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# Green Synthesis of Silver Nanoparticles using *Centella asiatica* and Evaluation of its Anticancer Activity

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**Abstract:** Gotu Kola, scientifically known as *Centella asiatica*, has a long history of use as traditional medicine due to its numerous health benefits, which include anti-inflammatory, antioxidant effects, and wound healing. In this study, the researchers aimed to produce silver nanoparticles (AgNPs) using a green synthesis method that utilized *C. asiatica* extract. This method is a more eco-friendly alternative to conventional chemical synthesis methods. The AgNPs produced were analyzed using various techniques such as UV-visible spectrophotometry, XRD, FT-IR, SEM, and TEM to determine their size, shape, and structure. We observed that the AgNPs had a size of 390 nm, were crystalline in nature, had a FT-IR peak at 3437 cm<sup>-1</sup>, and had a size of 37±0.75 nm and 34±0.64 nm based on SEM and TEM analysis, respectively. Furthermore, the liquid-liquid extraction (LLE) method was used to extract various compounds from *C. asiatica*, including saponin, phenolic compounds, flavonoids, terpenoids, steroids, glycoside, and alkaloids. The extraction process resulted in different layers that contained phenolic compounds. The researchers found that the green synthesized AgNPs had better radical scavenging activity at their highest concentration, with an IC<sub>50</sub> value of 385.364 µg/ml. Moreover, the minimum inhibitory concentration (MIC) of *C. asiatica* AgNPs on A549 lung cancer cells was found to be 216.972 µg/ml, indicating their potential as natural anticancer agents. Overall, this study highlights the potential of *C. asiatica* extract and its green synthesized AgNPs as natural remedies for cancer treatment. The green synthesis method used to produce AgNPs is also a promising approach to developing environmentally friendly methods for nanoparticle synthesis.

**Keywords:** *Centella asiatica*, Silver nanoparticle, A549 lung carcinoma cell line, Radical scavenging activity, Phenolic compounds, Antioxidant, Anticancer

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## Introduction

The Apiaceae family includes *Centella asiatica* (L.) Urban. The plant has a long history of being

revered as a miracle elixir of life (Fard *et al.*, 2018). It is a native plant that is widely found in

both hemispheres tropical regions (Bandara *et al.*, 2011). The plant includes substances that have medicinal use, such as madecassic acid, asiatic acid, and various triterpene ester, glycoside derivatives including asiaticoside, sicefloside, and madecassoid (Fard *et al.*, 2018). In addition, it includes alkaloids, volatile and flavonoid chemicals, steroids, phenolic acid, and triterpene glycosides like *Centella saponin* (Sabaragamuwa *et al.*, 2018).

In past, it has been demonstrated that *C. asiatica* extract inhibits the development of lung cancer cells in the culture medium. The death rate for lung cancer has recently fallen by 48% in males and 23% in females due to the implementation of screening standards and a decrease in cigarette usage (Alexander *et al.*, 2020). In 2023, around 103,000 of the 127, 070 lung cancer deaths (81%) will occur (Siegel *et al.*, 2023). Today, surgery, chemotherapy, and radiation are used to treat cancer (Hickey *et al.*, 2013). For instance, administering chemotherapy and, to a lesser extent, surgery results in hypoxia and perhaps cell death, which eventually harms the healthy non-cancerous cells (Wang *et al.*, 2019).

The use of AgNPs for medical diagnostics, drug delivery, therapies, anti-oxidants, anti-bacterial, and cytotoxic purposes are all significant uses (Ivanova *et al.*, 2018). There are different ways to make AgNPs: physically, chemically, and biologically. The hazardous reactants are frequently used in the chemical processes used to create nanoparticles. As a result, several researchers have become interested in using plants as readily available, sustainable sources to create biocompatible nanoparticles recently (Rajan *et al.*, 2015).

Recently, it has been suggested that using plant extracts to make metal nanoparticles is a quick and acceptable alternative to chemical and physical procedures (Prakash *et al.*, 2013). Low cost, eco-compatibility, and ease of synthesis in large numbers are benefits of the green synthesis process used to create nanoparticles (Allabaksh *et al.*, 2010). Also, in the biosynthesis approach, the

surface of the nanoparticles generated is modified and improved by the binding of some of the chemicals present in the plant extract during synthesis to the nanoparticles (Park *et al.*, 2011).

Recent research has shown that plant-derived nanoparticles may have the ability to inhibit the proliferation of tumour cells while also improving cytotoxicity owing to the existence of secondary metabolites and other non-metallic substances in the reaction environment (Özkan *et al.*, 2021). The current study has demonstrated cytotoxic effect of *C. asiatica* synthesized silver nanoparticle on A549 cancer cells.

## Materials and Methods

### *Collection and Extraction of Plant Extract:*

Locally-sourced fresh *C. asiatica* pieces were prepared according to prior reports. The plant sample was rinsed with reverse osmosis water after being cleaned with tap water. The pieces were first air-dried for 12 h to remove moisture before being further oven-dried for 12 h at 60 °C. For subsequent extraction, the dry material was crushed into a fine powder and kept in an opaque vial at -20 °C. 50 g of dried *C. asiatica* powder were extracted for 2 h with an overhead stirrer using 500 ml of an ethanol/water combination (80:20 v/v). Using a Buchner funnel and Whatman filter paper No. 1, the mixture was filtered. Using a rotary evaporator at 40 °C, the combined filtrate was reduced to one-third of its initial volume. The filtrate was then kept overnight at 4 °C (Eze *et al.*, 2019).

### *Liquid-Liquid Extraction:*

The 80% crude ethanol extract was suspended in water and further subjected to liquid-liquid extraction. Polyphenols in the dry ethanol extract of the plant were identified using established standards using qualitative phytochemical analysis. Then polyphenol were partitioned with double the amount of chloroform to remove non-polar bioactive components from the crude ethanol extract and then the aqueous dissolved layer was purified further with ethyl acetate to

obtain fraction containing polyphenols (Herrero *et al.*, 2012).

#### *Qualitative Phytochemical Analysis:*

Using a standard protocol, a qualitative analysis of *C. asiatica* was done to determine whether any phytoconstituents or secondary metabolites, such as alkaloids, glycosides, saponins, proteins and amino acids, phytosteroids, flavonoids, and phenolic compounds, were present that were responsible for the therapeutic effects of the drug (Gray *et al.*, 2018).

#### *Green Synthesis of Nanoparticles:*

About 4 ml of the plant extract was combined with 100 ml of a 0.01 mM aqueous solution of silver nitrate (EMD Millipore, Billerica, MA, USA), and the mixture was continuously agitated for 5 min at room temperature. After the colorless solution became dark brown and revealed the reduction of Ag<sup>+</sup> to Ag<sup>0</sup> NPs, the combination was left alone. Centrifugation was then used to separate the particles for 20 min at 13,000 rpm. The biosynthesized AgNPs were then dried for 4 h at 37°C. AgNPs were created using a sedimentation technique that was based on the bioreduction of Ag ions (AgNO<sub>3</sub>; Merck, Germany) by *C. asiatica* extract (Mirzaie *et al.*, 2022).

#### *Characterization of Nanoparticle:*

Using a UV-Vis Perkin Elmer instrument in the 200-800 nm range, AgNPs synthesized by using *C. asiatica* extract were spectroscopically analyzed (Lavakumar *et al.*, 2015). Similarly XRD technique was used to determine the crystallographic structures of the green synthesized AgNPs. The Cu K $\alpha$  radiation used for the XRD test ranged from 2 = 10° to 80° (Garibo *et al.*, 2020). Using spectrum RX 1 equipment, Fourier-transform infrared (FT-IR) spectroscopic experiments were performed. The transmittance mode was used to scan the FT-IR spectra between 4000 and 400 cm<sup>-1</sup> at a resolution of 4 cm<sup>-1</sup> (Jaiswal *et al.*, 2010). Finally, FE-SEM (FE-SEM, SIGMA; Carl Zeiss Meditec AG; Jena, Germany) and HR-TEM were used to conduct morphological analyses and size evaluation of the synthesized AgNPs (Leo 906, Zeiss100 KV model,

Germany)(Wang *et al.*, 2020).

#### *Antioxidant Activity:*

The DPPH assay was done according to the method of Brand-Williams *et al.* (1995) with some modifications. By dissolving 24 mg of DPPH in 100 ml of methanol, the DPPH stock solution was prepared and kept at -20°C until required. The working solution was prepared by diluting 10 ml stock solution with 45 ml methanol to obtain an absorbance of 1.27  $\pm$  0.02 units at 515 nm using the spectrophotometer. Silver nanoparticles (different concentrations – 25, 50, 100, 250, 500, 1000  $\mu$ g/ $\mu$ l) were allowed to react with 2 ml of the DPPH solution for 1 h in the dark. Then the absorbance was taken at 515 nm. Radical scavenging activity of the samples was expressed as IC<sub>50</sub> (the concentration required to inhibit 50 % of DPPH) (Rop *et al.*, 2012).

#### *Anticancer Activity:*

The human lung carcinoma cell line (A549) was used for the investigation (IBRC). In DMEM 1640 media with 10% FBS (fetal bovine serum), 1% penicillin-streptomycin, and 37°C in a humid environment with 5% CO<sub>2</sub>, the A549 cell line was grown. An inverted microscope was used to examine the cells shape, health, and number. When the cells had grown by at least 70%, 0.05% trypsin was used to trypsinize the cells from the flask before they were centrifuged at 1500 rpm for 5 min. The resultant precipitate was created as a suspension, and optical microscopy and a Neubauer chamber were used to calculate the proportion of live cells. Cells having a viability of at least 90% were employed for the experiment after checking for contamination (Montes-Gutiérrez *et al.*, 2022).

#### *MTT Assay and Cell Viability:*

The MTT assay was employed to assess the cytotoxicity of A549 cells. 10,000 A549 cells were loaded individually onto 96-well plates in a 100  $\mu$ l culture medium, and the plates were then incubated for 24 h. The wells were then individually filled with various doses of AgNPs (512, 256, 128, 64, 32, 16, 8, 4, 2, 1  $\mu$ g/ml), and the



plates were incubated for 24 and 48 h. The wells were then filled with 100 µl of MTT (3, 4, 5-dimethylthiazolyl-2,5-diphenyltetrazolium bromide) and incubated for 4 h at 37°C with a 5% CO<sub>2</sub> atmosphere. Pure DMSO solution was added to the wells to dissolve the purple formazan crystals that had developed in the cytoplasm of the cells. Optical absorbance was measured at 570 nm with an ELISA reader. The viability % and IC<sub>50</sub> value of the findings were presented (Sun *et al.*, 2019).

## Results and Discussion

### Liquid-Liquid Extraction:

Several phenolic chemicals are extracted using the liquid-liquid extraction (LLE) techniques. Soxhlet extraction, maceration, and hydro distillation are the three ways for extracting phenolic compounds utilizing the LLE method. The type, polarity, temperature, ratio of the solvents, extraction duration, as well as the chemical make-up and physical features of the materials, are the crucial variables in these extraction techniques (Garcia-Salas *et al.*, 2010). Based on the findings of this research, several classes of phenolic compounds were extracted using different polarity solvents, and the optimum solvents for extracting flavonoids, polyphenols, and tannins, respectively, were found to be ethyl acetate, hexane, and methanol. In the liquid-liquid extraction, 1-30 g of phenolic compounds may typically be extracted for 6–24 h (Cheynier, 2012). The benefits of this method include simple extraction procedures and the ability to extract a variety of phenolic compounds using organic solvents of different polarities, but the drawbacks include the high solvent consumption, prolonged extraction times, risk of exposure to organic vapors, low extraction yields, and degradation of target compounds during the extraction method (Ji *et al.*, 2017).

### Qualitative Phytochemical Analysis:

Phenols, Flavonoids, Saponin were discovered to be present in Ethanol layer, whereas Chloroform layer contains terpenoids, steroids and saponins and ethyl acetate layer has glycoside and alkaloids

(Table 1, Figs. 1-3). These substances are known to have curative qualities against different pathogens, including those that cause gastrointestinal infections, and have substantial applicability against human pathogens. This suggests that they may be used in the treatment of various disorders (Markowiak and Śliżewska, 2017). In general, the total phenolic compounds in the plant's leaf, root, and petiole make up the largest portion of the plant's antioxidant activity. Sulaiman and Balachandran (2012) did not find saponin in *C. asiatica*, but they did find alkaloids in all the extracts they examined. Their investigation also revealed when combined with vincristine from *Catheranthus roseus*, the asiaticoside and asiatic acid present in *C. asiatica* extracts showed great potential for preventing and treating cancer (Sulaiman and Balachandran, 2012). As per the research conducted by Wrońska *et al.* (2022), triterpenoids present in *C. asiatica* have advantageous antibacterial properties and have the potential to treat several bacterial infections, such as *Salmonella*, *Shigella*, *Pasteurella multocida* and *S. aureus*. (Wrońska *et al.*, 2022). Although phytochemical screening is the first step in anticipating the sorts of potentially active molecules from plants, it is necessary since phytochemicals frequently play a significant part in a plant's defense against predation, microorganisms, stress, as well as interspecies protections (Ndezo Bisso *et al.*, 2022).

### Biosynthesis of AGNPs using *C. asiatica*:

The green synthesis of silver nanoparticle using *Centella asiatica* was demonstrated in current study. The transformation of the solution's colour from clear to yellow served as the first indication that AgNPs had been successfully synthesized. Following the reduction of Ag ions and their concentration as AgNPs, the color changed to brown. Wide range of parameters can influence nanoparticle formation. The nature of the reductant and the medium in which the reduction is occurring determine how quickly particle formation occurs (Sudeep and Kamat, 2005). The surface plasmon resonance (SPR) samples was

Table 1: Qualitative phytochemical analysis of *Centella asiatica*

| Extracts      | Phenol | Flavonoids | Tannins | Terpenoids | Saponin | Steroids | Glycosides | Alkaloids |
|---------------|--------|------------|---------|------------|---------|----------|------------|-----------|
| Ethanol       | ++     | +          | +       | +          | +       | +        | -          | -         |
| Chloroform    | -      | -          | +++     | +++        | +++     | +++      | -          | -         |
| Ethyl acetate | -      | -          | -       | -          | -       | -        | +          | +         |

+ trace amount, ++ slightly present, +++ highly present, and - absence of phyto compound



Fig. 1: Qualitative analysis of phytochemical-Ethanol layer.

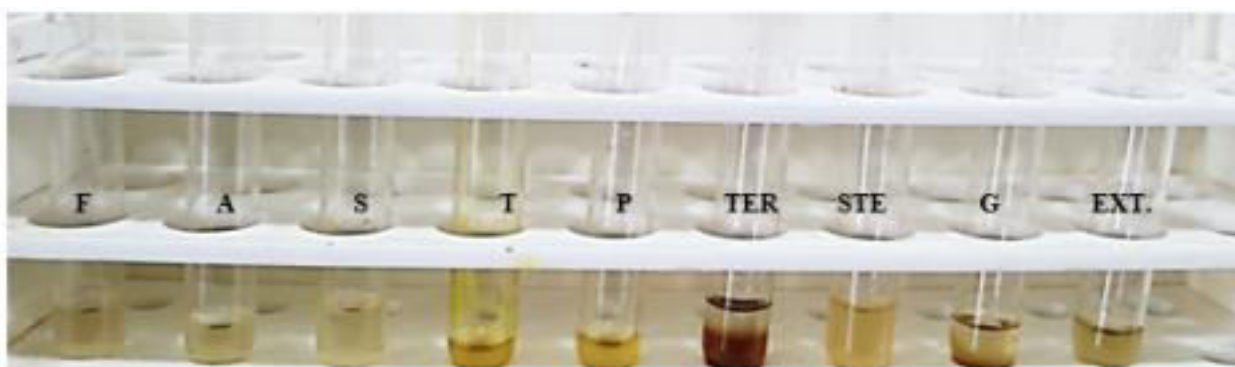


Fig. 2: Qualitative analysis of phytochemical- Chloroform layer.

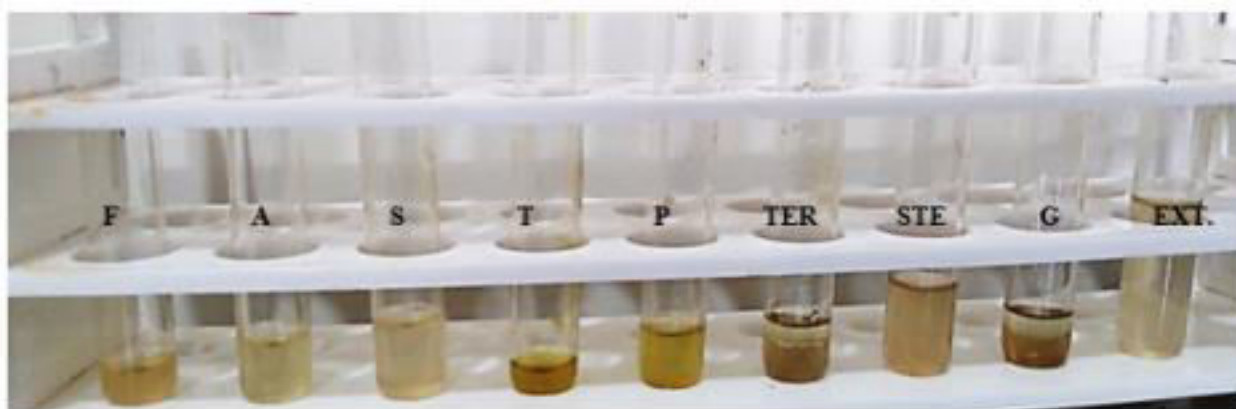


Fig. 3: Qualitative analysis of phytochemical-Ethyl acetate layer.

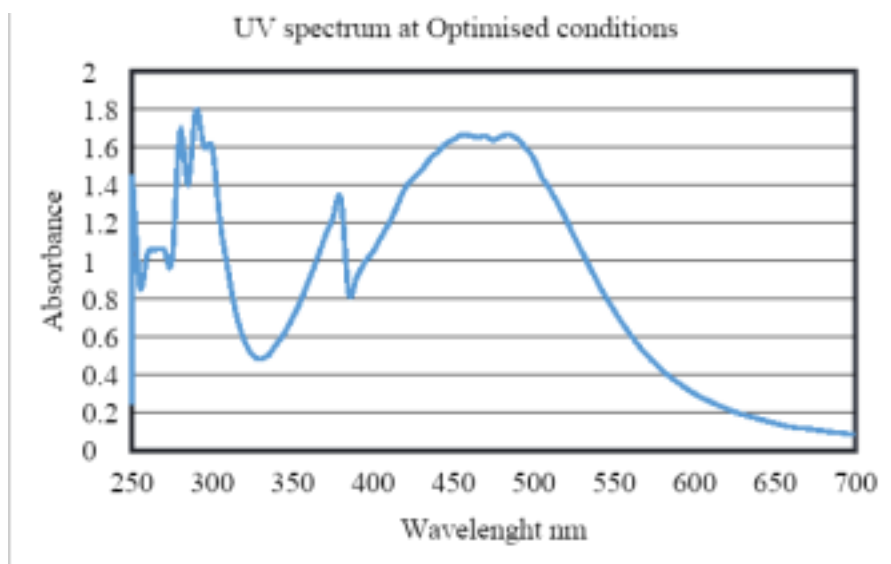


Fig. 4: UV- Visible Spectroscopy of silver nanoparticles synthesized from *C. asiatica* leaf extract.

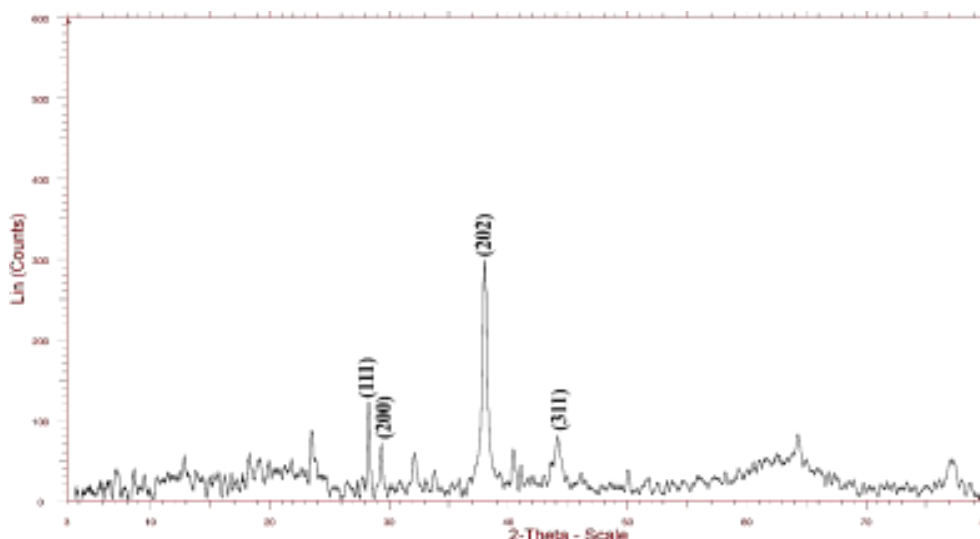


Fig. 5: XRD Analysis of silver nanoparticle from *Centella asiatica* extract.

excited, which resulted in the shift in color to brown (Arunachalam *et al.*, 2012). The phytocompounds (alkaloids, glycosides, flavonoids, amino acids, phenolic compounds, saccharides, and tannins) in the extract may serve as reducing agents to reduce silver ions into AgNPs (Bharathi *et al.*, 2018).

#### UV-VIS Spectroscopic Analysis:

UV-Vis spectroscopy is a helpful method that provides information on the synthesis of nanoparticles (Nazeruddin *et al.*, 2014). AgNPs

made from *C. asiatica* extract had a major absorption peak at 390 nm in the UV-visible absorption spectrum (Fig. 4). The AgNPs in the SPR region of around 450 nm can be attributed to spherical nanoparticles, according to earlier research (Gaddam *et al.*, 2014; Bhakya *et al.*, 2016).

#### XRD Analysis:

The presence of elemental silver signal of AgNPs was established by analysis using Energy Dispersive X-ray (EDX) spectrometers. The horizontal axis shows energy in KeV, whereas the

vertical axis shows the quantity of X-ray counts. There is confirmation that silver has been accurately recognized since identification lines for the main emission energies of silver (Ag) are presented and they correlate with peaks in the spectrum. Figure 5 displays the AgNPs' XRD patterns. The face-centered cubic (FCC) silver crystal had four major diffraction peaks which corresponded to the 111, 200, 202, and 311 planes, respectively (Fig. 5). The number of Bragg reflections obtained in the previous studies study 200, 111 and 311 corresponds to the diffraction facets of silver and indexed for the occurrence of crystalline silver nanoparticles (Dubey *et al.*, 2010; Sukirtha *et al.*, 2012).

#### FTIR Spectroscopy Analysis:

Silver nanoparticle synthesized from *C. asiatica* extract was subjected to FTIR analysis (Fig. 6). The major peaks at  $3420\text{ cm}^{-1}$ ,  $2922\text{ cm}^{-1}$  and  $1636\text{ cm}^{-1}$  were identified from FTIR analysis. The stretching vibrations of the O-H alcohol and phenolic groups in the extracts were responsible for a powerful peak ( $3437\text{ cm}^{-1}$ ) in the spectra. The C-H groups (aliphatic groups) were responsible for the absorption peak at  $2922\text{ cm}^{-1}$ . The protein's carbonyl amide group was indicated by a high absorption peak at  $1636\text{ cm}^{-1}$ . Bands corresponding to -OH stretching vibrations at  $3240.7\text{ cm}^{-1}$  and aromatic compound CH stretching at  $2924.5\text{ cm}^{-1}$  of the phenol group were observed. The vibration stretch recorded at  $1608\text{ cm}^{-1}$  is caused by the aromatic group's C-C stretch. The C-O stretching vibrations of the IR spectra were seen at  $1090.2\text{ cm}^{-1}$  and  $1032.9\text{ cm}^{-1}$ , respectively. The peak at  $1442.2\text{ cm}^{-1}$  corresponded to the O-H bend of polyphenol (Chen and Mu, 2002). Previous studies also suggest that these functional groups are attributed to polyphenols and aldehydes, which are the primary components of walnut aqueous extract (Oliveira *et al.*, 2008; Harshiny *et al.*, 2015). The bioreduction of  $\text{Ag}^+$  to Ag NPs may be caused by functional groups such the A-OH and C=O groups present in the sample. *Erythrina* sp. contains a high concentration of alkaloids and phenols. As a result, the capping agents might be

alkaloids, phenols, and their A-OH groups (Sre *et al.*, 2015).

#### TEM and SEM Analysis:

The synthesized silver nanoparticle was spherical in shape with an average size of  $37 \pm 0.75\text{ nm}$ , according to the SEM image analysis. Synthesized AgNPs from the *Urtica dioica* plant had a spherical shape and varied in size from 20 to 30 nm (Jyoti *et al.*, 2016). Moreover, AgNPs with average sizes of 30-50 and 42 nm were produced using aqueous *C. asiatica* extract (Rout *et al.*, 2013; Logeswari *et al.*, 2013). TEM analysis revealed that the silver nanoparticles were spherical in shape with ideal size of  $34 \pm 0.64\text{ nm}$ . The TEM pictures of the AgNPs synthesized using *Allium saralicum* revealed that they exhibited almost spherical morphology with high monodispersity and no aggregation in the 20-40 nm range (Zangeneh *et al.*, 2019). Figures 7 and 8 display the TEM and SEM images, respectively.

#### Antioxidant Activity:

The DPPH test was used to determine the ability of antioxidants to scavenge free radicals from DPPH (Fig. 9). The DPPH reduction was measured spectrophotometrically by measuring the drop in absorbance caused by the development of a stable DPPH-H molecule (reduced form). The free radical scavenging activity of AgNPs rose steadily as the concentration of AgNPs increased. At the maximum dose of 1 mg/ml, the silver nanoparticle examined demonstrated 60.63% inhibition and reference compound-ascorbic acid showed 88.50% inhibition. The  $\text{IC}_{50}$  concentration for silver nanoparticles was  $385.36\text{ }\mu\text{g/ml}$ . Previous study revealed that green synthesized AgNPs showed lower minimum inhibitory concentration at its highest concentration. The extract of *Centrocercas clavulatum* showed minimum inhibition at  $15.08\text{ }\mu\text{g/ml}$ . *Centrocercas clavulatum* extract appears to have a high concentration of hydrogen donor molecules, which may aid in the reduction of free radicals in DPPH scavenging experiments (Murugan *et al.*, 2016). According to Saravanan and Parimelazhagan (2014), several solvent



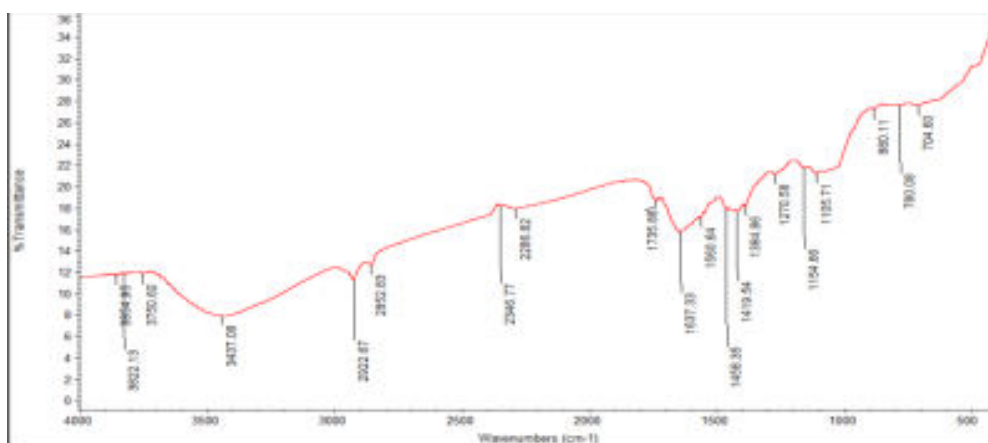


Fig. 6: FTIR of silver nanoparticle synthesis from *Centella asiatica* extract.

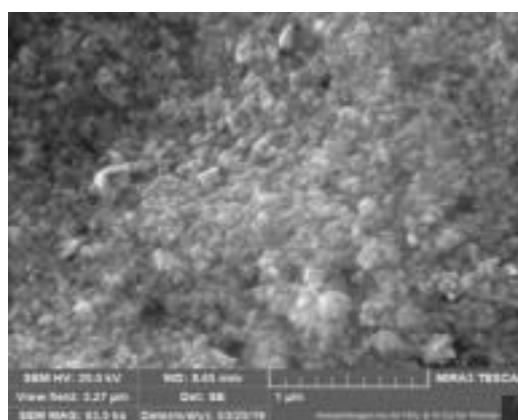


Fig. 7: SEM analysis of silver nanoparticles synthesized from *C. asiatica* leaf extract.

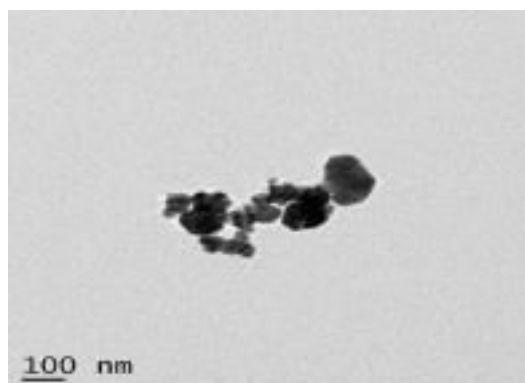


Fig. 8: TEM analysis of silver nanoparticles synthesized from *C. asiatica* leaf extract.

extracts of *Passiflora ligularis* fruits displayed DPPH scavenging capabilities.

#### **Cell Viability Analysis:**

AgNPs has gained attention in recent years as a potential anticancer therapeutic agent, because of their potential cytotoxic activities (Mohanta *et al.*, 2016). The *in vitro* cytotoxicity activity results of the nanoparticle significantly inhibited the growth

of cancer cells which were analyzed in different concentrations (512, 256, 128, 64, 32, 16, 8, 4, 2, 1 µg/ml) against A549 cell line. However, an increase in the sample concentrations showed increase in cytotoxicity and the results are given in Tables 2. It was evident that the tested silver nanoparticle at high concentration 512 µg/ml showed high inhibition percentage of 40.93 %

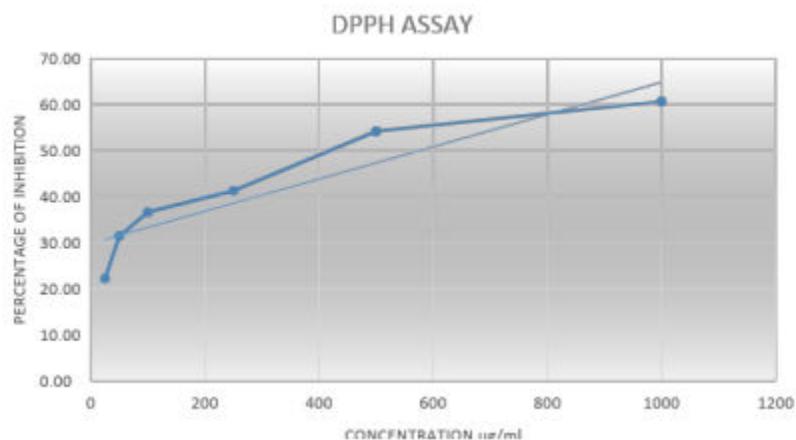


Fig. 9: DPPH Assay.

Table 2: Cytotoxicity activity of silver nanoparticle against A549 cell line

| S. No. | Concentration (µg/ml) | % cell viability |
|--------|-----------------------|------------------|
| 1      | 1                     | 77.40            |
| 2      | 2                     | 73.24            |
| 3      | 4                     | 67.93            |
| 4      | 8                     | 60.80            |
| 5      | 16                    | 55.05            |
| 6      | 32                    | 52.18            |
| 7      | 64                    | 47.08            |
| 8      | 128                   | 45.27            |
| 9      | 256                   | 42.57            |
| 10     | 512                   | 40.93            |

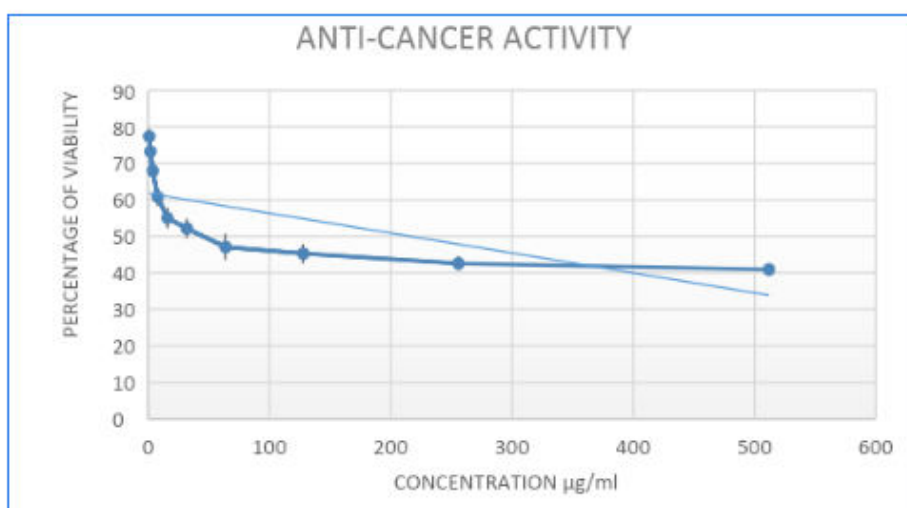


Fig. 10: Cytotoxicity of silver nanoparticles.

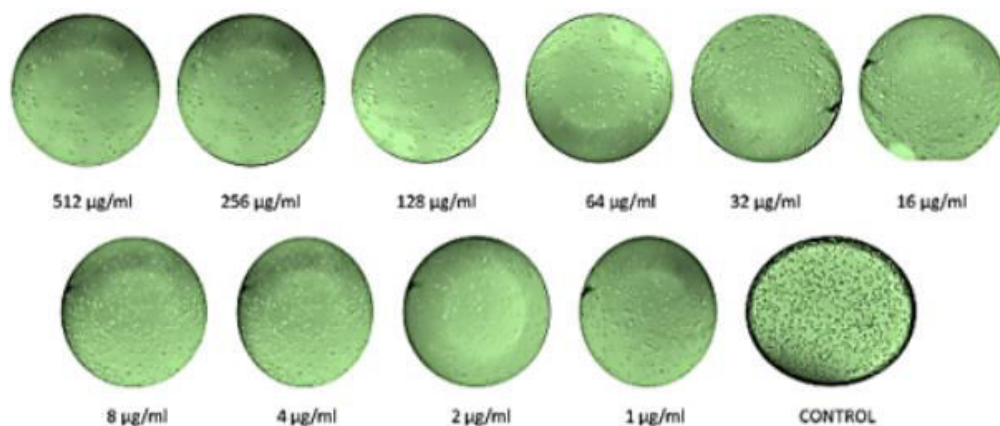


Fig. 11: Cytotoxicity activity of AgNP synthesized from *Centella asiatica*.

against A549 cancer cell line. It was evident from the result that the high cytotoxicity effect of the test sample showed cell disintegration after 24 h of treatment against the selected tested cell line even at lower concentrations. The  $IC_{50}$  calculated for sample was  $216.97 \pm 2 \mu\text{g/ml}$  (Fig. 10). The anticancer activity of silver nanoparticle with increasing concentration is represented in Figures 10 and 11. The increased activity of the produced AgNPs was due to their smaller particle size and extensive surface area, which allowed for the efficient endocytosis of AgNPs molecules into the nucleus, followed by DNA damage and apoptosis (Govindaraju *et al.*, 2015).

At a dosage of 10 g/ml, AgNPs synthesized from *Euphrasia officinalis* inhibited the growth of the A549 cell line by up to  $11 \pm 0.5\%$  and the growth of the HeLa cell line by  $13.5 \pm 2.2\%$  (Singh *et al.*, 2018). Cell viability was reduced to less than 70% in L929 fibroblast cells treated with CA-AgNPs at concentrations ranging from 2 to 10 mM (Bozkaya *et al.*, 2023). Biosynthesised AgNPs from *C. fistula* flower extract had a dose-dependent cytotoxic effect on breast cancer and the Vero cell line. The researchers observed 90.5 and 89.7% cell death against MCF-7 and Vero cell lines following incubation at 1000 g/ml, respectively (Remya *et al.*, 2015). AgNPs derived from *C. asiatica* extract exhibit anti-proliferative effect by inducing cell death via the caspase-dependent intracellular pathway, as demonstrated by enhanced activity of caspases 3 and 9 (Fard *et al.*, 2018).

## Conclusion

The importance of the green synthesized AgNPs utilizing *C. asiatica* leaf extract was emphasized in this work. The technique might be viewed as an environmentally friendly and economical method because it doesn't require the use of any reducing or capping chemicals (usually employed in physicochemical synthesis) to enable the biosynthesis of nanoparticles. The quick reaction time, minimal cost, and simple accessibility of this technology are its advantages. Green synthesized AgNPs, which have an average size of 36 nm, have the ability to suppress cell growth and cause apoptosis in A549 cancer cells in a manner that depends on the concentration and treatment period. Consequently, the synthesized nanoparticles can be utilized as a possible cancer therapy alternative after being confirmed by additional testing and *in vivo* research.

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