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RESEARCH ARTICLE

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Antibacterial effect of *Lavandula stoechas* and *Origanum majorana* essential oils against *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Escherichia coli*

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ABSTRACT

This research examined the antimicrobial effect of *Lavandula stoechas* (lavender) and *Origanum majorana* (marjoram) essential oil against three pathogens: *Staphylococcus aureus, Streptococcus agalactiae*, and *Escherichia coli*. Gas chromatography-mass spectrometry (GC/MS) analysis revealed that the main components of the lavender and marjoram oils were 17-Pentatriacontene, Linalyl acetate, Eucalyptol, linalool and 3-Cyclohexene-1-ol,4-methyl-1-(1-methylethyl)-,(R)-, α-terpineol, P-cymene, respectively. Broth dilution testing was performed using autoclaved whole milk instead of broth to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oils alone and in combination. In addition, time-kill assay of lavender and marjoram oils were determined in milk up to 24 h. MIC values ranged from 3.12 - 4.37% v/v and MBC between 6.25 - 8.75% v/v for the lavender. The MIC and MBC of the marjoram ranged from 0.62 - 1.87% v/v and 1.25 - 3.75% v/v, respectively. The MIC ranged from 2.5 - 5% v/v and MBC between 5 - 10% v/v for lavender + marjoram combination. In time-kill assays, the presence of lavender and marjoram oils at a sub-MIC concentration significantly reduced the bacterial population in 4, 10 and 24 h. Generally, essential oil of marjoram had greater antibacterial activity than lavender against all mastitis-causing pathogens tested and has the potential to be evaluated as an alternative or adjunct to antibiotics in the treatment of bovine mastitis.

Keywords

Antibacterial activity, Lavender, Marjoram, Organic farm

Abbreviations

MIC: minimum inhibitory concentration
MBC: minimum bactericidal concentration
GC/MS: gas chromatography/ mass spectrometry

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Introduction

Treatment of bacterial diseases are often encountered problems of increase in drug resistance and side effects of conventional medication [1]. In this context, natural products have a key role in discovery of alternative drugs [2]. Secondary metabolites of medicinal and aromatic plants present key candidates for discovering antimicrobial agents to fight against numerous microbial diseases.

Essential oils (EOs) have antibacterial, anti-fungal and antiviral activity and have been studied for finding new antimicrobial compounds, alternatives to cure microbial diseases [3]. Essential oils show antibacterial effects, therefore, the study of EOs antibacterial effects against bacterial agents is justifiable [4].

Lavandula is a medicinal plant from the family of *Lamiaceae* which is traditionally utilized to overcome diseases [1]. Antimicrobial [1,5,6] and antioxidant [7] activities of *L. stoechas* EO have demonstrated in several studies.

Origanum majorana (marjoram) from the *Lamiaceae* family has been used in traditional and folklore medicines for many disorders of gastrointestinal, respiratory, cardiac, and nervous system. Chemical constituents such as monoterpene hydrocarbons, oxygenated monoterpenes, and phenolic compounds have been isolated from marjoram essential oil. In pharmacological studies of marjoram, antibacterial, antifungal, antiprotozoal, and antioxidant activities have been reported in modern medicine [8].

Different diseases are caused by *Staphylococcus aureus, Streptococcus agalactiae*, and *Escherichia coli*. Mastitis is a common and important disease that can be produced by all these bacteria. The main causative agents of bovine mastitis are bacteria such as *Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Klebsiella pneumonia* [9] and Coagulase-negative staphylococci (CNS) [10].

There is an increasing need for new antibacterial agents to treat and control bovine mastitis, so we investigated the antimicrobial activity of these EOs in milk instead of synthetic laboratory medium for future application as an intra-mammary infusion in cows. Bacteria must survive and replicate in mammary gland to induce an infection [11]. Moreover, albumin, starch, and fat of milk can potentially interact with the antimicrobial constituents and reduce bioavailability of EOs [12]. Therefore, in the present study, milk was selected as the in vitro model for evaluating the antibacterial effect of lavender and marjoram EO for mastitis treatment. The antibacterial activity of EOs was determined on *Staphylococcus aureus (S. aureus), Streptococcus agalactiae (S. agalactiae)*, and *Escherichia coli (E. coli).*

Results

Chemical composition of the essential oils

GC/MS analysis revealed that the main components of the lavender and marjoram oils were 17-Pentatriacontene (42.15%), linalyl acetate (26.82%), eucalyptol (18.87%), linalool (5.7%), and 3-Cyclohexene-1-ol,4-methyl-1-(1-methylethyl)-,(R)-(44.84%), α-terpineol (6.83%), P-cymene (6.75%), respectively. (Tables 1 and 2).

MIC and MBC

The MIC and MBC of lavender and marjoram EOs on the mastitis bacteria are shown in Table 3. Although lavender, marjoram, and marjoram + laven-

Table 1
Chemical composition (relative % of peak area) of essential oil of lavender determined by GC-MS analysis.

RT	Compound	%
3.635	17-Pentatriacontene	42.15
4.117	1R-α-Pinene	0.73
5.909	m-Cymene	1.73
6.024	D-Limonen	2.4
6.099	Eucalyptol	18.87
7.667	Linalool	5.7
9.765	p-Menth-1-en-4-ol, (R)-(-)-	1.11
11.577	Linalyl acetat	26.82
12.433	Lavandulol acetate	0.48

RT: Retention time on HP-5MS column in minutes

Table 2
Chemical composition (relative % of peak area) of essential oil of marjoram determined by GC-MS analysis.

RT	Compound	%
4.11	Bicyclo[3.1.1]hept-2-ene, 3,6,6-trimethyl-	1.39
4.83	Sabinen	1.5
5.739	Terpinolen	1.63
5.909	P-Cymene	6.75
6.011	D-Limonene	1.55
6.086	Eucalyptol	2.58
6.676	γ-Terpinene	4.96
6.961	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1α,2α,5α)-	2.17
7.667	Linalool	5.12
7.722	Terpineol, cis-β-	4.58
8.312	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-	1.97
8.76	4-Isopropyl-1-methylcyclohex-2-enol	1.41
9.792	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	44.84
10.145	α-Terpineol	6.83
11.544	Linalyl acetate	3.45
12.209	trans-Ascaridol glycol	1.5
12.677	1,4-dihydroxy-p-menth-2-ene	1.46
13.438	4,4-Dimethylpent-2-enal	1.46
15.97	Caryophyllene	2.03
19.758	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aα,4aα,7β,7aβ,7bα)]-	1.42
19.873	Caryophyllene oxide	1.38

RT: Retention on HP-5MS column in minutes

Table 3
MIC and MBC of lavender and marjoram essential oils against *Staphylococcus aureus, Streptococcus agalactiae,* and *Escherichia coli*

	Bacterium	MBC (%V/V)	MIC (%V/V)
Lavender	*Escherichia coli*	6.25	3.12
	Staphylococcus aureus	8.75	4.37
	Streptococcus agalactiae	7.50	3.75
Marjoram	*Escherichia coli*	3.12	1.56
	Staphylococcus aureus	1.25	0.62
	Streptococcus agalactiae	3.75	1.87
Lavender + Marjoram (1:1)	*Escherichia coli*	5	2.5
	Staphylococcus aureus	10	5
	Streptococcus agalactiae	10	5

RT: Retention on HP-5MS column in minutes

der essential oils displayed antibacterial effects, marjoram oil was the most effective against the bacteria. Marjoram had the lowest MIC and MBC for the three pathogens compared to the other two essential oils.

Bactericidal kinetics of the oils

The bactericidal kinetics of lavender and marjoram against mastitis bacteria in milk were shown in Figures 1, 2, 3. The initial population of bacteria in the control and treatment groups for the three bacteria was approximately 6.0 log10 cfu/ml. The population of bacteria reached about 12 log10 cfu/ml in the control group during the 24 h incubation period. The presence of lavender and marjoram at a sub-MIC concentration significantly reduced the bacterial population to 3.59 to 4.68 log10 cfu/ml in 4, 10 and 24 h except for lavender that insignificantly decreased *E. coli* population to 5.31 log10 cfu/ml in 4 h. However, the time-kill assay and MIC and MBC experiments showed the antibacterial effect of lavender and marjoram in milk.

Discussion

Bovine Mastitis is an important disease in organic farms due to its prevalence and lack of efficient cures for that. There is a growing need for organic antibacterials that can be used in dairy farms. A growing need is for organic antibacterials, which can be used in organic dairy farms, that do not emerge antibiotic resistance. It seems that the possibility of drug resistance decreases when a dairy herd transits from conventional to organic status [13].

The broth dilution method is frequently used to study antimicrobial efficacy of different essential oils [14], but milk was used instead of broth in the present study to mimic the udder environment. Moreover, hydrophobic properties of lipids and other lipophilic molecules of milk may reduce the antibacterial activity of essential oils on mastitis-causing bacteria [15].

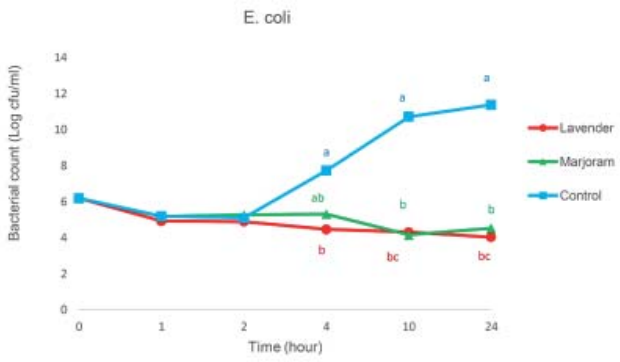

Figure1
Survival curve of E. coli in milk containing 0% (control, blue) and sub-MIC concentration
of essential oil of lavender (red) and marjoram (green).
[a-c]Values that are significantly ($P < 0.05$) different within the same time are indicated by different letters.

This work has demonstrated that the major components of the lavender EO were 17-Pentatriacontene, linalyl acetate, eucalyptol, and linalool. Main components of lavender were reported to be fenchone, camphor, and terpineol from Morocco [1] and fenchone, eucalyptol, and camphor from Spain [7]. Ashghari et al. reported camphor, 1,8 cineol, linalool, borneol and Mashak et al. found 1,8 cineol, borneol, camphor, linalool as main components of lavender EO in Iran [16,17]. Some of the aforementioned compounds were absent in our study that was in agreement with Carrasco et al. who declared that the main components of lavender had high variability even in the common constituents, i.e., camphor (0–49%) and fenchone (0–66%) [7]. This shows that study of biochemotypes existing in the different locations is important.

In the present study, main constituents of marjoram were 3-cyclohexene-1-ol,4-methyl-1-(1-methylethyl)-(R)-, P-cymene and linalool. Hajlaoui et al. reported that the Tunisian marjoram EO mainly consisted of terpinene-4-ol, followed by cis-sabinene hydrate, g-terpinene, and P-cymene [18]. The main components of the Venezuelan Andes marjoram EO were reported cis-sabinene hydrate, terpinene-4-ol, g-terpinene, a-terpineol, trans-sabinene hydrate, linalool acetate and a-terpinene [19]. In a study, terpinolene-4-ol, γ-terpinene, and α-terpinene were reported as the main constituents of marjoram EO in Iran [20]. Factors such as species, location of herb, growth stages, climatic conditions, distillation conditions, and the analyzed part of the plant are involved in variation of lavender and marjoram EO components [18].

Lavender and marjoram oil had antibacterial activity against the bacteria in the present study which are prevalent bacteria on organic farms [13]. Antibacterial effects of Lavender and marjoram oil has been reported against *E. coli* [1,18,19] and *S. aureus* [1,8,21,22]. Thus we hypothesized that lavender and marjoram oil are effective antibacterial agents against pathogens causing bovine mastitis. In the current study, we showed that lavender and marjoram oil could kill all tested bacteria. The MIC of marjoram oil for all tested bacteria obtained was lower than that of lavender and lavender + marjoram. In general, marjoram oil exhibited stronger activity than did lavender and lavender + marjoram oil. In agreement with our results, Dadalioglu and Evrendilek reported that Spanish lavender essential oils had a weak antibacterial effect [5].

In this research, MIC and MBC of lavender were 3.12% and 6.25% for *E. coli* and 4.37% and 8.75% for *S. aureus*, respectively. Also, MIC and MBC of marjoram were 0.62% and 1.25% for *E. coli* and 1.56% and 3.2% for *S. aureus*, respectively. Different values have

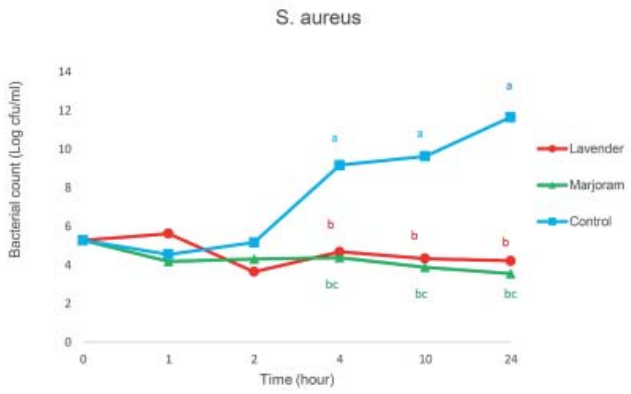

Figure 2
Survival curve of S. aureus in milk containing 0% (control, blue) and sub-MIC concentration of essential oil of lavender (red) and marjoram (green).
[a-c]Values that are significantly ($P < 0.05$) different within the same time are indicated by different letters.

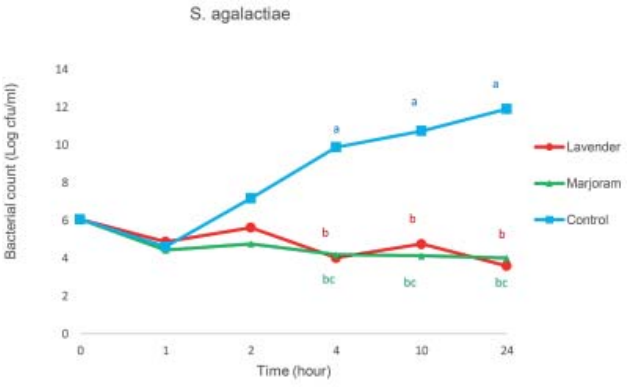

Figure 3
Survival curve of S. agalactiae in milk containing 0% (control, blue) and sub-MIC concentration of essential oil of lavender (red) and marjoram (green).
[a-c]Values that are significantly ($P < 0.05$) different within the same time are indicated by different letters.

reported for MIC and MBC of lavender and marjoram. Bouyahya et al. and Gayatri et al. obtained MIC values of 0.5% and 0.25% against *E. coli* for lavender and 0.5-2% and 0.5% against *S. aureus*, respectively [1,23]. MIC and MBC values of 7.8% and 15.6% against *E. coli* and 1.9% and 7.8% against *S. aureus* for marjoram have reported, respectively [18]. In another study, MBC value of marjoram for *S. aureus* was obtained 0.25% v/v [24]. The different values of MIC and MBC in various studies might be because of the variable components of EOs and susceptibility of strain.

Antimicrobial effects of EO is not due to one specific mechanism because there are several different chemical groups in the structure of EO. Hydrophobicity of essential oils or their components helps them to target the cell membranes of bacteria that contain lipid. This property increases the permeability of membranes, thus contents of cell leak [12]. An outer membrane is present in Gram-negative bacteria that prevent penetration of essential oils into cells. Moreover, periplasmic extracellular enzymes might deactivate anti-microbial components of essential oil [25]. Thus we expected that essential oils to be more effective against Gram-positive *S. agalactiae* and *S. aureus* than Gram-negative *E. coli* but, MIC and MBC of S. aureus and S. agalactiae was higher than E. coli in all treatments except marjoram that MIC and MBC of *S. aureus* were lower than *E. coli*. Our results from the MIC and MBC indicated that the most sensitive microorganism against lavender and lavender + marjoram was *E. coli* and against marjoram was *S. aureus*. In agreement with our findings, in a previous study, MIC of lavender EO was higher against *S. aureus* than *E. coli* [1]. Similar to our finding, Tunisian marjoram EO showed a higher MIC and MBC against *E. coli* than *S. aureus* [18]. In contrast, MIC of marjoram against *S. aureus* was one fold upper than that of *E. coli* [26]. Essential oils of Plant potentially have several antimicrobial constituents. Comparing the findings of different researches is difficult because they use different bacterial strains, test methods, and source of antimicrobial samples. The great variability of composition of the essential oil can be attributed to the extraction method of the EO, geographical region, variety, and plant age [27].

We further carried out a time-kill curve set of tests, to ascertain time of inhibition or killing these pathogens. Lavender and marjoram oil displayed a bacteriostatic effect in the first 2 h and bactericidal effect between 4 and 24 h. Lavender and marjoram oil at sub-MIC caused a ~2.0 log10 cfu/ml reduction of *S. aureus, S. agalactiae* and *E. coli* within 24 h (Figures 1, 2, and 3).

Mullen et al. reported the antimicrobial effect of an herbal intra-mammary product on mastitis-causing bacteria and declared that the antibacterial effect of the formula might be due to *Thymus vulgaris* (thyme) [28].

In conclusion, essential oil of marjoram had greater antibacterial activity than lavender on the mastitis-causing bacteria (*S. aureus, S. agalactiae,* and *E. coli*). Results of this research showed that marjoram EO might be effective as an alternative or adjunct to antibiotic therapy to control bovine mastitis. However, further in vivo tests are needed to evaluate the efficiency on treatment of bovine mastitis and potential side effects on the mammary gland tissue.

Material and methods

Essential oils

Lavender and marjoram EO were purchased from Barij Es-

sence Pharmaceutical Company, Kashan, Iran and Giah Essence Agro-Industry & Phytopharm Company, Gorgan, Iran, respectively.

Analysis of Chemical Composition of the Essential Oils

GC/MS analysis was performed using an Agilent 7890B gas chromatograph coupled to a mass detector (Model 5977A, Agilent Technologies, USA) and a HP-5MS capillary column (phenyl methyl siloxane, 30 m × 0.25 mm ID 0.25 um, Agilent Technologies). The temperature of injector was 270°C, and the temperature of 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 (1ul). The interface temperature was set at 280°C and mass range was 35 - 500 m/z.

Bacterial strain

The activity of the EOs was tested toward three major mastitis bacteria including *Staphylococcus aureus* (ATCC 9144), *Streptococcus agalactiae* (ATCC 13813), and *Escherichia coli* (ATCC 25922). These bacteria were obtained as a lyophilized culture from Persian Type Culture Collection, Tehran, Iran (PTCC). The lyophilized cultures were grown twice in tubes containing 10 ml of Tryptic Soy Broth (TSB) (Biolife, Milano, Italy) at 37°C for 18 - 20 h (overnight). Afterwards, cultures were diluted with sterile glycerin (1:5) and stored in micro tubes at - 20°C for our research. To obtain fresh bacteria, 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 [29].

Preparation of Inoculum

Cells were transferred from working cultures to tubes of TSB and incubated at 35 °C for 18 h to obtain Bacterial inoculum. Next subcultures were performed and incubated at 35°C for 18 h. A spectrophotometer (Libra S12, Biochrom Ltd., Cambridge, London) was used to set the bacterial broth cultures to optical density (OD) of 0.1 at 600 nm, and a cell concentration of 2.4×10^{11} cfu/ml for *E. coli*, 3.4×10^{10} for *S. aureus* and 1.64×10^{11} for *S. agalactiae* were obtained. The number of cells in the suspensions was estimated by duplicate plating from tenfold serial dilutions on TSA and counting the colonies after 24 h incubation at 35°C [29]. The working inoculum was prepared by 1: 500 dilutions of the primary inoculum.

Milk Preparation

Milk without antibiotic residues was collected and then autoclaved for 15 min at 121°C.

Determination of MIC and MBC

Essential oils were diluted (1:1) in dimethylsulphoxide (DMSO, Sigma, Germany) and filter sterilized. This dilution was used in the antibacterial analysis. Herbal oils alone or in combination (1:1) were tested using a modified protocol for broth dilution testing according to the Clinical & Laboratory Standards Institute (CLSI) instruction [30]. Whole autoclaved milk was used as the growth medium. Twofold serial dilutions of the oil dilution were performed for the determination of MIC. Treatments were added to milk and were vortexed. Total volume of test vials was 1 mL. Then 100 µl of inoculum of each bacterium was inoculated into each tube. The vials again were vortexed and incubated at 37°C for 24 h. Eight 10-fold dilutions were prepared using sterile 0.85% saline solution. The samples were plated on a TSA plate and incubated at 37°C for 24 h for enumeration of inoculated bacteria. The MBC was defined as the lowest concentration without visible growth and subsequent concentration was taken as the MIC. Milk was cultured alone as a negative control to ensure that autoclaing was successful. Milk + bacteria were included as positive control to document growth of bacteria in milk. Vehicle control was DMSO to evaluate possible antibacterial effect of this solvent.

The occurrence of synergism/antagonism in antibacterial action among the EOs of lavender and marjoram was evaluated against mastitis pathogens. For this purpose, the essential oils were mixed volume to volume (1:1).

Bactericidal kinetics of the oils

Sterile milk was inoculated the sub-MIC of EOs with each pathogen in the same way as the above MIC tests. Control samples were inoculated milk without EO. Bacterial populations were counted at 1, 2, 4, 10, and 24 h of incubation at 37°C for 24 h on TSA plates. Each treatment was done in duplicate. Time-kill curves were constructed by plotting log10 cfu/ml against time (hour).

Statistical analysis

All tests were carried out in duplicate. The data were evaluated by analysis of variance (ANOVA) and the Tukey's test using the SPSS 18 statistical software (IBM Corp., Armonk, NY, USA) at $p < 0.05$ statistical level.

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Author Contributions

Conceived and designed the experiments: RR, JBK. Performed the experiments: SN. Analyzed the data: RR, FBB. Research space and equipment: RR, JBK, FBB. Contributed reagents/materials/analysis tools: RR, SN. Wrote the paper: RR.

Conflict of Interest

The authors declare that there is no conflict of interest regarding to publication of this paper.

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تاثیر ضد باکتریایی اسانس اسطوخودوس و مرزنجوش بر استافیلوکوک آرئوس، استرپتوکوک آگالاکتیه و اشریشیاکلی

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چکیده

در این مطالعه اثر ضد باکتریایی اسانس اسطوخودوس و مرزنجوش بر سه باکتری بیماریزا شامل استافیلوکوک آرئوس، استرپتوکوک آگالاکتیه و اشریشیاکلی بررسی شد. آنالیز اسانس ها به روشGC/MS نشان داد که مهمترین ترکیبات اسطوخودوس 17-pentatriac-ontene، linalyl acetate، eucalyptol، linalool و مرزنجوش α-terpineol، 3-Cyclohexen-1-ol,4-methyl-1-(1-methylethyl)-(R)- و P-cymene بودند. روش رقت سازی لوله ای با استفاده از شیر کامل اتوکلاو شده به جای محیط کشت مایع برای تعیین حداقل غلظت مهاری و حداقل غلظت کشندگی اسانس ها به تنهایی و در ترکیب با هم استفاده شد. علاوه بر آن منحنی رشد باکتری ها در حضور اسانس ها و در محیط شیر تا ۲۴ ساعت رسم شد. محدوده حداقل غلظت مهاری و حداقل غلظت کشندگی به ترتیب برای اسطوخودوس ۳/۱۲-۴/۳۷ ٪، ۶/۲۵-۸/۷۵ ٪، مرزنجوش۱/۷۸-۰/۶۲٪، ۱/۲۵-۳/۷۵ ٪ و ترکیب آنها ۵-۲/۵٪، ۱۰-۵٪ بود. از نظر منحنی رشد، اسانس اسطوخودوس و مرزنجوش باعث کاهش معنی دار تعداد باکتری ها در ساعت های ۴، ۱۰ و ۲۴ شدند. در مجموع اسانس مرزنجوش اثر ضدباکتریایی قوی تری نسبت به اسطوخودوس علیه سه باکتری مورد مطالعه داشت و می تواند در مطالعات آینده به عنوان آنتی بیوتیک یا به همراه آنتی بیوتیک در درمان ورم پستان گاو مورد بررسی قرار گیرد.

واژگان کلیدی

اثر ضد باکتریایی، اسطوخودوس، دامداری ارگانیک، مرزنجوش