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APPENDIX 1

DETERMINATION OF DPPH RADICAL SCAVENGING ACTIVITY

(Blois, 2019; Brand Williams *et al*., 1995)

Principle

Antioxidants react with DPPH and convert it to diphenyl-picryl hydrazine by donating its OH group. The degree of discoloration from purple to yellow colour can be measured at 519nm, which is a measure of the radical scavenging potential of the extracts.

Reagents

1. DPPH – 2,2' – diphenyl-2-picryl hydrazyl hydrate (0.3mM in methanol)
2. Methanol

Procedure

The different solvent extracts and crude aqueous extract (20µl corresponding to 10mg) were added with 0.5ml of methanolic solution of DPPH and 0.48ml of methanol. The mixture was then allowed to react at room temperature for 30 minutes. DPPH methanol solution was used as positive control and methanol alone acted as blank. After incubation, the discolourisation of the purple colour was read at 518nm in a spectrophotometer. The per cent inhibition was calculated using the following formula;

$$\text{Scavenging activity (\%)} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX 2

DETERMINATION OF ABTS RADICAL SCAVENGING ACTIVITY

(Re *et al.*, 1999)

Principle

The ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) radical cation decolourisation assay was employed to evaluate the radical-scavenging potential of the plant extracts. ABTS is a chromogenic compound that is converted into a blue-green monocation radical ($ABTS^{+}$) in the presence of an oxidising agent, exhibiting a characteristic absorbance at 750 nm. Antioxidants present in the extracts reduce $ABTS^{+}$ back to its colourless form, and the extent of this decolourisation corresponds to the percentage reduction of the radical.

Reagents

1. Ethanol
2. ABTS working solution (7 mM ABTS mixed with 2.45 mM ammonium persulfate and incubated at room temperature for 12–16 hours before use)

Procedure

For the assay, 500 µL of each extract was mixed with 300 µL of ABTS working solution, and the final volume was adjusted to 1 ml using ethanol. The reaction mixture was allowed to stand, and the absorbance was measured at 745 nm. The percentage inhibition of the ABTS radical was determined using the following formula:

$$\text{Scavenging activity (\%)} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX 3

Determination of Ferric Reducing Antioxidant Potential (FRAP) Activity

(Benzie and Strain, 1996)

Principle

The Ferric Reducing Antioxidant Potential (FRAP) assay was employed to evaluate the reducing capacity of the plant extract. This method is based on the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) by antioxidants present in the sample. The reduced Fe^{2+} subsequently forms a blue-coloured complex with 2,4,6-tripyridyl-s-triazine (TPTZ), known as the Fe^{2+}–TPTZ complex, which exhibits a strong absorbance at 593 nm. The intensity of the colour formed is directly related to the reducing power of the sample.

Reagents

1. Acetate buffer
2. TPTZ solution
3. Ferric chloride ($FeCl_3$) solution
4. Standard antioxidant: Ascorbic acid

Procedure

For the assay, the FRAP reagent was freshly prepared by mixing acetate buffer, TPTZ solution, and $FeCl_3$ in the required proportions. Various concentrations of extracts were added to this freshly prepared FRAP reagent, and the reaction mixture was allowed to stand. The absorbance was measured at 593 nm at the initial time point (t = 0) and again after incubation (t = 4 minutes). A sample blank was used for baseline correction, and ascorbic acid served as the positive control. The increase in absorbance corresponded to the ferric-reducing antioxidant potential of the samples.

APPENDIX 4

ASSESSMENT OF *IN VITRO* ALPHA AMYLASE INHIBITORY ACTIVITY

(Ishwarya *et al*., 2022)

Reagents

1. Alpha amylase (0.5mg/ml).
2. 20mM Phosphate buffer (pH 6.9).
3. Starch (0.5%).
4. 96mM DNS

Procedure

20-100µg/ml plant sample extract and standard (Acarbose) was allowed to react with 200µl of 20mM phosphate buffer pH 6.9, containing porcine alpha amylase were incubated at 25^0C for 10 min. After pre incubation, 250µl of starch solution in 20Mm phosphate buffer pH 6.9 was added. The reaction mixture was then incubated at 25^0C for 10 minutes. The reaction was stopped with 500µl of dinitro salicylic acid (DNS) colouring reagent. The tubes were then incubated in boiling water bath for 5 minutes and cooled to room temperature. Absorbance (A) was measured at 540nm and the per cent inhibition was calculated using the formula,

$$\text{Inhibition (\%)} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX 5

ASSESSMENT OF *IN VITRO* ALPHA GLUCOSIDASE INHIBITORY ACTIVITY

(Peytan *et al*., 2021)

Reagents

1. 10mM para nitro phenyl -alpha-D-glucopyranoside
2. 1mM Phosphate buffer (pH 6.8).
3. Alpha glucosidase (1mg/ml).
4. 100 mM Sodium carbonate

Procedure

The assay mixture contains 0.3ml of 10mM para nitro phenyl alpha-D-glucopyranoside, 1ml of 1mM Phosphate buffer (pH 6.8), 0.2ml of alpha glucosidase and 20-100µg/ml plant extract all in a final volume of 1.7ml. The tubes were incubated for 30 min at 37°C. The reaction was terminated by the addition of 2ml of sodium carbonate. The liberated p-nitrophenol was determined at 400nm. The per cent inhibition was calculated using the formula,

$$\text{Inhibition (\%)} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX 6

DETERMINATION OF NON-ENZYMATIC GLYCOSYLATION OF HEMOGLOBIN

(Premanath *et al*., 2012)

Procedure

The non-enzymatic glycosylation of hemoglobin assay was conducted to assess the antiglycation potential of EBdAgNPs. Glycation involves the reaction of glucose with hemoglobin, leading to the formation of chromogenic products detectable spectrophotometrically. Solutions of glucose (2%), hemoglobin (0.6%), and gentamycin (0.02%) were prepared using 0.01 M phosphate buffer (pH 7.4). Equal volumes (1.0 ml each) of these solutions were mixed with 1.0 ml of EBdAgNPs at different concentrations (10, 20, 40, 60, 80, and 100 µg/ml). The reaction mixtures were incubated in the dark at room temperature for 72 hours. After incubation, the extent of hemoglobin glycation was determined colorimetrically at 520 nm. Acarbose served as the standard reference drug. The inhibition percentage was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX 7

INHIBITION OF i*n vitro* PROTEIN GLYCATION ASSAY

(Avwioroko *et al*., 2022)

Procedure

The in vitro protein glycation assay was used to evaluate the ability of EBdAgNPs to inhibit the formation of advanced glycation end products (AGEs). A fructose solution (1000 mM) prepared in 200 mM phosphate buffer (pH 7.4) was incubated with bovine serum albumin (BSA, 20 mg/ml) in the same buffer. A total of 4.0 ml of fructose solution was combined with 5.0 ml of BSA solution, followed by the addition of 1.0 ml of EBdAgNPs at concentrations of 10, 20, 40, 60, 80, and 100 µg/ml. The reaction mixtures were incubated, and the formation of fluorescent AGEs was monitored using fluorescence spectroscopy with an excitation wavelength of 360 nm and emission readings ranging from 370 to 650 nm. Higher fluorescence intensity indicated increased AGE formation. Metformin was used as a positive control at final concentrations of 1.25, 0.75, and 0.25 mg/ml. The percentage inhibition of AGE formation was calculated.

APPENDIX 8

ESTIMATION OF GLUCOSE UPTAKE BY YEAST CELLS

(Madiwalar *et al*., 2022)

Commercial baker's yeast was washed by repeated centrifugation (4200r/min, 5 minutes) in distilled water until the supernatant fluids were clear and a 10% (v/v) suspension was prepared in distilled water. Various concentrations of extracts (1-5mg) were added to 1ml of glucose solution (5-25mmol/L) and incubated together for 10 minutes at 37 °C. The reaction was started by adding 100µl of yeast suspension, vortexed and further incubated at 37°C for 60 minutes. After 60 minutes, the tubes were centrifuged (3800r/min, 5 minutes) and glucose was estimated in the supernatant. The percent increase in glucose uptake by yeast cells was calculated using the following formula,

$$\text{Increase in glucose uptake (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample) and Abs sample is the absorbance of the test sample.

APPENDIX 9

GLUCOSE DIFFUSION ASSAY

(Ansari *et al*., 2022a)

To evaluate *in vitro* glucose diffusion, a cellulose ester dialysis tube (CEDT) was filled with 2 ml of a solution containing 0.15 M NaCl and 0.22 mM glucose. This solution was divided into two groups: the treated group, which included EBdAgNPs (50 µg/ml), and the control group, which did not contain the nanoparticles. The CEDT was then placed inside a 50 ml centrifuge tube, with both ends securely sealed. Subsequently, 45 ml of 0.15 M NaCl solution was added to the centrifuge tube. The entire assembly was placed in an orbital shaker, set to a constant temperature of 27°C. Glucose concentration in the external solution surrounding the CEDT was measured at 60-minute intervals to observe and monitor the diffusion process.

$$\text{Inhibition (\%)} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX 10

ASSESSMENT OF PANCREATIC LIPASE INHIBITORY ACTIVITY

(Zhang *et al*., 2008)

A suspension containing 1% (v/v) triolein and 1% (v/v) Tween 40 in 0.1M phosphate buffer (pH 8) was prepared and emulsified. Assay was then initiated by adding 800µl of the triolein emulsion to 200µl of porcine pancreatic lipase (0.5 gm pancreatin in 15ml of 0.1M phosphate buffer at pH 8.0) and 200µl of plant extract. The contents were mixed and absorbance measured immediately at 450nm and designated as T0. The test tubes containing reaction mixture were incubated at 37°C for 30 minutes and then the absorbance was recorded at 450nm and designated as T30. The variation in absorbance = [A450 (T0) - A450 (T30)] was calculated for both control and test groups and the % inhibition was calculated using the following formula.

$$\% \text{ inhibition} = \frac{A_{450}\,(\text{Control}) - A_{450}\,(\text{Extract})}{A_{450}\,(\text{Control})} \times 100$$

APPENDIX 11

ESTIMATION OF RED BLOOD CORPUSCLES

(Sahastrabuddhe, 2016)

Principle

The total erythrocyte count was determined accurately by diluting a measured quantity of red blood corpuscles with a fluid isotonic solution by the method of Huxtable.

Reagents

Red blood cell diluting fluid (Hayem's fluid) - 5g of sodium sulphate, 1g of sodium chloride, 0.5g of mercuric chloride were dissolved in 200ml of distilled water.

Procedure

Blood was sucked exactly up to the 20µl mark in the RBC pipette and the diluting fluid was drawn immediately up to the mark and the blood mixed thoroughly with the diluting fluid. It was left for 2-3 minutes for proper mixing. The Neubauer counting chamber was placed along with the cover glass in position. The capillary stem of the pipette was emptied which contains only the diluting fluid. This was done by discarding first 3-5 drops.

Charging of the counting chamber

One drop of diluted blood was released into the groove of the Neubauer counting chamber. It was left for cells to settle for 2-3 minutes, the counting chamber was put under the microscope and the ruled area was located. Erythrocytes were counted in the 5 squares of the counting area of 1mm square. The number of cells in the 4-corner square was counted.

Calculation

The total number of cells found in 5 groups of 16 squares is multiplied by 10,000 to give the number of cells in millions/mm of blood.

APPENDIX 12

ESTIMATION OF WHITE BLOOD CORPUSCLES

(Enos and Moore, 2022)

Principle

WBC diluting fluid or Turk's fluid was used as the diluents which can destroy RBC'S.

Reagents

White blood cells diluting fluid - Glacial acetic acid, Gentian violet 1%, Water 95ml

Procedure

The method of counting is similar to RBC counting except that the count is made in 4 large (1mm) cover squares of the Neubauer counting chamber.

Calculation

The total number of cells in 4 squares is multiplied by a factor of 2500 to give the count/mm of blood.

APPENDIX 13

ESTIMATION OF TOTAL CHOLESTEROL

(Martins *et al*., 2023)

Reagents

1. Ferric chloride-acetic acid reagent: 0.05% ferric chloride in acetic acid.
2. Concentrated sulphuric acid.
3. Cholesterol standard

Procedure

0.1ml of extract was evaporated to dryness and 5ml ferric chloride-acetic acid reagent was added, mixed and centrifuged. To the supernatant 3ml of concentrated sulphuric acid was added and the absorbance was read after 20 minutes at 560nm against a reagent blank. A standard in the concentration range of 40-200µg was treated similarly. Values were expressed as mg/dL serum.

APPENDIX 14

DETERMINATION OF TRIGLYCERIDES

(Toth and Simko, 2019)

Principle

Triglycerides were determined by the following method. Triglycerides were extracted by isopropanol, which upon saponification with potassium hydroxide yield glycerol and soap. The glycerol liberated is treated with meta periodate, which releases formaldehyde, formic acid and iodide. The formaldehyde released reacts with acetyl acetone and ammonia forming yellow colored compound, the intensity of which is measured at 420nm.

Reagents

1. Isopropanol
2. Activated aluminium oxide (Neutral)
3. Saponification reagent - 5g of potassium hydroxide was dissolved in 60ml of distilled water and 40ml of isopropanol was added to it
4. Sodium meta- per iodate reagent - 77g of anhydrous ammonium acetate was dissolved in about 700ml of distilled water, 60ml glacial acetic acid was added to it followed by 650mg of sodium meta- periodate. The mixture was diluted to 1liter with distilled water
5. Acetyl acetone reagent - 0.75ml of acetyl acetone was dissolved in 60ml of distilled water and 40ml of isopropanol was added to it
6. Standard triolein solution - 1g of triolein was dissolved in 100ml isopropanol. 1ml of stock standard was diluted to 100ml to prepare a working standard 100µg of triolein/ml.

Procedure

An aliquot of serum/lipid extract was evaporated to dryness. 0.1ml of methanol was added, followed by 4ml of isopropanol. 0.4g of alumina was added to all the tubes and shaken well for 15 minutes. Centrifuged and then 2ml of the supernatant was transferred to labeled tubes. The tubes were placed in a water bath at 65°C for 15 minutes for saponification after adding 0.6ml of the saponification reagent followed by 0.5ml of acetyl acetone reagent. After mixing, the tubes were kept in a water bath at 65°C for 1 h, the contents were cooled and absorbance was read at 420nm. A series of standards of concentrations 8-40µg triolein were treated similarly along with a blank containing only the reagents. All the tubes were cooled and read at 420nm. The triglyceride content was expressed as mg/dl – serum

APPENDIX 15

ESTIMATION OF HDL-CHOLESTEROL

(Lopes-Virella *et al*., 1977 and Warnick and Albers, 1978)

Principle:

The common classification of lipoproteins – HDL, LDL and VLDL comes mainly from ultra centrifugation of serum or plasma. HDL (specific gravity more than 1.063) can be separated by using polyionic substances along with bivalent metal ions. HDL is separated from other protein fractions by treating serum with phosphotungstic acid and magnesium chloride. HDL remains in solution while all other lipoprotein fractions are precipitated. Centrifugation separates high density lipoproteins as a clear supernatant. Cholesterol content of which is estimated by enzymatic method as described earlier in estimation of total cholesterol.

Reagents

1. Enzyme reagent
2. Diluent buffer
3. Precipitating reagent PEG-6000
4. Standard – 200 mg/dL

The working reagent is prepared by dissolving enzyme reagent with 25ml of diluent buffer and kept for at least 10 min before use. The working reagent is stable for 4 weeks at 2- 80°C.

Procedure

0.5 ml of serum was taken into a glass tube and added 0.05ml of precipitating reagent. Mixed well, and kept at room temperature for 10 min, the mixture was then centrifuged for 15 minutes at 4000rpm. Separated the clear supernatant and immediately determined cholesterol content.

Estimation of LDL-Cholesterol and VLDL-Cholesterol

The amounts of LDL-cholesterol and VLDL-cholesterol were calculated by using the Friedwald formula.

VLDL-Cholesterol = Triglycerides / 5

LDL-Cholesterol = Total-Cholesterol – (HDL + VLDL)

The levels of HDL, LDL and VLDL-cholesterol are expressed as mg/dl.

APPENDIX 16

ESTIMATION OF SERUM GLUTAMIC OXALOACETIC TRANSAMINASE (SGOT)

(Reitman and Frankel, 1957)

Principle

Serum glutamine oxaloacetate transaminase (SGOT) catalyses the reversible transfer of an amino group from aspartate to α-keto glutarate forming glutamate and oxaloacetate. SGOT catalyses the following reaction:

$$\text{L – Aspartate} + \alpha\text{ – Keto glutarate} \xrightarrow{\text{SGOT (pH 7.4)}} \text{Oxaloacetate} + \text{L – Glutamate}$$

$$\text{Oxaloacetate} + \text{2,4 DNPH} \xrightarrow{\text{Alkaline medium}} \text{2,4 dinitrophenyl hydrazine (brown colored)}$$

Reagents

1. Tris buffer, pH 7.5 - 100mmol/l
2. L-aspartate - 500mmol/l
3. 2-oxoglutarate - 15mmol/l
4. 2, 4 dinitrophenyl hydrazine reagent
5. Working sodium hydroxide (4N)

Procedure

Five hundred microlitre of buffered substrate was incubated at 37°C for 3 minutes and 0.1ml of serum was added, mixed well and incubated at 37°C for 30 minutes. Then 0.5ml of 2, 4 - dinitrophenyl hydrazine (DNPH) reagent was added, mixed well and kept at room temperature for 20 minutes and 0.5ml of 4N working sodium hydroxide was added and kept at room temperature for 10 minutes. Blank and standards were also processed in a similar way and the absorbance was measured spectrophotometrically at 505nm. Activity of SGOT was expressed as U/L.

APPENDIX 16a

ESTIMATION OF SERUM GLUTAMATE PYRUVATE TRANSAMINASE (SGPT)

(Reitman and Frankel, 1957)

Principle

SGPT catalyses the reversible transfer of amino group from L-alanine to alpha ketoglutarate with the formation of pyruvate and glutamate. The pyruvate so formed is allowed to react with 2-4 dinitrophenylhydrazine (DNPH) to produce 2, 4- dinitrophenyl hydrazone derivative, which is measured spectrophotometrically.

$$\alpha\text{ – Keto glutarate + L Alanine} \xrightarrow{\text{SGPT (pH 7.4)}} \text{L - Glutamate + Pyruvate}$$

$$\text{Pyruvate + 2,4 DNPH} \xrightarrow{\text{Alkaline Medium}} \text{2,4 dinitrophenyl hydrazine (Brown Colored)}$$

Reagents

1. Tris buffer, pH 7.5 - 100mmol/l
2. L-alanine - 500mmol/l
3. 2-oxoglutarate - 15mmol/l
4. 2, 4 dinitrophenyl hydrazine reagent
5. Working sodium hydroxide (4N)

Procedure

Five hundred microlitre of buffered substrate was incubated at 37°C for 3 minutes and 0.1ml of serum was added, mixed well and incubated at 37°C for 60 minutes. Then 0.5ml of DNPH reagent was added, mixed well and kept at room temperature for 20 minutes and 0.5ml of 4N working sodium hydroxide was added and kept at room temperature for 10 minutes. Blank and standards were also processed in a similar way and the absorbance was measured spectrophotometrically at 505nm. Activity of SGPT was expressed as U/L.

APPENDIX 17

ESTIMATION OF ALKALINE PHOSPHATASE (ALP)

(Tietz *et al*., 1983)

Principle

Alkaline phosphatase (ALP) is an enzyme which catalyses the splitting of phosphoric acid from certain monophosphoric esters. In this method disodium phenyl phosphate was hydrolyzed with the liberation of phenol and formation of sodium phosphate. The amount of phenol formed was estimated in a spectrophotometer at 650nm.

Reagents

1. Disodium phenyl phosphate (0.01M) – 1.09g of disodium phenyl phosphate was dissolved in water and made up to 500ml. It was then boiled, cooled and little chloroform was added and kept in refrigerator (Solution A).
2. Sodium carbonate-sodium bicarbonate buffer (0.1M) - 3.18g of anhydrous sodium carbonate and 1.68g of sodium bicarbonate was dissolved in water and made up to 500ml (Solution B).
3. Buffered substrate for use - Equal volume of solution A and solution B was mixed which has pH of 10.
4. Trichloro acetic acid (20%) – Acid molybdate reagent - 5g of ammonium molybdate dissolved in 5N sulphuric acid.
5. 1, 2, 4 – ANSA : 0.25% of 1,2,4 – ANSA was prepared by adding 0.5g of dry powder ANSA to 190 ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite, stoppered the bottle and shaken until it dissolved.
6. Stock phosphate solution – 2.194g of pure potassium dihydrogen phosphate was dissolved in water and made up to 500ml. A few drops of chloroform was added to it (1mg/1ml of phosphate).
7. Working standard: Two ml of stock standard was diluted to 500ml.

Procedure

Six ml of buffered substrate was pipette out in a test tube and placed in water bath at 370°C for few minutes. Then, 0.3ml of serum was added, mixed well and incubated for 15 minutes. At the same time control and blank were also kept. For blank, 0.3ml of water was added to 6ml buffered substrate. For control, 0.3ml of serum was added to 6ml of distilled water. Later, 1.2ml of 20% TCA was added and shaken well. Five ml of the filtrate was taken in separate test tubes. To the blank and control, 0.8ml of acid molybdate was added followed by 0.2ml of ANSA. It was then mixed well and allowed to stand for 10 minutes at 370°C and the colour developed was read at 650nm.

Pipetted out 1.0 to 4.0 ml of standard solution and made up to 5ml with distilled water. Acid molybdate (0.8ml) was added followed by 0.2ml of ANSA. Standards were also read at 650nm. Alkaline phosphatase activity in serum was expressed as U/L. The activity in tissue homogenate was expressed as mole of phenol liberated/min/mg protein.

APPENDIX 18

DETERMINATION OF TOTAL BILIRUBIN

(Malloy and Evelyn, 1937)

To 0.2ml of serum added 5.4ml of water. Pipetted out 2.8ml of this into a second tube for blank. To the test added 0.7ml of diazo reagent [0.3ml of solution A (0.5% of sodium nitrite) + 10ml of solution B (1g of sulphanilic acid in 15ml of conc. HCl and made upto 1litre with water)] and to the blank 0.7ml of 1% sulphanilic acid solution. Mixed, allowed to stand for 5 minutes and added 3.5ml of methanol to each tube. To the standard bilirubin (20-100µg/ml of chloroform) added 3.5ml of methanol, 0.7ml of diazo reagent and 1.8ml of water read at 540 nm.

APPENDIX 19

ESTIMATION OF TOTAL PROTEIN

(Lowry *et al*., 1951)

Principle

The aminoacid tyrosine and tryptophan present in the protein will react with the Folin- Ciocalteau reagent. By the reduction of phosphomolybdic acid and phosphotungstic components it will produce blue colour. Also the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate was measured by Lowry's method.

Reagents

1. Solution A: 1 % Copper sulphate
2. Solution B: 2% Sodium potassium tartarate.
3. Solution C: 2% Sodium carbonate in 0.1 N NaOH.
4. Solution D: Mixed just before use, 1 ml of solution A, 1ml of solution B and 100ml of solution C.
5. Solution E: Folin-Ciocalteau reagent (Dilute the commercial reagent with an equal volume of water on the day of use. This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid).

Procedure

A sample of 500mg was extracted with 5-10ml of 0.1M potassium phosphate buffer (pH 7.4). It was centrifuged and an aliquot was pipetted out and made upto 1.0ml with 0.1N NaOH. Standard bovine serum albumin solution (40-200µg) was also pipetted out and made upto 1.0ml with 0.1N NaOH. To this, 5ml of alkaline copper reagent was added to all the tubes and allowed to stand for 10 minutes. Folin-Ciocalteau reagent (0.5ml) was added to each tube and mixed well. The tubes were allowed to stand for 30 minutes at room temperature. The blue colour developed was measured at 660nm.

APPENDIX 20

ESTIMATION OF UREA

(Netelson, 1957)

Principle

Urea present in the sample undergoes hydrolysis in the presence of urease, producing ammonia and carbon dioxide. The liberated ammonia reacts with 2-oxoglutarate in the presence of NADH, leading to its oxidation, which is measured spectrophotometrically at 340 nm. The intensity of the reaction corresponds to the urea concentration in the sample.

Reagents

1. 10% Trichloroacetic acid (TCA)
2. **Reagent A:** 50 mg ferric chloride dissolved in 0.2 ml water, followed by addition of 1 ml O-phosphoric acid and 2.5 ml water
3. **Reagent B:** 50 ml concentrated sulphuric acid diluted to 450 ml with water
4. **Reagent C:** 1 g diacetyl monoxime dissolved in 50 ml water
5. **Reagent D:** 250 mg thiosemicarbazide dissolved in 50 ml water
6. **Reagent I:** 0.25 ml of Reagent A mixed with 500 ml of Reagent B
7. **Reagent II:** 33.5 ml of Reagent C mixed with 33.5 ml of Reagent D and diluted to 500 ml

Procedure

For the estimation, 0.2ml of blood was mixed with 1.8 ml of 10% TCA, allowed to stand for 10 minutes, and then centrifuged. From the resulting supernatant, 0.5ml was taken and the volume was made up to 3ml using water. To this mixture, 2 ml of Reagent I was added, followed by 2ml of freshly prepared Reagent II. The tubes were stoppered with marbles and heated vigorously in a boiling water bath for 20 minutes along with blank and standard solutions (10–50 μg) treated similarly. After heating, the tubes were cooled and the absorbance was read at 340 nm against the reagent blank.

APPENDIX 21

ESTIMATION OF URIC ACID

(Caraway, 1955)

Principle

Uric acid in the sample is oxidised by the enzyme uricase to produce allantoin, generating one mole of hydrogen peroxide for every mole of uric acid oxidised. The released hydrogen peroxide reacts with 3,5-dichloro-2-hydroxybenzene sulfonic acid and 4-aminoantipyrine in the presence of peroxidase to form a quinoneimine dye. The intensity of the resulting colour is directly proportional to the uric acid concentration and is measured spectrophotometrically.

Reagents

1. **Enzyme reagent:** 4-Aminoantipyrine (4 mM), 3,5-dichloro-2-hydroxybenzene sulfonate (2 mM), microbial uricase (150 U/L), horseradish peroxidase (10,000 U/L)
2. **Standard uric acid:** 5 mg/100 mL

Procedure

For the assay, 25 µl of plasma was added to 1 ml of the enzyme reagent and mixed gently by inversion. Standard uric acid (25 µl) and distilled water (25 µl) served as the standard and blank, respectively, and were processed simultaneously. The reaction mixtures were incubated at 37°C for 5 minutes, after which the colour developed was measured at 510 nm. Uric acid levels were expressed as mg/dL of blood.

APPENDIX 22

ESTIMATION OF CREATININE

(Trinder, 1969)

Principle

Creatinine reacts with picrate ions in an alkaline medium to form a yellow–orange coloured complex. The intensity of this chromogenic product correlates with the creatinine concentration and is measured spectrophotometrically at 492 nm.

Reagents

1. Picric acid (35 mmol/L)
2. Sodium hydroxide (0.32 mmol)
3. Creatinine standard (2 mg/dL)
4. Sodium tungstate
5. Sulphuric acid

Procedure

For the estimation, 0.2ml of serum was mixed with 3ml of water, followed by the addition of 1ml of 10% sodium tungstate and 2ml of 2/3 N sulphuric acid. The mixture was allowed to stand for 10 minutes and then centrifuged. From the resulting clear supernatant, 3ml was taken and treated with 1ml of 0.04 M picric acid solution and 1ml of 0.75N sodium hydroxide. The reaction mixture was left undisturbed for 20 minutes to allow complete colour development. Blank and standard solutions (10–50 µg) were processed in the same manner. The absorbance of the developed yellow–orange complex was measured at 492 nm against the reagent blank.

APPENDIX 23

ESTIMATION OF PLASMA INSULIN

(Hales and Randle, 1963)

Plasma insulin was assayed by the solid phase enzyme linked immunosorbent assay (ELISA) using kit obtained from Monobind microwells Inc., CA., USA. Standards or samples containing insulin are allowed to react with capture antibodies coated on plastic wells and with monoclonal antibodies labeled with horseradish peroxidase. The plates were washed and then treated with revelation solution. The fluorescent product formed was measured at 450nm.

Reagents

1. Microtitre plate with anti-insulin coated wells
2. Anti-insulin horseradish peroxidases (HRP) conjugate in Tris-HCI buffer with bovine serum albumin and preservatives.
3. Standards containing insulin (5-500µU/ml) in sodium merthiolate
4. Washing solution - 20% tween 20. This was diluted with distilled water prior to use.
5. Revelation solution - 0.2ml of the chromogen tertramethyl benzidine (TMB) was mixed with 1 vial (21ml) of H_2O_2 in acetate/citrate buffer.
6. Arresting agent - 1N sulphuric acid, H_2SO_4

Procedure

Accurately 50µl each standard, control or sample was dispensed into the appropriate wells. Time between distribution of first standard and last sample was kept minimum. 50µl of anti-insulin HRP conjugate was dispensed into all wells and incubated for 30 min at room temperature on a horizontal shaker set at 700rpm. The plates were washed after aspirating the liquid from the well. Then, 0.4ml of washing solution was dispensed into each well and the contents were aspirated. Washing was repeated twice. Then, 200µl of the freshly prepared revelation solution was added into each well within 15 minutes after washing. Then the plate was incubated for 15 minutes on a horizontal shaker set at 700rpm at room temperature, avoiding direct sunlight. 50µl of arresting agent was added into each well. The absorbance was read within one hour at 450nm. Insulin concentrations were expressed in µU/ml.

APPENDIX 24

ESTIMATION OF C - REACTIVE PROTEIN (CRP)

(Wadsworth, 1977)

C-reactive protein (CRP) is an acute phase protein synthesized in the liver. Its rate of synthesis increases within hours of acute injury or the onset of inflammation and may reach as high as 20 times the normal levels. Serum CRP concentration provides useful information in patients with myocardial infarction there being an excellent correlation between peak levels of CRP and creatine phosphokinase.

Principle

The turbidimetric immunoassay for the determination of C-reactive protein in human serum is based on the principle of agglutination reaction.

Procedure

The test specimen is mixed with activation buffer and Quantia - CRP reagent and allowed to react. Presence of CRP in the test specimen results in the formation of an insoluble complex producing turbidity, which is measured at 340 nm. The increase in turbidity corresponds to the concentration of CRP in the test specimen.

The calibrator dilution is as shown below:

Test tube No	1	2	3	4	5
Calibrator dilution No	D1	D2	D3	D4	D5
Volume of saline (µl)	-	100	375	880	940
Volume of calibrator (µl)	100	100	125	120	60
Concentration of CRP (mg/dl)	10	5	2.5	1.2	0.6

APPENDIX 25

DETERMINATION OF GLUCOSE-6-PHOSPHATASE

(Hikaru and Toshitsugu, 1959)

The enzyme catalyses the reaction

Glucose-6-phosphate $+H_2O$ ⟶ glucose + Pi

Reagents

1. 0.1M citrate buffer, pH 6.5
2. Substrate Glucose-6-phosphate, 0.01M in distilled water
3. 2.5% ammonium molybdate solution
4. ANSA
5. 10% TCA

Procedure

The incubation mixture in a total volume of 0.1ml contained 0.3ml of buffer, 0.5ml of substrate, and 0.2ml of enzyme solution. Incubation was carried out at 37^oC for 60 minutes. The reaction was terminated by the addition of 1ml of 10% TCA solution. The suspension was centrifuged and the phosphorus content in the supernatant was estimated by the method of Fiske and Subbarow (1925). The enzyme activity is expressed as nmoles of Pi liberated/min/mg protein

APPENDIX 26

ESTIMATION OF FRUCTOSE - 1, 6-BISPHOSPHATASE

(Gancedo and Gancedo, 1971)

This enzyme catalyses the reaction

Fructose-1, 6-diphosphate + H_2O ⟶ fructose-6-phosphate + Pi.

Reagents

1. 0.1M tris-HCl buffer, pH 7.0
2. Substrate 0.05M Fructose-1,6-diphosphate solution
3. 0.1M $MgCl_2$
4. 0.1M KCl
5. 0.001M EDTA
6. 10% TCA
7. 2.5% ammonium molybdate solution
8. ANSA

Procedure

The assay medium in a final volume of 2ml contained 1.2ml of buffer, 0.1ml of substrate solution, 0.25ml of $MgCl_2$, 0.1ml of KCl solution, 0.25ml of EDTA solution and 0.1ml of enzyme. The incubation was carried out at 35^{o}C for 15 minutes. The reaction was terminated by the addition of 1ml of TCA. The suspension was centrifuged, and the phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow (1925). The enzyme activity is expressed as nmoles of Pi liberated/min/mg protein.

APPENDIX 27

ESTIMATION OF GLUCOKINASE

(Branstrup *et al.*, 1957)

Reagents

1. 0.005M glucose
2. 0.72M ATP
3. 0.05M MgCl2
4. 0.0125M KH2PO4
5. 0.1MKCl
6. 0.5M sodium fluoride
7. 0.01M tris-HCl buffer, pH 8.0
8. 10% TCA

Procedure

The incubation mixture containing 2.5ml buffer, 1ml of substrate, 0.5ml ATP, 0.1ml each of MgCl2 and sodium fluoride and 0.5ml each of KH2PO4 and KCl was preincubated at 37^{o}C for 5 minutes room temperature. The reaction was initiated by the addition of 2ml of enzyme extracts. 1ml of aliquot of the reaction mixture was removed immediately (zero time) and added to tubes containing 1ml of 10% TCA. After 30 minutes incubation, 1ml of aliquot of the above reaction mixture was added to a separate set of tubes and the reaction was stopped by the addition of 1ml of TCA. After the samples precipitated and centrifuged, the supernatants were used for the estimation of glucose by ortho toluidine method.

APPENDIX 28

ATHEROGENIC INDEX

(Friedewald *et al*., 1972)

Principle

The Atherogenic Index (AI) is a useful indicator of cardiovascular risk and reflects the balance between atherogenic and protective lipoproteins. It is calculated from serum lipid parameters, particularly total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C). Serum TC is estimated by the cholesterol oxidase–peroxidase method, triglycerides (TG) by the glycerol phosphate oxidase method, and HDL-C after precipitation of non-HDL lipoproteins. Low-density lipoprotein cholesterol (LDL-C) is computed using the Friedewald equation. The AI is derived from the ratio of serum total cholesterol to serum HDL cholesterol and reflects the degree of lipid-induced atherogenicity.

Procedure

Blood samples were collected from experimental rats after a 12-hour fasting period. Serum was separated by centrifugation and used for lipid profile analysis. Total cholesterol was measured using the cholesterol oxidase–peroxidase enzymatic method, whereas triglycerides were estimated using the glycerol phosphate oxidase method. HDL-C was determined after precipitating non-HDL lipoproteins using the reagent supplied in the enzymatic kit. LDL-C was subsequently calculated using the Friedewald formula. After obtaining TC and HDL values, the Atherogenic Index (AI) was computed by dividing the serum total cholesterol level by the serum HDL-cholesterol concentration. The resulting value represented the degree of atherogenic risk in each experimental group.

APPENDIX 29

ESTIMATION OF SUPEROXIDE DISMUTASE (SOD) IN LIVER

(Kakkar *et al*., 1984)

Principle

Superoxide dismutase (SOD) is a key endogenous antioxidant enzyme responsible for catalyzing the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen. The assay is based on the ability of SOD to inhibit the spontaneous oxidation of adrenaline to adrenochrome. The reduction of nitro blue tetrazolium (NBT) in this reaction system is monitored, and the degree of inhibition produced by the enzyme corresponds to SOD activity.

Reagents

1. Sodium pyrophosphate
2. Phenazine methosulfate
3. Nitro blue tetrazolium (NBT)
4. NADH
5. Glacial acetic acid
6. n-Butanol

Procedure

The reaction mixture consisted of 1.2ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of 186 μM phenazine methosulfate, 0.3ml of 300 μM NBT, 0.2ml of 780 μM NADH, an appropriately diluted liver enzyme preparation, and distilled water to make a total volume of 3.0ml. The reaction was initiated by adding NADH and the mixture was incubated at 30°C for 90 seconds. The reaction was terminated by adding 1.0ml of glacial acetic acid. The tubes were then vigorously mixed and shaken with 4.0ml of n-butanol. After standing for 10 minutes, the mixture was centrifuged and the butanol layer containing the chromogen was collected. The colour intensity was measured at 560 nm against a butanol blank. A reaction mixture without the enzyme served as the control. One unit of SOD activity was defined as the amount of enzyme that produced 50% inhibition of NBT reduction per minute under the assay conditions and was expressed as specific activity in units per mg protein.

APPENDIX 30

ESTIMATION OF CATALASE IN LIVER

(Sinha, 1972)

Principle

Catalase causes rapid decomposition of hydrogen peroxide to water.

$$H_2O_2 \xrightarrow{CAT} H_2O + O_2.$$

Dichromate in acetic acid was converted to perchloric acid and then to chromic acetate when heated in the presence of H_2O_2. The chromic acetate thus produced is measured spectrophotometrically at 610nm. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at specific time interval by the addition of dichromate- acetic acid mixture, and the remaining H_2O_2 is determined by measuring chromic acetate.

Reagents

1. Dichromate/acetic acid reagent (5% solution of potassium dichromate in acetic acid at 1:3 ratio
2. 0.01M Phosphate buffer, pH 7.0
3. 0.2M Hydrogen peroxide

Procedure

The assay mixture contained 4ml of hydrogen peroxide, 5ml of phosphate buffer and 1ml of homogenate. One portion of the reaction mixture were withdrawn and blown into 2ml of dichromate/acetic acid reagent at 1 minute intervals. Then the mixture was incubated for 30 minutes later the OD was measured at 570nm. The activity of catalase was expressed as µmole of H_2O_2 consumed/min/mg protein.

APPENDIX 31

ESTIMATION OF GLUTATHIONE PEROXIDASE IN LIVER

(Rotruck *et al.*, 1973)

Principle

Glutathione peroxidase (GPx) catalyses the following reaction Se-GPx

$2GSH + H_2O_2 \longrightarrow GSSH + 2H_2O.$

Reagents

1. 0.32M Phosphate buffer, pH 7.0
2. 0.8mM EDTA
3. 10mM Sodium azide
4. 3mM reduced glutathione
5. 2.5mM H_2O_2
6. 10% TCA
7. 0.3M Disodium hydrogen phosphate
8. DTNB solution (40mg of DTNB in 100ml of 1% sodium citrate)
9. Reduced glutathione

Procedure

The reaction mixture consisted of 0.2ml each of EDTA, sodium azide, H_2O_2, 0.4ml of phosphate buffer, 0.1ml homogenate/mitochondria and was incubated at 37°C at different time intervals. The reaction was arrested by the addition of 0.5ml of TCA and the tubes were centrifuged at 2000rpm. To 0.5ml of supernatant, 4ml of disodium hydrogen phosphate and 0.5ml DTNB were added and the colour developed was read at 420nm immediately. The activity of GPx was expressed as µmoles of glutathione oxidized/min/mg protein.

APPENDIX 32

ESTIMATION OF REDUCED GLUTATHIONE (GSH) IN LIVER

(Moron *et al.*, 1979)

Reagents

1. 10% TCA
2. 0.6mM 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) in 0.2 M sodium phosphate
3. 0.2M Phosphate buffer, pH 8.0

Procedure

One ml of homogenate was precipitated with 1ml of TCA, and centrifuged and removed the precipitate. To 0.5ml of supernatant, 2ml of DTNB was added and the total volume was made up to 3ml with phosphate buffer. The absorbance was read at 412nm.

The level of glutathione was expressed as µg/mg protein.

APPENDIX 33

ESTIMATION OF VITAMIN C IN LIVER

(Omaye *et al.*, 1979)

Reagents

1. 5% TCA
2. DTC reagent (3g of 2,4-dinitrophenylhydrazine, 0.4g of thiourea and 0.05g of copper sulphate were dissolved in 100ml of 9N sulphuric acid)
3. 65% sulphuric acid
4. Ascorbic acid

Procedure

To 0.5ml of homogenate, 0.5ml of water and 1ml of TCA were added, mixed thoroughly and centrifuged. To 1ml of the supernatant, 0.2ml of DTC reagent was added and incubated at 37^{o}C for 3 hrs. Then 1.5ml of sulphuric acid was added, mixed well and the solutions were allowed to stand at room temperature for another 30 minutes. The colour developed was read at 520nm. The level of ascorbic acid was expressed as µg/mg protein.

APPENDIX 34

ESTIMATION OF VITAMIN E IN LIVER

(Sarisozen *et al*., 2002)

Reagents

1. Ethanol
2. Petroleum ether
3. 0.2% 4, 6-diphenyl-1, 10-phenanthroline in ethanol
4. 0.001M Ferric chloride in ethanol
5. 0.001M o-phosphoric acid in ethanol
6. α-Tocopherol acetate

Procedure

To 1ml of homogenate, 1ml of ethanol was added and thoroughly mixed. Then 3ml of petroleum ether was added, shaken rapidly and centrifuged. 2ml of supernatant was taken and evaporated to dryness. To this 0.2ml of bathophenanthroline was added. The assay mixture was protected from light and 0.2ml of ferric chloride was added followed by 0.2ml of phosphoric acid. The total volume was made up to 3ml with ethanol. The colour developed was read at 530nm.

The level of vitamin E was expressed as µg/mg protein.

Annexure

भारतसरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA

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सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2022/Tech. /402

दिनांक/Date: 10th February 2022

पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as

Boerhavia diffusa L. – NYCTAGINACEAE.

The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

10/2/2022

डॉ. एम. यु. शरीफ़/DR. M.U. SHARIEF
वैज्ञानिक 'ई' एवं कार्यालयाध्यक्ष/
SCIENTIST 'E' & HEAD OF OFFICE

वैज्ञानिक 'ई' एवं कार्यालय अध्यक्ष
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दक्षिणी क्षेत्रीय केन्द्र
SOUTHERN REGIONAL CENTRE
कोयंबत्तूर / COIMBATORE - 641 003

सेवा में / To

Miss. Reena Joy A
Research Scholar
Department of Biochemistry, Biotechnology & Bioinformatics
Avinashilingam Institute for Home Science &
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COIMBATORE – 641 043

Avinashilingam Institute for Home Science and Higher Education for Women
(Deemed to be University under Category 'A' by MHRD, Estd. u/s 3 of UGC Act, 1956)
Re-accredited with 'A' grade by NAAC, Recognised by UGC under Section 12 B
Coimbatore – 641 043, Tamil Nadu, India

Dr.P.R.Padma
Dean, School of Biosciences
Professor and Head
Department of Biochemistry, Biotechnology and Bioinformatics

CERTIFICATE

This is to certify that the project title "Antidiabetic and Antiobesity activities of silver nanoparticles encapsulated ethanolic extracts of *Boerhaavia diffusa* in rats" has been approved by the IAEC.

Name of the chairman, IAEC
Dr.P.R Padma

Name of the CPCSEA Nominee
Dr. C. Gunasekaran

Signature with date

Chairman, IAEC: 30/1/19

CPCSEA Nominee: 30/1/19

Avinashilingam Institute for Homescience and Higher Education for Women * Institutional Animal Ethics Committee * Coimbatore

Avinashilingam Institute for HomeScience and Higher Education for Women
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Coimbatore - 641 043, Tamil Nadu, India

Appendix L2

(Item No 5 of Check List) Details of Research Publications

S.No	Article	Journal	Other Details Vol/No/Page No/ Year	Published in UGC-CARE / Scopus Indexed/ Web of Science
1	Green Synthesis of Silver nanoparticles using Ethanolic Extract of Boerhavia diffusa and its invitro Antioxidant activity	The Indian Journal of Nutrition and Dietetics	Vol.61(4) October to December 2024	✓ UGC - CARE I
2	Exploring the Therapeutic Potential: Investigating the Synthesis, characterization and invitroantidiabetic Efficacy of silvernano particle Ethanolic Extract from Boerhavia diffusa	Research Journal of Biotechnology	Vol.20(10) October 2025	Scopus Indexed

*Proof of list of Journals from Internet to be attached along with copies of reprints.

Scholar : REENA JOY. A Reenajoy

Supervisor : Dr. S. GAYATHRI DEVI 24/6/25

Checked By 24/6/25

HoD/Dean of Respective School

The scholar Miss. Reena Joy, A. has published her research articles in the following journals:

1. The Indian Journal of Nutrition and Dietetics - indexed in Care List,
2. Her paper accepted in "Research Journal of Biotechnology" - indexed in Scopus.

This may be considered.

24.06.25

The Indian Journal of Nutrition and Dietetics
ISSN: 0022-3174; eISSN: 2348-621X
www.ijndonline.org

DOI:10.21048/IJND.2024.61.4.32564

Green Synthesis of Silver Nanoparticle using Ethanolic Extract of *Boerhavia diWusa* and it's *in vitro* Antioxidant Activity

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(Received 15th March, 2024)

Abstract

The prospective uses of biological entities in the green production of silver nanoparticles (AgNPs) in nanomedicine are attracting significant interests. The current study focuses on synthesizing AgNPs using the ethanolic extract of Boerhavia diffusa (B.diffusa) (EAgNPsBd), characterized by SEM-EDAX and Zeta-potential and the antioxidant activity of EAgNPsBd was assessed using DPPH, ABTS and FRAP assays. SEM-EDAX analysis confirmed the spherical and nano-sized nature of the synthesized EAgNPsBd, while Zeta-potential indicated a single peak of (-20.2 mV). The DPPH and ABTS assays, EAgNPsBd exhibited significantly lower IC50 values (20.8 µg/mL and 62.1 µg/mL, respectively) compared to standard ascorbic acid (65.6 µg/mL) and quercetin (84.8 µg/mL), as observed in the FRAP assay. Significantly higher antioxidant activity is implied by lower IC50 values. The in vitro antioxidant activities demonstrated the excellent radical scavenging capabilities of EAgNPsBd. This study emphasises the safe and environmentally responsible use of EAgNPsBd in prospective therapeutic, nanobiotechnological and biomedical applications, especially in context to diabetes mellitus.

Keywords: *Silver nanoparticle, Boerhavia diffusa, antioxidant, green synthesis, DPPH assay, SEM*

Introduction

Nanotechnology, an emerging scientific field, focuses on the investigation of small particles or materials at the nanoscale, ranging in size from 1 to 100 nanometer. This discipline involves manipulating and understanding materials at the molecular and atomic levels, offering potential applications across various industries, from medicine to electronics. The unique properties exhibited by nanoscale materials contribute to the

development of innovative technologies with significant implications for future advancements[1]. In the context of this study, the focus is on analysing silver nanoparticles (AgNPs).

Silver nanoparticles (AgNPs), belonging to noble metal nanoparticles, serve as effective substrates for Surface Enhanced Raman Scattering (SERS) to detect single molecules and act as catalysts for diverse chemical reactions. Their synthesis methods include chemical, electrochemical, radiation and biological techniques. Notably, biological synthesis offers an eco-friendly approach, reducing the use of harmful substances[2]. AgNPs can be synthesized using either top-down or bottom-up strategies. The top-down strategy involves reducing bulk materials to nano-sizes using techniques such as laser ablation and sputtering. In contrast, the bottom-up technique entails creating nanoparticles from smaller entities *via* mechanisms such as chemical and biological processes[3]. This study especially employs a bottom-up strategy, emphasising the use of biological processes, often known as green synthesis. Green synthesis, which is frequently performed using medicinal plants, distinguishes itself from chemical and physical processes by being cost-effective, environmentally friendly, and readily available.

AgNPs are widely recognized for their extensive applications, this refers to a broad range of applications spanning curative, optoelectronic, catalytic, antiviral, chemotherapy and antibacterial fields. Recent research focuses on their effectiveness in controlling infections and deterioration, making them potent antibacterial agents in medicine. Scientists are currently exploring AgNPs for their diverse applications in sensors, computations, pigment degradation, skincare products and pharmaceuticals[4]. Over all metals, pure silver has the lowest contact resistance greatest electrochemical potential the conductivity. Studies and reports indicate potential adverse effects of nano-silver on both humans and the environment. Nevertheless, the green approach provides a synthesis of AgNPs that is free from toxic chemicals and environmentally friendly[5].

Plant-mediated green synthesis of AgNPs is a growing field in nanotechnology, known for its lower toxicity compared to chemical methods. Plant-mediated AgNPs combine the advantages of both nanoparticles and plant active ingredients, exhibiting more biological activities than those synthesized chemically[6]. The primary technique for isolating plant antioxidant components is solvent extraction. However, the attributes of the extracting

solvent have considerable effects on extract volumes and antioxidant activity. This dependence is due to the diverse chemical characteristics and polarities of antioxidant compounds in plant materials, which may or may not dissolve in each solvent. The solvent utilised in this experimental work is ethanol which is notable in extracting antioxidant compounds from diverse plants such as root, shoot, leaves and stem[7].

Boerhavia diffusa **(***B. diffusa***)** is a member of the family Nyctaginaceaea medicinal plant. It is a native to India but is also found in various other parts of Asia, Africa and America. It typically grows in moist and marshy areas. Scientific studies have explored Punarnava's potential benefits, including its anti-inflammatory effects, diuretic activity and its impact on various health conditions. However, more research is needed for a comprehensive understanding of its efficacy[8].

This study delves into the essential features of AgNPs and their potential therapeutic applications, particularly in addressing antioxidant a significant health concern. The research involves characterizing these nanoparticles and evaluating their antioxidant activity *in vitro*. Additionally, the study provides detailed insights into the medicinal plant used in the process. Given the profound health impacts of diabetes, the research aims to uncover key aspects related to assessing the antioxidant potentials of AgNPs derived from the ethanol extract of *B.diffusa* **(**EAgNPsBd**).**

Materials and Methods

B.diffusa plants (Figure 1) was gathered from the local region of Coimbatore. The entire plant underwent authentication (BSI/SRC/5/23/2013-14/ Tech/1041)[9] at the Botanical Survey of India (BSI), TNAU, Coimbatore, India.

The entire *B.diffusa* plant was harvested, washed extensively, and air-dried in the shade for the required duration. Following this, the entire plant was crushed into coarse powder and the ethanolic extract of *B.diffusa* was obtained using a Soxhlet apparatus. The crude extract underwent lyophilization, during which the powdered samples were collected for subsequent analysis in the study[10].

Figure 1
Boerhavia diffusa

2a **2b**

Figure 2

(a) Ethanolic Extract of *B. diffusa* (b) AgNPs synthesised in *B. diffusa*

Examining EAgNPsBd produced using environmentally friendly

Dissolving 500 mg of the ethanolic extract of *B.diffusa* in 100 ml of deionized water, 10 ml of the ethanolic extract was then mixed with 90 ml of deionized water containing 1 Mm silver nitrate Figure 2(a). After thorough mixing, the solution was exposed to sunlight for 5 to 10 minutes, resulting in a colour change from greenish to brown, indicating successful silver nanoparticle synthesis Figure 2(b). The solution was then transferred to a centrifugation tube and subjected to refrigerated centrifugation at 13,000 rpm for 20 minutes. After discarding the supernatant, the pellet was mixed with deionized water and subjected to centrifugation in a repetitive cycle three times. Following this, the pellet underwent lyophilization, and the resulting powder sample was collected for subsequent analysis[11].

The synthesized EAgNPsBd were characterized using SEM with EDAX (MIRA 3 TESCAN and EDAX APEX) and Zeta potential analysis (Malvern Instruments Ltd) *in vitro* Antioxidant activity of Ethanolic extract of synthesized AgNPs in *B.diffusa* (EAgNPsBd).

DPPH radical scavenging activity in EAgNPsBd

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was assessed using the method by Brand Williams[12] to determine *B. diffusa's* maximal antioxidant activity. The plant extract shifted shade from deep violet to bright yellow at 517 nm, implying radical scavenging excitement against stable DPPH radicals. Different

concentrations (1-500 µg/mL) of EAgNPsBd extract were tested and the antioxidant activity, expressed as IC50, represents the concentration needed to inhibit DPPH radical formation by 50%.

$$\% \text{ DPPH radical scavenging activity} = \frac{(A \text{ control} - A \text{ sample})}{A \text{ control}} \times 100\%$$

In this context, A control signifies the absorbance of the control sample lacking the plant extract or reference compound (Ascorbic acid), while A sample (EAgNPsBd) represents the absorbance of the sample containing the plant extract or reference compound[13].

ABTS assay in EAgNPsBd

The ABTS assay (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), following Chen and Kang's[14] protocol, utilized dissolved ABTS and potassium in distilled water, with subsequent incubation in the dark. After dilution with ethanol to achieve a specific absorbance, the mixture was combined with EAgNPsBd extracts. Trolox served as a control for assessing absorbance at 734 nm, and the ABTS radical scavenging activity was computed in accordance with the outcomes[15].

$$\text{ABTS Scavenging activity (\%)} = [1- A_i\text{-}A_j/ A_0] \times 100\%$$

where A_0 represents the blank absorbance (ethanol without any sample), A_i symbolises the EAgNPsBd absorbance, and A_j denotes the control absorbance (ethanol without ABTS).

FRAP radical scavenging activity in EAgNPsBd

The FRAP assay (Ferric Reducing Antioxidant Potential), based on the method by Benzie and Strain[16], gauges the reducing power of antioxidants. Antioxidants lower ferric ions (Fe3+) to ferrous ions (Fe2+) in this process, triggering the development of the blue-coloured complex ferric-tripyridyl triazine (Fe2+/TPTZ), which has a greater absorbing capacity at 593 nm. The FRAP reagent, a blend of acetate buffer, TPTZ solution, and FeCl3, was mixed with the EAgNPsBd sample. The initial (t=0) and post-incubation (t=4) absorbances were measured at 593 nm against a sample blank. Quercetin served as the standard, following a parallel procedure[17].

Results and Discussion

Characterization of EAgNPsBd

The characterization of medicinal plants entails a comprehensive analysis and identification of the various components and properties present in the plant. This intricate process aims to understand the chemical composition, pharmacological activities and potential therapeutic benefits of the plant. It employs a multidisciplinary approach, integrating botanical, chemical and biological methods to uncover the medicinal potential, ensuring the development of herbal remedies that are both safe and effective.

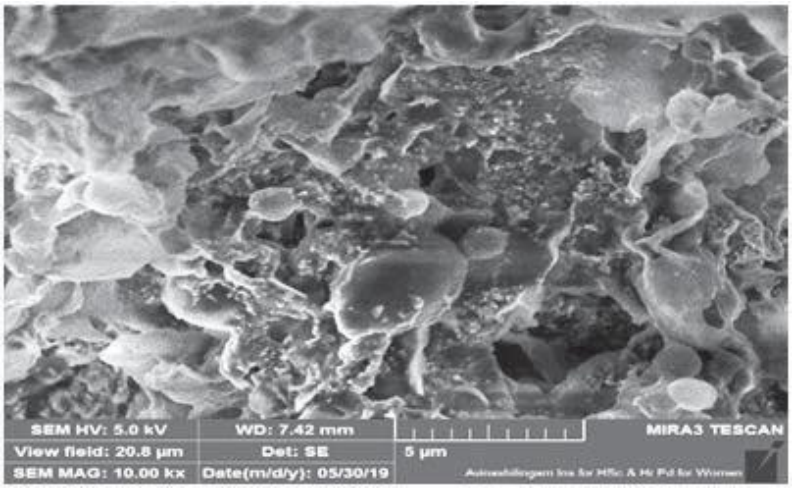

Figure 3
Scanning Electron Microscopy (SEM) analysis in EAgNPsBd

Scanning Electron Microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDAX) in EAgNPsBd

The size and shape of the synthesized silver nanoparticles was investigated by SEM. Figure 3 shows that the EAgNPsBd consists of spherical like structure with the length of 25 nm. Similar study was carried out in sprouted *Z. officinale* and *C. amada* AgNPs were found to be spherical, with particle sizes ranging from 25 to 30 nm[18].

SEM plays a crucial role in adequately defining the characteristics of biological specimens. To extract the maximum information from such materials, a common practice involves the simultaneous use of both light and electron microscopy. Researchers often make a deliberate effort to apply SEM, as it provides high-resolution images that reveal intricate surface details not easily observable through other microscopy techniques. The collaborative use of light microscopy for an overall view and electron microscopy, particularly SEM, for detailed surface morphology significantly enhances our understanding of the structural aspects of biological specimens[19].

The existence of pure silver material in the synthesised AgNPs can be observed by EDAX. Figure 4 shows the silver (30.6%) was a major constituent element compared to copper (47.6%), oxygen (10.9%) and chlorine (10.9%). The EDAX profile demonstrates a robust signal for silver, coupled with a subtle oxygen peak, possibly arising from the biomolecules attached to the surface of AgNPs. This finding implies the conversion of silver ions into elemental silver through reduction.

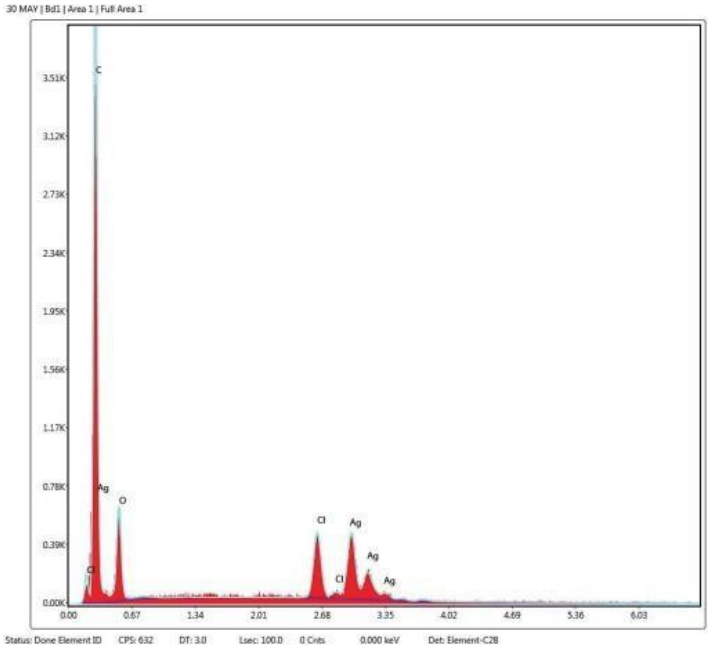

Figure 4
EDAX analysis in EAgNPsBd

Zeta potential analysis in EAgNPsBd

Zeta potential is a crucial parameter that provides valuable insights into the electrostatic forces influencing particle behaviour and dispersion. This understanding holds significance for a range of applications, including drug delivery and therapeutic interventions using plant-based approaches. Figure 5 illustrates that EAgNPsBd displayed a negative charge with a zeta potential value of -20.2 mV. This value falls within the moderately stable range of -20 to -30 mV, indicating the moderate stability of EAgNPsBd. Other studies have also reported comparable findings, where the zeta potential signifies stability at ambient temperature by providing an estimated potential difference in negative polarity. For example, AgNPs derived from *P. guajava* leaves demonstrated a zeta potential of -18.2 mV[20].

The in vitro antioxidant activity of AgNPs synthesized from the ethanolic extract of B. diffusa (EAgNPsBd)

The term "*in vitro* antioxidant activity" indicates that the study aimed to gauge the capability of these AgNPs to manifest antioxidant effects within a controlled laboratory environment. Antioxidants are vital for counteracting Reactive Oxygen Species (ROS) and shielding cells from the detrimental impacts of oxidative stress[21].

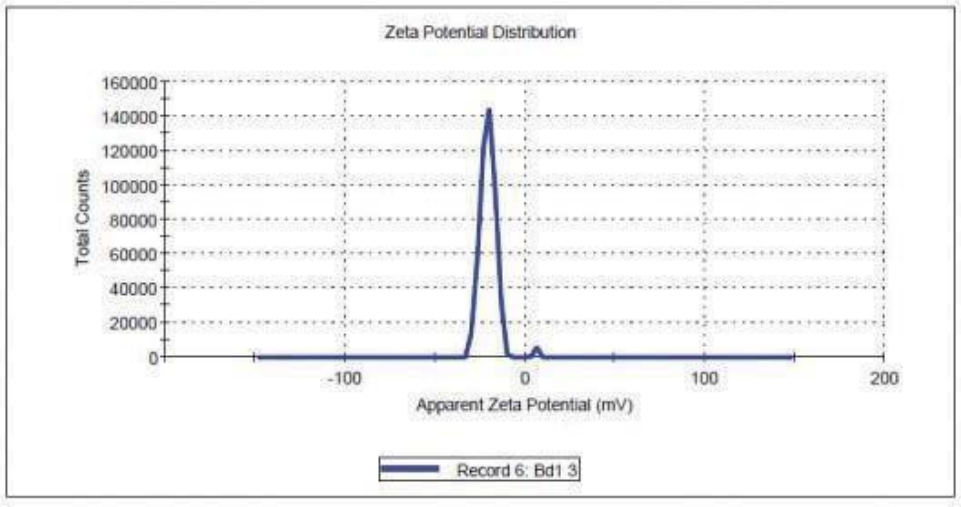

Figure 5
Zeta potential distribution rate

DPPH and ABTS Radical scavenging activity EAgNPsBd

The investigation highlighted the DPPH and ABTS radical scavenging capabilities of EAgNPsBd. Furthermore, the results demonstrated that the efficiency of EAgNPsBd in scavenging DPPH and ABTS radicals increased in direct proportion to the escalating concentrations, ranging from 10 to 100 µg/ml. This suggests that there was a commensurate rise in EAgNPsBd capacity to neutralise DPPH and ABTS radicals when its concentration rose within this range. The antioxidant findings are further delineated through IC50values (Figure 6). In the DPPH assay, EAgNPsBd exhibited a notably lower IC50 value (20.8 µg/mL) compared to the standard ascorbic acid (65.6 µg/mL). Similarly, in the ABTS assay Figure 7, EAgNPsBd displayed a lower IC50 value (62.1 µg/mL) compared to the standard quercetin (84.4 µg/mL). A lower IC50 value is indicative of heightened antioxidant activity[22].These findings indicate the potential application of AgNPs as an alternative antioxidant for treating conditions caused by free radicals. Several studies have showcased the robust antioxidant activity of AgNPs derived from plant extracts *Thymus vulgaris*[23], *Myrsine Africana*[24], *Scoparia dulcis*[25]. This suggests a promising direction for exploring the therapeutic uses of AgNPs in addressing conditions associated with oxidative stress.

FRAP radical scavenging activity in EAgNPsBd

The Ferric Reducing Antioxidant Power (FRAP) activity demonstrated an

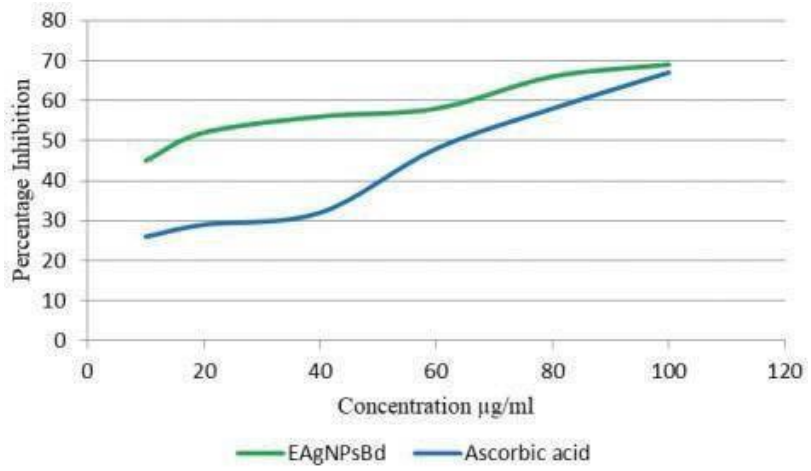

Figure 6
DPPH radical scavenging activities EAgNPsBd

increase in a dose-dependent manner within the range of 10 µg/ml to 100 µg/ ml, both for standard ascorbic acid and EAgNPsBd. During this range, the IC50 value for EAgNPsBd was recorded at
86.8 µg/mL, while for standard Quercetin, it was 91.3 µg/mL Figure 8. A lower IC50 value signifies a higher level of antioxidant activity. Similar trends were observed in the investigation of *Clinacanthus nutans*[26], where FRAP activity similarly increased in a dose-dependent manner.

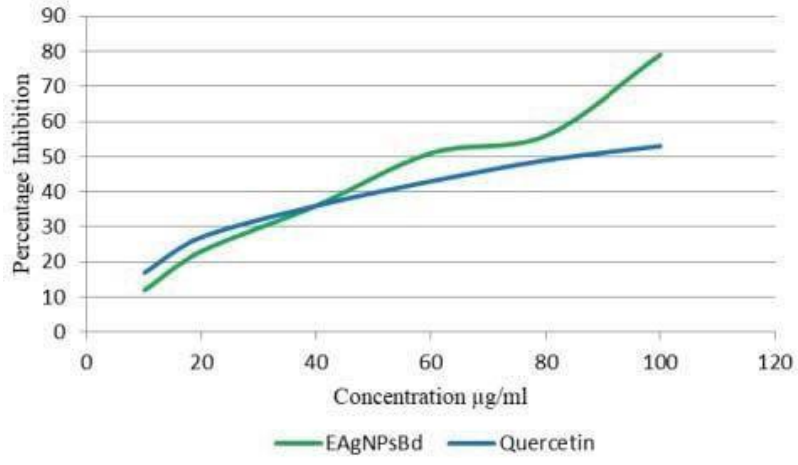

Figure 7
ABTS radical scavenging activities EAgNPsBd

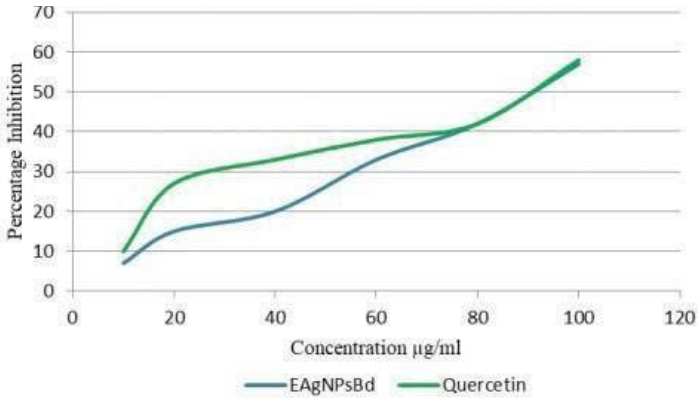

Figure 8
FRAP radical scavenging activities EAgNPsBd

Conclusion

This research concentrated on utilizing plant materials for nanoparticle production, an emerging approach in nanotechnology. The aim was to devise a method that is economical and environmentally sustainable for synthesizing AgNPs, utilizing the ethanolic whole plant extract extracted from *B.diffusa* (EAgNPsBd). The characterization of EAgNPsBd was carried out utilizing various techniques such as SEM, EDAX and Zeta potential, all of which indicated positive outcomes. Furthermore, *in vitro* assays including DPPH, ABTS and FRAP revealed the potential antioxidant activities of EAgNPsBd. The green synthesis method utilized in this study shows promise as a more sustainable alternative to traditional approaches. Further research involving animal and cell line models is essential to investigate the potential biological activities of EAgNPsBd.

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Exploring the Therapeutic Potential: Investigating the Synthesis, Characterization and *in vitro* Anti-Diabetic Efficacy of Silver Nanoparticle Ethanolic Extract from *Boerhavia diffusa*

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Abstract
Many studies explore the effects of Boerhavia diffusa (Bd) combined with synthesized silver-nanoparticles extract (AgNPs) for treating a variety of conditions, although rarely for diabetes. To address this, we investigate the inhibitory effect of BdAgNPs in standard in vitro antidiabetic assays using the whole plant extract dissolved in ethanol (EBdAgNPs). EBdAgNPs was characterized by UV-Visible, XRD and FT-IR spectroscopy techniques. The assays consisted of a-amylase, a-glucosidase, protein glycation, non-enzymatic glycosylation of hemoglobin, glucose uptake by yeast and glucose diffusion. The formation of AgNPs was revealed by observing a surface Plasmon resonance peak at 410 nm.

Notably, EBdAgNPs exhibited robust inhibitory effects in comparison to standard control drugs within the assays. These compelling findings strongly indicate the potential of EBdAgNPs as a viable avenue for treating diabetes mellitus. Further clinical investigations are warranted to fully explore EBdAgNPs potential in novel therapeutic approaches for managing diabetes and related conditions.

Keywords: Silver nanoparticles, *Boerhavia diffusa*, XRD, Diabetes Mellitus, Antidiabetic activity.

Introduction

Nanotechnology has brought transformative innovations to science and industry by employing nanoparticles[32]. However, producing silver nanoparticles (AgNPs) through biosynthesis remains a long-standing challenge[10]. Medicinal plant extracts, widely used in green AgNP synthesis, act as reducing and stabilizing agents[35]. In recent years, nanotechnology has significantly advanced diabetes diagnosis and management, addressing various issues[20,27,34]. Notably, certain plants like *Boerhavia diffusa* (*B. diffusa*) have biologically active compounds promoting natural nanoparticle biogenesis[39]. *B. diffusa*, a tropical plant, is valued in traditional medicine[33]. Our study focuses on using whole *B. diffusa* for antidiabetic treatment. We assess the characterization and antidiabetic potential of ethanolic extract of *B. diffusa*synthesized silver nanoparticles (EBdAgNPs).

Material and Methods

Plants belonging to the species *B. diffusa* (Figure 1) were collected from in and around Coimbatore. The entire plant was authenticated by the Botanical Survey of India (BSI), TNAU, Coimbatore (BSI/SRC/5/23/2013-14/Tech/1041). Extraction and synthesis of AgNPs ethanolic extract of the whole *B. diffusa* plant were using Soxhlet equipment. The extract was then subjected with AgNPs, characterized and analyzed for *in vitro* antidiabetic activity[36].

Figure 1: *Boerhavia diffusa* L.

Synthesis of Silver Nanoparticles: 500mg ethanolic extract of *B. diffusa* was dissolved under 100ml of deionized water. From the above solution, 10ml of ethanolic extract was obtained and mixed with 90ml of deionized water containing 1mM silver nitrate (Figure 2a). Once mixed, they were exposed to the sunlight for 5 to 10 minutes. The greenish solution turned brown indicating that the silver nanoparticle was synthesized (Figure 2b). Furthermore, the solution was added to the centrifugation tube and processed at 13,000rpm for 20 minutes. The supernatant was discarded with the remaining pellet being mixed with deionized water for further processing. This was repeated three times[36]. The resultant pellet was lyophilized and the powdered sample was collected.

Characterization of green synthesized AgNPs particles in ethanolic extract of B. diffusa: The characterization of synthesized silver nanoparticle was analyzed by XRD (X'pert Pro X-ray diffractometer), FT-IR (FTIR spectroscopy – miracle 10, SHIMADZU) and UV-Vis (Shimadzu Bio Spec-nano).

(a) **(b)**

Figure 2: (a) Plant extract (*B. diffusa*) + $AgNO_3$ (b) Synthesized AgNPs of *B.diffusa*

***In vitro* antidiabetic activities**

The a-amylase inhibition assay: From 1 mg/ml stock solution, different concentrations of plant extracts were prepared in phosphate buffer. 10, 20, 40, 60, 80 and 100 μg/ml of EBdAgNPs and acarbose were combined with 500μl of *a*-amylase (0.5 mg/ml) and incubated for 10 minutes at room temperature. Following that, 500μl of 1% starch solution was added and incubated for 10 minutes. The reaction mixture was then treated with 1 ml of dinitro salicylic acid (DNS), a colouring reagent and heated for 15 minutes in a boiling water bath before adding 10 ml of distilled water. A blank was prepared by replacing the enzyme with the phosphate buffer for each concentration of the sample set to quantify the absorbance of the coloured extracts. The absorbance was determined at 540nm. With the help of equation 1, the inhibition percentage was computed as described in Ishwarya et al[12].

$$\% \text{ inhibition} = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} 100 \quad (1)$$

The a-glucosidase Assay: The *a*-glucosidase inhibitory activity was determined using the spectrophotometer[26]. Yeast *a*-glucosidase was dissolved at 0.1U/ml in 100mM phosphate buffer, pH 7.0, including 2000 mg/l bovine serum albumin and 200 mg/ml sodium azide as an enzyme source. As a substrate, para-nitro phenyl-*a*-Dglucopyranoside was utilized. EBdAgNPs (5%) plant extract was weighed, with serial dilutions of 62.5, 31.25, 15.6, 7.8, 3.9 and 1.95 mg/ml dimethyl sulfoxide with distilled water being prepared in equal volume. For 5 minutes, ten microliters of plant extract dilutions were incubated with a 50 microliter enzyme source. Following the incubation, 50 microliters of substrate were added and incubated for 5 minutes at room temperature. A microtitre reader was used to measure the absorbance, with a setting of 405 nm. The inhibition percentage was also calculated using equation 1.

Non-Enzymatic Glycosylation of Haemoglobin Method: In a 0.01 M phosphate buffer at pH 7.4, solutions of glucose (2%), haemoglobin (0.6%) and gentamycin (0.02%) were made. 1.0 ml of each of the above solutions were combined with 1.0 ml of the EBdAgNPs extract of varying concentrations 10, 20, 40, 60, 80 and 100 μg/ml. The reaction mixture was incubated for 72 hours at room temperature in the dark. Subsequently, colorimetric analysis at 520 nm revealed that much of the haemoglobin had been glycosylated. Metformin was used as a standard comparison drug. The inhibition percentage was determined using formula according to Gupta et al[11].

Inhibition *in vitro* protein glycation: Fructose at 1000 mM in 200 mM phosphate buffer at pH 7.4 (4.0 ml) was incubated for 24 hours with 5.0 ml of Bovine serum albumin (BSA) at 20 mg/ml in 200 mM phosphate buffer, pH 7.4. Additionally, 1.0 ml of EBdAgNPs at concentrations of 250, 500, 750 and 1000 μg was added. After incubation, the fluorescence intensities of the reaction mixtures were measured in the emission range of 370-650 nm, with an excitation wavelength of 360 nm. The amount of fluorescent advanced glycation endproducts (AGEs) produced was correlated with the fluorescence intensity. Pioglitazone, a common anti-glycation drug, served as a positive control at final concentrations of 1.25, 0.75 and 0.25 mg/ml. The percentage inhibition of fluorescent AGE formation was calculated using equation 2 as reported by Avwioroko et al[5]:

$$\% \text{ inhibition} = \frac{(FC - FB) - (FS - FSB)}{FC - FB} 100 \quad (2)$$

where FC is the fluorescence intensity of the control, FCB is the fluorescence intensity of the control blank, FS is the fluorescence intensity of the sample and FSB is the fluorescence intensity of the sample blank.

Glucose uptake by yeast cell: A 10% v/v suspension of commercial baker's yeast was prepared in distilled water. The yeast suspension was repeatedly centrifuged at 3,000 × g for 5 minutes until the supernatant fluids became clear. Next, 1 ml of glucose solution at concentrations of 5, 10 and 25 mM was added to different concentrations of EBdAgNPs at 20, 40, 60, 80 and 100 μg/ml. The mixture was incubated at 37 °C for 10 minutes. Subsequently, 100 μl of the yeast suspension was added to the above mixture, which was then vortexed. The reaction was allowed to proceed for 60 minutes at 37 °C. After 60 minutes, the tubes were centrifuged at 2,500 × g for 5 minutes to separate the supernatant. The amount of glucose in the supernatant was

then determined. Metronidazole was used as the standard drug for comparison. The percentage increase in glucose uptake by yeast cells was calculated using the formula defined by Gaddala and Nataru[9].

Glucose diffusion assay: To assess *in vitro* glucose diffusion, a cellulose ester dialysis tube (CEDT) was filled with 2 mL of a solution containing 0.15 M NaCl and 0.22 mM glucose. This solution was divided into two groups: one with the addition of plant extract (50μg/ml) as the treated group and the other without the extract as the control group. Following this, the CEDT was placed inside a 50 ml centrifuge tube and both ends of the tube were tightly sealed. 45 ml solution of 0.15 M NaCl was added to the centrifuge tube. The entire setup was then placed in an orbital shaker, maintaining a constant temperature of 27°C. At regular intervals of 60 minutes, the concentration of glucose in the external solution surrounding the CEDT was measured to monitor and to track the diffusion of glucose[4].

Results and Discussion

Characterization of EBdAgNPs: In this study, we comprehensively characterize EBdAgNPs using a multi-technique approach. UV-Visible spectroscopy, X-ray diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR) analyzed the nanoparticles' structure and optical properties. This integrated analysis offers insights into EBdAgNPs' composition, crystalline structure and potential applications.

UV Visible spectroscopic measurements: The EBdAgNPs was monitored by recording absorption spectra at wavelengths spanning from 250 to 800nm (Figure 3). In UV Visible spectroscopy the excitation of surface plasmon resonance in EBdAgNPs indicates strong characteristic peak observed around 410nm. This peak corresponds to AgNPs standard surface plasmon resonance absorption band.

One of the most successful strategies for assessing nanoparticles is ultraviolet visible spectroscopy[30]. An identical study was presented reporting that, the synthesis of AgNPs is facilitated by the presence of many phenols, proteins, tannins, flavonoids, alkaloids, quinones, sterols, carbohydrates, amino acids, terpenoids and coumarins in the *Phagnalon niveum* plant extract. Similar to this, AgNPs UV-Vis absorbance spectrum showed a maximum absorbance peak between 360 and 410 nm at varied concentrations and time intervals[42].

X- ray diffraction (XRD): The XRD pattern of EBdAgNPs was examined with the results shown in figure 4. The diffracted intensities varied between 10 and 80. Particularly strong Bragg reflections were observed at 2θ values of 27.99, 32.48, 46.46, 55.00 and 57.60 degrees, corresponding to the lattice planes of (111), (200), (220), (311) and (222) respectively, within the face-centered cubic structure of silver. The expansion of Bragg's peaks demonstrates the organisation of nanoparticles and Debye-Scherrer's[24] condition determines that the average size of AgNPs was 24nm.

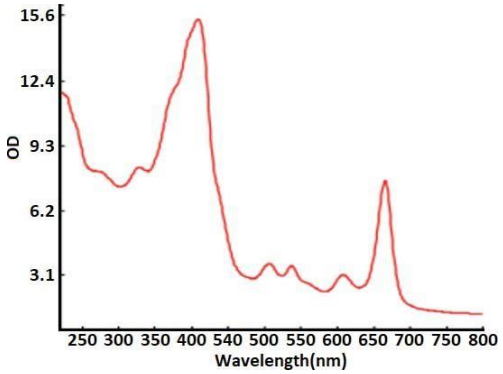

Figure 3: UV – Visible Spectrum of EBdAgNPs

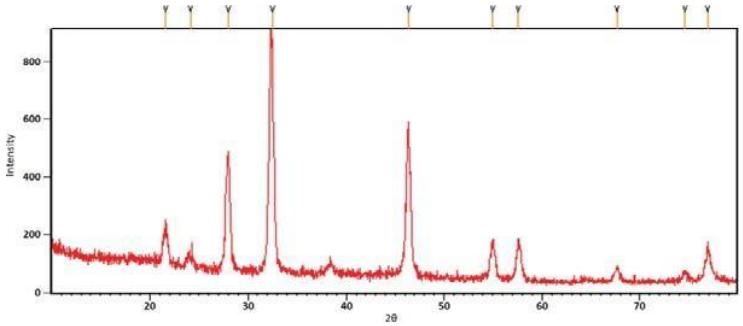

Figure 4: X-Ray Diffraction of EBdAgNPs

XRD is often used to analyse the composition or crystalline structure of the sample at specific conditions as shown by Ahmed et al[3]. The absorption spectra obtained at a higher pH (10) revealed that quercetin was around 19nm[25] and the average crystalline nature of the AgNPs of *Capparis zeylanica* L, leaf of extract was shown to be 35nm[13].

Fourier Transform Infrared Spectroscopy (FTIR) analysis: FTIR was performed in the range 1000 to 4000 cm^{-1} to determine which characteristic functional groups were present as shown in figure 3. The peaks at 1566 cm^{-1} and 1666 cm^{-1} reveal the C=C stretching of alkene[8]. The 1975 cm^{-1} and 2090 cm^{-1} peaks show the N=C=S stretching of isothiocyanate[46]. The 2399cm^{-1} peak shows the O=C=O stretching of carbon dioxide[37] whereas 2677 cm^{-1} indicates the C-H stretching of aldehydeade[1]. The distinct peak at 2877 cm^{-1} is attributed to C-H stretching vibrations of alkane[29]. The peak 3116 cm^{-1} is of O-H carboxylic stretch[43]. Avwioroko et al[5] showed that FTIR spectroscopy can be used to indicate when EBdAgNPs have reduced and stabilised silver nanoparticles (Figure 5).

Furthermore, similar analyses were performed on other natural extracts, such as fresh leaf Lemongrass[9], Mulberry leaves[6] and *Tabernaemontana ventricosa*[19]. The functional biomolecules containing carboxylic group have been found to be involved in the reduction of silver ions. This was confirmed by analyzing the FTIR spectrum[2].

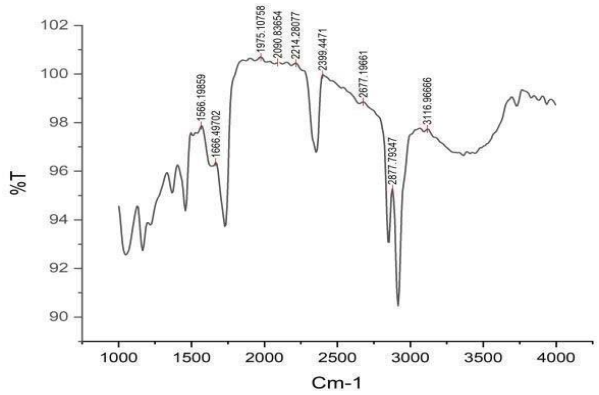

Figure 5: Fourier Transform Infrared Spectrum of EBdAgNPs

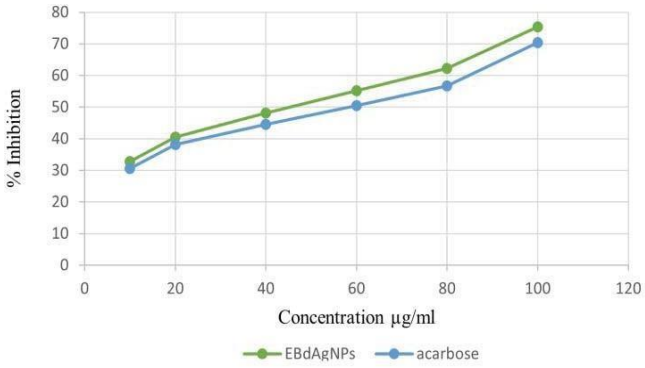

Figure 6: Alpha Amylase Activity of EBdAgNPs

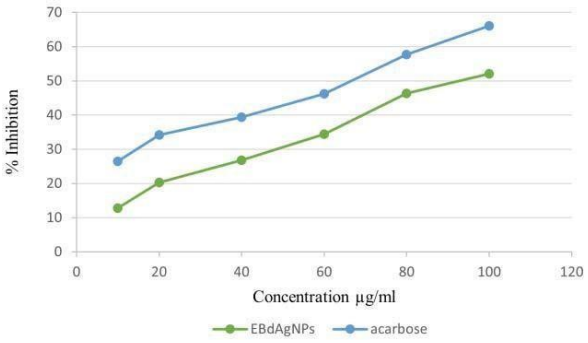

Figure 7: Alpha Glucosidase Inhibitory Activity of EBdAgNPs

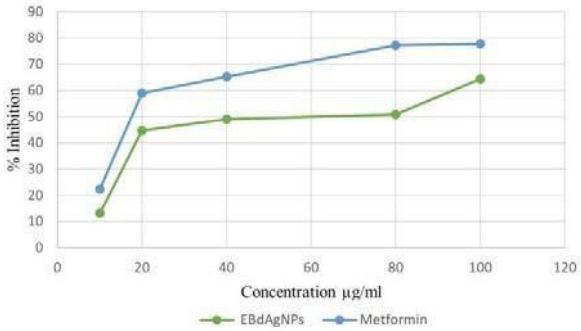

Figure 8: Non-enzymatic Glycosylation of EBdAgNPs

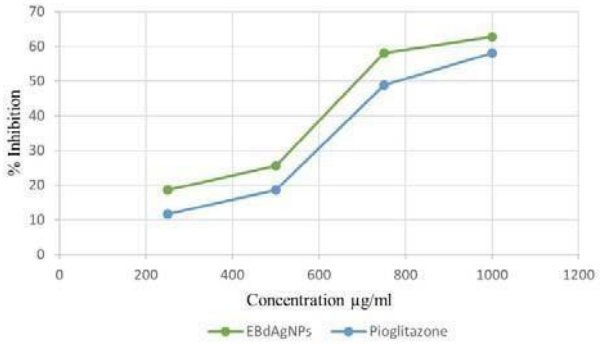

Figure 9: Protein Glycation in EBdAgNPs

Antidiabetic Study: We introduce comprehensive anti-diabetic assays that gauge EBdAgNPs' potential as a diabetes management agent. These assays target vital aspects of glucose metabolism. We analyze EBdAgNPs' inhibitory effects on *a*-amylase, starch hydrolysis enzyme and *a*-glucosidase, carbohydrate breakdown enzyme. Additionally, we study their impact on non-enzymatic glycosylation, protein glycation, yeast cell glucose uptake and inhibition of glucose diffusion through cell membranes. These assays collectively reveal EBdAgNPs' capacity to influence critical diabetes pathways, yielding insights for future therapeutics.

Inhibition of a-amylase activity of EBdAgNPs: Hyperglycemia defines diabetes mellitus, a group of metabolic disorders. Blood sugar is managed using insulin, oral hypoglycemic drugs, or carbohydrate enzyme inhibitors. Due to insulin and hypoglycemic side effects, researchers seek novel anti-diabetic sources[38]. *a*-amylase, vital for carbohydrate metabolism, is targeted for natural inhibitors[21]. For instance, Kifle and Enyew[17] found *Bersama abyssinica fresen* inhibiting *a*-amylase concentration-dependently (100-1000 μg/ml). *Bersama abyssinica fresen*'s crude extract showed lower IC_{50} compared to acarbose, suggesting its potential as an anti-diabetic *a*-amylase inhibitor source.

EBdAgNPs was assessed for their inhibitory effect on *a*-amylase in a dose-dependent manner, ranging from 10 μg/ml to 100 μg/ml (Figure 6). EBdAgNPs displayed significant inhibitory efficacy against the enzyme *a*-amylase, with a 30–70% inhibition range when synthesized from the entire plant. At the maximum doses of 100 μg/ml, acarbose and EBdAgNPs inhibited 70.4% and 75.31% of the enzyme activity respectively with IC_{50} values of 55.4 μg/ml and 46.2 μg/ml. Overall, EBdAgNPs showed higher dose-dependant inhibitory effects on *a*-amylase when compared with arcobose. These findings demonstrate that EBdAgNPs effectively suppressed the activity of the *a*-amylase enzyme under *in vitro* conditions.

Inhibition of a-glucosidase enzyme activity of EBdAgNPs: In this study, we explored plant-based *a*-glucosidase inhibitors. We examined inhibitory effects using EBdAgNPs-derived *a*-glucosidase enzymes. Blocking carbohydrate-hydrolyzing enzymes like *a*-glucosidase is a strategy to mitigate glucose absorption in diabetes. However, the common *a*-glucosidase inhibitor, acarbose, triggers gastrointestinal issues. Recognizing plants' therapeutic potential, recent research has focused on *a*-glucosidase inhibitors from plants[7,33].

In comparison to acarbose, the EBdAgNPs showed weaker *a*-glucosidase inhibitory activity, as illustrated in figure 7. The IC_{50} value for acarbose, the standard drug, was determined to be 63.4μg/ml, whereas EBdAgNPs yielded 93.0μg/ml. Despite the higher IC_{50} value for EBdAgNPs compared to the the standard drug, it still exhibited notable inhibitory effects as evidenced by increasing inhibition at higher concentrations. Further enhancement of these effects could potentially be achieved through the isolation and purification of their active constituents.

Inhibition of non-enzymatic glycosylation of EBdAgNPs: Non-enzymatic bonding between hemoglobin (Hb) and glucose causes glycated Hb formation, heightened in hyperglycemic conditions[31]. Plant extracts mitigate glucose-Hb complex, elevating free Hb levels. This was observed in *O. forskolei* leaves and stems[15] and *C. viscosa*[45]. Ukwuani-Kwaja et al[41] noted that plant extracts can surpass standard drugs in inhibiting hemoglobin glycosylation.

In this study, we observed a dose-dependent increase in the percentage inhibitory effect on hemoglobin glycosylation as the concentrations of EBdAgNPs increased (Figure 8). This suggests that the plant extracts enhance the amount of free hemoglobin by reducing the formation of the glucose-hemoglobin complex. Among the plant extracts tested, the most significant inhibitions were observed in EBdAgNPs (64.32%) at the highest concentration (100μg/ml), which was comparable to the inhibitory effect of Metformin (77.72%). EBdAgNPs demonstrate a robust dose-dependent response akin to the standard drug, albeit with slightly lower inhibition values, as evidenced by the IC_{50} values of 63.97 μg/ml and 28.64 μg/ml for EBdAgNPs and metformin, respectively. There is potential to enhance the inhibitory response of EBdAgNPs through additional extraction techniques and processing.

Inhibition of protein glycation of EBdAgNPs: Diabetes complications stem from advanced glycation end product (AGE) formation via protein glycation, posing health risks. This assay seeks inhibitors to curb protein glycation, yielding insights for diabetes research and care. Identifying effective inhibitors may open avenues for future investigations in diabetes management. In this study, the *in vitro* inhibitory efficacy of EBdAgNPs extracts on protein glycation was assessed using the model system of bovine serum albumin and fructose. As depicted in figure 9, the inhibitory effect improved with increases in concentration from 250 to 1000 μg/ml.

At a concentration of 1000 μg/ml, Pioglitazone exhibited 58.14% inhibitory effects on protein glycation activity, with an IC_{50} value of 856.25μg/ml. Meanwhile, the EBdAgNPs showed 62.79% inhibitory activity, with an IC_{50} value of 756.35μg/ml. In comparison to the widely used anti-glycation medication, aminoguanidine (IC_{50} = 138 mg/L), the etnanolic extracts of dandelion, roseroot and water extract of Myrica gale demonstrated significant suppression of AGE production with IC_{50} values of 69.4, 74.0 and 70.4 mg/ml respectively[32].

The leaf extracts of *C. bullatus*, *C. zabelii* and *C. integerrimus* were identified as the most potent inhibitors of protein glycation, with IC_{50} values ranging from 32.6 to 36.5 μg/ml. These extracts displayed a capacity twice as high as that of aminoguanidine (IC_{50} = 71.1 μg/ml), a synthetic drug used to treat diabetic complications and known to inhibit the formation of AGEs. Furthermore, conventional polyphenols, particularly procyanidin B2 and (-) -epicatechin, exhibited substantial anti-AGE activity in addition to the extracts, outperforming aminoguanidine significantly in this regard[16].

Glucose uptake assay by yeast cells of EBdAgNPs: Testing yeast cell glucose uptake informs diabetes research. Yeast cells, akin to humans, offer insulin-independent glucose transport insights. It reveals drug targets, clarifies glucose control and enables cost-effective screening. Manipulable yeast strains aid gene and protein studies, enhancing diabetes care. *Centella asiatica* was assessed for glucose absorption in yeast cells at 5mM and 10mM concentrations[44]. Comparing 10mM to 5mM, inhibition increase was lower. *L. hastata* leaves exhibited rising glucose uptake with 25mM, 10mM, 5mM fractions[41]. The impact of the EBdAgNPs samples on glucose transport across yeast cell membranes was investigated in an *in vitro* method that included yeast cells suspended in variable amounts of glucose solution (5, 10 and 25mM) in the presence of extracts at various concentrations (Table 1).

Table 1
Antidiabetic activity of Glucose uptake assay by yeast cells

Glucose conc. (mM)	Sample conc. (μg/ml)	Standard (%)	EBdAgNPs (%)
5	20	5.17±0.294	14.2±0.274
	40	19.5±0.312	32.4±0.49
	60	38.2±0.664	43.4±0.262
	80	60.2±0.64	56.5±0.0987
	100	65.2±2.43	66.5±0.0867
10	20	15.5±0.0917	22.2±0.63
	40	32.3±1.01	36.7±2.35
	60	49.3±1.97	53.1±1.79
	80	50.4±4.98	55.7±2.54
	100	62.3±2.67	64.5±1.13
25	20	32±0.947	21.3±0.643
	40	44.5±1.33	36.6±1.04
	60	49.9±1.98	33.8±2.15
	80	62.3±1.87	41.9±0.898
	100	63±1.76	52.4±1.1

Table 2
Antidiabetic activity of Glucose Diffusion Assay

Sample	Glucose in external solution (mM)				
	30mins	60mins	90mins	120mins	180mins
Absence of drug (Control)	51.9±0.638	61.5±0.57	61.6±0.521	107±0.831	135±0.944
Metformin	18.6±0.309	19.3±0.483	19.8±0.823	19.3±0.248	20.7±0.36
EBdAgNPs	19±0.28	20.4±0.527	21.2±0.351	21.6±0.416	26.5±0.343

The inhibition caused by metronidazole was marginally higher compared to EBdAgNPs, except at 25mM where EBdAgNPs showed higher inhibition. EBdAgNPs showed dose dependent inhibitory behaviour at all glucose concetrations. These findings suggest that the plant extract may effectively enhance glucose absorption, thereby potentially improving glucose utilization and managing blood glucose levels, as supported by previous research[29].

Inhibition of glucose diffusion of EBdAgNPs: Inhibiting glucose diffusion manages diabetes, controlling blood glucose, reducing hyperglycemia risk, enhancing insulin sensitivity and supporting heart health. It assists in postprandial glucose management and potential weight control. This study investigated the impact of EBdAgNPs extract incorporated with synthesized silver nanoparticles, on inhibiting glucose diffusion across a dialysis membrane (Table 2). Glucose diffusion inhibition was measured at intervals of 30, 60, 90, 120 and 180 minutes.

In comparison to EBdAgNPs, the effect of conventional metformin on glucose diffusion showed a less pronounced significance over different time intervals. Notably, the inhibitory level increased progressively from the initial to the final minutes in the sample.

The glucose diffusion inhibition measurements were conducted at intervals of 30, 60, 90, 120 and 180 minutes. Comparatively, the inhibitory effect of conventional acarbose was slightly lower than that of EBdAgNPs in the glucose diffusion assays. To explore the influence of different leaf extract fractions on glucose retardation within the dialysis tube, a glucose diffusion experiment was performed. Notably, significant differences were observed between the fractions in glucose diffusion at various time points. The glucose diffusion in *A. nilgiricum* leaves exhibited an increase from 30 to 180 minutes, as shown by Konappa et al[18]. This experiment provides valuable insights into the impact of leaf extract fractions on glucose diffusion inhibition, offering potential avenues for future research and development of antidiabetic agents.

Conclusion

The current study explores the activity of the whole plant extract of EBdAgNPs through three characterization studies. UV-Vis analysis revealed the rapid synthesis of AgNPs with a characteristic peak at 410nm, indicating successful nanoparticle formation due to the reaction of phytochemicals with silver nitrate solution. XRD analysis demonstrated the presence of lattice planes (111), (200), (220), (311) and (222) in the face-centered cubic structure of silver. FTIR evaluation confirmed the adsorption of an aldehyde group on the nanoparticle surface.

The EBdAgNPs exhibited inhibitory effects on several key activities related to diabetes mellitus including *a*-amylase, *a*-glucosidase, non-enzymatic glycosylation of hemoglobin, glucose absorption by yeast cells, glucose diffusion and protein glycation. These findings suggest the potential of EBdAgNPs for the treatment of diabetes mellitus. The method of synthesizing AgNPs through this approach proves to be safe, eco-friendly, cost-effective and efficient, with promising biological characteristics in in vitro tests. The presence of antidiabetic qualities in EBdAgNPs is evident.

However, further research through *in vivo* assays and clinical trials is required to validate the complete curative potential of diabetic illness. Overall, this study presents a promising avenue for the development and application of EBdAgNPs in diabetes treatment and it opens up new possibilities for future investigations in this field.

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(Received 10th April 2025, accepted 19th June 2025)