

Alstonia scholaris Leaves: A Biochemical Marvel for Eco-friendly Copper Nanoparticles Synthesis

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Abstract

The utilization of plants for the production of metallic nanoparticles is gaining significant attention in research. In this study, we conducted phytochemical screening of *Alstonia scholaris* (*A. scholaris*) leaves extracts using various solvents, including chloroform, ethyl acetate, n-hexane, methanol, and water. Our findings revealed higher proportions of flavonoids and alkaloids in both solvents compared to other phytochemical species. In the methanol, extract proteins, anthraquinone and reducing sugar were not detected. On the other hand, the aqueous extract demonstrated the presence of amino acids, reducing sugar, phenolic compounds, anthraquinone, and saponins. Notably, ethyl acetate and chloroform extracts displayed the highest levels of bioactive compounds among all solvents. Intrigued by these results, we proceeded to investigate the antibacterial properties of the leaf extracts against two major bacterial strains, *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). All extracts exhibited significant zones of inhibition against both bacterial isolates, with *S. aureus* showing higher susceptibility compared to *E. coli*. Notably, the methanol extract displayed the most potent inhibitory effect against all organisms. Inspired by the bioactivity of the methanol extract, we employed it as a plant-based material for the green synthesis of copper nanoparticles (Cu-NPs). The synthesized Cu-NPs were characterized using Fourier infrared spectroscopy (FT-IR), UV-visible spectroscopic analysis, and scanning electron microscopy (SEM). The observed color changes confirmed the successful formation of Cu-NPs, while the FTIR analysis matched previously reported peaks, further verifying the synthesis. The SEM micrographs indicated the irregular shapes of the surface particles. From the result obtained by energy dispersive x-ray (EDX) spectroscopic analysis, Cu has the highest relative abundance of 67.41 wt.%, confirming the purity of the Cu-NPs colloid. These findings contribute to the growing field of eco-friendly nanotechnology and emphasize the significance of plant-mediated approaches in nanomaterial synthesis and biomedical applications.

1. Introduction

Nowadays, incorporating green chemistry into nanoparticle research is a key area of development in material science and engineering¹. Undoubtedly, the rise in demand of fabricating nanoparticle using straightforward, economic, and non-toxic routes, have been vital in overcoming the adverse effects of the existing sources, especially for clinical applications. In this regard, the utility of plant-based extracts would be very desirable in the fabrication and architecture of nano-sized materials and other nanoparticle-embedded products as it brings a crucial synergy between nanotechnological tools and plant sciences. Among various classes of nanomaterials, metallic nanoparticles have gained tremendous scientific interests because of their unique properties^{2,3,4}. Many of their outstanding applications include catalysis^{5,6}, biological labeling^{7,8}, drugs delivery^{9,10}, tissue engineering^{11,12}, and optoelectronics^{13,14}. However, most reported synthetic approaches use organic solvents, hydrazine¹⁵, sodium borohydride¹⁵, as surfactants, stabilizers as well as the reducing agents¹⁶. These reagents are regarded as hazardous to the environment. In addition, most of the preparation methodologies necessitate intricate controls and conditions, leveling up the cost of production.

Today, biosynthesis approaches for metal nanoparticles production are tremendously followed and optimized as it does not involve the use of hazardous chemicals. However, synthesis approach from extracts from plant sources confirmed the advantages of the method to the microbial sources, associated with economic burdens, and the need for skilled personnels¹⁷. The growth and development of microbes' resistant antibiotics prompted the research on finding alternative materials from a variety of sources. Among many potential medicinal plants, *Alstonia scholaris* (*A. scholaris*) commonly known as blackboard tree or devil tree is a native plant species found in various countries such as India (locally called saptaparni) Cambodia (called popeal khe), China (locally referred as Tang jiao shu) Indonesia (pulai as popularly called) Laos (called as dtin pet) in Malaysia (Popularly called Rejang or Pulai) Myanmar (Toungmayobeng s the local name), Philippines (Ditaa popularly called), and Vietnam (Suà, mo cua as the local name)^{18,19}. It is a member of Apocyanaceae that contains various metabolites such as monoterpenoid and indole alkaloids. It is described as a sinister tree, growing up to a to a height of 18 m and mostly founds n the forests of India, Pacific Asia, Queensland as well as South China. According to a myth of Hindu, the tree is an abode of witches and source of evil spirits^{20,21}. It is widely used for herbal medication against various diseases such as malaria, fever, gastrointestinal disorders, ulcer dysentery, diarrhea, inflammation, cardiac and respiratory ailments. It is also used for the treatment of cancer²². Despite various phytochemical screening research have indicated the antimicrobial activity of the *Alstonia scholaris*, further studies regarding identification of its potentiality is essential for its usage, especially for the antimicrobial effects. Given their catalytic, plasmonic, magnetic, and thermal properties, Copper nanoparticles (Cu-NPs) gained particular interest for various applications²³. Cu-NPs also spark fascinating research activity up to the high expectations it generates, especially in biochemistry. A key advantage of copper is its affordability and availability, rendering the production of CuNPs cost-effective. So far, CuNPs have been synthesized from several plant extracts^{24,7}. However, the potentiality of using *A. scholaris* extracts is still not yet explored.

Herein, we aimed at investigating the effects of solvents extracts in phytochemical screening of the *A. scholaris* and determination of its antimicrobial activities (Fig. 1). Considering the green synthesis benefits of Cu-NPs and their potentials in the antimicrobial studies, we also investigated the usability of *A. scholaris* extracts as an alternative bio-source for reducing Cu ions to form Cu-NPs. The *A. scholaris* extracts was chosen due to its relative content of as previously reported. The as-made NPs are characterized thoroughly to determine the properties of the synthesized nanostructures. The Cu-NPs have found versatile applications in biomedical, environmental and other potential applications.

2. Materials and Methods

2.1 Collection of plant leave and preparation of aqueous and organic extract

The leaves of *Alstonia scholaris* was collected from Mewar University, Chittorgarh Rajasthan India and verified by the department of Life science Mewar University Rajasthan, India. The experiments were

performed according to the guidelines and legislation of Indian Institute of Interrogative Medicine.

To prepare the extract, the fresh piece of *A. scholaris* leaves was thoroughly washed using tap water, then with distilled water for the dust removal. It was then cut and set for drying at ambient temperature for a period of five days. The dried leaves were then grinded using a sterile blender, and about 40 g was weighed and subjected to 800°C boiling in a distilled water at (400 mL) for the duration of 15 minutes. After cooling down, the sample was filtered (using Muslin paper) to get the required amount ²⁵. 250 mL Erlenmeyer flasks was used for the extract collection and kept at 4°C for later usage. Additional extracts were made in an identical fashion, except that the water was substituted with the selected solvents. For solvent extraction of phytochemicals *A. scholaris* leaves, the finely powdered leaves (100 g) were placed in a 500 mL Soxhlet apparatus and various solvents such as chloroform, ethyl acetate, n-hexane, methanol as well as aqueous solvents were used for the extraction for 24 hours in each case.

2.2 Phytochemical screening of crude extracts

Phytochemical screening was conducted to test for chemical constituents such as reducing sugar, steroid, glycoside, flavonoids, alkaloids, saponins, phenolic compounds/ tannins, anthraquinone, proteins, and amino acids. Thus, the analysis for determining these compounds in the leaves sample of the *A. scholaris* is in the following sections. To carry out the analysis of steroids and terpenes (referred to as Salkowski test), 0.5 g of the sample in a test tube (20 mL) was used. 1 mL of the chloroform as a solvent was introduced, then dropwise of concentrated sulfuric acid (1 mL), resulting in two phases partitioning. The yellowish precipitate formation conclude the likelihood of steroids/terpenes in the sample ²⁶.

2.2.1 chemical constituents screening tests

To the sample of 0.5 g in the test tube, 5 mL of 1% solution of hydrochloric acid is introduced followed by stirring in hot water and filtration. The obtained filtrate is divided in three portions. Some drops of Dragendoff reagent were introduced to the first portion, and the formation of precipitate, a brownish observed Subsequently, 1 drop of Mayer's reagent was dropwise introduced to the portion number two, resulting into yellowish precipitation. For the last portion, droplet of Wagner's reagent was introduced, giving precipitate reddish- brown in color and confirming the alkaloids presence. **To test for the flavonoids**, Stock solution of 1 mL was used and added to it a few drops dilute NaOH. Yellow precipitate was formed. Adding a few drops of diluted acid the color disappeared, a confirmation for flavonoids ²⁶.

Then, **for protein testing (Million's test)**, small quantity of the plant leaf extract was weighed and mixed with million's reagent in few drops. of The precipitate formation, white in color indicates the presence of protein ²⁷. **For amino acid testing**, 4% of NaOH was introduced in drop into 500 µL of the extract and then 1% of CuSO₄ was added. The formation of precipitate, violet or pink in color is a confirmation of amino acids ²⁷.

For reducing sugars testing, 2 mL of crude extract was weighed, and distilled water of 5 mL was bestowed before the filtration. Boiling the filtrate with Fehling's solution addition in drop of 4–5 resulted

in the precipitate with orange red coloration which signified the reducing sugars formation ²⁷.

To test for tannins, 3 mL of the stock solution was diluted with chloroform and acetic anhydride was added to a test tube (1 mL). Finally, sulphuric acid was introduced gently to the solution. A green precipitate was observed which confirmed the tannins ²⁶. **For anthraquinones testing**, a tiny of the sample was mixed with benzene (10 mL), and subject to filtration. Next 10% ammonia (5 mL) solution was dropwise introduced to the filtrate and agitated. Non color formation in the layer of the ammonia (the lower part) confirmed the anthraquinone ²⁶. **For saponins (Frothing Test)**, Na₂HCO₃ was dropwise introduced to the 500 µL of the extract and agitated for 5 minutes duration. The formation of foams indicates the saponins process ²⁷. Finally, **for glycosides test (Ferric chloride test)**, extract of about 200 µL of was used to dissolved glacial acetic acid of 100 µL. 5% ferric chloride was drop wisely introduced and then concentrated sulfuric acid. The formation of precipitate, a greenish blue indicated the glycosides ²⁸.

2.3 Antimicrobial activity of crude extracts

All procedures were carried out aseptically. The pathogenic strain of *E. coli* and *S. aureus* were sourced from the laboratory at the Life Sciences Department of the University of Mewar. The medium was made by adding 28 g of nutrient agar in distilled water of 1000 mL. Using hot magnetic stirrer, this substance was heated to fully dissolve the metal. The dissolved media was autoclaved for 15 minutes at 121°C and 15 psi. Following autoclaving, the sterilized medium made of 15 mL distributed into the Petri plate and set to crystallized for culturing. The pathogenic microorganism, (*E. coli* and *S. aureus*) were subculture on the media and put in an incubator incubated at 37°C.

In sterilized Glass bottles, different concentration of the extracts was made by weighing 40, 20, 10, 5, 2.5 and 1.5 mg of each of the extract into 1mL of 10% DMSO for a better stability of the extract. It was then covered with foil paper. The antimicrobial activity of the extracts was analyzed against the strains of the bacteria *E. coli* (the Gram-negative) and *S. aureus* (the Gram-positive) via diffusion technique. A colony of 1000,000 forming units (CFU) of the microorganisms (*E. coli* and *S. aureus*) were inoculated on the plate. At the prepared concentrations (40, 20, 10, 5, 2.5 and 1.25 mg/mL) each extract was introduced to the nutrient agar plate containing the bacteria strains. The plates were subjected to incubation at 37°C for a night. After the period of incubation, each of the plates were analyzed for the zone of inhibition. The antibacterial activity of prepared extracts was measured by the zones of inhibition after the incubation period (24 hours) at temperature of 37°C.

2.4 Synthesis and Characterization copper nanoparticles (Cu-NPs)

The green synthesis of Cu-NPs was carried out. For the procedure, the *A. scholaris* leaves broth of known concentration (10 mL) was mixed with 0.1M CuSO₄ solution by mixing at a specific ratio (1:9). Gradual transformation of mixture from light blue to light green to dark green indicated the formation of copper nanoparticles ²⁹. The as-synthesized Cu-NPs was then characterized by UV-vis spectrophotometer recorded at Lambda max 750 (Perkin Elmer), Fourier Transformed Infra-red (FTIR) analyzed at 4000–400

cm⁻¹ using 2 (Perkin Elmer) and Scanning Electron Microscopic analysis with Nova Nano SEM 450 (dwell 10µs). The Energy Dispersive X-ray spectroscopy (EDX) was also analyzed for elemental composition in the sample

3. Results and Discussion

Figure 2 depicted the tree and the leaves of the *A. scholaris*. The bark of the tree is dark-brown in color with smooth texture and milky latex ²⁰. The leaves are non-stipules, with about 5–8 whorls. The petiole has a width of about 2 cm diameter and stout and flowers are ephemeral, with axillary cymes arrangement made of greenish-white color ²¹. The leaf is made up of medium to large size dorsiventral blade. The venation of the leaf is pinnate, consisting of large number of veins ³⁰.

The result of phytochemical analysis of the *A. scholaris* leaf extracts in the various solvents are presented in Table 1. It has shown flavonoids and alkaloids presence at higher proportions, in both solvents in comparison to other phytochemicals. The methanol extract has shown the presence of all metabolites, with exception of proteins, anthraquinone and reducing sugar. The aqueous extract contained reducing sugar, amino acid, phenolic compounds, anthraquinone and saponins. Of all, ethyl acetate and chloroform contained the lowest amount of the bioactive molecules. According to Anthony et al., the extracts of the leaf contained the most essential compounds in the ethyl acetate, butanol and water as the extracting solvents ²⁷. Additionally, higher proportions are present in the bark and leaves of the aqueous. The most detected ones were; proteins, steroids, phenols and tannins ²⁷. In a qualitative comparative review presented by Khyade et al., for the phytochemical screening of *A. scholaris* leaves, most of the studies conducted have majorly reported carbohydrates, oils and fats, alkaloids, flavonoids, tannins, terpenoids, saponins and steroids. For instance, Misra et al., reported that extract of n-hexane has alkaloids and steroids as metabolites. However, the isopropanol extract of the leaf has shown the terpenoids in smaller quantity in comparison to root, misty and bark of the plant. Mistry and Parekh observed oils and fats, alkaloids, flavonoids, terpenoids, glycosides, saponins, mucilage and gums in both bark, leaves and stem of the plant ³¹.

Table 1
Screening of phytochemical in the extracts of *A. scholaris*

Analytical parameters	n-hexane	Chloroform	Ethyl acetate	Methanol	Water
Steroids	-	-	+	+	-
Alkaloids	+	+	-	-	-
Dragendoff test	+	+	+	+	+
Mayer's test	-	-	-	-	-
Flavonoids	+	+	+	+	+
Protein	-	-	-	-	-
Amino acid	-	-	-	+	+
Reducing sugar	-	-	-	-	+
Tannins	-	-	-	+	+
Anthraquinone	+	+	+	-	+
Saponins	-	-	+	+	+
Glycosides	+	+	-	+	-
Key: (+) indicates presence, while (-) indicates absence					

Thus, we analyzed the antimicrobial properties of the *A. scholaris* plant. The results from the assessment of the antibacterial properties *E. coli* and *S. aureus* has been determined. It has shown the effects of extracts on the chosen bacterial pathogens under the zone of inhibitory study.

Table 2–5 have shown the antibacterial activities on the crude extracts. The results revealed the excellent inhibition against the growth of all the bacteria tested at predetermined studied concentrations. For n-hexane (Table 2), chloroform (Table 3), ethyl acetate (Table 4) and methanol (Table 5) extracts, *S. aureus* has the highest activity while *E. coli* has the lowest. Methanol extract also demonstrated the highest inhibitory effect for the studied organisms. Previous finding from Thankamani et al., revealed the effect of extract of methanol which possessed the highest inhibition against the microorganism and was more pronounced at the inhibitory zone of 15–25 mm. Whereas chloroform extract has less inhibition effect for the *S. aureus*³². Accordingly, the inhibitory effect of n-hexane and ethyl acetate extract was closely the same with only slight variation for the studied organisms. Study conducted by Anthony et al., reported that methanol extracts of *A. scholaris* leaf exhibited much significant antibacterial activities in comparison to other extracts employed³³.

Table 2
Antibacterial effect of extract of n-hexane for *A. scholaris* leave against the tested bacteria

Microorganisms	Concentration (mg/mL)					
	40	20	10	5	2.5	1.25
Zone of inhibition (mm)						
<i>E. coli</i>	6	4	3	2	2	2
<i>S. aureus</i>	7	6	6	4	3	3

Table 3
Antibacterial activities of extract of chloroform for *A. scholaris* leave against the tested bacteria

Microorganisms	Concentration (mg/mL)					
	40	20	10	5	2.5	1.25
Zone of inhibition (mm)						
<i>E. coli</i>	15	12.5	10	8	5	3
<i>S. aureus</i>	13	13	12	10	8	5

Table 4
Antibacterial effect of extract of ethyl acetate for *A. scholaris* leave against the tested bacteria

Microorganisms	Concentration (mg/mL)					
	40	20	10	5	2.5	1.25
Zone of inhibition (mm)						
<i>E. coli</i>	10	9	7	5	3	1
<i>S. aureus</i>	9	8	6	4	3	2

Table 5
Antibacterial effect of extract of methanol for *A. scholaris* leave against the tested bacteria

Microorganisms	Concentration (mg/mL)					
	40	20	10	5	2.5	1.25
Zone of inhibition (mm)						
<i>E. coli</i>	23	21	20	17	13	9
<i>S. aureus</i>	21.5	20	19.5	18	15	11

Biological route for the nanoparticle's synthesis using plant materials has a lot of advantages over synthesis techniques. It is ideal for a large-scale synthesis of the nanoparticles. The biosynthetic route is suitable and economical for the Cu-NPs synthesis for environmental, medical, as well as industrial applications. In addition, it eliminates the use of toxic chemical substances which are potentially hazardous to the living organisms and the surrounding environments. Thus, an aqueous extract of the *A. scholaris* leave was employed for the Cu-NPs biosynthesis. The Cu-NPs was confirmly synthesized by the color changes reacting species according to the UV-visible spectroscopy³⁴. Addition of the leaf broth to the $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution, resulted in the color changes from light to dark green, confirming the successful formation of the Cu-NPs (Fig. 3). In a previously study, the formation of the dark green color which eventually changed to dark brown, reaffirmed the successful biosynthesis of the Cu-NPs. Also, the color change was attributed to the excitation resulting from surface Plasmon resonance (SPR)³⁵. The spectrum was determined at 200–800 nm wavelength range (Fig. 3). The spectrum from UV-vis has 243 nm as the stable wavelength for the formation of Cu-NPs³⁶.

The FTIR spectrum revealed the various functionalities in the Cu-NPs. It revealed various interactions as well as compositions of the leave extract (Fig. 4). The presence of broad peak at 3137.35 cm^{-1} highlighted the stretching due O–H from the alcohols and carboxylic acid present in the compounds³⁷⁶. The intense peak appeared around 1733.46 cm^{-1} represents C = O bending vibrations. Also, appearance of the IR peak at 1639.03 cm^{-1} shows the stretching occurred for C = C in the compounds. The peak at 1372.06 cm^{-1} was due to S = O vibration, while the peak at 1071.52 cm^{-1} indicated the stretching due to C–O band of the primary alcohols. Peak at 870.44 cm^{-1} shows C–H bending in the molecules, while the peak at 599.21 cm^{-1} was a result of C–I vibration of the halo species. The occurring of peak around 449 cm^{-1} corresponding on the of Cu ion specie. The FT-IR spectrum confirmed the possibility of the various interactions between the copper ion and the various functional groups contained in the extract³⁰. Thiruvengadam et *al.*, stated that the interaction between the Cu-NPs and the metabolites in the *A. scholaris* resulted in the possible reduction the NPs to copper as confirmed by the FTIR result³⁶.

The images from SEM result depicted the synthesized CuNPs, with the surface particles having irregular shapes consisting of triangular, cylindrical, polygonal, and nearly spherical as shown in Fig. 5. Similar outcomes were reported, in which, SEM images of the synthesized Cu-NPs contained irregular particles arranged in clusters comprising of different structures from polygons to spherical shapes³⁶. The EDX spectrum explained the elements composition on the surface of the Cu-NPs. The mapping result highlighted the major elements present (Fig. 6). The carbon and oxygen are appeared as the lowest peaks compared to the Cu. They resulted from the phytochemical metabolites present in leave extract¹². Thus, they also served as the evidence for the for the reduction of Cu-NPs to Cu ions. The compositions of the elements from the EDX spectrum were depicted in Fig. 7. The Cu peak indicated that Cu-NPs has the purest form of copper ion with the relative abundance of 67.41%, followed by the O and C with the relative abundance of 21.17 and 11.42%, respectively. This is contrary to the previous findings with much lower

abundance of the Cu with the Cu content of 26.06% and 8.04%, respectively ³⁴³⁸. Thus, this result findings indicated that *A. scholaris* could be well utilized for the biosynthesis of Cu-NPs.

4. Conclusions

A. scholaris has been a widely known medicinal plant containing variety of bioactive compounds. In this work, leave extract of the *A. scholaris* has been analyzed for phytochemical composition and antimicrobial significance. The leave extracts have screened for various metabolites such as reducing sugar, amino acids, alkaloids, saponins, steroids tannins, phenolic glycosides, flavonoids, and anthraquinone. The antimicrobial activity of the extracts has been investigated against bacterial species, the *S. aureus* and *E. coli*. The extracts have shown significant zones of inhibition against the two bacterial isolates. *S. aureus* has shown an average highest activity while *E. coli* has shown an average lowest activity in all extracts. Methanol extract showed the highest inhibitory effect on all organisms. Biological synthesis of Cu-NPs has been investigated using the aqueous leave extract of the *A. scholaris*. It has been well identified by UV–vis analysis, FT-IR and SEM (including the EDX). The Cu-NPs was confirmed formed by the color changes in from light to dark green. The FT-IR has revealed the major functional groups present in the *A. scholaris* as well as their chemical interaction. The images according to the SEM analysis revealed the irregular shapes the particles have consisting of triangular, cylindrical, polygonal spherical shapes. The EDX spectrum indicated the formation of the Cu-NPs based on the sharp and strong peak of the Cu ions.

Declarations

Declaration of Interest:

The authors declare no competing of Interest.

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Author Contribution

The authors confirm contribution to the paper as follows: A.N.L., Conceptualization, Conceived design, Original draft. Z.U.Z., Conceptualization, Supervision, Writing – Review & Editing, Validation, Investigation, Visualization. G.T., Original draft, Writing – Review. A.A., Writing – Review, Editing. F.U, Writing – Review, Formal Analysis, Investigation. M.T.M., Original draft, Writing – Review. A.M.G., Data Curation, Formal Analysis. R.A., Editing, Data Curation, Visualization, Funding acquisition. K.H.I., Editing, Data Curation,

Formal Analysis, Investigation. O.A., Writing – Review & Editing, Supervision. Visualization. All authors reviewed the results and approved the final version of the manuscript.

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Figures

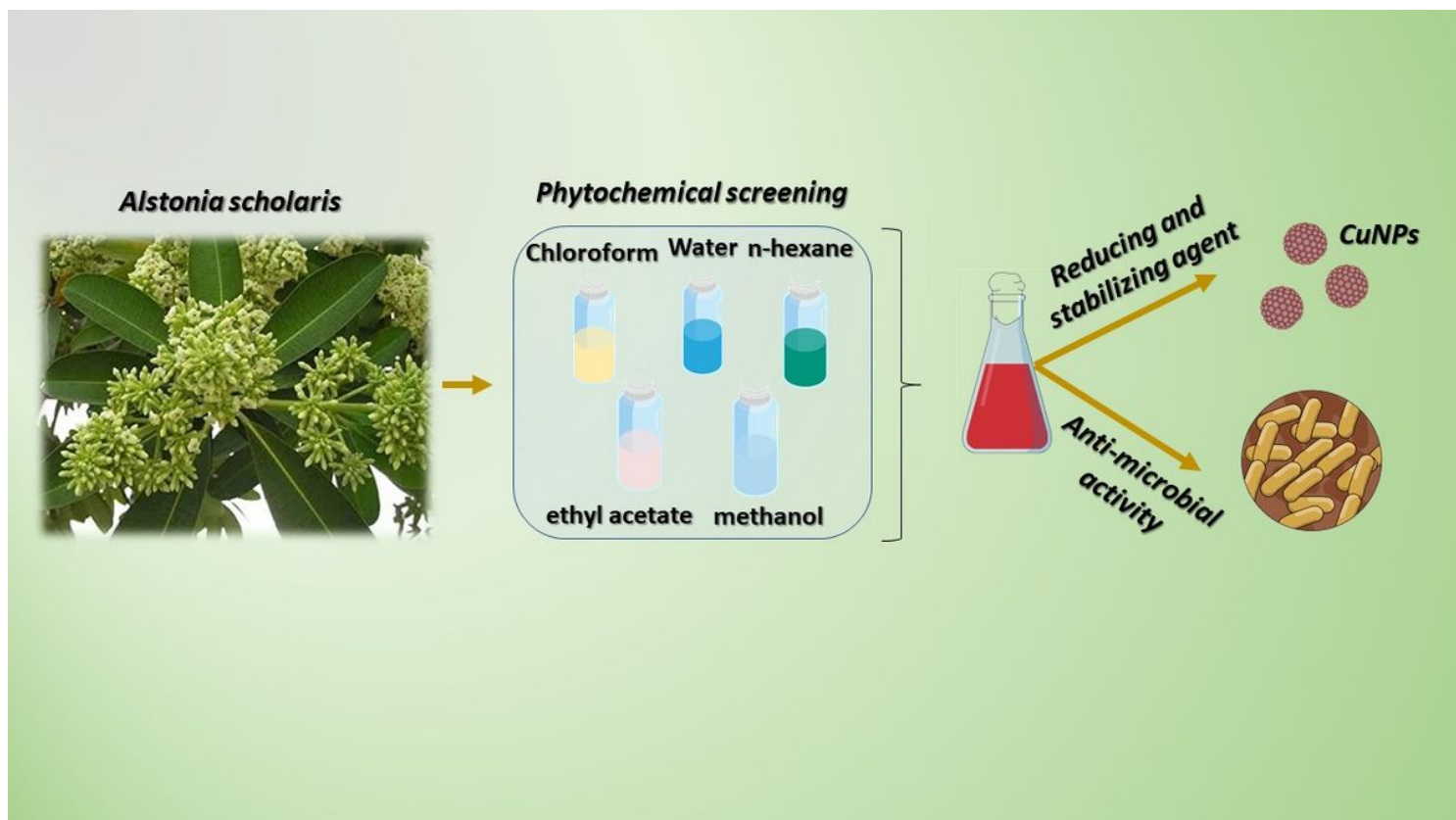


Figure 1

Schematic diagram, presenting the general aim of the research findings.



Figure 2

Digital photographs of (a) image of tree and (b) image of *A. scholaris* leaves.

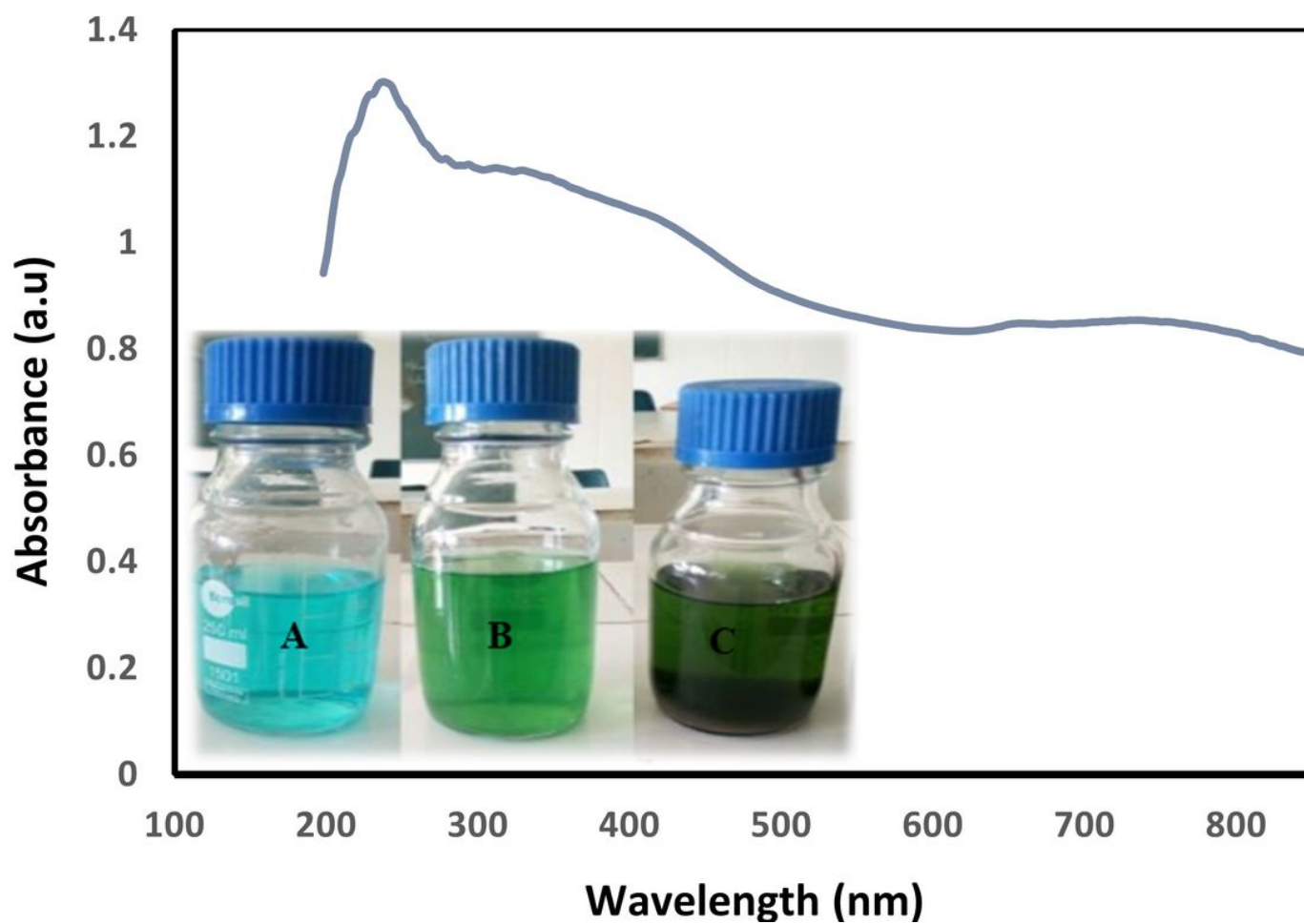


Figure 3

Optical profile of CuNPs produced using *A. scholaris* leaf aqueous extract. Color changes during synthesis of Cu-NPs (A-C), which indicates Cu-NPs formation.

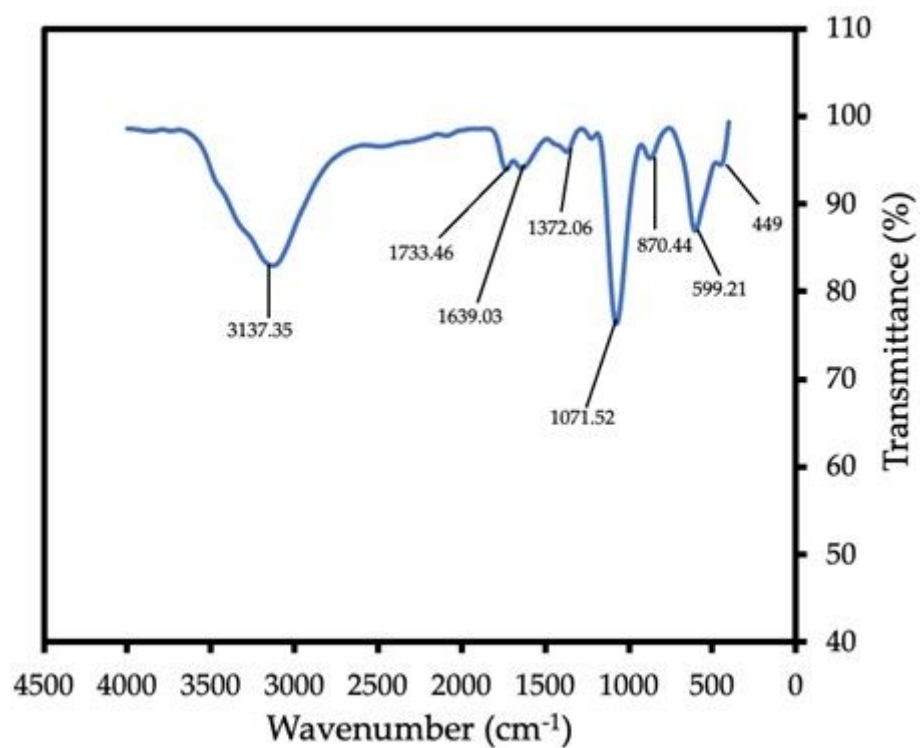


Figure 4

FTIR spectrum of Cu-NPs synthesized from *A. scholaris*

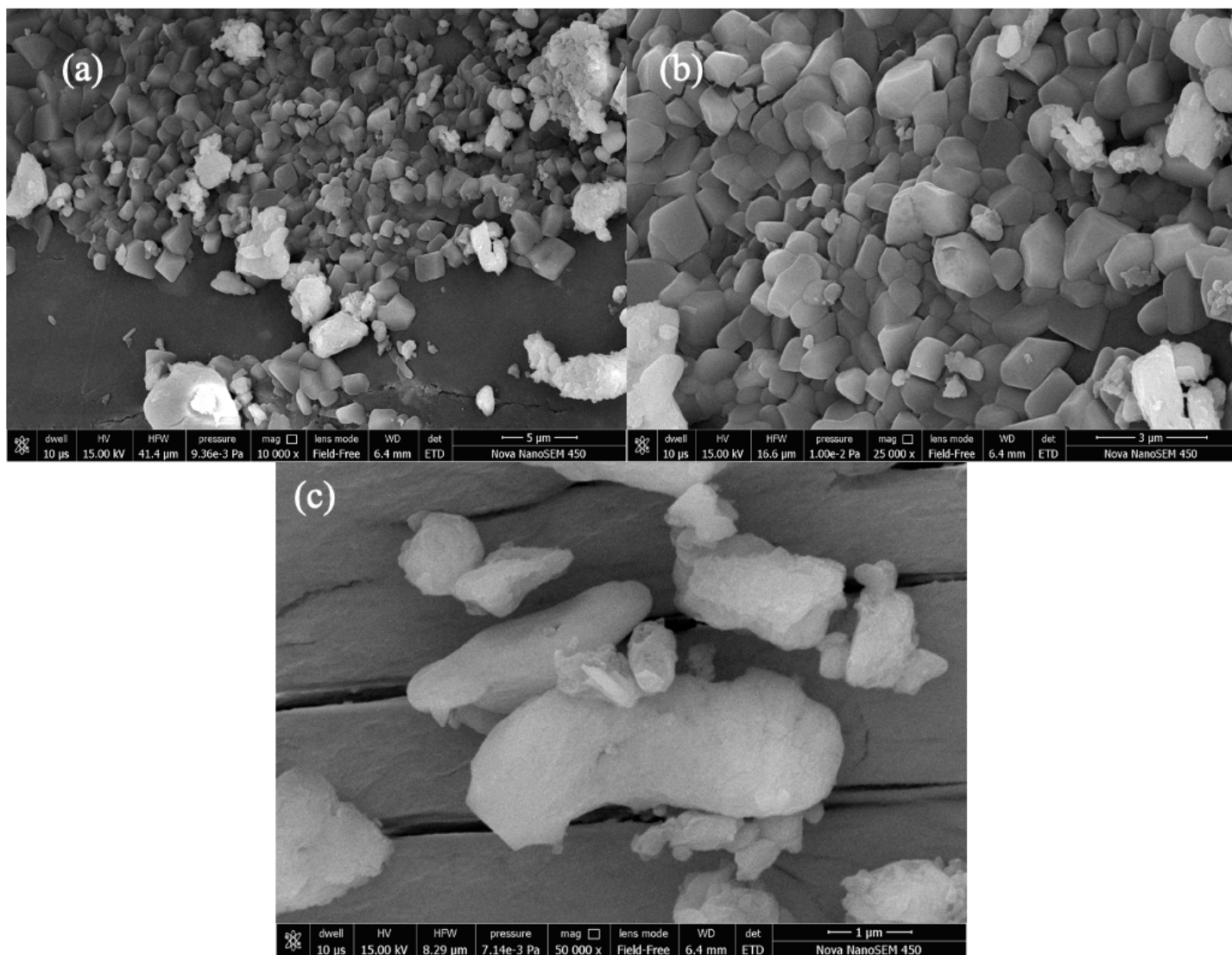


Figure 5

SEM micrographs of CuNPs at (a) 10K, (b) 25K and (c) 50K magnifications.

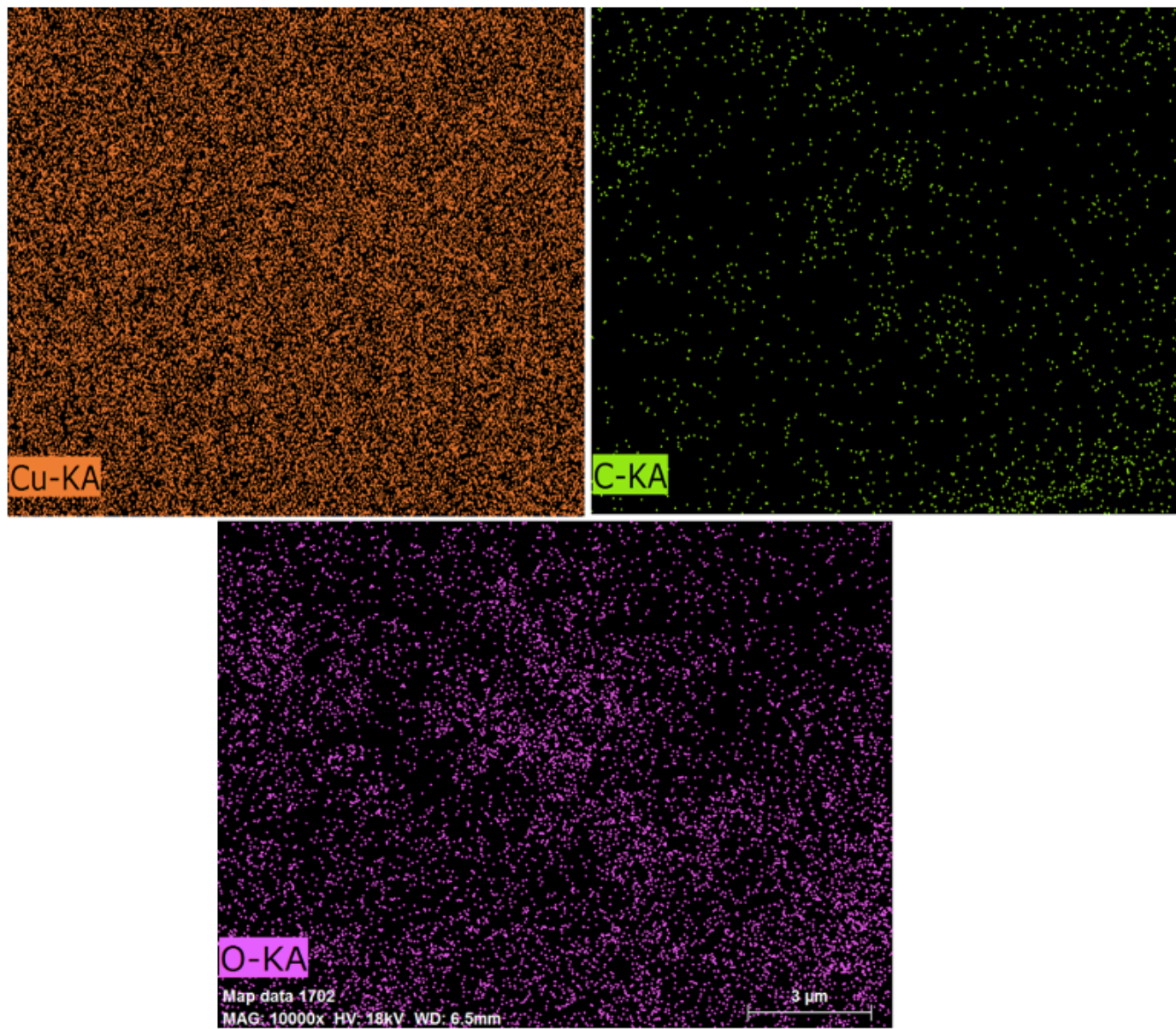


Figure 6

EDX mapping of the CuNPs

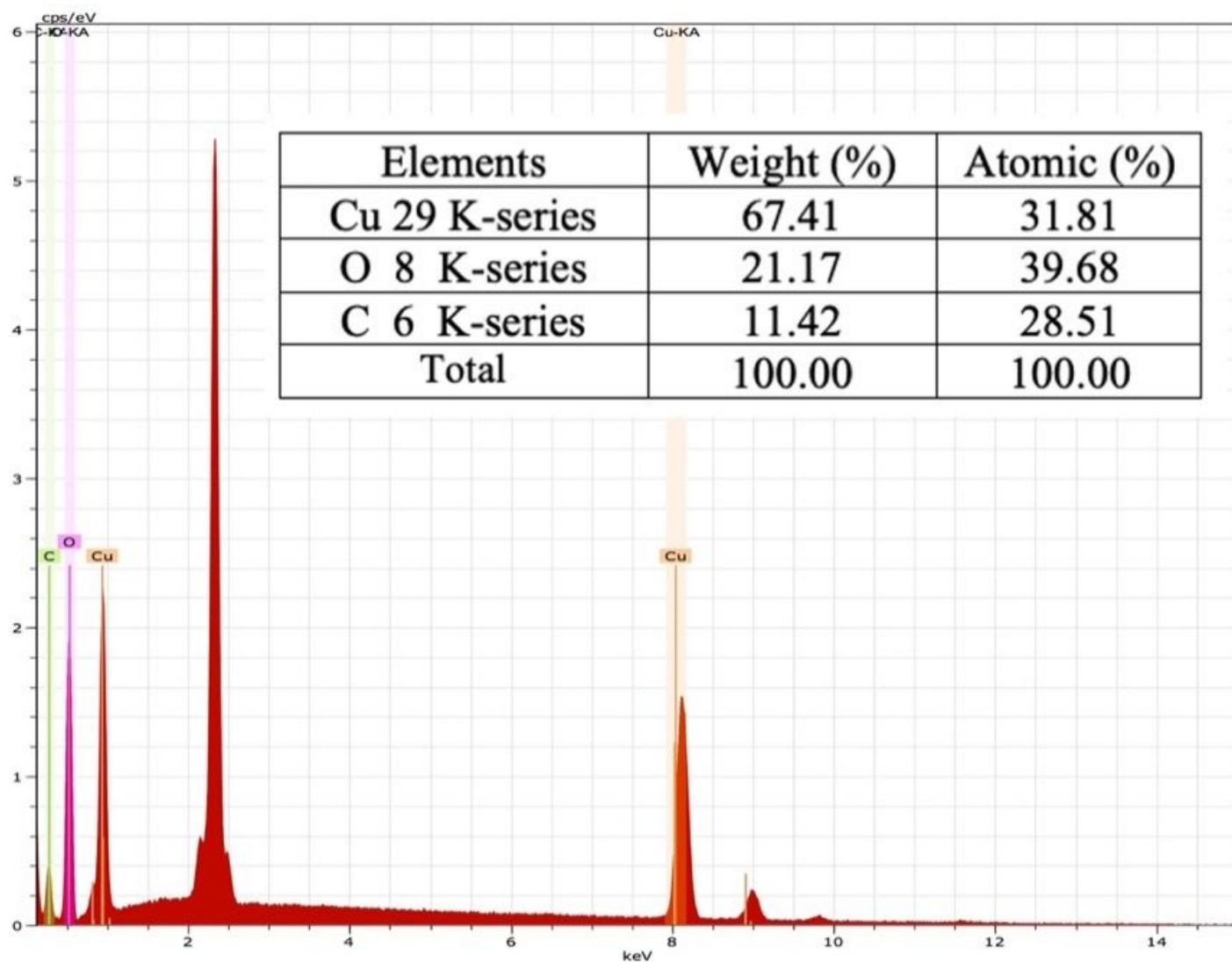


Figure 7

Cu-NPs Energy Dispersive X-Ray Analysis (EDX) spectrum