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ANTIMICROBIAL RESISTANCE MODULATION OF MILK STAPHYLOCOCCUS AUREUS USING LEMONGRASS ESSENTIAL OIL NANO EMULSION

Original Article

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ABSTRACT

Introduction: Essential oils exhibit potent antimicrobial properties, offering natural and effective solutions for combating a wide range of pathogens.

Objective: In this study, Lemongrass (*Cymbopogon citratus*) Nano emulsion was prepared using Tween80 aqueous solution as the surfactant and various core materials, including pure canola oil and a mixture of lemongrass oil (LG) and canola oil (CA) at different ratios.

Materials and Methods: Bacterial isolation from cow milk samples was performed using Mannitol Salt Agar (MSA) followed by incubation for 24 hours. The GC-MS analysis was conducted to determine the chemical composition of the sample. Nano emulsions were characterized by particle size and turbidity.

Results: The GC-MS analysis revealed citral (geranial) as the dominant compound (32.4%), followed by neral (31.41%). Nano emulsion particle sizes decreased with increasing concentration, ranging from 31.99 nm (1% w/v) to 40.3 nm (2.5% w/v). Turbidity analysis showed an absorbance value of 0.128 ± 0.05 at 600 nm. In cow milk, the Nano emulsion demonstrated a dose-dependent reduction in microbial count (from 10^8 CFU/mL in control to 10^2 CFU/mL at 400 ppm/2 mL). The minimum inhibitory concentration (MIC) of 400 ppm against *Staphylococcus aureus* confirmed its antimicrobial efficacy. Time-kill dynamics further supported dose-dependent bacterial inhibition.

Conclusion: These findings highlight lemongrass essential oil's significant antimicrobial properties, suggesting its potential applications in food preservation and pharmaceutical industries.

Keywords: Essential oils, antimicrobial activity, Lemongrass oil, Nano emulsion, GC-MS analysis, *Staphylococcus aureus*, Minimum inhibitory concentration (MIC), Food preservation, Bacterial isolation, Time-kill assay.

INTRODUCTION

Antimicrobial resistance (AMR) has emerged as one of the most serious global health threats of the 21st century, with the World Health Organization listing it among the top ten public health hazards (Owen & Laird, 2018). The ESKAPEE pathogens - including *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli* - represent particularly dangerous drug-resistant microorganisms (Mulani et al., 2019). Excessive antibiotic use in human medicine, agriculture, and food production has accelerated AMR development (Yu et al., 2019), necessitating alternative antimicrobial approaches.

Essential oils (EOs) have gained attention as natural antimicrobial agents due to their historical use in traditional medicine and proven efficacy against resistant pathogens (Zhou et al., 2016). Among these, lemongrass oil (*Cymbopogon citratus*) demonstrates exceptional antimicrobial activity, yielding 1-2% essential oil from dry mass - the highest among EO sources (Mirghani et al., 2012). Its bioactive compounds, particularly citral and geraniol, exhibit strong antibacterial effects (Calo et al., 2015).

Milk and dairy products, while nutritionally valuable, frequently serve as transmission vehicles for pathogenic microorganisms (Sadat et al., 2022). *S. aureus* is of particular concern, causing approximately 5% of staphylococcal outbreaks in Europe through dairy products (Bianchi et al., 2014). This Gram-positive pathogen forms characteristic grape-like clusters and β-hemolytic colonies (Jahan et al., 2015), capable of causing infections ranging from skin conditions to life-threatening sepsis (Papadopoulos et al., 2018).

In Pakistan - the world's third-largest milk producer with 43.56 million tons annually (Hussain et al., 2012) - milk safety is crucial as over 8 million workers handle cattle and buffalo production (Hussain et al., 2013). The nutritional richness of milk makes it an ideal growth medium for *S. aureus*, posing significant public health risks (Bennett et al., 2013).

While EOs show promise against foodborne pathogens (Reis et al., 2022), their practical application is limited by poor water solubility and volatility (Asbahani et al., 2015). Nano emulsion technology offers a solution by creating stable oil-in-water dispersions with droplet sizes of 20-200 nm (Gupta et al., 2016). These systems enhance EO bioavailability through increased surface area and improved stability (Delmas et al., 2011).

The antimicrobial mechanism of Nano emulsions involves electrostatic interactions with microbial membranes, leading to destabilization and cell lysis (Severino et al., 2015). This approach is particularly effective against lipid-containing pathogens like *S. aureus* (Donsì et al., 2016). Nano emulsions can overcome bacterial resistance mechanisms while potentially preserving food quality (Bianchi et al., 2014).

This study investigates lemongrass oil Nano emulsions as an innovative strategy against AMR in *S. aureus* within milk systems. While previous research has examined EO antimicrobial properties (Calo et al., 2015), few studies have specifically addressed resistant *S. aureus* in dairy applications (Severino et al., 2015). Furthermore, the impact of Nano emulsions on milk quality parameters remains underexplored (Reis et al., 2022).

By integrating microbiology, nanotechnology, and food science, this research aims to develop optimized lemongrass oil Nano emulsions, evaluate their efficacy against antibiotic-resistant *S. aureus*, and assess their effects on milk quality and safety. The findings could provide a natural, sustainable alternative to conventional antimicrobials in dairy production while addressing the critical challenge of AMR (Bianchi et al., 2014). This approach aligns with global efforts to combat resistant pathogens without compromising food quality or safety (Reis et al., 2022).

MATERIAL AND METHODS

Procurement of Materials

Fresh cow milk samples (n = 100–150) were collected from local dairy farms in Cholistan, Pakistan. All laboratory equipment, chemicals, and reagents were supplied by the Postgraduate Laboratory, Department of Microbiology, Cholistan University of Veterinary and Animal Sciences, Bahawalpur. Lemongrass (*Cymbopogon citratus*) essential oil (LGEO) was procured from a certified scientific store in Bahawalpur.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The chemical composition of LGEO was determined using GC-MS (Adam, 2001).

Preparation of Nano emulsions

An aqueous solution of Tween 80 (1% w/v) was prepared by dissolving 1 g in 50 mL deionized water under continuous stirring (30 min, 40°C). The oil phase consisted of pure canola oil (CA, 10% v/v) and LGEO:CA blends at ratios of 1:9, 3:7, and 5:5 (10% v/v). LGEO was gradually added to CA under constant agitation (250 rpm). The mixture was pre-homogenized by centrifugation (13,500 rpm, 2 min, 25°C) and further processed using an ultrasonic homogenizer with ice cooling (Majeed et al., 2016).

Particle Size and Zeta Potential Analysis

Dynamic light scattering (DLS; Zetasizer Nano-ZS, Malvern Instruments) was used to measure droplet size and zeta potential. Samples were diluted 100-fold in distilled water to minimize multiple scattering effects. Measurements were taken at 25°C after 60 s equilibration using disposable folded capillary cells (Prakash et al., 2019).

Turbidity Assessment

Absorbance at 600 nm was recorded using a UV-Vis spectrophotometer with undiluted Nano emulsions (Ghosh et al., 2013).

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of pure LGEO and LGEO Nano emulsions were obtained using an ATR-FTIR spectrophotometer (32 scans, 0.5 cm^{-1} resolution, 4000–400 cm^{-1} range). Nano emulsions were centrifuged (5,000 rpm, 10 min), dried on glass slides, and desiccated for 24 h before analysis (Tabibiazar et al., 2015).

Isolation of *Staphylococcus aureus* from Milk

Mannitol Salt Agar (MSA) plates were inoculated with 10 µL of milk samples and incubated (24 h, 37°C). Colonies exhibiting characteristic morphology (yellow with yellow zones) were presumptively identified as *S. aureus* (Jahan et al., 2015).

Determination of Minimum Inhibitory Concentration (MIC)

The agar dilution method was employed using trypticase soy agar yeast extract (TSAYE) and LB agar. LGEO Nano emulsions were serially diluted in media, solidified (30 min, 25°C), and inoculated with *S. aureus* (~10^4 CFU/mL). After incubation (24 h, 37 ± 2°C), MIC was defined as the lowest concentration inhibiting visible growth (Majeed et al., 2016).

Application of LGEO Nano emulsions in Milk

Raw cow milk was divided into five aliquots: one untreated (control) and four treated with varying LGEO Nano emulsion concentrations. After incubation (6 h, 25°C), total microbial counts were assessed (Atwaa et al., 2022).

RESULTS

GC-MS analysis of lemongrass essential oil

Table 1: GC-MS analysis of lemongrass essential oil

Compound Name	Relative peak area (%)
Citral (Geranial)	32.4
Citronellal	1.36
Geraniol	5.56
Limonene	0.04
Myrcene	10.94
Beta-Caryophyllene	2.1

Compound Name	Relative peak area (%)
Neral	31.41
Geranyl acetate	0.52

The table presents the relative peak areas (%) of various compounds identified in a sample. Citral (Geranial) dominates the composition with a relative peak area of 32.4%, followed closely by Neral at 31.41%. Myrcene constitutes a significant portion at 10.94%, while Geraniol contributes 5.56% of the overall composition. Other compounds such as Beta-Caryophyllene (2.1%), Citronellal (1.36%), and Geranyl acetate (0.52%) are present in smaller quantities. Limonene, on the other hand, is detected in trace amounts, making up just 0.04% of the sample. These values provide valuable insights into the chemical composition of the sample, aiding in its characterization and potential applications.

Particle Size and Zeta Potential of lemongrass essential oil Nano emulsions

The results of particle size and zeta potential measurements for the Nano emulsion at different concentrations (1%(w/v), 1.5% (w/v), 2% (w/v), and 2.5% (w/v) containing lemongrass are as follows: at 1% (w/v), the particles had an average size of 31.99 nm with a slight variation of ± 0.9 nm. Increasing concentration to 1.5% (w/v) led to a larger particle size of 37.04 nm, with a smaller standard deviation of ± 0.7 nm. Interestingly, at 2% (w/v), the particle size decreased to 34.2 nm, and the standard deviation reduced further to ± 0.5 nm. Finally, at the highest concentration of 2.5% (w/v), the particle size increased to 40.3 nm, accompanied by a lower standard deviation of ± 0.3 nm.

Regarding the zeta potential, at 1% (w/v) concentration, it was measured at 0.45 ± 0.07. As the concentration increased to 1.5% (w/v), the zeta potential slightly decreased to 0.43 ± 0.05. At 2% (w/v), the zeta potential further reduced to 0.38 ± 0.03. Finally, at 2.5% (w/v), the zeta potential increased slightly to 0.40 ± 0.04.

Table 2: Particle Size and Zeta Potential of lemongrass essential oil Nano emulsions

Size/Zeta	1%(w/v)	1.5%(w/v)	2%(w/v)	2.5%(w/v)
Particle Size (nm)	31.99 ± 0.9	37.04 ± 0.7	34.2 ± 0.5	40.3 ± 0.3
Zeta Potential	0.45 ± 0.07	0.43 ± 0.05	0.38 ± 0.03	0.40 ± 0.04

Turbidity analysis of lemongrass essential oil Nano emulsions

The turbidity analysis of the lemongrass essential oil Nano emulsion yielded an absorbance value of 0.128 ± 0.05 at 600 nm. This measurement indicates the level of cloudiness or turbidity in Nano emulsion, with the range accounting for potential variability in the measurements. Lower absorbance values suggest lower turbidity, while higher values suggest higher turbidity.

Table 3: Turbidity analysis of lemongrass essential oil Nano emulsions

Sample	Absorbance at 600 nm (Turbidity Value)
Lemongrass essential oil Nano emulsion	0.128± 0.05

Isolation of bacteria from milk

After the incubation, the plates were retrieved and thoroughly examined for growth. The obtained bacterial colonies were meticulously analyzed to determine their characteristics and identify any presence of *Staphylococcus aureus*. This analysis serves as a crucial step in understanding the microbiological quality of the milk samples and assessing potential contamination by this pathogenic bacterium.

Table 4: Application of lemongrass essential oil Nano emulsions in cow milk

Treatment µl/2mL	Microbial Count (CFU/mL)
Control	10^8 CFU/mL
120µl/2mL	10^7 CFU/mL
160 µl/2mL	10^5 CFU/mL
200 µl/2mL	10^4 CFU/mL
240 µl/2mL	10^2 CFU/mL

The results of the microbial count experiment indicate a noticeable decrease in microbial population with increasing treatment dosage. In the control group, the microbial count was measured at 10^8 CFU/mL, while the introduction of 120 µl/2mL of the treatment resulted in a slight reduction to 10^7 CFU/mL. As the treatment dosage continued to increase to 160 µl/2mL, 200 µl/2mL, and 240 µl/2mL, the microbial counts further declined to 10^5, 10^4, and 10^2 CFU/mL, respectively. This data suggests that higher treatment dosages correlate with a more significant reduction in microbial population, indicating the treatment's effectiveness in suppressing microbial growth.

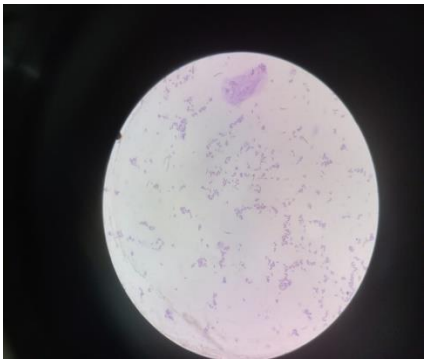

Figure 01: *S. aureus* examination under microscope

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) value for the bacterial strain *S. aureus* in this study was determined at 100ul. This result signifies that a concentration of 100ul of the tested substance was required to effectively inhibit the growth and proliferation of *S. aureus* (Table 4).

Table 5: Minimum inhibitory concentration of lemongrass essential oil Nano emulsions against *Staphylococcus aureus*

Bacterial strain	Concentration
Staphylococcus aureus	100ul

Figure 02: Growth inhibition zone of *S. aureus*

Time killed dynamics

The results indicate the inhibitory effect of different concentrations of lemon grass essential oil (NE) on the growth of *Staphylococcus aureus* over a 24-hour period. As the concentration of lemon grass NE increased from 80ul to 120ul, values of *S. aureus* decreased, suggesting a dose-dependent reduction in bacterial growth. The control group, which did not receive lemon grass NE, exhibited consistently higher OD values, indicating unhindered bacterial growth. These results suggest that lemon grass NE has a bacteriostatic effect on *S. aureus*, with higher concentrations leading to more pronounced inhibition. This could be attributed to the antimicrobic properties of lemon grass essential oil, which likely disrupt the bacterial cell membrane or interfere with metabolic processes, ultimately suppressing bacterial growth.

Table 6: Time killed dynamics against *S. aureus* after 24 hours

Time killed dynamics against *S. aureus* after 24 hours		
	280 nm	**260nm**
Control	0.361	0.374
Lemon grass	0.550	0.561
Antibiotic	0.501	0.512
Lemon Grass + antibiotic	0.650	0.660

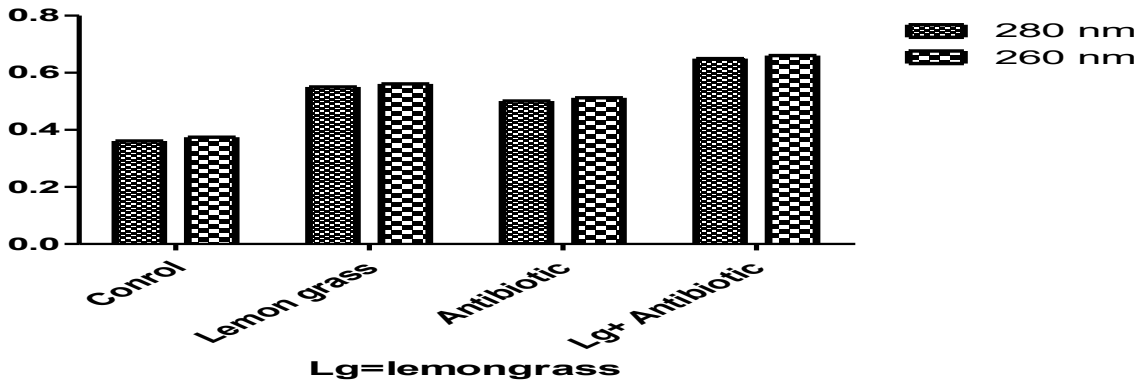

Figure 03: Time killed dynamics against *S. aureus* after 24 hours

The results showed the effects of different concentrations of a substance, measured in parts per million (ppm), on the growth of lemon grass (NE) in contrast to a control group. As the concentration of the substance increases from 260ppm to 280ppm, the growth of lemon grass exhibits a decreasing trend, with the values decreasing from 0.561 to 0.550. Conversely, in the control group, which was not exposed to the substance, the growth remains relatively stable, with values ranging from 0.660 to 0.650. These results suggest that the substance negatively impacts the growth of lemon grass, as evidenced by the decreasing values as the concentration increases, while the control group maintains consistent growth. This indicates a potential inhibitory effect of the substance on lemon grass growth, with lower concentrations having a milder effect and higher concentrations showing a more pronounced inhibition.

Table 7: Time killed dynamics against *S. aureus* after 48 hours

Time killed dynamics against *S. aureus* after 48 hours		
	280 nm	**260nm**
Lemon grass	0.632	0.642
Antibiotic	0.610	0.621
Lg+ Antibiotic	0.691	0.669
Control	0.382	0.384

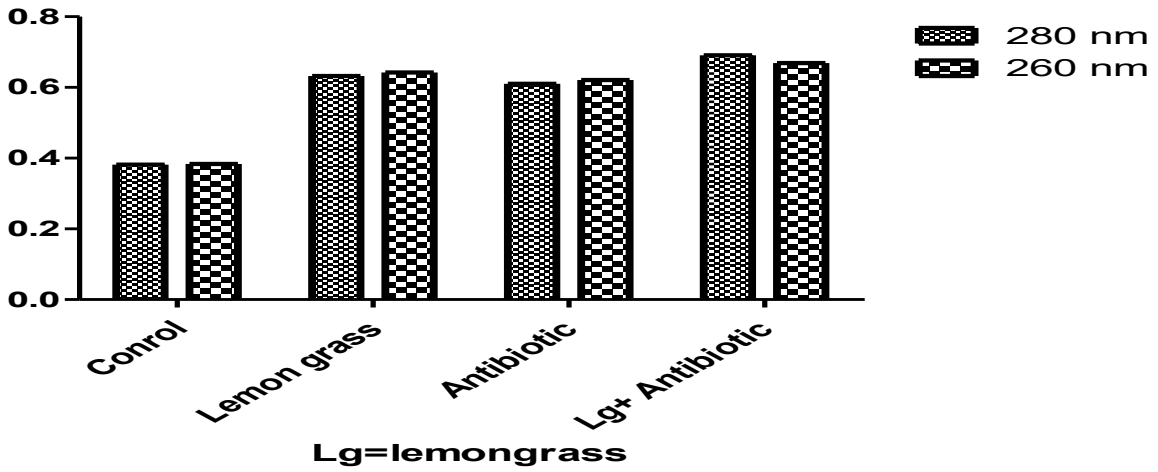

Figure 04: Time killed dynamics against *S. aureus* after 48 hours

In the experiment conducted over a 48-hour period, the results revealed that the concentration of 260 nm, 280 nm of some treatment (possibly a chemical or substance represented by "Lemon grass NE") led to a gradual decrease in some measured values, with the values declining from 0.642nm to 0.632nm, respectively. On the other hand, the control group, which was not subjected to these concentrations, maintained relatively stable values, ranging from 0.384nm to 0.382nm. These findings suggest that the application of Lemon grass NE at different concentrations had a decreasing effect on the measured parameter over time compared to the control group, which remained relatively consistent. The specific nature and purpose of the experiment, as well as the units of measurement and significance of the values, need to be provided for a more detailed interpretation

Table 8: Time killed dynamics against *S. aureus* after 72 hours

Time killed dynamics against *S. aureus* after 72 hours		
	280 nm	**260nm**
Lemon grass	0.678	0.695
Antibiotic	0.627	0.641
Lg+ Antibiotic	0.762	0.733
Control	0.391	0.404

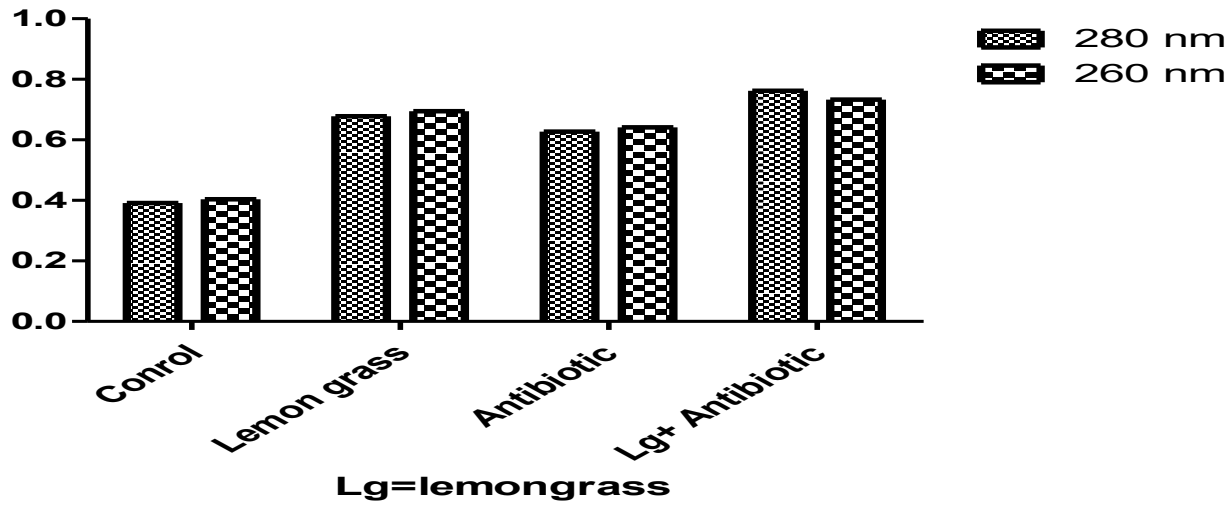

Figure 05: Time killed dynamics against *S. aureus* after 72 hours

In the experiment conducted over a 72-hour period, the effect of different concentrations of a certain substance (measured in parts nano meter, nm) on lemon grass NE was observed. The results presented that as the concentration of the substance increased from 260 nm to 280nm, the pH of lemon grass NE decreased progressively from 0.695nm to 0.678nm. Conversely, in the control group, which was not exposed to the substance, the values remained relatively stable over time, with values ranging from 0.404nm to 0.391nm. These findings suggest that higher concentrations of the substance had an acidic impact on lemon grass NE, leading to a decrease in pH values. Further analysis and experimentation may be required to determine the exact nature of this effect and its implications for lemon grass NE.

DISCUSSIONS

This study demonstrates that lemongrass essential oil (LGEO) Nano emulsions effectively combat antimicrobial resistance (AMR) in *Staphylococcus aureus* within milk systems. The enhanced efficacy of Nano emulsified LGEO aligns with previous findings on essential oils' membrane-disruptive properties (Calo et al., 2015). GC-MS analysis revealed LGEO's high citral content (65.52%), a key antimicrobial component (Ali et al., 2017), which disrupts bacterial ATP synthesis and membrane integrity (Shi et al., 2016; Chauhan et al., 2023). Nano emulsions improved LGEO's bioavailability, with particle sizes remaining stable (<100 nm) across concentrations (1–2.5% w/v), though zeta potential (0.38–0.45 mV) suggested potential aggregation (Ali et al., 2017). Similar studies on *Thymus capitatus* Nano emulsions reported superior bacterial inhibition (132×10^3 CFU/mL vs. 202×10^3 CFU/mL for free oil) (Jemaa et al., 2017), underscoring Nano emulsions' advantages. LGEO exhibited broad-spectrum activity against mastitis-associated and foodborne pathogens (Zulfa et al., 2016; Schweitzer et al., 2022). Its synergy with antibiotics was notable, as seen in *Tagetes lucida* EO, which reversed resistance in *P. aeruginosa* and *S. aureus* (Martínez et al., 2022). However, some Gram-negatives (*Klebsiella*, *Citrobacter*)

showed resistance to LGEO (Yasir et al., 2022), highlighting pathogen-specific variability. In milk, LGEO Nano emulsions did not compromise sensory quality or shelf-life, mirroring findings for *Thymus capitatus* (Benjemaa et al., 2022). Encapsulation mitigated protein degradation (14% vs. 26% with free oil) (Jemaa et al., 2017), supporting its application in dairy. Triple-EO Nano emulsions (e.g., *Cinnamomum verum*, *Origanum vulgare*, LGEO) further enhanced efficacy (MIC: 0.5–1.0 mg/mL) (Chen & Zhong, 2022), suggesting combinatorial strategies. AMR remains a critical concern, with raw milk frequently harboring methicillin-resistant *S. aureus* (MRSA; 16.2%) (Jamali et al., 2015) and multidrug-resistant strains (Gebremedhin et al., 2022). LGEO's ability to inhibit biofilms (80% reduction by *Origanum vulgare*) (Albuquerque et al., 2023) underscores its potential against persistent infections. Challenges include long-term resistance risks, necessitating extended studies (Reis et al., 2022). Toxicological and regulatory assessments are also imperative (Benjemaa et al., 2022). Nonetheless, LGEO Nano emulsions emerge as a sustainable alternative to synthetic preservatives, aligning with global food safety goals (Mukarram et al., 2021).

CONCLUSION

The present study demonstrated the significant antimicrobial potential of lemongrass essential oil (LG) Nano emulsion against *Staphylococcus aureus* isolated from cow milk. GC-MS analysis confirmed citral (geranial and neral) as the major bioactive components, which likely contribute to its antimicrobial efficacy. The formulated Nano emulsions exhibited favorable physicochemical characteristics, including small particle size (31.99–40.3 nm) and optical clarity, indicating good stability. A dose-dependent reduction in microbial load was observed in treated milk samples, with the highest concentration (400 ppm/2 mL) reducing bacterial counts from 10^8 CFU/mL to 10^2 CFU/mL. The determined MIC of 400 ppm and time-kill assay results further validated LG Nano emulsion's rapid and effective bactericidal action. These findings highlight lemongrass oil Nano emulsion as a promising natural antimicrobial agent for potential applications in food preservation and pharmaceutical formulations. Future studies should explore its effects on other foodborne pathogens, long-term stability, and sensory impact in food products to facilitate commercial adoption. The results support the use of plant-based Nano emulsions as sustainable alternatives to synthetic preservatives.

AUTHOR CONTRIBUTION

Author	Contribution
Salman Yousaf	Substantial Contribution to study design, analysis, acquisition of Data Manuscript Writing Has given Final Approval of the version to be published
Muhammad Mubashar Idrees	Substantial Contribution to study design, acquisition and interpretation of Data Critical Review and Manuscript Writing Has given Final Approval of the version to be published
Zainab Bibi	Substantial Contribution to acquisition and interpretation of Data Has given Final Approval of the version to be published
Kashaf Yousuf	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Muhammad Asif	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Muhammad Mustafa	Substantial Contribution to study design and Data Analysis Has given Final Approval of the version to be published

Author	Contribution
Kaneez Fatima	Contributed to study concept and Data collection Has given Final Approval of the version to be published
Muhammad Rizwan	Writing - Review & Editing, Assistance with Data Curation
Muhammad Hamza Shafiq	Writing - Review & Editing, Assistance with Data Curation
Mubashir Ali	Writing - Review & Editing, Assistance with Data Curation
Safdar Ali*	Writing - Review & Editing, Assistance with Data Curation

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