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Plant mediated synthesis of silver nanoparticles from leaf extract of *Phyllanthus amarus* L. and their antibacterial activity

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Abstract
Aqueous leaf extract of Phyllanthus amarus L. was used for the synthesis of silver nanoparticles to prevent human pathogenic bacteria. The silver nanoparticles formation was confirmed by the color change of plant extracts (SNPs) and further observed with the help of UV – Vis spectroscopy and Fourier Transform Infra-Red (FTIR). The absorption spectra of silver nanoparticles studied using the UV-VIS spectroscopy at 420 nm. The antibacterial activities of silver nanoparticles were studied against human pathogenic bacterial strains such as Escherichia coli, Klebsiella pneumonia, Bacillus subtilis, Pseudomonas aeruginosa, Salmonella paratyphi, and Staphylococcus aureus. The zone of inhibition was higher in Klebsiella pneumonia, Proteus vulgaris and Salmonella paratyphi, the zone of inhibition was lower in Staphylococcus aureus. The antimicrobial activity of the synthesized nanoparticles is studied using the disc diffusion method, which indicates that both Gram positive and Gram-negative microorganisms have been affected by the silver nanoparticles. The observed antibacterial activity could be find important applications in medicine, biology and industry.

Keywords: *Phyllanthus amarus L.* Silver nitrate, UV, FT-IR, FE-SEM, microorganisms

Introduction

Silver nanoparticles have unique optical, electrical and thermal properties and are being incorporated into products that range from photovoltaics' to biological and chemical sensors. Examples include conductive inks, pasts and filler which utilize silver nanoparticles for their high electrical for conductivity, stability, and low sintering temperatures. Additional applications include molecular diagnostic and photonic devices, which take advantages of the novel, optical properties of these nanoparticles for antimicrobial coatings, and many textiles, keyboards, wound dressings and biomedical devices now contain silver nanoparticles that continuously release a low level of silver ions to provide protection against bacteria.

The development of green process for the synthesis of nanoparticles is developing into an important branch of nanotechnology, because biological methods are considered safe and ecologically sound for nanomaterial's fabrication as an alternative to conventional physical and chemical methods. The green synthesis techniques are generally synthetic routes that utilize relatively non-toxic solvents such as water, biological extracts, and biological systems and microwave assisted synthesis. The plant constituents such as geranial possess reducing property and reduce Ag+ to silver nanoparticles with a uniform size and shape in the range of 1 to 10 nm with an average size of 6 nm.

Medicinal plants have been used all over the world for the treatment and prevention of various ailments, particularly in developing countries, where, infectious diseases are endemic and modern health facilities and services are inadequate (Zaidan *et al.*, 2005). The medicinal actions of plants unique to particular plant species or groups are consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Wink, 1999; Janakiraman *et al.*, 2012). Screening of active compounds from plants leads to the invention of new medicinal drugs which have efficient protection and treatment roles against various diseases including cancer and alzheimer's disease (Sheeja *et al.*, 2007; Mukherjee *et al.*, 2007). Plants remain a vital source of drugs and now-a-days much emphasis has been given to nutraceuticals.

The *Phyllanthus* genus of the family Euphorbiaceae was first identified in Central and Southern India in 18th century. It is commonly called carry me seed, stone-breaker, windbreaker, gulf leaf flower or gala of wind (Bharatiya, 1992) [5]. There are over 300 genera with over 5000 species in the Euphorbiaceae worldwide. *Phyllanthus* has about 750-800 species, including *P. amarus* (Schumm and Thonn) found in tropical and subtropical regions worldwide. Green medicine is safe and more dependable than the costly synthetic drugs, many of which have adverse side effects (Joseph and Raj, 2010a) [10].

Several compounds including alkaloids, flavonoids, lignans, phenols and terpenes were isolated from this plant and some of them interact with most key enzymes. In traditional medicine, it is used for its hepatoprotective, anti-diabetic, antihypertensive, analgesic, anti-inflammatory and antimicrobial properties (Adeneye *et al.*, 2006a, b) [1, 2]. *Phyllanthus amarus* leaf extract as a hepatoprotective agent. The plant is also used in the treatment of stomach disorders, skin diseases and cold (Kokwaro, 1976; Iwu, 1993) [15, 8]. It has anti-diarrhea effect (Odetola and Akojenu, 2000) [20]. Its anti-viral activity against hepatitis B virus has been established (Thiyagarajan *et al.*, 1988; Wang *et al.*, 1995) [36] anti-carcinogenic (Joy and Kuttan, 1998) [12] and antimutagenic activities (Joy and Kuttan, 1998) [12]. It also

has anti-nociceptive and anti-inflammatory activities (Kassuya *et al*., 2003) [13] antidiabetic and antilipidemic potentials (Adeneye *et al*., 2006a, b) [1, 2]. *P. amarus* has been reported to include antioxidant, antiviral, antibacterial, hypoglycemic, cancer suppressive and anthelmintic effects (Fig. 1).

Phyllanthus has been used in Ayurvedic medicine for over 2,000 years and has a wide number of traditional uses. This includes employing the whole plant for jaundice, gonorrhea, frequent menstruation and diabetes and using it topically as a poultice for skin ulcers, sores, swelling and itchiness. The plant is bitter, astringent, cooling, diuretic, stomachic, febrifuge and antiseptic. It is useful in dropsy, jaundice, diarrhea, dysentery, intermittent fevers, diseases of urino-genital system, scabies ulcers and wounds. The young shoots of the plant are administered in the form of an infusion for the treatment of chronic dysentery. Its efficacy in the field of gastro intestinal disorders like dyspepsia, colic, diarrhea, constipation and dysentery is undisputed. In females it is used as a galactagogue, in leucorrhoea, menorrhagia and mammary abscess. In skin conditions, especially scabby or crusty lesions, bruises, wounds, scabies, offensive ulcers and sores, edematous swellings, tubercular ulcers and ringworm, it has been utilized with good effect since many years. It is applied effectively in intermittent fevers and gonorrhea as well as in ophthalmia and conjunctivitis. It has a urolithic property, dissolving renal calculi. Also, used in cough, asthma and other bronchial affections. It's antifungal, antiviral and anti-cancerous properties have also been demonstrated in experimental animals.

The powdered leaves of *P. amarus* were used in clinical studies evaluating its usefulness in patients suffering from chronic damage to the liver due to the protracted hepatitis B virus infection. This type of infection results in inability of the body's immune system to eliminate the virus from the liver cells. This condition is described as a carrier state, because a continuously harbors the virus. Some of the components of the virus detectable in the carrier state in the blood are: HBsAg or the surface antigen of the virus and HBeAg or the envelope antigen of the virus. In addition, the carrier state may be confirmed by the presence of antibodies directed against the core of the virus or the anti-HBc antibodies. The powdered leaves of *P. amarus* were given in form of capsules to the patients with chronic viral hepatitis B in a dose of 200 mg three times a day for 30 days. Which resulted to reduce the viral antigen after15-20 days treatment. Due to its antiseptic, styptic, carminative, deobstruent, coolant, febrifugal, stomachic, astringent and diuretic properties of this plant it is very much utilized in traditional medicine.

P. amarus primarily contains lignans (e.g., phyllanthin and hypophyllanthin) (Sharma *et al*., 1993; Somanabandhu *et al*., 1993) [28] geraniin and 5 flavonoids (quercertin, astralgin, quercertrin, isoquercitrin and rutin). It also contains minor compounds like hydrolysable tannins like phyllanthusin D (Houghton *et al*., 1996) [7], amarin (Foo, 1993) [6] amarulone (Rao and Bramley, 1971) [24], amarinic acid and alkaloids like ent-norsecurinine, sobubbialine, epibubbialine; diarylbutane, nyrphyllin and a neolignan, phyllnirurin. The present study was aimed to synthesis silver nanoparticles from *Phyllanthus amarus* and determine their pharmacological efficacy against human pathogens.

Materials and Methods

Plant Taxonomic Positions

Kingdom: Plantae
Division: Angiosperms
Class: Dicotyledoneae
Order: Tubiflorae
Family: Euphorbiaceae
Genus: *Phyllanthus*
Species: *amarus*
Hindi: Jamgliamli, Jaramla
Malayalam: Kilarnelli, Kilukanelli
Tamil: Kilanelli, Kilakkainelli
Sanskrit: Bhumyamalaki
Telugu: Nelausirika

Fig 1

Preparation of leaf extract

Fresh leaves of *P. amarus* were collected, then washed thoroughly with tap water and distilled water several times to remove the dust and dried under room temperature for 2 weeks. The dried leaves were cut into small pieces and ground to very fine powder. 5 g of *P. amarus* leaf powder was boiled in 100 mL of distilled water at 80°C for 10 mins and filtered in Whatmann No: 1 filter paper. Finally, the prepared extract solution was cooled at 4°C and stored for further synthesis of nanoparticles.

Synthesis of silver nanoparticles

1 mM aqueous solution of silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles. 100 ml of leaf extract was taken in a separate conical flask and 1 mM $AgNo_3$ww added and then heated after 20 min until colour changed from the light green yellow to dark or golden brown colour which indicates the formation of Ag NPs.

For as a control, the same procedure is followed without leaf extract.

Characterization of silver nanoparticles by using UV – Vis Spectrophotometer Analysis

The reduction of pure Ag+ ions and biosynthesized silver nanoparticles were analyzed by using UV – Vis spectrum of the reaction medium after 3 hrs diluting small aliquot of the sample into distilled water.

UV-vis spectroscopy is the most important technique and simplest way to confirm the formation of nanoparticles synthesis. Absorbance spectra of colloidal sample were taken in the range of 300 to 600 nm ranges, with the help of UV-vis spectrometer was carried out on UV-VIS Spectrophotometer: 119 Absorption Spectroscopy, with distilled water as a reference.

Fourier Transform Infra-Red Spectroscopy Analysis of Silver Nanoparticles

After complete reduction of $AgNo_3$ by leaf extract sample: the solution was centrifuged at 15,000 rpm for 15 mins to separate nanoparticles from the other bio-organic compounds. Vibernational bonding in the samples was analyzed by using FTIR. The Ag+ based nanoparticles pellet obtained after centrifugation were responded in water and washed thrice with distilled water. The sample was finally observed in FTIR and recorded the chemical bonding formed during nanoparticles biosynthesis. FTIR analysis was performed for leaf extract and silver nanoparticles using FTIR RX1- Perkin Elmer in the wave length range of 400 to 4000 cm^{-1}. The emission spectra were recorded using a LF-45 fluorescence spectrophotometer (Perkin Elmer).

FE - SEM analysis of Silver Nanoparticles

Field Emission Scanning Electron Microscopy (FE-SEM) analysis was done by using Sigma model CARL ZEISS. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min. The SEM grid was allowed to dry and the images of nanoparticles were taken. Spherical and relatively uniform shape of nanoparticles formation was observed.

Antibacterial activity study of AgNP

1. Micro-organisms

The following microbial strains were obtained from the culture collection center, Department of Microbiology KAP Medical College, and Department of Microbiology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. *Escherichia coli*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, and *Staphylococcus aureus* were used for the study. The bacteria were maintained on nutrient broth (NB) and also nutrient agar medium (NAM) at 37^0C.

2. Preparation of Inoculums

The Gram positive and Gram-negative bacteria were pre-cultured in NB at 37^0-C. After overnight incubation, the culture was centrifuged at 10,000 rpm for 5 min, the pellet was suspended in distilled water (DW). The Petri dishes were flooded, 50 ml of sterile distilled water, using sterile cotton buds, micro tips and forceps.

3. Disc diffusion method

A suspension of the organisms was poured in NAM in sterile Petri dishes. The mixture was transferred to sterile Petri dishes and allowed to solidify. Sterile disc (6mm) (Hi-Media Sterile Disc) was dipped in different concentration of silver nanoparticles solution (10 - 50 µl) separately, and a blank were placed on the surface of agar plate. The plates were incubated at 37^0C for 24 hrs. Zone of inhibition for control, SNPs and silver nitrate were measured.

Results and Discussion

UV – Visible Spectrum

The reduction of silver ions into Ag particles during exposure to the plant extract could cold be followed by colour changes. Light yellowish to dark brown or golden brown colour changes in Ag nanoparticle aqueous solution was due to surface Plasmon Resonance phenomenon (Fig - 2). The results obtain in this investigation of very interesting in terms of identification of potential plant for synthesizing the Ag particles. UV Vis spectrograph of the colloidal solution of Ag nanoparticle has absorption spectra of Ag nanoparticle formed in the reaction media has absorbance peak 420nm, broadening of peak (Fig - 2).

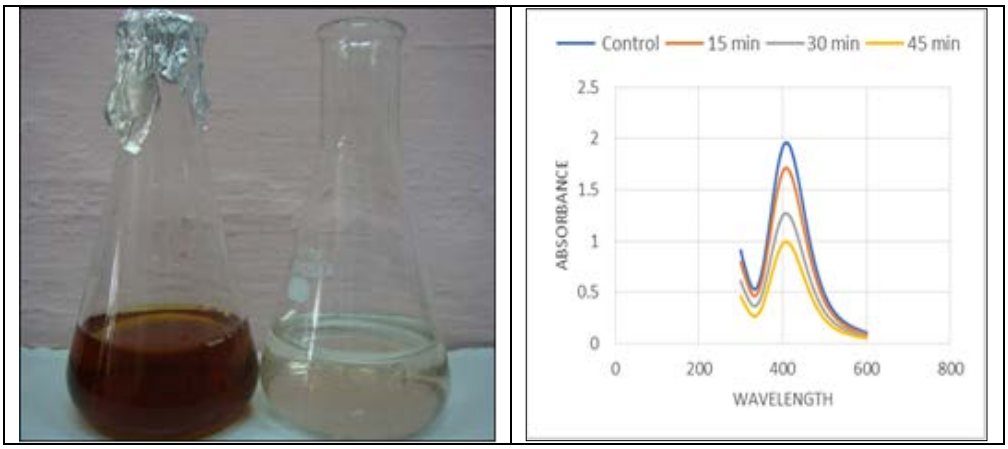

Fig 2: (a) Reduction of silver nanoparticles of *P. amarus*. (b) UV – Visible Spectra of Synthesized Silver Nanoparticle using Black Leaf Extract.

FT-IR Spectroscopy

FTIR measurement was carried out to identify the possible bimolecular accountable for the sterilization and for the reduction of the Ag+ ions and the capping of the bio reduced silver nanoparticles synthesized by the leaf extract after complete reduction of Ag+ ions was centrifuged at 10,000 rpm for 15 min to isolate the silver nanoparticle free from proteins or other compounds present in the solution. The band seen at the 4000–400 cm^{-1} were identified the peak value is 3441.62, 2614.14, 2404.24, 2085.43, 1637.18, 1420.66, 1112.58, 1035.15, 706.46, 623.35cm^{-1}. These absorbance bands were known to be associated with stretching vibrations for –C C=C O, respectively in particular, new peaks were presents in the analysis in 623.35 cm^{-1} bands were most probably from the C=C reduction of silver ions. The total disappearance of this band after the bio reduction might be due to the fact that the polyols were mainly responsible for the reduction of silver ions, whereby they themselves got oxidized to unsaturated carbonyl groups leading to a broad peak at 1637 cm^{-1} for reduction group of

aromatic hydroxyflavones and catechins. It is confirmed the fact that to identify the biomolecules for reduction and efficient stabilization of the metal nanoparticles, the band at 3441.62cm^{-1} corresponds to O–H, as also the H-bonded alcohols and phenols. The peak at 623.35cm^{-1}indicates carboxylic acid. The band at 1637.18cm^{-1} states primary amines. The band at 1637.18cm-1 corresponds to C–C stretching aromatics, while the peak at, 623.35cm^{-1} states C–H rock alkenes and bands at 1637.18 and 623.35cm^{-1}indicate the presence of C–O stretching alcohols, carboxylic acids, esters and ethers. An immediate reduction of silver ions in the present investigation might have resulted due to water soluble phytochemicals like flavones, quinones, and organic acids present in the callus, silver reduction and fabrication accomplished due to Phytochemicals (flavonoids or other polyphenols), some proteins and metabolites such as terpenoids having functional groups of alcohols, ketones, aldehydes and carboxylic acids present in callus may be considered as a significant advance in this direction.

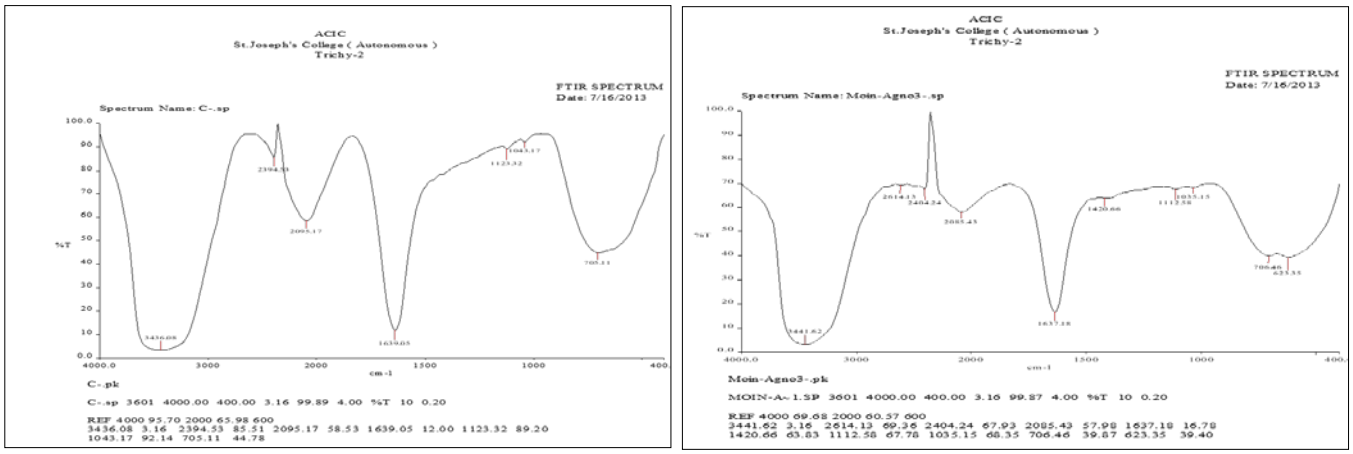

Fig 3: FTIR spectrum of silver nanoparticles synthesized using *P. amarus*a). a) Control b). Synthesized silver nanoparticles using plant.

Table 1: FT – IR Interpretation

S. No	Wavelength	Functional Class	Intensity	Assignment
1.	3441.62	Amines	Weak	N-H (Primary Amines)
2.	2614.13 & 2404.24	Alkanes	Strong	CH_3, CH_2& CH
3.	2085.43 & 1637	Alkanes	Strong	CH_3, CH_2& CH
4.	1420.66, 1112.8, 1035.15, 706.46 & 623.35	Carboxylic Derivatives	Medium	(N-H) Very Broad

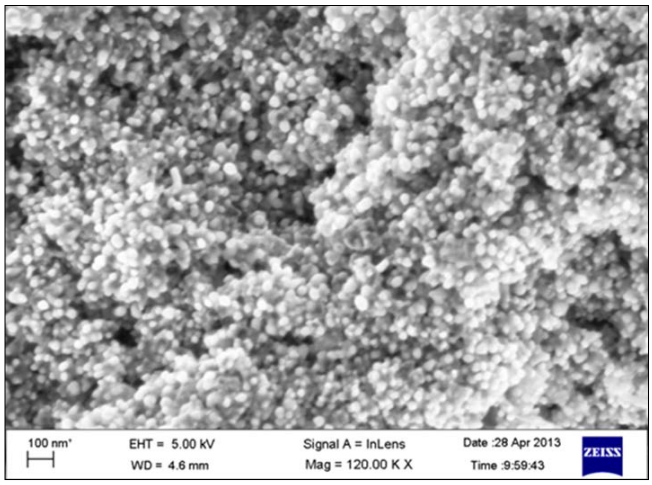

Fig 4: FE-SEM image of $AgNO_3$ by *Phyllanthus amarus*

FE-SEM analysis of Silver nanoparticles

FE-SEM has been employed to characterize the size, shape and morphology of formed silver nanoparticles. The resolution of high-resolution FE-SEM approaches a few nanometers and the nanometers and the instruments can operate at magnifications that are easily adjusted from -10 to over 30,0000. FE-SEM analysis was done using ZEISS-FE-SEM instruments. Thin film of the sample was prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and thin film on the FE-SEM grid was allowed to dry by putting it under normal lamp for 5 hrs. From the images, it is evident that the morphology of silver nanoparticles is nearly spherical it is in agreement with the shape of surface plasma resonance band in the UV – Visible spectra. The average particle size analyzed from the SEM images is observed to be 60 – 100 nm in *P. amarus* (Figure - 4).

Antibacterial Microbial Susceptibility Test (Disc diffusion assay)

Silver is well known as one of the most universal antimicrobial substances. The antimicrobial activity AgNP of *P. amarus* was assayed against *E. coli, K. pneumonia, P. vulgaris, P. aeruginosa* and *S. typhi*. The synthesized silver nanoparticle was highly effective in their activity against six bacterial strains which showed clear inhibition zone. Standard antibiotic disc Streptomycin was used as a control. It's clearly indicates that antibacterial effect of silver nanoparticle obeyed a dual action mechanism against bacteria.

The 6 mm diameter discs of Hi-Media sterile discs were used this technique. The dilutions of biosynthesized AgNps varying from 30 µl, 40 µl and 50 µl were prepared in the silver nanoparticle synthesized sample. 20 mL of molten sterilized Nutrient Agar solution was poured into each Petri plates and six organisms were grown in them.

Table 2: Antibacterial activity of silver nanoparticles synthesized using *P. amarus* against some human pathogenic organisms.

S.No	Tested Pathogenic Microbial Strains	Antibiotic Disc (Streptomycin)	Silver nanoparticles synthesized using Leaf extract of *P. amarus* Zone of Inhibition (mm)				
			$AgNO_3$ Stock	10 µl	20 µl	30 µl	40 µl
1.	*Escherichia coli*	Resistant	13	Resistant	Resistant	11	13
2.	*Klebsiella pneumonia*	Resistant	12	Resistant	Resistant	12	14
3.	*Bacillus subtilis*	Resistant	14	Resistant	10	11	14
4.	*Pseudomonas aeruginosa*	Resistant	11	Resistant	12	12	14
5.	*Salmonella paratyphi*	Resistant	12	Resistant	10	10	13
6.	*Staphylococcus aureus*	Resistant	11	Resistant	Resistant	10	11

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) values of the leaf extract against tested bacteria were shown in table – 1. The values were present in 40 µg/ml and respectively, against the tested Gram-positive bacteria, ranged from 20 to 40 µg/ml and against Gram-negative bacteria. Potency of antibacterial activity of plant extract against these bacteria exposed in MIC indicated the plant extract is more effective against Gram-positive at lower concentration than that against Gram negative bacteria.

Conclusion

The Ag nanoparticles were synthesized using leaf extract of *P. amarus.* Further, the above Ag nanoparticles revealed to possess an effective antibacterial property against human pathogenic organisms. The present study emphasizes the use of plant medicine for the synthesis of Ag nanoparticles with potent antibacterial effects. The synthesized AgNPs exhibits the best antibacterial activity on gram-positive and gram-negative bacteria.

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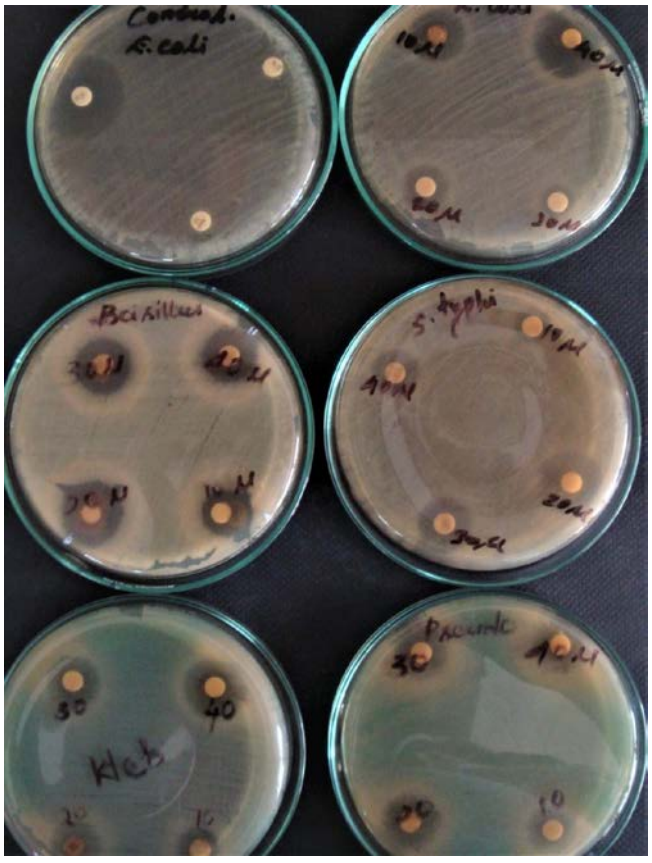

Fig 5: Antibacterial activity of silver nanoparticles synthesized by leaf extract of *Phyllanthus amarus* against various pathogenic bacterial strains.

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