Antinociceptive Activity of Certain Dihydroxy Flavones

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Four dihydroxy flavone derivatives viz 5,3′-, 7,3′-, 2′,3′- and 2′,4′-dihydroxy flavones were evaluated for their antinociceptive response in doses ranging from 3 to 200 mg/kg (s.c). Antinociception in mice was studied employing acetic acid induced abdominal constrictions, formalin induced nociception and tail immersion assay procedures. Involvement of opioid mechanism in the antinociceptive action of dihydroxy flavones was investigated using naloxone in acetic acid assay. All the four tested dihydroxy flavones exhibited a significant, dose related antinociceptive response in these assay procedures. The inhibition of nociceptive response was more than 98% in acetic acid writhing assay and 100% in the chronic phase of formalin assay. Naloxone almost completely antagonized the antinociceptive response of the four-dihydroxy flavone compounds. In conclusion, all the four investigated dihydroxy flavones exhibited opioid mediated antinociceptive response in mice.

Flavonoids are polyphenolic compounds ubiquitously present in almost all parts of flowering plants. Many interesting pharmacological actions have been reported for this group of compounds. Many flavone compounds have been found to possess antinociceptive[1-4], antiinflammatory[5,6] and antiulcer properties[7,8]. This unique combination of the above pharmacological properties in a single nucleus, flavone (fig. 1) is quite interesting. The antinociceptive activity of flavone and its monohydroxy derivatives[3], certain flavonol glycosides like hydroxy ethyl rutoside[1] and gossypin[2] have been reported earlier. Girija *et al.*[9] recently synthesized a few dihydroxy flavone derivatives and reported the antinociceptive activity of these compounds. In their study a maximum of 75% inhibition of acetic acid induced writhing was reported for the tested compounds.

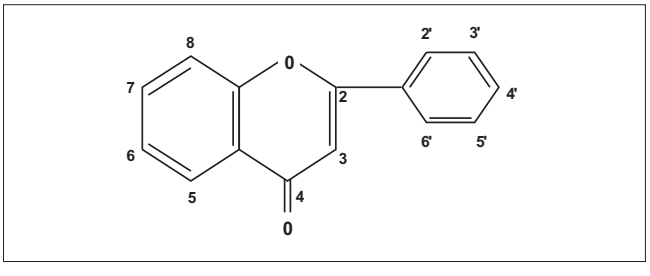

Fig. 1: Structure of flavone

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It is well known that structural modifications can improve the pharmacological actions of an active molecule. With this view in mind some more dihydroxy flavones were synthesized in the present study and investigated for their antinociceptive action by a battery of tests. Earlier reports indicate the involvement of opioid mechanism in the antinociceptive action of some flavonoid compounds[2,3]. Such a possibility was also investigated in the present study.

MATERIALS AND METHODS

Male Swiss albino mice weighing between 25-30 g (Animal house, Sri Ramachandra Medical College and Research Institute) were housed under 12:12 h light:dark cycle at controlled temperature (25°) with free access to pellet feed (Gold Mohar Ltd., Bangalore) and water. The experiments were conducted between 9.00 and 13.00 h. The experimental protocol was approved by the Institutional Animal Ethical Committee.

Dihydroxy flavones:

The dihyroxy flavones used in the study (7,3′-dihydroxy

flavone, 5,3′-dihydroxy flavone, 2′,3′-dihydroxy flavone and 2′,4′-dihydroxy flavone) were synthesised adopting standard procedures at Research Organics, Chennai. The authenticity of these compounds was confirmed in comparison with standard samples by melting points and UV spectra.

Toxicity:
A preliminary acute toxicity testing was carried out in mice according to the OECD guideline 423. No mortality was observed in mice up to a dose of 2 g/kg s.c. for all the dihydroxy flavones tested.

Acetic acid-induced abdominal constriction assay[10]:
This assay procedure is considered as very sensitive with minimal noxious stimulus[11]. The number of abdominal constrictions (writhings) in mice for a period of 15 min was counted following i.p. injection of 0.6% acetic acid in a dose of 10 ml/kg. Any significant reduction in the number of abdominal constrictions when compared with vehicle-treated animal was considered as antinociceptive response. The degree of antinociceptive response was also expressed as percentage inhibition of abdominal constrictions after different treatments.

Formalin assay:
Formalin test as described by Takahashi *et al.*,[12] was followed. Each mouse was placed in an observation chamber 5 min before the test for acclimatization to the new environment and 50 µl of 1% formaldehyde in saline was administered s.c in the left hind paw. The animal was then returned to the observation chamber and nociceptive response was recorded for a period of 30 min. Summation of time (s) spent in licking and biting of formalin injected paw during each 5 min block was measured as indicator of pain response. Duration of responses in first 10 min and that from 10 to 30 min represent acute and chronic phases, respectively.

Tail immersion method[13]:
The tail of the mouse was immersed to a constant level (3 cm) in a water bath maintained at 55 ± 0.5°. The time to flick the tail from water (reaction time) was recorded. A maximum immersion time of 30 sec. was maintained to prevent thermal injury to the animals. A significant increase in reaction time compared with control animals was considered a positive analgesic response.

Drug administration:
Dihydroxy flavone derivatives were prepared as uniform suspension in 1% carboxy methyl cellulose and injected s.c in doses of 3, 6, 12, 25, 50, 100, and 200 mg/kg 60 min prior to the test procedure. Morphine (5 mg/kg, s.c) was included in all the studies as a reference drug for comparison and administered 30 min before the test procedure.

Mechanism of antinociception:
The mechanism of antinociceptive action of the test compounds was investigated by employing an opioid antagonist naloxone in the acetic acid induced writhing assay. Naloxone (5 mg/kg, s.c) was administered 10 min prior to dihydroxy flavone or morphine treatment. The antinociceptive activity was then documented either 60 min after dihydroxy flavones or 30 min after morphine treatment as described earlier.

Statistical analysis of the results was performed by employing ANOVA followed by Dunnett's[14] t-test.

RESULTS AND DISCUSSION

The mean number of abdominal constrictions after i.p. injection of acetic acid was 32.5 in vehicle treated control animals. Morphine (5 mg/kg) treatment produced 100% inhibition of writhing response. A dose dependent reduction in the number of abdominal constrictions was observed in animals treated with different dihydroxy flavones. All the tested compounds produced nearly 100% inhibition of writhing response in a dose of 200 mg/kg (Table 1).

In the vehicle treated control animal, the mean paw licking response time was 48.17 sec. in acute phase and 79.67 sec. in chronic phase (Table 2). Morphine treatment resulted in a marked reduction of paw licking response time to 2.67 and 1.67 sec. in acute and chronic phases, respectively. Treatment with 2′, 3′ dihydroxy flavone, significantly reduced the paw licking response time in acute and chronic phase in a dose dependent fashion. A similar pattern of response was also observed after treatment with 2′,4′-dihydroxy flavone, 5,3′-dihydroxy flavone and 7,3′-dihydroxy flavone (Tables 2 and 3). The percentage reduction in paw licking response appears to be more in the chronic phase compared to the acute phase. The maximum percentage of inhibition of paw licking response during acute phase ranged between 73 and 78% among the compounds tested. However, all the dihydroxy flavones produced 100% inhibition of paw licking response in the chronic phase in a dose of 200 mg/kg.

TABLE 1: EFFECT OF DIHYDROXY FLAVONES ON ACETIC ACID-INDUCED ABDOMINAL CONSTRICTIONS IN MICE

Dose of test compound	Number of abdominal constrictions			
mg/kg s.c	2′,3′ DHF	2′,4′ DHF	5,3′ DHF	7,3′ DHF
3	23.83 ± 0.31*(26.67)	23.5 ± 0.22*(27.69)	24.0 ± 0.37*(26.15)	29.15 ± 0.31(10.30)
6	21.50 ± 0.56*(33.84)	19.67 ± 0.42*(39.47)	21.0 ± 0.37*(35.38)	28.17 ± 0.31*(13.32)
12	16.17 ± 0.31*(50.24)	16.00 ± 0.3*(50.67)	15.67 ± 0.33*(51.78)	25.83 ± 0.31*(20.52)
25	11.83 ± 0.31*(63.6)	10.0 ± 0.37*(69.2)	11.6 ± 0.33*(64.1)	24.0 ± 0.37*(26.15)
50	5.5 ± 0.22*(83.1)	4.5 ± 0.22*(86.15)	9.33 ± 0.21*(71.3)	13.67 ± 0.21*(57.9)
100	0.5 ± 0.22*(98.5)	0.33 ± 0.21*(98.98)	3.5 ± 0.2*(89.2)	2.17 ± 0.17*(93.32)
200	0.0 ± 0.0*(100)	0.0 ± 0.0*(100)	0.0 ± 0.0*(100)	0.0 ± 0.0*(100)

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 ± 0.22. The number of abdominal constrictions after morphine (5 mg/kg) treatment was 0.0 ± 0.0 with 100% inhibition. *P<0.01 compared with vehicle treatment, DHF - Dihydroxy flavones

TABLE 2: EFFECT OF 2′, 3′ AND 2′, 4′ DIHYDROXY FLAVONES (DHF) ON FORMALIN-INDUCED NOCICEPTION

Dose mg/kg s.c	Biting / Paw licking response time (s)			
	2′,3′ DHF		2′,4′ DHF	
	Acute phase (0-10 min)	Chronic phase (10-30 Min)	Acute phase (0-10 min)	Chronic phase (10-30 min)
3	32.17 ± 0.31*(33.21)	31.5 ± 0.22*(60.46)	32.57 ± 0.21*(32.38)	33.67 ± 0.21*(57.73)
6	31.00 ± 0.26*(35.64)	29.67 ± 0.49*(62.75)	31.17 ± 0.48*(35.29)	30.67 ± 0.33*(61.50)
12	26.00 ± 37*(46.02)	23.67 ± 0.21*(70.289)	27.83 ± 0.31*(42.23)	24.50 ± 0.43*(69.25)
25	21.00 ± 0.26*(56.41)	19.0 ± 0.45*(76.17)	23.67 ± 0.42*(44.6)	20.00 ± 0.26*(74.73)
50	16.83 ± 0.31*(65.07)	9.50 ± 0.22*(88.71)	18.67 ± 0.56*(61.25)	8.5 ± 0.22*(89.26)
100	16.00 ± 0.37*(66.79)	4.33± 0.33*(94.60)	15.50 ± 0.22*(67.83)	8.33 ± 0.21*(89.48)
200	12.83 ± 0.31*(73.37)	0.00 ± 00*(100%)	12.17 ± 0.31(74.41)	0 ± 0*(100)

Each value represents mean ± SEM of six observations. The values in parenthesis indicate the percentage inhibition of formalin-induced nociception. The biting/paw licking response in vehicle treated animals was 48.17 ± 0.3 s in acute phase and 79.67 ± 0.21 s in chronic phase. The biting/paw licking response for morphine (5 mg/kg) treatment was 2.67 ± 0.21 s in acute phase and 1.67 ± 0.33 s in chronic phase. *P<0.01 compared to vehicle treatment

TABLE 3: EFFECT OF 5,3′ AND 7,3′ DIHYDROXY FLAVONES (DHF) ON FORMALIN-INDUCED NOCICEPTION

Dose mg/kg s.c	Biting / Paw licking response time (sec.)			
	5,3′ DHF		7,3′ DHF	
	Acute phase (0-10 min)	Chronic phase (10-30 min)	Acute phase (0-10 min)	Chronic phase (10-30 min)
3	33.17 ± 0.31*(31.13)	42.33 ± 0.33*(46.80)	32.5 ± 0.22*(32.53)	26.67 ± 0.21*(66.52)
6	30.83 ± 0.31*(35.99)	41.33 ± 0.33*(48.123)	30.83 ± 0.31*(35.9)	24.17 ± 0.31*(69.66)
12	25.17 ± 0.31*(47.74)	38.67 ± 0.42*(51.46)	25.50 ± 0.43*(49.13)	18.67 ± 0.42*(75.56)
25	20.83 ± 0.31*(56.75)	36.33 ± 0.56*(54.54)	19.17 ± 0.31*(60.21)	14.83 ± 0.31*(81.39)
50	18.5 ± 0.22*(61.46)	18.17 ± 0.60*(81.33)	15.33 ± 0.21*(68.18)	9.33 ± 0.21*(88.28)
100	15.67 ± 0.21*(67.47)	18.33 ± 0.49*(81.83)	14.00 ± 0.26*(70.94)	4.67 ± 0.33*(94.14)
200	12.00 ± 0.37*(75.09)	0 ± 0*(100%)	10.83 ± 0.31*(77.83)	0 ± 0*(100%)

Each value represents mean ± SEM of six observations. The values in parenthesis indicate the percentage inhibition of formalin-induced nociception. The biting/paw licking response in vehicle treated animals was 48.17 ± 0.3 s in acute phase and 79.67 ± 0.21 s in chronic phase. The biting/paw licking response for morphine (5 mg/kg) treatment was 2.67 ± 0.21 s in acute phase and 1.67 ± 0.33 s in chronic phase. *P<0.01 compared to vehicle treatment

In tail immersion assay, the mean reaction time in vehicle treated animals was 1.67 sec., which was significantly increased to 18.1 sec. in morphine treated animals. Increasing doses of 2′, 3′ -dihydroxy flavone produced a dose dependent increase in the reaction time. A similar response was also noted after administration of 2′,4′-dihydroxy flavone, 5,3′-dihydroxy flavone and 7,3′-dihydroxy flavone (Table 4). For most of the compounds the increase was statistically significant in doses above 6 mg/kg. In naloxone pre-treated animals morphine treatment failed to produce any reduction in abdominal constrictions. In a similar fashion, naloxone pretreatment antagonised the reduction in writhing response induced by treatment with different dihydroxy flavones in mice (Table 5).

Even though the present day armamentarium is rich in potent analgesic agents, the search for novel and safe analgesic drugs continues and vigorously pursued in many parts of the world. The reasons are very obvious; the most potent opiate group of analgesics are associated with many undesirable side effects and also carry a potential for drug addiction. The other prominent group of analgesics viz. NSAIDS are notorious for their ulcerogenic[15] and nephrotoxic potential.[16] In this regard it is interesting to note that many flavonoid derivatives

TABLE 4: EFFECT OF DIHYDROXY FLAVONES (DHF) ON WARM WATER TAIL IMMERSION IN MICE

Dose mg/kg/s.c	Reaction time (s)			
	2′,3′ DHF	2′,4′ DHF	5,3′ DHF	7,3′ DHF
3	2.0 ± 00	2.00 ± 0.00	2.33 ± 0.21	2.33 ± 0.21
6	2.67 ± 0.21	3.0 ± 0.00*	3.50 ± 0.22*	3.5 ± 0.22*
12	3.33 ± 0.21*	3.50 ± 0.22*	5.33 ± 0.21*	4.00 ± 0.26*
25	3.83 ± 17*	4.17 ± 0.31*	6.67 ± 0.21*	5.00 ± 0.26*
50	6.50 ± 0.22*	5.67 ± 0.21*	9.17 ± 0.17*	9.00 ± 0.37*
100	10.33 ± 0.33*	8.50 ± 0.22*	10.83 ± 0.31*	11.00 ± 0.37*
200	15.67 ± 0.81*	13.83 ± 0.75*	14.17 ± 0.3*	15.50 ± 0.22*

Each value represents the mean ± SEM of six observations. The reaction time after vehicle treatment was 1.67 ± 0.21 s. The reaction time after morphine (5 mg/kg s.c) treatment was 18.1 ± 0.31 s. *P<0.01 compared to vehicle treatment

TABLE 5: EFFECT OF NALOXONE ON DIHYDROXY FLAVONE (DHF) 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	32.5 ± 0.22	32.4 ± 0.22
Morphine 5	0.0 ± 0.00*	29.5 ± 0.22†
2′,3′ DHF 50	5.67 ± 0.21*	30.00 ± 0.63†
2′,4′ DHF 50	4.5 ± 0.22*	14.33 ± 0.21†
5,3′ DHF 50	9.33 ± 0.21*	31.67 ± 0.49†
7,3′ DHF 50	13.5 ± 0.22*	33.00 ± 0.45†

Each value represents mean ± SEM of six observations.*P<0.05 compared with vehicle treatment. †P<0.05 compared with respective value without naloxone

isolated from various plants exhibited potent analgesic and anti-inflammatory action. Among them the effect of gossypin[2,7] and hydroxy ethyl rutoside[1,7] are worth mentioning.

In a subsequent study Thirugnanasambantham *et al.*[3,4] made an attempt to investigate a possible structure activity relationship among these compounds by studying the antinociceptive effect of the basic flavone nucleus and many monohydroxy[4] and monomethoxy derivatives.[3] The results of the above studies revealed that the flavone nucleus *per se* has inherent analgesic action; the C_2-C_3 double bond in the flavone nucleus appears to be essential for analgesic effect and substitution of different groups (hydroxyl or methoxyl) at different positions in the flavone nucleus altered the analgesic potency of flavone to varying degrees. In another recent study, a few dihydroxy flavone derivatives were synthesised and investigated for antinociceptive action.[9]

In most of the above studies the investigational compounds (flavone, mono and disubstituted flavones and flavonol glucosides) produced a maximum of 70% inhibition of nociception when tested by a standard and sensitive assay procedure namely acetic acid induced abdominal constriction assay. Hence, it was our interest to screen a few more compounds of this class to identify more effective analgesic agents. For this purpose four new dihydroxy flavone derivatives namely 2′,3′-dihydroxy flavone, 2′,4′-dihydroxy flavone, 5,3′-dihydroxy flavone and 7,3′-dihydroxy flavone were synthesised by adopting standard procedures and the structure confirmed by melting point and UV spectra in comparison with authentic samples.

The results of initial safety studies revealed that no mortality was observed for the tested dihydroxy flavones upto a dose of 2 g/kg in mice. This observation indicated the safe nature of the investigated compounds.

The antinociceptive effect of these compounds was investigated by three well-established assay procedures. The antinociceptive action of all the four-tested dihydroxy flavone was clearly evident by a dose dependent reduction in acetic acid induced abdominal constrictions (Table 1). An interesting observation is that almost 100% inhibition was achieved in a dose 200 mg/kg for all the four dihydroxy flavones. It is significant to point out that the previously tested compounds of this class exhibited only a maximum of 70% inhibition in this assay procedure[2-4,9]. The formalin assay procedure was included in the present study as it can assess both acute phasic pain and chronic inflammatory pain[12]. The first phase is considered probably due to direct chemical stimulation of nociceptors whereas the second phase is dependent on peripheral inflammation and changes in central processing. This procedure has also been employed for studying the antinociceptive response in diabetic animals to study chronic pain[17-19]. The results of the formalin test indicate that the tested dihydroxy flavones produced up to 78% inhibition of nociceptive response during the acute phase in this paradigm and 100% inhibition of nociceptive response during the chronic phase. This observation is very interesting as it reveals the potential use of these compounds in chronic inflammatory pain (including neuropathy). The present observation is

similar to that reported for flavone in an earlier study[20], which also reported a higher degree of inhibition in chronic phase than in acute phase of formalin-induced nociception.

The antinociceptive effect of dihydroxy flavones was also further confirmed by the results obtained in the tail immersion test. The reaction time was markedly elevated after treatment with different dihydroxy flavones. A similar pattern of dose dependent increment in the response time was noted for the tested compounds.

The antiniciceptive activity of all dihydroxy flavones was attenuated in animals treated with an opioid antagonist naloxone. Antagonism of the dihydroxy flavone-induced antinociception by naloxone clearly indicates the involvement of opioid mechanism in the action of dihydroxy flavones. The observation is similar to that reported for many flavone derivatives[2,9]. Even though gossypin and certain dihydroxy flavones involved opioid mechanism in their action, subsequent studies indicated the absence of either acute[21,22] or chronic[21] tolerance to the analgesic effect of these compounds. However this possibility remains to be investigated for the dihydroxy flavones reported in this study. Interestingly many flavonoid compounds have been also reported to possess powerful anti-inflammatory activity. The basic flavone nucleus, its monomethoxy derivatives[5] and monohydroxy derivatives[6] have been shown to inhibit carrageenan-induced paw edema and cotton pellet granuloma in albino rats. Flavonol glucosides like gossypin[7] and hesperidin[23] have also been found to possess anti-inflammatory activity in various experimental models. Moreover, gossypin was also shown to possess a marked degree of antiulcer activity[7] in contrast to conventional non-steroidal anti-inflammatory drugs which are ulcerogenic. Thus the flavone compounds appear to possess a unique combination of analgesic, anti inflammatory and antiulcer properties.

It is needless to say that their therapeutic potential is enormous considering the above spectra of pharmacological actions. The present study assumes significance in that it has identified certain dihydroxy flavones with maximum antinociceptive effect. Further investigation of these compounds for their anti inflammatory and ulcer protective effects may strengthen the pharmacological profile of flavones in general and dihydroxy flavones in particular.

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