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RESEARCH ARTICLE

Evaluation of Neuroprotective Effect of Salicin in an Experimental Animal Model of Streptozotocin Induced Diabetic Neuropathy

Ghanshayam B. Jadhav[1], Shubham J. Khairnar[1,2*], Simona E. D'Souza[2] and Pavan B. Udavant[2]

[1]Department of Pharmacology, NDMVP College of Pharmacy, Nashik – 422005, Maharashtra, India; sunnykhairnar62@gmail.com

[2]Department of Pharmacology, MET's Institute of Pharmacy, Nashik – 422003, Maharashtra, India

Abstract

Background: Diabetic neuropathy stands as the most prevalent secondary complication connected with diabetes mellitus. The susceptibility of mammalian nerves to oxidative stress is heightened due to their rich phospholipid content, leading to a reduced ability to counteract the free radicals responsible for neuropathy. While synthetic treatments can help alleviate oxidative stress, they often come with unwanted side effects. Shifting the focus towards natural pharmaceuticals could mitigate these negative effects. Phenolic compounds abundant in antioxidants may aid in reducing oxidative stress. **Aim:** Assess the preventive influence of Salicin, a phenolic compound against diabetic neuropathy induced by Streptozotocin (STZ). **Methods:** Four weeks following the injection of STZ into the peritoneal cavity, a noticeable reduction in thermal and mechanical hyperalgesia, cold allodynia, motor coordination and locomotor activity was noted. Natural antioxidants such as reduced glutathione and catalase were assessed along with lipid peroxidation levels on the 28th day and the sciatic nerve was subjected to histopathological examination. **Results:** Orally administering Salicin at dosages of 10, 15, and 20mg/kg over 28 days successfully mitigated the reduction in the nociceptive threshold and bolstered the levels of endogenous antioxidants. It also mitigated the unwanted histopathological changes effectively based on the dosage. **Conclusion:** Salicin having antioxidant properties, demonstrates potential in alleviating diabetic neuropathic pain and preventing associated complications.

Keywords: Antioxidant, Oxidative Stress, Peripheral Neuropathy, Polyphenols, Proinflammatory Mediators, Reactive Oxygen Species

Abbreviations: ANOVA: Analysis of variance; CAT: Catalase; CCSEA: Committee for Control and Supervision on Experiments on Animals); DPN: Diabetic Peripheral Neuropathy; DTNB: 5,5-dithio-bis-(2-nitrobenzoic acid); GSH: Glutathione/RGSH: Reduced Glutathione; i.p: Intraperitoneal; MAPK: Mitogen-Activated Protein Kinase; MDA: Malonaldehyde; NA: Nicotinamide; P.o: Per oral; PARP: Poly (ADP-ribose) Polymerase; PKC: Protein Kinase C; Remi C-24: Remi Centrifuge 24; ROS: Reactive Oxygen Species; SL: Salicine; SOD: Superoxide dismutases; STZ: Streptozotocin; TBARS: Thiobarbituric acid reactive substances

1. Introduction

Neuropathic pain, a commonly overlooked public health concern, impacts a large number of people worldwide and has significant socioeconomic ramifications. The International Association for the study of pain has lately modified the meaning of neuropathic pain as "pain caused by lesion or disease of the somatosensory system". This variety of pain can appear in a range of medical conditions and is classified as either peripheral neuropathic pain or central neuropathic pain[1]. Diabetes mellitus is associated with a range of complications,

*Author for correspondence

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including retinopathy, neuropathy, nephropathy, cardiomyopathy, vasculopathy, dermatopathy and encephalopathy. Diabetic neuropathy is specifically described as the presence of symptoms or signs indicating a dysfunction in the peripheral nerves of people with diabetes, after ruling out other potential reasons such as hereditary factors, trauma, compression, metabolic issues, toxins, nutritional deficiencies, infections, immune-related problems, neoplasms or complications stemming from other systemic illnesses[2]. Diabetic Peripheral Neuropathy (DPN) is marked by sensations such as pain, abnormal tingling (often described as "pins and needles"), shooting pain (similar to electric shocks) or stabbing sensations. This condition affects almost half of the diabetic population, leading to considerable morbidity, mortality and reduced quality of life. Approximately one-third of DPN patients and 20% of all individuals with diabetes experience symptoms like burning, tingling, shooting pain or stabbing sensations[3].

According to studies, Reactive Oxygen Species ()also commonly known as ROS have a notable responsibility in the onset and enduring nature of neuropathic pain. Because of its high lipid content, rapid rate of oxidative metabolic activity, strong creation of reactive oxygen metabolites and nonreplicating nature, neural tissue is particularly vulnerable to ROS damage[4]. Numerous ideas support hyperglycemia-induced nerve degeneration, which leads to diabetic neuropathy. Examples of these include the Aldol reductase pathway, heightened advanced glycation end-product pathway, increased oxidative-nitrosative stress, overactivity of PARP, activation of PKC, augmentation of the hexosamine pathway, MAPK activation and inflammatory harm. Many of these proofs point to oxidative stress as an attribute shared by all of these pathways. Inhibitors targeting these particular pathways are additionally known to reduce ROS and improve oxidative stress[5] which is considered a notable contributor to the emergence of neural and vascular issues. This is because when ROS are generated, they reduce the effectiveness of antioxidant defences like SOD, catalase and GSH. As a result, the cells and tissues affected become more vulnerable to oxidative harm. In individuals with diabetes, elevated blood sugar levels and oxidative stress lead to increased lipid peroxidation, which may accelerate the formation of advanced lipoxidation end products[6]. This process is thought to be a key contributor to the progression of neurovascular complications in diabetes[7,8]. Antioxidants, as a rule, endeavour to diminish the detrimental impact of free radicals by either inhibiting their formation, scavenging and deactivating them, or bolstering the body's innate protective mechanisms through the activation of antioxidant enzymes and the regeneration of other proteins involved in the antioxidant processes[9].

Polyphenols have recently been identified as possible neuroprotective agents in diabetes. Polyphenols, a diverse and extensive collection of phytochemicals containing phenol rings, are multi-target agents with the highest antioxidant and anti-inflammatory effects, with the potential to treat numerous illnesses such as diabetes and its consequences. Salicin is one such kind. Salicin is derived from the Latin word meaning willow, Salix. Salicin, a white bitter-tasting powder is derived from water extraction of willow bark and leaves. Salicin is a natural substance derived from numerous Salix (willow, bark, and leaves) and Populus (poplar) species, as well as *Gaultheria procumbens* (wintergreen) and *Betula lenta* (sweet birch) species, the volatile oils of which are virtually exclusively composed of methyl salicylate. Salicin which was discovered in 1828, is a phenolic glycoside that is chemically salicyl alcohol β-d-glucoside and is widely recognized as a B-glucosidase substrate. It is termed a natural aspirin since it is a prodrug and consequently a precursor of salicylic acid[10]. Salix bark is a good source of phenolic glycosides and polyphenols, such as flavonoids and condensed tannins. This compound has been shown to have antirheumatic, antipyretic, anti-inflammatory, hypoglycemic, antibacterial and antioxidant properties[11]. Considering the biological and physicochemical properties of Salicin, an attempt has been undertaken to assess its protective impact on diabetic peripheral neuropathy[12].

2. Materials and Methods

2.1 Materials

Salicin was purchased from Research Lab Fine Chem Industries, Gabapentin was procured from Intas Pharmaceuticals and Streptozotocin was procured from SRL. The other solvents and compounds used were all of analytical purity.

2.2 Experimental Animals

Male Wistar albino rats between the age of 6-8 weeks were obtained from LACSMI Bio Farms (Laboratory Animal Centre for Safe Medical Innovations) situated at #12, "Rachana Blossom", Jagdishnagar, Aundh, Pune – 411007, Maharashtra. These rats were registered under CCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals) with Registration Number 1344/PO/RE/S/10/CCSEA. They were sheltered in the animal facility at MET BKC Institute of Pharmacy, where they were placed in cages made from polypropylene under specified laboratory conditions, covering a temperature of 25 ± 2 °C, also a 12-hour light-dark cycle entailing a relative humidity of 55 ± 5%. The rats had unrestricted availability to food and water. The study strictly adhered to the procedures permitted by the ethics committee of MET BKC Institute of Pharmacy (ethical approval no. MET-IOP-IAEC/2022-23/04) and was executed in full compliance with the regulations established by CPSCEA (Committee for the Purpose of Control and Supervision on Experiments on Animals).

2.3 Induction of Diabetes

16 hours before prior induction of diabetes, the rats were not allowed food although they had access to drinking water. Diabetes was developed in rats by first administering Nicotinamide (NA) (100mg/kg i.p.), following a 15-minute duration. Streptozotocin (STZ) (50mg/kg i.p.) mixed in citrate buffer (0.1M)having a pH of 4.5 was injected to ensure Diabetes. Blood glucose levels were checked after 24, 48 and 72 hours. Rats were restrained and their tails were washed with a warm soap solution and cloth. Under light isoflurane anaesthesia, blood was obtained from the tail vein. Rats exhibiting a glucose level equal to 250mg/dl or more were chosen for the study. A glucometer (Accusure) was used to monitor blood sugar levels to confirm hyperglycemia[13].

2.4 Experimental Design

Following a quarantine period lasting two weeks, male Wistar albino rats were segmented into six sets, each comprising six rats. The experimental design below has been followed for the active phytoconstituent selected for the study. Adult Wistar albino rats weighing 150-200g were used as an experimental model.

Group I: The normal control group received oral administration of distilled water for a duration of 28 days.

Group II: The disease control group was administered with STZ (50mg/kg i.p.) and NA (100mg/kg i.p.) once.

Group III: The standard group was administered with STZ and NA once and after 72 hours with Gabapentin (300mg/kg p.o.) for 28 days.

Group IV: The test group was administered with STZ and NA once and after 72 hours with Salicin (10mg/kg p.o.) for 28 days.

Group V: The test group was administered with STZ and NA once and after 72 hours with Salicin (15mg/kg p.o.) for 28 days.

Group VI: The test group was administered with STZ and NA once and after 72 hours with Salicin (20mg/kg p.o.) for 28 days.

All groups, excluding the normal control group, were given Streptozotocin and Nicotinamide.

2.5 Behavioral Studies

2.5.1 Hot Plate Test (Heat Hyperalgesia)

On the heated surface of the hot plate, it is believed that the heightened sensitivity to pain is influenced by both cerebral and peripheral mechanisms. In this particular test, each animal was positioned on an Eddy's hot plate, where temperature was precisely maintained at a value of 55±1 °C. A 20-second limit was established to prevent any potential harm to the animals' paws. The precise time it took for the very first observable sign of either licking the paw or jumping in reaction to the heat was measured and utilised as an indicator for the threshold of pain[13].

2.5.2 Cold Plate Test (Cold Allodynia)

This is one of the simplest assays for determining behavioural responses in mice and rats to both painful and benign cold temperatures. The test involving the cold plate, like the hot plate test, can provide a variety of endpoints. Rats were placed on a chilled stainless-steel plate held at 4^0C for this test and the latency(s) to the initial lifting/shaking of the paw/avoidance was assessed. To avoid tissue injury, a cutoff time of 180 seconds was set[14,15].

2.5.3 Rota Rod Test (Motor Coordination)

This test was carried out utilising a Rota-rod instrument consisting of a rod made of metal having a diameter of 3cm rubber-coated to a motor at a speed of two rotations/minute. The length of the rod was 75cm with 5 compartments. The rod was set above the table at a distance of 50cm to prevent the rats from jumping from the rod. The rats were kept rotating on the rotating spindle at a pace of 25rpm. During 5 minutes, the time it took for each rat to fall off the rotating spindle as well as the number of free rides were recorded[15,16].

2.5.4 Open Field Test (Locomotor Activity)

To assess the motor activity of animals in an unfamiliar environment, a well-lit square enclosure measuring 50cm by 50cm by 50cm was utilised, with a 60-watt bulb illuminating the area. The base of the enclosure was partitioned into 16 even-sized squares, each measuring 12.5cm by 12.5cm. The rat was gently kept in the middle of this open field and the number of squares it traversed with all four paws for 5 minutes, indicating its locomotor activity was recorded. Furthermore, we observed and counted the instances of both assisted and self-assisted rearing on the hind feet as a measure of exploratory activity. After each test, the equipment's floor and walls were meticulously cleaned and dried[13].

2.5.5 Von Frey Hair Test (Mechanical Hyperalgesia)

Single rats were positioned on a raised maze inside an acrylic enclosure and exposed to a testing environment for a minimum of 15 minutes. Different-strength filaments (Von Frey hairs) were nudged to the bottom of their left hind paw through the mesh floor. A sufficient amount of filament force was applied to the paw (producing mild bending) and held for a few seconds. The application was repeated 5 times at 4-5 second intervals. The withdrawal of a paw was regarded as a favourable response[13-17].

2.6 *In Vivo* Assessment of Antioxidant Activity

2.6.1 Tissue Homogenate Preparation

The sciatic nerve was separated and promptly placed into an ice-cold Tris HCl buffer with a pH of 7.4 after the rats were sacrificed. Following that, the tissues were finely minced and blended in a Tris HCl buffer solution having 10% w/v concentration. Next, a high-speed cooling centrifuge, specifically the Remi C-24, was employed to spin at 10,000 revolutions per minute for a length of 15 minutes, sustaining a temperature of 4 degrees Celsius. The clear supernatant obtained from this process was employed for the assessment of reduced glutathione, lipid peroxidation and catalase activity[18].

2.6.2 Estimation of Reduced Glutathione (RGSH)

1 ml of a post-mitochondrial supernatant which was 10% in concentration, was combined with an equal volume of 4% sulpho-salicylic acid. This blend was then refrigerated at 4°C for a minimum of one hour before being exposed to centrifugation at 1200 x g for 15 minutes, also at a temperature of 4°C. The entire mixture used in the assay comprised 3ml in total, which included 0.1ml of the liquid above the sediment, 2.7ml of a phosphate buffer (0.1M) at pH 7.4 and 0.2ml of DTNB (Ellman's reagent, 100 mM) at pH 8.0. A yellow colour developed and its absorbance was promptly assessed at 412nm using a UV spectrophotometer[19].

Formula:

$$\text{Units/ml} = \frac{\text{Asample-Ablank)*(dilution factor)}}{\text{EmM*(Volume of sample in ml)}}$$

Dilution factor = 1

For TNB E^{mM}= 14.15 $mM^{-1}cm^{-1}$

Volume of sample in ml = 3

2.6.3 Estimation of Malonaldehyde (MDA) Activity

The assessment involved the examination of Thiobarbituric Acid Reactive Substances (TBARS). To perform this test, 0.5 mL of post-mitochondrial supernatant was mixed with 0.5 mL of Tris-HCl and left to incubate at 37°C for a period of 2 hours. Following this incubation period, 1 mL of a 10% trichloroacetic acid solution was included in the mixture and then subjected to centrifugation at 1000 x g for 10 minutes. Next, 1 mL of the resulting liquid above the solution was combined with an equal volume of 0.67% thiobarbituric acid, and the test tubes containing this mixture were immersed in heated water for 10 minutes. After its temperature was lowered, double distilled water (1 mL) was introduced, and the absorbance at 532nm was assessed using a UV spectrophotometer.

The quantification of TBARS was determined with the help of an extinction coefficient of 1.56 x 105 $M^{-1}cm^{-1}$, and the results were articulated in terms of nanomoles of malondialdehyde (MDA) equivalent per milligram of protein[20].

Formula:

$$\text{MDA conc}^{n}\text{(M)} = \frac{\text{Absorbance of sample}}{1.56\times10^{5}\text{cm}\times\text{path length}}\times\text{Dilution factor}$$

Dilution factor = 10^6 x 6.7

Path length = 1

2.6.4 Estimation of Catalase (CAT) Activity

In a volume of 0.1ml from the liquid above the sediment, 1.9 ml of phosphate buffer with a concentration of 50mm at a pH of 7.0 was introduced. Subsequently, 30mm H_2O_2 was added at a rate of units per 1.0ml, using a freshly prepared solution. The total volume of the resulting mixture reached 3ml. Using a UV spectrophotometer, alterations in absorbance were assessed at a wavelength of 240nm. Catalase activity was quantified as milligrams of protein per unit of measurement, as described in references[21].

Formula:

$$\text{CAT activity} = \frac{\text{Absorbance of solution}}{0.0494\,\frac{l}{\text{cm}}\times\text{Dilution Factor}}$$

Dilution Factor = 1

2.7 Histopathological Study of the Sciatic Nerve

After shaving the left thigh, the region was prepared for an operation. A cut was made in the skin and the gluteal muscle was divided to reveal the sciatic nerve. The nerve was then separated and placed in 10% formalin (fixation solution). Histopathological analysis was performed at the Biotox Laboratory in Nashik. Each group's sciatic nerve samples underwent conventional processing and were then embedded in paraffin. Thin sections, measuring 3-5 µ in thickness were then prepared and subjected to staining using the hematoxylin-eosin method. Following this, the cross-sectional slices were observed for signs of axonal degeneration and vascular irregularities with the help of a light microscope at a magnification value of 100×. The histopathological evaluation of all the organs was conducted by a toxicopathologist certified by the board[22].

2.8 Statistical Analysis

Values are exhibited as Mean ± S.E.M. (standard error of the mean) with a sample size of 6 in each group (n = 6). Statistical analysis was undertaken using One-way ANOVA (analysis of variance). Subsequently, Dunnett's test was conducted; a vs Disease Control; b vs Normal Control. *P<0.05, #P<0.01, +P<0.0001, SL: Salicin.

3. Results

3.1 Streptozotocin (STZ) Induced Diabetic Neuropathy

3.1.1 Blood Glucose Level Monitoring by Glucometer (Accusure)

After 72 hours of administering STZ, it was observed that blood glucose levels had significantly risen (*P<0.05) in every single group given the treatment when correlated to the group that served as the Normal Control group. However, the Gabapentin (300mg/kg) and Salicin (at quantities of 10, 15 and 20mg/kg) all given orally, treatment groups exhibited a remarkable anti-hyperglycemic effect (+P< 0.0001) in correlation to the Disease Control rats (Figure 1).

3.2 Body Weight:

All the groups experienced a rise in their final weights compared to their initial weights, except for the disease control group, which resulted in a decrease in the final weight relative to their initial weight (Figure 2).

3.3 Behavioral Studies

3.3.1 Hot Plate Test (Heat Hyperalgesia)

3.3.1.1 Paw Lick Latency (Sec)

Diabetic rats exhibited heat hyperalgesia starting from the first week after diabetes induction. This hyperalgesia was characterised by a notable decrease in paw lick latency (in seconds) in correlation to the Normal Control rats. However, diabetic rats receiving Gabapentin (300mg/kg) and Salicin (20mg/kg) both given orally, treatments displayed substantial and statistically significant improvements (P<0.01-0.0001)

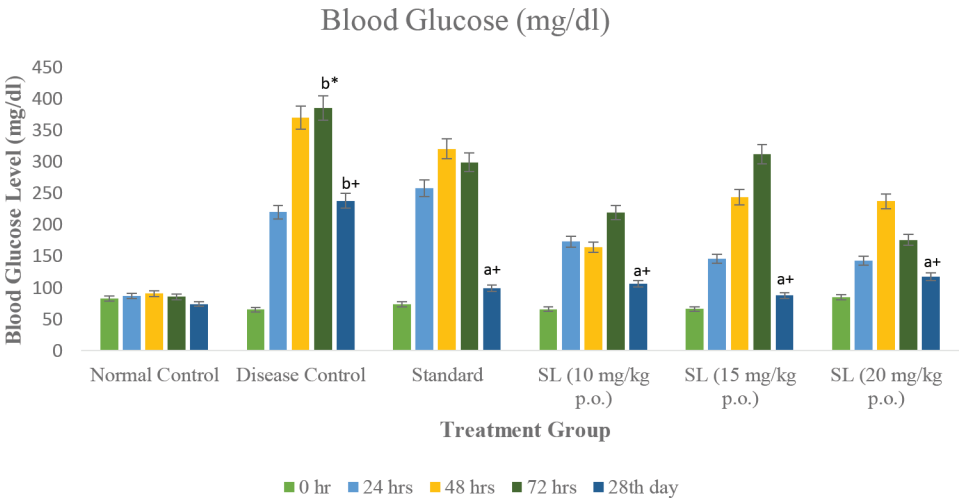

Figure 1. Effect of Salicin on blood glucose level of STZ induced diabetic neuropathy model of rats.

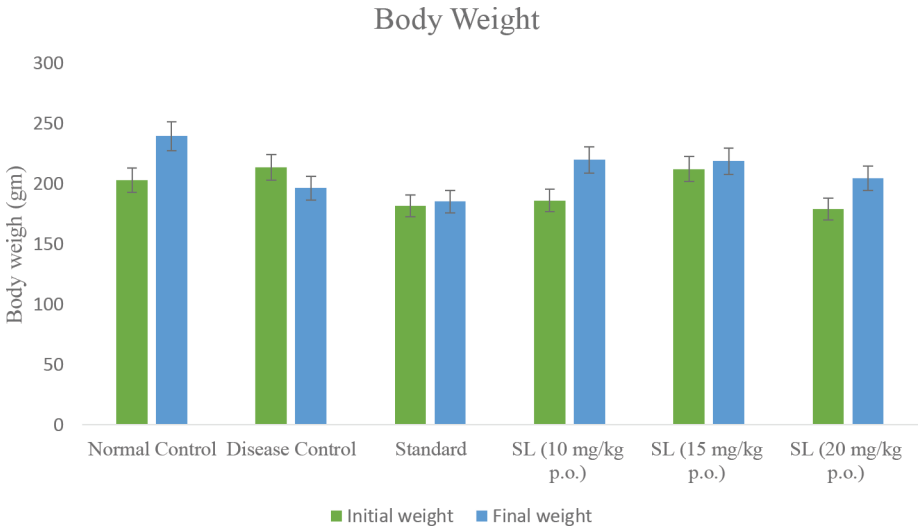

Figure 2. Effect of Salicin on the body weight of STZ-induced diabetic neuropathy model of rats.

in paw lick latency by the third week of the treatment regimen when correlated to the Disease Control rats (Figure 3).

3.3.1.2 Jump Response (Sec)

Heat hyperalgesia became evident in diabetic rats during the second week following diabetes induction. This hyperalgesia response was characterised by a substantial reduction (P<0.0001) in jump response latency (in seconds) when compared to the rats belonging to the Normal Control group. Diabetic rats that were administered Gabapentin (300mg/kg) and Salicin (20mg/kg) treatments both given orally, demonstrated a notable and statistically significant (P<0.0001) improvement in jump response latency (in seconds) by the fourth week of the treatment regimen in comparison to the Disease Control rats (Figure 4).

3.3.2 Cold Plate Test (Cold Allodynia)

In the second week following diabetes induction, diabetic rats exhibited signs of cold allodynia. This condition was characterised by a reduction in paw withdrawal latency (in seconds) or avoidance response when compared to the rats belonging to the Normal Control groups and this reduction was statistically significant (P<0.0001). In contrast, Disease Control rats treated with Gabapentin (300mg/kg) and Salicin (15 and 20mg/kg) both given orally, experienced notable and statistically significant improvements (P<0.01-0.0001) in paw withdrawal latency (in seconds) by the

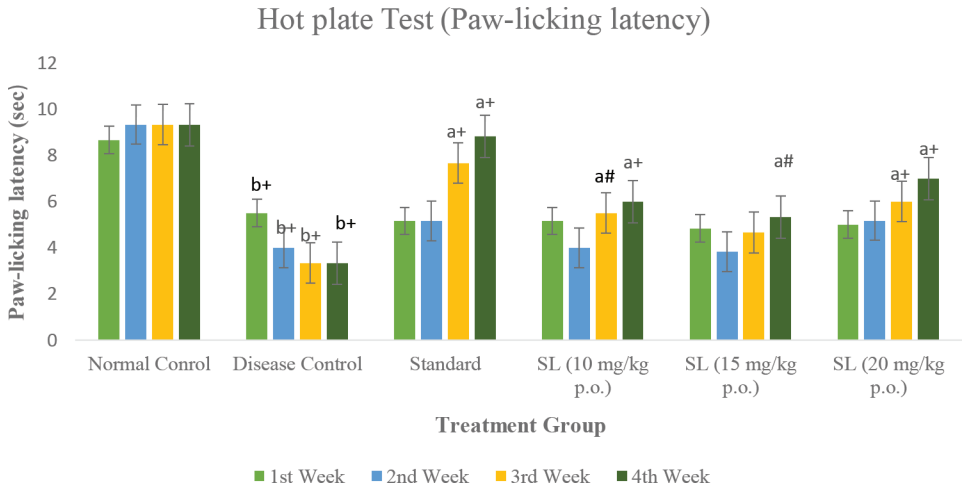

Figure 3. Effect of Salicin on heat hyperalgesia assessed by assessing paw lick response latency (sec) on a hot plate in STZ-induced diabetic neuropathy model of rats.

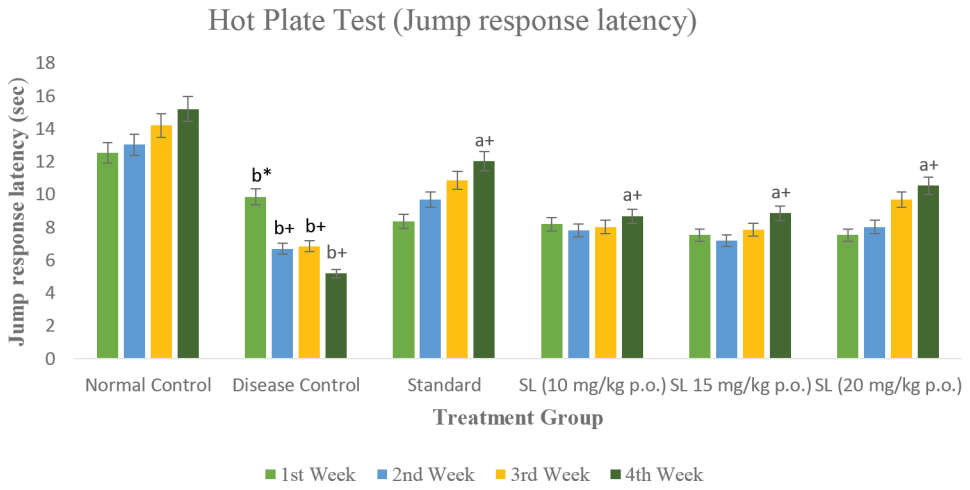

Figure 4. Effect of Salicinon heat hyperalgesia assessed by assessing jump response latency (sec) on a hot plate in STZ-induced diabetic neuropathy model of rats.

third week of their treatment regimen when correlated to the Diabetic Control rats (Figure 5).

3.3.2 Rota Rod Test (Motor Coordination)

3.3.2.1 Fall Off Time (Sec)

The diabetic rats displayed motor incoordination, which was evident through a significant decrease in falling latency when assessed using the Rota-rod apparatus (P<0.01-0.0001). However, diabetic rats receiving Gabapentin (300mg/kg) and Salicin treatment, both given orally, showed a non-significant improvement in motor coordination, reflected by an increase in falling latency (in seconds), when compared to that of the Disease Control rats after the third week of the treatment regimen (Figure 6).

3.1.1.2 Number of Free Rides

In diabetic rats, motor coordination issues were evident, as evidenced by a notable reduction (P<0.01) in the number of successful rides on the Rota-rod. However, diabetic rats treated with Gabapentin (300mg/kg) and Salicin both given orally, displayed a non-significant improvement in motor coordination, demonstrated by an increase in the count of successful rides when correlated to that of the Disease Control rats after the third week of the treatment schedule (Figure 7).

3.3.4 Open Field Test (Locomotor Activity)

3.3.4.1 Number of Square Crossings

The diabetic rats exhibited a decrease in locomotor and exploratory activity, as evidenced by a substantial

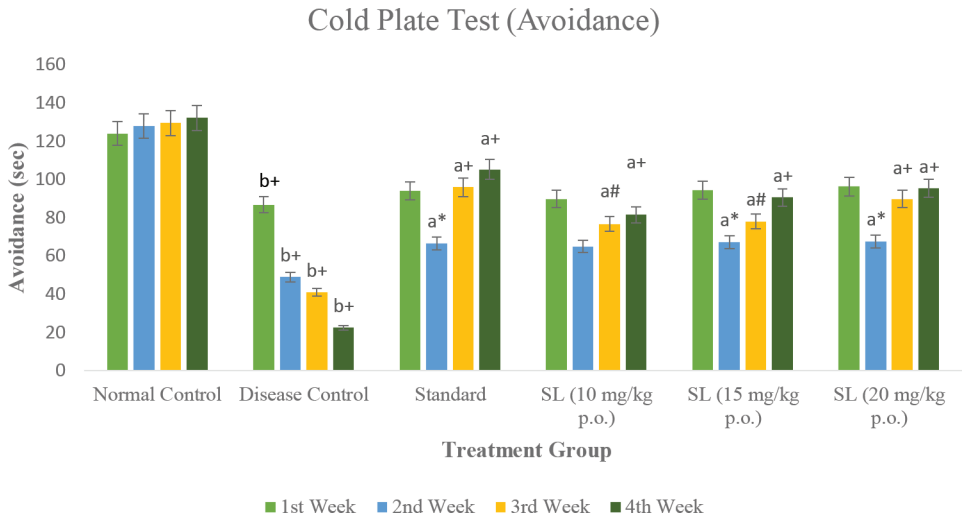

Figure 5. Effect of Salicin on cold allodynia assessed by the cold plate test in STZ-induced diabetic neuropathy model of rats.

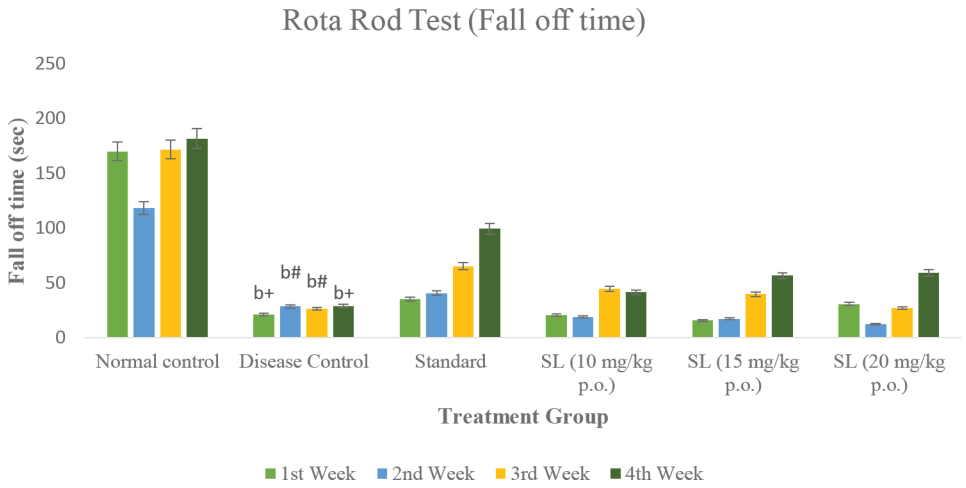

Figure 6. Effect of Salicin on motor coordination assessed by assessing fall off time (sec) on Rota-rod in STZ-induced diabetic neuropathy model of rats.

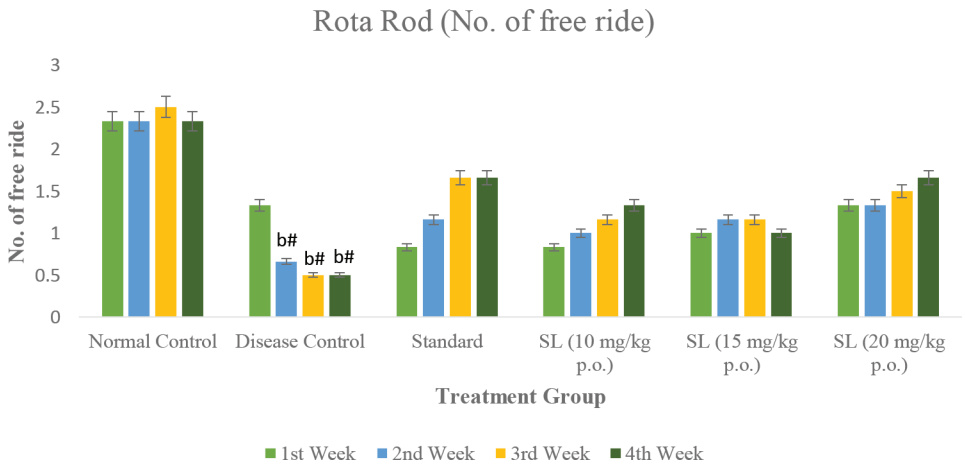

Figure 7. Effect of Salicin on motor coordination assessed by assessing number of free rides on Rota-rod in STZ-induced diabetic neuropathy model of rats.

decrease (P<0.0001) in the number of lines crossed in the open field apparatus. However, treatment with Gabapentin (300mg/kg) and Salicin (at quantities of 10, 15, and 20 mg/kg) both given orally led to a distinguished improvement (P<0.01-0.0001) in locomotor activity, as indicated by an increase in the count of squares crossed, when compared to that of the Disease Control rats after the third week of the treatment regimen (Figure 8).

3.1.1.2 Number of Assisted Rearing

The diabetic rats exhibited a decline in locomotor and exploratory activity, which was confirmed by a substantial reduction (P<0.05-0.0001) in assisted rearing during the second week. However, administration of Gabapentin (300mg/kg) and Salicin (20mg/kg) both given orally, led to a significant improvement (P<0.05-0.01) in locomotor activity, as demonstrated by an increase in the count of assisted rearing of the rats after the third week of the treatment regimen when correlated to the Diabetic Control rats (Figure 9).

3.3.4.3 Number of Self-Assisted Rearing

The diabetic rats displayed a decline in locomotor and exploratory activity, which was evident through a significant reduction (*P<0.01-0.0001) in self-assisted rearing. However, administration of Gabapentin (300mg/kg) and Salicin (20mg/kg) both given orally, resulted in a noteworthy improvement in locomotor

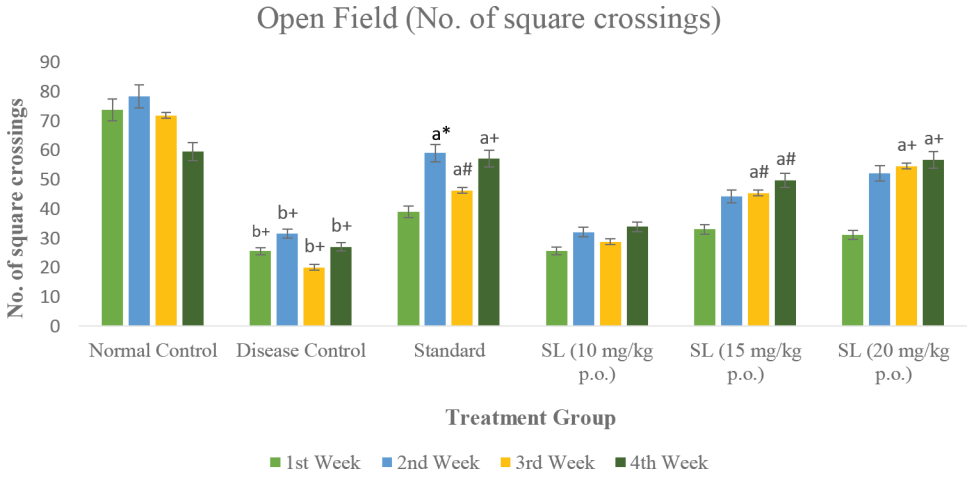

Figure 8. Effect of Salicin on locomotor activity assessed by assessing several square crossings in an open field maze in STZ-induced diabetic neuropathy model of rats.

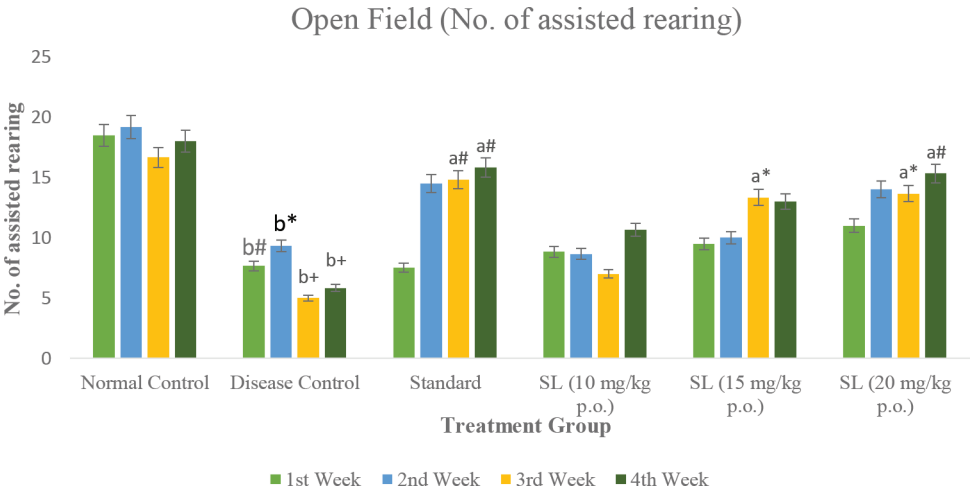

Figure 9. Effect of Salicin on locomotor activity assessed by assessing number of assisted rearing in open field maze in STZ-induced diabetic neuropathy model of rats.

activity, demonstrated by an increase in the number of self-assisted rearing by the rats after the third week of the treatment regimen when compared to the Disease Control rats (Figure 10).

3.3.5 Von Frey Hair Test (Mechanical Hyperalgesia)

In the Disease Control rats, tactile mechanical hyperalgesia became evident during the second week following the induction of the disease. This hyperalgesia was characterised by a decrease in the paw withdrawal threshold when correlated to that of the Normal Control rats, and this difference was statistically significant (P<0.0001). However, the diseased rats that received treatment with Gabapentin (300mg/kg) and Salicin (20mg/kg) both given orally, showed a notable improvement in paw withdrawal threshold, which was statistically significant in the range (P<0.05-0.01), after the fourth week of the treatment regimen when correlated to the Disease Control rats (Figure 11).

3.4 *In Vivo* Antioxidant Assay

3.4.1 Estimation of Reduced Glutathione (RGSH)

RGSH serves as the primary cellular antioxidant. Results displayed a highly substantial (P<0.0001) reduction in RGSH concentration noticed in the group which served as the Disease Control when correlated to the Normal Control group. However, treatment with Gabapentin (at a dose of 300mg/kg) and Salicin

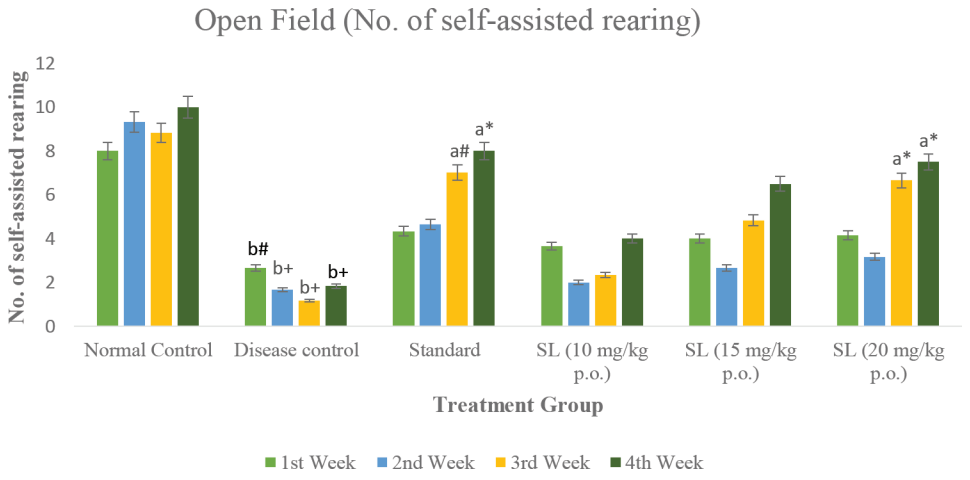

Figure 10. Effect of Salicin on locomotor activity assessed by assessing number of self-assisted rearing in open field maze in STZ-induced diabetic neuropathy model of rats.

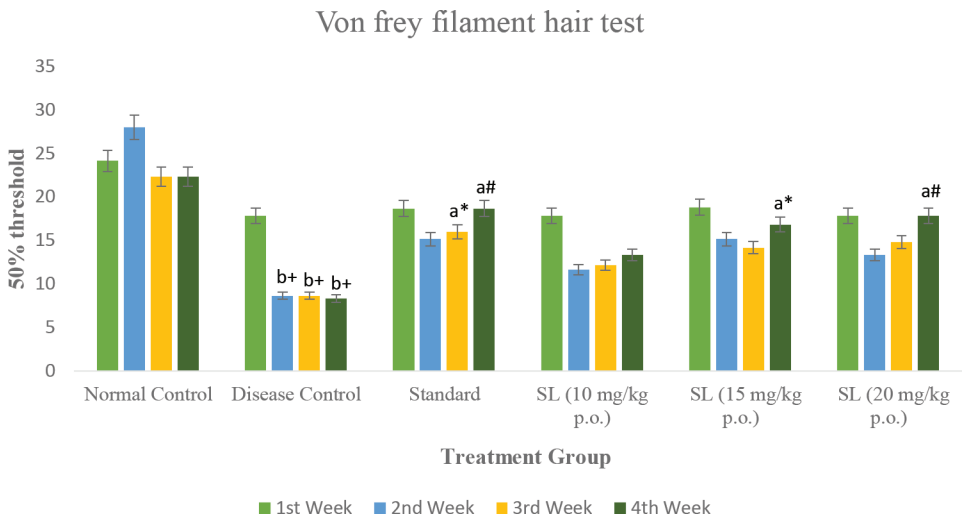

Figure 11. Effect of Salicin on mechanical hyperalgesia by the Von Frey hair test in STZ-induced diabetic neuropathy model of rats.

(at quantities of 10, 15, and 20mg/kg) both drugs given orally, resulted in a substantial increase (P<0.0001) in RGSH concentration when correlated to that of the Disease Control group (Figure 12).

3.4.2 Estimation of Malonaldehyde (MDA) Activity

A highly significant (P<0.0001) rise in lipid peroxidation levels was noted in the Disease Control group in correlation to the group that served as the Normal Control. Conversely, the administration of Gabapentin (at a dose of 300mg/kg) and Salicin (at quantities of 10, 15, and 20mg/kg) both drugs given orally, led to a substantial reduction (P<0.0001) in lipid peroxidation when correlated to the Disease Control group (Figure 13).

3.4.3 Estimation of Catalase (CAT) Activity

In the disease-control animals, CAT levels were found to be lower compared to those in the Normal Control animals. Results displayed a highly significant (P<0.0001) reduction in catalase concentration noted in the Disease Control group when correlated to the group that served as the Normal Control. However, treatment with Gabapentin (at a dose of 300mg/kg)

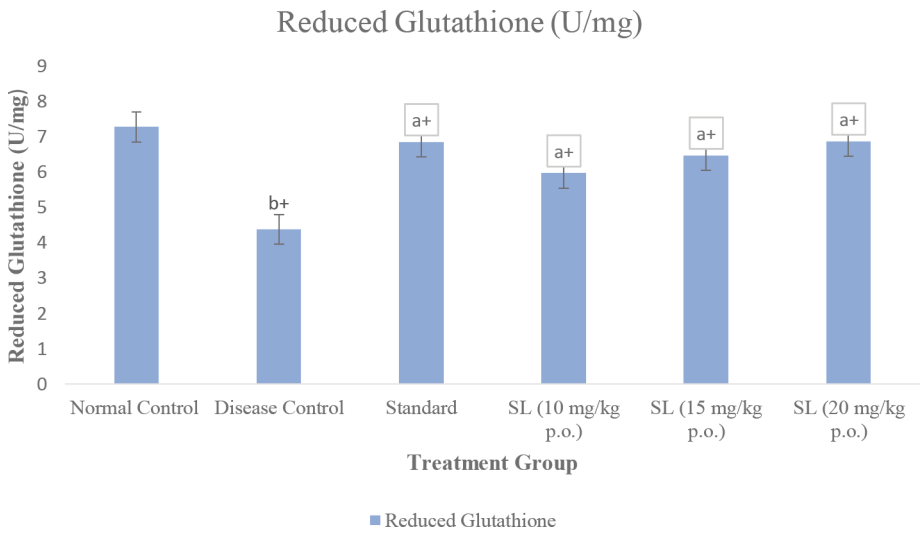

Figure 12. Effect of Salicin on reduced glutathione in sciatic nerve tissue homogenate of STZ-induced diabetic neuropathy model of rats.

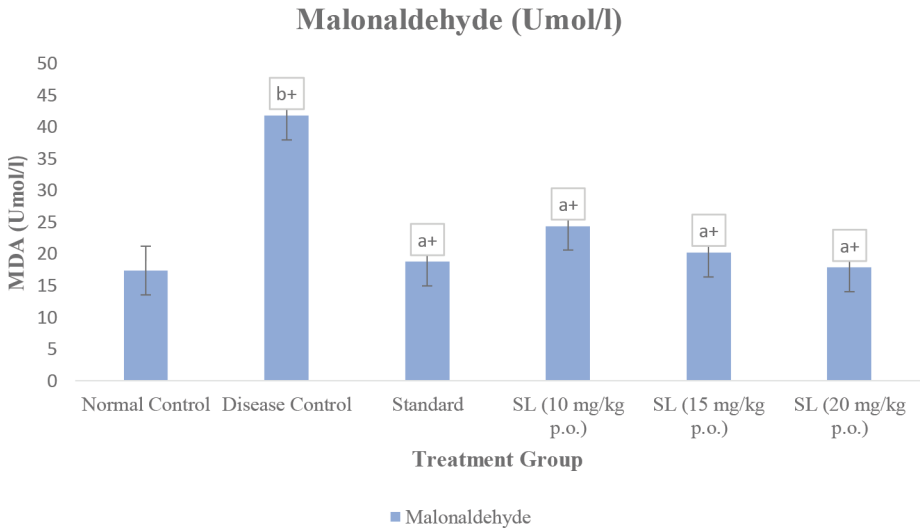

Figure 13. Effect of Salicin on lipid peroxidation/ malonaldehyde in sciatic nerve tissue homogenate of STZ-induced diabetic neuropathy model of rats.

and Salicin (at quantities ranging from 10 to 20mg/kg) both drugs given orally, showed a substantial increase (P<0.01-0.0001) in catalase concentration when correlated to that of the Disease Control group (Figure 14).

3.5 Histopathological Study of Sciatic Nerve

In histopathological examinations, it was observed that when examining microscopic samples of the sciatic nerve from the Normal Control group, everything appeared normal, including neuronal fibres, axons and nuclei. Conversely, in the Disease Control group, the sciatic nerve exhibited signs of neuronal degeneration, necrosis, vacuolation and inflammation when compared to the rats in the Normal Control group. However, it is noteworthy that the incidence and severity of these lesions, as recorded in the Disease Control group, decreased in a dose-dependent manner in the Salicin treatment groups (at quantities of 10, 15, and 20 mg/kg p.o.) when correlated to that of the Disease Control group, implying that these changes were potentially reversible.

Based on these histopathological findings, it can be concluded that animals treated with the test compound, specifically Gabapentin (300mg/kg) and Salicin (20mg/kg) both drugs given orally, exhibited an ameliorative effect and demonstrated significant protective effects on nerve tissue (Figure 15).

4. Discussion

The root cause of diabetic complications is primarily attributed to hyperglycemia. Diabetes encompasses a diverse range of conditions characterised by varying pathologies, with peripheral neuropathy being one of its common complications. Elevated levels of free radicals are typically observed in diabetes and recent findings highlight the significant role of hyperglycemia-triggered mitochondrial ROS production in driving the advancement of diabetic complications. This oxidative stress adversely affects proteins, cellular lipids and DNA directly[23].

Several animal models have been created to comprehend how diabetes develops. Many of these models have been utilised to test drugs that can treat diabetes. However, these models have been able to accurately replicate the intricate disease processes of human diabetes. When rats are given nicotinamide before STZ, it triggers diabetes in them along with consistent metabolic changes and decreased pancreatic insulin levels. In this current research, we employed the STZ/nicotinamide diabetic rat model to explore how the test compound can protect against diabetes-induced neuropathy by assessing its behavioural, biochemical and histopathological parameters[24].

In the current investigation, a substantial rise in blood glucose level (250mg/dl≥) was detected in all groups after 72 hours of STZ injection (50mg/kg) given

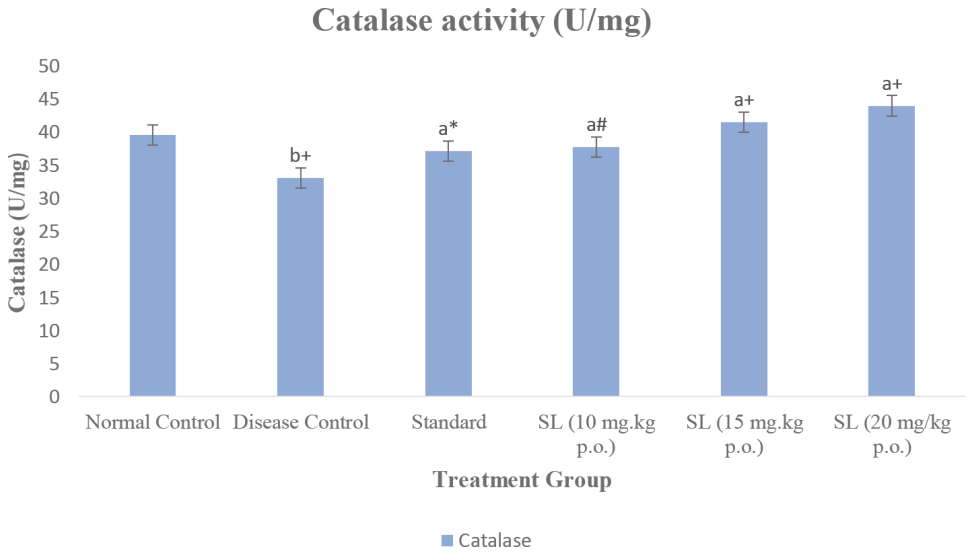

Figure 14. Effect of Salicin on catalase in sciatic nerve tissue homogenate of STZ-induced diabetic neuropathy model of rats.

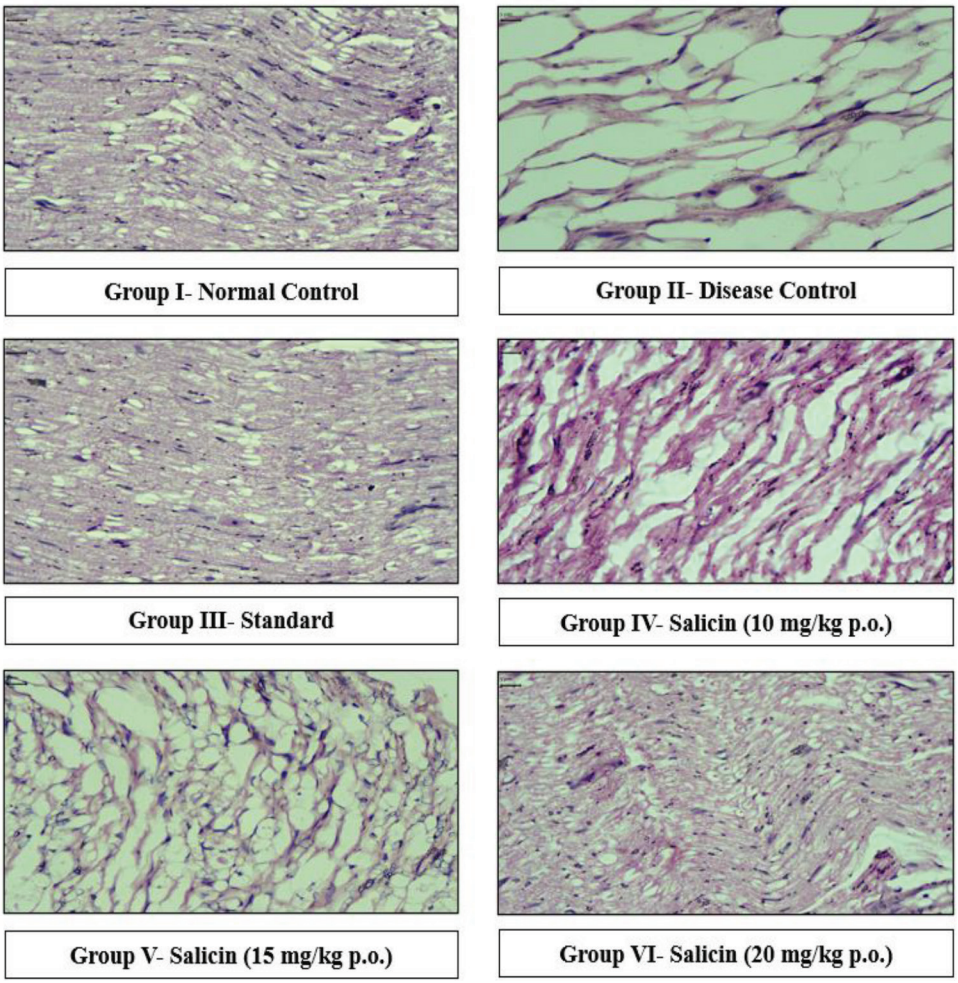

Figure 15. Effect of Salicin on histology of sciatic nerve of STZ- induced diabetic neuropathy model of rats.

intraperitonealy. Diabetic rats treated with Gabapentin (300mg/kg p.o.) and Salicin (10, 15 and 20mg/kg p.o.) exhibited a substantial drop in the blood sugar level as compared to the Disease Control rats after the fourth week, confirming Salicin's antihyperglycemic effect. After the second week of diabetes induction, peripheral nerve damage and behavioural and biochemical alterations indicating the start of neuropathy were detected in experimental rats.

While identifying "pain" is difficult due to its subjective nature in research models, there are several behavioural assessments like the hot-plate and Von Frey hair tests that enable consistent, quick and precise measurement of thermal and mechanical hyperalgesia in rats. Various parameters like paw licking and jump response were measured by hot plate test and a 50% paw withdrawal threshold was measured by von Frey hair test[25]. The current research revealed that rats with diabetes induced by STZ exhibited significant increases in sensitivity to both heat and mechanical stimuli. It has been recorded that heat hyperalgesia is linked to C-fibers, while mechanical hyperalgesia is associated with A-fibers. However, the administration of salicin dose-dependently can reverse this condition[25]. Apart from experiencing increased sensitivity to heat and mechanical stimuli, other indicators of neuropathic pain emerged during the second week following STZ induction. These included heightened sensitivity to cold temperatures, difficulties with motor coordination and a decrease in locomotor activity. However, these symptoms were alleviated when Salicin was administered as a treatment.

The main contributor to the onset of diabetes mellitus and its associated complications is the excessive presence of oxidative stress. This causes an elevated production of ROS and, as a result, damages

cellular lipids. The degree of lipid peroxidation serves as an indicator of the damage caused to cells by these ROS. In rats with diabetes induced by streptozotocin, there was an observed increase in lipid peroxidation, suggesting a rise in the generation of harmful free radicals. The majority of tissue damage occurred as these free radicals attacked cell membranes through the peroxidation of unsaturated fatty acids.

In our investigation using rats with induced diabetes, we observed a notable increase in MDA levels which serve as an indicator of lipid peroxidation, along with a decline in the performance of the antioxidant enzymes (Figure 13). Glutathione (GSH) is the most prevalent soluble antioxidant and is found in all cell compartments. The balance between reduced and oxidized GSH serves as a valuable indicator of oxidative stress (Figure 14). GSH plays a crucial role in acting as an antioxidant through multiple mechanisms. GSH-Peroxidase, for instance, helps in the detoxification of hydrogen peroxide and lipid peroxides[26], while catalase, being the most prevalent antioxidant enzyme with a rapid turnover rate, holds significance in living tissues. It plays a crucial role as a therapeutic enzyme in the transformation of hydrogen peroxide to molecular oxygen and water. Our investigation revealed that the treatment with Salicin led to a significant reduction in lipid peroxidation. Furthermore, after four weeks of Salicin treatment (10, 15 and 20mg/kg) given orally, we noticed a significant improvement in the functioning of the enzymes GSH and CAT in a manner that was dependent on the dosage[27].

In the histopathological examinations, it was noted that the microscopic analysis of sciatic nerve samples from the Normal Control group displayed the typical appearance of normal neuronal fibres, axons and nuclei. Conversely, the sciatic nerves from the Disease Control group exhibited signs of neuronal degeneration, necrosis, vacuolation and inflammation when compared to the rats in the Normal Control group (Figure 15). However, it is worth mentioning that the occurrence and severity of these lesions, as observed in the Disease Control group, decreased in a dose-dependent manner in the Salicin treatment groups (at quantities of 10, 15, and 20mg/kg orally) when correlated to that of the Disease Control group, indicating that these changes could potentially be reversed.

As a result, Salicin therapy has been shown to provide significant protection in cases of STZ-induced diabetic neuropathy. This effect is likely attributable to its antioxidant, antihyperglycemic and neuroprotective properties.

5. Conclusion

In the present study, Salicin has significantly reversed behavioural changes, antioxidant depletion, and histopathological studies. In conclusion, salicin therapy has demonstrated a notable protective effect against diabetic neuropathy generated by STZ, which may be due to its neuroprotective, antioxidant and antihyperglycemic properties.

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