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Research Article

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Preparation, characterization and synthesis of silver nanoparticles by using phyllanthusniruri for the antimicrobial activity and cytotoxic effects

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ABSTRACT

Silver nanoparticles are nanoparticles of silver, i.e. silver particles of between 1 nm and 100 nm in size. While frequently described as being 'silver' some are composed of a large percentage of silver oxide due to their large ratio of surface-to-bulk silver atoms.Silver nanoparticles are the most prominent one. Silver nanoparticles are nanoparticles of silver, i.e. silver particles of between 1 nm and 100 nm in size and have attracted intensive research interest. It is generally recognized that silver nanoparticles may attach to the cell wall, thus disturbing cell-wall permeability and cellular respiration. The nanoparticles may also penetrate inside the cell causingdamage by interacting with phosphorus and sulfur containing compounds such as DNA and protein. Generally, silver does not adversely affect viable cells and does not easily provoke microbial resistance. Hence silver containing materials were also employed in textile fabrics, as food additives, and in package and plastics to eliminate microorganisms. Because of such a wide range of applications, numerous methods concerning the fabrication of silver nanoparticles, as well as various silver-based compounds containing metallic silver (Ag0) have been developed.Silver nanoparticles (AgNPs) have emerged as an arch product from the field of nanotechnology.

INTRODUCTION

The field of nanoscience has blossomed over the last twenty years and the need for nanotechnology will only increase as miniaturization becomes more important in areas such as computing, sensors, and biomedical applications. Advances in this field largely depend on the ability to synthesize nanoparticles of various materials, sizes, and shapes, as well as to efficiently assemble them into complex architectures. (1) Nanotechnology provides the ability to engineer the properties of materials by controlling their size, and this has driven research toward a multitude of potential uses for nanomaterials.(2) Nanoparticle synthesis and the study of their size and properties of fundamental importance in the advancement of recent research. It is found that the optical, electronic, magnetic, and catalytic properties of metal nanoparticles depend on their size, shape and chemical surroundings. (Das.,*et al 2009).*

Silver nanoparticles (AgNPs) are reported to possess anti-fungal (Kim et al. 2009), anti-inflammatory (Nadworny et al. 2008), and anti-viral activity (Rogers et al. 2008). The concept of Ag either leaching or being released into water systems is of particular concern considering the many years of research showing that ionic Ag is highly toxic to various freshwater aquatic species with varying lethal concentrations depending on the species.(Thirunavukkarasu*et al ., 2011).* Chemical synthesis methods lead to presence of some toxic chemical absorbed on the surface that may have adverse effect in the medical applications. (Singh**.,***et al 2010).*Noble metal nanomaterials have been synthesized using a variety of methods, including hard – template, bio-reduction and solution phase – synthesis. In earlier reports, natural polymers like starch and chitosan (Huaung et al 2004) were shown to stabilize silver nanoparticles and separate reducing agents were used. Interest is now growing for synthesis of metalnanoparticles

using green chemistry principles for application in biology. Recently the concept of green nanoparticle preparation using B-D-glucose as the reducing agent was reported by Raveendran et al (2003) and later by Vigneshwaran et al (2006),(kirubha Daniel*et al ., 2011).*Silver has long recognized as having an inhibitory effect towards many bacterial strains and microorganisms commonly present in medical and industrial processes. The most widely used and known applications of silver and silver nanoparticles are in medical industry. These include topical ointments and creams containing silver to prevent infection of burns and open wounds. (Sathyavani*et al ., 2011).*

Phyllanthin

Phyllanthusniruri is a hepatoprotective.Theantihepatotoxic activity of Phyllanthus has been attributed to atleasttwo novel ligan phytochemicals named phyllanthin and hypophyllanthin.It has been demonstrated that Phyllanthus protects rats from liver damage induced by alcohol, and normalizes a "fatty liver".one in vitro study and four in vivo studies with rats and mice established that extracts of phyllanthus could effectively protect liver against damage caused by various chemical liver toxins.Similarily two clinical studies concluded that Phyllanthusniruri shown an antihepatotoxic effect in children with hepatitis and jaundice.

In India,HP-1 a herbal formulation comprising of Phyllanthusniruri and it was evaluated for hepatoprotective activity against carbon tetrachloride(CCl4)induced toxicity.Results showed that HP-1 reversed the leakage of lactate dehydrogenase(LDH) and glutamate pyruvate transaminase(GPT) and prevented the depletion of glutathione(GSH) levels in a primary monolayer culture of rat hepatocytes (in vitro).HP-1 attenuated serum toxicity as manifested in elevated levels of transaminase.this study concluded that HP-1 is a potential hepatoproctive formulation with an additional attribute of being anti-peroxidative.

EXPERIMENTAL SECTION

Plant Material:

The dried leaves of Phyllanthusniruri were collected from Chennai, Tamilnadu, India .

Organisms:

The following five organisms are used for checking antimicrobial activity

- *Escherichia coli*
- *Staphylococcus sp*
- *Salmonella sp*
- *Proteus sp*
- *Bacillus sp*

Medium: Blood Agar Base.

Reagents:

- 1mM solution of Silver nitrate
- Phosphate Buffered Saline
- Tri-Sodium citrate

Methods:

Preparation of Medium:

Blood Agar Base:

Composition:

Beef extract	500g/lt
Tryptose	10g/lt
Sodium chloride	5g/lt
Agar	15g/lt

Preparation:

8g of Blood agar base was taken and mixed with 200ml of sterile distilled water, and it is autoclaved for about 121 degree celcius for 15 minutes at 15Lbs.Then cool the solution at 45-50 degree celcius.

Phosphate buffered saline:
Composition:

Sodium chloride	8.50g/lt
Disodium hydrogen phosphate	1.91g/lt
Potassium dihydrogen phosphate	0.38g/lt
pH (at 25°C)	7.2+/-0.2

Preparation:
1.079g of Phosphate buffer saline was taken and mixed with 100ml of sterile distilled water,and it is autoclaved for about 121 degree celcius for 15 minutes at 15Lbs.

Trisodium citrate ($Na_3C_6H_5O_7.2H_2O$):
Composition:

Assay	99.0-100.05%
pH (5% solution in H_2O)	7.5-9.0
Water (K/F)	11-13%

Preparation:
10ml of 1% trisodium citrate prepared in triple distilled water.

Preparation of 1mM Silver nitrate solution:
Composition:

Silver nitrate	99.5%
Chloride	0.0005%
Sulfate	0.005%
Iron	0.002%

Preparation:
Molarity is moles per liter. Since the molar mass of $AgNO_3$ is 169.87g/mol, a 1 M solution of $AgNO_3$ would be 169.87 g (1 mole of $AgNO_3$) in 1 liter. For preparation of 200ml of solution 0.034g of silver nitrate is taken.

Protocol:
Preparation of plant material:
Dried leaves of Phyllanthusniruri were collected and made it to powder by using morter& pestle.

Preparation of Crude Extract:
5gram, Leaf powder of Phyllanthusniruri was taken in a sterile conical flask.50ml of distilled water was added to it. Then the mixer was kept in incubator for 48hours at Room Temperature. After incubation, the solution is subjected to centrifuge at 6000rpm for 10 minutes to obtain the pellet. Then the Supernatant was collected from the tube and it was kept for evaporation (to sediment the particles) until it get fully evaporated.

Synthesis of Silver Nanoparticles:
After effective evaporation, the settled powder was collected. Then that powder was taken as 25mg, 50mg, 75mg, and 100mg concentration and mixed with 44ml of Triple Distilled Water is taken in four sterile conical flasks. 1mM Silver nitrate solution is taken and introduced into the each conical flask and mixed well. The conical flask containing silver nitrate solution is kept in Magnetic stirrer. Then the 6ml of various concentrated sample solution is taken and made to add drop by drop into the silver nitrate solution. This process continues until the colour of the solution changes from green to brown. Then the obtained solution is kept for evaporation at room temperature or in incubator. After evaporation the product will be obtained as powder. Then the powder is washed twice with distilled water. Then the obtained solution is used for the further processes.

Chemical preparation of silver nanoparticles:
100 ml 1mM silver nitrate is taken and it is heated at boiling temperature using hot plate with magnetic stirrer. After attaining boiling temperature 10ml of trisodium citrate added drop by drop until it attains pale yellowish colour. Then the obtained solution is kept for evaporation at room temperature or in incubator. After evaporation the

product will be obtained as powder. Then the powder is washed twice with distilled water. Then the obtained solution is used for the further processes.

Characterisation of silver nanoparticles:
UV-Visible Spectroscopy analysis:
Synthesis of silver nanoparticles by reducing the silver ions solutions with *Phyllanthusniruri* leaves extract may be easily absorbed by UV-visible spectroscopy. The absorption spectra of leaves extract quantities and metal concentration were measured using 200-800range.

It uses Light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges. The absorption in the visible range directly. In this region of the electromagnetic spectrum, molecules undergo electronic transitions.

TEM analysis:
Transmission Electron Microscope (TEM), analysis was done using **Philips (technai 10)**. Thin films of sample were prepared on a carbon coated copper grid by just dropping a very small amount of sample on the grid, extra solution was removed using a blotting paper and then the film on the TEM grid were allowed to dry by putting it under incubator. In this technique, whereby a beam of electronics is transmitted through an Ultra-thin specimen, interacting with the specimen as it passes through. An image is formed from the interaction of the electrons transmitted through the specimen. The image is magnified and focused on to an imaging device.

Fluorescence Spectra analysis:
Fluorescence Spectra Analysis was done to confirm the silver nanoparticles. The reduction of silver ions was monitored by measuring the fluorescence spectrum by taking small amount of the sample in the U-bottom micro titter plate. This technique has proved to be very useful for the analysis of nano particles.

Fourier Transform Infrared Spectroscope
Synthesised silver nanoparticles were measured by using **Perkin Elmer Spectrum One FTIR** with 4000-400cm^{-1} of spectral range in KBr pellet. FTIR analysis was used for the characterisation of the extract and the resulting nanoparticles. Powder samples for the FTIR was prepared similarly as for powder diffraction measurements. The FT-IR spectra of plant extracts taken before and after synthesis of nanoparticles were analyzed which discussed for the possible functional groups for the formation of nanoparticles. FTIR measurements were carried out to identify the possible biomolecules in the leaf extract responsible for the reduction of ions and also the capping agents responsible for the stability of the biogenic nanoparticle solution.

Antibacterial activity
Antibacterial activity of leaf ethanol extract derived silver nanoparticles from *Phyllanthusniruri*was assessed using the standard well diffusion method with help of Blood Agar Base medium.100ml of blood agar base medium was prepared and sterilized using autoclave. Then the sterilized medium poured over on sterile Petri plates and it allowed to solidification. After solidification, wells of 5mm diameter were made on medium using gel puncture.

Then the respective organisms are swabbed uniformly into the individual plates using sterile cotton swabs. With the help of sterile micropipette 20µl of different concentration of the diluted particles were loaded on cut wells of pre-sterilized medium of each petriplates containing swabbed organisms and the plates were incubated at 37ºC for 24h.After incubation of nanoparticles were measured and tabulated.

Cytotoxicity assay
The Sheep Bone marrow cells was collected from Slaughter house and maintained in the Laboratory. The bone marrow was flushed with Dulbecco's Modified Eagles Medium (DMEM). The flushed out medium with cells, was centrifuged at 1,500rpm for 7-10min. After centrifugation the settled pellet was taken and again centrifuged for clarification at 1,500rpm for 10-15min. Then Supernatant was discarded. The pellet was resuspended in medium and then cultured into culture flask.

The cultured cells were taken in trypsinization. Again the cells were resuspended in the DMEM medium. With use of sterile micropipette the 100µl of medium along with cell suspension was added in each of the cells of sterile micro titter plate (96 wells). The inoculated plates were kept for incubation about 24H at 37ºC in presence of 5%CO_2.

After incubation, the sample i.e Silver nanoparticles and the plant extract were added to the wells at 100µl respectively serial dilution were made to successive wells in a pattern. Each well contains random number of cells. After addition, the microtittre plate containing sample was allowed to incubate for 48H.Then the sample of each well was loaded with 50µl of MTT dye and it is allowed to incubate for 1H. After incubation the wells were read at 630nm using ELISA reader.

RESULTS

Synthesis of silver nanoparticles

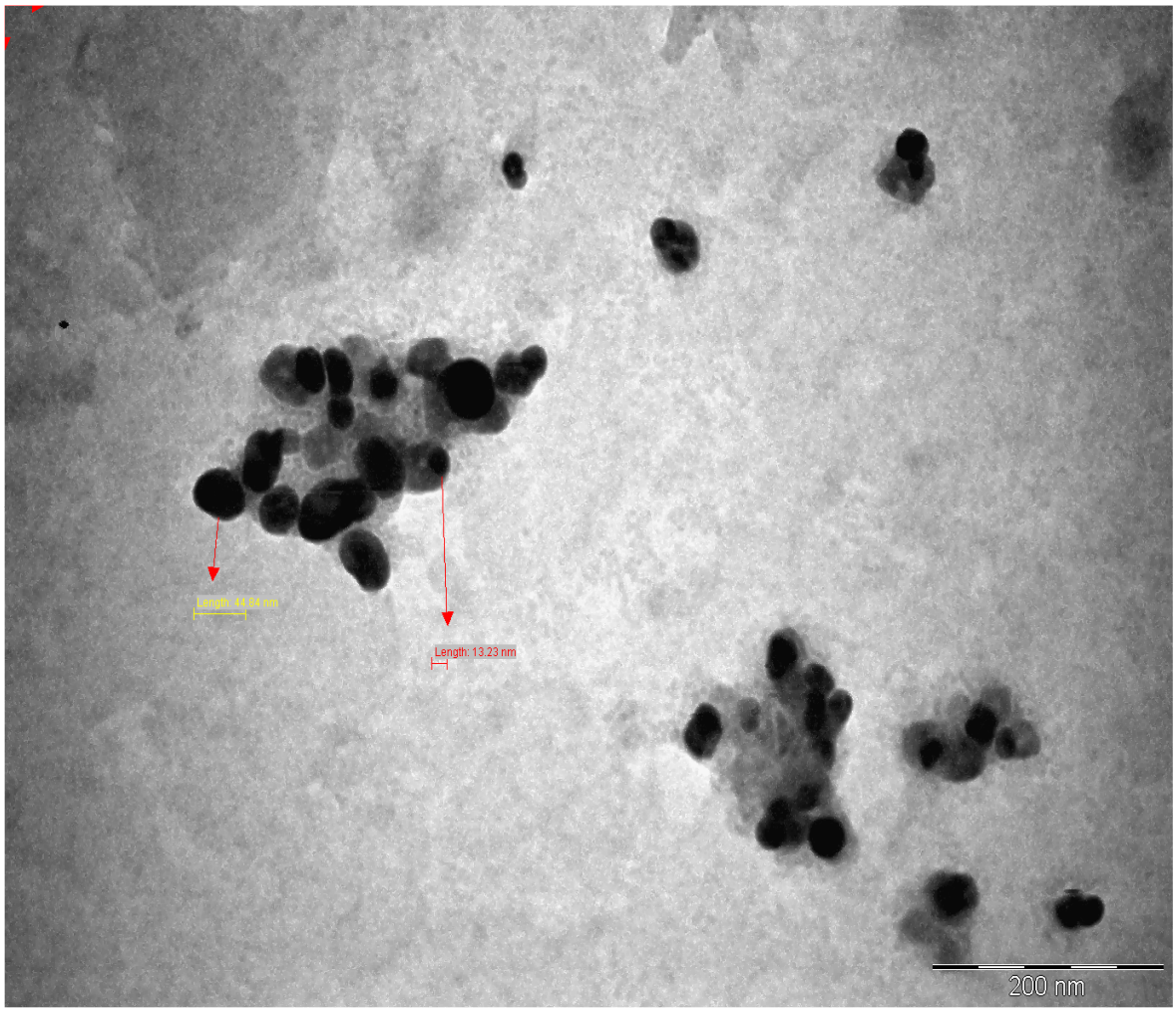
The Ethanolic extract of*Phyllanthusniruri* leaves were used to produce silver nanoparticles and the reduction of silver ions into silver particles during exposure to the plant extract is followed by colour change.

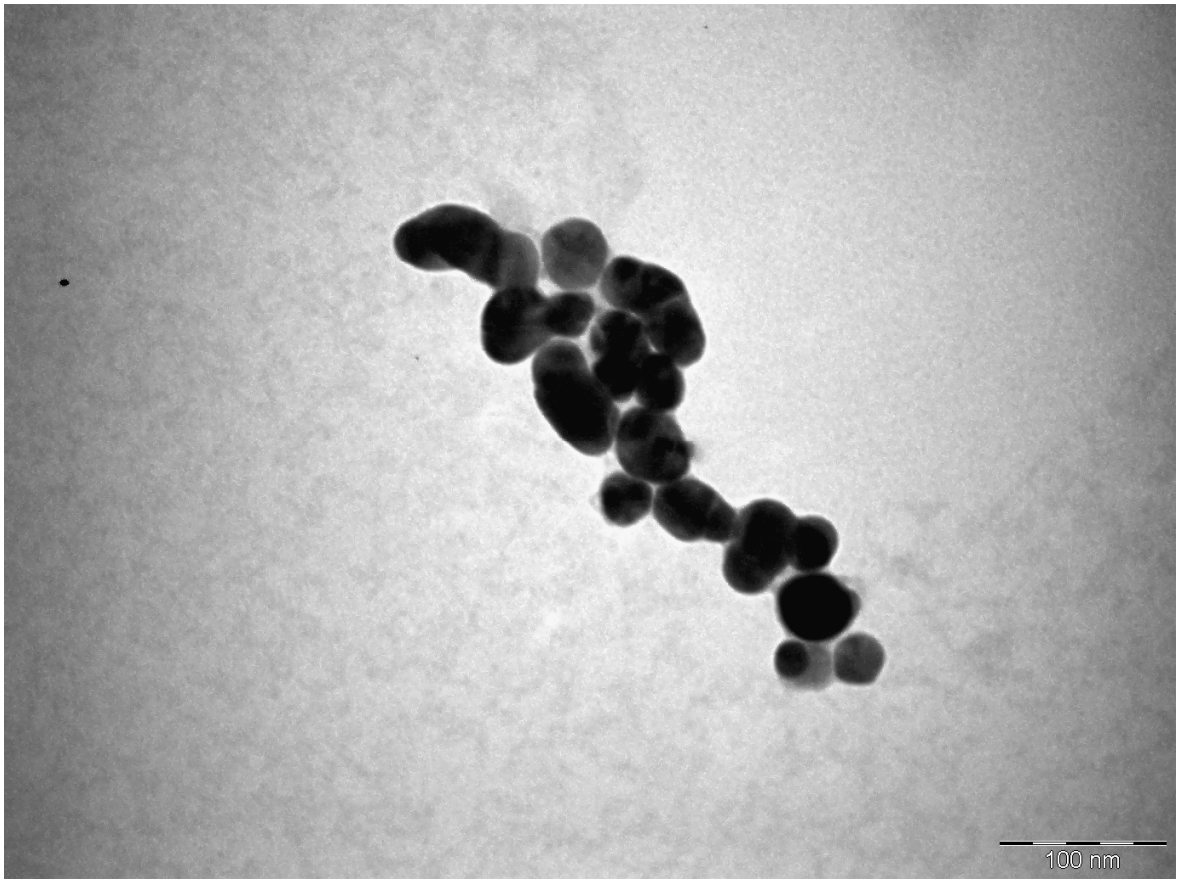
It is well known that silver nanoparticles exhibit yellowish brown colour in aqueous solution due to excitation of surface plasma vibrations in silver nanoparticles. As the ethanolic extract of *Phyllanthusniruri*leaf was mixed in the aqueous solution of silver ion complex, it started to change the colour from green to yellowish brown due to reduction of silver ion which may be the indication of formation of silver nanoparticles.

Almost all the herbal mediated silver nanosolutions after incubation time were showed the colour change from light to dark colour.

Fig 1: A)Green colour shows ethanolic extract of *Phyllanthusniruri* leaf. B) Brown colour shows the silver nanosolution.

TEM analysis





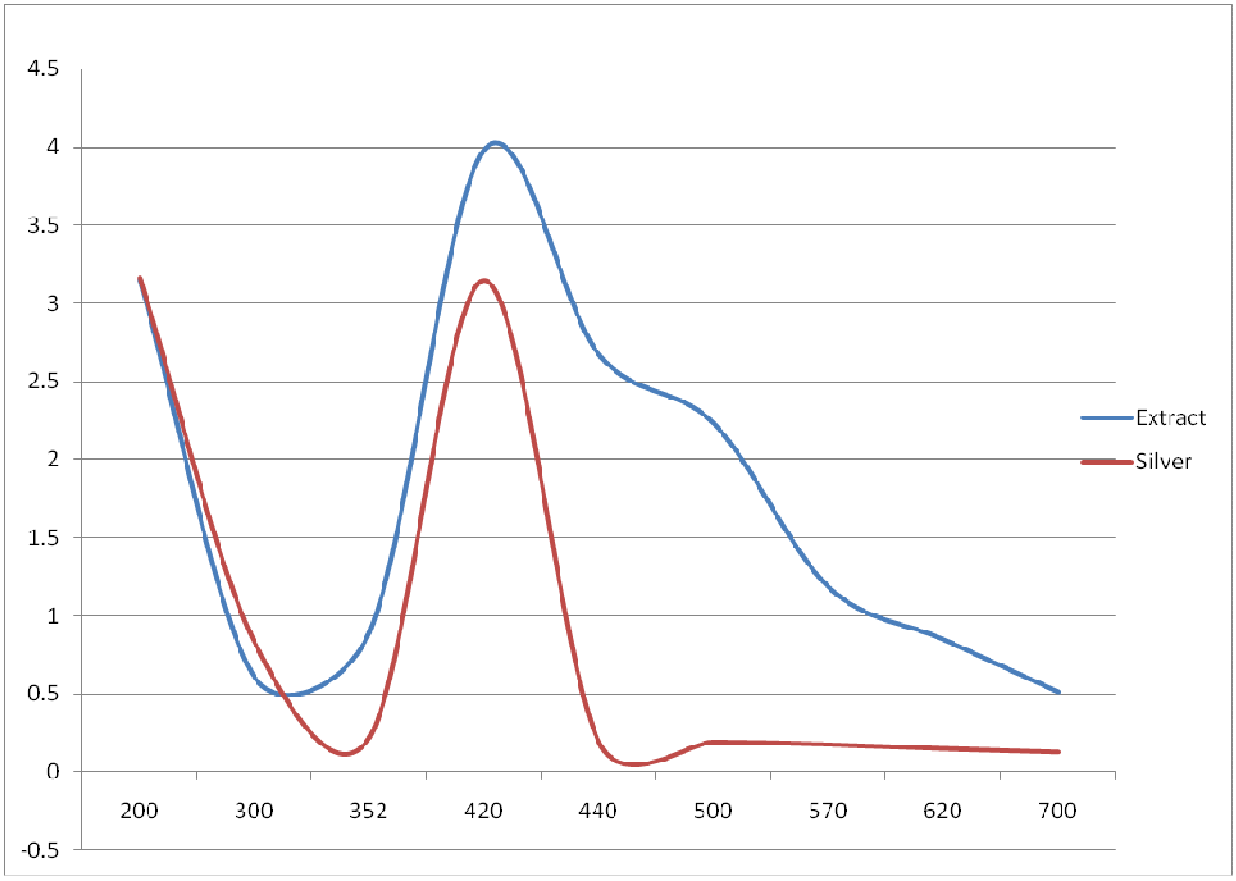
The TEM analysis was done and the sample were prepared on a carbon coated grid by just dropping a very small amount of sample on the grid extra solution was removed by using the blotting paper and it is allowed to dry by putting it under incubator.Thus by using this the result can be obtained at a range of 32-53nm in size of nanoparticles.

Fluorescence Spectra analysis

Spectrum read at nM/particulants	Ethanolic extract	Silver nanoparticles
200	3.156	3.166
300	0.609	0.830
352	0.899	o.222
420	3.989	3.150
440	2.676	0.190
500	2.236	0.192
570	1.188	0.176
620	0.853	0.149
700	0.517	0.128

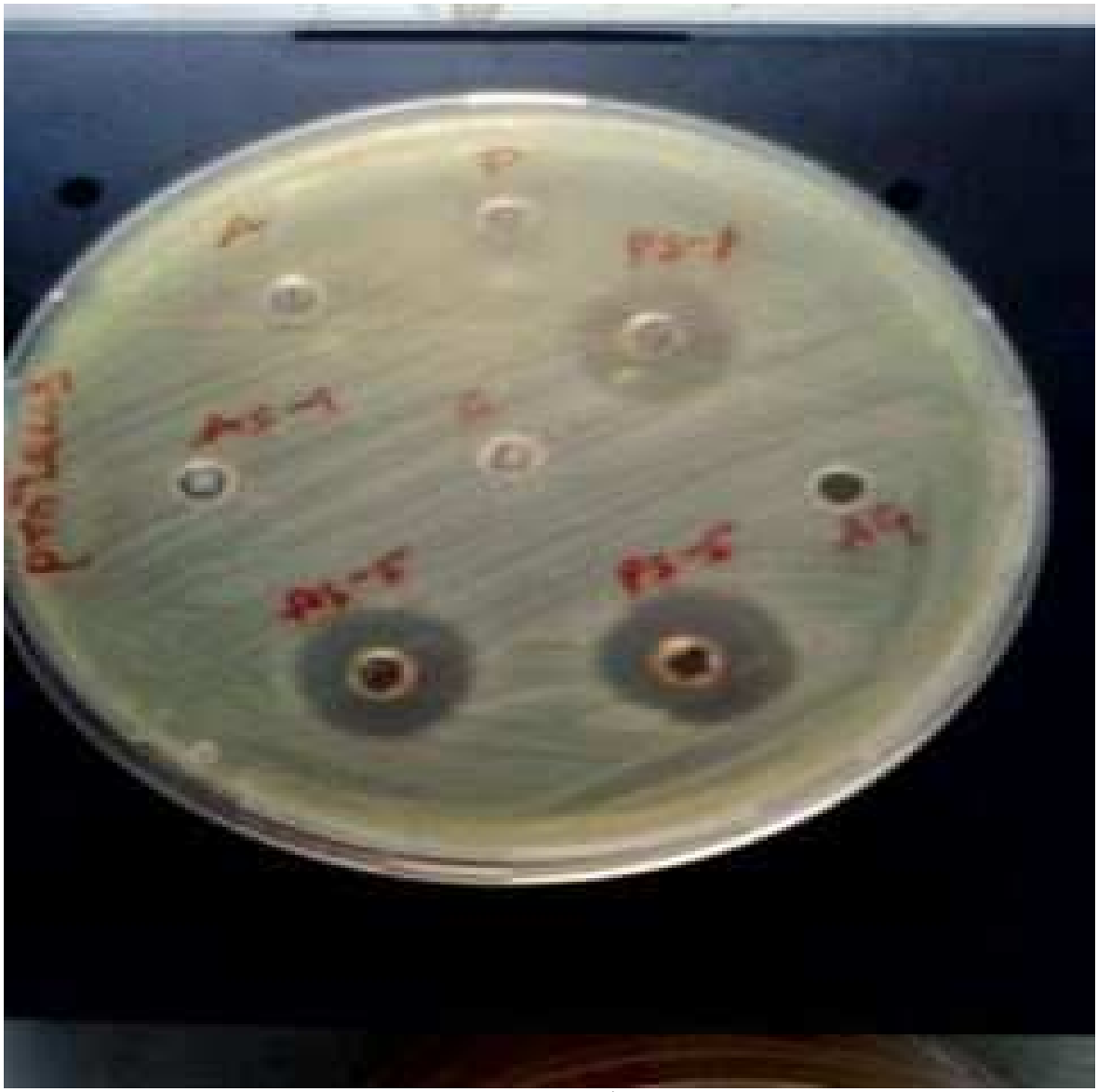
Antimicrobial activity:
The Fluoresence spectra analysis can be done by using Fluoresence spectrometer in which the wavelength are taken from 200-700nm.Ethanolic extract sample and the silver nanoparticles

The sample was taken in the spectrometer and the readings are noted for each wavelength by using those readings the graph can be plotted.This graph shows the variation among the ethanolic extract and the silver nanoparticles.

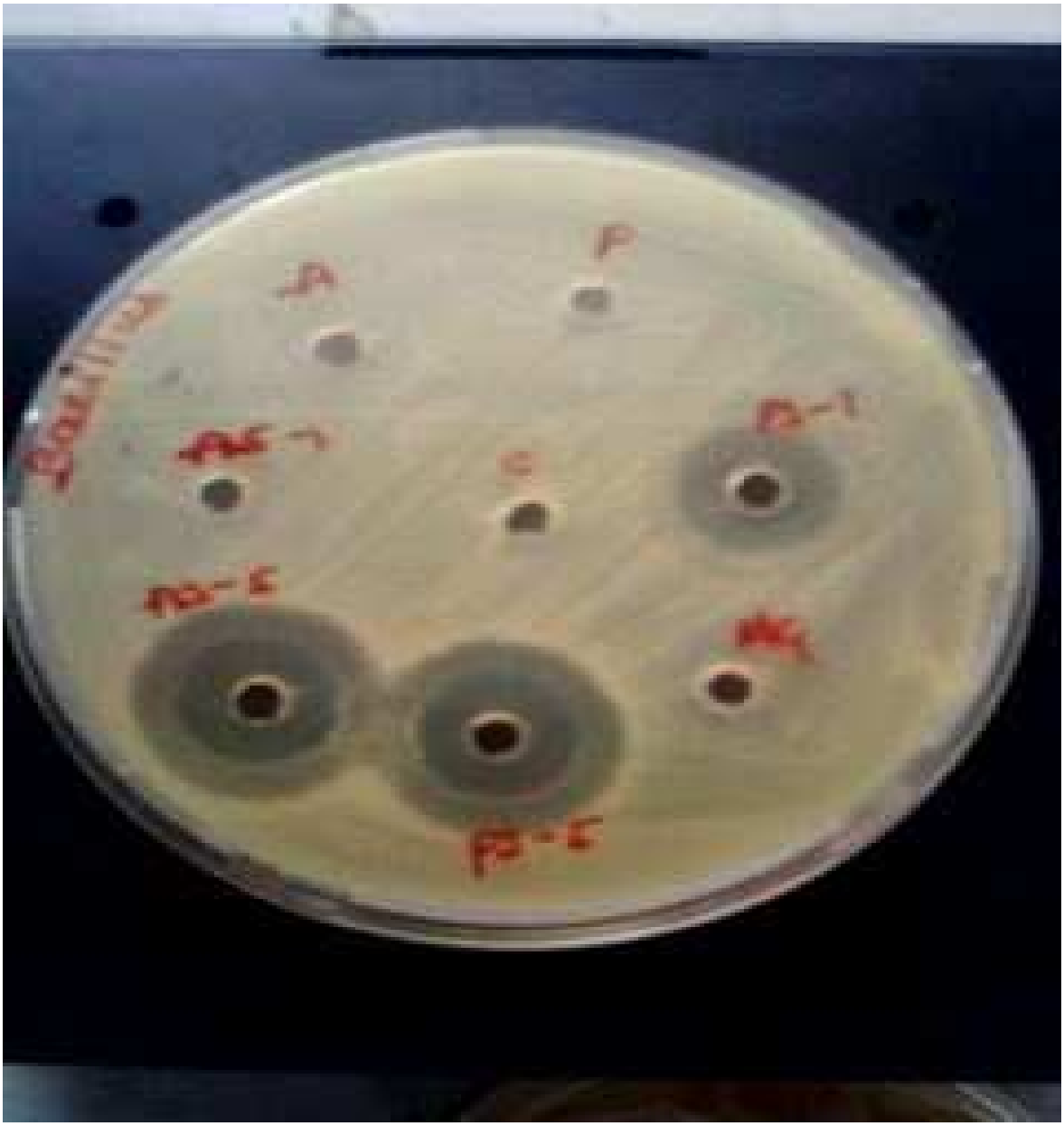


Antibacterial activity

The anti microbial activity of silver nanoparticles from *Phyllanthusniruri* was assessed using the standard well diffusion method with help of Blood Agar Base medium.Thus by using this base the organism were grown, measured and tabulated.



Proteus species



Bacillus species

ORGANISMS	INHIBITION ZONE(mm)		
	SILVER(5mg)	SILVER(1mg)	EXTRACT
Staphylllococcus	12	17	15
Proteus	15	15	16
Bacillus	15	32	30
Salmonella	12	13	13

DISCUSSION

The *Phyllanthusniruri* was found suitable for the synthesis of silver nanoparticles.In this the antimicrobial activity and cytotoxicity effect of silver is identified.Hence there are a number of studies in the field of silver nanoparticles by using different type of procedures and species *Dioscoreabulbifera, Phyllanthusamarus.*Silver ion and silver based compounds are highly toxic to microorganisms showing a strong biocidal effect against microbial species because these are highle reactive species with a large surfacearea.There has not been a consistent explanation for the antimicrobial mechanism of silver.although application of silver to burn wounds has been done for more than a century.Apronoumced antimicrobial effect of antibiotics in combination with silver nanoparticles was observed.This clearly indicates that enhancement of efficacy was due to the synergistic antibacterial action between antibiotics and silver nanoparticles.Silver nanoparticles facilitate the transport of antibiotics to the cell surface acting as a drug carrier.More recently it is shown that silver chelation prevents unwinding of DNA.Silver nanoparticles are composed of silver atoms.Silver nanoparticles are larger in size than silver ions, which makes them react with more molecules,leading to more antimicrobial activity.

CONCLUSION

Nnanotechnology has traditionally been thought of as something from science fictions or ana advance of the future.however more applications are being developed that can offer practical uses across several industries.The following takes a look at number of areas in which nanotechnology is making an impact.

Antimicrobials
Some of the earliest medical uses of nanotechnology have involved antimicrobial coating often made of nanoparticle silver,on wound dressing to prevent infection and on things like catheters to prevent the formation of biofilms.There has even been work on application of silver nanoparticles solution directly to wounds.

Orthopedics
Orthopedic implants are increasingly using nanostructures coating that allow cell to colonize their surface,this not only reduce the problem with rejection but also improve fixation in bone.Otherorthopaedic application include the use of nanotubes for elution of antibiotics and other drugs on implants .

Dentistry
It is not uncommon to think that only orthopedic surgeons get involved with fastening things to bone,but dental surgeons do it all the time.A traditional material for dental implants is Titanium,because its compatible with the body and give time .it mainly depend on the surface characterisation of the implant.

Soft tissue repair
Nano fiber based self assembling tissue scaffolding that seems to aid the repair of damaged spinal cord neurons,enabling paralyzed mice to walk again.display heparin chains and greatly stimulate angiogenesis to aid wound healing.

Other coating application
While most nanoparticulate coatings are designed to fight infection or enhance biocompatibility, Intended application include guide wirs in catheters,orthodontic wires and braces for teeth straightening and coating for artificial joints and hips.

*Phyllanthusniruri*leaf extract was found suitable for the synthesis of silver nanoparticles.The reduction of Silver ions by the leaf extract resulted in the formation of stable nanoparticles with spherical and cubic morphologies which range from 32-53nm in size.The concentration of leaf extract and metal ions play an important role in the green synthesis of Ag nanoparticles.The Spectroscopic characterisations using UV-Vis, SEM, and particle size analyzer were useful in proving the formation of nanoparticles and also in confirming their size, shape, and composition.FTIR evidenced the formation and stability of the biosynthesised silver nanoparticles which can be studied further to understand the chemical and molecular interaction which could be responsible for nanoparticle synthesis.Thus it can be further be applied in various biomedical and biotechnological fields and their properties and applications can be further explored.

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