Antimicrobial Activity of Various Extracts of *Satureja hortensis L.*

Mehlika BENLI[*1] Nazife YIĞIT[2] İlhan KAYA[3]

[1]Department of Biology, Faculty of Science, University of Ankara, Ankara, TURKEY
[2]Department of Biology, Faculty of Science and Arts, University of Kırıkkale, TURKEY
[3]Deparment of Plant Protection, Faculty of Agriculture, University of Yüzüncüyıl, Van, TURKEY

*Corresponding author
e-mail: benli@science.ankara.edu.tr.

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Abstract

The antimicrobial activities of ethyl acetate and methanol extracts of *Satureja hortensis L.* were studied. These extracts were tested in vitro against 10 bacterial species, 4 yeasts species and strains by the disc diffusion method. The results indicated that the methanol extract of *S. hortensis* is more effective against tested microorganisms than ethyl acetate. While ethyl acetate extract showed inhibition effect against *Pseudomonas aeruginosa* (ATCC 27853) and *Bacillus subtilis* (RSHI), methanol extract affected *Bacillus subtilis* (RSHI), *Escherichia coli* (ATCC 25922), *Shigella* (RSHI), *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 27853). The cells of microorganisms treated with plant extracts and normal microorganism cells were observed by scanning electron microscope. It was apparent that cells after treated with *S. hortensis* are damaged.

Key words: Antimicrobial activity, disc diffusion method, SEM, spices, *Satureja hortensis.*

INTRODUCTION

Some spices and their derivatives have been used to preserve foods, make them more tasty, as cure for diseases and to produce aromatic substance for many thousand of years. Spices have gained great importance due to their properties such as antimicrobial, antioxidants, antiviral, fungicidal, and have been the subject of many studies.

Spices extracts and their essential oils are a heterogeneous group of complex mixtures of organic compounds whose quality and quantity vary with the growth stages, ecological conditions and other factors of the plant. In addition to these, antimicrobial effects of the spices and derivatives depend also on test microorganisms, extraction techniques, and antimicrobial methodology [1]. Despite the development of antibiotics, bacterial and fungal infections are still a major issue in medicine, and the presence of numerous drug resistant strains poses a new challenge. Herbal drugs have been extensively used in this field for many centuries. Recently, there has been a growing interest in natural products due to their availability, fewer side effects or toxicity as well as better biodegradability as compared to the available antibiotics and preservatives [2-5]. The Lamiaceae is a plant family which can be found several spices with potential from microbial spoilage, primarily due to their essential oils. Especially, Lamiaceae family is well known for their antioxidant rich properties. The genus *Satureja* that belongs to this family provides various species, and *Satureja hortensis* L. is one of them. The essential oils isolated from various species of *Satureja* have certain biological properties such as antimicrobial, antiviral, antifungal and antioxidant activity [6,7].

Satureja hortensis (savory) is recognized as "Zeyturun", and is naturally distributed in the East Anatolia region in Turkey. The leaves of these plants are use in traditional food as spices. This plant is applied in flavoring condiments, relishes, soups, sausage, canned meats and spicy table sauces.

Various medicinal plants have been used for years in daily life to treat disease all over the world including our country. Turkey is an important floristic center internationally because of its geographic location, climate and the presence of nearly 10.000 natural plant species. For example, some endemic *Verbascum*, *Salvia* and *Stachys* species and certain plants used in Turkish traditional medicine were studied by Dulger and Gonuz [8,9]. The various extracts of *Spirulina platensis* have been screened for their activity against pathogenic microorganisms [10]. Also, antimicrobial activities of various medicinal and commercial plants extracts were studied by several investigators [11-13]. The aim of this study was to demonstrate antimicrobial activity of ethyl acetate and methanol extracts of *Satureja hortensis,* and compare the inhibitory effects of two different extracts. This study also showed cells damages after treatment with the most active plant extracts. The cell damages were observed by Scanning Electron Microscope (SEM).

MATERIALS AND METHODS

Plant material

Satureja hortensis L. was selected as a test plant. Fresh plants of *S. hortensis* were collected from Van (Turkey) during June-September of 2004. These plants were identified and preserved in Department of Plant Protection, Faculty of Agriculture, University of Yuzuncuyil, and then all plants were air-dried. The leaves were taken and used in this study to determine its antimicrobial activity against the test microorganisms.

Preparation of plant extracts

Dried leaves of plants were mechanically graded, and 2g of plant was extracted with 20 ml of ethyl acetate or methanol then was gently heated after rinsed for 24 h at room temperature. Then the extracts were filtered using Whatman filter paper no. 1 and the filtrates were then evaporated in incubator at 30 ºC. The dried extracts of ethyl acetate was resuspensed in 10 ml sterile distil water and also the dried extract of methanol was resuspensed in 5 ml sterile distil water [10-12].

Test microorganisms

In vitro antimicrobial studies were carried out ten bacteria strains (*Enterococcus gallinarum* CDC-NJ-4, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis, Escherichia coli*

ATCC 25922, *Shigella*, *Escherichia coli*, *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 29213, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 27853), and four yeast strains (*Saccharomyces cerevisiae*, *Candida albicans* ATCC 845981, *Candida crusei* ATCC 6258, *Candida albicans* ATCC 90028). These microorganisms were obtained from Microbiology Laboratory Culture Collection of Refik Saydam Hıfzıssıhha Institute (RSHI). The strains were inoculated on nutrient broth (Merck) and incubated for 24 h at +37º± 0.1 ºC.

Determination of antimicrobial activity

The disc diffusion method was used to determine the antimicrobial activities. Adequate amounts of autoclaved Muller Hinton Agar (Merck) was dispensed into sterile plates, and allowed to solidify under sterile conditions. The cultures of microorganisms growth for 24 h were used and diluted 10^{-1} with sterile serum physiological solution. 100 µl of test microorganisms were inoculated with a sterile spatula drigalski on the surface of solid medium in plates. Amikazin (30 µg/ml) (Eczacıbaşı), vancomycin (30 µg/ml) (Mayne), penicilline (10 U/ml)(I.E.ULAGAY), gentamisin (10 µg/disc) (I.E.ULAGAY), rifamicin (5 µg/ml)(Aventis), tetracycline (30 µg/ml) (SIGMA), ampiciline (10 µg/ml) (SELVA), chloromphenicol (30 µg/ml) (SIGMA), erythromycine (15 µg/ml) (SIGMA) were used positive controls. Acetone, methanol and chloroform were also used as negative controls.

The three sterile discs of 6 mm diameter were place onto each agar plates containing microorganisms with sterile forceps. Then 30 µl of extracts were absorbed onto discs under sterile conditions. Agar plates containing strains were incubated at +37 º ± 0.1 ºC for 24 h. After incubation, all plates were observed for inhibition zones, and the diameters were measured in millimeters. All experiments were done in three times [14].

The positive and negative control discs were tested on the same microorganisms under the same conditions. The stock antibiotics mentioned above that prepared appropriate amounts (µg/ml) of 20µl were absorbed onto discs (6mm). Disks injected with 20µl of pure ethyl acetate and methanol served as negative controls.

Scanning electron microscopy

Scanning electron microscopy observations were carried out on agar plates. The small parts of agar containing inhibition zone and microorganisms were cut with sterile bistouries. The small agar pieces were fixed in 3 % glutaraldehyde buffered with 0,1 M sodium phosphate buffer (pH 7.2) for 1 h at room temperature and then washed four times in sodium phosphate buffer, and postfixed in 1 % osmium tetroxide in the same buffer for 1 h and washed four in same buffer again. They were then dehydrated in a graded alcohol series. The last stages of dehydration were performed with propylene oxide. The specimens were then dried in the incubator at 30°C overnight. The dried specimens were mounted onto stubs by carbon double type. The specimens coated with a thin layer of gold by Polaron SC 502 sputter coater, and were examined in the Jeol JSM 6060 LV Scanning Electron Microscope [15].

RESULTS

Disc diffusion assay

The growth inhibition zones measured by disc diffusion method are shown in Table 1. Also, the inhibition zones formed by positive control of antibiotic disks are presented in Table 2. As can clearly seen from Table 1, the methanol as a solvent has higher efficiency than ethyl acetate in this study. The ethyl acetate extract of *S. hortensis* inhibited the growth of *P. aeruginosa* and *B. subtilis* but had no effect other microorganisms. However, the methanol extract showed inhibition effect of *B. subtilis, E. coli, Shigella, S. aureus* and *P. aeruginosa.*

Table 1. The antimicrobial activity of *S. hortensis* extracts against 14 microorganisms.

	Diameter of inhibition zone (mm)	
Microorganisms	**Ethyl Acetate**	**Methanol 5ml**
Enterococcus gallinarum CDC-NJ-4	-	-
Enterococcus faecalis ATCC 29212	-	-
Bacillus subtilis RSHI	10	17
Escherichia coli ATCC 25922	-	10,5
Shigella RSHI	-	12
Escherichia coli RSHI	-	-
Streptococcus pyogenes ATCC 19615	-	-
Staphylococcus aureus ATCC 29213	-	16
Listeria monocytogenes ATCC 7644	-	-
Pseudomonas aeruginosa ATCC 27853	11	11,5
Saccharomyces cerevisiae (Pakmaya)	-	-
Candida albicans 845981	-	-
Candida crusei ATCC 6258	-	-
Candida albicans 90028	-	-

The nine standard antibiotics were used as positive control in our study. Amikacin (30 µg), vancomycin (30 µg), penicillin (10 U), gentamicin (10 µg/disk), rifamicin (5 µg), tetracycline (30 µg), ampicilin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg) were treated against tested microorganisms (Table2).

Table 2. The inhibition zone formed by standard antibiotic discs at same conditions.

	Inhibition zone of diameter (mm)								
Microorganisms	**Amikacin**	**Vancomycin**	**Penicillin**	**Gentamicin**	**Rifocin**	**Tetracycline**	**Ampicilin**	**Chloramphenicol**	**Erythnomycin**
Enterococcus gallinarum CDC-NJ-4	-	12	-	15	13	-	-	-	11
Enterococcus faecalis ATCC 2921*2*	16	12	-	16	14	-	-	-	11
Bacillus subtilis RSHI	24	19	22	25	23	12	-	13	24
Escherichia coli RSHI	18	-	-	18	-	-	-	-	-
Shigella RSHI	20	-	-	19	-	-	-	-	-
Escherichia coli ATCC 25922	16	-	-	17	-	-	-	-	-
Streptococcus pyogenes ATCC 19615	13	12	-	16	15	-	-	-	12
Staphylococcus aureus ATCC 29213	17	15	19	17	27	12	-	-	18
Listeria monocytogenes ATCC 7644	25	16	-	27	39	-	-	-	19
Pseudomonas aeruginosa ATCC27853	17	-	-	15	-	-	-	-	-
Saccharomyces cerevisiae (Pakmaya)	-	16	-	-	17	10	8	-	11
Candida albicans 845981	17	12	-	19	15	-	-	-	12
Candida crusei ATCC 6258	14	11	-	17	17	-	-	-	11
Candida albicans 900628	17	11	-	16	15	-	-	-	11

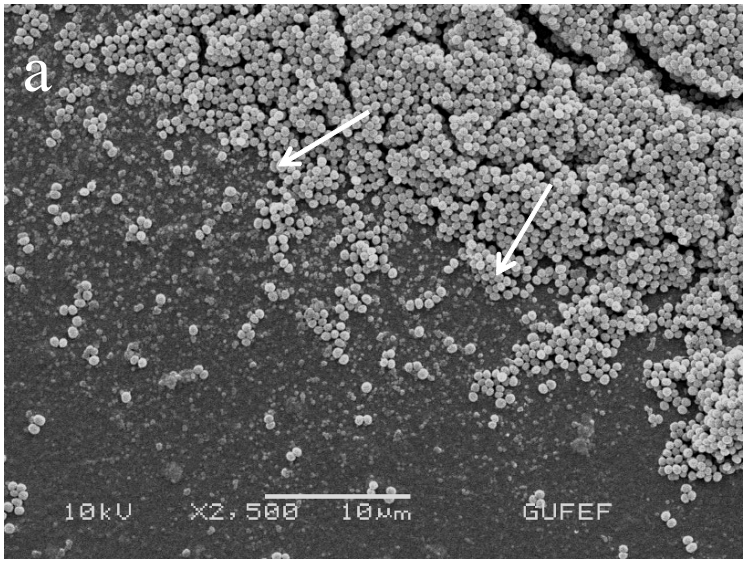
While *P. aeruginosa* were resistant to some tested antibiotics, two different extracts of *S. hortensis* had antimicrobial effects on *P. aeruginosa*. As can be clearly seen from Table 2, *Shigella*, *P. aeruginosa*, *Escherichia coli* ATCC 25922 were resistant to very much tested antibiotics but methanol extract of *S. hortensis* had antimicrobial activities on these microorganisms. On the other hand, *B. subtilis* and *S. aureus* were not only suspected to very much antibiotics but also extracts of *S. hortensis* was suspected.

Amikacin and Gentamicin affected *Shigella, P.aeruginosa* and *E. coli* ATCC 25922. These microorganisms are resistant to the other standard antibiotics (Table 2). Besides, the extracts of *S. hortensis* exhibited inhibition effects against the same microorganisms. The result showed that extracts of *S. hortensis* inhibited the growth of these bacteria. On the other hand, as can be seen from table 1, *S. aureus* and , *B. subtilis* are more susceptible to the standard antibiotics. Similarly, extracts of *S. hortensis* have inhibition effects against these microorganisms. This result is not surprising for us because these microorganisms are sensitive a lot of standard antibiotics.

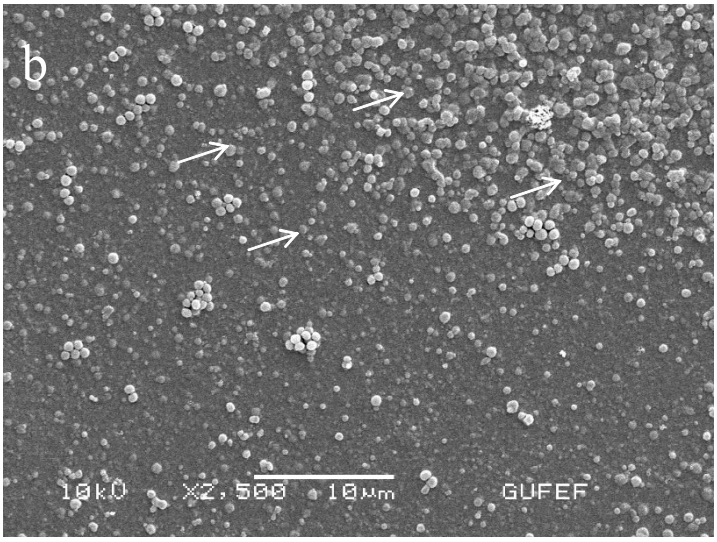
Scanning electron microscope observations

To observe morphological alterations after plant extracts treated microorganism cells we used scanning electron microscope. The plant extracts treated microorganisms cell with compared normal microorganism cells. The extract of *S. hortensis* exhibited antimicrobial properties against *B. subtilis, E. coli, Shigella, S. aureus* and *P. aeruginosa*. These microorganisms that were suspicious against *S. hortensis* extract were observed by SEM. The treated cells appeared shrinking, loss of cytoplasm and depression of the cell walls that is confronted with plant extract (Fig. 1,2,3). The morphology of control cells was normal. Similar results were found in all examined microorganisms by SEM.

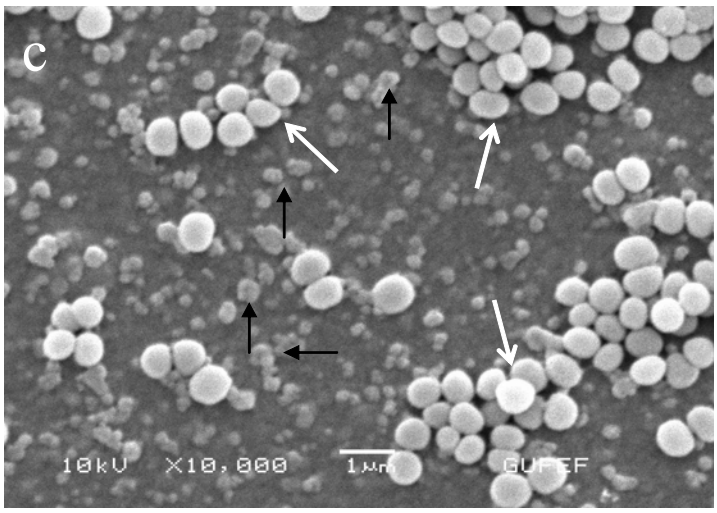
Figure. 1. Scanning electron microscope images of *S. aureus* cells after treatment with methanol extracts of *S. hortensis.*



(a) The region of inhibition zone (arrows) of *S. aureus* was treated by the methanol extracts of *S. hortensis*



(b) Damaged cells (arrows) in inhibition zone.



(c) The damaged cells (black arrows) and normal cells (white arrows) in inhibition zone region at higher magnification.

Figure 2. The inhibition zone (arrows) of *P. aeruginosa* was treated by the methanol extracts of *S. hortensis*

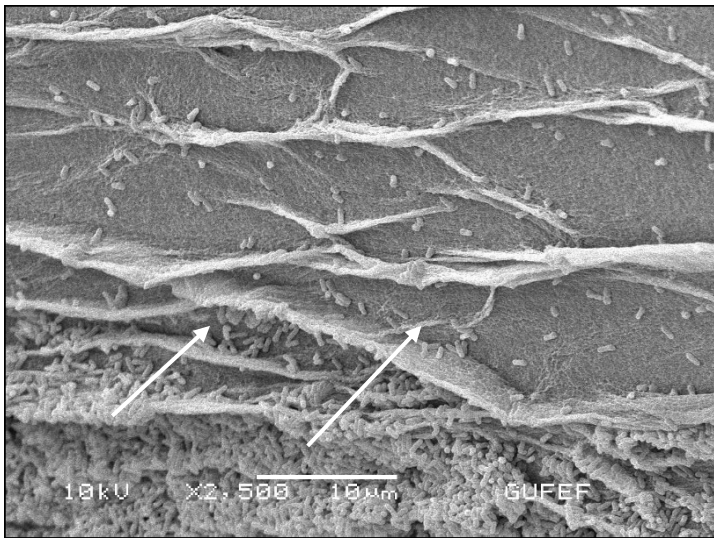
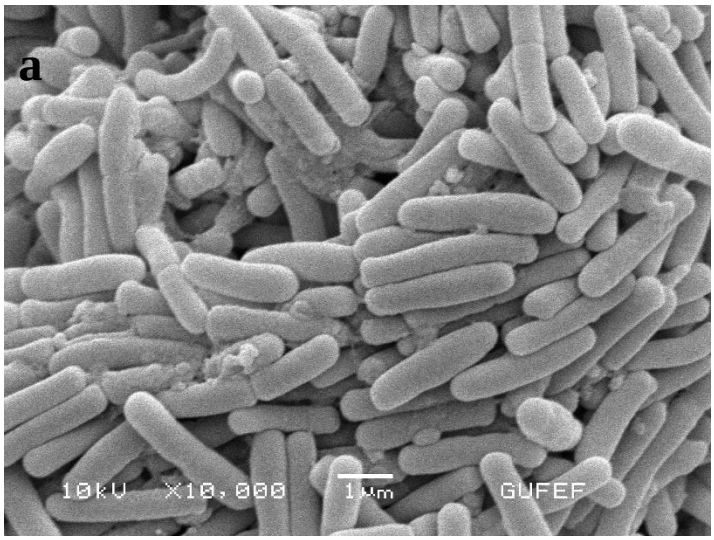
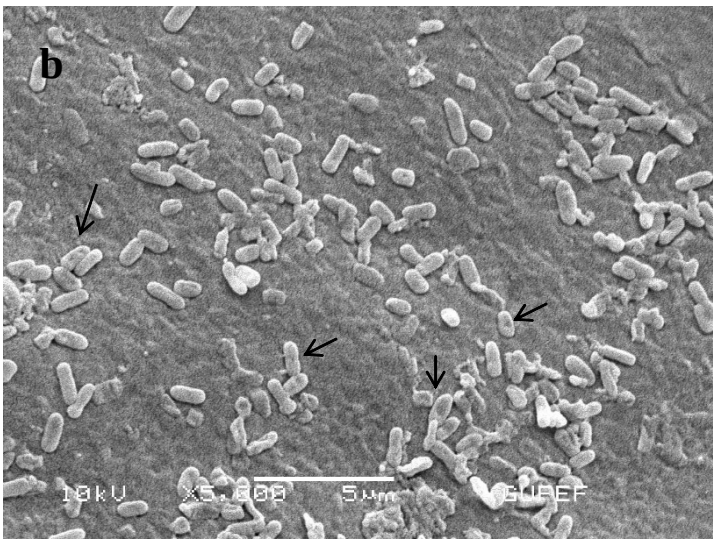


Figure 3. Scanning electron microscope images of *B. subtilis*



(a) Untreated cells of *B. subtilis*



(b) The cells of *B. subtilis* damaged after treatment with methanol extracts of *S. hortensis.*

DISCUSSION

The antimicrobial activities of *S. hortensis* (hexane and methanol) extracts against microorganisms were examined by Şahin et al. [1]. Their results showed that the hexane extract of *S. hortensis* did not have antimicrobial activity, except four strains belonging to three *Bacillus* species. However, the methanol extract showed inhibition effect of six *Candida albicans* isolates and 11 bacteria strains (*B. amyloliquefaciens, B. atrophaeus, B. macerans, B. megaterium, B. pumilus, B. sphaericus, B. substilis, E. coli, Kocuria varians, Micrococcus luteus* and *Pantoea agglomerans*). The tested microorganisms include *S. aureus* and *P. aeruginosa*, but methanol extracts of *S. hortensis* did not have antimicrobial activity against these microorganisms. While the treatments of *E. coli* and *B. substilis* were obtained similar results, in contrast our results showed that the methanol extracts affected against *S. aureus* and *P. aeruginosa* in our study.

In the another study, the antimicrobial and antioxidant activities of the essential oil, obtained by using a Clevenger distillation apparatus, water soluble (polar) and water insoluble (nonpolar) subfractions of the methanol extracts from aerial parts of *S. hortensis* was studied by Gulluce et al. [16]. Their antimicrobial test results showed that the essential oil of *S. hortensis* had great potential antimicrobial activities against all 23 bacteria and 15 fungi and yeast species tested. In contrast, the methanol extract from callus cultures and water soluble subfraction of the methanol extract did not show antimicrobial activities, but the nonpolar subfraction had antibacterial activity against only five out of 23 bacterial species, which were *Bacillus subtilis, Enterococcus fecalis, Pseudomonas aeruginosa, Salmonella enteritidis,* and *Streptococcus pyogenes.* The similar results on *Bacillus subtilis*, *Pseudomonas aeruginosa* were obtained in our study, but we not found effective results on *Enterococcus fecalis, Streptococcus pyogenes.*

A lot of studies have been performed the essential oils from Satureja plants up to now and these plants showed antimicrobial activity against a limited number of microorganisms [7, 17-21].

Burt and Reinders [22] found that oregano and thyme essential oil exhibit stronger antimicrobial properties against *E. coli* O157:H7 and observed cells damaged after treated with essential oil by SEM. On the other hand there have not been any studies that exhibited inhibition effect of S. *hortensis* by SEM. In our study, inhibition effect of *S. hortensis* was exhibited, and this mechanism was tried to demonstrate by SEM

Burt [23] tried to explain the mechanism of action for essential oils components in bacterial cells. The mechanism of action was thought to be degradation of the cell wall, damage to cytoplasmic membrane proteins, the binding of proteins, leakage of cell contents, and coagulation of cytoplasm and depletion of the proton motive force. Our studies have been continued to explain the mechanism of inhibition about plant extract.

Our result may suggest that *S. hortensis* extracts possess compounds with antimicrobial properties which can be used as antimicrobial agents in new drugs for threapy of infectious diseases in human beings.

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