Antimicrobial and determination of DPPH free radical scavenging activity of *Satureja hotensis L*

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Summary

This study was aimed at evaluating the antioxidant activity and efficacy of the ethanolic extract of the plant species in Satureja hotensis L inhibiting the development of selected fungi and bacteria. The highest susceptibility to the ethanolic extract of O. aucherianum among the bacteria was exhibited by B. subtilis and S. aureus (MIC = 15.62 µg/ml). Among the fungi, A. niger (MIC = 15.62 µg/ml) showed the highest susceptibility. Total phenolic and flavonoid contents were 106.46±1.68 mg GA/g, 25.91±0.88 mg RU/g, respectively. The results showed that the ethanolic extract of Satureja hotensis IC_{50} values were determined for each measurement: 21.45±1.55 µg/ml for DPPH free radical scavenging.

Key words*:* antimicrobial activity, antioxidant activity, *Satureja hotensis L.*

Introduction

The use of traditional medicinal plants for primary health care and other purposes has progressively increased worldwide in recent years. Plants communicate with their environment by producing a diverse range of chemicals. These secondary metabolites are a common feature of specific plants and plant families. Many plant secondary metabolites and extracts have antimicrobial properties that make plant extracts and products successful in the treatment of bacterial, fungal and viral infections (Gottschling et al., 2001; Zhou and Duan, 2005; Iqbal et al., 2005). The different parts of plants (root, leaf, flower, fruit, stem, bark) are used to effectively treat a number of diseases. Their antioxidant and antimicrobial properties affect a range of physiological processes in the human body, thus providing protection against free radicals and growth of undesirable microorganisms. However, synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), widely known for their ability to terminate the chain reaction of lipid peroxidation, have been proven to be carcinogenic and cause liver damage (Nobuyuki et al.,1985). Both bacterial resistance to a large number of antibiotics and the capacity of plants to synthesize biologically active substances are reasons for the increasing importance given to the use of plant-derived products in bacterial control. The use of plants in the food industry to replace synthetic preservatives, antioxidants or other food additives has increased significantly over the last years (Al-Bakri and Afifi, 2007). Many herb species are active antioxidants, mainly because of the content of phenolic compounds (Zeuthen and Bogh-Sorensen,2003). Phenolic compounds are ubiquitous in plants; flavonoids and other plant phenolics, such as phenolic acids, stilbenes, tannins, lignans, and lignins, are important for normal plant growth and development and defense against infection and injury. These compounds are commonly found in both edible and nonedible plants, and they have been reported to have multiple biological effects, including antioxidant activity (Kahkonen et al.,1999). Various investigations implied that total phenolic compounds are closely related to antioxidant activity (Duh and YED, 1997), with flavonoids and tannins being major plant compounds having antioxidant activity (Franke et al.,1998).

Materials and Methods

All standards for HPLC analysis were of analytical grade and were purchased from Sigma Chemical Co. (St Louis, MQ, USA) and Alfa Aesar (Karlsruhe, Germany). Acetonitrile and phosphoric acid were of HPLC grade (Tedia Company, USA). Ethanol was of analytical grade (Aldrich Chemical Co., Steinheim, Germany).
Spectrophotometric measurements were performed using a UV-VIS spectrophotometer MA9523-SPEKOL 211 (ISKRA, Horjul, Slovenia).
The test plant was collected at Ilijak Hill (Central Serbia) in May/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).
The air-dried aerial parts of the plant (90 g) were broken into small pieces by a cylindrical crusher, and extracted with ethanol (99.8%) using a Soxhlet apparatus. The ethanolic extract was filtered through filter paper (Whatman, No.1) and concentrated to dry mass (6.51g). The residues were stored in a dark glass bottle for further processing.

Determination of total phenolic and flavonoid content

Total phenols were estimated according to the Folin-Ciocalteu method (Singleton et al., 1999). The absorbance was measured at 765 nm using a spectrophotometer against a blank sample. Total phenols were determined as gallic acid equivalents (mg GA/g extract). Total flavonoids were determined according to Brighente method (Brighente et al., 2007). Total flavonoids were determined as rutin equivalents (mg RU/g dry extract).

Determination of DPPH free radical scavenging activity

The method used by (Takao et al., 1994), was adopted with suitable modifications from (Kumarasamy et al.,2007). Ascorbic acid (AA), gallic acid (GA) and butylated hydroxytoluene (BHT) were used as reference standards and dissolved in methanol to make the stock solution with the same concentration. Control sample was prepared containing the same volume without test compounds or reference antioxidants. Ninety-five percent methanol was used as a blank. The DPPH free radical scavenging activity (%) was calculated using the following equation:

$$\%\ \text{inhibition} = \frac{\text{Ac-As}}{\text{Ac}} \times 100$$

The IC_{50} value, defined as the concentration of the test material that leads to 50% reduction in the free radical concentration, was calculated as µg/ml through a sigmoidal dose-response curve.

Test microorganisms

The antimicrobial activity of the plant extract was tested in vitro against the following Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Micrococcus lysodeikticus* (ATCC 4698) and *Bacillus mycoides* (FSB1); and the following Gram-negative bacteria: *Klebsiella pneumoniae* (FSB26), *Pseudomonas glycinea* (FSB40) and *Escherichia coli* (ATCC 25922) and fungi Candida albicans (ATCC 10259), *Fusarium oxysporum* (FSB91), *Penicillium canescens* (FSB24), *Aspargillus glaucus* (FSB32), *Alternaria alternata* (FSB51), *Penicillium verrucocum* (FSB21), *Aspergillus niger* (FSB31), *Trichoderma viride* (FSB11) and *Phialophora fastigiata* (FSB81). The fungi were cultured on potato-glucose agar for 7 days at room temperature of 20ºC under alternating light and dark conditions. Then, they were cultured on a new potato-glucose substrate for another 7 days. The culturing procedure was performed four times until pure culture was obtained. Identification of the test microorganisms was confirmed by the Laboratory of Mycology, Department of Biology, Faculty of Science, University of Kragujevac, Serbia.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MIC) of the extract and cirsimarin against tested bacteria were determined using a microdilution method in 96 multi-well microtiter plates (Satyajit, et al., 2007). All tests were performed in Muller–Hinton broth (MHB), with the exception of yeast, in which case Sabouraud dextrose broth was used. A volume of 100 µl stock solutions of oil (in methanol, 200 µl/ml) and cirsimarin (in 10% DMSO, 2 mg/ml) was pipetted into the first row of the plate. Fifty µl of Mueller Hinton or Sabouraud dextrose broth (supplemented with Tween 80 at a final concentration of 0.5% (v/v) for oil analysis) was added to the other wells. Fifty 50 µl from the first test well 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. Ten µl of resazurin indicator solution (prepared by dissolving a 270-mg tablet in 40 ml of sterile distilled water) 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 amracin was used to control the sensitivity of the tested bacteria, whereas ketoconazole 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℃ for 24 h for the bacteria and at 28℃ for 48 h for the yeast. Color change was th en 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. The average of 3 values was calculated, and the obtained value was taken as the MIC for the tested compounds and standard drug.

Statistical analysis

The results are presented as mean ± standard deviations of three determinations. Statistical analyses were performed using Student´s t-test and one way analysis of variance. Multiple comparisons of means were done by LSD (least significant difference) test. A probability value of 0.05 was considered significant. All computations were made by employing the statistical software (SPSS, version 11.0). IC_{50} values were calculated by nonlinear regression analysis from the sigmoidal dose-response inhibition curve.

Results and discussion

Phenolic compounds and flavonoids have been reported to be associated with antioxidant action in biological systems, mainly due to their red-ox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Saha et al.,2008). One of the more prominent properties of flavonoids is their excellent radical scavenging ability, which makes them valuable for therapeutic and prophylactic applications, e.g., after infection, inflammation, burns, or radiation injury (Havsteen, 2002). The activity of crude methanol extracts is due to the presence of flavonoid monomers and polymers (condensed tannins), hydrolyzable tannins, and phenolics. Recently, polyphenolic compounds from plants such as condensed and hydrolyzable tannins have been shown to be powerful antioxidants (Beninger and Hosfield, 2003). Furthermore, tannins are reported to be 15-30 times more effective in quenching peroxyl radicals than simple phenolics. Total

phenolic and flavonoid contents were 106.46±1.68 mg GA/g, 25.91±0.88 mg RU/g, respectively. The results showed that the ethanolic extract of *Satureja hotensis* IC_{50} values were determined for each measurement: 21.45±1.55 µg/ml for DPPH free radical scavenging (Table 1).

Sample	[a]IC_{50} (µg/ml)		
	DPPH scavenging activity	Total phenolics (mg GA/g)	Flavonoids (mg RU/g)
Etahnol extract of *O. aucherianum*	21.45±1.55*	106.46±1.68	25.91±0.88
Gallic acid	3.79±0.69	-	-
Ascorbic acid	6.05±0.34	-	-
BHT	15.61±1.26	-	-
α-Tocopherol	-	-	-

Table 1. Total phenolics, flavonoids and DPPH scavenging activity of the ethanol extract of ***Satureja hotensis L***

*Results are mean values ± SD from three experiments.

The results on antimicrobial activity obtained by the dilution method are given in Table 2. Minimum inhibitory concentrations were determined for 8 selected indicator strains. The results presented in Table 2. reveal antimicrobial activity of the ethanolic extract of *Satureja hotensis L* within the concentration range of 15.62 *µg/ml* to 62.50 *µg/ml*. The highest susceptibility to the ethanolic extract of *O. aucherianum* among the bacteria tested was exhibited by *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923 (MIC = 15.62 *µg/ml*), followed by strains of *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 (MIC = 31.25 *µg/ml*), and *P. vulgaris* ATCC 13315 and *P. mirabilis* ATCC 14153 (MIC = 62.50 *µg/ml*). Among the fungi, *A. niger* ATCC 16404 (MIC = 15.62 *µg/ml*) showed the highest susceptibility, and *C. albicans* ATCC 10231 (MIC = 31.25 *µg/ml*) the lowest.

MIC µg/ml			
Microbial strains	Ethanolic extract of *Satureja hotensis L*	Amracin	Ketoconazole
Staphylococcus aureus ATCC 25923	15.62	0.97	/
Klebsiella pneumoniae ATCC 13883	31.25	0.49	/
Escherichia coli ATCC 25922	31.25	0.97	/
Proteus vulgaris ATCC 13315	62.50	0.49	/
Proteus mirabilis ATCC 14153	62.50	0.49	/
Bacillus subtilis ATCC 6633	15.62	0.24	/
Candida albicans ATCC 10231	31.25	/	1.95
Aspergillus niger ATCC 16404	15.62	/	0.97

Table 2. Minimum inhibitory concentrations (MIC) of the ethanolic extract of *Satureja hotensis L*

Conclusions

Antioxidant and antimicrobial properties of various extracts of many plants are of great interest in both fundamental science and alternative medicine, since their potential use as natural extracts has emerged from a growing tendency to replace synthetic antioxidants by natural ones. The present study, for the first time confirmed the antimicrobial and antioxidant activities of the ethanolic extract of the Serbian plant *Satureja hotensis L.* The obtained results suggest that the extract of the endemic species *Satureja hotensis L* shows antimicrobial activity under *in vitro* conditions against the test bacteria and fungi as well as antioxidant activity relative to the control antioxidants.

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