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Biofilm targeting with chitosan-based nanohydrogel containing *Quercus infectoria* G. Olivier extract against *Streptococcus mutans*: new formulations of a traditional natural product

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Abstract

Background Biofilm formation has a crucial role in the cariogenic virulence of *Streptococcus mutans*, which leads to resistance to common antibacterials. The antimicrobial resistance crisis has led to increased research about traditional natural products.

Purpose *Quercus infectoria* extract (QI extract) and nano hydrogels containing QI extract (QI-NH) and tannic acid (TA-NH) were evaluated against this pathogen.

Methods QI extract was analyzed by HPLC and the physiological characteristics of nanohydrogels were assessed by SEM, FTIR, zeta potential, DLS and determination of release kinetics and encapsulation efficiency. Determination of MIC and MBC of the material and their anti-biofilm effect was done by the microtiter method and on the extracted tooth surface. The properties of extracts and nano hydrogels in the expression of genes codifying glucosyltransferases (*gtf*B, *gtf*C and *gtf*D) and glucan binding protein B (*gbp*B) were quantified. Their toxicity was tested by the MTT method against the KB cell line.

Results According to HPLC, 55.18% of QI extract contained TA. The encapsulation efficiency of QI-NH and TA-NH was equal to 60% and 80%, respectively. SEM and FTIR exhibited that QI extract and TA were successfully entrapped in the networks resulting from the chemical bonding of chitosan and TPP. The average size of QI-NH and TA-NH was 70.45 and 58.43 nm, and their zeta potential was 6.17 ± 2.58 and 0.25 ± 0.03 mv, respectively. PDI < 0.3 of nano hydrogels indicated the favorable polydispersity of nanohydrogels. MIC of QI extract, QI-NH and TA-NH were 937.5, 30 and 10 µg/ml, respectively. Also their MBIC50 was 35.1, 2.1 and 0.95 µg/ml, respectively, and the extracts and nano hydrogels restrained the biofilm maturation on enamel. The pivotal genes of *S. mutans* in biofilm formation were significantly less expressed by treatment with QI-NH and TA-NH than others. Based on the MTT test, QI-NH had less

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acute toxicity than QI extract and TA-NH. IC50 of QI-NH was calculated as 775.4 µg/ml, while it was equal to 3.12 µg/ml for chlorhexidine as a common antibacterial agent.

Conclusion QI-NH, a new formulation derived from traditional anti-caries, can be a safe and efficient option to combat dental biofilm.

Keywords *Streptococcus mutans*, Biofilm, Persian medicine, *Quercus infectoraia*, Nanohydrogel

Introduction

Oral diseases are among the most important diseases that have attracted the attention of the health systems worldwide. One of the reasons is the high prevalence of these diseases. As studies have shown 3.5 billion people, i.e. 38% of the world's population, suffer from oral diseases. Dental caries is of great importance because 29.4% of adults and 7.8% of children with deciduous teeth have untreated dental caries [1].Among hundreds of oral species, *Streptococcus mutans* is one of the critical pathogens of the oral cavity because it is considered the main pathogen in dental caries. *S. mutans* as a leading pathogen shapes the oral microbiota in patients with dental caries [2]. Biofilm formation is the crucial virulence factor of *S. mutans* in the initiation of carious lesions. The conversion of dietary sucrose into glucans (or mutans) by glucosyltransferases including *gtfB*, *gtfC*, and *gtfD* is the basis of microbial biofilm formation [3]. Cariogenic pathogens are highly resistant to antimicrobial agents and local immune responses in the exopolysaccharide barrier and other components of the biofilm [4].

Despite all the advances in oral therapeutics in recent years, dental caries is still considered a global problem for healthcare systems due to its high prevalence and economic burden [5, 6]. Resistance of oral pathogens including *S. mutans* and other streptococci species to common antimicrobial agents has been a serious concern in recent decades [7, 8]. According to microbiological studies, chlorhexidine (CHX), as one of the most common oral antimicrobial agents, leads to an increase in the resistance of oral pathogens, including *S. mutans* [9, 10]. Besides these, clinical complications such as dental staining, burning sensation, enamel destruction, and oral ulcers are other restricting factors of CHX [11].

The mentioned limitations of antimicrobials have caused more and more attention to methods such as Immunomodulation and Anti-Virulence therapy [12]. The use of natural products is another non-antibiotic strategy that has high biocompatibility, low side effects, and excellent anti-biofilm properties [13]. The requirement of global attention to the increasing threat of antimicrobial resistance has caused the return of researchers' interest in traditional medicinal plants [14, 15].

One of the anti-biofilm natural agents is *Quercus infectoria* G. Olivier (QI) [16], which is used in Persian medicine (PM) as one of the main treatments for dental caries [17]. QI is from the Fagaceae family, commonly known as gall oak. Because it is mainly found in Iran, Greece, and Asia Minor [18], QI (*Mazoo* in PM) has long been considered in PM by great physicians such as Avicenna (980 to 1037 AD) and Rhazes (860 to 932 AD) [17]. QI gall is mainly composed of phenolic compounds, so tannins make up 50–70% of this gall extract [19]. Tannic acid (TA) is the simplest hydrolyzable form of tannins whose safety has been approved. TA has many physiological properties, including antimicrobial, anti-inflammatory, anti-tumor, etc [20].

Chitosan, as a natural biopolymer, has been noted for its biocompatibility, biodegradability, and muco-adhesivity in oral diseases related to microorganisms [21]. One of the applications of chitosan is encapsulating antibiofilm agents, including synthetic antibiotics and natural antimicrobials [22]. Nanoencapsulation of natural products or other bioactive agents can significantly enhance biofilm targeting due to their very small size, because the extracellular matrix limits access of common antimicrobials due to its physical and chemical characteristics [23]. In this study, the effect of chitosan-based nano hydrogels containing QI extract and TA (QI-NH and TA-NH) on *S. mutans*, its biofilm formation, and the expression of genes related to this process were appraised.

Materials and methods

Preparation of QI extract and TA

First, the oak gall was collected from the oak forest in Zagros Mountains, Lorestan, Iran, with the permission of Lorestan Natural Resources and Watershed Management Organization. Identification and verification of the collected samples were done in the herbarium department of the Faculty of Pharmacy, University of Tehran (Voucher number: PMP-1852). Air-dried QI galls were powdered, and then 100 g were extracted in 500 ml of 70% methanol for seven days. QI extraction was done at 25 °C without agitation. The extraction efficiency was 12.7%.

TA (Sigma-Aldrich, St. Louis, MO, USA) was also purchased for the chemical analysis of QI extract and nano hydrogel formulation.

HPLC quantification of TA in QI extract

Quantitation of TA in QI extract was done by reversed-phase High-performance liquid chromatography

(HPLC) method that was conducted on a Shimadzu (model 10Avp) instrument. Isocratic HPLC method with 0.05 mol/L acetate buffer pH 5.0 in water (solvent A) and acetonitrile (solvent B) was used (Acetate buffer: acetonitrile ratio 70:30) with a flow rate of 1 ml/min. This process was performed on a 25 cm × 4.6 mm i.d. RP-8 column with the temperature maintained at 30 °C. The peaks of the QI sample were detected compared to the TA solution as a standard at 280 nm. 1 mg of QI extract was dissolved in 10 ml of distilled water and after filtration with 0.45 µm nylon filter membrane, it was injected into the device. Finally, the percentage of TA in the QI extract was calculated based on the calibration curve of standard samples (5–200 µg/ml TA).

Preparation of nano hydrogels

To synthesize QI-NH, TA-NH, and unloaded-NH, 20 ml of 0.5% w/v chitosan solution containing 1% acetic acid (Merck, Darmstadt, Germany) was used. Low molecular weight chitosan (50,000-190,000 Da) was used for the formulations. After stirring for 24 h, 100 mg QI extract and 20 mg TA were added separately to 2 chitosan solutions gradually. No bioactive agent (QI or TA) was used for unloaded-NH formulation. After 4 h and complete dissolution of QI extract and TA, 5 ml of 0.3% w/v tripolyphosphate (TPP) solution was dropped to all three samples within 30 min. To separate the insoluble particles, the solutions were filtered using filter paper with 0.45 µ pore size in the synthesis steps. Prepared nano hydrogels were kept at 4 °C until analytical, antibacterial, and cytotoxic assays.

Encapsulation efficiency (EE)

5 ml of 2 M acetic acid was added to 0.3 mg of freeze-dried nano hydrogels (QI-NH and TA-NH) in test tubes. These two solutions were placed in a water bath (Memert, Germany) for 30 min at 90 ℃ to release the encapsulated natural products by breaking the bonds between chitosan and TPP. After the paper filtration of the solutions, the volume of each solution was doubled (10 ml) by adding distilled water, and finally, the absorbance of the samples was measured at 280 nm with a UV/Vis spectrophotometer (Shimadzuo, Japan). EE was calculated based on the following formula:

$$\mathrm{EE}\,(\%) = \left(\frac{\text{Loaded QI or TA amount (mg)}}{\text{Initial QI or TA amount (mg)}}\right) \times 100$$

Physicochemical analysis of the nanohydrogels

Scanning electron microscopy (SEM)

SEM was used to examine the morphology of nano hydrogels. For this purpose, freeze-dried nano hydrogels (at -50 °C for 24 h) were coated with gold layers with a thickness of 5 nm. Then, SEM images were taken with a 6300 field emission scanning electron microscope (Hitachi, S-4160) at 15 kV.

Fourier-transform infrared (FT-IR) spectroscopy

In this method, the main functional groups around the samples including QI extract, TA, chitosan, TPP, QI-NH, and TA-NH were found based on infrared wavelength absorption between 4000 and 400 cm^{-1}.

Dynamic light scattering (DLS) and zeta potential

The emulsions were diluted 1:1000 in distilled water, and the samples were filtered through 0.45 µ pores. The size distribution of QI-NH and TA-NH was measure using the DLS method. Polydispersity index and zeta potential to evaluate the stability of nanohydrogels were also determined with Malvern Instruments Ltd., Malvern, UK.

Release kinetics

To evaluate the release rate of QI extract and TA at different times, the dialysis bag was immersed in phosphate-buffered saline (PBS) (PH=7.4). After 24 h, 5 ml PBS solution containing 0.2 g QI-NH or TA-NH was completely sealed in two separate bags. These bags were kept in 100 ml of PBS while being stirred, and 1 ml of PBS was sampled in 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 210, and 240 min and immediately replaced with 1 ml of fresh PBS. The absorbance of 12 samples of each nano hydrogel was measured by UV/Vis spectrophotometer (Shimadzu, Japan) at 280 nm. Finally, the percentage of QI extract and TA released from nano hydrogels was calculated separately based on the following formula [11]:

$$\text{Cumulative release}(\%) = \sum_{t=0}^{t} \frac{Mt}{M0}$$

Bacterial strain and culture condition

The standard strain of *S. mutans* (ATCC 35668) was acquired from the Iranian Biological Resource Center (IBRC, Tehran, Iran). *S. mutans* was cultured in Brucella broth (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C under anaerobic conditions (5% CO2, 80% N2) for 24 h.

Antibacterial assay

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined by microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines [24]. Briefly, different concentrations of QI extract (29.29 to 15000 µg/ml), QI-NH (0.93 to 480 µg/ml), TA—NH(0.62 to 320 µg/ml), and unloaded-NH (117.18 to 60000 µg/ml) were prepared by serial dilution method in 96-well polystyrene plates. Then, 1.5×10^5 (CFU/mL) bacteria were added to the wells and incubated for 24 h at 37 °C and under

anaerobic culture conditions. Finally, MIC was determined using 2,3,5-Triphenyltetrazolium chloride to indicate bacterial viability.

To determine MBC, 10 µl of supra-MIC concentrations were subcultured in Brucella agar (Sigma-Aldrich, St. Louis, MO, USA) for 24 h under the same conditions. The minimum concentration of extract or nano hydrogels that inhibited visible growth in the culture medium was equal to MBC.

Anti-biofilm assay

Quantitative anti-biofilm assay

The minimum biofilm inhibition concentration ($MBIC_{50}$): $MBIC_{50}$ was determined by the microtiter method, by investigating the inhibition of biofilm formation at MIC and sub-MIC concentrations. $MBIC_{50}$ is the minimum concentration that inhibits biofilm formation by 50% or more. Briefly, 1.5×10^6 CFU/mL bacteria were added to the wells containing extract or nano hydrogels in the mentioned concentrations in the flat-bottom 96-well plate. In this method, Brucella broth contained 1% sucrose. After 48 h of cultivation in anaerobic conditions and 37 °C. Free cells were discarded by gently washing the wells twice with PBS. Then, by adding 200 µl of 10% formaldehyde, the attached bacteria were fixed. The biofilms formed on the surface of the wells were stained with crystal violet for 30 min. After its dissolving with ethanol, absorbance was measured at 595 nm [25].

Qualitative anti-biofilm assay on the dental surface

Evaluation of biofilm formation on the dental surface was done according to Ashrafi et al.'s study with a slight change [11]. Seventeen healthy premolar teeth extracted in the orthodontic department of dental school, Shahed University, were first washed with normal saline. In the next step, 25.5% sodium hypochlorite and then 20% H_2O_2 were used to remove the smear layer. After they were sterilized by autoclaving, the teeth were immersed in 4 mL of culture medium containing 1.5×10^6 CFU/mL of bacteria and extracts or nano hydrogels at concentrations of MIC, 1/2 MIC, and 1/4 MIC. Finally, they were cultured for 48 h in a shaker incubator under anaerobic conditions at 37 °C.

RNA extraction and real-time PCR

Total RNA was extracted from untreated *S. mutans* and *S. mutans* treated with sub-MIC concentrations of extract and nano hydrogels for 48 h with DNAbiotech kit (DNAbiotech, Iran). RNA extraction steps proceeded according to the manufacturer's instructions. The quantity and integrity of purified RNA samples were analyzed by spectrophotometry (Nanodrop, USA) and agarose gel electrophoresis (1.5% agarose gel), respectively. Purified RNA was stored at -80 °C until use. cDNA synthesis kit (Parstous, Iran) was used for reverse transcription of 2 µg of total RNA of each sample to cDNA.

The expression level of 4 essential genes in biofilm formation and *16SrRNA* as a reference gene was determined in triplicate through quantitative Real-Time PCR. Samples were analyzed using a SYBR Green master mix (Amplicon, Denmark) with an ABI 7500 detector (Applied Biosystems, USA). Informations of primers and PCR conditions are described in Table 1. The conditions of the real-time PCR amplification reactions were as follows:

95 °C for 10 min for initial pre-cycling heat activation, then 40 cycles of 95 °C for 20s, annealing at 60 °C for 45 s for *gtf*B, *gtf*D and *gbp*B and at 58 °C for 40 s for *gtf*C and *16SrRNA*.

The 2-ΔΔCT standard method was used to determine relative expression levels [26].

Cytotoxicity assay

In this study, for the first time, the cytotoxicity of QI extract, QI-NH and TA-NH against KB cells was assessed by MTT method. The toxicity of CHX as one of the most common antimicrobial agents in dentistry was investigated too. In this regard, KB cell line was purchased from National cell bank of Iran (NCBI, Pasteur Institute, Tehran). RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin was employed. Cells (3×10^4 cells/well) were cultured at 37 °C with 5% CO2 in 96-well plates. To evaluate cell viability, five

Table 1 Characteristics of primers for genes related to S.mutans biofilm formation

Gene	Gene description	Primer sequences (5 – 3)		Annealing T (°C)
gtf B	Glucosyltransferase-I	Forward	AGCAATGCAGCCAATCTACAAAT	58
		Reverse	ACGAACTTTGCCGTTATTGTCA	
gtf C	Glucosyltransferase-SI	Forward	GGTTTAACGTCAAAATTAGCTGTATTAGC	60
		Reverse	CTCAACCAACCGCCACTGTT	
gtf D	Glucosyltransferase-S	Forward	TGTCTTGGTGGCCAGATAAAC	58
		Reverse	GAACGGTTTGTGCAGCAAGG	
gbp B	Secreted antigen GbpB/SagA	Forward	ATGGCGGTTATGGACACGTT	58
		Reverse	TTTGGCCACCTTGAACACCT	
16SrRNA	16 S rRNA	Forward	CCTACGGGAGGCAGCAGTAG	60
		Reverse	CAACAGAGCTTTACGATCCGAAA	

different concentrations of the studied substances were mixed in culture medium in triplicate. After 24 h, the culture medium was removed and equal (20 ml) MTT or 2,5-diphenyl-2 H-tetrazolium bromide (Sigma-Aldrich, St. Louis, MO, USA) was added to the wells and incubated again for 1 h at 37 °C in the dark. The supernatants were removed and again by adding DMSO to the wells and gently shaking for 10 min, the remaining sediments were removed from the wells. Finally, the absorption of samples in different concentrations was measured with a microplate reader and, cell viability was calculated based on the following formula:

$$\text{Cumulative release (\%)} = \frac{\text{Average absorbance of each concentration of the samples}}{\text{Average absorption of control samples}}$$

Statistical analysis

In this study, Graph Pad Prism (Version 9.5.1) was used for data analysis. One-way ANOVA test was used for statistical analysis of data and, Tukey's test was used to compare means. Differences were considered statistically significant at $p<0.05$.

Results

HPLC analysis

HPLC was performed to measure the total amount of TA in the QI extract. Based on the analysis and the chromatograms shown in Fig. 1, TA constituted 55.18% of the QI extract.

Encapsulation efficiency

Based on UV-Vis spectroscopy of nano hydrogels, EE of QI-NH and TA-NH was 17.63% and 28.28%, respectively.

Physicochemical analysis of the nanohydrogels

SEM

Morphological evaluation of freeze-dried nano hydrogels was done with SEM. The creation of electrostatic bonds between chitosan and TPP has led to regular networks in the form of a porous structure, visible in Fig. 2 (Image A). SEM images show the successful synthesis of the samples. The non-porous structures of QI-NH and TA-NH (Images B and C), unlike Unloaded-NH, indicate the successful entrapment of QI extract and TA, respectively.

FTIR

Figure 3 shows the formation of electrostatic bonds between amine (N-H stretching) and phosphate (P-O stretching) functional groups in chitosan and TPP,

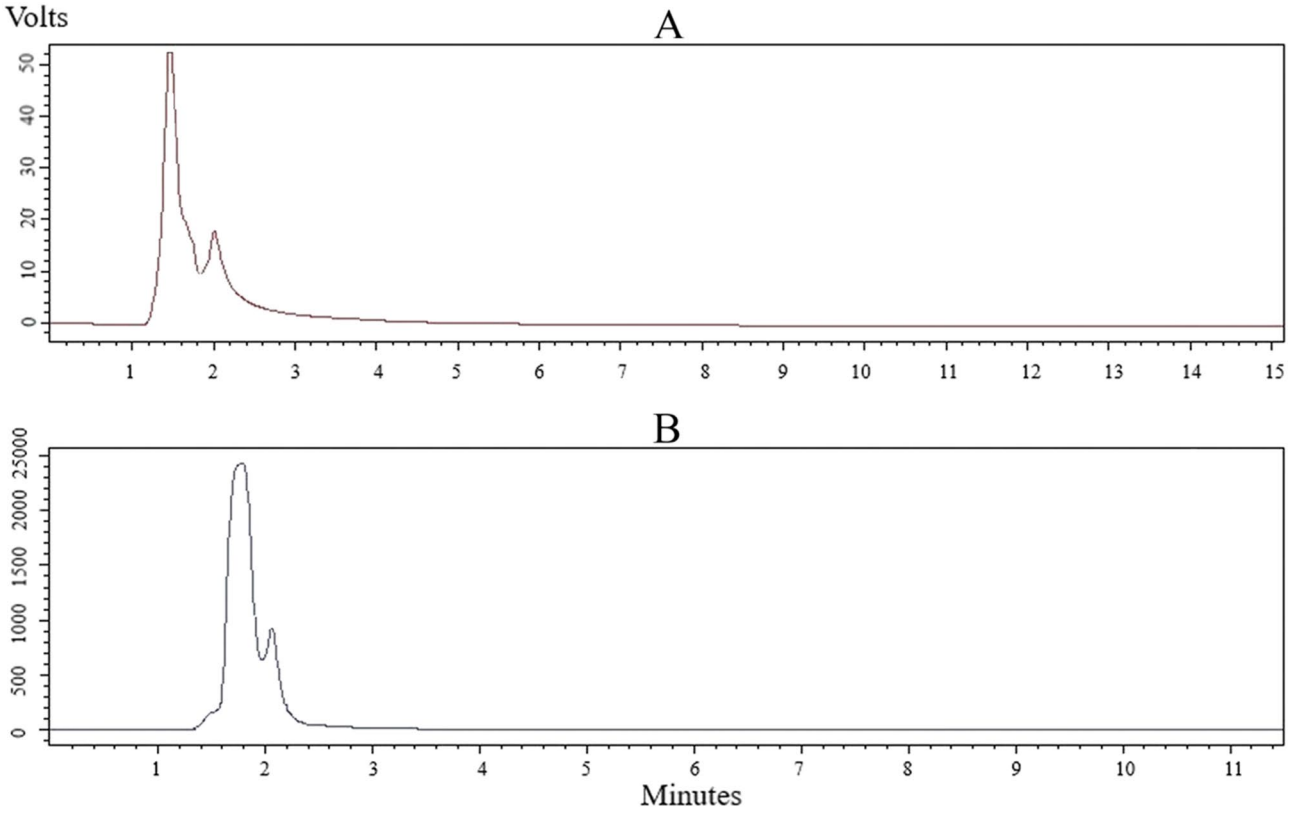

Fig. 1 HPLC analysis of methanolic extract of *Quercus infectoria* G. Olivier. Chromatograms of QI extract (**A**) and TA standard solution (**B**)

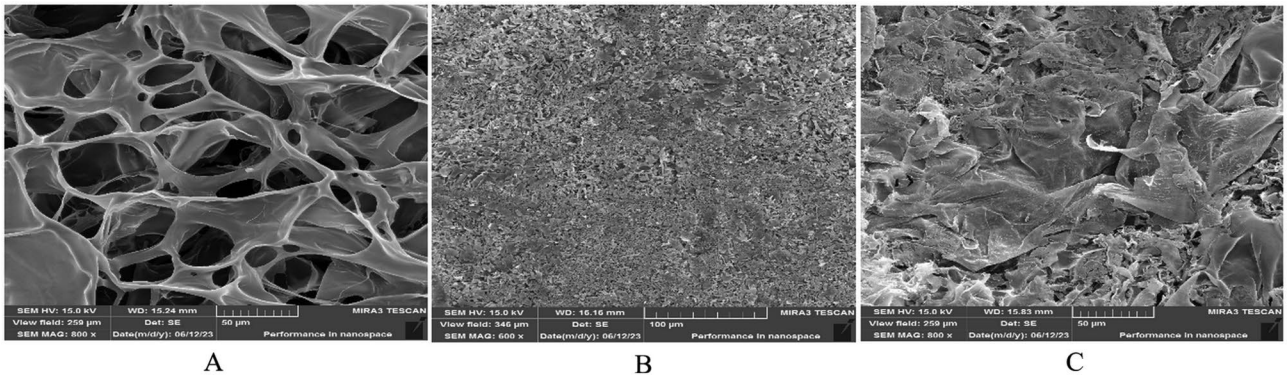

Fig. 2 Scanning electron microscopy (SEM) of Unloaded-NH (**A**), QI-NH (**B**), and TA-NH (**C**)

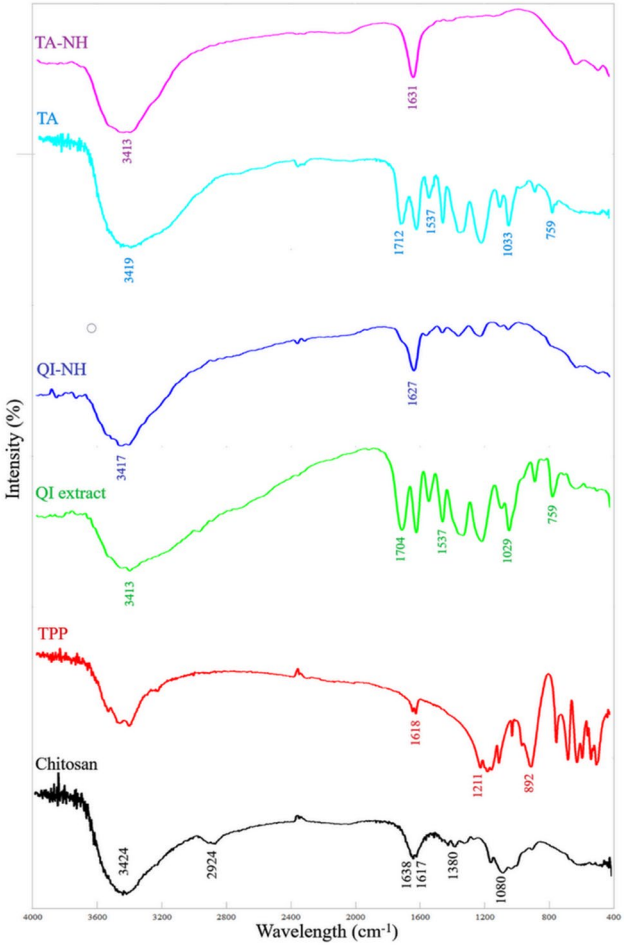

Fig. 3 FTIR of Chitosan, TPP, QI extract, QI-NH, Tannic acid and TA-NH

Table 2 Bioactive groups of materials based on FTIR

Material	Frequency (Wave number, cm-1)	Functional groups	Intensity
Chitosan	3500 – 3200	O-H and N-H stretching	Broad, Strong
	2924	C-H stretching	Strong
	1638	Amid I (C = O stretching)	Strong
	1617	Amid II (N-H bonding)	Strong
	1080	C-O-C	Weak
	1380	Symmetric or asymmetric CH_2 stretching vibration attributed to pyranose ring	Weak
TPP	1211	P-O stretching	Strong
	1618	Symmetrical and asymmetric stretching vibration of the PO_3 groups	Strong
	892	P-O-P asymmetric stretching	Strong
TA	3500 – 3200	Wide stretched band of OH	Broad, Strong
	1712	aromatic stretched band of C = O	Strong
	1537	stretch of aromatics	Strong
	1033	vibration of the benzene ring	Strong
	759	vibration of the OH band	Weak
QI extract	3413	stretched band of OH	Broad, Strong
	1704	stretched band of C = O	Strong
	1535	stretch of aromatics	Strong
	1029	vibration of the benzene ring	Strong
	759	vibration of the OH band	Strong

respectively, which led to the preparation of hydrogel networks. Some of peaks in QI extract and TA FTIR at wavelengths of 1712 – 1704, 1537, 1033 – 1029 and 759 cm^{-1} are not seen in QI-NH and TA-NH graphs. In addition, the decrease in intensity in the signal range represents the band of OH (3500 – 3200 cm^{-1}). These differences indicate the encapsulation of QI extract and TA in the hydrogel networks. The complete information of known functional groups in different samples is described in Table 2.

DLS and Zeta potential

The particle size distribution in nano hydrogels is shown in Fig. 4. The average particle size of QI-NH and TA_NH was 70.48 and 58.43 nm, respectively. In addition, the zeta potential of nano hydrogels was determined to be 2.58 and – 0.03, respectively. The complete results of DLS and zeta potential findings are reported in Table 3.

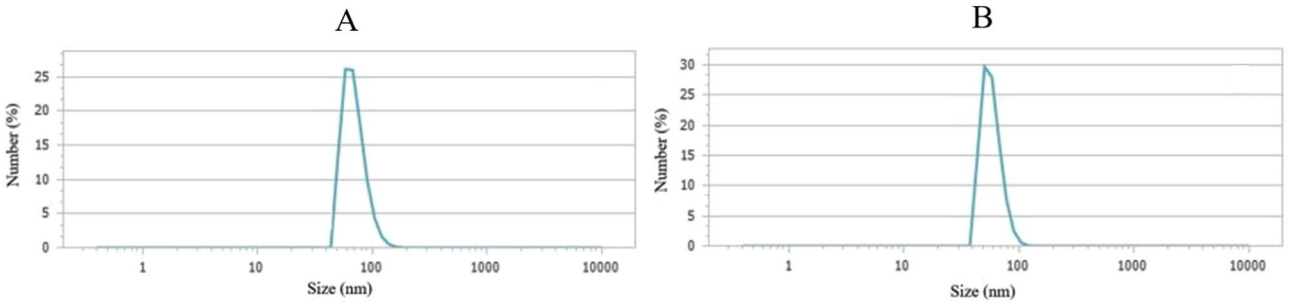

Fig. 4 Particle size of QI-NH (**A**) and TA-NH (**B**)

Table 3 The physicochemical characteristics of nano hydrogels

Nano hydrogels	Size (nm)	Polydispersity index	zeta potential (mv)
QI-NH	70.48 ± 16.72	0.164	2.58 ± 6.17
TA-NH	58.43 ± 11.72	0.209	-0.03 ± 0.25
Unloaded-NH	180 ± 44.28	0.792	0.04 ± 1.7

Release kinetics

The dialysis bag method were used to evaluate the releasing mechanism. In this method, the dialysis bags (12 KD) containing QI-NH and TA_NH were immersed in a specific volume of PBS while stirring. By examining the absorbance (at the wavelength of 280 nm) of separated samples in the time intervals specified in Figs. 5 and 6, the release of QI extract and TA from polymers was calculated separately. As shown in Fig. 5, the amount of QI extract released from QI_NH after 3000 min is about 19.78%, while in TA-NH polymer, after 300 min, 69.66% of initial tannic acid was released (Fig. 6). Then the Korsmeyer-Peppas mathematical model was used to calculate n. n was equal to 1.7 and 0.69 for QI-NH and TA-NH, respectively.

Antibacterial assay

Antibacterial properties of the extract and synthesized nano hydrogels against *S. mutans* were performed by microdilution method. The findings showed an increase in the antibacterial activity of QI extract in nanoformulation so that the MIC of QI extract and QI-NH was equal to 937.5 and 30 µg/ml, respectively. But TA-NH had the most potent inhibitory effect against *S. mutans* because

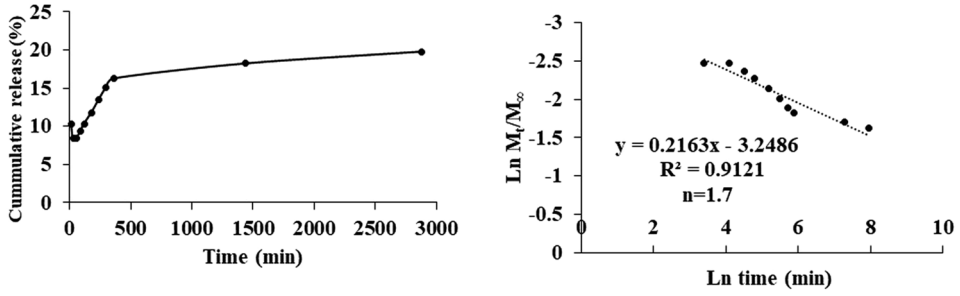

Fig. 5 Kinetic release of QI extract from QI-NH in PBS by dialysis bag method. (Left): The cumulative release of TA in 3000 min. (Right): the Korsmeyer–Peppas mathematical model for calculating n

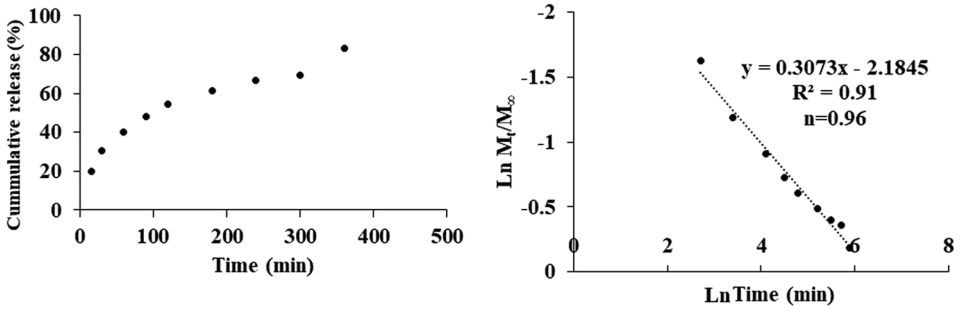

Fig. 6 Kinetic release of TA from TA-NH in PBS by dialysis bag method. (Left): The cumulative release of TA in 400 min. (Right): the Korsmeyer–Peppas mathematical model for calculating n

Table 4 MIC, MBC and MBIC50 of extracts and nano hydrogels against S. mutans

Sample	MIC (µg/ml)	MBC (µg/ml)	$MBIC_{50}$
QI extract	937.5	3750	35.1
QI-NH	30	30	2.1
TA-NH	10	20	0.95
Unloaded-NH	30,000	60,000	5915.7

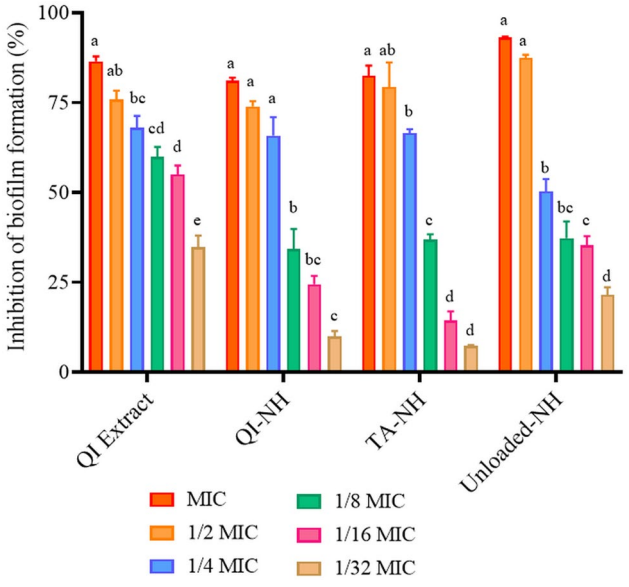

Fig. 7 Inhibition of *S. mutans* biofilm formation at sub MIC concentrations of extract and nano hydrogels

its MIC was 10 µg/ml. The complete results of MIC and MBC of all formulations are shown in Table 4.

Anti-biofilm assay

The effect of sub-MIC concentrations of nano hydrogels was quantitatively tested on the inhibition of biofilm formation. TA-NH showed the most potent anti-biofilm properties. This nano hydrogel at a concentration of 5 µg/ml resulted in 83% inhibition of biofilm formation, while QI-NH at a concentration of 15 µg/ml showed 81% inhibition. Also, the formulation of QI extract as nano hydrogel greatly enhanced its anti-biofilm effect so that the concentrations of 234 and 7.5 µg/ml of QI extract and QI-NH, respectively, inhibited about 75% of biofilm formation. MBIC50 of samples are reported in Table 4. Their inhibition of biofilm formation in sub-MIC concentrations is shown in Fig. 7.

Also, as shown in Fig. 8, the studied natural products, especially TA-NH and QI-NH, prevented the adhesion of *S. mutans* to crown enamel and thus the formation of dental biofilm, compared to the control group and Unloaded-NH.

Gene expression

In real time PCR, we used 16SrRNA gene as internal control to evaluate gene expression in sub-MIC concentrations of extracts and nano hydrogels. 4 genes *gtf*B, *gtf*C, *gtf*D and *gbp*B were studied, of which *gtf* genes are related to glucosyltransferases and as a result glucan synthesis by this enzyme. As shown in Fig. 9, QI-NH significantly inhibited the expression of all studied genes. In such a way that QI-NH led to the reduction of the average expression level of *gtf*B, *gtf*D and *gbp*B to below 1.5% and the average expression of *gtf*D to 6% While QI extract only affected the expression *gbp*B significantly. 27% reduction of *gtf*D expression level in *S. mutans* treated with QI extract was not significant. TA-NH suppressed the expression of the mentioned genes more than other natural products. Its difference with QI-NH was not significant except for the *gbp*B gene.

Cytotoxicity assay

In this study, for the first time, the cytotoxicity of QI extract and TA encapsulated in chitosan-based nano hydrogel against KB cells was investigated. Different concentrations of extract, nano hydrogel and CHX were tested by MTT assay. The effect of extract and nano hydrogels on the viability of KB cell line as KERATIN-forming tumor cell line HeLa was dose dependent. Based on IC50 results in Table 5, QI-NH had the least toxicity. In fact, the encapsulation of QI extract in chitosan has led to a reduction in acute toxicity. On the other hand, CHX showed cytotoxic effect in much lower concentrations than others.

Also, cell viability was checked at concentrations equivalent to the MIC of nano hydrogels, i.e., 10 and 30 µg/ml. As seen in Fig. 10, the KB cell line had the highest viability in the presence of QI-NH concentrations. Based on the results, KB cell viability in concentrations of 10 and 30 µg/ml of QI_NH was 96.7 and 95.5%, respectively, while in the case of cells treated with QI extract, it was 95.4 and 73%, respectively. As shown, the toxicity of QI-NH at concentration of 30 µg/ml is significantly

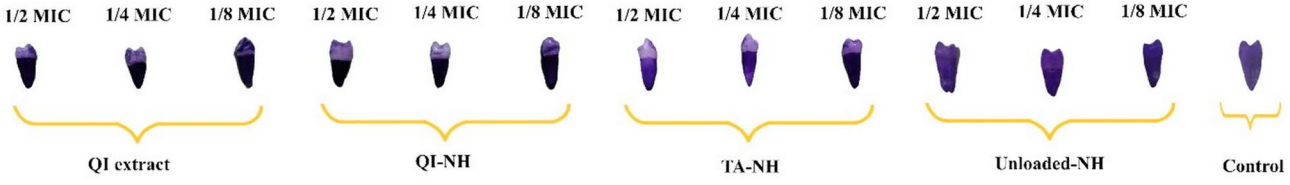

Fig. 8 Qualitative anti-biofilm assay on dental surface

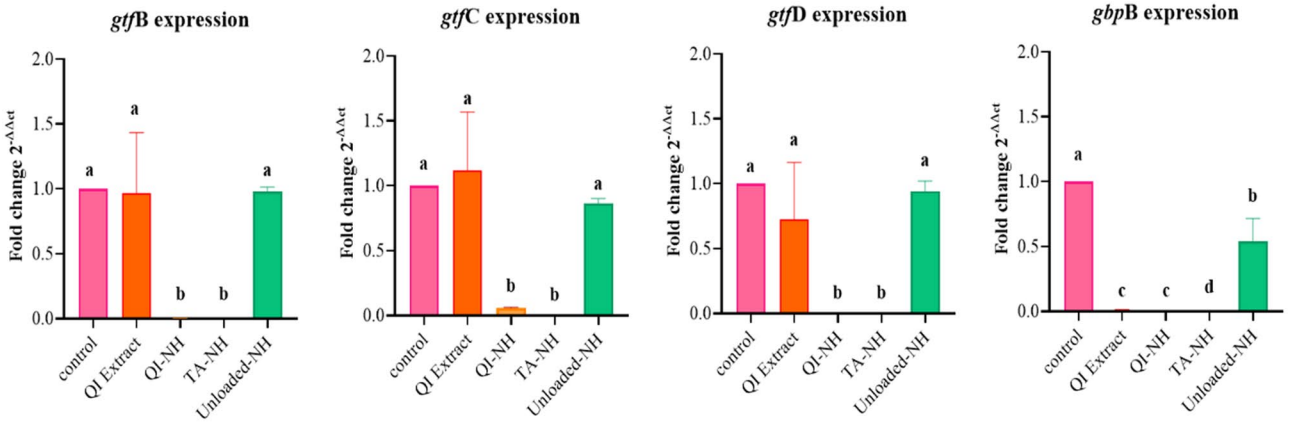

Fig. 9 The relative expression of *gtf*B, *gtf*C, *gtf*D and *gbp*B in *S. mutans* treated with sub-MIC concentrations of QI extract, QI-NH, TA-NH and Unloaded-NH. Mismatched letters (a, b, c, d) above the bars indicate significant difference ($p < 0.05$)

Table 5 IC50 of extract, nano hydrogels, and CHX based on MTT assay

Materials	IC_{50} (µg/ml)
QI extract	109.33
QI-NH	775.4
TA-NH	54.05
Unloaded-NH	25.93
CHX	3.12

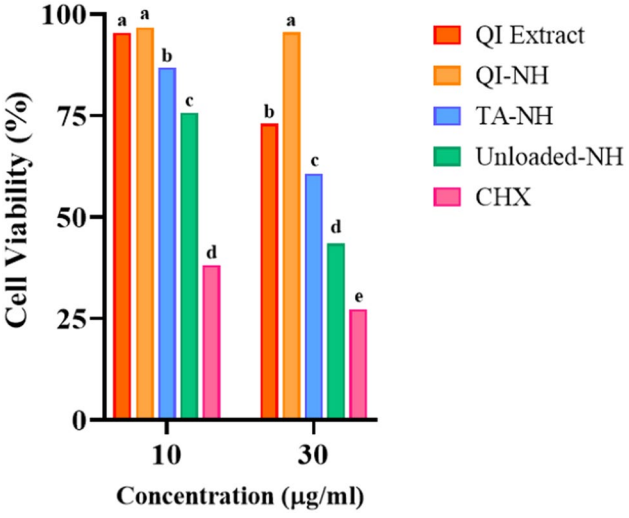

Fig. 10 Cell viability in concentrations equivalent to MIC of QI-NH (30 µg/ml) and TA-NH (10 µg/ml). Mismatched letters (**a**, **b**, **c**, **d**, **e**) above the bars indicate significant differences ($p < 0.05$)

lower than QI extract. As it is clear, in these two concentrations, CHX had significantly higher cytotoxicity than others. Cell viability in the aforementioned concentrations of CHX was 38.1 and 27.3%, respectively.

Discussion

In this study, an attempt was made to introduce traditional natural products with new formulations as a complementary method along with common antimicrobial agents. According to studies, the use of integrative medicine can improve antibiotic usage practices of the population [27]. Based on this, studying on traditional natural antimicrobials can reduce global concerns about pathogen resistance in medical or oral diseases.

The results of SEM and FTIR showed the proper synthesis of chitosan based nanohydrogels and the loading of QI extract and TA in the hydrogel network was done successfully. Very small sizes and acceptable polydispersity were among the advantages of these natural nano-formulations. According to DLS findings, the size of both, especially TA-NH, was below 100 nm. The comparison of Ashrafi et al.'s study [11] and their synthesized nanogel based on chitosan and TPP containing *Mentha piperita* essential oil makes these properties more apparent. Particle size is one of the most fundamental factors in the biological properties of drug-carrying nanohydrogels. Reducing the size of nanohydrogels leads to a significant increase in the permeation and penetration of natural products or other drugs loaded in the nanostructure into the bacterial cell wall [28]. It seems that one of the reasons for the stronger anti-biofilm effects of QI-NH and TA-NH and their lower MBIC50 compared to the mentioned study is the smaller size of the nanohydrogels. Because their small size can increase the permeability of bioactive agents to bacterial biofilm. PDI less than 0.3 indicates high monodispersity [29]. According to the PDI results of QI-NH and TA-NH, the monodispersity of the synthesized formulations is desirable. Also, the results of dialysis bag method and calculation of n through Korsmeyer-Peppas mathematical model showed that the release mechanism of QI-NH was relaxation (super casell transport) and the release mechanism of tannic acid from

TA-NH was relaxation (non-Fickian transport). Based on studies, the QI-NH release type, in the form of an initial burst release and then a controlled release over several days, is ideal for oral diseases, including dental and periodontal diseases [28, 30]. But in contrast, the low EE of nanohydrogels should be considered. It seems that in future studies, more time should be considered for loading QI extract and TA into the nanohydrogel network.

In this study, for the first time, we evaluated the antibacterial and anti-biofilm effects of chitosan-based QI-NH and TA-NH compared to QI extract against *S. mutans*. The results of microbiological tests showed that QI extract and QI-NH have preventive and therapeutic potential in the pathogenesis of dental caries. Antibacterial and anti-biofilm assays showed that the formulation of QI extract in the form of nanohydrogels has significantly potentate its properties against *S. mutans*. TA-NH inhibited the growth and formation of biofilm by *S. mutans* at lower concentrations than others. For more certainty, the anti-biofilm effect of the formulations was investigated ex vivo on the extracted tooth. Compared to the control groups, extracts and nano hydrogels prevented the formation of streptococcal biofilm on crown enamel. In recent studies, the properties of TA extracted from QI extract have been revealed in dental caries. As shown in the study of Schestakow et al. TA, due to the anti-adherent effects close to CHX, has been introduced as a promising antibiofilm [31].

The effect of prepared natural products on the down-regulation of *gtf*B, *gtf*C, *gtf*D and, *gbp*B expression was another part of this study. As the results showed, QI extract did not significantly affect the expression of *gtf* genes. On the other hand, the reduction of *gbp*B expression is one of the reasons for its anti-biofilm properties, shown in streptococcal biofilm tests. The *gbp*B gene is responsible for synthesizing of GBPC protein, which as a cell receptor leads to the binding of *S. mutans* to glucan and thus bacterial adhesion in the dental biofilm structure [32]. The potent inhibition of the mentioned genes by QI-NH and TA-NH can lead to the suppression of sucrose-dependent mechanism in biofilm formation by *S. mutans* [33]. *gtf* genes play a crucial role in the initial adherence of *S. mutans* to the dental surface due to the production of extracellular polysaccharides. Therefore, inhibition of their expression can effectively suppress the formation of dental plaque [34]. In several studies, inhibition of *gtf* gene expression has been proposed as the dominant mechanism of some natural products for their anti-cariogenic properties. For example, in the study of Vahid-Dastjerdi et al., the extract of *Punica Granatum L.* Flower (*Jolnar* in Persian medicine) caused the inhibition of these genes and thus restrained the formation of streptococcal biofilm [35]. Compared to Ashrafi et al.'s study, QI-NH and TA-NH inhibited the average expression level, especially *gtf* genes, more than chitosan-based nano hydrogel containing *Mentha piperita* essential oil [11]. It seems that one of the main underlying factors for the lower MBIC50 of these two compared to *Mentha piperita* nano hydrogel could be more potent suppression of genes related to biofilm formation.

Nanoformulation of QI extract based on chitosan, in addition to intensifying the antibacterial properties, has led to a noticeable decrease in acute cytotoxicity compared to pure QI extract. In general, reducing cellular damage and optimal safety are essential advantages of using biopolymer nanohydrogels as drug carriers for antimicrobial agents [36]. Although the anti-biofilm properties of TA-NH were more than QI-NH, its significantly higher toxicity should be considered. The important point is that although TA constituted 55.18% of QI extract, the IC50 difference of two nano hydrogels (775.4 µg/ml vs. 54.05 µg/ml) was noticeable. The comparison of cell viability in concentrations equal to the MIC of QI-NH (30 µg/ml) and TA-NH (10 µg/ml) in Fig. 10 shows the difference in the toxicity of natural products. Cell viability at a concentration of 30 µg/ml in the treatment with QI extract, QI-NH and TA-NH was equal to 73, 95.5 and 60.7%, respectively. CHX results also showed that KB cell line was 27.3% viable at this concentration.

The limitation of this study is in investigating the effect of nanohydrogels and other raw natural products on the oral microbiome. In addition, it is suggested to study the effectiveness of QI-NH and TA-NH on dental remineralization and demineralization, because according to Persian medicine, these formulated natural products can strengthen the dental mineral tissue.

Conclusion

QI extract is one of the important traditional anti-caries in Persian medicine. For the first time, a new formulation of traditional Persian remedies for dental applications was studied. This study showed the anti-biofilm properties of this extract. Its chitosan-based nano-formulation not only remarkably intensifies biofilm targeting and biofilm genes suppressing but also reduces its toxicity. TA-NH was cytotoxic at lower concentrations than QI-NH, despite its strong antibacterial properties. It seems that QI-NH can be a safe and efficient option to combat dental biofilm. Due to the trend towards natural products, formulations derived from traditional medicine can be used clinically as complementary therapies soon. Of course, to be sure, more researches are indispensable to check the efficiency and safety of these therapies for dental caries.

Abbreviations

S. mutans	Streptococcus mutans
QI	Quercus infectoria G. Olivier

TA	Tannic Acid
QI-NH	Quercus infectoria Nanohydrogel
TA-NH	Tannic Acid Nanohydrogels
Unloaded-NH	Unloaded Nanohydrogels
EPS	Exopolysaccharide
CHX	chlorhexidine
PM	Persian Medicine
HPLC	High-Performance Liquid Chromatography
TPP	Tripolyphosphate, EE; Encapsulation Efficiency
SEM	Scanning Electron Microscopy
FTIR	Fourier-Transform Infrared
DLS	Dynamic Light Scattering
PBS	Phosphate-Buffered Saline
UV/Vis	Ultraviolet–Visible
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MBIC50	Minimum Biofilm Inhibition Concentration
gtf	Glucosyltransferase
gbp	Glucan Binding Protein

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Author contributions
Yasin Karimi: Conceptualization, Investigation, Data collection, Writing - Original Draft; Marzieh Rashidipour: Project designer and consulting in nanotechnology and phytochemical data; Maryam Iranzadasl: Corresponding author, Project designer, Supervisor, Methodologist, Manuscript editing; Mohammad Hossein Ahmadi: Data interpretation, Review and consulting in microbiological data; Mostafa Moradi sarabi: Data analysis, Review and consulting in Real time PCR data; Fatemeh Farzaneh: Monitor data related to dentistry, Review and editing.

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Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The study was approved by the Ethical Committee at the Shahed University (Approval No. IR.SHAHED.REC.1401.019) in agreement with relevant guidelines and regulation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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