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Anti-Tumor Activity of Eco-Friendly AgNPs Derived from Musa Paradisiaca Pseudo Stem Methanolic Extracts and their Antibacterial and Antioxidant Activities in Ovarian Cancer Cell Line-PA1

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Abstract: ***Musa Paradisiaca, commonly known as Banana, is a gigantic herb. Its main upright stem is called as Pseudostem. Banana plant have lots of medicinal uses.This piece of work describes the anti-cancerous activity of methanolic extracts made from pseudo stem of Musa paradisiaca. Anti-tumour activity of biogenic AgNPs has not been digged in the field of Ovarian cancer. The synthesized silver nanoparticles were identified by the formation of light-yellow colour solution and U.V-Visible spectrophotometer analysis which showed maximum absorbance at 423nm. The presence of ketones, methyl groups, nitrosamines and aromatic rings as functional groups in AgNPS was identified using FTIR. The antibacterial studies were performed by Agar Diffusion method against different strains of bacteria. The AgNPs showed antioxidant activity through DPPH assay. The antiproliferative activity of AgNPs was demonstrated against ovarian cancer cell line Pa 1 with MTT assay and confirmed using PI staining. In the toxicity study, a significant mortality rate was observed with an IC50 concentration of 250 µg, so they are cytotoxic at high concentrations of AgNPs.***

Keywords: Biosynthesis; Silver nanoparticles; Characterization; Antibacterial; Antioxidant; Anticancer; Flowcytometry.

I. INTRODUCTION

Cancer is not a disease in general, yet group of diseases in which cells lost their ability to control proliferation and divide uncontrollably. Cancer can spread either locally or through bloodstream and lymphatic system to other parts of body. Uncontrolled and Abnormal proliferation of cells result in tumour formation. Concisely, Tumour may be of two types; Benign and Malignant. In most scenarios, benign tumours are not considered as cancerous, whereas malignant ones are more prominent in sloppy division. As in[1]. Both benign and malignant tumor are classified according to the type of tissue from which they originate. Carcinoma (cancer related to epithelial tissue), Sarcoma (cancer of mesodermal connective tissue), Leukaemia (cancer arising from blood forming tissue) [2], Lymphoma (cancer that affect the lymphatic tissue) [2], Myeloma (cancer that begins in bone marrow), Blastoma (cancer that begins in embryonic tissue). Highest percentage of cancer risk factors (90-95%) are caused due to the lifestyle and environmental concerns which in turn createthe genetic mutations. Slightest risks are obtained through inherited diseases [3]. Environmental factor contributors include radiation (10%), obesity and diet (30-35%) and smoking (25-30%) [3],[4]. Three main classes of carcinogenic agents are known: Radiations like UV radiation and gamma ray, Chemicals like benzopyrene and benzene, Biological like oncovirus. Tobacco smoking and chewing is associated with many forms of cancer such as lung, mouth, larynx, kidney .90% of lung cancer is caused due to tobacco smoking [5]. Tobacco smoke contains many carcinogens like nitrosamines and polycyclic aromatic compounds [6]. Prolonged expression to UV radiation results in melanoma and other skin cancer. Viruses are also responsible for cancer worldwide.

Ovarian cancer is a cancer which develops in ovary and produce abnormal cells which intrude to other parts of the body. Ovarian cancer is the 7th most gynaecological cancers in woman worldwide. The number of estimated new cases are around 239,000 and estimated deaths all over the world are 152,000 annually. 1 out of 75 women is at the risk of developing ovarian cancer and 1 out of 100 women has the chance of dying of the disease [7]. Genetic factors are in the verge of causing the disease is around 10% including the germ line mutations in genes BRCA1 and BRCA2**.** The risk of ovarian cancer increases by age. Women who take the diet containing red meat are at high risk [8].

Bloating, Pelvic or abdominal pain, Difficulty in eating or indigestion, Urination more frequently, Fatigue, Constipation, Pain during intercourse, Changes in menstrual cycle [9]. Lower energy X-rays are used for treatment of skin cancer while invasive cancers are generally diagnosed by high energy X-rays [10]. Immunotherapy treatment is used to stimulate the body immune system against cancer. Chemotherapy is considered as the primary treatment regimen which has a high efficacy in destroying the tumour cells. Several chemotherapeutic drugs are synthesized using both the natural products and the synthetic sources, yet these sources are broadly classified into alkylating agents, antimetabolites and platinum drugs [11]. Caspase 3 is effector caspase which cause apoptosis of cells [12]. The banana plant is a gigantic herb with juicy stem that grows from an underground stem or rhizome. The banana plant attains a height of 6-7.5 metres (20-25ft)[13]. These plants are tall and their main upright stem is actually a pseudo stem (false stem). Banana pseudo stem is a rich natural resource in subtropical and tropical regions. It has potential for providing products like manureand feed. Banana pseudo stem is also the abundant source for fibres which have been used for decades as raw materials for textiles in production of clothes.Banana pseudo stem has potential value for pulping. Banana pseudo stem is rich in potassium and vitamin B which helps in the production of insulin and haemoglobin. Its inner part is edible and it keeps high blood pressure under control[14]. Banana stem is also beneficial during kidney stones treatment. The banana stem is also beneficial in curing stomach upset and diabetes as it promotes insulin production. The banana flower is useful in curing diarrhoea and dysentery [15]. The *Musa paradisiaca* flower is also known for its anti-cancerousproperties [16].

Nanotechnology is the rapidly growing field with its applications in the synthesis of nanoparticle of noble metals like gold, silver and their characterizations. Biological method is eco-friendly and also cost effective, plants are most widely used for green synthesis of nanoparticles. Silver nanoparticles has many diverse properties like catalysis, electrical conductivity, antimicrobial activity, antioxidant activity andsurface enhancedRaman scattering (SERS)[17]. Nanoparticles can be used for target specific delivery of drug and are expected to revolutionized cancer diagnosis and therapy.

II. MATERIALS AND METHODS:

A. *Materials Utilised*

Pseudo stem, distilled water, conical flask, beaker, measuring cylinder, Petri plates, test tubes, glass rods, methanol, silver nitrate, Levofloxacin antibiotics strips, DPPH, MTT dye, PA 1 cell lines, Rotary evaporator, Soxhlet apparatus, Spectrophotometer, Particle size analyser, MTT assay kit, Flow cytometer, Co2 incubator, Laminar air flow chamber, Propidium iodide, Cell culture dishes, 96 well plate, 6 well plate, Nutrient agar media, Petri plates, RPMI and DMEM media and other lab equipment.

B. *Collection of Sample and Extract Preparation*

The pseudo stem of *Musa paradisiaca* was obtained from the local market, Tirupati, Andhra Pradesh. Stem was thoroughly washed with flowing tap water and thenusing distilled water and chopped into tiny pieces and shade dried for 2 weeks. The shade dried material was grinded into coarse powder using a grinding machine and stored in air tight Zip lock cover. 13.3 grams of powder was used for performing series of extractions in Soxhlet apparatus using 500 ml of methanol for 7 cycles. These extracted samples were further processed using flash evaporation. The methanolic extract were taken at a concentration of 10mg per ml, thus total of 100mg per 10ml by adding distilled water.

C. *Phytochemical Analysis*

1) *Test for Alkaloids:* 5 ml of synthesized sample were taken in a testube and few drops of Wagner's reagent was added (Wagner's test).
2) *Test for Terpenoids:* Extracts were treated with chloroform and filtered. The fine product was treated with few drops of conc. solution of sulphuric acid (Salkowski test)
3) *Test for Flavonoids:* 1 ml of methanolic extract was added to 1 ml of 10% lead acetate solution (lead acetate test)
4) *Test for Tannins:* Boiled extract of 0.5 gm in 10 ml of distilled water was done and filtered, 5ml filtrate was taken in testube and 5 drops of 0.1% ferric chloride was added.
5) *Test for Saponins:* In a test-tube, 0.5 gm of extract was shaken with 10 ml distilled water, froth formation occurrence was observed when kept in water bath for 5 min which confirms the presence of saponins.
6) *Test for Phenols:* 50ml of extract was dissolved in 5ml of distilled water. Neutral ferric chloride was added drop wise to the sample.
7) *Test for Glycosides:* 0.5 gms of extract was agitated with 5ml distilled water, glacial acetic acid containing 3-4 drops of ferric chloride was added followed by sulphuric acid along sides of testube.

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D. Green Synthesis of Silver Nanoparticles

Freshly prepared 2 ml crude sample was taken and madeup to the volume 5 ml by adding distilled water, 10 ml of 2 mM silver nitrate was added to the sample, it was kept in water bath for reduction reaction at 70-80 degree Celsius for 30 minutes, the colour change was observed after 30 minutes.

E. Characterization Study

The bio synthesized silver nanoparticles were monitored periodically by sampling of the 1 ml aliquots and the optical absorbance was recorded by U.V-Visible spectrophotometer (Nanodrop 8000 series Thermofisher) in 300-800nm wavelength range. The FTIR was carried to detect the functional groups present in AgNPs. Particle size and zeta potential measurement were carried out by using HORIBA SZ-100 scientific to detect the size and charge of AgNPs.

F. Antibacterial Activity

The antibacterial activity of synthesized silver nanoparticles of methanolic extract of *Musa paradisiaca*pseudo stem and crude methanolic extract were evaluated by using Disc Diffusion Method. 2-gram negative bacteria (*Escherichia coli,Klebsiella pneumonia*) and 2-gram positive bacteria (*Bacillus subtilis, Staphylococcus aureus* were obtained from microbiology department, SPMVV, Tirupati. The strains were maintained in agar plates for 24 hrs. Nutrient agar plates were prepared and 100µl of culture were inoculated into it. Antibiotics levofloxacin strips were placed in plates, it is used as positive control. Sterile paper discs dipped in different concentration (10,20,30 and 40 microlitre) of nanoparticles and paper disc containing crude (30 microlitres) were then placed in different plates. The plates were then incubated for 24 hrs at 37 degree Celsius and inhibition zone were measured after 24 hrs. The inhibition zone was compared with inhibition zone of levofloxacin strips.

G. Antioxidant Activity

DPPH free radical scavenging assay is highly recommended to evaluate the antioxidant activity of silver Nano particles. DPPH free radical scavenging method working principle ison electron transfer that develops a violet solution in methanol. This DPPH free radical will get reduced in the presence of an antioxidant molecule present in the nanoparticles. Stock solution was made with a concentration of 1mM DPPH and working solution of 0.1mM concentration was made to perform the experiment. The samples were taken as 100, 200, 300, 400 and 500ul and made up to 1ml with methanol. Then 1ml of DPPH was added in it. Positive control was prepared as 1mg/ml by taking 1mg of ascorbic acid in 1ml of methanol. The blank was taken as 2ml of methanol. Negative control was prepared by adding 1ml of methanol and 1ml of DPPH. After that it was incubated in dark for 30 minutes. During incubation DPPH reacts with antioxidant compound. The change in colour was observed after 30 min of incubation and the absorbance of sample were taken at 517nm using a U.V- visible spectrophotometer. The percentage of inhibition of the sample was calculated by using the formula

$$\% \text{ of inhibition} = \frac{Ac - As}{Ac}$$

Where,
Ac = Absorbance of control, As = Absorbance of sample.

H. Anticancer Study

The measurement of cell viability and proliferation forms the basis of numerous invitro assays for anticancer study. The MTT assay is one of the invitro assay which is used to check viability. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) is a water-soluble yellow dye that is taken up by viable cells and reduced by the action of mitochondrial dehydrogenase present in the cells. The reduced product is a crystal of purple coloured Formazan which is water insoluble as in [4]. The purple formazan can be dissolved using DMSO and used for colorimetric measurement.

1) *Cell Line:* Ovarian cancer cell line, PA-1 was procured from NCCS, Pune and were used to analyse the anticancer activity of AgNps biosynthesized from methanolic extract of Musa paradisiacal Pseudo stem. Cell lines were cultured in DMEM media containing 15% FBS and Penicillin-streptomycin antibiotic. Passages were performed every 3 days and all the experiments were done with 4-8 passages.

2) *Cell Treatment Procedure:* After reaching the 80% confluency the monolayer cells were trypsinized and pellet was formed. The pelleted cells were treated with 1ml of DMEM. The total no. of cells was counted in neubar chamber and seeded in a 96- well plate. $1*10^5$ cells were seeded in each well, containing 100 ul of medium and incubated for 24 hrs at 37°C, 5% CO_2 in CO_2 incubator. After 24 hrs the cells, PBS wash was done and treated with various concentration of AgNps (25µg/ml-400µg/ml).
3) *MTT Assay:* Post 24 hrs ,10µl of MTT solution was added to each well including control and wrapped with aluminium foil. It was incubated for 2-4 hrs. Post incubation, 100µl of solubilisation buffer solution was added and gently stirred on rotary stirrer for 1 hr. The absorbance was taken on ELISA reader at 570 nm with a reference wavelength higher than 650 nm.

$$\frac{\text{No. of cells in each well} * \text{No of wells added} * 100\mu l}{\text{Average} * 10^5}$$

I. Flow Cytometry

Flow cytometry technique is highly recommended to study the physical and chemical characteristics of cells such as size, shape, presence of tumour markers on cell surface. [5][6]. PA1cells were seeded in a 6 well plate at a density of $1*10^5$ cells per well and grown at 37^0c for 24 hrs in a CO_2 incubator. After 24 hrs the cells were treated with AgNps of Musa by considering the IC50 value. No treatment was given to the cell control. Treatment with AgNps was given for two different time periods i.e 24hrs and 48 hrs. After the incubation at 2 different time periods, cells were trypsinized and centrifuged at 8000 rpm for 5 minutes and pellet was collected. Cells were then washed with cold 1X PBS and 1ml of 70% chilled ethanol was added dropwise (fixing of cells) and stored at -20^0c. 70% chilled ethanol was removed by centrifuging at 2000 rpm for 15 min at 4^0c followed by a PBS wash. After centrifugation pellet was separated and to the pellet 50µl of RNaseA was added and incubated at RT for 30 min, 450 µl of PI solutions was added to the cells. After PI staining cells were filtered into FACS tubes using strainer and cell cycle profile for 10,000 events were determined using FACS calibrator.

III. RESULTS AND DISCUSSION:

A. Phytochemical Screening of Musa Paradisiaca Extract

By performing the phytochemical screening, methanolic extract of Musa paradisiaca indicated the presence of Alkaloid, terpenoid, flavanoid, Tannins, saponins, phenol, glycosides as shown in TABLE I

TABLE I : Phytochemical Analysis of Methanolic Extract of *Musa paradisiaca* Pseudostem.

Plant constituent	Result	Inference
Alkaloids	+	Yellow precipitate
Terpenoids	+	Golden yellow colour
Flavanoids	+	Colourless
Tannins	+	Bluish black colour
Saponins	+	Foam formation
Phenols	+	Black colour
Glycosides	+	Yellow colour

B. Biosynthesis and Characterizations of Silver Nanoparticles using musa Paradisiaca Pseudostem Extract

1) *Visual Inspection:* The visual inspection is the preliminary analysis to confirm the synthesis of silver nanoparticles synthesized using pseudostem extract. The silver ions get reduced to silver nanoparticles by plant extracts in 30 minutes and as a result colour changes from white to light yellow which is the indicative of AgNps synthesis. In the present study I have used the 2mM concentration of AgNps. The results are shown in Fig.1

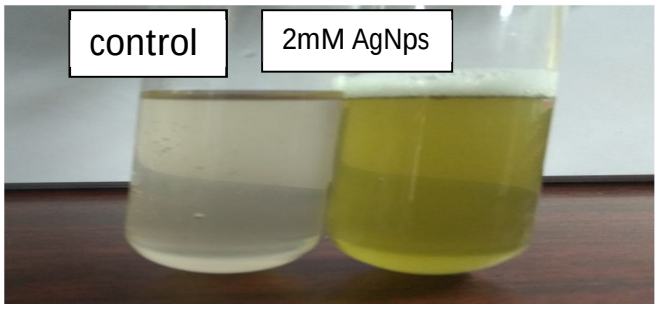


Fig. 1: Aqueous extract of *Musa paradisiaca* and AgNPs solution after addition of AgNO3 to Plant extract.

2) *UV- Visible Spectral Analysis:* The surface Plasmon resonance spectrum of biosynthesized AgNPs with 2mM concentration was observed at 423 nm wavelength as shown in Fig. 2. The metal Nanoparticles have free electrons which give the SPR absorption band due to the vibration of electrons of Nanoparticles with light wave. U.V- Visible spectral analysis was used for the confirmation of synthesis, size and shape of nanoparticles.

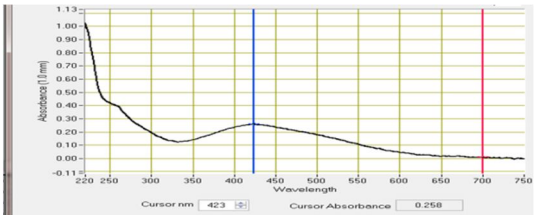


Fig. 2: UV-Visible absorption spectra of synthesized silver nanoparticles

3) *FTIR Spectral analysis:* The FTIR spectral analysis confirms that the methanolic extract of Musa paradisiaca pseudostem contains alkene as a unique functional groups while silver nanoparticles synthesized from methanolic extract contains ketones, methyl groups, nitrosamines and aromatic rings as functional groups as shown in TABLE II. Thus alkene group of pseudostem methanolic extract might be involved in reduction and stabilization of synthesized nanoparticles.

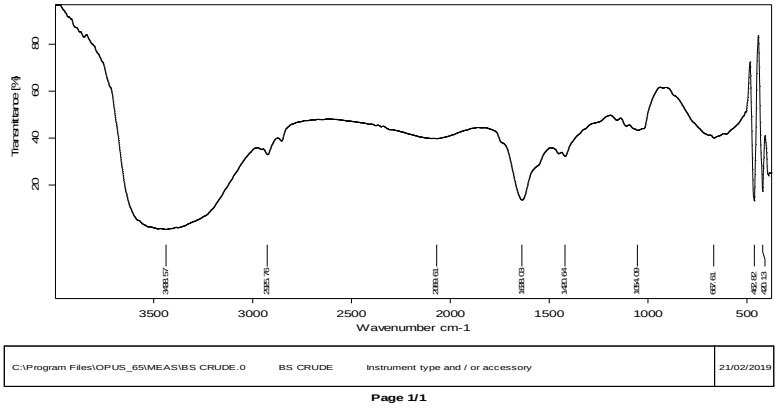


Fig. 3: FTIR spectra of methanolic extract of *Musa paradisiaca* Pseudo stem

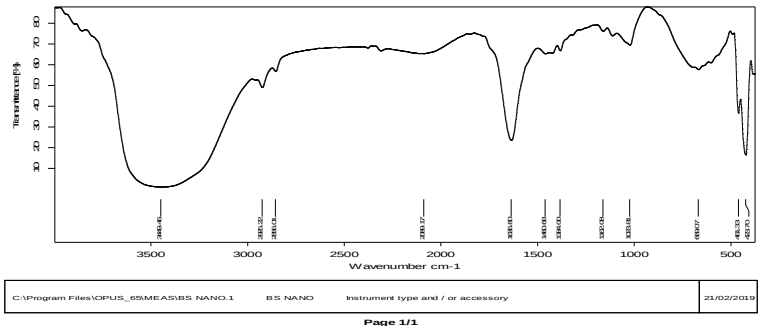
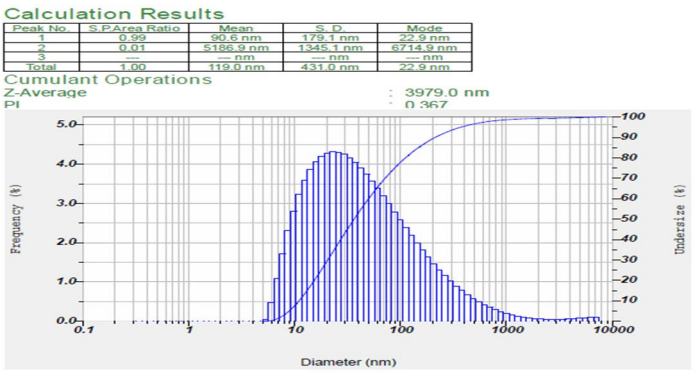


Fig. 4: FTIR spectra of synthesized silver nanoparticles derived from m methanolic extract

Table II: Functional groups present in methanolic extract and AgNPs.

Functional groups	Methanolic extract	AgNPs
Halogen compound	667.61	669.07
Alcohol	1054.09, 3438.57	1023.81, 3449.46
Carboxylic acid	1054.09, 2925.76	1023.81, 2856.01,2925.22
Ether	1054.09	1023.81
Alkanes	1420.64	1460.68, 2856.01,2925.22
Aldehydes	1638.03	1636.80
Amine	1054.09, 3438.57	1162.08,3449.46
Ketones		1636.80
Aromatic ring		1460.68
Methyl groups		2856.01, 2925.22
Phenols	3438.57	3449.46
Alkene	1638.03	
Ester	1054.09	1023.81
Nitrosamines		1460.68

4) *Particle Size Analysis:* The particle size of the synthesized silver nanoparticles ranges from 10 to 120 nm and the average diameter of the nanoparticles is 119 nm.



5) *Zeta Potential:* The zeta potential is a indicator of the stability of colloidal dispersion. The magnitude of the zeta potential indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in dispersion. The electrostatic repulsive forces protect them from forming an association between similarly charged particles. The zeta potential of synthesized silver nanoparticles is -33.7 mV. The zeta potential between ±30 to ±40 have moderate stability, hence the synthesized nanoparticles have moderate stability.

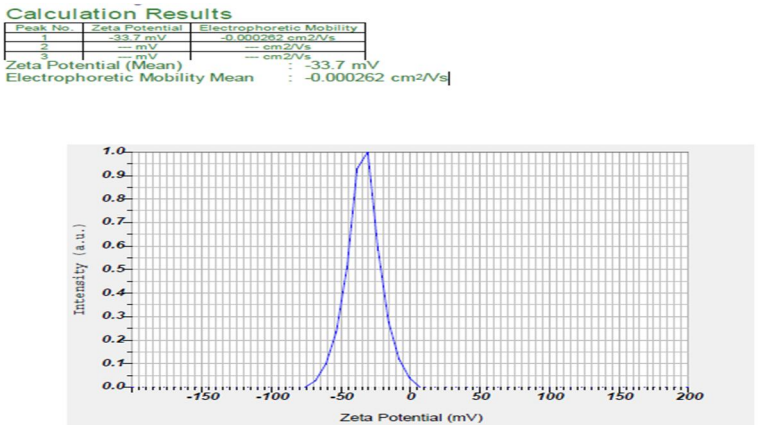


Fig. 6: Zeta potential of synthesized nanoparticles.

C. Antimicrobial Activity

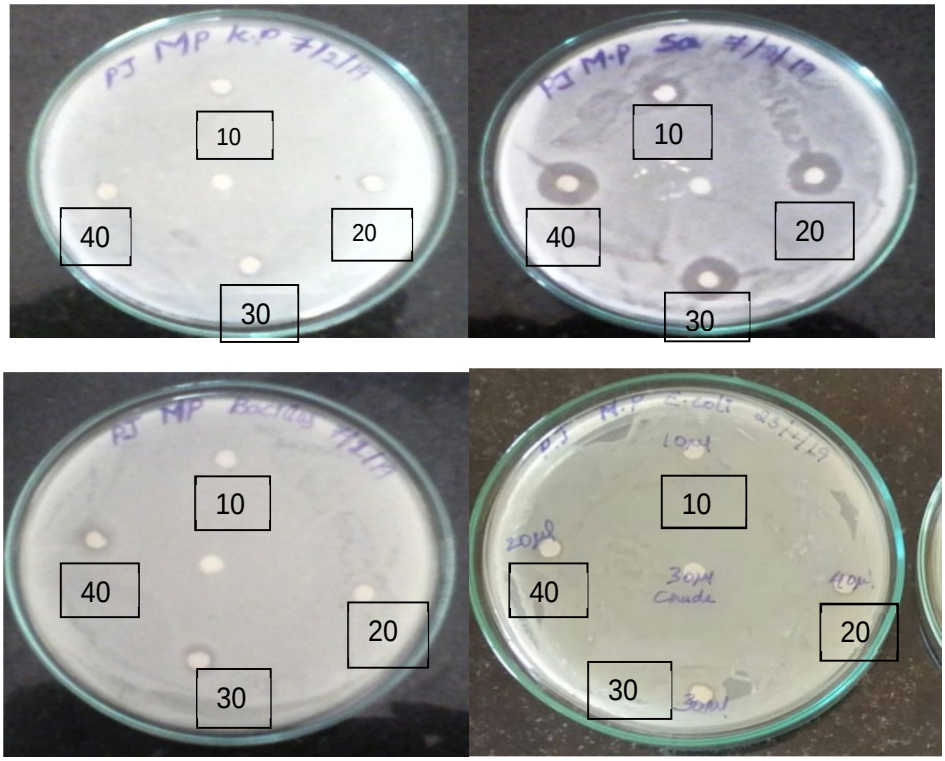


Fig. 7: Bactericidal activity of AgNPs against different strains

Table III: Inhibtion area shown against different strains of bacteria.

	Inhibition area in mm			
	E.coli	*B.subtilis*	*S.aureus*	*K.pneumoniae*
Laevofloxin	10	26	22	34
10 µg/ml	-	-	10	6
20 µg/ml	5	6	12	6
30 µg/ml	6	8	13	7
40 µg/ml	-	9	13	7

A substance showing antibacterial activity will prevent the growth of bacteria. In this study, I have observed that AgNPs at 10 µg/ml and 40 µg/ml against E.coli and AgNPs at 10 µg/ml against B.subtilis did not showed clear zone of zone of inhibition while rest all concentrations of AgNPs showed clear zone of inhibition against all strains of bacteria. Highest zone of inhibition was observed at 30 µg/ml and 40 µg/ml against Staphylococcus aureus as shown in tableIII and Fig.7.

D. Antioxidant (DPPH) Assay

The antioxidant activity of *Musa paradisiaca*pseudostem methanolic extract and synthesized silver nanoparticles from methanolic extract were evaluated by DPPH radical scavenging assay. Synthesized silver nanoparticles showed better antioxidant activity as shown in graph Fig. 8.

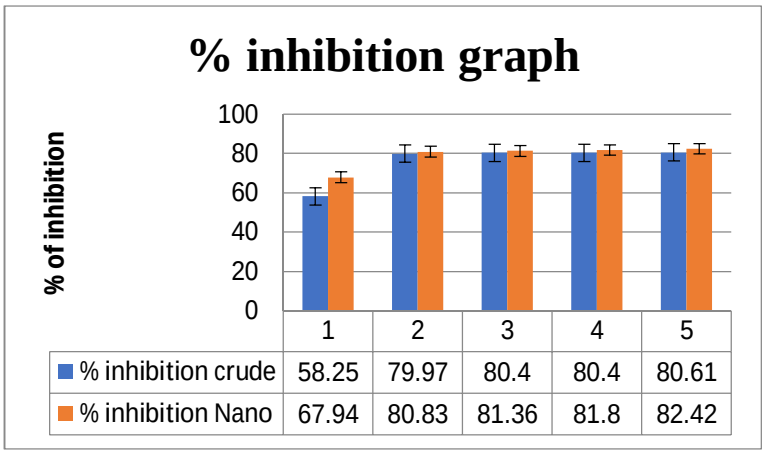


Fig. 8: Inhibition graph showing the percentage of inhibition at different concentrations of crude methanolic extract and AgNPs

E. Anticancer Study

In vitro cytotoxic activity against PA1 cell line was evaluated and compared with the standard drug Cisplatin. The anticancer activities of the synthesized silver nanoparticles were performed with the different concentrations such as 50µg, 100 µg**,** 150 µg**,** 200 µg, 250 µgand 300 µg**.** Result showed the increase in anticancer activity with the increase in concentration of AgNPs and viability of cells decreases as shown in TABLE IV.

TABLE IV: Effect of AgNPs at different concentrations against cell line

	Blank	Untreated	Cisplatin	50µg	100 µg	150 µg	200 µg	250 µg	300 µg
Reading1	0.06	0.857	0.465	0.847	0.742	0.679	0.562	0.438	0.305
Reading2	0.04	0.853	0.462	0.844	0.746	0.681	0.565	0.439	0.302
Mean	0.05	0.855	0.4635	0.8455	0.744	0.68	0.5635	0.4385	0.3035
Stand. Deviation		0.805	0.0021	0.0021	0.0028	0.0014	0.0021	0.0007	0.0021
Viability%		0.0028	51.36	98.81	86.211	78.26	63.78	48.26	31.49

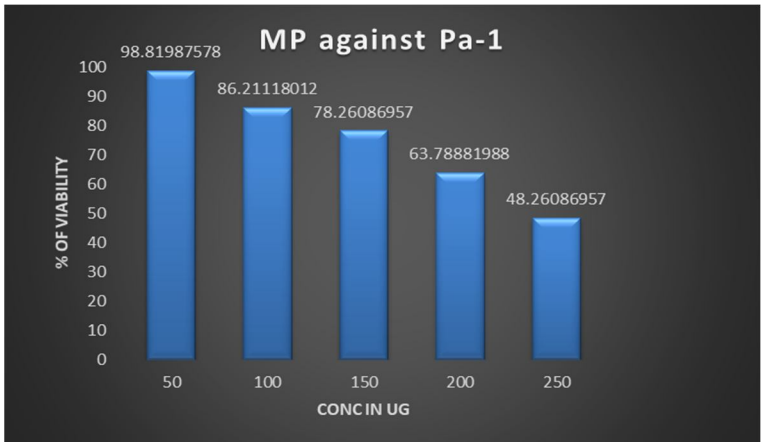


Fig. 9 : % inhibition graph *of Musa paradisiaca* against PA 1 cell line

F. Flow Cytometry

By using IC_{50} value flow cytometry analysis were performed. Most of the cells were arrested at S and G_2/M phase when standard drug were used. Cancer cells treated with *Musa paradisiaca* also get arrested at S and G_2/M phase(Fig. 10). Thus there is clear evidence that *Musa paradisiaca* is showing antiproliferative activity against PA 1 cell line. Hence it can be used as an anticancer drug.

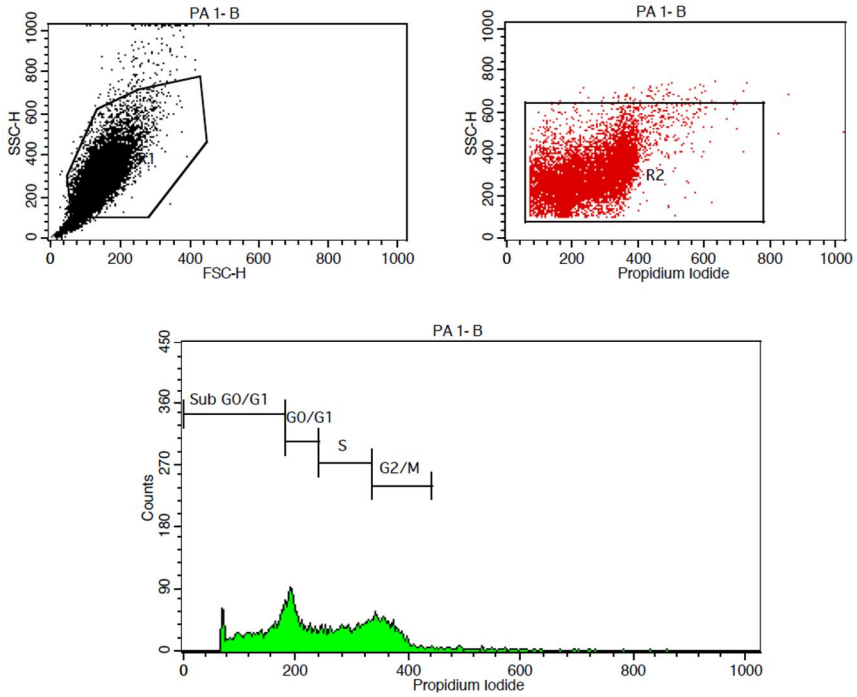


Fig. 10: Represents the cell cycle analysis of *Musa paradisiaca* AgNPs

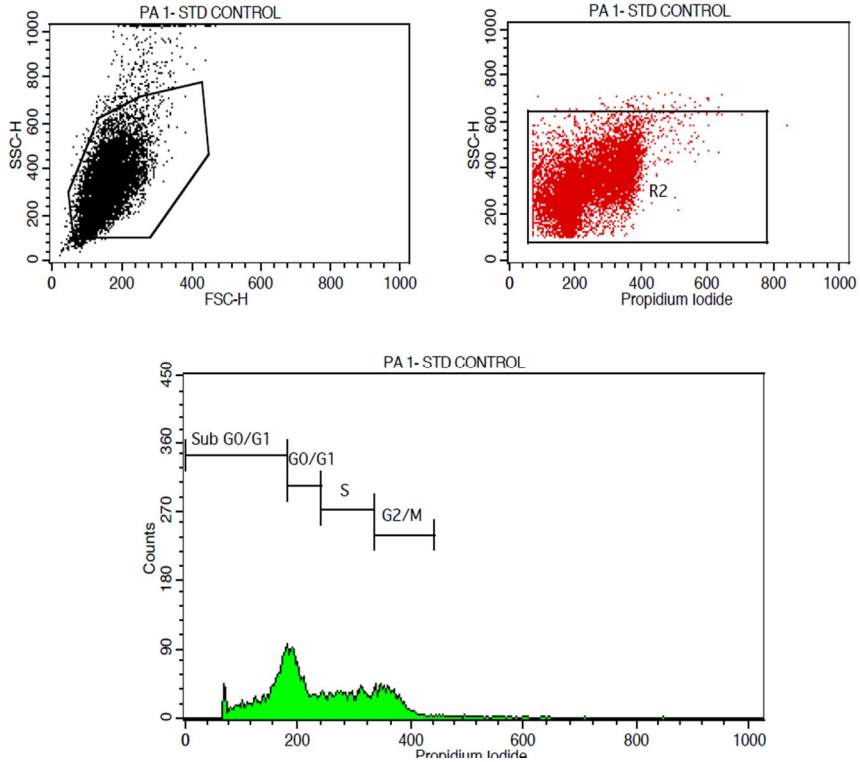


Fig. 11 : Represents the cell cycle analysis of Standard drug cisplatin

IV. CONCLUSION

The phytochemical analysis confirms the presence of various phytochemical like saponins, tannins, flavanoids, terpenoids, phenols. The silver nanoparticles synthesized using *Musa paradisiaca* pseudo stem extract provides simple, environmental friendly and efficient route of synthesis within short time of 30 minutes. AgNPs were initially identified by the formation of light yellow colour and U.V-Visible spectrophotometer analysis shows the surface plamon resonance band at 423nm. The synthesized silver nanoparticles have size ranges between 10nm to 120nm and has a surface charge of -33.7mV which prevent it from coagulation and gives stability. AgNPs derived from *Musa paradisiaca* pseudostem methanolic extract showed the significant antimicrobial, antioxidant and anticancer activity. In this study increased anticancer activity against PA 1 cell line was found to be at increased concentration of AgNPs. Flow cytometry analysis reveals that most of the cancer cells were arrested at S and G_2/M phase of cell cycle. This biosynthesis approach was easy, ecofriendly and can serve as a safer and better source in anticancer drug development. Thus*Musa paradisiaca* pseudo stem derived silver nanoparticles can be used as a natural drug for the treatment of cancer in future after the associated clinical trials.

V. ACKNOWLEDGEMENTS

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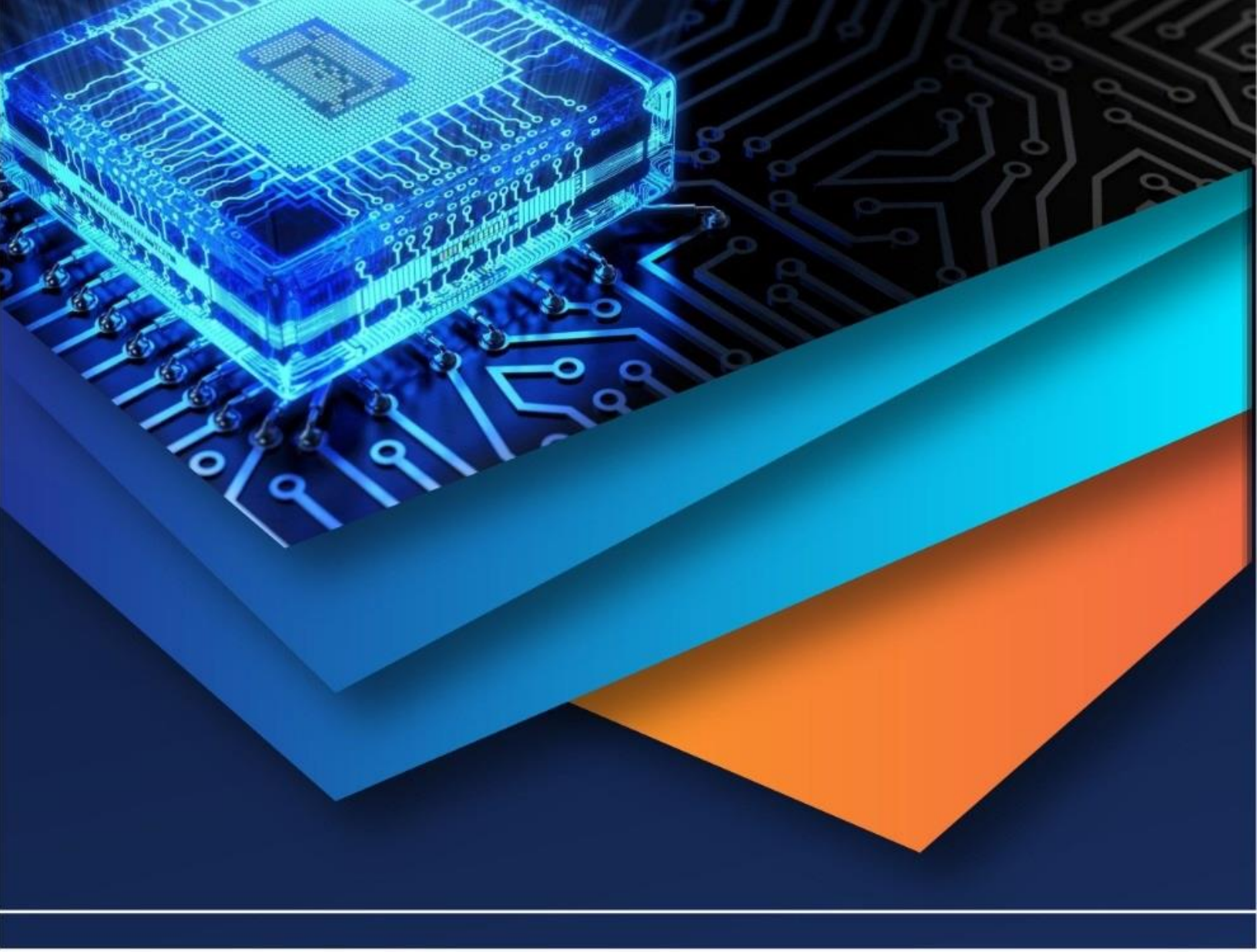

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