CHANGES OF MEMBRANE FATTY ACIDS OF *Salmonella enteritidis* TREATED WITH THE ESSENTIAL OIL OF *Satureja hortensis*

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ABSTRACT

There are many studies focused on antibacterial and antifungal activity of the essential oil or extracts of *Satureja hortensis*. But their modes of action against microorganisms are poorly understood. The first objective of this study was to determine the antibacterial activity of *Satureja hortensis* essential oil (500 µl/ml) against *Salmonella enteritidis* strains on the growing media (Triptic Soy Agar) at 35 °C. The second objective was to determine the effect of the oil on the fatty acid composition of the bacterium to identify the mechanism of antimicrobial action. Antibacterial activity assays were carried out by disc diffusion method. Analyses of the fatty acid compositions were performed according to the method described by the manufacturer's manual (Sherlock Microbial Identification System, MIDI, Inc., Newark, DE, USA). For fatty acid analysis, the essential oil (60 µl/ml) was added to bacterial strain inoculations, simultaneously and after 24-h bacterial inoculation in the growing media (Triptic Soy Broth). Our results showed that the essential oil of *Satureja hortensis* has a stronger antibacterial activity than that of Streptomycin antibiotic against *Salmonella enteritidis*. In addition, it was observed that the proportion of the total saturated fatty acids increased in the *Salmonella enteritidis* cells, induced by addition of this oil in the growing media of both applications. But, the proportion of the total unsaturated fatty acids decreased. The changes were observed especially on decreasing of the proportion of the long-chain saturated fatty acids. To the best of our knowledge, this is the first study about the modes of actions and antimicrobial effect mechanisms of the essential oil of *S. hortensis*.

KEYWORDS: Antibacterial activity, essential oil, fatty acid, *Salmonella enteritidis*, *Satureja hortensis*

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1. INTRODUCTION

Salmonella serotype *Enteritidis (SE)* is one of the major food-borne pathogens [1]. During the last decade, antibiotic resistance and multiresistance of *Salmonella* species have also increased a great deal [2, 3]. Therefore, researchers are increasingly turning their attention to folk medicine looking for new leads to develop better drugs against microbial infections [4].

Satureja hortensis L. is a well-known aromatic and medicinal plant, which is widely distributed in the Eastern Anatolia region of Turkey, and locally named as 'Koc Otu'. A lot of references on the antimicrobial activity of *S. hortensis* essential oil or extracts are available in the literature [5-13]. Furthermore, the previous studies have shown that its extracts and essential oil possess antimicrobial activity against *SE*, and could serve as a source for antimicrobial agents against food-borne pathogens [7, 14, 15].

The prospect of using natural antimicrobial compounds as preservatives in food raises the need for more information on the behavior of spoilage and pathogenic microorganisms. The major active essential oil components are phenols, terpenes and aldehydes, and it is also well-known that the action of these substances is principally performed against the cell cytoplasmic membrane [16]. Di Pasqua et al. [17] reported that substantial changes were observed on the long-chain unsaturated fatty acids when some tested bacterial strains grew in the presence of limonene, cinnamaldehyde, carvacrol and eugenol. To the best of our knowledge, efficacy of the essential oil of *S. hortensis* on membrane fatty acids of *S. enteritidis* has never been studied before, and there is not any information about physiological changes in bacteria exposed to the essential oil of *S. hortensis*.

The first objective of the present study was to determine the antibacterial activity of *S. hortensis* essential oil against *SE* strains. The second objective was to determine the effect of the oil on the fatty acid composition of the bacterium to identify the mechanism of antimicrobial action.

2. MATERIALS AND METHODS

2.1. Pathogenic bacterium

A total of 20 *SE* strains tested were provided by the Biotechnology Research and Application Centre at Atatürk University, Erzurum, Turkey. Identification of the strains was confirmed by using Microbial Identification System (MIS). The bacterial cultures were maintained in Luria Broth and a 15% glycerol solution at -80 °C.

2.2. Plant materials

The aerial parts of *S. hortensis* were collected from Erzurum province in Eastern Anatolia region of Turkey in July 2007, at the beginning of the flowering stages, and dried in shade. The plant samples were identified by Dr. Cakmakci, and deposited at the Herbarium of the Atatürk University, Erzurum (Turkey).

2.3. Isolation of the essential oil

Air-drying of plant material was performed in a shady place at room temperature for 10 days. The dried and powdered plant samples (500 g) were subjected to hydrodistillation using a Clevenger-type apparatus for 4 h. The resulting essential oil was extracted with $CHCl_3$, then dried over anhydrous sodium sulfate (Na_2SO_4), and stored under N_2 atmosphere at 20 ºC in a sealed vial until use.

2.4. Determination of antibacterial activity

Antibacterial activity assays were carried out by disc diffusion method [18] with a minor modification. The diluted essential oil (500 µl/ml) was prepared using 10% dimethylsulfoxide (DMSO), and sterilized by filtration through 0.45-µm Millipore filters. Bacterial suspensions (100 µl) containing approximately 1 x 10^8 cfu/ml of bacterial strains were spread separately by a sterile swab on Triptic Soy Agar (TSA) medium. The discs (6 mm in diameter) were impregnated with 10 µl of the essential oil, and put on the middle of the inoculated plates. The bacterial cultures were incubated at 35±2 °C for 48 h, and then, inhibition zones were measured in diameter (mm) around the discs. DMSO (10%) and streptomycin antibiotic dissolved in sdH_2O (500 mg/ml) were used as negative and positive control, respectively.

2.5. Growth conditions of the bacterial cells induced by the essential oil

Each of the SE strains was grown in 20-ml Erlenmeyer flasks containing 10 ml of Triptic Soy Broth (TSB) medium, and were incubated on a rotary shaker at 35 °C for 24 h. Absorbance values of the bacterial suspensions were measured spectrophotometrically at 600 nm and appropriately diluted to 1x108 cfu/ml in sdH_2O. These bacterial suspensions (20 µl) were separately added to each 20-ml Erlenmeyer flask containing 10 ml of TSB medium. The essential oils (500 µl/ml) were dissolved to prepare stock solutions in 10% DMSO. The stock solution of the essential oil (1200 µl) was separately added to Erlenmeyer flasks when bacteria were inoculated, and also after 24-h inoculation. The bacterial culture broths without the essential oil were used as control. All bacterial cultures were incubated on a rotary shaker at 35 °C for 72 h.

2.6. Fatty acid (FA) extraction from bacterial cells induced by the essential oil

After 72-h incubation, the bacterial cultures applied with and without the essential oil (control) were centrifuged for 10 min at 5000 rpm, followed by washing twice with sdH_2O, and the cell pellets were harvested and submitted for membrane fatty acid extraction. Approximately 40 mg cells (wet weight) were transferred to 13x100 mm glass tubes fitted with Teflon-lined screw caps, and fatty acids were extracted using methods described by Miller [19].

2.7. Analysis of the fatty acid composition

Analyses of FA composition were performed according to the method described by the manufacturer's manual (Sherlock Microbial Identification System version 4.5, MIDI, Inc., Newark, DE, USA) [20, 21]. FAs were separated by gas chromatography (Hewlett Packard HP 6890 Series GC system) with an Ultra 25% phenyl-methyl siloxane capillary column (25 m x 0.2 mm ID x 0.33 µm df). FA profiles were identified by comparing the commercial databases (TSBA 40) with the MIS software package.

2.8. Statistical analysis

The results of this study were analyzed using SPSS (Statistical Package for Social Sciences, Windows 10.0) Statistical Analysis System. Analysis of variance (Duncan`s multiple range test at $p < 0.01$) was used for data analysis. All treatments consisted of three replicates. Experiments were repeated three times and averages of the repeated experimental results were determined.

3. RESULTS

In the present study, the essential oil of *S. hortensis* was tested for antibacterial activity against a total of 20 *SE* strains at 35 °C on Petri plates. The mean inhibition zone values for 20 bacterial strains are given in Table 1. The results showed that the oil displayed stronger antibacterial activity than that of streptomycin against the pathogens, based on the zones of inhibition. The essential oil and positive control streptomycin showed 32 and 30 mm of mean inhibition zone, respectively. Negative control DMSO did not show any inhibition zone against the pathogen.

The changes in proportion of the fatty acids of *SE* cells induced by addiction of the oil in the growing media are listed in Table 2. According to these results, substantial changes were observed on the proportion of the saturated and unsaturated fatty acids when the *SE* strains grew in the presence of the essential oil. Substantial changes, especially the decrease of the proportion of long-chain saturated fatty acids (14:0; 16:0; 17:0 cyclo and 19:0 w8c cyclo) were

TABLE 1 - Antibacterial activity of *Satureja hortensis* essential oil against a total of 20 *Salmonella enteritidis (SE)* strains at 35 °C.

Treatments	Mean inhibition zone (mm)
Negative control (DMSO 10%)	06.00 [a]
Essential oils (500 µl/ml)	32.00 [b]
Positive control (Streptomycin 500 mg/ml)	30.00 [d]

* Data given as mean of three replicates in columns.

TABLE 2 - The changes in the percentage of principal fatty acids of *Salmonella enteritidis* cells in the growing media treated with the essential oil of *Satureja hortensis* at two different times (oil applied with bacterium simultaneously and after 24 h).

Fatty acids (FAs)**	The changes in fatty acid composition (%)*		
	Oil applied simultaneously	Oil applied after 24 h	Oil not applied (control)
Saturated FAs			
9:0	7.56±1.67 [b]	17.39±1.47 [c]	0.00±0.00 [a]
11:0	3.88±0.80 [b]	2.36±0.36 [ab]	0.00±0.00 [a]
12:0	11.77±0.45 [b]	10.45±0.35 [b]	0.00±0.00 [a]
14:0	17.07±0.73 [b]	13.38±1.12 [ab]	12.00±2.33 [a]
16:0	15.20±2.69 [a]	16.59±0.66 [a]	43.68±0.56 [b]
12:0 2OH	6.86±2.06 [c]	3.21±0.53 [ab]	0.00±0.00 [a]
12:0 iso 3OH	9.78±2.60 [c]	4.80±0.99 [ab]	0.00±0.00 [a]
13:0 anteiso	0.00±0.00 [a]	1.68±0.32 [b]	0.00±0.00 [a]
17:0 cyclo	0.00±0.00 [a]	2.36±0.33 [b]	9.50±1.23 [c]
19:0 w8c cyclo	0.00±0.00 [a]	0.00±0.00 [a]	2.83±0.30 [b]
The others	1.38 [NS]	6.26 [NS]	0.00 [NS]
Total	**73.53 [b]**	**78.50 [b]**	**68.01 [a]**
Unsaturated FAs			
16:1 iso	0.00±0.00 [a]	0.84±0.13 [b]	0.00±0.00 [a]
18:1 w7c	0.00±0.00 [a]	0.00±0.00 [a]	22.95±2.67 [b]
The others	8.78 [NS]	7.39 [NS]	0.00 [NS]
Total	8.78 [a]	8.23 [a]	22.95 [b]
Unknown FAs	17.69 [ND]	13.27 [ND]	9.04 [ND]

* Values are means ± standard deviation, data in line with different letters are statistically different according to Duncan's multiple range tests at $p<0.01$; ** All treatments consisted of three replicates and experiments were repeated three times; NS: Means followed within each line are not significantly different according to $P < 0.01$; ND: Not determined.

observed when the *SE* strains grew in the presence of the oil. The proportion of total saturated fatty acids of the *S. enteritidis* cells induced by addition of this oil increased in both applications, especially when the oil was added after 24-h incubation period of the bacterial cells in the growing media.

4. DISCUSSION

Previous studies have shown that the extracts and essential oil of *S. hortensis* possess strong antimicrobial activity against pathogenic micro-organisms, and could serve as a source of antimicrobial agents against food-borne pathogens [7, 8, 13-15]. Our data confirm the findings of these studies. We also suggest the possibility of using the essential oil of *S. hortensis* as natural food preservative because of its strong antibacterial activity against *S. enteritidis*. The main compounds of the essential oil from *S. hortensis* were characterized by high amounts of thymol (29.0%) and carvacrol (26.5%), γ-terpinene (22.6%) and ρ-cymene (9.3%) [7], evidencing a strong antimicrobial activity.

In both applications, the proportion of the total saturated fatty acids increased, while that of the total unsaturated fatty acids decreased in *SE* cells, induced by addition of this oil in the growing media. The addition of the antimicrobial compounds in growing media causes the changes in fatty acid levels of the bacterial cells using an adaptation mechanism to maintain membrane structure and function. Bayer et al. [22] stated that the unsaturated fatty acids give the membrane a high degree of fluidity, whereas the saturated fatty acids tend to be relatively rigid. Therefore, the bacterial cells responded to plant essential oils by increasing the saturated fatty acids resulting in membrane fluidity changes [17]. The same effect was found as an adaptation mechanism to a rise in growth temperature, where an increase in the proportion of long-chain and saturated fatty acids within the membrane was observed [23]. Yuk and Marshall [24] as well as Sampathkumar et al. [25] found that adaptation to pH in *E. coli* O157:H7, and *S. enterica* resulted in a decrease of unsaturated fatty acid concentration, suggesting that the adaptation mechanism depends on the type of stress factor to which the cells have been exposed. The same adaptation mechanism probably occurs when the cells grow in the presence of antimicrobial compounds. The changes observed were especially a decrease of the proportion of long-chain saturated fatty acids (C16:0, C17:0 and C19:0) but an increase of the proportion of short-chain saturated fatty acids (C9:0, C11:0, C12:0, C13:0

and C14:0) of bacterial cells induced by the oil, with regard to the control. The findings of this study also confirmed that of Di Pasqua et al. [17], who found that the presence of a sublethal concentration of thymol and carvacrol during growth of *Salmonella enterica* resulted in decrease of the proportion of saturated palmitic acid (C16:0).

Consequently, this study indicated that the essential oil of *S. hortensis* showed the strong antibacterial activity against *S. enteritidis*, and may be used for the development of novel food preservation systems. However, further studies are necessary to investigate the possible interaction between oil and food components for its use in food. Furthermore, we observed that the addition of the essential oil of *S. hortensis* in growing media causes changes in the proportion of both saturated and unsaturated fatty acids of *S. enteritidis* cells. To the best of our knowledge, this is the first study about the modes of actions and antimicrobial effect mechanisms of *S. hortensis* essential oil.

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