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Original article

In vitro antibacterial efficiency of some herbal extracts and theirs blends against subclinical mastitis pathogens (*S. aureus* and *E. coli*)

Hanifi Aydin

Balikesir Metropolitan Municipality, Rural Services Department Farmer Training Center, Balikesir, Turkey

Abstract. *Study Objectives:* The current research will form the basis for the production of new herbal mastitis drugs as an alternative to antibiotics. In this context, the aim of the study was to evaluate the *in vitro* antibacterial activity of *O. onites* and *O. sanctum* essential oil, solutions prepared in fixed oils of *N. sativa* and *O. europaea*, against *S. aureus* and *E. coli* bacteria which are the main agents of mastitis. Methods: Microorganism isolation and antibiotic susceptibility analyzes of milk samples taken from 43 subclinical mastitis dairy cows were performed. The antibacterial properties of the solutions were analyzed by a disk diffusion method. Results: In the bacterial isolation results, *S.aureus* was determined 97.7% and *E.coli* 34.9% positive of cows with mastitis. Occur that extract of *O. onites* showed maximum inhibition against *S. aureus* (23 mm) and *E. coli* (15 mm). At the same time, *E.coli*, which is already known to be multi-resistant to drugs, was susceptible only to the extract from *O.onites*. When the mean ± SD zone areas for the susceptible solutions (C, C1, C2, O, S, O+S, and Z2) and the control group were compared, determined that there was no statistically significant difference for *S.aureus* and *E.coli* ($p>0.05$). Conclusion: It was determined that the effectiveness of olive oil as a solvent against S. *aureus* was not sufficient, therefore, a 50% solution (15mm zone diameter) with *N. sativa* fixed oil was more effective.

Key words: *O.sanctum*, *O.onites*, Subclinical mastitis, Dairy cows, *In vitro*

Introduction

Mastitis is a complex disease caused by various bacterial pathogens, mainly *Staphylococcus aureus,* and *Escherichia coli.* It is also reported to be the most common reproductive disease in dairy cattle and a heavy burden disease in dairy farms worldwide (1,2). It is believed that the bacterial infection in mastitis cases is related to the disruption of alveolar cell-integrity, sloughing of cells, induced apoptosis, and increase of poorly differentiated cells. Since the ability of ruminant mammary glands to produce milk is determined by the number and activity levels of milk-secreting cells, the amount of milk produced and the protein, lactose, and fat concentrations in milk can be affected by the level of inflammation in the mammary gland (2).

Drug residues in milk cause allergic reactions for the consumer, interference in the intestinal flora, and resistant bacterial populations, and accordingly, they can destroy the effect of antibiotic treatment (3). The World Health Organization (WHO) stated that *E. coli* and *S. aureus* are the priority pathogens for overcoming antimicrobial resistance and for the research and development of new antibiotics (4-8). In terms of antimicrobial resistance, alternative, safe, effective and fewer side effects are needed for mastitis control.

Recently, new treatment alternatives based on the use of natural resources have been intensively researched. In this context, it is becoming more and more necessary to investigate the antimicrobial and antiseptic properties of essential oils and their contents obtained by various extraction methods from plants that can be used for therapeutic purposes. EOs are rich mixtures of different chemical compounds, including terpenes, aldehydes, alcohols, esters, phenols, ethers, and ketones. Therefore (EOs) are agents used in the control of pathogens causing bovine mastitis (1, 6, 9).

Origanum species are also known as 'kekik' in Anatolia and *Origanumonites* L. is the dominant species in the northwest of Turkey and a member of the family Lamiaceae. Carvacrol and thymol found in Origanum essential oil have antimicrobial effects. However, *O. onites* is gaining wide applications in the pharmaceutical and food industry due to its biological properties including antibacterial, antifungal, antioxidant, anticancer, antiangiogenic, analgesic, and acaricidal. In total 28 components belonging to polyphenols, flavonoids, terpenoids, organic acids, and esters have been identified (10, 11).

Ocimum sanctum (known as tulsi) is a member of the family Lamiaceae. Literally, Tulsi means "Matchless one". Also known as "Queen of Herbs" (incomparable one) (BabitaLabhKayastha). It is one of the holiest plants which exhibits tremendous healing potential. Therapeutically it is used in anticancer, anti-oxidant, anti-diabetic, radiations, infertility, and for many other major and minor diseases (12). *O. sanctum* is a valuable herbal medicine being used in a wide spectrum of animal diseases. It possesses immunomodulatory and anti-inflammatory properties attributed to its active constituents such as volatile oil (eugenol, 80%), flavonoids, and triterpene, which are largely responsible for its therapeutic potential (13, 14).

The *Nigella sativa* plant is native to Western Asia and the Mediterranean region. The seeds contain 40% fixed oil, a saponin, and up to 1.4% volatile oil (15). The fact that the fixed oil in the seeds is especially rich in polyunsaturated fatty acids contributes to its effects on antioxidant, antiallergic, anti-inflammatory, immunomodulatory, antibacterial, antiviral, antitumor, antidiabetic, hepatoprotective, cardiovascular system-blood, and gastrointestinal system (16, 17).

The result of research *in vivo* and *in vitro* have demonstrated that extra virgin olive oil (*Olea europaea*) phenolic compounds have potentially beneficial biological effects resulting from their antimicrobial, antioxidant, and anti-inflammatory activities. Phenolic compounds in olive oil have been shown to exert beneficial effects on lipid oxidation, DNA oxidative damage, and in general oxidative stress, *in vitro* and *in vivo* (18, 19).

In this context, the aim of this study was to evaluate the in vitro activity of solutions of essential oils of *O. onites* and *O. sanctum* prepared in *N. sativa* and *O. europaea*fixed oils against *S. aureus* and *E. coli* bacteria which are the causative agents of mastitis.

Materials and Methods

Collection of Plants

Medicinal aromatic plants used in the study were harvested from the parcels on Balikesir Metropolitan Municipality Farmer Training and Production Center (BACEM), which is our institution and the plants were washed with water and kept in an oven at 40°C for 3day their weight stabilized and turned into powder.

Extraction of Plants and Preparation of the Solution

Extraction of Essential Oil. Herba parts of *O. sanctum* and *O. onites* plants, the region with the highest essential oil, were used. The powdered material (500 g) was extracted exhaustively with water in a soxhlet apparatus. After Soxhlet extraction, the prepared crude extract was completely evaporated with water under reduced pressure with a rotary evaporator (Heidolph-instruments, Rotavapor, Germany). Finally, conserved in an air-tight container at 4°C in the refrigerator.

Extraction/Providing of the Fixed Oil. Ground 10 g of *Nigella sativa* seeds were extracted with hexane in a Soxhlet apparatus for 4 hours. After the hexane extract was evaporated to dryness at 40°C in a rotary evaporator, it was weighed, the % yield was calculated and stored at +4°C until used. The olive oil used in the research was obtained by the cold pressing method by providing service procurement from the olives

Table 1. Preparation of solutions according to their percentages.

Solutions Oils	S	O	S+O	Z	Z1	Z2	C	C1	C2
Fixed oils	-	-	-	100%	80%	50%	100%	80%	50%
Essential oils	100%	100%	100%	-	20%	50%	-	20%	50%

S: *O. sanctum*, **O**: *O. onites*, **S+O**: *O. sanctum*ve*O. onites* blend, **Z**: %100 *O. europaea*, **Z1**: %20 lik(%10 *O. sanctum*ve %10 *O. onites*) %80 *O. europaea*, **Z2**: %50 (%25 *O. sanctum* ve%25 *O. onites*) %50 *O. Europaea,* **C**:%100*N.sativa* fixed oil, **C1**:%80 *N.sativa* fixed oil, **C2**:%50 (%25 *O. sanctum*ve %25 *O. onites*) %50 *N.sativa* fixed oil.

harvested from the plots of our institution.Essential oils extracted from plants were diluted with fixed oils (Table 1).

Detection of Mastitis

Balikesir Edremit, Cikrikci Village BurakBey Farm, 80 Holstein breeds with 25-30 kg/day yield were fed with rations containing hay, concentrate and silage in the 3rd and 4th lactations, with feeding systems based on TMR (Total Mixed-Ration). The milk sample taken from each udder of the cow was evaluated by performing CMT. In the study, 43 cows evaluated as CMT +: 1, ++: 2, +++: 3 and without clinical endometritis, laminitis were accepted as the experimental group (20).

Milk Sample Collection

According to the CMT results, after milking a few shots of foremilk from any of the healthy udder lobes after milking, milk samples were taken into a tube of approximately 20 ml.

Isolation of Bacteria, Antibiogram, and In vitro antimicrobial activity of the extract Microorganism determination and antibiotic susceptibility analyzes were performed on milk samples in Balıkesir Cattle Breeding Association. According to the antibiogram results, Amoxicillin/ Clavulanic Acid and Cefoperazone antibiotics with the highest sensitivity on *S. aureus* and *E. coli* were selected as the control group. Then, the herbal solution samples were sent to Konya Selcuk University Veterinary Faculty Microbiology Department Laboratories. Antibiograms of the solutions were performed on *S. aureus* and *E.coli* strains in the same laboratory. Antibacterial activities of the materials/extracts were evaluated against the *S. aureus* ATCC 22923 and E. coli ATCC 25922. Bacteria were grown aerobically in brain-heart infusion (BHI) broth at 37°C for 24-h. Cells were harvested by centrifuging and resuspended in sterile physiological saline. Bacteria concentrations were adjusted according to McFarland no: 0.5 (1,5 x 108 CFU/ml).

The 10 ml sterilized Brain Heart Infusion (BHI) Agar (Oxoid) poured into 20 mm x 90 mm sterilized Petri dishes. Four wells (diameter 7 mm, depth 2mm) were made by the removal of agar at equidistant points. The 0,1 ml of the tested microorganisms were seeded into BHI agar. The wells on the agar were filled with 0,1 ml of materials/extracts and the plates were maintained at room temperature for 2 h for pre-diffusion of the materials, and then incubated at 37 C for 24 h (21). The diameter of the zone was measured at the inhibition zone with a millimeter (mm) ruler. Data were evaluated according to Table 2 (22).

Statistical analysis

Descriptive statistics of the data obtained from the study are given with mean, standard deviation, median, and quartiles (Q1, Q3) for numerical variables, and frequency and percentage analysis for categorical variables.

Table 2. Zone diameters used in the evaluation of inhibition activity (21).

Zone Diameter (mm)	Inhibition Activity
Not seen	(-)
<10	(+)
10-14	(++)
15-19	(+++)
>20	(++++)

(-): resistant; (+): less resistant; (++): medium sensitive, (+++): sensitive; (++++): very sensitive

The Mann-Whitney U test was used to compare the zone values obtained from the solutions and the control group. Analyzes were carried out with the help of the SPSS 22.0 program. p<0.05 significance level was chosen.

Results

Bacteria isolation data

The causative isolation of microorganisms was made from 43 samples of milk with mastitis (Table 3). *S. aureus* was isolated and identified in 42 cows (97.7%) and *E. coli* (34.9) in 15 cows with mastitis. The study was carried out on *S. aureus* and *E.coli* bacteria.

Bacteria zone diameters

While some of the materials were found to have different levels of antibacterial activity on the tested microorganisms, some were found to be ineffective (Table 4), (Figures 1-3).

Sensitivity of Solutions

Among the solutions, 2 solutions were found resistant for *S. aureus*, while 7 solutions were found to be sensitive (moderate and very sensitive). For *E.coli*, 8 solutions were found to be resistant, while 1 solution was found to be sensitive (Tables 5 and 6).

Table 3. Mastitis microorganism determination results.

Microorganism		Number	Percent (%)
Staphylococcus aureus	Negative	1	2,3
	Positive	42	97,7
E. coli	Negative	28	65,1
	Positive	15	34,9
Klebsiella Pneumonia	Negative	43	100,0
	Positive	0	,0
Pseudomonas aeruginosa	Negative	18	41,9
	Positive	25	58,1
Proteus mirabilis	Negative	41	95,3
	Positive	2	4,7
Yeast	Negative	35	81,4
	Positive	8	18,6
Mold	Negative	38	88,4
	Positive	5	11,6

When the mean ± sd zone areas for the susceptible solutions and the control group were compared, it was determined that there was no statistically significant difference for *S. aureus* and *E.coli* (p>0.05), (Figure 4).

Discussion and Conclusion

Essential oils have a bacteriostatic or bactericidal effect on microorganisms (8). To lessen the adverse effects of antibiotics, a different approach of preventing

Table 4. Antibacterial activities of solutions on test microorganisms

Solutions	**Bacteria and Zone diameter (mm)**			
	S. aureus	**Act**	***E. coli***	**Act**
C	17	(+++)	(-)	(-)
C1	10	(++)	(-)	(-)
C2	15	(+++)	(-)	(-)
S	10	(++)	(-)	(-)
O	23	(++++)	15	(+++)
O+S	15	(+++)	(-)	(-)
Z	(-)	(-)	(-)	(-)
Z1	(-)	(-)	(-)	(-)
Z2	10	(++)	(-)	(-)
Amoxicillin/ Clavulanıc Acid	15	(+++)	17	(+++)
Cefoperazone	22	(++++)	20	(++++)

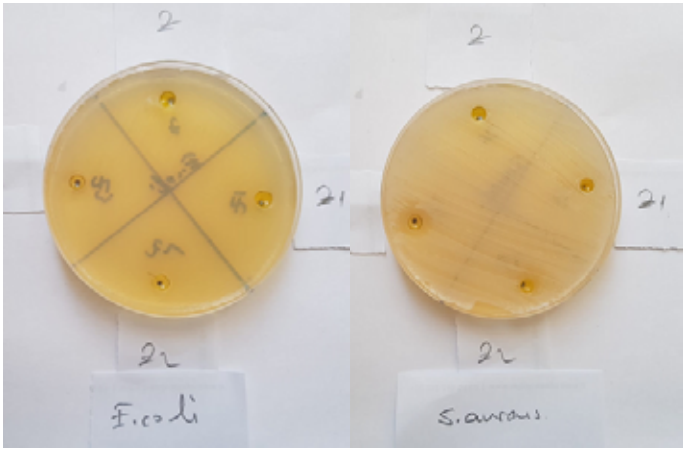

Figure 1. Bacterial zone diameters of Z, Z1, and Z2 solutions

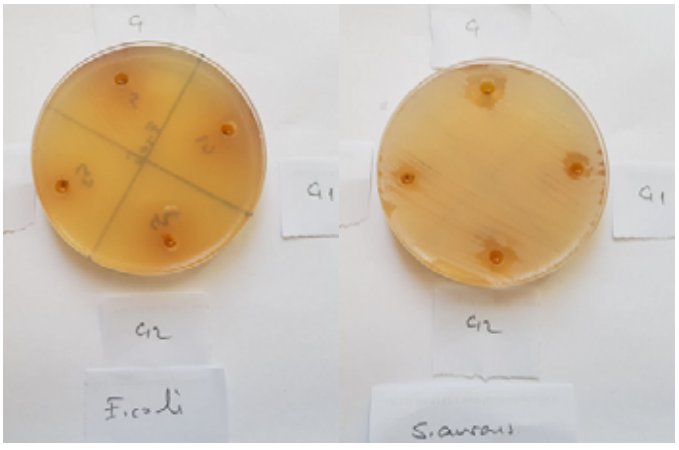

Figure 2. Bacterial zone diameters of C, C1, and C2 solutions

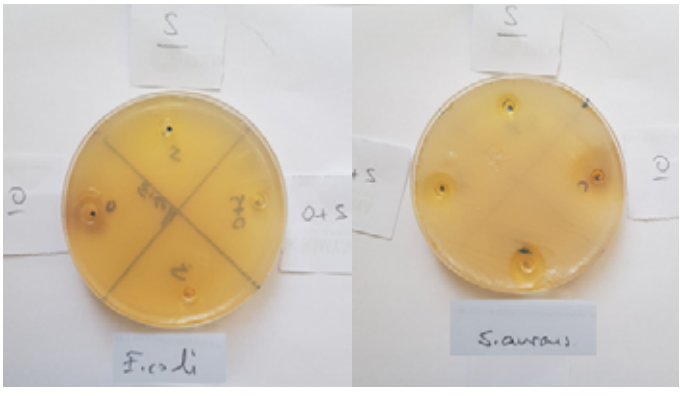

Figure 3. Bacterial zone diameters of O, S, and O+S solutions

Table 5. Solution and control group sensitivity

		Group			
		Solution		Control	
		Number	Percent (%)	Number	Percent (%)
S. aureus	Resistant	2	22.2	0	.0
	Sensitive	3	33.3	1	50.0
	Medium sensitive	3	33.3	0	.0
	Very sensitive	1	11.2	1	50.0
E. coli	Sensitive	1	11.2	2	100.0
	Resistant	8	88.8	0	.0

Table 6. Zone diameters of the solution and control groups that were found susceptible to S. aureus and E.coli.

Variables	Solution		Control		p
	Mean±SD	Median (Q1-Q3)	Mean±SD	Median (Q1-Q3)	
S. aureus	14.29 ± 4.82	15 (10 -17)	18.5 ± 4.95	18.5 (15 -22)	0.500
E. coli	15 ± 0	15 (15 -15)	18.5 ± 2.12	18.5 (17 -20)	0.667

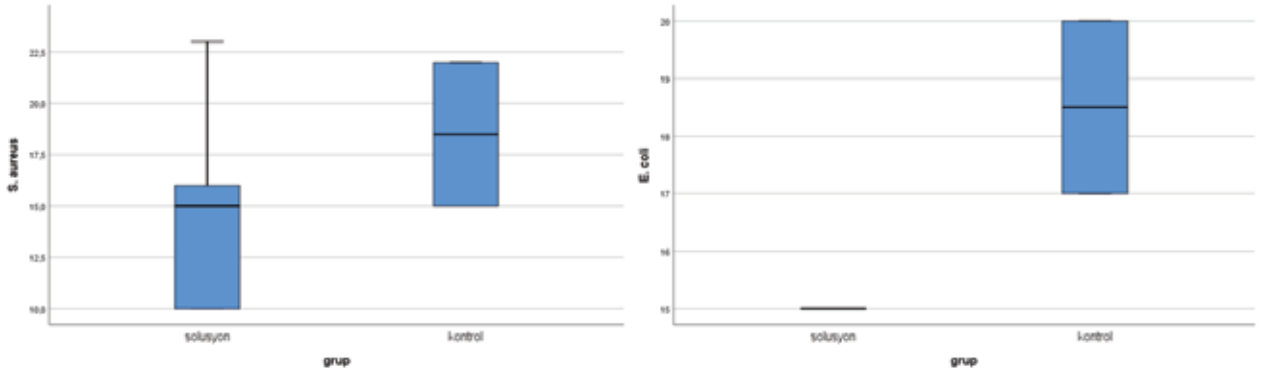

Figure 4. (A) Comparison of antibacterial Activities of solutions and control group against S. aureus, (B) and E.coli.

mastitis, *O. onites,* and *O. sanctum* administration, was evaluated *in vitro* for its therapeutic potential in specific subclinical mastitis of dairy cows.

Plants store some of the energy they get from the sun in essential oil. The synthesis of terpenes takes place, and in some plants, this synthesis process goes beyond terpenes to the production of steroids (cortisone, cholesterol, vitamin D) with hormonal properties. Phenylpropanoids found in essential oils are precursors of certain amino acids necessary for the synthesis of proteins and aid in healing where tissue damage has occurred. Essential oils need to be diluted as the absorption will be rapid in the tissue damaged area. Fixed oils are the best medium for the dilution of essential oils. Because essential oils dissolve easily and completely in fixed oil. The use of fixed oils reduces the absorption rate of essential oils, thus reducing the effect of essential oils but increasing their yield (23).

S. aureus, which is known to be highly resistant to antibiotic treatment in mastitis, lives in the host's cells and becomes chronic by forming microabscesses or granulomas in the mammary gland tissues (24). In the bacterial isolation results, *S.aureus* bacteria were found to be 97.7% and *E.coli* 34.9% positive. It was determined that the effects of these two bacteria in the formation of mastitis were intense in the cows

studied. Especially, *in vitro*solution trials were conducted against these two bacteria. The values found were similar to other research results (25-27).

Although all extracts showed antimicrobial activity against nearly all of the microorganisms tested, *O.onites* alone was found to exhibit broad-spectrum activity against selected bacterial pathogens isolated from clinical mastitis in dairy cows. The study found that extract of *O.onites* showed maximum inhibition against *S. aureus* (23 mm) and *E. coli* (15 mm). *E.coli*, which is already known to be multi-resistant to drugs, was susceptible only to the extract from *O.onites*. In the antibacterial study of Dinçoğlu (2019) with *O.onites* EO, the antibacterial activity of the plant was determined with the zone diameters of *S.aureus* 17mm and *E.coli* 13mm. The results of the researcher support the results of the study (10). Paiano (2020), It was determined that cinnamon, clove, oregano, and thyme essential oils showed inhibition zone diameters of 36 mm, *E. coli* 20 mm, especially for *S. aureus* causing endometritis (28).

Researchers have found that olive oil and its active ingredient oleuropeinare natural antibiotic agents. However, the precise mechanism of oleuropein's antimicrobial activity is still not fully understood (29). Unfortunately, the olive oil we had was less or no effective than other researches and other studied plants. Olive oil showed either negative (Z, Z1) or maximum 10 mm (Z2) inhibition zone against *S.aureus*, respectively. There was no indication of inhibition against *E. coli*. In the study conducted by the researchers, the antioxidant and antimicrobial activities of the phenolic components of the phenolic extracts of olive oil varieties obtained from 11 Algerian varieties against various bacteria were investigated. Results obtained in BouchoukSoummam varieties showed an inhibition zone diameter of 10.33±0.57 in *S.aureus*, but no activity against *E.coli*. The results of the researcher support the results of the study (19).

N.sativa is used as a natural medicine for multiple therapeutic purposes in animals. It has been observed that *N. sativa* essential oil inhibits the growth of Gram (-) microorganisms, especially Gram (+) bacteria. It has been reported that the biological activity of the oil is due to its phenolic content (16, 24). Azad et al. (2011) found that *in vivo* injections of *N.sativa* oil reduced bacterial growth and milk SCC in cows infected with *S. aureus* bacteria with subclinical mastitis (24). Consistent with our findings, *N. sativa* showed 17 mm (C), 10 mm (C1), and 15 mm (C2) inhibition zones against *S.aureus*, respectively. There was no sign of inhibition against *E. coli*.

It has been reported that *O. sanctum* oil showed good antibacterial activity against *S. aureus* (zone of inhibition 8.0 mm), but less active against *E. coli* (30). In the study results, *S.aureus* showed 10 mm zone inhibition, while there was no indication of inhibition against *E.coli*. The results of the study are consistent with the results of other studies. Jayati et al (2013) Discs containing four different concentrations of *O. sanctum* hot aqueous leaves extract were used to study the antimicrobial activity *O. sanctum* Ext./disc amount at 5 mg *S. aureus* Inhibition zone (8-12 mm), *E. coli* zone was no detected (31).In the study of Mukherjee et al Intramammary infusion of *O.sanctum* extract significantly increased the level of leukocyte lysosomal enzyme (myeloperoxidase) that is an important component of the oxygen-dependent antimicrobial activity of cells. The researchers concluded that intramammary infusion of *O. sanctum* extract showed antimicrobial and immunomodulatory activity. Both activities were associated with increased activity of PMN cells in the bovine mammary gland (32).

In Aydın 2020 study, SHS of 20% *O.sanctum* and *O. onites* was measured as 2185.78 cells/ml before *in vivo* intramammary administration and 809.72 cells/ml after administration. In addition, on the 5th day of the application (7715.67±2877.07 cells/ml), somatic cell increase was observed due to the inflammatory reaction (33). In order to see the antimicrobial efficacy of the current working solution, depending on the previous study, agent isolation was performed, and *S.aureus* 97.7% and *E.coli* 34.9% were found to be positive. While it showed C1 (10mm), Z1 (negative) activity against *S.aureus* in 20% solutions, it was determined with 15mm, 10mm, and 15mm zone diameters, respectively, against C2, Z2, O+S *S.aureus* in 50% solutions were done. Considering the current study results, it is thought that the efficacy of 50% solution *in vivo* will be better.

It was determined that while *N. sativa* fixed oil alone was moderately sensitive to *S.aureus* (17mm).

While olive oil alone was resistant to *S. aureus*, the solution (Z2) became sensitive as the essential oil concentration (50%) increased. *O.onites* 100% concentration has the best susceptibility to *S.aureus* and *E.coli* (23mm, 15mm zone diameter). Such high concentrations may irritating and cause damage to mammary tissues (34).The 100% concentration of *O.sanctum* was found to be sensitive (10mm) only to *S.aureus*. It was determined that the low zone diameter of *O.sanctum* reduced the sensitivity of all solutions. However, in solutions, *O.sanctum* has been used for *in vivo* studies to remove damage in the tissue and for the antioxidant and immunomodulatory effect of the plant, rather than its antimicrobial properties (32, 33).

When the zone diameters were compared between the 2 antibiotic groups and solutions used as the control group in the study, it was determined that there was no statistically significant difference ($p>0.05$). It was determined that 63.7% of the solutions were susceptible to *S.aureus* and 36.4% were resistant.The results of this study show that the solution in *N.sativa* fixed oil of *O. onites* and *O. sanctum* essential oils have an antibacterial effect against *S.aureus* "high priority", which is the causative agent of subclinical mastitis, and for the production of new herbal mastitis drugs alternative to antibiotics in the intramammary application. It shows that it will form the basis of further research in veterinary medicine in vitro as well as *in vivo.* Current research will also provide scientific validation of traditional knowledge and increase local farmers' confidence in ethnoveterinary practices.

References

1. Alonso MM, Salazar JCL, Robles SO, Guerrero IC, García FL, Marrero JG. *In vitro* antimicrobial activity of mexican plants on bovine mastitis bacteria: preliminary studies. Bioscience Journal 2020; 36(1): 183-190.
2. Harjanti DW, Sambodho P. Effects of mastitis on milk production and composition in dairy cows. In IOP Conference Series: Earth and Environmental Science IOP Publishing 2020 September; 518(1): 012032.
3. Amber R, Adnan M, Tariq A, Khan SN, Mussarat S, Hashem A, AbdAllah EF. Antibacterial activity of selected medicinal plants of northwest Pakistan traditionally used against mastitis in livestock. Saudi Journal of Biological Sciences 2018; 25(1): 154-161.
4. Klaas IC, Zadoks RN. An update on environmental mastitis: challenging perceptions. Transboundary and Emerging Diseases 2018; 65: 166-185.
5. Suleiman TS, Karimuribo ED, Mdegela RH. Prevalence of bovine subclinical mastitis and antibiotic susceptibility patterns of major mastitis pathogens isolated in Unguja island of Zanzibar, Tanzania. Tropical Animal Health and Production 2018; 50(2): 259-266.
6. Lopes TS. Estudo sobre a ação de óleos essenciais no controle de microrganismos causadores de mastite bovina. Universidade de Caxias do Sul, Master Thesis 2020; pp 158
7. Radzikowski D, Kalińska A, Ostaszewska U, Gołębiewski M. Alternative solutions to antibiotics in mastitis treatment for dairy cows-a review. Animal Science Papers & Reports 2020; 38(2): 117-133
8. Tepeli, SÖ. Origanum onites ve Ocimum basilicum'un blaCTX-M Pozitif Enterobacteriaceae Üzerine Antimikrobiyal Etkisi. Journal of Advanced Research in Natural and Applied Sciences 2020; 6(2), 206-216.
9. Gucwa K, Milewski S, Dymerski T, Szweda P. Investigation of the antifungal activity and mode of action of *Thymus vulgaris*, *Citrus limonum*, *Pelargonium graveolens*, *Cinnamomum cassia*, *Ocimumbasilicum* and *Eugenia caryophyllus* essential oils. Molecules 2018; 23(5): 1116, 1-18.
10. Dinçoğlu AH. Mercan köşk (*OriganumOnites* L.) bitkisinin bazı gıda patojenleri üzerine antibakteriyel etkinliğinin saptanması. Mehmet Akif Ersoy Üniversitesi Sağlık Bilimleri Enstitüsü Dergisi 2019; 7(2): 139-146.
11. Napoli E, Siracusa L, Ruberto G. New tricks for old guys recent developments in the chemistry, biochemistry, applications and exploitation of selected species from the Lamiaceae family. Chemistry & Biodiversity 2020; 17(3): e1900677.
12. Bano N, Ahmed A, Tanveer M, Khan GM, Ansari MT. Pharmacological evaluation of *Ocimum sanctum*. Journal of Bioequivalence and Bioavailability 2017; 9(3): 387-492.
13. Shafi TA, Bansal BK, Gupta DK. *In vitro* antibacterial activity and minimum inhibitory concentration of *Ocimum sanctum* leaves against common bovine mastitis pathogens. J Dairy Vet Anim Res 2018; 7(6): 322-324.
14. Sharma D. Studies on therapeutic potential of *Ocimum sanctum* on subclinical mastitis in goats (*Capra hircus*). Doctoral dissertation, Rajasthan University of Veterinary and Animal Sciences Bikaner 2018; 1-113.
15. Rakhshandeh H, Vahdati-Mashhadian N. *In vitro* and *in vivo* study of the antibacterial effects of *Nigella sativa* methanol extract in dairy cow mastitis. Avicenna Journal of Phytomedicine2011; 1(1): 29-35.
16. Uras SS. *Nigella sativa* L. (Ranunculaceae) bitkisi üzerinde farmakognozik araştırmalar Mersin Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmakognozi Anabilim Dalı YL 2009; p.135.
17. Salem AY, El-Awady HG, El-Dein MAT,Eisa DA. Effect of supplementation of aromatic plants oils on immunity, udder health and milk production of Friesian cows. Slov. Vet. Res. 2019; 58(22): 523-530.

18. Cicerale SRSJ, Lucas LJ, Keast RSJ. Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. Current Opinion In Biotechnology 2012; 23(2): 129-135.
19. Laincer F, Laribi R, Tamendjari A, Arrar L, Rovellini P, Venturini S. Olive oils from Algeria: Phenolic compounds, antioxidant and antibacterial activities. Grasas y Aceites2014; 65(1): 001.
20. Alkan H, Baştan A, Salar S, Özdal M, Kaymaz M. Kuru döneme çıkarken enfekte ve sağlıklı meme loblarında california mastitis test ve somatic hücre sayısı ile bakteriyolojik muayene sonuçlarının karşılaştırılması. Ankara Üniversitesi Veteriner Fakültesi Dergisi 2014; 61(3): 179-183
21. Cobankara FK, Altinoz HC, Erganis O¸ Kav K, Belli S. *In vitro*antibacterial activities of root-canal sealers by using two different methods. Journal of Endodontics 2004; 30 (1): 57-60.
22. Rodriguez E, Gonzalez B, Gaya P, Nunez M, Medina M. Diversity of Bacteriocins Produced by Lactic Acid Bacteria Isolated From Raw Milk. International Dairy Journal 2000; 10: 7-15
23. Price L. Traditional use, modern research, Aromatherapy for health professionals E-book Shirley Price, & Len Price, Elsevier Health Sciences 2011; 77-130.
24. AzadiHG, Farzaneh N, Baghestani Z, Mohamadi A, Shahri SAM.Effect of intramammary injection of *Nigella sativa* on somatic cell count and *Staphylococcus aureus* count in Holstein cows with *S. aureus* subclinical mastitis. American Journal of Animal and Veterinary Sciences 2011; 6(1): 31-34.
25. Yeşilmen S, Özyurtlu N, Bademkıran S. Diyarbakır yöresinde subklinik mastitisli ineklerde etken izolasyonu ve duyarlı antibiyotiklerin belirlenmesi. Dicle Üniversitesi Veteriner Fakültesi Dergisi 2012; (1): 24-29.
26. GülmezSA, Otlu S, Çoşkun, MR, Çelik, E, Büyük F, ahinM. İneklerde subklinik mastitis prevalansı, etken izolasyonu ve antibiyotik duyarlılıklarının belirlenmesi. Eurasian Journal of Veterinary Sciences 2018; 34(2): 92-98.
27. Doss A, Soris PT, Mohan VR. Evaluation of antibacterial effect of medicinal plant extracts on bacteria isolated from subclinical bovine mastitis. International Journal of Veterinary Science and Agriculture Research 2019; 1(3): 1-8.
28. Paiano RB, Bonilla J, de Sousa RLM, Moreno AM, Baruselli PS. Chemical composition and antibacterial activity of essential oils against pathogens often related to cattle endometritis. The Journal of Infection in Developing Countries 2020; 14(2):177-183.
29. Al Laham SA, Al Fadel FM. Antibacterial efficacy of variety plants against the resistant Streptococcus which cause clinical mastitis in cows. Asian Journal of Pharmaceutical Research and Health Care 2013; 5(1): 32-41
30. Singh S, Malhotra M, Majumdar DK. Antibacterial activity of *Ocimum sanctum* L. fixed oil. Indian Journal of Experimental Biology 2005; 43: 835-837.
31. Jayati AK, Bhatia AK, Kumar A, Goel A, Gupta A, Rahal A. Antibacterial activity of hot aqueous extract of *Ocimum sanctum* leaves against common bacterial pathogens of animals. Pharma Science Monitor 2013; 3(3): 279-285.
32. Mukherjee R, Dash PK, Ram GC. Immunotherapeutic potential of *Ocimum sanctum* (L) in bovine subclinical mastitis. Research In Veterinary Science 2005; 79(1): 37-43.
33. Aydin H. Phytotherapy with *O. sanctum* and *O. onites* in cows with subclinical mastitis. Kocatepe Veterinary Journal 2020; 13(4): 420-425.
34. Gupta R, Kumar S, Khurana R. Essential oils and mastitis in dairy animals: a review. Haryana Vet.2020; 59: 1-9.

Correspondence
Hanifi Aydın
Balikesir Metropolitan Municipality, Rural Services Department Farmer Training Center, Balikesir, Turkey
E-mail: haydin.vet@gmail.com