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Limonene encapsulated alginate/collagen as antibiofilm drug against *Acinetobacter baumannii*

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Abstract

This work examined the antibacterial and antibiofilm properties of alginate/collagen nanoparticles containing limonene. The multi-drug resistant (MDR) strains were screened, and the morphological features of the produced nanoparticles were determined utilizing SEM, DLS, and FTIR. Additionally, the encapsulation effectiveness, stability, and drug release were assessed. The levels of *OmpA* and *Bap* biofilm genes were assessed using qRT-*PCR*. At the same time, the antibacterial and cytotoxic activities of the nanoparticles were evaluated using well diffusion and MTT techniques, respectively. LAC nanoparticles measuring 300 ± 9.6 nm in size, 83.64 ± 0.19% encapsulation efficiency, and 60-day stability at 4 °C were synthesized. The biological investigation demonstrated that LAC nanoparticles had potent antibacterial capabilities. This was shown by their ability to significantly decrease the transcription of *OmpA* and *Bap* biofilm genes at a statistically significant level of $p \leq 0.05$. The nanoparticles exhibited reduced antibiotic resistance compared to free limonene and alginate/collagen. Compared to limonene, LAC nanoparticles exhibited negligible cytotoxicity against HEK-293 at doses ranging from 1.56 to 100 µg/mL ($p \leq 0.01$). The findings underscore the potential of LAC nanoparticles as a breakthrough in the fight against highly resistant pathogens. The potent antibacterial effects of LAC nanoparticles versus *Acinetobacter baumannii (A. baumannii*) MDR strains, considered highly resistant pathogens of significant concern, could inspire new strategies in antibacterial research.

Keywords *Acinetobacter baumannii*, Multi drug resistant, Limonene, Alginate/collagen, Antibiofilm

Introduction

A nosocomial infection is an infection that a person does not have when admitted to the hospital and during their recovery period [1]. Nosocomial infections are prevalent, resulting in a high risk even in well-equipped hospitals [1, 2]. *Acinetobacter baumannii (A. baumannii*), a well-known pathogen, poses a significant risk to human health due to the low effectiveness of standard antibiotics in treating its clinical illness [3]. Over the last several years, many antibiotic-resistant strains have been identified and documented. The infections with these bacteria may be difficult to treat [2, 3]. Recently, due to the widespread and uncontrolled consumption of antibiotics, these bacteria have developed resistance to several antimicrobial substances, leading to the emergence of antimicrobial resistance (AMR), which poses a significant risk to public health [4]. The prevalence of AMR results in the emergence of multi-drug resistant (MDR) traits, rendering standard medicines ineffective and limiting the available medical treatment choices [5]. Pathogenic bacteria use

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several alternative techniques to develop resistance to different categories of antibiotics, one of which involves the creation of biofilms. Biofilms consist of a collective of bacterial cells enclosed by an extracellular matrix, which enables them to firmly attach to medical surfaces and hinder the penetration of antibiotics into the microbial cells. Furthermore, biofilms also significantly impact the development of diseases by evading the immune system and preventing drugs from entering microbial cells [6].

Given the scarcity of novel antimicrobial agents for combating pathogenic bacteria that form biofilms and the constraints in developing new antibiotics [7], it is imperative to explore natural products as a viable alternative for treating these infections [7]. The newest study [8] suggests that current therapy options, such as polymyxins, may lead to the development of unique dependent tolerance. In order to control the fast spread of *A. baumannii*, scientists worldwide are working on developing several possible medicinal compounds, including nanoparticles, antibiotic conjugates, and biological small molecules [8, 9].

Medical plants containing many secondary chemicals are essential sources of herbal medications that may effectively combat bacteria that create biofilms. Of all the potential options, antibacterial compounds have consistently garnered attention [10]. It was shown that natural compounds have strong antibacterial properties and do not harm cells [11]. Essential oils are regarded as natural substances that may preserve food and act as agents to kill microorganisms. Essential oils are extensively used in food preservation [12]. Terpenoids have been identified as a crucial constituent of essential oils [13]. Limonene, specifically 1-methyl-4-(1-methyl phenyl), is a very prevalent terpene in the natural world. Limonene is a potent chemical in over 300 plant species, including black pepper, lemon, and orange [14]. It has been scientifically shown to possess antibacterial properties [13, 14].

Additionally, limonene serves as the primary constituent of citrus essential oils. Limonene's extensive bactericidal properties, safety, and low toxicity make it very promising for various applications, particularly in the food industry [15]. Furthermore, limonene has notable inhibitory effects on Gram-negative and Gram-positive bacterial and fungal activity [16, 17]. Limonene has been scientifically shown to efficiently hinder the development of spoilage microorganisms, including *Aspergillus niger*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* [16, 17].

Although limonene has good antibacterial properties, its poor solubility, quick breakdown, and chemical and biological instability owing to its volatile nature have restricted its utility as an antimicrobial agent [16, 17]. One proposed approach to address the restrictions is encapsulating limonene in nanocarriers [17]. Due to their tiny size, nanocarriers may enhance the stability of compounds and have the capacity to transport substantial quantities of drugs dependent on their physicochemical qualities [18]. Alginate has advantageous attributes such as low cytotoxicity, biodegradability, and biocompatibility, which render it acceptable for use as a vehicle for drug administration. Alginate is a biodegradable nanocarrier that may adhere to microbial cells, imparting antibacterial characteristics [17–19].

Alginate is a naturally occurring polysaccharide obtained from brown algae. Alginate has advantageous characteristics like abundant availability, cost efficiency, favorable biocompatibility, low cell toxicity, and the capacity to form a gel when exposed to divalent cations. Furthermore, alginate possesses distinct characteristics compared to other substances [19, 20]. These include (1) the capability to generate a non-reactive watery environment, (2) the ability to transform from a solution to a gel under gentle conditions, enabling the practical preservation of bioactive substances and encapsulation, (3) the presence of pores that permit adjustable diffusion of molecules, (4) the ability to break down naturally in a physiological setting, and (5) a structural resemblance to the extracellular matrix [21]. Alginate's many qualities make it suitable for extensive tissue engineering and biomedical science research. These applications include cell encapsulation, wound dressing, tissue regeneration, and medicinal uses [22]. Collagen is the predominant protein present in both soft and hard connective tissue. Because of its exceptional biocompatibility and low immunogenicity, collagen-based hydrogel has been extensively researched in tissue engineering and biomedical sciences [22, 33]. Nevertheless, its inadequate mechanical qualities restrict its widespread use [22, 33]. Enhancing collagen's mechanical characteristics is common by integrating other biomaterials such as alginate, chitosan, and hyaluronic acid [22, 33].

This work investigated the encapsulation of limonene in alginate/Collagen nanoparticles as a potential pharmaceutical formulation. The study focused on evaluating the formulation's encapsulation efficiency, in-vitro drug release pattern, and stability aspects. Furthermore, the produced alginate/Collagen nanoparticle was assessed for its antibacterial, anti-biofilm, and cytotoxic characteristics.

Materials and methods

Materials

Limonene and a phosphate buffer solution (PBS) were acquired from Merck (Germany). Alginate, collagen, and chloroform were purchased from Merck, a company based in Germany. The compounds penicillin/streptomycin 100X, Trypsin-EDTA, Trypan blue, Medium RPMI-1640, and Fetal Bovine Serum (FBS) were acquired from

Gibco, USA. The dialysis membrane with a molecular weight cut-off (MWCO) of 12,000 Da and MTT (dimethylthiazol-2-yl-)-2,5 was obtained from Sigma Aldrich (USA). The Mueller Hinton Broth, Mueller Hinton Agar, and H2SO4 were obtained from Merck, Germany. The strain *A. baumannii* ATCC 17,978 was acquired from the Pasteur Institute of Iran.

Preparation of limonene-loaded alginate/collagen nanoparticle (LAC)

One gram of alginate powder (Merck, Germany) was combined with 1 mL of purified water and 1 mL of hydrogen chloride (pH=4.5). The mixture was stirred for 1 h at 800 rpm utilizing a magnetic stirrer. Subsequently, the mixture was centrifuged at 1000 revolutions per minute for 5 min. A solution containing 50 mg of myristic acid, 100 mg of Ethyl-carbo-di-imide hydrochloride (EDC; $C_8H_{18}ClN_3$), and N-Hydroxysuccinimide (NHS) dispersed in 1 ml of ethanol was slowly added to alginate over 24 h. The pH of the chemical process was further elevated using a weak amount of sodium hydroxide (0.1 M) to cause the alginate solution to form a solid precipitate at a pH of 8.5. The alginate solvent precipitate was ultimately isolated by centrifugation and then cleansed with ethanol and distilled water. The addition of weak hydrochloric acid dispersed the alginate solution. The resulting combination was then subjected to sonication for 30 min utilizing an ultrasonic homogenizer. A solution was prepared by dissolving 100 µg limonene (Merck, Germany) in 1 mL of myristate. Collagen was then added drop-wise while the alginate solution was being sonicated. The sonication process was extended for 15 min to encapsulate limonene inside the alginate/collagen mixture. The concentration of synthesized nanoparticles was 100 $\mu g.ml^{-1}$ [4].

Characterization of LAC

The microscopic size and charge of LAC were measured utilizing dynamic light scattering (DLS) and palladium electrodes of the ZetaPals device (Brookhaven Instruments Corp., USA) at a light scattering range of 90° and a temperature of 25 °C. The form and structure of LAC were examined utilizing field scanning electron microscopy (Fe-SEM) utilizing the MIRA3 model (TESCAN, Czech Republic). The LAC was characterized using Fourier transform infrared spectroscopy (TENSOR II, BRUKER, Germany). The desiccated nanoparticles were measured to be about 2 mg and combined with the anhydrous potassium bromide (KBr) by grinding. A tablet press was used to create a uniform sheet. The wavelength spectrum was configured to span from 400 to 4000 cm^{-1}, with a precision of 2 cm^{-1} [4, 35].

Encapsulation efficiency

The effectiveness of encapsulation (EE%) of limonene in alginate/collagen was assessed after encapsulation. In summary, a volume of 1 mL of LAC was centrifuged at a speed of 14,000 rpm for 1 h at a temperature of 4 °C. The entrapped limonene content in each sample was determined by determining the precipitate's highest adsorption at an emission wavelength of 653 nm [4, 35]. The proportion of the encapsulation effectiveness was then computed utilizing the following equation:

EE%= Total amount of initial limonene entrapped into the alginate/collagen formulations – the amount of free limonene in supernatant/ total amount of drug × 100.

In vitro drug release study of LAC and stability

The medication was released in a laboratory using a dialysis bag with a molecular weight cut-off (MWCO) of 12 kDa. The dialysis bag was filled with 2 mL of limonene and 2 mL of LAC to achieve this objective. The whole mixture was then immersed in a 50 mL PBS solution at a pH of 7.4. The resulting mixture was gently stirred at 37 °C at 50 rpm. Subsequently, it was divided into equal portions at certain intervals, and a fresh environment was introduced. Furthermore, the same process was carried out for the limonene, which was not bound. This technique was used to monitor the diffusion stability for various LAC formulations. Measurements were collected at 0, 15, 30, 45, and 60 days over a two-month storage period at temperatures of 4 and 25 °C [35, 37].

Analysis of clinical samples using microbiological techniques

Bacterial collection and isolation

This investigation involved the purchase of 50 strains of *A. baumannii*, which were obtained and identified by Parsian BioProducts Company (Shahrekord, Iran). Microbiological and biochemical examinations were conducted by Parsian BioProducts Company (Shahrekord, Iran) to separate disease-causing *A. baumannii* species.

Testing for the sensitivity of bacteria to antibiotics

As outlined in the CLSI recommendation, the disk diffusion technique was used to conduct susceptibility tests to antibiotics for all samples. 3–5 colonies were chosen from pure culture and transferred to a tube comprising 5 ml of sterile normal saline solution. The mixture was gently incorporated and kept at 37°C until the suspension achieved a clarity level of 0.5 McFarland. The aseptic cotton swab was introduced into the bacterial solution and utilized to spread the germs across the Mueller-Hinton agar evenly. The antibiotic disks, which consisted of imipenem (IMI, 10 µg), ceftazidime (CAZ, 30 µg), cefepime (FEP, 30 µg), piperacillin-tazobactam (TZP, 110 µg), ampicillin-sulbactam (SAM, 10 µg+10 µg), kanamycin

Table 1 Classification of bacteria based on biofilm formation capacity

Biofilm Class	Results
OD > 4×ODc	Strong biofilm
2×ODc < OD ≤ 4×ODc	Medium biofilm
ODc < OD ≤ 2×ODc	Poor biofilm
OD ≤ ODc	Negative biofilm

(K, 30 µg), and Colistin (COL, 10 µg) from MAST in England, were placed on the surface of the plate. The *A. baumannii* ATCC 17,978 isolates were employed as a reference control [35, 37].

Biofilm formation assay

Congo red agar (CRA)

We created CRA, which consisted of BHI (37 g/l), sucrose (50 g/l), agar (10 g/l), and Congo red solution (0.8 g/l). Bacterial strains were then transferred to plates using this CRA medium and incubated for 24 h at 37 °C. Under these conditions, bacteria that can build biofilms generate colonies that seem black, whereas other bacteria make red colonies.

Microtiter plate assay (MPA)

In this experiment, after the cultivation of separated bacteria, 200 µl of bacterial culture was introduced into each well of a sterile 96-well polystyrene plate and then cultivated for 24 h at 37°C. A volume of 200 µl of crystal violet dye with a concentration of 1% was applied for staining. After 15 min, the dye was rinsed off using Phosphate-buffered saline (PBS). Following the addition of 200 µl of acetic acid (33%), the optical density at 570 nm (OD570) was determined utilizing the ELISA Reader Stat Fax2100 (Awareness Technology, Ukraine). The bacteria that formed biofilms were classified based on the information provided in Table 1 [35, 37].

Broth microdilution assay

The broth microdilution technique was employed to identify the minimum inhibitory concentration (MIC). In summary, varying amounts of limonene and LAC, ranging from 1.56 to 100 µg/mL, were introduced to the 96-well plate. Subsequently, 100 µL of microbial solution was introduced into each well, and the plates were incubated for one night at 37 °C. Ultimately, the turbidity of all wells was assessed utilizing the ELISA Reader Stat Fax2100 (Awareness Technology, Ukraine) device at a wavelength of 630 nm. Two wells were chosen as the negative control, containing just culture media, and the positive control, containing culture medium with standard bacteria. The MIC amounts, which refer to the minimum medication levels that hinder bacterial development, and the sub-MIC amounts, which represent the most significant levels of drugs that have no impact on bacterial growth, were established [4, 35, 37].

Activity against biofilm formation

The anti-biofilm efficacy of limonene and LAC was assessed using a microtiter plate. The strains capable of generating biofilms were cultivated on 96-well microtiter plates for 24 h at 37 °C. The strains were then exposed to sub-MIC (sub-minimum inhibitory concentration) levels of limonene and LAC for a 24-hour incubation period at 37 °C. Afterwards, methanol was used to fix the wells, while crystal violet was used to colorize them. The absorbance was then measured at 570 nm [35, 37].

Biofilm gene expression analysis

The impact of limonene and LAC on *Bap* and *OmpA* biofilm gene transcription of *A. baumannii* strain was evaluated using quantitative Real-Time PCR. The extraction of total RNA was performed utilizing a total RNX-Plus kit (Cinnagen Co., Iran) and samples treated with sub-MIC concentrations of limonene and LAC. The complementary DNA (cDNA) was manufactured using the YTA Kit Protocol provided by Yekta Tajhiz, a company based in Iran. A real-time PCR procedure was conducted using the YTA SYBR Green master mix from Yekta Tajhiz, Iran. The 16SrRNA gene was employed as an internal control, as shown in Table 2. A total of 15 µL of the reaction mixture was prepared for Real Time-PCR. This volume included 0.5 µL of cDNA, 0.5 µL of forward primer, 0.5 µL of reverse primer, 10 µL of master mix, and 3.5 µL of double sterile distilled water. The temperature-dependent cycle program consisted of an initial denaturation step at 95 °C for 10 min, afterwards performing 40 cycles at 95 °C for 30 s and 60 °C for 40 s. The sequence of the primers for the target genes, which include *Bap*, *OmpA*, and *16 S rRNA* (used as an internal control), may be found in Table 2 [4, 35, 37].

Table 2 Sequence of primers used for real-time PCR

Name	5'----------->3'	Product Size	Tm (°C)	GenBank	Reference
16SrRNA	F: TGTTTTGAAGGGGTTTGGAG R: GCCTCCTCCTCGCTTAAAGT	177 bp	60.0 °C	LN611374.1	This study
OmpA	F: GACAGCCAACACAACAATGG R: CCGTCTACGTCGCCTTTAAC	155 bp	60.0 °C	KJ363323.1	This study
Bap	F: AATACATCAGGCCCAGCAAC R: ACTGCCGGATAGTCAACACC	161 bp	60.0 °C	LC576828.1	This study

Cytotoxicity assay

The cytotoxicity of limonene and LAC was assessed utilizing the MTT colorimetric test conducted by Kalazist in Iran. 20,000 normal HEK 293 cells were grown in 96-well plates using RPMI1640 medium supplemented with 10% fetal bovine serum. The plates were then incubated at 37∘C in a CO2 incubator. The cells were exposed to limonene and LAC at various doses ranging from 1.56 to 100 µg/mL. Following incubation, 20 µL of MTT solution (5 mg/mL) in the colorless solution PBS (Kalazist, Iran) was introduced into each well. Following a 4-hour incubation period, the medium was extracted, and 100 mL of DMSO was introduced into the 96-well plates. The plates were subjected to agitation at a speed of 400 revolutions per minute for 6 min to dissolve the formazan crystals that had formed in DMSO fully. The amount of color was quantified using an ELISA Reader Stat Fax2100 (Awareness Technology, Ukraine) at a wavelength of 570 nm. The cell viability was reported as the mean ± standard deviation (SD) ($n=5$). For control, HEK-293 cells were cultured with RPMI1640 medium that did not include the test sample [35, 37]. The fraction of viable cells was determined utilizing the following equation:

$$\text{Cell survival rate}\% = \frac{\text{treated cells OD}}{Control\ cells\ OD} \times 100$$

Statistical analysis

The statistical evaluation in this research was performed using SPSS software version 20, and the obtained findings were analyzed using one-way analysis of variance (ANOVA). The levels of transcription of target genes in both the control and treatment samples were determined using Tukey's HSD post-statistical technique.

Results

The LAC nanoparticle has a uniform and spherical morphology

The morphological characteristics of alginate/collagen and LAC nanoparticles were investigated (Table 3). Each formulation exhibited unique characteristics in terms of size, polydispersity index (PDI), and entrapment efficiency (EE). Table 3 shows that the particle size of the free alginate/collagen and LAC nanoparticle was 250 and 300 nm, respectively. These nanoparticles had spherical shapes, as depicted in Fig. 1A. The mean diameters of LAC nanoparticle and free alginate/collagen were measured by DLS and found to be 263 ± 2.12 nm and 308 ± 3.42 nm, respectively (Table 3; Fig. 1B). According to Table 3, the PDI of the free alginate/collagen and LAC nanoparticle was 0.35 ± 0.017 and 0.28 ± 0.011, respectively. The zeta potential values for both free alginate/collagen and LAC nanoparticles can be found in Table 3; Fig. 1C, respectively.

Fourier transform infrared (FTIR) analysis

The FT-IR spectra of limonene and LAC nanoparticle clearly depict the majority of the principal distinctive peaks. Figure 1D displays the FTIR spectra of limonene and LAC nanoparticle, revealing alterations in some signals that have resulted in the creation of LAC nanoparticle. The limonene molecule (Fig. 1D) exhibits broad signals at 3560.95 cm^{-1} and 2424.74 cm^{-1}, which correspond to the peaks associated with the C-H bond. The peak at 2922.70 cm^{-1} corresponds to the amino group of the limonene, while the peaks at 1712.42 cm^{-1}, 1639.08 cm^{-1}, 1384.45 cm^{-1}, and 1033.20 cm^{-1} are associated with the carboxyl group. The limonene molecule (Fig. 1D) exhibits signal at 894.86 cm^{-1}, 616.70 cm^{-1}, and 518.45 cm^{-1}, which correspond to the peaks associated with the C-O bond. The FTIR spectra of the LAC nanoparticle (Fig. 1D) exhibit a peak at 3560.95 cm^{-1}, as well as four distinct stretching vibrations of C-H in the benzene ring at 3160.05, 2922.70, 2525.75, and 1969.68 cm^{-1}. These vibrations serve as indicators of the presence of the LAC nanoparticle. The 1812.42 cm-1 peak in Fig. 1D corresponds to the O-H functional group. The existence of this peak in the LAC nanoparticle confirms the accurate incorporation of limonene in the alginate/collagen material (Fig. 1D). Furthermore, a signal was observed at 1639.08 cm^{-1}, indicating the presence of electrostatic interactions between the amino groups of limonene and the collagen units of alginate/collagen. These interactions have played a role in the formation of LAC nanoparticles.

Encapsulation efficiency and in-vitro drug release

The objective of the present research was to investigate the impact of alginate/collagen on the encapsulation of limonene and the release behavior of limonene. The drug release pattern was examined using dialysis bags that contained free limonene and LAC nanoparticles. The encapsulation efficiency (EE%) of LAC nanoparticles was 83.64 ± 0.19, as indicated in Table 3.

Figure 2A illustrates the patterns of limonene emission from the dialysis bag that contains the limonene mixture and LAC nanoparticles. Figure 2A demonstrates that the

Table 3 Characterization of synthesized nanoparticles. Data are represented as mean ± SD, $n=3$

Nanomaterials	Polydispersity index (PDI)	Surface charge (mv)	Fe-SEM (nm)	DLS (nm)	EE (%)
Alginate/collagen	0.35 ± 0.017	-8.3 ± 0.41	250 ± 5.7	263 ± 2.12	---
LAC	0.28 ± 0.011	-24.1 ± 0.73	300 ± 9.6	308 ± 3.42	83.64 ± 0.19

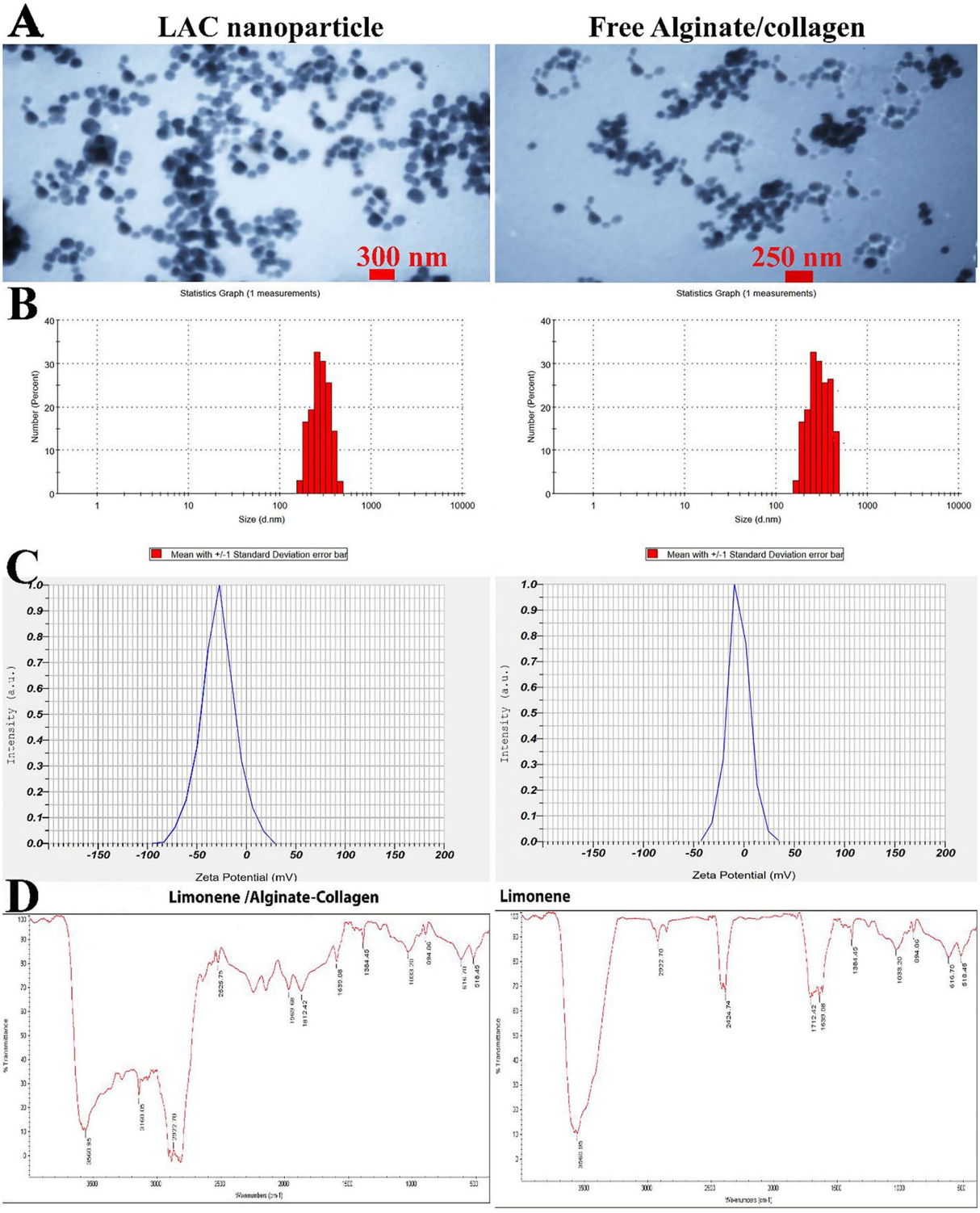

Fig. 1 **(A)** Field emission scanning electron microscopy (FE-SEM) of synthesized LAC nanoparticles and alginate/collagen. **(B)** The DLS test reveals the distribution of particles with sizes ranging from 130 to 500 nm for LAC nanoparticles and alginate/collagen. **(C)** Zeta potential was −8.3 ± 0.41 and −24.1 ± 0.73 for LAC nanoparticles and alginate/collagen respectively. **(D)** Fourier Transform Infrared FTIR Spectra of LAC nanoparticles and Free limonene

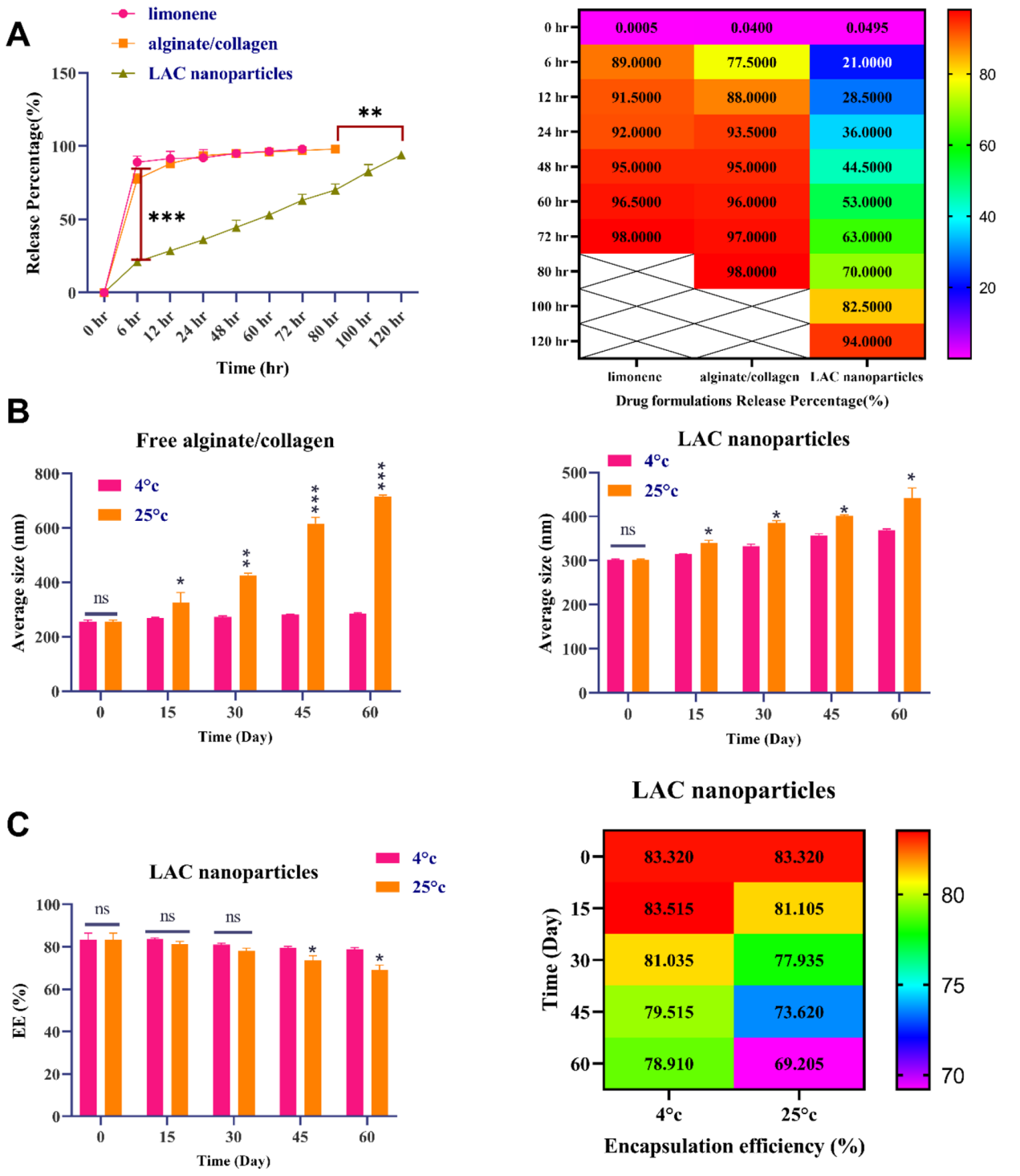

Fig. 2 **(A)** The amount of controlled drug release in of LAC nanoparticles and alginate/collagen compared to Free limonene. **(B)** The effect of temperature on the mean size of LAC nanoparticles and alginate/collagen. **(C)** The effect of temperature on the EE (%) of LAC nanoparticles

encapsulating of limonene in alginate/collagen effectively prevents burst release. The release pattern was characterized by an initial rapid release period lasting up to 6 h, followed by a subsequent slower release phase lasting up to 120 h. According to the data presented in Fig. 2A, the release of free limonene is 89% within the first 6 h. After that, the release remains very consistent, with 100% of the limonene being released within 72 h. The medication

release percentage in the LAC nanoparticles formulation over a period of 72 h is 63%, and it increases to 94% after 120 h.

Physical stability study of nanoparticles

The structural integrity of alginate/collagen and LAC nanoparticles were evaluated via determining the size of the encapsulated particles and the percentage of medication persisting after a period of storage at refrigerator (4±2 °C) and room temperatures (25±2 °C). By observing the change in mean diameter, it is evident that the movements were slower at a temperature of 4±2 °C compared to 25±2 °C. The drug retention in LAC nanoparticles formulation demonstrates a drug leakage rate of less than 15% from the original amount under both conditions (Fig. 2B). The findings demonstrate the robust physical stability of the evaluated LAC nanoparticles and suggest that these nanoparticles could serve as an efficient formulation to prevent medication leakage. Based on the findings shown in Fig. 2B, the size of the free alginate/collagen increases as the storage time increases ($p<0.001$). The sample held at 4±2 °C exhibits greater stability compared to the sample stored at 25±2 °C. This could be attributed to the increased rigidity of the hydrophobic component of alginate/collagen at lower temperatures.

Antibacterial tests

Isolation and antibiotic susceptibility profile

This investigation involved the purchase of 50 strains of *A. baumannii*, which were obtained and identified by Parsian BioProducts Company (Shahrekord, Iran). The results of antimicrobial susceptibility testing indicate that among the 7 selected antibiotics, kanamycin exhibited the highest resistance (94%, 47 strains), followed by ceftazidime (80%, 40 strains), cefepime (64%, 32 strains), piperacillin-tazobactam (58%, 29 strains), ampicillin-sulbactam (58%, 29 strains), imipenem (48%, 24 strains), and the lowest resistance was observed with Colistin (16%, 8 strains). Based on the information provided in Table 4, all isolates that showed resistance to three or more classes of antibiotics were categorized as MDR.

Biofilm formation assay

Out of the 50 MDR strains, 13 strains (26%) exhibited the formation of rough black colonies, whereas the remaining 37 strains (74%) were categorized as non-producers with smooth white colonies. The microtiter plate results indicated that out of the 13 strains tested, 6 strains (46.15%) were classified as strong biofilm producers, 2 strains (15.38%) were classified as moderate biofilm producers, and 5 strains (38.46%) were classified as poor biofilm producers (Table 5).

PCR amplification of OmpA and Bap gene

Table 5 depict the polymerase chain reaction (PCR) amplification of the *OmpA* and *Bap* genes. Furthermore, the presence of *OmpA* and *Bap* genes was detected in all 13 strains of *A. baumannii* that create multidrug-resistant biofilms. PCR amplification of genes resulted in the production of an amplicon with a size of 155 base pairs for the *OmpA* gene and 161 base pairs for the *Bap* gene (Fig. 3A).

Determination of MIC and sub-MIC

The MIC and sub-MIC properties of free alginate/collagen, free limonene, and LAC nanoparticles versus biofilm-formation *A. baumannii* were examined employing a microtiter plate. The free limonene MIC measurement findings revealed that all strains of *A. baumannii* had MIC values ranging from 25 to 12.5 µg/mL (Table 6). The LAC nanoparticles MIC analysis findings showed that all strains of *A. baumannii* had MIC values ranging from 0.781 to 1.56 µg/mL (Table 6). Table 6 demonstrates that the MBC values for LAC nanoparticles were frequently two or four times greater than the MIC values. Nevertheless, LAC nanoparticles exhibited MIC and MBC values against the investigated bacterial strains that were twice as high as those of free limonene. Table 6's data indicate that the MIC of free alginate/collagen against *A. baumannii* was 50 µg/mL. However, strain 3, strain 5, strain

Table 4 Antibiotic resistance pattern to *A. Baumannii*

Class	symbol	Antimicrobial Agent	Disk content (µg)	Susceptible (S)	Intermediate(I)	Resistant(*R*)	Number of resistant strains	Percentage of resistant strains	Total
Carbapenems	IMI	Imipenem	10 µg	≥22	19–21	18 ≥	24	48%	50 Strain
Ureidopenicillins	PTZ	Piperacillin-tazobactam	100/10µg	≥21	18–20	17 ≥	29	58%	
Sulfonamide + inhibitor	SAM	Ampicillin-sulbactam	10 µg + 10 µg	≥15	12–14	11 ≥	29	58%	
Aminoglycosides	K	Kanamycin	30 µg	≥17	15–16	14≥	47	94%	
Cephalosporins	CAZ	Ceftazidime	30 µg	≥18	15–17	14 ≥	40	80%	
	FEP	Cefepime	30 µg	≥18	15–17	14 ≥	32	64%	
Colistin	COL	Colistin	10 µg	2≥	-	≥4	8	16%	

Table 5 Biofilm formation results in isolated MDR *A. Baumannii*

Isolated bacteria	Biofilm formation ability				Biofilm gene		MDR
	CRA test	MPA TEST					
		OD_C	OD	Biofilm Class	*OmpA*	*Bap*	
Strain 1	+	0.006	0.261	Strong	+	+	+
Strain 2	+	0.008	0.028	Medium	+	+	+
Strain 3	+	0.004	0.006	Poor	+	+	+
Strain 4	+	0.004	0.007	Poor	+	+	+
Strain 5	+	0.005	0.006	Poor	+	+	+
Strain 6	+	0.009	0.085	Strong	+	+	+
Strain 7	+	0.011	0.131	Strong	+	+	+
Strain 8	+	0.008	0.024	Medium	+	+	+
Strain 9	+	0.013	0.116	Strong	+	+	+
Strain 10	+	0.007	0.011	Poor	+	+	+
Strain 11	+	0.013	0.213	Strong	+	+	+
Strain 12	+	0.008	0.013	Poor	+	+	+
Strain 13	+	0.010	0.206	Strong	+	+	+

6, strain 9, strain 10, and strain 12 exhibited no minimum inhibitory concentration (MIC) when exposed to free alginate/collagen. Nevertheless, concentrations of 0.195 and 0.390 µg/mL of LAC nanoparticles did not exhibit any growth inhibition. Thus, the greatest sub-MIC value of LAC nanoparticles was determined to be 0.390 µg/mL, which was utilized as a concentration for the experiments that prevent biofilm formation. Furthermore, the use of LAC nanoparticles led to a noteworthy decrease in the MIC compared to free alginate/collagen and free limonene, with a range of 2 to 4 times lower. This indicates that LAC nanoparticles effectively increased the antibacterial activity of limonene.

Anti-biofilm activity

The efficacy of free alginate/collagen, free limonene, and LAC nanoparticles in suppressing the proliferation of *A. baumannii* isolates by adhering to biofilm after a brief exposure to drug formulations was compared to that of free limonene alone. In contrast to the minimal biofilm inhibitory concentration (MBIC) investigation, the biofilm growth inhibition condition was more challenging. In this case, the biofilm was initially exposed to drug formulations or free limonene for only two hours. Subsequently, the biofilms were washed and cultured in a medium without drugs for 24 h. This analysis was conducted using MIC doses for each unique strain. The drug formulations' concentration was determined relative to the minimum inhibitory concentration (MIC) of free limonene. The results were expressed as the percentage of biofilm growth inhibition (BGI%) and presented in Fig. 4.

Relative transcription of biofilm genes

In order to further validate the ability of free alginate/collagen, free limonene, and LAC nanoparticles to suppress biofilm formation, the mRNA levels of biofilm-associated genes (*OmpA* and *Bap*) were analyzed using Real-Time PCR after treating *A. baumannii* strains with sub-MIC quantities of the medicines. The results indicate that the mRNA levels of *OmpA* and *Bap* were significantly decreased after treatment with free limonene and LAC nanoparticles, compared to the PBS group. This suggests that both free limonene and LAC nanoparticles have anti-biofilm activity against *A. baumannii* strains. Furthermore, the outcomes demonstrate that a greater reduction in *OmpA* and *Bap* transcription was achieved with sub-MIC doses of LAC nanoparticles compared to free limonene (Fig. 3B).

Cytotoxicity

The cytotoxicity effects of alginate/collagen, limonene, and LAC nanoparticles were evaluated in comparison to PBS on the HEK 293 normal cell line. The LAC nanoparticles demonstrated little cytotoxicity when compared to free limonene across all tested concentrations. Approximately, 80±1.28% of the cells treated with limonene remained viable 24 h after exposure to MIC concentration (12.5 µg/ml) treatment, whereas more than 90±2.41% of the cells remained viable 24 h following incubation with the 12.5 µg/ml of LAC nanoparticles. Furthermore, the cell survival rates after 72 h were measured to be 88.5±1.32%, 74±2.32%, and 87.5±3.54% for the 12.5 µg/ml of alginate/collagen, limonene, and LAC nanoparticles, respectively (Fig. 5). Conversely, the percentage of cells that remained alive after 72 h after being treated with PBS was 92.5±3.45%.

Discussion

The identification of antibiotics for the treatment of infections caused by bacteria was a significant milestone in the field of medicine. Regrettably, bacteria possess a high degree of adaptability, and the overuse of

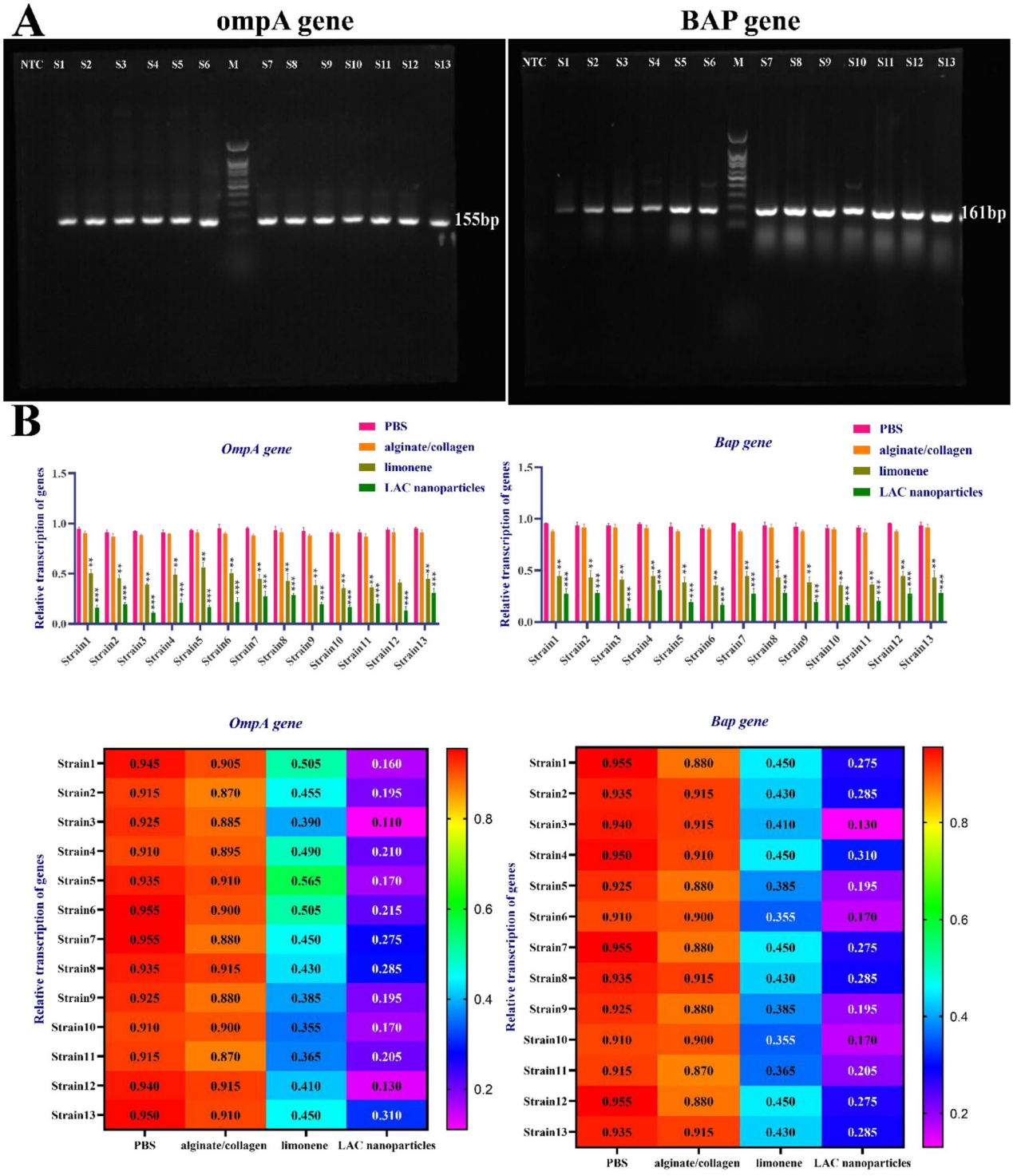

Fig. 3 **A)** PCR confirmation of the *OmpA* and *Bap* biofilm genes' existence in *A. baumannii*
B) The impact of alginate/collagen, limonene, and LAC nanoparticles on the transcription of *OmpA* and *Bap* biofilm genes. Data are mean ± SD of three independent experiments. The significance level was determined as *** $p < 0.0001$, ** $p < 0.01$, * $p < 0.0.5$

antibiotics has resulted in antibiotic resistance in several bacteria [23]. The rise in antibiotic resistance has led to an increased prevalence of MDR bacteria in hospitals, resulting in a new problem known as nosocomial infections [24]. Based on clinical findings, *A. baumannii* is a prominent bacterium resistant to many antibiotics. Prior research has shown a rise in antibiotic resistance in these specific bacteria [25].

Table 6 MIC, SubMIC and MBC value of free alginate/collagen, free limonene, and LAC nanoparticles in pathogenic strains ($n = 3$)

Sample	A. baumannii								
	Free alginate/collagen (µg/ml)			Free limonene (µg/ml)			LAC nanoparticles (µg/ml)		
	MBC	MIC	Sub-MIC	MBC	MIC	Sub-MIC	MBC	MIC	Sub-MIC
Strain 1	100	50	25	25	12.5	6.25	1.56	0.781	0.390
Strain 2	100	50	25	25	12.5	6.25	1.56	0.781	0.195
Strain 3	---	---	---	50	25	12.5	3.125	1.56	0.781
Strain 4	100	50	25	25	12.5	6.25	1.56	0.781	0.390
Strain 5	---	---	---	50	25	12.5	3.125	1.56	0.781
Strain 6	---	---	---	50	25	12.5	3.125	1.56	0.781
Strain 7	100	50	25	25	12.5	6.25	1.56	0.781	0.390
Strain 8	100	50	25	25	12.5	6.25	1.56	0.781	0.390
Strain 9	---	---	---	50	25	12.5	3.125	1.56	0.781
Strain 10	---	---	---	50	25	12.5	3.125	1.56	0.781
Strain 11	100	50	25	25	12.5	6.25	1.56	0.781	0.390
Strain 12	---	---	---	50	25	12.5	3.125	1.56	0.781
Strain 13	100	50	25	25	12.5	6.25	1.56	0.781	0.195

In contrast, the findings of the current investigation demonstrated that all 50 strains of the bacteria exhibited resistance to three types of antibiotics, had a MDR phenotype, and could build biofilms. The findings of this study align with earlier research, suggesting that there is a rise in antibiotic resistance caused by heightened transcription of biofilm genes and, therefore, better biofilm-forming capability [26]. In this investigation, 26% of the bacteria were shown to be able to create biofilms, while the other microorganisms did not. In this research, out of the biofilm-producer bacteria examined, 6 strains (46.15%) were identified as strong biofilm producers, 2 strains (15.38%) were identified as moderate biofilm producers, and 5 strains (38.46%) were identified as weak biofilm producers.

Studies have shown that bacteria utilize various biological, physical, and genetic elements to generate resistance mechanisms. The most significant form of resistance is associated with the formation of biofilms and the transcription of genes relevant to biofilm production [27]. This work demonstrated the active presence of *OmpA* and *Bap* genes in MDR bacteria, which enhance the capacity of biofilms to develop and contribute to microbial resistance to different antibiotics. Prior research suggests that the Bap protein plays a significant role in triggering the biofilm reaction during periods of stress [28]. *OmpA* has been identified as a contributing role in antibiotic resistance and the production of biofilms, according to several studies [29]. There is a growing number of nosocomial infections, a high occurrence of MDR bacteria, and a sluggish development of new antibiotics. As a result, there is a greater need to employ natural antibacterial agents [23].

Consequently, most research focuses on different approaches to introduce novel antibacterial medicines or overcome bacterial resistance [23]. Hence, in this research, alginate/collagen was prepared, and limonene was encapsulated inside the alginate/collagen structure to introduce a novel antibacterial substance. Limonene, as an antibacterial substance, is well described in the literature [30]. Alginate polymer has a strong affinity for biological membranes and enhances the drug transport capacity [19]. Alginate has unique qualities, including non-toxicity, biodegradability, and emulsifying capabilities. These properties enable it to transform oily and water-insoluble medicines into a soluble state [31]. Prior research has shown that collagen has a crucial role in several aspects, including controlled drug release, mucosal adherence, enhanced penetration, and inhibition of bacterial growth and efflux pump activity [32]. Alginate polymer and collagen may be transformed into nanoparticles to improve their chemical properties and boost drug delivery efficiency [33].

Thus, encapsulating natural agents like limonene has the potential for developing novel antibacterial chemicals. Encapsulation efficiency plays a crucial role in the production of nanomaterials. Various parameters, such as nanoparticle sizes, directly impact the effectiveness of encapsulation and, subsequently, the capacity to release drugs [34]. Our work revealed that encapsulating limonene with alginate/collagen enhances antibacterial efficacy. The findings of this work align with other research indicating that the antimicrobial effects of natural agents are enhanced at the nanoscale [35]. The investigation established the homogeneity of nanoparticles using DLS. Alginate/collagen and LAC nanoparticles were calculated, and SEM analysis indicated a reduced size for the nanoparticles. DLS is a technique used to measure the hydrodynamic diameter of nanoparticles and any ions or molecules that may be associated with them. SEM is the precise technique used to estimate nanoparticle dimensions while in a dry state. Consistent with other research,

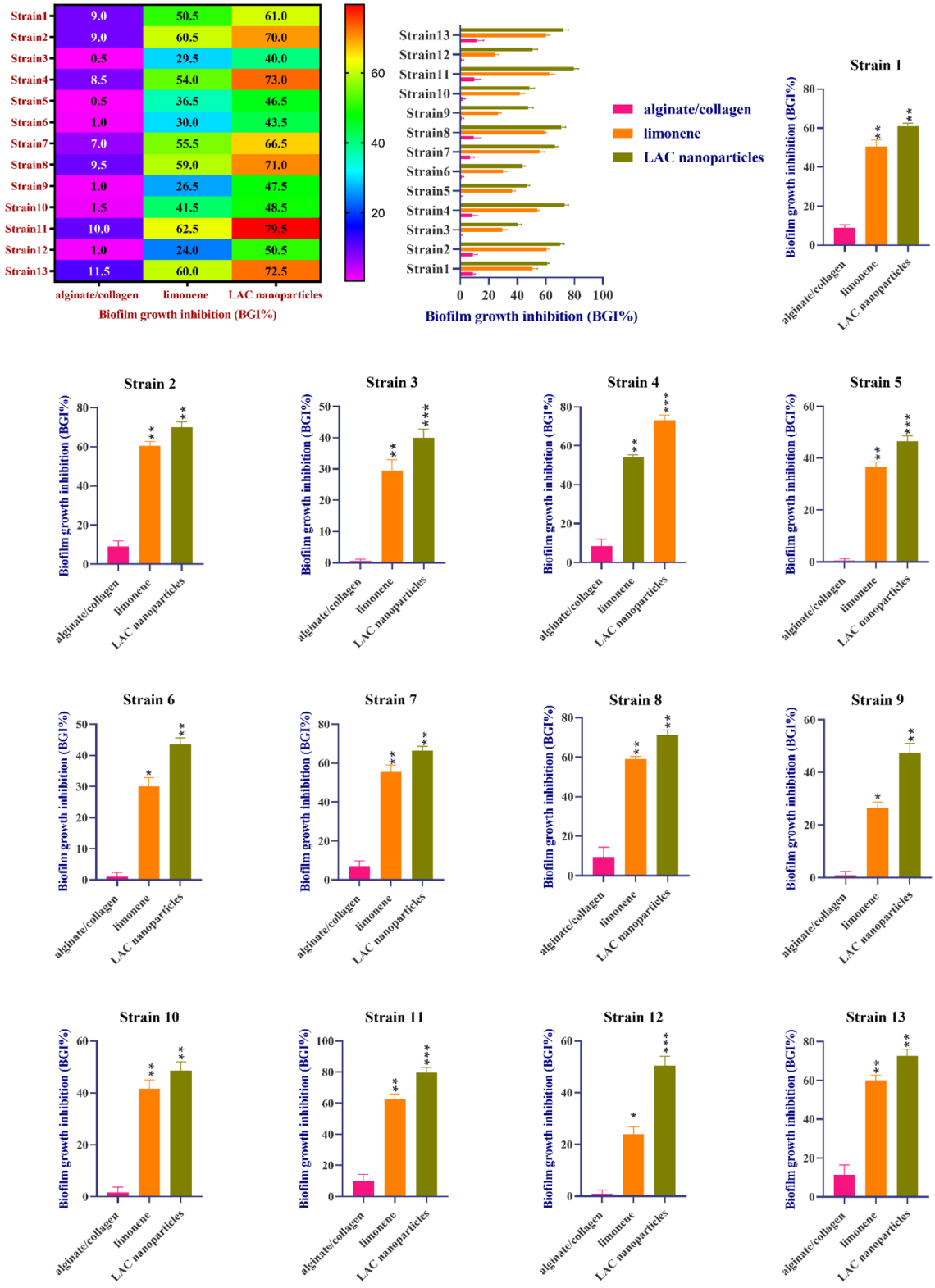

Fig. 4 The percentage of biofilm growth inhibition (BGI%) was measured for free alginate/collagen, free limonene, and LAC nanoparticles. Data are mean ± SD of three independent experiments. The significance level was determined as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

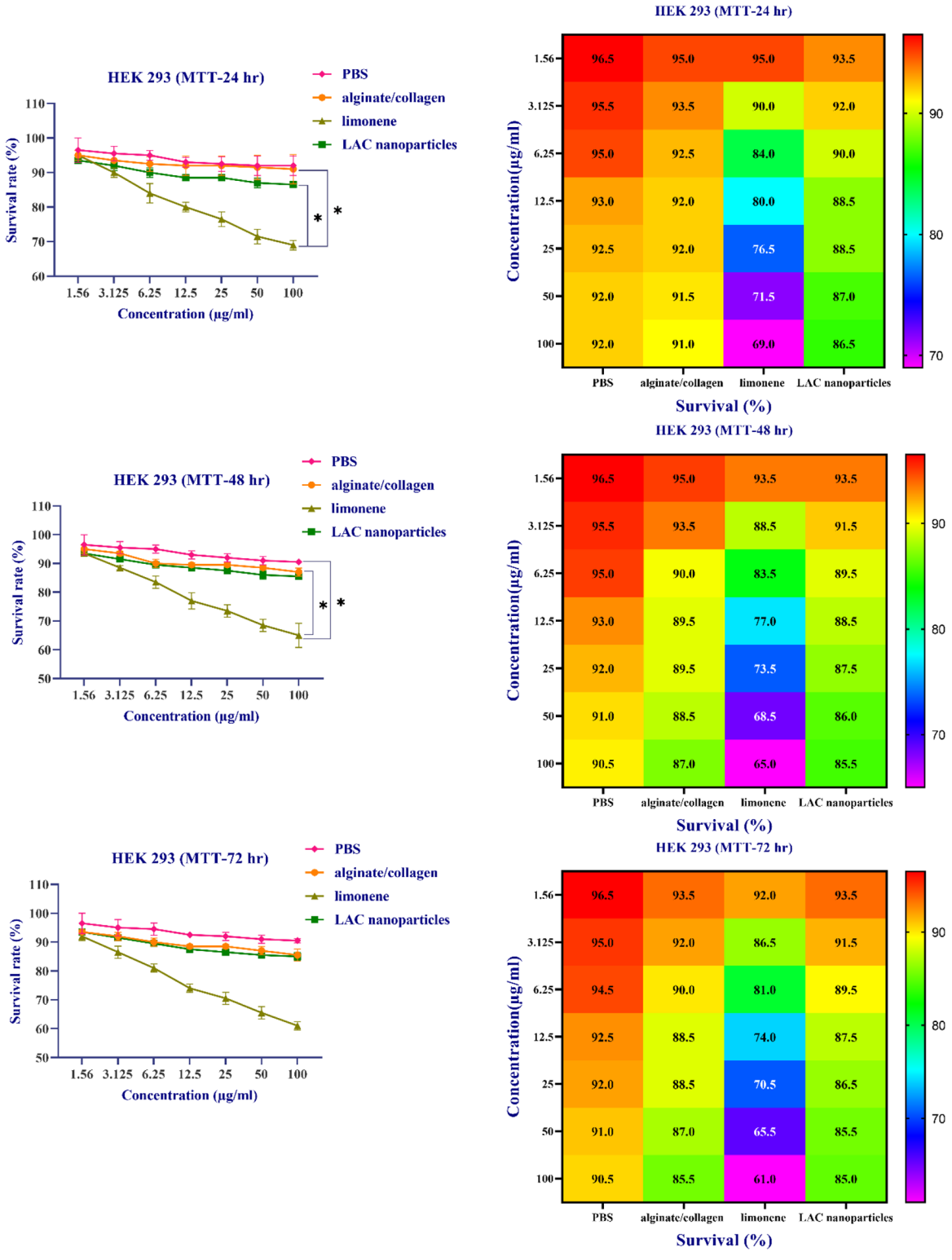

Fig. 5 Cell viability percentage of HEK-293 cells treated with alginate/collagen, limonene, and LAC nanoparticles in comparison with PBS over 24, 48 and 72 h

our findings demonstrate the impact of scattering on the accuracy of particle measurement using DLS [35, 36].

Additionally, the analysis of the drug release pattern demonstrated that drug diffusion occurred throughout 120 h. The initial diffusion rate may be related to the liberation of medicines from the surface. Over time, the medications contained inside the surface layer are gradually released. After 6 h, the concentration of limonene on the surface is decreased, and the pharmaceuticals in the lower layers start to be released. This hinders the rate at which the medication is released. Prior research has included drug release regulating agents into polymer nanoparticles to study their diffusion and biodegradation. These studies have seen a fast release of the drug during the first hours, referred to as "explosion diffusion" [37]. This investigation's findings and other research demonstrate the rapid spread of encapsulated limonene release [38]. The stability investigation showed that temperature, as a detrimental factor, significantly influences the preservation of drugs [39]. Over 60 days, the morphological features of nanoparticles changed due to rising temperatures, resulting in a decline in their efficiency.

Nevertheless, the shape and effectiveness of nanoparticles are hardly impacted by the passage of time. Consequently, keeping LAC nanoparticles at a temperature of 4 °C may extend the storage capacity beyond 60 days. Prior research has also identified temperature as a constraining element in drug storage, wherein elevating the temperature leads to the lubrication of fat and subsequent settling of the medication [40]. The primary objective of this research was to assess the efficacy of LAC nanoparticles in inhibiting biofilm formation and combating bacterial growth.

The findings indicate that LAC nanoparticles exhibited the most potent growth inhibitory capabilities against MDR bacteria. Nevertheless, the free limonene medication had the lowest level of inhibitory action. The MBC findings validated the MIC results. Studies have shown that alginate may both attach to the outer membrane of gram-negative bacteria and enter the microbial plasma membrane. Alginate's capacity to attach to the surface of bacteria and release encapsulated drugs at the target region is a key strength in targeted drug delivery [41]. Alternatively, the study examined the pace at which bacteria grew during a 24-hour period. The findings indicated that even at the lowest levels of LAC nanoparticles, there was a noticeable inhibition of microbial growth compared to the control group.

The antibiofilm activity was assessed by measuring the transcription levels of *OmpA* and *Bap* biofilm genes in subMIC concentrations of nanoparticles. The findings demonstrated that LAC nanoparticles exhibited the most significant decrease in gene expression. This suggests that LAC nanoparticles interacted with transcription factors, resulting in the suppression of gene expression. Several studies have shown a clear correlation between the creation of biofilms and the development of antibiotic resistance in bacteria [42]. Recent research identified biofilm as the primary factor contributing to bacterial pathogenicity [43]. Another research explicitly elucidated the correlation between the creation of biofilms and the development of antibiotic resistance in bacteria [44]. Several studies have examined the impact of several natural antibacterial compounds and nanoparticles on the regulation of biofilm formation in bacteria [45]. In their study, *Piri Gharaghieh* et al. (2018) identified silver nanoparticles as effective agents for controlling biofilms in multi-drug-resistant strains of *A. baumannii* [46]. The final objective of this research was to examine the lack of toxicity of LAC nanoparticles in human cells. To assess this, an MTT test was conducted, which revealed little or no toxicity of the nanoparticle on HEK-293 cells. The remarkable qualities of alginate/collagen and its lack of toxicity are responsible for these achievements. Alginate/collagen can decrease the toxicity of pharmacological agents [47]. Several investigations have shown the toxicity-altering characteristics of alginate nanoparticles [47]. Research demonstrated that the cellular harm caused by iron oxide nanoparticles was notably reduced after their encapsulation in alginate [48, 49]. The current research has also shown that the application of alginate/collagen coating on limonene decreased cell damage, leading to a substantial reduction in the cytotoxic impact of free limonene. Our investigation was constrained by the unavailability of a laboratory animal facility, necessitating the use of laboratory animals. Hence, it is recommended that an in vivo investigation of this chemical be conducted inside the mouse body.

Conclusion

Alginate/collagen, a polymer-based medication system, shows promising outcomes in enhancing the antibacterial efficacy of medical or natural antibacterial compounds. Consequently, the process of enclosing limonene in alginate/collagen nanoparticles not only enhance the antibacterial and anti-biofilm characteristics of limonene but also mitigate its cytotoxic effects and improve its effectiveness through targeted binding to the bacterial surface and controlled release of the drug. LAC nanoparticles have potent antibacterial activities against MDR strains of *A. baumannii*, a very problematic bacterium of the current day. Hence, applying LAC nanoparticles is an innovative approach in the pharmaceutical sector.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12896-024-00888-9.

Supplementary Material 1

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Author contributions
Conceptualization, H.S., F.GH.; methodology, H.S.; software, F.GH. and L.R.; All authors reviewed the manuscript.

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Data availability
The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent
The study was approved by the Ethics Committee of the Islamic Azad University of Shahrekord Branch in Iran (IR.IAU.SHK.REC.1402).

Consent to publish
Not applicable.

Competing interests
The authors declare no competing interests.

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