ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Green Synthesis of Copper Nanoparticles from *Hibicus Rosasinensis* and their antimicrobial, antioxidant activities.

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ABSTRACT

Green synthesis of nanoparticle is a novel way to synthesis nanoparticles by using biological sources. It is gaining attention due to its cost effective, ecofriendly and large scale production possibilities. In this present study the plant is *Hibiscus rosasinensis*, was taken to investigate their potential for synthesizing copper nanoparticle. The copper nanoparticles synthesized were confirmed by their change of colour to dark brown due to the phenomenon of surface plasmon resonance. The characterization studied was done by UV-Vis spectroscopy, Fourier Transmission infrared spectroscopy (FT-IR), Transmission Electron Microscope (TEM). Leaf extract with CuNPs was shows good anti-oxidant activity from FRAP and Hydrogen peroxide scavenging assay. *Hibiscus rosasinensis* plant leaf synthesized copper nanoparticle show good antimicrobial activity against clinically important pathogens like *Bacillus subtilis* and *E.coli.* The study illustrated the biologically synthesized copper nanoparticle was act as an effective drug to treat the lung cancer.

Keywords: Copper nanoparticle, *Hibiscus rosasinensis*, TEM, UV-spectroscopy, FT-IR, Antioxidant assay, Antibacterial activity.

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INTRODUCTION

Nanotechnology

Nanotechnology is the study and application of small object which can be used across all fields such as chemistry, biology, physics, material science and engineering. As the name indicates nano means a billionth or 10^{-9} unit. Its size range usually from 1-100nm. Plants can be described as nano factories which provide potential pathway to bioaccumulation into food chain and environment. Among the different biological agents plants provide safe and beneficial way to the synthesis of metallic nanoparticle as it is easily available so there is possibilities for large scale production apart from this the synthesis route is eco-friendly, the rate of production is faster in comparison to other biological models such as bacteria, algae and fungi From the various literature studies it can be stated that the amount of accumulation of nanoparticle varies with reduction potential of ions and the reducing capacity of plant depends on the presence of various polyphenols and other heterocyclic compounds (Nair *et al*., 2010). Due to small size it occupies a position in various fields of nano science and nanotechnology. So the main aim to study its minute size is to trigger chemical activity with distinct crystallography that increases the surface area (Osaka *et al*., 2006, & Sinha *et al*., 2009). Thus in recent years much research is going on metallic nanoparticle and its properties like catalyst, sensing to optics, antibacterial activity, data storage capacity (Nair *et al*., 2010 & Sharma *et al*., 2009).

Nanotechnology is a fast growing area in the field on science which is an interdisciplinary field of both science and technology that increase the scope of investing and regulating at cell level between synthetic material and biological system (Sinha *et al*., 2009). Nanotechnology proceeds by three processes - separation, consolidation, deformation of material by one atom or molecule (Taniguchi *et al* 1974).

There are two methods of synthesis of metallic nanoparticles which are chemical method and physical method. In chemical approach it include chemical reduction (Guzman *et al*., 2009), electrochemical technique, photochemical reduction (Sharma *et al*., 2009).

The biological synthesis of nano material can solve the environmental challenges like solar energy conservation, agricultural production, catalysis (Kumar *et al*., 2011), electronic, optics (Evanoff *et al*, .2005), and biotechnological area. Biological synthesis utilizes naturally occupying reducing agent such as plant extract, microorganism, enzyme, polysaccharide which are simple and viable which is the alternative to the complex and toxic chemical processes (Xu. *et al*., 2006).

Synthesis of nanoparticle

The nanoparticles have been intensively studied over the last decade due to their characteristics: physical, chemical, electronic, electrical, mechanical, magnetic, thermal, dielectric, optical and biological properties (Schmid *et al*., 1992; Daniel and Astruc 2004). The oxides of transition metals are an important class of semiconductors, which have applications in magnetic storage media, solar energy transformation, electronics, gas sensors and catalysis (Ramgir *et al*. 2013). Although various physical and chemical methods have been extensively used to produce nanocrystalline copper oxide such as microemulsion method (Nassar and Husein 2007), arc-submerged nanoparticle synthesis system (Kao *et al*. 2007), flame-based aerosol methods (Chiang *et al*. 2012), sonochemical (Vijayakumar *et al*. 2001), hydrothermal (Zhang *et al.* 2006) and solid-state techniques (Wang *et al*. 2004), the stability and the use of toxic chemicals are subjects of paramount concern. A wider range of compounds has been reported from the brown alga of the genus *Bifurcaria bifurcata*, when diterpenoids were the predominantly reported compound classes (Blunt *et al*. 2005).

Copper nanoparticles

Copper based nanoparticles (Cu-based NPs) of different composition and sizes have been hydrothermally synthesized by varying the reaction temperature and in the presence of biocompatible surfactants; polyoxyethylene (20) sorbitan laurate, polyethylene glycol 1000 and polyethylene glycol 8000.

Organic medicines from the plants

India is the largest producer of medicinal plants and is rightly called the "Botanical garden of the World". Medicinal plants have been stated to comprise about 8000 species and account for approximately 50% of all the higher flowering plant species of India. In other words, there are about 400 families of the flowering plants; at least 315 are represented by India. Several reports describe that the anticancer activity of these plants is due to antioxidants such as vitamins (A, C, E), carotene, enzymes (e.g., superoxide dismutase, catalase and glutathion peroxidase), minerals (e.g., Cu, Mn, Se and Zn), polysaccharides, polyphenols (e.g., ellagic acid, gallic acid and tannins), flavonoids (e.g., quercetin, anthocyanins, catechins, flavones, flavonones and isoflavones), lignins, xanthones, etc. Many medicinal plants mentioned in and contain several of these antioxidant.

MATERIALS AND METHODS

Collection of Plant Materials

- The plant leaves which is collected from the local area of garden. The plant leaves was washed with distilled water.
- Then the washed leaves were dried at room temperature for 2 days. Then it was crushed with the help of mortar and pestle dispensed in 10 ml of sterilized distilled water.
- The extract was then filtered using Whatman filter paper. The filtrate was collected in a clean and dried conical flask by standard sterilized filtration method and was stored.

Synthesis of copper nanoparticle:

- For the synthesis of copper Nanoparticles, both the precursor and the reducing agent were mixed in a clean test tube in 1:1 proportion.
- For the reduction of Cu ions, 5ml of filtered leaf extract was mixed to 5ml of freshly prepared 0.05 M aqueous of CuNo3 solution. It was then kept for incubation for 2 days in dark room.
- After 2 days the synthesized copper nano particle in the leaf extract was lyophilized to get the powder form sample.

Characterization Techniques: UV-visible spectroscopy

The reduction of pure Cu to nanoparticle was monitored by measuring the UV-Vis spectrum the most confirmatory tool for the detection of surface Plasmon resonance property (SPR) of CuNPs, by diluting a small aliquot of the sample in distilled water UV-Vis spectral analysis was done by using UV-Vis spectrophotometer Systronics 118 at the range of 400-700.

FTIR (Fourier Transform Infrared Spectroscopy)

The characterization of functional groups of surface of Cu nanoparticles by plant extracts were investigated by FTIR analysis and the spectra was scanned in the range of 4000–450 cm^{-1}. The sample were prepared by dispersing the Cu NPs uniformly in a matrix of dry KBr, compressed to form an almost transparent disc. KBr was used as a standard analyze the samples.

Transmission electron microscope (TEM)

Transmission electron microscopy (TEM), selected-area electron diffraction, and high-resolution TEM were used as the main characterization tools. Upon exposure to air, these nanoparticles are oxidized at different levels depending on their sizes. The occurrence of these different levels of oxidization demonstrates the reactive nature of Cu nanoparticles and the effect of size on their reactivity.

Antimicrobial Activity

- The Mueller Hinton agar medium is originally designed for the isolation of pathogenic *Neisseria* species. Now this medium widely for antibiotic susceptibility testing.

- Then the medium and other glass wares are placed in sterilization process at 121ºCfor 15 min. After sometimes the medium was taken and pour into 2 sterile petriplates.
- Three well was taken for sample, control and antibiotics. The clinical pathogenic strains of *Escherichia coli, Bacillus subtilis* were used to determine the antibacterial activity of the copper nanoparticles.

Ampicillin is used as antibiotic to against bacteria and it was in the disc. Copper-free plant extract was poured in the disc and that cultured were used as a control. The plates were incubated under 16-18 hrs at room temperature in sterile room.

Antioxidant assay: Hydrogen Peroxide Scavenging Assay

The hydrogen peroxide scavenging assay was assessed by the method using methanolic extract of *H.Rosasinensis* leaf of 200,500,1000µg/ml were added to a 0.6 ml 0f hydrogen peroxide(40mM)with the already prepared phosphate buffer(pH 7.4).The reaction mixture were placed in at room temperature for 10 minutes. After incubation, the reaction mixture read at 230 nm against the blank solution with phosphate buffer solution. The percentage of inhibition was calculated based on the formula,

$$\% \text{ of inhibition} = (A1-A2)/A1*100$$

Where A1- absorbance of H_2O_2
A2-absorbance of the reaction mixture with extract

FRAP Method

The antioxidant capacity of each sample was estimated by FRAP assay, following the procedure described in the literature (Gohari *et al.,* 2011) with modifications. A 2.7 ml of freshly prepared FRAP reagent (TPTZ, $FeCl_3$ and acetate buffer) at 37ºC was mixed with 90µl of leaf extract and 270µl of distilled water. Using a blank containing FRAP reagent as reference, absorbance at 593 nm was determined at 10 min. Aqueous solutions of known Fe (II) concentrations in the range of 200,500,1000µg/ml (Fe_2So_4) were used for calibration.

RESULTS

Synthesis of copper nanoparticle

After the 2 days of incubation the colour changes into green colour into brown colour. So, that the change of colour changes indicates the nanoparticle synthesized in leaf extract of *H.rosasinensis.* Because of the phenolic compounds which is present in the leaf which reduced the copper ($CuNo_3$) ions to copper nanoparticle.

Characterization of Copper Nanoparticle

The UV absorption peak of copper nanoparticles range from 550nm – 650nm. Figure 1, shows the UV absorption peaks of *H. rosasinensis*. UV-Vis spectra shows the peaks approximately at 421.00nm, clearly indicating the formation of spherical CuNPs in all the plants extracts. The occurrence of the peak at 610 nm is due to the phenomenon of surface Plasmon resonance, which occurs due to the excitation of the surface plasmons present on the outer surface of the silver nanoparticles which gets excited due to the applied electromagnetic field.

Fourier Transform Infrared Spectroscopy

The copper nanoparticles synthesized using *Hibiscus rosasinensis* showed strong band at 3220.50 cm-1, 3294.00 cm-1, 3462.00 cm-1 corresponds to stretched in OH hydrogen band (3200-3600).The level of free stretched OH bond ranges are 3617.50 cm-1, 3631.00cm-1.The bands at 1072.83cm-1, 1109.92 cm-1 corresponds to stretched CO(1050-1150). In alkane functional group of (–C–H) showed band at 1414.89 cm-1. In alkene the bands was absorbed 677.48 cm^{-1}, 780.18 cm^{-1} corresponds to bending CH band (675-1000). The peaks which was obtained from the range at 1647.36 cm-1 corresponds to stretched C=C (1620-1680) in alkene functional group. The band which is observed at 3462.50 cm-1 for the stretched N–H (3300-3500 cm-1) and the band absorbed at range of 1109.50 cm-1, 1157.14 cm-1, 1271.14 cm-1 for the stretched C–N (1080-1360 cm-1) in amine functional groups. The aromatic stretched C=C band was absorbed at the 1414.89 cm-1 in the aromatic functional groups. From this method of FTIR the functional group of alcohol, alkane, alkene, amine, aromatic band was absorbed by using the solvent of KBr from the *H.rosasinensis* plant leaf.

Figure 1: UV-Visible absorption peak of *Hibiscus rosasinensis* synthesized copper nanoparticles at 610 nm.

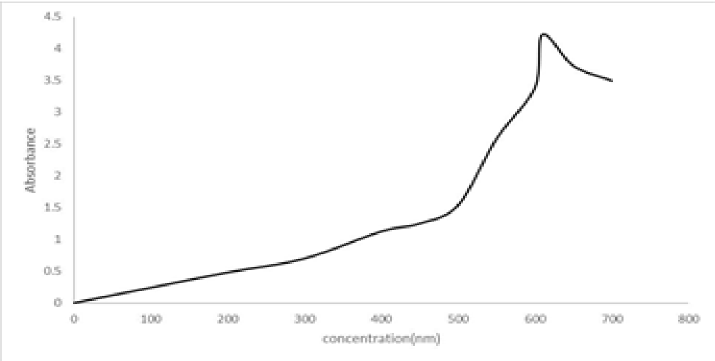
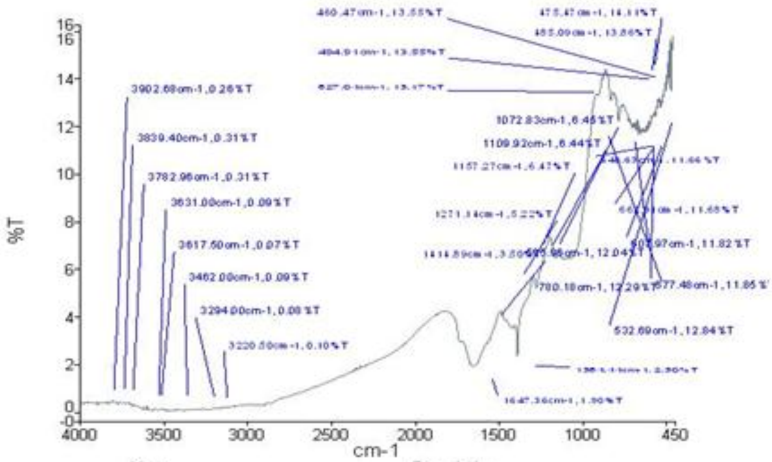


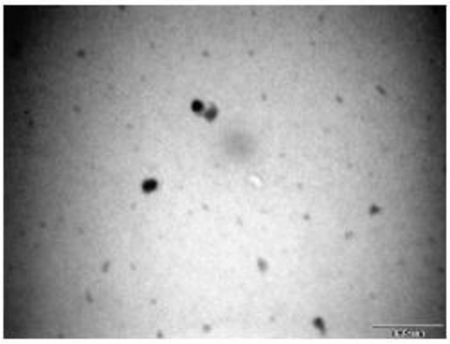
Figure 2: FTIR spectroscopic analysis of synthesized copper nanoparticles using *Hibiscus rosasinensis*



Transmission electron microscopy

From the figure 3, the particle size of the copper nano particle was size was analyzed and the morphological character was determined from the TEM. The diameter of silver nano particle was range from maximum resolution. The silver nano particle were observed in the TEM observation at 500nm magnification.

Figure 3: Particle size analyzed by TEM



Antimicrobial activity

Antimicrobial activity of the synthesized CuNPs from the leaf extract testing was done against *E. coli, Bacillus subtilis*, After 16-18 hrs, the zone of inhibition was absorbed in figure 4. In *E.coli* zone of inhibition indicate that the leaf sample of *H.rosasinensis* is against the bacteria. In figure 5, zone of inhibition was seen in *Bacillus subtilis.* The control has no inhibition of both *E. coli,* and *Bacillus subtilis*. The antibiotics was also against the microbes. Finally, the antimicrobial activity which is highly against the *Bacillus subtilis* than the *E. coli in* the leaf sample of *H.rosasinensis.*

Figure 4: Antimicrobial activity against the *E.coli*

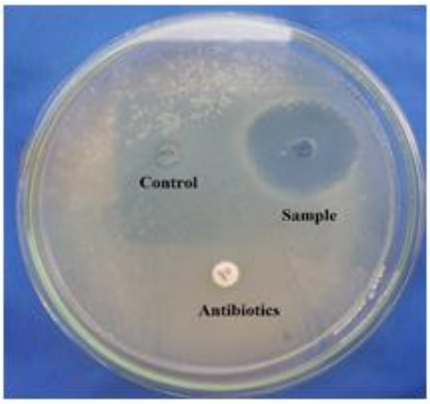
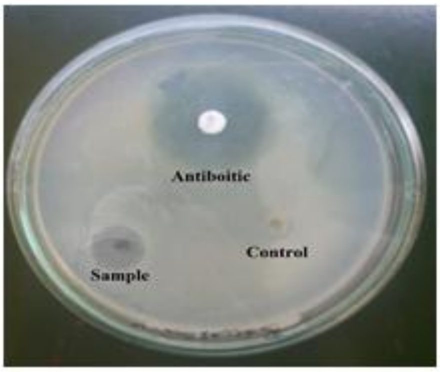


Figure 5: Antimicrobial activity against the *Bacillus subtilis*



Anti-Oxidant Activity

Hydrogen Peroxide scavenging assay

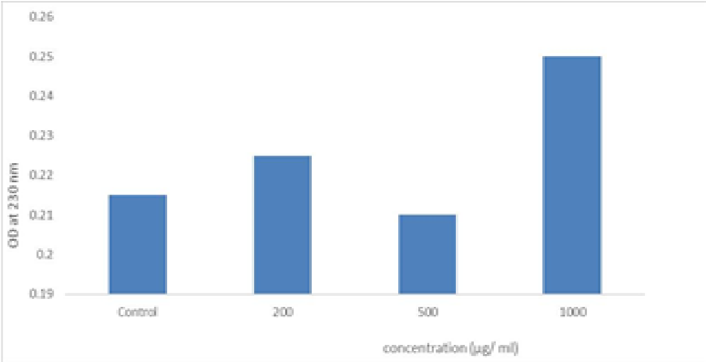
After the incubation the reading was absorbed at 230nm through UV-spec. Above figure 6, shows the concentration of the sample is (200, 500, 1000µg/ ml). OD values were taken through UV-Spectrophotometer and the % of inhibition was calculated below table 1.

Table 1: calculation for % of inhibition in H2O2 assay

Sample Concentration	% of inhibition=(A1-A2)/A1*100
Control	Nil
200	57.3
500	68.5
1000	41.6

Above Table: 1, shows the % of inhibition of the leaf sample at various concentrations from the result, it shows that control have 64.6 percent of inhibition in the leaf sample and synthesized nanoparticle contained leaf sample has high anti-oxidant property at 500 µg/µl when compared to control. So, the synthesized copper nanoparticle in leaf extract has high anti-oxidant property.

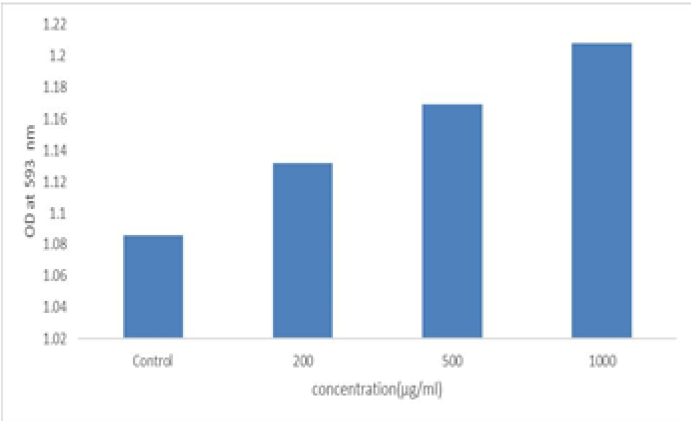
Figure 6: Anti-oxidant property by hydrogen peroxide scavenging assay



FRAP Method

After 10 minutes of incubation the OD value was absorbed at 593nm by using UV-Spectrophotometer, From the figure 7, the graph represents the various concentrations (200, 500, 1000µg/ml). The control has low anti-oxidant property when compare to various concentrations of leaf extract which synthesize the nanoparticle. So, the anti-oxidant property is high in the *H.rosasinensis* sample.

Figure 7: Anti-oxidant activity by FRAP method



DISCUSSION

H.rosasinensis Plant sample were collected and washed, dried and crushed for further procedure. Crushed plant samples were subjected to copper nitrate for the synthesis process. After the synthesis process colour change was observed. Characterization was carried out to different level for the purpose of identification of the nanoparticles.

CONCLUSION

Green synthesis of copper nanoparticles by the help of green plants is a very cost effective, safe, non-toxic, eco-friendly route of synthesis which can be manufactured at a large scale.

Hibiscus rosasinensis is showed great capability to synthesis CuNPs at optimum temperature conditions. The UV absorption peak at 610nm clearly indicates the synthesis of CuNPs. The TEM studies were helpful at deciphering their morphology and distribution. FTIR studies confirmed the biofabrication of the CuNPs by the action of different phytochemicals with its different functional groups present in the extract solution. The copper nanoparticle was synthesized from plant source and to analyze anti-oxidant activity assay by the FRAP and hydrogen peroxide scavenging assay. From this method knows to analyze the anti-oxidant property is high or low when compare to the plant extract. The CuNPs have great antimicrobial activity against *Bacillus subtilis* and *E.Coli*. By comparing the different characteristic data we could conclude that CuNPs synthesized from *H. roasinensis* is against bacterial activity by the formation of zone of inhibition (ZOI) in the agar medium.

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