Ameliorative potential of *Ocimum sanctum* in chronic constriction injury-induced neuropathic pain in rats

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ABSTRACT

The present study was designed to investigate the ameliorative potential of *Ocimum sanctum* and its saponin rich fraction in chronic constriction injury-induced neuropathic pain in rats. The chronic constriction injury was induced by placing four loose ligatures around the sciatic nerve, proximal to its trifurcation. The mechanical hyperalgesia, cold allodynia, paw heat hyperalgesia and cold tail hyperalgesia were assessed by performing the pinprick, acetone, hot plate and cold tail immersion tests, respectively. Biochemically, the tissue thio-barbituric acid reactive species, super-oxide anion content (markers of oxidative stress) and total calcium levels were measured. Chronic constriction injury was associated with the development of mechanical hyperalgesia, cold allodynia, heat and cold hyperalgesia along with an increase in oxidative stress and calcium levels. However, administration of *Ocimum sanctum* (100 and 200 mg/kg *p.o.*) and its saponin rich fraction (100 and 200 mg/kg *p.o.*) for 14 days significantly attenuated chronic constriction injury-induced neuropathic pain as well as decrease the oxidative stress and calcium levels. It may be concluded that saponin rich fraction of *Ocimum sanctum* has ameliorative potential in attenuating painful neuropathic state, which may be attributed to a decrease in oxidative stress and calcium levels.

Key words: chronic constriction injury, *Ocimum sanctum*, Saponin rich fraction, calcium, oxidative stress.

INTRODUCTION

The International Association for the Study of Pain defines neuropathic pain as "initiated or caused by a primary lesion or dysfunction in the nervous system" and defines neuropathic due to disordered peripheral or central nerves (Merskey et al. 1994). Neuropathic pain is not a single entity; it is a heterogeneous group of conditions that differs in not only aetiology, but also in location (Woolf and Mannion 1999), and symptoms respect neither cause nor anatomical site. Neuropathic pain is generally characterized by 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, Jaggi and Singh 2011).

Chronic constriction injury is a novel model of peripheral neuropathy as the alterations produced in this model of peripheral neuropathy are analogous to the human beings and the symptoms in this rat model are parallel to causalgia or complex regional pain syndrome (Bennett and Xie 1988). Chronic constriction injury produces unilateral peripheral neuropathy due to compression of the sciatic nerve

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and hence, it has been extensively used in research for the analysis of sensory abnormalities associated with entrapment neuropathy (Nakamura and Atsuta 2006). Furthermore, the behavioral changes observed in this model are the most sustained and generalized ones with regards to other models of peripheral neuropathy (Dowdall et al. 2005).

Ocimum sanctum (L.), also known as tulsi is an aromatic plant in the family Lamiaceae which has a number of medicinal and non-medicinal organic compounds (oleanolic acid, ursolic acid, rosmarinic acid, eugenol, carvacrol, linalool, - caryophyllene - elemene and germacrene) (Ahmad et al. 2012, Venuprasad et al. 2013). All the parts of the *Ocimum sanctum* plant like fresh leaves, juice, seeds and volatile oil are very beneficial to the health of humans. It possesses diverse protective effects including hepato-protective, immuno-modulatory, anti-ulcer, anti-diabetic, anti-hypercholesterolaemic, nerve tonic, chemo-protective, nootropic, antitussive, anti-inflammatory, wound healing, anti-tumorigenesis, anti-convulsant, anthelmintic, anti-bacterial, anti-anxiety, and anti-stress activities (Jaggi et al. 2003, Yanpallewar et al. 2004, de Almeida et al. 2007, Bhattacharyya et al. 2008, Baliga et al. 2013). Recently, our laboratory documented that *Ocimum sanctum* has ameliorative potential in attenuating sciatic nerve transection and vincristine-induced neuropathic pain (Muthuraman et al. 2008b, Kaur et al. 2010).

Saponins are important phyto-constituents present in different plants including *Ocimum sanctum* in which these constitute an important chemical class and include pentacyclic triterpenoids saponins such as ursolic acid, oleanoloc acid. A number of studies have shown that saponins exert diverse biological actions such as anti-hypertensive (Rhiouani et al. 1999), anti-cancer (Shibata 2001), anti-convulsant (Pal et al. 2005), anti-diabetic (Chen et al. 2008), anti-amnestic (Wang et al. 2006), hypocholesterolaemic (Al-Habori and Raman 1998) and neuroprotective (Kim et al. 2005). Furthermore, saponins have also been shown to exhibit anti-nociceptive actions

(Moharram et al. 2007, Wang et al. 2008); alleviate neuropathic pain in long standing diabetes (Yin et al. 2004) and nerve entrapment induced facial paralysis (Bell's palsy) (Liu et al. 2008).

Therefore, the present study was designed to investigate the ameliorative role of *Ocimum sanctum* in another model of neuropathic pain i.e. chronic constriction-induced neuropathy and to further explore the contributory role of saponins in *Ocimum sanctum* mediated beneficial effect in neuropathic pain.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Wistar albino rats weighing 180-250 g, maintained on 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 normal cycle of light and dark. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and the care of the animals was 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.- 107/ 1999/ CPCSEA).

INDUCTION OF NEUROPATHIC PAIN BY CHRONIC CONSTRICTION INJURY

Peripheral neuropathic pain was induced in rats by chronic constriction injury (CCI) (Bennett and Xie 1988, Muthuraman et al. 2008a). In brief, rats were deeply anesthetized with thiopental sodium (35 mg/kg *i.p.*). 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 an approximate 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 were immediately sutured with thread, and topical antibiotic was applied. Due to the distinct development of postural defects in the paw of chronic constriction injury control animals, the behavioral studies could not be blinded for comparing normal control; sham control and chronic constriction injury control groups. However, for all other groups the behavioral tests were blinded.

PLANT MATERIAL

Fresh leaves of *Ocimum sanctum* were collected from Patiala and authenticated by Botany Department, Punjabi University, Patiala. The Plant sample has been kept in Voucher specimen (PUP-039/2008–2009) at Punjabi university, Patiala. The fresh leaves of *Ocimum sanctum* were shed dried at room temperature and reduced to coarse powder. The powder was extracted with mixture of methanol: water (3:1). The solvent was completely removed at 50°C under reduced pressure. The yield of the extract was 13% (w/w) in terms of dried starting material. The saponin rich fraction was extracted from the concentrated hydro-alcoholic extract as described earlier (Ebata et al. 1996, Sadraei et al. 2003). The yield of saponin fraction was 6.8% (w/w). Briefly, the hydro-alcoholic extract was decanted by n-hexane followed by extraction with n-butanol. The butanol was completely removed at 50°C under reduced pressure (Table I).

BEHAVIORAL EXAMINATION

Paw Cold-Allodynia (Acetone Drop Test)

The cold allodynia was assessed by spraying a 100 μL of acetone onto the planter surface of the paw, without touching the skin. The duration of withdrawal response was recorded with *an arbitrary minimum value of 0.5 s and a maximum value of 20 s* (Decosterd et al. 2000).

TABLE I
HPTLC fingerprinting profile of hydro-alcoholic extract of *Ocimum sanctum* taking chloroform and methanol (80:20) as mobile phase and using anisaldehyde as spraying agent. The values indicate the R_f of the separated bands.

S. No of Resolving Bands	UV 254 nm	UV 366	Under white light
1.	-	-	0.44
2.	-	-	0.60
3.	-	-	0.71
4.	-	-	0.78
5.	-	-	0.85

Paw Heat-Hyperalgesia (Hot Plate Test)

The thermal nociceptive threshold, as an index of thermal hyperalgesia, was assessed by the Eddy's hot plate, which is an instrument designed by Eddy and Leimbach (1953) to assess thermal sensitivity. The plate was pre-heated and maintained at a temperature of $52.5 \pm 2.0°C$. The rat was placed on the hot plate and nociceptive threshold, with regards to licking of the hind paw, was recorded in seconds. The cut-off time of 20 s was maintained (Jain et al. 2009).

Mechanical hyperalgesia: (Pin prick test)

The mechanical hyperalgesia was assessed by the pinprick test as described by Erichsen and Blackburn-Munro (Erichsen et al. 2002). The surface of the injured hind paw was touched with the point of the bent gauge needle (at 90° to the syringe) at an intensity sufficient to produce a reflex withdrawal response in normal non-operated animals, but at an intensity which was insufficient to penetrate the skin. The duration of the paw withdrawal was recorded in seconds with a stopwatch. A cut-off time of 20 s was maintained. The duration of withdrawal response was recorded with an arbitrary minimum value of 0.5 s and a maximum value of 20 s.

The contra-lateral paw was not used to compare the hypersensitivity changes with the injured ipsi-lateral paw because there have been reports suggesting the hypersensitivity changes in the contralateral paw

on subjecting the animal to the unilateral nerve injury (Arguis et al. 2008). Furthermore, the contra-lateral pain development has also been reported in human beings (Koltzenburg et al. 1999).

Tail Cold-Hyperalgesia Test (Tail Immersion Test)

In peripheral nerve injury, initial burst from peripheral site and later sustained activation of peripheral nociceptors leads to a central sensitization of the dorsal horn neurons in the spinal cord. This central sensitization is an important feature in inducing long-term thermal allodynia and hyperalgesia, which may be assessed by noting down the changes in thermal sensitivity in the tail (Mika et al. 2008, 2009, Muthuraman et al. 2008a). The tail cold-hyperalgesia was noted by immersing a terminal part of the tail (1 cm) in the water, maintained at a temperature of $0 - 4°C$. The tail withdrawal latency was recorded and a cut-off time of 20 s was maintained (Necker et al. 1978).

BIOCHEMICAL PARAMETERS

All the groups of animals were sacrificed after the 14th day by cervical dislocation and the sciatic nerve was isolated immediately. The uniformity among the different nerve samples were maintained by taking the constant weight of the respective samples. The excised sciatic nerve homogenate (10% w/v) was prepared with 0.1 M Tris HCl buffer (pH 7.4). The tubes with homogenate were kept in ice water for 30 minutes and centrifuged at 4°C (2500 rpm, 10 min). The supernatant of homogenate was separated, and employed to estimate total protein content, TBARS, superoxide anion concenteration and total calcium content.

Estimation of the protein content

The protein concentration in the sciatic nerve was estimated according to the method of Lowry et al. (1951). using bovine serum albumin as a standard. The absorbance was determined spectrophotometrically at 750 nm.

Estimation of thio-barbituric acid reactive substances

The estimation of lipid peroxidation in the sciatic nerve was done by measuring the thio-barbituric acid reactive substances by the method of Okhawa et al. (1979). The absorbance was measured spectrophotometrically at 532 nm. The concentration was expressed in terms of nmol of thio-barbituric acid reactive substances/mg protein.

Estimation of superoxide anion generation

The superoxide anion generation in the sciatic nerve was estimated in terms of measuring reduced nitroblue tetrazolium (NBT) (Wang et al. 1998). The absorbance of formazan was determined spec-trophotometerically at 540 nm.

Estimation of total calcium

The total calcium levels were estimated in the sciatic nerve as described earlier (Severinghaus and Ferrebee 1950, Muthuraman et al. 2008a, b). Briefly, the sciatic nerve homogenate was mixed with 1 ml of trichloroacetic acid (4%) in ice cold conditions and centrifuged at 1500 g, for 10 minutes. The clear supernatant was used for the estimation of total calcium ion by atomic emission spectroscopy at 556 nm.

EXPERIMENTAL PROTOCOL

Ten groups, each group comprising six wistar albino rats, were employed in the present study.

Group I: Normal control group

Rats were not subjected to any surgical procedure and were kept for 14 days. The behavioral tests were employed on different days i.e., day 2nd, 4th, 6th, 8th, 11th and 14th. All the animals were sacrificed at end of the 14th day and the biochemical analysis was done for estimation of protein content, TBARS, superoxide anion and total calcium.

Group II: Sham control group

Rats were subjected to surgical procedure to expose the left sciatic nerve, on day one, without any nerve

ligation. The behavioral tests were employed after surgery on different days as described in group I and the biochemical analysis was done at the end of the 14th day as described in group I.

Group III: CCI control group

Rats were subjected to surgical procedure to expose and ligate the left sciatic nerve as described earlier. The behavioral tests and the biochemical parameters were assessed as described in group I.

Group IV: Vehicle in CCI group

After subjecting the rats to CCI, carboxyme-thylcellulose suspension (0.5% w/v, *p.o.*) was administered for 14 consecutive days, starting from day one just prior to surgery. The behavioral tests and the biochemical parameters were assessed as described in group I.

Group V: Hydro-alcoholic extract of *Ocimum sanctum per se*

The hydro-alcoholic extract of *Ocimum sanctum* (200 mg/kg *p.o.*) was administered to normal rats for 14 consecutive days, starting from day one prior to surgery. The behavioral tests and the biochemical parameters were assessed as described in group I.

Group VI: Saponin rich extract of *Ocimum sanctum per se*

The saponin rich extract of *Ocimum sanctum* (200 mg/kg *p.o.*) was administered to normal rats for 14 consecutive days, starting from day one prior to surgery. The behavioral tests and the biochemical parameters were assessed as described in group I.

Group VII and VIII: Hydro-alcoholic extract of *Ocimum sanctum* (100; 200 mg/kg *p.o.*) in CCI group

The hydro-alcoholic extract of *Ocimum sanctum* (100; 200 mg/kg *p.o.*) was administered for 14 consecutive days, starting from day one just prior to surgery. The behavioral tests and the biochemical parameters were assessed as described in group I.

Group IX and X: Saponin rich fraction of *Ocimum sanctum* (100; 200 mg/kg *p.o.*) in CCI group

The saponin rich fraction of *Ocimum sanctum* (100 and 200 mg/kg *p.o.*) was administered for 14 consecutive days, starting from day one just prior to surgery. The behavioral tests and the biochemical parameters were assessed as described in group I.

STATISTICAL ANALYSIS

All the results were expressed as mean ± S.E.M. The data of behavioral results were statistically analyzed by two-way ANOVA followed by Bonferonni's *post hoc* test by using Graph pad prism Version-5.0 software. The data of biochemical results were statistically analyzed by one-way ANOVA followed by Tukey's multiple range test by using Sigmastat Version-2.0 software. The *p-value* < 0.05 was considered to be statistically significant.

RESULTS

EFFECT OF *Ocimum sanctum* AND ITS SAPONIN RICH FRACTION ON COLD ALLODYNIA

The chronic constriction injury of the sciatic nerve resulted in the development of cold allodynia as reflected by an increase in the duration of hind paw withdrawal, when compared to normal control group. However, administration of *Ocimum sanctum* (100 and 200 mg/kg *p.o.*) and its saponin rich fraction (100 and 200 mg/kg *p.o.*) significantly attenuated the chronic constriction injury-induced increase in withdrawal duration of the hind paw in response to non-noxious cold stimuli. The effect of saponin rich fraction in attenuating cold allodynia was significantly higher than the hydro-alcoholic extract at the same dose levels. Vehicle administration did not modulate chronic constriction injury-induced alterations in behavior in response to non-noxious cold stimulus. *Per se* administration of *Ocimum sanctum* and its saponin rich fraction also did not produce alterations in the normal rats (Figure 1).
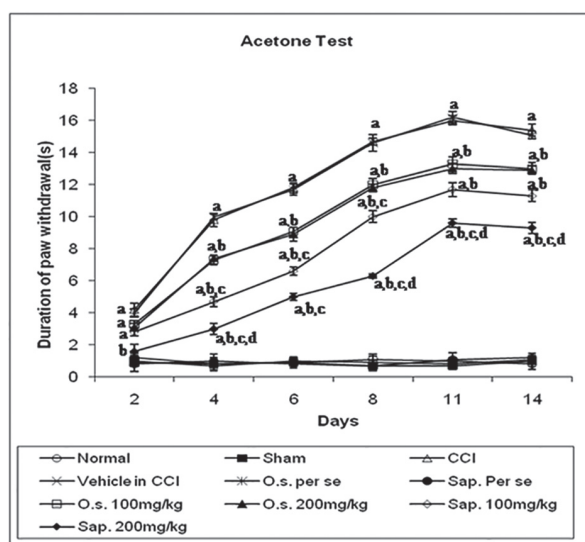
Figure 1 - Effect of *Ocimum sanctum* and its saponin rich fraction on cold allodynia in chronic constriction injury-induced neuropathic pain. CCI: chronic constriction injury; O.s.: *Ocimum sanctum;* Sap.: saponin rich extract of *Ocimum sanctum.* Data were expressed as mean ± S.E.M., n=6 rats per group. a= p<0.05 Vs normal control group, b= p<0.05 Vs CCI control group, c= p<0.05 Vs *Ocimum sanctum* 100 and 200 mg/kg group, d=p<0.05 Vs saponin rich fraction 100 mg/kg group.

Figure 2 - Effect of *Ocimum sanctum* and its saponin rich fraction on mechanical hyperalgesia in chronic constriction injury-induced neuropathic pain. CCI: chronic constriction injury, O.s.: *Ocimum sanctum,* Sap.: saponin rich extract of *Ocimum sanctum.* Data were expressed as mean ± S.E.M., n=6 rats per group. a= p<0.05 Vs normal control group, b= p<0.05 Vs CCI control group, c= p<0.05 Vs *Ocimum sanctum* 100 and 200 mg/kg group, d=p<0.05 Vs saponin rich fraction 100 mg/kg group.

EFFECT OF *Ocimum sanctum* AND ITS SAPONIN RICH FRACTION ON MECHANICAL HYPERALGESIA

The chronic constriction was associated with the development of mechanical hyperalgesia as reflected by an increase in the hind paw withdrawal duration, when compared to normal control group. However, administration of *Ocimum sanctum* (100 and 200 mg/kg *p.o.*) and its saponin rich fraction (100 and 200 mg/kg *p.o.*) significantly attenuated chronic constriction injury-induced increase in withdrawal duration of the hind paw in response to noxious mechanical stimuli. The effect of saponin rich fraction in attenuating mechanical hyperalgesia was significantly higher than the hydro-alcoholic extract at the same dose levels. Vehicle administration did not modulate chronic constriction injury-induced alteration in behavior in response to noxious mechanical stimulus. *Per se* administration of *Ocimum sanctum* and its saponin rich fraction also did not produce alterations in the normal rats (Figure 2).
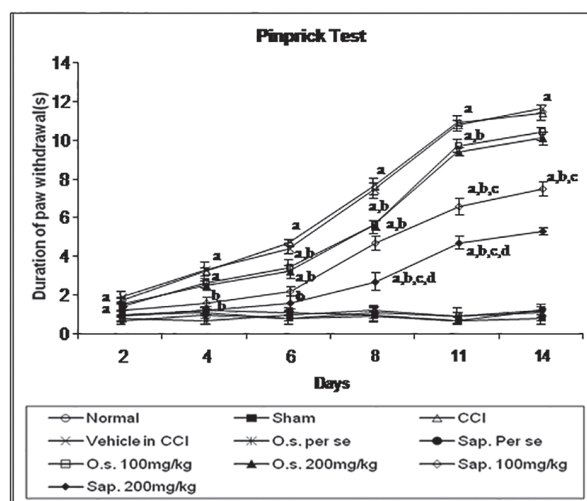
EFFECT OF *Ocimum sanctum* AND ITS SAPONIN RICH FRACTION ON PAW HEAT AND TAIL COLD HYPERALGESIA

The chronic constriction injury led to the development of paw heat and tail cold hyperalgesia as reflected by decrease in the threshold withdrawal latency of hind paw and tail respectively, when compared to normal control group. However, administration of *Ocimum sanctum* (100 and 200 mg/kg *p.o.*) and its saponin rich fraction (100 and 200 mg/kg *p.o.*) significantly attenuated the chronic constriction injury-induced decrease in the withdrawal latency in response to noxious thermal stimuli. The effect of saponin rich fraction in attenuating thermal hyperalgesia was significantly higher than the hydro-alcoholic extract at the same dose levels. Vehicle administration did not modulate chronic constriction injury-induced alteration in behavior in response to noxious thermal stimulus. *Per se* administration of *Ocimum sanctum* and its saponin rich fraction also did not produce alterations in the normal rats (Figures 3 and 4).
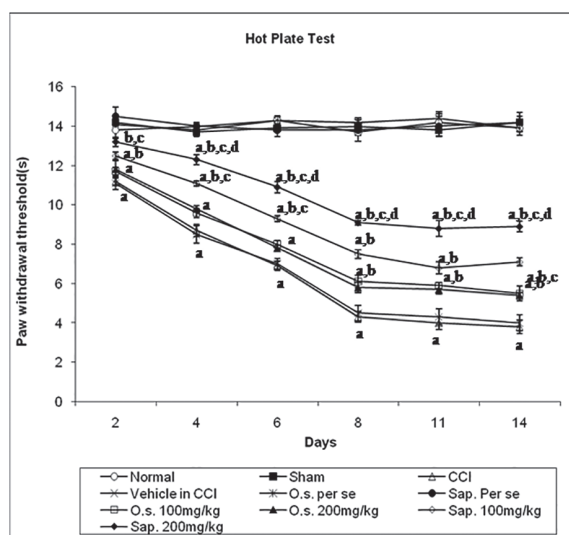
Figure 3 - Effect of Ocimum *sanctum* and its saponin rich fraction on paw heat hyperalgesia in chronic constriction injury-induced neuropathic pain. Data were expressed as mean ± S.E.M., n=6 rats per group. CCI: chronic constriction injury, O.s.: *Ocimum sanctum,* Sap.: saponin rich extract of *Ocimum sanctum.* a= p<0.05 Vs normal control group, b= p<0.05 Vs CCI control group, c= p<0.05 Vs *Ocimum sanctum* 100 and 200 mg/kg group, d=p<0.05 Vs saponin rich fraction 100 mg/kg group.
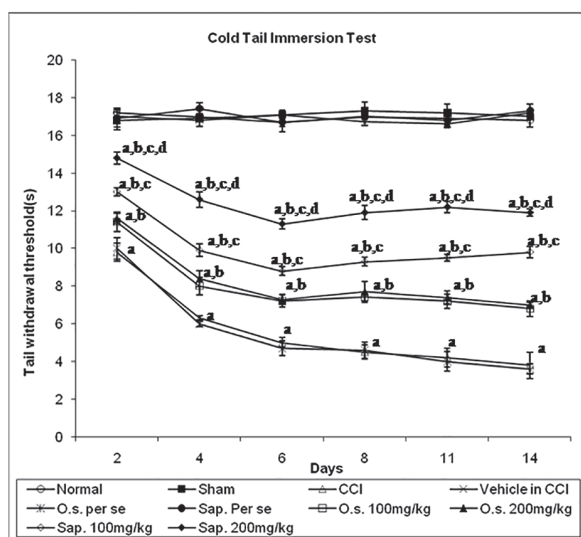

Figure 4 - Effect of *Ocimum sanctum* and its saponin rich fraction on tail cold hyperalgesia in chronic constriction injury-induced neuropathic pain. Data were expressed as mean ± S.E.M., n=6 rats per group. CCI: chronic constriction injury, O.s.: *Ocimum sanctum,* Sap.: saponin rich extract of *Ocimum sanctum.* a= p<0.05 Vs normal control group, b= p<0.05 Vs CCI control group, c= p<0.05 Vs *Ocimum sanctum* 100 and 200 mg/kg group, d=p<0.05 Vs saponin rich fraction 100 mg/kg group.

EFFECT OF *Ocimum sanctum* AND ITS SAPONIN RICH FRACTION ON OXIDATIVE STRESS MARKERS AND TOTAL CALCIUM CONTENT

The chronic constriction injury resulted in an increase in the oxidative stress markers and total calcium content as reflected by an increase in the tissue thio-barbituric acid reactive substances, superoxide anion content and total calcium levels, when compared to normal control group. However, administration of *Ocimum sanctum* (100 and 200 mg/kg *p.o.*) and its saponin rich fraction (100 and 200 mg/kg *p.o.*) significantly attenuated the chronic constriction injury-induced increase in oxidative stress markers and total calcium levels. The effect of saponin rich fraction in attenuating the rise in the levels of oxidative stress markers and the calcium levels was significantly higher than the hydro-alcoholic extract at the same dose levels. Vehicle administration did not modulate chronic constriction injury-induced alteration in oxidative stress markers and total calcium content. *Per se* administration of *Ocimum sanctum* and its saponin rich fraction also did not produce any biochemical alterations in the normal rats (Table II).

DISCUSSION

In the present study, chronic constriction injury led to significant development of cold allodynia, mechanical, tail cold and paw heat hyperalgesia. These behavioral changes were evident from the 2nd day of surgery and were at peak after 12-14 days of surgery. The chronic constriction injury model has been characterized by significant development of mechanical hyperalgesia, cold allodynia, mechanical allodynia, thermal hyperalgesia and thermal allodynia, and these behavioral alterations are much more significant in this model as compared to other models (Dowdall et al. 2005, Jaggi et al. 2011). Ligation of the sciatic nerve produces compression leading to mechanical injury of capillary endothelial cells and epineural edema, which subsequently exacerbates the nerve

TABLE II
Effect of *Ocimum sanctum* and its saponin rich fraction on thio-barbituric acid reactive subatances,
superoxide anion content and total calcium. CCI: chronic constriction injury, O.s.: *Ocimum sanctum*,
Sap.: saponin rich fraction of *Ocimum sanctum*. Data were expressed as mean ± S.E.M., 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 *Ocimum*
***sanctum* 100 and 200 mg/kg group, d=*p*<0.05 Vs saponin rich fraction 100 mg/kg group.**

Groups	Total Protein (mg/ml)	TBARS (nmol/mg of protein)	Superoxide Anion Content (pmol/min/mg)	Total Calcium (ppm/mg of protein)
Normal Control	5.17±0.23	5.97±0.26	0.15±0.02	4.97±0.21
Sham Control	5.23±0.21	6.01±0.23	0.17±0.01	5.10±0.24
CCI Control	5.36±0.13	8.90±0.24[a]	0.91 ± 0.05[a]	41.21±0.17[a]
Vehicle in CCI	5.29±0.17	8.93±0.22[a]	0.93±0.04[a]	40.0±00.22[a]
O.s. *per se*	5.21±0.15	6.00±0.24	0.12 ±0.01	5.09±0.15
Sap. *per se*	5.30±0.14	6.06±0.21	0.14 ±0.02	4.92±0.17
O.s. 100 mg/kg	5.33±0.22	8.13±0.14[a,b]	0.67 ±0.03[a,b]	25.21±0.20[a,b]
O.s. 200 mg/kg	5.26±0.16	8.06±0.17[a,b]	0.69 ±0.03[a,b]	25.47±0.14[a,b]
Sap. 100 mg/kg	5.35±0.13	7.20±0.21[a,b,c]	0.41 ±0.03[a,b,c]	19.71±0.19[a,b,c]
Sap. 200 mg/kg	5.29±0.14	6.40 ±0.23[a,b,c,d]	0.28 ±0.02[a,b,c,d]	12.52±0.21[a,b,c,d]

compression. Furthermore, the material used to ligate sutures also initiates immune reaction and hence, invasion by immune cells along with the production of immune modulators contribute significantly in inducing the pain behavior (Kleinschnitz et al. 2006, Morin et al. 2007).

The precise details of initiation and maintenance of hyper-responsiveness of injured paw to noxious as well as non-noxious stimuli are not clear. However, it has been documented that following the loose ligation mediated nerve injury, the nerve segment distal to ligature comprises of both injured as well as non-injured intact nerve fibers. Within the nerve segment, the injured nerve fibers trigger incompletely understood biochemical changes, which alter the chemical milieu of the non-injured nerve fibers and thus eventually result in significant changes in the responsiveness of non-injured nerves with respect to noxious and non-noxious stimuli (Klusáková and Dubový 2009).

In the present study, pre-treatment with hydro-alcoholic extract of *Ocimum sanctum* significantly attenuated the chronic constriction injury-induced behavioral alterations in neuropathic pain, thus suggesting its potential in ameliorating the painful state of neuropathy. In chronic constriction injury model, the nerve trauma is associated with an initial discharge that results in the development of neuroma and sustained behavioral alterations. There have been reports suggesting that pre-emptive treatment with drugs minimizes the initial discharge and thus, help alleviate the neuropathic manifestations (Sotgiu et al. 1994, Mika et al. 2007). Therefore, in the present study, the treatment was given prior to surgery to prevent injury discharge. Traditionally, *Ocimum sanctum* has been used as a nerve tonic to alleviate disorders related to nerves. In our previous studies, the hydroalcohlic extract of Ocimum sanctum was shown to attenuate neuropathic pain in axotomy model i.e., sciatic nerve transection model (Muthuraman et al. 2008b). In another study, the saponin rich fraction was shown to produce beneficial effects in chemotherapeutic agent-induced neuropathy in a vincristine model (Kaur et al. 2010). This present study is the extension of our previous studies to evaluate the neuropathic pain attenuating effects of Ocimum sanctum in chronic constriction-induced neuropathy.

Ocimum sanctum is a rich source of a number of saponins and the most important of them are ursolic acid and oleanolic acid. These saponins have been reported to possess beneficial effects in a number of disorders such as amnesia (Lu et al. 2007), hypertension (Somova et al. 2003), myocardial ischemia (Senthil et al. 2007), and cancer (Manu et al. 2008). Furthermore, the saponins have also shown to be beneficial effects in relieving nociceptive pain (Moharram et al. 2007, Wang et al. 2008) as well as neuropathic pain in diabetes (Yin et al. 2004) and facial paralysis (Bell's palsy) due to nerve entrapment (Liu et al. 2008). Therefore, to explore the chemical class of *Ocimum sanctum* responsible for its beneficial effect, its saponin rich fraction was evaluated in neuropathic pain. In the present investigation, pre-treatment with the saponin rich fraction of *Ocimum sanctum* also attenuated chronic constriction injury-induced behavioral alterations in neuropathic pain. Therefore, it may be proposed that saponins are the main components responsible for the noted beneficial effects of *Ocimum sanctum* in neuropathic pain.

In the present study, the same doses of hydroalcohlic and saponin rich fraction were collected to compare their relative potency of neuropathic pain attenuation. At the same dose the saponin rich fraction exhibited greater potency in attenuating neuropathic pain as compared to corresponding hydroalcohlic extracts. The difference in the potency of two fractions could have also been evaluated by comparing the doses at which the comparable effects were produced. However, the authors selected the former method to evaluate the difference in potency by comparing the effects at the same dose levels. The major limitation of comparing the dose at which same effects are produce is requirement of more number of groups in selecting the dose of saponin rich fraction which corresponds to hydroalcohlic extracts. However, due to animal ethic constraint, the number of animals and groups could not exceed certain limits.

Furthermore, in this study chronic constriction injury was associated with the rise in the oxidative stress (rise in thio-barbituric acid reactive substances and superoxide anion content) and total calcium content. These results are in line with earlier reports suggesting the key role of oxidative stress in chronic constriction injury (Tan et al. 2009, Zeinab and Bereha 2001, Muthuraman et al. 2008a). In the chronic constriction injury model of rat neuropathic pain, heat hyperalgesia was reduced by systemically injected antioxidants (Kim et al. 2004, Khalil et al. 1999). Calcium accumulation has been well documented to play an important role in chronic constriction injury-induced neuropathy (Kawamata et al. 1996, Muthuraman et al. 2008a, Jagodic et al. 2008). Calcium influx through different neuronal calcium channels including L-, and T-type, plays a fundamental role in excitation–transcription coupling, triggering changes in gene expression and potentiating the phosphorylation of the ERK1/2 MAP kinases, the p38 MAPK, or the CaM kinase II to activate the CRE/CREB-dependent transcription (Dolmetsch et al. 2001, West et al. 2001). The activation of the CREB transcription factor contributes to long-term modifications in the processing of nociceptive information in the spinal cord (Song et al. 2005, Crown et al. 2006). Activation of these signalling pathways in the dorsal horn participates in pain hypersensitivity (Kawasaki et al. 2004, Song et al. 2005). Treatment with *Ocimum sanctum* and its saponin rich fraction, attenuated the chronic constriction injury associated increase in oxidative stress and calcium levels. *Ocimum sanctum* has a well documented antioxidant effect (Yanpallewar et al. 2004, George and Chaturvedi 2008) and also decreases the calcium levels (Muthuraman et al. 2008b).

The increase in free radical during neuropathic pain may be attributed to the rise in intracellular calcium levels. Although the precise mechanisms are unknown, it may be speculated that increased release of glutamate during neuropathic pain.

may increase intracellular Ca^{2+} (Sekiguchi and Kawabata), which may contribute in activating NADPH oxidase to generate free radicals (Abramov et al. 2007). Furthermore, calcium may potentiate oxygen free radical injury to mitochondria, and Ca^{2+} induced potentiation of oxygen free radical injury has been linked to activation of phospholipase A2 (Malis and Bonventre 1986). On the other hand, free radicals have also been well documented to increase calcium levels (Doan et al. 1994, Kawakami and Okabe 1998). Free radicals may increase calcium ionic homeostasis due to alteration in membrane phospholipids leading to an increase in membrane permeability (Burton et al. 1990). Therefore, it may possible that free radicals and calcium may induce a vicious cycle to increase the levels of each other, which may contribute in producing neuropathic pain.

Based on above findings, it may be proposed that *Ocimum sanctum* reduces calcium levels indirectly due to decreased free radical generation. However, it may also be possible that direct action of *Ocimum sanctum* is responsible for the observed decrease in the calcium levels. Therefore, the observed decrease in calcium levels due to *Ocimum sanctum* may be due to either direct action or secondary to decrease in oxidative stress. However, data of the present study is still insufficient to elaborate the precise mechanism of *Ocimum sanctum* mediated decrease in calcium levels. Furthermore, saponins have also been reported to decrease oxidative stress (Feresin et al. 2002) and calcium levels (Tao et al. 2005, Li et al. 2009 a, b).

Based on above findings, it may be proposed that *Ocimum sanctum* has potential in ameliorating the painful symptoms in chronic constriction injury-induced peripheral neuropathy, and saponins may be the main chemical class responsible for its beneficial effect in neuropathic pain. Furthermore, the pain relieving effects of *Ocimum sanctum* and its saponin rich fraction may be due to attenuation of nerve injury inciting agent-induced increased levels of calcium and free radicals.

CONCLUSION

Ocimum sanctum has ameliorative potential in attenuating painful neuropathic state, which may be attributed to a decrease in oxidative stress and calcium levels. Furthermore, saponin rich fraction of *Ocimum sanctum* may be responsible for its noted beneficial effect in neuropathic pain in rats.

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RESUMO

O presente estudo foi delineado para avaliar-se o potencial efeito analgésico de extratos de *Ocimum sanctum* e uma fração enriquecida em saponinas na dor neuropática induzida pela lesão por constrição crônica de nervos periféricos em ratos. A lesão foi induzida pela colocação de quatro ligaduras apostas ao nervo ciático, proximalmente à sua trifurcação. Hiperalgesia mecânica, alodínia ao frio, hiperalgesia ao calor nas patas e hiperalgesia ao frio na cauda foram determinadas usando-se testes de picada de agulha, exposição à acetona, placa quente e teste de imersão em solução gelada, respectivamente. Análises bioquímicas com conteúdo de espécies reativas ao tiobarburato em tecidos e conteúdo de ânion superóxido (ambos marcadores de estresse oxidativo) e os níveis totais de cálcio foram realizadas. A lesão por constrição crônica do nervo ciático foi acompanhada de desenvolvimento de hiperalgesia mecânica, alodínia ao frio, hiperalgesia ao calor e ao frio, acompanhadas de aumento de marcadores de estresse oxidativo e níveis de cálcio. A administração de extratos de *Ocimum sanctum* (100 e 200 mg/kg, via oral) e sua fração enriquecida em saponinas (100 e 200 mg/kg, via oral) por 14 dias atenuou significativamente a dor neuropática associada à constrição crônica do nervo ciático, bem como associou-se à diminuição de estresse oxidativo e níveis de cálcio. Conclui-se que a fração

enriquecida em saponinas de *Ocimum sanctum* tem potencial atenuante da dor neuropática, que poderia ser atribuída à diminuição do estresse oxidativo e níveis teciduais de cálcio.

Palavras-chave: lesão por constrição crônica, *Ocimum sanctum*, saponinas, cálcio, estresse oxidativo.

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