

GREEN SYNTHESIS OF COPPER NANOPARTICLES USING GINGER OLEORESIN AND ITS ANTICANCER ACTIVITY AGAINST LIVER CANCER CELL LINE

ABSTRACT :

INTRODUCTION - Nanotechnology is getting used in developing countries to assist, treat disease, and stop health issues. Nanotechnology in addition to nanomedicine is being applied to or developed for application to a spread of commercial and purification processes. Ginger, scientifically known as *zingiber officinale*, belongs to the family *Zingiberaceae*. The preparation is done by solvent extraction of dried rhizomes.

AIM

The aim of the study was green synthesis of copper nanoparticles using ginger oleoresin and to evaluate its cytotoxic activity against liver cancer cells.

MATERIALS AND METHODS -

The Ginger oleoresin was obtained from Synthetic Industries, with a product code: 4010000370 was used for the study. Copper nanoparticles were prepared from oleoresin and confirmed using UV-Visible spectroscopy. The prepared copper nanoparticles were then evaluated for the anticancer effect on liver cancer cells using the method of Mosmann. The cells (1×10^5 cells per ml) were seeded in a 96 well microtiter plate (100 μ l per well) with replications. Treatment was conducted for 24 and 48 hours with different concentrations (control, 10, 20, 30, 40, and 60 μ M) of ginger oleoresins. The percentage of cell viability was calculated.

RESULT: Dose-dependent anticancer activity was observed with ginger Oleoresins mediated copper Nanoparticles. The IC 50 value was 30 mg / ml

CONCLUSION: In the present study, ginger oleoresin showed a good activity cytotoxic effect on liver cancer cell lines. However, more research is needed to understand the underlying mechanisms of the cytotoxic property of the ginger plant.

KEYWORDS: Anticancer activity, ginger oleoresin, innovation, liver cancer, copper nanoparticles, green synthesis

Running Title - Anticancer activity of liver cancer cells

Comment [M1]: Arrange alphabetically

INTRODUCTION :

Comment [M2]: Put [] for the references

Cancer, the most prevalent moderately curable group of diseases involving abnormal growth potential of cells to multiply and spread across the body parts leading to functional impairment of the essential process (1). It causes significant morbidity and mortality and is a major health problem worldwide. Chemotherapy is there for the treatment of cancer but still exhibits low specificity and is restricted by dose-limiting toxicity. It is a challenge to seek out the therapy and medicines for the treatments of varied sorts of cancer. So, conventional methods require a mixture of controlled release technology with targeted drug delivery which is simpler and less harmful. Nanomaterials are expected to revolutionize cancer diagnosis and therapy (2). A liver cancer cell is a useful method employed to understand properties such as regulation of cellular events, uncontrollable cell proliferation, minimal inhibitory concentration, evasion of cell death (3).

Nanoparticles are particles with sizes starting from 1 to 100 nanometers. These have gained popularity in recent years and are shown to possess a good range of technological and biological applications. Among the metallic element nanoparticles, silver nanoparticles have unique properties like chemical stability, anti-inflammatory, antiseptic, antimicrobial, antioxidant, antiviral, and antifungal activity which has made it the particle of interest to the scientific community (4),(5). These particles are usually prepared by physical or chemical methods which aren't environment friendly and are related to various biological risks. Hence, there's a requirement to follow other methods which are non-hazardous to the environment and lead to the green synthesis of nanoparticles using varied biological agents like bacteria, fungi, plant extract, or plant biomass (6), (7).

Ginger is mainly known as *Zingiber officinale*, it belongs to the family of *Zingiberaceae*. The preparation is done by solvent extraction of dried rhizomes. It contains a volatile oil in a composition of about 30 - 35 ml/100 g. Ginger has been known to have anti-inflammatory effects since the early 1970s. Ginger inhibits cyclooxygenase and suppresses prostaglandin secretion, hence bringing about anti-inflammatory effects. Ginger also inhibits 5-lipoxygenase, suppresses leukotriene production, hence inhibits the pro-inflammatory effects of leukotrienes. Ginger extract from the *Zingiberaceae* has been shown to inhibit several genes that are liable for inflammatory responses (8). 6-Gingerol and shogaol are the active constituents of ginger and are responsible, not only for their anti-inflammatory effects but also for the antitumor and

antioxidant effects of ginger. The content of the oleoresin may vary with the solvent used for the extraction (9).

Previous studies were done on *Symplocos racemosa* bark assisted copper nanoparticles and their antibacterial activity against *Staphylococcus aureus* and *Lactobacilli* species (10), herbal product using nanoparticles and antibacterial activity (11), ~~in vitro~~ cytotoxic effects of copper nanoparticles synthesized from avocado seed extract (12), Green synthesis of copper oxide nanoparticles using tamarind extract and its alpha-amylase inhibitory activity (13), cytotoxic activity study of copper oxide nanoparticle mediated through tamarind extract (14).

~~Our team has extensive knowledge and research experience that has translate into high quality publications(15–19)(20)–~~ The aim of the study was to prepare copper nanoparticles by green synthesis, using ginger oleoresin and to evaluate its cytotoxic activity against liver cancer cells. (15–19)(20).

MATERIALS AND METHODS :

Study Setting: Cell line Research was conducted in Blue Lab, Saveetha Dental College, Chennai, India. The study was ethically approved by the Scientific Review Board, Saveetha Dental College, Chennai. [Ethical approval number: IHEC/SDC/UG-1926/21/91].

Collection and Preparation :

The Ginger oleoresin extract ~~is was~~ collected from Synthetic Industries, with a product code: 4010000370. Ginger, scientifically known as *zingiber officinale*, belongs to the family Zingiberaceae.

Synthesis of Cu Nanoparticles :

~~20 mM CuSO₄~~ Copper sulphate (CuSO₄) 20 mM was prepared initially and mixed with ginger oleoresin solution to prepare nanoparticles. The solution was kept in a magnetic stirrer for nanoparticle synthesis. The color change was observed visually and photographed. The solution containing the nanoparticles was centrifuged using Lark refrigerated centrifuge. The Cu nanoparticles solution was centrifuged at 8000 rpm for 10 min and the pellet collected was washed with distilled water twice. The final purified pellet was collected and dried in a hot air oven at 100 – 150°C for 24 hrs, and finally, the nanoparticles powder was collected and stored in an airtight Eppendorf tube.

Comment [M3]: Was copper sulphate pentahydrated.

Confirmation of Cu Nanoparticles :

The synthesized nanoparticles solution ~~is was~~ preliminarily characterized using ultraviolet

(UV)- visible spectroscopy; 3 ml of the solution ~~is~~ was taken in the cuvette and scanned in a double-beam UV-visible spectrophotometer from 300 nm to 700 nm wavelength. UV-Visible spectrometer readings were taken every 2 hours and recorded. The peak in the graph was noticed after 72 hours which coincided with a visible color change from pale pink to brown, indicating the formation of nanoparticles (Figure 1).The results were recorded for the graphical analysis (Figure 2).

Comment [M4]: How about experiment for particle size. UV only is not conformatory about particle size.

CELL LINE STUDY:

Chemical :

DMEM medium, 0.25% Trypsin-EDTA solution, sodium bicarbonate solution, bovine serum albumin (BSA), low melting agarose, MTT from Sigma Chemicals Co., St. Louis, USA. fetal bovine serum (FBS) and antibiotic/antimycotic solution, DMSO was from Himedia, Sodium phosphate monobasic and dibasic, sodium chloride, sodium hydroxide, sodium carbonate, hydrochloric acid, and methanol were purchased from Sisco Research Laboratories (SRL) India.

Cell structure :

The liver cell line was procured from National Centre for Cell Science (NCCS, Pune), India. The cells were grown in T255 culture flasks containing DMEM medium supplemented with 10% FBS. Upon reaching confluence, the cells were detached using Trypsin-EDTA solution.

MTT ASSAY ANALYSIS:

In vitro cytotoxic activity assay: The effect of ginger oleoresin on cell viability was measured by MTT assay following the method by Mosmann. Briefly, the cells (1×10^5 cells per ml) were seeded in a 96 well microtiter plate (100 μ l per well) with replications. Treatment was conducted for 24 hours with different concentrations (control,10,20,30,40,60,80 μ M) of ginger oleoresin. After incubation, 20 μ l of 5 mg/ml MTT stock solution was added to each well and incubated for 4 h at 37 $^{\circ}$ C. The obtained formazan crystals were solubilized with DMSO and the absorbance was measured at 570 nm using a microplate reader (SpectraMax M5, Molecular Devices, USA). Cell viability (%) has been shown as a ratio of absorbance (A₅₇₀) in treated cells to absorbance in control cells (0.1 % DMSO) (A₅₇₀). The IC₅₀ was calculated as the concentration of sample needed to reduce 50 % of the absorbance in comparison to the DMSO-treated control.

Comment [M5]: Add reference

Cell viability estimation :

Cell viability ~~is~~ was calculated using the formula:

$$\text{Cell viability (\%)} = \{A_{570} \text{ of (sample)}/A_{570} \text{ of (control)}\} \times 100$$

MORPHOLOGICAL STUDY:

Based on the MTT assay we selected the IC₅₀ value of ginger oleoresin for the future study. The characterization of morphological changes in liver cancer cell lines before and after treatment with ginger oleoresin was observed under a phase-contrast microscope.

Statistical analysis :

All data obtained were analyzed and represented as mean \pm SE. The results were computed statistically (SPSS/10 Software Package; SPSS Inc., Chicago, IL, USA) using one-way ANOVA. In all tests, the level of statistical significance was set at $p < 0.05$.

RESULT :

In the present study, the cytotoxic activity effect of ginger oleoresin against liver cancer cells was evaluated using an MTT assay. MTT assay provided a quick, simple, and cost-effective way for testing the cytotoxic activity of ginger oleoresin. In this study, liver cancer cells were treated with different concentrations of the extract of ginger oleoresin for 24 hours. As the concentration of the extract increased, the percentage of cell viability decreased which depicted significance in the cytotoxic activity of ginger oleoresin against liver cancer cells (Figure 3). The IC₅₀ value was found to be 30 $\mu\text{g/ml}$. The cell viability was also found to be 10%. Thereby the results showed that as the drug concentration increases, the percentage of cell viability decreases proving ginger oleoresin's cytotoxic effect. Ginger oleoresin extract caused a dose-dependent increase in the cytotoxic activity of liver cancer cells. Morphological changes and apoptosis of liver cancer cells were observed using ginger oleoresin under an inverted phase-contrast microscope at 20x magnification (Figure 4).

Comment [M6]: Also mention about copper nano particle.

Comment [M7]: Add reference

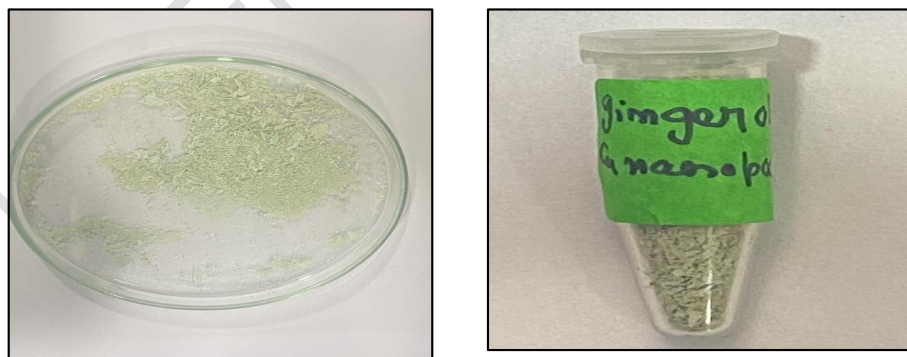


Figure 1: Ginger oleoresin mediated Copper oxide nanoparticles

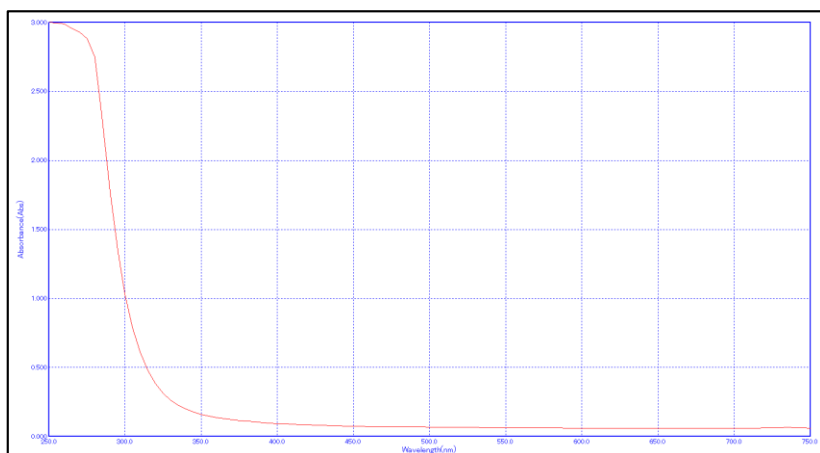


Figure 2: Graph showing the UV spectroscopy of ginger oleoresin mediated copper nanoparticles.

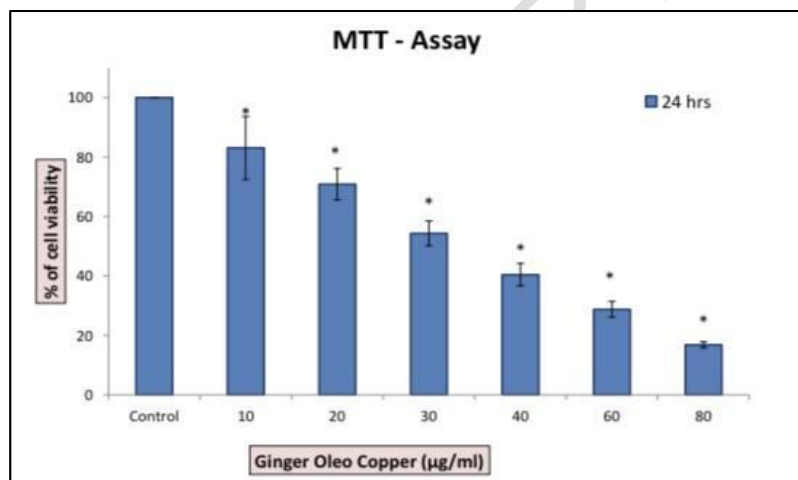


Figure 3: The graph represents the cytotoxic activity effects of ginger oleoresin mediated copper nanoparticles on HEPG2 cells. Cells were treated with ginger oleoresin (10, 20,30,40,60, and 80 µg/ml) for 24 hours and cell viability was evaluated by MTT assay. Blue denotes the control groups and other concentrations of ginger oleoresin. The X-axis denotes controls of the different concentrations of ginger oleoresin-mediated copper nanoparticles and the Y-axis denotes the percentages of cell viability. Percentage inhibition of cell viability .Data shown as means ± SE

(n = 3). * Compared with the control, $p < 0.05$. The p value was 0.007 at $40\mu\text{g/ml}$ and considered as significant as it was < 0.05

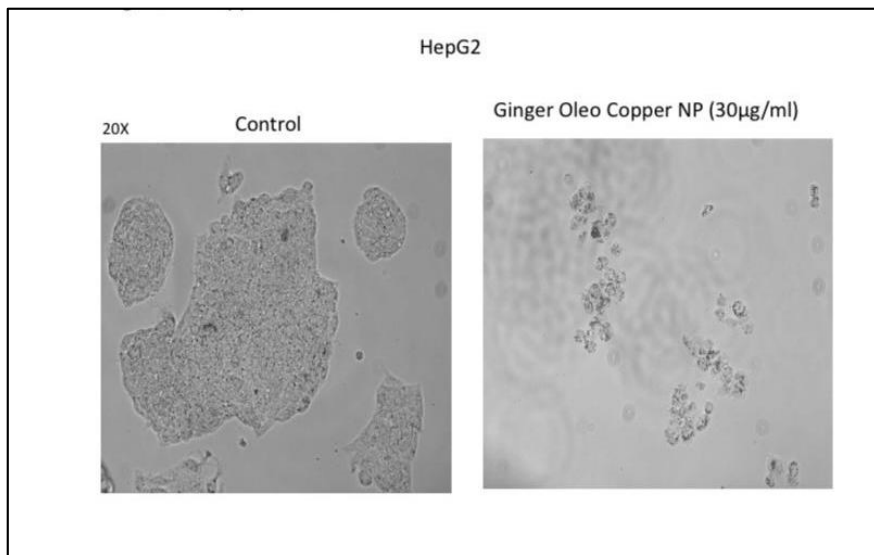


Figure 4: Assessment of cell morphology of HepG2 treated without or with ginger oleoresin. Cells were treated with ginger oleoresin ($30\mu\text{g/ml}$) for 24 hours along with the control group. Images were obtained using an inverted Phase contrast microscope at 20x magnification.

DISCUSSION :

In the present study, we evaluated the cytotoxic activity potential of ginger oleoresin in HEPG2 cells by MTT assay. MTT assay was employed to assess the viable cells to measure the growth modulation of cells *in vitro*. The *in vitro* cytotoxic assay is an economical method as well as a reliable method. The HepG2 cells were treated with different concentrations of ginger oleoresin ($10\mu\text{g/ml}$ to $80\mu\text{g/ml}$) for 24 hours. The ginger oleoresin caused a dose-dependent increase in concentration.

In the present study, liver cancer cells were treated with ginger oleoresin at different concentrations and evaluated by MTT assay (Figure 3, Figure 4) depict clearly that there was good level of anti-cancer activity exhibited by ginger oleoresin against liver cancer cell line when compared with the previous *in vitro* study performed by (21,22) to analyze the anticancer activity of ginger against cholangiocarcinoma also proved that there is a remarkable anticancer

activity for the extract, but both the studies were with different cell line, parameter but both the studies results were quite similar.

Shenani *et al* in the year 2017 stated that the ethanolic leaf extract of *Caralluma fimbriata* is capable of reducing cell proliferation by inducing anticancer activity of COLO 320 cells when we compared with our study we found that 30 µg/ml can lead to apoptosis (23). The activity of ginger extracts performed by different researchers found that the plant is with good pharmacological activity(24,25). Jennifer *et al* have used KB cells to evaluate the anticancer activity with increasing concentrations of the ethanolic extract of *C. fimbriata* (100 – 300µg/ml) and has shown a dose-dependent increase in anticancer activity in KB cell lines (26) (27)-(28) .

The Cytotoxic Activity effect of ginger oleoresin on liver cancer cells was proved; when the drug concentration was increased, the percentage of cell viability decreased proving cytotoxic activity effect. The limitation seen in the present study was that the mechanism of cytotoxic activity with ginger oleoresin was not explored and future studies should be done with animal models to take it to the next level of **research**.

Comment [M8]: Discussion about copper nanoparticle as anticancer agent is missing.

CONCLUSION :

In the present study, ginger oleoresin showed a good cytotoxic effect on liver cancer cell lines which may be helpful in the treatment of liver cancer (29-38). However, more research is needed to understand the underlying mechanisms of the cytotoxic property of the **plants**.

Comment [M9]: Elaborate with findings

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