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Antinociceptive and anti-inflammatory effect of Naringenin in different nociceptive and inflammatory mice models

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ABSTRACT

Inflammation is the vital defensive response triggered by our immune system against an infection or deleterious stimuli. This response in due course affects its own biological system leading to serious diseases like arteriosclerosis, osteoporosis, pancreatitis, cancer etc. Currently, researchers focused on utilizing phytochemicals as anti-inflammatory drugs since the drugs presently available in the market causes serious side effects and are less potent. Flavonoids are polyphenols which imparts colours to the plants and fruits. These flavonoids serve as phytonutrients to plants and they also possess antioxidant, anti-inflammatory and anti-cancer properties. Naringenin is one such flavonoid classified under flavones groups present in citrus fruits and vegetables. The present study is aimed to identify and confirm the antinociceptive and anti-inflammatory efficacy of Naringenin in different mice models. The antinociceptive effect of Naringenin was analyzed by both thermal induced and chemical induced nociceptive mice models. Carrageenan-induced paw edema test was performed to detect the anti-inflammatory effect of Naringenin and it is confirmed by analyzing the leukocyte infiltration in peritoneal cavity. Air pouch model test is performed to estimate the inhibitory property of Naringenin against proinflammatory cytokines. The potency of drug Naringenin was confirmed by treating along with opioid inhibitors naloxone and the results compared with standard drugs. To assess the muscle relaxant property of Naringenin open field test was performed. The overall results of Naringenin in different nociceptive and inflammatory mice models suggest that, Naringenin is a potent anti-inflammatory drug which relieves pain effectively and can be used in pain management therapy.

1. Introduction

Inflammation is a defensive response exhibited by the biological systems against the noxious stimuli like infection, tissue damage etc., [1]. The prolonged response of immune cells to a stimuli increases the causative risk of various disease like rheumatoid arthritis, type 2 diabetes, cancer, cirrhosis, Alzheimer's and several other neurological diseases. This defensive response of biological system is multifaceted and one of most common physiological outcome of inflammation is pain. Pain management had become a global issue and it has predicted that for every 1 in 5 adults are suffering with either acute or chronic pain [2]. Since pain not only impairs the normal well-being and but also it affects the health, productivity and the economic status of an individual and also the country [3].

Hyperalgesia, a condition caused by the inflammatory modulators by sensitizing nociceptors and somatosensory neurons leading to prolonged pain persistence [4]. Therefore the anti-inflammatory drugs like non-steroidal anti-inflammatory drugs (NSAID) are globally prescribed drugs for the patients to reduce pain and to overcome inflammation, which is the major causative of pain [5]. Even though these drugs effectively subsides the pain the prolonged usage of analgesic drugs leads to serious side effects. Hence it is current need to develop a cost effect potent anti-inflammatory drug with nil side effects. The diversified chemical structure and the immense pharmacological properties of plants triggered the research to formulate herbal based drugs to various diseases. The herbal derivatives like morphine, saliciate, and capsaicin possess neuro modulating property which effectively suppresses the pain sensation [6], these phytochemicals are potent, safe and also cost effective.

One such compound is Naringenin, 4′,5,7-thrihydroxyflavanone present in citrus fruits and grape fruits. It possess antioxidant, anti-inflammatory properties, increased bioavailability and less toxic hence it

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can used as effective drug to treat various deadly diseases [7,8]. It has proven to possess anticancer, antiatherogenic activities and researches also established it can be used to treat cardiovascular disease, osteoporosis etc. Naringenin and its metabolite Naringenin effectively inhibit the secretion of proinflammatory cytokines induced by lipopolysaccharides [9,10]. Reports suggest that Naringenin prevents rats from cognitive deficits induced by kanic acid [11] and 3 nitropropionic acid [12] in epilepticus and Huntington's models respectively. It also improves the long term memory in mice model of Alzheimer's disease [1].

However the anti-inflammatory activity and its influence on nociceptive effect of Naringenin were not yet studied. Hence the study was aimed to analyze the nociceptive effect of Naringenin in different mice model. The anti-inflammatory property was assessed by estimating the peritoneal leukocyte infiltration and the levels proinflammatory cytokines at inflammation site. To confirm whether Naringenin exerts any behavioral changes open field test was performed

2. Materials & methods

2.1. Drugs

The drugs Naringenin 98% (W530098), Indomethacin, naloxone, diclofenac sodium, capsaicin, formalin, morphine, carrageenan, dexamethasone were obtained from Sigma Aldrich, USA.

2.2. Animals

Male Swiss Albino mice weighing about 20–30 g were used for the present study. The mice were housed in sterile plastic cages at temperature of 22–25 °C with standard light dark cycle for 12 h each. The relative humidity of the animal house was at maintained at 55–60%. The mice were allowed to free access for food and water. Before initiation of experiment the mice were acclimatized for 14 days at the standard laboratory conditions. The mice were fasted overnight before the behavioral analyses and the analyzed were performed between 8.00 am to 12.00 am. All the animal procedures were approved by the Institutional Ethical committee (Xi'an Jiaotong University, Xi'an, Shaanxi province, China) and all the experiment performed on mice was done with extra care and concern (No.2018067).

2.3. Naringenin antinociceptive activity

2.3.1. Hot plate test

The antinociceptive effect of Naringenin was assessed by performing Eddy's hot plate method [13]. The animals which shown quick responses like jumping and withdrawal within 15 s to thermal stress were chosen for the hot plate test analysis. The animal selection was done 24 h prior to the experiment. The experimental mice were grouped into ten each group consisting of 6 mice. Group I treated with 1% tween 80 and Group VI treated with naloxone, opioid antagonist 2 mg along with saline. Groups II, III and IV mice were treated with Naringenin 25, 50, 75 mg respectively and Groups VII, VIII, IX were treated with naloxone along with Naringenin with different doses. As positive control the Group V rats were treated with morphine and the Group X was treated with morphine + naloxone. The mice from each group were placed on the plate at the temperature of 50 °C for 20 s to prevent any damage to the paw of animals. The behaviors of the mice were recorded before treatment and after 30, 60, 90, 120 min of drugs treatment.

The percentage of the maximal possible effect of each mouse was calculated using the equation.

$$\%MPE = [\{(Postdrug\ latency) - (Predrug\ latency)\}\{(Cut - off\ time) - (Predrug\ latency)\}] \times 100.$$

2.3.2. Tail immersion test

Same experimental group of mice analyzed for hot plate method was maintained for tail immersion test also. The analgesics morphine delays the tail withdrawal of mice from hot water maintained at 55 °C whereas the naloxone reverses the activity of drugs like morphine. Hence the present experimental setup is done in such a way to detect the antinociceptive effect of Naringenin comparing it with positive control morphine and also treating along with opioid inhibitor naloxone. The mice were observed for the tail withdrawal before the initiation of experiment and those who illustrate withdrawal time between 1.5 and 2.5 s were chosen for the experiment [14]. The mice were pretreated with different concentration of Naringenin, morphine and naloxone + Naringenin, naloxone + morphine before performing tail immersion test, the cut off time was set as 20 s to avoid any injury. The tail deflection time was recorded for every 30 min till 120 min and the percentage of MPE was calculated.

2.3.3. Acetic acid induced nociception test

The mice were grouped into five groups control (1% tween 80), Naringenin (25, 50, 75 mg/kg) and diclofenac sodium (10 mg/kg) as positive control. The Naringenin and diclofenac sodium were treated 15 min prior to the experiment after pretreatment the mice were subjected to experiment with 1% acetic acid at dose of 10 ml/kg [15]. The mice were place in the observation chamber for 1 h and the number of writhing performed by the mice were counted and recorded.

2.3.4. Glutamate induced nociception test

The same experimental group of mice as that of acetic acid induced nociception test was maintained for glutamate induced nociception test also. The mice were pretreated with Naringenin (25, 50, 75 mg/kg) and diclofenac sodium (10 mg/kg) before 15 min of the commencement of experiment. After pretreatment the mice were treated with 10 μm glutamate by injecting it to the ventral surface of left hind paw of the mice and kept for observation for 15 min [16]. The number of licks performed by the mice were counted and recorded.

2.3.5. Capsaicin-induced paw licking test

The mice were subjected to capsaicin induced nociceptive test to detect the effect of Naringenin against neuropathic nociception. Before 30 min of experiment initiation the mice were pretreated with different concentrations of Naringenin and diclofenac sodium. 20 μl of capsaicin dissolved in 5% ethanol and 95% phosphate buffered saline was injected into the left paw of the mice as that each paw receives 1.6 μg of capsaicin [17]. The mice were kept in the observation cage for 5 min and the time of licking on the injected paw was recorded which indicates as the presence of nociception.

2.3.6. Formalin induced paw licking test

Formalin induced paw licking test was performed to confirm the antinociceptive efficacy of Naringenin [18]. The mice were grouped into five and pretreated before 30 min via subcutaneous injection with control (1% Tween 80), Naringenin (25, 50, 75 mg/kg) and as positive control the group V rats were treated with morphine (5 mg/kg). After pretreatment the mice were injected with 3% formalin in the right hind paw plantar surface and kept for observation chamber for 30 min. The numbers of lickings performed by mice on first phase to smoothen the neurogenic pain (0–5 min) and on second phase (15–30) due to inflammation were recorded.

2.4. Anti-inflammatory effect of Naringenin

2.4.1. Carrageenan-induced paw edema test

The anti-inflammatory effect of Naringenin was assessed by carrageenan induced paw edema test [19]. The mice were grouped into five and pretreated with control (1% Tween 80), Naringenin (25, 50, 75 mg/kg) and as positive control the group V rats were treated with

Table 1
Antinociceptive effect of Naringenin and reversal effect of naloxone in hot plate induced nociception mice model.

Treatment (mg/kg)	Pretreatment	Response time(s) (%MPE)			
		30 min	60 min	90 min	120 min
Control	7.12 ± 0.26	7.25 ± 0.72	7.51 ± 0.54	7.93 ± 0.12	8.08 ± 0.37
Naringenin (25 mg)	7.61 ± 0.14	9.33 ± 0.19 (13.25)	11.26 ± 0.20 (25.41)#	11.83 ± 0.73 (32.88)*	12.01 ± 0.61 (40.29)*
Naringenin (50 mg)	7.39 ± 0.36	9.52 ± 0.42 (17.96)	11.43 ± 0.73 (33.41)#	12.27 ± 0.11 (44.30)*	12.63 ± 0.16 (48.57)*
Naringenin (75 mg)	7.86 ± 0.41	10.51 ± 0.14 (22.40)	12.97 ± 0.54 (46.38)#	13.83 ± 0.96 (54.47)*	14.42 ± 1.10 (58.97)*
Morphine (5 mg)	7.22 ± 0.74	12.36 ± 0.35 (43.22)	14.87 ± 0.11 (55.36)#	15.97 ± 0.80 (61.33)*	17.23 ± 1.31 (69.41)*
NLX (2 mg) + control	7.55 ± 0.01	7.63 ± 0.77	8.11 ± 0.43	8.41 ± 0.62	8.96 ± 0.71
NLX (2 mg) + Naringenin (25 mg)	7.78 ± 0.11	8.02 ± 0.41 (9.82)	8.72 ± 0.69 (11.30)#	9.13 ± 0.33 (16.67)*	10.33 ± 0.71 (22.14)*
NLX (2 mg) + Naringenin (50 mg)	7.33 ± 0.35	7.79 ± 0.74 (10.76)	8.69 ± 0.23 (14.42)#	9.41 ± 0.64 (19.36)*	10.27 ± 0.83 (26.41)*
NLX (2 mg) + Naringenin (75 mg)	7.21 ± 0.58	7.96 ± 0.30 (11.66)	8.87 ± 0.65 (19.77)#	10.21 ± 0.86 (26.71)*	11.66 ± 0.14 (33.32)*
NLX (2 mg) + morphine (5 mg)	7.53 ± 0.12	7.78 ± 0.36 (5.62)	9.22 ± 0.47 (13.51)#	10.36 ± 0.10 (19.96)*	13.83 ± 0.77 (36.44)*

Each bar represents mean ± SEM of 6 animals, #, *represents statistical significance between control Vs other groups at $p < 0.05$, $p < 0.01$ level respectively using Dunnett's test.

indomethacin (10 mg/kg) before 1 h of experiment initiation. 50 ml of carrageenan (1%) were injected in the right paw and 50 ml of 0.9% saline was injected to left paw of the pretreated mice. The edema formed in both paws were measured up to 4 h at interval of 1 h each and the percentage of MPE was calculated

2.4.2. Peritoneal cavity leukocyte infiltration test

The leukocyte infiltration into the peritoneal activity after the injection of inflammatory agent carageenan and the anti-inflammation efficacy of Naringenin was detected by the method of Vinegar et al. [20]. The mice were grouped into five and pretreated with control (1% Tween 80), Naringenin (25, 50, 75 mg/kg) and as positive control the group V rats were treated with morphine (5 mg/kg) before 30 min of experiment initiation. 500 µg of carrageenan (1%) were injected intraperitoneally and the leukocytes infiltrations were analyzed after 6 h. The mice were euthanized, the peritoneal cavity was washed with 2 ml of PBS containing 1 mM EDTA to harvest the cells. The solution was then centrifuge and examined for the total leukocyte and differential cell counts. The number of total leukocytes, mono and polymorphonuclear cells were recorded.

2.4.3. Effect of Naringenin on proinflammatory cytokines

The mice were given anesthesia with mild ether and the back skin was shaved, 5 ml of sterile air was subcutaneously injected twice at same site at an interval of 3 days to form a pouch [21]. The mice with pouches were divided into six groups and treated with 1% Tween 80 (control), 0.5 ml carageenan (Carageenan control), Carageenan with different concentration of Naringenin (25, 50, 75 mg/kg) and carageenan with dexamethasone (positive control). The mice were sacrificed by cervical dislocation after 1 h, the pouch tissue was cut open and 2 ml saline was injected inside the cavity and sucked back to harvest the cells. The exudates were centrifuged and the cell pellet was subject to analysis of proinflammatory cytokines TNF-α, IL-1β, and IL-6.

2.4.4. Open field test

The sedative effect of Naringenin was assessed by performing open field test. The mice were treated with 1% Tween 80 (control), with different concentration of Naringenin (25, 50, 75 mg/kg) and morphine (positive control, 5 mg/kg). After 60 min, the mice were allowed inside the open field apparatus which is a box of 50 cm × 50 cm × 50 cm, the box were divided into 25 equal squares. The mice were allowed to explore the open field apparatus for 2 min, the number of squares crossed by the mice with all the paws was recorded. The apparatus were cleaned each time with mild ethanol before performing the experiment with new mice.

2.5. Statistical analysis

The obtained data were statistically analyzed using Graph pad prism software and expressed as mean ± standard deviation. One way Analysis of Variance followed by Dunnet's post hoc test was performed to analyze the significant difference between the groups. p values were considered as $p < 0.05$, $p < 0.01$ respectively.

3. Results

3.1. Naringenin antinociceptive activity

3.1.1. Hot plate test

Hot plate test was performed to detect the antinociceptive effect of Naringenin against the thermal stimulus. The Naringenin antinociceptive effect was confirmed with morphine and its efficacy was confirmed by treating along with opioid antagonist naloxone. Naringenin significantly delayed the response time compared to the control; the maximal response time was observed in 75 mg Naringenin treated rats even when treated along with naloxone. Compared to control and Naringenin treated mice morphine treated mice showed significantly increased delayed response time (Table 1).

3.1.2. Tail Immersion test

Table 2 depicts the response of Naringenin, morphine treated mice to the thermal stimulus in tail immersion test. The response time of Naringenin treated rats increased in dose dependent manner. Morphine showed highest latency when compared to control and Naringenin, whereas in naloxone co treated mice, the Naringenin and naloxone treated rats showed increased latency time.

3.1.3. Acetic acid induced nociception test

The abdominal writhing test was performed to analysis the efficacy of Naringenin to subside pain. The mice were treated with acetic acid to induce abdominal writhing and the number of writhes or abdominal stretches performed by mice was counted. The number of writhes was significantly reduced in Naringenin treated mice compared to control. 75 mg treated Naringenin treated mice showed reduced writhes which are near to the value of standard non-steroidal anti-inflammatory drug diclofenac sodium treated mice (Fig. 1)

3.1.4. Glutamate induced nociception test

Fig. 2 illustrates the antinociceptive effect of Naringenin in glutamate treated mice model. The decrease in number of licks indicates the antinociceptive action of the drug. 75 mg of Naringenin oral administered mice performed 55.67 ± 1.25 licks which is comparably equal to the standard drug diclofenac sodium treated mice which performed 45.18 ± 1.10 licks. Compared to control mice 25 mg and 50 mg

Table 2
Antinociceptive effect of Naringenin and reversal effect of naloxone in tail immersion induced nociception mice model.

Treatment (mg/kg)	Pretreatment	Response time(s) (%MPE)			
		30 min	60 min	90 min	120 min
Control	2.99 ± 0.33	3.27 ± 0.40	3.49 ± 0.26	3.67 ± 0.29	2.74 ± 0.17
Naringenin (25 mg)	2.86 ± 0.23	3.44 ± 0.35 (4.32)	3.73 ± 0.43 (6.46)	3.96 ± 0.49 (7.75)	4.27 ± 0.27 (8.89)
Naringenin (50 mg)	2.05 ± 0.43	3.91 ± 0.26 (5.87)#	4.39 ± 0.22 (8.56)*	4.74 ± 0.24 (10.32)*	4.89 ± 0.52 (11.26)*
Naringenin (75 mg)	2.01 ± 0.31	3.16 ± 0.20 (6.47)#	4.69 ± 0.28 (9.98)*	5.10 ± 0.20 (12.78)*	5.12 ± 0.33 (12.38)*
Morphine (5 mg)	2.85 ± 0.16	4.23 ± 0.41 (8.69)#	4.94 ± 0.32 (22.19)*	5.48 ± 0.38 (25.54)*	5.59 ± 0.15 (26.12)*
NLX (2 mg) + control	2.60 ± 0.16	2.99 ± 0.32	2.97 ± 0.24	3.22 ± 0.12	3.34 ± 0.33
NLX (2 mg) + Naringenin (25 mg)	2.82 ± 0.27	3.24 ± 0.26 (3.47)#	3.41 ± 0.14 (4.36)*	3.57 ± 0.17 (5.21)*	3.98 ± 0.38 (7.99)*
NLX (2 mg) + Naringenin (50 mg)	2.90 ± 0.46	3.42 ± 0.25 (3.42)#	3.56 ± 0.24 (4.14)*	3.65 ± 0.29b (4.68)*	4.31 ± 0.20 (8.32)**
NLX (2 mg) + Naringenin (75 mg)	2.76 ± 0.36	3.61 ± 0.39 (5.75)#	3.77 ± 0.28 (6.61)*	4.19 ± 0.30 (8.91)*	4.42 ± 0.29 (9.16)*
NLX (2 mg) + morphine (5 mg)	1.96 ± 0.21	2.18 ± 0.28 (3.30)#	2.69 ± 0.36a (7.28)*	2.98 ± 0.31 (7.98)*	3.35 ± 0.32 (9.70)*

Each bar represents mean ± SEM of 6 animals, #,**, *represents statistical significance between control Vs other groups at $p < 0.05$, $p < 0.01$, $p < 0.01$ level respectively using Dunnett's test.

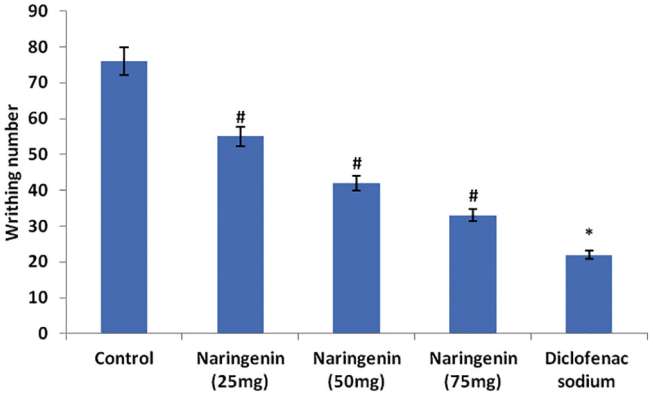

Fig. 1. Antinociceptive effect of Naringenin and diclofenac sodium in the acetic acid induced nociception mice model.
Each bar represents mean ± SEM of 6 animals, #, *represents statistical significance between control Vs other groups at $p < 0.05$, $p < 0.01$ level respectively using Dunnett's test.

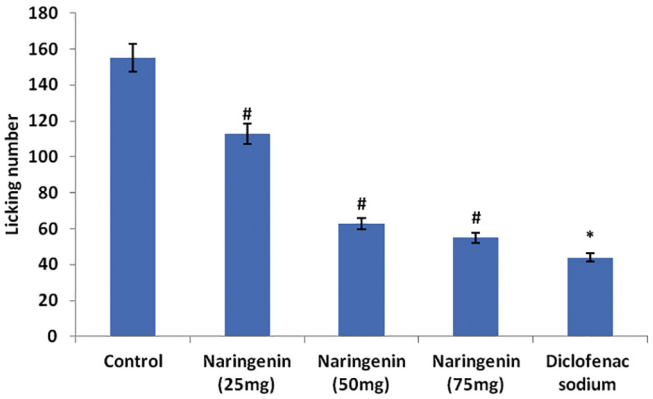

Fig. 2. Antinociceptive effect of Naringenin and diclofenac sodium in the glutamate induced nociception mice model.
Each bar represents mean ± SEM of 6 animals, #, *represents statistical significance between control Vs other groups at $p < 0.05$, $p < 0.01$ level respectively using Dunnett's test.

Naringenin treated mice also showed decreased numbers of licks after glutamate injection.

3.1.5. Capsaicin-induced paw licking test

The mice injected with irritant capsaicin, performed paw licking behavior which was maximal till 5 min of post injection period. Compared to standard reference drug diclofenac sodium (45 ± 2.1 licks), 75 mg Naringenin pretreated mice execute less number of licks (41 ± 1.78 licks). The maximum numbers of licks were executed by the control mice, whereas it is significantly decreased in 25 and 50 mg Naringenin treated mice (Fig. 3).

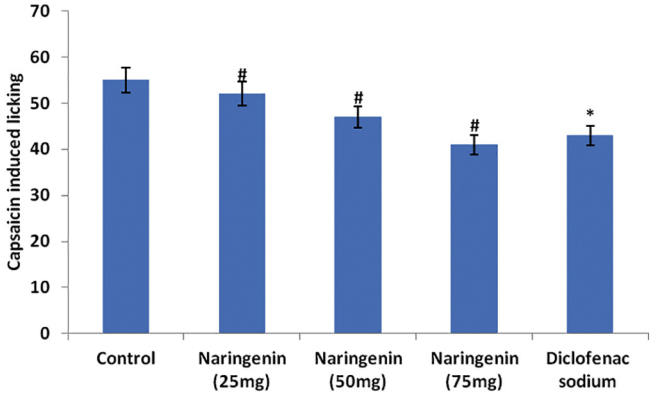

Fig. 3. Antinociceptive effect of Naringenin and diclofenac sodium in the capsaicin induced nociception mice model.
Each bar represents mean ± SEM of 6 animals, #, *represents statistical significance between control Vs other groups at $p < 0.05$, $p < 0.01$ level respectively using Dunnett's test.

3.1.6. Formalin induced paw licking test

Fig. 4 shows the effect of Naringenin and morphine, an opiate pain medication against the formalin pain induced model. Formalin induced paw licking test was performed to confirm the antinociceptive effect of Naringenin at biphasic stages. Compared to control all the three doses of Naringenin pretreated mice licking performance were significantly decreased in both the phase of study. But the licking number of Naringenin pretreated mice was increased at Phase B (15–30 min) compared to Phase A (5 min) licking number. Morphine, standard drug treated mice exhibited significant decrease in licking number compared to control and Naringenin treated mice

3.2. Anti-inflammatory effect of Naringenin

3.2.1. Carrageenan-induced paw edema test

The anti-inflammatory effect of Naringenin was analyzed by performing carrageenan induced paw edema test. The size of carrageenan induced paw edema was measured at an interval of 1 h in the control, Naringenin and indomethacin pretreated mice. The values were converted into maximal possible effect of each drug and tabulated in Table 3. 75 mg Naringenin pre-treated and indomethacin, positive control treated mice decreased edema size at 4th h, which indicates as like that of potent synthetic anti-inflammatory drug indomethacin,

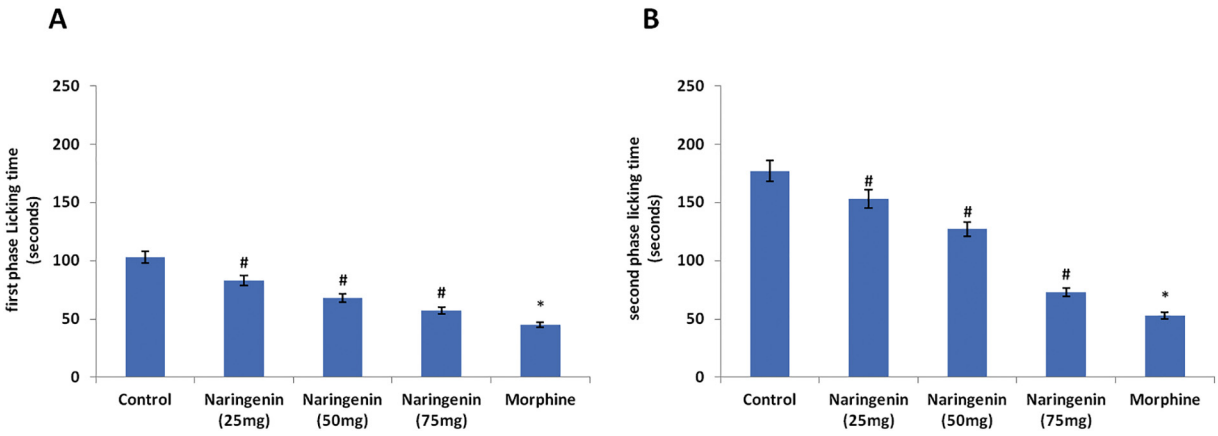

Fig. 4. Antinociceptive effect of Naringenin and morphine in biphasic formalin induced nociception mice model.
Each bar represents mean ± SEM of 6 animals, #, *represents statistical significance between control Vs other groups at $p < 0.05$, $p < 0.01$ level respectively using Dunnett's test.

Naringenin have significantly inhibited the inflammatory reactions induced by carrageenan and protected mice from inflammation.

3.2.2. Peritoneal cavity leukocyte infiltration test

The number of total leukocytes, mononuclear and polymorphonuclear infiltrated cells in the peritoneal cavity of carageenan alone treated, Naringenin (25, 50, 75 mg) and morphine pretreated mice were assessed and represented in the Fig. 5. Compared to carageenan alone treated, the mice pretreated with Naringenin and morphine showed decreased number of leukocyte infiltration. The minimal infiltration of leukocytes were seen in the morphine pretreated mice, 75 mg Naringenin treated mice also showed minimal infiltration of leukocytes which are comparable to the standard drug morphine.

3.2.3. Effect of Naringenin on proinflammatory cytokines

The proinflammatory cytokines TNF-α (Fig. 6A), IL-1β (Fig. 6B), IL-6 (Fig. 6C) were estimated in the air pouch induced by carageenan in carageenan alone, Naringenin and dexamethasone pre-treated mice. Compared to TNF-α and IL-1β, the IL-6 levels were significantly increased in carageenan alone treated mice. All three concentrations of Naringenin and dexamethasone drastically inhibited the concentration of TNF-α (Fig. 6A).

3.2.4. Open field test

The sedative effect of Naringenin was estimated by assessing the behavior of mice in open field apparatus. Compared to control, the Naringenin 25 and 50 mg treated mice don't show any significant changes whereas the number of squares crossed by the Naringenin 75 mg treated mice and morphine treated mice were decreased (Fig. 7).

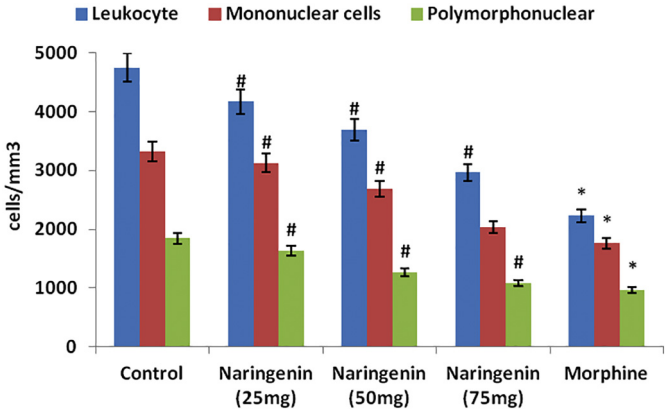

Fig. 5. Anti-inflammatory effect of Naringenin and morphine against peritoneal leukocyte infiltration in carageenan induced inflammatory mice model.
Each bar represents mean ± SEM of 6 animals, #, *represents statistical significance between control Vs other groups at $p < 0.05$, $p < 0.01$ level respectively using Dunnett's test.

4. Discussion

In the present study we analyzed the dose dependent antinociceptive and anti-inflammatory effect of Naringenin using different in vivo models. The effect of Naringenin was compared with the standard drugs and the reversal effect was also estimated by treating it with opiate inhibitor naloxone. The anti-inflammatory action of Naringenin was confirmed by estimating the peritoneal leukocyte infiltration and by assessing the levels of proinflammatory cytokines.

Nociception is the reflex response produced by the organism to an external stimulus. The nociceptive tests were performed to assess the

Table 3
Anti-inflammatory effect of Naringenin and indomethacin in carageenan induced inflammatory mice model.

Treatment (mg/kg)	Response time(s) (%MPE)				
	Basal	1st h	2nd h	3rd h	4th h
Control	25.65 ± 3.12	147.37 ± 13.96	134.86 ± 9.63	128.72 ± 7.27	119.41 ± 5.46
Naringenin (25 mg)	28.53 ± 9.41	96.72 ± 7.24 (47.26%)	94.06 ± 5.77 (36.04%)#	91.86 ± 4.43 (29.31%)*	83.52 ± 6.41 (19.03%)*
Naringenin (50 mg)	26.43 ± 4.86	93.48 ± 7.32 (43.64%)	90.72 ± 9.53 (41.72%)#	82.14 ± 5.33 (36.33%)*	79.65 ± 6.37 (33.79%)*
Naringenin (75 mg)	29.32 ± 3.21	90.21 ± 5.34 (36.77%)	77.26 ± 4.72 (34.96%)#	65.43 ± 7.67 (31.87%)*	61.68 ± 4.15 (32.93%)*
Indomethacin (10 mg)	25.53 ± 4.72	77.26 ± 7.33 (42.11%)	75.92 ± 6.23 (40.76%)#	68.33 ± 8.82 (38.62%)*	62.73 ± 5.33 (33.48%)*

Each bar represents mean ± SEM of 6 animals, #, *represents statistical significance between control Vs other groups at $p < 0.05$, $p < 0.01$ level respectively using Dunnett's test.

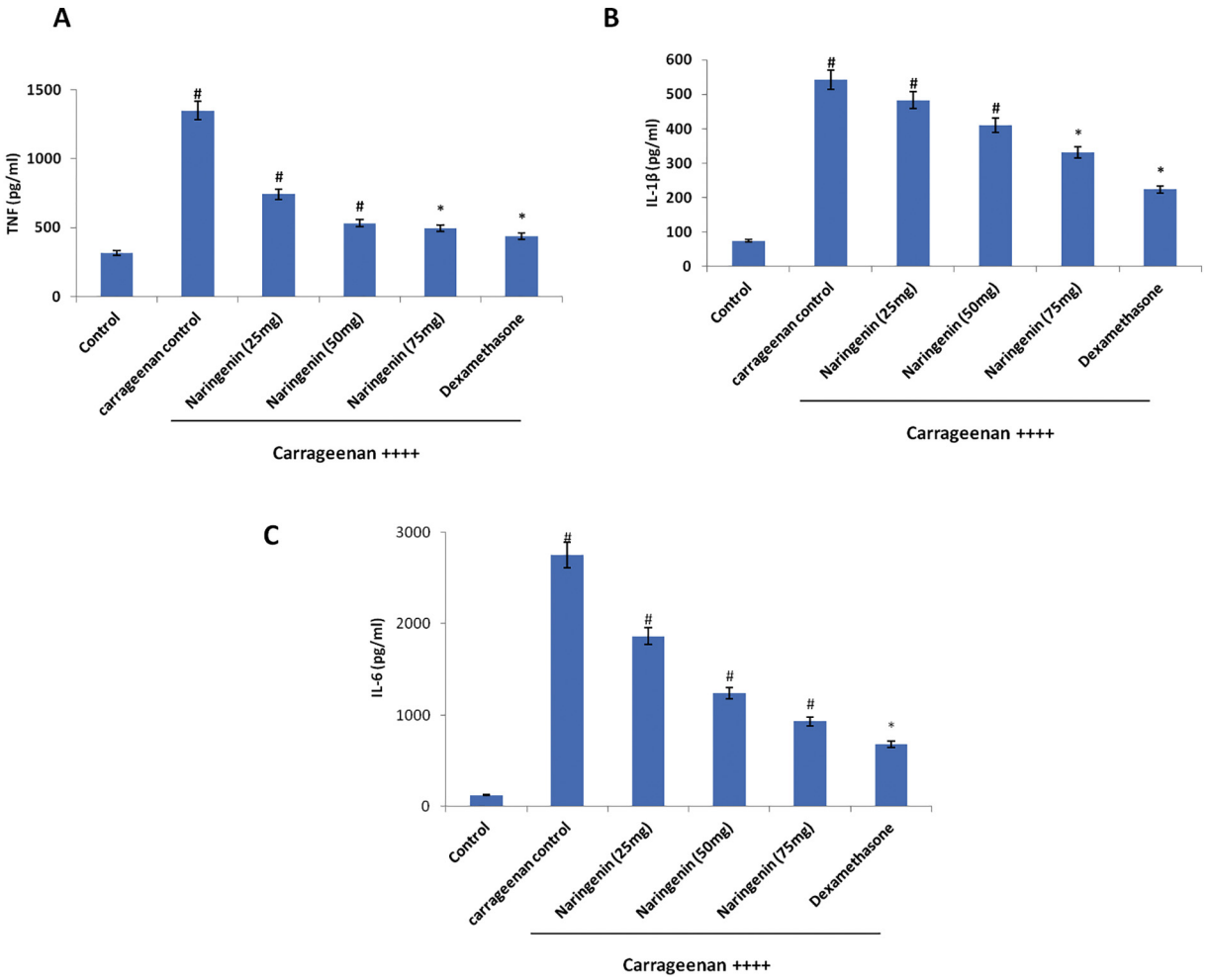

Fig. 6. Anti-inflammatory effect of Naringenin and morphine against proinflammatory cytokines in air pouch mice model.
Each bar represents mean ± SEM of 6 animals, #, *represents statistical significance between control Vs other groups at $p < 0.05$, $p < 0.01$ level respectively using Dunnett's test.

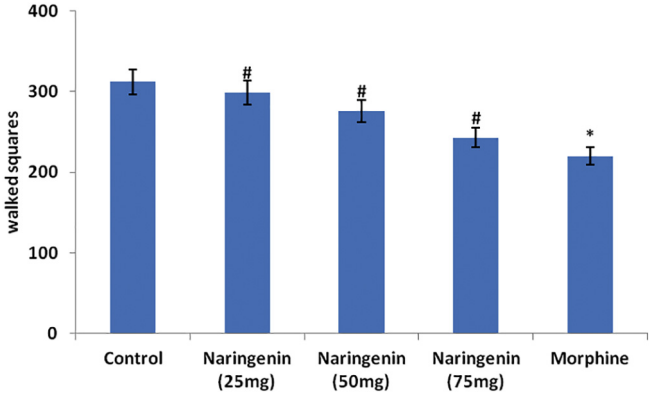

Fig. 7. Effect of Naringenin and morphine on behavior of mice in open field.
Each bar represents mean ± SEM of 6 animals, #, *represents statistical significance between control Vs other groups at $p < 0.05$, $p < 0.01$ level respectively using Dunnett's test.

therapeutic potential of drug to reduce pain. These tests were performed by using thermal, mechanical or electrical stimulus [22]. The antinociceptive effect of Naringenin against the thermal stimuli induced nociception was assessed by performing hot plate and tail immersion tests, which are the commonly used test to detect the analgesic property of drug [23,24]. Naringenin at the dosage of 75 mg/kg body weight significantly delayed the response of mice to the external stimuli both in hot plate and tail immersion test which is comparably equal to the response induced by the standard drug morphine. This may be due to neuromodulatory effect of Naringenin on the spinal and supraspinal reflexes which are triggered by the opioid receptors [23,25]. It was further confirmed by treating the Naringenin pretreated mice with naloxone, an opioid receptor antagonist. Compared to control the reversal effect of naloxone against Naringenin was significantly lower which confirm Naringenin potentially modulates the spinal and supraspinal reflex thereby exerts antinociceptive property.

The sensitization of nociceptors can be assessed by the induction of irritants like acetic acid. These irritant induces writhes which causes abdomen retraction and hind limb stretching in an episodic manner. The acetic acid induced writhing test was performed to detect the effect of analgesic drug to inhibit the signals transmitted to central nervous system by prostaglandins which increases the sensitivity of nociceptors [26]. In the current study there is decrease in number of writhes performed by mice treated with different concentration of Naringenin which states that Naringenin would have inhibits the production of prostaglandins thereby inhibition the sensitization of nociceptors.

The nociceptive effect of Naringenin against the excitatory amino induced pain was assessed by glutamate induced nociception in mice. The two major amino acids which modulate the pain perception are excitatory amino acids glutamate and aspartate. Glutamate carries out the reaction via two types of receptors in peripheral and spinal nervous system i.e. *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor

[27]. It also triggers the peripheral neurons by releasing proinflammatory cytokines. In present study Naringenin pretreated mice the number of licks which is the indicator of glutamate induced pain was reduced which signifies that Naringenin would have inhibited the activation of both NMDA and non NMDA receptors thereby suppressing the pain induction.

To identify the effect of Naringenin against no evoked pain behaviors like biting, flinching, capsaicin induced mice model was performed. This test is done to identify the inhibitory effect of antinociceptive drug against a chemogenic pain [28]. Naringenin pretreated exhibited significantly decreased licking performance when treated with capsaicin which confirms that Naringenin inhibited the inflammatory mediators there by suppressed the pain. The biphasic release of prostaglandins E2 induces nociceptive in formalin nociception mice model [29], and the induction of pain are triggered by the proinflammatory cytokines on the afferent sensory neurons. In the present study Naringenin significantly decreased the number formalin induced licks in both neurologic phase and inflammatory phase of the study which implies the potency of Naringenin as an antinociceptive drug

Naringenin inhibited the carageenan induced leukocyte infiltration in the peritoneal cavity. The increase in leukocyte generation may be due to the Myeloperoxidase activity in the paw edema which caused by the ROS generated by carrageenan. Naringenin would have scavenged the ROS via its antioxidant property which would inhibit the increase in leukocyte infiltration rate. Tumor necrosis factor α and the interleukin 1β produced by the macrophages are the key molecules which mediates inflammatory reactions [30]. A spinal glial cell secretes potent pro inflammatory cytokines like TNF-α, IL-1β and IL-6 which sensitizes the pain receptors [31,32]. In the current study the anti-inflammatory effect of Naringenin was confirmed by analyzing its effect in air pouch model test. The levels of proinflammatory cytokines in Naringenin pretreated mice were decreased compared to carageenan control treated mice. Reports suggest that Naringenin is an agonist of aryl hydrocarbon receptor and activates an Nrf2 transcription factor which inhibits the production of reactive oxygen species and inflammatory cytokines [33], which are involved in sensitization of nociceptors. Hence inhibition of ROS and inflammatory cytokine production by Naringenin may be the reason for its antinociceptive property.

Open field test was performed to assess the behavioral changes induced by Naringenin in mice. The results form open field test just that the Naringenin treated mice showed better performance than the morphine treated mice suggesting that it is potent antinociceptive drug with less side effects.

5. Conclusion

The overall results from different nociceptive and inflammatory mice models suggest that Naringenin is a potent antinociceptive and anti-inflammatory drug. The inhibition of proinflammatory cytokines and the explorative behavior exhibited by Naringenin treated mice confirms that the Naringenin is potent anti-inflammatory drug with no side effects.

Conflict of interest

We declare that no conflict of interest.

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