including gallic acid, quercetin, and the derivatives, quercetin-3-glucopyranoside, quercetin-3-galactopyranoside, and ellagic acid as representatives with high antioxidant properties (Giedrius, 2006). Major non-volatile secondary metabolites of *G. macrorrhizum* include gallic acid, ellagic acid, 4-galloylquinic acid, quercetin, quercetin-3-β-glucopyranoside, quercetin-3-β-galactopyranoside, and quercetin-4'-β-glucopyranoside (Giedrius, 2006). Sesquiterpene germacrone is the major volatile secondary metabolite of the essential oil.

Previous studies on the chemical constitutions of *G. macrorrhizum* tissues from Bulgaria (Ivancheva *et al.*, 2006; Tsankova and Ognyanov, 1976), Serbia (Radulovic *et al.*, 2010) and Spain (Barrero *et al.*, 2006), have been reported. To our knowledge, however, the phytochemistry and biological activity of *G. macrorrhizum* from ornamental plants grown in Germany have not been reported.

MATERIALS AND METHODS

Plant material. *G. macrorrhizum* plants used in this study were collected from an ornamental garden in Heidelberg on December 8, 2013. Voucher specimens of the collected *G. macrorrhizum* plants were deposited in the Institute of Pharmacy and Molecular Biotechnology, Heidelberg University (accession number P8474). The constituents of the plant include sesquiterpenes (Figure 1), along with flavonoids and phenolics.

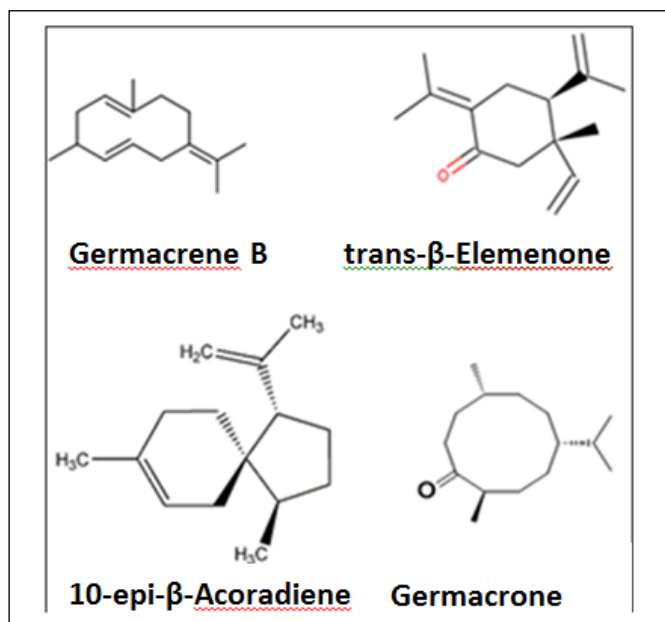


Figure 1. Chemical structures of the main secondary metabolites of *Geranium macrorrhizum*.

Tissue extraction. Approximately 200 g of fresh plant tissue of *G. macrorrhizum* was used to obtain essential oils by hydrodistillation using a Clevenger type apparatus. Methanol extracts were produced by maceration of 30 g of fresh plant material in 600 mL methanol for 48 h at room temperature.

GLC-MS analysis. The essential oil of *G. macrorrhizum* was analyzed by GLC-MS using a Shimadzu GCMS-QP2010 Ultra operated in the EI mode [electron energy = 70eV; scan range = 3.0 scans/sec], and GCMS solution software. The GC column was a DB-5 fused silica capillary column with a (5% phenyl)-polymethyl siloxane stationary phase a film thickness of 0.25 mm. The carrier gas was helium with a column head pressure 80 psi and flow rate of 1.37 mL/min. Injector temperature was 250 °C, the ion source temperature 200 °C increased at a temperature rate of 2 °C/min to 260 °C. The GC oven temperature was programmed for 50 °C

initial temperature, with an increase in rate of 2 °C/min to 260 °C. A 5% w/v solution of the sample in CH₂Cl₂ was prepared and 0.1 μL was injected in a split mode (30:1).

Identification of the oil components was based on retention indices determined by reference to a homologous series of *n*-alkanes (Kovats retention index) and by comparison of the mass spectral fragmentation patterns with those reported in the literature (Adams, 2007) and stored in the MS library.

HPLC analysis. For a phytochemical analysis of methanol extracts, HPLC was employed using a YL9100 HPLC system (Younglin Co., Korea). Samples and standard (gallic acid) were injected automatically by using a Midas autosampler with an injection loop of 100 μL from which only 20 μL was injected via partial loop fill. Standard and sample solutions were filtered through a Sartorius syringe filter before injection. The separation was achieved by using a C-18 reversed-phase column (Merck Lichrocart 250-4, Lichrospher 100 RP-18e, 5 μm, Sorbent lot number L010077333). The mobile phase consisted of acetonitrile and water/formic acid 0.1%; gradient elution from 10 to 100 percent in 45 min, 100 percent acetonitrile for 5 min. The initial condition was reconditioned by keeping the flow rate at 1.0 mL for 10 min. All analyses were done at 30 °C and detection was completed at several wavelengths (220 nm, 254 nm, 280 nm, 320 nm, and 350 nm) using a UV-DAD detector.

Total phenolics, total flavonoids, and anti-oxidant assays. The determination of total phenolics, total flavonoids, and anti-oxidant assays using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) following previously published protocols (Mamadalieva *et al.*, 2014; Sharopov *et al.*, 2015).

Cytotoxicity of the samples in human leukemia cells was determined in triplicate using an MTT assay. Parent CCRF-CEM (human T lymphoblast leukemia) and CEM/ADR5000 adriamycin resistant leukemia cells were supplied by Professor T. Efferth, Department of Pharmaceutical Biology, University of Mainz, Germany. The cells were maintained in RPMI1640 supplemented with 10 percent fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% L-glutamine. The cells were cultivated at 37°C, 5% CO₂, and 95% humidity. Once a week the CEM/ADR5000 cells, which grew as a suspension, were cultured for 24 h in RPM I1640 medium with 5 μg/mL doxorubicin. The methanol extracts were

seriallyly diluted from 5.0 mg/mL to 0.004 mg/mL, and 50 μ L liquid of each concentration was applied to wells in a 96-well plate. For CCRF-CEM and CEM/ADR5000 cells were seeded 3×10^4 cells per well in a 96-well microplate. The cells containing samples were incubated for 48 h. Then 50 μ L MTT (0.5 mg/mL) was added to the wells for formation of formazan. The medium was removed and the formazan crystals were dissolved in 100 μ L DMSO after 4 h; the absorbance was then measured at

570 nm with a Biochrom Ays UVM 340 Microplate Reader. Doxonbicin was used as a positive control.

RESULTS

The essential oil was dominated by germacrone (60.14%), followed by *trans*- β -elemenone (5.34%), α -eudesmol (3.9%), germacrene B (3.62%), and 10-*epi*- β -acoradiene (3.59%) (Table 1).

Table 1. Chemical composition of the essential oil of *Geranium macrorrhizum*

RT	RI	%	Compound	RT	RI	%	Compound
15.243	978	0.10	β -Pinene	59.805	1669	1.25	Unidentified
18.127	1024	0.09	<i>p</i> -Cymene	59.923	1671	0.80	Unidentified
20.339	1058	0.18	γ -Terpinene	60.163	1675	0.84	Unidentified
42.885	1389	0.34	β -Elemene	60.230	1676	0.26	Germacrone isomer
43.924	1405	0.09	Italicene	60.406	1680	1.03	Unidentified
45.428	1429	0.74	γ -Elemene	60.503	1681	1.21	Unidentified
48.474	1477	3.59	10- <i>epi</i> - β -acoradiene	60.703	1685	0.93	Unidentified
48.677	1480	1.01	<i>ar</i> -Curcumene	60.829	1687	1.10	Eudesma-4(15),7-dien-1 β -ol
50.203	1505	0.18	(<i>Z</i>)- α -Bisabolene	60.946	1689	0.83	Unidentified
52.732	1547	0.11	α -Elemol	61.173	1693	60.14	Germacrone
53.399	1558	3.62	Germacrene B	61.391	1697	0.34	Juniper camphor
55.693	1597	5.34	<i>trans</i> - β -Elemenone	62.726	1721	0.55	Unidentified
56.342	1608	0.46	Unidentified	63.076	1728	1.09	Unidentified
57.129	1622	0.64	Unidentified	64.855	1761	0.75	Unidentified
57.655	1631	0.73	γ -Eudesmol	65.080	1765	0.11	Benzyl benzoate
58.245	1642	0.26	Muurolol	65.306	1769	0.11	Hydroxy muurolole
58.567	1647	0.58	Unidentified	59.805	1669	1.25	Unidentified
58.994	1655	3.90	α -Eudesmol	59.923	1671	0.80	Unidentified
59.158	1658	0.81	Selin-11-en-4 α -ol	60.163	1675	0.84	Unidentified
59.474	1663	2.53	Germacrone isomer				

RT = Retention Time; RI = Retention Index; % = Percent of total peak area

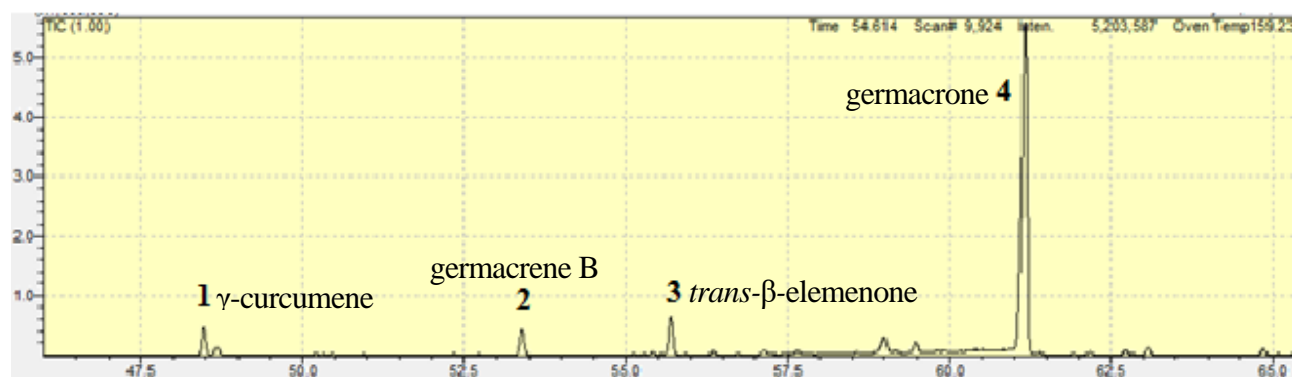


Figure 2. GLC-MS profile of the essential oil from *Geranium macrorrhizum*.

HPLC profiles of methanol extracts from leaves and roots of *Geranium macrorrhizum* indicated that gallic acid was a major component in both leaves and root extracts. (Figures 3 and 4) Other major components in leaf extracts, however, were qualitatively and quantitatively different from those in the root extracts. The total phenolic content of the methanol extracts, determined spectrophotometrically following the Folin-Ciocalteu procedure, ranged from 753 (leaves) to 993.5 (roots) mg caffeic acid equivalent (CAE) in 1 g of extract. Total

flavonoid content of extracts was determined by aluminium chloride colorimetric assay and ranged from 49.0 mg in leaves to 4.5 mg in roots quercetin equivalents (QE) in 1 g extract. The antioxidant activity of methanol extracts from leaves and roots were evaluated by using DPPH, ABTS and FRAP methods. The total phenolic and flavonoid content, along with an antioxidant activity of methanol extracts from roots and leaves, contained substantial amounts of phenolic compounds and exhibited substantial antioxidant activity (Table 2).

Table 2. Total phenol, total flavonoid, and antioxidant activity of methanol extracts of *G. macrorrhizum*.

Sample	Total phenol content (mg CAE/g extract)	Total flavonoid content (mg QE/g extract)	DPPH, IC ₅₀ (µg/mL)	ABTS, IC ₅₀ (µg/mL)	FRAP µM Fe ²⁺ /mg sample
Leaves	753.0	49.0	14.1	21.2	2419.8
Roots	993.5	4.5	5.5	4.7	2566.4
Caffeic acid	---	---	1.7	2.0	3383.5

CAE = caffeic acid equivalent; QE = quercetin equivalents; DPPH = 2,2-diphenyl-1-picrylhydrazyl; ABTS = 2,2'-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid; FRAP = ferric reducing antioxidant power

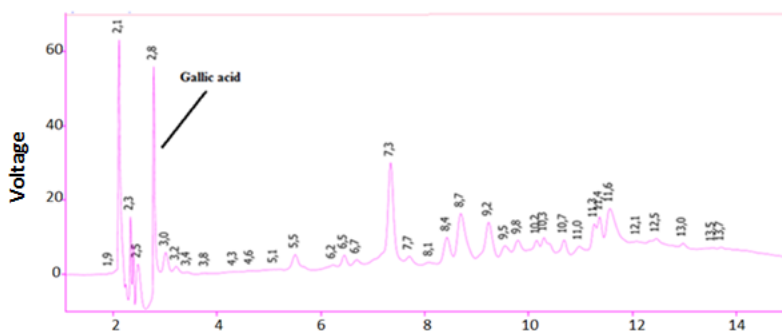


Figure 3. HPLC profile of the methanol extracts from leaves of *G. macrorrhizum*.

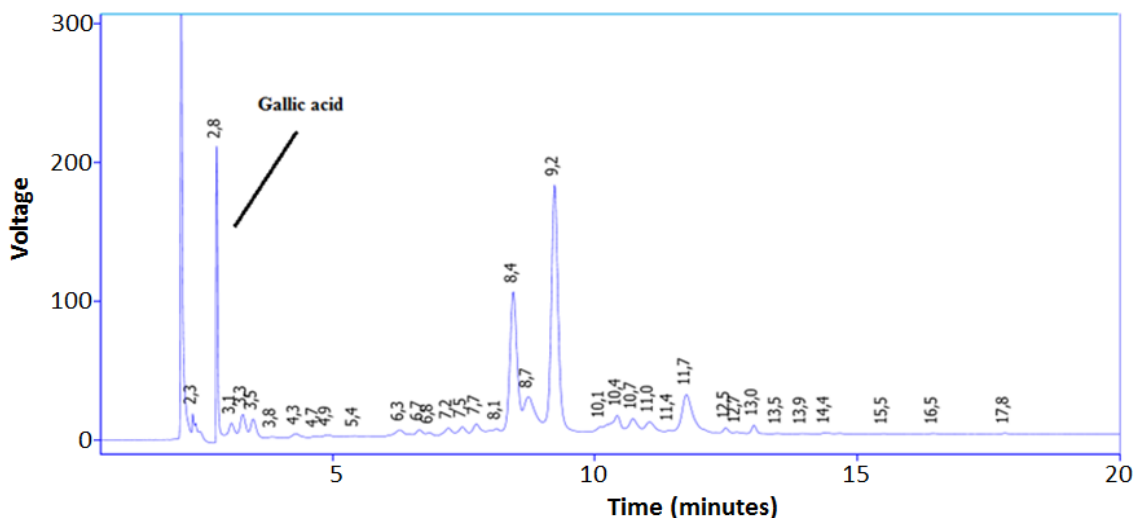


Figure 4. HPLC profile of the methanol extracts from roots of *Geranium macrorrhizum*.

The methanol extracts from leaves and roots showed a moderate cytotoxicity in two human leukemia cell lines (CCRF-CEM and CEM/ADR 5000) with IC₅₀ values of 22.4 and 98.3 µg/mL for CCRF-CEM and 112.3 and 154.2 µg/mL for CEM/ADR 5000 cells, respectively (Table 3). The IC₅₀ values of the known chemotherapeutic drug doxorubicin were 2.3 µg/mL for CCRF-CEM and 5.2 µg/mL for CEM/ADR 5000 cells, respectively. Apparently, the extracts are less toxic in CEM/ADR 5000 cells that over express p-gp, an important ABC transporter which exports lipophilic drugs that have entered the cells by diffusion. The results indicate that germacrone or other constituents of the essential oil either inhibit p-gp or are a substrate (Eid *et al.*, 2012, 2015).

Table 3. Cytotoxicity of methanol extracts of *Geranium macrorrhizum*.

Sample	CCRF-CEM, IC ₅₀ (µg/mL)	CEM/ADR 5000 IC ₅₀ (µg/mL)
Leaves	22.4	112.3
Roots	98.3	154.2
Doxorubicin	2.3	5.2

DISCUSSION

This study demonstrated the chemical profiles of the essential oil from *G. macrorrhizum* that had germacrone, *trans*-β-elemenone, α-eudesmol, germacrene B and 10-*epi*-β-acoradiene as major compounds. The methanol extract from the roots of *G. macrorrhizum* contained several phenolic compounds and exhibited strong antioxidant activity. The extracts exhibited moderate cytotoxicity in leukemia cells, apparently affecting ABC transporters. Our results are consistent with previous reported studies (Giedrius, 2006).

Experimental findings were similar to those reported by Radulovic *et al.* (2010). The 283 constituents were identified in the essential oils of *G. macrorrhizum* with germacrone (49.7% in the oil from aerial parts) and δ-guaiene (49.2% in rhizome from the oil) as main oil components (Radulovic *et al.*, 2010). Germacrone has also been detected in relatively high amounts in *Curcuma leucorrhiza* (Devi *et al.*, 2012), *Eugenia uniflora* (Bicas *et al.*, 2011), *Siparuna grandiflora* (Setzer *et al.*, 2008), and *Baccharis latifolia* (Barrero *et al.*, 2006) essential oils. Germacrone, an odorless compound with a germacrane skeleton, is an interesting substance for use in perfumery as a fixative (Barrero *et al.*, 2006).

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