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Nouran H. Moustafa

Inas T. Motawae

Rania E. Bayoumi

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Nouran H. Moustafa [a,*], Inas T. Motawae [b], Rania E. Bayoumi [c]

[a] Assistant Lectuer at Department of Dental Biomaterials, Faculty of Oral and Dental Medicine, Misr International University, Cairo, Egypt
[b] Dean of Faculty of Dentistry and Professor of Dental Biomaterial Department, Faculty of Dental Medicine, Al-Azhar University for Girls, Cairo, Egypt
[c] Assistant Professor of Dental Biomaterial Department, Faculty of Dental Medicine, Al-Azhar University for Girls, Cairo, Egypt

Abstract

Purpose: To evaluate the effect of prepared chitosan nanoparticles loaded with grape seed extract on the antibacterial efficiency against Streptococcus mutans of dental adhesive impregnated with these nanoparticles. **Materials and methods:** Chitosan nanoparticles (CsNps) were prepared and loaded with grape seed extract (GSE) by ionic gelation method with a ratio 2:1, then freeze-dried to obtain CsNps loaded with GSE powder. Characterization of prepared nanoparticles was done using Transmission electron microscope and Fourier transmission infrared spectroscopy. The CsNps loaded with GSE powder was added to the dental adhesive and the antibacterial activity was evaluated via direct contact test. Data were collected, tested for normality, and subjected to repeated measures ANOVA followed by post-Hoc tests for pair-wise multiple comparisons. **Results:** Transmission electron microscope and fourier transmission infrared spectroscopy ensured the encapsulation between GSE and CsNps. The particle size of CsNps with GSE ranged from 135 to 239 nm. After adhesive modification with prepared nanoparticles, the antibacterial evaluation showed a significant increase in bacterial inhibition ($P < 0.001$). **Conclusion:** The incorporation of CsNps loaded with GSE is a promising approach to enhance the antibacterial activity of the tested dental adhesive.

Keywords: Antibacterial activity, Chitosan nanoparticles, Chitosan, Dental adhesive, Grape seed extract

1. Introduction

A durable, long-lasting seal between restoration and the tooth structure remains the ultimate goal for adhesive dentistry. Surface modifications for chemical adhesion and surface roughness for micro-mechanical retention were promoted to enhance bond durability. However, the main reason for tooth-colored restoration failure and replacement is the tooth/restoration interface, especially the dentin/resin bond that leads to marginal gap formation, secondary caries development, and subsequent restoration loss [1].

Dental adhesives, as the main part of the adhesive procedure, have gone through tremendous improvements starting from total-etch moving to self-etch systems. Self-etch adhesives were introduced as a one-bottle system to achieve a proper bond to dentin. This simple application known as 'All in One' resulted in demineralization and infiltration of resin into dentin to the same depth, reduction in application time, and decrease in technique-sensitivity. Moreover, self-etch systems have specific functional monomers (10-MDP, 4-MET) as a part of their composition by which a satisfactory chemical bonding to dentin can be achieved [2,3].

Two significant clinical benefits were achieved by using dental adhesives; the first is minimizing microleakage between the restoration and the teeth by enhancing the interfacial bond strength and preventing bacteria from gaining access to this interface. While the second one is changing the

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* Corresponding author at: Department of Dental Biomaterials, Faculty of Oral and Dental Medicine, Misr International University, Cairo, Egypt.
E-mail address: nourann.husseinn@gmail.com (N.H. Moustafa).

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concept of cavity preparation by shifting to minimally invasive intervention with minimal removal of healthy tooth tissues and maximum preservation of the healthy structure. The primary aim of dental adhesives is to provide retention to the restoration and to withstand mechanical forces, especially the shrinkage stress from restoration. Moreover, a good adhesive should provide adequate sealing and prevent leakage along the restoration's margins [4].

These modifications improved not only the physical properties but also the durability of adhesives. Despite these improvements, the polymerization shrinkage and contraction gaps between tooth/restoration interfaces remain a significant problem. The primary aim of dental adhesive is the retention between the restoration and tooth structure and to withstand the mechanical forces. Moreover, a good adhesive should provide adequate sealing and prevent leakage along the restoration's margins [3].

As reported, one of the main causes of marginal leakage at tooth/restoration interface is the cariogenic bacteria, especially Streptococcus mutans (S. mutans), by the adherence to the dentinal surface, reproduction and production of an acidic media which can be mainly responsible for marginal leakage, recurrent caries, hypersensitivity, and restoration failure. Thus, modification of restorative materials to possess inherent antibacterial properties, which can eliminate the residual bacteria causing damage, became a great demand. *In-vitro* antibacterial activity against different pathogens can be evaluated through various laboratory tests. The most common methods are agar-diffusion test, minimum inhibitory concentration test, flow cytofluorometric test, and direct contact test [5].

Significant trials of incorporating synthetic antibacterial components into dental adhesive systems have been investigated. This was achieved by incorporating different concentrations of methacryloyloxydodecylpyridinium bromide, methacryloyloxyethylcetyl dimethyl ammonium chloride, chlorhexidine, and cetylpyridinium chloride with varying degrees of success [5].

Owing to the drug resistance caused by a variety of antibiotics used to treat infections caused by bacteria, natural antibiotics are in the process of being discovered as alternatives to synthetic products. Several natural alternatives have been reported in literature as potential antibacterial agents, such as, propolis, neem, clove oil, green tea, chitosan, aloe vera and grape seed extract. The antimicrobial properties of these extracts have undergone extensive evaluations in clinical microbiology, owing to their antibacterial activity. These preparations are referred to as 'nonantibiotic' antibacterial agents and might be used as antibiotics [6].

Chitosan is a natural polysaccharide reported in literature as a biocompatible, biodegradable, and water-soluble biopolymer. Chitosan can be blended with various materials and shaped into several physical forms as hydrogel, microspheres, fibers, and nanoparticles. Chitosan nanoparticles (CsNps) have shown antibacterial activity against different types of bacteria. Antibacterial activity of CsNps was reported due to interaction with bacterial cell wall or cell membrane via the electrostatic interaction between the positively charged amino group of chitosan and negatively charged cell membrane of bacteria. This interaction influences membrane permeability and results in cell death [7].

Grapes (Vitisvinifera) and grape seeds as dietary supplements are used in herbal medicine and proposed to provide antimicrobial activity, antioxidant effect, aids in wound healing, and prevention of cardiovascular diseases. Polyphenolic fractions such as tannins (catechins), flavonoids, and gallic acid derivatives are reported to have antibacterial properties. The antibacterial activity of grape seed extract (GSE) is reported to be due to the ability to damage microbial cells by exerting an influence on the selective permeability of the plasma membrane, which results in the leakage of vital intracellular substances [8].

The null hypothesis was that there would be no difference between the modified adhesive using chitosan nanoparticles loaded with grape seed extract and the commercial adhesive regarding antibacterial activity.

2. Material and methods

This study has been approved by the Research Ethics Committee (REC-MA-22-02), Faculty of Dental Medicine for Girls, Al-Azhar University, Cairo, Egypt.

2.1. Preparation of chitosan nanoparticles (CsNps) loaded with grape seed extract (GSE)

CsNps were prepared by the 'Ionic Gelation' method. The chitosan powder of low molecular weight (Chitosan: 100 000–300 000) (Across, Thermo Fisher Scientific, Belgium, UK) and grape seed extract powder (Mega Natural, Polyphenolics, Madera, California, USA) were used in this preparation with a ratio 2:1 according to a pilot study which ensured homogeneity. After preparation the precipitate was removed, washed three times with distilled water, then frozen at −4 °C and freeze-dried

using a lyophilize (Freezone 6 benchtop, LABCONO 4.5/Freeze Drier) and then characterization was done [7,9].

2.2. Characterization of prepared chitosan nanoparticles (CsNps) loaded with grape seed extract (GSE)

2.2.1. Transmission electron microscope

The morphology of the freeze-dried powder CsNps with GSE and the confirmation of the encapsulation were observed under the transmission electron microscope [transmission electron microscope (TEM), JEOL JEM-2100, Japan] at (10–100 k-Fold enlargements at an accelerating voltage of 100 KV).

2.2.2. Fourier transmission infrared spectroscopic analysis (FTIR)

Chitosan nanoparticles, grape seed extract, and loaded nanoparticles were chemically characterized using FTIR spectroscopy (Thermo Scientific, USA) within range of 600–4000 cm^{1}. FTIR analysis was performed to demonstrate the grape seed extract and polymer interaction and to ensure the encapsulation of grape seed extract into the CsNps [10].

2.3. Assessment of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration of GSE, chitosan nanoparticles (CsNps) and chitosan nanoparticles loaded with GSE against S. mutans was assessed by 'Serial Dilution' method. This was done to determine the exact weight % of the tested material needed for adhesive modification. S. mutans (ATCC 25175) was cultured overnight in brain heart infusion at 37 °C in complete anaerobic conditions. After 24 and 48 h of incubation, turbidity, the key sign of bacterial growth was observed. The lowest dilution at which inhibition took place was considered the MIC. For more confirmation, a loop of the dilutions was taken and spread over blood agar plates where bacterial growth was observed after 48 h of incubation at 37 °C. The same procedure was repeated for CsNps and CsNps with GSE to assess their MIC [11].

2.4. Samples' grouping

2.4.1. Sample size calculation

The effect size was found to be (0.61) [12]. Sample size was calculated using G*Power version 3.1.9.2 for sample size analysis at $\alpha = 0.05$ and 80% power, which yields a sample size of 7 samples per group.

2.4.2. Preparation of modified adhesive groups

Based on minimum inhibitory concentration assessment; 6 mg of GSE, 12 mg of CsNps with GSE and 12 mg of CsNps were weighted, added to 1 ml of self-etch adhesive (G-premio Bond) and mixed vigorously using vortex (XH-C, Hinotek) to prepare homogenous suspension. The prepared suspensions were then placed in dark tubes and experimental groups (n = 7) were classified.

2.5. Experimental groups

Group 1: Adhesive only (Control).
Group 2: Adhesive + Grape Seed Extract.
Group 3: Adhesive + Chitosan Nanoparticles.
Group 4: Adhesive + Chitosan Nanoparticles loaded with Grape Seed Extract.

2.6. Evaluation of antibacterial activity

The antibacterial activity of experimental groups against S. mutans was tested by means of the 'Direct Contact test' [11]. The direct contact test measures the optical density (OD) which is the turbidimetric determination of bacterial growth in 96-well microtiter plate at 37 °C. The readings were recorded at 620 nm in 0, 12, 24, 48, and 72 h time intervals, using a spectrophotometer (ELISA Reader, Thermo lab systems, Multi scan Technology, Inc., Finland). The 96 well micro-plate reader was divided into four groups of 7 wells each. Sterile microplate was held vertically and coated at the base (side wall) of each well with 25 μl of material of each experimental group. After that, a light-emitting diode (LED) (Elipar S10 free light, 3 m Espe, USA) curing device with light intensity of 650–850 mW/cm^2 was used to cure the adhesive for 20 s (from each side), at a tip distance of 2 mm from the sample and with 90° angulations. Then 10 μl of bacterial suspension was added in direct contact with tested material and the plate was left in vertical position for 1 h at 37 °C. After the1hour contact, the plate was placed in horizontal position and 300 μl of brain heart infusion was added to each well and gently mixed for 2 min. The microplate was placed for incubation at 37 °C and readings of the OD in each well was taken at the mentioned time intervals.

2.7. Statistical analysis

Numerical data were explored for normality using Kolmogorov–Smirnov and Shapiro–Wilk tests. Data showed normal (parametric) distribution and were presented as mean and standard deviation

values. Repeated measures ANOVAs and pair-wise comparisons were used to explore the effect of different materials and time intervals on mean OD. Results were analyzed using IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp. The significance level was set at P less than or equal to 0.05.

3. Results

3.1. Characterization of chitosan nanoparticles (CsNps) loaded with grape seed extract (GSE)

3.1.1. Results of transmission electron microscope (TEM)

TEM (Fig. 1) showed uniform spherical nanoparticles with polymeric shell, with particle size range from 135 to 239 nm.

3.1.2. Results of fourier transmission infrared spectroscopy (FTIR)

FTIR of CsNps, GSE, and CsNps with GSE (Fig. 2) revealed identical peaks due to the similarity of the functional groups. The characteristic peak at ~3340 cm^{-1} assigned for the stretching OH vibration mode was present in all three compounds. The peaks at ~1440 cm^{1}, ~1519 cm^{-1} and ~780 cm^{-1} represents the stretching of the aromatic phenolic compounds which are characteristic for GSE, slight shifting of these peaks at ~1404 cm^{-1}, ~1539 cm^{-1} and ~880 cm^{-1} indicates the interaction between the ammonium group of chitosan and GSE to form the CsNPs with GSE compound. The ~1026 peak of C—O stretching indicating the presence of alcohol functional group in chitosan in all three compounds.

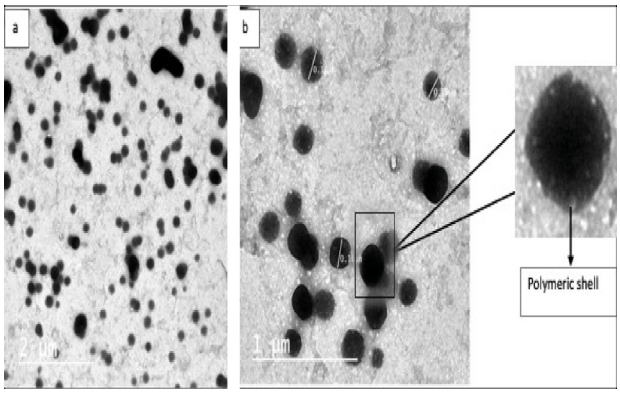

Fig. 1. Transmission electron microscope micrograph for chitosan nanoparticles with GSE (a) Magnification 10 000× (b) Magnification 20 000×.

3.2. Results of minimum inhibitory concentration (MIC) assessment

No turbidity was observed at 12.5% concentration of GSE solution which is equivalent to 6 mg/ml; thus, this was considered as the MIC of GSE. No turbidity was observed at 12.5% concentration of CsNps which is equivalent to 12 mg/ml and 12.5% concentration of CsNps with GSE solutions which is equivalent to 12 mg/ml; thus, this was considered as the MIC of CsNps and CsNps with GSE. Bacterial growth was observed in the control group.

3.3. Results of antibacterial evaluation

Results of antibacterial evaluation by direct contact test (DCT) are shown in Table 1 and (Figs 3 and 4). The results revealed that the addition of CsNps with GSE to adhesive resulted in a statistically significant decrease in the OD compared with other groups at different time intervals. Moreover, the adhesive containing CsNps loaded with GSE showed constant and continuous inhibition of bacterial growth throughout different investigation periods with the highest inhibition of the bacterial growth recorded at 72 h ($P < 0.001$). There are no significant differences between experimental groups

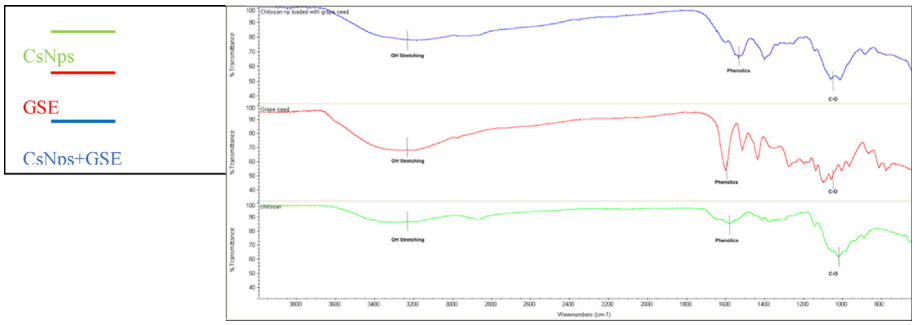

Fig. 2. Fourier transmission infrared spectroscopy for Chitosan nanoparticles, grape seed extract and chitosan nanoparticles with grape seed extract.

Table 1. The mean, standard deviation (SD) values and results of repeated measures ANOVA test for comparison between optical density (OD) values of different materials at each time interval.

Material	Base line		12 h		24 h		48 h		72 h	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Adhesive	0.823^{A}	0.096	0.815^{A}	0.062	0.558^{A}	0.026	0.374^{A}	0.014	0.172^{A}	0.018
Adhesive + GSE	0.143^{C}	0.002	0.149^{C}	0.003	0.156^{D}	0.002	0.159^{D}	0.002	0.166^{A}	0.003
Adhesive + CsNps	0.149^{C}	0.017	0.155^{C}	0.017	0.162^{D}	0.017	0.166^{D}	0.018	0.172^{A}	0.018
CsNps + GSE + Adhesive	0.153^{C}	0.009	0.147^{C}	0.009	0.140^{D}	0.010	0.137^{E}	0.010	0.131^{B}	0.010
P value	<0.001[a]		<0.001[a]		<0.001[a]		<0.001[a]		<0.001[a]	
Effect size (Partial eta squared)	0.979		0.989		0.973		0.916		0.662	

[a] Significant at *P* less than or equal to 0.05, Different superscripts in each column indicate statistically significant difference between materials.

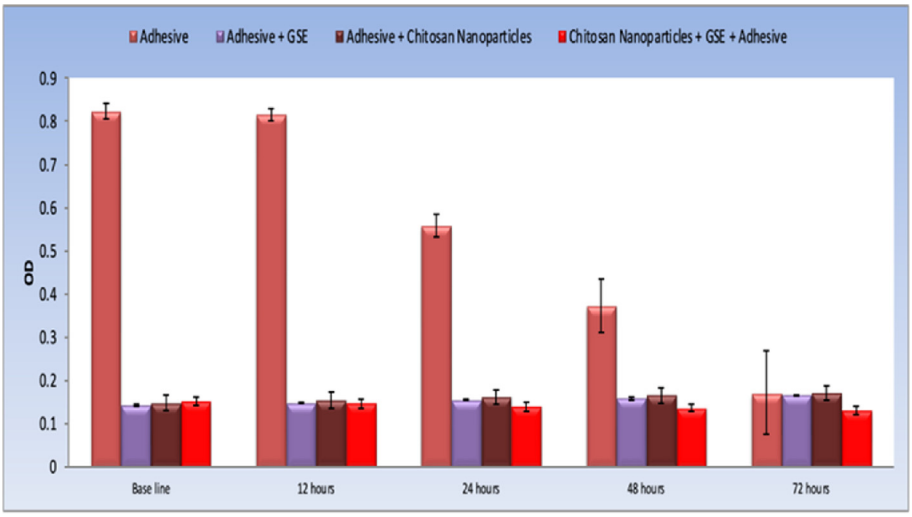

Fig. 3. Bar chart representing mean and standard deviation values for optical density of different materials regardless of time.

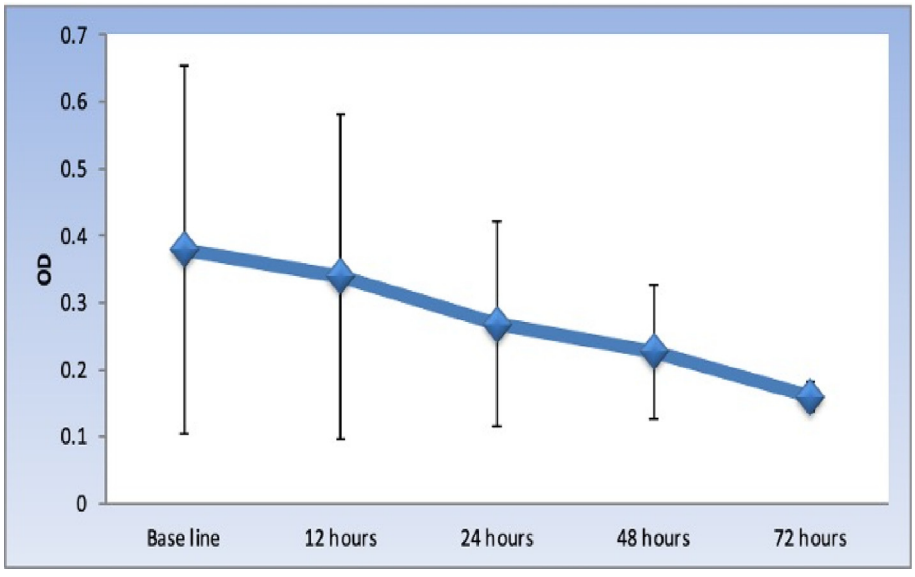

Fig. 4. Line chart representing mean and standard deviation values for optical density at different times regardless of material.

at 12 and 24 h only significant differences was at 48 and 72 h.

4. Discussion

Dentin bonding agents were created primarily to seal cavity borders and stop microleakage, the most recent bonding systems are still not able to achieve a complete seal. It was reported that residual bacteria in the prepared cavity have been shown to multiply within the smear layer even in presence of a good seal from the oral cavity [13].

Self-etching adhesives were introduced, as an attempt of simplifying the clinical procedure, reducing the technique sensitivity and decreasing the risk of contamination of the primed surface. However, residual bacteria may persist at the interface between tooth and restoration due to the non-rinsing procedure [13].

Since the dentin bonding agent is the component that will be in direct contact with dentin, therefore, owing antibacterial activity may eliminate the residual bacteria and prevent secondary caries. Thus, the aim of this study was to modify dental adhesive with Grape Seed Extract encapsulated by chitosan nanoparticles and evaluate its antibacterial activity on cariogenic bacteria.

GSE was chosen in this study due to being effective antibacterial agent. Grape seed proanthocyanins extract and polyphenols are considered rich source of various bioflavonoids. Grape seeds are documented for their antimicrobial activities against a huge number of pathogenic bacteria [6].

The 'chitosan nanoparticles loaded with grape seed extract' powder was prepared by means of the 'ionic gelation' method. This is a simple method which does not rely on the use of harmful organic solvent and retains the bioactivity of macromolecules during preparation. Moreover, it is a safe method where no heat or strong shaking is used that may destruct any sensitive component. This method was performed as an attempt to increase the concentration of loaded GSE and enhance through sustained release by encapsulation with CsNps [14].

Characterization of the prepared freeze-dried CsNps loaded with grape seed extract, was performed by means of TEM and FTIR. These methods were chosen owing to the easy sample preparation, excellent sample to sample reproducibility and minimal operator-induced variations [15].

The uniform spherical nanoparticles with polymeric shell showed by TEM could also confirm the successful loading of chitosan nanoparticles with grape seed extract with particle size range (135–239) which lied within the normal range reported for ion gelation method. The appearance of the main functional groups' peaks for both chitosan nanoparticles (C–O Stretching) and GSE (stretching of the aromatic phenolic compounds) with slight shifting indicates the interaction between the ammonium group of chitosan and GSE to form the CsNPs with GSE and this ensured the encapsulation of GSE with chitosan with no chemical reaction between them.

The antibacterial activity of modified adhesive was evaluated against S. mutans, the main pathogen responsible for the initiation of caries and the development of secondary caries. The DCT applied in this study was chosen owing to its advantages over the agar diffusion test. DCT depends on a direct and intimate contact between both tested material and microorganism and independent of the diffusion properties of both the tested material and the media. It is reproducible, quantitative, and allows testing of water insoluble materials. It also facilitates precise measurements of large number of specimens and their reproducibility on the same micro plate [16].

The results of DCT showed that the unmodified adhesive group (group 1) had the lowest bacterial inhibition (Table 1). As previously reported, minimal antibacterial activity of dental adhesives depends on several factors: mainly their composition and acidity [13,17]. The antibacterial activity of G-premio Bond used in this study may be due to its high percent of acidic monomers which showed to have limited antibacterial effect and viscosity of the material which influence its penetration ability. Moreover, the antibacterial effect of MDP, one of components of G-premio Bond, was reported and attributed to the disturbance of bacterial membrane function through their cationic and hydrophobic binding to bacterial cell wall and subsequently, induces leakage of the cytoplasmic material [18–20].

On the other hand, a significant increase in bacterial inhibition was observed in adhesive incorporated with CsNps loaded with GSE group (group 4) indicating enhancment of its antibacterial activity. This result may be attributed to the dual effect of adding chitosan and GSE, the bactericidal effect of grape seed extract is attributed to the presence of 60–70% polyphenols; mainly monomeric catechin, epicatechin and procyanidins, which are well documented to have microbicidal activities against a huge number of pathogenic bacteria. The mechanism of polyphenols toxicity against microbes may be related to the inhibition of hydrolytic enzymes (proteases and carbohydrolases) or other surface interactions to inhibit microbial adhesions, cell envelope transport proteins and nonspecific interactions with carbohydrates [21,22].

Moreover, chitosan is reported to possess antimicrobial activity owing to its cationic nature. The low molecular weight of chitosan allows its penetration to bacterial cell walls, binding with DNA and inhibition of DNA transcription and mRNA synthesis. Furthermore, the significant antibacterial inhibition of adhesive incorporated with CsNps loaded with GSE (group 4) may be due to the indirect effect of the acidic component of adhesive, which may provide an acidic environment owing to the presence of functionalized phosphoric acid ester in its components. As a result, acidic media may have increased the positive charge on chitosan, thus improved its degradation and release GSE in a direct contact with S. mutans causing the inhibition of its growth. Also, it was reported that a lower degree of chitosan acetylation and a lower pH enhance the antibacterial activity of chitosan [23]. These findings were in accordance with other studies [21,24] who reported that grape seed extract was effective against S. mutans and is considered a successful antimicrobial agent.

4.1. Conclusion

Within the limitations of the present study, the incorporation of CsNps loaded with GSE is a promising approach to enhance the antibacterial activity of the tested dental adhesive.

4.1.1. Recommendations

Additional *in-vitro* and *in-vivo* studies are recommended to evaluate the long-term antibacterial activity and clinical performance of the modified adhesive.

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Conflicts of interest

The author declares that there was no conflict of interest.

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