3

MATERIALS AND METHODS

Diabetes mellitus and obesity represent two of the most widespread and critical metabolic disorders across the globe. Because of their high correlation with cardiovascular illnesses, hypertension and several types of cancer, their rising incidence is a serious public health concern. Type 2 diabetes mellitus (T2DM), in particular, is marked by persistent hyperglycemia that results from insulin resistance and/or dysfunctional insulin secretion, ultimately leading to chronic damage in various organ systems (Dixit *et al*., 2023). Obesity, which commonly co-occurs with diabetes, is primarily driven by an imbalance between caloric intake and energy expenditure, resulting in excessive fat storage. Dysfunctional adipose tissue, in turn, promotes insulin resistance, low-grade chronic inflammation and lipid metabolism disturbances (Bonifácio *et al*., 2020). The convergence of these two conditions forms the foundation of metabolic syndrome, a major contributor to reduced lifespan and diminished quality of life.

Although standard treatments for diabetes and obesity including oral antidiabetic agents and pharmaceutical weight-loss drugs have been widely used, they often present limitations such as side effects and suboptimal long-term effectiveness (Ahmed and Alsayed.2024). This has catalyzed growing interest in alternative therapeutic strategies derived from medicinal plants, which offer multi-targeted mechanisms of action and typically display improved safety profiles. Numerous plant derived bioactive compounds have shown encouraging antidiabetic and antiobesity potential through modulation of glucose metabolism, lipid homeostasis, oxidative stress and inflammatory signaling (Barros *et al*., 2025).

Recent progress in nanotechnology has further enhanced the clinical potential of plant-based compounds by enabling their delivery through nanoparticle formulations. This approach improves solubility, bioavailability, cellular uptake and overall therapeutic efficacy (Kanthi *et al*., 2022). Among various nanocarriers, silver nanoparticles (AgNPs) synthesized via green chemistry approaches have shown particular promise in improving antidiabetic and

antiobesity outcomes (Nandan *et al*., 2024).

In light of these developments, the present research focuses on the synthesis and evaluation of silver nanoparticles derived from the ethanolic extract of *Boerhavia diffusa* L. (EBdAgNPs) as a novel therapeutic agent targeting both diabetes and obesity. *B. diffusa* is known for its diverse pharmacological properties and when incorporated into nanoparticle form, its bioactive compounds are expected to exhibit enhanced efficacy (Jadhav and Yadav, 2023). This investigation employs a comprehensive approach including *in vitro*, *in vivo* and gene expression analyses to systematically assess the therapeutic potential of EBdAgNPs in mitigating hyperglycemia, dyslipidemia, chronic inflammation and insulin resistance.

Phase I involved the collection and extraction of *B. diffusa*, followed by the green synthesis of silver nanoparticles using its ethanolic extract. The synthesized AgNPs were then characterized to confirm their formation and properties using various techniques, including UV-Vis spectroscopy, X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Energy Dispersive X-ray Spectroscopy (EDAX) and zeta potential analysis. Phase II focused on the *in vitro* evaluation of the antidiabetic and antioxidant potential of EBdAgNPs. Antidiabetic activity was assessed through α-amylase and α-glucosidase inhibition assays, nonenzymatic glycosylation, protein glycation, glucose uptake by yeast cells and glucose diffusion studies to evaluate their ability to inhibit key carbohydrate-metabolizing enzymes, prevent protein damage and enhance cellular glucose absorption. Antioxidant activity was further examined using DPPH, ABTS and FRAP assays to determine the free radical scavenging capacity of the nanoparticles.

Phase III includes the *in vivo* evaluation of the antidiabetic and antiobesity potential of EBdAgNPs using rat models. The animals were divided into control and experimental groups, with diabetes and obesity induced in specific groups. EBdAgNPs were administered over a defined treatment period, with regular monitoring of body weight and blood glucose levels. Hematological and biochemical parameters were analysed. Histopathological examinations of the pancreas, liver, kidney and adipose tissue were conducted to assess the protective effects of the treatment.

Phase IV aimed at assessing the impact of EBdAgNPs on gene expression related to diabetes and obesity in target tissues. Real-time PCR is used to analyze genes involved in insulin production and glucose regulation in pancreatic tissue, while genes linked to lipid metabolism and fat storage are studied in adipose tissue to evaluate antiobesity effects. Primers for these key genes are carefully designed, standardized and validated to ensure accurate measurement of gene expression levels. This phase, combined with earlier *in vitro* and *in vivo* studies, provides a thorough understanding of the molecular mechanisms behind the therapeutic benefits of EBdAgNPs.

PHASE I

3.1 Characterization of Ethanolic Extract of *Boerhavia diffusa* L. Synthesized Silver Nanoparticles EBdAgNPs

3.1.1 Collection, Identification and Preparation of the Ethanolic Extract of *B. diffusa*

The experimental plant, *B. diffusa*, was collected from and around Coimbatore, Tamil Nadu, India. It was duly authenticated by the Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore and the authentication number was assigned BSI/SRC/5/23/2013-14/Tech/1041.

The whole plant of *B. diffusa* was collected, thoroughly washed, dried, shredded into small piece and powdered, was then extracted using ethanol in a Soxhlet apparatus for 72 hours. The obtained extract was filtered and the solvent was removed using a rotary evaporator. The resulting extract was then used for further study

PLATE 1

***Boerhavia diffusa* L.**

SCIENTIFIC CLASSIFICATION

Kingdom	Plantae
Class	Magnoliopsida
Order	Caryophyllales
Family	Nyctanginaceae
Genus	*Boerhavia*
Species	*diffusa*

3.1.2 Synthesis of silver nanoparticles of *B. diffusa*

About 500 mg of ethanolic extract of *B. diffusa* was dissolved in 100ml of deionized water. From this solution, 10 ml was taken and mixed with 90 ml of deionized water containing 1 mM silver nitrate. The mixture was then exposed to sunlight for 5 to 10 minutes. The solution turned from greenish to brown, indicating the synthesis of silver nanoparticles.

The solution was centrifuged at 13,000 rpm for 20 minutes. The supernatant was discarded and the pellet was resuspended in deionized water. This washing process was repeated three times. The final pellet was lyophilized to obtain the powdered sample (Subramanian *et al*., 2013). The obtained ethanolic extract of *B. diffusa* synthesized with silver nanoparticles will be termed EBdAgNPs.

3.1.3 Characterization of EBdAgNPs

The characterization of the synthesized EBdAgNPs was performed using multiple analytical techniques. X-ray Diffraction (XRD) was conducted with an X'pert Pro X-ray diffractometer to determine the crystalline structure. Fourier Transform Infrared Spectroscopy (FTIR) analysis was carried out using a SHIMADZU miracle 10 to identify functional groups. UV-Visible spectroscopic measurements were obtained with a

Shimadzu Bio Spec-nano to assess the optical properties. The nanoparticles were further characterized using Scanning Electron Microscopy (SEM) with Energy-dispersive X-ray Spectroscopy (EDAX) using a MIRA 3 TESCAN and EDAX APEX to analyze their morphology and elemental composition. Finally, Zeta Potential analysis was performed using Malvern Instruments Ltd. to evaluate the surface charge and stability of the nanoparticles.

PHASE II

3.2 *in vitro* Antioxidant, Antidiabetic and Antiobesity Activities of EBdAgNPs

3.2.1 DPPH Radical Scavenging Activity of EBdAgNPs

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

The antioxidant activity of EBdAgNPs was estimated using the DPPH radical scavenging method. The reduction in absorbance at 517 nm indicated radical neutralization. Ascorbic acid acted as the standard antioxidant for comparison. The detailed procedure was given in Appendix 1.

3.2.2 ABTS Radical Scavenging Activity of EBdAgNPs (Re *et al.*, 1999)

The ABTS assay was used to assess the electron-donating and free radical scavenging potential of EBdAgNPs. The reduction in absorbance at 734 nm reflected the scavenging efficiency, with ascorbic acid as the positive control was given in Appendix 2.

3.2.3 Ferric Reducing Antioxidant Power (FRAP) Assay of EBdAgNPs

(Benzie and Strain, 1996)

The reducing ability of EBdAgNPs was determined using the FRAP method, which involves the conversion of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions, producing a blue complex measurable at 593 nm. The antioxidant potential was compared to that of ascorbic acid was described in Appendix 3.

3.2.4 α-Amylase Inhibition Assay of EBdAgNPs (Ishwarya *et al*., 2022)

The α-amylase inhibitory activity of EBdAgNPs was determined at concentrations ranging from 10 to 100µg/ml, using acarbose as the standard reference. After incubation of

the enzyme with the starch substrate, absorbance was measured at 540 nm to determine the inhibition percentage and the detailed procedure was given in Appendix 4.

3.2.5 α-Glucosidase Inhibition Assay of EBdAgNPs (Peytan *et al*., 2021)

The inhibitory effect of EBdAgNPs on α-glucosidase was evaluated using para-nitrophenyl-α-D-glucopyranoside (PNPG) as the substrate. Following incubation, absorbance was recorded at 405 nm and enzyme inhibition was expressed as a percentage relative to the control sample and the detailed explanation was given in Appendix 5.

3.2.6 Non-Enzymatic Glycosylation of Hemoglobin of EBdAgNPs (Premanath *et al*., 2012)

The potential of EBdAgNPs to prevent glucose induced glycosylation of hemoglobin was assessed after a 72 hour incubation period. Absorbance was measured at 520 nm and inhibition was compared against acarbose as the standard reference drug and the detailed procedure described in Appendix 6.

3.2.7 *in vitro* Protein Glycation Inhibition of EBdAgNPs (Avwioroko *et al*., 2022)

The anti-glycation capacity of EBdAgNPs was examined using a bovine serum albumin (BSA) fructose model. Fluorescence intensity was monitored between 370–650 nm and the results were compared with those obtained for metformin, which served as the positive control and it is explained in Appendix 7.

3.2.8 Glucose Uptake by Yeast Cells of EBdAgNPs (Madiwalar *et al*., 2022)

The influence of EBdAgNPs on glucose uptake was studied using yeast cell suspensions incubated with varying concentrations of glucose. After incubation, the amount of glucose was quantified spectrophotometrically and the increase in uptake was calculated with metformin as the reference and the detailed procedure is given in Appendix 8.

3.2.9 Glucose Diffusion Assay of EBdAgNPs (Ansari *et al*., 2022a)

A cellulose ester dialysis setup containing glucose NaCl solution was employed to evaluate the effect of EBdAgNPs on glucose diffusion. The concentration of glucose diffused through the membrane was monitored at fixed time intervals to estimate the percentage of diffusion inhibition and the procedure is described in Appendix 9.

3.2.10 Pancreatic Lipase Inhibitory Activity of EBdAgNPs (Zhang *et al*., 2008)

The pancreatic lipase inhibitory potential of EBdAgNPs was tested using porcine pancreatic lipase (PPL) and p-nitrophenyl palmitate as the substrate. The release of p-nitrophenol was measured at 405 nm, with sibutramine serving as the reference compound and the procedure is presented in Appendix 10.

PHASE III

3.3 *in vivo* Antidiabetic and Antiobesity Potential of EBdAgNPs

The EBdAgNPs were evaluated to determine their antidiabetic and antiobesity activities using male albino rats.

3.3.1 Housing and Maintenance of Laboratory Experimental Rats

Adult male albino animals weighing between 150-200g were sourced from the Animal House at KMCH College of Pharmacy, Coimbatore, India. Animal experimentation was conducted following approval by the Institutional Animal Experimentation Committee (reference number: 623/02502CBEA19/02/2019). Standard grouping techniques were adhered to the animals were housed in the animal facility for 15 days under normal room temperature with a 12 hour light-dark cycle. Throughout the trial period, the animals were provided unrestricted access to commercial pellet food and water. Following the acclimatization period, all animals underwent induction of diabetes using streptozotocin-nicotinamide (STZ-NI) and (OECD guidelines for testing of chemicals, 1981; 1987).

The STZ-NI model is a widely employed approach for inducing type 2 diabetes mellitus (T2DM) in experimental rats. Streptozotocin, a β-cell cytotoxic agent, selectively impairs pancreatic β-cells by causing oxidative stress and DNA damage, leading to decreased insulin secretion. The co-administration of nicotinamide, a form of vitamin B3, offers partial protection to β-cells by mitigating oxidative damage and enhancing DNA repair, thereby producing a diabetic phenotype characterized by moderate hyperglycemia and partial insulin deficiency (Ali *et al*., 2019; Gurunathan *et al*., 2020). This combination effectively simulates key pathophysiological aspects of human T2DM, including insulin resistance and progressive β-cell dysfunction, making it a highly relevant

and reproducible model for evaluating antidiabetic therapies (Patel *et al*., 2021; Peshattiwar and Muthuraman, 2019).

3.3.2 Grouping of Animals and Treatment Regimen

The animals were divided into six groups, each consisting of six individuals (Table 3.1). Following an overnight fast, initial fasting blood glucose levels were measured using the tip of the rat's tail vein. Non-insulin dependent DM was induced by administering a single intraperitoneal injection of 60 mg/kg b.w STZ, followed by an intraperitoneal dose of 120 mg/kg b.w NI. Hyperglycemia was confirmed by elevated blood glucose levels after 72 hours, with animals exhibiting levels exceeding 250 mg/dl selected for the study. The EBdAgNPs were administered to the rats for duration of 42 days via intragastric tubes.

TABLE 3.1

GROUPING OF EXPERIMENTAL RATS

GROUP	DESCRIPTION
I	Control 0.05% Carboxymethyl cellulose
II	EBdAgNPs (10 mg/kg b.w/day)
III	Streptozotocin STZ (60 mg/kg b.w) – Nicotinamide NI(120mg/kg b.w)
IV	STZ-NI (60 mg/kg b.w -120mg/kg b.w) + EBdAgNPs (10 mg/kg b.w/day)
V	STZ-NI (60 mg/kg b.w -120mg/kg b.w) + Glibenclamide (10 mg/kg b.w/day)
VI	High fat diet (HFD) (Cholesterol -2 %, Cholic acid - 1 %, Dalda-20 % and Coconut oil - 6%)
VII	HFD +EBdAgNPs (10 mg/kg b.w/day)
VIII	HFD +Sibutramine (10 mg/kg b.w/day)
IX	STZ-NI (60 mg/kg b.w -120mg/kg b.w) + HFD
X	STZ-NI (60 mg/kg b.w -120mg/kg b.w) + HFD + EBdAgNPs (10 mg/kg b.w/day)

3.3.3 Body Weight of Experimental Rats

During the designated intervention period, fasting animal body weight was recorded on days 0, 21st and 42nd of the treatment regimens using a digital weighing balance.

3.3.4 Collection of Blood and Tissue Samples of Experimental Rats

The blood samples were drawn from the tip of the rat tail vein and analyzed for glucose levels using a glucometer and glucose oxidase-peroxidase reactive strips (Accu-chek, Roche Diagnostics, USA). Glucose levels were assessed on days 0, 21st and 42nd of the treatment regimens. At the end of treatment, following an overnight fast, rats were anesthetized with ketamine hydrochloride. Blood samples were collected from the retro-orbital sinus using a capillary tube. For hematological parameters, EDTA was added to the centrifugation tube, while serum biochemical measurements were conducted without EDTA. The blood was allowed to clot at room temperature and serum was separated by centrifugation at 10,000 rpm for 10 minutes. After euthanasia the pancreas, liver, kidney and adipose tissues were carefully excised and rinsed with normal saline. Portions of these organs intended for histopathological evaluation were immediately fixed in 10% formalin. For biochemical analysis, 10% tissue homogenates were prepared in chilled Tris-HCl buffer (0.1 M, pH 7.4) and centrifuged at 12,000 × g for 30 minutes at 4°C to obtain the supernatant for further assays. Additionally, separate sections of the pancreas and adipose tissue were aseptically collected, snap-frozen in liquid nitrogen and stored at –80°C for subsequent gene expression studies (Alex *et al*., 2020).

3.3.5 Determination of Haematological Parameters of EBdAgNPs Treated Rats

The red blood cell (RBC) and white blood cell (WBC) counts were determined using standard laboratory procedures. The Neubauer counting chamber was used to determine the RBC and WBC counts. The detailed procedures for each assay are described in Appendices 11 and 12.

3.3.5.1 Assessment of Red Blood Cell (RBC) Count of EBdAgNPs Treated Rats (Sahastrabuddhe, 2016)

The red blood cell count was determined using the hemocytometric method with Hayem's diluting fluid and a Neubauer chamber. The calculation of RBCs per cubic millimeter of blood followed standard procedures outlined in Appendix 11.

3.3.5.2 Assessment of White Blood Cell (WBC) Count of EBdAgNPs Treated Rats (Enos and Moore, 2022)

Total white blood cell count was performed using Turk's diluting fluid in a Neubauer chamber. The method and calculation steps for determining the WBC concentration are detailed in Appendix 12.

3.3.6 Determination of Biochemical Parameters of EBdAgNPs Treated Rats

Biochemical parameters were comprehensively evaluated in experimental rats to assess the impact of treatments on metabolic health. The lipid profile was analyzed by measuring total cholesterol, triglycerides and key lipoproteins including high-density lipoproteins (HDL), low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL). Liver function was assessed through the activities of serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), alkaline phosphatase (ALP), as well as levels of total bilirubin and total protein. Kidney function markers, including urea, uric acid and creatinine, were also estimated to evaluate renal status and any potential nephroprotective effects of the administered treatments.

3.3.6.1 Determination of Lipid Profile in Experimental Rats Treated with EBdAgNPs

Serum lipid levels, including total cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL), were estimated using standard enzymatic colorimetric methods. The comprehensive experimental procedures for each lipid assay are described in Appendices 13–15.

3.3.6.1.1 Evaluation of Total Cholesterol in Experimental Rats Treated with EBdAgNPs (Martins *et al*., 2023)

Total cholesterol in the serum was quantified using an enzymatic reagent-based colorimetric method and absorbance was measured at 500 nm against a reagent blank. The stepwise procedure and reagent composition are detailed in Appendix 13.

3.3.6.1.2 Estimation of Triglycerides in Experimental Rats Treated with EBdAgNPs (Toth and Simko, 2019)

Triglyceride levels were determined by enzymatic hydrolysis followed by colorimetric detection of the end product at 500 nm. The specific protocol, including incubation and reagent composition, is outlined in Appendix 14.

3.3.6.1.3 Assessment of High-Density, Low-Density and Very Low-Density Lipoproteins in the Lipid Profile of Experimental Rats Treated with EBdAgNPs (Lopes-Virella *et al*., 1977 and Warnick and Albers 1978)

The classification and estimation of HDL, LDL and VLDL were performed based on selective precipitation and enzymatic quantification techniques. HDL was analyzed from the supernatant following precipitation of LDL and VLDL fractions and the calculations for each lipoprotein type were derived accordingly and the detailed methodology is presented in Appendix 15.

3.3.6.2 Evaluation of Liver Function Markers in Experimental Rats Treated with EBdAgNPs

The assessment of liver function in experimental rats was carried out by analyzing the activities of key hepatic biomarkers. These includes Serum Glutamate Oxaloacetate Transaminase (SGOT), also known as Aspartate Aminotransferase (AST), Serum Glutamate Pyruvate Transaminase (SGPT), also known as Alanine Aminotransferase (ALT) and Alkaline Phosphatase (ALP). Additionally, levels of Total Bilirubin (TB) and Total Protein (TP) were measured to evaluate overall hepatic health and synthetic function. These parameters serve as important indicators of liver integrity and metabolic activity. The detailed methodologies are presented in Appendices 16–19.

3.3.6.2.1 Estimation of Serum Glutamic-Oxaloacetic Transaminase (SGOT) and Serum Glutamic Pyruvic Transaminase (SGPT) in Experimental Rats Treated with EBdAgNPs (Reitman and Frankel, 1957)

The enzymatic activities of SGOT and SGPT were measured using standard colorimetric methods based on the formation of pyruvate hydrazone with 2,4-dinitro phenyl hydrazine (DNPH). Serum samples were incubated with appropriate substrates

under controlled pH and temperature conditions and absorbance was recorded between 500–550 nm to quantify enzyme activity. The detailed assay protocol and reagent composition are provided in Appendix 16 and 16a.

3.3.6.2.2 Estimation of Alkaline Phosphatase (ALP) in Experimental Rats Treated with EBdAgNPs (Tietz *et al*., 1983)

Alkaline phosphatase activity was assessed spectrophotometrically using disodium phenyl phosphate as a substrate and the color intensity developed with molybdate ANSA reagent was measured at 650 nm. Enzyme activity was expressed in U/L, reflecting the rate of phenol liberation. The complete procedure is described in Appendix 17.

3.3.6.2.3 Estimation of Total Bilirubin (TB) in Experimental Rats Treated with EBdAgNPs (Malloy and Evelyn, 1937)

Serum bilirubin levels were determined using the diazo coupling reaction, where bilirubin forms a chromogenic complex with sulfanilic acid under acidic conditions. Absorbance was read at 540 nm and results were expressed in mg/dL. The stepwise method is outlined in Appendix 18.

3.3.6.2.4 Estimation of Total Protein (TP) in Experimental Rats Treated with EBdAgNPs (Lowry *et al*., 1951)

Total protein concentration was estimated using the Lowry method, based on the reaction of peptide bonds with copper ions and subsequent color development with Folin–Ciocalteu reagent. The absorbance was measured at 670 nm and protein content was calculated against a standard curve of bovine serum albumin. The detailed procedure is given in Appendix 19.

3.3.6.3 Assessment of Kidney Function Markers in Experimental Rats Treated with EBdAgNPs

Renal function was analyzed to determine the effect of treatment on kidney integrity and filtration capacity by estimating urea, uric acid and creatinine levels in serum samples. These parameters serve as sensitive indicators of renal performance and possible nephroprotective effects of the tested formulations. The detailed analytical procedures are provided in Appendices 20–22.

3.3.6.3.1 Estimation of Urea in Experimental Rats Treated with EBdAgNPs (Netelson, 1957)

Serum urea concentration was determined colorimetrically following protein precipitation and reaction with diacetyl monoxime in an acid medium, forming a pink chromogen measurable at 540 nm. The results were expressed in mg/dL, reflecting nitrogenous waste levels in circulation. The complete experimental steps are outlined in Appendix 20.

3.3.6.3.2 Estimation of Uric Acid in Experimental Rats Treated with EBdAgNPs (Caraway, 1955)

Uric acid levels were quantified using an enzymatic uricase–peroxidase method, in which uric acid is oxidized to allantoin with subsequent chromogen formation detected at 510 nm. The concentration was calculated from a standard calibration curve and detailed assay conditions are presented in Appendix 21

3.3.6.3.3 Estimation of Creatinine in Experimental Rats Treated with EBdAgNPs (Trinder, 1969)

Creatinine concentration was assessed based on the Jaffe's alkaline picrate method, involving color development through the reaction of creatinine with picric acid in an alkaline medium. Absorbance was recorded at 500 nm and results were expressed in mg/dL. The stepwise methodology is described in Appendix 22.

3.3.7 Determination of Insulin and C-Reactive Protein in Experimental Rats Treated with EBdAgNPs

The quantification of plasma insulin and C-reactive protein (CRP) was conducted to evaluate the metabolic and inflammatory responses in experimental rats. These biomarkers provide valuable insights into pancreatic function, glucose homeostasis and the degree of systemic inflammation associated with diabetic and obese conditions.

3.3.7.1 Estimation of Plasma Insulin in Experimental Rats Treated with EBdAgNPs (Hales and Randle, 1963)

Plasma insulin levels were estimated using the modified immunoassay method of Hales and Randle (1963) with the Boehringer Mannheim kit. This method measured insulin concentration based on the color intensity formed during the enzymatic reaction, read at 420 nm and expressed as μU/ml of plasma. The detailed procedure is presented in Appendix 23.

3.3.7.2 C-Reactive Protein Estimation in Experimental Rats Treated with EBdAgNPs (Wadsworth, 1977)

C-reactive protein (CRP) levels were determined according to the method of Wadsworth (1977) using the Quantia-CRP reagent. The test involved the formation of an antigen–antibody complex producing turbidity proportional to CRP concentration, measured spectrophotometrically at 340 nm. The detailed procedure is presented in Appendix 24.

3.3.8 Assessing the Activities of Specific Enzymes in Carbohydrate Metabolism

The activities of key enzymes involved in carbohydrate metabolism including glucokinase, glucose-6-phosphatase and fructose-1, 6-bisphosphatase were assessed to understand the metabolic alterations in experimental rats under diabetic and treated conditions.

3.3.8.1 Assessment of Glucose-6-Phosphatase Activity in Experimental Rats Treated with EBdAgNPs (Hikaru and Toshitsugu, 1959)

The enzyme activity was estimated according to the method of Hikaru and Toshitsugu (1959) and the liberated inorganic phosphorus was quantified using the standard procedure of Fiske and Subbarow (1925). The detailed procedure is presented in Appendix 25.

3.3.8.2 Assessment of Fructose-1, 6-Bisphosphatase Activity in Experimental Rats Treated with EBdAgNPs (Gancedo and Gancedo, 1971)

Fructose-1, 6-bisphosphatase activity was measured based on the method of Gancedo and Gancedo (1971), with phosphorus estimation carried out as described by Fiske and Subbarow (1925). The detailed procedure is presented in Appendix 26.

3.3.8.3 Assessment of Glucokinase Activity in Experimental Rats Treated with EBdAgNPs (Branstrup *et al.*, 1957)

Glucokinase activity was determined following the method of Brandstrup *et al*. (1957). The assay measured glucose formation after enzymatic reaction and precipitation with trichloroacetic acid. The detailed procedure is presented in Appendix 27.

3.3.9 Atherogenic Index in Experimental Rats Treated with EBdAgNPs (Friedewald *et al*., 1972)

The atherogenic index (AI) was calculated from serum lipid parameters including total cholesterol, triglycerides, HDL and LDL, measured using enzymatic kits according to manufacturer's instructions. LDL-C was derived using the Friedewald formula (Friedewald *et al*., 1972) and the AI was expressed as the ratio of total cholesterol to HDL cholesterol. The detailed procedure is presented in Appendix 28.

3.3.10 Determination of Enzymatic and Non-Enzymatic Antioxidants of EBdAgNPs Treated Rats

The activities of enzymatic antioxidants (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic antioxidants (reduced glutathione, vitamin C and vitamin E) were analyzed in tissue homogenates to evaluate oxidative stress and the protective role of treatments in experimental rats.

3.3.10.1 Superoxide Dismutase Activity in Experimental Rats Treated with EBdAgNPs (Kakkar *et al*., 1984)

Superoxide dismutase (SOD) activity was determined following the procedure of Kakkar *et al*., (1984). The assay involved quantifying the inhibition of nitroblue

tetrazolium reduction at 560 nm, with enzyme activity expressed as units per milligram of protein. The detailed procedure is presented in Appendix 29.

3.3.10.2 Activity of Catalase in Experimental Rats Treated with EBdAgNPs (Sinha, 1972)

Catalase (CAT) activity was estimated according to the method described by Sinha (1972), based on the decomposition rate of hydrogen peroxide. The reaction product was measured at 620 nm and enzyme activity was expressed as µM of H_2O_2 decomposed per minute per mg protein. The detailed procedure is presented in Appendix 30.

3.3.10.3 Glutathione Peroxidase Activity in Experimental Rats Treated with EBdAgNPs (Rotruck *et al.,* 1973)

Glutathione peroxidase (GPx) activity was assayed following the method of Rotruck *et al*. (1973), using hydrogen peroxide as a substrate and quantifying residual GSH after reaction termination. The enzyme activity was expressed as µg of GSH consumed per minute per mg of protein. The detailed procedure is presented in Appendix 31.

3.3.10.4 Activity of Reduced Glutathione in Experimental Rats Treated with EBdAgNPs (Moron *et al*., 1979)

Reduced glutathione (GSH) levels were estimated using the method of Moron *et al*. (1979), where the yellow chromogen formed by the reaction of GSH with DTNB was measured at 412 nm. The concentration of GSH was expressed as µmol per gram of tissue. The detailed procedure is presented in Appendix 32.

3.3.10.5 Levels of Vitamin C in Experimental Rats Treated with EBdAgNPs (Omaye *et al.,* 1979)

Vitamin C concentration was measured according to Omaye *et al*. (1979). The method involved the formation of a red-orange complex with DNPH and absorbance measurement at 530 nm. Values were expressed as µg of ascorbic acid per gram of tissue. The detailed procedure is presented in Appendix 33.

3.3.10.6 Vitamin E in Experimental Rats Treated with EBdAgNPs (Sarisozen *et al*., 2002)

Vitamin E estimation followed the method of Sarisozen *et al*. (2002), involving colorimetric detection of the ferric dipyridyl complex at 520 nm. Results were expressed as µg of tocopherol per gram of tissue. The detailed procedure is presented in Appendix 34.

3.3.11 Histopathological analysis in Experimental Rats Treated with EBdAgNPs

The pancreas, liver, kidney and adipose tissue were quickly dissected and cleaned in ice-cold saline. Portions of these organs were preserved in a 10% neutral formalin fixative solution. Following this, they were dehydrated with alcohol and embedded in paraffin. Thin slices, about 4-5µm thick, were prepared using a rotating microtome. These sections were stained with hematoxylin and eosin (HE) to examine histological changes (Dias *et al*., 2008).

PHASE IV

3.4 Assessment of Gene Expression in Diabetic and Obese Rat Models

3.4.1 Animal Housing and Tissue Collection for Gene Expression

In continuation with the tissue collection procedure described earlier in 3.3.4, a designated portion of the pancreas and adipose tissues was separated shown in the table 3.1a. during dissection specifically for molecular analysis.

TABLE 3.1.1

GROUPING OF EXPERIMENTAL RATS IN GENE EXPRESSION

GROUP	DESCRIPTION
I	Control 0.05% Carboxymethyl cellulose
III	Streptozotocin STZ (60 mg/kg b.w) – Nicotinamide NI (120mg/kg b.w)
IV	STZ-NI (60 mg/kg b.w -120mg/kg b.w) + EBdAgNPs (10 mg/kg b.w/day)
V	STZ-NI (60 mg/kg b.w -120mg/kg b.w) + Glibenclamide (10 mg/kg b.w/day)
VI	High fat diet (HFD) (Cholesterol -2 %, Cholic acid - 1 %, Dalda-20 % and Coconut oil - 6%)
VII	HFD +EBdAgNPs (10 mg/kg b.w/day)
VIII	HFD +Sibutramine (10 mg/kg b.w/day)

These sections were aseptically harvested, immediately snap-frozen in liquid nitrogen and stored at –80°C for subsequent gene expression studies. The preserved tissues were later utilized to assess the differential expression of PPAR-α, PPAR-γ and RBP4 using real-time quantitative PCR (RT-qPCR) following established protocols (Arya *et al*., 2005).

3.4.2 Isolation and Quantification of Total RNA

A 50 mg pancreas and adipose tissue sample was rinsed with 1x PBS (Phosphate-buffered saline) and lysed with 2ml of Trizol reagent. The lysate was centrifuged at 12,000 x g for 5 minutes and the supernatant was mixed with 200 µl of chloroform and centrifuged again for 15 minutes. The aqueous phase, containing RNA (Ribonucleic acid), was transferred to a new tube, mixed with 500 µl isopropanol and centrifuged for 10 minutes. The RNA pellet was washed with 75% ethanol, air-dried, resuspended in 50 µl RNase-free water and stored at 55-60°C. RNA concentration and purity were measured using a NanoDrop spectrophotometer at 260 and 280 nm. (Thermo Fisher Scientific, Waltham, MA, USA) (Oñate-Sánchez and Vicente-Carbajosa, 2008).

3.4.2.1 First-Strand cDNA Synthesis

For cDNA (Complementary DNA) synthesis using the IScript cDNA Synthesis Kit (Cat No. 1708890, Bio-Rad, CA), reverse transcription reactions were assembled in an RNase-free environment with clean automatic pipettes designated for PCR (Polymerase chain reaction) and aerosol-resistant barrier tips. RNA templates were thawed and all reagents were kept on ice and gently mixed by vortexing. The reaction mixture prepared on ice included 5 µl of 5X IScript reaction mix, 3 µl of nuclease-free water, 15 µl of RNA (2 µg) and 2 µl of reverse transcriptase enzyme represented in the table 3.2. The tubes were then incubated in a PCR cycler with the following conditions shown in the table 3.3: priming for 5 minutes at 25°C, reverse transcription for 20 minutes at 46°C and inactivation for 1 minute at 95°C. The newly synthesized first-strand cDNA was subsequently stored at -20°C (Ramalho *et al*., 2004).

TABLE 3.2

cDNA SYNTHESIS REACTION MIX

Reagent	Volume (µl)
5X IScript reaction mix	5
Nuclease free water	3
RNA (2 µg)	15
Rev Transcriptase enzyme	2

TABLE 3.3

PCR CYCLER CONDITIONS

Description	Reaction condition
Priming	5 min at 25 ºC
Reverse transcription (RT)	20 min at 46 ºC
RT inactivation	1 min at 95 ºC

3.4.3 Primer Design and Validation

The primers for the gene expression studies were designed as outlined in the table 3.4. All primers, of HPLC grade, were synthesized based on existing literature.

TABLE 3.4

THE PRIMER SEQUENCES

S. No.	Gene Symbol	NM ID	Primer	Sequence	BP	Tm
1	PPAR-α	NM_013196.2	FP	ACTGAAGCGACGCTGGG	17	59.69
			RP	GCACCAATCTGTGATGACAACG	22	60.41
2	PPAR-γ	NM_013124.3	FP	GGAGATCCTCCTGTTGACCC	20	59.17
			RP	TGGGTCAGCTCTTGTGAACG	20	60.25
3	RBP4	NM_013162.1	FP	GCTTGCACGCGGCTTCT	17	61.46
			RP	GATGGCATACCAGAGCCCAG	20	60.25
4	β-Actin	NM_031144.3	FP	CAACCTTCTTGCAGCTCCTC	20	59.86
			RP	AGGGTCAGGATGCCTCTCTT	20	60.08

3.4.3.1 PCR Standardization

A gradient PCR was conducted to determine the optimal annealing temperature for the designed primers using 50 ng of synthesized cDNA. The temperature range tested was between 50-60°C, with the optimal temperature identified as 59°C for all primers.

3.4.3.2 Primer Validation

The specific primers for PPAR alpha, PPAR gamma, RBP4 genes and the housekeeping gene β-actin were validated by PCR using a mixed pool of cDNA from the provided cells. These primers were further validated with SYBR reactions to check amplification and melt curves. All primers successfully amplified the expected product sizes without any self-annealing or self-dimerization.

3.4.4 Quantitative Real-Time PCR (RT-qPCR) for Gene Expression Analysis

Gene expression was quantified using Real-Time PCR with a Quant Studio3 system and SYBR Green Chemistry. Reactions were prepared in a 25 µl volume, including 1 µl

cDNA, 12.5 µl SYBR Green Master Mix, 1 µl each forward and reverse primers and 9.5 µl water. PCR conditions were: initial denaturation at 95°C for 15 seconds, denaturation at 95°C for 10 seconds, annealing at 60°C for 60 seconds and extension at 95°C for 15 seconds. Primers were used at 200 nM with a Tm of 80-95°C. Each reaction had 50 ng cDNA, with 40 cycle's total. Data were analyzed by averaging triplicates and fold changes were calculated using the ΔΔCt method, where values >1 indicate upregulation and <1 indicate downregulation (Skiljaica *et al*., 2022).

3.5 Statistical Analysis

Data were analyzed with GraphPad Prism 5 and expressed as mean ± standard deviation (SD). One-way ANOVA was applied, followed by Dunnett's post-hoc test. Statistical significance was defined as a p-value less than 0.05 ($p < 0.05$), with values below this threshold considered significant.