Contents lists available at UGC-CARE

International Journal of Pharmaceutical Sciences and Drug Research

[ISSN: 0975-248X; CODEN (USA): IJPSPP]

Available online at www.ijpsdronline.com

Research Article

Study of 18β Glycyrrhetinic Acid for the Prevention of Progression of Diabetes Induced Neuropathy in Laboratory Animals

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ARTICLE INFO

Article history:

Received: 27 May, 2020

Revised: 13 February, 2021

Accepted: 24 February, 2021

Published: 30 March, 2021

Keywords:

Glycyrrhetinic acid,
Hyperalgesia,
Mitochondria,
Motor coordination exploratory,
Neuroprotective,
Nicotinamide,
STZ.

DOI:

10.25004/IJPSDR.2021.130201

ABSTRACT

Diabetes is a metabolic disease that is associated with oxidative stress and the dysfunction of mitochondria. Long-term diabetes may cause different associated complications. One of the most common complications of diabetes mellitus is neuropathy. The present study aimed to investigate the possible neuroprotective effects of 18β glycyrrhetinic acid (GA), the metabolite of glycyrrhizic acid, which is the main active component commonly used in traditional Chinese medicine on oxidative damage and the sciatic nerve of diabetes-induced neuropathy in laboratory animals. Diabetes was induced in male Wistar rats (200–260 g) by injecting a single dose of nicotinamide (110 mg/kg, intraperitoneally) and streptozotocin (STZ) (55 mg/kg, intraperitoneally). The rats were divided into six groups. Pregabalin (10 mg/kg, p.o.) and 18β GA (50, 100, and 200 mg/kg, p.o.) were administered daily after four weeks of nicotinamide-STZ injection to the rats of groups III to VI respectively for four weeks. Various behavioral (heat and mechanical hyperalgesia, allodynia, etc.), hemodynamic and biochemical parameters were investigated, and the histological examination of the sciatic nerve was carried out. In the present study, the 18β GA (100 and 200 mg/kg) significantly ($p < 0.01$, $p < 0.001$) increased in the tail withdrawal latency and increased in paw withdrawal threshold, and increased in systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure. Furthermore, 18β GA (100 and 200 mg/kg) significantly ($p < 0.01$, $p < 0.001$) restored altered blood glucose level, total protein, antioxidant status, and histological abnormalities. Pregabalin (10 mg/kg) showed a maximum protective effect, but a low dose of 18β GA (50 mg/kg) was not enough to show the protective activity in diabetic rats. In most of the tests, non-significant differences were observed between the control and 18β GA 200 mg/kg groups. Moreover, 18β GA lowers the inflammation and demyelination in sciatic nerves. Beyond its antioxidant role possessed neuroprotective effect via modulation of endogenous enzymes in nicotinamide-STZ induced diabetic in rats.

Introduction

Diabetes Mellitus (DM) is an endocrinological disorder. It is a group of metabolic or heterogeneous disorders resulting from the lack of insulin secretions and insulin actions or both. Absence or reduced insulin leads to an abnormal increase in blood sugar level than normal (fasting 80–100 mg/dL). It is a global health issue affecting children, adults, and adolescents.[1,2]

There appear to be more men in India than women with diabetes mellitus (DM), and about 50% of the diabetics live in towns and cities, but in the West, there are almost twice as many women as men with DM. King *et al*. observed that DM occurs at a younger age in developing countries. In the developed world, most diabetics are over 65 years, while in developing countries, the majority is in the age group of 45 to 64–another development of enormous public health implications. In a previous study by Kokiwar *et al*., it was found that there was a high prevalence of diabetes (3.67%) as compared to that in the WHO report (2.4%) for rural India.[3,4]

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Diabetes is not only an endocrine but also a vascular disease. Diabetes affects both large and small vessels and hence diabetic complications are broadly classified as microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular (heart disease, stroke, and peripheral arterial disease) complications. Evidence suggests that small vessel disease is important for heart disease, stroke, and neurodegenerative diseases such as dementia and Alzheimer's disease in patients with diabetes.[5]

Diabetic neuropathy is the symptoms of dysfunction of the peripheral nerve, which resembles pain. Changes in the blood vessels supplying the peripheral nerves underlie the mechanisms involved in microvascular damage and hypoxia. These disorders frequently affect lower extremity sensation and can cause lower-extremity pain in people with Lotfy *et al*., and Thaifa *et al*.[6,7]

The drugs derived from Natural plant are mostly considered to be less toxic and have very few side effects than synthetic drugs. Licorice (Glycyrrhiza glabra L.) and its main water-soluble constituent glycyrrhizin (GL) have been widely used plants as an antidote, demulcent, and as a folk medicine for generations most common in Asia and Europe. Glycyrrhizin is a pentacyclic triterpene derivative of the L-amyrin type (oleanane). Mostly it is used as a flavoring and sweetening agent in food products also. By taking it orally or intravenously, GL has been shown to be hydrolyzed by the glucuronidase in intestinal bacteria to its active principle aglycone. 18β-glycyrrhetinic acid (GA) is absorbed into the blood, which is a major metabolite of glycyrrhizin.[8]

The 18β-glycyrrhetinic acid is metabolized in the liver to 18β-glycyrrhetinic acid monoglucuronide, which is predominantly excreted in the feces, the urinary excretion of 18β-glycyrrhetinic acid monoglucuronide is considered less than 1% of the dose administered. Glycyrrhetinic acid is absorbed from the gut in a dose-independent fashion. It exhibits a fast initial elimination phase from plasma, which, for doses exceeding 500 mg, is followed by a clearly slower second elimination phase. However, in experimental animals, the half-life of the second elimination phase ranged from l to 2 hours only. Depending on the dose, the second elimination phase in humans has a half-life of 3.5 hours for glycyrrhizin and between 10 and 30 hours for glycyrrhetinic acid.[8,9]

GL and 18β-GA have been shown the several common pharmacological activities, which include an anti ulcerative effect,[9] anti-inflammatory activity,[10] direct and indirect antiviral activity[11], interferon inducibility,[12] and an anti-hepatitis effect.[13] In addition, 18β-GA can delay the development of autoimmune disease[14] and decrease body fat mass.[15] Recent studies indicate that GA enhanced glucose-stimulated insulin secretion and induced mRNA expression of insulin receptor substrate-2, pancreas duodenum homeobox-1, and glucokinase.[16]

Kalaiarasi *et al*. previously reported the antidiabetic and hypolipidemic effect of 18β -GA in STZ-diabetic rats. Based on clinical and experimental evidence, it suggests the involvement of free radical-mediated oxidative processes in the pathogenesis of diabetic complications. In the present study, we have determined the protective effect in neuropathy due to diabetes on the defense system against oxidative stress in STZ-diabetic rats and studied the influence of the treatment with 18β -GA lipid peroxidative markers and antioxidant system.[17,18]

Materials and Methods

Wistar rats with body weight ranging from 200–250 gm were procured from the National Institute of Biosciences, Pune (1091/abc/07/CPCSEA) and were maintained in an air-conditioned room (25±1°C) with 12 hours light/12 hours dark cycle. The animals had access to food pellets (by Nutrivet Pvt. Ltd., Pune, India) and water ad-libitum. The Institutional Animal Ethics Committee approved the experimental protocol (IAEC) constituted as per guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), at Sinhgad Institute of Pharmacy, Pune, India. The IAEC approval number is SIOP/IAEC/2017/02/02.

Drugs and Chemicals

Streptozotocin was purchased from Cayman Chemicals, USA. Pregabalin and nicotinamide were purchased from Fine-Chemicals, Mumbai, India. 18β glycyrrhetinic acid was purchased from Sigma-Aldrich chemical company the USA. Absolute alcohol (Changshu Yangyuan Chemicals, China) was purchased from the respective vendors. Urethane, citric acid, hydrochloric acid, sodium citrate, and sodium chloride of analytical grade were purchased from Fine-Chemicals, Mumbai, India.

Preparation of Drug Solution and Selection of Dose

Pregabalin was dissolved in distilled water, and 18β GA was dissolved in 5% dimethyl sulfoxide (DMSO). This study was carried out using three doses of 18β GA (i.e., 50, 100, and 200 mg/kg, p.o.) and one dose of pregabalin (i.e., 10 mg/kg, p.o.).

Experimental Induction of Diabetes

Male Wistar rats (200–250 g) were used for the study. Diabetes was induced by intraperitoneal (i.p.) injection of STZ (55 mg/kg) in the overnight fasted adult Wistar rats (200–250g). Nicotinamide (110 mg/kg) was administered i.p.15 minutes before injection of STZ. Animals were fed with glucose solution (5%) for 12 hours to avoid hypoglycemia. Hyperglycemia was confirmed after 3 days. Steady-state of hyperglycemia reached after 15 days. Blood glucose level was determined by the glucose-oxidase peroxidase method. Rats having serum glucose levels of more than 300 mg/dL were selected for the study. As well after 3 weeks, the 'heat hyperalgesia by hot water

tail immersion test, cold allodynia, thermal hyperalgesia, mechanical hyperalgesia, mechano-tactile allodynia, motor coordination and, exploratory motor activity has been performed to confirm the neuropathy in the Wistar rats. Water and feed were provided ad-libitum.[18,19]

Experiment Design

After three days the rats were randomly divided into the following six groups, each comprising six rats:

Group I: Normal control (vehicle 5% DMSO, p.o.)
Group II: Nicotinamide (110 mg/kg, i.p.) + STZ (55 mg/kg, i.p.) + 5% DMSO
Group III: Nicotinamide (110 mg/kg, i.p.) + STZ (55 mg/kg, i.p.) + standard drug pregabalin (10 mg/kg, p.o.)
Group IV: Nicotinamide (110 mg/kg, i.p.) + STZ (55 mg/kg, i.p.) + 18β GA (50 mg/kg, p.o.)
Group V: Nicotinamide (110 mg/kg, i.p.) + STZ (55 mg/kg, i.p.) + 18β GA (100 mg/kg, p.o.)
Group VI: Nicotinamide (110 mg/kg, i.p.) + STZ (55 mg/kg, i.p.) + 18β GA (200 mg/kg, p.o.)

18β GA and pregabalin were administered to the rats orally using an oral feeding needle after four weeks of nicotinamide-STZ induction, daily for a period of four consecutive weeks. The normal control and diabetic control (nicotinamide-STZ) rats received vehicle 5% DMSO. At the end of the study, period blood was collected from each rat by a retro-orbital puncture to measure blood glucose level and biochemical parameters.[20,21]

Estimation of Serum Glucose

Animals were anesthetized using anesthetic ether and blood was withdrawn from retro-orbital plexus from overnight fasted rats using a micro-capillary technique. The serum was obtained by centrifuging at 3000 rpm for 30 minutes and serum was pipette out 100 µL in a clean and dry test tube containing 1000 µL of glucose reagent, mixed well, and was incubated for 10 mats 37°C. The absorbance was measured for test and standard against blank.[22]

Estimation of HbA_1C[23]

The percent glycosylated hemoglobin is determined by measuring the absorbance of the glycosylated hemoglobin fraction (GHb) and total hemoglobin (THb) fraction the ratio of absorbance GHb and THb fraction of the control and the test were estimated using commercially available measurement kits (Delta Lab Pvt. Ltd., Mumbai, India).

Estimation of Body Weight

At the end of the study on the 56th day, body weight was measured by using an electronic weighing balance in gram (g).[24]

Estimation of Heat Hyperalgesia by Hot Water Tail Immersion Test

In the hot water tail immersion test, heat hyperalgesia was measured by immersing the terminal part of the tail (1 cm) in warm water (52.5 ± 0.5°C). The duration of tail withdrawal reflex (in sec.) was recorded to respond to thermal heat sensation. A Cut-off time of 15 seconds was maintained. Shortening of tail withdrawal time is an indication of thermal hyperalgesia.[25]

Estimation of Cold Allodynia by Using Cold Water Paw Withdrawal Latency

In cold allodynia, assessment of neuropathic pain was performed by immersing the left hind paw up to the ankle of rats in cold water maintained temperature at 4 ± 1°C. The ankle marked the paw of rat was submerged gently in cold water and the time required to withdraw of rat paw from cold water was recorded. Cut off time of 20 seconds was maintained.[25,26]

Estimation of Thermal Hyperalgesia by using Eddy's Hot Plate Method

The nociceptive threshold for heat was the index for thermal hyperalgesia—Eddy's hot plate, which is an instrument designed by Eddy and co-workers to assess thermal sensitivity. The plate was preheated and maintained at a temperature of 52.5 ± 2.0°C. The rats were placed on the hot plate, and the nociceptive threshold was recorded in seconds with respect to licking of the hind paw or jumping. The cut-off time of 15 seconds was maintained.[21,27]

Estimation of Mechanical Hyperalgesia by using Randall–Selitto Paw Pressure Test

The nociceptive threshold was determined by using the Randall-Sellito paw pressure apparatus (UGO Basile SRL Biological Research Apparatus, Italy) as per the method described by Chaplan *et al*., (1994). By increasing mechanical force (g) to the dorsum of the rat hind paw. The nociceptive threshold (expressed in g) was entitled by increasing pressure to the hind paw until squeak (vocalization threshold). The rat's paw was placed under the tip and the progressive pressure was applied until the rat vocalized. The nociceptive threshold was measured three or four times to obtain two consecutive values that differed more than 10% and respecting an interval of at least 10 min between two measures.[28,29]

Estimation of Mechano-tactile Allodynia by using Von-Frey Hair Test

Mechanical allodynia was determined by using Von-Frey hair. Wistar rats were placed individually on an elevated mesh in a clear plastic box and adapted to the testing environment for at least 15 minutes. Von-Frey hairs 9IITC, Woodland Hills, USA) with calibrated bending forces (g) of different intensities were used to deliver mechanical stimuli of varying intensity. Starting with the lowest filament force, Von-Frey hairs were applied from below the mesh floor to the plantar surface of the hind paw, with sufficient force to cause slight bending against the paw and hold for 1-second. The stimulation will apply five times

with and simultaneous interval of 4 to 5 seconds. Care was taken to stimulate random locations on the planter surface. A positive response was noted if the paw was robustly and immediately withdrawn.[29,30]

Estimation of Motor Coordination by using Rota-rod apparatus

For the measurement of motor coordination, Rota-rod was used. Briefly, rats were placed for 1-minute on the rotating rod (20–25 rpm). The time was taken for the falling from the roller of Rota-rod, during one minute period to be recorded.[30]

Estimation of Exploratory Motor Activity by using Actophotometer

Photoactometer test can be used to assess spontaneous motor activity. Each animal was observed for a period of 5 minutes (300 sec), a square closed field arena (30 × 30 × 30 cm) equipped with six photocells in the outer wall. Interruption of photocell beam (exploratory locomotor action) recorded by means of the digital counter.[31]

Histological examination of Sciatic Nerve

At the end of the 8th week, rats were sacrificed under deep anesthesia and sciatic nerves (Kumar *et al*., 2007; Volkan *et al*., 2017) were carefully removed. Isolated organs were kept in fixative solution (10%) formalin. It was then cut in sections of 3–5 µm in thickness by microtome and stained by hematoxyline-eosin (H&E) stain. H&E staining was performed to analyze the nerve section quantitatively under the light microscope for histopathological alterations such as necrosis, swelling, and congestion.[32,33]

Estimation of Endogenous Antioxidant Enzymes

At the end of the experimental period, the rats were humanely euthanized. The sciatic nerve was removed for further experiments. The portion of sciatic nerve tissue was individually homogenized in 10% ice-cold Tris-hydrochloride buffer (10 mmol/L; pH 7.4) in tissue homogenizer (Remi, India) and centrifuged at 7500 rpm for 15 minutes at 0°C. The clear supernatant was collected after centrifugation and used for assay of endogenous antioxidant enzyme viz., SOD, glutathione (GSH), and nitric oxide (NO) according to previously reported methods.[34]

Collection of Serum and Plasma Sample

At the end of the study period, blood was collected from each rat by retro-orbital puncture. The collected blood was separated into a centrifuge tube for separation of serum and blood plasma. For serum collection, the blood collected without anticoagulant was centrifuged at 3000 rpm for 15 minutes. For blood plasma, the anticoagulant was added to the centrifuge tubes, and then blood was added in it and centrifuged at 3000 rpm for 15 minutes by using a micro-centrifuge machine for the measurement of biochemical parameters.[34,35]

Estimation of Biological Serum Markers[35]

The total protein (TP) was estimated using commercially available measurement kits (Delta Lab Pvt. Ltd., Mumbai, India).

Estimation of Food and Water Intake

At the end of the study, on the 56th day, the rats were placed in the metabolic cages. The equal amount of water (ml) and food (g) given to the animals for 24 hours. After 24 hours, the amount of water and food intake was measured.[35]

Estimation of Urine Output

At the end of the study, on the 56th day, the rats were placed separately into the metabolic cages for 24 hours and urine was collected. The total amount of urine collected in the bottle was measured.[35]

Statistical Analysis

Data analysis was performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA) for Windows 10. Statistical comparisons were made between drug-treated groups and diabetic control animals. Data were statistically analyzed using two-way repeated ANOVA. Bonferroni's multiple range tests were applied for post hoc analysis. Using one-way ANOVA, Dunnett's multiple range tests were applied for post hoc analysis. A value of $p < 0.05$ was considered to be statistically significant.

Results

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Blood Sugar Level of Male Wistar Rats in Experimentally Induced Diabetic Neuropathy

Before diabetic induction, on day 0, there was no significant change in blood sugar level in diabetic control rats as compared to normal group rats. After 3 days from the intraperitoneal administration of nicotinamide-STZ, there was a significant ($p < 0.001$) increase in blood sugar level as compared to the normal group. Treatment 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) from the 5th week significantly ($p < 0.001$) decreased in the blood sugar level as compared to diabetic control group rats. Rats treated with 18β GA (200 mg/kg) and PREG (10 mg/kg) showed maximum effect ($p < 0.001$) on 56th day in STZ-nicotinamide induced diabetic rats (Fig. 1).

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on HbA1C Level

On the last day of the experiment (56th day), diabetic control rats showed a significant ($p < 0.01$) increase in HbA1C level as compared to normal group rats. Treatments with 18β GA (100 and 200 mg/kg) and PREG (10 mg/kg) significantly ($p < 0.05$) for 4 weeks decreased the level of HbA1C as compared to diabetic control rats. However, treatment with 18β GA (50 mg/kg) did not show any

significant decrease in HbA1C level on the 56th day (Fig. 2).

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Body Weight (g):

In the present study, the body weights were observed at the end of the 1st, 4th, 6th and, 8th week of the experiment. The bodyweight of diabetic control animals started to decrease significantly ($p < 0.001$) from the 6th week of nicotinamide-STZ treatment compared to the normal group. However, treatment with 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) prevented weight loss from the 6th week of treatment. 18β GA (100 and 200 mg/kg) and PREG (10 mg/kg) showed significant ($p < 0.01$) inhibition of body weight loss at the 8th week. 18β GA (50 mg/kg) did not show any significant inhibition of body weight loss at the end of the 6th week, but it showed significant ($p < 0.001$) inhibition at the end of the 8th week (Fig. 3).

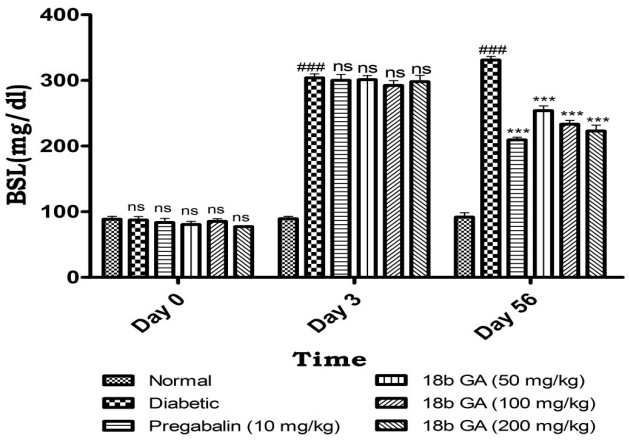

Fig. 1: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on blood sugar level of male Wistar rats in experimentally induced diabetic neuropathy.

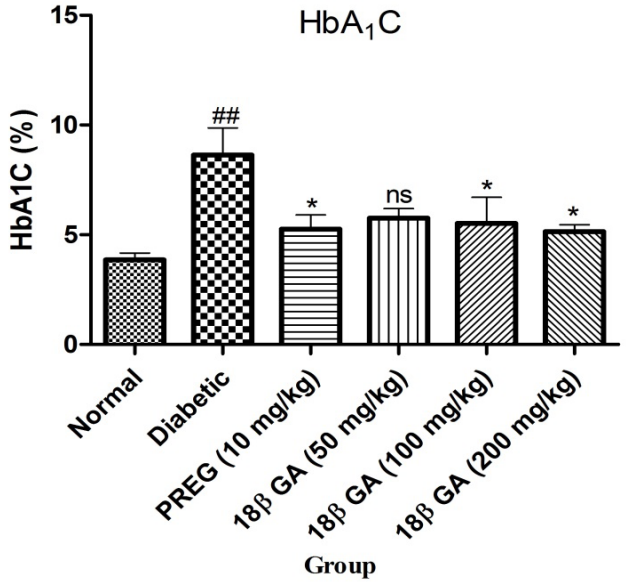

Fig. 2: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on HbA_1C level of male Wistar rats in experimentally induced diabetic neuropathy.

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Hot Water Tail immersion test

In tail withdrawal latency of diabetic control rats on 4th week after induction of diabetes was significantly ($p < 0.001$) decreased compared to normal rats. Treatment with 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) from the 5th weeks significantly ($p < 0.001$) attenuated this decrease in tail withdrawal latency as compared to diabetic control rats. However, this inhibition of decrease in tail withdrawal latency was more significant ($p < 0.001$) in 18β GA (100 mg/kg) treated rats. There was no significant change in the tail withdrawal latency of normal rats (Fig. 4).

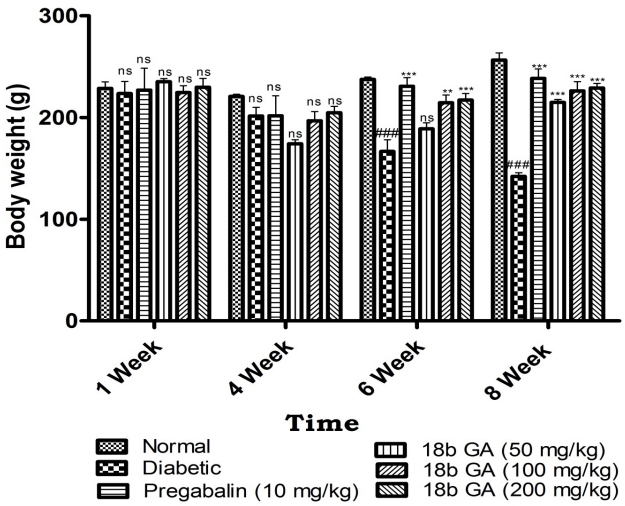

Fig. 3: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on body weight level of male Wistar rats in experimentally induced diabetic neuropathy.

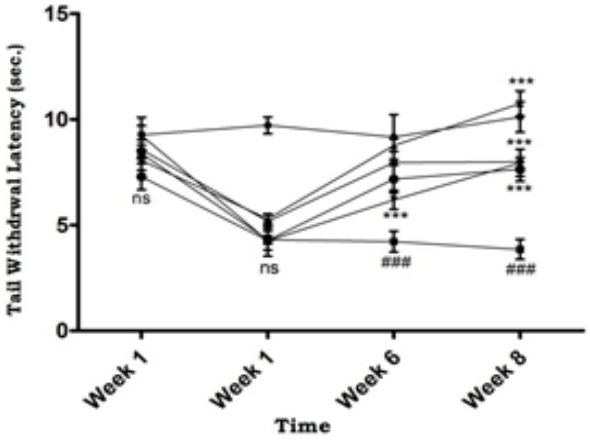

Fig. 4: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Hot water tail immersion test (Tail withdrawal latency) male Wistar rats in experimentally induced diabetic neuropathy.

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Cold Allodynia

From the 4th week after induction of diabetes, the paw withdrawal latency of diabetic control rats was significantly ($p < 0.001$) decreased in 6th and 8th weeks as compared to normal rats. Treatment with 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) for 8 weeks significantly ($p < 0.001$) increased the paw withdrawal latency at the 6th and 8th week as compared to diabetic control rats. Inhibition to decrease in paw withdrawal latency due to 18β GA (200 mg/kg) was more significant ($p < 0.001$) than the other two at the end of the 8th week (Fig. 5).

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Thermal Hyperalgesia

There was a significant ($p < 0.001$) reduction in mean paw withdrawal latency in diabetic control animals from the 4th week as compared to normal rats. Treatment with 18β GA (50, 100 and 200 mg/kg) and PREG (10 mg/kg) significantly ($p < 0,001$) inhibited the decrease in mean paw withdrawal latency at the end of the 6th and 8th week of treatment compared to diabetic control rats. Rats treated with 18β GA (200 mg/kg) and PREG (10 mg/kg) have shown a significant ($p < 0.001$) increase in mean paw withdrawal latency at the 8th week of treatment as compared to diabetic control rats (Fig. 6).

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Mechanical Hyperalgesia

After induction of diabetes mean paw withdrawal threshold of diabetic control rats at the end 4th week was significantly ($p < 0.001$) decreased as compared to normal control rats. Chronic administration of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) for 4 weeks (from 5th week) resulted in a significant ($p < 0.001$) increase in paw withdrawal threshold as compared to diabetic control rats. Inhibition to decrease in the mean paw withdrawal threshold by 18β GA (200 mg/kg) treatment was more significant ($p < 0.001$) at the end of the 6th and 8th week of treatment (Fig. 7).

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Mechano-tactile Hyperalgesia:

For the assessment of mechano-tactile hyperalgesia, Von-Frey hair apparatus was used. The mean paw withdrawal threshold of diabetic control rats at the end of the 4th week after induction of diabetes was significantly ($p < 0.001$) decreased as compared to normal rats. Chronic administration of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) for 4 weeks (from 5th week) resulted in a significant ($p < 0.001$) increase in paw withdrawal

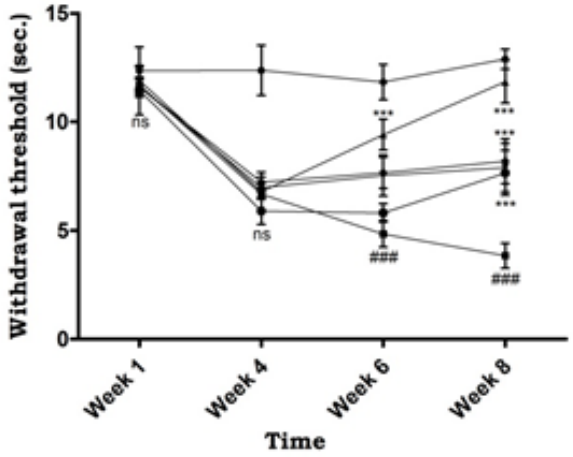

Fig. 6: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Thermal hyperalgesia of male Wistar rats in experimentally induced diabetic neuropathy.

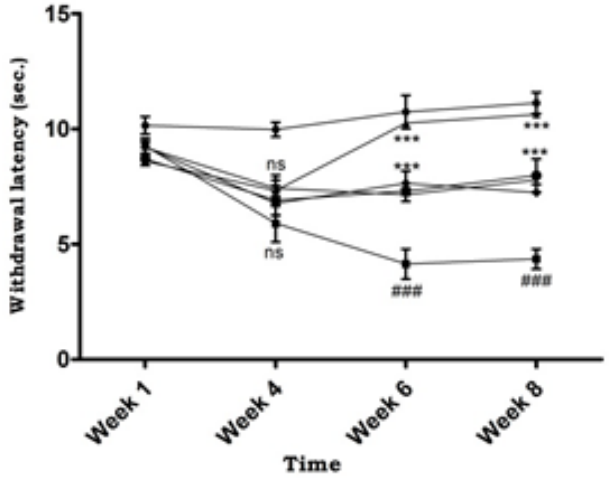

Fig. 5: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Cold allodynia of male Wistar rats in experimentally induced diabetic neuropathy.

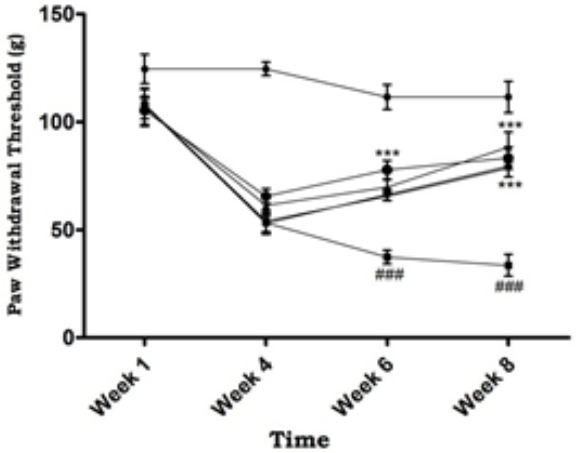

Fig. 7: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Mechanical hyperalgesia of male Wistar rats in experimentally induced diabetic neuropathy.

threshold as compared to diabetic control rats. Rats treated with 18β GA (200 mg/kg) and PREG (10 mg/kg) showed more significant ($p < 0.001$) inhibition to decrease in the mean paw withdrawal threshold at the end of 6th and 8th week of treatment (Fig. 8).

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Motor co-ordination:

Rats treated with various doses of 18β GA and PREG doses were shown significant ($p < 0.001$) improvement in muscle grip strength compared with diabetic control. Improvement in motor coordination activity was significantly ($p < 0.001$) achieved with the dose of 100 mg/kg in the 8th week. There was no significant change in the mean paw withdrawal threshold of normal rats (Fig. 9).

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Locomotor Activity

Rats treated with 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) were shown non-significant ($p < 0.001$) improvement in locomotor activity as compared with diabetic control. However, more improvement in locomotor activity was non-significantly achieved with the dose of 100 mg/kg after 4 weeks of treatment (Fig. 10).

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Histopathological Studies of Male Wistar rats in Experimentally Induced Diabetic Neuropathy

Histopathological examination of rat sciatic nerve sections under a light microscope with Hematoxyline and eosin stain were carried out on the last day (56th day) of the study period. The isolated sciatic nerve of non-diabetic (Fig. 9-A) showed normal architecture of sciatic nerve of, i.e., normal rat evidence by the absence of infiltration of neutrophils as well as macrophages, necrosis of nerve. Intraperitoneal administration of nicotinamide-STZ resulted in significant histopathological changes assessed in the cross-sectional section of the sciatic nerve (Fig. 9-B). It showed the presence of neutrophils and macrophages, congestion, and swelling in the nerve cells. It also showed the necrosis in the nerve cell, which results in swelling of non-myelinated and myelinated nerve fibers. Chronic administration of 18β GA (50, 100 and 200 mg/kg; Fig. 9-D to F) and PREG (10 mg/kg; Fig. 9-C) for 4 weeks resulted in inhibition of neutrophilic as well as macrophages infiltration, congestion, swelling and necrosis in the sciatic nerve. It also attenuated the swelling of non-myelinated and myelinated nerve fibers, axonal degeneration produced by intraperitoneal administration of nicotinamide-STZ (Fig. 11).

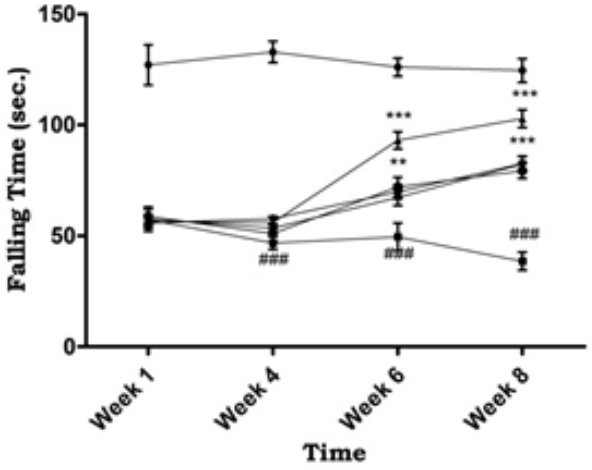

Fig. 9: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Motor coordination of male Wistar rats in experimentally induced diabetic neuropathy.

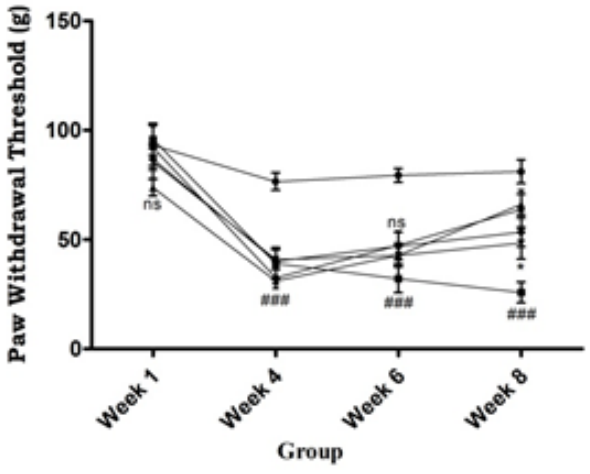

Fig. 8: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Mechano-tactile hyperalgesia (paw withdrawal threshold) of male Wistar rats in experimentally induced diabetic neuropathy.

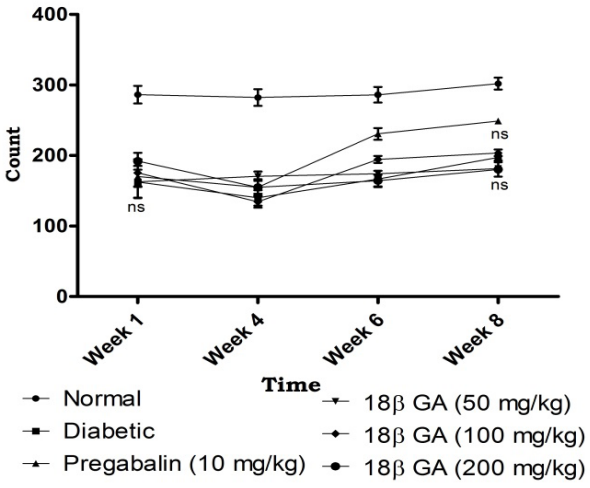

Fig. 10: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Locomotor activity of male Wistar rats in experimentally induced diabetic neuropathy.

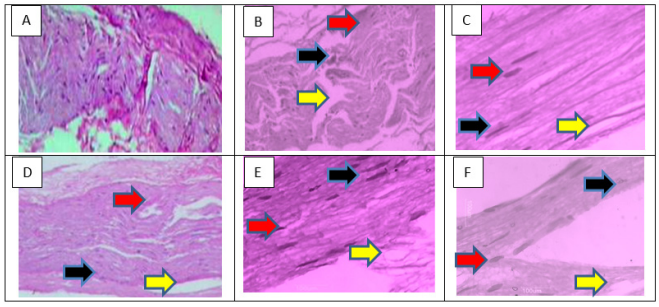

Fig. 11: Histopathological examination of sciatic nerve of male Wistar rats in experimentally induced diabetic neuropathy. Where, Congestion, Swelling, Necrosis. Whereas, Image A- Normal group, B- Diabetic control group, C- Standard i.e. PREG (10 mg/kg), D- Test I (18β GA 50 mg/kg), E- Test II (18β GA 100 mg/kg) and F- Test III (18β GA 200 mg/kg).

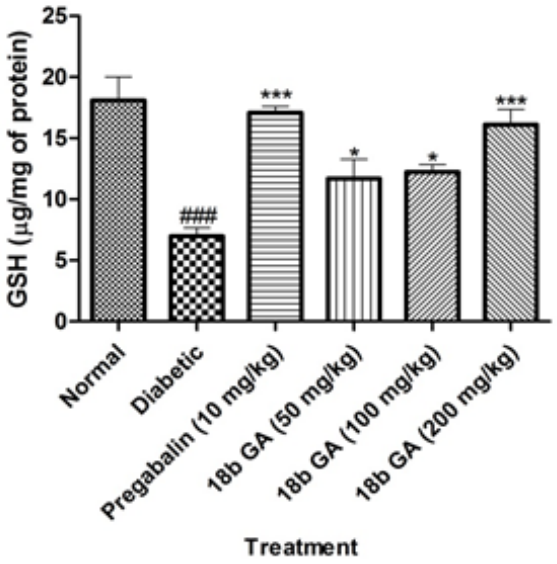

Fig. 12: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on GSH- glutathione of male Wistar rats in experimentally induced diabetic neuropathy.

Effect of 18β GA (50, 100, and 200 mg/kg) Endogenous Antioxidant Enzymes of Male Wistar Rats in Experimentally Induced Diabetic Neuropathy

After 8 weeks, there was significant ($p < 0.001$) decrease in activities of GSH (Fig. 12) and SOD (Fig. 13) in the tissues (sciatic nerve) in the diabetic control rats; 18β GA (200 mg/kg) treated rats showed significant ($p < 0.001$ and $p < 0.01$ respectively) restoration of the activities of GSH and SOD. 18β GA (100 mg/kg) also showed significant ($p < 0.05$) restoration of GSH and SOD, whereas 18β GA (50 mg/kg) did not show any significant restoration in SOD level; but 18β GA (50 mg/kg) showed significant ($p < 0.05$) restoration in GSH level. NO level (Fig. 14) in the tissues (sciatic nerve) of diabetic control rats increased significantly ($p < 0.001$), whereas the 18β GA (200 mg/kg) treated groups had significantly lower level ($p < 0.001$)

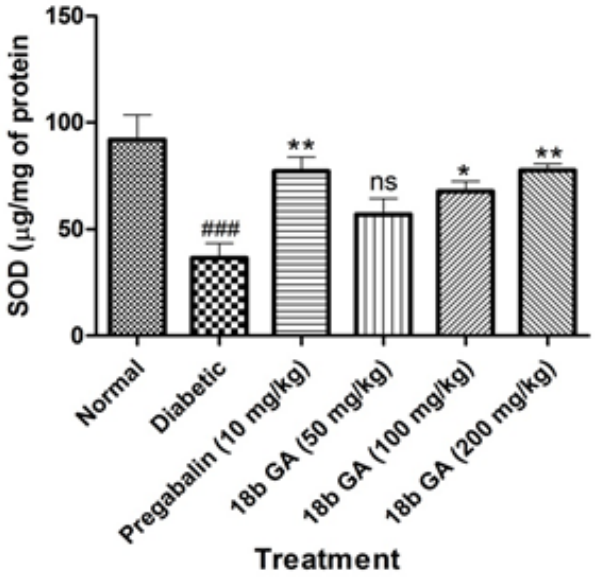

Fig. 13: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on SOD- superoxide dismutase of male Wistar rats in experimentally induced diabetic neuropathy.

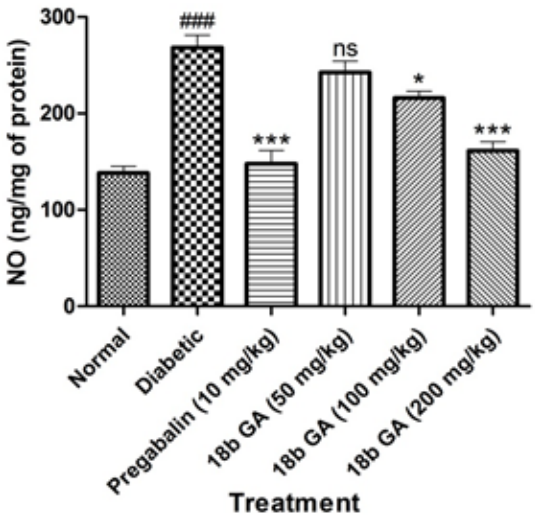

Fig. 14: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on NO- nitric oxide of male Wistar rats in experimentally induced diabetic neuropathy.

of NO than in the 18β GA (100 mg/kg) treated group ($p < 0.05$). However, 18β GA (50 mg/kg) treated group did not show any significant restoration

Effect of 18β GA (50, 100, and 200 mg/kg) on Total Protein

There was significant ($p < 0.001$ each) decrease in level of total protein in diabetic control group. After the treatments with 18β GA (200 mg/kg) showed significant ($p < 0.001$) increase total protein level. 18β GA (100 mg/kg) showed similar activity (i.e. $p < 0.001$) in total protein as compared to higher dose (200 mg/kg) of 18β GA. Lower

dose of 18β GA (50 mg/kg) also showed non-significant ($p < 0.05$) in total protein level (Fig. 15).

Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Food Intake (g), Water Intake (mL) and Urine Output (mL)

There was a significant increase ($p < 0.001$) in the food intake (Fig. 16), water intake (Fig. 17), and urine output (Fig. 18) in diabetic control rats after the 8th week of intraperitoneal administration of nicotinamide-STZ as compared to normal rats. Rats treated with 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) for 4 weeks significantly ($p < 0.001$) prevented this increased food intake, water intake, and urine as compared to diabetic control rats (Fig. 8).

Discussion

The present discussion is based upon the results obtained with the optimum dose of 18β GA (50, 100, and 200 mg/kg) once daily for 4 weeks. The present investigation selection of 18β GA doses (50, 100, and 200 mg/kg) and PREG (10 mg/kg) was based on previous.[9,17]

Kalaiarasi and Pugalendi in 2009 were reported antihyperglycemic activity of 18β GA on STZ-diabetic rats. In diabetic rats, there was degeneration of β-cells which leads to insulin resistance. Because of this, Insulin integrates hepatic carbohydrate metabolism by increasing the biosynthesis of enzymes of glycolysis, glycogenesis, and pentose oxidative pathway and by inhibiting gluconeogenesis. And after treatment with 18β GA, the gluconeogenic enzyme activities back to near-normal levels, which may be due to increased secretion of insulin. In another diabetic study, the scientist investigated the hypolipidemic activity of 18β GA in diabetic rats. In this study, the phospholipids are vital components of

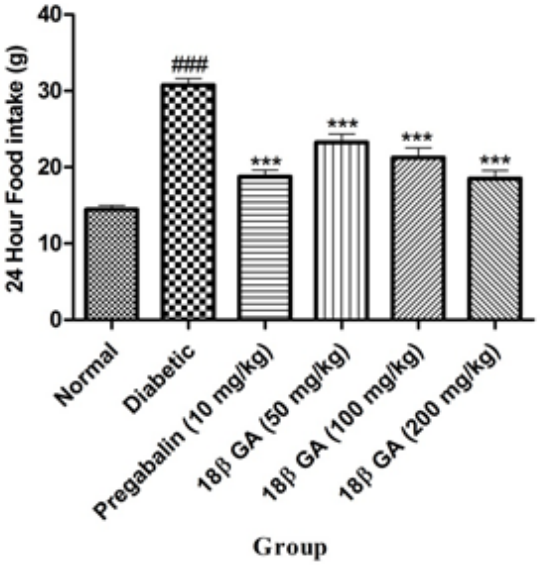

Fig. 16: Effect of 18β GA (50, 100, and 200 mg/kg) on food intake of male Wistar rats in experimentally induced diabetic neuropathy. Values are mean ± SEM; data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests; ns = non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with diabetic control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ as compared with normal group.

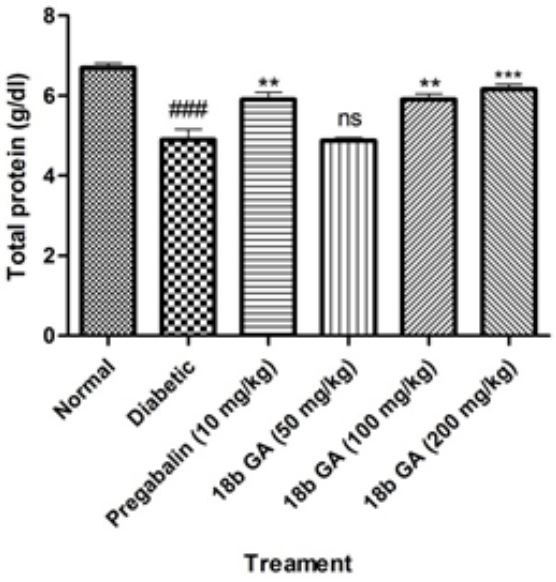

Fig. 15: Effect of 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) on Total protein of male Wistar rats in experimentally induced diabetic neuropathy.

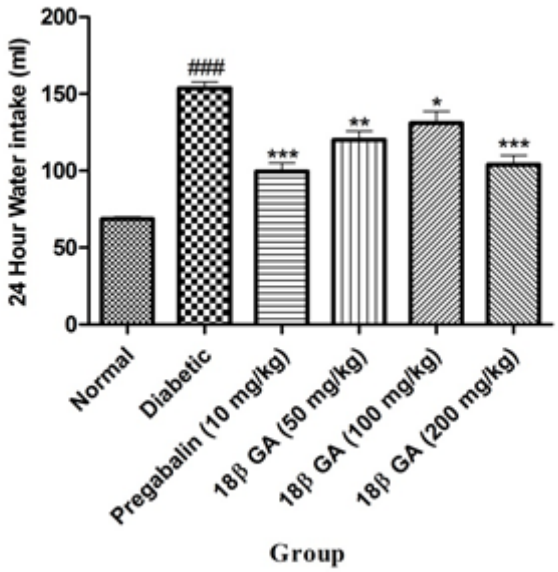

Fig. 17: Effect of 18β GA (50, 100, and 200 mg/kg) on water intake of male Wistar rats in experimentally induced diabetic neuropathy. Values are mean ± SEM; data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests; ns = non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with diabetic control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ as compared with normal group.

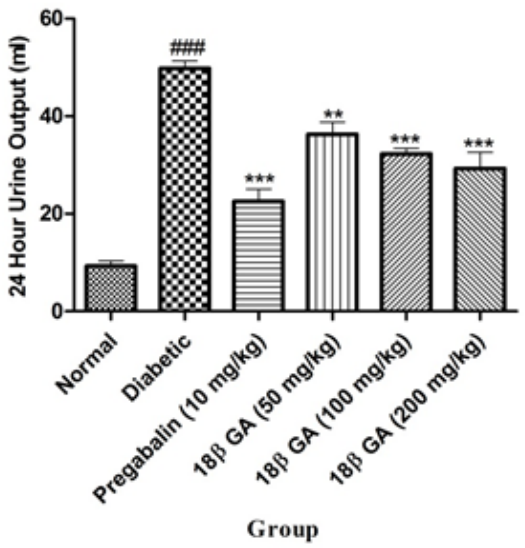

Fig. 18: Effect of 18β GA (50, 100, and 200 mg/kg) on urine output of male Wistar rats in experimentally induced diabetic neuropathy. Values are mean ± SEM; data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests; ns = non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with diabetic control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ as compared with normal group.

bio-membranes rich in polyunsaturated fatty acids, which are susceptible substrates for free radicals, such as O_2^- and OH^- radicals. These phospholipids are more important for the maintenance of cellular integrity, micro-viscosity, and survival. The level of phospholipids increased in diabetic rats and after treatment with 18β GA, these elevated levels were prevented decreased.[17,18] Similarly, Maitraie and co-scientist in 2009, showed the antioxidant activity of 18β GA. They studied; the ability of 18β GA derivatives to inhibit the DNA damage caused by O_2 was studied in-vitro by agarose gel electrophoresis. Because the reactive oxygen species (ROS) was well known to damage many biological macromolecules, with DNA being a significant target. Since there have been no studies of 18β GA on diabetic complications, on the basis of the previous research, we designed to investigate the effect of 18β GA on diabetic neuropathy in nicotinamide-STZ induced diabetes Wistar rats.[36]

Clinical features like allodynia confirm diabetic neuropathy, hyperalgesia due to elevated nociceptive response, reduced threshold to painful stimuli, reduced blood pressure. Intraperitoneal administration of STZ rats exhibits clinicopathological features, including biochemical, oxidative, and metabolic changes, presented in humans.[37]

In the present study, 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg) were given administered to see whether they prevent nicotinamide-STZ induced diabetic neuropathy. The development of these complications was observed at the 4th week after nicotinamide-STZ induction, which was consistent with previous reports. STZ is well known to cause pancreatic B-cell damage, whereas nicotinamide is administered to rats to partially protect insulin-secreting cells from STZ. STZ is transported into B-cells via the glucose transporter GLUT2 and causes DNA damage leading to increased activity of poly (ADP-ribose) polymerase (PARP-1) to repair DNA. However, the exaggerated activity of this enzyme results in depletion of intracellular NAD (+) and ATP, and the insulin-secreting cells undergo necrosis. The protective action of nicotinamide is due to the inhibition of PARP-1 activity. Nicotinamide inhibits this enzyme, preventing depletion of NAD (+) and ATP in cells exposed to STZ. Moreover, nicotinamide serves as a precursor of NAD (+) and thereby additionally increases intracellular NAD (+) levels. The severity of diabetes in experimental rats strongly depends on the doses of STZ and nicotinamide given to these animals; STZ intern triggers multiple biochemical pathways such as polyol pathway, hexosamine pathway, protein kinase C pathway (PKC), advanced glycation end (AGE) product and poly adipose ribose polymerase (PARP) pathway all of these pathways contribute towards oxidative stress by generating ROS in mitochondria results in nerve damage and neuropathy.[37-39]

In this study, diabetic rats showed a significant increase in blood glucose levels and decreased body weight. When rats with developed neuropathy were treated with 18β GA (50, 100, and 200 mg/kg) and PREG (10 mg/kg), the blood glucose level and prevented the weight loss which might be as a result of its ability to decrease blood glucose level by increasing insulin production.[9] Hyperglycemi a influences proteolysis in skeletal muscle and lipolysis in adipose tissues, resulting in severe weight loss in the animal models.[24,25]

In diabetic rats, we observed an increase in glycosylated hemoglobin (HbA_1C). Insulin generally has an anabolic effect on protein metabolism. It stimulates protein synthesis and retards protein degradation (Kalaiarasi and Pugalendi in 2009). Thus, increased glycation of protein has been found to be a consequence of diabetic complications. The 18β GA acid and PREG treated rats showed a significant reduction in HbA_1C due to improved glycemic control.

In the present study, behavioral parameters which distinguish nociceptor functions in diabetic rats were used. For behavioral studies, heat hyperalgesia, cold allodynia, Randall Selitto, and Von Frey hairs for paw withdrawal threshold are reported methods to measure mechanical hyperalgesia, thermal hyperalgesia in preclinical studies.[30] The damage of sensory and motor fibers results in a reduction in pain threshold due to the STZ. The damage of sensory and motor fibers results in a reduction in pain threshold due to the STZ. In this study, significant increases in pain threshold were observed in the 18β GA, and PREG treated group. The previous study of