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ANIBACTERIAL, ANTIBIOFILM AND ANTIOXIDANT ACTIVITY OF *Potamogeton nodosus* Poir. EXTRACTS

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ABSTRACT. Antibacterial, antibiofilm and antioxidant activity of acetone, ethanol, ethyl acetate and diethyl ether extracts of *P. nodosus* Poir. were tested.
In vitro antibacterial activity was investigated by microdilution method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) have been determined. The values were in the range from 5 to > 5 mg/ml.
Antibiofilm activity was tested by crystal violet assay. The biofilm inhibitory concentration (BIC) values were from 2.03 to > 10 mg/ml. The best results against biofilm formation had ethanol and ethyl acetate extracts.
The total phenolic content was established by using Folin-Ciocalteu method. The highest total phenol content was found in the ethyl acetate extract (28.45 mg GA/g) while the lowest content in the ethanol extract (22.74 mg GA/g). The concentrations of flavonoids were determined using aluminium chloride method. The concentration of total flavonoid content was 102.09 mg RU/g for the ethyl acetate extract, while the lowest content was noticed in diethyl ether extract (32.93 mg RU/g).
The antioxidant activity was determined by the DPPH free radicals assay. Antioxidative efficiency was in the range from 641.77 to 8811.63 μg/ml. The tested extracts showed weak antioxidant activity (AAI < 0.5).

Keywords: *Potamogeton nodosus*, phenolics, flavonoids, DPPH, antibacterial activity, biofilm.

INTRODUCTION

Longleaf pondweed (*Potamogeton nodosus* Poir., fam. Potamogetonaceae) is a species of aquatic plants. Longleaf pondweed has two different kinds of leaves. The submerged (underwater) leaves are long and thin, growing up to 50 cm long. The floating leaves are oval shaped and grow up to 12 cm long. It is widely distributed in continental and subtropical zones of both hemispheres (Europe, Asia, North America and Africa) (JOSIFOVIĆ *et al.*, 1975).

Pseudomonas aeruginosa is a common bacteria that can cause disease not only in animals but also in humans. The symptoms of such infections are generalized inflammation and sepsis. If such colonizations occur in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal. They often cannot be treated effectively with traditional antibiotic therapy, because of resistance to antibiotics.

Acetone, ethanol, ethyl acetate and diethyl ether extracts of *P. nodosus* were tested. These solvents were chosen due to their different polarity, and therefore the various possibilities of the secondary metabolites to dissolve. This analysis was first done on this plant extracts.

Biological activities of the *P. nodosus*haven't been sufficiently researched. Previous chemical investigations on different species of *Potamogeton* have shown the presence of alkaloids, flavonoids, and a labdane diterpenoid (QAIS *et al.*, 1998; RAMESH *et al*, 2014).

The objective of this study was to evaluate antibacterial activity of ethanol, ethyl acetate, acetone, diethyl ether extracts of *P. nodosus*, as well as the effect of these extracts on biofilm formation against *Pseudomonas aeruginosa* ATCC 27853 and *Pseudomonas aeruginosa.* Also the aim of this study was the determination of total phenolic content, flavonoid content and antioxidant activity by DPPH radicals scavenging capacity assay.

MATERIALS AND METHODS

Chemicals

Organic solvents (methanol, ethanol, ethyl acetate, acetone, diethyl ether) were purchased from Zorka Pharma (Šabac, Serbia). 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid and Folin-Ciocalteu phenol reagent were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Gallic acid, rutin hydrate and aluminium chloride hexahydrate ($AlCl_3x6H_2O$) were purchased from Acros Organics (New Jersey, USA). Sodium carbonate (Na_2CO_3) was obtained from MP-Hemija (Belgrade, Serbia). Dimethyl sulfoxide (DMSO) was purchased from Centrohem (Stara Pazova, Serbia). Resazurin was obtained from Alfa Aesar GmbH & Co. (KG, Karlsruhe, Germany). Nutrient agar and Mueller-Hinton broth were purchased from Liofilchem (Italy). Doxycycline was from Galenika A.D. (Belgrade, Serbia) and crystal violet stain was from Fluka AG (Buchs, Switzerland).

Plant materials

P. nodosus was collected in April 2013, from the region of Ovčar-Kablar Gorge, in the western Serbia, part of the composite valley of the West Morava River. Identification and classification of the plant material was performed at the Faculty of Science, University of Belgrade. Voucher specimens (16756) are deposited in Herbarium of the Institute of Botany and Botanical garden "Jevremovac", University of Belgrade (BEOU) (Thiers 2013). The collected plant material (stems and leaves) was air-dried in darkness at ambient temperature (20°C). The dried plant material was cut up and stored in paper bags until needed.

Preparation of plant extracts

Dried, ground plant material was extracted by maceration with acetone, ethanol, ethyl acetate and diethyl ether. Briefly, 50 g of plant material was soaked with 250 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. The amounts of crude extracts obtained from *P. nodosus* are presented in Table 1. The obtained extracts were kept in sterile sample tubes and stored at 4°C.

Determination of total phenolic content

The total phenolic content was determined using Folin-Ciocalteu method (WOOTTON-BEARD *et al.*, 2011). The reaction mixture was prepared by mixing 0.2 ml of methanolic solution of extracts (1 mg/ml) and 1.5 ml of 10% Folin-Ciocalteu reagent dissolved in water. The mixture

was allowed to equilibrate for 5 min and then it was mixed with 1.5 ml 6% Na_2CO_3 solution. After being in incubation for 90 min at room temperature and in darkness, the absorbance of the mixture was read at 725 nm against a blank using spectrophotometer. The blank was prepared with methanol instead of extract solution. The samples were prepared in triplicate and the mean value of absorbance was obtained. The same procedure was repeated for gallic acid which was used for calibration of standard curve. Total phenol content is reported as gallic acid equivalent by reference to linear equation of the standard curve. Then the total phenolic content was expressed as milligram of gallic acid equivalent per gram of extract (mg GAE/g of extract).

Determination of total flavonoid content

The concentrations of flavonoids were established by using aluminium chloride method (QUETTIER-DELEU *et al.*, 2000). The mixture contained 2 ml of methanolic solution of extract (1 mg/ml) and 2 ml of 2% methanolic $AlCl_3x6H_2O$ solution. The mixture was vigorously shaken, and after 10 min of incubation at room temperature, the absorbance versus a prepared blank was read at 430 nm using spectrophotometer. The samples were prepared in triplicate and the mean value of absorbance was obtained. Rutin was used as a standard for calibration of standard curve. The concentrations of flavonoids were calculated from the linear equation of standard curve. Then the concentrations of flavonoids were expressed as milligram of rutin equivalent per gram of extract (mg of RUE/g of extract).

DPPH radicals scavenging capacity assay

The ability of the plant extract to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals was assessed (TAKAO *et al.* 1994), along with antioxidant activity index (AAI) (SCHERER and GODOY, 2009). The stock solution of the plant extracts was prepared in methanol to achieve the concentration of 2000 mg/ml. Further, two-fold dilutions were made to obtain concentrations from 1000 mg/ml to 15.6 mg/ml. Diluted solutions of extract (2 ml each) were mixed with 2 ml of DPPH methanolic solution (80 μg/ml). After 30 min in darkness at room temperature, the absorbance was read in a spectrophotometer at 517 nm. The control samples consisted of 2 ml of methanol added to 2 ml of DPPH solution. Ascorbic acid was used as a positive control. The experiment was performed in triplicate. Scavenging activity is expressed as the inhibition percentage calculated using the following equation:

$$\text{Scavenging activity}(\%) = 100 \times [(A_{control} - A_{sample}) / A_{control})]$$

where $A_{control}$ is the absorbance of the control 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. Low IC_{50} value indicates strong ability of the extract to act as DPPH scavenger. The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated using the following equation:

$$AAI = \text{final concentration of DPPH } (\mu g/ml) / IC_{50} (\mu g/ml)$$

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 bacteria

Two bacterial strains have been tested. The clinical isolate, *Pseudomonas aeruginosa* was a generous gift from the Institute of Public Health, Kragujevac. The bacterium *P. aeruginosa*

ATCC 27853 was provided from a collection held by the Laboratory of Microbiology, Faculty of Science, University of Kragujevac.

Antibacterial assay

Antibacterial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using microdilution method with resazurin (SARKER *et al.*, 2007). Bacterial suspensions were prepared by direct colony method. The turbidity of initial suspension was adjusted by comparing it with 0.5 McFarland's standard on densitometer. Initial bacterial suspensions contain about 10^8 colony forming units (CFU)/ml and then 1:100 when diluted in sterile 0.85% saline. Twofold serial dilutions of plant extract were made in a concentration range from 5 mg/ml to 0.0195 mg/ml in sterile 96-well plates containing Mueller–Hinton broth. A 10 µl of diluted bacterial suspension was added to each well to give a final concentration of 5 x 10^5 CFU/ ml. Finally, 10 µl of resazurin solution, as an indicator of microbial growth, was added to each well. The inoculated plates were incubated at 37°C for 24 h. MIC was defined as the lowest concentration of tested compounds that prevented resazurin color change from blue to pink. MBC was determined by plating 10 µl of samples from wells, where no indicator of color change was recorded, on nutrient agar. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as minimum bactericidal concentration.

Doxycycline, dissolved in nutrient liquid medium was used as positive control. Solvent control test was performed in order to study an effect of 10% DMSO on the growth of bacteria. It was observed that 10% DMSO did not inhibit the growth of bacteria. Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

Biofilm formation assay and quantification

The ability of bacteria to form biofilms was assayed as described by O'TOOLE and KOLTER (1998) with some modifications. In sterile 96-well tissue culture plates (Sarstedt, Germany) containing 50 µl of Mueller–Hinton broth per well, a 50 µl of fresh bacterial suspension (1.0 McFarland) was added. After incubation at 37°C for 48 h, the content of each well was gently removed by tapping the plates. The wells were washed with 200 µl of sterile saline to remove free-floating bacteria. Biofilms formed by adherent cells in plate were stained with 0.1% crystal violet and incubated at the room temperature for 20 min. Excess stain was rinsed off by thorough washing with deionized water and plates were fixed with 200 µl of 96% ethanol. Optical densities (OD) of stained adherent bacteria were measured at 630 nm using an ELISA microplate reader. The cut-off optical density (ODc) was defined as three standard deviations above the mean OD of the negative control (culture medium). Strains were classified as follows: OD ≤ ODc no biofilm producer, ODc < OD ≤ 2 × ODc weak biofilm producer, 2 × ODc < OD ≤ 4 × ODc moderate biofilm producer and 4 × ODc < OD strong biofilm producer (STEPANOVIĆ *et al.*, 2000).

Effect on biofilm formation

A modified crystal violet assay was employed to test the effect of plant extracts on biofilm formation. Twofold serial dilutions of plant extract were made in sterile 96-well tissue culture plates containing 50 µl of Mueller–Hinton broth per well. The tested concentration range was from 10 mg/ml to 0.039 mg/ml. A 50 µl of fresh bacterial suspension (1.0 McFarland) was added to each well. Growth control (cells + broth), media control (only broth) and blank control (broth + extract) were included. After being in incubation at 37°C for 48 h, the biofilm biomass was assayed using the crystal violet staining assay as described above. The percentage of biofilm inhibition was calculated using the following formula: [(OD growth control – OD sample) / OD growth control] × 100. The biofilm inhibition concentration (BIC_{50}) was defined as the lowest

concentration of extract that showed 50% inhibition on the biofilm formation (CHAIEB *et al*., 2011).

RESULTS AND DISCUSSION

Total phenol and flavonoid content in plant extracts

Total phenol and flavonoid content was determined in acetone, ethanol, ethyl acetate and diethyl ether extract of *P. nodosus*. Various solvents were used in order to achieve extraction of active substances with diversity in their polarity. The content of phenolic compounds was measured spectrophotometrically by using method with Folin-Ciocalteu reagent for phenols and method with aluminium chloride for flavonoids. The results are presented in Table 1. The highest total phenol content was found in the ethyl acetate extract (28.45 mg GA/g) while the lowest content was in the ethanol extract (22.74 mg GA/g), and the similar case was with the total flavonoid content, up to 102.09 mg RU/g for the ethyl acetate extract, while the lowest content was noticed in diethyl ether extract (32.93 mg RU/g). Genus *Potamogeton* was manifested by different flavonoid glycoside distributions between floating and submersed foliage of morphologically heterophyllous species and according to LES and SHERIDAN (1990) it had been reported for the first time.

Table 1. Yield, content of total phenolic and flavonoid content in the extracts of *Potamogeton nodosus.*

Type of extract	Yield (g/50 g dw)	Total phenolic content[1] (mg GA/g of extract)	Flavonoid content[1] (mgRU /g of extract)
Ethanol	2.78	22.74 ± 0.12	47.96 ± 0.10
Acetone	1.32	24.20 ± 2.28	69.10 ± 0.19
Ethyl acetate	1.24	28.45 ± 0.19	102.09 ± 1.10
Diethyl ether	0.74	26.28 ± 0.26	32.93 ± 0.06

[1]Each value shown is the mean value ± standard deviation.

Antioxidant activity

The antioxidant activity of different plant extracts of *P. nodosus* is determined by use of methanol solution of DPPH reagent. DPPH method is used in the study of antioxidant effects of complex biological compounds and of their ability to reduce the free radicals' activity. Activity is measured as a decrease in absorbance of the sample relative to the standard solution of DPPH. This research of *P. nodosus* extracts has not been investigated until now.

The results expressed in the form of IC_{50} values are shown in Table 2. Antioxidative efficiency was in the range from 641.77 to 8811.63 μg/ml. The ethanol extract with the IC_{50} value at 641.77 μg/ml showed the most active reaction. Antioxidant activity index (AAI) is a number that indicates the success of a compound in the effects of antioxidation. The tested extracts showed weak antioxidant activity (AAI<0.5), while the control substance, as expected, showed a very strong antioxidant activity (AAI>2).

Antibacterial activity

The results of *in vitro* antibacterial activities of acetone, ethanol, ethyl acetate and diethyl ether extracts of *P. nodosus* are shown in Table 3. For comparison, the tables also give the results of the activities of doxycycline. The solvent (10% DMSO) had no effect on the growth of the tested microorganisms. Antimicrobial activities of tested extracts were assessed by determining the MIC and MBC values of these species of microorganisms.

Table 2. Antioxidant activity of the extracts from *P. nodosus* (IC_{50} and AAI).

Type of extract	$IC_{50value\ (\mu g/ml)}$[1]	AAI value
Ethanol	641.77 ± 7.12	0.13
Acetone	1066.61 ± 128.99	0.08
Ethyl acetate	8811.63 ± 46.48	0.01
Diethyl ether	1016.26 ± 12.49	0.08
Ascorbic acid	5.25	15.24

[1]Each value shown is the mean value ± standard deviation.

Table 3. Antibacterial activity of extracts from *P. nodosus.*

Species	Ethanol extract		Acetone extract		Ethyl-acetate extract		Diethyl ether extract		Doxycycline	
	MIC[1]	MBC[2]	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
P. aeruginosa	>5	>5	5	>5	>5	>5	5	>5	250	>250
P. aeruginosa ATCC 27853	>5	>5	5	>5	>5	>5	5	>5	62.5	125

[1]MIC values for plant extracts are given as mg/ml, for antibiotic as µg/ml – means inhibitory activity.
[2]MBC values for plant extracts are given as mg/ml, for antibiotic as µg/ml – means bactericidal activity.

The values of minimum inhibitory concentrations and minimum bactericidal concentrations obtained in this experiment were 5 mg/ml and > 5 mg/ml. Efficacy of antibacterial activity depended on both the type of extract and the strains of tested bacteria. Among the tested extracts, the diethyl ether and acetone showed the greatest inhibitory effects against the tested bacteria, although the overall effectiveness was weak. Extractives from ethanol extract of *P. nodosus* did not inhibit *Pseudomonas aeruginosa* according to ALAM *et al.* (1999).

Antibiofilm activity

The results of *in vitro* antibiofilm activity of acetone, ethanol, ethyl acetate and diethyl ether extract of *P. nodosus* are presented in Table 4. The bacteria used in this part of investigation have been selected as the bacteria used for antibacterial activity with their biofilm formation potential.

The influence on biofilm formation varied among the tested strains and between the extracts. Both strains formed thick biofilm (strong producers). Completely different than the results for planktonic bacterial cells (MIC and MBC), the best results against biofilm had ethanol and ethyl acetate extracts. It is obvious that bacterial cells inside the biofilm are different and act differently than the free floating bacteria, which is already known (DONLAN, 2002). The BIC

values varied from 2.03 to > 10 mg/ml. Antibiofilm properties of *P. nodosus* extracts of any kind have not been investigated before this evaluation.

Table 4. Antibiofilm activity of extracts from *P. nodosus.*

	Ethanol extract		Acetone extract		Ethyl acetate extract		Petroleum ether extract	
Species	**BIC_{50}[1]**	**BIC_{90}[2]**	**BIC_{50}**	**BIC_{90}**	**BIC_{50}**	**BIC_{90}**	**BIC_{50}**	**BIC_{90}**
P. aeruginosa	2.03	>10	3.32	6.3	4.79	>10	4.53	>10
P. aeruginosa ATCC 27853	9.75	>10	>10	>10	7.95	>10	>10	>10

[1]BIC_{50} values for plant extracts are given as mg/ml – means 50% inhibitory activity.
[2]BIC_{90} values for plant extracts are given as mg/ml – means 90% inhibitory activity.

CONCLUSION

P. nodosus was extracted by four different solvents. Extracts have shown various results in phytochemical analysis. The highest total phenol content was found in the ethyl acetate extract (28.45 mg GA/g) while the lowest content was in the ethanol extract (22.74 mg GA/g), and the similar case was with the total flavonoid content. Antioxidative efficiency was in the range from 641.77 to 8811.63 μg/ml. The ethanol extract with the IC_{50} value at 641.77 μg/ml showed the most active reaction. Antibacterial potential of extracts was weak, as well as antibiofilm potential, but 50% biofilm inhibitory concentrations were prominent. Altogether *P. nodosus* extracts should be investigated further.

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