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IN-VIVO INVESTIGATIONS OF CHRONIC INFLAMMATORY PAIN MODULATING POTENTIAL OF NIRANTHIN

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ABSTRACT

Background: It has been well referred that lignan family of natural products has goodpharmacological potential. A lignan, Niranthin, 6-[(2*R*,3*R*)-3-[(3,4-dimethoxyphenyl)methyl]-4-methoxy-2-(methoxymethyl)butyl]-4-methoxy-1,3-benzodioxole**]** is a common phytoconstituents from various *Phyllanthus* species.

Objectives: This study aims to investigate chronic pain modulatory potential of Niranthin.

Materials and Methods: We have investigated the effects of Niranthin on chronic thermal and mechanical hypersensitivities in rats, which were injected with 3% carrageenan in the left gastrocnemius muscle and hyperalgesia to heat and mechanical stimuli was assessed before and at varying times after injection, till end of 22 days after muscle insult. Histological changes and the determination of prostaglandin E2 (PGE2) concentration were performed after the completion of drug treatment protocol.

Results: Our finding noted that Niranthin causes hypersensitivityactivity, when administered intraperitoneally. There was also reduction in prostaglandin E2 (PGE2) concentration observed during our analysis.

Conclusion: To summarize, we wish to report chronic inflammatory pain modulating potential of Niranthin in current pain model and are thought to be mediated via spinal or supraspinal neuronal mechanisms, mainly inhibiting PGE2. Since, our current study is preliminary in nature, further in detailed molecular investigation will be needed to predict drug-like candidature of Niranthin as pain modulating agent.

Keywords: Niranthin; lignan; Anti-hyperalgesic activity; chronic muscle pain; pain modulating potential

1. INTRODUCTION

Chronic musculoskeletal pain can become an intolerable and debilitating clinical condition and the major source of this unnecessary pain may be chronic inflammation **[1]**. This pain is particularly arising from muscles of our body. Since many years, researchers are establishing and reporting various experimental animal models for the investigations of various mechanisms of chronic pain **[2, 3]**. It has been worthy to note that, pain originating from the muscles are considered as main symptom of various severe disease conditions associated with musculoskeletal system **[4, 5]**. A study reported by Radhakrishnan *et al*. **[6]** gives more details on animal models for chronic hyperalgesia induced by muscle inflammation. It has been well reported that some natural agents from plants can modulate inflammatory signals **[7]**. Various plants including, *Withania somnifera*, *Commiphora wightii*, *Zingiber officinale*, etc. have also been investigated for their experimental and clinical efficacy in chronic pain diseases **[8]**. As our routine pharmacological management i.e., Pharmacotherapy for the pain management has lots of side effects, there is an indeed need to investigate naturally occurring phytoconstituents for their safe, effective, and cost-effective analgesic effects. We believe that the use of naturally occurring phytoconstituents as pain managing agents and their developments into drug candidates would be our near future. The phytoconstituents of the lignan class are believed to possess exciting pharmacological potential and they have been reported for various bioactivities including anti-inflammatory, antimicrobial, anxiolytic potentials, etc. **[9-19]**.

Niranthin, a lignan, is common constituent of the plant Phyllanthus *amarus*and of various Phyllanthus subspecies, also. It has been reported for anti-viral, anti-allodynic properties, cytotoxic effects (K-562 cell line) **[7-16]**. Recently, anti-leishmanial activity of Niranthin was also reported **[17]**. Moreover, one study confirmed its potential as anti-leishmanial and *anti-trypanosome* agent **[18]**. Recently, our group has also reported the potential of Niranthin alone as

an anxiolytic agent by utilizing various in-vivo and in-silico methodologies **[14]**.

Previously studies from our lab have reported preliminary molecular docking hints of various phytocompounds from *Phyllanthus* species including Niranthin as chronic pain modulators docked with various inflammatory targets like COX-2, PGE synthase, TNF-Alpha, IL-1 and N-methyl-D-aspartate receptor. From thesestudies by our lab, we had several hints forplausible roles of Niranthin in chronic pain modulation **[11-15]**. Which encouraged us to initiate the present study oftheevaluation of chronic pain modulatory potential of pure, well characterized and isolated Niranthin by using well reported in-vivo chronic inflammatory pain model **[11-15]**. We believe that our current study of pure Niranthin phytoconstituent as a pain modulating agent will shade more lights for its pharmacological potential.

2. MATERIAL AND METHODS

2.1 Animals used- For the current investigation, we used adult male Wistar rats of 5-6 weeks of age (200– 240 gm.). Throughout entire study, we maintained standard environmental conditions along with the provision of standard diet feeding and water ad libitum. We have made food and water freely available throughout entire investigation. A prior approval from institutional animal ethics committee (from the Animal Ethics registered under Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA), Government of India) was obtained referenced **RCP/ 18-19/ CPCPSEA/P-20**. Allethical guidelines from the International Association for the Study of Pain **[22]** and the CPCSEA were followed. As forclarity, we have dipped both paw ipsilateral (injected paw) as well as contralateral paw (non-injected one). We handled and acclimatized the rats as per CPCSEA guidelines. The CPCSEA method is not stressful to the animals.

2.2 Chemicals- The standard pure, isolated and well characterized Niranthin [6-[(2*R*,3*R*)-3-[(3,4-dimethoxyphenyl)methyl]-4-methoxy-2-(methoxymethyl)butyl]-4-methoxy-1,3-benzodioxole] compound was purchased from Natural Remedies Pvt. Ltd., Bangalore (Product code : N006, Lot. No. T18C277). The purity of procured Niranthin was was certified above 95.0%. Standards like dimethylsulphoxide (from stock of DMSO0.1 %), and sodium chloride (NaCl) were procured from Merck.

2.3 Induction of carrageenan chronic inflammatory muscle hyperalgesia- For our current study, we modified the procedure described by Radhakrishnan *et al*. **[6]** and validated as per our previous reports **[4]**. Freshly prepared 100 µL of 3% carrageenan solution dispersed in normal saline was injected in left gastrocnemius

muscle to induce chronic inflammation in rats. This procedure was performed with light pentobarbital anesthesia **[4, 6]**. We have noticed initial pain behavioral signs in rats after 24 h intramuscular carrageenan injection. Further, we also recorded PWLs (Paw withdrawal latencies) for heat and mechanical stimuli for all groups under investigation. The setting of chronic muscle pain model required a period of 14 days post ipsilateral carrageenan injection the time point when we noticed decrease in PWLs of the contralateral paw such as the non-carrageenan injected side.

2.4Experimental protocol (Dosing study of Niranthin and standard drug)- The rats (n=30) were divided into five groups of six animals each as follows:

• group I – control group [normal healthy non-hyperalgesic rats, n=6] treated with vehicle DMSO 0.2 ml intraperitoneally;

• group II – inflammatory control group [hyperalgesic rats, n=6] treated with vehicle 10% DMSO 0.2 ml intraperitoneally;

• group III – Carrageenan induced chronic muscle pain (mechanical / heat hypersensitivity) in rats n=6, treated with standard Acceclofenac (preferential selective COX-2 inhibitor) 10 mg/kg, intraperitoneally;

• group IV – Carrageenan induced chronic muscle pain (mechanical alloydynia / heat hyperalgesia) in rats n=6, treated with Niranthin 5 mg/kg, intraperitoneally;

• group V – Carrageenan induced chronic muscle pain (mechanical alloydynia / heat hyperalgesia) in rats n=6, treated with Niranthin 10 mg/kg, intraperitoneally;

As per the previously used procedure in section 2.3, we induced the chronic inflammatory muscle pain to all the animals except for group I normal control.

The rats were administered two different doses of Niranthin (5 and 10 mg/kg), intraperitoneally in order to investigate the various its effects on chronic mechanical and thermal hypersensitivity. Over the period of 7 dayssuch as from 14th day to 22nd day, we administered Niranthin (twice/day; every 12 Hrs. apart) to the rats. Similarly, standard aceclofenac was administered twice daily in another group. The treatment was interrupted for couple of days on the 18th and 19th day and reinitiated on 20th day to investigate the possible development of tolerance. Further, we have carried out the evaluations of nociceptive responses30–40 min after the 1st treatment (daily). A vehicle DMSO, 0.2mL intraperitoneally, was administered to the inflammatory control group (hyperalgesic rats) which assisted us to make comparative evaluation with respect to Niranthin and Acceclofenac treated groups. We have also kept evolutionary analysis of healthy rats (normal control) for their assessments of level of muscle inflammation, changes in PGE2

(prostaglandin E2) and histopathological changes. We have tested all animals for their PWLs to heat and mechanical stimuli before each carrageenan injection and thereafter, continued till the end of the day. For the study of muscle histology, animals were sacrificed on 22nd day.

2.5 Behavioral testing for evaluation of thermal/heat hyperalgesia and mechanical allodynia- Spontaneous pain behavior signs observed as per reported procedure. From the measurements of PWLs of carrageenan injected paw by dipping it in the water bath (47 ± 1 °C), responses for inflammatory hyperalgesia were noted **[4]**. The BLPW (Baseline latency to paw withdrawal) from the heat or thermal source was recorded three times, 5 min apart, and those were averaged. Furthermore, we have kept 15 seconds cut-off time, so to avoid any severe injury to the paw. The PWLCP (PWL for contralateral paw) was recorded for current chronic model at 1Hr-1.3 Hrs. after drug administration on subsequent dosing. Till the end of the study, i.e., till 22nd day, we measured responses for heat stimuli before and after carrageenan intramuscular injection. For establishing and testing of mechanical allodynia, all animals were allowed to be placed on an elevated metal grid allowing stimulation of the plantar surface of the paw using a series of von Frey Nylon filaments (2–20 g), which were applied in increasing force until the rat withdrew its hindpaw **[4, 6]**.

2.6 Measurement of muscle circumference- In order to confirm the developments of inflammation, we had measured circumferences of the inflamed as well as non-inflamed gastrocnemius muscle (over the skin using a measuring tape) after a period of 2 weeks. We further also measured circumferences among each subsequent group after drug treatments to establish the relationship between PWL and inflammation. Muscle circumferences were measured before induction of chronic inflammation and on 13th and 22nd day after intramuscular injection of carrageenan as per reported literature procedure **[4, 6]**.

2.7 Histopathological Analysis- As per previous literature **[4, 6]**, we followed the same protocol after the injection of carrageenan in control and drug treatments and thus, all animals (from each group) were sacrificed at the end of 23days of protocol. A solution of 10 % formalin was used for fixing of dissected Ipsilateral muscle tissues. Further, these dissected muscles were embedded in the paraffin. Then, these sections of paraffin were stained with hematoxylin and eosin (H and E) and we followed the light microscopy examination. For our current study, we have kept our histological findings descriptive and those were examined by professional pathologist.

2.8 Measurement of prostaglandin E-2- We have added **2** mL KOH-methanol solution (**0.5** mol/L) with **0.5** mL supernatant inflammatory immersion of the left muscle and moreover, for isomerization about 20 mins were carried out by keeping the solution in a water bath (50°C). Later, we added methanol (**5**mL) to a total capacity and mixed thoroughly **[4]**. Then, after the duration of 5 min, we recorded the absorbance at 278 nm using the Shimadzu 1800 ultraviolet spectrophotometer. The prostaglandin E-2 (PGE2) content was corresponding with the optical density value of per milliliter inflammatory exudates.

2.9 Statistical Analysis- All statistical calculations were analyzed and performed with the help of a Graph pad Prism software version 6.01©, 1992–2012 version. All the data reported in this current investigation is in the form of mean ± SEM (standard error of the mean) and statistical significance was also recorded along with controls (P-value < 0.001). We have carried out one way ANOVA (one-way analysis of variance) and Dunnett's multiple comparison test.

3. RESULTS

3.1 Induction of gastrocnemius muscle inflammation, thermal and mechanical hyperalgesia- Carrageenan injected in left gastrocnemius muscle produces acute muscle inflammation after 24 hours which subsequently is transformed after 2 weeks (14 days) in chronic model. The chronic inflammation of muscle is accompanied with significantly reduced paw withdrawal latency particularly to mechanical and heat stimuli after muscular insult. The spontaneous pain behavior signs observed being initial guarding of ipsilateral paw and weight bearing on the contralateral paw. After 48 hours of muscle insult with carrageenan, no indications of spontaneous pain were observed exception for the curling of the ipsilateralpaw finally unto development of chronic model until 14th day.

The basal withdrawal latencies to heat / thermal stimuli in all the experiment groups wereapproximately 8.49 ± 0.21 seconds (n = 30) before carrageenan injection reducing in a significant manner post two weeks 2.88 ± 0.14 seconds. While basal withdrawal latencies to mechanical stimuli was approximately 16.27 ± 0.64 grams (n = 30) carrageenan injection prior which reduces after 2 weeks significantly to 8.55 ± 0.94 grams.

3.2 Effects of Niranthin on heat hyperalgesia- Niranthin reversed significantly carrageenan induced chronic inflammatory muscle hyperalgesia as comparison with hyperalgesic control. The evaluated doses of Niranthin (5 & 10 mg/kg), significantly attenuates thermal hyperalgesia compared with inflammatory

control in both paws. The lowest effective dose 5 mg/kg also significantly attenuated thermal hyperalgesia. Treatment of Niranthin was interrupted for couple of days (such as 18th to 19th day post carrageenan injection), thermal hypersensitivity being reestablished. The intraperitoneal administration of Niranthin significantly reduces muscle hyperalgesia returning it to near normal threshold in about 50-70 minutes. The maximal anti-hyperalgesic effect was observed in 60 - 90 min time frame prior intraperitoneal administration of the Niranthin and effect started neutralizing thereafter. **Figure 1** show effects of Niranthin on thermal chronic inflammatory muscle hyperalgesia in rats.

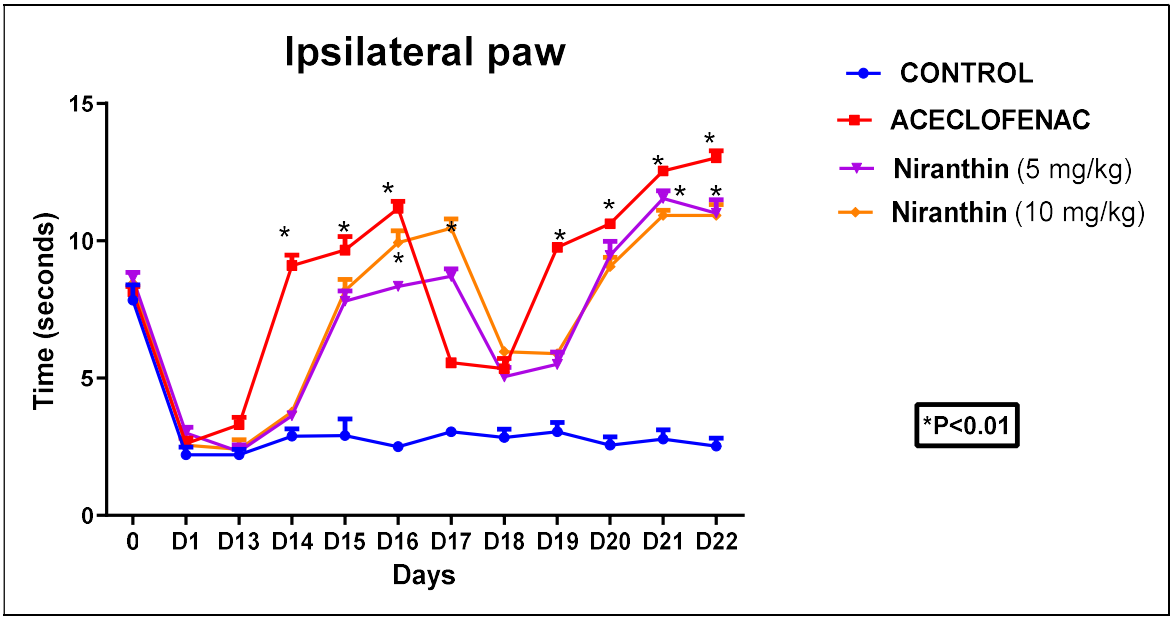
3.3 Effect of Niranthin on mechanical hyperalgesia- Figure 2 shows effects of Niranthin on mechanical chronic inflammatory muscle hyperalgesia. Niranthin (5 & 10 mg/kg) significantly modulates mechanical hyperalgesia induced by carrageenan. Maximal reduction in mechanical threshold was observed in the ipsilateral as well as contralateral paws in animals treated with vehicle alone. Treatment of Niranthin was interrupted for couple of days (such as 18th to 19th day post carrageenan injection), mechanical allodynia being reestablished. On 20th day Niranthin treatment was reinitiated and it was observed that Niranthin still significant reduction in mechanical hyperalgesia, excluding possibilities of tolerance.

3.4 Effects of Niranthin on muscle inflammation- Chronic muscle inflammation was produced by carrageenan as clearly observed in hyperalgesic controls, which is indicative of distinct inflammatory response. Consistent doses of Niranthin caused marked significant inhibition of chronic muscle inflammation observed by decrease in muscle circumference in comparison to inflammatory control for details see **Figure 3**.

3.5 Effects on concentration of PGE2 level- PGE2 basal levels in (normal rats) was 0.186 ± 0.26 in comparison to 8.736 ± 0.18 for animals grouped of inflammatory control. Niranthin treatment decreased PGE2 level in the edema exudates as compared with inflammatory control. The PGE2 inhibitory potency of Niranthin was better as compared to the control for details see Figure 4. Niranthin and aceclofenac shows significant ($p < 0.01$) attenuation in PGE2 concentration in muscle exudates.

3.6 Muscle Tissue Histopathology- Histopathological muscle tissue examination indicated inhibition of inflammatory muscle changes running paralleling the long-lasted hyperalgesia observed particularly among inflammatory control. The acute inflammation is marked

by myonecrosis and neutrophil presence. Chronic muscle inflammation characterized with epimysial or perimysial, with presence of macrophage and scattering mast cells. Niranthin treated rats showed decrease inflammatory tissue response with absenting of neutrophils, macrophages and mast cells on chronic administration as compared to hyperalgesic control for detailed slides.



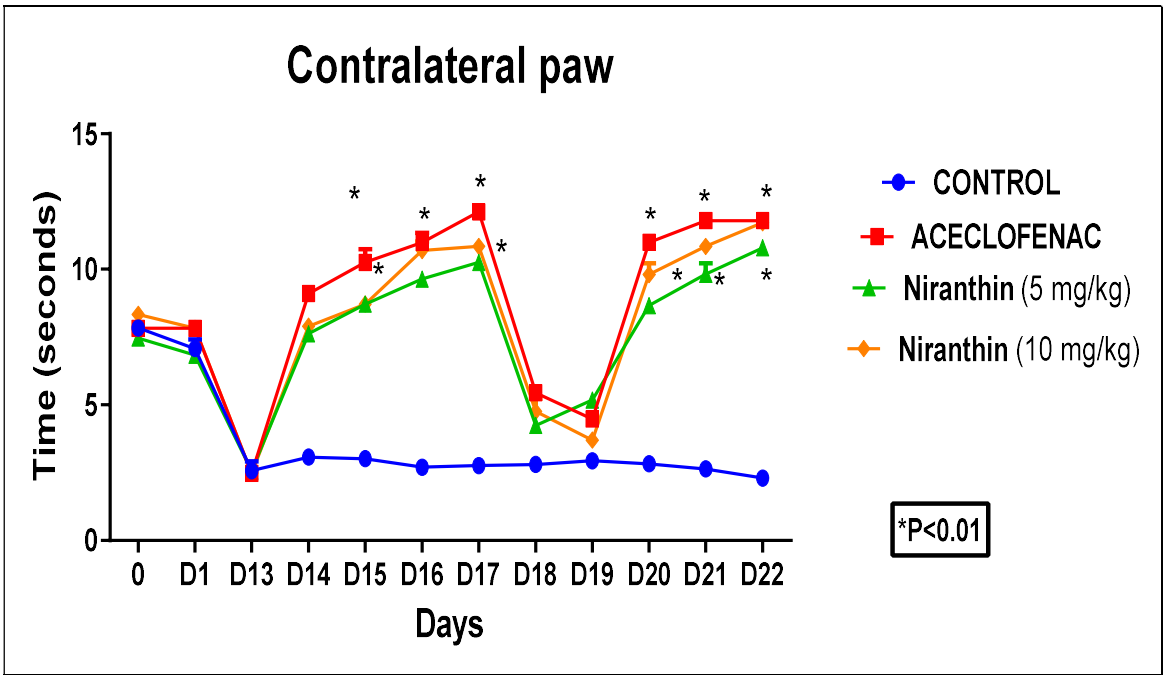


Figure 1: Modulatory effects of Niranthin on paw withdrawal latency to heat stimuli in chronic inflammatory muscle hyperalgesia.The treatment initiated after initiation of chronic heat hyperalegia such as on 14th day post carrageenan injection.[The mean thermal withdrawal latency (in seconds) measured in ipsilateral and contralateral paws (n = 6 for each group). Each point represented as mean ± standard error of mean of the paw withdrawal threshold (seconds) in response to heat stimuli. Data analyzed by one-way analysis of variance using multiple comparison test. P < 0.01 considered significant when compared with inflammatory control]

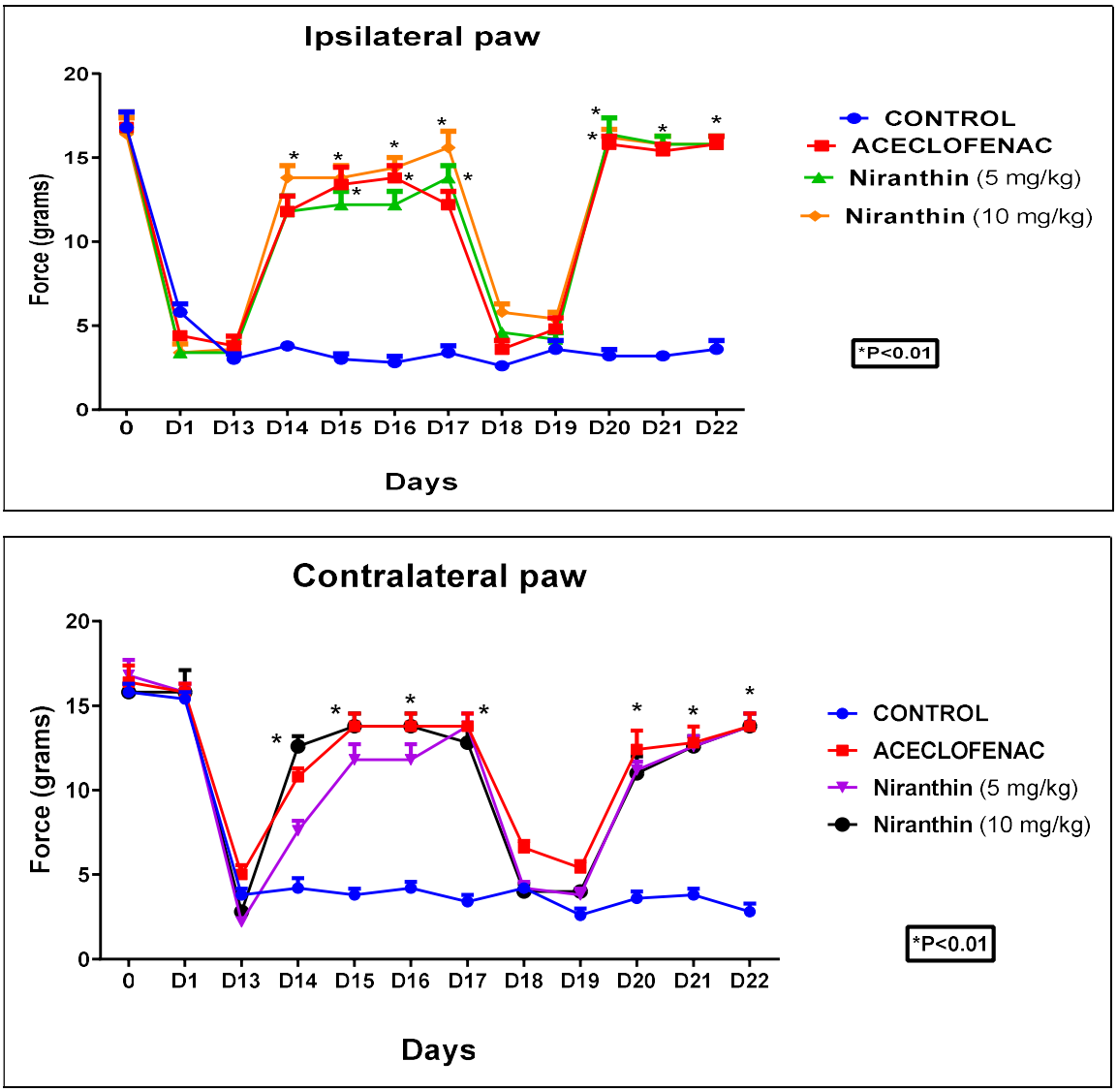


Figure 2: Modulatory effects of Niranthin on paw withdrawal latency to mechanical stimuli in chronic inflammatory muscle pain. The treatment initiated after initiation of chronic mechanical alloydynia such as on 14th day post carrageenan injection.[The mean mechanical withdrawal latency (grams) measured in ipsilateral and contralateral paws (n = 6 for each group). Each point represented as mean ± standard error of mean of the paw withdrawal threshold (grams) in response to mechanical stimuli. Data analyzed by one-way analysis of variance using multiple comparison test. P < 0.01 considered significant when compared with inflammatory control]

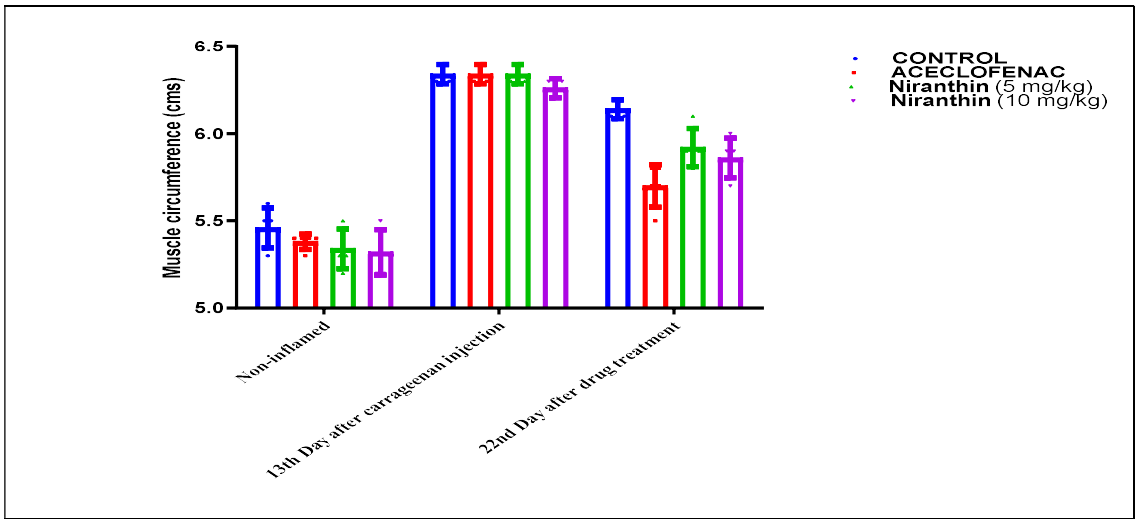


Figure 3: Effect of Niranthin on muscle inflammation in chronic inflammatory hyperalgesia. [Each point in SI.Figurerepresents mean ± standard error of mean of muscle thickness/diameter (in centimeters) before carrageenan injection (non-inflamed) and on 13th and 22nd day post intramuscular injection Carrageenan. Data were analyzed by one-way analysis of variance using multiple comparison test]

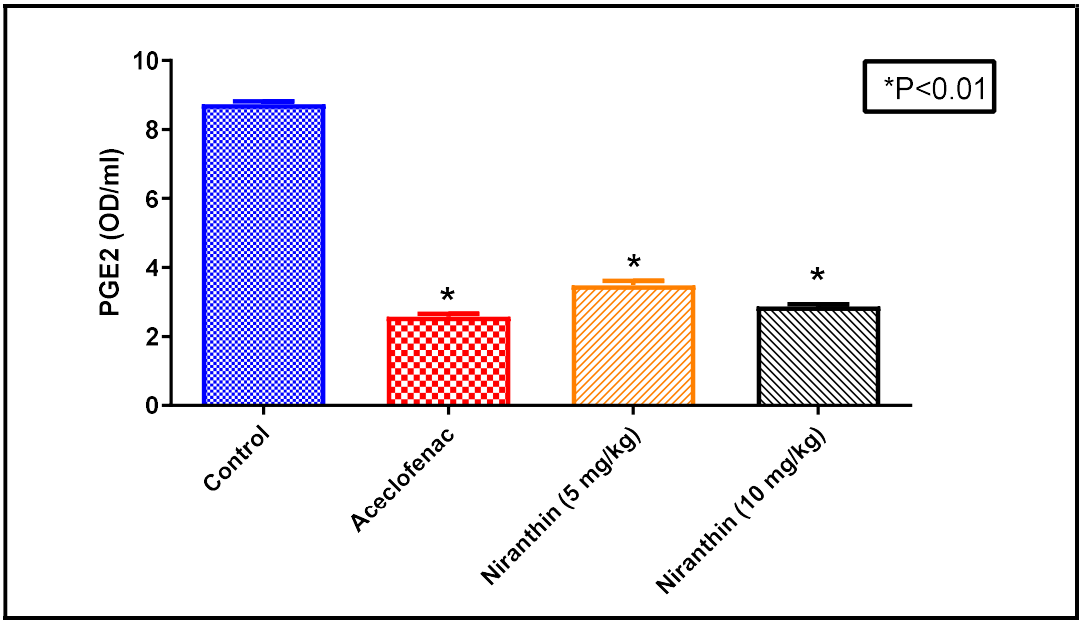


Figure 4: Effects of Niranthin on PGE2 concentration in muscle exudates induced by Carrageenan in rats. [PGE2 concentration was measured only in ipsilateral carrageeenan-injected muscle exudates on 22nd day after intramuscular carrageenan injection. Each bar represents the mean ± standard error of mean of the PGE2 concentration (in optical density/mL). Data analyzed by one-way analysis of variance multiple comparison test. p < 0.01 considered significant in comparison with inflammatory control]

4. DISCUSSION

Antihyperalgesic effects of Niranthin in chronic pain model

Present study evaluates thermal hyperalgesia and mechanical allodynia after systemic administration of the Niranthin, in chronic model of inflammatory muscle pain evoked by intramuscular administration of carrageenan. Interesting previous facts majorly reports data dealing with preventive hyperalgesia measure but to date no one have evaluated effects of Niranthin on the established mechano-thermal hypersensitivityinvolving spinal mediation in lab experiments. In the present model spinal plus or supraspinal neuronal mechanisms are significant in maintaining the chronic state of mechano-thermal hyperalgesia. Results of present study indicate the ability of Niranthin in modulating established chronic mechano-thermal hypersensitivity. Important observation to mention in present study of observed with Niranthin antihyperalgesic effect is not susceptible to the tolerance, as it is effectiveness is retained post repeated intraperitoneal administration.

Spinal COX-2 is known to play crucial role in maintaining mechano-themal hyperalgesia induced specifically by carrageenan **[21]**. It is also reported that after chronic inflammation, occurrence of mechano-thermal is due to increase of PGE2 levels of inflamed tissue as well as spinal cord, which associates with induction and activating spinal COX-2 **[22]**. The COX-2 is a main source for PGE2 in present inflammatory pain model, as in previous study selective COX-2 inhibitors has showed potent antihyperalgesia **[4, 22]**. Pulichino *et al*. have suggested significance of inhibiting PGE2 synthesis by non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors contributing their effectiveness in treating chronic muscular inflammatory pain **[23]**.

Whereas, in the present studies there is a significant decrease of PGE2 level in muscular edematous exudates of Niranthin and Aceclofenac treated groups as compared with inflammatory controls. Also, supported by histopathological examined reports of gastrocnemius muscle tissues in that parallel the changes observed in long lasting inflammatory hyperalgesia.

Lignans have been reported to show antioxidant, analgesic, anti-inflammatory, immunomodulatory and anti-arthritic activity **[10]**. Purified isolated lignans like niranthin, phyltetralin and nirtetralin, from Phyllanthus species showed anti-inflammatory potential in vivo and in vitro studies **[17]**. To mention especially lignan fraction rich which contained niranthin was effective to interfere with the platelet activating factor induced inflammatory response **[17]**. The toxicity profiling of

Niranthin as per reported literature, chemical database and our labs previous studies indicates its relative safety potential. Moreover, previously reported molecular docking studies from our labs demonstrates various phytocompounds including niranthin as chronic pain modulators docked with important inflammatory targets COX-2 and PGE synthase receptor **[11-15]**.

The results of current study predict potential anti-hyperalgesic effects of the Niranthin. The molecular docking analysis also support these effects of Niranthin could be due to the inhibition of PGE2 at the peripheral as well as spinal or supraspinal levels. As Niranthin have the ability to cross the blood brain barrier **[12]**. Our present study strongly predicts a possible chronic pain modulating role of Niranthin via inhibition of prostaglandins in relieving chronic mechano-thermal hyperalgesia.

In-depth experimental evaluation of the Niranthin, by advanced clinical approaches would prove it's clinically effectiveness in treatment of various chronic pain disorders.

CONCLUSION

In the present study, we attempted to identify a possible role of Niranthin in modulating the state of chronic mechano-thermal hyperalgesia suggesting a probable involvement a peripheral reduction of the inflammation suggesting peripheral effects.

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