ORIGINAL ARTICLE

A Dual Therapeutic Approach to Diabetes Mellitus via Bioactive Phytochemicals Found in a Poly Herbal Extract by Restoration of Favorable Gut Flora and Related Short-Chain Fatty Acids

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Abstract

Diabetes mellitus (DM), a metabolic and endocrine condition, poses a serious threat to human health and longevity. The emerging role of gut microbiome associated with bioactive compounds has recently created a new hope for DM treatment. UHPLC-HRMS methods were used to identify these compounds in a poly herbal ethanolic extract (PHE). The effects of PHE on body weight (BW), fasting blood glucose (FBG) level, gut microbiota, fecal short-chain fatty acids (SCFAs) production, and the correlation between DM-related indices and gut microbes, in rats were investigated. Chebulic acid (0.368%), gallic acid (0.469%), andrographolide (1.304%), berberine (6.442%), and numerous polysaccharides were the most representative constituents in PHE. A more significant BW gain and a reduction in FBG level towards normal of PHE 600 mg/kg treated rats group were resulted at the end of 28th days of the study. Moreover, the composition of the gut microbiota corroborated the study's hypothesis, as evidenced by an increased ratio of *Bacteroidetes* to *Firmicutes* and some beneficial microbial species, including *Prevotella copri* and *Lactobacillus hamster*. The relative abundance of *Bifidobacterium pseudolongum*, *Ruminococcus bromii*, and *Blautia producta* was found to decline in PHE treatment groups as compared to diabetic group. The abundance of beneficial bacteria in PHE 600 mg/kg treatment group

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was concurrently associated with increased SCFAs concentrations of acetate and propionate (7.26 nmol/g and 4.13 nmol/g). The findings of this study suggest a promising approach to prevent DM by demonstrating that these naturally occurring compounds decreased FBG levels by increasing SCFAs content and SCFAs producing gut microbiota.

Graphical Abstract

Flow chart summarizing research on the dual therapeutic approach to diabetes mellitus via bioactive chemicals found in a poly herbal extract and the management of gut microbiota in relation to DM. (SCFAs, short chain fatty acids; SMB53, a genus of bacterial microbiota of small intestine; LPS, lipopolysaccharide)
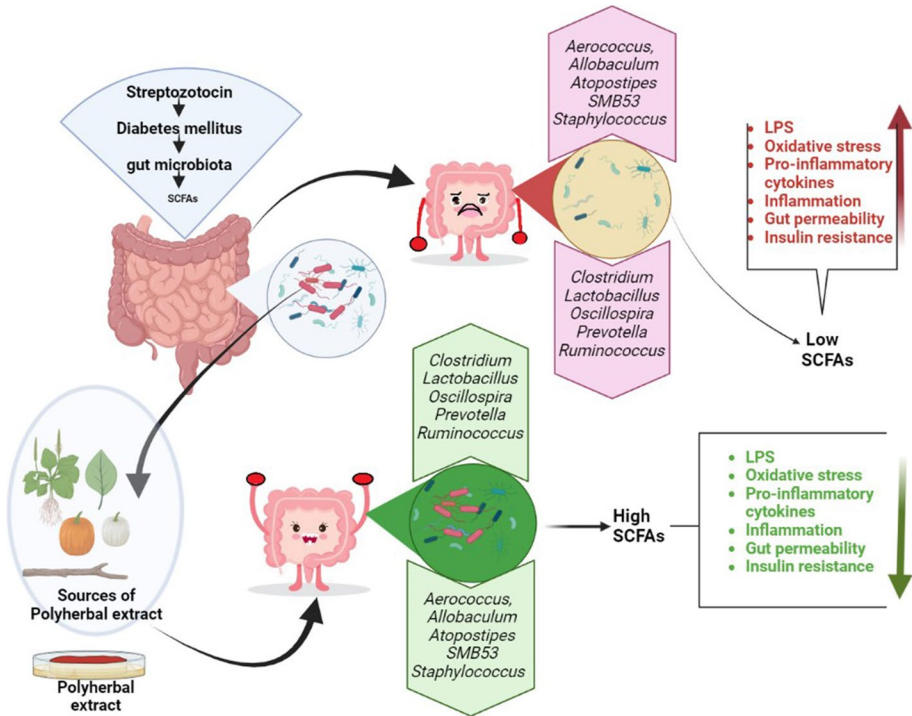
Introduction

Diabetes mellitus (DM) is a serious and complicated metabolic condition that lasts for a long time [1]. There are currently over 536.6 million people with diabetes globally, despite the availability of numerous therapeutic approaches, according to a recent report released from the International Diabetes Federation for 2022 [2]. A recent study predicts that without quick and efficient interventions, the occurrence of diabetes will grow sharply, reaching 11.3% (642.7 million individuals) by 2030 and 12.2% (783.2 million people) by 2045 [2]. The prevalence of diabetes in India is rising rapidly, and it now affects approximately 8.7%

of people aged 20–70. A longer life expectancy, unhealthy lifestyles, poor diets, smoking, and rapid urbanization are all factors in the rise in diabetes cases [3]. It costs at least $966 million a year, which is 9% of all global health spending [4]. Drugs like pioglitazone, acarbose, metformin, and others [5], which are sold on the market, can help prevent diabetes. The potential adverse side effects of these anti-diabetic medicines, however, must not be neglected [6]. Innovative, effective, and secure anti-diabetic medications are desperately needed to combat the rising prevalence of diabetes. Therefore, finding alternative active substances to treat this condition that are non-toxic and have fewer side effects is an urgent priority.

Ayurveda is recognized as one of the oldest of the traditional systems of medicine (TSMs) accepted worldwide because it has drawn considerable attention in recent years due to its holistic approach to disease management [7–9]. Ancient Indian medicines, which are defined through systematic and fundamental regulation by the utilization of multiple medicinal plants and their polyherbal formulations, contain multiple active components, act through multiple pathways and targets, and thus have an increasing impact on the development of anti-diabetic drugs [10–13]. Here we texted some of the medicinal plants with reported anti-diabetic activity potential (*Aegle marmelos*, *Andrographis paniculata*, *Citrullus colocynthis*, *Gymnema sylvestre*, *Momordica charantia*, *Ocimum sanctum*, *Swertia chirayita*, *Trigonella foenum graceum*, etc.) [9]. The Indian traditional medical system has developed several effective anti-diabetic medicines for the treatment of diabetes in accordance with advances in medicinal technology [12], for example, Madhumeh churna, Triphala churna, Vilva kudineer, Diabet, Diasol, Dianex, Diakyur, Diasulin, Diabecure, and others [3, 9]. Here, we formulated the PHE from our earlier study that summarizes the pharmacological actions like antimicrobial, anti-inflammatory, and antidiabetic, as well as the antioxidant potential that would be due to the bioactive molecules of the ingredient plants [3]. The crude source, short name, and family of six medicinal plants in PHE are illustrated herewith, with their voucher specimens granted: fruits of *Terminalia chebula* Retz. (TC), Combretaceae (No. Combreta. 2020/02); fruits of *Terminalia bellerica* (Gaertn.) Roxb. (TB), Combretaceae (No. Combreta. 2020/02); whole herb of *Andrographis paniculata* (Buem.f.) Nees. (AP), Acanthaceae (No. Acantha. 2020/01); stem of *Berberis aristata* DC. (BA), Berberidaceae (No. Berberida. 2020/02); leaves of *Nyctanthes arbor-tristis* L. (NA), Oleaceae (No. Olea. 2020/01); and leaves of *Premna integrifolia* L. (PI), Lamiaceae (No. Lamia. 2020/01) [3].

Modern metabolic profiling techniques are highly sensitive and selective, allowing for the successful acquisition of novel insights into the medicinal compounds of plants [14]. Metabolomics, which is defined as both the quantitative and qualitative examination of all small metabolites in a biological specimen whose presence is influenced at a specific time by specific conditions [15], grows our understanding of daily life by highlighting how the variety of chemicals in herbal remedies is still far beyond the researchers' thoughts, even on plant matrices, which are thought to have already undergone extensive research [16]. Therefore, in this paper, ultra-high-performance liquid chromatography (UHPLC) techniques were applied to PHE in conjunction with a high-resolution accurate mass spectrometry system (HRMS), as the examination of the chemical constituents of plant extracts stands as an important component for understanding their bioactivity, both in terms of efficacy and safety. The method has the potential to be much more sensitive than the current standard for disclosing secondary metabolites because of its ability to simultaneously measure mass with high precision and identify characteristic fragments. The phytoconstituents may influence our body physiology through various mechanisms, even through modulation of the gut microbiota and

the synthesis of micronutrients [17]. The gut microbiome is made up of eukaryotes, archaea, and bacteria. Bacterial phyla are the most common type [18]. Maintaining the diversity and abundance of the human gut microbiota has been found to be important for maintaining intestinal homeostasis [19, 20]. This is because the microbiota protects the host from pathogens by competing for niches and nutrients, boosting immune functions, and regulating metabolism. The gut microbiota is a dynamic ecosystem that is constantly changing and interacting with the host in a mutualistic manner. It may, in fact, have an impact on a number of physiological processes linked to human health, including immune system function, defense against infections, synthesis of micronutrients, and control of glucose and fat metabolism [17]. Therefore, modulating the gut microbiota may be an effective means of warding off metabolic and inflammatory disorders. Obesity, beta-cell exhaustion, insulin resistance, and T2DM are all conditions that have been linked to changes in the gut microbiota, according to animal studies [21]. Furthermore, observational investigations have demonstrated that there is evidence of altered microbial communities in the gut in prediabetic or DM patients in comparison to healthy subjects, a condition referred to as "gut microbiota dysbiosis" [22, 23]. Furthermore, "gut microbiota dysbiosis" may disrupt the equilibrium between commensal species and different types of pathogens, and it may also reduce the release of metabolic SCFAs and antimicrobial molecules like bacteriocins [24]. However, there is now more evidence on the activities of microbial species associated with the gut microbiota's protective effect against DM as a metabolic disorder [25].

The management of DM through gut microbiota may be based on the following mechanisms: (1) keeping the intestinal barrier strong; (2) stopping the movement of bacteria and, as a result, reducing systemic inflammation (endotoxemia); and (3) generation of SCFAs (acetate, propionate, and butyrate), which can change metabolic pathways [26]. In addition to SCFAs and bacterial toxins, the microbiota may also affect the gut barrier through the endocannabinoid system [27, 28] and the metabolism of bile acids (BAs) [27]. There is growing evidence that DM is strongly linked to gut microbiota dysbiosis, damages the gut wall, and encourages the movement of endotoxemia from the gut into the bloodstream, which leads to inflammation, autoimmune responses, and oxidative stress [29, 30]. Numerous studies showed that people with different types of DM had an increased abundance of *Firmicutes* and a decreased abundance of *Bacteroidetes*, which had a negative impact on their metabolic regulation of nutrients, their glucose tolerance, and their inflammatory responses [31]. Anti-inflammatory SCFA levels, specifically butyrate, were lower in people with diabetes because they had a decreased abundance of SCFA-producing microorganisms such as *Roseburia intestinalis* and *Faecalibacterium prausnitzii* [30, 32, 33]. The mechanism of action of SCFAs mainly depends on the intestinal bacteria, as illustrated in the flow chart figure, which may be more likely to enter the systemic circulation if SCFA synthesis is impaired, which may trigger or exacerbate integrated inflammatory responses [34]. Positive effects of SCFAs for diabetics include lowering serum glucose levels, improving insulin sensitivity, decreasing inflammation, and increasing the production of the protective hormones glucagon-like peptide 1 (GLP-1) [35] and peptide YY (PYY) that increase satiety feelings through the gut-brain axis [36]. In the liver, SCFAs have been shown to decrease glycolysis and gluconeogenesis and increase glycogen synthesis and fatty acid oxidation. Additionally, SCFAs have been shown to improve glucose uptake in skeletal muscle and adipose tissue by elevating the expression of GLUT4 through AMP kinase (AMPK) activity. Moreover, in the skeletal muscle, SCFAs reduce glycolysis with a consequent accumulation of glucose-6-phosphate and an increase in glycogen synthesis [36].

It makes sense to think that these results will help us understand how these bioactive compounds work together to prevent DM. They will likely provide strong evidence for at least some of the metabolic diseases that this PHE could potentially prevent. In this study, we used high-throughput 16S rRNA gene sequencing to look at how PHE affected the gut microbiota of DM rats and find key genera that were closely linked to PHE treatment. This helped us figure out if the gut microbiota was an appropriate target for PHE during the improvement of DM symptoms. Additionally, we correlated diabetic parameters with gut microbiota enrichment linked to increased SCFA content in order to ameliorate DM.

Materials and Methods

Chemicals and Drugs

Details of all the chemicals utilized for the preparation of polyherbal extract (PHE) and further required chemicals and drugs for evaluations were described in our previous study [3]. The analytical-grade solvents used in the study were bought from Sigma Aldrich through local vendors.

Preparation and Evaluation of PHE

All the crude materials for the preparation of PHE were scientifically collected from the medicinal garden at BHU, Varanasi. The crude materials were identified and authenticated taxonomically. The preparation steps, preliminary qualitative phytochemical tests, GC–MS fingerprint analysis of the prepared PHE, and their in-vitro anti-inflammatory activity with potential antioxidant values were reported in our previous study [3]. The already-prepared PHE from the previous study was used for the present study as well.

UHPLC-HRMS Analysis

Analyses were performed using a modified version of the high-resolution accurate mass spectrometry system (HRMS) published by Abu-Reidah [37], 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 utilized 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. Another factor, like the ion transfer tube temperature of 325 °C, the sheath gas flow rate of 50 arb, the vaporizer temperature of 350 °C, the aux gas flow rate of 10 arb, as well as the sweep gas flow rate of 1 arb, were also the corresponding determinants of the present study. 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 maximum achievable in high-energy collision-induced dissociation (HCD). The data was tracked, processed with Xcalibur 4.1 software from Thermo Fisher Scientific SII, and collected.

Ethical Approval

The laboratory animal care and use manual was followed for all experimental rats utilized in this investigation. All experimental protocols were approved by the Central Animal Ethical Committee of Banaras Hindu University (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.

Induction of Diabetes Mellitus

Rats were induced with DM using the established animal model outlined by Hilary [38]. Male Wistar rats ($n = 36$) weighing between 180.32 and 230.54 g were acclimated and then starved on water alone for 12 h. Prior to receiving streptozotocin, the rats' fasting blood sugar levels were measured and recorded. After that, we induced insulin resistance in all rats except normal group rats by administering for 24 h orally a 5% glucose solution to prevent mortality in rats after an I.P. injection of streptozotocin single at pH 4.5 dissolved in citrate buffer at a dose of 60 mg/kg. After 72 h of inducing DM in rats, we tested their FBG levels to confirm the diagnosis. Diabetic rats had a FBG level higher than 250 mg/dl.

Animal Groups and Treatment Protocol with PHE

Thirty-six male Wistar rats chosen in this study were divided into six groups of six. These classifications were created below:

GP-1: Normal rats + drinking water
GP-2: Diabetic control untreated rats
GP-3: Diabetic rats + 250 mg/kg BWt of metformin (oral gavage)
GP-4: Diabetic rats + 300 mg/kg BWt of PHE (oral gavage)
GP-5: Diabetic rats + 600 mg/kg BWt of PHE (oral gavage)
GP-6: Diabetic rats + 1700 mg/kg BWt of DMR (oral gavage)

Each rat group ($N = 6$)'s feces was collected at the completion of the entire study and frozen at -80 °C for later assessment. Image (Fig. 1) shows the treatment plan of the study.
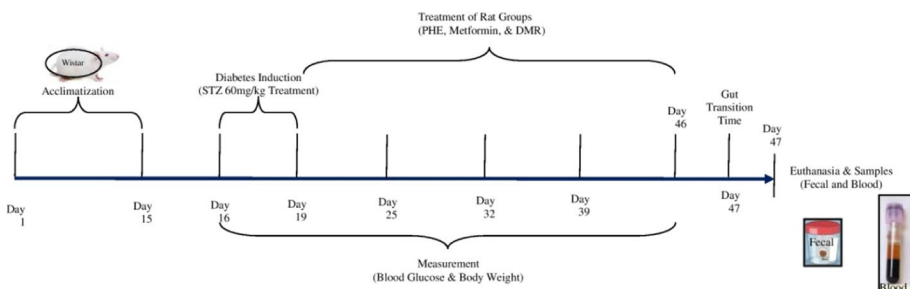

Fig. 1 Illustrated image shows the treatment plan of the study

Body Weight and Glucose Estimation

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) concentrations were measured weekly using a glucometer (One Touch Select Plus, Lifescan, Europe) based on the glucose oxidase method [39].

Fecal DNA Extraction and Sequencing

By using a DNA extraction kit (Clevergene Biocorp 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™ 2000 Spectrophotometer (ND2000, Thermo Scientific). The bacterial 16S rRNA gene was amplified using a forward and a reverse primer like 5′-CCTACG GGNGGCWGCAG-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 HiFi HotStart Ready Mix [40]. 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 Illumina MiSeq, and FastQC and MultiQC software were used to assess its quality.

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. Alpha diversity refers to diversity on a local scale, describing the species diversity (richness) within a functional community. Beta diversity, on the other hand, describes the amount of differentiation between species communities. Different indices are used to characterize the alpha diversity of the gut microbiota. In this study, species richness was characterized by the Chao1 estimator, the ACE estimator, and observed species indices; species diversity was characterized by the Shannon, Simpson, InvSimpson, and Fisher indices. The fecal gut flora was identified by sequencing the 5′ and 3′ untranslated regions of the 16S rRNA gene of bacteria on the Illumina MiSeq technology. GreenGenes v.13.8–99 [41] 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.

Determination of SCFAs by HPLC in the Fecal Sample

The fecal sample SCFA concentration was determined using a modified version of the procedure described by Sassaki [42]. Each of the six groups of rats was sampled for its feces. After centrifuging each feces pellet at 4 °C over 5 min at 13,000 rpm, we mixed in 1 ml of sterile 18Ω de-ionized water and mixed at 3200 rpm to homogenize the mixture. Whatman™ UNIFLO™ 13 mm sterile syringe filters with 0.2 μm PES Filter Media were used to collect and filter the supernatant. The filtrate underwent HPLC-based SCFA analysis.

Aminex HPX-87H carbohydrate column (4.6×250 mm cartridge), particle size 4 μm, flow rate 0.6 ml/min, run time 60 min, HPLC system (Waters 2000). The eluent employed was 5 mM H_2SO_4, and the column temperature was adjusted to 50 °C. Injection standards were generated by serially diluting a 10 mM stock solution of acetate and propionate and then filtering the resulting solution using Whatman™ UNIFLO™ 13 mm sterile syringe filters with 0.2 μm PES Filter Media. The concentration of each sample was determined by plotting and analyzing the customary plots. GraphPad Prism 5 was used to generate the graphs representing the results of the various test groups.

Statistical Analysis

GraphPad Prism 5 was used for statistical analysis. With the help of Tukey's test and one-way analysis of variance (ANOVA) for various comparisons, the significance of differences between groups was examined. Additionally, the Fishers exact test significance was run to evaluate the impact of each differentially abundant taxon, or OUT. A significant difference was defined as one with a p value of < 0.05. To find out whether there are any connections between metabolic parameters related to diabetes and gut microbiota, CCA analysis was performed using XLSTAT 2023.03.23.

Result

Bioactive Components and UHPLC-HRMS Metabolite Profiling of PHE

Through early qualitative tests and GC–MS analyses, it was determined that PHE exhibited synergistic antioxidant capacity as well as strong in vitro anti-inflammatory activity because of the presence of several bioactive components in our previous study [3]. Therefore, PHE can be utilized as a complementary therapy for the treatment of inflammatory conditions and metabolic diseases like diabetes. Through UHPLC-HRMS analysis of bioactive chemicals and regulation of gut bacteria and SCFAs in diabetic rats, we were able to extend the results of prior work on PHE.

Metabolic profiling led to the identification of numerous phytochemicals in PHE, including phenolic acids, organic acids, flavonoids, triterpenes, fatty acids, sesquiterpenes, coumarins, lignans, and sugars (Supplementary Table S1). Annotation sources included predicted composition, mzCloud search, chemSpider search, and massList search (Arita Lab 6549 Flavonoid Structure Database). The UHPLC-HRMS metabolite fingerprint revealed numerous bioactive compounds provided in the supplementary file along with the article. Out of 1759 screening checks, flavonoids were identified in 361 checks, with 9.9% calculated on the basis of the total peak percent area (Supplementary Table S1). Biomarker compounds like chebulic acid (0.368%), gallic acid (0.759%), ellagic acid (0.469%), andrographolide (1.304%), berberin (6.442%), kaempferol (0.069%), quercetin (0.020%), apigenin (0.040%), and other bioactive compounds like luteolin (0.034%), oxyberberine (0.141%), rutin (0.017%), genistein (0.022%), ferulic acid (0.148%), hesperidin (0.336%), and isorhamnetin (0.015%) were identified in good proportion from PHE shown in supplementary Table S1. The total metabolite profile of PHE was expressed in a total ion chromatogram at the various retention times (RT) for bioactive compounds obtained in both the negative and positive modes of analysis (Fig. 2). In this study, MS chromatograms with MS1 and MS2 peaks of well-known biomarker compounds like chebulic acid
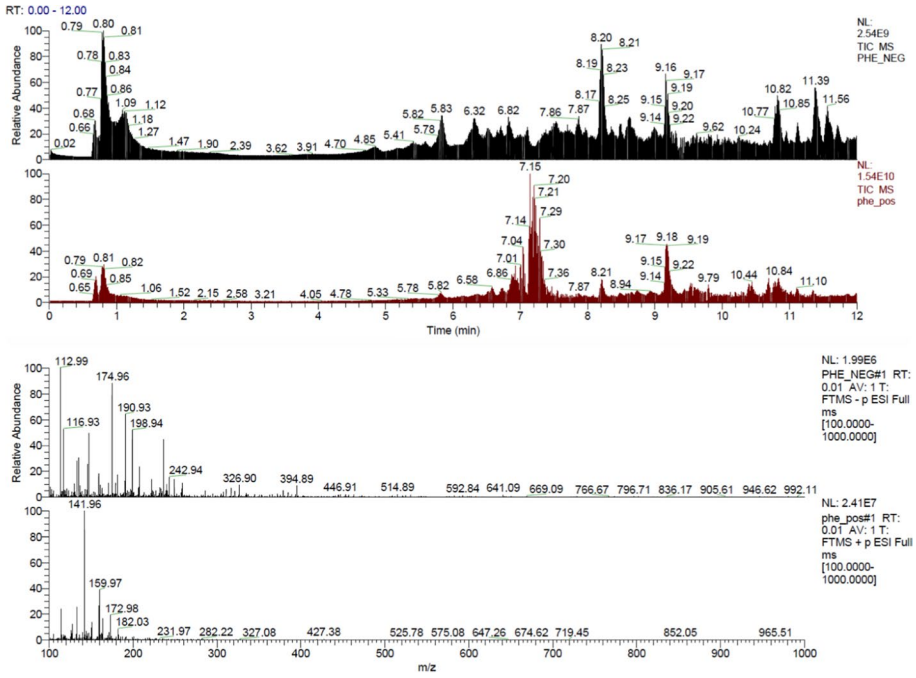
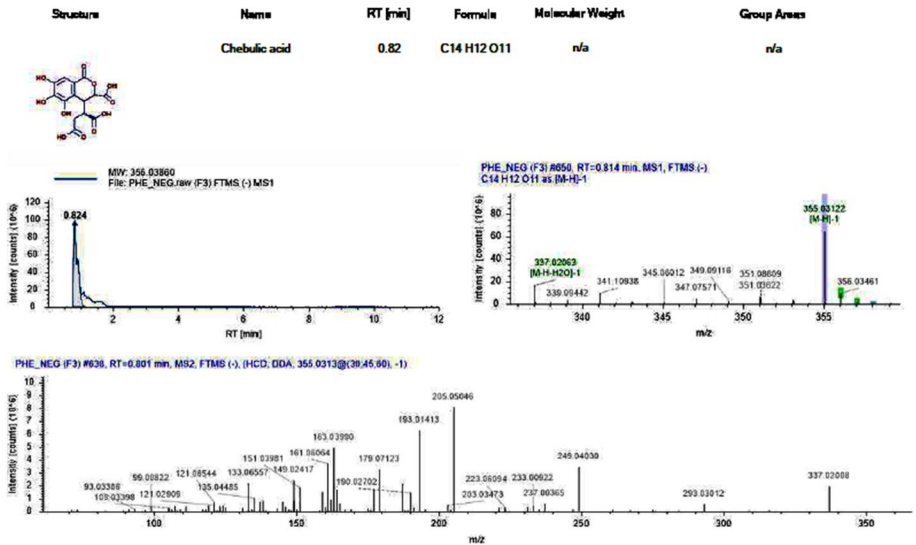**Fig. 2** Chromatogram of whole metabolites from PHE with relative abundance

Fig. 3 Identification of chebulic acid (C14H12O11) in full scan MS of negative ionization mode with raw, MS1, and MS2 Chromatograms
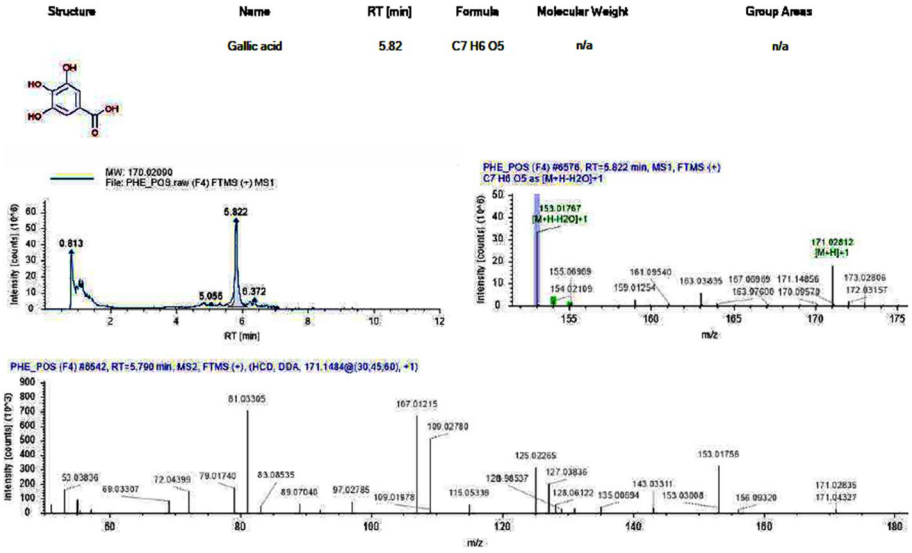
Fig. 4 Identification of gallic acid (C7H6O5) in full scan MS of negative ionization mode with raw, MS1, and MS2 Chromatograms
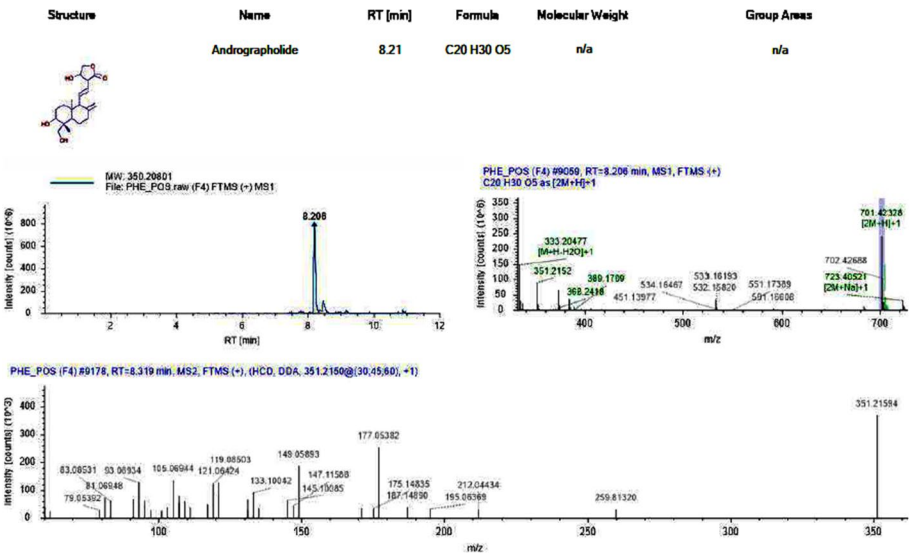

Fig. 5 Identification of andrographolide (C20H30O5) in full scan MS of positive ionization mode with raw, MS1, and MS2 Chromatograms

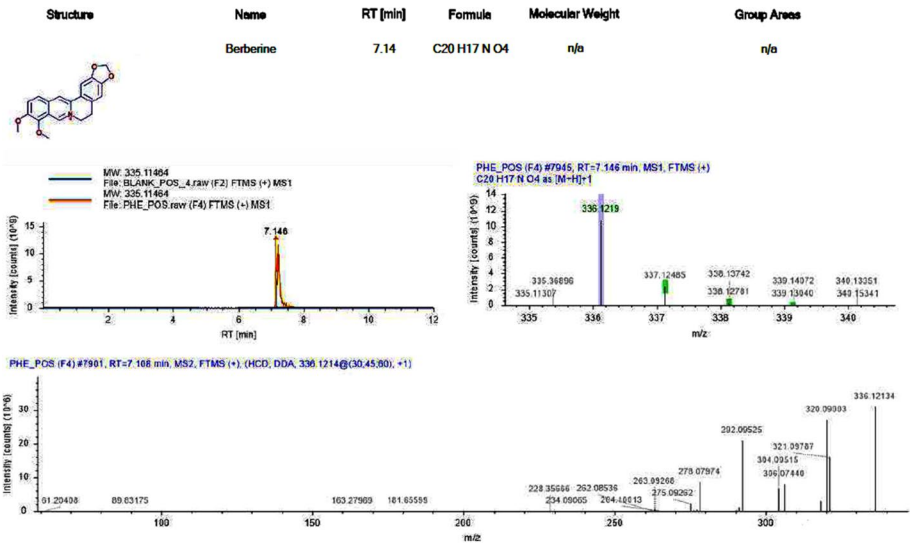**Fig. 6** Identification of berberine (C20H17NO4) in full scan MS of positive ionization mode with raw, MS1, and MS2 Chromatograms
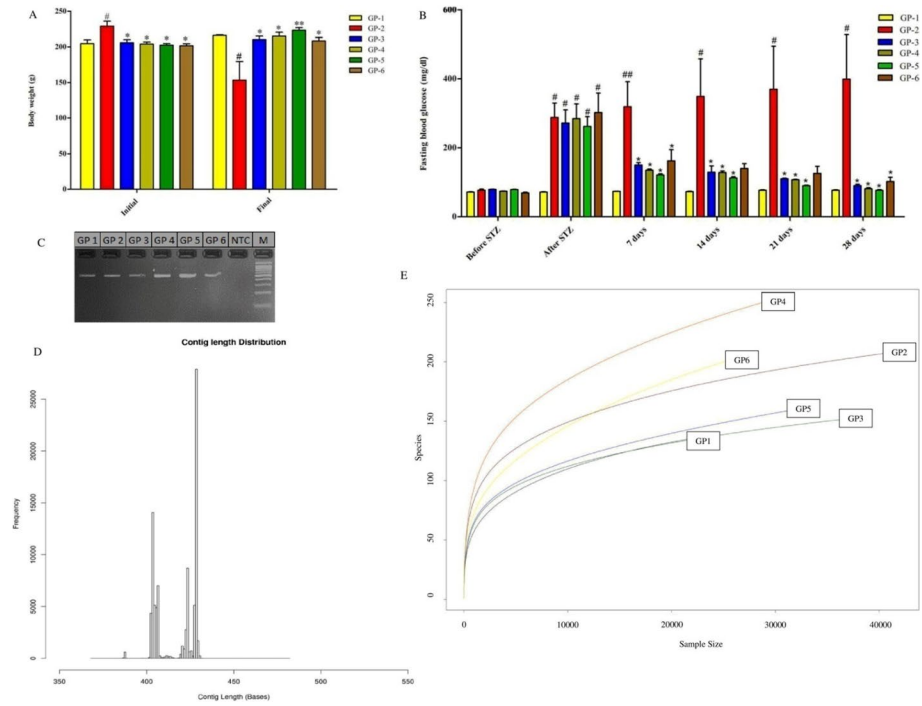

Fig. 7 Anti-diabetic effect of PHE on **A** body weight and **B** fasting blood glucose levels. Histogram of sequence diversity. **C** Gel image showing the amplicon size along with 100 bp ladder, **D** contig length distribution, and **E** rarefaction curve

(Fig. 3), gallic acid (Fig. 4), andrographolide (Fig. 5), and berberine (Fig. 6) were of particular interest.

Body Weight of Diabetic Rats Administered PHE

The animals' body weights dropped significantly after streptozotocin treatment (Fig. 7A), in contrast to the normal control group, which gained weight. Diabetic rats treated with PHE at doses of 300 mg/kg and 600 mg/kg gained weight. The body weight of GP-4 and GP-5 rats increased more significantly than that of GP-3 and GP-6 rats (Supplementary Table S2). Diabetic rats exhibited a therapeutic change, as evidenced by a dose-dependent alteration in body weight across treatment groups.

Fasting Blood Glucose Level

Male rats in the untreated group had FBG levels above 250 mg/dl for the entirety of the experiment, even after 72 h of streptozotocin induction (Fig. 7B). PHE at 300 mg/kg, 600 mg/kg, metformin at 250 mg/kg, and DMR at 1700 mg/kg body weight all reduced FBG levels, as seen in Supplemental Table S3. FBG levels were reduced to levels similar to those in the non-diabetic group in the groups of rats given PHE at 300 mg/kg and 600 mg/kg, as well as metformin and DMR (Fig. 7B). PHE was found to be more effective than metformin and DMR in lowering FBG levels in the present animal models. At 600 mg/kg of body weight, we saw the greatest change in FBG levels.

Effect of PHE on Gut Microbiota

Sequence score cards (Supplementary Figs. S1A and S1B) were used to verify that an entire amplification at around 450 bp was present in each of the six samples (Supplementary Table S4). The recorded gel picture (Fig. 7C) demonstrates the QC of samples from each group of experiments in our study. High coverage was attained across all rat groups, as shown by the distribution plot of sequence (contig) length (Fig. 7D) and the trend for each of the rarefaction curves (Fig. 7E). The diversity estimators and summary statistics showed that the gut microorganisms in the normal group (GP-1), diabetes group (GP-2), and treatment groups like metformin (GP-3), PHE (GP-4 and GP-5), and DMR (GP-6) were significantly different from one another. The diversity of the treated rats was significantly higher than that of the normal and diabetic rat groups. Based on the results of the microbial diversity analysis, we can see in Fig. 8 that there are 10 main phyla covered in the present study of gut microbiota.

Alpha Diversity

Table 1 demonstrates the non-significant prevalent alpha diversity indices in the different groups of experiments (GP-1 to GP-6). GP-5 rats' feces demonstrated similar ACE and Chao1, as well as observed species indices towards GP-1 but not GP-2. GP-4 and GP-6 got similar outcomes as GP-2 in these tests (supplementary Fig. S2). Thus, GP-5, as well as other investigational treatment groups, had rich microbial communities. GP-2 diabetic rats had higher Fisher indices than GP-1 control rats. Fisher indices for the treatment animals (GP-5) were equivalent to those of metformin-treated rats (GP-3) and restored the diversity of bacteria to
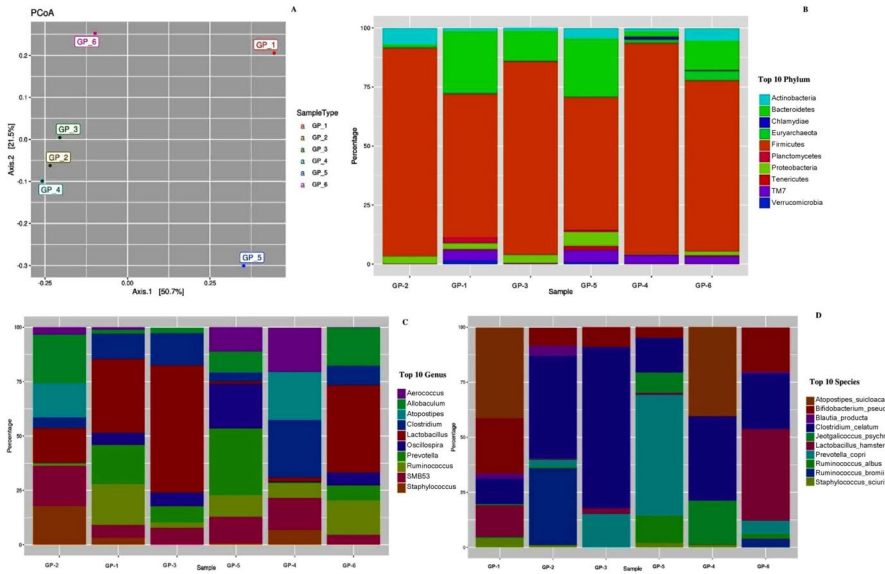
Fig. 8 The taxonomic composition relative abundance of gut microbial in rat fecal sample of different experimental (GP-1 to GP-6) groups ($n=6$). **A** PCoA plot, **B** phylum level, **C** genus level, and **D** species level

Table 1 Alpha diversity measurement of gut microbiota for different treatment groups

S. No	Sample ID	Alpha diversity of gut microbiota						
		Observed	Chao1	ACE	Shannon	Simpson	InvSimpson	Fisher
1.	GP-1	137	160.62 ± 11.00	171.27 ± 6.73	3.17	0.93	15.34	19.34
2.	GP-2	209	262.71 ± 20.74	263.06 ± 7.97	3.70	0.96	25.56	28.65
3.	GP-3	152	189.19 ± 16.76	194.43 ± 7.16	2.91	0.87	7.45	20.24
4.	GP-4	251	354.88 ± 33.08	362.74 ± 10.36	3.70	0.96	24.28	37.73
5.	GP-5	160	290.67 ± 56.91	234.66 ± 7.78	3.35	0.95	18.44	21.97
6.	GP-6	203	283.90 ± 26.15	315.43 ± 10.18	3.41	0.95	18.67	29.97

Data are expressed as mean $\pm$ SE ($n=6$)

normal (GP-1). Table 1 shows that the treatment groups GP-4 and GP-6 have opposite Fisher indices. Thus, sequencing was sufficient to assess gut microbiota diversity. Table 1 shows that the Simpson, InvSimpson, and Shannon indices for both GP-5 and GP-1 did not differ significantly. Community richness and rarely-seen amplicon sequence variations (ASVs) affect the Shannon index more. A total of 28 days of high-dose PHE (GP-5) treatment may improve rat feces species diversity.

Beta Diversity

This study has many microbial species. PCoA cluster analysis was used to investigate gut microbial populations in the feces of experimental rats. Based on taxonomical distribution, the principal coordinate analysis (PCoA) was employed to compare microbiota architectures. Gut microbial structure differed among rat groups. The two-axis principal component assessments explained 50.7% and 21.5% of the total variances (Fig. 8A). Axis 1 shows that GP-1 and GP-5 have a significant difference in PCoA1 compared to GP-2. GP-1 and GP-5 exhibit similar gut microbiota diversity at PCoA1, while GP-3, GP-4, followed by GP-6 remain close to GP-2. In addition, GP-3 and GP-4 were nearer to GP-2 on PCoA2, but GP-1, GP-5, followed by GP-6 were significantly different on Axis 2. Thus, the GP-3 and GP-4 in the present study had fewer microbial differences on PCoA2 and PCoA1 (Fig. 8A). The results of GP-1 and GP-5 were more similar, suggesting that rats' intestinal microbial communities might be altered by gavage with 600 mg/kg body weight of PHE for 28 days.

Comparison of the Gut Microflora Community Composition

According to the results of the metagenomic study, the 10 phyla of the microbiome are the representatives of the present study like *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Euryarchaeota*, *Firmicutes*, *Planctomycetes*, *Proteobacteria*, *Tenericutes*, TM7, as well as *Verrucomicrobia* (Fig. 8B). The phylum *Firmicutes* was present in the highest percentage among samples. The results showed that a bloom of *Actinobacteria* was associated with normal (GP-1), diabetic control (GP-2), metformin (GP-3), PHE (GP-4 to GP-5), and DMR (GP-6)-treated groups samples. An increase of *Firmicutes* and a reduction of *Bacteroidetes* along with *Euryarchaeota*, *Planctomycetes*, *Proteobacteria*, *Tenericutes*, *TM7*, and *Verrucomicrobia* in GP-2 rats were detected compared with GP-1 rats. In contrast, overall treatment groups reduced the prevalence of *Firmicutes* relative to *Bacteroidetes* as compared to diabetic rats of GP-2. However, treatment groups GP-3, GP-4, and GP-6 as compared to GP-5 remarkably enriched the amount of *Firmicutes*. The treatment GP-5 showed a reduction of *Firmicutes*, *Chlamydiae*, *and Euryarchaeota* associated with an elevation of the amount of *Bacteroidetes*, *Planctomycetes*, *Proteobacteria*, *Tenericutes*, and *TM7* in comparison to GP-2. The microbial diversity in GP-3, GP-4, and GP-6 was endorsed by microbiota phyla lateral to GP-5 and restored the microbial phyla abundance compared to GP-1, as seen in Fig. 8B.

In our investigation, we found that there were substantial differences between the groups of rats in terms of 10 different genera of bacterial microbiome. Figure 8C showed the gut microbiota: *Aerococcus*, *Allobaculum*, *Atopostipes*, SMB53, and *Staphylococcus* genera, which obviously increased, while *Clostridium*, *Lactobacillus*, *Oscillospira*, *Prevotella*, and *Ruminococcus* groups decreased in GP-2 rat samples in comparison to GP-1 rat samples. The gut microbiota: *Allobaculum*, *Atopostipes*, *Lactobacillus*, SMB53, and *Staphylococcus* declined, while *Oscillospira*, *Prevotella*, and *Ruminococcus* increased in the GP-5 rats in comparison to the GP-2 and restored microbiota abundance as in the GP-1. The microbial diversity at the genus level in GP-3, GP-4, and GP-6 was also regulated, as seen in Fig. 8C. The gut microbiota diversity of [*Prevotella*], *Bacteroides*, *Desulfovibrio*, *Adlercreutzia*, and *Bifidobacterium* (Supplementary Table S5) was also regulated for treatment groups including PHE to rehabilitate good health.

Furthermore, as shown in Fig. 8D, there were notable differences in the abundance of species-level gut microbiota among the various rat groups. Expression in various conditions is controlled by the species composition and abundance of gut bacteria. The result revealed that the abundance of *Atopostipes suicloacalis*, *Bifidobacterium pseudolongum*, *Lactobacillus hamsteri*, *Jeotgalicoccus psychrophilus*, *Staphylococcus sciuri*, and *Blautia producta* was increased, while the abundance of *Clostridium celatum*, *Prevotella copri*, and *Ruminococcus bromii* was decreased in GP-1 as compared to GP-2. *Clostridium celatum*, *Atopostipes suicloacalis*, *Jeotgalicoccus psychrophilus*, *Staphylococcus sciuri*, and *Ruminococcus albus* showed regulated abundance in different treatment groups as compared to group of diabetic rats. The relative abundance of *Bifidobacterium pseudolongum* was found to decline in PHE treatment groups as compared to diabetic, metformin, and DMR groups. The relative abundance of *Lactobacillus hamsteri* was elevated in PHE treatment groups as compared to diabetic while it was lower to metformin, and DMR groups. *Prevotella copri* abundance was also found to increase in PHE treatment groups as well as metformin and DMR groups as compared to the diabetic group. *Ruminococcus bromii* and *Blautia producta* showed almost similar declines in abundance in different treatment groups as compared to the diabetic group. At the species level, 10 species shown in Fig. 8D had markedly changed abundance levels with the treatment doses (supplementary Table S5). The taxonomic composition with relative abundance heat map of gut microbial in rat fecal sample of different experimental groups was also shown a significant diversity between groups (supplementary Fig. S2 and S3). The results indicated that the administration of 600 mg/kg body weight of PHE (GP-5) for 28 days was sufficient to alter the gut microbiota composition of the rat. Therefore, the PHE groups were selected for the subsequent study of SCFAs in this study.
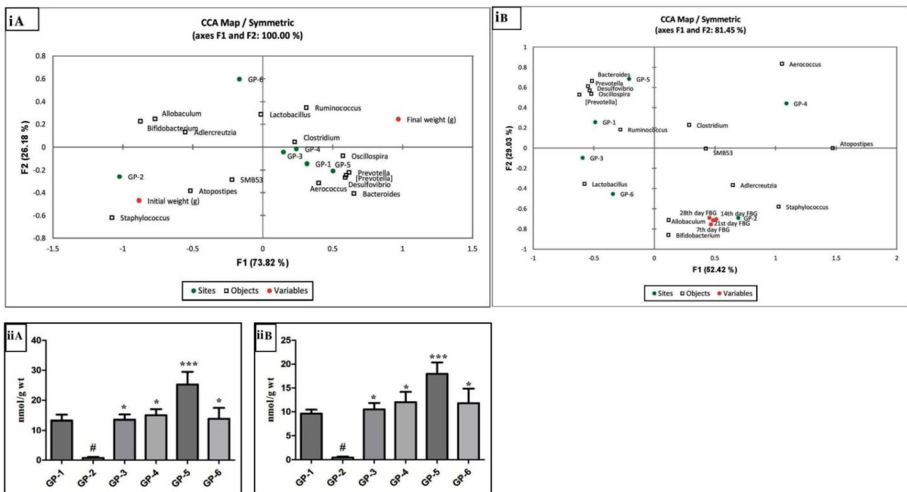

Fig. 9 i Correlation analysis between DM-related parameters (body weight and FBG) and gut microbiota at genus level. Canonical correspondence analysis. (**A**) Body weight Vs gut microbiota and (**B**) FBG vs gut microbiota. **ii** SCFAs analysis in the fecal sample of different rat groups. (**A**) The concentration of acetic acid and (**B**) the concentration of propionic acid

Correlation of Gut Microflora with Metabolic Parameters

DM-related indices (body weight and FBG) are shown in Fig. 9i along with their correlations with the microbiota of the gut (markedly modified ten genera of microorganisms by PHE) using canonical correspondence analysis (CCA) on multiple samples. The resultant ordination plots showed that the first axis for both body weight and FBG against gut microbiota explained the majority of the variance (73.82 and 52.42 percent, respectively), while the corresponding second axis accounted for only 26.18 and 29.03 percent, respectively. Based on CCA, we found that *Lactobacillus*, *Oscillospira*, *Prevotella*, *Clostridium*, *Ruminococcus*, *Desulfovibrio*, [*Prevotell*], *Bacteroides*, and *Aerococcus* were positively correlated with body weight (final weight) on the first axis. In contrast, the other bacteria (*Adlercreutzia*, *Bifidobacterium*, *Allobaculum*, *Atopostipes*, *Staphylococcus*, and SMB53 group) were negatively correlated with the body weight on the same axis (Fig. 9i (A)). Furthermore, the gut microbiota *Lactobacillus*, *Oscillospira*, *Prevotella*, *Ruminococcus*, *Desulfovibrio*, [*Prevotell*], and *Bacteroides* were associated with a negative correlation with the level of fasting blood glucose (FBG) on the first axis, while the other bacteria (*Adlercreutzia*, *Bifidobacterium*, *Allobaculum*, *Clostridium*, *Atopostipes*, *Aerococcus*, *Staphylococcus*, and SMB53 group) were positively correlated with FBG on the same axis (Fig. 9i (B)). Both T2DM-related indicators were found to be more significantly correlated with the gut microbiota species *Lactobacillus*, *Oscillospira*, *Prevotella*, *Ruminococcus*, *Desulfovibrio*, [*Prevotell*], and *Bacteroides*.

SCFAs Content of Fecal Sample

Standard graphs from 5 dilutions (50–5000 nM) were plotted for peak area versus concentration. A linear relationship was established with regression coefficients of 0.998 and 0.996 for acetate and propionate, respectively. Details of molecular weight (g/mol), density (g/ml), standard equation, retention time, molecular formula, and R^2 values of standards run on HPLC are shown in Table 2, with the standard graph in supplementary Figure S4. Subsequent calculations of SCFAs concentration in the sample groups were done using the standard linear equation derived and summarized in Table 2. Fecal SCFAs can indirectly reflect colon SCFAs, which are linked to intestinal health. The content two dominant main SCFAs in rat feces were affected through PHE as shown in Fig. 9ii (A, B). After 28 days of treatment with different doses of GP-3, GP-4, GP-5, and GP-6, all groups had higher SCFAs concentrations than GP-1 at the end of the experiment. GP-2 had lower SCFAs concentrations among other groups. GP-4 and GP-5 had significantly higher acetate content than GP-1 ($p < 0.05$ and 0.01, respectively). GP-4 and GP-5 increase feces acetate levels (Fig. 9ii (A)). GP-3 and GP-6 doses also increased acetic acid concentrations in feces ($p < 0.05$), but GP-2 decreased acetate (Fig. 9ii (A)). Both PHE dosages increased fecal

Table 2 HPLC-based estimation of standard SCFAs

Standards	Molecular weight (g/mol)	Molecular formula	Density (g/ml)	Retention time (minute)	Standard equation	R^2 value
Acetic acid	60.052	CH_3COOH	1.053	17	$Y = 11.27x + 39565$	0.998
Propionic acid	74.08	CH_3CH_2COOH	0.992	22	$Y = 47.13x + 38316$	0.996

acetic acid concentrations after 28 days of continuous administration in rats from various groups. A consistent finding for propionate concentration for both doses of PHE within GP-4 as well as GP-5 was also seen, with large and highly significant increases in propionic acid concentration in feces compared to other groups (Fig. 9ii (B)). This indicated that both dosages of PHE administered to rats for 28 days could also influence the intestinal environment. These studies demonstrated that PHE's effect on the gut is dose-dependent; particularly that 600 mg/kg body weight of PHE-treated rats for 28 days increased the primary SCFA content of their feces.

Discussion

With the recent advancement of metabolic and other analytical technologies in the field of scientific research, phytoconstituents introduce a new era of medicinals that may influence our body physiology through various mechanisms utilizing several microneutrients. Thus, the botanical products enriched with bioactive compounds known from earlier investigations can prevent diabetes by changing gut microbiota dysbiosis and inducing microbiome-associated SCFA production. The UHPLC-HRMS analysis of PHE examined numerous biomarkers and bioactive substances. The bioactive substances restored the gut microbiota in rats with DM. Previous investigations (GC–MS and LC–MS) on the bioactive compounds of PHE were consistent and supported our conclusion in the present study. Chebulic acid, chebulagic acid, and ellagic acid identified in PHE may be used as hepatoprotective agents due to the enrichment of metabolites in the gut through gut-microbial interaction [43, 44]. Gallic acid and andrographolide therapy in animals were also supported to restore the metabolic profile and eliminate the risk of developing diabetes [45, 46]. An anti-diabetic effect of berberine, the most concentrated bioactive compound in the current study, has been postulated to occur via regulation of the gut microbiota in an earlier study [47]. A list of bioactive compounds such as berberine [48], oxyberberine [49], kaempferol [50], quercetin [51], apigenin [52], luteolin [53, 54], rutin [55], genistein [56–58], hesperidin [59], isorhamnetin [60], ferulic acid [61], and several others have been shown to have anti-diabetic activity by altering gut microbial structures in the studies. These bioactive compounds, their derivatives, and several others were identified (tentatively) in good proportion in PHE through UHPLC-HRMS analysis, especially chebulic acid, gallic acid, andrographolide, and berberine. We found that the above-described bioactive compounds improved diabetes-related insulin resistance and the ability to tolerate glucose by strengthening the integrity of the intestinal membrane barrier and restructuring the microbiota of the gut [62], which would be responsible and consistent for the effective anti-diabetic activity of PHE in the current study.

As stated earlier, diabetes has been linked to gut dysbiosis, increased intestinal permeability, and alterations in the overall structure of the gut microbiota [63]. Therefore, altering the composition of the microbiota of the gut is a potential method of reducing hyperglycaemic index in the DM [64]. In the current study, we carried out sequencing of the 16S rRNA using Illumina by the V3 and V4 regions genes and determined the fecal microbiota composition at different taxonomic levels from the studied rat groups. DM rats had increased gut microbial diversity, as measured by Chao1, ACE, and the observed species estimator. These findings corroborated earlier studies showing that a host disease might increase the variety of microorganisms in the digestive tract [65]. The results indicated that the relative abundance of groups like *Lactobacillus*, *Clostridium*, *Oscillospira*, *Prevotella*, and

Ruminococcus was restored in DM rats via PHE treatment, while the relative abundance of groups like *Aerococcus*, *Staphylococcus*, *Allobaculum*, *Atopostipes*, and SMB53 was reduced. The study of gut microbiota dysbiosis found that *Firmicutes* and *Bacteroidetes* were two of the most common bacterial phyla in both rats and humans. A growing amount of literature shows that the *Bacteroidetes* are enriched for numerous pathways involved in glucose uptake, while the *Firmicutes* contribute to glucose transport systems [66]. The ratio of *Bacteroidetes* to *Firmicutes* was expected to rise after andrographolide treatment, as shown in the study [67], and was consistent in this study. Overall, our findings are consistent with the hypothesis that PHE promotes metabolic health by increasing the abundance of beneficial *Bacteroidetes* microorganisms in the body. Increasing data revealed that higher levels of *Bacteroidetes* and *Firmicutes* were linked to higher BMIs, lower glucose levels, and less insulin resistance, as also stated by Clemente [68]. Diabetic rats had an increased abundance of certain microbiota, including *Adlercreutzia*, *Bifidobacterium*, *Allobaculum*, *Clostridium*, *Atopostipes*, *Aerococcus*, *Staphylococcus*, and the SMB53 group, all of which linked positively with FBG level and negatively with final body weight in the current study. Concurrently, the other bacteria, including *Lactobacillus*, *Oscillospira*, *Prevotell*, and *Bacteroides*, were associated positively with the final body weight of the rats and inversely with their FBG levels. As their inverse correlation with FBG and positive correlation with body weight attest, these microbiotal communities have a therapeutic impact [69]. All of these results converged on the conclusion that the PHE treatment would be beneficial for the reshaping of gut microbiota dysbiosis.

In addition, PHE was able to significantly alter the diversity of the microbiota in the intestines of DM rats, specifically influencing certain bacteria associated with SCFAs production as well as anti-inflammation. Bacterial fermentation produces the majority of SCFAs in the major gastrointestinal tract [70] as acetate, propionate, and butyrate. Several positive impacts on host metabolism were mediated by SCFAs. As we know, strong cellular junctions and permeability are both negatively affected by a lack of SCFAs [71]. Total SCFAs, especially butyric, acetic, propionic, valeric acids, and other SCFAs, are reported to increase after treatment with bioactive compounds such as chebulic acid [43], gallic acid [45], andrographolide [67], and berberine [48]. The elevation of SCFAs content with the treatment was also approved in the studies on other bioactive compounds of PHE described and referenced earlier. In addition to the above, the SCFAs appear to promote epithelial regeneration and prevent the inflammation that is partly produced by LPS [72]. SCFAs can also enhance glucose homeostasis as well as insulin sensitivity by altering the function of skeletal muscle, the liver, and adipose tissue [73–75]. Diabetic rats had an increased generation of butyric, propionic, and acetic acids due to the enrichment of SCFAs-producing bacteria [63]. Therefore, the hypoglycemic effects of PHE treatment in this study could possibly be attributable to the elevated levels of SCFAs and the growing number of bacteria capable of generating SCFAs. Another study [76] showed that a high concentration of SCFAs was inversely related to the prevalence of overweight, diabetes, cardiovascular disease, and metabolic syndrome. The symbiotic bacteria genus bacterium known as *Prevotella copri* can generate SCFAs [77]. In another study, it was stated that the commensal obligate anaerobic gram-negative bacterium (*Prevotella copri*) in the gut has been shown to reduce blood glucose levels in rodents and healthy humans [78], making it a promising candidate to prevent or treat obesity and DM. At the mechanistic level, *Prevotella copri* enhanced the bile acid (BA) metabolism signal [79]. Despite the fact that *Prevotella copri* was shown to be the primary species driving the relationship between branch-chain amino acid (BCAA) production and insulin resistance, it was also found that BCAAs themselves can cause insulin resistance and exacerbate glucose intolerance in DM [62]. As stated, the

presence of *Prevotella copri* is an indicator of food, lifestyle, and health status [80]. Thus, collectively, in a mechanistic way, it increased hepatic glycogen through modulating BA metabolism [79], which was consistent with our result. From our data, it was identified that *Blautia*, a gut microbe, belonged to the *Lachnospiraceae* family within the *Firmicutes* phylum and that it exhibited a counterintuitive variation trend among distinct groups, many of which were known to be powerful SCFAs producers [81]. *Blautia producta*, an organism involved in the formation of acetate or propionate, was discovered in significantly higher amounts in this study, with similar findings in HFD-fed rats compared to control animals [82]. The findings are also consistent with those of Zhang [83], who found that rats given an HFD had considerably higher amounts of acetic acid as well as propionic acid. Our findings suggested that this family could have a major beneficial effect on PHE's ability to treat DM. *Blautia* may convert undigested carbs and proteins into acetate, which could subsequently be used to provide energy for the organism [84].

Bacteroidetes, *Proteobacteria*, *Firmicutes*, and *Verrucomicrobia* have all been found to increase in abundance in studies showing that berberine can alter the composition of gut microbiota in normal animals [85]. There is a wide variety of metabolites that can be produced and released by the gut microbiota, including but not limited to SCFAs, BCAAs, BAs, and TMA [86]. Berberine-deprived species that mainly create single sugars, or SCFAs from metabolizing polysaccharides or oligosaccharides, such as *Ruminococcus bromii* and *Bifidobacterium* spp. [87], were often observed to cross-feed with other saccharide degraders [88]. Metformin treatment generated the same species enrichment seen with berberine [89], including two *Bacteroides* spp. and many taxa of γ-*Proteobacteria*. Herbal extracts have been shown to be more effective at treating diabetes than the commonly used drugs acarbose and metformin, according to the literature [89, 90]. Researchers also found that BA metabolism was controlled by *Ruminococcus* strains [91]. This suggests that *Ruminococcus bromii* in the gut microbiome could be the objective of berberine in order to decrease the microbial synthesis of secondary BA, which is linked to the successful glucose management produced with berberine [87]. Multiple *Bifidobacterium* spp. were inhibited by berberine in both humans and animals [92]. Therefore, *Bifidobacterium* strains may have an especially positive effect on elderly people with DM who are taking Berberine. *Bifidobacterium pseudolongum* was also shown to control the number of bacteria in the gut, implying that its presence was essential for the proliferation of the genus [93]. Overgrowth organisms like *Staphylococci coliforms* were prevented by berberine, but native *lactobacilli* and *bifidobacteria* were unaffected [47]. The quantity of gut microbes such as *Actinobacteria*, *Lactobacillus*, and *Firmicutes* was decreased in an animal study with DM [48], and berberine was able to effectively manage glucose metabolism and reestablish glucose homeostasis. Previous research revealed that a novel gut microbiota-mediated metabolite of berberine called oxyberberine, which is also an important bioactive constituent of PHE, was able to keep the intestinal microbiota in equilibrium by altering the structure of the gut microbiota [49]. Andrographolide enhanced *Prevotella* and *Adlercreutzia* at the genus level [67]. Kaempferol, quercetin, luteolin, and rutin administration in the experimental animals with a specific protocol individually was found to be effective in reversing the gut dysbiosis associated with metabolic diseases, as determined in the different studies by sequencing the fecal microbiota [51, 53, 54]. Thus, PHE containing bioactive compounds like andrographolide, kaempferol, quercetin, luteolin, and rutin may change the composition of the gut microbiota by raising the number of good bacteria. Together with each other, our results showed that genistein [56–58], hesperidin [59], isorhamnetin [60], and ferulic acid [61] are the important contents of the PHE in the present study, which may have a direct interaction on the gut microbiota and can ameliorate diabetic symptoms in

DM rats by up-regulating SCFA concentrations. Our results were consistent with those of previous studies.

Our analysis of SCFAs in fecal samples from all rats groups revealed that PHE treatment compared with metformin and DMR could significantly alter the composition and function of the beneficial gut microbiota. This finding of the study would be due to the presence of numerous biomarkers and bioactive compounds in PHE [69]. In the current study, more than ten bacterial phyla controlled by PHE-contents exhibited a significant link with being healthy (body weight) or with beneficial glycemic effects (FBG levels). Thus, the collective presence of numerous beneficial bacteria like *Lactobacillus*, *Oscillospira*, *Prevotella*, *Desulfovibrio*, [*Prevotella*], and *Bacteroides* in the PHE has the potential to produce SCFAs such as acetate and propionate in good proportion, which could activate gut gluconeogenesis via complementary mechanisms [69] and reduce blood glucose levels as well as increase body mass index (good health). These findings were supported according to a correlation analysis between the abundance of gut bacteria with PHE treatment and a couple of DM-related indices. We generalized our prior study's [3] findings that ingesting PHE in DM rats had a hypoglycemic effect by targeting particular gut microbiota, which is the study's supportive approach for the treatment of DM at multiple pharmacological sites. Our results demonstrated that PHE, an antidiabetic medicine, was able to reduce and mitigate inflammation as well as hyperglycemia, insulin resistance, and lipid metabolism abnormalities brought on by DM in the present experimental rat groups through modulation of the gut microbiome.

Conclusion

In conclusion, the presence of several bioactive substances such as chebulic acid, ellagic acid, gallic acid, andrographolide, berberine, kaempferol, quercetin, luteolin, rutin, apigenin, and others in PHE suggested that it could alleviate the hyperglycemia in DM rats. Furthermore, PHE was able to change the gut microbiome and enhance SCFAs, both of which were significant in the pathophysiology of DM. Finally, we hypothesized that SCFAs-producing as well as anti-inflammatory bacterial genera like *Lactobacillus*, *Oscillospira*, *Prevotella*, *Desulfovibrio*, [*Prevotella*], and *Bacteroides* were involved in PHE's beneficial effects on DM rats. Specifically, the enrichment of bacterial strains like *Prevotella copri* and *Lactobacillus hamster* and the decline in relative abundance of *Bifidobacterium pseudolongum*, *Ruminococcus bromii*, and *Blautia producta* through PHE treatment produce health benefits because the generated metabolites increase epithelial barrier function, improve gut permeability, inhibit inflammation, reduce insulin resistance, and reduce the severity of DM by modulating the multiple metabolic pathways with multiple targets involved. We found unanticipated impacts of PHE's systemically hypoglycemic effects using dual therapy. PHE phytochemicals, regenerated gut microbiota, and SCFAs treated DM in this study. In the future, using a mechanistic approach to study the gut microbiota's anti-diabetic properties in vivo and apply them to clinical practice may help to treat DM.

Author Contribution Amit Kumar Singh: conceptualization, methodology, writing-original draft preparation. Pradeep Kumar: methodology, writing—original draft preparation. Vishnu D Rajput: conceptualization, formal analysis, validation. Sunil Kumar Mishra: conceptualization, methodology, formal analysis, supervision. Kavindra Nath Tiwari: conceptualization, formal analysis, validation. Anand Kumar Singh: formal analysis, investigation, writing—review and editing. Tatiana Minkina: formal analysis, writing—review and editing, validation. Ajay Kumar Pandey: formal analysis, validation. Prabhat Upadhyay: formal analysis, validation. All authors have read and agreed to the final version of the manuscript.

Data Availability Not applicable.

Declarations

Ethical Approval All of the tests were carried out in compliance with Institutional animal experimentation rules. The Central Animal Ethics Committee, as per CPCSEA guidelines at IMS BHU Varanasi 221005 (Reg. No. 542/GO/Rebi//S/02/CPCSEA dated 26.5.2017), approved this study (Approval number: Dean/2021/IAEC/2560). The CPCSEA guidelines were fully followed in the experiment.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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