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RESEARCH ARTICLE

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# Antibacterial effect of *Satureja hortensis* and *Salvia officinalis* essential oils against major bovine mastitis bacteria

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## ABSTRACT

Treatment of bacterial diseases such as bovine mastitis with antibiotics has problems such as antibiotic resistance and drug residue in animal products. Essential oil of medicinal plants have antibacterial activity and are suitable alternatives. This study examined the antimicrobial activity of *Salvia officinalis* (sage) and *Satureja hortensis* (savory) essential oils on major mastitis-causing bacteria, including *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Escherichia coli*. Chemical compositions of essential oils were determined by gas chromatography-mass spectrometry. Minimum inhibitory concentration and minimum bactericidal concentration of oils were determined with serial broth dilution method using autoclaved whole milk rather than synthetic broth. The effect of sub-minimum inhibitory concentrations of essential oils on the growth curve of tested bacteria in milk was obtained in 0, 1, 2, 4, 10, and 24 hours. Major compositions of sage and savory essential oils were carvacrol (61.01%), thymol (20.41%), 1R-α-pinene (7.88%), eucalyptol (32.45%), thymol (28.24%), and α-pinene (13.42%), respectively. The minimum inhibitory concentration and minimum bactericidal concentration ranged 1.25-2.5% and 2.5-5% for savory, and 0.625-1.25% and 1.25-2.5% for sage, respectively. Savory and sage significantly decreased the *S. aureus* and *S. agalactiae* population in 4, 10, and 24 h ($p < 0.05$) and *E. coli* population in 10 and 24 h ($p = 0.01$). The sage and savory essential oils had antibacterial effects against three tested bacteria, and sage had a stronger effect than savory because of stronger antibacterial components (carvacrol and thymol). Further *in vivo* tests are recommended to evaluate the efficiency of these essential oils on the treatment of bovine mastitis.

**Keywords**

*Antibiotic, medicinal plants, sage, savory*

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## Abbreviations

*MIC: minimum inhibitory concentration*
*MBC: minimum bactericidal concentration*
*TSA: tryptic soy agar*
*DMSO: dimethyl sulfoxide*
*GC/MS: gas chromatography/mass spectrometry*
*EO: essential oil*



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## Introduction

Bovine mastitis is the most common disease of the dairy industry worldwide that has very economic losses, including discarded milk, some ineffective treatments as well as concerns about animal welfare and public health [1]. Mastitis is commonly treated with intra-mammary infusion of antibiotics, but cure rates are usually poor and various for some pathogens of mastitis, such as the cure rates of 20-57% for *Staphylococcus aureus* mastitis. Moreover, the appearance of resistance to an antibiotic in bacteria is a potential consequence of antibiotic usage [2]. According to these problems and concerns, alternative approaches are needed to control bovine mastitis. Essential oils are volatile secondary metabolites of low molecular weight derived from plants. These oils have antibacterial properties, with no reports of resistance after prolonged exposure to bacteria, and no side effects on human health, which makes them a potential weapon against bacterial diseases [3].

*Salvia officinalis* (sage) is one of the oldest and still the most popular medicinal plant that can grow wildly or in cultivation. Besides many other therapeutical properties, *S. officinalis* has anti-inflammatory, antiseptic, and broad-spectrum antibacterial activity [4,5]. *Satureja hortensis L.* (summer savory; savory) is a main *Satureja* species that has some applications in medicine and nutrition. Savory has different pharmacological effects, including antispasmodic, antimicrobial, antidiarrheal, and sedative [6,7].

There is some data on the antibacterial effects of sage and savory using synthetic media. In this study, we tested the antimicrobial efficacy of *Salvia officinalis* and *Satureja hortensis* oils in inhibiting the growth of major bovine mastitis pathogens (*Staphylococcus aureus, Streptococcus uberis*, and *Escherichia coli*) in milk. There is a complex food matrix in milk that nutrients such as proteins and fat may preserve pathogens of mastitis or decrease the antimicrobial effect of molecules [1], thereby for the usage of an antimicrobial agent for intra-mammary infusion it is necessary to assess the antimicrobial properties of these plant oils in milk.

## Results

### *Chemical compositions of the essential oils*

Major components of the savory oil were eucalyptol (32.45%), carvacrol (28.24%), α-pinene (13.42%), and thymol (9.71%), and those of sage were carvacrol (61.01%), thymol (20.41%) and 1R-α-pinene (7.88%) (Tables 1, 2).

### *MIC and MBC*

The MIC and MBC of sage and savory essential oils on bacteria are provided in Table 3. Antimicrobial activity was confirmed against all tested microorganisms. The MIC and MBC ranged 1.25-2.5% and 2.5-5% for savory, and 0.625-1.25% and 1.25-2.5% for sage, respectively. Although savory, sage, and savory + sage essential oils showed an antibacterial effect against the three bacteria, sage oil was the strongest. It can be seen from the data in Table 3 that MBC and MIC of sage had the lowest effect compared with the other two essential oils.

### *Bactericidal kinetics of the oils*

Figures 1-3 provide the effect of savory and sage on the growth curve of bacteria in milk. The initial bacterial count in the control and treatment samples for three bacteria was approximately 5.0 $\log_{10}$ cfu/ml. The bacterial population increased after 24 h to 12 $\log_{10}$ cfu/ml for *E. coli* and *S. agalactiae* and 10 $\log_{10}$ cfu/ml for *S. aureus* in the control samples. The bacterial population of *E. coli* significantly reduced in 2 (4.11 vs. 6.59 $\log_{10}$ cfu/ml) ($p = 0.03$), 10 (7.28 vs.10.25 $\log_{10}$ cfu/ml) ($p = 0.01$), and 24 (7.11 vs. 11.48 $\log_{10}$ cfu/ml) ($p = 0.01$) h with sage oil and in 10 (6.3 vs. 10.25 $\log_{10}$ cfu/ml) ($p = 0.01$) and 24 (7.44 vs. 11.48 $\log_{10}$ cfu/ml) ($p = 0.01$) h with savory oil. The population of *S. aureus* significantly decreased with sage and savory oil in 4 (5.94 and 5.27 vs. 8.47 $\log_{10}$ cfu/ml, respectively) ($p = 0.02$), 10 (6.32 and 6.38 vs. 9.4 $\log_{10}$ cfu/ml, respectively) ($p = 0.02$), and 24 (6.46 and 7.3 vs. 10.53 $\log_{10}$ cfu/ml, respectively) ($p = 0.01$) h. Also, the sage and savory oil significantly decreased bacterial population of *S. agalactiae* in 4 (6.2 and 6.81 vs. 9.44 $\log_{10}$ cfu/ml, respectively) ($p = 0.01$), 10 (7.22 and 6.52 vs. 10.59 $\log_{10}$ cfu/ml, respectively) ($p = 0.01$) and 24 (6.74 and 6.5 vs. 11.79 $\log_{10}$ cfu/ml, respectively) ($p = 0.03$) h.

**Table 1.**
Chemical composition (relative % of peak area) of essential oil of sage determined by GC-MS analyses.

| No. | Components | Retention time (min) | Area sum% |
|---|---|---|---|
| 1 | 1R-α-Pinene | 4.117 | 7.88 |
| 2 | o-Cymol | 5.909 | 3.61 |
| 3 | Eucalyptol | 6.092 | 4.57 |
| 4 | γ-Terpinene | 6.676 | 2.52 |
| 5 | Thymol | 12.664 | 20.41 |
| 6 | Carvacrol | 12.935 | 61.01 |

## Discussion

Mastitis is a serious and prevalent problem and does not have effective treatments in organic dairy cattle. There is a need for organic antimicrobials for the production of organic foods and the prevention of antibiotic resistance [8]. Herein, we investigated the antibacterial property of savory and sage essential oils on three bacteria responsible for this pathology.

Considerable studies have been done on the antimicrobial effects of essential oils using model broth systems. The antimicrobial activity of plant extracts decreased when used in complicated systems or foods [2]. The antimicrobial activity of plant-derived molecules in laboratory media is significantly greater than that in complicated foods such as dairy products, fish, meat, and vegetables [9]. Similarly, the composition of milk, especially the amount of fat, reduced the antimicrobial effect of eugenol in milk [10]. According to these reports, the present study evaluated the MBC and MIC of the oils on the mastitis bacteria in milk instead of laboratory medium.

Thirteen constituents were recognized in the *S. hortensis* oil in this research. The most abundant chemical constituents in *S. hortensis* oil were eucalyptol (1,8-cineole) (32.45%), carvacrol (28.24%), α-pinene (13.42%), and thymol (9.71%). Major constituents of savory were reported carvacrol (41%), p-cymen (10%), and thymol (10%) from Bosnia Herzegovina [11]. Jafari et al. [7] analyzed essential oils of savory from Ardebil province of Iran, and 25 components were shown, and major oil components were γ-terpinene (37%), carvacrol(32%), and p-cymen (13%) while in a sample from Shiraz province of Iran, 22 compounds were identified and major com-

**Table 2.**
Chemical composition (relative % of peak area) of essential oil of savory determined by GC-MS analyses.

| No. | Components | Retention time (min) | Area sum% |
|---|---|---|---|
| 1 | α-Pinene | 4.117 | 13.42 |
| 2 | m-Cymene | 5.909 | 3.54 |
| 3 | p-Menth-8-en-1-ol, acetate | 6.018 | 2.3 |
| 4 | Eucalyptol | 6.099 | 32.45 |
| 5 | γ-Terpinene | 6.676 | 1.01 |
| 6 | Linalool | 7.66 | 2.58 |
| 7 | (+)-2-Bornanone | 8.889 | 1.41 |
| 8 | Isoborneol | 9.324 | 1.06 |
| 9 | p-Menth-1-en-4-ol, (R)-(-)- | 9.765 | 1.42 |
| 10 | α-Terpineol | 10.138 | 1.49 |
| 11 | Linalyl acetate | 11.554 | 1.38 |
| 12 | Thymol | 12.636 | 9.71 |
| 13 | Carvacrol | 12.874 | 28.24 |

**Table 3.**
MIC (%V/V) and MBC (%V/V) of savory and sage essential oils against tested bacteria

| Bacterium | Savory | | Sage | | Savory+ Sage (1:1) | |
|---|---|---|---|---|---|---|
| | MIC | MBC | MIC | MBC | MIC | MBC |
| *E. coli* | 2.5 | 5 | 1.25 | 2.5 | 1.25 | 2.5 |
| *S. aureus* | 2.5 | 5 | 0.625 | 1.25 | 1.25 | 2.5 |
| *S. agalactiae* | 1.25 | 2.5 | 1.25 | 2.5 | 1.25 | 2.5 |

MIC: Minimum inhibitory concentration
MBC: Minimum bactericidal concentration

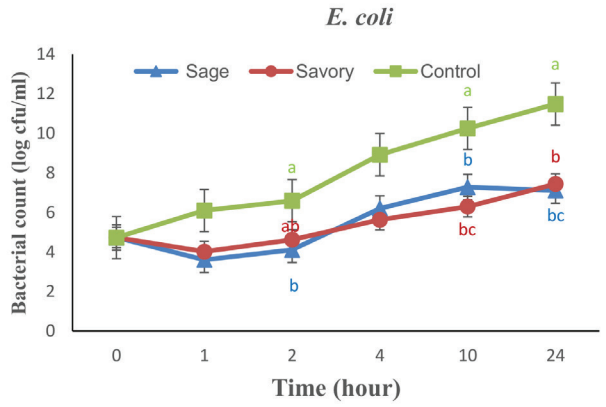


**Figure 1.**
Survival curve of *E. coli* in milk containing 0% (control, ■) and sub-MIC concentration of essential oil of savory (●) and sage (▲). [a-c] Values that are significantly ($p < 0.05$) different within the same time are indicated by different letters.

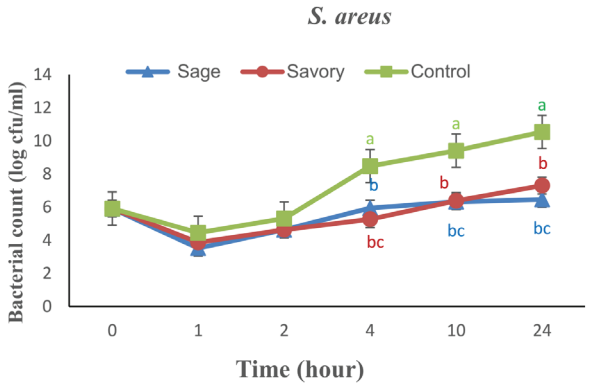


**Figure 2.**
Survival curve of S. aureus in milk containing 0% (control, ■) and sub-MIC concentration of essential oil of savory (●) and sage (▲). [a-c] Values that are significantly ($p < 0.05$) different within the same time are indicated by different letters.

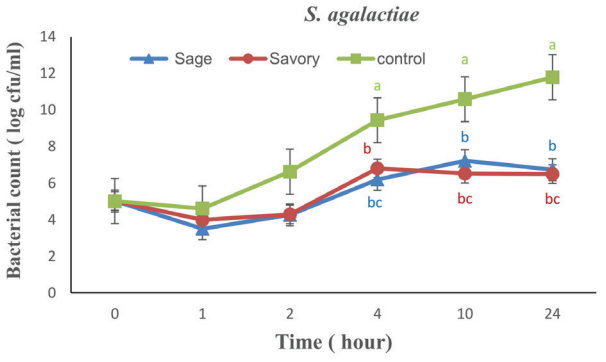


**Figure 3.**
Survival curve of S. agalactiae in milk containing 0% (control, ■) and sub-MIC concentration of essential oil of savory (●) and sage (▲). [a-c] Values tht are significantly ($p < 0.05$) different within the same time are indicated by different letters.

ponents were carvacrol (54%) and γ-terpinene (26%) [6]. In another study in Iran, major constituents of savory were reported carvacrol (56%), γ-terpinen (24%), and p-cymen (5%) [12]. In our study, eucalyptol (32.45%) was higher and γ-terpinen (1.01%) was lower than other studies while other components somewhat similar to other works.

In the present study, six compounds were recognized in the *S. officinalis* oil, and the main constituents were carvacrol (61.01%), thymol (20.41%), and 1R-α-pinene (7.88%). In one study, sage was raised at 18 experimental places in Italy, and major constituents were α-thujone, camphor, borneol, c-muurolene, and sclareol [13]. In another study from Italy, the primary substances of sage were cis-thujone (23.90%), camphor (19.22%), and 1.8-cineole (10.62%) [14]. Aumeeruddy-elalfi et al. [15] reported that the Mauritius sage EO mainly consists of thujone, followed by camphor and aromadendrene. Compositions of sage in the present work were somewhat different from most researches and amount of carvacrol + thymol was high that both compositions have potent antibacterial effect.

The variation in the content and composition of oils in different studies may be due to species, growth stages, plant origin and adaptive metabolism, conditions of climate, drying, distillation, and the part of the plant being analyzed [16].

Savory and sage oils had antibacterial activity against the bacteria in the present study, and we showed that sage and savory oil could kill all tested bacteria. The MIC and MBC of sage oil for *S. aureus* was lower than that of savory and savory+ sage. The MIC and MBC of three groups were similar for *S. agalactiae*. Sage oil exhibited stronger activity than savory and savory+ sage oil.

Most researchers declared that the MIC is a measure of the antimicrobial activity of Eos [9]. Different values have been reported for MIC and MBC of savory and sage. Jafari et al. [7] obtained MIC and MBC values of 0.01% and 0.8 % against *E. coli* and 0.2% and 0.2% against *S. aureus* for savory, respectively. MIC and MBC values of 0.03% and 0.1% against *E. coli* and 0.03% and 0.06% against *S. aureus* for savory have been reported, respectively [6]. In another study, the MIC value of savory for *E. coli* was obtained to be 5% [17].

Regarding sage, obtained MIC and MBC values of 5% against *S. aureus* and 0.8% against *E.coli* reported by Raffaella et al. [14] and Moghimi et al. [18], respectively. Also, a MIC value of 0.2% has been reported against *E. coli* and *S. aureus* for sage [15]. Moreover, a MIC value of 0.03% against *E. coli,* and MIC and MBC values of 12.5% and 6.1% have been reported for sage [5,19].

The different values of MIC and MBC in various studies might be because of the variable components of EOs and susceptibility of strain. Moreover, our MIC and MBC values are more than most of these researches which might be due to different media used in studies (synthetic medium of others versus milk of ours). The MBC/MIC ratio is used to determine the antimicrobial activity of EOs. The ratio of greater than 4 shows bacteriostatic and lower or equal to 4 shows the bactericidal characteristics [16]. This ratio showed a bactericidal effect of the oils on the strains tested.

In the present study, the miscibility and antimicrobial activity of savory and sage essential oils were tested in milk to obtain useful information for their application in bovine mastitis. Furthermore, a time-kill curve set of experiments was conducted to determine the time of inhibition or elimination of these pathogens. Savory and sage oils at sub-MIC concentrations caused a ~4.0 $\log_{10}$ cfu/ml reduction of *E. coli*, *S. aureus*, and a ~5.0 $\log_{10}$ cfu/ml of *S. agalactiae* reduction within 24 h. Significant reduction of bacteria started 2 (*E. coli*) or 4 (*S. aureus* and *S. agalactiae*) h after exposure to sub-MIC concentration. Higher concentrations may result in an early reduction of the bacterial population.

Essential oils have different components, and their antimicrobial activity cannot be contributed to one compound [20]. The antibacterial effect of essential oil may be attributed to the main compounds of essential oil and the synergism between main and minor compounds [21].

In this study, compounds such as eucalyptol (1,8-cineole) (a monoterpene hydrocarbon), carvacrol, thymol, and α-pinene (oxygenated monoterpenes) contributed to more than 70% of the chemical composition of oils. Oxygenated compounds, especially phenolic compounds as carvacrol and thymol, have higher antibacterial activity, while hydrocarbon monoterpenes possess the lowest potential because they have limited diffusion through the medium due to their low water solubility [22]. In this study, sage oil exhibited stronger activity than savory, an effect which might be due to a higher amount of stronger antibacterial components (carvacrol+ thymol) in sage (81.42%) than savory (28.24%).

In conclusion, the essential oil of sage and savory had antibacterial activity, and sage had higher activity than savory on the mastitis-causing bacteria (*S. aureus, S. agalactiae*, and *E. coli*). Sage EO might be effective in the treatment of mastitis as an alternative or adjunct to antibiotics. However, further *in vivo* tests are needed to evaluate the efficiency on the treatment of bovine mastitis and potential side effects on the mammary gland tissue.

## Materials & Methods

This study was performed in Gonbad Kavous University (Gonbad Kavous, Iran) from October 2017 to June 2018.

### *Chemical composition analysis of the essential oils*

Essential oils of sage and savory were purchased from Dorrin Golab Co. (Kashan, Iran). Gas chromatography/mass spectrometry (GC/MS) analysis was performed employing a gas chromatograph connected with a mass detector (Model 5977A, Agilent Technologies, USA) and equipped with an HP-5MS capillary column (phenylmethyl siloxane, 30 m × 0.25 mm ID 0.25 um, Agilent Technologies). The temperature of the injector was 270 °C, and the temperature of the oven was raised from 60 °C (0 min) to 200 °C by a rate of 5 °C/min. The analysis was performed using helium as a carrier gas, while the flow rate was adjusted to 1 mL/min and injection volume (1 ul). The interface temperature was set at 280 °C, and the mass range was 35 - 500 m/z.

### *Bacterial strains*

The activity of the EOs was tested toward three different major mastitis pathogens, including *Staphylococcus aureus* (PTCC 1113), *Streptococcus agalactiae* (PTCC 1768), and *Escherichia coli* (PTCC 1399). These bacteria were obtained as a lyophilized culture from Persian Type Culture Collection, Tehran, Iran (PTCC). The lyophilized cultures were grown twice in tubes contaning 10 ml of tryptic soy broth (TSB) (Biolife, Milano, Italy) at 37 °C for 18-20 h (overnight). Sterile glycerin (1:5) was used to dilute the cultures, and then the cultures were stored in microtubes at -20 °C. To achieve fresh bacterium, it was cultured twice in TSB at 37 °C for 20 h followed by streaking on tryptic soy agar (TSA) (Biolife, Milano, Italy) slants and incubation under the same conditions. The cultures were stored at 4 °C and sub-cultured monthly [23].

### *Preparation of Inoculum*

Cells from working cultures were transferred to tubes of TSB to obtain bacterial inoculum. The cultures incubated 18 h at 35 °C, and then second subcultures were provided. The bacterial broth cultures were adjusted to optical density (OD) (absorbance) of 0.1 at 600 nm, using a spectrophotometer (Libra S12, Biochrom Ltd., Cambridge, London). These adjustments gave a cell concentration of $2.4 \times 10^{11}$ cfu/ml for *E. coli*, $3.4 \times 10^{10}$ cfu/ml for *S. aureus*, and $1.64 \times 10^{11}$ cfu/ml for *S. agalactiae*. The cell counts in the suspensions were estimated by duplicate plating from tenfold serial dilutions on TSA and counting the colonies after 24 h incubation at 35 °C [24].

### *Milk Preparation*

Raw milk with no antibiotic residues was collected and autoclaved at 121 °C for 15 min.

### *Determination of MIC and MBC*

1/2 dilution of the oils was made with dimethyl sulfoxide (DMSO, Sigma, Germany) to increase solubility in the culture medium and filter sterilized. This dilution was used in the antibacterial analysis. Herbal oils alone or in combination (1:1) were investigated according to a modified protocol for broth dilution testing [25]. Autoclaved milk was utilized rather than synthetic broth medium as the growth medium. Twofold serial dilutions (10, 5, 2.5, 1.25, and 0.625 %) of the oil were performed for the determination of MIC. Treatments were added to milk, then vials were vortexed for 90 s. The total volume of test vials was 1 mL

of liquid. Afterward, 100 µl of 1:300 dilution of inoculum of each bacterium was inoculated into each tube. The vials were vortexed for 15 s and incubated for 24 h at 37 °C. Following incubation, vials were vortexed for 15 s. Bacterial counts were determined with serial dilution employing a 0.1-mL aliquot of the vortexed vial and sterile normal saline to create eight 10-fold dilutions. Dilutions were plated on eighths of a TSA plate and incubated for 24 h at 37 °C. Bacterial populations of dilutions were counted. The occurrence of synergism/antagonism in antibacterial action between the essential oils of sage and savory was tested with mixing of oils volume to volume.

Several controls were in treatments. Milk was a negative control to evaluate autoclavation. Milk + bacteria was a positive control to check bacterial growth in the milk. A positive control containing the bacterial culture and DMSO without the EO was performed as well. MBC was the lowest concentration that inhibited bacterial growth following subculture on TSA. Ante-MBC concentration was taken as the MIC. Each experiment was repeated twice on at least two separate occasions and was repeated if results differed by more than one doubling dilution.

### *Growth curve of bacteria*

Sterile milk containing the sub-MIC of EOs with each pathogen was inoculated in the same way as the above MIC tests to assess the bactericidal kinetics of oils. Control samples contained inoculated milk without EO. The samples were incubated at 37 °C for 24 h. Bacterial counts were enumerated in 1, 2, 4, 10, and 24 h of incubation by plating 0.1 mL portions of the samples with or without serial dilutions (1:10 in normal saline). Each experiment was done in duplicate. Bacterial counts ($\log_{10}$ cfu/ml) against time (hour) were plotted for time-kill curves construction.

### *Statistical analysis*

All the tests were carried out in duplicate. The data were evaluated to a one-way analysis of variance (ANOVA) and *Tukey's* test using the SPSS statistical software (SPSS, Chicago, USA). $p$ values $< 0.05$ were considered significant.

## Authors' Contributions

R.R. and F.G. conceived and designed the experiment. S.Z. performed the experiments. R.R. and A.K. analyzed and interpreted the data. R.R. and F.G. drafted and critically revised the manuscript.

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## Competing Interests

The authors declare that there is no conflict of interest.

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