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ORIGINAL ARTICLE

Synthesis, Characterization, and Biological Activity of Silver Nanoparticles Synthesized from *Aristolochia bracteolata* Lam.

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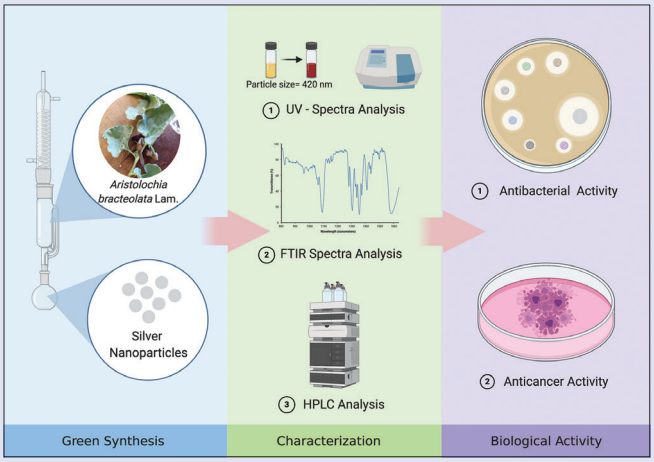
ABSTRACT

The aim of this research is to analyze the antimicrobial activity and cytotoxicity of biologically synthesized silver nanoparticles against A549 cancer cell line. Silver nanoparticles were synthesized from the leaf extract of *Aristolochia bracteolata*. For the characterization of silver nanoparticles, Fourier transform infrared, high-performance liquid chromatography, and ultraviolet techniques were adopted. The silver nanoparticle was found to show better antimicrobial activity when compared to the crude extract of the plant. Noteworthy cytotoxic effects against A549 cell line were shown by the nanoparticles derived from the plant extract. Following the potential of biologically synthesized silver nanoparticles, they can be a valuable addition to the treatment of human laryngeal carcinoma and can be categorized as a potent anticancer agent.

Key words: *Aristolochia bracteolata* Lam., infrared and A549, Silver Nanoparticles, ultraviolet

SUMMARY

- The extract from *Aristolochia bracteolata* Lam showed promising action against the tested organism and cell line. Hence, drug from this plant can be developed into new drug after standardization of the doses, concentractions etc. The active principal of the crude drug should be identified to understand the exact role of the extract.



Abbreviations used: FTIR: Fourier Transform Infrared, HPLC: High Performance Liquid Chromatography, UV: Ultraviolet–visible, $AgNO_3$: Silver nitrate, KBr: Potassium bromide, DMSO: Dimethyl sulfoxide, TBES: Trypan blue exclusion studies, E coli: *Escherichia coli*

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INTRODUCTION

In current recent trends, nanotechnology, due to its biological uses, has become a fast-rising technology for the creation and development of nano-sized particles.[1,2] Nanoparticles can be manufactured by different methodologies, for instance, biological, physical, and chemical methods.[3] Nanoparticle synthesis from chemical or physical methods deals with functional aspects of nanomaterials.[4] The biological method of nanoparticle creation is considered safe for environmental and public health as compared to other approaches.[5] Various types of metal nanoparticles such as gold, silver, zinc, copper, titanium, magnetite, and nickel are synthesized using different parts of the plant

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portions. The extracts of plants work as capping and reducing mediators having fundamental importance for the development of crystal-like nanoparticles with a variety of shapes and sizes.[6] Silver nanoparticles are used for diagnostic purposes and for bimolecular discovery.[7,8] These nanoparticles have been used for different biomedical purposes, for instance, antimicrobial, anti-inflammatory, antidiabetic, and anticancer activity or diagnosis.[9-12]

Aristolochiaceae family *Aristolochia bracteolata* Lam. is an herb which has glabrous and weak stems and 12 ribbed, glabrous capsule-type fruits. Seeds are deltoid in shape. The leaf structure is simple, with base cordate (up to 7.5 cm in diameter), broadly ovate or reniform, with prominent veins like pattern while the flower of this herb has orbicular bract at its base and a cylindrical perianth tube with dusky purplish tip.[13]

One other noticeable species from the *Aristolochia* genus is *Aristolochia indica* Linn. It can be commonly found in the plains and low hilly areas of South India spreading to West Bengal and Nepal. The chemicals of this plant are categorized as primary and secondary metabolites. Primary metabolites occur in all organisms in one form or another. The compounds are needed for primary cell metabolism and physiological development and are concentrated in seeds and vegetative organs for storage in higher plants.[14] Secondary metabolites include rotenone, pyrethrin, and nicotine. They are used in pesticides and in the manufacturing of alkaloids and steroids by the pharmaceutical industry.[15-20] The juice of the plant leave has antiperiodic properties. It is saturated with white peppercorns and is used in situation of diarrhea and cholera. It is also used as an antidote to poisonous insect's bites, for example, scorpion or a snake.[21]

The progress of assessment procedures for the separation of bioactive natural products should also deliver the means of identifying adaptogenic, antiviral, immunostimulating, and antitumor agents.[22]

In the current investigation, a natural method of the synthesis of silver nanoparticles from *A. bracteolata* Lam. plant extract was studied. The processes carried out were economical, environmentally safe, cost-effective, and not harmful for public health. The characterization method was carried out by ultraviolet (UV), infrared, and high-performance liquid chromatography (HPLC). The biological study was done by an antibacterial, antifungal, and cytotoxicity study.

MATERIALS AND METHODS

Collection of plant material

The plant used belongs to the family Aristolochiaceae, *A. bracteolata* Lam., which was collected from Pondicherry, India.

Plant extract preparation

With the use of Soxhlet apparatus, 25 g of fresh leaf powder (powder is made using room temperature-dried fresh leaves) is mixed with ethyl acetate (500 ml), methanol, and ethanol until the solvent becomes colorless, and then, using a rotary evaporator, the extracted material is dried out at 30°C. To get a concentrated solution (20 ml), the weighed extract is then dissolved in a particular solvent.

Preparation of silver nanoparticles

3.5 mm of $AgNO_3$ was prepared by mixing 0.6 g in 100 ml of deionized H_2O and was kept under UV for overnight. 1 ml of plant extract was then taken and added to 19 mL of 3.5 mm aqueous solution of AgO. It was kept in the dark after shaking well. This solution changes its color to reddish brown. The characterization of silver nanoparticles is done by noting the change of color of the solution.

Ultraviolet-visible spectroscopy

Extracted and synthesized nanoparticles were observed to cross-check the synthesized silver nanoparticle through absorption spectrum recorded between 400 nm and 500 nm. The peak between this 400 and 420 nm confirms the synthesis of silver nanoparticle.

Fourier transform infrared spectroscopy

For the Fourier transform infrared (FTIR) spectroscopy measurement, the biotransformed product was frozen, dried, and then diluted using KBr (ratio 1:100) and recorded in Nicolet Impact 400 FT-IR spectrophotometer instrument (with the range of 400–4000 cm at a resolution of 4 cm) using diffuse reflectance mode (DRS 8000) attachment.

Microbial cultures

Pathogenic microbial cultures of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Candida albicans* (ATCC 90028), and *Klebsiella pneumoniae* (ATCC 700603) were obtained from CMC, Vellore.

Preparation of antimicrobial discs

The extracts were loaded into sterile readymade discs (HiMedia) in different volumes of 50 µl, 100 µl, and 150 µl/disc, respectively, and left at room temperature to dry for 24 h. Similarly, Ag-conjugated plant extract was also loaded onto the sterile discs in different volumes of 50 µl, 100 µl, and 150 µl/disc, respectively, and left at room temperature to dry for the same time.

Antifungal assay

The antifungal activity was accomplished using the agar disc diffusion method. The Czapek Dox Agar (HiMedia) in molten form after inoculating with 50 µl of inoculum (1×10^8 CFU) is poured in the sterile Petri dishes. After the saturation and drying, the plates are left overnight at 37°C in the incubator. By measuring the zone diameter, the inhibition of microbial growth was calculated.

Antibacterial assay

The antibacterial activity was accomplished using agar disc diffusion method with HiMedia being the molten Mueller-Hinton agar (HiMedia) and following the same procedure as for antifungal assay. The diameter of the zone of inhibition indicates the microbial growth.

Anticancer activity

Vero cell line

The cell line made from the kidney of a normal green African monkey is called Vero cell line. It is prone to various viruses, for instance, arboviruses, rubella, and polio.[23] This cell line is used to produce viral vaccines on an industrial scale (an example is the polio vaccine) because of the reduced risk of contamination by the endogenous viruses.[24] Vero cells have an anchorage-dependent cell line which helps them attain high densities. Cell density is known to influence the manufacturing of poliovirus antigen.[25]

A549 cell line

Using the human carcinoma of larynx, the cell line was first established by Moore.[26] The larynx cancer cell line has been utilized the production of virus, cytotoxicity, virology, virus titration, bacterial adhesiveness, and tumor.

Note: Hep2 is not a laryngeal cell line but is contaminated by HeLa.

Cell culture

Vero cell line and A549 were obtained from King Institute, Chennai. Dulbecco's modified Eagle's medium is used to cultivate A549 cells which is supplemented with 10% of calf bovine serum, and antibiotics (streptomycin and penicillin each 100unit/ml in quantity) at a temperature of 37°C in a humidified incubator with 5% of carbon dioxide. Vero cells were protected in Dulbecco's modified Eagle's medium without serum and maintained as above.

Cell counting by hemocytometer

Cell counting was done using a Neubauer hemocytometer. The total number of cells was counted using the following formula:

Total cell count $N = M \times Tb \times V \times 10^4$

Where

M = Mean of cell count of 4 corners

Tb = Trypan blue correction value

V = Volume of cell suspension

10^4 = Conversion factor for counting chamber.

3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide

3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazoliumbromide (MTT) stock solution was prepared by adding 5 mg/ml in phosphate-buffered saline (PBS), and pH was maintained at 7.5. This was then filtered through a filter of 0.22 µ size to eliminate the insoluble remnants.

0.2% phenol red solution

1 g of phenol red was crushed using mortar and pestle, and 15 ml of N/10 sodium hydroxide was added to dissolve it. Using autoclaved Millipore water, the volume was increased to 500 ml. The obtained solution was then autoclaved at a pressure of 15 lbs and temperature of 121°C for 15 min. For storage, 4°C temperature was maintained.

Antibiotics (penicillin and streptomycin)

100 mg/500 ml of medium.

Sodium bicarbonate

1 g of sodium bicarbonate solution was crushed using a mortar and pestle, and 15 ml of N/10 sodium hydroxide was added to dissolve it. The volume was made up to 25 ml.

Trypsin solution

1% ethylenediaminetetraacetic acid (EDTA) was obtained by solving 2 g of EDTA in 200 ml of Millipore filtered water; to this, 0.5 g of trypsin was added.

Trypan blue solution

Trypan blue – 0.5% in saline.

Drug exposure

The powdered drugs were liquefied in 100% of dimethyl sulfoxide (DMSO) solution to a standard concentration of 1 mg/µl. Using 8 dissimilar concentrations of *A. bracteolata* (initial concentration of drug as 0.6 mg), cells obtained from log phase culture were then put in a carbon dioxide (CO_2) incubator and incubated for 24 h at 37°C. For storage, the drugs were kept at 4°C.

Trypan blue exclusion studies

After 24 h of incubation, using trypan blue exclusion studies (TBES), cells' viability was calculated.

The viability of cells was assessed by TBES after 24 h of incubation.

Morphological analysis

Light microscopy

The Vero and A549 cells after incubation of 48 h were rinsed using PBS. Using a phase-contrast microscope at 40X, the cells were then observed closely for any morphological changes and photographed.

After 48 h incubation with drugs, the Vero and A549 cells were washed with PBS. The cells were observed for morphological changes under a phase-contrast microscope at 40X and photographed.

Cell viability assessment

The method used to calculate cell viability was MTT. IC_{50} or the cytotoxic index was calculated using the mentioned method.

RESULTS

Ultraviolet-visible spectrum analysis

Using *A. bracteolata* Lam. leaf, ethanol extract as a reducing agent to form silver nanoparticles changes the color from pale yellow to reddish brown while the size range starts from 420 nm.

Fourier transform infrared analysis

Partially purified compound of ethanol extract [Figure 1] exhibits the band at 3391 cm^{-1}, 2929 cm^{-1}, 1637 cm^{-1}, 1516 cm^{-1}, 1386 cm^{-1}, and 1267 cm^{-1}. A broad peak at 3391 cm^{-1} is due to the presence of –OH– group with strong hydrogen bond while the peak stretching up to 2929 cm^{-1} is due to the presence of $-CH_3-$. The presence of –C═C– bonds forms a peak at 1637 cm^{-1}. Asymmetric stretching of NO_2 gives NO_2 group peak at 1516 cm^{-1}. The peak at 1386 cm^{-1} is due to the NO_2 symmetric stretching confirming the presence of –C–O– bond. This peak is due to –C–O– stretching at 1267 cm^{-1}.

Partially purified compound of silver nanoparticle synthesized ethanol extracts exhibits [Figure 2] the band at 3400 cm^{-1}, 2130 cm^{-1}, 1645 cm^{-1}, 1393 cm^{-1}, 1051 cm^{-1}, and 718 cm^{-1} confirming the presence of –OH– group with strong hydrogen bond (at 3400 cm^{-1}). A sharp peak is due to the presence of –C═N– which occasionally elongates at 2130 cm^{-1}. Intense peak at 1645 cm^{-1} may be due to the presence of carbonyl group due to –C–O– bonds. A peak stretching to 1393 cm^{-1} is due to –C–N– bond. It is also confirmed by the broad peak at 1051 cm^{-1}. Upon conjugation, N can be considered to form H bond, leading to give –N–H– out of plane stretching 718 cm^{-1}.

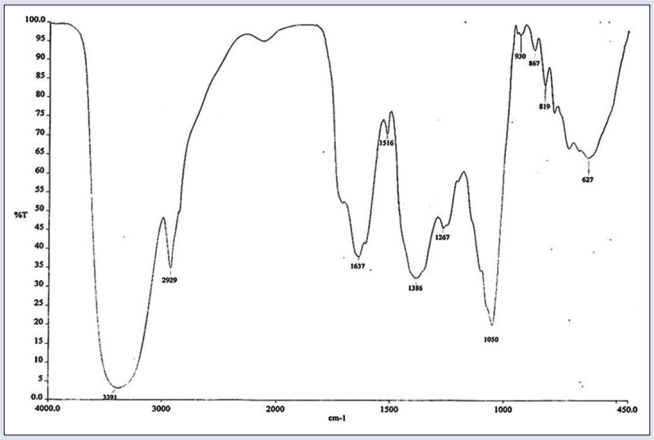


Figure 1: Infrared spectroscopy analysis of *Aristolochia bracteolata* Lam. extract

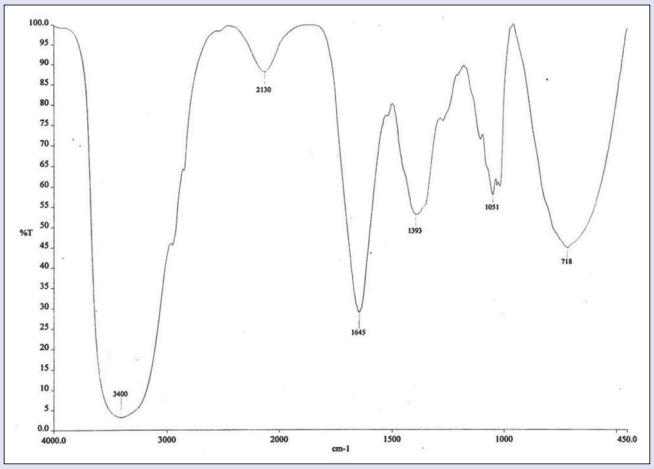


Figure 2: Infrared spectroscopy analysis of silver nanoparticle synthesized *Aristolochia bracteolata* Lam. extract

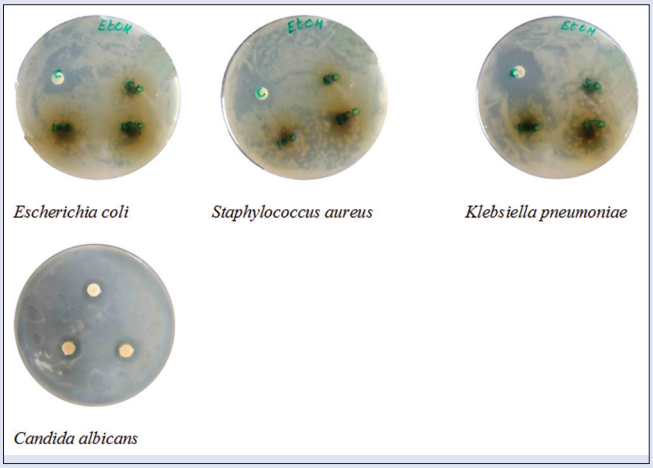


Figure 5: Antimicrobial Activity analysis of Aristolochia bracteolate extract

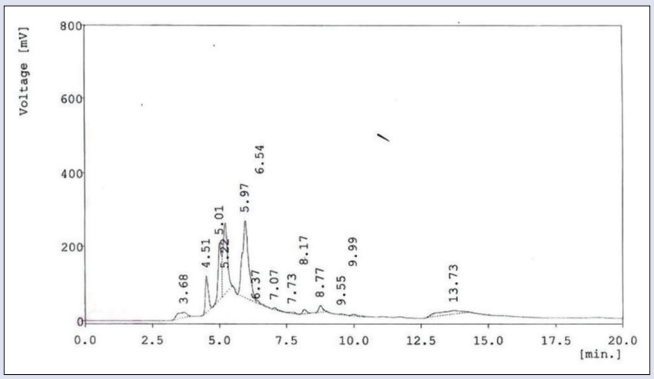


Figure 3: High-performance liquid chromatography analysis of *Aristolochia bracteolata* Lam. extract

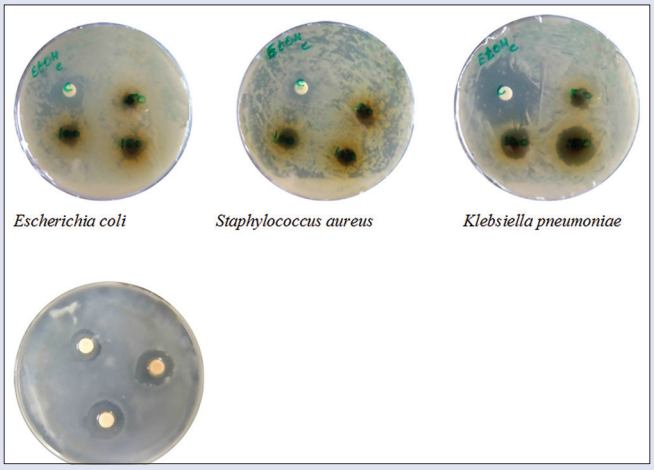


Figure 6: Antimicrobial activity of synthesized silver nanoparticle

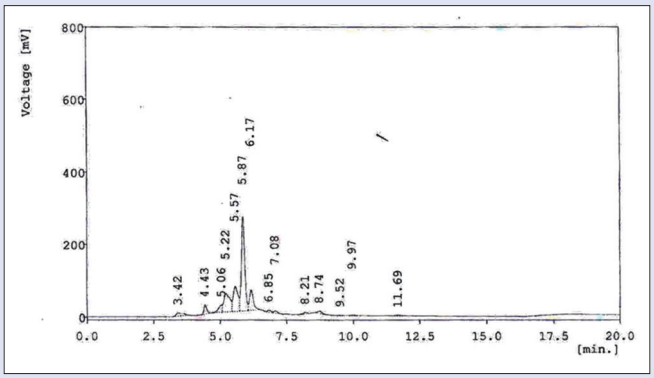


Figure 4: High-performance liquid chromatography analysis of silver nanoparticle synthesized *Aristolochia bracteolata* Lam. extract

High-pressure liquid chromatographic analysis

Chromatography was performed on a Shimadzu LC 10AT VP system while separation was on a 250 × 4.60 mm. Phenomenex C_{18} column at a mobile phase flow rate of 0.5 ml/min. The mobile phase gradient was used as acetonitrile and water ratio of 80:20, with the injection volume of 20 µl. To reduce molecular ionization, the water and acetonitrile consist of 0.1% (v/v) trifluoroacetic acid. The retention time for aristolochic acid II was approximately 5.97 min for *A. bracteolata* Lam. extract [Figure 3] and 5.87 min for synthesized *A. bracteolata* Lam. extract [Figure 4].

Antimicrobial activity analysis

Several of the available antibiotics have become obsolete as pathogens are developing resistance to the effects of antibiotics over time which enforces the search of new antibiotic.[27-29]

The antimicrobial activities of the drug of *A. bracteolata* Lam. crude extract [Table 1] and synthesized silver nanoparticle extract [Table 2] were studied on bacteria and fungus, namely *E. coli, S. aureus, K. pneumoniae,* and *C. albicans,* which have been documented. The ethanol extract of *A. bracteolata* for antimicrobial activity studies carried out on *E. coli, S. aureus, K. pneumoniae,* and *C. albicans* has shown [Figure 5] maximum inhibition for the growth of organism followed by showed some activities against these pathogens. The synthesized nanoparticles have shown [Figure 6] more control of organism than the crude extract samples, which shows that the compounds were enhanced by the silver ions resulting in a large growth inhibition zone.

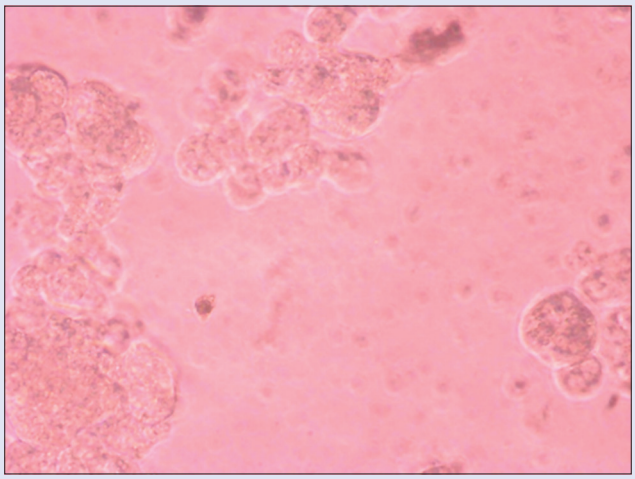


Figure 7: Cytotoxicity of *Arictolochia bracteolata* on Vero cell line

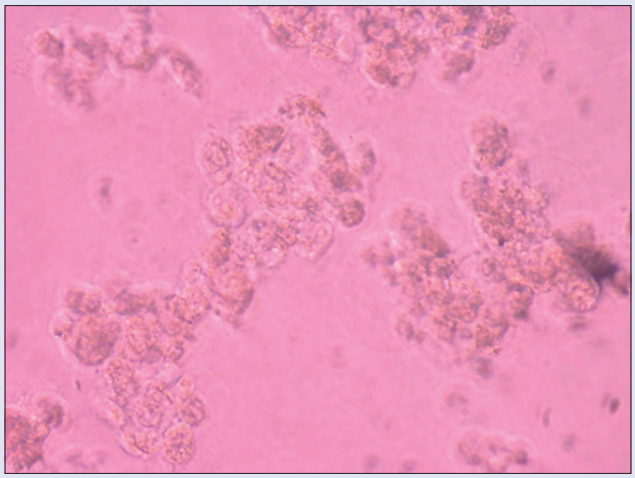


Figure 8: Anticancer studies of the drug of *A. bracteolata* on A549

Anticancer activity

The ethanol extract was diluted at different concentrations and was plated on a 24-well plate along with Vero cell line and minimum dilution of medium.

The cell cytotoxicity was observed at 1:2 dilution on Vero cell lines. The higher dilution of ethanol extract was used for cancer cell line A549 [Table 3 and Figure 7].

The synthesized silver nanoparticle was diluted in different concentrations and was placed on the 24-well plate containing medium along with cancer cell (A549) [Table 4 and Figure 8].

The dilution of 1:128 was observed to be toxic on the A549 cell line.

Anticancer studies of the drug of *A. bracteolata* on A549 showed that it has the ability to constrain the spread of larynx tumor cells. The result reveals that the extract of ethanol (1:128) and ethanol conjugate (1:128) showed maximum antiproliferation activity on A549 cancer cell lines.

The results indicate that *A. bracteolata* crude extract effectively controls the larynx carcinoma in *in vitro* condition. A further study has to be carried out to identify the specific compound present showing activity against carcinoma in *in vitro* system. This could be an alternative herbal drug which could be formulated for the larynx carcinoma treatment.

Table 1: Antimicrobial activity of *Aristolochia bracteolate* extract

Organisms	Concentration		
	50 µl	100 µl	150 µl
Escherichia coli	12	12	15
Staphylococcus aureus	13	15	15
Klebsiella pneumoniae	Nil	12	13
Candida albicans	12	15	15

Table 2: Antimicrobial activity of synthesized silver nanoparticle from *Aristolochia bracteolate* extract

Organisms	Concentration		
	50 µl	100 µl	150 µl
Escherichia coli	10	13	20
Staphylococcus aureus	8	10	15
Klebsiella pneumoniae	10	10	10
Candida albicans	15	20	20

Table 3: Schematic representations for cytotoxicity of *Aristolochia bracteolata* on Vero cell line

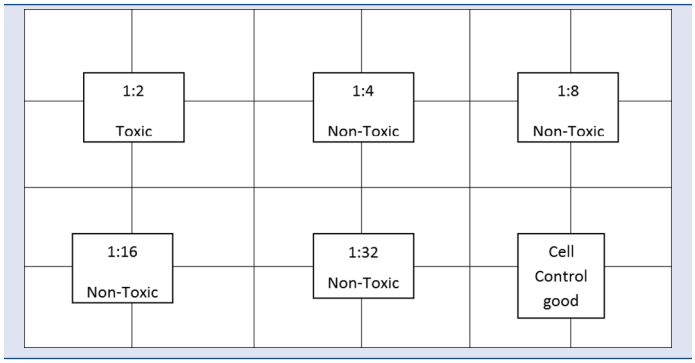
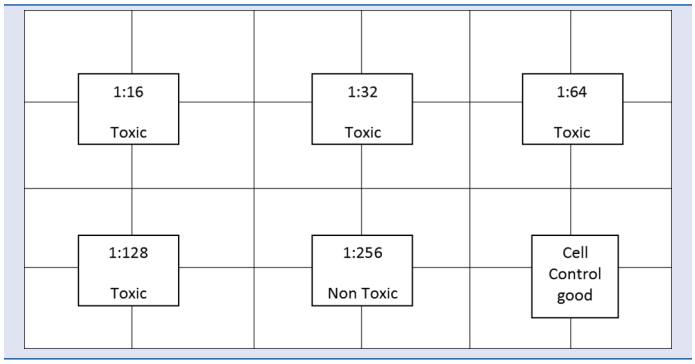


Table 4: Schematic representations for cytotoxicity of synthesized silver nanoparticle on A549 cell line



DISCUSSION

In previous studies, green synthesis of silver nanoparticles has been carried out using *A. indica* extract. The synthesized nanoparticle was found to be effective against *Anopheles* vectors.[30] Silver nanoparticles synthesized from the leaf extract of *A. indica* exhibited good disinfectant and antioxidant activity.[31] Biologically synthesized nanoparticles have always shown enhanced bioactivities when compared to chemically synthesized nanoparticles and are always cost-effective. Due to the

slow kinetics depicted by green synthesized nanoparticles, they hold a better control over the crystal growth.[32] In the current study, anticancer activity was exhibited by the nanoparticles synthesized from *A. bracteolata* extracts. The green synthesized nanoparticles provide a safer method to treat laryngeal carcinoma. Further studies can reveal the true potential and broad application of the green synthesized nanoparticles.

CONCLUSION

The study carried out on the drug *A. bracteolata* Lam. extract has confirmed its antimicrobial and anticancer activity and holds significance in environmental sciences. A suitable drug dose level for antimicrobial and proper dilution for anticancer activity exists between the cytotoxic activities of the drug on normal and cancerous cells, which can be used for designing the drug. The drug is a formulation and not just a single compound; it can induce cell death in several pathways. The lower concentration reveals the cytotoxic effect on cancer cells, but the higher concentration may induce cancer cell growth. Further study is required to confirm the drug action on the microbial and cancer cells and to identify the role of extract compound on the cancer cell proliferation and also identify the compounds present in the extract which inhibit the action against the cell proliferation or cytotoxicity in a certain concentration.

Data availability

The data used to support the findings of this study are included within the article such as cell line A549, HPLC, IR, cytotoxicity of *A. bracteolata* on Vero cell line, antimicrobial activity of synthesized silver nanoparticle from A*ristolochia bracteolate* extract, and so forth.

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Conflicts of interest

There are no conflicts of interest.

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