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THIAGARAJAN, VENKATA R.K.; SHANMUGAM, PALANICHAMY; KRISHNAN, UMA M.;
MUTHURAMAN, ARUNACHALAM

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Ameliorative potential of *Vernonia cinerea* on chronic constriction injury of sciatic nerve induced neuropathic pain in rats

VENKATA R.K. THIAGARAJAN^{1,2}, PALANICHAMY SHANMUGAM³,
UMA M. KRISHNAN⁴ and ARUNACHALAM MUTHURAMAN⁵

¹School of Chemical and Biotechnology, Sastra University,
Thanjavur-613402, Tamilnadu, India

²Department of Pharmacognosy, College of Pharmacy,
Madurai Medical College, Madurai-625011, Tamilnadu, India

³Department of Pharmaceutical Sciences & Research, Sankaralingam Bhuvaneshwari
College of Pharmacy, Thiruthangal, Sivakasi-626130, Tamilnadu, India

⁴Center for Nanotechnology & Advanced Biomaterials, School of Chemical & Biotechnology
Sastra University, Thanjavur-613402, Tamilnadu, India

⁵Department of Pharmaceutical Sciences & Drug Research,
Punjabi University, Patiala-147002, Punjab, India

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ABSTRACT

The aim of the present study is to investigate the ameliorative potential of ethanolic extract of whole plant of *Vernonia cinerea* in the chronic constriction injury (CCI) of sciatic nerve induced neuropathic pain in rats. Behavioral parameters such as a hot plate, acetone drop, paw pressure, Von Frey hair and tail immersion tests were performed to assess the degree of thermal, chemical and mechanical hyperalgesia and allodynia. Biochemical changes in sciatic nerve tissue were ruled out by estimating thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) and total calcium levels. Ethanolic extract of *Vernonia cinerea* and pregabalin were administered for 14 consecutive days starting from the day of surgery. CCI of sciatic nerve has been shown to induce significant changes in behavioral, biochemical and histopathological assessments when compared to the sham control group. *Vernonia cinerea* attenuated in a dose dependent manner the above pathological changes induced by CCI of the sciatic nerve, which is similar to attenuation of the pregabalin pretreated group. The ameliorating effect of ethanolic extract of *Vernonia cinerea* against CCI of sciatic nerve induced neuropathic pain may be due to the presence of flavonoids and this effect is attributed to anti-oxidative, neuroprotective and calcium channel modulator actions of these compounds.

Key words: antioxidant, calcium, chronic constriction injury, reduced glutathione, thiobarbituric acid reactive substance.

INTRODUCTION

Pain is initiated or caused by a primary lesion or dysfunction in the peripheral nervous system. This

pain is called as Peripheral neuropathic pain (PNP). Neuropathic pain is manifested clinically as various sensory abnormalities such as spontaneous pain, hyperalgesia, hypothesia, dyesthesias and allodynia (Woolf and Mannion 1999). Diseases like Cancer,

Correspondence to: Venkata Rathina Kumar Thiagarajan
E-mail: vrkmmcmadurai@gmail.com

AIDS, diabetes, leprosy, multiple sclerosis, and stroke are associated with neuropathic pain. Traumatic injury due to lumbar disc syndrome, traumatic spinal cord and brain injury and occupational nerve entrapment (i.e., computer typing work) injury are also associated with neuropathic pain (Alston and Pechon 2005, Koltzenburg and Scadding 2001). Chronic constriction injury of the sciatic nerve is the common model for the evaluation of anti-neuralgic agents and CCI is clinically resemble to Complex Regional Pain Syndrome (CRPS) (Kramer et al. 2009, Nagler 2010, Muthuraman et al. 2008b). Drugs currently used for treating neuropathy are gabapentin, pregabalin, carbamazepine, amitriptyline, duloxetine, topical treatments (lidocaine patch, capsaicin) and opioids which are unable to alleviate the neuropathic pain (de Leon-Casasola 2013, Kerstman et al. 2013). Adverse effects have been reported for these medicaments which limit their full clinical exploitation in the management of painful neuropathy (Hammersla and Kapustin 2012). Moreover, randomized controlled studies revealed that these drugs were ineffective in CRPS (Kalita et al. 2006). Therefore, pharmacotherapy requires newer drug molecules from alternative medicine for effective management of neuropathy particularly in CRPS.

Herbal medicines such as *Aconiti tuber*, *Lindera angustifolia*, *Teucrium polium*, *Phyllanthus emblica*, *Vochysia divergens*, *Cannabis sativa*, *Nigella sativa*, *Ocimum sanctum* and *Ginkgo biloba* have been reported to produce the beneficial effect on the management of painful neuropathy (Muthuraman et al. 2008a). Clinical reports have also been documented a beneficial effect of herbal drugs in neuropathic pain management (Ellis et al. 2009). *Vernonia cinerea* belongs to the family *Asteraceae* which is a common weed distributed throughout India. This plant is also called “Sahadevi”, Naichette or Mukuthipundu. The ethnomedical information reveals that this plant is used for malaria fever, worms, pain, inflammation,

nervous disorders, infections, wounds, cancer, edema, abortion and various gastrointestinal disorders. It is also used as an astringent and tonic. Flowers are used for conjunctivitis and rheumatism (Vaidyaratnam 1994, Thiagarajan et al. in press). This plant has been reported to possess analgesic, anti-pyretic, anti-inflammatory and anti-arthritis activity (Mazumder et al. 2003, Iwalewa et al. 2003, Latha et al. 1998). Antioxidant and anti-inflammatory activity have also been reported for both in vitro and in vivo assays (Kumar and Kuttan 2009, Pratheeshkumar and Kuttan 2010).

Other *Vernonia* species that shared some of these medicinal values include *Vernonia brachycalyx*, *Vernonia brasiliensis*, *Vernonia herbacea*, *Vernonia subligera*, and *Vernonia coloralia*. This plant has been reported in the presence of secondary metabolites like flavonoids, tannins, sesquiterpene lactones, sterols and triterpenoids (Misra et al. 1993). Flavonoids such as luteolin, apigenin, chrysoeriol, quercetin, rutin, stigmasterol-3-O-beta-D-glucoside, (+)-lirioresinol B, stigmasterol and coumaric acids like caffeic acid, ferulic acid and terpenoids like lupeol acetate were reported from this plant (Rajamurugan et al. 2011, Zhu et al. 2008). Fresh decoction of *Vernonia cinerea* has been commonly used to relieve muscular pain, knee pain and severe headache in some areas of Palani, Vadalur and Ramanathapuram regions in Tamil Nadu, India. Ayurvedic formulations namely chandrakalarasa and Alamottadi khasayam are prepared from this plant (Anonymous 2003). Although ethnomedical information indicates that this plant is used for nervous disorder. However, this plant has not been evaluated for neuropathy in experimental animals. It has also been reported for analgesic and anti-inflammatory activity. However, experimentally, its analgesic potential in neuropathic pain remains unexplored. Therefore, the present study was designed to investigate the anti-nociceptive potential of *Vernonia cinerea* on chronic constriction injury induced painful

neuropathy in rats. Pregabalin (Lyrica®) binds to the $\alpha 2\text{-}\delta$ site of an auxiliary subunit of voltage-gated calcium channels (N-type) in the central nervous system, inhibiting excitatory neurotransmitter release, and it has been reported to possess the potential management of neuropathic pain (Muthuraman and Sood 2010). Therefore, pregabalin served as a positive control in this study.

MATERIALS AND METHODS

DRUGS AND CHEMICALS

The gift sample of pregabalin was provided by Pfizer Company, India. Vincristine 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), bovine serum albumin and reduced glutathione (GSH) were purchased from Sisco Research Laboratories, Mumbai. Thiobarbituric acid was purchased from Loba Chemie (Mumbai). All other reagents were used as an analytical grade in the present study.

PLANT MATERIAL

Fresh Whole plant of *Vernonia cinerea* was collected from Madurai and authenticated by Dr. D. Stephen, Asst. Prof., Department of Botany, American College, Madurai. Plant sample has been kept in the Department of Pharmacognosy (Voucher specimen no: VC. 002/2007-2008 MMC, Madurai), Madurai Medical College, Madurai.

EXTRACTION

The fresh whole plant of *Vernonia cinerea* was shade dried at room temperature and reduced to a coarse powder (sieve no. 10/40). The dried powdered plant material of *Vernonia cinerea* (500 g) was defatted with petroleum ether and then extracted with ethanol (95%) in a Soxhlet apparatus (Süzgeç-Selçuk and Birteksöz 2011). Ethanolic extract of *Vernonia cinerea* (VC) was concentrated under reduced pressure to dryness (yield 15.12% w/w). The purity of flavonoids in VC was analyzed by High Performance Liquid Chromatography with VWD (Agilent Technologies

1220 infinity LC USA-Variable wavelength UV detector). Apigenin and kaempferol were used as (external standard) marker compounds. The chromatographic analysis was performed on a C18 column (4.6×150 mm). Mobile phase comprised of solvent A and solvent B and these two solvents were used with a constant flow rate of 1.0ml/min. Solvent A consisted of 19% acetonitrile, 5% methanol and 1% THF in water (pH 3.0), solvent B included 55% acetonitrile and 15% methanol in water (pH 3.0). The 20 μ l of VC was injected into an HPLC column and detection was performed at 352 nm according to the standard operating procedure (Oncina et al. 2000). The retention time and spectrum of the VC were compared with specific marker compounds.

DETERMINATION OF TOTAL PHENOLIC CONTENT

The total phenolic content of *Vernonia cinerea* was determined by spectrophotometric method (Harborne 1980, Siddique et al. 2010). Approximately, 1 ml (0.5 and 1 mg/ml in ethanol) of ethanolic extracts of *Vernonia cinerea* was separately mixed with 0.5 ml of Folin Ciocalteu reagent (1 N) and allowed to stand for 15 minutes. Then 1 ml of 10 percentage sodium carbonate solution was added to the above solution. Finally the mixtures were made up to 10 ml with distilled water and the mixture of extract and reagent incubated at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 760 nm spectrophotometrically. Gallic acid was used as a reference standard and the gallic acid was prepared in a variable concentration range i.e., 0, 2, 4, 6, 8 and 10 μ g / ml of ethanol. The reaction mixture without sample was used as blank. Total phenolic content of ethanolic extract of *Vernonia cinerea* was expressed in terms of mg of gallic acid equivalent per gram of extract (mg GAE / g).

DETERMINATION OF TOTAL FLAVONOID CONTENT

The total flavonoid content of *Vernonia cinerea* was determined by spectrophotometric method (Harborne 1980). Ethanolic extract *Vernonia cinerea* (0.5 ml

of 1:10 g/ml in ethanol) was separately mixed with 1.5 ml of ethanol, 0.1 ml of 10% w/v aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The mixture of extract and reagent were incubated at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm. Quercetin was used as a reference standard and the quercetin was prepared in a variable concentration range i.e., 0, 10, 20, 30, 40 and 50 µg/ml of ethanol. The content of total flavonoids was expressed as quercetin equivalents (mg of quercetin equivalents / g of VC extract).

ANIMALS

Wistar rats of both sex weighing 180-250 g, maintained on a standard laboratory diet (Kisan Feeds Ltd., Mumbai, India) and having free access to tap water were employed in the present study. They were housed in the departmental animal house and were exposed to 12 hour cycles of light and dark. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and care of the animals were carried out as per 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:- 874/ac/05/CPCSEA and Ref. No. 2360/E2/4/2010/IAEC), Madurai Medical College, Madurai.

INDUCTION OF PERIPHERAL NEUROPATHY

Painful peripheral neuropathy was induced in experimental animal by chronic constriction injury of the sciatic nerve in rat (Bennett and Xie 1988), with slight modification (Sommer and Schafers 1998). Basically, rats were anesthetized with thiopental sodium (35 mg/kg, i.p.). 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. Four loose ligatures (silk 4-0), were placed around the nerve proximal part of the trifurcation with a distance of

1 mm 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 at once. Nociceptive threshold was assessed before and after performing surgery on different days i.e. 0, 1, 3, 6, 9, 12, 15, 18 and 21st day.

EXPERIMENTAL DESIGN

Ten groups, each comprising six Wistar rats were employed in the present study.

Group I (*Normal control group*): Rats were not subjected to any surgical procedure and were kept for 21 days.

Group II (*Sham control group*): Rats were subjected to the surgical procedure to expose the left sciatic nerve without any nerve ligation process.

Group III (*CCI control group*): Rats were subjected to the surgical procedure to expose and ligate left sciatic nerve.

Group IV (*CCI + Vehicle treated group*): After subjecting the rats to chronic constriction injury of the sciatic nerve, 1% carboxy methyl cellulose was administered orally for 14 consecutive days from the day of surgery.

Group V (*VC per se*): Rats were subjected to the administration of ethanolic extract of *Vernonia cinerea* (400 mg/Kg, p.o.) for 14 consecutive days from the day of surgery.

Group VI (*Pregabalin per se*): Rats were subjected to administration of pregabalin (10 mg/Kg, p.o.) for 14 consecutive days from the day of surgery.

Group VII to IX (*CCI + VC treated group*): After subjecting the rats to CCI, ethanolic extract of *Vernonia cinerea* (VC 200, 300 and 400 mg/kg, p.o. for 14 consecutive days) was administered from the day of surgery to Group VII, VIII and IX animals respectively.

Group X (*CCI + Pregabalin treated group*): After subjecting the rats to CCI, pregabalin (10 mg/kg, p.o. for 14 consecutive days) was administered from the day of surgery.

All the groups of animals were employed to assess behavioral tests to determine the degree of nociceptive threshold on certain day intervals i.e., 0, 1, 3, 6, 9, 12, 15, 18 and 21st day. All the animals were sacrificed at the end of the 21st day and biochemical analysis was carried out in sciatic nerve tissue homogenate for estimation of total protein content, thiobarbituric reactive substance (TBARS), reduced glutathione and total calcium levels.

BEHAVIORAL STUDIES

Heat hyperalgesic test

Heat thermal sensitivity of the hind paw was assessed by using Eddy's hot plate method (Eddy et al. 1950), with slight modification, for assessing the degree of noxious thermal sensation. The rats were placed on the top of a preheated ($52.5 \pm 0.5^{\circ}\text{C}$) hot plate surface, allowing access to the left hind paw withdrawal response to the degree of the nociceptive threshold. The cut-off time of 20 s was maintained.

Cold chemical allodynic test

Cold chemical thermal sensitivity of the hind paw was assessed using acetone drop methods (Choi et al. 1994), with slight modification, for assessing the reactivity to non-noxious cold chemical stimuli. The rats were placed on the top of a wire mesh grid, allowing access to the hind paws. Acetone (100 μl) was sprayed on the plantar surface of the left hind paw of rat. Cold chemical sensitive reaction with respect to paw licking, shaking or rubbing the left hind paw was observed and recorded as a paw withdrawal threshold. The cut-off time of 20 s was maintained.

Mechanical hyperalgesic test

The mechanical sensation of the hind paw as an index of the mechano-hyperalgesic test was assessed by pressure stimulation method (Randall

and Selitto 1957). Mechanical nociceptive threshold, expressed in grams, was measured by applying increasing pressure to the left hind paw. Withdrawal of the left hind paw was used to assess the mechanical nociceptive threshold. The cut-off pressure 450 g was maintained.

Mechanical allodynia test

The mechanical sensation of the hind paw was assessed as an index of the mechano-allodynia by filaments touching method (Chaplan et al. 1994). Calibrated nylon filaments, in terms of different bending forces, was applied to the mid plantar surface of the 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 as a positive response. The criterion for the threshold value, in grams, was equal to the filament evoking a withdrawal of the paw 5 times out of 10 trials i.e., 50% response. The cut-off pressure 30 g was maintained.

Tail heat hyperalgesic test

Spinal thermal sensitivity was assessed by the tail immersion test method (Necker and Hellon 1978). The terminal part of the tail (1 cm) of the rat was immersed in a heat-noxious temperature ($52 \pm 0.5^{\circ}\text{C}$), until the tail was withdrawn. The duration of the tail withdrawal reflex was used to assess the thermal heat hyperalgesia. The cut-off time of 10 s was maintained.

BIOCHEMICAL ESTIMATION OF OXIDATIVE STRESS MARKERS

Twenty-one days after surgery, animals were sacrificed by cervical dislocation and sciatic nerve was immediately isolated from the body. The proximal part of sciatic nerve tissue homogenate (10% w/v) was prepared with 0.1 M Tris-HCl buffer (pH 7.4) and supernatant of the homogenate was employed to estimate total protein content, TBARS, reduced glutathione and total calcium content.

Estimation of tissue protein

Protein concentration was estimated by copper-protein complex reaction method (Lowry et al. 1951), using bovine serum albumin (BSA) as a standard. The absorbance was determined spectrophotometrically at 750 nm.

Estimation of lipid peroxidation

Estimation of lipid peroxidation was done by measuring the levels of malondialdehyde [MDA: thiobarbituric acid reactive substances (TBARS)] by spectrophotometric method (Ohkawa et al. 1979). The concentration of TBARS in tissue homogenate was expressed in terms of nmol of malondialdehyde per mg of protein. 1,1,3,3-Tetramethoxypropane (1–10 nmol) was used as the standard.

Estimation of reduced glutathione

Reduced glutathione (GSH, endogenous anti-oxidant molecule) was measured by spectrophotometric method (Ellman 1959). Equal quantity of the sciatic nerve homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate proteins. To 10 µl of this supernatant, 2 ml of phosphate buffer (pH 8.4), 500 µl of 5, 5'-dithio, bis (2-nitrobenzoic acid) and 400 µl double distilled water was added. The mixture was vortexed and the absorbance was taken at 412 nm within 15 min. The concentration of reduced glutathione was expressed as µg per mg of protein in sciatic nerve tissue.

Estimation of total calcium

Total calcium levels were estimated from the sciatic nerve tissue by atomic emission spectroscopic method (Severinghaus and Ferrebee 1950) with slight modification (Muthuraman et al. 2008a). The sciatic nerve tissue homogenate was mixed with 1 ml of trichloroacetic acid (4%) in ice cold

conditions and centrifuged at 2000 rpm for 10 minutes. The clear supernatant was used for the estimation of total calcium ion by atomic emission spectroscopy at 556 nm.

HISTOPATHOLOGICAL ASSESSMENT

Samples of sciatic nerve were stored in the fixative solution (10% formalin) and cut into 4 µm thickness. Staining was done by using hematoxylin and eosin (H & E) method (Sudoh et al. 2004). Nerve sections were analyzed qualitatively under light microscope (450 X) for axonal degeneration.

STATISTICAL ANALYSIS

All the results were expressed as Standard Error Mean (SEM). Data obtained from behavioral tests was statistically analyzed by using two-way repeated ANOVA, while the data of biochemical parameters was analyzed using one way ANOVA. In both cases, Tukey's multiple range tests was applied for *post-hoc* analysis. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

PHYTOCHEMICAL ANALYSIS OF ETHANOLIC EXTRACT OF *Vernonia cinerea*

The yield of ethanolic extract of *Vernonia cinerea* (VC) is found to be 14.06 grams per 100 grams of coarse powder of *Vernonia cinerea*. In addition, HPLC chromatogram analysis indicates 98% purity of ethanolic extract of *Vernonia cinerea* with reference to flavonoids marker compound (i.e., apigenin and kaempferol). The R_t value for apigenin is 41.40 and for kaempferol is 44.28 minutes (Fig. 1). Total phenolic and total flavonoids content of ethanolic extract of *Vernonia cinerea* (VC) found to contain 79.11 mg of gallic acid equivalent / gram of VC extract and 40.68 mg of quercetin equivalents / g of VC extract respectively.

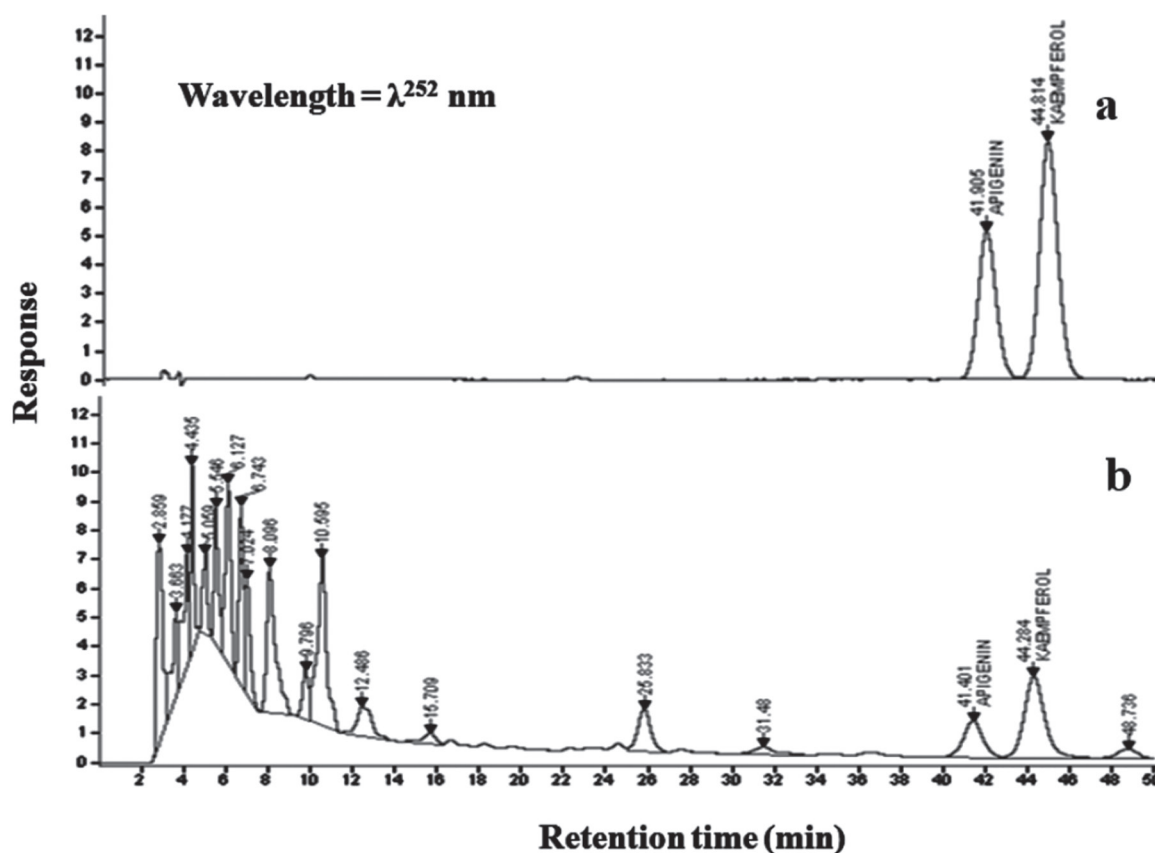


Fig. 1 - HPLC chromatogram of *Vernonia cinerea* and standard compounds.

HPLC peak in (Fig. 1a and 1b) indicates the retention time and peak area of major biomarker compounds (i.e., apigenin and kaempferol) and *VC* extract respectively. Furthermore, Fig. 1b indicates the presence of flavonoids in *VC* (Rt value for apigenin is 41.40 and for kaempferol is 44.28 minutes).

EFFECT OF *Vernonia cinerea* ON HEAT HYPERALGESIC TEST

Chronic constriction injury (CCI) of sciatic nerve resulted in significant development of noxious thermal hyperalgesia, indicated by decrease in left hind paw withdrawal threshold, after 3rd day of surgery as compared to sham control. Administration of ethanolic extract of *Vernonia cinerea* (*VC*- 200, 300, and 400 mg/kg, *p.o.*) attenuated CCI induced decrease in the nociceptive threshold for thermal hyperalgesia in a dose dependent manner. Treatment of pregabalin also produced similar effects. However, statistically significant attenuation was recorded only with medium and high dose of *VC*. Further, vehicle, *VC per se* and pregabalin did not show any significant effect on heat hyperalgesic test (Fig. 2).

EFFECT OF *Vernonia cinerea* ON COLD CHEMICAL ALLODYNIC TEST

Chronic constriction injury of sciatic nerve resulted in significant development of non-noxious cold chemical allodynia, indicated by decrease in left hind paw withdrawal threshold, after 3rd day of surgery as compared to sham control. Administration of ethanolic extract of *Vernonia cinerea* (*VC*- 200, 300, and 400 mg/kg, *p.o.*) attenuated CCI induced decrease in the nociceptive threshold for thermal allodynia in a dose dependent manner. Treatment of pregabalin also produced similar effects. However, statistically significant attenuation was recorded only with medium and high dose of *VC*. Further, vehicle, *VC per se* and pregabalin did not show any significant effect on cold chemical allodynic test (Fig. 3).

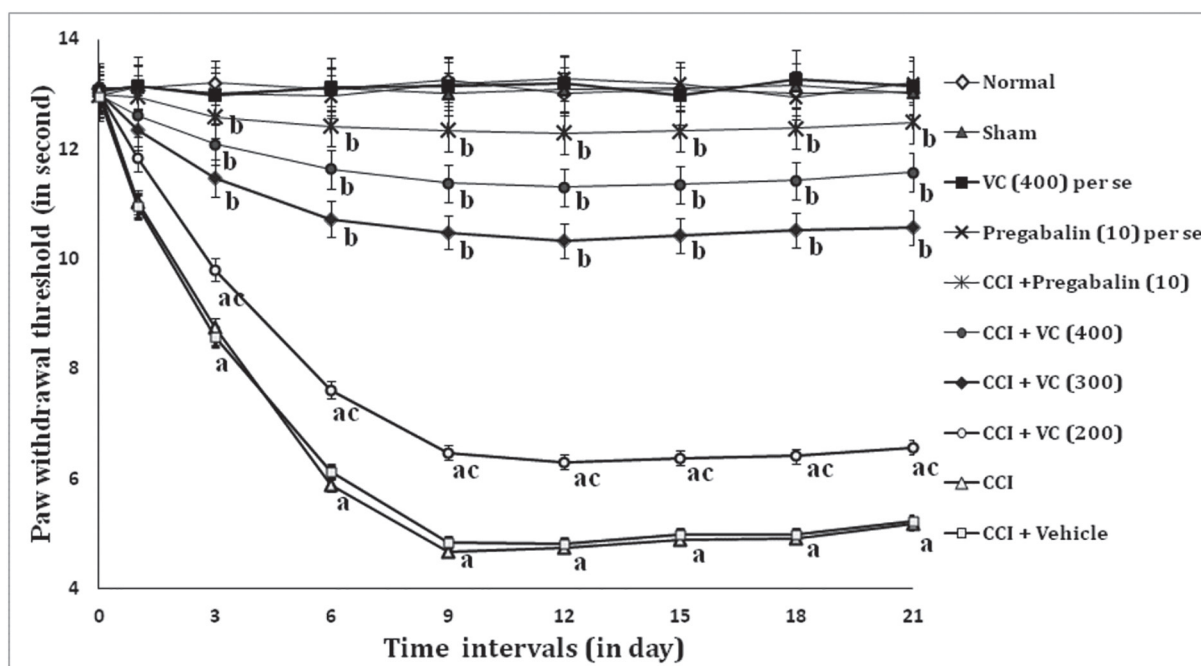


Fig. 2 - The effect of *Vernonia cinerea* on paw heat hyperalgesic test. Digits in parenthesis indicate dose in mg/kg. CCI, chronic constriction injury; VC, *Vernonia cinerea*. Data were expressed as mean \pm SD, n=6 rats per group. ^a p <0.05 vs sham control group. ^b p <0.05 vs CCI control group. ^c p <0.05 vs pregabalin treated group.

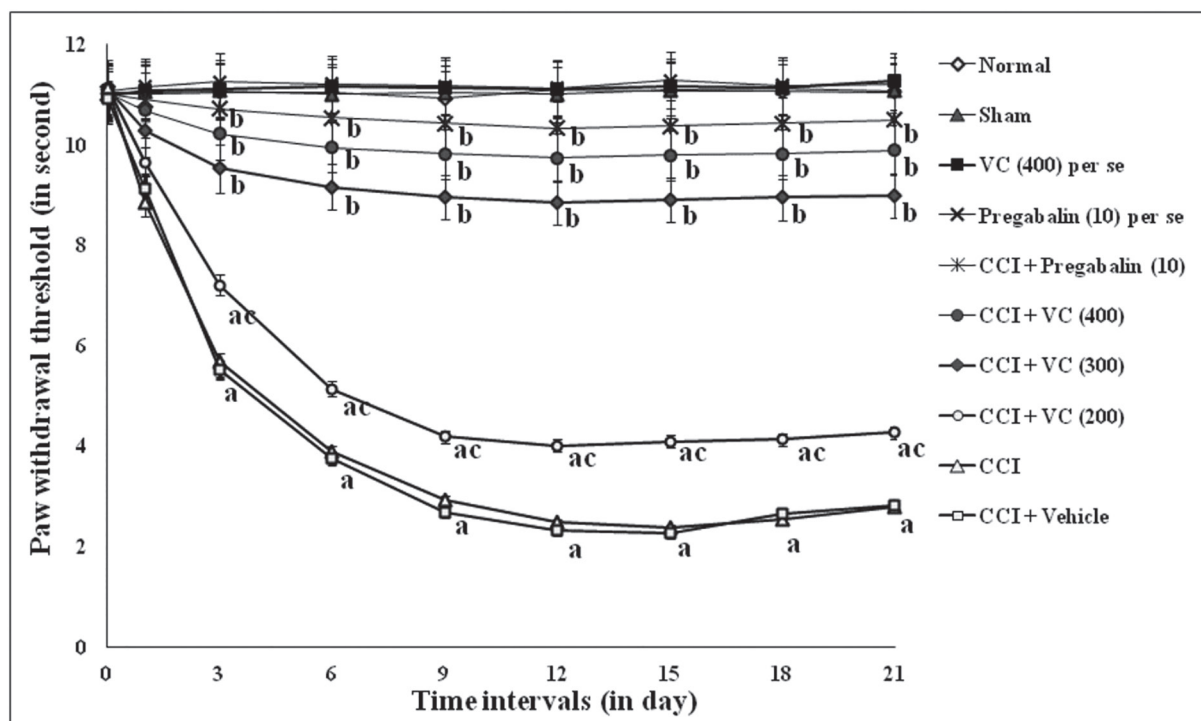


Fig. 3 - The effect of *Vernonia cinerea* on paw cold allodynia test. Digits in parenthesis indicate dose in mg/kg. CCI, chronic constriction injury; VC, *Vernonia cinerea*. Data were expressed as mean \pm SD, n=6 rats per group. ^a p <0.05 vs sham control group. ^b p <0.05 vs CCI control group. ^c p <0.05 vs pregabalin treated group.

EFFECT OF *Vernonia cinerea* ON MECHANICAL
HYPERALGESIC TEST

Chronic constriction injury of sciatic nerve resulted in significant development of noxious static mechanical hyperalgesia, indicated by decrease in left hind paw withdrawal threshold, after 3rd day of surgery as compared to sham control. Administration of ethanolic extract of *Vernonia cinerea* (VC- 200,

300, and 400 mg/kg, *p.o.*) attenuated CCI induced decrease in the nociceptive threshold for mechanical hyperalgesia in a dose dependent manner. Treatment of pregabalin also produced similar effects. However, statistically significant attenuation was recorded only with medium and high dose of VC. Further, vehicle, VC *per se* and pregabalin did not show any significant effect on mechanical hyperalgesic test (Fig. 4).

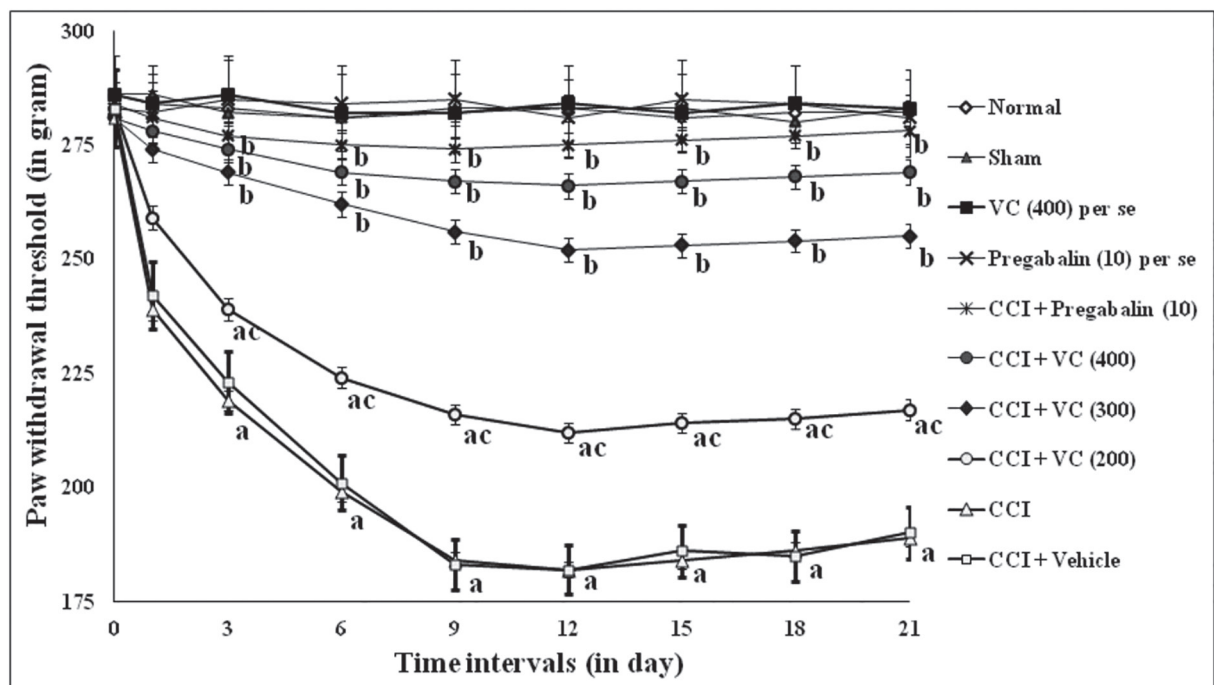


Fig. 4 - The effect of *Vernonia cinerea* on paw mechanical hyperalgesia. Digits in parenthesis indicate dose in mg/kg. CCI, chronic constriction injury; VC, *Vernonia cinerea*. Data were expressed as mean \pm SD, $n=6$ rats per group. ^a $p<0.05$ vs sham control group. ^b $p<0.05$ vs CCI control group. ^c $p<0.05$ vs pregabalin treated group.

EFFECT OF *Vernonia cinerea* ON MECHANICAL
ALLODYNIC TEST

Chronic constriction injury of sciatic nerve resulted in significant development of non-noxious tactile mechanical hyperalgesia, indicated by decrease in left hind paw withdrawal threshold, after 3rd day of surgery as compared to sham control. Administration of ethanolic extract of *Vernonia cinerea* (VC-

200, 300, and 400 mg/kg, *p.o.*) attenuated CCI induced decrease in the nociceptive threshold for mechanical hyperalgesia in a dose dependent manner. Treatment of pregabalin also produced similar effects. However, statistically significant attenuation was recorded only with medium and high dose of VC. Further, vehicle, VC *per se* and pregabalin did not show any significant effect on mechanical hyperalgesic test (Fig. 5).

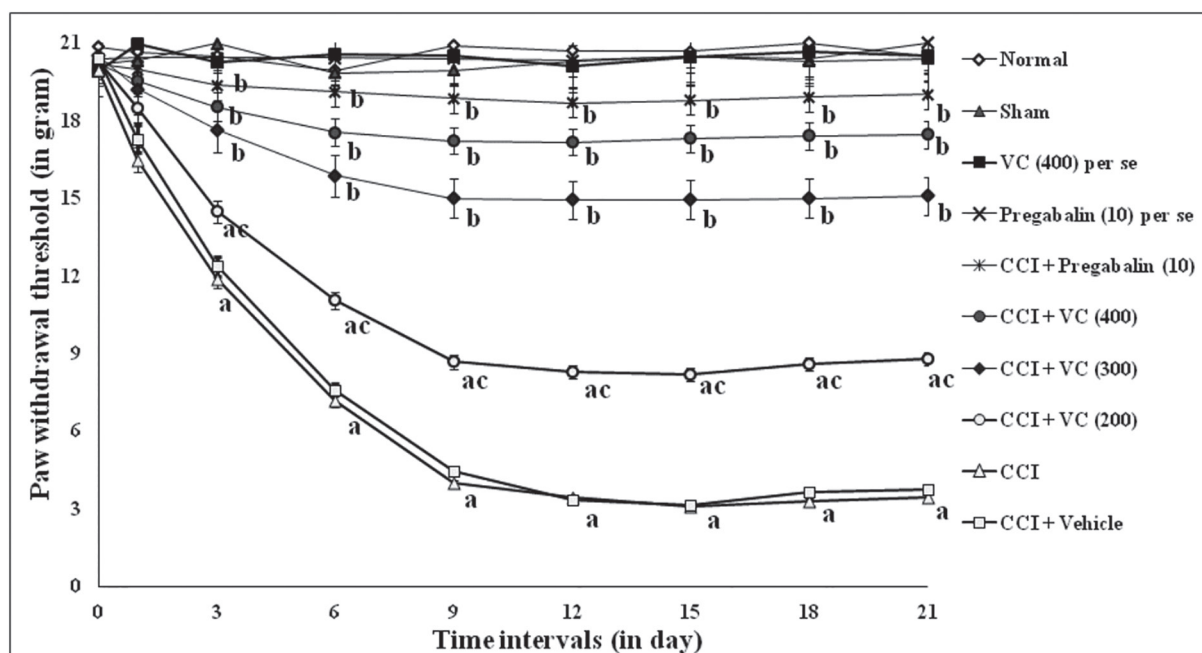


Fig. 5 - The effect of *Vernonia cinerea* on paw mechanical allodynia. Digits in parenthesis indicate dose in mg/kg. CCI, chronic constriction injury; VC, *Vernonia cinerea*. Data were expressed as mean \pm SD, $n=6$ rats per group. ^a $p<0.05$ vs sham control group. ^b $p<0.05$ vs CCI control group. ^c $p<0.05$ vs pregabalin treated group.

EFFECT OF *Vernonia cinerea* ON TAIL HEAT

HYPERALGESIC TEST

Chronic constriction injury of sciatic nerve resulted in significant development of noxious thermal hyperalgesia, indicated by decrease in tail withdrawal threshold, after 3rd day of surgery as compared to sham control. Administration of ethanolic extract of *Vernonia cinerea* (VC- 200, 300, and 400 mg/kg, *p.o.*) attenuated CCI induced decrease in the nociceptive threshold for thermal hyperalgesia in a dose dependent manner. Treatment of pregabalin also produced similar effects. However, statistically significant attenuation was recorded only with medium and high dose of VC. Further, vehicle, VC *per se* and pregabalin did not show any significant effect on tail heat hyperalgesic test (Fig. 6).

EFFECT OF *Vernonia cinerea* ON OXIDATIVE STRESS

MARKERS AND CALCIUM LEVELS

Sciatic nerve ligation resulted in a significant rise in TBARS, total calcium levels and in a decrease in the levels of reduced glutathione, after the 21st day of

surgery as compared to sham control. Administration of the ethanolic extract of *Vernonia cinerea* (VC-200, 300, and 400 mg/kg, *p.o.*) attenuated CCI induced rise in sciatic nerve tissue thiobarbituric reactive substances (TBARS), total calcium and in a decrease in GSH levels in a dose dependent manner. Treatment of pregabalin also produced similar effects. Further, vehicle, VC *per se* and pregabalin did not show any significant effect on biochemical levels (Table I).

EFFECT OF *Vernonia cinerea* ON HISTOPATHOLOGICAL CHANGES

Chronic constriction injury of sciatic nerve resulted in significant histopathological changes assessed in a transverse section of the sciatic nerve. In transverse section, nerve derangement, axonal swelling, increase in the number of Schwann and satellite cells were also noted. Administration of the ethanolic extract of *Vernonia cinerea* (200, 300, and 400 mg/kg, *p.o.*) significantly attenuated CCI induced axonal degeneration and histopathological alterations (Fig. 7).

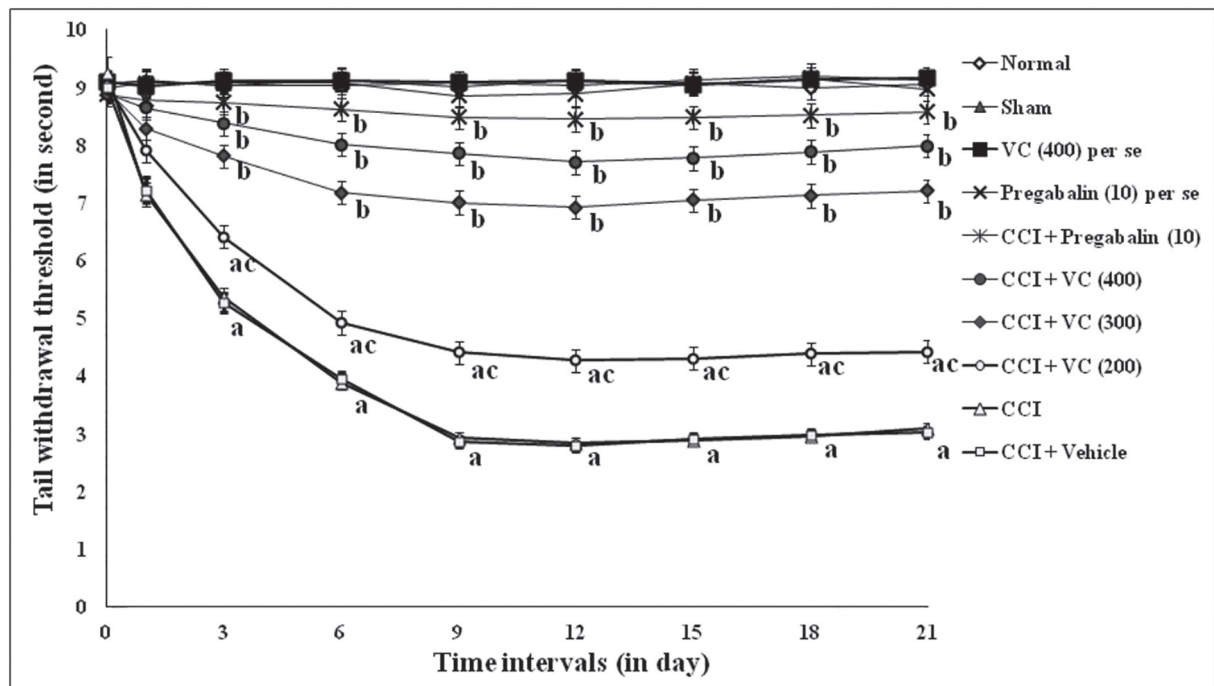


Fig. 6 - The effect of *Vernonia cinerea* on tail heat hyperalgesia. Digits in parenthesis indicate dose in mg/kg. CCI, chronic constriction injury; VC, *Vernonia cinerea*. Data were expressed as mean \pm SD, n=6 rats per group. ^a p <0.05 vs sham control group. ^b p <0.05 vs CCI control group. ^c p <0.05 vs pregabalin treated group.

TABLE I
The effect of *Vernonia cinerea* on tissue biomarker changes.

Groups	MDA (nmol/mg of protein)	GSH (μ g/mg of protein)	Total calcium (ppm/mg of protein)
Normal	3.09 \pm 0.33	74.42 \pm 2.36	2.83 \pm 0.22
Sham	3.12 \pm 0.39	74.26 \pm 2.58	2.94 \pm 0.31
CCI	4.39 \pm 0.27 ^a	48.25 \pm 4.21 ^a	20.32 \pm 0.47 ^a
Vehicle in CCI	4.42 \pm 0.29 ^a	47.96 \pm 3.63 ^a	19.49 \pm 0.32 ^a
VC (400) per se	3.16 \pm 0.21	73.56 \pm 2.71	2.73 \pm 0.39
Pregabalin (10) per se	3.09 \pm 0.34	74.38 \pm 2.58	2.85 \pm 0.39
VC (200) in CCI	4.37 \pm 0.24 ^{ac}	53.37 \pm 2.31 ^{ac}	18.63 \pm 0.19 ^{ac}
VC (300) in CCI	3.46 \pm 0.29 ^b	64.19 \pm 2.84 ^b	10.69 \pm 0.34 ^b
VC (400) in CCI	3.24 \pm 0.16 ^b	68.38 \pm 2.03 ^b	7.83 \pm 0.42 ^b
Pregabalin (10) in CCI	3.27 \pm 0.19 ^b	72.51 \pm 2.43 ^b	5.02 \pm 0.19 ^b

Digits in parenthesis indicate dose in mg/kg.

MDA, malondialdehyde; GSH, reduced glutathione; CCI, chronic constriction injury; VC, *Vernonia cinerea*.

Data were expressed as mean \pm SD, n= 6 rats per group.

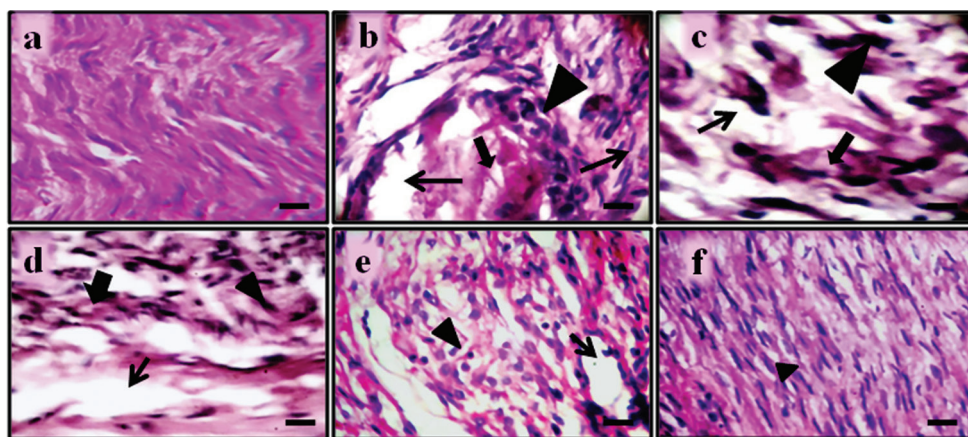


Fig. 7 - The effect of *Vernonia cinerea* on histopathological changes.

Figures 7a-7f show transverse-section of sciatic nerve of sham, CCI, *VC* (200, 300 and 400 mg/kg, *p.o.*) and pregabalin (10 mg/kg, *p.o.*) pretreated groups respectively. In figure 7, thin arrow shows axonal swelling, thick arrow shows fiber arrangement and arrow head shows CCI induced expression of satellite cells and Schwann cells. Figure 7b shows CCI induced axonal swelling, derangement of nerve fibers and expression of neuroglial cells (i.e., satellite and Schwann cells). Figures 7c-7f, pretreatment of *Vernonia cinerea* (200, 300 and 400 mg/kg, *p.o.*) and pregabalin (10 mg/kg, *p.o.*) show the ameliorative effect in CCI induced histopathological changes (i.e., axonal swelling, derangement of nerve fibers and expression of neuroglial cells). Microscopic examination was performed under 450 × light microscopy, scale bar 35 μm.

DISCUSSION

In the present study, *Vernonia cinerea* attenuated chronic constriction injury of sciatic nerve induced behavioral [i.e., heat (paw and tail) hyperalgesia, cold chemical allodynia, mechanical hyperalgesia and allodynia], biochemical (TBARS, total calcium and reduced glutathione) and histopathological changes. Furthermore, this plant has also been used as a nerve tonic to alleviate disorders related to nerves and as an analgesic in muscular pain, joint pain and severe headache.

Pathophysiological changes in diabetes and their complications i.e., diabetic neuropathy, Freund's adjuvant induced inflammation, CCI and vincristine mediated neuropathy were also mediated by reactive oxygen and reactive nitrogen species (Honda et al. 2004, Gao et al. 2007, Otto et al. 2003). In the present study, elevation of TBARS (an index of lipid peroxidation) and total calcium and fall in the reduced glutathione

(GSH, an endogenous anti-oxidant level) were noted in the CCI induced animals thus supporting the contention that free radicals may contribute to the pathogenesis of neuropathy. CCI induced peripheral and central behavioral changes associated with oxidative stress marker alterations were attenuated by the administration of ethanolic extract of *Vernonia cinerea* (Bandyopadhyay et al. 1999, Stanciu et al. 2000). Intracellular Ca^{2+} concentration was elevated by free radicals along with activation of NMDA receptor, which has been considered as having consanguineous relation with pain modulation (Stanciu et al. 2000). Present and other laboratory reports suggested that CCI induced increases in the calcium level in sciatic nerve (Muthuraman and Singh 2011). Electrical hyper-excitability, depletion of ATP and activation of calpains were noted with increased calcium ions (Xie and Barrett 1991). Calcium induced activation of calpain is also associated with the generation

of reactive oxygen species from mitochondria (Carriedo et al. 2000). Calcium-induced activation of calpains has been shown to be responsible for the axonal degeneration by alteration of stability of axonal cytoskeleton protein (Glass et al. 2002).

Administration of *Vernonia cinerea*, attenuated CCI induced rise in calcium ion and oxidative stress markers and it plays a critical role to produce the anti-nociceptive effects in painful peripheral neuropathy. The noted decrease in calcium levels with *Vernonia cinerea* may be attributed to its anti-oxidant effects and free radicals are well reported to increase calcium ions (Glass et al. 2002). However, the possibility of direct action of *Vernonia cinerea* decrease in calcium level remains to be explored. Moreover, increase in calcium ions is also associated with an increase in oxidative stress (Carriedo et al. 2000). So, the noted antioxidant effects of *Vernonia cinerea* may be the secondary effect in decrease of calcium ion level in sciatic nerve. Similar results were obtained in the pregabalin treated animals. Pregabalin is a potential voltage dependent calcium channel ($\alpha 2-\delta$ subunit) antagonist. It has also been reported to possess the potential role in the management of painful neuropathy in human and in experimental animals (Kumar et al. 2010). *Vernonia cinerea* extract was found to possess free radical scavenging activity and proinflammatory cytokine and TNF-alpha inhibition activity. Administration of *Vernonia cinerea* extracts in mice significantly increases the level of anti-oxidant markers such as catalase, superoxide dismutase, glutathione, glutathione peroxidase and glutathione-S transferase in blood and liver, whereas lipid peroxidation activity was significantly decreased (Pratheeshkumar and Kuttan 2010, Kumar and Kuttan 2009, Rajamurugan et al. 2011). Recent reports suggested that *Vernonia cinerea* has analgesic, anti-inflammatory, anti-oxidant, radical scavenging and inhibition of pro inflammatory cytokines like TNF-alpha (Pratheeshkumar and Kuttan 2010, Rajamurugan et al. 2011, Thiagarajan et al. in press).

CONCLUSION

The ameliorative potential of *Vernonia cinerea* against chronic constriction injury of sciatic nerve induced neuropathic pain may be due to its potential anti-oxidative, neuroprotective and inactivation of calcium channel opening actions. Nevertheless further studies are needed to substantiate these findings.

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RESUMO

O objetivo do presente estudo é investigar o potencial benéfico do extrato etanólico da planta inteira de *Vernonia cinerea* sobre a dor neuropática induzida pela lesão por constrição crônica (CCI) do nervo ciático em ratos. Parâmetros comportamentais, tais como uma placa quente, uma gota de acetona, a pressão na pata, teste do filamento de Von Frey e teste de imersão da cauda foram realizados para avaliar o grau da hiperalgesia e alodinia térmica, química e mecânica. Alterações bioquímicas no tecido do nervo ciático foram descartadas estimando substâncias reativas ao ácido tiobarbitúrico (TBARS), glutathione reduzida (GSH) e os níveis totais de cálcio. Extrato etanólico de *Vernonia cinerea* e pregabalina foram administrados por 14 dias consecutivos a partir do dia da cirurgia. Mostramos que CCI do nervo ciático induziu mudanças significativas em parâmetros comportamentais, bioquímicos e histopatológicos quando comparado com o grupo simulado controle. *Vernonia cinerea* atenuou, de uma maneira dependente da dose, as alterações patológicas acima descritas, induzidas por CCI do nervo ciático, o que é semelhante à atenuação do grupo pré-tratado com pregabalina. O efeito de melhora do extrato etanólico

de *Vernonia cinerea* contra a dor neuropática induzida pela CCI de nervo ciático pode ser devido à presença de flavonóides, e este efeito é atribuído às ações antioxidativa, neuroprotetora e moduladora de canais de cálcio destes compostos.

Palavras-chave: antioxidante, cálcio, lesão por constrição crônica, glutatona reduzida, substância reativa ao ácido tiobarbitúrico.

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