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Original Article

BIOSYNTHESIS OF COPPER OXIDE NANOPARTICLES AND EVALUATION OF THEIR ANTIMICROBIAL PROPERTIES

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ABSTRACT

Objective: This research was carried out to synthesize and characterize copper oxide nanoparticles (CuONPs) using *Vernonia amygdalina* leaf extract and investigate the *in vitro* antimicrobial properties using clinical microbial isolates.

Methods: The CuONPs were synthesized by heating a mixture of copper sulfate pentahydrate and *V. amygdalina* aqueous extract. The CuONPs were characterized by Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and particle size analysis. Phytochemical analysis of *V. amygdalina* was carried out to determine the bio-molecules that served as a reducing agent during the synthesis of CuONPs. The antimicrobial activities of CuONPs and *V. amygdalina* were evaluated by the agar disc diffusion method against *Staphylococcus aureus*, *Escherichia coli,* and *Candida albicans*. Ampicillin and fluconazole were used as reference antibacterial and antifungal agents, respectively.

Results: The nanoparticles were in the nanometer dimension and exhibited significant antimicrobial activity (P<0.05) against the tested microbes. However, the standard antibacterial drug, ampicillin, showed higher antibacterial activity against *S. aureus* and *E. coli* with the inhibition zone diameter of (IZD) of 13.10±0.38 mm and 11.80±0.12 mm, respectively. Fluconazole had no antifungal activity against *C. albicans* while *V. amygdalina* demonstrated good antibacterial activity against *S. aureus* and *E. coli* but lacked antifungal activity against *C. albicans*. However, the combination of CuONPs and plant extract exhibited significant antifungal activity with an IZD of 10.37±0.72 mm.

Conclusion: An eco-friendly, simple, reproducible, and economical CuONPs have been synthesized using *V. amygdalina* leaf extract. The findings indicate that CuONPs could be used as an antimicrobial agent.

Keywords: Nanoparticles, Green synthesis, *Vernonia amygdalina*, Plant extract, Antibacterial, Antifungal

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INTRODUCTION

Nanotechnology can be applied in several areas of medicine such as the treatment of disease, diagnosis of disease, and drug delivery systems for potentially toxic and ineffective drugs. Nanoparticles, which are products of nanotechnology, are in the nanometer range and they exhibit improved physical, chemical, and biological activities because of their increased surface area. Generally, the synthesis of nanoparticles involves three methods, namely: chemical methods, physical methods, and biosynthesis [1]. Biosynthesis or green synthesis employs an environment-friendly, cost-effective, low-toxic, and simple procedure to synthesize nanoparticles. Green synthesis has the edge over chemical and physical methods because it does not utilize toxic chemicals, high pressure, temperature, and energy.

Treatment of microbial infections is becoming a challenge due to the resistance exhibited by several antimicrobial agents. Copper oxide nanoparticles are known to exert antibacterial activities [2]. Copper oxide nanoparticles are potential antimicrobial agents because when they are synthesized, they have the high surface area and uncommon crystal morphologies [3].

Vernonia amygdalina (VA) is a shrub having a petiolate leaf of about 6 mm in diameter and a height of about 1 to 10 m. scientifically, it belongs to the family *Compositae* or *Asteraceae* and the genus *Vernonia*. It is the most commonly planted shrub among the *Vernonia* genus. In English, it is called bitter leaf because of its bitter taste. The bitter taste could be reduced by either boiling or washing it several times in water. Its bitter taste is due to the presence of its anti-nutritional constituents, such as saponins, alkaloids, tannins, and glycosides [4]. The VA is known by different native names in Nigeria, such as "Onugbu" in the Igbo language, "Fatefate or Chusar doki" in the Hausa language, and Ewuro" in the Yoruba language. It is found predominantly in tropical Africa, such as Nigeria, South Africa, and Zimbabwe [4]. Traditionally, VA is used as food in the famous bitter leaf soup by abating the bitter taste through boiling or washing in water. The VA is also included in herbal remedies in the treatment of different illnesses such as malaria, parasite infestation, diabetes mellitus, and microbial infections.

The objective of this work is to evaluate the antimicrobial activities of copper oxide nanoparticles (CuONPs) and *V. amygdalina*, separately and in combination, against *Staphylococcus aureus*, *Escherichia coli,* and *Candida albicans*. This is the first time that the antimicrobial activities of the combination of CuONPs synthesized using *V. amygdalina* with plant extract, ampicillin, and fluconazole were determined.

MATERIALS AND METHODS

Collection and identification of plant material

The *Vernonia amygdalina* leaves were collected in Benin City, Nigeria. The plant leaves were identified by Dr. Akinnibosun Henry Adewole, a plant ecologist and taxonomist in the Department of Plant Biology and Biotechnology, Faculty of Life Science, University of Benin. A voucher specimen was deposited in the University of Benin Herbarium and Voucher Number: UBH_v342 was assigned to the plant sample.

Materials

Analytical grade copper sulfate pentahydrate ($CuSO_4.5H_2O$) was obtained from Merck Company (Merck, Germany), ampicillin (Jigs Chemical Limited, India), fluconazole (Globela Pharma Private Limited, India). All other reagents used in this study were of analytical grade.

Preparation of the plant extract

The fresh leaves of *Vernonia amygdalina* were washed with tap water and then with distilled water to remove dust particles. They

were dried in a shaded place at room temperature (25-30 °C). The leaves were pulverized using a commercial blender and sieved in a 0.6 mm mesh sieve. Eight-gram weight of the dried leaf powder was mixed with 100 ml of distilled water in a conical flask. It was boiled in a water bath for ten minutes at 100 °C. The mixture was cooled and filtered through Whatman No. 1 filter paper. The color of the mixture was noted. The filtrate was refrigerated (10 °C) and used for further experimental procedures.

Phytochemical analysis of *Vernonia amygdalina aqueous extract (VAE)*

A. Test for saponins

Five milliliters of distilled water was added to 0.5 g of leaf extract in a test tube. The test tube was stopped and shaken vigorously. It was observed for the presence of froth or foam. The foam was mixed with three drops of olive oil which was shaken vigorously and observed for emulsion [5].

B. Test for flavonoids

Two drops of 1% aluminum solution were added to an aliquot portion of the filtrate. A yellow coloration showed the presence of flavonoids [5].

C. Test for starch

Ten cubic centimeters of the filtrate were added to a test tube. Five drops of iodine solution were added to the test tube. The color change was recorded [5].

D. Test for phenol

Two milliliters of the filtrate were added to a test tube. Two drops of 5% Ferric chloride solution ($FeCl_3$) were added to the test tube. A blue coloration indicated the presence of phenol [6].

E. Test for glycosides

A small portion of the extract (0.05 g) was hydrolyzed with concentrated hydrochloric acid for two hours in a water bath. It was filtered. Subsequently, the hydrolysate was subjected to Borntrager's test.

Borntrager's test

Three milliliters of chloroform were added to 2 ml of the filtered hydrolysate and the mixture was shaken. The chloroform layer was separated and 10% ammonia solution was then added to it. A pink coloration showed the presence of glycosides [7].

F. Test for terpenoids (Salkowski test)

Two milliliters of chloroform were used to dissolve 0.5 g of the extract. Subsequently, 3 ml of concentrated H_2SO_4 were carefully added to form a layer. The presence of terpenoids could be indicated if there is a reddish-brown coloration of the interface [5].

G. Test for tannins

The extract (0.5 g) was boiled in 10 ml of water using a test tube. It was filtered. A few drops of 0.1% ferric chloride were added to the filtrate. It was observed for a blue-black or brownish-green coloration [5].

H. Test for alkaloids

The leaf extract (0.5 g) was made up to 10 ml with acid alcohol, boiled, and filtered. Dilute ammonia (2 ml) was added to 5 ml of the filtrate. Five milliliters of chloroform were then added and shaken gently so as to extract the alkaloidal base. Ten milliliters of acetic acid were used to extract the chloroform layer. This was divided into two parts. To the first part, Mayer's reagent was added, while draggendorff's reagent was added to the other part. The formation of a cream precipitate with Mayer's reagent and a reddish-brown precipitate with draggendorff's reagent indicated the presence of alkaloids [5].

Synthesis of copper oxide nanoparticles (CuONPs)

Aqueous *Vernonia amygdalina* leaf extract (20 ml) was added to 80 ml of 0.01 M $CuSO_4.5H_2O$. It was mixed well by shaking and stirring vigorously for 5 min. The suspension was heated in a water bath at 60 °C for 30 min. The suspension was then removed from the water bath. Two control experiments were carried out; the first was carried out with only 0.01 M $CuSO_4.5H_2O$, while the second was performed with only *V. amygdalina* filtrate.

Characterization of nanoparticles

The synthesized copper oxide nanoparticles were characterized using the following analytical techniques:

Fourier-transform Infrared Spectroscopy (FTIR)

The functional groups of the copper oxide nanoparticles were characterized using an FTIR spectrophotometer (Spectrum BX, PerkinElmer, England). The potassium bromide (KBr) method was used during the FTIR analysis. A hundred mg of dried KBr salt and 5 mg of the sample (CuONPs) were weighed. The sample was mixed uniformly with the dry KBr powder. The sample was placed in an evacuable KBr die and a 13 mm clear disk was pressed in a hydraulic press to form a KBr pellet. After placing the pelletized sample (which was formed inside an evacuable chamber) in a cell holder (demountable universe cell), it was inserted into the FTIR spectrophotometer (Spectrum BX, PerkinElmer, England) and scanned at a range of 800 to 4000 cm^{-1}.

Particle size analysis

The size of the copper oxide nanoparticles was measured using a Zetasizer Nano (Malvern Instruments Ltd, United Kingdom). The size measurement was performed in a disposable polystyrene cuvette. The sample was inserted into the cell (cuvette). After filling the cell, it was inserted into the instrument and the measurement was taken.

Scanning electron microscopy (SEM)

The morphological features of the CuONPs were determined using the JEOL 5800LV scanning electron microscope. The sample was coated in a conductive material such as gold in a process known as sputter coating before it was available for viewing. The sputter coating lets a sample be grounded, restricting the sample from being destroyed by the electron beam. The specimen inserting tool was used to load the sample into the chamber and the scanning was determined.

Collection of microorganisms

Clinical isolates (*Staphylococcus aureus, Escherichia coli,* and *Candida albicans*) were collected from the Microbiology Laboratory, University of Benin Teaching Hospital, Benin City, Edo State, Nigeria. They were identified in the Mycofarms laboratory, Isiohor, Benin City, using Gram staining and specific biochemical tests.

Gram staining of the organisms

The wire loop was sterilized by flaming and was allowed to air cool. A drop of sterile water was placed on a glass slide. A loopful of the culture of the organism in the nutrient agar slant labeled *S. aureus* was mixed with the sterile water in the glass slide to form the smear. The smeared glass slide was allowed to dry. Thereafter, the smeared glass slide was heat fixed and treated with crystal violet and iodine solution. The smear was counterstained with safranin after rinsing with water and alcohol and allowed to air dry. A drop of oil immersion was put on the smear and viewed with the microscope using the oil-immersion objective lens. The Gram character of the organism in the nutrient agar slant labeled *E. coli* was also ascertained following the above procedure.

Biochemical tests for the identification of microorganisms

Catalase test

A syringe was used to collect a drop of hydrogen peroxide which was placed on the glass slide. A wire loop was sterilized by heating in the flame and was allowed to air cool. The sterile wire loop was used in collecting a small portion of the culture of the test organism from the nutrient agar slant labelled *S. aureus*. The culture of the test organism was placed in the glass slide containing the hydrogen

peroxide and mixed. The above method was used in carrying out the catalase test for the other organism in the nutrient agar slant labeled *E. coli*. The result was recorded as either effervescence (+) or no effervescence (-).

Coagulase test

A drop of distilled water was placed on a clean slide using a Pasteur pipette. The wire loop was flamed; a colony of the test organism from the nutrient agar slant was lifted into the distilled water in the slide. It was emulsified in the drop of distilled water on the slide. The wire loop was flamed and a loopful of undiluted human plasma was added. It was mixed by tilting the slide to and fro. The result was recorded as either clumping (+) or no clumping (-).

Indole test

A loopful of the culture of *E. coli* was inoculated in a test tube containing 3 ml of sterile peptone water. It was incubated at 37 °C for up to forty-eight hours. A 0.5 ml volume of Kovac's reagent was added to the inoculated peptone water and shaken gently. It was examined for a red color in the surface layer within ten minutes. The result was recorded.

Lactophenol cotton blue mount

A drop of lactophenol cotton blue was placed on a clean slide. The wire loop was sterilized by heating in the flame to redness and cooling in air. A small portion of the colony of the *C. albicans* in the inoculated potato dextrose agar slant was transferred to the drop of lactophenol cotton blue on the slide using a sterile wire loop. Using a sterile wire loop, the organism was emulsified in the lactophenol cotton blue drop on the slide. A clean cover slip was placed gently on the preparation. The preparation was examined using x40 objective lens. The observations were recorded.

Germ tube test

Using a sterile Pasteur pipette, 0.5 ml of human serum was transferred into a test tube. A pinpoint portion of growth from the culture of *C. albicans* was collected using a sterile cooled wire loop. The collected culture of *C. albicans* was emulsified with the serum in the test tube. It was incubated at 37 °C for three hours. A loopful of the suspension was transferred onto a clean slide. The preparation was covered with a cover slip and examined under x40 objective lens. The observation was recorded.

Dilution of CuONPs and plant extract

Twenty percent of dimethyl sulphoxide (DMSO) was prepared by adding 20 ml of 100% DMSO in a 100 ml beaker and adding sterile water up to the 100 ml mark. Four beakers were autoclaved. Each of the four beakers was labeled as follows: 20 mg CuONPs, 10 mg CuONPs, 5 mg CuONPs, and 2.5 mg CuONPs. Five milliliters of 20% DMSO was added into each of the above four beakers. Zero point one gram (0.1 g), 0.05, 0.025, and 0.0125 g of CuONPs were added into each of the four beakers respectively and shaken to get the following concentrations of CuONPs: 20, 10, 5, and 2.5 mg, respectively. Sterile discs were soaked into each of the four beakers containing CuONPs suspension. The beakers were covered with foil and left for 24 h. The above procedure was used when preparing the different concentrations of the plant extract.

Evaluation of antimicrobial activities

The antimicrobial activities were evaluated on clinical isolates (*Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*) by using agar disc diffusion method. *S. aureus* represented Gram-positive bacteria, while *E. coli* represented Gram-negative bacteria. *C. albicans* was a representative of the fungi. For positive control, ampicillin was used as a reference antibacterial agent, while fluconazole was used as a reference antifungal agent. Mueller Hinton agar was used to subculture bacteria. The clinical isolates were adjusted to 0.5 McFarland standard turbidity. The fresh overnight standardized broth cultures (0.1 ml) were spread on the Mueller Hinton agar plates using a sterile spreader to cultivate bacteria. Sterile paper discs of 0.5 cm (5 mm) saturated with synthesized copper oxide nanoparticles (CuONPs) and plant extract were placed in each mueller hinton agar plates and incubated at 37 °C for 24 h. The inhibition zone diameter around the disc impregnated with synthesized CuONPs and plant extract was measured in millimeters to determine antibacterial activity.

For the antifungal studies, potato dextrose agar was used to subculture *C. albicans*. Fresh overnight standardized broth cultures of *C. albicans* (0.1 ml) were spread on the potato dextrose agar plates to cultivate fungi. Sterile paper discs of 0.5 cm saturated with synthesized CuONPs and *V. amygdalina* leaf extract were placed in each of the potato dextrose agar plates and incubated at 37 °C for 24 h. The inhibition zone diameter was measured in millimeters.

The antimicrobial evaluation of the combination of CuONPs with ampicillin, fluconazole and *V. amygdalina* leaf extract was determined using the above culture media for subculturing bacteria and fungi. The above procedure was used. Sterile water (5 ml) was added to 12.5 mg of Ampicillin to get a concentration of 2.5 mg/ml. Fluconazole (2.5 mg/ml) was also prepared using sterile water.

Statistical analysis

All statistical analyses were conducted using the computer software, Statistical Package for the Social Sciences (SPSS). Three analytical replicates were performed on each sample. The results were averaged. The results were given as mean±standard error of the mean (SEM). The data were analyzed by one-way analysis of variance (ANOVA). $P<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Phytochemical analysis of *V. amygdalina* aqueous leaf extract (VAE)

The phytochemical analysis was carried out to determine the biometabolites that were present in the leaf extract, which might have served as reducing agents during the synthesis of the copper oxide nanoparticles. In a study, the different plant biometabolites that could aid in the synthesis of nanoparticles based on their role as reducing and capping agents were reported [1]. The reducing agents include the following: terpenoids, tartaric acid, tannic acid, amino acids, sesquiterpenes, saponin, secondary metabolites, reducing sugars, citric acid, flavonoids, quercetin, protein, peptide, phenolics, hydrogenase, heterocyclic compounds and functional groups (amines, alcohols, ketones, sulfhydryl, and carboxyl acids). On the other hand, the capping agents are tannic acid, pralines, tartaric acid, peptides, extracellular proteins, functional groups (aldehydes, carboxylic acid, alcohols, ketones, sulfhydryl, and amines), enzymes and citric acid. The biometabolites present in the VAE are shown in table 1. In this study, the phytochemical analysis showed the presence of saponin, flavonoid, phenol, glycoside, terpenoid, and alkaloid and the absence of starch and tannin. From table 1, it can be inferred that the saponin, flavonoid, terpenoid, and phenol present in the leaf extract served as the reducing agents during the synthesis of the CuONPs using *V. amygdalina* leaf extract.

Table 1: Phytochemical constituents of *V. amygdalina* aqueous leaf extract

Phytochemical constituent	*Vernonia amygdalina*
Saponin	+
Flavonoid	+
Starch	-
Phenol	+
Glycoside	+
Terpenoid	+
Tannin	-
Alkaloid	+

Key: +signifies present, — signifies absent

Observation during the synthesis of copper oxide nanoparticles (CuONPs)

The *V. amygdalina* filtrate was brown, while the 0.01 M $CuSO_4.5H_2O$ was colorless. As soon as the *V. amygdalina* filtrate was added to the 0.01 M $CuSO_4.5H_2O$, the color changed to dark green. During the

heating of the mixture of the copper salt and plant extract, a dark brown precipitate was observed. The gradual change of color from brown to dark green to dark brown showed the formation and stabilization of the copper oxide nanoparticles (CuONPs). The copper oxide nanoparticles appeared dark brown after freeze-drying. The control experiments did not show any evidence of the formation of CuONPs because there was no color change.

Fourier-transform infrared spectroscopy (FTIR)

In the present study, the FTIR spectrum (fig. 1) showed the functional groups that were present in the CuONPs synthesized using *V. amygdalina* leaf extract. Peaks were observed at 3425 cm^{-1} for the hydroxy (OH) group (alcohol); 2392.5 $cm^{-1}$for the OH group (acids), 2092 cm^{-1} for alkyne (C≡C), 1642.95 $cm^{-1}$for alkene (C=C), 1289 $cm^{-1}$for strong C-O and 423.5 cm^{-1} is located at the fingerprint region for various functional group [8, 9]. This result also shows the biomolecules present in the *V. amygdalina* leaf extract that were responsible for the stabilization of the CuONPs.

The organic functional groups found in the synthesized CuONPs indicate the interaction between copper sulfate pentahydrate and *Vernonia amygdalina* leaf extract. The reduction of copper ions to metallic copper arose as a result of the strong affinity of the phytochemicals in *V. amygdalina* towards the copper ions to donate free electrons.

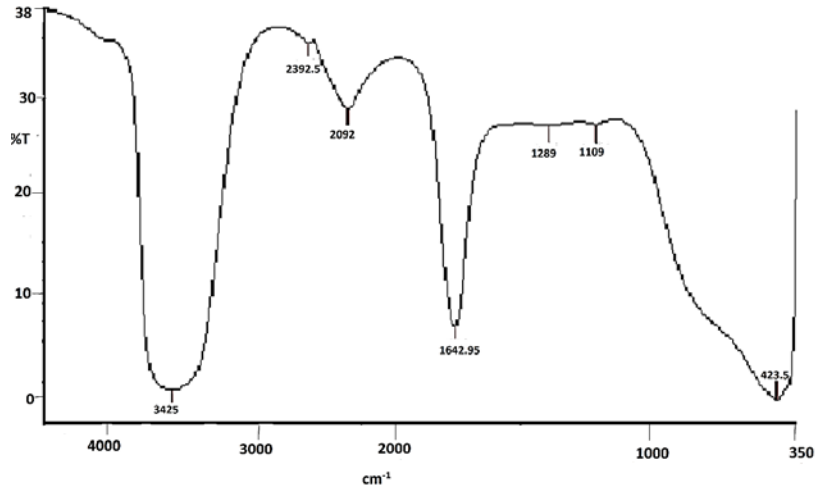

Fig. 1: FTIR spectrum of the synthesized copper oxide nanoparticles

Particle size analysis

From fig. 2, the particle size of the synthesized Copper oxide nanoparticles was highly heterogeneous. The heterogeneous size of the nanoparticles showed the inability of the phytochemicals to effectively stabilize the nanoparticles. A study reported that a drawback of nanoparticles was their tendency to aggregate upon lyophilization [10]. It is established that the disadvantages of silver nanoparticles, such as quick aggregation and instability, prevent its usage [11].

The polydispersity index (PDI) of the synthesized copper oxide nanoparticles was 0.615. The size distribution of nanoparticles could be expressed through the polydispersity index value. Nanoparticle with a PDI value less than 0.1 is regarded to be highly monodisperse (homogenous), while a PDI value in the range of 0.1 to 0.4 is considered to moderately disperse distribution and a PDI value greater than 0.4 is regarded to be highly polydisperse (heterogenous) distribution [11]. The synthesized copper oxide nanoparticles were highly polydisperse and, therefore, unstable. The appropriate formulation of a nanocarrier, such as nanoparticles for a specific route of drug administration relies on their mean diameter, size stability, and PDI [12]. Regulatory agencies such as Food and Drug Administration (FDA) have not stated the criteria for an acceptable PDI range for different routes of administration. There is, therefore, a need for regulatory agencies to mention the guidelines for the formulation of the different routes of drug administration based on their PDI range. Copper poisoning is known to cause different toxicities such as cancer, diabetes, heart attacks, anemia, and kidney disorders [13]. Considering the toxicity of copper, it would be safer for the synthesized copper oxide nanoparticles to be administered topically.

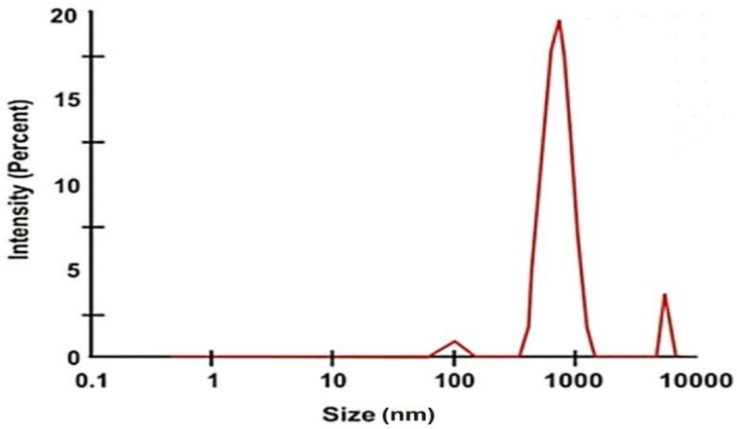

Fig. 2: Particle size analysis image of the synthesized CuONPs

Scanning electron microscopy (SEM)

The morphological features of the CuONPs were determined using SEM analysis. The SEM image revealed copper oxide nanoparticles with different morphology. Fig. 3 shows copper oxide nanoparticles with leaf-like and undefined shape morphology. The nanoparticles were highly polydisperse. The majority of the copper oxide nanoparticles were available in the agglomerated form. Hence, the SEM image confirmed the nanoparticle characteristic of the synthesized copper oxide nanoparticles. The result conforms with previous reports in which some nanoparticles showed undefined shapes while most were present in the agglomerated form but with some differences as a result of varying chemical constituents [2, 3].

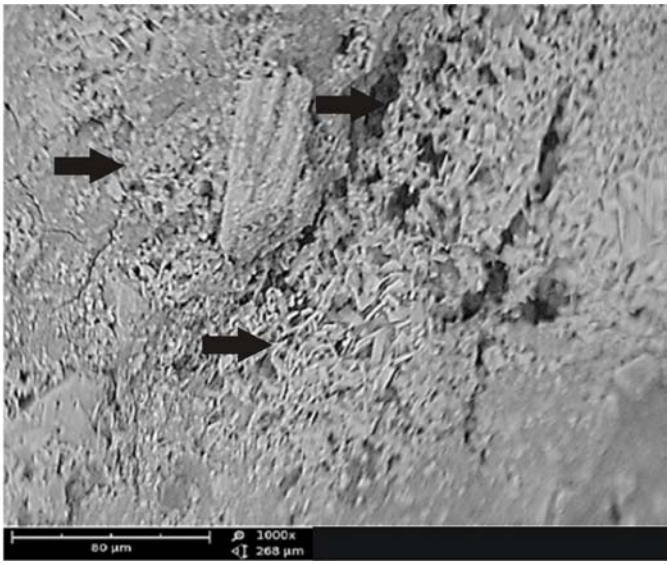

Fig. 3: SEM image of the synthesized copper oxide nanoparticles, the scale bar corresponds to 80 µm

Antimicrobial studies

The three organisms used in this study were *Staphylococcus aureus, Escherichia coli,* and *Candida albicans*. The gram staining alone was not enough to identify the presence of *Staphylococcus aureus* (*S. aureus*). This is because *Enterococcus*, *Streptococcus*, and *Lactococcus* species are also Gram-positive cocci. As a result of this, specific biochemical tests (catalase and coagulase tests) were carried out for the identification and confirmation of *S. aureus.* Similarly, the Gram staining was also insufficient to identify the presence of *E. coli*. This is because other members of the *Enterobacteriaceae,* such as *Klebsiella pneumonia* and *Pseudomonas aeruginosa* are also Gram-negative bacteria having rod-shaped (bacilli). The indole test was used to confirm the presence of *E. coli*. For the *Candida albicans*, it was confirmed by the lactophenol cotton blue mount and germ tube test. From table 2, it was certain that the three organisms used in this study were *Staphylococcus aureus, Escherichia coli,* and *Candida albicans*.

Table 2: Results of specific biochemical tests

Organism	Test	Observation	Inference
S. aureus	Catalase test	Presence of Effervescence	*S. aureus* is identified
S. aureus	Coagulase test	Presence of clotting	*S. aureus* is identified
E. coli	Indole test	Presence of red surface area	*E. coli* is identified
C. albicans	Lactophenol cotton blue mount	Budding cells were observed	*C. albicans* is identified
C. albicans	Germ tube test	Germ tube formation	*C. albicans* is identified

Antimicrobial activity of the copper oxide nanoparticles (CuONPs)

The inhibition zone diameter of CuONPs against the test organisms is shown in table 3. The 20 mg CuONPs exhibited marked antimicrobial activity against all the test organisms. The 10 mg CuONPs exhibited marked antimicrobial activity against *S. aureus* and *c. albicans* but minimal antibacterial activity against *E. coli*. In the case of the 5 mg CuONPs, there was minimal antimicrobial activity against *s. aureus* and *e. coli* but a marked antimicrobial activity against *C. albicans*. The 2.5 mg CuONPs did not show any antimicrobial activity against *s. aureus,* but it exhibited minimal antimicrobial activity against *E. coli* and marked antimicrobial activity against *c. albicans*.

The synthesized CuONPs showed antibacterial activity on both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. This conforms with a previous report [14]. The nanoparticles also exhibited antifungal activity against *C. albicans*. There is no established mechanism for the antibacterial action of CuONPs. It could be possible that CuONPs were able to disrupt the cell enzyme function [15]. Copper ions might have competed with non-copper metal ions for essential binding sites on proteins which led to decreased cellular functions [16]. The size of nanoparticles plays an essential role in evaluating antimicrobial activities. It is established that the efficiency of antimicrobial activities of nanoparticles increases with a reduction in the size of nanoparticles [16]. This is due to the large surface area of nanoparticles that is made available for interaction with microorganisms. The synthesized CuONPs enhanced antimicrobial activity because of the reduced size of CuONPs that interacted with the test microorganisms. The synthesized CuONPs tend to carry out their killing effect by introducing reactive hydroxyl radicals, which caused irrespirable destruction such as the separation of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) molecules, oxidation of proteins, and mutilation of the cell membrane as a result of lipid peroxidation [2].

The mechanism by which copper materials act to kill fungi is not well understood [17]. The proposed mechanism of antifungal

activity of synthesized CuONPs could be that CuONPs might have induced stress on *C. albicans* which led to the endogenous generation of reactive oxygen species (ROS) and subsequently caused damage to the cell membrane of *C. albicans* [18]. It might also be possible that the synthesized CuONPs inhibited ergosterol biosynthesis in *C. albicans* thus leading to the death of the fungi. This is because ergosterol is an essential component of the fungal cell membrane and functions to maintain its growth, development, permeability, and stability [19]. Any agent that affects ergosterol could lead to the death of the fungi.

Table 3: Inhibition zone diameter (mm) of CuONPs against test isolates

Microorganisms	CuONPs (mg)				Amp. (2.5 mg/ml)	Flu (2.5 mg/ml)
	20	10	5	2.5		
S. aureus	8.66±0.88	7.50±1.00	6.50±0.00	0.00±0.00	13.10±0.38	-
E. coli	7.83±0.16	6.50±0.00	6.50±0.00	6.50±0.00	11.80±0.12	-
C. albicans	9.50±0.76	9.00±1.04	8.33±1.58	8.00±0.76	-	0.00±0.00

Mean±SEM (n = 3), CuONPs = copper oxide nanoparticles, Amp= ampicillin, Flu= fluconazole

Antimicrobial activity of the vernonia amygdalina aqueous leaf extract (VAE)

From table 4, the four different concentrations of VAE did not show any activity against the fungi, *Candida albicans*. This is in agreement with a previous report in which the aqueous, ethanolic, and methanolic extracts of *Vernonia amygdalina* did not elicit inhibitory activity against *c. albicans* [20]. On the other hand, the result contradicted a study in which it was found that methanol, n-hexane, and ethylacetate crude extracts of leaves of *v. amygdalina* elicited antifungal activity against *Candida albicans* [21]. This might be a result of bioactive substances in the leaves of *v. amygdalina* that have antifungal potential against *c. albicans*.

The four different concentrations of *V. amygdalina* showed antibacterial activity against *S. aureus* and *E. coli*. This is in conformity with previous studies in which *V. amygdalina* exerted antibacterial activity against *S. aureus* and *E. coli* [22-24]. *Vernonia amygdalina* has a broad-spectrum antibacterial activity because it exhibited antibacterial activity against gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria.

Vernonia amygdalina might have elicited its antibacterial activity against the tested bacteria by disrupting their cell wall thereby inhibiting the growth of or killing the bacteria. This is justified in a report in which it was stated that the effects of *V. amygdalina* extracts on the organism are suggestive of its activity against cell wall components of the organism [25]. *Vernonia amygdalina* could have disrupted the peptidoglycan of *S. aureus* because peptidoglycan is the main component of the cell wall of a Gram-positive organism. It might also be possible that *V. amygdalina* disrupted the lipopolysaccharide of Gram-negative (*E. coli*) bacteria. This is because lipopolysaccharide, which is commonly known as endotoxin, is a vital component of Gram–negative bacteria and is important for bacterial cell integrity and defense against environmental stress [26]. Any agent that disrupts the lipopolysaccharide of Gram-negative bacteria could either inhibit the growth of or kill the bacteria. The phytochemicals in the leaves of *V. amygdalina* were responsible for its antibacterial activities.

It was observed that the reference antibacterial drug, ampicillin, possessed a higher antibacterial activity than *V. amygdalina*. If the active component of *V. amygdalina* is isolated, it might have a higher antibacterial activity than the reference drug. *Vernonia amygdalina* could be used as a safer antibacterial agent than conventional antibiotics when treating bacterial infections. This is because conventional antibiotics are known to possess several adverse effects [27].

Table 4: Inhibition zone diameter (mm) of *Vernonia amygdalina* aqueous extract (VAE) against test isolates

Microorganisms	VA (mg)				Amp. (2.5 mg/ml)	Flu. (2.5 mg/ml)
	20	10	5	2.5		
Staph. aureus	10.67±2.81	8.25±0.90	8.16±0.33	6.50±0.00	13.10±0.38	-
E. coli	7.16±0.66	7.08±0.22	6.66±0.16	6.75±0.14	11.80±0.12	-
C. albicans	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-	0.00±0.00

Mean±SEM (n = 3), VA = *Vernonia amygdalina*, Amp= ampicillin, Flu= fluconazole

Antimicrobial activity of the combination of CuONPs with *Vernonia amygdalina*, ampicillin, and fluconazole against test isolates

In this study, the antimicrobial activities of the combination of 20 mg CuONPs with 20 mg *Vernonia amygdalina,* 2.5 mg Ampicillin and 2.5 mg Fluconazole were evaluated. The combined effect of CuONPs and *V. amygdalina* against *S. aureus* was antagonistic. This is because there was a decrease in the inhibition zone diameter when CuONPs and *V. Amygdalina* were tested against *S. aureus* (table 5) compared to when only *V. amygdalina* was tested against *S. aureus* (table 4). Antagonism is when the effect of a drug or active ingredient is decreased or masked by another drug or other compounds in a complex mixture [28, 29]. The mechanism of the antagonistic effect of the combination of CuONPs and *V. amygdalina* against *S. aureus* is not too clear. This is because of the different constituents or phytochemicals that are present in the plant extract. It would be difficult to identify the particular phytochemical that is responsible for this antagonism. It might be possible that CuONPs inhibited the antibacterial activity of *V. amygdalina* against *S. aureus*. The combination of CuONPs and *V. amygdalina* should not be used in the treatment of bacterial infections caused byGram–positive (*S. aureus*) bacteria.

When CuONPs were combined with *V. amygdalina* and tested against *E. coli*, there was a slight increase in the inhibition zone diameter. The interaction of CuONPs and *V. amygdalina* against *E. coli* was synergistic. This is in conformity with the previous study but with little modification in which copper oxide nanoparticles were combined with cephalexin and tested against *E. coli* led to a synergistic effect [30]. CuONPs and *V. amygdalina* might have acted through different mechanisms that led to the increased inhibition zone diameter when they were tested against *E. coli*. It might be possible that both combinations (CuONPs and *V. amygdalina*) led to bacterial cell membrane permeability of *E. coli* which enabled the leakage of proteins and this led to the destruction of the cell membrane.

During the combination study of CuONPs and *V. amygdalina* against *C. albicans*, there was a significant synergistic effect (table 5). From table 4, it would be seen that aqueous extract of *V. amygdalina* had no antifungal activity against *C. albicans.* From table 3, the inhibition zone diameter of CuONPs against *C. albicans* was 9.50±0.76 mm. interestingly, after the combination of CuONPs and *V. amygdalina* against *C. albicans*, the inhibition zone diameter was 10.37±0.72 mm (table 5). The mechanism of this synergistic activity of CuONPs and

V. amygdalina against *C. albicans* might not be fully explained because of the several phytochemicals present in *V. amygdalina*. It might be possible that V. amygdalina contained phytochemical(s) that potentiated the antifungal action of CuONPs against *C. albicans*.

The combined effect of CuONPs and ampicillin against *S. aureus* and *E. coli* was antagonistic (table 5). This is because there was a decrease in the inhibition zone diameter after the combination study compared to when ampicillin was used alone (table 3). This result is contrary to the previous study in which a combination of CuONPs with ampicillin led to a synergistic effect against *E. coli* and *S. aureus* [31]. From the same study, the copper nanoparticles used were synthesized using a chemical method [31], while in the present study, the CuONPs used were synthesized using biosynthesis. It might be possible that the type of synthesis of nanoparticles could determine the type of combined effect of nanoparticles with an antibacterial agent whether it is antagonistic or synergistic. Ampicillin is a member of aminopenicillins, which exert bactericidal activity. It is known that combination therapy using a bactericidal antibiotic and bacteriostatic drug would lead to antagonism [32]. From this work (table 5), it can be hypothesized that ampicillin exerted bactericidal activity against tested bacteria (*S. aureus* and *E. coli*) while CuONPs exerted bacteriostatic activity against *S. aureus* and *E. coli*. The combination of ampicillin, a bactericidal agent, and copper oxide nanoparticles, proposed bacteriostatic agents, led to an antagonistic effect. The combination therapy of CuONPs synthesized using *V. amygdalina* and ampicillin should be avoided.

The combined effect of CuONPs and fluconazole was synergistic (table 5). This is because when fluconazole was used against *C. albicans* (table 3), there was no inhibition zone diameter (0.00±0.00 mm) but after the combination study (table 5), there was an increased inhibition zone diameter (11.29±0.57 mm). The result justified the previous report though with some changes in which *C. albicans* was resistant to fluconazole but with the combination of silver nanoparticles and fluconazole; there was a remarkable reduction in fluconazole minimum inhibitory concentration (MIC) for *C. albicans,* thus increasing the antifungal effect [33]. In a study, it was reported that the resistance of *C. albicans* to fluconazole was due to overexpression of genes that encode efflux pumps [33]. It might be proposed that CuONPs could cause disruption of fungal cell walls and allow increased permeability of cell membrane, which led to the entrance of fluconazole that interfered with the synthesis of ergosterol thereby leading to the death of the fungi. It would be advocated for the future combination therapy of copper oxide nanoparticles and fluconazole against fungal infections caused by *C. albicans* because of the promising synergistic effect against *C. albicans*.

Table 5: Inhibition zone diameter (mm) of the combination of CuONPs with *vernonia amygdalina* (VA), ampicillin (AMP), and fluconazole (FLU) against test isolates

Microorganisms	CuONPs 20 mg+VA 20 mg	CuONPs 20 mg+AMP 2.5 mg	CuONPs 20 mg+FLU 2.5 mg
Staph. aureus	8.08±0.83	10.00±1.00	9.80±0.16
E. coli	7.58±0.36	8.17±0.28	10.67±0.33
C. albicans	10.37±0.72	13.00±1.56	11.29±0.57

Mean±SEM (n = 3), CuONPs = copper oxide nanoparticles, VA = *Vernonia amygdalina*, Amp= ampicillin, Flu= fluconazole

CONCLUSION

An eco-friendly, simple, reproducible, and cost-effective copper oxide nanoparticles were synthesized using *Vernonia amygdalina* leaf extract. The biometabolites present in *V. amygdalina* served as the reducing and stabilizing agents during the synthesis of the CuONPs. The CuONPs possessed significant antibacterial activities though the standard drug, ampicillin, had a higher antibacterial activity. The findings showed that *V. amygdalina* possessed effective antibacterial activity but was devoid of antifungal activity. It was evident that *V. amygdalina* could be used in the treatment of bacterial infections. The combination of CuONPs with *V. amygdalina* led to antifungal activity against *C. albicans.* The combination of CuONPs with ampicillin led to an antagonistic effect and should be avoided. It would be advocated for the future combination therapy of copper oxide nanoparticles and fluconazole against fungal infections caused by *C. albicans* because of the promising synergistic effect against *C. albicans*. The CuONPs would serve as a potential antimicrobial agent and drug delivery system.

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AUTHORS CONTRIBUTIONS

Okoye designed the work. He also carried out synthesis and antimicrobial studies. Prof. Okhamafe supervised the work. Prof. Arhewoh interpreted the results. He also corrected and organized the manuscript.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest in this work.

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