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Review article

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Green Synthesis of Copper (Cu) Nanoparticles Using Marine Brown Algae *Turbinaria Ornata* and Its Brine Shrimp Lethality Bioassay

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Abstract

Objective: *The synthesis of copper nanoparticles using marine macro algae has been unexplored, which resulted to nano sizes having the greatest potential for biomedical applications. In this investigation, we present the synthesis and brine shrimp lethality bioassay of Copper nanoparticles using Turbinaria ornate, marine algae.*

Methods: *Fresh T. ornata was collected from the Mandapam, Southeast coast of India. The seaweed extract was sued for the synthesis of CuNPs at room temperature. The detail characterization of the nanoparticles was carried out, using Uvvis spectroscopy at 420nm. SEM analysis confirmed the range of particle size between 100-200nm. Fourier Transform Infra-Red (FTIR) spectroscopy analysis showed that the synthesized Copper nanoparticles are capped with biomolecule compounds which are responsible for reduction Copper ions.*

Results: *The synthesized CuNPs were tested in vivo for their cytotoxic effect against the brine shrimp lethality bioassay and related toxicity result with them know cytotoxic activities. In the concentration of 0.75 mg/ml of Copper Nanoparticles of T. ornata about 50% shrimp remain survived after 24 hours. So LC50 value was seems to be 0.75 mg/ml.*

Conclusions: *The above eco-friendly synthesis procedure of CuNPs could be easily scaled up in future for industrial and therapeutic needs.*

Introduction

Marine bio-nanotechnology is an exciting and upcoming area of research. The biologically diverse marine environment has a great promise for nanoscience and nanotechnology. Marine organisms produce remarkable nanoparticles of 1-100nm size which constitute nanofabricate structure such as seahells, pearls and fish bones. Diatoms and sponges are constructed with nanostructure cover of silica and coral reef with calcium, arranged in remarkable architectures [1]. Nanobiotechnology, an emerging trend in material sciences which provide new improved nanoscale structures with different physio-chemical properties for various therapeutic applications. Nanoparileas anticancer drug delivery system is beneficial in intra cellular infiltration and hydrophobic solubility. Nanotechnology affects drug circulation time, reduces non-specific uptake, and decreases the toxic effect of anticancer drugs. They act specifically against tumor cells and spare normal cells [2]. Pathogenic bacteria are playing an important role in the creation of unknown disease, and the development of antibiotic resistance which are the major problems in the current scenario. The applications of nanoparticles are gaining an important function in the current scenario as they possess well defined chemical, visual and mechanical attributes. For this purpose, several researches have made attempts for synthesis of CuNPs using chemical reduction, electrochemical reduction, and photochemical reduction. These methods employ harsh reducing and stabilizing agents making them unsuitable for biological applications. At nanoscale material behave differently

compared to their micro-size counterparts, due to increased specific area and thus, higher reactivity. Due to their toxic properties metal-containing NMs are good candidates for antimicrobial consumer and patient care products. The current study was to develop a simple and reliable method for the evaluation and comparison of biocide potency of NMs to different types of unicellular target organisms such as bacteria, fungi, and algae in the same test condition. Sandra suppi, Kaja kasemets and Anne kahru,2014. [3,4].

Recent environmental fate models underline that Nano wastes will end up in the aquatic environment, thus potentially affecting natural ecosystems and human health Bystrzejewska-Piotrowska *et al*,2009; Liu et al,2014;) [5, 6].

Seaweeds have been reported that to possess biological activity of potential medicinal value [8]. It was reported that seaweeds are rich source of bioactive compounds, such as terpenoids, phlorotanins, fucoidand, sterols and glycolipids, and the extracts or isolated pure components from seaweeds possess a wide range of pharmacological properties such as anticancer, antibacterial, antifungal, anti-viral, anti-inflammatory, anticoagulant, antioxidant, hypoglycemic, hypolipidemic, ant melanogenic, Antione loss, hepatoprotective and neuroprotectivities [7]. Earlier reports indicated that the extracts of brown seaweeds belonging to *Turbinaria* spp. were found to have antioxidant and anti-inflammatory activities [8].

This present investigation deals with a simple, eco-friendly synthesis of Copper sulphate nanoparticle by the reeducation of aqueous $CuSo_4$ in to nanoparticle using extract of marine algae *Turbinaria ornata*. CuNPs were tested in vivo for their cytotoxic effect against the brine shrimp lethality bioassay and related toxicity result with them know cytotoxic activities. In vivo lethality test successfully used as a preliminary study of cytotoxic and antitumor agent, Thus, the findings of this present work would give baseline information on the most promising marine macro algae and CuPNs that could be use as a basis for the development of new tools of great therapeutic Importance

Materials and Methods

Collection and Processing of Marine Algae

The brown algae *T. Ornata* were collected from costal area, Ramesvaram, Tamilnadu. The algal sample was washed in clean sea water to remove epiphytes and salts attached to the surface of the sample. The sample was transported to the laboratory in sterile polythene bags. It was then rinsed with tap water, shade dried for a week, powdered and stored in a room temperature.

Plant source selected for the present study was *Turbinaria Ornata* aerial parts of the selected plant was identified with the help of Marine Algal Research Station (CSIR-Central Salt & Marine Chemicals Research Institute, Mandapm Camp, Tamil nadu and authenticated with the specimen deposited at RAPINAT Herbarium, Department of Botany, St. Joseph's college, Truchirappalli.

Preparation of Extract

Synthesis of copper nanoparticles, 10 g leaf powder was taken and mixed with 100 ml of distilled water and kept in boiling water bath at 60 ◦C for 10 minutes. After cooling, the extracts were filtered with Whatman filter paper No. 1 and stored in refrigerator at 4 ◦C for further studies [9].

Biosynthesis of Copper Sulphate Nanoparticles from Turbinaria Ornata Extract

The aqueous solution of 10 mM Copper sulphates ($CuSo_4$) was prepared and used for the synthesis of Copper nanoparticles. 100ml of *Turbinaria Ornata* extract was added into 100 ml of aqueous solution of 10mM Copper sulphate for reduction into Copper+ ions and kept in Room temperature. The reaction mixtures were monitored spectrophotometrically at 24 h interval. The appearance of dark brown color indicated the formation of CuNPs.

Characterization of CuNPs

Synthesized Copper nanoparticles were initially characterized by sampling small aliquotes of sample into UV–visible spectrophotometer absorption spectra at 300–700 nm using JASCO V-650 spectrophotometer. The biosynthesized CuNPs were purified by repeated centrifugation at 12,500 rpm for 15 min and freeze dried. The crystalline nature of the purified CuNPs was coated on to the glass substrate and X-ray diffraction (XRD) measurements were carried out using Shimazdu, model LabX-XRD-6000 instrument operated at a voltage of 20 to 30 keV and a current of 30 mA with Cu K α radiation with a wavelength of 1.5418 Å. Scanning electron microscope Jeol JSM-6480 LV SEM machine were used tcharacterize mean particle size, morphology of nanoparticles. The CuNPs powder sample and freeze dried sample of CuNP solution were sonicated with distilled water and a small drop of this sample was placed on a glass slide and allowed to dry. A thin layer of platinum was coated to make the samples conductive. Jeol JSM-6480 LV SEM machine was operated at a vacuum of the order of 10-5 torr. The accelerating voltage of the microscope was kept in the range 10^{-20} kV.

Fourier transforms infrared spectroscopy (FTIR) spectroscopic studies were carried out to find possible bio-reducing agents present in the plant leaves. The wavelength spectrum of the leaf extracts before and after the addition of CuSo4, the samples were mixed with KBr powder and pelletized after drying the spectra were recorded using Perkin Elmer make model spectrum RX1 (wavelength range between 4000 cm−1 and 400 cm−1).

Brine shrimp Lethality Bioassay

1 g of *Artemia salina* (Linnaeus) cysts was aerated in 1000 ml capacity glass container containing Artificial saline water (34gm/l NaCl solution, pH about 8.2) was maintained. After 48 hours of incubation at room temperature (25-29°C), under continuous illumination newly hatched free swimming nauplii were harvested from the bottom. As the cyst capsules floated on the surface, this collection method ensured pure harvest of nauplii. The freshly hatched free-swimming nauplii were used for the bioassay [11].

The assay system was prepared with 10 ml of filtered seawater containing chosen concentration of extract and 1% yeast extract (for feeding) in test tubes. In each test tube, 10 nauplii were transferred and the setup was allowed to remain for 24 h, under constant illumination. Numbers of survived nauplii were counted with a hand lens. Three replicates were prepared for each dose level and after 24 hours LC50 values were determined, based on the per cent mortality, using probit regression by statistical software acts by

treating nauplii with different concentrations for Copper Nanoparticles of Turbinaria *ornate* [12].

LC50 determination

The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes. LC50 values were obtained from the best-fit line plotted log concentration verses Probit values of percentage lethality. LC50 is indicative of toxicity level of Copper Nanoparticles of *T. ornata* to Brine [13].

Results and Discussion

The seaweed was evaluated for synthesis of copper nanoparticles in short reaction time. *T. ornate* has been known to contain protein, tannin, sterol, flavonoid and alkaloids which may possibly helpful in the synthesis of nanoscale value particles. *T. ornate* extract to copper sulphate solution resulted in color change of solution from transparent to dark brown due to the production of copper nanoparticles.

Copper nano particles were prepared by chemical reduction of copper salt. The change in colour of the copper sulphate solution was captured at different stages during synthesis of copper nano colloid. The synthesis of Copper nanoparticles using aqueous leaf extracts of *Turbinaria ornate* were used for the reduction of Copper sulphate ($CuSO_4$) into nanoscale. The biosynthesis reaction started with in few seconds, it was confirmed based on the colour change from colorless to dark brown Fig 1a,1b,1c. The formation of nano sized Cu was noticed by the change in the optical properties of the reaction solution. The blue green colours of $CuSO_4$ solution gradually change to intense yellow when the particle size of copper was reduced to nano level. Temperature dependent reduction was observed by change in the colour of reaction mixture with an excitation of (SPR) Surface Plasmon Resonance of Copper nanoparticles produced a peak centered new 420nm in Uv-visible spectra indicates the presence of silver nanoparticles.

(a) (b) (c)

Figure 1: (a) 10 mM CuSO4 solution (b) Turbinaria ornate leaf extract (c) After addition of leaf extract at 5 minutes

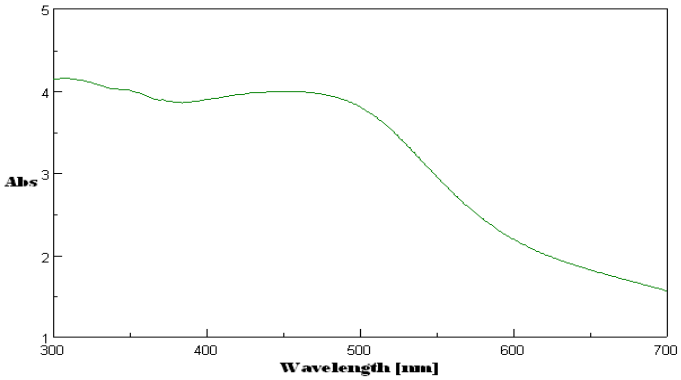

Figure 2: UV spectra of plant extract and reduced CuNPs

The green synthesized nanoparticle in crystalline nature was clearly analyzed using XRD patterns. Fig (6) shows the XRD patterns of the CuNPs prepared using the algal extract and its simulated solution. The characteristics peak observed in X-ray diffraction pattern of Copper nanoparticles synthesized from seagrass leaf extract of *Turbinaria ornate* further demonstrated and confirmed the presence of Copper nanoparticles. The XRD peaks at 31.1, 32.8, 40.4, 50.4, 66.3, and 73.7, correspond to the (111), (200), (220), and (311) planes are observed which may be indexed as the band for centered cubic structure of Copper (Fig.6). The presence of these four intense peaks corresponding to the nanoparticles was in agreement with the Bragg's reflections of gold identified with the diffraction pattern. [10]. The XRD pattern of T. *conoides*-derived AuNPs shows some unidentified peaks that if raised with standard Au peaks reveals the association of algal biomolecules with the synthesized AuNPs. Similar result of XRD for gold nanoparticles synthesized using brown algae *S. marginatumwas* found by [14]. XRD analysis showed three distinct diffraction peaks at 38.080º, 64.560º and 77.640, that indexed the planes 111, 113 and 080 of face centered cubic structure of silver [15]. The XRD pattern thus clearly illustrates that the silver nanoparticles synthesized by the present green method are crystalline in nature. Scherer's formula disclosed the of the nanoparticles is about 10 nm subtending electron microscopic studies. The average size distribution of copper nanoparticles in colloidal solution was found to be 100nm. The XRD pattern thus clearly illustrates that the Copper nanoparticles synthesized by the present green method are crystalline in nature.

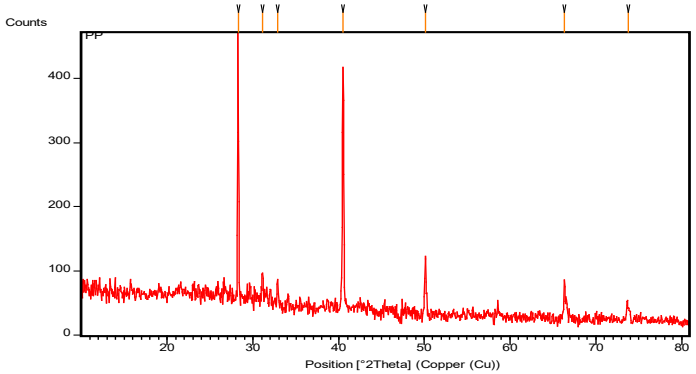

Figure 3: XRD pattern showing peaks of CuNPs

FTIR analysis was used for the characterization of Copper nanoparticles from the absorbance bands are known to be associated with the stretching vibration for N-H stretching vibration in the amide linkage. The band at 2924cm-1 can be assigned absorption peaks of SH structure (organo silicon compound), On the other hand, the shift of band from 1409cm^{-1} is attributed to the binding of C=C (Alkanes) with nanoparticles. Bands at 1244cm^{-1} can be assigned as absorption peaks of S=O stretching vibration (Sulfonamides). The bands at 1085cm-1 in Copper nano may be attributed to C-O (Thiocarbonyl group). Bands at 1037cm- can be assigned to O-C-C (Aromatic esters of primary alcohols). The weak bands at 887cm^{-1} 601cm^{-1} can be assigned absorption peaks of C=S (Stretching vibration sulfides). This study also confirms that the carbonyl group from amino acids or proteins has stronger ability to bind copper so that the proteins or enzymes could most possibly cap the Copper nanoparticles to prevent the agglomeration of the particle. Reported that the presence of carboxylic, amine, phosphate, and hydroxyl functional groups is involved in the reduction of Copper ions in the algae extract of *Turbinaria ornata.*

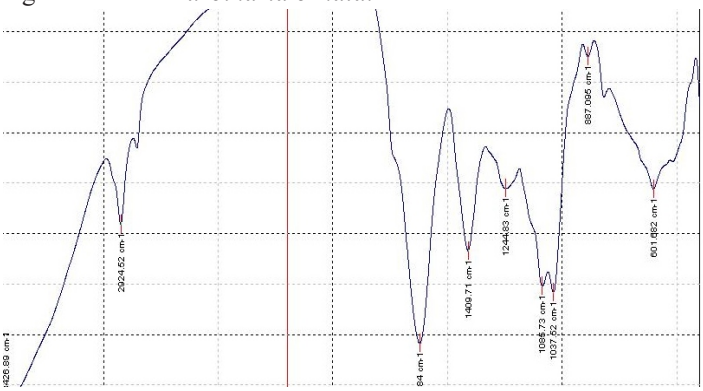

Figure 4: FTIR spectra of CuNPs and T.ornata extract

According to the Scanning electron microscope, the morphology of plant extract and Copper nanoparticles was observed spherical in shape with aggregation (Fig 4). When reaction mixtures were incubated for 24 hours some nanoparticles aggregated. The size of Colloid Copper nanoparticles was found to be 50 nm with average of 100 nm. We agree to take as true that, higher concentration of bioactive compounds in the colloidal solution might cause the formation of nanoclusters. Moreover, the results suggested that the Copper nanoparticles are synthesized due to the action of *Turbinaria ornata* (seagrass) plant extract, which act as good bioreductant for biosynthesis. Aquatic seaweed leaf extracts of *Ipomoea aquatica, Enhydra fluctuan* and *Ludwigia adscendens* were used in the synthesis of silver nanoparticles. This enables the development of value added products from the weed leave, SEM studies showed the formation of spherical and cubic nanoparticles by *I. aquatic* and spherical nanoparticles [16].

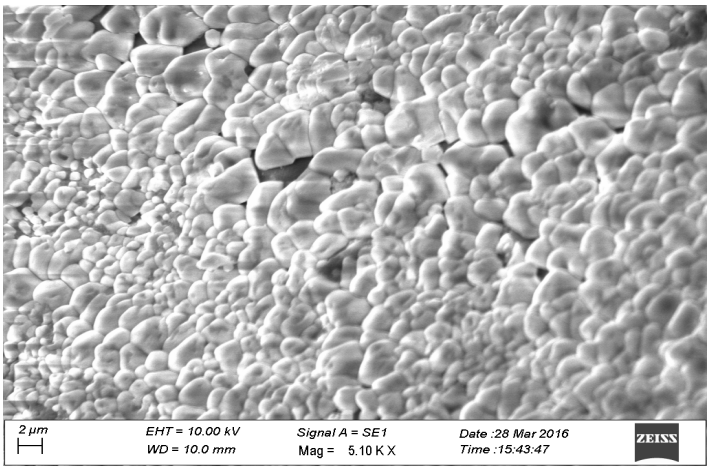

Figure 5: Scanning Electron Microscopic image shows the polydispersed CuNPs with the mean size of 150 nm

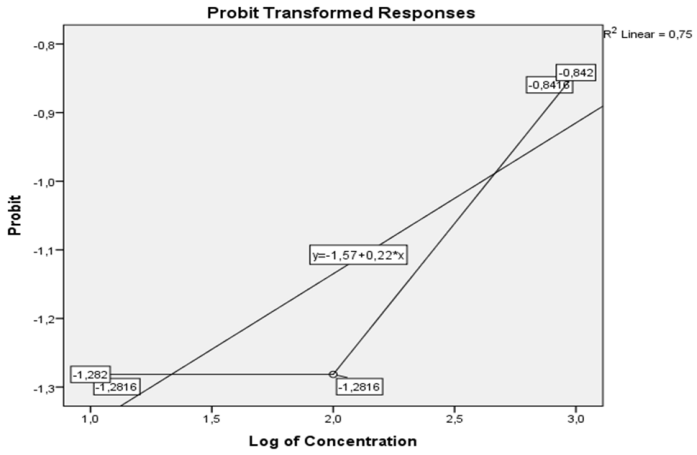

Figure 6: Brine Shrimp Lethal assay

Brine Shrimp Lethal Bioassay

The LC 50 value of brine shrimp lethality bioassay obtained for these algal extracts and that of the positive control, CuNPs have been presented in Table 1 and 2 respectively. CuNPs extracts exhibited significant toxicity towards brine shrimps. In the concentration of 0.75 mg/ml of Copper Nanoparticles of *Turbinaria ornata* about 50% shrimp remain survived after 24 hours. So LC50 value was seems to be 0.75 mg/ml.

The lethality concentration (LC50) of *Lantana camara, Chromolaena odorata*, and *Euphorbia hirta* extracts were 55 ppm (µg/mL), 10 ppm, and 100 ppm respectively (table 1). The degree of lethality was directly proportional to the concentration of the extract. Maximum mortalities (100%) were observed at a concentration of 1000 ppm in both *Lantana camara and Euphorbia hirta* extracts

while that of *Chromolaena odorata* was at 100 and 1000 ppm. Based on the results, the brine shrimp lethality of the three plant extracts was found to be concentration-dependent. The observed lethality of the three plant extracts to brine shrimps indicated the presence of potent cytotoxic and probably antitumor components of these plants [17].

Table 1: Brine Shrimp Lethal assay of Copper Nanoparticles of Turbinaria ornata

S.No	Concentration ppm	Total Number of survivors(24 hours)			Total no: of survivors	%Mortality
		T_1	T_2	T_3		
1	0	10	10	10	30	100%
2	1	10	10	10	30	100%
3	10	9	9	9	27	90%
4	100	9	9	9	27	90%
5	1000	8	8	8	24	80%

Table 2:Cell Counts and Residuals

Number	Concentration	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability
1	,000	10	0	,230	-,230	,023
2	1,000	10	1	,547	,453	,055
3	2,000	10	1	1,139	-,139	,114
4	3,000	10	2	2,084	-,084	,208

Table 3:Chi-Square Tests

		Chi-Square	dfb	Sig.
PROBIT	Pearson Goodness-of-Fit Test	,655	2	,721a

a. Since the significance level is greater than, 150, no heterogeneity factor is used in the calculation of confidence limits.
b. Statistics based on individual cases differ from statistics based on aggregated cases.

LC50 Determination

The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes. LC50 values were obtained from the best-fit line plotted log concentration verses Probit values of percentage lethality described by Finney. LC50 is indicative of toxicity level of Copper Nanoparticles of Turbinaria ornata to Brine Shrimp.

Conclusions

In the present investigation, we described environment friendly synthesis of CuNPs using *Turbinaria ornata* Seaweed extract. The characterization with UV- visible spectroscopy, Fourier Transform Infrared (FT-IR), Scanning Electron Microscope (SEM) and X-ray Diffraction (XRD) analysis evidence the formation of nanoparticles. The synthesized Cu nanoparticles showed promising were tested in vivo for their cytotoxic effect against the brine shrimp lethality bioassay and related toxicity result with their cytotoxic activities. These particles were observed by SEM technique, it reveals that with the adopted method of synthesis and stabilization it is possible to keep the prepared nano below 200nm size, a qualifying limit to be called as nanoparticles. Thus, the findings of this present work would give baseline information on the most promising marine macro algae and CuPNs that could be use as a basis for the development of new tools of great therapeutic Importance. *In vivo* lethality test successfully used as a preliminary study of cytotoxic and antitumor.

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