

## Determination of Anti-Inflammatory and Analgesic Activities of a Citrus Bioflavanoid, Hesperidin in Mice

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### Abstract

Inflammation is one of the important responses elicited by organisms to counteract obnoxious stimuli. However, continuous inflammation has been responsible for the induction of several diseases. Therefore, it is essential to combat excess inflammation by devising countermeasures to neutralize excess inflammation. The present study was undertaken to investigate the analgesic and anti-inflammatory activities of hesperidin, a citrus flavonoid in mice using standard procedures employed for these activities including hotplate, acetic acid, tail immersion, xylene and formalin-induced edema tests. Treatment of mice with different doses of hesperidin revealed that hesperidin induced analgesic and anti-inflammatory activities in a dose dependent manner as indicated by pain inhibition and reduced inflammation. The maximum effect was observed for 300 mg/kg b. wt. hesperidin. Our study demonstrates that hesperidin has analgesic as well as anti-inflammatory action.

**Keywords:** Mice; Hesperidin; Analgesic; Anti-inflammatory

### Introduction

Inflammation is a complex biological response of body tissues, which is elicited against the harmful stimuli, pathogenic attacks, and irritants. The inflammation is characterized by redness, warmth, swelling and pain [1,2]. The sustained inflammation causes rheumatoid arthritis, atherosclerosis, hay fever, ischemic heart diseases and other disorders [3-5]. Inflammation is a common manifestation of infectious diseases including leprosy, tuberculosis, syphilis, asthma, inflammatory bowel disease, nephritis, vascularitis, celiac diseases, and numerous auto-immune diseases [6]. Inflammation is known for a generic response, and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen [7]. The non-steroidal anti-inflammatory drugs (NSAIDs) employed in the treatment of inflammation are one of the most widely used drugs throughout the globe. However, the use of NSAIDs as anti-inflammatory agents is limited due to induction of undesirable side effects on gastric mucosa, kidney, bronchus and cardiovascular system [8,9]. The NSAIDs are mainly used to alleviate the inflammation related swelling and pain and their persistent use are accompanied by the risk of gastrointestinal, cardiovascular and other toxicities.

Hesperidin is a naturally occurring flavonoid present in citrus fruits and was first discovered by Lebreton in 1827 [10]. Hesperidin occurs in all parts of plants including fruit, vegetables, nuts, seeds, leaves, flowers and bark. It is an abundant and inexpensive byproduct of citrus family [11,12]. Hesperidin is also present in plants belonging to family *Fabaceae* apart from the plants belonging to citrus family including *Betulaceae* and *Lamiaceae* [13-15]. The citrus peel flavonoids were found effective in preventing capillary bleeding associated with scurvy as early as 1938 [16], since then hesperidin has undergone several investigations. Hesperidin has been found to protect against inflammation, oxidative stress, hypotension, nitric oxide synthase inhibition, apoptosis and infection [17-21]. Hesperidin has been reported to protect against neurotoxicity by normalizing oxidative stress and inflammation [22,23]. It also acts as an anti-hypercholesterolemic and anticarcinogenic agent [24-27]. Hesperidin has been found to be useful in inflammatory bowel disease and it has been reported to be antiarthritic, antiatherogenic and also to protect against platelet and erythrocyte aggregation [28-32]. Subchronic

administration of Hesperidin for 13 weeks has been reported to be non-toxic up to 5% in mice receiving [33]. The humans can orally tolerate as high as less than 150 g of hesperidin. However, reports regarding the anti-inflammatory activity of hesperidin in animal model are scanty. Therefore, the present study was carried out to obtain an insight into the anti-inflammatory and analgesic efficacy of the hesperidin in mice treated with different doses of hesperidin.

### Materials and Methods

#### Chemicals

Hesperidin PG 95% was purchased from Himedia Laboratories, Mumbai, India, whereas diclofenac sodium was procured from NEON Laboratory Ltd, Mumbai, India. The acetic acid, formaldehyde, Xylene and other routine chemicals were supplied by Merck India Ltd., Mumbai, India.

#### Animal care and handling

The animal care and handling were carried out according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Usually, 6 to 8 weeks old healthy male Swiss albino mice weighing 30-35 g were culled from an inbred colony maintained under the controlled conditions of temperature ( $25 \pm 2^\circ\text{C}$ ) and humidity (55-60%) and 12 hours of light and dark cycle, respectively. The animals were housed in a sterile polypropylene cage containing wood powder (procured locally) as bedding material. The animals had free access to standard rodent diet and water. All animal experiments were carried out according to NIH, USA and Indian National Science Academy, New

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Delhi, India guidelines, after getting the approval of the Institutional Ethics Committee of the Mizoram University, Aizawl, Mizoram, India.

### Preparation of drug and mode of administration

Hesperidin was weighed and dissolved in distilled water, henceforth it will be called as HPD. HPD was administered orally using an oral gavage (Popper and Sons, New Hyde Park, USA).

### Experimental

The anti-inflammatory and analgesic activities were determined by dividing the animals into the following groups:

#### Saline group

The animals of this group did not receive any treatment except sterile physiological saline (SPS).

#### Diclofenac group

The animals of this group were injected with 20 mg/kg b. wt. of diclofenac sodium (DIF) intraperitoneally.

#### Hesperidin group

The animals of this group were administered with 100, 200, 300 and 400 mg/kg b. wt. hesperidin. The analgesic and anti-inflammatory activities were determined 30 minutes after the administration of SPS or diclofenac (DIF) or hesperidin (HPD) as the case may be.

#### Analgesic activity

The analgesic activity of hesperidin was determined by carrying out the following tests:

**Hot-plate test:** The hot plate test was carried out as described earlier [34] where the grouping and other conditions were essential similar to that described above. The hotplate contained metallic surface (diameter 20 and 10 cm high) and its temperature was set at 55°C. Briefly, each mouse was placed onto the hotplate and covered with a glass beaker to avoid heat loss. Each mouse also acted as its own control. The time taken to lick the fore paws or jump was recorded. The latency is defined as the reaction time taken by each mouse to respond to licking of the fore paws or jumping. Untreated animals exhibited a latency of 5-20 seconds. Thirty minutes after administration, the latency period/reaction time for all groups was recorded. Usually 10 mice were used for each group.

The pain inhibition (%) was calculated as follows:

$$\frac{\text{Post treatment latency (s)} - \text{Pre treatment latency (s)}}{\text{Pre treatment latency (s)}} \times 100$$

**Acetic acid induced writhing test:** A separate experiment was conducted to evaluate the analgesic activity by the acetic acid-induced writhing test as described earlier [35]. The grouping and other conditions were essentially similar to that described in the experimental section. The mice of all groups were administered intraperitoneally with 0.7% v/v acetic acid (volume of acetic acid did not exceed 10 µl/g b. wt.). Immediately after acetic acid administration, the mice were individually placed into glass beakers and the number of writhes induced in these animals was counted up to 30 min after five minutes of acetic acid administration. The stretching of the abdomen with simultaneous stretching of at least one hind limb was scored as a writhe. Usually 10 mice were used for each group.

Inhibition of writhing (%) was calculated as:

$$\frac{\text{Control} - \text{Treated}}{\text{Control}} \times 100$$

**Tail-immersion test:** A separate experiment was performed to evaluate the analgesic activity of HPD by the tail immersion test. The grouping and other conditions were similar to that described above in experimental section. The tail-immersion test was carried out in a hot water bath set at a temperature of 55 ± 0.5°C, where 3 cm of animal tail was immersed into the hot water and tail withdrawal reaction was recorded as time in seconds in all groups using a digital stopwatch at 0, 0.5, 1, 2, 3, 4 and 6 hours after administration of HPD or DIF. A minimum of three observations were made for each animal in control group, immediately and 10 min after the initial reading. Usually 10 mice were used for each group.

#### Anti-inflammatory activity

The anti-inflammatory activity of hesperidin was investigated by undertaking the following tests:

**Xylene-induced ear edema:** A separate experiment was conducted to evaluate the anti-inflammatory activity of hesperidin by xylene-induced ear edema, where the grouping and other conditions were essentially similar to that described above in the experimental section. Mice were divided into three groups of 10 each. Thirty minutes after administration of SPS, HPD or DIF, the ear edema was produced by applying 0.03 ml of xylene on the inner surface of the right ear, whereas untreated left ear served as control. Fifteen minutes after the application of xylene, the mice were killed under ketamine anesthesia. Circular sections of both the ears were taken, using a cork borer with a diameter of 6 mm and weighed.

$$\text{Inhibition (\%)} = \frac{\text{Difference in ear weight (control)} - \text{Difference in ear weight (test)}}{\text{Difference in ear weight (control)}} \times 100$$

Difference in ear weight (control)

**Formalin induced inflammation:** The anti-inflammatory activity of hesperidin was investigated by formalin induced inflammation in a separate experiment, where the grouping and other conditions were similar to that described above in experimental section. The assessment of anti-inflammatory activity using the formalin induced inflammation was carried out as described earlier [36]. The inflammation was produced by subaponeurotic administration of 0.1 ml 2% formaldehyde in the right hind paw of the mice on the first and third day. The animals were intraperitoneally administered daily with the HPD or DIF for 10 days. Alteration in paw size was estimated daily by wrapping a piece of cotton thread around the paw and measuring the circumference with a meter scale. Ten mice were utilized for each group.

#### Statistical analysis

Student's *t*-test was used for analysis of statistical significance using statistical software Origin Pro 8 SRO v8.0724 (B724), Northampton, MA, USA. A *p* value of ≤ 0.05 was considered to statistically significant.

#### Results

The results of analgesic and anti-inflammatory activities of hesperidin have been expressed as mean ± SEM (Tables 1-5; Figures 1-4).

#### Acute toxicity

The hesperidin has been found to be non-toxic in mice up to 2

g/kg. b. wt. orally and dissolution of higher doses precluded further experimentation (data not shown).

### Analgesic activity

**Hot-plate test:** The results of analgesic activity, which was assessed using the hot plate method, are presented in Table 1. The mice treated with different doses of hesperidin showed a significant analgesic activity at all doses (Figure 1). However, the maximum analgesic effect was observed at a dose of 300 mg/kg b. wt. hesperidin, which showed a maximum pain inhibition of 62.22% as compared to other doses i.e., 100 (25.54%), 200 (34.2%), and 400 (61.85%) mg/kg b. wt. The diclofenac (positive control) treated animals exhibited a pain inhibition of 76.31% at a dose of 20 mg/kg b. wt. (Figure 1).

**Acetic acid induce writhing test:** The analgesic effect of hesperidin was further studied by acetic acid induced writhing test and the data are shown in Figure 2. The administration of acetic acid in control mice produced  $66.2 \pm 1.16$  writhes, whereas pretreatment of mice with 100, 200, 300 and 400 mg/kg b. wt. of hesperidin reduced the number of writhes in a dose dependent manner i.e.,  $50.6 \pm 1.45$  (23.56%),  $45.3 \pm 1.87$  (31.62%),  $24.52 \pm 0.16$  (62.86%), and  $26 \pm 1.38$  (60.67%) for 100, 200, 300 and 400 mg/kg HPD, respectively (Figure 2) when compared with the saline treated controls. The standard anti-inflammatory drug, 20 mg/kg b. wt. diclofenac reduced the number of writhes to  $10.8 \pm 0.74$  (83.68%) (Table 2).

**Tail immersion test:** The analgesic activity was also estimated by tail immersion test and the results are depicted in Table 3. This test revealed that hesperidin as well as the positive controls exhibited a significant analgesic activity as compared to the negative saline control. However, diclofenac treatment was superior to the hesperidin treatment. The 300 mg/kg b. wt. hesperidin post treatment and diclofenac showed 56.98% and 72.89% inhibition, respectively.

### Anti-Inflammatory activity

**Xylene induced ear edema:** The saline treated control mice showed  $13.98 \pm 0.60$  mg increase in ear weight when compared to untreated ear indicating that xylene induced inflammatory changes (Figure 3). Treatment of mice with 100, 200, 300 and 400 mg/kg b. wt. HPD inhibited the induction of ear edema by 26.03% ( $10.34 \pm 1.05$  mg), 34.54% ( $9.15 \pm 1.09$  mg), 47.21% ( $7.38 \pm 1.2$  mg) and 43.63% ( $7.88 \pm 0.63$  mg), respectively, whereas the positive control diclofenac treatment at a dose of 20 mg/kg b. wt. inhibited the development of edema by 52.7182% ( $6.61 \pm 0.49$  mg) which was greater when compared to all the doses of the hesperidin tested (Table 4).

**Formalin induced inflammation:** The anti-inflammatory activity of hesperidin was further confirmed by formalin induced inflammation in the mouse paw. The treatment of mice with formalin induced inflammation in the mouse paw as evidenced by increased paw diameter (Figure 4). However, treatment of mice with 100, 200, 300 and 400 mg/kg b. wt. hesperidin significantly reduced the paw diameters (Table 5). Diclofenac treatment also reduced the formalin-induced paw diameter significantly (Table 5).

### Discussion

Inflammation is a natural defense process, which is evoked by a cascade of events in response to disturbances caused by agents that are unwelcome by the body and its principal role is to neutralize the cause of disturbance, remove damaged cells/tissues and restore normal state [37,38]. Despite this fact, persistence of inflammation is a great cause of concern as it leads into the development of numerous human diseases

including asthma, cardiovascular diseases, allergy, type 2 diabetes, autoimmunity, atherosclerosis, Alzheimer disease, obesity, rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, cancer, certain psychiatric disorders and many more [39-42]. Although many anti-inflammatory agents are in vogue, their constant use is not without harmful side effects implying that a continuous search is needed to screen newer and safer agents, which can reduce inflammation without any side effect or minimum side effects. Therefore, the present study was designed to evaluate the anti-inflammatory and analgesic activities of a citrus bioflavanoid, hesperidin in mice.

The classical methods of hot plate and tail immersion techniques are suitable to evaluate the analgesic activity of any substance that acts on the central nervous system [43]. The pain is regarded as unpleasant sensory, emotional and cognitive experience elicited by nociceptors against pain-inducing physical or chemical stimuli [44]. The management of chronic pain has been a constant problem in humans as it has a deleterious impact on the sufferers [45]. Pain can be alleviated by administering analgesic drugs, which will interrupt nociceptor pathways. The opioid and non-steroidal anti-inflammatory drugs have been used for the clinical management of pain in humans since a long time, however, their constant use make them ineffective and moreover, they have been reported to induce adverse effects [46]. The evaluation of analgesic effect of hesperidin by different methods revealed that it possessed analgesic activity at a dose of 300 and 400 mg/kg b. wt. in the analgesic animal model used to evaluate centrally acting analgesic drugs. The hot plate test allows precise determination of the analgesic activity of drugs that act on the central nervous system. The analgesic activity of hesperidin was also tested by the acetic acid-induced abdominal constriction method, where the pain is indirectly initiated via endogenous mediators like prostaglandins, which stimulates peripheral nociceptive neurons. The hesperidin inhibited both the heat and acetic acid-induced pain in the animals indicating

Treatment	Dose (mg/kg b. wt.)	Mean $\pm$ SEM		Inhibition (%)
		Pre-treatment Reaction-latency (s)	Post-treatment reaction-latency (s)	
Control (SPS)	0	7.60 $\pm$ 0.58	7.60 $\pm$ 0.45	00.00
Diclofenac	20	7.60 $\pm$ 0.55	13.40 $\pm$ 0.84	76.31
Hesperidin	100	8.00 $\pm$ 0.32	10.05 $\pm$ 1.57	25.54
	200	8.52 $\pm$ 0.65	11.45 $\pm$ 1.89	34.2
	300	6.50 $\pm$ 0.55	14.60 $\pm$ 1.35	62.22
	400	9.00 $\pm$ 0.32	14.55 $\pm$ 1.31	61.85

N=10

**Table 1:** Effect of hesperidin on the analgesic activity in mice by hot plate test.

Treatment	Dose (mg/kg b. wt.)	Writhing No. (Mean $\pm$ SEM)	Writhing Inhibition (%)
Control (SPS)	0	66.2 $\pm$ 1.16	0.000
Diclofenac	20	10.8 $\pm$ 0.74	83.68
Hesperidin	100	50.6 $\pm$ 1.45	23.56
	200	45.3 $\pm$ 1.87	31.62
	300	24.52 $\pm$ 0.16	62.86
	400	26 $\pm$ 1.38	60.67

N=10

**Table 2:** Alteration in the analgesic activity by acetic acid induced writhing in mice treated with different doses of hesperidin.

Treatment	Dose (mg/kg b. wt.)	Response time in seconds (Mean ± SEM)					
		Assessment time (h)					
		0	0.5	1	2	4	6
Control (SPS)	0	4.84 ± 0.19	4.86 ± 0.22 (0.5)	4.89 ± 0.18 (1.07)	4.96 ± 0.18 (2.44)	4.91 ± 0.19 (1.32)	4.82 ± 0.20 (-0.54)
Diclofenac	20	4.28 ± 0.04	5.38 ± 0.07 (25.7)	6.69 ± 0.67 (56.3)	7.40 ± 0.24 (72.89)	6.82 ± 0.19 (59.34)	5.50 ± 0.31 (28.5)
Hesperidin	100	4.85 ± 0.29	4.88 ± 0.22 (0.62)	4.94 ± 0.18 (1.85)	4.98 ± 0.18 (2.68)	4.99 ± 0.19 (3.92)	5.05 ± 0.20 (3.54)
	200	5.01 ± 0.15	5.18 ± 0.30 (3.39)	5.40 ± 0.33 (7.78)	5.68 ± 0.37 (13.37)	5.75 ± 0.29 (14.77)	5.38 ± 0.39 (7.38)
	300	5.30 ± 0.29	6.01 ± 0.33 (13.39)	6.25 ± 0.82 (17.92)	8.32 ± 0.48 (56.98)	6.58 ± 0.08 (24.15)	6.26 ± 0.35 (18.11)
	400	5.32 ± 0.29	6.00 ± 0.33 (12.78)	6.24 ± 0.82 (17.29)	7.25 ± 0.48 (36.27)	6.37 ± 0.08 (19.73)	6.19 ± 0.35 (16.35)

Inhibition (%) is shown in brackets

**Table 3:** Alteration in the response time in mice treated with hesperidin before tail immersion test.

Treatment	Dose (mg/kg b. wt.)	Ear weight (mg) Mean ± SEM	Inhibition (%)
Control (SPS)	0	13.98 ± 0.60	0.000
Diclofenac	20	6.61 ± 0.49	52.71
Hesperidin	100	10.34 ± 1.05	26.03
	200	9.15 ± 1.09	34.54
	300	7.38 ± 1.2	47.21
	400	7.88 ± 0.63	43.63

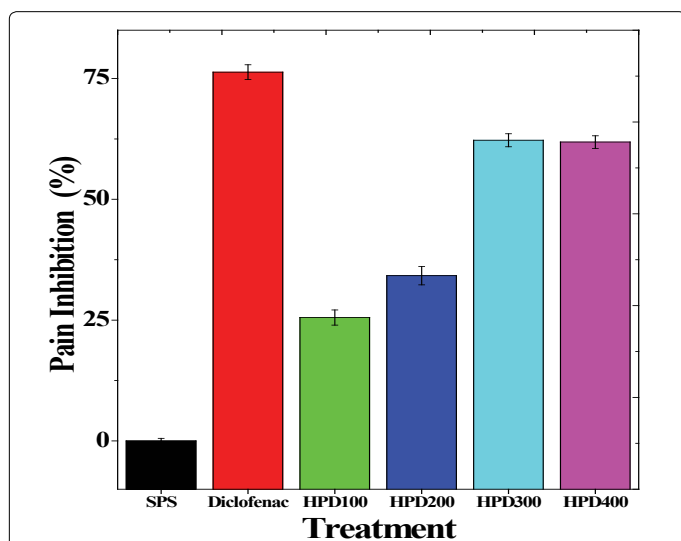
N=10

**Table 4:** Effect of hesperidin on xylene induced ear edema in mice.

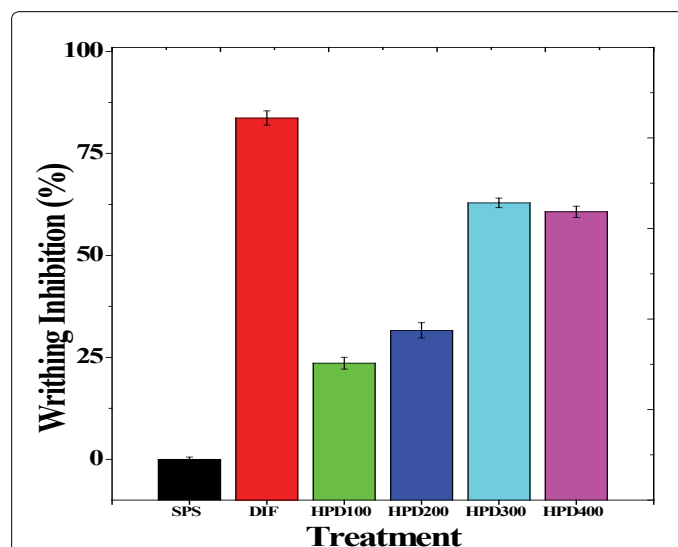
Treatment	Dose (mg/kg b. wt.)	Paw diameter in mm (Mean ± SEM)					
		Assessment time (day)					
		1	2	4	6	8	10
Control (SPS)	0	1.50 ± 0.02	1.39 ± 0.02	1.52 ± 0.033	1.46 ± 0.02	1.36 ± 0.02	1.32 ± 0.02
Diclofenac	20	1.48 ± 0.02	1.28 ± 0.02	1.42 ± 0.024	1.16 ± 0.04	0.88 ± 0.03	0.64 ± 0.04
Hesperidin	100	1.42 ± 0.02	1.38 ± 0.01	1.48 ± 0.012	1.41 ± 0.02	1.22 ± 0.02	1.18 ± 0.02
	200	1.46 ± 0.03	1.35 ± 0.03	1.48 ± 0.015	1.33 ± 0.03	1.18 ± 0.05	1.12 ± 0.05
	300	1.47 ± 0.03	1.37 ± 0.03	1.43 ± 0.036	1.23 ± 0.02	1.02 ± 0.08	0.92 ± 0.03
	400	1.45 ± 0.03	1.39 ± 0.04	1.44 ± 0.015	1.28 ± 0.03	1.15 ± 0.05	1.10 ± 0.06

N=10.

**Table 5:** Effect of hesperidin on xylene induced paw edema in mice.



**Figure 1:** Effect of hesperidin on the heat-induced pain (analgesic activity) in mice.



**Figure 2:** Alteration in the analgesic activity by acetic acid induced writhing in mice treated with different doses of hesperidin.



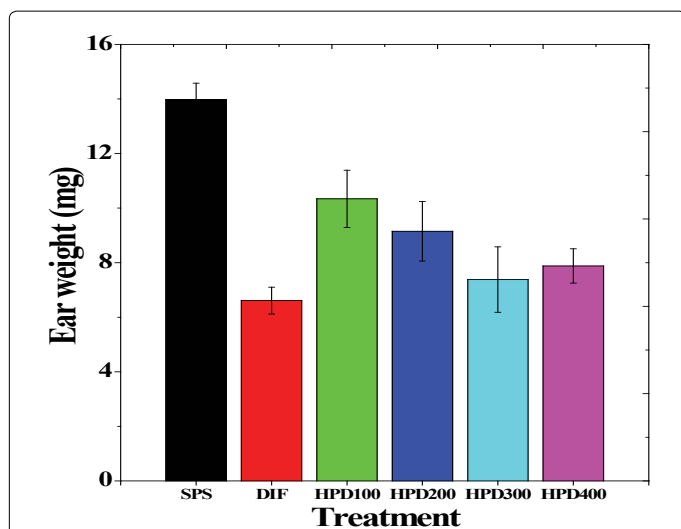


Figure 3: Alteration in the xylene induced ear edema in mice by hesperidin.

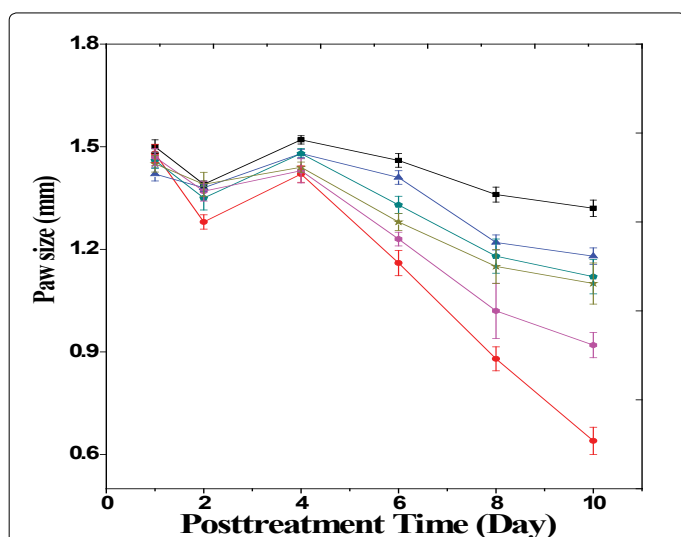


Figure 4: Effect of hesperidin on formalin induced paw edema in mice. Squares: SPS (saline); Triangle: Hesperidin 100 mg; pentagon: Hesperidin 200 mg; Diamonds: Hesperidin 300 mg Stars: Hesperidin 400 mg and Circles: Diclofenac.

that it possessed analgesic activity. A similar effect has been observed for quercetin earlier [47]. The neuronal fibers respond equally to both narcotics and non-steroidal anti-inflammatory drugs [48].

Inflammation is all pervasive phenomenon and its main function is to neutralize the cause, destroy the source, repair the damaged tissue and regain the homeostatic state of the tissues [37,38]. It may or may not be associated with pain depending on the stimuli of inflammation. A neurogenic and inflammatory pain model, the formalin-induced paw edema test, was used to assess further the antinociceptive properties of hesperidin. Formalin administration evokes behavioral effects, which are related to the direct chemical stimulation of nociceptors. The pain induced by inflammatory phase involves a combination of stimuli, including inflammation of peripheral tissues and mechanisms of central sensitization [49,50]. The drugs that act on central nervous system including opioids, inhibit both phases equally, however drugs that interact with peripheral nervous system such

as NSAIDs and corticosteroids, only inhibit the second phase [49]. Hesperidin seems to be effective on both the central and peripheral nervous systems as it is able to desensitize neurons of both central and peripheral nervous systems equally as indicated by the attenuation of pain and inflammation. Hesperidin has been reported to exert anti-inflammatory effect in rat and *Aeromonas hydrophila* earlier [51,52]. The anti-inflammatory effect of hesperidin was further confirmed by Xylene, induced mouse ear edema, which causes serious edematous changes in the skin when applied to the ear surface [53]. The ear edema model induced by xylene has certain advantages in the evaluation of anti-inflammatory activity of steroids as well as non-steroidal anti-inflammatory agents [54,55]. The reduction in ear edema by hesperidin indicates that it has anti-inflammatory potential.

The direct comparison of the results obtained for analgesic and anti-inflammatory activities of diclofenac in the present study with that of other studies may not be feasible due to differences in experimental protocol, dosage and animal species used. However, we have observed analgesic and anti-inflammatory activities in the present study. Similarly, diclofenac has been reported to possess analgesic and anti-inflammatory activities earlier and it has been used as a standard drug while evaluating the analgesic and anti-inflammatory activities of new natural products/drugs [35,56-58].

The exact mechanism of analgesic and anti-inflammatory action of hesperidin is not known. However, inflammation has been reported to be induced by secretion of proinflammatory cytokines like TNF- $\alpha$ , IL1 $\beta$  [59]. It seems that hesperidin has been able to inhibit the secretion of proinflammatory cytokines including TNF- $\alpha$ , IL-6 and IL1 $\beta$  leading to alleviation in the inflammatory response. In fact hesperidin treatment has been reported to bring the 2,3,7,8-tetrachlorodibenzo- $\rho$ -dioxin-induced TNF- $\alpha$ , and IL1 $\beta$  levels to normal in rats [59]. Cyclooxygenases (COX-1 and COX-2) are involved in inflammation and hesperidin has been reported to suppress the expression of COX-II gene earlier [60]. The inhibition of COX-II gene by hesperidin may have blocked the production of prostaglandins leading to the suppression of inflammatory response in the present study. The NF- $\kappa$ B arouses proinflammatory pathway by stimulating the expression of various inflammatory cytokines, chemokines and adhesion molecules [61]. Therefore, inhibition of NF- $\kappa$ B by hesperidin may have blocked the NF- $\kappa$ B-induced inflammatory pathway and subsequently invoked the analgesic and anti-inflammatory action. Hesperidin has been reported to suppress the transcription of NF- $\kappa$ B earlier [62].

## Conclusions

Our study demonstrates that hesperidin has been able to exert analgesic and anti-inflammatory actions in mice by reducing pain and inflammatory changes, which may be due the inhibition of inflammatory cytokines like TNF- $\alpha$ , IL1 $\beta$  and IL-6 and also the suppression of transcription of NF- $\kappa$ B and COX-II genes that eventually blocked the production of prostaglandins and inflammatory pathway.

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