

Essential role of calcium oxide nanoparticles in the infection of methicillin-resistant *Staphylococcus aureus*

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Abstract

Calcium oxide nanoparticles (CaONPs) exist as an alternative therapeutic option in treating infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) strains, which do not respond to antibiotic therapy. The synthesis of nanoparticles from *Carica papaya* leaf (CPL) extract has been proven considerably green due to its environmentally friendly process. From the previous effort, it is essential to identify the external factors that may influence the antibacterial activity against MRSA. The present study focused on the effects of CaONPs in the entire infection process of MRSA. Various optimum conditions to produce CaONPs are evaluated by using UV-VIS, FTIR and FESEM. The antibiotic and sensitivity were evaluated via the disk diffusion method, minimum inhibitory concentration (MIC), while the minimum bactericidal concentration (MBC) was estimated using turbidimetry. Results showed that the size of nanoparticles was in the range of 50 to 250 nm spheres, but agglomerate structure. Results also revealed that the presence of functional groups of the synthesised CaONPs was confirmed via FTIR analysis (400 – 4000 cm⁻¹). Interestingly, the turbidimetry findings showed MIC and MBC were 200 and 250 µg/mL against MRSA. In conclusion, CPL functioned as a good bio-reductant to produce CaONPs by exhibiting an acceptable size and shape of NPs and effective antibiotic sensitivity towards MRSA.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged in the last 30 years and tends to occur in waves of infections (Zainol Abidin *et al.*, 2020). It has been classified by the World Health Organization (WHO) as one of the priority pathogens that pose the greatest threat to human health (Ismail *et al.*, 2021). Nowadays, cefoxitin is used as a substitute for methicillin because the Clinical and Laboratory Standards Institute (CLSI) recommends the cefoxitin disk as an alternative method for screening. Although methicillin was initially widely used, it is no longer recommended for the general population due to its toxicity and is being replaced by similar, more stable penicillin antibiotics such as oxacillin, flucloxacillin and dicloxacillin (Lee *et al.*, 2018).

In the hospital, patients infected with MRSA are isolated and treated with chlorhexidine gluconate 4% (CHG) and nasal mupirocin instead of administering antibiotics. CHG is usually given to patients because it can effectively reduce bacterial count, topical antiseptic

solution and is used as a disinfectant agent to remove microorganisms (Amirov *et al.*, 2017). Although CHG has more advantages, it also has disadvantages such as the potential for cross-resistance with antibiotics and the possibility of a severe allergic reaction (Van den Poel *et al.*, 2022). Our study aimed to investigate the essential role of CaONPs against MRSA infections and provide an alternative treatment instead of using CHG.

In order to solve the various environmental problems, the search for new solutions, such as the use of nanoparticles, is very helpful in combating these infections. Medicinal plants have been subjected to a green process for the synthesis of nanoparticles due to their high potential for therapeutic purposes and proven good bacterial efficacy (Dhivya *et al.*, 2020). The green synthesis of nanoparticles by an environmentally friendly method is valuable and has shown significant advantages compared to other biological methods.

Papaya is the common name for *Carica papaya*, which belongs to the Caricaceae family and is known as

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one of the medicinal plants that can treat various diseases. Papaya leaf extracts contain many proteolytic enzymes, phenols and vitamins that act as excellent antioxidants (Kale, 2018). It also consists of secondary metabolites such as alkaloids, steroids, tannins, saponins and flavonoids (Callixte *et al.*, 2020). The phytochemicals contained in *C. papaya* leaf treat various kinds of diseases like dengue fever, urinary tract infections, anti-oxidant and anti-cancer properties (Devmurari *et al.*, 2018). In this study, the properties of the nanoparticles were investigated by UV-Vis Spectroscopy (UV-vis), Fourier Transform Infrared Spectroscopy (FTIR) and Field Emission Scanning Electron Microscopy (FESEM). Antibiotic activity and sensitivity were investigated using disk diffusion techniques.

2. Materials and methods

2.1 Materials

The leaves of *C. papaya* were collected from the local fields in Batu Pahat, Johor. Reagents, such as Nutrient Agar, were purchased from the OXOID company. Clinical MRSA samples were obtained from the Laboratory of Microbiology, Hospital Sultanah Nora Ismail, Batu Pahat, Johor.

2.2 Preparation of *Carica papaya* leaf extract and calcium nitrate

Fresh leaves of *C. papaya* (20 g) were cut into small pieces and placed in a sterile 500 mL beaker. Then, 200 mL of distilled water was added to the beaker and allowed to stir at room temperature for 24 hrs. The extracts were filtered with Whatman No. 1 filter paper, and the filtrate was stored at 4°C for further use. A total of 10 g of 5 M $\text{Ca}(\text{NO}_3)_2$ was added to 100 mL of distilled water and dissolved thoroughly.

2.3 Determination and synthesis of calcium oxide nanoparticles

The aqueous leaf extract of *C. papaya* and $\text{Ca}(\text{NO}_3)_2$ (1M, 3M, 5M) were mixed in a 1:1 ratio and heated at 50°C for 2 hrs until a colour change was observed. The precipitating agent, sodium hydroxide solution (NaOH) of 20 mL, was added dropwise. Then, the prepared

solution was stirred for 2 hrs. The colour change to yellow indicated the formation of calcium oxide nanoparticles (CaONPs) by the extract of *C. papaya* leaves. The precipitate was obtained by centrifuging the reaction mixture, and the filtrate was washed twice with distilled water to remove other organic matter and secondary metabolites. The precipitate was dried in an oven at 60°C for 6 hrs and then calcined in a muffle furnace at 450°C for 2 hrs. The product was ground with mortar and pastel to obtain the fine yellow powder. The obtained yellowish calcium oxide nanopowder was used for further analysis. The synthesis process of calcium oxide nanoparticles (CaONPs) using bulk $\text{Ca}(\text{NO}_3)_2$ and papaya leaf extract is illustrated in Figure 1.

2.4 Characterization of calcium oxide nanoparticles from *Carica papaya* leaf

2.4.1 UV-visible spectrometric analysis of calcium oxide nanoparticles

The spectrometric measurement of the biosynthesised CaONPs was carried out using an ELICO UV-visible spectrophotometer from India. At regular intervals, measurements of the decrease of calcium oxide were at 200-800 nm. A spectrum of CaO nanoparticles was plotted with a wavelength on the x-axis and absorbance on the y-axis to display the absorption peaks.

2.4.2 Fourier transform infrared analysis of calcium oxide nanoparticles

The vibrational frequencies between the bonds of the atoms in the nanoparticles are represented by absorption peaks in the FTIR spectrum. FTIR is also the best technique for qualitative analysis; the intensity of the peaks is a clear indication of the materials present (Torres-Rivero *et al.*, 2021). To remove the biological substances or the ligands that decapped the nanoparticles, the 200 mL residual solution of CaONPs was centrifuged at 10000 rpm for 30 mins, and the precipitate was then resuspended in 10 mL ethanol and finally in sterile distilled water. To obtain the powder, the resuspension and centrifugation processes were dried in an oven before FTIR analysis. The FTIR spectrum of the synthesised nanoparticles was created in the range of 400 – 4000 cm^{-1} using an alpha FTIR spectrometer.

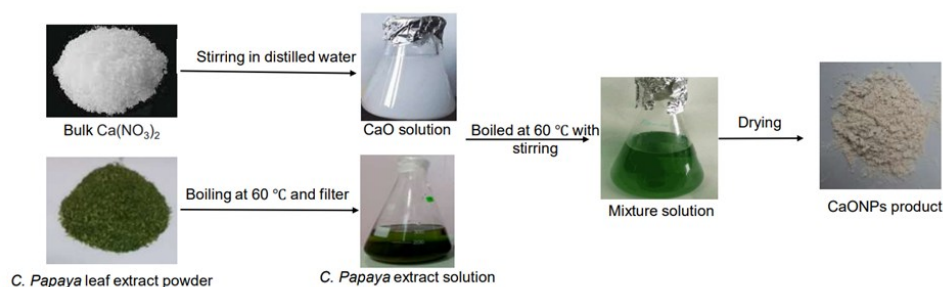


Figure 1. Schematic of green synthesis of CaONPs using *C. papaya* leaf.

2.4.3 Field emission scanning electron microscopy and energy-dispersive x-rayspectrometry

Field emission scanning electron microscopy (FESEM) analysis was carried out using a Zeiss EVO 18-EDX special model compatible with an Energy Dispersive X-ray Spectrometry (EDX) instrument. The small particles of the dried samples were prepared on a copper grid with carbon coating and analysed for size determination. The particle size and texture of the nanoparticles can be examined using image magnification software compatible with FESEM, which helps identify the presence and production of CaONPs. By using EDX analysis, the elemental composition of the synthesised sample was investigated.

2.4.5 Evaluation of antibacterial activity using disc diffusion method

Antibacterial assays were performed on ATCC MRSA and 10 other human pathogenic MRSA strains. The standard disc diffusion method was used. Peptone water broth was used to culture bacterial strains. A fresh inoculum (100 μ L) of each culture was spread on Mueller-Hinton agar plates overnight. Sterile Whatman No. 1 paper discs of 5 mm diameter containing 10 μ L of *C. papaya* leaf extract (5 μ g/mL) and 10 μ L of CPL-CaONPs (1M, 3M, 5M) were added to each plate in a serial arrangement. Distilled water was used as a negative control, and 10 μ L of ampicillin served as a positive control. All plates were incubated at 37°C in an incubator for approximately 24 hrs. The zone of inhibition was measured in diameter (mm) using an

antibiotic ruler. Measuring the clear zone around the test and control disc allowed for the estimation of the inhibition zone. The larger the zone of inhibition, the greater the bacterial susceptibility activity.

2.4.6 Minimum inhibitory concentration and minimum bactericidal concentration

The concentration of biosynthesised CaONPs that limit the growth of bacterial strains was ascertained using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The MIC that was necessary to kill bacteria was observed by two-fold serial dilution and incubated at 37°C for 2 hrs. The MBC is referred to as the test material concentration at which no detectable bacterial colonies are present. Samples from each culture tube for each bacterial strain were applied to Nutrient agar plates and incubated overnight after the initial 24-hour incubation period.

3. Results and discussion

3.1 UV-vis analysis

UV-vis is a crucial tool for characterising these materials. It may also be used to assess the stability of nanoparticle colloidal solutions. Analysis of the UV-visible spectra of 5 M CPL-CaONPs showed that absorbance spectra between 450 nm to 500 nm indicated bioreduction of Calcium nitrate into CaONPs (Figure 2). The optical band gap energy of the biosynthesised 5M CPL-CaONPs is identified by using the formula $E=H=hc/\lambda$, where H is Planck's constant. This report,

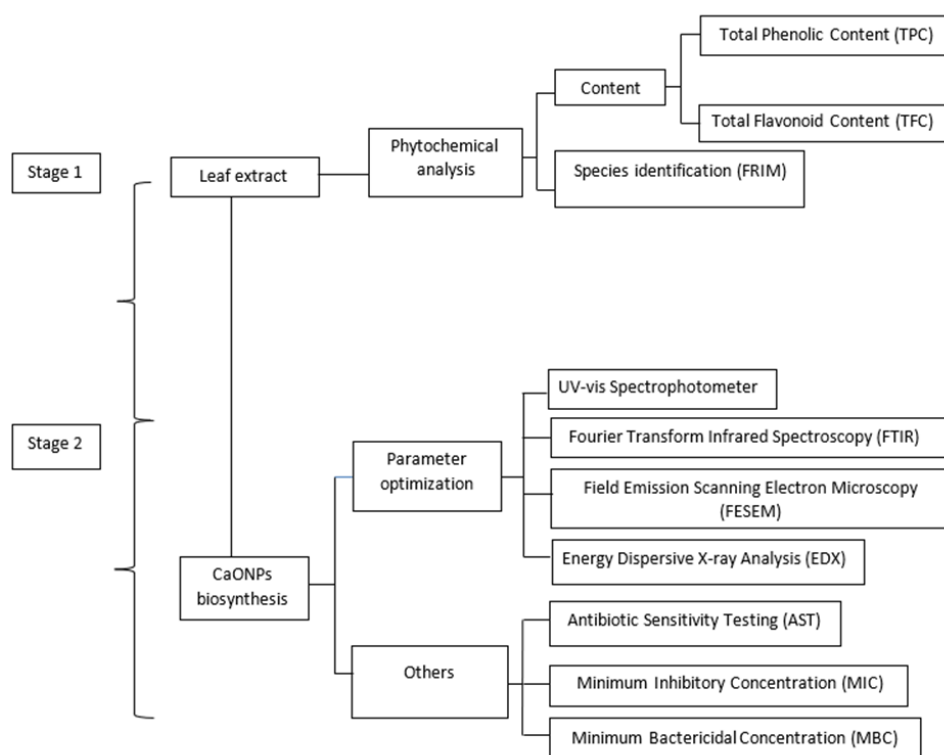


Figure 2. Flowchart synthesis of CaONPs from *C. papaya* leaf extracts.

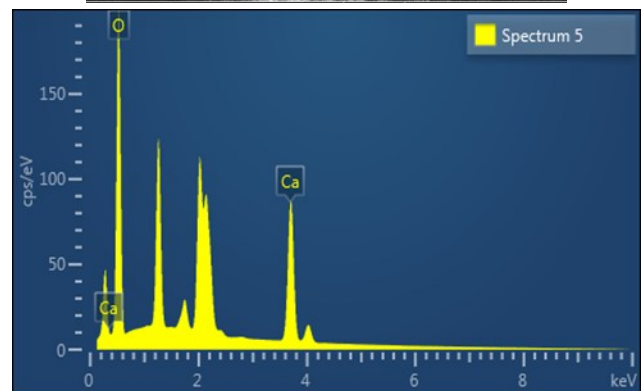
based on the evidence from Figure 3, indicates that the leaf contains various substances such as flavonoids, steroids, alkaloids, alkenes, and aromatic compounds, which act as reducing, capping, and stabilising agents in

previous study using *C. papaya* leaves in the synthesis to evaluate the bactericidal properties also showed an approximately pattern similar drawn in this study (Banala et al., 2015).

3.3 Field emission scanning electron microscopy analysis

According to the FESEM investigations, CaONPs with sphere-shaped yet agglomerate-structured nanoparticles had a particle size between 50 and 250 nm (Figure 5). The quantitative analysis using EDX revealed a high calcium content of 42.27%. The spectrum also indicated the presence of oxygen, which is 57.53% (Figure 4). Due to EDX analysis's high sensitivity in detecting elements in bacterial cells, it reveals the presence of pure calcium and other elements, supporting the manufacture of CaONPs (Scimeca et al., 2018).

The energy-dispersive X-ray spectroscopy (EDX) spectrum and elemental composition analysis of the synthesised calcium oxide nanoparticles are shown in Figure 5.



Element	Line Type	Apparent concentration	K Ratio	Wt%
O	K series	57.29	0.19279	57.53
Ca	K series	40.59	0.36262	42.47
Total				100

Figure 5. FESEM and EDX for CaONPs.

3.4 Antibiotic sensitivity testing

The result of the disc diffusion method showed the effect of CPL extract, CPL-CaONPs (1 M, 3 M, 5 M) and ampicillin on ten MRSA bacterial strains. Ampicillin was used as a standard antibiotic during the test. The results can be seen in Table 2. The antibacterial activity

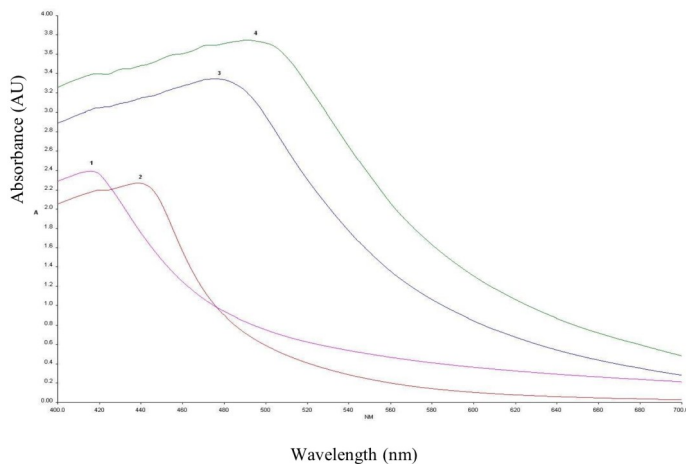


Figure 3. UV-vis of 1. *C. papaya* leaf, 2. 1M CPL-CaONPs, 3. 3M CPL-CaONPs, 4. 5M CPL-CaONPs.

the biosynthesis of CaONPs (Vergheese et al., 2018).

3.2 Fourier transform infrared spectroscopy analysis

The FTIR study spectrum showed strong absorbance visible between 400 and 4000 cm^{-1} . Other peaks in the spectrum can be found at 580, 1552, 1700, 2100 and 3310, which are alkyl halides, aromatic, carbonyl, alkynes and amines compounds (Figure 4). These results confirm that these compounds act as capping agents and reductants in the synthesis of nanoparticles and contribute to stability. Table 1 shows the stretching and bending vibrations from each peak of CPL-CaONPs. The

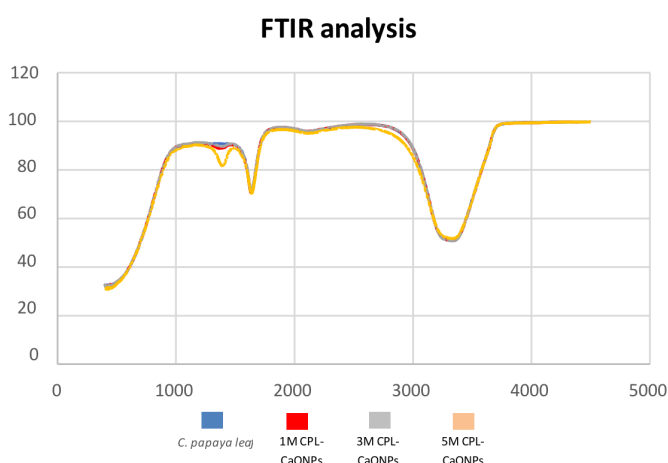


Figure 4. FTIR Analysis

Table 1. Stretching and bending vibrations of biosynthesised CaONPs.

No.	Band Range	Type of Vibration	Appearance
1	580	C-Br (Alkyl halides)	Strong
2	1552	NO ₂ (Nitro compounds)	Strong
3	1700	C=O (Carbonyl compounds)	Strong
4	2100	-C≡C- (Alkynes)	Medium
5	3310	N-H (Amines)	Medium

of the synthesised CPL-CaONPs, as indicated by the inhibition zones of varying diameters (a: 7 mm, b: 8 mm, c: 10 mm), is illustrated in Figure 6. Based on the results, 5M CPL-CaONPs showed good antibacterial activity against MRSA, and the zone of inhibition was 10 mm compared to 1M and 3M CPL-CaONPs. The mechanism at this stage was that the bacterial cells aggregated with the CPL-CaONPs. These interactions resulted in breakdown of the cell membrane, cytoplasm leakage and abnormal morphology and inhibited motility activity. Moreover, these nanoparticles induced the formation of Reactive Oxygen Species (ROS) and once they entered the cytoplasm, they damaged and fragmented DNA, ultimately leading to cell death (Wang *et al.*, 2017).

Table 2. Antibacterial capacity of CPL extract, CPL-CaONPs and ampicillin against MRSA.

Microorganism	Zone of inhibition in mm				
	CPL (5 μ L/mL)	CPL-CaONPs (5 μ L/mL)			Amp (10 μ g/mL)
Disc No.	1	2	3	4	5
Control positive	6	8	10	14	16
MRSA samples	7	7	8	10	16

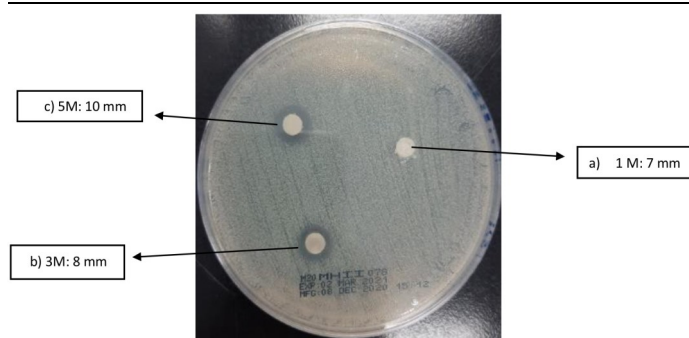


Figure 6. Antibiotic sensitivity in diameter (mm) of CPL-CaONPs at different concentrations against MRSA.

3.6 Minimum inhibitory concentration and minimum bactericidal concentration

The turbidimetry analysis of CPL-CaONPs showed good antibacterial activity, with MIC and MBC of MgONPs were 200 and 250 μ g/mL against MRSA. This showed that nanoparticles from *C. papaya* can be advantageously used for the treatment of MRSA compared to commercial CaONPs powder, as they exhibit potent antimicrobial activity against pathogens (Cai *et al.*, 2018).

4. Conclusion

The *C. papaya* leaf has significant properties for health and therapy. This green approach to the production of CaONPs is very advantageous because it makes the process economical and more environmentally

friendly compared to chemical synthesis. In this study, the extract of *C. papaya* leaves was used as a reducing and stabilising agent. The synthesised nanoparticles were confirmed by UV-Vis, FTIR, FESEM, EDX and XRD. The CaONPs exhibited good antibacterial activity against MRSA. This is an effective method that can serve as an alternative for antibacterial applications where a lower amount of chemicals is used.

Conflict of interest

The authors declare no conflict of interest.

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