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PROTECTIVE EFFECTS OF *COCCINIA GRANDIS* LEAF EXTRACT: BEHAVIOURAL, ELECTROPHYSIOLOGICAL, BIOCHEMICAL AND HISTOLOGICAL FEATURES OF DIABETIC NEUROPATHY

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Keywords:

Coccinia grandis, Streptozotocin, Nerve conduction velocity, Aldose reductase, Diabetic neuropathy

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ABSTRACT: Present study aimed to evaluate the nociceptive, neuro-muscular coordination, electrophysiological, biochemical, and histological features of diabetic neuropathy in streptozotocin-induced diabetic rats and the protective effects of *Coccinia grandis* leaf methanolic extract. *Coccinia grandis (*200 mg/kg per day) was given to diabetic rats for 3 weeks. Metformin (150 mg/kg body weight) was used as standard reference drug. *Coccinia grandis* showed its protection against allodynia test. NCV was also attenuated by treatment of Coccinia. The significant decrease in AR shows its protection against diabetic complications. Histological alterations induced by diabetes in the sciatic nerve were restored with *Coccinia grandis* leaf extract treatment. These results suggest that *Coccinia grandis* has attenuated progression of diabetic neuropathy in STZ-induced diabetic rats.

INTRODUCTION: Diabetic neuropathic pain is one of the most recognized most difficult types of pain, which is a symptom of diabetic neuropathy (DN). DN is the microvascular complication associated with diabetes [1]. Peripheral nerve injury is associated with neuropathic pain and is characterized by the sensory abnormalities such as unpleasant abnormal sensation (dysesthesia), an increased response to painful stimuli (hyperalgesia), and pain in response to a stimulus that does not normally provoke pain (allodynia) [2]. The various proposed mechanism which leads to pathogenesis of diabetic neuropathy is activated polyol pathway [3], AGE's formation [4], PKC activation [5,] and Hexosamine pathway [6].

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Hyperglycemia is the primary culprit for diabetic neuropathy. There have been major advances in the control of hyperglycemia (diabetes), through dietary changes, hypoglycemic agents, insulin and islet transplantation, even though the long-term complication of diabetes, such as neuropathy remains a serious problem. Although, the oxidative stress is a major determinant in diabetic complications including diabetic neuropathy, which is a result of cross-links between above pathways, which was proposed by several, studies [6, 7, 8]. Therefore, agents or compounds that exert multiple actions, such as antioxidant, antidiabetic/ hypoglycemic, AR inhibitory, and antiglycation properties, could be more effective than agents with a single action.

Coccinia grandis, the ivy guard, of the family Cucurbitaceae is distributed in tropical Asia, Africa and is commonly found in India, Bangladesh, and Srilanka. Since, long before the leaves are consumed to control of hyperglycemia as an

indigenous system of medicine. Aqueous fractions significantly inhibited inflammation, which can be thought to possess antiproliferative and antiarthritic activities similar to cyclooxygenase inhibitor [12]. Every part of this plant is valuable in medicine, and various preparations have been mention in indigenous system of medicine for various skin diseases, bronchial catarrh, bronchitis and Unani systems of medicine for ringworm, psoriasis, smallpox, and scabies and other itchy skin eruptions and ulcers [13]. The leaves of this species are widely used in Indian folk medicine for reducing the amount of sugar in the urine of patients suffering from diabetes mellitus. Literature suggests the use of this plant in the treatment of diabetes [14].

The present study reports the possible protective effects of *Coccinia grandis* leaf extract on serum glucose, neuronal protein carbonyls, allodynia, motor coordination, nerve conduction velocity, Aldose reductase, and Na^+K^+ ATPase activity.

MATERIAL AND METHODS:

Plant Material and Extraction: The fresh leaves of *Coccinia grandis* were collected locally. Leaves were then shade dried at room temperature. The dry material was coarsely pulverized to powdered form. The powder was extracted with boiling water, and ethanol using rotary evaporator, and the crude extracted was used for an experiment. A voucher specimen (No. 018) was deposited at Department of Botany, University College of Science, Osmania University, Hyderabad-500007.

Animals: Adult male albino rats of Wistar strain (NIN) aged 11–12 weeks (100-200g wt.,) obtained from National center for laboratory animal sciences, NIN, Hyderabad were used. The animals were housed in plastic cages, which were maintained in the climate-controlled animal facility (Department of Zoology, Osmania University, Hyderabad) with a 12-h light/12 h dark cycle at a stable temperature 18-22 ◦C with standard pellet diet (NIN) and water *ad libitum*. All experiments involving animals were conducted according to guidelines of the Institutional Animal Ethics Committee (CPCSEA No: 383/01/a/ CPCSE).

Chemicals: STZ was obtained from Sigma Chemical (USA). Metformin drug procured from Hetero drugs, INDIA. Other essential chemicals were obtained from SRL biochemical, INDIA.

Experimental Design: The animals were divided into five groups, each containing six animals each.

1. **Groups I:** This group served as untreated control and received physiological saline.
2. **Group II:** This group served as the positive control (diabetic) treated with STZ (Streptozocin 50 mg/Kg body weight in 100mM Citrate buffer pH 4.5.
3. **Group III:** This group served as Metformin, wherein STZ induced diabetic animals treated with Metformin drug (150mg/kg body weight in RO water).
4. **Group IV:** This group served as Coc+D, STZ induced diabetic animals treated with *Coccinia grandis* leaf extract (200mg/kg body weight in RO water).
5. **Group V:** This group served as Coc+C, control animals were treated with *Coccinia grandis* leaf extract (200mg/kg body weight in RO water).

The experiments were carried out to see the short-term effect of diabetes on the sciatic nerve. The animals were sacrificed after 21 days, and various biochemical and histological studies were conducted on the sciatic nerve.

Analgesic Test:

1. **Allodynia Test:** Allodynia test was conducted as described by Bennet and Xie, 1998 [15], to observe the sensitivity of animals to cold.
2. **Neuro-muscular Coordination:** Neuro-muscular coordination and balance were measured on Roto rod as described Carter *et al.* 1999 [16], which were used to assess the ability of an animal to balance on a rotating rod at the specific speed of 24 RPM. The time taken was recorded in seconds [17].
3. **Nerve Conduction Velocity:** *In-vivo* nerve conduction experiments were performed in deep anesthesia with 60 mg/kg body wt., of

sodium pentobarbitone, supplemental pentobarbitone was provided during the entire experiment through i.p. injection. Sciatic nerve about 4-6 mm at mid-thigh region was exposed and freed from adherent tissue. Animal body temperature maintained at 37 °C with a warm blanket and provided the O_2, CO_2 supply with 90:10 ratios.

Nerve perfused with mammalian ringer solution to prevent the drying of tissue. The electrodes place at two sites of the sciatic nerve; one is placed at the site of injury (for recording) another placed near the ischium or just proximal to the injury site (stimulator). The setup is ready for recording. The nerve conduction velocity (NCV) was calculated by the ratio distance between the two sites of stimulation in mm divided by the difference between proximal and distal latencies in ms, giving a value for NCV in meters per second (m/s) [18].

Biochemical Estimations:

Preparation of Tissue Extracts: The animals were sacrificed by cervical dislocation after the 21st day, and sciatic nerves carefully dissected out avoiding extraneous tissue, washed with normal saline, blotted dry, the nerves were immediately transferred and kept at 80 ºC to be used later.10% tissue (sciatic nerve) homogenate was prepared in 50mM potassium phosphate buffer pH 7.2 and centrifuged at 25,000g for 30 min at 4°C and the supernatant is used for Aldose reductase (AR) activity.

Estimation of Aldose Reductase (AR, EC.1.1.1.21): The oxidation of NADPH is measured as an index of Aldose reductase activity by Hayman and Kinoshita [19].

Other Estimations: Blood glucose was estimated in the plasma using glucose measuring kit from (Beacon Diagnostics Pvt., Ltd.), New Delhi India., utilizing glucose oxidase-peroxidase (GOD-POD) method.

The extent of protein oxidation was determined by measuring the protein carbonyl content of soluble protein of tissues (sciatic nerve) spectrophoto-metrically using 2, 4,-dinitro phenyl-hydrazine [20].

Histological Processing: Fixation of nerves and staining was done as explain by Federica Di Scipio, *et al*., 2008 [21].

Statistical Analysis: Results are presented as mean ± S.E., six in each group. Statistical difference between control and various groups was determined by one-way ANOVA, followed by post Hoc test (Multiple comparisons). *p*-values less than 0.05 were considered significant.

RESULTS: The body weight, serum glucose levels, and protein carbonyls of all the experimental groups with diabetes are shown in **Fig. 1, Fig. 2** and **Fig. 3**. The body weights of STZ induced diabetic rats were found to be reducing throughout the study.

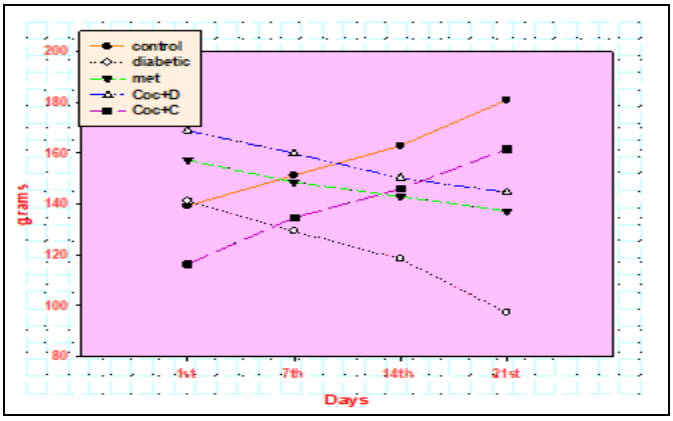

FIG. 1: EFFECT OF *COCCINIA GRANDIS* LEAF EXTRACT ON BODY WEIGHT IN RATS. (Body weight in grams) (Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at p<0.05.Significance Control *vs.* Diabetes is <0.005; Control Vs Met is <0.77; Control *vs.* Coc+C is < 0.006; Diabetes *vs.* Coc+D is <0.6; Diabetes *vs.* Coc+C is < 0.009; Met *vs.* Coc+D is <0.1; Met *vs.* Coc+C is < 0.3; Coc+D *vs.* Coc+C is < 0.01 respectively)

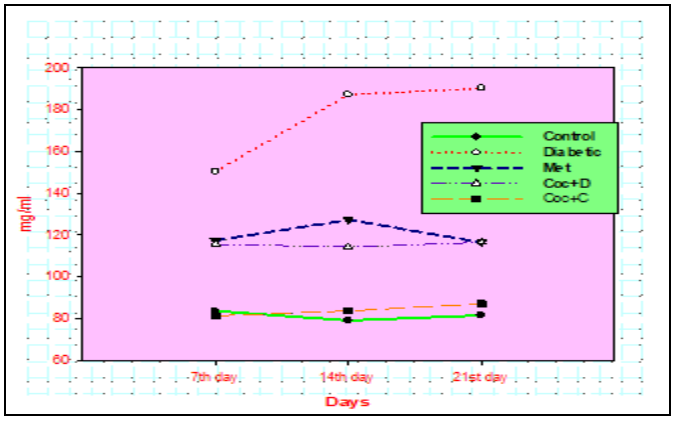

FIG. 2: EFFECT OF *COCCINIA GRANDIS* LEAF EXTRACT ON SERUM GLUCOSE LEVELS IN RATS. (Serum glucose levels expressed in mg /dl) (Values are given as mean ± Std. E for groups of six animals each. Values are statistically significant at p<0.05.Significance Control *vs.* Coc+C is < 0.3; Met *vs.* Coc+D is < 0.315 respectively).

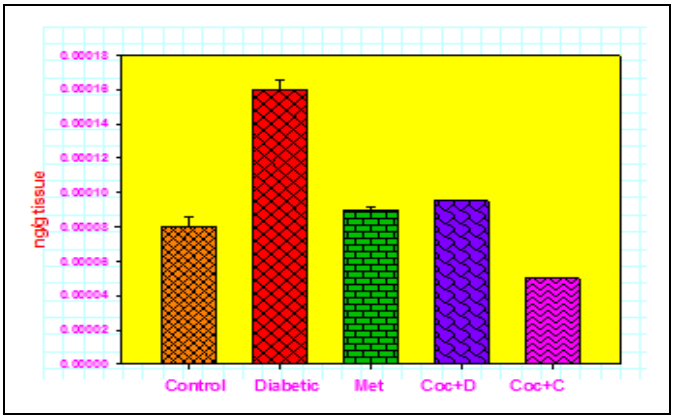

FIG. 3: EFFECT OF *COCCINIA GRANDIS* LEAF EXTRACT ON PROTEIN CARBONYLS LEVELS OF SCIATIC NERVE IN RATS. (Protein carbonyls are expressed in ng/gram tissue) (Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at p<0.05. Significance Control *vs*. Diabetes is <0.006; Control *vs*. Met is <0.329; Control *vs*. Coc+D is <0.004; Control *vs*. Coc+C is < 0.151; Diabetes *vs*. Met is <0.04; Diabetes *vs*. Coc+D is <0.8; Diabetes *vs*. Coc+C is <0.1; Met+D *vs*. Coc+D is < 0.02; Met+D *vs*. Coc+C is < 0.6; Coc+D *vs*. Coc+C is 0.06 respectively).

However, the percentage changes in body weights of metformin-treated and *Coccinia grandis* leaf extract treated diabetic rats were not significantly different compared to the diabetic control rats. Blood glucose levels were persistently high STZ-induced diabetes in rats (169%) in comparison to the control group, which was restored to 43% in metformin-treated animals and 43% in *Coccinia grandis* leaf extract treated animals. Levels of protein carbonyls an important marker of the long-term AGE's state was found to be significantly elevated in short-term (p<0.05) diabetic animals compared to control. The protein carbonyls levels in the Met group were found significantly nearer to the control group, and that of Coc+D is -42% compared to the control group.

Measurement of Antinociceptive Activity: At the end of the 3rd week, the nociceptive threshold was significantly lower in diabetic rats from non-noxious stimuli as compared to control **Fig. 4**.

Treatment with *Coccinia grandis* by the end of 3rd week significantly increased the pain threshold in allodynia test. The rotarod latencies of all experimental rats are shown in **Fig. 5**.

The STZ induced diabetic rats have shown a progressive decrease in coordination with the periods. The decrease in coordination was moderate on the 14th day with thereupon it has shown a further decline in the coordination indicating neuropathy on the 21st day. The subsequent treatment with *Coccinia grandis* leaf extract was found reverse the coordination on the 14th day; thereafter, the coordination almost started to decline on 21st day respectively.

Nerve Conduction Velocity: After 3 week's experimentation, sciatic nerves NCV was measured. Diabetic rats had shown 27% lower NCV than controls **Fig. 6**.

Coccinia grandis leaf extract treatment of diabetic rats (Coc+D) resulted in -14% lower NCV compared to control. However, the NCV of diabetic rats treated with metformin (met) was -22% lower than control.

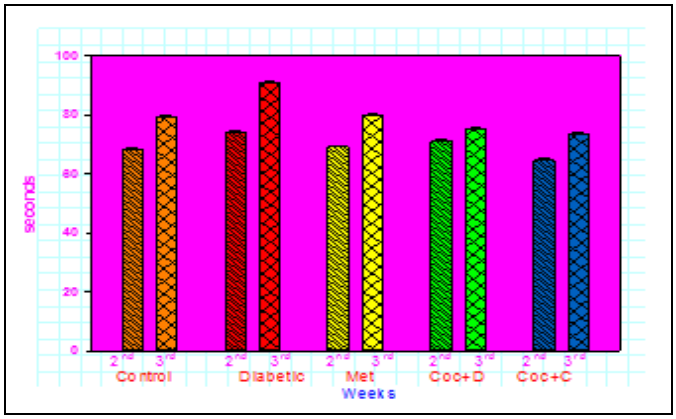

FIG. 4 EFFECT OF *COCCINIA GRANDIS* LEAF EXTRACT ON ALLODYNIA TEST IN RATS. (Allodynia latency is expressed in seconds) (Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at p<0.05 Significance Control *vs*. Met is < 0.1; Control *vs*. Coc+D is <0.2; Met *vs*. Coc+D is < 0.01; Control *vs*. Cur+D is <0.03 respectively).

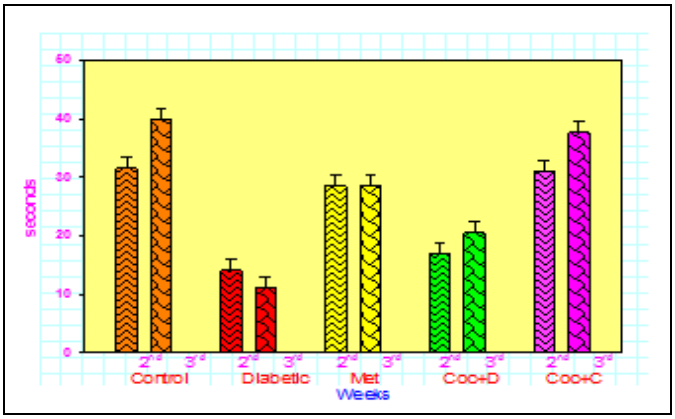

FIG. 5: EFFECT OF *COCCINIA GRANDIS* LEAF EXTRACT ON NEUROMUSCULAR COORDINATION TEST IN RATS ON GIVEN WEEKS. (Coordination test is expressed in seconds) (Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at p<0.05. Significance Control *vs*. Met is < 0.01; Control *vs*. Coc+C is < 0.4; Diabetes *vs*. Coc+D is <0.003; Met *vs*. Coc+C is < 0.004; respectively)

Aldose Reductase and Na^+-K^+ ATPase Activity of Sciatic Nerve: There was a significant increase in aldose reductase enzyme activity in the sciatic nerve of diabetic animals (+83%) as compared to normal animals. Diabetic rats treated with *Coccinia grandis* leaf extract showed a decrease in aldose reductase activity by +50% **Fig. 7**.

Percentage of variation of a metformin-treated diabetic was +61%, and that of control animals treated with *Coccinia grandis* leaf extract was +1%. Sciatic nerve Na^+-K^+ ATPase activity **Fig. 8** was significantly reduced in diabetic rats (-77%) as compared to normal control. This was largely corrected by *Coccinia grandis* leaf extract treatment by +8%. Percentage of variation of a metformin-treated diabetic was -46%, and that of control animals treated with *Coccinia grandis* leaf extract was -6%.

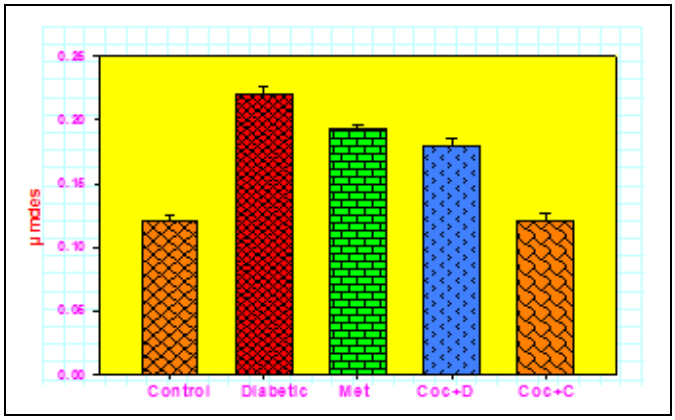

FIG. 7: EFFECT OF *COCCINIA GRANDIS* LEAF EXTRACT ON AR ACTIVITY OF SCIATIC NERVE IN RATS ON 21ST DAY. (Expressed as µ moles of NADPH oxidized/ hour/100 mg of protein) (Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at $p<0.05$ Significance Met *vs.* Coc+C is < 0.015 respectively)

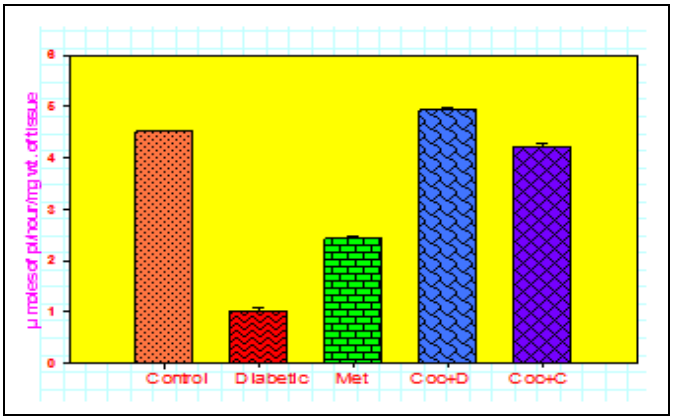

FIG. 8: EFFECT OF *COCCINIA GRANDIS* LEAF EXTRACT ON Na^+K^+ ATPASE ACTIVITY OF SCIATIC NERVE IN RATS ON 21ST DAY. (Expressed as µ moles of pi/ hour/mg wt. of tissue) (Values are given as mean ± Std.E for groups of six animals each. Values are statistically significant at $p<0.05$ Significance control *vs.* diabetes is ns, con *vs.* met is ns; con *vs.* Coc+D is ns; con *vs.* Coc+c is ns; diabetes *vs.* met is ns; diabetes vs. Coc+D is ns; diabetes *vs.* Coc+C is ns respectively)

Histological Changes in Sciatic Nerve:

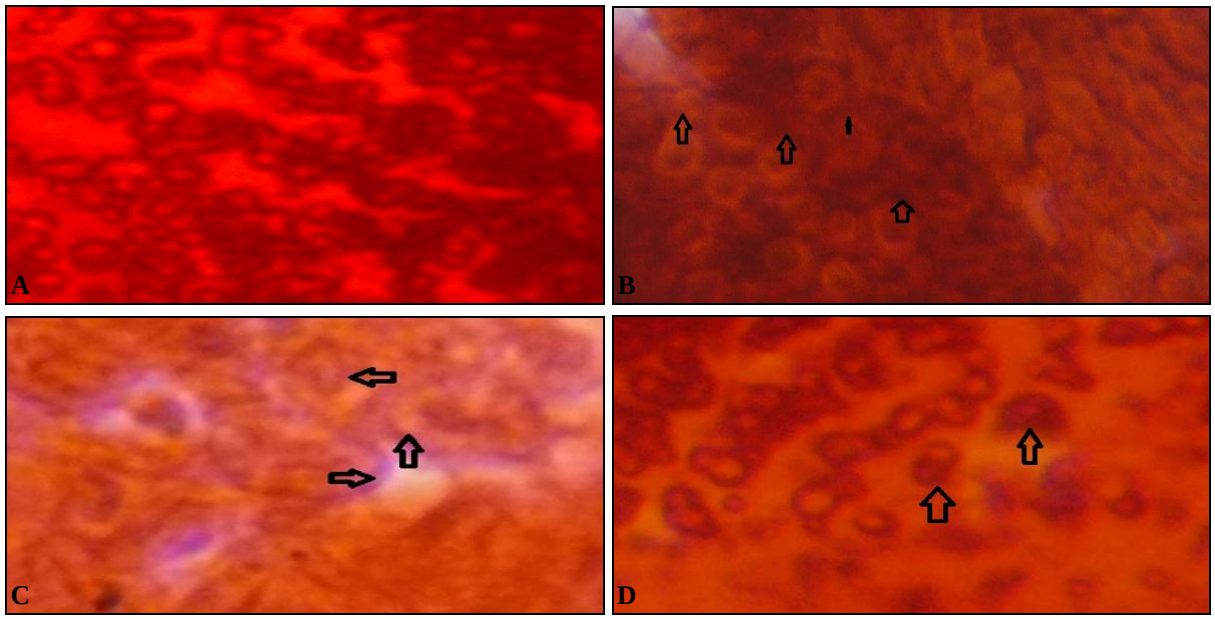

FIG. 9: PHOTOMICROGRAPHS SHOWING THE TRANSVERSE SECTIONS OF THE SCIATIC NERVES. Fig. A is of Control rat (5µm thick, 100X+Immersion oil) showing normal histological features with thick myelin membrane. B is the photomicrograph of T.S of Sciatic nerve of STZ-induced diabetic rat, showing axonal atrophy (swelling), and Increased extra-axonal space among nerve fibers (↑), deposition of electron-dense material within the axons. In Fig.C STZ-induced diabetic rats treated with metformin showed onion bulb formation (→) and also segmental demyelination. STZ-induced diabetic rats treated with Curcumin (Cur+D) are shown in Fig.D, no demyelination, and no onion bulb formation is seen, and also myelin membrane is intact (↑). Control animals treated with Curcumin have not shown any significant histological changes (Fig not shown).

DISCUSSION: The present work is undertaken to assess the alterations in serum glucose levels and nociceptive tests after systematic treatment with *Coccinia grandis* leaf extract. The STZ induced diabetic rats have shown a marked increase in the serum glucose levels after 7^{th},$14^{th,}$ and 21^{st} day indicating the hyperglycemia when compared with controls. The increase in serum glucose levels was observed due to diabetogenic action of STZ in diabetic rats (Diabetic group) on all the periods [22].

After simultaneous treatment, *Coccinia grandis* leaf extract the serum glucose levels were decreased significantly. And the antidiabetic activity of *Coccinia grandis* may be due to various steps which result in the increased glucose tolerance. Reduction of sugar absorption from the gut increased insulin production from the pancreas, reduction of the release of glucose from the liver, increasing glucose uptake by fat and muscle cells are probable mechanisms which may be involved [24].

Neuropathic pain associated with peripheral nerve injury is characterized by the sensory abnormalities such as unpleasant abnormal sensation (dysesthesia), an increased response to painful stimuli (hyperalgesia), and pain in response to a stimulus that does not normally provoke pain (allodynia) [25]. Spontaneously diabetic mice with hyperglycemia have shown a decreased sensitivity to the antinociceptive effects of morphine [26]. The behavioral alterations start on the 3^{rd} day after STZ-induced diabetes in rats and last throughout the experimental period showing hyperalgesia. These observations were reported earlier findings [27, 28].

In the present study, STZ-induced diabetic rat group has shown, a significant increase in hind paw cumulative withdrawal latencies in allodynia response showing hyperalgesia. Administration of Metformin and *Coccinia grandis* resulted in consequent decrease was observed in allodynia response, compared to diabetic rats suggesting a reduction of hyperalgesic condition. The antinociceptive effect of *Coccinia grandis* may be attributed to its powerful antioxidant activity. *Coccinia grandis* leaf extract treatments were efficacious in reversing and preventing impairment of normal motor function as assessed on the Roto rod. These behavioral abnormalities are hypothesized to be a consequence of the irregularities and variability in muscle spindle group Ia innervation.

Neuropathic pain is characterized by decreased nerve conduction velocities. The change in microenvironment of the nerve involving metabolic and vascular processes have been proposed, that is leading to early reductions in nerve conduction velocities [29]. Further slowing of nerve conduction velocity may be due to structural changes. The key findings of the present study were to report that *Coccinia grandis* leaf extract has prevented diabetes-induced deficit in NCV. This protection occurred in line with that of obvious alteration in the biochemical indexes measured, which included Na^+-K^+-ATPase activity and polyol pathway enzyme such as AR. Altered polyol metabolism and an associated decrease in neuronal myo-inositol content were reported to be associated with diabetes [30].

This finding suggests that lower tissue myo-inositol concentrations may result in abnormal neuronal Na^+-K^+-ATPase activity through a reduction in the formation of diacylglycerols and inositol trisphosphate and incomplete activation of the sodium pump by activated protein kinase C [31]. Lower Na^+-K^+ ATPase activity may underpin the depressed NCV in diabetes. Also, inhibition of aldose reductase has been reported to restore neuronal polyol metabolite content (principally the myo-inositol) and the nerve conduction deficit in diabetes.

In the present study, *Coccinia grandis* leaf extract treatment ameliorates diabetes-associated changes in Na^+-K^+ ATPase and NCV. These effects were associated with attenuated anatomical damage and preventive effect on the bimodal distribution of myelinated fiber diameters. The improved NCV was strongly correlated with Na^+-K^+ ATPase.

CONCLUSION: In conclusion, data from the present study show that Coccinia exhibited antinociceptive action. There is a significant decrease in levels of AR, Na^+-K^+ ATPase treated groups indicating the protective role of Coccinia against STZ induced alignments, could be beneficial in preventing the progression of diabetic neuropathy. Further studies are anticipated for

phytochemical investigations to isolate the active compounds and lead to their further clinical use.

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CONFLICT OF INTEREST: Nil

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