Research Article

In Vitro Essential Oils (*Thymus vulgaris* and *Mentha piperita*) as an Antibacterial Activity Against *Staphylococcus aureus* Isolated from Ovine Mastitis

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Abstract | This study investigated the antibacterial potential of *Thymus vulgaris* and *Mentha piperita* essential oils against *Staphylococcus aureus* isolated from the milk of lactating Awassi ewes in Baghdad. Milk samples were collected and examined by culturing, microscopic analysis, and biochemical tests for bacterial confirmation. Essential oils were extracted using hydrodistillation and analyzed by GC-MS to determine phytochemical composition. The pharmacodynamic activity was evaluated by minimum inhibitory concentration (MIC) determination, well diffusion assays, and time-kill kinetics, compared with amoxicillin. *T. vulgaris* oil showed the lowest MIC (3.125 µg/ml) compared with *M. piperita* (6.25 µg/ml) and amoxicillin (25 µg/ml). Antibacterial activity was quantitatively notable producing the largest zones. The post-antibiotic effect (PAE) was longest for *T. vulgaris* (MIC: 7.1 ±0.38 h, sub-MIC: 6.0 ±0.25 h), followed by *M. piperita* (MIC: 5.8 ±0.23 h, sub-MIC: 4.4 ±0.19 h), and amoxicillin (MIC: 3.8 ±0.17 h, sub-MIC: 3.1 ±0.11 h). These quantitative differences clearly demonstrate that *T. vulgaris* exhibits the strongest and most prolonged antibacterial effect against *S. aureus* from mastitic ewes, followed by *M. piperita* and amoxicillin. In conclusion, this study showed that *T. vulgaris* and *M. piperita* oils possess promising natural antibacterial properties and may serve as alternative or complementary agents for controlling *S. aureus* infections in ovine mastitis.

Keywords | *Thymus vulgaris*, *Mentha piperita*, Antibacterial, *Staphylococcus aureus*, Mastitis, Ovine

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INTRODUCTION

Mastitis in sheep is considered a major concern due to its detrimental effects on animal welfare, the substantial economic losses associated with reduced milk yield and quality, and its potential risks to human health. Because sheep milk is predominantly utilized in traditional cheese production, preventing mastitis and ensuring optimal hygienic quality of milk are fundamental goals in dairy-sheep management and in systems aimed at producing both milk and lambs. Antimicrobial agents continue to play a key role in mastitis prevention and control programs (Yass *et al*., 1992; Saleem *et al*., 2021).

Antimicrobial resistance poses a serious challenge in the treatment of mastitis, often leading to treatment failure and chronic infections. In many regions, pathogens such as *Staphylococcus aureus* isolated from mastitic milk show high resistance rates to commonly used antibiotics, including penicillin and ampicillin. As resistance spreads, traditional

antibiotic therapy becomes increasingly unreliable, highlighting the urgent need for alternative control strategies and stricter antibiotic stewardship in dairy farms (Sharifi *et al*., 2023).

Staphylococcus aureus is a common commensal organism in many mammalian species. However, under favorable conditions, it can act as an opportunistic pathogen, causing infections that range from mild to severe, including mastitis. Consequently, *S. aureus* is among the most prevalent pathogens associated with both clinical and subclinical ovine mastitis worldwide (Safana, 2003; Gharaibeh *et al*., 2025).

Essential oils (EOs) are composed of a complex mixture of volatile molecules, which are specific to each plant, including their range of bioactivities; these molecules include alkaloids, monoterpenes, carotenoids, flavonoids, isoflavones, phenolic acids, oxygen-containing and non-oxygenated terpene hydrocarbons, and aldehydes (Maleš et al ., 2022). The mechanisms of antimicrobial activity of EOs include the degradation of the cell wall and cytoplasmic membrane, cytoplasm coagulation, the inhibition of toxic bacterial metabolites, and the inhibition of the bacterial efflux system. However, the efficacy of antimicrobial activity can vary depending on the pathogen and the composition of the EO (Zhang *et al*., 2018; Salman *et al*., 2024).

This study aims to investigate the *in vitro* effectiveness of *Thymus vulgaris* and *Mentha piperita* essential oils as antibacterial agents against *Staphylococcus aureus* isolated from mastitic sheep.

MATERIALS AND METHODS

Bacterial isolation and identification

Milk sampling

Twenty-five lactating Awassi ewes (>2 years) in Latifa, Baghdad were sampled for *S. aureus* isolation. From each, 10 ml milk was collected based on clinical signs of mastitis characterized by hard udder by palpation, pain during milking in addition restricted movement, congested mucous membranes, and watery milk appearance and the California Mastitis Test was performed using 5 ml milk mixed with 5 ml reagent on a plastic paddle (Al-Rasheed *et al*., 2022).

Bacteriological examination

Culturing

Milk samples were cultured on nutrient agar, and suspected *S. aureus* colonies were purified and confirmed by hemolysis on blood agar and growth on mannitol salt agar (Markey et al., 2014).

Microscopic examination

A single blood agar colony was gram-stained, heat-fixed, and examined under a light microscope (Olympus, Japan) (100x) to observe bacterial cells (Markey et al., 2014).

Biochemical tests

All test were done according to (Alshammary and Galfoori, 2013).

Mannitol salt agar

A suspected *S. aureus* colony from nutrient agar was inoculated onto mannitol salt agar, a selective and biochemical medium.

Coagulase test

For the coagulase test, a colony was mixed with rabbit plasma on a slide; agglutination within 10 seconds indicated a positive result.

Catalase test

A blood agar colony was mixed with 3% H_2O_2 on a slide; bubble formation indicated a positive catalase reaction.

Plant extracts preparation

T. vulgaris and *M. piperita* plant materials were obtained from a certified herbal supplier in Baghdad, where each batch was accompanied by information on origin, harvest period, and handling conditions. The botanical identity of both plants was confirmed by a specialist in plant taxonomy at the College of Agriculture/University of Baghdad through morphological. Both plants were identified and classified by the Al-Razi Center for Alternative Medicine under reference numbers 230 and 231, respectively, on 9/9/2025.

Essential oil extraction

T. vulgaris and *M. piperita* oils were extracted from 100 g of dried leaves using hydrodistillation with a Clevenger (Jeulin, France) unit, mixing with 1000 mL water in a 2 L flask and heating at 100 °C (El-Kharraf *et al*., 2020; Kadhim, 2025).

Analysis of essential oil by gas chromatography–mass spectrometry

GC/MS was used to analyze *T. vulgaris* and *M. piperita* essential oils for phytochemicals with therapeutic effects. Samples (1 µL) were injected using a Thermo Trace GC Ultra/TSQ Quantum GC-MS with an Agilent HP-5ms column. The phytochemical analysis was performed using an Agilent HP-5ms Ultra Inert capillary column (30 m × 0.25 µm film thickness). The temperature program consisted of four ramps: ramp 1 at 60 °C with a 3-minute hold; ramp 2 from 60–180 °C with a 7-minute hold; ramp 3 from 180–280 °C with an 8-minute hold; and ramp 4 at 280 °C with a 3-minute hold. Operating conditions included helium

(99.99%) as the carrier gas, with both injector and detector temperatures set to 250 °C. The chemical constituents of the thyme and peppermint essential oils were identified by comparing the obtained GC–MS spectra and retention times with those in the NIST database (Yassin *et al.*, 2020; Al-Tememmi *et al.*, 2025).

Determination of the MIC of *T. vulgaris* and *M. piperita* oil extract

The micro-dilution assay (Veiga *et al.*, 2019) was performed in 96-well plates with Mueller-Hinton broth. Serial dilutions of *T. vulgaris* and *M. piperita* oils (100–0.781 µg/ml) and amoxicillin (amoxicillin salt, USA) (µg/ml) were prepared across wells. Each well received *S. aureus* inoculum (5×10^5 cfu/ml), while column 12 served as the negative control with broth only. Plates were incubated at 37 °C for 24 h, then treated with resazurin dye to detect bacterial growth (Najem and Abed, 2017).

Antibacterial activity

Preparation of different concentration of plant extract

Stock solutions were made by dissolving 1 g of oil in few drops of DMSO (0.20 µm filtered), then diluted with phosphate buffer to the concentration 1, 2, 4, 6, and 8 µg/ml. The method of oil homogenisation by vortex to ensure uniform exposure. The final concentration of DMSO in the medium was controlled, and a separate DMSO control was included to rule out any inherent inhibitory or toxic effects.

Sensitivity test

The antibacterial activity of *T. vulgaris* and *M. piperita* was tested using a well diffusion assay. *S. aureus* (5×10^5 CFU/ml) was mixed into Mueller-Hinton agar, poured into plates, and wells were filled with extract concentrations (1–8 µg/ml). After 2 h diffusion, plates were incubated at 37 °C for 24 h, and inhibition zones were measured (Alsterholm *et al.*, 2010; Zhang *et al.*, 2017).

Time kill curve kinetics

The time-kill and post-antibacterial effects of *T. vulgaris*, *M. piperita* oil, and amoxicillin against *S. aureus* were evaluated following NCCLS guidelines. Bacterial suspensions (10^6 CFU/ml) were exposed to 0.25–4× MIC concentrations and incubated at 37 °C for 24 h. Colony counts were taken at 0, 2, 4, 8, 12, and 24 h, and the area under the killing curve (AUC) was calculated.

$$AUC_{Kill} = \Sigma\left(\frac{\log(Cn)+\log(Cn+1)}{2}.\ (\Delta t)\right)$$

Post antibacterial effect (PAE)

The post-antibacterial effect (PAE) and post-antibacterial sub-MIC effect (PA-SME) of *T. vulgaris*, *M. piperita* oil, and amoxicillin were measured by exposing *S. aureus* to MIC and 0.5× MIC for 90 min, removing the agents, washing, and resuspending in fresh broth. Cultures were incubated at 37 °C, and Spectrophotometer (Scot – Tech, Germany) OD_{600} was monitored for 28 h. PAE/PA-SME was calculated as the delay in reaching 50% of the control OD (Odenholt, 1993; Miles *et al.*, 1938; Chiou, 1978).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS, 2019) was used to analyze the effects of different treatments and time periods on the study parameters. Means were compared using two-way ANOVA followed by the Least Significant Difference (LSD) test to determine significant differences.

RESULTS

Isolation of *S. aureus*

The results of isolation and culturing of bacteria including biochemical tests showed that a total of 25 milk samples which were collected from ewes were diagnosed with clinical mastitis based on clinical signs in different local breeders in Latifiya district/ Baghdad governorate, 18 specimens, exact number of isolates 3 specimens of each test indicated positive results for the existence of *S. aureus* with percentage 72% (Table 1).

Table 1: Prevalence of *S. aureus* in collected samples.

Microorganism	No. of sample	Percentage %
Staphylococcus aureus	18	72
Other	7	28
Total	25	100

Identification of *S. aureus*

Culturing milk samples on blood agar revealed β-hemolysis, characterized by clear zones surrounding convex, round, mucoid, greyish-yellow colonies (Figure 1). All *Staphylococcus aureus* isolates fermented mannitol, producing acidic byproducts that changed the phenol red indicator to yellow (Figure 2). Microscopic examination showed Gram-positive cocci, violet in color, arranged in clusters (Figure 3).

The catalase test confirmed the presence of *S. aureus*, as air bubbles appeared immediately after adding 3% hydrogen peroxide (H_2O_2) to the colonies. This reaction occurs because *Staphylococci* produce the enzyme catalase, which breaks down hydrogen peroxide into water and oxygen (Figure 4).

The coagulase test was used to differentiate pathogenic from non-pathogenic staphylococci. Production of coagulase,

a hallmark of pathogenic *S. aureus*, was confirmed by the formation of clumps of agglutinated particles within 90 seconds after mixing the isolates with rabbit plasma (Figure 5).

Figure 1: *S. aureus* on blood agar.

Figure 2: *S. aureus* on mannitol salt agar.

Extraction of *T. vulgaris* and *M. piperita* oil

Thymus vulgaris and *Mentha piperita* oils were extracted by steam distillation, and their yields were calculated using the following equation.

$$\text{Yield (\%)} = \text{EO (g)/Dry matter (g)} \times 100$$

The yield of *T. vulgaris* essential oil was 1.2% (v/w) from 100 g of dried herb, producing a pale-yellow oil with a strong spicy odor, while *M. piperita* yielded 0.5% (v/w) of a colorless to light yellow greenish oily with menthol odor (Table 2).

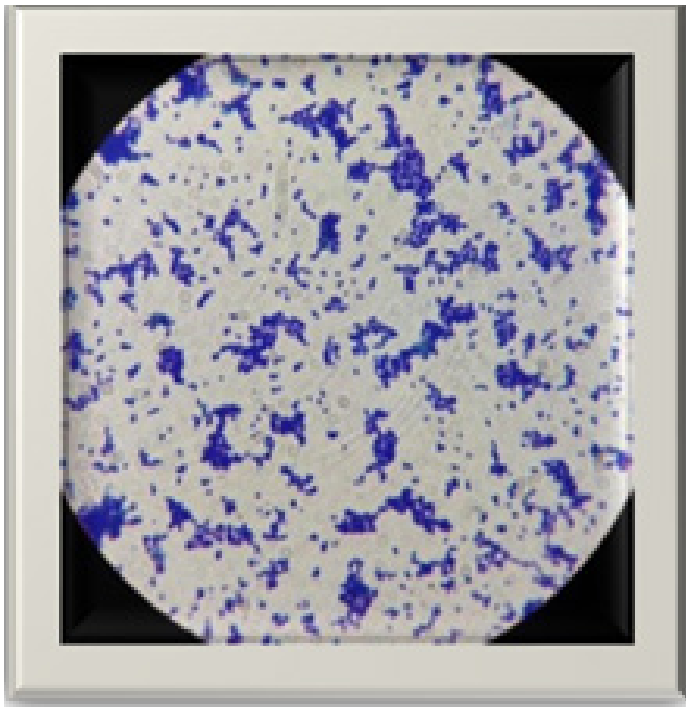


Figure 3: Gram staining of *Staphylococci* under the light microscope (100 X).

Figure 4: Catalase test.

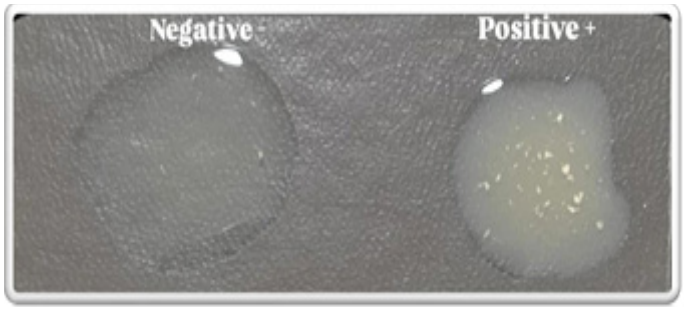


Figure 5: Coagulase test.

Table 2: Yields of the essential oils extract.

Essential oils	Yield (%)	Oil specifications
T. vulgaris oil	1.20	Pale yellow color and a strong spicy odor
M. piperita oil	0.5	Light yellow greenish oily with menthol odor

Analysis of *T. vulgaris* and *M. piperita* oil extract by chromatography-mass spectrometry (GC-MS)

Using GC/MS analysis showed that *T. vulgaris* oil was mainly composed of thymol (78.49%), carvacrol (6.18%), caryophyllene (3.89%), cymene (3.48%), γ-terpinene (1.76%), and thymoquinone (1.35%) (Table 3, Figure 6). While *M. piperita* oil contained menthol (31.3%), menthone (23.2%), cyclohexene (14.97%), eucalyptol (10.39%), menthyl acetate (8.19%), caryophyllene (3.95%) and β-pinene (3.70%), (Table 4, Figure 7).

Table 3: Analysis of the phytochemicals in *T. vulgaris* essential oil.

Compounds	Retention time	Percentage of total
Thymol	12.415	78.49
Carvacrol	23.908	6.18
Caryophyllene	14.536	3.86
Cymene	6.711	3.48
Gamma.-Terpinene	7.335	1.76
Thymoquinone	11.342	1.35
Olean-12-ene	28.704	1.30
Terpinen-4-ol	9.862	0.94
Hydrazinecarboxylic acid	8.243	0.78
Diethyl sulfate	7.611	0.72

Sensitivity and antibacterial activity

The results of MIC showed that *T. vulgaris* oil extract relatively had low MIC (3.125 µg/ml) against *S. aureus* compare to (6.25 µg/ml) and (25 µg/ml) for *M. piperita* oil and amoxicillin respectively (Figure 8). The MIC was determined based on the presence or absence of purple color; the lowest concentration that showed no color development was considered the MIC.

Different concentrations of *Thymus vulgaris* and *Mentha piperita* oils were tested using the agar well diffusion assay, producing varying degrees of inhibition against *Staphylococcus aureus*. The size of the inhibition zones increased proportionally with higher concentrations of the oil extracts (Table 5, Figure 9).

Table 4: Analysis of the phytochemicals in *M. piperita* essential oil.

Compounds	Retention time	Percentage of total
Menthol	9.440	31.30
Menthone	11.830	23.20
Cyclohexene	7.603	14.97
Eucalyptol	5.231	10.39
Menthyl acetate	10.018	8.19
Caryophyllene	12.485	3.95
Beta.-Pinene	4.392	3.70
Pulegone	9.100	1.83
Gamma-Terpinene	5.638	0.60
1,6-Octadien-3-ol, 3,7-dimethyl	6.443	0.57
Beta.-Farnesene	12.961	0.53

Pharmacodynamic analysis

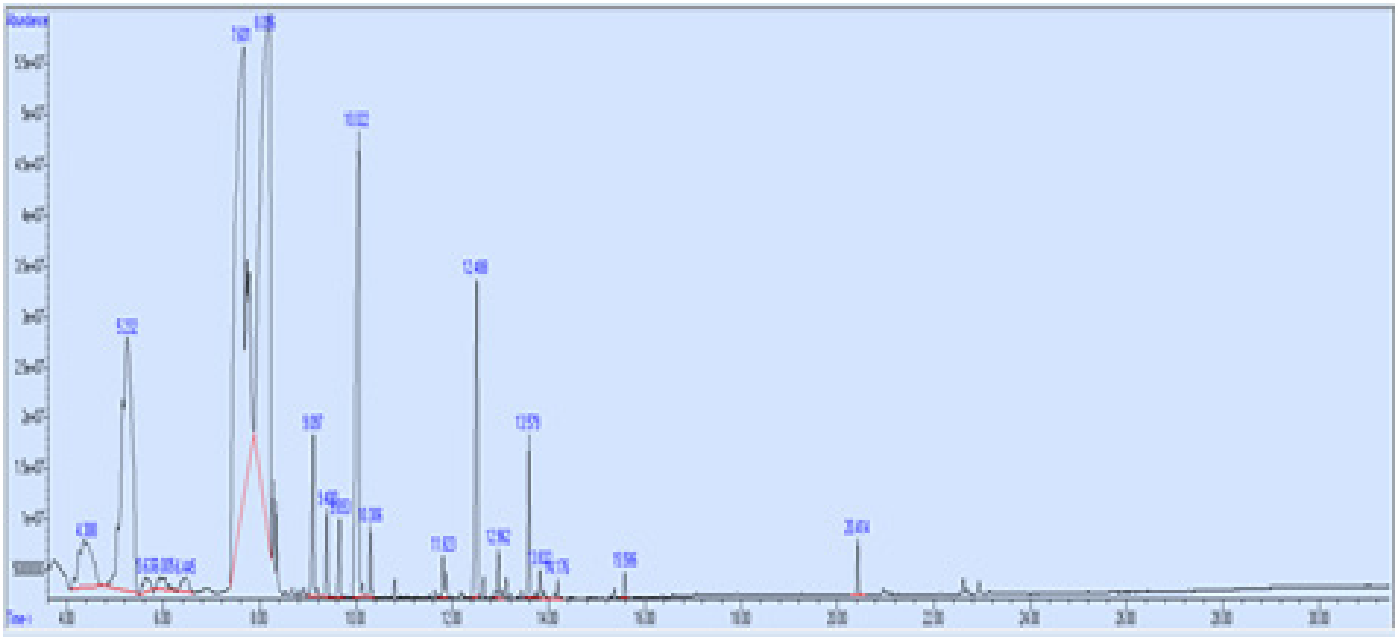


Figure 6: Phytochemicals analysis of *T. vulgaris* essential oil by GC-MS.

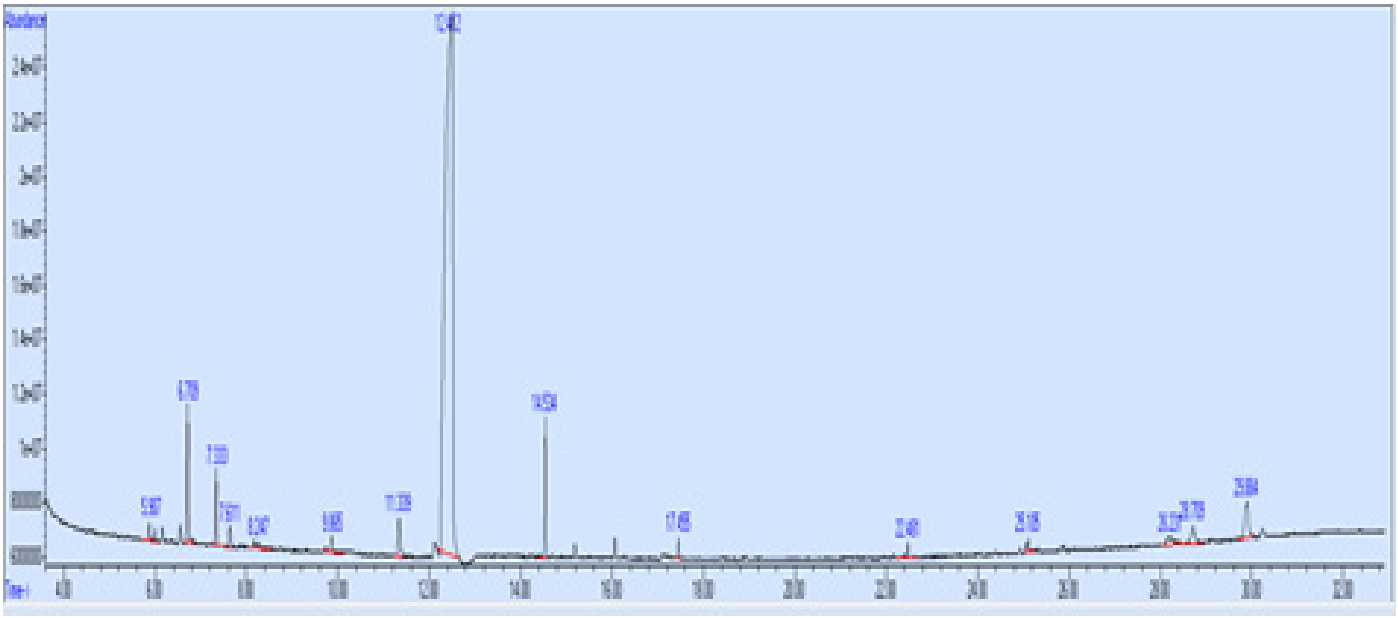


Figure 7: Phytochemicals analysis of *M. piperita* essential oil by GC-MS.

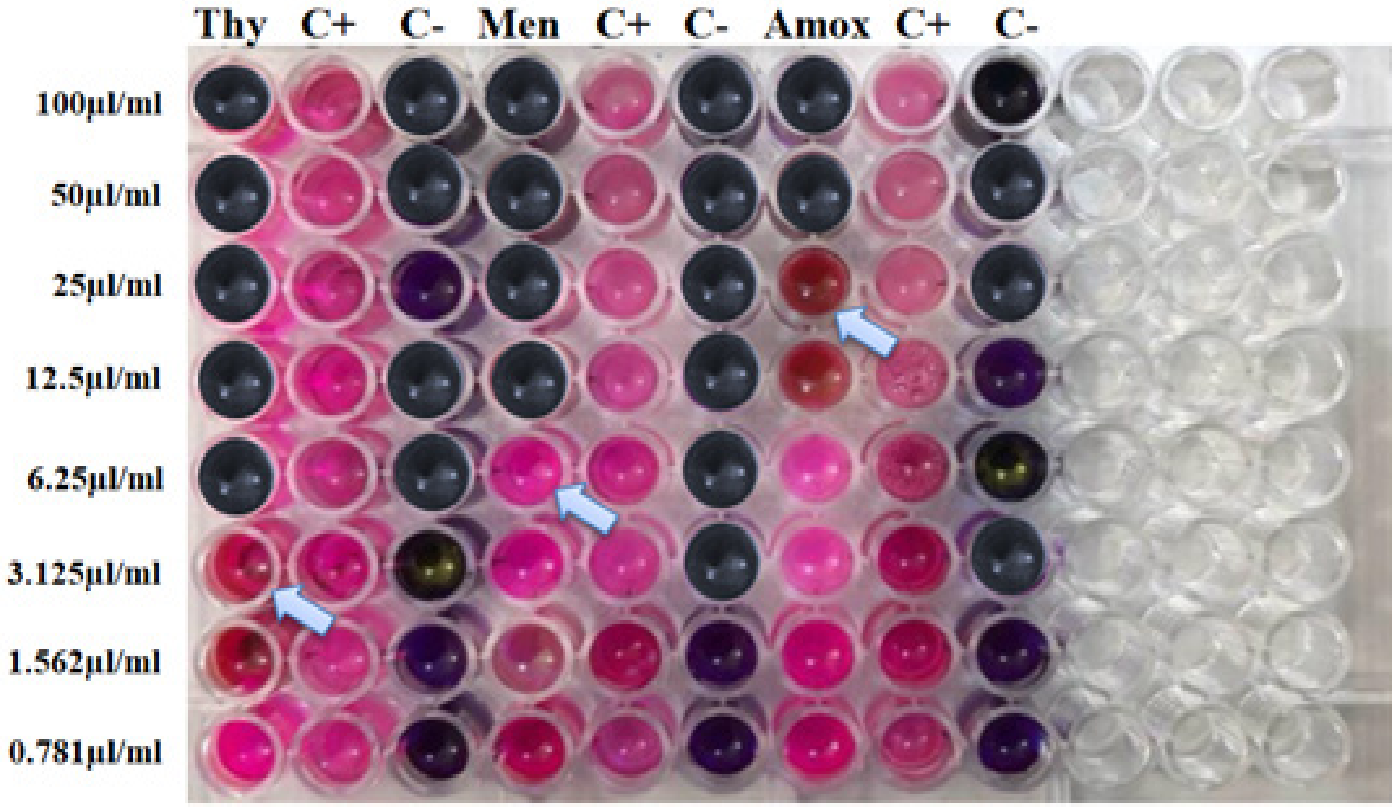
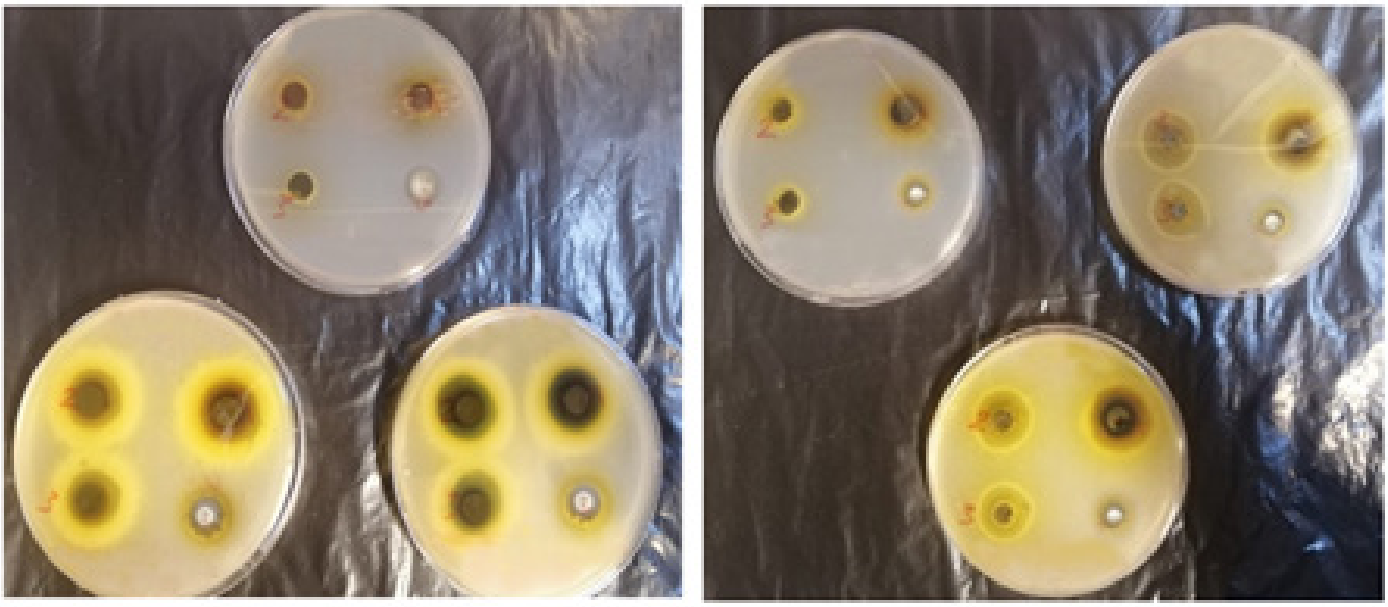


Figure 8: Minimum inhibitory concentration of *T. vulgaris and M. piperita oil* and Amoxicillin against *S. aureus* Isolates {purple (no growth) pink (growth)}.

Table 5: Inhibitory zone of *T. vulgaris and M. piperita* oil antibacterial efficacy at various concentration against *S. aureus.*

Concentration (µg/ml)/ Groups	Zone of inhibition (mm)				
	1.0 µg	2.0 µg	4.0 µg	6.0 µg	8.0 µg
T. vulgaris oil	19 ±1.26^{Ad}	22.2 ±1.56^{Ac}	26 ±2.15^{Ab}	27 ±2.41^{Ab}	29.8 ±2.63^{Aa}
M. piperita oil	18 ±0.76^{Ac}	20.5 ±1.07^{Ab}	22 ±0.94^{Bab}	23 ±1.57^{Ba}	24.5 ±2.19^{Ba}

LSD: 2.071; Means having with the different capital letters in same column and small letters in same row differed significantly (P≤0.05). Values are mean ± SD, n = 5.



A. Sensitivity of *S. aureus* to different concentrations of *T. vulgaris* oil

B. Sensitivity of *S. aureus* to different concentrations of *M. piperita* oil

Figure 9: Sensitivity of *S. aureus* to different concentrations of *T. vulgaris* (A) and *M. piperita* oil (B).

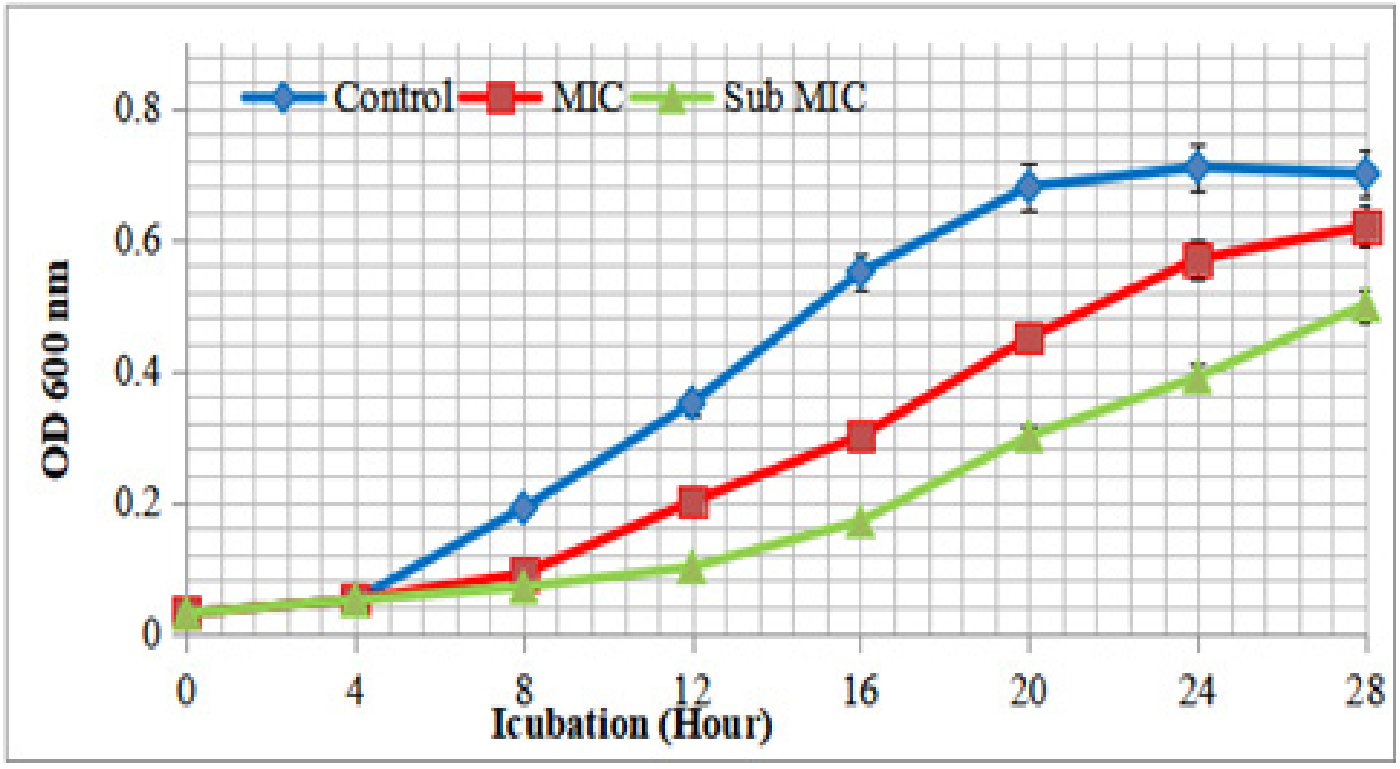


Figure 10: Post-antibacterial effect and post-antibacterial sub-MIC effect of *T. vulgaris* oil against *S. aureus.*

Post antibiotic effect

As shown in Figures 10–12, the post-antibiotic effect (PAE) of *T. vulgaris* and *M. piperita* oils was evaluated based on the time required for treated cultures to reach 50% of the OD_{max} of the control. Exposure of *S. aureus* to MIC and sub-MIC concentrations of the oils significantly ($P < 0.05$) increased the regrowth time. The PAE times recorded after removal of *T. vulgaris* oil and *M. piperita* oil were 7.1 ± 0.38, 6.0 ± 0.25, 5.8 ± 0.23, and 4.4 ± 0.19 hours, respectively, which were higher than those observed for amoxicillin-treated cultures (3.8 ± 0.17 and 3.1 ± 0.11 hours) (Table 6).

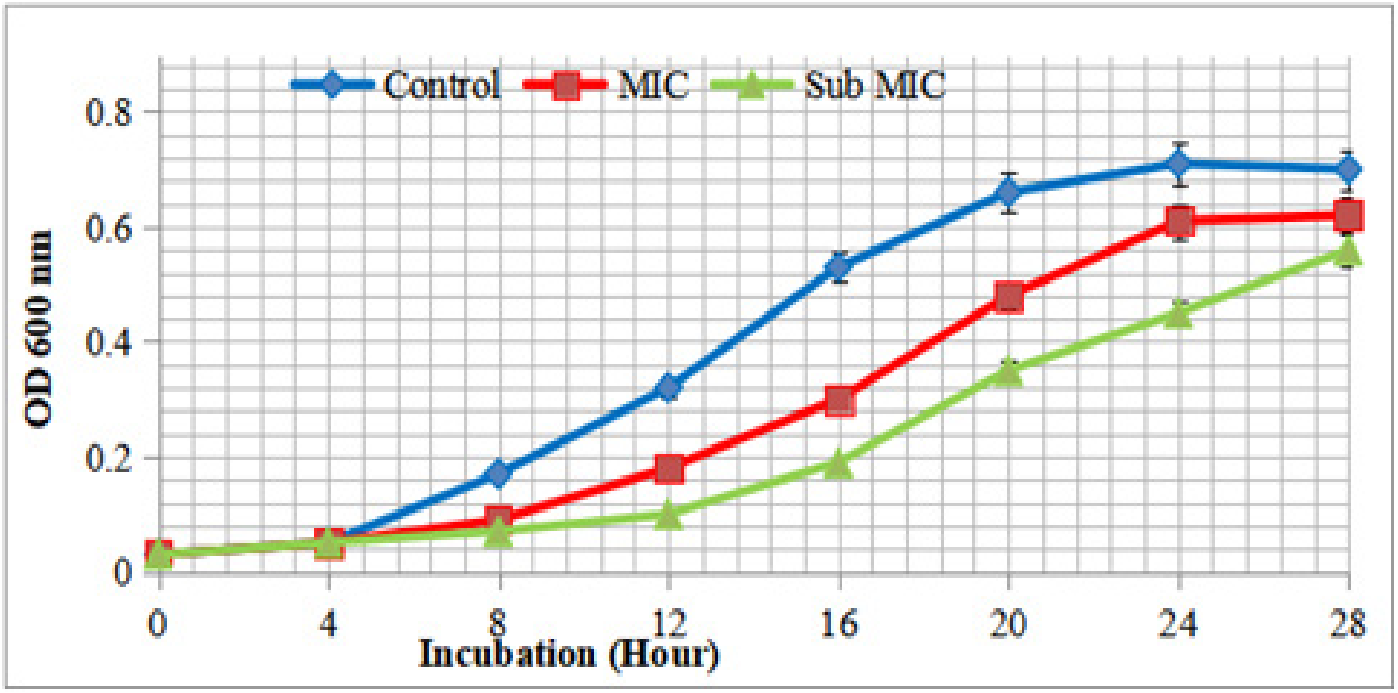


Figure 11: Post-antibacterial effect and post-antibacterial sub-MIC effect of *M. piperita* oil against *S. aureus.*

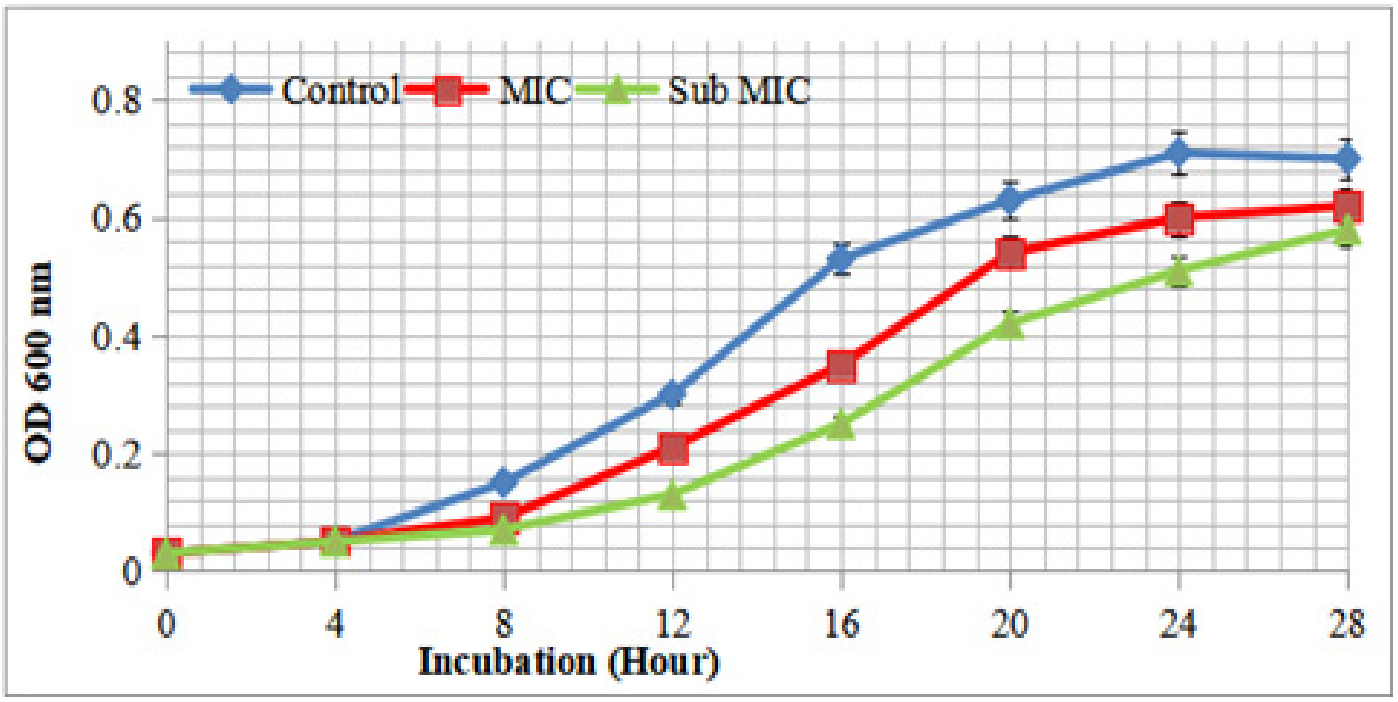


Figure 12: Post-antibacterial effect and post- antibacterial sub-MIC effect of amoxicillin against *S. aureus.*

Table 6: Post Antibacterial duration of *T. vulgaris* and *M. piperita* oil and amoxicillin against *S. aureus.*

Groups	MIC (µg/ml)	Post Antibacterial duration (hours)	
		MIC level	Sub - MIC level
T. vulgaris	3.125	7.1 ±0.38^{Aa}	6.0 ±0.25^{Aa}
M. piperita	6. 25	5.8 ±0.23^{Ba}	4.4 ±0.19^{Bb}
Amoxicillin	25.0	3.8 ±0.17^{Ca}	3.1 ±0.11^{Ca}

LSD: 1.283; Means having with the different capital letters in same column and small letters in same row differed significantly (P≤0.05). Values are mean ± SD, n = 5

DISCUSSION

Culturing *S. aureus* on blood agar allows observation of hemolysis for identification and provides a rich, non-selective medium for microbial growth (Srisrattakarn et al., 2024). Bacterial colonies on blood agar show α (partial), β (complete), or γ (no) hemolysis. The double β-hemolysis zone around *S. aureus* colonies results from α- and β-hemolysin activity (Al-Izzi *et al.*,1989; Turista and Puspitasari, 2019).

Staphylococcus aureus is a Gram-positive bacterium that retains the crystal violet stain, appears purple or blue under a light microscope due to its thick peptidoglycan cell wall (Yu *et al*., 2023; Latif and Yousif, 2025).

A key virulence factor of *S. aureus* is coagulase, which converts fibrinogen to fibrin, forming plasma clots that protect the bacteria and may delay phagocytosis in tissues (Javed *et al*., 2024).

These results align with previous studies: *T. vulgaris* hydrodistillation yields ranged from 0.62% to 1.40% (Shallal and Ahmed, 2022), while *M. piperita* yields varied from 0.63–1.36% depending on conditions (Ibrahim *et al*., 2021). Steam distillation efficiency is influenced by water quality, equipment, temperature, and duration (Shrestha *et al*., 2025).

Thymol, a phenolic monoterpene, is well documented as the principal antimicrobial and antioxidant constituent of thyme oil. Its high abundance confirms that the oil belongs to the "thymol chemotype," which is particularly effective against Gram-positive bacteria, including *Staphylococcus aureus*, a major causative agent of ovine mastitis. The presence of γ-terpinene and p-cymene is also significant, since they act as precursors in thymol biosynthesis and may enhance the antimicrobial efficacy (Rani *et al*., 2022).

Menthol and menthone, key components of peppermint oil, provide analgesic, cooling, and antibacterial effects. Eucalyptol enhances anti-inflammatory action, while caryophyllene and β-pinene add antimicrobial and antioxidant properties. Variations in composition depend on origin, climate, harvest time, and extraction method (Burt, 2004;Yassin *et al*., 2020).

Pharmacodynamic studies help optimize antibiotic dosing (Yoo and Lee, 2020). In this study, *T. vulgaris* oil showed MIC of 3.125 µg/ml against *S. aureus*, consistent with Sienkiewicz *et al.* (2012), who reported MICs of 0.25–1.0 µg/ml for resistant strains. *Thymus digenesis* also showed strong activity against *S. aureus* (MIC 6.25 µg/ml) due to flavonoids (Rashid *et al*., 214). *M. piperita* oil had higher MICs, in agreement with Li *et al.* (2011), who reported 64–256 µg/ml against clinical *S. aureus* isolates. Singh *et al.* (2015) demonstrating MIC values of essential oil of *M. piperita* indicate that *S. aureus* is more susceptible which record 5µg/ml.

Based on our results showing a proportional increase in the inhibition zones with higher concentrations of *T. vulgaris* (Thyme) and *M. piperita* (Peppermint) oils against *S. aureus*, it is important to note the technical limitations of the agar well diffusion assay for these specific substances (Donaldson *et al*., 2005). Inhibition zones size increased proportionally with higher extract concentrations. *T. vulgaris* oil showed strong activity against *S. aureus*, mainly due to aromatic monoterpenes such as thymol, carvacrol, and p-cymene (Daouk *et al*., 1995).

Thyme oil's effectiveness is due to hydrophobic thymol and carvacrol, which disrupt bacterial membranes, increasing permeability (Chaichi *et al*., 2021). Peppermint oil showed moderate inhibition against *S. aureus*, attributed to secondary metabolites like essential oils, glycosides, and flavonoids (Okmen *et al*., 2017). Its activity aligns with studies showing plant oils rich in phenols, terpenes, and aldehydes are potent antimicrobials (Di Matteo *et al*., 2024).

Pharmacodynamic parameters like the post-antibiotic effect (PAE) are vital for understanding antimicrobial action and optimizing dosing. Many antibiotics induce PAE and SME in various bacteria (Yoo and Lee, 2020). PAE estimation best reflects in vivo antibiotic behavior. PAE likely results from non-lethal bacterial damage or drug persistence at binding sites (Zhanel *et al*., 1991). Its duration depends on the drug type, bacterial strain, concentration, and assessment method (Farooq et al., 2025). Thymol showed strong antibacterial activity (Mith *et al*., 2014). This study confirmed its MIC and half-MIC effects and, Moderate claims and contextualize with previous literature, revealed PAE and PA-SME against *S. aureus*. Agents lacking PAE require more frequent dosing (Zarrini *et al*., 2010).

CONCLUSIONS

This study demonstrated that *Thymus vulgaris* and *Mentha piperita* essential oils exhibit strong in vitro antibacterial activity against *Staphylococcus aureus* isolated from mastitic Awassi ewe milk. Both oils showed concentration-dependent inhibitory effects, as evidenced by measurable inhibition zones, low MIC values, and significant bacterial reduction in time-kill assays. GC–MS analysis revealed the presence of bioactive constituents that likely contribute to the observed antibacterial activity. Additionally, the post-antibacterial effect (PAE) and post-antibacterial sub-MIC effect (PA-SME) assays confirmed their ability to delay bacterial regrowth under controlled laboratory conditions. However, these findings are limited to in vitro experiments. Further *in vivo* studies, including safety assessments, pharmacokinetic evaluations, formulation optimization, and controlled clinical trials in ewes with mastitis, are necessary to validate the therapeutic potential of these essential oils.

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NOVELTY STATEMENT

This study provides a comprehensive pharmacodynamic evaluation of *Thymus vulgaris* and *Mentha piperita* essential oils against *Staphylococcus aureus* isolated from ovine mastitis. By combining GC–MS profiling with MIC, time-kill kinetics, and post-antibiotic effect analyses, it demonstrates that *T. vulgaris* exhibits superior antibacterial activity compared with *M. piperita* and amoxicillin, supporting its potential as a natural therapeutic alternative

AUTHORS' CONTRIBUTION

HMA and OMS designed the study. HMA conducted the experimental work and collected the samples. NAR performed the statistical analysis and interpretation of data. HMA wrote the manuscript. All authors read and approved the final version of the manuscript. The final manuscript draft was reviewed by all authors.

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Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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