

Evaluation of anti-biofilm properties of *Mentha piperita* essential oils loaded in chitosan nanoparticles against *Acinetobacter baumannii* in the Catheter surface

Pegah Shakib

Lorestan University of Medical Sciences

Behnam Ashrafi

Lorestan University of Medical Sciences

Faranak Rezaei

Lorestan University of Medical Sciences

Somayeh Delfani (✉ Somayehdelfani@gmail.com)

Lorestan University of Medical Sciences

Research Article

Keywords: *Mentha piperita*, Essential oils, Nanoparticles, Biofilm formation, *Acinetobacter baumannii*, Catheter surface

Posted Date: June 1st, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2937257/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Multidrug-resistant (MDR) Gram-negative bacteria such as *Acinetobacter baumannii* pose an increasingly severe threat. Finding new therapeutic strategies such as herbal remedies is a vital priority, especially in combination with nano gels which have recently been widely used in medical purposes and drug delivery systems.

Objective

In this study, evaluated the physicochemical properties and effects of mutagenicity synthesizing Mentha Piperita Essential Oils loaded in chitosan nanoparticles (MPEO- CsNPs), which is referred to as an antimicrobial agent for inhibiting biofilm formation of *Acinetobacter baumannii* on the catheter surface.

Method

Mentha piperita essential oils loaded in chitosan nanoparticles (MPEO- CsNPs) and the physicochemical properties of MPEO-CsNPs were analyzed by Scanning Electron Microscopy (SEM) and Differential Calorimetry Scanning (DCS). The Minimum Inhibitory Concentration (MIC), the Minimum Bactericidal Concentration (MBC), and the Inhibitory Concentration (MBIC50) of MPEO- CsNPs against the standard strain of *Acinetobacter baumannii* were investigated. The amount of biofilm formation in the presence and absence of MPEO- CsNPs on the catheter surface was analyzed. The *bap* gene expression was studied as the primary leading indicator of biofilm formation using the real-time PCR technique. The effects of mutagenicity according to the Ames test were investigated.

Results

The results showed that the synthesized MPEO- CsNPs at equivalent concentrations had lower MIC than sodium azide. MIC and MBC levels were reported to be 3.12 and 12.48 µg / ml, respectively. MBIC50 was calculated to be 2.69%, which it was approximately twofold compared to the control that was treated with no MPEO concentration. The adherence of bacterial cells to the catheter surface had high sensitivity to the MPEO- CsNPs, while when treated with unloaded CNPs, it had no inhibitory effect on adherence activity. It was observed that with increasing the concentration of the MPEO- CsNPs, expression of the *bap* gene decreased.

Conclusion

This study showed that chitosan-peppermint nanoparticles could be used as a safe, compatible, and preventive coating in the design and manufacture of hospital catheters and other medical devices.

1. Introduction

Biofilms are a collection of surface microbial cells enclosed in an extracellular polymer matrix [1]. According to vital many studies, *A. baumannii* biofilm plays an important role in infectious diseases such as cystic fibrosis, bloodstream, and *urinary tract infection (UTI)* due to their ability to deploy medical equipment [2]. For example, in many cases, *A. baumannii* bacteremia is associated with catheter infection [3]. Factors such as biofilm-associated protein (bap), Outer membrane protein A (OmpA), iron uptake mechanism, and the CsuA/BABCDE chaperone-usher pili assembly system are involved in the formation of biofilms [4]. Members of the bap family contain proteins with high molecular weight at the bacterial level and have the main range of tandem repeats [5]. Bap production in *A. baumannii* affects the biological volume and thickness, and the initial binding to non-living surfaces. It is a significant factor in biofilm formation, thus helping the survive in various environments. Furthermore, biofilm formation in bacteria increases antibiotic resistance and host defenses up to 1000-fold [6].

Also, today widespread resistance of *A. baumannii* isolates to various families of antibiotics, including aminoglycosides and cephalosporins is occurring because they carry genes that encode inhibitory enzymes. The presence of multi-resistance isolates limits antibiotic treatment, which increases treatment barriers, including the length of treatment and treatment costs for patients [7–9].

Since ancient times plants have been used as antimicrobial agents in the treatment of many infections [10]. Since, in many cases, herbal medicines are cheaper and have fewer side effects than chemical drugs, today, biological compounds of plant origin are considered an important branch of drug treatment of infectious diseases. With the development of nanotechnology, these plants are used to synthesize nanoparticles and nanogels. with increasing antibiotic resistance and the effect of plants on microbial agents, researchers are encouraged to research new antimicrobial agents [11, 12]. Therefore, nanoparticles and nanogels are among the most promising new antimicrobial agents that have shown widespread antimicrobial activity in recent studies. These particles target several biological molecules simultaneously and prevent the formation of resistant microbial isolates [11, 13]. Nano-gels are polymer networks with a three-dimensional structure capable of absorbing large amounts of water and biological fluids, which have recently been widely used in medical purposes and drug delivery systems. To this end, these compounds must have acceptable biocompatible and biodegradable properties [14]. Chitosan is a cationic polymer derived from chitin acetylation. It has an excellent capacity to load bioactive compounds, including plant extracts and other effective antimicrobial, anticancer, and vaccine compounds [15]. Therefore, plant extracts encapsulated in CsNPs will have a more effective effect on microbial agents. *Mentha piperita* essential oils have anti-cancer, anti-seizure, anti-inflammatory, anti-bacterial, and anti-fungal properties [16]. The active ingredients of this plant are resins, flavonoids, phenol, carotene, betaine, and tannin [17]. Therefore, considering the properties of *Mentha piperita* and CsNPs, the present study aimed to investigate the anti-biofilm properties of MPEO-encapsulated CsNPs against *Acinetobacter baumannii* in the catheter surface and its mutagenic potential.

2. Materials and Methods

2.1. Preparation of MPEO- CsNPs

Synthesis of nanoparticles containing peppermint essential oil (*Menthapiperita*) and chitosan was performed in previous projects entitled "Mentha piperita essential oils loaded in a chitosan nanogel with inhibitory effect on biofilm formation against *S. mutans* on the dental surface " [14]. In short, it includes the following steps: a) Preparation of aqueous essential oil from peppermint by distillation, b) Identification of chemical compounds of essential oil using gas chromatography coupled with a mass spectrometer, c) Preparation of separate solutions of chitosan and Tri-polyphosphate with different percentages, d) Adding the solution containing essential oil slowly to the chitosan solution being stretched, e) Centrifuging at low speed and separating the supernatant containing the MPEO- CsNPs from the precipitated phase, f) Using As an emulsifier, g) the drug was loaded and encapsulated on the substrate surface. Morphology of MPEO- CsNPs was considered by scanning electron microscopy (SEM).

2.2. Differential Calorimetry Scanning of MPEO- CsNPs Formation

The thermal properties of the samples were investigated by the US-made DSC910S. 3 mg of each sample was placed in a closed aluminum pan and cooled and cooled at a rate of 10 °C per minute in the temperature range of 25–400 °C in the presence of nitrogen gas at a flow rate of 50 ml 1 min. An empty aluminum pan was used as a control sample [18, 19].

2.3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC of MPEO- CsNPs against *A.baumannii* ATCC19606 isolate was determined using the micro-dilution broth method in 96 sterile plates according to the CLSI method [20].

2.4. Biofilm production by light absorption method

Biofilm production was investigated by the light absorption method using violet crystal dye in microplates [21].

2.5. Bacterial adherence on the catheter surface

The effect of MPEO-CsNPs on bacterial adhesion on the catheter surface was investigated. Briefly, the catheters were removed from the package under sterile conditions and immersed in a bacterial suspension equivalent to half-McFarland turbidity equal to 1.5×10^8 CFU / ml at various concentrations and incubated at 37 ° C for 24 h. It was then gently shaken at 37 ° C for 90 minutes to induce the binding of bacterial cells. The samples were then washed with PBS to remove unbound bacteria and transferred to a new tube. Finally, the biofilm covering the catheter surface was measured qualitatively by staining with 0.1% safranin [22]. Tubes containing washed and non-adherent bacteria were examined by real-time PCR to quantify of *bap* gene in *A.baumannii* ATCC19606.

2.6. Evaluation of *bap* gene expression by Real-Time PCR

RNA was extracted from *A. baumannii* to study the expression of *DNA gyrase A* and *bap* genes. RNA extraction was performed according to the instructions prepared by *Geneall* co, Korea. The concentration of RNA used was considered to be about 1 to 2 micrograms. For this purpose, its light absorption was measured at a wavelength of 260 nm. Also, light absorption at 280/260 nm was measured to ensure no protein contamination, and light absorption at 260/230 nm was measured to ensure no salt contamination. Preparation of cDNA from the extracted RNA was performed, after determining the amount and concentration of RNA from *A. baumannii ATCC19606* by the Nanodrop spectrophotometer (Thermo Scientific) by kit protocol (Pishgam Co, Iran). Quantitative real-time PCR was carried out by a real-time PCR system.

The *DNA gyrase A* was used as an internal control to study the expression of the *bap* gene. The sequence of the forward and reverse primers of the two *bap* and *DNA gyrase A* genes is shown in Table 1. The reaction volume was 25 µl and included 2 µl of cDNA, 10 pM of forward and reverse primers for both *bap* and *DNA gyrase A* genes, 10 µl of master-mix, and 11 µl of double distilled water (DDW). The temperature cycle consisted of initial denaturation at 95 ° C for 10 min, followed by 40 cycles at 95 ° C for 20 s, and 58 ° C for 40 s. The final step was performed to draw the Melting Curve at 95 ° C for 15 seconds, 60 ° C for 30 seconds, and 95 ° C for 15 seconds [23]. Melting point data and their curves were recorded after qRT-PCR and checked for each well. A control reaction without cDNA was included in each run as a no-template control. The tests were repeated three times.

Table 1
Characteristics of sequence primers of *bap* and *DNA gyrase A* genes in *A. baumannii ATCC19606*

Gene target	primer sequence	Size(bp)	Ref
<i>Bap</i>	R: 5'- TGCAACTAGTGGAATAGCAGCCCA-3'	121	[23, 24]
	F: 5'-TGCTGACAGTGACGTAGAACCACA-3'		
<i>DNA gyrase A</i>	R: 5'-AACCGTACCAGAAGCTGTC < G-3'	110	[23, 24]
	F: 5'-AAGGCCGTCCAATCGTGAA < T>-3'		

2.7. Assessment of mutagenic potential using Ames test

Increasing the number of colonies in the culture medium compared to the negative control sample will be a positive sign of the Ames test. To perform the experiment, the strains were first enriched in nutrient broth. With the sample in top agar and minimum glucose (GM agar) in the presence of different concentrations of MPEO- CsNPs in an incubator at 37 ° C. We put it for 72 hours. After that time, we examined the plates to count the returned colonies. Also, all the above steps were performed to test the activator system by adding 0.5 ml of S9 buffer obtained from rat Wistar liver extract and added to the previous mixture [25]. Finally, the mutability of the material was determined using the following formulas.

$$\text{Mutagenic index (MI)} = 100 \times \frac{\text{Number of his+ revertants induced in the samples}}{\text{Number of his+ revertants induced in the negative control}}$$

Statistical methods

A CRD (completely randomized design) was used for the study. Data were analyzed using the one-way ANOVA procedure of SPSS [24]. Least-square means were computed and tested for differences by the Tukeys test. Differences in least-square means were considered to be significant at $P \leq 0.05$. The data were analyzed using the following Statistical model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where: Y_{ij} = dependent variable, μ = overall mean, T_i = fixed effect of treatments, e_{ijkl} = residual error.

3. Results

3.1. Physical description of the MPEO- CsNPs

The scanning electron microscopy (SEM) images of MPEO- CsNPs were depicted in Fig. 1. It is apparent that resulting CsNPs are more and less uniform in size and shape.

3.2. Differential Calorimetry Scanning (DSC)

The thermograms for chitosan (CHE), triphosphate (TPP), peppermint essential oil (MP), peppermint essential oil (NF), and hydrogel base (BNF) are shown in Fig. 2, respectively. The thermal spectrum of chitosan shows an exothermic peak at 332/65°C. The reaction starts temperature for chitosan was 290/76°C. The endothermic spectrum observed at 124.48°C can be related to the decomposition of the structure due to water loss. The tri-polyphosphate thermogram showed an endothermic peak at 198/18°C. The starting temperature for the decomposition of triphosphate was 171/87°C. The thermal spectra of peppermint essential oil (NF) showed an endothermic peak related to the evaporation of the essential oil at 123/65 °C and a transfer temperature related to the oxidation of the essential oil at 146/96°C. The starting temperature of the essential oil decomposition reaction was 119/24°C. The thermogram of peppermint (BNF) non-essential oil hydrogel showed an exothermic peak at 210/39°C. The reaction start temperatures were 136/79°C, respectively. While in the thermogram, the hydrogel containing peppermint essential oil showed an endothermic peak at 226/80°C. The reaction starts temperature for the hydrogel containing peppermint essential oil was 213/83°C. This reaction occurred for the encapsulated samples at a higher temperature and indicated an increase in thermal resistance in the hydrogel containing the essential oil loaded into the base of the chitosan particles.

3.3. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Minimum Biofilm Inhibitory Concentration (MBIC50)

MIC, MBC, and MBIC value of MPEO-CsNPs and unloaded-nano gel against *A.baumannii* was determined and presented in Table 2. As shown in the table, the MIC and MBC of MPEO-CsNPs were 3.12 and 12.48 µg / mL, respectively. Antibiofilm inhibition of *A.baumannii* ATCC19606 occurred at 2.69µg/mL as the

minimum dose of MPEO- CsNPs. This value for CsNPs without essential oil was obtained at the highest dose tested, namely 400 µg/mL. The lowest dose of unloaded-nano gel was observed at about 7.38% biofilm inhibition, while MPEO- CsNPs in the same dose were able to inhibit 2.69% of biofilm formation. Therefore, MBIC for MPEO- CsNPs was approximately twofold compared to the control that was treated with no MPEO concentration.

Table 2

Minimum inhibitory concentration (MIC), Minimum Bactericidal Concentration (MBC), and Minimum Biofilm Inhibitory Concentration (MBIC) against *A.baumannii* ATCC19606 (µg/mL).

<i>Acinetobacter baumannii</i>				
Test composition	MIC (µg/mL)	MBC (µg/mL)	D.D (mm)	MBIC50 (µg/mL)
MPEO- CsNPs	3.12	12.48	14	2.69
unloaded CsNPs (µg/mL)	400	> 400	10	7.38
Gentamicin	200	> 200	15	2.90
* MIC; Minimum inhibitory concentration, MBC; Minimum Bactericidal Concentration, MBIC; Minimum Biofilm inhibitory Concentration, D.D; Disc Diffusion				

3.4. Bacterial adherence

The adherence of the bacterial cells to the catheter surface was tested in the presence of different doses of MPEO- CsNPs. As seen in Fig. 3, the adherence of the bacterial cells was influenced in all the experiments that were treated with MPEO- CsNPs 7.5 to 250µg/l. The results revealed that the adherence of bacterial cells had high sensitivity to the nanoformulation of MPEO, while when treated with unloaded CsNPs (blank), it had no inhibitory effect on adherence activity.

Catheter treatment with gentamicin was used as a positive control, and bacterial catheter without any treatment was used as the negative control.

3.5. The result of the evaluation of bap gene expression

Expression of the *bap* gene in samples treated with Gentamicin as a positive control was reduced and statistically significant compared to a negative control without any treatment (NTC) and unloaded CsNPs (Blank)(P = < 0.0001). It was observed that the expression of this gene decreased in the presence of concentrations of 100, 200, and 400 µg/mL MPEO-CsNPs. MPEO-CsNPs from controls are not significantly different at concentrations of 400, 200, and 100 µg/mL, but there are significant differences at concentrations of 50 µg/mL (P = < 0.0001). MPEO-CsNPs from Blank differ significantly at concentrations of 400, 200, and 100 µg/mL (P = < 0.0001), but not significantly at concentrations of 50 µg/mL (P = 0.006). MPEO-CsNPs from gentamicin differ significantly at concentrations of 400, 200, 100,

and 50 µg/mL ($P = < 0.0001$). MPEO-CsNPs at concentrations of 50 µg/mL are significantly different from MPEO-CsNPs at concentrations of 400, 200, and 100 µg/mL ($P = < 0.0001$) (Fig. 4).

3.6. Assessment of mutagenic potential using the Ames test

The mutagenic potential of the different doses of MPEO- MPEO-CsNPs was tested on the bacterial cells. As seen in Table 3. There was a significant difference between treatments of 200 and 400 µg compared to treatment of 50 µg ($P < 0.05$) in the presence of MPEO-CsNPs with liver extract. Also, there is a significant difference between treatments 200 and 400 µg compared to treatments 100 µg ($P < 0.05$) in the presence of MPEO- MPEO-CsNPs without liver extract. No significant difference was observed between experimental treatments ($P > 0.05$). There was a significant difference between treatments 200 and 400 µg compared to treatment 50 ($P < 0.05$) in the presence of Blank with liver extract: There is also a significant difference between treatments 100 and 400 µg ($P < 0.05$). There was a significant difference between treatments 200 and 400 µg compared to treatment 50 ($P < 0.05$). Also, there is a significant difference between treatments 200 and 400 µg compared to treatment 100 and treatments 200 and 400 µg ($P < 0.05$) in the presence of Blank without liver extract.

Table 3
Mutagenic potential in the presence of MPEO-CsNPs and Unloaded CsNPs

<i>Salmonella</i> Strains	S9	Mutagenic index (MI)				SEM	P- Value	LSD $P \leq 0.05$
		0.5 µg per plate	1.0 µg per plate	2.0 µg per plate	4.0 µg per plate			
TA100 (Unloaded CsNPs)	+	1.15 ± 0.042	1.28 ± 0.035	1.52 ± 0.014	1.46 ± 0.063	0.056	0.003	0.118
TA100(Unloaded CsNPs)	-	0.94 ± 0.091	1.2150 ± 0.530	0.91 ± 0.558	1.04 ± 0.106	0.113	0.865	1.086
TA100(MPEO- CsNPs)	+	0.41 ± 0.056	0.43 ± 0.007	0.56 ± 0.014	0.59 ± 0.084	0.032	0.045	0.143
TA100 (MPEO- CsNPs)	-	0.24 ± 0.014	0.28 ± 0.035	0.59 ± 0.042	1.05 ± 0.155	0.124	0.002	0.229

* LSD; List Square Difference, SEM; Standard Error of Means

4. Discussion

Nowadays, with the spread of antibiotic resistance in bacteria, new therapies are necessary to combat bacterial infections [26]. Therefore, with the advances in nanotechnology, opportunities have been provided to find the antimicrobial effects of nanoparticles stenciled with plants with medicinal value common side effects and low cost [27, 28]. In addition to inhibiting bacterial proliferation by attaching to

bacterial membranes and preventing bacteria from binding to each other, nanoparticles delay the formation of bacterial biofilms [29, 30]. So far, much research has been done on the effect of nanoparticles synthesized by medicinal plants on the biofilm of various bacteria [30–33]. The present study was conducted to investigate the anti-biofilm effect of MPEO-CsNPs against *A. baumannii*. The results showed that MPEO-CsNPs could inhibit the formation of *A. baumannii* biofilm (at concentrations $> 3.12 \mu\text{g/ml}$). In a study investigating the anti-biofilm effect of MPEO-CsNPs against *Streptococcus mutans*, the researchers showed that MPEO had anti-biofilm effects against the pathogen *S. mutans*, so that the rate of biofilm inhibition (MBIC) at a concentration of $400 \mu\text{g} / \text{ml}$ was reported to be 72% [14].

But in the present study, the MIC and MBC of MPEO-CsNPs against *A. baumannii* were 3.12 and 12.48 $\mu\text{g/mL}$, respectively. The difference in MIC and MBC is affected by the concentration, type, and diameter of nanoparticles and the sensitivity of the organism under study, so gram-negative bacteria are more resistant to the positive charge of nanoparticles than gram-positive bacteria. Samuggam et al., in Malaysia, described that Spondias mombin-mediated silver nanoparticles are effective against biofilm-producing bacteria. Synthesized silver nanoparticles (AgNPs) are effective against biofilm-producing bacteria, such as *S. haemolyticus*, *S. epidermidis*, *B. subtilis*, *S. aureus*, *S. pyogenes*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhi* was effective in causing structural changes that resulted in cell death and less cytotoxicity [30]. Hosseini et al., in Iran, based on the result of in vitro anti-biofilm activity of curcumin nanoparticles, MIC for curcumin was $128 \mu\text{g/mL}$ in *A. baumannii* they suggest that curcumin nanoparticles can be helpful as a candidate for inhibiting the formation of *A. baumannii* biofilms [34]. Pirigharaghie et al., based on Real-time PCR results of *Bap* gene expression in *A. baumannii* treatment by AgNPs reported that all the strains had a significant downregulation in gene expression compared to the control gene ($P < 0.05$), and described that AgNPs can be used as drug candidates in pharmaceutical industries according to inhibitory effects on *bap* gene expression in *A. baumannii* strains [35]. Our study's results indicate the successful biofilm reduction results in *A. baumannii* isolates used after MPEO-CsNPs treatment, which is consistent with the results of the above studies and is a suitable candidate for the treatment of infections caused by biofilm-producing *A. baumannii*.

In the present study the results of Real-time PCR for gene expression for the *bap* gene in biofilm-producing *A. baumannii* showed that after treatment with 100, 200, and $400 \mu\text{g/mL}$ of MPEO-CsNPs, there was a significant decrease in expression in *bap* gene compared to the control gene ($P = < 0.0001$). In Bahador et al., study investigated the Effect of Satureja khuzestanica Essence against the *bap* gene in *A. baumannii*. It showed Satureja khuzestanica essential oil 42.40 times reduced *bap* expression, but no effect on *DNA gyrA* expression [36].

The Ames test is based on the induction of reverse mutation using *Salmonella typhimurium* auxotrophic strains relative to the amino acid histidine (His⁻), which presents different mutations. In the operon, this amino acid is made to detect mutations such as template change or base pair replacement in DNA. These mutant strains will not be able to grow in the minimal culture medium without histidine unless new mutations occur at the site of these pre-existing mutations, or in the vicinity of genes that restore gene function and enable cell processing. These mutant cells can grow to a minimum and form a colony in the

absence of histidine in a culture medium, so this test is called a reversal test [37]. In the present study, the recurrent Kant of Salmonella was lower than the control of sodium azide, which was used as a potential mutagen, at equivalent concentrations in the presence of both the microsomal activating metabolite (S9) and in the absence (S9). The results showed that with increasing concentration in both groups, the amount of recurrent Kant increases. In general, it can be concluded that the amount of MI in different concentrations of nano gels is less than sodium azide and has far fewer mutagenic effects.

5. Conclusion

Due to the effect of MPEO-CsNPs on decreasing the biofilm formation of *A. baumannii*, this substance can be used to treat infections caused by *A. baumannii*. Also suggested that in future studies, the effect of MPEO-CsNPs on other bacterial virulence factors such as quorum sensing be investigated to achieve more comprehensive results for the use of MPEO-CsNPs in treatment.

Declarations

Acknowledgments

The authors would like to thank all the staff of Raze Herbal Medicines Research Center, Loreta University of Medical Sciences.

Funding/Support

This study was supported in part by grant IR.LUMS.REC.1398.192 (<https://ethics.research.ac.ir/>) from the lorestan university of medical sciences.

Ethical Approval

[https://ethics.research.ac.ir/EthicsProposalView.php? &code=IR.LUMS.REC.1398.192](https://ethics.research.ac.ir/EthicsProposalView.php?&code=IR.LUMS.REC.1398.192).

Availability of Data and materials

Not Applicable.

Author Contribution

Shakib P; Writing an article Data review, Ashrafi B; Data collection, Rezaei F; Data analysis, Delfani S; Research design and Project Management.

Conflict of interests

The authors declare that they have no conflict of interest.

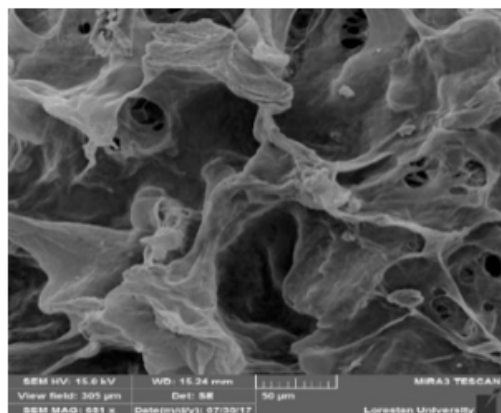
References

1. Pakharukova N, Tuittila M, Paavilainen S, Malmi H, Parilova O, Teneberg S, et al. Structural basis for *Acinetobacter baumannii* biofilm formation. *Proc Natl Acad Sci*. 2018;115(21):5558-63. <http://dx.doi.org/10.1073/pnas.1800961115>. PMID: **29735695**.
2. Longo F, Vuotto C, Donelli G. Biofilm formation in *Acinetobacter baumannii*. *New Microbiol*. 2014;37(2):119-27. PMID: **24858639**
3. Brossard KA, Campagnari AA. The *Acinetobacter baumannii* biofilm-associated protein plays a role in adherence to human epithelial cells. *Infect immun*. 2012;80(1):228-33. <https://doi.org/10.1128/IAI.05913-11>
4. Gaddy JA, Actis LA. Regulation of *Acinetobacter baumannii* biofilm formation. *Future Microbiol*. 2009;4(3). <https://doi.org/10.2217/fmb.09.5>
5. Lasa I, Penadés JR. Bap: a family of surface proteins involved in biofilm formation. *Res microbiol*. 2006;157(2):99-107. <https://doi.org/10.1016/j.resmic.2005.11.003>
6. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol*. 2004;2(2):95-108.
7. Shirmohammadlou N, Zeighami H, Haghi F, Kashefieh M. Resistance pattern and distribution of carbapenemase and antiseptic resistance genes among multidrug-resistant *Acinetobacter baumannii* isolated from intensive care unit patients. *J Med Microbiol*. 2018;67(10):1467-73. <https://doi.org/10.1099/jmm.0.000826>.
8. Huang H, Chen B, Liu G, Ran J, Lian X, Huang X, et al. A multi-center study on the risk factors of infection caused by multi-drug resistant *Acinetobacter baumannii*. *BMC Infect Dis*. 2018;18(1):1-6. <https://doi.org/10.1186/s12879-017-2932-5>
9. Levy-Blitchtein S, Roca I, Plasencia-Rebata S, Vicente-Taboada W, Velásquez-Pomar J, Muñoz L, et al. Emergence and spread of carbapenem-resistant *Acinetobacter baumannii* international clones II and III in Lima, Peru. *Emerg Microbes Infect*. 2018;7(1):1-9. <https://doi.org/10.1038/s41426-018-0127-9>
10. Knezevic P, Aleksic V, Simin N, Svircev E, Petrovic A, Mimica-Dukic N. Antimicrobial activity of Eucalyptus camaldulensis essential oils and their interactions with conventional antimicrobial agents against multi-drug resistant *Acinetobacter baumannii*. *J Ethnopharmacol*. 2016;178:125-36. <https://doi.org/10.1016/j.jep.2015.12.008>
11. Tiwari V, Mishra N, Gadani K, Solanki PS, Shah N, Tiwari M. Mechanism of antibacterial activity of zinc oxide nanoparticle against carbapenem-resistant *Acinetobacter baumannii*. *Front Microbiol*. 2018;9:1218. <https://doi.org/10.3389/fmicb.2018.01218>
12. Alharbi FA, Alarfaj AA. Green synthesis of silver nanoparticles from *Neurada procumbens* and its antibacterial activity against multi-drug resistant microbial pathogens. *Journal of King Saud University-Science*. 2020;32(2):1346-52.
13. Wintachai P, Paosen S, Yupanqui CT, Voravuthikunchai SP. Silver nanoparticles synthesized with Eucalyptus citriodora ethanol leaf extract stimulate antibacterial activity against clinically multidrug-resistant *Acinetobacter baumannii* isolated from pneumonia patients. *Microb Pathog*. 2019;126:245-57. <https://doi.org/10.1016/j.micpath.2018.11.018>

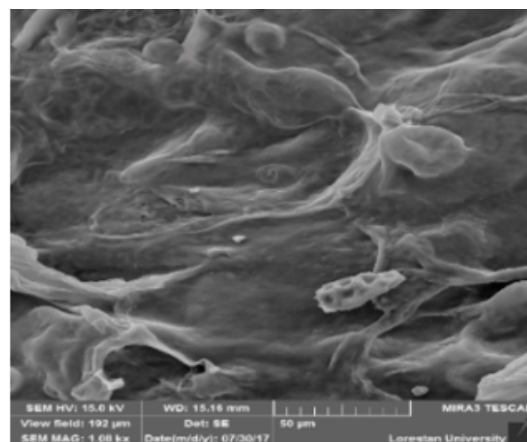
14. Ashrafi B, Rashidipour M, Marzban A, Soroush S, Azadpour M, Delfani S, et al. *Mentha piperita* essential oils loaded in a chitosan nanogel with inhibitory effect on biofilm formation against *S. mutans* on the dental surface. *Carbohydr Polym.* 2019;212:142-9. <https://doi.org/10.1016/j.carbpol.2019.02.018>
15. Kumari A, Yadav SK, Yadav SC. Biodegradable polymeric nanoparticles-based drug delivery systems. *Colloids Surf B Biointerfaces.* 2010;75(1):1-18. <https://doi.org/10.1016/j.colsurfb.2009.09.001>
16. Saeidi S, Mohsenbeygi M, Roustakhiz J, Javadian F, Hassanshahian M. Antimicrobial and anti-biofilm effects of *Mentha piperita* and *Zataria multiflora* on pathogenic bacteria. *J Med Bacteriol.* 2019;8(1-2):37-44.
17. Hoffmann BG, Lunder LT. Flavonoids from *Mentha piperita* leaves. *Planta med.* 1984;51(4) : 361. <https://doi.org/10.1055/s-2007-969736>
18. Rashidipour M, Maleki A, Kordi S, Birjandi M, Pajouhi N, Mohammadi E, et al. Pectin/chitosan/tripolyphosphate nanoparticles: efficient carriers for reducing soil sorption, cytotoxicity, and mutagenicity of paraquat and enhancing its herbicide activity. *J agric food chem.* 2019;67(20):5736-45. <https://doi.org/10.1021/acs.jafc.9b01106>
19. Amiri A, Mousakhani-Ganjeh A, Amiri Z, Guo Y-g, Singh AP, Kenari RE. Fabrication of cumin loaded-chitosan particles: Characterized by molecular, morphological, thermal, antioxidant and anticancer properties as well as its utilization in food system. *Food Chem.* 2020;310:125821. <https://doi.org/10.1016/j.foodchem.2019.125821>
20. Zhou T, Xiao C, Fan J, Chen S, Shen J, Wu W, et al. A nanogel of on-site tunable pH-response for efficient anticancer drug delivery. *Acta biomater.* 2013;9(1):4546-57. <https://doi.org/10.1016/j.actbio.2012.08.017>
21. Hendiani S, ABDI AA, Mohammadi P. Comparison of two methods for quantification of *Acinetobacter baumannii* biofilm formation. *Biologic J Microorganism.* 2014;2(8):51-56.
22. Kim B-S, Park S-J, Kim M-K, Kim Y-H, Lee S-B, Lee K-H, et al. Inhibitory effects of *Chrysanthemum boreale* essential oil on biofilm formation and virulence factor expression of *Streptococcus mutans*. *Evid.-based Complement Altern Med.* 2015;2015. <https://doi.org/10.1155/2015/616309>
23. Stickler DJ. Bacterial biofilms in patients with indwelling urinary catheters. *Nat clin pract urol.* 2008;5(11):598-608.
24. Bahador A, Saghii H, Ataee R, Esmaeili D. The Study of inhibition effects *Satureja khuzestanica* essence against gene expression *baumannii* with real-time PCR technique. *Iran J Med Microbiol .* 2015;9(1):42-9. <http://ijmm.ir/article-1-313-en.html>
25. Abdul-Hafeez EY, Karamova N, Ilinskaya O. Evaluation of mutagenic and antimutagenic potential of stem bark aqueous extracts of eight trees by the bacterial reverse mutation assay. *Ecol Genet.* 2018;16(3) :55-61. <https://doi.org/10.17816/ecogen16355-61>.
26. Jain N, Jansone I, Obidenova T, Simanis R, Meisters J, Straupmane D, et al. Antimicrobial Resistance in Nosocomial Isolates of Gram-Negative Bacteria: Public Health Implications in the Latvian Context. *Antibiotics.* 2021;10(7):791. <https://doi.org/10.3390/antibiotics10070791>.

27. Mandal S, Marpu SB, Hughes R, Omary MA, Shi SQ. Green Synthesis of Silver Nanoparticles Using Cannabis sativa Extracts and Their Anti-Bacterial Activity. *Green Sustain Chem.* 2021;11(1):38-48. <https://doi.org/10.4236/gsc.2021.111004>
28. Akintelu SA, Yao B, Folorunso AS. Green synthesis, characterization, and antibacterial investigation of synthesized gold nanoparticles (AuNPs) from Garcinia kola pulp extract. *Plasmonics.* 2021;16(1):157-65. <https://doi.org/10.1007/s11468-020-01274-9>.
29. LewisOscar F, Nithya C, Vismaya S, Arunkumar M, Pugazhendhi A, Nguyen-Tri P, et al. In vitro analysis of green fabricated silver nanoparticles (AgNPs) against Pseudomonas aeruginosa PA14 biofilm formation, their application on urinary catheter. *Prog Org Coat.* 2021;151:106058. <https://doi.org/10.1016/j.porgcoat.2020.106058>
30. Samuggam S, Chinni SV, Mutusamy P, Gopinath SC, Anbu P, Venugopal V, et al. Green Synthesis and Characterization of Silver Nanoparticles Using Spondias mombin Extract and Their Antimicrobial Activity against Biofilm-Producing Bacteria. *Molecules.* 2021;26(9):2681.
31. Barabadi H, Mojab F, Vahidi H, Marashi B, Talank N, Hosseini O, et al. Green synthesis, characterization, antibacterial and biofilm inhibitory activity of silver nanoparticles compared to commercial silver nanoparticles. *Inorg Chem Commun.* 2021;129:108647. <https://doi.org/10.1016/j.inoche.2021.108647>
32. Naidi SN, Khan F, Tan AL, Harunsani MH, Kim Y-M, Khan MM. Green synthesis of CeO₂ and Zr/Sn-dual doped CeO₂ nanoparticles with photoantioxidant and antibiofilm activities. *Biomater Sci.* 2021;9, 4854-4869. <https://doi.org/10.1039/D1BM00298H>
33. de Lacerda Coriolano D, de Souza JB, Bueno EV, Medeiros SMdFRdS, Cavalcanti IDL, Cavalcanti IMF. Antibacterial and antibiofilm potential of silver nanoparticles against antibiotic-sensitive and multidrug-resistant Pseudomonas aeruginosa strains. *Braz J Microbiol.* 2021;52(1):267-78.
34. Hosseini A, Nejadsattari T, Zargar M. In vitro anti-biofilm activity of curcumin nanoparticles in Acinetobacter baumannii: a culture-based and molecular approach. *Arch Clin Infect Dis.* 2019;14(4).
35. Pirigharaghie T & Sadatshandiz A. The Inhibitory Effects of Silver Nanoparticles on Bap Gene Expression in Antibiotic-Resistant Acinetobacter baumannii Isolates using Real-Time PCR. *Sci J Ilam Univ Med Sci.* 2018; 26(4): 175-185.
36. Bahador A, Saghii H, Ataee R, Esmaeili D. The Study of inhibition effects Satureja khuzestanica essence against gene expression bap acinetobacter baumannii with real time PCR technique. *Iranian J Med Microbiol.* 2015;9(1):42-9. <http://ijmm.ir/article-1-313-en.html>
37. de Cássia Proença-Assunção J, Constantino E, Farias-de-França AP, Nogueira FAR, Consonni SR, Chaud MV, et al. Mutagenicity of silver nanoparticles synthesized with curcumin (Cur-AgNPs). *J Saudi Chem Soc.* 2021;25(9):101321. <https://doi.org/10.1016/j.jscs.2021.101321>

Figures



A



B

Figure 1

SEM image of MPEO- CsNPs micrographs at 15 kV of unloaded chitosan nanogel and MPEO- chitosan nanogel (A and B)

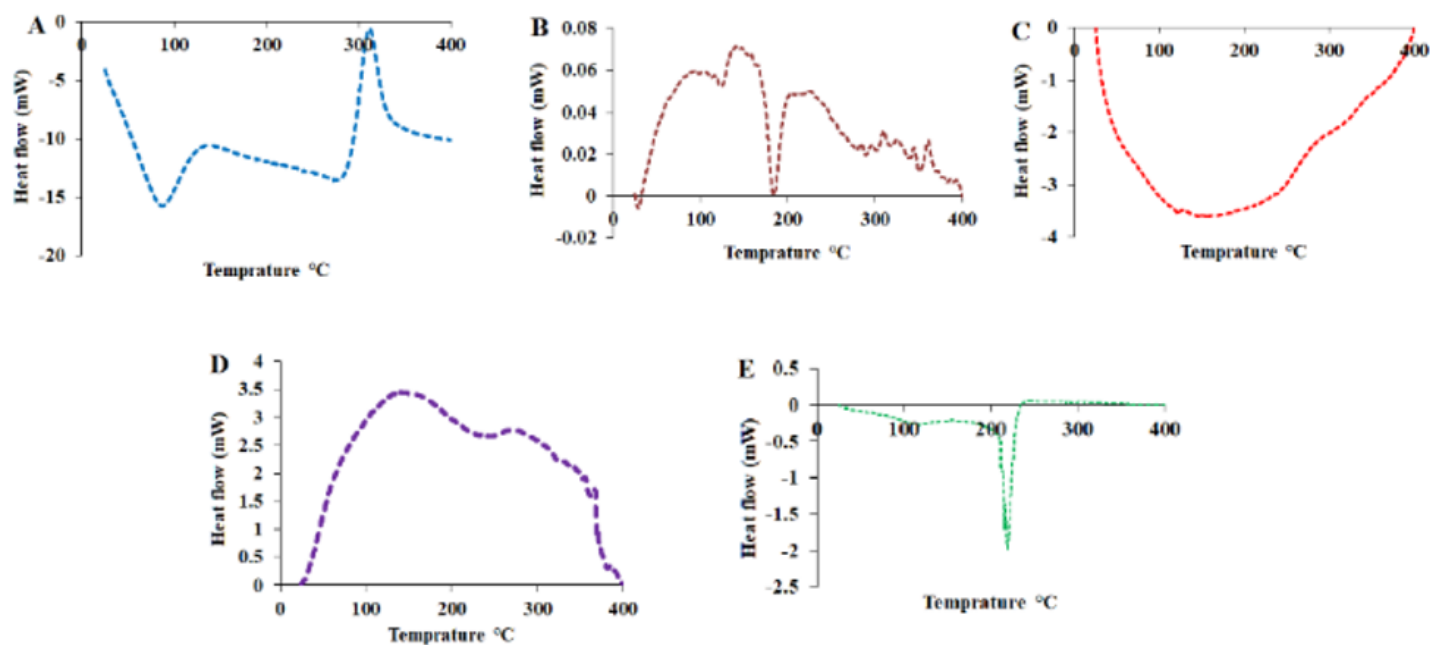


Figure 2

Thermal spectra of Differential Scanning Calorimetry (DSC) hydrogel components and final formulation of chitosan (A), triphosphate (B), peppermint essential oil (C), hydrogel without peppermint essential oil (D), a hydrogel with peppermint essential oil loaded on chitosan particles (E).

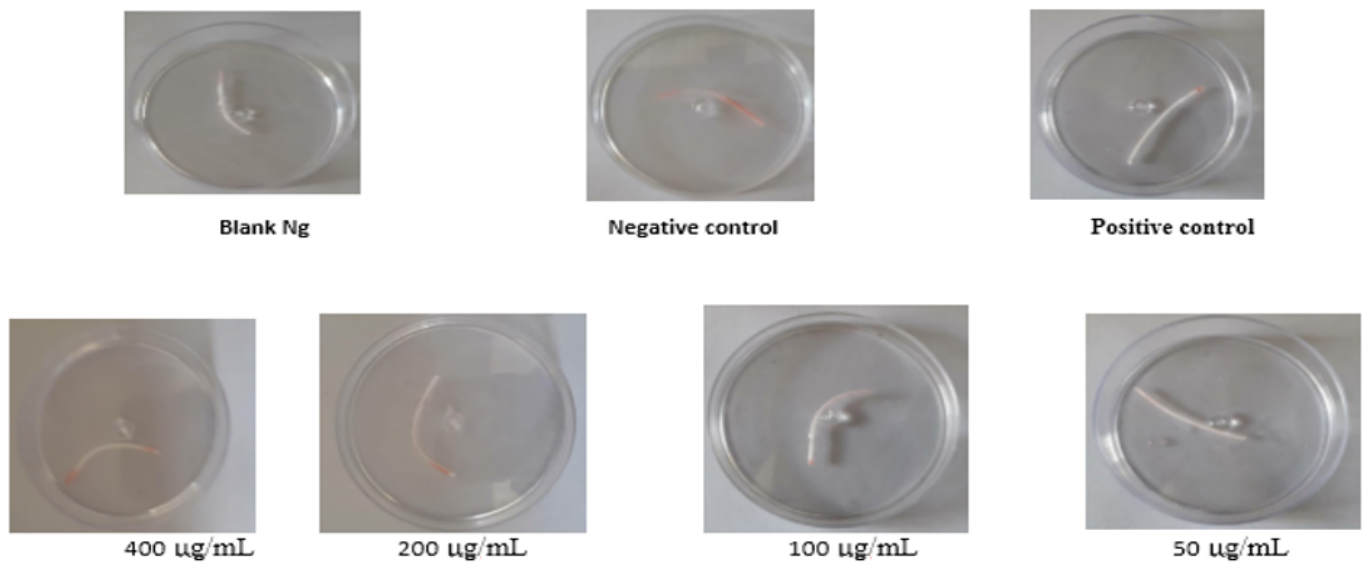


Figure 3

Antibiofilm activity of MPEO-CsNPs against *A. baumannii*. Growth of the bacterium in the presence of different concentrations of MPEO-CsNPs and the effect of MPEO-CsNPs on biofilm formation by the bacterium on the catheter surface

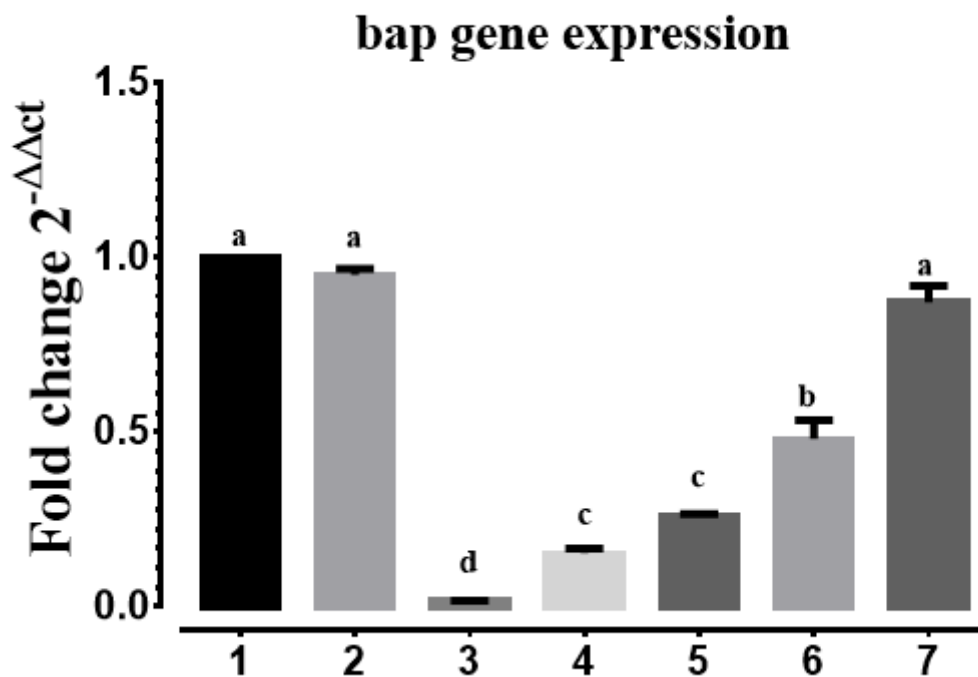


Figure 4

Real-time PCR analysis of *bap* gene expression. 1. NTC, 2. Unloaded CsNPs (blank), 3. Gentamicin, 4. MPEO-CsNPs 400 µg/mL, 5. MPEO-CsNPs 200 µg/mL, 6. MPEO-CsNPs 100 µg/mL, 7. MPEO-CsNPs 50 µg/mL