Effects of naringenin on allodynia and hyperalgesia in rats with chronic constriction injury-induced neuropathic pain

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OBJECTIVE: To study the analgesic effects of naringenin on chronic constriction injury (CCI) model of neuropathic pain.

METHODS: After inducing of neuropathic pain by CCI, treatment with 25 and 50 mg/kg of naringenin and 10 mg/kg of pregabalin was given. Rats were evaluated for behavioral tests using Hargreaves apparatus for thermal hyperalgesia, pin prick test for tactile mechanical hyperalgesia and cold water-induced allodynia on days 0, 3, 5, 7, 14 and 21. At the end of study, oxidative stress parameters were measured.

RESULTS: Naringenin showed ameliorating action against CCI-induced neuropathic pain in all the tested models. Also, naringenin attenuated the elevated levels of lipid peroxidation and nitric oxide, and restored the level of reduced glutathione.

CONCLUSION: The results of the present investigation suggest that naringenin exhibits analgesic effect in sciatic nerve injury model.

KEYWORDS: analgesics; sciatica; hyperalgesia; flavonoids; pain; oxidative stress

Neuropathic pain initiated by a primary lesion or dysfunction of the nervous system may be peripheral (peripheral nerve, plexus and nerve route) or central. Peripheral injuries lead to spontaneous pain followed by allodynia and hyperalgesia. Neuropathic pain is one of the chronic painful and debilitating conditions which affects large population worldwide (7% to 18%), and that could disturb the daily activities. The etiology and underlying mechanisms of such pains are poorly understood and several pharmacological and non-pharmacological approaches such as opioid analgesics, tricyclic antidepressants, anticonvulsants, local anesthetics, as well as acupuncture and electrical stimulation have been used to treat neuropathic pain. Nevertheless, these are not completely effective to relieve neuropathic pain, and novel options are thereby necessary to investigate. A number of animal nerve injury models have been developed to study the mechanisms underlying neuropathic pain. But chronic constriction injury (CCI) animal model appears to be one of

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DOI: 10.3736/jcim20121223
http://www.jcimjournal.com


Received August 3, 2012; accepted October 28, 2012; published online December 15, 2012.


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ISSN 1672-1977. Published by JCIM Press, Shanghai, China.
the most frequently used for the study of neuropathic pain and its treatments, due to similarities of this model with human behavioral responses [25, 26].

Herbal medicines have been used in treatment of many problems and disorders. Bioactive ingredients offer some specific therapeutic benefits and less adverse effects. Therefore, finding new medicines with herbal origin is well worth. In present context, beneficial effects of some plants in treatment of different kinds of preclinical neuropathic pain models have been studied [26-31]. Recently, preclinical investigation of naringenin-rich extract of Lippia gracilis showed analgesic and anti-inflammatory activities [22]. Naringenin, a flavanone, is biologically active molecule abundantly found in citrus fruits such as grapefruits, oranges and tomatoes possessing wide range of pharmacological effects [25] such as peroxisome proliferator-activated receptor (PPAR) activation [26], antidepressant, neuroprotective [25, 26], anti-oxidant [25], anti-inflammatory [25, 26], anti-atherogenic [25, 26], antidiabetic [25, 26], immunomodulator [25], antitumor [25], DNA protective [25] and hypolipidemic [25] activities.

PPARY agonists have been documented to ameliorate the painful state in the diabetic, tibial and sural nerve transaction and spared nerve injury-induced neuropathy [25, 26]. Interestingly, naringenin is reported to activate PPARγ [31]. Nitric oxide (NO) plays a pivotal role in many biological processes including neuropathic pain [1, 37]. It is suggested that inhibition of nitric oxide synthase (NOS) could be effective drug strategy to enhance the clinical efficacy of therapeutic agents against neuropathic pain [18, 19], whereas naringenin is reported to exhibit nitric oxide synthase inhibitory activity [40, 41]. Further analgesic effect of the antidepressant (dual norepinephrine and serotonin reuptake inhibitor) is partially due to inhibition of NOS [42] and naringenin possesses potent antidepressant-like property via the inhibition of central serotonergic and noradrenergic systems [25]. These evidence points that naringenin may have potential in neuropathic pain but, literature is silent on such possibility. Therefore, present study was designed to investigate the protective effect of naringenin in the CCI-induced neuropathic pain model in rats.

1 Material and methods

1.1 Experimental animals Male Sprague-Dawley rats weighing 150 to 200 g were procured from National Institute of Biosciences, Pune, India. The animals were maintained under standard laboratory conditions at temperature 23 °C ± 2 °C, relative humidity 55% ± 10% and 12 to 12 h light-dark cycle maintained throughout the experiment. Animals had free access to water and standard laboratory feed. The animal studies were approved by the Institutional Animal Ethics Committee (Protocol NO. SCOP/2011-12/12), constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, India. Animals were naive to drug treatments and experimentation at the beginning of all studies. All tests were conducted between 8 AM to 2 PM.

1.2 Drugs and reagents Naringenin (Sisco Research Lab, India) was suspended in 1% carboxy methylcellulose and pregabalin (Hetero Labs, India) was dissolved in saline. Drug suspensions and solutions were prepared fresh before the administrations. All the other chemicals used were of analytical grade. The doses of naringenin were selected on the basis of previous reports and our preliminary observations in present study [40].

1.3 CCI-induced neuropathic pain Peripheral neuropathic pain was induced in rats by CCI injury method [39]. In brief, rats were deeply anesthetized intramuscularly with the mixture of 60 mg/kg of ketamine and 10 mg/kg of xylazine. The hairs of lower back and thigh were removed using commercially available depilator and the skin was sterilized with 0.5% chlorhexidine. The skin of the lateral surface of right thigh was incised and a cut was made directly through the biceps femoris muscle to expose the sciatic nerve and four ligatures were placed around the nerve proximal part of the trifurcation with an approximate distance of 1 mm between each ligature with non-absorbable suture (catgut No. 4-0). The ligatures were loosely tied
until a short flick of the ipsilateral hind limb was observed. After performing nerve ligation, muscle and skin layers were immediately sutured with silk thread and the topical antibiotic was applied. All surgical procedures were carried out under normal sterile conditions and were performed by the same experimenter.

1.4 Groups and treatments Five groups with 6 rats in each were employed in the present study. Animals in treatment group orally received 25 and 50 mg/kg of naringenin or 10 mg/kg of pregabalin once daily. Treatments were started from day 0 (before CCI) to the end of experimental period (after CCI). The grouping was as follows: sham control group was the normal rats treated with 1% carboxy methylcellulose (vehicle); CCI control group was the model rats treated with vehicle; treatment groups were the CCI model rats respectively treated with 25 and 50 mg/kg of naringenin and 10 mg/kg of pregabalin.

1.5 Behavioural examination

1.5.1 Assessment of thermal hyperalgesia using Hargreaves apparatus The nociceptive activity was assessed in terms of paw withdrawal latency in the Hargreaves apparatus. The apparatus described by Hargreaves[40] consists of rectangular glass surface with plexiglass walls on four sides[41]. The temperature of the glass plate was maintained at 30 °C ± 1 °C with the help of thermocouples attached to the bottom of the chamber. The rats were allowed to acclimate for the period of 10 min. Thereafter, a radiant heat source consisting of a high intensity bulb (12 V, 50 W) located 20 mm below the glass floor and projecting through a small round aperture of 5 mm diameter was directly targeted at the hind paw of the sham or neuropathic rats and the paw withdrawal latency was recorded as the time interval between the application of the heat beam and the first overt behavioural sign of noiception as described earlier[42,43]. The cut-off time was set at 30 s. Following these treatments, the rats were tested in Hargreaves apparatus and three readings were taken at intervals of 10 min each.

1.5.2 Cold alldynia The method was performed as described earlier[1,30]. In brief, 2 h after assessment of thermal hyperalgesia, cold alldynia was assessed by measuring paw (ipsilateral) withdrawal latency. Ice cold water 4 °C ± 1 °C was taken in beaker and the paw of rat was submerged gently in it and the withdrawal time was measured on days 0, 3, 5, 7, 14 and 21 after CCI. A cut-off time was maintained throughout the experiment at 20 s.

1.5.3 Mechanical hyperalgesia (pin prick test) The mechanical hyperalgesia was assessed by the pinprick test as described by Erichsen and Blackburn-Munro[44]. The surface of the injured hind paw was touched with the point of the bent 18 gauge needle (at 90° angle to the syringe) at intensity sufficient to produce a reflex withdrawal response. The paw withdrawal (lifting) duration was recorded in seconds[20,26].

1.5.4 Assessment of locomotor activity (exploratory behaviours) The locomotor activity was recorded to determine the possible interference of motor activity on analgesic effect of naringenin. The exploratory behaviour of rats was measured 30 min after administration of agents on days 0, 2, 4, 6, 13 and 20. Rats were placed on the central square and as soon as animal entered a square with four legs, the number of squares that animal crossed was recorded during a 5 min session. This test was performed one day prior to surgery as referred to day 0 and days 2, 4, 6, 13 and 20[21].

1.6 Biochemical estimations All the rats were sacrificed by decapitation on day 21 immediately after behavioural assessments. A segment of sciatic nerve, approximately 1.5 cm in length, 5 mm proximal and 5 mm distal to the injured site was used for preparing homogenates for biochemical estimations. The sciatic nerve homogenate (10% w/v) was prepared with 0.1 mol/L Tris-HCl buffer (pH 7.4), and centrifuged for 20 min at 15 000 r/min. Supernatants were used for estimation of lipid peroxidation, reduced glutathione (GSH) and NO levels.

1.6.1 Lipid peroxidation Estimation of lipid peroxidation was done by measuring the levels of malondialdehyde (MDA)[45] as described earlier[46]. The absorbance was measured spectrophotometrically at 532 nm. The concentration of MDA in sciatic nerve homogenates was expressed in nmol MDA/g wet tissue.

1.6.2 GSH GSH was determined by Ellman’s method[47] as described previously[48]. Equal quantity of sciatic nerve homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate proteins. To 0.01 mL of this supernatant, 2 mL of phosphate buffer (pH 8.4), 0.5 mL of 5,5'-dithio, bis(2-nitrobenzoic acid) and 0.4 mL double-distilled water was added. Mixture was vortexed and the absorbance was taken at 412 nm within 15 min. The concentration of GSH was expressed as nmol/(L • g) wet tissue.

1.6.3 Nitrite The accumulation of nitrite in the supernatant, an indicator of production of NO was determined with a colorimetric assay with Greiss reagent [(0.1% N-(1-naphthyl) ethylene-diamine dihydrochloride, 1% sulphanilamide and 2.5% phosphoric acid)[49]. The concentration of nitrite in the supernatant was determined from a standard curve and expressed as nmol/(L • g) wet tissue.

1.7 Statistical analysis The data were expressed as mean±standard error of mean. The results obtained from behavioural tests were analyzed using two-way analysis of variance followed by Bonferroni’s post test or one-way analysis of variance followed by Tukey’s multiple comparison test. P < 0.05 was considered statistically significant in all cases.

2 Results

2.1 General behavioral observations The rats with
CCI showed abnormal gait, posture, licking of hind paw of the ipsilateral side of sciatic nerve ligation from day 3 onwards. The rats could not put weight on affected side and the hind limb of affected side was drawn close to body with distinctive guarding posture.

2.2 Effect of naringenin on CCI-induced neuropathic pain in thermal hyperalgesia model
CCI control group exhibited significant reduction in paw withdrawal latency on days 3, 5, 7, 14 and 21 when compared with sham-operated group ($P < 0.01$). CCI model rats treated with 25 and 50 mg/kg of naringenin significantly tolerated the radiant heat when compared with CCI control group on days 5, 7, 14 and 21 ($P < 0.01$). Treatment with naringenin on day 3 did not show any effect on paw withdrawal latency, whereas treatment with 10 mg/kg of pregabalin substantially increased paw withdrawal as compared with CCI control group on days 3, 5, 7, 14 and 21 ($P < 0.01$ or $P < 0.05$). See Figure 1.

![Figure 1 Effect of naringenin on CCI-induced neuropathic pain in thermal hyperalgesia model](image1)

Data are expressed as mean ± standard error of mean, and results obtained were analyzed by repeated two-way analysis of variance followed by Bonferroni’s post test. $n = 6$. ** $P < 0.01$, vs sham-operated group; $\Delta P < 0.05$, $\Delta \Delta P < 0.01$, vs CCI control group. CCI: chronic constriction injury.

2.3 Effect of naringenin on CCI-induced neuropathic pain in cold allodynia model
CCI control group showed significant cold allodynia compared with sham-operated group ($P < 0.01$). Chronic treatment with naringenin (25 mg/kg) significantly increased paw withdrawal latency on days 14 and 21 ($P < 0.05$). Further, treatment with naringenin (50 mg/kg) showed significant effect against cold allodynia compared to CCI control group on days 5, 7, 14 and 21 ($P < 0.01$ or $P < 0.05$). Also, treatment with pregabalin (10 mg/kg) substantially increased paw withdrawal latency compared to CCI-induced neuropathic pain control group on days 5, 7, 14, 21 ($P < 0.01$). See Figure 2.

![Figure 2 Effect of naringenin on CCI-induced neuropathic pain in cold allodynia model](image2)

Data are expressed as mean ± standard error of mean, and results obtained were analyzed by repeated two-way analysis of variance followed by Bonferroni’s post test. $n = 6$. ** $P < 0.01$, vs sham-operated group; $\Delta P < 0.05$, $\Delta \Delta P < 0.01$, vs CCI control group. CCI: chronic constriction injury.

2.4 Effect of naringenin on CCI-induced neuropathic pain in tactile mechanical hyperalgesia model
Paw lifting duration was significantly increased in CCI group when compared to that of sham-operated rats ($P < 0.01$). Further, treatment with 25 mg/kg of naringenin in CCI-induced neuropathic pain showed significant decrease in paw lifting duration on days 14 and 21, and 50 mg/kg of naringenin significantly reduced the paw lifting duration on days 5, 7, 14 and 21 as compared with CCI control group ($P < 0.01$). Treatment with pregabalin also significantly reduced paw lifting duration on days 5, 7, 14 and 21 ($P < 0.01$). See Figure 3.

![Figure 3 Effect of naringenin on CCI-induced neuropathic pain in mechanical hyperalgesia model](image3)

Data are expressed as mean ± standard error of mean, and results obtained were analyzed by repeated two-way analysis of variance followed by Bonferroni’s post test. ** $P < 0.01$, vs sham-operated group; $\Delta \Delta P < 0.01$, vs CCI control group. CCI: chronic constriction injury.
2.5 Effect of naringenin on CCI-induced neuropathic pain in exploratory behavior (Locomotor activity) test

Treatment with 25 and 50 mg/kg of naringenin as well as 10 mg/kg of pregabalin did not affect locomotor activity. See Figure 4.

![Figure 4](image)

Figure 4 Effect of naringenin on CCI-induced neuropathic pain in exploratory behavior (Locomotor activity) test

Data are expressed as mean±standard error of mean. Repeated measure of two-way analysis of variance followed by Bonferroni post test.

2.6 Effect of naringenin on oxidative stress markers in CCI-induced neuropathic pain model

MDA level in CCI control group was significantly elevated when compared with sham-operated rats, whereas treatment with naringenin (25 and 50 mg/kg) and pregabalin (10 mg/kg) significantly reduced MDA level in CCI-induced neuropathic pain model ($P<0.01$). CCI-induced neuropathic rats exhibited significantly lower level of GSH, whereas treatment with naringenin and pregabalin significantly increased GSH level ($P<0.01$). CCI-induced neuropathic rats showed substantially higher NO level. Treatment with naringenin and pregabalin significantly decreased NO level ($P<0.01$). See Figure 5.

3 Discussion

The study was designed to evaluate the effect of naringenin against neuropathic pain. This is the first report to show that naringenin exhibits analgesic effect in animal model of nerve injury. Naringenin attenuated CCI-induced neuropathic pain in a dose-dependent manner. CCI rats exhibited thermal hyperalgesia, cold allodynia, tactile mechanical hyperalgesia and foot deformity which were associated with increased oxidative stress, whereas naringenin treatment counteracted these physical, behavioral and biochemical changes.

CCI is the most commonly employed animal model with nerve damage-induced allodynia/hyperalgesia [9, 54]. Neuropathic pain was induced by entrapping the sciatic nerve by four loose ligatures. This produced persistent neuropathic pain, allodynia and hyperalgesia. Several studies employed CCI-induced neuropathic pain model for screening of drugs that are effective in treatment of neuropathic pain [50, 55].

In present study, we evaluated the effect of naringenin treatment on cold allodynia by using cold water test. The ipsilateral paw withdrawal latency on exposure to cold water was determined. Naringenin improved the pain threshold and attenuated cold allodynia in hind limb of CCI-induced neuropathic rats, suggesting its potential against allodynic state. CCI-induced neuropathic pain also leads to thermal hyperalgesia. We examined the effect of naringenin on thermal hyperalgesia in Hargreaves apparatus. Exposure to radiant heat-produced thermal hyperalgesia in rats indicates neuropathic pain which is well in accordance with earlier reports [30, 21]. In this study, an increase in hind paw withdrawal latency in CCI-induced thermal hyperalgesia by naringenin indicates its potential in treatment of neuropathic pain. Previous reports showed the utility of tactile mechanical hyperalgesia for evaluation of mechanical hyperalgesia condition [50]. Chronic treatment with naringenin showed decreased paw lifting duration after exposure to tactile mechanical stimulus.

CCI produces significant oxidative damage in sciatic nerve as indicated by rise in MDA and nitric oxide concentration and depletion of GSH levels. Evidence indicates that reactive oxidants

![Figure 5](image)

Figure 5 Effect of naringenin on oxidative stress markers in CCI-induced neuropathic pain model

A: effect of naringenin on MDA level; B: effect of naringenin on GSH level; C: effect of naringenin on NO level. Data are expressed as mean ± standard mean of error, and results obtained were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison, $n=6$.

*** $P<0.01$, vs sham-operated group, $\triangle\triangle P<0.01$, vs CCI control group. MDA: malondialdehyde; GSH: reduced glutathione; NO: nitric oxide; CCI: chronic constriction injury.
species are critically involved in the development and maintenance of neuropathic pain\textsuperscript{[50]}. In present study, naringenin and pregabalin treatment significantly reduced oxidative damage by attenuating the elevation of lipid peroxides and nitrite concentration and restoring the depletion of glutathione. These results indicate that the beneficial effects of naringenin in present study could be because of its antioxidant property. Earlier evidence also indicated the antioxidant potential of naringenin and pregabalin\textsuperscript{[27,57-59]}. In addition previous reports point towards the role of PPAR\textgamma receptors and its activators in neuropathic pain and nociception\textsuperscript{[58,61]}. Interestingly, naringenin is reported to have PPAR\textgamma agonistic action\textsuperscript{[56]}.

4 Conclusion

The findings of present study and previous reports point towards the possible effect of naringenin in neuropathic pain through mechanisms such as inhibition of nitric oxide pathway and ameliorating oxidative stress, and this warrants further investigation in order to establish its mechanisms at molecular level.

5 Conflict of interests

The authors declare that they have no competing interests.

6 Acknowledgements

The authors would like to thank Sinhgad Technical Education Society for providing the facilities of this work. Authors would like to thank AICTE for providing the financial assistance (Shyam Kaulskar).

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柚苷配基对慢性压迫性损伤引起的大鼠触摸痛及触觉过敏的影响

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目的：研究柚苷配基对慢性压迫性神经损伤模型的镇痛效果。

方法：慢性压迫性损伤建立神经性疼痛大鼠模型后，分别给予模型大鼠 25 和 50 mg/kg 的柚苷配基以及 10 mg/kg 的普拉加巴林。在第 0, 3, 5, 7, 14 和 21 天采用 Hargreaves 方法评估大鼠的行为变化。实验测试触觉性疼痛和疼痛性感觉的异常性疼痛，最后测定氧化应激反应参数。

结果：柚苷配基能缓解慢性压迫性损伤诱发的神经性疼痛，减轻脂质氧化反应，降低氧化应激水平。

结论：柚苷配基能缓解坐骨神经损伤引起的疼痛。

关键词：镇痛药；坐骨神经痛；触觉过敏；类黄酮物质；疼痛；氧化性应激

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