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Characterization and Antimicrobial activity of Copper nanoparticles synthesized using Anthocyanin-rich aqueous extracts of *Costus afer*

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Abstract

Copper nanoparticles (CuNPs) were synthesized using anthocyanin-rich aqueous extracts of *Costus afer* flowers as reducing and capping agents. The reduction process of Cu^{2+} to Cu^{0} was determined by the change of colour from red to brown after the addition of copper nitrate to the aqueous extract of *Costus afer* flowers. The formation of copper nanoparticles was monitored by UV and IR spectrophotometry. Simple qualitative tests were carried out on the *Costus afer* flower extract to determine the presence of anthocyanins and other phytochemicals which could be responsible for the reduction process. The size and morphology of the CuNPs were further assessed through XRD and SEM. The CuNPs showed spherical morphology with an average size of 1.3 nm. Thermogravimetric analysis of the nanoparticles revealed that the bioactive capping agents decomposes between 220 and 280 °C and is 51% of the mass of the nanoparticles. The antimicrobial activities of the nanoparticles were evaluated against some pathogenic microorganisms.

Keywords: Synthesis, Copper nanoparticles, *Costus afer*, FTIR, XRD, SEM and TGA analyses, Antimicrobial activity.

Introduction

Nanoparticles are particles with sizes between 1 nm and 100 nm in one, two or three dimensions [1]. Nanoparticles are being evaluated for use in many fields because of their peculiar properties compared to large-sized particles. Transition metal nanoparticles have wide application in chemistry, physics, biotechnology, and other disciplines [2] and copper nanoparticles are widely used due to their electrical, optical, catalytic, antimicrobial, and biomedical properties [3]. Copper nanoparticles are very reactive due to their high surface-to-volume ratio, and interact easily with other particles [4]. They also act as antimicrobial agents as they are highly toxic to microorganisms such as *Escherichia coli*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*. Several studies have established the potentials of copper-containing therapeutics in biomedical fields using *in vitro* and *in vivo* models [5,6]. Furthermore, copper nanoparticles are used as catalyst in water–gas shift reactions and detoxification processes [7]

Nanotechnology plays an important role in modern research. The technology can be

applied in almost all fields, including electronics, chemical industry, energy science, space industries, mechanics, cosmetics, environmental health and health care, food, biomedical science, pharmaceutical, drug, and gene delivery [8]. Biosynthetic processes have lots of advantages in the synthesis of nanoparticles due to cost-effectiveness, eco-friendliness and easier approach than physical and chemical methods. This biosynthetic method (use of plants, bacteria, fungi) offers the cheapest and simplest method for synthesis of nanomaterials [9]. Synthesis of nanoparticles by plants is a green chemistry approach; plant extracts are used for the metal ion reduction to form nanoparticles. It has been demonstrated that plant metabolites such as terpenoids, alkaloids, phenolic compounds, sugars and proteins play important roles in metal ion reduction into metallic nanoparticles and in stabilizing the nanoparticles. Using plants for the synthesis of nanoparticles is advantageous over other environmentally benign biological systems.

Many plants have been used for the synthesis of nanoparticles. The nanoparticles synthesized using plant extracts were found to contain many phytochemicals and have the advantage of the medicinal properties of the plant extracts which could be used as drugs, in targeted drug delivery and cosmetic applications [10]. *Costus afer*, also known as monkey sugar cane, contains many potentially bioactive compounds such as alkaloids, flavonoids, saponins, phenols, tannins, anthraquinones, cardiac glycosides and terpenoids. These phytochemicals in the plant makes it a very beneficial one. Alkaloids are known to have antimicrobial, antifungal and anti-inflammatory effects [11] and also act as anti-hypertensive agents [12]Extracts of the flowers of *Costus afer* contain additional valued water-soluble pigments known as anthocyanins.as well as small units of sugar at the base of the fresh flower. Thus, the presence of these two important substances (anthocyanins and sugars) as well as other phytochemicals present in this plant makes it a unique asset for drug discovery. It has many medicinal values such as simple prevention and treatment of venereal diseases; it reduces and serves as eye drops, reduces stomach ache and other inflammatory symptoms [13,14].

Materials and Methods **Materials**: Cu $(NO_3)_2 2.5H_2O$ and 0.2 µm membrane filter paper were from VWR chemicals and deionized water was used for all experiments. The FTIR spectra were recorded in the solid phase at a range of 3900 – 400 cm^{-1} on Bruker Tensor 27 at a resolution of 1 cm^{-1}. Ultraviolet−visible (UV−Vis) spectroscopy was acquired with a JASCO V-670 spectrophotometer.

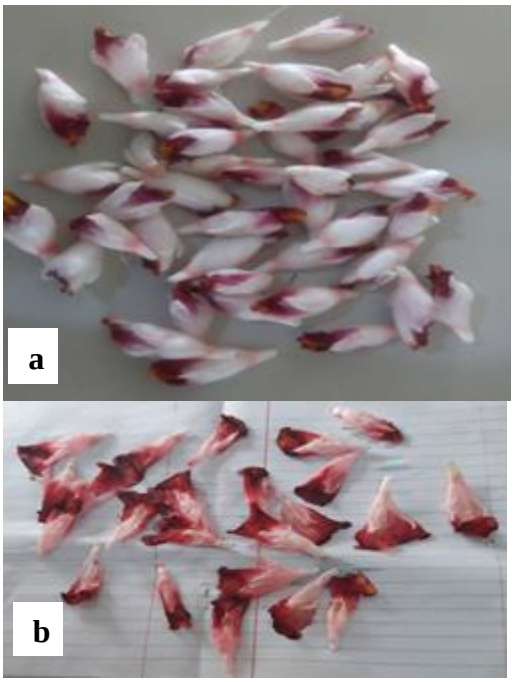

Figure 1 (a) fresh and (b) dried *Costus afer* flowers

Harvest and Preparation of *Costus afer* flowers: Fresh *Costus afer* flowers were harvested around the premises of Federal University Otuoke, Nigeria and authenticated at University of Port Harcourt reference Herbarium with reference number UPH/V/1289. The flowers were dried at 40 ^{o}C then stored in a polyethylene bag. The copper nitrate has 99% purity.

Preparation of Flower Extracts: The flower extracts were prepared by taking 4 g of dried flowers into a 250 ml beaker with the addition of 100 ml of distilled water and then stirred for 15 minutes. The mixture was left to stand incubated in a cupboard for 1 hour at 25 ^{o}C. The extract was collected by filtration using Whatman filter paper

a

Figure 2: (a) *Costus afer* aqueous solution and (b) After addition of 0.1 M copper nitrate solution

Synthesis of Copper Nanoparticles

For the reduction of Cu^{2+} ions, 1 ml of *Costus afer* flower extract was added into a clean test-tube and then 5 ml of 0.1 M aqueous $Cu(NO_3)_2$ $2.5H_2O$ solution was added to the extract. On addition of copper nitrate solution to the extract, a colour change was observed after 5 minutes from red to brown colour and became darkened as time progresses. After one hour, precipitates were formed at the base of the test-tubes.

Figure 3: progressive formation of copper nanoparticles after the addition of copper nitrate solution to aqueous extract of *Costus afer*. Beakers labelled B_R and B_L contain precipitated CuNPs solution and plant extract respectively. Each of the test-tubes labelled 1 to 6 from right contain copper nanoparticles, 2 ml of extract, 2 ml of extract and 0.1 ml of copper solution, 2 ml of copper solution and 2ml of extract, 2 ml of extract and 0.5 ml of copper solution, 2 ml of extract and 1 ml of copper solution

and 0.5 ml of extract and 2ml copper solution respectively.

Table 1: Table showing the contents of beakers and test-tubes in figure 3.

S/N	LABEL	CONTENT
	B_L	precipitated CuNPs solution
	B_R	plant extract only
	1	2 ml of extract
	2	2 ml of extract and 0.1 ml of copper solution
	3	2 ml of copper solution and 2 ml of extract
	4	2 ml of extract and 0.5 ml of copper solution
	5	2 ml of extract and 1 ml of copper solution
	6	0.5 ml of extract and 2ml copper solution

Qualitative Phytochemical Analysis for *Costus afer*

Test for Saponins: 2 ml of aqueous extract of *Costus afer* was transferred into a test-tube and was vigorously shaken; the formation of froth (foam) indicates the presence of saponins

Test for Phenols: 5 ml of ethanol was added to 5 ml of *Costus afer* filtrate and 5-drops of iron (III) chloride. A yellow-greenish precipitate indicates the presence of phenols

Test for Carbohydrates: Few drops of iodine were added to fresh *Costus afer* flower extract. A blue-black solution indicates the presence of carbohydrates.

Test for Flavonoids: 2 ml of dilute sodium hydroxide was added to 2 ml of the extract, the appearance of a yellow colour indicates the presence of flavonoids.

Test for Anthocyanins: drops of H_2SO_4 was added to 1 ml crude extract. A deep red coloration indicates the presence of anthocyanin.

Tests for Steroids: 2 ml of acetic anhydride was added to 0.5ml of ethanolic extract with subsequent addition of 2 ml of conc. H_2SO_4. A violet color which changed to green indicates the presence of steroids.

Antimicrobial Studies: Pure cultures of the test organisms were inoculated into broth medium and incubated at 37 ^{o} C for 24 hours and 28 ^{o} C for 72 hours for bacteria and fungi respectively. All Gram-positive and fungi isolates were serially diluted to factor 3 using the 10-fold dilution; Gram negative isolates were serially diluted to factor 5. The last dilution for each isolate was compared to MacFarland standard to match their turbidity. Well in agar diffusion method was used to test the antimicrobial activities of the nanoparticles on the test isolates. 0.5 ml of each of the diluted test organisms was aseptically spread on the surface of the Mueller-Hinton agar plates using sterile hockey stick. Two concentrations of the test nanoparticles (0.5mg/ml and 0.25 mg/ml), prepared in DMSO, were added to 5 mm holes bored on the agar plates. All plates were then incubated at 37 ^{o} C. for 34-48 hours and 72

hours – 5 days for bacterial and fungal isolates respectively. Antimicrobial activities of the complexes against microbial isolates were determined by measuring the zones of inhibition in millimeter. Control experiments were set up using DMSO while streptomycin and nystatin were used as reference drugs for bacteria and fungi assay respectively.

RESULTS AND DISCUSSION

Phytochemical Analysis of Costus *afer* flower extracts

Table 2. Phytochemicals Present in *Costus afer* Flower Extracts

Phytochemicals	Present	Absent
Phenols	++	
Steroids	++	
Carbohydrates	++	
Tannins		-
Flavonoids	++	
Saponins	++	

Phytochemical constituents of aqueous *Costus afer* extracts are presented in Table 2. This screening indicates phytochemicals such as phenols, carbohydrates, saponins, flavanoids, and steroids [15,16] to be present in the flowers which were responsible for the reduction of metal ions and capping of the nanoparticles.

UV-Vis Spectroscopy Analysis

The aqueous extract of *Costus afer* flowers contain chromophores which were able to absorb light within the 400-800 nm range, as shown in Figure 4.

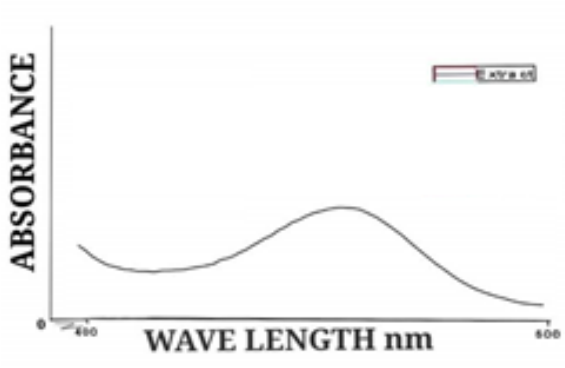

Figure 4. The UV-Vis spectra of *Costus afer* extract showing an absorbance maxima of 515 nm

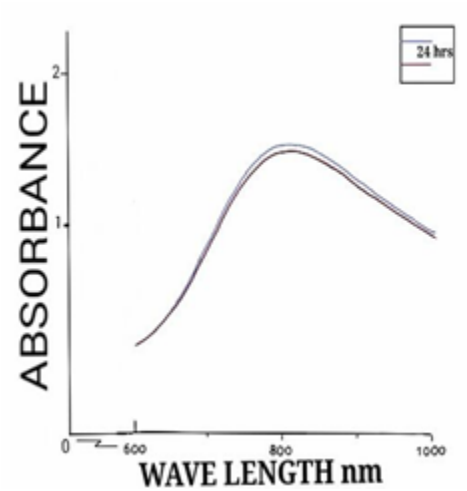

Figure 5. The UV-Vis spectrum of 0.1M aqueous solution of copper nitrate and *Costus afer* flower extracts as a function of time of reaction after 24 hours.

It is evident that the more the reducing agent and/or the capping agent reacts with the copper nitrate, the more the copper nanoparticles are formed. That is, the higher the reduction of copper nitrate by the flower extracts, the more nanoparticles are formed and are stable with time at 0.1 M concentration of copper nitrate solution.

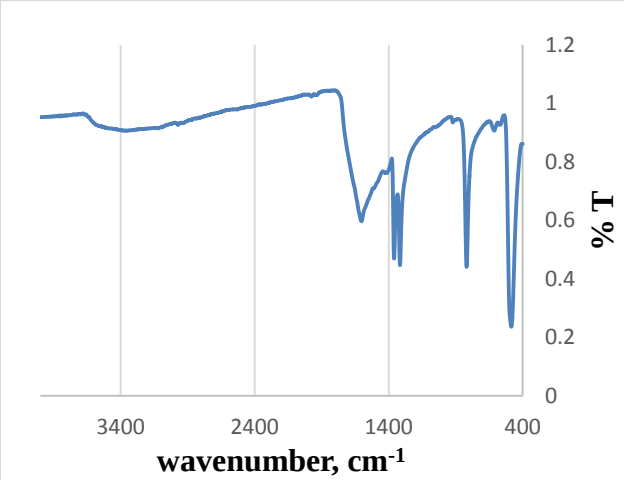

Figure 6. FTIR Spectra of synthesized copper nanoparticles

The multifunctional groups present in the synthesized copper nanoparticles and the *Costus afer* flower extract were identified using FTIR analysis. The characteristic peak at 1587 cm-^{1} is due to –C=O stretching and C=C groups of capping agents from the extracts present on the surface of the copper nanoparticles. The peak at 1317 cm-^{1} can be assigned to in-plane OH bending of carboxylic acids. The peak at 1315 cm^{-1} can be assigned to C-O stretch while the peak at 480 cm^{-1} can be assigned to C-N bond.

These peaks suggested the presence of flavonoids and other polyphenolic compounds in the aqueous leaf extract

Costus afer, which could be responsible for the reduction of copper ions to their corresponding copper nanoparticles [17]. The above results confirm the role and importance of bioactive compounds in the synthesis of copper nanoparticles [10]. The absence of the frequency mode at around 610 cm^{-1} excludes the formation of Cu_2O [18]. The absence of three absorption peaks at 588, 534 and 480 typical of CuO also precludes the formation of CuO.

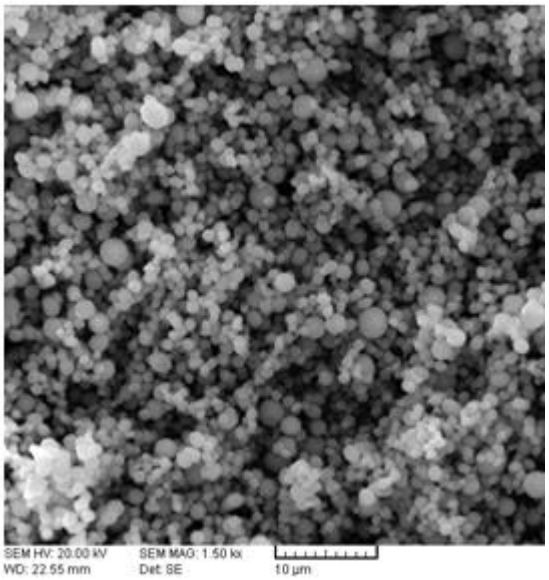

Figure 7. SEM image of the as-synthesized copper nanoparticles

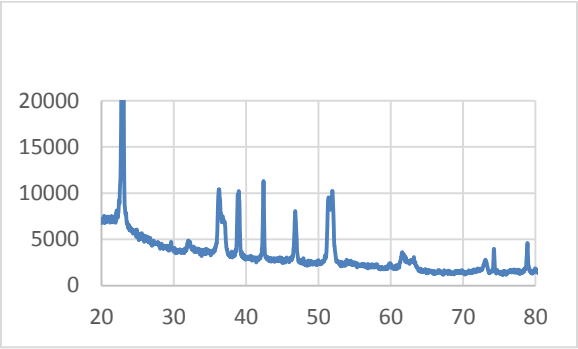

Figure 8. Powder XRD of the as-synthesized copper nanoparticles

Characterization of Size and Morphology of the Nanoparticles

The SEM image (Figure 7) shows the average particle size of the nanoparticles to be in the range of 1.3 nm; they are spherical and have smooth surfaces. It is noteworthy that the SEM images which were taken more than one month after their preparation show that the final nanoparticles are very stable and therefore, can be used without any further stabilizers. In addition, the powder diffraction pattern (Figure 8) of the nanoparticles confirm the crystallinity of the nanoparticles.

TGA was employed to measure the relative composition of the organic capping agents on the nanoparticles. Figure 9 shows the TGA curve obtained for the as-synthesized copper nanoparticles that were heated from 40 to 900 °C under nitrogen. The decrease in mass due to the removal of the capping agents took place between 220 and 280 °C. The decrease in mass below 220 ^{o} C was due to evaporation of solvent (water) molecules. Increase in mass observed at temperatures beyond 350 °C was due to the oxidation of the copper metal to copper oxides. The remaining mass of the particles above 280 ^{o} C is from copper particles and is 42 %. About 51 % of the sample mass is due to the capping shell while 7 % of the mass was solvent.

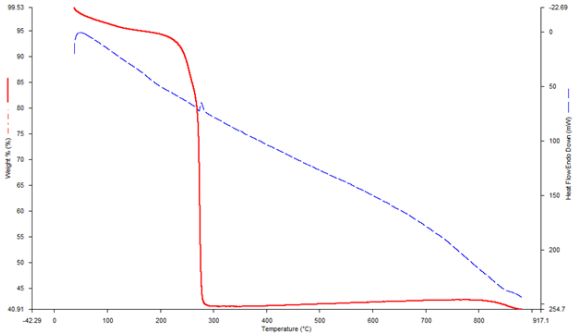

Figure 9: TGA curve for as-synthesized copper nanoparticles

Antimicrobial data

Table 3. Zones of inhibition diameter (mm) of as-synthesized copper nanoparticles

Concentration / Organisms	Compound		
	CHR		Reference drug
	C_1	C_2	0.2 mg
Candida Albicans	10	8	30
Staphylococcus aureus	15	12	25
Pseudomonas	6	N_A	20
Bacillus Subtilis	10	N_A	11
A. niger	10	N_A	18
E. coli	25	15	22

C_1 = 0.5 mg/ml C_2 = 0.25 mg/ml N_A = no activity

The synthesized nanoparticles were screened for antimicrobial susceptibility against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Aspergillus niger* and *Candida albicans.* Results of the antimicrobial susceptibility tests are shown in Table 3. At concentration that doubles that of the reference drug (streptomycin), the nanoparticles displayed similar inhibitory activity against *Bacillus subtilis* and better activity against *E. coli* compared to the reference drug, streptomycin. This activity of the nanoparticles can be ascribed to the

synergistic effect of the nano-sized copper and the phytochemical capping agents.

CONCLUSION

In this study, copper nanoparticles were successfully synthesized using the aqueous extracts of *Costus afer* through a cost-effective, environmentally benign and simple method. Synthesis of nanoparticles using this method was completed after 24 hours. These copper nanoparticles showed characteristics decreasing absorption peaks at 815 nm in visible spectra region as Cu^{2+} ions were being reduced to Cu^{0}. It was clearly seen that the extracts successfully reduced copper ions (Cu^{2+}) to copper nanoform (Cu^{0}). FTIR spectroscopy confirmed the functional groups present on the surface of the synthesized copper nanoparticles. Thermogravimetric analysis showed that 51% of the particles were of the bioactive capping agents.

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