IJPSR (2024), Volume 15, Issue 10 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 26 April 2024; received in revised form, 26 May 2024; accepted, 09 July 2024; published 01 October 2024

SYNTHESIS OF GOLD NANOPARTICLES USING LEAF AQUEOUS EXTRACT OF *PHYLLANTHUS VIRGATUS* (PHYLLANTHACEAE), ANTIMICROBIAL AND CYTOTOXIC ACTIVITY AGAINST A549 CELL LINE

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Keywords:

Gold Nanoparticles, Cell line, *Phyllanthus virgatus*, cytotoxicity

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ABSTRACT: The local tribes have used *Phyllanthus virgatus* for Malnutrition, Contagious infections, Inflammations (Gond Tribe), Hepatitis, Cold, Stomach-ache, Gonorrhoea, Fever, Headache, and septic ailments (Chenchus and Yanadis), etc. The gold nanoparticles have a highly specific surface area, and their unique physiochemical characteristics include catalytic activities, optimal properties, antimicrobial activity, etc. Aqueous leaf extract of *Phyllanthus virgatus* is used to synthesize gold Nanoparticles. The confirmation of biosynthesis of gold nanoparticles was characterized using UV Spectrophotometer, with the maximum scale observed at 551nm. SEM and EDX characterized the morphology of Gold Nanoparticles used to detect the presence of the element gold. The synthesized Nanoparticles were observed to be roughly spherical with a range of 2-50 µm. The dynamic light technique measured the hydrodynamic diameter (28.7nm) and a Zeta potential (-18.6mV). The FTIR analogue shows that the functional group assigned as=C-H, C-C, O-H, –C=C- and –C=O capping agents. The synthesized nanoparticles have shown strong inhibitory action against pathogenic microbial Strains using the Disc diffusion method. *Bacillus cereus* exhibited more significant results than other microorganisms. The cytotoxicity of gold nanoparticles was studied using an MTT assay against a human lung cancer cell line (A549) at different concentrations at 24 hrs. Anticancer activity of crude aqueous leaf extract and green synthesized nanoparticle extracts exhibited different responses against the A549 cell line. *P. virgatus* aqueous leaf extract and the green synthesized nanoparticles exhibited cytotoxic potential properties with the IC_{50} concentrations at 497.55 (µG/mL) and 189.97(µG/mL), respectively. The results revealed that gold nanoparticles could be an efficient therapeutic agent for lung cancer treatment.

INTRODUCTION: The Genus *Phyllanthus* is well known to possess medicinally active compounds and has been used as traditional antitumor remedies throughout the world [1] *Phyllanthus* species are used in Ayurvedic medicine to treat liver and kidney diseases.

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DOI: 10.13040/IJPSR.0975-8232.15(10).3026-34

This article can be accessed online on www.ijpsr.com

DOI link: https://doi.org/10.13040/IJPSR.0975-8232.15(10).3026-34

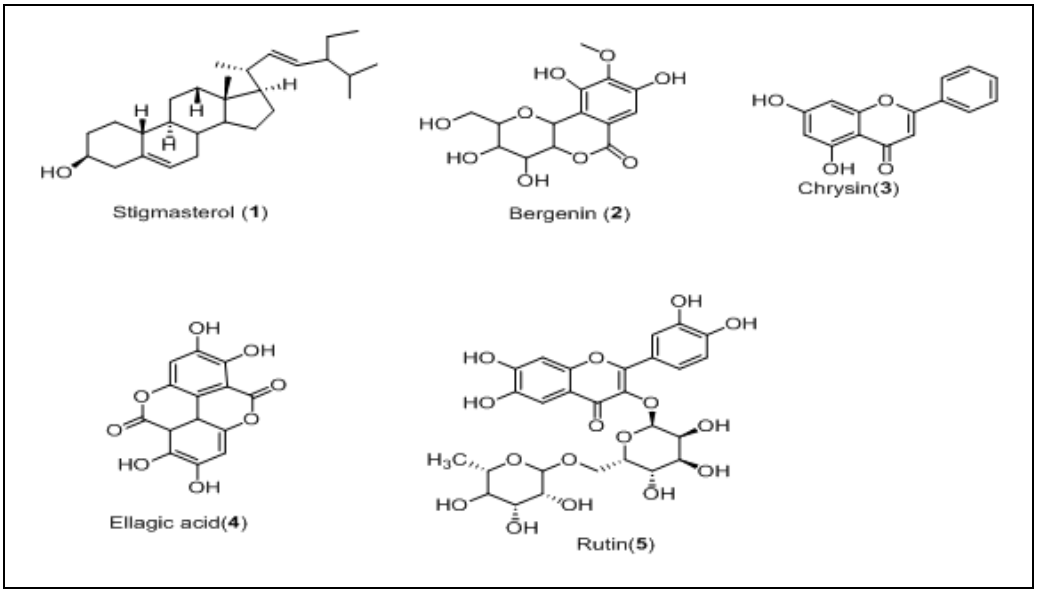
Although some herbaceous *Phyllanthus* species resemble similar ethnomedical uses, Pharmacology activities, and chemical composition are quite different.

The secondary metabolites like alkaloids, coumarins, lignans, and terpenes contribute to antioxidant, anticancer, hepatoprotective, antidiuretic, and anti-inflammatory antiviral activities [2]. *P. virgatus* is an antiseptic and anti-inflammatory agent used by the Gond tribe, inhabited in the Eastern Ghats of India [3]. *P. virgatus* extracts have diversified phenol

compounds and exhibits high antioxidant and anti-inflammatory properties [4]. *Phyllanthus simplex* Retz. synonym for *Phyllanthus virgatus* G.Forst.[5] Synthesized ZnoNs with plant extract of *Phyllanthus emblica* exhibits potential antimicrobial activity [6]. Synthesized and characterized Ag-Cu-Co nanoparticles with the Phyllanthus niruri extract stabilizes and capping agent for larvicidal, anti-bacterial, antioxidant and photodegradation activities[7]. Fruit extract of AgNPs of *P. emblica* acts as a biogenic reducing agent antioxidant and antimicrobial activity [8]. Leaf extract with AgNPs of *P. emblica* shows antioxidant, anti-leishmania, and anti-inflammatory activity [9].

Among the metal nanoparticles, Au, Ag and Fe NPs have been widely used in medical applications. AuNPs are used in drug delivery, bioimaging and photothermal therapy [10]. AgNps and AuNps are synthesized from different plant parts of families like Phyllanthaceae, Lamiaceae, Rutaceae, and Euphobiaceae, which are wide range of therapeutic uses[11]. AuNPs have garnered the most attention among the numerous metallic nanoparticles because of the exceptional surface plasma resonance, coupled with a variety of molecules, include proteins, enzymes, antibodies etc.[12]. AuNPs can also be synthesized using a variety of microbes and plants [13]. Gold Nanoparticles has been playing vital role in biomedicine for convenient surfaces with biomolecular probes and remarkable plasma resonant optical properties [14, 15]. Gold Nanoparticles have a significant function in the delivery of proteins, nucleic acids, *in-vivo* delivery, gene therapy, targeting, etc, [16]. Neither size nor shape of the AuNPs was determined to induce cytotoxicity in the human lung cancer cell line A549. Intracellular modification of Nanoparticles is determined by the cellular environment [17]. *Phyllanthus* species were used to synthesize AgNPs, and AuNPs showed antimicrobial and antioxidant activities. The predominant secondary metabolites like stigmasterol, bergenin, chrysin, ellagitanins, rutin, galanin-8-sulfate, kaempferol-8-sulfonate, (structures shown below) etc., recorded in *Phyllanthus virgatus* [18, 19, 20. 21].

Phyllanthus sps. found to contain alkaloid securinine and euginole (terpenoids) as a principal role in the bioreduction of $AgNo_3$ to $HAuCl_4$. The hydrometholic extract of *P. virgatus* exerted greater antioxidant and cytotoxic effects on human hepatoma HepG2 cells than *P. amarus* extract [22]. Green synthesis of gold nanoparticles using diverse plant extracts has been reported [23, 24, 25] *P. virgatus* showed a stronger cytotoxic effect than *P. amarus* [26]. The anisotropic gold and spherical silver nanoparticles were synthesized by reducing aqueous choloroauric acid and silver nitrate solution with the extract of Phyllanthin at room temperature. The reduction rate of $HAuCl_4$ is greater than the $AgNo_3$ at the same amount of phyllanthin extract [27]. The present study focused on green synthesis of gold Nanoparticles using P. virgatus leaf extract and its antimicrobial activity. The human lung cancer cell line (A549) was assessed with different concentrations.



MATERIAL METHODS:

Collection of Plant Material: The plant was collected from the fields of Kalasamudram, Anantapuramu district, Andhra Pradesh, India. The *Phyllanthus virgatus* was identified, and a voucher specimen (No.50202) was housed at Sri Krishnadevaraya University herbarium SKU. *P. virgatus* leaves were washed with tap water, followed by distilled water to remove dust and other contaminants, and then they were allowed to shade dry at room temperature for a day.

Preparation of Leaf Extract: 10 g of dried leaves were weighed, and 100 ml of double distilled water was added and boiled for 30 min at 60˚C. After cooling, the extract was filtered using Whatman no.1 filter paper and stored at 4°C for further use.

Preparation of 1mM HAucl4 Solution: An accurate concentration of 1mM of Gold Nanoparticles prepared by dissolving 0.03639 g $HAucl_4$ in 100 ml of double distilled water and stored in amber colour bottle to prevent auto-oxidation of Gold particles

Green Synthesis of *Phyllanthus virgatus* Leaf Nanoparticles: 2ml of *P. virgatus* leaf extract was added to 200 ml of Aucl4 (1mM) Solution and exposed to the sunlight for half an hour. Leaf extract acted as reducing agent and the stabilised solution leading to the formation of nanoparticles.

Analysis of Bioreduced Gold Nanoparticles:

UV-Vis Spectroscopy: Synthesis of Gold nanoparticles was confirmed through a UV-Visible Spectrophotometer. The absorption was scanned at 470-700nm.

SEM analysis of Gold Nanoparticles: SEM images investigated the Morphology and size of the Gold Nanoparticles. Thin films of the sample were prepared on a carbon-coated copper grid by just dropping a very small amount of the sample on the grid.

EDAX: The presence of elemental gold was determined, and the samples were dried at room temperature. Then, the sample composition of the synthesized NPs was analyzed.

Particle size: Particle Size analysis of NPs were carried out on HORIBA SZ-100 Laser light Scattering analyzer with the following measurement parameters: Refractive index fluid – 1.330, Angle – 15.00, average count rate- 5.2 kcps with run completed 3 times.

Zeta Potential: The Zeta potential of gold NPs was carried out on HORIBA SZ-100 Laser light scattering analyzer with the following measurement parameters, Conductivity -0.279 ms/cm, Dispersive medium viscosity-0.892mPa.s, electrode voltage-3.3 Vat 25.2˚C measurement was completed thrice. The magnitude of the zeta potential is predictive of the colloidal stability. Nanoparticles with Zeta Potential values greater than +25 mV or less than -25 mV typically have high degrees of stability. In the case of a combined electrostatic and steric stabilization, a minimum zeta potential of ± 20 mV is desirable [28].

Fourier Transform Infrared (FTIR) Studies: Fourier transform infrared spectroscopy (FTIR) measurements were performed using Bomem MB-3000 (make: Canada) spectrophotometer equipped with KBr disc method. Each sample was finely grounded with KBr to prepare the pellets under a hydraulic pressure of 400 kg and spectra were scanned between 4000to 400 cm^{-1}.

Antimicrobial Activity: *In-vitro* activity of synthesized Nanoparticles was determined using disc diffusion method [29]. The test microorganisms were obtained from the Microbial type culture collection center, Institute of Microbial Technology (IMTECH), Chandigarh, India. Bacterial and Fungal strains. Approximately 20 ml of molten and cooled media was poured in sterilized Petridishes. The plates were left over of 30 min. 30-50 µl of the stock solutions were applied to each sterilized filter paper discs of 5mm. Discs were dried and preserved for antimicrobial study. The plates containing the test organism and AuNPs were incubated at 37ºC for 24-30 hrs. The plates were examined to measure the zone of inhibition at different concentrations (20µl, 30µl and 40µl).

Anticancer Studies: Nanoparticles and crude form of drug obtained from leaf extract were added separately into 200 µl cell suspension (24 hours incubated cells) containing 96 well plates at required cell density (20,000 cells per well), by different concentrations. The culture plates were

incubated for 24 hrs at 37°C in a 5% CO_2 atmosphere. After incubation period, spent media was removed, and MTT reagent with a final concentration of 0.5mg/ml of total volume was wrapped on the plate with aluminum foil to avoid exposure to light. The plates were incubated for 3 hours, and then MTT reagent was replaced by 100 µl solubilizing solution (DSMO) for dissolution purposes in a gyratory shaker.

MTT to insoluble Formosan crystals, which upon dissolution into an appropriate solvent exhibits purple colour, the intensity of color proportional to the number of viable cells and measured with spectrophotometrically or an ELISA reader at 570nm and 630nm wavelength. The IC_{50} value was determined using a linear regression equation i.e., Y =Mx+C Here, Y = 50, M and C values were derived from the viability graph.

RESULTS AND DISCUSSION:

UV-Vis Spectroscopy: Synthesized Gold nanoparticles were confirmed through a UV-visible spectrophotometer. The absorption was recorded at 551nm. Gold loses its yellow color when dispersed in the form of gold nanoparticles in solution and adopts a ruby-red color. The intensity of the peak steadily increases with the density of the Nanoparticles with respect to time. **Fig. 1** & **Fig. 2.**

FIG. 1: (A) *PHYLLANTHUS VIRGATUS* LEAF EXTRACT (B) 1MM AQUEOUS HAUCL$_4$ AND (C) AUNPS FIGURES

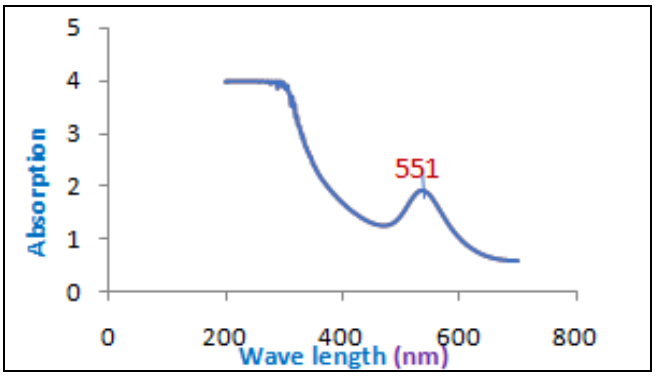


FIG. 2: UV-SPECTRUM

SEM and EDAX: The present experimental investigation reports the green synthesis of nanoparticles using *P. virgatus* leaf aqueous extract. The formation of ruby-red colour conforms to the reduction of Auto AuNps. The synthesized Nanoparticles were observed to be roughly spherical with a range of 2-50µm.

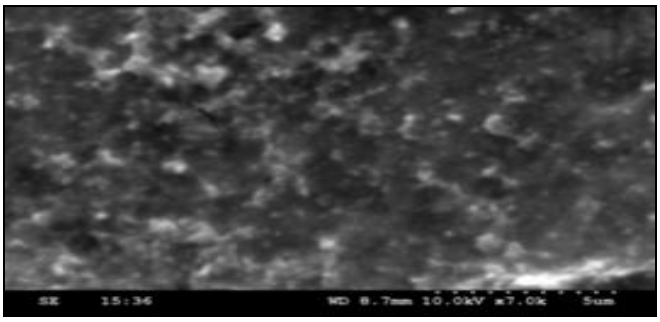


FIG. 3: SEM

Fig. 3 The surface peaks were observed for carbon, oxygen and chlorine, suggesting the presence of biomolecules at the surface of AuNps or its proximity. The significant peak at 2 Kev is characteristic for the element Gold **Fig. 4.**

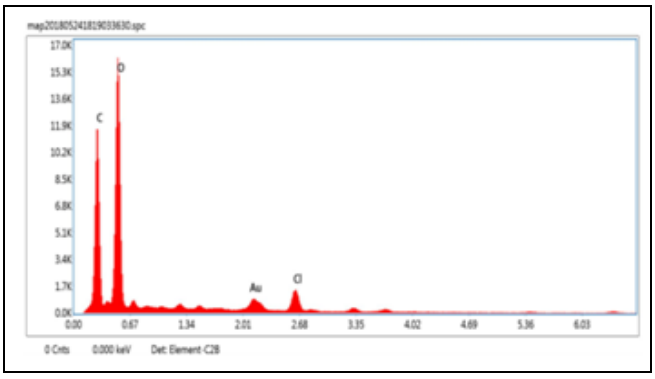


FIG. 4: EDAX SPECTRA OF NANOPARTICLES SYNTHESISED FROM *PHYLLANTHUS VIRGATUS*

Particle Size and Zeta Potential Analyser: Laser diffraction particle size analyzer provides the details about the particle's nature, such as monodispersed, dispersed, and polydispersed.

The results revealed that nanoparticles show monodisperse at 0.146 indexing and various sizes of Nanoparticles ranging with an effective diameter around 28.7 nm, and the Average zeta potential was -18.6Mv **Fig. 5 & Fig. 6.**

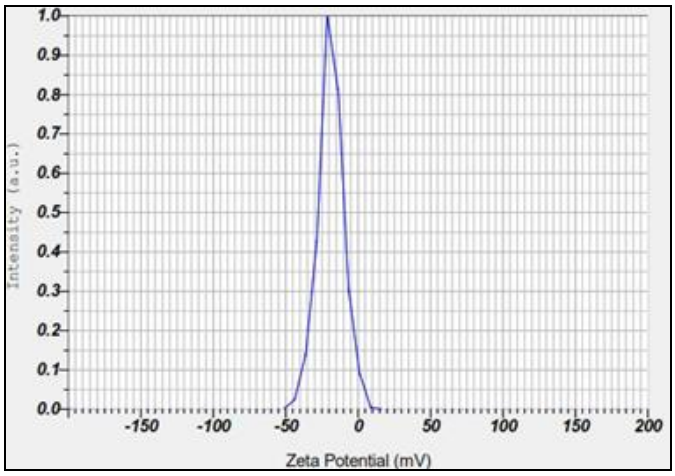


FIG. 5: ZETA POTENTIAL

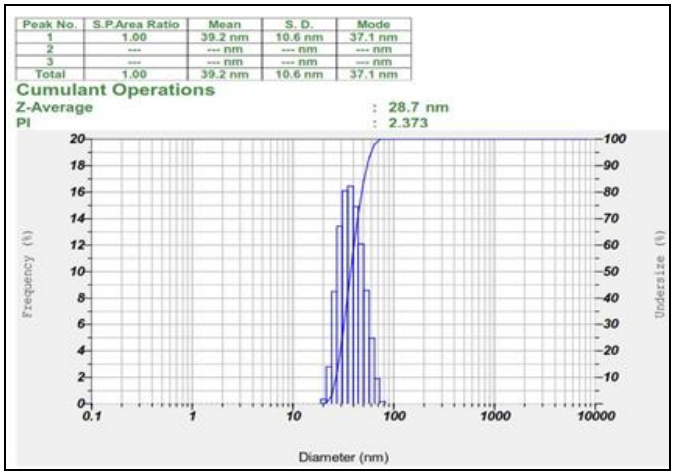


FIG. 6: PARTICLE SIZE

Fourier Transforms Infrared Spectroscopy: The FTIR spectrum of gold NPs, as shown in **Fig. 3**, reveals that the gold NPs show a broad peak at 3421 cm-1 is due to the presence of O-H stretching frequency, the peak at 1595 cm-1 indicates the presence of C=O stretching frequency, the peak 1387 cm-1 indicates the presence of C=C stretching frequency and the peak 1127 cm-1 corresponds C-O stretching frequency, the peaks at 1068 cm-1 indicates C-C stretching, the peak at cm-1indicates the presence of =C-H bending frequency. The stretching frequencies indicate that amino acid residues and protein peptides can effectively bind to the surface of metals, thereby functioning as a coating agent on the surface of the nanoparticles (preventing agglomeration) and serving as a reducing agent. The results suggest that the biological molecules can synthesize AuNPs in an aqueous medium **Fig. 7.**

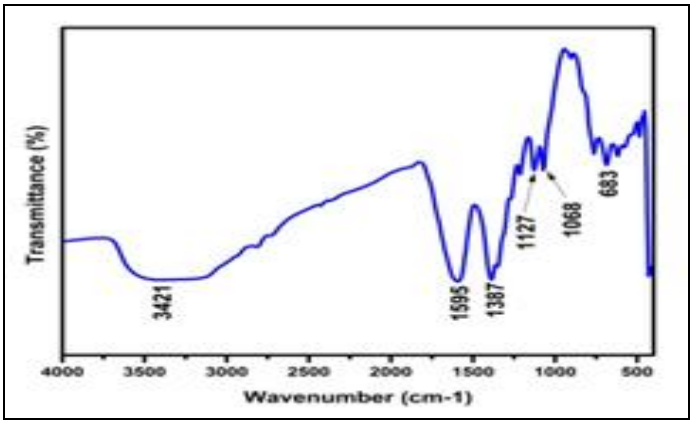


FIG. 7: FTIR SPECTRA OF NANOPARTICLES SYNTHESISED FROM *PHYLLANTHUS VIRGATUS*

Antimicrobial Activity Study: *In-vitro* activity of gold nanoparticles was tested against *Staphylococcus aureus* (7443), *Salmonella enteric* (98), *Pseudomonas aeruginosa* (7296), *Bacillus subtilis* (1133) *Klebsiella pneumonie* (7028), *Candida albicans* (854), *Bacillus cereus* (1272), *Micrococcus liteus* (2470), *Salmonella typhe* (3224), *Fusarium oxysporum and Escherichia coli* (1668) by disc diffusion method and the results were tabulated. The gold nanoparticles exhibit significant antimicrobial activity against all tested microorganisms. The maximum zone of inhibition was found against Bacillus cereus, moderate in Salmonella enterica, and no effect on fungal strains (*Fusarium oxysporum* and *Candida albicans*). Comparing with *P. virgatus* aqueous extract, synthesized Gold Nanoparticles have shown better results against test microorganisms. Zone of Inhibition was increased with increasing the concentration of AuNps but in *Salmonella enterica* at 20 and 30 µg/ml concentration. Only *Bacillus subtilis* didn't show any results on Plant extract, but P. virgatus Nps showed significant results in increasing the concentration of NPs **Fig. 8** & **Table 1.**

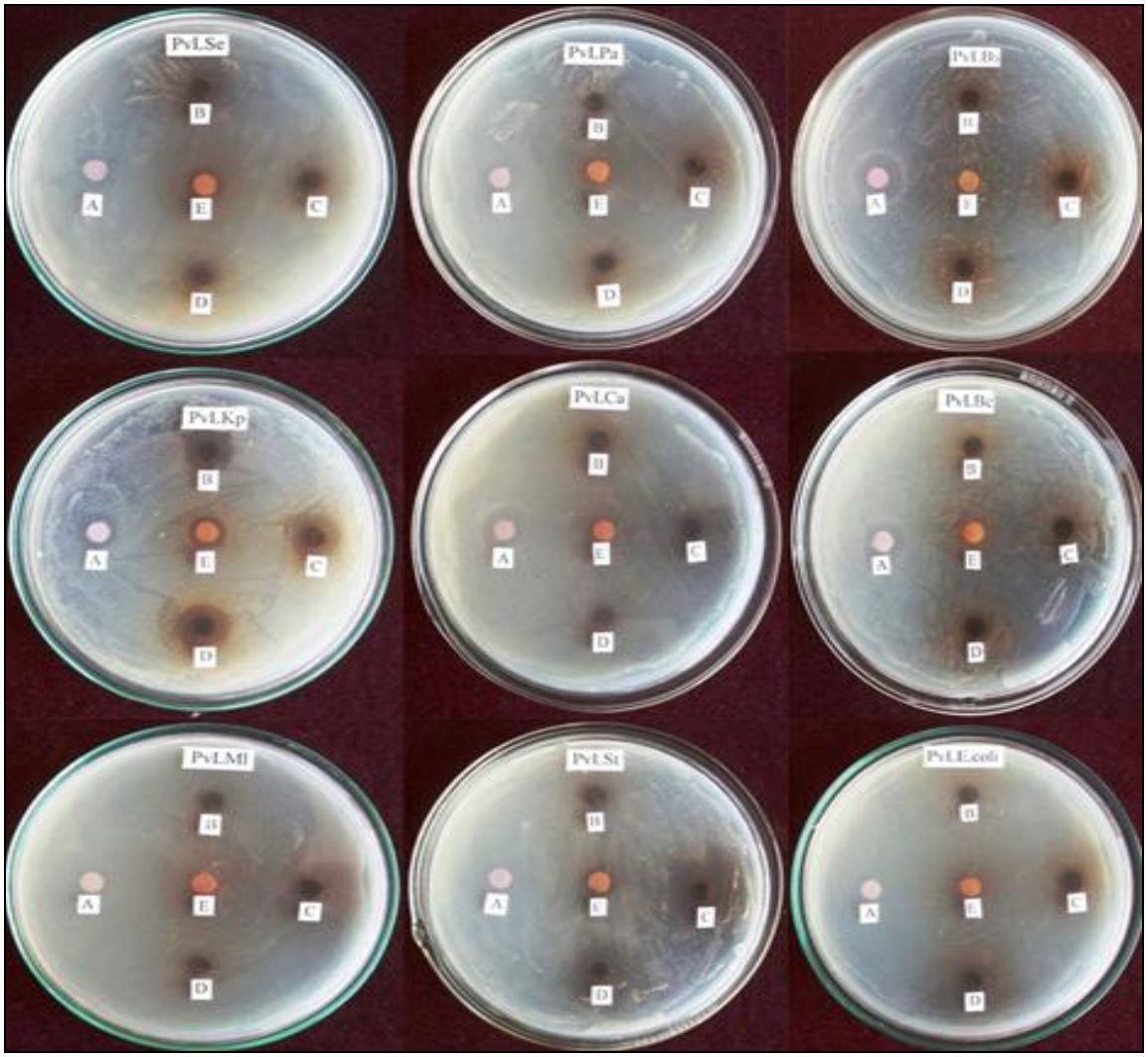


FIG. 8: ZONES OF INHIBITION OF (A) HAUCL4 (B) *PHYLLANTHUS VIRGATUS* AQUEOUS EXTRACT (C) 20 µL OF AUNPS (D) 30 µL AUNPS (E) 40 µL AUNPS

TABLE 1: ZONES OF INHIBITION OF (A) HAUCL4 (B) *PHYLLANTHUS VIRGATUS* AQUEOUS EXTRACT (C) 20 µL OF AUNPS D) 30 µL AUNPSE) 40 µLAUNPS

S. no.	Name of the Bacteria	Zone of Inhibition (mm)					
		Type	$Aucl_4$ (20µl)	*P. virgatus* aqueous leaf extract (20µl)	AuNPs		
					20µl	30 µl	40 µl
1	*Staphylococcus aureus*	+Ve	7	-	7	8	12
2	*Salmonella enterica*	-Ve	11	12	15	15	12
3	*Pseudomonas aeruginosa*	-Ve	10	-	-	-	-
4	*Bacillus subtilis*	+Ve	11	-	7	7	8
5	*Klebsiella pneumoniae*	-Ve	8	9	12	13	14
6	*Escherichia coli*	-ve	11	-	-	-	-
7	*Bacillus cereus*	+Ve	15	15	16	20	20
8	*Micrococcus luteus*	+Ve	9	-	-	-	-
9	*Salmonella typhe*	-Ve	7	9	9	9	12
10	*Fusarium oxysporum*	F	11	-	-	-	-
11	*Candida albicans*	F	8	-	-	-	-

Percentage of Cell Viability: Different concentrations of aqueous leaf extracts of *P. virgatus* were subjected to determine the percentage of cell viability study using A549 at 48 hrs. All were found to be concentration dependent, while a decreased in cell viability was observed in the treatment of aqueous leaf extract of *P. virgatus* **Fig. 9 Table 2 & Graph 1.**

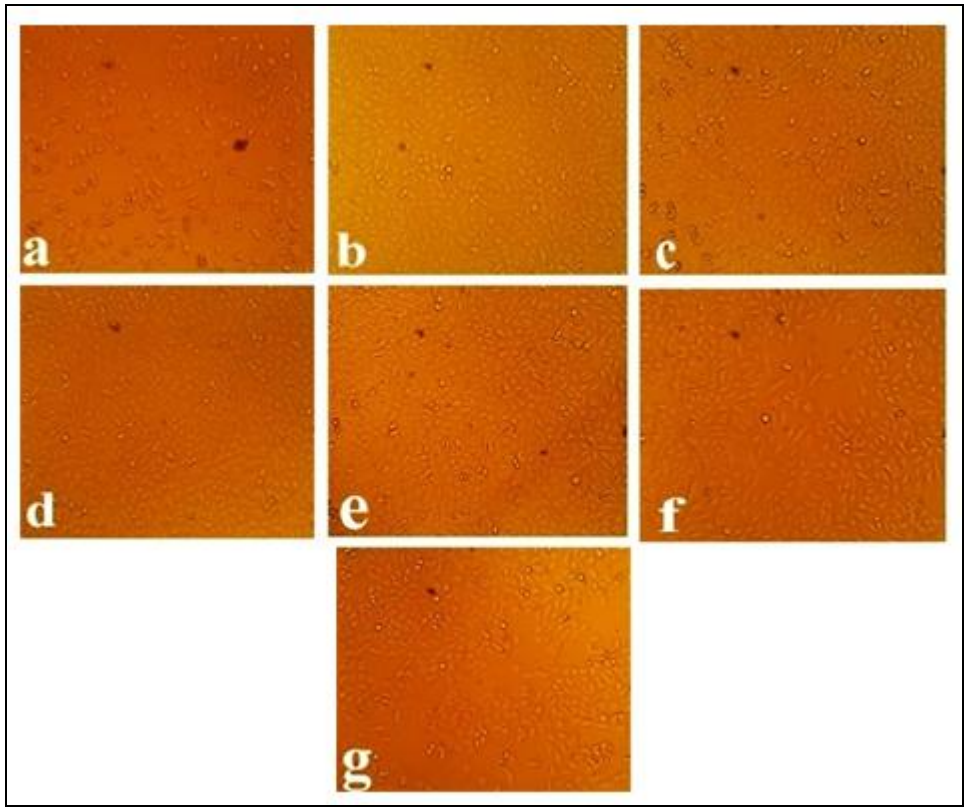
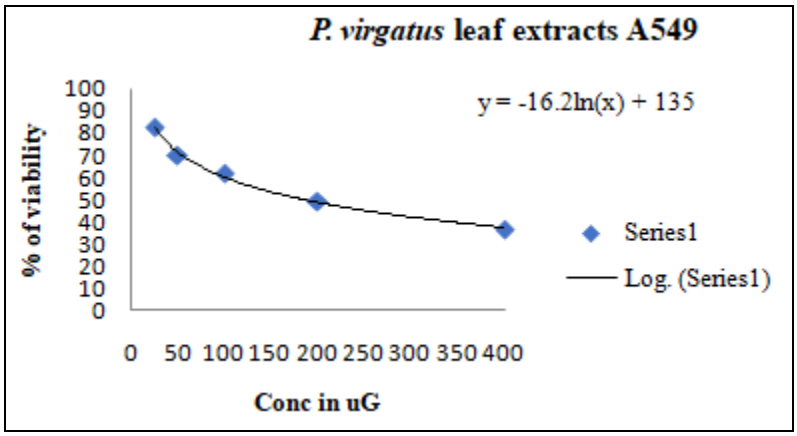


FIG. 9: PHOTOGRAPHS OF A549 CELLS AT 48 HRS (A) STANDARD (B) CONTROL (C) 25µG/ML (D) 50µG/ML (E) 100 µG/ML (F) 200µG/ML AND (G) 400µG/ML

TABLE 2 & GRAPH 1: ABSORBANCE READINGS AT 570NM IN ELISA PLATE READER OF THE *P. VIRGATUS* LEAF EXTRACT AGAINST THE A549 CELL LINE

Concentration (uG)	Abs Reading 1	Abs Reading 2	Mean Abs	Mean Abs (Sample-Blank)	% Viability
Cell Control	1.422	1.451	1.4365	1.4015	100
Std Control	0.728	0.722	0.725	0.69	49.23296468
25	1.426	1.423	1.4245	1.3895	99.14377453
50	1.405	1.406	1.4055	1.3705	97.7880842
100	1.363	1.364	1.3635	1.3285	94.79129504
200	1.164	1.151	1.1575	1.1225	80.09275776
400	0.883	0.838	0.8605	0.8255	58.90117731

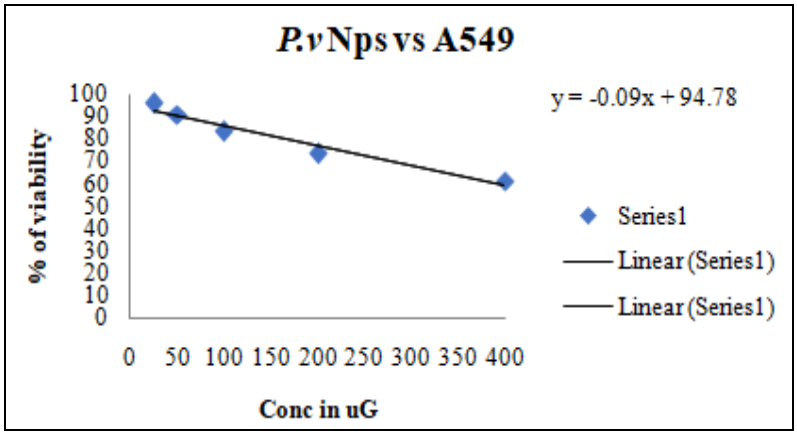


Cytotoxicity Study of the *P. virgatus aqueous* Leaf Extracts and its Nanoparticles against A549 Cell Line: The observations were statistically analyzed, which revealed that data of cell cytotoxicity study by ELISA reader against A549 cells, *P. virgatus* aqueous leaf extract and its Nanoparticles showing cytotoxic potential properties with the IC_{50} Concentrations at 497.55 (µG/mL) and 189.97 (µG/mL) compared to the Standard Drug, Cisplatin with IC_{50} concentration at

15µM used for the study. The results strongly reveal that the Nanoparticles synthesized with the leaves of *P. virgatus* have possible therapeutic potential against human lung cancer cell lines (A549) based on the dosage of the drug after the incubation period of 24 hours **Fig. 10 Table 3 & Graph 2.**

TABLE 3 &GRAPH 2: ABSORBANCE READINGS AT 570NM IN ELISA PLATE READER OF THE *PV* NPS AGAINST THE A549 CELL LINE

Concentration (uG)	Abs Reading 1	Abs Reading 2	Mean Abs	Mean Abs (Sample-Blank)	% Viability
Blank	0.02	0.05	0.035	0	0
Cell Control	1.88	1.874	1.877	1.842	100
Std Control	0.952	0.963	0.9575	0.9225	50.08143322
25	1.797	1.798	1.7975	1.7625	95.68403909
50	1.712	1.714	1.713	1.678	91.09663409
100	1.571	1.565	1.568	1.533	83.2247557
200	1.388	1.381	1.3845	1.3495	73.26275787
400	1.145	1.167	1.156	1.121	60.8577633



Sl. no.	Sample	IC 50
1	*P. virgatus* leaf aqueous extract	497.55µG/mL
2	Nanoparticles	189.97 µG/mL

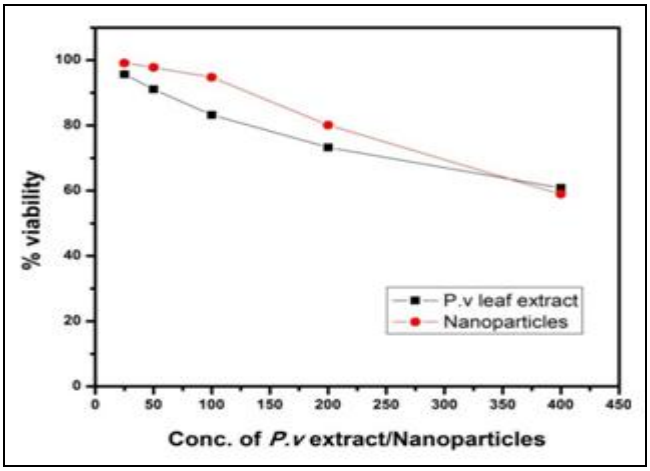


FIG. 10: CYTOTOXICITY STUDY OF THE *P. VIRGATUS AQUEOUS* LEAF EXTRACTS AND ITS NANOPARTICLES AGAINST A549 CELL LINE

CONCLUSION: *Phyllanthus virgatus* leaf extract was found suitable for green synthesis of AuNPs. The reduction of gold ions by the leaf extract forms stable Nanoparticles with spherical and ranging from 2-50µm. The concentrations of leaf extract and metal ions play an important role in the green synthesis of AuNPs. The spectroscopic characterization using UV-Vis, SEM, EDX and Particle size analyzer was useful in proving their minute characteristics like size, shape and, composition, etc. FTIR evidenced the formation and stability of the bio-synthesized AuNPs which can be studied further to understand the chemical and molecular interactions which could be responsible for Nanoparticles synthesis. The presence of Phenol compounds acts as a reducing and capping agent for the preparation of NPs. AuNps cannot affect fungal strains, but they promise inhibition against test bacterial strains. Prepared nanoparticles could be used to make medicinal devices and to treat human lung cancer.

ACKNOWLEDGEMENT: The authors are grateful to the Department of Biotechnology, New Delhi, for financial assistance and thankful to the

Andhra Pradesh Forestry for permission and help during field exploration trips.

CONFLICTS OF INTEREST: The authors declare that there is no conflict of Interest

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How to cite this article:
Akkulanna S, Nagendra C, Srinath A and Reddy AM: Synthesis of gold nanoparticles using leaf aqueous extract of *Phyllanthus virgatus* (Phyllanthaceae), antimicrobial and cytotoxic activity against a549 cell line. Int J Pharm Sci & Res 2024; 15(10): 3026-34. doi: 10.13040/IJPSR.0975-8232.15(10).3026-34.