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Green Synthesis of Copper Nanoparticles using Aqueous Extract of Yerba Mate (Ilex Paraguarients St. Hill) and its Anticancer Activity

Alyaa Majid* and Hadeel Rashid Faraj

Department of Chemistry, College of Science, University of Thi-Qar, Nasiriyia, Iraq

(*) Corresponding author: aliaa.s_mschem@sci.utq.edu.iq
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Abstract

Nanotoxicology is a major field of study that exposes dangerous effects of nanomaterials on the living cells. In the present study, an extract of llex paraguarients St. Hill in distill water was firstly prepared. This extract was added to 1 mM of Cupric sulfate solution, and the change in the color of the solution from colorless to colored solution was detected. This change in color indicates that there is a formation of CuNPs. Secondly, Copper nanoparticles (CuNPs) were prepared by the chemical reduction method and characterized by the utility of different techniques such as: UV-Vis spectrometer, FTIR spectroscopy and SEM with EDAX. The evaluation of the toxicity of CuNPs was performed on human lung carcinoma cell (A549). The half-maximal inhibitory concentration IC50 of CuNPs for human lung carcinoma cell (A549) was determined. CuNPs suppressed proliferation and viability of carcinoma lung cells. Overall, the results show that the IC50's of the prepared Cu NPs were cytotoxic to cancerous lung cells.

Keywords: *Anticancer activity, Biosynthesis, Copper nanoparticles, llex paraguarients.*

1. INRODUCTION

In recent years, nanotechnology has got the concertation of many researchers from various fields such as biotechnology, physics, chemistry, material sciences, engineering, and medicine. Nanoparticles (NPs) are produced by physical and chemical methods. These methods are suffering from drawbacks such as hazardous reaction condition, longer time, expensive reagent, tedious process to isolate NPs [1,2]. The word Nano is originated from a Greek word that means too small or the little thing infinitely [3].

Hence, there is a choice to develop new methods for the synthesis of NPs which should be requiring less drastic reaction condition, inexpensive reagent, and eco-friendly. In recent years, copper (Cu) NP gained worthy attention among researchers because of its application in wound dressings and biocidal properties [4, 5]. In literature, the CuNPs are synthesized from vapor deposition [6], electrochemical reduction [7], radiolysis reduction [8], thermal decomposition [9], chemical reduction of Cu metal salt [10], and room temperature synthesis using starch and hydrazine hydrate [11]. In recent years, some methods have been developed for preparing the novel nanostructure of metal oxides which can be synthesized by a number of preparative methods that are typically described as physical and chemical methods [12]. In addition to that green synthesis of CuNPs was achieved using microorganisms [13] and plant extract [14]. These nanoparticles are described as safe and less toxic; hence it has been used to treat diseases caused by bacteria, fungi as well infections of the urinary tract [15].

Nanotechnology is the engineering of substances at subatomic and sub-molecular scales. It is the process deals with the design, fabrication, characterization, and the utility of nanomaterials in different

fields. Nanotechnology is widely used in the late twentieth century [16]. Consequently, the green synthesis of nanoparticles has gained a significant interest as it utilizes non-toxic phytochemicals. Moreover, Nanoparticles diminish the hazardous substances which could be encountered in chemical synthesis. Nanotechnology deals with the use of bio-extracts as reducing agents, which in turn reduces the cost of the process significantly [17]. The motivation of this branch of technology that it has an impact on different types of industries including medicine, food, energy, space, sports, textiles, electronics, environmental management. Nanotechnology deploys the coordination between size and shape of particles to improve the quality of products or to enhance a process [18].

Ilex paraguariensis is a dioecious evergreen tree, which can grow to a raise of up to 8–15 m. The 8 cm long olive-green leaves are perennial, alternate, coriaceous, obovate with slightly crenate dentate margins and obtuse apex, and have a wedge shaped base. The petioles are up to 15 mm long. The flowering period occurs during spring season, generating small, unisexual flowers which have 4 white petals. In some tropical or subtropical species, the number of petals may be 5, 6 or 7. These may be clustered in groups of 1–15 flowers that appear in the axil of the leaves. The fruits are red-colored berries containing 4–5 seeds. The term "mate" actually refers to a gourd made from the dry and hollowed fruit of *Lagenaria vulgaris Ser. (Cucurbitaceae)*, a plant of widespread use across the global. In Uruguay and Southern Brazil, it is frequent to see gourds averaging 12–15 cm wide with openings of 10 cm. In Argentina the gourds commonly used are smaller: approximately 7–10 cm wide and have an opening of 2.5–3.0 cm at the top which is used to fill it with mate leaves and water in order to prepare the infusion. Once the infusion is ready, a special drinking straw, a narrow tube which has a flattened open end which helps as a mouth piece and finishes in a closed perforated bulblike filter is inserted into the mate [19]. *Yerba-maté* is a matrix that is rich in nutrients and bioactive composites, such as phenolic, flavonoids, and phenolic acids that have high antioxidant activity [20,21]. Its infusion is known to have stimulating, anticonvulsant, and neuroprotective effects on the central nervous system, mainly due to its high content of bioactive composites, such as phenolic acids, flavonoids, and saponins [22].

Medicinal plants are part and parcel of human society to warfared diseases, from the dawn of civilization [23]. The world health organization has evaluated that over 75% of the world's population still depends on plant derived medicines, usually get from traditional healers, for its basic health care needs [24,25]

The configurations of *yerba mate* has been partially characterized and it includes a variety of polyphenols, xanthines, caffeoyl derivatives, saponins, and minerals that may be responsible for pharmacological activity [26, 27]. Studies have proposed that yerba mate leaves may have antioxidant [28, 29], antiobesity [30], antidiabetic [31]. It may also aid in digestion [32]. *Yerba mate* has been recognized for a variety of pharmacological activities, but limited research has been conducted on its antimicrobial properties [33, 34]. A considerable number of these plants/plant based products have been widely used. Therefore, interest in the examination of plants as potential sources of new drugs is increasing. In India, medicinal plants are traditionally used in the treatment of cardiovascular disease, as they are inexpensive, efficacious and safe [35]. Phenolic compounds exhibit multiple pharmacological properties such as anti-microbial, anti- allergenic and antioxidant [36]. The present study aims to study the cytotoxic effects of CuNPs on carcinoma lung cells (A549).

In the present study, it is aimed to investigate for the first time the use of plant extract from *Ilex paraguariensis* with a nanoparticle size in the treatment of lunge cancer. This is an endeavor to utilize green chemistry in the nanotechnology.

2. EXPERIMENTAL PART

2.1. The Collection of *Ilex paraguariensis*

Samples of *Ilex paraguariensis* were collected from the local market of Nasiriyia City, Thi-Qar, Iraq. They were cleaned, broke and finally grinded by electric grinder.

2.2. Preparation of *Ilex paraguariensis* Extract

The weight of *Ilex paraguariensis* powder was 5g. This powder was dissolved in 100ml of distilled water and boiled for 20 min at 50 C. The extract was filtered by Whatmann No1 filter Paper. Then the filtrate was stored in a tight seal pack under 4 °C for further use.

2.3. Synthesis of Copper Nanoparticles.

A mixture of 80 ml of 1mM $CuSO_4$ and 20ml of Plant Extract was added for the reaction. Blank was prepared by the addition of 80 ml of distilled water to 20 ml of plant. The reduction of Cu+ was indicated by the color change from light color to dark color.

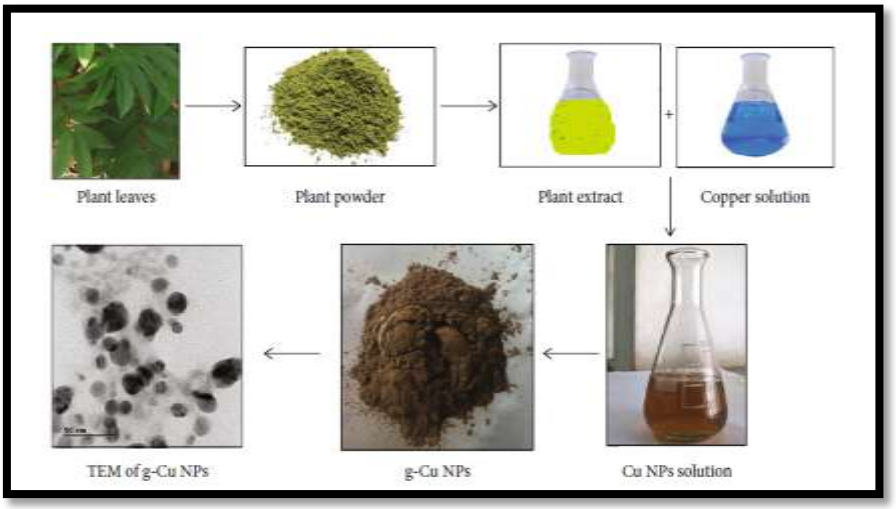


***Figure 1**. The scheme of synthesis of CuNPs.*

2.4. Characterization of Copper Nanoparticles

The synthesized CuNPs were characterized through UV-Vis spectrophotometer UV-1700 (Shimadzu, Tokyo, Japan) that was operated in the scanning range of 250-750 nm. Synthesized CuNPs were characterized by FTIR to detect the biomolecules that were responsible for the reduction of CuNPs Shimadzu model with the wavelength range from 400 to 4000 cm^{-1} were used. The morphology and chemical structure of the synthesized nanoparticles were examined by scanning electron microscopy (SEM, JEOL JSM-6490A) equipped with an energy-dispersive X-ray spectrometer (EDX) (6490 LA). EDX was carried out at an acceleration voltage of 20.0 kV.

2.5. Maintenance of Cell Cultures

A549 Cells were maintained in MEM supplemented with 10% Fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged using Trypsin-EDTA reseeded at 80% confluence twice a week, and incubated at 37 °C [37].

2.6. Cytotoxicity Assays

For the limitation the cytotoxic effect, the MTT cell viability assay was

conducted on 96-well plates [38]. Cell lines were seeded at 1 × 10^4cells/well. After 24 hrs. or a confluent monolayer was achieved, cells were treated with tested compound. Cell viability was measured after 72 hrs of treatment by removing the medium, adding 28 µL of 2 mg/mL solution of MTT (and incubating the cells for 1.5 h at 37 °C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 130 µL of DMSO (Dimethyl Sulphoxide) followed by 37 °C incubation for 15 min with shaking [39]. The absorbency was determined on a microplate reader at 492 nm (test wavelength); the assay was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as the following equation [40]:

% Cell viability = (Absorbance of treated cell / Absorbance of non-treated cell) x 100
% Cytotoxicity = 100 – cell viability

The obtained data were statically analyzed using an unpaired t-test with GraphPad Prism 6 [41]. The values were offered as the mean ± SD of triplicate measurements [42].

3. RESULTS AND DISCUSSION

3.1. UV-Analysis

UV-Vis spectroscopy measures the extinction (scatter + absorption) of light passing through a sample. NPs have unique optical properties that are sensitive to the size, shape, concentration, agglomeration state, and refractive index near the NP surface, which makes UV-Vis a valuable tool for identifying, characterizing, and studying nanomaterials. The biosynthesized CuNP was monitored by UV-spectrophotometer range of absorbance from 250 to 800 nm. The spectroscopic analysis of synthesized CuNPs showed the maximum absorbance at 400 nm indicating the presence of biosynthesized CuNPs in the reaction mixture. These experimental investigations were found to be in good agreement with the results that already presented in the literature by Elisma *et al*., 2019 as Figure 2 [43].

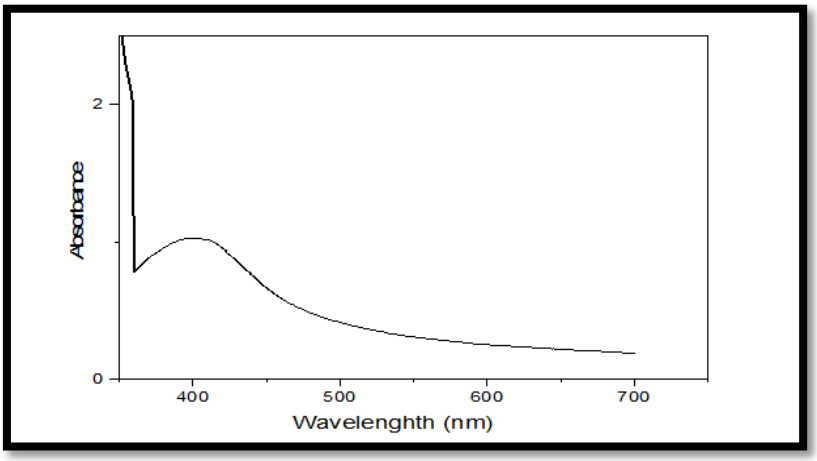


Figure 2. *Ultraviolet-visible spectrum of copper nanoparticles.*

3.2 FTIR Analysis

FTIR measurements were performed for both the aqueous fresh *Ilex paraguariensis* extract and the synthesized dried CuNPs to recognize the possible phytoconstituents responsible for the bio reduction, capping and efficient stabilization of the synthesized CuNPs. The FTIR spectra of the leaf extract and the synthesized CuNPs are shown in (Figure 3a and b). The spectrum was recorded in the wavelength region between 400 and 4000 cm^{-1}. The spectrum of aqueous fresh *Ilex paraguariensis* extract (Figure 3a) shows the peaks at wave numbers 3282cm^{-1} and 1688cm^{-1}. The peak at 1688 cm−1 was due

to the presence of C=O. The peak at 3282cm−1 was due to the presence of an OH bond of the phenolic group [44]. FTIR measurement of CuNPs showed the absorption peaks at 3367 cm-1, 1697 cm-1 and 613 cm^{-1} in (Figure 3b). Peak at 1697 cm^{-1} corresponds to C=O and 3367 cm^{-1} corresponds to O-H of phenolic compound. (Figure 3b) showed a peak at 613 cm-1 which indicates the presence of Cu ion. The formation of a new peak at 752 cm-1 is due to the peak shifting from 613 to 752 cm-1 which indicates the formation of CuNPs [45]. It is concluded that biosynthesized CuNPs might be surrounded by any one of these bioactive molecules such as polyphenols,xanthines, caffeoyl derivatives, saponins, and minerals that may be responsible for pharmacological activity [25-26]. The FTIR spectrum of the CuNPs indicates the lowering of peak intensity for O-H stretch of phenolic compounds, confirming the reduction of CuNPs, which have been possibly proceeded via these groups. This indicates that water soluble compound such as polyphenols are present in extract. Therefore, it can be inferred that the biomolecules present in leaf extract, namely, flavonoids and alkaloids might be responsible for the reduction of Cu ions to CuNPs due to their markable reducing capacity. Burris *et al*.[46] studied the Composition of *Ilex paraguariensis* and reported the presence of caffeic acid, caffeine, caffeoyl derivatives, caffeoylshikimic acid, chlorogenic acid, feruloylquinic acid, kaempferol, quercetin, quinic acid, rutin, andtheobromine in enormous amount, which further supports our analysis for concluding flavonoids as one of the major reducing and capping agent as Figure 3.

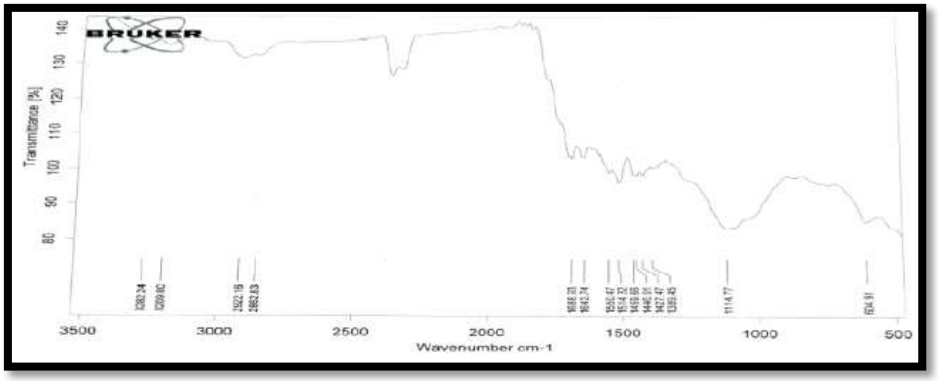


Figure 3 a. *Fourier transform infrared (FTIR) spectrum of aqueous fresh Ilex paraguariensis extract.*

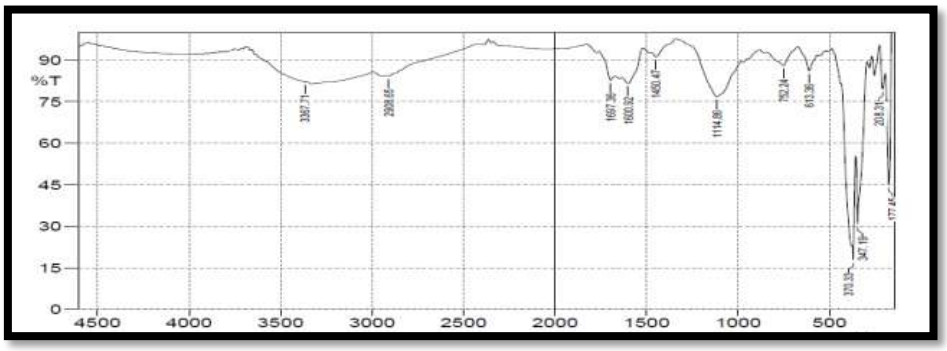
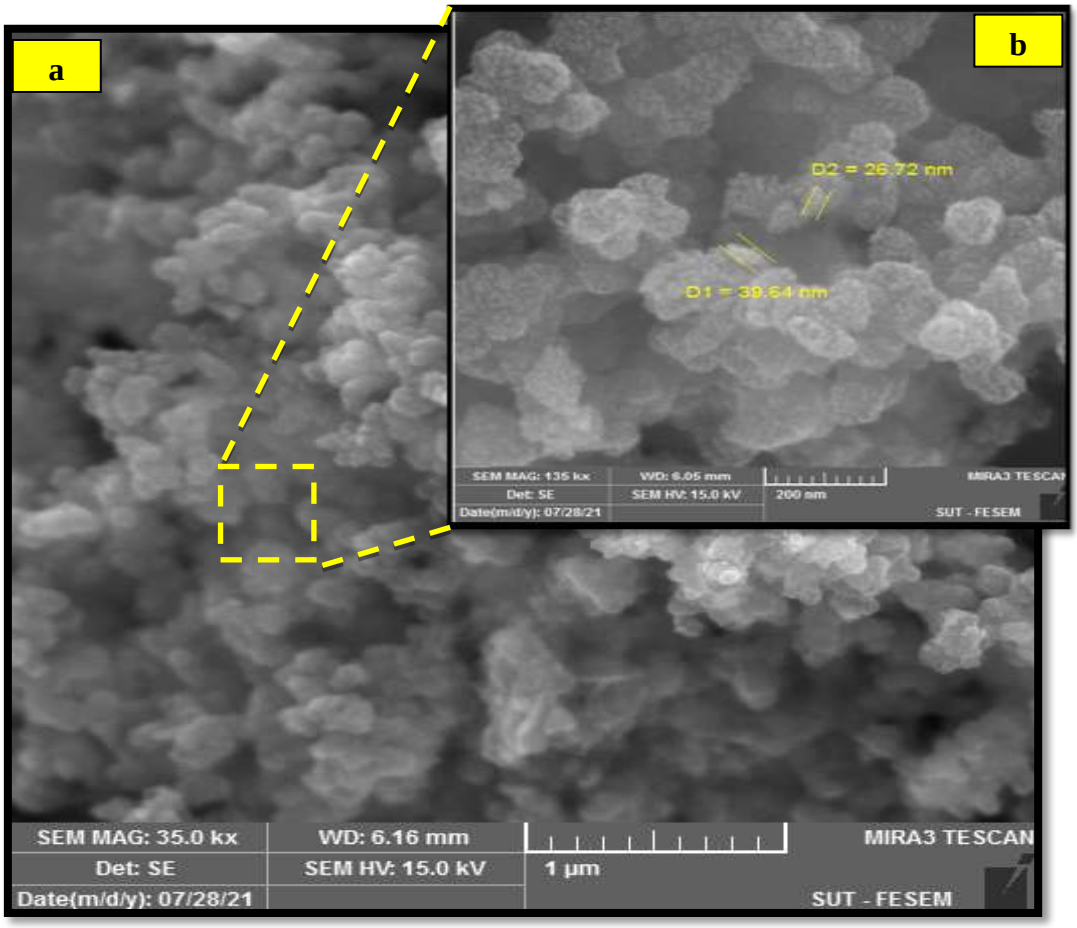


Figure 3b. *FTIR spectrum of copper nanoparticles*.

3.3. SEM and EDX Analysis

The size, morphology structure and the elemental composition of the synthesized CuNPs of CuNPs was studied by Scanning Electronic Microscopy (SEM) and Energy-dispersive X-ray (EDX). SEM micro-graphs showed that the nanoparticles are agglomerated in some amount due to

sticky nature of the plant extract. The SEM micrographs shows average size of (26-40) nm of CuNPs indicating well established synthesized nanoparticles. The SEM micrographs were taken at 1µm (low resolution) and 200 nm (high resolution) as depicted in the inset of Fig. 4a-b. [46]. EDX spectrum confirms the presence of copper and oxygen in the cuprous oxide nanoparticles [47]. As Figure 5.



Scanning electron microscope image of biosynthesized

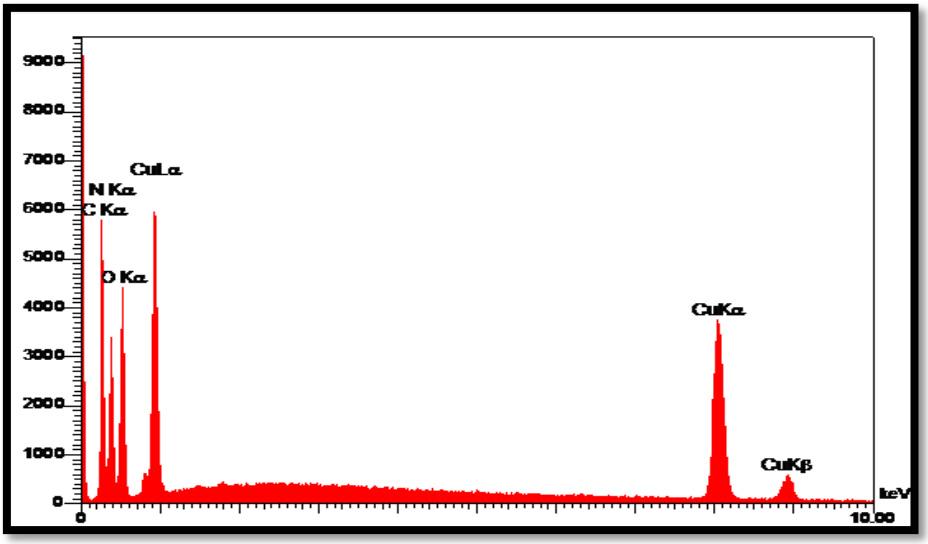


Figure 5. *Energy dispersive analysis X-ray analysis of copper nanoparticles.*

3.4. Anticancer Activity of CuNPs

The cytotoxic effect of CuNPs against A549 cells was studied. The anti-proliferative activity of the CuNPs was tested by studying their ability to inhibit the proliferation of A549 cell line. The results of this study showed that there is a cytotoxic activity of CuNPs against the A549 cell line and the results is concentration dependent manner. By increasing the concentration of CuONPs from 3.125 to 100 µg/ml the rate of inhibition increase from 29% to 60 %. The maximum rate of inhibition of A549 cell line at 100 µg/ml was 36.89 % after 72 hrs time exposure. As showed in Figures. (6-8(a-b)). Cu/CuO NPs can gain easy entry into the body through the skin and the respiratory system. They can then be retrograde transported to the neurons innervating the skin or airways [48]. Due to this fact, lung cells were chosen to be the target cells of the present study on which the cytotoxicity of Cu/CuO NPs were evaluated. The present results are in line with the results of Ahamed and his collaborators which studied the impact of CuO NPs on human pulmonary epithelial cells (A549), for short term exposure time (24 h). The study of Ahamed and his collaborators demonstrated that CuO NPs induced cytotoxicity in a dose-dependent manner. Also, the authors suggested that CuO NPs possessed a DNA damaging potential in human lung epithelial cells (A549) [48].

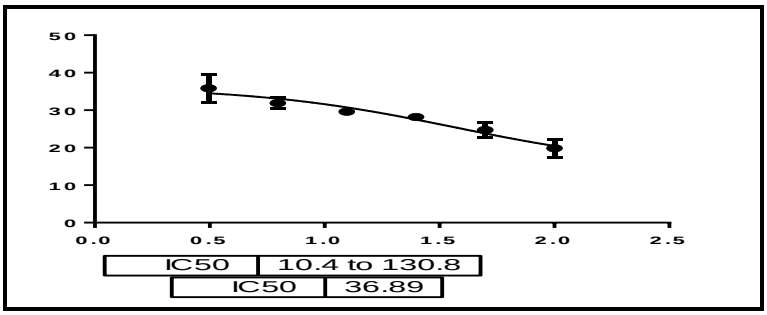


Figure 6. *IC_{50} of Copper nanoparticles on A549 cell line.*

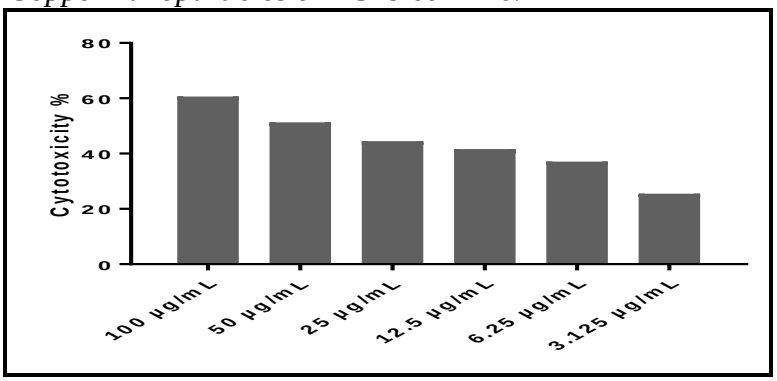


Figure 7. *Cytotoxic effect of Copper nanoplarticles on A549 cell.*

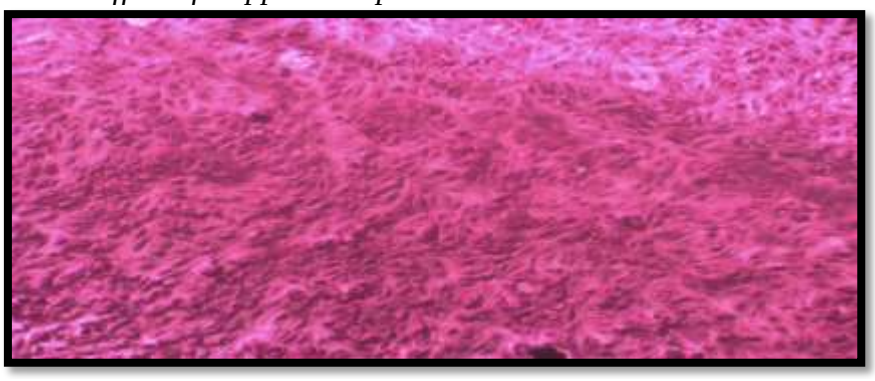


Figure 8a. *Control A549 cells untreated under inverted microscope (X10).*

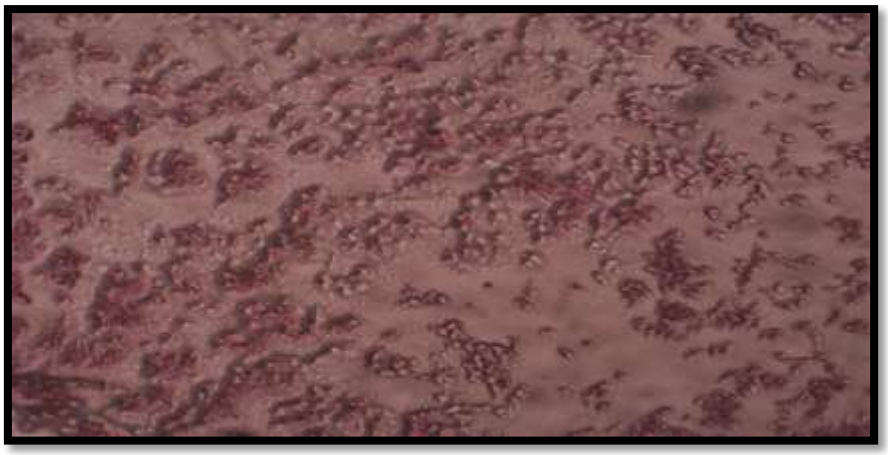


Figure 8b. *Treated under inverted microscope (X10).*

4. CONCLUSION

CuNPs were synthesized using aqueous extract *of Ilex paraguariensis* through green synthesis route. The extract of *Ilex paraguariensis* was found efficient for the synthesis of CuNPs. This method has merits over other reported methods such as easy availability of starting materials, inexpensive process, ease of conduction at laboratory, simple reaction conditions, avoidance of use of expensive, hazardous and toxic reagents and pollution free. The synthesized CuNPs were characterized and assessed by UV-Vis spectrometer, FTIR spectroscopy, SEM with EDAX. UV surface plasmon peak reveals the formation of CuNPs in the reaction mixture, which is further confirmed by EDAX analysis. FTIR interpretation gives an insight about the probable bioactive molecules which are acting as reducing and capping agents during the biosynthesis of CuNPs. which is in accordance with SEM result and EDAX result indicate the presence of Cu oxide NPs. The biologically synthesized CuNPs shows excellent anticancer activity in the reaction mixture. Therefore, from the present results, it can be concluded that the IC50 value of CuNPs having a size<20 nm resulted in cytotoxicity in cancer A549 lung cells. It is worth to mention here that the toxicological effects of Cu NPs on cultured lung cancer cells (A549) can be useful in designing and developing delivery carriers for cancer cell targeting.

Conflict of Interest

The authors have to state that no conflict of interest.

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