Discussion
DISCUSSION

Biological significance of flavonoids

Among the myriad classes of phytochemicals, flavonoids assume a special status. This is not only because of their importance as dietary constituents but also due to many therapeutic benefits that are emerging from recent investigations. Many flavonoid compounds like rutin, diosmin and hydroxy ethyl rutoside were introduced in medicine to protect vascular integrity (Beretz and Cazenav., 1988).

Flavonoids and related polyphenolics are widely acclaimed for their hepatoprotective properties. Silymarin, a flavone lignan remains the gold standard for hepatoprotective action. The protective effects of silymarin against many liver toxins like carbon tetrachloride, ethanol, halothane, thioacetamide, galactosamine and paracetamol have been well established (Dixit et al., 2007). This observation has been extrapolated and confirmed by many clinical studies in mushroom poisoning, hepatitis and cirrhosis (Dixit et al., 2007). This property is also shared by many flavonoids like bicalein (Hwang et al., 2005), catechin (Kalender et al., 2005), quercetin (Janbaz et al., 2004) and rutin (Janbaz et al., 2002).

Another therapeutically useful action exemplified by flavonoid group of compounds is their marked anti inflammatory potential. The anti inflammatory activity of gossypin was extensively studied by Parmar and Ghosh (1978). The structure activity relationship of flavone and several methoxy
(Muthiah et al., 1993) and hydroxy flavones (Arivudainambi et al., 1996) was also analysed. The free radical scavenging action and COX-2 inhibition have been reported to be responsible for the anti inflammatory effect of luteolin and chrysin (Harris et al., 2006). Many recent studies reveal the potent anti inflammatory activity exhibited by hyperin (Lee et al., 2004), isoflavones (Jun et al., 2005) and wogonin (Jang et al., 2002).

A unique antinociceptive effect of hydroxy ethyl rutoside was reported by Ramaswamy et al., (1980). Further investigations identified flavonol glycosides like gossypin (Viswanathan et al., 1984) and rutin (Thirugnanasambantham et al.,1985) and many hydroxy and methoxy derivatives of flavone (Thirugnanasambantham et al.,1993) possessing antinociceptive property. Another interesting observation reported for these compounds added more value to the therapeutic utility of flavonoids. Generally compounds exhibiting analgesic and antiinflammatory activity cause extensive gastric mucosal injury and ulceration. Surprisingly many flavonoid compounds possessing both the above properties did not cause any gastric mucosal damage or ulceration.

In fact a potent anti ulcer activity was identified for many flavonoids. The anti ulcer properties of gossypin and hydroxy ethylrutoside (Parmar and Ghosh 1981), apigenin derivative (Viswanathan et al., 1981), kaempferol (Goel et al., 1988), quercetin (Dicarlo et al., 1999) and wogonin (Park et al., 2004) underscores the importance of these compounds in gastric mucosal
protection. The combination of analgesic, anti-inflammatory and ulcer-protective properties among flavonoids is very unique.

The foregoing paragraphs highlight the importance of flavonoids as potential therapeutic agents in many common ailments where pain and inflammation manifest. The margin of safety for the therapeutic use of flavonoids in humans is considered to be very large and probably much superior to any other drug in current use (Havsteen. 2002).

Moreover studies carried out by Rajendran (1998), Venkataraman et al., (2000) and Arivudainambi (2000) have suggested the potential utility of flavonoid substances in the management of chronic pain situations like diabetic neuropathy. This was supported by another study of quercetin on thermal hyperalgesia in mouse model of diabetic neuropathic pain (Anjaneyulu et al., 2003). The above reasons necessitate a much deeper/closer investigation on flavonoids for a detailed understanding and identification of most effective therapeutic agents among them. The present investigation is one such attempt aimed in this direction.

Antinociceptive effects of flavones

A few isolated reports indicated the antinociceptive effects of certain flavonol glycosides like hydroxy ethylrutoside (Ramaswamy, 1980), gossypin (Viswanathan et al., 1984) and rutin (Thirugnanasambantham et al., 1985). However, a detailed structure activity study was reported by Thirugnanasambantham et al., (1990, 1993) wherein the flavone nucleus, several
monohydroxy flavones, monomethoxy flavones and a few dihydroxy flavones and flavonol glycosides were investigated. The flavone nucleus *per se* exhibited a reasonable degree of antinociception which was differentially modified by hydroxyl, methoxyl or glycosyl substitutions. Many of the above reported compounds utilized opioid pathways in mediating their antinociceptive action. However, even in maximally tested doses complete inhibition of nociception (acetic acid induced abdominal constriction) was not achievable with any of the above flavone derivatives, in contrast to morphine, which was able to produce total inhibition of nociception in the above paradigm. In order to identify more effective compounds, Girija *et al.*, (2002) screened six newer dihydroxy flavone derivatives. But they also reported a ceiling effect in the antinociceptive efficacy exhibited by these compounds.

Many other recent evidences also strengthen the above reports on the antinociceptive action of flavones. The potent antinociceptive action of kaempferol 3,7-α - dimethyl rhamnoside (Toker *et al.*, 2004), quercetin (Kaur *et al.*, 2005), myricitrin (Meotti *et al.*, 2006) and naringenin derivatives (Orhan *et al.*, 2006) also support the need for a focused investigation on this beneficial effect of flavone derivatives. Hence in the present study four new dihydroxy flavone derivatives, hitherto not subjected to any biological evaluation were selected. These compounds were subjected to a battery of tests to identify their pharmacological profile.
Study pattern

The pharmacological profile of selected four dihydroxy flavones with close structural relation was carried out in different phases.

1. Since all the chosen dihydroxy flavones were new compounds for biological screening, a preliminary screening and acute toxicity testing were considered imminent.

2. The effect of these compounds on locomotor activity and motor coordination was tested in mice since the subsequent experiments on antinociception essentially involved the assessment of some kind of motor activity of animals.

3. The main focus of the investigation was the assessment of antinociceptive potential of the chosen di-hydroxy-flavones. This was attempted by employing three different well established methodologies viz acetic acid induced abdominal constriction, formalin induced nociception and hot water tail immersion methods.

4. The role of opioid system in the antinociceptive action of di-hydroxy-flavones was examined by using an opioid antagonist naloxone. Since dihydroxy flavone induced antinociception involved opioid mechanism, possible development of tolerance to the above action was considered essential.
5. The potent antinociceptive activity observed for these compounds enlarged the scope of the study to screen these compounds for possible anti inflammatory action. This was achieved by selecting a time tested screening method viz- carageenan induced hind paw edema in rats.

6. The effect of dihydroxy flavones on some mediators of pain and inflammation was investigated in a subsequent study. The influence of di-hydroxy flavones on nitrergic system, prostaglandins, interleukin-6 and tumour necrosis factor-alpha (TNF-α) and reactive oxygen species /free radicals was studied using suitable in vivo and in vitro experiments.

7. The antinociceptive and anti inflammatory activities for a combination of different dihydroxyflavones with standard analgesic and antiinflammatory drugs was investigated to delineate any possible synergism.

8. The effect of the test compounds per se and with aspirin treatment on gastric mucosa was also investigated in rats.

The above battery of tests yielded interesting results and are discussed below in relation to the existing literature in this area of research.
Preliminary screening and acute toxicity

The results of the acute toxicity tests revealed that the tested dihydroxy flavones did not produce any mortality in mice even in a dose of 2g/kg indicating the safe nature of these compounds. This observation is in line with many earlier reports on flavonoids. Flavone and its monohydroxy compounds did not also exhibit any toxicity upto 2g/kg in mice (Thirugnanasambantham (1987). In a subsequent study Girija (2000) also investigated a few dihydroxy flavones and reported a similar observation. It is pertinent to reiterate that “the margin of safety for the therapeutic dose of flavonoid in human is considered to be very large, probably much superior to any other drug in current use” (Havsteen., 2002). One of the main objectives of the present study is to identify safe and effective compounds for the management of pain and inflammation. The present results revealing the safe nature of the investigational compounds is the first step achieved in the above goal.

Effect on motor activity

The available experimental models for antinociception in animals essentially involve the assessment of some kind of motor activity in response to a noxious stimulus. The abdominal constrictions induced by acetic acid, lifting of tail from hot water and the biting response to the intraplantar injection of formalin require co-ordinated movements. Any substance interfering with motor activity is likely to yield false positive results. Therefore in the present study the effect of dihydroxyflavones on the spontaneous motor activity in mice was tested using an actophotometer and the effect on muscle co-
ordination was tested using a rotarod. The motor activity of mice and the balancing time on a rotarod were not altered by treatment with different doses of dihydroxy-flavones. Thus it can be inferred that the test compounds may not influence the motor activity in the doses employed. The findings of the present results are akin to that recorded for other flavonoid substances such as gossypin (Viswanathan et al., 1984) flavone, its mono hydroxy and monomethoxy derivatives (Thirugnana sambantham, 1987) and a few dihydroxy flavones (Girija. 2000). It can also be presumed that any response recorded in the antinociceptive assay procedure may be considered a true response.

Methods for studying antinociception

The selected dihydroxy flavone derivatives were subjected to three different assay procedures to assess the anti nociceptive action. Acetic acid induced abdominal constriction (Koster et al., 1959) is regarded as a very sensitive method which employs a chemical to induce minimal noxious stimulus. The advantage of this method is that even weaker analgesics can be detected from the results of this test. Hot water tail immersion (Sewell and Spencers., 1976) is also a well established procedure for antinociceptive assay which employs a high degree of thermal nociception. Hence compounds exhibiting good antinociceptive effect in this method may be considered as potent analgesics.

Another evaluation method selected for assessing antinociceptive effect of dihydroxy flavones in the present study was the formalin test. This
method measures the ability of a substance to attenuate moderate continuous pain generated by injured tissue. Thus it differs from other traditional tests of nociception which depend upon brief stimuli of threshold intensity where the nociceptive experience is short lasting (Tjolsen., 1992). This test was introduced by Dubisson and Dennis (1977) and modified by Takahashi et al. (1984) and Hunskaar et al., (1985). Intraplantar injection of formalin results in licking and biting of the injected paw. This nociceptive behaviour appears in two distinct phases which seem to involve two distinctly different stimuli. The first phase starts immediately after injection of formalin and lasts for about 5 minutes. It is probably due to direct chemical stimulation of nociceptors (Dubisson and Dennis., 1977) with selective stimulation of C fibres and not A δ afferents (Heapy et al., 1987). This is followed by a quiescent period of 10-15 minutes when the animals may not exhibit any nociceptive behaviour. The second phase starts approximately 15-20 minutes after formalin injection and may extend for 20-40 minutes. The second phase 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).

This method has been employed to investigate a variety of compounds including opioids, NSAIDS, and monoamines (Calcagnetti et al., 1988, Hunskaar and Hole., 1987, Rosland et al., 1990). Formalin test was employed by Rajendran et al., (2000) to investigate the antinociceptive activity of flavone and to analyze the mechanism of action. Hence in the present study formalin test was also included to assess the antinociceptive activity of dihydroxy flavones. Earlier investigations for the assessment of
antinociceptive action of several monohydroxy flavones, monomethoxy flavones (Thirugnanam-Sambantham et al., 1990, 1993) and a few dihydroxy flavones (Girija et al., 2002) have restricted mainly to acetic acid assay and hotplate methods. This is the first attempt to extend the investigations involving another assay model for the antinociceptive evaluation of flavone derivatives. It was considered essential to employ “different tests which differ with regard to stimulus quality, intensity and duration so as to obtain as complete a picture as possible of the antinociceptive properties of any new substance using behavioural nociceptive tests” (Tjolsen., 1997). Thus the selection of three antinociceptive testing procedures in the present study is justified.

Antinociceptive effect of dihydroxyflavones

The findings of the present study indicate that, subcutaneous administration of dihydroxy flavones produced consistent and dose related antinociception when assessed by acetic acid-induced visceral nociception in mice. In a dose of 200mg/kg all the tested dihydroxy flavones exhibited nearly 100 percent inhibition of abdominal constriction response (Fig 5-12, Tables 9-12). The ED50 values observed for 2', 3'- dihydroxy flavone (13.8mg) 2', 4'- dihydroxy flavone (12mg), and 5, 3'- dihydroxy flavone (11.48 mg) lie very close to each other indicating equipotent anti nociceptive activity in this assay model (Table-21).

Eventhough the ED50 of 7, 3'- DHF was 43.8mg, maximum efficacy in inhibiting the abdominal constrictions was achieved in a dose of 200mg
similar to other dihydroxy flavones. It is pertinent to note that morphine in a dose of 5mg was able to produce near 100 percent inhibition of abdominal constrictions. Although the dihydroxy flavones apparently are less potent than morphine, maximum efficacy (100 percent inhibition) was achievable with all the tested dihydroxy flavones.

The results obtained from formalin test also substantiate the antinociceptive effect of dihydroxy flavones. A significant and dose related antinociception was evident for all the tested dihydroxy flavones against both neurogenic (early phase) and inflammatory (late phase) pain responses caused by formalin injection in mice. All the dihydroxy flavones in a maximum dose of 200 mg/kg produced nearly 75-80% inhibition of early phase (neurogenic) of formalin nociception (Table13-16, Fig13-20). However, in the same dose range almost 100% inhibition of the late phase (inflammatory) of the pain response was demonstrable for all the tested dihydroxy flavones.

The ED$_{50}$ values of different dihydroxy flavones for the abolition of early phase of formalin pain response ranged between 13-26 mg (Table -21). But the late phase of formalin response was abolished by these compounds even at a much lower range. The ED$_{50}$ of various dihydroxy flavones in this phase ranged between less than 3 to 6.25mg. This finding indicates a much more favourable effectiveness of dihydroxy flavones on inflammatory phase of formalin response.

Morphine in a dose of 5mg produced near 100% inhibition of both the phases of formalin pain response. Earlier reports indicate that morphine
(5mg/kg s.c) could inhibit both the phases of formalin induced nociception in mice and naloxone could effectively antagonize the effect of morphine in both the phases (Ardenghi et al., 2006). The present observations on morphine confirm the earlier reports. In an earlier study Rajendran et al. (2000) reported the antinociceptive effect of flavone in formalin induced pain. Flavone was shown to inhibit both the phases of the above pain response. Flavone in a dose of 50mg/kg produced more reduction (89.1%) in the late phase than in the early phase (60.9%) of formalin response. A similar pattern of response has been recorded in the present study for the dihydroxy flavones. While 100% inhibition was attainable in the late phase of formalin nociception, a maximum of 79 % inhibition could only be recorded in the early phase. Significant inhibition of pain response by dihydroxy flavones in both the phases of formalin nociception indicates the effectiveness of these compounds against both neurogenic (early phase) and inflammatory (late phase) nociception. Moreover 100% inhibition of the inflammatory phase of formalin nociception may suggest more preferential / predominant effect of dihydroxy flavones on inflammatory pain.

The results of thermal model of nociception provide further support to the antinociceptive efficacy of dihydroxy flavones. A significant increase in the reaction time was evident in a dose dependent fashion for all the tested dihydroxy flavones (Tables 17-20). While morphine (5mg) treatment produced 91 percent inhibition of thermal nociception, different dihydroxy flavones in the maximum dose (200mg) tested produced 68-78% inhibition of the thermal nociceptive response (Fig 21-28).
The ED$_{50}$ values for different dihydroxy flavones ranged between 90 and 120mg suggesting almost equipotent activity in this nociceptive assay. Previous studies by Thirugnana sambantham et al., (1990, 1993) demonstrated the antinociceptive effect of certain hydroxy and methoxy derivatives of flavone in thermal nociception. The present study also confirms the ability of flavone derivatives in attenuating thermally induced pain.

**Maximum efficacy**

The results of the different antinociceptive assays when considered together reaffirm the analgesic potential of dihydroxy flavones. Perusal of literature indicates that flavone nucleus *per se* has inherent analgesic action which was modified to varying degrees by different substitutions (Thirugnana sambantham et al., 1990, 1993). In another recent study a few dihydroxy flavones were also shown to exhibit significant antinociceptive activity (Girija et al., 2000). In most of the above studies, the investigational compounds (mono and di substituted 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. A salient finding of the present study is that almost 100% inhibition was achieved in a dose of 200mg for all the four new dihydroxy flavones screened in the above procedure.

One of the aims of the present study was to identify more effective antinociceptive compounds from among the flavonoid family. The present investigation has succeeded in this search and has identified more effective
antinociceptive flavones than those previously reported. This assertion is further corroborated by the results of formalin assay, where maximum inhibition of the late phase of nociception could be demonstrated for the tested compounds. The acetic acid induced nociception is considered as a typical model of inflammatory pain (Tjolsan and Hole, 1997) and the late phase of formalin pain is also considered to represent the inflammatory pain response (Tjolsan, et al 1992). The maximum inhibitions of both the above responses suggest that, the investigated dihydroxy flavones may be very effective in inflammatory pain. However significant inhibition of the early phase of formalin nociception (neurogenic pain) and thermal pain by the above compounds indicates that the screened compounds will also be effective in pain of different origin.

**Structure activity relationship of flavones**

A few studies carried out earlier have investigated the antinociceptive effect of several flavone derivatives and also attempted to suggest a possible relation between the structure of flavones and antinociceptive activity. In a carefully designed in-depth study, Thirugnanasambantham (1987) synthesised and investigated the pharmacological profile of the basic flavone nucleus, many of its monohydroxy, monomethoxy, two dihydroxy flavones and two flavone glucosides. In another extensive work Girija (2000) screened six dihydroxy flavone derivatives for their antinociceptive and other pharmacological actions.
In addition to these reports several poly hydroxy flavones and flavonol glucosides like hydroxy ethyl rutoside (Ramaswamy et al., 1980), gossypin (Viswanathan et al., 1984), morin and rutin (Thirugnana sambantham et al., 1987), quercetin (Anjaneyulu et al., 2003; Kaur et al., 2005), kaempferol 3,7-o-α-di rhamnoside (Toker et al., 2004) and myricitrin (Meotti et al., 2006a) were also found to exhibit significant antinociceptive activity in various experimental models.

All the above data clearly suggest that flavone nucleus by itself may possess inherent antinociceptive activity which could be modified by various substituents in different positions. In fact the studies of Thirugnana sambantham (1987) established this primary fact and conclusively proved the antinociceptive activity of flavone nucleus by two different assay models.

After elaborately studying several flavone derivatives he suggested certain structural determinants for the antinociceptive activity of flavones. These can be stated as follows.

1. Flavone nucleus per se possesses antinociceptive action which was found to involve opioid mechanism.

2. Several monohydroxy flavones, mono methoxy flavones, two dihydroxy flavones and two flavone glucosides also exhibited varying degrees of antinociceptive activity.
3. Substitution with either hydroxyl or methoxyl group at 5, 7 or 2’ position of flavone nucleus involve opioid mediated antinociception.

4. Saturation of the double bond at 2, 3-position in the flavones ring abolished the antinociceptive effect.

In a subsequent study Girija., (2000) reported the antinociceptive property of six dihydroxy flavone derivatives. These compounds were structurally related to the monohydroxy flavones studied earlier (Thirugnana sambantham., 1987). Among the various compounds studied Girija reported enhanced antinociceptive potency for 7, 2’ - dihydroxy flavone when compared to its respective monohydroxy compounds. The other compounds investigated by Girija (2000), 3, 2’ - dihydroxy flavone, 3, 6 -dihydroxy flavone, 3,4’ -dihydroxy flavone, 6,7 dihydroxy flavone and 3’4’-dihydroxy flavone were found to be less effective in antinociceptive activity than their respective monohydroxy flavones. These findings are intriguing. Thirugnana sambantham., (1987) postulated that dihydroxylation of flavone nucleus could augment antinociceptive activity based on his studies. However this could not be corroborated by further studies carried out by Girija (2000), who reported enhanced antinociceptive efficacy only for 7, 2’- dihydroxy flavone compared to its respective monohydroxy flavones. Moreover, many of the flavone compounds reported in the above studies were found to exert only upto 70% of maximum inhibition of nociceptive activity in the acetic acid induced pain. The above contradictory findings of the earlier workers and the keen desire to
identify more effective flavone derivatives motivated the present investigation with some more dihydroxy flavone compounds.

In earlier studies though several dihydroxy flavones were synthesised and investigated, compounds with a hydroxyl group at 3’-position were not attempted so far except 3’,4’-DHF (Girija., 2000). In the present study a few compounds with one hydroxyl group in 3’-position and another hydroxyl substitution in a carefully chosen second position of the flavone nucleus were selected for investigation. In addition, another compound 2’, 4’- DHF which has not been earlier investigated was also included for antinociceptive studies.

The antinociceptive activity of dihydroxy flavones may be considered in comparison with the antinociceptive activity of respective mono hydroxy flavones studied by the same method and reported earlier by Thirugnanasambantham., (1987).

When another hydroxyl group was introduced at the 3’-position of 2’-hydroxy flavone, the resultant 2’, 3’- DHF offered enhanced antinociceptive efficacy compared to 2’-hydroxy flavone reported earlier. A maximum of 100% inhibition of nociception was achieved with an ED50 of 13.8 mg/kg compared to 2’-hydroxy flavone which could exhibit only 66% maximum inhibition in a dose of 200 mg/kg with an ED50 of 87 mg.

Another compound selected in the present study was 5, 3’- di-hydroxy-flavone. 5- Hydroxy flavone per se in a dose of 100mg produced a maximum
of 82% inhibition of nociception with an ED$_{50}$ of 31 mg. Introduction of another hydroxyl group in the 3' position of this compound enhanced the antinociceptive efficacy. 5, 3'-DHF produced 100% inhibition of nociception (200mg/kg) and the ED$_{50}$ was found to be 11.5 mg.

Hydroxylation at, 3’- position of 7-hydroxy flavone markedly improved the antinociceptive efficacy of 7-hydroxy flavone. In a dose of 200 mg/kg, 7-hydroxy flavone offered a maximum of 62% inhibition of nociceptive response with an ED50 of 125 mg/kg. 7, 3’ - DHF produced 100 % inhibition of nociception in a dose of 200 mg and ED$_{50}$ was 44mg.

Another new compound studied in the present investigation was 2', 4'-DHF. 2'- hydroxy flavone and 4'- hydroxy flavone in a maximal dose of 200 mg produced 66% and 72% inhibition of acetic acid induced nociception with an ED$_{50}$ value of 87mg and 34.6mg respectively. The results of the present study indicate that when both these positions are hydroxylated the resultant compound 2’, 4’ - DHF produced almost 100% inhibition of acetic acid - induced nociception with an ED$_{50}$ value of 12.5 mg.

To sum up the structural correlations, it can be stated that introduction of a hydroxyl group at the 3'- position of many mono hydroxy flavones has improved the antinociceptive efficacy of the respective mono hydroxy flavone. The suggestion put forth by Thirugnana sambantham (1987) that, introduction of additional hydroxyl groups in the flavone ring may result in more efficacious compounds, has been substantiated in the present study. Augmented antinociceptive efficacy of the dihydroxy flavones reported in the present
study when compared to their respective mono hydroxy flavones, adds credence to the above proposal.

**Mechanism of dihydroxy flavone induced anti nociception**

Previous results on the antinociceptive action of many flavone derivatives described the participation of opioid like mechanism in mediating their antinociceptive action. Compounds like epicatechin and gossypin were first reported to mediate antinociceptive action through opioid mechanism (Viswanathan.,1984). In subsequent studies many monosubstituted flavones were also reported to possess opioid mediated antinociceptive activity (Thirugnana sambantham et al., 1990, 1993).

The involvement of opioid mechanism in the antinociceptive action of quercetin (3, 5, 7, 3′, 4′ -penta hydroxy flavone) was reported by Naidu et al (2003) and Anjaneyulu et al (2003). Moreover opioid mediated delaying of small intestinal transit was also established for many flavone derivatives (Viswanathan., 1984, Thirugnana sambantham et al., 1998, Girija., 2000).

Hence in the present study the role of opioid system in the antinociceptive action of dihydroxy flavones was investigated. Naloxone a non selective opioid antagonist was able to reverse the antinociceptive activity of all the presently investigated dihydroxy flavones. This observation confirms the earlier reports and conclusively suggests a role for opioid mechanism in the antinociceptive action of dihydroxy flavones.
Flavone structure and opioid interaction

It was reported that substitution of a hydroxyl group at 5, 7 or 2’-position of the flavone nucleus favoured opioid mediated antinociception and flavone compounds with substitution of hydroxyl group at 3, 6, or 4’-position exhibited antinociceptive effect independent of opioid mechanism. (Thirugnanam Sambantham et al., 1990). However this specific structural requirement for opioid mediated antinociception was not proved in further studies. In fact, Girija et al., (2000) have reported opioid mediated antinociceptive effect of various dihydroxy flavones which had hydroxyl substitutions at different positions of the flavone nucleus including 3, 6 or 4’ position. Moreover in the present study all the investigated dihydroxyflavones (including 2’, 4’-DHF) involved opioid mechanism in mediating antinociception. Thus introduction of two hydroxyl groups in the flavone nucleus confers a more favourable affinity towards opioid receptor binding than monohydroxy substitution.

Further studies pertaining to the molecular interaction between flavones and opioid receptors may throw more light on the opioid agonistic properties of flavones. With the presently available limited data it may not be possible for an elaborate discussion on the flavone-opioid receptor interaction.

Moreover participation of other mechanisms in the antinociceptive activity of dihydroxy flavones can not be excluded. This suggestion stems from the fact that the investigated dihydroxy flavones were very effective in alleviating the nociception induced by formalin and acetic acid which are
primarily considered as inflammatory nociceptive agents. Myricitrin was found to possess pronounced antinociception against chemical and mechanical models of pain in rodents. Myricitrin has been suggested to involve an interaction with nitric oxide-L-Arginine and protein kinase C pathways and excluded the participation of opioid system (Meotti et al., 2006). The role of opioid, dopaminergic (D2) (Naidu et al., 2003 a) and α2 adrenergic mechanisms (Kaur et al., 2005) in the antinociceptive action of quercetin has been reported. The above reports also suggest multiple pathways in the antinociceptive action of flavonoids.

The observation of opioid receptor involvement in the antinociceptive action of dihydroxy flavones raises an important question regarding tolerance development upon repeated administration of dihydroxy flavones. This was addressed by designing suitable experiments to investigate the acute as well as chronic tolerance to dihydroxy flavone induced antinociception.

The results indicated that the antinociceptive effect of the investigated compounds did not change significantly after repeated administration, either in acute or chronic fashion. This observation indicates the absence of either acute or chronic tolerance to the antinociceptive action of dihydroxy flavones. A similar observation has been reported for gossypin (Viswanathan., 1985), monohydroxy flavones Thirugnana sambantham, (1987) and certain dihydroxy flavones (Girija et al., 2002).

It is difficult to explain the lack of tolerance to the antinociceptive effect of dihydroxy flavones despite the involvement of opioid mechanism in the
above action. Perhaps the participation of other multiple pathways in the antinociceptive action of many flavone compounds as described earlier, might be responsible for the absence of tolerance noted for many flavone derivatives.

**Peripheral mechanism**

In recent times the action of both endogenous and exogenous opioids at many sites outside the central nervous system has been also considered to be involved in opioid mediated analgesia. Pain due to the inflammation appears to be very sensitive to peripheral opioid action (Gutstein and Akil., 2006).

Functional μ receptors have been identified on the peripheral terminals of sensory neurons. Decrease in sensory neuron activity and release of transmitters have been coupled to the stimulation of peripheral μ receptors.

During inflammation, immune cells capable of releasing endogenous opioids are present near sensory nerves (Stein., 1993). All the above findings provide a strong evidence for the peripheral action of opioid like agents in alleviating pain especially due to inflammation.

The results of the present study reveal a prominent antinociceptive action of flavonoids especially on inflammatory models of pain. Blockade of these responses by naloxone reveals the involvement of opioid receptors in the above action. It may be suggested that the investigated dihydroxy
flavones may preferentially act in the periphery at inflammatory sites and recruit opioid receptors to mediate this effect.

The absence of tolerance to the flavonoid induced antinoiciception may possibly be due to such a peripheral mode of action. This suggestion stems from the fact that administration of the dihydroxy flavones did not produce any behavioral alteration in the experimental animals indicating lack of central effects. However these aspects require further detailed investigation. It can also be suggested that the lack of tolerance to the antinociceptive action may be therapeutically exploited.

**Anti inflammatory effect of dihydroxy flavones**

One of the earliest therapeutic applications of flavonoids is in the treatment of some inflammatory diseases. The beneficial effects of flavonoids in rheumatoid arthritis (Rinehart., 1955) and in gingival inflammatory conditions (Carvell and Halperin., 1961) are some of the earliest reports in this regard. Subsequently, many flavonoid compounds have been reported to possess significant antiinflammatory activity in several animal models of acute and chronic inflammation; taxifolin (Gupta et al., 1971), gossypin (Parmar and Ghosh., 1978), hesperidin (Shahidi et al., 1998), naringin (Havsteen.,2002), titonine and its derivatives (Carvalho et al.,1999) and silymarin (Gupta et al.,2000 ).

In two detailed studies, the anti inflammatory activity of monomethoxy flavones (Muthiah et al., 1993) and mono hydroxy flavones (Arivudainambi,
were investigated. It was reported that hydroxy derivatives of flavone were potent anti-inflammatory agents than their corresponding methoxy derivatives (Arivudainambi., 1996). Since the earlier studies indicated marked antiinflammatory activity for monohydroxy flavones, the higher homologous series viz; the dihydroxy flavones were also studied for the effect on acute inflammation. Moreover in the present study all the four dihydroxy flavones were found to exert potent antinociceptive activity especially in inflammatory models of pain like acetic acid writhing and formalin induced nociception. Hence an investigation on their antiinflammatory activity was considered imminent.

The compounds were screened for their effect on acute inflammation using carageenan induced paw edema, a well established animal model. Edema represents the early phase of inflammation and the above method is the simplest and most widely used model for studying the antiinflammatory activity of new compounds (Ghosh., 2005). Substances that are found to significantly reduce the paw edema have been therapeutically correlated to possess good antiinflammatory activity.

The results of the present study indicate a dose and time dependent antiinflammatory activity of all the tested dihydroxy flavones. The marked reduction in paw edema was clearly evident in doses of 10 and 50 mg upto 5 hours of observation period. All the four dihydroxy flavones produced nearly 81-88 % inhibition of inflammation when employed in a dose of 50mg/kg. Thus they can be considered as equally effective in their antiinflammatory activity.
activity. In an earlier study, monohydroxy flavones like 2’- hydroxy flavone and 4’ -hydroxy flavone showed less than 50% inhibition of paw edema (Arivudainambi et al., 1996). However 2’, 4’- DHF investigated in the present study inhibited rat paw edema to the extent of 88 percent. In general the antiinflammatory efficacy of dihydroxy flavones appear to be greater than monohydroxy flavones. The hypothesis of Arivudainambi et al., (1996) that polyhydroxy flavones may exert potent antiinflammatory activity has been confirmed by the results of the present study. The present result further supports the earlier findings on the antiinflammatory effect of flavonoid compounds. The novel antiinflammatory activity of dihydroxy flavones coupled with their antinociceptive property has enormous therapeutic applications.

The currently available drugs used to treat inflammation mainly belong to two important groups; glucocorticoids and NSAIDs. Glucocorticoids though potent in the antiinflammatory effect can also cause serious adverse effects. Long term use of steroids may affect almost all systems of the body and may result in metabolic derangement, immunosuppression, osteoporosis, myopathy, cataract, peptic ulceration and cushing syndrome (Schimmer and Parker., 2006). The other group of drugs NSAID also on regular use produce many intolerable side effects. Similar to corticosteroids NSAID also can produce extensive gastric mucosal damage and ulceration. Induction of gastric or intestinal ulcer is known to affect 15 to 30% of NSAID users. The concurrent use of corticosteroid or alcohol consumption further aggravates the above risk.
Eventhough COX-2 selective inhibitors have much less propensity to induce gastric ulcers, cardiovascular complications resulting from the use of COX-2 inhibitors are limitations for their regular use (Bresalier et al., 2005). Regular use of NSAID may result in salt and water retention, edema, and decreased efficacy of antihypertensive drugs or diuretics. The inhibitory effect of NSAID on platelet function increases the risk of hemorrhage. In addition, many CNS adverse effects like vertigo, dizziness and lowering of seizure threshold and many hypersensitivity reactions are also associated with NSAID usage (Burke et al., 2006).

The aforementioned adverse effects of conventional anti inflammatory drugs necessitate the search for newer and safer anti inflammatory agents. Flavonoid group of drugs appear to be promising in this regard. This proposition arises from the fact that potent anti inflammatory flavonoids were found to be devoid of ulcerogenic potential, the major adverse effect of NSAID or glucocorticoids. In addition gastric antiulcer effect of catechin, naringin, naringenin, gossypin, β-hydroxy ethyl rutosides, and (+) cyanidanol-3 has been established in several experimental animal models. The above flavonoids protected the animals from ulcers induced by pyloric ligation, restraint and drugs (Parmar., 1977, Parmar and Parmar., 1998). The potent antiulcer activity of kaempferol was also reported (Goel et al., 1988). Alcohol induced gastric mucosal damage was prevented by wogonin (Park et al., 2004) which also exhibited potent anti inflammatory activity (Jang et al., 2002).
Thus many flavonoid compounds while exhibiting potent anti-inflammatory activity are devoid of ulcerogenic property. Such an ulcer-sparing effect of dihydroxyflavones requires confirmation. Experiments were designed to investigate this possibility and the results are discussed in a subsequent section.

**Effect of dihydroxy flavones on certain mediators of inflammation and pain**

Various types of endogenous chemical mediators play a significant role in bringing about the different reactions of the inflammatory process like vascular permeability, vasodilation, chemotaxis, cellular migration and tissue damage and also stimulate a variety of nociceptors. Since the dihydroxy flavones exhibited a potent anti-nociceptive and anti-inflammatory activity it was considered interesting to investigate the effect of these compounds on certain mediators of inflammation and pain.

Prostaglandins are an important group of lipid derived mediators implicated in pain and inflammation. Many cytokines like TNF-α and IL-6 mediate various reactions in the cascade of inflammation and in sensitization of pain fibers. Reactive oxygen species/free radicals have also been found to play a crucial role in many types of inflammation and arthritis. Moreover, many recent evidences indicate a potential role for nitric oxide in the development of inflammation and pain. Hence in the present study, the effect of dihydroxy flavones on the above mediators was studied by employing suitable *in vitro* and *in vivo* experiments.
Effect of dihydroxy flavones on cyclooxygenase

Cyclooxygenase (COX) is the first enzyme in the synthetic pathway to form prostaglandins from arachidonic acid. A good correlation has been reported between the potency of an agent as COX inhibitor and its antiinflammatory activity. Among the two well understood isoforms of cyclooxygenase, COX-1 is primarily a constitutive in nature that maintains tissue homeostasis while COX-2 is an inducible form released from inflammatory cells and is considered responsible for the synthesis of prostanoid mediators of inflammation (Vane and Botting., 1998). Most of the NSAID inhibit COX-1 and COX-2 with little selectivity. However COX-2 inhibitors have been developed with markedly less gastric irritation potential.

It was considered interesting to investigate the effect of dihydroxy flavones on cyclooxygenase activity. The results of the present equipments revealed marked inhibitory activity of cyclooxygenase enzyme by the tested dihydroxy flavone derivatives. Both the isoforms of cyclooxygenase COX-1 and COX-2 were inhibited to varying degrees by the tested dihydroxy flavones. The inhibition of COX-1 ranged between 38 and 57 percent for all the different dihydroxy flavones. The inhibition of COX-2 ranged from 58 to 68 percent for the above compounds in the same concentration. It is very obvious that the dihydroxy flavones exert a significant inhibitory action on cyclooxygenase responsible for generating prostaglandins which is an important mediator of inflammation and pain. Though potent inhibition of COX-1 and COX-2 was evident for dihydroxy flavones, a higher degree of
COX-2 inhibition was apparent than COX-1 inhibition for the same concentration of dihydroxy flavone.

Many flavone compounds have earlier been shown to inhibit prostaglandin synthesis as detailed in an earlier section luteolin and chrysin (Harris et al., 2006), 5,7-dihydroxy ,7-methoxy flavone ( Daott et al.,2003), 2,4,7-trimethoxy flavone ( Chang et al.,2005) and wogonin ( Park et al., 2001 and Chi et al., 2005) are widely reported for their inhibitory effect on prostaglandin synthesis.

The present studies have also identified a homologous series of dihydroxy flavones with potent inhibitory action on cyclooxygenase. This finding brings out the important mechanism by which the dihydroxy flavones exert their potent antinociceptive and anti inflammatory actions.

**Effect of dihydroxy flavones on certain cytokines (TNF-α and IL-6 )**

Cytokines are a large group of biologically active proteins released during tissue injury or infection. They are secreted by monocytes, macrophages and other types of cells like adipose cells. The cytokine super family includes interferons, several interleukins, tumour necrosis factor and various growth factors. In a complex co-ordinated net work, they act on leucocytes, vascular endothelial cells, mast cells, fibroblasts, haemopoetic stem cells and osteoclasts (Rang et al., 2003). Some cytokines have pro inflammatory actions and some other cytokines have anti inflammatory actions. The primary pro inflammatory cytokines are tumour necrosis factor-
alpha (TNF-α) and interleukins (IL-1 and IL-6) which are implicated in many inflammatory and immunological diseases and induce the formation of other cytokines. The anti-inflammatory cytokines include IL-4, IL-10, IL-13 and tumour growth factor β (TGF-β). The concentrations of many inflammatory cytokines are increased in the synovium of patients with inflammatory arthritis. At the site of inflammation, peptides like substance P are also elevated which promotes the firing of pain fibres. Glucocorticoids are known to interfere with the synthesis and actions of cytokines such as IL-1 or TNF-α (Burke et al., 2006).

**Tumour necrosis factor -alpha (TNF-α)**

Tumour necrosis factor-alpha (TNF-α), a pro inflammatory cytokine is involved in the pathophysiology of a number of disorders including crohn’s disease, rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis. Monoclonal antibodies like infliximab and soluble TNF-receptors etanercept directed against TNF-α are used in treating rheumatoid arthritis and ankylosing spondylitis (Singh and Suruchi., 2007).

TNF-α is a polypeptide primarily produced by activated monocytes and macrophages. This poly peptide mediates many immune and inflammatory responses including activation and differentiation of monocytes and macrophages and expression of adhesion molecules on the endothelial cells and can stimulate PGE2 synthesis which in turn mediates its own effects and those of TNF-α and IL-1 thereby perpetuating the inflammatory response by releasing a cascade of cytokines.
TNF-α inhibition by dihydroxy flavones

In the present study, the basic flavone nucleus exhibited a minimal inhibitory action on TNF-α. However, introduction of two hydroxyl groups at various positions appears to potentiate the TNF-α inhibitory activity of flavone. A dose dependent inhibition of TNF-α activity was clearly evident for all the dihydroxy-flavones. 7, 3'-DHF and 5, 3'-DHF produced a higher inhibition of TNF-α compared to other tested compounds.

Inhibition of TNF-α has been previously reported for many flavone derivatives. The over production of TNF-α and nitric oxide by activated macrophages was markedly inhibited by quercetin (Manjeet and Ghosh., 1999).

Luteolin (3’, 4’, 5, 7 tetrahydroxy flavone) has been shown to inhibit TNF-α production in vitro and in vivo and to exert good anti-inflammatory activity (Ueda et al., 2004). Luteolin has been further shown to possess inhibitory effect on TNF-α –induced IL-8 production in intestinal epithelial cells (Kim et al., 2005b).

The presence of hydroxyl groups in the flavone nucleus has been suggested as an important determinant in this activity. Many flavonols like apigenin, luteolin, chrysin, kaempferol, quercetin, bicalaein and flavone have been shown to inhibit TNF-α induced upregulation of inter cellular adhesion molecule-1 which has been implicated in inflammation and carcinogenesis (Chen et al., 2004).
Amoradisin isolated from Amorpha fusicosa significantly inhibited TNF-α production in LPS-stimulated macrophages (Cho et al., 2000). Dosmalfate a derivative of diosmin is considered to possess antioxidant and cytoprotective effects. Dosmalfate significantly decreased the colonic mucosal production of TNF-α in an experimental model of colitis (Villegas et al., 2003). Morin was earlier shown to exert a potent antinociceptive action (Thirugnanasambantham et al., 1985). Recent studies highlight the use of morin and its derivatives in inflammatory diseases by their inhibitory action on TNF –α and nitric oxide production from activated macrophages (Fang et al., 2003).

Certain kaempferol glucosides isolated from Cinnmomum osmophloeum inhibited LPS-induced TNF–α besides nitric oxide and IL-12 in a dose dependent manner (Fang et al., 2005). The inhibitory activities of flavonoids from Caesalpinia pulcherimma on nitric oxide, TNF–α and IL-12 has been demonstrated by Rao et al., (2005). Naringinin chalcone which is present in tomatoes, dose dependently inhibited the production of pro inflammatory mediators especially TNF–α and nitric oxide (Hirai et al., 2007). The result of the present study is in agreement with the earlier reports revealing an inhibitory effect on one of the pro inflammatory cytokine, TNF –α.

Effect of dihydroxy flavones on Interleukin-6 (IL-6)

Interleukin-6, which was earlier identified as a B-cell differentiation factor has been found to exert many functions in regulating the immune response, haemopoiesis, acute phase response and inflammation. Increased IL-6 levels are observed in rheumatoid arthritis, osteoporosis and psoriasis.
(Ishihara and Hirano, 2002). The definite role of IL-6 in inflammation has been confirmed by the ability of auto antibodies of IL-6 to protect experimental animals against collagen induced arthritis and allergic encephalitis (Galle et al., 2007).

In a recent study involving rheumatoid arthritis patients, IL-6 production was analysed in LPS stimulated peripheral blood mononuclear cell cultures. It was found that chronic inter personal stress is associated with greater stimulated cellular production of IL-6 (Davis et al., 2008).

In the present study the effect of dihydroxy flavones on IL-6 production was investigated. The results of the present study indicate that flavone per se in a dose of 25 µM inhibited IL-6 activity by 29%. Different dihydroxy flavones inhibited IL-6 activity in a dose dependent fashion and the activity of these compounds was much superior to flavone nucleus.

Among the dihydroxy flavones, 7, 3’-DHF exerted a maximum inhibitory activity. The results also indicate that dihydroxylation favours the inhibitory effect on IL-6. It can be appreciated that 7, 3’-DHF and 5, 3’-DHF exert maximum inhibitory activity on both the cytokines compared to other tested compounds.

Nobiletin has been identified as a novel immunomodulatory and antiinflammatory drug based on its inhibitory effect on the production of proinflammatory cytokines like IL-6 and TNF –α in mouse macrophage (Lin et al., 2003). A few polyhydroxy flavones like chrysin, apigenin, and luteolin were
evaluated for their effect on pro inflammatory cytokines by LPS stimulated human peripheral blood mononuclear cells. The above flavones dose dependently inhibited the pro inflammatory cytokine production (TNF –α and IL-6 ) and the metabolic activity of LPS stimulated peripheral blood mononuclear cells (Hougee et al., 2005). Apigenin decreased TNF –α induced production of IL-6 and nitric oxide in osteoblasts (Choi et al., 2007a). Luteolin has been shown to decrease the production of nitric oxide, PGE$_2$, TNF–α and IL-6 in osteoblasts (Choi et al., 2007b). Both these compounds have been shown to promote osteoblasts function and suggested for the treatment of osteoporosis.

The present results reveal that the investigated dihydroxyflavones share the IL-6 inhibitory activity with other flavone derivatives reported. The inhibitory action exerted on important inflammatory cytokines TNF–α and IL-6 may affectively contribute to the antiinflammatory actions of dihydroxyflavones.

**Oxidant- antioxidant system**

**Role and significance in human body**

Several types of reactive oxygen species are generated in the body in the form of free radicals as a result of metabolic reactions. These species may be either oxygen derived or nitrogen derived and called pro oxidants. They attack macromolecules including proteins, DNA and lipid etc, causing cellular /tissue damage. To counter their effect, the body is endowed with
another category of compounds called antioxidants. These antioxidants are produced either endogenously or received from exogenous sources.

Endogenous antioxidants include enzymes like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, minerals like Se, Mn, Cu and zinc, and vitamins like A, C and E. Other compounds with antioxidant activity include glutathione, flavonoids, bilirubin and uric acid etc.

In a healthy body, pro-oxidants give rise to oxidative stress. This oxidative stress may be the cause of several diseases such as cardiovascular diseases, neurological diseases, malignancies, renal diseases, diabetes, inflammatory disorders, skin diseases, ageing, respiratory diseases, liver diseases and different types of viral infections (Irshad and Chowdary, 2002).

The oxidants/ free radicals are species with very short half life, high reactivity and damaging activity towards macromolecules. The oxygen derived species include $O_2^-$ (superoxide), HO$^-$ (Hydroxyl), HO$_2$ (Hydroperoxyl), ROO$^-$ (Peroxy), RO$^-$ (alkoxyl) as free radicals and H$_2$O$_2$ (Hydrogen peroxide), HOCl (Hypochlorous acid), O$_3$ (Ozone), and O$^•$ (Singlet oxygen) as non-radicals. Similarly, nitrogen derived oxidant species are mainly NO (Nitric oxide), ONOO$^-$ (Peroxy nitrite), NO$_2$ (Nitrogen dioxide) and N$_2$O$_3$ (Dinitrogen trioxide).

Reactive oxygen species generated from activated phagocytes have been implicated in inflammation and tissue destruction (Krane et al., 1990). Increased levels of peroxides generated from leucocytes accumulate at inflammatory sites (Burke et al., 2006). Reactive oxygen species produced by
neutrophils and macrophages are implicated in tissue damage. During arthritis granulocytes and macrophages accumulate at inflammatory sites and generate large quantity of superoxide and hydrogen peroxide radicals (Holley and Cheeseman., 1993). NSAID like sulindac that possess both COX inhibitory and strong oxygen radical scavenging effect may decrease tissue damage during inflammation (Rang et al., 2003).

Many drugs used in rheumatoid arthritis mediate their therapeutic actions by multiple mechanisms; an important effect being reduction of oxidative damage at inflammatory sites either by inhibiting reactive oxygen production by phagocytes or by scavenging such noxious radicals (Aruoma., 1996). Flavonoids which are abundant in fruits, vegetables and many medicinal plants are effective free radical scavengers and extensive work on the anti oxidant nature of several flavonoid compounds have been recorded. Moreover many beneficial effects of flavonoids have been attributed to the anti oxidant and free radical scavenging properties (Burda and Oleszek., 2001).

In the present study the investigated dihydroxy flavones exhibited potent antinociceptive and antiinflammatory activities. The effect of these compounds on free radical generation/scavenging was investigated against DPPH, hydroxyl and nitric oxide radicals by in vitro methods. A dose dependent inhibition of DPPH free radical generation activity was recorded for all the dihydroxy flavones.
The free radical scavenging activity was further corroborated by the ability of the tested dihydroxy flavones in scavenging hydroxyl and nitric oxide radicals. The activity of different dihydroxy flavones was almost comparable to the standard antioxidant vit E in the above assay procedures. The IC\textsubscript{50} values for scavenging nitric oxide and hydroxyl radicals by different dihydroxy flavones ranged between 10-25 µg/ml whereas the IC\textsubscript{50} values for inhibiting DPPH ranged between 25-50 µg/ml.

Many structure activity relationship investigations have been performed on the antioxidant activity of flavonoids. The reports suggest that, the antioxidant activity of flavonoids strongly depends on the number and position of hydroxyl groups in the molecules. The antioxidant capacity is suggested to be high in compounds with dihydroxylated B-ring (catechol structure), presence of unsaturation and of 4-oxo function in the C-ring (Rice-Evans et al., 1996; Heim et al., 2002; Harborne and Williams., 2000). The antioxidant activity of 28 natural and synthetic hydroxy flavonoids was estimated by DPPH radical superoxide scavenging and inhibition of lipid peroxidation (Furusawa et al., 2005). The above study showed that hydroxylation pattern of the flavone molecule significantly altered the antioxidant nature of the compounds.

This study also revealed the presence of C2, C3 double bond as essential for antioxidant activity. Furusawa et al (2005) proposed that 3’, 4’-dihydroxylation augmented the antioxidant nature of flavone compounds. Tri hydroxy flavones like apigenin (5, 7, 4’-tri hydroxy flavone), galangin (3, 5,
7-trihydroxy flavone), Baicalein (5, 6, 7-trihydroxy flavone) and 3, 3', 4'- trihydroxy flavone, tetrahydroxy flavones like luteolin, kaempferol, fisetin and pentahydroxy flavone like quercetin, morin and hexahydroxy flavones like myricitrin also exhibited potent antioxidant activity in the tested methods (Furosawa et al., 2005).

The above report suggests that dihydroxylation or polyhydroxylation of flavone nucleus favouring the antioxidant activity. The results of the present study are in agreement with the above proposal. All dihydroxy flavones investigated in the present study exhibited potent antioxidant/free radical scavenging activity suggesting that this may be one of the mechanisms for mediating their biological actions.

**Role of nitric oxide in the antinociceptive effect of dihydroxy flavones**

**Nitric oxide and Pain**

Nitric oxide has been implicated in many cellular events and even as a transmitter. It is also considered to play a role in vasodilation, inflammation and immune reactions. Nitric oxide has been shown to be involved in neurogenic inflammation and also plays a role in the genesis of migraine pain (Lincoln *et al.*, 1997).

Recent studies suggest that nitric oxide- CGMP- pathway may be involved in modulation of pain perception (Kulkarni and Manu., 2000). Nitric oxide may be involved in the perception of pain at many levels of nociceptive neural pathways. The presence of nitric oxide synthase (NOS) has been
suggested in the primary afferent neuron, dorsal root ganglia, brainstem, thalamus and several other sensory areas (Lincoln et al., 1997). It has been suggested that nociceptive reflexes involve a glutamatergic NMDA receptor mediated pathway that involves the production of nitric oxide and this leads to the enhancement of the processing or spinal facilitation of afferent input that is ultimately conveyed to the cortex and subsequently manifest in behavioral responses (Meller and Gebhart., 1993).

An inhibitor of nitric oxide synthase L-NAME (L- Nitro Arginine Methyl Ester) has been shown to exhibit dose related antinociceptive activity against formalin induced, acetic acid-induced and thermal pain responses (Kulkarni and Manu., 2000). The antinociceptive effect of diclofenac, paracetamol or ibuprofen was reversed by L-Arginine implying that nitric oxide is involved in central pain pathways (Bjorkman., 1995). A synergistic antinociceptive effect was recorded for a NOS inhibitor, L-NAME and flurbiprofen when employed in sub-threshold doses (Morgan et al., 1992).

Myricitrin, a flavonoid exhibited pronounced anti nociception against chemical and mechanical models of pain and L-Arginine -Nitric oxide pathway is likely to be involved in the antinociceptive effect of myricitrin (Meotti., 2006a). Quercetin is a common flavonoid occurring in many plant species. The antinociceptive activity of quercetin has been reported by many workers (Naidu et al., 2003a, Kaur et al., 2005, and Anjaneyulu and Chopra., 2003). The ability of quercetin to suppress nitric oxide synthase activity has been implicated in the reversal of morphine tolerance and dependence by this
flavonoid (Naidu et al., 2003b). These evidences suggest a role for nitric oxide in the antinociceptive action of flavone compounds. Hence in the present study the role played by nitric oxide in the dihydroxy flavone induced antinociception was studied. The results of present study revealed that L-Arginine pretreatment annulled the antinociceptive effect of dihydroxy flavones. Moreover minimally effective doses of a NOS inhibitor, L-NAME and dihydroxy flavone produced a potentiated antinociceptive effect in combination. These evidences suggest a possible role for nitric oxide in the antinociceptive effect of dihydroxy flavones.

Myricitrin has been found to be effective in many models of persistent inflammatory and neuropathic pain (Meotti et al., 2006 b). It has also been shown to inhibit nitric oxide production and reduce the over expression of nitric oxide synthase (Chen et al., 2000). In a recent work, Matsuda et al., (2003) have examined the structure activity relationship of flavonoids for inhibiting nitric oxide production in lipopolysaccharide (LPS) activated mouse peritoneal macrophages. Several compounds were found to be very effective in inhibiting nitric oxide production and were also shown to inhibit the induction of inducible nitric oxide synthase without iNOS enzymatic inhibitory activity (Matsuda., 2003). Further studies may help to identify the role of dihydroxy flavones in nitric oxide synthesis and the possible involvement in pain modulation.
Nitric oxide is a modulator of many body functions. It is synthesized endogenously from its precursor L-Arginine by an enzyme nitric oxide synthase (NOS). Nitric oxide plays an important role in many physiological processes such as vasodilation, memory, peristalsis, neuroprotection, penile erection and immune defence. Production of large quantities of nitric oxide is implicated in cytotoxic effects observed in various disorders like AIDS, cancer, Alzheimer's and arthritis etc (Shinde et al., 2000).

Nitric oxide has mainly pro inflammatory actions. It is a potent vasodilator; it increases vascular permeability and it increases the vascular production of pro inflammatory prostaglandins. The inducible form of nitric oxide synthase is mainly involved in the inflammatory reactions. All inflammatory cells express the inducible form of the enzyme in response to cytokine stimulation. Nitric oxide synthase has been identified in the mucosa of the colon in patients with ulcerative colitis and in synoviocytes in inflammatory joint disease (Rang et al., 2003).

The pro inflammatory action of nitric oxide seems to play an important role in tissue damage and elevated levels of nitrite has been detected in the synovial fluid of patients with rheumatoid arthritis and osteo arthritis (Nussler and Billiar., 1993, Stefanovic et al., 1993). Similarly, elevated levels of nitric oxide or inducible nitric oxide synthase activity have been associated with many other inflammatory diseases like ulcerative colitis, irritable bowel syndrome and asthma etc (Lincoln et al., 1997).
Increased level of nitric oxide synthesis has been demonstrated in experimental models of arthritis and selective inhibitors of type-II nitric oxide synthase suppress the joint inflammation (Connor et al., 1995). Nitric oxide has been implicated in the destruction of pericellular and extra cellular matrix of cartilage in arthritis (Stephenovic et al., 1993).

A study by Salvemini et al., (1996) investigated the role of nitric oxide derived from constitutive and inducible nitric oxide synthase in carageenan induced paw edema. The increase in paw edema was accompanied by neutrophils infiltration, increased levels of nitrite/nitrate and PGE2 in the paw exudates. The results suggested that nitric oxide produced by constitutive nitric oxide synthase (c-NOS) was involved in the development of inflammation at the early stages and nitric oxide produced by iNOS is involved in the maintenance of inflammatory response at later stages. The possibility of nitric oxide promoting neutrophils infiltration and PGE2 production was also suggested (Salvemini et al., 1996).

Since the previous set of experiments suggested a role for nitric oxide in the antinociceptive activity of dihydroxyflavones, the role of nitric oxide in the anti inflammatory activity of dihydroxyflavones was investigated in the present study. Pretreatment with a nitric oxide precursor L-Arginine markedly attenuated the antiinflammatory activity of various dihydroxyflavones investigated in the present study. This observation indicates a putative inhibition of nitric oxide by dihydroxyflavones while exerting their antiinflammatory activity. This suggestion is further supported by the
observation that combination of minimal antiinflammatory dose of dihydroxyflavones with NOS inhibitor L-NAME exerted a markedly potentiated anti-inflammatory effect. This potentiation was evident from the first hour of observation, and was maintained for extended periods (5 hours). The antagonism of the anti-inflammatory effect of dihydroxyflavones by L-Arginine (NO donor) and its potentiation by L-NAME (iNOS inhibitor) definitely implicates a role of nitric oxide in the actions of dihydroxyflavones. Infact many flavonoids like apigenin, diosmetin and tetra-o-methyl luteolin compounds were found to be potent inhibitors of nitric oxide synthesis (Matsuda et al., 2003). Wogonin, a potent anti inflammatory flavonoid was shown to inhibit iNOS induction and suppress nitric oxide production (Kim et al.,2001). Many derivatives of flavonols, flavones and flavanones inhibited the induction of (iNOS) in LPS treated macrophages (Olszanecki et al.,2002). Synthetic wogonin derivatives were shown to suppress LPS induced nitric oxide production in microglial cells (Chen et al., 2004).

In another study, in-vitro and in-vivo experiments were performed to study the inhibitory activity of rutin, wogonin and quercetin on LPS induced nitric oxide and PGE₂ production. The data suggested that wogonin and quercetin inhibited LPS induced nitric oxide production through suppression of iNOS expression (Shen et al., 2002). Kim et al., (1999) investigated 27 flavone compounds for their effect on NO production. Apigenin, wogonin and luteolin were found to be very active. They suggested the requirement of C- 2, 3 double bond as essential for this inhibition and the potency of inhibition was also dependent upon the substitution of the flavonoid molecule. The
inhibitory effect of flavonoids was not due to direct inhibition of iNOS enzyme activity but might be due to the reduction of iNOS enzyme expression (Kim et al., 1999).

The C-2, 3 double bond is considered to be essential for many biological activities. The C-2, 3 double bond was also reported to be essential for phosphodiesterase inhibition (Beretz et al., 1988). Saturation of C-2, 3 double bond alters the planarity of the molecule which might be a reason for the loss of above biological action (Ferrell et al., 1979). In fact, saturation of C-2, 3 double bond abolished the antinociceptive activity of flavone (Thirugnana sambantam et al., 1990, 1993). In yet another study Kim et al., (2004) suggested certain structural requirements of flavonoids to inhibit nitric oxide production. Some optimal chemical structures suggested by them were A-ring 5,7-DHF having the B-ring 2′,3′ dihydroxy/methoxy groups. Further as proposed by these authors earlier (Kim et al., 1999), these compounds were found to be down regulators of iNOS induction, but not direct iNOS inhibitors. Kim et al., (1999) have further proposed that some tetrahydroxy flavone compounds like luteolin as potential anti inflammatory agents. The results of the present study are in agreement with these findings. The 2′, 3′-DHF (the essential structural requirement suggested by Kim et al., 1999) induced anti inflammatory activity observed in the present study was attenuated by L-Arginine and potentiated by L-NAME.
In a recent study Huang et al., (2007) have investigated certain hydroxy and methoxy flavones and wogonin (5, 7 dihydroxy 8-methoxy flavone) for their effect on nitric oxide production in macrophages.

5, 7 DHF and dimethoxy flavone significantly inhibited LPS induced iNOS protein expression and NO production while 5- hydroxy flavone and 7-hydroxy flavone were inactive. Huang et al., (2007) further suggested that hydroxylation at both C5 and C7 (dihydroxy flavone) is essential for nitric oxide inhibition of flavonoids.

The results discussed in the earlier part of this work conclusively proved the involvement of nitric oxide in the antinociceptive action of dihydroxy flavones in inflammatory model of pain. It is unequivocally clear that inhibition of nitric oxide essentially plays a major role in mediating the antinociceptive and anti inflammatory activities of dihydroxy flavones.

Further studies may identify whether dihydroxy flavones, similar to other reported flavonoids, inhibit the generation of nitric oxide (iNOS expression) or antagonise the cellular effects of nitric oxide.

**Effect of dihydroxy flavones on gastric mucosa**

After conclusively establishing the antiinflammatory activity of dihydroxy flavones it was felt interesting to study their effect on gastric mucosa. The dihydroxy flavones were administered (50mg/kg) subcutaneously as well as orally two for consecutive days to fasting rats. The
dihydroxy flavones in the above dose have already been shown to exhibit potent antinociceptive and antiinflammatory effects. When these animals were sacrificed 5 hours after the second dose, the examination of gastric mucosa did not reveal any ulcer or hyperemia. This was comparable to vehicle treated control animals. This observation indicated that the dihydroxy flavones themselves did not cause any gastric mucosal damage in rats.

In another set of experiment aspirin was administered (200mg/kg) to rats pretreated with various dihydroxy flavones for two days. The results revealed a marked protective effect of dihydroxy flavones against aspirin induced ulceration.

This observation confirms the results obtained with many other flavonoids in earlier studies. The lack of ulcerogenic effect and protection against aspirin induced ulceration opens up two new possibilities. Flavonoids exhibiting high efficacy against pain and inflammation may be identified and used alone to treat the suffering. In addition flavonoids may be useful as an adjuvant with any of the currently used NSAID. This can, on the one hand improve the therapeutic efficacy of NSAID and on the other hand can blunt/offset gastric ulceration, the most feared adverse effect of NSAID.

**Anti ulcer activity**

The results of present investigations indicate a protective role of dihydroxy flavones against aspirin induced gastric ulceration. The gastric mucosal damage induced by ulcerogenic NSAID like aspirin has a
multifactorial basis. Though the inhibition of cytoprotective COX-1 is considered important in the ulcerogenic NSAID, other prostaglandin independent events are also suggested to be involved in the ulcerogenic effect of NSAID.

Besides COX I inhibition free radical generation by NSAID has also been suggested as one of the contributing factors for gastric mucosal damage (Andrew and Soll., 1990; Desai et al., 1997). The free radicals may cause lipid peroxidation, after membrane integrity of surface epithelia cells resulting in formation of gastric ulcers (Naito et al.,1995). The antiulcer activity of quercetin was attributed to the cytoprotective effect through increased mucous production and antioxidant properties (Martin., et al 1998). The gastroprotective effect of naringin involved an increase in the glycoprotein content and viscosity of gastric mucosal gel. The free radical scavenging is also suggested to contribute to the ulcer protective effect of naringin (Martin et al., 1994 ).

The gastric anti ulcer effect of catechin, naringin, Gossypin, β-hydroxy ethyl rutoside and (+) cyanidanol -3 has been established in several experimental animal models (Parmar., 1977, Parmar and parmar., 1988). The antiulcer effect of kaempferol was reported by Goel et al., (1988). A direct action of flavonoids on mucosal capillaries has been postulated to be responsible for the anti ulcer effect of flavonoids (Parmar and Ghosh., 1980) Rutin was shown to exert a cytoprotective effect on rat gastric mucosa against
ethanol induced gastric lesions. But this property did not appear to be mediated by endogenous prostaglandins (Guerro et al., 1994).

The free radical scavenging action of flavonoids has been suggested as a mechanism to protect gastric mucosa (Slater and Eakins., 1975). The dihydroxy flavones investigated in present study exhibited a high degree of radical scavenging action against DPPH, hydroxyl, and nitric oxide. The free radical scavenging effect of dihydroxy flavones may be responsible for the gastric mucosal protection.

**Unique nature of flavonoids**

From the above discussion, it is clear that flavone compounds while exhibiting good antiinflammatory activity are not ulcerogenic in nature. On the contrary they possess a good anti ulcer effect. This unique behavior of flavonoids is very interesting and opens a new avenue in the identification of safe anti-inflammatory drugs

**Synergism with other drugs**

**Anti nociceptive synergism**

In many diseases, a combination of drugs is employed to achieve maximal therapeutic benefit and at the same time reduced adverse effects. In chronic pain situations NSAID are usually combined with opioids to reduce the tolerance development. In the present study various dihydroxy flavones have been proved to exert potent antinociceptive and antiinflammatory activities
utilising multiple pathway. It may be suggested that combination of dihydroxy flavones with currently used analgesic or anti-inflammatory drugs may enhance their efficacy.

The suggestion proposed above that dihydroxy flavones may augment the antinociceptive and anti-inflammatory activity of currently employed drugs required confirmation. This has been attempted by studying the combination of sub analgesic doses of morphine or diclofenac with a minimum dose of dihydroxy flavones on antinociception.

Morphine (0.1mg/kg) *per se* inhibited acetic acid nociception to an extent of 32.5 %. The inhibition of nociception produced by different dihydroxy flavones in a dose of 6mg/kg ranged between 20-40% (Fig-56). A marked potentiated inhibitory response on nociception was clearly evident when the sub effective dose of morphine was combined with the minimal doses of various dihydroxy flavones. The high order of inhibition (86-94%) obtained in this combination clearly indicates a synergistic effect of flavones on morphine induced antinociception. The main deterrent in using higher doses of morphine for treating chronic pain is the fear of development of tolerance and dependence to morphine. This apprehension dissuaded many physicians from employing the adequately required dose of opioids to control chronic or terminal pain. This results in inadequate management of pain and hence exaggerated suffering. Literature documents that nearly 40-50% of patients suffering from cancer pain receive inadequate analgesia (Brennan *et al.*, 177)
Any adjuvant that can potentiate the analgesic effect of morphine may reduce the requirement of high dose of opioids.

In general to treat chronic pain, it is recommended that opioids always be combined with other analgesic agents such as NSAID. Evidences are available that a combination of sub analgesic dose of morphine and aspirin can exert potentiated antinociceptive action (Sandrini et al., 1998). The additive analgesic effect reduced the dose of opioids and hence the undesirable side effects are the advantages of such a combination (Gutstein and Akil., 2006). The “opioid sparing” strategy is considered the back bone of “Analgesic ladder” for pain management proposed by World Health Organization (1990). The combination of codeine with aspirin or paracetamol is usually considered to provide superior efficacy than 60mg of codeine itself. The combination of certain antidepressants like amitriptyline and desimipramine has been suggested for enhancing opioid analgesia in some types of neuropathic pain (Mcquy., 1998). Many other classes of drugs like anti histamines, anticonvulsants (such as carbamazepine and phenytoin) and glucocorticoids are suggested as potentially useful adjuvants to opioid analgesia (Gutstein and Akil., 2006). In this context, the flavonoids that exhibit reasonably good analgesic and anti inflammatory efficacy without possessing serious adverse effects also emerge as potentially useful adjuvant to opioid analgesics.

In another set of experiments the antinociceptive effect was examined for combination of minimal doses of diclofenac and different dihydroxy
flavones. Diclofenac in a dose of 1mg/kg inhibited nociception to an extent of 26 %. Different dihydroxy flavones in a dose of 6mg/kg inhibited nociception to an extent ranging between 18-38%. A well marked potentiation was noticed when diclofenac (1mg/kg) was administered to the animals pretreated with different dihydroxy flavones (6mg/kg). In combination they produced an inhibition of nociception ranging between 69 - 87 % (Figure- 55).

Thus the present results reveal a synergistic antinociceptive effect of dihydroxy flavones with either morphine or a NSAID. Further studies may reveal the potential clinical application of such a combination.

**Anti inflammatory synergism**

Since the dihydroxy flavones potentiated the antinociceptive effect of diclofenac, it was considered interesting to study the anti inflammatory efficacy of such a combination.

For this purpose minimal doses of diclofenac (1mg/kg) and various dihydroxy flavones (5mg/kg), which themselves did not induce any significant anti inflammatory activity were chosen. The inhibition of paw edema did not exceed 20% when these compounds were used alone in the above mentioned dose. However in combination a marked reduction in paw edema was evident during all the periods of observation. The inhibition of paw edema with a combination of diclofenac and various dihydroxy flavones ranged between 63-80%. Thus the present results indicate a synergistic effect of dihydroxy flavones on the antinociceptive and anti inflammatory effect of
NSAID. This synergism paves way for a possible combination of the above classes of agents to achieve maximum therapeutic efficacy with minimal side effects. It is not uncommon to employ a combination of NSAID and serratio peptidase for achieving better clinical efficacy. The results of the previous studies on various flavonoids and the results of the present study indicate the gastric mucosal protection offered by flavonoid compounds. The main side effect that preludes the regular use of NSAID is gastric ulceration. The combination of a dihydroxy flavone may protect against the mucosal damage resulting from NSAID use. In addition the reduced requirement of NSAID may also help to avoid other adverse effects too.