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"SYNTHESIS, CHARACTERIZATION & ANTIBACTERIAL EFFICACY OF SILVER NANOPARTICLES USING A HERBS *SPHARANTHUS INDICUS* FRUIT EXTRACT AND *PERILLA FRUTESECENE* FLOWER EXTRACT AGAINST PATHOGENIC BACTERIA"

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KEYWORDS:

Nanotechnology, *perilla frutsecene, Sphaeranthus indicus*, AgNPs, antibacterial activity, *E.coli,S.aureus*.

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ABSTRACT

Phyto-mediated synthesis of nanomaterial used as a reducing agents due to its ecofriendly nature and low cost. This is a cost-effective, stable for a longtime and repeatable aqueous synthesis process to acquire a self-assembly Ag Nanotechnology widely used in different areas for example drug delivery, imaging, control of bacterial growth. These having various advantages including short reaction time, excellent yield and green condition for various plant mediated synthesis. The aim of present study silver nanoparticle synthesized from flower and fruit aqueous extract of *perilla frutsecene (Laminaceae)* and *Sphaeranthus indicus (Asteraceae)* which act as a caping agent and reducing caping agent that reduce silver ions into AgNPs. Silver nano particle prepared by mixing 10mm silver nitrate solution with aqueous plants extracts. The silver nanoparticle formation was characterized by a technique UV-Vis absorption spectroscopy in the range from 350-650nm, further confirmed by their change of color to dark brown. The phytochemical screening of both plants extract was carried out for detection of the bio active marker like alkaloids, flavonoids, saponin, tannin and polyphenols. The antibacterial activity of synthesized AgNPs against dissimilar pathogenic bacteria was determined via well diffusion method the silver nanoparticle were found to exihibit effective activity against both pathogenic bacteria *E.coli, S.aureus*.

INTRODUCTION:

According to Traditional Indian healthcare systems medicinal plants are used to cure human disease. Ayurveda and Siddha are still in practice where plant-based medicines are used to cure various diseases. These medicines widely used because of its more effectiveness and less side effects. (1) The phytoconstituents of herbal medications have more advantage when its combined with several other substances that appear to be inactive. That is much superior then of its isolated and pure active components. They play an important to enhance immunity against the pathogenic bacteria. (2) Now a days Nanotechnology have a excessive impact in many research areas in the natural science. (3) Due to their major characteristics for using in several applications including pharmaceutics and chemical reactions. (4) Various methods have been used for the preparation of silver nanoparticles which can be physical, chemical, or biological methods. Physical process required high energy consumption and chemical processes required toxic chemicals, that can cause unfavorable effects on the environment. Biological methods for synthesis of AgNPs using microorganisms, fungus, and plant extract have been suggested as ecofriendly than the chemical and physical methods. Thus, the benefits of using plant extract are because it provides accessible, safe and non-toxic compounds. (5) The biologically synthesized silver nanoparticles were more toxic against different multi-drug resistant pathogens.(6) The properties of metallic nanoparticles vary according to their shape,size and morphology.(7) The techniques were used for obtaining nanoparticles including plant extracts, and microorganisms as reductants and capping agents attractive for nanotechnology which involving for the plant mediated synthesis.(8) In general, nanoparticles size less than 100 nm are referred to as NPs such important member of the noble metal (Ag NPs).(9) Silver nanoparticles (Nps) have showed most effective due to their great antimicrobial effective against bacteria, viruses and other micro-organisms.(10) Silver is a basic element on planet and slightly harder than gold. Silver having three different oxidation states: Ag0, Ag2+, Ag3+.Metallic silver is insoluble in water, but metallic salts such as AgNO3 soluble in water. (11). The Noble-metal nanoparticles exhibit incredible physicochemical, and biochemical characteristics. Medicinal plants have served as rich sources of pharmacologically active substances. Herbs have been used in a various range of purposes, including medicine, nutrition, flavoring, dying, repellents, fragrances, cosmetic, smoking and industrial uses. (12)

Biological Samples

1. ***Sphaeranthus indicus***

Fig 1: *Sphaeranthus indicus*(2)

Sphaeranthus indicus is an annual spreading herb. It belonging to family Asteraceae. It has a round purple flowers. It's scattered all over the plains and wet lands in India and cultivates as weed in paddy fields. (13)

Phytochemical constituents: The various of phytoconstituents were isolated from plant, and flowers of *S.indicus.* Aerial parts of this plant showed presence of an , glycosides, and an alkaloid sphaeranthine and an isoflavone 5, 4'- dimethoxy-3'-prenylbiochanin 7-o-β-galctoside. the flower of *S.indicus* which afforded four new sesquiterpenes one new sesquiterpenes glycosides, one known sesquiterpenes and one known steroid. And another new compound identified. (2)

The essential oil, obtained by steam distillation of the whole herb, contains ocimene, α-terpinene, methyl-chavicol, α-citral, geraniol, α-ionone, β-ionone, d-cadinene, p-methoxy cinnamaldehyde. (14)

S. indicus has long been used in the indigenous medicine. The herb is bitter and hot with a sharp sweet.

Medicinal uses: *S.indicus* has used in the native medicine. The whole herb is used in medication to treat epilepsy, mental disorder & hepatitis. it's also useful for, Spleen diseases , anemia. (13) The flower has various medicinal value, moreover the application of its whole plant is externally beneficial in edema & pain like arthritis, gout & cervical adenopathy, internally is widely usrd in various human diseases such as strong stimulant for digestive system such as appetizer, digestant, laxative. (2)

S.Indicus L. also have medicinal properties such as antiviral, antifungal antibacterial,antipyretic, hepatoprotective, antidiabetic, antioxidant, and anticancer are reported. (14)

2. ***Perilla frutescens***

Fig 2: *Perilla frutescens*(15)

Perilla frutescens is an herbaceous plant.its belongs to the Lamiaceae family. (15) *Perilla* plants have distinctive square stems and four stamens as with most species in the family Lamiaceae. (16)

Phytochemical constituents: *Perilla frutescens* plant contains a number of important phytochemicals such as Rosmarinic acid, Luteolin, Chrysoeriol, Quercetin, Catechin, Caffeic acid and Ferulic acid. (17)

The individually and total phenolic contents were remarkably different, especially those of rosmarinic acid and rosmarinic acid-3--glucoside, which were the predominant compounds in all the perilla cultivars. The other isolated and identified five phenolic compounds (apigenin, luteolin, caffeic acid-3--glucoside, rosmarinic acid, and rosmarinic acid-3--glucoside) from the seeds of *Perilla frutescens*. (18)

Medicinal use: *Perilla frutescens* (L.) have been used as an important herbal medicine for treating various disease including depression, anxiety, tumor, cough, antioxidant, allergy, intoxication, and some intestinal disorders. (19)

Biological analysis of *Perilla frutescens* plant revealed that this plant showed anti-microbial, anti-allergic, anti-cancer, anti-tumor, anti-depression , anti-viral , anti-asthmatic and antioxidant activities.(20). *Perilla frutescens* also used such as antioxidant, anti-allergic, anti-inflammatory, and anti-HIV-1 activity. (21).

Materials and methods:

Collection of plant material: The identification and authentication, fruits of *Sphaeranthus indicus* and flower of *perilla frutsecene* collected from The Himalaya drug company in Dehradun(Uttarakhand), India. The plant was identified and authenticated by Dr. Maya Ram Uniyal. The flower and fruits of selected plants were cleaned and, washed, dried below 60ºC in an oven, grounded to a fine powder. The drug powder was reserved in an airtight container with a level showing the common name, botanical name; part used and date of powder preparation for further analysis.(22)

Preparation of plant extract: 10 g powder is added 100 mL of DW in a 250 ml of flask and then boiled at 50°C for 30 min. Subsequently, the test solution was allowed to cool at a room temperature and filtered by employing simple filtration process through Whatman No.1 filter paper. Filtrate is stored at 4°C temperature for further use for analysis. (23)

Synthesis of Ag Nanoparticles: AgNO3 powder was dissolved in DW to prepare 10mM AgNO3 stock solution from which a series of 1 mM, 2 mM, 3 mM, 4 mM, and 5mM AgNO3 solutions were prepared. AgNO3 solutions were mixed with the aqueous extract of, fruits of *Sphaeranthus indicus* and flower of *perilla frutsecene* at a ratio of 1 :1 (v/v) to a volume of 50mL in a flask. flask was covered with an aluminium foil and was then heated on water bath at 60°C for 5 hours. Furthermore, the sample mixture was stored in the refrigerator for the antibacterial activity and further analysed by using UV Vis spectrophotometer.

Preformulation Study

Solubility Test

Fruits of *Sphaeranthus indicus* and flower of *perilla frutsecene* extract powder about 1mg was taken in a test tube and solubility in Water, Acetone, Ammonia, Ether, hexane and, methanol,were checked.

Characterization of Ag Nanoparticles: The Reduction of pure Ag+ ions were characterized by measuring the UV-Vis spectrum of the reaction after diluting a small aliquot of the sample into DW.

a) **By observing color change:**

The Plants extract added to silver nitrate solution, the color of the sample solution changed from pale yellow to reddish-brown after 5h because of the process of reduction of Ag^+ to Ag° nanoparticles. Colour changes were possible because of Ag ions is reduced due to the effects of heat and produces Ag+ complex. This complex was responsible for changing colour from pale yellow to reddish-brown. This colour change indicates the formation of Ag nanoparticles.

b) **Ultraviolet-visible spectral analysis:**

UV–vis spectra of the AgNPs and the aqueous extract of the each plants give a sharp peak at 420 nm after 3 h incubation. UV–visible spectroscopy is t important techniques for characterization of nanoparticles. (2) This Ag nanoparticles synthesized in each extract solution was analysed using UV-Vis spectroscopy. This was done to determine the characteristics of the peak spectrum of the Ag nanoparticle wavelength prepared for each different $AgNO_3$ concentrations (1mM–5mM) (Figure). This characteristic of Ag nanoparticles normally appears at a wavelength interval of 400–600nm. UV-Vis spectra of Ag nanoparticles synthesized using the fruits of *Sphaeranthus indicus* and flower of *perilla frutsecene* show the absorption peak spectrum with increasing $AgNO_3$ concentration. This results shows that the Ag nanoparticles have formed in the both extract, where the Ag^+ has been reduced to Ag^0.This Ag nanoparticles confined in the extract of *Sphaeranthus indicus* and *perilla frutescence* also exhibit similar characteristics, where the shift of the absorption peak with increasing $AgNO_3$ concentrations. *Sphaeranthus indicus* and *perilla frutsecene* aqueous extract, where the absorption peak is centered on 450–440 nm. (24)

Preliminary Phytochemical screening of synthesized silver nanoparticles

The Presence of secondary metabolites such as Saponins, Tannins, Phenolic Compounds, Flavanoids, Alkaloids and glycosides test was performed for screening the bioactive marker present in silver nanoparticles using the standard procedure. (25)

Test for alkaloids: 2 mL of sample, 1 mL of Meyer's reagent was added. The presence of pale-yellow ppt indicated the presence of alkaloids. (Meyer's test)

Test for flavonoids: 1 ml of sample was taking and 1 ml of 5% liquid ammonia solution was added and mixed. Presence of flavonoids yields a yellow colour.(26)(Alkaline reagent)

Test for Tannins: 5ml of the sample, a few drops of 0.1% Ferric chloride were added to 5ml of the extract. presence of a blue-black colour or brownish green indicates the presence of Tannins.

Test for Saponin: In a test tube about 5ml of an aq. extract of the drug was taken and a few drops of sodium carbonate solution were added, the mixture was shake continuously and was left for three minutes. Honey combs like froth was formed indicating the presence of saponins. (27)

Tests for glycosides: Baljet's Test: Treat the test solution with picric acid or sodium picrate, orange colour formed indicates the presence of glycosides. es.(28)

HPTLC fingerprints profile of *Sphaeranthus indicus* fruits extract and *perilla frutescence* flower extract*:*

Sample preparation: Measure accurately 5gm of sample in a 250ml flat bottomed flask add Methanol and reflux it by immersing in a water bath at 80 °C for 30 minutes. Filter the extract through Whatman no.1 filter paper into a conical flask.

Application: Apply the sample as band,on a pre coated thin layer silica gel plate in a distance of 12mm from the bottom , mark up to a distance of 8.5 cm as a development mark by using pencil.

Mobile phase: Chloroform: Methanol (90:10)(29)

Preparation of development tank: Use camag twin trough development tank (10/10cm). Cover a side of the inside chamber with required size of Whatman no.1 filter paper measure 20 ml of mobile phase and transfer into the chamber along the cover side of the chamber .

Visualization and Documentation: Visualize the dried plate at UV 254 nm and 366nm using Camag Reprostar 3 .The image of the plate capture at UV 254 nm and 366nm.(30)

Antibacterial activity

The bacterial strain *Staphylococcus aureus* and *E.coli were used for the* antibacterial activity. These bacterial cultures were continued on Nutrient agar slants at first incubated at 37 °C for about 18-24 hours and then stored at 4 °C as stock for antibacterial activity. Fresh culture was obtained by transferring a loop full of cultures into Nutrient broth for dilution and then diluted Nutrient broth was transferred to Nutrient agar which was then incubated at 37 °C over night. To test antibacterial activity, the well diffusion method was used.

Culture media Preparation:

The microbiological media preparation was done as per standard instruction provided by the Himedia Laboratries, Mumbai. The media used for antibacterial activity were Nutrient agar and Nutrient broth. The prepared media sterilized at 121 °C at 15 PSI for 20 mins in autoclave.

Plate Preparation: 25 ml of pre autoclaved Nutrient agar was poured into 90 mm diameter pre sterilized petri-plates. These petri-plates were allowed to solidify at room temperature.

Well Diffusion Method:

In vitro antibacterial activities of Aqueous extracts of *Sphaeranthus indicus* and *perilla frutsecene* aqueous extract were determined by standard agar well diffusion method [13]. After the plates solidified , the freshly prepared microbial broth culture suspension (about 20 µL) was spread over the Nutrient agar media using l shaped sterilized glass spreader separately under the aseptic condition using Laminar Air Flow. Well were made in each plate with the help of borer of 6 mm diameter. In these well 50 mc L of Aqueous extract were individually loaded.

The plates were then incubated upright position at 37º C for 18 hours. Two replicates were carried out for each extract against each of the test organism. Simultaneously addition of the solvents in its place of extracts was carried out as negative controls and the Ciprofloxacin(Standard) was used for positive control. After incubation the diameters of the results and growth inhibition zones were measured.(33)

Result and Discussion:

Synthesis of Ag Nanoparticles:

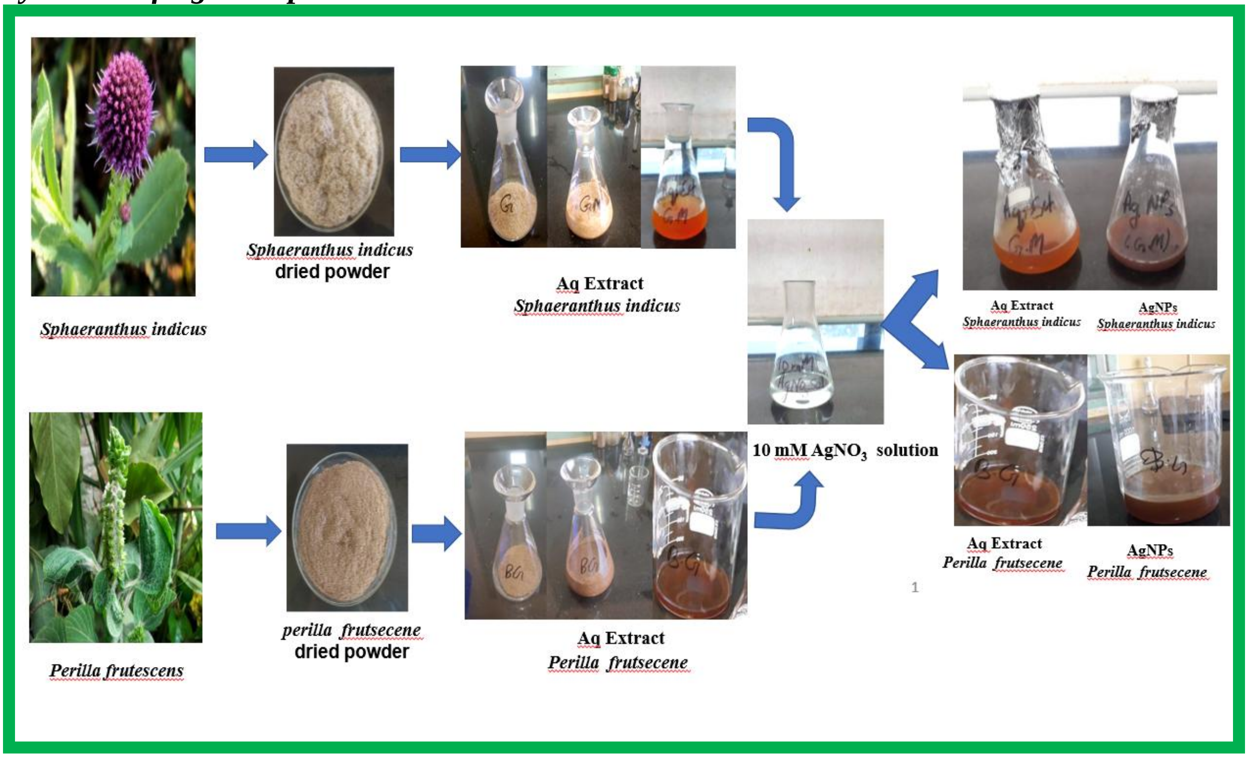


Fig 3: preparation of *Sphaeranthus indicus* and *perilla frutsecene* Aqueous extract and Synthesis of Ag Nanoparticles of GMNPs and BGNPs

PREFORMULATION STUDY

Solubility Test

Solubility test for powdered fruits of *Sphaeranthus indicus,flower of perilla frutsecene* were carried out in different solvents such as Water, Acetone, Ammonia, Ether, Glycerol hexane, chloroform ,methanol, were given in table:

Table: Solubility of fruits of *Sphaeranthus indicus and flower of perilla frutsecene.* in different solvents-

S.no	Solvents	Soluble	Sparingly soluble	Insoluble
1	Water	✓	-	-
2	Acetone	✓	-	-
3	Ammonia	✓	-	-
4	Ether	-	✓	-
5	Hexane	✓	-	-
6	Methanol	✓	-	-

From the solubility test analysis it was found that powdered fruits of *Sphaeranthus indicus,flower of perilla frutsecene* are soluble in Water,methanol,hexane, Ammonia and Acetone and sparingly soluble in Ether. Hence the powdered fruits of *Sphaeranthus indicus* and *flower of perilla frutsecene* extract is more soluble in polar solvents and sparingly soluble in non polar solvents.

Characterization Techniques:

Visible Observation:

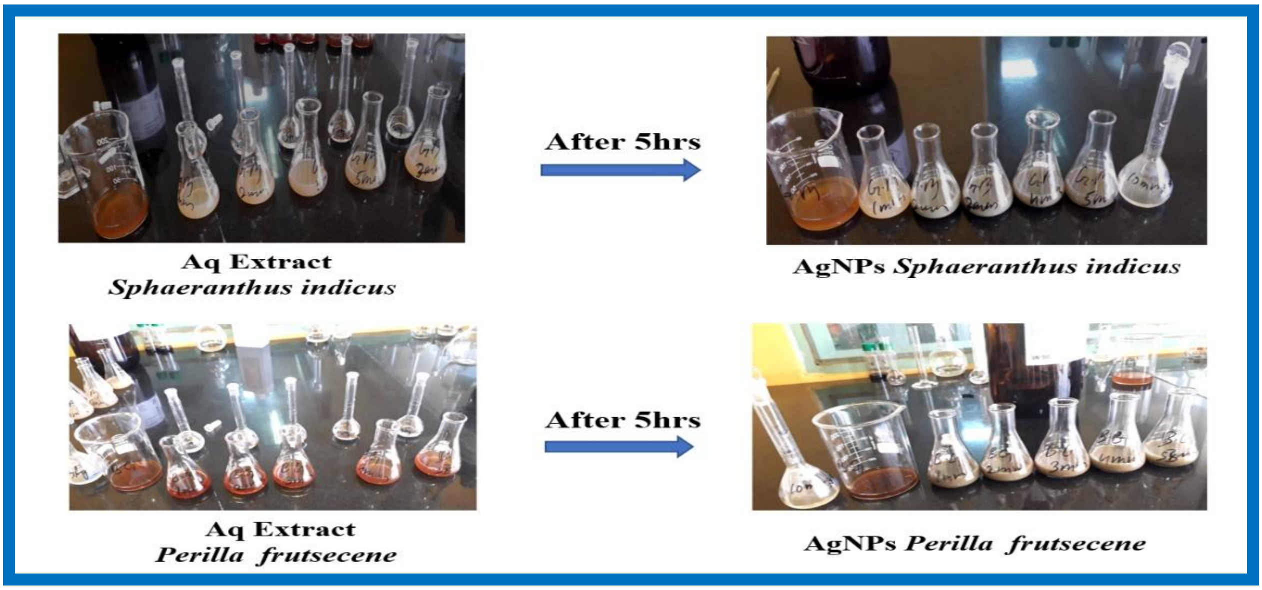


Fig 4: Colour change of plant extract before after addition of $AgNO_3$ (a) ***Sphaeranthus indicus****(b)* ***Perilla frutsecene***

UV-vis spectra analysis:

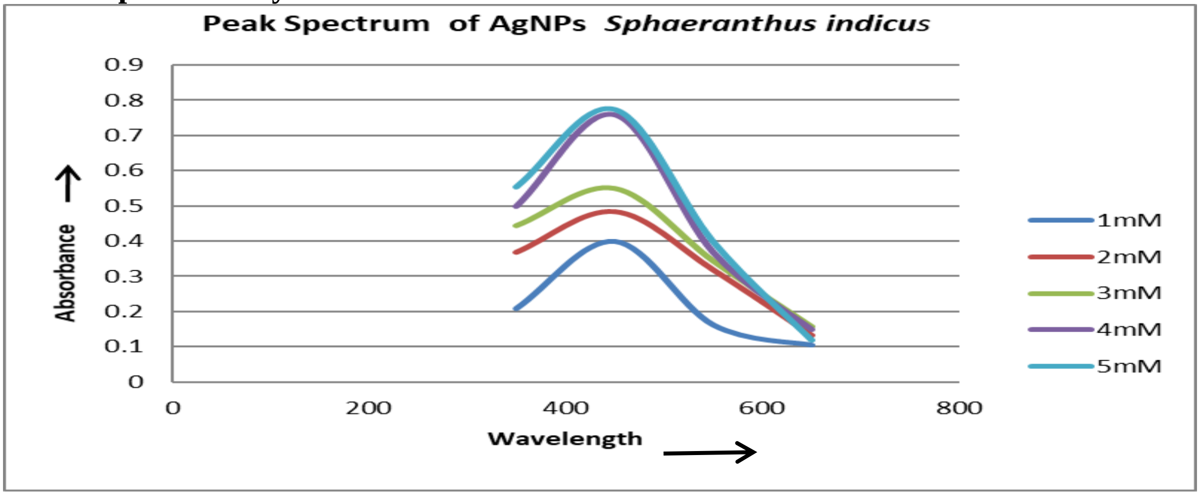


Fig 5: UV-visible absorption peak of *Sphaeranthus indicus* synthesized silver nanoparticles approximately at 421nm.

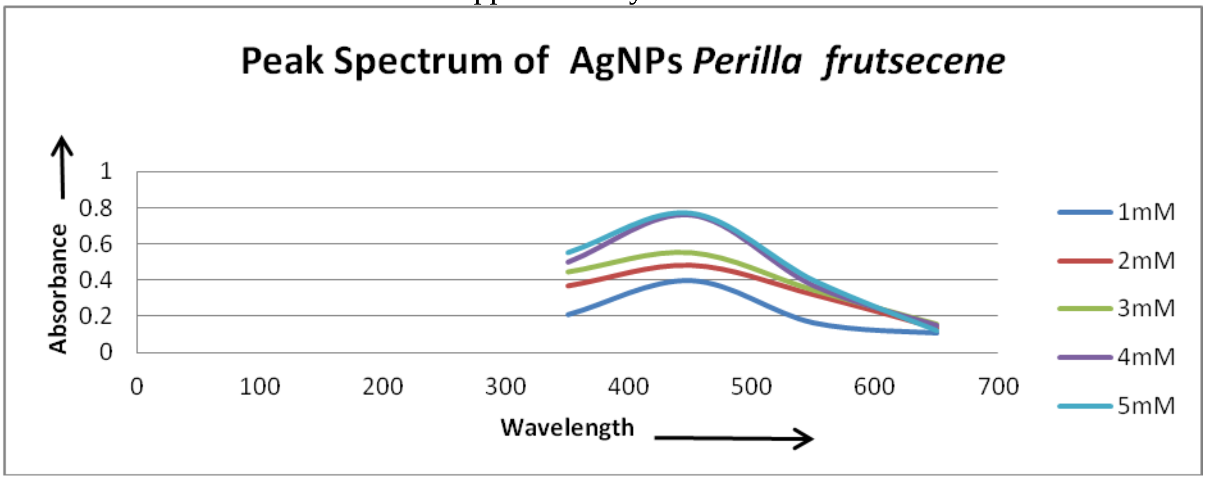


Fig 6: UV-visible absorption peak of *perilla frutsecene* synthesized silver nanoparticles approximately at 421nm

Table 1: Preliminary Phytochemical screening of synthesized silver nanoparticles:

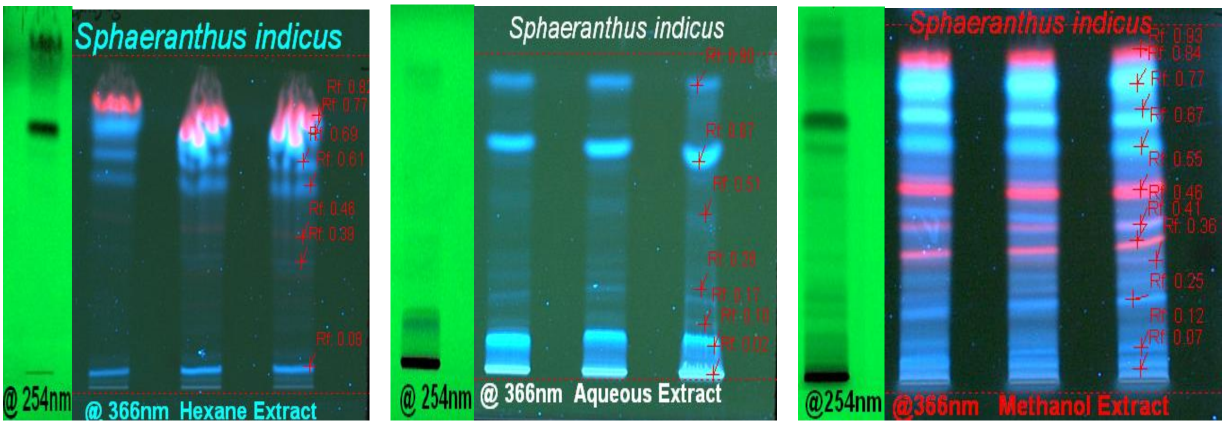
S.NO	Phytoconstituents	GMNPs	BGNPs
1	Alkaloid	(+)	(+)
2	Tannin	(+)	(+)
3	Flavonoid	(+)	(+)
4	Glycosides	(-)	(-)
5	Saponin	(+)	(+)

Presence of phytochemical constituents: (+)

Absence of phytochemical constituents: (-)

HPTLC Fingerprints of plants extract:

a)



b)

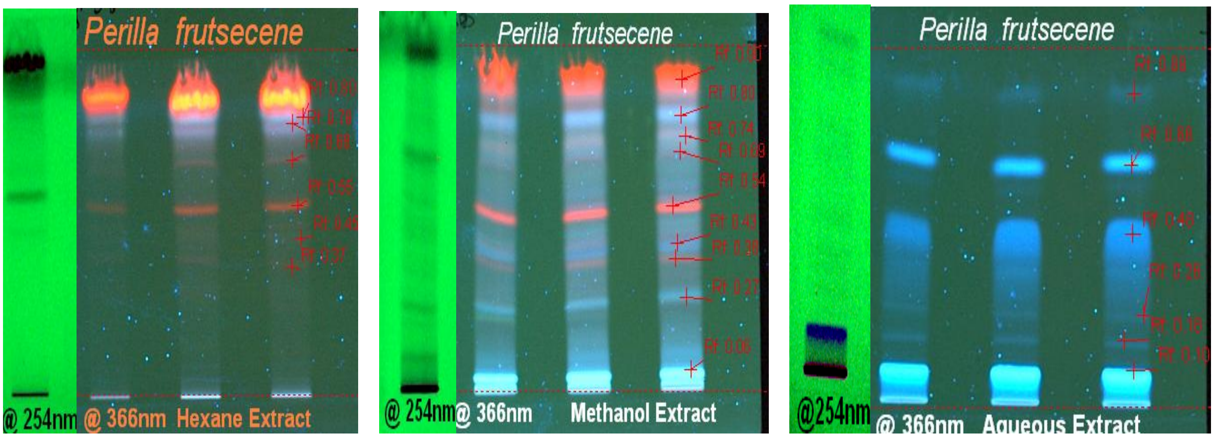


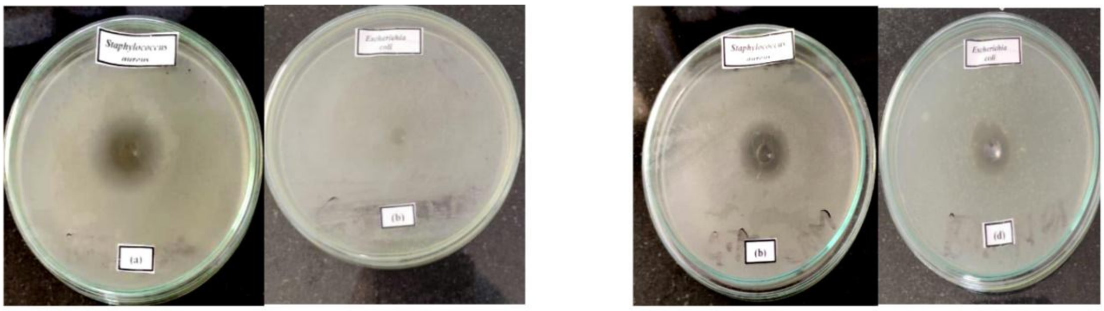
Fig 7: General HPTLC fingerprints of Sphaeranthus *indicus* fruits extract *and perilla frutescence* flower extract. Panel a and b of the Figure shows the chromatogram of the extracts from *Sphaeranthus indicus* and *perilla frutescence* of different solvents separated by using solvent system (chloroform: methanol; 9:1)

Table 2: Rf values and color of the resolved bands of test solutions of *Sphaeranthus indicus and perilla frutsecene at 366nm*

Mobile phase: (chloroform: methanol; 9:1)

Solvents	A	b
Hexane	0.77 (pink), 0.69(blue), 0.61 (blue), 0.46 (red), ,0.39(red),0.06(blue))	0.80 (red), 0.78 (blue), 0.78 (blue), 0.55(blue), 0.45 (blue), 0.37 (blue)
Methanol	0.90 (blue), 0.0.67(blue), 0.61 (blue), 0.28(blue), 0.17 (blue), 0.10 (blue),0.02(red)	0.90 (red), 0.80(blue), 0.74 (blue), 0.69(blue), 0.64 (red), 0.43 (blue),0.36(red),0.27(blue),0.06(blue)
Aqueous	0.93 (pink), 0.84(blue), 0.77 (blue), 0.67 (blue), ,0.55(blue),0.46(blue) , 0.41(blue), 0.36 (red), 0.25 (red), ,0.12(blue),0.07(blue)	0.88(blue), 0.68 (blue), 0.48(blue), 0.28 (blue),0.18(red),0.10(blue)

Antibacterial activity:



A1 **A2**

Fig 8: Antibacterial activity of GM Plants Extract(A1) and BG Plants Extract(A2) Against *Staphylococcus aureus* and *Escerichia coli*

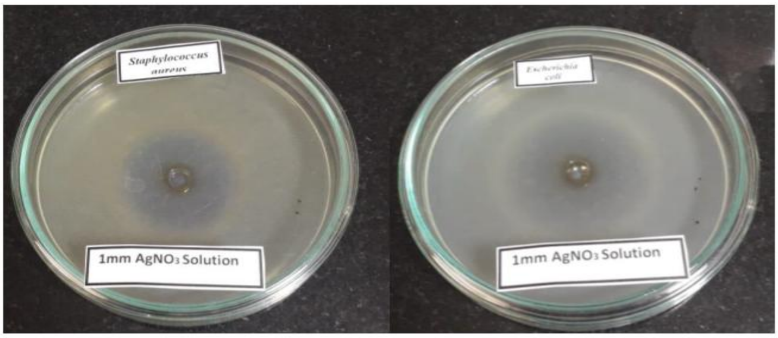


Fig 9: Antibacterial activity of 10mM AgNO3 Stock Solution Aagainst *Staphylococcus aureus* and *Escerichia coli*

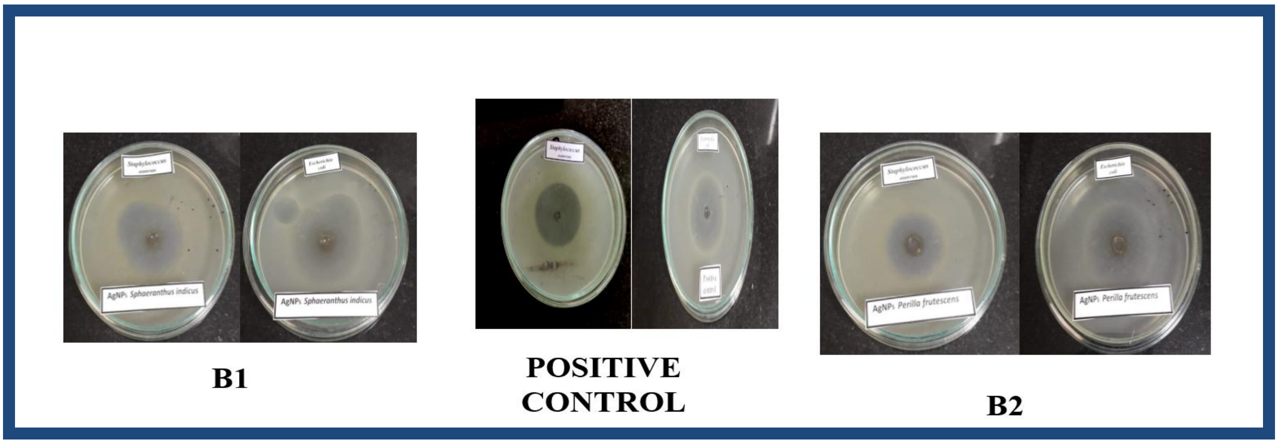


Fig 10: Antibacterial activity of Synthesized Ag Nanoparticle of GMNPs(B1) and BGNPs Plants Extract (B2) Aagainst *Staphylococcus aureus* and *Escerichia coli*.

Table 3: Zone of inhibititon diameter of test samples against *staphylococcus aureus* and *Escerichia coli.*

S.NO	Name	Zone of inhibition against *staphylococcus aureus*	Zone of inhibition against *Escerichia coli*
1	GM Sample	18mm	11mm
2	BG Sample	17mm	15mm
3	AgNO3 soln	31mm	30mm
4	GMNPs	32mm	27mm
5	BGNPs	32mm	25mm
6	Ciprofloxacin(Standard positive control)	31mm	29mm

Conclusion:

It's concluded that green synthesis is an innovative technology in which natural products are used against infectious pathogens. Current research shows the significant antibacterial effect against infectious agents. In this study, the phyto-mediated synthesis of AgNPs using extracts of both plants (*Sphaeranthus indicus and perilla frutescence)* which showed strong antibacterial activities against *S. aureus* and *E. coli*. These biogenic silver nanoparticles, which particles size less than 100 nm were further characterized with visible observation and UV-Vis's spectroscopy. These silver nanoparticles showed stronger antibacterial activities through zone-of-inhibition studies. This results proved that these synthesized silver nanoparticles were more competent than plants extract alone. HPTLC Fingerprint profile indicated the presence different

Phytoconstituents (Fig.7), which confirming the synergistic action. The phytochemical screening with qualitative chromatographic analysis shows the fruits and flowers of the *Sphaeranthus indicus and perilla frutescence* are rich in alkaloids, tannins, saponins,and flavonoids, which are popular phytochemical constituents. The present studies conclude these extract could inhibit human pathogens growth. The results are good as well as the most active extracts can be subjected to isolation of the therapeutic antimicrobials and undergo secondary pharmacological evaluation.

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