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RESEARCH ARTICLE

Green Synthesis and Characterization of Copper Nanoparticles Using *Urginea wightii* and its Biological Activities

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Abstract

Objectives: To investigate the potential of copper and copper oxide nanoparticles synthesized from the bulb extract of *Urginea wightii* (*U. wightii)*. **Methods:** Copper and copper oxide nanoparticles (Cu-Cu_2O NPs) were synthesized by the reduction method and were characterized by using UV-vis spectroscopy, FTIR, XRD, SEM, and EDX. Antioxidant activity was calculated by DPPH and ABTS method. *In vitro* antimicrobial activity by agar well diffusion method and cytotoxic activities by MTT assay. **Findings:** The presence of Cu-Cu_2O NPs was confirmed by visible colour change, XRD pattern showed distinctive peaks corresponding to 110, 111, 200, 220, and 222 planes revealing the crystalline nature; FTIR spectra showed the presence of different functional groups such as alcohol (OH-stretch), alkene (C=C stretching) and amines (NH bending), etc. SEM studies revealed the morphological and structural characteristics of biosynthesized NPs and the percentage of inhibition was 92% and 90% at 100 μg/mL respectively. Further studies of *in vitro* antimicrobial activity of different concentrations of Cu-Cu_2O NPs of *U. wightii*, showed the highest inhibition zone against *Pseudomonas aeruginosa* (22 mm). The NPs exhibited cytotoxic activity against the A549 lung cancer cell line in a dose-dependent manner with IC_{50} of 83.629 μg/mL. **Novelty:** This study showed that Cu-Cu_2O NPs synthesized from *U. wightii* are a good antioxidant agent, potent antimicrobial, and cytotoxicity, hence can be used in clinical therapeutic applications.

Keywords: Urginea wightii; green synthesis; antioxidant; antimicrobial; cytotoxicity

1 Introduction

Nanotechnology is a fast-booming branch in modern science that involves the synthesis of nanoparticles. It aims at producing lighter, faster, cheaper, and small devices which consume less energy but have greater ability while using less raw materials. Currently, much attention is paid to metal oxide nanostructures, a particular class of nanomaterials. Their physical, chemical and catalytic properties are slowly affected by the size, shape, structure, and composition of the nanomaterials [(1)].

Numerous studies have reported the green synthesis of metal/metal oxide nanoparticles such as silver, copper, iron, zinc, gold, selenium, etc. The combination of metal or metal oxide nanoparticles is known to have a wide range of plant biodiversity due to the presence of phytochemicals such as flavonoids, phenols, terpenoids, and alkaloids[2]. These compounds are found to possess antibacterial, antioxidant, and anti-inflammatory properties[3].

Among all the metals, copper is one of the trace elements that have a vital role in growth, immunity, hematopoiesis, melanin and makes the thyroid gland function normally[4]. Copper and copper oxide nanoparticles have been successfully synthesized using various plant parts such as roots, stems, leaves, fruits, and barks of plants[5]. Plant extract is considered as the best material for synthesis of nanoparticles, since plants contain various phytochemicals which can replace high toxicity and also can reduce the harm caused to the Environment[6]**.** Recent research has proved that the assessment of photocatalytic and other biological activities of copper oxide nanoparticles is better compared to other metal oxide nanoparticles[7]. Raizada et al.[8] reported that among the various metal oxides, copper oxide (CuO) has been studied widely since it exhibits properties like large surface area, electrochemical activity, good redox potential, and excellent conductivity and stability.

There are different physical and chemical methods of synthesizing nanoparticles, due to the use of harmful substances leading to several biological threats and are expensive. To counter the limitations, the most effective and emerging technology is the green synthesis method which is eco-friendly, cost-effective, less toxic, and more efficient than the other methods. One of the major advantages of using plant extracts is that they provide a biological route for various metal nanoparticles[9]. During the synthesis of nanoparticles, various parameters such as pH of the reaction mixture, reaction time, reaction temperature, concentration of the plant extract, and metal precursor were carefully investigated.

Medicinal plants are used since ancient times to treat several diseases, the bioactive compounds present in them are responsible for their curative and preventive effects. The genus *Urginea wightii* belongs to the family Hyacinthaceae commonly known as wild onion or Indian Squill distributed in India, Africa, and Mediterranean regions[10]. Due to its rich medicinal properties, it is included in the British and European Pharmacopoeia. The identified chemical compounds in *U. wightii* are stigmasterol, hexadecenoic acid and methyl ester, etc. [11]. A thorough survey of the literature indicated that not much work has been done on U. wightii in the field of nanotechnology. The present study was carried out to synthesize Cu-Cu2O NPs from *U. wightii* and their characterization to study the physicochemical properties such as crystalline nature, functional groups, structure, and particle size by using XRD, FTIR, SEM, EDX and also to determine antibacterial and antioxidant efficacy.

2 Methodology

Most of the plants having antioxidant properties are used for the synthesis of nanoparticles only such plant parts have reducing properties which help in the reduction process. The biomolecules like phenols, flavonoids, proteins, tannins, and terpenoids found in the plant extracts are responsible for the reduction and capping of the nanoparticles[12].

2.1 Preparation of *Urginea wightii* bulb extract

The fresh *U. wightii* bulbs were collected from Kolar, Karnataka during the monsoon season. The bulbs were washed repeatedly with water to get rid of the soil particles and later washed in water and were taken away into small pieces. It was dried on a paper towel for 3-4 days at room temperature and was ground to powder employing a commercial mixer and sieved to get a particle size up to 30 μm. The powder was dried in an oven at 45 °C for 2 h and stored for further use.

2.2. Synthesis of copper nanoparticles

The Cu-Cu_2O NPs were synthesized by the reduction method. 10 mM solution of copper sulphate was prepared in double-distilled water. Plant extract of 10 mg/mL concentration was prepared in methanol. Both the above solutions were mixed in equal volumes and initial colour and pH changes were recorded. The nanoparticles were confirmed by measuring the wavelength in UV Spectrophotometer. The solution containing the specified nanoparticles was filtered by a 0.22-micron syringe filter. The filtered solution was dried and powdered. These purified nanoparticles were used for further characterization and for various other biological activities[13].

2.3. Characterization of nanoparticles

Cu-Cu_2O NPs were primarily confirmed using UV-vis spectroscopy at a scan range from 200 to 800 nm wavelength (UV-1800 Shimadzu). The crystalline property of NPs was characterized using an X-ray diffractometer (XRD, Siemens' D5005) using monochromatic Cu Kα radiation (λ=1.5406 A°) at a voltage of 40kV and a current of 40mA with a scan rate of 20°/min and step size of 0.05°over 2θ range of 10° to 80° and measurement temperature at 25 °C. Different functional groups within the sample

were detected by the FTIR spectrophotometer[14]. The morphological and structural features were determined using scanning electron microscopy (SEM) and transmission electron microscope with high resolution (HRTEM), elemental composition were analysed with EDX using TESCAN VEGA. The zeta potential analysis was carried out in Litesizer 500 equipment.

2 .4. Antioxidant activity

2 .4.1. DPPH radical scavenging activity

The proton removal activity was determined by the DPPH method reported by Keshari with slight modifications[15]. Different concentrations (1, 10, 25, 50 and 75 μg/mL) of plant extract were made up to 1 mL and treated with 3mL DDPH solution. All the solutions were mixed thoroughly and were allowed to stand in dark for 30 min at room temperature. After incubation, the absorbance of the mixture solution was noted at 518 nm employing a UV-vis spectrophotometer. Each sample was done in triplicates. Control was prepared without adding NPs and ascorbic acid was used as standard. Percentage scavenging activity was calculated using the subsequent formula.

% DPPH scavenging activity= (OD Control –OD Sample/ OD Control) x 100

2 .4.2. ABTS radical scavenging activity

The antioxidant activity of the samples was measured by ABTS decolorization assay according to the method reported by Igor[16] with slight modifications. The stock solutions contain 7 mM ABTS and a couple of 4 mM potassium persulfate solution. The working solution was then prepared by mixing the 2 stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. 2mL of Cu-Cu_2O NPs solution in methanol was prepared in different concentrations (1, 10, 25, 50, and 75 μg/mL). Then 2mL of ABTS was added to each tube and were thoroughly mixed and was kept in dark for 10 min. Then OD values were recorded at 734 nm. Percentage scavenging was calculated using the subsequent formula.

Total scavenging activity= (OD Control –OD Sample/ OD Control) x 100

2 .5. Antimicrobial activity

The antibacterial activity of the synthesized Cu-Cu_2O NPs was investigated on four bacterial strains, namely *Bacillus cereus* (ATCC 14579), *Klebsiella pneumonia* (MTCC 3384), *Pseudomonas aeruginosa* (MTCC 424), and *Staphylococcus aureus* (MTCC 737) all the bacterial strains were procured from the Department of Microbiology, Bangalore University, Bengaluru. Fungal strains *Alternaria solani* and *Candida albicans* (ATCC10231) were procured from Cellkraft Biotech, Bengaluru.

2.5.1. Antimicrobial activity by Agar well diffusion method

Pathogenic organisms from fresh cultures were smeared evenly on the sterilized Muller Hinton Agar plates employing a cotton swab and allowed to dry. Wells of roughly 8 mm diameter were cut in agar plates by using sterile gel puncture. 10 - 30 μg/ml of Cu-Cu_2O NPs with different concentrations of test sample and standard were poured within the wells. Ciprofloxacin was used as a standard and DMSO was used as negative control against all pathogens. The experiments were carried out in triplicate. to observe the zone of clearance, the plates were incubated at 37 °C for 24 h and zones of inhibition were measured and photographed the following day[17].

2 .5.2. Antifungal activity

The antifungal activity was determined by the well diffusion method[18]. About 25mL of potato dextrose agar was poured into a sterile Petri plate. The plates were allowed to solidify, after which 100 μL of fungal spore suspension of mycelia fungal pathogens were swabbed using a sterile cotton swab on plates, and wells were made. Test samples of various concentrations such as 10, 20, and 30μg/ml were loaded into the wells, and clotrimazole was used as a positive control. All the drug-loaded Petri plates were incubated at 37 °C for 72 h. The antifungal activity was determined by measuring the zone of inhibition around the wells.

2.6. Anticancerous activity

To determine the cytotoxic effect of Cu-Cu_2O NPs and *U. wightii* bulb extract, cell viability was measured with the MTT reduction assay[19]**.** **A**549 carcinoma cell lines were procured from the National Centre of Cell Science (NCCS), Pune. These cell lines were seeded in a 96-well plate for 24 h, in 200 μL of DMEM with 10% FBS. After that, the media was removed and replaced with a suspension of varied concentrations of Cu-Cu_2O NPs 10 to 100 μg/ml, and therefore the cells were incubated for 48 h. Then MTT was added and incubated at 37 °C for an additional 4 h. Then the media was removed and 200 μL of DMSO was added to every well. OD value was subjected to calculate the percentage of viability by using the subsequent formula.

Percentage of cell viability = (OD Control –OD Sample/ OD Control) x 100

2.7 Statistical analysis

Mean ± standard deviation (SD) was used to interpret the results. All the experiments were done in triplicates, and data were analyzed by one-way analysis of variance followed by Duncan's multiple range tests using SPSS software. $p<0.05$ was considered statistically significant.

3 Results and Discussions

In the present study, Cu-Cu_2O NPs were successfully synthesized in a simple and eco-friendly way by reducing Cu^{2+} ions present in the aqueous solution of copper sulphate with the assistance of *U. wightii* bulb extracts. The formation of Cu-Cu_2O NPs occurs with an observable change in the color of the bulb extract when copper salt was added shown in Figure 1 .Free electrons which are present on the surface of the metal nanoparticles gives surface plasmon resonance (SPR) absorption band due to the vibrations of electrons of metal nanoparticles with the light wave. Many studies revealed that the biomolecules present in the plant first forma complex with the copper salt and then reduce the ions to form nanoparticles [20]. In our study bulb extract mediated synthesized Cu-Cu_2O NPs were fast and was stable for several months due to the stabilizing agent present in the plant extract.

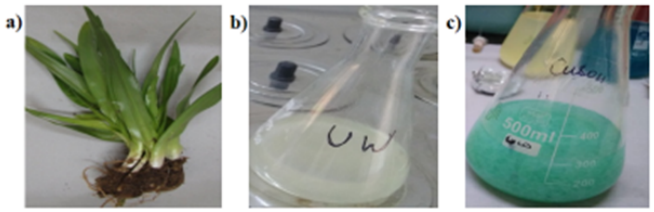

Fig 1. (a) *U. wightii* bulb; (b) *U. wightii* bulb extract; (c) Formation of Cu-Cu_2O NPs

3.1 Characterization of nanoparticles

3.1.1 XRD Data analysis of Cu-Cu2O NPs

X-ray diffraction technique (XRD) was accustomed examine the structure of synthesized Cu-Cu_2O NPs and the XRD graph is depicted in Figure 2 . Diffraction peaks at 43.26°, 50.32°, and 74.05° are indicated as (111), (200), and (220) planes of copper in the synthesis of CuNPs, diffraction peaks at 29.67°, 36.57°, 42.40°, 61.50°and 73.59°corresponding to (110), (111), (200), (220) and (222) reflections, respectively indicating the formation of copper oxide nanoparticles (JCPDS No.05-0667) [21]. Mixed NPs and impurities are present in the sample. Later calculated the size of NPs using Scherrer's equation, which was found to be 10-40 nm and it's well-matched with our study.

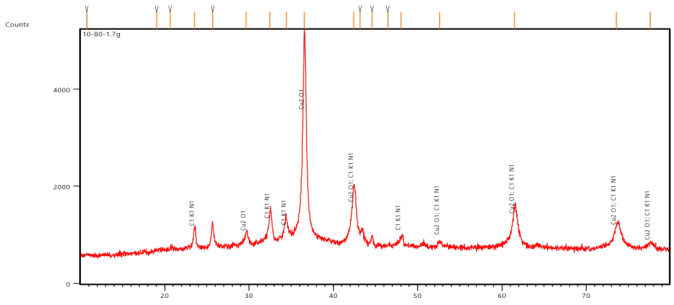

Fig 2. XRD pattern of Cu-Cu_2O NPs from bulb extract of *U. wightii*

3.1.2 FTIR Data analysis of Cu-Cu2O NPs

FTIR spectroscopy was further analysed to the molecular structure to identify the functional groups involved in the reduction and stabilization of copper ions. The FTIR spectra obtained from Cu-Cu_2O NPs are shown in Figure 3. FTIR spectrum revealed the presence of chemical bonds responsible for the formation of Cu-Cu_2O NPs. The FTIR spectrum was usually measured within the range 512-4000 cm^{-1}. The strong peaks at 3756 cm^{-1} represent the presence of OH stretch, whereas 2917 cm^{-1} corresponds to the C-H group, 1452 cm^{-1} and 1258 cm^{-1} identified as an acid group. The absorption band at 507.95 cm^{-1} related to vibrations of Cu-Cu_2O confirms the formation of Cu-Cu_2O NPs. The prominent peaks are the characteristics of Phytocompounds like flavonoids and terpenoids present in the bulb extract of *U. wightii*. The differences in the peak locations represent that the protein's obligation for the synthesis of Cu-Cu_2O NPs is diverse. These protein molecules control the agglomeration of the particles [22].

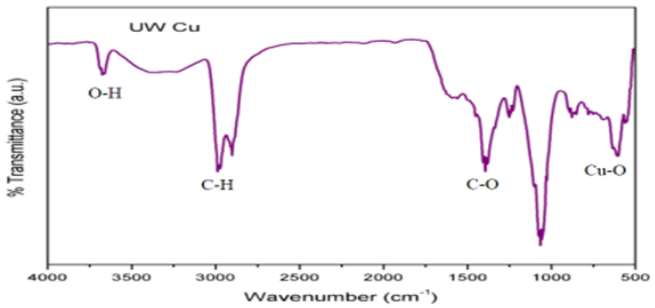

Fig 3. FTIR graph of Cu-Cu_2O NPs from bulb extract of *U. wightii*

3.1.3 SEM, HR TEM and EDX Data analysis of Cu-Cu2O NPs

The morphological characterization and size of the biosynthesized Cu-Cu_2O NPs were analysed using SEM-EDX and HRTEM analysis. Reports from the SEM analysis revealed the presence of spherical nano particles with some agglomeration because of sample preparation, the average size of the nanoparticles was found to be 71.91nm shown in Figure 4a. Similar spherical morphology was found in *Celastrus paniculatus* [23]**. M**ajority of the nanoparticles observed from the micrograph were found to be spherical in shape and they showed variation in the particle size shown in Figure 4b. The EDX analysis confirmed the composition and stability of synthesized Cu-Cu_2O NPs shown in Figure 4b. together with copper, there are many weak signals like oxygen, sulphur, and sodium. There are few weak signals, this could be due to the X-ray emission from the macromolecules like flavonoids, carbohydrates, phenols, tannins, and steroids present in the plant extract.

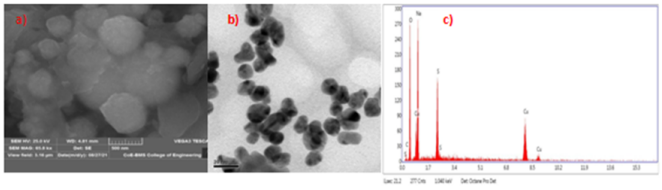

Fig 4. (4.a) SEM Micrograph (4.b) TEM Micrograph (4.c) EDX spectrum of Cu-Cu_2O NPs from bulb extract of *U. wightii*

3.1.4 Zeta potential

The Zeta Potential of Cu-Cu_2O NPs is shown in Figure 5. The zeta potential obtained from the nanoparticles showed a negative surface charge with a value of −31.5 mV. The obtained result suggests good colloidal stability of the Cu-Cu_2O NPs and can be attributed to the high coating content and the negative value of zeta potential may be due to the formation of hydroxyl groups on the surface of the particle upon dispersion in water [24]**.** The solution containing the test sample maintained good stability, as there was no aggregation when kept for an extended period.

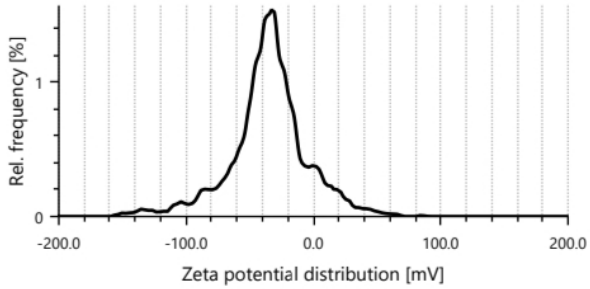

Fig 5. Zeta potential analysis of Cu-Cu_2O NPs from bulb extract of *U. wightii*

3.2 Antioxidant activity

3.2.1 DPPH radical scavenging activity

DPPH could be a stable chromogenic and lipophilic free radical that's ready to act as an initiator in lipid peroxidation and oxidation chain reactions. The antioxidant strength depends on the reduction percentage of the initial dark purple to yellow colour. Figure 6 represents the DPPH atom scavenging activity of Cu-Cu_2O NPs compared with the standard water-soluble vitamin. The activity exhibited a dose-related relationship with 92% inhibition at 100 μg/mL. IC_{50} values of ascorbic acid and Cu-Cu_2O NPs were found to be 22.92 μg/mL and 31.99 μg/mL respectively.

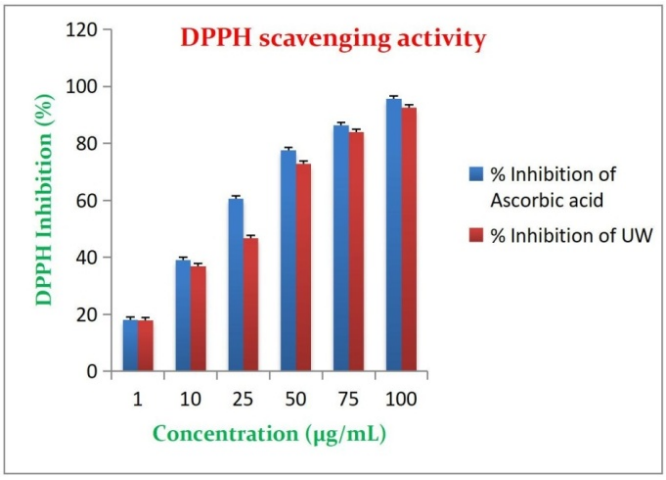

Fig 6. DPPH scavenging activity of Cu-Cu_2O NPs from bulb extract of *U. wightii*

3.2.2 ABTS radical scavenging activity

ABTS is a super tool for detecting hydrogen donating antioxidants. The percentage of inhibition showed dose-dependent activity as shown in Figure 7 with IC_{50} values of ascorbic acid and Cu-Cu_2O NPs were 25.91 and 32.13 μg/mL respectively. ABTS is a widely used technique which includes a radical which is shaped chemically to remove the color in its non-radical system [25].

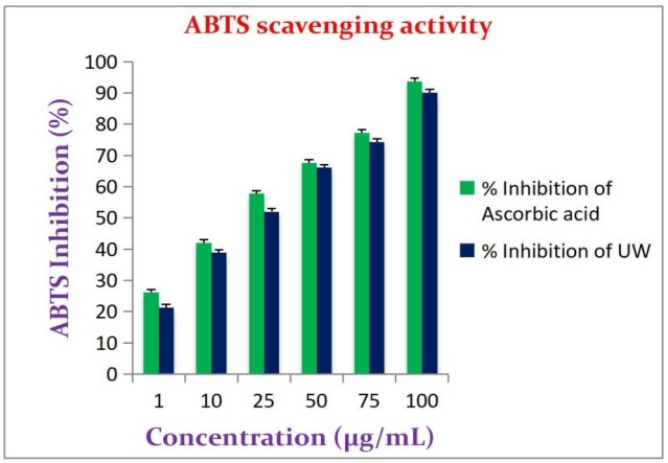

Fig 7. ABTS scavenging activity of Cu-Cu_2O NPs from bulb extract of *U. wightii*

3.3 Antimicrobial activity

3.3.1 Antibacterial activity by Agar well diffusion method

The antimicrobial activity of the synthesized Cu-Cu_2ONPs was evaluated against *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus* and *Klebsiella pneumonia* shown in Figure 8. Cu-Cu_2O NPs showed a pronounced antimicrobial activity against all the tested microorganisms the highest zone of inhibition (22 mm) was recorded by *Pseudomonas aeruginosa* (Gram -ve), and the least zone of inhibition (11 mm) was recorded with *Staphylococcus aureus* (Gram +ve) this might be due to the nature of the structure of cell wall. The efficient antibacterial activity is due to a high number of amine groups in Cu-Cu_2O NPs also as a large surface area which provides contact between bacteria and nanoparticles surface, exhibiting required antibacterial results which confirms their potential as a remedy for different diseases caused by the tested bacterial strains [(26)] .

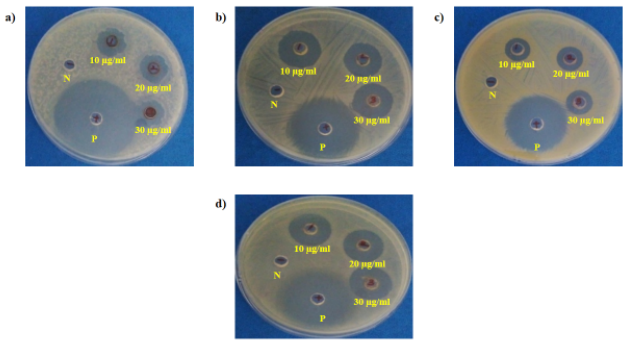

Fig 8. Zone of inhibition with different concentration of Cu-Cu_2O NPs by Well Diffusion method (a) *Bacillus cereus* (b) *Klebsiella pneumonia* (c) *Pseudomonas aeruginosa*

4 Antifungal activity

4.1 Antifungal activity by well-diffusion method

Antifungal analysis of green synthesized Cu-Cu_2O NPs was carried out using different concentrations (10, 20, and 30μg/mL) against *Alternaria solani* and *Candida albicans*. Dose-dependent responses were observed against the fungal strains, C. albicans did not show much activity; whereas, A. solani showed activity in all the concentrations. The highest zone of inhibition of 15 mm was seen at 30μg/mL (Figure 9).

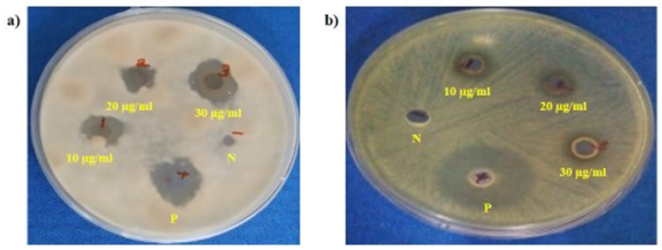

Fig 9. Zone of inhibition with different concentration of CuNPs by well Diffusion method (a) Alternaria alternata (b) *Candida albicans* **(N - Negative & P - Positive)**

4.2 Anticancer activity

4.2.1 MTT assay

MTT is a colorimetric assay that's widely used to assess cytotoxicity and cell viability. Over a decade, copper-based NPs had a robust biological impact because it plays an important role as anticancer agents. In the present study, the green synthesized Cu-Cu_2O NPs showed cytotoxic effect against the A549 lung cancer cell line during a dose-dependent manner; whereas, the crude plant extract failed to show any activity. The Inhibitory Concentration (IC_{50}) of the phytomediated Cu-Cu_2O NPs was found to be 83.629 μg/mL against the A549 lung cancer cell line (Figure 10). Copper oxide nanoparticles obtained using *Acalypha indica* leaf extract incorporated with graphene oxide showed 70% cytotoxic activity at 100 μg/mL [27]**.** The secondary metabolites like alkaloid, tannin, terpenoid, glycoside, steroid, and flavonoid in the plant extracts lead to an increase in cytotoxicity.

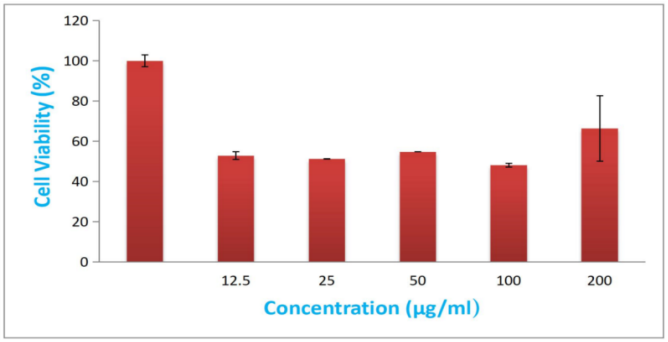

Fig 10. Cytotoxic activity of CuNPs from bulb extract of *U. wightii* against A549 lung cancer cell line

5 Conclusions

The plant extract of *U. wightii* revealed a very simple, fast, affordable, efficient, and eco-friendly green method for the synthesis of Cu-Cu2O NPs as a bio reducing and capping agent. The findings proved that *U. wightii* is a good source of metallic copper oxide nanoparticles, which is evidenced by various characterization techniques viz. XRD, FTIR, TEM, EDX and Zeta potential. The green synthesized NPs also possessed potent free radical scavenging, and antimicrobial and anticancer activity against the A549 lung cancer cell line supporting its broad-spectrum biological effects. Further, *in vitro* and *in vivo* studies will help to maximize the applications of Cu-Cu2O NPs in the field of medicine and industry.

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