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

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**ABSTRACT**

A hydrodistilled 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 disk-diffusion method and broth microdilution method. Essential oil of *S. hortensis* L. showed significant activity against wide spectrum of Gram (-) bacteria (MIC/MBC=0.025–0.78/0.06–0.78 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). Therefore, the present results indicate that this oil can be used in food conservation, treatment of different deseases of humans, and also for the treatment of the plants infected by phytopathogens.

**Keywords:** *Satureja* L., *S. hortensis*, essential oils, antimicrobial activity


**Introduction**

Aromatic plants are known for a very long time and owing to their aromatic and antiseptic properties, they are used in many ways – as spices and natural food conservances, but also in perfume industry, for aromatherapy and 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 southeastern Europe, Asia Minor, and northern Africa, with the centre of the genus area predominantly in the Mediterranean. According to Silic (28), there are only 12 species of the genus *Satureja* reported from Europe (2, 28). 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 annual species - *Satureja hortensis* L. Besides its use in cookery and conservation of food, it is traditionally used for treatment of many deseases. For this reason, many scientists studied chemical composition of extracts and essential oils isolated from the aerial parts of this plant (leaves, stalks and flowers). It is well known that chemical composition and yield of essential oils are affected by exogenous factors such as geophysical position, altitude, climate and soil composition. During comparative analysis of the literature data, Baser et al. (4) 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%). Beside high content of the essential oil, *S. hortensis* L. cultivated on the territory of the former Yugoslavia contained 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% (oil from *S. hortensis* of Russian origin), to 73.0% (oil samples of Crimean origin). Beside carvacrol, essential oil also contain 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 analisys 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 dominated thymol as the main component (29.0-43.0%) (4).

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. (26) concluded that drying of the aerial parts of *Satureja hortensis* in the oven at 45°C and extraction of their essential oil by hydrodistillation 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 (26).

Pharmacological and biological investigations confirmed traditional application of *S. hortensis* L. as 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 (17), due to its antispasmodic, anti-diarrhoeal (13), antioxidant (12), antibacterial (1, 7) and antifungal properties as reported
in literature (1, 6). Essential oil of S. hortensis L. showed high activity against clinical multiresistant isolates from wounds (18). Yazdanparast and Shahriary (33) proved the effects of Satureja hortensis L. methanol extract on inhibition of blood platelet adhesion, aggregation and secretion. This explains its traditional use in treatments of cardiovascular diseases and thrombosis. Hajhashemi et al. (14) suggested that hydro-alcoholic extract, polyphenolic fraction and essential oil of the aerial parts of S. hortensis L. have antinociceptive and anti-inflammatory effects. Also, they supposed that probably mechanism(s) other than involvement of opioid and adenosine receptors mediate(s) the antinociception. This claim was confirmed by the results of the Uslu et al. (32) study, where they demonstrated, on a rabbit model, that water extract of S. hortensis can be used for the treatment of rhinosinusitis diseases.

In previous papers, the results of antimicrobial activity of S. hortensis L. extract were published, while the effect of essential oil was investigated by more or less precise disk-diffusion method (8), or it was limited to foodborne pathogens (1, 22).

For this reason, in this paper, disk-diffusion method was used only for preliminary screening, while more precise broth microdilution method was employed to determine antimicrobial activity of S. hortensis L. essential oil. The testing was performed against 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. (surrounding of Nis, Malca village), 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 cutted and subjected to hydro-distillation 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 0.4x10^3-5x10^4 spore/ml for fungal strains(19).

Disc-diffusion assay

This method is presented as a consensus standard by the NCCLS (19). Essential oils were diluted in ethanol to different test concentrations (2%, 5% and 10%). Antimicrobial tests were carried out by 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°C for 2 h and incubated at 37°C (24 h) for bacterial strains and at 28°C (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 (19). 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 (500μl/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 10^6 of spores for fungal strains). The plate was incubated for 24 h at 37°C for bacteria and for 48 h at 25°C for fungal strains. Chloramphenicol, Streptomycin and Nystatin served as positive controls, 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 620 nm using the universal microplate reader (ThermoLabystems, 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 24 h at 37°C for bacteria or in malt extract agar (MEA) for 48 h at 25°C for fungal strains.
The highest dilution without growth is the minimum inhibitory concentration – MIC (19). The MBC/MFC is defined as the lowest concentration of the essential oil at which inoculated microorganisms were 99.9% killed.

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

Results and Discussion
From the collected plant material of S. hortensis L., by the process of hydro-distillation, 2.05% (v/w) of essential oil has been isolated. The oil was intensively yellow, with characteristically strong and pleasant odour. The yield of essential oil collected in Serbia, is in accordance with the previous results of Baser et al (4). Thirty six components (86.14%) were identified as constituents of this essential oil by combining GC/FID and GC/MS analyses. The major components were carvacrol (67.00%), γ-terpinene (15.3%), and p-cymene (6.73%). In 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 oil (82.33%) was evident, while the most abundant were oxygenated monoterpenes (69.14%). In addition, sesquiterpenes hydrocarbons (3.15%) and oxygenated sesquiterpenes (0.46%) were isolated (18).

The results from the disk-diffusion method assays 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. 1A). Gram (-) bacteria were slightly more resistant, while 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. 1B). 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. 1C).

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

Against tested Gram (+) bacteria, essential oil was active in the range from MIC/MBC=0.05–0.39/0.05-0.78 μl/ml, which is higher activity in relation to the referent antibiotic, streptomycine (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

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by the fact that the fenol compound carvacrol (67.00%) is the main constituent of the oil, present in very high percentage (3, 5, 9, 11, 15, 16, 18, 20). Most of the studies on the mechanism of this phenolic compound focused on its effects on cellular membranes, where the compound is altering the membrane function and in some instances its structure, causing swelling and an increase in its 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 (31). Biological precursor of carvacrol, p-cymene, is hydrophobic and causes expansion of the cytoplasmic membrane. When combined with carvacrol in vitro, p-cymene incorporates into cytoplasmic membranes, facilitating transport of carvacrol across them (30). Thus, antimicrobial activity of carvacrol is increased by the presence of its precursor p-cymene, owing to described synergistic effect. Determined high antimicrobial activity of the tested oil can also be explained by 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 (1, 6, 8, 25). Activity against *B. subtilis* of this oil 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 mentioned oil from Turkey (MIC=15.62-62.50 μl/ml, respectively) (1). The result for *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) (1). The determined high susceptibility of *Erwinia amylovora* is very significant from the standpoint of this oil’s application as natural and non toxic substance in the protection of economically important plants.

In previous studies, essential oil of *S. hortensis* L. showed antifungal activity against phytopathogenic fungi (6) and against food spoilage fungi (1). The dominant component of this oil, carvacrol, is able to inhibit aflatoxin production by *A. Parasiticus* (23) and *A. flavus* in liquid medium and tomato paste (10, 21). The same authors suggested that carvacrol may be useful to control aflatoxin contamination of susceptible crops in the field. 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), as well as against *C. albicans* (MIC/MBC=0,20 μl/ml) (Fig. 4.).
the investigated essential oils, which showed much slighter 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. It’s MIC/MBC values are very low (lower than these of the referent antibiotics) and in most cases they are at the same concentration.

Conclusions
Essential oil of S. hortensis L., collected in Serbia, yielded 2.05%, which is higher than previously reported (4). The oil exhibited very high antibacterial and antifungal activity, owing to high content of monoterpen carvacrol, which is well known antimicrobial compound. Active concentrations were much lower than those of the referent antibiotics. These values, together with high yield and lack of toxicity (29) economically justify the use of essential oil derived from S. hortensis L. for many purposes: food conservation, treatment of different human deseases, and also for the treatment of phytopathogens which infect economocaly important plants.

REFERENCES