Original Article

Neuroprotective Role of Wheatgrass Powder in Experimental Diabetic Neuropathy via Modulating Oxidative Stress Markers in Rat Sciatic Nerves

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ABSTRACT

Objective: To evaluate the neuroprotective role of Wheatgrass Powder in Experimental Diabetic Neuropathy.
Methods: An experimental rat model of Diabetic Neuropathy was developed using 50 mg/kg i.v dose of Streptozotocin (STZ). Development of Experimental Diabetic Neuropathy by the end of 8 weeks of STZ injection was confirmed by assessing behavioral parameters like neuropathic pain, allodynia and motor deficits which are the important hallmarks of Diabetic Neuropathy. The effect of 500 mg/kg Wheatgrass powder on Blood glucose levels, serum lipid profile, behavioral biomarkers was assessed before and after the treatment. At the end of the study (8 weeks) sciatic nerves of rats were isolated and oxidative stress markers and structural biomarkers of DN in treated and untreated groups were estimated.
Results: STZ caused hyperglycemia induced oxidative damage of sciatic nerves and developed symptoms of Diabetic Neuropathy. Wheatgrass treatment in diabetic rats prevented the development of neuropathic pain, hot allodynia, cold allodynia and motor deficits, the important signs of Diabetic Neuropathy. Wheatgrass treatment ameliorated the oxidative damage of sciatic nerves by increasing the antioxidant enzymes SOD, Catalase, GSH levels and by decreasing the formation of TBARS (Thio Barbituric Acid Reactive Substances) in diabetic rat sciatic nerves. The neuroprotective role of Wheatgrass was also evident from electron microscopic studies.
Conclusion: The present study revealed the neuroprotective role of Wheatgrass in Diabetic Neuropathy through modulating oxidative stress markers in Diabetic rat sciatic nerves and its beneficial role as a supplement in Diabetic Neuropathy.

Keywords: Diabetic neuropathy, Wheatgrass, Streptozotocin, Oxidative stress, Electron microscopy.

INTRODUCTION

Diabetes is one of the largest providers of Neuropathy in the world. Diabetic Neuropathy (DN) consists of several clinical syndromes affecting motor, sensory and autonomic nerves. Of the various types, symmetrical poly neuropathy, usually referred as Diabetic Poly Neuropathy (DPN), is more common amongst the diabetic population[1]. DPN often presents with neuropathic pain but can also present with decreased balance or a change in gait[2]. The generation of free radicals is an important factor in development of Diabetic Neuropathy. Simultaneous with generation of free radicals during the glycolytic process, oxidative stress harms mitochondrial DNA, proteins, and membranes. Oxidative stress and reactive oxygen species (ROS) link the physiological mediators and metabolic initiators implicated in progressive nerve fiber damage, dysfunction, and loss in Diabetic Neuropathy[3,4]. In DN, oxidative stress persists by which anti-oxidant defense mechanisms decline[5].

Current therapeutic interventions for the treatment of DPN are less satisfactory. At present, treatment alleviates pain and can control some associated symptoms, but they cannot halt the progression of Diabetic Neuropathy. According to assumption that oxidative stress may mediate several complications in diabetes, antioxidant therapy remains a vital therapy[6] that needs to be exploited. A great number of medicinal plants have been used in the treatment of diabetes in different parts of the world. Evaluation of the anti-diabetic potential of these plants becomes necessary to provide scientific proof and justify their uses in ethnomedicine[7]. Wheatgrass has been used as traditional herbal medicine and is highly valued for its therapeutic and nutritional properties[8]. Regular ingestion of the Wheatgrass can improve the digestive system, treats constipation and believed to prevent some cancers, diabetes and heart diseases. It can detoxify heavy metals from the bloodstream, cleanse the liver, prevent hair loss and promotes general well-being [9]. But its antioxidant role in diabetic complications is not explored till now. So in the present research, we investigated the protective role of Wheatgrass powder in sciatic nerve damage of Experimental Diabetic Neuropathy and the possible mechanism of its activity.

Wheatgrass powder's health benefits range from providing supplemental nutrition to having unique curative properties. Wheat (*Triticum aestivum* L.) belongs to the Family Poaceae. This plant is consumed as a health food in various forms such as tablet, powder and juice. We had taken commercially available standardized Wheatgrass powder (Maddi pharmaceuticals, AP, India) that is said to be having an array of clinically beneficial actions.

MATERIALS AND METHODS

Animals

Age matched young male Albino Wistar rats weighing about 180-220 g were employed in the present study and were procured from Albino Research and Training Institute, Hyderabad. They were fed on a standard chow diet and water ad libitum. The animal experiments in this study were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forest, Government of India. The experimental protocol has been approved by the IAEC Reg. No 1722/RO/Ere/S/13/CPCSEA.

Experimental induction of Type 1 Diabetes Mellitus

Diabetes was experimentally induced in adult rats by administering a single intravenous dose of 50 mg/kg STZ (Streptozotocin). Control animals received an equal volume of citrate buffer (p^H 4.5). STZ induced diabetic animals were given 10 % (w/v) glucose in drinking water for 24 h. The animals which have shown plasma glucose levels more than 300 mg/dl after 72 h of STZ injection were considered diabetic and included in this study.

Experimental design

Animals were randomly divided into four groups having eight animals each. Control, Diabetic (Vehicle treated), Standard (Diabetic + 15 mg/kg Pregabalin), and Wheatgrass treated (Diabetic + 500 mg/kg Wheat grass powder) groups. Commercial grade Wheatgrass powder (Maddi Pharmaceuticals, Secunderabad, India) was used for this study. Pregabalin at a dose of 15 mg/kg (PREG 15) and Wheat grass (WG 500) at a dose of 500 mg/kg was suspended in water and given orally for seven weeks from the 8th day of STZ induction in diabetic rats.

Behavioral assessment of neuropathic pain

Neurobehavioral tests for nociception and motor coordination were performed on each animal before and after the treatment.

Assessment of thermal hyperalgesia (Eddy hot plate method)

Thermal hyperalgesia was assessed by Eddy hot plate. The temperature of the hot plate should be maintained at 55^0 C+1^0C throughout the experimental period. The latency to first sign of paw licking or jumping response to avoid thermal pain was taken as an index of pain threshold. A cut off time of 15 Sec was maintained[10].

Assessment of cold and hot allodynia by tail immersion method

The tail of each rat was immersed in cold (10^0C) or warm (48^0C) water and tail flick latency was recorded until tail withdrawal or signs of struggle were observed (cut off time 15 Sec)[11,12].

Assessment of Sensorimotor deficit

Motor in coordination of the animals was evaluated by the Rotarod treadmill test[13]. The rats were tested for three times at 25 RPM speed with an interval of 20 min between each trial. An average of 3 readings was taken as the final latency to fall[14]. The spontaneous motor (exploratory) activity was evaluated using an actophotometer which operates with photoelectric cells, which are connected in circuit with a counter[15].

Estimation of Oxidative stress markers

All animals were sacrificed at the end of the study by decapitation under light ether anesthesia and both the sciatic nerves were immediately isolated carefully and weighed. The sciatic nerve homogenate (10%) was prepared with 0.25 M Tris buffer using Potter-Elvehjam type glass homogenizer. The homogenate was centrifuged at a temperature of 4^0 C at 4000 RPM for 10 minutes. The supernatant was collected and again centrifuged at 10000 RPM for 20 minutes to get post mitochondrial supernatant. Various biochemical assays were performed in homogenate and post mitochondrial fraction[16].

Estimation of Reduced Glutathione (GSH)

The acid soluble sulfhydryl groups in postmitochondrial portion, form a yellow colored complex with dithionitrobenzene (DTNB). The absorbance was measured at 412 nm against a blank contained TCA instead of supernatant. The amount of

glutathione is expressed as n.mole/mg protein[17].

Estimation of Superoxide Dismutase (SOD)

The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction can be extracted into butanol layer upon inactivation of the reaction with acetic acid and measured at 560 nm. The SOD level was expressed as Units/mg protein[18].

Estimation of catalase

H_2O_2 decomposition by CAT (Catalase) was monitored spectrophotometrically by following decrease in absorbance at 240 nm at an interval of 30sec.Catalase was expressed as µmoles of H_2O_2 metabolized/mg protein /min[19].

Estimation of lipid peroxidation

Thiobarbituric acid reacts with malondialdehyde to yield fluorescent product. The amount of the tissue Melondealdehyde (MDA), product of lipidperoxidation formed was measured with Thiobarbituric acid at 532 nm. The results were expressed as nano mol/mg protein[20].

Estimation of protein

The protein content was estimated according to the method of Lowry *et al*.[21] using albumin as standard.

Electron microscopy

Samples are fixed in 2.5% - 3% glutaraldehyde in 1 M phosphate buffer (P^H7. 2) for 24 hours at 4^0c and washed with PBS for 3-4 times each 30-45 minutes, then post fixed in 1% aqueous Osmium Tetroxide for 2 hours later washed with deionized distilled water for 4-6 times each 40-45 minutes, dehydrated in series of graded alcohols, infiltrated and embedded in araldite 6005 resin and incubated. Ultra-thin (50-70 nm) sections were made and counter stained with Reynolds lead citrate (LC) [22] and viewed under a Transmission Electron Microscope.

Statistical analysis

All values were expressed as Mean ± SEM (n = 8/group). Data was analyzed using one way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple pairwise comparisons between the various groups. Values with $P < 0.05$ or less were considered as statistically significant.

RESULTS

The effect of 500 mg/kg Wheatgrass powder on body weights, food and water intake in Type 1 Diabetes Mellitus

During the 8 weeks of the study period, the body weight of control rats was increased by 38. 81 %, whereas diabetic rats showed significantly reduced body weight. There was a 25.25%, 17. 39 % and 19.68 % reduction in body weights of diabetic control, diabetic + PREG 15 and diabetic + WG 500 rats respectively. Water and food consumption were significantly higher in diabetic rats, when compared to control rats, suggesting polyphagia, and polydipsia, hallmarks of clinical diabetes. PREG 15 and WG 500 rats also showed an increase in water consumption compared to the control group. But interestingly Pregabalin decreased the food intake in diabetic rats. Wheatgrass had not shown any significant effect on type 1 diabetes induced weight loss, polyphagia and polydipsia.

The effect of 500 mg/kg Wheatgrass powder on blood glucose levels in diabetic rats

Control rats were normoglycemic throughout the study period. After 72 hours of the STZ injection, the rats in diabetic control, diabetic + PREG 15 and diabetic +

WG 500 groups showed blood glucose levels > 300 mg/dl. After 8 week study period the untreated diabetic rats showed still increased blood glucose levels. Diabetic rats treated with PREG 15 had not shown significantly reduced the blood glucose levels, but the diabetic rats treated with 500 mg/kg Wheatgrass showed significant reduction (39.61%) in blood glucose levels. Although Wheatgrass significantly reduced the glucose levels, the blood glucose is still above 300 mg/dl indicating persistence of Type 1 Diabetes Mellitus at the selected dose.

The effect of 500 mg/kg Wheatgrass powder on the serum lipid profile in Type 1 diabetes

At the end of the study and there was a significant increase in cholesterol ($p<0.05$) and triglyceride ($p<0.001$) levels and fall in HDL cholesterol levels in diabetic control rats. Pregabalin had no effect on diabetes induced changes in serum lipid profile. However, treatment with Wheatgrass significantly lowered the serum cholesterol ($p<0.01$) and triglyceride ($p<0.001$) levels in diabetic rats but no significant effect on HDL cholesterol.

The effect of 500 mg/kg Wheat grass powder thermal hyperalgesia

After 8 weeks of induction, diabetic rats showed decreased reaction latency compared to the control animals. Diabetic rats showed a significant decline in reaction time, even compared to baseline values. Pregabalin treatment in diabetic rats improved the reaction latency significantly. Reaction time significantly increased in diabetic rats treated with Wheatgrass. Diabetic animals treated with 500 mg/kg Wheatgrass powder showed almost same baseline reaction time after seven weeks of treatment.

Effect of 500 mg/kg Wheatgrass powder on hot and cold allodynia

Tail flick latency of untreated diabetic animals at hot temperature (48 ± 1^0C) and cold temperatures was significantly less when compared to the normal animals. Diabetic rats also showed significant decline in tail flick latency after 8 weeks, even when compared to the baseline values indicating the symptoms of Diabetic Neuropathy. The tail flick latency of WG 500 treated group showed significantly increased hot and cold allodynia threshold which is comparable with that of standard drug Pregabalin.

Effect of 500mg/kg Wheatgrass powder on Motor coordination and loco motor activity

Rotarod treadmill test and locomotor count in actophotometer revealed a marked impairment of motor coordination in the diabetic animals. Pregablin improved motor coordination and locomotor activity of diabetic rats. Treatment with 500mg/kg Wheatgrass also increased the retention time and locomotor activity of diabetic rats significantly.

The effect of 500 mg/kg Wheatgrass powder on Oxidative stress markers in sciatic nerves

The anti-oxidant enzyme levels (GSH, SOD and Catalase) significantly decreased in the sciatic nerves of diabetic rats as compared to the control group. MDA (end product of lipid peroxidation) levels increased significantly ($p<0.001$) in the sciatic nerves of DN rats. 500 mg/kg Wheatgrass powder significantly increased the levels of SOD and catalase in the sciatic nerves of diabetic rats. It moderately increased the level of GSH in the sciatic nerves of diabetic rat.

Effect of 500mg/kg Wheatgrass powder on sciatic nerve morphology

Transmission electron microscopy study of sciatic nerve sections showed the normal structure and morphology of myelinated fibers in the control group. Some evidences of axonal degeneration such as increased number of abnormal myelinated fibers, demyelination, Wallarian degeneration and onion-bulb formation was observed in Diabetic group. WG 500 treatment prevented the development of most of these structural abnormalities.

DISCUSSION

The STZ induced Type 1 diabetic rats exhibit loss of body weight, increased food and water intake which could be due to the excessive breakdown of tissue proteins, dehydration and catabolism of fats and proteins[23]. Signs of marked hyperglycemia include polyuria, polydipsia, weight loss, polyphagia, and blurred vision[24] and 500 mg/kg Wheatgrass powder in diabetic rats had not shown any recovery from these features of Type 1 Diabetes. Wheatgrass powder lowered the blood glucose levels in Type 1 Diabetic rats. Previous works also demonstrated the blood glucose lowering capacity of Wheatgrass in Type1 Diabetic mice[25].

In the present study Type 1 diabetes model was used where there was lack of insulin due to pancreatic β cell destruction with STZ. So the blood glucose lowering capacity of Wheatgrass in Type I diabetic rats might be due to its insulinomimetic or due to its regenerating activity of pancreatic β cells of STZ - induced diabetic rats. However in the present study the hyperglycemia is maintained in the diabetic rats through out the study period at the selected dose of Wheatgrass powder (500 mg/kg). Potentially modifiable risk factors of DPN include levels of total cholesterol, low-density lipoprotein cholesterol, and triglycerides[2]. Bioactive plant compounds flavonoids and triterpenoids, were reported to modulate lipid levels. The presence of flavonoids and triterpenoids in Wheatgrass powder might have contributed in lipid lowering effect[26,27]. The other phytochemical constituents of Wheatgrass, tannins are reported to increase in activity of the endothelial bound lipoprotein lipase activity which hydrolyzes triglycerides[28]. This action of Wheatgrass renders its lipid lowering action in diabetic condition and hence prevents diabetes-related complications[29,30].

Neuropathic pain is one of the most common complications in Diabetic Neuropathy. Almost 50% of diabetic patients develop neuropathy with symptoms including spontaneous pain, allodynia and hyperalgesia[31]. The decreased reaction latency to thermal stimuli in diabetic rats is hyperalgesia, one of the characteristic symptoms of DN. Increased nociception and sensitivity during hyperglycemic hypoxia could be a mechanism in painful neuropathy[32]. The sensimotor deficits resulting in diabetic peripheral neuropathy can lead to significant motor impairment with increased risk of falls due to decreased postural control and altered gait and balance[33]. Wheatgrass treatment for seven weeks prevented the development hyperalgesia and motar deficits in diabetic rats and the effect is comparable with that of standard drug Pregabalin.

Development of Diabetic Neuropathy involves hyperglycemia induced oxidative stress in peripheral nervous system[34]. The possible sources of oxidative stress in Diabetes Mellitus might include auto-oxidation of glucose, shifts in redox balances, decreased tissue concentrations of low molecular weight antioxidants, such as reduced glutathione (GSH) and vitamin E, and impaired activities of antioxidant defense enzymes such as superoxide

dismutase and catalase[35]. Lipid peroxidation is one of the characteristic features of chronic diabetes. The increased free radicals produced may react with polyunsaturated fatty acids in cell membranes leading to lipid peroxidation. Lipid peroxidation will in turn result in the elevated production of free radicals[36]. Lipid peroxide-mediated damage has been observed in the development of type 1 Diabetes Mellitus. Low levels of lipoxygenase peroxides stimulate the secretion of insulin, but when the concentration of endogenous peroxides increases, it may initiate uncontrolled lipid peroxidation leading to cellular infiltration and islet cell damage in type I diabetes[37]. The most commonly used indicator of lipid peroxidation are TBARS[38]. In present study increased lipid peroxidation, reduced SOD, GSH and Catalase levels were observed in STZ induced diabetic rats. These changes may be due to the glucose oxidation, formation of free radical generation and nitric oxide donor property of STZ[39].

Wheatgrass treatment in the present study improved the SOD, Catalase and GSH levels in Diabetic rat sciatic nerves and decreased the MDA levels indicating the depletion of lipid peroxidation in diabetic sciatic nerves. Wheatgrass contains at least 13 vitamins (several of which are antioxidants) including B12, abscisic acid, superoxide dismutase (SOD), cytochrome oxidase, mucopolysaccharide [40]. It also has a high content of Chlorophyll, bioflavonoids like Apigenin, Quercetin and Luteonin[41]. All of these contribute to its antioxidant activity. Wheatgrass is a natural source of SOD. SOD scavenges the superoxide radical by converting it to H_2O and molecular oxygen[42]. CAT is a hemeprotein, which catalyzes the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals[43]. An increase in the SOD activity may protect CAT by scavenging superoxide radical which have been shown to inactivate CAT[44]. Thus, the increase in SOD activity may indirectly play an important protective role in preserving the activity of CAT. This antioxidant effect of Wheatgrass might be responsible to prevent the thermal nociception, motor deficits, thereby showing the neuroprotective role in experimental Diabetic Neuropathy.

Many natural products possess antioxidant properties for improving the structural integrity of cell membranes and alleviating the biochemical perturbations[45]. From experiments in animals, and by analogy with other neuropathies, It can be suggested that the pathogenesis of DN is likely to be associated with demyelination, axonal atrophy and degeneration (Figure 1b)[46]. The demyelination may be attributed to decreased protein synthesis in Schwann cell in the diabetic state. However Wheatgrass supplementation prevented most of these structural abnormalities (Figure 1d). From this it was evident that 500 mg/kg Wheatgrass prevented the structural abnormalities of the sciatic nerve that may occur from oxidative damage.

In summary present results revealed that WG 500 treatment ameliorated thermal hyperalgesia, motor in coordination and showed an increase in the antioxidant defense mechanism in diabetic rats. The action of Wheatgrass to restore the altered antioxidant enzymes in the STZ induced diabetic rats indicates its free radical scavenging potential. This antioxidant role of Wheatgrass is responsible for interrupting many pathogenic pathways, which involves oxidative stress as a common factor in the development of PDN. This could offer a rationale for the use of Wheatgrass as a neuroprotective supplement in the treatment of Diabetic Neuropathy.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Table 1. Effect of Wheatgrass powder on body weight, water and food intake in Type 1 Diabetes Mellitus

	Bodyweight in grams		Water intake In ml/day	Food intake in grams/day
	Before treatment	After treatment		
Control	192.83 ± 6.65	267.67 ± 10.87	12.75 ± 1.41	18.24 ± 1.63
Diabetic	184.17 ± 2.86	137.67 ± 4.64***	37.34 ± 8.93***	29.19 ± 1.35***
PREG 15	184.00 ±1.98	152.00 ± 4.49***	24.25±3.74**	16.63 ± 1.42#
WG 500	196.50 ± 5.85	157.83 ± 9.31***	33.15 ± 8.58**	25.53 ± 1.24**

Values are Mean ± SEM n=8/group. *significantly different from Control group #significantly different from the diabetic group (*** P<0.001, **P<0.01, *P<0.05 ###P<0.001, ## P<0.01, #P<0.05).

Table 2. Effect of Wheatgrass powder on serum glucose and lipid profile of Diabetic rats

	Serum blood glucose (mg/dl)		Serum Total Cholesterol (mg/dl)	Serum triglycerides (mg/dl)	Serum HDL cholesterol (mg/dl)
	Start of study	End of study			
Control	104.65±4.46	106.35±8.38	73.27±4.82	99.74±6.26	36.70±1.73
Diabetic	378.33±14.92***	406.45±7.89***	114.55±7.52*	188.91±10.25***	20.35±1.72**
PREG 15	397.28±11.63***	322.05±15.88	108.79±5.23*	182.75±10.43***	25.78±2.13
WG 500	495.4±11.95***	306.37±12.14##	63.73±3.85##	117.32±8.17###	26.26±1.55

Values are Mean ± SEM n=8/group. *significantly different from Control group #significantly different from the diabetic group (*** P<0.001, **P<0.01, *P<0.05 ###P<0.001, ## P<0.01, #P<0.05).

Table 3. Effect of 500 mg/kg Wheatgrass powder on Oxidative stress markers

	GSH (nmole/mg protein)	CAT (units/mg protein)	SOD (unit/mg protein)	MDA (n moles /mg protein)
Control	3.55 ± 0.16	2.29± 0.28	2.64 ± 0.21	1.28 ± 0.17
Diabetic	1.78 ± 0.15***	1.11 ± 0.10**	0.99 ± 0.17**	3.78 ± 0.24***
PREG 15	2.47 ± 0.26#	2.09 ± 0.15#	2.65 ± 0.29###	3.25 ± 0.08
WG 500	2.03 ± 0.14	2.03 ± 0.29#	2.43 ± 0.21###	1.79 ± 0.14###

Values are Mean ± SEM n=8/group. *significantly different from Control group #significantly different from the diabetic group (*** P<0.001, **P<0.01, *P<0.05 ###P<0.001, ## P<0.01, #P<0.05).

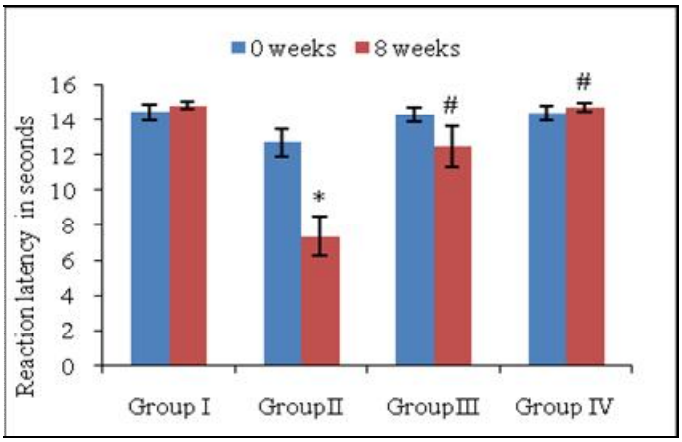

Figure 1. Effect of Wheatgrass powder on Thermal Hyperalgesia

Values were expressed in Mean ± SEM (n=8/group). * Significantly different from Normal control group ($p < 0.05$). # Significantly different from Diabetic control group ($p < 0.05$). **Group I:** Normal control. **Group II:** Diabetic control **Group III:** 15 mg/kg Pregabalin treated group (PREG 15). **Group IV:** 500 mg/kg Wheatgrass powder treated group (WG 500).

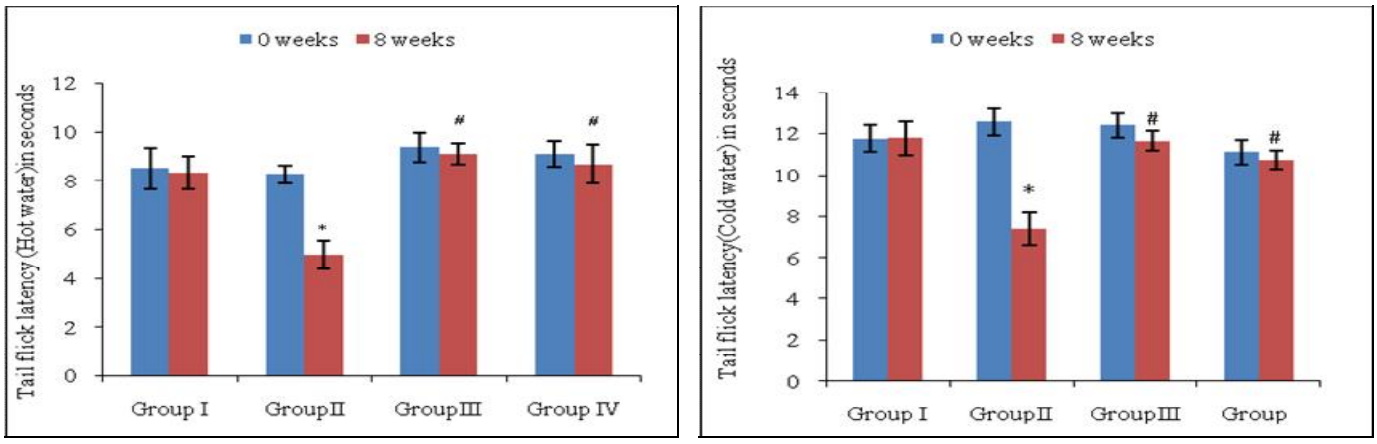

Figure 2. Effect of Wheatgrass powder on Hot and Cold Allodynia

Values were expressed in Mean ± SEM (n=8/group). * Significantly different from Normal control group ($p < 0.05$). # Significantly different from Diabetic control group ($p < 0.05$). **Group I:** Normal control. **Group II:** Diabetic control **Group III:** 15 mg/kg Pregabalin treated group (PREG 15). **Group IV:** 500 mg/kg Wheatgrass powder treated group (WG 500).

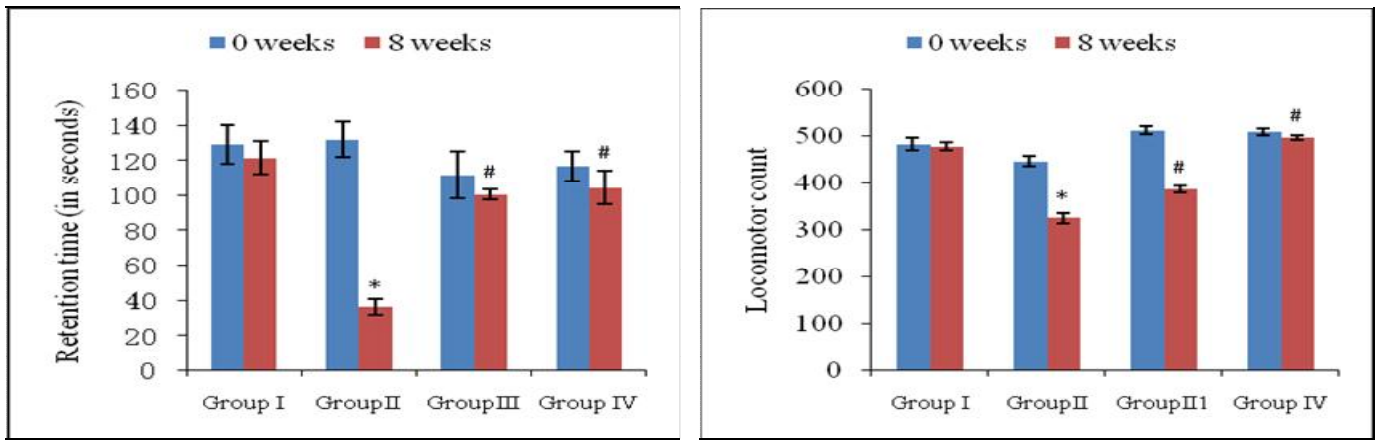

Figure 3. Effect of Wheatgrass powder on Motor coordination and loco motor activity

Values were expressed in Mean ± SEM (n=8/group). * Significantly different from Normal control group ($p < 0.05$). # Significantly different from Diabetic control group ($p < 0.05$). **Group I:** Normal control. **Group II:** Diabetic control **Group III:** 15 mg/kg Pregabalin treated group (PREG 15). **Group IV:** 500 mg/kg Wheatgrass powder treated group (WG 500).

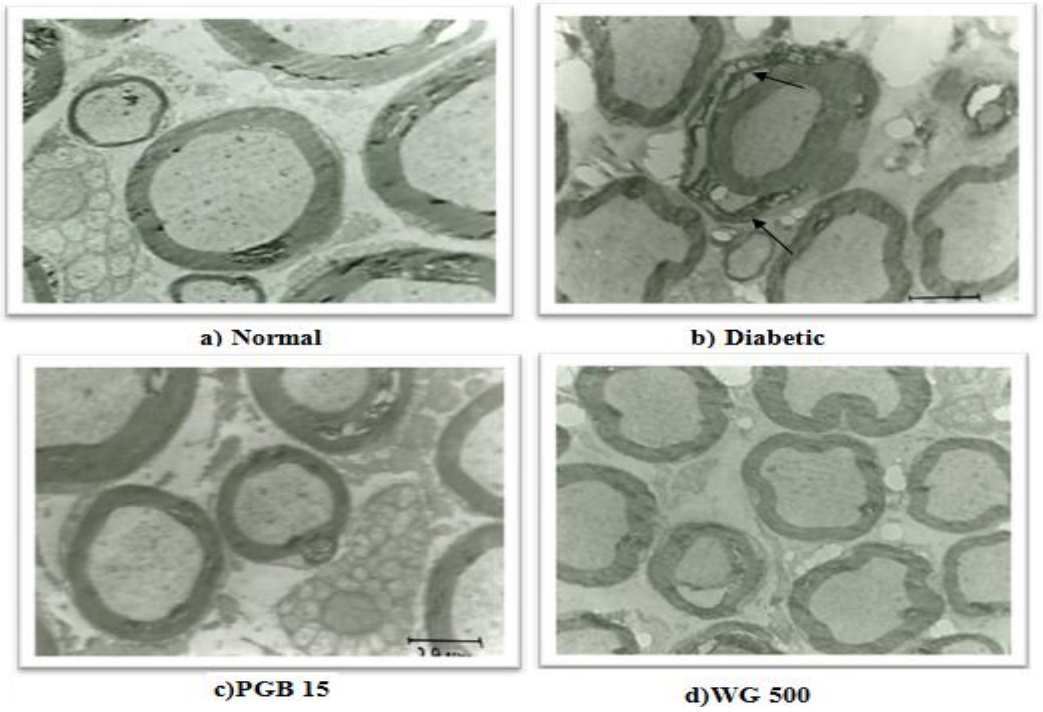

Figure 4. Transmission Electron microscopy of rat sciatic nerves

a) Normal rat **b)** Diabetic rat with Wallarian degeneration and Demyelination
c) Pregabalin treated **d)**WG 500 treated