Abstract

Chronic pain such as peripheral neuropathy is the major complication in long term hyperglycemia. The present context was conducted to explore the protective effect of ethanolic extract of S. robusta resin (SRE) in diabetes associated pain in rats as resin has phenolic components, hypoglycemic and anti-oxidative properties. Pure resin was extracted with 70% ethanol using a Soxhlet apparatus and evaporated under rotary vacuum evaporator. Diabetic peripheral neuropathy rat model was generated by single intraperitoneal injection of streptozotocin (STZ). Rats were treated for 8 week as follows: normal control with vehicle, normal with SRE (300 mg/kg), Diabetic control with vehicle and Diabetic with SRE (30,100 and 300 mg/kg). To evaluate the effects various parameters, including nociceptive response (tail flick and formalin test), oxidative stress status (Super oxide dismutase (SOD), catalase, lipid peroxidation (MDA) and nitric oxide (NO)) and histopathology (H&E) were assessed. Behavioral assessment showed significant hyperalgesia in diabetic rats but long term administration of SRE caused a concentration dependent increase in pain threshold, restored the activity of SOD and catalase activity, as well as reduced the MDA and NO level significantly (p<0.05). Furthermore diabetic rats exhibited loss of myelin sheath, loose and disorganized sciatic nerve fibers these changes were also ameliorated in treatment groups. In conclusion the results suggest that S. robusta resin can limit neurodegenerative changes in sciatic nerve by suppression of STZ-induced hyperglycemia associated oxidative stress may represent promising agents to improve peripheral nervous system functioning.

Keywords: Diabetes, Shorea robusta, resin, pain, oxidative stress

Introduction

Diabetes is a metabolic syndrome characterized by hyperglycemia caused impaired metabolism of proteins and lipids [1]. Excessive entry of glucose during long term hyperglycemia associated with polyol pathway and advanced glycation end products (AGEs) formation facilitate the production of large amount of oxidants cause microvascular and macrovascular complications, which led to a series of detrimental reactions [2]. Chronic pain such as peripheral neuropathy is the major complication characterized by loss of sensation, pain perception, hyperesthesia and allodynia. Recently, there has been an upsurge of interest in the therapeutic potential of plants as antioxidants in reducing oxidative tissue injuries. The Indian ethnomedicine Shorea robusta Gaertn. f. (Dipterocarpaceae) is a large sub-deciduous tree, found extensively in parts of North-East and Central India known as Sal or Shala, was documented by Edward J. Waring in Pharmacopoeia of India (1866) indicates S. robusta officinal in British pharmacopoeia which is resembles to Pine resins. Interestingly, monograph on S. robusta has been mentioned in the Ayurvedic Pharmacopoeia of India (2008) introduced by Department of AYUSH, Government of India. Besides the plaster of fumigation, resin is an astringent used therapeutically, in diarrhea, dysentery, and gonorrhea. In addition of that resin has, Anti-inflammatory [3], wound healing [4], analgesic [5] Anti obesity [6] and microbicidal [7] activity in rat model. Since, there is no report available on the use of ethanolic extract of S. robusta resin in diabetes associated pain. Therefore behavioral experiments and oxidative stress measurement were conducted to describe the ameliorative effect of S. robusta in diabetes associated nerve injury.

Materials and Methods

Plant material and preparation of extract

Pure resin of S. robusta was purchased from a local market of Bhubaneswar, India. It was ground into powder then extracted with 70% ethanol under reflux for 72 h using a Soxhlet.
apparatus. Hydro alcoholic extract was filtered and evaporated to dryness under reduced pressure in rotary vacuum evaporator at 40°C temperature and further kept in vacuum desiccators for complete removal of solvent and the yield of the extract was calculated \( [8] \). Hereafter, extract is to be considered as ‘SRE’ was stored at 4°C until use.

**Animals**

Apparently healthy adult male Wistar rats (200-220g) were procured from Laboratory Animal Resource Section, ICAR - Indian Veterinary Research Institute, Izatnagar, India. The animals were housed in polypropylene cages and kept for a week under acclimatization period with free access to standard feed and water and maintained on a 12 hour dark-to-light cycle in an air controlled room (temperature 22±2°C, humidity 55±5%). The experimental protocols involved in this study were according to the guidelines for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Induction of diabetes

Diabetes was induced by streptozotocin (STZ) freshly prepared in citrate buffer pH 4.5 administered single injection intraperitoneal @ 60 mg/kg body weight to overnight starved rats those having fasting blood glucose level in a normal range. Similarly, control group received only equal volume of citrate buffer to nullify its effect. Initially 2% sucrose solution was given in drinking water for 48 h to alleviate the sudden hypoglycemic phase. After 72 h blood glucose was monitored by tail pricking using digital glucometer (On-Call-Plus, ACON Biotech). Rats having blood glucose on or above 300 mg/dl were selected for further study and experiment was started after 15 days of observation period.

**Experimental design**

The animals were randomly divided into 6 groups of 6 animals as follows: NC (normal control rats treated with vehicle), NE (normal rats treated with SRE 300 mg/kg), DC (diabetic rats treated with vehicle), DE1, DE2 and DE3 (diabetic rats treated with SRE 30, 100 and 300 mg/kg, respectively). SRE was dissolved in aqueous vehicle containing 0.5% carboxymethylcellulose and administered orally once in a day by using 16G gastric gavages for 8 weeks, control groups received only vehicle. Evaluation of nociceptive response to stimuli was performed on the end of study.

**Evaluation of nociceptive parameters**

**Tail flick test**

The central analgesic activity of SRE was studied by tail withdrawal assay, as described by D’Amour and Smith (1941) \( [9] \). After 30-min acclimatization of rats in the testing environment, radiant heat was applied to the ventral surface right from 2.25 cm tip of the tail using tail flick unit (Ugo-Basile, Italy) and the latency time for removal of the tail from the stimulus was recorded. The intensity of the heat stimulus was set to elicit a tail flick within 10-12 sec. A cut off time of 16 sec was used to prevent tissue damage. Two recordings were taken at an interval of 30 min for each rat and the mean value was calculated for statistical analysis.

**Formalin-induced nociception**

The peripheral analgesic activity of SRE was studied by formalin induced hypernociception or hind paw licking assay as described by Corea and Calixto (1993) \( [10] \). After 30-min acclimatization of rats in the testing environment, the animals were injected 50µl formalin (2.5%), intraplantar region of one hind paw thereafter immediately placed in a glass cylinder and the mean of the time spent on licking the injected paw as index of nociception in each group was recorded with a chronometer for the next 30 min. The biphasic response is characterized by an early / neurogenic phase (0-5 min), a brief quiescent period, than late / inflammatory phase (15-30 min).

**Sample Collection**

Immediately after nociceptive tests at the end of 8th week animals were sacrificed with over dose of diethyl ether. Thereafter, sciatic nerve was removed bilaterally. One portion was preserved in 10% neutral buffer formalin for histopathological evaluation. The second portion of the tissue was homogenized in ice-cold PBS. Protein concentration was estimated according to the protein estimation kit by Lowry’s method (GeNei, Merck). The aliquots were prepared and stored at -80°C for antioxidant parameters.

**Biochemical parameters**

**Super oxide dismutase (SOD) activity**

SOD activity was measured by the method of Madesh and Balsubramaniam (1998) \( [11] \). It involves the generation of superoxide by pyrogallol auto oxidation and inhibition of superoxide dependent reduction of the tetrazolium dye (MTT) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to its formazan. The reaction mixture contained 0.65 ml PBS (pH 7.4), 30µl MTT (1.25 mM), 10 µl sample and 75µl pyrogallol (100mM). The mixture was incubated at room temperature for 5 min and the reaction was stopped by adding 0.75 ml of DMSO which solubilizes formazan. The absorbance was read at 570 nm against the blank and the activity was expressed as U/mg protein.

**Catalase (CAT) activity**

CAT activity was assayed as described by Aebi (1984) \( [12] \). Sample (0.1ml) was added to 1.9 ml of 50 mM phosphate buffer (1:1.5 v/v of 50mM KH₂PO₄ and 50mM Na₂HPO₄ pH 7) in a test tube then content is transferred to cuvette. The reaction was started after adding 1 ml of H₂O₂ (10mM: 0.1 ml of 30% H₂O₂ was diluted to 100ml phosphate buffer) directly into cuvette. Optical density was recorded at every 15 sec for 1 min at 240 nm against distilled water (blank). The catalase activity was calculated using mimol extinction coefficient of H₂O₂ (0.071 nmol cm⁻¹) and the activity was expressed as U/mg protein.

**Malondialdehyde (MDA)**

Lipid per oxidation in sciatic nerve was estimated by thiobarbituric acid reactive substances TBARS method of Beuge and Aust (1978) \( [13] \). In short, 0.1 ml of sample in 200 mM Tris-HCl buffer, pH 7.5 was treated with 2 ml of (1:1:1) TBA-TCA-HCl reagent (TBA 0.37%, 0.25% HCl and 15% TCA) and placed in water bath for 15 min and cooled then centrifuged at 2000 rpm for 10 min. The absorbance of clear supernatant was measured at 535 nm against the blank. Concentration was calculated using molar extinction coefficient of MDA which is 1.56 X 10⁵ M⁻¹ cm⁻¹ and expressed as nmol/mg protein.

**Nitric oxide (NO)**

Nitrite measurement in the tissue lysate is an indirect indicator of NO production \( [14] \). Briefly, it was measured in sample(100 µl) taking equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). After 10 min incubation at room
temperature, the absorbance was measured at 550 nm in a microplate reader (SpectraMax Multi-mode). The nitrite concentration was determined from extrapolation of the calibration curve was made with standard sodium nitrite (0-100 µM).

**Hematoxylin and eosin (H&E) staining.**
The sciatic nerve tissue was fixed for 72 h in 10% neutral buffer formalin and embedded in paraffin. 5 µm thick tissue sections were obtained and stained with H&E as per standard method Sudoh et al. (2004) [15]. The gross histopathological changes in the sciatic nerve were visualized and analyzed qualitatively under light microscope (Olympus, BX41, USA) at 10x magnification.

**Statistical analysis**
Results were expressed as mean ± S.E. The Statistical significance was analyzed by applying one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test using the GraphPad Prism v5.03 software program (San Diego, California USA) and the difference between the control and treatment groups were considered statistically significant at p<0.05.

**Results**

**Extraction of S. robusta resin**
The hydro alcoholic extract of *S. robusta* resin was dark brown in color with a pasty consistency and the per cent yield was 54.3% w/w dry matter.

**Nociceptive parameters**

**Tail flick test**
Radiant heat induced tail withdrawal latency response after 8 weeks of diabetic rats treated with vehicle showed marked reduction in threshold to noxious stimuli (3.2±0.21 s) than normal control (6.08±0.24 s), which exhibits significant nociceptive response in diabetic animals compared with the control. Long term administration of SRE caused a concentration dependent increase in threshold as found highest at higher concentration in diabetic (5.21±0.30 s) and normal rats (8.26±0.40 s) compared with diabetic control and normal control, respectively (Fig. 1A).

**Formalin-induced nociception**
Formalin induced biphasic response after 8 weeks of diabetic rats treated with vehicle showed marked greater mean of licking time in early and late phase (97.22±4.51 and 240.2±6.02 s, respectively) than normal control (63.37±3.72 and 120.0±5.22 s, respectively), which exhibits significant nociceptive response in diabetic animals compared with the control. Long term administration of SRE caused a concentration dependent decrease in the paw licking time as found highest at higher concentration in diabetic (69.79±3.7 and 167.7±6.82 s, respectively) and normal rats (39.62±3.44 and 53.21± 6.35 s, respectively) compared with diabetic rats and normal rats, respectively (Fig. 1B, C).

![Fig 1: Effects of alcoholic extract of S. robusta resin (SRE) on tail withdrawal latency (A) and formalin-induced paw licking time (B and C) in different groups after at 8 week treatment. Data are presented as mean ± S.E. Different superscripts (a, b, c, d, e, f, g) differ significantly (p<0.05).](image-url)
Effect of SRE on SOD and CAT activity
There was significant reduction in SOD and CAT activity in diabetic rats as compared to non diabetic rats. Results revealed that the in vivo antioxidant effect of SRE produced a significant dose dependent increase in SOD and CAT activity in diabetic rats treated with SRE compared to control diabetic rats (P<0.05). However, higher dose of SRE maintained SOD and CAT activity in NE group. (Fig. 2A, B).

Effect of SRE on lipid per oxidation
There was significant increase in MDA level in diabetic control as compared to normal control. The in vivo antioxidant effect of SRE produced a significant dose dependent decrease in the MDA level. Long term highest dose exposure to NE group, exhibited reduced MDA level but statistically was non-significant with NC group (Fig. 2C).

Effect of SRE on NO
There was significant increase in NO level in diabetic control as compared to normal control. In diabetic treated goups, SRE produced a significant dose dependent decrease in the NO level. (Fig. 2D).

Fig 2: Effects of alcoholic extract of S. robusta resin (SRE) on superoxide dismutase (SOD), catalase, Malondialdehyde (MDA) and nitric oxide (NO) level in sciatic nerve lysate from different groups after at 8 week treatment. Data are presented as mean ± S.E. Different superscripts (a, b, c, d) differ significantly (p<0.05).

Histopathologic changes
Histological evaluation of the sciatic nerves of diabetic rats exhibited loss of myelin sheath, loose and disorganized fibers. While treatment with S. robusta resin extract reduced the changes. There was no detrimental effects were observed in normal control and extract treated normal group (Fig. 3)
The streptozotocin-induced diabetic rat model is most widely used to study diabetic neuropathy associated pain and to evaluate natural products as a herbal medicine [16]. Natural products have been extensively studied on streptozotocin-induced diabetic neuropathic pain models [17]. Neuropathic pain is the most common consequence of diabetes. Diabetic neuropathy (DN) is a complex process associated with sensory symptoms in peripheral nervous system especially sciatic nerve (SN). Advancement in proteomic and metabolomic provide pathogenesis and revealed altered polyol pathway causes dysregulation of sugar and lipid consequent enhanced advanced glycation end-products formation and exaggerated oxidative stress predominantly affects SN because of aldose reductase is one of the enzyme which regulate polyol pathway is highly expressed in SN. Moreover, tissue composition might be different as the dorsal root of ganglion and trigeminal ganglia have dense neurons, whereas high proportion of Schwann cells found in the SN [18]. Biomarkers of neuropathy in the rat models of diabetes may be behavioral include mechanical/chemical hyperalgesia, allodynia and thermal nociception [19] as well as oxidative stress related lipid peroxidation, impaired antioxidant systems viz. SOD and catalase activities [20]. In present context nociceptive threshold for thermal noxious stimuli was decreased in diabetic rats as compare to normal rats indicating augmented pain response, this is in the line of earlier reported studies [21]. However, some of the studies reported hyperalgesia [22] while others have reported hypoalgesia in the STZ model [23]. Long term administration of ethanolic extract of S. robusta resin for 8 weeks in diabetic rats exhibited significant increase in threshold for thermal noxious stimuli. Interestingly, anti-inflammatory and analgesic effect of S. robusta resin have been reported in rodents [3, 4]. In accordance with earlier findings, the antioxidant level such as SOD and catalase activity has reduced in diabetic control in comparison to normal control. Malondialdehyde is the most commonly used biomarker which can be considered as a direct index of oxidative injuries. MDA gets substantially elevated in diabetes revealing the associated lipid peroxidation [24]. Similarly MDA level was significantly raised in sciatic nerve homogenate from diabetic rats. In fact, significant defects of antioxidant defenses which may raise susceptibility to oxidative damage and consequent diabetes complications [25]. In favor, numerous studies showed peripheral chronic pain like pathological conditions might be due to high glucose induced oxidative stress-induced nerve injury such as nerve degeneration, demyelination and loss of nerve fiber [26]. Diabetic rats treated with ethanolic extract of S. robusta resin exhibited significant lower level of MDA against control group. In contrast to diabetic control, the antioxidant system was strengthened in terms of increased SOD and catalase activity after 8 week treatment with resin extract. Earlier reports [27, 28] as well as our previous findings (under publication) showed that the treatment with SRE improved significant positive outcome in diabetic rats probably by hypoglycemic effect and scavenging the reactive oxygen species (ROS) which strengthen antioxidants system owing to resin loaded with phenolic and flavonoid contents. Diabetic complications like neuropathy development could be prevented by use of antioxidant compounds-mediated inhibition of hyperglycemia and associated ROS production [29, 30]. In contrast to conventional antioxidants, SOD/catalase mimetic are beautiful choice to revert the hyperglycemia-induced mitochondrial superoxide overproduction which is the paramount factor to activate major five damaging pathways (AGE formation, RAGE expression, PKC activation, increased polyol flux and hexosamine flux) by inhibiting GAPDH [31].

In conclusion the results suggest that S. robusta resin can limit neurodegenerative changes in sciatic nerve by suppression of STZ-induced hyperglycemia associated oxidative stress and SRE may represent promising agents to improve peripheral nerve damage.
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References