4. Materials and Methods
4.I. Experimental Animals

Swiss mice (either sex, 20-30 g) and Wistar rats (either sex, 200–230 g) procured from disease free small animal house, CCS-Haryana Agricultural University, Hisar and Punjab Agriculture University, Ludhiana were employed in the present study. They were housed in institute animal house with free access to water and standard laboratory pellet chow diet. The animals with cages were kept in the departmental animal house and were exposed to normal cycles of light and dark. The experimental protocol was duly approved by the Institutional Animal Ethics Committee (IAEC) and the care of the animals was carried out as stated in the guidelines of the Committee for the Purpose of Control and supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No.-107/1999/CPCSEA).

4.II. Drugs and Chemicals

Vincristine sulfate (Chandra Bhagat Pharma Pvt. Ltd. Mumbai), pregabalin (gift sample obtained from Ranbaxy Research Laboratories, Gurgaon), Ehrlich reagent (SRL, Mumbai), NBT (Nitro Blue Tetrazolium) (Sigma Aldrich, USA), hexadecyl trimethyl ammonium bromide (HETAB), O-dianisidine hydrochloride (S.D. Fine, Mumbai India), Folin-Ciocalteu's phenol reagent (Merck Limited, Mumbai), 5, 5'-dithio, bis (2-nitrobenzoic acid) (DTNB), bovine serum albumin (BSA), (Sisco Research Laboratories Pvt. Ltd. Mumbai), were procured for the present study. All the reagents used in the present study were of analytical grade. Pregabalin and Acorus calamus extracts were suspended in 0.5 % w/v carboxy methyl cellulose solution and vincristine was diluted with normal saline.

4.III. Plant Materials

The fresh rhizome parts of Acorus calamus (AC) were collected at Kodaikanal of Tamilnadu, India and authenticated by Dr. D. Stephen, Assistant Professor, Department of Botany, American College, Madurai district, Tamilnadu. Plant sample has been kept in voucher specimen (PUP-218/2009-2010) at Punjabi University, Patiala for future reference. After authentication, fresh rhizomes of AC were collected, cleaned thoroughly with distilled water and dried under shade.
4.IV. Preparation of Extracts

4.IV.A. Preparation of Hydro-Alcoholic Extract of Acorus calamus (HAE-AC)

The shade dried rhizomes of Acorus calamus were pulverized in a mechanical grinder to obtain coarse powder (sieve no.10/40). The coarsely powdered plant material was subjected to extraction with mixture of ethanol:water (1:1, 50%) at room temperature as described by Shukla et al. (2002) as well as Parab and Mengi (2002). After completion of extraction, the solvent was completely removed by vacuum drying at low temperature (<50ºC). The yield of hydroalcoholic extract was found to be 26.4% (w/w).

4.IV.A.1. Phytochemical Analysis

Phytochemical analysis was carried out in hydroalcoholic extract of Acorus calamus for identification of flavonoids, saponins, tannins, steroids, alkaloids, and phenolic compounds as described by Evans (2009), and Harborne (1998). Briefly, Mg-HCl and Zn-HCl were used for flavonoids, foam test was used for saponins, ferric chloride and gelatin were used for tannins, acetic anhydride and sulphuric acid were used for steroids, Wagner’s and Heger’s reagents were used for alkaloid, and ferric chloride was used for phenolic compounds (data not shown). The experiments were carried out three times for each phytochemical constituent. Further, the hydroalcoholic extracts was also evaluated for absence of β-asarone by measuring the extinction at 253 and 303 nm using HPLC method. β-asarone a phyto-constituent of Acorus calamus has been reported to be responsible for psychedelic (hallucinogen), sedative and carcinogenic actions (Zanoli et al., 1988; Chen et al., 2009). Hence AC free from β-asarone was selected for the present study.

4.IV.B. Preparation of Saponin Rich Extract of Acorus calamus From HAE-AC (SRE-AC)

Saponins rich extract was prepared from hydroalcoholic extract of Acorus calamus as described in the method of Harborne (1988) with some modification. Briefly, the aqueous extract of Acorus calamus (100 g) was refluxed with n-butanol for 2 h and n-butanol soluble constituents were separated by filtration. The n-butanol layer was sequentially washed with distilled water, alkali (2% KOH) and distilled water again. The n-butanol layer was evaporated and dried under vacuum to obtain a clear powder of crude saponins.

4.IV.B.1. Determination of Total Saponins
The total saponins content of SRE-SD was determined by the vanillin-sulfuric acid method as described by Hiai *et al.* (1976). This extract was mixed with vanillin (8%, w/v) and sulfuric acid (72%, w/v). The mixture was incubated at 60°C for 10 min, cooled in an ice water bath for another 15 min followed by absorbance measurement at 538 nm. Ursolic acid was used as a reference standard and the content of total saponins was expressed as ursolic acid equivalents (UA mg/mg extract).

4.V. Toxicity Study

4.V.A. Acute Oral Toxicity Test in Mice

Acute toxicity tests were performed in both male and female mice in order to evaluate the toxic effect of hydro-alcoholic extract of *Acorus calamus* (2500, 5000, 7500, and 10000 mg/kg, *p.o.* ) in different gender groups. All deviations in general behavior i.e., convulsions, disorientation, hypoactivity, hyperventilation, pilo-erection and mortality rate in mice were monitored continuously for 24 hours after dosing completed. The median lethal dose (LD$_{50}$) was calculated according to the method of Litchfield and Wilcoxon, (1949), and Miller and Tainter, (1944). The experimental procedure for toxicity studies was followed according to the Organization for Economic Co-operation and development (OECD) guideline no. 401 and 425 (Combes *et al.*, 2006; Lipnick *et al.*, 1995; Schlede *et al.*, 1995).

4.V.B. Sub-Acute Oral Toxicity Test in Rat

Sub-acute toxicity tests were performed in rats of both sexes, in order to evaluate the toxic effect of the HAE-AC in different gender groups. Based on median lethal dose (LD$_{50}$) values (5070.59 mg/kg) obtained from acute toxicity studies, the selection of doses for sub-acute toxicity study was carried out. The dose selected for sub-acute toxicity studies ranges from 200 mg/kg, *p.o.* to 1000 mg/kg, *p.o.* The dosing was done daily and the rats were observed for 28 days for signs of sub-acute toxicity i.e. mortality, and the body weight changes. On the 28$^{th}$ day, the animals were anaesthetized with chloroform and sacrificed. Whole blood samples were collected by cardiac puncture and transferred into ethylenediaminetetraacetic acid (EDTA), sodium fluoride and gel containing tubes for hematological and biochemical analysis. Tissue sample i.e, liver, heart, and kidney were
collected from the rats. Further, tissue samples were weighed, observed for color changes of the vital organs and then subjected to histopathological examinations.

4.VI. Induction of Neuropathic Pain in Rats

4.VI.A. Painful Peripheral Neuropathy by Tibial and Sural Nerve Transection

Peripheral neuropathic pain was induced by tibial and sural nerve transection as described by the method of Lee et al., (2000). Briefly, the rat was anesthetized with thiopental sodium (35 mg/kg i.p.). The skin layer of the lateral surface on the left thigh was incised and a cut was made directly through the biceps femoris muscle to expose the sciatic nerve and its three terminal branches (the sural, common peroneal and tibial nerves) were identified. Thereafter, the tibial and sural nerves were ligated, with silk suture and transection was made at 2 mm from the ligation at distal side. The common peroneal nerve was left intact and no contact was made with it. The muscle and the skin layers were closed immediately. Sham control group was performed by exposing the sciatic nerve and its branches without inducing any lesion.

Fig. 4. Induction of neuropathic pain by tibial and sural nerve transection (TST).  

4.VI.B. Painful Peripheral Neuropathy by CCI of Sciatic Nerve

Peripheral neuropathy was induced in rats by chronic constriction injury (CCI) as described by the method of Bennett and Xie (1988), with slight modifications (Sommer and Schafers, 1998). In brief, rats were deeply anesthetized with thiopental sodium (35 mg/kg
The hair of the rat’s lower back and thigh were shaved, and the skin was sterilized with 0.5% chlorhexidine. The skin of the lateral surface of the left thigh was incised and a cut was made directly through the biceps femoris muscle to expose the sciatic nerve and four ligatures (silk 4-0), were placed around the nerve proximal part of the trifurcation with a distance of one millimeter between each ligature. The ligatures were loosely tied until a short flick of the ipsilateral hind limb was observed. After performing nerve ligation, muscular and skin layer was immediately sutured with thread, and topical antibiotic was applied.

Fig. 5. Induction of painful peripheral neuropathy by chronic constriction injury (CCI) of sciatic nerve.

4.VI.C. Peripheral Neuropathy by Vincristine

Peripheral neuropathy was induced in rats by administration of vincristine sulfate (50 and 75 µg/kg, i.p.) for 10 consecutive days as described previously (Siau and Bennett, 2006).

Fig. 6. Induction of painful peripheral neuropathy by vincristine administration.
4.VII. Behavioral Assessment for Painful Neuropathic Parameters

All the behavioral tests were carried out on the different time intervals as per study protocol, i.e., 0, 1, 3, 6, 9, 12, 15, 18 and 21st day. Spontaneous motor activity, motor coordination test, body and organ weight changes were performed in the acute and sub-acute oral toxicity study protocol to find behavioral, motor function, whole body and vital organ mass changes.

4.VII.A. Motor Co-ordination Test (Rota-Rod Test)

Motor co-ordination (grip muscle strength) was evaluated by rota-rod device as described by Jones and Roberts (1968) with slight modification of Muthuraman et al. (2008). Rats were placed for one minute on the rotating rod (25 rpm). The time taken for the falling from the roller, during one minute period was recorded.

4.VII.B. Spontaneous Locomotor (Exploratory) Activity Test (Photo-Actometer Test)

Photo-actometer test was employed to assess the effect of drug treatment on spontaneous motor (exploratory) activity. Each animal was observed for a period of 5 min in a square closed field arena (30 x 30 x 30 cm) equipped with 6 photocells in the outer wall. Interruptions of photocell beams (locomotor / exploratory action) were recorded by means of a 6 digits counter (Goddard et al., 2008).

4.VII.C. Cold-Allodynia Test (Acetone Drop Test)

Cold-allodynia of the hind paw was assessed using acetone drop method as described by Choi et al. (1994), with slight modification (de la Calle et al., 2002) for assessing the reactivity to non-noxious cold chemical stimuli. The rats were placed on the top of a wire mesh grid, which allowed access to the hind paws. Acetone 0.1 ml (100 µl) was sprayed on the plantar surface of hind paw of rat. Cold sensitive reaction with respect to either paw licking, shaking or rubbing the hind paw was observed and recorded as paw lifting duration for 20 s test period.
4.VII.D. Heat (Radiant) Hyperalgesia Test (Plantar Test)

Radiant heat hyperalgesia of the left hind paw was assessed using the radiant heat lamp source as described by the method of Hargreaves et al. (1988), for assessing the reactivity to noxious radiant heat stimuli. The intensity of the radiant heat stimulus was maintained at 25 ± 0.1°C. Response of left hind paw withdrawal threshold was noted. Cut-off time of 20 s was maintained.

4.VII.E. Heat (Conduction) Hyperalgesia (Hot Plate Test)

Thermal nociceptive threshold, as an index of thermal-hyperalgesia, was assessed using Eddy’s hot plate as described by the method of Eddy et al. (1950). Eddy’s hot plate was pre-heated and maintained at temperature of 52.5±0.5°C. Rats were placed on the hot plate and nociceptive threshold, with respect to jumping or hind paw of licking was recorded in seconds. The cut-off time of 15 seconds was maintained.

4.VII.F. Tactile Mechanical Allodynia Test (Von Frey Hair Test)

Mechanical allodynia (non-noxious mechanical stimuli) was assessed as described by the method of Chaplan et al. (1994). Briefly, calibrated nylon filaments [Von Frey Hair, total 20 filaments numbered 1.65, 2.36, 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, 4.17, 4.31, 4.58, 4.74, 4.83, 5.07, 5.18, 5.48, 5.88, 6.10, 6.45, and 6.65], in terms of different bending forces [0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, 2, 4, 6, 8, 10, 15, 26, 60, 100, 180, 300 g], were applied to the mid plantar surface of left hind paw. The filaments were applied ten times, starting with the softest and continuing in ascending order of stiffness. A brisk withdrawal of the left hind limb was considered a positive response. The criterion for the threshold value, in grams, was equal to the filament evoking a withdrawal threshold of the left hind paw 5 times out of 10 trials i.e., 50% response.

4.VII.G. Tactile Mechanical Hyperalgesia Test (Pinprick Test)

Mechanical (tactile) hyperalgesia was assessed using pinprick test as described by the method of Erichsen and Blackburn-Munro (2002). The surface of the injured hind paw was touched with the point of the bent 18 gauge needle (at 90° angle) at intensity sufficient to produce a reflex withdrawal response in normal non-operated animals, but at an intensity
which was insufficient to penetrate the skin in all other group. The duration of the paw withdrawal was recorded in seconds. A cut-off time of 20 s was maintained.

4.VII.H. Static Mechanical Hyperalgesia Test (Randall Selitto Test)

Mechanical (static) nociceptive threshold as an index of mechano-hyperalgesia were assessed by pressure stimulation method as described method of Randall and Selitto, (1957). Briefly, nociceptive threshold, expressed in grams, as measured by applying increasing pressure to the left hind paw. Withdrawal of left hind paw or vocalization response was noted to assess the static mechanical nociceptive threshold. The cut-off pressure of 450 g was maintained.

4.VII.I. Neuronal Functional Index Test

The SFI was subsequently measured from the images, using the formula derived by Bain et al. (1989). Briefly, animals were subjected to walking-track analysis and measurement of the sciatic functional index (SFI) using a method similar to that described by de Medinaceli et al. (1982). The trials were done in an 8.2 x 42 cm corridor darkened at one end and covered with a sheet of white paper. The rat hind paws were dipped in black India- ink and then each animal was allowed to walk freely in above mentioned corridor. In most cases, a single walk by each animal was enough to obtain adequate prints on paper. From the analysis of the footprints of the feet, the following lengths were obtained: (I) Distance from the heel to the third toe, the print length (PL); (II) Distance from the first to the fifth toe, the toe spread (TS); and (III) Distance from the second to the fourth toe, the intermediary toe spread (ITS). All three measurements were taken from the experimental (E) and normal (N) sides. The factors were calculated as follows: (I) Print length factor (PLF) = (EPL - NPL)/NPL; (II) Toe spread factor (TSF) = (ETS - NTS)/NTS; (III) Intermediary toe spread factor (ITF) = (EIT - NIT)/NIT. These factors were then incorporated into the following formula: for sciatic functional index (SFI) = -38.3 × PLF + 109.5 × TSF + 13.3 × ITF - 8.8; for tibial functional Index (TFI) = -37.2 × PLF + 104.4 × TSF + 45.6 × ITF - 8.8 and for peroneal functional index (PFI) = 174.9 × PLF + 80.3 × TSF - 13.4. A sciatic tibial and peroneal functional index of 0 is normal. A functional index of -100 indicates total impairment, such as would result from an injured and uninjured tibial, peroneal and sciatic nerve function.
4.VIII. Electrophysiological Studies

The study of nerve conduction velocity (NCV) was carried out by applying the electrophysiological approach in the rat. After complete assessment of various behavioral parameters on 21\textsuperscript{st} day, the animal was anaesthetized for the evaluation of NCV. The measurement of sensory and motor nerve conduction velocity is a major hallmark to detect neurological disorders especially neuropathic pain. It is also important to differentiate and for confirmation of the either nerve or muscular related disease.

4.VIII.A. Motor Nerve Conduction Velocity (MNCV) Test

Motor nerve conduction velocity was assessed as an index of the speed of conduction of an electrical impulse through a nerve by using student physiograph (INCO Pvt. LTD, Ambala, India) with bio-potential coupler and stimulator device as per method of Thomsen \textit{et al.} (2002) and Saini \textit{et al.} (2007). Briefly, on 21\textsuperscript{st} day rats were anaesthetized by administering chloral hydrate (350 mg/kg, i.p.). Motor nerve conduction velocity was measured by stimulating the sciatic (proximal to sciatic notch) and tibial (distally to ankle) nerve using bipolar needle (26 1/2 gauge) electrodes with supramaximal stimulus i.e., 3 V, single stimulus and square-wave pulses with a duration of 0.2 ms. Paired steel needle electrodes were inserted percutaneously with a distance of 1 cm between the reference electrode and the stimulating or recording electrode. Stimulating electrodes were inserted at the sciatic notch and at the knee. Recording electrodes were placed over the small muscles of the dorsum of the hind paw. Surface electrodes connected to bio-potential coupler were placed on paw to detect motor response. A ground electrode was attached to the calf muscle with an alligator clip. The recording was a typical biphasic response with an initial M-wave,
which is a direct motor response due to stimulation of motor fibers. Motor nerve responses were amplified and recorded by student physiograph. During the study, body temperature of rat was maintained at 37°C using homeo-thermic blanket system (Ascent Healthcare, Mumbai, India). Motor nerve conduction velocity recordings were measured twice at a time and results were averaged. Latency was measured from the point of stimulation to the onset of the response. The latency was measured in milliseconds and distance between the two stimulating electrodes measured in millimeters using steel rule. Motor nerve conduction velocity was calculated by using the following formula: Motor nerve conduction velocity = (Distance between the nerve stimulation point) / (Latency period difference).

4.VIII.B. Sensory Nerve Conduction Velocity (SNCV) Test

The determination of sensory nerve conduction velocity (SNCV) was recorded orthodromically by using student physiograph with bio-potential coupler and stimulator device as per method of Oh (2003) and Kurokawa et al. (2004) with slight modification of Saini et al. (2007) and Renn et al. (2011). The recording electrodes were placed subcutaneously at the ankle behind the medial malleolus, and the reference electrode placed 1 cm proximal to the active electrode. The thin stimulating ring (0.5 mm diameter) electrodes were placed in the middle (3rd) digital nerve of hind limb and stimulated with a square-wave pulse of 0.05 ms in duration, at the rate of 1HZ and 10 pulses per train intensity current, which resulted in a maximal amplitude response. The amplitudes were measured from peak to peak. The distance between recording and stimulating electrodes approximately 25 mm and 10 mm between indifferent and active recording needles were maintained. During the study, body temperature of rat also maintained at 37°C using homeo-thermic blanket system (Ascent Healthcare, Mumbai, India). Approximately 10 responses were averaged to obtain the position of the negative peak. Latencies were measured from the point of stimulation to the onset of the response. The maximal sensory nerve action potential was calculated by using the following formula: sensory nerve conduction velocity also known as H-reflex = Onset of latency / Peak of the initial negative deflection (Distance between stimulating and recording electrodes).
4.IX. Biochemical Estimations

After behavioral; nerve functional and electrophysiological assessments, animals were sacrificed on 21st day with chemical euthanasia (50 mg/kg thiopentone sodium, i.p.). Skin and muscle layers were removed from the thigh region of hind limbs in the rat. The portions of the sciatic nerve and the tissue beneath the sciatic nerve were isolated immediately as described earlier (Muthuraman et al., 2008; 2008a). The sciatic nerve portion, proximal to the point of transection up to its point of emergence from the spinal cord, and distal to the point of transection up to its ending, was excised. The tissue 1 cm diameter, exactly beneath the point of the sciatic nerve transection and ligation were taken. The uniformity among the different nerve and the tissue samples was maintained by taking the constant weight of the respective samples. Superoxide anion measurement was carried out immediately in sciatic nerve as described method of Wang et al. (1998). The rest of the samples were kept in the humidity chamber (maintained at 85% relative humidity and 37ºC temperature) to remove and maintain the moisture content of the collected samples. The sciatic nerve homogenate (10%, w/v) was prepared with 0.1 M Tris–HCl buffer (pH 7.4), and deionised water for total protein and total calcium estimation respectively. The tubes with homogenate were kept in ice water for 30 minutes and centrifuged at 4°C (2500 rpm for 10 min). Further, surrounding muscular tissue was homogenated with phosphate buffer (pH 7.4) and employed for myeloperoxidase (MPO) estimation. In addition, blood sample and histopathological assessments were carried out in sub-acute oral toxicity study protocol to find any hematological; organ dysfunction and microscopical changes respectively.

4.IX.A. Blood Analyses

For sub-acute oral toxicity study, hematological and biochemical analyses were performed at a commercial laboratory. Complete blood cell count i.e. red blood cell count (RBC), white blood cell count (WBC), platelet count, lymphocytes, haemoglobin, hematocrit (packed cell volume, PCV) mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) were determined on a fully automated analyzer. Blood chemistry tests were performed on an auto analyzer (Beckman Coulter CX7, Fullerton, CA, USA) using a kinetic rate method for the enzymes i.e. alanine transaminase (ALT), aspartate transaminase (AST) modified rate Jaffe method for creatinine;
the timed endpoint method for urea, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and glucose oxidase peroxidase method for glucose (Slot, 1965; Kirschner and Woods, 2001).

4.IX.B. Estimation of Tissue Total Protein Content

Sciatic nerve protein content was determined by Lowry’s method (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard. 0.3 ml of supernatant of tissue homogenate was diluted to 1 ml. The 100 µl of diluted supernatant was made up to 1 ml using distilled water. To this, 5 ml of Lowry’s reagent was added. The contents were mixed thoroughly and the mixture was allowed to stand for 15 min at room temperature. Then 0.5 ml of Folin-Ciocalteu reagent was added and the contents were vortexed vigorously and incubated at room temperature for 30 min. The protein content was determined spectrophotometrically at 750 nm and expressed as mg per ml of 10% sciatic nerve homogenate.

➢ Preparation of Reagents

Preparation of Lowry’s Reagent

Lowry’s reagent was freshly prepared by mixing 1% w/v copper sulphate solution, 2% w/v sodium-potassium tartrate and 2% w/v sodium carbonate in 0.1 M sodium hydroxide, in the ratio of 1:1:98.

Preparation of 0.1 M Sodium Hydroxide Solution

0.1 M sodium hydroxide was prepared by dissolving 4 g of sodium hydroxide in distilled water and volume was made up to 1 liter with distilled water.

Preparation of 1% Copper Sulphate Solution

1% CuSO₄ was prepared by dissolving 1 g of copper sulphate in 0.1 M sodium hydroxide and volume was made up to 100 ml with sodium hydroxide.

Preparation of 2% Sodium Potassium Tartrate Solution

2 g of sodium potassium tartrate was dissolved in 0.1 M sodium hydroxide and final volume was made up to 100 ml with the same.

Preparation of 2% Sodium Carbonate Solution

2 g of sodium carbonate was dissolved in 0.1 M sodium hydroxide and volume was made up to 100 ml with 0.1 M sodium hydroxide.
4. IX.C. Estimation of Superoxide Anion Generation

The superoxide anion generation was estimated in terms of measuring reduced nitroblue tetrazolium (NBT) (Wang et al., 1998). Weighed amount of tissue (10 mg) was taken in 5 ml phosphate buffered saline containing 100 µM of NBT and incubated at 37 °C for 1.5 h. The NBT reduction was stopped by adding 5 mL of 0.5 M HCl. Then, the tissue was taken out and was minced and homogenized in a mixture of 0.1 M sodium hydroxide and 0.1% sodium dodecyl sulphate in water containing 40 mg/L diethylene triamine penta acetic acid. The mixture was centrifuged at 20,000 g for 20 min and the resultant pellet was suspended in 1.5 mL of pyridine and kept at 80 °C for 1.5 h to extract formazan, an adduct formed after reaction of NBT with superoxide anions. The mixture was again centrifuged at 10,000 g for 10 min and absorbance of formazan was determined spectrophotometerically at 540 nm. The amount of reduced NBT was calculated using the formula

\[
\text{Amount of reduced NBT} = \frac{A \times V}{T \times Wt \times \varepsilon \times l}
\]

where A is absorbance, V is volume of pyridine (1.5 mL), T is time for which the tissue was incubated with NBT (1.5 h), Wt is blotted wet weight of tissue (25 mg), \( \varepsilon \) is extinction coefficient (0.72 L/mmol/mm) and l is length of light path (1 cm). Results were expressed as reduced NBT picomole per min per mg of wet tissue.

- **Preparation of Reagents**

  **Preparation of Phosphate Buffered Saline**

  2.5 g of sodium dihydrogen phosphate, 2.523 g of disodium hydrogen phosphate and 8.2 g of sodium chloride were dissolved separately in sufficient quantity of distilled water. The three solutions were mixed and final volume was made to 1 L with distilled water.

  **Preparation of 100 µM of Nitroblue Tetrazolium**

  8.17 mg of nitroblue tetrazolium was dissolved in 100 mL phosphate buffered saline to make 100 µM solution.

  **Preparation of 0.5 M Hydrochloric Acid**
4.34 mL of 11.5 M HCl was diluted to 100 mL with distilled water.

**Preparation of 0.1 M Sodium Hydroxide Solution**

400 mg of sodium hydroxide was dissolved in 100 mL of distilled water.

**Preparation of 0.1% Sodium Dodecyl Sulphate Solution**

100 mg of sodium dodecyl sulphate was dissolved in 100 mL distilled water.

**4.IX.D. Estimation of Total Calcium**

Total calcium levels were estimated in sciatic nerve as described by Severnghaus and Ferrebee (1950). Briefly, 2 mg of sciatic nerve was homogenated with 1ml of deionised triple distilled water under ice cold conditions and homogenate was mixed with 1 ml of trichloroacetic acid (4 %). The tubes were centrifuged at 2500 rpm, for 10 minutes. The clear supernatant was used for the estimation of total calcium ion by atomic emission spectroscopy. A standard calibration curve was prepared by using 0-1000 parts per million (ppm) of calcium chloride. The total calcium value was expressed as parts per million per mg of protein.

**Preparation of Reagents**

**Preparation of 10% Trichloroacetic Acid**

4 g of trichloroacetic acid was dissolved in 100 ml of distilled water.

**Preparation of 1000 ppm Calcium Chloride Solution**

0.3676 g of calcium chloride was dissolved in 100 ml of deionised triple distilled water.

**4.IX.E. Estimation of Myeloperoxidase Activity**

The myeloperoxidase activity was measured by a method of Grisham et al. (1994) and Patriarca et al. (1977). The inflammatory reactions are characterized by the recruitment of inflammatory cells from the blood capillaries to the connective tissue, adjacent to the point of injury. Therefore, the tissue surrounding the nerve lesion was excised as a measure of degree of inflammation. The tissue samples were homogenized in 10 volumes of ice-cold potassium phosphate buffer (pH 7.4), using a tissue homogenizer. The homogenate was centrifuged at 5000 × g for 10 min at 4°C. The supernatant was discarded and 10 mL of ice-cold 50 mM potassium phosphate buffer (pH 6.0), containing 0.5% hexadecyl trimethyl ammonium bromide (HETAB) and 10 mM EDTA was then added to the pellet. It was then subjected to
one cycle of freezing and thawing and a brief period (15 s) of sonication. After sonication, the solution was centrifuged at 19,000 \( \times \) \( g \) for 15 min. 100 µl of supernatant was combined with 2.9 ml of phosphate buffer [It contains 0.167 mg/ml of O-dianisidine hydrochloride and 0.0005% hydrogen peroxide (\( H_2O_2 \))]. The myeloperoxidase activity was measured spectrophotometrically at 460 nm. One unit of the myeloperoxidase activity is defined as that which would produce a change in absorbance of 1.0 unit/min at pH 7.0 and 25ºC, calculated from the initial rate of reaction with peroxide (1μM) as the substrate. The results were expressed as myeloperoxidase activity units per milligram of protein at one minute. The amount of MPO activity was calculated using the formula

\[
\text{MPO activity (U/g)} = \frac{X}{\text{Weight of the piece of tissue taken}}
\]

Where

\[
X = \frac{10 \times \text{change in absorbance per minute}}{\text{Volume of supernatant taken in the final reaction (mL)}}
\]

**Preparation of Reagents**

**Preparation of 0.2 M Potassium Phosphate Buffer (pH 7.4)**

Potassium dihydrogen orthophosphate (27.22 g) was dissolved in 930 mL of distilled water. pH was then adjusted with 30 % w/v potassium hydroxide solution and sufficient distilled water was added to make volume upto one liter.

**Preparation of Potassium Phosphate Buffer (pH 6.0), Containing 0.5 % Hexadecyl Trimethyl Ammonium Bromide (HETAB) and 10 mM EDTA**

Disodium hydrogen phosphate (60.5 g) and potassium dihydrogen phosphate (46 g) were dissolved in one litre of distilled water to make potassium phosphate buffer (with pH 6.0). 5 g of hexadecyl trimethyl ammonium bromide (HETAB) and 3.72 g of EDTA were dissolved in one litre of potassium phosphate buffer (pH 6.0) to make final solution.

**Preparation of Phosphate Buffer Containing 0.167 mg/mL O-dianisidine Hydrochloride and 0.0005 % \( H_2O_2 \)**

O-dianisidine hydrochloride (16.7 mg) was dissolved in 100 mL of potassium phosphate buffer (pH 6.0) solution to this 1 µL of \( H_2O_2 \) was added to make desired solution.
4.IX.F. Estimation of Tumor Necrosis Factor-Alpha (TNF-α) Level

Sciatic nerve samples were utilized for determination of TNF-α level. TNF-α levels (sensitivity: 25 pg/ml) were determined by using rat TNF-alpha ELISA kit (RayBio, Inc., USA) method and procedure was followed as per manufacturer instructions (see below).

- **Available Reagent in Rat TNF-Alpha ELISA Kit**
  

- **Preparation of Reagent for Estimation of TNF-Alpha Level**
  
  1. All reagents and samples were bought to room temperature (18-25º C) before use for further preparation of solution as required.
  2. Assay diluent B (Item E) was used for dilution of tissue supernatants.
  3. Assay diluents B was diluted 5-fold with distilled water.
  4. Preparation of standard: Item C vial was spin and then added 400 µl of 1x assay diluent B into Item C vial to prepare a 100 ng/ml standard TNF-alpha solution. Powder was dissolved thoroughly by a gentle mixing.
  5. 100 µl TNF-alpha standard solutions was added from the vial of Item C, into a tube with 400 µl 1x assay diluent B to prepare a 20,000 pg/ml stock standard solution. Pipette 400 µl 1x assay diluent B into each tube. This stock solution was used for the further dilution series (shown below). Each tube was mixed thoroughly before the next transfer. 1x assay diluent B was serves as the zero standard (0 pg/ml).
Fig. 8. Illustration for TNF-alpha standard solutions preparation.

6. Preparation of Wash Concentrate (20x): If (Item B) was appear to visible crystals, vial was warmed to room temperature and mixed gently until dissolve the crystal. 20 ml of wash buffer concentrate was diluted with distilled water to get 400 ml of 1x wash buffer.

7. Preparation of Detection Antibody: Vial (Item F) was spinned for preparation of detection antibody before use for further dilution. 100 µl of 1x assay diluent B was added into the vial to prepare a detection antibody concentrate. This preparation was mixed gently by pipette up and down of the solution 3-5 times and the concentrate was stored at 4º C for 5 further days utilization. The detection antibody concentrate was diluted 80-fold with 1x assay diluent B and used in step 4 of part VI assay procedure.

8. Preparation of HRP-Streptavidin Concentrate: Vial (Item G) was spinned and pipette up and down to make uniform mixing. HRP-Streptavidin concentrate was diluted 10,000-fold with 1x assay diluent B.

[For example: Vial (Item G) was spinned and pipetted up and down to make uniform mixing and added 2 µl of HRP-Streptavidin concentrate into a tube with 198.0 µl 1x assay diluent B to prepare a 100 fold diluted HRP-Streptavidin solution (Note: This diluted solution was not stored for next day use). Further, 120 µl of prepared 100-fold diluted solution was pipetted out and added into a tube with 12 ml 1x assay diluent B to prepare a final 10,000 fold diluted HRP-Streptavidin solution].
Assay Procedure for Estimation of TNF-Alpha Level in Tissue Sample

1. All standards and samples were brought to room temperature (19-25º C) for further estimation. All standards and samples were estimated two times for accuracy.
2. 100 µl of each standard dilution (see reagent preparation step 2) and samples were added into appropriate wells. All wells were covered and incubated for 2.5 hours at room temperature with gentle shaking.
3. The micro plate solution was discarded and washed 4 times with 1x wash solution (Item B). Washing was carried out by filling each well with wash buffer (300 µl) using a micropipette. Complete removal of liquid at each step was carefully performed.
4. 100 µl of 1x prepared biotinylated antibody (Reagent preparation step 6) was added into each well. Incubation was maintained for 1 hour at room temperature with gentle shaking.
5. For discarding of micro plate well solution was repeated as in step 3.
6. 100 µl of prepared Streptavidin solution (see reagent preparation step 7) was added into each well. Incubation was maintained for 45 minutes at room temperature with gentle shaking.
7. For discarding of micro plate well solution was repeated as in step 3.
8. 100 µl of TMB one-step substrate reagent (Item H) was added into each well. Incubation was maintained for 30 minute at room temperature in the dark with gentle shaking.
9. 50 µl of stop solution (Item I) was added to each well. Absorbance reading was recorded immediately at 450 nm after addition of stop solution.

Calculation procedure for test results

Recombinant anti-Rat TNF-alpha was used to generate the standard curve (range 0-20,000 pg/ml) with rat TNF-alpha ELISA diagnostic kit. Mean absorbance for each set of duplicate standards, controls and samples was calculated by subtracting the average zero standard optical density. Standard plot curve was estimated by using sigma plot software inbuilt in ELISA reader, with standard concentration of the X-axis and absorbance on the Y-axis. Best-fit straight line was drawn through the standard points. The results were expressed as pictograms of TNF-α per mg of total protein in the supernatant.
Summary of Assay Procedure

1. Coat antigen onto microplate.

2. 100 µl of standard or samples was added and incubated for 2.5 hours at room temperature.

3. 100 µl of prepared biotin antibody was added and incubated for 1 hour at room temperature.

4. 100 µl of prepared HRP-Streptavidin solution was added and incubated for 45 minutes at room temperature.

5. 100 µl TMB one step substrate reagent was added and incubated for 30 minutes at room temperature to produce colorimetric reaction.

6. 50 µl of stop solution was added to each well and absorbance reading measured at 450 nm immediately.

Fig. 9. Summary of TNF-alpha assay procedure.

4.X. Histopathological Studies

For sub-acute toxicity study, the liver, heart, and kidney tissue samples; and for neuropathic pain study, the sciatic nerve tissue samples were stored in the fixative solution (1% formalin), dehydrated in graded concentrations of ethanol, immersed in xylene and then embedded in paraffin. The tissue sections about 4 µm thick (forward mode wax ribbon cutting method) were cut by using a microtome device (Radical Scientific Equipments Pvt. Ltd.,
Ambala, India) and placed on slide using commercial Baker’s mounting fluid. Paraffin wax was removed by warming the slide gently, until the wax melted and then was washed with xylene. The slide was followed by washings with absolute alcohol and water to hydrate the sections and stained with haematoxylin-eosin (H & E) method.

- **Haematoxylin-Eosin (H & E) Method**
  
  The haematoxylin-eosin staining of nervous tissue was carried out as described by Sudoh et al. (2004). The hydrated sections were stained with haematoxylin for 15 min. The stained sections were washed with water and treated with 1% acid alcohol mixture for 15-20 s. The acid alcohol mixture was washed off with water and sections were counterstained with 1% aqueous solution of eosin for 2 min. After washing with water to remove excess of eosin, sections were dehydrated using absolute alcohol and then mounted using Canada balsam as mounting agent. Microscopic slides were analyzed qualitatively under light microscope (450 X) for neuronal pathological changes assessment.

- **Preparation of Reagents**

  **Preparation of Haematoxylin Solution**
  
  2 g of haematoxylin was dissolved in a mixture of absolute alcohol, distilled water and glycerin 100 mL each, followed by addition of 10 mL glacial acetic acid and in excess addition of sodium potassium sulphate.

  **Preparation of 1% Eosin Solution**
  
  1 g of eosin was weighed and dissolved in 100 mL of distilled water.

  **Preparation of Acid Alcohol Mixture**
  
  The acid alcohol mixture was made by adding 1 mL of concentrated hydrochloric acid (HCl) in 99 mL of 70% alcohol.

**4.XI. Experimental Protocol**

Twenty seven groups, each comprising of six Wistar rats (n=6), were employed in the present study.

**Group I (Normal Control Group):** Rats were not subjected to any surgical procedure or drug administration and all animals were kept for 21 days. Behavioral tests were performed before (i.e. day 0) and after surgery on the different time intervals, i.e., 1, 3, 6, 9, 12, 15, 18 and 21st
day. On 21st day sciatic functional index and nerve conduction velocity were measured as per method of Bain et al. (1989) and Kurokawa et al. (2004) respectively. Thereafter, all the animals were sacrificed and subjected to biochemical analysis for estimation of total protein, tumor necrosis factor-alpha (TNF-α), superoxide anion generation and total calcium in sciatic nerve tissue and myeloperoxidase (MPO) activity in the surrounding muscular tissue sample. Further, histopathological study was also performed on 21st day as described by Sudoh et al. (2004).

**Group II (Sham Control Group):** Rats were subjected to surgical procedure to expose left hind paw sciatic, tibial and sural nerve without any nerve ligation and transection respectively. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group III (TST Control Group):** Rats were subjected to surgical procedure to expose and transect tibial and sural nerve as described earlier. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group IV (CCI Control Group):** Rats were subjected to surgical procedure to expose and ligate left hind paw sciatic nerve as described earlier. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group V and VI (Vincristine Control Groups):** Vincristine (50 and 75 µg/kg; i.p.), was administered to normal rats for 10 consecutive days. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group VII (Vehicle in TST Group):** After subjecting the rats to TST, normal saline (0.9 % w/v; i.p.) was administered for 14 consecutive days, starting from the day of surgery. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group VIII (Vehicle in CCI Group):** After subjecting the rats to CCI, normal saline (0.9 % w/v; i.p.) was administered for 14 consecutive days, starting from the day of surgery. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group IX (Vehicle in Vincristine Treated Group):** Rats were administered normal saline (0.9 % w/v, i.p.) one hour before vincristine injection, for 10 consecutive days. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.
**Group X (HAE-AC per se):** Rats were administered hydro-alcoholic extract of *Acorus calamus* (HAE-AC, 200 mg/kg; *p.o.*) for 14 consecutive days, starting from the day 1st. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group XI (SRE-AC per se):** Rats were administered saponin rich extract of *Acorus calamus* (SRE-AC, 40 mg/kg; *p.o.*) for 14 consecutive days, starting from the day 1st. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group XII (Pregabalin per se):** Rats were administered pregabalin (10 mg/kg; *p.o.*) for 14 consecutive days, starting from the day 1st. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group XIII and XIV (HAE-AC in TST):** Hydro-alcoholic extract of *Acorus calamus* (100 and 200 mg/kg; *p.o.*) was administered for 14 consecutive days in rats subjected to tibial and sural nerve transection, starting from the day 1st. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group XV and XVI (HAE-AC in CCI):** Hydro-alcoholic extract of *Acorus calamus* (100 and 200 mg/kg; *p.o.*) was administered for 14 consecutive days in rats subjected to CCI of sciatic nerve, starting from the day 1st. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group XVII and XVIII (HAE-AC in Vincristine Treated Group):** Hydro-alcoholic extract of *Acorus calamus* (100 and 200 mg/kg; *p.o.*) was administered (one hour before) for 14 consecutive days in rats subjected to vincristine injection, starting from the day 1st. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group XIX and XX (SRE-AC in TST):** Saponin rich extract of *Acorus calamus* (20 and 40 mg/kg; *p.o.*) was administered for 14 consecutive days in rats subjected to tibial and sural nerve transection, starting from the day 1st. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

**Group XXI and XXII (SRE-AC in CCI):** Saponin rich extract of *Acorus calamus* (20 and 40 mg/kg; *p.o.*) was administered for 14 consecutive days in rats subjected to CCI of sciatic nerve, starting from the day 1st. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.
Group XXIII and XXIV (SRE-AC in Vincristine Treated Group): Saponin rich extract of Acorus calamus (20 and 40 mg/kg; p.o.) was administered (one hour before) for 14 consecutive days in rats subjected to vincristine injection, starting from the day 1\textsuperscript{st}. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

Group XXV (Pregabalin in TST): Pregabalin (10 mg/kg; p.o.) was administered for 14 consecutive days in rats subjected to tibial and sural nerve transection, starting from the day 1\textsuperscript{st}. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

Group XXVI (Pregabalin in CCI): Pregabalin (10 mg/kg; p.o.) was administered for 14 consecutive days in rats subjected to CCI of sciatic nerve, starting from the day 1\textsuperscript{st}. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

Group XXVII (Pregabalin in Vincristine Treated Group): Pregabalin (10 mg/kg; p.o.) was administered (one hour before) for 14 consecutive days in rats subjected to vincristine injection, starting from the day 1\textsuperscript{st}. Behavioral tests, biochemical and histopathological parameters were assessed as described in group I.

In addition to above acute and sub-acute oral toxicity potential of HAE-AC was evaluated in mice (six groups, n=10) and in rats (six groups, n=10) respectively. Our previous studies have documented that 0.5 % w/v carboxy methyl cellulose did not show any significant changes in the neuropathic pain behaviour and biochemical marker alterations (Muthuraman et al., 2008a, 2008b). Therefore, in the present research protocol 0.5 % w/v of carboxy methyl cellulose vehicle treatment group is not added.

4.XII. Statistical Analysis

All the results were expressed as mean ± standard deviation. The Student’s t-test was used for statistical comparison of data between groups in toxicological study. The LD\textsubscript{50} was calculated according to method of Litchfield and Wilcoxon (1949), and Miller and Tainter, (1944). The data of behavioral results was statistically analyzed by two-way analysis of variance (ANOVA) followed by Bonferonni’s post test and the data of biochemical results was statistically analyzed by one-way ANOVA followed by Tukey’s multiple range tests by using Sigmastat Version-2.0 software. The P-value < 0.05 was considered to be statistically significant.