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Elsholtzia ciliata Leaf Extract Mediated Silver Nanoparticles: Synthesis and their Antioxidant, Antidiabetic and Anticancer Activities

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This study deals with silver nanoparticle synthesis utilizing the leaf extract of the medicinal plant *Elsholtzia ciliata* and evaluated its antioxidant, antidiabetic and anticancer activities. The characterization of the synthesized *E. ciliata* silver nanoparticles (Ec-AgNPs) were done by using UV-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), dynamic light scattering (DLS) and transmission electron microscopy (TEM). The formation of face-centred cubic silver nanocrystals was confirmed by XRD data. During the formation of Ec-AgNPs, the phytochemicals present in the leaf extract act as reducing and capping agents as confirmed by FTIR spectra. The zeta potential and polydispersity index values from DLS measurements were -16.4 mV and 0.261, respectively. The high content of flavonoids, phenols, tannins and coumarins was confirmed by the phytochemical studies of the plant extract. The antioxidant activity was shown by the synthesized Ec-AgNPs. The inhibition of carbohydrate digestive enzyme α-glucosidase with IC_{50} values of 29.32 ± 0.42 µg/mL was found for the biosynthesized silver nanoparticles. MTT assay was performed on A549, HCT116 and HeLa Cell lines for evaluation of the antiproliferative activities of the Ec-AgNPs and have shown IC_{50} values of 189 ± 8.7, 192.5 ± 0.5 and 51.7 ± 7.8 µg/mL in A549, HCT116 and HeLa Cell lines, respectively. These findings proved that the biosynthesized Ec-AgNPs had good antioxidant, antidiabetic and anticancer activities and hence can be a competent candidate for an antioxidant, antidiabetic and anticancer agents in the pharmaceutical sector.

Keywords: Silver nanoparticles, *Elsholtzia ciliata*, Antiproliferative activity, Cancer cell line, Antioxidant activity, Antidiabetic activity.

INTRODUCTION

Metal nanoparticles are used in a variety of medical research domains for having certain better characteristics of chemical, physical and biological capabilities over their macroscopic counterparts. Among the numerous types of metal nanoparticles, significant importance is given to silver nanoparticles since they have biological applications like antibacterial, antifungal and antidiabetic activities [1-3]. The technique used most frequently for producing silver nanoparticles is chemical reduction. Chemical techniques are commonly used to produce silver nanoparticles; however, the use of toxic chemicals as starting materials and the production of hazardous byproducts are major problems with this approach, prompting many researchers to search out cleaner and sustainable alternatives. The synthesis of silver nanoparticles with plant extract is an improved option because it makes use of a natural stabilizing agent and has no potentially harmful compounds.

Silver nanoparticles were synthesized from different plants' leaf extracts of *Azadiracta indica*, *Taxus baccata*, *Oscimum sanctum*, *Aloe vera*, *etc.* [4-7]. Flowering *Elsholtzia ciliata* (Thunb.) Hylander, a member of the Lamiaceae family, can be found both in its native Asia and Europe [8]. The plant *E. ciliata*'s crude extract contains elsholtzia ketone, flavonoids, steroids, triterpenes and essential oil [9]. Studies have shown that the natural antioxidants found in *E. ciliata* are valuable bioactive sources [10]. In present work, a new and green route is explored for the synthesis of silver nanoparticle from the leaf extract of *E. ciliata* and characterized with various sophisticated techniques. The synthesized Ec-AgNPs were analyzed for the comparative antioxidant, antidiabetic and antiproliferative activities on cancer cell lines.

EXPERIMENTAL

Silver nitrate, 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), α-glucosidase, acarbose were supplied by Sigma-Aldrich, USA. Other chemicals/solvents used were of analytical grade and purchased from reputed companies. Milli-Q water was used throughout the experiment for the preparation of solutions.

Synthesis: From the Ema Market of Imphal West district, Manipur, India (Latitude: 24.807565º, Longitude: 93.92945º), fresh leaves of *E. ciliata* were collected and cleaned with distilled water and dried beneath a shed. The dried leaves were powdered by using an electric grinder. A mixture of 10 g of dried leaf powder and deionized water (200 mL) was heated for 10 min at 60 ºC and then cooled to room temperature followed by filteration (Whatman No. 1). The filtrate was placed in an Amber bottle and stored at 4 ºC for AgNPs synthesis. A 20 mL of *E. ciliata* extract was added to 200 mL of silver nitrate solution (10 mM) and heated at 70 ºC for 25 min in a water bath before making it cool to room temperature to form silver nanoparticles. The Ec-AgNPs formation was observed visually by detecting changes of colour and also by monitoring UV-vis spectra every 5 min. When the absorbance achieved a steady state, the process could no longer be observed. The resulting colloidal suspension was centrifuged for 15 min in a REMI Centrifuge R24 at 10000 rpm and the supernatant liquid was discarded once the residue had been collected. The residue was purified with repeated washing with deionized water and centrifuged three times and finally, the purified residue was collected. The purified residue was dried at 45 ºC and stored in an Amber bottle for further characterization.

Qualitative phytochemical analysis: Phytochemical analysis of *E. ciliata* leaf extract was performed to assess the presence or absence of alkaloids, flavonoids, glycosides, phenols, saponins, tannins, terpenoids, steroids, coumarins by following standard protocol with slight modifications [11].

Characterization of Ec-AgNPs: The absorption bands of synthesized Ec-AgNPs were monitored using Beckman Coulter DU 720 UV-vis spectrophotometer with a resolution of 0.5 nm and wavelength range from 350 to 800 nm at room temperature. By making a thin layer of Ec-AgNPs on a glass slide, the obtained sample was subjected to an X-ray diffraction (XRD) measurement using a BRUKER (eco D8, Advance) with Cu*K*α radiation in the 2θ range of 20-80º. Analysing the XRD data by Origin software, the crystallite size and phase composition of the synthesized nanoparticles were determined. Particle size and morphology of Ec-AgNPs were ascertained using a transmission electron microscope (JEM-2100, JEOL). The surface charge on Ec-AgNPs correlates with its stability. Malvern Instrumental's Nano ZS90 Zeta sizer was employed to ascertain the surface charge of Ec-AgNPs. Further, the polydispersity index (PDI) value was also determined from the DLS micrograph obtained. The functional groups found in the phytochemicals are accountable for undergoing reduction and stabilizing of Ec-AgNPs were examined using an FTIR spectrometer (Perkin-Elmer, Spectrum 2) operating at room temperature and measuring wavelength between 4000 and 400 cm^{-1}.

DPPH free radical scavenging assay: The scavenging activity of the green synthesized Ec-AgNPs was performed according to reported method [12] with slight modifications. 100 µL of DPPH (0.2 mM prepared in methanol) and 100 µL of different concentrations of Ec-AgNPs (8.5, 17, 25, 35, 50 µg/mL) were mixed. The reaction mixture was thoroughly shaken well accompanied by 30 min of dark incubation and followed by the recording of absorbance at 517 nm with a Varioskan LUX multimode microplate reader (ESW version 1.00.38) make from Thermo-Fisher Scientific. The percentage of DPPH free radical scavenging capacity was calculated using eqn. 1:

$$\text{Scavenging capacity } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (1)$$

Experiments were performed in triplicate and L-ascorbic acid was served as standard. The IC_{50} value of L-ascorbic acid and Ec-AgNPs was determined by linear regression analysis.

ABTS cation radical scavenging assay: The ABTS radical cation scavenging capacity of Ec-AgNPs was assessed with ABTS following Floegel *et al.* [13] with slight modifications. A solution of the ABTS radical cation was prepared by combining 7.4 mM ABTS and 2.6 mM potassium persulfate. After mixing 100 µL of the prepared ABTS solution with 100 µL of Ec-AgNPs at various concentrations (8.5, 17, 25, 35 and 50 µg/mL), with the help of Varioskan LUX multimode microplate reader, the absorbance was recorded at 734 nm. By using the following equation, the percentage of ability to scavenge ABTS radical cation was determined:

$$\text{Scavenging capacity } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (2)$$

The experiments were performed in triplicate. For standard, L-ascorbic acid was used. The IC_{50} value of Ec-AgNPs and L-ascorbic acid was calculated using a plot of concentration *versus* % scavenging capacity and linear regression analysis.

α-Glucosidase inhibition activity of Ec-AgNPs: A modified approach was used to measure the inhibition of α-glucosidase [3]. The assay mixture, included 150 µL of 0.1 M sodium phosphate buffer (pH 6.9, 6 mM NaCl), Ec-AgNPs of 5, 10, 20, 30, 40 and 50 µg/mL and α-glucosidase (0.1 units), was prepared to incubate at 37 ºC for 10 min. Next, 50 µL of 2 mM *p*-nitrophenyl-α-D-glucopyranoside in buffer sodium phosphate was added to the mixture and again incubation for 20 min was performed at 37 ºC. After the addition of 50 µL of 0.1 M Na_2CO_3, the reaction was stopped and recorded the absorbance at 405 nm. To serve as positive control acarbose was used. A solution containing α-glucosidase with 100% enzyme activity but no Ec-AgNPs served as control. Thermo-Fisher Scientific's Varioskan LUX multimode microplate reader (ESW version 1.00.38) was employed to record the absorbance at 405 nm and calculate the IC_{50} value of nanoparticles. The inhibition percentage was evaluated from eqn. 3:

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (3)$$

Cell cytotoxicity and anticancer activity assessment: The viability assay was performed by MTT assay following Lang *et al*. method [14]. In a 96-well plate, 5×10^3 cells were seeded and incubated at 37 ºC for 24 h in a humidified 5% CO_2 cell culture incubator. Before treatment, Ec-AgNPs were first dissolved in 10% DMSO as a stock solution and then diluted with growth media to concentration ranges (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µg/mL). Different doses of Ec-AgNPs concentrations were administered to the cells and then incubated for 48 h. Each well received 15 µL of MTT reagent (5 mg/mL in PBS) and left to incubate for 3 h. After incubation, 100 µL of DMSO as solubilizing agent was added and mixed thoroughly to dissolve the purple formazan product and then measured the absorbance at 570 nm. Instead of Ec-AgNPs, the cells with culture media were used as a negative control. The cytotoxic effects of Ec-AgNPs in terms of % cell viability were calculated by using eqn. 4:

$$\text{Cell viability (\%)} = \frac{OD_{sample}}{OD_{control}} \times 100 \quad (4)$$

The IC_{50} of cell viability was analyzed by GraphPad Prism statistical software.

RESULTS AND DISCUSSION

UV-Vis studies: The colourless silver nitrate solution changed its colour after the addition of *E. ciliata* leaf extract because Ag^+ was reduced to Ag^0 and turned the solution into a dark brown-coloured colloidal solution and this demonstrated Ec-AgNPs biosynthesis. Before 5 min of reaction time, no observable surface plasmon resonance (SPR) band was found, indicating that not enough Ec-AgNPs had been formed. However after 5 min, the silver nanoparticles' colour changed to dark brown accompanied by a distinctive SPR band [15,16]. The highest absorption band of the nanoparticles exhibited around 456 nm, indicating the formation of Ec-AgNPs and its intensities increased as reaction time progressed from 0 to 150 min (Fig. 1).

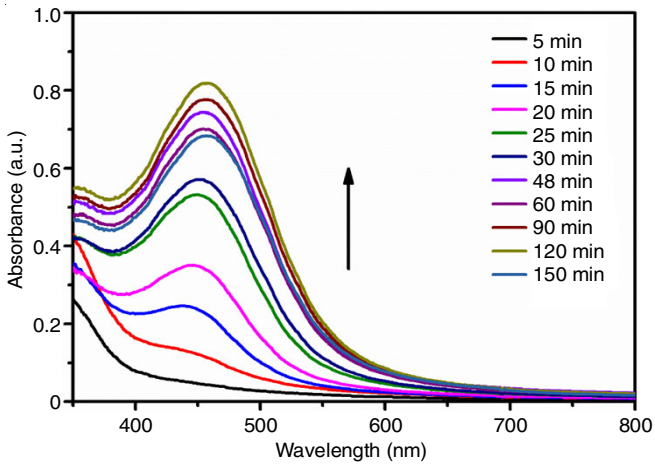

Fig. 1. UV-spectra of Ec-AgNPs showing different SPR bands with the progress over time

XRD studies: Fig. 2 displays the XRD diffractogram of Ec-AgNPs and JCPDS File No. 04-0783. No extra peaks exist in the diffractogram because of the inclusion of impurities. The high crystallinity of the synthesized Ec-AgNPs is demonstrated by their strong peak strengths. Nanoparticle formation is evidenced by the broadening of Braggs peaks [17] and the most intense peak lies at the (111) plane. Four peaks at 2θ values 37.9976º (111), 18.8079º (200), 11.9646º (220) and 10.8167º (311) are clearly shown by XRD diffractogram and attributed to the silver fcc structure. The diffraction pattern matched with the silver fcc crystal structure of JCPDS file No. 04-0783. The average crystallite size, D was determined by applying the formula of Debye-Scherrer:

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

where Scherrer's constant, K = 0.94; λ (0.154 nm) is X-ray wavelength, β (radian) is fullwidth at half maximum (FWHM) of the XRD peaks and found to be 14.24 nm [18].

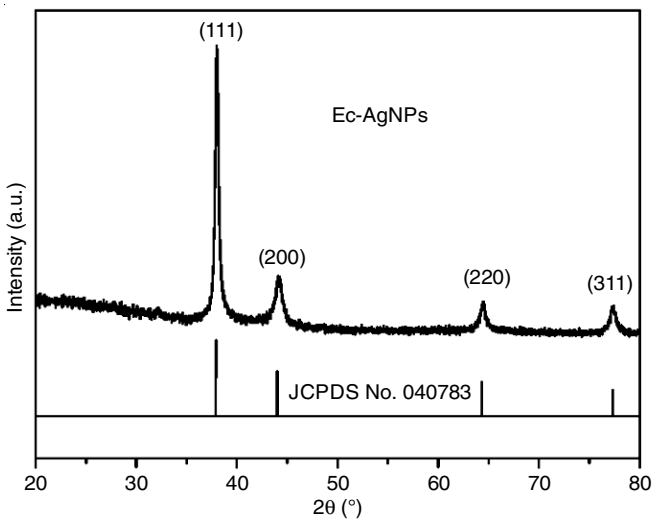

Fig. 2. XRD diffractogram of Ec-AgNPs with JCPDS file No. 040783

TEM studies: The TEM micrograph of the synthesized Ec-AgNPs (Fig. 3a) shows two unique morphologies, namely triangular and quasi-spherical shapes. Quasi-spherical shapes of an average size of 65.14 ± 21.14 nm are the majority of the nanoparticles formed. Fig. 3b reveals the SAED patterns of Ec-AgNPs with circular bright spots, which correspond to (111), (200), (220) and (311) planes and are well matched with XRD pattern. The presence of bright circular rings shows that the nanoparticles are polycrystalline [19]. Fig. 3c indicates the clear lattice fringes with a distance of 0.265 nm, whereas Fig. 3d shows the histogram showing the particle diameter size distribution of Ec-AgNPs.

FTIR studies: Fig. 4 shows the FTIR spectra of *E. cialiata* leaf extract and the synthesized Ec-AgNPs. The characteristic absorption peak at 3170 cm^{-1} of *E. ciliata* is due to the stretching vibration by O-H present in phenol on flavone rings and a peak at 2888 cm^{-1} attributes to C-H stretching of alkane. A peak at 1590 cm^{-1} is responsible for C=C groups from aromatic rings [20] while a peak at 1390 cm^{-1} is due to the O-H bending

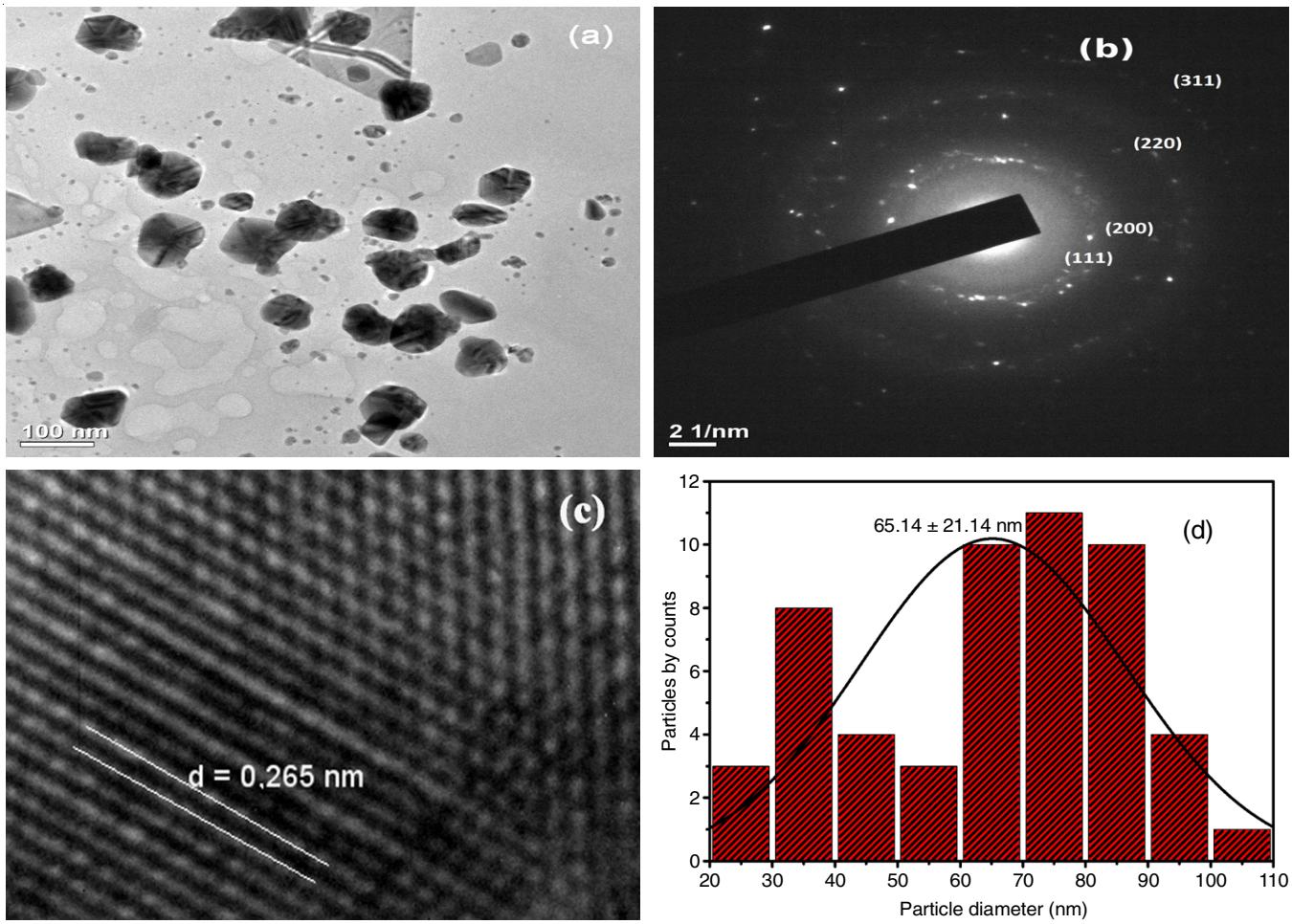

Fig. 3. TEM image (at100 nm scale bar) (a), SAED pattern (b), lattice fringes (c) and histogram of particle diameter size distribution of the synthesized Ec-AgNPs (d)

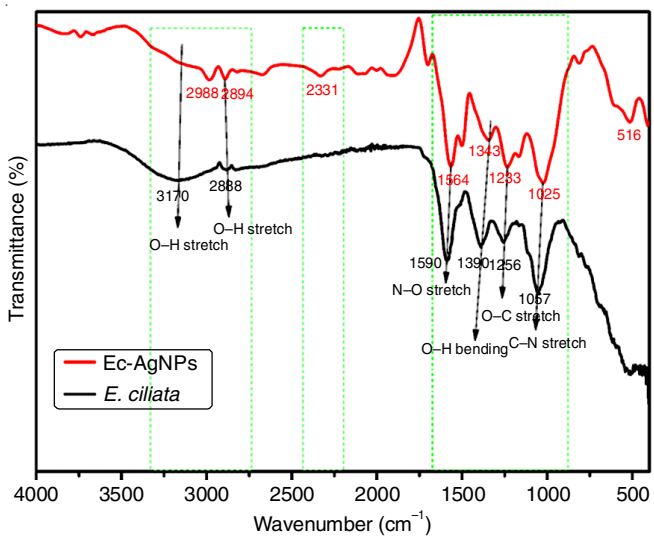

Fig. 4. FTIR spectra of synthesized Ec-AgNPs and *E. ciliata*

vibration of phenols/polyphenols, 1256 cm^{-1} evidence for O-C stretching vibration and 1057 cm^{-1} is due to C-N stretching vibration. FTIR spectrum of Ec-AgNPs shows some shifting, appearance and disappearance of some peaks. The peaks at 2888, 1590, 1390, 1256 and 1057 cm^{-1} shifted to 2894, 1564, 1343, 1233 and 1025 cm^{-1}, respectively. The appearance of new peaks at 2988, 2331 and 516 cm^{-1} indicated that the functional groups present in the phytochemical are bonded to the synthesized Ec-AgNPs. The peaks at 3170 cm^{-1} disappeared in FTIR spectra of Ec-AgNPs which suggests binding between the Ec-AgNPs and leaf extract. All these peaks supports the evidence of the role of functional groups present in *E. ciliata* as reducing and stabilizing agent for the synthesized Ec-AgNPs.

DLS studies: Fig. 5a shows the ζ-potential of -18.2 mV , whereas Fig. 5b shows the average hydrodynamic particle size distribution of 156.4 nm and polydispersity index (PDI) of approximately 0.261 of biosynthesized Ec-AgNPs. The size measured from the DLS is typically bigger than the size determined by the TEM pictures because the nanoparticles have a hydration layer on their surface [17]. Since leaf extract contains negatively charged functional groups, the synthesized Ec-AgNPs have a negative ζ-potential value on their surface, which facilitates in their stability [21]. The negatively charged nanoparticles repelled each other and prevented aggregation and stabilized the synthesized nanoparticles [22,23].

Qualitative phytochemical studies: Table-1 revealed the presence of pharmacologically active groups of chemical compounds like flavonoids, tannins, carbohydrates, glycosides, phenols, coumarins and quinone. It is clear that the leaf extract

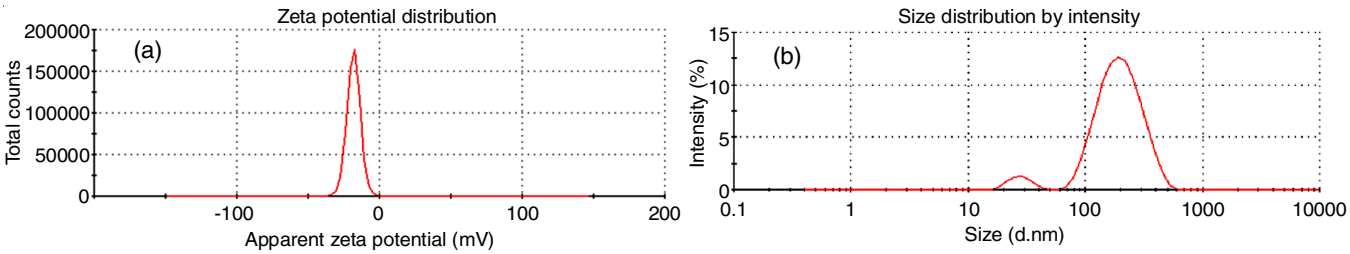

Fig. 5. The ζ-potential (a), average hydrodynamic particle size distribution and polydispersity index (PDI) (b) of synthesized Ec-AgNPs

TABLE-1
PRELIMINARY PHYTOCONSTITUENTS IN THE AQUEOUS LEAF EXTRACT OF *E. ciliata*

Phytochemicals	Tests performed	Extract
Alkaloids	Mayer's test	Absent
Flavonoids	NaOH test	Highly present
Tannins	$FeCl_3$ test	Highly present
Carbohydrates	α-Naphthol test	Low concentration
Glycosides	Borntrager's test	Moderately present
Saponins	Foam test	Absent
Triterpenes	Salkowski's test	Absent
Phenols	$FeCl_3$ test	Highly present
Coumarins	NaOH test	Highly present
Quinones	H_2SO_4 test	Low concentration
Terpenoids	Salkowski's test	Absent
Acids	$NaHCO_3$ test	Absent

is abundant in flavonoids, tannins, phenols and coumarins. Many previous studies have reported important parameters for antioxidant capacity depending on the emphasis on total flavonoids and phenols [24]. The silver may have been reduced by these phytochemicals, which also act as agents for capping to prevent them from aggregating and thus make them more stable nanoparticles. Most phytochemicals are polar (that are extracted using polar solvents) and important in nanoparticle synthesis [25,26].

Antioxidant activity: The % scavenging capacity of the synthesized Ec-AgNPs and ascorbic acid were determined by DPPH and ABTS assays in a concentration dependent way and are depicted in Fig. 6a-b, respectively. The calculated IC_{50} values were found to be 31.4 ± 0.12 µg/mL and 14.04 ± 0.09 µg/mL for Ec-AgNPs whereas, 32.45 ± 0.77 µg/mL and 14.51 ± 0.1 µg/mL for ascorbic acid (Table-2). It was found that the Ec-AgNPs have a lower scavenging capability than the standard compound. This can be explained on the basis that a synergistic effect might have occurred from the excessive quantity of total flavonoid and phenolic compounds present in the leaf extract which is attributed to their antioxidant capacity and this may also be correlated with the antiproliferative properties of the Ec-AgNPs [27].

TABLE-2
IC_{50} VALUES OF DPPH AND ABTS RADICAL SCAVENGING CAPACITY FOR Ec-AgNPs AND ASCORBIC ACID

Antioxidant assay	IC_{50} (µg/mL)	
	Ec-AgNPs	Ascorbic acid
DPPH	31.40 ± 0.12	32.45 ± 0.77
ABTS	14.04 ± 0.09	14.51 ± 0.10

***In vitro* inhibition of α-glucosidase activity:** The synthesized Ec-AgNPs were analyzed and found that possessed a high potential level of inhibition with an IC_{50} value of 29.372 ± 0.42 µg/mL (Fig. 7a). The percentage of inhibition by Ec-AgNPs likewise rises as the concentration of synthesized Ec-AgNPs does. The IC_{50} value of the reference compound (acarbose 42.1 ± 0.8 µg/mL) as shown in Fig. 7b is not lower than that of synthesized Ec-AgNPs. It is evident from IC_{50} values that Ec-AgNPs (29.372 ± 0.42 µg/mL) showed strong anti-diabetic efficacy.

Cytotoxic activity: The cell lines HeLa (cervical cancer), A549 (lung adenocarcinoma) and HCT 116 (colon cancer) were treated with the synthesized Ec-AgNPs sample at different concentrations and after 48 h of incubation and assayed with the MTT colorimetric assay for the antiproliferative activity.

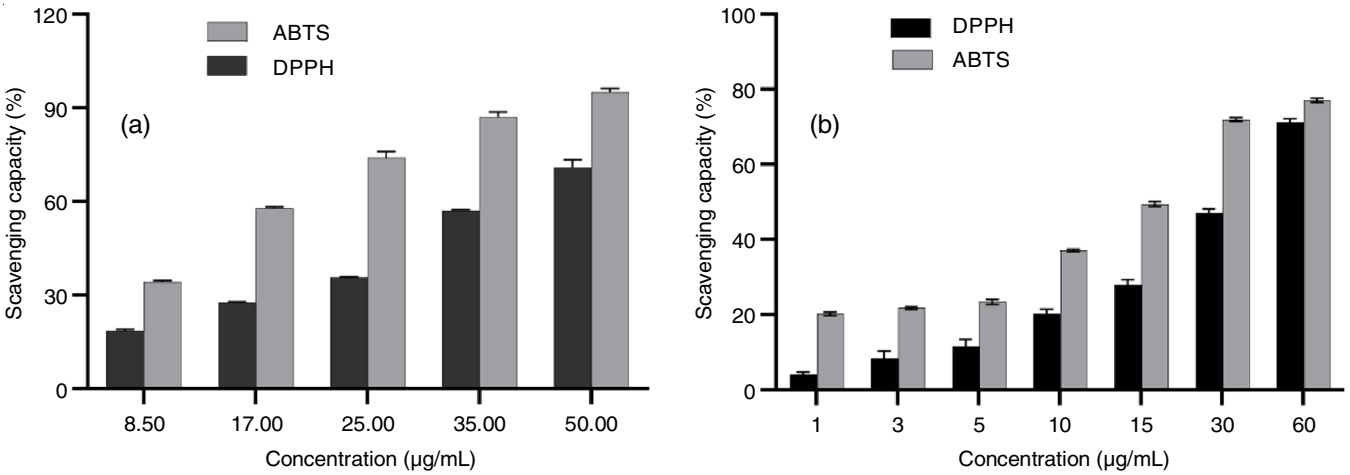

Fig. 6. DPPH radical and ABTS cation radical scavenging activity % of Ec-AgNPs (a) and ascorbic acid (b)

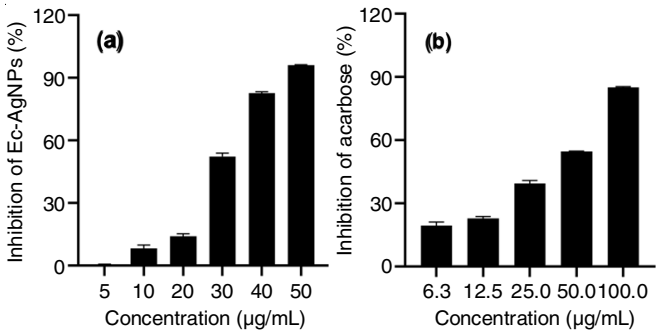

Fig. 7. α-Glucosidase inhibitory % of Ec-AgNPs (a) and acarbose (b)

Cellular viability and proliferation of the cell lines decreased with concentration (Fig. 8). The sample Ec-AgNPs showed strongest cytotoxic and anti-proliferative effects against HeLa cell line with inhibitory concentration (IC_{50}) of 51.7 ± 7.8 µg/mL, compared to A549 (IC_{50} = 189 ± 8.7 µg/mL) and HCT 116 (IC_{50} = 192.5 ± 0.5 µg/mL) (Table-3). Data has been represented as Mean ± SD of three different experiments. Cytotoxicity has been classified into four categories, based on Geran and American National Cancer Institute *in vitro* cytotoxic test; highly cytotoxic if IC_{50} < 20 µg/mL, cytotoxic moderately when IC_{50} value is 20-200 µg/mL, weakly cytotoxic when IC_{50} range between 201-500 µg/mL and non-cytotoxic when IC_{50} > 500 µg/mL. Thus, Ec-AgNPs possess moderate anticancer activities against HeLa, A549, HCT 116 cell lines [28].

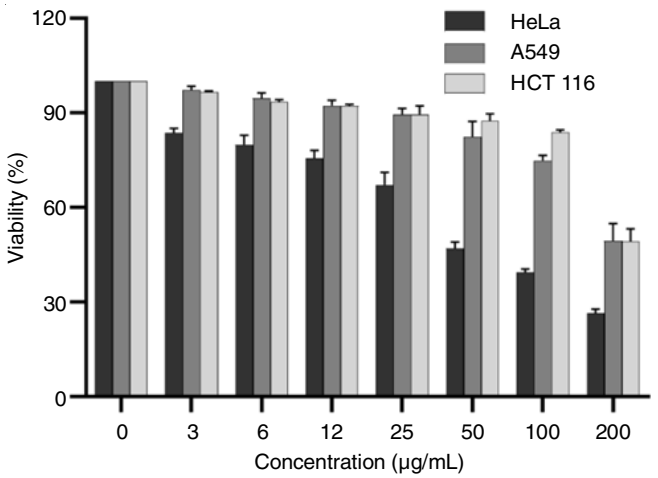

Fig. 8. Cytotoxic effect of Ec-AgNPs on HeLa, A549 and HCT 116 cell lines

TABLE-3
CYTOTOXICITY ACTIVITY EVALUATION BY MTT ASSAY OF Ec-AgNPs AGAINST VARIOUS CELL LINES

Cell line	IC_{50} (µg/mL)
HeLa	51.7 ± 7.8
A549	189 ± 8.7
HCT116	192.5 ± 0.5

Conclusion

Using leaf extract from plant *E. ciliata*, triangular and quasi-spherical silver nanoparticles were synthesized. The stabilization of the nanoparticles and reduction of silver are both caused by the phytochemicals found in the leaf extract of *E. ciliata.* The as-prepared AgNPs have antioxidant, antidiabetic and anticancer properties. Compared to A549 and HCT 116, the HeLa cell line was more sensitive to its cytotoxic and anti-proliferative effects. In the pharmaceutical industry, the green synthesized Ec-AgNPs may be used in the production of antioxidant, antidiabetic and anticancer drugs.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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