Chapter 3
MATERIALS AND METHODS

3.1 Collection

The ripe fruits of *Phyllanthus emblica* and *Punica granatum* were obtained from a local market, while *Hodgsonia heteroclita* was taken from the forest in the Kokrajhar district. The leaves of *Bambusa balcooa* were collected from the Bambusetum at Bodoland University, located in Kokrajhar, BTR, Assam, India. (Table 3.1).

3.2 Scientific classification of *Bambusa balcooa* Roxb.

Kingdom: Plantae

Family: Poaceae

Order: Poales

Genus: *Bambusa*

Species: *B. balcooa*

Description: *Bambusa balcooa* has a variety of phytoconstituents, such as flavonoids, saponins, resins, fixed oils, phytosterols, phenols, and tannins. These

Table 3.1. List of plants with information on parts used, vernacular names and GPS coordinates of the collection site.

		Vernacular names		GPS coordinates	
Botanical name	**Part used**	**Bodo name**	**Assamese name**	**Latitude**	**Longitude**
Hodgsonia heteroclita Hook.f. & Thomson	Fruit plup	Hagrani jwgwnar	Not Known	26.4011°N	90.2729°E
Bambus balcooa Roxb.	Leaf	Owa burkha	Bhaluka bah	26.4694332°N	90.292971°E
Punica granatum L.	Peel	Dalim	Dalim	26.5288799°N	90.2495364°E
Phyllanthus emblica L.	Fruit plup	Amlai	Amalaki	26.4720043°N	90.2979632°E

compounds have the potential to be employed in the treatment of illnesses and the development of drugs (Wani et al., 2018). *B. balcooa* has been documented to exhibit antidiabetic properties (Goyal et al., 2017).

3.3 Scientific classification of *Phyllanthus emblica L. (syn. Emblica officinalis)*

Kingdom: Plantae

Family: Euphorbiaceae

Order: Geraniales

Genus: *Emblica*

Species: *E. officinalis*

Description: Various plant components have been documented to possess therapeutic properties for a range of ailments, including but not limited to the common cold, fever, inflammation, hair health, diabetes, lipid regulation, bacterial infections, oxidative stress, stomach ulcers, liver protection, gastrointestinal health, cancer prevention, and prevention of genetic mutations (Singamaneni et al., 2020).

3.4 Scientific classification of *Hodgsonia heteroclita* (Roxb.) Hook.f, & Thomson

Kingdom: Plantae

Family: Cucurbitaceae

Order: Cucurbitales

Genus: *Hodgsonia*

Species: *H. heteroclita*

Description: *Hodgsonia heteroclita* is a significant medicinal plant that has a bitter taste and is utilized in the traditional medical system of the Bodo tribe for the purpose of treating diabetes (Swargiary et al., 2013). It has been claimed that H. heteroclita possesses the ability to reduce the risk of developing diabetes (Narzary et al., 2017).

3.5 Scientific classification of *Punica granatum* L.

Kingdom: Plantae

Family: Punicaceae

Order: Myrtales

Genus: *Punica*

Species: *P. granatum*

Description: In traditional medicine, *P. granatum* is a common therapeutic fruit. Several studies have shown that *P. granatum* possesses anti-oxidant and therapeutic properties. Along with its anti-inflammatory, anti-hypertensive, anti-anemic, anti-cancer, anti-diabetic, anti-hepatoprotective, and cardioprotective effects, this medicinal fruit plant also has immunomodulatory and anti-cancer capabilities (Isha et al., 2021).

3.6 Authentication of plants

The collected plant materials were pressed dried, processed, tagged, recorded as the specimen at the departmental herbarium. *Bambusa balcooa* Roxb., *Hodgsonia heteroclita* Hook. f. & Thomson, *Phyllanthus emblica* L., *Punica granatum* L., by a complete proved identification in the BSI Howrah. The voucher specimens were deposited at Botanical Survey of India, Central National Herbarium, Howrah and was identified and authenticated vide letter no. CNH/ Tech.II/2021/43 date 26-11-2021.

3.7 Preparation of the polyherbal formulation (PHF) and its naming

Each of the above ingredients was blended in various ratios to obtain effective formulation (data not published). The novel formulation is named as HOPE where the word 'H' is derived from *Hodgsonia heteroclite, 'O' from Bambusa balcooa (vernacular name Owa burkha in Bodo), 'P'* from *Punica granatum* and 'E' is obtained from *Emblica officinalis*.

3.8 Preparation of PHF-HOPE extract

All the individual plant parts such *Bambusa balcooa* (leaf), *Phyllanthus emblica* (fruit), *Hodgsonia heteroclita* (fruit pulp) and *Punica granatum* (fruit peel) were dried at room temperature. They were then ground, passed through 600 µm mesh size sieve and to obtain fine powder and stored in airtight glass bottles for analysis. Polyherbal formulation (HOPE) (50g) was then subjected to Soxhlation (for 72 hrs i.e. 3 cycles) using solvent aqueous extract. Using rotary evaporator (Reduced pressure) obtained extract were concentrated at 4° C in air tight bottles for further use.

3.9 Determination of plant extract yield

Based on dry weight, the yields of extracts were calculated using following equation:

$$Y = W1 \times 100 / W2$$

where, Y= Yield (g/100 g of dry plant material), W1 = the weight of the extract after the solvent evaporation and W2 = the weight of the dry plant material.

3.10 Preliminary phytochemical screening

The presence or absence of phytoconstituents in powdered plant material was determined using the following standard procedures by Trease and Evans with slight modification by Shaikh et al. (2020).

3.10.1 Detection of alkaloids

i. *Dragendroff's test:* A few milliliters of extract were mixed with milliliters of Dragendroff's reagent. The observation of a reddish-brown precipitate indicates the existence of an alkaloid.

ii. *Hager's test:* Few milliliters of extracts, The reagent of Hager Alongside the test tube, one to two droplets of Hager's reagent were added. The formation of a creamy-white precipitate indicates the presence of an alkaloid.

iii. *Mayer's test:* On the test tube's sides, a few milliliters of extract and one to two droplets of Mayer's reagent were added. The presence of alkaloids is denoted by the formation of a yellow or creamy-white precipitate.

iv. Vitali-Morin test: Add concentrated nitric acid and alcoholic KOH to 1 mL of extract.

3.10.2 Detection of carbohydrates

i. *Molisch's test:* Three droplets of Molisch's reagent were added to 1 mL or 2 mL of extract, followed by vigorous shaking of the mixture and the slow addition of 1 mL of concentrated H_2SO_4 along the test tube's sides before allowing the solution to stand. An indicator of the presence of carbohydrates was a violet ring.

ii. *Test for Starch:* A 5 mL solution of 5% KOH was added to 1 mL of extract. When carbohydrates are present, a cinary coloration is observed.

iii. *Test of Pentose:* Add a small quantity of phloroglucinol to 2 ml of concentrated HCl, followed by an equal volume of extract, and heat the solution over a flame. A precipitate of a red hue indicates the existence of carbohydrates.

3.10.3 Detection of reducing sugars

i. *Benedict's test:* After adding 1 mL of Benedict's reagent to 1 mL of extract, the mixture was heated over boiling water for two minutes. A precipitate with a

green, yellow, or red hue suggested the existence of sugar.

ii. *Fehling's test:* A few seconds of boiling occurred after adding one milliliter each of Fehling's solutions A and B to one milliliter of extract. An indicator of sugar's presence was a red precipitate.

iii. *Aqueous NaOH test:* Take a little amount of alcoholic extract, dissolve it in 1 mL of water, and then add a few drops of an aqueous NaOH solution. A yellow precipitate indicates the presence of sugar.

iv. *Conc. H_2SO_4 test:* A few mL of concentrated H_2SO_4 were added gradually along the test tube's sides after five mL of extract, two mL of glacial acetic acid, and one drop of 5% $FeCl_3$. The test tube was then left to stand.

3.10.4 Detection of cardiac glycosides

i. *Keller-Killani test:* Pour 1.5 mL of glacial acetic acid and 1 drop of 5% ferric chloride into a test tube containing 1 mL of extract. Gently pour a few milliliters of concentrated sulfuric acid down the edge of the test tube. The presence of glycosides is indicated by a solution that has a blue hue.

3.10.5 Detection of proteins and amino acids

i. *Biuret test:* A solution containing one drop of 2% copper sulphate was added to 2 ml of extract. Following the addition of 1 ml of 95% ethanol, an excess of potassium hydroxide granules is introduced. The ethanolic layer exhibited a pink hue, which signified the existence of proteins.

ii. *Millon's test:* A few drops of Millon's reagent were added to 1 millilitre of extract. The observation of a white precipitate signifies the existence of proteins.

iii. *Ninhydrin test:* Add 2 drops of ninhydrin solution to 1 milliliter of extract and apply heat for a duration of 5 minutes. The presence of amino acids is indicated by a distinct purple hue.

3.10.6 Detection of flavonoids

i. *Alkaline reagent test:* Mix 1 ml of the extract with 2 ml of a 2% NaOH solution and a little amount of diluted hydrochloric acid. The mixture will develop a strong yellow hue, which will become colourless with the addition of a few drops of diluted acid. This colour change confirms the presence of flavonoids.

ii. *Lead acetate test:* Add a small amount of 10% lead acetate solution to 1 ml of the extract. The presence of flavonoids is indicated by a yellow fluorescence.

iii. *Shinoda's test:* Take a little strip of magnesium ribbon and add a few drops of concentrated hydrochloric acid. Then, add 1 milliliter of the extract. Actual indication of the presence of flavonoids is indicated by a solution that is pink to crimson in hue.

iv. *Shibata's reaction:* To1mL extract, dissolved in 1-2 mL 50% methanol by heating, + metal magnesium, add 5 drops of conc. HCl. A red, orange colour indicates presence of flavonoids.

v. *Ferric chloride test:* To 1mL extract, add few drops 10% ferric chloride solution and observed for intense green color.

vi. *Pew's test:* To a 1 mL extract, solid zinc, and a few 1 mL of concentrated H_2SO_4 on the side. Flavonoids are present when the colour turns red.

vii. *Zinc-hydrochloride reduction test:* To a milliliter of extract, add a pinch of zinc dust and a few drops of concentrated HCl along the side of the test tube. Flavonoids are present when the colour is magenta.

viii. *Ammonia test:* Add 1 ml of diluted ammonia solution to 1 ml of extract, and then add 1 ml of concentrated H_2SO_4 to the side. Flavonoids are present when the colour is yellow.

3.10.7 Detection of phenolic compound

i. *Ferric chloride test:* To 1 mL of extract. Add a few drops of a 10% $FeCl_3$ solution to this. The presence of phenolic substances was shown by a dark green colour.

3.10.8 Detection of tannins

i. *Braymer's test:* Mix 3 ml of pure water with 1 mL of extract, and then add 3 drops of a 10% ferric chloride solution. The presence of phenolic chemicals was shown by the blue-green colour.

3.10.9 Detection of phlobatannis

i. *HCl test:* Add 2 mL of aqueous extract to 2 mL of 1% HCl and boil them together. There was a red residue that indicated the presence of phlobatannis molecules.

3.10.10 Detection of saponins

i. *Foam test:* The 2 ml of extract was diluted with distilled water and then brought up to a total volume of 10 ml. The suspension was agitated in a graduated

cylinder for a duration of 15 minutes. The detection of saponins was suggested by the presence of a 2-centimetre layer of foam.

3.10.11 Detection of phytosterol

i. *Salkowski's test:* Add 5 mL of chloroform to a 1 mL extract, and treat the sample with a small amount of concentrated solution. The presence of steroids is indicated by the red colour in the bottom layer of H_2SO_4, whereas the creation of a yellow-coloured lower layer shows the presence of phytosterol.

ii. *Libermann-Burchard's test:* To 2 ml of acetic acid, add 2 ml of chloroform. Then, add 1 ml of extract. Finally, slowly add 1-2 drops of concentrated sulfuric acid along the sides of the test tube. Phytosterols can be seen by the different colour changes they cause.

3.10.12 Detection of triterpenoids

i. *Horizon test:* Add 1 mL of extract to 2 mL of trichloroacetic acid.

3.10.13 Detection of lignins

i. *Labat test:* Add gallic acid to the side of a 1 mL extract. The presence of lignins is indicated by an olive-green hue.

3.10.14 Detection of quinines

i. *Alcoholic KOH test:* Mix 1 milliliter of extract with a little amount of alcoholic potassium hydroxide.

3.10.15 Detection of anthraquinones

i. *Borntrager's test:* Add a small amount of filtrate to a 10 mL solution of ammonia that has a concentration of 10%. Shake the mixture vigorously for 30 seconds. The presence of anthraquinones is indicated by the formation of a solution that is pink, violet, or red in colour.

3.10.16 Detection of coumarins

i. *NaOH paper test:* A test tube containing a wet extract is coated with filter paper and treated with 1N NaOH. The mixture is then heated for a short period of time. The emergence of yellow fluorescence from paper when exposed to UV light signifies the existence of coumarins.

ii. *NaOH test:* Add 1 mL of 10% NaOH and 1 mL of chloroform to 1 mL of plant extract. The presence of coumarins was shown by a yellow colour.

3.10.17 Detection of resins

i. *Turbidity test:* The plant extract, dissolved in acetone, was added to 1 mL of distilled water.

3.10.18 Detection of fixed oil and fats

i. *Spot/ stain test:* A small amount of plant extract is placed between two filter sheets and squeezed. The existence of a spot is shown by the oil stain on the paper.

3.11 Microscopic study

The powdered samples were individually affixed to a transparent glass slide using water and then covered with a protective coverslip. The slides were seen using a binocular microscope (Labomed Vision 2000), and the images were captured using a Samsung Galaxy phone.

3.12. Determination of physical characteristics of powder (Kaushik et al., 2011; Madhavi et al., 2019)

3.12.1 Angle of repose

The angle of repose was calculated using the funnel approach. In a funnel, the fifteen grams of powder were allowed to flow until the accumulation of powder reached the tip of the funnel, which was then set above the graph paper on a flat surface. We measured the powder cone's diameter and used the following formula to determine its angle of repose:

$$\theta = tan^{1}\frac{h}{r}$$

h = height of powder cone formed

r = radius of the powder cone formed.

3.12.2 Bulk density

After the powder was sieved through muslin cloth, the apparent bulk density was calculated by pouring 15 grams of powder into a measuring cylinder that was 100 millilitres in capacity without compacting the powder. The original measurement was then recorded. For the purpose of calculating the bulk density, the following formula was utilized:

$$Bulk\ density = \frac{W}{V0}$$

where, W= powder mass, V0= untapped volume

3.12.3 Tapped density

After the bulk density was determined, the cylinder that contained the powder was manually tapped five hundred times until there was no longer any discernible change in volume. The following formula was used to determine the tapped density:

$$Tapped\ density = \frac{W}{Vf}$$

Where, W= mass of powder, Vf = tapped volume

3.12.4 Carr's index

It indicates the characteristics of the powder's flow. The calculation of this value is expressed as a percentage and is determined by employing the following formula (Barbosa-Ferreira et al., 2005):

$$Carr's\ index = \frac{Tapped\ density - Bulk\ density}{Tapped\ density} \times 100$$

3.12.5 Hausner ratio

Powder can be indirectly measured using the Hausner ratio. It was calculated using this formula:

$$Hausner's\ ratio = \frac{Tapped\ density}{Bulk\ density}$$

3.13 Determination of the total phenolic content

Following Singleton and Rossi's (1965) method, Goyal et al., (2010) used a slightly modified Folin-Ciocalteu (FC) reagent to measure the total phenolic content. In brief, 1 ml of 2% Na_2CO_3 solution was added to 0.5 ml of extract after incubating it at room temperature for 5 minutes with 0.5 ml of FC reagent that had been diluted 1:1 with distilled water. After incubating at room temperature for 10 minutes, the absorbance was measured at 730 nm. Every experiment was repeated three times. A monohydrate of gallic acid was used as a control. Grams of gallic acid equivalents (GAE) per 100 g of extract were used to determine the total phenolic content.

3.14 Determination of the total flavonoid content

The quantification of total flavonoid content was conducted using the methodology

described by Goyal et al. (2010), utilizing the aluminum chloride ($AlCl_3$) technique and employing rutin as the standard. The plant extract (0.25 ml) was diluted with 1.25 ml of distilled water (DDW) and then combined with 75 µl of a 5% solution of sodium nitrite ($NaNO_2$). After 5 minutes at room temperature (RT), 0.15 ml of $AlCl_3$ (10%) was introduced. Following a further 6 minutes at ambient temperature, the reaction mixture was subjected to 0.5 mL of 1 mM NaOH. Finally, 275 microliters of distilled deionized water (DDW) were added to the reaction mixture. Following an additional duration of 20 minutes at ambient temperature, the level of light absorption at a wavelength of 510 nm was quantified. The experiments were conducted in groups of three. The quantification of flavonoid content was conducted by employing a rutin standard curve.

3.15. DPPH radical scavenging activity

The extracts and standard were evaluated for their antioxidant activity by measuring their ability to scavenge the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical using the method described by Goyal et al., (2010). A 0.006% w/v solution of DPPH was prepared using a 95% solvent. The PHF-HOPE extract and a 95% concentration of solvent were mixed to create the stock solution (1 mg/ml). The freshly prepared DPPH solution was placed in test tubes, and extracts were added. Subsequently, successive dilutions ranging from 100 to 1000 g were made in each test tube until the total volume reached 2 ml. After an incubation period of 30 minutes in the dark, the degree of discoloration was evaluated at a wavelength of 517 nm using a Themo UV1 spectrophotometer (manufactured by Thermo Electron Corporation in England, UK). Measurements were conducted on a minimum of three occasions. Ascorbic acid served as the standard and was diluted in distilled deionized water (DDW) to create the first solution at a concentration of 1 mg/ml. To serve as a control sample, an equivalent volume was prepared without any extract, and a blank was created using a solvent consisting of 95% concentration. The equation provided was utilized to compute the percentage of DPPH-free radical scavenging.

$$S = \frac{A_0 - A_1}{A_0} \mathrm{X}(100)$$

Where, Ao is the absorbance of the control and A1 is the absorbance in the presence of the sample.

In order to determine if the test chemicals actually reduced absorption, we compared them to the positive controls. Using the dosage inhibition curve, the IC_{50} value was determined.

3.16 Reducing power assay

The reducing capacity of the PHF-HOPE extract was assessed following the method described by Oyaizu (1986), with some modifications. The extract was mixed with different quantities ranging from 250 to 2,500 g in 1 ml of DDW. This mixture was then blended with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$] (1%). The solution was subjected to incubation at a temperature of 50°C for a duration of 20 minutes. The mixture was thereafter subjected to centrifugation at a speed of 3,000 revolutions per minute for a duration of 10 minutes, with the addition of 2.5 milliliters of trichloroacetic acid (10%). The top layer solution (2.5 ml) was combined with DDW (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was determined at a wavelength of 700 nm. The increased absorbance of the reaction mixture showed a greater capacity for reducing substances. Ascorbic acid was used as a reference standard. Phosphate buffer (pH 6.6) was used as a control. The mean absorbance of the final reaction mixture in the two parallel experiments was computed, together with its standard deviation.

3.17 Hydrogen peroxide scavenging

The experiment was conducted with a prexisting methodology with minor adjustments. A portion of H_2O_2 (2 mM) and various sample concentrations (100–1000 g/ml) were mixed together in a ratio of 1:0.6 v/v and left to incubate at room temperature for 10 minutes. After the period of incubation, the measurement of the amount of hydrogen peroxide was done by assessing its absorbance at a wavelength of 230 nm. This measurement was compared to a blank solution that included phosphate buffer but did not contain any hydrogen peroxide. A distinct blank sample was employed for background removal at each concentration. The scavenging activity of hydrogen peroxide in the PHF-HOPE leaf extract was assessed using the following method:

$$S = \frac{A_0 - A_1}{A_0} \mathrm{X}(100)$$

Where, Ao is the absorbance of the control and A1 is the absorbance in the presence of the sample.

3.18 Phytoconstituent profiling of PHF-HOPE extract by GC-MS analysis

The study was carried out using a gas chromatogram-mass spectrometer (Agilent Technologies 7890B GC and Triple Quadrupole Mass Spectrometer 7000D series) coupled with a capillary column (30 m 0.25 mm 1 m) and a blend silica column comprised of 5% biphenyl and 95% dimethylpolysiloxane (Elite-5MS). The test sample was broken down into its individual parts at a pressure of 8.745 psi and steady flow rates of 2.25 mL/min for helium quench gas and 1.5 mL/min for nitrogen collision gas. A 2 µL volume of the 100× diluted sample was injected into the instrument with a split ratio of 10:1 during the chromatographic run, which included an injector temperature of 260°C. The oven's temperature may be set anywhere from 20 to 260 degrees Celsius. In the mass detector, the parameters were as follows: a temperature of 300 °C for the transfer line, 260 °C for the ion source, and 70 eV for electron impact ionization mode. A scan interval of 0.1 seconds and a scan duration of 0.2 seconds were both configured. The mass spectrometer measured the sample's mass from fifty million to one thousand million. The overall operating duration of the GC-MS/MS system was 44 minutes, with a solvent delay of 0 to 2 minutes being employed.

All peaks in the total ion chromatogram had their mass spectra compared to the spectral databases of the NIST 14 MS Library, which has a database of spectra for a huge number of compounds (276,248 compounds) for the purpose of identification. The IUPAC names, molecular formulas, molecular weights, and structures of the metabolites in the High Molecular Polarity Gum Lignin (HMPGL) sample were compared to find out what they were (Usha et al., 2021).

3.19 In vivo studies

3.19.1 Experiment approval

The experimental methods were carried out with the consent of the Animal Ethical Committee (1368/ac/10/CPCSEA/BT-SKM/06) and in strict adherence to the protocol authorized by the Maharani Lakshmi Ammanni College Ethical Committee, Bangalore, for the care and utilization of laboratory animals.

3.19.2 Acclimatization

Veterinary treatment Rats were procured from the Indian Institute of Science in Bangalore, India; they were four-month-old male Wistar albino rats weighing 200–250 kg. Animals were housed at the animal house of MLACW, Bengaluru, for seven days before the experiment. They were maintained in groups of four in cages made

of polypropylene, polycarbonate, or stainless steel. The environmental conditions were as follows: temperature 23 °C, relative humidity 68%–1%, lighting 350 lx, and a 12-hour light/dark cycle. According to Middha et al., (2015) and Middha et al. (2019), the animals in the experiment were provided with ordinary food and plenty of water. The National Institutes of Health (2010) suggested changing the husks and cages every other day to keep the animals dry and clean. The cages measured 421 mm, 290 mm, and 190 mm, with a 7 mm space between the wires. No harmful situations occurred during the daily supervision of the animals' health.

3.19.3 Acute toxicity test

A group of Swiss albino mice weighing between 25 and 30 grams was divided into five groups, each consisting of 10 animals. The animals had an overnight fasting period, during which they were supplied with water prior to the experiment. The experimental groups were administered different doses of HOPE's aqueous extract, ranging from 5.0 to 9.0 g/kg BW/ml. The mice had a 24-hour examination, during which their mortality was recorded. Then, in accordance with the OECD standard, Aliyu and Nwude modified the Karber arithmetic approach to calculate the median lethal dosage (LD_{50}) (Middha et al., 2011). The formula was utilized to calculate the acute toxicity (LD_{50}).

$$LD_{50} = LDy - {}^{I\text{-}r}\sum (Dd \times Md) / n,$$

where LD_y = highest dose and n = number of animals per group (n = 10), Dd = dose difference, Md = mean dead.

3.19.4 Induction of diabetes

Following an overnight fast, rats were given a single intraperitoneal injection of freshly made alloxan monohydrate in citrate buffer (150 mg/mL, 0.1M, pH 4.0) at a volume of one mL/kg. This procedure was done in accordance with Middha et al. (2013)'s protocol, which excluded the normal control group. The animals' diabetes was verified using blood samples taken using the tail vein technique, following two days of alloxan injections. An electronic glucometer called Accu-Chek (Roche, USA) was used to test the fasting blood glucose (FBS).

3.19.5 Diabetic study group

Following habituation, mice were given (150 mg/kg IP) of alloxan monohydrate and then starved for 16 hours to induce diabetes. Hyperglycemic animals were selected for additional experiments when their fasting blood glucose levels were 9.7 mmol/L

or higher. After the animals were given diabetes, they were randomly divided into six groups, as shown below.

Group I Normal control: The normal control group was given one milliliter of saline (NC). Alloxan was not administered to the animals, and they were not even given medicine extract. Instead, they were given a functional diet consisting of chow chow pellets (Lipton India) and water from the tap, which was available to them at any time.

Group II Diabetic control (DC): functioned as a control for diabetes. This group of rats received an intraperitoneal injection of alloxan but did not receive any treatment other than water and rat pellets; they were not given any medication extract.

Group III Low dose PHF-HOPE (200 mg/kg): At a dosage of 200 mg/kg BW (HOPE), the extract was administered to the diabetic rats in Group III.

Group IV high dose PHF-HOPE (400 mg/kg): The extract was administered to diabetic rats in Group IV at a dosage of 400 mg/kg BW (HOPE).

Group V DG: Rats in Group V that had diabetes were given glibenclamide (600 µg/ kg BW) in saline solution every day through an intragastric tube for 45 days (DG).

The technique of weighing the amount of feed and the volume of water was continued for a period of forty-five days from the beginning of the medication administration. Additionally, blood was drawn from the retro orbital puncture on a weekly basis, and the blood glucose level was determined using an Ames One Touch Glucometer (Accu Check, Roche, Germany) (Middha et al., 2019).

3.19.6 Tissue preparation

Following the completion of the trial time, the animals were administered a sedative consisting of a combination of ketamine and xylazine (80–120 mg/kg; 10–16 mg/kg IP) and euthanized using a CO_2 chamber (Conlee et al. 2005). The pancreas was excised, purified, and washed in a saline solution. Subsequently, the sample was measured and mixed uniformly using a 50 mM phosphate buffer with a pH of 7.0. The homogenate underwent centrifugation at a speed of 600 g for a duration of 15 minutes at a temperature of 4°C using the (RV/FM superspin centrifuge manufactured by Platocraft in India). The liquid portion was collected and utilized for enzymatic antioxidant analysis.

3.19.7 Biochemical assays

In this experiment, the activities of various enzymes were assessed using standard

(Amplification diagnostic) kits from Graz, Austria. The primary function of these assays was to evaluate the tissues function and damage. Specifically, liver glycogen, urea, creatinine , the serum enzymatic levels of SGOT (Serum Glutamic Oxalo-acetic Transaminase), SGPT (Serum Glutamic Pyruvic Transaminase), total protein, HDL-cholesterol, LDL-cholesterol, total cholesterol (TC), and triglycerides (TG) were measured to ensures the effectiveness of treatments, accuracy and reliability of the results obtained.

3.19.8 Total protein assay

The total protein assay was done by the method of Lowry et al., (1975), taking BSA as standard. In brief, to the supernatant of 100 µL, 500 µL of alkaline copper sulphate was added and allowed to incubate for 10 minutes at room temperature. After incubation, 60 µL of diluted Folin Catecholamine reagent (1:1) was added and incubated for another 30 minutes at room temperature. The absorbance was measured at 660 nm against the reagent blank.

3.20 Enzymatic antioxidant assays

Studied enzymatic antioxidants involve, Superoxide dismutase (SOD), CAT Catalase assay (CAT) and Malondialdehyde (MDA).

3.20.1 Superoxide dismutase (SOD, EC 1.15.1.1)

Using the technique developed by Misra and Fridovich (1972), the SOD activity was measured at room temperature (RT). Add 100 µL of tissue extract to 880 µL of carbonate buffer at a concentration of 0.05 M, a pH of 10.2, and 0.1 mM EDTA. A spectrophotometer was used to record the change in absorbance for 4 minutes at 480 nm after adding 20 µL of 30 mM epinephrine in 0.05% acetic acid to the mixture. A unit of enzyme is defined as the quantity that inhibits epinephrine auto-oxidation by 50%.

3.20.2 Malondialdehyde (MDA)

The malondialdehyde (MDA) concentration was determined by applying the methodology described by Ohkawa et al., (1979), with 1, 1, 3, and 3-tetramethoxypropane (TMP) serving as the reference standard. Thiobarbituric acid-reactive substances (TBARS) is a spectrophotometric assay that typically measures MDA generation. It measures a chromogen that is formed when MDA reacts with thiobarbituric acid. MDA frequently indicates the rapid oxidation of lipids by reactive oxygen species (ROS). A standard graph was used to show the

concentration in moles of malondialdehyde per milligram of protein, and the final TBA-MDA chromogen was found at 532 nm.

3.20.3 Catalase assay (CAT)

The determination of catalase activity was conducted using the Bears and Sizer technique from (1952). The catalase activity reaction solution consisted of 1 mL of hydrogen peroxide (H_2O_2) at a concentration of 59 mmol/L, diluted in 50 mL of phosphate buffer at pH 7.0. Additionally, 0.1 mL of hepatic supernatant was added to 1.9 mL of deionized water. The variations in the absorbance of the reaction solution at a wavelength of 240 nm were measured at one-minute intervals for a duration of three minutes, utilizing a Kinetics spectrometer. A single unit of catalase activity was defined as a 0.01 absorbance change per minute.

3.21 Histopathology

After the investigation concluded, the whole pancreas of the slaughtered animals was utilized to examine the properties of islet cells using a binocular compound microscope. The organ specimens were preserved in a solution of 10% buffered formalin. The specimens were put in a plastic container and treated in an automated tissue processing machine. The tissues were immersed in liquefied paraffin wax. The trimmed portions were precisely fitted and sliced to a thickness of 5 µ using the rotary microtome. The ribbon pieces were immersed in a buoyant water bath for a duration of 10–15 minutes at a temperature of 40 degrees Celsius. The sections were immersed in xylene and alcohol solutions of increasing concentration and then treated with hematoxylin stain. Following the treatment with 1% acid alcohol and 2% potassium acetate, the slices were then stained with eosin stain. The slides were affixed to the DPX and appropriately labelled. Examined with the pathological binocular microscope.

3.22 Statistical analysis

Data were expressed as means ± SE comparison between different groups was done using one-way ANOVA followed by Turkey's Multiple comparison test's ($p < 0.05$) was considered to be statistically significant.