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Biological activities of phenolic compounds and ethanolic extract of *Halacsya sendtneri* (Boiss) Dőrfler

Research Article

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Abstract: The objective of this study was to evaluate the efficacy of the ethanolic extract of endemic plant *Halacsya sendtneri* in inhibiting the growing of the test fungi and bacteria as well as to determine its genotoxic potential and toxicity using the *Allium* anaphase-telophase assay. Minimum inhibitory concentrations (MIC) were determined for 15 indicator strains of pathogens, representing both bacteria and fungi. The highest susceptibility to the ethanolic extract of *H.sendtneri* was exhibited by *Pseudomonas glycinea* (FSB4), (MIC=0.09 mg/ml) among the bacteria, and by *Phialophora fastigiata* (FSB81), (MIC=1.95 mg/ml) among the fungi. The composition of *H. sendtneri* extracts was also determined using HPLC analysis. Rosmarinic acid was found to be the dominant phenolic compound. The *Allium* anaphase-telophase genotoxicity assay revealed that the ethanolic extract of *H. sendtneri* at concentrations of 31.5 mg/l and below does not produce toxic or genotoxic effects. This is the first report of chemical constituents, genotoxic and antimicrobial activities of the endemic species, *H. sendtneri.*

Keywords: *Antimicrobial activity • Genotoxicity • Halacsya sendtneri • Phenolic compounds*

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1. Introduction

The use of traditional medicinal plants for primary health care and other purposes has progressively increased worldwide in recent years. Much attention has been given to various plant secondary metabolites that are a common feature of specific plants and plant families. Many plant secondary metabolites and essential oils have antimicrobial properties that make plant extracts and products successful in the treatment of bacterial, fungal and viral infections [1-3]. The *Boraginaceae* family occurs worldwide, and it consists of about 100 genera with more than 2000 species, (one of the species is *Halacsya sendtneri*) [4]. Many members of the Boraginaceae family produce secondary metabolites such as alkaloids, naphthoquinones, polyphenols, phytosterols and terpenoids [5,6]. Polyphenols, including flavonoids and phenolic acids, produced by the family Boraginaceae, have a wide range of pharmaceutical activities, including anti-inflammatory, anti-viral and anti-bacterial activities [7-9].

Halacsya sendtneri is a member of the monotypic genus *Halacsya* of the family Boraginaceae, its range

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being limited to parts of the habitat in the central Balkans. The species inhabits open serpentine rocky landscapes at altitudes ranging from 190 to 1500 m [10]. In Serbia, it is found along the serpentines of certain mountains, as well as in some gorges. *H.sendtneri* is considered a Tertiary relict [11]. So far, antimicrobial properties have not been indicated for this endemic species. It is qualified as a vulnerable species (V) in the European Red List (marked as +) [12]. Apart from being highly important in terms of world plant gene pool preservation, endemic plants can also contribute substantially to studies on antimicrobial activity [13]. In order to minimize the risk of using new natural antioxidants as preservers or for other purposes (as drugs or food supplements) it is necessary to have data on their potential toxicity and genotoxicity. For evaluation of genotoxicity, a significant number of assays using different biological systems (plants, fruits, vegetables *etc*.) have been developed. Plant bioassays, characterized by generally higher sensitivity in comparison to other systems, proved useful in different situations and for different end points [14]. Among them, the *Allium (A.cepa)* test has been used to assess a great number of chemical compounds [15], wastewater [16], waters from rivers and lakes [17,18], drinking water [19] and melted snow [20]. The advantages of this assayinclude high sensitivity, similarity in chromosome organization to that in humans, good correlation to other test systems and the possibility of studying the effects under a wide range of conditions [21]. So far, only one study reported on the activities of *H. sendtneri.* This research is based on studies of antioxidant activity *H. sendtneri* extracts [22].

No previous studies on the biological activity or chemical constituents of *Halacsya sendtneri* have been reported in the literature. The objective of the study was to determine the antimicrobial, antioxidant, and genotoxic activities of *Halacsya sendtneri* growinig is Serbia. We also identified the major phenolic consitutes of *H. sendtneri* using HPLC.

2. Experimental Procedures

2.1 Chemicals used

Standards for HPLC (chlorogenic, caffeic, ferulic, rosmarinic, protocatechuic, gallic and p-coumaric acid, myricetine, quercetine, resveratrol, rutin, epigallocatechine, catechine, apigenine) analysis were of analytical grade and were purchased from Sigma Chemical Co. (St Louis, MQ, USA). Acetonitrile gradient grade (J. T. Baker, Philipsburg, NJ, USA), phosphoric acid p.a. grade and 18 M deionized water (Millipore, Bedford, MA) were used. Ethanol was of analytical grade (Aldrich Chemical Co., Steinheim, Germany).

2.2 Plant material

Halacsya sendtneri (Boiss) Dőrfler. was collected at Ilijak Hill, Central Serbia (214 m above sea, coordinates: 43°52′02"N, 20°31′28"E) from May to June 2008. The species was identified and the voucher specimen was deposited at the Department of Botany, Faculty of Biology, University of Belgrade (16336 BEOU, Lakušić Dmitar).

2.3 Preparation of extracts

The air-dried parts of the above-ground of plant (90 g) were broken into small pieces by a cylindrical crusher, and extracted (60°C, 5 h and, 1:5, g/ml (drug:solvent ratio)) with ethanol (99.8%) using a Soxhlet apparatus. The ethanolic extract was filtered through filter paper (Whatman, No.1) and concentrated to dry mass (6.51 g). The residues were stored in a dark glass bottles for further processing.

2.4 Test microorganisms

The antimicrobial activity of the plant extract was tested *in vitro* against the following Gram-positive bacteria: *Staphylococcus aureus* (American Type Culture Collection (ATCC) 12600), *Micrococcus lysodeikticus* (ATCC 4698) and *Bacillus mycoides* (ATCC 6462); and the following Gram-negative bacteria: *Klebsiella pneumoniae* (ATCC 6462), *Pseudomonas glycinea* (Faculty of Biological Sciences, Serbia (FSB) 40) and *Escherichia coli* (ATCC 11775) and fungi *Candida albicans* (ATCC 10259), *Fusarium oxysporum* (FSB91), *Penicillium canescens* (FSB24), *Aspergillus glaucus* (FSB32), *Alternaria alternata* (FSB51), *Penicillium verrucosum* (FSB21), *Aspergillus niger* (FSB31), *Trichoderma viride* (FSB11) and *Phialophora fastigiata* (FSB81). Pure cultures were generated by subculturing four times on the same media for seven days. All test microorganisms used are from the Institute for Immunobiology and Virology, Torlak, Belgrade, Serbia. Identification of the test microorganisms was confirmed by the Laboratory of Mycology, Department of Biology, Faculty of Science, University of Kragujevac, Serbia.

2.5 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MIC) of the extracts were determined using a microdilution method in 96 multi-well microtiter plates [23]. All tests were performed in Müller–Hinton broth (MHB), with the exception of yeast, in which case Sabouraud dextrose broth was used. A volume of 100 µl stock solutions of extract (in methanol, 200 µl/ml) was pipetted into the first row of the plate. 50 µl of Müller–Hinton or Sabouraud dextrose broth (supplemented with Tween 80 at a final concentration of 0.5% (v/v) for extract analysis) was added to the other wells. 50 µl from the first test well

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was pipetted into the second well of each microtiter line, and then 50 μl of scalar dilution was transferred from the second to the twelfth well. 10 μl of resazurin indicator solution (prepared by dissolving a 270-mg tablet in 40 ml of sterile distilled water, 6.75 mg/ml) and 30 μl of nutrient broth were added to each well. Finally, 10 μl of bacterial suspension (10^6 CFU/ml) and yeast spore suspension (3×10^4 CFU/ml) was added to each well. For each strain, the growth conditions and the sterility of the medium were checked. Standard antibiotic tetracycline was used to control the sensitivity of the tested bacteria, whereas ketoconazol was used as control against the tested yeast. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37°C for 24 h for the bacteria and at 28°C for 48 h for the yeast. Color change was then assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. Final MIC values were caluclulated as the average of 3 values.

2.6 Genotoxic examination

All samples were analyzed using the procedure of Fiskesjö [24] as modified by Rank and Nielsen [25] and known as the *Allium* anaphase-telophase genotoxicity assay. Commercial onion bulbs of *Allium cepa*, weighing 2-4 grams, obtained from local companies, were selected for the assay. The yellow shallows and the dry center of the primordia were carefully removed prior to placement of the onions into the samples. The test procedure included two parts, and both general toxicity and genotoxicity were determined. The following concentrations of 250, 125, 62.5 and 31.5 mg/l of samples I, II, III and IV were evaluated respectively. For each sample, as well as the positive and negative controls, twelve onions were placed in test tubes filled with test liquids. Methyl methanesulfonate (MMS) at a final concentration of 10 μg/l, was used as the positive control and commercial bottled water as the negative. Fresh solution was added each day. For the first 24 hours the onions were grown in fresh commercial bottled water and subsequently exposed for two days to the test samples. To assess toxicity, roots were cut off on the fourth day and the length of each root was measured to the nearest mm in all groups. Statistical significance of differences in root length was assessed using Tukey HSD for unequal N (Statistica 6.0). For genotoxicity evaluation, root tips were hydrolyzed in 1 N HCl at 60°C for 12 minutes. Five apical parts of the root tips from each onion were placed on a slide, stained with 2% orcein and squashed in 45% acetic acid. The slides were coded and examined blind. Chromosome aberrations were scored on slides with a mitotic index higher than 1%. About one hundred mitoses per slide (only anaphase and telophase stages) were examined for five slides in each group. The following aberrations were scored: bridges, fragments, vagrant chromosomes, multipolarity and c-mitoses. A 2x2 contingency χ^2 statistical test (Statistics 6.0) was employed to determine the significance of differences between the analyzed groups and controls.

2.7 HPLC analysis

Quantification of individual phenolic compounds was performed using reversed phase HPLC analysis. Samples were injected into the Waters HPLC system consisting of 1525 binary pumps, a thermostat and a 717+ autosampler connected to a Waters 2996 diode array detector (Waters, Milford, MA, USA). Chromatograms were gathered in the 3D mode with extracted signals at specific wavelengths for different compounds (368, 324 and 254 nm, respectively). Separation of phenolics was performed on a 125x4 mm Symmetry C-18 RP column (Waters, Milford, MA, USA) with 5 μm particle size connected to an appropriate Symmetry C-18 guard column (Waters, Milford, MA, USA).Two mobile phases, A (0.1% phosphoric acid) and B (acetonitrile), were used at a flow rate of 1 ml min^{-1} at 30°C, with the following gradient profile: the first 20 minutes from 10 to 22% B; next 20 minutes of linear rise up to 40% B, followed by 5 minute reverse to 10% B and additional 5 minutes of equilibration time. Peaks of detected compounds were located and identified in the HPLC-UV analyses through comparison of separated standards with authentic samples by combining their retention times and UV spectra obtained by DAD (Diode-Array Detection) (Figure 1). Each component was analyzed quantitatively by the external standard method using pure compounds as references for concentration, retention time and characteristic UV spectra, respectively. DAD response was linear in the whole concentration range for all standard compounds and method validation procedure as well as data acquisition and spectral evaluation for peak confirmation was successfully carried out by Empower 2 software (Waters, Milford, MA, USA).

3. Results and Discussion

3.1 Antimicrobial activity of ethanolic extract of *H. sendtneri*

Antimicrobial activity expressed as minimum inhibitory concentrations (MIC) were obtained by the dilution method for 15 indicator strains of pathogens (Table 1). The

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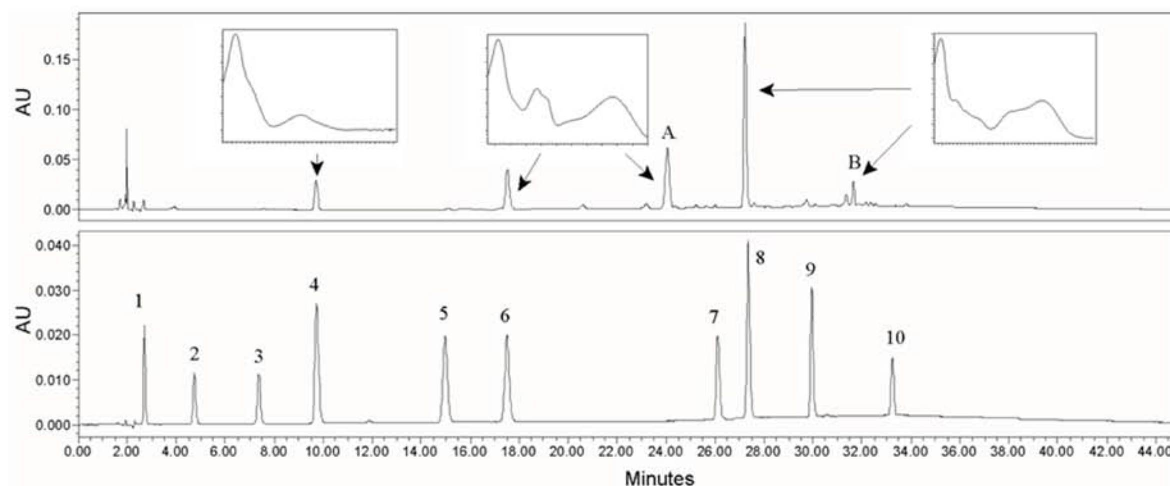

Figure 1. HPLC chromatograms of the ethanolic extract of *H. sendtneri* showing peaks of dominant components and their corresponding UV spectra (boxed, range from 200 to 420 nm) and of selected standards recorded at 254 nm. Peak numbers correspond to gallic (1), protocateuchic (2) and chlorogenic (3) acid, epicatehin gallate (4), *p*-coumaric acid (5), rutin (6), myricetine (7), rosmarinic acid (8), resveratrol (9) and apigenine (10). Peaks A and B are unidentified compounds with spectra similar to rutin and rosmarinic acid as shown by arrows.

Microorganisms	MIC		
	Ethanolic extract (mg/ml)	Tetracycline (µg/ml)	Ketoconazol (µg/ml)
Staphylococcus aureus ATCC12600	0.19	6.25	/
Micrococcus lysodeikticus ATCC4698	0.19	6.25	/
Bacillus mycoides ATCC6462	0.19	1.25	/
Klebsiella pneumoniae ATCC13883	0.19	6.25	/
Pseudomonas glycinea FSB40	0.09	0.19	/
Escherichia coli ATCC11775	0.78	2.50	/
Candida albicans ATCC 10259	3.90	/	1.95
Fusarium oxysporum FSB91	3.90	/	3.90
Penicillium canescens FSB24	6.25	/	3.90
Aspargillus glaucus FSB32	6.25	/	7.80
Alternaria alternata FSB51	3.90	/	7.80
Penicillium verrucocum FSB21	3.90	/	3.90
Aspergillus niger FSB31	6.25	/	7.80
Trichoderma viride FSB11	3.90	/	7.80
Phialophora fastigiata FSB81	1.95	/	3.90
ATCC – American Type Culture Collection FSB- Faculty of Biological Sciences			

Table 1. Antimicrobial activity expressed as minimum inhibitory concentration (MIC) determined using dilution method.

ethanolic extract of *H. sendtneri* showed antimicrobial activity within the concentration range of 0.09 mg/ml to 6.25 mg/ml (Table 1). The highest susceptibility to the ethanolic extract of *H. sendtneri* was exhibited by *Pseudomonas glycinea* (FSB4), (MIC=0.09 mg/ml), among the test bacteria, and by *Phialophora fastigiata* (FSB81), (MIC=1.95 mg/ml), among the test fungi. Conversely, the lowest susceptibility was observed in the bacterium *Escherichia coli* (ATCC 11775), (MIC=0.78 mg/ml) and in the fungi *Penicillium canescens* (FSB24), *Aspergillus glaucus* (FSB32) and *Aspergillus niger* (FSB31), the measured MIC value being 6.25 mg/ml.

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3.2 *Allium* anaphase-telophase genotoxicity assay (toxicity and genotoxicity) of the ethanolic extract of *H. sendtneri*

The degree of toxicity of the analyzed samples was assessed from the mean root lengths expressed as a percentage of the mean root length of the negative control. Significant inhibition of growth compared to the negative control was observed for samples I (P=0.001) and II (P=0.008). The relative number of total chromosome aberrations was the highest for sample II (12.3%) and the lowest for sample IV (Table 2). Overall, the most frequent single aberrations were cells with multipolarity (43.4%), and vagrant chromosomes (32.7%), while bridges were less frequent (11.5%). Fragments and C-mitosis were found in less than 3% of aberrant cells. Statistical analysis of aberration frequencies scored in anaphase and telophase indicated that all samples differed significantly from both the positive and the negative controls, except for sample IV that did not differ significantly from the negative control (Table 3). Sample IV also differed significantly from all other samples. Comparison of the results on toxicity and genotoxicity showed that samples I and II, which showed significant inhibition of growth, had an increased level of genotoxic potential which was, however, still far away from the mutagenicity obtained for the positive control. Two groups of aberrations were detected in the analyses. One type was produced by spindle disturbance and it included vagrant chromosomes, multipolar configurations and C-mitoses, whereas the other type was produced by action on the chromosomes and it covered bridges and fragments. Both types of aberrations occurred at the same time in some cells. The first group of aberrations was much frequent among aberrant cells (78.3%), indicating that the tested samples were predominantly acting by disturbing the spindle.

3.3 Quantification of dominant metabolites in this plant

HPLC was used for identification and quantification of dominant metabolites in *H. sendtneri*. HPLC analysis showed rosmarinic acid (t_R=27.3 min) to be the dominant component of the extract, with its content being 736.6±22.4 μg/g dry weight of extract. Besides rosmarinic acid, flavonoid rutin (t_R=24.2 min) was also identified in the extract by comparison of its retention times to this of standard (Figure 1). The content of rutin was 152.1±2.1 μg/g dry weight of extract (Table 4). Rosmarinic acidwas found to be the dominant phenolic component of the extractand was previously reported as being a characteristic phenolic component of the *Boragniceae* family [26]. Rosmarinic acid is well known for its biological activities, such as: adstringent,

Sample	BR	FR	MP	Vch	Cm	MA	Total number of analyzed cells	Aberrant cells (%)
I	6	-	30	20	3	9	629	10.8
II	4	1	31	30	1	7	601	12.3
III	12	-	22	15	1	3	618	8.6
IV	4	-	15	9	-	3	598	5.2
K-	4	-	13	9	-	1	692	3.9
K+	26	4	10	22	8	52	521	23.4

Table 2. The results of genotoxicity assay in *H. sendtneri* extracts determined by Allium anaphase-telophase tests. Negative (K-) and positive control (K+). BR – bridges; FR – fragments; MP – multipolarity; Vch – vagrant chromosomes; Cm – C-mitosis; MA – cells with multiple aberrations.

Samples	I	II	III	IV	K-
II	X^2=0.79 P=0.375				
III	X^2=1.78 P=0.183	X^2=4.28 P<0.05			
IV	X^2=13.09 P<0.001	X^2=19.55 P<0.001	X^2=5.44 P<0.05		
K-	X^2=16.60 P<0.001	X^2=32.23 P<0.001	X^2=12.44 P<0.001	X^2=1.23 P=0.268	
K+	X^2=32.83 P<0.001	X^2=7.91 P<0.005	X^2=47.88 P<0.001	X^2=78.41 P<0.001	X^2=105.05 P<0.001

Table 3. Statistical analysis of aberration frequencies scored in anaphase and telophase for all samples and controls. (Negative control – K-; positive control – K+)

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antioxidative, anti-inflammatory, antimutagen, antibacterial and antiviral activities [26]. Compounds similar to rutin (A) and rosmarinic acid (B) were also abundant and probably represented different glycoside isomers (Figure 1). It is well known that rosmarinic acid can provide protection against cancer and contribute to the antioxidant activity of plants [26,27]. It is therefore possible that rosmarinic acid, found in high amounts in *H. sendtneri* extracts, could be a source of antimicroial and/or antioxidant activity Further research is required to establish the roles for the most abundant phenolic compounds found in *H. sendterni*. Details on the mechanisms behind the antioxidant and antimicrobial properties of *H. sendtneri* remain unknown.

Phenolic compound	concentration (μg/g dry weight)
Epicatehin gallate	83.2 ± 4.2
Rosmarinic acid	736.6 ± 22.4
Gallic acid	24.2 ± 3.1
Rutin	152.1 ± 2.1
Resveratrol	7.2 ± 0.8

Table 4. Phenolic compounds in *H. sendtneri* extracts determined using HPLC.

4. Conclusions

In this study, we showed effects (toxic and genotoxic) and biological activities of ethanolic extract of *H. sendtneri*. The ethanolic extract of *H. sendtneri* at concentrations of 31.5 mg/l and below does not produce toxic or genotoxic effects. The highest susceptibility to the ethanolic extract of *H. sendtneri* was exhibited by *Pseudomonas glycinea,* among thebacteria, and by *Phialophora fastigiata*, among the fungi. Determination of polyphenolic components revealed the presence of high amounts of rosmarinic acid in ethanol extract of *H. sendtneri*. The results will help in the search for alternative drugs to be used in pharmacotherapy, and will contribute to establish safe and effective use of phytomedicines in the treatment of diseases.

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