Pharmacogn. Mag.
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ORIGINAL ARTICLE

Polyherbal Formulation Improves Glucose-Lipid Metabolism and Prevent Hepatotoxicity in Streptozotocin-Induced Diabetic Rats: Plausible Role of IRS-PI3K-Akt-GLUT2 Signaling

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Submitted: 15-Jul-2021 Revised: 23-Aug-2021 Accepted: 07-Dec-2021 Published: 28-Mar-2022

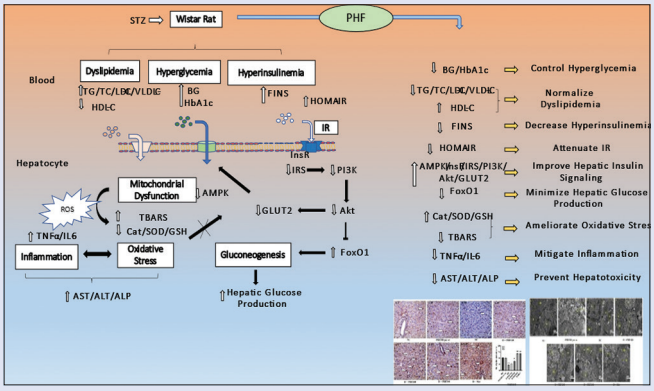
ABSTRACT

Background: Type 2 diabetes mellitus is a progressive polygenic disorder requiring a multi-targeted therapeutic approach. A polyherbal formula (PHF) comprised of four traditional herbs known for their anti-diabetic, anti-oxidant, and hepatoprotective activities were used in this study. **Objectives:** The purpose of this research was to find out the plausible mechanisms underlying the synergistic effects of PHF in an experimental setting of diabetes mellitus. **Materials and Methods:** PHF was evaluated for the presence of residual toxins, acute oral toxicity, and its effects on carbohydrate digestive enzymes. PHF (100, 200, and 300 mg/kg/p. o., respectively) was used to treat streptozotocin-induced diabetic rats for 12 weeks followed by glycemic, histopathological, biochemical, and ultrastructural changes measurement. mRNA levels of AMPK, insulin receptor substrate (IRS), phosphoinositide-3-phosphate kinase (PI3K), Akt, and glucose transporter 2 (GLUT2) were measured using real-time polymerase chain reaction. **Results:** In PHF, the residual toxins were absent. PHF inhibited the α-amylase (IC_{50} 152.2 ± 9.3 μg/mL), and α-glucosidase (IC_{50} 103.0 ± 0.81 μg/mL) activities *in-vitro*. Further, up to a dose of 2000 mg/kg, PHF showed no signs of mortality. PHF treatment significantly maintained the glycemic state, antioxidant status, lipid profile, hepatic-architecture, and ultrastructural alterations in diabetic rats. Similarly, diabetic rats showed detrimental effects on the mRNA level of AMPK and IRS-PI3K-Akt-GLUT2 signaling which were attenuated by PHF treatment. Immunohistochemical analysis also revealed an improvement in p-Akt expression after PHF treatment. **Conclusion:** In streptozotocin-induced diabetic rats, PHF improves glucose and lipid metabolism by attenuating hyperglycemia, hyperinsulinemia, dyslipidemia, insulin resistance, oxidative stress, inflammation, and deterioration of hepatic architecture possibly through AMPK mediated improvement in hepatic IRS-PI3K-Akt-GLUT2 signaling pathway.

Key words: Glucose-lipid metabolism, hepatic insulin signaling, oxidative stress, polyherbal formula, streptozotocin-induced diabetes, transmission electron microscopy

SUMMARY

- Type 2 diabetes mellitus is a progressive polygenic disorder requiring a multi-targeted therapeutic approach. A polyherbal formula (PHF) comprised of four traditional herbs *viz Nigella sativa, Trigonella foenum graecum, Chichorium intybus,* and *Azadirachta indica* used in this study to ascertain the plausible mechanisms underlying the synergistic effects in an experimental model of diabetes mellitus. PHF was evaluated for the presence of residual toxins, acute oral toxicity, and its effects on carbohydrate digestive enzymes. Streptozotocin-induced diabetic rats were treated with PHF (100, 200, and 300 mg/kg/p. o. respectively) for 12 weeks followed by glycemic, biochemical, histopathological, and ultrastructural changes measurement. Furthermore, the effects of PHF were also examined on the hepatic insulin signaling. In the present study, PHF proved to be effective in improving glucose-lipid metabolism in diabetic rats by normalizing hyperglycemia, hyperinsulinemia, and dyslipidemia, enhancing insulin sensitivity, attenuating oxidative stress, mitigating inflammation, and preventing the deterioration of hepatic architecture, possibly through AMPK mediated improvement in hepatic insulin receptor substrate-phosphoinositide-3-phosphate kinase-Akt-glucose transporter 2 signaling.



Abbreviations used: AIP: Atherogenic index of plasma; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AMPK: AMP-activated protein kinase; ANOVA: Analysis of variance; AOT: Acute oral toxicity; API: Ayurvedic Pharmacopoeia of India; AST: Aspartate aminotransferase; CAD: Coronary artery diseases; CAT: Catalase; DC: Diabetic control; DNS: Dinitro salicylic acid; FBG: Fasting blood glucose; FI: Fasting insulin; FoxO1: Forkhead box protein O1; GLUT2: Glucose transporter 2; GSH: Reduced glutathione; H and E: Hematoxylin and eosin; HbA_{1c}: Glycosylated haemoglobin; HDL-C: High-density lipoprotein cholesterol; HMPs: Herbal medicinal plants; HOMA-IR: Homeostasis model of insulin resistance; IL-6: Interleukins-6; INSR: Insulin Receptor; IRS: Insulin receptor substrate; LD_{50}: Median lethal dose; LDL-C: Low-density lipoprotein cholesterol;

Cite this article as: Haye A, Ansari MA, Saini A, Ahmed Z, Munjal K, Shamsi Y, *et al.* Polyherbal formulation improves glucose-lipid metabolism and prevent hepatotoxicity in streptozotocin-induced diabetic rats: Plausible Role of IRS-PI3K-Akt-GLUT2 Signaling. Phcog Mag 2022;18:52-65.

MDA: Malondialdehyde; Met: Metformin; Na-CMC: Sodium-carboxymethyl cellulose; NAD: Nicotinamide; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; NC: Normal control; OGTT: Oral glucose tolerance test; PI3K: Phosphoinositide-3-phosphate kinase; qRT-PCR: Quantitative real-time polymerase chain reaction; PHF: Polyherbal formula; PHFH: PHF high dose; PHFL: PHF low dose; PHFM: PHF moderate dose; RI: Refractive index; ROS: Reactive oxygen species; S. E. M: Standard error mean; SOD: Superoxide dismutase; STZ: Streptozotocin; TEM: Transmission electron microscopy; TNF-α: Tumor necrosis factor-α.

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DOI: 10.4103/pm.pm_318_21

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a medical emergency that threatens both the global economy and quality of life. In 2019, T2DM affected around 463 million adult individuals worldwide, and this figure is projected to rise by 25% and 51% by 2030 and 2045 respectively.[1] With the increasing magnitude of T2DM, its associated complications are also expected to rise parallelly.[2] The liver is a target area of insulin functions, which plays an important role in glucose and lipid homeostasis.[3] In T2DM, hepatocytes fail in responding adequately to the physiological level of insulin, resulting in an imbalance in glucose-lipid metabolism. Consequently, Insulin resistance is exacerbated by hyperglycemia and dyslipidemia, which decrease hepatic insulin signaling.[4]

In normal physiological conditions, insulin's ability to attach to its receptors at the hepatic membrane activates insulin receptor substrate (IRS) leading to phosphoinositide-3-phosphate kinase (PI3K) activation. Once activated, PI3K phosphorylates Akt (Protein kinase B is another name for this enzyme) at Thr308 residue. Activation of Akt governs several physiological activities such as (1) glucose transporter 2 (GLUT2) translocation from the cytosol to the plasma membrane for glucose absorption (2) Forkhead box protein O1 (FoxO1) suppression limits gluconeogenesis; (3) promotion of glycolysis to ensure energy supply; (4) glycogen synthesis in glycogen synthesis kinase 3 dependent and independent manner; and (5) de novo lipogenesis. However, in T2DM, inappropriate action of insulin hampers insulin signaling and exaggerates hepatic insulin resistance.[5] Previous studies have confirmed that up-regulated p-Akt plays a vital role in improving insulin sensitivity and glucose-lipid metabolism.[6]

Insulin resistance exaggerates mitochondrial dysfunctions by elevating lipid peroxidation and subsequent an increase in reactive oxygen species (ROS). The increase in ROS production may lower the activity of antioxidant protein activities directly and parallelly amplify pro-inflammatory cytokines production such as Tumor necrosis factor-alpha (TNF-α) and interleukins (IL)-6.[7] The chronic production of pro-inflammatory cytokines and ROS promotes insulin resistance by impairing the hepatic insulin signaling pathway. This progressive rise in inflammation and oxidative stress and subsequent impairment in the hepatic insulin signaling gradually damages the function and integrity of hepatocytes.[7,8] These pathological characteristics involved in the progression of insulin resistance augments morbidity and mortality in patients with T2DM, thus repression of these causative factors can be pivotal to prevent and treat T2DM.

Considering the development of drug tolerance and adverse effects associated with conventional therapies, alternative and traditional medicines are growing interest in metabolic disease prevention and treatment due to their multifunctional physiological activities with minimal adverse effects.[9] Previous studies have supported the use of polyherbal formulation over a single drug due to the synergistic effects of the constituents against disease progression.[10-12] In this investigation, the polyherbal formula (PHF) used contains *Nigella sativa, Trigonella foenum graecum, Chichorium intybus,* and *Azadirachta indica*. These herbal medicinal plants contain various pharmacologically active phytochemicals including alkaloids, flavonoids, phenolic acids, terpenoids, which are widely reported to exert antidiabetic, hepatoprotective, antioxidant, and anti-inflammatory activities.[13-18] Findings from a meta-analysis suggested that *Trigonella foenum graecum* and *N. sativa* were effective in improving glucose-lipid homeostasis and controlling complications in patients with T2DM.[13,15] *C. intybus* exhibited hepatoprotective, insulin-sensitizing, and anti-inflammatory effects in diabetes mellitus.[14,16,18] Saleem *et al*. reported that *A. indica* can repair damaged hepatocytes and prevent nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) in diabetes mellitus.[17] The probable synergistic effects of these herbal constituents during T2DM progression are still unexplored.

In this study, PHF's effects and underlying mechanism of action have been studied using streptozotocin and nicotinamide (NAD-STZ) induced diabetic Wistar rats. NAD-STZ animal model is widely reported to present syndromes including hyperglycemia, hyperinsulinemia, hyperlipidemia, insulin resistance, and hepatic impairments in the disease pathogenesis, which are similar to the features of patients with T2DM.[19]

MATERIALS AND METHODS

Materials

The ingredients of PHF were procured from vendor and metformin (Met) was received as gift samples from Lupin Limited, Maharashtra, India, respectively. Nicotinamide (Purity, >98%), and streptozotocin (Purity, >98%) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Na-CMC (Sodium-carboxymethyl cellulose) was procured from S. D. Fine Chemicals (Mumbai, India). The calibration standards for analysis of residual toxins were purchased from Merck, Germany. All the other chemicals used for the study were of analytical grade.

Physicochemical evaluation

The part of the plant used and composition (%) of herbal medicine available in PHF is mentioned in Table 1. The organoleptic properties of PHF herbs such as the pH, ash values, foreign matter, extractive values, and moisture content were evaluated as per protocols given by Ayurvedic Pharmacopoeia of India.[12] Short-term laboratory-based accelerated stability testing can provide preliminary data to translate into real-time stability studies. Therefore, to examine the stability of PHF accelerated

Table 1: Composition of polyherbal formula

Name of herb	Family	Part used	Quantity used (500 mg) (%)
Nigella sativa	Ranunculaceae	Seed	48
Trigonella foenum-graecum	Fabaceae	Seed	24
Chichorium intybus	Asteraceae	Seed	24
Azadirachta indica	Meliaceae	Leaf	4

stability study was performed as described previously, at different intervals (0, 24, 48, 72, 96, and 120 h.) by maintaining 30°C ± 2°C and at 65% relative humidity.[10]

Determination of residual toxic contaminants

The analysis of residual toxins was performed as described by Telapolu *et al*. for the evaluation of toxic contaminants such as aflatoxins, heavy metal contaminants, residues, pesticide, and microbial load using calibration standards.[12]

Preparation of extract

The powder (5 g) of PHF was extracted with 70% methanol (3 × 25 mL) thrice through cold maceration for at least 24 h. The filtrate obtained was concentrated using a rotary vacuum evaporator (Superfit, India) at 60°C (yield 16.32% w/w). The PHF methanolic extract was used for the evaluation of the effects on carbohydrate digestive enzymes activity (*in-vitro*) and in STZ-induced diabetic Wistar rats (*in-vivo*).

Determination of digestive enzymes inhibition activity

The enzymes, α-amylase, and α-glucosidase that digest carbohydrates in the gastrointestinal tract were determined as described previously.[12] To see if PHF extract has any influence on the activity of α–amylase (EC 3.2.1.1), 10 µM enzyme solution (200 µl) was incubated for 10 min at 25°C with a reaction mixture containing 200 µl of PHF extract (1.95–1000 µg/mL in DMSO), 0.2% starch solution (250 µl), and 250 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl). The reaction was halted after incubation by boiling for 1 min at 90°C–100°C following the addition of di-nitro salicylic acid color reagent (500 µl). The tubes were then placed in a hot water bath for 5 min. The absorbance of the mixture was estimated at 540 nm and the percentage (%) inhibition of α-amylase activity was calculated using the formula: % inhibition = ([A540 control-A540 extract]/A540 control) × 100. As a positive control, acarbose was used.

To measure the effect of PHF extract on the activity of α-glucosidase (EC 3.2.1.20), 10 mg/mL of enzyme solution was preincubated for 5 min with 200 µl extract of PHF (1.95–1000 µg/mL in DMSO). For reaction to initiate, 200 µl sucrose (37 mM) was added into the mixture. After incubation at 37°C for 15 min at 90°C–100°C, the reaction was stopped and liberated glucose was measured using the available commercial kit (Accurex, India). The absorbance of the mixture was determined at 405 nm and the % inhibition of α-glucosidase activity was estimated using the formula: % inhibition = ([A405 control-A405 extract]/A405 control) × 100. As a positive control, acarbose was used.

Experimental animals

The Jamia Hamdard institutional animal ethics committee (Reg. No.: 173/GO/Re/S/2000/CPCSEA), New Delhi, examined and approved the study protocol (India). Wistar albino rats (200–250 gms) were procured from Central Animal House Facility, Jamia Hamdard, New Delhi (India). Rats were placed in polypropylene cages (4/cage) for 1 week to acclimatize to the normal conditions (Temp. 23°C ± 2°C; RH 60% ± 5% and 12 h light: dark cycle). Experimental rats were permitted to feed standard rat laboratory chow (Nav Maharashtra Chakan Oil Mills Ltd., Delhi, India) and water *ad libitum*. Guidelines of the Committee for Control and Supervision of Experimentation on Animals were followed to perform the study.

Acute oral toxicity testing

Acute oral toxicity (AOT) study was performed according to the Organization of Economic Cooperation and Development (OECD) Test guidelines (AOT-OECD Test guideline 425) using a statistical program (AOT425StatPgm, version 1.0). Up and down procedure was performed, using different doses of up-to 2000 mg/kg (p. o.) of PHF extract in Wistar rats. Rats were kept fasted for 4 h before doses administration with access to water *ad libitum*. The bodyweight of the fasted rats was considered to calculate the doses of PHF. When the first rat survived after administration of 2000 mg/kg dose, then sequential doses were administered to the remaining rats. During the AOT study, rats were closely observed with special attention for any specified signs of acute toxicity for the first 30 min and then at regular intervals for 14 days.[20]

Induction of diabetes

Experimental diabetes was induced chemically in overnight fasted rats by single-dose intraperitoneal (i. p.) administration of nicotinamide (230 mg/kg) followed by administration of streptozotocin (STZ 65 mg/kg, i. p.) both freshly prepared in normal saline (0.9%) and citrate buffer (pH 4.5) with a difference of 15 min.[21] After 1 week, diabetes was confirmed using Accu-Chek Blood Glucose Meter (Roche Diagnostics, Mannheim, Germany). Animals with fasting blood glucose (FBG) levels >200 mg/dl were measured for the experiment. Vehicle (0.5% Na-CMC) and drug treatment were given orally after confirmation of diabetes through gastric gavage for 12 weeks.

Experimental design

A total of 56 animals were used for this study. All the acclimatized animals ($n = 8$) were randomly divided into seven groups:

I. Normal Control (NC): received 1 ml of 0.5% Na-CMC (vehicle);
II. *Per SE* PHF high dose (PHF300): received PHF 300 mg/kg;
III. Diabetic Control (DC): received a single injection of NAD-STZ followed by 1 ml of vehicle;
IV. Diabetic + PHF low dose (D + PHF100): diabetic rats received PHF 100 mg/kg;
V. Diabetic + PHF moderate dose (D + PHF200): diabetic rats received PHF 200 mg/kg;
VI. Diabetic + PHF High dose (D + PHF300): diabetic rats received PHF 300 mg/kg;
VII. Diabetic + metformin (D + Met): diabetic rats received metformin 250 mg/kg.

The diabetic animals in groups III to VII received a single injection of NAD-STZ (230 and 65 mg/kg i. p., respectively) before starting the vehicle and drug treatment. The vehicle and drug treatments were given for 12 weeks. PHF and Met were dissolved in the vehicle (0.5% Na-CMC). At the end of the experiment, overnight fasted animals were re-weighed, samples of blood were collected by retro-orbital puncture, blood samples were processed for biochemical analysis. For glycosylated hemoglobin (HbA_{1c}) determination a small amount of blood was stored in EDTA tubes. Animals were then euthanized by cervical dislocation under CO_2, and the liver was removed and cleaned in normal saline. Small sections of liver samples were preserved 10% formalin solution for histopathological and protein estimations and in 2.5% glutaraldehyde plus 2% paraformaldehyde solution in 0.1M sodium phosphate

buffer (pH 7.2) for ultra-structural studies. The remaining samples of excised liver tissues were stored at −80°C for real-time polymerase chain reaction (PCR) analysis.

Determination of blood glucose, glycated hemoglobin (HbA_{1c}), and serum insulin

FBG levels were estimated by using a glucometer (Roche Diagnostics, Mannheim, Germany). The levels of whole blood HbA_{1c} and serum fasting insulin (FI) were measured as per the manufacturer's instructions using ELISA kits from CrystalChem Inc. (Downwrs Grove, USA) and Mercodia (Uppsala, Sweden), respectively.

Determination of oral glucose tolerance test and homeostasis model of insulin resistance

The method reported by Pari and Saravanan was used to estimate oral glucose tolerance test (OGTT).[22] Before sacrificing the animals, glucose solution (2 g/kg) was administered orally to overnight fasted rats through gastric gavage. After glucose administration, blood samples were collected from the tail vein at different time intervals (0, 15, 30, 60, and 120 min). Glucose levels were measured using a glucometer. FBG and FI levels were used for the calculation of the homeostasis model of insulin resistance (HOMA-IR) by using the formula: HOMA-IR = FI (μIU/ml) × FBG (mg/dl)/405.

Determination of oxidative stress markers

The levels of the thiobarbituric acid reactive substrate (TBARS),[23] superoxide dismutase (SOD) activity,[24] catalase (CAT) activity,[25] and reduced glutathione (GSH) activity[26] in hepatic tissue were estimated as described previously.

Determination of serum lipids

The serum levels of triglycerides (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) were determined by commercially available kits (Sigma-Aldrich, Switzerland) according to the instructions of the manufacturer. Low-density lipoprotein cholesterol (LDL-C) and very-LDL cholesterol (VLDL-C) were calculated using Friedewald's method.[27] The atherogenic index of plasma (AIP), a strong marker of predicting the risk of coronary artery diseases (CAD) was calculated using the following formula: AIP = log10 (TG/HDL-C). The risk of CAD can be classified based on valves obtained as −0.3–0.1 for low risk of CAD, 0.1–0.24 for medium risk of CAD, and above 0.24 for high risk of CAD.[28]

Determination of liver marker enzymes

The hepatic enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) are well-accepted markers of hepatotoxicity. The activities of AST, ALT, and ALP in serum were assayed using commercially available kits as per the protocol given by the manufacturer (Sigma Aldrich, USA).

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA from liver tissue was separated, purified, and allowed to treat with DNAse-I using RNeasy mini kit (Qiagen) as per the supplier's instructions. A Nano-Drop 1000TM (Thermo Scientific) was used to estimate the quality and quantity of the total RNA. The integrity of the total RNA was evaluated using 23S/16S banding pattern visualization. Total RNA (1 μg) was used to prepare cDNA following the manufacturer's instructions using the Script cDNA synthesis kit (Bio-Rad). The sequence of the primers and their accession number for the targeted and housekeeping genes are given in Table 2. The primers' efficiency was estimated by dilution and temperature gradient method. A 20 μl of reaction volume, containing SsoFast EvaGreen supermix qPCR (Biorad), 1 μl cDNA, and 500 ng/μl final concentration of each primer, was performed in real-time PCR. The following steps were performed by qRT-PCR on a CFX 96 (Bio-Rad)– (a) 95°C for 30 s, (b) 39 cycles of 95°C for 5 s, (c) Tm for 15 s and (d) 72°C for 15 s. Relative fold change in each target mRNA transcript was calculated using the $2^{-\Delta\Delta Ct}$ method.[29] All experiments were performed in triplicates.

Histopathological, immunohistochemical, and ultrastructural (transmission electron microscopy) analysis

For hematoxylin and eosin (H and E) staining and immunohistochemical analysis liver tissues were fixed in 10% formalin, sliced, and embedded in paraffin wax. 5 μm thick sections were sliced and processed as per the method described previously.[29] Slides were prepared and examined under light microscopy with the help of a pathologist blinded to the study groups. Photomicrographs were captured using a computer-enabled Motic microscope. Semi-quantification of protein expression (p-Akt) was done using Fiji (ImageJ) software. For the ultrastructural study, the sections of liver tissue were directly fixed into modified Karnovasky's (2.5% glutaraldehyde and 2% paraformaldehyde solution in 0.1M sodium phosphate buffer, pH 7.2) solution.[30] After fixation, tissue sections were embedded in CY212. Ultrathin sections were stained with 2% uranyl acetate and 2% lead acetate and examined under Transmission Electron Microscope TECNAI 200 kV TEM (Fei, electron optics, Hillsboro, USA).

Statistical analysis

The data were presented as mean ± standard error of the mean ($n = 8$). Data of different groups were compared using one-way analysis of variance followed by Dunnett's t-test. The value of $P < 0.05$ was considered statistically significant in the results. Statistical analysis was carried out using Graph Pad Prism 8.0 software (Graph Pad Software, San Diego, California, USA).

RESULTS

Physiochemical analysis of ingredients in polyherbal formula

Morphological and physicochemical parameters are used as key references during scaling up the production during quality control. Parameters such as macroscopic and microscopic features, loss on drying at 105°C, foreign matter, total ash, acid insoluble ash, water-soluble extractive, alcohol soluble extractives for each ingredient of PHF is represented in Table 3. After comparing all the parameters with the dry weight of the standard powders no visible evidence of mold growth, insect contamination, and any sign of the presence of foreign matter was found.

Accelerated stability study of polyherbal formula

There were no significant changes observed in color, odor, appearance, pH, surface tension, viscosity, specific gravity, and refractive index (RI) throughout the study. Furthermore, the microbial load in PHF was absent throughout the testing with 4726.8 ± 159.0 CFU/g total bacterial counts. The data is represented in Table 4.

Table 2: Primer sequence for quantitative real-time PCR

Gene	Primer	Sequence (5'-3')	Size (bp)	Accession number
TNF-α	Forward	GTCCCAACAAGGAGGAGAAGTT	125	NM_012675.3
	Reverse	GTTTGCTACGACGTGGGCTA		
IL-6	Forward	CATTCTGTCTCGAGCCCACC	91	NM_012589.2
	Reverse	GCTGGAAGTCTCTTGCGGAG		
AMPK	Forward	AGCCGACTTCGGTCTTTCAA	113	NM_019142.2
	Reverse	GGCCTGCGTACAATCTTCCT		
INSR	Forward	GGACCAGGCATCCTGTGAAA	178	NM_017071.2
	Reverse	ATCCTGCCCGTCAAACTCTG		
IRS-1	Forward	CTCCCTGCATCGGACTCTAC	150	NM_012969.1
	Reverse	TAAGGGCGTTTCATGCCCAG		
PI3K	Forward	CCCGGGTAGGTTTGAATTCGTT	95	NM_001371300.1
	Reverse	ATGCCCTAGGTGACCTGACA		
Akt	Forward	TGGAGTGTGTGGACAGTGAAC	275	XM_006240631.2
	Reverse	AACCCCAGGATAGTTTTCCTCCT		
GLUT-2	Forward	CATCAATACGCAGCCTTGGTTA	258	XM_006232207.3
	Reverse	CAGCTCAGTCCTCACGATGT		
FoxO1	Forward	GTGCCCCAGGACTCTTGAAA	98	NM_001191846.2
	Reverse	ACTGTTGGGTTGAGCCACTC		
GAPDH	Forward	AGTGCCAGCCTCGTCTCATA	248	NM_017008.4
	Reverse	GATGGTGATGGGTTTCCCGT		

TNF-α: Tumor necrosis factor-α; IL-6: Interleukins-6; AMPK: AMP-activated protein kinase; INSR: Insulin receptor; IRS: Insulin receptor substrate; PI3K: Phosphoinositide-3-phosphate kinase; GLUT-2: Glucose transporter 2; FoxO1: Forkhead box protein O1; PCR: Polymerase chain reaction

Table 3: Morphological and physicochemical parameters of polyherbal formula herbs

Parameters	*Nigella sativa*	*Trigonella foenum-graecum*	*Chichorium intybus*	*Azadirachta indica*
Macroscopic features	Seed flattened oblong, angular, rugulose tubercular, small, funnel-shaped, 0.2 cm long and 0.1 cm wide, black; odor slightly aromatic; taste bitter	Seed oblong, rhomboidal with deep furrow running obliquely from one side, dividing seed into a larger and smaller part, 0.2-0.5 cm long, 0.15-0.35 cm broad, smooth, hard, dull yellow; odor pleasant, taste bitter	The seed brown weed shaped, gradually tapering towards base, 0.20-0.30 cm long, 0.10-0.15 cm wide, roughly 4-5 ridged, pappus present, sepals five, thin, membranous, seed one anatropous on basal placentation; dicotyledonous, ex-albuminous, taste and odor indistinct	Leaf compound, alternate, rachis 15-25 cm long, 0.1 cm thick; leaflet with oblique base, opposite, exstipulate, lanceolate, acute, serrate. 7-8.5 cm long and 1.0-1.7 cm wide, slightly yellowish green; with a characteristic odor, taste bitter
Microscopic	Complies as per standard	Complies as per standard	Complies as per standard	Complies as per standard
Thin-layer chromatography	Complies as per standard	Complies as per standard	Complies as per standard	Complies as per standard
Loss on drying at 105°C	6.6% w/w	6.5% w/w	6.4% w/w	7.6% w/w
Foreign matter	0.3% w/w	0.3% w/w	0.3% w/w	0.5% w/w
Total ash	4.9% w/w	3.2% w/w	5.3% w/w	8.0% w/w
Acid insoluble ash	0.1% w/w	0.4% w/w	3.7% w/w	0.7% w/w
Water soluble extractive	19.5% w/w	30.15% w/w	10.4% w/w	23.7% w/w
Alcohol soluble extractive	21.3% w/w	5.6% w/w	5.4% w/w	14.1% w/w

Table 4: Accelerated stability study of polyherbal formula

Parameters	Observations and time interval						
	Times (0 h)	Times (24 h)	Times (48 h)	Times (72 h)	Times (96 h)	Times (120 h)	Mean±SD
Color	Brown	Brown	Brown	Brown	Brown	Brown	
Odor	Pleasant	Pleasant	Pleasant	Pleasant	Pleasant	Pleasant	
Appearance	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	
pH	4.7	4.7	4.8	4.7	4.6	4.7	4.7±0.06
Viscosity	1.07	1.09	1.08	1.09	1.11	1.08	1.09±0.01
Surface tension (*n*/m)	115.2	115.65	116.25	115.52	115.85	116.95	115.9±0.57
Specific gravity	1.52	1.56	1.52	1.53	1.54	1.51	1.53±0.02
RI	1.492	1.485	1.521	1.519	1.534	1.539	1.52±0.02
Bioburden level (microbial load)							
Total aerobic plate count (CFU/g)	4900	4888	4795	4726	4598	4454	4726.8±159.0
Escherichia coli	Absent	Absent	Absent	Absent	Absent	Absent	
Staphylococcus aureus	Absent	Absent	Absent	Absent	Absent	Absent	
Salmonella	Absent	Absent	Absent	Absent	Absent	Absent	
Klebsiella	Absent	Absent	Absent	Absent	Absent	Absent	
Clostridium botulinum	Absent	Absent	Absent	Absent	Absent	Absent	

CFU: Colony-forming unit; RI: Refractive index; SD: Standard deviation

Residual analysis of polyherbal formula

The data obtained from the residual analysis of PHF are represented in Table 5. The data showed the absence of (a) heavy metal contaminants such as lead (Pb), mercury (Hg), arsenic (As), and cadmium (Cd), (b) aflatoxins (B1, B2, G1, G2), and (c) pesticide classes including organochlorine pesticides, organophosphorus pesticides, and pyrethroids.

PHF inhibits carbohydrate digestion enzyme (*in-vitro*)

Carbohydrate digestive enzymes, α-amylase, and α-glucosidase are responsible for glucose uptake into blood circulation by transforming polysaccharides and disaccharides into monosaccharides.[10] In this study, PHF demonstrated dose-dependent inhibition of the activity of α-amylase with an IC_{50} 152.2 ± 9.3 μg/mL, whereas reference standard acarbose showed IC_{50} 179.8 ± 9.56 μg/mL [Figure 1a]. Similarly, PHF and acarbose demonstrated strong inhibition of α-glucosidase activity in a dose-dependent manner with an IC_{50} 103.0 ± 0.81 μg/mL and IC_{50} 115.8 ± 1.68 μg/mL, respectively [Figure 1b]. The α-amylase inhibitory action was similar to acarbose with a maximum of 70.83% ± 0.11% activity, whereas α-glucosidase inhibitory action was stronger ($P < 0.05$) than acarbose with a maximum of 84.39% ± 0.02% activity.

Table 5: Analysis of heavy metals, aflatoxins, and pesticide residue of polyherbal formula

Metallic contaminants	Acceptable limit	Result
Lead (Pb)	NMT 10 ppm	ND
Arsenic (As)	NMT 3 ppm	ND
Mercury (Hg)	NMT 1 ppm	ND
Cadmium (Cd)	NMT 0.3 ppm	ND
Aflatoxin		
B1	NMT 5 ppb	ND
B2	NMT 5 ppb	ND
G1	NMT 5 ppb	ND
G2	NMT 5 ppb	ND
Pesticide residue analysis		
Organochlorine pesticides		
DDT	NMT 1 ppm	ND
Endosulfan α	NMT 1 ppm (sum of isomers)	ND
Endosulfan β	NMT 1 ppm	ND
Organophosphorus pesticides		
Chlorpyrifos	NMT 0.2 ppm	ND
Pyrethroids		
Cypermethrin	NMT 1 ppm	ND

NMT: Not more than; ND: Not detected; DDT: Dichloro-diphenyl-trichloroethane

Acute oral toxicity study

The PHF did not cause any death up to the 2000 mg/kg dose, therefore, LD_{50} could not be calculated. However, mild writhing was recorded in the rats administered with a 2000 mg/kg dose of PHF.

Polyherbal formula controls blood glucose and body weight in streptozotocin-induced diabetic rats

The blood glucose levels at-3, 0, and last day, and body weight at 0 and the last day of the study are presented in Table 6. There was no significant difference in blood glucose levels was recorded in the groups at-3 days (i.e., before STZ administration). However, DC rats showed a significant increase ($P < 0.001$) in the blood glucose level at 0 days when compared to NC rats. Treatment with different doses of PHF and Met for 12 weeks significantly decreased blood glucose levels ($P < 0.05$ and 0.001) when compared with DC rats. Similarly, at 0 days, there was not any significant difference observed in the bodyweight of rats among the groups. However, after 12 weeks, diabetic rats have shown a significant decrease in body weight vs normal control rats ($P < 0.001$). Treatment with high dose and Met showed significant improvement in the bodyweight loss ($P < 0.05$ and 0.01 respectively), however, treatment with a low dose and moderate dose of PHF did not show any significant difference when compared with the DC group.

Effect of polyherbal formula on oral glucose tolerance test in streptozotocin-induced diabetic rats

After administration of glucose (2 gms/kg) in experimental rats, OGTT at 0, 15, 30, 60, and 120 min is represented in Table 7. The blood glucose level of DC rats was significantly increased ($P < 0.001$) when compared with NC rats. However, treatment with different doses of PHF and Met significantly reduced the blood glucose level ($P < 0.05$ and 0.001) when compared with DC rats.

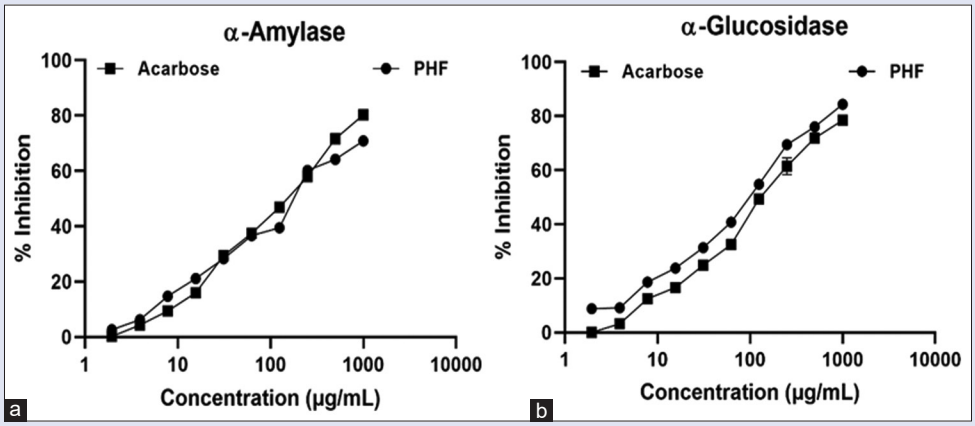

Figure 1: Polyherbal formula inhibits carbohydrate digestion enzyme (*in-vitro*). Effect of polyherbal formula on % inhibition of (a) α-amylase and (b) α-glucosidase activities; Values are expressed as mean ± standard error mean ($n = 3$)

Table 6: Polyherbal formula controls blood glucose and body weight in streptozotocin induced diabetic rats

Groups	BG (mg/dl)			BW (g)	
	At - 3 days	At 0 day	At the last day	At 0 day	At the last day
NC	86.16±2.49	91.25±3.06	90.75±3.21	232.63±3.06	299.75±6.07
PHFH per se	89.25±3.09	89.00±2.17	87.38±2.59	229.25±3.77	303.00±5.76
DC	85.63±3.07	291.88±7.23***	391.38±6.69***	231.63±3.48	221.50±3.27***
D + PHFL	88.88±3.15	298.75±5.43***	363.00±7.86#	230.63±3.82	233.25±3.43
D + PHFM	91.50±3.61	295.25±6.97***	310.50±9.41###	230.38±3.11	235.13±3.09
D + PHFH	89.25±3.35	290.13±7.61***	244.63±8.65###	232.63±4.2	243.50±4.10#
D + MET	86.13±3.12	293.23±7.60***	239.75±6.23###	231.5±3.78	244.50±4.94##

***P<0.001 as compared to NC group; #P<0.05; ##P<0.01 and ###P<0.001 as compared to the DC group; Data are expressed as mean±SEM (n=8). BW: Body weight; BG: Blood glucose; SEM: Standard error of mean; NC: Normal control; PHF: Polyherbal formula; PHFH: PHF high dose; PHFL: PHF low dose; PHFM: PHF moderate dose; DC: Diabetic control; MET: Metformin; D: Diabetic; FINS: Fasting insulin

Table 7: Effect of polyherbal formula on oral glucose tolerance test in streptozotocin induced diabetic rats

Groups	OGTT (mg/dl)				
	At 0 min	At 15 min	At 30 min	At 60 min	At 120 min
NC	89.33±3.68	117.5±3.42	155.83±3.86	124.17±3.66	93.67±3.84
PHFH per se	90.67±3.27	117.0±2.71	154.33±3.72	123.17±3.49	90.83±3.54
DC	393.17±4.30***	427.33±4.62***	463.67±6.98***	435.67±7.14***	400.83±7.94***
D + PHFL	370.0±6.01	397.33±4.99##	450.17±7.32	419.05±6.29	376.5±4.53#
D + PHFM	308.17±5.77###	346.0±4.27###	399.00±4.31###	368.0±5.96###	307.50±5.05###
D + PHFH	246.17±5.68###	294.33±6.23###	355.83±6.06###	306.33±5.65###	249.0±5.01###
D + MET	240.83±5.78###	288.17±5.87###	357.50±4.30###	299.0±3.87###	239.67±4.62###

***P<0.001 as compared to NC group; #P<0.05; ##P<0.01 and ###P<0.001 as compared to the DC group; Data are expressed as mean±SEM (n=6). OGTT: Oral glucose tolerance test; NC: Normal control; PHF: Polyherbal formula; PHFH: PHF high dose; PHFL: PHF low dose; PHFM: PHF moderate dose; DC: Diabetic control; MET: Metformin; D: Diabetic; SEM: Standard error of mean

Table 8: Polyherbal formula normalizes fasting insulin, hemoglobin A1c, and homeostasis model of-insulin resistance in Streptozotocin induced diabetic rats

Groups	FI (μIU/ml)	HbA_{1c} (%)	HOMA-IR
NC	13.95±0.73	5.40±0.08	3.14±0.22
PHFH per se	13.12±0.85	5.28±0.06	2.83±0.21
DC	23.12±0.64***	9.76±0.09***	22.37±0.82***
D + PHFL	22.66±0.75	9.63±0.10	20.33±0.87
D + PHFM	20.19±0.84#	9.38±0.07##	15.44±0.69###
D + PHFH	18.48±0.57###	8.19±0.04###	11.10±0.35###
D + MET	17.52±0.46###	7.84±0.11###	10.36±0.34###

***P<0.001 as compared to NC group; #P<0.05; ##P<0.01 and ###P<0.001 as compared to the DC group; Data are expressed as mean±SEM (n=6). FI: Fasting insulin; HbA_{1c}: Hemoglobin A1c; HOMA-IR: Homeostasis model of insulin resistance; NC: Normal control; DC: Diabetic control; D: Diabetic; PHF: Polyherbal formula; PHFH: PHF high dose; PHFL: PHF low dose; PHFM: PHF moderate dose; SEM: Standard error of mean; MET: Metformin

Polyherbal formula normalizes fasting insulin, HbA_{1c}, and homeostasis model of insulin resistance in streptozotocin-induced diabetic rats

Serum insulin, HbA_{1c} (%) levels, and HOMA-IR index of all experimental rats are presented in Table 8. Levels of serum insulin, HbA_{1c}, and HOMA-IR index were significantly elevated (P < 0.001) in the DC rats when compared with NC rats. Treatment with moderate and high doses of PHF, and Met significantly reduced (P < 0.05, 0.01, and 0.001) the levels of serum insulin, HbA_{1c}, and HOMA-IR when compared with DC rats.

Polyherbal formula ameliorates oxidative stress in streptozotocin-induced diabetic rats

TBARS level was determined in experimental rats by evaluating the hepatic malondialdehyde content. A significant (P < 0.001) increase in the TBARS level was observed in DC rats when compared with NC rats. Treatment with different doses of PHF and Met significantly (P < 0.05, 0.01, and 0.001) decreased the elevated level of TBARS as compared to the DC rats. On the other hand, DC rats showed a significant (P < 0.001) reduction in anti-oxidant parameters (SOD, CAT, and GSH) when compared with NC rats. Treatment with PHF and Met significantly (P < 0.05, 0.01, and 0.001) increased the levels of SOD, CAT, and GSH when compared to DC rats. However, treatment with a low dose of PHF did not show any significant effect on CAT and GSH levels when compared with DC rats. The data of oxidative stress markers are presented in Table 9.

Polyherbal formula normalizes lipid profile and atherogenic index in streptozotocin-induced diabetic rats

Dyslipidemia is a key feature of T2DM. The levels of lipid parameters (TG, TC, HDL-C, LDL-C, and VLDL-C) and the AIP are represented in Figure 2a and b, respectively. The levels of TG, TC, LDL-C, and VLDL-C were significantly (P < 0.001) increased in DC rats when compared with NC rats. Treatment of diabetic rats with PHF and Met showed a significant (P < 0.05, 0.01, 0.001) reduction in the levels of TG, TC, LDL-C, and VLDL-C as compared to DC rats. Similarly, a significant (P < 0.001) reduction was observed in HDL-C in diabetic control rats when compared with NC rats. Treatment diabetic rats with PHF and Met significantly (P < 0.05, and 0.01) increased the level of HDL-C as compared to DC rats.

AIP was significantly (P < 0.001) higher in DC rats as compared to NC rats and the valves were above 0.24 suggesting the DC rats were at high risk of CAD. Treatment of diabetic rats with moderate and high doses of PHF and Met demonstrated a significant (P < 0.01 and 0.001) decline in AIP.

Table 9: Polyherbal formula ameliorates oxidative stress in Streptozotocin induced diabetic rats.

Groups	TBARS (nmol MDA/mg protein)	SOD (unit/mg of protein)	CAT (nmol H_2O_2/min/mg protein)	GSH (µmol GSH/mg protein)
NC	0.29±0.05	5.25±0.22	6.68±0.31	3.91±0.21
PHFH per se	0.28±0.06	5.17±0.19	6.60±0.42	3.93±0.14
DC	1.37±0.17***	1.60±0.11***	0.95±0.13***	0.87±0.07***
D + PHFL	0.99±0.09#	2.44±0.11#	2.01±0.11	1.35±0.13
D + PHFM	0.83±0.08##	2.64±0.15##	2.28±0.26##	1.59±0.16#
D + PHFH	0.70±0.08###	2.88±0.23##	2.76±0.20###	1.76±0.13##
D + MET	0.77±0.09###	2.76±0.18###	2.66±0.22###	1.72±0.15##

***P<0.001 as compared to NC group; #P<0.05; ##P<0.01 and ###P<0.001 as compared to the DC group; Data are expressed as mean±SEM (n=8). TBARS: Thiobarbituric acid reactive substrate; MDA: Malondialdehyde; NC: Normal control; DC: Diabetic control; D: Diabetic; PHF: Polyherbal formula; PHFH: PHF high dose; PHFL: PHF low dose; PHFM: PHF moderate dose; SEM: Standard error of mean; MET: Metformin; SOD: Superoxide dismutase; CAT: Catalase; GSH: Reduced glutathione; H_2O_2: Hydrogen peroxide

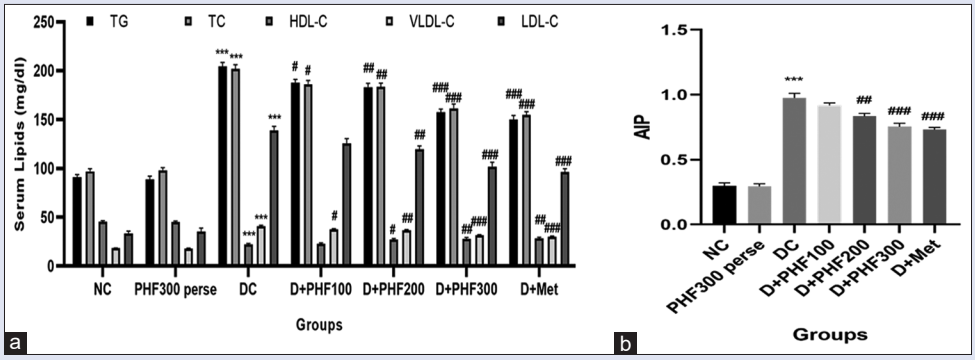

Figure 2: Polyherbal formula normalizes lipid profile (a) and atherogenic index (b) in Streptozotocin induced diabetic rats. Data are expressed as mean ± Standard error mean (n = 8). ***P < 0.001 as compared to NC group; #P < 0.05, ##P < 0.01 and ###P < 0.001 as compared to the DC group

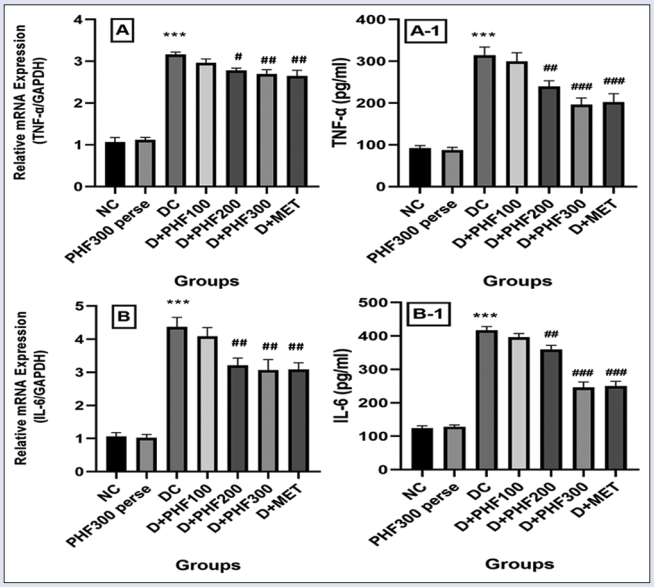

Figure 3: PHF mitigates inflammatory markers; TNF-α [3 A & A-1] and IL-6 [B & B-1] in Streptozotocin induced diabetic rats. Data are expressed as mean ± Standard error mean (n = 8). ***P < 0.001 as compared to NC group; #P < 0.05, ##P < 0.01 and ###P < 0.001 as compared to the DC group

Polyherbal formula alleviates hepatic marker enzymes in streptozotocin-induced diabetic rats

As shown in Table 10, the level of hepatic marker enzymes (AST, ALT, and ALP) was increased significantly (P < 0.001) in DC rats as compared to NC rats. Treatment of diabetic rats with moderate and high doses of PHF significantly (P < 0.05, 0.01, and 0.001) reduced the elevated level of hepatic enzymes as compared with DC rats.

Polyherbal formula mitigates inflammation in streptozotocin-induced diabetic rats

The plasma gene and protein levels of TNF-α and IL-6 in DC rats were significantly (P < 0.001) elevated as compared to NC rats. In diabetic conditions, treatment with moderate and high doses of PHF significantly reduced the gene and protein expressions of proinflammatory cytokines compared to DC rats. Data are depicted in Figure 3a and b.

Polyherbal formula improves hepatic insulin signaling in streptozotocin-induced diabetic rats

The mRNA expression of genes involved in hepatic insulin signaling is illustrated in Figure 4a-g. In DC rats, the mRNA expression of INSR, IRS-1, PI3K, AKT, GLUT2, and AMPK was significantly (P < 0.001) reduced, whereas mRNA expression of FoxO1 was increased when compared with NC rats, suggesting impairments in hepatic insulin signaling. Treatment of diabetic rats with moderate and high doses of PHF significantly up-regulated the mRNA expression of INSR, IRS-1, PI3K, AKT, GLUT2, and AMPK whereas reduced the overexpression of FoxO1.

Immunohistochemistry of p-Akt in hepatic tissue

As shown in Figure 5a I-VII and b, when compared with NC rats, the DC group resulted in significantly (P < 0.001) reduced expression of p-Akt. Whereas treatment with PHF200 and PHF300 significantly (P < 0.01 and 0.001) increased the expression of p-Akt when compared with DC rats.

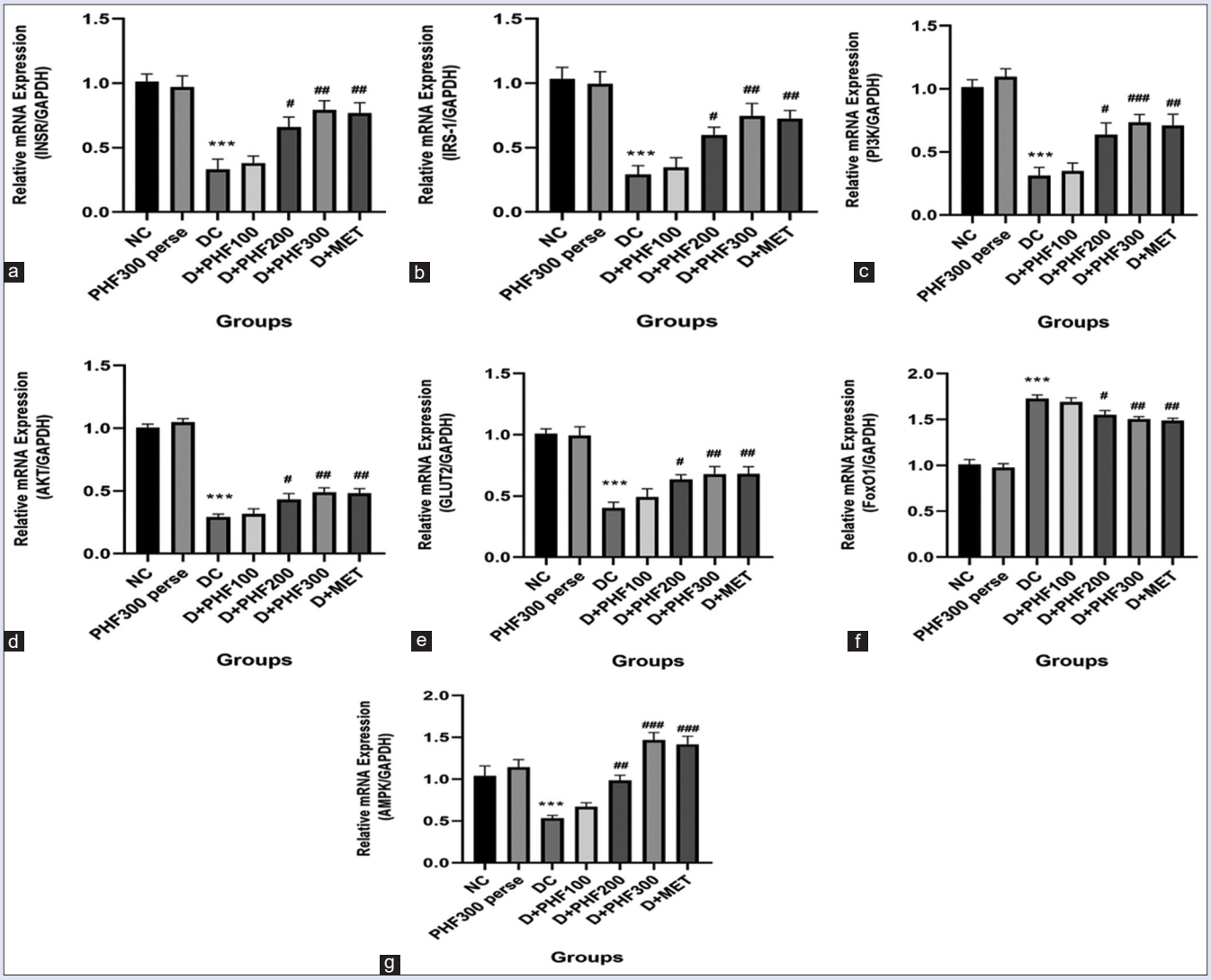

Figure 4: Polyherbal formula improves hepatic insulin signaling in Streptozotocin induced diabetic rats. (a) INSR; (b) IRS-1; (c) PI3K; (d) Akt; (e) GLUT2; (f) Foxo-1; (g) AMPK. Data are expressed as mean ± Standard error mean ($n = 8$). ***$P < 0.001$ as compared to NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ as compared to the DC group

Histopathological and ultrastructural analysis by hematoxylin and eosin staining and transmission electron microscopy

Figure 6 shows the histopathological changes in hepatic tissues of various groups. NC rats showed normal liver architecture with normally distributed hepatocytes containing prominent central veins in the hepatic lobule. DC rats showed significant deterioration of hepatic architecture with apparently congested and dilated central vein with infiltration of inflammatory cells around the portal tract, disturbed portal vein and sinusoids, presence of pyknotic nuclei, and vacuolization of hepatocytes. Treatment of diabetic rats with PHF100 and PHF200 improved the architecture of hepatocytes with lesser vacuoles and inflammatory cell infiltrates. Treatment of diabetic rats with PHF300 and Met prevented the deterioration of the hepatic tissues and restored the architecture of the hepatocytes.

Figure 7 illustrates the ultrastructure of hepatic tissues of various groups. Severe ultrastructure alterations were observed including heterochromatic nucleus with the deteriorated nuclear envelope, mild dissolution of cytoplasm with the appearance of several swollen mitochondria, disorganized rough endoplasmic reticulum, lipid droplets, vacuoles, collagen fibers, and process of Von Kupffer cells containing lysosomes in DC rats when compared with the normal hepatic architecture of NC rats. Treatment of diabetic rats with PHF100 and PHF200 improved the architecture of hepatocytes. Further, the treatment of diabetic rats with PHF300 and Met prevented the deterioration of the hepatic ultrastructure and restored the architecture of the hepatocytes.

DISCUSSION

Considering the complexity of T2DM pathogenesis and its deleterious effect on various organs, multidrug options are accepted over single components as a promising therapeutic strategy for the prevention and treatment of T2DM.[10] In recent years, the polyherbal formulation is considered to be an effective prevention and treatment option with no or minimal side effects for various diseases including T2DM.[12]

As per WHO guidelines for Good Agricultural and Collection Practices, we ensured the absence of these toxic residues to maintain the quality

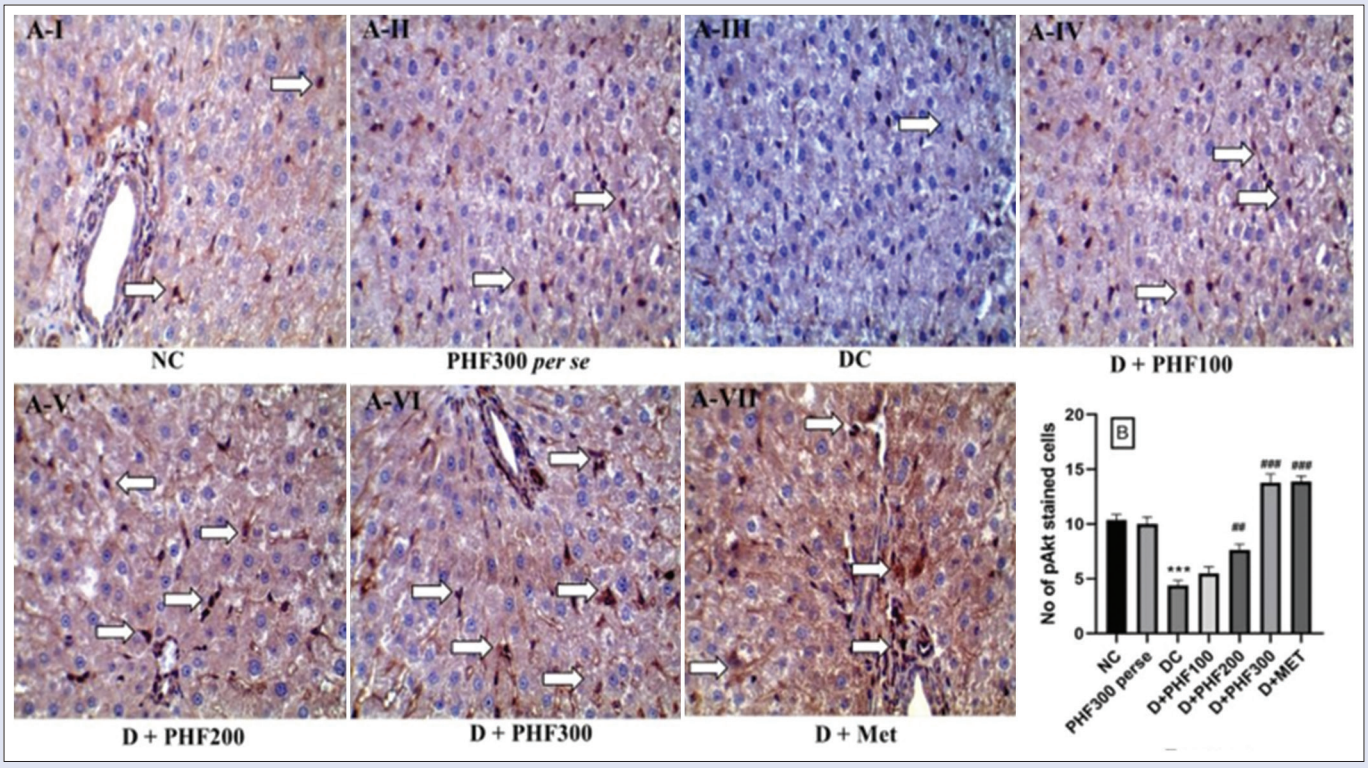

Figure 5: Immunohistochemistry of p-Akt protein expression in hepatic tissue. Representative images showing of p-Akt (a-I to a-VII) staining of hepatic tissues after treatment (Scale bar-100 μm). (b) Showing the semi-quantitative analysis of the number of p-Akt stained cells in different groups. DC group showing reduced expression of p-Akt whereas treatment with polyherbal formula moderate dose, polyherbal formula high dose, and Met significantly increased the expression. Treatment with polyherbal formula low dose however, was found to be ineffective. Values are expressed as mean ± Standard error mean (n = 8). *** P < 0.001 as compared to NC group; ## P < 0.01 and ### P < 0.001 as compared to the DC group

Table 10: Polyherbal formula alleviates hepatic marker enzymes in Streptozotocin induced diabetic rats

Groups	AST (moles of pyruvate/min/mg of protein)	ALT (moles of pyruvate/min/mg of protein)	ALP (moles of phenol liberated/min/mg of protein)
NC	14.71±0.71	20.71±0.87	0.50±0.08
PHFH per se	15.15±0.63	21.36±0.80	0.54±0.06
DC	64.76±1.80***	55.70±1.30***	1.50±0.16***
D + PHFL	60.30±1.85	50.36±1.22#	1.43±0.17
D + PHFM	57.24±1.49##	49.36±1.98#	0.95±0.08#
D + PHFH	43.52±1.33###	37.47±1.01###	0.90±0.08##
D + MET	44.63±1.90###	38.40±1.45###	0.93±0.12##

***P<0.001 as compared to NC group; #P<0.05; ##P<0.01 and ###P<0.001 as compared to the DC group; Data are expressed as mean±SEM (n=8). AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; NC: Normal control; DC: Diabetic control; D: Diabetic; PHF: Polyherbal formula; PHFH: PHF high dose; PHFL: PHF low dose; PHFM: PHF moderate dose; SEM: Standard error of mean; MET: Metformin

of medicinal herbs present in PHF before using it as a treatment option [Table 5]. We also performed a real-time stability study at an interval of 0, 24, 48, 72, 96, and 120 h. by maintaining 30°C ± 2°C and at 65% relative humidity. No change was observed in color, odor, appearance, pH, viscosity, surface tension, specific gravity, and RI of individual ingredients of PHF [Table 4]. One of the key therapeutic strategies for reducing postprandial hyperglycemia is the inhibition of carbohydrate digestive enzymes in the intestinal lumen.[12] Previous studies have suggested that α-amylase and α-glucosidase enzymes inhibition can be useful in reducing elevated blood glucose levels.[10] Consistent with these findings, PHF significantly inhibited α-amylase and α-glucosidase enzyme activities [Figure 1a and b].

NAD-STZ treated rats showed significant hyperglycemia, hyperinsulinemia, dyslipidemia, impaired hepatic insulin signaling, insulin resistance, increased oxidative stress, elevated expressions of proinflammatory cytokines, upregulated liver marker enzymes, histological and ultrastructural changes, which were in line with previous studies.[10,31,32]

T2DM is manifested by a progressive imbalance of glucose and lipid metabolism.[31] Besides, bodyweight reduction is also evident in NAD-STZ induced diabetic model.[33] These manifestations are suggested to exacerbate diabetes progression. In the present study, quantitative analysis revealed that a high dose of PHF treatment ameliorated bodyweight reduction significantly. This effect was not observed with a low and moderate dose of PHF. Additionally, fasting and postprandial blood glucose levels are key diagnostic markers and important causes of T2DM complications. Controlling increased blood glucose levels is a primary target of antidiabetic drugs in the therapeutic avenue. A dose-dependent reduction in the level of FBG was found in diabetic rats after PHF treatment. Similarly, findings from OGTT and HbA_{1c} examinations revealed a dose-dependent control in blood glucose level in PHF treated diabetic rats.

In response to increased blood glucose levels, the β-cells of pancreatic islets produce a considerably large amount of insulin that results in hyperinsulinemia. In the present study, diabetic rats showed increased levels of FI, and HOMA-IR index indicating hyperinsulinemia and insulin resistance, which are in line with previous findings.[33] In diabetic rats, PHF treatment improved insulin sensitivity as well as reduced circulatory FI levels in diabetic rats [Table 8]. It is reported that *N.*

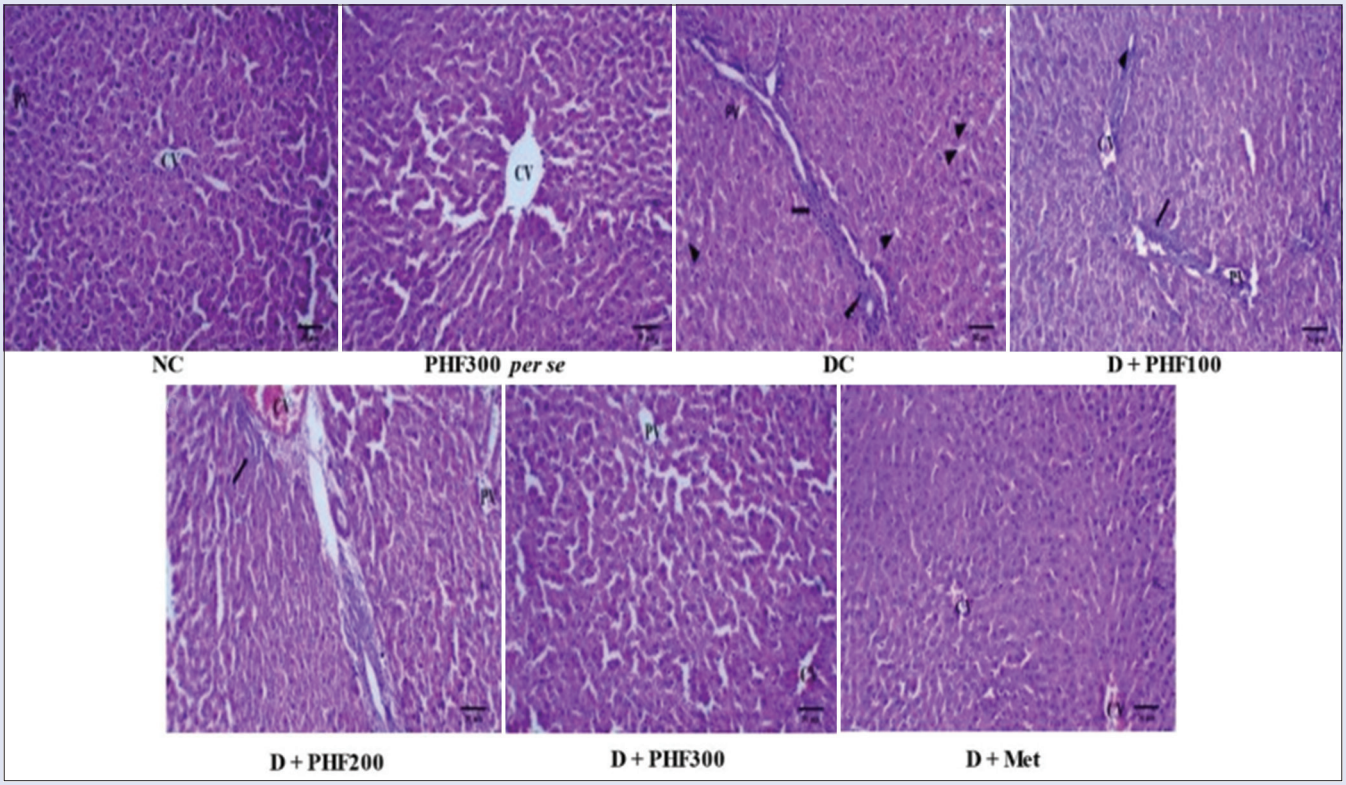

Figure 6: Hematoxylin and eosin staining of hepatic tissues. Photomicrograph of Hematoxylin and eosin stained hepatic tissues of different groups (scale bar: 50 µm, X20): NC and polyherbal formula high dose per SE rats showing normal liver architecture with normally distributed hepatocytes containing central vein in hepatic lobule; DC rats showing deteriorated liver architecture with apparently congested and dilated central vein with infiltration of inflammatory cells (arrow) around portal tract, disturbed portal vein and sinusoids (bolt), pyknotic nuclei, and vacuolization of hepatocytes (arrowhead). Treatment of diabetic rats with polyherbal formula low dose, and polyherbal formula moderate dose showing the improved architecture of hepatocytes with lesser vacuoles and inflammatory cell infiltrates. Treatment of diabetic rats with polyherbal formula high dose and Met showing a reversal in the deterioration of the hepatic tissues and restored the architecture of the hepatocytes with normal-looking central vein, portal vein, and hepatocytes

sativa,[13] *Trigonella foenum graecum*,[15] *C. intybus*,[16] and *A. indica*,[34] exerts antidiabetic effects through their diverse action on glucose metabolism by improving insulin sensitivity and glucose utilization in diabetes mellitus.

We also tested our hypothesis by examining the effects of PHF treatment on the molecular pathways involved in hepatic insulin signaling during the disease progression. During metabolic disturbances, AMPK activation serves various regulatory effects and is considered as a metabolic "master switch."[35] It is evident that *N. sativa*,[36] *Trigonella foenum graecum*,[37] *C. intybus*,[38] and *A. indica*,[34] improve glucose metabolism and insulin sensitivity by activating AMPK phosphorylation. Consistent with the previous results, gene expressions of AMPK, INSR, IRS, PI3K, Akt, and GLUT2 were increased and FoxO1 was decreased after PHF treatment. Similarly, IHC results confirmed that the PHF treatment was positively correlated with the protein expression of p-Akt in diabetic rats [Figure 5].[39,40] The amelioration of hepatic insulin signaling by PHF might have resulted in improvement in insulin sensitivity in diabetic rats possibly through increased expression of AMPK.[41] AMPK activation improves impaired insulin signaling and insulin sensitivity by alleviating IRS/PI3K/Akt pathway and subsequently improves glucose-lipid metabolism.[35] Wei Y. *et al*. have reported that by upregulating IRS/PI3K/Akt insulin signaling, insulin resistance can be prevented.[41] Furthermore, several studies have confirmed that Akt plays a pivotal role in glucose-lipid homeostasis via acting on hepatic gluconeogenesis and lipogenesis.[6] In addition, AMPK activation attenuates oxidative stress and inflammation by targeting the downstream signaling axis.[35] The hepatocytes respond to insulin and induce the GLUT2 expression at the membrane to facilitate glucose uptake and utilization.[32] Furthermore, impaired insulin signaling promotes enhanced glucose production from the liver via nonglucose precursors by upregulating FoxO1 expression.[11] The increased mRNA and protein expression of Akt is correlated positively with GLUT2 and negatively with FoxO1.[42]

Previous studies have aimed to prevent dyslipidemia as treatment approaches in insulin-resistant diabetic models.[40,41] Besides, human and animal studies have presented increased serum levels of TG, TC, LDL-C, VLDL-C, and reduced serum levels of HDL-C in diabetic conditions.[43] Consistent with these results, oral administration of PHF showed a significant reduction in TG, TC, LDL-C, VLDL-C, and remarkably increased levels of HDL-C in diabetic rats. Additionally, the disorder in lipid profile might be prevented by the improvements in hepatic insulin signaling and subsequent reduction in de novo lipogenesis.[42] A significant increase in AIP predicted the high risk of CAD in DC rats, which was significantly reduced post-PHF treatment.

It is reasonable to deem that by exhibiting synergistic effects, polyherbal formulations can offer benefits against the progression of T2DM via demonstrating potent antioxidant effects in diabetic rats. Additionally, clinical and preclinical studies provide evidence that increased oxidative stress contributes to a disproportion in ROS production and the antioxidant defense system.[44,45] In the present study, NAD-STZ-induced diabetic rats showed a remarkable increase in expression of an oxidative marker, TBARS, and simultaneously reduced expression of anti-oxidant proteins SOD, CAT, and GSH in hepatic tissue, which is consistent

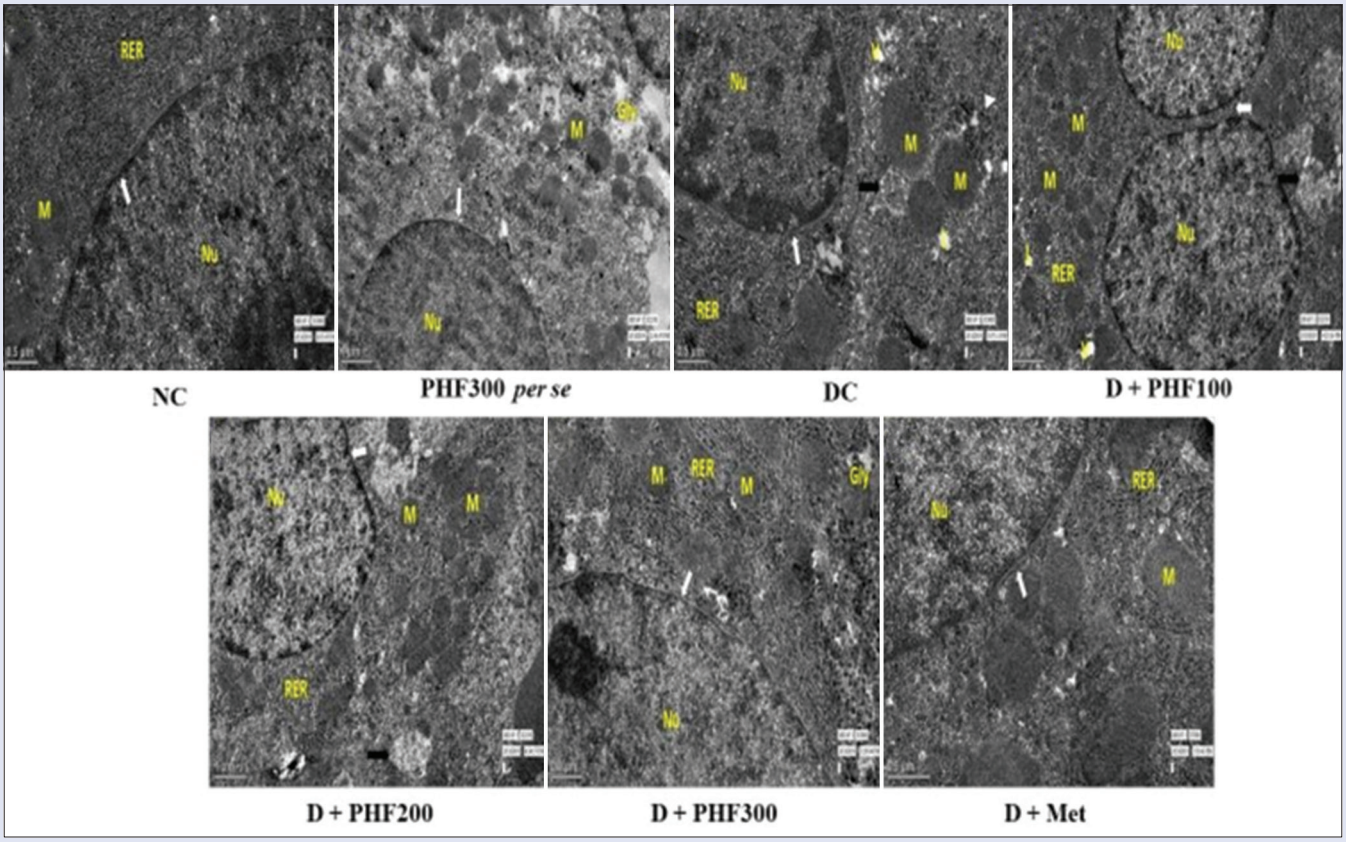

Figure 7: Transmission electron microscopy of the hepatic ultrastructure. Transmission electron microscopic images of the hepatic ultrastructure: Normal ultrastructure of hepatocytes were observed including the normal nucleus (Nu) with a prominent nucleolus and normal nuclear envelope (white arrow), active mitochondria (m) with normal cristae, the appearance of glycogen (Gly) content in the cytoplasm in NC rats (a. scale bar: 1 μm and X5000) and PHF high dose per SE rats (b. scale bar: 1 μm and X2550). Severe ultrastructure alteration was observed including the heterochromatic nucleus (Nu) with the deteriorated nuclear envelope (white arrow), mild dissolution of cytoplasm with the appearance of several swollen mitochondria (m), disorganized rough endoplasmic reticulum, lipid droplets (l), vacuoles (v), collagen fibers (arrowhead), and process of Von Kupffer cells containing lysosomes (black arrow) in DC rats (c. scale bar: 0.5 um and X5000). Treatment of diabetic rats with PHF low dose (d. scale bar: 1 μm and X2550), polyherbal formula moderate dose (e. scale bar: 1 μm and X2550) showing an improved architecture of hepatocytes. Treatment of diabetic rats with PHF high dose (f. scale bar: 0.5 μm and X5000) and Met (f. scale bar: 0.5 μm and X7000) showing a reversal in the deterioration of the hepatic ultrastructure and restored the architecture of the hepatocytes

with previous results.[31] Moreover, treatment of diabetic rats with PHF reduced the oxidative stress by significantly decreasing hepatic tissue level of TBARS and elevating antioxidant enzymes such as SOD, CAT, and GSH levels in hepatic tissue [Table 9]. Similarly, previous results suggest that *N. sativa*, *Trigonella foenum graecum*, *C. intybus*, and *A. indica*, possess strong antioxidant effects.[13-18] Therefore, the balance observed in the oxidant-antioxidant system can be attributed to the synergistic effects of PHF by alleviating hepatic insulin signaling and glucose-lipid metabolism in diabetic rats.[45]

Diabetic rats in the present study showed remarkably increased plasma expression of TNF-α and IL-6 when compared to normal rats which were significantly reduced by the oral administration of PHF. Previous studies have confirmed that tissue-specific insulin resistance, oxidative stress, and inflammation are closely associated with the development of diabetes mellitus and related complications.[31] In addition, insulin-resistant diabetic rat exhibits increased tissue and plasma expression of proinflammatory cytokines such as TNF-α and IL-6.[31,45] Moreover, natural products are considered to be potential candidates in ameliorating hyperglycemia-induced oxidative stress and inflammation.[46]

Diabetic rats in the present study have shown elevated levels of AST, ALT, and ALP and histological disturbances in hepatic tissue.[46] Treatment with PHF significantly reduced the elevated levels of liver enzymes. Hepatic insulin resistance mediated gluconeogenesis, and dyslipidemia in T2DM is closely associated with liver dysfunctions including NAFLD, that can progress to NASH.[40] Liang *et al*. have suggested that NAFLD and NASH can be the early complications associated with insulin resistance in patients with T2DM.[43] Histopathological and ultrastructural analysis in this study revealed that PHF restored the integrity of hepatic tissues. PHF might have shown this effect by ameliorating the paradox of impaired hepatic insulin signaling and free radical scavenging activity.[31,43] In T2DM, the aggravation of insulin resistance, increased expression of inflammatory cytokines, and oxidative stress can produce deleterious effects on the structure and functions of the liver.[43]

CONCLUSION

This study provides evidence that PHF can improve glucose-lipid metabolism in STZ-induced diabetic rats by normalizing hyperglycemia, hyperinsulinemia, and dyslipidemia, enhancing insulin sensitivity, attenuating oxidative stress and inflammation, and reversing the deterioration of hepatic architecture, possibly through AMPK mediated improvement in hepatic insulin signaling. These results of PHF were comparable to metformin. As evident from the aforementioned studies, synergistic effects from herbal formulations can be beneficial through their multitargeted potential on various underlying

pathological mechanisms for the management and treatment of T2DM and its related complications. Therefore, further preclinical and clinical studies are needed to completely elucidate the molecular mechanisms impacted in the anti-diabetic effects of PHF.

Acknowledgements

The first author (AH) is grateful to Sun Pharmaceutical Industries Limited, India, for assisting with this study under the collaboration with Jamia Hamdard, New Delhi, India for the Ph. D. program.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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