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Dentistry Section

Eco-friendly Synthesis of Copper Sulphate Nanoparticles using Citrus Sinensis Extract and their Antimicrobial Properties: An In-vitro Study

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ABSTRACT

Introduction: The extensive use of nanoparticles underscores the importance of developing sustainable synthesis methods. There is a growing demand for green synthesis, which prioritises clean, safe, and environmentally friendly methods devoid of high temperature, pressure, energy consumption, and toxic chemicals.

Aim: To produce copper sulfate nanoparticles through the utilisation of Citrus sinensis extract and assess their antibacterial efficacy against *Streptococcus mutans*, *Staphylococcus aureus* and *Enterococcus faecalis*. This addresses the requirement for innovative and environmentally friendly antimicrobial agents for dental purposes.

Materials and Methods: This in-vitro study was conducted at the Department of Conservative Dentistry and Endodontics at Saveetha Dental College, located in Chennai, Tamil Nadu, India, during the timeframe of April 2023 to June 2023 following the acquisition of ethical clearance from the Scientific Review Board Committee. Utilising *Citrus sinensis* extract, a green synthesis method was employed to synthesise copper sulfate nanoparticles. Characterisation procedures included Fourier-transform Infrared Spectroscopy (FTIR) analysis, Scanning Electron Microscopy (SEM) imaging, Energy Dispersive X-ray Analysis (EDAX) analysis, and antibacterial testing. The antibacterial effectiveness of the nanoparticles was evaluated against *Streptococcus mutans*, *Staphylococcus aureus* and *Enterococcus faecalis* using the agar well diffusion method. The data were subjected to statistical analysis using Statistical Package for Social Sciences (SPSS) version 23.0 to assess antibacterial effectiveness. Analysis of Variance (ANOVA) was employed to analyse the Zone of Inhibition (ZOI) regarding antibacterial efficacy.

Results: The copper sulfate nanoparticles, synthesised through a green approach, displayed a spherical morphology as affirmed by SEM and exhibited characteristic peaks in FTIR spectra. EDAX analysis confirmed the elemental composition of the nanoparticles. Notably, the nanoparticles showcased the most substantial antibacterial activity against *Enterococcus faecalis* followed by *Staphylococcus aureus*, and the least activity was seen against *Streptococcus mutans*.

Conclusion: Copper sulfate nanoparticles synthesised through *Citrus sinensis* mediation show promising antibacterial efficacy against *Enterococcus faecalis*, highlighting their potential for dental applications. However, further investigations are necessary to assess their cytotoxicity, evaluate their clinical viability, and explore broader applications in antimicrobial contexts.

Keywords: Agar well diffusion, Antibacterial efficacy, Green synthesis, Orange

INTRODUCTION

In the realm of dental research, it is well-established that dental caries rank as the most widespread chronic affliction on a global scale, while periodontal disease emerges as the predominant oral condition, serving as a primary contributor to tooth loss [1]. Cariogenic bacteria, which contribute to the deterioration of the structure of the tooth, include *Streptococcus sanguinis* (*S. sanguinis*), *Streptococcus salivarius* (*S. salivarius*), *Streptococcus sobrinus* (*S. sobrinus*), *Streptococcus mutans* (*S. mutans*), and *Enterococcus faecalis* (*E. faecalis*) [2]. The use of antimicrobial agents has emerged as a practical and cost-effective strategy for preventing oral diseases. However, their widespread use has been associated with various complications, including discoloration of the teeth, a burning sensation, dryness of the mouth, toxicity, alteration in taste, and the development of microbial resistance. Given the global need for novel, readily accessible, cost-efficient, and effective antimicrobial agents that minimise complications, attention has shifted towards green products [3]. In this context, nano drugs have been unveiled and rapidly acknowledged for their improved substantivity, precise tissue targeting, and diminished likelihood of causing adverse side-effects [4,5].

In recent times, there has been a growing utilisation of Copper Nanoparticles (CuNPs) as agents with antibacterial properties. The techniques employed for synthesising CuNPs encompass both chemical and physical methods, including sol-gel processes, physical vapor deposition, chemical reduction, microwave irradiation, and thermal decomposition. However, these conventional approaches are associated with significant drawbacks such as high expenses, substantial energy requirements, and the generation of hazardous and toxic byproducts [6]. The shift from physical and chemical methods to green synthesis methods is gaining momentum [7] due to concerns associated with high energy consumption, the release of toxic substances, and the complexity of equipment and synthesis conditions [8]. Presently, green synthesis predominantly employs microorganisms such as fungi, bacteria, and algae, or extracts derived from leaves, flowers, roots, peelings, fruits, and seeds of various plants [9,10]. Previous studies have underscored the advantages of green synthesis over chemical methods and highlighted the potential and promise of green synthesis [11]. Among these approaches, the utilisation of plant extracts has garnered greater attention, largely due to its advantages, such as the absence of constraints related to microorganism culture and the ease of sourcing plant materials [12]. Furthermore, employing plants for nanoparticle synthesis offers additional benefits, including the utilisation of safer solvents, more moderate reaction conditions, increased feasibility, and diverse applications in fields like surgery and pharmaceuticals [13].

The succulent orange fruit, a member of the Rutaceae family, is scientifically referred to as Citrus sinensis. Citrus sinensis ranks as an extensively cultivated fruit crop globally, boasting a total reported global production of approximately 120 million tons. Orange trees are extensively cultivated for their delectable juice and recognised medicinal properties [14]. Orange peel extract has been associated with numerous medicinal properties, including its efficacy against conditions such as colic, upset stomach, cancer, diuretic effects, carminative properties, immune enhancement, stomachic qualities, and its role as a digestive and immune system tonic. Additionally, it is utilised for preventing and treating various bacterial and viral infections [15]. Notably, research conducted by Mehmood B et al., demonstrated significant antimicrobial activity, particularly against enteric pathogens, using extracts derived from orange peels. Furthermore, Akdemir Evrendilek G found orange peel extract to be effective in combating *Klebsiella pneumonia* [16,17]. Obtaining citron juice is a relatively straightforward process compared to extracting juices from other plants. The method involves squeezing pre-washed citron fruits and filtering the resulting juice through muslin cloth. The CuNPs synthesised using citron juice demonstrated significant antibacterial and antifungal properties against plant pathogenic fungi [18]. The synthesis of CuNPs using citron juice is not only convenient but also cost-effective, making it a valuable method for practical application.

Recognising the critical role of plaque control in maintaining oral health, coupled with concerns regarding the complications associated with chemical products, the potential advantages of nanoparticles, and the disinfecting properties of Citrus sinensis, the present study aimed to synthesise and characterise copper sulfate nanoparticles utilising Citrus sinensis extract as a mediator, and subsequently, assess their antimicrobial effectiveness against *Staphylococcus aureus*, *Streptococcus mutans* and *Enterococcus faecalis*.

MATERIALS AND METHODS

The in-vitro study was conducted within the Department of Conservative Dentistry and Endodontics at Saveetha Dental College, located in Chennai, Tamil Nadu, India, during the timeframe of April 2023 to June 2023. The present study received thorough review and approval from the Scientific Review Board under the reference number SRB/SDC/ENDO-2103/23/009.

Study Procedure

Preparation of Citrus sinensis extract: Following meticulous washing with distilled water upon collection, the fruits underwent meticulous preparation. The peels of the oranges were carefully separated, and 20 mL of pure juice extract was obtained by pressing the fruit halves. All these procedures were conducted under strict aseptic conditions [19]. The extracted juice served as the undiluted stock solution.

Preparation of Copper Sulphate Nanoparticles: Green synthesis facilitated by orange extract was employed in the production of copper sulphate nanoparticles. This synthesis process involved the following steps:

Preparation of Diluted Copper Sulphate Solution: A total of 100 mL of copper sulphate suspension was prepared by diluting copper sulphate in distilled water to achieve a concentration of 20 mL. Subsequently, 80 mL of the copper sulphate suspension was transferred to a flask, and 20 mL of orange extract was added to the solution. The mixture was stirred continuously to ensure thorough blending, resulting in the formation of a diluted copper sulphate solution [20].

Orbital shaking and centrifugation: The blend comprising copper sulphate and orange extract underwent orbital shaking using an orbital shaker for a specified duration, typically ranging from 2 to 3 hours. The reduction of metallic ions and the subsequent formation of nanoparticles were enabled by the orbital shaking process. Following the shaking phase, the mixture underwent centrifugation at 8000 rpm for 10 minutes. This centrifugation step served to effectively isolate the nanoparticles from the residual solution.

Visual observation played a crucial role in monitoring the progress of the synthesis. A notable characteristic feature was the change in the solution's colour, which signified the transformation of metal salts into nanoparticles through reduction. The solution was continuously observed until this change in colour became evident, serving as a clear indicator of the successful nanoparticle synthesis [21].

The copper sulphate nanoparticles underwent various diverse characterisation techniques to assess their properties and efficacy against *Enterococcus faecalis*, *Streptococcus mutans*, and *Staphylococcus aureus*.

Fourier-transform Infrared (FTIR) Spectroscopy: FTIR analysis was conducted using Thermo Nicolet Avatar 330, USA, which was used to discern the functional groups existing in the nanoparticles. To facilitate FTIR analysis, a pellet was formed by combining the nanoparticles with Potassium Bromide (KBr) [22].

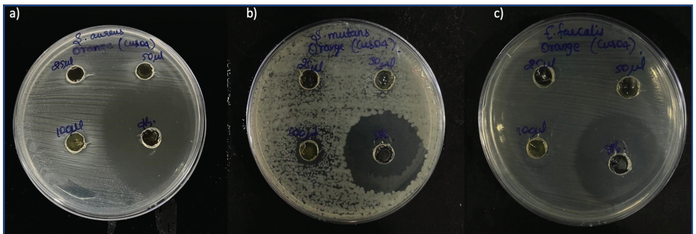
Energy Dispersive X-ray Analysis (EDAX): Elemental composition analysis of the synthesised nanoparticles was carried out through EDAX analysis. The nanoparticles, deposited on a suitable substrate, were examined using a SEM equipped with an EDAX detector (Bruker Germany, D8 Advance diffractometer).

Scanning Electron Microscopy (SEM): SEM analysis, conducted with a JEOL USA Inc. instrument, was utilised to explore the morphology and size of the nanoparticles. For SEM analysis, the synthesised nanoparticles were positioned on a sample holder, coated with conductive material, and scrutinised using a high-resolution SEM.

Antimicrobial activity of CuNPs against oral pathogens: The agar Well diffusion method was employed on Mueller-Hinton Agar (MHA) plates to evaluate the antibacterial activity of different doses of CuNPs against *Enterococcus faecalis*, *Streptococcus mutans*, and *Staphylococcus aureus*. The experimental procedure was as follows: After subculturing the test organisms onto a fresh and suitable broth medium, the broth cultures were incubated at 37°C until reaching a turbidity equivalent to 0.5 McFarland standard, approximately 1.5×10^{8} Colony Forming Unit (CFU)/mL. MHA plates were used as the bacterial medium, and each culture medium was appropriately incubated according to the requirements of the respective test organisms. One milliliter of this standardised suspension was used to uniformly coat the surface of solid MHA plates, allowing it to dry. One milliliter of this standardised suspension was used to uniformly coat the surface of solid MHA plates, allowing it to dry [23]. Using a micropipette, three wells (9 mm in diameter) were made in the MHA medium. These wells were filled with different volumes (25 μL, 50 μL, and 100 μL) of the final nanoparticle solution and a control antibiotic solution containing 0.2% chlorhexidine, which was then inoculated into the agar containing the test organisms. Subsequently, the agar plates were placed in an incubator at 37°C for 18-24 hours. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone surrounding each well, as shown in [Table/Fig-1] [19]. The experimental procedure was conducted in triplicate, and the obtained data were presented as the mean value along with the Standard Deviation (SD) for further analysis and interpretation [24].

STATISTICAL ANALYSIS

The collected data underwent statistical analysis using the SPSS version 23.0 to assess the Zone of Inhibition (ZOI) regarding



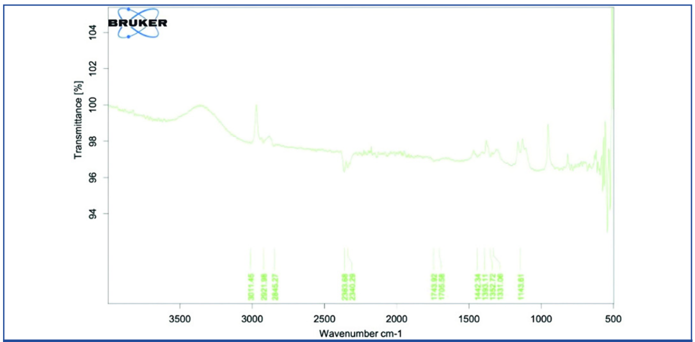
[Table/Fig-1]: Represents the Zone of Inhibition (ZOI) growth of: a) *Staphylococcus aureus*; b) *Streptococcus mutans*; c) *Enterococcus faecalis* against copper sulphate nanoparticles.

antibacterial effectiveness. ANOVA was employed due to the normal distribution of data sets among the groups. Statistical significance was defined as a p-value of ≤0.05.

RESULTS

The formation of nanoparticles during exposure to plant extracts could be monitored by observing a colour change. CuNPs display a green colour in an aqueous solution, attributed to the surface plasmon resonance phenomenon. The synthesis of CuSO4 was confirmed by the observed colour change.

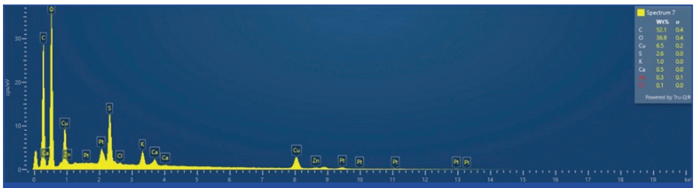
Fourier-transform Infrared Spectroscopy (FTIR): FTIR analysis was conducted to discern the functional groups existing in the Copper Sulfate nanoparticles synthesised with the aid of Citrus sinensis extract [Table/Fig-2]. In the FTIR spectrum, distinctive peaks were observed, corresponding to various functional groups. These specific peaks at wavenumbers provided confirmation of effective nanoparticle synthesis. The infrared spectra of the $CuSO_4$ sample exhibit absorption peaks ranging from 1100 to 3010 cm-1. Notably, peaks at 3011 cm-1 and 2845 cm-1 indicated the presence of Alkane C-H stretching and Aliphatic C-H stretching, respectively, suggesting the involvement of organic compounds from the citrus extract in the synthesis process. The peak at 1743 cm-1 is typically associated with C=O stretching, indicating the presence of carbonyl functional groups such as ketones or aldehydes. The absorption peak at 1143 cm-1 indicated C-O stretching (carbonyl), while the peak at 1743 cm-1 suggested C=O stretching (ketone or aldehyde), potentially originating from the Citrus sinensis extract. These findings contribute to the interpretation of the FTIR spectrum and provide insights into the chemical composition and structure of the synthesised nanoparticles.



[Table/Fig-2]: Demonstrates the FTIR analysis of orange-derived copper sulphate nanoparticles.

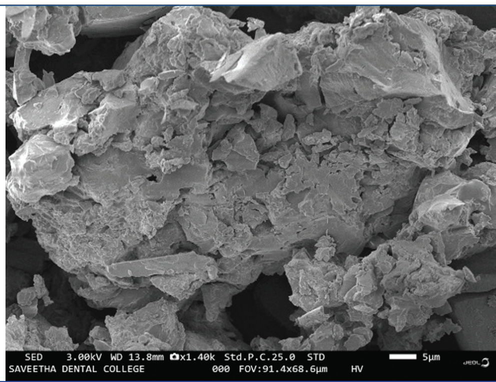
Energy Dispersive X-ray Analysis (EDAX): The elemental composition analysis through EDX Analysis was conducted to determine the composition of the synthesised nanoparticles. The results of the EDX analysis confirmed the presence of Copper (Cu) and Sulfur (S) elements, providing evidence for the successful production of Copper Sulfate nanoparticles as seen in [Table/Fig-3]. This analysis, carried out using the JEOL JSM 7600F instrument, revealed the elemental composition of the nanoparticles: Carbon (C) at 52.1%, Oxygen (O) at 36.9%, Copper (Cu) at 6.5%, Sulfur (S) at 2.6%, Potassium (K) at 1%, and Calcium (Ca) at 0.5%. The composition of the nanoparticles suggests a predominantly carbon and oxygen-based structure, likely originating from organic compounds involved in the synthesis process. Additionally, the presence of copper, along with trace amounts of sulfur, potassium, and calcium, further contributes to the understanding of the nanoparticle composition and the chemical compounds accountable for both the production and stability of the nanoparticles.

Scanning Electron Microscopic Analysis: SEM analysis was carried out to assess both the structure and dimensions of the synthesised nanoparticles. Examination of the SEM images unveiled nanoparticles that were round and conical in shape and exhibited an inconsistent size distribution in [Table/Fig-4]. The determination of the average particle size involved taking measurements on numerous nanoparticles, from which the mean size was subsequently calculated.



[Table/Fig-3]: Energy-dispersive X-ray (EDAX) analysis describing the elements detected in the sample with corresponding spectra values.

This SEM analysis not only visually validated the nanoparticle structure but also provided insights into their surface attributes.



[Table/Fig-4]: Scanning Electron Microscopy (SEM) of samples showing nanoparticles of conical and round shape.

Antibacterial efficacy: The nanoparticles were subjected to assessment for their antibacterial efficacy against *Enterococcus faecalis*, *Streptococcus mutans*, and *Staphylococcus aureus*, employing the agar Well diffusion method to measure the ZOI. The mean and SD of growth inhibition zone diameters were assessed and are presented in [Table/Fig-5] below. *E. faecalis* demonstrated the highest mean inhibition zone sizes of 13.03 mm, 14.02 mm, and 16.03 mm at concentrations of 25 μL, 50 μL, and 100 μL, respectively. In contrast, *S. mutans* exhibited the lowest mean inhibition zone sizes of 9.8 mm, 9.9 mm, and 12.2 mm at the corresponding concentrations, while *S. aureus* displayed inhibition zone sizes of 9.9 mm, 11.2 mm, and 14.03 mm at the same concentrations. Notably, all groups demonstrated some level of antibacterial activity at the higher concentration of 100 μL. A one-way ANOVA revealed a significant difference (p <0.001) between the groups.

Groups	25 μL (mm)	50 μL (mm)	100 μL (mm)	Control (mm)	p-value
S. mutans	9.8±0.5	9.9±0.3	12.2±0.2	22.1±0.1	<0.001*
S. aureus	9.9±0.4	11.2±0.2	14.03±0.4	30.4±0.5	<0.001*
E. faecalis	13.03±0.4	14.2±0.2	16.03±0.3	34.9±1.0	<0.001*

[Table/Fig-5]: Comparison of mean diameters (mm) ±SD of growth inhibition zones of different concentrations of copper sulphate nanoparticles against *S. mutans*, *S. aureus*, *E. faecalis*, and the control assessed using the agar disc diffusion method (*denotes significance p<0.05).

DISCUSSION

Citrus sinensis (L.) Osbeck, commonly referred to as sweet orange, holds the distinction of being the most extensively cultivated and commercially traded citrus species across the globe. Citrus sinensis is renowned primarily for its rich vitamin C content. Moreover, it functions as a noteworthy reservoir of diverse phytochemicals, encompassing phenolics and carotenoids, both recognised for their potential health-promoting properties [25,26]. Oranges are rich in various essential compounds, including vitamin B6, folic acid, riboflavin, thiamine, niacin, and pantothenic acid, as well as essential minerals like potassium, phosphorus, calcium, iron, magnesium, and sodium. They also contain significant amounts of ascorbic acid (vitamin C), flavonoids, amino acids, and phenolic compounds, as reported by the US Department of Agriculture in 2019 [27]. In a carried-out investigation, it was noted that the extract derived from Citrus sinensis peels demonstrated in-vitro antimicrobial effectiveness against microorganisms linked to dental caries [28]. Considerable attention has been focused on investigating the antimicrobial properties of different plant extracts and their constituent compounds. Certain extracts from these sources have exhibited significant inhibitory effects against pathogenic bacteria, viruses, fungi, and yeasts [29]. Citrus fruit extracts, in particular, have garnered significant interest in this regard owing to their diverse array of phytochemical components [30]. Copper has long been acknowledged as a hygienic material throughout history, leading to the utilisation of copper compounds as antimicrobial agents. Particularly in its nanoscale form, copper exhibits noteworthy antibacterial activity, and the synthesis of CuNPs is highly cost-effective [30]. Although the precise mode of action of CuNPs remains unclear, it is understood that copper ions disrupt biochemical processes within bacterial cells. Additionally, research thus far has not identified the development of resistant bacteria to copper, as observed in the case of antibiotics [31].

Green synthesis adheres to principles that prohibit the use of harmful substances in both the production and application of chemical products. This approach is crucial given the growing environmental concerns, emphasising the need for "green" substances that are eco-friendly [32,33]. Utilising plant extracts in the environmentally friendly synthesis of nanoparticles provides a method that is both straightforward and swift, with large-scale production emerging as a notable area of research. In the nanotechnology field, this method is recognised as eco-friendly, cost-effective, and a preferred substitute for chemical methods [34]. Additionally, plant extracts, renowned for their significance in treating microbial infections, represent a valuable source for sustaining human health [35]. Following the synthesis process, it is imperative to thoroughly characterise nanoparticles, especially if they are intended for use to enhance human well-being in areas such as the healthcare industry, specifically in nanomedicine. Various methods have been utilised to characterise synthesised nanoparticles. These techniques encompass FTIR, SEM, Transmission Electron Microscopy (TEM), UV spectroscopy, X-ray Photoelectron Spectroscopy (XPS), and X-Ray Diffraction (XRD). Such comprehensive characterisation is essential to ensure the safety and efficacy of nanoparticles in their intended applications, particularly in the realm of nanomedicine, where human health is paramount [36]. Evaluating cytotoxicity is crucial as it plays a significant role in determining the safety and potential adverse effects of the substance being assessed.

Amiri M et al., conducted a study examining the antibacterial properties of CuNPs, indicating significant bactericidal activity of CuO NPs against various cariogenic bacteria [37]. Several studies have explored similar methods for the green synthesis of CuNPs using different sources such as Persea americana seed, Cissus arnottiana plant, Punica granatum, and others [31,38]. In a similar study conducted by Singh S et al., Copper Oxide Nanoparticles (CuONPs) were synthesised using plant extracts derived from Camellia Sinensis (CS) and Citrus Limon (CL). The research revealed notable antimicrobial effects, along with reduced cytotoxicity. Moreover, these nanoparticles demonstrated enhanced anti-inflammatory and antioxidant properties, particularly at concentrations exceeding 50 μL [39]. A study by Amer MW and Awwad AM revealed that green-synthesised CuNPs exhibited significant efficacy against two tested pathogenic strains, E. coli and *S. aureus* [40]. Overall, the results obtained from other studies demonstrate significant antibacterial properties of CuNPs, consistent with the findings of present study.

In present study, the investigation focused on the antimicrobial properties of copper sulphate nanoparticles production. Different concentrations, specifically 25 μL, 50 μL, and 100 μL, were examined. The antimicrobial efficacy was evaluated through the agar disc diffusion method, and the resulting ZOI diameter was calculated in millimeters. A notable antibacterial effect was observed at 100 μL, with the highest impact against *E. faecalis* (16.03 mm), followed by *S. aureus* (14.03 mm), while the least effect was recorded against *S. mutans* (12.2 mm) as depicted in [Table/Fig-5]. The level of inhibition of bacterial growth observed in this study was found to be influenced by the concentration of nanoparticles present in the medium. This finding aligns with a study conducted by Jadhav S et al., which similarly concluded that the antibacterial effect exhibited dose-dependency [41]. Prior research has indicated that CuNPs have an impact on the depolarisation of bacterial cell membranes, leading to cell filamentation. Moreover, CuNPs were observed to markedly increase cellular Reactive Oxygen Species (ROS) levels, leading to protein oxidation and lipid peroxidation, DNA degradation, and ultimately, the death of bacterial cells [42,43]. The study results clearly demonstrate the successful production of copper sulphate nanoparticles through a green synthesis approach facilitated by Citrus sinensis extract. The use of orange extract as both a stabilising agent and a reducing agent in the nanoparticle production process offers several advantages. Citrus fruit extract proved highly effective in synthesising CuNPs at the nanoscale, with the prepared nanoparticles demonstrating promising antimicrobial activity [40].

The present study stands out for evaluating the impact of green-synthesised CuNPs using citrus extracts, particularly in comparison to various pathogenic strains, notably *E. faecalis*. To author's knowledge, no previous study has specifically examined the effects of citrus-based nanoparticles on *E. faecalis*. This aspect holds significant importance as such a combination could potentially be utilised in the development of endodontic irrigants or intracanal medicaments, offering promising effects against *E. faecalis*, a known resilient organism.

Limitation(s)

A limitation of present study is its confinement to in-vitro experimentation, indicating the necessity for subsequent in-vivo studies to broaden the scope of understanding. Another limitation is the lack of assessment of the cytotoxicity of the citrus-mediated CuNPs. Evaluating the cytotoxic effects on human cells is essential to ensure the safety and efficacy of these nanoparticles for potential dental applications. Additionally, the study may not thoroughly explore the mechanisms or specific antimicrobial actions involved, thereby limiting the assessment of nanoparticle dosages and toxicity. By acknowledging these limitations, future dental research can aim to address these gaps and advance understanding of green nanoparticle synthesis for antimicrobial applications. Future studies should prioritise assessing the cytotoxicity of these nanoparticles to ensure their safety and efficacy for clinical use. Understanding the potential adverse effects on human cells is crucial for determining the overall biocompatibility of these nanoparticles. Furthermore, research prioritising precise dosages and assessing the practical effectiveness of the extract should be done.

CONCLUSION(S)

There is an urgent need to enhance the screening of natural products and plant components to lay the groundwork for future exploration in terms of pharmacological and phytochemical aspects. This approach holds the potential to unveil novel, clinically effective antibacterial compounds, addressing not only dental caries but also other bacterial pathogens that may exhibit resistance. From present study, it can be concluded that successful green synthesis of CuNPs was achieved using Citrus sinensis extract. The present study demonstrates the substantial antibacterial efficacy of citrus-mediated CuNPs, showing the highest activity against *E. faecalis*, followed by *S. aureus*, and least against *S. mutans* with a dose-dependent effect. These findings suggest the potential utility of these nanoparticles in various dental formulations, including irrigants, intracanal medicaments, mouthwashes, and other applications. Moving forward, further research efforts should focus on assessing the toxicity of these nanoparticles to ensure their safety and efficacy in clinical settings.

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