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Original article

Polyherbal formulation: The studies towards identification of composition and their biological activities

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ABSTRACT

Synergistic strategy is always supported by nature due to multi-targets synergistic mode. Spices are one of the most nutritional products present in our diet, and dietary manipulation has been implicated in managing various medical conditions. Our interest was to study the effect of polyherbal formulation (PHF), composed of fenugreek, black seed, cumin, and flaxseed, in the experimental model of diabetic nephropathy (DN). After induction of diabetes (streptozotocin 55 mg/kg) in male Wistar rats, PHF (200 mg/kg) was given for 56 days. Biochemical and histopathological indicators were used to study nephritis. The diabetic group had elevated glucose, dyslipidemia, and impaired renal function. PHF treatment improved glycemic control, renal function, and dyslipidemia. Consecutively, oxidative damage marked as the elevated level of 8-hydroxy-2′-deoxyguanosine (8-OHdG), transforming growth factor-beta1 (TGF-β1) and malonaldehyde (MDA) with concomitantly decreased glutathione (GSH), glutathione reductase (GR) and superoxide dismutase (SOD) depicted in the diabetic group were restored after the administration of PHF. Further histological analysis was done and explored as glomerulosclerosis and interstitial fibrosis in the diabetic group. Administration of PHF significantly modulated renal histological variations in diabetic rats. A current investigation concluded that PHF exhibited a strong nephroprotective effect by preserving renal damage due to its antidiabetic and antioxidant actions. Thus, PHF may find a pharmaceutical candidature to manage diabetes-related complications requiring more studies.

1. Introduction

Plants are considered safe and natural therapeutics for mankind, exploring various pharmaceuticals and health-promoting products (Ivanova et al., 2005). Herbs and spices derived from the plant have been used in food and pharmaceutics for centuries (Embuscado, 2015). Over the last few decades, research has been growing to investigate the protective effects of plants towards curing chronic human diseases, e.g., cancer, heart disease, arthritis, neurological, obesity, and diabetes (Forni, 2019; Kausar et al., 2021). Spices have been shown to have antioxidant, immunomodulatory, and anti-inflammatory properties. Many health advantages are attributed to active ingredients (primarily polyphenols) targeting certain signaling pathways. Elevated blood glucose levels characterize type 2 diabetes and increased glucose intolerance

Abbreviations: MDA, Malonaldehyde; GSH, Gglutathione; GR, Glutathione reductase; SOD, Superoxide dismutase; DM, Diabetes Mellitus; BUN, Blood Urea Nitrogen; PHF, polyherbal formulation; STZ, Streptozotocin.

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owing to inadequate insulin production, action, or both. Diabetic nephropathy (DN) is a serious consequence of Type 2 Diabetes Mellitus (DM) that affects 50% of those with type 2 diabetes and is characterized by elevated serum creatinine, blood urea nitrogen (BUN), and glomerular filtration rate (GFR) (Shahidi and Ambigaipalan, 2015; Afkarian et al., 2016; Varì et al., 2021). DN is one of the major causes of End-Stage Renal Disease (ESRD) globally. Many therapies exist and most of them are synthetic. Adverse reactions are a part of every treatment, including kidney ailments. Thus, discovering a natural drug with fewer side effects is becoming more critical (Lv and Zhang, 2019; Kumar et al., 2021).

Indigenous medicine is consistently encouraged owing to its superiority over contemporary medicine. The notion of polyherbal formulation (PHF) is gaining popularity as the obstacles of standardization are addressed by current science and technology. PHF treatments are widely accepted worldwide due to their potency, low cost, easy availability, clinical efficacy, safety, patient tolerance, and success in chronic conditions (Petchi et al., 2014). Due to synergistic effects, PHF has a superior therapeutic impact than a single herb (Ghorbani, 2014). The current study looked into the therapeutic effects of PHF by using a mixture of four spice seed extracts that have already been shown to help treat diabetes in both animals and people. The research selected fenugreek, black seed, cumin, and flaxseed for therapeutic and pharmacological qualities. The present study investigated whether PHF therapy affected lipids, oxidative damage, renal function, and architecture in a type 2 diabetic rat model.

2. Materials and methods

2.1. Preparation of PHF

The PHF was prepared using a 1:1:1:1 ratio of methanolic extracts of black seed (*Nigella sativa*), Fenugreek (*Trigonella foenum*), Flax seeds (*Linum usitatissimum*), and Cumin (*Cuminum cyminum*). The dried seed of plant material was crushed into a coarse powder to perform the extraction technique. The extraction was done for three days using methanol and soxhlet equipment. The extraction was cooled and filtered to eliminate any residue. The extraction process was repeated using a rotary evaporator working at reduced pressure to concentrate further and get a fine dry powder. PHF stands for crude extract yield, and it was retained in an airtight container for future use. The PHF was tested for quality control in compliance with WHO regulations (http://apps.who.int/medicinedocs/documents/h1791e/h1791e.pdf). For the animal experiment, the dry powder was dissolved in normal saline.

2.2. Acute toxicity investigations

The working dosage of PHF was determined by conducting an acute toxicity investigation in 6 to 8-week-old male Wistar rats under OECD recommendations. PHF was administered intraperitoneally (i.p.) to overnight fasting rats at various dosages. Miller and Tainter Method was used to calculate the LD50. Due to lack of mortality at larger dosages (2000 mg/kg, p.o), the current investigation used a 200 mg/kg dose of PHF (Ghosh, 2007).

2.3. Gas chromatography-mass spectrometry (GC–MS) analysis

GC–MS analysis of PHF was done using a Shimadzu GCMS-QP-2010 Ultra machine. The system uses an Rtx-5 MS low bleed column with 30 mm × 0.25 mm ID × 0.25 μm films. At 1.0 ml/min, helium was used as a carrier gas. The column was operated as follows: The oven temperature was configured from 140 to 280 °C in 5 °C min^{-1} increments for 56 min. The injector was held at 260 °C. The injection volume was 0.3 μl at 107.4 kPa. The overall flow was 28.4 ml min^{-1}, with a column flow of 1.21 ml min^{-1} and a purge flow of 3.0 ml min^{-1}. The ion split ratio was retained at 230 °C. The mass scan range (m/z) was 40–600 at 270 °C. The GC–MS data were interpreted using the National Institute of Standard and Technology (NIST) database of approximately 62,000 patterns. The molecules in each sample were assessed by comparing their spectra to the NIST database of known compounds. The components of the test solutions were identified by name, molecular weight, and structure.

2.4. Experimental animals

The experiment employed male Wistar rats (150 ± 10 g) of age 7–8 weeks from the Animal House run by Jamia Hamdard in New Delhi, India. Before the trial, the animals were given a week to adjust to life in the animal house. Animals were fed and watered ad libitum throughout the laboratory research. IAEC, which is registered with the Committee for the Control and Supervision of Experimental Animals (173/CPCSEA), assessed and authorized all techniques of animal usage.

2.5. Experimental design

In order to conduct a proper comparison, two distinct nondiabetic groups (n = 8) were designed to receive either normal saline or PHF for 56 days. These groups were designated as Control and Control + PHF, respectively. Streptozotocin (STZ) (55 mg/kg body weight) was used intraperitoneally (i.p.) to induce diabetes in rats. Three days following STZ injection, fasting blood glucose (FBG) levels were tested by a glucometer. The research only included animals whose FBG levels were above 250 mg/dl. After successfully developing diabetes in the rats, they were divided into two groups of eight each at random. The animals were separated before administering PHF orally in one group for 56 days (200 mg/kg body weight). In contrast, the diabetic group was maintained untreated for 56 days to monitor the course of kidney impairment.

2.6. Sample collection

After 56 days, blood was withdrawn from the retro-orbital venous plexus of rats for centrifuging at 2500 rpm/10 min to extract serum. This serum was used to determine the biochemical characteristics present in the serum. FBG levels and 24-hour urine samples were taken at the end of the study to measure 8-hydroxy-2′ deoxyguanosine (8-OHdG)., an indicator of DNA oxidative damage.

2.7. Tissue processing

The rats were sacrificed, and the kidneys were quickly taken out. One kidney was used for homogenate preparation. The second kidney was immersed in a 10% buffered formalin solution for histopathological examinations. Phosphate buffer was used to prepare the homogenate. Malondialdehyde was determined in a small amount of supernatant collected after centrifuging the samples to eliminate nuclear debris. The post-mitochondrial supernatant (PMS) was obtained by centrifuging the remaining supernatant.

2.8. Assay for dyslipidemia

Serum triglyceride levels were assessed using the Stein, Myers, method (1995) and the Wybenga et al. one-step methodology (1970) for total cholesterol (TC) and HDL cholesterol (HDL-C). Friedewald's equation was used to determine LDL cholesterol (LDL-C) and very-low-density lipoprotein cholesterol (VLDL-C).

2.9. Evaluation of renal function

In this study, the sarcosine oxidase method was used to detect the levels of creatinine in both urine and serum by adopting a standard commercial test kit. The concentration of BUN in serum was measured using the urease technique provided in commercial assay kits and was represented as mmol/l. The standard equation for calculating creatinine clearance was used by Arreola-Mendoza (2006). GFR was determined from creatinine clearance.

2.10. Urinary 8-OHdG level

Centrifugation at 2000*g* for 20 min was used to measure the 8-OHdG level in urine samples, and then dilution was used to evaluate the 8-OHdG level using an ELISA kit (Elabscience, Texas, U.S.A.). Concentration was expressed as the total amount excreted in 24 h.

2.11. Transforming growth factor-beta1 (TGF-β1) level in the serum

TGF- β1 is critical for maintaining renal architecture by suppressing fibrosis. TGF-β1 serum level was measured as per the manufacturer's procedure using an ELISA kit (Elabscience, Texas, USA).

2.12. Assessment of oxidative stress in renal tissue

The MDA test was performed according to Ohkawa et al. (1979) protocol. The total GSH concentration was measured using the DTNB reagent. The glutathione reductase (GR) activity was determined using a modified Carlberg and Mannervik (1975) procedure. In a total of 2.0 ml, the test solution included phosphate buffer, GSSG, EDTA, NADPH, and PMS ions (10 percent). At 340 nm, the enzyme activity was determined and represented as nmol NADPH/min/mg protein. SOD activity was assessed as previously reported. Bradford's (1976) technique was used to determine the total protein content in the homogenate and PMS.

2.13. Histopathological analysis of tissues

The formalin solution to fix the kidneys was buffered to a 10% concentration. A portion of renal tissue was dehydrated at various time intervals with varying percent variations of alcohols. After xylene wash, the tissue part infiltrated in paraffin. The tissue blocks were cut by employing a microtome to obtain 3–4 μm thickness slices. After mounting the tissue slices with DPX mountant and drying them, Jones periodic acid-Schiff was used to stain the sections. The PAS stain was done to note the alteration in renal architecture. An Olympus BX50 microscope (bright field) was used to view the slides.

2.14. Statistical analysis

The statistical programme SPSS 23 was used to conduct the analyses. For multiple comparisons, the ANOVA test, accompanied by the Tukey–Kramer post-analysis test, with a significance level of $P < 0.05$ considered statistically significant. Accordingly, the results were reported as a standard error of the mean ± SEM.

3. Results

3.1. Phytochemical analysis

In this paper, we reported the identification of 69 phytoconstituents (Table 1) in the polyherbal methanolic extract from Fenugreek (*Trigonella foenum*), Flax seeds (*Linum usitatissimum*), Cumin (*Cuminum cyminum*), and Black seed (*Nigella sativa*). Ten dominant compounds were identified by the GC–MS technique, with an area ranging from 2.70% to 12.59%. The main compounds are Palmitic acid (12.59%), 9-Octadecenoic acid (12.01%), 9,12-Octadecadienoic acid (11.62%), 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (7.81%), 11-Octadecenoic acid, methyl ester (6.51%), 2-(2-Butoxyethoxy) ethanol (6.36%), Hexadecanoic acid, methyl ester (5.18%), 2-Oleoylglycerol (2.70%), *cis*-2-Penten-1-ol (2.54%), and Stearic acid (2.01%).

The chromatogram obtained (Fig. 1) showed the main compounds identified in the tested polyherbal methanolic extract together with their chemical structure.

3.2. Effect of PHF on hyperglycemia

In the diabetic group, elevated glucose levels were shown to constitute direct proof of hyperglycemia. A decreased blood glucose level was observed in the PHF-supplemented group; however, no changes were observed in healthy rats with concurrent administration of PHF (Table 2).

3.3. PHF and lipid profile

The lipid profile parameters (TC, TG, HDL-C, LDL-C, and VLDL-C) changed significantly during the trial, as shown in Table 3. These parameters were considerably restored in the treated group of rats compared to the diabetic rats after PHF. In comparison to the normal control group, diabetic rats consistently had higher TC, TG, LDL-C, VLDL-C and lower HDL-C levels.

3.4. The effect of PHF on markers of renal function

As the data demonstrate, the diabetic category experienced a concomitant rise in BUN levels compared to the controls (Table 2). In the treatment group, PHF therapy considerably decreased BUN levels. It is well known in clinical and experimental studies that GFR decreased with the development of DN. The diabetic group exhibited an abruptly raised creatinine level in serum with steeply lower creatinine clearance in urine than the control group, indicating elevated values of GFR as equated with creatinine clearance (Fig. 2A & B). Administration of PHF in the diabetic group reverted these altered values of GFR compared with the diabetic group. The diminish in GFR may be due to glomerular damage supported by the histological analysis in diabetic and treated groups linked to DN progression.

3.5. The effect of PHF on the concentration of 8-OhdG

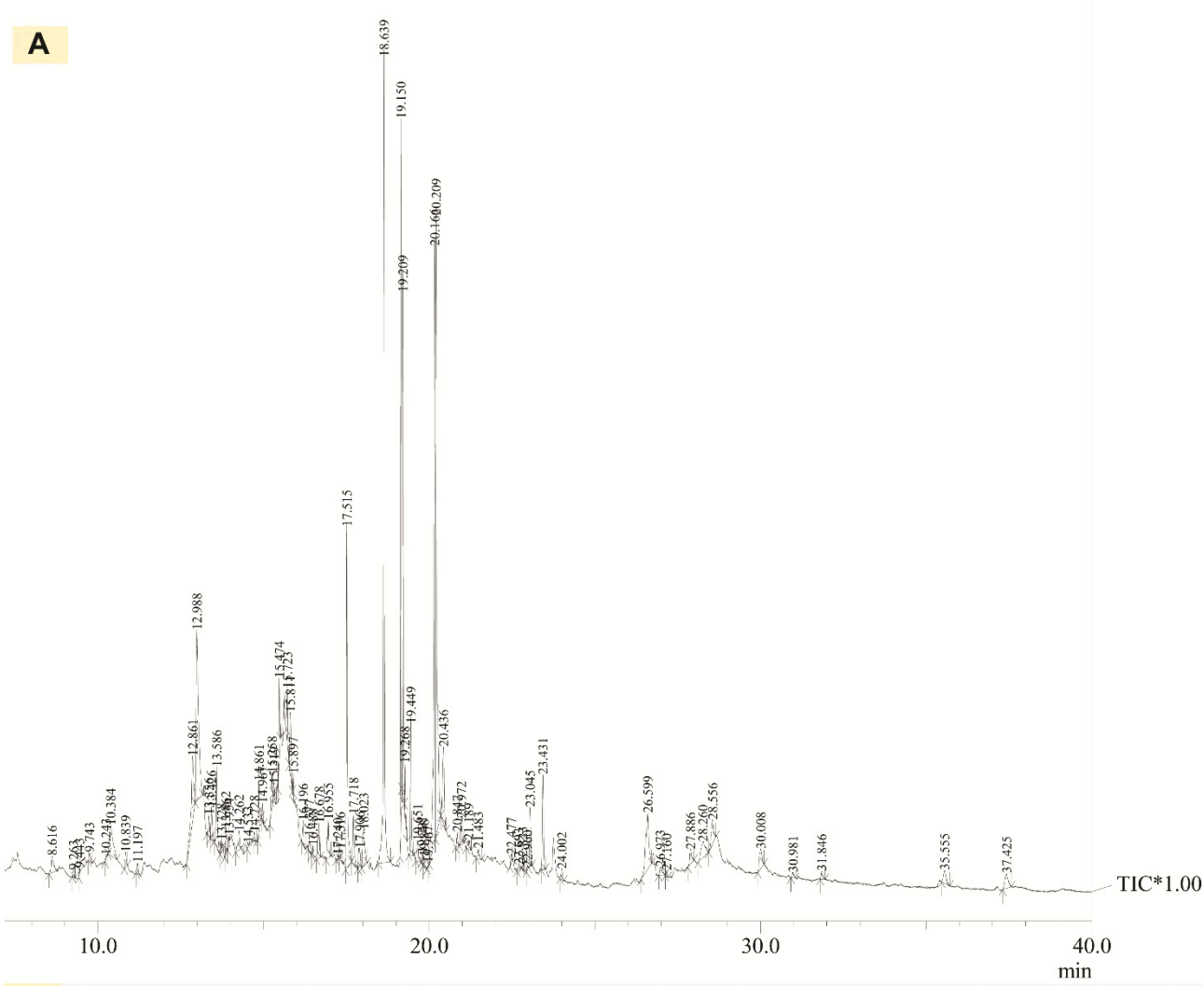
Urinary 8-OHdG excretion is a critical indicator of the oxidative DNA modification patterns produced by ROS.. As a consequence, detection of 8-OHdG in urine can be employed as a viable biomarker for detecting oxidative damage associated with diabetes. The diabetic group had a significantly higher value of 8-OHdG than the control group (Fig. 3). PHF treatment for 56 days resulted in a significant decrease in 8-OHdG levels in diabetic rats treated vs untreated. The concentration of 8-OHdG in normal rats fed PHF was not significantly different from that in the control group.

3.6. PHF has an effect on the level of TGF- β1:

TGF- β1 is a well-established fibrogenic cytokine that has been implicated in triggering the hypertrophic changes related to the development of DN. The diabetic group had considerably higher TGF-1 levels than the control group (Fig. 4). As opposed to the diabetic category, treatment with PHF resulted in a statistically significant decrease in TGF-β1.

Table 1
Phytochemical profiling of the polyherbal methanolic extract by using GC–MS technique.

No.	Compounds Identified	RT [min]	Area (%)	Molecular Weight	Formula
1	Phloroglucinol	8.616	0.63	126.11	$C_6H_6O_3$
2	Benzene, 1,3-bis(1,1-dimethylethyl)-	9.263	0.10	190	$C_{14}H_{22}$
3	Octanoic acid	9.443	0.07	144.21	C8H16O2
4	9H-Purine	9.743	0.25	120.11	$C_5H_4N_4$
5	5-(2-bromoethyl)-4h-pyrazole-3-carbonitril	10.243	0.07	199	$C_6H_6BrN_3$
6	2-O-Acetyl-1,3-dideoxy-4,5-O-(1-methylethylidene) hexitol	10.384	1.63	232.28	$C_{11}H_{20}O5$
7	Nonanoic acid	10.839	0.25	158.24	C9H18O2
8	1,3-Pentanediol	11.197	0.20	104.15	$C_5H_{12}O_2$
9	*cis*-2-Penten-1-ol	12.861	2.54	86.13	$C_5H_{10}O$
10	2-(2-Butoxyethoxy) ethanol	12.988	6.36	162.23	$C_8H_{18}O_3$
11	1-Propanol, 2-methyl	13.356	0.33	74.12	$C_4H_{10}O$
12	7-Nonynoic acid, methyl ester	13.426	0.60	170.25	$C_{10}H_{18}O_2$
13	2-ketohexanoic acid	13.586	1.40	130.14	$C_6H_{10}O_3$
14	2′-Hydroxy-4′-methoxyacetophenone	13.728	0.11	166.17	$C_9H_{10}O_3$
15	Hexanoic acid	13.862	0.34	116.16	$C_6H_{12}O_2$
16	Tetradecane	13.954	0.10	198	$C_{14}H_30$
17	2-Hydroxyhexanoic acid	14.262	0.46	132.16	$C_6H_{12}O_3$
18	Dodecanoic acid	14.533	0.05	200.32	$C_{12}H_{24}O_2$
19	Uridine	14.728	0.09	244.2	$C_9H_{12}N_2O_6$
20	D-Ribofuranose	14.861	1.06	150.13	$C_5H_{10}O_5$
21	D-Erythrose	14.967	0.22	120.10	$C_4H_8O_4$
22	5-methyl-1,3-dioxane-5-carboxylic acid	15.258	0.74	146.14	$C_6H_{10}O_4$
23	1,6-Anhydro-2,3-O-isopropylidene-.beta.-D-mannopyranose	15.319	0.22	202.20	$C_9H_{14}O_5$
24	Methyl alpha-D-glucopyranoside	15.474	1.30	194.18	$C_7H_{14}O_6$
25	3-O-Methyl-d-glucose	15.723	0.68	380	$C_7H_{14}O_6$
26	3,3-Dimethyl-1,4-dioxane-2,5-dione	15.811	1.29	144.12	$C_6H_8O_4$
27	3-Deoxy-L-*ribo*-hexonic acid	15.897	0.22	180.15	$C_6H_{12}O$
28	Hexadecanoic acid	16.196	0.36	256.42	$C_{16}H_{32}O_2$
29	2-Mono-isobutyrin	16.377	0.25	162.09	$C_7H_{14}O_4$
30	Pentacosanoic acid, methyl ester	16.488	0.15	396	$C_{26}H_{52}O_2$
31	Myristic acid	16.678	0.83	228.37	$C_{14}H_{28}O_2$
32	Succinic acid, tridec-2-yn-1-yl 4-heptyl ester	16.955	0.84	394	$C_{24}H_{42}O_4$
33	3,5,7-Nonatrien-2-one, 8-methyl-7-(1-methylethyl)-, (E,E)-	17.240	0.05	192	$C_{13}H_{20}O$
34	9-Hexadecenoic acid, methyl ester, (Z)-	17.316	0.15	268	$C_{17}H_{32}O_2$
35	Hexadecanoic acid, methyl ester	17.515	5.18	270	$C_{17}H_{34}O_2$
36	3,3,8a-Trimethyl-6-oxode cahydro-1-naphthalenyl acetate	17.718	1.11	252	$C_{15}H_{24}O_3$
37	Dibutyl phthalate	17.906	0.57	278	$C_{16}H_{22}O_4$
38	9-Tetradecen-1-ol, acetate, (Z)-	18.023	1.27	254	$C_{16}H_{30}O_2$
39	Palmitic acid	18.639	12.59	256.43	$C_{16}H_{32}O_2$
40	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	19.150	7.81	280.40	$C_{18}H_{32}O_2$
41	11-Octadecenoic acid, methyl ester	19.209	6.51	296.50	$C_{19}H_{36}O_2$
42	Methyl stearate	19.449	1.77	298.50	$C_{19}H_{38}O_2$
43	Octadecan-1-ol	19.651	0.51	270.49	$C_{18}H_{38}$
44	Phytol	19.791	0.11	296.53	$C_{20}H_{40}O$
45	9-(3,3-dimethyl-2-oxiranyl)-2,7-dimethyl-2,6- nonadien-1-ol	19.848	0.11	238	$C_{15}H_{26}O_2$
46	Hexanoic acid, 2-ethyl-, dodecyl ester	19.967	0.05	312.50	$C_{20}H_{40}O_2$
47	9,12-Octadecadienoic acid	20.166	11.62	280.40	$C_{18}H_{32}O_2$
48	9-Octadecenoic acid	20.209	12.01	282.50	$C_{18}H_{34}O_2$
49	Stearic acid	20.436	2.01	284.50	$C_{18}H_{36}O_2$
50	3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester	20.847	0.21	213	$C_{12}H_{23}NO_2$
51	12-Hydroxy-9-octadecenoic acid methyl ester	20.972	0.75	312	$C_{19}H_{36}O_3$
52	2-Hydroxyethyl palmitate	21.189	0.56	300.50	$C_{18}H_{36}O_3$
53	Fumaric acid, decyl 2-heptyl ester	21.483	0.27	354	$C_{21}H_{38}O_4$
54	Fumaric acid, 2-dimethylaminoethyl heptyl ester	22.477	0.12	285	$C_{15}H_{27}NO_4$
55	4-Cyanobenzoic acid, undec-10-enyl ester	22.693	0.13	299	$C_{19}H_{25}NO_2$
56	2-Undecanone, 6,10-dimethyl-	22.823	0.11	198	$C_{13}H_{26}O$
57	Pentadecane, 2,6,10,14-tetramethyl-	22.960	0.06	268	$C_{19}H_{40}$
58	10-Nonadecenoic acid, methyl ester	23.045	1.05	310	$C_{20}H_{38}O_2$
59	1,2-benzenedicarboxylic acid	23.431	1.76	390	$C_{24}H_{38}O_4$
60	1-Monopalmitin	24.002	0.20	330.50	$C_{19}H_{38}O_4$
61	2-Oleoylglycerol	26.599	2.70	356.50	$C_{21}H_{40}O_4$
62	Oxalic acid, 3,5-difluorophenyl nonyl ester	27.886	0.43	328	$C_{17}H_{22}F_2O_4$
63	Stearic acid triglyceride	28.260	1.07	890	$C_{57}H_{110}O_6$
64	9, Nonadecanoic acid-glycerine-(1)-monoester	28.556	0.98	372.59	$C_{22}H_{44}O_4$
65	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-hydroxymethyl ester	30.008	0.77	354	$C_{21}H_{38}O_4$
66	24-Norursa-3,12-diene	30.981	0.15	394	$C_{29}H_{46}$
67	Cholesterol	31.846	0.22	386.70	$C_{27}H_{46}O$
68	beta.-Sitosterol	35.555	0.54	414.71	$C_{29}H_{50}O$
69	Lupeol	37.425	0.75	426.72	$C_{30}H_{50}O$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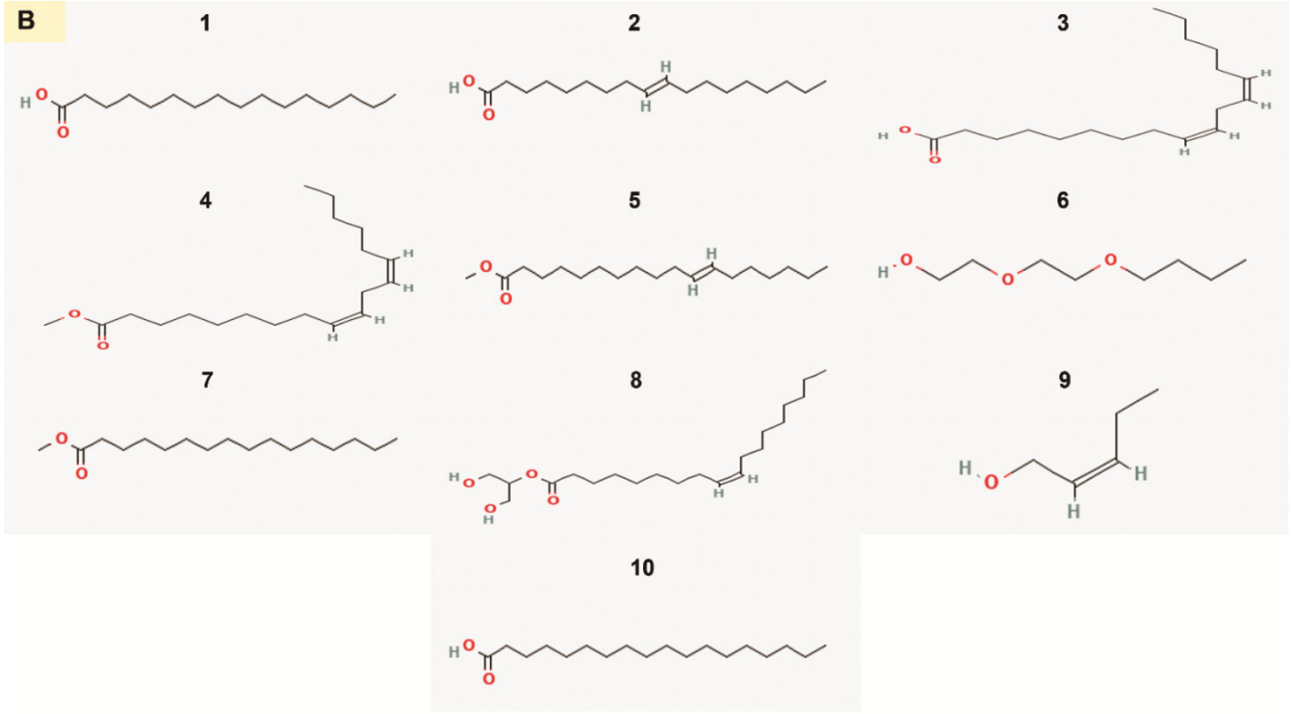
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Table 2
The effect of PHF therapy on FBG and BUN levels in patients with diabetes.

	Control	Diabetic	Diabetic + PHF	Control + PHF
FBG (mg/dl)	85.14 ± 2.98	383.92 ± 9.21* (+283.92%)	203.87 ± 7.3** (−46.89%)	99.54 ± 3.88 (+16.91%)
BUN (mmol/l)	8.22 ± 1.3	16.43 ± 1.9* (+49.96%)	13.02 ± 1.4** (−20.75%)	7.11 ± 1.2 (−13.50%)

The data is presented as a mean, standard error of the mean (S.E.M.). FBG and BUN levels were significantly higher in the diabetic group. PHF treatment preserved the value of these parameters significantly. *P < 0.05 Diabetic group vs Control OR Control + PHF group; **P < 0.05 Diabetic + PHF group vs Diabetic group.

Table 3
Effect of PHF treatment on dyslipidemia in the diabetic group.

	Control	Diabetic	Diabetic + PHF	Control + PHF
TC (mg/dL)	114.67 ± 3.7	189.58 ± 5.2* (+65.32%)	138.27 ± 3.5** (−27.06%)	118.31 ± 3.2 (+3.17%)
TG (mg/dL)	127.88 ± 3.3	195.87 ± 4.3* (+53.16%)	157.99 ± 4.8** (−19.33%)	130.22 ± 3.4** (+1.82%)
HDL-C (mg/dL)	48.38 ± 1.7	22.80 ± 1.4* (−52.87%)	36.22 ± 1.6** (+58.85%)	50.11 ± 1.8** (+3.57%)
LDL-C (mg/dL)	40.71 ± 1.1	127.60 ± 2.6* (+213.44%)	70.45 ± 1.2** (−44.79%)	42.15 ± 1.8** (+3.54%)
VLDL-C (mg/dL)	25.57 ± 0.94	39.17 ± 0.89* (+53.19%)	31.59 ± 1.1** (−19.35%)	26.04 ± 0.99** (+1.83%)

mean ± S.E.M values are shown. The TC, TG, LDL-C, and VLDL-C levels were all significantly higher in the diabetic group, whereas HDL-C levels were lower. PHF treatment preserved the lipid profile significantly. *P < 0.05 Diabetic group vs Control OR Control + PHF group; **P < 0.05 Diabetic + PHF group vs Diabetic group.

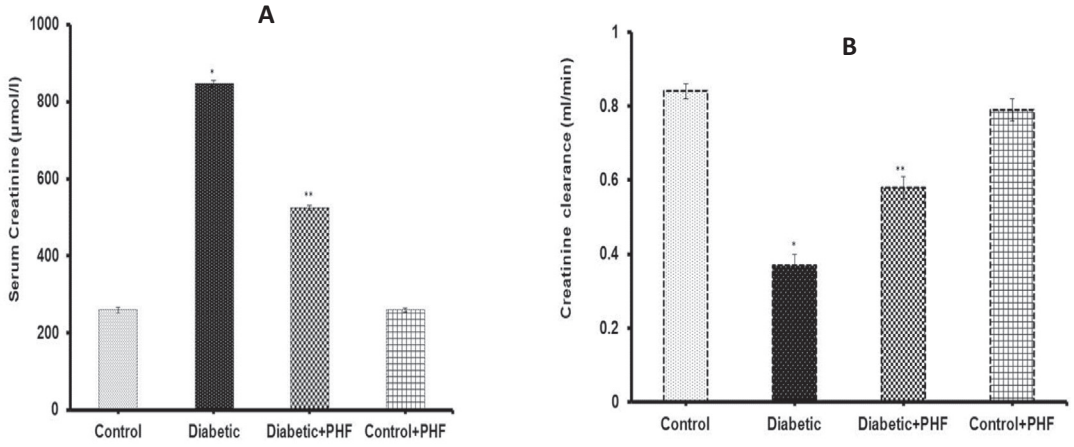

Fig. 2. (A) PHF therapy affects the serum creatinine level in people with diabetes. (B)Effect of PHF on creatinine clearance in diabetic group. Data expressed as mean ± S.E.M. (*P < 0.05 diabetic group vs Control OR Control + PHF group; **P < 0.05 Diabetic + PHF group vs Diabetic group).

3.7. Peroxidation of lipids (LPO) as a result of PHF exposure

In renal tissue, lipid peroxidation (LPO) was linked to the production of MDA. The diabetic group's MDA concentration was significantly higher than the control group's. As shown by the findings, treatment with PHF suppressed MDA generation in diabetic rats and resulted in a decreased MDA concentration in the treatment group (Fig. 5).

3.8. Effect of PHF on antioxidant system in the kidney

The antioxidant state of hyperglycemia-induced oxidative stress was also assessed using GSH, GR, and SOD levels. Compared to the control group, the diabetic group had lower GSH, GR, and SOD (Figs. 6 and 7, respectively). PHF treatment raised GSH and antioxidant enzyme levels in kidney tissues relative to the diabetic group.

Fig. 1. GC–MS chromatogram of the 69 identified phytoconstituents in the polyherbal methanolic extract (A) and the main identified compounds (B). Compounds name: (1): Palmitic acid (12.59%), (2): 9-Octadecenoic acid (12.01%), (3): 9,12-Octadecadienoic acid (11.62%), (4): 9,12-Octadecadienoic acid (Z,Z)-, methyl ester (7.81%), (5): 11-Octadecenoic acid, methyl ester (6.51%), (6): 2-(2-Butoxyethoxy) ethanol (6.36%), (7): Hexadecanoic acid, methyl ester (5.18%), (8): 2-Oleoylglycerol (2.70%), (9): *cis*-2-Penten-1-ol (2.54%), and (10): Stearic acid (2.01%).

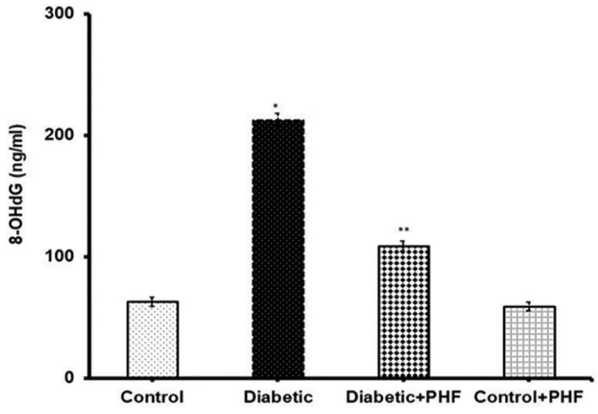

Fig. 3. Urine 8-OHdG excretion in diabetics as a result of PHF treatment. 8-OHdG levels in the urine of diabetics rose significantly. In the Diabetic + PHF group, PHF therapy considerably lowered this level. Data expressed as mean ± S.E.M. (*P < 0.05 Diabetic group vs Control OR Control + PHF group; **P < 0.05 Diabetic + PHF group vs Diabetic group).

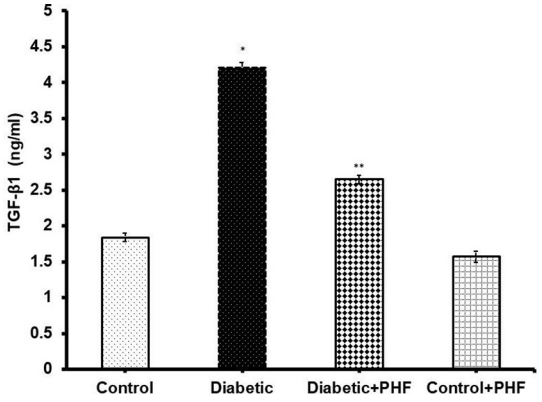

Fig. 4. Effect of PHF administration on TGF-β1 level in diabetic group. Diabetic group showed a significant rise in TGF-β1 level. While supplementation with PHF significantly reduced its level in Diabetic + PHF group. Data expressed as mean ± S.E.M. (*P < 0.05 Diabetic group vs Control/Control + PHF group; **P < 0.05 Diabetic + PHF group vs Diabetic group).

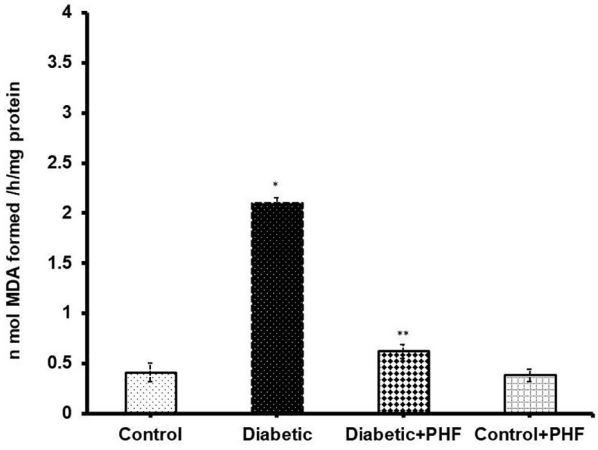

Fig. 5. Effect of PHF treatment on MDA content in the diabetic group. Data expressed as mean ± S.E.M. Diabetic group showed a significant increase in MDA level that was reversed after PHF treatment in the treatment group in kidney tissue*P < 0.05 diabetic group vs Control OR Control + PHF group; **P < 0.05 Diabetic + PHF group vs diabetic group.

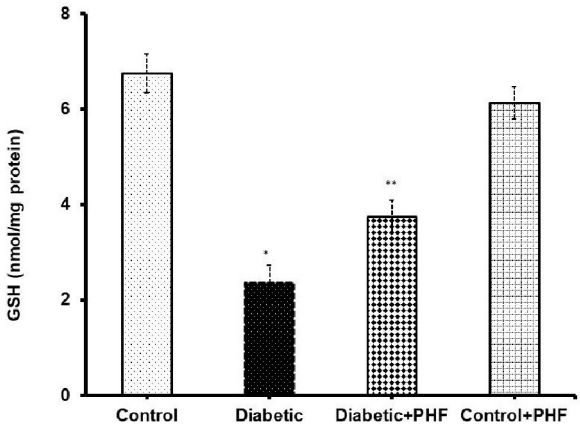

Fig. 6. PHF therapy affects GSH content in diabetics. Data are shown as mean ± S.E.M. Diabetic group exhibited considerably lower GSH content in kidney tissue, which was restored by PHF therapy. *P < 0.05 diabetic group vs control OR Control + PHF group; **P < 0.05 Diabetic + PHF group vs diabetic group.

3.9. Histopathological findings

The parenchyma of the kidneys in the control group was well-preserved via PAS. Bowman's capsule and renal tubules enveloped the normal glomeruli. In contrast, the diabetic group had many regions of thickening of the glomerular basement membrane. Consecutively, sclerotic changes in glomeruli, accumulation of plasma proteins between the glomerular endothelium and glomerular basement membrane (i.e., fibrin cap) and fibrinous deposits in the renal interstitium were also observed focally. Findings showed that administration of PHF reverted these maladies of renal tissue showed as well documented renal parenchyma (Fig. 8).

4. Discussion

DM is the most typical and most serious disorder of carbohydrate metabolism, decreasing a ten-year life span globally (Kirkman et al., 2012). Hyperglycemia, lipid abnormalities, and oxidative stress have been considered the main factors to accelerate the pathological condition associated with diabetes (Poznyak et al., 2020). Diabetes also causes chronic kidney disease in both industrialized and developing economies. To treat diabetes and its complications, early therapeutic intervention and care are required. The scientists now support the PHF strategy due to its multi-targets synergistic nature. Various phytoconstituents in the PHF act synergistically to employ their therapeutic efficacy to manage the disease condition (Nayak et al., 2018). PHF prepared from methanolic extracts of *Trigonella foenum*; *Linum usitatissimum*, *Cuminum cyminum*, and *Nigella sativa* seeds were tested for its impact on STZ-induced DN in rats, given its potential health advantages. The present study evaluated that STZ-induced diabetes resulted in hyperglycemia, lipid abnormalities followed by renal oxidative damage, and dysfunctionality in rats, similar to previous research (Clozel et al., 2006). Treatment with PHF for 56 days was enough to mitigate these alterations in experimentally induced DN due to the multi-target approach employed by the individual spices present in it. Beneficial effects of these spices were substantiated by prior research indicating that either of the plants contributed to the restoration of normal metabolism in diabetic studies (Soltanian and Janghorbani, 2018).

The GC–MS analysis of PHF revealed the presence of phytoconstituents such as phenolics, sterols, esters, alcohols and fatty acids. These diverse groups of phytochemicals have gained much attention as potential natural antidiabetic agents and antioxidants.

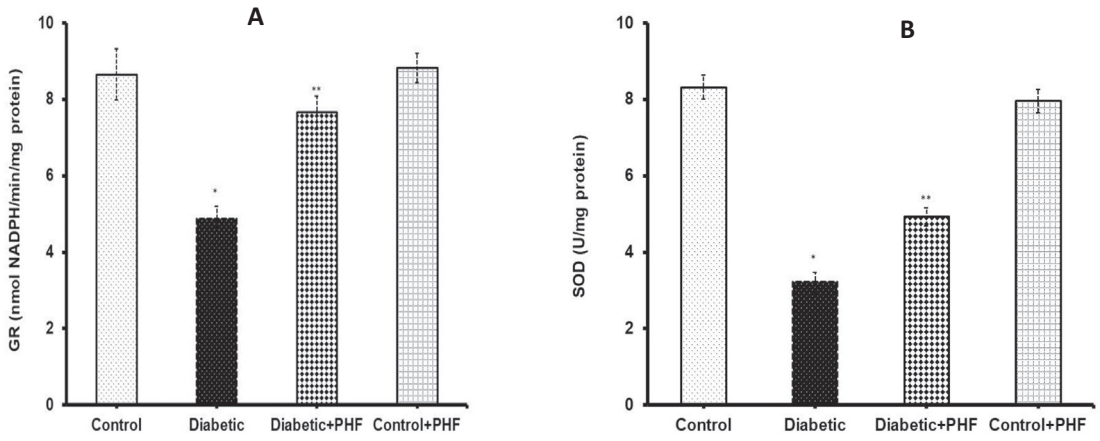

Fig. 7. Supplementation with PHF has an effect on the activity of GR and SOD. (A)Significant diminished level of GR was noted in the diabetic group that was augmented after PHF treatment in the treatment group in kidney tissue. (B) The significantly lowered activity of SOD was observed in the diabetic group, while PHF treatment was restored in the treatment group in kidney tissue. Data expressed as mean ± S.E.M. *P < 0.05 diabetic group vs control OR Control + PHF group; **P < 0.05 Diabetic + PHF group vs diabetic group.

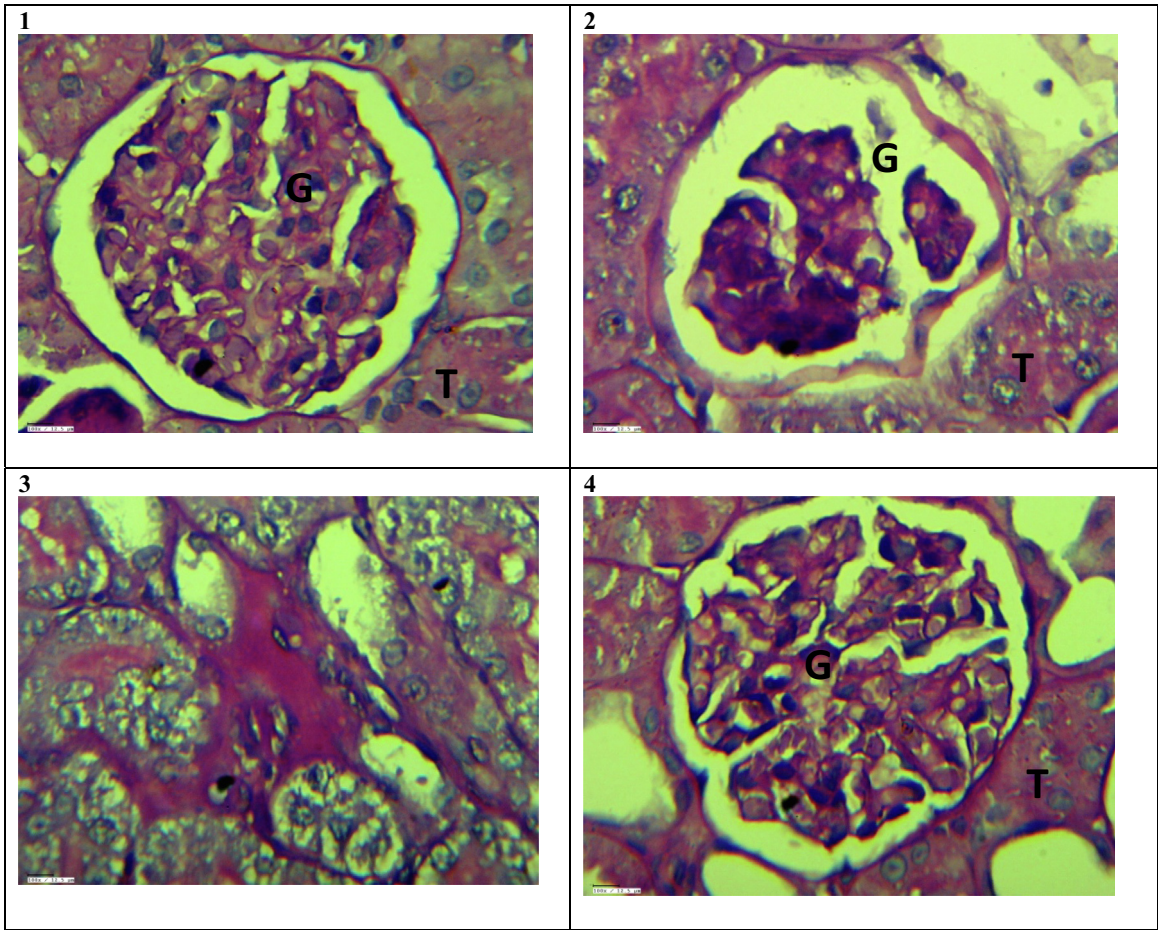

Fig. 8. The PAS-stained area of the kidney (at 40×) is seen.1) Control Kidney – Glomerulus and renal tubules seem to be normal. 2) Diabetic Kidney – Moderately thickened glomerular basement membrane and sclerotic changes in glomeruli. 3) Diabetic kidney – Fibrinous deposits in renal interstitium. 4) The glomerulus and renal tubules seem to be normal in the treated kidney.

Major compounds were identified as sterols, polyunsaturated fatty acids (PUFA), phenol and esters. Previous studies have suggested that phenols are potent free radical scavengers, sterols are insulin secretagogues by regulating ATP sensitive K+ channel and PUFA controlling hyperglycemia (Coelho et al., 2017).

As data depicted that the diabetic group revealed a considerable increase in blood glucose level, the treated group showed an improvement in glucose level after receiving PHF. The favorable impact of PHF may be attributed to the presence of a significant component of individual spices, which decreased hyperglycemia

in diabetics by increasing glucose absorption, correcting insulin action/insulin sensitivity, and maintaining cell mass, corroborated by earlier research (Gray, 2016).

Diabetes is widely documented to compromise kidney function, making it difficult to properly filter blood and produce urine due to increased cell membrane permeability and loss of functional integrity (Abtahi-Evari, 2017). Deterioration of GFR occurs rapidly, measured by evaluating the creatinine and BUN levels, which are indications of renal impairment (Vaidya and Aeddula, 2022). Similar to a prior study, creatinine and BUN levels were higher with diminished creatinine clearance in the current investigation in the diabetic group (Zeng, 2014). We evaluated that administration of PHF significantly ameliorated GFR by improving creatinine clearance in the treatment group due to its anti-inflammatory and antioxidant potential, which were similar to the prior reports (Sayed et al., 2012; Bouzghaya et al., 2020; Sharma et al., 2006).

Dyslipidemia has also been demonstrated to be important in the development and progression of DN (Rutledge et al., 2010). Dyslipidemia therapy is crucial in DN patients because they have a high risk of dying from cardiovascular disease (Kawanami and Utsunomiya, 2016). Increases in VLDL-C and LDL-C, as well as a reduction in HDL-C, are produced by abnormalities in lipoprotein metabolism (Hirano, 2014). So, lipid profiles must be assessed to regulate diabetes and its consequences. PHF-treated diabetics had reduced TC, TG, LDL-C, and VLDL-C levels than diabetics who were not treated. The fact was supported by prior evidence indicating that each PHF plant has been documented to improve lipid anomalies (Mnif and Aifa, 2015). The estrogenic component of fenugreek, which indirectly elevates thyroid hormone T4 levels, may also play a role in its extraordinary protective effect on lipid profiles (Khan et al., 2018). Other methods include sapogenins, which promote biliary cholesterol excretion and reduce serum cholesterol (Bogoriani, 2015). Lignan complex of flaxseed comprising secoisolariciresinol diglucoside (SDG), 3-hydroxy-3methylglutaric acid (HMGA) and cinnamic acid have been described as a potent antioxidant and hypocholesterolemic agent (Prasad, 2000).

Significant evidence has suggested that persistent hyperglycemia is the primary determinant in advancing the early stages of DN. It is the chief source of increased oxidative stress to carry out the pathological alterations during diabetes and diabetes-associated renal complications (Forbes et al., 2008). Reactive oxygen species (ROS) are synthesized either directly or indirectly by hyperglycemia (glycolysis, polyol pathway, PKC, NAD(P)H oxidase, and so on). According to growing research, controlling oxidative stress is crucial to limit the severity of kidney damage; because of this, antioxidant therapy is chosen (Mohebbati et al., 2020).

Our findings revealed that the diabetic group had a much higher level of oxidative DNA damage marker 8-OHdG, raised MDA content and the fibrotic cytokine TGF-β1. We found modification of these elevated oxidative stress markers (8-OHdG, MDA) and TGF-β 1 in the treatment group and other positive effects of PHF in this research. The effectiveness of PHF on the endogenous antioxidant system as one of the strategies to reduce diabetes-induced oxidative kidney damage was also investigated. Like previous research (Bouzghaya et al., 2020; Mohebbati et al., 2020), diabetic rats received PHF demonstrated enhanced GSH, GR, and SOD in the treated group. As a result of the findings, PHF administration for 56 days was enough to relieve oxidative stress-prompted renal damage due to their antioxidant ability in a model of experimental DN in rats. Substantially, our findings were supplemented by enhanced histological characteristics. Renal glomeruli and tubules of diabetic rats showed significant structural changes. PHF's beneficial effect is due to the presence of a large number of phytoconstituents that work synergistically to prevent renal damage and maintain renal integrity under oxidative stress conditions, most likely by inhibiting the formation of AGEs and free radicals and suppressing TGF-1/CTGF signaling (Mahmoodi and Mohammadizadeh, 2020).

Considering hyperglycemia is the main culprit in DN, many potential mechanisms of each individual herb present in PHF have been proposed and investigated in animal and human subjects to manage glucose levels. Fenugreek's antidiabetic activities have been related to an insulin signaling pathway based on earlier studies on the reduction of intestinal glucose absorption, delayed stomach emptying, and/or insulinotropic action. This unique fenugreek amino acid, 4-Hydroxyisoleucine (4-HIL), improves glucose absorption by increasing GLUT4 phosphorylation of AKT (Jaiswal et al., 2012). In contrast, *Nigella sativa* and its active constituents have preserved pancreas β- cell mass by inhibiting COX-2 mRNA expression under oxidative stress conditions (Al Wafai, 2013). Cuminaldehyde and cuminol, two key components of *C. cyminum*, which is one of the components of PHF, are insulin secretagogues with substantial inhibition effect on aldose reductase and R-glucosidase, and a protective effect on the β–cell. Likely, SDG isolated from flaxseed has been proposed as a strong antioxidant with β-cell regeneration properties (Slavova-Kazakova et al., 2015).

5. Conclusion

To summarise, the current investigation supports the nephroprotective effects of PHF by alleviating hyperglycemia, dyslipidemia, antioxidant status, and the degree of renal dysfunction and pathological changes, therefore presenting a multi-target therapy for DN prevention. Hence, it would facilitate the preservation of nephritic integrity and the hyperglycemia-prompted oxidative stress condition. Their antioxidant and anti-inflammatory qualities, thereby defining their antidiabetic potential against DN, may play a significant role in treating diabetes complications and may ultimately acquire therapeutic significance, supporting these positive effects. In this investigation, we concluded that a PHF was both efficacious and safe in the treatment of diabetic nephropathy. However, the long-term effectiveness and side effects of PHF use must be assessed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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