

Original research article

ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF ECO-FRIENDLY HYDROGEL *MORINGA OLEIFERA*– CHITOSAN FORMULATION AGAINST *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS*

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Abstract

Escherichia coli and *Staphylococcus aureus* represent the dominant etiological agents of pyometra in cattle. Their strong biofilm-forming ability critically contributes to therapeutic failure and escalating antibacterial resistance. This study aimed to develop and evaluate an eco-friendly chitosan hydrogel formulation containing *Moringa oleifera* leaf extract as an alternative antibacterial and antibiofilm agent. The ethanolic extract of *M. oleifera* was characterized using Gas Chromatography–Mass Spectrometry (GC–MS). Hydrogel formulations (10,

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20, 30, and 40%) were prepared and assessed for bacterial growth inhibition and biofilm degradation over 24, 48, and 72 h using microplate spectrophotometry. The formulations exhibited clear concentration- and time-dependent activities. The 10% formulation produced the strongest inhibition against *E. coli*, whereas *S. aureus* responded most effectively to the 30% formulation, which maintained stable inhibitory effects across all incubation periods. Biofilm assays revealed transient suppression in *E. coli*, with maximal degradation at 48 h, while *S. aureus* showed more sustained inhibition, particularly with the 30% formulation. Correlation analysis demonstrated a stronger alignment between growth inhibition and biofilm reduction in *E. coli*, indicating more synchronized antibacterial and antibiofilm effects ($p < 0.05$). The *M. oleifera*–chitosan hydrogel formulation exhibited promising dual activity against planktonic and biofilm-associated bacteria, highlighting its potential as an eco-friendly therapeutic candidate against bovine uterine infections and reducing reliance on conventional antibiotics.

Keywords: *Moringa oleifera*, chitosan, *Escherichia coli*, *Staphylococcus aureus*, biofilms

INTRODUCTION

Pyometra is a serious uterine disorder in cattle, characterized by purulent accumulation resulting from secondary bacterial infection, along with hormonal imbalance and suboptimal reproductive performance (Amin et al., 2021). This condition contributes to delayed estrus, prolonged calving intervals, reduced fertility, and increased systemic risks, ultimately causing substantial economic losses (Kasimanickam et al., 2025).

In Aceh cattle, various opportunistic bacteria, including *E. coli*, *Corynebacterium* sp., *Enterobacter* sp., and *Pseudomonas* sp. are frequently isolated from the uterus (Thasmi et al., 2021). Among these, *E. coli* and *S. aureus* are commonly reported as associated with uterine infections, along with other pathogens (Talib et al., 2023). The ability of these bacteria to adhere to host tissues and form biofilms contributes to their persistence. Biofilm development enhances bacterial resistance, rendering infections more difficult to treat.

Conventional therapies, which include antibiotics, prostaglandins, and uterine irrigation, are increasingly limited due to rising antibacterial resistance driven by alterations in bacterial targets, enzymatic degradation, and biofilm-mediated protection (Iancu et al., 2025; Mekibib et al., 2024). These challenges underscore the need for alternative treatments that can effectively target both planktonic and biofilm-associated bacteria. Herbal-based approaches have gained attention, and *M. oleifera*, rich in antibacterial and antioxidant compounds, shows promising potential for managing uterine infections (Armansyah et al., 2023; Rosmaidar et al., 2021).

Several medicinal plants have been reported to exhibit antibacterial, anti-inflammatory, and immunomodulatory properties relevant to infection control

(Japheth et al., 2021). Among these, *M. oleifera* has attracted considerable attention due to its rich content of bioactive compounds, including flavonoids, alkaloids, and saponins, which contribute to its antibacterial and antioxidant activities (Armansyah et al., 2023). These compounds are known to inhibit bacterial growth through mechanisms such as disruption of cell membranes and interference with bacterial metabolic processes, highlighting the potential of *Moringa oleifera* extract as a natural antibacterial agent (Wen et al., 2022).

In this study, an eco-friendly hydrogel combining *M. oleifera* extract and chitosan was developed. Chitosan, a biodegradable and biocompatible biopolymer, exhibits intrinsic antibacterial activity due to its polycationic nature, which enables the interaction with bacterial cell membranes and inhibits biofilm formation (Ardean et al., 2021). The incorporated *M. oleifera* is expected to enhance the responsiveness and functionality of the chitosan matrix, improve its physicochemical and biological properties, including strength, biocompatibility, and biodegradability (Gani et al., 2023). This combination may also facilitate controlled release of active compounds, leading to enhanced antibacterial and antibiofilm effects (Jadhav et al., 2022; Jaferník et al., 2023).

Sustainable nanotechnology through green synthesis offers a novel strategy to enhance the stability, delivery, and bioactivity of plant-derived compounds (Jadhav et al., 2022). Chitosan nanoparticles possess inherent antibacterial properties, are biocompatible, effectively penetrate cell membranes, and have controlled release behavior (Herdiana et al., 2021; Jayathilaka et al., 2022). The antibiofilm activity of the *M. oleifera*–chitosan hydrogel is supposed to involve multiple complementary mechanisms, including disruption of bacterial cell membranes due to chitosan's polycationic interactions, inhibition of initial bacterial adhesion, and interference with biofilm matrix integrity. In addition, bioactive compounds from *M. oleifera* may penetrate the extracellular polymeric substances (EPS) of biofilms, interfere with quorum sensing, and enhance bacterial susceptibility through sustained release from the hydrogel system (Menon et al., 2026; Suhartono et al., 2019). However, an eco-friendly *M. oleifera* extract combined with a chitosan-based hydrogel formulation targeting *E. coli* and *S. aureus* has not yet been thoroughly investigated. Therefore, this study aimed to develop and evaluate an eco-friendly *M. oleifera*–chitosan hydrogel as an antibacterial and antibiofilm agent utilizing an *in vitro* experimental approach.

MATERIAL AND METHODS

Experimental design

This study was a randomized laboratory *in vitro* investigation. Ethanolic extracts of *M. oleifera* leaves were first subjected to phytochemical profiling using gas chromatography–mass spectrometry (GC–MS). The extracts were subsequently

formulated into chitosan-based hydrogels at different concentrations (10%, 20%, 30%, and 40%). Next, each was evaluated for its antibacterial and antibiofilm activities against the tested bacterial strains.

Preparation, extraction, and GC-MS analysis of M. oleifera

One kilogram of *M. oleifera* leaves was washed, air-dried for two days, and oven-dried at 50°C for 48 h before being powdered and stored airtight. The powder was macerated in 96% ethanol (100 mL), with solvent replaced every three days, and the filtrate was concentrated in a rotary evaporator at 50°C and 75 mmHg (BUCHI R-300) (Permadi et al., 2018). GC–MS analysis was conducted using a Shimadzu QP2010PLUS equipped with a 30 m × 0.25 mm polymethyl silicone column, employing an oven ramp of 80–200°C in 5 min, held for 20 min. FID and injection temperatures were 300°C and 220°C; nitrogen served as the carrier gas at 1 mL/min (116.9 kPa) with 50 mL/min column flow (Thasmi et al., 2024).

Preparation of chitosan–Moringa oleifera hydrogel formulation

The chitosan–*M. oleifera* hydrogel formulation was prepared by dissolving 1% chitosan in distilled water according to the designated volume for each treatment group (10–40%). Under continuous magnetic stirring, 70% ethanol and 2% glycerol were added sequentially, with each component mixed for 30 minutes to ensure uniform dispersion. The *M. oleifera* extract was then incorporated into the chitosan solution and stirred until a homogeneous hydrogel matrix was formed. All formulations were allowed to stand at room temperature to ensure complete gel formation and stabilization, as summarized in Table 1.

Table 1. Composition of eco-friendly *Moringa oleifera*–Chitosan hydrogel formulations

Ingredients	Hydrogel concentration			
	10%	20%	30%	40%
Chitosan 1% (ml)	25	25	25	23
Ethanol 70% (ml)	2	2	2	2
Glycerol 2% (ml)	5	5	5	5
<i>M. oleifera</i> extract (ml)	5	10	15	20
Distilled water (q.s.) (ml)	13	8	3	0

Preparation of Staphylococcus aureus and Escherichia coli

Staphylococcus aureus ATCC 25923 and *Escherichia coli* ATCC 25922 (Culti-Loops™, Thermo Scientific, Lenexa, USA) were used in this study. A single colony

of *S. aureus* and *E. coli* was inoculated separately into 5 mL of nutrient broth and incubated at 37°C for 24 h. Following incubation, the bacterial suspensions were adjusted to the 0.5 McFarland standard, equivalent to approximately 1.5×10^8 CFU/mL (Pitjamit et al., 2024).

Inhibition assessment of E. coli and S. aureus

The antibacterial activity of chitosan-*M. oleifera* hydrogel against *E. coli* and *S. aureus* was evaluated using a modified broth microdilution method according to El-Sherbiny et al. (2024). Briefly, 50 µL of tryptic soy broth (TSB) was dispensed into each well of a sterile 96-well microplate, followed by the addition of test samples at concentrations of 10%, 20%, 30%, and 40%, with penicillin-streptomycin as positive controls and distilled water as the negative one. A standardized bacterial suspension (0.5 McFarland) was diluted to obtain a final inoculum of $\sim 5 \times 10^5$ CFU/mL, and 100 µL was added to each well (final volume 200 µL). The plates were incubated at 37°C for 24, 48, and 72 h. Bacterial growth was quantified spectrophotometrically at 620 nm using a microplate reader (Bio-Rad, USA), where optical density (OD) values of 0.08–0.10 correspond to the 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL). The inhibitory activity was defined as the lowest concentration that did not lead to a significant increase in turbidity compared to the negative control.

Biofilm formation assay

Inhibition of biofilm formation was evaluated using a modified crystal violet assay as described by Adeyemo et al. (2022). Bacterial suspensions of *E. coli* and *S. aureus* were adjusted to the 0.5 McFarland standard and further diluted to approximately 1×10^6 CFU/mL in TSB. A hundred µL of the bacterial inoculum was co-incubated with 100 µL of chitosan-*M. oleifera* hydrogel at concentrations of 10%, 20%, 30%, and 40% in 96-well microplates at 37°C for 24, 48, and 72 h, with penicillin-streptomycin and distilled water as the positive and negative controls, respectively. Following incubation, the wells were gently washed three times with distilled water to remove non-adherent cells, air-dried, and stained with 0.1% crystal violet for 15 min. Excess stain was removed, and the wells were rinsed and dried. The bound dye was subsequently solubilized with 96% ethanol, and absorbance was measured at 595 nm with a microplate reader (Bio-Rad, USA). All experiments were performed in duplicate with two independent replications.

Statistical analysis

Data were analyzed using IBM SPSS Statistics version 24.0 (IBM Corp., Armonk, NY, USA). Data on growth inhibition and biofilm formation were analyzed using

the Kruskal–Wallis test. The relationship between growth inhibition and biofilm formation was evaluated based on the frequency distribution at each *M. oleifera* concentration. The correlation analysis was performed using Pearson's correlation coefficient. Statistical significance was set at $p < 0.05$.

RESULTS

Figure 1 illustrates the GC–MS chromatogram of the *M. oleifera* leaf extract, showing the overall distribution of its volatile and semi-volatile constituents. GC–MS analysis identified 45 compounds in the extract. The chromatographic profile is characterized by a series of small early peaks followed by a cluster of more prominent peaks between 22 and 28 minutes, indicating that the extract is largely composed of moderately non-polar compounds such as fatty acids, fatty alcohols, and ester derivatives. Consistent with this pattern, Table 2 summarizes the major compounds identified within this retention window, most of which are lipophilic metabolites known for their antibacterial, antifungal, or anti-inflammatory activities. The GC–MS profile of *M. oleifera* leaf extract is dominated by *cis*-9-tetradecen-1-ol (RT 23.53 min, 23.74% of TIC), with several other medium-abundance fatty acids and esters (including octadecanoic acid, glyceryl oleate, and a hexadecanoic acid ester) and minor amounts of phytol.

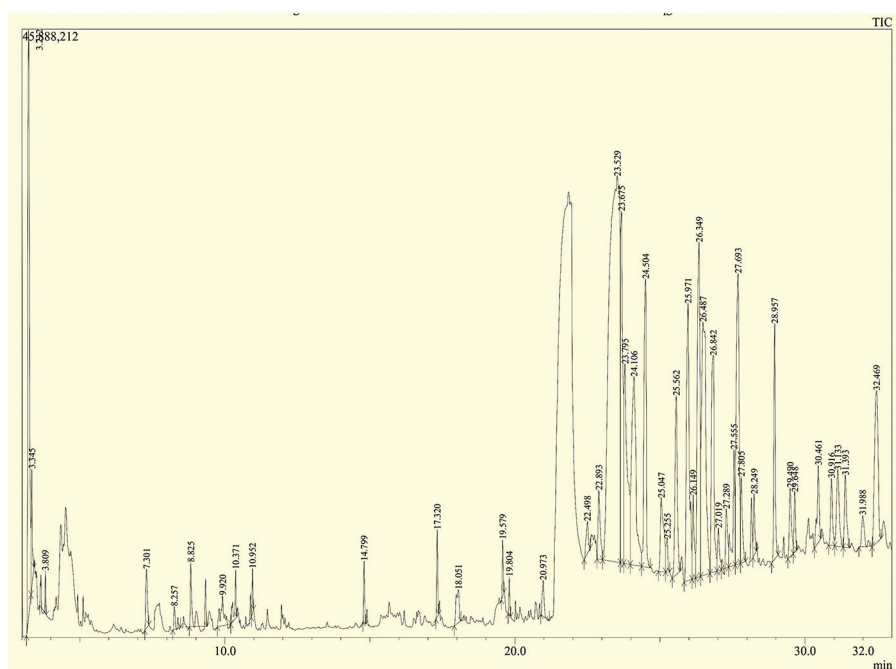


Figure 1. GC–MS chromatograms of *M. oleifera* leaf extract.

Table 2. Chemical composition (major compounds) of *M. oleifera* extract

R. Time	Compound	Chemical formula	Area%	Bioactivity	Reference
22.893	Phytol	C ₂₀ H ₄₀ O	00.87	Antibacterial	(Almeida-Bezerra et al., 2024)
23.529	cis-9-Tetradecen-1-ol	C ₁₄ H ₂₈ O	23.74	Antifungal, and antibacterial	(Mugayi & Mukanganyama, 2024)
23.675	Octadecanoic acid	C ₁₈ H ₃₆ O ₂	40.65	Antibacterial	(Casillas-Vargas et al., 2021) (Ghareeb et al., 2022); (Thasmi et al., 2024)
25.971	9-Octadecenoic acid, 1,2,3-propanetriyl ester	C ₅₇ H ₁₀₄ O ₆	40.65	Antibacterial, antiviral, anticancer, anti-spasmodic, and immune modulators	(Al-Marzoqi et al., 2016); (Ojo et al., 2021); (Najah & Alshawish, 2023)
26.349	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₁₉ H ₃₈ O ₄	50.31	Antibacterials, antifungal, anti-Alzheimer's	(Subash & Srinivasa Rao, 2021); (Idris et al., 2022)
26.487	Bicyclo[4.2.0]oct-1-ene, exo-7-(1-cyclohexen-1-yl)-	C ₁₄ H ₂₀	60.58	Antibacterial	(Sundarajan & Abdurahman, 2019)
27.693	Propylene glycol monooleate	C ₂₁ H ₄₀ O ₃	40.97	Anti-inflammatory, antiviral, and antioxidant	(Qadir et al., 2018)

Table 2 presents the GC–MS analysis of the *M. oleifera* extract, which identifies several bioactive compounds with potential antibacterial properties. The GC–MS profile shows that cis-9-tetradecen-1-ol is the predominant component, contributing substantially to the extract’s antibacterial and antifungal effects, while other compounds—such as hexadecanoic acid derivatives, octadecanoic acid, bicyclo[4.2.0]oct-1-ene, propylene glycol monooleate, and 9-octadecenoic acid propanetriyl ester—provide additional antibacterial, antioxidant, anti-inflammatory, and immunomodulatory activities. Although present in smaller amounts, phytol may further enhance the extract’s antibacterial activity, suggesting that its overall bioactivity likely results from the synergistic action of these metabolites.

Chitosan–*M. oleifera* hydrogel produced significant ($p < 0.05$) antibacterial effects against both *E. coli* and *S. aureus* at 24, 48, and 72 h (Table 3). In *E. coli*,

the 10% concentration consistently yielded the lowest OD values, indicating the strongest and most stable inhibition compared with higher concentrations. By contrast, *S. aureus* was most sensitive to 30%, which was reflected in the lowest OD readings after all incubation periods, while the 10%, 20%, and 40% groups were less suppressed. These findings demonstrate that the optimal inhibitory concentration of the hydrogel depends on the bacterial species: 10% was most effective against *E. coli*, and 30% produced the highest inhibition of *S. aureus*.

Figure 2 demonstrates that chitosan-*M. oleifera* hydrogel produced concentration-dependent antibacterial effects against *E. coli* (Figure 2A) and *S. aureus* (Figure 2B) across all incubation periods. The 10% formulation showed the strongest and most sustained inhibition of *E. coli*, consistently outperforming the positive control at 24 and 48 h and remaining comparable at 72 h. By contrast, *S. aureus* displayed a different response pattern: the 30% and 40% concentrations yielded the highest inhibition at 24 h, though only the 30% treatment maintained relatively stable activity at later time points. The 10% and 40% groups showed marked reductions over time, and the 20% concentration exhibited minimal inhibition throughout. In summary, the findings indicate that the 10% formulation is optimal for *E. coli*, whereas the 30% concentration provides the most durable inhibition of *S. aureus*.

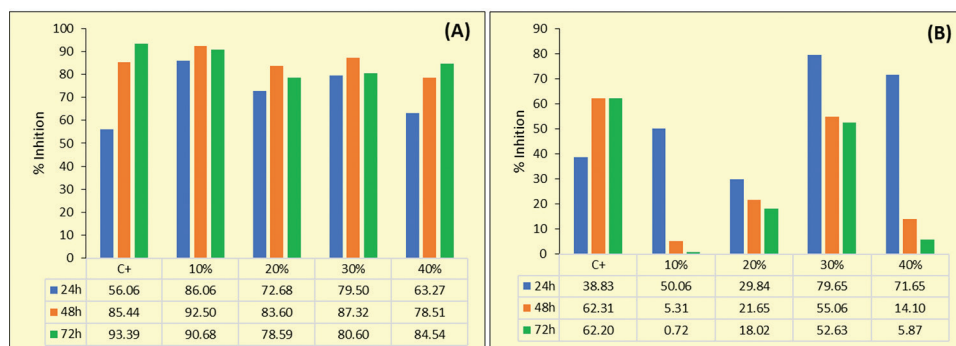


Figure 2. Growth inhibition (%) of *E. coli* (A) and *S. aureus* (B) after treatment by chitosan-*M. oleifera* hydrogel at 24, 48, and 72h

The antibiofilm activity of chitosan-*M. oleifera* hydrogel in *E. coli* and *S. aureus* after treatment is shown in Table 4. In *E. coli*, all concentrations showed limited inhibition at 24 h, a clearer reduction at 48 h, and renewed biofilm growth by 72 h, with the 40% formulation performing best. *S. aureus*, however, exhibited a clearer dose-dependent pattern: the 30% formulation consistently produced the lowest OD values and the most stable inhibition. The 40% treatment weakened over time, while the 10% and 20% concentrations remained ineffective. Significant differences appeared at 24 and 48 h only. Overall, antibiofilm effects were short-lived in *E. coli* but more sustained in *S. aureus*, especially with the 30% formulation.

Table 3. Optical density (OD) of *E. coli* and *S. aureus* inhibition after treatment by chitosan-*M. oleifera* hydrogel at 24, 48, and 72 h

Treatment Groups	n	OD (Mean ± SD)							
		<i>E. coli</i>			<i>p-value</i>	<i>S. aureus</i>			<i>p-value</i>
		24h	48h	72h		24h	48h	72h	
C+	3	1.00±0.03	0.31±0.32	0.13±0.01		0.48±0.05	0.12±0.00	0.12±0.00	
10%	3	0.32±0.03	0.16±0.01	0.18±0.02		0.39±0.07	0.31±0.11	0.32±0.11	
20%	3	0.62±0.02	0.35±0.07	0.41±0.07	0.038	0.55±0.06	0.26±0.02	0.27±0.02	0.009
30%	3	0.47±0.13	0.27±0.04	0.37±0.03		0.16±0.05	0.15±0.02	0.15±0.02	
40%	3	0.84±0.21	0.45±0.07	0.29±0.16		0.22±0.03	0.28±0.04	0.30±0.04	
p		0.007	0.038	0.015		0.009	0.033	0.03	

Statistical test: Kruskal–Wallis ($p < 0.05$).

Table 4. Optical density (OD) values of *E. coli* and *S. aureus* biofilm after treatment by chitosan-*M. oleifera* hydrogel at 24, 48, and 72 h

Treatment	n	OD (Mean±SD)							
		<i>E. coli</i>			<i>p</i> -value	<i>S. aureus</i>			<i>p</i> -value
		24 h	48 h	72 h		24 h	48 h	72 h	
C+	3	0.10±0.00	0.01±0.00	0.10±0.03		0.48±0.05	0.12±0.00	0.12±0.00	
10%	3	0.59±0.04	0.03±0.01	0.20±0.17		0.39±0.07	0.31±0.11	0.32±0.11	
20%	3	0.59±0.06	0.04±0.05	0.64±0.11	0.064	0.55±0.06	0.26±0.02	0.27±0.02	0.019
30%	3	0.50±0.32	0.02±0.01	0.41±0.47		0.16±0.05	0.15±0.02	0.15±0.02	
40%	3	0.36±0.11	0.01±0.01	0.39±0.19		0.22±0.03	0.28±0.04	0.30±0.04	
p		0.064	0.087	0.074		0.019	0.015	0.085	

Statistical test: Kruskal–Wallis ($p < 0.05$)

Figure 3 illustrates the inhibition of biofilm formation of chitosan-*M. oleifera* hydrogel against *E. coli* and *S. aureus*. In *E. coli*, degradation was minimal at 24 h but peaked at 48 h, especially in the 30% and 40% groups, before declining at 72 h, with moderate residual activity in the 10%, 30%, and 40% treatments. *S. aureus* showed a similar trend: early degradation appeared in the 10% and 20% groups, maximal removal occurred at 48 h with the 30% and 40% formulations, and activity decreased by 72 h, although the 40% and 10% groups showed higher remaining effects. Overall, biofilm degradation was strongest at 48 h, with the 30% and 40% formulations demonstrating the most consistent performance against both species.

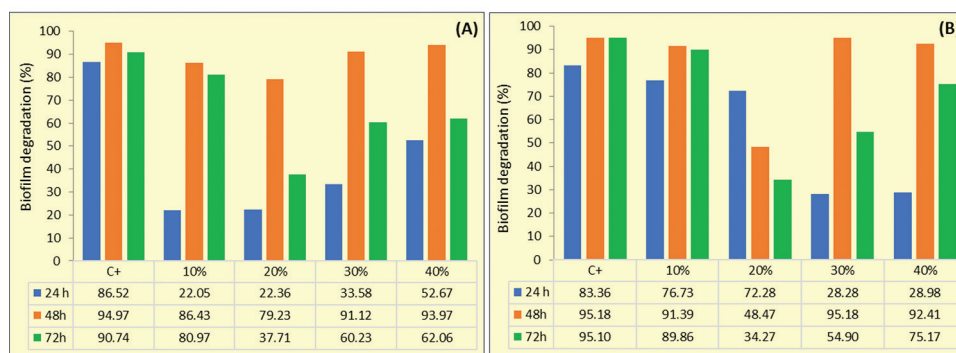


Figure 3. Biofilm degradation (%) of *E. coli* (A) and *S. aureus* (B) after treatment by chitosan-*M. oleifera* hydrogel at 24, 48, and 72 h

Figures A, B, and C represent *E. coli* at 24 h, 48 h, and 72 h, respectively, and D, E, and F represent *S. aureus* at 24 h, 48 h, and 72 h, respectively. At 24 h, both bacteria showed varying patterns between inhibition activity and biofilm percentage across concentrations. At 48 h, a closer relationship between inhibition and biofilm values was detected at specific concentrations (indicated by boxed areas). At 72 h, certain concentrations demonstrated a stronger inverse relationship, suggesting improved antibiofilm performance at these points. The boxed areas highlight concentrations with the closest inhibition-biofilm relationship.

The relationship between inhibition and biofilm formation in *E. coli* and *S. aureus* becomes more coordinated over time (Figure 4). At 24 h, correlations were weak across concentrations, but by 48 h, a clearer pattern emerged—especially at 30%, where both species showed more aligned inhibition and biofilm responses. At 72 h, *E. coli* displayed a stronger inverse relationship, particularly at 10%, while *S. aureus* remained less consistent. However, the 40% concentration produced the most stable and closely aligned patterns against both bacteria species. These trends indicate that the formulation achieved its most synchronized antibacterial and antibiofilm activity between 48 h and 72 h.

Table 5 shows that *E. coli* exhibits more consistent relationships between inhibition and biofilm responses than *S. aureus*. In *E. coli*, strong correlations across incubation periods—particularly at 48–72 h—indicate stable antibacterial activity that increasingly aligns with biofilm reduction. By contrast, *S. aureus* displays weaker and irregular correlations, with significant links appearing only at certain time points, although a notable biofilm correlation at 24–48 h suggests a more coordinated early response. Overall, the nanoencapsulation achieves more synchronized antibacterial and antibiofilm effects in *E. coli* than in *S. aureus*.



Figure 4. Relationship between growth inhibition and biofilm formation of *E. coli* and *S. aureus* treated with chitosan-*M. oleifera* hydrogel at different incubation times.

Table 5. Correlation between inhibition and biofilm formation of *E. coli* and *S. aureus* treated by chitosan-*M. oleifera* hydrogel

		<i>E. coli</i>						<i>S. aureus</i>					
		Inhibition			Biofilm			Inhibition			Biofilm		
		24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h
Inhibition	24 h	<i>r</i>											
			0.940**	0.898**	0.226	0.516*	0.555*			0.165	-0.101	0.443	0.497*
	48 h	<i>p</i>	0.000	0.000	0.367	0.028	0.017	0.193	0.066	0.514	0.691	0.066	0.036
		<i>r</i>								0.483*	0.190	0.211	0.207
			0.940**	0.962**	0.436	0.600**	0.587*	0.322	0.211	0.042	0.449	0.401	0.411
	72 h	<i>p</i>	0.000	0.000	0.071	0.008	0.010	0.193	0.005	0.042	0.365	0.550*	0.043
Biofilm	24 h	<i>r</i>											
			0.962**		0.497*	0.565*	0.674**	0.165	0.483*		0.137	0.018	0.867
	48 h	<i>p</i>	0.000	0.000	0.036	0.015	0.002	0.514	0.042	0.365		0.634**	0.148
		<i>r</i>								0.190		0.005	0.557
			0.226	0.436		0.166	0.265	-0.101	0.190	0.137	0.634**		0.104
	72 h	<i>p</i>	0.367	0.071		0.511	0.288	0.691	0.449	0.005	0.005	0.682	
Biofilm	24 h	<i>r</i>											
			0.516*	0.565*	0.166		0.005	0.443	0.211	0.550*	0.634**		
	48 h	<i>p</i>	0.028	0.008	0.511		0.985	0.066	0.401	0.018	0.005		
		<i>r</i>								0.043	0.148	0.104	
			0.555*	0.587*	0.265	0.005		0.497*	0.207	0.043	0.557	0.682	
	72 h	<i>p</i>	0.017	0.010	0.288	0.985		0.036	0.411	0.867	0.557	0.682	

Significant correlations are indicated by * ($p < 0.05$) and ** ($p < 0.01$)

DISCUSSION

Figure 1 and Table 2 show that *M. oleifera* extract contains predominantly lipophilic compounds, particularly cis-9-tetradecen-1-ol, with additional fatty acids, esters, and their derivatives, which are widely reported to exhibit antibacterial and antioxidant properties (Casillas-Vargas et al., 2021; Mugayi & Mukanganyama, 2024). Minor metabolites such as phytol may further enhance antibacterial action through terpenoid-mediated stress modulation (Almeida-Bezerra et al., 2024). Together, these compounds support a synergistic, multi-target mechanism consistent with lipid-based agents that disrupt membranes, metabolism, and biofilm formation processes (Pérez-Flores et al., 2025). These bioactive components may contribute to membrane perturbation and metabolic interference in bacterial cells. Previous studies have also highlighted that *M. oleifera* contains diverse functional compounds, including polysaccharides and fatty acid derivatives, which are associated with antibacterial and biofunctional activities (Dzuovor et al., 2022; Yang et al., 2022).

Nanoencapsulation enhanced antibacterial activity, potentially by improving the stability and dispersion of bioactive compounds. In *E. coli*, the 10% formulation showed the most consistent inhibitory effect (Table 3 and Figure 2A), whereas in *S. aureus*, the 30% formulation demonstrated more sustained activity (Figure 2B). These differences may be related to variations in bacterial cell wall structure and permeability between Gram-negative and Gram-positive bacteria (Mulat et al., 2025). A reduction in activity at higher concentrations may be associated with aggregation effects that limit effective interaction with bacterial cells.

The antibiofilm assay in this study specifically evaluated inhibition of biofilm formation rather than disruption of preformed biofilms. The results demonstrated time-dependent and species-specific responses. In *E. coli*, inhibition peaked at 48 h before declining at 72 h (Table 4 and Figure 3A), suggesting an effect on early-stage biofilm development. This observation is consistent with previous findings indicating that *Moringa*-based materials may inhibit the initial attachment of *E. coli* to abiotic surfaces (Bessalah et al., 2024). Similarly, plant-derived compounds have been reported to interfere with bacterial adhesion and early biofilm formation processes (Liu et al., 2019; Neto et al., 2017).

In *S. aureus*, the 30% formulation showed relatively stable inhibition at 48 h (Figure 3B), which may reflect interference with early biofilm matrix formation. Previous studies have reported that bioactive compounds from *M. oleifera*, including fatty acids such as behenic acid, can inhibit biofilm formation in *S. aureus* (Kim & Kim, 2025). However, these effects should be interpreted within the context of early-stage biofilm inhibition, as mature biofilm disruption was not assessed in the present study.

The current results are also consistent with previous findings that chitosan effectively inhibits biofilm formation by *Streptococcus mutans*, *Streptococcus*

sanguinis, and *Candida albicans* (Djunaidy & Putri, 2020; Garcia et al., 2023; Haniastuti et al., 2023). In this study, the combination of chitosan and *M. oleifera* extract suggested a potential synergistic effect: chitosan offered physical antibacterial properties, while *Moringa* bioactive interfered with quorum sensing (Haris & Ahmad, 2024) and oxidative stress pathways (González-Romero et al., 2022).

The relationship between antibacterial activity and biofilm formation showed a clear pattern in *E. coli*, where stronger growth inhibition was associated with reduced biofilm production (Figure 4), suggesting that antibacterial effects may contribute to limiting early-stage biofilm development. By contrast, this relationship was less consistent in *S. aureus*, possibly due to differences in cell wall structure and biofilm architecture. These findings are consistent with previous reports on variability in biofilm formation and susceptibility among bacterial species (Jayathilaka et al., 2022). The observed effects may be associated with bacterial membrane perturbation and subsequent interference with early biofilm formation processes (Ghareeb et al., 2022).

Table 5 shows that *E. coli* exhibited a clearer and more consistent relationship between growth inhibition and biofilm reduction, particularly at 48–72 h, suggesting sustained antibacterial activity and its association with reduced biofilm formation. By contrast, *S. aureus* showed a weaker and less consistent relationship, which may be related to its thicker peptidoglycan layer and more complex biofilm structure (Jayathilaka et al., 2022; Mulat et al., 2025). The correlation observed at 24–48 h may reflect an effect on early-stage biofilm development before maturation.

Taken together, the findings suggest that chitosan–*M. oleifera* hydrogel exhibits antibacterial activity and inhibits early-stage biofilm formation under *in vitro* conditions, particularly at concentrations of 10–30% and at 48 h. These effects may be associated with interactions between chitosan and the bioactive compounds present in *M. oleifera*, which could influence bacterial viability and the initial adhesion process. However, the present findings are limited to *in vitro* observations and do not directly reflect clinical or therapeutic efficacy. Therefore, further studies, including mechanistic investigations and *in vivo* evaluations, are required to elucidate the potential applications and limitations of this formulation.

CONCLUSION

Chitosan–*M. oleifera* hydrogel demonstrated concentration- and time-dependent antibacterial activity: the 10% formulation showed greater inhibition against *E. coli* and the 30% formulation against *S. aureus*. The strongest bacterial inhibition and reduction in biofilm formation were observed at 48 h. These results demonstrate that the hydrogel formulation possesses antibacterial characteristics and can impede early-stage biofilm development under *in vitro* conditions. However, as this study was conducted in *in vitro* conditions, further studies are required to confirm its broader applicability.

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Authors' contribution

CNT and HH substantially contributed to the conception and design of the study, methodology development, and drafting of the initial manuscript. SR, NNN, AF, and SLA made substantial contributions to data acquisition and laboratory investigation. HZ, AS, and TNS contributed to data curation, interpretation of the results, and critical revision of the manuscript for important intellectual content. HA, FJ, and DD facilitated project administration, oversight, and fundraising efforts. All authors have read and approved the final manuscript.

Ethical statement

This study did not involve experimental research on live animals. All procedures were conducted in accordance with applicable institutional and ethical standards. ARRIVE guidelines are not applicable to this study.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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ANTIBAKTERIJSKA I ANTIBIOFILM AKTIVNOST EKOLOŠKI PRIHVATLJIVE HIDROGELNE FORMULACIJE MORINGA OLEIFERA-HITZANA PROTIV *ESCHERICHIA COLI* I *STAPHYLOCOCCUS AUREUS*

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Kratak sadržaj

Escherichia coli i *Staphylococcus aureus* predstavljaju dominantne uzročnike piometre kod goveda, a njihova izražena sposobnost formiranja biofilma značajno doprinosi neuspehu terapije i eskalaciji antibakterijske rezistencije. Cilj ovog ispitivanja bio je razvoj i procena ekološki prihvatljive hidrogel formulacije hitzana koja sadrži ekstrakt lista biljke *Moringa oleifera* kao alternativnog antibakterijskog i antibiofilmskog agensa. Etanolni ekstrakt *M. oleifera* je analiziran korišćenjem GC-MS. Formulacije hidrogela (10, 20, 30 i 40%) su pripremljene i njihova sposobnost inhibicije rasta bakterija i razgradnje biofilma tokom 24, 48 i 72 h procenjena korišćenjem spektrofotometrije mikroploče. Formulacije su pokazale jasne aktivnosti zavisne od koncentracije i vremena. Koncentracija od 10% dovela je do najjače inhibicije *E. coli*, dok je *S. aureus* najbolje reagovao na formulu od 30%, koja je održala stabilne inhibitorne efekte tokom svih perioda inkubacije. Testovi biofilma su otkrili prolaznu supresiju kod *E. coli*, sa maksimalnom razgradnjom nakon 48 h, dok je inhibicija *S. aureus* bila dugotrajnija, naročito u prisustvu 30% formulacije. Korelaciona analiza pokazala je jaču usklađenost između inhibicije rasta i smanjenja biofilma kod *E. coli*, što ukazuje na sinhronizovanje antibakterijske i antibiofilmske efekte ($p < 0,05$). Hidrogel *M. oleifera*-hitzan pokazao je obećavajuću dvojaku aktivnost protiv planktonskih i bakterija koje stvaraju biofilm, čime se ističe njegov potencijal kao ekološki prihvatljivog terapijskog kandidata protiv infekcija materice kod goveda i smanjuje zavisnost od konvencionalnih antibiotika.

Ključne reči: *Moringa oleifera*, hitzan, *Escherichia coli*, *Staphylococcus aureus*, biofilmovi