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EVALUATION OF ANTIBACTERIAL AND BIOCOMPATIBILITY PROPERTIES OF PHYTO-FABRICATED GREEN CALCIUM NANOPARTICLES

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ABSTRACT

The upsurge in antibiotics and other chemotherapeutic agents' resistance relative to different microbial infections has necessitated and intensify the search for effective antimicrobial agents. The present study was undertaken to evaluate the antibacterial and biocompatibility properties of phyto-fabricated green calcium nanoparticles. *Azadirachta indica* (Neem plant) was collected, room dried, pulverized and extracted using aqueous and methanolic solvents. Qualitative phytochemical analysis was carried out t and the aqueous extracts were further utilized to synthesize calcium oxide nanoparticles under alkaline condition which were indicated by the colour change formations. The dried nanopowders were characterized using conventional nanotechnological techniques The biosynthesized were tested for the antibacterial potencies using agar well diffusion, MIC and MBC while their biocompatibility was ascertain using haemolytic assay. The results revealed that extract exhibited higher presence of tannins, saponins, terpenoids and glycosides. The elucidated nanopowders were found to conform to calcium oxide nanoparticles properties. The *Azadirachta indica* CaONP had the highest zone of inhibition of 20.00 20.00±0.39 mm against *Pseudomonas aeruginosa* while ciprofloxacin had the lowest zone of inhibition of 0.00±0.00 mm against *Staphylococcus aureus* and *Escherichia coli*, respectively. *Azadirachta indica* CaONP recorded the lowest MIC and MBC values of 0.47 and 0.94 mg/mL against *Staphylococcus aureus*. The result of the haemolytic activity revealed that biosynthesized calcium nanoparticles are safe for human usage at lower doses as LC_{50} (median lethal concentration) >100 are relatively not acutely toxic. The aqueous leaf extract of the *Azadirachta indica* (Neem plant) possess reducing agents for the biofabrication of CaONPs, with noteworthy antibacterial activities as well as biocompatibility.

Keywords: Antibacterial, Antibiotic resistance, Biocompatibility, Calcium oxide nanoparticle, Nanotechnology,

INTRODUCTION

Nanotechnology is gaining enormous attention as a new area of research dealing with the development of nanomaterials and nanoparticles (NPs) for their utilization in diverse fields such as catalysis, electrochemistry, biomedicines, pharmaceuticals, sensors, food technology, cosmetics, etc. Nanoparticles (NPs) are nanometer-sized (< 100 nm) atomic or molecular scale solid particles having some excellent physical properties compared to the bulk molecules depending on their size and morphology. Among all types of NPs, metal and metal oxide nanoparticles have been thoroughly examined using science and technology due to their excellent properties such as high surface to volume ratio, high dispersion in solution, etc. Owing to these, metal and metal oxide nanoparticles display enhanced antimicrobial properties (Vanlalveni et al., 2021).

Calcium oxide (CaO) is the most important materials which has been popularly used in many fields, such as catalyst, cosmetic and ceramic. It is also applied as inorganic antimicrobial material for controlling microorganisms. Based on its chemical properties, CaO exist in the alkaline earth group on periodic table. On the other hand, concerning the rich biodiversity of tropical plants found in Indonesia, the uses of tropical biomasses or their extracts for CaO biosynthesis will be a good scientific challenge due to more efficient and eco-friendly method for CaO biosynthesis. It has been well known that metabolite compounds found in plant materials (biomasses or their extracted compounds) can act as biological reductor on metal synthesis, where flavonoid compounds had been noticed as one of the most useful groups of secondary metabolite in plant tissues applied as reductive agent for metal ions. Most flavonoid compounds naturally contained natural pigment with various colours, such as red, pink and yellow depending on the kind of the plants (Ramli et al. 2019).

The leaf extract of *Piper betel* extract-derived calcium oxide nanoparticles (CaONPs) has strong antibacterial, antioxidant, and antibiofilm properties. *Thymbra spicata* leaf extract-derived biogenic metallic nanoparticles have strong bactericidal activity that is comparable to industry standards. These metallic nanoparticles have an antioxidant activity of about 79.67% (Mazher et al. 2023). *Moringa olifera* leaf extracts can be used to easily synthesize CaONPs that have powerful antibacterial and antioxidant properties. Calcium oxide nanoparticles produced from *Trigona* sp. show great antifungal potential and pose less toxicity. Metallic nanoparticles show great fabrication by phytochemicals and prove to be less toxic. Calcium apatite nanoparticles show significant apoptosis in some cancer cell lines. The cytotoxic activity displayed by calcium apatite nanoparticles is comparable to the standards. The CaONPs prepared from *Linum usitatissimum* leaf extracts are found to be safer for in vivo treatments. Recently, a study on rats has reported different concentrations of calcium oxide nanoparticles that were administered for 60 days, with no toxicity evidence. Due to the fabrication of phytochemicals on the surface of metallic nanoparticles, they show great biocompatibility.

The Neem tree, is primarily cultivated in the southern regions of Asia and Africa, where it has been seen used through many ages, in medical folklore. We should note that various parts of the Neem tree, including the leaves, bark, fruit, flowers, oil, and gum are associated with the aforementioned medical folklore in the treatment of certain medical conditions such as cancer, hypertension, heart diseases, and diabetes. The potential effects that are seen when using these extracts can certainly be attributed cellular and molecular mechanisms, these mechanisms include free radical scavenging, detoxification, DNA repair, cell cycle alteration, programmed cell death mitigation and autophagy, immune surveillance, anti-inflammatory, anti-angiogenic, and anti-metastatic activities and the ability to modulate of various signaling pathways (Islas et al. 2020). Previous studies mostly focused on antibacterial activities without emphasis on the *in vivo* biocompatibility or safety study of these metallic particles and hence justifies the present study. The present study was undertaken to evaluate the antibacterial and biocompatibility properties of phyto-fabricated green calcium nanoparticles.

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MATERIALS AND METHODS

Chemicals and Reagent

Mueller Hinton Broth (MHB) or Mueller Hinton Agar (MHA), calcium nitrate and ciprofloxacin antibiotics and all other chemicals and reagents were purchased from Himedia, Loba Chem India and also be of analytical grade, unless otherwise stated.

Microbial Strains

Two Gram-positive bacterial strains (*Bacillus subtilis, Staphylococcus aureus*) and two Gram-negative bacterial strains (*Escherichia coli, Salmonella* sp.) were used (Alagesan and Venugopal, 2018). These local pathogenic strains were obtained from Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu Campus and use in this study.

Plant Specimen Source and Preparation of Aqueous Seed Extract

The leaves of Neem plants that were used for the phyto-assisted synthesis of calcium oxide nanoparticle were collected at Mr Dimejesi Compound Umuikpa Oka village Uga town in Aguata Local Government Area of Anambra State and transported to the laboratory. The leaves were washed with tap water and finally washed in distilled water. Thereafter, the leave was dried for 1 week at room temperature. The dried leaves were blended into powder using industrial blender. Fifty grams of the grounded leaves was weighed and dissolved in 100 mL deionized water and methanol. The aqueous suspension was boiled for 5 min until a colour change was observed while the methanol was left for 72 hrs under shaking conditions. The boiled suspensions were cooled at room temperature and used in the calcium nanoparticle synthesis while methanolic extract was used phytochemical determination after double filtration with muslin cloth and Whatman no. 1 filter paper (Acay *et al*., 2019).

Phytochemical Analysis

The qualitative phytochemical screening for alkaloid, tannin, anthraquinone, phenol, terpenoid, glycoside, saponin, flavonoid, steroid, protein and carbohydrate of the methanolic leave extract of *Azadirachta indica* (Neem plant) was performed by the following standard method as reported by Okaiyeto *et al.* (2019) and Karthigaiselvi and Rameshwari (2016) with slight modifications. The obtained results were qualitatively expressed as positive (+) or negative (-).

Synthesis of Calcium Nanoparticles (CaONPs)

The calcium nanoparticle was synthesized by following the protocol of Mazher *et al*. (2023) and 50 mL of 0.2 M $CaCl_2.2H_2O$ solution was added to 50 mL of *Azadirachta indica* (Neem plant) extracts. To keep the pH at 10.5, 10 mL of 2.0 M aqueous NH_4OH was added dropwise and agitated for 30 min, until a white, milky precipitation of $Ca(OH)_2$ was visible. The resultant mixture was centrifuged for 15 min at 10,000 rpm. To eliminate any unreacted starting material, the product was rinsed repeatedly with distilled water. The white $Ca(OH)_2$ particle was then calcinated at 700 °C for 3 hrs. A white powder was obtained, which was CaONPs and stored for characterization (Mazher *et al*., 2023).

Physicochemical Characterization of Calcium Oxide Nanoparticle

The physical and chemical characteristics of the synthesized green silver nanoparticle was determined using standard nanotechnological techniques as described by Dokubo *et al*. (2023), Okafor *et al.* (2023) and Uba *et al*. (2023).

Anti-Bacterial Activity

The standard agar well diffusion method will be employed to study the antibacterial property of CaO-NPs against human clinical pathogens such as *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus* and *Staphylococcus aureus* (Alagesan and Venugopal, 2018). A 6 mm wells will be made on Muller–Hilton agar plates using sterile cork borer. Amounts of 100 uL of the biosynthesized CaONPs from a stock concentration of 60 mg/mL and ciprofloxacin (0.1 g/mL) as well as dimethyl sulphoxide as negative controls were introduced into the wells using a micropipette. The experiments were replicated three times. The different levels of the inhibition zones were recorded after incubation at 37 °C for 24 h. The diameters of the inhibition zones around each well were determined (Vu *et al*., 2022).

Determination of Minimal Inhibitory and Bacterial Concentration of Calcium Nanoparticle and Antibiotics

The method of Okaiyeto *et al.* (2019) was adopted in order to determine the minimal inhibitory and bactericidal concentrations of calcium oxide nanoparticles or positive control antibiotics using micro-dilution procedure. The bacterial strains were cultured in Mueller Hinton Broth (MHB). Cell suspensions were adjusted to obtain standardized populations (0.5 McFarland 1 X 10^8 CFU/mL) by measuring the turbidity with a spectrophotometer. Susceptibility tests were performed by transferring 500 µL Mueller Hinton broth (MHB) into microcentrifuge tubes. Stock solutions (60 mg/mL) of CaONPs were prepared in dimethyl sulfoxide (DMSO) and then different concentrations ranging from 0.47 – 30.00 mg/mL were prepared by two-fold serial dilutions in MHB. Subsequently, 20 µL of each standardized test bacteria will be added into the mixture and vortexed followed by incubating at 37 °C for 24 h. The positive and negative controls used were ciprofloxacin and 2 % DMSO, respectively. Afterward, the minimum bactericidal concentration (MBC) was determined by plating out those broths without visible growth on fresh Mueller Hinton agar, and further incubating the plates at 37 °C for 24 h.

Haemolytic Test

The haemolytic assay was carried to determine the toxicity of CaONPs on human erythrocytes by adopting the method of Katva *et al.* (2017). Prior to the assay, 10 mL of blood was taken from human volunteer and dispensed into ethylene diamine tetraacetic acid (EDTA) tube. Consequently, the blood sample was centrifuged at 3,000 rpm for 15 min and decanted. The RBC pellet left at the bottom of tube was washed with phosphate-buffered saline (PBS) and afterward added to CaONPs ranging from 10 µg/mL to 100 µg/mL. RBCs mixed with 1.5 mL Triton X-100 was taken as positive control. All the samples were incubated at 37 °C for 1 hr, followed by centrifugation at 3,000 rpm for 15 min and the supernatant was analyzed spectrophotometrically at 540 nm. The percentage of haemolysis was calculated from the formula: OD_{540} (sample) − OD_{540} (0 % lysis)/OD_{540} (100 % lysis) − OD_{540} (0 % lysis) × 100 %

Statistical Analysis

All assays were conducted in triplicate and mean ± standard deviation were calculated. All experimental data were analyzed with GraphPad Prism Version 8.1.0 using the ANOVA test with Dunnet multiple comparison test. A *p* value less than 0.05 were considered as statistically significant at 95 % confidence interval.

RESULTS AND DISCUSSION

Table 1: Qualitative phytochemical profile of the methanolic leave extract of *Azadirachta indica*

Component	*Azadirachta indica*
Anthroquinone	-
Tannins	++
Terpenoids	+++
Flavonoids	+
Carbohydrate	+
Alkaloids	+
Protein and amino acid	+
Cardiac glycoside	++
Saponins	++
Glycoside	++

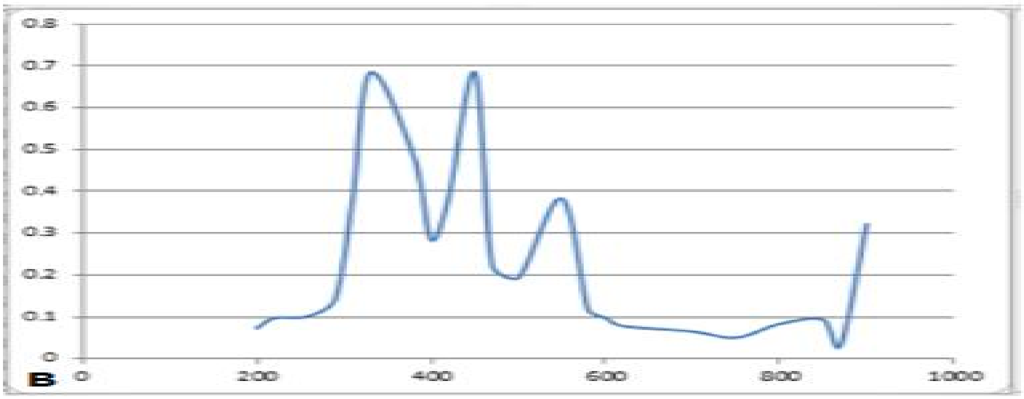

Figure 1: UV - VIS spectral profile of *Azadirachta indica* biosynthesized calcium oxide nanoparticle

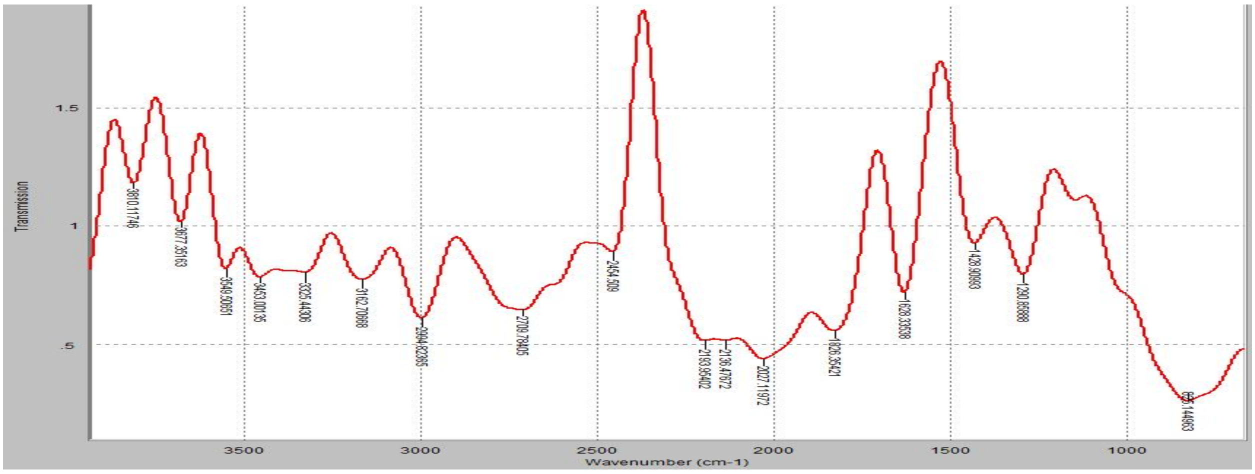

Figure 2: Infra-red spectral profile of *Azadirachta indica* biosynthesized calcium oxide nanoparticle

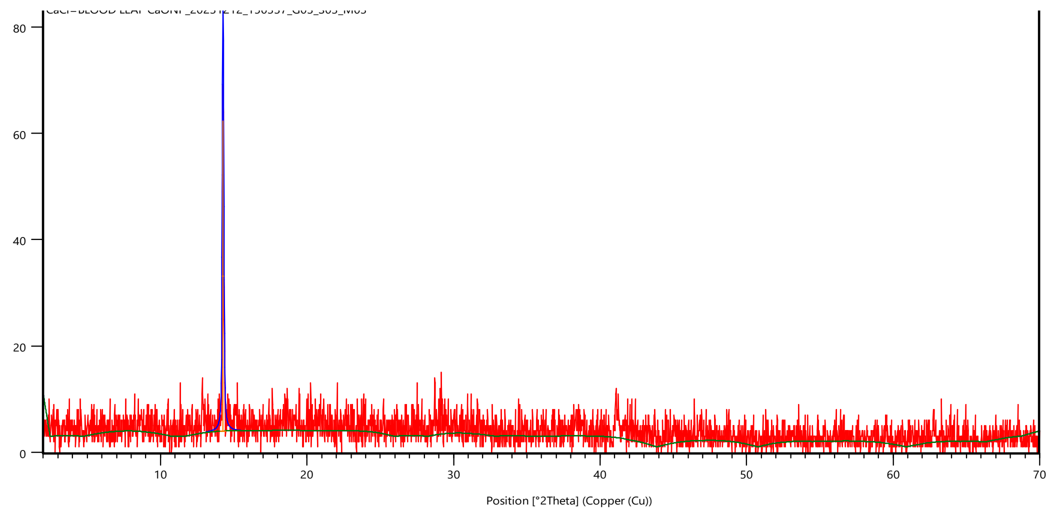

Figure 3: X ray diffraction profile of a) *Azadirachta indica* biosynthesized calcium oxide nanoparticle

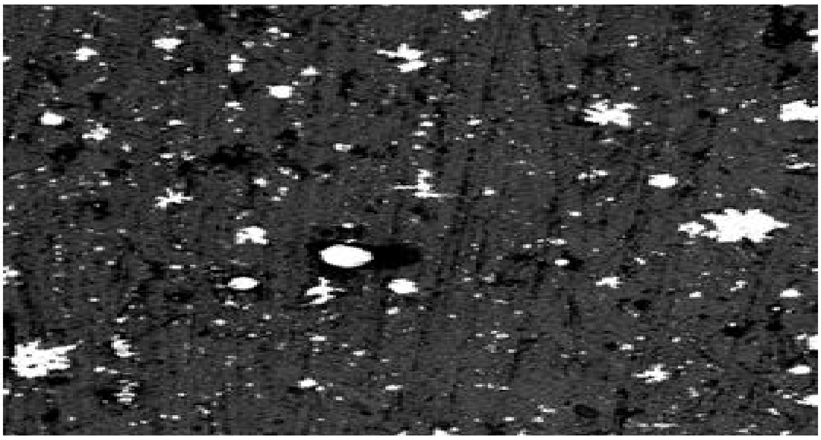

Figure 4: Scanning electron micrograph profile of *Azadirachta indica* biosynthesized calcium oxide nanoparticle

Table 2: Antimicrobial activity of the green calcium oxide nanoparticles against tested bacterial pathogens

Agent	*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas Aeruginosa*
Azadirachta indica CaONP	13.20±0.21	18.80±0.29	15.00±0.00	20.00±0.39
Ciprofloxacin	9.14±0.11	0.00±0.00	0.00±0.00	6.83±0.51
DMSO	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Key: CaONP = Calcium oxide nanoparticles; DMSO = Dimethylsulphide; Values represent mean standard deviation of triple determination

Table 3: Minimium inhibitory (MIC) and bactericidal concentration (MBC) of the *Azadirachta indica* aqueous leave extract mediated calcium oxide nanoparticle

S/N	Dilution	Concentrati-on (mg/mL)	*Bacillus cereus*	*Staphyloco-ccus aureus*	*Escherichia coli*	*Pseudomonas Aeruginosa*
1	Neat	60.00	_	_	_	_
2	1:2	30.00	_	_	_	_
3	1:4	15.00	_	_	+	+
4	1:8	7.50	+	_	++	++
5	1:16	3.75	++	_	++	++
6	1:32	1.88	++	_	++	++
7	1:64	0.94	++	_	++	++
8	1:128	0.47	++	+	++	++
9	1:256	0.23	++	++	++	++
10		3% DMSO	++	++	++	+++
MIC (mg/mL)			7.50	0.47	15.00	15.00
MBC (mg/mL)			15.00	0.94	30.00	30.00

Key: S/N= Serial number; CaONPs= calcium oxide nanoparticle; mg/mL= Milligram per millilitre; %= Percent; DMSO= Dimethylsulphide; ++= Dense growth; MIC= + Slight growth; MBC= - No growth

Table 4: Haemolytic safety studies of calcium oxide nanoparticles on red blood cells

Treatment	*Azadirachta indica* CaONP
Control RBC	0.00
RBC+Triton X-100	100.0±0.00
RBC+CaONPs 10µg/ mL	0.82±0.01
RBC+CaONPs 20µg/ mL	2.23±0.01
RBC+CaONPs 40µg/ mL	6.50±0.01
RBC+CaONPs 60µg/ mL	8.67±0.06
RBC+CaONPs 80µg/ mL	12.00±0.01
RBC+CaONPs 100µg/ mL	16.22±0.01
LC_{50} (µg/mL)	304.89

Key: Values represent mean standard deviation of triple determination; RBC-Red blood cells; CaONPs- calcium oxide nanoparticles; µg/mL-microgram per milliliter; LD_{50} (Lethal dose)-media; LC_{50} (Lethal concentration)>100= relatively not acutely toxic; LC_{50} 10-100= Minor acutely toxic; LC_{50} 1-10= Moderately acutely toxic; LC_{50}<1= Very toxic.

The results of the qualitative phytochemical profile of the methanolic leave extract of *Azadirachta indica* is presented in Table 1. The extract exhibited higher presence of tannins, saponins and phenolics, terpenoids, flavonoids, alkaloids and absence of anthraquinone, respectively. Similar observation was reported by Ujah *et al.* (2021) on their phytochemical screening of *Azadirachta indica* methanolic leave extract.

Figure 2 showed the wavelength (horizontal axis) and absorbance (vertical axis) in the UV spectral. It showed that *Azadirachta indica* biosynthesized calcium oxide nanoparticle had wavelength and absorbance values of 0.69 nm and 400, respectively. Mazher et al. (2023) in their publication reported that greenly synthesized nanoparticles had an average size of 35.93 ± 2.54 nm and showed an absorbance peak at 325 nm. An absorbance peak in this range depicts the coating of phenolic acids, flavones, flavonols, and flavonoids on the surface of CaONPs. The infra-red spectral results shown on Figure 2 showed that the wavenumber (on x-axis) and wave peak (on y-axis) are the same *Azadirachta indica* biosynthesized calcium oxide nanoparticle. The FTIR analysis of the CaONPs showed a coating of phytochemicals on their surface, due to which they showed great stability. The vibrations present at 3639 cm^{-1} for alcohols or phenols, 2860 cm^{-1} for carboxylic acids and aldehydes show adsorption of phytochemicals on the surface of CaONPs. The CaONPs for alkanes, 2487 cm^{-1} for alkynes, 1625 cm^{-1} for amines, and 1434 cm^{-1}. Figure 3 showed the chromatograph of the theta (horizontal) and peak of the electron count (vertical) of the x-ray diffractogram. The result showed high theta and peak in the *Azadirachta indica* biosynthesized calcium oxide nanoparticle. The working principle of the XRD method involves the scattering of X-rays due to the revolution of electrons in the atom's nucleus when the rays strike on the nanoparticles. The scattered X-rays are reflected in various directions, which cause interference patterns. These patterns are either destructive or constructive (Fultz and Howe, 2013) but only the scattered X-rays that undergo constructive interaction result in diffraction. Since nanoparticles have a large surface area-to-volume ratio, their properties are significantly altered with size, which makes the characterization an important step to understand their properties at different molecular levels. Other properties, such as texture, strain, shape anisotropy, crystalline phase, crystal defects, and crystal size impact the chemical, electronic, mechanical, and optical attributes of the nanoparticles. Without the proper characterization, the applicability of the specific nanoparticle would meet an immense challenge (Thanh *et al.,* 2014). The result of the scanning electron micrograph profile of *Azadirachta indica* biosynthesized calcium oxide nanoparticle is shown in Figure 4. From the result, it was observed that there were porous, layered, non – uniform and conglomerate particles and corroborated with finding of Koca et al (2020).

Table 2 displayed the antimicrobial activity of the green calcium oxide nanoparticles against tested bacterial pathogens. *Azadirachta indica* CaONP had the highest zone of inhibition of 20.00 20.00±0.39 mm against *Pseudomonas aeruginosa* while ciprofloxacin had the lowest zone of inhibition of 0.00±0.00 mm against *Staphylococcus aureus* and *Escherichia coli*, respectively. There was no inhibition recorded by dimethylsulphoxide against all the tested bacterial pathogens. This showed that, the biosynthesized silver nanoparticle is more potent to the inhibition of microbes compared to drugs as previously reported in the literature (Rai *et al*., 2014). Tables 3 showed the minimium inhibitory (MIC) and bactericidal concentration (MBC) of the *Azadirachta indica* aqueous leave extract mediated calcium oxide nanoparticles. *Azadirachta indica* CaONP recorded the lowest MIC and MBC values of 0.47 and 0.94 mg/mL against *Staphylococcus aureus,* respectively. Statistically, there was significant ($P < 0.05$) inhibition among the means of *Azadirachta indica* CaONP and ciprofloxacin treatment doses on the tested bacterial strains. The possible reason for these significant inhibitions by *Azadirachta indica* could be attributed to the adhesion of their larger surface area to the tested bacterial strains leading to inhibition of numerous physiological and biochemical processes such as disturbing cell-wall permeability and cellular respiration in the cell (Kim *et al*., 2012). These observations are consistent with previous reports that have demonstrated the potential of CaNPs as antimicrobial agent (Mazher et al. 2023; Uba et al., 2024; Elee et al., 2024).

Table 4 showed the haemolytic safety studies of calcium oxide nanoparticles on red blood cells. The results demonstrated that *Azadirachta indica* CaONPs recorded the high LC_{50} value of 304.89 µg/mL. The percentage haemolytic activity was dose dependent ($P < 0.05$). The results revealed that biosynthesized calcium nanoparticles are safe for human usage at higher doses as LC_{50} (median lethal concentration) >100 are relatively not acutely toxic (Elee et al. 2024).

Conclusion

The whole study revealed that the *Azadirachta indica* aqueous leave extracts contained significant phytochemical components. They were potential producers of calcium oxide nanoparticles as elucidated by UV-visible spectroscopy, XRD, SEM and FTIR studies. The biosynthesized calcium oxide nanoparticle exhibited antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus and Staphylococcus aureus*; based on its haematological assay, calcium oxide nanoparticle synthesized using *Azadirachta indica* is non-toxic to human since it didn't affect the red blood cell. This green inexpensive and simple method can be used as alternative to other mediated methods used for production of calcium oxide nanoparticles. Future should focus on exploring the production of calcium oxide nanoparticle using other plant materials.

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