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Green synthesis of Copper Nanoparticles and Investigation of its Antimicrobial Properties

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ABSTRACT

Nanoparticles containing copper metal ions and their derivatives are being used for different medical purposes to prevent infections, ulcers etc. The aim of this study was to qualitatively determine the phytochemicals present in the ethanolic extract of *Kigelia africana* fruit (which are responsible for the reduction of the copper ions as well as stabilization of the nanoparticles), the synthesis of copper nanoparticles (CuNPs) through a green synthesis route from the ethanolic extract of *Kigelia africana* fruit and the anti-microbial assessment of the synthesized CuNPs. The synthesized nanoparticles were characterized by UV-Vis Spectrophotometer. Phytochemical screening revealed the presence of alkaloids, glycosides, flavonoids, phenols, steroids, tannins, carbohydrate and terpenoids. The synthesized CuNPs were confirmed by the change in colour from dark yellow to dark green. The CuNPs displayed promising antibacterial activity on *Pseudomonas aeruginosa, Shigella sp., Staphylococcus aureus, Salmonella typhi, E. coli*. The highest inhibition activity was exhibited by *Pseudomonas aeruginosa* (17.0 ± 4.24 mm). The CuNPs showed considerable antifungal activity against *Aspergillus flavus* and *Aspergillus niger* with inhibition activity of 8.0 ± 2.83 mm and 3.0 ± 4.24 mm respectively. Conclusively, the synthesized CuNPs from *Kigelia africana* should be incorporated as a therapeutic drug for microbial infectious disease and other health associated disorders.

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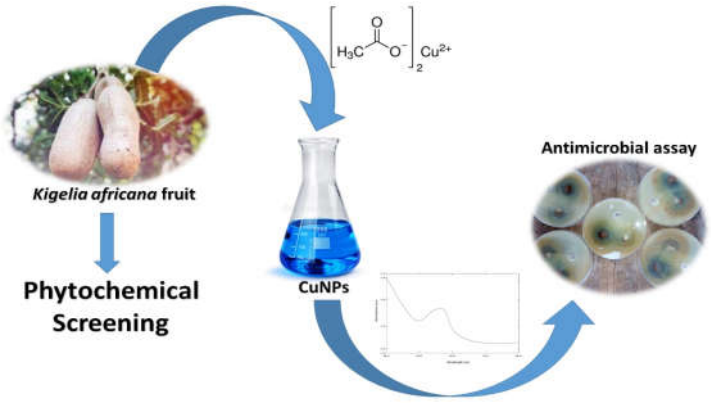
GRAPHICAL ABSTRACT



INTRODUCTION

Over the years, scientists from various disciplines have carried out researches extensively on nanotechnology in a bid to either satisfy their curiosity or to solve an already existing problem which over the year has led to its sustainable growth [1]. In trying to do this, new materials (with a size ≤ 100 nm) [2-4] have been fabricated through the knowledge of nanotechnology that have applications in the medical, engineering and even in our daily facets of life. The fabrication of these materials especially through the bottom-up approach are established through one of three routes, namely; Chemical route, Physical route and Biosynthetic route [5, 6]. In comparison to the biosynthetic method, which is safer, more biocompatible, and ecologically friendly, the physical and chemical routes of synthesis are more costly and less environmentally favourable [7-9]. Chemical and physical techniques of nanoparticle production are further limited by their high energy requirements, the use of toxic and costly ingredients, and the time and effort required for preparation [10, 11]. In addition, biosynthetic route (which utilizes Fungi, algae, bacteria, plants, and other organisms as precursors) is gaining traction due to their capacity to overcome toxicity.

Plant-mediated nanoparticle synthesis is advantageous than the bacteria-, algae-, and fungi-mediated nanoparticle production since the latter are time-consuming due to the high maintenance culture and continuous sterile conditions required. In addition, plants are also more widely available in useable forms and plant-assisted nanoparticle production takes less time, similar to the chemical route of synthesis [12-14]. Plant components such as the leaves, stem, root, and fruit have been utilized for the green synthesis of nanoparticles because they contain phytochemicals which aid in the bio-reduction of metallic ions [15].

Kigelia africana (**Figure 1**) belongs to the *Bignoniaceae* family and is a tropical plant. It is a medium to big semi-deciduous tree with a thick rounded crown that may grow up to 25 m in height, which is very common in the African continent [16, 17]. The leaves form an opposing extension with 3-5 pairs of leaflets plus a terminal leaflet towards the ends of branches; the lower leaflets have short petioles, while the terminal pair does not [18, 19]. The dark-red cup-shaped flowers bloom at night on long, rope-like stalks that dangle down from the tree limbs and fall off before daylight. The fruit's cylindrical form gave it the common name "sausage tree" [16, 17]. Different tribes of the world have various native names for this plant.

For example, it is referred to as *Uturubein*, *Pandoro* or *Iyan*, *Rawuya*, *Bechi*, *Ntabinim*, and *Ketete* respectively by the Igbo, Yoruba, Hausa, Nupe, Ibibio, and Bette tribes in Nigeria [20]. Outside Nigeria, it is referred to as *Umfongothi* by the Zulu tribe in South Africa, *Mwegea* in Kenya and Tanzania, and *Balmkheera* in India [20, 21]. This plant has been confirmed to possess medicinal and pharmacological properties such as anti-diarrhoeal properties, anti-leprotic properties, anti-malarial properties, anti-inflammatory properties, anti-cancer activity, anti-microbial properties, anti-oxidant, anti-implantation activities, and as a stimulant for the Central nervous system, which have made it common as a herb for the treatment of various diseases and ailments, especially in Africa [18, 22].

Different parts of the plant have been utilized for the synthesis of various nanoparticles. Such as its leaves [24-26], stem [27], flowers [28-30], and fruit. Very few studies have been carried out on the synthesis of nanoparticles from the fruits of *Kigelia africana*. Ashishie, Anyama [20] Utilized the aqueous extract of its fruit for the synthesis of silver nanoparticles and copper-silver bimetallic nanoparticles and the assessment of the anti-microbial properties of the synthesized nanoparticles. From the authors' extensive research, *Kigelia africana* fruit or its ethanol extract has never been investigated as a precursor for the green production of copper nanoparticles (CuNPs) and published. This is the novelty of this study. As a result, the current study is an examination of the technical feasibility of a chemical process combined with the anti-microbial assessment of the chemical product. The goal of this research is to qualitatively examine the phytochemicals present in the ethanolic extract of *Kigelia africana* fruit (which are responsible for the reduction of the copper ions as well as stabilization of the nanoparticles), the synthesis of copper nanoparticles from the ethanolic extract and the assessment of the anti-microbial properties of the synthesized nanoparticles.

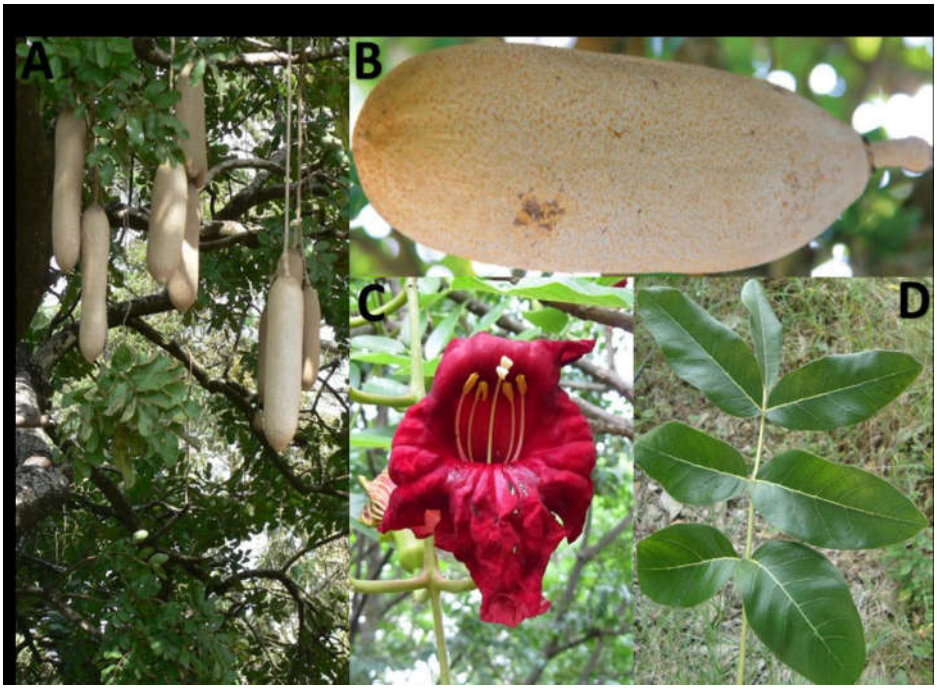


Figure 1: Pictures showing Kigelia africana tree with hanging fruits (A) fruit (B) flower (C) and leaves (D) [23]

EXPERIMENTAL

Reagent and materials

Copper acetate purchased from Pascal Scientific Ltd was utilized as the starting material. Fresh *Kigelia Africana* fruits were collected from Oja Odo-peturu in Ondo State, Akure. All reagents were dissolved with distilled water throughout the experiment and no further purification was carried out as all chemicals were of analytical grade.

Preparation of the aqueous Kigelia Africana fruit extract

Kigelia Africana fruits were surface cleaned and repeatedly washed with distilled water to completely remove all dust particles. The thoroughly washed fruits were sliced, and seeds were separated. The sliced fruits were sundried for five days to ensure complete moisture removal, and thereafter ground using a mechanical grinder. This was subsequently sieved and stored in an airtight container. The extraction was carried out by measuring 50 g of the powdered plant fruit into a 1000 ml beaker after which 500 ml of ethanol was added. The beaker containing the ethanol and powdered fruit – covered with an aluminum foil was left for 48 hours with repeated stirring with the aid of magnetic stirrer every day to ensure complete extraction. The extract was subsequently filtered and stored in an airtight container.

Phytochemical Screening

Chemical tests were carried out on the aqueous extract and on the powdered specimens using standard procedures to identify the constituents.

Test for Tannins

5.0 g of the samples each was added to 20 ml of distilled water and filtered. A few drops of 0.1% $FeCl_3$ was added in filtrate and observed for colour change; brownish green or a blue-black coloration was taken as evidence for the presence of tannins [31].

Test for Saponins

The emulsion test and froth tests were used. The ability of saponins to produce emulsion with oil was used for the screening test. 2 g of sample was boiled in 20 ml of distilled water in a water bath for five minutes and filtered. 10 ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for froth formation. Three drops of olive oil were mixed with froth, shaken vigorously and observed for emulsion development [31].

Test for Flavonoids

5.0 g of the samples each was suspended in 100 ml of distilled water to get the filtrate. 5 ml of dilute ammonia solution was added to 10 ml of filtrate followed by few drops of concentrated H_2SO_4. Presence of flavonoids was confirmed by yellow colouration [31].

Test for Glycosides

Few drops of ferric chloride and concentrated sulfuric acid were added to 5 ml of aqueous extract in glacial acetic acid. A reddish-brown coloration at the junction of two layers and the bluish green colour in the upper layer indicated the presence of glycosides.

Test for Anthraquinones

10% ammonia solution was added to 1 ml of the aqueous extract of the plant; the deposition of a pink precipitate indicated the presence of anthraquinones.

Test for Terpenoids

5 ml of each extract was mixed in 2 ml of chloroform, and 3 ml of concentration H_2SO_4 was carefully added to form a layer. A reddish-brown colouration of the interface was formed indicating the presence of terpenoids.

Test for Sterols

1 ml of concentrated H_2SO_4 was added to 1 ml of each extract. A red colouration was an indication of presence of sterols [31].

Test for Alkaloids

The Mayer's, Dragendroff's, Wagner's, and Picric acid tests were used to test for alkaloids. 1 g of the plant material was boiled for almost 2 minutes with 5 ml of 2% hydrochloric acid in a steam bath and the material filtered. 1 ml portion of the filtrate was treated with 2 drops of any of the following reagents and for precipitate. Turbidity or precipitate with either of the reagents was taken as evidence for the presence of alkaloids [31].

Test for Carbohydrate

To 2 mL of extract, 1 mL of Molisch's reagent and few drops of concentrated sulphuric acid was added. Formation of purple or reddish colour indicated the presence of carbohydrate.

Synthesis of the Cu-NPs using *Kigelia Africana* fruit extract

A 0.25M aqueous solution of $Cu(CH_3COO)_2$ was prepared and stored in a container. 50 ml of the already prepared 0.25M copper acetate solution was transferred into a 250 ml Erlenmeyer flask containing the 25 ml of the *Kigelia Africana* fruit extract under constant stirring for 3 hours (with the aid of a magnetic stirrer) to ensure thorough mixing. The mixture was then allowed to stand in the absence of light for 24 hours. The mixture was centrifuged at 10000 rpm for 15 minutes and the precipitate dispersed into distilled water to wash off any remain of biological extract. The precipitate was transferred into an oven for 4 hours at 80^{o}C to ensure it is completely dried.

Antibacterial Activity of the synthesized nanoparticle

The antibacterial potential of the synthesized copper from *Kigelia Africana* against some test microorganisms was carried out using the agar well diffusion method. An 18-24 hr old culture of each test isolate was inoculated into 5ml normal saline in a test tube and standardized. A sterile swab stick was used to apply the suspension to the surface of already prepared Nutrient Agar (NA) plates. A sterile 8mm cork borer was used in boring holes on the agar and a micropipette was used in dispensing 100 µg/mL of the nanoparticles and 100 µg/mL of the antibiotic into the respective labelled holes, Co-trimoxazole (480 µg/mL) solution was used as control. The antimicrobial activities were then determined by measuring the diameter of the zones of inhibition in millimetre. The MIC of the nanoparticles on the test isolates was determined using two-fold dilution method. Sterile 8 mm cork borer was used to bore three holes into prepared Nutrient Agar plates seeded with the nanoparticles and an antibiotic as standard. Different concentrations of the nanoparticles and the antibiotics (100 %, 50 %) were dispensed into each well and labelled.

RESULTS AND DISCUSSION

Phytochemical screening of *Kigelia africana fruit*

Preliminary screening of the phytochemical content of the ethanolic extract of the fruit (**Table 1**) revealed the presence of alkaloids, glycosides, quinones, saponins, flavonoids, phenols, carbohydrate, steroids, tannins, terpenoids and anthraquinone. Flavonoids and tannins were found to be strongly present, while glycosides, terpenoids and anthraquinone were the least present.

Table 1. Result of qualitative phytochemical analysis

Phytochemicals	Inference
Alkaloids	++
Anthraquinone	+
Carbohydrate	++
Flavonoids	+++
Glycosides	+
Phenols	++
Quinones	++
Saponins	++
Steroids	++
Tannins	+++
Terpenoids	+

(+) Least present (++) Moderately present (+++) Strongly present

The ethanolic extract were qualitatively analyzed for the presence of secondary metabolites. Secondary metabolites, otherwise known as phytochemicals are non-nutritive chemical compounds that occur naturally in plants, and have protective or disease preventive properties [32]. The medicinal values of plants and their derivatives are dependent on the compositions of these secondary metabolites [33, 34]. Among the phytochemicals present in the ethanolic extract, flavonoids and tannins are most abundantly present. Flavonoids, tannins, alkaloids and saponins are known to display various biological properties and possess anti-microbial activity against several pathogenic organisms [35], as well as anti-inflammatory and antispasmodic effects [36]. Similar results have been obtained from findings of previous studies on the phytochemical content of *K. africana*. The result of the phytochemical compounds obtained in the present study (irrespective of abundance) was compared with some previous findings in literature (**Table 2**).

Priya, Menkudale [37] reported the presence of alkaloids, flavonoids, steroids, saponins, tannins, glycosides and phenols in the methanolic extract of *K. africana* leaves. Similar result was reported by Awere, Githae [35], with the addition of terpenoids and alkaloids being below the limit of detection. Furthermore, the presence of tannins, terpenoids, flavonoids and phenols in the ethanol extract of *K. africana* was recently reported by [Obianagha, Okafor [34]], thus revealing the medicinal potentials of the plant. It can be seen from **Table 2** that different phyto compounds can be obtained from different studies. This is because the phytochemical compounds present in a particular plant is dependent on several factors that includes their growing conditions, the plant species, location of the plant, extraction methods, the type of soil, age of the plant, among others[32]. Notwithstanding, the ethanolic extract of *K. africana* fruit showed the presence of these phyto compounds more abundantly that the plant extracts, as shown in **Table 2**.

Table 2. Comparison of phyto-compounds present in *K. africana*

Phytochemicals	**Awere, Githae [35]~**	**Priya, Menkudale [37]#**	**Obianagha, Okafor [34]***	**Vaidegi, Padmapriya [38]***	**Saini, Chauhan [39]***	**Fagbohun, Babalola [36]***	**This work***
Plant parts	Fruit	leaves	Fruit	Fruit	Fruit	Fruit	Fruit
Alkaloids	-	+	+	+	+	+	+
Anthraquinone	NA	NA	NA	NA	NA	NA	+
Carbohydrate	NA	NA	NA	NA	NA	+	+
Flavonoids	+	+	+	+	+	+	+
Glycosides	+	+	NA	-	+	+	+
Phenols	+	+	+	-	NA	+	+
Quinones	NA	NA	NA	+	NA	NA	+
Reducing	NA	NA	NA	NA	+	NA	NA
sugars	+	+	NA	-	-	+	+
Saponins	-	+	NA	+	NA	+	+
Steroids	+	+	+	-	+	+	+
Tannins	+	NA	+	-	+	-	+
Terpenoids							

(+) present; (-) below detectable limit; (NA) not available (#) methanolic extract; (*) ethanolic extract; (~) water extract

One of the phytochemical groups present in high amounts in the fruits of the plant under study is the flavonoids which are known for their antioxidant nature [40]. The anti-oxidant properties of the flavonoid group makes them good reducing agents for the green synthesis of metallic nanoparticles [15]. This makes the fruit of *K. africana* a good precursor for the green synthesis of nanoparticles, especially metallic nanoparticles like CuNPs.

Copper Nanoparticle Synthesis and Characterization

The change of colour of the reaction mixture during the reaction period is the primary indication of nanoparticles synthesis [41]. Upon the addition of the plant extract to aqueous $Cu(CH_3COO)_2$ solution, a change from yellow to green was observed, indicating the formation of copper nanoparticles, (CuNPs). This colour change is as a result of interaction between conduction electrons of metal NPs and incident photons [42].

The UV-spec absorption peak was observed in the region of 560 cm^{-1} – 600 cm^{-1} at an absorbance range of 0.175 abs (**Figure 2**). In addition, a peak around 265 – 300 nm was observed, indicating the presence of phenolic group which is attributed to phenolic compound in the extract used in preparing the NPs, at an absorbance of 0.190 abs.

Antimicrobial properties of the synthesized CuNPs

The in-vitro antimicrobial potential of the synthesized CuNPs was examined against five (5) selected human pathogenic bacteria, namely: *Escherichia coli, Pseudomonas aeruginosa, Shigella sp., Staphylococcus aureus* and *salmonella typhi*, (**Table 3** and **Figure 3**), and 2 pathogenic fungi; *Aspergillus flavus* and *Aspergillus niger* (**Table 4** and **Figure 4**). The CuNPs showed varying activities against all the test organisms. Among the anti-bacterial test, it was found that the synthesized CuNPs showed the highest inhibitory zone against *P. aeruginosa* (17.0±4.34 mm), followed by *Shigella sp.* (16.0±5.66 mm), *E. coli* and *S. aureus* (both 8.0±2.83 mm), and *S. typhi* (6.0±2.83 mm); while among the pathogenic fungi, *A. flavus* showed the highest sensitive than *A. niger*. The clear zones of inhibition in the regions where the organisms were treated with the synthesized NPs as shown in **Figures 5** and **6** justifies the results. In **Figure 5**, the clearest zone of inhibition was observed in the CuNPs-impregnated disc against *P. aeruginosa*, indicating the organism is more sensitive to the antimicrobial strength of the CuNPs; while in **Figure 5**, a clearer zone of inhibition was observed in the copper region where *A. flavus* was treated with the NPs. These results are similar to previous findings obtained on the treated of both human and plant pathogens using plant-synthesized metal nanoparticles [41, 43-47].

Table 3. Result of antibacterial screening

Bacteria	**Zone of inhibition (mm)**
Escherichia coli	8.0 ± 2.83
Pseudomonas aeruginosa	17.0 ± 4.24
Salmonella typhi	6.0 ± 2.83
Shigella sp.	16.0 ± 5.66
Staphylococcus aureus	8.0 ± 2.83

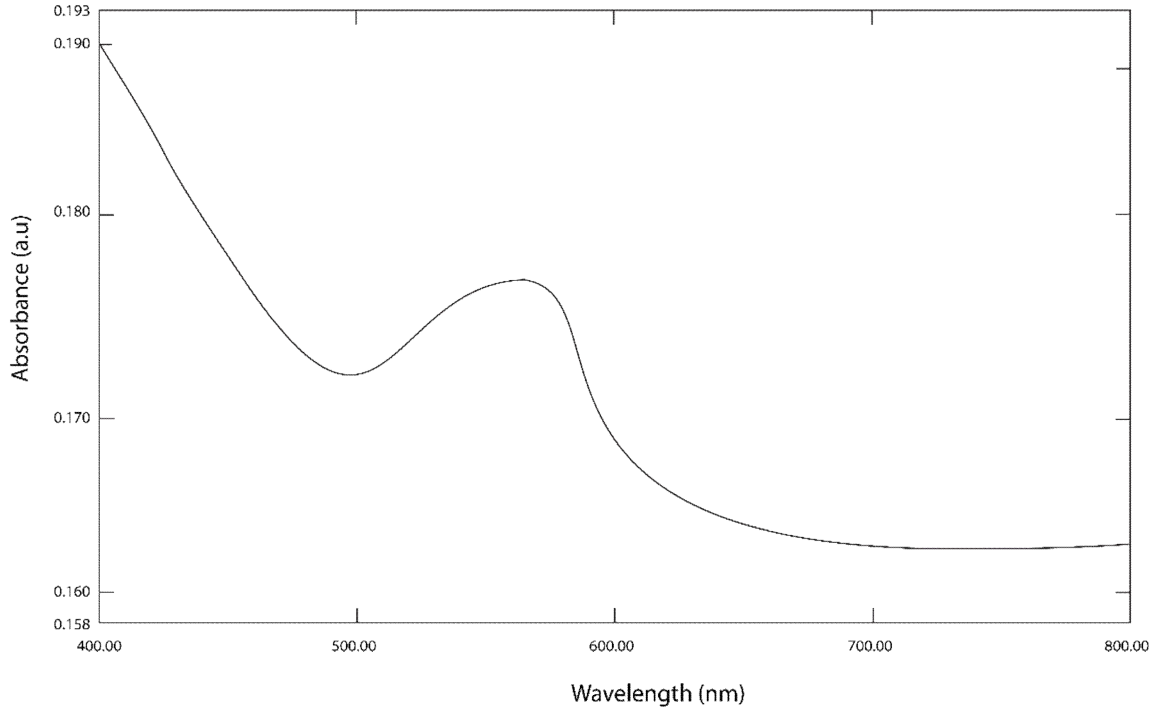


Figure 2. UV spectroscopy result for the synthesized copper nanoparticles

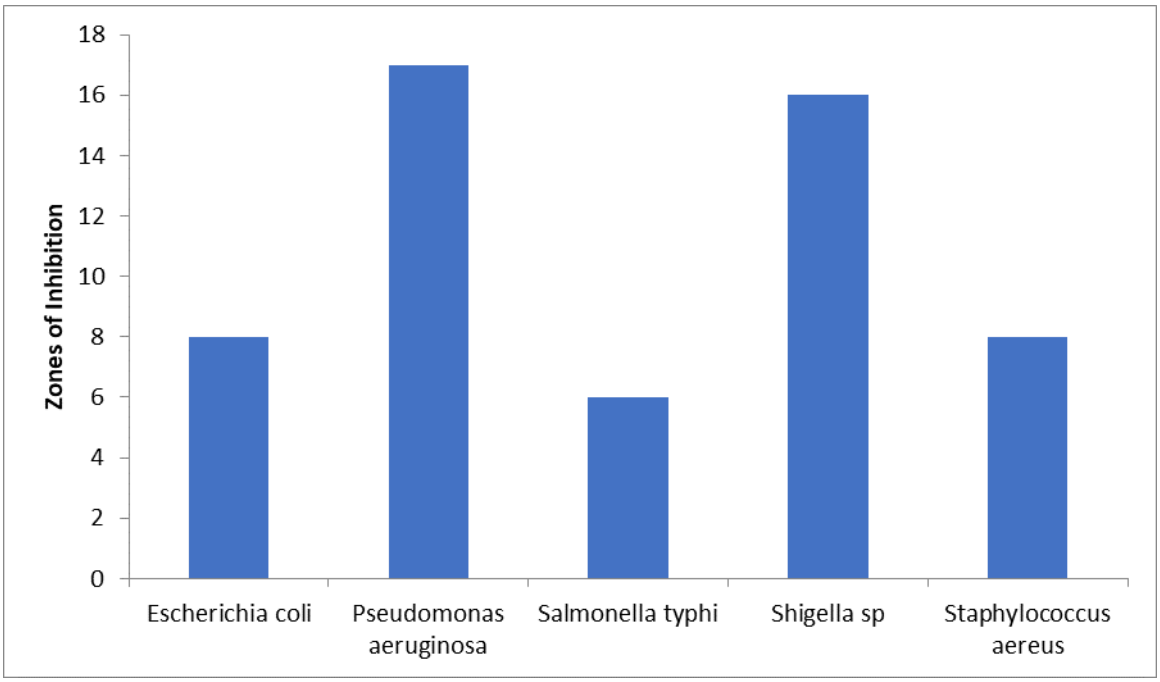


Figure 3. Graphical representation of antibacterial activity of CuNPs against selected pathogenic bacteria

Table 4. Result of anti-fungal screening

Fungi	Zone of inhibition (mm)
Aspergillus flavus	8.0±2.83
Aspergillus niger	3.0±4.24

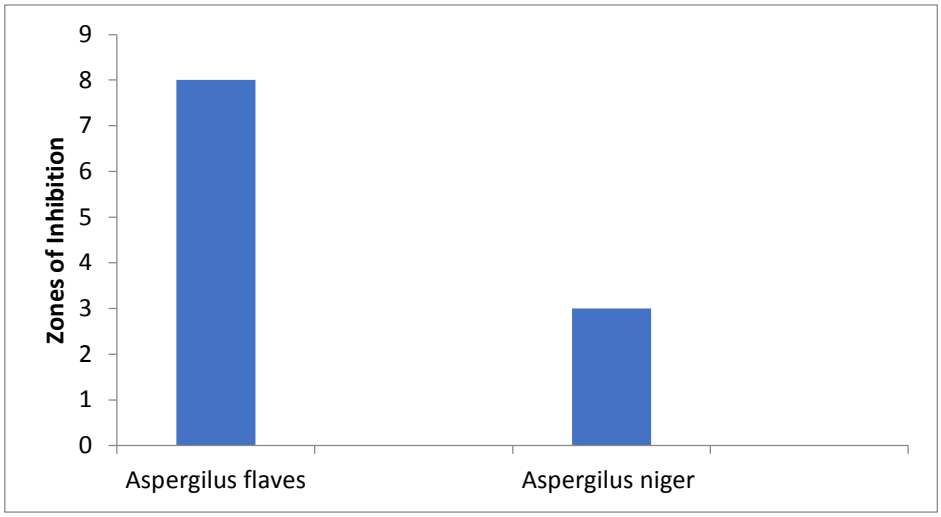


Figure 4. Graphical representation of antifungal activity of CuNPs against selected pathogenic fungi

The excellent adaptation of bacteria coupled with the illegal use of herbal medications has resulted to an increase in the number of drug resistant organisms [48]. The major challenge facing the global public healthcare is development of novel and more potent microbial drugs to overcome this [44]. The technology of nanoparticles provides a novel step towards improving drug development in this regard, and in particular, plant-mediated NPs with many biological properties than the chemically-synthesized NPs, providing several profound advantages, such as easy of operations in drug delivery, bio-labelling, sensing, food preservation, wound healings, water purification and cosmetics [49]. In general, smaller NPs with higher surface area interact more with bacteria compare to bigger particles, hence their higher antibacterial activity [50].

The biosynthesis of CuNPs using ethanolic extract of *K. africana* provides a simple, efficient and environment friendly approach for the synthesis of nanoparticles. The synthesized CuNPs showed promising anti-microbial activities against all the test organisms, especially against *P. aeruginosa* and *shigella sp*. of the human pathogenic bacteria. The antibacterial property of CuNPs is largely as a result of the release of copper ions (Cu^{++}) which are attached to the bacteria cell wall by electrostatic attraction [50]. Shende, Ingle [51] reported the in-vitro anti-microbial activity of synthesized CuNPs using Citron juice (*Citrus medica* Linn) against some selected species of bacteria, viz*: Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Propioni acnes* and *Salmonella typhi*; and 3 plant pathogenic fungi: *Fusarium culmorum, Fusarium oxysporum* and *Fusarium graminearum*.

The synthesized CuNPs showed significant activity against all the test organisms, with *E. coli* the most sensitive bacterium, and *F. culmorum* the most sensitive fungus.

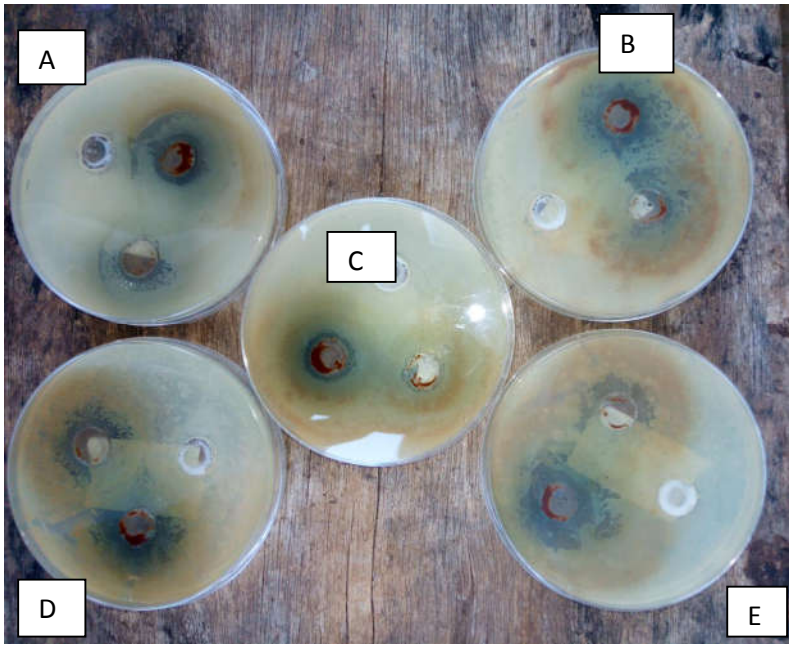


Figure 5. Zone of inhibition around discs impregnated with copper nanoparticles against: (A) *E. coli;* (B) *Salmonella typhi;* (C) *Pseudomonas aeruginosa;* (D) *Shigella sp; (E) Staphylococcus aureus*

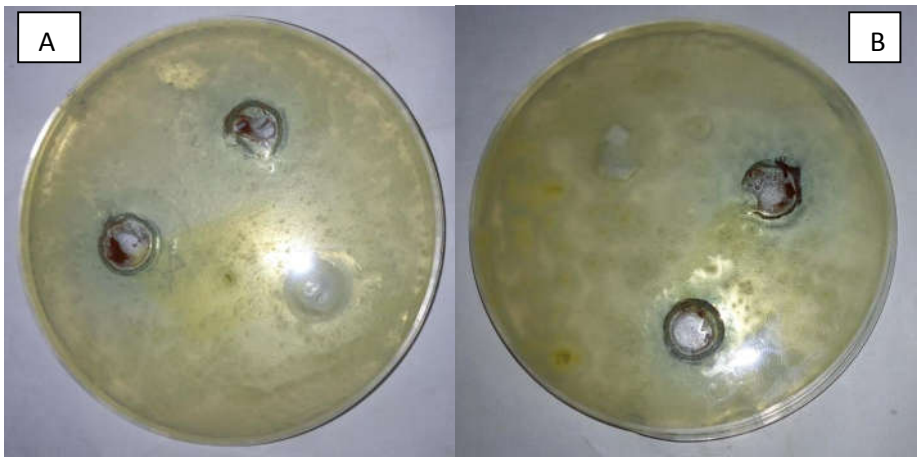


Figure 6. Zone of inhibition around discs impregnated with copper nanoparticles against: (A) *Aspergillus niger;* (B) *Aspergillus flavus*

This anti-microbial property of the CuNPs justifies its applications in different formulations such as nano-fungicides, nano-antimicrobials and nano-fertilizers.

Fruit extract of *Ziziphus spina-christi* (L) Willd was used to synthesized CuNPs by Khani, Roostaei [52]. Among other applications, the CuNPs were used to study the anti-bacterial activity of Gram-negative organism, *Escherichia coli* and Gram-positive *staphylococcus aureus*. The extracts at high concentrations (78% and 100%) and methanolic extract were found to exhibit high resistance against the test organisms, but only showed moderate inhibition in low concentrations (25% and 50%). In another study, *K. africana* fruit extract was used to synthesized copper-silver bimetallic nanoparticles and evaluated for their antimicrobial activities [20]. The fabricated AgCuNPs showed high inhibition zones against

all test bacteria (*K, pneumoniae, E. coli, S. aureus* and *P. aeruginosa*) and the fungus, *Candida albicans*, with *S. aureus* showing the highest zone of inhibition (27 mm).

Rajesh, Ajitha [50] reported that the synthesis of CuNPs prepared from *Syzygium aromaticum* (clove) bud extract showed positive test results against selected pathogens. The bio-synthesized CuNPs showed prominent bactericidal activity against *Staphylococcus sp., Escherichia coli, Pseudomonas sp.,* and *Bacillus sp.,* with the highest zone of inhibition of 8 mm at 16 µl of CuNPs volume observed in Bacillus sp. Antifungal activities of the bio-CuNPs were also studied against *Aspergillus niger, Aspergillus flavus* and *Penicillium sp.* fungal test pathogens, with the CuNPs reportedly showing a pronounced fungicidal activity against *Penicillium sp.* with a zone of inhibition of 6 mm at 16 µl of CuNPs volume. With the present study clearly showing higher inhibitory zones in all test organisms except *A. niger*, it can be surmised that the *K. africana*-synthesized CuNPs has excellent antimicrobial activities.

CONCLUSION

Several key conclusions were derived from this study. Based on the observation of the study, the research methodology implemented was successfully able to synthesize CuNPs using the fruits of *Kigelia africana* as a precursor. Preliminary phytochemical analysis revealed the presence of flavonoids which are good reducing agents for the synthesis of metallic nanoparticles. The in-vitro anti-microbial potential of the synthesized CuNPs was examined against five (5) selected human pathogenic bacteria, namely: *Escherichia coli, Pseudomonas aeruginosa, Shigella sp., Staphylococcus aureus* and *salmonella typhi*, and 2 pathogenic fungi; *Aspergillus flavus* and *Aspergillus niger*. The highest inhibition activity was exhibited by *Pseudomonas aeruginosa* (17.0 ± 4.24 mm). The CuNPs showed considerable antifungal activity against *Aspergillus flavus* and *Aspergillus niger* with inhibition activity of 8.0 ± 2.83 mm and 3.0 ± 4.24 mm respectively. Conclusively, the green synthesized CuNPs using *Kigelia africana* should be incorporated as a therapeutic drug for microbial infectious disease and other health associated disorders.

Disclosure statements

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Declaration of Interest: none

Conflict of Interest: The authors declare that there are no conflicts of interest.

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