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Protective effect of *Acorus calamus* L. in rat model of vincristine induced painful neuropathy: An evidence of anti-inflammatory and anti-oxidative activity

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ABSTRACT

The study investigates the protective effect of *Acorus calamus* L. (*AC*) in vincristine-induced painful neuropathy. Vincristine (75 μg/kg, *i.p.* for 10 consecutive days) was administered to induce painful neuropathy in rats. Various tests were performed to assess the degree of painful neuropathy at different days i.e., 0, 1, 7, 14, and 21st day. Sciatic nerve TNF-α, superoxide anion generation, total calcium, and myeloperoxidase activity level were also estimated after 21st day of study. Hydro-alcoholic extract of *AC* (HAE-*AC*, 100 and 200 mg/kg, *p.o.*) and pregabalin (10 mg/kg, *p.o.*) were administered for 14 consecutive days. Vincristine significantly induced neuropathic pain manifested in the terms of thermal hyperalgesia and allodynia (increase in hind paw licking, lifting or jumping from hot plate); mechanical hyperalgesia (increase in left hind paw lifting duration in pin-prick test) and allodynia (left hind paw withdrawal reflexes to non-noxious stimuli in Von Frey test); and sciatic functional index (analysis of footprints of the feet) along with rise in the levels of various biochemicals. HAE-*AC* attenuated vincristine induced behavioral, and biochemical changes comparable to that of pregabalin (positive control). HAE-*AC* attenuated vincristine induced painful neuropathy, which may be attributed to its multiple effects including anti-oxidative, anti-inflammatory, and calcium inhibitory actions.

1. Introduction

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) (Woolf and Mannion, 1999). Peripheral neuropathic pain is frequently observed in patients with long standing diabetes, cancer, AIDS, leprosy, cervical disc protrusion and foraminotomy and after surgery (Weisz et al., 2010; Koltzenburg and Scadding, 2001; Shaladi et al., 2009). Various anti-cancer chemotherapeutic agents including vincristine, oxaliplatin, paclitaxel and cisplatin are well reported to produce peripheral neuropathic pain as their main side effect (Sioka and Kyritsis, 2009; Gomber et al., 2010; Kiguchi et al., 2009; Argyriou et al., 2008). Dying-back neuropathy is commonly known as distal axonopathy, and is observed in some metabolic or toxin mediated derangement of neurons. Dying-back neuropathy has reported to be associated with metabolic disorders like diabetes, renal failure, malnutrition, alcoholism, and toxic disturbance caused by chemotherapeutic agents like vincristine (Argyriou et al., 2008).

Conventional analgesic agents like non-steroidal anti-inflammatory drugs (NSAIDs) and opioids are clinically in-effective for attenuation of painful neuropathy. Usefulness of tricyclic antidepressants and anti-convulsants in management of neuropathic pain is indicated but these medications exhibit a wide spectrum of adverse effects which limit their full clinical exploitation (Dworkin et al., 2010). Several herbal medicines such as *Aconiti tuber*, *Lindera angustifolia*, *Teucrium polium*, *Phyllanthus emblica*, *Vochysia divergens*, *Cannabis sativa*, *Nigella sativa*, *Ocimum sanctum*, and *Ginkgo biloba* are shown to have potential in different types of experimentally induced neuropathic pains (Muthuraman et al., 2008a; Kim et al., 2009; Wirth et al., 2005). Some clinical reports have also advocated beneficial effect of drugs from plant origin in neuropathic pain conditions (Ellis et al., 2009; Zareba, 2009). Therefore, ample scope of the new herbal medicine to combat the management of neuropathic pain syndromes is expected.

Acorus calamus L. is an indigenous medicinal plant. Traditionally, it is used as an ingredient of various cocktail preparations employed for the treatment and management of headache, migraine, body ache and severe inflammatory pain (Muthuraman et al., 2011). Phytochemically *Acorus* species have shown to possess the presence of glycosides, flavonoids, saponins, tannins, polyphenolic compounds, mucilage, volatile oil and bitter principles (Mittal et al., 2009; Zhu et al., 2010; Dong et al., 2010). The aqueous and hydro-alcoholic extracts have also been shown to possess the lipid

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lowering and neuropharmacological actions (Parap and Mengi, 2003; Martis et al., 1991). Moreover, *AC* has also been reported in various neurological disorder i.e., amnesia, epilepsy including neuropathic pain (Muthuraman et al., 2011; Lee et al., 2010; Hazra et al., 2007). However, beneficial effect of *AC* in the painful neuropathy remains to be explored. Therefore, the present study has been designed to investigate the ameliorative potential of hydro-alcoholic extract of *AC* in vincristine induced painful neuropathy in rats. Pregabalin (Lyrica™) a selective N-type voltage dependent calcium channel [Ca_v 2.2 ($\alpha 2$–δ subunit)] blocker an anti-convulsant with significant analgesic, and neuroprotective actions served as positive control in this study (Gilron and Flatters, 2006; Xiao et al., 2007).

2. Materials and methods

2.1. Plant material

The fresh rhizome part of *AC* were collected at Kodaikanal of Tamilnadu (India) and authenticated through department of botany, American college, Madurai, Tamilnadu. Plant sample has been kept in voucher specimen (PUP-218/2009–2010) at Punjabi University, Patiala for future reference. The shade dried rhizome was pulverized in a mechanical grinder to obtain coarse powder (Sieve No. 10/40).

2.2. 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 was suspended in 0.5% w/v carboxymethyl cellulose solution and vincristine was diluted with normal saline. Our previous studies indicate that 0.5% w/v carboxy methyl cellulose did not show any change in the neuropathic pain behavior and biochemical markers (Muthuraman et al., 2008a, 2008b, 2011).

2.3. Extraction

The coarsely powdered plant material was subjected to extraction with mixture of ethanol: water (1:1, 50%) at room temperature followed by vacuum drying at low temperature (<50 °C). The yield of hydro-alcoholic extract was found to be 26.4% (w/w). The extract was suspended in 0.5% w/v carboxymethyl cellulose solution for oral administration.

2.4. Experimental animals

Wistar rats of either sex, weighing 200–230 g, were employed in the present study. The animals were given free access to water and standard laboratory diet (Kisan Feeds Ltd., Mumbai, India). The rats were exposed to 12 h light and dark cycles. The experimental protocol was duly approved by the Institutional Animal Ethics Committee.

2.4.1. Induction of peripheral neuropathy by vincristine

Peripheral painful neuropathy was induced in rats by administration of vincristine sulfate as per the method of Authier et al. (1999). Briefly, 75 μg per kg of vincristine sulfate was administered intraperitoneally for 10 consecutive days.

2.5. Behavioral examination

The animals were subjected to the assessment of behavioral tests such as hot plate test, Von Frey hair and pin prick tests as degree of thermal and mechanical, hyperalgesia and allodynia respectively. Sciatic functional index and motor nerve conduction velocity were also performed for the assessment of neuronal functional changes.

2.5.1. Determination of hot plate test

Heat hyperalgesia (noxious thermal stimuli) and allodynia (non-noxious thermal stimuli) of the hind paw were assessed using Eddy's hot plate as described method of Eddy et al. (1950), for assessing the reactivity to noxious and non-noxious thermal stimuli respectively. The rats were placed on the top of a controlled preheated (52.5 ± 0.5 °C for hyperalgesia; 45 ± 0.5 °C for allodynia) and maintained hot plate surface, allowing access to the hind paw withdrawal response to degree of the nociceptive threshold. The cut-off times of 20 s for hyperalgesia and 30 s for allodynia were maintained.

2.5.2. Determination of pin prick test

Mechanical (noxious mechanical stimuli) hyperalgesia was assessed by the pin prick test as described in the method of Erichsen and Blackburn-Munro (2002). The plantar surface of the left 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.

2.5.3. Determination of Von Frey hair test

Mechanical allodynia (non-noxious mechanical stimuli) was assessed as described by Chaplan et al. (1994). Briefly, calibrated nylon filaments (Von Frey Hair), in terms of different bending forces, 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.

2.5.4. Determination of sciatic functional index (SFI)

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 × 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 SFI formula: SFI = −38.3 × PLF + 109.5 × TSF + 13.3 × ITF − 8.8. An SFI of 0 is normal. An SFI of −100 indicates total impairment, such as would result from a complete transaction of the sciatic nerve.

2.6. Biochemical estimation

All the animals were sacrificed on 21st day after surgery with chemical euthanasia. The portions of the sciatic nerve and the tissue beneath the sciatic nerve were isolated immediately. For superoxide anion measurements, samples were processed immediately. Further, the rest of the samples were kept in the humidity chamber (maintained at 85% relative humidity and 37 °C 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), deionised water, and phosphate buffer (pH 7.4) for total protein, total calcium, tumor necrosis factor-aplha (TNF-α) estimation respectively. Superoxide anion and myeloperoxidase (MPO) activity were also estimated in the sciatic nerve tissue sample.

2.6.1. Estimation of total protein content

Protein concentration was estimated according to the method of Lowry et al. (1951), using Bovine serum albumin (BSA) as a standard. The absorbance was determined spectrophotometrically at 750 nm.

2.6.2. 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) and procedure was followed according to the manufacturer instructions. A sample of sciatic nerve homogenate was tested in duplicate. Recombinant anti-Rat TNF-alpha was used to generate the standard curve (range 0–20,000 pg/ml) as per diagnostic kit. The absorbance was determined spectrophotometrically at 450 nm. The results were expressed as pictograms of TNF-α per mg of total protein in the supernatant.

2.6.3. Estimation of superoxide anion generation

The sciatic nerve superoxide anion generation was estimated in terms of measuring reduced nitroblue tetrazolium (NBT) as described in the method of Wang et al. (1998). Briefly, sciatic nerve homogenate react with NBT under certain chemical environment to form formazan as an index of superoxide anion generation. The absorbance of formazan was determined spectrophotometrically at 540 nm.