

AMYLASE INHIBITORY POTENTIAL OF SILVER NANOPARTICLES BIOSYNTHESIZED USING BREYNIA RETUSA LEAF EXTRACT

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ABSTRACT

Biological methods are less cumbersome and much ecofriendly and this paper reports the green synthesis of silver nanoparticles using the extract of *Breynia retusa* leaves extract. Biosynthesis of silver nanoparticles was assisted by microwave irradiation to minimize the time taken for the reduction of silver ions. The nanoparticles thus synthesized were subjected to physical characterization. The UV-Visible spectra, Fourier Transform Infra-Red Spectroscopy spectra, X-ray diffraction pattern were studied. The size and surface morphology of the nanoparticles were determined using Scanning Electron Microscopy. The silver nanoparticles were also analyzed for amylase inhibitory activity. The nanoparticles proved to be potential inhibitors of amylase and thus promising anti-diabetic agents.

KEY WORDS: silver nanoparticles, *Breynia retusa*, XRD, FTIR, amylase inhibition, anti-diabetic.

INTRODUCTION

Breynia retusa, a plant belonging to the family Euphorbiaceae, has found a predominant use in indigenous, traditional folklore medicines. The plant is used to treat bowel disorders, inflammation, nervous system disorders, vitality, insect bites and conjunctivitis.^[1] The roots and leaves of *B. retusa* are used in traditional medicine for various ailments. The leaves are effective in antimicrobial treatment and are used as poultice to relieve suppuration.^[2, 3] *B. retusa* is used in traditional herbal formulations to treat complications of diabetes, skin

problems.^[4] Macerated leaf juice is taken for body pain, skin inflammation, hyperglycemia, diarrhoea and diuresis and the bark as astringent and diuretic. Also, the fruits have been used for dysentery, roots for fits and meningitis, twigs for tooth ache.^[5, 6, 7]

Nanotechnology has emerged into a promising field of research with potent applications in medicine. Nanobiotechnology utilizes biological principles alongside physical and chemical processes to synthesise nanoparticles with biological applications. Microorganisms and plant extracts have been employed to synthesise nanoparticles and thus help to avert the undesirable effects posed by chemical production of nanoparticles. Biological methods are also cost-effective, less energy consuming and ecofriendly. The nanoparticles of silver, gold etc., have found profound applications as anti-bacterial, anti-viral, anti-cancer, anti-inflammatory and anti-diabetic agents, biopesticides, biosensors, components of textiles etc.^[8] The green synthesis of nanoparticles using extracts from plants like *Calotropis*, *Eucalyptus*, *Azharidacta*, *Hibiscus*, *Aloe vera* have been reported.^[9]

The extracts of the leaves of *B. retusa* were found to inhibit alpha-amylase and were suggested for usage as anti-diabetic agents.^[2] Herbal medicines with antidiabetic potential have different modes of action - mimic insulin, act on insulin secreting beta cells, or modify glucose utilization. Glucose utilization can be modified by altering the viscosity of gastrointestinal contents, delaying gastric emptying, or delaying glucose absorption. Glucose absorption can be delayed by reducing the rate of digestion of starch. This could be achieved by inhibiting of the mammalian alpha amylase enzyme in the intestine. This would decrease the absorption of glucose and consequently reduce postprandial blood glucose.^[10] In the present study, silver nanoparticles were synthesized using the extract of *B. retusa* leaves and the nanoparticles thus synthesized were evaluated for their amylase inhibitory potential.

MATERIALS AND METHODS

Collection of plant materials

B. retusa plant leaves were collected from Coimbatore, Tamil Nadu. The plant was identified and duly authenticated by Dr. J. Jayaraman, Plant Anatomy Research Center, Tambaram, Chennai, Tamil Nadu (Voucher No. PARC/2012/1441).

Breynia Retusa Extract Preparation

A 10 g portion of thoroughly washed fresh *Breynia retusa* leaves were cut and 200ml of sterile distilled water was added and exposed to microwave irradiation for 3 min to subdue

the active plant constituents. The solution was then filtered in hot condition to remove the solid fibrous residues. The clear filtrate which is the extracellular extract of the leaf was used for nanoparticles synthesis.

Preparation of Silver Nitrate Solution

Analytical grade silver nitrate procured from Hi Media labs (RM 638) was prepared as 10^{-3} M and 10^{-6} M solutions and used in this experiment.

Synthesis of silver nanoparticles

For the synthesis of silver nanoparticles using *B. retusa*, 10 ml of extract was added to 50ml of silver nitrate. The solution was subjected to microwave irradiation for 3 min and the color change was observed. Then the solution was centrifuged at 3000 rpm for 20 min. The deposited residue was dried on a hot plate. The silver nanoparticles were isolated and concentrated by repeated (5 times) centrifugation of the reaction mixture at 3,000 g for 20 min by replacing the supernatant with distilled water each time. The nanoparticles were washed well to remove any residue particles that were not the capping agents. The suspension was dried and stored as a crystalline powder for optical measurements, characterization studies and biological assay.

Characterization of Silver Nanoparticles

The physical characterization of silver nanoparticles was done at SAIF, IIT Madras.

UV- Vis spectroscopy

The UV-Vis spectrum of the synthesized nano silver particles was studied with Cary 5E UV-Vis Spectrophotometer, from 300-800 nm.

XRD

The X-ray diffraction (XRD) pattern of the silver nanoparticles synthesized was studied by Bruker Kappa AXE XII, with Cu $K\alpha$ ($\lambda=1.54 \text{ \AA}$) radiation, scanning range from $10^\circ - 90^\circ$.

FTIR

The Fourier Transform Infra-Red Spectroscopy (FTIR) of the dried nanoparticles were studied using Perkin-Elmer Spectrum-One instrument. 256 scans of Silver nanoparticles were taken in the range of 400-4000 cm^{-1} and the resolution was kept as 4 cm^{-1} .

SEM

Scanning Electron Microscopic (SEM) analysis was done using Quanta 200 FEG scanning electron microscope. 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 grid, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

Assay of Alpha Amylase Inhibition

Alpha amylase (α) inhibitory activity of each extract was analysed by the method of Bernfeld, 1995.^[11] In brief, 100 μ l of the test extract was allowed to react with 200 μ l of α -amylase enzyme (Hi media Rm 638) and 100 μ l of 2 mM phosphate buffer (pH 6.9). After 20 min incubation, 100 ml of 1% starch solution was added. The same was performed for the control where 200 μ l of the enzyme was replaced by buffer. After incubation for 5 min, 500 μ l of dinitrosalicylic acid reagent was added to both control and test. They were kept in boiling water bath for 5 min. The absorbance was recorded at 540 nm using spectrophotometer and the percentage inhibition of α - amylase enzyme was calculated using the formula

$$\text{Inhibition (\%)} = 100 (\text{control} - \text{test} / \text{control})$$

Activity Staining of Amylase

Activity staining of amylase was done according to the method of Scandalios, 1974.^[12] The gel consisted of 1% agar in 0.4 M phosphate buffer of pH 7.5. The plant extracts (1mg/ml) that were pre-incubated with the enzyme were loaded in to different wells. Untreated enzymes served as a positive control in a separate well. The buffer used in the gel was also used in the electrode compartments. A stabilized current of 100 V was passed through the gel for 2 h at 4 °C. For visualization of the amylase bands, the gel was immersed in 0.5% soluble starch and incubated at 37 °C for 30 min. The excess starch was then washed and then flooded with iodine potassium iodide solution for 1 min. Colorless bands against deep blue background indicate amylase activity.

RESULTS AND DISCUSSION

Synthesis and Characterization of Nanoparticles

Nanoparticles have a wide range of applications such as magnetic devices, sensors, and optoelectronics and drug delivery systems. Different biomolecules like proteins, polyphenols and flavonoids are used for the synthesis and surface modifications of metal nanoparticles. These nanoparticles can also be functionalized with different bio-moieties that can be used

for bio-specific assays. The physical and chemical methods of nanoparticles synthesis involve top-down approach and these methods produce enormous quantity of byproducts which are hazardous to health. As the demand for nanomaterials in the industrial field is growing, newer innovative novel biological methods are being established.^[13] In this study, the silver nanoparticles were synthesized and studied using the extract of the leaves of *B. retusa*. To fasten the process, heating using microwaves was adopted. The formation of homogenous silver nanoparticles is best achieved by an even heat transfer. This is provided by microwave heating.

Visual Inspection

After the addition of *B. retusa* leaf extract to silver nitrate solution, a light yellowish color was observed which changed to dark brown colour on subjecting to microwave irradiation. This colour change indicated that there was reduction of silver particles. The same treatment was given to silver nitrate solution without the addition of plant extract. There was no change in colour and this showed that the reduction of silver was by the plant extract and not by the microwaves. The microwave-assisted method is much faster than the earlier conventional studies. The time required for the conventional synthesis of silver nanoparticles using plant extracts ranged from several minutes to few hours and thus are rather slow.^[8] At room temperature, the stability of the synthesized silver nanoparticles was observed for 10 weeks during the period of study and this was much greater than the particles produced from other biological methods.

Uv- Vis Spectroscopy

This is one of the most widely used techniques for structural characterization of silver nanoparticles. The position and shape of plasmon absorption of silver nanoclusters are strongly dependent on the particle size, dielectric constant of the medium and surface adsorbed species. In the present study, a single peak at 430 – 460 nm was observed with the silver nanoparticles synthesized with 10^{-3} M AgNO_3 , while multiple peaks were observed with 10^{-6} M AgNO_3 (Fig 1a-c). Thus higher concentration of silver nitrate results in synthesis of nanoparticles of diverse shapes.

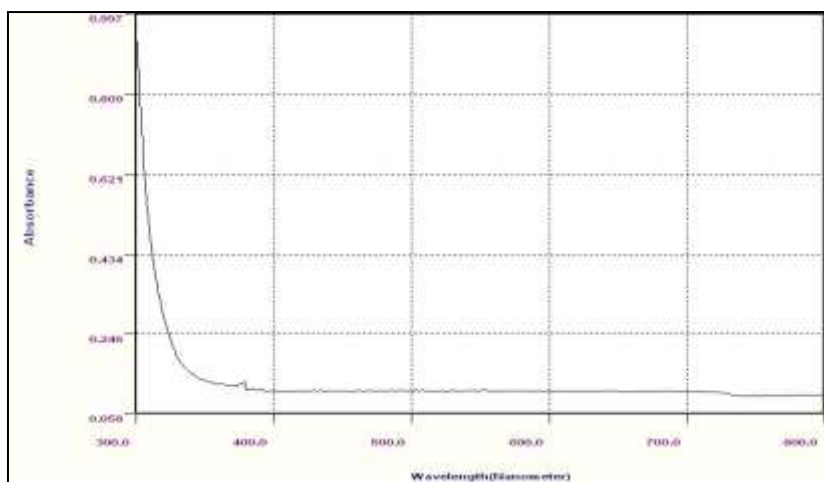


Figure 1a. UV-Vis spectrum of colloidal silver solution without the addition of *B. retusa* leaf extract

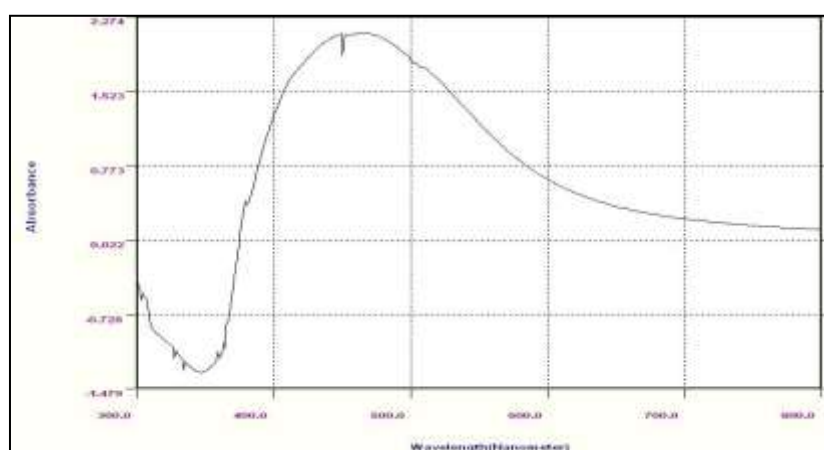


Figure 1b. UV-Vis spectrum of silver nano particles synthesised using *B. retusa* leaf extract and 10^{-3} M AgNO_3

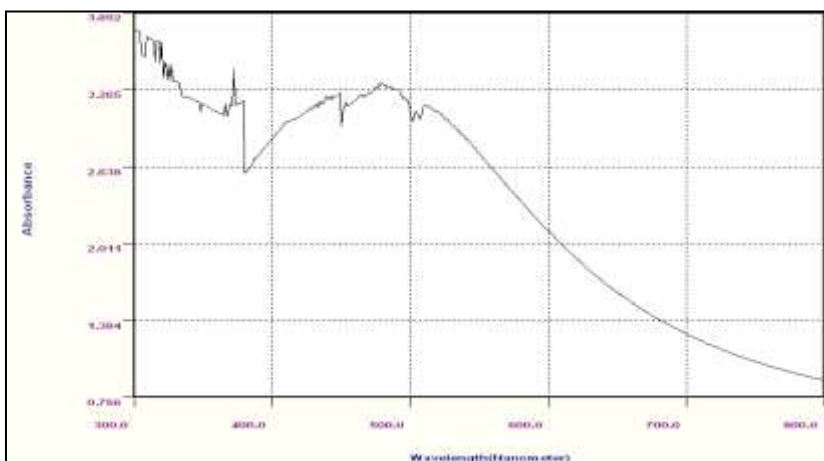


Figure 1c. UV-Vis spectrum of silver nano particles synthesised using *B. retusa* leaf extract and 10^{-6} M AgNO_3

The absence of a peak in the 400-500 nm region analysed with the colloidal silver nitrate solution which was devoid of plant extract indicated the absence of nanoparticles and thus ensured the effect of plant extract in the biogenic synthesis of nanoparticles.

According to Mie's theory, only a single Surface Plasmon Resonance (SPR) band is expected in the absorption spectra of spherical nanoparticles, whereas anisotropic particles could give rise to two or more SPR bands depending on the shape of the particles. The number of SPR peaks increases as the symmetry of the nanoparticle decreases. Thus, spherical nanoparticles, disks, and triangular particles of silver show one, two, and more peaks respectively.^[14]

X-ray Diffraction

It was carried out to confirm the crystalline nature of the particles and the x-ray diffraction (XRD) pattern was obtained as shown in Figure 2. The XRD pattern shows the intense peaks in the whole spectrum of 22 values ranging from 10 to 100. It is important to know the exact nature of the silver particles formed and this can be deduced from the XRD spectrum of the sample. XRD spectra of pure crystalline silver structures have been published by the Joint Committee on Powder Diffraction Standards (file no. 04-0783).

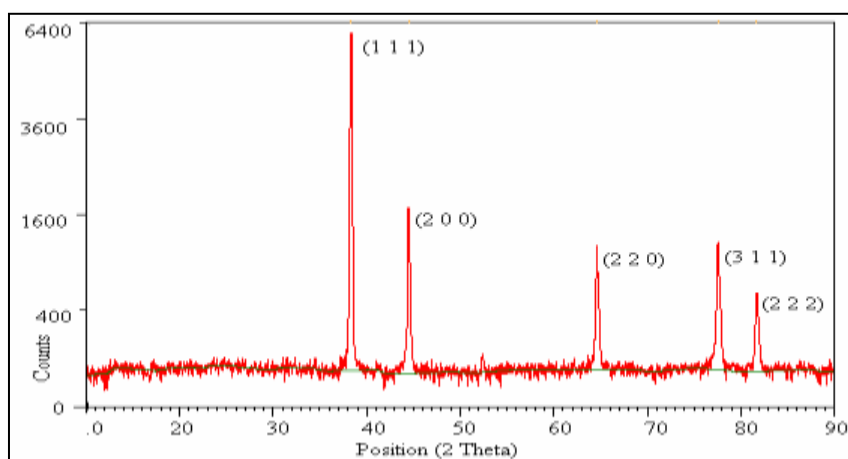


Figure 2. XRD Patterns of Ag nanoparticles synthesized using *B. retusa* leaf extract

A comparison of our XRD spectrum with the Standard confirmed that the silver particles formed in our experiments were in the form of nanocrystals, as evidenced by the peaks at 38.43° , 44.40° and 67.57° , corresponding to 111, 200 and 220 planes for silver respectively (Figure 2). The XRD pattern clearly shows that the silver nanoparticles are crystalline in nature. A Bragg reflection corresponding to the (111) sets of lattice planes are observed which may be indexed based on the face-centered cubic (fcc) structure of silver.^[15] Apart from the Bragg peak, no additional peaks were observed. This suggests the absence of

crystallization of bio-organic phase which otherwise occurs on the surface of the silver nanoparticles.^[16]

FTIR

FT-IR spectroscopic studies were carried out to investigate the plausible mechanism behind the formation of these silver nanoparticles and offer information regarding the functional groups. The representative spectra of stabilized silver nanoparticles obtained using *B. retusa* and the leaf extract of *B. retusa* alone has been given in Figure 3a and b.

The very strong absorption peaks at 1634, and the strong absorption peaks at 1384 represents the presence of NO_2 which may be from AgNO_3 solution, the metal precursor involved in the silver nanoparticles synthesis process. Strong interaction of water with the surface of Silver could be the reason for the O-H stretching mode peaks at 2925^[17].

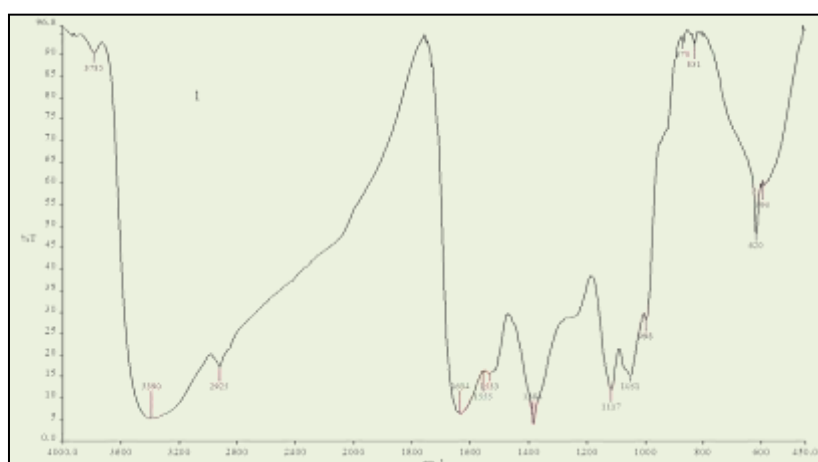


Figure 5a. FTIR spectrum of silver nanoparticles synthesised using *B. retusa* leaf extract

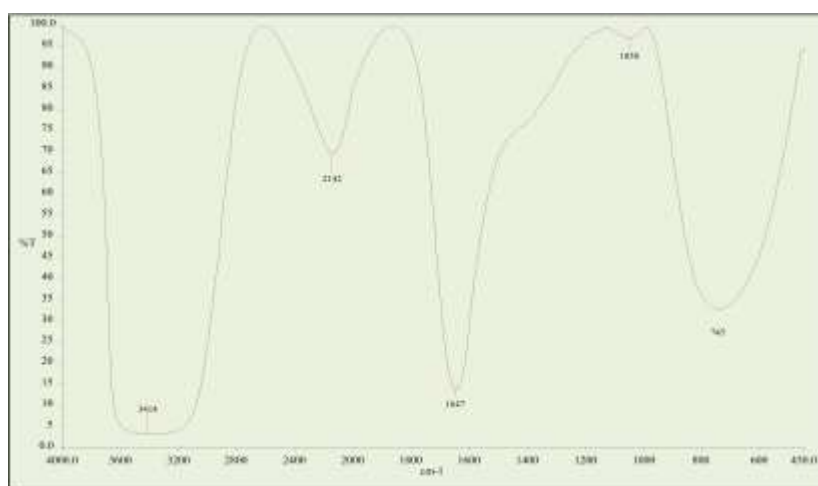


Figure 5b. FTIR spectrum of *B. retusa* leaf extract

Appearance of broad peak at wavenumber 3390 in the nanoparticle spectrum and 3414 in the plant extract spectrum indicates presence of phenolic hydroxyl group (OH) which represent the stretching vibration because of presence of hydrogen bonding belonging to flavonoids and tannins. This along with the peak of 870 cm^{-1} which represents the aromatic ring C-H vibrations, indicate the involvement of free catechin. This suggests the attachment of some polyphenolic components on to silver nanoparticles. This means the polyphenols attached to silver nano particles may have atleast one aromatic ring. Among them, the absorption peak at 1020 cm^{-1} can be assigned as absorption peaks of C-O-C- or -C-O- or to C-N stretching vibrations of aliphatic amines. The peaks at $1000\text{-}1200\text{ cm}^{-1}$ indicate C-O single bond and peaks at $1620\text{-}1636\text{ cm}^{-1}$ represent carbonyl groups (C = O) from polyphenols such as catechin gallate, epicatechin gallate and theaflavin.^[18, 19] Studies have confirmed the fact that the carbonyl group form amino acid residues and proteins has the stronger ability to bind metal. The proteins could therefore form a layer over the metal nanoparticles to prevent agglomeration.

SEM

The size and structure of silver metal nanoparticles was further characterized using SEM analysis. The surface deposited silver nanoparticles are clearly seen at high magnification ($\times 100,000$) in the micrograph. This was further confirmed by SEM analysis. The morphology of silver nanoparticles was more clearly seen and the particles are being predominantly spherical, polydispersed and ranged in size from 30-40 nm (Figure 4).

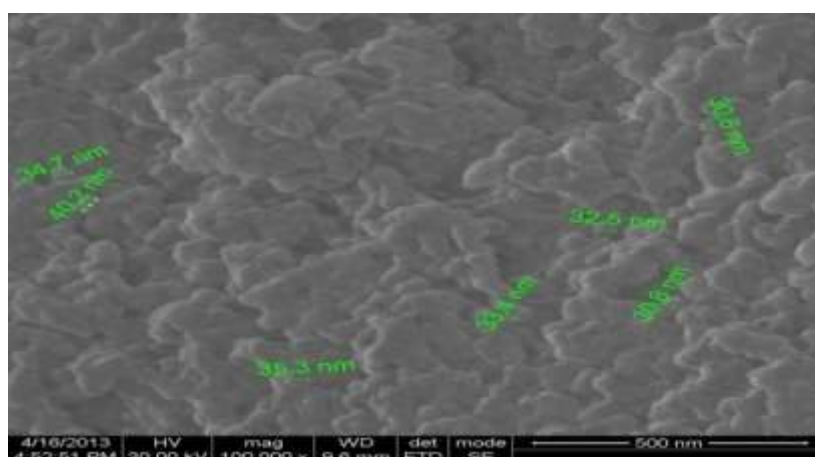


Figure 4. SEM micrograph of silver nanoparticles

Alpha Amylase Inhibition

α -amylase is a key enzyme involved in starch breakdown, by catalysing the hydrolysis of α -1,4 glycosidic linkages in starch and related polysaccharides. The main challenge involved in

managing diabetes is maintaining blood glucose levels close to normal levels. The inhibition of the enzyme α -amylase has significant implications in controlling hyperglycemia and therefore may be useful in managing diabetes. The percentage inhibition of α -amylase by the silver nano particles synthesized using the extracts of *Breynia retusa* leaves was studied in a concentration range of 10-640 μ g/ml. The IC₅₀ of the nano particles was found to be 100 μ g/ml (Figure 5).

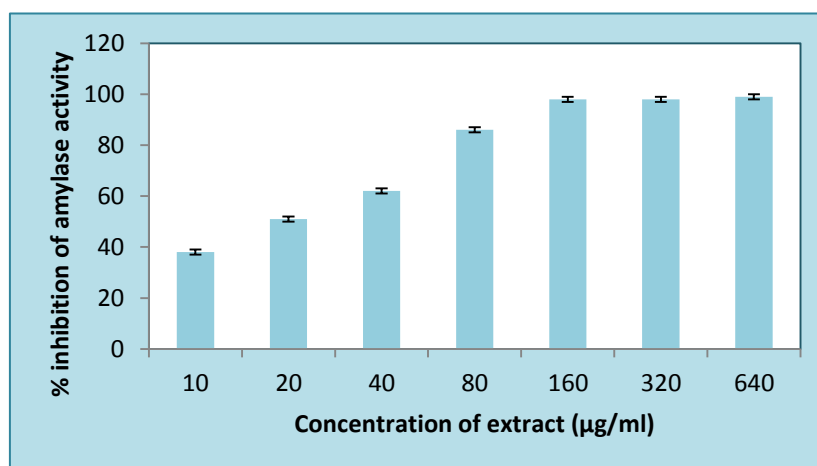


Figure 5. Alpha amylase inhibitory activity of silver nanoparticles synthesised using *B. retusa* leaf extract

Complete inhibition of amylase was observed when the concentration of the nanosilver particles was above the IC₅₀ value. The activity of the nanosilver particles was compared with the enzyme control which exhibited a distinct achromatic band against a dark blue background on the agar gel (Figure .6).



At concentrations below the IC₅₀ value, faint colourless bands could be observed and this indicates partial inhibition of α -amylase. With concentration above the IC₅₀ value, complete inhibition of α -amylase was observed with no colourless bands on the gel. This indicates

inhibition of amylase activity and utilization of starch substrate. In the traditional system of Indian medicine, *B. retusa* is used for treatment as a diuretic, anti-microbial and analgesic. A thorough study of literature shows the folklore claim of *B. retusa* in treatment of diabetes. The amylase inhibitory activity of the leaves of *B. retusa* has been scientifically established by Kripa *et al.*, 2011.^[2] The present study established the amylase inhibitory potential of the silver nanoparticles synthesised using the leaves of *B. retusa*. Enzyme inhibitors are either proteinaceous or polyphenolic in nature. The FTIR analysis of the silver nanoparticles indicates the presence of proteins and polyphenols in the bioorganic cap of the nanoparticles. This establishes the amylase inhibiting efficiency of the nanosilver particles.

CONCLUSION

Green synthesis of nanoparticles of silver was achieved using the extract of *B. retusa* leaves. The nanoparticles thus synthesized were characterized and analysed for amylase inhibitory potential. The efficacy of the nanoparticles to inhibit amylase and serve as an anti-diabetic agent has been ascertained and thus these nanoparticles can be studied as anti-diabetic agents *in vivo*.

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