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EXCLI Journal 2015;14:496-503 – ISSN 1611-2156
Received: November 03, 2014, accepted: March 02, 2015, published: March 30, 2015

Original article:

PHYTOMEDICAL INVESTIGATION OF *NAJAS MINOR* ALL. IN THE VIEW OF THE CHEMICAL CONSTITUENTS

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http://dx.doi.org/10.17179/excli2014-662

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ABSTRACT

Plants are an abundant natural source of effective antibiotic compounds. Phytomedical investigations of certain plants haven't still been conducted. One of them is *Najas minor (N. minor)*, an aquatic plant with confirmed allelopathy. Research conducted in this study showed the influence of water and ethyl acetate extracts of *N. minor* on microorganisms, in the view of chemical profiling of volatile constituents and the concentrations of total phenols, flavonoids and tannins. Antimicrobial activity was defined by determining minimum inhibitory and minimum microbicidal concentrations using microdilution method. Influence on bacterial biofilm formation was performed by tissue culture plate method. The total phenolics, flavonoids and condensed tannins were determined by Folin-Ciocalteu, aluminum chloride and butanol-HCl colorimetric methods. Chemical profiling of volatile constituents was investigated by GC and GC-MS. Water extract didn't have antimicrobial activity below 5000 µg/mL. Ethyl acetate extract has shown strong antimicrobial activity on G+ bacteria - *Staphylococcus aureus* PMFKGB12 and *Bacillus subtilis* (MIC < 78.13 µg/mL). The best antibiofilm activity was obtained on *Escherichia coli* ATCC25922 (BIC50 at 719 µg/mL). Water extract had higher yield. Ethyl acetate extract had a significantly greater amount of total phenolics, flavonoids and tannins. As major constituent hexahydrofarnesyl acetone was identified. The ethyl acetate extract effected only G+ bacteria, but the biofilm formation of G- bacteria was suppressed. There was a connection between those *in vivo* and *in vitro* effects against pathogenic bacterial biofilm formation. All of this points to a so far unexplored potential of *N. minor*.

Keywords: *Najas minor,* antimicrobial, antibiofilm, total phenolic content, flavonoid and tannin content, volatile constituents

INTRODUCTION

Najas minor All. 1785 Fl. Pedem. 2:221 belongs to the family Najadaceae. *N. minor* is aquatic, submersed, invasive plant that grows in fresh water (calm waters such as rivers, slow-flowing aquatic ecosystems, ponds and lakes) and it's widespread in central and southern Europe, northern and tropi-

cal Africa and Asia. It is usually found in depths of 0.6-4.5 m and prefers to live in moderately warm waters (8 °C at the coldest) and can live in the hot summer water too. Stem is to 40 cm long with branched shells. The leaves are opposite, narrow and toothed, up to 2 cm, and the flowers are a unipolar, individually or few in axils list. It's a true annual, grows anew from seeds each spring. Seeds form in the leaf axils from July through September (Josifović, 1974).

Relevant literature review has shown little data about the biological activity of *N. minor*. The most commonly study is its allelopathic effect in the community where it is located. The allelopathic effect of different extracts of *N. minor* have been studied on other plants (El-Shahawy, 2012) or some harmful algae (He et al., 2008; Wang et al., 2010).

Since the other biological activities of *N. minor* have not been investigated so far, the aim of this study was this plant's antimicrobial and antibiofilm activity applying *in vitro* methods, also the determination of total phenol, flavonoid and tannin content from water and ethyl acetate extracts. For seeing the substances possibly responsible for the activity, the chemical profiling of volatile constituents of *Najas minor* ethyl acetate extract has been set.

MATERIALS AND METHODS

Chemicals

All chemicals were commercially available and used as received. A Mueller–Hinton broth, which was used as nutrient liquid medium, was obtained from Liofilchem (Italy); Sabouraud dextrose broth from Torlak (Belgrade, Serbia); an antibiotic, tetracycline from Sigma Chemicals Co. (St. Louis, MO, USA) and antimycotic, fluconazole, was purchased from Pfizer Inc. (USA).

Plant material

In June-July 2012, of whole plant *N. minor* were collected, in Međuvršje Reservoir, central Serbia (position: 43°51´- 43°56´ N, 17°47´- 17°55´ E, altitude: 273m). Identification and classification of the plant material was performed at the Institute of Botany and Botanical garden "Jevremovac", University of Belgrade (No. 16758). Voucher specimens are deposited in Herbarium of the Institute of Botany and Botanical garden "Jevremovac", University of Belgrade (BEOU) (Thiers, 2013; continuously updated). The collected plant material was air-dried in darkness at ambient temperature.

Preparation of plant extracts

Dried, ground plant material was extracted by maceration with water and ethyl acetate. Firstly, we immersed 50 g of plant material in 250 mL of solvent. Then, the plant material was moistened by fresh solvent at ambient temperature on a daily basis – one time for three days. Afterwards, thus gained filtrates were combined and then put into rotary evaporator at 40 °C to dry. Final extracts were held in sterile sample tubes and conserved at -20 °C.

In vitro antimicrobial assay

Table 1 shows the list of tested microorganisms. Complete clinical isolates were donated by the Institute of Public Health in Kragujevac. Other microorganisms used were from the collection of Microbiology Laboratory of Faculty of Science, University of Kragujevac.

The direct colony method was used in preparation of bacterial and yeast suspensions. The regulation of initial suspension turbidity was conducted by comparison with 0.5 McFarland's standard (Andrews, 2005). Around 10^8 colony forming units (CFU)/mL are held within initial bacterial suspensions. In addition, 1:100 dilutions of initial suspension were made into sterile 0.85 % saline. The process of preparing the suspensions of fungal spores included gentle stripping of spore from agar slants with growing aspergilli. The outcome were the suspensions 1:1000 diluted in sterile 0.85 % saline.

The process of testing antimicrobial activity included determination of minimum inhibitory concentration (MIC) and mini-

EXCLI Journal 2015;14:496-503 – ISSN 1611-2156
Received: November 03, 2014, accepted: March 02, 2015, published: March 30, 2015

mum bactericidal concentration (MBC) which was conducted by microdilution method with resazurin (Sarker et al., 2007). Tested plant extracts were dissolved in 10 % DMSO to a concentration of 10000 µg/mL. Sterile 96-well plates were used for making twofold serial dilutions of plant extracts with liquid media for microorganisms. The range of tested concentration was from 5000 to 78.13 µg/mL. Detailed description of this method is presented in previous paper (Vasić et al., 2012). As positive controls we used tetracycline for bacteria and fluconazole for fungi. As negative control we used 10 % DMSO which did not influence the growth of microorganism. Growth control and sterility control were present in all tests. Also, the tests were carried out in duplicate and MICs were constant.

Tissue culture plate method (TCP)

The TCP assay described by Christensen et al., (1985) 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., (2000). Antibiofilm activity was calculated in form of the biofilm inhibitory concentration (BIC) at 50 and 90 percent.

The tissue culture 96-well plates (Sarstedt) were filled with Mueller–Hinton broth for bacteria at 50 µL per well. Then extract stock solution (10000 µg/mL), that was tested, was filled in plate's first row and twofold serially diluted. A 50 µL of fresh bacterial suspension was added to each well. Initial bacterial suspensions contain about 10^8 CFU/mL. 1:100 dilutions of initial suspension were additionally prepared into Mueller–Hinton broth. The obtained extract concentration range was from 5000 to 78 µg/mL. After 24 h at 37 °C of incubation, for inoculated plates, the plates were leisurely tapped in order to remove the present content. Sterile 0.85 % saline was used for washing the wells and removing the unattached bacteria. Empty plates containing only biofilms were filled with 0.1 % w/v crystal violet solution for staining 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. All test values were reduced by OD numbers read for sterile medium with extracts (fixed and dyed) in order to recompense the background absorbance.

Phytochemical analysis

Determination of total phenolic content

Folin-Ciocalteu's method was used in determining the concentrations of total phenols (Wootton- Beard et al., 2011). The preparation of samples was performed in triplicate and the mean value of absorbance was calculated. Gallic acid was used as a standard for calibration of standard curve. The linear equation of standard curve ($y = 0.008x + 0.0077$, R2 = 0.998) was used in calculating the total phenolic content. Finally, the total phenolic content was shown by form - milligram of gallic acid equivalent per gram of extract (mg of GAE/g of extract).

Determination of total flavonoid content

Aluminium chloride method was used in determination of the concentrations of flavonoids (Quettier-Deleu et al., 2000). The preparation of samples was performed in triplicate and the mean value of absorbance was calculated. Rutin was used as a standard for calibration of standard curve. The linear equation of standard curve ($y = 0.021x + 0.040$, R2 = 0.999) was used in calculating the concentrations of total flavonoids. Finally, the concentrations of total flavonoids were shown by form - milligram of rutin equivalent per gram of extract (mg of RU/g of extract).

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Determination of condensed tannins (proanthocyanidins)

Butanol-HCl method was used in determination of condensed tannins (Porter et al., 1986). The preparation of samples was performed in triplicate and the mean value of absorbance was calculated. Cyanidin chloride was used as a standard for calibration of standard curve. The linear equation of standard curve (y ¼ 0. 0094 x þ 0.006, R2 ¼ 0.999) was used in calculating the concentrations of proanthocyanidins. Finally, the concentrations of proanthocyanidins were shown by form - milligram of cyanidin chloride equivalent per gram of extract (mg of CChE/g of extract).

GC-FID and GC-MS analyses

Chemical composition of the extract was conducted by GC and GC-MS. The GC-MS analyses (in triplicate) were carried out on a HP 6890N gas chromatograph coupled with a 5975C mass selective detector using a DB-5 capillary column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies, USA). The injector and interface temperature were 250 and 320 °C, respectively, while the oven temperature was programmed from 70 to 315 °C at a rate of 5 °C/min and then held isothermally for 10 min. Helium was used as a carrier gas at flow rate of 1.0 ml/min, while 1 µl of the extract solutions were injected in a pulsed split mode in a split ratio of 40:1. The MS was operated at ionization energy of 70 eV in a acquisition mass range of 35–650 amu, with a scanning period of 0.34 s. The same experimental conditions as described above were used for the GC-FID analyses.

The extract constituents were identified

(i) by linear retention indices matching (calculated relative to the retention times of C8–C34 n-alkanes (Van den Dool and Kratz, 1963) to those reported in the literature (Adams, 2007),
(ii) by comparison of mass spectra to those of authentic standards, as well as those from Wiley 6, NIST05 and MassFinder 2.3 library and homemade MS library with the spectra corresponding to pure compounds and
(iii) by coinjection, wherever possible, with an authentic sample. The percentage composition was obtained by integration of the GC-FID peak area without the use of correction factors.

Data analysis

All data were presented as means ± standard deviations (mean ± SD) where appropriate. All statistical analyses were performed using Microsoft Excel software.

RESULTS AND DISCUSSION

The results of *in vitro* antimicrobial activity of two extracts from *N. minor* against 27 strains of bacteria and fungi, with control results, determined by microdilution method, is presented in Table 1.

The intensity of antimicrobial action varied depending on the type of plant extract and on the groups of microorganisms. MICs and MMCs values were in range from < 78.13 µg/mL to > 5000 µg/mL. Water extract didn't had antimicrobial activity below 5000 µg/mL. The ethyl acetate extract has shown the strongest antimicrobial activity on G+ bacteria while the activities on other species were moderate. The tested extract showed high antibacterial activity against species from the genus *Bacillus*. MICs values were in range from < 78.13 µg/mL to 625 µg/mL. Significant effect extract showed in food spoilage isolate *Staphylococcus aureus*, and clinical isolate and standard strain of *S. aureus*. The influence on G- bacteria was either very low or was not observed at all within the tested concentrations (MIC and MMC ranged from 5000 µg/mL to > 5000 µg/mL). The influence of tested extracts on fungi was generally weak. The exception is the ethyl acetate extract on the species *Aspergillus restrictus* (MIC and MMC were < 78.13 µg/mL).

Ethyl acetate extract had influenced the formation of bacterial biofilm (Table 2). The maximum concentrations of extract where the biofilm could develop were interesting.

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Table 1: Antimicrobial activity of water and ethyl acetate extracts of *N. minor* against tested microorganisms based on microdilution method

Species	Water extract		Ethyl-acetate extract		Tetracycline / Fluconazole	
	MIC[1]	MMC[2]	MIC	MMC	MIC	MMC
Bacillus subtilis	> 5000	> 5000	< 78.13	< 78.13	<0.5	<0.5
***Bacillus pumilus* NCTC 8241**	> 5000	> 5000	625	5000	<0.5	<0.5
Bacillus cereus	> 5000	> 5000	625	5000	<0.5	3.906
Staphylococcus aureus	> 5000	> 5000	156.25	> 5000	0.976	15.62
***S. aureus* PMFKGB12**	> 5000	> 5000	< 78.13	> 5000	0.122	1.953
***S. aureus* ATCC 25923**	> 5000	> 5000	312.50	> 5000	0.244	1.953
Enterococcus faecalis	> 5000	> 5000	> 5000	> 5000	7.812	125
***E. faecalis* ATCC 29212**	> 5000	> 5000	5000	> 5000	7.812	62.5
Pseudomonas aeruginosa	> 5000	> 5000	5000	> 5000	500	1000
***P. aeruginosa* ATCC 27853**	> 5000	> 5000	5000	> 5000	7.812	62.5
Proteus mirabilis	> 5000	> 5000	5000	> 5000	500	500
***P. mirabilis* ATCC 12453**	> 5000	> 5000	5000	> 5000	125	125
Escherichia coli	> 5000	> 5000	> 5000	> 5000	1.953	3.906
***E. coli* ATCC 25922**	> 5000	> 5000	> 5000	> 5000	0.976	3.906
Salmonella enterica	> 5000	> 5000	> 5000	> 5000	1.953	3.906
Salmonella typhimurium	> 5000	> 5000	> 5000	> 5000	0.976	3.906
Rhodotorula sp.	5000	> 5000	1250	2500	62.50	1000
Candida albicans	5000	> 5000	5000	> 5000	62.50	1000
***C. albicans* ATTC 10231**	5000	> 5000	5000	> 5000	31.25	1000
Saccharomyces boulardii	> 5000	> 5000	5000	> 5000	31.25	1000
Penicillium italicum	> 5000	> 5000	> 5000	> 5000	1000	1000
Penicillium digitatum	> 5000	> 5000	5000	> 5000	31.25	31.25
Aspergillus restrictus	> 5000	> 5000	< 78.13	< 78.13	500	2000
Aspergillus flavus	5000	5000	5000	> 5000	1000	1000
Aspergillus niger	5000	> 5000	5000	> 5000	500	1000
***A. niger* ATCC 16404**	5000	> 5000	5000	> 5000	62.5	62.5
Botrytis cynerea	5000	> 5000	5000	> 5000	31.25	500

[1] MIC values for plant extracts and antibiotic are given as µg/mL – means inhibitory activity;
[2] MMC values for plant extracts and antibiotic are given as µg/mL – means microbicidal activity

Table 2: Antibiofilm activity of ethyl acetate extracts of *N. minor* against tested microorganisms

Species	Ethyl acetate extract		Tetracycline	
	BIC50[1]	BIC90[2]	BIC50	BIC90
***Escherichia coli* ATCC 25922**	719	1262	36	125
Proteus mirabilis	1238	4478	42	666
Pseudomonas aeruginosa	3344	5284	746	2000
***Pseudomonas aeruginosa* ATCC 27853**	1058	6111	156	305

[1] Minimum biofilm inhibition concentration for plant extracts and antibiotic are given as µg/mL – means 50 % inhibition on the biofilm formation;
[2] Minimum biofilm inhibition concentration for plant extracts and antibiotic are given as µg/mL – means 90 % inhibition on the biofilm formation

In this case *Escherichia coli* ATCC 25922 biofilm formation was the most sensitive of all tested strains (BIC90 at 1262 µg/mL) and the less sensitive was *Pseudomonas aeruginosa* ATCC 27853 in forming the biofilm (BIC90 at 6111 µg/mL).

The percent yields of crude extracts, concentration of total phenolic and flavonoid obtained from *N. minor* are presented in Table 3. Although with noticeably lower yield than water extract, ethyl acetate extract has in itself a significantly greater amount of total phenolics and flavonoids. In Table 3 is presented concentration of tannins in the both extracts of *N. minor*. As with the previous (total phenols) much higher amount of the tannins is in the ethyl acetate extract.

Table 4 lists the identified volatile constituents of *N. minor* ethyl acetate extract by means of a detailed GC and GC/MS. The analyses allowed the identification of 23 volatile compounds, accounting for 87.3 % of the detected GC peak areas. The most abundant constituents were hexahydrofarnesyl acetone, (*E*)-phytol and neophytadiene (isomer I) (17.6, 11.9 and 10.1 %, respectively).

Since, until now, antibiofilm and antimicrobial activity was not performed, as well as the content of total phenols, flavonoids and tannins in the extracts of *N. minor,* obtained results can be considered in relation to the different chemical compounds isolated from this plant. It is noticed that the ethyl acetate extract had no effect below 5000 µg/mL in MIC for G-bacteria (Table 1). The results have pointed that the ethyl acetate extract had microdilution effect only on G+ bacteria, probably because of the difference in cell wall structure, but the biofilm formation of G-bacteria was suppressed (Table 2).

These results are showing the potential of this allelopathically confirmed plant. We have noticed that there is some connection between those *in vivo* effects and *in vitro* effects against biofilm formation by pathogenic bacteria that were tested in this investigation. It can be a starting point for further examination.

Significant antimicrobial activity on most G+ bacteria can be attributed to the action of hexahydrofarnesyl acetone. Hexahydrofarnesyl acetone, the most abundant volatile in our extract, was already proposed to be a potential antimicrobial agent (Radulović et al., 2006). The same compound was also the main volatile constituent of the essential oil isolated from the aquatic plant *Sagittaria trifolia* (Xiangwei et al., 2006). The antimicrobial assays of the essential oil isolated from this plant also showed the significant antimicrobial activity on G+ bacteria (Xiangwei et al., 2006). Some of the terpenoids identified in our sample, such as phytol, have been reported in the literature for their recognized antimicrobial properties (Inoue et al., 2005). The terpenoids also could be responsible and contribute to the observed activity since it was known that these compounds possess the antimicrobial properties.

Other authors by studying allelopathic activity of *N. minor* also found that the water extract had significantly less activity than methanol extract (El-Shahawy, 2012) or ethyl acetate fraction (Wang et al., 2010).

Water and methanol extracts isolated from the whole plant of *N. minor* showed significant allelopathic effect in suppressing

Table 3: Yield, concentration of total phenolic, flavonoid and tannin content in the extracts of *N. minor*

Type of extract	Yield (% dw)	Total phenolic content* (mg GA/g of extract)	Flavonoid content* (mgRU/g of extract)	Tannin content* (mgCCh/g of extract)
Water	7.70	2.46 ± 0.11	1.25 ± 0.03	0.10 ± 0.09
Ethyl acetate	1.54	20.58 ± 0.11	89.72 ± 0.61	1.93 ± 1.83

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

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Table 4: Volatile constituents of *N. minor* ethyl acetate extract

RI calc.[a]	Compound	Content [%][b]	Identification Method
1535	Dihydroactinidiolide	1.5	RI,MS
1700	Heptadecane	t	RI,MS,Co-GC
1727	Methyl tetradecanoate	t	RI,MS,Co-GC
1772	Loliolide	t	MS
1800	Octacosane	t	RI,MS,Co-GC
1842	Neophytadiene (isomer I)	10.1	RI,MS
1846	Hexahydrofarnesyl acetone	17.6	RI,MS
1866	Neophytadiene (isomer II)	3.4	RI,MS
1884	Neophytadiene (isomer III)	7.9	RI,MS
1900	Nonadecane	2.1	RI,MS,Co-GC
1928	Methyl hexadecanoate	6.1	RI,MS,Co-GC
1996	Ethyl hexadecanoate	t	RI,MS,Co-GC
2000	Eicosane	t	RI,MS,Co-GC
2096	Methyl linoleate	8.3	RI,MS
2100	Heneicosane	7.9	RI,MS,Co-GC
2102	Methyl linolenate	7.0	RI,MS
2106	γ-Hexadecalactone	t	RI,MS
2117	(*E*)-Phytol	11.9	RI,MS,Co-GC
2128	Methyl octadecanoate	t	RI,MS,Co-GC
2200	Docosane	t	RI,MS,Co-GC
2220	(*E*)-Phytyl acetate	t	RI,MS
2300	Tricosane	2.2	RI,MS,Co-GC
2353	5-Methyl-5-(4,8,12-trimethyltridecyl)dihydro-2(3*H*)-furanone	1.3	RI,MS
3292	Unidentified component[c]	2.4	
3353	Unidentified component[c]	7.2	
	Identified	87.3	
	Unidentified (> 0.5 %)	9.6	
	Total	96.9	

[a] Linear retention indices experimentally determined on the DB-5 column; [b] Values are means of three individual analyses; RI – Retention indices matching with literature data; MS – mass spectra matching; Co-GC – co-injection with pure reference compound; [c] MS, 70eV, 230 ºC: RI 3292, *m/z* (rel. int.): 412 (73), 271 (53), 205 (57), 159 (55), 145 (55), 105 (55), 83 (86), 81 (84), 69 (71), 55 (100); RI 3353, *m/z* (rel. int.): 414 (100), 145 (73), 107 (73), 105 (78), 95 (68), 91 (61), 81 (66), 57 (63), 55 (69), 43 (99); t – Trace amounts (<0.05 %).

seedling root and shoot growth of the assayed species (*Lolium perenne, Corchorus olitorius* and *Amaranthus viridis*) (El-Shahawy, 2012). The results of the other study showed that the ethyl acetate fraction of *N. minor* aqueous extracts had activity to inhibit the growth of *Microcystis aeruginosa* (Wang et al., 2010).

CONCLUSION

Although the antimicrobial and antibiofilm activity of *N. minor* have not been investigated so far, the study showed that this plant has notable antimicrobial effect on G+ bacteria and that it reduces the forming of biofilm at G- bacteria. Ethyl acetate extract had great amounts of phenols and basic volatile substance was identified as hexahydro-

farnesyl acetone. All of this points to a so far unexplored potential of *N. minor*.

Acknowledgements

This investigation was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant No. III 41010, OI 173032 and OI172061. The authors declare that they have no conflict of interest.

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