



Green Synthesis of Copper Oxide Nanoparticles Using *Coix lacryma jobi* Leaves Extract and Screening of its Potential Anticancer Activities

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Copper oxide nanoparticles (CuO NPs) have been powerful evidence in several in vitro studies such as cytotoxicity and antimicrobial compared with other metal oxide. Here, we have synthesized green CuO NPs using *Coix lacryma jobi* leaves extracts.

Place and Duration of Study: Department of Chemistry Manipur University, Manipur, India and Regional Institute of Medical Sciences, Imphal, India between February 2019 to March 2021.

Methodology: Green CuO NPs nanoparticles were synthesized from Copper chloride dihydrate (CuCl₂·2H₂O) using *Coix lacryma jobi* leaves extract, and the synthesized green CuO NPs were characterized using Field Emission Scanning Electron Microscopy (FESEM) - Energy Dispersive Spectroscopy, IR Spectroscopy, UV-Visible Spectroscopy, Powder X-Ray diffraction Spectroscopy and HR-TEM where FESEM-EDS determined the purity of CuO NPs.

Results: No other impurities present were observed in EDS, and the PXRD spectra show the crystallite size of CuO NPs with respect to the (002) plane is found to be 25.2 nm, and the

presence of CuO NPs at adsorption spectrum with a distinct peak at 282 nm was determined by UV-Visible spectroscopy and the homogenous morphology and crystalline nature of the CuO NPs were determined from TEM micrograph and SAED pattern. In applications, the substantial anticancer activity of green CuO NPs (synthesized using *Coix lacryma jobi* leaves extract) was proved on human cervical and lung cancer cell lines with IC50 values of 31.88 µg/ml and 15.61 µg/ml, respectively

GRAPHICAL ABSTRACT

Green CuO NPs were synthesized using *Coix lacryma jobi* leaves extract. It has been characterized by different techniques. CuO NPs were screened with its anticancer activities in which shows a potent drug target.



Keywords: CuO NPs; *Coix lacryma jobi*; human cervical cancer cell line; lung cancer cell line.

1. INTRODUCTION

Green chemistry routes of synthesizing copper oxide nanoparticles (CuO NPs) using various plant's parts (stem, roots, leaves, etc.) extract have been attracted by many researchers in recent years because of its potential applications as antibacterial [1-15], catalytic [16-28], anticancer against HCT-116 cell line [29], human cervical carcinoma cells [30], breast cancer cell lines [31], MCF-7 cells [32], AMJ-13 cells, SKOV-3 cells [33]. HL-60 cell line, PC-3 cell line [34], and other anticancer activity were explained and reviewed [35-36]. The researchers using different salt of copper compounds such as copper sulphate, copper chloride, copper nitrate, copper acetate with plant parts extract for the green

synthesized of CuO NPs are explained in detail following different physical and chemical methods of preparation with appropriate morphologies that have been reported [37]. In addition, the green approach of preparing nanoparticles provides an inexpensive and efficient nanoparticle with unique properties [38].

The non-toxic form of nanoparticles have been used in cancer therapy [39,40]. Considering the biological activities of *Coix lacryma jobi*, which acts as antioxidant [41], cytotoxic activities [42] and its leaves extracts at a different medium that inhibits various cancer cells such as Hela, HepG2, and SGC-7901 [43,44], hepatoprotective and anti-inflammatory activity [45] antitumor activities in human cervical cells [46] antibacterial

and anthelmintic activities [47] were observed. However, the preparation of green CuO NPs using *Coix lacryma jobi* leaves has not yet been reported to the best of our knowledge.

In this work, we have synthesized green CuO NPs using leaves extract of *Coix lacryma jobi* as the reducing agent from $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ using an eco-friendly solvent such as ethanol and distilled water. Substantial anticancer activity of green CuO NPs on human cervical and lung cancer cell line was carried out. And the present study explores the green synthesis of CuO NPs, their characterization, and investigation of in vitro cytotoxic activities.

2. MATERIALS AND METHODS

2.1 Materials

Copper (II) chloride dihydrate ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$) was purchased from Sigma-Aldrich. Absolute ethanol 99.9% OMNIS AR ACS was used.

2.2 Preperation of the Extract

Fresh leaves of *Coix lacryma jobi* were collected locally from Imphal West, Manipur, India. The leaves were repeatedly washed with distilled water and air-dried for one day, and the leaves were cut into small pieces. About 30 g of *Coix lacryma jobi* leaves were soaked in 100 ml of doubled distilled water in a conical flask and reflux at 100 °C for 30 min. And then cooled at room temperature and filtered with whatman No. 1 filter paper to remove the plant residues, and then the extract was stored at room temperature for future use.

2.3 Green Synthesis of CuO NPs

30 ml of plant extract was mixed with 70 ml of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (10 mmol) solution in a round bottom flask and the reaction mixture was refluxed for about 1 hour at 100 °C with continuous stirring. The reaction mixture's color was changed to dark brown. The solution was kept at room temperature overnight, and then the black powder CuO NPs was collected by annealing at 300 °C for 1 hour after centrifuging at 8000 rpm for 10 min followed by continuous washed with distilled water and absolute ethanol.

2.4 Characterization Technique

The purity of the CuO NPs was analyzed using Energy Dispersive X-ray analysis and FESEM

(Zeiss Sigma model). IR-spectra were recorded using Perkin Elmer FT-IR spectrum 400 in the range of 4000-400 cm^{-1} using activated KBr pellets. The UV-Visible spectra were recorded using Shimadzu 2450 Spectrophotometer, and the diffraction pattern was recorded using PANalytical (X'Pert Pro) X-ray diffractometer with Cu $K\alpha$ (1.54060 Å) radiation. To access the morphology and the size of CuO NPs was found using JOEL JEM-F200 HR-TEM.

2.5 Cell Lines and Culture Condition

Human cervical (HeLa) and lung (A549) cancer cell lines were obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in RPMI 1640 (Gibco, USA) media with 10% heat-inactivated fetal bovine serum (Gibco, USA) and 1% PenStrep (Gibco, USA). Cell lines were incubated in a humidified atmosphere with 5% of CO_2 at 37 °C. The cell lines were maintained at 37 °C in 5% CO_2 , and media was changed frequently.

2.6 Cell Viability Assay

For evaluation of potential cytotoxic effects of synthesized CuO NPs leave extracts of *Coix lacryma jobi*, we used two human cell lines: HeLa and A549. Upon reaching confluency, they were trypsinized, and the concentration of viable cells was determined using the Trypan blue assay. Cells were cultured at a density of 1×10^4 cells per well in 200 μl RPMI (without phenol red), 10% FBS, and incubated with 5% CO_2 at 37°C in a 96-well tissue culture plate. The final concentration of DMSO in all the experiments did not exceed 0.5% (v/v). Untreated cells served as a control and were assumed to be 100% viable. In the present toxicity analysis, various concentrations (3.12, 6.25, 12.5, 25, 50, 100, and 200 $\mu\text{g/ml}$) of CuO NPs were tested for 24 hr in triplicates. According to the manufacturer's protocol, the cytotoxic effect of synthesized CuO NPs leaves extracts was evaluated using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, USA). These assays are based on the cellular conversion of a tetrazolium salt into a formazan product which the plate reader detects. After 24 hr incubation, a premixed optimized Dye Solution was added to each well, and the cells were further incubated at 37 °C for 4 hr. During 4 hour incubation, living cells convert the tetrazolium component of the Dye Solution into a formazan product. The plates were shaken, and the optical density was measured at 570 nm using a microplate reader (Thermo

Scientific Multiskan Spectrum, Thermo Fisher Scientific, Inc., Waltham, MA). The IC50 values of CuO nanoparticles on various cell lines were determined using Graph Pad Prism version 8.0.1 (GraphPad, San Diego, CA).

3. RESULTS AND DISCUSSION

3.1 FESEM-EDX (Field Emission Scanning Electron Microscopy-Energy Dispersive X-Ray Spectroscopy) Analysis

FESEM determined the phase purity of CuO NPs with EDX analysis, and it is clear that no other impurity peaks were observed with the EDX taken at different spectrums. FESEM with EDX images of green CuO NPs is reported in Fig. 1.

3.2 Infra-Red Spectra Study

Infra-Red spectra of green CuO NPs are shown in Fig.2. FT-IR (solid KBr pellet v/cm^{-1}): 3454.62, 3357.22, 3223.46, 1651.33, 1618.33, 1542.14, 851.60, 492 cm^{-1} . The peak at 3454 and 3357.22 cm^{-1} was related to -OH stretching frequency. The slight broadband at 3223.46 cm^{-1} corresponds to the N-H stretch due to the amine group, and the peak exhibit at 1651.33 and 1618.33 cm^{-1} shows the presence of medium C=C stretching and carbonyl stretching frequency. 1542.77 cm^{-1} peak corresponds to C=C bending and the peak at 851.14 cm^{-1} shows the presence of =C-H bending due to the alkene group. The presence of Cu-O vibration in the green CuO NPs shows a prominent peak at 492.83 cm^{-1} and 611 cm^{-1} .

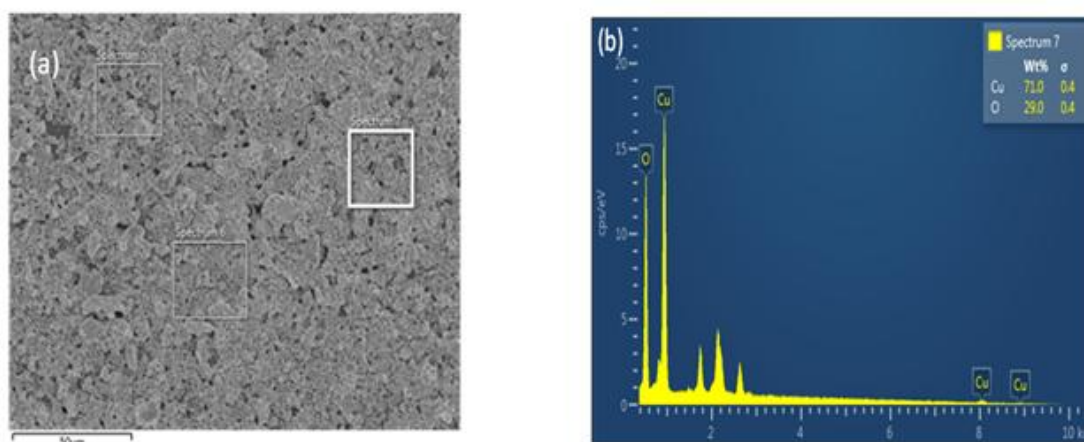


Fig. 1. (a) FESEM (b) EDX images of CuO NPs

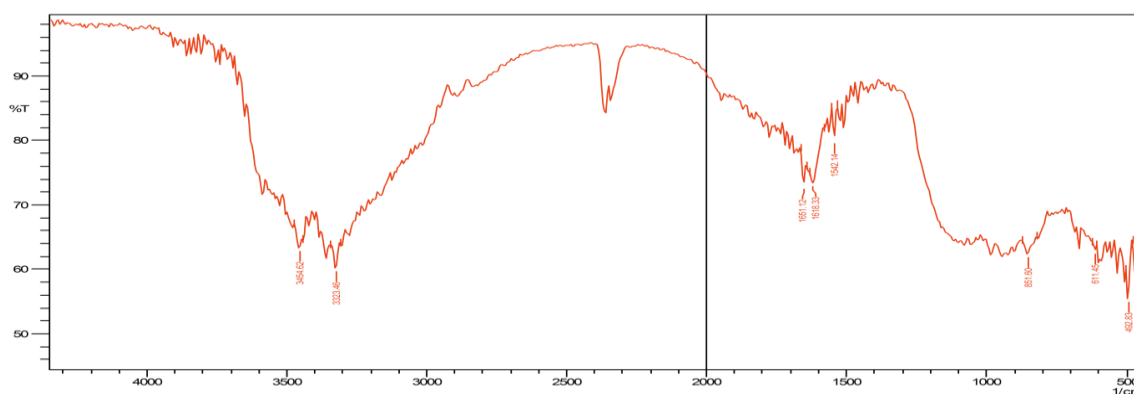


Fig. 2. Infra-Red Spectra of green CuO NPs using *Coix lacryma jobi* leaves extract

3.3 UV-Visible(Ultra Violet) Spectra Study

UV-visible spectra of green synthesized CuO NPs using *Coix lacryma jobi* leaves Extract shown in Fig.3. A broad peak from 300-340 nm was observed in the absorption spectrum for the leaves extract and the absorption spectrum with a distinct peak centred near 282 nm (λ_{max}) indicates the presence of CuO NPs, which was confirmed from the maximum Surface Plasmon absorption band with a maximum at 250-350 nm [48,49].

48.754, 53.503, 58.144, 61.570, 65.844, 66.284, 68.144, 72.472, 75.062 can be indexed to (110), (002), (111), (-202), (020), (202), (-113), (022), (-311), (220), (311), and (004) respectively. It reveals that the sample has monoclinic crystal structure (space group C2/c, ICSB: 16025). The calculated lattice parameters are $a = 4.68370$; $b = 3.42260$; $c = 5.12880$. The average crystallite size was calculated using Debye-Scherrer equation [50].

$$D=0.9\lambda/\beta \cos \theta$$

where, $\lambda = 0.154$ nm, $\theta =$ Bragg's angle of (002) plane, $\beta =$ full width half maximum. The crystallite size of the (002) plane for CuO NPs is found to be 25.2 nm.

3.4 XRD(X-Ray Diffraction) Analysis

The X-ray diffraction displayed in Fig.4. shows the formation of the CuO NPs. The peaks observed at $2\theta = 32.534, 35.468, 38.754,$

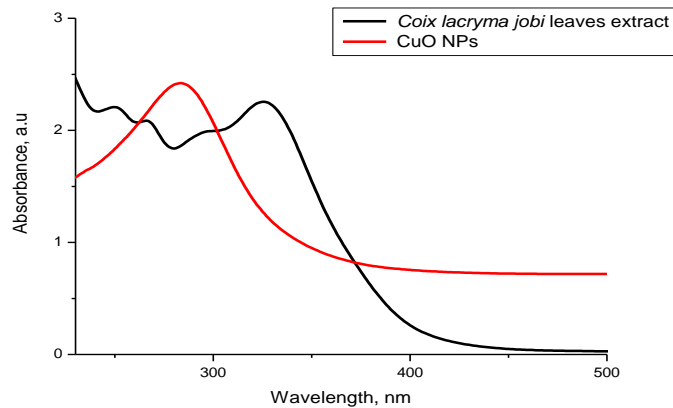


Fig. 3. UV-Visible spectra showing absorption of 10^{-3} M aqueous solution of green CuO NPs (red curve) and *Coix lacryma jobi* leaves extract (black curve)

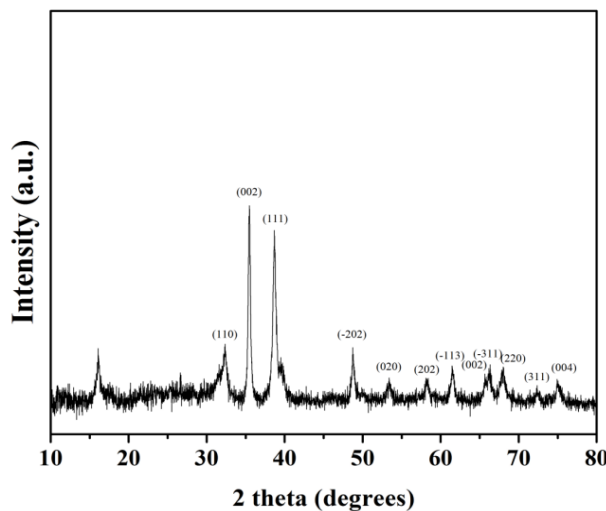


Fig. 4. Powder XRD pattern of CuO NPs using *Coix lacryma jobi* leaves extract

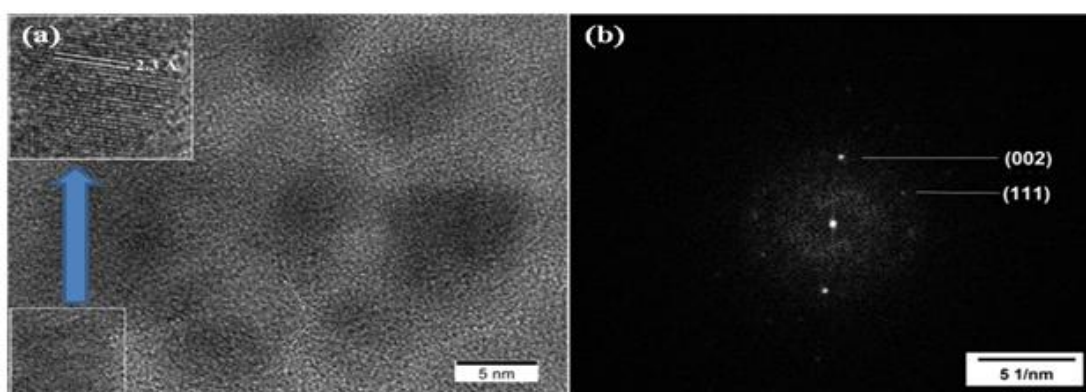


Fig. 5. (a) HRTEM image of CuO NPs with inset showing d spacing (b) SAED pattern

3.5 HR-TEM(High Resolution Transmission Electron Microscopy) Analysis

The HR-TEM micrograph is shown in Fig. 5a. Suggests the formation of CuO NPs with good lattice fringes. The spacing in this region is 2.3 Å which corresponds to (111 planes) (space group C2/c, ICSB: 16025). Fig. 5b shows the SAED pattern of the green synthesized CuO NPs. It confirms the crystalline nature of the prepared sample [51, 52].

3.6 Assessment of Cell Cytotoxicity by CuO NPs Leave Extracts of *Coix lacryma jobi*

HeLa and A549 cells were treated with various doses of CuO NPs for 24 hr. Morphological changes (rounding up), cell detachment, cell death, and decrease of cell viability were observed following CuO NPs treatment for 24 hr [Fig. 6(a-d)]. The CuO NPs treated cancer cells appeared irregular and rounded, whereas the untreated cells were normal in shape. The cell viability was concentration-dependent, which decreased with an increase in the concentration of the samples. Further, cells were treated with various doses of synthesized CuO NPs (final concentration; 3.12, 6.25, 12.5, 25, 50, 100, and 200 µg/mL) for 24 hr, and an MTT assay was performed to determine the IC₅₀ (half maximal inhibitory concentration). The result shows a decrease in proliferation rate with an increase in the concentration of CuO NPs. The half-maximal inhibitory concentration (IC₅₀) was calculated as the concentration requires inhibiting the growth of tumour cells in culture by 50% compared to the untreated cells. Also the anti-proliferative activity

expressed by the IC₅₀ values of CuO NPs and Doxorubicin (standard) on Human cervical cell line (HeLa) and Lung cancer cell line (A549) after 24 hr. incubation were shown in Table 1.

Table 1. Anti-proliferative activity (IC₅₀ values) of the CuO NPs and Doxorubicin on HeLa and A549 cell line after 24 hr incubation

Cell Line	Sample/Standard	IC ₅₀ (µg/ml)
HeLa	CuO NPs	31.88
	Doxorubicin	13.21
A549	CuO NPs	15.61
	Doxorubicin	8.24

Cytotoxicity of CuO NPs against HeLa cell was measured. IC₅₀ value of CuO NPs was found to be 31.88 µg/ml, but less effective than Doxorubicin (13.21 µg/ml) of 2.41 times which is used as standard. (Fig. 7) Further cytotoxicity of CuO NPs against A549 cell line with IC₅₀ value of Doxorubicin (8.24 µg/ml) was found, the corresponding the IC₅₀ value of CuO NPs was 15.61 µg/ml (Fig. 8). Our results showed that the synthesized copper oxide nanoparticles have proved significant cytotoxicity effect on both HeLa and A549 cells with IC₅₀ value of 31.88 µg/ml and 15.61 µg/ml respectively Fig 6 (e). The percentage of cell viability was declined further when the concentration of the CuO NPs was gradually increased to a maximum of 200 µg/ml on the cancer cell line tested. The reduced cell viability of HeLa and A549 cells observed in this study suggest anticancer effects of CuO NPs. Further studies are required to understand the process of cell death by apoptosis or necrosis pathway.

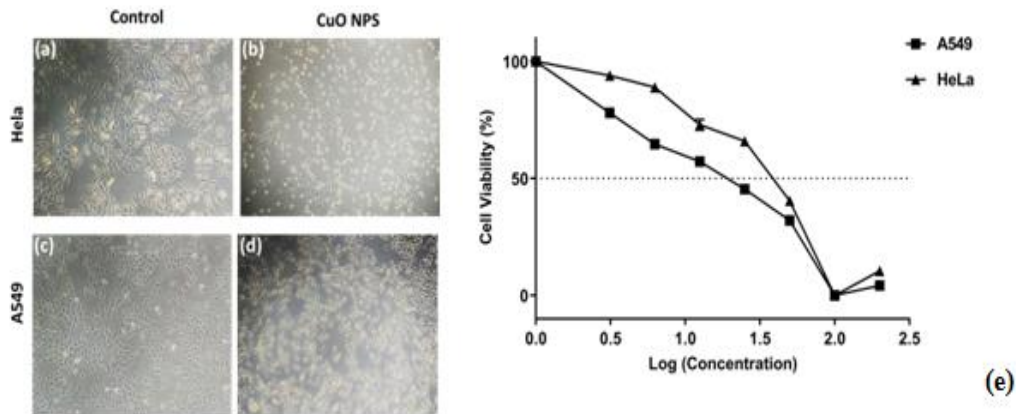


Fig. 6. Cytotoxicity of CuO Nanoparticles (CuO NPs) synthesized from leaves of *Coix lacryma jobi*: CuO NPs induces cytotoxicity in HeLa and A549 cells: Morphological changes observed under a simple microscope and acquired pictures; (a & b) HeLa, (c & d) A549 cells. (e) Cell viability of HeLa and A549 cells measured by MTT assay and IC50 values determined following treatment with different doses of CuO NPs

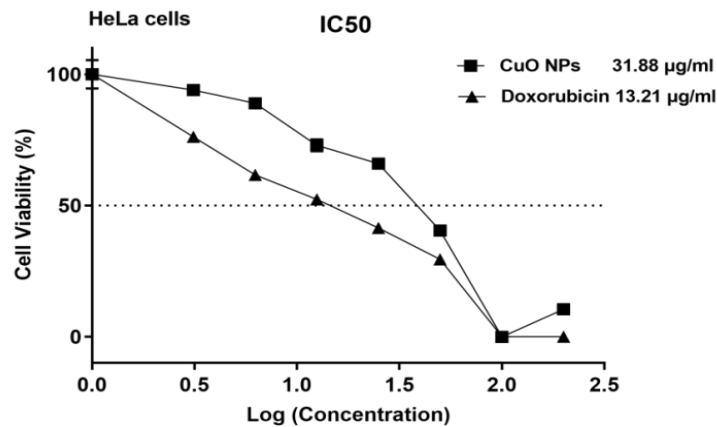


Fig. 7. The viability of HeLa cells after exposed to the different concentrations of CuO NPs and Doxorubicin for 24 hr as determined using MTT assay

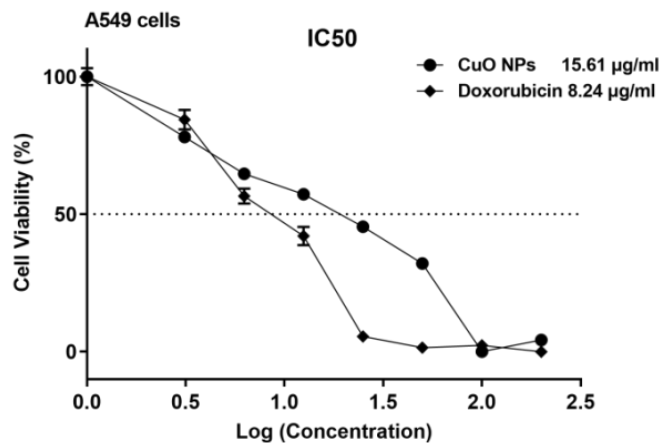


Fig. 8. The viability of A549 cells after exposed to the different concentrations of CuO NPs and Doxorubicin for 24 hr as determined by MTT assay

Anticancer efficiency of green synthesized CuO NPs has been reported in various cancer cell lines, including breast cancer (MCF-7), cervical cancer (HeLa), epithelioma (Hep-2), and lung cancer (A549) [53]. Recently, nanoparticles-based cancer research has emerged as one of the potential areas of research. Several studies towards validating the impacts of the various forms of nanoparticles on various cancerous cells have been reported [54-57]. The CuO NPs toxicity results in our study clearly showed a dose-dependent response in the tested cancer cells. This demonstrates a significant increase in cell death with an increased dosage of the nanoparticles treatment. In depth study will be required to understand the real mechanism behind anticancer activity of the synthesized copper nanoparticles

4. CONCLUSION

The present study has demonstrated that the leaves of *Coix lacryma jobi* can be an unconventional resource for the green synthesis of CuO NPs. The nature of the synthesized CuO NPs was characterized by analytical techniques like UV-Visible, XRD, SEM, and HR-TEM. The CuO NPs synthesized using the leaves of *Coix lacryma jobi* have proved the substantial anticancer activity on human cervical and lung cancer cell line with IC50 values of 31.88 µg/ml and 15.61 µg/ml, respectively. The potent efficacy of green CuO NPs may be induced from phytochemicals present in the extract of *Coix lacryma jobi* leaves. The above findings can be a future drug target in the near future. Further identification and isolation of the biologically active compound from *Coix lacryma jobi* leaves would give rise for discovery of novel anticancer drug. Such CuO NPs are expected to serve as potent anticancer agents and thus can be used in biomedical applications.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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