CHAPTER 6

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Bioactive compounds can be extracted using a variety of techniques, such as microwave, soxhletion, decoction, cold or hot percolation, and maceration. Even now, research frequently uses the cold maceration procedure since it is a relatively simple technique [253-254]. The biological properties of bioactive phytochemicals derived from plants need to be assessed in relation to their presence in different plant components that are separated in particular organic solvents [255–256]. The polyherbal formulation's alcoholic extract produced the most total phenolic compounds due to its high polarity [257]. Alcohol may be collected more easily than other solvents, and phenolic components may have a role in the production of bioactive compounds through proteins, carbohydrates, aromatic compounds, and inorganic compounds [258]. The application of ethanol as a solvent for extraction has been extensively studied, which is well reported [255, 257].

Flavonoids are a class of polyphenolic compounds that consist of a range of naturally occurring molecules with diverse molecular structures and potent biological activity. Fruits, vegetables, and fungi are major sources of flavonoids, which exist as secondary metabolites [259]. Flavonoids are abundant in fruits (especially berries and citrus fruits), vegetables, nuts, roots, stems, flowers, some beverages (tea, coffee, and red wine), cereals, legumes, extra-virgin olive oil, and Chinese herbal medicines [260–261]. Their fundamental structure is made up of a pair of aromatic rings (A and B), fifteen carbon skeletons, and three carbon chains connecting them. Six subclasses of flavonoids are mostly recognized: anthocyanidins, isoflavones, flavonols, flavanols, and flavones.The basis for classification is the substitution of side groups such as hydroxyl, methoxy, acrylamide, and glycoside for the conjugation between rings [262].

The crude extract (PHE) prepared using ethanol as a solvent from traditional Indian medicinal plants possessed excellent *in vitro* antioxidant capacities as well as inhibitory activities against inflammation caused by its enrichment in major bioactive phytochemicals [82]. Bioactive components such astannins, flavonoids, and phenolics, whichare components of PHE, have been linked to the anti-diabetic effects of various medicinal plants [263]. Over the years, scientists have investigated these substances in traditional medicinal plants for the treatment of several diseases [264].

Flavonoids were a reliable, simple, and cost-effective way to prevent and manage DM patients based on *in vitro* models, animal models, and clinical trials [265-266]. Flavonoids could ameliorate long-term complications of diabetes, which included cardiovascular diseases, neuropathy, kidney diseases, and retinopathy [267-270]. Multiple randomized control trials (RCTs) indicated that consumption of flavonoid-rich fruits, vegetables, cocoa, and beverages was negatively associated with the risk of T2DM [271-272].

Based on in vitro models, animal models, and clinical trials, flavonoids were a dependable, easy-to-use, and affordable method of managing and preventing diabetic patients [265–266]. Long-term diabetic adverse effects, such as neuropathy, renal disease, retinopathy, and cardiovascular illnesses, may be improved by flavonoids [267–270]. Consuming fruits, vegetables, cocoa, and beverages that contain flavonoids was linked to a lower incidence of type 2 diabetes (T2DM), according to several randomized control studies (RCT) [271–272].Flavonoids may enhance blood vessel functions, improve the metabolism of adipose tissue, decrease pro-inflammatory cytokines and gastrointestinal permeability, increase the absorption of glucose, shield pancreatic cells from glucose toxicity, and act through other mechanisms to prevent the development of advanced glycosylated end products (AGE).

Recent interest in phenolic compounds has come from the idea that their anti-radical and anti-inflammatory properties, which depend on concentration, could make them useful as new

biological products [222, 273]. Numerous experiments have shown the biological characteristics of flavonoids, a prominent class of secondary metabolites in which these compounds have found a prime position [223, 274].The findings of the current study showed that the total phenolic and flavonoid contents of the substance were influenced by the concentration of PHE.

A number of disorders, including diabetes, have been associated with free radical pathophysiology [275]. Scientists proved that hyperglycemia, the prime DM index, causes oxidative stress by releasing free radicals that activate the body's antioxidant defenses. The ability to scavenge free radicals is one way that the bioactive compounds of medicinal plants and their derivatives can be evaluated for their antioxidant capacity [226]. Recent research has shown that medicinal plants and formulations containing high concentrations of antioxidants can be effective in mitigating the stress-related health consequences of conditions like DM [276]. In our research [82], we found that PHE had the ability to suppress free radicals in a concentration-dependent manner.

Since natural supplements are widely available, it is crucial to regulate and monitor them for the presence of potentially harmful heavy metals. Previous research [277] provided permissible heavy metal concentrations based on regulatory documents in various nations. All heavy metals (As, Pb, Cd, Ni, and Hg) summarized in the result section of this study have been measured below the reference. Thus, PHE was established as safe in reference to the above heavy metals.

The ability of current medications or locally sourced natural components to reduce inflammation has long been ascertained using assays of erythrocyte membrane stability [278]. Hemolysis and hemoglobin destruction occur when red blood cells are subjected to saline media or high temperatures, which lyze the red blood cell membranes. In saline solution, hemolysis—the breakdown of extra fluid inside the cell—occurs, resulting in membrane

rupture. An increase in body temperature beyond normal causes hemolysis and the rupture of the RBC membrane [279]. The organelles' lysosomal membranes lyse, releasing enzymes that set off the inflammatory response. A stable inflammatory membrane prevents its contents from seeping out. In cases where the lysosome membrane becomes stable or the enzyme discharge is suppressed, non-steroidal anti-inflammatory drugs may prove beneficial.Because of the above-mentioned parameters, we discovered a dose-dependent relationship between PHE and hemolysis in our experiment. Heat treatment was applied to egg albumin to induce conformational changes as a component of the protein precipitation-mediated test. Proteolysis has been linked to rheumatoid arthritis along with other chronic inflammatory diseases, particularly during the later stages of the illness [280]. NSAID drugs have the ability to inhibit heat-induced proteolysis [281]. Additionally, it was shown that PHE could preserve membrane integrity and reduce heat protein precipitation, both of which were comparable to the reference aspirin employed in this study. Future research on PHE's effectiveness in vivo is encouraged by the observation of an anti-inflammatory effect in vitro.

In order to effectively manage DM, treating hyperglycemia must be a primary focus. Another one of the most important and helpful techniques is limiting carbohydrate digestion and absorption after a meal to lower postprandial hyperglycemia [282]. So, anti-diabetic drugs work by blocking the α-amylase enzyme, which is involved in DM [226]. Since they limit or obstruct the hydrolysis of 1,4-glycosidic linkages in starch and other oligosaccharides that produce maltose and maltriose, notably other simple sugars, these α-amylase inhibitors are often referred to as starch blockers [226]. Due to its ability to inhibit α-amylase, PHE shows promise as an anti-diabetic therapy. By blocking these enzymes, PHE is able to slow the breakdown of carbs, keeping FBG levels stable. PHE contains components with anti-diabetic effects, including polar moieties and phenolics (kaempferol, quercetin, and rutin). It is

interesting to note that acarbose, which blocks the enzyme's activity, is used as a comparison for PHE's α-amylase activity [226].

Many studies have pointed to the potential role that medicinal plant extracts play in inhibitingα-glucosidase, indicating that the ability of extracts to handle hyperglycemia is a real possibility. According to Oh et al. (2021), the extract's action can be attributed to the abundance of bioactive compounds [164]. In the present study, PHE explained a remarkable inhibition potential against the α-glucosidase enzyme. Moreover, synergistic good antioxidant values with enrichment of medicinal compounds were discovered in PHE, making it a potent anti-inflammatory drug [82], corroborating our earlier statement. Therefore, PHE containing bioactive compounds was found to be a significant and synergistically effective antidiabetic drug for the treatment of DM.

The aforementioned results demanded that the antidiabetic activity be performed in an in vivo model. As a result, research was done on the antihyperglycemic impact of PHE on the FBG of orally glucose-loaded normal rats and diabetic rats caused by STZ. The longer fasting period between measures is the cause of the small decrease in FBG in the control group. A medication that effectively treats diabetes will be able to regulate the rise in blood sugar levels through several mechanisms, and the glucose-loaded hyperglycemic mode may be used to assess an extract's capacity to avoid hyperglycemia [283]. Rats were fasted for 14 hours prior to glucose loading in order to test the antihyperglycemic activity during the oral glucose tolerance test. Due to the fact that the animal's blood glucose level is momentarily raised without causing harm to the pancreas, this technique is known as the physiologic initiation of DM [283]. Prior to administering glucose, fasting is necessary to establish a stable baseline glucose level and to remove variations in FBG caused by meal consumption [284]. Moreover, it increases insulin sensitivity brought on by glucose [285]. Either sex was chosen for OGTT because studies on the relationship between sex preference and the susceptibility of glucose-

induced insulin release are inconsistent [286-287]. PHE's ability to reduce FBG after OGTT may indicate that it can induce the release of insulin. The released insulin then promotes peripheral glucose uptake and regulates glucose synthesis via various pathways. It takes more than two hours for released insulin to return the blood glucose level to its baseline level in animal models undergoing OGTT [287]. In this investigation, the PHE and standard reduced FBG to baseline in less than two hours, even though it took more than two hours to return FBG to normal in the control group. FBG began to decline with the start of the metformin and PHE doses, approximately one hour and ninety minutes, respectively.

Additionally, the effects of the conventional PHE dosage remained for at least one hour, but the DMR dose only lasted for thirty minutes. Given that PHE decreased fasting blood glucose (FBG) after glucose loading and that glucose triggers the release of insulin, the extract's antihyperglycemic action may be due to an insulin-like action that either increases peripheral glucose uptake or increases β-cell sensitivity to glucose. The antidiabetic action of PHE may be related to suppression of glucose absorption due to the presence of numerous polyphenolic and flavonoid components, through in vitro α-amylase and α-glucosidase inhibitory activity and in vivo OGTT findings.

The ethanol extracts of PHE contained many bioactive compounds, as revealed by the GC-MS chromatogram. Numerous pharmacological properties have been linked to the main compounds discovered in this work in earlier publications [255, 288-289]. Consequently, results indicated that these PHE active metabolites may have maintained β-cell function while suppressing intestinal α-glucosidase and α-amylase activity in vitro. ATA was found to be a novel compound in PHE [82]. ATA and alpha-tocospiro B are tocopherols and have been proven to show antioxidant properties [290]. Thus, ATA is a compound of the alpha-tocopherol group. Alpha-tocopherol (vitamin E) is identified in *Andrographis paniculata* crude extract [291], which is one of the ingredients of PHE in the present study. Alpha-

tocopherol content is also reported in different parts of the *Andrographis paniculata* [292] plant in several other studies. Considering the outcomes of a GC-MS study that was conducted on extracts of *Andrographispaniculata* that had been made public in the past, it has been hypothesized that ATA may play a significant role in DM and a wide variety of diseases. Numerous studies have all come to the same conclusion, which is that ATA is a therapeutic component of the extracts that they investigated. Some examples of these studies were included in our earlier report [82].

A phytochemical investigation of PHE with UPLC-Q-TOF-MS/MS demonstrated the existence of several biologically active flavonoids. The identified flavonoids in PHE are important and maybe accountable for the high antioxidant potential, anti-inflammatory activity, and several pharmacological activities in the Indian traditional medicine system and also in TCM [293-294]. The three flavonoids puerarin [294], homoorientin [295], and vitexin [296] were common in both modes of UPLC-Q-TOF-MS/MS analysis. Vitexin is a flavone glycoside derived from the plant compound apigenin, which is used both in food and medicine. Its pharmacological effects include anti-inflammatory, antioxidant, anti-cancer, analgesic, and neuroprotective effects [296-297].It is primarily used in the ayurvedic medicinal system and in TCM. In addition, puerarin, an isoflavone glycoside, is frequently used in the management of numerous disorders, including diabetic cardiovascular problems, hypertension, heart failure, myocardial infarction, stroke, cardiac hypertrophy, and atherosclerosis [294-298]. Moreover, homoorientin is a naturally produced C-glucosyl flavone with a wide range of potential medical applications. In more and more experiments, homoorientin [295-299] has been observed to have strong antioxidant potential and anti-inflammatory activities. Several other chief bioactive flavonoids, like eriodictyol, kaempferide, acacetin, neodiosmin, diosmin, luteolin, taxifolin, saponarin, catechin,

quercetin, neohesperidin, and isorhamnetin derivatives, have been used as food and medicine in the ayurvedic medicinal system and in TCM[294].

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 micronutrients. 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, 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 may be used as hepatoprotective agents due to the enrichment of metabolites in the gut through gut-microbial interaction [300-302] in previous studies and also the reshaping of the gut microbiome in the current study. Gallic acid therapy in animals was also supported to restore the profile of metabolism[303] and eliminate thepossibility of acquiring diabetes,which was also found as a study conclusion of andrographolide [304]. 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 [305]. A list of bioactive compounds such as berberine [306], oxyberberine [307], kaempferol [308], quercetin [309], apigenin [310], luteolin [311-312], rutin [313], genistein [314-316], hesperidin [317], isorhamnetin [318], ferulic acid [319], 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 in good proportion in PHE through UHPLC-HRMS analysis, especially chebulic acid, gallic acid, andrographolide, and berberin. We found that the above-described bioactive

compoundsimproved diabetes-related insulin resistance and the ability to tolerate glucose through restoring the intestinal microbiota's structure and fortifying the intestinal membrane permeability[320], which would be responsible and consistent for the effective antidiabetic activity of PHE in the current study.

HPTLC analysis of PHE showed that kaempferol, quercetin, and rutin were all present in this crude extract. Kaempferol, a compound found in plants, has recently become the subject of research. Kaempferol decreases glucose toxicity by regulating lipid metabolism, enhancing IR to decrease lipotoxicity, enhancing insulin signaling, and reestablishing homeostasis between glucose use and production. In conclusion, kaempferol protects cells by reestablishing the equilibrium between autophagy and apoptosis. As a result, the mechanisms of kaempferol's anti-diabetic effects consist of comprehensively preventing the advancement of diabetes and diabetic complications [321].To date, the antioxidant profile of quercetin is its most well-known feature. It protects us from harmful free radicals and is the most potent antioxidant flavonoid found in nature. Its animal safety profile as an antibacterial, anti-inflammatory,anti-diabetic, anti-cancer, and antioxidant drug has been extensively characterized. Because it works well with anticancer, antibacterial, anti-diabetic, and anti-inflammatory drugs, quercetin is an interesting chemical to study for new ways to treat acute and chronic diseases in humans that are less harmful and work better [322]. Recent research lends credence to rutin's ability to improve glycemic control, lipid profiles, and diabetes-related micro- and macro-vascular problems. Gluconeogenesis, promoting glucose uptake by tissues, making the pancreas make more insulin, and keeping the Langerhans islet from shrinking have all been linked to this effect [323].Thus, the presence of these bioactive compounds in PHE, identified through these modern analytical techniques, has verified its therapeutic efficiencies.

In the healthcare system, herbal medicines can be used to treat diseases that affect both humans and animals. Traditional medicine has sustained significantly greater popularity around the world, and its use is increasing rapidly. Despite this, reports of illness and death, hepatotoxicity and nephrotoxicity, and other side effects have led some to question the safety of herbal medicine [324]. Only a few of the several traditional herbal medicines on the market have been proven safe and effective in clinical trials, and patients continue to question their safety and efficacy. Toxicity testing of herbal medicines is sparsely performed. Because it has been demonstrated that combining a number of herbal biologically active substances and secondary metabolites can have adverse effects, traditional medicine toxicity assessments are required [325-328].Accordingly, the toxicity of PHE was tested in a Wistar rat model for lethality and sub-acute toxicity. It was expected that the results would help in making a toxicology profile for PHE thatcould be applied to a variety of diseases.Compared to normal control rats, PHE-treated rats showed no significant variations in body weight, food orwater intake, which was in line with earlier research in the field [329]. At the LD_{50} of 2000 mg/kg, PHE did not cause any lethality in the rats after 14 days of administration. According to these findings, lethal doses of PHE must be higher than the highest standard value of 2000 mg/kg body weight,which is consistent with several reports on safety evaluations [236, 330]. However, in this study, rats were given extracts of ingredient plants at the dosages described above and below and had no adverse effects on health [236, 330-331]. Thus, the NOAEL of PHE was revealed to bemore than 2000 mg/kg.

Further histopathological examinations were necessary to ensure that no risks were occurring at the cellular level, even though the rats' physical appearances suggested that extract doses below 2000 mg/kg body weight were not lethal. A sub-acute toxicity assessment was carried out in order to accomplish this. The clinical findings were an important factor in understanding the effects of doses on animals. No physical abnormalities were found among

the established clinical signs during sub-acute toxicity assessments. Animals exposed to doses of less than 2000 mg/kg body weight did not observe any significant signs oftoxic effects. The findings of the sub-acute toxicity study can be extrapolated to humans by studying the histopathological evaluation of hepatic-renal cells or cellular structures treated with PHE doses in rats. In comparison to the control group, the treated rats' liver and kidneys were found to be macroscopically normal [332].The degree to which a substance harms cells or tissues is used to determine its toxicity. Toxins can be ingested or produced by the body's natural processes. Since the liver and kidney play such important roles, they have been used as toxicity indicators for pharmaceutical drugs and plant extracts. Toxicants administered to the liver and kidneys, for example, are the first targets because they are detoxifying organs [325]. Hepatic vacuolation, necrosis, and hyperplasia are all signs that toxic substances are in the organs that get rid of them [325, 333].ALT, AST, and ALP are biomarker enzymes that indicate the health of the liver and kidneys [334]. The main place where the transaminases ALT and AST are made is in the hepatocytes of the liver. ALP, on the other hand, is found in the plasma membrane and endoplasmic reticulum of all tissues. Damaged liver tissue is indicated by an elevated level of these enzymes [335].

These organs may be infected or damaged by toxic substances. The levels of these enzymes in the treated groups were not significantly altered by PHE administration when compared to the controls. Blood albumin and bilirubin levels are other biochemical indicators of hepatocellular function. A decrease in these two indicators indicates a problem with liver function. The amounts of albumin and bilirubin in the treatment and control groups do not differ significantly from each other.In the bodies of organisms, xenobiotics have the potential to produce reactive oxygen species (ROS), which can alter the amounts of antioxidants.As a result of these stressors, antioxidant enzymes were inactivated, proteins were denatured, and DNA was damaged as a result of cellular membrane lipid peroxidation [334]. It is possible

that plant extracts may harm organisms by releasing ROS and reducing their own antioxidant enzymes.

SOD, GSH, and CAT levels in a tissue are good measures of its antioxidant ability, while MDA is a sensitive and precise measure of lipid peroxidation [111]. Following oral delivery, PHE had no obvious impact on the amounts of MDA and GSH or on the levels of antioxidant enzymes (CAT and SOD) in both the treatment and control groups.The study of PHE in the liver as well as pancreatic tissues confirmed its non-toxic effects. Prior research has demonstrated that plant extracts can cause the production of ROS, which damages the liver tissue of the hosts. As we all know, waste products are filtered out of the body by the kidneys [336]. It is possible for plant extracts to cause kidney toxicity when given to animals [336]. A good way to assess renal function is to measure BUN, uric acid, and serum creatinine levels. The presence of elevated levels of these biomarkers suggests damage to the kidneys [335]. BUN, uric acid, and serum creatinine concentrations were not statistically different within the PHE-treatment groups and the control group. PHE was found to be safe for the kidneys of the test animals. Elevations in cholesterol levels can lead to chronic conditions, including diabetes and hypertension [337]. The cholesterol levels of the PHE-treated group and the control group did not differ statistically significantly. This finding supports the hypothesis that PHE can be used to treat and prevent hyperlipidemia. The liver and kidneys were found to be in good health by histological examination. Because of this, it can be stated that PHE had no ill effects on the rats studied. PHE may have excellent antioxidant potential and free radical scavenging capacities, which could explain its nontoxic effects [338-339]. *In vitro*, the antioxidant properties of PHE were demonstrated *in vivo* and confirmed for its use in the treatment of free radicals generated by xenobiotics in the host [336].

Before a doctor can prescribe an herbal medicine, it must first undergo a toxicological evaluation. The degree of toxicity of the medicine is determined by the deterioration of

tissues or cells in key organs, especially the liver and kidneys.According to the Wistar rats' in vivo toxicity analysis of PHE, there were no fatalities up to 2000 mg/kg body weight. The histopathology study revealed that PHE was safe at the dose that was used. The findings showed that neither a single dose of PHE nor a series of doses caused harm to rats. As a whole, the enrichment of many bioactive compounds in PHE and the results of acute and sub-acute testing of PHEas a drug suggest that it could be used safely in the treatment of several complications.

As we all know, DM is a polygenic disease resulting from an imbalance in metabolism and/or hormones between how beta cells make insulin and how sensitive other cells are to insulin [340]. The pathogenesis of DM frequently begins with insulin resistance, which is followed by a compensatory elevation of insulin levels, which leads to reduced insulin secretion and hyperglycemia due to β-cell dysfunction [341]. The glucosamine-nitrosourea compound streptozotocin (STZ), discovered in *Streptomycesachromogenes* and used as a treatment with chemotherapy for pancreatic cancer, destroys pancreatic cells, causing diabetes [342]. To reliably induce both T1DM and T2DM, STZ is widely employed nowadays to cause β-cell death via DNA alkylation [343]. The elevated FGB concentration reported after 72 hours of STZ intake builds support for the hypothesis that glucose-induced insulin generation is reduced by localized inhibition of the enzyme hexokinase and a particular toxicity to β-cells [1]. Metformin, the reference anti-diabetic medication, decreases glucose production, improves oxidation of fats in liver tissue, and/or enhances glucose absorption in the body [344]. Evidence suggests that phytoconstituents such as kaempferol, quercetin, and rutin may be responsible for PHE's beneficial features, as FBG was lower among the PHE, metformin, and DMR groups relative to the diabetic group. PHE may have been able to lower serum glucose levels because of enhanced glucose uptake, as seen above, as a result of an enhanced glycolytic pathway.

The main feature of STZ-induced diabetes is usually a considerable decrease in body weight. A change in body weight is caused by a disparity between the amount of energy consumed and that utilized [238]. Reduced glucose metabolism, increased fat metabolism, or the structural destruction of proteins providing an alternative resource of energy [2] are all possibilities for explaining the weight loss observed in diabetic rats. Diabetic rats given PHE at the most effective amount likely gained weight due to better glycemic control brought on by enhanced insulin production. This anti-diabetic effect suggests that PHE may encourage insulin synthesis from the remnant beta cells or repaired beta cells, whichin turn may stimulate the hormones responsible for fat accumulation. DM is characterized by other different DM indices, as discussed above, along with a substantial increase in HbA1c [238]. Levels of glucose in rats with diabetes that received treatment with PHE, metformin, and DMR dropped drastically, insulin levels went up, and glucose tolerance (HbA1c) was dramatically enhanced. Histopathological studies corroborated the findings, demonstrating a considerable increase in the islet area of diabetic rats treated with PHE. These findings demonstrate the protective effect of PHE through its anti-diabetic capability on pancreatic β-cells, allowing them to resume normal functioning [345]. The effects of insulin levels are almost identical to those caused by taking metformin and PHE at a 300 mg/kg dose. As a result of ameliorating these metabolic abnormalities, PHE has been shown to be effective in preventing diabetes complications. In this study, insulin resistance (IR) as well as β-cell function (HOMA-β) weremeasured, and their evaluation was done with the help of the homeostasis model assessment (HOMA-β) equations [346]. PHE's capacity to enhance HOMA-IR and HOMA-β has been demonstrated by its effect on insulin secretion and sensitivity. Bioactive substances (kaempferol, quercetin, and rutin) in PHE have been proven to stimulate insulin release and support the regeneration of pancreatic β-cells in STZ-induced diabetic rats [346].

Liver enzymes (ALP, ALT, andAST) and TP were elevated in the serum of diabetic rats, indicating hepatic impairment. When liver cells are damaged, enzymes that help the liver work leak out of the cytosol and into the bloodstream. An increase in these marker enzymes [238] is evidence of liver damage. Increased levels of ALP, ALT, AST, TP, and others in the blood of rats with diabetes were previously reported [238], and the current results confirm these findings. These changes in the marker enzymes were confirmed by other changes in the livers of diabetic rats, such as necroinflammation, hepatocyte ballooning, steatosis, an increased liver index, and a lower glycogen content. This shows how important PHE is for fixing the damage diabetes does to the liver, since PHE, metformin, and DMR all drastically reduced changes and fixed enzymes and functions in diabetic rats' livers. Evaluation of kidney functional variables is also a useful method to acquire knowledge about kidney health. There was a significant change from normal group rats-kidney performance to clearing waste through the kidneys of the diabetic rats, as measured by higher serum levels of BUN, CRE, and UA in the study [238]. However, the renoprotective benefits of PHE were clearly seen in the recovery of all kidney functioning indices in rats with diabetes treated with PHE, metformin, and DMR. Dyslipidemia is a problem for diabetic patients because it is marked by high levels of TC and TG, as well as abnormalities in lipoprotein components. In addition, STZ causes upregulation in the levels of LDL and VLDL, which result in a higher chance of cardiovascular disease. Rats with diabetes have a higher TC because lipoprotein lipase becomes inactive [21]. Clinical research, on the other hand, has shown a negative correlation between low HDL levels and the progression of atherogenic dyslipidemia in poorly managed DM [347]. PHE, metformin, and DMR may be able to correct metabolic imbalances because they slow the rate at which lipids are degraded—that is, the rate at which free fatty acids are biotransformed into phospholipids—by inhibiting lipase via insulin. This action of PHE could

be due to the occurrence of kaempferol, quercetin, and rutin, all of which have been observed to slow lipid metabolism [348].

Antioxidants, enzymatic (CAT, SOD, and GSH) and non-enzymatic (MDA), perform a crucial function in keeping oxygen and hydrogen peroxide amounts in the body stable [21]. They do this by dismuting O_2 radicals and getting rid of organic peroxides that are generated when STZ is ingested. Recent research has shown that STZ-induced diabetes disrupts the equilibrium among the enzymes linked to the liver. Due to the toxicity caused by STZ [349], when inducible NO-synthase is turned on, the amount of NO goes up, and the amount of H_2O_2 produced goes up. This causes redox intermediate states and oxidized glutathione (GSSG) to be made, which in turn make radicals such as the OH radical, the most hazardous type of ROS [350]. This might explain why reactive oxygen species (ROS)-related damage is difficult for the immune system to prevent or treat. The gap between ROS production and enzymatic antioxidant activity in diabetic rats appears to have been minimized as a result of PHE, metformin, and DMR's effects on these enzymes. These antioxidant enzymes not only eliminate radicals, but they also inhibit ROS production. Therefore, PHE's radical scavenging potential is demonstrated by its ability to minimize the oxidized antioxidant enzyme levels in rats given STZ-induced diabetes. This could be because PHE contains phenolics and other bioactive chemicals with radical-scavenging properties, such as kaempferol, quercetin, and rutin [348].

As a whole, metabolic disorders, including diabetes, are characterized by persistent inflammation. Diabetes regulates the way in which signs of inflammation are expressed, like TNF-α, SOX-9, IL-18, IL-4, and COX-2, all of which are strongly connected with the endocrine system's inflammatory response. Taken as a whole, these changes are indicative of the involvement of inflammatory triggers in the development of diabetes [351]. Earlier research has demonstrated that diabetic patients' islets undergo a unique inflammatory

response marked by the presence of cytokines, immune cell infiltration, and ultimately necrosis. Hence, it seems like a promising treatment strategy to target intra-islet inflammation-related mediators such as SOX-9, TNF-α, IL-18, COX-2, and IL-4. Strong associations have been found between insulin resistance, lipid abnormalities, and anti-inflammatory markers like TNF-α, IL-18, and IL-4 [352-353]. Arachidonic acid is converted into prostanoids by cyclooxygenase (COX), and COX-2 is thought to be triggered by stimuli that promote inflammation. Studies in rodents and mammals have shown that elevated blood sugar levels induce an increase in COX-2 expression in the islets. Overproduction of inflammatory substances that are then deposited in pancreatic tissue contributes to β-cell dysfunction [351] and is another consequence of hyperglycemia. As discussed before, protecting the pancreas from inflammatory agents is helpful in avoiding islet destruction and keeping diabetes under control. SOX9 is needed for healthy pancreatic development. It acts as a gatekeeper, letting pancreatic progenitor cells turn into specialized cells or stopping them from doingso along different pathways [354].

In the current investigation, the expression of SOX-9, TNF-α, IL-18, and COX-2 at the protein level was significantly elevated in the rat with diabetes in contrast to that in the normal rat. This indicates that a process of inflammation was gradually initiated in the STZ-induced diabetic rat. According to the current study, increased Sox-9, TNF-α, IL-18, and COX-2 expressionin the pancreas and liver was effectively reversed due to the PHE, metformin, and DMR treatments. Activation of these inflammatory markers with their grounded pathways was hypothesized to improve protein, carbohydrate, and lipid metabolism, which in turn may have been the mechanism behind the prevention of abnormal inflammatory mediator expression. Bcl-2, an anti-apoptotic molecule, plays a crucial role in the apoptosis pathway [21]. However, PHE therapy restored normal Bcl-2 expression within the liver as well as the pancreas of STZ-induced rats with diabetes, and it, like metformin and

DMR, protected cell death in the liver and pancreas at all tested doses in this study. Earlier investigations [355-357] indicated that the bioactive substances (kaempferol, quercetin, and rutin) found in the treatment group PHE protected the pancreatic β-cells. These results corroborate previous research reporting increased radical production leading to hepatic and pancreatic cell death in diabetic rats (increased MDA concentration and decreased SOD activity in hepatic as well as pancreatic tissues).

As previously mentioned, dysbiosis of the gut, elevated intestinal permeability, and modifications to the general composition of the gut microbiota have all been associated with diabetes [358]. Therefore, altering the composition of the microbiota of the gut is a potential method of reducing hyperglycaemic index in the DM [359]. In the current work, we used Illumina to sequence the 16S rRNA via the V3 and V4 sections genes and examined the genetic makeup of the fecal microbiota at various taxonomic levels in the rat groups that were the subject of the investigation. 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 [360]. The results indicated that the proportionate 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 in both rats and humans, the two most prevalent bacterial phyla were *Firmicutes* and *Bacteroidetes*.According to an increasing body of research, numerous pathways are enriched in the *Bacteroidetes*, which areinvolved in glucose uptake, while the *Firmicutes* contribute to glucose transport systems [361]. The ratio of *Bacteroidetes* to *Firmicutes* was expected to rise after andrographolide treatment, as shown in the study [362], and was consistent in this study. Overall, our findings are consistent with the

hypothesis that PHE promotes metabolic health by boosting the number of advantageous*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 [363]. 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 [364]. All of these results converged on the conclusion that the PHE treatment would be beneficial in order to modify the dysbiosis of the gut microbiota.

In addition, PHE was able to significantly modify the intestinal microbiota's diversity in DM rats, specifically influencing certain bacteria associated with SCFA production as well as anti-inflammation. Bacterial fermentation produces the majority of SCFAs in the major gastrointestinal tract [365] 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 [366]. Total SCFAs, especially butyric, acetic, propionic, valeric acids, and other SCFAs, are said to rise after treatment with bioactive compounds like chebulic acid [301], gallic acid [303], andrographolide [362], and berberine [306]. The elevation of SCFA content with the treatment was also approved in the studies on other bioactive compounds of PHE described and referenced earlier. In addition to the aforementioned benefits, SCFAs seem to encourage epithelial regeneration and inhibit inflammation, which is partially caused by LPS[367]. SCFAs can also improve insulin

sensitivity and glucose homeostasis by modifying how the liver, skeletal muscle, and adipose tissue operate[368-370]. Diabetic rats had an increased generation of butyric, propionic, and acetic acids because of the enrichment of SCFAs-producing bacteria [358]. 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 [371] showed that elevated levels of SCFAs were inversely related to the prevalence of overweight, diabetes, cardiovascular disease, and metabolic syndrome. The symbiotic bacteria genus bacterium known as *Prevotellacopri* can generate SCFAs [372]. Another study reported that in both rodents and healthy humans, the gut's commensal obligate anaerobic gram-negative bacterium, *Prevotella copri*, has been demonstrated to lower blood glucose levels [373], making it a promising candidate to prevent or treat obesity and DM. At the mechanistic level, *Prevotellacopri* enhanced the bile acid (BA) metabolism signal [374]. Even though it was demonstrated that *Prevotella copri* is the main species influencing the relationship between the synthesis of branch-chain amino acids (BCAAs) and insulin resistance, it was also found that BCAAs themselves can cause insulin resistance and exacerbate glucose intolerance in DM [320]. As stated, the presence of *Prevotellacopri* is an indicator of food, lifestyle, and health status [375]. Thus, collectively, in a mechanistic way, it increased hepatic glycogen through modulating BA metabolism [374], 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 SCFA producers[376]. *Blautiaproducta*, 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 [377]. The findings are also consistent with those of Zhang [378], 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 [379].

Bacteroidetes, *Proteobacteria*, *Firmicutes*, and *Verrucomicrobia* have all been found to increase in abundance in studies showing that berberine can alter the structure of the intestinal microbiota in normal animals [380]. There is a wide variety of metabolites that can be produced and released by the gut microbiota, which include but are not restricted to BAs, BCAAs, SCFAs, and TMA [381]. Berberine prevented organisms like *Ruminococcus bromii* and *Bifidobacterium spp.*, which primarily produce single sugars or SCFAs, from breaking down polysaccharides or oligosaccharides [382], which were often observed to interconnect with other saccharide degraders [383]. Metformin treatment generated the same species enrichment seen with berberine [384], including several taxa of γ-*Proteobacteria* and two species of *Bacteroides*. Herbal extracts have been shown to be more effective at treating diabetes than the commonly used drugs acarbose and metformin, according to the literature [384-385]. Researchers also found that BA metabolism was controlled by *Ruminococcus* strains [386]. This suggests that *Ruminococcusbromii* 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 [382]. Multiple *Bifidobacterium* spp. were inhibited by berberine in both humans and animals [387]. Therefore, *Bifidobacterium* strains may have an especially positive effect on elderly people with DM who are taking Berberine [388]. *Bifidobacteriumpseudolongum* was also shown to control the number of bacteria in the gut, implying that its presence was essential for the proliferation of the genus. Overgrowth organisms like *Staphylococcicoliforms* were prevented by berberine, but native *lactobacilli* and *bifidobacteria* were unaffected [305]. The quantity

of gut microbes such as *Actinobacteria*, *Lactobacillus*, and *Firmicutes* was decreased in an animal study with DM [306], and berberine was able to effectively manage glucose metabolism and re-establish glucose homeostasis. Previous research revealed that a new metabolite of berberine mediated by the gut microbiome called oxyberberine, which is also an important bioactive constituent of PHE, was able to keep the intestinal microbiota in equilibrium via modifying the gut microbiota's structure [306]. Andrographolide enhanced *Prevotella* and *Adlercreutzia* at the level of genera[362]. 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 [309, 311-312]. Thus, PHE containing bioactive compounds like andrographolide, kaempferol, quercetin, luteolin, and rutin may alter the gut microbiota's composition by raising the number of good bacteria. Together with each other, our results showed that genistein [314-316], hesperidin [317], isorhamnetin [318], and ferulic acid [319] are the important contents of the PHE in the present study, which may have a direct interaction with the gut microbiotaand 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 revealed that PHE treatment compared with metformin and DMR could significantly alter the structure and function of the beneficial intestinal microbiota.This finding of the study would be because of the presence of numerous biomarkers and bioactive compounds in PHE [364]. 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 hasthe potential to produce SCFAs such as acetate

and propionate in good proportion, which could induce gut gluconeogenesis via supportive mechanisms [364] and reduce blood glucose levels as well as increase body mass index (good health). These findings were supported by a correlation study between the enrichment of gut bacteria with PHE treatment and a couple of DM-related indices. We generalized our prior study's [82] 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 anti-diabetic medicine, was capable of diminishing and mitigating 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.

Further, we used data-driven analysis to look into the association between gut microbiota and metabolites to find out more about how PHE could be used to treat diabetes. Prior studies have demonstrated a strong relationship between the gut microbiota and metabolite structure in the treatment of DM, but the descriptions of the relevant metabolites and related target genes are still not clear. Recent studies have demonstrated that the gut microbiome's transcriptional activity and metabolome are significantly altered by STZ-induced hyperglycemia. It only takes induced hyperglycemia to change the amount of intestinal metabolites, increase dysbiosis, and lessen colonization resistance, as evidenced by the association between hyperglycemia-related alterations and worsened STZ-induced microbiome dysbiosis [389]. In this context, eubiosis in gut microbiome structure can exhibit a positive role in reducing reactive species through metabolites (acetate, butyrate, propionate, l-isoleucine, proline, creatine, 3-phenyl propionic acid, ursodeoxycholic acid, and 3-indole propionic acid), while dysbiosis may contribute to systematic inflammation, increased intestinal permeability, and β-cell damage. In the present study, ursodeoxycholic acid was defined as the most important metabolite among others in reducing the pathogenesis of DM.

The results indicated that the relative abundance of microbiome families like Desulfovibrionaceae, Lactobacillaceae, Prevotellaceae, Bacteroidaceae, and Ruminococcaceae while reducing Clostridiaceae, Aerococcaceae, Staphylococcaceae, and Turicibacteraceae attenuated DM progression and insulin resistance in rats. At the genus level, *Oscillospira*, *Desulfovibrio*, *Lactobacillus*, *Prevotella*, and *Ruminococcus* were restored in the gut of DM rats via PHE treatment, while a lowering in the relative abundance of groups like *Aerococcus*, *Staphylococcus*, *Atopostipes*, and SMB53 indicated the function of polyphenols in maintaining the intestinal lining. Kaempferol [308], quercetin [309], and rutin [313] have been shown to have anti-diabetic activity by altering gut microbial structures in studies, and as these bioactive compounds are enriched in the PHE obtained through HPTLC analysis, their consistent anti-diabetic activity in rats in the currentresearch was revealed. The correlation between outcomeswithinthe gut microbiota and lipid metabolism DM indexes supports the conclusion of the present study that PHE may build glucose homeostasis, including lipid metabolism, through reshaping the intestinal flora. In the recent past, target compounds and medical diagnosis have both been accomplished using network-based systems pharmacology [128]. The findings of network-based systems pharmacology studies confirm the effectiveness of this technique in our experimental study for the therapeutic management of DM, and the current study showed that significant microbiome-derived metabolites could be discovered using this method.

In the PPI networks, the core genes (CASP3, MPO, PARP1, CTSG, MMP14, MMP2, SHBG, and ALB) were identified as the important targets. The TNF signaling pathway and the AGE-RAGE signaling pathway were found to have more MMP2, MMP14, and CASP3 than other pathways. Upregulation of the AGE-RAGE signaling pathway speeds up the bad effects of diabetes, such as liver damage, inflammation, and fibrosis of the liver. Because of this, limiting AGEs might be a way to treat diabetes. TNF-α expression was elevated in DM

patient samples; nevertheless, rats with TNF receptor deletions also exhibited reduced hepatocyte fibrosis, inflammation, and steatosis in the PHE treatment groups. According to the findings of the GO enrichment analysis, the majority of the DM targets for gut microbiota metabolites are connected to endopeptidase and peptidase activities, metallo and serin-type endopeptidase activities, antioxidant activity, response to oxidative stress, responses to UV, chemical, abiotic stimulus, and reactive oxygen species, and enrichment of these genes in the lumens of secretory granules as well as the cytoplasmic vesicle to relieve DM. This investigation clarifies the roles played by metabolites generated by the gut microbiome in the management of DM. Especially *Prevotella* and *Ruminococcus* display an excellent preventive effect on DM rats by improving IR, dyslipidemia, oxidative stress, inflammation, and protecting beta-cell and liver function [374]. It also offers excellent potential for use in the pharmaceutical sector to halt the development of diabetes. This revealed that inhibition of MMP2, MMP14, and CASP3 genomic-linked pathways through antagonistic activity of metabolites like ursodeoxycholic acid generated from gut microbiota families showed a direct link with the family Ruminococcaceae, which primarily connected with other families like Prevotellaceae, Bacteroidaceae, and Desulfovibrionaceae, and this might be a good approach to glucose homeostasis. In our study, ursodeoxycholic acid is a promising antagonist that binds stably to TNF (PDB ID: 1U5Z) and FGFR4 (PDB ID: 4XCU), which also shows strong binding to core targets from the human genome database like MMP2 (PDB ID: 3AYU), MMP14 (PDB ID: 4QXU), and CASP3 (PDB ID: 4JQZ). The production of ursodeoxycholic acid by several Firmicutes suggested the potential health benefits of PHE on the hosts via the activation of intestinal membrane receptors. Ursodeoxycholic acid, a therapeutic bile acid, improves intestinal barrier function and was discovered to shield colonic epithelial cells against oxidative stress and apoptosis in a recent study, suggesting PHE could regulate metabolic disorders via modification of the gut microbiota [390]. All things considered, our

findings imply that PHE might modify the environment of the intestinal microbiome by encouraging the growth and metabolism of the resulting gut microbiota. Diabetes could be treated by regenerating pancreatic β-cells through a PHE-mediated metabolic environment. There were increases in IL-4 and Bcl-2-positive cells and decreases in TNF-α, COX-2, IL-18, and SOX-9-positive cells in different PHE's treatment groups in contrast to the diabetic rats group, suggesting that PHE treatment at both doses significantly stimulated the re-growth of β-cells in diabetic rats. Therefore, PHE-treated groups maintained β-cell mass of pancreas, morphometry, and insulin enrichment in comparison to normal control rat groups, and similar results were concluded in the bioinformatic approach [128, 357]. This was likely due to the enrichment of kaempferol, quercetin, and rutin in PHE.

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