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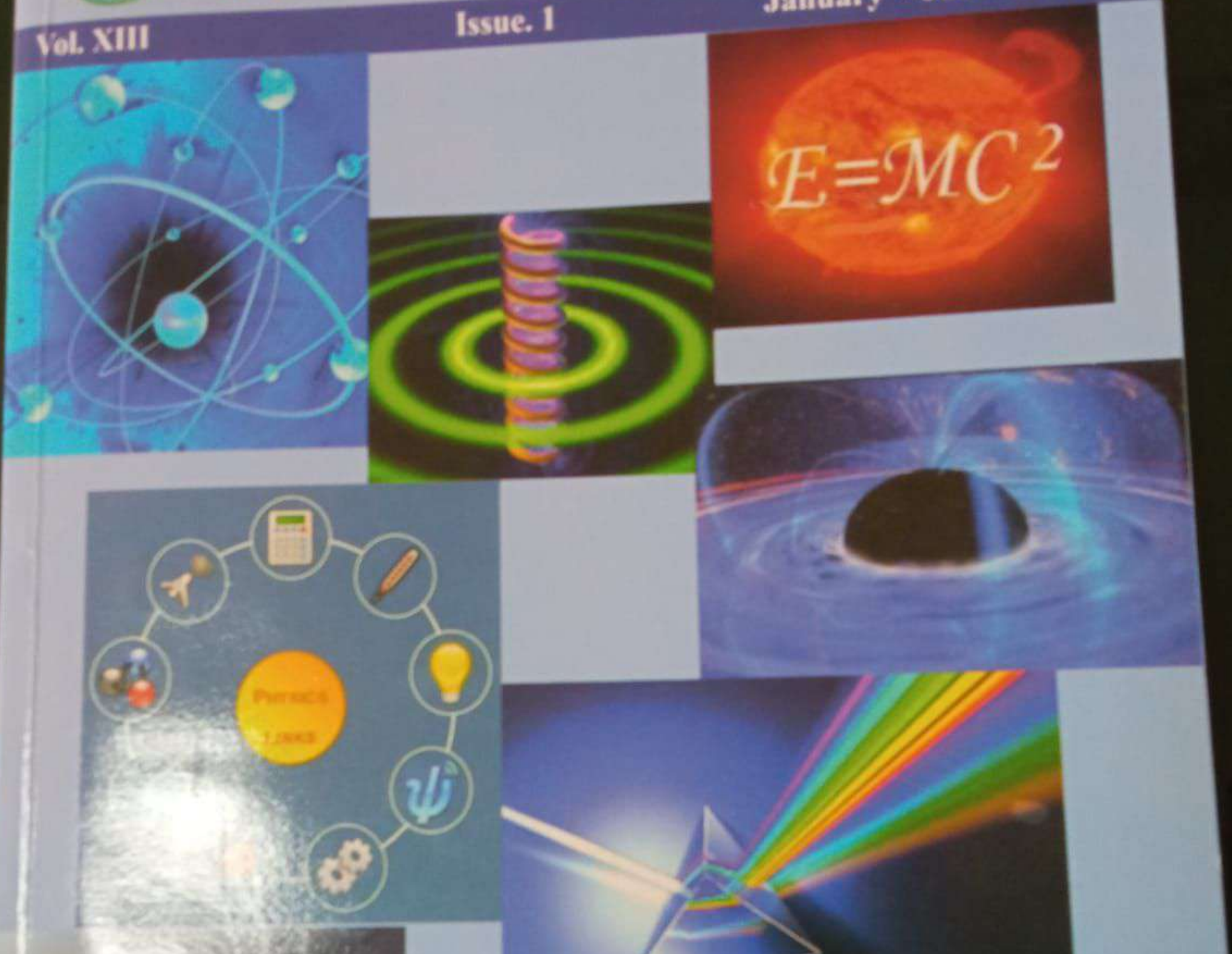

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Synthesis of AgNPs using Biogenic Route and Studied their Effective Application in Vitro Antibacterial, Anticancer and Drug Delivery

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Abstract

A cost-effective and eco-friendly technique was proposed for synthesis of silver nanoparticles (AgNPs) using silver nitrate and extract of *Glycosmispentaphylla*plant leaves served as combined bio-reducing and capping agents.The biological synthesis of AgNPs is eco-friendly, single step. These NPs were fully examined using XRD, FT-IR, UV-Vis, HR-SEM and HR-TEM techniques. UV-Visible profile showed the narrow surface plasmonic resonance (SPR) band with high-intensity peak located at 410 nm, signifies the formation of mono-dispersed AgNPs. XRD analysis of the AgNPs corresponding to (111), (200), (220) and (311) planes confirms fcc cubic morphology. The functional groups present in the plant extract and spherical morphology were determined by FT-IR and HR-SEM analyses respectively. HR–TEM studies signify that the average particle size of AgNPs is 30 nm. Further the antibacterial activity was screened against Gram-positive (*Streptococcus mutans*) and Gram-negative (*Pseudomonas aeruginosa*) bacterial strains which was carried out by agar well diffusion process. The result strongly confirms the synthesized AgNPs has promising candidate for antibacterial activity with significant zone of inhibition and less effective in *Streptococcus mutans* as compared to *Pseudomonas aeruginosa*. In addition to this *in-vitro* anticancer activity against HepG2 cell line and *in-vitro* drug release studies were also performed. This study indicated that the greener synthesized AgNPs using *Glycosmispentaphylla* have good anticancer activity towards HepG2 cell line and drug releasing property at lower pH condition. Further the synthesized nanoparticles are meritoriously employed in plethora of biomedical and its related applications.

Keywords: *AgNPs, Antibacterial, Anticancer, Drug release studies.*

1. Introduction

Over a decade, the research community has witnessed tremendous developments in the areas of nanoscienceand nanotechnology. Nanoparticles (NPs) have been used as flexible tools in a range of different application, due to their extraordinary physicochemical properties with constrained size and shape [1, 2]. Several metal nanoparticles (NPs) such as Ag, Au, Pt, Ni, Pd, Iron were extensively utilized in diversified fields. Amongst these AgNPs have been received a considerable interest in the areas of bio-medicine, biosensor, nanosensors, electronic chip making, energy and bio-technology applications [3 - 6]. So far numerous techniques such as photochemical [7], electrochemical [8], sonochemical [9], microwave [10], UV-irradiation [11], laser irradiation [12], ionizing radiation [13], radiolysis [14] have been used to develop the AgNPs and applied for clinical applications. Nevertheless, the chemical synthetic procedures are associated with hazardous chemicals that may pose adverse effects to the human health [11]. Recently, usage of biological methods has proven reliable and eco-friendly approach to overcome the drawbacks of chemical methods. Alternatively, medicinal plants are considered as extremely attractive system[15] due to have less toxicity, cost effectiveness, ecofriendliness, very less time consumption and produce the lower size of NPs. The plant parts have numerous phytochemicals which can reduce and stabilize the NPs[16].Several works have been reported in the last decades on plant extract assisted synthesis of AgNPs. For example *J. sambac* [17], *Lansiumdomesticum* [18], *Cocosnucifera* [19], *Olive leaves* [20], *Azadirachtaindica* [21], *A. Indicum leaf* [22], *Gum Karya*[23], *T. Pentaphyllaleaves* [24], *Dodonaeaviscosa leaves* [25], *Hibiscus cannabinus* [26], and *Averrhoabilimbi* Fruit [27] were extracted and used to synthesize AgNPs for the treatment of variety of diseases including cancer and bacterial infection.

Glycosmispentaphylla is a shrub found in Indiansub-continent, isrich in terpenoids, amides, imides, alkaloids, coumarins, and flavonoids [28]. The extract of *G. pentaphylla* leaves exhibited many pharmacological activities like antibacterial, antifungal, anthelmintic, antipyretic, antioxidant, anti-tumour, anti-inflammatory, anti-hepatocellular carcinoma (HCC), hepatoprotective, wound healing activity, insecticidal and larvicidal property [29]. However, based on our knowledge,there is no scientific report on the synthesis of Silver (Ag) NPs with *Glycosmispentaphylla* leaf extract followed by its bio-reducing property is ponder as suitable for medical applications as of now. Here in this work, we reported the biosynthesis and characterization of AgNPs. Further antibacterial activity, anticancer studies were reported against HepG2 cell lines and drug release activity.

2. Experimental section

2.1 Chemicals

Glycosmispentaphylla extract were procured locally in Andhra Pradesh, India. Silver Nitrate ($AgNO_3$), Dimethyl sulphoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were procured through Merck chemical company.

Bacterial strains such as Gram-positive (*Streptococcus mutans*) and Gram-negative (*Pseudomonas aeruginosa*) were obtained from Koneru Lakshmaiah Education Foundation, India and used for antibacterial activity studies. Dulbecco's modified Eagle's medium (DMEM), Muller-Hinton agar (MHA) medium, Trypsin-EDTA,Fetal bovine serum (FBS) and antibiotic-antimycotic solution were purchased from HiMedia chemical company, India. Human liver cancer cell line (HepG2) was obtained from National Centre for Cell Science (NCCS), Pune, India.

2.2 Preparation of *Glycosmispentaphylla* leaves extract

Initially *Glycosmispentaphylla* leaves were washed with distilled water followed by drying at room temperature for 24h. Then 15 g of dried leaves were crushed using a mortar and dispersed in 45 mL of de-ionized (DI) water in soxhlet apparatus and heated for 15 min at 60°C and filtered. The resulted mixture was used for further analysis.

2.3 Silver Nanoparticles preparation

Firstly $AgNO_3$(0.5 mL from 0.01M) solutionis added to 5 mL *G.pentaphylla* leaves extract followed by stirred at room temperature. During this process the solution colour is gradually changed, this strongly signifies reduction of Ag ions into its corresponding NPs and was observed within 10 min. The appearance of a stable colour strongly confirms the formation of finite AgNPs.

2.4 Physical characterization analysis

The structural and morphological features of prepared NPs were examined with different spectroscopy and microscopy studies. Where SPR bands of Ag NPs were observed using JASCO-V-530 model UV-Vis spectroscopy. Crystallite parameters are examined with XRD analysis. To identify the nature of bond, FT-IR spectroscopic analysis is carried using BRUKER Optik GmbH-TENSOR 27 instrument. Further shape and morphological features of AgNPs are investigated with HITACHI S300H model SEM analysis operated at 15kV and HR-TEM with JEOL-JEM 2100 MODEL operated at 200 kV.

2.5 In-vitro antibacterial studies

Antibacterial efficacy of pure plant extract, kanamycin and extract containing AgNPs was conducted by agar well diffusion methods against respective bacterial strains (Gram-positive, *Streptococcus mutans* and Gram-negative, *Pseudomonas aeruginosa*). The antibacterial studies are carried by using agar well diffusion assay method for AgNPs. Briefly, the agar medium was placed on Petri plates surface and solidified. After that four wells were created and 100 μL each of pure plant extract, kanamycin and extract containing AgNPs were poured into the wells individually. All the Petri plates were incubated at 28ºC for one day. Finally the zone of inhibition was enumerated and indicated in terms of millimetre diameter after 24h [30].

size of the AgNPs. UV-Vis results correspond to the *G. pentaphylla* leaves extract has reduced all $AgNO_3$ solution to AgNPs [23].

3.2 XRD analysis

The crystallite parameters of synthesized AgNPs are examined using XRD. Fig. 1B corresponds to the XRD of *Glycosmispentaphylla* plant leaves extract tethered AgNPs. Form the figure it clearly observed that AgNPs have four diffraction intensity peaks appeared at 2θ value of 38.45, 46.35, 64.75 and 78.05 correspond to the Bragg reflections from (111), (200), (220) and (311) [34]. These peaks are strongly signifies that the AgNPs have fcc structure. The average particle size is enumerated from Debye Scherrer formula using all intensity peaks and is estimated to be 25 nm. The size of the NPs strongly depends upon the influence of the peak patterns.

3.3 FT-IR analysis

The presence of possible functional groups in *G. pentaphylla* leaves extract responsible for the reduction of AgNPs were analysed by FT-IR method and depicted in Fig.2. The peaks located at 3090, 1400, 1295, and 1115 cm^{-1} are corresponds to the AgNPs synthesized from leaves extract and peaks at 3441, 3135, 1559, 1400, 1295, 1115 cm^{-1} are represented for blank leaves extract. The broad peak appeared at 3441 cm^{-1} indicates the -OH stretching mode of vibration. The shifting of -OH position from 3135 to 3090 cm^{-1} (shifted to lower wavenumber) confirms the formation of AgNPs and these results were in good agreement with the previous report [35]. The carboxylic acid -OH bending peak observed at 1400 cm^{-1}. The peaks at 1295 and 1115 cm^{-1} are strongly attributed to the characteristic absorption peaks of -C-O stretching [36]. The hydroxyl and

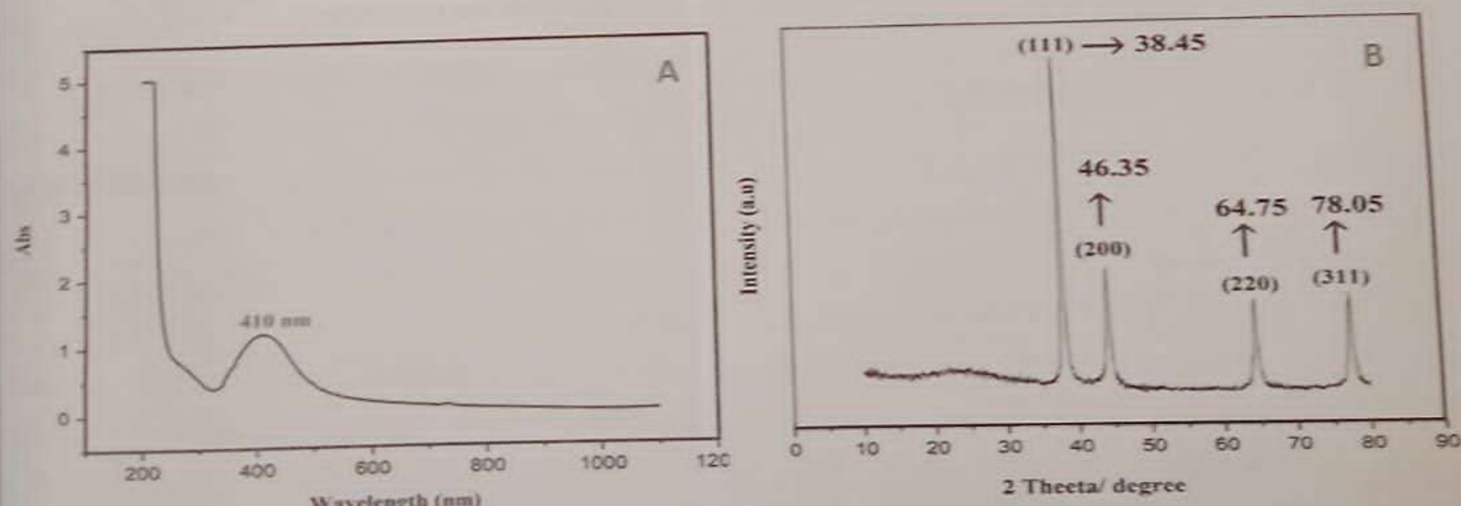

Fig. 1.(A) UV-Vis absorption pattern of neat extract reaction mixture with $AgNO_3$. (B) XRD pattern of extract of AgNPs synthesized from $AgNO_3$.

2.6 In-vitroanticancer activity test

The AgNPs anticancer activity was performed quantitatively by MTT reduction assay was carried by adopting modified Mosmann technique. The composition of following materials such as 10 % heat-inactivated fetal bovine serum contain DMEM, 100 units/mL of penicillin and streptomycin, 25 mM HEPES, 2 mM sodium bicarbonate are used for cull culture in growth medium (37 °C in 5% carbon dioxide, with 95 % humidity) in a carbon dioxide incubator. Further cytotoxicity was examined using almost 95% of cells viability. Cells are plated at in range of $1x10^4$ cells per well on a 96 well plate with in 100 μL standard. Further each plate was swapped another concentration of new medium contain (25, 50 and 75 μg/mL) of metal colloids. At last stage this process 5 mg/mL of MTT solution is sedately added to all the wells followed by incubated at 37°C in 5% carbon dioxide for 240 min. Later optical density was observed after adding DMSO to each well at 570 nm with 650 nm as a reference filter with microtiter plate reader and this result is expressed as % viability [31]. Exponentially growing cells are place on 6 well plates at a concentration of $1x10^5$ cells/mL/well, then 1 mL of this sample was added to the wells followed by incubated at 37 °C, 5% CO_2. Finally after completion of process, the images are taken with microscope to assess the morphology of reacted cells [31].

2.7 In-vitro drug released experiment

Dialysis bag technique was used to explore the release profile of Kanamycin and Letrozole after loading AgNPs. These analyses are studied using various pH solution such as pH-7.2 and pH-1.2 are served as simulated intestinal fluid and gastric fluid. Kanamycin and letrozole loaded AgNPs were taken in beakers and dispersed into 3 mL pH solution separately. This solution was loaded into 1kDa MWCO dialysis bag at 37°C separately with kind agitation. The time interval of each sample is evaluated using UV-Vis analysis.

3. Results and discussion

3.1 UV-Visible spectra

As the aqueous silver nitrate solution was mixed with aqueous *Glycosmispentaphylla* leaves extract, the yellow colour solution was changed into reddish-brown with in 3h due to the reduction of Ag ions to AgNPs. UV-Vis analysis is finite and promising method for the determination of NPs formations. In Fig.1A the UV-Visible absorption spectra of AgNPs exhibited a prominent peak around 410 nm after 3h stirring clearly confirms the formation of AgNPs owing to the surface plasmon resonance (SPR) situated on the surface of NPs. In general AgNPs absorption bands are observed in between 400-500 nm [24, 32, 33]. Thus time-taken for completion of reaction at neutral pH and concentration are key for accurate determination of SPR, and it ishighly sensitive to the shape and carbonyl groups present in the *G. pentaphylla* leaves extract acts as a bio-reducing agent and binds metal ions, further prevents chelation or agglomeration, which leads to size and shape of the NPs [37].

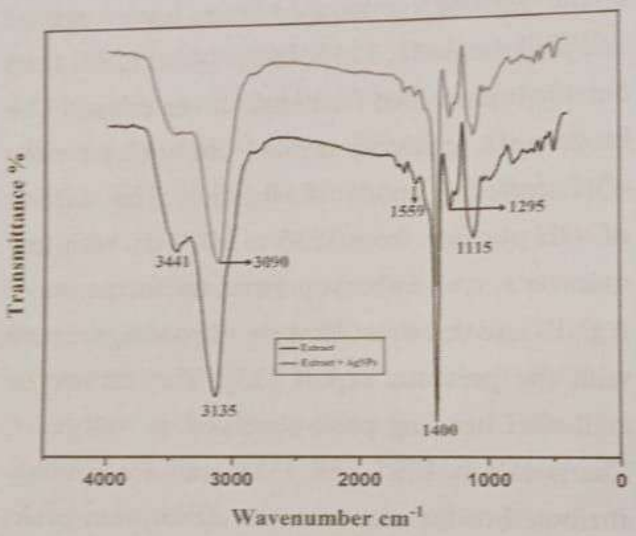

Fig. 2. FT–IR spectra of Pure extract (Black colour) and Extract containing AgNPs (Red colour).

3.4 SEM and HR-TEM studies

The SEM image of AgNPs is shown in Fig. 3A which revealed the small spherical shape sized particles that are randomly distributed [27,38]. HR-TEM images of AgNPs are represented in Fig. 3B&C. Spherical shaped AgNPs with the medium distribution are obtained.The particle size of AgNPs is found to be 30 nm evidenced by both SEM and HR-TEM analysis. The SAED shape of AgNPs indicates the face centred cubic (FCC) structure and in good agreement with XRD analysis. Fig. 3D shows sunny type of do rings are strongly signifies to the AgNPs are als appeared in SAED pattern.

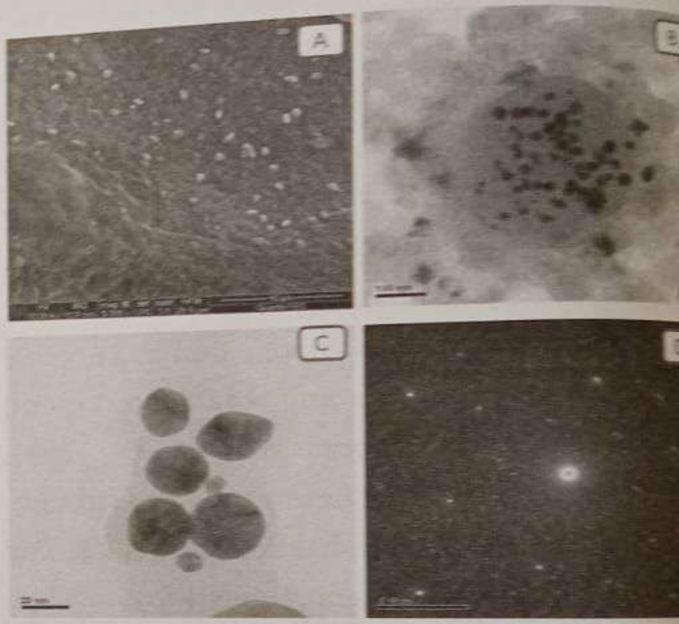

Fig. 3.(A) SEM, (B, C) HR-TEM images different magnifications (D) SEAD pattern AgNPs synthesized from *G. pentaphylla* lea extract.

3.5 Antibacterial activity of AgNPs

Disk diffusion susceptibility met has been adopted to study the antibacte activity of AgNPs specified time duration 24 h for two different bacterial strains such *Pseudomonas aeruginosa* and *Streptococ mutans*. Antibacterial activity of aque *Glycosmispentaphylla* plant leaves ext antibiotics and extract containing Ag are showed in Fig.4. The extract contain AgNPs confirm very high antibacterial acti in expressions of zone of inhibition aga both stains when compared to aqueous no other morphological change is observed. While the different concentration of AgNPs treated with HepG2 cell line, the morphological results are smoothly changed. When increase the concentration, the structural features are drastically changed. Majority of HepG2 cells (images 25–75 µg/mL) were harshlyslanted, found to form long filaments, while few cells twisted round in shape when matched with controlled cells. This is because of bonding between AgNPs and HepG2 cells. Dead cells are indicated by arrows. Fig. 6 represents the Fluorescence image of the Liver cells. The physicochemical bonding of AgNPs with intracellular proteins and DNA of HepG2 liver cancer cell lines exhibits cytotoxic effects [31].

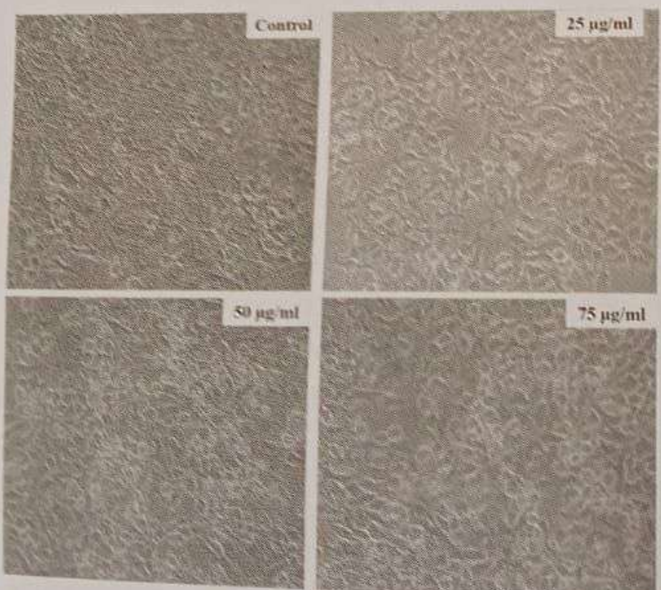

Fig. 5. Bright field images of control, AgNPs (Control, 25µg/mL, 50µg/mL, 75µg/mL concentrations).

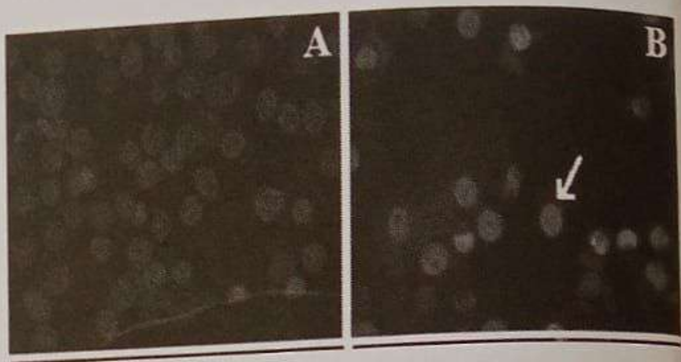

Fig. 6. Fluorescence images of control, AgNPs with (A) Control image and (B) Treated dead cell indicated by arrow).

3.7 Drug Release study

Fig. 7 represents the *in-vitro* drug releas sketches of Kanamycin and Letrozole from plan mediated AgNPs were determined in simulate gastric fluid (pH 1.2) and simulated intestina fluid. At pH 1.2, Kanamycin and Letrozole wer rapidly released. For kanamycin loaded AgNPs 40% of the adsorbed drug is released within 6 h in the intestine at a gentle slope. For Letrozol loaded AgNPs 80 % adsorbed drug is within 6h. After that, the drug is moderately increased in 10h [41], indicating that Letrozole loaded AgNP remain stable in the physiological condition du to the letrozole drug is localized on the NP surface and partition of drug into the surface active agent layer adsorbed at emulsion surfac of droplets. From this finding Letrozole releas of the AgNPs is faster at base pH compared t neutral pH which may depends upon the p value.

extract and kanamycin as an antibiotic. The maximum zone of inhibition is observed in *Streptococcus mutans* bacterial strains (Fig. 4B). The aqueous *Glycosmispentaphylla* leaves extract has important antibacterial activity and which is found to be reduced when applied alone as related to extract containing AgNPs. The finite AgNPs antibacterial activity is highly marked in case of *Pseudomonas aeruginosa* as Gram-negative bacteria when compared to the *Streptococcus mutans* as Gram-positive bacteria. This is because of various arrangements of their cell wall, membrane and single layer peptidoglycan of Gram-negative bacteria. The plausible mechanism may be providing the better antibacterial activity. AgNPs released the silver ions which may attract to the negatively charged bacterial cell wall and form the coulombic electrostatic force of interaction between bacterial cell wall and NPs. NPs can transfer and bonded to cell wall to improve a change in the composition by affecting cell wall absorptivity [39, 40].

3.6 Anticancer activity

To check the anticancer activity of AgNPs, a series of concentrations ranging from 25 to75 μg/mL of AgNPs were added with HepG2 cell lines. Fig.5 provides the *in vitro* anticancer study has been carried by MTT assay on HepG2 cell lines using biosynthesized AgNPs. When the concentration of AgNPs increases from 25 to 75 μg/mL, the cell viability of HepG2 cell lines is decreased. The morphological change of HepG2 cell lines clearly observed in brigh field and fluorescence microscopy, these a mainly used to study the morphological chang of HepG2 cells. In control image, there

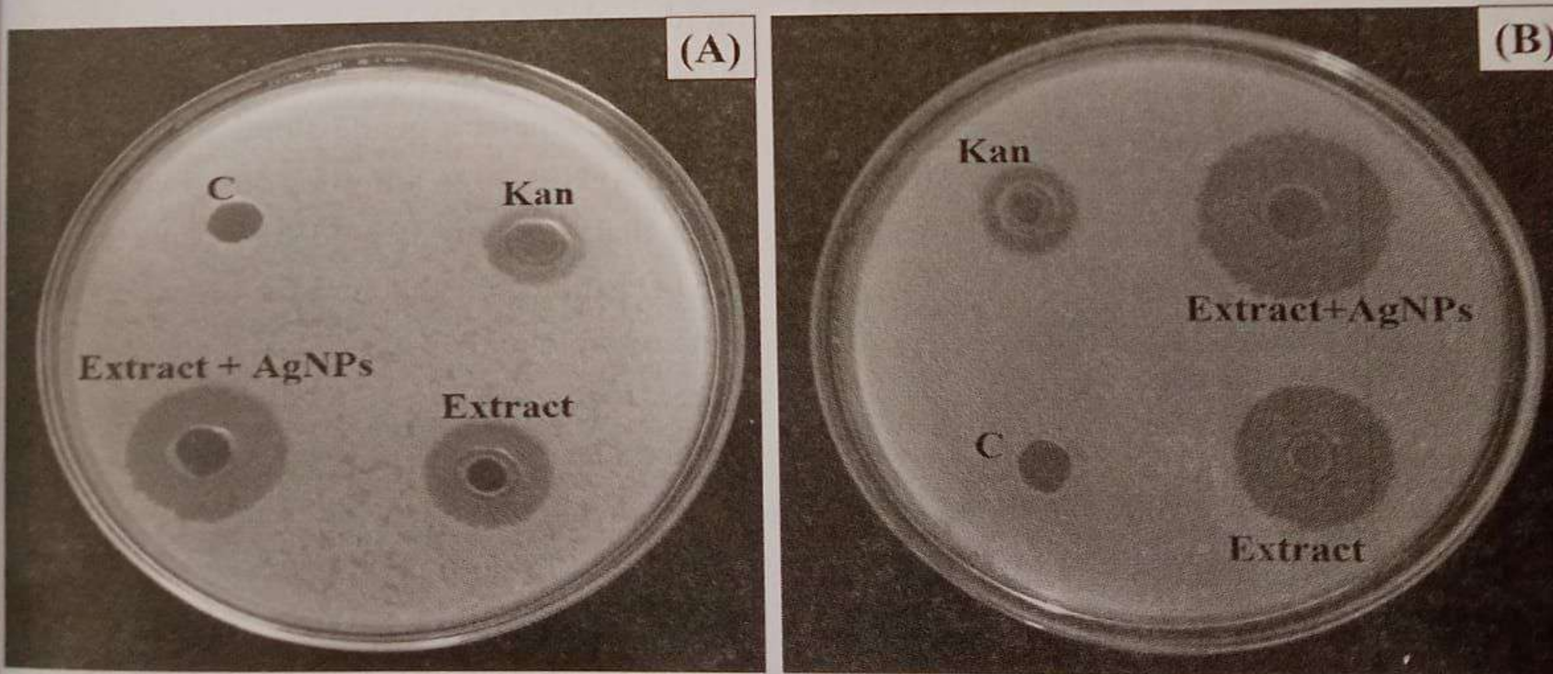

Fig. 4. Interaction of AgNPs with Bacterial strains of (A) *Pseudomonas aeruginosa*, (B) *Streptococ mutans* [**Kan** is Kanamycin, **C** is control].

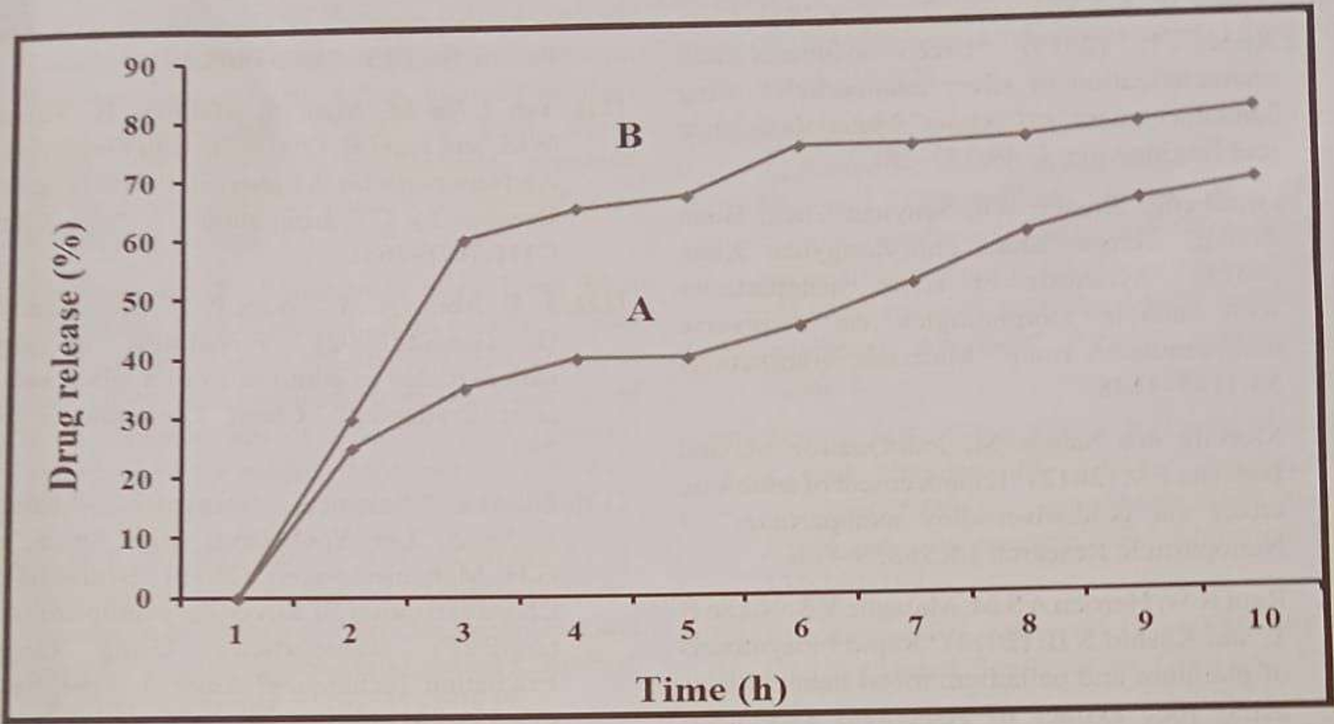

Fig. 7. Kanamycin antibiotic (A) and Cancer drug Letrozole (B) release profile in intestinal fluid and in simulated gastric fluid (pH 1.2).

4. Conclusion

Bio synthesis of AgNPs using *G. pentaphylla* leaves extract through a greener method with numerous rewards including cost-effective, efficient and eco-friendly, which can protect the human health, leading to less wastage and safe products in the environment. Several functional groups are reported in plant extract which are involved during AgNPs formation due to presence of stabilizing and reducing agents. The as prepared AgNPs have an average particle size of 30 nm which can be obtained from supporting studies of XRD, HR-TEM. The NPs can be exhibiting better antimicrobial activity against two different bacterial strains and anticancer activityof HepG2 cell line due to the silver ions released from the NPs. AgNPs using *G. pentaphylla* leaves extract could be used for biomedical applications especially antibacterial and anticancer properties.

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