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ORIGINAL ARTICLE

Antimicrobial and antibiofilm effects of *Satureja hortensis* essential oil against *Escherichia coli* and *Salmonella* isolated from poultry

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ABSTRACT

Background and Objectives: *Escherichia coli* and some *Salmonella* serovars cause various disease manifestations in poultry leading to significant economic losses. The widespread and imprudent use of antibacterial agents in poultry flocks have increased resistant to many antibacterial agents which has become a major public health concern. Some medicinal plants may be alternative to antibacterial agents. The purpose of this study was to investigate the antibacterial and anti-biofilm activity of summer savory essential oil against *E. coli* and *Salmonella* isolated from poultry.

Materials and Methods: The essential oil was extracted using a Clevenger apparatus and subsequently its compounds were determined using GC-MS. Antibacterial properties of essential oil were determined by disc diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). To evaluate the anti-biofilm properties the Microtiter plate test was used. Herbal essential oil was extracted and its compounds were identified correctly.

Results: The major components of *Satureja hortensis* essential oil were thymol (41.28%), γ-terpinene (37.63%), p-cymene (12.2%) and α-terpinene (3.52%). The inhibition zone diameter in the disc diffusion test for *E. coli* and *Salmonella* were 32 ± 3 and 38 ± 4 mm, respectively, which was confirmed by MIC and MBC values. Regarding anti-biofilm activity, the MIC/2 concentration of *S. hortensis* significantly inhibited biofilm formation of *E. coli*. However, inhibition of biofilm formation of *Salmonella* was shown at concentration of MIC/2 and MIC/4.

Conclusion: Based on our results, *S. hortensis* essential oil showed the growth inhibition and bactericidal activity against *E. coli* and *Salmonella*. Moreover, this study demonstrated anti-biofilm activity of *S. hortensis* essential against both tested bacteria.

Keywords: Antibacterial agents; Biofilm; *Escherichia coli*; Essential oils; Medicinal plants; *Salmonella*; *Satureja*

INTRODUCTION

Natural products such as plant extracts and plant essential oils have been used from ancient times for treatment of various diseases. These natural products have different compounds with therapeutic functions (1). *Satureja hortensis* L., known as summer savory belongs to the *Lamiaceae* family is an aromatic herb, spice and natural food preservative. This plant is traditionally used as a stimulant, stomachic, carminative, expectorant, anti-diarrheic, and aphrodisiac herb. In addition, extracts and essential oils of this plant have antioxidant, antibacterial and antifungal activities (2).

Escherichia coli is an important human and animal

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pathogens which is responsible for a serious public health concern with a significant economic burden. Avian pathogenic *E. coli* (APEC) causes extraintestinal infections, known as colibacillosis, which causes extensive mortality in poultry flocks with high economic losses (3). The pathogenicity of this bacterium is facilitated by broad range of virulence factors encoded by virulence-associated genes (4). One of the most important invasive factors associated with the pathogenicity of this bacterium is the ability to form biofilms (5).

Salmonella is a human pathogen causes a worldwide major public health concern. In addition, this bacterium frequently infects poultry flocks. Consumption of raw or undercooked poultry products contaminated by *Salmonella* can induce acute gastroenteritis in humans (6). It is well known that the biofilm formation enhances the effective colonization of *Salmonella* and subsequently increases its pathogenicity potential (7).

Biofilms known as specialized cellular communities that produce an extracellular matrix adhering to biotic or abiotic surfaces, support bacterial survival under adverse environmental conditions (8). Both Gram-positive and Gram-negative bacteria have the ability to form biofilm with varying degrees. It was demonstrated that, *E. coli* and *Salmonella* spp. can easily switch between planktonic and biofilm forms. In the biofilm phase, the bacteria can avoid from immune system and also bacterial cells in this phase are resistant about 1000 times more than their planktonic phase (9).

Today, several anti-biofilm strategies are available for the prevention of biofilm formation in bacteria including inhibition via interference in the quorum sensing pathways; adhesion mechanism; and disruption of extracellular DNA, protein, lipopolysaccharides, exopolysaccharides and secondary messengers involved in various signaling pathways (10). Also, plant-derived components often exhibit remarkable anti-biofilm ability, especially when these agents are combined with antibacterial agents (11). A wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids which their antimicrobial properties have been confirmed in different studies have been found in various plants (12).

Accordingly, the purpose of this study at first was determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *S. hortensis* essential oil against *E. coli* and *Salmonella* isolated from poultry sources. At the second step of the study, the anti-biofilm activity of the *S. hortensis* essential oil was evaluated against tested bacteria.

MATERIALS AND METHODS

Bacterial strains. Two bacterial strains, *Escherichia coli* O78:K80 and *Salmonella* Enteritidis ATCC 13076, were obtaiend from bacterial collection of the Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran. Both strains had been previously isolated from poultry infections.

Essential oil and GC-MS analysis. *Satureja hortensis* was obtained from Medicinal Plants and Drugs Research Institute of Shahid Beheshti University of Iran. The extraction of essential oils was performed by Clevenger apparatus for four hours as described previously (13). Determination of their main components of the plant essential oil was done according to the standard procedure by Gas chromatography–mass spectrometry (GC-MS) analysis (14).

Antibacterial susceptibility test. The susceptibility of both bacterial strains to *S. hortensis* essential oil was determined by the agar disk diffusion method according to the Clinical & Laboratory Standards Institute (15). To prepare paper disks, 10 µl of the *S. hortensis* essential oil was inoculated onto a sterile Whatman filter paper disk (6 mm diameter). After that Mueller–Hinton agar plates was cultured by surface culture plating with *Escherichia coli* and *Salmonella*. Essential oil-inoculated paper discs was transferred on the plates and then the plates were incubated at 37°C for 18-24 h. Antibacterial susceptibility was evaluated by measuring the growth inhibition zones after the incubation period. In addition to *S. hortensis* essential oil-inoculated paper discs, gentamicin (10 µg) disk was used as a positive antibacterial control.

Determination of MIC and MBC values. Minimum inhibitory concentration of *S. hortensis* essential oil was determined using broth microdilution method as previously described by Kerekes et al. (16) with some modifications. The dilution of the essential oil was made in Tryptic Soy Broth (TSB) culture media (Merck, Germany) in combination with 0.1% of

dimethyl sulfoxide (DMSO) (Merck) to increase solubility of the essential oil. Concentration of essential oil ranged from 0.19 to 25 µl.ml^{-1} in DMSO-TSB medium prepared into wells of 96-well microtiter plates (SPL, South Korea). One hundred microlitres of fresh cell suspension (5 × 10^5 CFU.ml^{-1}) in liquid culture medium was added to the wells. Positive controls contained the inoculated growth medium without any essential oil and negative controls contained essential oil in sterile medium. Plates were aerobically incubated at 37°C for 24 h. The MIC was considered as the lowest concentration of the essential oil at which no visible growth was detected. To determine MBC, 5 µl of each well with no growth in MIC test was cultured on blood agar medium followed by incubation at 37°C for 24 h. The MBC was defined as the lowest concentration at which the original growth was reduced by ≥ 99.9%. The MIC and MBC were performed for each two tested bacteria.

Effect of *Satureja hortensis* essential oil on the biofilm formation. Inhibition of biofilm formation of *S. hortensis* essential oil on *E. coli* and *Salmonella* cells were investigated using the microtiter plate assay. In detail, sterile 96-well polystyrene plates were filled with 98 µl of TSB contained MIC/2 and MIC/4 concentrations of the *S. hortensis* essential oil (six wells for each concentration). Then, for each of two bacteria, two µl of the inoculum (~1.5 × 10^8 CFU/ml) was added to each well. The negative and positive controls contained TSB-DMSO with bacteria and TSB-DMSO with bacteria and gentamicin (0.1 mg/ml), respectively. After 24 h incubation at 37°C, the supernatant and un-attached cells were removed, and the wells were rinsed with physiological saline for three times. Then, 100 µl Safranin dye (0.1%) solution was added to all wells, and after 20 minutes, the excess dye was removed and washed by physiological saline. Finally, the bound dye was released by adding 100 µl ethanol (95%) and optical density values (OD) were measured using a microplate reader (ELx808, BioTek, USA) at 490 nm. Each assay was repeated three times. As a measure of efficacy, the mean ODs of treated wells were compared with those of negative control (without essential oil) and the inhibition percentage was calculated using the following formula:

Percentage of inhibition = 100 - [($OD_{490\ nm}$ of the treated wells) / (mean $OD_{490\ nm}$ of the negative control wells contained no antimicrobial agent) × 100)] (14). Figs. 1 to 4 demonstrate the data as the mean ± SD (standard deviation).

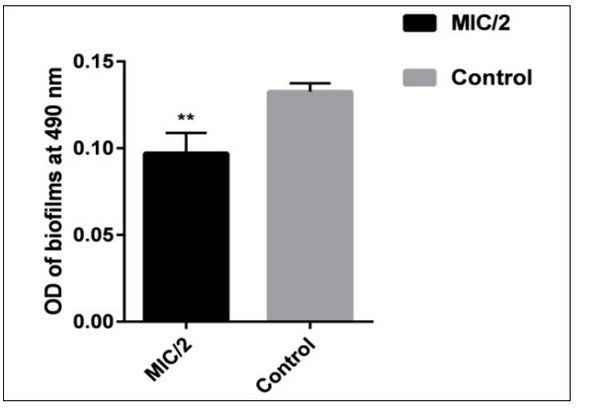

Fig. 1. Anti-biofilm activity of *S. hortensis* essential oil at MIC/2 concentration against *E. coli*
(**: $P < 0.01$)

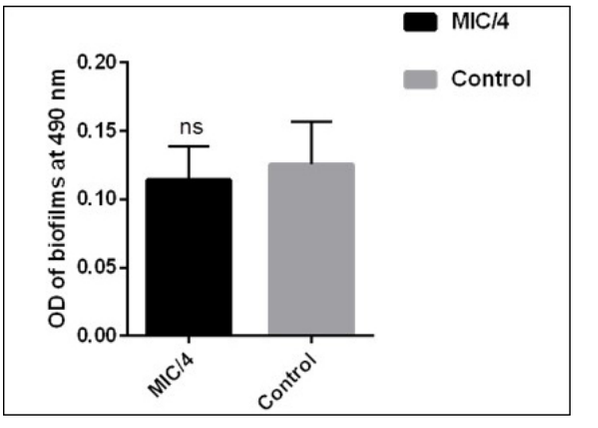

Fig. 2. Anti-biofilm activity of *S. hortensis* essential oil at MIC/4 concentration against *E. coli*
(ns: Not Significant)

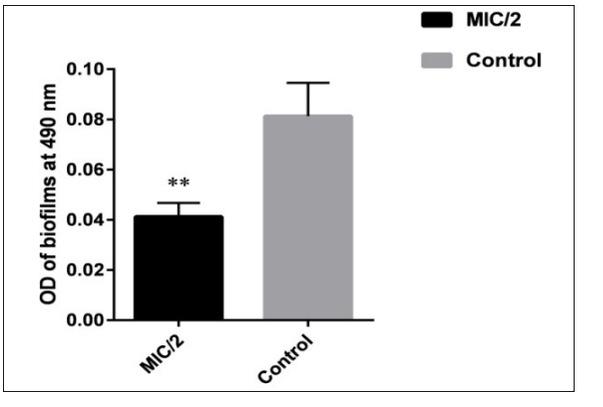

Fig. 3. Anti-biofilm activity of *S. hortensis* essential oil at MIC/2 concentration against *Salmonella* (**: $P < 0.01$)

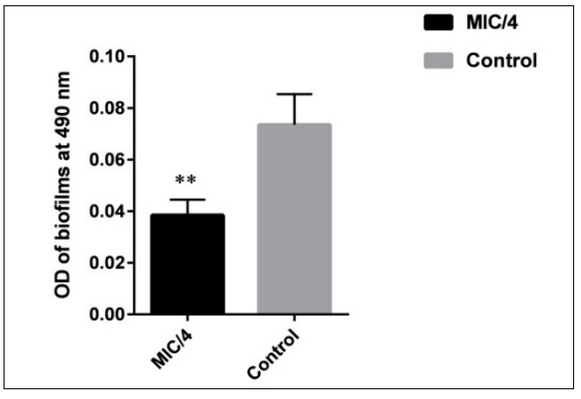

Fig. 4. Anti-biofilm activity of *S. hortensis* essential oil at MIC/4 concentration against *Salmonella* (**: $P < 0.01$)

Statistical analysis. The statistical analysis was done using SPSS, ver. 17. All the experiments were performed in triplicate and the data are expressed as the mean ± SD. A Student's t-test was used to compare treated well versus negative control. A $P < 0.01$ was considered to be statistically significant.

RESULTS

Chemical composition of the essential oil. The major components of *S. hortensis* essential oil was determined as thymol (41.28%), γ-terpinene (37.63%), p-cymene (12.2%) and α-terpinene (3.52%).

Antibacterial susceptibility test. The mean inhibition zone for the *E. coli* and *Salmonella* bacteria were 38 ± 4 and 32 ± 3 mm, respectively. These findings show that essential oils from *S. hortensis* do possess antibacterial activity at tested concentrations against *E. coli* and *Salmonella*.

Determination of MIC and MBC values. The MIC values of *S. hortensis* were ranged from 0.07 to 0.15 µl/ml for *E. coli*, and 0.31 to 0.62 µl/ml for *Salmonella*. The MBC values were 0.15 and 0.625 µl/ml for *E. coli* and *Salmonella*, respectively. These results revealed that all tested bacteria were sensitive to the *S. hortensis* essential oil. In addition, our data indicated that *E. coli* isolated were a little more sensitive to this oil compared to *Salmonella*.

Effect of *Satureja hortensis* essential oil on the biofilm formation. Biofilm inhibitory properties were performed at MIC/2 and MIC/4 concentrations of the essential oil. The effect of the *S. hortensis* essential oil on the biofilm formation at MIC/2 concentration on *E. coli* is shown in Fig. 1. The percentage of inhibition of biofilms according to the mentioned formula was 26.8 at MIC/2 concentration. The inhibitory effects on biofilm formation were statistically significant compared to that of control ($P < 0.05$). At MIC/4, the tested essential oil did show significant anti-biofilm activity compared to that of control (Fig. 2) and the percentage of biofilm inhibition at MIC/4 concentration was 12.31 (Fig. 2). In case of *Salmonella*, our results revealed that each two tested concentrations (MIC/2 and MIC/4) of essential oil significantly inhibited the biofilm formation (Figs. 3 and 4). It should be noted that the comparative results between two tested bacteria by *t*-test indicated that the anti-biofilm effect of the essential oil on *Salmonella* is significantly ($P < 0.01$) higher than that of *E. coli*.

DISCUSSION

Emergence of antibiotic-resistant bacteria has created the necessity of replacement of antibiotics with other products like probiotics, prebiotics, organic acid botanicals, and herbal compounds such as essential oils. Essential oils are important aromatic components of herbs and spices that have been traditionally used in human medicine for their pharmaceutical properties including anti-inflammatory, antimicrobial, and immunomodulatory (17). Currently, due to possession of antimicrobial activity, some essential oils have been used as food additives (18). Some plant essential oils have been analyzed for their antimicrobial activity and used as disinfectants. The potential of these compounds as an alternative to the antibiotic therapy in human and veterinary medicine, especially for diseases caused by multidrug-resistant and biofilm producer bacteria is under investigation (19).

In the present study, we examined the antimicrobial and anti-biofilm effects of *S. hortensis* L. essential oil against *E. coli* and *Salmonella* bacteria isolated from poultry infections. Our results showed good antimicrobial activity of *S. hortensis* essential oil against both tested bacterial strains.

The antibacterial properties of *S. hortensis* essential oil has been investigated by previous researchers (20). In a study carried out by Mihajilov-Krstev et al. (21), the antibacterial properties of the *S. hortensis*

essential oil was shown on both gram-negative and gram-positive bacteria and MIC/MBC values were reported in the range of 0.78-25 µl/ml. In a similar study (22), strong growth inhibitory effects of the *S. hortensis* essential oil against common periodontitis-associated bacteria was reported by Gursoy et al. These researches showed the effects of *S. hortensis* essential oil on both aerobic and anaerobic bacteria.

Based on previous studies, different MIC and MBC values have been reported for this essential oil (20, 23, 24). It is well known that both plant geographic area and genetic factors of studied strain are deeply effective in these observed variations (25). The GC-MS analysis results revealed that thymol, γ-terpinene, p-cymene and α-terpinene were the most prevalent components of the tested essential oil. The high antimicrobial activity can be explained by the presence of phenol compound thymol and also terpenes (26). The strong antimicrobial properties of these compounds have been reported by other researchers (21, 27, 28). According to Pei et al. (2009), MIC of thymol against *E. coli* was 400 µg/ml (29). In addition, Ivanovic et al. (2012) have reported that *E. coli* and *S.* Enteritidis are inhibited by thymol at concentrations between 160 and 320 µg/ml (30).

In the second part of the study, using the microtiter plate assay, the anti-biofilm properties of the essential oil were studied against *E. coli* and *Salmonella* isolates. Our results showed that essential oil of the *S. hortensis* inhibited the formation of biofilms at concentrations lower than MIC. This effect on *Salmonella* was stronger than *E. coli.* In similar previous studies, the anti-biofilm properties of *S. hortensis* essential oil was studied and confirmed on *Staphylococcus aureus* (23) and *Streptococcus pneumoniae* (24). In this study, both MIC and MBC were 0.125 µl/ml and the biofilms were significantly inhibited at MIC/2, MIC/4 and MIC/8 concentrations (23). Therefore, we can conclude that the *S. hortensis* essential oil is effective on the biofilms of both tested bacteria. Similarly, these anti-biofilm results were shown on staphylococcal cells (31), *Streptococcus pneumoniae* (24) and periodontal bacteria (22).

Considering the application of essential oils in various fields, such as adding to food products, treating human and even animal infections from one side, and a dramatic increase in antibiotic resistance on the other side, today, many studies have been conducted to find compounds with anti-biofilm properties (10). In order to find these anti-biofilm compounds, researchers have focused on herbal compounds such as extracts and essential oils as these agents are inexpensive and available and have low toxicity to mammalian cells (32). For example, essential oil such as *Origanum majorana, Thymus zygis, Rosmarinus officinalis, Juniperus communis* and *Zengiber officinale* have been recorded for their antibiofilm activities (33). The anti-biofilm properties of the plant essential oils may be due to anti-adhesive activity of the essential oil compounds such as thymol (31), inhibition of structure formation such as reduction of exopolysaccharide production (34) or altering the biofilm related gene expression (35).

Residence in the biofilm phase offers various advantages to avian pathogenic *E. coli* (APEC); the first one is the ability to acquire transmissible genetic elements, such as plasmids, at high level rates and subsequently, increasing ability to cause disease (36). The second one is that, bacteria living in biofilm exhibit enhanced resistance to cleansing and sanitation. This matter is especially significant in poultry production and processing; where biofilms can be developed on environmental surfaces (37). Therefore, the use of a biofilm inhibitor such as *S. hortensis* essential oil can help to reduce bacterial pathogenesis as well as remove bacteria by antimicrobial agents (36).

CONCLUSION

Satureja hortensis essential oil with its antimicrobial and especially anti- biofilm properties may play a role in reducing the prevalence of important poultry diseases such as colibacillosis and salmonellosis or decreasing the severity of these diseases. This essential oil can help to strengthen the poultry immune system because it does not cause bacterial death at concentrations lower than MIC. This essential oil can be used as a poultry dietary supplement for both prevention and control purposes. Generally, essential oils, unlike antibiotics, do not cause microbial resistance. However, additional and *in vivo* studies are required to determine the suitability of this essential oil in clinical applications or as a feed additive in poultry rations.

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