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Enhanced Antimicrobial Efficacy of Propolis Solid Lipid Nanoparticles Against Methicillin-Resistant Staphylococcus aureus Isolated from Raw beef

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ABSTRACT

Methicillin-Resistant *Staphylococcus aureus* (MRSA) in raw beef poses a serious public health threat by facilitating resistant infections, spreading through food chains, and enabling animal to human transmission. Addressing this issue requires coordinated efforts across food safety, agriculture, and healthcare sectors. A total of 112 raw beef samples were collected from the boneless outer surface of shoulder and thigh regions of beef carcasses. Methicillin-resistant S. aureus then were isolated by traditional methods. Propolis-loaded solid lipid nanoparticles (LNPs-PL) were prepared using ultra-sonication and high-shear homogenization techniques. The manufactured formulation's encapsulation efficiency, loading capacity, and physicochemical characteristics were evaluated. Morphology was examined using scanning electron microscopy (SEM), and chemical makeup of the tested regular oil and LNPs-PL was analyzed using GC/MS. The antibacterial activity of LNPs-PL was compared with that of free propolis (PL) using in vitro assays. The prevalence of MRSA among raw beef samples was 50/112 (44.6%). among which 20 isolates (40%) were confirmed as MRSA. LNPs-PL demonstrated significantly enhanced antimicrobial activity, with an inhibition zone of 36.0 mm compared to 14.0 mm for PL. The findings indicate that modifications should be made in the loading capacity, encapsulation efficiency, and physicochemical properties. Also the findings confirmed that SLNs are effective PL carriers for controlling bacterial and fungal diseases. These findings suggest that LNPs-PL could be developed as natural antimicrobial agents for food preservation or surface sanitization in the food industry

Keywords

Methicillin-Resistant S. aureus, Raw beef, Propolis nanoparticles, Antimicrobial efficacy, Foodborne pathogens.

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Abbreviations

LNPs-PL: Propolis Solid Lipid Nanoparticles

MRSA: Methicillin-Resistant Staphylococcus aureus

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Introduction

Meat is an essential component of human nutrition, providing high quality protein, essential and non-essential essential amino acids, minerals and vitamins [1]. However, it also serves as an ideal medium for the growth and proliferation of spoilage microorganisms and serious pathogens as *Bacillus cereus*, *Escherichia. coli* O157:H7, Salmonella spp., Listeria monocytogenes and *S. aureus* [2]. Food of animal origin, such as meat and meat products, can be contaminated with *methicillin-resistant S. aureus* (MRSA) during various stages of production. Contamination may occur during slaughtering or milking processes from colonized or infected animal. This can lead to contamination of food products and environment, and ultimately the transmission of MRSA to humans through direct contact with contaminated meat [3]. The emergence of antimicrobial resistance among foodborne pathogens is largely attributed to the extensive use of antibiotics. The spread of MRSA germs to the human population can be attributed to improper hygienic standards and procedures throughout the slaughtering, transportation, and marketing processes, as evidenced by meat contamination [4]. Propolis, a natural resinous substance collected by honeybees from plant parts, buds, and exudates, has gained attention due to its potent biological activities. Its chemical composition varies depending on climates and geographical origin but is generally rich in polyphenols and flavonoids, which are responsible for its antimicrobial properties. In recent years, the use of natural antimicrobial agents has become increasingly important. Furthermore, because nanoparticles may pass through bacteria and bacterial biofilms, they may be a useful tool for limiting the spread of bacterial infections [5]. The goal of this study was to evaluate the enhanced antimicrobial efficacy of propolis-loaded solid lipid nanoparticles (LNPs-PL) against methicillin-resistant Staphylococcus aureus (MRSA) isolated from raw beef, and to compare its performance with free propolis.

Results

Prevalence of MRSA in raw beef samples

The microbiological analysis of 112 raw beef collected From different markets in Wasit city during the summer season revealed that four (MRSA) isolates, were detected from the pooled samples from different brand and locations. 50 samples (44.6 %) were positive for *S. aureus,* and among these, 20 isolates (40%) were confirmed as MRSA (Table 1, and figure 1). Also the Conventional PCR screening revealed that all the isolates were positive for intrinsic methicillin resistance (mecA) gene, producing a 163 bp amplicon (Figure 2).

Preparation and Characteristics of LNPs-PL

DLS analysis

Particle Size and Morphology

Dynamic light scattering (DLS) analysis revealed an increase in nanoparticle size from 204.16 nm to 244 nm after loading with propolis extract, confirming successful encapsulation. The SEM image of LNPs-PL

Table 1.
Prevalence of MRSA in Raw Beef Samples (n=112)

Sample Type	Total Samples (N)	*S. aureus* Positive (%)	MRSA Positive (%) (95% CI)
Raw Beef	112	50 (44.6 ± 4.7%)	20/50 (40.0 ± 6.9%)

Data analyzed by Chi-square test; $p < 0.05$
S. aureus prevalence calculated as (positive samples/N) × 100.

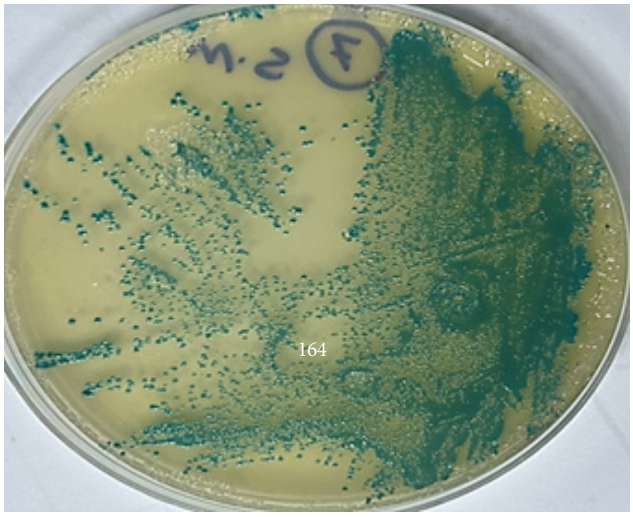

Figure 1.
Methicillin-Resistant *S. aureus* (MRSA) on HiCrome MeReSa Agar Base medium

Abbreviations-Cont'd

SEM: Scanning electron microscopy
GC-MS: Gas Chromatography-Mass Spectrometry
EE: Encapsulation efficiency
LC: Loading capacity
ICMSF: The International Commission on Microbiological Specification for Foods
ATCC: The American Type Culture Collection
UV–Vis: Ultraviolet–visible spectrophotometry
Au–Pd: Gold-Palladium
DSC: Differential Scanning Calorimetry
MHA: Mueller-Hinton agar
ANOVA: Analysis of Variance
PCR: Polymerase Chain Reaction
PDI: Polydispersity Index
mV: millivolt
HERP: Hydroethanolic extract of red propolis
PLGA: Poly(lactic-co-glycolic acid)
MIC: Minimum Inhibitory Concentration
3D: A three-dimensional

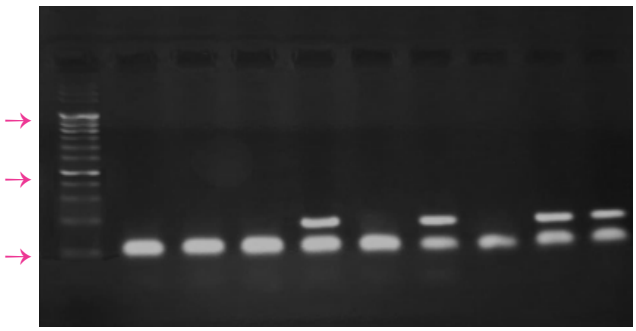

Figure 2.
Agarose gel electrophoresis image shows PCR product analysis of *S. aureus*. Lane M Marker ladder (50-1000bp), lanes (1-10): mecA gene of *S. aureus* isolate with 163bp.

is displayed in Figure 3. SEM scan revealed that the nanoparticles were spherical in shape.

Polydispersity Index (PDI)

The PDI values provide critical insights into the size distribution and homogeneity of the nanoparticle populations. A PDI of 0.421 for the free LNPs (Figure 3) suggests a moderately broad size distribution, indicating some variability in particle size. Conversely, a PDI of 0.277 for the LNPs-PL (Figure 4) indicates a more homogeneous and uniform size distribution compared to the free LNPs. This suggests that the incorporation of propolis extract has contributed to a more uniform population of nanoparticles. Generally, a PDI below 0.3 is considered acceptable for pharmaceutical applications, indicating a relatively homogeneous population. The figures 4, 5 and 6 representation clearly demonstrates the size increase post propolis loading. The peak corresponding to LNPs-PL is shifted to the right, indicating a larger size compared to the free LNPs. The narrower peak width for LNPs-PL, which is consistent with the lower PDI value, suggests a more uniform size distribution. Additionally, the presence of a small peak at the higher size range in the LNPs-PL sample indicates the aggregation of a minor fraction of the nanoparticles. In summary, the data suggests that the incorporation of propolis extract into the lipid nanoparticles has resulted in a size increase and a more uniform particle size distribution. The lower PDI value for LNPs-PL supports the notion of enhanced homogeneity, which is advantageous for pharmaceutical applications. The size distribution data, corroborated by the intensity graph, further validates these findings.

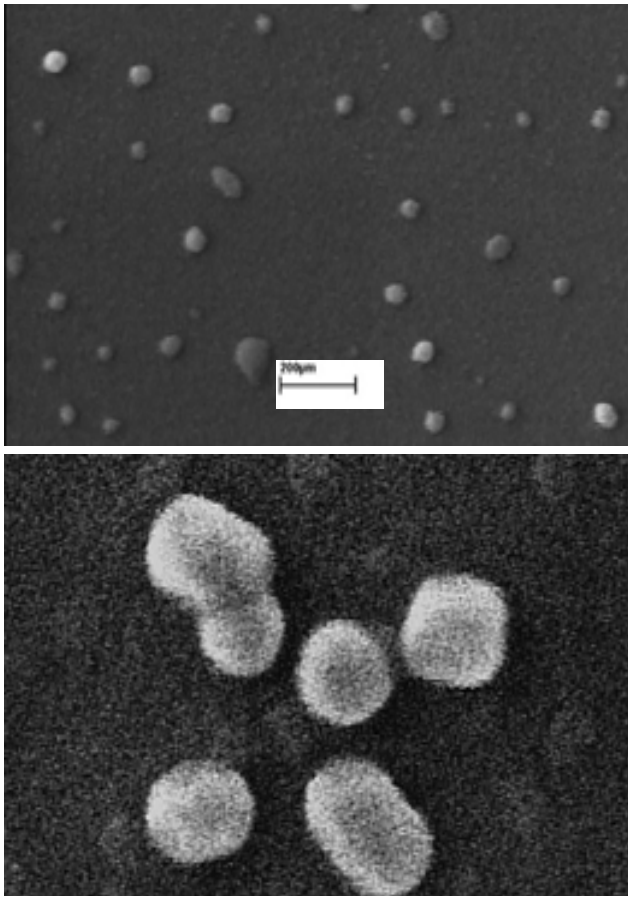

Figure 3.
Appearance of LNPs-PL under scanning electron microscopy(SEM image of LNPs-PL at 50,000× magnification. Scale bar: 200 nm)

Zeta Potential

The zeta potential of the lipid nanoparticles becomes more negative and shifted from -36.7 mV to -50.7 mV after loading with propolis extract. This increase in negative surface charge indicates a significant alteration in the surface properties of the nanoparticles. The propolis extract likely contributed negatively charged components to the nanoparticle surface, and thus enhanced the overall negative charge.

The antimicrobial activity of LNPs-PL

According to the antimicrobial activity data, LNPs-PL exhibited 74%, inhibition on the growth of MRSA. In addition, LNPs-PL exhibited significantly higher antibacterial activity against tested microorganisms compared to free PL. The inhibition zone for LNPs-PL was 36.0 ± 1.2 mm, while free propolis showed only 14.0 ± 0.8 mm (Table 2 and Figure 7). The 2.6-fold larger inhibition zones of LNPs-PL ($p < 0.001$, Cohen's d = 3.2) confirm not just statistical but practical superiority over free propolis.

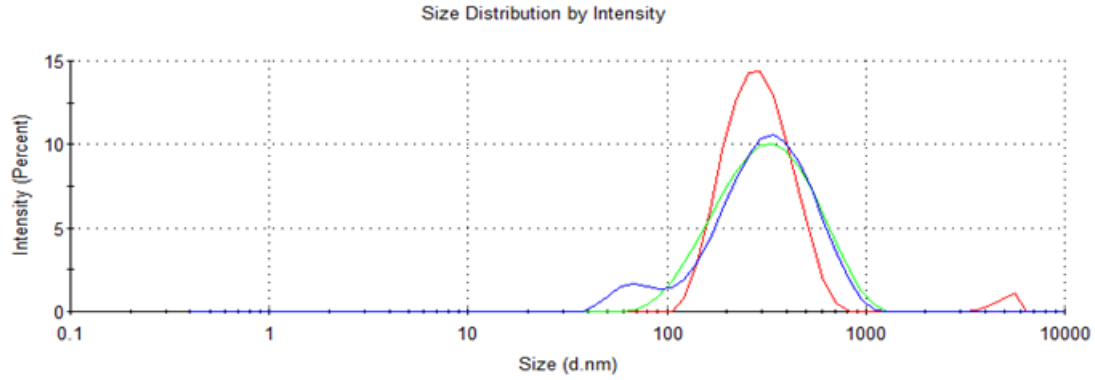

Figure 4.
(PDI) to LNPs (Size distribution profile of free LNPs showing polydispersity index (PDI = 0.421).

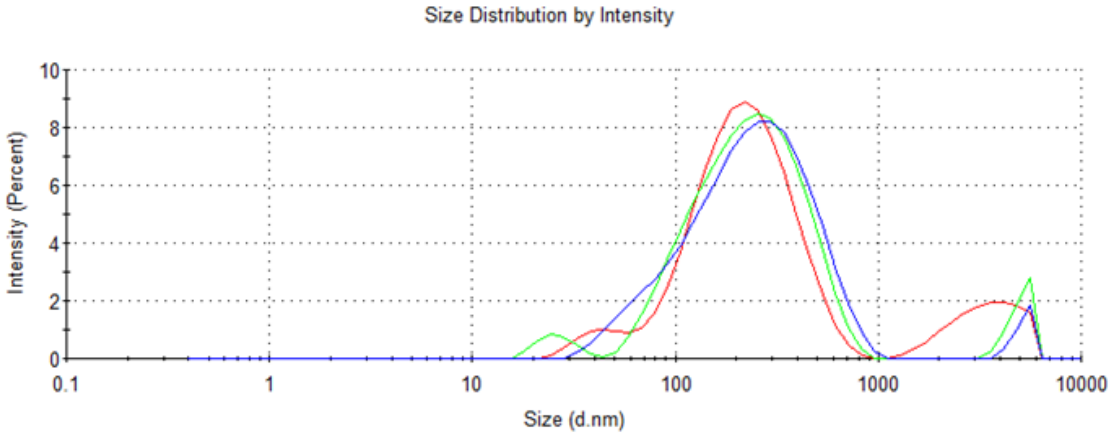

Figure 5.
(PDI) LNPs-PL (Size distribution profile of LNPs-PL showing improved homogeneity (PDI = 0.277).

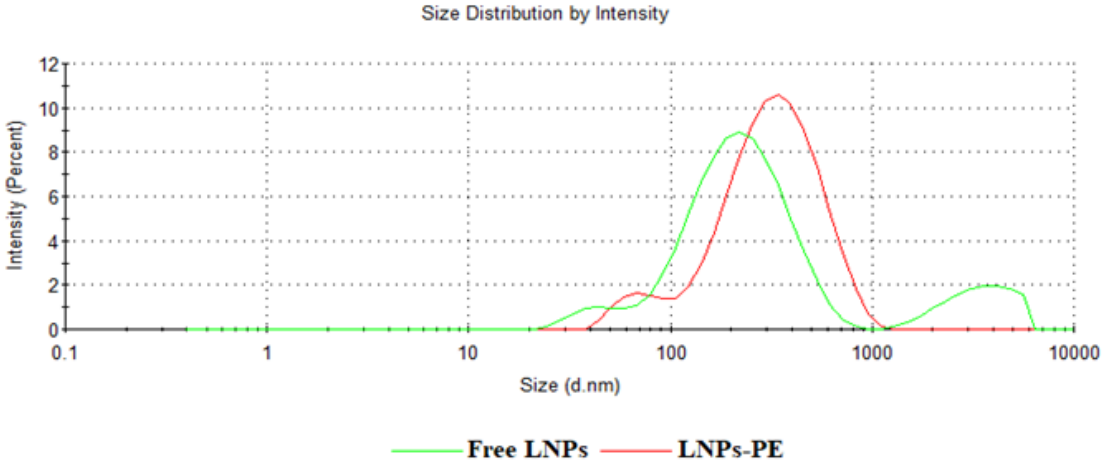

Figure 6.
LNPs-PL (Intensity-based particle size distribution of LNPs-PL demonstrating increased average hydrodynamic diameter post-propolis loading).

Table 2.
Antimicrobial Activity of LNPs-PL vs. Controls

Treatment	Zone of Inhibition (mm, Mean ± SD)	p-value (vs. PL)	Statistical Test
LNPs-PL	36.0 ± 1.2**	<0.01	One-way ANOVA
Propolis (PL)	14.0 ± 0.8	—	(Tukey's post-hoc)
Empty SLNs	0 (no activity)	—	

Data represent mean ± standard deviation of triplicate experiments.
Asterisk (**) indicates statistical significance ($p < 0.01$) vs. PL.
ANOVA assumptions (normality, homogeneity of variance) were verified.

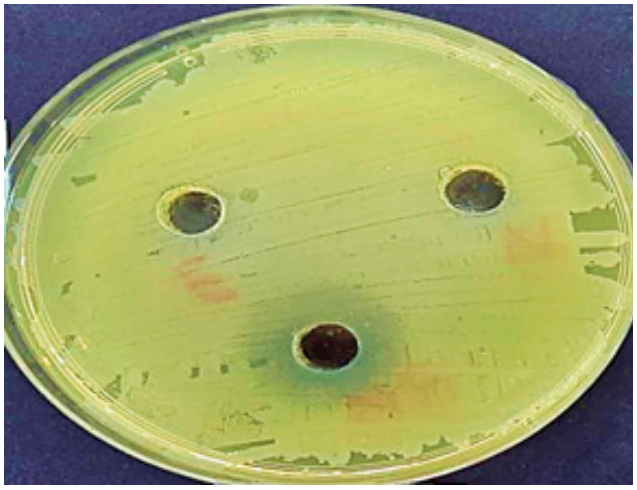

Figure 7.
Inhibition zone as shown by LNPs-PL and regular PL on MRSA

Discussion

In this study, we aimed to compare the antimicrobial activity of propolis-loaded solid lipid nanoparticles (LNPs-PL) with that of free propolis against MRSA isolated from raw beef.

The high prevalence of MRSA in raw beef observed in this study suggests contamination from multiple sources, including infected animals and improper handling during slaughtering and processing (animal meat or workers who handled these meat may carried these bacteria as normal flora in their nasal cavity and their skin). These findings are consistent with previous studies reporting contamination sources. Scott et.al [6], reported that the meat can be contaminated with *S. aureus* during manufacture and handling. Waters et.al [7] reported a high prevalence of S. aureus isolated from fresh bovine meat, and Porpino et.al [8], reported that contamination of meat may occur through the different stages of food preparation as production, distribution, storage in the retailing supermarkets and during improper refrigeration temperature, as a results an enhance in bacterial growth can occur, leading to productions of enterotoxins. In the study done by Mohammed et.al, reported that food handlers who work with meat also can be the sourse of meat contamination by *S. aureus* [9].

This size augmentation implies that the propolis extract has been effectively integrated into the lipid matrix or adsorbed onto the surface of the LNPs. Such an increase is anticipated and confirms the presence of the propolis extract within the nanoparticle system [10].

The SEM scan revealed that the prepared SLNs had a spherical shape. Particle size distribution was similar to DLS. After loading, the nanocapsules had a spherical form, high dispersion, and a limited size distribution [11].

Because of their spherical morphology, the SLNs have the finest ability to have controlled release and to protect against encapsulated essential oil. This is because, compared to other types of nanoparticles, the spherical shape has the lowest contact surface with the aqueous medium of the dispersed phase and a longer path for the essential oil contained in the nanoparticles to flow [12].

Zeta potential is a critical parameter in assessing the stability of colloidal dispersions. Typically, zeta potential values exceeding +30 mV or below -30 mV are indicative of good colloidal stability, as the electrostatic repulsion between particles mitigates aggregation. Based on this, in the current study both the free LNPs and LNPs-PL exhibited zeta potential values within this range. Notably, the more negative zeta potential of the LNPs-PL (-50.7 mV) implies even greater stability compared to the free LNPs. This enhanced stability can be attributed to the additional negatively charged components provided by the propolis extract, which increase electrostatic repulsion and prevent particle aggregation [13, 14].

The significantly higher antimicrobial activity of LNPs-PL, in this study and in the study done by Jaiswal et.al [15], compared to free propolis can be attributed to improved growth inhibition of the tested bacteria, even at lower concentrations. The may be because the

PL is efficiently delivered by the vehicle (SLNs) and had better interaction with the bacteria [16].

Some of its constituents appear to be connected to the mechanism of propolis' antimicrobial action. Propolis's combination activity, which inhibits protein synthesis and bacterial growth by stopping cell division, is linked to its strong bacteriostatic and bactericidal actions [17]. Propolis contains high concentration of flavonoids, such as galangin, pinocembrin, and pinobanksin, which contribute to its antimicrobial (antibacterial and fungicidal) properties. These compounds may be the primary cause of the Propolis activity. For example, galangin and caffeic acids, two enzymatic inhibitory agent, are in charge of preventing the growth and multiplication of bacteria. Furthermore, several of the active ingredients in propolis have the potential to partially bacteriolyze the cell wall and cytoplasmic membrane. Flavonoids alter the permeability of the inner membrane of microorganisms and have an impact on the bacterial membrane potential [18]. Because smaller particles have better penetration and are therefore more effective, PNs' antimicrobial activity is validated. Johnson et.al [19] examined the impact of PLGA nanoparticles containing a hydroethanolic extract of red propolis (HERP) (PLG-AHERP-NPs) on the breakdown of harmful biofilms. According to this research, PLG-AHERP-NPs outperformed gram-positive bacteria in terms of MIC and biofilm formation. Variable cell wall and membrane structure in organisms can be linked to this discrepancy [19]. We mean the ingredients of propolis, specifically the flavonoids and polyphenols (such as galangin, pinocembrin, pinobanksin, and caffeic acids) that are naturally present in propolis.We do not mean adding extra or external chemicals. We mean that when propolis is loaded into nanoparticles, its natural active ingredients (flavonoids and polyphenols) are delivered more effectively, which enhances antibacterial activity.

It is also useful against abnormalities of the mouth, particularly dental problems. Propolis's polyphenols and flavonoids interact with a variety of microbial proteins to establish ionic and hydrogen connections, changing the protein's three-dimensional (3D) structure and, its function [20]. Consequently, in comparison to propolis, the addition of these chemicals in propolis nanoparticles can enhance their ability to prevent bacterial growth.

Materials & Methods

Declaration on AI use

The authors declare that no artificial intelligence (AI)-assisted technologies were used in the preparation, writing, editing, data analysis, or illustration of this manuscript.

Meat Sampling Technique

In the present research, a systematic and extensive sampling protocol was employed to examine the contamination of S. aureus and MRSA in raw beef. A total of 112 beef samples (500 g each) were collected aseptically from the boneless outer surfaces of shoulder muscles (n=56) and thigh muscles (n=56). The samples were collected from randomly chosen carcasses in three main slaughterhouses and ten licensed retail butchers across Wasit City, Iraq, to ensure proper representation of both the production and consumer points. Sampling was randomized and occurred over spring and summer periods (April to October 2024) to provide for potential temperature-dependent microbial variation, using sterile knives and pre-labeled polyethylene bags to prevent cross-contamination. The samples were transported to the laboratory in insulated iceboxes at 4 °C and processed within 6 hours to promote microbial viability. Prior to analysis, the samples were all aseptically trimmed of fat and connective tissue and homogenized in 0.1% peptone water for microorganism enumeration. To validate the integrity of sampling, negative controls (sterile swabs/blank peptone water) and positive controls (ATCC 43300 MRSA and ATCC 25923 S. aureus) were run in parallel.

This protocol, standardised in accordance with ICMSF (2002) guidelines, enabled the detection of MRSA prevalence that was statistically significant with control of confounding variables by randomisation and stratification by anatomical cut and source (60% abattoir/40% retail). Strength in the methodology provides confidence in follow-on antimicrobial efficacy testing of propolis nanoparticles on isolated MRSA strains.

Isolation of S.aureus

The bacteria were cultured on a variety of media, such as mannitol salt agar, MacConkey agar, and blood agar, which is specific to *S. aureus*. Isolated bacteria were identified based on colony morphological shape, size, colour, and pigment synthesis after incubating the samples at 37˚C for 24 hours.

Propolis exhaustive extraction (PL)

Frozen propolis samples were first put through a drying procedure in a dark environment kept at a certain temperature of 60 °C. This drying step was crucial to remove moisture content while preserving the bioactive compounds within the propolis. Following the drying process, the propolis was finely milled to obtain a uniform powder weighing 100 g. This powder was then homogenized in 100 ml of 70% ethanol, ensuring thorough mixing and extraction of the bioactive constituents. The homogenization process was allowed to proceed for 48 hours, facilitating the efficient extraction of bioactive compounds into the ethanol solvent. After the 48 hours extraction period, the homogenized sample was subjected to sonication to further enhance the extraction efficiency by disrupting cell walls and releasing intracellular contents. The sonicated mixture was then centrifuged at 10,000 rpm for 10 min to separate the solid residues from the liquid extract. The resulting supernatant was carefully filtered through Whatman No. 2 filter paper to remove any remaining particulate matter. The filtered extract was then concentrated using rotary evaporation at 60 °C, a step designed to remove the ethanol solvent and concentrate the bioactive compounds. The concentrated extracts were subsequently lyophilized, a freeze-drying process that removes water content while preserving the integrity and activity of the bioactive compounds. The final lyophilized extract, weighing 20 g, was carefully weighed and stored at 4 °C in a desiccator to prevent moisture absorption and degradation. This meticulously prepared dried extract was then stored under controlled conditions until it was required for further experimental analyses, this way the stability and consistency of the bioactive components for subsequent research applications was ensured [21].

Characterization of LNPs-PL

Particle size and zeta potential

The dynamic light scattering method (ZetaSizer Nano-ZS) was used to evaluate the SLNs formulations for particle size, polydispersity index, and zeta potential [22].

Determination of Encapsulation and Loading Efficiency

The percentage of the total amount of PL that was obtained in the formulation at the end of the process is known as the encapsulation efficiency (EE). The loading capacity (LC) was calculated by dividing the entrapped PL mass by the lipid (stearic acid) total mass. The EE and LC were calculated. Following careful weighing, 10 mg of LNPs-PL formulations were dissolved in 10 ml of methanol, and then centrifuged at 9,000 rpm for 30 minutes. The amount of PL in the supernatant was measured at 274 nm using a UV–Vis spectrophotometer (T80+ UV/VIS Spectrophotometer, PG Instruments Ltd.). To calculate the percentage of PL, a calibration curve was developed using a range of pure garlic oil concentrations. Measurements of each concentration was repeated for three times.

The sormula for calculation of the loading and encapsulation efficiency comes as follows:

$$A\text{-}B/A \times 100 = \%EE, \text{ and } A\text{-}B/C \times 100 = \%LC$$

Where:

A: The percentage of GO that was added to the formulation overall.

B: The supernatant's measured GO content.

C: The formulation's total weight of lipid (1% w/w stearic acid).

Morphology Study

The morphology of the nanoparticles has been examined using scanning electron microscopy (SEM). A thin layer of Au-Pd was sputter coated onto the nanoparticles, which were then placed on aluminum stubs and analyzed using a scanning electron microscope (SEM XL30, Philips, Netherlands) [23].

Differential scanning calorimetry (DSC)

For PL and LNPs-PL DSC scans, a Mettler DSC 821e (Mettler Toledo, Germany) was used. Five milligrams of the samples were put into pans made of aluminum oxide, sealed, and then examined. An empty aluminum pan served as a guide. The melting point of SLN dispersions was compared to that of the bulk lipid using DSC, which was carried out at a rate of 5 °C/min under N2 flow in a temperature range of 25 to 250 °C [24].

The antimicrobial activity of LNPs-PL

Mueller-Hinton agar (MHA) was the culture medium that was used for bacterial culture grow, also *S. aureus* was cultivated on mannitol salt agar. The well diffusion method was used to assess the antimicrobial activity of the tested compounds on MHA and mannitol salt agar. In the solid agar medium, 8 mm-diameter wells were made. 100 µL aliquots of every tested formulation were introduced into the wells. Using a metal caliper, the diameter of the zone of inhibition was measured and recorded in millimeters following a 24-hour stay in the incubator. The experiment was repeated for three times for each bacterium. The average zone of inhibition has been determined for each test LNPs-PL and the regular PL [25].

Statistical analysis

One-way ANOVA and Tukey's post-hoc test for inhibition zones were used to examine the data, and unpaired t-tests was used for microbial counts (α=0.05). Proportions (e.g., MRSA prevalence) included 95% Wilson score confidence intervals. All tests assumed normality ($p > 0.05$, Shapiro-Wilk) and equal variance (Levene's test).

Authors' Contributions

Conceptualization and study design, Data analysis and interpretation: AS, JA.

Methodology and laboratory work: JA, BJM.

Writing – original draft: JA.

Writing – review & editing, Supervision and project administration, Resources and ethical coordination: AS, BJM.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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