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Bioactive extracts of *Gentiana asclepiadea*: antioxidant, antimicrobial, and antibiofilm activity

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ABSTRACT: Extracts of the aerial parts and roots of the wild-growing medicinal plant *Gentiana asclepiadea* were analysed for their antimicrobial, antibiofilm, and antioxidant activity with quantification of the total phenolic and total flavonoid content. Antimicrobial activity was tested against pathogenic and spoilage bacteria, yeasts, and moulds using the microdilution method. The strongest antibacterial activity was detected on *Bacillus* species, where minimum inhibitory concentrations (MICs) of from 0.16 mg/mL to 5 mg/mL were obtained, while antifungal activity was low to moderate, with MICs between 1.25 and 20 mg/mL. In the crystal violet assay, the extracts inhibit 50% biofilm formation in the concentration range of from 2.12 to 37.04 mg/mL. *Staphylococcus aureus*, *S. aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 biofilms were the most sensitive to the presence of extracts. The extracts rich in phenolic compounds showed good DPPH-scavenging activity, with EC_{50} values between 181.3 and 614.3 μg/mL for extracts of aerial parts and from 426.67 to >1000 μg/mL for root extracts. Even though *G. asclepiadea* has long been traditionally used, its biological activity is still insufficiently explored, so the obtained results are significant for contributing new knowledge about the plant's medicinal properties.

Keywords: *Gentiana asclepiadea*, extracts, antimicrobial agents, antioxidants, biofilm

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INTRODUCTION

Gentiana asclepiadea L. (fam. Gentianaceae) is a known medicinal plant. The root of *G. asclepiadea* has been traditionally used as medicine for hepatitis A virus infections or as a gastric stimulant (Sarić 1989). On the basis of their chemical composition, Szücs *et al*. (2002) have suggested that the roots of *G. asclepiadea* could be a potential replacement for *G. lutea*, a well-known medicinal plant and spice. Also, because of its bitter taste, *G. asclepiadea* is used as a flavouring agent in food and beverages. In recent years, several studies have indicated biological activities of *G. asclepiadea* (Nićiforović *et al*. 2010; Mihailović *et al*. 2011, 2013; Hudecova *et al*. 2012a, b, c). The biological activities of plants depend on the content and composition of bioactive compounds. The main bioactive compounds detected in extracts of *G. asclepiadea* are: secoiridoid glycosides (swertiamarin, gentiopicroside, sweroside), xanthone-C-glycosides (mangiferin), alkaloids, flavones-C-glucosides (isovitexin, homoorientin), and phenolic acids (Kitanov & Spasov 1992; Hudecova *et al*. 2012b; Mihailović *et al*. 2013).

Considering the fact that the content of plant bioactive compounds depends on climate, environmental conditions, and geographical location, we assumed that plant material collected from characteristic arid limestone substrates should be rich in biologically active

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compounds. In addition, because only methanolic and *n*-butanolic extracts have been studied in terms of antimicrobial and antioxidant activity, aqueous, ethanol, ethyl acetate, acetone, and diethyl ether extracts were chosen for evaluation in this paper. Accordingly, the aims of the present study were to evaluate and compare the antimicrobial, antibiofilm, and antioxidant activity of different extracts from roots and aerial parts of wild-growing *G. asclepiadea* and determine the total phenol and total flavonoid content in the extracts.

MATERIAL AND METHODS

Plant materials. In August of 2012, aerial parts and roots of *G. asclepiadea* were collected in the Mokra Gora Mountains of Southwest Serbia (position: 42˚ 51' N, 20˚ 27' E, altitude: 1550 m, exposure: SE, habitat: dry, sunny, rocky land). Identification of the plant material was done by Prof. Dr. Dragana Pavlović-Muratspahić. The voucher sample is deposited in the herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia. Plant extracts were prepared by maceration with water, ethanol, ethyl acetate, acetone, and diethyl ether. After filtration, the solvent was removed using a rotary evaporator under reduced pressure at 45°C.

Determination of total phenol and flavonoid content. Total phenol content (Wootton-Beard *et al.* 2011) and total flavonoid content (Quettier-Deleu *et al.* 2000) were determined spectrophotometrically and the results expressed as gallic acid equivalents (mg of GAE/g of extract) and rutin equivalents (mg of RUE/g of extract), respectively.

DPPH radical-scavenging capacity assay. The ability of *G. asclepiadea* aerial part and root extracts to scavenge DPPH free radicals was assessed using the method described by Takao *et al.* (1994). The tested concentration range of plant extracts was from 15.62 μg/mL to 1000 μg/mL. Ascorbic acid was used as a positive control. Radical-scavenging activity is expressed as the EC_{50} value, calculated from the nonlinear graph of scavenging activity (%) versus concentration of the samples. The EC_{50} value is the effective concentration at which 50% of DPPH radicals were scavenged. A lower EC_{50} value indicates a stronger DPPH–scavenging ability.

Determination of antimicrobial activity. Antimicrobial activity was tested against 16 strains of bacteria and nine strains of fungi (Table 1). All clinical isolates of the bacteria were a generous gift from the Institute of Public Health, Kragujevac, while the fungi and ATCC strains were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac, Serbia. The bacterial and yeast suspensions were prepared by the direct colony method. Turbidity of the initial suspension was adjusted by comparing with 0.5 McFarland's standard. Suspensions of fungal spores were prepared by gentle stripping of the spores from agar slants with growing fungi.

Minimum inhibitory concentrations (MICs) were determined using the microdilution method with resazurin (Sarker *et al.* 2007). The tested concentrations ranged from 0.156 to 20 mg/mL. Twofold serial dilutions of the extracts were prepared with Mueller-Hinton broth (Torlak, Serbia) for the bacteria and Sabouraud dextrose broth (Torlak, Serbia) for the fungi at a volume of 0.1 mL per well in sterile 96-well flat-bottom microtitre plates. The microtitre plates were inoculated with suspensions to give a final concentration of 5×10^5 CFU/ mL for the bacteria and 5×10^3 CFU/mL for the fungi. The inoculated microtitre plates were incubated at 37°C for 24 h for the bacteria, at 28°C for 48 h for the yeasts, and at 28°C for 72 h for the filamentous fungi. The MIC was defined as the lowest concentration of the tested substance that prevented resazurin colour change from blue to pink. Resazurin is an oxidation–reduction indicator used for evaluation of microbial growth. For the filamentous fungi, MIC values of the tested substance were determined as the lowest concentration that inhibited visible growth of mycelia. Doxycycline (Galenika A.D., Belgrade) and fluconazole (Pfizer Inc., USA) were used as positive controls. Stock solutions of crude extracts were obtained by dissolving in 10% DMSO, which did not inhibit microorganism growth in a control test. Each test included a growth control and a sterility control. All tests were performed in duplicate and MICs were constant.

Effect on Biofilm Formation. The bacteria chosen for the antibiofilm assay were: *Staphylococcus aureus*, *S. aureus* ATCC 25923, *Pseudomonas aeruginosa*, *P. aeruginosa* ATCC 27853, and *Proteus mirabilis*. Testing of the ability of bacteria to adhere to an abiotic surface *in vitro* and quantification of biofilm formation were done according to Stepanović *et al.* (2000). The effect of tested extracts on biofilm formation was determined using a crystal violet staining assay. Twofold serial dilutions of plant extracts with concentrations ranging from 0.31 to 40 mg/mL were made in sterile 96-well tissue culture microtitre plates (Sarstedt, Germany). Optical densities (ODs) of stained adherent bacteria were determined with an ELISA microplate reader at 630 nm. The percentage of inhibition was determined using the following formula:

Inhibition of biofilm formation (%) = $100 \times [(OD_{control} - OD_{sample}) / OD_{control}]$ where $OD_{control}$ is optical density of the growth control and OD_{sample} is optical density of the biofilm biomass in the presence of the extract. The biofilm inhibition concentration (BIC_{50}) was defined as the lowest concentration that prevented 50% biofilm formation.

Table 1. Antibacterial and antifungal activity of *Gentiana asclepiadea* extracts.

Species	Aqueous extract		Ethanol Extract		Acetone extract		Ethyl acetate extract		S μg/mL
	Aerial parts	Roots	Aerial parts	Roots	Aerial Parts	Roots	Aerial parts	Roots	
	MIC (mg/mL)								
Gram-positive bacteria									
Bacillus subtilis	5	>20	0.63	20	0.16	20	0.31	5	0.12
Bacillus cereus	5	>20	2.5	20	0.63	20	2.5	5	0.98
B. subtilis ATCC 6633	5	2.5	0.63	2.5	0.63	<0.16	1.25	<0.16	1.95
Enterococcus faecalis	20	20	20	>20	> 20	10	20	10	7.8
E. faecalis ATCC 29212	5	10	10	20	10	2.5	5	1.25	7.8
Staphylococcus aureus	10	>20	> 20	>20	10	20	10	5	0.45
S. aureus ATCC 25923	5	>20	5	>20	5	20	2.5	2.5	0.22
Gram-negative bacteria									
Escherichia coli	20	20	20	>20	> 20	10	20	10	7.8
E. coli ATCC 25922	20	20	20	>20	> 20	10	10	10	15.6
Proteus mirabilis	2.5	>20	20	20	> 20	10	10	10	250
P. mirabilis ATCC 12453	20	>20	> 20	>20	> 20	10	20	10	15.6
Pseudomonas aeruginosa	> 20	20	> 20	20	> 20	10	20	10	250
P. aeruginosa ATCC 27853	> 20	20	> 20	20	> 20	10	20	10	62.5
Klebsiella pneumoniae	20	10	20	>20	> 20	20	20	10	n.t.
Salmonella enterica	20	20	> 20	>20	> 20	10	20	10	15.6
Salmonella typhimurium	20	20	20	>20	> 20	10	20	10	15.6
Fungi									
Candida albicans	>20	>20	>20	2.5	20	20	20	10	62.52
C. albicans ATCC 10231	>20	>20	>20	2.5	10	20	10	20	31.2
Penicillum digitatum	20	10	20	2.5	1.25	<0.16	1.25	5	31.2
Penicillium italicum	20	20	10	10	10	20	10	5	1000
Aspergillus restrictus	20	20	10	<0.16	2.5	10	2.5	2.5	500
Aspergillus flavus	10	5	5	<0.16	5	5	10	5	1000
Aspergillus fumigatus	20	5	2.5	2.5	5	5	0.63	5	500
Aspergillus niger	>20	20	20	20	20	10	20	10	500

MIC- minimum inhibitory concentration; S – doxycicline/fluconazole; n.t. – not tested.

Table 2. Total phenol content, total flavonoid content, and DPPH radical-scavenging ability expressed as EC_{50}.

Plant extract	Total phenol content (mg GAE/g)		Total flavonoid content (mg RUE/g)		EC_{50} values (μg/mL)	
	Aerial parts	Roots	Aerial Parts	Roots	Aerial parts	Roots
Aqueous extract	27.11 ± 0.91^{a,b}	9.94 ± 0.47^{a}	24.63 ± 1.77^{a}	2.32 ± 0.02^{a}	356.3 ± 11.01^{a}	> 1000
Ethanol extract	35.59 ± 0.43^{c}	12.83 ± 0.70^{b}	55.02 ± 1.74^{b}	3.61 ± 0.27^{a,b,c}	181.3 ± 1.53^{b}	> 1000
Acetone extract	33.52 ± 0.18^{d}	24.07 ± 0.79^{c}	68.63 ± 1.39^{c}	4.53 ± 0.10^{c}	363.3 ± 3.51^{a}	783.33 ± 2.52^{a}
Ethyl acetate extract	29.32 ± 0.17^{a}	27.88 ± 0.13^{d}	85.07 ± 1.31^{d}	3.85 ± 0.16^{b}	614.3 ± 12.5^{d}	795.3 ± 3.78^{a}
Diethyl ether extract	30.16 ± 0.10^{b}	63.42 ± 0.12^{e}	83.78 ± 2.46^{d}	20.16 ± 1.52^{d}	420 ± 10.00^{c}	426.67 ± 41.6^{b}
Ascorbic acid						5.23 ± 0.23

Values are expressed as means ± standard deviation. Means in the same column with a superscript with different letters are significantly different at $p < 0.05$; GAE- gallic acid equivalents; RUE – rutin equivalents; EC_{50} - effective concentration at which 50% of DPPH radicals were scavenged.

Statistical analysis. The experiments, except for those testing antibacterial and antifungal activity, were carried out in triplicate. Statistical analysis was performed with the SPSS 20.0 software program (SPSS Inc., Chicago, IL, USA). Differences between means were tested through Student's test and one-way analysis of variance (ANOVA). A difference was considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Total phenol and total flavonoid content. The quantities of total phenols and total flavonoids, determined by spectrophotometric analyses, are presented in Table 2. In root extracts, the highest total phenolic and flavonoid content was measured in the diethyl ether extract (63.42 ± 0.12 mg of GAE/g of extract and 20.16 ± 1.52 mg of RUE/g of extract, respectively), while in aerial part extracts, the ethanol extract was the richest in total phenols (35.59 ± 0.43 mg of GAE/g of extract) and the ethyl acetate and diethyl ether extracts in flavonoids (85.07 ± 1.31 and 83.78 ± 2.46 mg of RUE/g of extract, respectively). The aerial part extracts contained significantly higher concentrations of flavonoids than in root extracts ($p < 0.05$).

DPPH radical-scavenging activity. The antioxidant activity of *G. asclepiadea* extracts depended on the used plant part, the type of extract, and the tested concentrations (Table 2). The aerial part extracts showed significantly better antioxidant activity than root extracts ($p < 0.05$). A statistically significant difference of activity was also noticed in different types of extracts ($p < 0.05$). Activity was expressed in the form of EC_{50} values, which were in the range of 181.3 to 614.3 μg/mL for aerial part extracts and from 426.67 to >1000 μg/mL for root extracts. The most active extracts were the ethanol aerial part extract (181.3 μg/mL) and the diethyl ether root extract (426.67 μg/mL). In this study, potent free radical-scavenging activity was exhibited by extracts which, at the same time, were more highly enriched with phenolic compounds. Previous investigators reported radical-scavenging ability for extracts of the aerial parts of differeent plants (Nićiforović *et al*. 2010), and the results obtained in the present study extend findings of antioxidant activity to different types of *G. asclepiadea* extracts.

Antimicrobial activity. The *in vitro* antibacterial and antifungal activities of aqueous, ethanol, acetone, and ethyl acetate extracts from roots and aerial parts of *G. asclepiadea* were tested against pathogenic and food-spoilage microorganisms (including bacteria, yeasts, and moulds) in order to evaluate the presence of broad-spectrum antimicrobial activity (Table 1).

Regarding bacteria, the tested extracts were more effective against Gram-positive than Gram-negative bacteria. A marked effect was exhibited against *Bacillus* species, especially by the ethanol, acetone, and ethyl acetate extracts of aerial parts. For these extracts, the MICs ranged from 0.16 to 5 mg/mL. On the other hand, Gram-

Table 3. Antibiofilm activity of *Gentiana asclepiadea* extracts.

	Aqueous extract		Ethanol extract		Acetone extract	
	Aerial parts	Roots	Aerial parts	Roots	Aerial parts	Roots
Species	BIC_{50} (mg/ml)					
Staphylococcus aureus	8.93	4.10	20.00	17.54	17.86	17.24
S. aureus ATCC 25923	5.32	2.12	20.41	8.93	18.87	9.26
Pseudomonas aeruginosa	30.30	32.79	35.09	36.36	37.04	36.36
P. aeruginosa ATCC 27853	20.00	22.73	26.32	24.39	4.46	37.04
Proteus mirabilis	20.41	25.00	21.48	21.74	12.20	16.66

BIC_{50} – concentration that prevented 50% biofilm formation.

negative bacteria showed low sensitivity to the tested *G. asclepiadea* extracts. Similar results were obtained in the study of Mihailović *et al.* (2011). Those researchers tested the methanol extract from aerial parts and the n-butanol extract from roots of *G. asclepiadea* and found them to be more active against the Gram-positive bacteria *S. aureus* ATCC 25923, *Enterococcus faecalis*, and *Micrococcus lysodeikticus* than against the Gram-negative bacteria *Klebsiella pneumonia* and *Escherichia coli*.

According to published data, this is the first report on antifungal activity of aqueous, ethanol, acetone, and ethyl acetate extracts of *G. asclepiadea*. The tested extracts exhibited low to moderate antifungal activity. Aqueous extracts affected fungal growth at the highest tested concentrations or else were inactive. The most active was the ethanol root extract. *Candida* species were more resistant than the tested filamentous fungi. The ethanol, acetone, and ethyl acetate extracts manifested good inhibitory effects on the growth of *Penicillium digitatum*, *Aspergillus restrictus*, *A. flavus*, and *A. fumigatus*.

Effect on Biofilm Formation. Bacterial biofilms are defined as a surface-attached community of bacteria embedded in an organic polymer matrix of bacterial origin. The antibiofilm activity displayed by aqueous, ethanol, and acetone extracts of the roots and aerial parts of *G. asclepiada* was investigated for the first time in the present study. The extracts prevented development of the tested bacterial biofilms. As presented in Table 3, the extracts inhibited 50% of biofilm formation in the concentration range of from 2.12 to 37.04 mg/mL. The biofilms of *Staphylococcus aureus* and *S. aureus* ATCC 25923 were the most sensitive to the presence of extracts, a concentration of 2.12 mg/mL of the aqueous extract of roots inhibiting 50% of biofilm formation. In addition, the ethanol extract of roots showed the best result on the biofilm of *S. aureus* ATCC 25923, while the acetone extract of aerial parts showed the best result on the biofilm of *P. aeruginosa* ATCC 27853. The ability of plant extracts to control bacterial biofilms has been reported before, suggesting that the application of bioactive plant extracts could be a promising tool for reducing microbial colonization of surfaces or epithelial mucosa leading to subsequent infections (Quave *et al.* 2008; Stefanović *et al.* 2015; Teanpaisan *et al.* 2017).

Plants belonging to the genus *Gentiana* are well-known for their pharmacological activities, including hepatoprotective, anti-inflammatory, cytotoxic, antitumor, antimicrobial, antioxidant, cholinesterase inhibitory, and immunomodulatory activities. *Gentiana* sp. are especially known for their anti-inflammatory and hepatoprotective effects, which are attributable to secoiridoid glycosides, the most significant bioactive compounds in representatives of the genus *Gentiana*. Hepatoprotective activity was reported for root and rhizome extracts of *G. cruciate*, *G. manshurica*, *G. scabra*, and *G. lutea* (Mihailović *et al.* 2013, 2014; Pan *et al.* 2016). Niiho *et al.* (2006) confirmed gastroprotective effects of the methanol extract of *G. lutea* roots. The extracts from roots of *G. macrophylla* and *G. straminea*, acetone extract from *G. striata*, ethanol extract from flowers of *G. kurroo*, and ethanol and petroleum ether extracts of *G. lutea* rhizomes possessed potent anti-inflammatory activities (Pan *et al.* 2016; Mirzaee *et al.* 2017). Antioxidant activity has also been proven. The extracts of roots, flowers, and leaves of several species (*G. cruciata*, *G. verna*, *G. septemfida*, *G. olivieri*, *G. lutea*, and *G. scabra*) showed antioxidant activity, which was verified by the DPPH, hydroxyl radical, lipid peroxidation, and metal-chelation assays (Senol *et al.* 2012; Pan *et al.* 2016). Inhibition of cholinesterase by *G. campestris*, *G. verna*, and *G. cruciata* has also been reported. Flower and leaf extracts of *G. lutea* showed broad-spectrum antimicrobial activity. Moreover, the butanol fraction of *G. olivieri* extracts was shown to possess immunomodula-

tory activity, while *G. aristate, G. kurroo,* and *G. kochiana* exhibited cytotoxic activity against human cancer cell lines (Pan *et al*. 2016; Mirzaee *et al*. 2017).

CONCLUSION

The results obtained in the present work contribute to a better understanding of the antioxidant, antimicrobial, and antibiofilm activity of the tested *G. asclepiadea* extracts. To judge from published data, this is the first report on antibiofilm activity, as well as on the antimicrobial and antioxidant activity of certain extracts obtained with different solvents. The ethanol extract of aerial parts and the diethyl ether extract of roots could be potential natural antioxidant agents. The tested extracts obtained with organic solvents showed better antibacterial and antifungal activity than aqueous extracts. The extracts were active against *Bacillus* species and *S. aureus*, bacteria which cause food poisoning or human infection. Moreover, the extracts inhibited development of bacterial biofilms, especially that of *S. aureus*. The determined total content of phenols and flavonoids confirmed that the intensity of biological activities of extracts is correlated with the content of bioactive compounds.

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REZIME

Bioaktivni ekstrakti vrste *Gentiana asclepiadea*: antioksidativna, antimikrobna i antibiofilm aktivnost

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Analizirana je antimikrobna, antibiofilm i antioksidativna aktivnost, kao i izmerena koncentracija ukupnih fenola i flavonoida u ekstraktima pripremljenim od nadzemnog dela i korena samonikle, lekovite biljke *Gentiana asclepiadea*. Antimikrobna aktivnost je testirana u odnosu na patogene bakterije, kvasce i plesni, kao i na uzročnike kvarenja životnih namirnica. Najjača antibakterijska aktivnost je uočena u odnosu na *Bacillus* vrste sa detektovanim minimalnim inhibitornim koncentracijama (MIK) od 0,16 mg/ml to 5 mg/ml, dok je uočen nizak do srednji intenzitet antifungalne aktivnosti sa MIK vrednostima između 1,25 i 20 mg/ml. Primenom kristal violet testa pokazano je da su koncentracije ekstrakata od 2,12 - 37,04 mg/ml inhibirale formiranje biofilma. Biofilmovi bakterija *Staphylococcus aureus*, *S. aureus* ATCC 25923 i *Pseudomonas aeruginosa* ATCC 27853 su bili najosetljiviji. Testirani ekstrakti, bogati fenolnim jedinjenjima ispoljili su značajnu antioksidativnu aktivnost. Efektivne koncentracije (EC_{50}) su bile u intervalu 181,3 – 614,3 μg/ml za ekstrakte od nadzemnog dela biljke i u intervalu 426,67 - >1000 μg/ml za ekstrakte od korena biljke. Iako se biljka *G. asclepiadea* koristi u tradicionalnoj medicini, njene biološke aktivnosti su još uvek nedovoljno istražene, tako da dobijeni rezultati doprinose boljem upoznavanju njenih lekovitih svojstava.

Ključne reči: *Gentiana asclepiadea*, ekstrakti, antimikrobna aktivnost, antioksidansi, biofilm