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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *PIPER BETLE* (L) VAR. SIRUGAMANI 1(SGM1) LEAF EXTRACT: CHARACTERIZATION, ANTIDIABETIC AND ANTI- INFLAMMATORY ACTIVITIES

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ABSTRACT

Fabrication of biologically synthesized nanoparticles played a vital role in the catalytic method of green synthesis. In the current study, a simple and environmentally convenient method was used for the synthesis of silver nanoparticles using the leaf extract of Piper betle (L) var. sirugamani 1 (SGM1). The biosynthesized silver nanoparticles were characterized by using scanning electron microscopy (SEM), X-ray diffraction analysis (XRD), Energy dispersive X-ray spectroscopy (EDAX) technique, Ultra-violet spectroscopy (U.V), and Fourier transform infrared spectroscopy (FTIR).

The FTIR confirms the modes of spherical phase due to the stretching and bending vibrations with the spectral peaks of 3450.80, 1634.74, 1519.97, and 682.83cm^{-1}. The surface plasma absorption band occurred at a maximum average of 200-800 nm indicating the presence of spherical Ag nano particles through UV-Vis spectroscopy. The elemental analysis of the

bio synthesized silver nanoparticles was confirmed by EDAX spectroscopy. Scanning Electron Microscope technique was employed to visualize the size and shape of silver nano particles. The average sizes were found to *be 18.24nm, 20.73nm, 25.81nm, 32.17nm in Sirugamani-1. The* antidiabetic activity of silver nanoparticles was confirmed by α-Amylase Inhibition Assay, α-Glucosidase Inhibition Assay, and anti-inflammatory activity was assessed using Albumin Denaturation Technique and HRBC stabilization method.

Keywords: Biosynthesized Silver nanoparticles, Sirugamani-1 piper betel cultivar, Anti-diabetic activity, and Anti-inflammatory activity.

INTRODUCTION

The conformist approach through the relieve of using which the plants were used can on the other hand be pragmatic in tribal communities, handed down by way of natural history, and even so subsist unbeaten to be a strike contour of medication, within nastiness of the beginning of progressive medicine **[1]**. Based on the literature, scientific evidence through worldwide the utilization of medicinal and aromatic plants for the medical purpose has been recorded less than 50% for their phytochemical and pharmacological potential **[2]**. Aromatic medicinal plants contain highly scent qualities within accumulation headed for having medicinal habitats and are "chemical goldmines" owing near the frequent assortment of secondary metabolites that they have possession of and the wide-ranging selection of pharmacological activities **[3]**. The use of scented medicinal deposited living has been upward with time to implausible use withinside the pharmaceutical, cosmetic, and meals industries **[4]**. Considering the adverse effects of synthetic drugs the Western population is looking for natural remedies to various diseases, which are safe, and effective without any side effect**s [5-7]**. Many plant living and their rudiments are used for the medication of miscellaneous illness in exclusive vicinities of the World and are being screened for phytochemical and unrefined sports and the cost obtained from individuals of medical studies have aided withinside the clearing up of the conservative medicinal use of those plant existence **[8-10]**.

Although the chemical method of synthesis requires a short period for the synthesis of a gigantic choice of nanoparticles, this come close to calls used for capping sellers for stabilization of the extent of nanoparticles **[11]**. The chemical

essence has been used for nanoparticles synthesis and stabilization of noxious material and origin non-green commodities. The desire for environmental non-poisonous reproduction protocols for nanoparticles synthesis results inside the increasing diversion in crude processes which are movable from using chemical substances which are poisonous as by-merchandise **[12]**. There has been an ever-growing leisure pursuit in herbal merchandise as options reasserts for artificial components of pharmacologically relevant sellers in current years **[13]** and vital oils from medicinal and scented plant life have won a superior treaty hobby for his or her use in numerous foods, fitness drinks, cosmetics, and pharmaceutical merchandise.

They have pervasive use as flavouring resources and arise as "green synthesis" option in agricultural, pharmaceutical, neutraceutical, and several former applicable fields. Based on the customary information and scientific background, the leaves of P. betel was designed to experiment for their phytochemical property, green synthesis, and characterization of nanoparticles and their biological activities which helps in the identification of novel compounds for basic medicine formulation. In the Piperaceae family Piper sarmentosum, Piper longum, Piper nigrum, and Piper betle are identified as potential anti-diabetic agents and effectively in the treatment of diabetes **[14]**. Sirugamani-1 (SGM-1) is one of the varieties of *Piper betle* (L). The *Piper betle* leaf extract is useful in controlling glucose and lipid levels of diabetic rats and also used the pan to cure diabetes **[15]** reported Eco-friendly synthesis, characterization, in vitro, and in vivo anti-inflammatory activity of silver nanoparticles-mediated Selaginell-amyosurus. The present research is employed to synthesize silver nanoparticles and the synthesized nanoparticles were subjected to various biological activities for novel drug synthesis.

MATERIALS AND METHODS

Chemicals:

Chemicals and reagents, including Gallic acid, Catechin, 1, TPTZ (Tri pyridyl tri-azine), 1-diphenyl-2-picrylhydrazyl (DPPH), and Ferric chloride were purchased from Sigma Aldrich Chemical Co. Ltd., (MO, and USA). Aluminium chloride, Sodium nitrite, Sodium acetate, Folin-Ciocalteu reagent, Sodium hydroxide, and Glacial acetic acid were purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India.

Reagents:

5% Bovine serum albumin, 1N HCl, Phosphate buffer saline, Alsevers solution, 2% dextrose, 0.8% sodium citrate, 0.5% citric acid, 0.42% NaCl, 0.15M Phosphate buffer (pH 7.4), 0.36% Hypo saline, HRBC suspension (10% v/v), α-glucosidase (0.5 mg/ml), 0.1 M phosphate buffer (pH 6.9), 5M p-nitrophenyl-α-D-glucopyranoside, 0.02M Sodium phosphate buffer (pH 6.9), 0.006M NaCl, 1% Starch, Dinitrosalicylic acid were prepared from Sigma Aldrich and Himedia company chemicals.

Leaf Extract preparation:

The fresh and mature leaves were collected from IIHR (Indian Institute of horticulture research, Bangalore), then compacted the use of pestle and mortar. Further, the samples had been soaked and extracted (bloodless extract) with methanol. After that methanol turn into evaporated then semi-stable samples have been used for studying the experiments.

Synthesis of Piper silver Nanoparticles:

The silver nanoparticles encompass be synthesized the exploit of a standard amount of the plant extract under varied experimental conditions. The aqueous responses of 1mM $AgNO_3$ grow to be ordered and used for the synthesis of silver nanoparticles. 5 ml of aqueous plant extract is blended with 95 ml of $AgNO_3$ for the synthesis of silver nanoparticles. The formation of silver nanoparticles is showed with the aid of using coloration alternate from greenish to darkish brown. The look of a darkish brown coloration after three hrs suggests the formation of silver nanoparticles.

Separation of Sirugamani silver nanoparticles

The synthesized silver nanoparticles were separated by centrifugation (Spectrofuge 7M) at 13,000 rpm for 15 mins. The process was repeated by dispersion of pellets in water, to obtain colored supernatant solutions. The sample was then stored at -4^0C for further use.

Characterization methods:

The reduction of pure Ag^+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium at 5, 10-12 hours. UV-Vis spectral analysis was done by using UV-Vis spectrophotometer UV- 2450 (Shimadzu) in the range of 200 to 800 nm.

Scanning Electron Microscope (SEM) appraisal became completed the tradition of the Hitachi S – 4500 SEM machine. Thin films of the prototype have been planned on a carbon-covered copper grid among the support of using only behind a entirely small measure of the pattern at the grid, a advance

react became eradicated the procedure of a blotting paper behind which the films at the SEM grid have been allowed to dry among the help of use putting (placing) them underneath a mercury lamp for five minutes.

The silver nanoparticles respond arriving are purified among the support of using repeated centrifugation at 5000 rpm for 30 minutes accompanied by way of the relieve of using redispersion of the pellet of silver nanoparticles into 10 ml of deionised water. After the freeze-drying of the purified silver particles, the contour and composition have been analyzed with the aid of using XRD and SEM analysis. The dried combination of silver nanoparticles turned into gathered for the resolve of the formation of Ag nanoparticles with the support of using an X' Pert Pro X-ray Diffract meter (PAN analytical BV, The Netherlands) operated at a voltage of 40 kV and a fashionable of 30 mA with Cu K α radiation in a configuration. Energy-dispersive X-ray analysis, the aqueous extract of Piper betle were dehydrated and plunge coated on to carbon film and performed on JEOL-MODEL 6390 SEM instrument equipped with a Thermo EDX attachments. Fourier transformed infrared (FTIR) spectrum of the sample was recorded by Fourier transform infrared (Nicolet b6700 FT-IR, Thermo Scientific) spectrophotometer. The FTIR spectrum ranged from 4000 to 450 cm^{-1} at a resolution of 4 cm^{-1} by making a KBr pellet with AgNP'S.

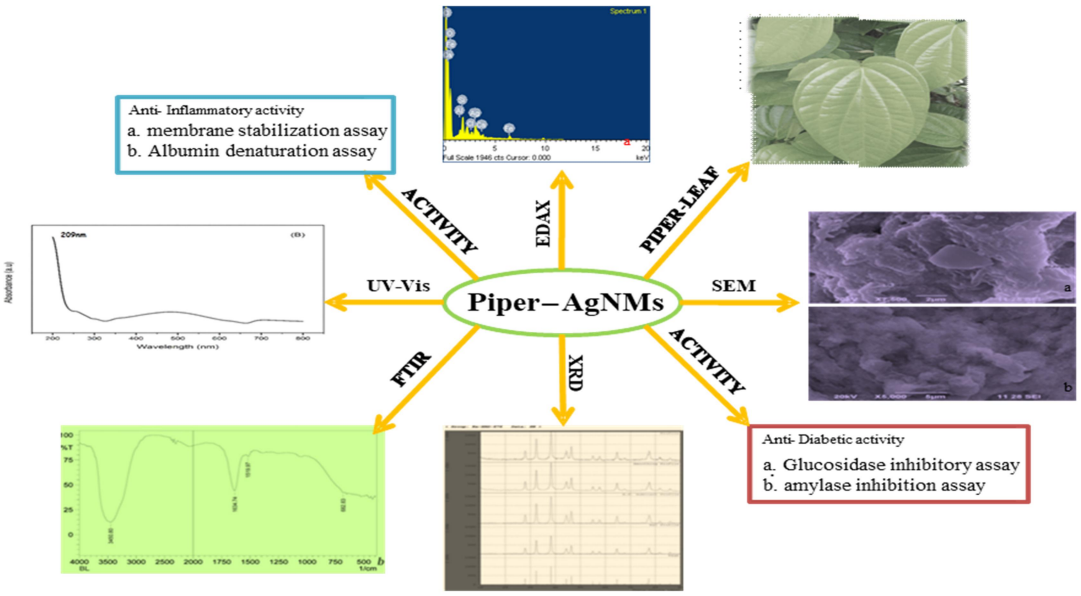

Figure 1: Graphical abstract: Schematic representation of formation AgNMs by green synthesis

Anti-inflammatory activity

Albumin denaturation technique

The anti-inflammatory activity of plant extract was studied by using inhibition of albumin denaturation technique which was studied according to **[16, 17]** followed by slight modifications were carried out. The reaction mixture (0.5 ml; pH 6.3) consisted of 0.45 ml of bovine serum albumin (5% aqueous solution) and 0.05 ml of distilled water. The pH was adjusted at 6.3 using a small amount of 1N HCl. The changed concentrations of silver nanoparticles (five different concentrations) be further to the mixture reaction and were incubated at 37^0C for 20 min and then heated at 57^0C for 5 min after cooling the samples; 2.5 mL of phosphate buffer saline was added. *Turbidity was measured spectrophotometrically* at 600 nm. The percentage of inhibition of protein denaturation was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{(Abs_{Control} - Abs_{sample}) \times 100}{Abs_{Control}}$$

HRBC Membrane stabilization method

HRBC Membrane Stabilization method is the stabilization of the human red blood mobileular membrane with the aid of using hypo tonicity impelled membrane lysis. Since HRBC (human red blood mobileular) membrane is just like the liposomal membrane; they have a look at became undertaken to test the stableness of the HRBC membrane with the aid of using the extracts to are expect the *in vitro* anti inflammatory activity. The various silver nanoparticles extracts on the consciousness of 50, 100, 150, 200, and 250μg/ml respectively, have been incubated one after the other with the HRBC solution.

Blood was collected (2 mL) from healthy volunteers and was mixed with an equal volume of sterilized Alsevers solution (2 % dextrose, 0.8 % sodium citrate, 0.5 % citric acid, and 0.42 % NaCl in distilled water) and centrifuged at 3000 rpm. The packed cells were washed with isosaline solution and a 10 % v/v suspension was prepared with normal saline and kept at 4^0C undisturbed before use. Different concentrations of silver nanoparticles (50, 100, 150, 200 and 250 µg /0.5 ml) in normal saline, Aspirin as standard (50, 100, 150, 200 and 250 µg / 0.5 ml) and control (distilled water instead of hypo saline to produce 100 % haemolysis) were separately mixed with 1 ml of phosphate buffer, 2 ml of hypo saline and 0.5 ml of 10% HRBC suspension was added to prepared. All the assay mixtures were incubated at 37^0C for 30 min and

centrifuged at 3000 rpm for 20 min and the haemoglobin content of the supernatant solution was estimated spectro-photometrically at 560 nm.

Antidiabetic activity:

α-amylase inhibition assay

Different concentrations of silver nanoparticles of the cultivars and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing porcine pancreatic α-amylase enzyme (EC 3.2.1.1) (0.5 mg/ml) were incubated at 25^0C for 10 min. After the incubation, 500µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to the reaction mixture. Subsequently, the reaction mixture was incubated at 25^0C for 10 min. followed by the addition of 1.0 ml of dinitrosalicylic acid (DNSA). Finally, the retort become stopped by the use of revenue of incubation in boiling water for 5 min and cooled to room temperature. The retort mixture became diluted with 10 ml distilled water, and the absorbance becomes measured at 540 nm in a spectrophotometer. The combination of all diverse reagents and the enzyme in addition to the prototype become used as a control. The α-amylase inhibitory sideline became expressed as percent inhibition.

$$\text{Inhibition (\%)} = \frac{(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{Control}}} \times 100$$

The IC_{50} assessment was distinct as the concentration of the silver nanoparticles to inhibit 50% activity of α-amylase under assay conditions.

α-Glucosidase inhibition assay

A choice of concentrations of silver nanoparticles (50-250µg/ml) and 100µl of α-glucosidase (0.5 mg/ml) in 0.1 M phosphate buffer (pH 6.9) solution were incubated at 25^0C for 10 min. Then, 50 µl of 5M p-nitro phenyl-α-D-glucopyranoside in 0.1M phosphate buffer (pH6.9) solution was added. Reaction mixtures were incubated at 25^0C for 5 min. and the absorbance was taken at 405 nm by a spectrophotometer. The mixture of all other reagents and the enzyme except the sample was used as a control and the results of α-glucosidase inhibition activity were expressed in terms of inhibition percentage. The IC_{50} value was defined as the concentration of the silver nanoparticles to inhibit 50% of α-glucosidase activity under assay conditions.

The percentage of α-glucosidase inhibitory activity is calculated by the following formula:

$$\text{Inhibition (\%)} = \frac{(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{Control}}} \times 100$$

RESULTS AND DISCUSSION

UV-visible analysis:

The fresh leaf extract of *P. Betle* varieties was green but after the addition of $AgNO_3$ solution and stirring at room temperature, gradually the solution color changed into dark brown. In other words, bypassing the incubation time, the color intensity increased which confirmed Ag ion reduction and the formation of AgNPs **[18]**. Silver nanoparticles surface Plasmon excitation causes a color change in the solution **[19]**, which is the primary and notable evidence for the formation of AgNPs **[20]**. These exterior Plasmon vibrations of silver nanoparticles twisted a peak at 209 nm as shown in (**Figure 2)**, which indicates the reduction of $AgNO_3$ into silver nanoparticles. It is an eminent reality that the optical properties of metallic nanoparticles are strongly dependent on their appearance and size **[21]**. It may be due to the excitation of the Surface Plasmon Resonance (SPR) effect and the reduction of $AgNO_3$ **[22]**. The intensity of brown color increased in direct proportion to the incubation period **[23]** reported that *Ocimum sanctum* extracts have taken 1 hr to synthesize the AgNPs. However, the report of Shankar *et al*., 2004 shows that the AgNPs are formed in 10 min in the solution of *Azadirachta indica* **[24, 25]**. Also reported that Green Synthesis and Characterization of Silver Nanoparticles using *Piper betle* leaf extract and these results were following the present findings **[26]**.

FT-IR analysis:

FT-IR analysis reveals that bio molecules with carbonyl, hydroxyl, and amine functional groups have the potential for metal ion reduction and for capping the newly formed nanoparticles. Fourier transform infrared (FTIR) spectrophotometer was obtained using in the range of 3600-400 cm^{-1}.The peaks are observed in (**Figure 3)**. The spectral peak of the Sirugamani-1 proved spectral peak of 3450.80 cm^{-1}, 1634.74 cm^{-1}, 1519.97 cm^{-1}, and 682.83cm^{-1}.

SEM analysis:

The SEM results revealed that the experiment confirmed the silver colloids present in the sample and it also clearly illustrated that the silver nanoparticles are crystalline in the present synthesis and size was approximately 25nm - 37nm as shown in (**Figure 4)**. The angiosperm plant species of *Lantana camera* extracts have been used for the synthesis of silver nanoparticles and the size of nanoparticles observed by SEM **[27]**. In the present findings observed the SEM micrograph of the dry mass showed silver nanoparticles of size approximately above

39.60 nm. The SEM image showed relatively spherical shape nanoparticles formed with a diameter range of 40-50 nm. A similar phenomenon was represented by Chandran *et al*., **[18]**. The size of silver nanoparticles size allotment, the morphology particles of nano-sized, and surface morphology in solution are imperative factors in evaluating the biosynthesized of nanoparticles toxicity **[28-29]**.

X-Ray diffraction analysis:

The shape of the silver nanoparticles proved to be spherical shaped was shown by XRD analysis and as shown in (**Figure 5)**. Similarly observed in nano wires of synthesized nanoparticles of *Piperbetle* aqueous extract by using XRD and SEM studies confirmed the formation of metallic silver nanoparticles with diameters in the range of 40-60 nm **[30]**. The portion of particle size by extent gives a spherical shaped group. In an earlier study, synthesized nanoparticles size distributions were obtained in the range of 18.03 nm to 148.7 nm by using *Saururus chinensis* leaf extract **[31]**.

EDX Analysis:

The EDX spectrum revealed that the strong signal in the silver particle region and confirms the formation of AgNP s indicated as in (**Figure 6).** The energy dispersive spectroscopy (EDX) data show very strong silver and weak signals of chloride and carbon peaks, which indicate that the reduction of silver ions to elemental silver possibly originated from the molecules attached to the surface of the AgNPs. The EDX spectrometers confirmed the presence of an elemental silver signal of the silver Nanoparticles **[32]** reported the energy dispersive spectral analysis, the leaf extracts of *Putranjiva roxburghii* weight percent of gold nanoparticles was found to be 48%. In our investigation, the energy dispersive spectral analysis of silver nanoparticles by *Piper betle* has the weight percentage of silver is 62.58%.

***In-Vitro* anti-inflammatory activity:**

The *in vitro* anti-inflammatory activity of spherical silver nanoparticles were determined using Albumin Denaturation Technique and HRBC stabilization method which as shown in (**Table 1** and **Table 2)**. The *in-vitro* anti-inflammatory activity of spherical silver nano particles and Mono disperse hexagonal gold nanoparticles were observed using fruit extract of *Prunus serrulata* **[33]**. A number of studies have made to known that these cytokines are decisively concerned in inflammation, different auto-immune disorders, and tumor production. Due to their exclusive responsibility in the progress of

inflammation, down regulation of iNOS, COX-2, and NF-jB is an important therapy for proper treatment and prevention of inflammatory diseases. As the inflammatory process progressed, various inflammatory mediators and growth factors are released. Previous studies were evidence that, nitrite and COX-2are basic inflammatory components in the development of the inflammatory process **[34, 35]**.

Anti- diabetic activity:

The maximum anti-proliferative activity was shown by petroleum ether extract of *Piper betel* and its modulation of oxidative stress parameters was investigated by using streptozotocin-induced diabetic rats. The bio-fabricated Nanoparticles of Piperbetle leaves showed anti- diabetic activity with Aspirin as standard by using α-Amylase Inhibition Assay, α-Glucosidase Inhibition Assay as depicted in (**Table 3** and **Table 4)**. Eugenol was found to be one of the isolated molecules present in the ethyl acetate extract of *Piper betle*, also showed significant antiproliferative activity. The anti-proliferative and anti-cancerous diversion of Eugenol in resistance en route for assorted mobileular injure has been whispered earlier. The mechanism, through the aid of using which Eugenol acts, consists of Eugenol-precipitated cell apoptosis. Where one of the components is with the aid of using activating reactive oxygen radical's knowledge with contained by the cancerous cells **[36]**.

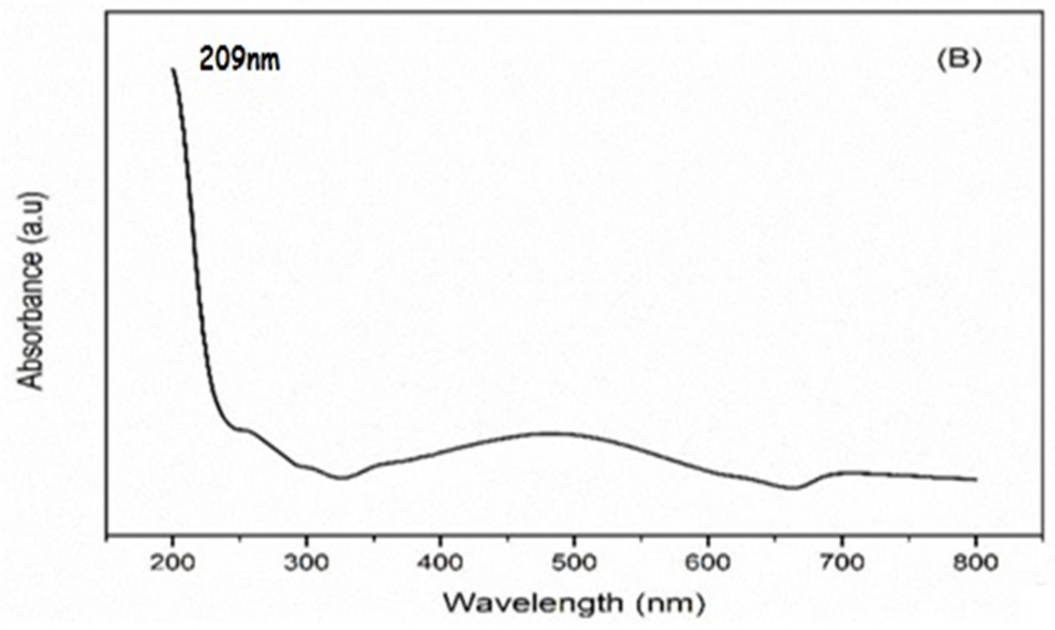

Figure 2: The UV-visible absorption spectrum of aqueous extract of silver nitrate with Leaf extracts of *Piper betle* variety-Sirugamani-1

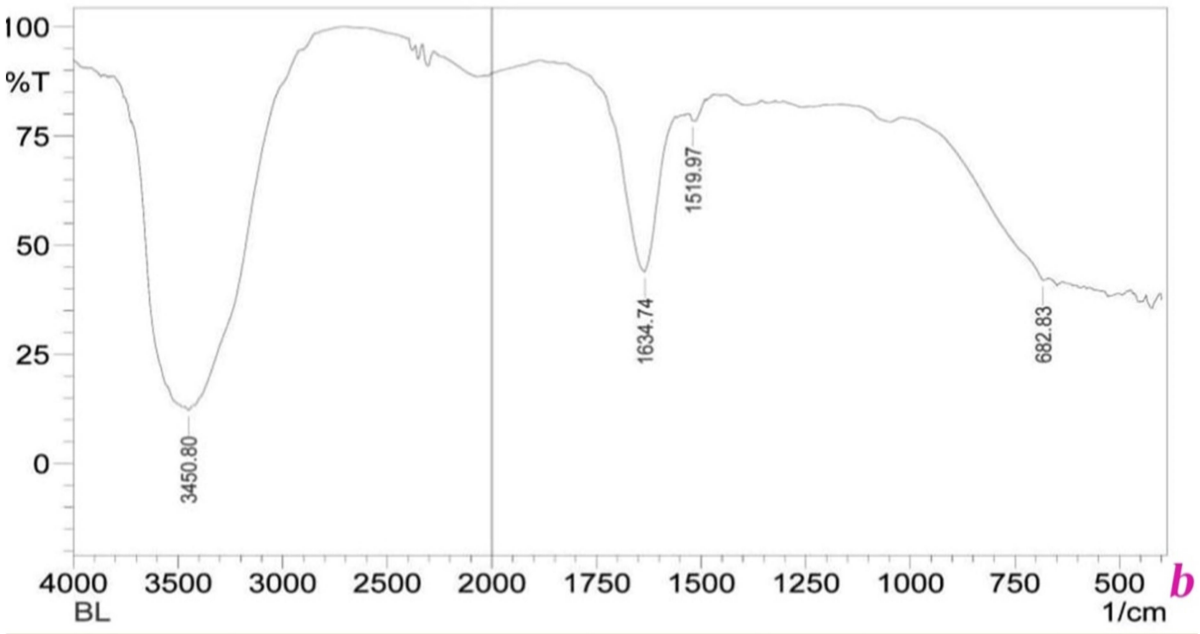

Figure 3: FT-IR spectra of nanoparticles synthesized by *Piper betle* extract solution; Sirugamani-1

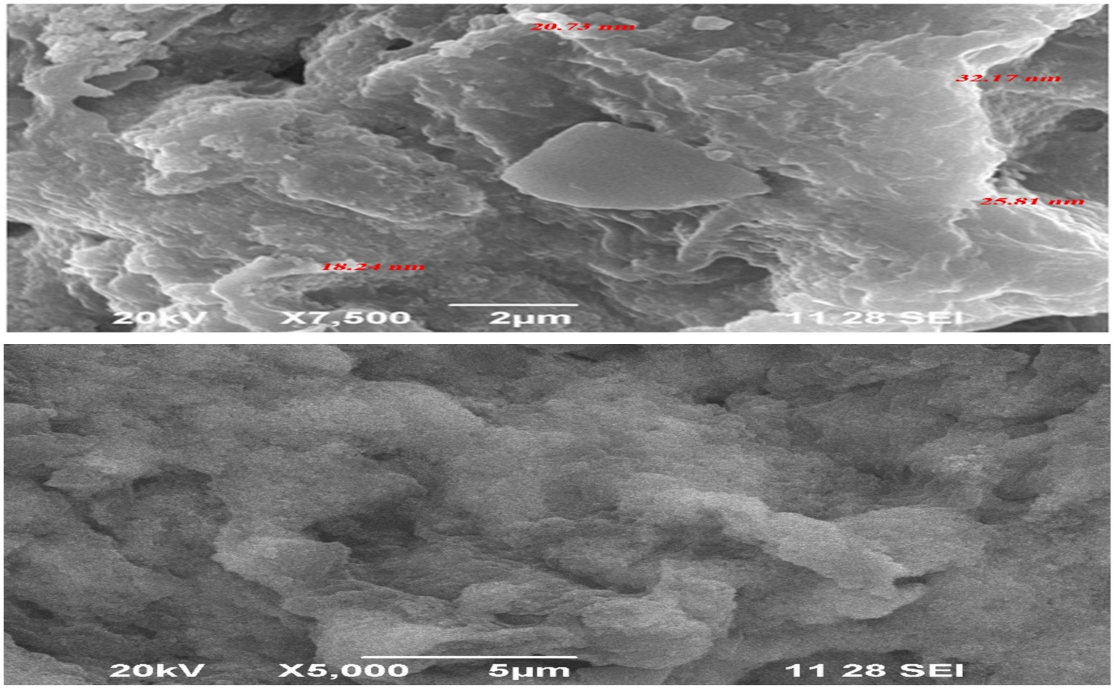

Figure 4: Scanning electron microscopic images of silver Nanoparticles formed in *Piper betle* of Sirugamani-1 cultivar

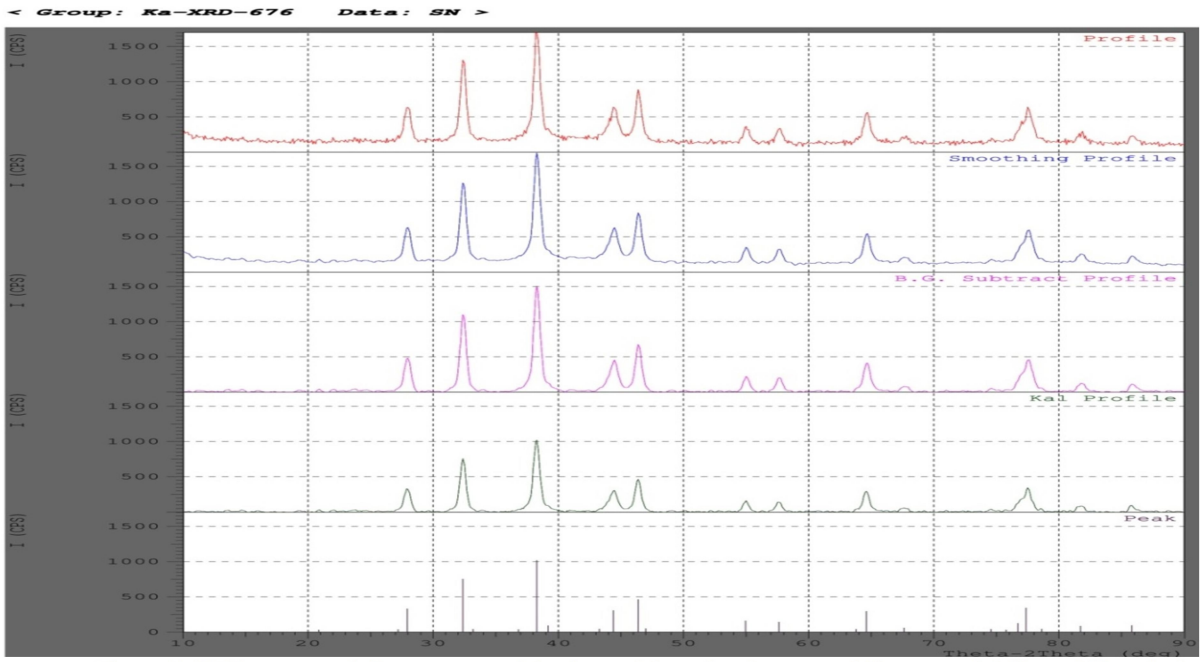

Figure 5: XRD pattern of silver nanoparticles formed from Leaf extract of Sirugamani-1 Var. of *Piper betle*

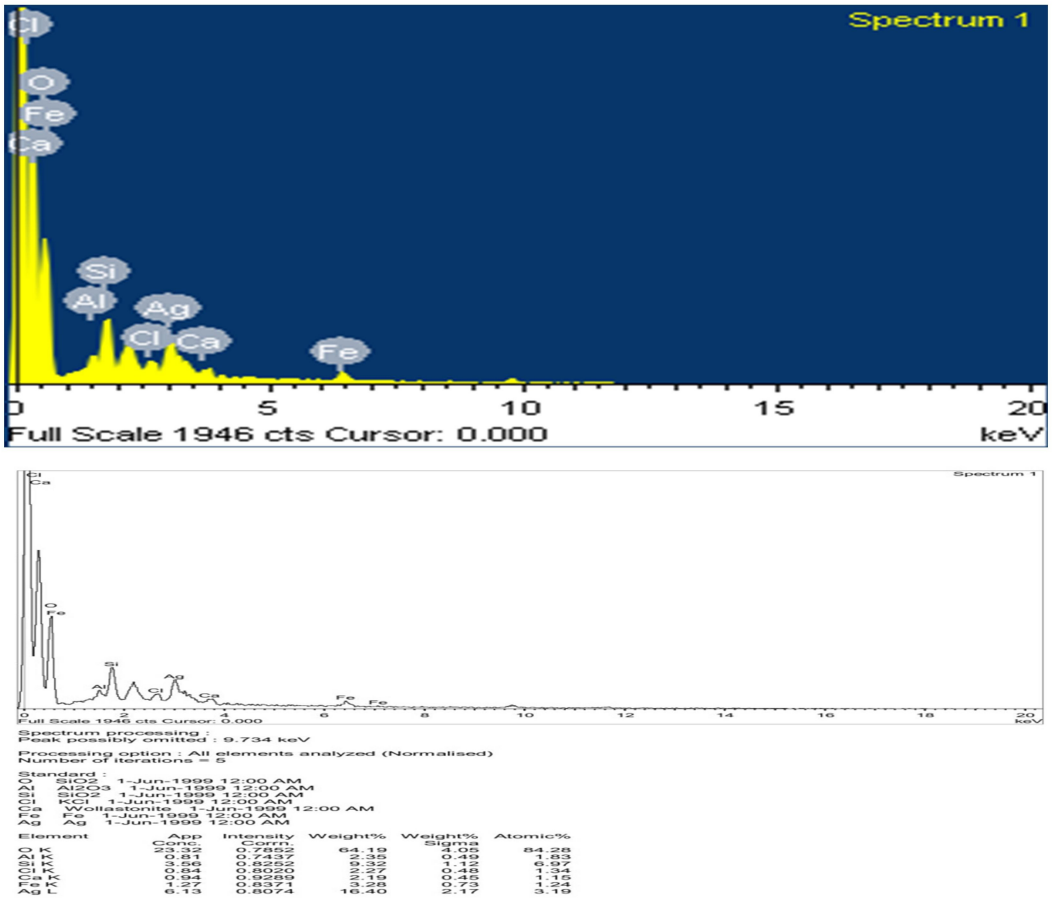

Figure 6: a-b-EDAX spectra recorded from a film, after synthesis of silver noparticles with different X-ray emission peaks of Sirugamani 1 Var. of *Piper betle*

Table 1: *In-vitro* anti-inflammatory activity of membrane stabilization Assay of (Sirugamani -1) Ag NP's

Concentration (µg/ml)	AgNPs (Sirugamani 1)	Aspirin
50	21.40 ± 0.15	25.78 ± 0.84
100	28.50 ± 0.30	37.49 ± 0.58
150	40.30 ± 0.50	47.11 ± 0.24
200	52.50 ± 0.42	59.34 ± 0.52
250	64.39 ± 0.45	68.55 ± 0.56

Values are expressed as mean ± SD (n=3)

Table 2: *In-vitro* anti-inflammatory activity of albumin denaturation assay (Sirugamani -1) Ag NP's

Concentration(µg/ml)	AgNPs (Sirugamani 1)	Aspirin
50	10.30 ± 0.80	18.30 ± 0.40
100	24.30 ± 0.50	28.40 ± 0.35
150	36.30 ± 0.30	39.30 ± 0.40
200	48.23 ± 0.20	52.30 ± 0.50
250	62.20 ± 0.30	65.40 ± 0.50

Table 3: *In- vitro* α-amylase inhibition assay for AgNPs extract in piper betle

Concentration (µg/ml)	AgNPs (Sirugamani -1)	Aspirin
50	10.30 ± 0.80	18.30 ± 0.40
100	24.30 ± 0.50	28.40 ± 0.35
150	36.30 ± 0.30	39.30 ± 0.40
200	48.23 ± 0.20	52.30 ± 0.50
250	62.20 ± 0.30	65.40 ± 0.50

Values are expressed as mean ± SD (n=3)

Table 4: *In-vitro* activity of α-Glucosidase inhibitory assay for Ag NPs extract in *piper betle*

Sample concentration (µg/ml)	AgNPs (Sirugamani 1)	IC_{50} value(µg/ml)
50	20.25 ± 0.30	167.36
100	32.05± 0.20	
150	45.25 ± 0.30	
200	58.50 ± 0.40	
250	69.50 ± 0.20	

Values are expressed as mean±SD (n=3)

CONCLUSIONS

Thus review of the literature suggests that the leaves of *Piper betle* have tremendous potential sources of bioactive constituents used for novel therapeutic usage and pharmacological properties reveal it to be fit for its future practice as a promising supply for treating various ailments. Synthesis of silver nanoparticles was carried out using medicinal and aromatic plants, which is an eco-friendly method of synthesis of nanoparticles. Using plants for the same is advantageous as they are freely available and do not require elaborate maintenance like that required for microbial cultures. The selected plants are known to have various secondary plant metabolites like alkaloids, phenols, flavonoids, and terpenoids which play an important role in antimicrobial action against various pathogens. Hence, it was promising that these secondary metabolites would help in the reduction of silver to silver

nanoparticles. The selected plant was capable of synthesizing silver nanoparticles at room temperature as per the protocols.

The *Piper betle* was finalized for further study to investigate the antioxidant and anti-cancer properties of synthesized nanoparticles of the nanomedicine. The anti-diabetic, anti-inflammatory, and cytotoxicity of synthesized silver nanoparticles showed uppermost activity against tested biological exploration. In the future, the alternative of such medicinal plant being strength also in addition create a variety of new platform for spotting the in all probability herbal drugs in nano science for biomedical applications. Hence the sophisticated process has numerous reimbursement comprehensive of controlling the scale of metallic nanoparticles being, eco-friendly, and cost-powerful technology.

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