Nanomed Res J 6(3):279-286, Summer 2021

RESEARCH ARTICLE

In-vitro Antioxidant and Cytotoxicity (sk-mel-3 cell) Activity of Green Synthesised Copper Nanoparticle using *P. pellucida* Plant Aqueous Extract

Shilpa V P[1, 2], Vilgeena Wilson[1], Bhagyasree Sureshkumar[3], Alby Babu E[1], Nidhina Davis[1], Meena Kurup[4], Muddukrishnaiah K[5]

[1] Department of Pharmaceutics, Sanjo College of Pharmaceutical Studies, Vellapara, Palakkad, Kerala, India.

[2] Department of Pharmaceutics, Karuna College of Pharmacy Iringuttoor, Palakkad, Kerala, India.

[3] Department of Pharmaceutics, KVM College of Pharmacy, cherthala, Kerala, India

[4] Vinayaka Mission College of Pharmacy, Salem, Tamil Nadu.

[5] Department of Technology, Anna University, Chennai

ARTICLE INFO

Article History:
Received 16 Apr 2021
Accepted 05 Jul 2021
Published 01 Aug 2021

Keywords:
P. Pellucida
CuNPs
Antioxidant
Cytotoxicity
FE-SEM
EDAX

ABSTRACT

Green synthesis of metallic nanoparticles using medicinal plant extract treated to environmentally friendly, stable, cost-effective. Present work to study *In-vitro* antioxidant and cytotoxicity of green synthesised CuNPs using *P. Pellucida* plant extract. Copper nanoparticles were synthesised biogenically using *P. Pellucida* plant aqueous extract. CuNPs Characterised by using FE-SEM, U.V., FT-IR, EDAX. Biogenically synthesised copper nanoparticles conducted *in-vitro* Antioxidant (DPPH) and cytotoxicity (SK-MEL-3 cell). The results CuNPs were characterised by using U.V. spectroscopy absorbance 575 nm. Scanning electronic microscope showing the distribution and shape (5-20 nm) of the nanoparticles. X-ray spectrum showed different peaks for CuNPs detected at 2θ values 35.45°, 44.32°, and 65.25°. EDAX elemental spectroscopy conformed to the copper metal inside the nanoparticle (69.7%). The synthesised CuNPs showed good free radical scavenger activity than the ascorbic acid, and it also showed significant cytotoxicity (16µg/mL) against human skin cell lines (SK-MEL-3 cell). The current study conclusion recommended that green synthesised (*P. Pellucida*) CuNPs be used for therapeutic application.

How to cite this article
Shilpa V P. , Vilgeena W., Bhagyasree S., Alby Babu E., Davis N., Kurup M., Muddukrishnaiah K. In-vitro Antioxidant and Cytotoxicity (sk-mel-3 cell) Activity of Green Synthesised Copper Nanoparticle using P. pellucida Plant Aqueous Extract. Nanomed Res J, 2021; 6(3): 279-286. DOI: 10.22034/nmrj.2021.03.008

INTRODUCTION

There are numerous techniques for the biogenic development of metallic nanomaterials. Because of their fascinating physical characteristics and possible application in various fields (medicine, pharmacy, biotechnology, chemistry etc.), technical and scientific research on the synthesis and development of metallic nanoparticles has augmented. Principally tiny size and large volumes to surface area ratio. Special attention was paid to copper nanoparticles production within metallic nanoparticles due to its low price of the noble metal compared to Ag, Au and Pt and their varied research and industrial (1, 2). Synthesis of nanoparticles through a green approach, non-toxic and biodegradable chemicals have been used. Nanoparticles are synthesised from different plant sources (3,4).

Medicinal plant extractions for copper nanoparticles have generous benefits, such as catalysis, photocatalytic activity (5) and bactericidal (6), DNA binding and sensors (7), anticancer (8), free radical scavenging, etc. (9, 10).

* Corresponding Author Email: *Krishna123muddu@gmail.com*

The green synthesis of copper nanoparticles with medicinal plants extracts is biodegradable, profitable and stable (11). Biomolecules such as carbohydrates, phenols, tannins, flavonoids, and others in plant extract have been found to play a significant factor in nanocomposites reduction, aggregation and capping (12). Presently, the market for herbal supplements for health care is growing with each day.

Pellucida peperomia (shiny bush, silver bush) annual herb, and it belong to the Piperaceae family. The monsoon season grows to a height of 15-46 cm in humid soil, particularly underneath the trees (13). It is usually observed in southeast and southwest Nigeria and several tropical Asian and South American countries in the West African rainforest region. *Pellucida peperomia* used amazon region humans for the treatment of cardiac arrhythmia, diuretic, dementia disease. The leaves and stem aqueous blend is used to treat bleeding, fever, headache, stomach pain, injuries, and a cough suppressant recorded in the Ayurveda (14, 15, 16). The present study investigates in-vitro antioxidant and cytotoxicity of green synthesised copper nanoparticles using *P. pellucida* plant extract.

MATERIALS AND METHODS

Source of chemical and reagents

Cu $(NO_3)_2 \cdot 3H_2O$, acridine orange, ethidium bromide (AO/EB), PBS (1%), DMEM (Dulbecco's Modified Eagle's Medium), streptomycin, penicillin-G, L-glutamine, phosphate-buffered saline, MTT (3-(4,5 dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide), ethylene diamine tetraacetic acid, ethanol, ethidium bromide and acridine orange (EB/AO) and DMSO (dimethyl sulfoxide), Dehydrated methanol obtained from Sigma Aldrich Chemicals Pvt. Ltd (India).

Plant collection

P. pellucida was obtained from Patambi, Kerala, India, in August 2020. A plant taxonomist (Dr Dhanapal V, Professor and Principal of Sri Sasta College of Pharmacy) authenticated the plant (Fig. 1).

Preparation of plant extract

Plant leaves are cleaned with distilled water to eliminate any particulate matter. Plant leaves make a fine powder using mortar and pestle. The extract (aqueous) was prepared using a cold maceration process. 100 g powder of P. pellucida Linn was saturated in 1 litre of deionised water and held in a shaker at 30 ° C for 24 hrs for constant stirring at 100 rpm. It then dried out at normal temperature. The extract was then purified and processed at - 4 ° C for additional study (17, 18).

Phytochemical screening

Preliminary phytochemical examination of the extract was carried out to classify active elements using basic methods (19).

Green synthesis of copper nanoparticle (CuNPs)

The aqueous plant extract was used to reduce Cu $(NO_3)_2 \cdot 3H_2O$. The Cu $(NO_3)_2 \cdot 3H_2O$ was mixed with the aqueous plant extract and stirred continuously for at least one hr. The reaction process was repeatedly observed, and the colour change was noted. The reaction mixture was centrifuged at 12,000×g for 15 min, and the nanoparticle pellet was cleaned with distilled water (20)

Characterisation of CuNPs

CuNPs were initially studied by ultraviolet spectroscopic analysis within a 200 – 800 nm range. FTIR was used to identify the structural features and

Fig. 1. Hole plant of *P. pellucida*

selective phytochemical components. The results estimated 4000–400 cm−1 range. The powder form of the nanoparticles exposed to CuKα1X-Ray diffractometer radiation (λ = 1.5406 A°) at 40 kV and 30 mA with 2θ rang. CuNPs were placed on the sample holder and sputter-coated using gold, following that, the nanoparticles' average particle size and shape were studied by using TESCAN MIRA3 LMH Schottky FE-SEM (Japan) (21, 22)

Determination of antioxidant activity (CuNPs)

The ascorbic acid and CuNPs sample stock solutions prepared have a strength of 1.0 mg/ml. CuNPs and ascorbic acid concentrations of 10, 20, 40, 60, 80, 100 µg/mL, in methanol solution. CuNPs (0.5 ml), ascorbic acid transformed into 0.5ml of 0.5 mM DPPH in methanol solvent. After 30 min of storage at in the dark place at room temperature, the optical density was measured at 517 nm using Stat Fax 4200 Elisa reader (USA). Each experiment was performed in duplicate. Inhibition (I%) calculated as follows: (23)

$$\text{I\% antioxidant activity} = \frac{\text{Abs control} - \text{Abs of antioxidant}}{\text{Abs control}} \times 100$$

Cell culture maintenance

Human skin cancer cells (SK-MEL-3) were obtained from the National Centre for Cell Sciences, Pune, India. Dulbecco's Modified Eagle's medium was used to preserve the cell line, complemented by 10% of Fetal Bovine Serum. Penicillin (100 U/mL) and streptomycin (100 µg/ml) was added to prevent microbial contamination. The cell culture medium was preserved in a pressurised environment with 5 per cent CO2 at 37°C.

Cytotoxicity activity

SK-MEL-3 cells were seeded in 96 well plates and incubated in a CO2 incubator for 24hr to facilitate the adhesion. Cells were treated with the control and CuNPs at various concentrations and incubated cell culture incubator after 24hr cell were washed with the cell culture media and added MTT dye (Incubated 4hr 37°C). After 4hr the cells were treated with formazan and observed cell viability using a multi-well plate reader (540 nm). In comparison to the control, the proportion of stable cells was calculated. CuNPs IC50 value was calculated, and the effective dose was analysed at a different period (24).

$$\text{Inhibitory of cell growth}(\%) = \frac{\text{absorbance of the control mean} - \text{absorbance of the sample Mean}}{\text{absorbance of the control Mean}} \text{X10}$$

IC_{50} of nanoparticles measured from the dose-responsive curve inhibit 50% cytotoxicity compared to control cells. Experiments were carried out three times (25).

Measurement of apoptotic induction using acridine orange/ethidium bromide (AO/EB) dual staining method

Microscopic fluorescence analysis of apoptotic cell inhibition was carried out according to Liu et al. (26). SK-MEL-3 cells were seeded at 5 x 10^4 cells/well in a 96 six-well plate and incubated for 24 hours. After treatment with CuNPs for 24 hrs, the cells were detached, washed with cold PBS and then stained with a mixture of A.O. (100µg ml−1)/ E.B. (100µg ml−1) ratio (1:1) at room temperature for 5 min. Treated cells were collected and rinsed three times with PBS. The plates were stained with acridine orange/ethidium bromide (AO/EB 1:1 ratio; 100 µg/mL) for 5 minutes and examined immediately under fluorescent microscope 40x magnification. A fluorescence microscope observed the stained cells at 40x magnifications.

Statistical analysis

Results descriptive as mean ± S.D. They restricted comparing statistical differences carried by (ANOVA) one-way analysis of variance accompanied by Duncan's Multiple Range Test, using SPSS version 12.0 for windows. Values tested for significance if the p-value was less than 0.05.

RESULTS

Phytochemical analysis

All the findings of the phytochemical investigation are shown in Table 1. Aqueous extract gave promising outcomes for steroids, terpenoids confirmed by Salkowski and Liebermann-Burchard's test in the present study. The presence of terpenoids, phenols and flavonoids were also confirmed in *P. pellucida* aqueous extract.

Evaluation of CuNPs

UV - Vis spectroscopic analysis

U.V. spectroscopy within the 200-800 nm range initially verified the development of CuNPs. A characteristic peak of 575 nm showed the absorption spectrum of green synthesised CuNPs.

Table 1. Phytochemical constituents of *P. pellucida* aqueous leaf extract.

S. No	Phyto-Constituents	Chemical Test	Inference
1	Alkaloids	Dragendorff's reagent	+
2	Tannins	Ferric chloride	+
3	Saponins	Forthing	+
4	Flavonoids	Lead ethanoate	+
5	Steroids	Salkowski test	+
6	Terpenoids	Liebermann-Buchard's	+
7	Flavanols	Shinoda	+
8	Anthraquinones	Borntrager's	-
9	Carbohydrates	Molish's	+

(+) present, (-) absent

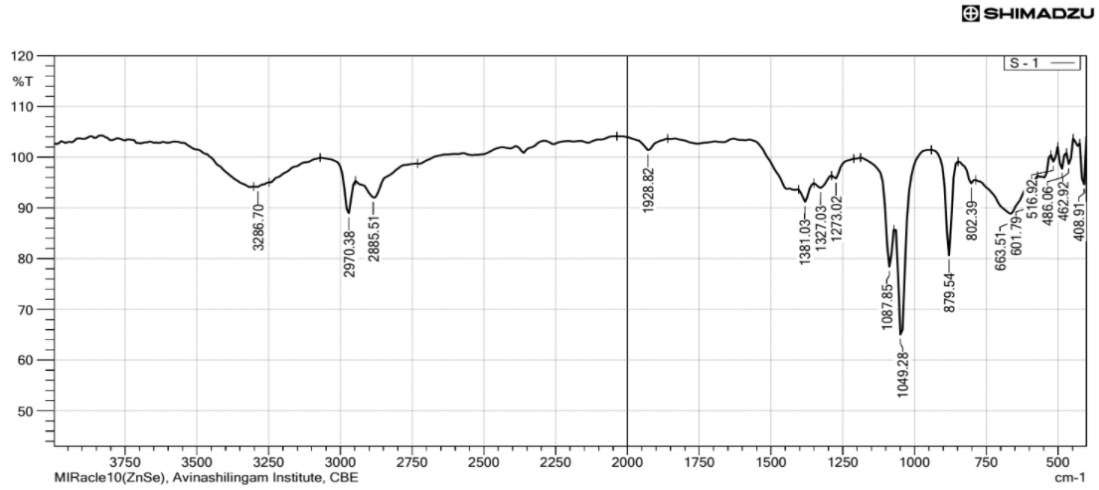

Fig. 2. FTIR analysis of CuNPs

FTIR spectroscopic analysis

A Fourier transform infrared spectroscopy was utilised as an affirmative method for the construction of nanoparticles (Fig. 2). This study offers an imprint of current molecules vibrational and rotational modes, thus helping to classify the functional and potential plant molecules involved in CuNPs nanoparticles reduction.

XRD Analysis

The structure of the nanoparticles is indicated by the XRD analysis of the synthesised CuNPs. At 2θ values of 35.45°, 44.32°, and 65.25° degrees, diffraction peaks were observed (Fig. 3).

FE-SEM analysis

The SEM study was carried out using the Schottky FE-SEM (Japan) TESCAN MIRA3 LMH model. FE-SEM analysis was used to classify the structure and scale of CuNPs. Microscopy of CuNPs has shown that they have a nano-range particle size (500nm) spherical and homogeneous in distribution under the FE-SEM Microscope (Fig. 4).

EDAX analysis

The elemental analysis of the CuNPs from the EDAX spectrum of the FE-SEM image is shown in Fig-5. The percentage of molecular mass and atomic value of Cu respectively 69.7%. The EDAX spectrum is consistent with the presence of copper in the nanoparticle.

Antioxidant activity

The antioxidant properties of CuNPs using DPPH were evaluated and compared to ascorbic acid. The ratio of free radical inhibition of CuNPs was observed at different concentrations. The free radical inhibition visually detected colour transformation from purple to yellow suggests that

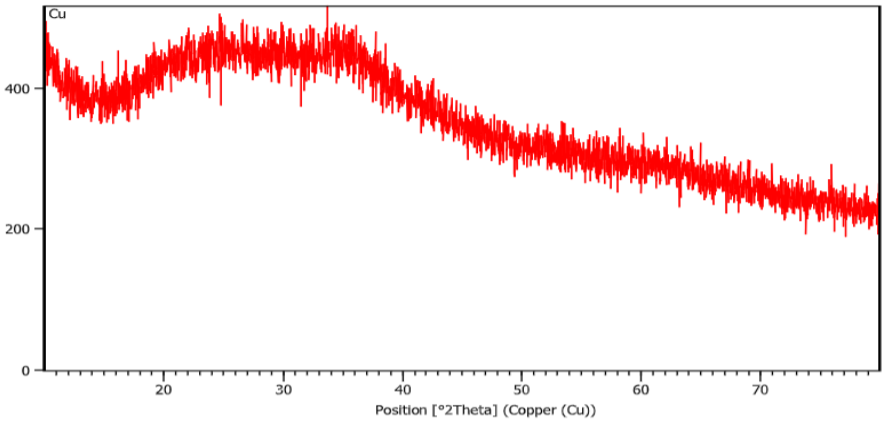

Fig. 3. XRD analysis of CuNPs

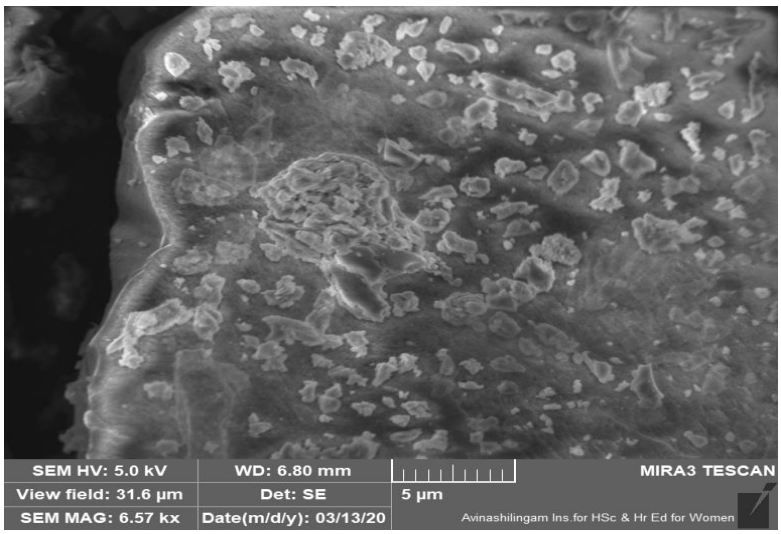

Fig. 4. FE-SEM images of CuNPs

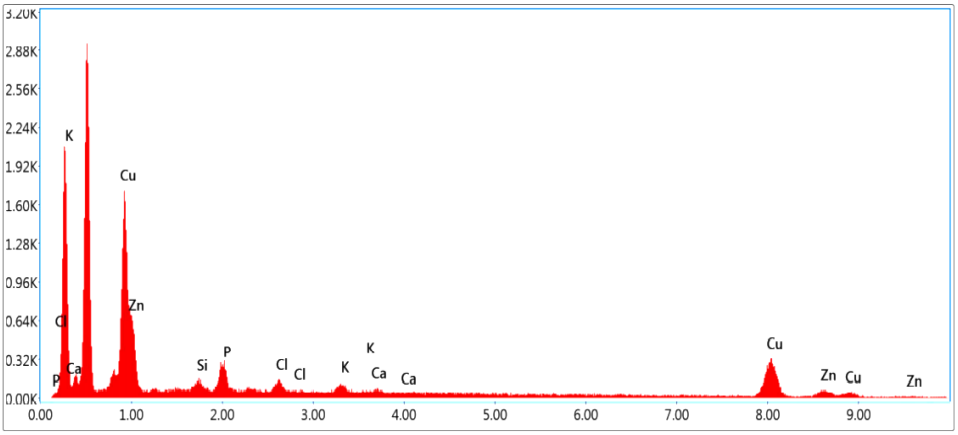

Fig. 5. EDAX analysis of green synthesised CuNPs

DPPH reduced exhibiting better scavenging action (Fig. 6).

Cytotoxic activity (MTT assay)

Cytotoxic activity against SK-MEL-3 cancer cells with different concentrations ranging from 5µg/mL, 10µg/mL, 15µg/mL, 20µg/mL, 25µg/mL and 30µg/mL tested *P. Pellucida* mediated CuNPs. The In-vitro cytotoxicity evaluation was evaluated after 48hrs. Fig 7 and 8 shows the altered structure of SK-MEL-3 cells after dose-dependent treatment with CuNPs. Compared with control cell viability, CuNPs (16µg) significantly reduced the proliferation of SK-MEL-3 cells.

DISCUSSION

In the present investigation, CuNPs were bio-synthesised from the *P. pellucida* plant extract. Microscopical observation of CuNPs have shown that they have a particle size in the nano range; they are irregular in distribution. The dimensions, shape of the CuNPs were observed under FE-SEM. Cu particles grow slowly, form small structures.

Several investigations described biogenic preparation copper nanoparticles as environmentally approachable effective antioxidant and antitumor agents (27). The nanoparticle has a wide surface nature, permeability and biodegradability (28). Green synthesised CuNPs studied antioxidant activity using the DPPH method and showed antioxidant activity (standard ascorbic acid). Further analysis of copper nanoparticles studied cytotoxicity against SK-MEL-3 cell lines and showing good cytotoxic activity (IC50 value 16µg/mL).

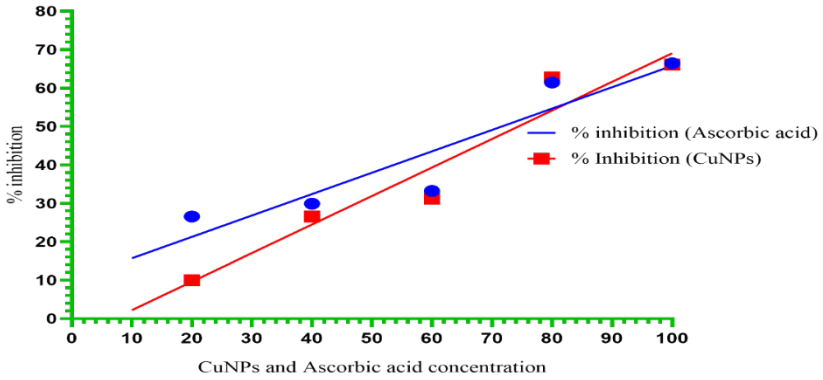

Fig. 6. Antioxidant activity of CuNPs

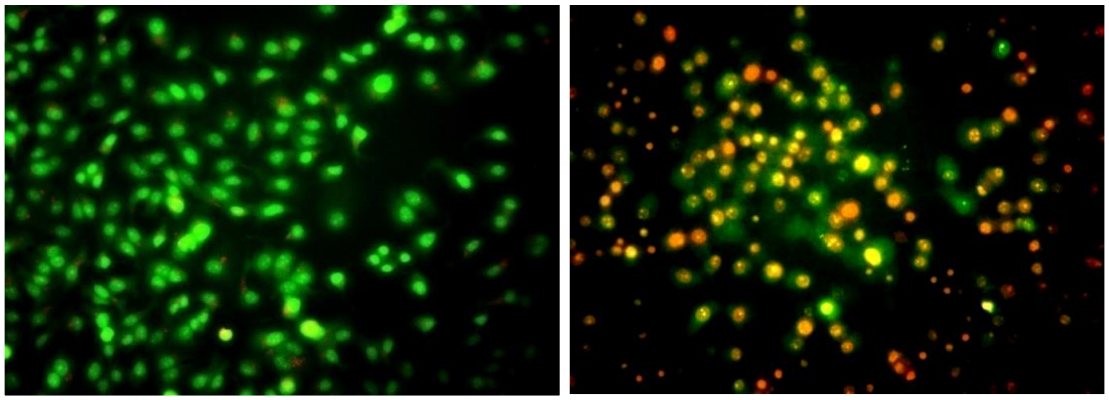

Fig. 7. Regulated human melanoma cells (SK-MEL-3) and CuNPs (16µg/mL) at 24 hrs, marked with dual stain AO/EB, were observed under fluorescence microscopy. Green nucleus appeared in living cells, an early disjointed form of the yellow nucleus with chromatin, late apoptotic condensation of chromatin of orange nuclei and necrotic cells.

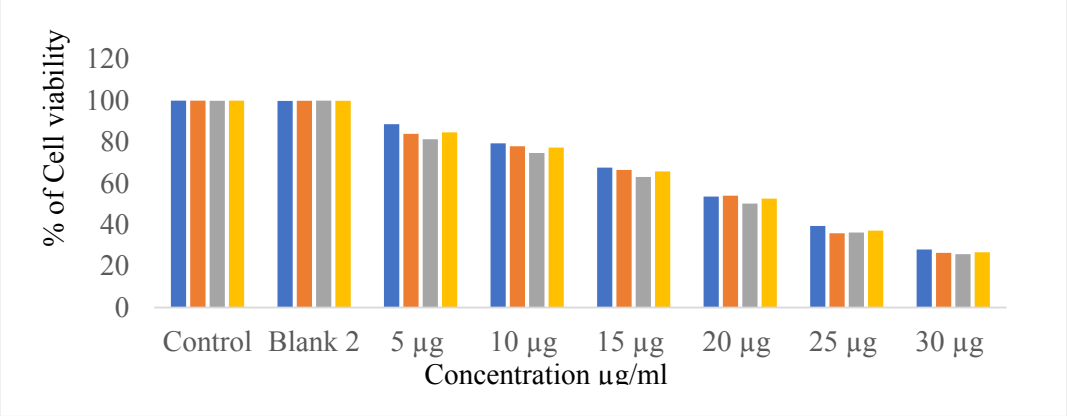

Fig. 8. Percentage of SK-MEL-3 cell viability of control and CuNPs by MTT assay at different concentrations from 5-30 µg/mL.

CONCLUSION

The convenient and environmentally friendly method to prepare CuNPs using reducing Cu (NO3)2·3H2O with *P. pellucida* aqueous extract was established. *P. pellucida* aqueous extract acting as a better reducing and covering agent. Green synthesised CuNPs characterised using EDAX, U.V, FT-IR, FE-SEM and XRD techniques. The development of CuNPs shows the deviations in the colour of the solution. U.V. spectroscopy showed the 200-800 nm range initially verified the story of CuNPs. A characteristic peak of 575 nm showed the absorption spectrum of CuNPs. The crystalline nature of the synthesised nanoparticles identified by XRD analysis has diffraction values at 35.45°, 44.32°, and 65.25° degrees. FE-SEM analysis conforms to the nanoparticle size and shape. The CuNPs investigated antioxidant and cytotoxicity. The experimental results concluded that biogenic synthesised nanoparticles had significant antioxidant (DPPH) and cytotoxicity activity (SK-MEL-3 cell).

ACKNOWLEDGEMENT

The authors acknowledge the Sanjo College of Pharmaceutical Studies and Centre for Biotechnology and Phyto-Pharmacognosy Research (CBPPR), Coimbatore, to provide necessary facilities to carry out the antioxidant cytotoxicity studies.

CONFLICT OF INTEREST

Nil

FUNDING SUPPORT

Nil

REFERENCES

1.V. Kulkarni, S. Suryawanshi, P. Kulkarni, Biosynthesis of copper nanoparticles using aqueous extract of Eucalyptus sp. plant leaves, *Curr. Sc.* 2015;109 (2): 255–257.
2.J. Yang, S. Yang, T. Okamoto, Synthesis of copper monolayer and particles at the aqueous-organic interface, *Surf. Sci.* 600 (24) (2006) 318–320.
3. Ghosh MK, Sahu S, Gupta I, Ghorai TK. Green synthesis of copper nanoparticles from an extract of Jatropha curcas leaves: characterization, optical properties, CT-DNA binding and photocatalytic activity. RSC Advances. 2020;10(37):22027-35.
4. Molnár Z, Bódai V, Szakacs G, Erdélyi B, Fogarassy Z, Sáfrán G, et al. Green synthesis of gold nanoparticles by thermophilic filamentous fungi. Sci Rep. 2018;8(1):3943-.
5. Nazar N, Bibi I, Kamal S, Iqbal M, Nouren S, Jilani K, et al. Cu nanoparticles synthesis using biological molecule of P. granatum seeds extract as reducing and capping agent: Growth mechanism and photo-catalytic activity. International Journal of Biological Macromolecules. 2018;106:1203-10.
6. Vasantharaj S, Sathiyavimal S, Saravanan M, Senthilkumar P, Gnanasekaran K, Shanmugavel M, et al. Synthesis of ecofriendly copper oxide nanoparticles for fabrication over textile fabrics: Characterization of antibacterial activity and dye degradation potential. Journal of Photochemistry and Photobiology B: Biology. 2019;191:143-9.
7. Duman F, Ocsoy I, Kup FO. Chamomile flower extract-directed CuO nanoparticle formation for its antioxidant and DNA cleavage properties. Materials Science and Engineering: C. 2016;60:333-8.
8. Ingle AP, Duran N, Rai M. Bioactivity, mechanism of action, and cytotoxicity of copper-based nanoparticles: A review. Applied Microbiology and Biotechnology. 2013;98(3):1001-9.
9. Ghosh MK, Jain K, Khan S, Das K, Ghorai TK. New Dual-Functional and Reusable Bimetallic Y(2)ZnO(4) Nanocatalyst for Organic Transformation under Microwave/Green Conditions. ACS Omega. 2020;5(10):4973-81.
10. Yallappa S, Manjanna J, Sindhe MA, Satyanarayan ND, Pramod SN, Nagaraja K. Microwave assisted rapid synthesis and biological evaluation of stable copper nanoparticles using T. arjuna bark extract. Spectrochimica Acta Part A: Mo-

lecular and Biomolecular Spectroscopy. 2013;110:108-15.
11. Yugandhar P, Vasavi T, Uma Maheswari Devi P, Savithramma N. Bioinspired green synthesis of copper oxide nanoparticles from Syzygium alternifolium (Wt.) Walp: characterization and evaluation of its synergistic antimicrobial and anticancer activity. Applied Nanoscience. 2017;7(7):417-27.
12. Mukhopadhyay R, Kazi J, Debnath MC. Synthesis and characterization of copper nanoparticles stabilized with Quisqualis indica extract: Evaluation of its cytotoxicity and apoptosis in B16F10 melanoma cells. Biomedicine & Pharmacotherapy. 2018;97:1373-85.
13.Habsah M, Yosie A, Baker K, Siang CC, Syamsumir DF, Alias A, et al. Effect of drying method on antimicrobial, antioxidant and isolation of bioactive from P. pellucida. *J Chem Pharm Res*. 2015; 7:578-84.
14. Wei LS, Wee W, Yong Fu Siong J, Syamsumir DF. Characterization of antimicrobial, antioxidant, anticancer properties and chemical composition of Sauropus androgynus stem extract. Acta medica Lituanica. 2011;18(1):12-6.
15. de Fátima Arrigoni-Blank M, Dmitrieva EG, Franzotti EM, Antoniolli AR, Andrade MR, Marchioro M. Anti-inflammatory and analgesic activity of Peperomia pellucida (L.) HBK (Piperaceae). Journal of Ethnopharmacology. 2004;91(2-3):215-8.
16. Okoh SO, Iweriebor BC, Okoh OO, Okoh AI. Bioactive Constituents, Radical Scavenging, and Antibacterial Properties of the Leaves and Stem Essential Oils from Peperomia pellucida (L.) Kunth. Pharmacogn Mag. 2017;13(Suppl 3):S392-S400.
17. Zhang Q-W, Lin L-G, Ye W-C. Techniques for extraction and isolation of natural products: a comprehensive review. Chin Med. 2018;13:20-.
18. Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA. Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. Plants (Basel). 2017;6(4):42.
19. Senguttuvan J, Paulsamy S, Karthika K. Phytochemical analysis and evaluation of leaf and root parts of the medicinal herb, Hypochaeris radicata L. for in vitro antioxidant activities. Asian Pac J Trop Biomed. 2014;4(Suppl 1):S359-S67.
20. Sarkar J, Chakraborty N, Chatterjee A, Bhattacharjee A, Dasgupta D, Acharya K. Green Synthesized Copper Oxide Nanoparticles Ameliorate Defence and Antioxidant Enzymes in Lens culinaris. Nanomaterials (Basel). 2020;10(2):312.
21. Abbas HS, Krishnan A, Kotakonda M. Fabrication of Iron Oxide/Zinc Oxide Nanocomposite Using Creeper Blepharis maderaspatensis Extract and Their Antimicrobial Activity. Front Bioeng Biotechnol. 2020;8:595161-.
22.Arzani, Hossein, et al. "Preparation of curcumin-loaded PLGA nanoparticles and investigation of its cytotoxicity effects on human glioblastoma U87MG cells." *Biointerface Research in Applied Chemistry* 9.5 (2019): 4225-4231.
23. Wu S, Rajeshkumar S, Madasamy M, Mahendran V. Green synthesis of copper nanoparticles using Cissus vitiginea and its antioxidant and antibacterial activity against urinary tract infection pathogens. Artificial Cells, Nanomedicine, and Biotechnology. 2020;48(1):1153-8.
24. Alishah H, Pourseyedi S, Ebrahimipour SY, Mahani SE, Rafiei N. Green synthesis of starch-mediated CuO nanoparticles: preparation, characterization, antimicrobial activities and in vitro MTT assay against MCF-7 cell line. Rendiconti Lincei. 2016;28(1):65-71.
25.V P, S., B, S., E.R, R., Vijayan U, S., Panicker, L., S, B., G, J., K, M. Green synthesis Zinc Oxide nanoparticle using Allamanda cathartica leaf extract and their cytotoxic and antibacterial activity. *Nanomedicine Research Journal*, 2020; 5(3): 298-305.
26.Liu K, Liu PC, Liu R, Wu X. Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. *Med Sci Monit Basic Res*. 2015; 21:15-20.
27. Yaqub A, Malkani N, Shabbir A, Ditta SA, Tanvir F, Ali S, et al. Novel Biosynthesis of Copper Nanoparticles Using Zingiber and Allium sp. with Synergic Effect of Doxycycline for Anticancer and Bactericidal Activity. Current Microbiology. 2020;77(9):2287-99.
28. Hassanien R, Husein DZ, Al-Hakkani MF. Biosynthesis of copper nanoparticles using aqueous Tilia extract: antimicrobial and anticancer activities. Heliyon. 2018;4(12):e01077-e.