European Review for Medical and Pharmacological Sciences 2021; 25: 6283-6294

Polyphenol rich extracts of *Geranium* L. species as potential natural antioxidant and antimicrobial agents

M. ILIĆ[1], S. SAMARDŽIĆ[1], J. KOTUR-STEVULJEVIĆ[2], D. UŠJAK[3], M. MILENKOVIĆ[3], N. KOVAČEVIĆ[1], M. DROBAC[1]

[1]Department of Pharmacognosy, University of Belgrade-Faculty of Pharmacy, Belgrade, Serbia
[2]Department of Medical Biochemistry, University of Belgrade-Faculty of Pharmacy, Belgrade, Serbia
[3]Department of Microbiology and Immunology, University of Belgrade-Faculty of Pharmacy, Belgrade, Serbia

Abstract. – OBJECTIVE: Plants and plant extracts are of great scientific interest due to the chemical diversity and pharmacological properties of present bioactive molecules. The *Geranium* L. species are widely used in ethnomedicine. In the current study, the total phenolic and tannin content, antioxidant and antimicrobial activity of methanol extracts of eight *Geranium* species were investigated.

MATERIALS AND METHODS: The total phenolic and tannin content were determined by the FC method. Antioxidant capacity was evaluated in FRAP, DPPH, and biochemical assays, while antimicrobial activity was examined using the broth microdilution method.

RESULTS: The high total phenolic (170.64-636.32 mg GAE/g dry extract) and tannin content (37.80-414.02 mg GAE/g DE), along with significant total antioxidant (FRAP values 1.13-8.80 mmol Fe2+/g) and DPPH radical scavenging activity (SC50 values 4.24-34.52 μg/mL) were observed. The prominent antioxidant capacity was confirmed in biochemical assays (OS values -1.47 – -13.02). The extracts exhibited significant antimicrobial activity against ATTC strains (MICs dominantly in the range of 12.5-200 μg/mL) as well as against clinical isolates of *E. coli* (MICs mostly 50 and 100 μg/mL). The pronounced antioxidant and antimicrobial activity can be due to the high phenolic content, particularly due to the presence of hydrolyzable tannins.

CONCLUSIONS: Based on the high content of polyphenols, pronounced antioxidant and antimicrobial activities, the examined extracts are promising natural antioxidant and antimicrobial agents with the potential medicinal purpose and use as a functional food.

Key Words:
Geranium species, Polyphenols, Antioxidant, Antimicrobial.

Introduction

Nature is an inexhaustible source of biologically active substances with potential medicinal purposes. It is estimated that more than a half of existing therapeutics are natural or nature-related which is especially emphasized among anti-infective and anticancer drugs[1,2]. However, the growing antimicrobial resistance to existing drugs is one of the major threats to public health. According to the UK government, there could be 10 million deaths per year due to antibiotic resistance by 2050 resulting in urgent necessity for new antimicrobials[3]. The current trends in research include the discovery and introduction of new antimicrobials as effective alternatives to the existing ones, especially those of natural origin.

Oxidative stress is one of the key factors in the development of many chronic diseases. The presence of pro-oxidant compounds and/or other risk factors, including smoking, stress, and excessive physical activity can cause free radicals overproduction, which exceed the capacity of the endogenous antioxidant system consisting of enzymes and thiol-containing molecules[4]. If not neutralized in time, free radicals can cause oxidative stress and damage to DNA, lipids, proteins, and other biomolecules contributing to the development of different diseases including cardiovascular, neurological, immunological, and metabolic disorders[5,6]. To enhance the overall antioxidant capacity, there is a continuous demand for exogenous antioxidants, which could ameliorate the oxidative damage by inhibiting the oxidative chain reaction or scavenging free radicals[7].

Corresponding Author: Milan Ilić, MD; e-mail: ilicmilan88@yahoo.com

Furthermore, natural food additives with antimicrobial and antioxidant properties have been gaining more interest recently, due to rising concern on the safety of synthetic antioxidants and preservatives as some studies[8-11] indicated their hazardous effects.

In this sense, plants and plant extracts are of great scientific interest due to the chemical diversity and pharmacological properties of bioactive molecules among which polyphenols have special significance[12]. It is demonstrated that phenolic compounds possess various activities, including antimicrobial, antioxidant, anti-inflammatory, anticancer activity, and hence have different medicinal purposes[13-22].

Geranium L. species have significant use in traditional medicine. The underground parts of *G. macrorrhizum* L. are used against intestinal mucositis[23], while aerial parts have astringent and antiseptic properties[24]. *Geranium robertianum* L. is especially valued in Western Europe. According to earlier data, its aerial parts were used to enhance fertility and as an anticancer agent[25]. It is also used for the treatment of gastrointestinal and liver disorders, inflammatory conditions, and diabetes, as well as a diuretic, antihypertensive and antispasmodic agent[26]. Regarding chemical composition, previous studies[27-30] revealed that these plants are rich in polyphenols, especially ellagitannins with geraniin as the most prevalent in the genus.

In line with the significance and potential of natural products, this study aimed to evaluate the antioxidant and antimicrobial activity of extracts of eight *Geranium* species aerial and underground parts and to define their potential uses as antioxidant and antimicrobial agents. It should be emphasized that some of the species have been examined for the first time regarding selected pharmacological activities. Furthermore, this is the first time that *Geranium* species have been tested for antioxidant activity in a biologically relevant environment.

Materials and Methods

Chemicals

All solvents and reagents were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), Aldrich Chemie (Steinheim, Germany), Acros (Geel, Belgium), and Merck (Darmstadt, Germany).

Plant Material

The aerial and underground parts of eight *Geranium* species: *G. macrorrhizum* L., *G. phaeum* L., *G. sanguineum* L., *G. robertianum* L., *G. palustre* L., *G. pyrenaicum* Burm. f., *G. columbinum* L. and *G. lucidum* L. were collected in South Eastern Serbia (Vlasina plateau) in the blooming stage in June 2017. The voucher specimens are deposited in the Herbarium of the Department of Biology and Ecology, University of Niš-Faculty of Sciences and the Department of Botany and Herbarium of the Department of Botany University of Belgrade-Faculty of Pharmacy.

Preparation of the Extracts

The powdered, air-dried flowering aerial parts and roots were firstly defatted by extraction with petroleum ether at room temperature (by maceration for 24 hours), and then, extracted with methanol (by bimaceration for 24 hours, drug/solvent ratio 1:10). After filtration, the methanol extracts were evaporated to dryness using a rotary vacuum evaporator.

Determination of Total Phenolic and Tannin Content in Methanol Extracts

The total phenolic content (TPC) in dry methanol extracts of aerial and underground parts was determined using the FC method[31]. Briefly, 100 μL of extract (0.2 mg/mL in methanol) was mixed with 750 μL of FC reagent (10-fold diluted). The 750 μL of Na_2CO_3 (60 g/L) was added to the mixture after 5 minutes at room temperature. The absorbance was measured at 725 nm after 90 minutes in the dark at room temperature. The blank was prepared in the same manner using 100 μL of methanol instead of extract solution.

The content of tannins (TC) in extracts was determined after their adsorption on hide powder[32]. The hide powder (100 mg) was added to 10 mL of extract solution (0.2 mg/mL in methanol) and the mixture was stirred for 1 hour and filtered. The non-tannin phenolics were determined in the filtrate identically as total phenolics. Tannin content was calculated as a difference between the total and non-tannin phenolic content.

The tests were done in triplicate and the calibration curve of gallic acid (GA) (1-10 mg/mL) was used to express the results as GA equivalents (GAE) in dry extract (DE) (mg GAE/g DE).

Antioxidant Activity

FRAP Assay

Ferric Reducing Antioxidant Power (FRAP) assay was used for the determination of the total antioxidant activity (TAA) of extracts[33,34]. FRAP reagent was prepared by mixing 25 mL of acetate buffer (300 mmol/L, pH 3.6), 2.5 mL TPTZ solution (10 mmol/L TPTZ in 40 mmol/L HCl) and 2.5 mL $FeCl_3$ solution in water (20 mmol/L). Depending on the tested extract, different volumes (10 µL, 25 µL, or 50 µL) of sample solution (0.5, 1, or 2 mg/mL) were mixed with 3 mL of FRAP reagent and stirred. After 30 min at 37°C, the absorbance was measured at 593 nm against a blank (100 µL of methanol mixed with 3 mL of FRAP reagent). L-ascorbic acid, a well-known antioxidant compound, was used as a positive control. The tests were done in triplicate. The results were expressed in mmol Fe^{2+}/g DE using the calibration curve of ferrous sulphate (200-1000 mmol/L).

DPPH Radical Assay

The DPPH assay, as described previously[35], was used to determine the radical scavenging ability of extracts. The different aliquots of stock solutions of the methanol extract (0.5 mg/mL or 1 mg/mL in methanol, depending on the tested extract) were diluted to 2 mL with methanol and 0.5 mL of DPPH solution (0.5 mM) was added. The mixture was intensively shaken. After incubation in the dark at room temperature, the absorbance was measured at 517 nm using methanol as a blank. 1 mL of DPPH solution diluted with 4 mL of methanol was used as a negative control. L-ascorbic acid was used as a positive control. Scavenging of DPPH radical, $SC_{(\%)}$, was calculated using the following formula:

$$SC_{(\%)} = 100 \times (A_0 - A_S)/A_0$$

where A_0 is the absorbance of the negative control and A_S is the absorbance of the tested extract. The results, expressed as SC_{50} value, represent the concentration of the extract that leads to the scavenging of 50% of DPPH radicals. The tests were done in triplicate.

Biochemical Assays

Antioxidant activity was examined *in vitro* in the human serum pool after the induction of oxidative stress using exogenous oxidant *tert*-butyl hydroperoxide (TBH). The serum was collected from healthy volunteers who had their regular checkups at the Military Medical Academy in Belgrade and had given written consent that the serum could be used in the study. Only the samples within reference ranges of basic biochemical parameters were used for preparing the serum pool. The aliquots of the serum pool were frozen and kept at -80°C until analysis.

Sample Preparation

The same volumes (25 µL) of dimethyl sulfoxide (DMSO) solutions of methanol extracts (concentration range 0.25-1 mg/mL) and TBH (0.25 mmol/L) were added to serum (450 µL). The mixture was stirred and incubated at 37°C for 2 h. The samples were prepared in duplicate.

Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) was determined using Erel's method with some modifications[36,37]. The reduced 2,2-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) was oxidized to ABTS radical cation ($ABTS^{\bullet+}$) using hydrogen peroxide (2 mmol/L) in the acetate buffer (30 mmol/L, pH 3.6) – ABTS solution. The deep green colour of $ABTS^{\bullet+}$ spontaneously and slowly bleaches when diluted with acetate buffer solution (0.4 mol/L, pH 5.8). Antioxidants present in the samples accelerate the bleaching of $ABTS^{\bullet+}$ proportionally to their concentrations. Briefly, 200 µL of acetate buffer (0.4 mol/L, pH 5.8), 12.5 µL of the sample, and 37.5 mL of ABTS solution were mixed and incubated for 10 minutes at room temperature. The absorbance was measured at 660 nm using deionized water as blank and DMSO as control. The reaction was calibrated using Trolox and the results were expressed in mmol Trolox equiqualents/L.

Total Oxidant Potency (TOP)

The total oxidant status (TOP) was determined using optimized Erel's method[37,38]. The assay is based on the oxidation of ferrous ion-*o*-dianisidine complex to ferric ion by oxidants present in the samples. The ferric ion forms a coloured complex with xylenol orange in the acidic medium whereby the colour intensity is proportional to the amount of oxidants. Briefly, 225 µL of reagent 1 (xylenol orange 150 µM, NaCl 140 mM and glycerol 1.35 M in 25 mM H_2SO_4 solution, pH 1.75), 11 µL of reagent 2 (ferrous ammonium sulphate 5 mM and *o*-dianisidine 10 mM in 25 mM H_2SO_4 solution) and 35 µL of the sample was mixed and the absorbance was measured at 560

nm after 3-4 minutes. The results were expressed in μmol H_2O_2/L using a calibration curve (H_2O_2 10-200 μmol/L). Deionized water and DMSO were used as blank and control.

Prooxidant-Antioxidant Balance (PAB)

Prooxidant-antioxidant balance (PAB) was measured as previously published[37,39]. The assay is based on the determination of hydrogen peroxide in the presence of antioxidants due to the property of 3,3′,5,5′-tetramethylbenzidine (TMB) to change colour depending on its oxidation state (the reduced molecular form is colourless, while the cation is blue). TMB simultaneously reacts with hydrogen peroxide and antioxidants. The reaction with hydrogen peroxide is catalyzed by peroxidase, whereas the reaction with antioxidants is non-enzymatic. The intensity of TMB cation's blue colour is proportional to the concentration of oxidants in the sample. The working solution was prepared by mixing 1 mL of TMB cation (1 mL) and TMB reagent II (10 mL). The 1 mL of TMB reagent I (the solution of TMB in DMSO 6 g/L) was added to 50 mL of acetate buffer (0.05M pH 4.5) along with 175 μL of chloramine T (100 mmol/L) for the preparation of TMB cation. To prepare TMB solution II, 200 μL of TMB reagent I was dissolved in 10 mL of acetate buffer (0.05M pH 5.6). The tested samples (10 μL) were mixed with the working solution (180 μL) and incubated at 37°C in the dark. After 10 min the reaction was interrupted by the addition of 40 μL of HCl (2M) and the absorbance was measured at 450 nm. The results were expressed in hydrogen peroxide concentration (%) using a calibration curve constructed by mixing different proportions (0-100%) of hydrogen peroxide (oxidant) and uric acid (antioxidant).

Total Sulphydryl Groups Content (SHG)

The levels of sulphydryl groups were determined using the previously described method with some modifications[37,40]. The assay is based on a reaction between dinitrodithiobenzoic acid (DTNB) and aliphatic thiol compounds in a basic medium whereby 1 mol of coloured *p*-nitrophenol anion per mol of thiol is generated. Briefly, 15 μL of samples were mixed with 270 μL of phosphate buffer (0.2 mol/L pH 9) and 10 μL of DTNB solution (10 mmol/L in phosphate buffer: 50 mmol/L pH 7). The absorbance was measured at 412 nm after 25 min incubation at room temperature. The method was calibrated using reduced glutathione (0.1-1.0 mmol/L).

Oxy Score

Oxy score was calculated as the difference between prooxy and antioxy score. Prooxy and antioxy scores present the average value of Z scores of determined oxidant (TOP and PAB) and antioxidant parameters (TAC and SHG), respectively. Z score was calculated as the difference between the sample and control value divided by SD of control values. The lower oxy score values indicate better antioxidant activity. The results were expressed as medians and 25^{th}-75^{th} percentile values (in brackets) as the distribution of parameters was non-normal. The results were compared to the Trolox – water-soluble analog of α-tocopherol.

Antimicrobial Activity

The antimicrobial activity of methanol extracts of aerial and underground parts was tested against standard strains of Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212) and *Bacillus subtilis* (ATC 6633), Gram-negative bacteria *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 9027) and *Salmonela abony* (NCTC 6017), as well as against the yeast *Candida albicans* (ATCC 10231). Additionally, the antimicrobial activity was tested against 10 clinical isolates of *E. coli* and 7 clinical isolates of *K. pneumoniae*. Standard strains were provided by the Institute of Immunology and Virology, Torlak, Belgrade, while clinical isolates were obtained from Clinical Center of Serbia, Belgrade and Clinical-Hospital Center Zvezdara, Belgrade. The minimal inhibitory concentrations (MICs) were determined using the broth microdilution method according to Clinical and Laboratory Standards Institute guidelines[41]. The tests were performed in Müller-Hinton broth for bacterial strains and Sabouraud dextrose broth for *C. albicans*. The extracts were dissolved in methanol and the serial dilutions of tested samples in broth (100 μL) were prepared in 96-well microtiter plates so the final concentrations of extracts were 12.5-200 μg/mL. To each well, 100 μL of an overnight broth culture of each strain, prepared at concentrations of 2×10^6 CFU/mL for bacteria and 2×10^5 CFU/mL for *C. albicans*, was added. The incubation period was 24 h at 37°C for bacteria and 48 h at 26°C for *C. albicans*. 2,3,5-Triphenyl-2*H*-tetrazolium chloride (TTC) was used as a growth indicator in the final concentration of 0.05%. The MIC

Table I. Total phenolic content (TPC), tannin content (TC), DPPH radical scavenging (SC_{50}), and total antioxidant (TAA) activity of tested samples.

Sample		TPC (mg GAE/g)	TC (mg GAE/g)	DPPH SC_{50} [a] (μg/mL)	TAA [b] (mmol Fe^{2+}/g)
G. macrorrhizum	1a[c]	523.96 ± 2.28	372.66 ± 6.92	4.92 ± 0.21	5.68 ± 0.10
	1u[d]	553.40 ± 8.72	414.02 ± 9.46	5.34 ± 0.11	6.33 ± 0.02
G. robertianum	2a	425.31 ± 3.37	270.72 ± 3.67	6.81 ± 0.16	5.30 ± 0.23
	2u	390.29 ± 6.10	233.00 ± 3.12	7.54 ± 0.07	4.92 ± 0.14
G. phaeum	3a	170.64 ± 1.08	37.80 ± 1.33	34.52 ± 0.91	1.13 ± 0.03
	3u	586.34 ± 6.14	376.32 ± 5.33	5.18 ± 0.06	7.02 ± 0.17
G. sanguineum	4a	547.38 ± 5.83	398.36 ± 5.47	5.18 ± 0.04	6.32 ± 0.19
	4u	523.52 ± 5.60	295.87 ± 2.71	5.80 ± 0.03	5.85 ± 0.18
G. palustre	5a	515.24 ± 1.15	371.73 ± 1.35	4.34 ± 0.09	6.64 ± 0.24
	5u	636.32 ± 7.51	389.77 ± 8.87	4.24 ± 0.05	8.80 ± 0.09
G. pyrenaicum	6a	298.73 ± 6.47	161.14 ± 6.34	9.20 ± 0.04	3.31 ± 0.12
	6u	397.69 ± 4.00	266.33 ± 0.72	9.15 ± 0.23	3.15 ± 0.18
G. lucidum	7a	413.45 ± 5.85	257.53 ± 7.08	7.76 ± 0.24	4.93 ± 0.02
	7u	388.19 ± 5.24	253.74 ± 4.52	5.74 ± 0.10	5.19 ± 0.25
G. columbinum	8a	400.57 ± 4.99	239.88 ± 4.82	10.33 ± 0.31	4.03 ± 0.06
	8u	441.93 ± 3.87	261.19 ± 2.80	10.45 ± 0.32	3.88 ± 0.13
L-ascorbic acid				3.68 ± 0.05	6.55 ± 0.04

[a]Concentrations of the extract or ascorbic acid that inhibit 50% of DPPH radical obtained in three independent measurements; [b]Expressed as FRAP value (the mean ± SD obtained in three independent measurements); [c]Aerial parts; [d]Underground parts.

was defined as the lowest concentration of the tested sample in which the microorganism does not demonstrate visible growth. All the tests were performed in duplicate with methanol as a negative control for each microbial strain.

Statistical Analysis

Statistical analysis was performed using SPSS 18.0 (SPSS, INC. Chicago, IL, USA). In order to compare and determine whether there were statistically significant differences between TAA or SC_{50} values of tested samples, the Kruskal-Wallis test with Bonferroni correction was used. The OS values were compared using Friedman's test after the significant difference between different concentrations of tested samples was excluded. Differences were considered significant at $p < 0.05$.

Results

Total Phenolic and Tannin Content

The TPC and TC of investigated extracts are shown in Table I. The extracts of underground and aerial parts were rich in phenolic compounds with TPC values ranging from 170.64-636.32 mg GAE/g DE (Figure 1). The extracts of underground parts of *G. palustre* (636.32 mg GAE/g DE), *G. phaeum* (586.34 mg GAE/g DE), and *G. maccrorhizum* (553.40 mg GAE/g DE), as well as the extract of *G. sanguineum* aerial parts (547.38 mg GAE/g DE) were the most abundant in polyphenols, while the lowest content of total polyphenolics was determined in the extracts of aerial parts of *G. pyrenaicum* (298.73 mg GAE/g DE) and *G. phaeum* (170.64 mg GAE/g DE). It was observed that the content of total polyphenolics is higher in the extracts of underground organs than in aerial parts, with the exception of aerial parts of *G. robertianum, G. sanguineum,* and *G. lucidum*.

The content of tannins was in the range 37.80-414.02 mg GAE/g DE. Figure 1 shows the share

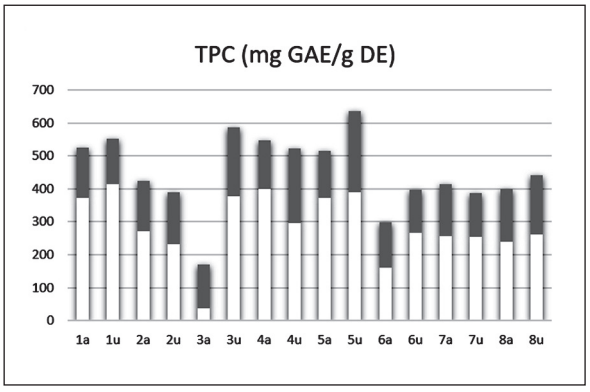

Figure 1. Share of tannin compounds in total polyphenols.

of tannin compounds in total polyphenols, and it can be noticed that tannins were the most abundant phenolics (161.14-414.02 mg GAE/g DE) in the extracts, except in *G. phaeum* aerial parts extract (37.80 mg GAE/g DE).

Antioxidant Activity

The antioxidant activity of extracts was evaluated *in vitro* using FRAP and DPPH assays and also by the determination of biochemical parameters in human serum. Biochemical assays enabled closer insight into the antioxidant activity of extracts in a biologically relevant environment and provided more relevant information on the potential medicinal application of tested samples.

The total antioxidant (TAA) and anti-DPPH activity of extracts and positive control (L-ascorbic acid) is presented in Table I.

Regarding the total antioxidant activity, no statistically significant difference in TAA between examined extracts and L-ascorbic acid as a known antioxidant was observed in FRAP assay, indicating a prominent total antioxidant capacity of the extracts. As expected, the underground parts of *G. palustre* and *G. phaeum* that had the highest TPC, exhibited the highest TAA (8.80 and 7.02 mmol Fe^{2+}/g DE, respectively), even stronger than L-ascorbic acid (6.55 mmol Fe^{2+}/g DE). On the other hand, *G. phaeum* aerial parts had the lowest TAA (1.13 mmol Fe^{2+}/g DE) which is also in accordance with determined lower TPC content.

High TPC and TAA values indicate that these extracts could have significant antiradical activity. In DPPH assay all of the tested extracts showed concentration-dependent activity (r^2 = 0.9902-0.999). The SC_{50} values of all tested extracts, except *G. phaeum* aerial and *C. columbinum* underground parts, were statistically comparable to the anti-DPPH activity of L-ascorbic acid (SC_{50}= 3.68 μg/mL). *G. palustre* exhibited the strongest anti-DPPH activity with the SC_{50} values of 4.24 and 4.34 μg/mL for underground and aerial parts extracts, respectively which is in accordance with their significantly high TPC and TAA. Analogously, the lowest anti-DPPH activity was exhibited by extract of *G. phaeum* aerial parts (SC_{50} = 34.52 μg/mL).

The antioxidant activity of samples was also examined in a biologically relevant environment by determining TAC, TOP, PAB, and SHG parameters and calculating prooxidative, antioxidative, and oxy score values (OS). The results are shown in Table II.

All of the examined extracts had OS values ranging from -1.47 to -13.02 and there was no significant difference between samples and Trolox as a proven antioxidant (OS value -7.01). The negative values indicate strong antioxidant potential and were more pronounced in underground parts extracts. The extracts of *G. macrorrhizum, G. phaeum, G. robertianum* and *G. pyrenaicum* underground parts had the strongest antioxidant potential (OS values -13.02, -10.93, -9.32, and -9.31, respectively), even higher than Trolox.

Table II. Oxy score (OS) values of tested extracts and positive control

Sample		Oxy score value[a]	Significant difference *vs.* ($p < 0.05$)
G. macrorrhizum	1a	-5.99 (-10.52 – -4.40)	
	1u	-13.02 (-18.67 – -6.16)	3a, 4a, 6a, 7a, 8a
G. robertianum	2a	-6.86 (-8.05 – -6.17)	
	2u	-9.32 (-10.57 – -6.71)	8a
G. phaeum	3a	-3.24 (-4.02 – -2.36)	1u, 3u
	3u	-10.93 (-18.43 – -5.84)	3a, 4a, 6a, 8a
G. sanguineum	4a	-3.54 (-4.82 – -1.50)	1u, 3u
	4u	-5.54 (-14.04 – -3.57)	
G. palustre	5a	-5.49 (-7.73 – -4.12)	
	5u	-6.25 (-12.74 – -5.68)	8a
G. pyrenaicum	6a	-2.51 (-5.69 – -0.50)	1u, 3u
	6u	-9.31 (-12.82 – -5.13)	8a
G. lucidum	7a	-4.53 (-6.76 – -1.74)	1u
	7u	-8.97 (-12.98 – -2.45)	
G. columbinum	8a	-1.47 (-2.98 – -0.69)	1u, 2u, 3u, 5u, 6u
	8u	-6.50 (-8.10 – -0.72)	
Trolox		-7.01 (-7.53 – -6.51)[42]	

[a]The results are expressed as medians and 25th-75th percentile values in brackets.

Antimicrobial Activity

Antimicrobial activity was tested against seven standard strains of Gram-positive and Gram-negative bacteria, one strain of yeast *C. albicans*, as well as against ten clinical isolates of *E. coli* and seven isolates of *K. pneumoniae*. The results are given in Table III. All of the tested extracts inhibited the growth of ATCC microbial strains with the MICs predominantly in the range of 12.5-200 μg/mL. Regarding clinical isolates, the MICs against *E. coli* isolates were mostly 50 and 100 μg/mL, whereas the MICs against *K. pneumoniae* clinical isolates were 200 μg/mL or higher.

The most susceptible strain was *E. faecalis,* especially to extracts of aerial parts of *G. phaeum* and *G. columbinum* and underground parts of *G. sanguineum, G. pyrenaicum,* and *G. columbinum* (MICs = 12.5 μg/mL). The MICs of examined samples against *E. coli* ATCC strain were 25-100 μg/mL with *G. robertianum* aerial and *G. palustre* underground parts extracts as the most active (MICs = 25 μg/mL). The significant antimicrobial activity with MICs = 50 μg/mL was determined for *G. pyrenaicum* aerial and *G. lucidum* underground parts extracts against *E. faecalis*, as well as for *G. macrorrhizum, G. sanguineum, G. palustre, G. lucidum,* and *G. columbinum* aerial and *G. macrorrhizum, G. robertianum, G. phaeum,* and *G. sanguineum* underground parts extracts against *E. coli*. The same activity was also observed against *S. aureus* (*G. palustre* and *G. pyrenaicum* aerial and *G. robertianum G. phaeum, G. palustre, G. pyrenaicum, G. lucidum,* and *G. columbinum* underground parts extracts), *K. pneumoniae* (*G. phaeum* aerial and *G. columbinum* underground parts extracts) and *S. abony* (*G. columbinum* aerial and *G. lucidum* underground parts extracts). The yeast *C. albicans* was also susceptible to all tested samples (MICs = 100-200 μg/mL) with the exception of *G. palustre* and *G. columbinum* underground parts extracts (MICs > 200 μg/mL).

Discussion

The results on phenolic and tannin content showed that extracts of aerial and underground parts of investigated *Geranium* species are very rich in polyphenols with tannins as predominant, which is in line with some previous findings[43,44] on plants of this genus. The antioxidant and antimicrobial activities of plant polyphenols are well documented[45-48]. The antioxidant activity of polyphenols is based on H atom transfer, single electron transfer, and chelation of metals, but also on the regulation of oxidoreductase enzyme system[49,50].

It should be noticed that this is the first time that the antioxidant activity of *Geranium* extracts, as well as plant extracts, was assessed in a biologically relevant environment (using human blood serum). In addition, to our knowledge, there is no information on the antioxidant activity of *G. phaeum* and *G. palustre* extracts up to date.

The oxy score values for tested extracts were calculated using TAC, TOP, PAB, and SHG parameters. The negative values of oxy score indicate strong antioxidant potential which was comparable or even higher than those of reference standard antioxidant Trolox. The effect can be attributed to high polyphenol content, particularly to the presence of hydrolyzable tannin geraniin and/or other chemically similar compounds. Geraniin is the principal phenolic constituent of some *Geranium* species and it was found that geraniin can attenuate oxidative stress by recovering oxidative stress biomarkers, serum antioxidants, and glutathione redox balance[51]. Also, geraniin exhibited stronger radical scavenging activity than L-ascorbic acid and was effective at enhancing the activity of superoxide dismutase[52].

High TPC, anti-DPPH, and TAA of *G. macrorrhizum* extracts or dry leaves and rhizomes were also reported in previous studies. Radulović et al[53] reported TPC of 160.2 and 85.7 mg GAE, the reductive capacity of 178.7 mg and 106.4 mg Trolox equivalents and TAA of 1.35 and 0.63 mmol Fe^{2+} all per g of dry leaves and rhizomes, respectively. According to Miliauskas et al[54] ethanol-butanol extracts of leaves exhibited high radical-scavenging capacity. Methanol extracts of aerial parts of several *Geranium* species including *G. macrorrhizum, G. sanguineum, G. pyrenaicum, G. robertianum, G. lucidum* and *G. columbinum* showed considerable radical scavenging activity, but lower than revealed in this study[55]. Compared with our findings, lower TPC (35.62 mg GAE per g of extract), but stronger anti-DPPH activity (IC_{50} = 1.86 μg/mL) of *G. lucidum* aerial parts methanol extract was reported by Wafa et al[56]. Khavrona et al[57] reported similar antioxidant activity (IC_{50} = 5.80 μg/mL) of an aqueous lyophilized extract of *G. palustre* aerial parts.

Antimicrobial activity was evaluated towards standard strains as well as clinical isolates. The obtained results are significant having in mind

Table III. Antimicrobial activity of *Geranium* spp. extracts.

Microorganism	Minimal Inhibitory Concentrations MICs (µg/mL)															
	G. *macrorrhizum*		G. *robertianum*		G. *phaeum*		G. *sanguineum*		G. *palustre*		G. *pyrenaicum*		G. *lucidum*		G. *columbinum*	
	a	u	a	u	a	u	a	u	a	u	a	u	a	u	a	u
ATCC strains *S. aureus* ATCC 6538	100	100	100	50	100	50	100	> 200	50	50	50	50	100	50	100	50
E. faecalis ATCC 29212	25	25	25	25	12.5	12.5	25	12.5	25	25	50	12.5	25	50	12.5	12.5
B. subtilis ATCC 6633	100	100	200	100	200	200	200	200	100	200	200	200	100	200	200	> 200
E. coli ATCC 10536	50	50	25	50	100	50	50	50	50	25	100	100	50	100	50	100
K. pneumoniae ATCC 13883	100	200	100	100	50	100	100	100	100	100	100	100	100	100	100	50
P. aeruginosa ATCC 9027	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
S. abony NCTC 6017	100	100	200	100	100	100	100	100	100	200	200	100	100	50	50	100
C. albicans ATCC 10231	100	50	100	100	200	100	100	100	100	> 200	100	200	100	100	100	> 200
E. coli clinical isolates nasopharynx	100	50	50	100	100	50	50	50	100	200	100	50	200	> 200	100	100
Blood	100	50	100	50	200	100	100	100	200	50	200	100	200	> 200	100	100
Wound	100	50	100	100	100	100	50	100	200	>200	100	100	200	100	100	100
Vagina	100	50	100	25	100	25	100	50	200	50	100	50	200	100	100	50
Wound	100	50	50	25	50	50	50	50	50	100	50	50	50	50	50	25
Wound	100	> 200	25	> 200	50	> 200	100	> 200	100	> 200	50	> 200	100	> 200	25	> 200
Vagina	100	100	100	100	100	100	100	100	200	> 200	100	100	100	200	100	100
Pharynx	50	50	50	50	100	100	50	100	50	> 200	50	100	100	50	50	100
Bronchi	200	100	100	100	100	100	200	50	200	> 200	100	100	200	200	100	50
Pharynx	100	100	100	50	100	100	100	100	100	> 200	100	100	200	100	100	50
K. pneumoniae clinical isolates																
Blood	200	> 200	> 200	> 200	200	200	200	> 200	> 200	> 200	> 200	> 200	> 200	> 200	> 200	> 200
Blood							> 200									
Urine	200	> 200	200	> 200	200	200	200	200	200	200	200	200	200	200	200	200
Peritoneal Fluid							> 200									
Blood							> 200									
Blood							> 200									
Blood							> 200									

that activity with MIC concentrations below 100 μg/mL is considered as significant and very interesting[58].

The previous results also demonstrated the antibacterial activity of some *Geranium* sp. extracts. Radulović et al[53] determined the MICs of *G. macrorrhizum* leaf and rhizome extracts against *S. aureus* (15.6 and 620.5 μg/mL, respectively), *B. subtilis* (250 and 150.6 μg/mL), *K. pneumoniae* (500 and 620.5 μg/mL), *E. coli* (>500 and 5000 μg/mL) and *C. albicans* (>500 and 310.2 μg/mL) which were significantly lower than reported in our study, with the exception of underground parts extract against *S. aureus*.

Özçelık et al[59] reported significant antimicrobial activity of ethanol extract of *G. pyrenaicum* aerial and underground parts against Gram-positive and Gram-negative standard strains and clinical isolates, as well as against *C. albicans* with the MICs in the range of 8-128 μg/mL. The strongest antibacterial activity of both extracts was against *S. aureus* and *E. faecalis* standard strains as reported in our study.

Antimicrobial activity of *G. lucidum* aerial parts methanol extract against *S. aureus, E. coli, P. aeruginosa, B. subtilis,* and *C. albicans* was examined by Wafa et al[56] using the disc diffusion method. *E. coli* was the most susceptible strain which is in correlation with our results.

According to Hamami et al[60], the MICs of methanol extract of *G. sanguineum* flowers against *B. subtilis, S. aureus,* and *P. aeruginosa* were 2, 6, and 6 mg/mL, respectively.

In the present study, *G. palustre* extracts were the most effective against *E. faecalis, S. aureus,* and *E.coli* (MICs = 25-50 μg/mL) among bacterial strains. Previously, the potent activity of aerial parts extract against *E. coli*, but using agar diffusion method was reported[57].

The discovery of new antimicrobials is a necessity due to continuing global concern involving drug resistance. Generally, Gram-negative bacteria including *E. coli* and *K. pneumoniae* are more resistant to antibiotics than Gram-positive. Data retrieved from European Antimicrobial Resistance Surveillance Network show the non-susceptible level of resistance of *E. coli* and *K. pneumoniae* against several classes of antibiotics which may be due to the diversity of resistance genes[61,62]. The antimicrobial activity of examined extracts was more pronounced against *E. coli* than *K. pneumoniae* clinical isolates. Having that in mind, the demonstrated strong activity of *Geranium* extracts towards the standard strain of *E. coli* and especially against some clinical isolates of *E. coli* could be of great interest.

The exhibited antimicrobial activity could be related to high polyphenol content. According to some studies[63], the antimicrobial activity of plant extracts is related not only to the concentration of phenolic compounds but also to phenolic profile. The antimicrobial mechanism of action of polyphenols is diverse. The hydroxyl group (OH) of phenolic compounds is important for antimicrobial activity and can induce cell death by initiating several mechanisms including disruption of the membrane structure leading to the leakage of cellular content, delocalization of electrons resulting in depolarization of bacteria, reducing the pH gradient across the membrane and the level of ATP[64]. *Geranium* species are characterized by the presence of hydrolyzable tannins, a group of polyphenolic constituents, that have been shown to possess antimicrobial properties[65]. Some studies hypothesized that the mechanism of antimicrobial activity of hydrolyzable tannins includes damaging the structure and activity of cell wall and membrane, interaction with cytoplasm, wall enzymes (e.g., oxidoreductases) and proline-rich proteins or cell-surface lipoteichoic acid as well as the inhibition of glucosyltransferase[66]. Furthermore, it is shown that polyphenols can prevent oxidative stress caused by bacterial infection[67].

Conclusions

The extracts of aerial and underground parts of eight *Geranium* species were rich in polyphenols among which tannins were dominant. The extracts exhibited significant antioxidant activity in DPPH and FRAP assay, as well as in biochemical assays which provided data on antioxidant capacity in the biological environment. The highest TPC and the strongest TAA and radical scavenging activity were determined for *G. palustre* underground parts extract. The biochemical assays revealed the remarkable antioxidant capacity of *G. macrorrhizum, G. phaeum, G. robertianum,* and *G. pyrenaicum* underground parts extract, even more pronounced than that of Trolox. The antimicrobial activity of extracts against *E. coli* should be noted especially due to the resistance of this strain and common treatment failures. Based on the results, the extracts of investigated *Geranium* species with high content of polyphe-

nols, pronounced antioxidant and antimicrobial activities represent significant and rich sources of phenolics and promising natural antioxidant and antimicrobial agents with potential medicinal purposes and use as a functional food.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

This research was supported by the Ministry of Education, Science and Technological Development, Republic of Serbia (No. 451-03-68/2020-14/200161).g.

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