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Phytochemical Profiles, Antioxidant and Antimicrobial with Antibiofilm Activities of Wild Growing *Potentilla visianii* Extracts

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We investigated the phytochemical profiles, antioxidant and antimicrobial with antibiofilm activities of methanol and ethyl acetate extracts from the whole plant of *Potentilla visianii* (Vis. et Panč.) Panč. The major compounds were: epicatechin and quercetin in both extracts. Total phenolic content was determined using spectrophotometric method with Folin-Ciocalteu reagent, the concentration of flavonoids with aluminum chloride and amount of tannins with butanol-HCl reagent. The methanol extract has a significantly higher yield, as well as the content of total phenols and condensed tannins. The antioxidant activity was monitored spectrophotometrically and expressed in terms of IC_{50} (for methanol extracts 23.8 μg/mL). *In vitro* antimicrobial activity was investigated by microdilution method against 17 microorganisms. The best results for MIC <0.02 mg/mL showed ethyl acetate extract against *Staphylococcus aureus*. The antifungal activity of extracts on *Penicillium chrysogenum* was in the range of positive control. The influence on biofilm formation and preformed biofilm was different. *Escherichia coli* ATCC 25922 and *Bacillus subtilis* ATCC 6633 were more sensitive as formed biofilms. *S. aureus* ATCC 25923 could be influenced by extracts only during the biofilm formation.

Keywords: *Potentilla visianii*, Phytochemical profile, Antioxidant, Antimicrobial, Antibiofilm.

The serpentine flora of the Balkans is characterized by a relatively high degree of endemism. The greatest concentration of serpentine endemic species in the Balkans is located in the mountains of western part of the peninsula in the territories of Bosnia, Serbia, Albania and North Greece [1]. The species *Potentilla visianii* (Vis. et Panč.) Panč.[2] grows in cliffs and dry grassland, usually on serpentine North Western part of Balkan Peninsula. The extracts from the aerial and/or underground parts of *Potentilla* species have been applied in traditional medicine and exhibit antioxidant [3–6], anticariogenic [7, 8] and antimicrobial [9–12] activities. *Potentilla* extracts are presumed to be safe and cause no toxic effects when applied to human [13, 14]. Tomczyk and Latté [15] reviewed phytochemical and pharmacological profile of *Potentilla* species.

In spite of the fact that there are a lot of data connected with issue of phytomedical research on different *Potentilla* species, we have not found published data about *P. visianii*. In order to define a phytochemical profile, total phenolic content, the concentration of flavonoids, the amount of tannins were determined using spectrophotometric method, and chromatographic analysis of phenolic compounds was determined using HPLC method. Yield, concentration of total phenols, flavonoids and condensed tannins in extracts of *P. visianii* are shown in Table 1.

All investigated compounds had responses at 290 nm, where they were successfully separated. The constituents under investigation were also identified by the recorded absorption spectra, which were comparable both for extracts of *P. visianii* and standard substances. Under the described chromatographic conditions (see Experimental), this method enabled accurate resolution of the mixture of 11 phenolics commonly encountered in plant material, including those that are most abundant. This method was applied in routine measurement of the phenolic contents of *P. visianii* cultivars.

Table 1: Yield, concentration of total phenolic, flavonoid and tannin content in the extracts of *P. visianii*.

Type of extract	Yield (%)	Total phenolic content (mg GA/g of extract)*	Flavonoid content (mg RU/g of extract)*	Tannin content (mg CCh/g of extract)*
Methanol	12.6	177.2 ± 0.6	30.1 ± 0.1	50.6 ± 0.1
Ethyl acetate	3.1	62.8 ± 0.1	53.6 ± 0.3	20.8 ± 0.1

* Each value shown is the mean value ± standard deviation.

The contents of investigated phenolic compounds are expressed in μg/mg dry extract weight (FW) as a mean value ± standard deviation (Table 2).

Table 2: Content polyphenolic compound (μg/mg dry extracts).

Phenolic acid	Peak No.	t_R (min)	Methanol	Ethyl acetate
p-Coumaric acid	1	8.15	2.7 ± 0.01	10.3 ± 0.00
Chlorogenic acid	3	13.25	5.4 ± 0.01	0.4 ± 0.01
Vanilic acid	5	16.47	13.6 ± 0.01	/
Syringic acid	6	17.33	4.1 ± 0.02	/
3-HBA	7	17.56	/	4.3 ± 0.03
3,5-DHBA	9	20.61	5.7 ± 0.02	0.9 ± 0.01
Ferulic acid	10	21.91	8.3 ± 0.03	4.8 ± 0.04
Flavonoids				
Catechin	2	9.96	3.7 ± 0.01	1.0 ± 0.00
Epicatechin	4	15.86	18.8 ± 0.01	1.9 ± 0.00
Rutin	8	18.48	/	0.9 ± 0.00
Quercetin	11	24.91	10.1 ± 0.01	6.8 ± 0.08

* Each value shown is the mean value ± standard deviation.

The results for phenolic acid contents revealed that ellagic acid was the most abundant phenolic acid in all samples, followed by trans-cinnamic and chlorogenic acids. Ellagic acid can be derived from other contents, such as geraniin described in investigation of two *Geranium* species [16]. The vanilic acid content in methanol extract was 13.6 mg/g. Experimental results have indicated that epicatechin (18.8 mg/g) and quercetin are a major component of *P. visianii*, and it has been proposed to exert anti-atherogenic, anti-carcinogenic and

antioxidative properties. In this study, some flavonoids were identified and quantified as aglycones. These high amounts of epicatechin and quercetin can contribute to the health benefits of *P. visianii*, apart from the potent antioxidant and free-radical scavenging activities [17, 18]. Numerous studies have assessed quercetin and proved that it is safe compound which has various beneficial effects [19, 20].

The antioxidant activity of two extracts of *P. visianii* and the control substance as a standard parameter was monitored spectrophotometrically in the experiment and expressed in the form of IC_{50} values (μg/mL). The methanol extract showed the prominent antioxidant activity. Antioxidant activity index (AAI) is a number that indicates the success of a compound in the antioxidation effects. The tested methanol extract showed a very strong antioxidant activity (AAI > 2). The results are shown in Table 3.

Table 3: Antioxidant activity of the extracts of *P. visianii* and positive control.

Type of extract	IC_{50} value*	AAI value
Methanol	23.8 ± 0.7	3.4
Ethyl acetate	127.7 ± 0.4	0.6
Ascorbic acid	5.3	7.6

* Each value shown is the mean value ± standard deviation (μg/mL).

In vitro antimicrobial activity was investigated by microdilution method against 17 microorganisms (Table 4). In general, the tested extracts demonstrated high antimicrobial activity, having shown more potent inhibitory effects on the growth of bacteria than on fungi. There is no difference in the antimicrobial activity of extracts between Gram-negative and Gram-positive bacteria. The best results for MIC <0.02 mg/mL showed ethyl acetate extract on *Staphylococcus aureus*. The tested extracts showed low antifungal activity. The exceptions were the extracts on the species *Penicillium chrysogenum*, where the MICs were 0.6/1.3 mg/mL which was in the range of positive control.

Anti-biofilm activity was calculated in form of the biofilm inhibitory concentration (BIC) which is the concentration that showed inhibition at different percentages. The influence on biofilm formation and preformed biofilm was different. *E. coli* ATCC 25922 and *B. subtilis* ATCC 6633 were more sensitive as formed biofilms, probably due to the maturity of the biofilm. *S. aureus* ATCC 25923 could be influenced by extracts only during the biofilm formation. The antibiofilm activity was stronger in the presence of methanol extract (Table 5).

Different biological effects that show some *Potentilla* species can be explained by the large amount of condensed tannins and flavonoids, which are present in all parts of these plants [21]. For example, compounds from *Potentilla fulgens* exhibited significant anti-oxidative activity, which is in accordance to our study [22]. Extracts of *Potentilla fruticosa* possess a relatively large amount of phenolic acids and flavonoids and also show a high degree of anti-oxidative activity [3, 5-7].Literature review of antimicrobial activity of *Potentilla* species gives different conclusions. According to various authors, extracts of this genus showed strong inhibition against bacteria, possible promising potential against *Candida* and no or only very weak effects against moulds[5, 9-12]. Compared to these researches, extracts of *P. visianii* in our research showed significantly higher antibacterial activity and similar results for fungi. Different *Potentilla* species have been used in form of aqueous extracts by Tomczyk *et al*. [7]. They found the extracts to be strong against oral cariogenic streptococci in terms of biofilm formation and mutant synthesis. In other investigation *P. recta* ethyl acetate extract fraction was the most prominent, between other fractions, in activity against the streptococcal biofilm [8]. Comparing to our results the antibiofilm effects in these investigations were stronger. These results make contribution to comprehension of phytomedical investigation of species from genus *Potentilla* L. *P. visianii* show that it has potent antioxidant and antimicrobial activities and can be used as pharmaceuticals and preservatives.

Experimental

Plant material and preparation of extracts: In June 2016, the whole plants of *P. visianii* were collected, during the flowering season, from the Gočmountain, in Central Serbia. The investigated site is at352–410 m above sea level, and is centered on 43°36’76”–44°04’34”N, 20°42’46”–20°32’24”E (read by GPS Garmin-etrex, vistaHCx). Voucher specimens (17421) are deposited

Table 4: Antibacterial and antifungal activity of extracts of *P. visianii* and positive controls.

Species	Methanol extract		Ethyl acetate extract		Doxycycline/Fluconazole	
	MIC[1]	MMC[2]	MIC	MMC	MIC	MMC
Bacillus cereus	0.04	0.3	0.3	0.3	1	7.8
Bacillus subtilis ATCC 6633	0.3	0.3	1.3	1.3	2	31.3
Bacillus subtilis	0.6	0.6	1.3	1.3	0.1	2
Staphylococcus aureus ATCC 25923	0.04	0.6	0.6	0.6	0.2	3.8
Staphylococcus aureus	0.3	0.6	<0.02	0.2	0.5	7.8
Pseudomonas aeruginosa ATCC27853	2.5	5	0.1	1.3	62.5	125
Proteus mirabilis ATCC 12453	1.3	5	5	10	15.6	62.5
Escherichia coli ATCC 25922	0.3	0.6	0.2	0.2	15.6	31.3
Salmonella enterica	0.04	0.2	0.3	0.3	15.6	31.3
Rhodotorulamucilaginosa	2.5	5	5	5	31.3	500
*Candida albicans*ATCC 10231	5	5	5	5	31.3	62.5
Penicilliumchrysogenum	0.6	>10	1.3	10	1000	1000
Penicilliumitalicum	2.5	>10	2	10	250	500
*Trichodermaviridae*ATCC 13233	10	>10	5	5	500	1000
*Aspergillusflavus*ATCC 9170	10	>10	5	>10	500	500
Aspergillus fumigatus ATTC 204305	5	>10	5	>10	1000	1000
*Aspergillusniger*ATCC 16404	10	>10	10	>10	1000	1000

[1]Minimal inhibitory concentration (MIC) values are given as mg/mL for plant extracts and μg/mL for antibiotic and antimycotic; [2] Minimum microbicidal concentration (MMC) values are given as mg/mL for plant extracts and μg/mL for antibiotic and antimycotic.

Table 5: Antibiofilm activity of methanol and ethyl acetate extracts of *P. visianii* and positive control against tested bacteria.

	Biofilm formation				**Preformed biofilm**					
	Methanol extract		Ethyl acetate extract		Methanol extract		Ethyl acetate extract		Tetracycline	
Species	BIC50[1]	BIC90[2]	BIC50	BIC90	BIC50	BIC90	BIC50	BIC90	BIC50	BIC90
Salmonella enterica	3.8	4.5	6.4	9.4	7.5	9	>10	>10	<15.6	180
Escherichia coli ATCC 25922	7.5	9	7.5	9	3.8	4.5	5	8.8	36	125
Staphylococcus aureus ATCC 25923	5.4	9	1.5	4.9	>10	>10	>10	>10	250	300
Bacillus subtilis ATCC 6633	3.8	4.5	7.5	9	1.1	2.1	1.8	2.4	<15.6	15.6

[1]Minimum biofilm inhibition concentration for plant extracts given as mg/mL, for antibiotic given as μg/mL – means 50% inhibition;[2]Minimum biofilm inhibition concentration for plant extracts given as mg/mL, for antibiotic given as μg/mL – means 90% inhibition.

In Herbarium of the Institute of Botany and Botanical garden "Jevremovac", University of Belgrade (BEOU). The collected plant material was air-dried in darkness at ambient temperature. Dried, ground plant material was extracted by maceration with methanol and ethyl acetate. Briefly, 25 g of plant material was soaked with 125 mL of solvent. The plant material was macerated three times at room temperature using fresh solvent every 24 h. The filtrates obtained were combined and then evaporated to dryness using a rotary evaporator at 40°C, water extract on a water bath. The obtained extracts were kept in sterile sample tubes and stored at -20°C.

Total phenols content: The total phenols content was determined using Folin-Ciocalteu's method [23] and expressed as milligram of gallic acid equivalent per gram of extract (mg GAE/g of extract).

Total flavonoids content: The concentrations of flavonoids were determined using aluminum chloride method [24] and were expressed as milligram of rutin equivalent per gram of extract.

Proanthocyanidins content: Condensed tannins were determined using the butanol-HCl method [25] and were expressed as milligram of cyanidin chloride equivalent per gram of extract.

HPLC equipment and chromatographic analysis of phenolic compounds: HPLC analyses were performed on a Shimadzu system (Kyoto, Japan) including a vacuum degasser DGU-20A3, analytical pumps LC-20AT, manual injector 7125 and SPD-M20A diode array detector and CBM-20A system controller and with LC-Solution system manager as data processor. A reversed-phase Hypersil GOLD aQ (150 × 4.6 mm, particle size 5μm) column (Bellefonte, PA, United States) was used for separation. The mobile phase consisted of aqueous formic acid solution, pH 2.8 (A) and acetonitrile (B). Samples were eluted with the following gradient: 10% B from 0 to 2 min, from 2 to 60% B in 25 min, 60% B for 2 min, from 60 to 10% B in 1 min, and 2 min 10% B to re-establish the initial conditions, before the injection of next sample. All gradients were linear. The flow rate was 1 mL/min and the injection volume was 20 μL. Column temperature was maintained at 30 °C. Chromatograms were acquired at three different wavelengths (254, 290 and 360 nm) according to absorption maxima of analyzed compounds. Each compound was identified by its retention time and by spiking with standards under the same conditions. Each compound was quantified according to the peak area measurements, which were reported in calibration curves of the corresponding standards. Data are reported as means ± standard deviations (μg/mg dry weight) of three independent analyses.

Determination of antioxidant activity (DPPH assay): The ability of the plant extract to scavenge DPPH free radicals was assessed using the method described by Takao et al. [26], with antioxidant activity index (AAI) by Scherer and Godoy [27]. Ascorbic acid was used as a positive control. The experiment was performed in triplicate. On the basis of the obtained results are determined % inhibition of DPPH radicals, the IC_{50} value and the antioxidant activity index (AAI).The percentage of inhibition was calculated using the following equation:

$$\%\ inhibition = ((A\ control - A\ sample) / A\ control) \times 100 \quad (1)$$

where A control was the absorbance of the control sample and A sample is the absorbance of the extract.

The IC_{50} value is the effective concentration at which 50% of DPPH radicals were scavenged. It was obtained from the graph of scavenging activity (%) versus concentration of samples. The antioxidant activity index (AAI) was calculated using the following equation:

$$AAI = final\ concentration\ of\ DPPH\ (\mu g/mL) / IC_{50}\ (\mu g/mL) \quad (2)$$

The estimation of AAI was: if AAI < 0.5 – poor antioxidant activity; AAI > 0.5–1 – moderate antioxidant activity; AAI > 1–2 – strong antioxidant activity and AAI > 2 – very strong antioxidant activity.

Test microorganisms and suspension preparation: Antimicrobial activity of methanol and ethyl acetate extract was tested against 9 strains of bacteria and 8 strains of fungi. The list of tested microorganisms is presented in Table 4. All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. The other microorganisms were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac. The suspensions were prepared by the direct colony method. The turbidity of the initial suspension was adjusted using 0.5 McFarland densitometer (DEN-1, BioSan, Latvia). The initial bacterial and yeast suspensions were additionally diluted in 1:100 ratio in sterile 0.85% saline. The suspensions of fungal spores were prepared by gentle stripping of spore from agar slants with growing aspergilli. The resulting suspensions were 1:1000 diluted in sterile 0.85% saline.

Microdilution method: The antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and the minimum microbicidal concentration (MMC) using the microdilution method with resazurin [28]. The stock concentration of tested plant extracts was 20 mg/mL. Twofold serial dilutions of plant extracts were made in sterile 96-well plates containing Mueller-Hinton broth for bacteria and Sabouraud dextrose broth for fungi. The tested concentration range was from 10 mg/mL to 0.02 mg/mL. Detailed description of this method is presented in previous paper[29]. Doxycycline and fluconazole, dissolved in nutrient liquid medium, were used as positive controls. Stock solutions of crude extracts were obtained by dissolving in 10% DMSO which was used as a negative control. Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

Tissue culture plate method (TCP): The TCP assay described by Christensen et al. [30] is most widely used test for detection of biofilm formation. We screened all strains for their ability to form biofilm by TCP method with some modifications. Each test included biofilm formation control. Bacterial biofilm formation properties were well described by O'Toole et al. [31]. The tissue culture 96-well plates (Sarstedt) were prepared by dispensing 50 μL of Mueller-Hinton broth for bacteria into each well. A 50 μL from the stock solution of tested extracts (concentration of 20 mg/mL) was added into the first row of the plate for influence on biofilm formation. For preformed biofilm a 100 μL of the extract solutions were added after the biofilm incubation. Twofold, serial dilutions of extracts were performed by using a multichannel pipette. A 50 μL of fresh bacterial suspension was added to each well. Initial bacterial suspensions contain about 10^8 CFU/mL and diluted 1:100. The obtained extract concentration range was from 10 to 0.3 mg/mL. The inoculated plates were incubated at 37 °C for 48 h and another 24 h for influence on preformed biofilm. After incubation, the content of each well was gently removed by tapping the plates. The wells were washed with 200 μL of sterile 0.85% saline to remove free-floating bacteria. Biofilms formed by adherent cells in plate were stained with crystal violet (0.1% wv) and incubated at the room temperature for 20 minutes. Excess stain was rinsed off thorough washing with deionized water and plates were fixed with 200 μL of ethanol. Optical densities (OD) of stained adherent bacteria were determined with a micro ELISA plate reader at wavelength of 630 nm (OD630 nm). Only broth or broth with

extracts served as control to check sterility and non-specific binding of media. To compensate for background absorbance, OD readings from sterile medium, extracts, fixative and dye were averaged and subtracted from all test values.

Statistical analysis: Data are presented as means ± standard deviations where appropriate. All statistical analyses were performed using SPSS package. (SPSS for Windows, ver. 17, 2008) (Chicago, IL, USA).

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