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Anti-Bacterial Activities of Green Synthesized ZnO and CuO Nanoparticles from Leaf Extracts of *Warburgia ugandensis*

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Abstract: This work reports for the first time the green synthesis of zinc oxide nanoparticles (ZnO NPs) and copper oxide nanoparticles (CuO NPs) using leaf extracts of *Warburgia ugandensis* as encapsulating, stabilizing, and reducing agent. The green method of synthesis proved easy and less costly. The methanolic extracts contained various secondary metabolites as analyzed using gas chromatography-mass spectrometer (GC-MS). The nanoparticles (NPs) were further characterized for the confirmation of their synthesis using various techniques. Ultra violet-Visible spectrometer (UV-vis) confirmed the successful synthesis of ZnO NPs and CuO NPs with a maximum peak at 367 nm and 307.5 nm, respectively. The X-ray diffractometer (XRD) results confirmed the formation of hexagonal wurtzite ZnO NPs and monoclinic structures of CuO NPs with an average size of 21.2 nm and 12.86 nm, respectively. In addition, the Fourier transform infrared (FTIR) analysis showed the presence of various functional groups responsible for the formation of the nanoparticles. The antibacterial activity of the formulated nanoparticles was also investigated against *Staphylococcus aureus (S. aureus)* and *Escherichia coli (E. coli)* bacterial strains with ZOI (Zones of Inhibition) measured in mm. The green synthesized ZnO nanoparticles using *Warburgia ugandensis* leaf extracts significantly revealed higher anti-bacterial potentials against *E. coli* (9.6 ± 0.9 mm) compared to both CuO NPs and ampicillin. This shows that they can be applied in the field of medicine to develop antibacterial agents to treat various ailments.

Keywords: Zinc Oxide, Nanoparticles, Copper Oxide, *Warburgia ugandensis*, Anti-Bacterial Activities, Green Synthesis

1. Introduction

In recent times, the field of nanotechnology have mostly emerged and developed in relation with medicinal sciences. The innovation has also found applications in rapid material detectors, sensors, magnetic imaging, diagnosis methods, agriculture and military. The nanoparticles are in the nanoscale range [1]. Nanomaterials have different biological, optical and physicochemical characteristics with a unique high surface to volume ratio with great reactive sites [2]. The metal oxide nanostructures are synthesized by various chemical and physical methods such as liquid-liquid interface, precursor thermal decomposition, metal alkoxide-based route, electrochemical, sonochemical, micro-wave irradiations and sol-gel means utilizing harmful reducing reagents. These techniques involve the production of toxic products or chemicals at high temperatures and pressures, from tedious processes, expensive methods, non-biodegradable stabilizing agents and toxic organic solvents [3, 4]. The methods (chemical and physical) are discouraging due to the requirements of more time, high energy intensity and are costly. Biogenic synthesis is therefore an alternative method as a green or facile methodology [5].

The term "green synthesis'' also known as biological method, has recently acquired popularity because the technique is environmentally friendly, economical, results in high yield of nanoparticles, and the process of reduction and capping processes occurs in a single step [6]. The process involves the application of bioactive molecules from fruit peel waste, microbes and mainly plants for reduction of metal ions to form nanoparticles of their correspondence [7]. The pertaining nanoparticles are yielded as a result of reduction of precursor salts facilitated by green methods due to presence of intrinsic properties. The ZnO nanoparticles synthesis has drawn much interest because of their unique chemical and physical properties applied in cancer and biomedical uses. The microbial cells are denatured or disintegrated when ZnO nanoparticles accumulate on the walls of microbes triggering Zn^{2+} release regarded as Reactive Oxygen Species (ROS) and production of hydrogen peroxide, hydroxyl radicals and superoxide anion [8].

The copper oxide-based nanomaterials are applied in treatment of diseases due to their possession of biocidal and antimicrobial activities. CuO NPs also have novel properties applied in nanotechnology including photovoltaic properties, gas sensors, catalytic properties, high adsorption capacity, photodegradation and semi-conductance at higher temperature [9-11]. Furthermore, the conductance of water thermally was improved by 20% when 4% of copper oxide nanostructures were added [12]. The air oxidation of biosynthesized metal oxide nanostructures compared to chemically fabricated ones was noted to be minimal remaining stable for over thirty days. This is due to the encapsulation of the green synthesized nanoparticles by phytochemicals present in plants [13].

The phytochemicals occurrence mostly affects the mode of reaction with the metal ions. The metal oxide-based biomolecules involved formulation of metal-biomolecules ion complexes, metal-hydroxide complexes and eventual metal oxide nanostructures [14]. The biosynthesized copper oxide nanoparticles possess unique characteristics ranging from magnetic, mechanical, electronic, chemical and optical properties [11]. The chemical degradation of contaminants is effective due to the larger surface areas to volume ratios of the nanoparticles during interactions [15]. The uniqueness of biological, chemical and physical properties of green synthesized metal oxide nanostructures has led to their wide application in environmental, industrial, agricultural and bio-medicinal study [16].

The biogenically formulated ZnO nanoparticles have gained various uses in antibacterial activities to treat illnesses in animals and humans, micro-electronics, diagnostics, cosmetics and textiles [17]. Plants, due to their vulnerability to nanotoxicology, are applied to test for the toxicity of the nanoparticles due to their tendency to gather absorbed nanomaterials from the air, water and soil. The rise in concern globally of AMR (Anti-microbial Resistance), like a resistance by *Acinetobacter baumannii*, has led to development of zinc oxide nanoparticles biologically. The ZnO nanomaterials through effective cell apoptosis prevent biofilm formation and cell growth of pathogens by causing oxidative stress as a result of generation of reactive oxygen species [18]. Biosynthesis of CuO NPs and ZnO NPs have been achieved using different plant extracts such as *Garlic Bulb (Allium sativum)* and *Zingiber officinale* [19], *Moringa Oleifera* [20], *Hibiscus cannabinus* [21], *Psidium guajava* [22], *Rosa indica* [23], *Syzygium guineense* [24] *and Catha edulis* [8].

*Warburgia ugandensis (*WU) is also called Ugandan greenheart, Pepper bark tree and East African greenheart. Its extracts were historically used in antibacterial studies [25]. It is among the popular medicinal-plant varieties in South Africa, Tanzania, Kenya and Uganda. It is used in treatment of bacterial diseases [26] and HIV treatments due to its toxicity levels [27]. In Ethiopia, WU is named Befti. It is among nine species of plants capable of aromatic oils production belonging to Canellaceae family. It is an important plant in African communities located in drier highlands and lower rainforests of Ethiopia, Uganda and Kenya [28]. Traditionally, the leaf, root and bark extracts of WU were applied in treatments of chest pains, pneumonia [29], intestinal worms, lung problems, ulcers, skin diseases, tuberculosis and malaria. Its diversified therapy activities result from possession of mannitol, tannins, drimane sesquiterpenes [30], flavonoids, saponins, steroids and terpenoids phytochemicals [25]. This study intended to evaluate the antibacterial properties of green synthesized ZnO NPS and CuO NPS using extracts of *Warburgia ugandensis* as a reducing and capping agent.

2. Materials and Methods

2.1. Materials

Oven, centrifuge, hot plate, magnetic stirrer, filter papers (Whatman No. 1), analytical balance (digital electronic), Petri dishes, UV-VIS spectrophotometer (UV 1800, Schimadzu model), Fourier Transform Infrared Spectrophotometer (IRAffinity-1S, SHIMADZU model) and XRD (D8 Advance X-ray Diffractometer system model).

2.2. Chemicals and Reagents

Bacterial strains, distilled water, Sodium hydroxide (NaOH), Zinc-acetate (Di-hydrate) and cupric nitrate (Tri-hydrate) were purchased at Reno Chemicals and Lab Equipment Suppliers, Eldoret, Kenya. The Nutrient Agar was purchased at Commercial Enterprises, KEMRI, Kenya.

2.3. Plant Collection

The fresh leaves of *Warburgia ugandensis* were collected at Kenya Plant Health Inspectorate Services, Kitale, Kenya.

2.4. Preparation of Crude Extracts

5 g of air-dried leaves powder of *Warburgia ugandensis* leaves was immersed in 100 mL of distilled water and extraction done at 78 ± 2 ^{o}C while stirring up for about 3

hours. The extract obtained was filtered using Whatman No. 1 filter papers. The fresh solution of *Warburgia ugandensis* leaf extract was refrigerated at 4°C for further use.

2.5. GC–MS Analysis

The samples were extracted using methanol. The prior sample clean-up was carried out to concentrate the analyte, change the sample matrix and removal of matrices that could cause the interference. Therefore, C18 cartilage was conditioned with 3 mL methanol then 3 mL of sample was loaded. It was allowed to flow slowly out of the cartilage giving it adequate time to interact with adsorbent. Then it was left to dry in a stream of air for twelve minutes. It was thereafter eluted with 3mL of methanol into a 4 mL vial. It was then concentrated using a genetic concentrator, reconstituted with 1mL of methanol, filtered using nylon micro filters size of 0.22 micro meters into 1.5 mL vials and taken to GC-MS for analysis.

The GC-MS analysis was carried out with GC-MS Qp2010SE model. The ion source and column oven temperature were 200°C and 55°C, respectively. The helium (carrier gas) flow rate was at 1.2 mL/min. The dimensions of capillary column were 30 m × 0.25 mm (internal diameter) and a split injection mode. EI mode was at 70 eV and a mass range of 40 – 400 m/z.

2.6. Preparation of Zinc Oxide Nanoparticles

In a conical flask, 75 mL of 100 mM Zinc Acetate Dihydrate solution was treated with 15 mL of plant extract while constantly stirring using a magnetic stirrer. The mixture (at pH of 8) was run for 20, 30, 40 and 60 minutes, respectively. The reaction mixture was heated under continuous stirring at temperatures of 50°C -60°C. The change in color to yellowish indicated the synthesis of zinc oxide nanoparticles as shown in *Figure 1*. The product was centrifuged and washed in two cycles with distilled water for the removal of precursor salt that unreacted. At temperatures of 40°C, the Zinc oxide nanoparticles were dried overnight [31]. The same procedure was repeated at varied pH of 9, 10 and 11 by applying 0.2M NaOH solution dropwise.

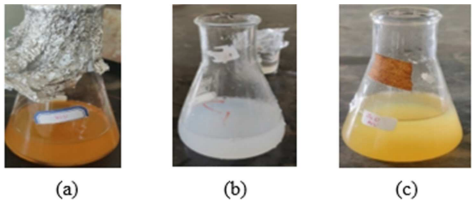


Figure 1. *Represents biosynthesis of Zinc Oxide Nanoparticles; (a) Leaf extracts of Warburgia ugandensis; (b) zinc acetate solution and (c) synthesis of ZnO NPs using Warburgia ugandensis extracts and characteristic color change.*

2.7. Preparation of Copper Oxide Nanoparticles

The biosynthesis of copper oxide nanoparticles was carried out adopting procedure developed by the author [12] with some modifications. At continuous stirring at 55°C - 60°C, 18 mL of plant extract was added to 80 mL of 0.01M Cu $(NO_3)_2.3H_2O$ solution in a conical flask. The mixture (at pH of 6.5) was run for 20, 30, 40 and 60 minutes, respectively. The supernatant after centrifugation was discarded and the final product purified using distilled water. The washing was repeated twice (two cycles). During the biosynthesis process, light green color observation from initial blue color indicated the synthesis of CuO NPs as shown in *Figure 2*. The final product was dried overnight at 35°C. The same procedure was repeated at varied pH of 6.5, 8.5 and 10.5 by applying 0.2M NaOH solution dropwise.

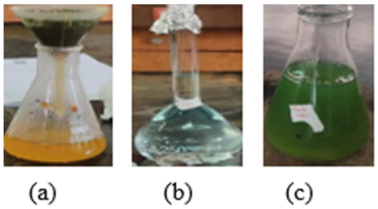


Figure 2. *Represents phytosynthesis of Copper Oxide Nanoparticles (a) Leaf extracts of Warburgia ugandensis, (b) cupric nitrate solution and (c) synthesis of CuONPs with Warburgia ugandensis leaf extracts and respective color changes.*

2.8. Antibacterial Assay Using Agar Disc Diffusion Technique

The test was carried out as reported by the research [32] with some modifications. The anti-bacterial potentials of CuO NPs and ZnO NPs were tested against two bacterial strains: *S. aureus* (Gram positive) and *E. coli* (Gram Negative). Ampicillin was used as a positive control. A sterile spreader was used to swab the pathogens uniformly on the petri dishes with agar nutrients. The filter paper discs made by a paper punch were soaked in concentrations of 500 ppm, 250 ppm and 125 ppm of the samples and placed on the inoculated dishes. The petri dishes were incubated at 37°C for 24 hours and the zones of inhibition results recorded.

3. Results and Discussions

3.1. GC-Ms Analysis of Warburgia Ugandensis Leaf Extracts

A qualitative study of the chemical compounds present in the extracts of *Warburgia ugandensis* was analyzed using GC-MS method. These compounds are responsible for the reduction and capping of zinc and copper oxide nanoparticles studied in this paper for the first time. *Figure 3* shows the GC-MS chromatogram of 40 chemical compounds present in the methanol extracts of *Warburgia ugandensis* and respective percentage chemical compositions as follows; Styrene (14.60%), undecane (1.86%), Cyclopropane (0.72%), caryophyllene oxide (1.29%), corymbolone (2.98%), isoshyobunone (0.51%), patchouli alcohol (1.19%), aristolene epoxide (1.51%) and others. The styrene (14.60%) were present at high percentage while isoshyobunone (0.51%) at low levels. *Table 1* shows the summary of chemical compounds in methanol extracts of *Warburgia*

ugandensis with reference to NIST 17 spectra information database.

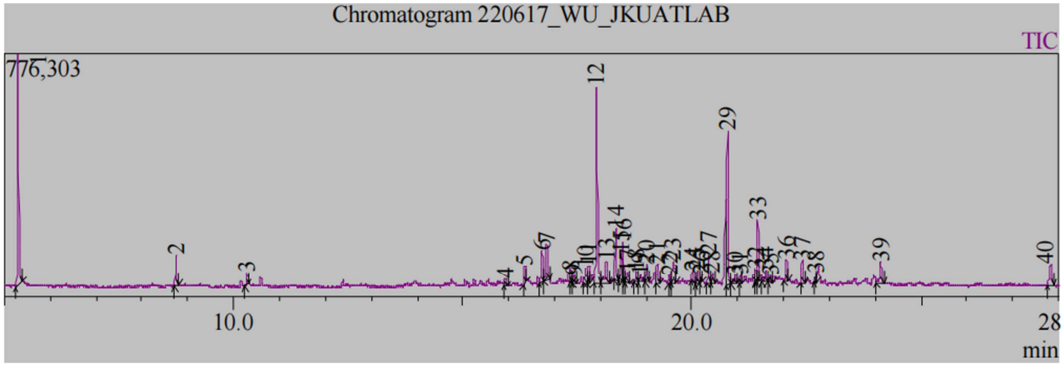


Figure 3. *GC-MS chromatogram of methanol extracts of Warburgia ugandensis.*

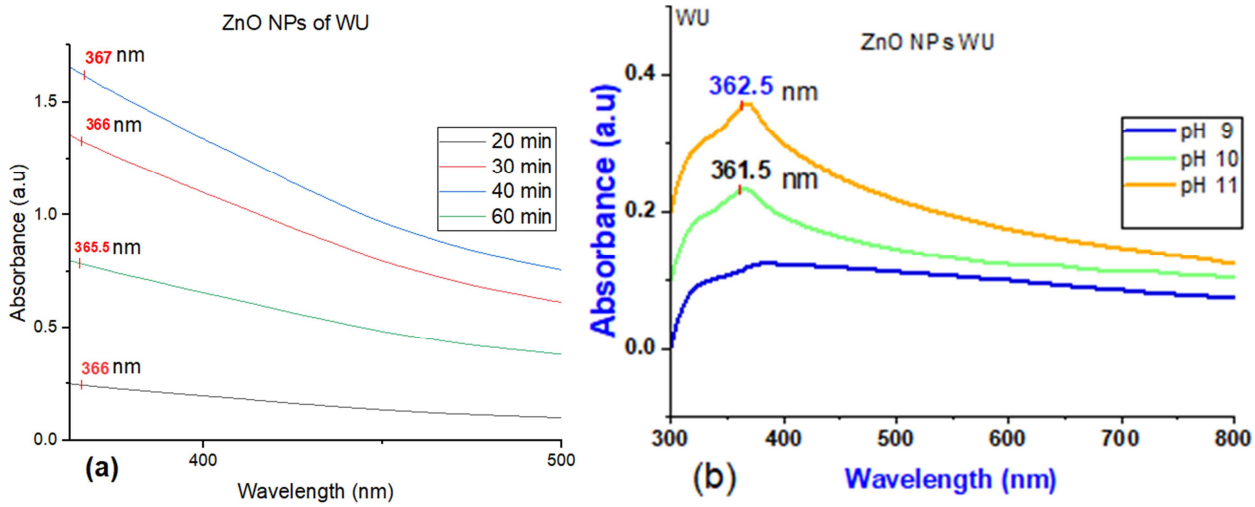
Table 1. *GC-MS Analysis of summary chemical compounds in methanol extracts of Warburgia ugandensis with reference to NIST 17 spectra information database.*

S/No	RT	Name of the compound	M.W	% Peak area	Class
1	5.294	Styrene	104.15	14.60	Hydrocarbon
2	8.742	Undecane	156.31	1.86	Alkanes
3	10.296	Cyclopropane	42.08	0.72	Cycloalkane
5	16.357	Caryophyllene oxide	220.35	1.29	sesquiterpenes
6	16.727	Corymbolone	236.35	2.98	Essential oil
9	17.424	Isoshyobunone	58.12	0.51	sesquiterpenoids
10	17.676	Patchouli alcohol	222.36	1.19	Tertiary alcohol
11	17.744	Aristolene epoxide	204.36	1.51	Essential oil

3.2. Characterizations of the ZnO and CuO NPs

The optical characteristics of the biosynthesized zinc oxide and copper oxide nanoparticles dispersed in distilled water were carried out where the spectrum of absorption was obtained using a double beam UV-Vis spectrophotometer (SHIMADZU model) at a range of 190-1100 nm wavelength scan.

The maximum absorption peak of ZnO nanoparticles (*Figure 4 (a) and (b)*) at varied pH was obtained at 362.5 nm after 30 minutes at a pH of 11. The maximum absorption peak at varied time shifted to 367 nm after 40 minutes from initial 366 nm at 30 mins both at a constant pH of 8. The red shifts of 362.5 nm at pH 11 (*Figure 4 (b)*) and 367 nm after 40 minutes (*Figure 4 (a)*) observed could be attributed by decrease in particle sizes. The band gap energy obtained from Tauc plot (*Figure 4 (c)*) was 2.53 eV at pH 11. This was consistent with a study applying water hyacinth and mango steen peels crude extracts to synthesize ZnO nanoparticles [7], where the absorbance peak was located at 365 nm and a band gap of 2.88 eV. Furthermore, the spectra do not contain other peaks signifying that the green synthesized nanoparticles were a pure product. The higher absorption peaks of 367 nm as reported by [34] can be attributed by inherent band gap absorptions by ZnO's as electrons transition to conduction band (Ec) from valence band (Ev). The sample obtained at varied pH of 11 was used for further characterizations.



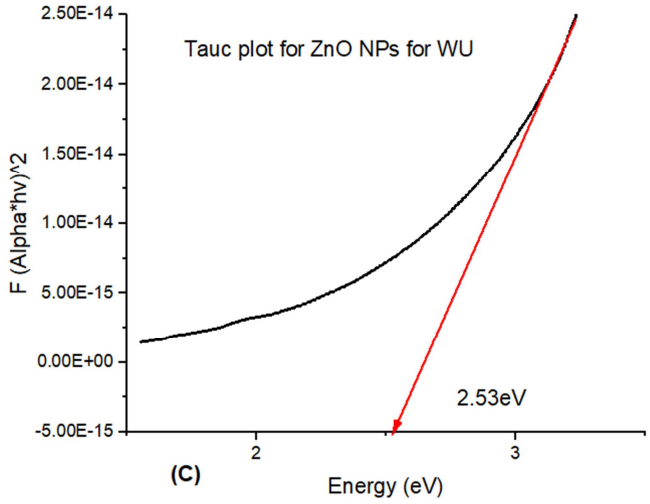


Figure 4. *(a) Absorption spectrum for ZnO-NPs of Warburgia ugandensis (WU) at varied time, (b) Absorption spectrum for ZnO-NPs of Warburgia ugandensis (WU) at varied pH and (c) Tauc plot.*

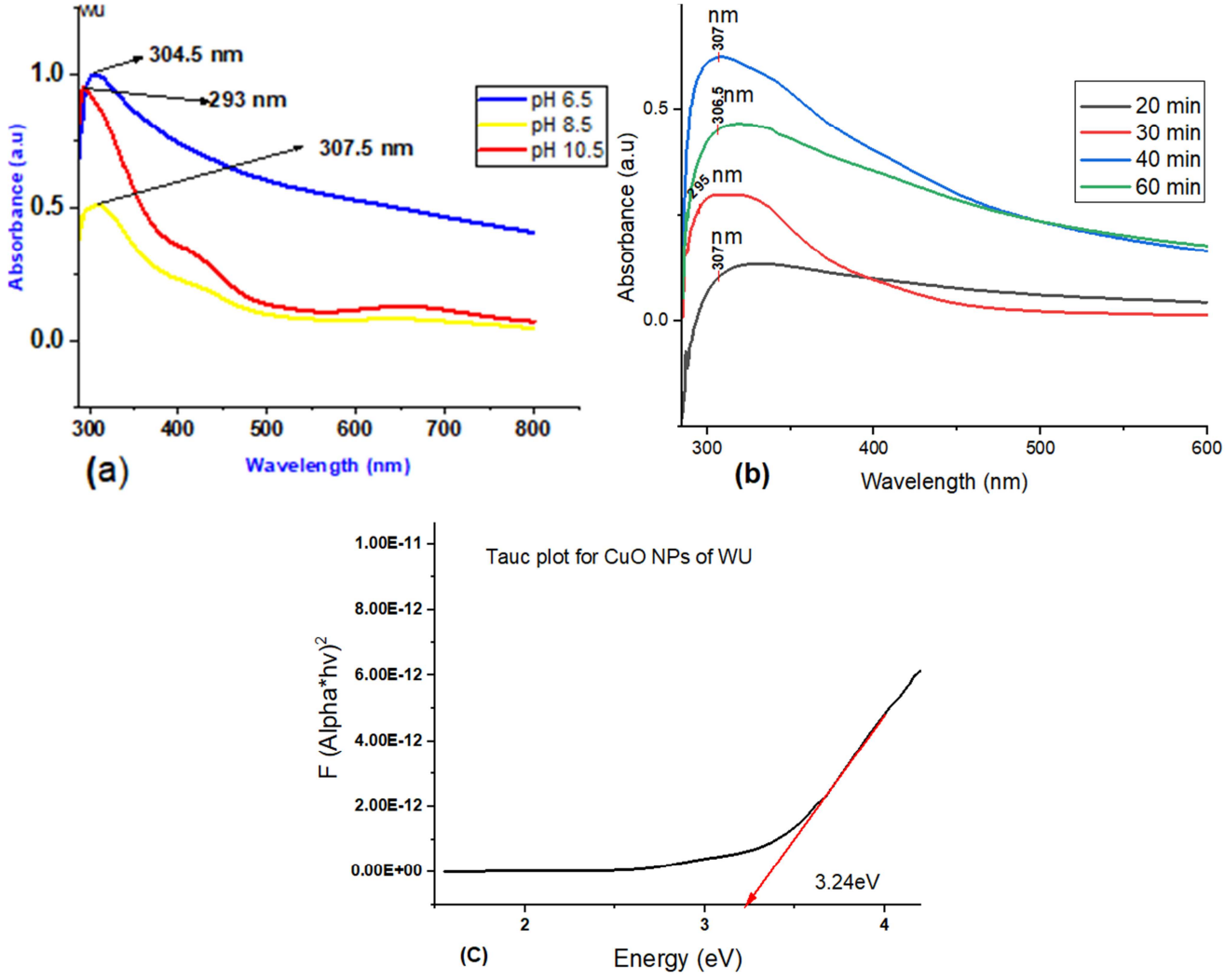


Figure 5. *(a) Absorption spectrum for CuO NPs of Warburgia ugandensis (WU) at varied pH, (b) Absorption spectrum for CuO NPs of Warburgia ugandensis (WU) at varied time and (c) Tauc plot of CuO NPs.*

In (*Figure 5 (a) and (b)*), the maximum absorption peaks at 307 nm and 307.5 nm at varied time and pH respectively indicated surface plasmon resonance of CuO nanoparticles with a band gap energy of 3.24 eV from Tauc plot (*Figure 5 (c)*) at pH 8.5. A blue shift of 293 nm at pH 10.5 was observed from 304.5 nm at pH 6.5 (*Figure 5 (a)*) while a red

shift of 307 nm at 40 minutes from initial 295 nm at 30 min at pH 6.5 (*Figure 5 (b)*) was observed. This clearly confirmed that with passage of time, there resulted red shifts and peak broadening. The optical results obtained in this paper agrees with previous results from other studies [12] that the maximum wavelength of CuO NPs was in the range of 250 nm to 395 nm. The maxima absorbance peak of CuO was at a wavelength of 310 nm [11]. The sample obtained at varied pH of 8.5 was used for further characterizations.

The FTIR was utilized to explore the presence of functional groups or compounds responsible for the green synthesis and stabilization of ZnO NPs and CuO NPs efficiently as also analysed in GC-MS. The spectra was obtained at a wavelength scan of between 4000–400 cm^{-1} as shown in *Figure 6* and *Figure 7*. In *Figure 6*, the bands that absorbed at lower wavenumbers ranging from 400 cm^{-1} - 600 cm^{-1} indicated existence of Zn-O bonds in the formulated ZnO nanoparticles [37]. Therefore, the stretching at 543 cm^{-1} shows the formation of ZnO NPs.

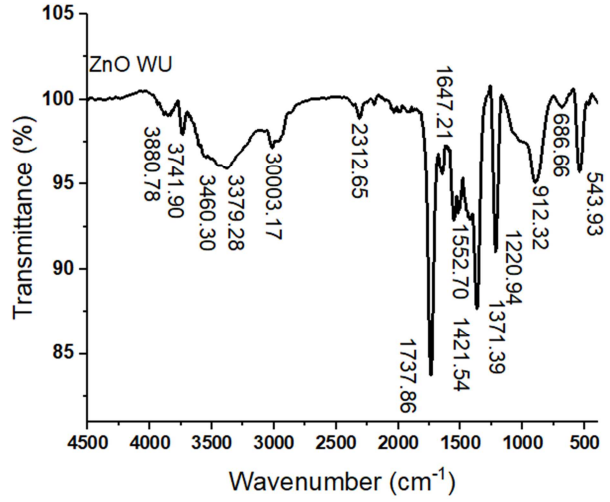


Figure 6. *FTIR spectrum of biosynthesized ZnO nanoparticles by Warburgia ugandensis leaf extracts.*

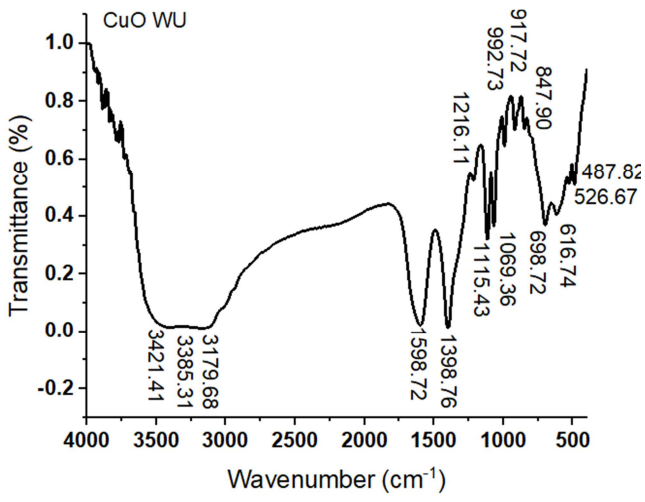


Figure 7. *FTIR spectrum of biosynthesized CuO nanoparticles by Warburgia ugandensis leaf extracts.*

The formation of Cu-O bond was indicated by small, low frequency sharp bands between 526 cm^{-1}-616 cm^{-1} [13, 35], C=O band stretching at 1598 cm^{-1} [36], C-N stretching at 1115 cm^{-1} correlates to aliphatic (amine) groups [10] and 1398 cm^{-1} absorption band indicates −COO group for carboxylic acids presence [24] as shown in *Figure 7*.

The biosynthesized ZnO and CuO nanoparticles were further analyzed for crystallite structure and particle sizes by using XRD (Brucker D8 Advance X-ray Diffractometer system). The intensity of the diffracted Cu-K_α radiation (λ =0.154nm, 40 kV and 40mA) was obtained in a 2θ range of 10°–90° with an increment of 0.0194°. The average particle size obtained from Scherrer's equation shown below was found to be 21.2 nm and 12.86 nm for ZnO and CuO nanoparticles respectively.

$D = \frac{0.9\lambda}{\beta \sin \theta}$ Scherrer's equation.

Where D is the particle average crystallite size, λ (0.154) as the X-ray wavelength, β as the FWHM (full width half maximum) intensity value in radian obtained from Gauss curve in Origin pro software and θ as the Bragg's angle in radians. The Bragg's 2θ values for ZnO NPs (*Figure 8*) were located at 31.77°, 34.40°, 36.26°, 47.53°, 56.60°, 62.84°, 67.95° and 69.06°. These peaks as reported by [34] corresponds to (1 0 0), (0 0 2), (1 0 1), (1 0 2), (1 1 0), (1 0 3), (2 0 0) and (1 1 2) respectively thus in relation to (JCPDS card No: 36-1451), indicates that the obtained nanoparticles are hexagonal wurtzite structures. The Bragg's diffraction 2θ values of CuO NPs (*Figure 9*) were observed at 12.94°, 21.79°, 25.81°, 32.20°, 33.96°, 36.63°, 39.72°, 43.75°, 53.5°, 58.57°, 61.20° and 62.79°. [11] Reports that the synthesized CuO NPs are monoclinic structures confirmed by 2θ at 39.72° and 43.75°. The location of 2θ values ranging from 30°-40° signifies CuO formation and their crystalline structure is affirmed in peak sharpness [9]. Crystallographic purity is indicated with the non-appearance of spurious diffractions [4].

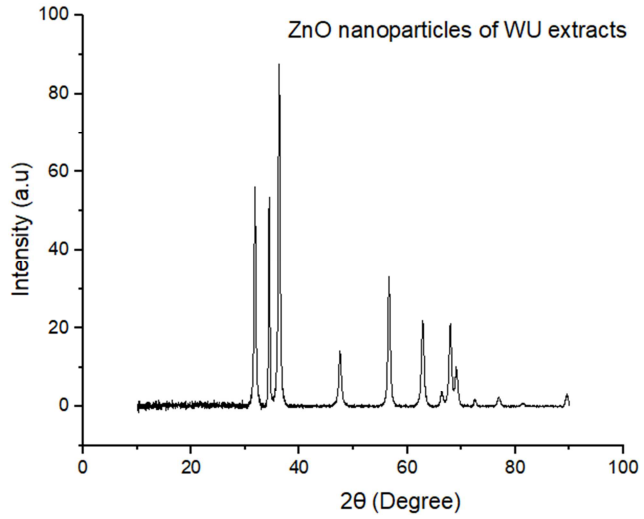


Figure 8. *XRD pattern of green synthesized ZnO NPs from Warburgia ugandensis leaf extracts.*

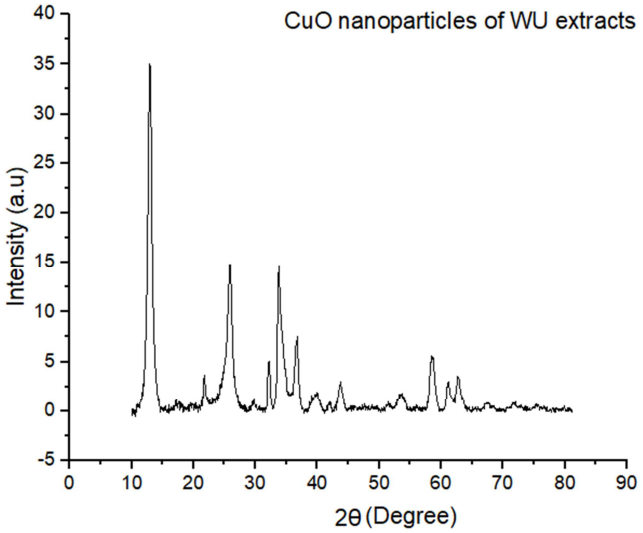


Figure 9. *XRD pattern of green synthesized CuO NPs from Warburgia ugandensis leaf extracts.*

3.3. Antimicrobial Study

The green synthesized zinc oxide and copper oxide nanoparticles from *Warburgia ugandensis* leaf extracts were tested for anti-bacterial activities against two bacterial strains, Gram- negative *(E. coli)* and Gram- positive (*S. aureus)* bacteria. The results are shown in *Figure 10, Table 2* and *Figure 11*. The green synthesized ZnO nanoparticles using *Warburgia ugandensis* leaf extracts significantly revealed higher anti-bacterial potentials against *E. coli* (9.6 ± 0.9 mm) while ampicillin was more effective on *S. aureus* (9.3±0.6 mm). In both bacterial strains, CuO NPs compared to ZnO NPs revealed to be slightly less effective. The differences in the bactericidal activities can be as a result of chemical and structural make up of cellular membranes of microorganisms [8].

The gram (-ve) bacteria (*E. coli*) possess thin-layer of lipopolysaccharides and peptidoglycan and ZnO nanoparticles positively charged will adhesively bind to negative charges of lipopolysaccharides. Hence, the nanoparticles easily adsorb on the walls of *E. coli* membranes disrupting ultimately permeability selectivity [18]. The secondary metabolites present in *Warburgia ugandensis*, containing of sesquiterpenoids, sesquiterpenes and essential oils possess therapeutic potentials in medicine [33]. From the antimicrobial analysis, it was found out that the extracts of *Warburgia ugandensis* inhibited the zones of *S. aureus* and *E. coli* due the presence of active compounds (Table 1) acting as reactive oxygen species against microbes.

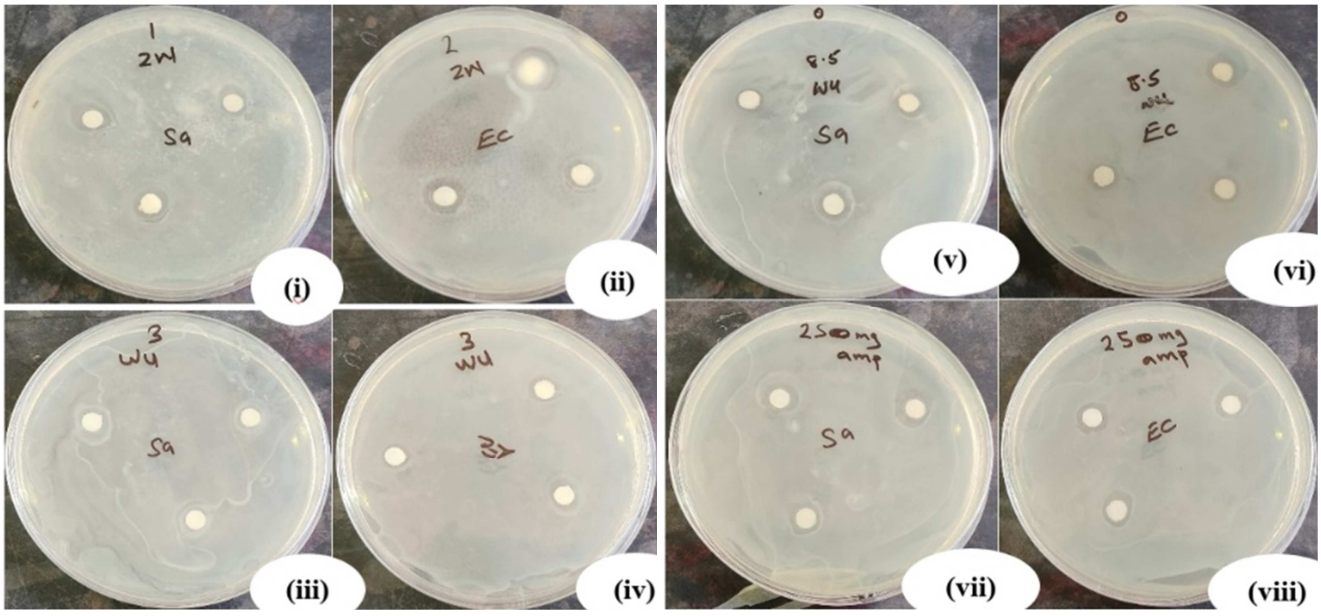


Figure 10. *Anti-bacterial images of ZnO NPs against. (i) Staphylococcus aureus and (ii) Escherichia coli, anti-bacterial images of Warburgia ugandensis leaf extracts against: (iii) Staphylococcus aureus and (iv) Escherichia coli, anti-bacterial images of CuO NPs against: (v) Staphylococcus aureus and (vi) Escherichia coli and anti-bacterial images of ampicillin against: (vii) Staphylococcus aureus and (viii) Escherichia coli.*

Table 2. *Inhibition Zones for ZnO-NPs, CuO-NPs, Warburgia ugandensis leaf extracts and ampicillin against S. aureus and E. Coli bacteria.*

Sample Identity	**Inhibition Zones (mm)**	
	S. aureus	***E. coli***
Zinc Oxide Nanoparticles	9.0±1.0	9.6±1.5
Copper Oxide Nanoparticles	8.3±0.6	7.3±0.6
Warburgia ugandensis leaf extracts	8.0±1.0	8.0±1.0
Ampicillin	9.3±0.6	9.0±1.0

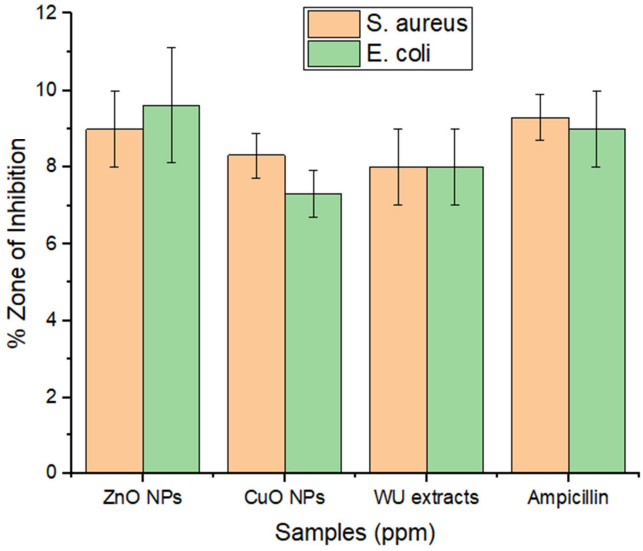


Figure 11. *Percentage inhibition results against the two bacterial strains.*

4. Conclusions

The green synthesis of ZnO and CuO nanoparticles using *Warburgia ugandensis* leaf extracts was for the first time highlighted in this study. The GC-MS data shows the presence of various compounds responsible for the formulation of nanoparticles. The characterization was carried out where the biosynthesized nanoparticles were optically characterized and the crystallinity and nanoparticle sizes were determined by the XRD pattern. The FTIR analysis showed the presents of various functional groups present in various molecules or secondary metabolites responsible for reduction, capping and stabilization of the green route synthesized ZnO and CuO nanoparticles. The nanoparticles were tested for antibacterial assays by disc diffusion technique. The nanoparticles proved to possess more potent inhibition characteristics of the pathogenic bacteria thus can be employed as stable and safe materials in the field of biomedicine and pharmacy.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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