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RESEARCH PAPER

Formulation, characterization and co-delivery of curcumin-rosemary loaded niosomes to enhance antimicrobial activity against staphylococcus aureus strains

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ABSTRACT

Objective(s): *Staphylococcus aureus* is a common human pathogen, that infects thousands of people every year. The growing resistance of this bacterium to antibiotics has increased the need for developing more effective and natural antimicrobial medications. In this study, niosomes loaded *Rosmarinus officinalis* (rosemary)@curcumin were synthesized, and their antimicrobial and anti-biofilm activity on clinical *S. aureus* strains was investigated.

Materials and Methods: *Rosmarinus officinalis* (rosemary) extract was prepared through maceration and its compounds were identified by Gas chromatography–mass spectrometry (GC/MS). Niosomes loaded rosemary@curcumin were synthesized through thin-film hydration. Their characteristics were analyzed with dynamic light scattering (DLS)-zetasizer, scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR); and drug release test was studied using a dialysis bag. Finally, the antimicrobial and anti-biofilm activity against clinical strains of *S. aureus* were investigated using minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and crystal violet methods.

Results: Formulated niosomes were spherical, averaged 442 nm in size, and had uniform particle distribution. FTIR results indicated the successful encapsulation of rosemary@curcumin inside niosomes with 94.23% encapsulation efficiency. *In vitro* drug release studies showed a slow-release pattern of rosemary@curcumin from niosomes. The MIC and MBC values of niosomes loaded rosemary@curcumin were between 32.5 and 62.5 µg/mL and showed higher antimicrobial activity. Also, the results of the biofilm inhibition test showed that niosomes loaded rosemary@curcumin reduced the rate of biofilm formation between 2 and 4 fold.

Conclusion: Niosomes loaded rosemary@curcumin have suitable structure and surface characteristics, and can successfully inhibit *S. aureus* growth and prevent its biofilm formation potency.

Keywords: *Antimicrobial, Curcumin, Niosome, Rosmarinus officinalis, Staphylococcus aureus*

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INTRODUCTION

Antibiotics have a long history of application in the treatment of bacterial infections. However, the increasing resistance of different bacterial strains to these medications has raised the need for new strategies to fight pathogens. *Staphylococcus aureus* is among the most concerning gram-positive bacteria that has shown resistance to common antibiotics. In addition to its ability to adapt to different environmental conditions, it can form biofilms and cause deadly infections [1]. Biofilms are organized bacterial communities, enveloped by a matrix that consists of proteins, DNA, and polysaccharides. Biofilm-forming *S. aureus* strains have reduced susceptibility to antibiotics compared to their planktonic counterparts. This makes it challenging to efficiently treat them with the existing medications [2]. To deal with these problems, the use of natural compounds from medicinal plants has emerged as a promising alternative. These plants' natural antimicrobial compounds are effective in treating many infectious diseases and have fewer side effects

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than synthetic drugs [3].

Rosmarinus officinalis (rosemary) is one of these medicinal plants. This Mediterranean plant from the *Lamiaceae* is used as a spice in cooking and as a natural preservative in the food and pharmaceutical industries. Its high phenolic content is responsible for its vast medicinal properties. Some of the rosemary extract's notable compounds include carnosol, carnosic acid, rosmarinic acid, rosmanol, camphor, ursolic acid, alpha-pinene, and 1,8-cineol [4]. These compounds have shown therapeutic effects against conditions like inflammation, depression, and diabetes. They have also been useful against parasites, fungi, and bacteria. Various studies have attributed the antimicrobial effects of rosemary extract to the synergy between its different compounds. This possible synergy between rosemary's phenolic components suggests that the presence of a second substance may increase the effectiveness of the extract [4].

Another natural compound has shown synergy with other antibiotics against pathogens such as *S. aureus* is curcumin. This active component of turmeric grows in the tropical regions of South Asia. This hydrophilic polyphenol acts as a natural pigment and is used as a spice in cooking. It has strong anti-inflammatory, anti-fungal, anti-viral, antioxidant, antibacterial, and anti-biofilm properties [5]. Studies show that curcumin suppresses gram-positive and gram-negative bacteria by damaging their cell walls or cell membranes, interfering with their cellular processes, targeting their DNAs and proteins, and inhibiting bacterial quorum sensing [6]. With the synergistic effects of these compounds, the joint use of rosemary extract and curcumin may be an effective solution in the fight against *S. aureus*. However, despite the immense potential of these natural compounds as antibiotics, their use is limited due to the need for higher doses as a result of their low hydrophilicity, physical/chemical instability, low absorption rate, poor pharmacokinetics, first-pass metabolism, poor penetration, and accumulation in the body. In addition, extraction with organic solvents can make their direct use problematic for humans [7]. Therefore, new methods have been developed to formulate herbal extracts and safely deliver them to the body while increasing their therapeutic efficiency. Polymeric nanoparticles and lipid-based nanocarriers are among these methods. They have extensive usage in the pharmaceutical industry and offer advantages over conventional formulations. These drug delivery systems can increase the antibacterial activity of extracts and enhance their solubility, stability, penetration, and delivery [8]. Niosomes are one of these systems, and these non-ionic surfactant-based carriers form when amphiphilic molecules self-assemble in aqueous media. Due to their bilayer vesicle structure, niosomes can be used to transport hydrophilic and hydrophobic drugs simultaneously and deliver them through different routes such as eyes, skin, lungs, and mouth [9]. They are promising systems for the continuous, controlled, and targeted delivery of drugs [10].

Currently, there are no studies formulating niosomes loaded rosemary@curcumin and evaluating their effects on *S. aureus* strains. This study attempted to encapsulate rosemary extract and curcumin in niosomes and examine their antibacterial and anti-biofilm effects on clinical *S. aureus* strains to create novel antibiotics with fewer side effects and better loading, drug release, shelf-life, and therapeutic efficiency.

MATERIALS AND METHODS

Materials

Dried rosemary powder was acquired from the Botanical Repository of the Iranian Biological Resource Center (Iranian Biological Resource Center, Iran). Curcumin was acquired from Bio Basic (Bio Basic Inc., Canada). Span 60, Tween 60, and dimethylsulfoxide were also used for niosome synthesis (Merck, Co., Germany).

Rosmarinus officinalis (rosemary) Extract Preparation

The maceration method was used to prepare rosemary extract [11]. 5 g of plant powder was combined with 100 ml of distilled water as solvent. This mixture was placed on the magnetic stirrer for 1 hr and the shaker at 147 rpm for 48 hr. After filtration, the mixture was first placed in a rotary evaporator and then in an incubator at 37 ºC for 24 hr for evaporation of the remaining solvent. Finally, rosemary extract was obtained in powder form and prepared at a 10 mg/mL concentration for further study. The extract's compounds were detected with a gas chromatography/mass spectrometer (GC/MS).

Curcumin preparation

Curcumin (100 mg) was dissolved in dimethyl sulfoxide (20 mL) and used for loading in niosomes.

Niosome synthesis

Curcumin-rosemary loaded niosomes were synthesized through the handshaking method

(thin-film hydration). Appropriate concentrations of Span 60, Tween 60, and cholesterol were added to a chloroform solvent (8 mL) and dissolved. The mixture was then transferred to the rotary evaporator. The solvent was completely evaporated at 60 ºC and 150 rpm. Rosemary extract and curcumin in phosphate buffer (1 mg/mL) with pH 7.2 were added to the thin film remaining at the bottom of the flask. The mixture was rotated at 120 rpm and 60 ºC for 30 min until it was completely hydrated. The resulting niosomes were homogenized in an ice bath for 7 minutes with a probe sonicator to reduce the size of the particles [12].

Characterization of niosomes

The size of niosomes was assessed with dynamic light scattering (DLS) through a Zetasizer. Their dynamic diameter was measured at 633 nm and 25 ºC. Their polydispersity index (PDI) was also calculated with the Zetasizer. The functional groups of niosomes were determined with Fourier-transform infrared spectroscopy (FTIR) [8]. Nanostructures' morphology was assessed using scanning electron microscopy (SEM) [13].

Entrapment efficiency

To determine the entrapment efficiency (EE%), niosomes were first centrifuged for 45 minutes at 4 ºC and 14,000 g to separate the free drugs. The loaded niosomes precipitated and the rest of the compounds remained in the supernatant. The solution's absorbance rate was read at 760 nm. EE% was calculated with the following equation:

$$EE\% = \frac{\text{Weight of initial drug} - \text{Weight of free drug}}{\text{Weight of initial drug}} \times 100$$

In vitro drug release studies

The release studies of niosomes were conducted using a dialysis bag, made of cellulose acetate. The bag was placed in a solution of 0.1% sodium azide. 2 mL of niosome and free drug solutions was placed in separate dialysis bags. Each dialysis bag was suspended in a graduated cylinder with 50 mL phosphate buffer at 37 ºC and placed on stirrers. Samples of 1 mL were taken in time intervals of 1, 2, 4, 8, 24, 48, and 72 hr and replaced with the same amount of phosphate buffer. Their optical absorption was read at 760 nm. The drug release chart was drawn [14].

Isolation of microbial strains

200 clinical samples were collected from different diagnostic and treatment centers across Tehran (Imam Khomeini, Pars, and Shohada Tajrish hospitals). Various tests were used to identify *S. aureus* strains. These tests included Gram staining, catalase test, growth on Baird-Parker agar, and mannitol salt agar culture media. Samples that were positive in all the above tests were isolated as *S. aureus* and preserved for further studies. Biofilm-forming strains were identified by culture in Congo red agar medium.

Antibiotic susceptibility testing

The antibiotic susceptibility of samples was assessed through Kirby-Bauer disk diffusion [15]. Each sample's uniform solution was cultured on Muller Hinton plates. Following the addition of antimicrobial discs, the plates were left to incubate for 24 hr. Each disk's inhibition halo diameter was measured and reported as sensitive, intermediate, and resistant according to the reference table of the discs. The following antibiotics were tested: cefoxitin, vancomycin, ciprofloxacin, penicillin, erythromycin, trimethoprim, amikacin, ampicillin, gentamicin, amoxicillin, chloramphenicol, and clindamycin. The positive and negative controls in all experiments were *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228, respectively.

Determination of minimum inhibitory concentration (MIC)

MIC of Curcumin-rosemary loaded niosomes against clinical strains of *S. aureus* was determined through the 96-well microtiter plate dilution method. Results were compared to those of rosemary extract and curcumin. The concentration range used for this purpose was 7.8-1000 µg/mL. 95 µL of Mueller Hinton broth was poured inside each of the plate's wells and 5 µL of microbial suspension (1×10^6 CFU/mL) was added. 100 µL of niosomes, rosemary extract, and curcumin were added to the first well of each row. Serial dilution was carried out and the last well of each row, containing 95 µL of Mueller Hinton broth, 100 µL of 0.5% DMSO, and 5 µL of microbial suspension, acted as a negative control. The final volume of each well reached 200 µL. A sterile lid was placed on the plates and the contents were mixed for 60 seconds with a shaker at 100 rpm. The microtiter plates underwent an incubation process at 37 ºC for 24 hr. The analysis of bacterial growth was conducted by measuring the absorbance rate at 620 nm [16].

Minimum bactericidal concentration (MBC)

Antibacterial properties of noisome loaded

curcumin-rosemary were assessed by determining their MBC. Using a loop, a sample from the well representing the MIC and one dilution before and after that was taken and cultured on a Mueller Hinton agar plate. The plates were left to incubate overnight at 37 ºC. The first dilution without bacterial growth was considered the MBC [17].

Anti-biofilm activity

Niosomes' anti-biofilm activity was determined through the 96-well microtiter plate method. Colonies from an overnight culture of bacteria were inoculated into a TSB medium containing 0.2% glucose to obtain a bacterial suspension with turbidity matching that of a 0.5 McFarland standard. Bacteria-free TSB medium was added to the wells of the microtiter plate. The well of column 12, which had no bacteria, was the negative control. The well of column 11, containing only bacterial suspension, was our positive control. To each well, 100 µL of bacterial suspension and 50 µL of sub-bactericidal concentrations of niosomes were added. The plate was left to incubate for 24 hr at 37 ºC. Afterward, supernatant solutions were removed. Each well was rinsed 3 times with sterile physiological serum. 250 µL of 96% ethanol was added for bacterial fixation. After 15 minutes, the wells' contents were discarded. The plate was dried at room temperature. 200 µL of crystal violet was incorporated and rinsed off 15 minutes later. 200 µL of 33% acetic acid was introduced and the plate's optical absorption was read at 570 nm [18].

Statistical analysis

We performed all tests three times to ensure accuracy. Statistical calculations were done with the SPSS software, version 16. Results were interpreted with a one-way analysis of variance. The data are shown as mean ± standard deviation with a statistical significance of 0.05.

RESULTS

GC/MS analysis of Rosmarinus officinalis extract

GC/MS test was utilized to detect the chemical composition of in *R. officinalis* extract.

Table 1. Compounds of rosemary extract identified using GC/MS

Peak No.	Compound	Rt (min)	Area %
1	α-Tocopherol	52.27	0.46
2	Camphor	12.85	3.6
3	Limonene	11.01	0.62
5	α-Pinene	6.44	1.52
6	1,8-Cineole	16.64	1.16
7	Linalool	13.49	0.65
8	α-Amyrin	55.40	9.64
10	Borneol	15.03	1.01

Interpretation of the results was done by referring to the Wiley 275 Mass Spectral Database and comparing the spectra of unknown compounds with the available spectra of compounds in the database. Isolated compounds with the highest concentrations were α-Amyrin (9.64%) and camphor (3.6%). Alpha-pinene and 1,8-cineole (eucalyptol) were also among the identified compounds (Table 1). Alpha-pinene is an organic terpene and one of the two isomers of pinene. This monoterpene has various medicinal qualities, including anti-inflammatory, anti-tumor, and anticoagulant effects. In addition, alpha-pinene has antibacterial properties that can inhibit bacterial growth [19]. It can also act as a synergist with antibiotics and regulate the antibiotic resistance of bacteria [20]. 1,8-Cineol is also a monoterpenoid with therapeutic properties. It is useful in treating inflammatory respiratory diseases like asthma [21]. 1,8-cineole also has antimicrobial activity and can negatively inhibit the formation of bacterial biofilm [22]. This compound has also been shown to have synergy with other substances like chlorhexidine gluconate against pathogens such as *S. aureus* and *E. coli* [23].

Formulation and characterization of niosomes

By altering the surfactant and cholesterol molar ratio, different formulations were developed (Table 2). The niosomes' size, PDI, and zeta potential were assessed with a zetasizer, and

Table 2. Niosomes loaded rosemary@curcumin formulations

Formulation	Type of Surfactant	Span60:Tween60 (mol ratio)	Lipid (µmol)	Rosemary-curcumin (mg/mL)	Sonication Time (min)	Surfactant: Cholesterol (molar ratio)
F1	Span 60	100:0	200	1	7	1:1
F2	Span 60	50:50	200	1	7	1:1
F3	Span 60	0:100	200	1	7	1:1
F4	Span 60	100:0	200	1	7	2:1
F5	Span 60	0:100	200	1	7	2:1
F6	Span 60	50:50	200	1	7	2:1

Table 3. Size, PDI, and encapsulation efficiency of niosomes loaded rosemary@curcumin formulations

Formulation	(nm)Size	PDI	(%)EE
F1	442	0.212	94.23
F2	590	0.163	67.52
F3	650	0.263	51.74
F4	635	0.279	60.32
F5	720	0.136	61.29
F6	550	0.356	78.28

the encapsulation efficiency was calculated (Fig. 1, Table 3). The F1 niosomal formulation with an average 442 nm size (Table 4). PDI of 0.212, and EE% of 94.23% was selected as the optimal formulation. According to Fig. 2 and Table 5, the zeta potential of the optimal formulation of niosomes was -29.3 mV. SEM examinations of niosomes showed uniform spherical conformations (Fig. 3).

FTIR analysis

To assess the surface functional groups and the probable interaction of components on a molecular level, FTIR spectroscopy was used. First, the FTIR spectroscopy for curcumin and rosemary extract was evaluated separately. In curcumin's

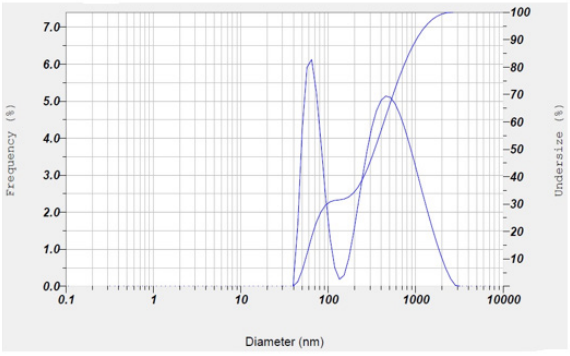

Fig. 1. The DLS analysis of niosomes loaded rosemary@curcumin

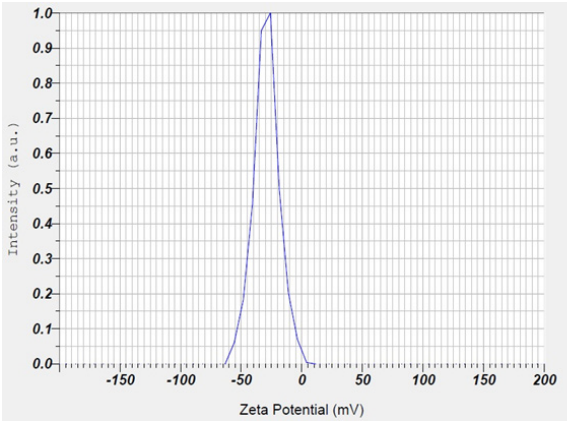

Fig. 2. Zeta potential of the optimal niosomes loaded rosemary@curcumin formulation

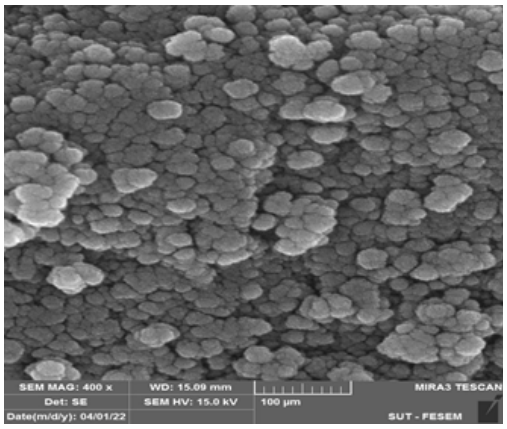

Fig. 3. SEM micrograph of niosomes loaded rosemary@curcumin

FTIR spectrum, significant peaks were seen at three points: 3509 cm^{-1}, 1628 cm^{-1}, and 1509 cm^{-1}. These three peaks belong to OH– and C=O stretching vibrations, and C=C of benzene. These functional groups are in accordance with the peaks reported in other studies [24]. In the FTIR

Table 4. The average size of niosomes loaded rosemary@curcumin

Peak No.	S.P. Area Ratio	Mean	S.D.	Mode
1	0.31	64.6 nm	15.7 nm	60.3 nm
2	0.69	615.4 nm	397.6 nm	428.2 nm
3	---	nm---	nm---	nm---
Total	1.00	442.4 nm	417.0 nm	60.3 nm

Table 5. Zeta potential of the optimal niosomes loaded rosemary@curcumin formulation

Peak No	Zeta potential	Electrophoretic mobility
1	-29.3 mV	-0.000227 cm^2/Vs
2	mV---	cm2/Vs---
3	mV---	cm2/Vs---

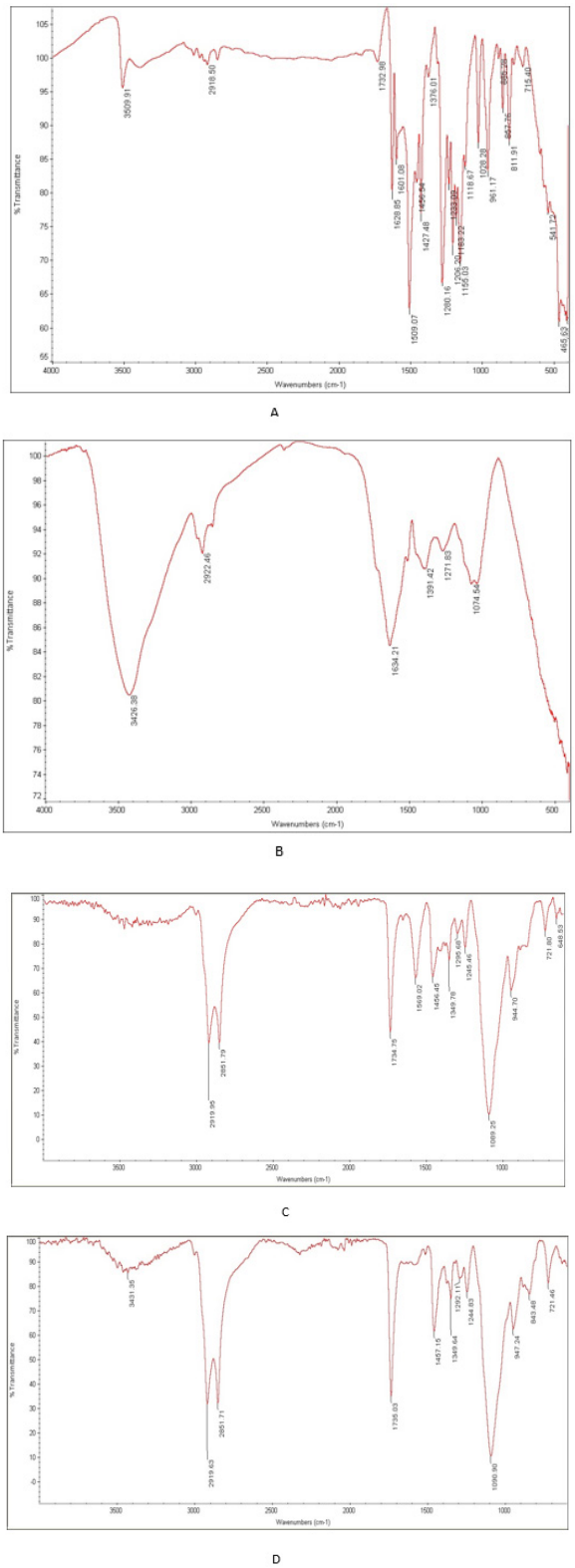

Fig. 4. FTIR spectra, A. curcumin, B. rosemary extract, C. unloaded niosome, D. of niosomes loaded rosemary@ curcumin

spectrum of rosemary extract, significant peaks were observed at three points: 3426 cm^{-1}, 2922 cm^{-1}, and 1634 cm^{-1}. These three peaks belong to OH–, C–H, and C=O stretching vibrations. These functional groups are in accordance with the peaks reported in other studies [25]. The FTIR spectrum of unloaded niosomes was also examined. In this spectrum, significant peaks were observed at 2919 cm^{-1}, 2851 cm^{-1}, 1734 cm^{-1}, and 1089 cm^{-1}. The first two peaks belong to C–H stretching vibrations. The rest indicates C=O and C–O stretching vibrations, respectively. Lastly, the loaded niosomes' FTIR spectrum was analyzed and compared to the other spectra. The first peak was observed at 3431 cm^{-1}, indicating the presence of OH–stretching vibrations. This peak was also observed in both the spectra of rosemary extract and curcumin, indicating the entry of these compounds into niosomes. The next two peaks at 2919 cm^{-1} and 2851 cm^{-1}, both of which belong to C–H stretching vibrations, can also indicate the presence of rosemary extract and curcumin since they present with less intensity in the FTIR spectra of these compounds. Slight changes in the 1735 cm^{-1} peak related to C=O stretching vibrations compared to the unloaded niosomes also indicate the entry of antimicrobial compounds into the niosomes. The peak at 1457 cm^{-1} with a slight change compared to unloaded niosomes indicates the entry of curcumin into the niosome and the presence of a bending vibration. Two peaks at 1292 cm^{-1} and 1090 cm^{-1} also indicate rosemary extract encapsulation and the presence of C–O stretching vibrations. These results not only indicate the encapsulation of rosemary extract and curcumin inside the niosomes, but the absence of new peaks proves that all compounds have kept their molecular natures and no chemical interactions have occurred between them (Fig. 4).

In vitro drug release profile

Drug release assessments revealed that the release rate of rosemary extract and curcumin in 72 hr was 39% lower than the free compounds. According to Fig. 5, during the first 8 hr, 56% of curcumin and 79% of rosemary extract were released. Meanwhile, the amount of cumulative drug released from curcumin-rosemary niosomes was equal to 31%. Thus, during 8 hr, 60% less rosemary and 44% less curcumin were released from the niosomes than the free form of the drugs. During 24 hr, 45% and 39%, and during 48 hr, 41% of rosemary extract and curcumin were released from niosomes compared to their free forms. These values indicate the slow

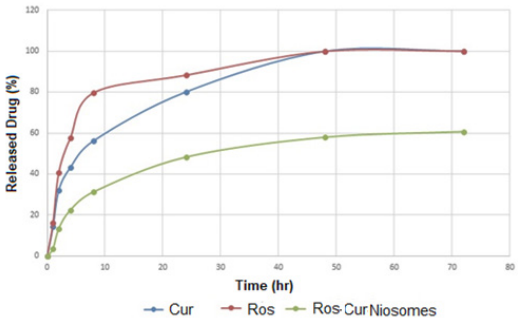

Fig. 5. *In vitro* release profile of the free curcumin, free rosemary and of niosomes loaded rosemary@curcumin

and continuous release of compounds from the niosomal formulations (Fig. 5).

Isolation of S. aureus strains

For this study, 200 samples were collected from Tehran hospitals in 2021. Samples were isolated from urine, blood, skin, and wounds. 50 samples were identified as *S. aureus* and used for further study (Table 6).

Table 6. The type of clinical samples

Gender	Female		Male		Total	
Type of Sample	No.	%	No.	%	No.	%
Blood	20	10	30	15	50	25
Wound	15	7.5	20	10	35	17.5
Skin	25	12.5	10	5	35	17.5
Urine	50	25	30	15	80	40

Antibiotic susceptibility of the isolates

Results from antibiotic susceptibility testing revealed that the strains had the highest resistance to ampicillin (84%), penicillin (82%), amoxicillin and trimethoprim (80%), cefoxitin (62%), and the

Table 7. Antibiotic resistance pattern of the isolated *S. aureus* strains

Antibiotic	Resistant Strains		Medium Strains		Sensitive Strains	
	No.	%	No.	%	No.	%
Methicillin (Cefoxitin)	31	62	0	0	19	38
Vancomycin	0	0	0	0	50	100
Ciprofloxacin	15	30	2	4	33	66
Penicillin	41	82	0	0	9	18
Erythromycin	20	40	8	16	22	44
Amikacin	20	40	4	8	26	52
Ampicillin	42	84	0	0	8	16
Gentamicin	15	30	2	4	33	66
Amoxicillin	40	80	0	0	10	20
Chloramphenicol	5	10	9	18	36	72
Clindamycin	20	40	9	18	21	42
Colistin	0	0	0	0	50	100

Table 8. Biofilm-forming strains and their antibiotic resistance pattern

Strain No.	Antibiotic Resistance Pattern	Biofilm-forming	MDR
2, 6, 9, 12	AMO, GEN, CIP	+	+
19, 20, 25, 27	PEN, CEF, CLI	+	+
31, 33, 35	CEF, AMI, TRI	+	+
39, 42, 43	CEF, GEN, CLI	+	+
45, 46, 48, 50	PEN, CEF, AMI	+	+

Table 9. The antimicrobial effects of curcumin, rosemary extract, and niosomes loaded rosemary@curcumin

Strain No.	MIC and MBC of Curcumin (µg/mL)	MIC and MBC of Rosemary Extract (µg/mL)	MIC and MBC of niosomes loaded rosemary@curcumin. (µg/mL)
2, 6	250, 500	500, 500	31.25, 62.5
9	125, 250	250, 500	15.62, 15.62
12, 19, 25	250, 250	500, 1000	31.25, 31.25
20, 27, 31, 33, 35	250, 500	500, 500	31.25, 62.5
39, 42, 43, 45, 46, 48, 50	250, 250	500, 1000	31.25, 62.5

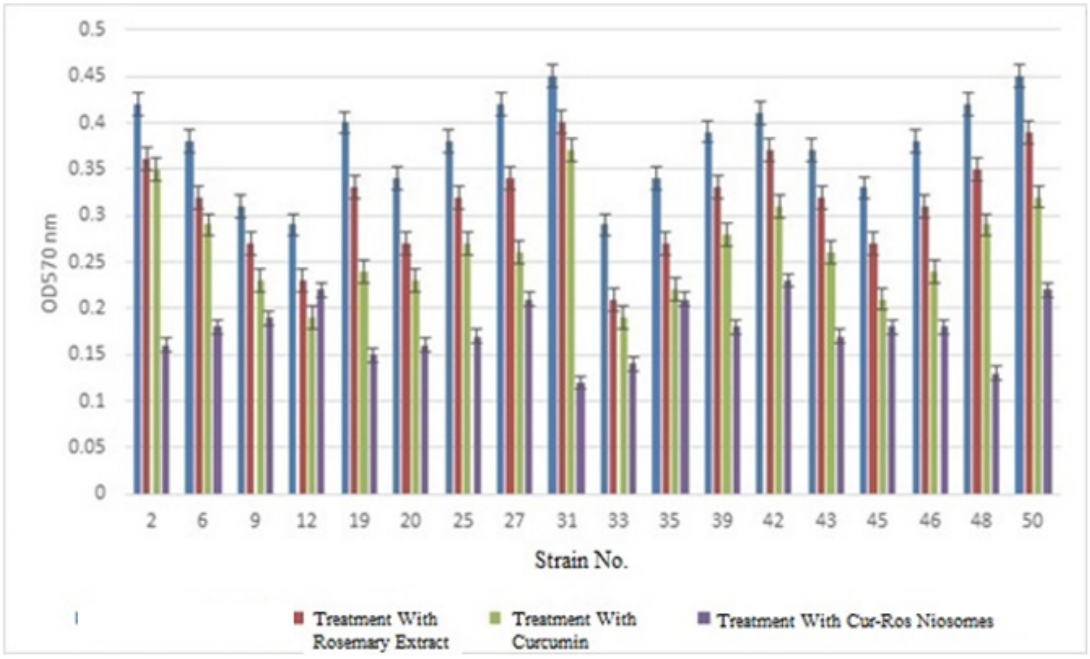

Fig. 6. Anti-biofilm activity of rosemary@curcumin niosomes

lowest resistance to vancomycin (100% sensitive) and colistin (100% sensitive) (Table 7).

Isolation of biofilm-forming strains

From the 50 clinical samples of *S. aureus*, 18 (36%) were biofilm-forming strains. All these strains were also multi-drug resistant (MDR) (Table 8).

MIC and MBC values of Niosomes

The antimicrobial properties of niosomes were studied through the microbroth dilution method. 7.8-1000 µg/mL of curcumin, rosemary extract, and niosomes were used. Our findings showed that the antibacterial effects of the compounds in niosomal formulations were significantly higher than their free forms (Table 9). The results showed that the MIC and MBC values of niosomes loaded rosemary@curcumin is reduced by 8 fold.

Anti-biofilm Activity of Niosomes

The anti-biofilm action of the niosomes loaded rosemary@curcumin was analyzed through a microtiter plate assay. Our findings showed that all strains could not form biofilm at sub-MIC concentrations of niosomes, (Fig.6).

DISCUSSION

The increase in antibiotic-resistant *S. aureus* strains has amplified the urgency for new treatment strategies. Since synthetic antibiotics have numerous side effects, attention has turned to natural plant compounds to fight this pathogen [26]. Rosemary extract and curcumin are among these compounds. According to our findings, these compounds can have significant anti-biofilm and antimicrobial effects against *S. aureus*. Rosemary extract's antimicrobial effects can be attributed to its secondary metabolites, such as rosmarinic acid, alpha-pinene, carnosic acid, camphor, 1,8-cineole, ursolic acid, etc. Each of these compounds possesses strong antimicrobial properties [27]. For instance, rosmarinic acid weakens the cell wall and makes pathogens such as *S. aureus* more vulnerable to environmental stresses by preventing the activity of enzymes involved in the synthesis of peptidoglycan. It also prevents certain enzymes like catalase and coagulase from working. By inhibiting these enzymes, it disrupts important cellular processes and prevents the growth and survival of bacteria. The antioxidant properties of this compound also help to reduce the pathogenicity of *S. aureus* [28]. Alpha-pinene is another effective compound in rosemary extract, which disrupts and destabilizes the cell membrane of bacteria, leading to membrane disintegration, leakage of cell contents, and ultimately cell death [29]. This compound also inhibits the activity of

bacterial enzymes such as DNA gyrase and RNA polymerase, which are necessary for bacterial proliferation and gene expression, and prevents the growth and survival of bacteria. Alpha-pinene can also induce oxidative stress in bacterial cells and damage various cellular components such as proteins, lipids, and DNA. It also reduces the pathogenicity of bacteria by interfering with the production and expression of virulence factors [30]. Other compounds of rosemary extract also play a role in its antimicrobial effect. For instance, carnosic acid affects important bacterial proteins and enzymes involved in cellular processes. Camphor and 1,8-cineole disrupt the bacterial cell membrane's integrity and interfere with the cell's essential functions. Ursolic acid also exerts its antimicrobial effects by interfering with cell wall synthesis and inhibiting bacterial growth [31]. The synergy of all these compounds and their different antibacterial mechanisms cause the inhibition and death of *S. aureus*. Additionally, the synergy of rosemary extract with curcumin increases this antimicrobial effect [32]. By damaging the cell membrane, curcumin causes the intracellular components to leak and the microorganism to die. It also prevents the activity of certain bacterial enzymes that take part in processes like protein synthesis and DNA replication. By interfering with these processes, it can suppress the proliferation and survival of bacteria. Curcumin also reduces the expression of virulence factors and lowers the bacteria's pathogenicity [33]. This substance also has an antioxidant capacity and affects L-tryptophan metabolism in *S. aureus*, leading to lipid peroxidation and bacterial DNA fragmentation [34]. Additionally, curcumin and rosemary extract both have anti-biofilm effects. Rosemary extract inhibits bacteria's initial attachment to surfaces, prevents biofilm formation, destroys the biofilm matrix, and makes bacterial biofilms more vulnerable to environmental conditions and therapeutic agents. Some of the rosemary extract's components, such as rosmarinic acid, carnosic acid, ursolic acid, and oleanolic acid reduce biofilm-related genes' expression and interfere with biofilm formation [35]. Curcumin also inhibits *S. aureus*' initial adhesion and colonization on surfaces and prevents biofilm formation. This substance also penetrates the biofilm matrix and disrupts the cellular network and extracellular polymeric substances (EPS), weakening the biofilm structure as a result and making the bacteria more vulnerable [36]. Rosemary extract and curcumin both disrupt and inhibit the quorum sensing (QS) system in bacteria, which is crucial for facilitating communication between bacteria and the maturation of biofilms. Curcumin also inhibits QS-dependent virulence factors [37]. Encapsulation of rosemary extract and curcumin in niosomes improves their antimicrobial and anti-biofilm properties and increases the effectiveness of these therapeutic substances against clinical strains of *S. aureus*. Niosomes also protect the antibacterial drugs from degradation by enzymes or harsh environmental conditions. Plus, niosomes ensure that a higher concentration of drugs reaches the bacteria by increasing their stability and solubility [38]. Due to its similarity to the bacterial cell membrane, the lipid bilayer structure of niosomes allows for easier fusion, more effective transport, and better absorption of drugs [38]. Also, with the controlled release of drugs, it continuously maintains a concentration of them in the body and increases the efficiency of antibacterial agents, thereby reducing the concentration required to achieve the desired effects [39].

Various studies have been conducted to investigate the antimicrobial and anti-biofilm effects of niosomes containing plant extracts and curcumin. Khaleghian et al, synthesized niosomes containing curcumin, investigated its antimicrobial and anti-biofilm effects. The results of this study showed that, in silico, niosomes containing curcumin can bind with genes involved in the biofilm formation, and can increase antimicrobial effects [40]. One of the mechanisms of increasing the antimicrobial effects of niosomes containing curcumin is the fusion of niosomes with the bacterial cell membrane and the targeted release of the drug into the bacterial cell. Also, due to the nanometer size of niosomes, they can penetrate the lower layers of biofilms and destroy microbial cells, leading to a decrease in the number of microbial cells and the absence of biofilm formation [41].

Ghumman et al, to develop an effective administration method, various niosomal formulations were optimized using the Box-Behnken method. To investigate niosomes loaded curcumin, size, zeta potential, entrapment efficiency, antioxidant potential, and cytotoxicity were performed. The optimized niosomes loaded curcumin had an average particle size of 169.4 nm, a low PDI of 0.189, and a high entrapment

efficiency of 85.4%. The release profile showed 79.39% of curcumin after 24 hr and significantly higher antioxidant potential compared to free curcumin. Cytotoxicity of niosomes loaded curcumin increased cytotoxicity in human ovarian cancer A2780. One of the limitations of this study is the low diversity of studied microbial strains, and therefore, it is suggested to use more diverse bacterial strains in future studies to investigate antimicrobial effects of niosome loaded rosemary@curcumin [42].

CONCLUSION

In this study, niosome loaded rosemary@curcumin were successfully synthesized and their effects were analyzed against clinical strains of *S. aureus*. The loaded niosomes had an average size of 442 nm, a PDI of 0.212, an encapsulation efficiency of 94.23%, a zeta potential of -29.3 mV, and uniform spherical conformations. By encapsulating both drugs simultaneously inside the niosomes, their synergistic effect reduced their MIC by 8-16 folds and their MBC by 16-32 folds. None of the strains could form biofilms at sub-MIC concentrations of niosomes and the *in vitro* drug release assessments showed the gradual release of antimicrobial compounds from niosomes. According to these findings, niosome loaded rosemary@curcumin has the potential to be used as a new antibiotics with optimal loading, controlled release, and enhanced therapeutic effects to inhibit and destroy *S. aureus* and prevent its biofilm from forming. Additional research needs to be conducted to analyze the impact of these niosomes on animal models and to evaluate their efficacy relative to currently available antibiotics.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This research was approved by the ethical board of the Faculty of Converging Sciences and Technologies, Department of Biology, Islamic Azad University, Research and Science Branch, Tehran, Iran (IR.IAU.PAR.REC.1401.033).

AVAILABILITY OF DATA AND MATERIALS

All data obtained and analyzed during the research are presented in this article.

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CONFLICTS OF INTERESTS

The authors declare no competing interest.

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