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PHARMACOLOGICAL EVALUATION OF *Passiflora incarnata* ON VINCRISTINE INDUCED NEUROPATHY IN RATS

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ABSTRACT

The present study was designed to explore the anti-nociceptive effects of hydroethanolic extract of *Passiflora incarnata* in vincristine induced painful neuropathy in rats. The intraperitoneal administration of vincristine 50 µg/kg; for 10 consecutive days has been employed for the induction of neuropathy. Hydroethanolic extract of *Passiflora incarnata* (HEEPI)(100mg/kg, 200mg/kg; *p.o)* and pregabalin (standard; 10mg/kg; *p.o*) has to be administered for 14 consecutive days two hour before vincristine injection. Behavioural tests such as paw heat hyperalgesic test, cold chemical allodynic test and tail heat hyperalgesic test were assessed starting from day 0 and subsequently on day 1, 3, 6, 9, 12, 15, 18 and 21st day. Further, the biochemical changes i.e., thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH)and total calcium levels has to be estimated in sciatic nerve tissue, on 21st day after sacrificing the animal and histopathological changes has to be observed in sciatic nerve tissue. Administration of vincristine resulted in significant changes in behavioural and biochemical parameters. Pretreatment of HEEPI ameliorated vincristine induced behavioral, biochemical and histopathological changes in a dose dependent manner, which is similar to that of pregabalin pretreated group. The results suggests that, the neuroprotective effect of *Passiflora incarnata* may be due to its potential of anti-oxidative, calcium channel modulating and neuroprotective action.

KEYWORDS: Vincristine, Biochemical changes, *Passiflora incarnata,* Calcium channel modulating effect, Oxidative stress.

INTRODUCTION

Neuropathic pain is defined as 'pain caused by a lesion or disease of somatosensory nervous system'(Ross and Wilson, 2001). Neuropathic pain 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). Peripheral neuropathic pain is frequently observed in patients with cancer, AIDS, long-standing diabetes, lumbar disc syndrome, herpes infection, traumatic spinal cord injury (SCI), multiple sclerosis and stroke. Moreover, post-thoracotomy, post-herniorrhaphy, post-mastectomy and post-sternotomy are some other conditions often associated with peripheral neuropathic pain (Jaggi AS et al., 2011). The pharmacotherapy of neuropathic pain has exhibited a limited success with little or no response to commonly used pain reducing drugs, such as NSAIDS and opiates (Hsiupei Chen et al., 2004).

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., 2008).

Passiflora incarnata (Passifloraceae) is a perennial, creeping herb, climbing by means of axillary tendrils. Leaves alternate, palmately three to five serrate lobes. Flowers large, solitary, with long peduncles, whitish, with a triple purple and pink crown. Fruits are ovate berries containing numerous ovoid, flattened seeds covered with a yellowish or brownish aril native to America. The ethanomedicinal information revealed that this plant is used to treat neuralgia, ulcer and asthma etc (Timothy Johnson 1998). Therefore, the present study was designed to investigate the role of hydroethanolic extract of *Passiflora incarnata* in vincristine induced neuropathic pain in rats. Pregabalin widely used for the management of neuropathic pain, and our earlier experimental report suggesting that, it can be used as a positive control for the neuropathic pain related studies.

MATERIALS AND METHODS

Chemicals and drugs

Hydroethanolic extract of *Passiflora incarnata*(HEEPI), vincristine sulphate (Chandra Bhagatpharma Pvt. Ltd., Mumbai), Pregabalin (Glenmark), Folin-ciocalteu's-phenol reagent, DTNB ([5, 5′-dithio, bis (2-nitrobenzoic acid)], Reduced glutathione, Bovine serum albumin, Thiobarbituric acid and 1, 1, 3, 3 Tetra Methoxy Propane.

Plant material

The leaves and stem of *Passiflora incarnata* were collected from Salem, Tamilnadu, India. The plant was identified and authenticated by senior plant Taxonomist at Plant Anatomy Research Centre (PARC).

Sample preparation

The shadow dried leaves and stem were powdered coarsely in a laboratory blender and defatted with petroleum ether (60-80 °c) by cold maceration for 72 h. The defatted marc was extracted with ethanol by cold maceration method for 72 h and filtered. The filtrate was concentrated under reduced pressure in rotary vacuum evaporator at 40 °c, which gave a green residue and it was designated as hydroethanolic extract of leaves and stem of *Passiflora incarnata*.

Animals

Wistar albino rats, weighing 200-250 g, were employed in the present study. The rats were exposed to 12 h light-dark cycles. The experimental protocol was duly approved by the Institutional Animal Ethics Committee.

Acute oral toxicity study

As per OECD 423 guidelines six animals were used for this study. *HEEPI* 2000 mg/kg of bodyweight (*b.w.*), was administered orally to three animals and mortality was not observed in any of these animals. Then the same dose of *HEEPI* 2000 mg/kg of *b.w.*, is administered to another three animals to confirm the toxic dose. All the test animals were kept under investigation for 14 days for the observation of various parameters (Combes et al., 2006).

Induction of peripheral neuropathy

Peripheral neuropathy was induced in rats by administration of vincristine sulfate (50 µg/kg; *i.p*. daily) for 10 consecutive days. Behavioral test were assessed on different days. i.e. 0, 1, 3, 6, 9, 12, 15, 18 and 21st day.

EXPERIMENTAL PROTOCOL

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

Group I (Normal control group)

Rats were not subjected to administration of vehicle and vincristine and were kept for 21 days. Behavioral tests such as hot plate test, acetone drop test and tail immersion test were employed to assess nociceptive threshold of the hind paw on different day's i.e. 0, 1, 3, 6, 9, 12, 15, 18 &21st day.

Group II (vincristine 50 µg/kg; i.p)

Vincristine (50 µg/kg; *i.p)* was administered to the rats for 10 consecutive days. Behavioral tests were assessed as described in group I.

The HEEPI extract and standard drug (Pregabalin) were prepared as suspension using 1% CMC.

Group III (HEEPI extract 100 mg/kg treated group)

HEEPI extract (100 mg/kg, *p.o)* was administered two hours before each vincristine injection (vincristine was administrated daily for 10 days; 50 µg/mg, *i.p*) for 14 consecutive days. Behavioral tests were assessed as mentioned in group I.

Group IV (HEEPI extract 200 mg/kg, treated group)

HEEPI (200 mg/kg, *p.o)* was administered two hours before each vincristine injection (vincristine was administrated daily for 10 days; 50 µg/mg, *i.p*) for 14 consecutive days. Behavioral tests were assessed as mentioned in group I.

Group V (Pregabalin 10mg/kg, treated group)

Pregabalin (10 mg/kg, *p.o)* was administered orally two hours before each vincristine injection (vincristine was administered once in three days) for 14 consecutive days. Behavioral tests were assessed as mentioned in group I.

Group VI (Per se group)

HEEPI (200 mg/kg, *p.o)* was administered for 10 consecutive days. Behavioral tests were assessed as mentioned in group I.

BEHAVIORAL ASSESMENT

Hot plate 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 ± 0.5 ºc) hot plate surface, allowing access to the paw licking or vocalization or jumping which one is first appearing to degree of the nociceptive pain threshold. The cut- off time of 20 s was maintained to avoid the tissue injury.

Tail immersion test

Spinal nociceptive pain sensitivity was assessed by using tail immersion test as per Necker and Hellon method (Necker and Hellon, 1978). Tip of the rat's tail (1 cm level from the terminal part of the tail) was immersed into the temperature (52 ± 0.05 ºc) controlled water chamber. Spinal thermal hyperalgesia was assessed by the time taken to withdrawal of tail (flick response) from the water path as indicated tail withdrawal reflex. The cut off time of 10 s was maintained to avoid the tissue injury.

Acetone drop test (Paw cold-allodynia)

Cold-allodynia of the hind paw was assessed using acetone drop method using standard procedure with slight modification, for evaluating the reactivity to noxious cold chemical stimuli. The rats were placed on the top of a wire mesh grid, allowing access to the hind paws. Acetone (0.1 ml) was sprayed on the plantar surface of hind paw of rat and time taken for withdrawing the hind paw from the mesh surface was noted. The cut off time of 20 seconds.

All the animals were sacrificed according to CPCSEA guidelines at the end of the 21st day. Sciatic nerve of the animals were isolated and a portion of the sciatic nerve was used to prepare a homogenate with Tris HCl buffer to estimate biochemical markers such as total protein, Thiobarbituric acid reactive substances (TBARS), reduced glutathione(GSH), and total calcium level. Histopathological studies also carried with the distal portion of the sciatic nerve.

BIOCHEMICAL ESTIMATION OF MARKERS OF OXIDATIVE STRESS

Freshly excised sciatic nerve homogenate (10 %) was prepared with 0.1 M Tris Hcl buffer (P^H-7.4) and the homogenate was kept in ice water for 30 min and centrifuged at 4˚C (2000 g,10 min).The supernatant of homogenate was separated and which was used to estimate following biochemical markers.

Estimation of total protein content

The tissue total protein concentration was estimated according to the Lowry's method (Lowry et al., 1951). The absorbance was determined spectrophotometrically at 750 nm. The bovine serum albumin was used as the standard. The concentration of total protein was expressed as mg of protein per gram of tissue.

Estimation of total calcium

The tissue total calcium level was estimated in sciatic nerve by using atomic emission spectroscopy method (Severinghaus and Ferrebee, 1950) with slight modification of (Muthuraman et al., 2008). The sciatic nerve homogenate was prepared with 1 ml of trichloroacetic acid (4 %) under ice cold condition and centrifuged at 1500 g for 10 min at 4 ºc. The clear supernatant was used for the estimation by atomic emission spectroscopy at 556 nm. The calcium chloride was used as the standard. The concentration of total calcium was expressed as ppm per mg of proteins.

Estimation of reduced glutathione

Reduced glutathione was measured according to the Ellman method (Ellman, 1959). The absorbance was determined spectrophotometrically at 412 nm. The reduced glutathione was used as the standard. The concentration of reduced glutathione was expressed as µg per mg of protein.

Estimation of TBARS

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

Histopathology studies

Samples of sciatic nerve were stored in fixative solution (10 % formalin) and cut in to 4 µm thickness. Staining was done by using hematoxylin and eosin as described by standard procedure. Nerve sections were analysed qualitatively with a light microscope (45x) for axonal degeneration.

Statistical analysis

All the results were expressed as mean ± standard error of means (SEM). The data from the behavioral results were statistically analysed by two-way analysis of variance followed by bonferroni's post hoc-test by using graph pad prism v.5.0 software. The data from the biochemical results were statistically analysed by oneway ANOVA followed by Tukey's multiple range tests. $p< 0.05$ was considered to be statistically significant.

RESULTS

Acute oral toxicity study

Acute oral toxicity study has been performed as per OECD 423 guidelines (Combes et al., 2006). Hydroethanolic extract of *Passiflora incarnata* has not produced any toxic symptoms or mortality at the dose level of 2000 mg/kg; *b.w.* in rat and hence the extract of this drug was expected as a potential pharmacological action.

Effect of HEEPI extract on paw heat hyperalgesic test

Administration of vincristine caused significant development noxious thermal hyperalgesia noted by decrease in hind paw withdrawal threshold after 3rd day of vincristine administration when compared to normal control group. Vincristine induced, decrease in nociceptive threshold for thermal hyperalgesia was improved by the administration of HEEPI (100 and 200 mg/kg, *p.o.*) in a dose dependent manner. Pregabalin treated animals also produced similar effects. Normal control and per se group of animals did not show any significant effect on paw heat hyperalgesic test (Figure 1).

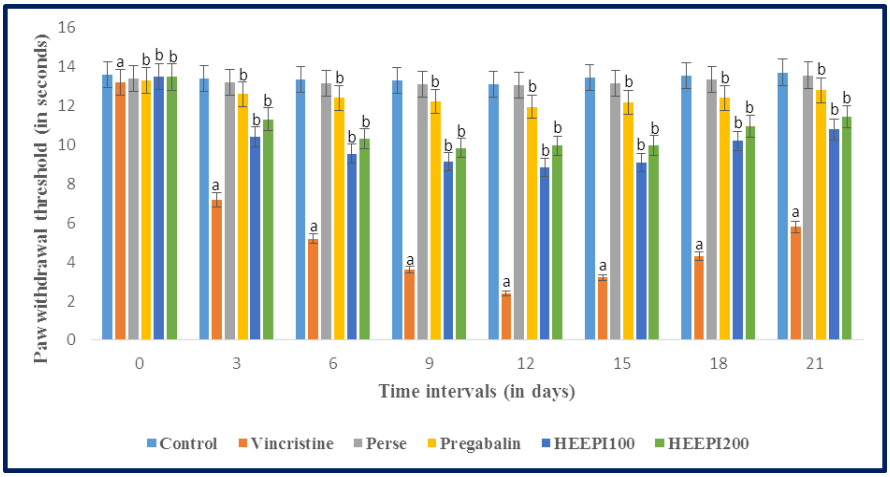


Figure 1: EFFECT OF HEEPI ON PAW HEAT HYPERALGESIA

Digits in *parenthesis* indicate dose in mg/kg. Data were expressed as mean ± SEM, n=6 rats per group. a- Indicate statistical significance (p<0.05) difference when compared to normal control group. b- Indicate statistical significance (p<0.05) difference when compared to vincristine control group.

Effect of HEEPI on paw cold allodynia test

Vincristine treatment lead to the development of paw cold-allodynia indicated by decrease in the nociceptive threshold, when compared to normal control group of animals. Treatment of HEEPI at 100 and 200 mg/kg, *p.o.* improved the nociceptive threshold in a dose dependent manner. Similar result was obtained with pregabalin treated animals. Normal control and per se animals did not show any effect on paw cold allodynia test (Figure 2).

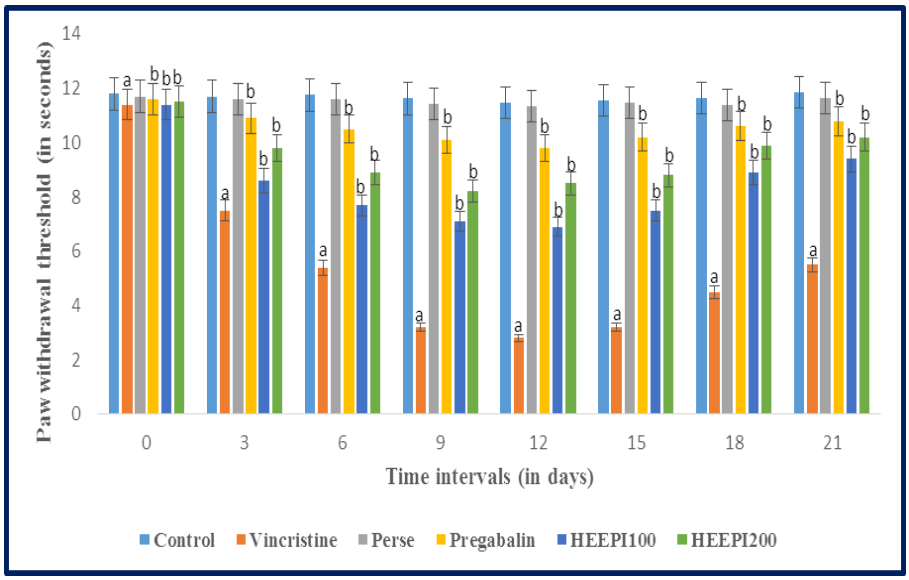


Figure 2: EFFECT OF HEEPI EXTRACT ON PAW COLD ALLODYNIA TEST

Digits in *parenthesis* indicate dose in mg/kg. Data were expressed as mean ± SEM, n=6 rats per group. a- Indicate statistical significance (p<0.05) difference when compared to normal control group. b- Indicate statistical significance (p<0.05) difference when compared to vincristine control group.

Effect of HEEPI on tail immersion test

Vincristine administration caused significant development of noxious thermal hyperalgesia noted by decrease in tail withdrawal threshold, after 3rd day of vincristine administration when compared to normal control animals. Vincristine induced, decrease in nociceptive threshold for thermal hyperalgesia was improved by administration of HEEPI at 100 and 200 mg/kg, *p.o.* in a dose dependent manner. Treatment of pregabalin also produced similar effects. Normal control and per se animals did not show any effect on tail immersion test (Figure 3).

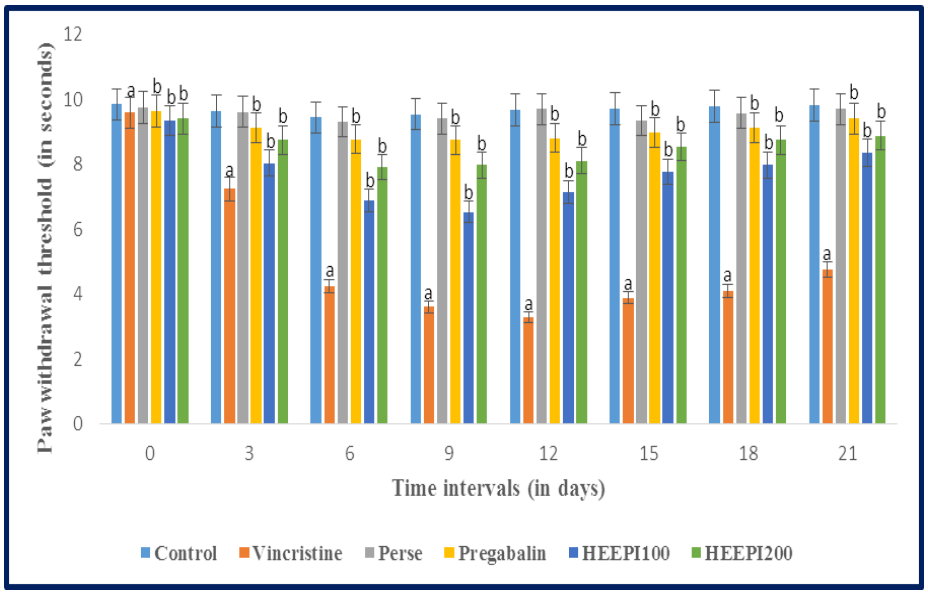


Figure 3: EFFECT OF HEEPI ON TAIL HEAT HYPERALGESIA

Digits in *parenthesis* indicate dose in mg/kg. Data were expressed as mean ± SEM, n=6 rats per group. a- Indicate statistical significance (p<0.05) difference when compared to normal control group. b- Indicate statistical significance (p<0.05) difference when compared to vincristine control group.

Effect of HEEPI on nerve tissue biomarkers changes

Elevated TBARS, calcium and decrease in GSH level were noted in the vincristine control group of animals. Administration of HEEPI 100 and 200 mg/kg, b.w, *p.o* caused significant

(p<0.05) decrease TBARS and calcium level and elevation of GSH level in a dose dependent. Treatment of standard drug pregabalin also produced similar effect as produced by the extracts. The per se group did not show any significant effect for the biochemical parameters (Table 2).

Table 1: EFFECT OF HEEPI EXTRACT ON TISSUE BIOMARKER CHANGES

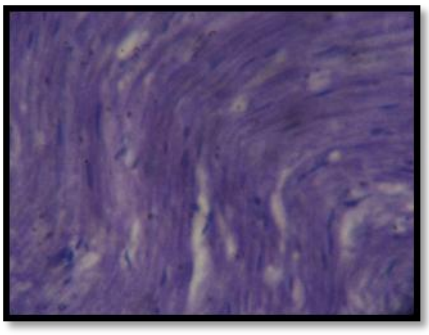
Groups	Total Protein (mg /g of tissue)	TBARS (nmol/mg of protein)	GSH (µg/mg of protein)	Total calcium (ppm/mg of protein)
Normal	0.38±0.006	2.36±0.02	81.05±0.31	2.86±0.03
Vincristine	0.40±0.01	5.18±0.01 [a]	33.95±0.74 [a]	22.03±0.41 [a]
Perse	0.43±0.005	2.56±0.04	78.12±0.55	3.41±0.05
Pregabalin (10)	0.42±0.01	2.68±0.01 [b]	75.61±0.62 [b]	5.28±0.23 [b]
HEEPI(100)	0.39±0.008	3.66±0.06 [b]	62.97±0.55 [b]	12.23±0.15 [b]
HEEPI(200)	0.41±0.01	3.21±0.05 [b]	71.12±0.28 [b]	6.56±0.14 [b]

*mean of six readings ± SEM

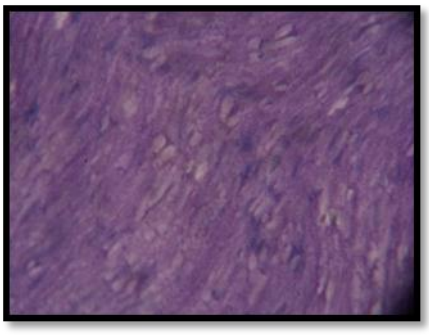
Digits in *parenthesis* indicate dose in mg/kg. Data were expressed as mean ± SEM, n=6 rats per group. a- Indicate statistical significance (p<0.05) difference when compared to normal control group. b- Indicate statistical significance (p<0.05) difference when compared to vincristine control group.

Histopathological studies

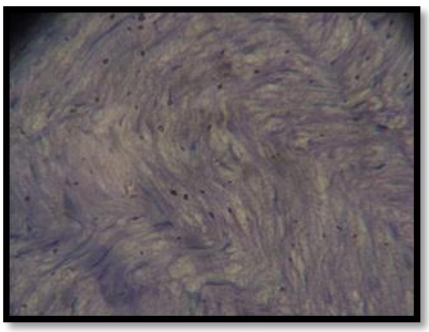
The sciatic nerve of vincristine group showed nerve derangement, axonal degeneration and axonal swelling. Treatment with hydroethanolic extract of HEEPI (100 and 200mg/kg, *p.o.*) significantly attenuated vincristine induced histopathological alterations. Similar effects were observed with pregabalin treatment (Figure 4).



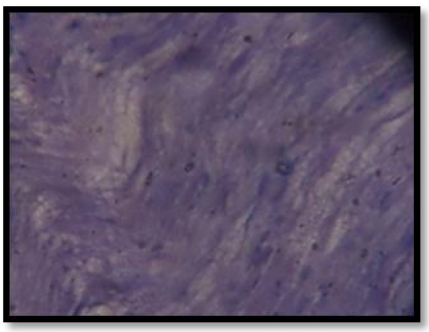
a) Normal Control group



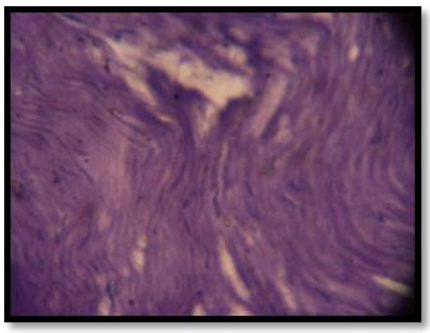
b) *Per se group*



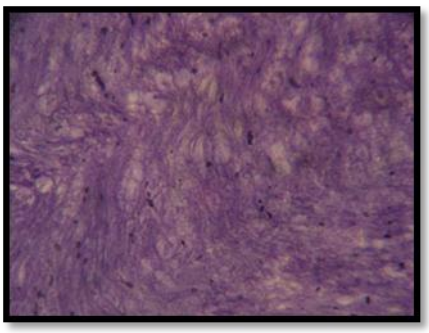
c) Vincristine group



d) HEEPI 100mg/kg group



e) HEEPI 200mg/kg group



f) Standard group

FIGURE: 4 EFFECT OF VINCRISTINE INDUCED HISTOPATHOLOGICAL CHANGES.

DISCUSSION

The results of the present study indicate that administration of vincristine produced a significant degree of painful neuropathy in rats, manifested as behavioural (thermal and mechanical hyperalgesia and mechanical allodynia), biochemical (TBARS, GSH and total calcium levels) and histopathological changes. Vincristine is a chemotherapeutic agent that often evoke a long-lasting painful peripheral neuropathy. It exerts antitumor activity largely by binding to β- tubulin and disrupting mitotic spindle formation in actively dividing cells. It has also been reported to affect Ca^{2+} movement through the mitochondrial membrane, reducing both the amount and rate of Ca^{2+} uptake and decreasing Ca^{2+} efflux and free radical generation (Tari et al., 1986). It is well-established that intracellular Ca^{2+} represents a key role for neurotransmitter release, cell membrane excitability, activation of intracellular proteins and pain thresholds (Silinsky et al., 1977). After vincristine treatment, the behavioural alterations started to show from 3rd day and the maximal nociceptive threshold was observed on 9th day. In this study vincristine produced a rise in the levels of calcium (Ca^{2+}), TBARS and decreased GSH levels, these observations support the contention that enhanced Ca^{2+} ions, free radicals together played a key role in producing vincristine-induced painful neuropathy.

Treatment with hydroethanolic extract of *Passiflora incarnata* (HEEPI) and pregabalin significantly attenuated vincristine-induced behavioural, biochemical and histopathological changes, and hence the painful neuropathy. HEEPI also showed invitro antioxidant activity in hydrogen peroxide, total phosphomolybdenum and FRAP assay methods. Oxidative stress is also implicated for the pathogenesis of painful neuropathy. The elevated GSH and decreased TBARS and calcium level were noted in the HEEPI extract treated rats. This study revealed that antioxidant is also one among the mechanism for the neuroprotective effect of this extract. The antioxidant activity may be due to the presence of flavonoids like myricetin.

Previous studies also suggests that Ca^{2+} channels as promising targets of myricetin. The analgesic effect of myricetin is due to its protein kinase C induced decrease in calcium channel currents of rat's dorsal root ganglia (Meotti et al., 2007). Hence it is concluded that the neuroprotective effect of HEEPI may be due to its calcium channel modulating effect as well as antioxidant activity.

CONCLUSION

The results of antineuropathic activity suggests that HEEPI at 100 and 200mg/kg b.w, *p.o*, alleviates neuropathic pain by virtue of its antihyperalgesic activity and antiallodynic activity. Treatment of this extract also augumented the level of GSH and decreases the elevated TBARS and calcium level in a dose dependent manner. This study revealed the antioxidant and calcium channel modulating property of this extract. Hence extensive studies required to explore the exact mechanism responsible for the management of neuropathy.

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