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PRODUCTION OF BIO-FABRICATED SILVER NANOPARTICLES; CHARACTERIZATION AND EVALUATION OF BIOLOGICAL APPLICATIONS EFFICACY USING AQUEOUS LEAF EXTRACT OF *POGOSTEMON MYOSUROIDES (ROTH) KUNTZE* Jayachandra N* and Vijaya T

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ABSTRACT: The huge demand for metallic nanoparticles synthesized by green protocols has increased because of their therapeutic clinical and different applications. The present work, aimed to synthesize and evaluate the potential of AgNPs synthesized from Pogostemon myosuroides (Roth) kuntze by a cost-effective way. Synthesized AgNPs with the leaf aqueous extract further characterization was done by recent spectroscopic tools like UV-Vis Spectrophotometer, DLS-Zeta potential, FT-IR (Fourier transform infra-red) spectra, Transmission Electron Microscopy, and X-ray Diffraction. The AgNPs formation was primarily confirmed through UV-Vis scan range from 190 to 750 nm acquired intensive peak at 445 nm. The DLS particle size and zeta potential analysis revealed the size and stability of the nanoparticle around 8.5 nm and -4.4 mV zeta potential values. The FT-IR was used to validate the presence of different functional groups accountable for stabilization and capping agents. TEM was used to find the morphology, size, and agglomeration pattern of the nanoparticles. TEM images exhibited an average size of the nanoparticles of about 22.16 nm size ranging between 10 to 38 nm. XRD was used to crystalline nature and size of the synthesized nanoparticles. Biologically synthesized nanoparticles from leaf extract of the Pogostemon myosuroides (Roth) kuntze had shown significant anti-bacterial activity on selected two gram-positive Bacillus subtilis (MTTC-441) and Staphylococcus aureus (MTTC -731); two gram-negative bacteria Klebsiella pneumonae (MTTC-741) and Escherichia coli (MTTC-443) bacteria; and anti-oxidants activities. PmL-AgNPs also evaluated on five selected human cancer cell lines (MDA-MB-231, SK-OV-3, PC3, PANC-1, and HeLa); exhibited magnificent activity. Keywords: Characterization, AgNPs, anti-bacterial activity, anti-oxidants, and human cancer cell lines.

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1. INTRODUCTION

The revolution in nanotechnology has marked a magnificent turning point in history. Nanotechnology involves the manufacturing, manipulation, and imaging of nanostructures with sizes ranging from 1 to 100 nm [1]. Nanoparticles have a plethora of applications in the fields of health care and cosmetics, food and feed, environmental health, mechanics, space industries, energy science, and photo-electrochemical field [2]. Metallic nanoparticles mainly consist of gold, silver, copper, magnetic (cobalt, nickel), and semiconducting materials. In contrast, non-metallic nanoparticles are primarily composed of carbon-based materials. Intensive research has been conducted on metallic nanoparticles because they possess distinctive electrical, optical, and catalytic properties [3]. Silver nanoparticles (AgNPs), among other nanoparticles; have received extensive attention because of their unique properties s [4-7]. AgNPs are essential nanomaterials studied extensively because of their electrical, optical, and biological properties. Consequently, these nanoparticles have been used for numerous applications, including bio-sensing, drug delivery, Nano device fabrication, and medicine [8]. Silver nanoparticles have different natural applications significantly antimicrobial, antimalarial, anti-inflammatory, wound recuperating, chemo preventive agent, and so on [9-10]. Silver-NPs and silver-based materials are exceptionally harmful to microorganisms. Silver is known for inhibiting a wide range of bacterial strains and pathogens commonly seen in clinical and mechanical settings [11]. Since Silver NPs have antibacterial and antifungal characteristics, they have a wide range of uses in medication development, burn infection prevention, and disinfection [12]. For the amalgamation of Silver-NPs, a variety of approaches are available, including chemical, physical, and bio-reduction procedures [13-14]. Physical and chemical techniques are rather hazardous and expensive, while biological techniques are eco-friendly, secure, and less difficult for nanoparticle synthesis [15]. Synthesizing of nanoparticles using plant extracts offers several advantages compared with other green synthesis methods because plants are eco-friendly and easy to handle [16]. Moreover, it offers energy efficiency, low toxicity, high yield, time-, cost-effectiveness, and availability. Further, plants are a good and easily available source of bioactive plant secondary metabolites such as polysaccharides, proteins, polyphenols, flavonoids, terpenoids, tannins, alkaloids, amines, ketones, and aldehydes, which act as reducing, stabilizing, and capping agents in the conversion of metal ions to metal nanoparticles, leading to the production of desirable nanoparticles with predefined characteristics [17]. In recent years different types of nanoparticles have been synthesized successfully by the greener approach using plant materials like Indium oxide nanoparticles from Aloe vera [18], Iron oxide nanoparticles from Medicago sativa [19], Palladium nanoparticles from Cinnamomum camphora [20], Copper nanoparticles from Magnolia kobus [21], Cadmium Oxide Nanoparticles from Achillea wilhelmsii [22]. Calcium nanoparticles from Boswellia ovalifoliolata [23], Zinc oxide nanoparticles from Vitex negundo [24], Silver nanoparticles from Flemingia wightiana [25]. Pogostemon myosuroides (Roth) kuntze is an herb, that belongs to the Lamiaceae family. The plant species contains the following synonyms i.e. Dysophylla myosuroides (Roth) Benth.exWall., Dysophylla myosuroides (Roth) Benth., Dysophylla myosuroides Benth., Eusteralis myosuroides (Benth.) M.R. Almeida, Mentha myosuroides Roth, Pogostemon myosurodes Kuntze, Pogostemon myosuroides (Roth) El Gazzar & L. Watson. Rootstock woody, stem terete, pale tawny or white silky tomentose. Leaves are thick and wrinkled. Flowers are very small, whorls close and compact. Calyx minute is 1.2 to 1.5 mm and the corolla 1.5 mm long. Pogostemon myosuroides (Roth) kuntze is common to open rocky crevices on hill slopes of Tirumala [26], the local healers and tribal peoples use to cure ailments of leaf extract is used to relieve anxiety and stimulation of brain [27].

2. MATERIALS AND METHODS

Pogostemon myosuroides (Roth) kuntze Plant leaves were collected from Japaliteertham, Tirumala Hills of the Eastern Ghats, Chittoor district, A.P., INDIA. Healthy and mature leaves were cleaned by running tap water and followed by distilled water. Then all leaves were wiped with tissue paper, later cut into 1.0x1.0 cm pieces, and dried up for 10 to 14 days under shade dried to evaporate moisture content and made ground fine powder with an electric blender and it was stored in the amber colour bottle until further work.

Preparation of plant extract

25 g. of powder was taken into 500 ml sterile Erlenmeyer conical flask and 250 ml of sterile Milli-Q water was added and boiled for 15 min at 100oC. After that the leaf extract was collected in a separate sterile conical flask through a standard filtration procedure, this filtrate was utilized for the characterization, antibacterial, anti-oxidants, and anti-cancer activities.

Chemicals and preparation of the 1 mM Ag (No₃)₂ solution

10 grams of Silver nitrate was purchased by Hi-media Company, and then 1 mM silver nitrate solution was prepared by sterile double distilled water, later this was taken into the amber-coloured bottle until the process of synthesis.

Synthesis of silver nanoparticles (AgNPs)

Synthesis of AgNPs was performed by the leaf aqueous extract of Pogostemon myosuroides (Roth) kuntze was added to 1 mM of Ag (No3)2. 20 ml of leaf aqueous extract were taken into a 250 ml sterile conical flask and it was titrated by 200 ml of 1 mM of Ag (No3)2 solution with heating at 600C to 800C for 30 min. Later it was centrifuged at 20,000 rpm for 15 min to remove the presence of biological admixture, and this was utilized for characterization and evaluation of its antimicrobial antioxidant, and anti-cancer activities.

Characterization of the Nanoparticles

Synthesized silver nanoparticles (PmL-AgNPs) were analyzed by the advanced tools. AgNPs of Pogostemon myosuroides (Roth) kuntze were performed by the UV-Vis spectrophotometer Nanodrop scan range from 190 to 750 nm to know which nanoparticles interfered in decreasing the nanoparticles by Surface Plasmon Resonance (SPR) Mechanism. To determine the size of the particle and size distribution in aqueous AgNPs solution carried out by the advanced tool Dynamic Light Scattering (DLS) Malvern-Zeta analyzer. To comprehend which phyto-constituents interacted in the capping and stabilization of the nanoparticles was analyzed by the Fourier Transform Infra-Red (FT-IR, EC ART), Bruker, Ettlingen, Karlsruhe, Germany by KBr pellet procedure. The crystalline nature and to calculation of the average size of nanoparticles was done by the XRD (Shimadzu, XRD-6000). To find out the nanoparticle's shape and size was performed with the Hr-TEM H-3300 advanced 300 kV from Hitachi.

Antibacterial activity

The antibacterial study was evaluated by using standard protocol followed by the disc diffusion assay [28]. Clinically isolated bacterial strains were acquired from the Department of Microbiology, Sri Venkateswara University, Tirupati. Biologically synthesized PmL-AgNPs were experimented for antibacterial activity against selected two gram-positive (Bacillus subtilis and Staphylococcus aureus) and two gram-negative bacteria (Klebsiella pneumonae and Escherichia coli). For this 20 μ l of plant extract, Ag (No3)2, AgNPs, and streptomycin were applied on separate sterile Whatman No.1 filter paper discs (7 mm diameter) and allowed to dry before being put on nutrient agar medium. The total process was done in triplicates and incubated at 370C for 24 hours. The results were tabulated after measuring the zone diameter in centimetres.

The antioxidant activity of PmL-AgNPs was tested by DPPH- (2, 2-Diphenyl-1-picry Hydrazyl radical Scavenging activity) [29]. For this, 1mM DPPH stock solution was prepared by adding 4 mg DPPH in 100 mL of methanol. 2 mL of DPPH stock solution was added to 1 mL of methanolic solution of PmL-AgNPs consisting of two different concentrations of PmL- AgNPs (50and 100 μ g/ml). Following 30 minutes of incubation time at room temperature, we recognize the absorbance (RSA) against blank at 517 nm. The DPPH assay expressed IC50 values. The assay was performed in triplicates. DPPH activity of the PmL-AgNPs was calculated by the following bellow mentioned formula.

% of formula = [(Absorbance of control - Absorbance of sample)/ Absorbance of control] X100.

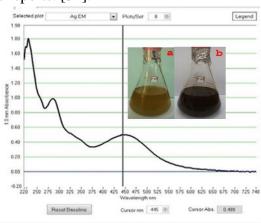
Anticancer studies (Cell proliferation assay using SRB (Sulforhodomine-B)

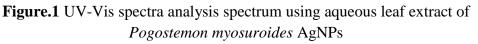
Different human cancer Cell lines (MDA-MB-231, SK-OV-3, PC3, PANC- 1, and HeLa) were purchased from the American Type Culture Collection. SRB assay is a quantitative colorimetric method used for the determination of cell survival and proliferation based on the measurement of cellular protein content. Cells were grown in Dulbecco's modified Eagle's medium (containing non-essential amino acids and 10%FBS). PC-3 was grown in RPMI with glutamine containing non-essential amino acids. Cell lines were maintained in a humidified atmosphere of 5% CO2 at 37°C. Cells were trypsinized when sub-confluent from T75 flasks/90mm dishes and seeded in 96 well plates at a concentration of 1×104 cells/mL in complete medium a day before treatments. Cells were incubated with different concentrations of Ag NPs (12.5-100µg/ml) in triplicates for 48h for potent compounds.

3. RESULTS AND DISCUSSIONS

Ultraviolet- Vissible (UV-Vis) Spectroscopy analysis PmL-AgNPs

The aqueous leaf extract of P. myosuroides mixed with the 1 mMof Ag(No3)2 solution, the color turned into deep brown from transparent yellow, which is the primary and valuable technique to confirm that the synthesized nanoparticles are silver. UV spectra analysis revealed that the broad peak at 445 nm (Fig.1), was due to the SPR Surface Plasmon Resonance mechanism of the AgNPs in the prepared samples. SPR is a collection of oscillations in the conduction of electrons on the material's surface. Obtained UV-Vis spectroscopic results indicate that the stability of the green synthesized AgNPs can be maintained for several months [30]. These types of results were in accordance with the previous reports. [31].





DLS (Dynamic Light Scattering) can be used to scrutinize the surface charge, size, and size distribution of the nanoparticles. This is an advanced tool; it depends on the interaction of the Brownian motion of spherical particles with the light passing by a colloidal solution [32]. The Zeta potential is utilized to estimate the stability, dissemination, and aggregation levels of biologically synthesized nanoparticles through repulsion effects around through fluctuations in charge densities. The PmL-AgNPs in this work expressed about average size is 8.5 nm and negatively charged zeta potential value of -4.4 mV, and 0.165 of the polydispersity index. The results PmL-AgNPs are elucidated in the figure (Fig.2 a &b). This type of result was monitored in the Biosynthesis of silver nanoparticles of Syzigium cumini leaves extract [33].

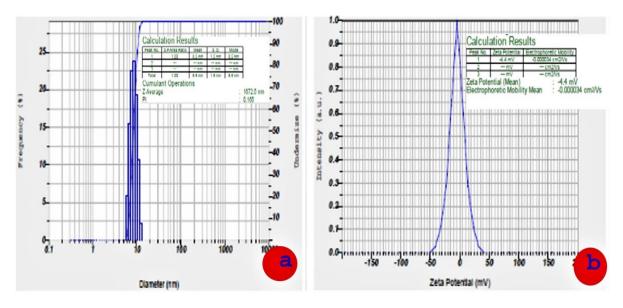


Figure.2 a). Particle size and b). Zeta potential *studies of Pogostemon myosuroides* AgNPs

FT-IR

Green synthesized nanoparticles were analyzed by FT-IR (Fourier transform infra-red) spectra with a scan range of 4000-500 cm-1 (ALPHA interfero meter ECO-ATR, Bruker Ettlingen, Karlsruhe Germany) was used to find the expedient phytochemicals responsible for capping and stabilization. By the FT-IR spectrum broad peaks obtained from leaf aqueous extract of PmL-AgNPs exhibited at 3282 – corresponds to O-H Stretch H Bonded Alcohol, Phenol, 1635 – assigned for N-C Stretch Alkenes and 514 – belongs to C-Br Stretch Alkyl halides (Fig.3). Based on these results cleared that the phytochemicals in the plant extract were essentially accountable for capping and stabilization of pmL-AgNPs. Therefore, FT-IR spectroscopy is a valuable and economical tool to determine the role of biomolecules in the synthesis and stabilization of biosynthesized AgNPs. These results accordance with previous results [34].

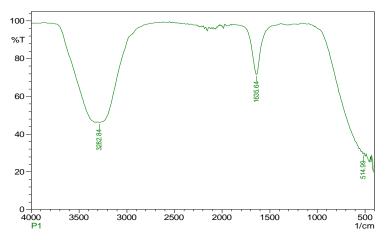


Figure.3 Frourier- Transform Infra-Red (FT-IR) spectra of bio-synthesized AgNPs from Pogostemon myosuroides

XRD

X-ray diffraction (Shimazdu XRD-6000) was analyzed to confirm the crystalline nature and average size, and measure the degree of crystallinity of the PmL-AgNPs. XRD is a powerful characterization technique for both qualitative and quantitative analysis of nanoparticles. The peaks obtained at 20 of X-axis 27.500, 37.890, 46.50, 63.770, and 76.270corresponds to 222,111, 200, 220, and 311 Bragg reflections of Y- axis respectively which may be indexed based on the end- centred monocrystalline structure of AgNPs (Fig. 4). Based on the X-ray diffraction reports indicates that the formed particles are AgNPs. The highest Bragg reflection was obtained at 2θ of 37.89 to predict FWHM (Full Width Half Maxima) value, i.e. 10 and 38 nm average sizes of the nanoparticles by using the Debye-Scherrer equation:

1.1. D=k $\lambda/\beta \cos \theta$

Where D is the diameter of NPs, k is the Scherrer constant, λ is the wavelength of X-ray radiation source, β is full-width half maximum value of XRD diffraction lines and θ is the half diffraction angle-Bragg angle.

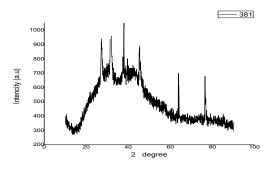


Figure.4 XRD pattern analysis of leaf sourced biologically synthesized AgNPs Pogostemon myosuroides

Jayachandra & Vijaya RJLBPCS 2024 www.rjlbpcs.com **TEM**

Nanoparticles can also be characterized by TEM (Transmission Electron Microscope). Quantitative measurements of the synthesized nanoparticles i.e. size of the particle, size distribution, and morphology of the nanoparticles can be acquired by TEM. TEM conducts imaging by passing an electron beam by a sample and the carbon copper grids must coat a single drop of the reaction mixture. TEM is an advanced tool and provides improved special resolution when compared with SEM and enables a much more in-depth analysis of nanoparticles. HR-TEM showed at 50 nm scale micrograph very small sized, spherical in shape and size range from 10 to 38 nm; Average size of the AgNPs 22.16 nm. These tiny-sized nanoparticles showed no physical contact and did not see any agglomeration between them (fig.5). Such type of results were seen in silver nanoparticles synthesized from plant extracts of Wrightia tinctoria and Acacia chundra [35].

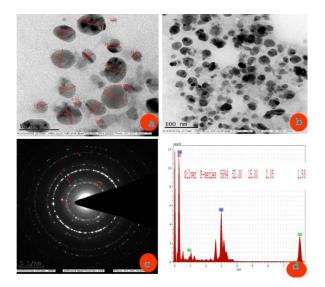


Figure.5 TEM images of Pogostemon myosuroides AgNPs

a). At 50 nm 0.24 nm, b). At 50 nm AgNPs are spherical in shape AgNPs. c). At 51 nm beam of TEM d). EDAX analysis

Antibacterial studies

The bio-fabricated PmL-AgNPs efficacy was evaluated against two selected two-gram-positive Bacillus subtilis MTTC-441 and Staphylococcus aureus MTTC -731; two-gram negative bacteria Klebsiella pneumonae MTTC-741 and Escherichia coli MTTC-443. To do this Streptomycin standard drug was used as a positive control, Ag solution as a negative control, and plant extract served as a control for comparison. The results showed significant anti-bacterial activity against gram-negative bacteria; sufficient activity was seen against gram-positive bacteria. The zone of inhibition was exhibited in the table (Figure 6). The activity revealed that the highest zone of inhibition was observed with PmL-AgNPs in Escherichia coli (Fig. 6 & 7). These kinds of results were found in synthesized AgNPs with leaf aqueous extract of Walsura trifoliata [36].

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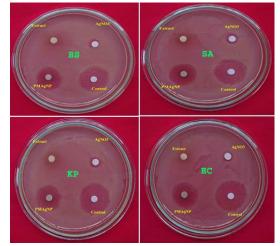
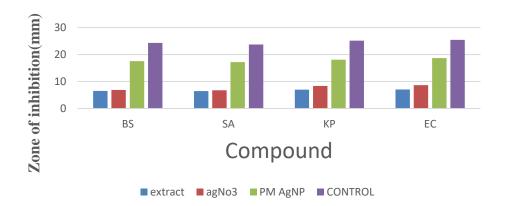


Figure.6 Antimicrobial activity of *Pogostemon myosuroides* aqueous leaf extract biologically synthesized PmL- AgNPs, Ag (No₃)₂ solution and antibiotic Streptomycin.

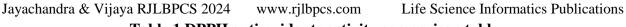


Pogostemon myosuroides AgNPs

Figure.7 Graphical representation of anti-bacterial activity using bio-synthesized PmL-AgNPs

Antioxidant activity (DPPH)

reduction of DPPH radical from DPPH-H, a hydrogen-donating anti-oxidant. The IC 50 values of in-vitro Pogostemon myosuroides leaf aqueous extract, sourced biosynthesized AgNPs anti-oxidant activity described in the table. The results divulged that the DPPH anti-oxidant activity was done by the increased concentrations of the test samples. Plants possess a wealth of bio-active compounds i.e. Flavonoids, tannins belong to the phenolic compounds along with other polyphenols, which are a significant group of phytochemicals that act as primary anti-oxidants of free radical scavengers [37-38]. The maximum free radical scavenging activity was monitored in the PmL- AgNPs with 58.78 % at 100 μ g/mL concentrations and the lowest activity was seen 41.04 % at 50 μ g/mL respectively (Fig.8; Table.1). Similar results were seen in earlier work AgNPs synthesized from Plantago lanceolata leaf extract [39].



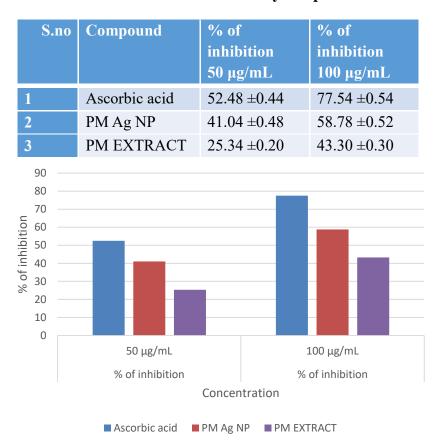
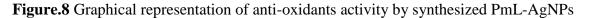


Table.1 DPPH anti-oxidants activity comparison table



Anti-cancer studies

Biologically synthesized PmL-AgNPs were screened out against diverse selected human cancer cell lines i.e. MDA-MB-231 an epithelial, human breast cancer cell line, SK-OV-3 -is an ovarian cancer, PC3 -human prostate cancer, PANC-1 -human pancreatic cancer, and HeLa -human epithelial cervical cancer cell lines. The experiment was done as a cell proliferation assay using SRB (Sulforhodomine-B) colorimetric method. Here served cell control, standard control Doxorubicin, and different concentrations of plant-mediated nanoparticles (i.e. 12.5 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml) to the growing cell lines, in the dose dependant manner. The cytotoxic results suggested the improvement of the aqueous leaves extract AgNPs of Pogostemon myosuroides (Roth) kuntze. Above mentioned assay clearly explained that the tiny-sized nanoparticles allow them to enter cells more easily through endocytosis. Not only do they enter cells, but also they interact with the functioning of cellular proteins, causing changes in cellular structure and chemistry. However, due to these nanoparticles are small and non-aggregated, they are also efficient in producing reactive oxygen species (ROS) in cells. Moreover, ROS can cause stress by damaging DN and causing morphological changes in cells, ultimately leading to cell death [37-38]. Among the activity on five selected human cancer cell lines (MDA-MB-231, SK-OV-3, PC3, PANC- 1, and HeLa) significant activity was noticed in all, lesser activity was seen 4.8 µg/ml in PANC- 1 and PC3 exhibited 4.83 µg/ml at lower concentration (12.5 µg/ml); whereas highest activity was observed in MDA-MB-231 (64.46 µg/ml) and SK-OV-3 (59 µg/ml) at 100 μ g/ml than remaining cell lines. The highest IC50 values were monitored PANC- 1 (87.08 μ g/ml) and HeLa (85.61 µg/ml) (Fig. 9 & 10). Similar types of results were observed in the Anti-cancer © 2024 Life Science Informatics Publication All rights reserved

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Jayachandra & Vijaya RJLBPCS 2024 www.rjlbpcs.com Life Science Informatics Publications potential of biologically synthesized AgNPs using Lantana camera leaf extract [40] and also by Cucumis prophetarum leaf-mediated synthesized AgNPs evaluated on different human cancer cell lines [41].

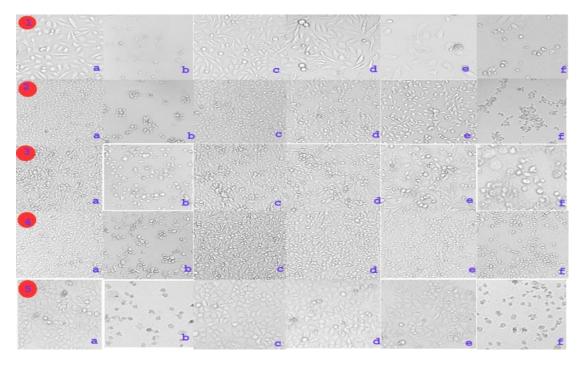


Figure. 9 Anti-cancer activity on selected human cancer cell lines by synthesized PmL-AgNPs

- 1). PC-3 2). MDA-MB-231 3). SK-OV-3 4). PANC-1 5). HeLa
- a). Cell control b). Standard control c). 12.5 μ g/ml d). 25 μ g/ml e). 500 μ g/ml and f). 100 μ g/ml

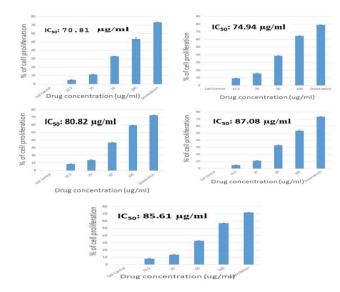


Figure. 10 Graphical representation of anticancer activity by using PmL-AgNPs against five selected human cancer cell lines

a). PC-3 b). MDA-MB-231 c). SK-OV-3 d). PANC-1 e). HeLa

The greener way is adapted rather than the heavy toxic synthesizing methods of synthesis of nanoparticles. This is a novel method to form nanoparticles in the reaction mixture. Our present work is intended for the easy synthesis of nanoparticles using a very small amount of chemicals and a small volume of aqueous plant leaves extract of Pogostemon myosuroides (Roth) kuntze by the green method. The synthesized PmL-AgNPs were characterized by advanced techniques i.e. UV-Visible spectroscopy, FT-IR, DLS: Zeta potential, TEM and XRD. Primarily, colorless Ag (No3)2 solution added to the aqueous leaf sourced extract got the deep brown color from transparent yellow; this indicates the formation of nanoparticles. Then these were observed through the UV-visible spectroscopy, acquired peak at 445 nm, this confirmed that the formed nanoparticles are silver in the solution. The DLS zeta potential study revealed about stability, dissemination, and aggregation levels of bio-synthesized nanoparticles and the size of the nanoparticles around 8.5 nm evenly distributed and -4.4 mV zeta potential values. The FT-IR analysis results expressed that the phyto-constituents in the plant extract help in the oxidation of silver nitrate solution into silver nanoparticles. TEM micrographs confirmed that the shape of the nanoparticle is spherical, and the average size of the particle was 22.16 nm size ranging from 10 to 38 nm in the reaction mixture. The EDS study done along with the TEM revealed that silver is a major element in the nanoparticles. X-ray diffraction suggests the crystalline nature and average size of the PmL-AgNPs. All the above studies regarding the synthesized nanoparticles are spherical with small size. The anti-bacterial studies of the PmL-AgNPs on selected two grampositive Bacillus subtilis (MTTC-441) and Staphylococcus aureus (MTTC -731); two gramnegative bacteria Klebsiella pneumonae (MTTC-741) and Escherichia coli (MTTC-443) showed significant activity, PmL-AgNPs also expressed effective DPPH activity with dose dependant manner. Anti-cancer activity was evaluated on five selected human cancer cell lines (MDA-MB-231, SK-OV-3, PC3, PANC- 1, and HeLa), showing remarkable activity through increasing concentration levels. Above mentioned biological properties were evaluated, and AgNPs were found to have potent anti-bacterial, anti-oxidant, and anti-cancer action. The advantages of this method to synthesis from plant extracts are a clean working environment, a healthy, and nonhazardous atmosphere, lesser wastage, and most stable products. The work suggests that the plantassisted nanoparticles can be employed in the forthcoming days for the treatment of diseases. The green synthesized nanoparticles from PmL-AgNPs will help to develop into efficacious drug delivery systems as they are smaller in size. The nanoparticles exhibited their correct capability as a pharmaceutical drug.

ABBREVIATIONS

PmL- Pogostemon myosuroides leaf material AgNPs- Silver nanoparticles XRD- X-ray diffraction FT-IR- Fourier transform infra-red spectra **DLS-Dynamic Light Scattering** TEM Transmission Electron Microscope EDAS- Energy-Dispersive X-ray Analysis

Jayachandra & Vijaya RJLBPCS 2024 www.rjlbpcs.com Life Science Informatics Publications mM- Millimolar ml- Milli litre mg- Milligram mm- Millimetre nm- Nano metre

µg- Micrograms

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declared that they have no interest of conflicts

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