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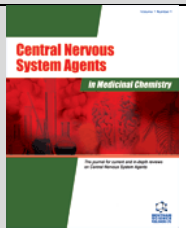
RESEARCH ARTICLE

Neuroprotective Effects of Isolated Mangiferin from *Swertia chirayita* Leaves Regulating Oxidative Pathway on Streptozotocin-induced Diabetic Neuropathy in Experimental Rats



Shivam[1,*] and Asheesh Kumar Gupta[1]

[1]*Faculty of Pharmacy, School of Pharmaceutical Sciences, IFTM University Delhi Road, NH-24 Moradabad, Lodhipur Rajput, Uttar Pradesh 244102, India*

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Abstract: ***Background*:** Oxidative stress has an important role in the pathogenesis and development of diabetic peripheral neuropathy (DPN), the most common and debilitating complication of diabetes mellitus. *Swertia chirayita* is a rich source of phenolic constituents and has hypoglycemic, anti-inflammatory, and antioxidant properties.

***Aims*:** This study was performed to evaluate the neuroprotective effect in diabetes by enhancing antioxidant defense against oxidative stress, which exhibits a neuroprotective effect in streptozotocin-induced diabetic rats.

***Objectives*:** The objective of this study was to elucidate the therapeutic potential of bioactive compounds of *Swertia chirayita* for diabetic complications.

***Methods*:** The present work focused on isolating the bioactive from the leaves of *Swertia absinthe* for acute toxicity studies, assessing its protective effects against diabetes and diabetic neuropathy as well as its mode of action in STZ-induced Wistar rats. The local area of Moradabad is the place from where the leaves *of Swertia chirayita* were gathered. Mangiferin was isolated and identified using spectroscopic techniques, such as UV, HPLC, 1H NMR, C13 NMR, MAS, and FTIR. Mangiferin was administered in doses of 15 and 30 mg/kg to test its effect on experimentally induced diabetes. The sciatic nerves of all groups were examined histopathologically. The protective effect of the drug against diabetes and diabetic neuropathy was demonstrated by measures, such as blood glucose level, body weight, food intake, thermal hyperalgesia, grip strength, spontaneous locomotor test, and lipid profile analysis. Sciatic nerve cells of the treated groups showed less inflammation, degeneration, and necrosis.

***Results*:** The results of this study confirmed that mangiferin alleviated diabetic neuropathic pain, possibly by reducing inflammatory cytokines (TNF-α, TGF-β1, IL-1β, and IL-6), strong antioxidant activity, and NGF in sciatic nerves. It may be a therapeutic agent.

***Conclusion*:** Our results suggested that active phytochemicals of *Swertia chirayita* showed preventive and curative effects against STZ-induced diabetic neuropathy in rats, which might be due to its antioxidant, anti-inflammatory, and anti-apoptotic properties.

Keywords: Diabetic neuropathy, mangiferin, diabetes, Swertia chirayita, oxidative stress, inflammation, inflammatory cytokines.

1. INTRODUCTION

The annual global cost of treating diabetes mellitus (DM), a serious endocrine disorder, and its complications can be in the trillions of dollars [1]. Medical research is of great importance in the improvement of Diabetes Mellitus (DM) Type-II. Achieving adequate glucose control and reducing the risk of developing microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (cardiovascular and cerebrovascular) problems are both dependent on self-management of diabetes [2]. Due to their safety and accessibility, natural extracts have been used to treat diabetes and its associated problems [3]. Since many plant species

*Address correspondence to this author at the Faculty of Pharmacy, School of Pharmaceutical Sciences, IFTM University Delhi Road, NH-24 Moradabad, Lodhipur Rajput, Uttar Pradesh 244102, India;
E-mail: shivamdmohit@gmail.com

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have hypoglycemic properties and are used in folk medicine, they can be used to treat diabetes mellitus [4].

Diabetes mellitus is associated with a nerve-damaging condition known as diabetic neuropathy (DN). According to the latest publication of the International Diabetes Federation (IDF), the incidence and prevalence of diabetes are increasing at an alarming rate in both developed and emerging countries, especially in India and China [5]. The outer nerves of the limbs, especially the nerves of the feet, are affected by peripheral neuropathy, which is the most prevalent type of diabetic neuropathy. It primarily affects sensory function, resulting in abnormal sensations and gradual numbness that promote ulcer development [6]. Swertia chirayita is unique among these herbal plants, which are highly renowned for their anti-hyperglycemic properties. The Swartia genus of the Gentianaceae family contains about 135 species of plants that have medicinal properties [7]. Among them, *Swertia absinthe*, which is similarly known as "salicylia or absinthe," is believed to have a variety of therapeutic properties, including anti-diabetic, anti-inflammatory, hypoglycemic, hepatoprotective, antibacterial, wound healing, antipyretic, anthelmintic, antioxidants, and antitussive [8]. Several research papers strongly support the growing importance and use of such an important medicinal plant in a variety of therapeutic areas [9]. Rich sources of xanthones, flavonoids, iridoids, terpenoids, and alkaloids are found in the Swertia genus [10]. Twenty priceless polyhydroxylated xanthones were found to be present in this plant extract. Many researchers conducted experiments to test the hypoglycemic properties of extracts of this plant made from bark, leaves, shoots, roots, and even the entire plant [11]. Under oxidative stress, cellular adaptive lesions occur, such as hyperplasia, metaplasia, hypertrophy, cell inflammation leading to neuropathy, *etc*. The use of antioxidants may contribute to reducing such cellular adaptations [12].

2. MATERIALS AND METHODS

Plant material was authenticated by a botanist under reference number NISCAIR/RHMD/consult/2021/4141-42-2. The reagents were obtained from the laboratory facility at SPS, IFTMU Moradabad. UV, TLC and FTIR were performed at SPS, IFTMU, Moradabad laboratory, while C13 NMR and 1H NMR were performed by Panjab University, Chandigarh. Animals were obtained from the animal house facility at SPS, IFTMU, Moradabad. The animals were caged and fed a regular diet according to the experimental protocol. SPS, IFTMU laboratory facility provided the necessary equipment for the analysis of blood glucose, locomotor activity, *etc*. Blood was taken from the tail vein, and Dr. Morepen® was used to measure FBG or fasting blood glucose. The lipid profile was analyzed using a semi-auto analyzer from Triveni Traders and Diagnostic Pvt. Ltd., Mumbai.

2.1. Plant Material

The plant was grown in the Moradabad Valley of Uttar Pradesh, a province of India. It was bought from the neighborhood market in September, 2022. The leaves were then crushed and allowed to air dry. Three different solvents, including the ethanolic fraction, were used to further extract them. The ethanol fraction was used in column chromatography for additional analysis.

2.2. Chemicals

Streptozotocin was taken from the chemical storage of the School of Pharmaceutical Sciences and used for induction of Type 1 diabetes in rats, IFTMU. Glibenclamide was procured from Sanofi India Limited. The entire chemical used was of analytical grade.

2.3. Animal Housing

At the School of Pharmaceutical Sciences at IFTM University, Moradabad, adult Wistar rats weighing 180-220 g were collected from the animal house (registration number 837/PO/ReBiBt/S/04/CPCSEA) with the reference number IFTM/IAEC/2022/. The rats were housed in huge, spacious polyacrylic cages. The ambient temperature in the room was approximately 12 hours of light and 12 hours of darkness. The rats were fed a regular pellet diet from Hindustan Liver Limited and had free access to water. The experiment was performed following the CPCSEA standards of the Ministry of Environment, Forest and Climate Change, Government of India, and the study received approval from the Institute Animal Ethics Committee [13].

2.4. Packing of Column for Column Chromatography

To get rid of any contaminants, if any, the residue was adsorbed on neutral alumina. It was then placed in a glass column with a neutral-alumina-to-petroleum-ether-ratio solution (1:2). The packed column was 3.5 x 50 cm in size, and 50 mL/fraction was separated as the elution rate was held constant at 2 mL/min. The resulting fractions were combined after being mixed with various solvents to look for any residues [14].

2.5. TLC

Fractions 8 to 12 were combined and evaporated before being dissolved in ethyl acetate to generate a yellow-brown residue. This was run on a TLC plate with silica gel G-form as the stationary phase and ethanol as the developing phase. The spraying agent was 3% methanolic H_2SO_4. A dark green patch was observed, and an RF value of 0.48 was recorded [15].

2.6. Spectroscopic Studies

After extraction and evaporation, the solid residue was collected and used for spectroscopic analysis. HPLC, FTIR, and UV were also performed. All spectroscopic investigations confirmed the existence of mangiferin, which has the molecular formula of C_{19} H_{18} O_{11}. The interpretations led us to undertake mass spectroscopy, which established that the material was mangiferin. The structure of mangiferin is shown in Fig. (**1**).

2.7. Acute Oral Toxicity Study of Leaves of *Swertia chirayita* and Dose Selection

A toxicity study was performed on normal rats to examine if Mangifera causes any harmful effects and to determine

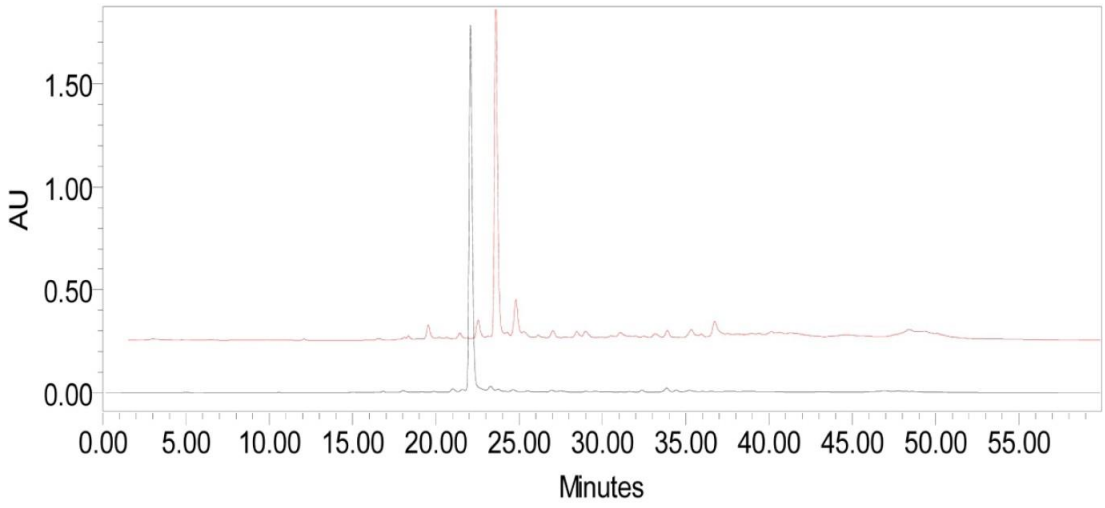

Fig. (1). HPTLC photographs and chromatograms of spectra of isolated compound (Mangiferin) obtained from leaves of *Swertia chirayita.*

the best dosage. After 12 h starvation, 15 healthy, normal rats were randomly separated into five groups (n = 6) and administered mangiferin starting at 5, 50, 300, and 2000 mg/kg body weight (BW). An oral dose was given. Individually housed animals were monitored for the first 8 h on day 1 and again for 14 days to look for signs of acute toxicity in animal behavior and neurological markers. For pharmacological evaluation, three dosages (15, 30, and 60 mg/kg/day oral administration) of mangiferin were chosen based on acute toxicity investigation [16]. All rats were fed a conventional pellet diet and unlimited access to tap water, and the extract's ability to cause death within this time frame was also noted [17].

2.8. Induction of Diabetes

Diabetes mellitus (type-1) was induced in overnight-fasted adult rats who were experimentally administered a single intravenous dose of 60 mg/kg freshly dissolved streptozotocin (STZ) in 0.1 mol/L citrate buffer (pH 4.5). Control animals were given a comparable volume of citrate buffer. To prevent early mortality from the sudden hypoglycemia period that occurred immediately after STZ-induced lysis of pancreatic islet cells, STZ-induced diabetic rats permitted 10% (w/v) glucose in drinking water for 24 h to overcome the initial drug-induced hypoglycemic death [18]. At 72 hours after STZ injection, diabetic rats were defined as those with a plasma glucose level greater than 300 mg/dl. These animals were included in the study [19, 20].

2.9. Treatment Schedule and Experimental Protocol

A total of thirty-six animals were used for the investigation, of which six were in the normal control group. All remaining animals were given diabetes through induction. Animals in the control group (Group I) (n = 6) received distilled water and were kept diabetic. The rats were divided into four groups (n = 6) three days after receiving a shot of streptozotocin. Animals in the Group II diabetic control group received vehicle (1 ml/kg, p.o.). For twelve weeks, group III was given glibenclamide (0.25 mg/kg), while groups IV and V were given separately mangiferin (15 mg/kg and 30 mg/kg) through oral route, respectively. As it has been previously documented that mangiferin exhibits activity at this concentration, this dose of mangiferin was chosen [21]. The study recorded the subjects' body weight and fasting blood glucose levels (from the study area). For each group, the average daily consumption of food (based on the number of pellets consumed) and water was calculated [22].

2.10. Development of Diabetic Neuropathy

To evaluate the effects of diabetic neuropathy, the drug was continued from week 0 to week 12. In the twelfth week, tests for mechanical hyperalgesia (pinprick test), grip strength, spectrophotometer, and rotarod were performed on all groups [20], respectively [23].

2.11. Fasting Blood Glucose

After 28 days of the experiment, rats in all groups were kept on strict fasting, and blood was drawn from the tail region to measure the blood glucose levels of the rats. After testing, body weight, food, and water consumption were also noted and examined [24].

2.12. Body Weight, Food Intake, and Water Intake

Animal body weight was measured on the first day of selection, the first day of experimentally induced diabetes, seven times while receiving treatment, and on the last day of the study. Depending on how many pellets were consumed each day, the amount of food consumed was calculated. Utilizing calibrated water bottles, the experiment estimated daily water intake.

2.13. Biochemical Parameters

Overnight fasting rats at the second, fourth, sixth, eighth, and twelfth weeks were taken out for blood collection. The retro-orbital plexus was punctured under ether anesthesia to retrieve blood, which was then collected in heparinized Eppendorf tubes (1.5 ml). The serum was centrifuged at

3000 rpm for 15 min at 30°C to estimate a number of biochemical parameters. Serum glucose level was measured using a Microlab 300 semi-automated clinical chemistry analyzer, and the concentration of cholesterol and triglycerides was assessed using commercially available kits.

2.14. Lipid Profile

Lipid profile measurements were performed after blood sampling using retro-orbital methods during the 12th week of the experiment. Centrifugation to separate serum from plasma was performed at Remi Industries Limited (Mumbai, India) for 10 min at a speed of 15000 rpm. The serum was used for tests, such as LDL, HDL, total cholesterol, and triglyceride analysis. The kit was procured from Span Diagnostics Limited India.

2.15. Anti-oxidants Parameters

To assess tissue malondialdehyde (MDA), glutathione (GSH), catalase (CAT), and Mn-superoxide dismutase (Mn-SOD) levels, the left sciatic vein was adequately washed and then rapidly frozen and placed in a -80°C freezer. MDA levels were calculated using thiobarbituric acid reactive substances (TBARS), which were detected spectrophotometrically by absorbance at 535 nm [25]. Concentration was reported in terms of micromoles per milligram of protein. According to the instructions of Ekerbaum and Cies, 10% trichloroacetic acid was used to estimate GSH levels [26]. This was determined by absorbance at 412 nm spectrophotometrically. Concentration was reported in terms of micromoles per milligram of protein. Spectrophotometric measurement of CAT activity was performed [27], following a decrease in absorbance at 240 nm in reaction media containing phosphate buffer and hydrogen peroxide and expressed as a ratio of Trox nanomoles per milligram of protein. Pyrogallol's inhibition of the superoxide radical process provided the basis for estimating Mn-SOD activity [12]. It was measured using spectrophotometry and reported as nanomoles Trox equivalents per milligram of protein using the absorbance at 420 nm.

2.16. Assessment of Thermal Hyperalgesia (Eddy's Hot Plate Method)

Eddy's hot plate was used to evaluate thermal hyperalgesia. Throughout the experiment, the temperature of the hot plate was maintained at 55°C plus or minus 1°C. It was decided to use the delay in the first symptom of paw licking or jumping response (an index of pain threshold) to prevent thermal pain [28].

2.17. Assessment of Cold and Hot Allodynia by Tail Immersion Method

The tail of each rat was immersed in water that was either cold (10°C) or warm (48°C), and tail-flick latency was measured until the tail retracted or evidence of struggle appeared [29].

2.18. Assessment of Sensorimotor Deficit

Neuromuscular coordination of rats from all groups was measured at weeks 2, 4, 8, and 12 using rotarods. At a rotational speed of 25 rpm, rats were placed on the rotarod device. During the first five minutes of the run, deceleration times were noted for each group [30, 31].

2.19. Grip Strength

Determination of neuromuscular strength included a grip strength test. The animals were suspended by their forelimbs using a small metal wire that was clamped tightly to the ends of a pole to test their grip strength. Their muscle strength was measured by timing how long they could hold onto the wire before falling to the ground. A muscle that is weak or injured will cause the animal to fall quickly. The length of the hanging was noted [32].

2.20. Spontaneous Locomotor (Exploratory) Test

With the use of an ectophotometer, spontaneous motor exploratory testing was performed to document and analyze animal behavior. The animals were accommodated in this actophotometer (30 x 30 x 30 cm), which was closed and had photocells on the outer wall. The computerized counter noted beam interruptions [32].

2.21. Estimation of Inflammatory Cytokines

Levels of inflammatory cytokines, interleukin-6 (IL-6), transforming growth factor (TGF)-β1, tumor necrosis factor-alpha (TNF-α), and nerve growth factor (NGF) levels in the sciatic nerve were determined using ELISA kits [33, 34].

2.22. Nerve Fiber Analysis (Histopathology)

The sciatic nerve was isolated from the thighs of rats in each group on the last day of the 12th week of the experiment. The sciatic nerve was isolated by holding the hind limb with the left hand and the base of the tail with the right hand, and the nerve was severed when the rat was killed on the final day. The thick, white, cord-like substance was then exposed and removed by cutting in the thigh area close to the back of the thigh (sciatic nerve) [35]. The isolated sciatic nerve was treated in buffered, pH 6.9 4% formaldehyde solution. After the sections were frozen for 24 hours, histopathology examinations were performed. After setting them in the resin, the parts were cut out with a small blade. Eosin dye was used to stain the samples [36].

2.23. Statistical Analysis

All values were presented as mean ± standard error of the mean (SEM) (n = 6). Graph Pad Prism software was used to analyze data using Student's t-test or one-way/two-way analysis of variance (ANOVA), followed by Tukey's-test for repeated pair-wise comparisons across different treated groups. The Kramer test was also performed. Statistics were considered significant for P-values of 0.05

3. RESULTS

3.1. Acute Toxicity Study

All animals survived the 14-day observation period after receiving 2000 mg/kg mangiferin orally, and all of them gained weight during this time. Only a transient acuity and

Table 1. Effect of mangiferin on fasting blood sugar level during the course of anti-diabetic study.

S. No.	Groups	Fasting Blood Glucose Level in mg/dl			
	Treatment	Day 7	Day 14	Day 21	Day 28
1	Group I	95.6 ±1.23	95.25 ± 0.43	95.51 ± 1.93***	95.24 ±1.65***
2	Group II	165.52 ± 1.36a	261.29 ± 2.36a	272.12 ± 0.28a	305.31 ±1.12a
3	Group III	149.45 ± 1.31***	193.50 ± 1.89***	137.12 ± 1.33***	119.12 ±3.21***
4	Group IV	148.21 ± 1.32***	145.50 ± 1.89***	132.12 ± 1.33***	118.10 ±3.21***
5	Group V	147.14 ±1.23***	136.25 ± 2.43***	128.51 ± 1.93***	115.24 ±1.65***

Note: Values are expressed as Mean ±SEM; n =6. two-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a P< 0.001 in comparison with normal control and ****P* < 0.001 in comparison with the diabetic control.

Table 2. Effect of mangiferin on the body weight in wistar albino rats.

S. No.	Groups	Body Weight (g)			
	Treatment	2nd week	4th week	8th week	12th week
1	Group I	164.59 ±1.990a	157.69 ±2.13a	148.52 ±0.62a	258.12 ±0.28a
2	Group II	158.11 ±0.91	145.2 ±0.33***	143.40 ±1.81***	135.31 ±1.22***
3	Group III	154.73 ±0.57	158.35 ±3.30***	161.14 ±2.32***	162.12 ±1.13***
4	Group IV	157.77 ±0.57	159.35 ±3.30***	160.14 ±2.32***	167.15 ±1.33***
5	Group V	159.97 ±2.456	160.53 ±1.59	163.52 ±0.62a	168.31 ±1.93***

Note: Values are expressed as Mean ±SEM; n =6. Two-way ANOVA; Tukey-Kramer multiple comparisons test: a *P*<0.001 in comparison with normal control; and ****P* < 0.001 in comparison with the diabetic control.

decrease in motor activity were observed in the first three hours after therapy. At necropsy, no overt pathological abnormalities were found, and no significant organ changes were observed.

3.2. Fasting Blood Glucose

The results are displayed in Table **1**. On day 28th of the experiment, tests were done, and the treated group showed a reduction in blood glucose with readings of 118.10 and 115.24 mg/dl in groups III and IV, respectively. When compared to the results at week 12 of group II (diabetes control), the findings were found to be significant.

3.3. Body Weight, Food Intake, and Water Intake

Rats in both groups IV and V gained weight from week two to week twelve. Both the treatment group, with an average weight of 167.15 and 168.31 grams and the diabetes group, with an average weight of 135.31 grams, showed improvement in health because diabetes is a condition where weight gain is the opposite of weight loss. Results are significant in comparison to data from group II (diabetes control) at week 12. The results are shown as a graph in Table **2**.

3.4. Lipid profile

Mangiferin supplements dramatically reduced blood triglyceride and cholesterol levels in diabetic rats compared to diabetic control rats in a dose-dependent manner. Compared to the control group, the diabetic rats had significantly lower HDL cholesterol levels. However, diabetic rats treated with mangiferin did not show a significant increase in HDL cholesterol levels (Table **3**).

3.5. Anti-oxidants Parameters

The malondialdehyde (MDA) levels in each group after the experiment are mentioned in Table **4**. The amount of lipid peroxidation increased significantly ($p < 0.001$) in the STZ group compared to the vehicle group after STZ treatment. The preventive and curative groups were not statistically different from each other ($p > 0.05$). However, MDA levels were significantly lower in the preventive and curative groups than in the STZ group ($p < 0.01$). After the experiment, the glutathione (GSH) concentration for each group is mentioned in Table **4**. After STZ administration, the GSH level in the STZ group dropped dramatically ($p < 0.001$) compared to that in the vehicle group. GSH levels in the preventive and curative group were significantly higher ($p < 0.01$) than those in the STZ group, even though the differences between the preventive and curative group and the STZ group were not statistically significant ($p > 0.05$). The catalase (CAT) activity levels for each group after the experiment are mentioned in Table **4**. Administration of STZ to the STZ group caused a significant ($p < 0.001$) decrease in CAT activity compared to the vehicle group. Although there was no statistically significant difference between the preventive and curative groups ($p > 0.05$), the CAT activities of the preventive and curative groups were significantly higher than those of the STZ group ($p < 0.01$). The Mn-superoxide

Table 3. Effect of mangiferin on serum lipid profile.

S. No.	Groups	Lipid Profile (mg/dl)			
	Treatment	Serum Cholesterol in	Serum Triglycerides	Serum HDL	Serum LDL
1	Group I	75.55±1.52	71.91 ± 1.25	30.35 ± 1.72	38.41± 1.23
2	Group II	150.48 ± 1.02**	145.89 ± 1.35***	15.43 ± 1.45**	86.21 ± 1.64***
3	Group III	78.78 ± 0.32***	69.89 ± 0.35***	31.83 ± 1.99***	36.41 ± 0.35 ***
4	Group IV	79.48 ± 1.02***	65.89 ± 1.35***	28.83 ± 1.69***	37.43 ± 0.33 ***
5	Group V	76.87 ± 1.56***	66.15 ± 1.65***	27.45 ± 1.89***	33.79 ± 2.53***

Note: Values are expressed as Mean ± SEM; n =6. One-way ANOVA; Tukey-Kramer multiple comparisons test: a $P< 0.001$ in comparison with normal control; and ***$P < 0.001$ in comparison with the diabetic control.

Table 4. Effect of the mangiferin on anti-oxidants parameters of rat sciatic nerve affected by STZ-induced diabetic neuropathy.

S. No.	Groups	Biochemical Parameters (µmol/mg-protein)			
	Treatment	GSH	MDA	CAT	Mn-SOD
1	Group I	24.67 ± 1.15	32.07 ± 3.44	11.35 ± 0.52	35.56 ± 2.54
2	Group II	18.95 ± 0.54**	5627 ± 6.25**	9.13 ± 0.10**	24.92 ± 5.15*#
3	Group III	16.94 ± 1.21***	52.45 ± 1.95**	9.34 ± 0.56**	20.64 ± 2.52***
4	Group IV	24.00 ± 1.00***	33.33 ± 2.85***	10.87 ± 1.24***	36.67 ± 2.08***
5	Group V	13.84 ± 1.03***	69.72 ± 1.69***	5.72 ± 1.60***	20.45 ± 1.81***

Note: Data are represented in Mean ± SD of 6 rats/group $p < 0.001$ *versus* vehicle and GRL group **$p < 0.01$ *versus* STZ group ***$p < 0.05$ *versus* STZ group by one-way ANOVA followed by Tukey's post-hoc tests.

Table 5. Effect on inflammatory mediators.

S. No.	Group	IL-1β (Pg/ml)	IL-6 (Pg/ml)	TGF-β1 (%)	TNF-α (Pg/ml)	NGF (Pg/ml)
1	Group I	162±2.70	176±2.50	15.2±3.70	35.5±3.60	146±2.60
2	Group II	355±4.32a**	365±6.5a**	27.2±3.60a**	252±30.5a**	116±1.12a**
3	Group III	185±4.52***	235±4.52***	18.2±1.75***	36.4±10.6***	165±2.32***
4	Group IV	240±5.62***	220±7.52***	15.5±1.50***	42.0±5.52***	163±3.52***
5	Group V	225±5.20***	185±5.50***	13.5±1.60***	36.5±3.32***	172±3.10***

Note: Data are represented in Mean ± SD of 6 rats/group * $p < 0.001$ *versus* vehicle and GRL group **$p < 0.001$ *versus* STZ group ***$p < 0.05$ *versus* STZ group by one-way ANOVA followed by Tukey's post-hoc tests.

dismutase (Mn-SOD) activity levels for each group after the experiment are mentioned in Table **4**. After STZ delivery, Mn-SOD activity decreased significantly ($p < 0.001$) in the STZ group compared to the vehicle group. The Mn-SOD activities of the preventive and curative group were not significantly ($p > 0.05$) higher than those of the STZ group.

3.6. Effect on Inflammatory Cytokines in Diabetic Rats

We investigated how the mangiferin affected the levels of inflammatory cytokines in the blood of diabetic rats induced by STZ. In comparison to the control group, STZ-treated rats had considerably lower concentrations of inflammatory cytokines (IL-6 and TGF-β1) and higher concentrations of an anti-inflammatory cytokine. Rats given STZ showed a significant increase in TGF-β, IL-1β, and IL-6 concentrations but a significant decrease in TNF-α release. The STZ-induced pro-inflammatory cytokines TGF-β, IL-1β, and IL-6, as well as the anti-inflammatory cytokine, were both considerably recovered by the administration of mangiferin. Sciatic levels of NGF were significantly ($P<0.05$ and $P<0.01$, respectively) reduced in the diabetic rats compared to the control rats. Group V rats exhibited significant ($P<0.05$) reductions in the levels of NGF compared to the untreated diabetic rats (Table **5**).

3.7. Thermal Hyperalgesia: Eddy's Hot Plate Method

Treatment groups IV and V responded at 12 weeks with mean response times of 4.79 and 4.42 seconds, respectively. When compared to the 12th week results of group II (diabetes control), the findings were found to be remarkable (Table **6**).

Table 6. Effect of mangiferin on thermal hyperalgesia.

S. No.	Groups	Thermal Hyperalgesia -Eddy's Hot Plate Method Response Time (s)			
	Treatment	2nd week	4th week	8th week	12th week
1	Group I	6.03 ± 0.48	5.11 ± 0.12	4.91 ± 1.31	4.79 ± 2.71
2	Group II	6.91 ± 2.31a	7.24 ± 0.22a	8.94 ± 0.21a	9.95± 0.19a
3	Group III	6.01 ± 0.14***	5.01 ± 0.30***	5.21 ± 2.91***	5.67 ± 0.48***
4	Group IV	5.01 ± 0.54***	4.01 ± 0.20***	4.43 ± 2.91***	4.79 ± 0.38***
5	Group V	4.12 ± 0.12***	4.60 ± 2.32***	4.01 ± 0.12***	4.42 ± 0.78***

Note: Values are expressed as Mean SEM; n =6. two-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a $P< 0.001$ in comparison with normal control; ***$P < 0.001$ in comparison with the diabetic control.

Table 7. Effect on cold allodynia.

S. No.	Groups	Paw Withdrawal Latency (s)			
	Treatment	0 day	7 day	14 day	21 day
1	Group I	27.50 ±2.25	25.50 ±2.22	23.50 ±2.15	26.30 ±1.25
2	Group II	4.50 ±0.25*	6.20 ±0.34*	8.30 ±0.13*	5.13 ±0.15*
3	Group III	4.40 ±0.25**	10.20 ±1.15**	18.20 ±1.12**	24.15 ±0.32**
4	Group IV	3.30 ±0.15**	6.10 ±1.11**	12.30 ±1.15**	16.25 ±0.22**
5	Group V	4.51 ±0.25**	12.10 ±1.25**	16.10 ±1.52**	21.55 ±0.42**

Note: Data are represented in Mean ± SD of 6 rats/group * $p < 0.001$ *versus* vehicle and GRL group **$p < 0.001$ *versus* STZ group ***$p < 0.05$ *versus* STZ group by two-way ANOVA followed by Tukey′s post-hoc tests.

Table 8. Effect on hot allodynia.

S. No.	Groups	Paw Withdrawal Latency (s)			
	Treatment	0 day	7 day	14 day	21 day
1	Group I	4.52 ±0.25	6.25 ±0.32	8.30 ±0.15	10.11 ±0.18
2	Group II	0.00 ±0.00*	3.32 ±0.22*	2.20 ±0.25*	1.12 ±0.58*
3	Group III	0.00 ±0.00	5.22 ±0.32**	6.10 ±0.55**	9.52 ±0.48**
4	Group IV	0.00 ±0.00	3.12 ±0.44**	4.50 ±0.65**	7.22 ±0.45**
5	Group V	0.00 ±0.00	3.48 ±0.24**	6.20 ±0.68**	9.48 ±0.25**

Note: Data are represented in Mean ± SD of 6 rats/group * $p < 0.001$ *versus* vehicle and GRL group **$p < 0.001$ *versus* STZ group ***$p < 0.05$ versus STZ group by two-way ANOVA followed by Tukey′s post-hoc tests.

3.8. Effect on Cold and Hot Allodynia

Cold and hot allodynia were assessed by paw withdrawal latency (PWL) using both cold water and hot water immersion tests. In both the models of thermal sensitivity, groups IV and V caused a significant decrease in the score of PWL in Group II compared to Group I on days 7, 14 and 21. Treatment with glibenclamide and variable doses (15 and 30 mg/kg, p.o.) of mangiferin significantly increased the score of PWL in thermal sensitivity of pain as compared to the diabetic control group (II) on days 7, 14 and 21, respectively (Tables **7** and **8**).

3.9. Assessment of Sensorimotor Deficit

Neuromuscular coordination recorded an improvement in Group IV and Group V animals. The results were compared with the responses from the diabetes control group (Table **9**).

3.10. Grip Strength

After the experiment, the grip strength of groups IV and V was 29.72 and 32.34, respectively. The results were significant compared to the responses of the diabetic control group (Group I) in the 12th week of the experiment (Table **10**).

3.11. Spontaneous Locomotor (Exploratory) Test

The results of the exploratory test are mentioned in Table **11**. After receiving the supplements for 12 weeks, the locomotor activity of the group increased to 116.54 and 117.56 in group IV and group V, respectively. The results were significant compared to the responses of the diabetic control group (Group II) in the 12th week of the experiment.

3.12. Nerve Fiber Analysis (Histopathology)

Histopathology of the sciatic nerve was observed as follows: a) Diabetic control group showed rearrangement of nerve fibres with significant axonal swelling, b) lipoid degeneration of axons and mild focal peripheral axonal loss were observed in the treatment group 1, c) minimal axon degenerative changes were observed without regenerative features in the treatment group 2, d) minimal axon degenerative changes were observed with the normal arrangement of fibre in normal control, and e) standard glibenclamide treated diabetic animals showed a normal arrangement of nerve fibres. Histological examination (Figs. **2** and **3**) and morphometric analysis of sciatic nerves in STZ-induced diabetic animals are presented in Table **12**.

Table 9. Assessment of sensorimotor deficit.

S. No.	Groups	Rotarod			
	Treatment	Counts (2nd week)	Counts (4th weeks)	Counts (8th weeks)	Counts (12th weeks)
1	Group I	118.45 ±1.25	125.5 ± 1.15	122.25 ± 1.25	127.15 ± 5.50
2	Group II	42.32 ± 2.55a	28.25 ± 4.52a	22.45 ± 5.50a	8.52 ± 5.22a
3	Group III	48.32 ± 5.15***	82.15 ± 2.15***	95.62 ±5.35***	108.15±1.35***
4	Group IV	54.50 ± 6.55***	65.32 ± 6.22***	75.15 ±6.25***	88.25± 2.15***
5	Group V	55.15 ± 2.22	78.15 ± 6.15***	88.22 ±6.30***	118.55±2.33***

Note: Data are represented in Mean ± SD of 6 rats/group * $p < 0.001$ *versus* vehicle and GRL group **$p < 0.001$ *versus* STZ group ***$p < 0.05$ *versus* STZ group by one-way ANOVA followed by Tukey's post-hoc tests.

Table 10. Effect of mangiferin on grip strength to record the response latency in wistar albino rats.

S. No.	Groups	Grip Strength for each animal in a group ± SEM, n = 6.			
	Treatment	2nd week	4th week	8th week	12th week
1	Group I	20.71 ± 1.36	21.44 ± 2.13	23.71 ± 1.36	23.12 ± 1.49
2	Group II	12.23 ± 0.21a	9.96 ± 1.35a	7.13 ± 0.52a	4.36 ± 1.13a
3	Group III	19.13 ± 0.21***	22.09 ± 0.18***	24.32 ± 1.42***	32.52 ± 0.91***
4	Group IV	18.45 ± 0.21***	23.49 ± 0.18***	26.32 ± 1.72***	29.72 ± 0.93***
5	Group V	22.26 ± 1.92***	21.19 ± 0.87***	23.23 ± 0.73***	32.34 ± 2.36***

Note: Values are expressed as Mean SEM; n =6. Two-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a $P < 0.001$ in comparison with normal control; ***$P < 0.001$ in comparison with the diabetic control.

Table 11. Effect of mangiferin on spontaneous locomotor (exploratory) test: actophotometer in Wistar albino rats.

S. No.	Groups	Actophotometer: Counts per 5 min			
	Treatment	2nd week	4th week	8th week	12th week
1	Group I	120.33 ± 7.236	126.98 ± 5.324a	120.17 ± 6.882a	123.36 ± 1.13a
2	Group II	22.67 ± 6.02	20.22 ± 6.25	15.17 ± 7.22	16.43 ± 5.28
3	Group III	58.67 ± 6.22***	84.24 ± 8.37***	94.63 ± 5.37***	119.34 ± 3.72***
4	Group IV	54.77 ± 5.32***	69.24 ± 8.37***	83.63 ± 5.57***	116.54 ± 3.36***
5	Group V	40.17 ± 6.492**	61.67 ± 6.25***	85.26 ± 5.262***	117.56 ± 3.71***

Note: Values are expressed as Mean ± SEM; n =6. two-way ANOVA; followed by Tukey-Kramer multiple comparisons test: a $P < 0.001$ in comparison with normal control; ***$P < 0.001$ in comparison with the diabetic control.

Table 12. Morphometric analysis of the sciatic nerve at the end of the 12th week.

S. No.	Experimental Groups	Fibre Area (μm^2)	G ratio (Axon Diameter/Fibre Diameter)	Axon Density (N/mm^2)
1	Group I	6458 ± 2366	0.38 ± 0.05	26.56 ± 8.78
2	Group II	5832 ± 1868	0.35 ± 0.06	38.53 ± 8.79
3	Group III	6085 ± 1052**	0.36 ± 0.08**	32.80 ± 7.62***
4	Group IV	6463 ± 2360***	0.39 ± 0.05***	28.67 ± 8.59***
5	Group V	4703 ± 1656***	0.45 ± 0.07***	34.60 ± 18.0***

Note: Data are represented in Mean ± SD of 6 rats/group * $p < 0.001$ *versus* vehicle and GRL group **$p < 0.001$ *versus* STZ group ***$p < 0.05$ *versus* STZ group by one-way ANOVA followed by Tukey's post-hoc tests.

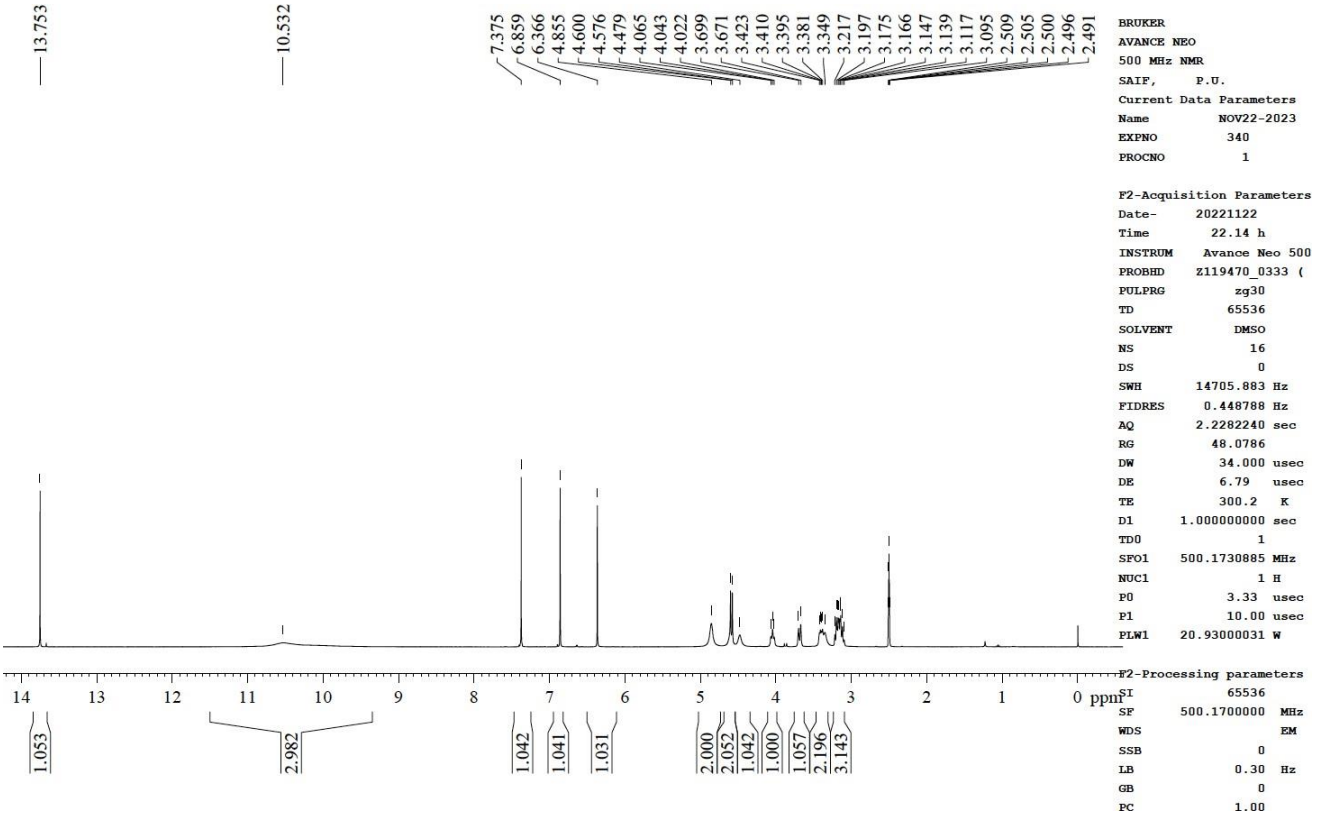

Fig. (2). 1H NMR (DMSO-d6, 400 MHz) spectra of isolated compound (Mangiferin) obtained from leaves of *Swertia chirayita.*

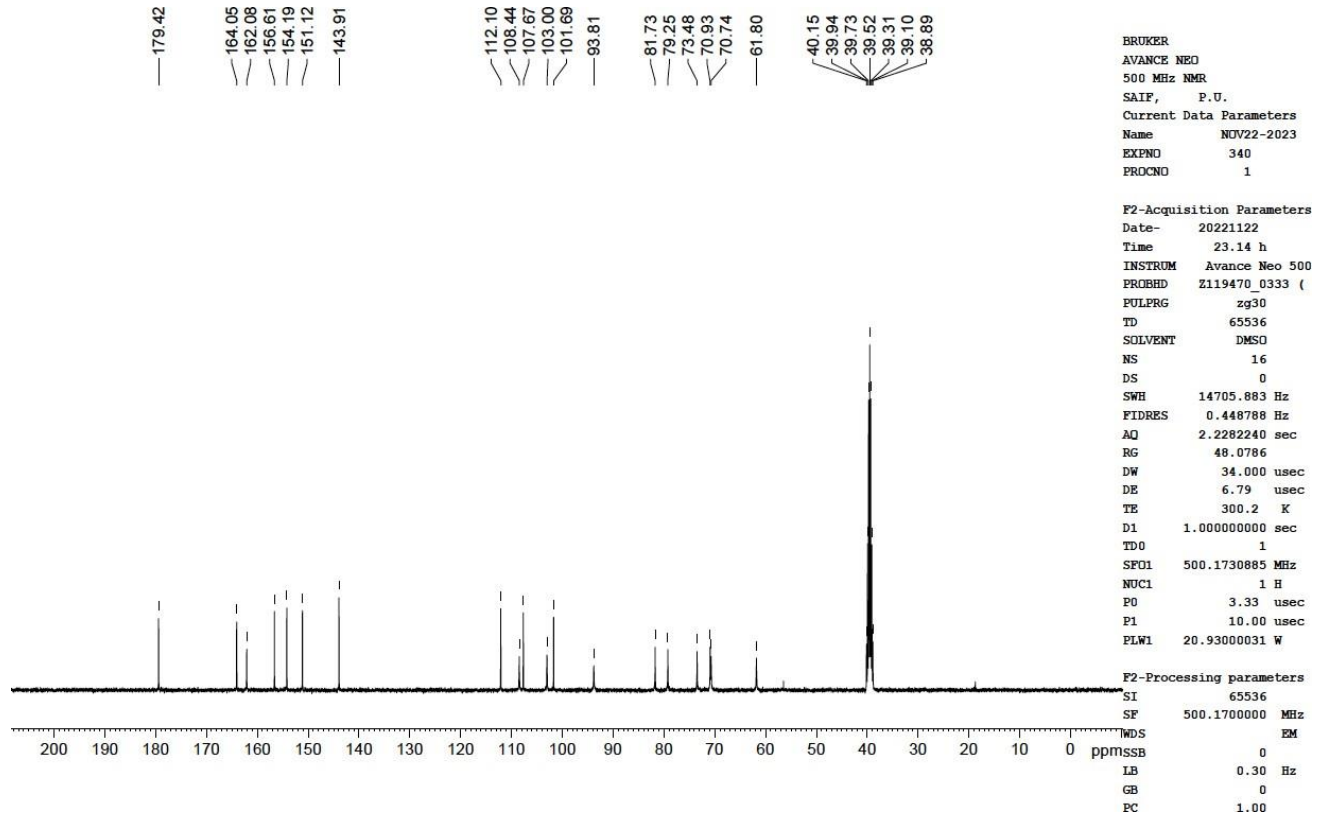

Fig. (3). 13C NMR Mangiferin (DMSO-d6, 100 MHz) spectra of isolated compound (Mangiferin) obtained from leaves of *Swertia chirayita.*

4. DISCUSSION

According to the unified hypothesis, oxidative stress caused by mitochondria is a fundamental pathophysiology shared by diabetes and its long-term consequences. There is little doubt that diabetes animal models cause significant oxidative status alterations [37]. With type 1 diabetes, diabetic neuropathies typically develop more reliably and quickly, resulting in a more severe neuropathy [38]. In hyperglycemic conditions, neurons lacking insulin regulation are more vulnerable to hyperglycemic injury. The risk and intensity of neuropathic pain are positively correlated with the severity of the neuropathy and poor glycemic control [39].

The tail and paw withdrawal latencies of diabetic rats in the study were delayed compared to control rats. This suggests that the rats had thermal and mechanical hyperalgesia, which slowed their reaction times and got worse with more research weeks. At 12 weeks in the treated groups, thermal algesia responses, writhing responses, and pinprick responses all improved, most dramatically in group V, showing the neuroprotective action of mangiferin. Treated groups displayed better grip strength, actophotometric locomotor responses, and rotarod responses than diabetic control groups. Animals become less active because of the development of diabetic neuropathy and the development of diabetes as they gradually develop blunted reflexes, inflammation of the nerves that cause pain in the limbs, and delayed locomotor responses [40].

As loss of body weight is a defining feature of the diagnosis of diabetes, we monitored the weight of the animals to test that the isolated mangiferin was acting as intended. The body weight of animals in the diabetic control group declined, which is a characteristic of diabetes, whereas the body weight of animals in the treatment groups increased. This shows whether the treated group can recover [41].

By the end of 8 weeks of STZ injection, the DC group in the present study developed sensorimotor deficits, thermal hyperalgesia (increased pain caused by a stimulus that usually provokes pain), and allodynia (pain caused by a stimulus that usually provokes pain). These are symptoms of diabetic neuropathic pain. The STZ-induced diabetes model exhibits hyperalgesia and allodynia, which are characteristic of neuropathic pain seen in diabetics. Changes in nociceptive responses are likely due to different pathophysiological mechanisms due to hyperglycemia [42]. Diabetes caused by STZ produces reactive oxygen species (ROS), including oxygen-free radicals, which damage nerves. Allodynia and hyperalgesia, two recognizable symptoms of neuropathic pain, occur due to damaged afferents. Diabetic animals showed muscle tremors in addition to hyperalgesia. Implications of gradual loss of lower extremity sensation over time include motor weakness, loss of balance, slips, and a numb, insensitive foot [43]. Peripheral diabetic neuropathy can be significantly hindered by these sensorimotor disorders. The results of this investigation suggest that mangiferin has neuropathic pain-relieving properties even in hyperglycemic settings, demonstrating the importance of mechanisms other than antidiabetic action in the prevention of DN.

Mn-SOD and MDA, two endogenous enzymes, are di- rectly associated with oxidative stress. Endothelial damage is caused by endogenous enzymes and superoxide. Superoxide anion resulted in increased protein kinase C and aldose reductase activity, and these changes were found to be associated with the sensation of pain. Mn-SOD converts superoxide anions to H_2O_2 to act as an antioxidant defense against them. By rearranging the bonds in the unsaturated FA (fatty acids) of the membrane and contributing significantly to the disruption of lipid membranes, MDA represents a state of high stress and causes tissue damage. Increased lipid peroxidation serves as an indicator of oxidative stress, and pharmacological therapy reduces its level. Nitric oxide, an intracellular messenger, plays an important role in many disease processes. Reactive oxygen species, which act as an antioxidant, react with it. This oxidation affects cell molecules and is nonspecific [36, 44].

A powerful endogenous antioxidant called glutathione serves as the first line of defense against free radicals. In the present investigation, the sciatic nerves of diabetic rats had significantly reduced GSH levels. Mangiferin therapy dose-dependently ameliorated a decrease in GSH levels in sciatic nerves. Mangiferin protects cells and neurons by maintaining GSH levels, increasing GSH levels, and perhaps promoting GCLC expression [45].

The superoxide anion is one of the primary mediators of oxidative damage caused by glucose. These highly reactive superoxide anions (O_2) are converted to H_2O_2 by the enzyme Mn-superoxide dismutase (Mn-SOD). The reduced Mn-SOD activity in the sciatic nerve in diabetic rats may be due to non-enzymatic glycosylation. Diabetes can reduce catalase activity, which can weaken the body's ability to fight free radicals. Sciatica is particularly vulnerable to oxidative stress caused by hyperglycemia because the diabetic condition simultaneously reduces SOD and CAT activities. Mangiferin at both doses markedly increased the level of Mn-SOD in diabetic rats. A high dose of mangiferin restored catalase levels in the sciatic nerves of diabetic rats. The findings showed that mangiferin restored Mn-SOD and CAT activity while protecting sciatic nerve damage from oxidative stress. Lipid peroxidation in the diabetic sciatic nerve leads to several cascades that ultimately culminate in cell deterioration (Figs. **4** and **5**) [46].

In the present investigation, mangiferin increased the levels of antioxidant enzymes and decreased lipid peroxidation in sciatic nerves. The tannins, flavonoids, and phenols found in Swertia absinthe are thought to be responsible for the antioxidant abilities of the plant. Swertia absinthe contains saponarin, an alpha-glucosidase inhibitor that has been found to have strong antioxidant activity. In a rat model, the polysaccharide arabinogalactan from Swertia absinta exhibited antioxidant abilities through protection against free radicals. Mangiferin alkaloids have been shown to enhance antioxidant status and inhibit lipid peroxidation in rats exposed to xenobiotic chemicals. Therefore, the antioxidant activities of saponins and other anti-oxidant components of Swertia salicylic extract may be the reason for the anti-nociceptive potential discovered in the present research [47].

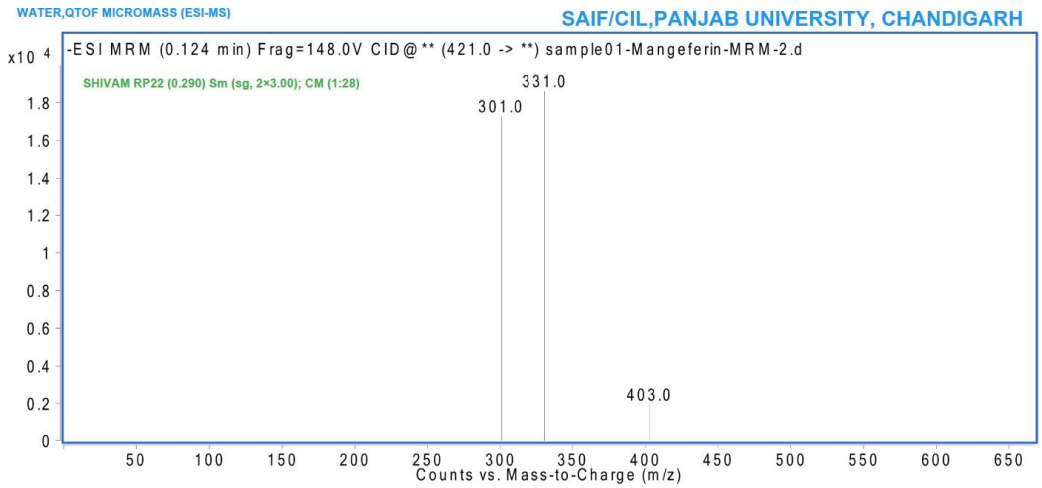

Fig. (4). Mass spectra of isolated compound (Mangiferin) obtained from leaves of *Swertia chirayita.*

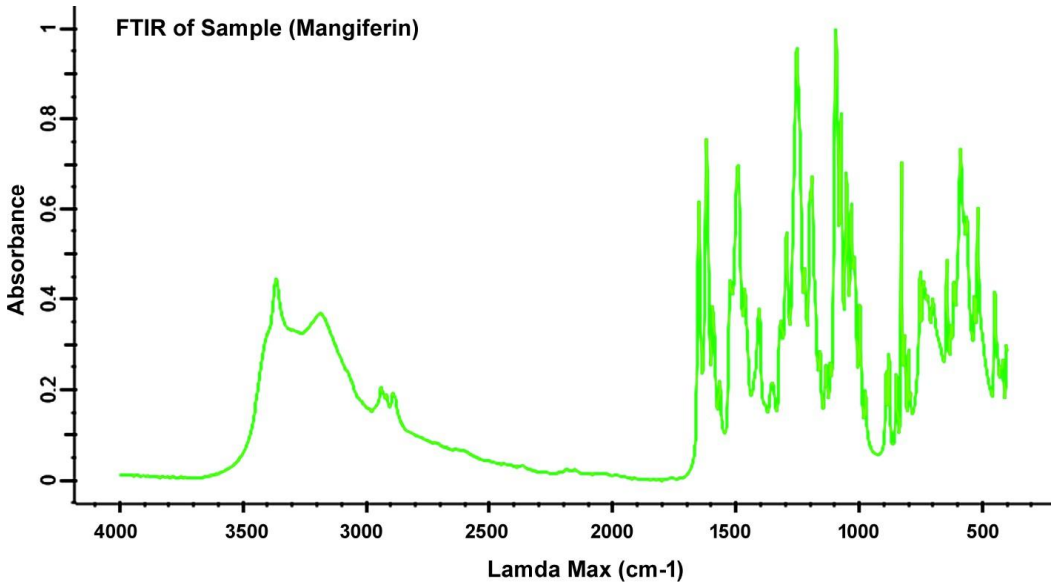

Fig. (5). FTIR spectra of isolated compound (Mangiferin) obtained from leaves of *Swertia chirayita.*

In diabetic rats, morphological abnormalities of axonal degeneration in the sciatic nerves were congruent with changes in gene expression in fibroblasts, endothelial cells, and adipocytes connected with the sciatic nerve [48]. One of the most important neurotrophic factors, NGF, was the first to be discovered. For the development and differentiation of neurons, it is important. NGF is responsible for nerve regeneration. It also protects against neuronal degeneration and death. People with diabetes have reduced levels or activity of NGF, which may significantly affect the etiology of DN. In the present study, mangiferin increased the expression of NGF in the sciatic nerve of the diabetic rat, protecting these nerves from axonal damage. It has been suggested that phytosterols, such as B-sitosterol, have neuroprotective properties. The presence of the sterols, B-sitosterol, D-sitosterol, G-sitosterol, B-hydroxygenase, ecdysterone, mecisterone, gylonesterolgetorin, and columbine in mangiferin is responsible for its neuroprotective effects [49].

Rats treated with mangiferin showed improvements in their walking, lipid profiles, and reaction times and had histological abnormalities in their liver and sciatic nerve. Therefore, it is hypothesized that by giving isolated mangiferin to Wistar rats with STZ-induced hyperglycemia at concentrations of 15 mg/kg and 30 mg/kg, respectively, an improvement occurred, and a reduction was observed in sciatic nerves as an indication, along with a reduction in neuropathy because of inflammatory injury, a long-term side effect of diabetes [50]. Such infiltration is indicative of hyperglycemia and diabetic neuropathy in the liver and sciatic nerve tissues of diabetic control group rats. The use of medicinal plants as a source to cure various disease problems is a common practice in many civilizations around the world. In both the early (7 days) and late (28 days) stages, the morphology was examined. In the sciatic nerves of group II rats, there was diffuse degeneration of the axons and myelin sheath. The normal concentric lamellar structure of the myelin sheath was disrupted, resulting in disorganized and frag-

mented lamellae. Axonal ballooning was obvious, and complete axonal degeneration occurred in some cases. Reductions in myelin sheath thickness, nerve fiber diameter, and axon diameter were also seen after light microscopic morphometric study of tissues. This approach confirmed the findings of the current study and exemplified the stages of regeneration following nerve degeneration.

CONCLUSION

The major results of the present investigation suggest that treatment with isolated mangiferin, in addition to hypoglycemic effects, ameliorated the parameters of neuropathy in STZ-induced diabetic rats. Moreover, the study findings showed that these positive effects persisted even after the onset of peripheral diabetic neuropathy. To put it another way, consumption of Mangiferin or Swertia chirayita extract has both therapeutic and preventive effects on STZ-diabetic neuropathy, which may be due to its antioxidant, anti-inflammatory, and anti-apoptotic properties.

LIST OF ABBREVIATIONS

DNP	= Diabetic Neuropathy
STZ	= Streptozotocin
UV	= Ultra violate
HPLC	= High-Performance Liquid Chromatography
NMR	= Nuclear Magnetic Resonance
FTIR	= Fourier Transform Infrared
IL	= Interleukins
NGF	= Nerve Growth Factor
DM	= Diabetes Mellitus
TGF-β1	= Transforming Growth Factor
TNF-α	= Tumor Necrosis Factor-alpha
OECD	= Organization for Economic Cooperation and Development
MDA	= Malondialdehyde
GSH	= Glutathione
CAT	= Catalase
SOD	= Mn-superoxide dismutase
ELISA	= Enzyme-Linked Immunosorbent Assay
ANOVA	= Analysis of Variance
IAEC	= Institute Animal Ethics Committee

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained to conduct pharmacological activity on animals from CPCSEA/Institutional Animal Ethics Committee (IAEC), with a registration number 837/PO/ReBiBt/S/04/CPCSEA and a reference number IFTM/IAEC/2022/02/03. These recommendations complied with the widely recognized standards for the usage and maintenance of laboratories.

HUMAN AND ANIMAL RIGHTS

The reported experiments in accordance with the standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animals and are reported in accordance with ARRIVE guidelines.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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