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RESEARCH PAPER

# Comparative Studies of Synthesized Copper Oxide Nanoparticles using Aqueous Extract of Leaves

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**ABSTRACT**

The use of plant extracts as reducing agents in the synthesis of metal and metal oxide nanoparticles has come into prominence due to their procedural simplicity, precursor's eco-friendliness, and cost effectiveness. The phytochemicals in plant extracts works as a reductant when mixed with metal salt solution. In this study, we compared the percentage yields of copper oxide (CuO) nanoparticles synthesized using aqueous leaf extracts of *Psidium guajava* (guava), *Terminalia catappa* (tropical almond), *Cymbopogon citratus* (lemon grass), and *Tithonia diversifolia* (mexican sunflower) at varying volume-ratios with copper sulphate standard solutions. The optimization study of the biosynthesis mixtures revealed that the mixture with the volume-ratio 1:1 of Cu salt : plant extract gave the highest yields in all samples. The nanoparticles were characterized using Fourier-transformed infra-red (FT-IR) to confirm characteristic bands expected of metal oxide particles at the finger print region. The transmission electron microscope (TEM) presented the average sizes within the range of 3.68 and 7.92 nm, while the energy dispersive X-ray Spectroscopy (EDS) confirmed their high purity due to the absence of other elements except carbon. The biosynthesized nanoparticles are highly pure with their small sizes and this makes them suitable for adsorption applications.

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## INTRODUCTION

In recent years, there has been a rapid increase in enthusiasm for nanotechnology and the use of nanoparticles in commercial applications. Nanotechnology is a branch of science that deals with the study of nanoparticles, particles or materials with the size in the range of 1-100 nm in at least one of its three possible dimensions [1]. The great potential of nanoparticles is due to unique properties of substances when they are reduced to nanosized. Nanoparticles have special and enhanced physical and chemical properties as compared to their bulk materials due to their large reactive and exposed surface area and quantum size effect resulting from specific electronic structures. These particles have been widely used in many fields such as electronics, photochemical industry, biomedicine, food industry, environmental applications, and cosmetics [2].

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Metal and metal oxide nanoparticles have drawn considerable attention due to their application in biomedical field in terms of biosensing, imaging, diagnose, and therapy [3]. Copper oxide nanoparticles (CuO NPs) are widely used in biomedical applications as antimicrobial, anti-fouling, antifungal, antibiotics, antioxidants, drug delivery, anticancer, and other applications such as biocide, photocatalysis, and electronic applications [4]. Although CuO NPs have proved their use in biomedical applications, they have potential toxic effects [5]. CuO NPs may increase the production of reactive oxygen species in mammalian cell, leading to oxidative stress and damage of DNA and mitochondria [6,7].

Different methods have been applied for the commercial production of CuO NPs which include sonochemical, precipitation, sol-gel, chemical reduction, chemical bath deposition, hydrothermal approach, electrothermal, and green chemistry routes [8].

The synthesis of nanoparticle using an efficient green chemistry method has become a major focus of researchers. Green method is more preferable in the synthesis of Cu NPs than other methods due to its cleanliness, nontoxicity, cost-effectiveness, and eco-friendliness. It utilizes biological materials such as bacteria, yeasts, fungi, algae and plant rather than harsh toxic and expensive chemicals used in other methods [3]. One of the commonest green methods of copper nanoparticles synthesis is the use of plant phytochemicals such as terpenes, phenolic compounds, and alkaloids as reducing and capping agents for synthesizing metal nanoparticles [9].

The physical and chemical properties of nanoparticles depend on their compositions, as well as crystal and surface structures. FT-IR (Fourier-transform infra-red) spectroscopy uses both its functional group and finger print regions to identify functional groups and metal-oxide bonds present in the nanostructure and plant extracts (phytochemicals) that play a role in the formation of the metallic nanoparticles [10]. Energy dispersive X-ray spectroscopy (EDS or EDX) is a powerful technique that is employed to analyze the elemental composition of nanoparticles. In addition, transmission electron microscopy (TEM) is a powerful and unique technique for analyzing structure morphology such as the size and shape [11].

In biosynthesis of nanoparticles, the yield and composition of synthesized nanoparticles depend on the plant species, which partially dictates the phytochemical composition of the plant, as well as the volume-ratio of the reaction mixtures [12].

Table 1: Plant extract label

| Plant leaf | Sample label |
|---|---|
| Mexican Sunflower | A |
| Tropical Almond | B |
| Guava | C |
| Lemon grass | D |

This research aimed to compare the percentage yields, FT-IR, EDS, and TEM spectra of the biosynthesized copper oxide nanoparticles (CuO NPs) using four different leaves aqueous extracts at varying mixture volume-ratios.

## MATERIALS AND METHODS

*Plant sample collection and preparation*

Fresh leaves of the plants, *Psidium guajava* (guava), *Terminalia catappa* (tropical almond) *Cymbopogon citratus* (lemon grass), *Tithonia diversifolia* (mexican sunflower) were collected from Osun State Polytechnic, Iree, Nigeria (Geographical coordinates, 7.55 North and 4.43 East). These leaves were cut, washed, and rinsed twice with distilled water.

*Preparation of plant extracts solutions*

The preparation of the leaves extract follows the method employed by Oyetola (2023)[12] according to which 'for each of the leaves, a 10 % w/v leaves concentration was prepared by soaking the washed leaves weighing 1000 g into 10 L of distilled water at room temperature i.e. 28°C, left for seven (7) days, after which a clean meshed cloth was used to separate the filtrate from the residue and 1 mm Whatmann filter paper was use to re-filter the filtrate. The leaves extracts were labelled according to the table 1 and kept at 4°C until further use.'

*Preparation of Copper salt and Base solutions*

A 0.01 M $CuSO_4$ was prepared by dissolving 1.6 g of the salt into 1 L of distilled water using 1000 mL volumetric flask, while 0.01 M NaOH was prepared by dissolving 0.4 g of the alkali into 1 L of distilled water using 1000 mL volumetric flask (i.e., 0.29 g, 0.44 g and 0.58 g are contained in 180 mL, 270 mL and 360 mL, respectively, in the copper salt standard solution). All chemicals and reagents in this work were of analytical grade and used

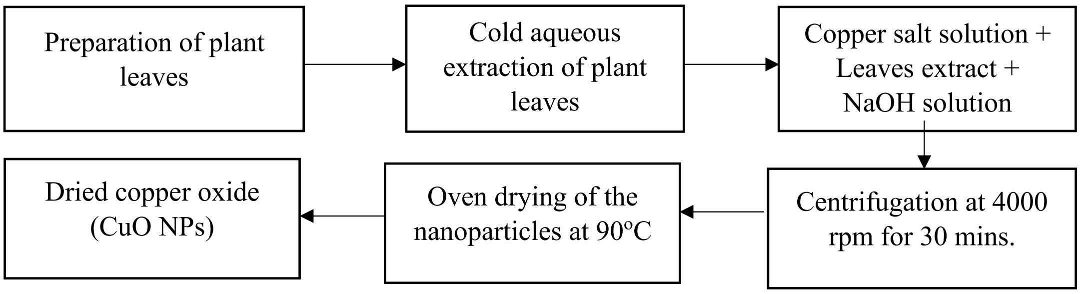


Fig. 1: Flow chart of the biosynthesis

without further purification. Standard solutions were prepared and used where required.

*Phytochemical Screening*

Standard procedures were employed to test for the presence of phytochemicals:

(i) Saponins: 5 mL of each leaves extract was mixed with 20 $cm^3$ of distilled water and then agitated in a graduated cylinder for 15 minutes. The formation of foam indicates the presence of Saponins [13].

(ii) Phenols: leaves extracts were mixed with 2 $cm^3$ of 2 % $FeCl_3$ solution. A black color indicated the presence of phenols [14].

(iii) Flavonoids: 5 drops of 20 % sodium hydroxide solution were added to each leaves extract. A change to yellow color, which on addition of acid changed to colorless solution, indicated the presence of flavonoids [14].

(iv) Anthraquinones: This was done by adding few drops of 2 % hydrochloric acid to each leaves extract. Appearance of red color indicated the presence of anthraquinone [12].

(v) Anthocyanins: 2 mL of extract was added to 2 mL of 2 M HCl and Ammonia. The appearance of pink-red which turns to blue-violet demonstrated the presence of anthocyanins [14].

(vi) Coumarins: 4 $cm^3$ of 10 % NaOH was added to 2 $cm^3$ of extract and the formation of yellow color indicated the presence of coumarins [14].

(vii) Emodins: To 3 mL of the extract, 3 mL of $NH_4OH$ and 5 mL of Benzene was added. The appearance of red color showed the presence of emodins [15].

(viii) Terpenoid: To 2 mL of the plant extract, 2 mL of acetic anhydride, and 2 mL of concentrated $H_2SO_4$ were added. The formation of blue green ring indicated the presence of terpenoids [14].

(ix) Steroids: 1 mL of the plant extract was dissolved in 10 mL of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turned red and sulphuric acid layer became yellow with green fluorescence. This indicated the presence of steroids in each of the plant leaves aqueous extracts [14].

*Biosynthesis and optimization process*

Table 2 presents the varying precursors volume combinations with the aim of knowing higher yields samples. The biosynthesis reaction took place at room temperature, and each mixture was thoroughly mixed by stirring with a glass rod for 2 minutes at the initial mixing and left for three (3) days to bring the biosynthesis process into completion (during the biosynthesis reaction, +2 oxidation state of copper in the $CuSO_4$ standard solution were being reduced to zero (0) i.e., $Cu^{2+} \rightarrow Cu^0$). The supernatants were decant off and the sediment i.e. the nanoparticles were centrifuged at 4000 rpm for 30 minutes, the centrifugation of each sample was carried out thrice with fresh distilled water so as to make nanoparticles free from unreacted components. The centrifuged samples were oven-dried at 90°C for 4 hours to obtain the dried nanoparticles since one of the ways for the formation of copper oxide nanoparticles from its copper nanoparticles is through heating and atmospheric oxygen interaction [12,16]. Fig. 1 shows the flow chart of the biosynthesis process.

*Characterizations of biosynthesized CuO nanoparticles*

The synthesized CuO nanoparticles were characterized using Fourier transform infra-red (FTIR), energy dispersive X-ray spectroscopy (EDS), and transmission electron microscopy (TEM). The FTIR spectra of the selected nanoparticles were acquired in the range of 380–4000 $cm^{-1}$ at a resolution of 4 $cm^{-1}$ using spectrum 100 infrared spectrometer equipped with universal

Table 2: Different Biosynthesis process mixture

| Sample Label | Reaction mixture (Metal salt: Leave extract: Stabilizing agent) | Reacting volume | Ratio |
|---|---|---|---|
| A1 | Copper salt: Mexican sunflower extract: Sodium hydroxide | 270 mL: 270 mL: 180 mL | 1:1 |
| A2 | Copper salt: Mexican sunflower extract: Sodium hydroxide | 180 mL: 360 mL: 180 mL | 1:2 |
| A3 | Copper salt: Mexican sunflower extract: Sodium hydroxide | 360 mL: 180 mL: 180 mL | 2:1 |
| B1 | Copper salt: Tropical almond extract: Sodium hydroxide | 270 mL: 270 mL: 180 mL | 1:1 |
| B2 | Copper salt: Tropical almond extract: Sodium hydroxide | 180 mL: 360 mL: 180 mL | 1:2 |
| B3 | Copper salt: Tropical almond extract: Sodium hydroxide | 360 mL: 180 mL: 180 mL | 2:1 |
| C1 | Copper salt: Guava extract: Sodium hydroxide | 270 mL: 270 mL: 180 mL | 1:1 |
| C2 | Copper salt: Guava extract: Sodium hydroxide | 180 mL: 360 mL: 180 mL | 1:2 |
| C3 | Copper salt: Guava extract: Sodium hydroxide | 360 mL: 180 mL: 180 mL | 2:1 |
| D1 | Copper salt: Lemongrass extract: Sodium hydroxide | 270 mL: 270 mL: 270 mL | 1:1 |
| D2 | Copper salt: Lemongrass extract: Sodium hydroxide | 180 mL: 360 mL: 180 mL | 1:2 |
| D3 | Copper salt: Lemongrass extract: Sodium hydroxide | 360 mL: 180 mL: 180 mL | 2:1 |

diamond crystal attenuated total reflection (ATR) accessory (Perkin Elmer, USA). JEM-ARM200F-G transmission electron microscope was employed for the TEM analysis to determine the size and shape of the synthesized CuO nanoparticle. The sample was made to be semi-transparent to allow the passage of electron beams through it; to achieve this semi-transparent nature, the sample is sectioned into fine sections using the glass attached to a device known as ultra-microtome. The device has a trough that is filled with distilled water; the section cuts were collected in this trough which were then moved to a copper grid to be viewed under the microscope. Energy dispersive X-ray spectroscopy (EDS) was applied to analyze the elemental constituents of the synthesized CuO nanoparticle using JEOL, Oxford-EDS system.

## RESULTS AND DISCUSSION

*Phytochemical screening*

Table 3 presents the results of the phytochemical screening which are in line with Oyetola (2023)[12] work on the same leaves species. The phytochemicals tested on the leaves extracts were anthraquinones, anthocyanins, coumarins, emodins, flavonoids, phenolics, terpenoids, saponins and steroids. The results indicate the presence of saponins and steroids in all four sample extracts. Anthocyanins, flavonoids and phenolics are present in all samples except *Cymbopogon citratus* (lemon grass) leaves extract. The presence of flavonoids, anthocyanins, and phenolics has positive effects on reducing the strength of the leaves extracts, which agrees with the results of Umar *et al.* (2016) [17]. *Psidium guajava* (guava) leaves extract has the highest phytochemicals presence followed by *Terminalia catappa* (tropical almond) and *Tithonia diversifolia* (mexican sunflower) while *Cymbopogon citratus* (lemon grass) leaves extract has the least. The fewer number of phytochemicals in the lemon grass leaves extracts might have led to its lower yields. It is suggested that the presence of terpenoids and phenolics plays a key role in its bio-reductive ability [18].

*Experimental Percentage Yield*

Table 4 shows the percentage yield of the nanoparticles synthesized. The percentage yield

$$Experimental\ \%\ yield = \frac{\text{weight of the dried nanoparticle}}{\text{calculated weight of the salt}} \times 100\% \quad \text{Eq. (i)}$$

Table 3: Phytochemical screening of the aqueous leaves extract

| Test | Mexican Sunflower (A) | Almond (B) | Guava (C) | Lemongrass (D) |
|---|---|---|---|---|
| Anthraquinones | - | - | - | - |
| Anthocyanins | + | + | + | - |
| Coumarins | - | - | - | - |
| Emodins | - | - | - | - |
| Flavonoids | + | + | + | - |
| Phenolics | + | + | + | - |
| Terpenoids | - | - | + | + |
| Saponins | + | + | + | + |
| Steroids | + | + | + | + |

NB: Present (+) / Absent (-)

Table 4: Percentage yield of the synthesized CuO nanoparticles

| Sample label | Mass of copper salt (g) | Weight of dried nanoparticle (g) | Percentage (%) |
|---|---|---|---|
| A1 | 0.44 | 0.43 | 97.7 |
| A2 | 0.29 | 0.28 | 96.6 |
| A3 | 0.58 | 0.21 | 36.2 |
| Average | | | 76.83 |
| B1 | 0.44 | 0.39 | 88.6 |
| B2 | 0.29 | 0.25 | 86.2 |
| B3 | 0.58 | 0.22 | 37.9 |
| Average | | | 70.90 |
| C1 | 0.44 | 0.42 | 95.5 |
| C2 | 0.29 | 0.27 | 93.1 |
| C3 | 0.58 | 0.26 | 44.8 |
| Average | | | 77.80 |
| D1 | 0.44 | 0.22 | 50.0 |
| D2 | 0.29 | 0.13 | 44.8 |
| D3 | 0.58 | 0.14 | 24.1 |
| Average | | | 39.63 |

was calculated using equation (i):

The optimization of the reaction mixture of each sample indicates that the mixture with the ratio 1:1 of Copper salt to aqueous leaves extract has higher yields when compared to the other two ratios 1:2 and 2:1 in all samples including A, B, C and D. Sample A1 has the highest yield with 97.7 %, followed by sample C1 with 95.5 %, sample B1 with 88.6 % while sample D1 has the lowest yield with 50.0 %. The mean value of the yields of samples A, B, C, and D are 76.83 %, 70.90 %, 77.80 %, and 39.63 %, respectively, implying that the reducing power trend of the aqueous extract against the copper salt solution is C, *Psidium guajava* (guava) > A, *Tithonia diversifolia* (mexican sunflower) > B, *Terminalia catappa* (tropical almond) > D, *Cymbopogon citratus* (lemon grass). This relates to the presence of phytochemicals in the aqueous leaves extract (Table 3), where the yield of nanoparticles is proportional to the reducing ability of the phytochemicals present in the aqueous extract. The absence of phenolics, flavonoids, and anthocyanins may account for the low yield observed in the *Cymbopogon citratus* (lemon

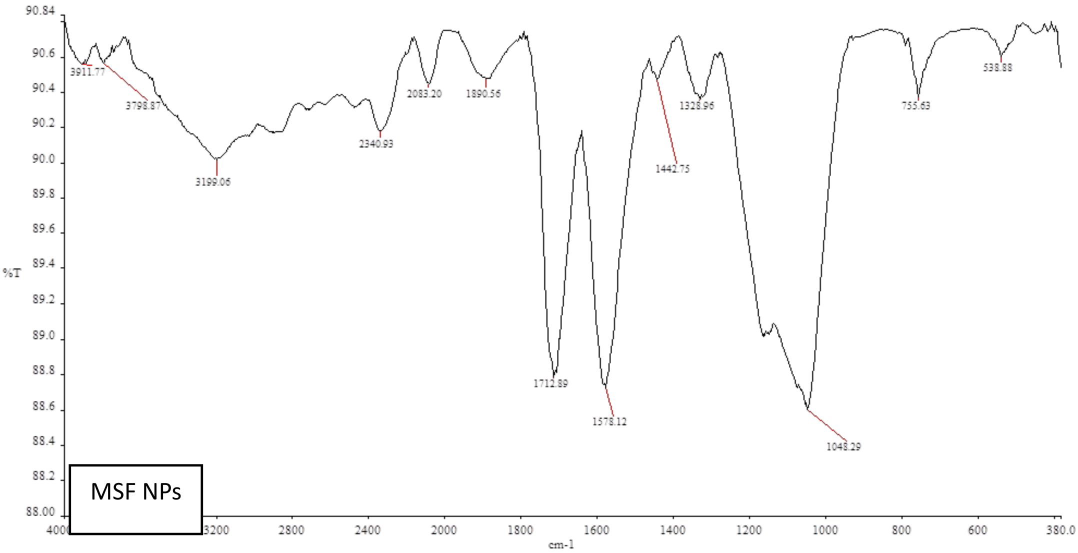


Fig. 2: FTIR Spectrum of $A_1$

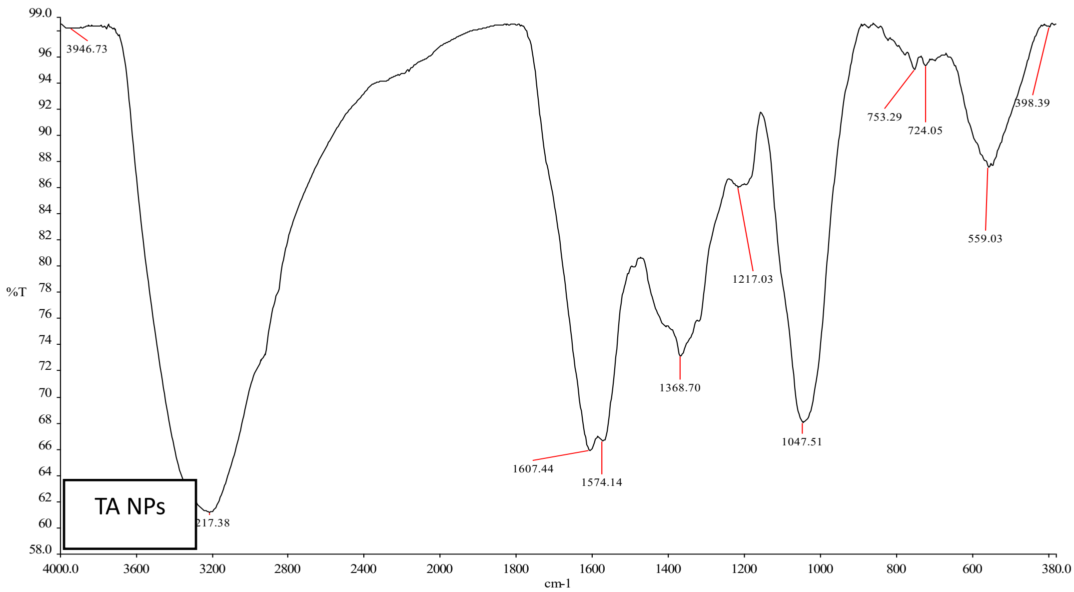


Fig. 3: FTIR Spectrum of $A_2$

grass) leaves extract.

*FTIR Characterization*

Figs. 2 to 5 show the FTIR spectrum for leaves of Mexican Sunflower mediated CuO nanoparticle-MSF NPs (A1), leaves of Tropical Almond mediated CuO nanoparticles-TA (B1), leaves of Guava mediated CuO nanoparticles-G (C1), and leaves of Lemon grass mediated CuO nanoparticles-LG (D1).

Tables 5 displays the characteristic peaks with their attributed functional groups and the finger print region bands present.

538 cm$^{-1}$ 559 cm$^{-1}$ 545 cm$^{-1}$ 542 cm$^{-1}$ are indicative of CuO, which confirms the presence of metal-oxide bonds as a characteristic feature expected of nanoparticles; this is in line with Subramaniyan *et al.*, (2018) [19] and Nakhaei and Akhbari (2020) [20]. The tropical almond mediated CuO nanoparticles gave a broad peak at 3217 cm$^{-1}$, indicating that O-H bond exists in the sample which can be due to the

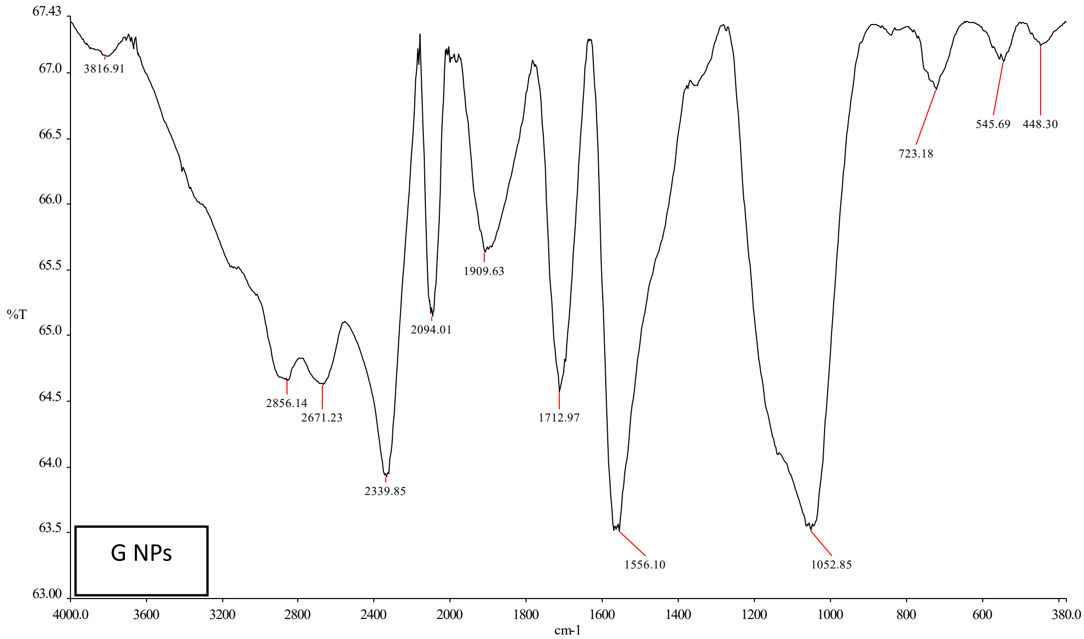


Fig. 4: FTIR Spectrum of $A_3$

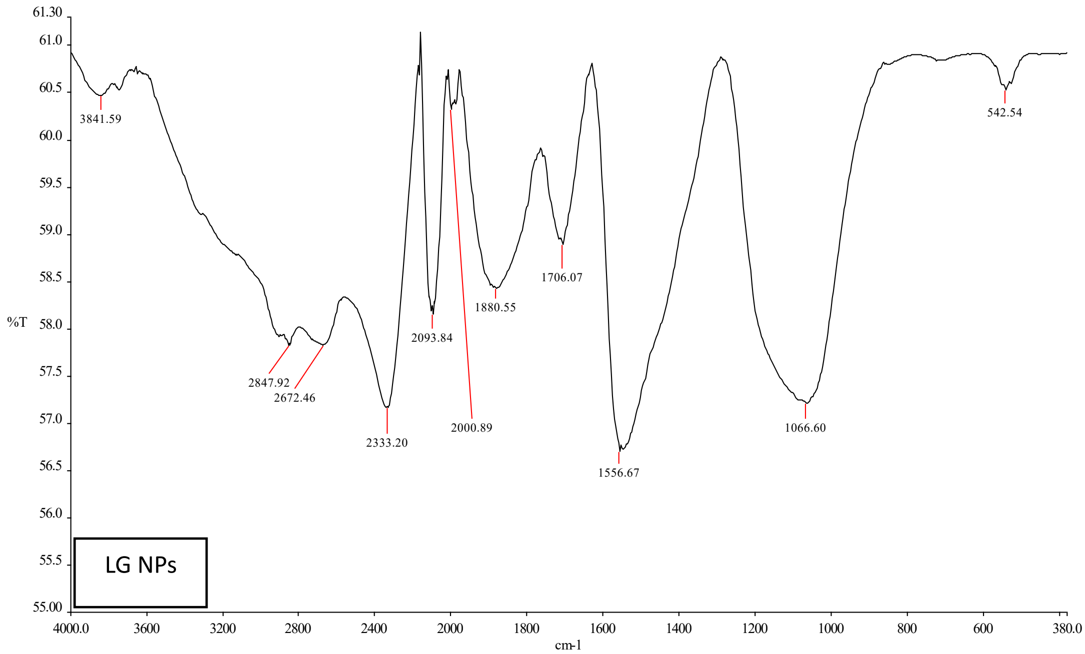


Fig. 5: FTIR Spectrum of $A_4$

absorption of water molecules.

In general, the presence of noticed peaks at various wave numbers represented functional groups belonging to phenolic compounds, carboxylic group, aromatic alkenes, aldehydes, and other groups which act as reducing and stabilizing agents for the biosynthesis of Copper nanoparticles.

*Elemental Composition of the nanoparticles*

The energy dispersive X-ray spectroscopy (EDS) analysis was applied for determining the elemental composition which invariably shows the purity level of the nanoparticles. Table 6 presents

Table 5: Summary of FT-IR spectral peaks in the four selected samples

| Nanoparticle | FT-IR peaks ($cm^{-1}$) with attributed functional groups |
|---|---|
| A1 | 3199 (O–H), 2340 (COO), 2083 (C≡C), 1890 (C═C═C), 1712 (C═O), 1578 (C=C), 1328 (C–H) bend, 1048 (C–O), 538 (Cu–O) |
| A2 | 3217 (O–H), 1607 (C═C), 1368 (C–H) bend, 1047 (C–O), 559 (Cu–O) |
| A3 | 2671 (C–H), 2339 (COO), 2094 (C≡C), 1909 (C═C═C), 1712 (C═O), 1556 (C=C), 1052 (C–O), 545 (Cu–O), 448 (C–C out of plane ring) |
| A4 | 2672 (C–H), 2333 (COO), 2093 (C≡C), 2000 (C═C═C), 1880 (aromatic compound), 1706 (C═O), 1556 (C=C), 1066 (C–O), 542 (Cu–O) |

Table 6: Elemental composition of biosynthesized CuO nanoparticles samples

| Sample label | Cu (%) | O (%) | C (%) |
|---|---|---|---|
| A1 | 60.96 | 25.67 | 18.34 |
| B1 | 89.87 | 10.13 | - |
| C1 | 73.30 | 20.40 | 6.30 |
| D1 | 79.66 | 20.34 | - |

Table 7: EDS Percentage Yield of the dried Nanoparticles

| Sample | Mass of copper salt (g) | Weight of dried nanoparticle (g) | EDS Weight of Cu + O | Percentage (%) |
|---|---|---|---|---|
| A1 | 0.44 | 0.43 | 86.63 | 84.5 |
| B1 | 0.44 | 0.39 | 100.00 | 88.6 |
| C1 | 0.44 | 0.42 | 93.70 | 89.5 |
| D1 | 0.44 | 0.22 | 100.00 | 50.0 |

the summary of the elemental composition of the synthesized nanoparticles as revealed by the EDS spectra of the 1:1 mixture-volume ratio of each biosynthesized nanoparticles.

The elemental compositions indicate the presence of copper (Cu), Carbon (C) and oxygen (O) in the samples (Figs. 6-9). Sample B1, *Terminalia catappa* (tropical almond) and sample D1, *Cymbopogon citratus* (lemon grass) consist of only copper and oxygen, which implies zero impurity. While sample A1, *Tithonia diversifolia* (mexican sunflower) and sample C1 *Psidium guajava* (guava) have carbon in addition to the expected copper and oxygen elements, the carbon contents are 18.34 % and 6.30 %, respectively.

A look into the four samples demonstrates that tropical almond leaves extract produced the purest nanoparticles with copper element alone taking an approximate value of 90 %. This compares well with the result of Manjunatha *et al.,* (2021) [21], who reported the presence of two expected elements of copper and oxygen in his work.

Considering the percentage of CuO as presented by the EDS analysis, the percentage yield of the pure copper oxide nanoparticles CuO NPs were determined by equation (ii) and the result is presented in Table 7. The results provide the yields of the pure CuO NPs synthesized from the plant

$$\text{EDS \% yield} = \frac{\text{weight of the dried nanoparticle} \times \text{\% weight of Cu and O from the EDS}}{\text{calculated weight of the salt}} \times 100\% \qquad \text{Eq. (ii)}$$

extract.

The deviation between the experimental and calculated EDS percentage yields gave an insight into the purity of the biosynthesized nanoparticles. Sample B1, *Terminalia catappa* (tropical almond) among others is seen to have the highest yield without the presence of carbon (an impurity), although on a general note, sample C1 *Psidium*

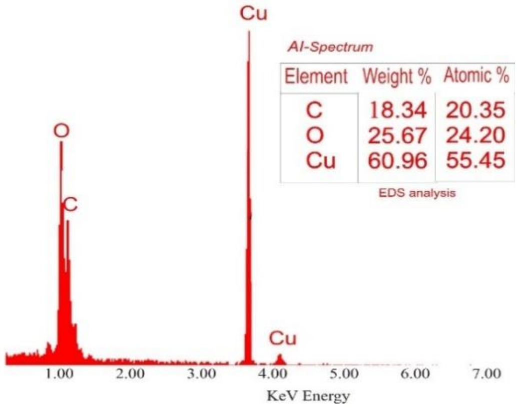


Fig. 6: EDS Spectrum of $A_1$

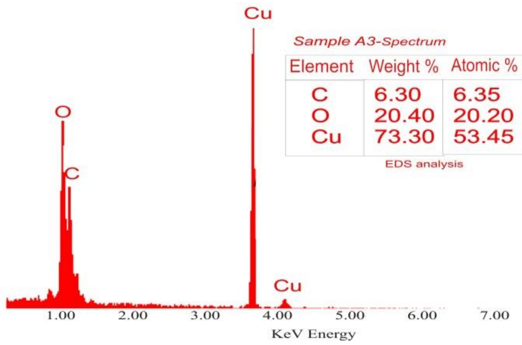


Fig. 8: EDS Spectrum of $C_1$

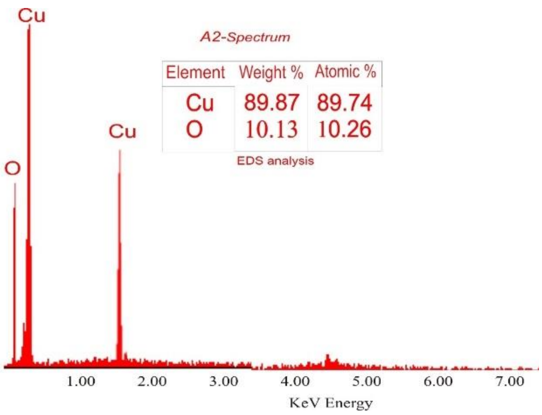


Fig. 7: EDS Spectrum of $B_1$

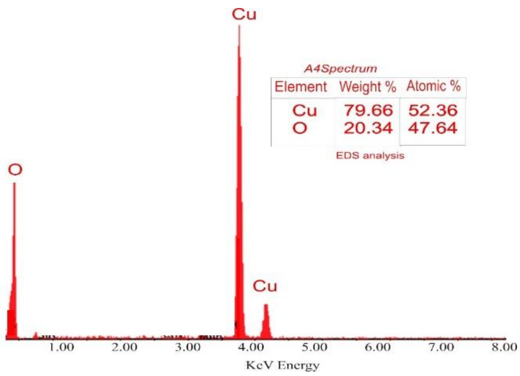


Fig. 9: EDS Spectrum of $D_1$

*guajava* (guava) has the highest yield but the presence of carbon will affect the true state of the nanoparticles as CuO.

*Transmission Electron Micrographs of the nanoparticles*

TEM analysis reveals the morphological nature of the synthesized copper oxide nanoparticles as an agglomerated cluster structure as shown in Figs. 10 -13. The four samples of CuO NPs are quite uniform in size with the average size within the range of 3.68 nm-7.92 nm (Table 8). The research results gave favorable small sizes when compared to the other literatures. For instance, Mali *et al.,* (2019) [22] reported a similar average size of 6.44 nm in the CuO nanoparticle biosynthesized using *Enicostemma axllare* leaf extract. Gunalan *et al.* (2012) [4] reported spherical shaped CuO nanoparticles with the average diameter of 20 nm in CuO nanoparticles synthesized with the leaf extract of *Aloe barbadensis.* Khatami *et al.,* (2017) [23] reported a uniform structure with a near-spherical morphology and mean diameter of 80 nm in the CuO nanoparticle biosynthesized using *Stachys lavandulifolia* aqueous leaf extract. Sankar *et al.,* (2014) [7] reported rod-shaped nanoparticles with the average size of 140 nm in the CuO nanoparticle biosynthesized using *Carica papaya* leaf extract. Sample B1, *Terminalia catappa* (tropical almond) has the lowest average size of 3.68 nm. These TEM micrographs confirm the nano sizes of the biosynthesized copper nanoparticles.

## CONCLUSION

The finger print region of the FT-IR spectra confirmed the presence of metal-oxide bands. In addition, the TEM micrographs revealed that the size of synthesized nanoparticles was in the range of 1 and 100 nm. However, the EDS presented a high level of purity approximately 100 % of the biosynthesized copper nanoparticle whose reducing agents are tropical almond and lemon

Table 8: Summary of TEM displayed particle sizes

| Sample | Captured particle sizes (nm) | Mean size (nm) |
|---|---|---|
| A1 | 3.22, 3.52, 8.33, 15.30, 2.72, 3.65, 9.70 | 6.63 |
| B1 | 5.32, 3.65, 1.32, 5.23, 2.70, 3.22, 4.33 | 3.68 |
| C1 | 3.22, 17.83, 5.80, 5.50, 15.47, 4.20, 15.47, 5.52, 5.80 | 7.92 |
| D1 | 5.20, 10.02, 6.55, 10.22, 5.84 | 7.57 |

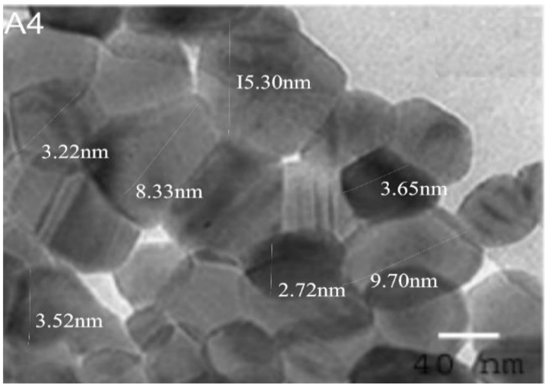

Fig. 10: TEM diagram of $A_1$

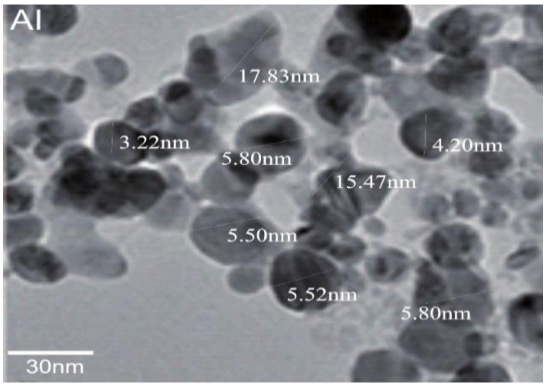

Fig. 12: TEM diagram of $C_1$

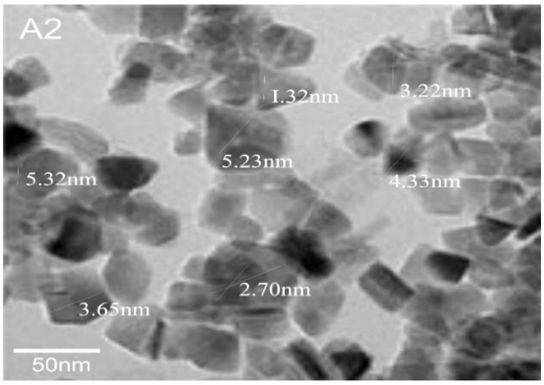

Fig. 11: TEM diagram of $B_1$

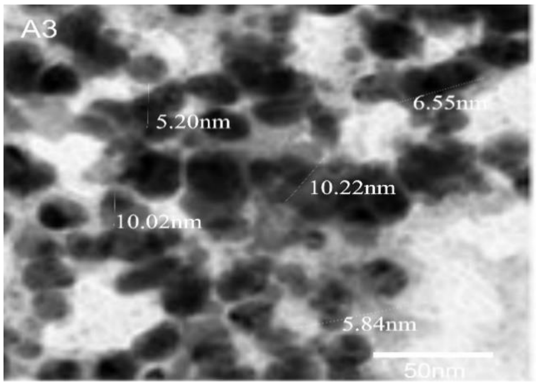

Fig. 13: TEM diagram of $D_1$

grass aqueous extract. The best yields were noted in the precursors i.e. copper salt-leaves extract, having the mixture-volume ratio of 1:1. In addition to the high purity from nanoparticles mediated by tropical almond, it was also found to have the smallest average size particle of 3.68 nm. The approach employed in this work for synthesizing nanoparticles using aqueous extracts opens up possibilities in the development of nanoparticles as adsorbents for domestic and commercial applications.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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