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Occurrence of *Staphylococcus aureus* in milk and antibacterial properties of some herbal extracts on milk and hospital-acquired strains

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Abstract: *Staphylococcus aureus* is a remarkable infectious agent, and with the development of antibiotic resistance by *S. aureus*, finding new, effective, and safe alternative therapeutic options in animals and humans is crucially demanded. In this study, the occurrence of *S. aureus* was explored by collecting nonmastitis bovine milk samples first, and then the effects of herbal extracts on methicillin-resistant *S. aureus* (MRSA) strains isolated from cow's milk and humans in the hospitals were investigated. The occurrences of *S. aureus* in bovine raw milk samples were 13.4%. All raw milk *S. aureus* isolates were fully susceptible to cefoxitin, ceftiofur, penicillin G, and enrofloxacin. However, resistance was recorded for methicillin, streptomycin, tylosin, and sulfamethoxazole-trimethoprim. The most antibacterial resistance was recorded for oxytetracycline with 40% (intermediate resistance) and 15% (full resistance) respectively. The hospital-acquired *S. aureus* isolates were entirely resistant to methicillin and penicillin and were susceptible to some antibiotics. Sulfamethoxazole-trimethoprim was the most effective antibiotic with 83.4%. The antimicrobial activities of five medicinal herbs against raw milk *S. aureus* and hospital-acquired *S. aureus* isolates were assessed with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The findings affirmed that *Rosmarinus officinalis* and *Nigella sativa* acted as potent antibacterial agents on *S. aureus* strain obtained for nonmastitis bovine raw milk and hospital-acquired MRSA strains. The efficiency of these herbs against *S. aureus* isolates indicates their applicability as an antibacterial agent for alternative therapy in dairy products and human health and provokes further investigations in this area.

Key words: Antimicrobial-resistant, essential oil, milk, nosocomial infection, phytotherapy

1. Introduction

Staphylococcus aureus is one of the globally crucial bacteria in human and animal health. It causes varied types of infections from mild to life-threatening situations and toxin-related syndromes that can affect tissues and organs. Consequently, employing different methods like risk analysis and management, critical control points, and decent hygienic preparations are the key factors to prevent contamination [1]. With the growth of public health concerns due to different strains of *S. aureus*, especially methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA), the development of alternative antimicrobial and antipathogenic compounds are required to prevent the resistance and pressures [2]. MRSA has been known as one of the major origins of hospital-acquired infections globally [3]. Moreover, in livestock animals, *S. aureus* has been recognized as an emerging pathogen and food poisoning pathogen [4]. Hospital-acquired and community-acquired *S. aureus* strains mainly affect humans, and commonly do not include food-producing animals; nevertheless, livestock-associated MSSA and MRSA may also be received by humans, especially where there is work-related contact with affected livestock [5]. In accordance with available investigations, the number of antibacterial resistance strains is increasing progressively compared with the total number of *S. aureus* isolates [6]. The widespread administration of different antibiotics is the main reason for expanded antimicrobial resistance that leads to major health concerns [7]. Accordingly, identifying and investigating alternative ways instead of current antibiotics such as compounds originating from plants to prevent and eliminate *S. aureus* infections is an important task [8]. In this study, five important herbs with antibacterial properties are selected to investigate their antibacterial properties against both strains of *S. aureus*.

Rosmarinus officinalis (RO) (Rosemary) is a medicinal Mediterranean plant used for many purposes. With many of its bioactive molecules and phytocompounds, this

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plant has certain pharmacological actions like antioxidant and antiinflammatory, antiproliferative, hormonal, and cellular defensive [9]. The antibacterial effects of this herb have been investigated in different studies [10,11]. Several investigations show that divergent components (flower, seed, fruit, and leaf) of *Piper nigrum* (PN) (black pepper) have antioxidant, anticancer, analgesic, and antiinflammatory properties. The antibacterial properties of PN on *S. aureus* have been reported in different studies. Most of these studies showed that several extractions of different parts of this herb can inhibit the growth of *S. aureus* [12, 13]. *Pistacia vera* (PV) (Pistachio), a member of the Anacardiaceae family, is an aromatic plant that is extensively disseminated in the Mediterranean region. It is very rich in linoleic and linolenic acids, fatty acids [14]. Additionally, PV has been previously notified to have different biological activities like antioxidant, antiinflammatory, antifungal, and insecticidal [15,16]. *Linum usitatissimum* (LU) (Flaxseed) which is also known as linseed is a valuable herb associated with the Linaceae family and has anticancer, antioxidant, and antibacterial effects [17,18]. The in vitro potential antioxidant and antibacterial activities of LU extract have been evaluated in a study [19]. *Nigella sativa* (NS) (black seed) belongs to the Ranunculaceae family and has been defined as a source of essential oils, saponins, alkaloids, unsaturated fatty acids, glycolipids, and lipid-soluble vitamins [20]. The broad spectrum of therapeutic effects of this plant including antioxidant, and antibacterial activity have been demonstrated [21,22].

The objective of this work is 1) to detect the occurrence of *S. aureus* in nonmastitis bovine milk samples collected from Ankara, Turkey, 2) to consider the antibiotic susceptibility tests for raw milk and hospital-acquired MRSA-confirmed samples and 3) to explore the chemical composition and antimicrobial effects of five medicinal herbs on *S. aureus* strains isolated from bovine milk and hospital-acquired strain of humans.

2. Material and methods

2.1. Sampling

One hundred and fifty milk samples were randomly collected between June 2019 and December 2020 from different dairy farms in Ankara, Turkey. The sampling excludes any additional practices or interventions. Milk samples were collected from cows with no elevated body temperature and normal symptoms on clinical examination. The milk sample was collected from all active lactating lobes, thus each sample is for all lobes of a cow. All cows with clinical or subclinical mastitis treated with antibiotics were excluded from our sampling. All samples were transferred according to cold-chain transport standards (in an ice bucket) to the laboratory and analyzed on the same day.

2.2. Isolation and identification of *S. aureus*

The process of isolation of the bacteria was carried out on the agar plate method [23]. Samples are diluted serially and 0.1 mL is transferred onto the surface of the sheep blood agar plates (Merck, Germany). For checking of morphological properties of bacteria, we prepared the smear of each colony on a glass slide and stained it according to Gram's staining method. Optical microscopy was used for checking the bacterial morphology of the samples. All obtained strains of sheep blood agar medium were subjected to the catalase test, as described by Reiner [24]. All detected *Staphylococcus* strains were checked in terms of purity. Mannitol salt agar (MS agar, Merck, Germany) was used as the selective medium for isolating *S. aureus* strain types.

2.3. Evaluating the antibiotic susceptibility of *S. aureus* strains

Susceptibility and resistibility of the isolates found in bovine raw milk to various frequently used antibiotics were tested using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (MHA, Merck, Germany in accordance with the Clinical and Laboratory Standards Institute. The ATCC 29213 standard strain of *S. aureus* served as quality control [25]. Mueller-Hinton agar plates were inoculated with a suspension (equivalent to a 0.5 McFarland standard) of each strain.

Nine antibiotic disks, methicillin (5 µg), cefoxitin (30 µg), streptomycin (10 µg), ceftiofur (30 µg), tylosin (15 µg), penicillin G (10 U), enrofloxacin (5 µg), sulfamethoxazole-trimethoprim (25 µg), oxytetracycline (30 µg) were used for the determination of antibiotic resistance. The strategy of antibiotics selection was built on their priority, the inhibitory activity, and continual management in pretreatment for *S. aureus*-related infections in veterinary and medicine. The antimicrobial disks were put on Mueller-Hinton agar and incubated at 37 °C for 18–24 h.

2.4. Plant materials and extractions

RO, PN, PV, LU, and NS, which are used as traditional herbal therapy and have strong antibacterial properties were purchased from commercial herbal firms (Biorganix Life). They were identified and certified by the Department of Pharmacology and Toxicology, Veterinary Faculty of Ankara University. The essential oils are extracted from RO, PN, PV, LU, and NS (each 100 g) by hydro-distillation method via a Clevenger-type apparatus with 3 L distilled water for 2.5 h according to the European Pharmacopoeia (until no more essential oil was acquired). The essential oils were dehydrated over anhydrous sodium sulfate and stocked at –21 °C up to the experimental processes. The essential oil yields of the RO, PN, PV, LU, and NS were 1.9%, 1.7%, 10.2%, 0.4%, and 0.2%, respectively.

2.5. Extracts composition analyses

The analysis of the essential oils was carried out by a Thermo Finnigan (USA) Trace GC gas chromatograph equipped with a Thermo Finnigan Polaris Q ion trap–mass spectrometric detector (electronic impact, ion source temperature, analyzed mass interval and interface temperature were 70 eV, 230 °C, 50–650, 280 °C, respectively) equipped with a DB-5MS capillary column (30mm × 0.25mm i.d.; film thickness 0.25 m). Helium (purity 99.9%) was the carrier gas, at a flow rate of 1.2 mL/min. Injector and detector MS transfer line temperatures were set at 160 and 265 °C, respectively. The oven temperature was initially kept at 80 °C for 2 min, 10 °C min^{-1} to 200 °C, 6° C min^{-1} to 280 °C, and held for 35 min. The ingredients were defined by comparison of their relative retention times and mass spectra with those of standards (for the major ingredients), NIST library input of the Gas Chromatography-Mass Spectrometry system, and literature info.

2.6. Disk diffusion method

The disk diffusion method for antibacterial susceptibility testing was carried out in accordance with the Kirby-Bauer disk diffusion standard test method [26] to assess the antibacterial properties of the chosen plant extracts. By using the 0.5 McFarland test standard, bacterial suspensions of each isolate were prepared turbidometrically and measured using a spectrophotometer adjusting the concentration to 0.600 OD (450 nm) and were used for lawn MHA plates evenly via a sterile swab. The disks impregnated with the series of the chosen antibiotics and plant extracts were placed on the MHA surface where the previously prepared bacterial suspensions were spread with a cotton swab. The standard commercial antimicrobial discs methicillin (5 µg), cefoxitin (30 µg), streptomycin (10 µg), ceftiofur (30 µg), tylosin (15 µg), penicillin G (10 U), enrofloxacin (5 µg), sulfamethoxazole-trimethoprim (25 µg), and oxytetracycline (30 µg) Bioanalysis^{Ò}, Turkey), the herbal extracts, 10% DMSO as solvent of the herbal extract and sterile distilled water as a negative control embedded discs were put onto the agar plates and incubated at 37 °C for 18 to 24 h under aerobic conditions. After that, the plates were checked for inhibiting the antibiotics and extracts by measuring the diameter of the inhibition zones around the disks using a caliper and recording. The tests were repeated thrice to ensure reliability.

2.7. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by microdilution method

All isolates of *S. aureus* were analyzed for MIC via the microdilution test based on guidelines of the Clinical and Laboratory Standards Institute [25]. The solvent dimethylsulfoxide (DMSO) showed no antibacterial effect. Overnight cultures were arranged by incubating the inocula and regulated to 0.5 McFarland standard. All extracts were dissolved in 10% DMSO. After that, serial dilutions were prepared in concentrations from 256 µg/mL to 0.25 µg/mL in 96-well plates, using Mueller–Hinton broth (MHB, Becton Dickinson, Sparks, MD, USA) in 96-well microplates. The bacterial suspension (0.5 McFarland standard) was arranged from a 24 h culture plate. A hundred µL of the suspension was inoculated into each well. The last well (negative control) contained MHB alone without any black seed oil. The total volume of each well was 200 µL. The plates were incubated (at 37 °C for 18–24 h). MIC is the lowest concentration endpoint of the extracts with no observable gain in the well. The MBC endpoint is the lowest level of an extract that can eradicate more than 99.9% of the bacterial population with no observable gain on the MHA.

2.8. Statistical analysis

All assays were performed in triplicate in 3 independent tests, and the quantitative data obtained were expressed as an average of the assays. For the determination of differences ($p < 0.05$), ANOVA, followed by Tukey's test was used.

3. Results

3.1. Occurrence of *S. aureus* in raw milk dairy cows

Table 1 presents the occurrence of *S. aureus* in the milk and hospital-acquired samples. Twenty samples from 150 cows' milk were detected as positive for *S. aureus*. Additionally, 6 samples that were hospital-acquired *S. aureus* (MRSA-confirmed) were prepared for further investigations.

3.2. Antibacterial susceptibility tests (AST)

Table 2 represents the antibacterial properties of nine antibiotics against S. *aureus* isolates from raw milk dairy cows and hospital-acquired isolates. All *S. aureus* isolates from milk showed full susceptibility to cefoxitin, ceftiofur, penicillin G, and enrofloxacin while some isolates demonstrated intermediate susceptibility to methicillin, streptomycin, tylosin, and sulfamethoxazole-trimethoprim. Additionally, the highest level of resistance was determined for oxytetracycline (15% of isolates were resistant and 40% were intermediate). On the other side, all MRSA samples exhibited full resistance to methicillin and penicillin.

3.3. Chemical composition of extracts

The chemical composition of the five extracts obtained was defined by gas chromatography-mass spectrometry (GC–MS). Table 3 represents the chemical composition of extracts. The major components were p-cymene (44.13%), linalool (20.49%), and gamma-terpinene (16.42%) for RO, limonene (22.87%), beta-pinene (15.57%), delta-3-Carene (8.67%), and alpha-pinene (6.57%) for PN, carvacrol (83.5%) for LU, alpha-pinene (74.59%) and beta-pinene (8.49%) for PV, and trans-anethole (37.22%), and p-cymene (13.4%) for NS.

Table 1. Occurrence of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) in bovine raw milk.

Sample	**Number of samples**	**Number of *Staphylococcus aureus* positive samples**
Bovine raw milk	150	20
Hospital-acquired *S. aureus* (MRSA-confirmed)	6	6
Total	156	26

Table 2. Antimicrobial susceptibility patterns of *Staphylococcus aureus* isolated from raw milk dairy cows (MSSA) and hospital-acquired samples (MRSA) tested by disk diffusion method.

Antimicrobial agent	Antibiogram pattern					
	Susceptible (%)		Intermediate (%)		Resistant (%)	
	MSSA[A] (n = 20)	MRSA[B] (n = 6)	MSSA (n = 20)	MRSA (n = 6)	MSSA (n = 20)	MRSA (n = 6)
Methicillin (5 µg)	18 (90)	-	2 (10)	-	-	6 (100)
Cefoxitin (30 µg)	20 (100)	-	-	2 (33.4)	-	4 (66.6)
Streptomycin (10 µg)	19 (95)	1 (16.6)	1 (5)	-	-	5 (83.4)
Ceftiofur (30 µg)	20 (100)	1 (16.6)	-	1 (16.6)	-	4 (66.8)
Tylosin (15 µg)	18 (90)	2 (16.6)	2 (10)	2 (16.6)		2 (16.6)
Penicillin (10 U)	20 (100)	-	-	-	-	6 (100)
Enrofloxacin (5 µg)	20 (100)	2 (33.4)	-	1 (16.6)	-	3 (50)
Sulfamethoxazole-Trimethoprim (25 µg)	19 (95)	5 (83.4)	1 (5)	1 (16.6)	-	-
Oxytetracycline (30 µg)	9 (45)	-	8 (40)	1 (16.6)	3 (15)	5 (83.4)

[A]MSSA:Methicillin-sensitive *Staphylococcus aureus*, [B]MRSA: Methicillin-resistant *Staphylococcus aureus*

3.4. Antibacterial activity

The average zone of inhibition of the extracts and selected antibiotics are shown in Tables 4 and 5. The negative control group disc containing 10% DMSO (diluent of the extract) produced no zone of inhibition. In the extracts, we employed both disc and well diffusion methods to compare the results. From the five selected extracts, RO and NS exhibited significant susceptibility in both MSSA and MRSA isolates. The inhibitory effects of PN, PV, and LU were weak and not significant compared with the control group. In MSSA isolates, there was a significant difference in the inhibitory zone between disc diffusion and agar well diffusion method ($p < 0.05$) while it was not in the MRSA group. For NS, there were no significant differences in both methods in MSSA and MRSA.

3.5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Table 6 represents the resistance, MIC, and MBC value of selected extracts and antibiotics to SA bovine raw milk (MSSA) and hospital-acquired (MRSA) isolates. The MIC and MBC mean values of MSSA isolates for RO were 49.3 ± 30 µg/mL and 68 ± 39.8 µg/mL and for NS were 61.5 ± 33.2 µg/mL and 103.3 ± 60 µg/mL, respectively. These values for MRSA isolates were 85.3 ± 30.1 and 213.3 ± 60.3 for RO and 170.6 ± 60.3 and 213.3 ± 60.3 for NS, respectively. The resistant MSSA isolates with no MIC and MBC values were 40% and 35% in RO and NS and 10% and 20% in penicillin and oxytetracycline groups respectively, while 50% of MRSA isolates were resistant.

4. Discussion

S. aureus is known as one of the noteworthy reasons for food poisoning, foodborne zoonotic disease around the world resulting from the effects of staphylococcal enterotoxins (SEs) [27]. Additionally, the economic impact of this infection is considerable [28]. This study investigated the occurrence, antimicrobial activities, and resistance along with testing the antimicrobial effects

Table 3. Chemical composition of *Rosmarinus officinalis*, *Piper nigrum*, *Linum usitatissimum*, *Pistacia vera*, and *Nigella sativa* essential oils. The compounds listed show a minimum concentration of 0.2% in at least one essential oil analyzed. Values are presented as percentages of normalized peak areas without correction factors.

Compounds	*Rosmarinus officinalis*	*Piper nigrum*	*Linum usitatissimum*	*Pistacia vera*	*Nigella sativa*
n-nonane	-	-	-	-	1.60%
3-Methyl nonane	-	-	-	-	0.40%
1,3,5-Trimethyl benzene	-	-	-	-	0.40%
n-Decane	-	-	-	-	0.30%
1-Methyl-3-propyl benzene	-	-	-	-	0.60%
1-Ethyl-2,3-dimethyl benzene	-	-	-	-	0.30%
n-Tetradecane	-	-	-	-	0.20%
n-Hexadecane	-	-	-	-	0.30%
Alfa-Thujene	0.34%	0.31%	-		2.30%
alfa-Pinene	2.81%	6.57%	-	74.59%	1.10%
Sabinene	-	0.79%	-	-	1.50%
Beta-Pinene	3.59%	15.57%	-	8.49%	1.40%
Myrcene	1.78%	2.45%	-	-	0.35%
alfa-Phellandrene	-	1.76%	-	-	0.51%
p-Cymene	44.13%	0.69%	0.49%	-	13.40%
Limonene	-	22.87%	-	1.30%	4.28%
gamma-Terpinene	16.42%	-	0.43%	-	0.54%
Fenchone	-	-	-	-	1.08%
Dihydrocarvone	-	-	-	-	0.25%
Carvone	-	-	-	-	3.95%
Thymoquinone	-	-	-	-	0.56%
Terpinen-4-ol	0.79%	0.68%	-	-	0.65%
Carvacrol	0.12%	-	83.50%	0.06%	1.54%
Longifolene	-	-	-	-	0.67%
Estragole	-	-	-	-	1.82%
Anisaldehyde	-	-	-	-	1.60%
Trans-Anethole	-	-	-	-	37.22%
Myristicin	-	-	-	-	1.36%
Dill apiole	-	-	-	-	1.71%
Apiole	-	-	-	-	0.97%
camphene	1.39%	0.19%	1.39%	-	-
alfa-terpinene	-	0.13%	0.18%	-	-
delta-3-carene	-	8.67%	-	-	-
beta-phellandrene	-	0.19%	0.12%	-	-
gama-terpinene	-	0.11%	-	-	-
terpinolene	-	0.48%	-	-	-
linalool	20.49%	3.84%	0.09%	-	-
alfa-terpineol	0.42%	0.76%	-	0.07%	-
eugenol	-	0.46%	0.19%	-	-
alfa-copaene	-	0.65%	-	-	-

Table 3. (Continued).

camphre	1.73%	-	-	-	-
Borneol	0.97%	-	-	0.12%	-
Thymol	1.77%	-	1.20%	-	-
beta-Caryophyllene	0.13%	-	-	-	-
Alfa-terpinolene	-	-	0.14%	-	-
carvacrol methyl ether	-	-	0.11%	-	-
trans-caryophyllene	-	-	0.42%	-	-
alfa-bergamotene	-	-	0.13%	-	-
neryl acetone	-	-	0.08%	-	-
beta-Bisabolene	-	-	2.69%	-	-
trans-pinocarveol	-	-	-	1.14%	-
trans-verbenol	-	-	-	2.98%	-

Table 4. Mean inhibition zone (mm) of *Rosmarinus officinalis*, *Piper nigrum*, *Linum usitatissimum*, *Pistacia vera*, and *Nigella sativa* on methicillin-sensitive *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* isolates

Inhibition diameters of extracts (Disc and well diffusion method) (zones in mm)				
Extracts	**MSSA[A]**		**MRSA[B]**	
	Disc diffusion (Mean ± SD)	Well diffusion (Mean ± SD)	Disc diffusion (Mean ± SD)	Well diffusion (Mean ± SD)
Rosmarinus officinalis	13.9 ± 6.1	9.9 ± 4.2	9.1 ± 2.1	13.5 ±5 .8
Piper Nigrum	0.5 ± 1.6	1.6 ± 4.8	NA	NA
Pistacia Vera	1.5 ± 3	2.1 ± 4.3	NA	NA
Linum Usitatissimum	0.9 ± 1.9	0.9 ± 2.2	NA	NA
Nigella Sativa	48.4 ± 9.6	48.2 ± 10.4	39.3 ± 14.5	35.8 ± 13.3

[A]MSSA:Methicillin-sensitive *Staphylococcus aureus*, [B]MRSA: Methicillin-resistant *Staphylococcus aureus*

of some herbal extracts to be an alternative way. The antimicrobial activities of herbal extracts were investigated in all isolated *S. aureus* from raw milk samples and hospital-acquired MRSA-confirmed isolates to obtain the antimicrobial resistance that helps prevent and control *S. aureus* infections in cows and protects humans.

In this work, although all bovine milk samples were obtained from dairy, nonmastitis, and nonmedicated cows, 13.3% of samples were found positive for *S. aureus*. This result corresponds to the isolation range of SA in some other studies from 10% to 50% [29–31]. The prevalence of SA in dairy cows with mastitis is normally more than 40% [32]. However, this contamination can be detectable in nonmastitis dairy cows. Overall, our study indicates that SA is a common bacteria that can be detected in nonmastitis dairy cows. Moreover, further research to investigate the prevalence and occurrence of SA and control methods in raw milk should be considered.

Recently, SA-related infections in animals and humans have become a great public health concern [33]. In this present work, all SA isolated from raw milk dairy cows are susceptible to cefoxitin, ceftiofur, penicillin G, and enrofloxacin, however, some antibiotic resistances were detected with high resistance to oxytetracycline that was approximately similar to previous studies [34]. Moreover, the 6 MRSA-confirmed hospitalized isolates displayed the highest resistance amount to the antibiotics with a the rate of 100% resistance to penicillin, and 83.4% to oxytetracycline and streptomycin. It was notable that oxytetracycline and tylosin are used at dairy farms and veterinary applications in Turkey. Additionally, penicillin and aminoglycosides are still the first-line treatment protocols in SA infections in Turkey. The infections of animals/humans with such resistance isolates can be an inevitable concern. It means that appropriate management and proper antibiotic prescriptions in food-producing livestock is very crucial

Table 5. Mean inhibition zone (mm) of antibiotics on methicillin-sensitive *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* isolates.

Inhibition diameters of antibiotic (Disk diffusion method) (zones in mm)		
Antibiotic	**MSSA[A]**	**MRSA[B]**
Methicillin (MET), 5 µg	22.5 ± 4.9	NA
Cefoxitin (FOX), 30 µg	33.2 ± 8.8	7.6 ± 8.5
Streptomycin (S), 10 µg	25.7 ± 3.8	4.6 ± 7.6
Ceftiofur (FUX), 30 µg	33.3 ± 9.1	7 ± 11
Tylosin (TY), 15 µg	26.3 ± 4.1	20 ± 4.7
Penicillin G (P), 10 U	43.8 ± 5.7	12.3 ± 6.2
Enrofloxacin (ENR), 5 µg	35.4 ± 5.9	20.8 ± 11.2
Sulfamethoxazole-Trimethoprim (SXT), 25 µg	32.9 ± 8.3	29.3 ±7.5
Oxytetracycline (T), 30 µg	25.4 ± 12.6	7.8 ±10

[A]MSSA:Methicillin-sensitive *Staphylococcus aureus*,
[B]MRSA: Methicillin-resistant *Staphylococcus aureus*

Table 6. Resistance, minimum inhibitory concentration, and minimum bactericidal concentration of *Rosmarinus officinalis*, *Nigella sativa*, penicillin, and oxytetracycline against methicillin-sensitive *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* isolates.

Herbs	**MSSA[A]**				**MRSA[B]**			
	n	**R[C] (%)**	**MIC[D] (µg/mL)**	**MBC[E] (µg/mL)**	**n**	**R (%)**	**MIC (µg/mL)**	**MBC (µg/mL)**
Rosmarinus officinalis	20	8 (40%)	49.3 ± 30	68 ± 39.8	6	3 (50%)	85.3 ± 30.1	213.3 ± 60.3
Nigella sativa	20	7 (35%)	61.5 ± 33.2	103.3 ± 60	6	3 (50%)	170.6 ± 60.3	213.3 ± 60.3
Penicillin	20	2 (10%)	0.4 ± 0.2	1.5 ± 1.9	6	1 (16.6%)	6 ± 2.8	39.2 ± 25.3
Oxytetracycline	20	4 (20%)	7.7 ± 4.4	76 ± 59.4	6	2 (33%)	28 ± 22.9	192 ± 64

[A]MSSA: Methicillin-sensitive *Staphylococcus aureus*, [B]MRSA: Methicillin-resistant *Staphylococcus aureus*, [C]R: Resistance, [D]MIC: Minimum inhibitory concentration, [E]MBC: Minimum bactericidal concentration

to prevent antibiotic-resistant SA [35]. André et al. [36] showed the resistance of MSSA and MRSA to beta-lactams, macrolides, and aminoglycosides, which is similar to our results in tylosin (macrolides) and streptomycin (aminoglycoside), however, we could not see resistance to penicillin (beta-lactam). For decades, the methicillin-susceptible *S. aureus* (MSSA) and its enterotoxins have had food safety importance. Thus, contamination of raw food by SA should always be monitored and controlled to prevent the possible danger of MRSA infections in humans whose antibiotic resistance is significantly higher [37]. With increasing antimicrobial resistance to different antibiotics that are routinely used in veterinary and human and the development of multidrug-resistant bacterial strains, investigating some alternative effective therapeutic ways like herbal medicine should be considered precisely [38].

In this study, the potential antibacterial effects of five herbs in SA isolates from raw milk dairy cows and MRSA-confirmed hospitalized samples were examined. Notably, more than 90% of raw milk strains were susceptible to methicillin while all hospitalized strains were resistant to it. The extracts used in this work exhibited divergent antibacterial activities against samples. Among the extracts, RO and NS were the most effective in both strains (bovine raw milk and hospitalized). The MIC and MBC values for RO were 49.3 µg/mL and 68 µg/mL for raw milk strains and 85.3 µg/mL and 213.3 µg/mL for MRSA strains. These values for NS were 61.5 µg/mL and 103.3 µg/mL for milk strains and 170.6 µg/mL and 213.3 µg/mL for MRSA strains. It is also considered that these two extracts demonstrated a significant difference in their antibacterial effect in both strains in this study ($p < 0.05$).

The antimicrobial properties of herbal products against SA have been investigated in many studies. The antimicrobial effects of RO on SA have been reported in some works [39,40]. Andrade et al. [41] displayed antibacterial effects against *S. aureus, Listeria monocytogenes,* and *Clostridium perfringens*. Mouwakeh et al. [42] reported the antibacterial effects of the NS essential oil and its bioactive ingredients in MRSA strains. Other studies confirmed the same inhibitory effects of NS in SA infections [43, 44].

Dairy sectors in Mediterranean and Middle East countries with hot climates always suffer from the hazard of raw milk and derived products, contaminations, and animal-related infections [45]. Dupas et al. [46] reviewed the antimicrobial and antioxidant activity of different plants in dairy processing. Other reports confirmed the antibacterial properties of RO on dairy products [47, 48]. Besides, for NS, previous studies show that this herb has antibacterial effects on SA strains. Rakhshandeh et al. [49] showed the significant in vitro and in vivo inhibitory effects of the NS on causative microorganisms in dairy cow mastitis compared with routine drugs and their positive effects on disease healing. It could suggest that NS could be a proper candidate as a natural antibiotic in food dietary systems [50].

We could not find the antimicrobial activities of PN, LU, and PV, in bovine raw milk and hospitalized-associated strains. Although in some previous studies, these herbs have positive effects on SA strain [51–53], our results showed that their inhibitory effects did not have significant differences with control groups. In a study, El Feghali et al. [54] reported that the methanolic extract of LU can enhance bacterial growth when the level of the extract was 0.15mg/mL or more. In another study, the methanolic extract of PN has shown no activity against *S. aureus* [55]. However, it should be considered that antibacterial activities and structural composition of herbs may be differently based on geographical location cultivation and harvesting methods, and commercializing processes. Additionally, different factors such as extraction methods, active components in extracts, and different bacterial strains might lead to different results from previous studies. A comparison of the results in other works could be a complicated challenge due to the absence of a standard method for considering the antibacterial properties of herbal compounds such as oils gained from various herbs [56]. In this study, the results showed that RO and NS have positive antibacterial effects on SA strain obtained for nonmastitis bovine raw milk and hospital-acquired MRSA-confirmed strains, which were resistant to some antibiotics.

5. Conclusion

In conclusion, the essential oils from the RO and NS were found to possess good antibacterial activities in MSSA and MRSA strains in vitro and might be good candidates as preservatives in the dairy sector and in humans. The finding of the current study indicates that further investigations for in vivo studies of the antibacterial effects of these herbs should be performed. Based on the results, it seems that these herbs could be evaluated as innovative therapeutic alternatives in food and drug industries particularly against staphylococcal infections.

Author's contributions

Conceptualization, funding acquisition, methodology, investigation, formal analysis, visualization, collection of samples: R.Ebrahimi Hariry, A.Filazi
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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Informed consent

This article does not contain any studies with human participants or animals performed by any of the authors.

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