CHAPTER 4

Materials, methods, instruments, and software used in the experiments

CHAPTER 4

4 Materials and methods

4.1 Chemicals and drugs

Local vendors procured analytical-grade solvents for the investigation from Sigma Aldrich. The substances and medications utilized were ascorbic acid (50-81-7), aspirin (50-78-2), bovine albumin (9048-46-8), and DPPH [1, 1-diphenyl,2-picrylhydrazyl] (1898-66-4). Analytical assay quality was maintained for every chemical used.Toluene, ethyl acetate, and formic acid were obtained from Merck in India, and acetone via Qualigens Fine Chemicals in Mumbai. 3,5dinitro salicylic acid (DNSA), *Aspergillusoryzae*-α-amylase, superoxide dismutase (SOD), streptozotocin, malondialdehyde (MDA), and glutathione peroxidase (GPx) were acquired from Sigma, and Sisco Research Laboratories Pvt. Ltd. provided α-glucosidase (maltase) ex. yeast. Sigma Aldrich, Bangalore, India, provided dimethylsulfoxide (DMSO), rutin, quercetin, and kaempferol. An instrumentation system for CAMAG HPTLCassociated with a LINOMAT 5 applicator, a CAMAG TLC plate-scanner, a (100 µl) syringe, and the winCATS software for analysis were employed. For analysis, a UV-visible spectrophotometer (Shimadzu 1800) was set up.

4.2Selection of plant materials

The use of medicinal plants to treat diabetes is being investigated once more. Certain herbal extracts have been shown to have a hypoglycemic impact in both human and animal diabetic models. Traditional medicinal plant research has been suggested by the World Health Organization Expert Committee on Diabetes [61]. Numerous medicinal plants are believed to be useful in treating diabetes mellitus in traditional systems, according to a thorough review of the literature. Numerous plants have only been studied for their anti-hyperglycemic properties; their effects on the molecular level of diabetes treatment have not been examined. Many plants are only assessed based on their first activity and not their thorough screening.

In light of the current study, the following medical plants have been shortlisted for additional literature review in order to identify 11 medicinal plants for further investigation.

Therefore, our research aims to develop 4polyherbal extract combinationsfrom a total of 11 traditional medicinal plants with anti-diabetic properties, containing in 6 plants in each combination of formulationsin equal proportions. These formulations will consist of ethanolic extracts derived from specific plant parts, namely:

1. The pericarp of matured fruit from *Terminaliachebula* Retz. (from the Combretaceae family)
2. The pericarp of dried fruit from *Terminaliabellerica*Roxb. (from the Combretaceae family)
3. The whole herb of *Andrographispaniculata*Nees. (from the Acanthaceae family)
4. The dried stem of *Berberisaristata* DC. (from the Berberidaceae family)
5. The dried leaves of *Nyctanthesarbor-tristis* L. (from the Oleaceae family)
6. The dried leaves of *Premnaintegrifolia* L. (from the Lamiaceae family)
7. The dried roots of *Cyperusrotundus* L. (from the Cyperaceae family)
8. The dried ripe fruit of *Emblicaofficinalis*Gaertn. (from the Euphorbiaceae family)
9. The dried rhizome of *Picrorhizakurroa*Royle ex Benth. (from the Plantaginaceae family)
10. The dried stem branch of *Tinosporacordifolia* (from the Menispermaceae family)
11. The dried root of *Citrulluscolocynthis* L. (from the Cucurbitaceae family)

4.3 Collection, authentification, and processingof plants source

Selected plant parts were collected from the Banaras Hindu University (BHU) Medicinal Garden in Varanasi. Prof. N. K. Dubey of BHU's Department of Botany performed the taxonomic identification and authentication of the raw materials. Pharmaceutical Engineering and Technology department of IIT, BHU stores vouchers specimens of the plants for future reference. All of the crude materials were washed, air dried in the shade, and coarsely

powdered (Fig. 3) in an electric blender before being packaged in a nonreactive plastic bag and placed in a separate card box at room temperature to be used further.

Figure 3 Coarse powder of ingredient medicinal plant of PHE

4.4 Method of preparation ofextract

The process of alcoholic cold maceration was used to create the extract. In this investigation, every component's crude material was ground into a coarse powder and defatted in a separate manner using hexane in conical flasks with random shaking for a full 72 hours. For that, 400 milliliters of hexane were used to treat around 50 grams of powdered material from each medication. Following defatting, the entire dried (defatted) powdered material was macerated for 72 hours with random shaking in 200 ml of ethanol as the solvent. The substance was filtered, and the residue was then evaporated using rota-vapor to a temperature below 50°C. After the dried extracts were finally complete, they were weighed to determine the yield (percent) precisely and refrigerated at 4°C for later research.

4.5 Phytochemical investigation

4.5.1 Preliminary phytochemical study

Using conventional techniques, a preliminary phytochemical analysis was conducted to ascertain the presence or absence of certain phytochemicals [114]. A range of qualitative tests were performed on all of the ethanolic extracts to determine the category of phytoconstituents.

4.5.2 Quantitative phytochemical study

To detect phytoconstituents, a qualitative analysis will be performed, and the results will thereafter be verified by the use of an appropriate, advanced chromatographic technology. Using accepted techniques, the total phenolic content (TPC) and total flavonoid content (TFC) of each ethanolic extract were calculated.

4.5.3 Total phenolic content determination

The earlier reported Folin-Ciocalteu method[222], was applied to calculate the TPC in all the extracts separately. Folin-Ciocalteu reagent diluted previously (1:1 ratio) with 1ml distilled water was mixed with extract (0.1ml, 1mg/ml) in a test tube. After that, the entire mixture was allowed to incubate for five to eight minutes at room temperature. Following the addition of 2 ml of a 7% sodium carbonate solution, the reaction's volume was kept at 3 ml. After an hour of room temperature incubation, the solution was collected, and the absorbance at 750 nm was measured. To ensure precision, each analysis was carried out three times, and the outcomes were expressed as micrograms per milligram of gallic acid (GAE).

4.5.4 Total flavonoid content determination

The colorimetric technique with $AlCl_3$ from a previous study was used to estimate the TFC [223]in all the extracts separately. A solution of 1 M potassium acetate and 2% AlCl3 were combined with the extract sample (0.1 mL, 2 mg/mL) in the same amount. 2.7 mL of ethanol was added to the mixture to keep its volume at 3 mL. After 30 minutes of incubation at room

temperature, the absorbance at 415 nm was measured using the Shimadzu 1800. Rutin was used as the reference material to compute the TFC.

4.6 Antioxidant capacity of each extract

4.6.1 DPPH free radical scavenging assay

A method previously published [224] with minor modifications was used to assess each extract's capacity to eliminate DPPH free radicals. Three milliliters of a 0.004% methanolic DPPH solution were mixed with varying quantities of extract (2 mg/ml), and the mixture was allowed to sit at room temperature for fifteen minutes. A Thermo Scientific UV1 spectrophotometer was used to measure the absorbance of the produced solution at 517 nm in comparison to the blank (methanol). In this experiment, ascorbic acid, also known as vitamin C, was used as a reference standard. Using the following equation, it was possible to calculate the specific quantity of free radical-suppressive activity that each extract possessed:

$$\text{Suppression activity (\%)} = \frac{\text{Absorbance (reference)} - \text{Absorbance(extract)}}{\text{Absorbance (reference)}} \text{x}100$$

4.7 Preparation of poly herbal extracts (PHE)

The phytochemicals in the therapeutic plants were classified into distinct categories.The medicinal plant extracts were evaluated for their TPC and TFC. Strong antioxidant qualities were demonstrated by these plant extracts when they were assessed using an antioxidant enzyme that is present in humans. These plants, which are part of our traditional knowledge, have long been utilized to treat disorders.Thus, on the basis of the above results of the studied extracts, the extracts were combined randomlyin equal proportion in 4 different formulations of 6 extracts, each taking the concept of triphala, a well-known ayurvedic remedy, to further check the best effectiveness between PHEs. The design of 4 different formulations of the polyherbal extract (PHE) that contains these medicinal plants is given in Table 2.

Table 2 Design of 4 different poly herbal combinations containing 6 medicinal plants each

S.No.	PHE1	PHE2	PHE3	PHE4
1.	*Terminaliachebula*	*Terminaliachebula*	*Terminaliachebula*	*Terminaliachebula*
2.	*Terminaliabellerica*	*Terminaliabellerica*	*Terminaliabellerica*	*Terminaliabellerica*
3.	*Emblicaofficinalis*	*Andrographispaniculata*	*Picrorhizakurroa*	*Picrorhizakurroa*
4.	*Berberisaristata*	*Berberisaristata*	*Tinosporacordifolia*	*Cyperusrotundus*
5.	*Citrulluscolocynthis*	*Nyctanthesarbor-tristis*	*Citrulluscolocynthis*	*Andrographispaniculata*
6.	*Cyperusrotundus*	*Premnaintegrifolia*	*Cyperusrotundus*	*Emblicaofficinalis*

4.8 Polyphenol content, flavonoid content and antioxidant capacity of PHE

PHE 1-4, formulated here, was evaluated for its TPC, TFC, and antioxidant capacity. The methods for these studies were utilized as described earlier for the standardization of extracts in the present study.

4.9 Selection of PHE

On the basis of higher TPC and TFC in PHE 1-4, PHE 2 was selected for further study. The selected PHE 2 in this study was verified by different in vitro as well as in vivo activities. Presence of good amount of TPC and TFC was the sign of higher antioxidant capacity [222] which is consistent with PHE 2 in this study. PHE 2 is replaced as PHE for the further studies shown below:

4.9.1 ICP-MS determination of heavy metals

The PHE was tested using the ICP-MS technique for quantitative assessment of heavy metals such as Pb, Cd, As, Hg, and Ni [225]. The digesting process (Table 3) and Table 4 contain details of the optimal ICP-MS settings for analyzing Pb, Cd, As, Hg, and Ni concentrations.

Table 3 Microwave digestion program for the digestion of PHE sample

Parameters	Steps				
	1	2	3	4	5
Power (%)	90	90	0	0	0
Time (min)	5	15	10	10	10
Temp (°C)	180	180	50	50	50
Pressure (bar)	60	60	50	50	50
Ramp (min)	36	10	10	10	10

Table 4 Operating conditions for ICP-MS determination of Pb, Cd, As, Hg and Ni in digested sample

Parameters	Conditions
RF Generator	40 MHz
RF Power	1000 W
Spray Chamber	Ryton Scott
Nebulizer	Cross-Flow
Plasma gas flow rate	15.0 L/min
Auxiliary gas flow rate	1.0 L/min
Nebulizer gas flow rate	0.60 L/min
Sampler and skimmer cone	Nickel
Resolution	0.7 ± 0.1 amu
Dwell time	250 ms
Sweeps/Reading	20
Reading/Replicates	3

4.9.2 *In vitro* anti-inflammatory activity of PHE

Using human blood RBC wall integrity and albumin denaturation suppression as in vitro models, PHE's anti-inflammatory efficacy was assessed using a slightly modified version of an earlier study [64]. For these investigations, the widely used medication aspirin was used as a baseline.

4.9.2.1 Membrane stabilization assay

Using heparinized centrifugation containers, 10 ml of NSAIDS-prohibited healthy human fresh blood was spun for 10 minutes at 3000 rpm. As soon as the supernatant was removed, a similar quantity of 0.9% saline solution was added, and the centrifuge was run once more. Three times, the identical technique was followed, and the results were the same. Using a 0.9% saline solution, red blood cells (RBCs) were reconstituted into a 10 percent volume-to-volume suspension. Along with the reference, a series of various dilutions of the PHE were taken. In order to conduct the laboratory experiments, the same volumes of RBC suspension with extract, aspirin, and saline solution were employed for the test, reference, and control [64]. For 30 minutes at 56 °C, all of these samples were kept, and afterwards they recovered to room temperature. Pouring the solution into the tubes and rotating them by centrifugation at a setting of 2500 rpm for 5 minutes. Further absorbance at 560 nm was measured and recorded. The membrane stabilization percentage was determined, and 50% inhibition was reported (IC_{50}) using the below equation:

$$\text{Membrane stabilization percentage } (\%) = \frac{\text{Absorbance (reference)} - \text{Absorbance(extract)}}{\text{Absorbance (reference)}} \text{x}100$$

4.9.2.2 Albumin denaturation inhibition

Because it is easily obtainable, bovine albumin (BSA) was employed in this investigation. Additionally, different amounts of PHE (2 ml) and phosphate buffered saline (pH 6.4) were used, with double-distilled (DD) water acting as a control. The ready samples were heated to 70°C for five minutes after being incubated for fifteen minutes at 37°C. After the sample was cooled, absorbance at 660 nm was measured. As a reference drug, aspirin was utilized [64], while albumin and phosphate buffer served as experiment blanks. The 50% inhibition (IC_{50})and percentage of suppression were computed using the following equation:

$$\text{Inhibition } (\%) = \frac{\text{Absorbance (reference)} - \text{Absorbance(extract)}}{\text{Absorbance (reference)}} \text{x}100$$

4.9.3 *In vitro* anti-diabetic activity of PHE

4.9.3.1 *In-vitro*α-amylase inhibition assay

The α-Amylase inhibition assay, known as 3,5-dinitrosalicylic acid (DNSA), was performed as an earlier reported procedure [226]. PHE was initially dissolved in DMSO (10%), and the solution was treated with buffers of Na_2HPO_4 and $NaHPO_4$ (0.02 M) in sodium chloride (0.006 M) at 6.9 pH.After adding 2 units/ml of α-amylase to the 200 µl PHE mixture, it was incubated at 30 oC for 10 minutes.Starch solution (1%, 200 µl) was mixed into each reaction solution in the test tube.After that, the reaction mixture was left to incubate for three minutes at room temperature.The mixture was further heated for 10 minutes in a boiling water bath (set between 85 and 90 degrees Celsius), and the reaction was mixed with DNSA reagent (200 µl). After allowing the reaction mixture to reach room temperature, 5 ml of distilled water was added to dilute it. A UV-visible spectrophotometer (Shimadzu 1800) was used to evaluate the sample and measure the absorbance at 540 nm. In this investigation, an acarbose-containing positive control sample was utilized.

$$\%\ \alpha\ \text{amylase inhibition}\ = 100 \times \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}$$

In order to determine the values for IC_{50}, the percentage of α-amylase inhibition versus the concentration of PHE was plotted.

4.9.3.2 *In-vitro*α-glucosidase inhibition assay

The present investigation employed the methodology expounded by Bhatia et al. (2019) [227] to determine whether or not PHE possessed α-glucosidase inhibitory activity. PHE (50 µL) of varying concentrations (from 20 to 140 µg/mL) was kept at 37 °C for 20 minutes for incubation with the α-glucosidase (maltase) ex. Saccharomyces enzyme solution (1 U/mL) of volume 10 µL, and then a 125 µL solution of 0.1M phosphate buffer was added to the mixture (pH 6.8).Following a 20-minute pause, 20 µL of 1M pNPG (substrate) was added to start the reaction, and the mixture was then given an additional 30 minutes to incubate. After

mixing 50 µL of 0.1 N Na_2CO_3, the reaction was halted. The absorbance of the final solution was then measured at 405 nm using a UV-visible spectrophotometer (Shimadzu 1800). Throughout the trial, acarbose was used as a standard medication at doses ranging from 20 to 140 µg/mL.The following is how the enzyme activity was calculated:

$$\%\ \alpha glucosidase inhibition\ = 100 \times \frac{Abs_{control} - Abs_{sample}}{Abs_{control}}$$

Each unit of the enzyme is the same as the quantity of α-glucosidase that is required to produce a quantity of one micromole per minute of the formula p-nitrophenol, which is synthesized from the substrate p-nitrophenyl-α-D-glucopyranoside. A regression equation was obtained by plotting concentrations with a range of 20-140 µg/mL (on the x-axis) and percent inhibition (on the y-axis) for PHE. This allowed for the calculation of IC_{50}, which is the concentration needed to inhibit 50% of the enzymatic activity.

4.9.4 Anti-hyperglycemic activity through oral glucose tolerance test (OGTT)

OGTT was executedin overnight-starved (14h) rat groups following the protocol described in a previous study with slight modifications [228]. Distilled water, PHE (300 mg/kg), metformin (250 mg/kg), and DMR (1700mg/kg) were administered to four different rat groups, respectively. After 30 minutes of the pretreatment doses, glucose at a concentration of 2 g/kg body weight was given orally. Glucose estimation [229] in the blood samples with the assistance of a glucometer (One Touch Select Plus, Lifescan, Europe) before and after glucose administration was taken from the tail vein at a -30, 0, 30, 60, 90, 120, and 180minute interval.

4.10 Chromatographic and spectroscopic analysis of PHE

4.10.1 GC-MS analysis

GC-MS analysis was carried out [114] at the PHE by means of a 0.2µm filter filtering and dissolving in methanol. On a GC-MS-QP2010 Plus (Shimadzu, Kyoto, Japan) system, a nozzle (AOC-20i) and headspace analyzer (AOC-20s) were utilized for GC-MS. At 230°C

was the ion source, and at 280°C was the contact. At 1000 eV, the solvent termination duration was 3.50 min. It was decided to set the mass range at 40–650 m/z. A 30 m long and 0.25 mm diameter Restek 5 MS capillary column (cross-bond 5 percent diphenyl and 95 percent dimethyl polysiloxane) was utilized for the separation (film thickness). To introduce the sample, the injector was programmed at 260°C, and the split mode was utilized at 10:1.The thermostat was accurately raised to 280°C (15°C/min) (24-minute hold) after being modified from 80°C (2 min) to 250°C (10°C/min). Helium (> 99.999%) was used as the working fluid, moving at 40.5 cm/s. The overall velocity was set at 1.21 mL/min, with a 16.3 mL/min flow rate. A total of 46 minutes were spent recording the MS. The contents were discovered by correlating retention indices (RI) with mass spectral cleavage patterns (Wiley Registry: Mass Spectral Library, 11th Edition) and comparable alkane series (Sigma, St. Louis, USA). When the RI and mass spectra matched nicely, authentication was declared complete.

4.10.2 UPLC-Q-TOF-MS/MSanalysis

Using a previously published technique [111], the produced PHE was examined using ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS) to identify specific flavonoids. Acquity UPLC system (Waters, Milford, MA, USA) with Acquity UPLC BEH C18 column (100mm×2.1 mm, 1.7 μm) was used to separate PHE, and column temperature was kept constant at 25 °C. 0.1% of acetonitrile (B), methanol (C), and formic acid (A) made up the mobile phase. The amount of fluid injected was 5 μl, and the mobile phase flow rate was 300 μl/min. The current investigation was conducted using the same default parameters as the previous study's compound analysis.As previously mentioned, both positive and negative modes of the ReSpect online phytochemical server

(http://spectra.psc.riken.jp/menta.cgi/respect/datail/datail?accession=PT211753) were utilized to obtain the precise mass and molecular formula denomination [230].

4.10.3 UHPLC-HRMS analysis

Analyses were performed using a modified version of the high-resolution accurate mass spectrometry system (HRMS) published by Abu-Reidah [231], and data were collected with the help of a Thermo Scientific Orbitrap Eclipse Tribrid mass spectrometer (MS) running on the small molecule application H-ESI mode. To achieve improved MS detection of ethanol extract derived from PHE, a suitable full MS/ddMS2 (OT HCD) technique was created, which included the use of a UHPLC system (Dionex Ultimate 3000 RS Series). These were the source parameters utilised here, such as formic acid at 0.1 percent in either water (A), acetonitrile (B), or methanol (C) as the mobile phase and a spray voltage of +ve 3.5 kV and -ve 2.5 kV.Additional factors that were corresponding determinants of the current investigation were the ion transfer tube temperature of 325°C, the vaporizer temperature of 350°C, the sheath gas flow rate of 50 arb, the aux gas flow rate of 10 arb, and the sweep gas flow rate of 1 arb.The initial charge state was determined to be 1. MS2 spectra of PHE glycosylated with various sugars were obtained by using the stepped collision energy mode (SCEM) at collision energies of 30, 45, and 60% of the maximumachievable in high-energy collision-induced dissociation (HCD). The data was tracked, processed with Xcalibur 4.1 software from Thermo Fisher Scientific SII, and collected.

4.10.4 Determination of quercetin, rutin and kaempferolby HPTLC

Quercetin, rutin, and kaempferol concentrations in the PHE were determined using the HPTLC technique [232]. HPTLC was carried out on silica gel 60F254, a late-ready-made product available on the market. Both the standard solutions and the sample solution were run on the same chromatographic plate in parallel 8 mm wide bands spaced 30 mm apart and located 10 mm from the plate's bottom line. Methanol, ethyl acetate, formic acid, and water

were mixed to prepare a solution in a proportion of 3:20:1:2 (v/v/v/v), and this solution was utilized as the mobile phase for standard solutions of quercetin and rutin. For the standard solution of kaempferol, a different ratio (4:6:1:6) of chloroform, toluene, formic acid, and ethyl acetate by volume (v/v/v/v) was taken as the mobile phase. After development, plates were air-dried, scanned at 254 nm and 366 nm using WINCAT software in the TLC scanner system, equipped with the deuterium lamp (Table 5). The standards were calibrated using a stock solution (20mg/mL) in methanol. The validity of the method was established according to ICH regulations [233]. LOQ, LOD were calculated, and constituents (quercetin, rutin, and kaempferol) were quantified in PHE using a standard curve.

Table 5 Parameters of HPTLC for quantification of quercetin, rutin and kaempferol in the PHE

S.N.	Parameters	Values
	Calibration parameters	
1.	Calibration mode	Single level
2.	Statistics mode	CV
3.	Evaluation mode	Peak height
	Linomat 5 application parameters	
1.	Spray gas	Inert gas
2.	Sample solvent type	Methanol
3.	Dosage speed	150 nl/s
4.	Predosage volume	0.2 µl
5.	Syringe size	100 µl
6.	Application position	10.0mm
7.	Band length	10.0mm
8.	Solvent front position	50.0mm
	Detection—CAMAG TLC scanner	
1.	Number of tracks	6
2.	Position of track X	15.0mm
3.	Distance between tracks	14.0mm
4.	Scan start position Y	5.0mm
5.	Scan end position Y	85. 0mm
6.	Slit dimensions	6.00 × 0.30 mm, micro
7.	Optimize optical system	Light
8.	Scanning speed	20 mm/s
9.	Data resolution	100 µm/step
	Integration: properties	
1.	Data filtering	Savitsky-Golay 7
2.	Baseline correction	Lowest slope
3.	Peak threshold min. slope	5
4.	Peak threshold min. height	10 AU
5.	Peak threshold min. area	50
6.	Peak threshold max. height	990 AU
7.	Track start position	33.2mm
8.	Track end position	40.5mm
9.	Display scaling	Automatic
	Measurement	
1.	Wavelength	254 nm and 366 nm
2.	Lamp	D2/Hg
3.	Measurement type	Remission
4.	Measurement mode	Absorption/fluorescence
5.	Optical filter	Second order/K400
6.	Detector mode	Automatic
7.	PM high voltage	332 V

4.11 Toxicity study of PHE

4.11.1 Experimental animals and doses

The laboratory animal care and use manual was followed for all experimental rats utilised in this investigation. All experimental protocols were authorized by Banaras Hindu University's Central Animal Ethical Committee (Reg. No. 542/GO/Rebi//S/02/CPCSEA dated 26.5.2017), which provided an approval number (Approval number: Dean/2021/IAEC/2560) for this research.

Toxicity experiments were completed in compliance with the guidelines423 and 407 of the OECD for the assessment of toxicity in animals [234], OECD 2002, and OECD 2008 [235]. Wistar rats aged 6–8 weeks were collected from the research center of the Institute of Medical Science (IMS), Banaras Hindu University, Varanasi, India. The rats had become used to 15 days before experimentation and were kept in specially designed cages with sawdust bedding. The rats were kept in a light-dark cycle for 12–12 hours at room temperature (23–25 °C) with a relative humidity of 55 ± 10%.Throughout the study, the bedding of cages and water bottles were cleaned on a regular basis. The rats were fed a recommended condensed diet and were allowed unlimited access to sterile tap water. To avoid starvation and death, rats were fed multiple times daily (in the morning, then in the afternoon, and at night). Thirst in the rats was induced by prohibiting drinking water, mostly in the early hours, for ease of drug administration via oral gavage. Since the ingredients used have been previously studied for their toxicity profiles and found to be safe at lower doses, OECD guideline 423 required the use of PHE as a kind of limit dose test at 2000 mg/kg body weight [234] OECD 2002; [236]. The dose for the acute toxicity study was calculated using the average weight of the animals as 150 ± 5 g. To obtain a dose equivalent to 2,000 mg/kg as per the OECD of PHE in rats, in one mL of distilled water,300 mg of PHE was added to

formulate the 300 mg/mL of PHE. The three sub acute toxicity doses were selected as per OECD $1/5^{th}$, $1/10^{th}$, and $1/20^{th}$ accordingly from the acute toxicity dose.

4.11.2 Acute oral toxicity assay

The LD_{50} was determined in an acute toxicity assay [234] (OECD 2002) with the two selected groups of 10 Wistar rats (150 g, 5 males and 5 females) in good health as per OECD 423 guidelines. The PHE was given to the rats at a limit of 2000 mg/kg body weight. A single dose of PHE was administered to experimental rats as part of the LD_{50} test, an *in vivo* test that evaluated the lethal dose range for the drug. Except for the normal control group (group I), all five animals in group II got the same amount of PHE. The rats were observed for clinical signs at the beginning of the study and for the next three hours, and then every 48 hours for evaluation of general activity for eyes and mucus membrane, changes in skin and fur, behavior pattern and vocal franticness, touch and tail grip responses, straightening reflex, contortion, force to grasp, posterior train position, body tone along with force to grasp, salivation, auricular reflex, diarrhea, lacrimation, corneal reflex, tremors, ataxia, anesthesia, convulsions, sleep, coma, urination, ptosis, breathing, defecation, cyanosis, piloerection, hyperemia, mortality, hypothermia, illness, or any visible reaction to treatment and death. For a period of 14 days, all experimental rats were kept under close observation.The behavioral changes, the total number of rats that died during that time, and water and food intake were recorded.

4.11.3 Sub-acute toxicity assay

A sub-acute oral toxicity investigation was conducted in accordance with OECD standards 407 [235] OECD 2008.As per guidelines, rats of both sexes (5 female and 5 male rats) were divided into 4 groups of 10 rats each, and weight was measured. One plastic cage was set up for each group to conduct treatment. The rats in group I were given distilled water, while those in groups II–IV received PHE at doses of 100, 200, and 400 mg/kg body weight,

respectively. The dose was given orally to each animal once a day for a total of 28 days.At noon, the doses were administered through oral gavage, and clinical signs were recorded. Toxicity (clinical) signs for all rats were noted at different time intervals of 10, 30, and 60 minutes, then at 4, 8, and finally at 24 hours after appropriate dosage administration. Symptoms and behavioral patterns were documented in a written protocol, along with a list of signs to be looked into further.

4.11.4 Hematological study

All rats were kept overnight for fasting on the 28th day after drug treatment with water *ad libitum*. Using isoflourane, fasted rats were anesthetized, and the retro-orbital piercing technique was used to obtain blood samples into heparinized microhematocrit tubes coated with potassium EDTA and tri-sodium citrate (TSC) to store the sample for further biochemical analysis. The differential leukocyte count (DLC) was determined by staining blood smears prepared from EDTA-containing blood samples with hemocolor quick dyeing of blood smears (E. Merck, Mumbai, India). Automatically, different parameters in a blood sample, like platelet count, platelet distribution width (PDW), hemoglobin (HGB), mean cell hemoglobin concentration (MCHC), hematocrit (HCT), mean red cell volume (MCV), red cell distribution width (RDW),white blood cell (WBC), red blood cell (RBC), and other measurements, were made using an automated hematology analyzer manufactured and designed as a Model PE 6000 by Rapid Diagnostics Pvt. Ltd., New Delhi, India.

4.11.5 Liver function test

Blood samples were put into a semi-automated biochemical analyzer (Model: Benespera C-61, Lablife India Pvt. Ltd., New Delhi) with the help of various biochemical kits (Accurex Biomedical Pvt. Ltd., India) and tested for glucose (FBG), triglycerides (TG), total cholesterol (TC), alkaline phosphates (ALP), aspartate transaminases (AST), alkaline transaminases (ALT), total bilirubin (TBIL), creatinine, urea, protein, and albumin.

4.11.6Histopathology

The rats were sacrificed by decapitation in order to obtain organ weights (heart, liver, kidney, lungs, brains, pancreas, spleen, testis, and ovaries, among others) compared to those of controls. Histopathology examinations required the liver and kidneys to be fixed in 10% formaldehyde in NaCl buffer. Histopathological evidence of the drugs' side effects, in particular their negative effects, was gathered and analyzed. To prepare for microscopic examination, a macroscopic examination was performed first with the specimen fixed and stained with a non-toxic solution. Paraffin embedding was done for the organs extracted for histological study. This was followed by a fixation step that involved section cutting of embedded tissue (4μ size) and staining (hematoxylin and eosin). An optical microscope was used to examine the stained tissues on slides to look for evidence of cellular degeneration, like inflammation and necrosis. The images were captured using a Motic B1 series microscope and scanned using a Moticam 480 micro-camera called Motic Images Plus 2.0,a Multi-Language Application Suite program. Organs from rats that had been treated were studied for histopathological toxicity effects and compared to those from controls [223].

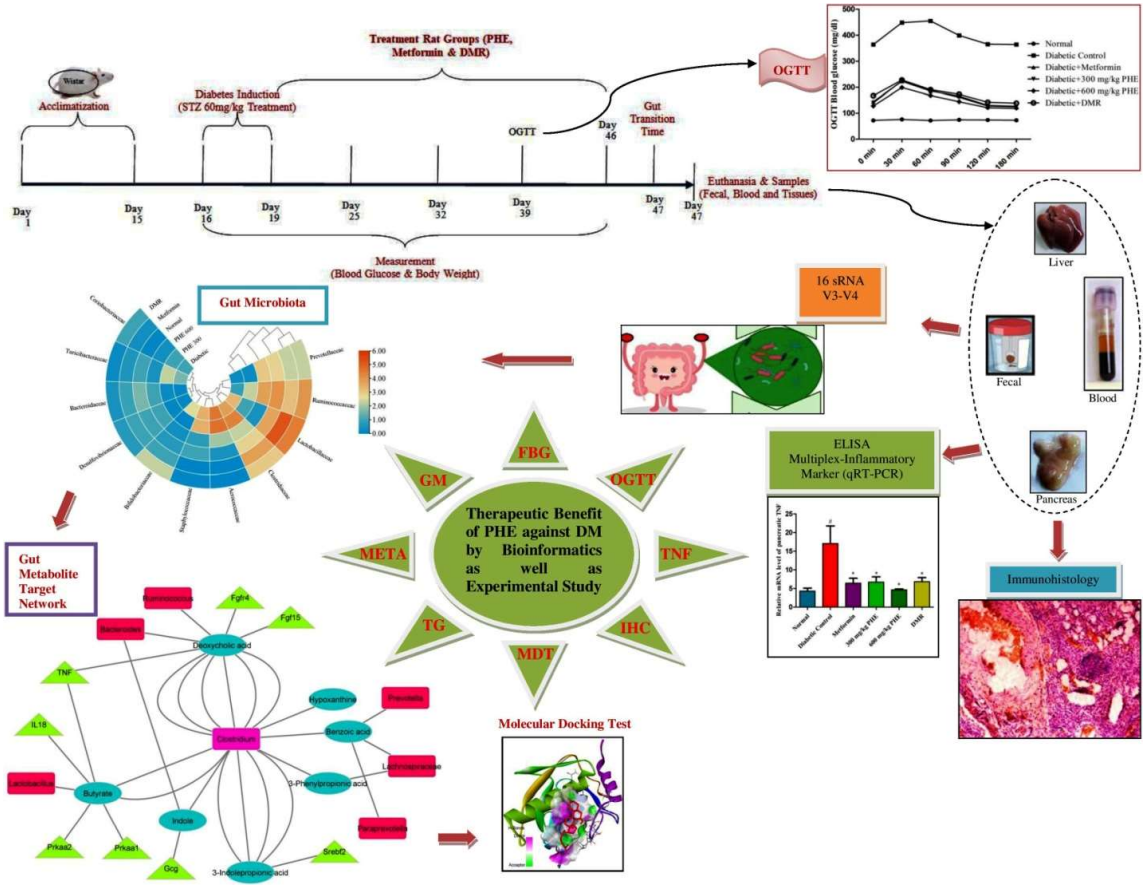

Figure 4 An illustration of the complete treatment plan and outcome of the present study

4.12 Anti-diabetic activity of PHE

4.12.1 Experimental animals and doses

Wistar rats (weighing 180.32g to 230.54g)were collected from the research center of the Institute of Medical Science (IMS), Banaras Hindu University, Varanasi, India (221005). The acclimatization of the collected experimental rats was done for 15 days. Ethical clearance number (Dean/2021/IAEC/2560) was issued by Banaras Hindu University's Central Animal Ethical Committee (Reg. No. 542/GO/Rebi//S/02/CPCSEA dated 26.5.2017)for all experimental protocols in the current work.

Ethno botanical surveys, personal communications with traditional medicine practitioners, and the toxicity study of PHE extrapolated for effective treatment of diabetes through 300 and 600 mg/kg body weight doses. Additionally, metformin (250 mg/kg body weight) and DabarMadhuRakshak (DMR) at a 1700 mg/kg body weight dose as marketed drugs were

used to compare against PHE in the present study. The entire treatment dose is administered once a day through oral gavage.

4.12.2 Diabetes induction, animal group and treatment protocol

The approach outlined in the previous work was used to induce DM in rats [237]. Male Wistar rats (36) were divided into groups of six, with each group having six rats. 12-hour-fasted rats (receiving water only) were tested and recorded for their fasting blood glucose (FBG) levels before administering streptozotocin. Male Wistar rats, except for the normal control, were injected intraperitoneally with 60 mg/kg of streptozotocin incorporated in citrate buffer at pH 4.5. A glucose solution (5%) in two consecutive days was given to establish insulin resistance and escape hypoglycemic shock. After 72 hours of diabetes induction, the FBG levels of all of the rats were tested to make sure that the disease was indeed induced and reported as diabetic rats with an FBG level higher than 250 mg/dl. The following are the rat groupings and treatments:

GP-1: Normal rats + drinking water

GP-2: Diabetic control untreated rats

GP-3: Diabetic rats + 250 mg/kg body weight of metformin

GP-4: Diabetic rats + 300 mg/kg body weight of PHE

GP-5: Diabetic rats + 600 mg/kg body weight of PHE

GP-6: Diabetic rats + 1700 mg/kg body weight of DMR

The duration of the experiment was a total of 28 days. FBG levels were assessed in the tail vein blood on a weekly basis after dose administration. The whole treatment plan for the study was illustrated in Figure 4. Rat weights were taken every seven days during the experiment, and the mean change was determined.

4.12.3 Oral glucose tolerance test (OGTT)of PHE in DMrats

OGTT was performed one week before the experiment ended, following the protocol as stated in a prior study [238]. After the treatment doses, overnight-starved rats were administered glucose at a concentration of 2 g/kg body weight via oral route. After glucose administration, blood samples for glucose estimation with the assistance of a glucometer were taken from the tail vein at 1 minute, 30, 60, 90, 120, and 180 minute intervals.

4.12.4 Body weight, glucose level and biochemical indicesestimation

Every seventh day, the body weight was measured. For blood glucose determination, blood was withdrawn using the tail snipping technique, and the rats' fasting blood glucose (FBG) levels were determined weekly with the use of a glucometer (One Touch Select Plus, Lifescan, Europe) based on the glucose oxidase method [21,238]. Lipid profiles, HbA1c, insulin, and biochemical parameters were estimated using an earlier reported method with slight modifications [21]. Clinical Chemistry AGD 2020 Analyzer, AGD BiomedicalsPLtd., was used with biochemical kits (AGD-Clinipack) for blood parameter analysis. The blood was collected by cardiac puncture of the overnight fasted (16–20 h) euthanized rats (isoflourane at 28 days). Blood was kept in a plain microcentrifuge tube from all the rats for at least 1 hour before centrifugation (3000×g) for 10 minutes. Then, samples of blood were put into a semi-automated biochemical analyzer and tested for various blood parameters like HbA1c, insulin, ALT, AST, ALP, TP, UA, CRE, BUN, TG, TC, HDL, VLDL, and LDL.

4.12.5 Insulin resistance and β-cell function scores

To evaluate the insulin resistance of the sample, the current diagnostic approach employs an important assay, the homeostasis model assessment of insulin resistance (HOMA-IR) [21]. Additionally, the study also stated the β-cell counts (HOMA-β) of the samples obtained from Wilson and Islam's equations to figure out the β-cell in pancreatic tissue.

$$\mathrm{HOMA-IR} = \frac{[\text{insulin } (\frac{\mathrm{U}}{\mathrm{L}}) \times \text{blood glucose } (\frac{\mathrm{mmol}}{\mathrm{L}})]}{22.5}$$

$$\text{HOMA} - \beta = \frac{[20 \times \text{insulin } (\frac{\text{U}}{\text{L}})]}{[\text{blood glucose } (\frac{\text{mmol}}{\text{L}}) - 3.5]}$$

Insulin (1 U/l) is equivalent to 7.174 pmol/l, while one mmol/l of blood glucose is equal to 18 mg/dl. These are the measurement factors for the units.

4.12.6 Organ harvesting and analysis of samples

Isoflourane was used to euthanize the rats after 28 days of treatment. Vital organs were harvested from euthanized animals, and samples were analyzedusing earlier reported methods [21]. Isolated organ tissues were homogenized in phosphate buffer in cold conditions and stored at a temperature of -4 ºC. Oxidative stress indicators were analyzed with homogenized and centrifuged (10 minutes at 3000×g) liver and pancreas tissues. Supernatants obtained after centrifugation wereemployed as a representation for the levelsof reduced glutathione (GSH), superoxide dismutase (SOD) capacities, contents of malondialdehyde (MDA), catalase (CAT), and other biomarkers in the liver and pancreatic tissue [239].

4.12.7 RT-PCR analysis

Total RNA from isolated liver and pancreatic tissues was extracted, followed by cDNA transformation [21]. Frozen (-80 ºC) liver as well as pancreatic tissues were lysed in cool (4 ºC) Trizol reagent (Biochemika), and total RNA was extracted by phase separation method (the Trizol-Chloroform-Isopropanol method). The absolute RNA obtained was distributed in chloroform solvent before being centrifuged at 15,000 rpm for 15 minutes. After that, the pellet was twice cleaned in a 70% ethanol solution (70 ml of pure ethanol to 30 ml of nuclease-free water). The washed pellet was then dissolved in RNA buffer at a pH of 6.4, containing 1 mM sodium citrate. The solution was used for cDNA transformation using the Thermo Scientific cDNA Synthesis Kit. Forward and reverse primers from Sigma in combination with SybrGreen master mix (G Biosciences) for the target genes were used with a Roche 400 light cycler machine. The reaction mixture (20 µl) contained SybrGreen 2x master mix (10 µl), antisense and sense strand binding primers (Table 6) (2 µl), 2 µl of cDNA

(10 ng), and PCR-grade water (6 µl). The 45 cycles of the PCR reaction were set up by one annealing cycle. Negative controls in all of the experiments were used where the mixture contained no cDNA. The Cq value is expressed as a fold change in comparison to the control groups, with the internal control, GAPDH, used to calculate the ΔΔCt value.

Table 6 The antisense and sense mRNA sequences (5'→3') of the primers for RT-PCR

S.No.	Marker	Forward	Reverse
1.	TNF	GCAGATGGGCTGTACCTTATC	GAAATGGCAAATCGGCTGAC
2.	COX-2	GGCCATGGAGTGGACTTAAA	GTCTTTGACTGTGGGAGGATAC
3.	Bcl-2	CATGCGACCTCTGTTTGATTTC	GACCATTTGCCTGAATGTGTG
4.	IL-18	GAATCCCAGACCAGACTGATAAT	GGTAGACATCCTTCCATCCTTC
5.	IL-4	CGGTCTGAACTCACTGAGAAG	GCAAGTATTTCCCTCGTAGGAT
6.	SOX-9	CTGCGACCTCAGAAGGAAAG	CAAATGTGCTTGGGCACTTAC
7.	GAPDH	GGAGAAACCTGCCAAGTATGA	TTGAAGTCACAGGAGACAACC

4.12.8 Histology and immunohistochemistry studies

The isolated pancreas from the animal was immediately preserved in 10% formalin for histological analysis [238]. To randomize the area of the perception zone, the formalin-fixed tissues of the pancreas were chopped into 5 µm-thick sections and fixed with paraffin. A stain with hematoxylin and eosin was done over the paraffin regions of the sections. The fine sections of tissues were subjected to immunohistochemistry [240]. Paraffin was removed from the tissue section, followed by hydration and washing in 0.1M phosphate buffered saline (PBS) for further study. Endogenous peroxidases were neutralized by treating the sample with H_2O_2 and methanol (peroxidase functions as a blocking solution) and then washing in Tris buffered saline (TBS).

The sections were treated with previously diluted primary antibodies (TNF, COX-2, SOX-9, Bcl-2, IL-4, and IL-18) and kept overnight at 4°C. The pieces underwent many washings and buffer rinses.Washed sections were further incubated with Vectastain ABC kits and diaminobenzidinetetrahydrochloride (DAB). After the establishment of the enzyme reaction, to aid in the morphological recognition of cells, the slides were counterstained with

hematoxylin. Section slides were treated with escalating concentrations of alcohol for dehydration, and finally,they were cleaned with xylene. The brownish red color of sections generated by substrate was focused on as immunoreactive areas under a fluorescence microscope by Thermo Fisher Scientific, USA (EVOS® FL cell imaging system).

4.12.9 Quantification of the beta-cell area in the intact pancreas

Pancreas isolated was divided into 150 μm-long, 5μm-thick parts, and the 30th section was stained for analysis. Pancreatic β-cell density was estimated in accordance with the earlier-described approach [241]. β-cell mass accumulation and size were estimated using the open-access software Fiji/ImageJ with Java Plugin, produced by the NIH, USA [241]. To prevent any inaccuracy in the topography measurement, the transverse section of the rat pancreas was inspected in several fields of focus. The total mass of the β-cells was subsequently taken out for the study.

4.13 Gut microbiotadysbiosis study of PHE in diabetic rat model

4.13.1 Fecal DNA extraction and sequencing

By using a DNA extraction kit (ClevergeneBiocorp Private Limited, Banaswadi, Bangalore, India), the complete genomic DNA of microorganisms in the fecal samples of several rat groups was retrieved. The concentrations of the DNA samples were tested for purity using a NanoDrop^{TM} 2000 Spectrophotometer (ND2000, Thermo Scientific). The bacterial 16S rRNA gene was amplified using a forward and a reverse primer like 5'-CCTACGGGNGGCWGCAG-3' and 5'-GACTACHVGGGTATCTAATCC-3', respectively, on the highly variable region V3–V4 using 25 ng of DNA. Designed 341F and 785R primers are present in the process at 100 nm final concentrations, together with KAPA HiFiHotStart Ready Mix [242]. In order to prepare the genome sequencing libraries, an additional 8 cycles of PCR utilizing Illumina barcoded adapters were carried out. The sequence data was

produced by IlluminaMiSeq, and FastQC and MultiQC software were used to assess its quality.

4.13.2 Composition and diversity of the gut microbiota

Alpha and beta diversity parameters were applied to characterize variations in species diversity within as well as between habitats, respectively, in order to conduct an all-encompassing evaluation of species overall diversity. The fecal gut flora was identified by sequencing the 5' and 3' untranslated sections of the 16S rRNA bacterial gene on the IlluminaMiSeq technology. GreenGenes v.13.8-99 [243] was used to filter, analyze, merge, and classify the acquired sequences into taxonomic outlines. Following this, 97% sequence similarity was used to group the contigs into OTUs.

4.13.3 Determination of SCFAs by HPLC in the fecal sample

The method reported by Sassaki was modified to determine the SCFA concentration in the fecal sample [244]. Each of the six groups of rats was sampled for its feces. After centrifuging each feces pellet at 13,000 rpm over 5 minutesat 4°C, we mixed in 1 ml of sterile 18Ω de-ionized water and mixed at 3200 rpm to homogenize the mixture. Whatman™ UNIFLO™ 13mm sterile syringe filters with 0.2µm PES Filter Media were used to collect and filter the supernatant. The filtrate underwent HPLC-based SCFA analysis. Aminex HPX-87H carbohydrate column (4.6 x 250mm cartridge), particle size 4µm, flow rate 0.6 ml/min, run time 60mins, HPLC system (Waters 2000). The eluent employed was 5mM H_2SO_4, and the column temperature was adjusted to 50°C. Injection standards were generated by serially diluting a 10mM stock solution of acetate and propionate and then filtering the resulting solution using Whatman™ UNIFLO™ 13mm sterile syringe filters with 0.2µm PES Filter Media. The concentration of each sample was determined by plotting and analyzing the customary plots. GraphPad Prism 5 was utilized to create the graphs that illustrate the outcomes of the different test groups.

4.14 Bioinformatics study of bioactive compounds in PHE

The list of bioactive phytoconstituents of PHE recognized byUPLC-Q-TOF-MS/MS analysis, UHPLC-HRMS analysis, gas chromatography-mass spectrometry (GC-MS) analysis, and HPTLC technique was utilized in a systematic computational study. Further, the identified bioactive compounds in PHE were predicted for their different biological activities through an in silico approach. Details of the study are given below:

4.14.1 Bioinformatic study for GC-MS identified bioactive compounds

4.14.1.1 Compounds database construction and drug-likeness filtering

For this investigation, the recognized bioactive substances found in PHE were employed. AdmetSAR2.0 (http://lmmd.ecust.edu.cn/admetsar2), MolSoft (http://molsoft.com/mprop/), and ADVERPred (http://www.way2drug.com/adverpred/) were used to calculate ADMET characteristics, drug likeness score, and probable side effects, respectively, in order to predict the ADMET, DLS, and side effects of individual phytoconstituents. The PubChem database (https://pubchem.ncbi.nlm.nih.gov/) was utilized to get the phytoconstituents' SMILES [245]. Alpha tocospiro A (ATA) of PHE was selected for further study on the basis of the above screening results as well as reported biological evaluations in a variety of peer-reviewed publications.

4.14.1.2 Target genes related to selected compounds and DM

Certain databases at web search engines with credentials (http://www.swisstargetprediction.ch/) were using the "*Homo sapiens*" option. One such engine is Swiss Target Prediction (STP),which predicts genes involved in the preferred compounds based on SMILES. Utilizing another database, DisGeNET (https://www.disgenet.org/), DM genes were retrieved by searching websites. The Venny 2.1 tool, online with credentials available (http://bioinfogp.cnb.csic.es/tools/venny/), was

accustomed topicking the genes encoding for compounds. The tool was also used for genes of DM, encoding, recognition, and visualization [246].

4.14.1.3 Network construction of interconnection between active ingredients and overlapping genes

The number of nodes and edges was determined by calculating the correlations among both compounds and the related genes based on STP results and the network visualization created by String (https://string-db.org/). Nodes in the network are representations of the compounds and genes, while associations among both are represented by edges [247]. The important active compounds as well as hub genes of PHE and individual compounds against diabetes were defined by implementing the "degree value" of bioactive compounds and genes acquired by establishing the geometrical features of networks with Cytoscape 3.8.2 [230]. The total number of edges for compounds as well as genes at the network scale is described mostly by the degree value obtained.The higher the degree values of compounds or genes, the greater the therapeutic benefit[248] of the ATA of PHE on diabetes was noted.

4.14.1.4 Pathways and interactive analysis of hub genes

The "*Homo sapiens*" mode of ShinyGO v0.741: “Gene Ontology Enrichment Analysis + More” database, with a search option for genes on the web (http://bioinformatics.sdstate.edu/go/), was used to conduct a KEGG pathway enrichment analysis of intersecting genes recognized from the venn diagram [249]. The results of the KEGG enrichment pathway analysis were used to infer the probable molecular mechanisms of PHE on DM [249]. The lollipop chart of pathways was plotted using the ShinyGO v0.741 “Gene Ontology Enrichment Analysis $^{+}$ More” database to represent the potential molecular mechanism pathways of the ATA of PHE to overcome DM.

4.14.1.5 Prediction of biological activity spectrum

By using the Prediction of Activity Spectra (PASS) database for compounds, it was possible to predict the anticipated biological spectrum of phytoconstituents. The probable functions were denoted by the letters "probable activity" (Pa) and "probable inactivity" (Pi). The activities associated with Pa > Pi were taken into consideration in the current analysis of biological spectrum interpretation [250].

4.14.1.6 Binding affinity energy analysis of the uttermost ingredient on a hub gene by molecular docking test

Autodock 4.2.6 software (http://autodock.scripps.edu/) was utilized to estimate the molecular docking energy of the primary ingredient on hub genes [251-252].

4.14.2 Bioinformatic study for HR-MS identified biomarker compounds

4.14.2.1 Compounds database construction and drug-likeness filtering

The identified biomarkers(andrographolide, apigenin, berberine, chebulic acid, gallic acid, and kaempferol) present in PHE were used for this study. The three prediction programs admetSAR2.0 (http://lmmd.ecust.edu.cn/admetsar2), MolSoft (http://molsoft.com/mprop/), and ADVERPred (http://www.way2drug.com/adverpred/) were used to calculate the ADMET characteristics, drug likeness score, and probable side effects, respectively, for all six of these phytoconstituents. The PubChem database (https://pubchem.ncbi.nlm.nih.gov/) was utilized to get the phytoconstituents' SMILES [245].

4.14.2.2 Collection of target genes related to biomarker compounds and DM

Swiss Target Prediction (STP, http://www.swisstargetprediction.ch/, accessed on November 30, 2023) employed the "Homo sapiens" option. Similarity ensemble approach (SEA, https://sea.bkslab.org/, accessed on November 30, 2023) predicted genes involved in the selected compounds based on SMILES. Traditional Chinese Medicine Systems Pharmacology (TCMSP, https://old.tcmsp-e.com/tcmsp.php, visited on November 30, 2023)

used a chemical name. Diabetes mellitus is the disease name used to search websites for DM genes. These databases, DisGeNET (https://www.disgenet.org/) and GeneCards (https://www.genecards.org/), were used to retrieve DM genes. The genes encoding for chemicals were selected using the Venny 2.1 program, which can be accessed online at http://bioinfogp.cnb.csic.es/tools/venny/ and requires a valid login.The tool was also used for genes of DM, encoding, recognition, and visualization [246].

4.14.2.3 Construction of protein-protein network

Biomaeker compoundtargets and disease targets were intersected using an online platform(Venny 2.1 tool, http://bioinfogp.cnb.csic.es/tools/venny/) to achieve common targetsof biomarker compounds against DM. Then, the common targets were uploaded to STRING(https://string-db.org/) to build the PPI network.The number of nodes and edges was determined by calculating the correlations among both compounds and the related genes based on STP, SEA, and TCMSP resultsand the STRING-created network visualization. Nodes in the network are representations of the compounds and genes, while associations among both are represented by edges [247]. The organism was determined to be "*Homo sapiens,*" with the highest score for interactions of 0.9. Finally,the data generated from STRING was sent online and imagined byCytoscape 3.8.2 software(Cytoscape Consortium, Seattle, WA, USA).

The hub genes of PHE against diabetes were defined by implementing the "degree value (DC), closeness (CC), and betweenness (BC) without weight" of bioactive marker compounds and genes acquired by establishing the topological features of networks with CytoNCA plugged into Cytoscape 3.8.2 [230]. The total number of edges for compounds as well as genes at the network scale is described mostly by the degree value obtained. The higher the degree valuesof genes, the greater the therapeutic benefit [248] of the biomarker compounds of PHE on diabetes was noted.

4.14.2.4 Pathways and interactive analysis of hub genes

An earlier method used in the present study was to conduct a KEGG pathway enrichment analysis of intersecting genes recognized from the venn diagram [249]. The results of the KEGG enrichment pathway analysis and gene annotation were used to infer the probable molecular mechanisms of PHE in DM.

4.14.2.5 Binding affinity energy analysis of the uttermost ingredient on a hub gene by molecular docking test

The Autodock 4.2.6 application (http://autodock.scripps.edu/) was utilized to estimate the molecular binding energy of the biomarker compounds on hub genes [251-252].

4.14.3 Network pharmacology approach via compound-microbiota-signaling pathways-targets-metabolite network analysis

4.14.3.1 Mining of metabolites from gut-microbiota

The metabolites generatedin the gut by the gut microbiota were extracted from the gutMGene v1.0 database utilizing bacterial genera, which was accessed at http://bio-annotation.cn/gutmgene/. Subsequently, the compilation of metabolites was sourced from PubChem, a reputable online database (https://pubchem.ncbi.nlm.nih.gov/). Finally, the beneficial genus of gut microbes was screened on the basis of metabolites useful in the treatment of diabetes, both in animals and humans.

4.14.3.2 Identification of the core gene of metabolite and DM

The targets of screened metabolites were obtained through databases like the similarity ensemble approach (SEA) and Swiss Target Prediction (STP), with logins at http://sea.bkslab.org/ and http://www.swisstargetprediction.ch/.Key target genes of the metabolite were obtained from online databases by selecting the option *Homosapiens*. Overlapped target genes found in the SEA and STP databases were prioritized for further investigation, providing valuable information. In addition, diabetes-related target genes were

taken from disease databases such as DisGeNET and Gene Card with credentials https://www.disgenet.org/ and https://www.genecards.org/, respectively. Metabolite-related targets and diabetes key target genes were overlapped with each other using the web tool Venny Tool 2.1 with credentials (https://bioinfogp.cnb.csic.es/tools/venny/).

4.14.3.3 PPI and compound target networks construction

The PPI network of the top-scored common gene of the gut microbe metabolite and diabetes was drawn by the online database STRING 11.5 with credential https://string-db.org/ and opting "*Homosapiens.*" The whole data set of the PPI network, metabolites, and phytochemicals was used to generate and visualize the compound target network with Cytoscape software.

4.14.3.4 Estimation of the gene ontology (GO) and KEGG pathway

ShinyGo examined the core target genes for gut microbiota metabolites.The core target genes responsible for biological function, cellular component, and molecular function (BF, CC, and MF, respectively) were subjected to KEGG pathway enrichment and GO analysis, which clarified their roles in the functioning of gut microbiota metabolites. To gain a better understanding of the signaling pathways connected to the ultimate objectives of the battle against diabetes, KEGG pathway enrichment assessment was employed.The dotplots were constructed using the fold change value of a screened gene of a gut microbiota metabolite.

4.14.3.5 Molecular docking of the gut microbiota metabolite with core gene

The SDF file of gut microbiota metabolites was extracted from the Pubchem database. It underwent additional conversion to the PDB file format on the Cactus online server (https://cactus.nci.nih.gov/translate/). To download the PDB file format of the key target genes, RCSB (https://www.rcsb.org/) was utilized. Using AutoDock software, a molecular docking analysis was carried out on the gut microbiota metabolite and the key target

gene.The interaction betweengut microbiota metabolites and key target genes was visualized by the Discovery Studio Visualize software.

4.15 Statistical Analysis

The standard error (SE) of the mean was used to report all results. The data analysis of DPPH radical scavenging activity was determined through ANOVA at a significance level of $p<0.05$, if there were any statistically significant differences between the group means, Tukey's post hoc test was employed to find them.The toxicity study of the control groups and test groups was performed through GraphPad Prism 5.The Student's t-test was used in the study to apply data analysis at a significance level of $p<0.05$. Additionally, the Fishers exact test of significance was run to evaluate the impact of each differentially abundant taxon, or OTU. A significant difference was defined as one with a p value of < 0.05. To find out whether there are any connectionsbetween metabolic parameters related to diabetes and gut microbiota, CCA analysis was performed using XLSTAT 2023.03.23.

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