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Antinociceptive effect of certain dihydroxy flavones in mice

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ABSTRACT

Objective: This study was designed to evaluate the antinociceptive action of four dihydroxy flavone derivatives; 3,3′-dihydroxy flavone, 5,6-dihydroxy flavone, 3,7-dihydroxy flavone and 6,3′-dihydroxy flavone and to investigate the mechanisms involved.
Materials and methods: The antinociceptive effect of dihydroxy flavones was investigated in mice employing acetic acid induced abdominal constrictions, formalin-induced nociception, and hot plate assay procedures. The effects following pretreatment with naloxone, yohimbine, ondansetron, haloperidol, bicuculline and glibenclamide were also studied by acetic acid assay to reveal the involvement of opioid, adrenergic, tryptaminergic, dopaminergic, GABAergic or potassium channels respectively in the antinociceptive action of these compounds.
Results: Dihydroxy flavone derivatives significantly reduced the number of abdominal constrictions in acetic acid assay. The paw licking response time during both the early and late phases of formalin-induced nociception was reduced in a dose dependent manner by dihydroxy flavones treatment. A significant increase in reaction time was also evident in hot plate assay after dihydroxy flavones treatment.
The antinociceptive effect of dihydroxy flavones in the acetic acid assay was significantly attenuated by pretreatment with either naloxone or bicuculline. However, pretreatment of animals with yohimbine, ondansetron, haloperidol, or glibenclamide did not alter the response.
Conclusion: All the four investigated dihydroxy flavones produced dose related antinociception through mechanisms that involve an interaction with opioid and GABAergic pathways.

1. Introduction

Many interesting pharmacological actions have been identified for naturally occurring flavonoid compounds as well as synthetic flavone derivatives. Recent studies reveal potent antinociceptive and anti-inflammatory effects for many flavone derivatives like monohydroxy flavones, monomethoxy flavones and a few dihydroxy flavone compounds (Thirugnanasambantham et al., 1990, Muthiah et al., 1993, Arivudainambi et al., 1996, Girija et al., 2002, Umamaheswari et al., 2006). In the present study four new dihydroxy flavone derivatives, 3,3′-dihydroxy flavone, 5,6-dihydroxy flavone, 3,7-dihydroxy flavone and 6,3′-dihydroxy flavone have been investigated for their antinociceptive action in mice. A second objective of this study was to explore the possible mechanisms that may be involved in the antinociceptive action of dihydroxy flavones, by use of selective agents that interact at various antinociceptive pathways.

2. Materials and methods

2.1. Animals

Adult Swiss male albino mice weighing 25–30 g bred in the institutional animal house facility were used. The animals were housed in a controlled environment, with free access to food and water and were maintained on a 12 h/12 h, day/night cycle. Each animal was used only once. All the experiments were carried out between 0900 and 1300 h to avoid circadian variations and to maintain uniformity. The experiments were performed after the approval of the protocol by the Institutional Animal Ethical Committee. In all the experimental studies each group consisted of six animals.

2.2. Drugs and chemicals

The dihydroxy flavones used in the study are; 3,3′-dihydroxy flavone, 5,6-dihydroxy flavone 3,7-dihydroxy flavone and 6,3′-dihydroxy flavone (Research organics—Chennai) (Fig. 1). Dihydroxy flavones were prepared as a fine suspension in 1% carboxy methyl cellulose and injected s.c. in doses ranging from 3 to 100 mg/kg, 30 min prior to test procedures. Morphine sulphate (Pharma Chemico

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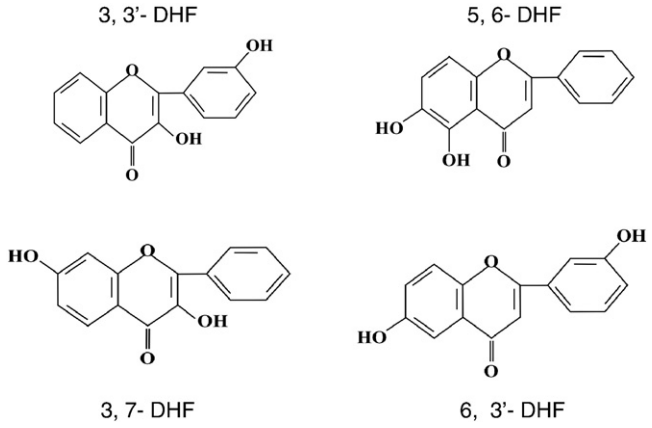

Fig. 1. Dihydroxy flavones used in the study.

Laboratories, India) 5 mg/kg or 10 mg/kg s.c. was used as a standard drug for comparison.

To carry out the antagonist assays the following chemicals were used: naloxone (Endo labs, USA); yohimbine hydrochloride (Sigma Chemical Co., USA); glibenclamide (Dr Reddy's laboratory, India); bicuculline (Sigma Chemical Co., USA); haloperidol (RPG Life sciences, India); ondansetron (Neomit Laboratories, India).

2.3. Acute toxicity study

Acute toxicity study was performed according to the Organization for Economic Cooperation and Development (OECD 423) guidelines (Ecobichon, 1997). Each test compound was administered in a dose of 2 g/kg by s.c. route to a group of three mice. The animals were continuously observed for changes in autonomic or behavioral responses for 6 h. The animals were kept under observation for 14 days to detect any mortality.

2.4. Rota-rod test

To evaluate the possible motor in-coordination, the mice were pretreated with maximum dosage of dihydroxy flavones (200 mg/kg) or vehicle, 30 min before being tested on the rotarod (Jurgensen et al., 2005). The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into four compartments. The bar rotated in a constant speed of 15 rpm and the ability of the animal to remain on the rotating rod was measured and compared with vehicle treated animals. The cut-off time used was 60 s.

2.5. Evaluation of loco motor activity

To assess the ambulatory behavior, the animals were tested in an open field apparatus as described by Rodrigues et al. (2002). The apparatus consisted of a wooden box, where the arena was divided into 16 equal squares, and the number of squares crossed with all paws was counted in a 5 min session. Mice were treated with dihydroxy flavones (200 mg/kg, S.C.) or vehicle 30 min beforehand.

2.6. Abdominal constrictions induced by acetic acid (Koster et al., 1959)

Acetic acid (0.6%, 10 ml/kg) was injected i.p. and the number of abdominal constrictions (writhings) during the following 15 min period was observed. A significant reduction in the number of abdominal constrictions by any treatment compared with vehicle treated animals was considered as an antinociceptive response. The percentage inhibition of writhings compared to vehicle treatment was calculated using the formula $(C-T/C)\times 100$, where C is the number of abdominal constrictions recorded in vehicle treated animals, and T is the number of abdominal constrictions in the treatment group.

2.7. Formalin assay

This was carried out as described by Tjolsen et al (1992). Each mouse was placed in an observation chamber 5 min before the injection to allow acclimatization to the new environment. Fifty microlitre of 1% formalin was administered s.c. into the plantar surface of the left hind paw and the time spent in licking and biting the injected paw was recorded every 5 min for a period of 30 min and considered as the quantitative indication of nociception. The early phase of nociceptive response normally peaks from 0 to 10 min and the late phase from 10 to 30 min after formalin injection.

2.8. The hot plate test

The hot plate test was carried out according to the method described by Eddy and Leimbach (1953). The hot plate was maintained at 55 ± 0.5 °C. Animals were placed on the hot plate and the time between placement and licking of the hind paws or jumping was recorded as the index of response latency. The reaction time was recorded 30 min after administration of various doses of dihydroxy flavones or morphine (5 mg/kg). A cut-off time of 30 s was maintained to minimize tissue damage.

2.9. Investigations on the mechanism of action

Further experiments were undertaken to elucidate the mechanisms by which dihydroxy flavones exerted their antinociceptive activity. A dose which produced nearly 50% inhibition of acetic acid induced nociception was selected for this purpose. (3,3′-dihydroxy flavone—100 mg/kg., 5,6-dihydroxy flavone—25 mg/kg., 3,7-dihydroxy flavone—25 mg/kg., and 6,3′-dihydroxy flavone—25 mg/kg).

In order to investigate the participation of the opioid system in the antinociceptive action of dihydroxy flavones, mice were pretreated with naloxone (5 mg/kg i.p., Rajendran et al., 2000) and after 15 min the animals received an injection of one of the dihydroxy flavones. The antinociceptive response was recorded 30 min after dihydroxy flavone treatment using acetic acid assay.

To assess the possible participation of the adrenergic system on the antinociceptive action of dihydroxy flavones, animals were pretreated with yohimbine (1 mg/kg i.p., Kaur et al., 2005), an α_2 adrenergic antagonist and after 15 min the animals received an injection of one of the dihydroxy flavones. Acetic acid challenge was made 30 min after the administration of dihydroxy flavones.

To examine the possible contribution of tryptaminergic or dopaminergic system, mice were pretreated with a 5-HT_3 antagonist ondansetron (0.5 mg/kg i.p., Pietrovski et al., 2006) or a dopaminergic antagonist haloperidol (1 mg/kg i.p., Naidu et al., 2003) 15 min prior to dihydroxy flavone treatments and were subjected to acetic acid test after 30 min.

Pretreatment with bicuculline, (1 mg/kg i.p., Filho et al., 2008) 15 min prior to dihydroxyflavone treatments was attempted to investigate the role of GABAergic pathway in the antinociceptive effect of these compounds.

Finally, to explore the role played by potassium channels in the antinociceptive effect caused by dihydroxy flavones, mice were pretreated with glibenclamide, (10 mg/kg i.p., Venkataramanan et al., 2000) a potassium channel blocker and after 15 min they received dihydroxy flavone injections before being subjected to acetic acid assay 30 min later.

The results were analyzed statistically by analysis of variance followed by Dunnett's t-test.

3. Results

3.1. Acute toxicity testing

There was no significant alteration in autonomic or behavioral responses in mice, treated with different dihydroxy flavones in a dose of 2 g/kg. No mortality was recorded in these animals up to 14 days.

3.2. Motor performance and loco motor activity

Treatment of animals with the four tested dihydroxy flavones in a dose 200 mg/kg did not alter the motor performance of mice in rotarod and loco motor activity in open field apparatus when compared to vehicle treated animals (data not shown).

3.3. Abdominal constriction assay

The mean number of abdominal constrictions after i.p. injection of acetic acid was 32.5 ± 0.47 in control animals. A significant reduction in abdominal constrictions was observed in morphine treated mice and the mean value being 1.56 ± 0.86 (Table 1).

All the four dihydroxy flavones elicited a dose proportionate reduction in the number of abdominal constrictions in mice (Table 1). Nearly 50% inhibition of nociception was observed with 25 mg/kg for three dihydroxy flavone derivatives (5,6-dihydroxy flavone, 3,7-dihydrodxy flavone , and 6,3′-dihydroxy flavone) and further increase in doses up to 100 mg/kg resulted in a maximum inhibition of nociception ranging from 75 to 94%. In contrast, 3,3′-dihydroxy flavone could produce 50% inhibition only in a dose of 100 mg/kg (Table 1).

3.4. Thermal nociception

The mean reaction time in the control group of animals was 8.41 ± 0.21 s which was increased to 28.47 ± 0.31 s in morphine (10 mg/kg) treated animals (Table 2). A dose dependent increase in reaction time was also observed in animals treated with all the four dihydroxy flavones (Table 2). A maximum of 60% inhibition was evident for a dose of 100 mg/kg of 5,6-dihydroxyflavone and 3,7-dihydroxy flavone whereas in the same dose 3,3′-dihydroxy flavone offered only 45% inhibition of thermal nociception. However 6,3′-dihydroxy flavone caused a significant and marked increase in the pain latency with an inhibition of 89% in a dose of 100 mg/kg (Table 2).

3.5. Formalin test

In vehicle treated control animals the mean paw licking response time was 47.55 ± 0.30 s in the acute phase and 87.56 ± 0.21 s in the chronic phase. Morphine treatment resulted in a marked reduction of response time to 8.67 ± 0.21 s and 2.67 ± 0.33 s in the acute and chronic phases, respectively (Table 3).

All the tested dihydroxy flavones in varying doses showed a dose dependent and statistically significant reduction in biting and licking response time after formalin injection compared to vehicle treatment (Table 3).

Even though a significant reduction in response time was seen in both the acute and chronic phases, it was evidently more in the chronic phase. Out of the four tested dihydroxy flavones, three compounds viz. 5,6-dihydroxy flavone, 3,7-dihydroxy flavone , and 6,3′-dihydroxy flavone, inhibited the response time to an extent of 90% in chronic phase compared to 35% inhibition in acute phase (Table 3). However under the same conditions, 3,3′-dihydroxy flavone produced 30% and 47% inhibition of nociceptive response in the acute and chronic phases, respectively.

3.6. Analysis of possible mechanisms of action of dihydroxy flavones

Various dihydroxy flavones in the selected doses offered a significant reduction in the number of abdominal constrictions in mice (Table 4). However the reduction in the number of abdominal constrictions brought out by all the four tested dihydroxy flavones was completely reversed by naloxone pretreatment(Table 4). In other words naloxone could effectively block the response of dihydroxy flavones on acetic acid induced abdominal constriction assay.

Pretreatment of mice with yohimbine, ondansetron, haloperidol, or glibenclamide did not modify the antinociceptive response elicited by dihydroxy flavones (data not shown).

In the absence of bicuculline, various dihydroxy flavones significantly protected mice from acetic acid induced abdominal constrictions (Table 5). However, pretreatment of mice with bicuculline significantly attenuated the antinociceptive effect of dihydroxy flavones in acetic acid induced pain (Table 5).

4. Discussion

Pain is the most common motivating factor to seek medical attention. Although adequate pain relief is achieved with the currently available analgesic agents like opioids or NSAIDs, some of their serious side effects are major limitations to their routine use in therapy. Flavonoids are an important group of compounds, presently undergoing extensive studies in search of safe analgesic and anti-inflammatory agents. Earlier studies have identified the potent antinociceptive and anti-inflammatory properties of several flavone derivatives. (Parmar and Ghosh, 1980; Thirugnanasambantham et al., 1985; Muthiah et al., 1993). To widen the spectrum of biologically active flavones and to identify the most effective compounds, the present study investigated the antinociceptive effect of four new dihydroxy flavone compounds viz.: 3,3′-dihydroxy flavone, 5,6-dihydroxy flavone, 3,7-dihydroxy flavone and 6,3′-dihydroxy flavone.

Table 1
Effect of dihydroxy flavones (DHF) on acetic acid induced abdominal constrictions in mice.

Dose of test compounds mg/kg; s.c.	Number of abdominal constrictions			
	3,3′-DHF	5,6-DHF	3,7-DHF	6,3′-DHF
3	29.48 ± 0.92 (10.81)	25.30 ± 0.75[a] (21.97)	27 ± 2.08[a] (16.89)	28.2 ± 0.73 (13.18)
6	26.67 ± 0.83[a] (17.61)	23.10 ± 0.79[a] (28.66)	25.5 ± 0.56[a] (21.52)	23.80 ± 1.01[a] (26.76)
12.5	24.83 ± 0.10[a] (25.11)	16.66 ± 0.33[a] (48.15)	20.6 ± 0.55[a] (36.40)	19.40 ± 0.40[a] (40.76)
25	23.33 ± 0.04[a] (28.39)	14.3 ± 0.49[a] (55.31)	15.5 ± 0.56[a] (52.81)	16.20 ± 0.58[a] (50.26)
50	17.50 ± 1.77[a] (45.30)	11.10 ± 0.47[a] (65.86)	12.3 ± 0.66[a] (62.04)	9.60 ± 0.6[a] (68.61)
100	16.33 ± 0.70[a] (50.40)	8.16 ± 0.47[a] (74.82)	4 ± 0.8[a] (87.68)	2 ± 0.44[a] (93.84)

Each value represents the mean ± SEM of six observations.
The values in parenthesis indicate the percentage inhibition of abdominal constrictions.
The number of abdominal constrictions after vehicle treatment was 32.5 ± 0.47.
The number of abdominal constrictions after morphine (5 mg/kg) treatment was 1.56 ± 0.86.
[a] $P<0.01$, compared with vehicle treatment. DHF—Dihydroxy flavones.

Table 2
Effect of dihydroxy flavones (DHF) on thermal nociception.

Dose of test compounds mg/kg; s.c.	Reaction time in seconds			
	3,3′-DHF	5,6-DHF	3,7-DHF	6,3′-DHF
3	8.14 ± 0.34 (26.74)	9.74 ± 0.31* (32.45)	8.96 ± 0.58 (28.89)	14.31 ± 0.84* (47.69)
6	8.33 ± 0.42 (27.12)	10.94 ± 0.76* (36.49)	8.61 ± 0.41 (29.45)	15.62 ± 0.60* (52.07)
12.5	9.77 ± 0.31* (32.56)	13.38 ± 0.40* (45.93)	10.13 ± 0.55* (33.84)	20.95 ± 0.44* (69.83)
25	10.67 ± 0.3* (35.57)	15.16 ± 0.51* (50.53)	14.80 ± 0.93* (49.34)	24.48 ± 0.80* (81.6)
50	11.83 ± 0.24* (39.45)	16.07 ± 0.71* (55.40)	16.61 ± 0.43* (55.51)	24.64 ± 0.60* (82.14)
100	13.67 ± 0.28* (45.55)	18.10 ± 0.56* (60.33)	17.17 ± 0.37* (57.24)	26.51 ± 0.73* (88.84)

Each value represents the mean ± SEM of six observations.
The values in parenthesis indicate the percentage inhibition of pain response.
The reaction time after vehicle treatment was 8.41 ± 0.21 s.
The reaction time after morphine (10 mg/kg) treatment was 28.47 ± 0.31 s.
* $P<0.01$ compared to vehicle treatment.

Selection of mice for the present study enabled us to investigate the antinociceptive effect of dihydroxy flavones in three different types of nociception; viz: visceral nociception (acetic acid induced abdominal constriction assay), thermal nociception (hot plate assay), and neurogenic and inflammatory nociception (formalin nociception).

In order to estimate the antinociceptive property of any new substance using behavioral nociceptive tests it is essential to employ different tests which differ in stimulus quality, intensity and duration (Tjolsen and Hole, 1997). Acetic acid induced abdominal constriction assay (Koster et al., 1959) is regarded as a very sensitive method employing minimal noxious stimulus and even weaker analgesics can be detected from the results of this test. Hot plate assay (Eddy and Leimbach, 1953) employs a high degree of thermal nociception and compounds exhibiting good antinociceptive effect in this method may be considered as potent analgesics. Formalin-induced nociception measures the ability of the substance to attenuate moderate continuous pain generated by injured tissue (Tjolsen et al., 1992). The acute and chronic phases of formalin nociception are considered to represent neurogenic and inflammatory pain behavior, respectively. Hence the dihydroxy flavones were tested for their antinociceptive effect employing the above three nociceptive assay procedures.

Since the test compounds did not produce any mortality in mice even in a dose of 2 g/kg, they may be considered to be relatively safe. The present study further demonstrates that, systemic administration of investigated dihydroxy flavones did not produce any motor dysfunction, sedation or alteration in locomotor activity of animals.

The finding of the present study shows a dose dependent reduction in the number of abdominal constrictions in acetic acid assay by pretreatment with these dihydroxy flavone derivatives. This clearly indicates the potent antinociceptive action of these compounds. In a dose of 100 mg/kg, 6,3′-dihydroxy flavone exhibits nearly 94% inhibition of pain response indicating higher efficacy than the other tested compounds. The dose which produces nearly 50% inhibition in this assay model is similar for three dihydroxy flavones viz: 5,6-dihydroxy flavone, 3,7-dihydroxy flavone and 6,3′-dihydroxy flavone.

The antinociceptive effect of dihydroxy flavones was also confirmed from the results of thermal nociceptive assay. A dose dependent increase in reaction time after treatment with the investigated dihydroxy flavones indicates the efficacy of these compounds in a model of thermal nociception. In this assay procedure also, 6,3′-dihydroxy flavone exerted a higher degree of inhibition of nociception than the other tested dihydroxy flavones.

Further, a marked reduction in the paw licking response time recorded in the formalin assay also substantiates the antinociceptive action of dihydroxy flavones. The nociceptive behavior after formalin injection was distinctly recorded in two phases. The first phase of paw licking/biting response starts immediately after injection and is considered probably due to direct stimulation of nociceptors (Dubuisson and Dennis, 1977). The second phase which appears little later is considered to be due to a combination of an inflammatory reaction in the peripheral tissue and changes in central processing (Tjolsen et al., 1992). A significant and dose related antinociceptive effect was clearly evident for all the tested four dihydroxy flavones against both neurogenic (early phase) and inflammatory (late phase) pain behavior caused by formalin injection in mice . The degree of

Table 3
Effect of dihydroxy flavones (DHF) on formalin-induced nociception (acute and chronic phases).

Dose of test compounds mg/kg; s.c.	Paw licking response time in seconds							
	3,3′-DHF		5,6-DHF		3,7-DHF		6,3′-DHF	
	Acute	Chronic	Acute	Chronic	Acute	Chronic	Acute	Chronic
3	49.17 ± 2.14 (0)	81.56 ± 1.12 (0)	48.34 ± 1.76 (0)	70.30 ± 2.21* (18.11)	44.33 ± 1.01 (6.85)	78.62 ± 1.47 (10.32)	50.09 ± 0.06 (0)	75.67 ± 0.55 (13.55)
6	49.15 ± 2.64 (0)	78.69 ± 0.17 (10.13)	47.71 ± 1.70 (0)	64.68 ± 2.42* (26.09)	41.72 ± 0.60 (12.26)	71.30 ± 1.50 (18.56)	46.4 ± 1.30 (4.73)	76.21 ± 0.64 (12.94)
12.5	44.99 ± 2.32 (6.6)	70.53 ± 0.50 (19.44)	42.69 ± 1.28 (11.41)	55.31 ± 1.67* (36.82)	41.47 ± 2.04 (12.78)	63.77 ± 1.09* (27.20)	44.85 ± 1.62 (8.45)	73.70 ± 1.46 (15.80)
25	43.55 ± 1.48 (8.71)	65.03 ± 1.03* (25.73)	37.43 ± 1.01* (17.45)	49.28 ± 1.45* (43.70)	39.74 ± 0.94* (16.76)	58.73 ± 0.77* (32.30)	41.51 ± 1.69* (12.96)	54.77 ± 1.51* (37.42)
50	39.85 ± 0.84* (15.35)	58.69 ± 1.41* (32.97)	35.69 ± 1.44* (24.94)	33.16 ± 1.07* (60.56)	36.98 ± 2.01* (22.23)	34.77 ± 1.65* (60.48)	31.59 ± 1.29* (33.56)	14.36 ± 1.83* (82.34)
100	33.72 ± 1.02* (30.35)	45.68 ± 0.67* (47.83)	29.38 ± 1.17* (38.17)	9.48 ± 1.25* (89.16)	31.98 ± 2.01* (32.23)	8.82 ± 1.00* (89.90)	30.87 ± 1.01* (35.07)	3.85 ± 1.44* (95.59)

Each value represents the mean ± SEM of six observations.
The values in parenthesis indicate the percentage inhibition of formalin-induced nociception.
The biting/paw licking response time in vehicle treatment were 47.55 ± 0.3 s in the acute phase and 87.56 ± 0.21 s in the chronic phase.
The biting/paw licking response time after morphine (5 mg/kg) treatment was 8.67 ± 0.21 s in the acute phase and 2.67 ± 0.33 s in the chronic phase.
* $P<0.05$ compared to vehicle treatment.

Table 4
Effect of naloxone on dihydroxy flavones induced inhibition of acetic acid writhing in mice.

Treatment mg/kg; s.c.	Number of abdominal constrictions	
	Without naloxone	With naloxone 5 mg/kg; i.p.
Vehicle	32.15 ± 0.15	30.01 ± 0.70
Morphine5	1.56 ± 0.56[a]	30.06 ± 0.57[b]
3,3′-DHF 100	15.83 ± 0.70[a]	30.40 ± 0.50[b]
5,6-DHF 25	14.33 ± 0.49[a]	30.16 ± 0.60[b]
3,7-DHF25	15.50 ± 0.56[a]	30.04 ± 0.51[b]
6,3′-DHF25	14.33 ± 0.49[a]	29.16 ± 0.30[b]

Each value represents the mean ± SEM of six observations.
[a] $P<0.05$ compared with vehicle treatment.
[b] $P\leq0.05$ compared with respective value without naloxone.

inhibition in the late phase of formalin nociception was much higher when compared to the early phase for all the dihydroxy flavones. This observation may suggest a more preferential and predominant effect of dihydroxy flavones on inflammatory pain.

Significant inhibition of acetic acid induced nociception, both the phases of formalin nociception, and thermal nociception by the investigated dihydroxy flavones indicate that these compounds may be effective in pain of different origins. The acetic acid induced nociception and the late phase of formalin nociception are considered to represent the inflammatory pain response (Tjolsen et al., 1992, Tjolsen and Hole, 1997). Significant attenuation of both the above responses by the investigated dihydroxy flavones suggests that these compounds may be more effective in inflammatory pain.

Previous studies have reported the potent antinociceptive activity exerted by some monohydroxy flavones (Thirugnanasambantham et al., 1993) and a few dihydroxy flavones (Girija et al., 2002; Umamaheswari et al., 2006). The present results are in agreement with the above previous reports and also have identified four new dihydroxy flavone derivatives with marked antinociceptive efficacy.

Many previous studies have reported the participation of multiple mechanisms in the antinociceptive effects of flavone derivatives. The present study analyzed some of those possibilities by employing suitable interacting drugs.

Evidences for the major participation of opioid mechanism in the antinociceptive action of flavone compounds are available in literature. Gossypin (Viswanathan et al., 1985), several monohydroxy and monomethoxy flavones (Thirugnanasambantham et al., 1990,1993), various dihydroxy flavone derivatives (Girija et al., 2002; Umamaheswari et al., 2006) and quercetin (Naidu et al., 2003) were found to utilize opioid pathways in mediating their antinociceptive effect. The present results reveal that nalaxone was able to significantly attenuate the antinociceptive activity of the investigated dihydroxy flavones. This observation confirms the earlier reports and conclusively suggests a role for opioid mechanism in the antinociceptive action of dihydroxy flavones.

The inhibitory GABAergic system has been found to play a major role at many sites in the neuronal pathway mediating nociception.

Table 5
Effect of bicuculline on dihydroxy flavones induced inhibition of acetic acid writhing in mice.

Treatment mg/kg; s.c.	Number of abdominal constrictions	
	Without bicuculline	With bicuculline 1 mg/kg; i.p.
Vehicle	32.15 ± 0.15	31.60 ± 1.08
3,3′-DHF 100	15.83 ± 0.70[a]	28.50 ± 0.50[b]
5,6-DHF 25	14.33 ± 0.49[a]	28.50 ± 1.60[b]
3,7-DHF 25	15.50 ± 0.56[a]	24.64 ± 0.81[b]
6,3′-DHF 25	14.33 ± 0.49[a]	25.56 ± 0.95[b]

Each value represents the mean ± SEM of six observations.
[a] $P<0.05$ compared with vehicle treatment.
[b] $P\leq0.05$ compared with respective value without bicuculline.

$GABA_A$ receptors are found in the spinal cord dorsal horn, where they regulate the pain signals from the periphery to higher central nervous system areas. Diminished inhibitory activity of GABA at this site has been suggested as a main factor in chronic pain syndromes (Zellhofer et al., 2009). The GABA receptor agonists, 4,5,6,7-tetra hydro isoxazole (5,4) pyridine 3-ol (THIP) and muscimol were found to exhibit antinociception and also potentiated opioid analgesia (Hill et al., 1981). Flavonoids like gossipin (Viswanathan et al., 1993) and quercetin (Filho et al., 2008) were found to utilize GABAergic mechanism in mediating their antinociceptive effects. Such a possibility in the antinociceptive action of dihydroxy flavones was investigated in the present study. Bicuculline, an antagonist of $GABA_A$ receptor significantly antagonized the antinociceptive action exerted by various dihydroxy flavones. This observation suggests a possible interaction of dihydroxy flavones with $GABA_A$ receptors to mediate the antinociceptive activity.

A role for alpha adrenergic system in opioid action was suggested by Ramaswamy et al. (1981) from the observation that, clonidine treatment could effectively antagonize the development of acute and chronic tolerance to morphine analgesia. Moreover, the ubiquitous bioflavonoid quercetin was reported to exert its antinociceptive effect primarily involving the modulation of adrenergic pathways (Kaur et al., 2005). Hence, in the present study, it was considered interesting to investigate the role of alpha-2 adrenergic system in the antinociceptive action of dihydroxy flavones. Pretreatment with yohimbine, an alpha-2 adrenergic receptor antagonist failed to modify the antinociceptive effect of dihydroxy flavones (data not shown).This observation suggests that alpha-2 adrenergic system may not be involved in the antinociceptive effect of investigated dihydroxy flavones.

Serotonin is an important neurotransmitter in modulating the nociceptive response at many stages in the pain pathway. The descending serotonergic pathways may directly modulate the activity of projection neurons and also via interneuron (Alchaider, 1991). Among the various subtypes of serotonin receptors, $5HT_{1A}$, $5HT_2$, and $5HT_3$ receptors are considered to play a major role in nociceptive modulation (Bardin et al., 2000, Sasaki et al., 2001, Millan, 2002).

The antinociceptive action of quercetin was found to involve serotenergic pathways in addition to GABAergic and nitric oxide pathways (Filho et al., 2008). In the present study ondansetron was employed to investigate the role of serotonergic system in the antinociceptive action of dihydroxy flavones. However, pretreatment with ondansetron did not alter the antinociceptive effect of these compounds revealing that the serotonergic system (especially $5HT_3$ receptor mechanism) may not be involved in the action of dihydroxy flavones (data not shown).

An interaction between dopaminergic, adrenergic and opioid systems has been suggested in the modulation of pain perception (Akil and Liebeskind, 1975, Pollard et al., 1978). Dopamine has been suggested to play an important role in the modulation of nociceptive information by basal ganglia (Chudler and Dong, 1995). Further a role for dopaminergic and alpha-2 adrenergic systems has been established in the antinociceptive effect of quercetin (Naidu et al., 2003). In the present study haloperidol (D_1/D_2 receptor antagonist) pretreatment failed to alter the antinociceptive effect of dihydroxy flavones (data not shown). This observation rules out the role of dopaminergic system in the antinociceptive action of dihydroxy flavones.

A role for ATP sensitive potassium channels in opioid induced antinociception has been documented (Ocana et al., 1990 and Wild et al., 1991). Various drugs acting on μ opioid receptor (Rodrigues and Duarte, 2000), δ opioid receptor, (Duarte and Pacheco, 2005) as well as an anti-inflammatory analgesic like diclofenac (Alves et al., 2004) and resveratrol (Granados-Soto et al., 2002) have been shown to interact with ATP sensitive potassium channels in mediating their antinociceptive action. In a previous study, the antinociceptive effect of 7-hydroxy flavone was attenuated by glibenclamide, thus

establishing a role for ATP sensitive potassium channels in this action (Venkataramanan et al., 2000). A possible role for ATP sensitive potassium channel in the antinociceptive effect of dihydroxy flavones was investigated in the present study. However, glibenclamide pretreatment failed to modify the analgesic action of dihydroxy flavones in mice (data not shown). This observation excludes the participation of ATP sensitive potassium channels in the action of dihydroxy flavones.

In summary, a novel antinociceptive action of 3,3′-dihydroxy flavone, 5,6-dihydroxy flavone, 3,7-dihydroxy flavone and 6,3′-dihydroxy flavone has been confirmed against three different models of nociception in mice. In addition, the results demonstrate that the antinociceptive effect of tested dihydroxy flavones involves an interaction with opioid and GABAergic systems, but not with adrenergic, dopaminergic, tryptaminergic or with potassium channels.

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