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Green Synthesis of Silver Nanoparticles using *Leea indica* (Burm. f.) Merr.: Phytochemical profiling, Characterization and Cytotoxicity Assessment

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Abstract

Silver nanoparticles have been the focus of extensive research in recent years because of their potential use in a variety of disciplines. The present study focused on the identification of phytochemical compounds and the synthesis of AgNPs using leaf extracts of *Leea indica* (Vitaceae), which is commonly known as bandicoot berry. GC-MS analysis of petroleum ether leaf extract of *L. indica* revealed the presence of five compounds. They include n-Hexadecanoic acid, dotriacontane, squalene, tetrapentacontane, and geranyllinalool. AgNPs were synthesized by using aqueous and ethanol leaf extracts of *L. indica.* The synthesized green AgNPs were characterized by using UV-visible spectroscopy, XRD, and SEM. The results showed that the AgNPs synthesized from the aqueous and ethanol leaf extracts, when exposed to sunlight and UV light, respectively, exhibited characteristic peaks at 415 nm and 410 nm. From XRD pattern the average crystalline size was estimated to be 58.433 nm and 30. 956 nm. SEM analysis was employed to examine the surface morphology of the AgNPs. The cytotoxicity of the synthesized AgNPs was evaluated *in vitro* using DLA cells through the trypan blue exclusion method. IC_{50} values for the AgNPs synthesized from the aqueous and ethanol extracts were determined to be 9.1 µl/mL and 12.4 µl/mL, respectively. The results demonstrated significant anticancer activity of the synthesized AgNPs against DLA cell lines..

1. Introduction

One of the most active study fields in contemporary material science is the topic of nanotechnology. The field of nanotechnology is developing day by day and has an effect on all aspects of human life (Singh *et al.,* 2010) and developing an increasing feeling of excitement in the life sciences, particularly biomedical devices and biotechnology (Prabhu *et al.,* 2010). Nanotechnologies have an impact on practically all scientific disciplines, including physics, material science, chemistry, biology, computer science, and engineering. Notably, nanotechnologies have recently been used to improve human health, particularly in the area of cancer treatment, with encouraging results (Bayda *et al.,* 2019).

As a bridge between bulk materials and atomic or molecular structures, nanoparticles are of tremendous scientific interest (Thakkar *et al.,* 2010). Particles up to 100 nm in size are considered to be nanoparticles (Simi *et al.,* 2007). Biosynthesis of nanoparticles is a type of bottom-up technique in which a biological system or one of its components is used to create nanoparticles, with the major

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reaction being the reduction of raw material into nanoparticles. The process of the biological route is due to the metal tolerance of biological entities (Li *et al.,* 2007). In comparison to other noble metal nanoparticles, AgNPs are intensively explored because of their optical, antibacterial, anticancer, antioxidant, and larvicidal properties, as well as their affordability (Suresh *et al.,* 2014; Mahmudin*et al.,* 2015; Vijayan *et al.,* 2018). Due to the quick, eco-friendly, non-pathogenic, inexpensive, and one-step method for biosynthetic processes, the use of plants as a production assembly for silver nanoparticles has attracted interest.

In the current study, we report the synthesis of AgNPs using simple, rapid and green technology from the aqueous and ethanol leaf extract of *Leea indica* (Burm. f.) Merr., an evergreen perennial herb, belonging to the family Vitaceae. The plant is distributed in forests of tropical and subtropical India, from Himalayas to Southward to the Peninsula (Kekuda *et al.,* 2018). *Leea indica* is a softwood shrub with glabrous stems that grows to a height of 8 metres. The leaves are 1-3 pinnate bearing 7 leaflets, with petioles 7-20 cm long. Leaflets are ovate -lanceolate with crenate to serrate margins. Flowers are greenish-white with 5 mm across. Fruits are purplish black, bearing six seeds 1 cm in diameter (Dalu *et al.,* 2014). The present study also investigates the *in vitro* cytotoxicity of AgNPs in Dalton's Lymphoma Ascites (DLA cells) using trypan blue assay. The study also screens the presence of bioactive compounds from petroleum ether extract of *Leea indica* using GC-MS.

2. Materials and methods

2.1. Collection and authentication of plant material

The fresh leaves of *Leea indica* were collected from Kizhuparamba, Malappuram in January 2023. It is located at Latitude 11.239777° and Longitude 76.017656°. The plant was authenticated by Jayakrishnan T., Assistant Professor, Department of Botany, Govt. Arts and Science College for Women, Malappuram.

2.2. Preparation of leaf extracts

The plant leaves were thoroughly washed with water thrice, chopped and dried for two weeks under the shadow at room temperature and ground into fine powder. The powdered sample was stored in an airtight jar for further analysis.

Three types of solvents with various polarity were used to carry out the extraction process and they were petroleum ether, ethanol and water. 20 g fine powder was let to soak in 200 ml petroleum ether, ethanol and water separately for 2 hours at room temperature with occasional stirring. Then it was boiled at 70-80°C for nearly an hour. The leaf extract was allowed to cool to room temperature and filtered through muslin cloth followed by Whatman No. 1 filter paper. The crude extract was preserved at 4°C for further use.

2.3. Gas Chromatography – Mass Spectrometry (GC-MS) analysis

GC-MS analysis of the petroleum ether leaf extract of *L. indica* was evaluated using GCMS QP2010S (Shimadzu Corporation, Japan) equipped with SH-I-5sil MS column (30 m length × 0.25 mm inner diameter × 0.25 µm film

thickness) and an AOC-30/20i autosampler. GC-MS analysis was performed by the split injection of 1 µLof the sample. The injector temperature was 260°C, with split ratio 40:1. High-purity helium gas (99.9995%) was used as the carrier gas with a flow rate 1.03 mL/min and constant pressure of 55.9 kPa. Ion source temperature was maintained at 230°C. The oven temperature was programmed from 50°C (hold for 2 minutes) to 280°C (hold for 4 minutes) at a rate of 7°C/min. The sample was then run for 32.5 minutes to complete GC-MS analysis. Data handling was done using GCMS solutions software. The identification of compounds was based on the comparison of obtained spectra with those of the NIST 20 library database and the results obtained have been tabulated.

2.4. Preparation of 2 mM silver nitrate ($AgNO_3$) solution

2 mM $AgNO_3$ solution was prepared by dissolving 0.0679 g $AgNO_3$ in 200 ml distilled water and stored in an amber-colored glass bottle.

2.5. Synthesis of AgNPs

2.5a. Using aqueous leaf extract

AgNPs were synthesized by mixing 90 ml of 2 mM silver nitrate solution and 10 ml of crude aqueous leaf extract in a ratio of 9:1. It was homogenized and taken in two beakers. One is kept undisturbed in sunlight and the other one in UV for 2 hours. The solution turned from yellow to dark brown color. The color change into dark brown indicates the formation of AgNPs. It was further confirmed by a UV-visible spectrophotometer. The synthesized AgNPs were centrifuged for 10 minutes and dried using a lyophilizer. The powdered form of AgNPs was stored in an airtight container for further studies. **Figure 1** illustrates the green synthesis of silver nanoparticles using leaf extracts.

2.5b. Using ethanol leaf extract

90 ml of 2 mM silver nitrate solution was added to 10 ml of crude ethanol leaf extract in a ratio of 9:1. It was homogenized and taken in two beakers. One is kept undisturbed in sunlight and the other one in UV for 2 hours. The solution turned from green to dark brown. The color change into dark brown indicates the formation of AgNPs. It was further confirmed by a UV-visible spectrophotometer. The synthesized AgNPs were centrifuged for 10 minutes and dried using a lyophilizer. The powdered form of AgNPs was stored in an airtight container for further studies.

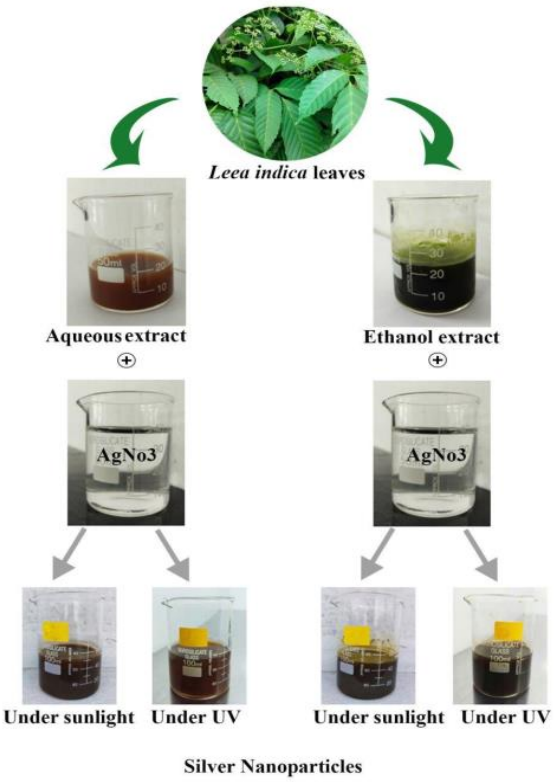


Fig. 1. Synthesis of AgNPs using *Leea indica* leaves

2.6. Characterization of AgNPs

2.6a. UV-visible spectroscopy

To confirm the synthesis of AgNPs, the sample was examined at a wavelength ranging from 300-800 nm using a UV spectrophotometer (UV1900I model).

2.6b. X-ray diffraction

The crystalline nature of synthesized AgNPs was analyzed by XRD (Bruker AXS) operated at 40 kV and with a current of 35 mA, with Cu-kα radiation (λ=1.54060 A°) in the range of 10°- 80° in 2θ angles. The average particle size of the synthesized AgNPs was calculated using the Debye-Scherrer equation.

$$D = \frac{k\lambda}{\beta cos\theta}$$

Where D is the average particle size, k is the Scherrer constant (0.94), λ is the wavelength of the X-ray, β is full width at half maximum of the peak in radians (FWHM) and θ is the diffraction angle.

2.6c. Scanning electron microscopy

The surface morphology of the synthesized AgNPs was analyzed by scanning electron microscope (Joel 6390LV) with an acceleration voltage of 20 kV.

2.7. Cytotoxicity of AgNPs in cancer cell lines

Short term *in vitro* cytotoxicity was assessed by the trypan blue exclusion method using Dalton's Lymphoma Ascites (DLA) cells. DLA cells were aspired from the peritoneal cavity of tumor-bearing mice and were washed thrice with a phosphate -buffered cell line (PBS) or normal cell line. Cell viability was determined by the trypan blue exclusion method. Viable cell suspension (1×10^6 cells in 0.1 ml) was added to tubes containing various concentrations (1.5, 2.5, 5, 10, 15 and 20 µl/mL) of AgNPs synthesized from aqueous and ethanol extracts and the volume was made up to 1 ml using PBS. The control tube contained only cell suspension. These assay mixtures were incubated for 3 hours at 37°C. The further cell suspension was mixed with 0.1 ml of 1% trypan blue and kept for 2-3 minutes and loaded on a hemocytometer. Dead cells take up the blue color of trypan blue while live cells do not take up the dye. The number of stained and unstained cells were counted separately. The percentage of cytotoxicity was calculated using the following formula:

Percentage of cytotoxicity = Number of dead cells / number of live cells + number of dead cells × 100

3. Results and Discussion

3.1. GC-MS analysis

The phytochemical compounds present in the petroleum ether leaf extract of *L. indica* were analyzed using GC-MS. The results of the analysis, including the names of the compounds along with their respective retention times (RT), peak areas (%), molecular formulas, and molecular weights, and their various bioactivities, are presented in **Table-1**. The GC-MS chromatogram, depicting the separation and identification of these compounds, is provided in **Fig. 2**.

Based on peak area, the major compounds were squalene (82.93%),

tetrapentacontane (10.69%), and geranyllinalool (2.33%). The minor compounds were n-Hexadecanoic acid (1.13%) and dotriacontane (0.54%). Bis(2-ethylhexyl) phthalate, detected with a peak area of 2.38%, is classified as a pthalate pollutant by the United States Environment Protection Agency (US EPA) (Ma *et al.,* 2015). Based on the GC-MS analysis, the identified compounds exhibit various activities, indicating the pharmaceutical importance of the plant.

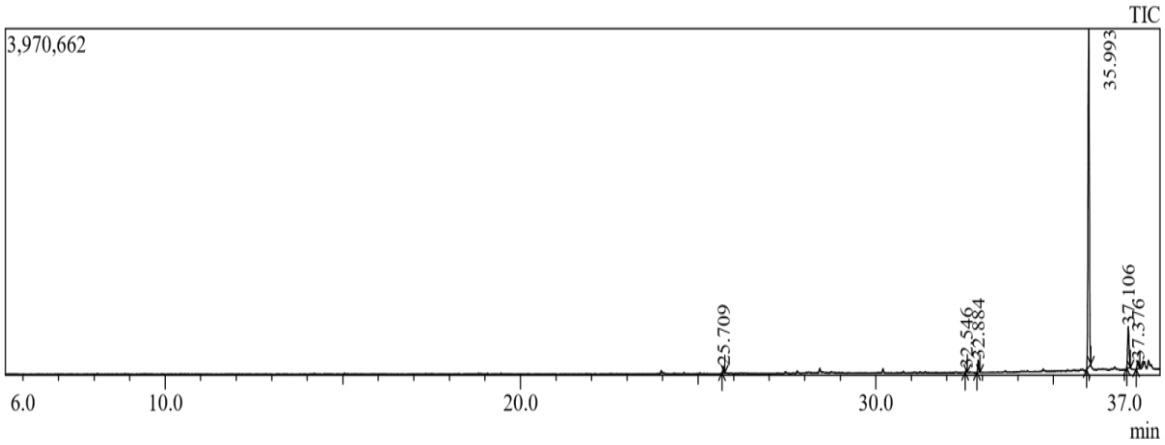
3.2. Synthesis and characterization of AgNPs

3.2a. Visual observation

The phytoconstituents play a crucial role in initiating and facilitating the reduction process, leading to the successful formation of AgNPs. The initial observation in nanoparticle synthesis is the change in color. In this study, after 2 hours of synthesis, the color of the solution prepared using the aqueous extract changed from yellowish to dark brown, while the solution prepared using ethanol extract changed from green to dark brown.

Fig. 3 illustrates the progression of color changes, which indicates the successful synthesis of AgNPs. The conversion of Ag^{+} ions to Ag^{0} was confirmed by the color change from colorless to dark brown upon theaddition of leaf extracts from *Leea indica*. This color transformation signifies the reduction process and the formation of AgNPs.

Fig. 2. Total ion chromatogram of petroleum ether extract of *Leea indica*



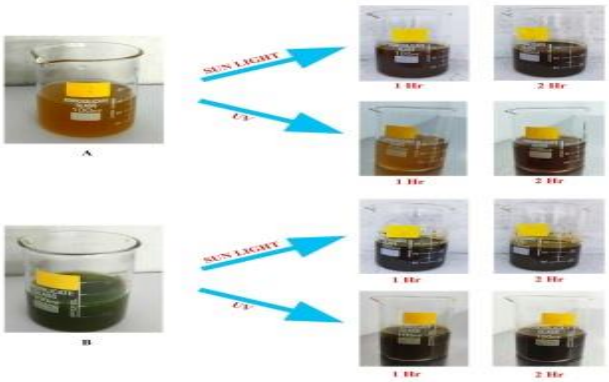


Fig. 3. Visual observation of AgNPs synthesis (A) from aqueous leaf extract (B) from ethanol leaf extract.

Table-1. Phytoconstituents identified from petroleum ether leaf extract of *Leea indica* by GC-MS analysis

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Sl. No.	RT	Name of the compound	Area %	Molecular formula	Molecular weight	Bioactivities
1.	25.709	n-Hexadecanoic acid	1.13	$C_{16}H_{32}O_2$	256.42	Anti-inflammatory, anti-spasmodic, anticancer, antiviral (Mohammed *et al.*, 2016), flavoring, antioxidant, hypocholesterolemic, nematicidal, pesticide, anti-androgenic [Henry *et al.*,2002;Kumar *et al.*, 2010), hemolytic, potent mosquito larvicidal and lubricating properties (Rahuman *et al.*, 2000)
2.	32.546	Dotriacontane	0.54	$C_{32}H_{66}$	450.86	Anti-spasmodic, antioxidant and antimicrobial activities (Soosairaj& Dons, 2016)
3.	35.993	Squalene	82.93	$C_{30}H_{50}$	410	Antioxidant, cholesterol control, emollient, detoxifying, anticancer (Gopinath *et al.*, 2013), pesticide and sunscreen properties (Ezhilan & Neelamegam, 2012)
4.	37.106	Tetrapentacontane	10.69	$C_{54}H_{110}$	759.45	Hair growth promoter, inhibits the production of uric acid and arachidonic acid, inhibitor of liver enzymes during phase I metabolism (Shunmugapriya *et al.*, 2017)
5.	37.376	Geranyllinalool	2.33	$C_{20}H_{34}O$	290.48	Anti-inflammatory, antioxidant and antitumor necrosis properties (Lakshmi & Bai,2015;Lapczynski*et al.*, 2008; Passos *et al.*, 2007;Fernandes *et al.*, 2007)

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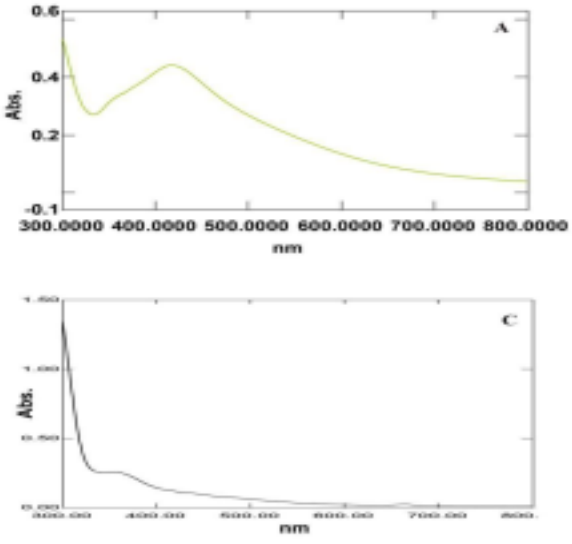
3.2b. UV-visible spectroscopy

The synthesized AgNPs were initially assessed using a UV-visible spectrophotometer within the wavelength range of 300-800 nm. Among the four samples tested, only the AgNPs synthesized from the aqueous and ethanol extracts, under sunlight and UV conditions respectively, exhibited a characteristic peak. The AgNPs synthesized using the aqueous extract of *L. indica* displayed a peak at 415 nm, while those synthesized using the ethanol extract showed the peak at 410 nm. The UV-visible spectra of the AgNPs are presneted in **Figure 4**, providing a visual representation of their absorbance patterns across the measured wavelength range.



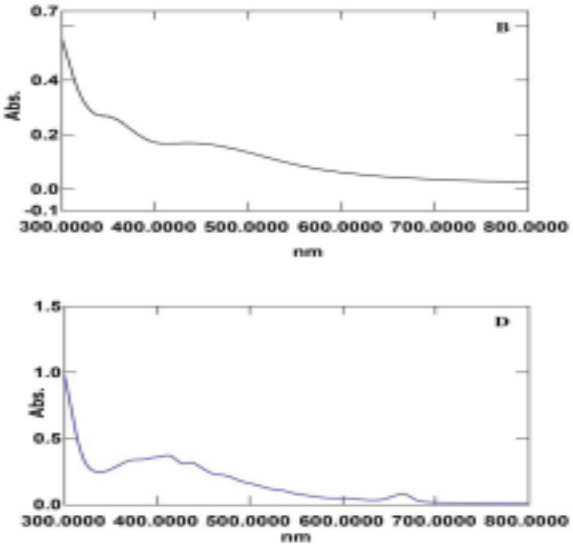
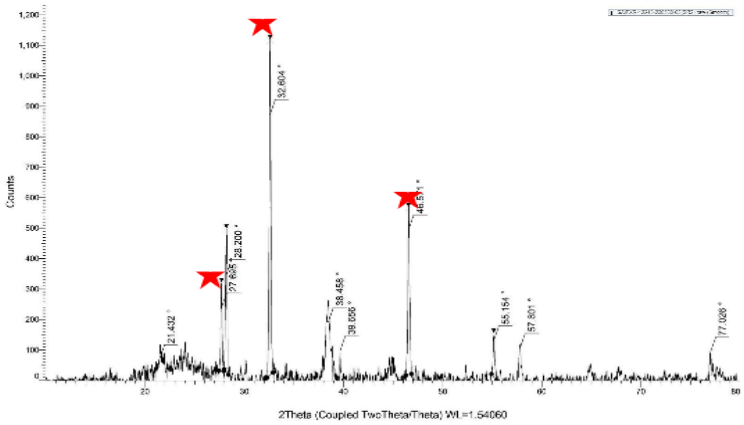


Fig. 4. UV-visible spectra of AgNPs (A) from aqueous extract under sunlight (B) from aqueous extract under UV (C) from ethanol extract under sunlight (D) from ethanol extract under UV.

3.2c. X-ray diffraction analysis

The crystalline nature of the synthesized AgNPs was analyzed using XRD. **Figure 5** presents the XRD diffractogram, which provides information about the crystal structure and the size of the nanoparticles. For the AgNPs prepared from the aqueous extract, the XRD pattern exhibited three prominent peaks at 27.695°, 32.604° and 46.571°, corresponding to the lattice planes (210), (122) and (231) of silver, respectively. On the other hand, the XRD pattern of AgNPs synthesized from the ethanol extract displayed three peaks at 38.171°,

44.369°, and 77.443°, corresponding to the lattice planes (111), (200), and (311) of silver, respectively. This pattern confirms a face-centered cubic crystal structure (Deepika*et al.*, 2020). Additionally, there were some unassigned peaks observed in the XRD pattern at 28.200°, and 55.154°, which are not included in the tables. These peaks are attributed to crystalline impurities from residual leaf extracts present on the surface of the resulting nanoparticles (Awwad *et al.*, 2013). The average crystalline size of the synthesized AgNPs from the aqueous and ethanol extracts was determined to be 58.433 nm and 30.956 nm, respectively, as shown in **Table-2** and **Table-3**.



A

Fig. 5. XRD spectra of AgNPs**(A)** from aqueous extract **(B)** from ethanol extract

Table-2. XRD results of AgNPs synthesized from aqueous leaf extract

FWHM (β)	Interplanar spacing (d) (nm)	Crystalline size (D) (nm)	Average crystalline size (nm)
0.141	0.321	64.40	58.433
0.163	0.274	56.32	
0.176	0.194	54.58	

Table-3. XRD results of AgNPs synthesized from ethanol leaf extract

FWHM (β)	Interplanar spacing (d) (nm)	Crystalline size (D) (nm)	Average crystalline size (nm)
0.251	0.235	37.18	30.956
0.347	0.204	27.45	
0.401	0.123	28.24	

3.2d. Scanning Electron Microscopy

The morphology of the synthesized AgNPs was analyzed using SEM, as depicted in **Figure 6**. The SEM images revealed that the nanoparticles exhibited a variety of shapes. Previous studies have reported the formation of AgNPs with different morphologies.

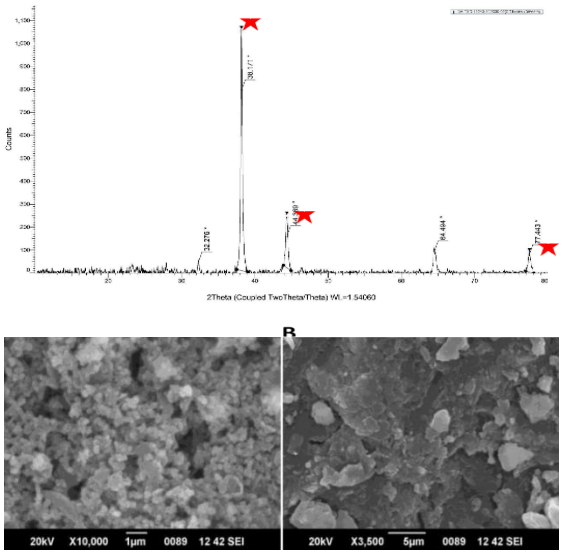


Fig. 6. SEM images of AgNPs**(A)** from aqueous extract **(B)** from ethanol extract

3.3 Anticancer activities of AgNPs

The cytotoxic properties of AgNPs synthesized from aqueous and ethanol leaf extracts of *L. indica* were investigated using DLA cells at concentration ranging from 1.5 to 20 µl/mL. The results of the cytotoxic assay showed that the synthesized AgNPs caused cell death up to 83.5±1.8% and 73.5±2.7% for the aqueous and ethanol extracts, respectively. The anticancer activity of AgNPs synthesized from aqueous extract was found to be higher compared to that of the ethanol extract. **Figure 7** graphically represents the cell death percentages. The results demonstrate a dose-dependent reduction in cell viability upon exposure to AgNPs. The IC_{50} values were determined as 9.1 µl/mL of AgNPs synthesized from the aqueous extract and 12.4 µl/mL for those synthesized from the ethanol extract. The AgNPs exhibited significant toxicity against the DLA cell line.

The cytotoxicity of AgNPs has been attributed to the active interaction between the phytoconstituents present in the leaf extracts and the silver atoms, particularly with functional groups of intracellular proteins, nitrogen bases, and phosphate groups in DNA (Jinu*et al.*, 2017).

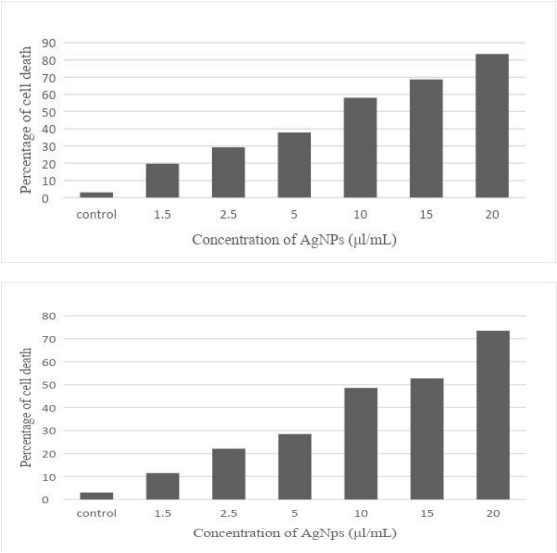


Fig. 7. Percentage of cell death of untreated and synthesized AgNPs treated DLA cell lines **(A)** from aqueous extract **(B)** from ethanol extract

4. Conclusion

Leea indica is a medicinal plant widely recognized for their pharmaceutical properties, and the current study focused on the identification of phytochemical compounds and the synthesis of AgNPs using its leaves. The GC-MS analysis revealed the presence of five phytochemical compounds in the sample, with squalene being the most abundant, followed by tetrapentacontane and geranyllinalool. These identified compounds possess diverse bioactivities. The AgNPs synthesized

from aqueous and ethanol leaf extracts, when exposed to sunlight and UV light, respectively, exhibited peaks at 415 nm and 410 nm. XRD analysis confirmed the FCC crystalline structure of the synthesized AgNPs. The average crystalline size of the AgNPs synthesized from the aqueous and ethanol extracts was determined to be 58.433 nm and 30.956 nm, respectively. Furthermore, the synthesized AgNPs demonstrated potent anticancer activity against DLA cell lines. Overall, the results suggest that *L. indica* could serve as a promising source of medicinal compounds, and its potential application in cancer therapy deserves further exploration.

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