RESEARCH PAPER

Evaluation of Metformin and Herbal Formulations for Treating Diabetic Neuropathy in a Rat Model

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Running title: Metformin and Herbal Treatments for Diabetic Neuropathy

ABSTRACT

Background and Objectives: Diabetic neuropathy (DN), which affects 60% of individuals with diabetes mellitus (DM), is characterized by spontaneous pain, allodynia, and hyperalgesia that considerably diminish their quality of life. Amla, Arjuna, Gudmar, Guduchi, and Ginger are herbs widely used in India for the treatment of diabetes and its related problems. This study examined the impact of the concurrent administration of polyherbal formulations and metformin on STZ-NA-induced diabetic neuropathy in rats.

Approaches: Streptozotocin (65 mg/kg, intraperitoneally) was injected to rats 15 minutes following nicotinamide (110 mg/kg, intraperitoneally) to develop diabetic neuropathy. Diabetic rats were administered daily oral doses of metformin; a polyherbal formulation (PHF) at 100, 200, and 400 mg/kg; and an allopolyherbal formulation (APHF) at 200 mg/kg for 60 days. Blood glucose levels (BGL) were assessed on days 0, 30, and 60, whereas blood levels of SOD, GSH, MDA, NO, and Ca were evaluated at the end of the trial. Motor coordination, grip strength, behavioral metrics, passive avoidance test results, ocular sensitivity, and mechanical hyperalgesia were also evaluated. Tail-flick latency assessments (both warm and cold) were performed at 4, 6, and 8 weeks of the research.

Results: Treatment with metformin, PHF, and APHF led to statistically significant reductions in blood glucose, malondialdehyde, nitric oxide, and calcium levels, with statistically significant increases in superoxide dismutase, glutathione, and body weight in treated diabetic rats. Post-treatment with the specified drugs, tail withdrawal latencies and corneal sensitivity were markedly increased relative to diabetic control rats. Moreover, the administration of PHF and metformin resulted in a statistically significant increase in activity ($p < 0.01$) compared to the diabetic control rats.

Conclusion: This study revealed that metformin, a polyherbal formulation (PHF), and an allopolyherbal formulation (APHF) significantly alleviated diabetic neuropathy in rats, as indicated by enhanced blood glucose levels, biochemical markers, and behavioral metrics.

Keywords: Herbal; Diabetes; metformin; Neuropathy, Peripheral polyneuropathy, Diabetic neuropathy, Diabetes mellitus, Polyherbal formulation, Allopolyherbal formulation, Streptozotocin, Nicotinamide, Metformin

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INTRODUCTION:

Diabetic neuropathy, characterized by spontaneous pain, allodynia, and hyperalgesia, is the predominant consequence of diabetes mellitus (DM), affecting 60% of patients and significantly affecting their quality of life[1-3]. The complex pathogenesis of diabetic neuropathy often renders existing treatments inadequate, highlighting the need to investigate novel therapeutic combinations.

Hyperglycemia triggers numerous physiological changes and activates several pathways, including the polyol pathway, depletion of myoinositol, increased accumulation of sorbitol, disrupted Ca2+ homeostasis, elevated levels of advanced glycation end-products, enhanced reactive oxygen species, and altered protein kinase C activity[4-5]. These changes may result in neuropathy. Multiple signaling pathways are implicated in the development of diabetic neuropathy, including increased activation of the polyol pathway, oxidative stress, formation of advanced glycation end products (AGEs), nerve hypoxia/ischemia, activation of protein kinase C, and reduced levels of nerve growth factors[6].

The polyol pathway plays a crucial role in the development of diabetic neuropathy. This route consists of two enzymes, aldose reductase (AR) and sorbitol dehydrogenase. AR, predominantly expressed in Schwann cells (SC), catalyzes the conversion of glucose to sorbitol using NADPH as a cofactor, whereas sorbitol dehydrogenase converts sorbitol to fructose using NAD+[7]. Increased sorbitol levels harm nervous tissue, and the lack of NADPH in the SC reduces glutathione concentration and increases vulnerability to intracellular oxidative stress[8].

Hyperglycemia leads to excessive synthesis of nitric oxide (NO), which interacts with superoxide to produce peroxynitrite, causing significant DNA damage[4]. Oxidative stress results in elevated levels of malondialdehyde (MDA) and reactive oxygen species (ROS), leading to oxidative damage in the pancreas, liver, and kidneys[9], these physiological alterations underlie specific conditions such as diabetic peripheral polyneuropathy (DPN), a prevalent long-term complication affecting both somatic sensory and autonomic neurons[10].

Despite current treatment options, there remains a pressing demand for more effective therapeutics to improve the management of diabetic neuropathy and its associated oxidative stress. Contemporary therapeutic approaches are constrained and mostly concentrate on symptomatic relief rather than on targeting fundamental pathophysiological processes.

Herbal remedies such as Amla, Arjuna, Gudmar, Guduchi, and Ginger have garnered significant attention because of their potential antidiabetic and antioxidant properties. These herbs have traditionally been used in various cultures, particularly in Ayurvedic medicine, to alleviate diabetic symptoms and complications. Their ability to modulate blood glucose levels, improve insulin sensitivity, and reduce oxidative stress make them promising candidates for adjunctive therapy in diabetes management. Moreover, these herbs have shown potential for addressing specific diabetic complications, such as neuropathy, through their multifaceted mechanisms of action.

Despite increasing interest in herbal remedies for diabetic neuropathy, comprehensive research on their combined effects with allopathic medications remains insufficient. This knowledge gap presents an opportunity for further scientific investigation. The objective of this study was to evaluate the effect of metformin, a polyherbal formulation, and its combined treatment on diabetic neuropathy in a rat model. We hypothesized that the concurrent administration of metformin and a polyherbal formulation will elicit a superior therapeutic effect on diabetic neuropathy compared to either treatment administered independently. The research question is: Does the co-administration of metformin and a polyherbal formulation produce a synergistic effect in the treatment of diabetic neuropathy in rats?

MATERIALS AND METHODS

Drugs and chemicals

Streptozotocin (STZ) was procured from Chemvenio, LIC, Gulbarga, India; Nicotinamide (NA) from SDS Neutraceuticals, Karad, India; and Metformin tablets from USV Pharma, Mumbai. The glucometer was acquired from AccuCheck (Roche Diagnostics). All other compounds used in this study were of analytical grade.

Holistic extracts

All extracts were obtained by supercritical fluid extraction (SFE) and provided as complementary

samples by Nisarga Biotech Pvt. Ltd., Satara, Maharashtra.

Preparation of polyherbal formulation

A polyherbal suspension was prepared using a mortar and pestle comprising supercritical CO2 extracts of five plants: Gymnemasylvestre (25%), Terminalia arjuna (25%), Emblica officinalis (20%), Zingiber officinale (20%), and Tinospora cordifolia (10%). The formulation incorporated Sodium carboxymethyl cellulose (1%) was used as the suspending agent, propylparaben (0.20%) as the preservative, glycerin (1 mL) as the wetting agent, and peppermint oil (0.15%) as the flavoring agent. Purified water was used as a vehicle to achieve the desired volume. The mixture was triturated to form a homogeneous suspension, and the quality of the final product was evaluated according to the World Health Organization (WHO) guidelines for quality control of herbal materials[11].

Identification of phytochemical constituents of natural products

The obtained extracts were subjected to preliminary phytochemical studies to identify the groups of chemicals (secondary metabolites). Plant extracts and polyherbal formulations (PHF) were prepared at a concentration of 100 mg/ml.

Phytochemical Analysis of polyherbal formulation

Polyherbal formulations and individual herbal CO2 extracts used in the formulation were subjected to systematic qualitative analyses to identify various phytoconstituents, including glycosides, phenolic compounds, tannins, saponins, steroids, flavonoids, alkaloids, carbohydrates, proteins, and lipids, in accordance with standard procedures[12-15].

Experimental animals

Albino Wistar rats (180-200 g ± 20 g, of either sex) were obtained from the National Institute of Biosciences (Dhangawadi, Bhor, Pune, India) and maintained under standard laboratory conditions (temperature: 22±2°C, relative humidity: 50±15%, 12-hour light-dark cycle)[16]. They were provided with ad libitum access to water and a rat pellet diet from Hindustan Lever Ltd., Bangalore. The rats were acclimatized for one week prior to the study[17]. The Institutional Animal Ethics Committee of GES's Satara College of Pharmacy in Satara, India sanctioned the experimental procedure (approval number: SCOP/IAEC/2011-12/38). All animal studies were performed in compliance with the CPCSEA rules.

Determination of LD_{50} of PHF

PHF was administered as a single dose via gavage in a stomach tube. Rats were fasted overnight before administration of an initial dose of 300 mg/kg[18]. Before administering the dose, the body weight of each animal was measured and the dose was calculated accordingly. Three female rats were used for each step and administered doses at 48-hour intervals. Animals were observed individually after dosing for the initial 30 min and periodically over the first 24 h, with a particular focus during the first 4 h and daily assessments thereafter for a total duration of 14 days[19]. An acute oral toxicity study was performed according to OECD Guideline 423[20].

Induction of diabetic neuropathy in rat

Diabetic neuropathy was established in overnight-fasted adult albino Wistar rats with a single intraperitoneal injection of 65 mg/kg streptozotocin (STZ) administered 15 minutes after an intraperitoneal injection of 110 mg/kg nicotinamide (NA). STZ was solubilized in citrate buffer (pH 4.5), while NA was solubilized in normal saline. Hyperglycemia was validated by increased blood glucose levels 72 hours after injection. Animals with fasting blood glucose levels over 250 mg/dl were categorized as diabetic and subsequently utilized for DCM investigations[21].

Experimental design

Experimental animals were randomly divided into seven groups, each consisting of eight animals.

Group I: Normal rats given a vehicle (10 ml/kg) containing 1% CMC solution

Group II: Diabetic rats given a vehicle (10 ml/kg) containing 1% CMC solution

Group III: Metformin (200 mg/kg)-treated diabetic rats

Group IV: PHF-A (polyherbal formulation, 100 mg/kg)-treated diabetic rats

Group V: PHF-B (polyherbal formulation, 200 mg/kg)-treated diabetic rats

Group VI: PHF-C (polyherbal formulation, 400 mg/kg)-treated diabetic rats

Group VII: APHF-treated diabetic rats (100 mg/kg PHF plus 100 mg/kg metformin)

Statistical Analysis

The results are presented as mean±standard error of the mean (mean±SEM). The two experimental groups were compared using Student's t-test (acute toxicity). One-way analysis of variance (ANOVA) and Dunnett's multiple comparison test (GraphPad Instat version 3.00) were used to identify differences between more than two groups. The probability values were deemed statistically significant at $p<0.05$.

RESULT

Induction of diabetic neuropathy in rats

Diabetic neuropathy was induced in adult albino Wistar rats through a single intraperitoneal injection of 65 mg/kg streptozotocin, administered 15 minutes following the intraperitoneal administration of 110 mg/kg nicotinamide. The rats had been fasted overnight prior to the procedure[21-22].

Table 4.15.1. Experimental design for neuro-protective activity

Group		Animals	Treatment, dose and route	Study Period
I	Normal control	Healthy rats	Vehicle, 1% CMC solution 10 ml/kg, p.o.	
II	Diabetic control	Diabetic rats	Vehicle, 1% CMC solution 10 ml/kg, p.o.	60 days
III	Metformin	Diabetic rats	Metformin 200 mg/kg/day, p.o.	
IV	PHF-A	Diabetic rats	Polyherbal formulation 100 mg/kg/day, p.o.	
V	PHF-B	Diabetic rats	Polyherbal formulation 200 mg/kg/day, p.o.	
VI	PHF-C	Diabetic rats	Polyherbal formulation 400 mg/kg/day, p.o.	
VII	APHF	Diabetic rats	Metformin 100 mg/kg + Polyherbal formulation 100 mg/kg/day, p.o.	

TREATMENT

One week following the STZ-NA injection, all of the previously specified treatments were started. For 60 days, each therapy was given to the corresponding animal groups once every day.

Measurement of serum glucose: Blood samples were collected from the experimental rats via the retro-orbital plexus method using capillary glass tubes. The collected blood samples were placed in Eppendorf tubes with a capacity of 1.5 ml. Serum glucose levels were quantified using the GOD-POD reagent kit (Span Diagnostics Ltd., India). Blood was removed from the retro-orbital vein through puncture on days 0, 15, 30, 45, and 60 of the treatment. The blood sample was centrifugation at

2500 rpm for 20 min to facilitate serum separation. Subsequently, the concentration of glucose in the serum was quantified using the glucose oxidase method. The measurement of serum glucose concentration was conducted through a specific procedure utilizing GOD-POD reagents, as detailed in (Table 4.2.5.1.1).

Assessment of motor coordination in diabetes rats: The rotarod apparatus was employed to evaluate motor coordination, operating at a rotational speed of 15 rpm and cutoff time of 180 s. Rats were acclimatized for two days, and tests were performed on the third day. The device comprised a horizontal metal rod with a diameter of 3 cm, which was coated with rubber and connected to a motor featuring a speed control knob. The occurrence of ataxia, defined as the tendency of rats to fall, has been documented in normal control, diabetic control, and drug-treated rats.

Assessment of behavioral parameters in diabetes rats

A. Open-field test: The open-field test was used to assess the effect of diabetic neuropathy on the explorative behavior of diabetic rats and the potential protective effects of PHF and APHF. After eight weeks of diabetes induction, each subject was positioned in the middle of a designated space. Their exploratory behavior was monitored for 5 min, including measurements of horizontal movement in both the central and peripheral zones, time spent in each area, and number of grooming and rearing instances.

B. Passive avoidance test: The passive avoidance test was conducted utilizing a shuttle-box contraption. The equipment comprised a two-compartment dark/light shuttle box including a guillotine door that separates the compartments. The dim chamber included stainless steel shock-grid flooring. Each rat was then placed in a room. Following a 60-second habituation period, the guillotine door was opened, and the latency for the animals to enter the dark chamber was documented. Upon entry into the dim room, the guillotine door was secured, and an electric foot shock (0.6 mA) was administered to the floor grids for a duration of 2 seconds. Five seconds later, the rat was extracted from the dark chamber and returned to its cage. Following a 24-hour period, the retention latency was assessed using the same methodology as the acquisition experiment; however, no foot shock was administered, and the latency was recorded for a maximum duration of 300 seconds. Reduced latencies indicated inferior retention.

C. Corneal sensation test Corneal sensation was measured using a Cochet-Bonnet filament esthesiometer (Luneau Ophthalmogie, France). The Aeshesiometer operates on the principle of axial pressure transmission utilizing a nylon monofilament with a fixed diameter, but can vary in length. Adjustment of the monofilament length was executed using the forefinger. The increase in the transmitted pressure correlates with the decrease in the length. The experiment commenced when the nylon filament was stretched to a maximum length of 6 cm. The end of the nylon filament makes contact with the cornea. Filament length was documented in instances where the rat exhibited a blink, indicating a positive response. In instances where the rat failed to blink, the nylon filament was systematically shortened by 0.5 cm, and the procedure was repeated until a positive response was documented. The procedure was repeated three times for each eye. The measurements are presented in centimetres.

Assessment of pain sensitivity in diabetes rats

A. Thermal hyperalgesia: Thermal hyperalgesia was assessed using hot (45 °C) and cold (10 °C) tail immersion tests. Tail-flick latency served as the endpoint of the tail immersion test. Flicking of the tail and signs of struggle were regarded as affirmative responses. A cut-off time of 15 second was established for both tests. Three consecutive readings were obtained at 30-minute intervals.

B. Mechanical hyperalgesia: Mechanical hyperalgesia was assessed by measuring the hind paw withdrawal response to a probe with a set of calibrated filaments (von Frey filaments, Chicago, IL, USA). The rats were individually housed in plexiglass boxes situated on a stainless steel mesh floor and allowed to acclimatize for a minimum of 20 min. A 0.5-mm diameter polypropylene rigid tip was used to exert force on the plantar surface of the hind paw. The van Frey filament responsible for eliciting a withdrawal response has been documented. The test was conducted four to five times at five-minute intervals for each animal, and the mean value was computed.

Biochemical estimations of aldose reductase activity in sciatic nerve protein extracts

The aldose reductase activity was measured spectrophotometrically by monitoring the decrease in NADPH during glyceraldehyde reduction. The reaction commenced with the addition of 100 mM NADPH to 100 µg of sciatic nerve protein extract in 100 mM sodium phosphate buffer, which included 1 mM D-glyceraldehyde and 400 mM lithium sulfate. The absorbance of NADPH at 340 nm was recorded at 37°C prior to and at 5, 10, and 15 minutes following the addition of NADPH. The results were quantified as nanomoles of NADP+ produced per minute per milligram of protein.

Biochemical estimation of SOD, GSH, MDA, NO and Ca

Sciatic nerve homogenate preparation: All rats were sacrificed at the end of the study, i.e. 8th week and the sciatic nerves were immediately isolated. Tissue homogenates were prepared with 0.1 M tris–HCl buffer (pH 7.4), and the homogenate supernatant was used to estimate the superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation (MDA content), nitric oxide (NO content), and total calcium (Ca) content.

A. Determination of SOD content: Neural pathological alterations occur owing to ROS overproduction. The superoxide dismutase (SOD) assay was performed as previously described by Misera and Fridovich (1972). SOD activity was expressed as U/mg protein.

B. Determination of GSH contents: Glutathione (GSH) assay was performed according to the method described by Moron et al. (1979). The amount of reduced glutathione was expressed as µg/mg of protein.

C. Determination of MDA content: Malondialdehyde (MDA) levels in neural tissue were determined using the method described by Slater and Sawyer (1971). The values are expressed as nanomoles per milligram of protein.

D. Determination of nitrite level: The NO level was estimated as nitrite using the acidic Griess reaction after the reduction of nitrate to nitrite by vanadium trichloride, as described by Miranda et al. (2001). The Griess reaction is based on a colorimetric interaction between nitrite, sulfonamide, and N-(1-naphthyl) ethylenediamine, resulting in the formation of a pink azo product with maximum absorbance at 543 nm. Sodium nitrate concentrations were determined using a standard curve, and the results were expressed in µg/ml.

E. Determination of total calcium: Total calcium levels were estimated in the sciatic nerve as described by Severinghaus and Ferrebee, 1950. Briefly, the sciatic nerve homogenate was combined with 1ml of trichloroacetic acid (4%) under ice-cold conditions and subsequently centrifuged at 2000 rpm for 10 min. The resulting clear supernatant was used to determine the total calcium content using flame photometry.

Effects of Metformin, PHF and APHF on BGL and Body weight in STZ-NA-induced diabetic rats.

After 60 days of daily administration of metformin, PHF-A, PHF-B, PHF-C, and APHF, a significant decrease in blood glucose levels ($P<0.01$) was observed in diabetic rats compared to untreated diabetic rats. A significant reduction in BGL ($P<0.01$) was observed when comparing days 0 and 60 within the treated groups. APHF (200 mg/kg) exhibited a significantly greater ($P<0.01$) anti-hyperglycemic effect than metformin-, PHF-A-, PHF-B-, and PHF-C-treated rats (Table 1). The initial body weights were approximately uniform across all groups. At the end of the experimental period, body weight was significantly reduced compared to that of the healthy control rats. Following treatment with Metformin, PHF, and APHF, diabetic rats showed a significant increase in body weight (Table 1).

Table 1.Effects of metformin, PHF, and APHF on BGL in STZ-NA-induced diabetic fasted rats.

Treatment	BGL (mg/dl) at different time interval after treatment			Body weight (gm)
	Initial	30th day	60th day	
Healthy control	71.16 ±4.47	79.83 ±3.24[b]	76.83 ±3.97[b]	301.10 ±4.15[b]

Diabetic Control	229.50 ±6.34	256.66 ±4.33	314.33 ±7.46	166.66 ±6.18
Metformin 200mg/kg	231.16 ±4.96	181.00 ±3.83[b]	144.50 ±5.81[b]	214.33 ±5.96[b]
PHF-A 100mg/kg	215.60 ±3.32	195.33 ±6.63[b]	161.50 ±8.81[b]	202.66 ±4.20[b]
PHF-B 200mg/kg	226.83 ±4.46	179.16 ±5.38[b]	147.33 ±5.04[b]	219.83 ±6.25[b]
PHF-C 400mg/kg	206.50 ±3.40	161.5 ±4.64[b]	139.83 ±7.63[b]	321.33 ±6.63[b]
APHF-200mg/kg	221.00 ±5.28	156.00 ±5.65[b]	131.66 ±6.33[b]	241.50 ±4.64[b]

Values are expressed as the mean±SEM (n=6) in each group. Where, c = P <0.05, b = P <0.01, a=P<0.001, when compared to diabetes control groups (One-way ANOVA followed by Dunnett's multiple comparison test), BGL: Blood Glucose Level, PHF: Polyherbal formulation, APHF: Allopolyherbal formulation (PHF100 mg/kg + metformin 100 mg/kg).

Effects of Metformin, PHF and APHF on motor coordination in rats with STZ-NA-induced diabetic neuropathy

The Rotarod test indicated notable deficits in motor coordination in diabetic rats relative to healthy controls. In healthy control rats, the muscle grip strength was within normal limits, whereas it was diminished in diabetic control rats. Diabetic rats administered PHF at a lower dose of 100 mg/kg did not exhibit significant improvements in muscle grip strength; however, doses of 200 mg/kg and 400 mg/kg resulted in significant effects. The combination of PHF (100 mg/kg) and metformin (100 mg/kg) significantly improved muscle grip strength compared with diabetic rats (P < 0.01). Nonetheless, the grip strength of all the treated animals was significantly higher than that of the diabetic control group. The retention time of diabetic rats decreased by 61.2% compared to that of healthy control rats. In contrast, treatment with PHF (100, 200, and 400 mg/kg) and APHF (400 mg/kg) increased the retention time to 83.6% of the control values (Table 2).

Table 2. Effect of Metformin, PHF and APHF on muscle grip strength (s) in STZ-NA-induced diabetic neuropathy in rats.

Group	Animal fall of time (s)
Healthy control	180.66±11.55[b]
Diabetic control	92.66±6.48
Metformin 200mg/kg	127.66±7.05[c]
PHF-A 100mg/kg	119.33±6.93
PHF-B 200mg/kg	132.33±7.31[c]
PHF-C 400mg/kg	145.00±6.35[b]
APHF-200mg/kg	159.00±6.65±[b]

Values are presented as mean ± SEM (n = 6) for each group. Statistical significance was observed with c=P<0.05, b=P<0.01, and a=P<0.001 when compared to diabetes control groups, as determined by One-way ANOVA followed by Dunnette's multiple comparison test. PHF refers to the Polyherbal formulation, while APHF denotes the Allopolyherbal formulation (PHF 100 mg/kg + Metformin 100 mg/kg).

Effects of Metformin, PHF and APHF on behavioral parameters

Significant differences in velocity, rearing, grooming, and duration of immobility were observed in the open field test between the treated and diabetic control groups. Moreover, treatment with PHF at a dose of 400 mg/kg and APHF in diabetic rats demonstrated more pronounced effects than in diabetic control rats (Table 3).

Table 3. Effects of Metformin, PHF and APHF on behavioral parameters in STZ-NA-induced diabetic neuropathy in rats.

Group	**Velocity (cm/s)**	**Rearing (N)**	**Grooming (N)**	**Immobile duration (s)**
Healthy cont	10.33 ±0.88[b]	14.66 ±0.91[b]	8.66 ±0.57[b]	143.66 ±11.40[b]

rol				
Diabetic control	4.33 ±0.33	5.33 ±0.21	3.66 ±0.57	296.00 ±10.58
Metformin 200 mg/kg	6.66 ±0.33[c]	9.33 ±0.21[b]	6.33 ±0.15[c]	199.00 ±19.73[b]
PHF-A 100 mg/kg	5.33 ±0.33	8.66 ±0.42[c]	5.33 ±0.52	213.66 ±14.83[c]
PHF-B 200 mg/kg	6.66 ±0.33[c]	9.33 ±0.91[b]	6.33 ±0.52[c]	186.66 ±8.38[b]
PHF-C 400 mg/kg	7.66 ±1.16[b]	11.33 ±0.21[b]	6.66 ±0.57[c]	175.33 ±11.83[b]
APHF-200 mg/kg	8.33 ±0.66[b]	12.33 ±0.38[b]	7.66 ±0.57[b]	162.00 ±12.74[b]

Values are presented as mean ± SEM (n = 6) for each group. Statistical significance was observed with c=P<0.05, b=P<0.01, and a=P<0.001 in comparison to diabetes control groups, as determined by One-way ANOVA followed by Dunnette's multiple comparison test. PHF refers to the Polyherbal formulation, while APHF denotes the Allopolyherbal formulation (PHF 100mg/kg + Metformin 100mg/kg).

Effects of Metformin, PHF and APHF on passive avoidance test in diabetic rats.

Diabetic rats at the 8th week demonstrated a notable decrease in avoidance response, as indicated by transfer latency, when compared to healthy control rats. The administration of metformin at a dosage of 200 mg/kg, along with PHF at doses of 100, 200, and 400 mg/kg, resulted in a notable enhancement in the avoidance response, as indicated by the transfer latency, when compared to the diabetic control rats. Additionally, the combination of metformin (100 mg/kg) with PHF (100 mg/kg) exhibited synergistic effects in the avoidance response tests (Table 4).

Table 4. Effects of Metformin, PHF and APHF on passive avoidance test in STZ-NA-induced diabetic neuropathy in rats.

Treatment	Transfer latency (s)		
	Acquisition trial	Retention trial	Difference
Healthy control	49.00±4.35[b]	247.00±14.22[b]	198
Diabetic control	20.33±3.75	79.33±10.39	44
Metformin 200mg/kg	35.33±4.91	161.33±7.53[b]	126
PHF-A 100mg/kg	28.66±6.38	126.00±6.65[c]	97.34
PHF-B 200mg/kg	37.00±3.21[c]	172.00±10.44[b]	135
PHF-C 400mg/kg	40.33±4.33[c]	191.33±7.53[b]	151
APHF-200mg/kg	45.33±4.91[b]	219.00±10.11[b]	173

Values are presented as mean±SEM (n=6) for each group. In this analysis, c represents a significance level of P<0.05, b indicates P<0.01, and a denotes P<0.001, in comparison to the diabetes control groups. This was determined using a One-way ANOVA followed by Dunnette's multiple comparison test. The terms PHF and APHF refer to the Polyherbal formulation and the Allopolyherbal formulation, respectively, with APHF consisting of PHF at 100mg/kg combined with Metformin at 100mg/kg.

Effects of Metformin, PHF and APHF on Corneal sensitivity in STZ-NAD-induced diabetic neuropathy in rats.

Diabetic rats at eight weeks demonstrated a statistically significant decrease in corneal sensitivity relative to healthy control rats. The administration of metformin (200 mg/kg) and PHF at doses of 100, 200, and 400 mg/kg led to a statistically significant enhancement in corneal sensitivity compared to diabetic control rats. Additionally, the combination of metformin (100 mg/kg) and PHF (100 mg/kg) demonstrated synergistic effects in the corneal sensitivity assessments (Table 5).

Table 5.Effects of Metformin, PHF and APHF on corneal sensitivity in STZ-NAD induced diabetic neuropathy in rats

Treatment	Corneal sensitivity (cm)
Healthy control	6.03±0.20[b]
Diabetic control	4.46±0.14
Metformin 200mg/kg	5.18±0.60[b]
PHF-A 100mg/kg	5.16±0.35[c]
PHF-B 200mg/kg	5.27±0.14[b]
PHF-C 400mg/kg	5.39±0.95[b]
APHF-200mg/kg	5.51±0.10[b]

Values are presented as mean ± SEM (n = 6) for each group. Statistical significance was observed with c=P<0.05, b=P<0.01, and a=P<0.001 when compared to diabetes control groups, as determined by One-way ANOVA followed by Dunnette's multiple comparison test. PHF refers to the Polyherbal formulation, while APHF denotes the Allopolyherbal formulation (PHF 100 mg/kg + Metformin 100 mg/kg).

Effects of Metformin, PHF and APHF treatment on diabetic pain threshold in tail immersion (warm water) tests.

After four weeks, diabetic rats showed a marked reduction in pain threshold in response to noxious stimuli compared to healthy control rats. Treatment with PHF (100, 200, and 400 mg/kg) and APHF (200 mg/kg) for four to eight weeks significantly increased tail withdrawal latencies ($p < 0.01$) compared to diabetic control rats (Table 6).

Table 6. Effects ofMetformin, PHF and APHF on tail flick latency (warm) in STZ-NA-induced diabetic neuropathy in rats.

Group	Tail flick latency warm (s)		
	4th weeks	6th weeks	8th weeks
Healthy control	13.33±0.88[b]	13.5±0.64[b]	13.25±0.47[b]
Diabetic control	4.33±0.33	4.0±0.57[c]	3.75±0.47
Metformin 200mg/kg	5.66±0.57	6.75±0.47	6.75±0.75[b]
PHF-A 100mg/kg	5.33±0.66	6.0±0.40	6.25±0.62[c]
PHF-B 200mg/kg	5.66±0.66	6.5±0.64[c]	6.75±0.47[c]
PHF-C 400mg/kg	6.33±0.33	7.33±0.33[c]	7.75±0.47[c]
APHF-200mg/kg	7.66±0.66[c]	8.66±0.88[b]	9.25±0.47[b]

Values are presented as mean±SEM (n=6) for each group. In this analysis, c represents P<0.05, b indicates P<0.01, and a signifies P<0.001, in comparison to the diabetes control groups. The statistical evaluation was conducted using One-way ANOVA followed by Dunnette's multiple comparison test. PHF refers to the Polyherbal formulation, while APHF denotes the Allopolyherbal formulation, which consists of PHF at 100mg/kg combined with Metformin at 100mg/kg.

Effect of Metformin, PHF and APHF treatment on diabetic pain threshold in tail immersion (cold water) tests

After a four-week period, diabetic rats showed a marked reduction in pain threshold in response to non-noxious stimuli when compared to healthy control rats. Treatment with PHF (100, 200, and 400 mg/kg) and APHF (200 mg/kg) resulted in a significant increase in tail withdrawal latencies when compared to diabetic control rats. The elevated doses and their combination resulted in a notably greater (p<0.01) increase in tail-flick latency (Table 7).

Table 7. Effects of Metformin, PHF and APHF on Tail flick latency (cold) in STZ-NA induced diabetic neuropathy in rats.

Group	Tail flick latency cold (s)		
	4th weeks	6th weeks	8th weeks
Healthy control	13.25±0.62[b]	13.0±0.40[b]	13.5±0.64[b]
Diabetic control	4.25±0.40	4.0±0.40	4.0±0.40
Metformin 200mg/kg	5.66±0.40	7.0±0.40[c]	7.25±0.47[c]
PHF-A 100mg/kg	5.5±0.28	6.5±0.64[c]	6.5±0.50[c]
PHF-B 200mg/kg	5.66±0.40	6.75±0.25[c]	7.0±0.40[c]
PHF-C 400mg/kg	6.25±0.25[c]	7.20±0.58[b]	7.75±0.47[b]
APHF-200mg/kg	8.0±0.40[c]	9.0±0.91[b]	9.5±0.64[b]

Values are presented as mean±SEM (n=6) for each group. In this analysis, c represents a significance level of P<0.05, b indicates P<0.01, and a denotes P<0.001, in comparison to the diabetes control groups. The statistical evaluation was conducted using One-way ANOVA followed by Dunnette's multiple comparison test. The terms PHF and APHF refer to the Polyherbal formulation and the Allopolyherbal formulation, respectively, with APHF consisting of PHF at a dosage of 100mg/kg combined with Metformin at 100mg/kg.

Assessment of Mechanical hyperalgesia:

Diabetic rats demonstrated heightened sensitivity to nonpainful mechanical stimuli in the hind paw following STZ-NA injection. Rats in the diabetic control group exhibited a notable decrease in paw withdrawal pressure, as assessed using the von Frey test (Wang et al., 2017). Administration of a polyherbal formulation (PHF) at a dosage of 100 mg/kg, in conjunction with metformin at 100 mg/kg, resulted in a significant increase in the pain threshold (p<0.01). Treatment with PHF (100 mg/kg) alone did not significantly affect paw withdrawal pressure, except at intermediate and high doses (200 mg/kg and 400 mg/kg, respectively), which resulted in a significant reduction in hyperalgesia (Table 8).

Table 8. Effects of Metformin, PHF and APHF on paw withdrawal pressure in STZ-NA induced diabetic neuropathy in rats.

Group	Paw withdrawal pressure (g)
Healthy control	83.66±6.11[b]
Diabetic control	37.33±1.45
Metformin 200mg/kg	55.66±3.5[c]
PHF-A 100mg/kg	47.00±2.5
PHF-B 200mg/kg	53.66±4.45[c]
PHF-C 400mg/kg	57.00±4.04[c]
APHF-200mg/kg	68.00±5.85[b]

Values are presented as mean±SEM (n=6) for each group. In this analysis, c represents a significance level of P<0.05, b indicates P<0.01, and a denotes P<0.001, in comparison to the diabetes control groups. The statistical evaluation was conducted using One-way ANOVA followed by Dunnette's multiple comparison test. The terms PHF and APHF refer to the Polyherbal formulation and the Allopolyherbal formulation, respectively, with APHF consisting of PHF at 100mg/kg combined with Metformin at 100mg/kg.

Aldose Reductase analysis: Studies have shown that Hyperglycemia results in elevated aldose reductase (AR) activity in animal models of diabetic neuropathy. Enzyme activity was assessed in sciatic nerve extracts from rats at eight weeks of diabetes and quantified as nanomoles of NADP+ produced per minute per milligram of protein. The enzyme activity observed in the diabetic control rats was markedly higher than that in the healthy control rats. Treatment with metformin, PHF, and APHF in diabetic rats resulted in a significant reduction in aldose reductase enzyme activity when compared to the diabetic control group (Table 9).

Table 9. Effect of Metformin, PHF and APHF on aldose reductase in STZ-NA induced diabetic neuropathy in rats.

Group	nmol of n/mg of protein
Healthy control	8.33±0.33[b]
Diabetic control	27.66±4.05
Metformin 200mg/kg	17.00±1.15[c]
PHF-A 100mg/kg	20.33±3.18
PHF-B 200mg/kg	16.33±2.60[c]
PHF-C 400mg/kg	15.00±2.08[c]
APHF 200mg/kg	13.33±1.20[b]

In each cohort, the mean±SEM is used to express the values. Where, c=P<0.05, b=P<0.01, and a=P<0.001 were compared to the diabetes control groups using a one-way ANOVA followed by Dunnette'smultiple comparison test. PHF is a polyherbal formulation, and APHF is an allopolyherbal formulation (PHF100mg/kg + Metformin 100mg/kg).

Effect of Metformin, PHF and APHF on STZ-NA-induced alterations in SOD, GSH, MDA, NO, and Ca levels.

A. Superoxide dismutase: Neural SOD in the sciatic nerve of diabetic control rats was substantially reduced ($P < 0.05$) compared to healthy controls 8 weeks after intraperitoneal injection of STZ-NA. Compared to diabetic control rats, neural SOD levels were markedly and dose-dependently elevated by PHF treatment (100, 200, and 400 mg/kg) ($P < 0.05$). The effects of APHF treatment (200 mg/kg) were more pronounced than those of diabetic controls (Table 5.5.3.9).

B. Reduced glutathione: Eight weeks post-STZ-NA injection, diabetic control rats demonstrated a marked reduction in reduced glutathione levels ($P < 0.05$) in the sciatic nerve relative to healthy controls. GSH levels in rats administered PHF (100, 200, and 400 mg/kg) were considerably elevated in a dose-dependent manner ($P < 0.05$) compared to those in diabetic control rats. The decreased level of GHS in metformin (200 mg/kg)-treated rats was considerably higher ($P < 0.05$) than that in diabetic control rats. The combination of PHF (100 mg/kg) and metformin (100 mg/kg) significantly elevated ($P < 0.05$) GSH levels compared to those treated with either PHF (100 mg/kg) or metformin (100 mg/kg) alone (Table 5.5.3.9).

C. Lipid peroxidation: Eight weeks after intraperitoneal STZ-NA injection, diabetic control rats demonstrated significantly elevated lipid peroxidation levels ($P < 0.05$) compared with healthy controls. Lipid peroxidation in treated rats (100, 200, and 400 mg/kg) was significantly and dose-dependently reduced ($P < 0.05$) compared to that in diabetic controls. The administration of metformin (100 mg/kg) resulted in a slight reduction in MDA levels compared with the diabetic control group. Combining metformin (100 mg/kg) with PHF (100 mg/kg) resulted in a significant reduction in lipid peroxidation compared to either treatment alone (Table 5.5.3.9).

D. Nitric oxide: A single intraperitoneal STZ-NA injection significantly increased ($P < 0.05$) sciatic nerve neural nitrite levels in diabetic rats (317.2 ± 8.45 g/ml) compared to healthy controls (97.86 ± 8.49 g/ml). PHF treatment at doses of 100, 200, and 400 mg/kg resulted in a significant and dose-dependent reduction in neural nitrite levels (245.9 ± 5.50 and 200.1 ± 13.88 g/ml) when compared to diabetic controls. The combination of metformin (100 mg/kg) and PHF (100 mg/kg) resulted in a significant reduction of neural nitrite levels (129.5±5.26 g/ml) when compared to either metformin (100 mg/kg) or PHF (100 mg/kg) administered individually (154.8±8.37 g/ml) (Table 5.5.3.9).

E. Total calcium content: Following eight weeks of STZ-NA injection, diabetic control rats demonstrated a significantly elevated neural calcium level (23.29 ± 0.90 ppm/mg of protein, $P < 0.05$) in comparison to healthy controls (5.47 ± 0.46 ppm/mg of protein). PHF treatment at doses of 100, 200, and 400 mg/kg resulted in a significant and dose-dependent reduction in neural calcium levels (16.42±0.93 and 13.86±0.84 ppm/mg of protein, $P < 0.05$) when compared to diabetic controls. Metformin (100 mg/kg) significantly reduced neural calcium levels (11.24±0.58 ppm/mg of protein, $P < 0.05$) in comparison to diabetic controls. The combination of metformin (100 mg/kg) and PHF (100 mg/kg) resulted in a further reduction of neural calcium levels (8.12±0.63 ppm/mg of protein, $P < 0.05$) compared to each treatment administered individually (Table 10).

Table 10. Effects of Metformin, PHF and APHF on STZ-NA-induced alterations in SOD, GSH, MDA, NO, and Ca levels.

Treatment	SOD (U/mg of protein)	GSH (µg/mg protein)	MDA (nM/mg of protein)	NO (µg/ml)	Ca (ppm/mg of protein)
Healthy control	27.33 ±3.40[b]	1.5 ±0.17[b]	2.78 ±0.17[b]	102 ±8.14[b]	6.08 ±0.36[b]
Diabetic control	6.33 ±0.75	0.28 ±0.04	10.33 ±1.45	325.33 ±12.9	24.33 ±2.90
Metformin 200mg/kg	17.33 ±2.33[c]	1.16 ±0.14[b]	6.5 ±0.28[c]	172.66 ±8.76[b]	15.00 ±1.15[b]
PHF-A 100mg/kg	14.66 ±2.02[c]	1.06 ±0.08[c]	7.0 ±0.76[c]	213.33 ±6.09[b]	16.33 ±1.45[c]
PHF-B 200mg/kg	20.33 ±2.60[b]	1.13 ±0.8[b]	6.33 ±0.42[b]	169.66 ±6.88[b]	13.23 ±2.04[b]
PHF-C 400mg/kg	23.00 ±3.21[b]	1.26 ±0.8[b]	5.0 ±0.57[b]	154.33 ±10.47[b]	12.33 ±0.66[b]
APHF 200mg/kg	25.00 ±4.61[b]	1.31 ±0.32[b]	4.16 ±0.72[b]	139.66 ±9.52[b]	10.66 ±1.20[b]

Values are presented as mean±SEM (n=6) for each group. In this analysis, the significance levels are indicated as follows: c=P<0.05, b=P<0.01, a=P<0.001, in comparison to the diabetes control groups. The statistical method employed was One-way ANOVA, followed by Dunnette's multiple comparison test. The abbreviations used are SOD for Superoxide Dismutase, GSH for Reduced Glutathione, and MDA for Lipid Peroxidation. NO refers to Nitric Oxide, while Ca denotes Calcium. PHF refers to a polyherbal formulation, while APHF denotes an allopolyherbal formulation consisting of PHF at a dosage of 100mg/kg combined with Metformin at 100mg/kg.

DISCUSSION

Plant-derived pharmaceuticals have gained significant research attention as alternatives to conventional medicines for the treatment of chronic illnesses[23]. Botanical medications are widely used because of their perceived effectiveness and long-standing history of patient care[24-25].

The concept of polyherbalism has been highlighted in Ayurvedic literature, Sarangdhar Samhita, for its improved therapeutic effectiveness. Phytochemicals isolated from individual plants are often inadequate for achieving the desired effects. However, the combination of multiple herbs in precise ratios offers several advantages, including enhanced therapeutic efficacy, mitigated toxicity[26] reduced amounts of individual herbs required, and minimized unwanted effects[27-28].

Emblica officinalis, Gymnemasylvestre, Terminalia arjuna, Tinospora cordifolia, and Zingiber officinale are significant herbs employed in traditional Indian medicine for managing diabetes and various ailments. Qualitative phytochemical analysis of the plant extracts revealed the presence of various bioactive secondary metabolites. The polyherbal formulation contained nearly all bioactive secondary metabolites, including flavonoids, terpenoids, phenolic compounds, saponins, alkaloids, tannins, carbohydrates, steroids, lipids, oils, glycosides, and proteins. The extensive presence of these bioactive components in polyherbal formulations enhances their protective effects against diabetes and its associated effects. Although the individual safety of these herbs has been established, the effects of their concurrent use remain ambiguous. Consequently, it is imperative to assess the safety and toxicity of polyherbal formulations (PHF). Preclinical tests were performed to evaluate various facets of PHF.

Diabetic neuropathy includes a range of illnesses with intricate pathophysiologies that affect both the somatic and autonomic nervous systems. It is the most common chronic consequence of diabetes mellitus and is characterized by spontaneous pain, allodynia, and hyperalgesia[29]. This neuropathy arises from extended uncontrolled hyperglycemia, which destroys nerve cells and hinders healing mechanisms[30]. Elevated levels of reactive oxygen species in peripheral nerves induce oxidative stress, mitochondrial malfunction, neuronal injury, and

death. Oxidative stress elevates substrates for advanced glycation end products (AGEs), glycoxidation, and lipoxidation precursors, thereby increasing free radical generation, which may be worsened by inadequate antioxidant and detoxification mechanisms[6]. Large-fiber diabetic peripheral neuropathy (DPN) causes sensory impairments that severely affect the affected individuals. Studies have demonstrated that patients with diabetic peripheral neuropathy (DPN) have an increased risk of falls, attributable to compromised postural control, modified gait, and reduced balance. Compromised muscle spindles, essential for proprioception, balance, gait, and postural responses, lead to motor incoordination[3]. The degree of diabetic neuropathy correlates with diminished muscle strength in individuals with diabetes mellitus[4].

Daily injections of PHF-A, PHF-B, PHF-C, and APHF for 60 days significantly ($P<0.01$) reduced blood glucose levels in diabetic rats compared to untreated diabetic rats. Diabetic rats demonstrated substantial gain in body weight following treatment with PHF and APHF. The current investigation noted substantial enhancements in motor behavior, especially grip strength, in diabetic rats after the administration of PHF or a combination of PHF and metformin. The combination of PHF and metformin treatment led to a notable enhancement in grip strength relative to that in the diabetic control group, whereas the diabetic control group exhibited a considerable reduction in grip strength.

Diabetic neuropathy is defined as pain manifesting as hyperalgesia and allodynia. Thermal hyperalgesia was evaluated using hot and cold immersion tests, whereas mechanical allodynia was measured by applying a von Frey monofilament to the plantar area of the foot[31]. In both assessments, the threshold for tail and paw withdrawal was significantly diminished after four weeks of STZ-NA dosing. Treatment with PHF and metformin partially ameliorated the altered thermal hyperalgesia and mechanical allodynia; however, the effects of APHF were superior to those of PHF and metformin alone[5].

The threshold of peripheral neurons for noxious stimuli is decreased as a consequence of intraperitoneal administration of STZ-NA. The up-regulation of reactive oxygen species is associated with chronic diabetes. Vascular and neuronal abnormalities induce edema, ischemia, and hypoxia, which subsequently induce multifocal axonal degeneration in the peripheral nerves. This results in a reduced pain threshold, decreased motor nerve conduction velocity, and decreased number of peripheral neurons[32].

Neural nociception is altered as a result of peripheral receptor sensitization, ischemic tissue injury, ectopic discharge from sprouting fibers, and alterations in dorsal root ganglia cells[32] observed a substantial reduction in tail withdrawal latency, paw withdrawal threshold, and paw withdrawal latency following four weeks of diabetic induction.

The development of neuropathy in human patients is primarily determined by the duration of diabetes and chronic hyperglycemia. Several mechanisms contribute to the development of diabetic neuropathy, such as increased polyol flux through the aldose reductase pathway, changes in nerve microvessel structure and function, hypoxia in the nerves and ganglia, oxidative and endoplasmic reticulum stress, non-enzymatic glycosylation, and reduced neurotrophic support for neurons and peripheral nerves. All these factors contribute to the intricate pathogenesis of diabetic neuropathy[7,33].

Diabetic control rats exhibited substantially higher aldose reductase activity than the healthy controls. The activity of aldose reductase was significantly reduced in diabetic rodents treated with PHF and APHF compared to that in untreated diabetic rats.

Corneal sensation was assessed using a Cochet-Bonnet filament esthesiometer. The aeshesiometer functions on the basis of pressure being transmitted axially by a nylon monofilament of a known diameter but variable length. The transmitted pressure increased as the nylon monofilament length decreased. Compared to diabetic control rats, corneal sensitivity was substantially enhanced by the administration of PHF and APHF[34].

In STZ-diabetic rodents, the accumulation of sorbitol and fructose, which is a consequence of increased aldose reductase and sorbitol dehydrogenase enzyme activities in the polyol pathway, reduces nerve conduction velocity. Neurophysiological and pathological dysfunctions are the consequences of an increase in sorbitol and fructose levels in the sciatic nerves of diabetic rodents[35]. The polyol pathway can be inactivated by drugs from the aldose reductase inhibitor class, which inhibit the conversion of sorbitol to glucose. Consequently, nerve conduction

velocity was enhanced. The elevated levels of aldose reductase were substantially reduced by treatment with PHF and APHF, resulting in the amelioration of neuropathy.

The activity of superoxide dismutase and GSH in diabetic control rats was substantially reduced in comparison to that in normal rats after eight weeks of intraperitoneal STZ-NA administration. The generation of superoxide and hydroxyl free radicals can lead to vascular endothelial injury, which, in turn, affects nociception[36]. Superoxide dismutase (SOD) is a critical mediator that catalyzes the conversion of superoxide anions to hydrogen peroxide (H2O2), thereby serving as an antioxidant. NADPH is a critical component of the polyol pathway, where it is involved in the formation of sorbitol and fructose, in conjunction with aldose reductase. Nevertheless, NADPH competes with glutathione disulfide reductase, leading to a reduction in the availability of glutathione (GSH), an enzyme that is crucial for safeguarding the sulfhydryl group of cysteine in proteins. Consequently, the antioxidant potential of the cells is reduced. SOD, in addition to GSH, is essential for safeguarding cells from oxidation[37-38]. Consequently, the regeneration of neurons in patients with DN may be enhanced by antioxidant treatment. The activity of endogenous antioxidant enzymes SOD and GSH was considerably enhanced by chronic administration of PHF and APHF for eight weeks, as demonstrated in this study[6].

In rodents with STZ-induced diabetes, malondialdehyde (MDA) levels are elevated, which is a defining characteristic of oxidative stress. MDA has been shown to cause the rearrangement of the double bond in unsaturated fatty acids in the lipid membrane, which leads to nerve injury and destruction of cell membranes[39]. Decreased levels of endogenous antioxidant defense enzymes, including glutathione peroxidase and superoxide dismutase, in peripheral nerve tissues may induce an increase in lipid peroxidation levels in diabetic control animals[40]. The MDA levels in rats treated with PHF and APHF were substantially elevated, which was in accordance with the increase in SOD and GSH levels[7].

Nitric oxide is a critical intracellular messenger; however, its production through inducible nitric oxide synthetase (iNOS) alters the blood supply to nerves, leading to microvascular alterations in the aftermath of nerve injury[41]. NO produces peroxynitrite in conjunction with the superoxide anion, which results in neuronal degeneration and nerve fiber dysfunction, thereby contributing to the development of neuropathic pain in patients with diabetes[42]. The neuroprotective effects of PHF and APHF in diabetic rodents may be attributed to their capacity to quench free NO moieties.

Uncontrolled excitotoxicity results from an elevated level of free radical generation, accompanied by an increased efflux of calcium to the extracellular matrix of the cell. A cascade of biochemical changes is initiated by increased calcium efflux, which leads to neuronal dysfunction by degranulating the axonal cytoskeleton and increasing the release of free radicals in addition to elevated calcium levels[43]. Elevation of calcium levels in the sciatic nerve was substantially attenuated by PHF and APHF, thereby preventing neuronal damage.

Under hyperglycemic conditions, the production of reactive oxygen species and cellular metabolism is elevated, which may affect the potential of the mitochondrial membrane. Under these conditions, poly (ADP-ribose) polymerase (PARP) is essential for the development of diabetic complications such as endothelial dysfunction, cardiomyopathy, retinopathy, nephropathy, and neuropathy[7].

CONCLUSION

The results from biochemical and pathological investigations demonstrated that PHF and APHF treatments exhibit beneficial effects in the management of diabetic neuropathy and its associated complications. This study concludes that PHF may function as an adjuvant in the management of diabetic neuropathy and oxidative stress associated with uncontrolled diabetes. Moreover, concurrent administration of polyherbal formulations and metformin enhanced therapeutic efficacy, reduced dosage requirements, and mitigated adverse effects.

FUTURE SCOPE

Future studies should aim to clarify the specific molecular pathways responsible for the observed therapeutic effects, and investigate the possible clinical applications of these formulations in humans.

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Declarations

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Data Availability declaration in the manuscript.

Data will be made available on request

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Author Contribution declaration

SB-Conceptualization; Study design; Experimental work; Supervision; Interpretation; Critical revision of the manuscript; Final manuscript editing, **DB**-Data acquisition; Drafting original draft, **RD**-Analysis; Validation; Statistical validation, **BB**- Data analysis and interpretation, **ST**: Review and editing; Review and editing, **VM**-Data curation; Writing original draft,.

Competing Interest declaration.

The authors declare that they have no competing financial or non-financial interests that could have influenced the work reported in this manuscript. The authors alone are responsible for the accuracy and integrity of the content of this paper.

REFERENCE

1. Sandkuhler, J. Models and Mechanisms of Hyperalgesia and Allodynia. Physiological Reviews, (2009) 89(2), 707–758. DOI: 10.1152/physrev.00025.2008.
2. Wei-Cheng Z., Bin Zhang., Mei-Juan L., Wen-Xuan Z., Wan-You H., Han-Bing W., Cheng-Xiang Y., Curcumin ameliorated diabetic neuropathy partially by inhibition of NADPH oxidase mediating oxidative stress in the spinal cord, Neurosci Lett, 560 (2014) 81-85. DOI: 10.1016/j.neulet.2013.12.019
3. Shaikh A.S., Somani R.S., Animal models and biomarkers of neuropathy in diabetic rodents, Indian J Pharmacol, 42 (2010) 129-134. DOI: 10.4103/0253-7613.66833
4. Jangra A., Datusalia A.K., Khandwe S., Sharma S.S., Amelioration of diabetes-induced neurobehavioral and neurochemical changes by melatonin and nicotinamide: Implication of oxidative stress–PARP pathway, Pharmacol Biochem Behav, 114 (2013) 43-51. DOI: 10.1016/j.pbb.2013.10.021
5. Negi G., Kumar A., Kaundal R.K., Gulati A., Sharma S.S., Functional and biochemical evidence indicating beneficial effect of Melatonin and Nicotinamide alone and in combination in experimental diabetic neuropathy, Neuropharmacology, 58 (2010) 585-592. DOI: 10.1016/j.neuropharm.2009.11.018
6. P Sytze van Dam, Oxidative stress and diabetic neuropathy: pathophysiological mechanisms and treatment perspectives. Diabetes Metab Res Rev. 2002; 18(3):176-84. doi: 10.1002/dmrr.287.
7. Ariza L., Pages G., Garcia-Lareu B., Cobianchi S., Otaegui P.J., Ruberte J., et al. Experimental diabetes in neonatal mice induces early peripheral sensorimotor neuropathy, Neuroscience, 274 (2014) 250-259. DOI: 10.1016/j.neuroscience.2014.05.015
8. Maekawa, K., Tanimoto, T., Okada, S., Suzuki, T., Suzuki, T., & Yabe-Nishimura, C. Expression of aldose reductase and sorbitol dehydrogenase genes in Schwann cells isolated from rat: effects of high glucose and osmotic stress. Molecular Brain Research, (2001) 87(2), 251–256. DOI:10.1016/S0169-328X(01)00009-2
9. Nagja, T., Kumar, V., & Sanjeev, A. Anti-Diabetic Activity of a Polyherbal Formulation in Streptozotocin Induced Type 2 Diabetic Rats. Journal of Natural Remedies, (2016) 16 (4), 148. DOI:10.18311/jnr/2016/15323
10. Kotova, O. V., Belyaev, A. A., &Akarachkova, E. S. Neurological complications of diabetes mellitus. Medical Council, (2019) 9, 40–44. DOI:10.21518/2079-701X-2019-9-40-44
11. Jain, A., Radiya, P., Bagul, S., Wadekar, R., & Khan, K. Polyherbal Formulation: Preparation, Standardization and Neuropharmcological Screening, (2019)

1(3), 15–18. DOI: https://doi.org/10.12300/rrjopc.v1i3.407

12. Kokate C.K., Practical Pharmacognosy, 4th ed., Vallabh Prakashan, New Delhi, (1994)106-112.
13. Harbone J.B., Phytochemical Methods, A guide to modern technique of plant analysis, 3rd ed., London, Chapmanand Hall, (1998)117-119.
14. Paliwal, S. Standardization and evaluation of two marketed polyherbal formulation (Gutikas). Asian Journal of Pharmaceutical and Clinical Research, (2020) 81–83. DOI: doi.org/10.22159/ajpcr.2020.v13i9.38236
15. Khandelwal K.R., Practical Pharmacognosy Techniques and Experiments, 15th ed., Nirali Prakashan, Pune, (2000) 234.
16. Ziółkowska, N., Rawicka, A., Lewczuk, B., Szyryńska, N., & Vyniarska, A. (2023). Low-Intensity Blue Light Exposure Reduces Melanopsin Expression in Intrinsically Photosensitive Retinal Ganglion Cells and Damages Mitochondria in Retinal Ganglion Cells in Wistar Rats. Cells, 12(7), 1014. doi.org/10.3390/cells12071014
17. Gothandam, K., Ganesan, V. S., Ayyasamy, T., & Ramalingam, S. (2019). Antioxidant potential of theaflavin ameliorates the activities of key enzymes of glucose metabolism in high fat diet and streptozotocin – induced diabetic rats. Redox Report, 24(1), 41–50. doi.org/10.1080/13510002.2019.1624085
18. Belhekar Santosh N, Chaudhari Pravin D. Acute and Sub-acute Oral Toxicity Assessment of the Polyherbal Formulation in Albino Wister Rats. Int J Pharm Pharm Sci, 2016, 8(7), 311-316.
19. Erik Walum. Cell and Molecular Biology. Acute Oral Toxicity. Environ Health Perspect. 1998;106 Suppl. 2: 497-503. DOI: 10.1289/ehp.98106497
20. Rameshwar, G. S., Vithalrao, N. A., Venkatrao, P. U., & Pramodrao, A. M. OECD Guidelines For Acute Oral Toxicity Studies: An Overview. International Journal of Research in Ayurveda and Pharmacy, (2023) 14(4), 137–140.
21. Shirwaikar A, Rajendran K, Barik R. Effect of aqueous bark extract of Garuga pinnata Roxb. in streptozotocin-nicotinamide induced type-II diabetes mellitus. J Ethnopharmacol.2006; 107(2): 285-290. DOI: 10.1016/j.jep.2006.03.012
22. Shirwaikar A., Rajendran K., Punitha I.S.R., Antidiabetic activity of alcoholic stem extract of Cosciniumfenestratum in streptozotocin-nicotinamide induced type 2 diabetic rats, J Ethnopharmacol, 97 (2005) 369-374. https://doi.org/10.1016/j.jep.2004.11.034
23. Kifayatullah M., Mustafa M.S., Sengupta P., Sarker M.M.R., Das A., Das S.K., Evaluation of the acute and sub-acute toxicity of the ethanolic extract of Pericampylus glaucus (Lam.) Merr. in BALB/c mice, J Acute Disease, 4 (2015) 309-315. https://doi.org/10.1016/j.joad.2015.06.010
24. Al-Achi, A. An introduction to botanical medicines: history, science, uses, and dangers. Choice Reviews Online, 2008; 46(09), 46–5051. DOI:10.5040/9798400671968
25. Ernst, E. American Herbal Pharmacopoeia: Botanical Pharmacognosy - Microscopic Characterization of Botanical Medicines. Focus on Alternative and Complementary Therapies, 2012; 17(1). https://doi.org/10.1111/j.2042-7166.2011.01131.x
26. Parasuraman S., Thing G.S., Dhanaraj S.A., Polyherbal formulation: concept of Ayurveda, Pharmacogn Rev, 8 (2014) 73-80. DOI: 10.4103/0973-7847.134229
27. Petchi R.R., Chockalingam V., Parasuraman S., Antidiabetic activity of polyherbal formulation in streptozotocin–nicotinamide induced diabetic wistar rats, J Tradit Complement Med, 4 (2014) 108-117. https://doi.org/10.4103/2225-4110.126174
28. Karole, S., Shrivastava, S., Thomas, S., Soni, B., Khan, S., Dubey, J., Dubey, S. P., Khan, N., & Jain, D. K. Polyherbal Formulation Concept for Synergic Action: A Review. Journal of Drug Delivery and

Therapeutics, (2019) 9(1-s), 453–466. DOI:10.22270/jddt.v9i1-s.2339

29. Singh R., Kishore L., Kaur N., Diabetic peripheral neuropathy: Current perspective and future directions, Pharmacological Research, 80 (2014) 21-35. DOI: 10.1016/j.phrs.2013.12.005
30. Jain D., Bansal M.K., Dalvi R., Upganlawar A., Somani R., Protective effect of Diosmin against diabetic neuropathy in experimental rats, J Integr Med, 12 (2014) 35-41. DOI: 10.1016/S2095-4964(14)60001-7
31. Sharma A.K., Sharma A., Rita K., Kunal K., Sharma D., Bharthu P.S., Sitagliptin, Sitagliptin and Metformin, or Sitagliptin and Amitriptyline attenuate streptozotocin-nicotinamide induced diabetic neuropathy in rats, J Biomed Res, 26 (2012) 200-210. DOI: 10.7555/JBR.26.20110054
32. Visnagri A., Kandhare A.D., Kumar S.V., Rajmane A.R., Adil M., Ghosh P., Elucidation of ameliorative effect of Co-enzyme Q10 in streptozotocin-induced diabetic neuropathic perturbation by modulation of electrophysiological, biochemical and behavioral markers, Biomedicine & Aging Pathology, 2 (2012) 157-172. Doi : 10.1016/j.biomag.2012.10.006
33. Mizisin, A. P. Chapter 27 - Mechanisms of diabetic neuropathy: Schwann cells. Handbook of Clinical Neurology, (2014) 126, 401–428. DOI: 10.1016/B978-0-444-53480-4.00029-1
34. Davidson E.P., Holmes A., Coppey L.J., Yorek M.A., Effect of combination therapy consisting of enalapril, α-lipoic acid, and menhaden oil on diabetic neuropathy in a high fat/low dose streptozotocin treated rat, Eur J Pharmacol, 765 (2015) 258-267. DOI: 10.1016/j.ejphar.2015.08.015
35. Oates P.J., Aldose reductase, still a compelling target for diabetic neuropathy, Current Drug Targets, 9 (2008)14-36. DOI: 10.2174/138945008783431781
36. Patil M.V.K., Kandhare A.D., Bhise S.D., Pharmacological evaluation of ethanolic extract of Daucus carota Linn root formulated cream on wound healing using excision and incision wound model, Asian Pac J Trop Biomed, (2012) 646–655. DOI:10.1016/S2221-1691(12)60290-1
37. Loven D., Schedl H., Wilson H., Daabees T.T., Stegink L.D., Diekus M., Oberley L., Effect of insulin and oral glutathione levels and superoxide dismutase activities in organs of rats with streptozotocin-induced diabetes, Diabetes, 35 (1986) 503–507. DOI: 10.2337/diab.35.5.503
38. Wohaieb S.A., Godin V., Alterations in free radical tissue defense mechanisms in streptozotocin-induced diabetes in rat, Effects of insulin treatment, Diabetes, 36 (1987)1014-1018. DOI: 10.2337/diab.36.9.1014
39. Kandhare A.D., Raygude K.S., Ghosh P., Ghule A.E., Bodhankar S.L., Neuroprotective effect of naringin by modulation of endogenous biomarkers in streptozotocin induced painful diabetic neuropathy, Fitoterapia, 83 (2012) 650–659. DOI: 10.1016/j.fitote.2012.01.010
40. Kishi M., Tanabe J., Schmelzer J.D., Low P.A., Morphometry of dorsal root ganglion in chronic experimental diabetic neuropathy, Diabetes, 51 (2002) 819–824. DOI: 10.2337/diabetes.51.3.819
41. Levy D., Zochodne D.W., NO pain: potential roles of nitric oxide in neuropathic pain. Pain Pract 4 (2004)11-18. DOI: 10.1111/j.1533-2500.2004.04002.x
42. El-Sakka A.I., Lin C.S., Chui R.M., Dahiya R., Lue T.F., Effects of diabetes on nitric oxide synthase and growth factor genes and protein expression in an animal model, Int J Impot Res, 11 (1999)123–132. DOI: 10.1038/sj.ijir.3900392
43. Kandhare, A. D., Bodhankar, S. L., Ghosh, P., & Ghule, A. E. (2012). Elucidation of molecular mechanism involved in neuroprotective effect ofCoenzymeQ10 in alcohol-induced neuropathic pain. Fundamental & Clinical Pharmacology, 27(6), 603–622. doi.org/10.1111/fcp.12003