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Orthodontic retainer, Caffeic-acid nanoparticles, Control release, Drug delivery system.

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Antibacterial efficiency of caffeic acid nanoparticles-based sanitizer on orthodontic retainers

Abstract
Inefficient cleaning methods could allow the build-up of bacteria on orthodontic retainers, which may cause severe periodontal diseases. Synthetic chemical compounds were used to sanitize the oral retainers; however, some of these compounds are not safe for human consumption. This study was aimed to develop a caffeic acid (CA) nanoparticle-based sanitizer for orthodontic retainers, that could overcome major oral infections in orthodontic retainer users. Polyvinyl alcohol (PVA) was used to create CA nanoparticles as an encapsulant. The nanoparticles were characterized using several spectroscopy approaches. The *in vitro* drug release behaviour, in addition to the assessment of its antibacterial activity on oral pathogens, was also studied. On average, the size of the nanoparticle was 133nm, with a surface charge of -11.70mV. Fourier-transform infrared spectroscopy spectra have revealed successful encapsulation of CA into PVA nanoparticles. On the CA release test, it reaches a plateau phase at 24 h, with a total release of 158.86 µg/mL from the nanoparticles. This steady and gradual drug release behaviour is desirable. On the Kirby Bauer assay, broad spectrum antibacterial activity was demonstrated by CA nanoparticles. *Staphylococcus aureus* demonstrated the lowest minimal inhibitory concentration and minimal bactericidal concentration. By immersing the Essix orthodontic retainer in the nanoparticle solution prior to the test, the load of *S. aureus* was reduced by 71.5% relative to growth control. Immersion in a CA nanoparticle solution is an effective way of cleaning and sanitizing the orthodontic retainer as CA nanoparticles effectively removed the oral pathogens present on orthodontic retainers.

1. Introduction
Oral infections caused by retainers have been a significant issue for orthodontic patients. More than 200 species of microorganisms with acid-producing capabilities can be found on the tooth surface or around the orthodontic retainer (Albanna et al., 2017). Thus, prolonged use of retainers will cause biofilms or plaque accumulation on enamel. Retainer hygiene plays a crucial role in maintaining oral health, particularly among children and adolescent orthodontic patients. The retainers are typically worn for extended period, and poor hygiene can lead to the formation of biofilms on retainers, which may subsequently increase the risk of dental caries, gingival inflammation, halitosis and periodontal complications (Chi et al., 2020).
Current methods for cleaning orthodontic retainers involve mechanical brushing or chemical soaking as cleanser. However, mechanical brushing can cause damage to the surface of the thermoplastic material due to its abrasiveness (Tsolakis et al, 2019). Moreover, most cleansers are formulated with persulfate, monopersulfate, and ethylenediaminetraacetic acid (EDTA), which could trigger allergy reactions and gastric perforation (Ingram et al., 2008). The use of zinc in some orthodontic products has been reported to cause severe injuries. Besides, excessive ingestion of zinc also causes nerve damage (Choi et al., 2020). Thus, using a less toxic organic compound in orthodontic products is necessary.

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Caffeic acid (CA) is categorized under hydroxycinnamic acid. It is best known for phenolic functional groups, which permit hydrogen donation and stabilize phenoxyl radicals (Birkova et al., 2020). CA exhibited a wide range of biological activities, including anti-diabetic (Xu et al., 2020), anti-inflammatory (Chao et al., 2010), immunoregulatory (Wang et al., 2016), anti-depressant (Monteiro et al., 2020), anti-microbial (Khan et al., 2021), anti-cancer (Kabala-Dzik et al., 2017) and anti-oxidant (Gulcin, 2006) activities. Besides, research has also found that CA is safe, even in relatively large doses (Khan et al., 2021). However, the application of CA in the pharmaceutical and nutraceutical industries is limited due to its poor water solubility. CA has also been reported to have poor chemical and physical stability, and it decomposes rapidly. CA could absorb moisture from the surrounding air and degrade once exposed to light at room temperature (Khan et al., 2021). To address these issues, CA can be encapsulated in polymeric nano-materials, which is an alternative approach to improve the compound's functionalities. By applying nanoencapsulation technology, it could improve the stability and biological properties of the bioactive compounds (Mohanraj et al., 2017). Thus, in the present study, we aimed to develop a CA nanoparticle-based sanitizer for orthodontic retainers, that could potentially prevent microbial growth on the retainers.

2. Material and methods

2.1 Chemicals

Pure CA (≥ 98% HPLC purity) and pluronic F127 were supplied by Sigma-Aldrich (USA). Nutrient agar (NA), Mueller Hinton agar, and Mueller Hinton broth were bought from Merck (USA). PVA and solvents (95% ethanol and methanol) were purchased from R&M (India) and Fisher (USA), respectively.

2.2 Synthesis of CA nanoparticles

This method was performed according to Sahadan et al. (2019). Firstly, 0.5 g of CA was added to 5 mL of ethanol (95%). After that, 50 mL of 2% PVA and 2% pluronic F127 were combined with the CA-ethanol solution. The combination was homogenized for 5 min at 10,000 rpm using a Silent Crusher M homogenizer (Heidolph, Germany). This translucent solution was stored at -80°C for 24 h and then subjected to a freeze-dryer (Labconco, USA) for 48 h. The freeze-dried particles, which are pronounced as CA/PVA nanoparticles, were kept in a desiccator prior to use. A blank control was set by replacing the CA solution with ethanol (95%).

2.3 Transmission electron microscope

The determination of the size and shape of CA nanoparticles was conducted using transmission electron microscopy (TEM) (Philips CM12, Eindhoven, Netherlands). In order to observe the sample, a small quantity of CA nanoparticle solution was applied onto a copper grid coated with carbon. Subsequently, a droplet of uranyl acetate stain was added. The sample was subjected to a drying process at 25°C in preparation for microscopy observation.

2.4 Dynamic light scattering

A zeta sizer (Nano-ZS90, Malvern, UK) was employed to determine the particle size and surface charge of the synthesized CA nanoparticles. The temperature was standardized at 25°C. Further analysis was done via Zetasizer V2.2 software. The data were expressed as mean ± standard deviation.

2.5 Identification of functional groups in CA/PVA nanoparticles

The functional groups were determined according to the infrared spectra of CA nanoparticles, PVA, and pure CA via Fourier transform infrared (FTIR, Thermo Scientific Nicolet IS10, USA). All spectra were generated at room temperature (25°C) with a wavenumber range of 4000-600 cm^{-1} (Tong et al., 2018).

2.6 *In vitro* release property

Firstly, 50 mg of CA nanoparticles were added to 10 mL of pH 7.4 phosphate buffer in a 100 mL Erlenmeyer flask. Then, the solution was statically incubated at 37°C for 1, 2, 4, 8, 24, and 96 h. At the fixed hour, a 500 µL sample was pipetted and subjected to a UV-vis spectrophotometer (Shimadzu, Japan) analysis at 340 nm (Iraz et al., 2006). CA standards were prepared at a range of 62.50 to 1000 µg/mL to construct a calibration curve, and the amount of CA released was calculated based on the calibration curve. A graph representing the amount of CA versus time was plotted to observe the CA release behaviour from the nanoparticles. All experiments were carried out in three replicates.

2.7 Test microorganisms

Tools for translating research articles tested by exposing them to oral bacteria, including *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus* sp. methicillin-resistant *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa*. To maintain viability, all test bacteria were inoculated on nutrient agar (NA) (Oxoid) fortnightly. To prepare the bacteria suspension, one loopful of fresh colonies was suspended in 10 mL of sterile physiological saline prior to vigorous mixing using a vortex (Tong et al., 2018). Subsequently, the turbidity of the suspension was matched to the 0.5 McFarland standard in order to gain 1×10^8 CFU/mL of bacterial load.

2.8 Antibacterial activity of CA nanoparticle sanitizer: Kirby Bauer assay

A method by Sahadan et al. (2019) was used to screen the antibacterial activity of CA nanoparticles sanitizer. To prepare the sanitizer, CA nanoparticles were dissolved to the desired concentration by suspending the nanoparticles in sterile distilled water. Five substances were tested, i.e., CA nanoparticles sanitizer (100 mg/mL), blank nanoparticles (100 mg/mL), positive control (chloramphenicol, 0.05 mg/mL), and a negative control (sterile distilled water). By using a sterile cotton swab, the abovementioned bacterial suspension was streaked on the surface of MHA. Next, 20 µL of each test substance were pipetted onto a sterile paper disc (6

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mm in diameter) and left to dry. Each disc was placed on the agar surface using sterile forceps. All plates were incubated at 37°C for 24 h. Then, the diameter of appearing clear zones was measured using a ruler. All experiments were carried out in three replicates.

2.9 Antibacterial activity of CA nanoparticle sanitizer: Broth microdilution assay

The assay was conducted in a flat bottom 96 well plate (Nest, China) in accordance with Sahadan et al. (2019). One milliliter of bacterial suspension (1×10^8 CFU/mL) was pipetted into 9 mL sterile of double strength Mueller Hinton broth (MHB) (Oxoid). Then, 100 µL of this bacterial suspension was transferred to the 96 well plates. At a variety of concentrations of 0.78 to 100 mg/mL, CA nanoparticle sanitizer was added to each well, which made the final volume equivalent to 200 µL. After that, the plate was statically incubated at 37°C for 24 h. Next, 40 µL of 0.2 mg/mL p-iodonitrotetrazolium violet salt was pipetted to the test well and kept under a dark condition for 1 h. The colour changes (yellowish to pinkish) denote the appearance of bacterial growth. The minimal inhibitory concentration (MIC) was evaluated as the CA nanoparticle's lowest concentrationsanitizer, which inhibits bacterial growth. A loopful of each sample from the 96 well plates was streaked on the Mueller Hinton agar (MHA) (Oxoid) agar plate's surface to determine the minimal bactericidal concentration (MBC). Prior to viability observation, all plates were incubated at 37°C for a 24 h period. The MBC was determined as the lowest concentration of CA nanoparticles necessary to kill the bacterial cells.

2.10 Antibacterial activity of CA nanoparticle sanitizer: Bacterial reduction on a contaminated commercial oral retainer

This *in vitro* study was conducted using a clear commercial retainer (Essix®, USA). The retainers were first immersed in a 1% sodium hypochlorite solution for 30 min for disinfection purposes. After the disinfection, the retainers were immersed in sterile neutralizing broth for 30 min using sterile forceps, and rinsed three times with sterile distilled water. To initiate the experiment, the retainers were placed in a sterile beaker containing 50 ml of MHB containing a bacterial inoculum of *S. aureus* at a final inoculum size of 1×10^7 CFU/mL. The cultures were incubated at 37°C for 72 hours. Then, the retainers were tested by immersing them in 40 mL of CA nanoparticle sanitizer (25 mg/mL) for 5 min at room temperature (25±3°C). Another set of retainers was immersed in a blank nanoparticle solution (25 mg/mL) for 5 min. After the treatment, the retainers were placed in a beaker containing sterile peptone water (Oxoid) and incubated at 37°C for 24 h. At pre-determined time points (0, 3, 6, 9, ….. 21, 24 h), one of the retainers was collected. The retainers were swabbed using a sterile cotton swab and suspended in 5 mL of phosphate buffered saline (PBS). Spread plating was carried out to enumerate the bacterial cells present in the sample. The samples were suitably diluted with sterile PBS and 100 µL of the diluent was pipetted and inoculated on MHA agar for viable cell counts using the spread plate method. The living bacterial cells were approximated according to the number of colony-forming units (CFU) per volume of sample. The study was done in triplicate.

3. Results

3.1 Nanoparticles: Synthesis, particle size, and surface charge

Based on DLS analysis, the particle size reported was 133.90±7.64 nm (Fig. 1a), with a surface charge of -11.70±3.4 mV (Figure 1b). TEM is used to quantify particle size, size distribution, and morphology in the study of nanomaterials. The size and morphology of CA nanoparticles were investigated using TEM in this study. CA nanoparticles had an average diameter of 94.3±8.2 nm (Fig. 2). CA nanoparticles are also spherical in shape and have smooth surfaces. Besides, a high-speed stirring of 10,000 rpm showed a sufficient mixing level during the encapsulation of CA to PVA and pluronic F-127 (Andrade et al., 2015; Chuang et al., 2000;; Yang et al., 2014). The process is crucial to accelerate the dispersion of CA nanoparticles and narrow the dispersity index by intensifying shear force.

3.2 FTIR: Identification of functional groups

The FTIR spectra of pure PVA and CA as well as CA nanoparticles are manifested in Fig. 3. In general, the FTIR spectra are partitioned into two regions, *viz.* functional groups and molecular fingerprints, with regional ranges of 4000-1500 cm^{-1} and 1500-500 cm^{-1}, respectively (Barra et al., 2021). In Fig. 3a, PVA nanoparticles exhibited a broad absorption at 3434.49 cm^{-1}, indicating a –OH stretching from the intra- and extra-molecular hydrogen bonding. The peaks at 2909.16 cm^{-1}, and 1643.27 cm^{-1} were corresponded to –CH stretching and –OH bending, respectively. Other peaks were recorded at 1459.69 cm^{-1} ($–CH_2$ bending), 1102.34 cm^{-1} and 950.59 cm^{-1} (C-O stretching) (Mandegari et al, 2019). In the spectrum of CA, it showed –OH stretching at 3429.62 and 3232.45 cm^{-1} (Fig. 3b). Additionally, characteristic peaks of CA were detected at 3061.00 and 3019.79 cm^{-1} (=C-H stretching), 1647.09 cm^{-1} (C=O stretching), along with aromatic and olefinic C=C stretching at 1624.53, 1528.31 and 1450.03 cm^{-1} (Catauro et al., 2020). In the present study, the doping of CA into PVA was successfully conducted (Fig. 3c). Chemical interaction was noticed in the nanoparticles of CA/PVA through a broad absorption at 3381.06 cm^{-1} (–OH stretching). Moreover, C=O stretching was shifted from 1647.09 cm^{-1} to 1701.06 cm^{-1}. Changes in the wavenumbers of C=C stretching were observed at 1635.74, 1598.04 and 1521.30 cm^{-1}.

3.3 Drug release behaviour of CA nanoparticles

A drug release study is crucial to ascertain the CA release behaviour of the nanoparticles. Fig. 4 shows the pattern of CA released from the nanoparticle system for a duration 96 h. A rapid burst release pattern was observed starting from the first two hours of the study. After that, the CA release was slow and steady, with an average rate of 6.8 µg/mL CA released per hour. The release follows first order of kinetic. Then, the release reached a plateau state at 24 h, with a total CA release of 158.86±5.00 µg/mL. There was a slow and gradual behaviour observed. The CA nanoparticles maintained this release pattern until the end of the experimental period. From the spectrophotometric analysis, the release rate was approximately 4.67 µg/mL per hour throughout the experiment period.

3.4 Antibacterial property

The CA nanoparticles-based sanitizer exhibited broad-spectrum antimicrobial activity on common oral bacteria, which are represented by Gram-positive and Gram-negative bacteria (Table 1). In Kirby Bauer assay, All of the test germs were prevented from growing by the sanitizer. , with the highest inhibition zone was demonstrated by *P. aeruginosa* (10.3±0.8 mm). It was followed by MRSA (9.1±0.7mm), *Streptococcus* sp. (8.4±0.6 mm), *S. aureus* (8.2±0.5 mm), and *M. luteus* (7.5±0.5 mm). The negative control sanitizer, without the addition of CA nanoparticles, has displayed no inhibitory effect against the bacteria.

The abovementioned result was further examined on the broth microdilution assay, represented in MIC and MBC (Table 2). *S. aureus* depicted the lowest MIC and MBC , of 12.5 mg/mL and 25.0 mg/mL, respectively. As for MRSA, *Streptococcus* sp., and *M. luteus*, similar concentrations were required to inhibit and kill these bacteria. By comparing with other microbes, *P. aeruginosa* was susceptible to the CA nanoparticle sanitizer at a higher concentration, with MIC=25.0 mg/mL and MBC=50.0 mg/mL. The results have proven the bactericidal effect of the CA nanoparticles sanitizer against oral bacteria.

A quantitative evaluation of the antibacterial activity of CA nanoparticle sanitizer was tested with Essix retainers (Fig. 5). By comparing with the control, the retainer model which was initially treated with CA nanoparticles, inflicted a significant ($P \leq 0.05$) growth reduction. PVA nanoparticles, which were used to encapsulate CAshowed no indications of inhibition on the proliferation of *S. aureus*, this finding suggests that the observed inhibitory activity can be attributed to the presence of CA. The sanitizer has successfully killed 71.5% of the bacterial population since the end of incubation. All the results indicated the exceptional efficacy of CA nanoparticle sanitizer for orthodontic oral usage.

4. Discussion

In this study, PVA was employed to encapsulate CA in the form of nanoparticles. PVA is widely applied in membrane development and synthetic medical tools due to its promising biocompatibility, biodegradability, and high-water solubility (Chuang et al., 2000). CA is lipophilic by nature (Andrade et al., 2015). Due to its non-cytogenetic effect on humans, PVA has been utilized in the teething structure as a part of composite materials (Abas et al., 2019). In regard to nanoparticles' study, several researchers, such as Yang et al. (2014), El-Aassar et al. (2016) and Kamboj and Verma (2018), have reported successful synthesis of nanoparticles once combined with PVA and pluronic F127. The study's findings are consistent with previous studies, where the combination of PVA and pluronic F127 provides an excellent delivery system for CA.

The use of nanoparticles as a medicine delivery mechanism has some benefits. First off, nanoparticles' surface properties and particle size may be readily adjusted to the nature of the drug (Gan et al., 2005). Secondly, nanoparticles can regulate and maintain medication release at the localization site and during transit (Chuang et al., 2000). It alters the organ distribution of the drug and the drug's subsequent clearance to boost the therapeutic effectiveness of the drug and reduce the side effects (Tong et al., 2018). Because of its subcellular size, it provides an effective approach by improving the physical stability of encapsulated active substances and increasing antimicrobial activity (Chuang et al., 2000). Thus, nanoparticles can be applied effectively to various antimicrobial agents to enhance their efficiency.

Generally, particles with ultrafine sizes, ranging from 1 to 100 nm in diameter are considered nanoparticles. In some cases, the term is also used to describe larger particles (>500 nm), for instance, colloidal particles and particulate dispersions (Tong et al., 2018). Most of the reliability and effectiveness of the drug's delivery system depend on the particle sizes. A small drug size provides a large ratio of surface area to volume, which drives great solubility, bioavailability, as well as rapid drug release (Rizvi et al., 2018). Additionally, according to Biswas et al. (2014), the preferred size for nanomedical applications should be less than 200 nm. Henceforth, the results of the current study, obtained through both TEM and zeta sizer, have shown a consensus outcome with Biswas et al. (2014). The nanosize of CA nanoparticles has primarily marked the suitability of the compound for nanomedical uses.

Zeta potential is a potent measurement to assess the surface charge of nanoparticles, which later leads to a better understanding of their physical stability (Jiang et al., 2009). In this experiment, the resulted surface charge was negative, and it was sufficient to sustain the physical stability of CA nanoparticle sanitizer. The use of PVA results in more stable nanoparticles, as the high negative surface charge prevents particle agglomeration. Owing to Brownian motion, particles with smaller sizes have faster movement and easier dispersal stability against gravity. However, it must be taken into account that it may also result in particle amalgamation since there are van der Waals forces acting between the particles, which could lead to physical instability (Fathi et al., 2013).

The stability and effectiveness of the integration CA nanoparticles in the developed sanitizer were proven via infrared spectra analysis. The presence of—–OH stretching and bending,–CH stretching and bending, C-O stretching and C-C stretching in PVA nanoparticles was reported previously (Bhat et al., 2020; Kharazmi et al., 2015). Catauro et al. (2020) reported the characteristic peaks of pure CA at a range of 4000 to 2600 cm^{-1}, which were assigned for –OH stretching and partially overlapped with C-H moieties. In the synthesis of CA nanoparticles, the hydroxyl group of PVA interacts with the hydroxyl and carbonyl groups of CA. It created an excellent interfacial attachment, providing a better dispersion of the sanitizer.

The CA release study provides a better understanding of the delivery behaviour of the nanoparticle system. Types of polymeric matrix and preparation methods of nanocarriers could affect the drug release behaviour. These factors could affect the drug's solubility, its dispersion through the matrix, and its degradation (Jain et al., 2019). In this study, the initial burst release of CA was observed, followed by a slow and gradual release

phase. This behaviour was consistent with the findings of Sahadan et al. (2019), who utilized PVA as an encapsulant. The initial burst release was due to the faster movement of weakly localized molecules that attach to the particle's surface. The phenomenon of burst release plays a crucial role in achieving an adequate concentration of CA within the aqueous system, thereby effectively inhibiting bacterial growth on retainers.. In contrast, the slow and gradual release pattern is associated with a slower migration of CA nanoparticles trapped in the internal area of the particles. This characteristic is desirable to ensure efficient sanitization of orthodontic retainers.

The developed CA nanoparticle sanitizer exhibited broad spectrum antibacterial activity. The findings are in line with those of previous studies by Lima et al. (2016), Pinho et al. (2015), and Vaquero et al. (2007), all of which found CA to have broad antibacterial activity. CA has antibacterial activity through two mechanisms: it disrupts cell membrane structure and inhibits bacterial nucleic acid synthesis. Antibacterial agents with these two modes of action tend to inhibit microorganisms that are both Gram positive and Gram negative

, which explains the findings of the current study. CA alters cell membrane permeability by disrupting membrane components and reducing membrane efflux activity, according to Khan et al. (2021). CA is also frequently used as an antibacterial adjuvant, where its presence improves the antibacterial efficacy of other antibacterial agents such as pyrogallol (Lima et al., 2016), chitosan (Kim et al., 2017), zinc oxide (Choi et al., 2017), and ultraviolet-A (Choi et al., 2017). Arasoglu et al. (2015) also used PLGA to develop CA nanoparticles, and they reported a long-lasting antibacterial effect on *S. aureus* in this study. Choi et al. (2017) made a similar observation, reporting excellent inhibitory effects of CA-functionalized zinc oxide nanoparticles on both *S. aureus* and *E. coli*. On the broth microdilution assay, the quantitative analysis showed that The CA nanoparticles' antimicrobial activity was significant on all test bacteria. Besides *S. aureus* and *P. aeruginosa*, the other three stains had the same MIC and MBC. This reflects good sensitivity to the CA nanoparticles, the action was bactericidal.

The antibacterial performance of the CA nanoparticle sanitizer observed in this study is comparable with findings reported in previous orthodontic and dental cleaning studies. Earlier investigations demonstrated that chemical disinfectants such as sodium hypochlorite, chlorhexidine, and effervescent cleansing tablets can significantly reduce bacterial colonies on orthodontic appliances and clear aligners. For instance, several studies reported that disinfectant solutions including 0.5% sodium hypochlorite and 0.12% chlorhexidine effectively decrease the growth of cariogenic bacteria such as *S. mutans* on orthodontic appliances (Silva et al., 2025). Furthermore, comparative analyses of disinfectants for thermoplastic retainers have shown that chemical cleaners and antimicrobial agents can substantially inhibit bacterial colonization and biofilm formation on retainer surfaces, highlighting the importance of chemical sanitation methods in addition to mechanical brushing (Kiatwarawut et al., 2022). In comparison with these conventional approaches, the CA nanoparticle sanitizer demonstrated notable antibacterial activity against oral pathogens, including *S. aureus*, and effectively reduced bacterial load on Essix retainers.

S. aureus was selected for further study, to evaluate the efficiency of the developed sanitizer using orthodontic retainers as a model. The test bacterium was selected because we reported the lowest MIC and MBC on the broth microdilution assay, which reflects its good sensitivity. *S. aureus* is a biofilm-producing bacteria. The biofilm formation acquires three general steps: adhesion on the abiotic or abiotic surface, proliferation, and dispersion. The ability of *S. aureus* to attach to surfaces of orthodontic retainers is remarkable (Eroglu et al., 2019). Owing to a high *S. aureus* growth reduction, the CA nanoparticle sanitizer is able to interrupt the biofilm formation processes on the retainer's surface. In general, by immersing the retainers in the CA nanoparticle solution, the CA nanoparticles were coated onto the surfaces of retainers. Then, by immersing the retainers in the liquid medium, the CA was gradually released from the nanoparticle system, and thereby inhibiting the growth of *S. aureus*. However, the results of the broth microdilution assay indicated that 99.9% of killing efficiency was not achieved at the concentration of MBC (25 mg/mL). The concentration of CA nanoparticles was insufficient to kill 99.9% of *S. aureus* cells. Nevertheless, upon the coating of retainers with CA nanoparticles, the culture of *S. aureus* did not exhibit any exponential growth. The inhibitory effect is significant. A cleaning regimen for biofilm removal can be executed via mechanical or chemical approaches (Chuang et al., 2000). In their work, there is approximately 99% bacterial removal on orthodontic retainers after a cleaning process using both techniques. The result is in consensus with our recent findings, and mechanical removal is also necessary to improve the sanitizing efficiency.

S. aureus is recognized as an opportunistic pathogen capable of forming biofilms on orthodontic retainer materials. Once adhered, *S. aureus* cells proliferate and produce extracellular polymeric substances that facilitate the development of structured biofilms (Al-Groosh et al., 2015). Consequently, orthodontic retainers may function as niches that support sustained microbial colonization within the oral cavity. Persistent colonization can promote microbial imbalance and increase the likelihood of gingival irritation, periodontal inflammation, and localized oral infections (Eroglu et al., 2019). Therefore, effective retainer sanitization strategies like using of CA are necessary to control *S. aureus* colonization and minimize the potential oral health complications associated with long term retainer use.

The developed CA nanoparticle-based sanitizer has potential applications in both orthodontic clinical practice and home care for maintaining retainer hygiene. In orthodontic settings, the sanitizer can be used as a disinfecting soaking solution for removable retainers to reduce microbial contamination before appliance

reinsertion. For patient home care, retainers may be immersed in the CA nanoparticle solution as part of routine cleaning to reduce microbial load. Importantly, CA is a naturally occurring phenolic compound that has been reported to possess a favorable safety profile and is generally recognized as safe, even at relatively high doses. This characteristic supports its potential suitability for oral applications, making CA nanoparticles a promising and biocompatible alternative to conventional chemical cleansers for controlling microbial colonization on orthodontic

5. Conclusions

A CA nanoparticle-based sanitizer was successfully developed using PVA as encapsulant. The sanitizer showed broad spectrum antibacterial activity against oral pathogens. The orthodontic retainers pre-treated with the sanitizer also showed significant reduction of *S. aureus* bacterial load. CA nanoparticles showed good potential for orthodontic application, as CA is classified as generally recognized as safe (GRAS). This study is important to provide an effective, green, and safe alternative to sanitize oral retainers. Further study should be done to determine the antibacterial efficiency in oral cavities.

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Table 1 Antibacterial activity of CA nanoparticles-based sanitizer on oral pathogens..

Test microorganisms	Diameter of inhibition zone (mm)		
	CA nanoparticles	Blank nanoparticles	Positive control
S. aureus	8.2±0.5	-	26.3±1.1
Streptococcus sp.	8.4±0.6	-	22.0±0.7
MRSA	9.1±0.7	-	22.3±1.8
M. luteus	7.5±0.5	-	14.3±0.8
P. aeruginosa	10.3±0.8	-	12.6±0.7

(-) no inhibitory activity

Table 2 MIC and MBC of CA nanoparticles on broth microdilution assay.

Test microorganisms	MIC (mg/mL)	MBC (mg/mL)
S. aureus	12.5	25.0
MRSA	25.0	25.0
Streptococcus sp.	25.0	25.0
M. luteus	25.0	25.0
P. aeruginosa	25.0	50.0