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GREEN SYNTHESIS OF SILVER NANOPARTICLES BY USING PHYLLANTHUS EMBLICA AND ADHATODA VASICA LEAF EXTRACT AND THEIR COMPARATIVE STUDY ON MICROBES.

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Abstract

Biosynthesis of silver nanoparticle is very useful because of eco-friendly in nature. AgNPs are specialized nanomaterials with many potential biomedical properties and applications. In this study, the two novel plant Phyllanthus emblica (Amla) Adhatoda vasica (Adusa) capable of producing biological silver nanoparticles were used.

The synthesized nanoparticles of Phyllanthus emblica and Adhatoda vasica plant extract were separately tested to examine their antimicrobial activities. The activities were tested against various microorganisms, including bacteria like Escherichia coli, and Staphylococcus aureus and fungus like mucor and aspergillus strain obtained by leaf ,water and soil source . The main aim of the present study is to evaluate the green synthesis of nanoparticles and their impact on microbes and their antimicrobial activity assessment against by using some commercial available antibiotics streptomycin, tetracycline.Aim of research is to focus our work on nature eco-friendly depend medicinal quality to kill microbes.

Keywords: Nanoparticle,
Phyllanthus emblica (Amla),
Adhatoda vasica(Adusa),
silver nano particle,
AgNPs,
Escherichia coli,
Staphylococcus aureus,
Mucor,Aspergillus.

Introduction

Antimicrobial resistance is now commonly acknowledged as a major worldwide health concern.Therefore, the scientific community is always searching for innovative, more efficient approaches to tackle Antimicrobial resistance. From ancient time silver has been used for many perspective like protection against microbial infection , burn healing , dental diseases , capping of teeth for protection against tooth decaying bacteria, for sterilization of medical equipment(Sokmen et al., 2017).In recent AgNPs are the most suggested nanoparticles for research proposal because of their unique and specific physico-chemical characteristics and exceptional antibacterial capabilities , numerous uses are present in food processing, cleaning and disinfection, textiles industry,silver mediated drugs manufacturing, construction materials, medication delivery , bio molecular detection , bio sensing, water treatment to remove impurities , catalysis, imaging in photo recovery, and many other biomedical and engineering applications (Banasiuk et al., 2020). Silver nanoparticles have also shown promise in other biological applications, including those that are antidiabetic, anticancer,antibacterial, anti-inflammatory, and antiparasitic (Sudha et al., 2017, Benakashani et al., 2016, Khan et al., 2016). Apart from AgNPs good applications there are a number of disadvantages are present , including high allergy, and dangerous chemical reactions that put human health and environment at serious risk . The green synthesis and production of AgNP from plant material has garnered a lot of attention in recent years research due to its many benefits, including its lack of harmful chemicals , less sideffects , affordability, ease of scaling, economic viability, and environmental friendly. AgNPs have been made from a variety of plant parts, such as fruit, seeds, bark, peel, roots, and leaves(Dutta et al., 2022; Abdullah et al., 2021; Rohaizad et al., 2020). Changes of silver salt to effective AgNPs depends on secondary metabolites , which are present in plant crude extract , it may be flavonoid,saponins , polyphenols , terpenoids ,vitamins and proteins (Alabdallah & Hasan, 2021).

Amla (*Phyllanthus emblica)* tree is a deciduous tree that grows to a height of 8 to 18 meters on average,has a thin layer of light grey bark that occasionally sheds in tiny, uneven flakes, revealing a fresh, different-colored surface beneath the older bark.In addition to vitamin C, amla is rich in minerals, amino acids, glutamic acid, aspartic acid, alanine, gallic acid, and tannin. Amla is well-known for its healing qualities and antioxidant qualities. Amla also possesses strong antioxidant, hepatoprotective, anti-diabetic, anti-cancer, anti-obesity, anti-ulcer, anti-

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hypercholesterolemia, anti-dyslipidaemia, and anti-mitochondrial qualities (Khan, 2009; and Reddy et al., 2010).The amla plant has been used extensively in complementary and alternative medicine systems such as Ayurveda and Unnani for over 6,000 years. Leaf, root, seed, bark, and fruit are all utilized to make different herbal remedies that are widely used for the therapy of a number of illnesses, such as diarrhea, constipation, indigestion, and acidity. Its goods are available as churna, oils, and powders. The churna, being an antioxidant, helps with acidity, detoxification, constipation, indigestion, and body function revival. Thus, frequently employed in Unani and Ayurvedic systems,Chemical constituents reported in Amla plant leaf are Ascorbic acid ,Gallic acid, ellagic acid, chebulic acid, chebulagic acid, chebulinic acid, phyllatidine and phyllantine, Phyllantine ,phyllantidine ,zeatin, zeatin nucleotide ,zeatin rioside, Quercetin leucodelphinidin kaempherol,these all provides specific medicinal quality to amla (Agrawal & Chopra, 2004).

Natural medicine has grown in popularity in recent years as a result of allopathic treatment's toxicity and adverse effects. These organic plant-based remedies work by preventing or even curing many forms of cancer and degenerative illnesses in people(Chanarat, 1992; Feig et al., 1994; Kohlmeier et al., 1995; Yen and Chan, 1995; Cragg et al., 2005).Additionally, some researchers have looked at the antibacterial phytochemicals inqualities in the essential oil that is derived from *Justicia adhatoda(Adhatoda vasica)* leaves(Sarker et al., 2011).Vasicine, vasicinone, vasicinol, deoxyvasicine, adhatonine, adhavasinone, and other phytochemical constituents with a wide range of important activities, including abortifacient, antimicrobial, antitussive, cardiovascular protection, anticholinesterase, and anti-inflammatory properties, have been isolated from J. adhatoda (Singh et al., 2011). Some antioxidant phytochemicals such as alkaloids, tannins, phenolics and flavonoids are also present in in *Justicia adhatoda*/ *Adhatoda vasica* as a secondary metabolites (Kumar et al., 2013).

So research of amla,adusa has been showed presence of secondary metabolites are capable to make AsNPs from their part like flower,stem,leaf ,seed and root,so we have used these quality to make silver nanoparticles from adusa and amla leaf for comparative study on antimicrobial activity.

Chemicals and instruments

Glass wares

Conical Flasks,Petri plates,watch glass,Beaker, slides, Test tubes,Measuring Cylinder, Funnel and Glass rods,dark brown bottle.

Apparatus

Weighting Machine,Magnetic Stirrer,grinder, Centrifuge Machine,Scanning electron microscope,XRD,UV-vis spectroscopy, Laminar air flow,Bacterial incubator, fungus incubator, Autoclave machine, Hot air oven,Spatula;forceps,spreader,cork borer, Tray,paraffin tape, Eppendorf tubes,Test tube stand, Gloves, Masks, Burner; Matchsticks, Silver foil,Newspapers,Rubber Bands.

Chemicals Used

Silver Nitrate ($AgNO_3$)Potato dextrose ,agar,MH agar, NAM agar),70% ethanol,distilled water,Czapek dox agar, MacConkey agar and PDA,Commercial antibiotics like streptomycin ,tetracycline.

Plant material/ Herbs

Plant leaves were collected from botanical garden of Kalinga university Raipur Chhattisgarh.Green and healthy leaves were collected for fresh preparation of extract .After collection, samples were stored in dried form and crushed them in a sterilized mortal pestle and stored them in plastic bags at room temperature for further research.

Fig (a) **Fig(b)**

Fig 1.1:-(a) Leaf collection from Amla plant from Botanical garden kalinga university (b) Amla leaf

Fig(a) **Fig(b)**

Fig1.2:-(a) Leaf collection from Adusa plant from Botanical garden kalinga university (b) Adusa leaf

Microorganisms Used

The microorganism like fungi *Mucor and Aspergillus* strain and bacteria *E.colli ,S.aureus* were used to observe Antimicrobial activity of the silver nanoparticles. The sample of these microbes were taken from soil ,leaf and pond water sample from the Kalinga university Raipur Chhattisgarh.

Fig:(a)water collection **Fig:(b)soil collection** **Fig(c)diseased leaf collection**

Fig2:- Collection of water sample,soil sample and leaf sample from Botanical garden of Kalinga university Raipur for microbes culture.

Methods

Cleaning of Leaves

Green and healhy leaves of plants *Phyllanthus emblica* (Amla) , *Adhatoda vasica*(Adusa) were collected from botanical garden then they were washed properly with normal tap water and then distilled water.

Drying of Leaves

After washing, leaves were placed for drying at least for 3-4 days in a cleaned room,dust free,dark room where direct contact of sunlight was absent .

Extract Formation by using water

The dried leaves of Amla and Adusa were converted into fine powder by using sterilized mortar pestle and then 25 gm amla and 25 gm of adusa powdered mixtures dissolved in 100ml of distilled water separately and stirred on a magnetic stirrer at 80 ^{o}C for 3 hours for extract formation.

Fig (a): Extract formation of Amla leaf **Fig(b):Extract formation of Adusa leaf**

Fig 3:-Leaf extract formation by using Magnetic stirrer

Purification of the Amla and Adusa leaf Extract

After stirring leaves with distilled water on magnetic stirrer for 3-4 hrs, at 80° C , leaf extracts of amla and adusa were filtered out with the help of Whatman No. 1 *filter paper* through glass funnel by using conical flask. We collected these liquid water leaf extract sample for further research work.

Fig 4.1:-Adusa leaf extraction collection

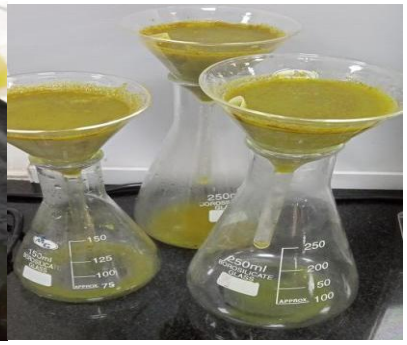

Fig4.2:-Amla leaf extraction collection

Preparation of Silver Nitrate($AgNO_3$) Solution

To prepare 0.1M silver nitrate solution , need to add 1.7gram silver nitrate into 90 ml distilled water.A solution of total 100ml was prepared by adding some distilled water as required by solution . This solution was placed in dark room for an hour to 24 hrs to prevent silver nitrate from chemical oxidizing reaction . And then it was ready for standardization and characterization for further research.

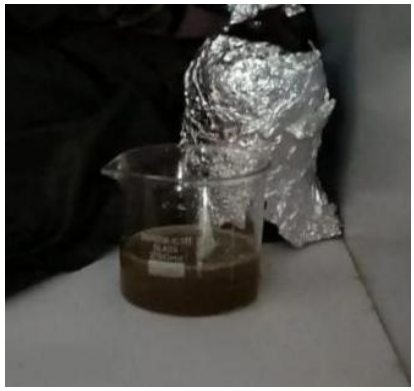

Fig 5.1 :-AgNPs solution in dark brown bottle covered by silver foil paper

Preparation of Amla and Adusa Nanoparticles.

To obtain silver nanoparticles we mixed aqueous solution of silver nitrate solution with our plant samples (amla and adusa) in ratio 1:10 The solution of silver nitrate and leaf extract were mixed properly and incubated overnight in the dark.

Both the samples were centrifuged at 12,000 rpm for 15 minutes separately for 2 two to three rounds,then light colour supernatant was discarded and dark pallet of Amla and Adusa were collected in two different Watch glass and kept in a dark and clean place for drying process.

Fig (a)centrifugation **Fig(b)AgNPs pellet formatiom**

Fig5.2:- Centrifugation of Plant extract+AgNPs mixture and pellet collection

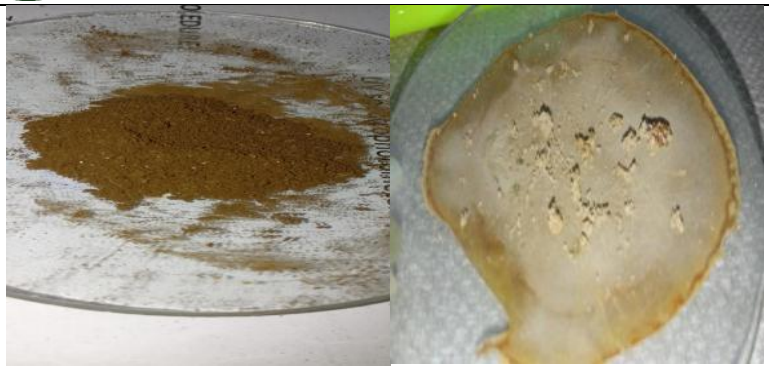

Fig5.3 Adusa AgNPs powder form **Fig5.4 AmlaAgNPs powder form**

Preparation of Bacterial and Fungal Culture plates.

Collection of Sample

The soil,water and leaf sample for microbial culture were collected from botanical garden and pond from kalinga university.

Serial dilution Process

Collected water,leaf and soil sample were measured by 1 gm separately and then mixed with 9 ml of distilled water ,then total volume made by 10 ml in each test tube by using of distilled water.Further we used 3+3+3 different test tubes for serial dilution Process where we used our water ,leaf and soil liquid sample with distilled water in ratio of 1:9 For further sequencing process .

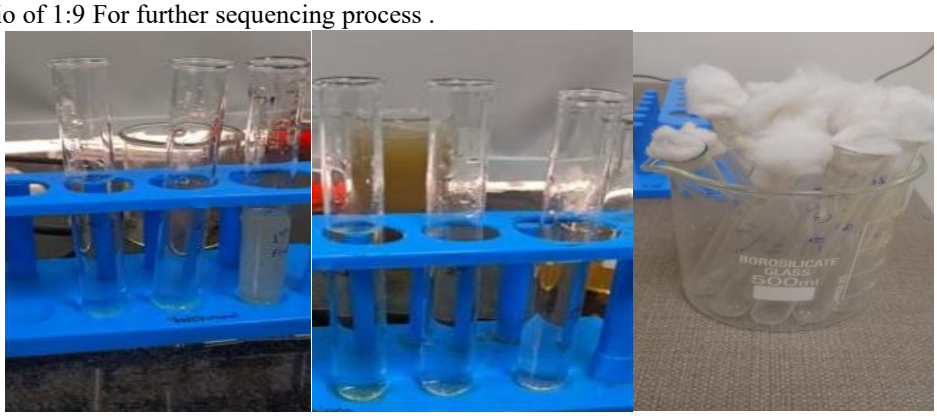

Fig (a):soil sample **Fig (b):water sample** **Fig (c):soil sample**

Fig 6 Serial dilution Process for collected sample of soil ,water and leaf

Preparation of Agar Solution

We made agar plates separately for both bacteria and fungus culture . For bacteria NAM and MH agar were used and for fungus Czapek and PDA were used.Measurement for all media was in NAM was 7 grams of agar powder was dissolved in 250ml distilled water,in second media muellar hinton agar 9.5gm in 250ml,in 3rd media Czapek Dox agar 12.25gm in 250 ml ,in 4th media MacConkey agar 12.88gm dissolved in 250 ml distilled water,in last media Potato dextrose agar 9.75gm dissolved in in 250 ml of distilled water.

Preparation of Culture plates for bacteria and fungus

Before poring plates , UV of Laminar airflow on at least for 45 min for sterilization of room and to prepare microbes free environment.The prepared autoclaved media then poured in clean and sterilized petri plates under laminar airflow and at least 1 hr time taken for solidifying of plate media . In cultured plates about 1µl of all different sample of pond water,soil ,leaf water sample was loaded with the help of micro- pipettes separately and spread all over the plate with the help of spreader under laminar airflow. And the plates were incubated for 2-3 days in the separate incubator by maintaining temperature at 34°C temperature for bacteria incubator and 25-28°C for fungus incubator .

Fig 7.1:- Performing plate poring method by using Agar solution and PDA for bacteria and fungus culture by using laminar air flow

Fig7.2:-After Incubation fungus culture developed on petriplate

Fig7.3:-After Incubation, bacteria culture developed on petriplate

Disc Diffusion Method

Specific Fungal and bacterial sample were taken from incubated petri plate and then spread all over the plate by using spreader or cotton swab. In Disc Diffusion Method,firstly small spherical disc formed from whatman filter paper no. 1 and then all disc dipped into Adusa and Amla leaf silver nanoparticles extract with the help of forceps. The petri- plates were marked outside into 4 equal part by using marker pen . Out of 4 we used 3 disc for our AgNPs and one for commercial antibiotic streptomycin which was used for comparative study with AgNPs.

Fig (a)using disc on plate (b)petriplate with disc (c)commercial antibiotics
Fig 8.1:- Disc Diffusion Method.

Well Method

In this, first fungal bacterial sample was spread all over the petri plate with the help of spreader or cotton swab. The plates were divided into four parts, by use of marker outside.With the help of cork borer 4 well created in petri plate,then out of four,three well filled by different concentration 0.2ul,0.4ul,0.6ul of amla and adusa AgNPs extracts,except of one filled by commercial antibiotic streptomycin for comparative study.

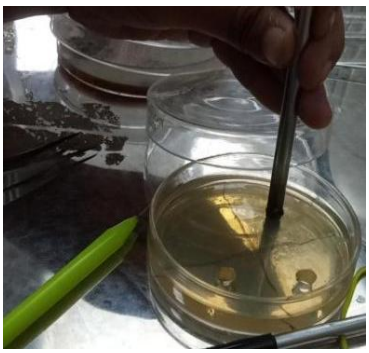

Fig8.2:- Well Method by cork borer

Comparative Analysis of Synthesized Nanoparticles from Adusa and Amla

Antibacterial Activity

The agar well and disc diffusion method was used to assess the antibacterial activity. Gram-positive *Staphylococcus* and gram-negative *Escherichia coli.* bacteria were used to test the antibacterial activity. Using the agar well diffusion method, the antibacterial activity of the produced AgNPs was assessed. It was found that the growth was gradually inhibited by increasing the concentration of AgNPs by 0.2ul, 0.4ul, 0.6ul, and 1 ul concentration. The zone of inhibition for gram-positive bacteria is less than that of gram-negative bacteria. In gram positive bacteria the peptidoglycan layer may be a factor for decreased zone of inhibition ,In gram negative bacteria have a significantly thinner peptidoglycan layer, which makes it easier for the bacteria to infiltrate cell walls and denature or kill them.All comparative study done with comparative observation with commercial antibiotics streptomycin also.

Fig 9.1:- Showing zone of inhibition by using Amla AgNPs extract on *E.colli* cultured plate by using method disc diffusion

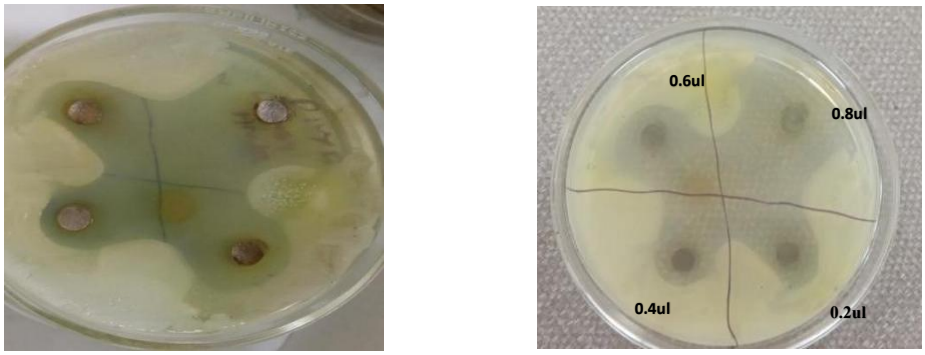

Fig 9.2:- Showing zone of inhibition by using Adusa AgNPs extract on *E.colli* cultured plate by using well diffusion method by increasing concentration of AgNPS

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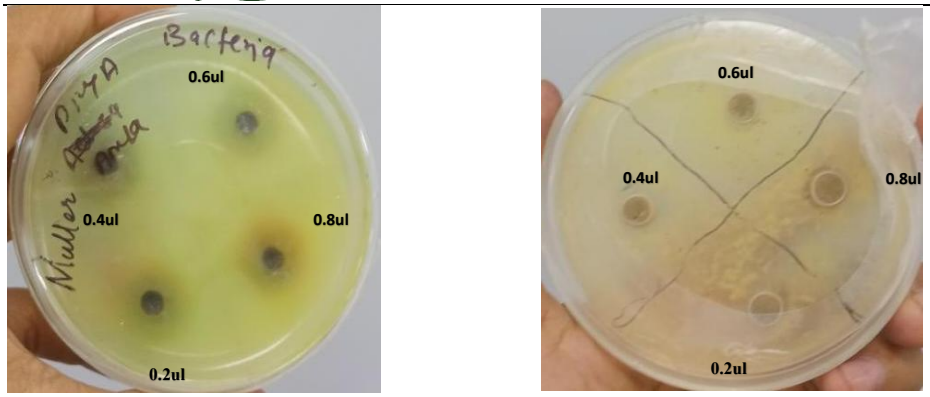

Fig 9.3:- Showing zone of inhibition by using amla AgNPs extract on *S.aureus* cultured plate by using well diffusion method by increasing concentration of AgNPS

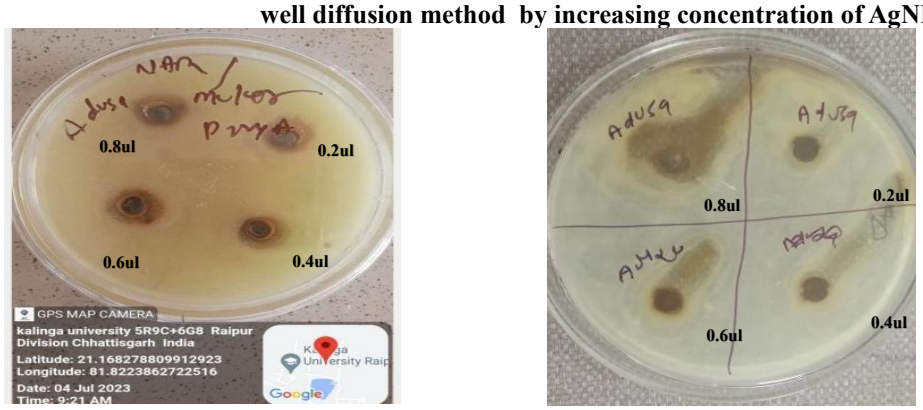

Fig 9.4:- Showing zone of inhibition by using adusa AgNPs extract on *S.aureus* cultured plate by using well diffusion method by increasing concentration of AgNPS

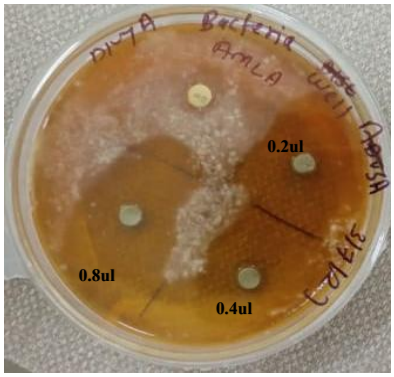

Fig 9.5:- Showing zone of inhibition by by increasing concentration of AgNPS using amla AgNPs extract on MacConkey mediated agar *E.colli* cultured plate by using disc diffusion method against streptomycin antibiotic.

Antifungal Activity

Antifungal activity were performed by using *Mucor* and aspergillus strain by using of well diffusion method and disc diffusion method. By using both method,zone of inhibition were observed for Amla and Adusa silver nanoparticle sample.

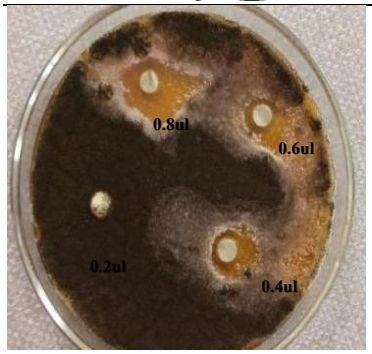

Fig 9.6:-Showing zone of inhibition by using amla AgNPs extract on *Aspergillus strain* cultured plate by using disc diffusion method by increasing concentration of amla AgNPS.

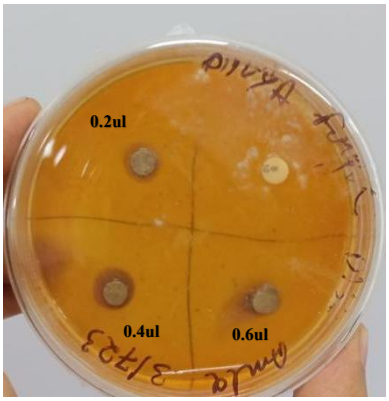

Fig 9.7:-Showing zone of inhibition by using amla AgNPs extract on *Mucor* strain cultured plate by using disc diffusion method by increasing concentration of amla AgNPS

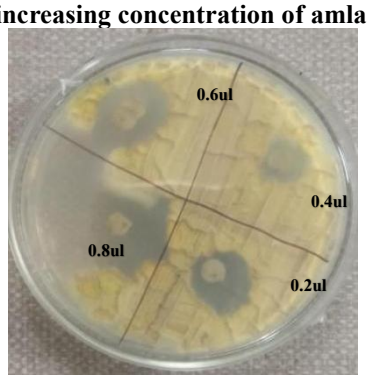

Fig 9.8:-Showing zone of inhibition by using adusa AgNPs extract on *Mucor strain* cultured plate by using disc diffusion method by increasing concentration of adusa AgNPS

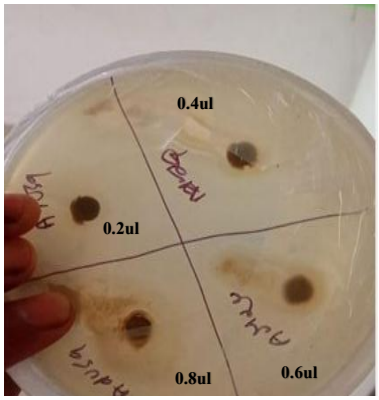

Fig 9.8:-Showing zone of inhibition by using adusa AgNPs extract on *Aspergillus strain* cultured plate by using well diffusion method by increasing concentration of adusa AgNPS

Results and discussion

Process optimization for the synthesis of AgNPs using *Phyllanthus emblica Adhatoda vasica* (Adusa)leaves extract

The extract obtained from leaves of dried Amla and Adusa in the presence of clear solution of 0.1M AgNO3,showed color change from light yellow to dark brown after 1-3 hrs at room temperature within 2hrs.

Fig10:-formation of amla and adusa leaf based AgNPS

To prepare stable AgNPs, diferent parameters were required such as pH (3–9), volume of plant extract (10-15 mL), AgNO3 concentration (0.1 M), reaction temperature (25–40 °C) and reaction time (5–25 min) were studied. The major constituents of Adusa and Amla leaves are Gallic acid, Methyl gallate, Ellagic acid, Trigallayl glucose tannins, and saponins alkaloids, favonoids ,other phenolic compounds and phytochemicals mainly responsible for the reduction of silver ions to AgNPs .all these biochemicals serve as natural surfactants and stabilizer agents in the formation of nanoparticles.

The chemical reactions that are involved in converting silver ions into AgNPs are as follows-

$Ag(NO_3)$ (colourless) + Aq. plant extract (pale yellow colour) $\rightarrow Ag^{+} + NO^{3-}$

Ag^{+} e_{aq}^{-} (from phytochemicals from amla and adusa) $\rightarrow$ Ag^{0} (brown colour)

The hydrated electrons e_{aq}^{-} act as a strong reducing agent and thus reduce Ag+ ions into zero-valent Ag atoms Ag^{0} .The solution containing Ag^{0} was centrifuged and after decantation of the supernatant, dark brown AgNPs pellet was obtained.

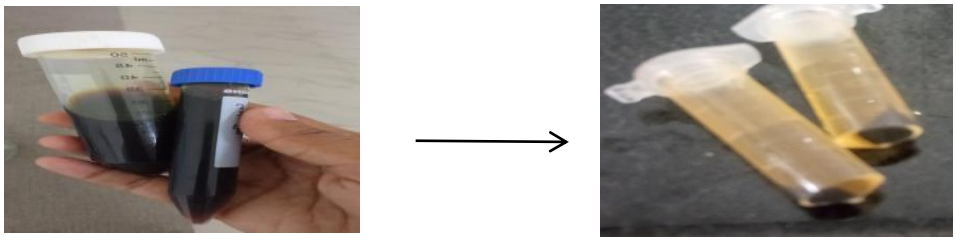

Fig 11:-formation of AgNPs in form of pellet

UV- Visible absorbance Spectroscopy

The plant extract of *Phyllanthus emblica* and *Adhatoda vasica* were mixed with silver nitrate in ration of 10:1 for 30 mins to 24hrs. The sample were examined, as the sample changed its color gradually from light yellow to dark brown. After 24 hours, UV-visible spectroscopy of the sample were measured..The range of absorption was between 250-350 nm.

Fig12.1:- Anlaysis of sample through UV-vis spectroscopy

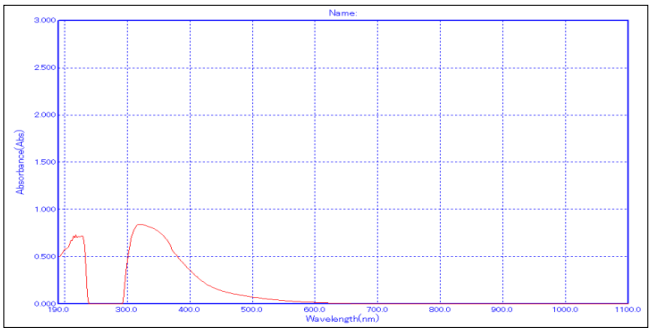

Fig 12.2 :-Result of UV-vis spectroscopy of Silver nanoparticles of *Adhatoda vasica* (Adusa) leaf extract.

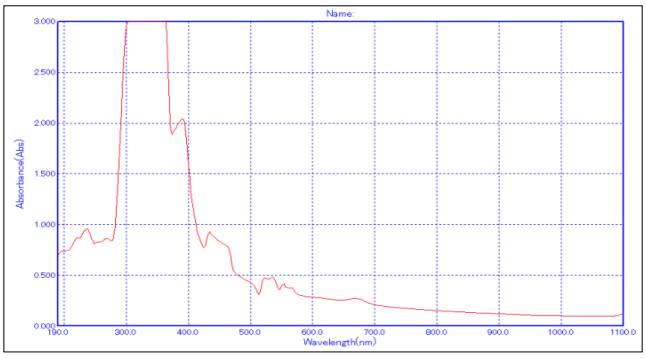

Fig12.3:-Result of UV-vis of Silver spectroscopy nanoparticles of *Phyllanthus emblica* (Amla)leaf extrac.

XRD analysis

For characterization of AgNPs ,XRD used , provided specific information regarding nanoparticle's structure ,crystalline size and shape and their chemical composition.

Fig12.3:-Result of XRD analysis of nanoparticles of amla and adusa leaf extract by using XRD machine

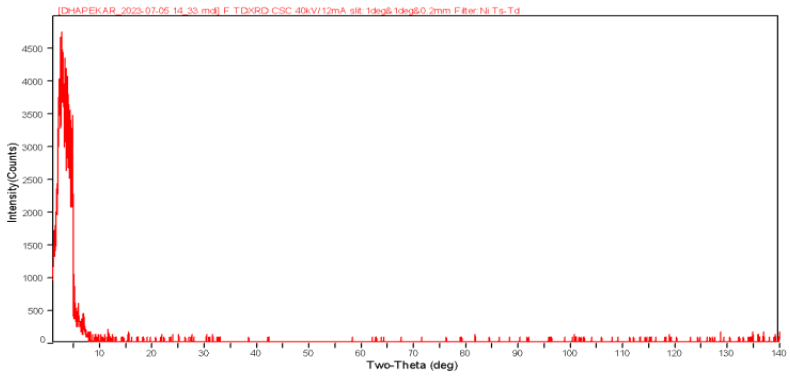

Fig12.4:- XRD anaylsis of (Adusa) leaf extract.

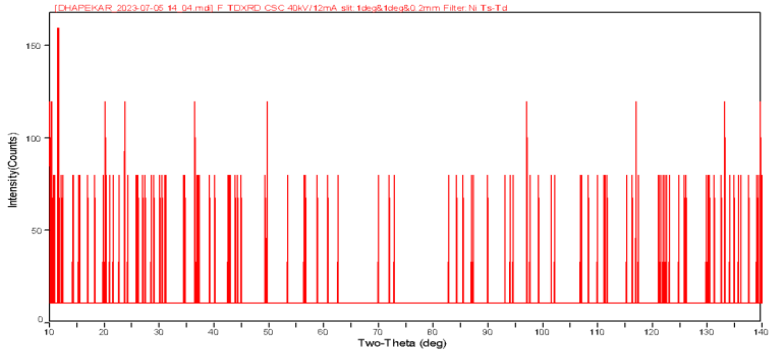

Fig12.5:-XRD analysis of nanoparticles of *Phyllanthus emblica* (Amla)leaf extract.

SEM (Scanning Electron Microscope)

The dried nanoparticles of *Phyllanthus emblica Adhatoda vasica* were used to study under the scanning electron microscope for Analysis of morphology of the nanoparticles. Following observation was examined (Fig:- a; b; c; d; and e).

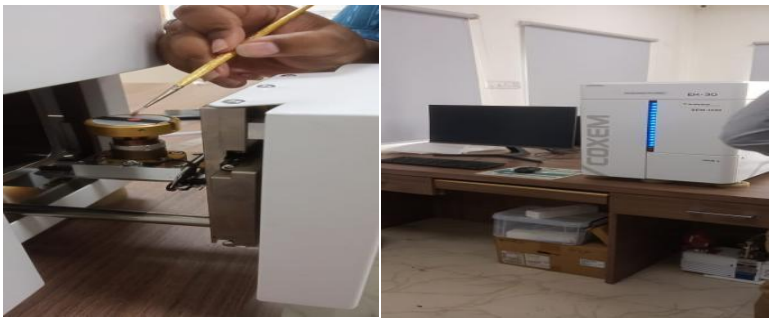

Fig12.6:- Amla and adusa based AgNPs Sample loading on SEM

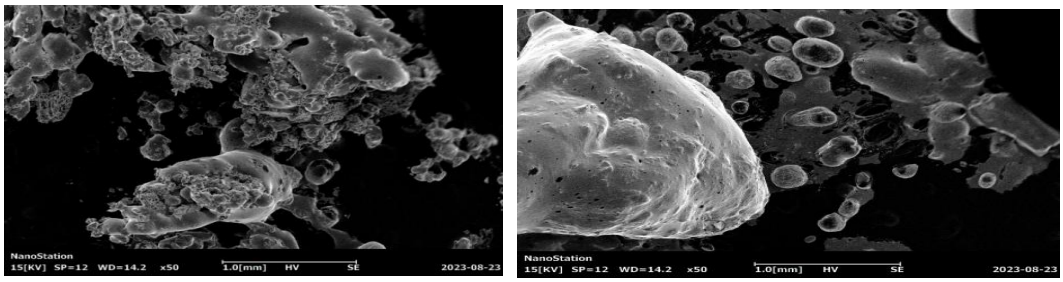

Fig (a) **Fig (b)**

Fig12.6:- Amla AgNPs Sample analysis (a&b)

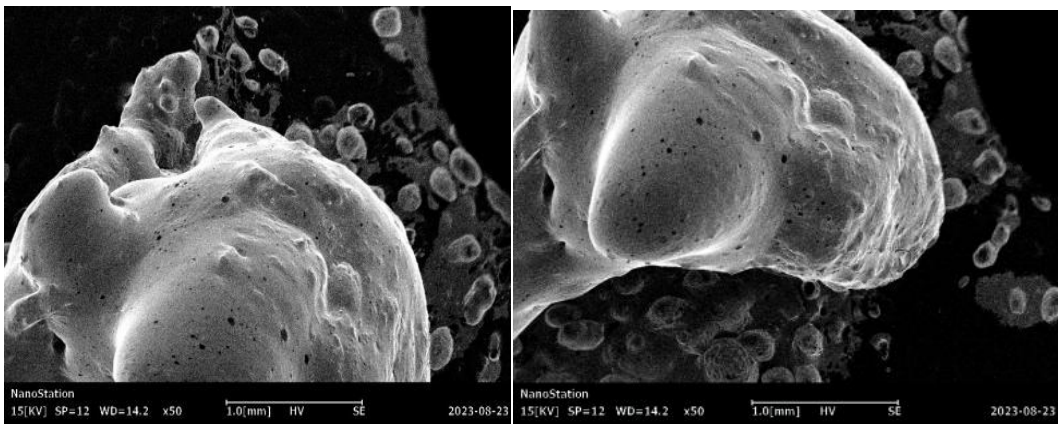

Fig (c) **Fig (d)**

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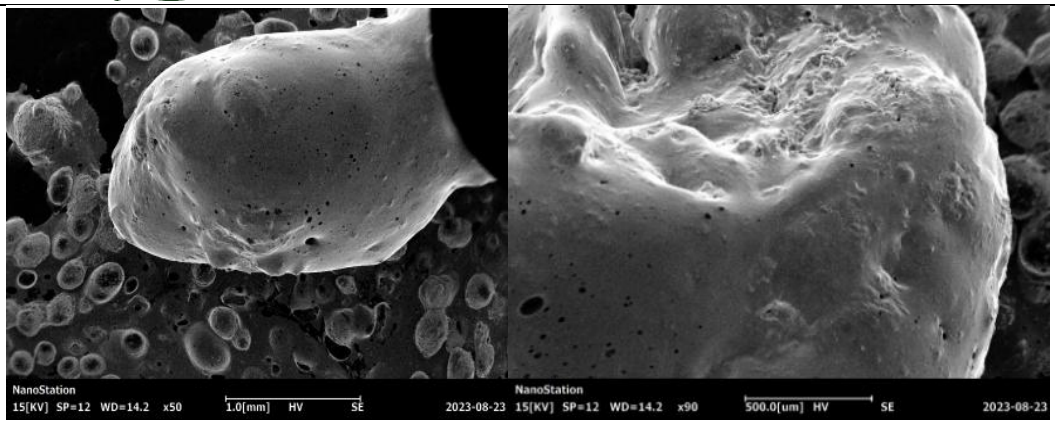

Fig (e) **Fig (f)**

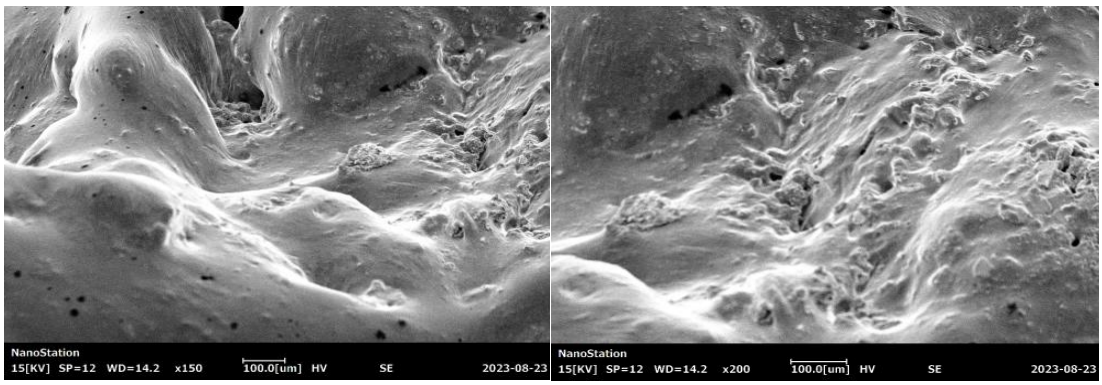

Fig (g) **Fig (h)**

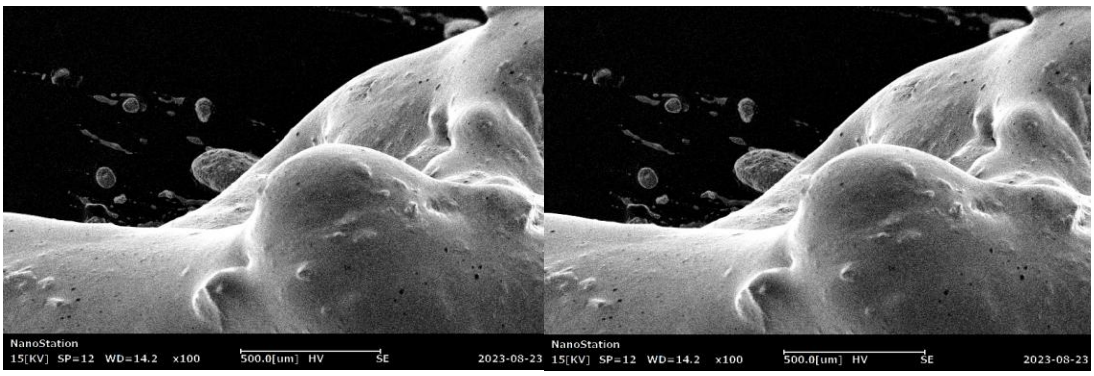

Fig (i) **Fig (j)**

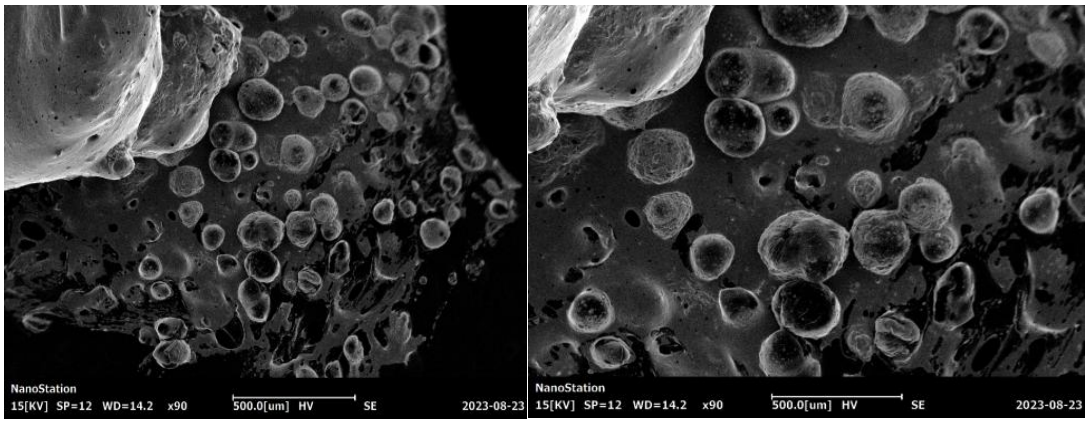

Fig (h) **Fig (k)**

Fig12.7:- fig(c) to fig(k)Adusa AgNPs Sample analysis (a&b)

Conclusion

This technique for synthesizing AgNPs by using plant source is simple, affordable, and eco-friendly and can be applied to a variety of projects like agriculture field and to design drug against many diseases. Furthermore, AgNPs produced using *Phyllanthus emblica* and *Adhatoda vasica* aqueous leaves extracts have strong antibacterial activity anti-fungal action against a number of human diseases that are resistant to many drugs, including *Staphylococcus aureus* and *Escherichia coli* and fungi of the *Mucor* and *Aspergillus* strain .Antimicrobial quality of these plant based nanoparcticles seen by zone of inhibition on petriplate.

XRD and SEM imaging demonstrated that the particles' size range , and characterization of shape, indicating that the biosynthesis of AgNPs using *Phyllanthus emblica* and *Adhatoda vasica leaf* extract was successful. Moreover, UV examination showed that these AgNPs did not require external stabilizing or capping agents. all the peaks of samples were detected between 250-350 nm of range.

In these research, zone of inhibition formed by amla and adusa leaf based AgNPs was larger as compared to zone of inhibition made by commercial antibiotic streptomycin so it was a good result to create ecofriendly AgNPs against chemical based medicine .

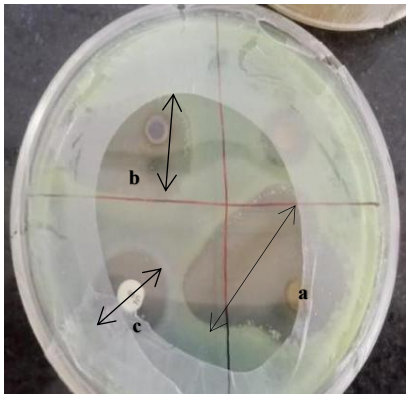

Fig 13:- Showing 3 different zone of inhibition (a) and(b) made by leaf based AgNPS was larger and effective as compare to 3rd zone of inhibition made by commercial and chemical based aantibiotic streptomycin (c)

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