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Yuvraj Singh Surana

Department of Pharmacology,
KLE College of Pharmacy,
Bengaluru, KLE Academy of
Higher Education and Research,
Belagavi, Karnataka, India

Purnima Ashok

Department of Pharmacology,
KLE College of Pharmacy,
Bengaluru, KLE Academy of
Higher Education and Research,
Belagavi, Karnataka, India

Rajendran R

Green Chem Herbal Extracts and
Formulation, Domlur, Bengaluru,
Karnataka, India

Krishna Rajendran

Massachusetts Institute of
Technology, Harvard Business
School, USA

Correspondence

Purnima Ashok

Department of Pharmacology,
KLE College of Pharmacy,
Bengaluru, KLE Academy of
Higher Education and Research,
Belagavi, Karnataka, India

Role of Polyherbal formulation in secondary complications like neuropathy and retinopathy in streptozotocin-nicotinamide induced diabetes in rats

Yuvraj Singh Surana, Purnima Ashok, Rajendran R and Krishna Rajendran

Abstract

Objective: To evaluate neuropathy, retinopathy and antioxidant properties of a polyherbal formulation (PHF) aqueous extract in Streptozotocin- nicotinamide induced diabetic rats.

Methods: Fasting blood glucose (FBG), serum insulin and glycated haemoglobin (HbA1C) levels were determined in normal and Streptozotocin- nicotinamide induced diabetic rats after oral administration of the PHF for 45 days. Neuropathic analgesia was assessed by tail-flick and hot-plate methods and to assess retinopathy, increase levels of vascular endothelial growth factor (VEGF) and intracellular adhesion molecule (ICAM-1) were measured. Antioxidant property was evaluated by estimating superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels in sciatic nerve and retina. Histopathological changes in sciatic nerve and retina were also observed after PHF treatment.

Results: Daily oral administration of PHF (200 and 400 mg/kg, b.w.) and metformin (5mg/kg, b.w.) showed beneficial effect on blood glucose levels ($P < 0.001$) of diabetic animals. The PHF treatment enhances serum insulin levels and body weight of diabetic rats as compared to diabetic control group. PHF treated animals showed decrease in tail immersion latency time, increase in pain sensitivity and significant decrease in levels of VEGF and ICAM-1, when compared to diabetic group. Furthermore, the PHF has a favourable effect on histopathological studies, in Streptozotocin- nicotinamide induced diabetic animals. Antioxidant enzyme levels were found to be significantly increased and that of MDA were decreased in PHF treated diabetic animals.

Conclusion: PHF showed potent anti-diabetic and protection against associated secondary complications of diabetes like neuropathy and retinopathy and also possesses antioxidant properties. Histopathological studies support these claims of PHF in Streptozotocin- nicotinamide induced diabetic animals.

Keywords: Streptozotocin, Nicotinamide, Metformin, Polyherbal formulation

1. Introduction

Diabetes mellitus (DM) comprises a group of common metabolic disorders that share the phenotype of hyperglycaemia. Type 2 DM is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion and increased glucose production [1]. The worldwide prevalence of DM has risen dramatically over the past two decades. The prevalence of type 2 DM is expected to rise more rapidly in future because of increasing obesity and reduce activity levels [2].

The chronic complications of DM affect many organ systems and are responsible for the majority of morbidity and mortality associated with the disease. Neuropathy and retinopathy are common complications of DM and are related to duration and severity of hyperglycaemia [3, 4].

Usually in neuropathy it will take two or more than two decades for the appearance of symptoms in 50% of the affected population. Diabetic neuropathy affects all peripheral nerves including pain fibers, motor neurons, and the autonomic nervous system [5].

More than 60% of Type 2 DM patients develop retinopathy after 20 years. It progresses from nonproliferative abnormalities to proliferative diabetic retinopathy and is characterized by retinal edema, haemorrhage, increased neovascularisation and neuronal degeneration in the retina. The major factors responsible for the development of diabetic retinopathy are hyperglycaemia and poor diabetic control. Apart from hyperglycaemia, other factors which are responsible for the development of diabetic retinopathy are increased activity of aldose reductase (AR) and protein kinase C (PKC), as well as promoting nonenzymatic glycation and glycooxidation of proteins like advanced glycation end products (AGEs).

It has been reported that the up-regulation of proinflammatory factors and angiogenic parameters, such as tumor necrosis factor alpha (TNF- α), vascular endothelial growth factor (VEGF), intercellular adhesion molecule-1 (ICAM-1) and interleukin-1 β (IL-1 β), contribute to the blood-retinal barrier (BRB) breakdown, which directly leads to macular edema in diabetic retinopathy (DR) [6].

Ethno-botanical information reports around more than 800 plants that may possess antidiabetic activity when assessed using the presently available experimental techniques [7]. India is a rich source of medicinal plants and in Ayurveda and Siddha system of medicines, number of plant extracts are available to manage diabetes. The advantage of traditional medicinal plant is fewer side effects with multiple therapeutic actions due to presence of different bioactive compounds. World Health Organisation (WHO) has also recommended that the research on the beneficial uses of medicinal plants in the treatment of DM have also gained momentum [8]. Moreover diet and spice therapies become the major approaches recently for the management of diabetes. A significant amount of work has been carried out with *Momordica charantia* Linn [9], *Coccinia indica* W. & A [10], and *Lagerstroemia speciosa* Linn [11], and all these herbs individually possess significant antidiabetic activity. This is an attempt to expose the possibility of these herbs in combination as antidiabetic and also explore their protective effect in secondary complications of DM in Streptozotocin-nicotinamide induced diabetic animals.

2. Materials and Methods

2.1. Chemicals

Streptozotocin and nicotinamide (Sigma-Aldrich, USA), serum insulin kit (Mercodia, Sweden), HbA1C kit (Accurex Biomedical PVT. LTD. Maharashtra, India) Intracellular adhesion molecule-1 Elisa kit (YH Biosearch laboratory, Shanghai, China), Vascular endothelial growth factor Elisa kit (RayBio, USA) and biochemical reagent for Fasting blood sugar Agapee diagnostics, India, were purchased.

2.2. Animals

Healthy albino Wistar rats of approximately same age group (200-250 g) were procured from, a registered breeder. Animals were housed at Institutes animal house facility in polypropylene cages and maintained under standard conditions (12 h light/dark cycle, 22 \pm 2^o C and 55 \pm 5% relative humidity). They were fed with standard rat pellet diet and water *ad libitum*. The animals were maintained in accordance with Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) guidelines for the care and use of laboratory animals. The study protocol was approved by Institutional Animal Ethics Committee (IAEC), KLE University's College of Pharmacy, Bengaluru (01/PA/2015).

2.3. Preparation of solutions

Test drug and metformin were dissolved in distilled water and administered orally for experimental purpose. All the test drugs were freshly prepared each time before use.

2.4. Determination of acute oral toxicity [12]

The acute oral toxicity of Polyherbal formulation (PHF) was carried out according to Organization of Economic Cooperation and Development (OECD) guidelines 425 by using female albino Wistar rats (150-200g), which were

maintained under standard conditions. Animals were kept under fasting 12 h prior to the experiment, water given *ad libitum*. Test drug was administered to all animals in a single dose of 2000 mg/kg by using a stomach tube and all the animals were observed individually for signs of toxicity.

Animals observed for first four hours and thereafter for total of 14 days.

2.5. Induction of diabetes and experimental design [13]

Diabetes was induced in overnight fasted animals (deprived of food 16 h but had been allow to free access to water) by a single intraperitoneal (i.p.) injection of freshly prepared Streptozotocin (STZ) dissolved in citrate buffer (65 mg/kg, b.w.) 15 min after the i.p. administration of 110 mg/kg, b.w. of nicotinamide (NA) dissolved in normal saline. Hyperglycaemia was confirmed by elevated glucose levels in plasma, determined after 72 h injection of STZ. Animals with blood glucose concentration more than 200 mg/dL were used for the study. The diabetic animals were randomly divided into five groups containing twelve in each group. All groups receive STZ - Nicotinamide except normal control and the treatment protocol is as follows:

Group I – Normal control (saline treatment)

Group II – Positive control (STZ -Nicotinamide treatment)

Group III – Aqueous PHF (STZ -Nicotinamide + Dose I, 200 mg/kg, b.w.)

Group IV – Aqueous PHF (STZ -Nicotinamide + Dose II, 400 mg/kg, b.w.)

Group V – Standard group (STZ -Nicotinamide + Metformin, 5mg/ kg, b.w.)

The test drugs were administered orally using an intragastric tube once daily for 45 days, continuously. Body weight of animals was measured throughout the experiment. After 2 h of Standard and PHF treatment on the last day, the animals were subjected to Eddy's hot-plate test and tail-flick method to assess the development of neuropathy [14]. At the end of the experiment, animals were fasted overnight and blood collected by retro-orbital puncture under light anaesthesia for various biochemical estimations.

The animals were then sacrificed (under the influence of overdosed isoflurane anaesthesia), after that rat eyes were collected and the left one was used for the measurements of vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1) using respective ELISA kits [6]. The sciatic nerve and retina were quickly excised, immediately rinsed in ice cold saline; a portion of the nerve was fixed in 10% neutral buffered formalin for histopathological study and remaining portion was stored for further biochemical estimations.

Estimation of Biomarkers of Oxidative Stress in Nerve:

Sciatic nerve was placed in 10% w/v potassium chloride (KCl) solution, homogenize and centrifuge at 5000 rpm for 10 min. The supernatant obtain was used for the following assay:

- SOD [15]
- CAT [16]
- MDA [17]

Measurements of SOD and MDA levels in Retina: At the end of the experiment, left eye was collected and from that retina was isolate and centrifuge at a speed of 3,500 rpm for 10 min, the supernatant obtain was used for the assay of SOD and MDA.

3. Results

Acute oral toxicity study

Acute oral toxicity study of polyherbal formulation (PHF) was performed as per the OECD guideline 425 and it showed the non-toxic nature of PHF at the limit test dose of 2000 mg/kg, b.w.p.o.

Effect of PHF and metformin on body weight, blood glucose level, serum insulin level and glycated haemoglobin level in STZ-nicotinamide induced diabetic animals

The body weight of the diabetic rats showed a significant ($P < 0.001$) decrease after the administration of STZ-nicotinamide. The treatment with PHF (Dose-I 200 mg/kg, Dose-II 400 mg/kg) and metformin showed mild reduction in the body weight as compared with diabetic control rats.

In the diabetic control rats, FBS level was significantly ($P < 0.001$) increased when compared to normal control rats. The diabetic rats treated with PHF (Dose-I 200 mg/kg, Dose-II 400 mg/kg) and metformin showed more significant ($P < 0.001$) reduction in FBS levels as compared to diabetic

control rats. The STZ-nicotinamide injection significantly decreases insulin levels.

In the diabetic control rats, HbA1C level was significantly ($P < 0.001$) increased when compared to normal control group. The diabetic rats treated with PHF (Dose-I 200 mg/kg, Dose-II 400 mg/kg) and metformin showed significant ($P < 0.001$) reduction in HbA1C levels as compared to diabetic control rats [18].

Effect of PHF on latency period in Hot-plate and Tail-flick methods in STZ-nicotinamide induced diabetic animals

Diabetic control rats showed a statistically significant ($P < 0.001$) increase in the tail-flick latency time and significant ($P < 0.001$) decrease in the response time with hot-plate method when compared with normal control group. The treatment of diabetic rats with PHF (Dose-I 200 mg/kg, Dose-II 400 mg/kg) and metformin showed a statistically significant ($P < 0.001$) increase in the tail-flick latency time and significant ($P < 0.001$) decrease in the response time with hot-plate method when compared with the positive control group [Table-1].

Table 1: Effect of PHF on latency period in Hot-plate and Tail-flick methods in STZ- nicotinamide induced diabetic animals

Groups	Hot-Plate Method (sec.)	Tail-Flick Method (sec.)
Normal	0.61±0.036	4.99±0.066
Positive control (STZ- Nicotinamide)	3.93±0.109***a	8.25±0.088***a
Standard (Metformin 5mg/kg, b.w.)+ STZ Nicotinamide-	1.11±0.048 ***b	5.95±0.052 ***b
Dose-I (200 mg/kg, b.w.)+STZ- Nicotinamide	2.07±0.034 ***b	7.17±0.039 ***b
Dose-II (400 mg/kg, b.w.)+STZ- Nicotinamide	1.56±0.071 ***b	6.12±0.048 ***b

Values are expressed as mean± SEM, n=6. ***p<0.001, when compared to normal control group (a) and ***p<0.001, when compared to positive control group (b).

Effect of PHF on SOD, CAT and MDA levels in sciatic nerve in STZ-nicotinamide induced diabetic animals

The content of MDA, end product of lipid peroxidation and marker of oxidative stress was significantly ($P < 0.001$) increased in sciatic nerve of diabetic control rats as compared to non-diabetic rats. There was a significant ($P < 0.001$) decrease in the levels of anti-peroxidative enzymes (SOD and CAT) in sciatic nerve as compared to normal control group (Fig.1). The treatment of diabetic rats with PHF (Dose-I 200 mg/kg, Dose-II 400 mg/kg) and metformin showed a significant ($P < 0.001$) decrease in the levels of MDA as compared to diabetic control rats (Fig.2) and showed a significant ($P < 0.001$) increase in SOD and CAT activities.

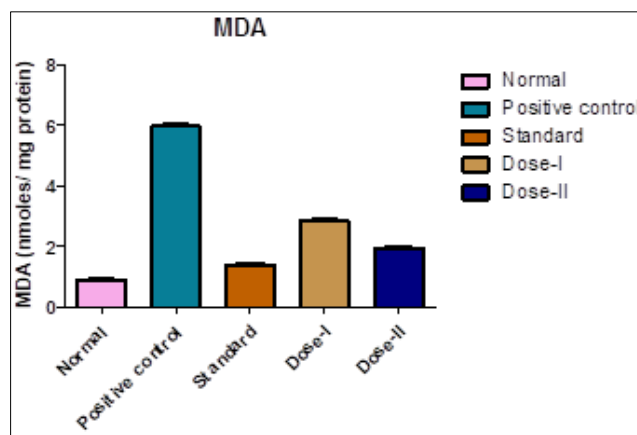


Fig 2: Effect of PHF on MDA levels in sciatic nerve in STZ-nicotinamide induced diabetic animals

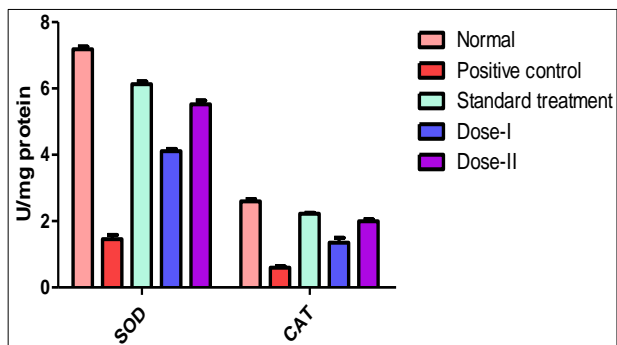


Fig 1: Effect of PHF on SOD and CAT levels in sciatic nerve in STZ-nicotinamide induced diabetic animals

Effect of PHF on retinal VEGF and ICAM-1 Levels in STZ-nicotinamide induced diabetic animals

Significantly increased levels of VEGF and ICAM-1 were observed in retina of diabetic rats at the end of the study. Treatment with PHF (Dose-I 200 mg/kg, Dose-II 400 mg/kg) and metformin significantly reduced the levels of retinal VEGF and ICAM-1 compared with diabetic control group ($p < 0.001$) and the effect of PHF (Dose-I 200 mg/kg, Dose-II 400 mg/kg) was comparable to that of standard metformin group [Table-2].

Table 2: Effect of PHF on retinal VEGF and ICAM-1 levels in STZ-nicotinamide induced diabetic animals

Groups	ICAM-1 (concentration in ng/ml)	VEGF (concentration in ng/ml)
Normal	4.45±0.168	12.18±0.216
Positive control (STZ)	10.23±0.077***a	28.99±0.365***a
Standard (Metformin 5mg/kg, b.w.) + STZ -Nicotinamide	5.39±0.080***b	12.29±0.081***b
Dose-I (200 mg/kg, b.w.)+STZ- Nicotinamide	7.57±0.056***b	20.77±0.283***b
Dose-II (400 mg/kg, b.w.)+STZ- Nicotinamide	5.85±0.043***b	15.74±0.239***b

Values are expressed as mean± SEM, n=6. ***p<0.001, when compared to normal control group (a) and ***p<0.001, when compared to positive control group (b).

Effect of PHF on SOD and MDA levels in retina in STZ-nicotinamide induced diabetic animals

The results showed that compared with rats in the normal control group, MDA levels in diabetic rats were significantly increased, while SOD activity was significantly decreased at the end of the study (p < 0.001). The treatment of diabetic rats

with PHF (Dose-I 200 mg/kg, Dose-II 400 mg/kg) and metformin showed a significant (P < 0.001) decrease in the levels of MDA as compared to diabetic control rats and showed a significant (P < 0.001) increase in SOD activities [Table-3].

Table 3: Effect of PHF on SOD and MDA levels in retina in STZ-nicotinamide induced diabetic animals

Groups	SOD (U/mg protein)	MDA (nmoles/mg protein)
Normal	41.83±0.891	0.49±0.023
Positive control (STZ- Nicotinamide)	24.14±0.420***a	5.37±0.115***a
Standard (Metformin 5mg/kg, b.w.) + STZ -Nicotinamide	34.86±0.281***b	1.16±0.045***b
Dose-I (200 mg/kg, b.w.)+STZ- Nicotinamide	28.68±0.298***b	3.14±0.056***b
Dose-II (400 mg/kg, b.w.)+STZ- Nicotinamide	33.21±0.206***b	1.58±0.075***b

Values are expressed as mean± SEM, n=6. ***p<0.001, when compared to normal control group (a) and ***p<0.001, when compared to positive control group (b).

Histopathological studies of sciatic nerve and retina

Histopathological analysis showed small myelinated fiber loss which was more prominent than large diameter fiber loss. Endoneurial vessel was also not thickened with diabetic control rats as compared to normal control group. However, the treatment with Dose-II 400 mg/kg showed intact myelinated fiber (arrow) density, the small myelinated fiber and large diameter fiber appear intact and the endoneurial vessel was also not thickened as compare to normal control

group [Fig-3].

Section studied in positive control rat shows extensive vacuolations in the plexiform layers and ganglion layer (arrow) as compare to normal control group (without any vacuolations in the plexiform layers and ganglion layer). Treatment with PHF (Dose-II 400 mg/kg) showed moderately reduced vacuolations [compared to positive control] in the plexiform layers and ganglion layer [Fig-4].

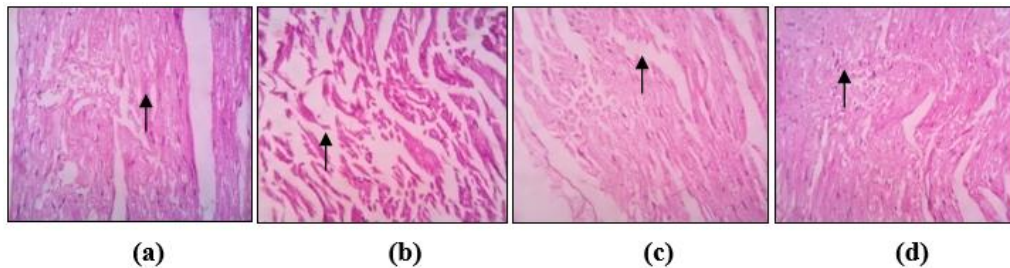


Fig 3: Histopathology of sciatic nerve from rats. (a) Normal control group, (b) Positive control group, (c) Standard (d) Dose-II 400 mg/kg (H&E 400x)

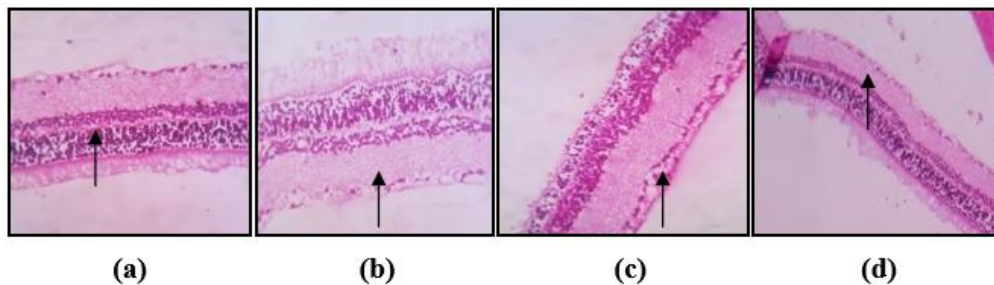


Fig 4: Histopathology of retina from rats. (a) Normal control group, (b) Positive control group, (c) Standard (d) Dose-II 400 mg/kg (H&E 400x)

4. Discussion

This study explored the protective effect of PHF on Streptozotocin-nicotinamide induced diabetes in Wistar albino rats.

STZ is a broad-spectrum antibiotic that is toxic to insulin-producing pancreatic islet β-cells, and it is a widely used experimental model to induce hyperglycemia. The intraperitoneal administration of STZ (65 mg/kg) partially

damage the insulin secreting pancreatic β -cells by breaking the DNA strand, which results in increased blood glucose levels and decreased in endogenous insulin release [19]. Oral administration of PHF for 45 days (200 and 400 mg/kg) resulted in significant reduction in fasting blood glucose levels. The increased serum insulin levels in PHF treated STZ- nicotinamide diabetic rats could be due to protection of functional β -cells from further deterioration. Increased levels of insulin might help in improving glycemic control in STZ-diabetic rats.

In this study, the body weight of STZ-nicotinamide induced untreated diabetic group showed significant decrease in body weight. Per Oral administration of PHF at dose of 400 mg/kg for 45 days showed an improvement in body weight in comparison to diabetic control and rats treated with metformin. The increased in body weight of PHF treated rats might be due to their improved glycemic control.

HbA1C levels are monitored as a consistent index of glycemic control in diabetes [20]. In this study administration of PHF decreased fasting blood glucose levels, further leading to significant reduction in HbA1C levels in diabetic rats.

The pain perception is significantly low in diabetic animals when compared to normal control animals may be due to nerve damage and induction of peripheral neuropathy [21, 22]. The PHF untreated animals showed a significant decrease in paw withdrawal magnitude, which indicates the development of hyperalgesia. This study revealed that treatment with PHF decreases the neuropathic pain in animals.

There are various theories of diabetic retinopathy have been proposed, increased oxidative stress induced by hyperglycemia seems to be the one of the mechanism of diabetic complications, which can lead to activate the polyol pathway, increase AGE formation, activate PKC and hexosamine pathways and all leading to the development of diabetic retinopathy (DR). Among all the cytokines involved in DR, VEGF, has been identified as a primary initiator of proliferative DR and as a potential mediator of nonproliferative retinopathy [23]. In the retina, VEGF can induce ICAM-1 expression and leucocyte adhesion, which together with VEGF lead to the BRB breakdown, and it has also been reported that retinal VEGF and ICAM-1 levels are strongly correlated with neovascularization in patients with DR [24]. In our present study, significantly increased levels of VEGF and ICAM-1 were observed in retina of diabetic rats at the end of the study. Treatment with PHF significantly reduced the levels of retinal VEGF and ICAM-1 compared with diabetic control group and this effect of PHF was comparable to that of standard metformin.

Oxidative stress plays an important role in the development of hyperglycaemia, which may result in generation of reactive oxygen species (ROS) causing cellular injury and several deleterious effects on the cellular physiology and these have important role in the development of secondary complications associated with diabetes. Increased level of MDA in diabetic put forward for consideration that peroxide injury may be involved in the diabetic complications.

In the present study, a significant increase in the levels of tissue malondialdehyde (MDA) content in STZ- nicotinamide induced diabetic rats leading to tissue injury and failure of antioxidant defence mechanism has been observed. The diabetic rats treated with PHF significantly decreased the levels of MDA in sciatic nerve and retina. Several studies showed that there is generation of oxygen free radicals in STZ-treated β -cells, and that the over expression of

antioxidant enzymes, such as SOD, CAT [25]. Reduced activities of SOD and CAT in sciatic nerve and retina have been observed during diabetes. SOD is vital defence enzyme which catalyses the dismutation of superoxide radicals. CAT is a heme protein which catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals [26]. In schizophrenia and atherosclerosis low activity of catalase has been reported [27], with same assumption that long-term oxidative stress may lead to development of type 2 diabetes mellitus. Our results correlates with these findings as treatment with PHF significantly increased the SOD, CAT levels in kidney and liver and also significantly increased the levels of GSH in vital organs of diabetic rats which is of vital importance in the treatment of DM.

Histopathological study of sciatic nerve showed that a degenerative change like small myelinated fiber loss was more prominent than large diameter fiber loss and endoneurial vessel was also not thickened with diabetic control rats as compared to normal control group. Treatment with PHF showed intact myelinated fiber density, the small myelinated fiber and large diameter fiber appear intact which reveals the protective effect of PHF in secondary complications DM.

Histopathological study of sciatic nerve also showed protection from extensive vacuolations in the plexiform layers and ganglion layer when treated with PHF, which can be considered as a promising effect. This lead to state that may be PHF can be considered in the treatment of secondary complications of diabetes mellitus specially in neuropathy and retinopathic complications.

5. Conclusion

In conclusion, data from the present study states that the PHF has potent antidiabetic activity and also shown protective effect in neuropathy and retinopathy, secondary complications associated with DM. The biochemical and histopathological results of present study also revealed the degree of protection offered by PHF to diabetic animals. Further studies are required for bioactivity guided drug discovery to isolate lead compounds, which may be responsible for these claimed activities.

6. Conflict of interest

The authors declare that they have no conflicts of interest.

7. Acknowledgements

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