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ANTIMICROBIAL ACTIVITY OF *SATUREJA HORTENSIS* L. ESSENTIAL OIL AGAINST PATHOGENIC MICROBIAL STRAINS

TATJANA MIHAJILOV-KRSTEV[1], DRAGAN RADNOVIĆ[2], DUŠANKA KITIĆ[3], ZORICA STOJANOVIĆ-RADIĆ[1] and BOJAN ZLATKOVIĆ[1]

[1]*Department of Biology and Ecology, Faculty of Science and Mathematics,University of Niš,* 18 000 Niš, Serbia
[2]*Department of Biology and Ecology,* 21000 Novi Sad, Serbia
[3]*Faculty of Medicine,* 18000 Niš, Serbia

Abstract – A hydro-distilled oil of *Satureja hortensis* L. was investigated for its antimicrobial activity against a panel of 11 bacterial and three fungal strains. The antimicrobial activity was determined using the disk-diffusion and broth microdilution methods. The essential oil of *S. hortensis* L. showed significant activity against a wide spectrum of Gram (-) bacteria (MIC/MBC=0.025–0.78/0.05-0.78 μl/ml) and Gram (+) bacteria (MIC/MBC=0.05–0.39/0.05-0.78 μl/ml), as well as against fungal strains (MIC/MBC=0.20/0.78 μl/ml). The results indicate that this oil can be used in food conservation, treatment of different diseases of humans, and also for the treatment of plants infected by phytopathogens.

Key words: *Satureja* L., *Satureja hortensis*, essential oils, antimicrobial activity

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INTRODUCTION

Aromatic plants have been known about for a very long time and owing to their aromatic and antiseptic properties they are used as spices and natural food preservatives, in the perfume industry, for aromatherapy and for different medical purposes. Among the aromatic plant species, together with *Origanum* and *Thymus*, the genus *Satureja* L. occupies a special position. The genus *Satureja* L. (savory, saturei) includes more than 30 species belonging to the family Lamiaceae, subfamily *Nepetoideae*, tribe *Mentheae*. Distribution of the genus *Satureja* overlaps the region of southern and south-eastern Europe, Asia Minor, and northern Africa, with the center of the genus area predominantly in the Mediterranean. According to Šilić (1979), there are only 12 species of the genus *Satureja* reported from Europe (Ball and Getliffe, 1973). Savory species produce antimicrobial secondary metabolites, essential oils, either as a part of their normal program of growth and development or in response to pathogens' attack or stress. The most famous species, which has the widest usage, is the annual species - *Satureja hortensis* L. Besides its use in cookery and conservation of food, it is traditionally used for treating many diseases. For this reason, scientists have studied the chemical composition of extracts and essential oils isolated from the aerial parts of this plant (leaves, stalks and flowers). It is well known that the chemical composition and yield of essential oils are affected by exogenous factors such as geographical position, altitude, climate and soil composition. During a comparative analysis of the literature data, Baser et al. (2004) pointed out that the plants cultivated on the territory of the former Yugoslavia had the highest yield of essential oil (2.7%), while the plant material cultivated in Italy had the lowest yield of oil (0.6%). In addition to the high content of the essential oil, *S. hortensis* L. cultivated on the territory of the former Yugoslavia contained a relatively high level of carvacrol (44.0%). This phenolic compound was present in the oil of this species as the dominant component in the range from 12.8% in oil from *S. hortensis* of Russian origin, to 73.0% in oil samples of Crimean origin. Beside carvacrol, the essential oil also

contains γ-terpinene - from 6.0% (South American oil), to 60.3% (oil from material of Lawrence), p-cymene - from 4.5% (Lawrence oil) to 35.8% (Russian oil) and thymol - from 8.6% (Russian oil) to 18.0% (South American oil). The same authors, according to their analysis of 20 samples from different localities in Turkey, concluded that cultivated forms of *S. hortensis* contained carvacrol as the dominant component of oil (42.0-63.0%), while in the oil of wild growing forms thymol dominated as the main component (29.0-43.0%) (Baser et al., 2004).

Together with exogenous factors, the quality and quantity of essential oils are also affected by endogenous factors (ontogenetic development stage), method of drying and method of essential oil isolation. Sefidcon et al. (2006) concluded that drying of the aerial parts of *Satureja hortensis* in an oven at 450C and extraction of their essential oil by hydro-distillation is most suitable and is recommended for fast drying and high oil yield (1.06%), as well as for a high percentage of carvacrol (48.1%). Beside this, the highest content of phenolic compounds was in the oil isolated from material collected during the full-flowering stage (Sefidkon et al., 2006a).

Pharmacological and biological investigations justified the traditional application of *S. hortensis* L. as a natural source of compounds for food conservation, as well as in the treatment of ailments including inflammatory diseases, cramps, muscle pains, nausea, indigestion, diarrhea, and infectious diseases (Leporatti and Ivancheva, 2003), due to its antispasmodic, anti-diarrhoeal (Hajhashemi et al., 2000), antioxidant (Dorman and Hiltunen, 2003), antibacterial (Chorianopoulos et al., 2004; Adiguzel et al., 2007) and antifungal properties as reported in literature (Boyraz and Ozcan, 2006; Adiguzel et al., 2007). The essential oil of *S. hortensis* L. showed high activity against clinical multiresistant isolates from wounds (Mihajilov-Krstev et al., 2009). Yazdanparast and Shahriyary (2007) proved the effects of *Satureja hortensis* L. methanol extract on the inhibition of blood platelet adhesion, aggregation and secretion. This explains its traditional use in treatments of cardiovascular diseases and thrombosis. Hajhashemi et al. (2002) suggested that the hydro-alcoholic extract, polyphenolic fraction and essential oil of the aerial parts of the *S. hortensis* L. have antinociceptive and anti-inflammatory effects. Also, they supposed that probably mechanism(s), other than the involvement of opioid and adenosine receptors, mediate(s) the antinociception. This claim was confirmed by the results of Uslu et al. (2003) who demonstrated on a rabbit model that the water extract of S. *hortensis* can be used for the treatment of rhino-sinusitis diseases.

In previous papers, the results of the antimicrobial activity of *S. hortensis* L. extract were published. The effect of the essential oil was investigated by the more or less precise disk-diffusion method (Deans and Svoboda, 1989). It was limited to food borne pathogens (Oussalah et al., 2006; 2007; Adiguzel et al., 2007).

For this reason, in this paper the disk-diffusion method was used only for preliminary screening while the more precise broth microdilution method was employed to determine antimicrobial activity of *S. hortensis* L. essential oil. The testing was performed against a wide spectrum of Gram (+) and Gram (-) bacteria. *S. crevisiae* and *C. albicans* were used as a model system for yeasts, while *A. niger* was used as a model system for fungi.

MATERIALS AND METHODS

Plant Material

The aerial parts of cultivated *Satureja hortensis* L. (in the area near Niš, the village Malča), were collected at the beginning of the flowering stage. Voucher specimens No. UTM34TEN89 were confirmed and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad.

Extraction of the essential oil

Air-drying of plant material was performed in a shady place at room temperature for 10 days. Dried

aerial parts (100 g) were cut and subjected to hydrodistillation for 3 h, using a Clevenger-type apparatus. The resulting essential oil was dried over anhydrous sodium sulfate and stored at 4°C.

Microbial strains

The antimicrobial activity of *S. hortensis* L. essential oil was evaluated using laboratory control strains, *Bacillus subtilis* ATCC 6633, *Clostridium perfringens* ATCC 19404, *Micrococcus flavus* ATCC 40240, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538 and *Sarcina lutea* ATCC 9341 (Gram (+) bacteria), *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enteritidis* ATCC 13076 and *Erwinia amylovora* NCPPB 595 [Gram (-) bacteria] and *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* 112 Hefebank Weihenstephan (fungal microorganisms) obtained from the American Type Culture Collection. The inocula of the bacterial and fungal strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity (corresponding to 10^7-10^8 CFU/ml for bacteria, depending on genera, and $0.4x10^4$–$5x10^4$ spore/ml for fungal strains (consensus standard by the NCCLS M38 [ISBN 1-56238-480-8]).

Disc-diffusion assay

This method is presented as a consensus standard by the NCCLS (National Committee for Clinical Laboratory Standards, 2001). Essential oils were diluted in ethanol to the test concentration (2%, 5% and 10%). Antimicrobial tests were carried out by the disc-diffusion method using 100 µl of suspension containing 2.0 x 10^8 CFU/ml of bacteria and 2.0 x 10^4 of fungal spores spread on Mueller-Hinton agar (MHA, Torlak) and malt extract agar (Torlak) in sterilized Petri dishes (90mm in diameter). The discs (6mm in diameter, HiMedia Laboratories Pvt. Limited) were impregnated with 15 µl of the oil dilution (2%, 5% and 10%) and placed on the inoculated agar. Negative controls were prepared using the same solvents to dissolve the essential oil (ethanol). Chloramphenicol (30 µg), Streptomycin (30 µg) and Nystatin (30 µg) were used as positive reference standards to determine the sensitivity of a strain of each tested microbial species. The inoculated plates were kept at 4^0C for 2 h and incubated at 37^0C for 24 h for bacterial strains, and at 28^0C for 48 h for fungal strains. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms.

Broth microdilution assay

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) according to the National Committee for Clinical Laboratory Standards (NCCLS, 2003). The inocula of the bacterial strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Methanol was used to dissolve the essential oil and then diluted to the highest concentration (500 µl/ml). A serial doubling dilution of the oil was prepared in a 96 well microtiter plate over the range of 50.00-0.02 µl/ml in inoculated nutrient broth (the final concentration in each well adjusted to 2.0 x 10^6 CFU/ml for bacteria and 2.0 x 10^5 of spores for fungal strains). The plate was incubated for 24 h at 37^0C for bacteria and for 48 h at 25^0C for fungal strains. Chloramphenicol, streptomycin and nystatin served as a positive control, while the solvent was used as a negative control. MIC was defined as the lowest concentration of essential oil at which microorganisms show no visible growth. The microbial growth was determined by absorbance at 620nm using the universal microplate reader (ThermoLabsystems, Multiskan EX, Software for Multiscan ver.2.6.). To determine MBC/MFC, broth was taken from each well and inoculated in Mueller Hinton agar (MHA) for 24h at 37^0C for bacteria or in malt extract agar (MEA) for 48 h at 25^0C for fungal strains. The highest dilution without growth is the minimum inhibitory concentration – MIC (NCCLS, 2003). The MBC/MFC is defined as the lowest concentration of the essential oil at which 99.9% of the inoculated microorganisms were killed.

Statistical analysis

Analysis of variance (ANOVA) was used to determine the significance ($p \leq 0.05$) of the data obtained in all experiments. All results were determined to be within a 95% confidence level for reproducibility.

RESULTS

Extraction of the essential oil

From the collected plant material of S. *hortensis* L., by the process of hydro-distillation, 2.05% (v/w) of essential oil was isolated. The oil was intensively yellow, with a characteristically strong and pleasant odor.

Disc-diffusion assay

The results of the disk-diffusion method showed very high activity against all tested strains of microorganisms. The higher susceptibility showed Gram (+) bacteria: *B. subtilis* (18-23-45 mm), *S. lutea* (19-23-38 mm), *M. flavus* (17-22-33 mm), *S. aureus* ATCC 8538 (18-20-30 mm), *C. perfringens* (16-18-27 mm) and *S. aureus* ATCC 25923 (15-15-20 mm, respectively) (Fig. 1, A). Gram (-) bacteria were slightly more resistant, where smaller, but similar inhibition zones were measured: *S. enteritidis* (18-19-19 mm), *E. coli* ATCC 25922 (16-16-20 mm), *E. coli* 8739 (18-17-18 mm) and P. *aeruginosa* (15-16-15 mm, respectively) (Fig. 1, B). All tested fungal strains were highly sensitive to the activity of *S. hortensis* L. essential oil: *A. niger* (18-34-41 mm), *C. cerevisiae* (18-27-31 mm) and *C. albicans* (18-19-26 mm) (Fig. 1, C).

Broth microdilution assay

The results of broth microdilution assay showed that the essential oil was active against all tested Gram (-) bacteria in the following range of concentrations: MIC/MBC=0.025–0.78/0.05-0.78 µl/ml, while the referent antibiotic chloramphenicol had an effect at MIC/MBC= 4.0-16.0 µl/ml. The lowest susceptibility showed *P. aeruginosa*, where the oil had a microbiostatic effect at low concentration (MIC=3.125 µl/ml), but showed a microbicidal effect at the highest tested concentration (MBC=50.0 µl/ml). Particularly susceptible was the phytopathogenic bacteria *Erwinia amylovora* (MIC/MBC=0.025/0.05 µl/ml) (Fig. 2).

The essential oil was active against tested Gram (+) bacteria in the range from MIC/MBC=0.05–0.39/0.05-0.78 µl/ml which exhibited higher activity relative to the referent antibiotic streptomycin (MIC/MBC=0.5-8.0/1.0-16.0 µl/ml). The oil showed the highest activity against the following strains: *C. perfringens* (MIC=MBC=0.05 µl/ml), *S. lutea* (MIC=MBC=0.10 µl/ml) and *M. flavus* (MIC/MBC=0.10/0.20 µl/ml) (Fig. 3).

The essential oil of *S. hortensis* L. also showed a very high antifungal activity against the fungal strains *A. niger* (MIC/MBC=0,78 µl/ml), *S. cerevisiae* (MIC/MBC=0,39/0,20 µl/ml), as well as against *C. albicans* (MIC/MBC=0,20 µl/ml) (Fig. 4).

DISCUSSION

The essential oil of *S. hortensis* L. collected in Serbia yielded 2.05% which is in accordance with the previous results of Baser et al. (2004). Thirty six components (86.14%) were identified as constituents of this essential oil by combined GC/FID and GC/MS analyses. The major components were carvacrol (67.00%), γ-terpinene (15.3%), and p-cymene (6.73%). In a smaller percent, α–terpinene (1.29%), β–caryophyllene (1.90%) and β–bisabolene (1.01%) were identified as constituents of the oil. The monoterpene prevalence in the oil (82.33%) was evident, while the most abundant were oxygenated monoterpenes (69.14%). In addition, sesquiterpene hydrocarbons (3.15%) and oxygenated sesquiterpenes (0.46%) were isolated (Mihajilov-Krstev et al., 2009).

The present results demonstrate high activity against a wide spectrum of pathogenic Gram (-) and Gram (+) bacteria and three fungal strains. The high antimicrobial activity is explained firstly by the

fact that the phenol compound carvacrol (67.00%) is the main constituent of the oil, present in very high percentage (Dorman and Deans, 2000; Lambert et al., 2001; Bennis et al., 2004; Nostro et al., 2004; Bagamboula et al., 2004; Di Pasqua et al., 2006; Ben et al., 2006; Krist et al., 2007 Mihajilov-Krstev et al., 2009). Most of the studies on the mechanism of this phenolic compound focused on its effects on cellular membranes which alters its function and, in some instances, structure, causing swelling as a result of its increased permeability. Increases in cytoplasmic membrane permeability appear to be a consequence of the loss of the cellular pH gradient, proton motive force and decreased ATP levels, resulting in the death of the cell (Ultee et al., 2000). The biological precursor of carvacrol, p-cymene, is hydrophobic and causes an expansion of the cytoplasmic membrane. When combined with carvacrol *in vitro*, p-cymene incorporates into the cytoplasmic membrane, facilitating transport of carvacrol across the membrane (Ultee et al., 2002). Thus, the antimicrobial activity of carvacrol is increased by the presence of its precursor p-cymene, owing to the described synergistic effect. The determined high antimicrobial activity of the tested oil can also be explained by the significant presence of p-cymene (6.73%).

Our results indicate that *S. hortensis* essential oil collected in Serbia, showed higher activity in relation to the results from previous studies. The activity of this oil against *B. subtilis* was in the range from MIC/MBC=0.39/0.78 µl/ml, while the oil collected from Yusufeli in Turkey did not show any effect against this strain. Also, it exhibited higher activity against two strains of *S. aureus* (MIC=0.20 and 0.39 µl/ml, respectively) in relation to the oil from Turkey (MIC=15.62-62.50 µl/ml, respectively) (Adiguzel et al., 2007). The result of *P. aeruginosa* susceptibility is in accordance with the effect of the Turkish *S. hortensis L.* essential oil against this strain (MIC=31.25, 62.50 and 125 µl/ml, respectively) (Adiguzel et al., 2007). The determined high susceptibility of *Erwinia amylovora* is very significant from the standpoint of this oil's application as a natural and non-toxic substance in the protection of economically important plants.

In previous studies, the essential oil of *S. hortensis* L. showed antifungal activity against phytopathogenic fungi (Boyraz and Ozcan, 2006) and against food spoilage fungi (Adiguzel et al., 2007). The dominant component of this oil – carvacrol, is capable of inhibiting aflatoxin production by *A. parasiticus* (Razzaghi-Abyaneh et al., 2008) and *A. flavus* in a liquid medium and tomato paste (Omidbeygi et al., 2006; Dikbas et al., 2008). The same authors suggested that carvacrol could be useful in controling aflatoxin contamination of susceptible crops. In the present paper, the oil exhibited very high antifungal activity against *A. niger* (MIC/MBC= 0.78 µl/ml), *S. cerevisiae* (MIC/MBC=0.39/0.20 µl/ml) and *C. albicans* (MIC/MBC= 0.20 µl/ml). Against *C. albicans*, the oil from Turkey did not show any effect (Adiguzel et al., 2007). According to this, savory oil can be considered as a potential natural agent for the treatment of candidiasis.

The determined disc-diffusion inhibition zones were very similar for different ethanol dilutions of oil. Comparison of these with the broth microdilution method results demonstrated that the disc-diffusion method is not a precise method, pointing out its significance only as a preliminary antimicrobial screening method. The more precise broth microdilution method showed that in relation to most of the investigated essential oils, which showed much lower activity against Gram (-) bacteria, the oil of *S. hortensis* L. exhibited the same high effect against both groups of bacteria, as well as against fungal strains. Its MIC/MBC values are very low (lower than the referent antibiotics) and in most cases they are at the same concentration. These values, together with high yield and non-toxicity (Stammati et al., 1999) justify its use for many purposes: food conservation, treatment of different human diseases and also for treatment of phytopathogens which infect economically important plants.

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АНТИМИКРОБНА АКТИВНОСТ ЕТАРСКОГ УЉА *SATUREJA HORTENSIS* L. ПРОТИВ ПАТОГЕНИХ СОЈЕВА МИКРООРГАНИЗАМА

ТАТЈАНА МИХАЈИЛОВ-КРСТЕВ[1], Д. РАДНОВИЋ[2], ДУШАНКА КИТИЋ[3], ЗОРИЦА СТОЈАНОВИЋ-РАДИЋ[1] и Б. ЗЛАТКОВИЋ[1]

[1]*Department of Biology and Ecology, Faculty of Science and Mathematics,* 18000 Niš
[2]*Department of Biology and Ecology,* 21000 Novi Sad, Serbia
[3]*Faculty of Medicine,* 18000 Niš, Serbia

Етарско уље врсте *S. hortensis* L., изоловано дестилацијом у апаратури по Clavenger-у, показало је високу антимикробну активност против типских сојева (11 бактеријских и 3 фунгална соја). Тестирање је вршено поређењем двеју метода: диск-дифузионе и микродилуционе. Микродилуционом методом је установљено да је етарско уље врсте *S. hortensis L.* деловало против свих тестираних грам-негативних бактерија, у концентрацији од MIC/MBC=0,025–0,78/0,05-0,78 μl/ml), на које је референтни антибиотик хлорамфеникол деловао у опсегу од MIC/MBC=4,0-16,0 μl/ml. На испитиване грампозитивне бактерије уље је деловало у опсегу од MIC/MBC=0,05–0,39/0,05-0,78 μl/ml), што је знатно боље него деловање референтног антибиотика стрептомицина MIC/MBC=0,5-8,0/1,0-16,0 μl/ml. Уље је испољило високу антифунгалну активност на сојеве *A. нигер* (МИЦ/МБЦ=0,78μл/мл), *S. cerevisiae* (MIC/MBC=0,39/0,20 μl/ml), као и на *C. albicans* (MIC/MBC=0,20 μl/ml). Овако добијена висока антимикробна активност етарског уља *S. hortensis* L. се може објаснити високим садржајем ароматичног монотерпенског алкохола карвакрола (67,00%) и угљоводоника γ-терпинена (15,3%) и п-цимена (6,73%), који испољавају синергистички ефекат.

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IN VITRO SYNERGISTIC ANTIBACTERIAL ACTIVITY OF *SALVIA OFFICINALIS* L. AND SOME PRESERVATIVES

DRAGANA STANOJEVIĆ[1*], LJILJANA ČOMIĆ[1], OLGICA STEFANOVIĆ[1] and SLAVICA SOLUJIĆ-SUKDOLAK[2]

[1]*Department of Microbiology,* and [2]*Department of Biochemistry*
Faculty of Science, University of Kragujevac, 34000 Kragujevac, Serbia

Abstract – The aim of this work was to investigate the antibacterial activity of aqueous extracts of the species *Salvia officinalis* L. and its synergistic action with the preservatives sodium nitrite, sodium benzoate and potassium sorbate *in vitro* against selected food spoiling bacteria. Synergism was assessed by the checkerboard assay method and quantitatively represented by the FIC index. Synergistic action was established for aqueous extract/sodium benzoate, aqueous extract/potassium sorbate, aqueous extract/sodium nitrite combinations. Synergism was detected in relation to: *Agrobacterium tumefaciens, Bacillus subtilis* and *Proteus* sp. Synergism was established at plant extract and preservative concentrations corresponding up to 1/8 MIC values.

Key words: *Salvia officinalis* L., plant extracts, preservative, MIC, synergism

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INTRODUCTION

Salvia officinalis L (commonly known as sage) of the Lamiaceae family is an aromatic, perennial plant widely distributed in Europe. Since ancient times it has been used as a flavoring agent in a variety of food preparations and it is integral part of the popular, healthy Mediterranean diet (Malamas et al., 1992). In the past few decades sage has been the subject of intensive studies for its diterpenoids, triterpenoids, flavonoids and phenolic glycosides, which have been isolated from the plant (Couladis et al., 2002; Durling et al., 2007; Länger et al., 1998). It is for this reason that sage has found increasing application in food formulations (Shahidi, 2000). Lima et al., (2005) tested the antioxidant potential of the Salvia tea *in vivo* and showed that following 14 days of drinking *Salvia* tea the liver antioxidant status improved. The aqueous extract of *S. officinalis* possesses an antioxidant (Geuenich et al., 2008) and antiviral effect (Lima et al., 2007).

In recent years there has been considerable interest in ways to reduce the incidence of food poisoning. As a result of scientific research and negative consumer perceptions of artificial preservatives, attention is shifting towards alternatives that consumers perceive as natural plant extracts (Fiorentino et al., 2008; Gutierrez et al., 2008a). Sodium benzoate has proved a controversial additive, as recent studies have highlighted health concerns from its use (Haaws et al., 2007; McCann et al., 2007) and the commonly used preservative sodium nitrite has been under the spotlight since 2007 (Jiang et al., 2007). The use of plant extracts as natural preservatives has been especially highlighted since The European Food Safety Authority's Pronouncement (2008) that rosemary extract is safe for use as an antioxidant in food. The antibacterial activity of sage against food spoilage bacteria has been investigated (Di Pasqua et al., 2005; Viuda-Martos et al., 2007), but there have been no studies to test the synergy between an aqueous extract and preservatives.