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# Biogenic Copper Nanoparticles from Combined leaf extracts of Catharanthus roseus (L.) and Abutilon Indicum(L.) and their biological effects – an In vitro study

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**Abstract:** Plant-based (Green) synthesis of Copper nanoparticles is of much interest to researchers currently because of its important applications in various fields of biotechnology. In this study, the production of copper nanoparticles (CuNPs) utilizing an ethanolic extract of *Catharanthus roseus* (L.) and *Abutilon indicum* (L.) leaves extract (CALE) and assess the therapeutical use of these nanoparticles. Preliminary phytochemical analysis was done by using this ethanolic crude extract of the combined plant. The phytochemical tests showed positive results for the phytochemicals alkaloids, phenols, tannins, terpenoids, and phytosterols. The leaf extract was used for the synthesis of copper nanoparticles and in the reduction and capping of the combined leaf extract CuNPs, it was discovered that the bioactive phytoconstituents condensed tannins, played a crucial role by the reduction of copper ions was identified by the change in color from blue to light green. SEM image showed the morphology and size of the nanoparticles and their binding ability with the combined leaf extracts. The spherical biogenic combined leaf extracts of CuNP's were measured to have a size between 5 and 25 nm by Scanning Electron Microscopy (SEM) examination, Ultraviolet-Visible spectroscopy (UV-Vis) showed maximum wavelength at 224nm and XRD showed three strong peaks for its crystallinity and maximum was observed at 2063.0. From their characterization of synthesized CuNPs anti-microbial and anti-inflammatory methods were assessed. The samples showed different diameters of zone of inhibition at different concentrations by using Ampicillin as positive control. The synthesized nanoparticles showed a concentration-dependent anti-inflammatory activity, and the protection percentage increased with an increase in the concentration of the samples.

***Keywords:*** Plant-based synthesis, Copper nanoparticles, Structural characterization, Antibacterial, Inflammation studies.

## 1. Introduction

The complexes of nano-particles from copper have been in existence by homosapiens for many millennia as antibacterial and antifouling agents, fungicides, water purifiers, and algaecides. Due to its benefits, including its

excellent conductivity and cost effective nature compared to other metals like gold and silver, plant-based (green) synthesis of copper nanoparticles (CuNps) is of great attention. Due to its varied applications in different fields of physical, chemical and biological science, copper oxide nanoparticles played a significant role. Having effective biocidal capabilities, copper-based compounds are frequently used in a variety of applications in the medical field. Plants are currently being developed to produce metal nanoparticles through biosynthesis [1].

Copper nanoparticles have a high surface volume ratio, which makes them highly reactive and enables them to rapidly interact with other particles to boost their antibacterial activity [2]. Compared with other metal particles copper (Cu) is the most frequently utilized substance by the mankind because of its varied therapeutical and electronic applications [3]. When compared to other conventional catalysts, it can provide higher yields and a faster rate of reaction in moderate reaction circumstances [4].

*Catharanthus roseus* is an angiospermic plant in the *Apocynaceae* family. It is also referred by various vernacular names across the earth [5]. It is indigenous to Madagascar and is an endemic species there, although it is also produced there for decorative and therapeutic purposes. The most important anticancer medications *vincristine* and *vinblastine* are derived from *C.roseus* plants are in use by allopathy doctors. *Vinca rosea was* the previous name for it in the *genus Catharanthus roseus* [6].

*Abutilon indicum* (also known as Indian abutilon or Indian mallow) is a shrub in the Malvaceae family is native to Indian climatic conditions. Its roots and leaves are used to treat fever, making this plant a significant medicinal and decorative plant. The plants have been widely imported outside of its natural area, and several tropical islands consider it invasive [7].

Both these plants are helpful in synthesizing of copper nanoparticle represents a potential commercial alternative for mass production. Previous studies reported that the microorganisms are helpful in biosynthesis of CuNPs. Plant based synthesis of CuNps are widely in use especially among the recent scientific industries [8]. The manufacture and characterization of CuNPs utilizing combined *Catharanthus roseus* and *Abutilon indicum* leaf extract are reported in this paper for the first time. The main study of our research work is to validate the ethnopharmacological acquaintance of *Catharanthus roseus and Abutilon indicum* on the biosynthesis of CuNPs using ethanolic combined leaf extracts and to investigate its structural characterization and anti-microbial activity of the nanoparticles against different microorganisms and its anti-inflammatory activity against HrBc Membrane.

## 2. Materials and methods

### Collection of plants:

The plants were collected from areas in and around Padur, Chennai. The plant materials were authenticated (Authenticated No. PARC/2021/9354) by the Late Professor P. Jayaraman, Director, Plant Anatomy Research Centre, Tambaram, Chennai-45, India [9]. After the collection of plants, it must be cleaned properly with normal faucet water to remove debris followed by double distilled water. The most important goal of drying is to get rid of the moisture content in plant and must be dried as soon as they are cleaned since failing to do so can cause plant parts to decay. Dried plants must be thoroughly pulverized for further investigation after being fully dried [10].

### Preparation of crude extracts:

The shade dried leaves was powdered with the facilitation of a mechanical pulverizer and soaked in 1000ml of ethanol. The ethanolic extract of combined leaf extract was filtered using Whatmann filter paper. This was repeated twice or thrice and similar extracts were pooled together and concentrated using soxhlet and cold extraction methods. The concentrated ethanolic extracts were subjected to a preliminary phytochemical analysis and biological activities [11].

### Soxhlet Extraction methods:

The combined leaf extract powder was extracted by the soxhlet apparatus with 80% ethanol [12]. After extraction by soxhlet the solvent was evaporated at room temperature and the pooled extracts were preserved and stored at 4˚C until further investigation of phytochemical screening, and biological evaluation [13].

**Qualitative screening of phytochemicals:**

Phytochemical screening of extracts obtained was performed after extraction with ethanol to profile various phytochemicals present in the sample. The phytochemical determination methods used were adapted from previous works on herbal analysis [14]. The method defines biologically active compounds which contribute to the flavor, color and other features of plant parts [15]. The samples were screened for different phytochemicals.

**Synthesis of copper nanoparticles (CuNPs):**

To 10mL of ethanol extract was mixed to 90 ml of 1mM copper sulphate ($CuSO_4$) solution with constant stirring under magnetic stirrer for 15 minutes at room temperature (25°C). After the complete addition, the $CuSO_4$-EtOH extract was incubated for 24 hrs [16]. The resultant were identified by the color change and the mechanism involve is conversion of copper sulphate ($CuSO_4$) to copper ions ($Cu^+$). In addition to $CuSO_4$-EtOH extract control was maintained without the addition of $CuSO_4$ to the plant extract. The synthesized CuNPs was further confirmed by spectral analysis by SEM, UV-Vis and XRD spectroscopical studies [17].

The CuNPs were obtained in pure condition by repeated centrifugation at 10,000rpm for 30 min and the pellets were dispersed in deionized water to remove impurities [18]. Purified CuNPs were air desiccated and stored up for further analysis.

**Characterization of copper nanoparticles:**

**UV- Visible spectroscopy:**

CuNPs characterized by UV- Visible spectroscopy using Jasco V-550 spectrophotometer instrument. The size of the Copper nanoparticles was analyzed with UV-Spectrometer in the range between 100-700nm in 1cm path length quartz cell of spectrometry [19].

**Scanning Electron Microscopy (SEM) analysis:**

The SEM analysis was established by using Hitachi-S 3400N. The surface morphology and mean particle size of the nanoparticles were obtained by Scanning Electron Microscopy (SEM) analysis [20]. The preparation of sample was by keeping a drop of $CuSo_4$ colloidal solution on a copper grid which is made out of carbon [21] and the sample was dried for further analysis. The sample was observed with a 130 Kv accelerated voltage microscope [22] (Hitachi-S 3400N).

**Anti microbial activity:**

The pathogenic study of the combined leaf extracts was determined on Muller and Hinton Agar by disc diffusion method [23]. The bacterial strain of *Staphylococcus aureus* was cultured in nutrient agar medium at 37°C for 24 hours and the culture was suspended in saline (0.85% NaCl) solution and the cell density was adjusted with a MacFarland turbidity standard of 0.5, resulting in 1.5 x $10^8$CFU/ml bacterial suspension [24]. The fungal strain *Candida albicans* was cultured at 48 to 96 hours at 28°C. A pure culture of inoculums was spread on Muller Hinton Agar (MHA) plates by spread plate techniques. Inoculated MHA plates were placed on sterile Whatmann No. 1 discs. Different concentrations of 1 filter paper disc (diameter approx. 5 mm) and crude extract containing ethanol and nanoparticles were loaded onto the discs. An antibiotic ampicillin 10mg was placed as a positive control for bacteria and nystatin 10 mg was placed as a positive control for fungus. Plates were incubated at 37°C for 24 hours. Three replicate plates were kept for the micro-organism [25].

The plates were observed for the zones around each disc for the inhibiton of bacterial and fungalgrowth [26]. The diameter of the zones of inhibition was measured in millimeters (mm) using a ruler. Both combined leaf extract and CuNPs zones were compared with standard antibiotics Ampicillin and nystatin (10mg). The zone of

inhibition was interpreted as the presence or absence of the activity. The organisms are predicted as either susceptible, intermediate or resistants to the combined leaf extracts and CuNPs that have been tested [27].

**Anti-inflammatory (HRBC membrane)**

To 10mL of human blood (10ml) was centrifuged at 3000 rpm for 10min and were washed thrice with 10mL of normal saline solution [28]. The sample (2mL) was prepared with 1mL of sample solution and 1 mL of 10% RBCs suspension, and in control only saline was taken instead of the test sample. Aspirin was used as a positive control [29]. All the samples and control tubes were incubated in a water bath at 56ºC for 30min. After incubation, the tubes were cooled immediately under normal running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples [30].

Percent membrane stabilization activity was calculated by the formula

Percentage Inhibition = (A of Control – A of Sample)/A of Control x100.

**3. Result and Discussions**

**Collection of plants:**

The leaves of *Catharanthus roseus* and *Abutilon indicum* was collected from Padur, Kelambakkam, Chennai in the month of June 2023 and were identified by eminent plant taxonomist Late P. Jayaraman, Plant Anatomy Research center, Tambaram, Chennai (Fig.1 &2). After the collection of leaves, it have to be cleaned properly to remove the debris followed by which the leaves to be shade dried until the moisture is completely evaporated and the dried sample is subjected to mechanical pulverizer and powdered .

Fig.1. *Catharanthus roseus* (L.)

Fig.2. *Abutilon indicum* (L.)

**Extraction of yield:**

The leaves of *C.roseus* and *A. indicum* (25g each) were powdered and extracted with the polar solvent ethanol by soxhlet apparatus and the obtained yield was 5.34g after being concentrated under vaccum and air-dried in a dessicator was stored at 4°C till further use (Fig. 3 & 4). Ethanol extract and soxhlet apparatus was used in the extraction of leaves to maintain the viability of heat resistant compounds without much degradation in the bioactive compounds [31].

**Fig**.3.Dried leaf of *C. roseus* (L).

**Fig**.4. Dried leaf of *A. indicum* (L.)

**Qualitative phytochemical analysis:**

The combined ethanolic leaf extracts of *C. roseus* and *A. indicum* was subjected to screen the secondary metabolites according to Harbone, 1973 method [32]. Qualitative phytochemical screening of *C. roseus* and *A. indicum* confirms the presence of secondary metabolites like alkaloids, terpenoids, phenols, saponins, tannins, and steroids (Table.1). The result suggests from previous studies that the various secondary metabolites possess potential bio-applications like antimicrobial and anti-inflammatory for the purification of novel compounds. Different phytochemicals has key role against various diseases where the phenols from the leaf sample of *C. roseus* and *A. indicum* has a strong anti-microbial agent [33] and anti-inflammatory agent [34]. Antimicrobial chemical constituent has the capacity to break the proteins present in the cell wall and interrupt the mechanism of DNA and RNA replication with the change of enzyme secretions [35]. Phenolic compounds and its derivatives like Gallo-tannins have the mechanism to stop the expression of mediators such as cytokines and COX-2 in inflammatory responses [36].

**Table** 1: Phytochemical analysis of leaf extract of *C. roseus* and *A. indicum*

| S.NO | CONSTITUENT | RESULT |
|---|---|---|
| 1. | Alkaloids | + |
| 2. | Terpenoids | + |
| 3. | Phenols and Tannins | +++ |
| 4. | Saponins | + |
| 5. | Flavonoids | - |
| 6. | Quinones | - |
| 7. | Steroids | + |

**Synthesis of Copper nanoparticles:**

The appearance of CuNPs was observed from the test by the change of colors from blue to dark reddish brown in the combined leaves ethanol extracts of *C. roseus* and *A. indicum*. Reduction of $CuSO_4$ to cuprous ions was confirmed by the appearance of color (Fig. 5). The combined leaf ethanol extract contains the secondary metabolites like tannins, saponins, phenol and alkaloids which act as capping and prevents the growth of the nanoparticles whereas the stabilizing agents prevent agglomeration for the green synthesized CuNPs from the combined leaf extract, and they are also responsible for the reduction [37].

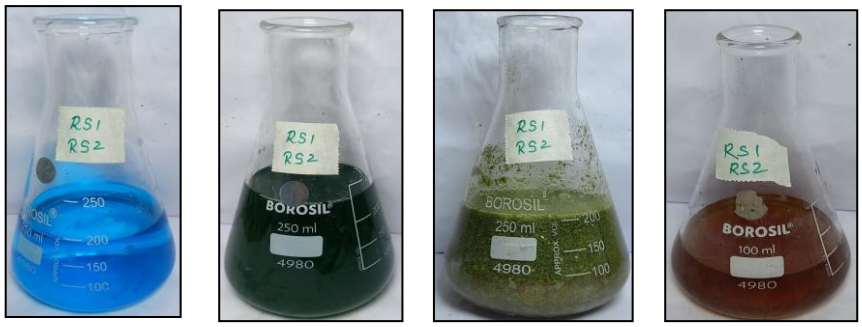


**Fig. 5:** Synthesing of CuNP's of Combined leaf extract of *C.roseus* and *A.indicum*

**Characterization of CuNP's by SEM analysis:**

The morphology of the leaf ethanolic extracts of *C. roseus* and *A. indicum* CuNPs was examined using SEM. Fig. 6 represent the SEM images of the CuNPs at different resolutions. SEM examination provided information on the nanoparticles size and surface shape. The synthesized CuNPs using plant extract is accomplished through the electric charges which are not in movement interacts with the hydrogen bonds between the capping molecules. It was established that copper nanoparticles between 60 and 100 nm in size had a spherical and largely homogeneous shape [38].

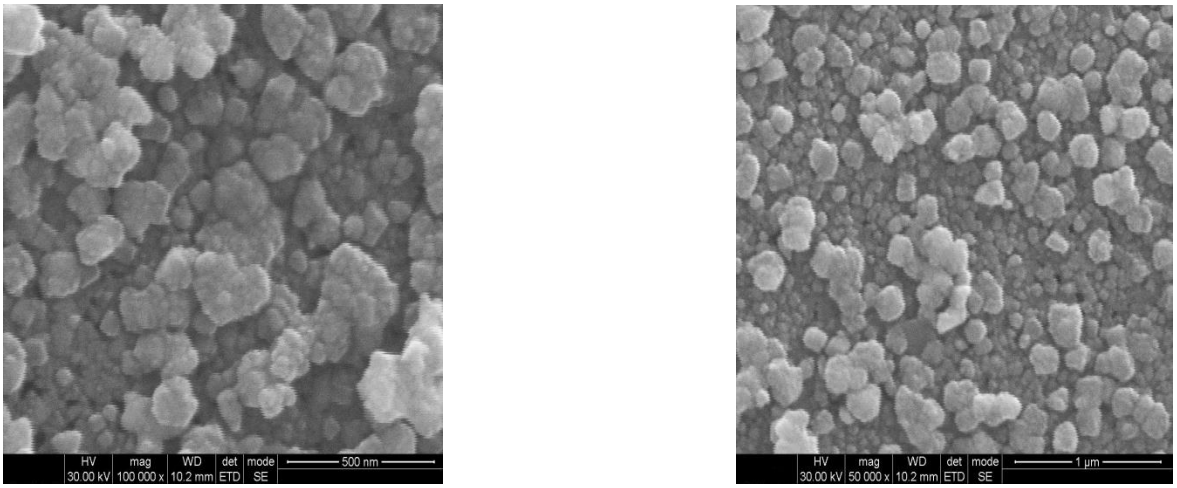


**Fig. 6.** SEM Analysis of CuNP's from the leaf extracts of *C. roseus* and *A. indicum*

**UV- Vis Analysis of CuNP's:**

The UV-Vis spectrum of leaf extract of *C. roseus* and *A. indicum* Fig. 7 showed maximum peak at 224 nm, indicating the $\pi \rightarrow \pi*$ transition of the existing polyphenolic compounds. UV-Vis spectral analysis of the combined leaf extracts was due to the transition of $\pi \rightarrow \pi*$ localized with the shift of solvent interaction from a less energy and longer wavelength to obtain the red color in the synthesis of CuNPs. The transition is specific to the double bonds present in the nuclei of extract compounds [39].

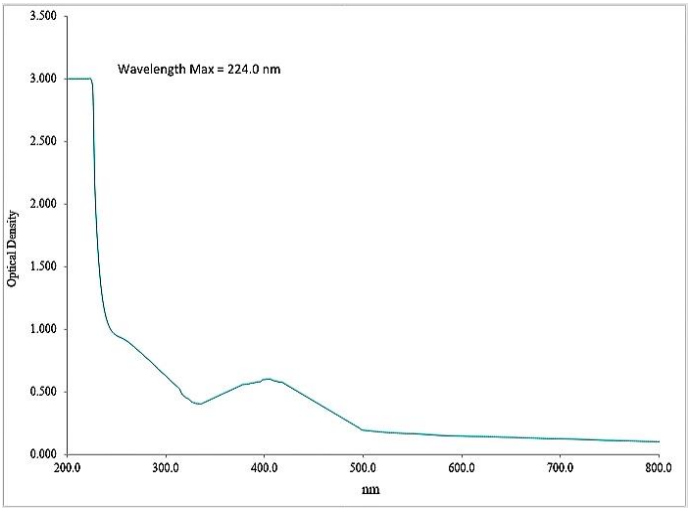


**Fig. 7.** UV-Vis spectral Analysis of CuNPs

When a semiconductor absorbs electromagnetic energy (Photons) in a large gap of semi conductor a shift of an electron occurs from the valence band to the conduction band. In that case there is a sudden increase in absorption of the material to the wavelength to bridge the band gap. The relation between the absorption coefficient to the incidental photon energy dependent on the type of electronic transitions [40].

**XRD Analysisof CuNP's:**

The crystal size of CuNPs synthesized was confirmed by XRD analysis as shown at Fig. 8. The three distinct diffraction peaks at 2θ values were observed at 1603.78°, 1664.3° and 2063.02°. The three diffraction peaks in the XRD shows the crystalline nature of the sample which is hexagonal structure according to the standard data base and no other peaks were observed which determines that other impurities were not detected. From the obtained results we interrupt that the peaks belonged only to the CuNPs [41].

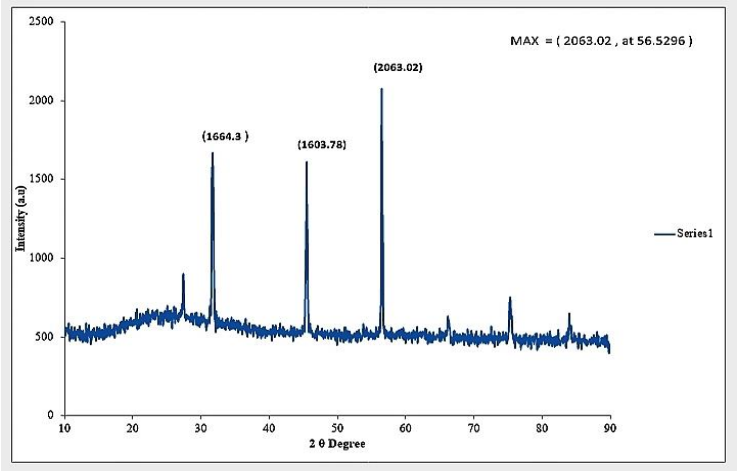


**Fig. 8.** XRD spectral Analysis of CuNPs

**Anti -microbial activity:**

**Disc Diffusion Method:**

The combined ethanolic leaf extract of *C. roseus* and *A. indicum* against the human pathogenic organisms was tested by Disc diffusion method have shown the maximum zone of inhibition. According to the findings of the extract, copper nanoparticles and ethanolic crude extract both significantly reduced *Staphylococcus aureus* and *Candida albicans* anti-bacterial and anti-fungal activity (Table 2).

| Sample | Zone of inhibiton (mm) | |
|---|---|---|
| | ***S.aureus*** | ***C.albicans*** |
| CuNP's (0.2 mg/ml) | 2.3±0.20 | 2.5±0.55 |
| Combined Leaf extracts | 1.8±0.45 | 2.0±0.5 |
| Negative control | 0 | 0 |
| Positive control | 3.2±0.41 | 3.6±0.88 |

**Table 2:** Antimicrobial effects of CuNP and combined leaf extracts by disc diffusion method

When comparing the two samples, copper nanoparticles demonstrated the greatest zones of inhibition against the test organisms. This is so because, as compared to crude extract, nanoparticles exhibit higher levels of antibacterial activity. The CuNP's synthesized leaf extract has the capacity to break the peptidoglycan wall of bacteria and fungus by the production of ROS and the other metabolic disruption of pathways to show the potent anti-bacterial effects [42] by the attraction of electrostatic law between negatively charged pathogen to positively charged nanoparticles [43].

**Anti-inflammatory Activity:**

The results obtained that ethanolic crude leaf extract and CuNPs were able to stop protein denaturation at different concentrations from 0–100µg/mL (Table.3). Inflammations are obtained as a result of damaged cells or tissue and are more prone to infection from the cuts [44]. Membrane Stabilization has strong anti-inflammatory activity due to the release of lysosomal enzymes from the damaged cells [45].

**Table 3.** Anti inflammatory activity of Combined Leaf extract

| S.No. | Concentration (µg) | Parameters | Optical density | % of inhibition |
|---|---|---|---|---|
| 1 | 0 | Membrane stabilization Assay | 0.501 | 0.00 |
| 2 | 20 | | 0.472 | 5.79 |
| 3 | 40 | | 0.421 | 15.97 |
| 4 | 60 | | 0.349 | 30.34 |
| 5 | 80 | | 0.287 | 42.71 |
| 6 | 100 | | 0.245 | 51.10 |

## 4. Conclusion

The combined leaf extract of *C.roseus* and *A.indicum* shows the presence of various secondary metabolites and among that phenols and their derivatives showed strong presence in the combined leaf extract. The synthesized CuNPs were characterized by SEM, Uv-Vis and XRD showed the affinity, structure and size of crystalinity. The characterized CuNP's leaf extract and only the combined leaf extract was tested against anti-microbial and anti-inflammatory activity and observed the positive efficacy of both the extracts. Therefore the mechanism of action is yet to be focused whereas the qualitative analysis showed strong activity of the extract which can be further uptake the research to the next level by the affinity of these nanoparticles which are ecofriendly in nature and can be utilized in large scale productions.

**Acknowledgement:**

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**Data Availability**

The data used to support the research findings are included within the article.

**Conflicts of Interest**

There are no conflicts of interest associated with the publication and among the authors.

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