PHARMACOLOGICAL INVESTIGATIONS ON CERTAIN TRIHYDROXY FLAVONES

Thesis submitted to the Tamil Nadu
Dr. M.G.R. Medical University
Chennai - 600 032

FOR THE AWARD OF THE
DEGREE OF DOCTOR OF PHILOSOPHY

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AUGUST – 2009

CERTIFICATE

This is to certify that, Mr. C.S. VIJAYAKUMAR carried out the work of his thesis on “Pharmacological investigations on certain trihydroxy flavones”, for the award of the degree of Doctor of Philosophy of the TamilNadu Dr. M.G.R. Medical University for the requisite period under the regulations in force and the thesis is a bonafide record of the work done by him under our supervision and guidance. The work is original and has not formed the basis of the award to the candidate of any degree, diploma, associateship, fellowship or other similar title.

We, further state that, the entire thesis represents the independent work of Mr. C.S. Vijayakumar and all the experimental techniques employed in this work were actually under taken by the candidate himself under our guidance.

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ACKNOWLEDGEMENT

I wish to express my profound thanks to my guide Dr. S. Viswanathan Assistant professor of pharmacology (retired), Madras Medical College and to my Co-Guide Dr. C.B. Tharani, former Director and professor, Institute of pharmacology, Madras Medical College, for their valuable guidance throughout my research work.

I am thankful to Dr.K.Babu Rao, Chief Medical officer (In-charge), Government Medical Stores Depot and former heads of the same organization for granting permission to pursue a research career.

I am grateful to Dr. V. Krishnamoorty and Dr. C.I. Mythirayee of Research Organics for generously gifting the tri-hydroxy flavones used in the present study.

My sincere thanks are due to the Dean, Madras Medical College and Dr. R.Nandhini, Director, Institute of Pharmocology, Madras Medical College for the permission granted to utilize the facilities in Madras Medical College.

I wish to express my sincere thanks to Dr. B. Suresh, vice – chancellor J.S.S University Dr. V. Ravichandran, Principal, Vels college of Pharmacy, Chennai for their timely and valuable suggestions during the course of the study.

The valuable suggestions offered by Dr.E.Sukumar, Dr.P.Thirugnanasambantham, Dr.N.N.Rajendran Dr.N.Narayanan, Dr.R. Govindarajan and Dr. Ramachandra Bhat provided great impetus to my work.

But for the earnest co-operation extended by my colleagues Mr.Natarajan, Mr.V.Balasubramanian, Ms.P.V.Vidhulatha, Mr.K.Chellayan, Mr. V. Mohan, Mr.K.Dhyaneswaran, Mr.N-Duraisamy, Mrs. Geetha Sundhar, Mr.R.Krishnankutty, Mr.V. Thangaraj and my work would not have been fruitful.

My Sincere thanks are due to Mrs.Umamaheswari Dhilipkumar and Mr. M. Dhilipkumar, Mr. K.Kamalakannan and Mrs. Vidhyalakshmi for their moral support.

The Co-operation and the technical assistance rendered by Mr. R.A.C Mathew, Mrs. Pavunthai and Mrs. Ramadevi and gratefully acknowledged.

The constant encouragement and moral support from my beloved mother, brother, wife and all family members were the catalyst for the successful completion of my research work.

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	-	Analysis of variance
AT	-	Acetyl transferase
ATP	-	Adenosine triphosphate
Aß	-	ß-amyloid
CGRP	-	Calcitonin gene related peptide
CNS	-	Central Nervous System
cm	-	Centimeter
CMC	-	Caboxy Methyl Cellulose
cNOS	-	Constitutive nitric oxide synthase
COPD	-	Chronic obstructive pulmonary disease
COX-1	-	Cyclooxygenase -1
COX-2	-	Cyclooxygenase -2
DHF	-	Dihydroxy flavone
DNA	-	Deoxyribo Nucleic Acid
DPPH	-	2,2 Diphenyl -1-picryl hydrazyl
ED_{50}	-	Effective dose that produces 50 percent of maximal effect
ELISA	-	Enzyme Linked Immuno Sorbent Assay
Fig	-	Figure
GABA	-	Gama amino butyric acid
GIT	-	Gastro intestinal tract
GM-CSF1	-	Granulocyte colony stimulating factor
h	-	hour
HETE	-	Hydroxy Eicosa Tetraenoic Acid
5-HT	-	5-Hydroxy tryptamine
i.p	-	Intraperitoneal
i.v	-	Intravenous
IC_{50}	-	Concentration that produces 50 percent inhibition
IL	-	Interleukin
iNOS	-	inducible nitric oxide synthase
IASP	-	International association for the study of pain

kg	-	kilogram
L	-	Litre
L-NAME	-	L-Nitro Arginine Methyl Ester
LO	-	Lipoxygenase
LPS	-	Lipopolysaccharide
LT	-	Leuckotrienes
mg	-	milligram
min	-	minutes
ml	-	milliliter
mm	-	millimeter
mM	-	millimole
NGF	-	Nerve Growth factor
NMDA	-	N-methyl D-aspartate
NO	-	Nitric oxide
NOS	-	Nitric oxide synthase
NRM	-	Nucleus Raphe magnus
NSAID	-	Nonsteroidal Antiinflammatory Drugs
PAF	-	Platelet activating factor
PAG	-	Periaqueductal gray
PG	-	Prostaglandin
PGE_2	-	Prostaglandin E_2
ROS	-	Reactive oxygen species
s.c	-	sub cutaneous
SEM	-	Standard Error of Mean
Sec	-	Seconds
TNF- α	-	Tumor necrosis factor alpha
THF	-	Trihydroxy flavone
uv	-	Ultra Violet
µg	-	microgram
µl	-	microlitre
µM	-	micromole
VR1	-	Vanilloid receptor

INTRODUCTION

The most common motivation to seek immediate medical attention is the suffering of pain. Acute pain due to any injury to the skin or superficial tissues is usually well defined and easily treatable. Visceral pain and neuropathic pain are refractory to conventional pain management. Improved understanding of the origin of pain, its transmission, modulation of the pain sensation and affective responses that usually accompany the sensation of pain has led to the development of treatment modalities that effectively mitigate the above components of the pain adequately.

The ideal treatment for any pain is to remove the cause. However symptomatic treatment of pain with a suitable analgesic provides relief of physical symptoms and also provides psychological benefit to the sufferer. Non steroidal anti inflammatory drugs which are conventionally employed to treat mild pain of musculo-skeletal origin are also now widely used to treat post-operative pain. Opioid analgesics remain the main stay of therapy in visceral pain, post-operative pain and pain of terminal illness.

Neuropathic pain constitutes a special category where the drugs of the above groups remain ineffective. Entirely different groups of agents like anti-convulsant drugs and anti depressant drugs provide partial relief in these situations.

Eventhough adequate relief from pain is achieved with many of the afore mentioned groups of drugs, many undesirable side effects preclude the

chronic usage of these drugs. Systematic research yielded non steroidal anti inflammatory analgesics with selective inhibition of cyclo oxygenase-2. The most common untoward effect of non steriodal anti inflammatory drugs (NSAID) viz: the gastric mucosal damage was not exhibited by these compounds. The structural modifications in the morphine nucleus and other opioid drugs are also rewarding with new molecules of lesser addictive potential.

Recent investigations on naturally occurring polyphenolics like flavones indicate their potential application in the treatment of inflammation and pain. A novel antinociceptive property was reported for hydroxy ethyl rutoside, by Ramaswamy et al (1980). Subsequent work by Viswanathan et al (1984a) revealed a potent antinociceptive action for gossypin in different experimental models. A detailed structure activity study was undertaken with flavone and many of its methoxy and hydroxy derivatives. The flavone nucleus itself was found to exert significant antinociceptive action which was modified to varying degrees by different substitutions (Thirugnanasambantham et al 1990, 1993). A series of dihydroxy flavone compounds were screened for potential antinociceptive action by Girija et al (2002) and Umamaheswari et al (2006). Most of the above compounds (methoxy and hydroxy flavones) also exhibited potent anti-inflammatory activity in several experimental models. (Muthiah et al 1993 and Arivudainambi et al 1996). Generally compounds exhibiting analgesic and anti inflammatory activity cause extensive gastric mucosal injury and ulceration. Surprisingly, many flavonoid compounds have been found to be devoid of these adverse effects. In fact, a potent antiulcer activity

was identified for many flavonoids. The antiulcer properties of gossypin and hydroxy ethyl rutoside (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.

A unique combination of antinociceptive, anti inflammatory and ulcer protective properties of flavonoids opens new vistas to the therapeutic potential of this novel group of poly phenolics. Moreover, 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).

It is well known that structural modifications can modify the pharmacological actions of an active molecule. With this objective in mind, a few tri-hydroxy flavones were selected for the present investigation. These compounds have been subjected to a battery of tests to identify their antinociceptive potential and the mechanisms involved therein.

REVIEW OF LITERATURE

PAIN

Pain results from many body ailments and develops whenever any tissue is being damaged. The purpose of pain may in fact, be considered as a protective mechanism of the body that cautions the individual to react and if possible to remove the pain stimulus.

The international association for the study of pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage.

The experience and expression of pain are different in different individuals (Oxenham et al 2006). The perception of pain and the distress response it evokes are greatly influenced by the individual's previous experiences. Moreover, the above definition also implies that, perception of a noxious stimulus is not the same thing as pain, which is a subjective experience that also includes an emotional component.

Types of Pain and their qualities (Guyton and Hall 2006)

Pain may broadly be classified into two major types; Fast pain and slow pain. Fast pain is felt within about 0.1 second after a pain stimulus is applied. Whereas slow pain begins after one second or more and then increases slowly over many seconds and some times even minutes.

Fast Pain:

Different terminology is employed to describe the fast pain such as acute pain, sharp pain or pricking pain. Acute or fast pain is experienced immediately when the body is injured or damaged. This type of pain alerts the individual to injury and helps in identifying the location of the damage. Though it is uncomfortable to experience, it is easy to treat and generally does not produce any persistent psychological reaction. Acute pain is felt by a needle prick, or when the skin is cut or acutely burned. Fast sharp pain is not felt in many of the deeper tissues of the body. Acute pain may initiate a stress reaction with an increase in blood pressure, heart rate, respiratory rate, enhance the alertness and increase the motor strength for a faster response. When fast pain is precipitated by trauma or acute medical conditions, diagnosis and treatment of the underlined casues are always the first priority. Sometimes untreated or under-treated acute pain may increase the risk of evolving in to a chronic pain situation (Fields and Joseph 1994).

Slow Pain

Slow pain is also termed as chronic pain, throbbing pain, aching pain or slow burning pain this is invariably associated with tissue destruction and can lead to prolonged and unbearable suffering. Slow pain can originate both in the skin as well as in any deeper organ. Several factors can initiate, perpetuate or exacerbate chronic or slow pain. Some disease conditions are characteristically painful for which presently there is no satisfactory cure such as arthritis, cancer, migraine head-aches, fibromyalgia or diabetic neuropathy.

Sometimes chronic pain can have a psychosomatic or psychogenic cause (Oxenham et al 2006).

Pain Receptors

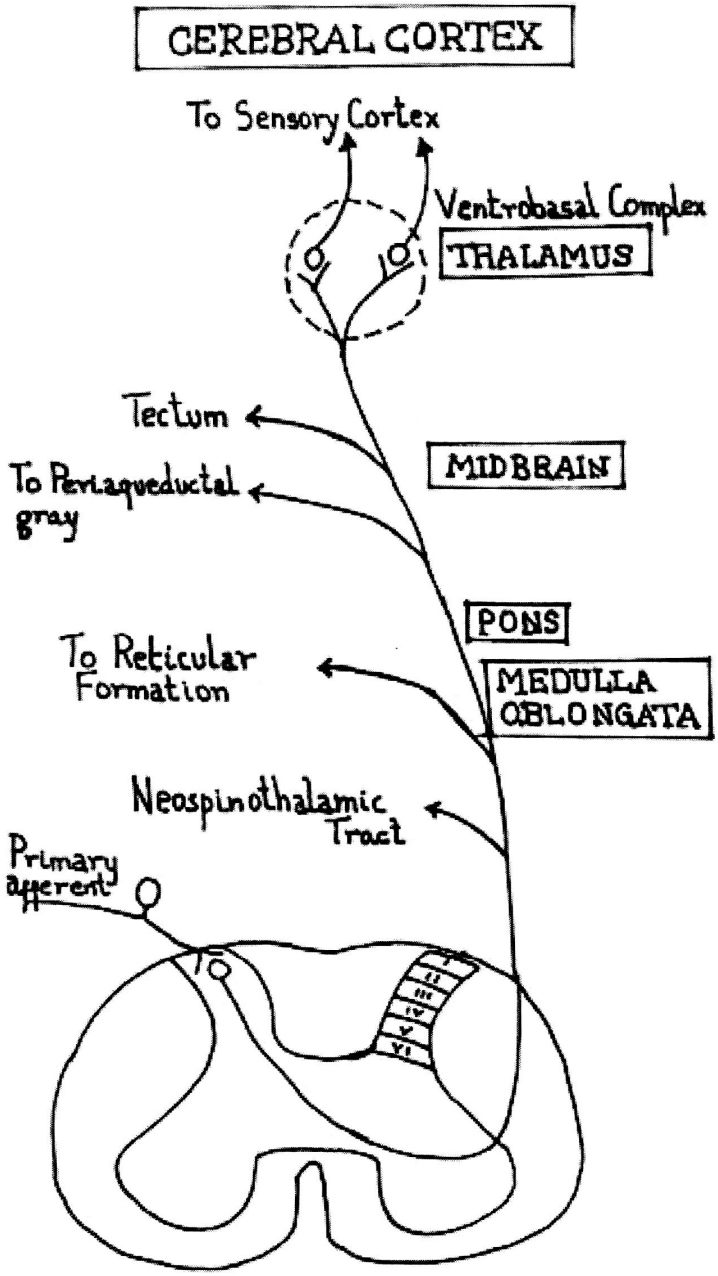
The existence of the receptors that respond to different types of noxious stimuli was first predicted by Sherrington a century ago (Szolesanyi, 2008). Concrete evidences for this hypothesis was offered by Bessou and Perl (1969) who described the subset of C – afferent fibres responding to a wide variety of noxious stimuli. These sense organs were termed as polymodal nociceptors or polymodal receptors which can respond to heat, mechanical stimuli, acid or other irritant chemicals (Bessou and Perl (1969).

The nociceptors responsible for pain sensation in the skin and other tissues are all free nerve endings. These sensory nerve endings are ubiquitously present in the superficial layers of the skin and in some internal tissues, whereas most of the deeper tissues do not have an extensive distribution of pain fibres and hence localisation of cutaneous pain is easier than visceral pain. A variety of stimuli can elicit pain; Mechanical, thermal and chemical. Fast pain is usually elicited by mechanical and thermal types of stimuli whereas the slow pain can be elicited by all three types.

Pain Pathway (Fig. A)

Although all the pain receptors are free nerve endings, two separate pathways are involved in transmitting pain signals into central nervous system. They correspond to two types of pain: a fast – sharp pain pathway

Figure A Pain Pathway



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and a slow-chronic pain pathway. The fast-sharp pain signals are elicited by mechanical or thermal pain stimuli. Small A-delta fibres transmit the signals along peripheral nerves to the spinal cord in a velocity of 6-30 M/sec. In contrast, the slow chronic type of pain is mainly elicited by chemical stimuli and sometimes by persisting mechanical or thermal stimuli. Slow chronic pain is transmitted by type 'C' fibres at velocities between 0.5 to 2 M/sec. The sharp pain alerts the individual immediately of a damaging influence and making him react at once to remove himself from the stimulus. The slow pain intensifies over time and may give intolerable suffering.

Both types of pain fibres enter the spinal cord via, dorsal spinal route and terminate on neurons in the dorsal horn. From the spinal cord the pain signals are transmitted to the higher centers through two different tracts, neo-spino thalamic tract and paleo spino thalamic tract. The fast type A delta fibres that terminate in lamina I of the dorsal horn excite second order neurons of the neo spino thalamic tract. Long fibres originating from second order neurons cross immediately to the opposite side of the cord and pass upward to the brain stem in the antero lateral columns.

Most of these fibres terminate in thalamus, while some of the fibres terminate in the reticular areas of the brain stem. From the thalamus these signals are transmitted to other basal areas of the brain and to the somatosensory cortex. Glutamate is considered to be the neuro transmitter substance secreted in the spinal cord at the A delta pain nerve fibre endings.

The slow-chronic type-C pain fibres terminate in laminae II and III of the dorsal horns which together are called substantia gelatinosa. Type C pain fibres entering the spinal cord release both glutamic acid and substance – P from the nerve endings. These signals then enter lamina-V through short fibre neurons within the dorsal horn. The axons arising from lamina V cross to the opposite side of the cord and travel upwards to the brain in the antero lateral pathway. Most of the nerve fibres carrying slow-chronic pain signals terminate widely in the brain stem and a portion of the fibres reach thalamus. Pain impulses entering the brain stem reticular formation, the thalamus and other lower centers can cause conscious perception of pain. The somato sensory cortex plays an important role in processing the intensity and location of the pain and helps in cognitive evaluation of the pain.

Spino thalamic tracts are also linked with frontal cortex and the limbic system. This pathway may be responsible for the affective or unpleasant emotional dimension of pain. The fibres mediating slow pain also have projections to reticular areas of the brain stem and terminate in the intralaminar nuclei of thalamus. These two areas constitute part of principal arousal system of the brain. This may be the reason why a subject remains sleepless while experiencing severe pain.

MODULATION IN THE PAIN PATHWAY

(a) Facilitation and hyper excitability

The nociceptive pathway is subjected to various types of positive and negative modulation at different levels. The primary afferent neurons contain

several peptides like substance – P and calcitonin gene related peptide (CGRP) which is released as mediator at peripheral and central terminals in pain pathway. The facilitation and thus the hyper excitability happens both peripherally and from central influences. The transmission at the synaptic relay in the dorsal horn is facilitated by several neuropeptides and inflammatory mediators.

Substance – P and CGRP released from the primary afferent neurons also act in periphery to sustain and amplify the inflammatory reaction and hence in further activation of nociceptive afferent fibres. N-Methyl – d – aspartate (NMDA) and nitric oxide (NO) also facilitate nociceptive transmission and this may be responsible for pathological hyperalgesia usually associated with inflammatory responses. Many central mechanisms also contribute to facilitate this hyperalgesia. Increased nerve growth factor (NGF) production, particularly in inflammation is considered to augment the electrical excitability, chemo sensitivity and peptide content of nociceptive afferent neurons. The increased levels of NGF may be an important mechanism by which nociceptive transmission becomes facilitated by tissue damage leading to hyperalgesia (Rang et al 2003a).

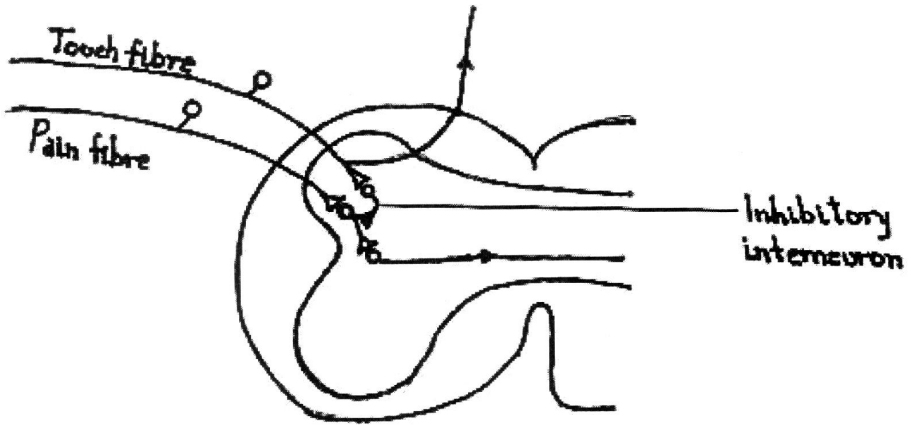
(b) Inhibitory Control

Regulation of nociceptive impulses is mediated at the spinal cord level as well as by descending inhibitory pathways from higher centres. These impulses attenuate and dampen the excitation of the primary afferent neurons or the activity of spinothalamic tract:

i) Gate Control Theory

The substantia gelatinosa cells of lamina II of the dorsal horn send short inhibitory inter neurons projecting to lamina I and V where the primary afferent neurons terminate. These inter neurons regulate the transmission at the first synapse of the nociceptive pathway between the primary afferent fibres and the spino thalamic tract. The regulation of the nociceptive pathway at the primary synapse is popularly known as "Gate Control theory" proposed by Wall and Melzack in 1965 (Fields et al 2006). It has been proposed that non nociceptive afferent input (from mechano receptors through A β fibres) and descending inhibitory neurons activate the inhibitory interneuron of the substantia gelatinosa cells. These interneurons originating from substantia gelatinosa cells act to inhibit the nociceptive transmission at the synapse between primary afferent neuron and spino thalamic tract.

Fig. B. Gate Control Theory



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ii) Descending inhibitory control

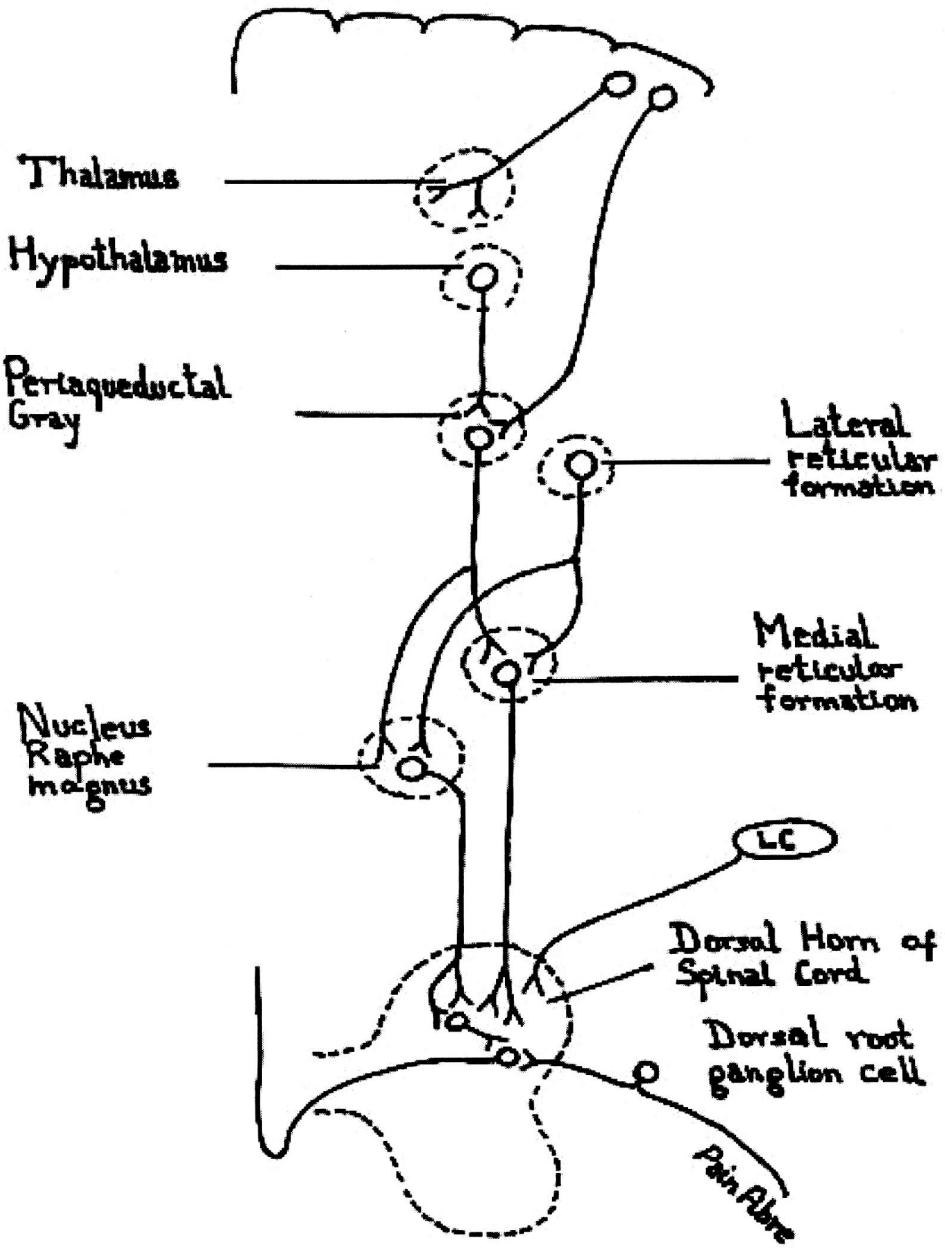
The impulse transmission in the dorsal horn is also subjected to descending inputs from higher centres. The peri aqueductal gray (PAG) area of the mid brain forms a key part of this descending regulatory mechanism. The PAG receives inputs from hypothalamus, cortex and thalamus and it may be the main mechanism by which cortical and other inputs act to control the nociceptive gate in the dorsal horn (Rang et al 2003a).

The neuronal pathway activated by stimulation of PAG passes through nucleus raphe magnus (NRM) and from there fibres run via dorsolateral funiculus of the spinal cord and synpse with dorsal horn interneurons. 5 – Hydroxy tryptamine (5-HT) is believed to be a major neuro transmitter in this synapse. Endogenous opioid peptides (Enkephalins) are also considered to play a role in the descending inhibitory pathway. In addition to the connection from PAG, input from spino thalamic neurons also activate NRM. The transmission in the dorsal horn is also subjected to an inhibitory control by a nor-adrenergic pathway from locus ceruleus. (Figure - C.)

Pain transmission and tactile sensation

Application of liniments and rubbing the skin near the areas affected by pain are sometimes effective in offering good relief from pain. Stimulation of large A β sensory fibres arising from the peripheral tactile receptors can dampen the transmission of pain signals. This is believed to result from local lateral inhibition in the spinal cord.

Figure : C Descending Pathway



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Chemicals involved in nociceptive pathway (Rang et al 2003a).

A variety of chemicals have been identified to play characteristic roles along the pain pathway. Some chemical substances have been established to stimulate the free nerve endings of the peripheral sensory neurons. Some other endogenous substances have been identified to sensitise the nerve endings to the action of the above nociceptive chemicals. Many endogenous compounds released locally as well as from descending pathways have been shown to modulate pain pathways. These agents either amplify the pain signals or suppress the pain transmission by acting at different sites.

Nociceptive Chemicals: Eventhough the polymodal nociceptors respond to thermal, mechanical and chemical stimuli, invariably they are also subjected to excitation by a variety of chemicals.

1) **Kinins:** Kinins are formed during tissue injury by the proteolytic cleavage of the precursor kininogen. Bradykinin and Kallidin are the two important vasodialator peptides formed from kininogen. Bradykinin is a potent pain producing chemical which acts by combining with specific G-Protein coupled receptors. Bradykinin B_1 receptors are believed to play a significant role in inflammation and hyperalgesia. Bradykinin may also release prostaglandins, which in turn enhance the direct actions of bradykinin.

2) **Products of tissue injury:** Many chemical substances and metabolites are released from injured or inflamed tissues that may affect nociceptive terminals. Histamine, 5-HT, adenosine triphosphate

(ATP), protons and lactic acid are prominent among them. Though a minor role has been suggested for 5-HT and histamine, these substances definitely cause excitation of sensory nerve endings. Low pH causes excitation of nociceptive afferents partly by opening acid-sensitive ion channels and partly by facilitation of vanilloid receptors (VR1).

3) **Prostaglandins:** Prostaglandins of the E and F series are released during inflammation and tissue ischemia. It has been proposed that prostaglandins *per se* may not cause pain but they may strongly enhance the pain producing effect of bradykinin or 5- HT. Prostaglandins may sensitise the nerve terminals to other agents partly by inhibiting potassium channels and partly by facilitating cellular depolarisation caused by noxious agents.

4) **Nitric Oxide (NO):** Nitric oxide has been implicated in many cellular events and also considered as a transmitter. A vital role for nitric oxide has been suggested 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). 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 identified in the primary afferent neurons, dorsal root ganglia, brain stem, thalamus, and several other sensory areas. Nitric oxide has been suggested to enhance the

processing or spinal facilitation of afferent nociceptive inputs (Meller and Gebhart 1993).

Exogenous Substances

Capsaicin is an active chemical present in chilly, pepper, ginger and black pepper. Capsaicin has been identified to bind to vanilloid receptors present on nociceptive afferent neurons and open ligand gated cation channels. Entry of sodium and calcium into the primary afferent neurons causes depolarisation and initiation of action potentials.

The vanilloid receptor is also activated by other capsaicin like agonists and by other stimuli including temperature in excess of about 45^{o}C (threshold for pain) and protons. Anandamide has been identified as an endogenous ligand for vanilloid receptors. Many other noxious substances like bradykinin also act by sensitising vanilloid receptor. (VR1).

Transmitters and Modulators in the nociceptive pathway

The origin of pain impulse from peripheral nerve endings and transmission via spinal cord to various higher centres involves the release of several transmitters at different junctions. The excitation of the nociceptors and the release of transmitters at different synapses are modulated by many local and central influences.

i. Tachykinins

Tachykinins (fast acting) are peptides formed by cleavage of larger protein precursors. Substance – P and neurokinin – A are widely distributed in nociceptive primary afferent neurons and in dorsal horn. When the nociceptive neurons are activated these neuro peptides are released both in the central as well as in the peripheral terminals.

In the spinal cord dorsal horn, substance P released is responsible for nociceptive signal transmission. Tissue inflammation through the action of nerve growth factor (NGF) increases the substance – P content of nociceptive neurons and thus potentiating excitatory responses in the spinal cord. Type – C pain fibre terminals entering the spinal cord have been shown to release both glutamate and substance – P. The glutamate transmitter acts instantaneously and lasts for only few milli seconds. In contrast, substance – P is released more slowly over a period of several seconds or even minutes. Substance – P and other related peptides are considered to be concerned with slow chronic pain.

ii) Glutamate

Glutamate has been identified as the neurotransmitter secreted in the spinal cord at the type A - delta pain nerve fibre endings. Glutamate is believed to act on α - amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and it is responsible for fast synaptic transmission.

MODULATORS

The signal transmission at the spinal cord dorsal horn from the primary afferent neurons is subjected to many regulatory influences some of which have been described earlier.

Endogenous Opioid Peptides

Several peptides with morphine like pharmacological actions are present in the body and have been termed as endogenous opioid peptides. These are formed from large molecular weight precursors like Preproopiomelanocortin, prepronenkephalin and preprodynorphin. From these precursors opioid peptides like β endorphin, met-enkephalin, leu-enkephalin and dynorphin are formed. These peptides are widely distributed in various parts of CNS and periphery. Pro-enkephalin peptides are present in areas of CNS that are considered to be related to pain perception; lamina I and II of spinal cord, spinal trigeminal nucleus and PAG. The presence of enkephalin in the above areas suggests a possible role for them in the descending pathway from the mid brain to the dorsal horn. Enkephalin is beleived to cause both presynaptic inhibition and postsynaptic inhibition of incoming type C and A delta pain fibres where they synapse in the dorsal horn. Presynaptic inhibition is probably achieved by blocking calcium channels in the membranes of the nerve terminals. In addition to blocking pain signal at the initial entry point to the spinal cord, pain transmission at other points in the pain pathway, especially in brain stem and thalamus can also be obtunded by endogenous opioids. Enkephalins have also been identified in brain areas like

amygdala hippocampus, locus ceruleus and frontal cerebral cortex which modulate the affective behaviour (Gutstein and Akil 2006). These evidences strongly indicate the essential role played by endogenous opioid peptides in the sensory perception of pain as well as in the consequent behavioural/emotional responses exhibited by an individual.

Gamma Amino Butyric Acid (GABA)

Spinal cord interneurons release GABA and inhibit transmitter release at the primary afferent terminals in the dorsal horn.

5 – Hydroxy tryptamine (5-HT)

This is considered to be the transmitter of inhibitory neurons running from nucleus Raphae magnus (NRM) to the dorsal horn.

Nor adrenaline

Nor-adrenalline is a transmitter of the inhibitory pathway from locus cereleus to the dorsal horn and possibly also in other antinociceptive pathways.

Adenosine

The descending inhibitory purinergic pathway is believed to act on pain transmission through adenosine A_1 receptors and produce analgesia. On the contrary, activation of A_2 receptors may induce pain.

SOME COMMON PAIN SITUATIONS

Cutaneous Pain

Cutaneous pain is caused by injury to the skin or superficial tissues. Cutaneous nociceptors terminate just below the skin and due to high concentration of nerve endings produce a well defined, localised pain of short duration. Minor cuts, minor burns and laceration are some of the injuries that produce cutaneous pain.

Somatic Pain

Somatic pain originates from ligaments, tendons, bones, blood vessels and even nerves themselves. It is perceived by somatic nociceptors and examples include sprains and broken bones.

Visceral Pain

This type of pain originates from body's visceral organs. In general viscera have sensory receptors mainly for pain and for no other modalities of sensation. Visceral pain differs from surface pain in many aspects. Visceral pain is extremely difficult to localise and several injuries to visceral tissue exhibit referred pain where the sensation is localised to an area completely unrelated to the site of injury. Myocardial ischemia is the best example of visceral pain.

Phantom limb pain

A type of referred pain from a limb that has been lost or from which a person no longer receives physical signals. It is an experience almost universaly reported by amputees and quadriplegics.

Neuropathic Pain

This can occur as a result of injury or disease to the nerve tissue itself. For example, damage to the peripheral nerves as occurs in diabetic neuropathy or primary afferents as in Herpes Zoster can result in pain that is referred to body region innervated by damaged nerves. Pain may also be produced by damage to central nervous system in particular the spino thalamic pathway. Neuropathic pains typically have an unusual burning, tingling, or electric shock like quality and even may be triggered by very light touch. A variety of mechanisms contribute to neuropathic pain. Damaged primary afferents including nociceptors become highly sensitive to mechanical stimulation and begin to generate impulses. Thus both central and peripheral nervous system changes may contribute to neuropathic pain (Fields and Joseph 1994).

Treatment of Pain

Even though the ideal treatment for any pain is to remove the cause, symptomatic treatment with a suitable analgesic offers relief of both physical symptoms and psychological benefit to the sufferer. Wide arrays of analgesic agents are currently employed to treat pain of different origin. Mild pain of

musculo skeletal origin is managed with non steroidal anti inflammatory drugs (NSAID). Visceral pain and post operative pain are mainly treated with opioid analgesics. Neuropathic pain constitutes a special category, which is refractory to treatment with the above drugs. Special groups of drugs like anticonvulsants, and antidepressants are employed in the management of neuropathic pain.

Non steroidal anti inflammatory drugs (NSAID)

NSAID are effective in the treatment of mild to moderate pain and are also useful adjuncts in the treatment of severe pain. All these compounds inhibit the enzyme cyclooxygenase and possess significant anti inflammatory action, especially at higher doses except acetaminophen. They are particularly effective in pain of musculoskeletal origin and to treat mild to moderate head ache.

The following NSAID are currently employed in therapy (Burke et al 2006).

Salicylates	Aspirin and Diflunisal
Para amino phenol derivative	Acetaminophen
Acetic acid derivatives	Indomethacin, Sulindac and Etodolac
Fenamates	Mefanamic acid, Meclofenamate Flufenamic acid. Tolmetin Ketorolac and Diclofenac
Propionic acid derivatives	Ibuprofen, Fenoprofen, Ketoprofen / Flurbi profen, Naproxen, Oxaprozin
Enolic acid derivatives	Piroxicam, Meloxicam, Nabumetone
Cox-2 selective inhibitors	Celecoxib, Valdecoxib, Rofecoxib Etoricoxib, Pavecoxib and Lumaricoxib.

Analgeic effect of NSAID

NSAID are mainly effective against pain associated with inflammation since they decrease the production of prostaglandins which sensitise the nociceptors to inflammatory mediators such as bradykinin. Therefore they are effective in arthritis, bursitis, and pain of muscular and vascular origin, toothache, dysmenorrhoea and all conditions that are related with increased prostaglandins. Some evidences also indicate a central effect of these compounds by an action mainly on spinal cord.

Mechanism of action

The main action of NSAID is inhibition of arachidonic acid metabolising activity of cyclo oxygenase (COX), (Vane et al 1971). The principal therapeutic effect of NSAID derives from their ability to inhibit prostaglandin production. The first enzyme in the prostaglandin synthetic pathway is prostaglandin G/H synthase, also known as cyclo oxygenase. This enzyme converts arachidonic acid to the unstable intermediates PGG2 and PGH2 and leads to the production of thromboxane- A2 and a vairety of prostaglandins. There are two isoforms of cycloxygenase, COX-1 and COX-2.

COX – 1 is primarily a constitutive isoform found in most normal cells and tissues, while cytokines and inflammatory mediators that accompany inflmmation induce COX-2 (Siebert et al 1997).

Opioid analgesics

Opioids are the most potent pain relieving drugs currently available. Furthermore, of all analgesics they have broader range of efficacy, providing the most reliable method for rapidly relieving pain. Opioids activate pain inhibitory neurons and directly inhibit pain transmission neurons. The following Opioid analgesics are employed in therapy (Gutstein and Akil, 2006).

Morphine and related opioid agonists

a. **Natural and synthetic agents:** Morphine, heroin, hydromorphone, oxymorphone, levorphanol, levallorphan, codeine, hydrocodone and oxycodone.

b. Piperidine and phenyl piperidine derivatives: Meperidine, fentanyl, sufentanil, alfantanyl, remi fentanil.

c. Methadone and congeners: Methadone hydrochloride and propoxyphene.

d. Opioid agonists / antagonists, partial agonists: Pentazocine, nalbuphine, butarphanol, buprenorphine.

Opioid Receptor

Three types of opioid receptors (μ, delta and kappa) have been studied in detail (Waldhoer et al.., 2004). The μ receptors are believed to be responsible for most of the analgesic effects of opioids, respiratory depression, euphoria, sedation and dependence. Most of the analgesic

opioids are μ – receptor agonists. The delta receptors may also contribute to analgesia at the spinal level and more importantly in the periphery. The kappa receptors contribute to analgesia at the spinal level and may elicit sedation and dysphoria. They produce few side effects and do not contribute to dependence. Some analgesics act through kappa receptors. A fourth subtype of sigma receptors was reported by Walker et al (1990). There is pharmacological evidence for further subdivisions of each of these subtypes. Akil and Watson (1994) have suggested the presence of one additional sub type of kappa receptor, with high affinity for the benzomorphan class of opiate alkaloids. Existence of delta opioid receptor subtypes (delta 1 and delta 2) was proposed from behavioral studies by Jiang et al., (1991). Occurrence of μ - opioid receptor subtypes (μ1 and μ2) were proposed after behavioral and pharmacological studies (Pasternack., 1986).

Mechanism of action

All opioid receptors are linked to G-protein coupled receptors. Opioids inhibit adenylyl cyclase leading to decrease in intracellular C- AMP with consequent decrease in cell excitability. Opioids activate K^+ channels and subsequent increase in K^+ conductance to produce hyper polarization of neurons and a decrease in their excitability. Opioids inhibit Ca^{2+} conductance by suppressing voltage gated N type Ca^{2+} channels. Since Ca^{2+} influx is needed for the stimulus induced neurotransmitter release, opioids decrease the release of neurotransmitter like substance P and glutamate from nociceptive terminals. Opioids are believed to act at several sites in the pain pathway. Even though the primary site of action of opioids is considered to be

the higher centres in brain, peripheral sites of opioid action are also suggested. Opioids may act on the peripheral terminals of nociceptive afferent neuron and also act directly on the dorsal horn exerting an inhibitory influence on pain transmission. The release of substance P from dorsal horn neurons is inhibited by opioids. Several studies have proved that, the opioids activate neurons in PAG and projections to NRM. The above two structures constitute the important descending controls of the pain pathway. Projections from, Nucleus Raphe Magnus run to the substantia gelatinosa of the dorsal horn and inhibit pain transmission. Enkephalins and 5-HT are considered to be important mediators in the descending pathways.

Other analgesic drugs

Besides opioids and NSAID, several other drugs are used as analgesics particularly to treat neuropathic pain states, which respond poorly to conventional analgesic drugs and cause a major clinical problem.

Tramadol is a metabolite of the anti depressant trazolone and is widely used for post operative pain. It is a weak agonist at μ - opioid receptors and also an inhibitor of nor adrenaline reuptake. Tricyclic anti depressants, imipramine and amitriptyline act centrally by inhibiting noradernaline reuptake and are highly effective in relieving neruopathic pain.

Antiepileptic drugs such as carbamazepine, gabapentin and occasionally phenytoin are sometime effective in neuropathic pain. Ketamine, a dissociative anesthetic which works by blocking NMDA receptor channels, has analgesic properties. Intravenous lidocaine, a local anaesthetic drug, can

give long-lasting relief in neuropathic pain states. It probably acts by blocking spontaneous discharge from damaged sensory nerve terminals.

Treatment of chronic pain

Antidepressant medications: The tricyclic antidepressants are extremely useful for the management of patients with chronic pain (Mcquay et al., 1996). The analgesic effect of tricyclics has a more rapid onset of action and occurs at a lower dose than is typically required for treatment of depression. Furthermore, patients with chronic pain, who are not depressed, obtain pain relief with antidepressants. There is evidence that tricyclic drugs potentiate opioid analgesia and are considered as useful adjuncts for the treatment of severe persistent pain such as occurs with malignant tumors (Luissier et al., 2004). Tricyclics are of particular value in the management of neuropathic pains such as painful diabetic neuropathy and post herpetic neuralgia.

Anticonvulsants and antiarrhythmics

These drugs are useful primarily for patients with neuropathic pain. Phenytoin and carbamazepine were first shown to relieve the pain of trigeminal neuralgia. This pain has a characteristic brief shooting, electric shock like quality (Mcquay et al., 1995). Anti arrhythmic drugs such as low dose lidocaine and mexiletine seem to be effective for pains that respond to anticonvulsants, as well as other conditions, including post operative and burn pain. These drugs block the spontaneous activity of primary afferent nociceptors when they are damaged. They are considered for use in patients

with pain associated with damage to peripheral nerves (Oskarsson et al., 1997).

Adverse effects to analgesic drugs

The availability of a wide range of analgesic drugs enables the physician to choose an effective drug to suit the requirements of the patient. However no analgesic drug is free from adverse effects whether employed for acute pain or chronic pain syndromes. A brief consideration of the adverse effects to different classes of analgesics is presented here.

Adverse effects of NSAID therapy

Effects on gastrointestinal tract

The most common symptoms associated with these drugs are gastrointestinal, including, anorexia, nausea, dyspepsia, abdominal pain and diarrhea (Hawkey, 2001). These symptoms may be related to the induction of gastric or intestinal ulcers. 15-30% of NSAID users suffer from these symptoms. Ulceration may range from small superficial erosions to full thickness perforation of the muscularis mucosa. There may be single or multiple ulcers and ulceration may be accompanied by gradual blood loss leading to anemia or life threatening hemorrhage (Burke et al., 2006). The concurrent use of corticosteroids, heavy alcohol consumption or H. pylori infection further aggravates the above risk. The selective COX -2 inhibitors have been shown to be less prone than fully efficacious doses of other NSAID to induce endoscopically visualized gastric ulcers (Deeks et al., 2002).

Gastric damage by NSAID can be brought about by two distinct mechanisms. Inhibition of COX-1 in gastric epithelial cells depresses mucosal cytoprotective prostaglandins, especially PGI2 and PGE2 These eicosanoids inhibit acid secretion by the stomach, enhance mucosal blood flow and promote the secretion of cytoprotective mucus. Another mechanism by which NSAID may cause ulceration is by local irritation from contact of orally administered drug with the gastric mucosa. Local irritation allows back diffusion of acid into the gastric mucosa and induces tissue damage. It is also possible that enhanced generation of lipoxygenase products (e.g. Leukotrienes) contributes to ulcerogenecity in patients treated with NSAID.

Renovascular complications: NSAID use may result in salt and water retention, edema and worsening of renal function in renal/cardiac and cirrhotic patients. Regular NSAID use may also decrease the effectiveness of antihypertensive medications and diuretic drugs. Many NSAID especially aspirin may reduce uric acid excretion and may also produce hyperkalemia.

CNS effects: Headache, vertigo, dizziness, confusion and lowering of seizure threshold are some of the CNS adverse effects of NSAID.

Cardiovascular effects:

The inhibitory effect of NSAID on platelet function increases the risk of hemorrhage. Even though COX-2 inhibitors do not share this effect, the cardiovascular side effect of coxibs acquires another dimension. The selective Inhibitors of COX-2 depress PGI_2 formation by endothelial cells without inhibiting platelet thromboxane. Selective inhibition of PGI_2 may increase the

risk of thrombosis. There is increased incidence of myocardial infarction and stroke in patients treated with rofecoxib (Bresalier et al.., 2005), valdecoxib (Nussmeier et al., 2005) and celecoxib (Solomon et al., 2005). Patients with increased risk of cardiovascular disease or thrombosis are particularly prone to cardiovascular adverse events of COX-2 inhibitors (Burke et al.., 2006).

Pregnancy and lactation: The use of NSAID to terminate pre-term labour has been associated with closure of ductus arteriosus and impaired fetal circulation. NSAID may delay labour and increase the risk of postpartum hemorrhage.

Hypersensitivity reactions: Many hypersensitivity reactions may be exhibited by individuals treated with NSAID. Vasomotor rhinitis with profuse watery secretions, angioedema, utricaria, asthma, flushing and hypotension and shock may be encountered with NSAID use. The hypersensitivity reactions may occur in 10-25% of patients with asthma, nasal polyps or chronic utricaria.

Adverse effects of opioid analgesics:

Morphine and related opioids produce a wide spectrum of unwanted effects including respiratory depression, nausea, vomiting, dizziness, mental clouding, dysphoria, pruritis, constipation, increased pressure in the biliary tract, urinary retention and hypotension.

Tolerance and dependence: Tolerance to opioids develops rapidly and is readily demonstrated. Dependence comprises of physical and psychological

dependence. (Ballantyne and Laforge, 2007). Tolerance develops rapidly accompanied by physical withdrawal syndrome. Tolerance extends to most of the pharmacological effects of morphine, including analgesia, emesis, euphoria, and respiratory depression. Physical dependence is characterized by a clear cut abstinence syndrome, fever, sweating, piloerection, nausea, diarrhoea and isomnia. Extreme restlessness and distress are accompanied by a strong craving for the drug.

Adverse effects of antiarrythmic drugs

The adverse effects of lidocaine are mainly manifestations of actions on the central nervous system and include drowsiness, disorientation and convulsions (Roden 1994).

Adverse effects of tricyclic antidepressants

Important side effects are sedation, (H1 block) postural hypotension, (α- adrenoceptor block) dry mouth, blurred vision, constipation (muscarinic block), occasionally mania and convulsions, impotence, risk of ventricular dysrythmias and interaction with other CNS depressants.

Adverse effects of Anti convulsants

Side effects of phenytoin are ataxia, vertigo, gum hypertrophy, hirsutism, megaloblastic anaemia, fetal malformation and hypersensitivity reactions. The important side effects of carbamazepine are sedation, blurred vision, water retention and hypersensitive reactions.

INFLAMMATION

Inflammation may be defined as the local tissue response to any injurious stimulus. It is a kind of defense mechanism of the body to eliminate or limit the spread of injurious substance and may also proceed to remove the consequent necrosed cells or tissue (Harsh Mohan., 2006). A wide variety of noxious agents may be responsible for inflammation, which includes physical noxious stimuli like heat, cold, radiation or mechanical trauma, chemical agents such as acid or alkali or any other micro organism, infective agents and toxins released from bacteria, virus or any other micro organism and endogenously released immunological agents due to antigen antibody reaction or specific cell types. The inflammatory response is essential for survival to overcome environmental pathogens and injury. However in certain situations and diseases the inflammatory response may be exaggerated and sustained without apparent benefit and even with severe adverse complications (Burke et al., 2006).

Phases of inflammatory reaction

Irrespective of the nature of initial stimulus, the classic inflammatory response includes calor (warmth), rubor (redness), tumor (swelling) and dolor (pain). Different mechanisms are likely to mediate three distinct temporal phases of the inflammatory response. The acute phase is characterized by transient local vasodilatation and increased capillary permeability. A delayed sub acute phase in which there is infiltration of leucocytes and phagocytes cells. A chronic proliferative phase, in which there may be tissue degeneration

and subsequent fibrosis. For an easy understanding, inflammatory response may be considered as acute and chronic based on certain distinct features.

Acute inflammation

Acute inflammation is generally of short duration representing the early body reaction which is usually followed by a quick tissue repair (Gaybay and Kushner., 1999).

Chronic inflammation

The inflammatory process is not characterised by the classical signs of acute inflammation. Instead, chronically inflamed tissue is characterised by infiltration of mononuclear immune cells, tissue destruction and attempts at healing which include angiogenesis and fibrosis. Many mechanisms are involved in the promotion and resolution of the inflammatory response. The process of vasodilatation, chemotaxis, cell adhesion, migration and other proliferative reactions involve the role of several substances collectively known as the mediators of inflammation (Sehran and Chirang., 2004; Kyriakis and Avuruch., 2001).

Chemical mediators of inflammation

Many endogenous chemical mediators play a significant role in mediating various reactions of the inflammatory process like vascular permeability, vasodialation, chemotaxis, cellular migration and tissue damage (Dale, 1994). These mediators may originate from different types of cells, plasma, or from the damaged tissue itself. They may be considered in two

groups: a) Mediators released by cells and b) Mediators originating from plasma.

Cell derived chemical mediators: (Dale, 1994)

(i) Vaso active amines (Histamine, 5 -Hydroxy tryptamine).

(ii) Arachidonic acid metabolites through cyclooxygenase pathway (prostaglandins, thromboxane A2 and prostacyclin) and metabolites through lipoxygenase pathway (HETE, Leukotrienes).

(iii) Lysosomal components.

(iv) Platelet activating factor.

(v) Cytokines (Interleukins, Tumor necrosis factor)

(vi) Nitric oxide and many types of free radicals.

Plasma derived mediators

These are products of the kinin system, the coagulation system, the fibrinolytic system and compliment system. The components of these cascades are proteases that are inactive in their native form; are active by proteolytic cleavage, each activated component then activating the next (Gaybay and Kushner., 1999)

Biological effects of various mediators of inflammation

Histamine

Histamine is a basic amine present in many tissues of the body particularly in lungs, skin and G.I.T. Mast cells and basophils are rich source of histamine, where it is stored in granules as a complex with an acidic protein and heparin. Histamine is released from mast cells by exocytosis during inflammatory or allergic reaction. Histamine produces many effects by acting on H_1, H_2, or H_3 receptors on target cells. The triple response after an intradermal injection of histamine reveals as a reddening of the skin, a wheal and a flare in surrounding area. The reddening results from vasodilatation of small arterioles and pre capillary sphincters and the wheal is caused by the increased permeability of post capillary venules. These effects are mainly mediated through activation of H_1 receptors .The flare is an axon reflex that involves activation of sensory nerves which in turn may release a peptide vasodialator (Rang et al ..,2003).

5 - Hydroxy tryptamine (5-HT)

5- HT is present in high concentration in platelets and is released when platelets aggregate at sites of tissue damage. 5-HT is considered to play a role in vasodilatation, sensitization of nociceptors and as an inflammatory mediator.

Arachidonic acid metabolites

The eicosanoids are not preformed and stored in tissues, but are generated de novo from phospholipids. The eicosanoids are important mediators of various reactions in the inflammatory process. The arachidonic acid released from tissue phospholipids may be acted upon by two forms of cyclooxygenase (COX); COX-1, a constitutive enzyme and COX-2, which is induced in inflammatory cells by inflammatory stimuli. Arachidonic acid may be alternatively metabolised by 5-lipoxygenase that can generate, several types of Leukotrienes. PGE_2, PGD_2 and prostacyclin (PGI_2) are powerful vasodilators. In areas of acute inflammation, the vasodilator eicosanoids PGE_2 and PGI_2 are generated by the local tissue and blood vessels and PGD_2 is released from mast cells. They act in combination with other mediators to dilate the precapillary arterioles and increase blood flow in areas of acute inflammation. Prostaglandins also sensitize afferent C fibres to the action of bradykinin for initiating the pain.Prostaglandins of E series are also implicated in the production of fever. Leukotrienes are the arachidonic acid metabolites derived by lipoxygenase pathway. Leukotriene B4 (LTB_4) is an important mediator in all types of inflammation. LTB_4 may cause adherence, chemotaxis and activation of polymorphs and monocytes. LTB_4 also stimulates proliferation and cytokine production from macrophages and lymphocytes. Leukotrienes are present in the tissues in many inflammatory diseases, like rheumatoid arthritis, psoriasis, ulcerative colitis and also in bronchial asthma.

Platelet activating factor (PAF)

PAF is believed to be an important mediator in many allergic and inflammatory reactions. PAF is generated and released from most inflammatory cells and also from platelets. PAF is released by the action of the enzyme phospholipase A2 on tissue phospholipids. PAF causes vasodialation, increases vascular permeability, chemotaxis, activation of leucocytes and activation and aggregation of platelets. It is spasmogenic for smooth muscles and mainly implicated in bronchial asthma (Rang et al., 2003).

Cytokines

Cytokines are mainly peptide mediators released by cells of the immune system. Important cytokines belong to the family of interleukins, chemokines, interferons, colony stimulating factors, growth factors and tumor necrosis factors. Cytokines have both pro inflammatory and anti inflammatory properties (Janeway et al., 2004).

Pro inflammatory cytokines

The important pro inflammatory cytokines are Tumor necrosis factor alpha (TNF- α) and interleukin -1 (IL-1) and IL-6. These are released from macrophages and many other types of cells and evoke the synthesis of various chemokines. Cytokine growth factors like platelet derived growthfactor, fibroblast growthfactor are important in tissue repair process.

Anti inflammatory Cytokines

Cytokines like tissue growth factor (TGF), IL-4, IL-10 and IL-13 can inhibit the production of chemokines and thus exert an inhibitory effect on inflammation.

Chemokines

These peptides control the migration of leukocytes in immune and inflammatory reactions.

Bradykinin

Bradykinin and kallidin are vaso active peptides derived from kininogens. Bradykinin causes vasodilatation and increased vascular permeability. It is also very effective in stimulating nociceptors.

Nitric oxide (NO)

The actions of nitric oxide on inflammation are complex in nature. Many cytokines stimulate the expression of inducible form of nitric oxide synthase (iNOS) by all inflammatory cells. NOS is also present in the bronchial epithelium (asthma subjects), in colon (ulcerative colitis) and in synovium (arthritis). Nitric oxide has pro inflammatory actions. It is a potent vasodilator, increases vascular permeability and increases the synthesis of pro inflammatory prostaglandins. Some actions of nitric oxide are anti inflammatory in nature. The nitric oxide released from endothelial cells inhibits adhesion of neutrophils and platelets and platelet aggregation.

Free radicals

Biologically derived free radicals also play a role in tissue injury and inflammatory response. These include superoxide anion, singlet oxygen, hydroxyl radical, hydrogen peroxide, peroxynitrite and hypochlorous acid. These oxidants are generated by phagocytic cells like neutrophils and macrophages and cause severe tissue damage (Woodfork and Dyke, 2004).

Steroidal and Nonsteroidal antiinflammatory drugs

Inappropriate and aberrant inflammatory or immune reactions are the main causes of several diseases and the scientific community is constantly exploring new avenues for the discovery of safe and effective anti inflammatory agents. Glucocorticoids and the Nonsteroidal anti inflammatory agents are the major classes of drugs used for this purpose. A brief sketch about these agents is felt appropriate here.

Corticosteroids

The corticosteroids secreted by adrenal cortex are glucocoticoids with primary effect on carbohydrate metabolism and mineralocorticoids with a primary role in regulating electrolyte balance. The physiological functions of corticosteroids involve almost all systems of the body and hence the effect of corticosteroids use in pharmacological doses may also have repercussions almost in all body functions. Among the various properties of glucocorticoids, their impartant action on inflammation and immune mechanism signifies them as ideal candidates in the treatment of chronic inflammatory diseases and for

immunosuppression. The immunosuppressive and anti-inflammatory actions of glucocorticoids are linked inseparably because both the above actions involve inhibition of leukocyte function (Chrouses., 1995).

Even though glucocorticoids do not treat the underlying cause of the disease, the anti-inflammatory activity of these drugs has enormous utility in many clinical situations. Glucocorticoids can prevent or suppress inflammation in response to a variety of provoking factors including radiant, mechanical, chemical, infectious and immunological stimulants. Multiple mechanisms are involved in the suppression of inflammation by glucocorticoids (Schimmer and Parker, 2006).

Almost all stages of inflammation are inhibited by corticosteroids. They decrease the release of vasoactive and chemo attractive factors, diminish the secretion of lipolytic and proteolytic enzymes, decrease extravasation of leukocytes to areas of injury and finally decrease fibrosis.

(a). The release of arachidonic acid and its metabolites is prevented by an inhibitory effect of glucocorticoids on phospholipase- A2. These drugs are believed to stimulate the production of lipocortin, an inhibitor of phosphoplipase-A2.

(b) The production and release of cytokines like, IL-1, IL-6, and TNF-α from macrophages and monocytes is inhibited by these compounds. ·

(c) The release of endothelial leukocyte adhesion molecule-1 and intracellular adhesion molecule-1, cytokines and acute phase reactants from endothelial cells is prevented by glucocorticoids.

(d) The IgE dependent release of histamine and leukotrienes from basophils is blocked by glucocorticoids.

(e) Glucocoticoids not only prevent the release of arachidonic acid metabolites from fibroblasts but also suppress growth factor induced DNA synthesis and fibroblast proliferation.

(f) Similar to their action on macrophages and monocytes, glucocorticoids inhibit also the synthesis and release of cytokinins from lymphocytes (IL-1, IL-2, IL-3, IL-6, TNF-α, granulocyte/monocyte colony stimulating factor (GM-CSF1) and interferon gamma.

Cortisol (hydrocortisone), dexamethasone, prednisone, prednisolone, methyl prednisolone and triamcinolone are the important corticosteroids available in different dosage forms. Beclomethasone, budesonide, flunesolide and triamcinolone are available as inhalation to be used in bronchial asthma.

Therapeutic uses

A. Substitution therapy:

1. Acute adrenal insufficiency: This life threatening emergency requires immediate management. Therapy includes large amounts of parenteral (I.V) cortisol, correction of fluid and electrolyte imbalance and treatment of precipitating factors.

2. Chronic adrenal insufficiency: Congenital adrenal hyperplasia

3. Therapeutic uses in non endocrine diseases

Rheumatoid Arthritis

Glucocorticoids are used in rheumatoid arthritis mainly in patients with progressive diseases who fail to respond to NSAID or who are unable to tolerate the side effects of other disease modifying drugs. In patients with major symptomatology confined to one or a few joints, intra articular steroid injections is considered. 5 to 20 mg of triamcinolone injected can control acute inflammation of a specific joint without causing systemic side effects.

Renal diseases

Patients with nephrotic syndrome due to systemic lupus erythmatosus or primary renal disease may be benefited with corticosteroid therapy.

Allergic diseases

Glucocorticoids suppress the allergic manifestations in hay fever, serum sickness, utricaria, contact dermatitis, drug reactions, bee stings and angioneurotic edema etc which are of limited duration. In life threatening situations dexamethasone sodium phosphate is administered intravenously.

Bronchial disease

Corticosteroids are often used in bronchial asthma and in chronic obstructive pulmonary disease (COPD). Parenteral glucocorticoids are given during severe asthma attacks. Inhaled steroids can decrease the need for oral corticosteroids or replace them entirely. In the treatment of children with mild asthma, glucocorticoids can be administered as inhalation in place of oral corticosterioids.

Infectious diseases

Glucocorticoids in combination with an antibiotic is used to treat AIDS patients with Pneumocystis carinii pneumonia . Glucocorticoids increase oxygenation and lower the incidence of respiratory failure. Glucocorticoids decrease the incidence of long-term neurological impairment associated with H. influenza type B meningitis in infants.

Ocular diseases

Corticosteroids find their frequent use in a number of inflammatory and allergic conditions of eye. For the disease of the outer eye and anterior

chamber they are instilled locally as eye drops, applied as ointments and sometimes administered as injections locally. However diseases of the posterior segment require their systemic administration.

Skin diseases

Corticosteroids as topical preparations are highly effective in the treatment of a wide variety of skin diseases. Atopic dermatitis (eczematous skin disease), dermatoses, lichen simple chronicus, seborrheic dermatitis, mycosis fungioides and pemphigus. Topical preparations vary in their concentration. They are also used systemically for severe episodes of acute skin disorders.

Gastro intestinal diseases

Corticosteroids are helpful in the treatment of chronic inflammatory bowel disease, ulcerative colitis, and Crohn's diseases. Hydrocortisone (100mg) can be administered as a retention enema for treating mild ulcerative colitis. In severe acute ulcer conditions oral prednisone (10-30mg/kg /day) can be administered as a retention enema. For severely suffering patients with fever, anorexia, anemia and impaired nutritional status large doses should be used (40mg to 60 mg prednisone per day).

Malignancies

Because of the anti lymphocytic effects, glucocorticoids are used in the chemotherapy of acute lymphatic leukemia, Hodgkin's and other lymphomas. Prednisone is the commonly used corticosteroid.

Cerebral edema

Corticosteroids have been found to be useful in the reduction or prevention of cerebral edema associated with neoplasm, especially the metastatic ones.

Miscellaneous diseases and conditions

Sarcoidosis

Sarcoidosis is treated with corticosteroids (1mg/day of prednisolone) to induce remission.

Thrombocytopenia

In thrombocytopenia, prednisolone (0.5mg/kg) is used to decrease the bleeding tendency. For severe cases daily doses of prednisone (1 to 1.5 mg/kg) are employed.

Auto immune destruction of erythrocytes

Patients with auto immune destruction of erythrocytes are treated with prednisone (1mg/kg per day). For treating severe hemolysis higher doses may be used. Small maintenance doses may be required for several months in patients who respond.

Organ transplantation

During organ transplantation higher doses of prednisone (50 to 100mg) are given along with other immunosuppressive agents.

Adverse effects of corticosteroids

Adverse effects resulting from withdrawal therapy

Sudden withdrawal of corticosteroids after prolonged therapy may result in acute adrenal insufficiency through suppression of the patient's capacity to synthesize corticosteroids. Glucocorticoids withdrawal syndrome consists of fever, myalgia, arthralgia and malaise.

Adverse effects resulting from continued use of glucocorticoids

Effect on fluid and electrolytes

Continuous use of corticosteroids can cause hyperkalemia, alkalosis, edema and hypertension, particularly in patients with primary hyperaldosteronism.

Effect on metabolic actions

Glucocorticoids may cause hyperglycemia.

Immune responses

Prolonged administration of glucocorticoids inhibits immune system and the inflammatory response and increase the susceptibility to infection.

Effect on G.I.T

Peptic ulceration risk will be increased. Bleeding and silent perforation of ulcers may occur. Dyspeptic symptoms may be frequent with high dose corticosteroid therapy either alone or when given along with NSAID.

Myopathy

Myopathy is seen in patients receiving long term steroid therapy. Myopathy is characterised by weakness of proximal muscles of arms, legs, shoulders and pelvis.

Behavioral changes

Mild euphoria frequently accompanies high dose steroid treatment. Nervousness, decreased sleep and mood changes are noted by few. Rarely a depressive illness may be precipitated.

Eye

Glaucoma may develop in susceptible individuals after prolonged topical therapy. Ocular complications include cataract, particularly in children with bronchial asthma who are receiving long term steroid therapy. Increased intraocular pressure leading to inducement of glaucoma is also seen.

Osteoporosis

Compression fractures of vertebrae and spontaneous fractures of long bones can occur, especially in elders. Osteoporosis occurs in patients of all ages who receive long term glucocorticoids therapy. It affects trabecular bone and cortical rim of the vertebral bodies and ribs.

Osteonecrosis

The necrosis of bone of femoral head is seen with long time therapy. In children, the metabolic effects may result in inhibition of growth, even with low doses. When the drugs are used in anti inflammatory and immuno suppressive therapy, the metabolic actions and the effects on water and electrolyte balance and organ systems are the side effects and Cushing syndrome may occur (Saag 2003).

Nonsteroidal antiinflammatory drugs (NSAID)

Of all the therapeutic agents in the world, NSAID are the most widely used compounds. They are primarily used for their anti inflammatory, a nalgesic and antipyretic actions. All these effects are observed due to the primary effect of these compounds in inhibiting prostaglandin synthesis. The first enzyme in the prostaglandin synthetic pathway is known as cyclo oxygenase or COX. There is good correlation between the potency of these drugs as COX inhibitors and their anti-inflammatory activity. There are two forms of cyclooxygenase enzyme, COX-1 and COX-2. COX-1 is primarily a constitutive isoform found in most normal cells and tissues and has a primary

effect in maintaining tissue homeostasis. COX-2 is an inducible form of cyclo oxygenase, which is released from inflammatory cells for activation by cytokines and other inflammatory mediators. Thus COX-2 may be considered responsible for the synthesis of prostanoid mediators of inflammation (Vane and Botting ., 1998)

Most NSAID inhibit both COX-1 and COX-2 with little selectivity. It is believed, that the antiinflammatory effect of NSAID is mainly related to their inhibition of COX-2 while their unwanted effects in particular gastro intestinal irritation are due to the inhibitory effect on COX-1. Based on this concept selective COX-2 inhibitors have been developed with markedly less gastric irritation potential. Aspirin and other NSAID inhibit the COX enzymes and prostaglandin production. These compounds do not inhibit the lipoxygenase pathways of arachidonic acid metabolism and hence do not suppress leukotrienes production.

Even though all NSAID inhibit prostaglandin synthesis, there are minor but important differences among them in the above action. Aspirin covalently modifies COX-1 and COX-2, irreversibly inhibiting cyclooxygenase (COX) activity. This irreversible inhibition has a major implication in inhibiting thromboxane A2 production in platelets which lasts for the lifetime of the platelet (8-12 days). Platelets being devoid of nucleus may not generate COX-1 after exposure to aspirin. Majority of NSAID are organic acids which act as reversible competitive inhibitors of cyclooxygenase activity.

Apart from the inhibition of cyclooxygenase, some NSAID possess other properties that may also contribute to their anti inflammatory effect. At higher concentrations NSAID are known to reduce production of super oxide radicals, decrease nitric oxide synthase, decrease pro inflammatory cytokines (TNF-α and IL-1), modify lymphocyte activity, alter cellular membrane compounds and inhibit the expression of adhesion molecules (Burke et al.,2006).

Therapeutic uses of NSAID

The inhibition of cyclooxygenase in inflammatory cells and at other sites has endowed them with significant anti-inflammatory, analgesic and anti pyretic effects. NSAID are mainly used against pain of low to moderate intensity. Their maximal efficacy is generally much less than the opioids. Pain due to inflammation and post operative pain are very well controlled by NSAID while the pain arising from the hollow viscera (except menstrual pain) is not usually amenable to NSAID treatment. Paracetamol remains the main choice among the NSAID to control fever of any origin. The important clinical application of NSAID is their use in the treatment of musculoskeletal disorders such as rheumatoid arthritis and osteo arthritis. NSAID provide only symptomatic relief from pain and inflammation in the above conditions and may not arrest the progression of pathological injury to tissues. Many types of mild arthropathies, ankylosis, spondylosis and gout are treated by any one of the NSAID. The property of COX inhibition by NSAID has made them useful in other conditions like closure of patent ductus in neonates, systemic mastocytosis and Barters syndrome. Recent reports indicate the potential use of aspirin or other NSAID, in cancer prevention (Burke et al 2006).

FLAVONOIDS

Flavonoids, the derivatives of benzopyrone are widespread in photosynthesizing plants. More than 4000 flavonoids have been reported since 1980 (Harborne *et al.,* 1988).

Chemistry of Flavonoids

The flavonoids are an important group of polyphenolic compounds derived from nature. The basic skeleton of flavonoid is the gamma - pyrone ring occurring as phenyl benzopyrone (Flavone). Various substitutions, mainly phenolic OH groups, take place in 3, 5, 7, 3' and 4' positions of the flavone 3',4' nucleus. Flavonoids are generally found in nature as glycosides with sugar moiety attached to the 3rd or 7th position. Depending upon the position of OH groups, saturation of OH groups and of the pyrone nucleus, flavonoids are classified into various sub groups, viz; isoflavones, flavonol, flavone, flavanones, flavans and chalcones (Fig - D) (Harborne *et al*., 1975).

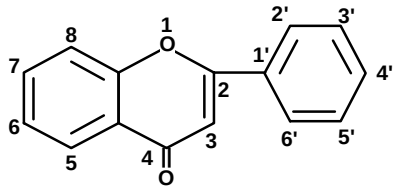
Isoflavones

The isoflavones have their phenyl group (B ring) attached to the 3rd position of the benzopyrone nucleus. Isoflavones have been shown to possess potential estrogenic actions.

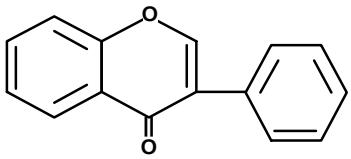
Flavonol

Flavonols have a OH group in the 3rd position of the ring. Gossypin and rutin are important members of this group.

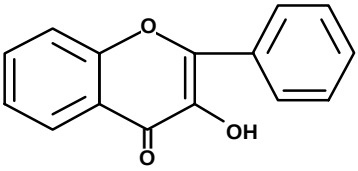
Figure D
Basic Structure of various flavonoids



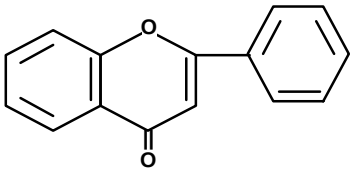
Flavone



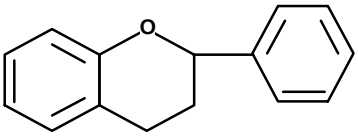
Isoflavone



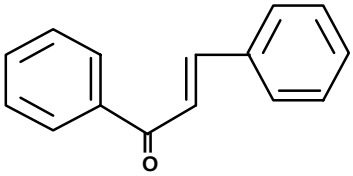
Flavonol



Flavanone



Flavan



Chalcone

Flavanones

Flavanones result from the saturation of the double bond in the 2-3 position of the benzopyrone nucleus.

Flavans

Reduction of the carbonyl group of the pyrone ring and subsequent saturation of this ring gives flavans; catechin and epicatechin are standing examples of this group.

Chalcones

Higher pH results in the opening of the pyrone ring in the 1-2 position of flavanone. Hesperidin methyl chalcone is the best known example of this class.

Natural occurrence of Flavonoids

Flavonoids are present in vascular plants (Harborne and Simmonds.,1964) , but more rare in bryophytes. Some flavonoid classes have a restricted distribution.e.g. Isoflavonoids occur predominantly in Fabaceae family. Flavonoids are present in different plant organs, such as leaves, stems, flowers, fruits, and within a plant, the flavonoid glycosides are found in the cell vacuole, while aglycones are located in the leaf outer wall, in leaf waxes and plant exudates.

Natural occurrence of Flavonoids

Compound	Substitution	Plant source
2-hydroxy flavone	2-OH	Primula and Diosmysia spp
5-hydroxy flavone	5-OH	Primula imperialis and other spp. (Primuletin)
5, 7–dihydroxy flavone	5, 7-(OH)2	Populus spp (Chrysin)
5, 8 –dihydroxy flavone	5, 8-(OH)2	Primula modestia and other spp (Primetin)
Apigenin 5, 7, 4_	(OH)3	Antirhinum spp, Scrophularacea and other spp
Bicalein 5, 6.7-	(OH) 3	Scutellaria spp, Oroxoxylum indicum, Plantago major
Galangin 3, 5, 7-	(OH) 3	Alpinia officinarum
Luteolin 5, 3, 3', 4'-	(OH) 4	Roseda luteola
Kaempferol 3, 5, 7, 4'	(OH) 4	Widespread
Gossypetin 3, 5, 7, 8, 3', 4'	(OH) 6	Widespread

Biological significance of flavonoids

The polyphenolic flavonoids and anthocyanins are present in various parts of the plant. These secondary metabolites have been ascribed with many important functions in plants. The different hues and colours resulting from a combination of several polyphenolics are considered important for pollination of flowers. Many flavonoids possess antiviral activity and also protect plants from predators. Regular consumption of these natural compounds by humans obviously has many nutritional and possibly

therapeutic implications. The biological effects of flavonoids are appreciated in recent times and many amazing facts are reported.

Capillary integrity

Rusznyak and Szent Gyorgi (1936) carried out the pioneering work in guiea pigs and reported that, crude preparations of vitamin-C containing flavonoids could improve the capillary resistance in scorbutic animals more effectively than pure vitamin C. This observation was later confirmed by Rusznyak and Benko (1941). Further studies conducted in rats revealed an unequivocal effect of flavonoids in maintaining capillary integrity in these species (Benko et al., 1970, Varkonyi et al., 1971). Hesperidine methyl chalcone increased the capillary resistance of small intestine, large intestine and kidney of guinea pigs kept on a scorbutic diet (Gabor et al., 1968)

Anti inflammatory effect

Among the various actions of flavonoids, the anti inflammatory action has been extensively studied by several workers (Rinehart., 1955; Gupta et al., 1971; Gabor, 1986 and Parmar and Ghosh., 1978). Flavonoids have been found to exert a beneficial effect in rheumatoid arthritis (Rinehart, 1955) and also in gingival inflammatory conditions (Carvel and Halperin, 1961). Experimental evidence for the anti inflammatory effect of taxifolin (Gupta et al., 1971) and gossypin (Parmar and Ghosh., 1978) have been provided. Gossypin significantly reduced the paw edema and was also effective against adjuvant and formalin induced arthritis in rats (Parmar and Ghosh., 1978).

Flavone derivatives such as apigenin and chrysin showed higher activity only in acute inflammation whereas luteolin, bicalein, spinosin, santin, ermanin, nepitrin and wogonin exhibited higher level of activity in acute as well as chronic inflammatory models (Lee et al., 2004, Martinez et al., 1997 and Agarwal.,1982).

Muthiah et al., (1993) reported the anti inflammatory effect of several methoxy flavones. In a study by Arivudai Nambi et al., (1996) on hydroxyl flavones, they suggested that, hydroxylation favoured anti inflammatory activity more than methoxylation.

Flavonol glycosides showed similar activity as exhibited by their aglycones when given orally. Flavanone glycosides like naringin and hesperidins when administered intraperitoneally showed good response in both acute and chronic inflammatory models (Perriera et al., 2007).

A flavone titonine and its methoxy and acetyl derivatives (7, 3' 4'_ trimethoxy Flavone and 7, 3 – dimethyl 4 – acetyl flavone) possess different degree of anti inflammatory potential (Carvalho et al., 1999). They suggested that the relative potencies of flavone / flavonol and isoflavone are dependent on the pattern and number of hydroxylation / methoxylation on A/B ring, similar to a report on the structure-activity study by Thirugnanasambantham et al., (1990, 1993) on the antinociceptive effect of flavonoids.

Significant anti inflammatory and anti arthritic activities of silymarin, a mixture of flavano lignans, was also reported in the papaya latex induced model of inflammation and mycobacterial adjuvant arthritis in rats. Gupta et

al., (2000) suggested a role for inhibition of 5- Lipoxygenase in the anti inflammatory and anti arthritic activities of silymarin. A number of other flavonoids also are reported to possess anti inflammatory activity. Hesperidin, apigenin, luteolin and quercetin have been reported to exhibit significant anti inflammatory activity (Alcaraz and Ferrandiz, 1987).

Mechanisms involved in the anti inflammatory action of flavonoids.

Flavonoids may produce their anti inflammatory effect by a multitude of ways to inhibit the inflammatory process. Formation and release of various mediators of inflammation like histamine and prostaglandin are affected by flavonoids (Lorenze et al., 1973; Fewtrell and Gomperts., 1977a), Baumann and Bruchhausen, 1979). Flavonoids inhibit the increased capillary permeability during inflammation (Parmar and Ghosh. 1978). The adhesion of leucocytes to endothelial surface and subsequent migration is influenced by flavonoids like hydroxyethye rutoside (Pearson and Gardon 1979).

Inhibition of cyclooxygenase

Phospholipid metabolism is catalyzed by enzymes such as phospholipase A2 (PLA2), cyclo oxygenase (COX), and lipoxygenase (LO) that lead to the production of inflammatory mediators such as prostaglandins (PGs) and Leukotrienes (LTs). Inhibition of these enzymes therefore remains an attractive target for development of anti inflammatory agents (Payan et al. 1995). Inhibition of prostaglandin E_2 and leukotriene C_4 in mouse peritoneal macrophages and thromboxane B2 production in human platelets by

flavonoids from *Stachys chrysanta* and *Stachys candida* was studied by Kaltsa et al., (2002).

5, 7 - di hydroxy 7 - methoxy flavone was reported to have moderate inhibitory activity of prostaglandins (Daott et al., 2003). Harris et al., (2006) reported that luteolin and chrysin suppressed PGE_2 formation equally well, despite differential effects on COX-2 protein expression and on superoxide and hydroxyl radical scavenging. These data indicate that flavones may display similar anti inflammatory activity via different mechanisms. Cheng and Harris (2004) have evaluated 2', 4', 7- trimethoxy flavone for its inhibitory activity of PGE_2 production from LPS treated mouse macrophage RAW 264.7 cell line.

Wogonin (5,7- di hydroxy 8-methoxy flavone) was found to suppress pro inflammatory enzymes including COX-2 (Park et al., 2001 and Chi and Kim 2005). Among flavonols, kaempferol, quercetin, myricitrin and 6-c-methyl quercetin 3, 7, 3' trimethyl ether were reported to inhibit COX-1 catalyzed PG biosynthesis in vitro. Prenylated flavonoids such as brousso chalcones A, and cycloheterophylline were reported to inhibit COX. Promising COX-2 inhibitors from prenylated flavonoids have also been identified (Kim et al., 2005, Jang et al., 2002).

Inhibition of lipoxygenase (LO)

Several flavonoids were investigated on various in vitro assays for LO inhibitory activity. Among them, flavonol, (3-hydroxy flavone), fisetin, quercetin and kaempferol possessed 5-LO inhibitory activity whereas oroxylin - A and

wogonin showed 12 - LO inhibition in vitro assay (Laughton et al., 1991), Luteolin, baicalein and cirsilol showed both 5-LO and 12- LO inhibition (Yoshimoto et al., 1983, Yamamoto et al., 1998). From the above reports it is clear that 5, 6, 7 or 5, 7, 8 tri substitution of A ring of flavone favors 12 - LO inhibition and hydroxyl subtituent at 4' favours 5 - LO inhibitory effects. Gupta et al., (2000) suggested a role for inhibition of 5- lipoxygenase in the anti inflammatory and antiarthritic activities of silymarin. Crisilol, a flavone when derivatized by introducing alkyl groups of 5-10 carbons at 5 or 6 position of A ring markedly decreased IC50 values for 5- lipoxygenase inhibition. From the recent structure activity relationship studies of flavonoids inhibiting rabbit reticulocyte 15-LO-1, it is evident that in parallel to the free radical scavenging properties, presence of a catechol arrangement in the B or A ring and a carbonyl group with C-2,3 double bond in C ring favours LO inhibition (Sadik et al., 2003).

Flavonoids such as hesperidin, naringin and quercitin when preincubated with H_2O_2 treated endothelial cells, demonstrated strong inhibition of acetyl transferase (AT) activity (Balestrier et al., 2003). AT is known to participate in platelet activating factor (PAF) biosynthesis, a potent inflammatory lipid.

Inhibition of cytokines

Flavonoids inhibit cytokine release from RAW 264.7 Cells (Xagorari et al., 2002) and may modulate the increasing number of cellular processes

involving redox titration including the regulation of tyrosine phosphatase activity (Gamet -payarastre et al., 1999).

Inhibition of free radicals

Several types or reactive species are generated in the body as a result of metabolic reactions in the form of free radicals or non radicals. 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. Flavonoids have been found to inhibit a wide range of enzymes involved in oxidation systems such as 5-lipoxygenase, cyclooxygenase, mono oxygenase and xanthine oxidase (Laughton et al., 1991; Siess et al., 1995; Cotelle et al., 1996; Cushman et al., 1991). These biological activities are related to their anti oxidative effects (Bors et al., 1990: Rice-Evans et al., 1995; 1996).

Flavonoids can exert their antioxidant activity by various mechanisms, for example, by scavenging radicals, which initiate lipid peroxidation and lipid peroxide radicals, by binding metal ions, and by inhibiting enzymatic systems responsible for free radical generation.

Modulation of Nitric oxide (NO) production

The relevance of NO as a mediator of inflammation is more recently known (Lidia., 2000). NO is biosynthesized in mammalian cells by nitric oxide synthase (NOS), which has, neuronal (NOS I) , inducible (NOS II / iNOS), and endothelial (NO III) isozymes. Both NOS III and NOS I are constitutively

expressed whereas NOS II is expressed on induction by lipopolysaccharide (LPS) and number of cytokines in different cell types. Once expressed, this isoform generates nanomolar amounts of NO for hours and days. NO has a role in both acute and chronic inflammation (Kulkarni et al., 2000).

Recent studies indicates that NO stimulates the synthesis of inflammatory PGs by activating COX-2. Thus inhibition of nitric oxide pathway might have beneficial effects on inflammatory diseases, including joint disease. Myricitrin reduces the over expression of nitric oxide synthase and nuclear factor kappa b (NF kb) activation induced by lipo polysaccharide on RAW 264.7 cells (Meotti et al 2006 a).

Wogonin, baicalin and baicaleine were examined for their effect on LPS induced nitric oxide production and i NOS and COX -2 expression and they inhibited LPS induced nitric oxide production (Kim et al., 2005).

5, 4' – Dihydroxy 6, 7, 8, 3', 5' - pentamethoxy flavone demonstrated dose dependent suppression of NO production (Liang et al., 2001). Wogonin, quercetin and luteolin inhibit NO production from LPS induced cells (Kim et al., 2001).Thus the above evidences indicate various mechanisms by which flavonoids could exert their anti-inflammatory action.

Antinociceptive activity of Flavonoids

A novel antinociceptive property was reported for hydroxy ethyl rutoside by Ramaswamy et al., (1980). Subsequent work by Viswanathan et al., (1984 a) revealed potent antinociceptive action for gossypin in different

experimental models. The effect was found to be mediated through opioid pathways and the possible development of tolerance to its antinociceptive action and dependence were investigated. Results revealed that, though gossypin utilized opioid pathways there was no tolerance development to gossypin analgesia either in acute or chronic experiments and no withdrawal signs were identified (Viswanathan et al., 1985). Further studies also indicated that gossypin pretreatment could significantly attenuate the acute tolerance development to morphine analgesia (Ramaswamy and Viswanathan 1997) and gossypin treatment suppressed the withdrawal signs in acutely as well as chronically morphine dependent mice (Viswanathan et al., 1985). Moreover gossypin antinociception was found to involve the cholinergic and GABA ergic systems which are important neurotransmitters in central nervous system (Viswanathan et al., 1993).

Thirugnanasambantham et al., (1985) reported that chrysin, morin and rutin exhibited antinociceptive action in mice. Later, a detailed structure activity study was undertaken with flavone and many of its methoxy and hydroxy derivatives. The flavone nucleus itself was found to exert a significant antinociceptive action which was modified to varying degrees by different substitutions (Thirugnanasambantham et al., 1990 and 1993). A series of dihydroxy flavone compounds were screened by Girija et al., (2002) for exploring potential antinociceptive action of these compounds.

A study by Anjaneyulu and Chopra (2003) investigating quercetin on thermal hyperalgesia in mouse model of diabetic neuropathic pain suggested that, the antinociceptive activity of quercetin may probably involve modulation

of opioidergic mechanism and its potential use in attenuating diabetic neuropathic pain. The antinociceptive action of quercetin against several chemical and mechanical models of pain was further confirmed by Gadotti et al., (2005). The study by Kaur et al., (2005) revealed that quercetin exertes its antinociceptive effect by involving the modulation of adrenergic pathways.

Naidu et al., (2003b) attempted to explore the possible involvement of nitric oxide (NO) System in quercetin reversal of morphine tolerance and dependence in mice. Co administration of L- nitro arginine methyl ester (L-NAME) or quercetin with morphine during the induction phase delayed the development of tolerance to the antinociceptive action of morphine and also reversed naloxone precipitated withdrawal jumps. The results of the study suggested that quercetin reversal of morphine tolerance and dependence may involve its ability to suppress nitric oxide synthase (NOS) activity (Gadotti et al., 2005). Kaempferol-3, 7-O- alpha-di rhamnoside and quercetin 3,7-O-alpha di rhamnoside isolated from the leaves of *Tilia argentea* were shown to possess potent antinociceptive and anti inflammatory activity (Toker et al.,2004).

Meotti et al., (2006a) investigated the antinociceptive effect of myricitrin. They provided evidence for a role of L-arginine- nitric oxide and protein kinase C pathways in the above effect. Five flavonoid derivatives of 5, 7-dihydroxy naringenin isolated from *Viscum album* were investigated and reported to have anti inflammatory and antinociceptive activity without inducing gastric damage. (Orhan et al., 2006).

Flavonoids and anxiolytic activity

In addition to the role in analgesia as described above, flavonoid compounds have also been found to exhibit another novel effect on CNS functions. Wolfman et al., (1994) have reported that 5, 7–dihydroxy flavone (chrysin) possessed anxiolytic actions without inducing sedation and muscle relaxation. They also postulated that this flavonoid is a partial agonist of the central benzodiazepine receptors.

Flavonoids from *Leptospermum scoparium* were found to have affinity for the benzodiazepine receptor. Dose related sedation and anxiolytic effect were exhibited in rats (Haberien et al., 1994).

Anxioselective properties of 6,3' –dinitro flavone, was tested in mice using elevated plus maze by Wolfman et al.,(1996) and it was found that 6,3' –dinitro flavone has a benzodiazepine partial agonist profile, with low selectivity for central benzodiazepine receptor type I and receptor type II. Paladini et al., (1999) studied the effect of halogen and nitro substituted flavones on affinity of ligands for the benzodiazepine receptor and reported that chemical modification of the flavone nucleus increases their anxiolytic potency. Two flavonoids from *Artemisia herba alba* Asso were reported for in vitro GABA-benzodiazepine receptor activity (Salah et al., 2005). Xu et al., (2006) have demonstrated the anxiolytic like effect of bicalein and compared its activity with other anxiolytics.

Neuroprotective actions

Yoon et al (2004) investigated the effects of gossypin on the toxicity induced by oxidative stress or ß-amyloid (Aß) in primary cultured rat cortical cells. The results indicated that gossypin exerted neuroprotective effects in the cultured cortical cells by inhibiting oxidative stress and Aß induced toxicity and that the antioxidant properties of gossypin may contribute to its neuroprotective action,

Anti ulcer effect of Flavonoids

Generally compounds exhibiting analgesic and anti inflammatory effects like NSAID induce gastric mucosal damage. However such an effect has not been reported for any flavonoid. But, in fact, many flavonoids have been documented to possess a protective effect against ulceration. Vogin and Rosi (1961) showed that a combination of orange bioflavonoid complex with vitamin C could protect the animals against histamine and reserpine induced ulcer. Gastric anti ulcer effect of catechin, naringin and gossypin in various experimental models has been extensively studied by Parmar (1977a). These flavonoids protected the animals from ulcers induced by pyloric ligation, restraint and drugs. They also reduced the gastric acid secretion. The effect of flavonoids on histaminergic system, viz; antihistaminic effect (Ramaswamy et al., 1979), histidine decarboxylase inhibition (Reimann et al., 1977) and mast cell stabilizing effects (Fewtrell and Gomperts, 1977a) may play an important role in the anti ulcer and anti secretory property of these compounds. In addition, flavonoids may protect gastric mucosa against free H^+ions by a

radical scavenging action (Slater and Eakins, 1975) and by a direct action on mucosal capillaries (Parmar and Ghosh., 1980).

ß – Hydroxy ethyl rutosides, gosyypin, naringin and (+) cyanidanol-3 were shown to exhibit anti ulcer activity (Parmar et al., 1978). Quercetin, kaempferol, morin, myricetin and rutin when tested were found to inhibit the mucosal content of PAF in a dose dependent manner suggesting the protective role of these substances may be mediated by endogenous PAF (Izzo et al.,1994).

Preventive effect of the flavonoid wogonin against ethanol –induced gastric mucosal damage in rats was tested by Park et al., (2004). They concluded that the flavonoid wogonin could be used as a preventive agent of alcohol induced gastropathy.

Effect on experimental cataract and retinopathy

The cataract observed in diabetic and galactosemic conditions was found to involve the enzyme aldose reductase in the lens. Flavonoids have been shown to inhibit cataract formation in diabetic and galactosemic cataracts (Varma et al., 1977; Parmar and Ghosh, 1979). The inhibition of the enzyme aldose reductase in the lens was found to be responsible for the above beneficial effect.

Cardiovascular actions

A coronary vasodilator action of flavonoids was reported by Setniker et al., (1961). Brkic and Laszt (1972) showed that HR and catechin could

develop collateral circulation after left coronary occlusion. Perflavone has been suggested to be useful in the treatment of angina, atherosclerosis and myocardial infarction (Wagner. 1977).

Parmar and Ghosh (1978, 1980) suggested that flavonoids reduce capillary permeability by decreasing the general susceptibility of the capillaries to various permeability factors. They also have a direct constrictor action on the capillary bed. HR has been reported to increase the affinity of endothelial cells to each other and make the inter-endothelial junctions tighter (Pearson and Gordon., 1979). Based on this action, flavonoids have been successfully used in dysfunctional uterine bleeding, periodontal disease, hemorrhoids and in Reynaud's syndrome (Parmar and Ghosh, 1980).

Flavonoid and anti tumor activity

Yusukava et al., (1990) have reported that the ability of myricitrin to inhibit tumor promotion is due to the activation of immune responses against tumors. Myricitrin's anti mutagenic effect is attributed to a free radical scavenger action (Edenharder and Grunhage.,2003).

The effect of a few methoxy flavones, 3', 4' - dimethoxy flavone, 5, 7, 4'- trimethoxy flavone and in particular 7,3' - dimethoxy flavone on cytochrome p450 1B1 in Scc-9(squamous cell carcinoma) cell was measured with quantitative DNA method. The flavones inhibited the CYP1B1-mRNA expression. Curcumin and quercetin were also found to inhibit CYP 1B1 m RNA expression (Walle and Walle., 2007).

Need for the present study

From the foregoing review, it is clear that flavonoid compounds exhibit multivarious biological actions and have enormous therapeutic utility. A significant anti nociceptive and anti inflammatory action of flavonoids merit closer investigation. Eventhough a wide variety of drugs are currently available to treat pain and inflammation their undesirable side effects necessitate the search for safe and effective compounds. Novel molecules that inhibit several mediators of inflammation and pain are being developed in different parts of the world. The polyphenolic flavonoids in addition to possessing significant antinociceptive and anti inflammatory effects are reported to be devoid of gastric irritation. In fact this is the most common side effect shared by all NSAID. Few previous investigations have been undertaken to analyse the antinociceptive activity of many flavone compounds. It is well known that, structural modification may alter the efficacy of any ative molecule. In this regard the hypothesis proposed by an earlier investigator. (Thiruganana Sambantham et al 1990, 1993) deserves active consideration., The postulations of the above author included that screening of polyhydroxy flavones may yield high efficacy antinociceptive agents. Hence in the present study it has ben envisaged to investigate certain trihydroxy flavones derivatives for their potential effect on nociception and inflammation.

AIM AND SCOPE OF THE STUDY

The main objective of the present study is to conduct a systematic screening of four trihydroxy flavone compounds for their antinociceptive and anti-inflammatory actions. These trihydroxy flavones have nitherto not been subjected to any biological screening. A preliminary screening and acute toxicity testing have been considered essential before subjecting these new compounds to further pharmacological evaluation. In the next stage it has been envisaged to investigate the antinociceptive potential of the selected trihydroxy flavones by different established evaluation techniques. Since the involvement of opioid mechanism has been established for previously investigated flavone compounds, such a possibility has also been considered for the presently chosen trihydroxy flavones. For this purpose, an opioid antagonist naloxone has been included in the antinociceptive paradigm. In addition it has been considered interesting to investigate the role of potassium channels, GABA ergic and α - adrenergic systems, in the antinociceptive action of trihydroxy flavones.

The scope of the study has further been expanded to investigate the trihydroxy flavones for their possible antiinflammatory effect. This has been carried out by a well established screening method namely carregenan induced hind paw oedema in rats.

More experiments have been designed to study the effect of trihydroxy falvones on a variety of mediators that initiate and sustain inflammation and

pain. The effect of these compounds on prostaglandins, interleukin 1β and tumor necrosis factor alpha has been investigated.

Many flavanoid compounds have been reported in the literature to possess high degree of anti-oxidant/ free radical scavenging potential. Suitable in vitro tests have been considered for studying the above property of the selected trihydroxy flavones.

In therapeutic practice it is common to combine different analgesic or antiflammatory drugs to achieve maximal efficacy, with limited side effects. Such a feasiblity has been explored in the present study by combining trihydroxy flavones with an established non steroidal anti inflammatory drug.

The afore mentioned scope of the study is expected to adequately fulfil the aim and help in understanding the antinociceptive and antiinflammatory potential of trihydroxy flavones.

MATERIALS AND METHODS

Trihydroxy Flavones

The four trihydroxy flavones (THF) used in the study, viz: 3, 3', 4' – trihydroxy flavone, 3,6,3' – trihydroxy flavone, 6,3',4' – trihydroxy flavone, and 7, 8,3' – trihydroxy flavone) (Fig E) were synthesized adopting standard procedures at Research Organics, Chennai. Melting point, thin layer chromatography (TLC), U.V spectra and I.R spectra of the synthesised compounds were compared with the standard samples and were found to be similar.

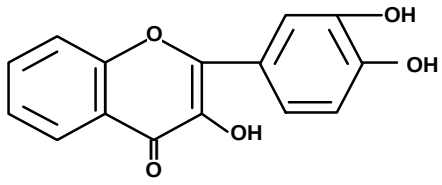
Animals

Male Swiss albino mice (25-30g) and male wistar albino rats (150-200g) were housed in the animal house, Madras Medical College under 12h: 12h light: dark cycle at controlled temperature (25^0 C) with free access to pellet feed (Gold Mohar Ltd., Bangalore) and water. The experiments were mostly conducted between 9.00 and 13.00 h. The experimental protocol was approved by the Institutional Animal Ethical Committee.

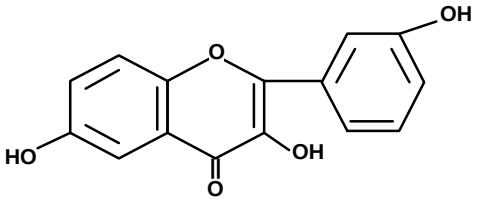
Drugs and Chemicals

The following drugs and chemicals were used. Acetic acid (E. Merck), Carboxy methyl cellulose (Glaxo laboratories, Bombay) Morphine sulphate (Government opium and alkaloid works Ghazipur), Naloxone hydrochloride (Endolabs NY, USA), Diclofenac sodium injection (Novartis), Carageenan

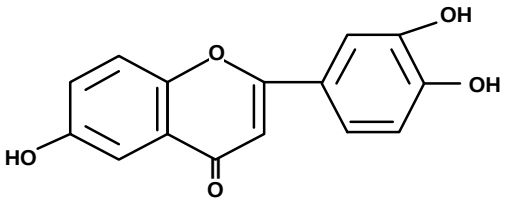
Figure E



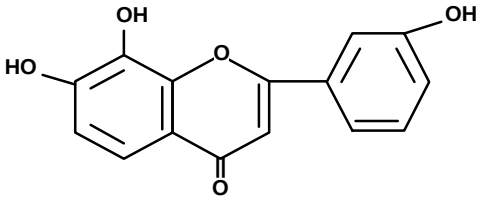
3,3'4' - Trihydroxy Flavone



3,6,3'-Trihydroxy Flavone



6,3',4' - Trihydroxy Flavone



7,8,3' - Trihydroxy Flavone

(Sigma),Glibenclamide(Dr Reddys Lab), Bicuculline (Sigma), Yohimbine (Sigma), Diazepam (Ranbaxy), Carboxy methyl cellulose (Loba) and Formalin (S.D.Fine Chemicals). Diagnostic kits for the assay of interleukin-1 β, Tumor necrosis factor alpha, and cyclooxygenase (Cayman chemicals, USA).

Drug administration

Trihydroxy flavone derivatives were prepared as a uniform suspension in 1% carboxy methyl cellulose (CMC) and injected by subcutaneous (s.c) route in doses of 25, 50, 100 and 200 mg/kg, 60 min prior to the test procedures. The route of administration and time interval for various tests were based on earlier studies involving various flavone derivatives (Girija et al., 2002, Uma Maheswari et al., 2006).

Preliminary screening and acute toxicity

A preliminary acute toxicity testing was carried out in mice. The animals were treated with different doses of various trihydroxy flavones and continuously monitored for two hours to detect any behavioral or autonomic changes and were under observation for two weeks to record any mortality. The highest dose employed was 2 g/kg s.c.

Assessment of locomotor activity

The effect of four trihydroxy flavones in a dose of 200mg/Kg on locomotor activity of mice was tested using an open field apparatus. The number of squares crossed was measured sixty minutes after the administration of trihydroxy flavones for a period of five minutes and

compared with vehicle (CMC) treated group. A group of mice treated with diazepam (5mg/Kg s.c) was also included in the study.

Assessment of balancing time (Motor performance test)

(Dunham and Miya., 1957)

The effect of trihydroxy flavones on the motor co-ordination in mice was tested using a rotarod (15 rpm) in a dose of 200 mg/kg s.c. The balancing time over a rotating rod was measured sixty minutes after administration of trihydroxy flavones. The ability of the animal to remain on the rotating rod was measured and compared with vehicle and diazepam (5mg/Kg s.c) treated animals.

Assessment of antinociceptive activity

Three methods were employed to assess the antinociceptive activity. **Acetic acid induced abdominal constriction assay (Koster et al., 1959).** This assay procedure is considered very sensitive with minimal noxious stimulus. The number of abdominal constrictions (writhings) in mice for a period of 15 minutes was counted following intraperitoneal (i.p) injection of 0.6% acetic acid in a dose of 10 ml/kg (Koster et al., 1959). Any significant reduction in the number of abdominal constrictions when compared with vehicle treated animal was considered as antinociceptive response. Separate groups of animals received different trihydroxy flavones (3,3', 4' – THF, 3,6,3' – THF, 6, 3',4' – THF and 7,8, 3' – THF) in doses of 25, 50, 100 or 200 mg/kg s.c, sixty minutes before acetic acid challenge. Morphine (5mg/kg s.c) was

included as a reference drug for comparison and administered 30 minutes before acetic acid challenge. The abdominal constrictions induced by acetic acid after trihydroxy flavones administration was compared with that of the vehicle treatment.

The percent inhibition of abdominal constrictions produced by different pretreatments was calculated using the formula: %inhibition=(C-T/ C) x100

C = Number of abdominal constriction in vehicle treated group.

T = Number of abdominal constriction in treatment group.

Formalin Assay

Formalin test as described by Dubuisson and Dennis, (1977) was followed. Each mouse was placed in an observation chamber five minutes before the test for acclimatization to the new environment and 50 µl of 0.5% formaldehyde in saline was administered s.c in the left hind paw. The animal was immediately returned to the observation chamber and nociceptive response was recorded for a period of 30 minutes. Summation of time (seconds) spent in licking and biting of formalin injected paw during each 5 minutes block was measured as an indicator of pain response. Duration of responses in first 10 minutes and that from 10 to 30 minutes represent acute and chronic responses respectively. The different trihydroxy flavones were administered in doses of 25, 50, 100 or 200 mg/kg s.c as CMC suspension, to various groups of animals. Formalin was injected to the left hind paw of animals, sixty minutes after different trihydroxy flavones treatment. Morphine

(5 mg/kg s.c) was used as a standard drug and the mice received formalin injection 30 min later. The paw licking time in mice after different dihydroxy flavones/morphine treatment was compared with that of vehicle treated mice. The percent inhibition of paw licking time compared with vehicle treatment was also calculated using the formula:

%inhibition= (C-T) / C x 100

C = Biting/Paw licking response time (seconds) in vehicle treated group

T = Biting/Paw licking response time (seconds) in treatment group.

Tail immersion Method

Antinociceptive activity was also evaluated by tail immersion method as described by Sewell and Spencer (1976). The tail of the animal was immersed in a waterbath maintained at 55 ± 0.5^0c and the time taken to flick the tail was taken as the reaction time. A maximum immersion time of 10 seconds was maintained to prevent thermal injury to the tail. Different trihydroxy flavones were administered s.c. in selected doses (25, 50, 100 or 200 mg/kg) to various groups of animals. The reaction time (in seconds) was observed 60 minutes after administration of trihydroxy flavones. Morphine 5 mg /kg s.c was included as standard drug and the reaction time was observed 30 min later.

The percentage inhibition of thermal nociception was calculated considering the maximum cut off time (10 sec) as 100 % inhibition using the formula: % Inhibition = 100 - (M- T / M)

M = Maximum cut off time (10 sec)

T = Reaction time observed in test group.

Calculation of ED_{50}

The results of the above experiments (acetic acid abdominal constriction assay, formalin and hot water tail immersion assays) were tabulated. The percent inhibition of nociception was calculated and a graph was drawn representing the percentage inhibition against log doses of respective trihydroxy flavone. From the log dose response curves, the ED_{50} values of trihydroxy flavones were computed in the above antinociceptive methods. For further investigations on the possible mechanism of antinoceptive action of trihydroxy flavones, acetic acid induced abdominal constriction assay alone was employed.

Involvement of Opioid System

The involvement of opioid system in the antinociceptive action of trihydroxy flavones was investigated by employing an opioid antagonist naloxone (Viswanathan et al., 1984) in the acetic acid induced writhing assay. Naloxone (5 mg/kg i.p) was administered 15 min prior to trihydroxy flavones (100 mg/kg s.c) or morphine (5 mg/kg s.c) treatment. The antinociceptive activity was then documented either 60 minutes after trihydroxy flavones or 30 minutes after morphine treatment as described earlier. The number of abdominal constrictions after trihydroxy flavones administration in naloxone

pretreated mice was recorded and compared with that obtained in the absence of naloxone pretreatment.

Role of Potassium Channel in trihydroxy flavone induced anti-nociception

ATP sensitive potassium channels are known to participate in the antinociception of opioid analgesics. The possible role of potassium channels in the antinociceptive effect of trihydroxy flavones was investigated in mice. The animals received a potassium channel blocker, glibenclamide (10mg/kg ip.) 15 minutes prior to treatment with various trihydroxy flavones which were administered in a dose of 100mg/Kg s.c. The number of abdominal constrictions after acetic acid injection was assessed in these animals after 60 minutes.

Role of GABAergic system in trihydroxy flavone induced antinociception

Bicuculline, a GABA receptor antagonist was employed to investigate the role of GABA ergic system in the antinociceptive action of trihydroxy flavones. Different groups of mice received bicuculline (1mg/Kg) i.p. 15 minutes prior to different trihydroxy flavone treatment (100mg/Kg s.c). The number of abdominal constrictions after acetic acid injection was recorded in these animals 60 minutes later.

Role of α- 2 adrenergic system on trihydroxy flavone induced antinociception

Yohimbine, an antagonist at α_2 adrenergic receptors was administered in a dose of 1mg/Kg i.p. to various groups of mice. Fifteen minutes later these animals received any one of the trihydroxy flavone in a dose of 100mg/Kg. The animals were challenged with acetic acid 60 minutes later and the number of abdominal constrictions was recorded.

Anti inflammatory effect of trihydroxy flavones

Carrageenan induced hind paw oedema in rats (Winter et al., 1962)

Male wistar albino rats (120-150g) were used in the study. Carrageenan (0.1 ml of 1% solution) was injected s.c. into the plantar surface of the right hind paw. Different trihydroxy flavones (3, 3', 4'–THF, 3,6,3'–THF, 6,3'4'–THF and 7,8,3'–THF) were administered in doses of 25, 50,100 or 200 mg/kg to different groups of animals 30 minutes prior to carrageenan injection. Diclofenac (1 or 10mg/kg i.p) was used as a standard anti inflammatory drug which was administered 30 minutes prior to carrageenan injection. The paw diameter was measured just prior to and thereafter every hour upto 5 hours after carrageenan injection using a digital vernier caliper. Any increase in the paw diameter was taken as a measure of oedema. The diameter of paw after treatment with trihydroxy flavones or diclofenac was compared with that of vehicle treatment. The percent inhibition of inflammation produced by different pretreatments was also calculated using the formula:

(%inhibition= (C-T)/ C x100

C=Paw diameter (cm) in vehicle treated group

T=Paw diameter (cm) in drug treatment group

Mechanisms involved in antinociception and anti inflammatory effect

The effect of trihydroxy flavones on several mediators of pain and inflammation was studied by various in vitro experiments. The role played by cyclooxygenase and cytokines like TNF- α and IL-1β and free radicals was investigated by suitable experiments.

Effect of Trihydroxy flavones on Cyclooxygenase (COX): Enzyme immuno assay (Warner et al., 1999)

The COX-1 and COX-2 immuno assays were carried out using a commercial assay kit according to the manufacturer's instruction (Cayman, USA). 50µl of Prostaglandin standard (15.6–2000 pg) and various trihydroxyflavones (diluted to 10-50µM) were incubated with 50µl of Prostaglandin Screening AChE (Acetylcholine Esterase) tracer and 50µl of Prostaglandin Screening Antiserum for 18 hours at 4ºC.The plate was washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate to AChE) was added to the wells. The intensity of yellow colour was measured at 412 nm. The intensity of this colour is proportional to the amount of tracer bound to the well, which is inversely proportional to the amount of COX-1 and COX-2 present in the well during the incubation.

Ibuprofen was used as standard COX-1 inhibitor and Celecoxib was used as standard COX-2 inhibitor.

Effect of Trihydroxy flavones on certain Cytokines (Warner et al., 1999)

Cytokines are an important group of peptides released from inflammatory cells and also from other types of cells. Tumor necrosis factor and interleukin-1β are primarily considered as pro inflammatory cytokines and are believed to induce the formation of other cytokines as well. They mediate several reactions in the cascade of inflammation and in sensitization of pain fibres. It has been considered worthwhile to investigate the effect of trihydroxy flavones on the above cytokines to understand the mode of action of trihydroxy flavones in detail.

Whole blood assay for cytokine inhibition

Freshly heparinised human whole blood was used for the assay. Trihydroxy flavones (10-500 µm) were diluted in dimethyl sulfoxide (DMSO) and further dilution was done in phosphate buffer saline. To the reaction well, 170µl of blood was added and to it 20µl of compound stock solution was added to get desired final concentration of compound in the reaction well. 10µl of LPS (1µg/ml concentration) prepared in phosphate buffer saline was added into each well. Plate was Incubated for 18 hrs in CO_2 incubator. After 18 hrs of incubation, the plate was centrifuged to collect plasma. The plasma collected was stored at -80^{0}C refrigerator until the sample analysis was done. ELISA assays for TNF- and IL-1 β were carried out using respective ELISA kits as per manufacturer's instructions. Dexamethasone was used as standard.

Cytokines % Inhibition = (Blank – Test / Blank) x 100

Blank = Cytokines measured in vehicle

Test = Cytokines measured with trihydroxy flavones

Reactive oxygen species/ Free radical scavenging activity

Reactive oxygen species / free radicals generated from activated phagocytes have been implicated in inflammation and tissue destruction (Krane et al., 1990). NSAID like sulindac, in addition to COX-inhibition also exerts strong oxygen radical scavenging effect which may decrease tissue damage during inflammation (Rang et al., 2003). Moreover, many beneficial effects of flavonoid compounds have been attributed to their antioxidant/free radical scavenging properties (Burda and Oleszek. 2001). The effect of trihydroxy flavones on free radical generation/ scavenging has been investigated in the present study against 2, 2 diphenyl 1-picryl hydrazyl (DPPH), and nitric oxide radicals by in vitro methods. The test compounds were dissolved in ethanol and employed in concentrations ranging from 4-250 µg /ml. The IC50 value of each compound for the above activity was calculated from the dose response curve.

DPPH scavenging activity (Cotelle et al., 1996)

2, 2 diphenyl-1-picryl hydrazyl (DPPH) is a stable free radical, showing a deep violet colour, characterised by an absorption band in ethanol solution at 517 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, the free radical DPPH is reduced to

corresponding hydrazine (Okawa et al., 2001). DPPH assay is considered a valid and easy method to evaluate the scavenging activity of antioxidants, since the radical compound is stable and does not have to be generated as in other radical scavenging assays (Sanchez-Moreno et al., 1998 and 1999).

3ml of reaction mixture containing 200 µl of DPPH and 2.8 ml of different trihydroxy flavones (3,3', 4' – THF, 3,6,3' – THF, 6, 3',4' – THF or 7,8,3' –THF) at various concentrations(1-200µg/ml) in ethanol was incubated at 37^0 C for 30 min and absorbance of the test mixture was read at 517 nm using a spectrophotometer (Shimatzu). A standard antioxidant vit C (1-200µg/ml) was used for comparison.

The percentage inhibition of DPPH radical was calculated comparing the results of the test with that of the blank using the following formula (Shirwaker et al., 2004).

Percentage scavenging = (Absorbance of blank / Absorbance of Test) X 100

Nitrogen derived radical scavenging activity (Sreejayan and Rao., 1997)

This assay is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions and the same can be estimated using Griess Illosvoy reaction. (Garrat ., 1964)

In the present investigation Griess Illovoy reagent is modified using 0.1% w/v 1-Napthyl ethylene diamine dihydrochloride, instead of 5% w/v 1-

napthylamine. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions (Marcocci et al., 1994).

Sodium niroprusside (5 µM) in standard phosphate buffer solution was incubated with different concentrations (1-200µg/ml) of trihydroxy flavones (3,3'4'- THF, 3,6,3'–THF, 6,3',4'–THF or 7,8,3'–THF) dissolved in ethanol and the tubes were incubated at 25^{o}C for 5 hours. Control experiments without test compounds but with equivalent amounts of buffer were conducted in an identical manner. After 5 hours, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent (1% sulphanilamide, 2% ortho phosphoric acid and 0.1% napthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with ethylene diamine was read at 546 nm. The control experiment was also carried out in a similar manner. The activity was compared with vitamin C (1-200µg/ml) which was used as a standard antioxidant. The percentage inhibition was calculated using the formula:

Percentage scavenging = (Absorbance of Control / Absorbance of test) X 100

Synergism

Effect of diclofenac on trihydroxy flavone induced antinociception

A dose of 1 mg/kg of diclofenac was selected for experiments involving combination of trihydroxy flavones and diclofenac. Separate groups of mice were pre treated with diclofenac 1 mg/kg s.c. and 15 minutes later were supplemented with different trihydroxy flavones (25mg/Kg.sc). These animals

were challenged with acetic acid one hour after trihydroxy flavones supplementation. The number of abdominal constrictions induced by acetic acid was assessed in these animals and the values were compared with those obtained in the absence of diclofenac.

Effect of diclofenac on trihydroxy flavone induced anti-inflammatory response

A dose of 1 mg/kg (i.p) of diclofenac was selected for experiments involving combination of trihydroxy flavone and diclofenac. Diclofenac (1mg/Kg) pretreated rats received various trihydroxy flavones (25mg/Kg, s.c), 15 minutes later. These animals received intraplantar injection of carrageenan and the paw diameter was measured at hourly intervals up to 5 hours. The diameter of paw observed after treatment with the combination of trihydroxy flavones and diclofenac was compared with respective individual treatment values. The percentage inhibition of inflammation was also tabulated.

Statistical Analysis

The data obtained from various experiments were subjected to analysis of variance (ANOVA) followed by Tukey's test for multiple comparison. A probability value less than 5% was considered to be statistically significant. The various data have been presented as tables and line graphs.

RESULTS

Preliminary screening and acute toxicity

The preliminary screening of different trihydroxy flavones (3,3',4' THF, 3,6,3'- THF, 6,3'4' – THF and 7,8,3' - THF) in mice indicated that, treatment with these compounds did not induce any significant alteration in autonomic or behavioural responses in mice. No mortality was observed in mice upto a dose of 2 g / kg, for the trihydroxy flavones tested.

Assessment of locomotor activity

The activity score in vehicle treated mice was 127 $\pm$ 5.2. A significant reduction in the activity score was noticed in animals treated with diazepam (table 1). The activity score after treatment with different trihydroxy flavones (3,3',4' – THF, 3,6,3' – THF, 6,3',4' – THF and 7,8,3' – THF) varied from 119 to 132. The activity scores were not significantly different from that of vehicle treatment.

Assessment of motor coordination

The balancing time over a rotating rod remained unchanged in vehicle treated mice. Diazepam treatment significantly reduced the balancing time of mice (Table 2). The balancing time of mice before treatment with any of the trihydroxy flavones ranged between 54 and 59 seconds, and it was not significantly altered by treatment with the investigational trihydroxy flavones (Table 2).

TABLE – 1

Effect of trihydroxy flavones (THF) on locomotor activity in mice

Treatment (mg/kg,sc)	Activity Score[$]
Vehicle	127 ± 5.2
Diazepam (5)	11.5 ± 2.1*
3,3'4' -THF (200)	119 ± 4.2
3,6,3' -THF (200)	132 ± 1.8
6,3',4'-THF (200)	123 ± 2.1
7,8,3' -THF (200)	128 ± 2.4

Each value represents the mean + SEM of six observations.

* P<0.05 compared with vehicle treatment

$ The activity score represents the number of squares crossed by an animal in five minutes

TABLE – 2

Effect of trihydroxy flavones (THF) on Motor Coordination in mice

Treatment mg/kg, s.c.	Balancing time (seconds)	
	0 minute	60 minutes
Vehicle	58.1 + 2.5	56.8 ± 1.91
Diazepam (5)	55.2 + 1.7	2.3 ± 1.1*
3,3',4' -THF (200)	56.8 + 2.1	53.9 ± 2.7
3,6,3' -THF (200)	59.1 + 1.8	56.3 ± 1.8
6,3',4'-THF (200)	54.8 + 2.7	57.3 ± 1.9
7,8,3' -THF (200)	57.3 + 1.3	55.3 ± 2.1

Each value represents the mean + SEM of six observations

* $P < 0.05$ compared with respective observations at 0 minute.

Antinociceptive activity

A. Acetic acid induced abdominal constrictions

3, 3',4' - trihydroxy flavone:

In vehicle treated animals, injection of acetic acid was found to produce 32 abdominal constrictions. (Table–3). In morphine (5 mg/kg) treated animals, no abdominal constriction response could be observed, thus producing 100% inhibition of this nociceptive behaviour. A dose dependent reduction in abdominal constrictions was observed after treatment with different doses (25, 50, 100 and 200 mg/kg) of 3, 3', 4' trihydroxy flavone. (Table-3). The reduction in abdominal constrictions was significant with 25 mg/kg and a maximum reduction was observed with 200 mg/kg of 3, 3',4'- THF. A maximum of 58% inhibition of nociceptron was recorded for 100 mg/kg of 3, 3'4' – THF and there was only a marginal increment (60.4%) of inhibition after treatment with 200 mg/kg of 3, 3', 4' – THF. The ED_{50} of 3, 3',4'- THF in this assay procedure was calculated from the dose response curve (fig.1) and found to be 69.18 mg/kg,s.c.

3, 6, 3' – trihydroxyflavone:

Treatment with 3,6,3'- trihydroxy flavone in different doses (25 – 200 mg/kg sc) decreased the number of abdominal constrictions induced by acetic acid in mice (Table 4). The percentage inhibition of nociception by different doses of 3,6,3'-THF are also presented in table–4. A maximum antinociceptive effect (58.34%) inhibition was observed in a dose of 200

Table - 3

Effect of 3, 3', 4'- trihydroxy flavone on acetic acid induced abdominal constriction in mice.

Treatment mg/Kg, sc	Number of Abdominal Constrictions
Vehicle	32.0 ± 0.89
Morphine – (5)	0 ± 0 * (100)
3,3',4'- Trihydroxy flavone	
25	23.83 ± 0.87* (25.53)
50	21.50 ± 1.34* (32.81)
100	13.33 ± 0.42* (58.34)
200	12.67 ± 0.80* (60.41)

Each value represents the mean ± SEM of six observations

*$p < 0.05$ compared with vehicle treatment.

The value in parenthesis indicates the percent inhibition.

Table - 4

Effect of 3, 6, 3'- trihydroxy flavone on acetic acid induced abdominal Constriction in mice.

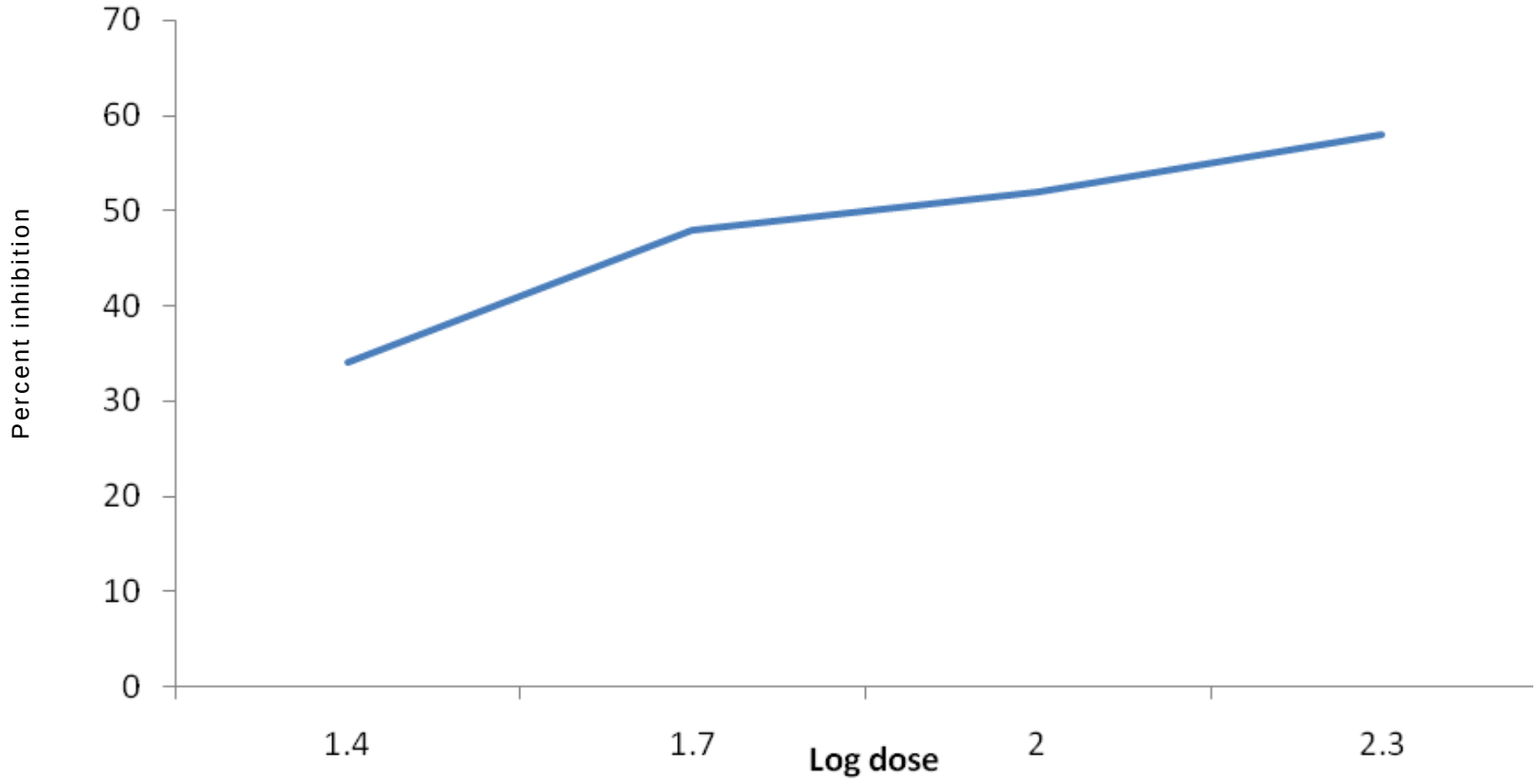
Treatment mg/Kg, sc	**Number of Abdominal Constrictions**
Vehicle	32.0 ± 0.89
Morphine-5	0 ± 0 * (100)
3,6, 3'- Trihydroxy flavones	
25	21.17 ± 1.40* (33.84)
50	16.67 ± 1.5* (47.90)
100	15.17 ± 1.08* (52.28)
200	13.33 ± 0.96* (58.34)

Each value represents the mean ± SEM of six observations.

*$p < 0.05$ compared with vehicle treatment.

The values in parenthesis indicate the percent inhibition.

Fig.1 Inhibitory effect of 3,3',4' – trihydroxy flavone on acetic acid induced abdominal constrictions in mice



mg/kg. The ED_{50} of 3, 6, 3'- THF in this assay was calculated from the dose response curve (fig.2) and found to be 67.60 mg/ kg,s.c.

6, 3' 4' – trihydroxy flavone

A similar reduction in the number abdominal constrictions was recorded after treatment with various doses (25 – 200 mg/kg sc) of 6,3'4' THF (Table –5). A maximum reduction in the number of abdominal constrictions was observed in a dose of 200 mg/kg. The percentage inhibition of nociception by different doses 6,3',4'- THF ranged from, 25.5% to 66%. The ED_{50} of 6,3'4' – THF for the inhibition of abdominal constrictions was calculated from the dose response curve (Fig.3) and found to be 104.7 mg/kg,s.c.

7,8,3'- trihydroxy flavone

Treatment with 7,8,3' - trihydroxy flavone in different doses reduced the number of abdominal constrictions in mice. A maximum of 54% inhibition of abdominal constrictions was evident in a dose of 100 mg/kg. (Table 6). However, there was no further reduction in the number of abdominal constrictions by increasing the dose to 200 mg/kg. The ED_{50} of 7,8,3' trihydroxy flavone for inhibition of abdominal constriction was calculated from the dose response curve (Fig.4) and found to be 79.43 mg/kg, s.c.

Table - 5

Effect of 6, 3', 4' - trihydroxy flavone on acetic acid induced abdominal constriction in mice.

Treatment mg/Kg, sc	Number of Abdominal Constrictions
Vehicle	32.0 ± 0.89
Morphine-5	0 ± 0 * (100)
6,3',4'- Trihydroxy flavones	
25	23.83 ± 0.40* (25.53)
50	19.0 ± 0.93* (40.63)
100	16.33 ± 1.02* (48.97)
200	10.83 ± 1.11* (66.16)

Each value represents the mean ± SEM of six observations.

*$p < 0.05$ compared with vehicle treatment.

The values in parenthesis indicate the percent inhibition.

Table – 6

Effect of 7,8, 3' - trihydroxy flavone on acetic acid induced abdominal constriction in mice.

Treatment mg/Kg, sc	Number of Abdominal Constrictions
Vehicle	32.0 ± 0.89
Morphine	0 ± 0 * (100)
7,8,3'- Trihydroxy flavone	
25	23.67 ± 1.15* (26.03)
50	19.33 ± 0.21* (39.59)
100	14.67 ± 0.56* (54.16)
200	14.83 ± 0.87* (53.66)

Each value represents the mean ± SEM of six observations.

*$p < 0.05$ compared with vehicle treatment.

The values in parenthesis indicate the percent inhibition.

Fig : 3 Inhibitory effect of 6,3',4'-trihydroxy flavone on acetic acid induced abdominal constrictions in mice

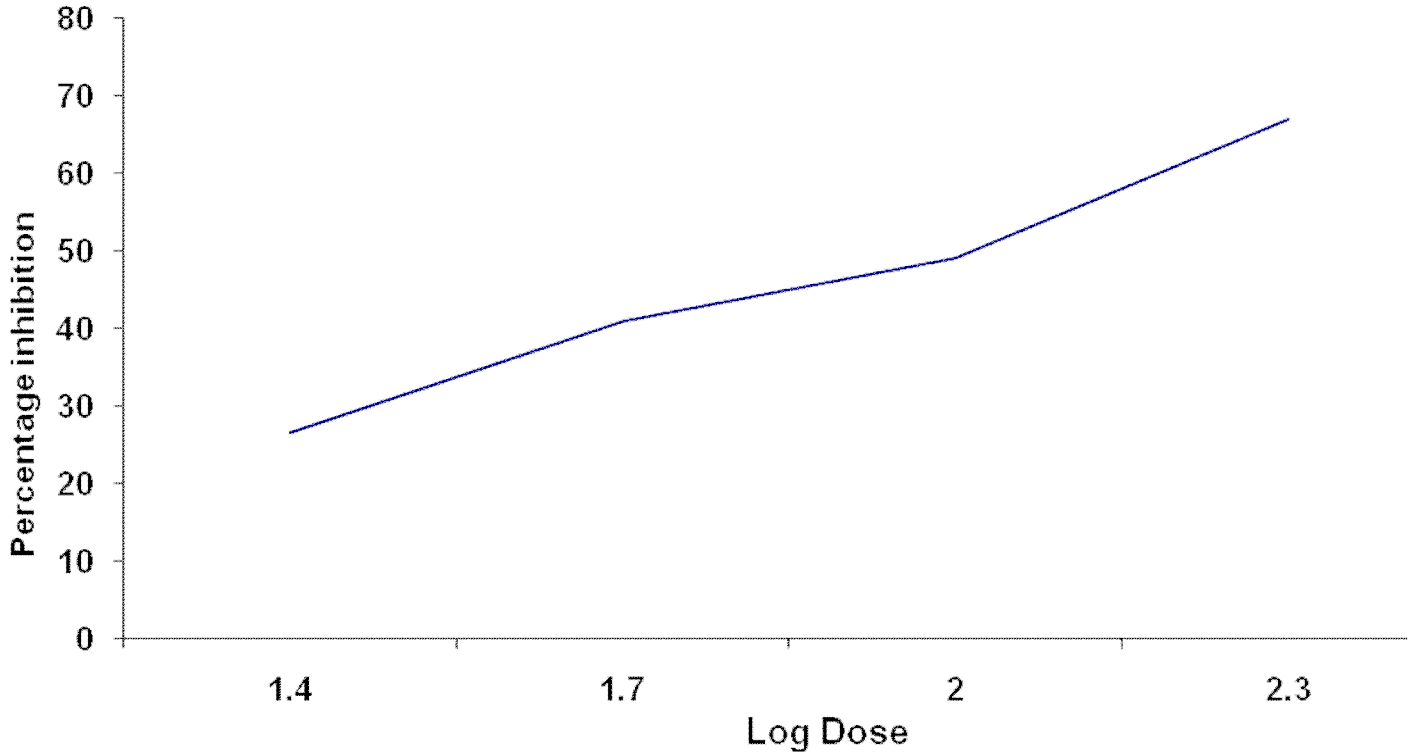
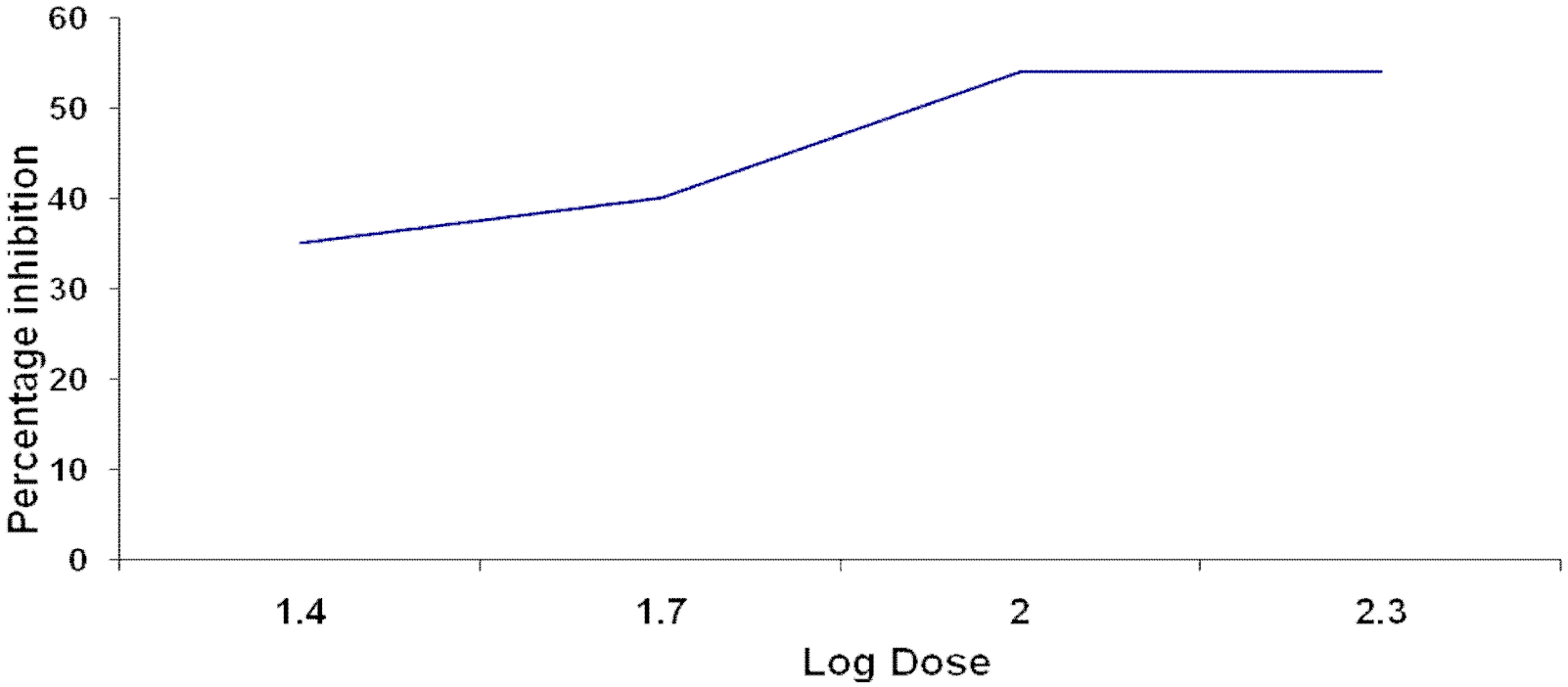


Fig: 4 Inhibitory effect of 7,8,3'-trihydoxy flavone on acetic acid induced
abdominal constrictions in mice



B.Formalin induced nociception:

3, 3',4' - trihydroxy flavone:

In vehicle treated control animals, mean paw licking response time was 58.83 seconds in acute phase and 86.67 seconds in chronic phase (Table 7). Morphine treatment (5 mg/kg) resulted in a marked reduction of paw licking response time to 3 and 8.83 seconds in acute and chronic phases respectively.

Treatment of mice with 3,3',4'- trihydroxy flavone significantly reduced the paw licking response time after formalin injection. The reduction in response time in acute and chronic phases was dose dependent. (Table 7). The inhibition was significant at 25 mg/kg dose and steadily increased with increase in dose of 3, 3',4'- THF.

The degree of inhibition of formalin nociception was more in the chronic phase than in the acute phase. This was clearly evident in doses of 100 and 200 mg/kg. The ED_{50} of 3,3',4'- THF in this assay procedure was calculated from the dose response curve (Fig.5,6) and found to be 42.66mg/kg in acute phase and 52.45 mg/kg in chronic phase.

3,6,3'- trihydroxy flavone

A dose dependent reduction in paw licking response time was recorded after treatment with different doses of 3, 6, 3' – THF (table.8). The reduction in paw licking time was significant even in the minimum dose of 25 mg/kg.

Table - 7

Effect of 3, 3, 4'- trihydroxy flavone on formalin induced nociception in mice.

Treatment mg/Kg, sc	Paw licking/biting response time (seconds)	
	Acute phase 0-10 min	Chronic phase 10-30 min
Vehicle	58.83 ± 0.36	86.67 ± 2.55
Morphine-5	3.00 ± 0.37 * (94.91)	8.83 ± 0.48* (89.83)
3,3',4'- tri hydroxy flavones		
25	41.00 ± 2.41* (30.31)	58.83 ± 3.40* (32.13)
50	27.00 ± 2.76* (54.11)	47.17 ± 2.54* (45.58)
100	21.83 ± 1.83* (62.90)	16.17 ± 0.95* (81.36)
200	19.17 ± 2.15* (67.42)	2.50 ± 0.85* (97.13)

Each value represents the mean ± SEM of six observations.

*$p < 0.05$ compared with vehicle treatment.

The values in parenthesis indicate the percent inhibition.

Table – 8

Effect of 3, 6,3' - trihydroxy flavone on formalin induced nociception in mice.

Treatment mg/Kg, sc	Paw licking / biting response time (seconds)	
	Acute phase 0-10 min	Chronic phase 0-10 min
Vehicle	58.83 ± 0.36	86.67 ± 2.55
Morphine – 5	3.00 ± 0.37 * (94.91)	8.83 ± 0.48* (89.83)
3,6,3'- trihydroxy flavone		
25	35.67 ± 1.96* (39.37)	56.50 ± 3.20* (34.82)
50	28.17 ± 1.49* (52.12)	24.33 ± 1.31* (71.94)
100	28.67 ± 2.95* (56.37)	8.33 ± 0.80* (90.40)
200	20.67 ± 2.03* (64.87)	6.33 ± 1.09* (92.71)

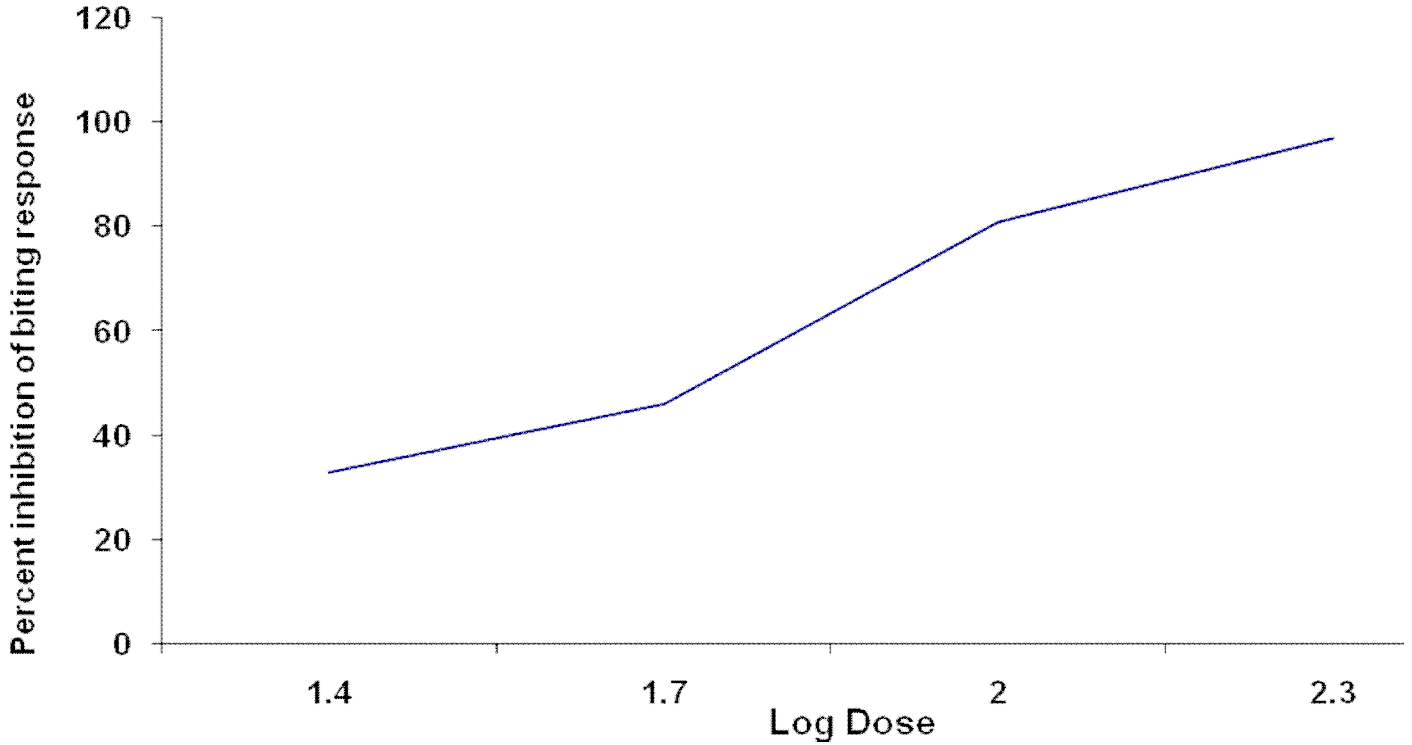
Each value represents the mean ± SEM of six observations.

*$p < 0.05$ compared with vehicle treatment.

The values in parenthesis indicate the percent inhibition.

Fig: 5 Inhibitory response of 3,3',4' - trihydroxy flavone on the biting response in acute phase of formalin nociception in mice

Fig: 6 Inhibitory response of 3,3',4' - trihydroxy flavone on the biting response in chronic phase of formalin nociception in mice



The reduction in paw licking time observed in the chronic phase after treatment with 100 mg and 200 mg/kg of 3,6,3' - THF was similar to that of morphine. The percentage inhibition of paw licking response time ranged from 39 – 65% in acute phase and 34 – 92% in the chronic phase. The ED_{50} of 3,6,3'- THF in this assay procedure was calculated from the dose response curve (Fig7,8) and found to be 45.70 mg/kg for the acute phase and 32.36 mg/kg for chronic phase.

6,3',4'- trihydroxy flavone:

6,3',4' – THF in the doses of 25 to 200 mg/kg produced a significant reduction in paw licking response time in a dose dependent fashion (Table 9). The percentage inhibition of nociception with various doses in acute phase ranged from 8 – 40%.

The percent inhibition of nociception in chronic phase ranged between 5 and 63%. The ED_{50} of 6,3',4'- THF to inhibit the chronic phase of formalin nociception was calculated from the dose response curve (Fig.10) and found to be144.5 mg/kg. The ED_{50} of 6,.3',4'- THF in acute phase of formalin nociception could not be calculated as a maximum of 40% reduction could only be recorded (fig.9) even in the dose of 200 mg/kg.

7, 8, 3' - trihydroxy flavone:

The mean paw licking response time in mice treated with 7,8,3'-trihydroxy flavone in the tested doses (25 – 200 mg/kg) was significantly less when compared with vehicle treated mice (Table 10). The percentage

Table - 9

Effect of 6, 3', 4' - trihydroxy flavone on formalin induced nociception in mice.

Treatment mg/Kg, sc	Paw licking / biting response time (seconds)	
	Acute phase 0-10 min	Chronic phase 10-30 min
Vehicle	58.83 ± 0.36	86.67 ± 2.55
Morphine-5	3.00 ± 0.37 * (94.91)	8.83 ± 0.48* (89.83)
6, 3',4' - trihydroxy flavone		
25	54.17 ± 2.07* (7.92)	82.83 ± 3.99* (5.01)
50	43.67 ± 3.22* (25.77)	75.83 ± 4.87* (12.51)
100	39.50 ± 2.47* (32.86)	56.67 ± 3.39* (34.62)
200	35.33 ± 1.02* (39.95)	32.17 ± 1.58* (62.90)

Each value represents the mean ± SEM of six observations.

*$p < 0.05$ compared with vehicle treatment.

The values in parenthesis indicate the percent inhibition.

Table - 10

Effect of 7, 8, 3'- trihydroxy flavone on formalin induced nociception in mice.

Treatment mg/Kg, sc	Paw licking/ biting response time (seconds)	
	Acute phase 0-10 Min	Chronic phase 10-30 Min
Vehicle	58.83 ± 0.36	86.67 ± 2.55
Morphine	3.00 ± 0.37 * (94.91)	8.83 ± 0.48* (89.83)
7,8,3' - Trihydroxy flavones		
25	41.5 ± 5.2* (29.46)	73.33 ± 2.89* (15.39)
50	40.67 ± 3.72* (30.87)	64.33 ± 5.6* (25.78)
100	35.87 ± 5.3* (39.0)	47.0 ± 6.90* (45.78)
200	29.0 ± 4.81* (50.71)	32.17 ± 4.69* (62.89)

Each value represents the mean ± SEM of six observations.

*$p < 0.05$ compared with vehicle treatment.

The values in parenthesis indicate the percent inhibition.

Fig: 7 Inhibitory response of 3,6,3' - trihydroxy flavone on the biting response in acute phase of formalin nociception in mice

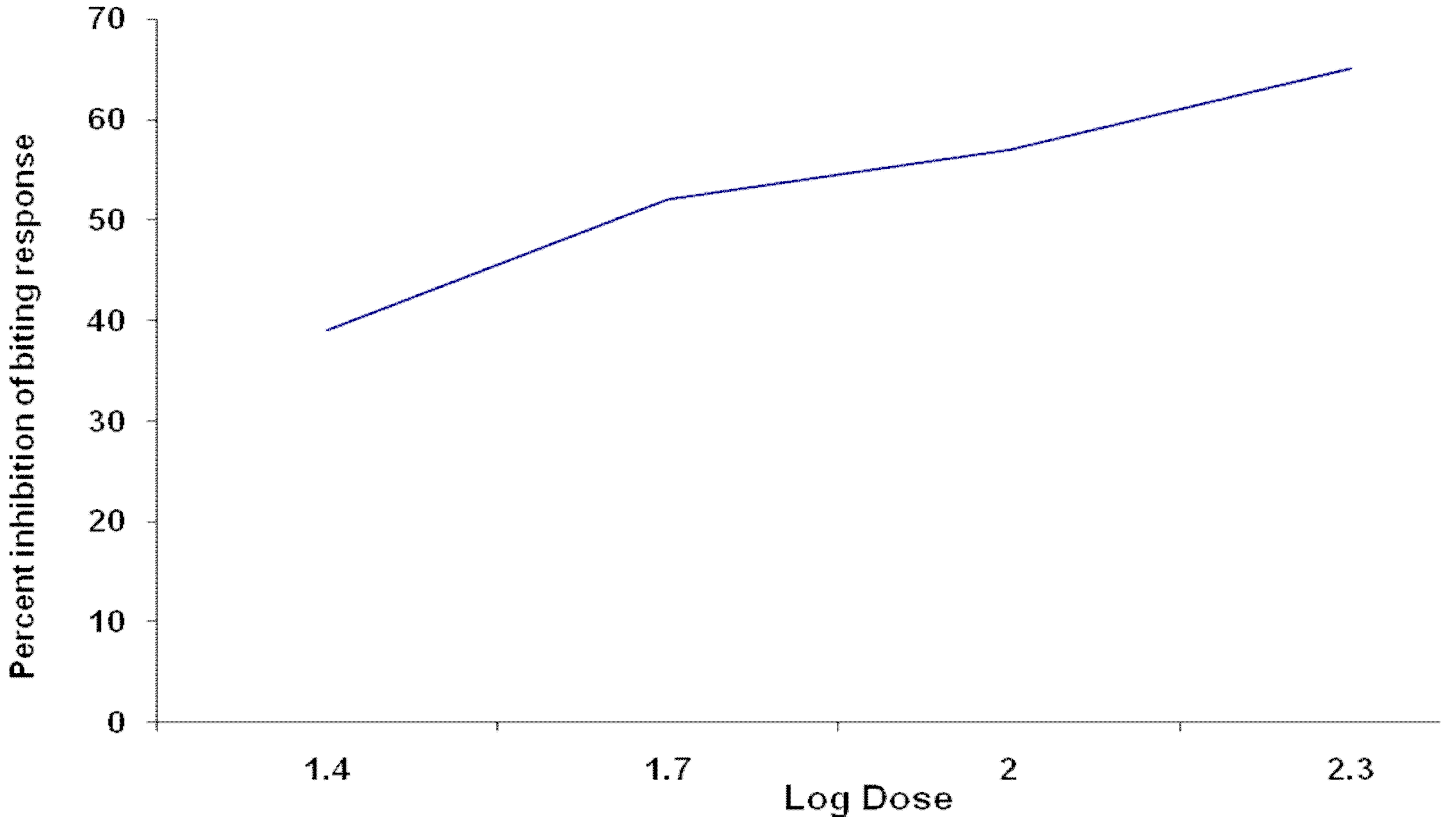


Fig: 8 Inhibitory response of 3,6,3'- trihydroxy flavone on the biting response in chronic phase of formalin nociception in mice

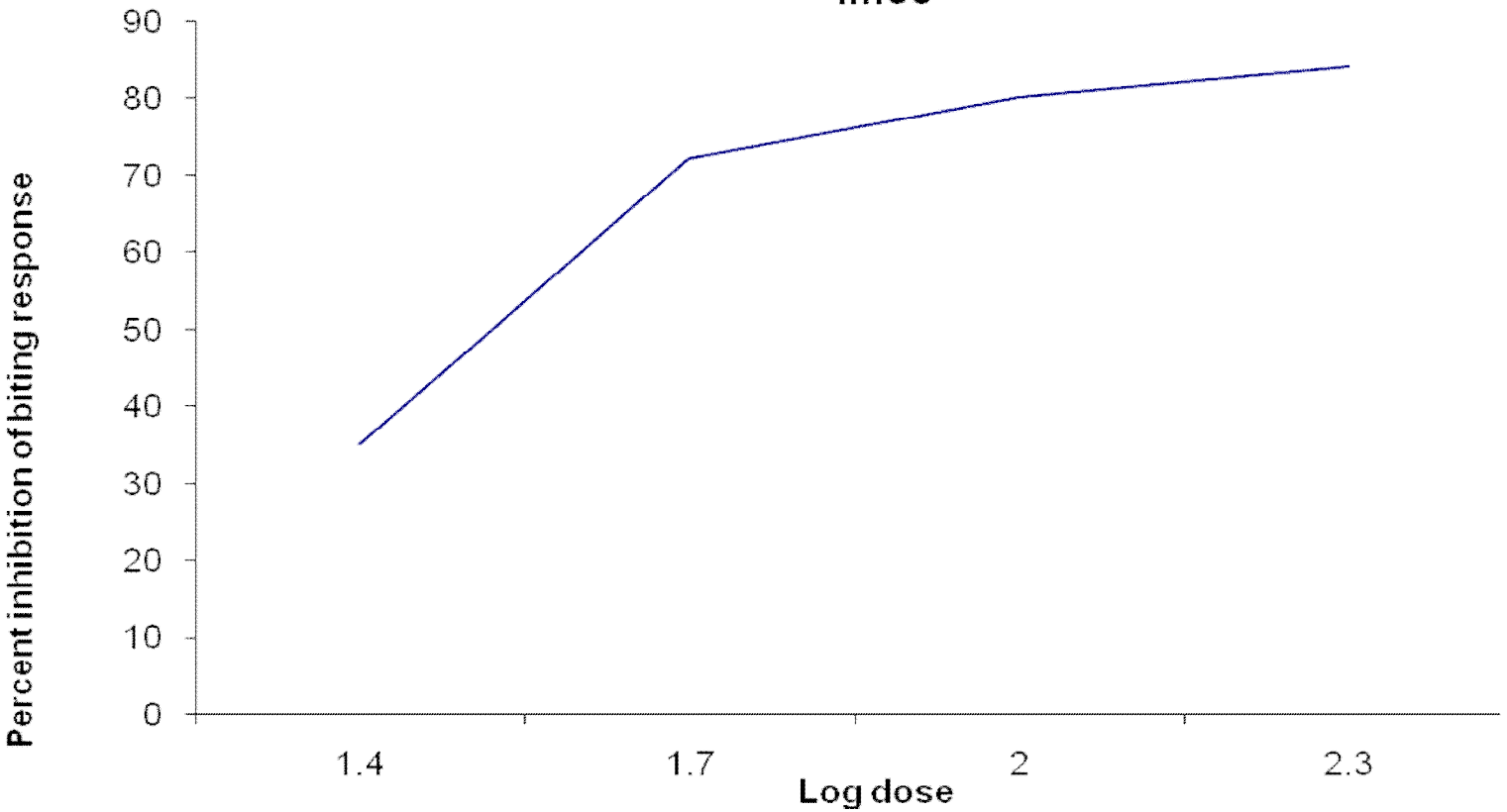


Fig: 9 Inhibitory response of 6,3',4' - trihydroxy flavone on the biting response in acute phase of formalin nociception in mice

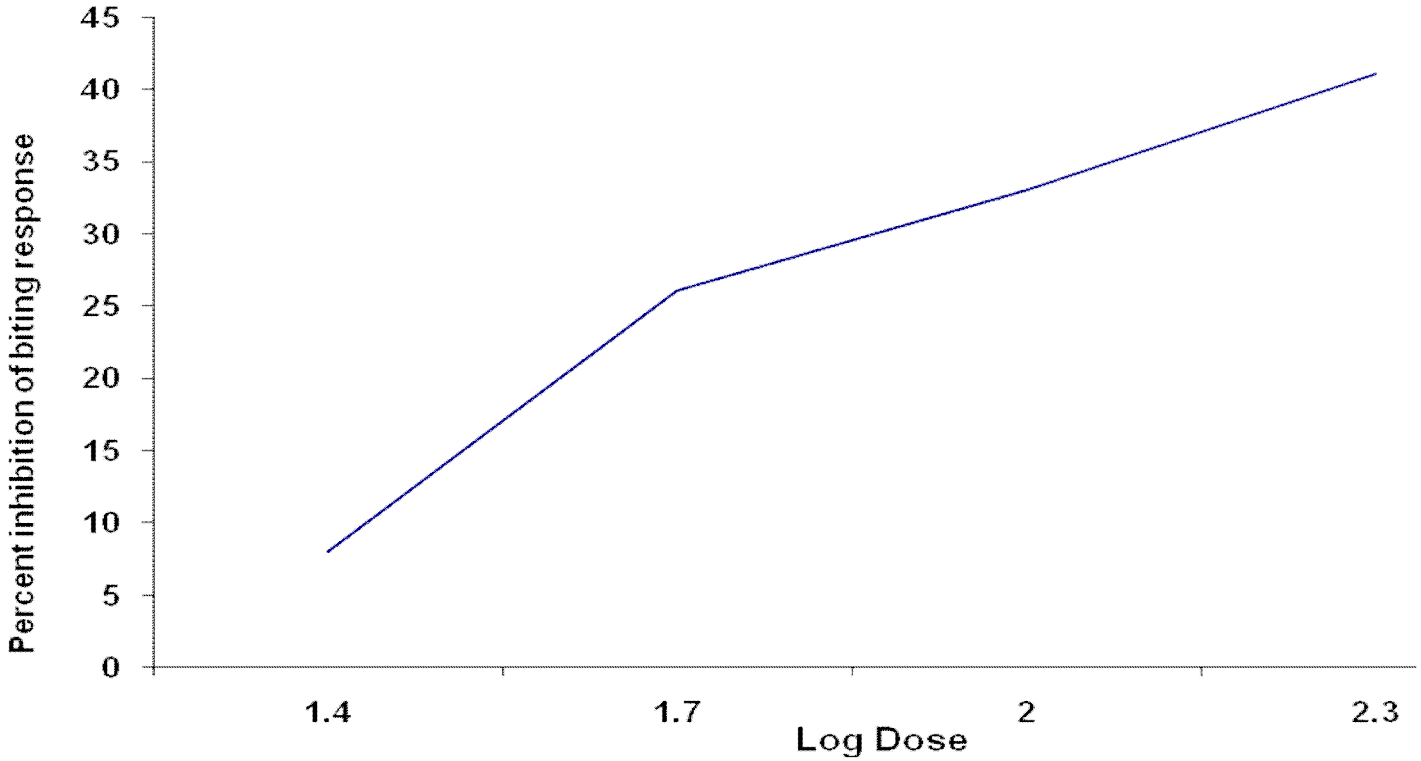
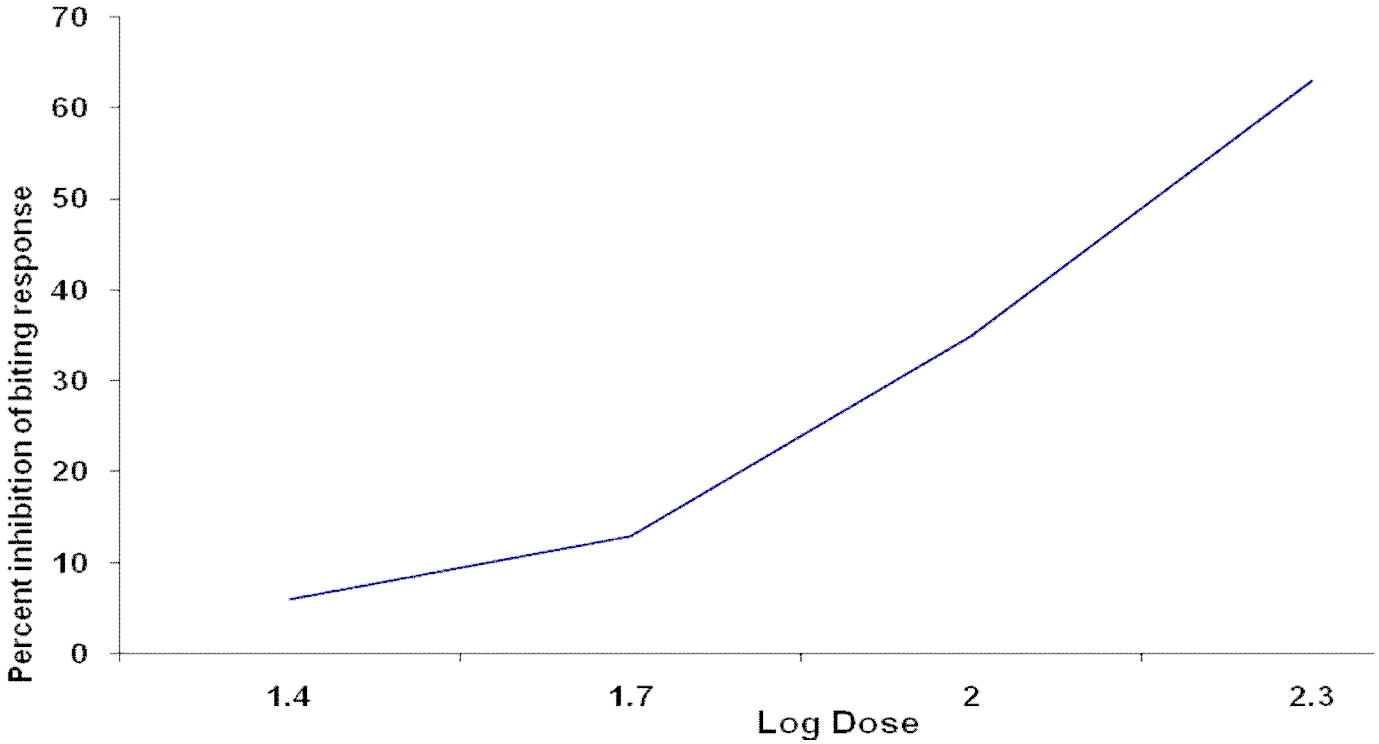


Fig: 10 Inhibitory response of 6,3',4' - trihydroxy flavone on the biting response in Chronic phase of formalin nociception in mice



inhibition of nociceptive response ranged from 29 to 51% in the acute phase and 15 to 63% in the chronic phase for various doses of 7, 8, 3' – THF. The ED_{50} of 7,8,3'- THF in this assay procedure was calculated from the dose response curves(Fig.11,12) and found to be 182.0 mg/kg in acute phase and 114.8 mg/kg in chronic phase:

C. Hot water tail immersion assay

3 3'4' - trihydroxyflavone:

The mean reaction time in vehicle treated mice was 0.30 seconds which was significantly increased to 7.36 seconds in morphine treated animals (table 11). A significant increase in reaction time was evident with increasing doses of 3, 3'4' – THF. A maximum of 14.6% inhibition was recorded for 200 mg/kg dose of 3, 3',4' – THF compared to 73% inhibition produced by morphine.

3,6,3'- trihydroxy flavone:

A significant increase in reaction time was observed with various doses of 3,6,3' – THF. The percentage inhibition of nociception ranged from 7.2%for 25 mg/kg to 18.3% for 200 mg/kg of 3,6,3' - THF (table 12).

Table – 11

Effect of 3,3'4'- trihydroxy flavone on hot water tail immersion in mice

Treatment mg/Kg, sc	**Reaction time (Seconds)**
Vehicle	0.30 ± 0.05
Morphine (5)	7.36 ± 0.34* (73.6)
3,3,'4' – trihydroxy flavone	
25	0.94 ± 0.06* (9.4)
50	1.04 ± 0.05* (10.4)
100	1.26±0.09* (12.6)
200	1.46±0.12* (14.6)

Each value represents the mean ± SEM of six observations.

*p < 0.05 compared to vehicle treatment.

The values in parenthesis indicate the percentage inhibition

Table – 12

Effect of 3, 6, 3' - trihydroxy flavone on hot water tail immersion in mice

Treatment mg/Kg, sc	**Reaction time (Seconds)**
Vehicle	0.30 ± 0.05
Morphine (5)	7.36 ± 0.34* (73.6)
3,6,3' – trihydroxy flavone	
25	0.72± 0.06* (7.2)
50	1.26 ± 0.14* (12.6)
100	1.56±0.11* (15.6)
200	1.83±0.12* (18.3)

Each value represents the mean ± SEM of six observations.

*$p < 0.05$ compared to vehicle treatment

The values in parenthesis indicate the percentage inhibition

Fig: 11 Inhibitory response of 7,8,3' - trihydroxy flavone on the biting response in acute phase of formalin nociception in mice

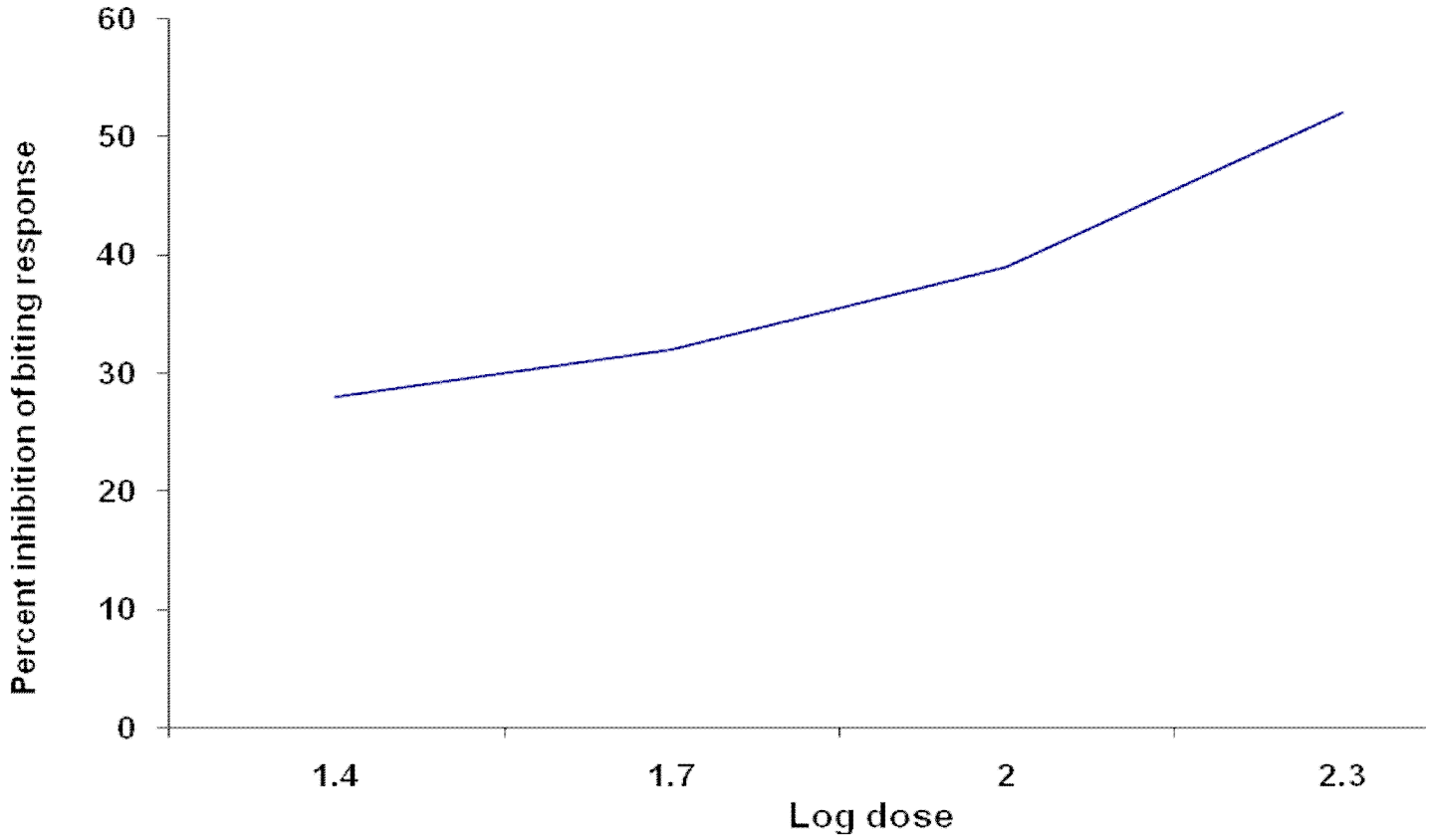
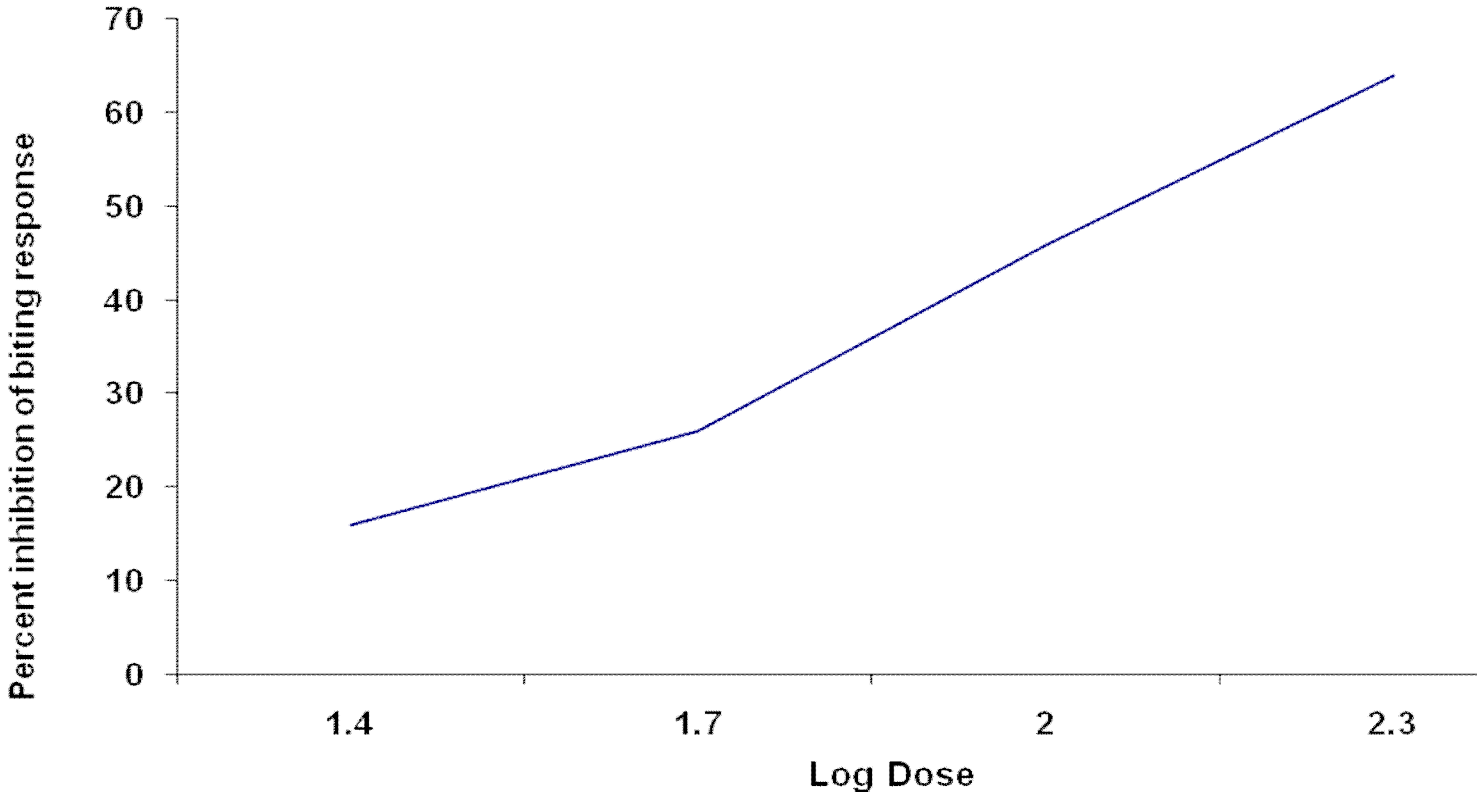


Fig: 12 Inhibitory response of 7,8,3' -trihydroxy flavone on the biting response in chronic phase of formalin nociception in mice



6,3',4'- trihydroxy flavone

Treatment with 6,3',4'- trihydroxy flavone in various doses produced a dose dependent increase in the reaction time. The percentage inhibition of nociception with 25 mg/kg of 6,3',4' – THF was 11.6% and a maximum of 22.1% inhibition was recorded for 200 mg/kg (Table 13).

7,8,3'- trihydroxy flavone

A significant and dose dependent increase in reaction time was evidenced after treatment with 7, 8, 3' – THF. A minimum of 6.3% inhibition of nociception was recorded in a dose of 25 mg/kg and a maximum of 28% inhibition was recorded in a dose of 200 mg/kg. (Table 14).

ED_{50} Values Of Trihydroxy Flavones In Different Nociceptive Procedures

The ED_{50} values obtained for various trihydroxy flavones in different nociceptive procedures are indicated in table 15. In acetic acid nociception the ED_{50} values for 3, 3',4' – THF and 3,6,3' – THF were almost similar (69.18 and 67.60 mg/kg s.c.). The ED_{50} value of 7,8,3'-THF was 79.43 mg/kg and a higher ED_{50} value of 104.7 mg/kg was recorded for 6,3',4'-THF.

In formalin induced nociception the ED_{50} values of trihydroxy flavones to inhibitt the acute and chronic phases were vastly different. In general the ED_{50} recorded to inhibit the chronic phase was considerably less compared to the ED_{50} of the same compound to inhibit the acute phase. However, this generalisation was not attributable to 3,3',4'-THF wherein the ED_{50} value of chronic phase was more than the acute phase.

Table – 13

Effect of 6, 3', 4' - trihydroxy flavone on hot water tail immersion in mice

Treatment mg/Kg, sc	**Reaction time (Seconds)**
Vehicle	0.30 ± 0.05
Morphine (5)	7.36 ± 0.34* (73.6)
6,3',4' – trihydroxy flavone	
25	1.16± 0.09* (11.6)
50	1.32 ± 0.06* (13.2)
100	1.37±0.09* (13.7)
200	2.21±0.20* (22.1)

Each value represents the mean ± SEM of six observations.

*p < 0.05 Compared to vehicle treatment.

The values in parenthesis indicate the percentage inhibition

Table – 14

Effect of 7, 8, 3' - trihydroxy flavone on hot water tail immersion in mice

Treatment mg/Kg, sc	**Reaction time (Seconds)**
Vehicle	0.30 ± 0.05
Morphine (5)	7.36 ± 0.34* (73.6)
7,8 ,3' – trihydroxy flavone	
25	0.63± 0.05* (6.3)
50	1.50 ± 0.07* (15)
100	1.91±0.19* (19.1)
200	2.80±0.15* (28.0)

Each value represents the mean ± SEM of six observations.

*p < 0.05 compared to vehicle treatment.

The values in parenthesis indicate the percentage inhibition

Table -15

ED_{50} Values of Trihydroxy flavones

Name of the Compound	ED_{50} by Acetic acid nociception mg/Kg s.c.	ED_{50} by formalin nociception mg/Kg s.c.	
		Acute Phase	Chronic Phase
3,3',4' – THF	69.18	42.66	52.45
3,6,3' – THF	67.60	45.70	32.36
6,3',4' – THF	104.7	>200	144.5
7,8,3' – THF	79.43	182	114.8

The ED_{50} values of different trihydroxy flavones in thermal nociception could not be ascertained because none of the compounds produced an inhibition greater then 28% in the doses studied.

Involvement of Opioid system

The influence of opioid system in the antinociceptive action of trihydroxy flavones was investigated by employing acetic acid induced abdominal constriction assay. Naloxone *per se* in a dose of 5 mg/kg did not alter the number of abdominal constriction when compared to vehicle treatment (Table 16). In animals that received only morphine, no abdominal constricton could be elicited by acetic acid injection thus revealing total protection by morphine in this type of nociception. In contrast, naloxone pretreatment completely annulled the protective effect of morphine. The number of abdominal constrictions in these animals (29.5) was similar to vehicle treatment. In a similar fashion the reduction in the number of abdominal constrictions elicited by different trihydroxy flavones was antogonised by naloxone pretreatment. This reversal was complete in respect of 3, 6, 3' THF, and partial in case of other trihydroxy flavones (Table 16).

Role of potassium channel in the antinociceptive action of trihydroxy flavones

Glibenclamide, a known potassium channel blocker did not alter the number of abdominal constrictions when compared to vehicle treatment. Pretreatment with glibenclamide did not affect the reduction in the abdominal

Table - 16

Effect of Naloxone on trihydroxy flavone

induced antinociception in mice

Treatment mg/Kg, sc	Number of abdominal constrictions	
	In the absence of Naloxone	In the presence of Naloxone[$]
Vehicle	32.0 ± 0.89	32.4 ± 0.22
Morphine (5)	0.0 ± 0.0*	29.5 ± 0.25[≠]
3,3',4'-THF (100)	13.33 ± 0.42*	23.0 ± 0.93*[≠]
3,6,3'-THF (100)	15.17 ± 1.08*	28.50 ± 0.76[≠]
6,3',4'-THF (100)	16.33 ± 1.02*	24.33 ± 0.99*[≠]
7,8,3'-THF (100)	14.67 ± 0.56*	18.50 ± 1.06*[≠]

Each value represents the mean ± SEM of six observations.

*p< 0.05 when compared with vehicle treatment.

≠p <0.05 when compared with respective value in the absence of naloxone

$ The animals received naloxone 5 mg/kg i.p,15 min prior to THF/ Morphine treatment

constriction elicited by 3,3'4' – THF, 3,6,3' – THF or 6,3',4' – THF. But the reduction in the number of abdominal constrictions produced by 7,8,3'- THF was significantly attenuated by glibenclamide pretreatment (Table 17).

Role of GABA ergic system in the antinociceptive effect of trihydroxy flavones

Bicuculline, an antogonist at $GABA_A$ – receptors did not alter the number of abdominal constrictions compared to vehicle treatment (Table 18). Bicuculline pretreatment did not affect the reduction in the number of abdominal constrictions elicited by 3,3',4'- THF and 3, 6, 3' – THF. However, bucuculline pretreatment significantly attenuated the reduction in the abdominal constriction produced by 6,3',4' – THF and 7,8,3'- THF.

Role of α -2 Adrenergic System in trihydroxy flavone induced antinociception

Yohimbine in a dose of 1 mg/kg did not change the number of abdominal constrictions compared to vehicle treatment (Table 19). Yohimbine pretreatment significantly attenuated the reduction in the number of abdominal constrictions elicited by 3,6,3'- trihydroxy flavone. However, the reduction in abdominal constrictions observed with 3,3',4' – THF, 6,3',4'- THF or 7,8, 3' – THF remained unaffected by Yohimbine pre treatment.

Table - 17

Effect of Glibenclamide on trihydroxy flavone

induced anti nociception in mice

Treatment mg/Kg, sc	Number of abdominal constrictions	
	In the absence of Glibenclamide	In the presence of Glibenclamide[$]
Vehicle	32.0 ± 0.89	29.83± 1.19
3,3',4'-THF (100)	13.33 ± 0.34*	16.83 ± 1.70*
3,6,3'-THF (100)	15.17 ± 1.08*	13.67 ± 1.09*
6,3',4'-THF (100)	16.33 ± 1.02*	16.83 ± 1.20*
7,8,3'-THF (100)	14.67 ± 0.56*	22.17 ± 1.22*[≠]

Each value represents the mean ± SEM of six observations.

*$p<0.05$ when compared with vehicle treatment.

≠$p<0.05$ when compared with respective value in the absence of glibenclamide

$The animals received glibenclamide (10mg/kg, i.p) 15 min prior to THF treatment

Table - 18

Effect of Bicuculline on trihydroxy flavone induced antinociception in mice

Treatment mg/Kg, sc	Number of abdominal constrictions	
	In the absence of Bicuculline	In the presence of Bicuculline $
Vehicle	32.0 ± 0.89	30.17 ± 0.60
3,3',4'-THF (100)	13.33 ± 0.42*	16.5 ± 1.40*
3,6,3'-THF (100)	15.17 ± 1.08*	17.83 ± 0.83*
6,3',4'-THF (100)	16.33 ± 1.02*	23.5 ± 0.67*≠
7,8,3'-THF (100)	14.67 ± 0.56*	30.33 ± 1.17≠

Each value represents the mean ± SEM of six observations.

*p < 0.05 when compared with vehicle treatment.

≠p< 0.05 when compared with respective value in the absence of bicuculline.

$The animals received bicuculline (1 mg/kg, ip) 15 min prior to THF treatment

Table - 19

Effect of Yohimbine on trihydroxy flavone induced antinociception in mice

Treatment mg/Kg, sc	Number of abdominal constrictions	
	In the absence of Yohimbine	In the presence of Yohimbine[$]
Vehicle	32.0 ± 0.89	29.33± 0.67
3,3',4'-THF (100)	13.33 ± 0.42*	13.83 ± 1.22*
3,6,3'-THF (100)	15.17 ± 1.08*	25.67 ± 0.88*[≠]
6,3',4'-THF (100)	16.33 ± 1.02*	16.00 ± 0.63*
7,8,3'-THF (100)	14.67 ± 0.56*	13.17 ± 0.70*

Each value represents the mean ± SEM of six observations.

* $p < 0.05$ when compared with vehicle treatment.

≠$p < 0.05$ when compared with respective value in the absence of yohimbine.

$The animals received yohimbine (1mg/kg, i.p) 15 min prior to THF Treatment

Effect of trihydroxy flavones on rat paw oedema:

3,3',4'- trihydroxy flavone:

In vehicle treated animals, carrageenan administration in the hind paw resulted in progressive increase in the paw diameter and a maximum of 1.38 cm was recorded after five hours. In diclofenac (10 mg/kg) pretreated animals the diameter of the paw increased after one hour but there was a gradual decline during the remaining periods of observation. When compared to vehicle treatment a significant reduction in paw diameter was recorded from the second hour onwards (Table 20).

In various groups of animals that received different doses of 3,3',4' – THF, there was no significant alteration in paw diameter compared to vehicle treatment during the first hour of observation. However, a significant reduction in paw diameter was clearly evident compared to vehicle treated group during subsequent periods of observations. Declofenac treatment produced a maximum of 72.5% inhibition of paw oedema (Table 20). 3,3',4'– THF in a dose of 100 mg/kg produced a maximum of 60% inhibition of paw oedema and further increment in dose did not produce any augmented effect.

3, 6, 3' - trihydroxy flavone

In 3, 6, 3' – THF treated animals the paw diameter recorded during the first hour of observation was not significantly diffeent from that of vehicle treated animals. But a significant, dose dependent reduction in paw diameter

TABLE - 20

Effect of 3, 3', 4' trihydroxy flavone on rat paw oedema

Treatment mg/Kg, sc	Paw Diameter (cm)					
	0 Hour	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Vehicle	0.38 ± 0.01	0.86 ± 0.02	0.97 ± 0.03	1.18 ± 0.06	1.33 ± 0.02	1.38 ± 0.02
Diclofenac – (10)	0.34 ± 0.01 (0)	0.87 ± 0.01 (0)	0.66 ± 0.02* (31.6)	0.47 ± 0.02* (60.17)	0.39 ± 0.01* (70.68)	0.38 ± 0.01* (72.46)
3,3',4'-THF (25)	0.39 ± 0.01 (0)	0.94 ± 0.02 (0)	0.87 ± 0.02 (10.31)	0.80 ± 0.02* (32.2)	0.69 ± 0.03* (48.12)	0.64 ± 0.03* (53.62)
3,3',4'-THF (50)	0.43 ± 0.01 (0)	0.79 ± 0.14 (0)	0.83 ± 0.03* (14.43)	0.69 ± 0.03* (41.53)	0.60 ± 0.02* (54.88)	0.59 ± 0.02* (57.24)
3,3',4'-THF (100)	0.41 ± 0.02 (0)	0.85 ± 0.03 (0)	0.92 ± 0.01 (5.15)	0.73 ± 0.02* (38.14)	0.71 ± 0.02* (46.6)	0.55 ± 0.02* (60.14)
3,3',4'-THF (200)	0.43 ± 0.01	0.93 ± 0.02 (0)	0.80 ± 0.03* (17.5)	0.69 ± 0.02* (41.5)	0.66 ± 0.02* (50.35)	0.62 ± 0.02* (55.07)

Each value represents the mean ± SEM of six observations.

* P < 0.05 compared with respective vehicle treatment.

The values in parenthesis indicate the percentage inhibition

was recorded during all other periods of observation after treatment with 3,6, 3'–THF (Table 21). A maximum of 72.46% inhibition of paw oedema was recorded in a dose of 200 mg of 3, 6, 3' – THF at five hours which was comparable to that of diclofenac (Table 21)

6, 3', 4'- trihydroxy flavone

When compared to vehicle treatment, 6, 3', 4' – THF treatment reduced the paw diameter in a dose and time dependent fashion. Maximum reduction in paw diameter could be observed in a dose of 100 mg/kg and further increment in dose did not offer any further reduction in paw diameter (Table 22). A maximum of 67.4% inhibition of paw oedema was recorded for 100 mg/kg of 6.3', 4' THF during the fifth hour of observation compared to 72.46% recorded for diclofenac (Table 22).

7,8,3'- trihydroxy flavone

Pre treatment with 7,8,3'- trihydroxy flavone resulted in a significant reduction in the paw diameter after carrageenan administration compared to vehicle treatment. The protective effect by 7, 8, 3' – THF was dose and time dependent (Table 23). 7,8,3'- THF in a dose of 200 mg/kg could offer 71% inhibition of paw oedema during the fifth hour which was almost comparable to the effect of diclofenac (Table 23).

TABLE - 21

Effect of 3, 6, 3' - trihydroxy flavone on rat paw oedema

Treatment mg/Kg, sc	Paw Diameter (cm)					
	0 Hour	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Vehicle	0.38 ± 0.01	0.86 ± 0.02	0.97 ± 0.03	1.18 ± 0.06	1.33 ± 0.06	1.38 ± 0.02
Diclofenac – (10)	0.34 ± 0.01 (0)	0.87 ± 0.01 (0)	0.66 ± 0.02 (31.96)	0.47 ± 0.02 (60.17)	0.39 ± 0.01 (70.68)	0.38 ± 0.01 (72.46)
3,6,3'-THF (25)	0.44 ± 0.01 (0)	0.89 ± 0.01 (0)	0.84 ± 0.01* (13.40)	0.80 ± 0.02* (32.2)	0.69 ± 0.02* (48.12)	0.61 ± 0.01* (55.79)
3,6,3'-THF (50)	0.44 ± 0.01 (0)	0.90 ± 0.1 (0)	0.77 ± 0.01* (20.62)	0.71 ± 0.01* (39.83)	0.64 ± 0.01* (51.88)	0.56 ± 0.01* (59.42)
3,6,3'-THF (100)	0.44 ± 0.02 (0)	0.85 ± 0.01 (0)	0.76 ± 0.01* (21.65)	0.69 ± 0.01* (41.53)	0.59 ± 0.02* (55.64)	0.45 ± 0.02* (67.39)
3,6,3'-THF (200)	0.43 ± 0.01 (0)	0.87 ± 0.01 (0)	0.64 ± 0.02* (34.62)	0.59 ± 0.01* (50)	0.39 ± 0.01* (70.68)	0.38 ± 0.01* (72.46)

Each value represents the mean ± SEM of six observations.

The values in parenthesis indicate the percentage inhibition.

*$p < 0.05$ compared with respective vehicle treatment

TABLE - 22

Effect of 6, 3', 4' - trihydroxy flavone on rat paw oedema

Treatment mg/Kg, sc	Paw Diameter (cm)					
	0 Hour	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Vehicle	0.38 ± 0.01	0.86 ± 0.02	0.97 ± 0.05	1.18 ± 0.06	1.33 ± 0.06	1.38 ± 0.02
Diclofenac – (10)	0.34 ± 0.01 (0)	0.87 ± 0.01 (0)	0.66 ± 0.02* (31.96)	0.47 ± 0.02* (60.17)	0.39 ± 0.01* (70.68)	0.38 ± 0.01* (72.46)
6,3',4'-THF (25)	0.43 ± 0.01 (0)	0.86 ± 0.01 (0)	0.79 ± 0.01* (18.56)	0.76 ± 0.01* (35.60)	0.66 ± 0.01* (50.38)	0.59 ± 0.01* (57.24)
6,3',4'-THF (50)	0.39 ± 0.01 (0)	0.83 ± 0.01 (0)	0.73 ± 0.01* (24.74)	0.63 ± 0.01* (46.61)	0.56 ± 0.01* (57.90)	0.49 ± 0.01* (64.50)
6,3',4'-THF (100)	0.40 ± 0.01 (0)	0.86 ± 0.03 (0)	0.66 ± 0.01* (31.96)	0.62 ± 0.02* (47.46)	0.50 ± 0.01* (62.41)	0.45 ± 0.01* (67.39)
6,3',4'-THF (200)	0.36 ± 0.01 (0)	0.84 ± 0.01 (0)	0.72 ± 0.02* (25.77)	0.65 ± 0.02* (44.92)	0.51 ± 0.01* (61.66)	0.46 ± 0.01* (66.66)

Each value represents the mean ± SEM of Six observations.

The values in parenthesis indicate the percentage inhibition.

*$p < 0.05$ compared with respective vehicle treatment.

TABLE - 23

Effect of 7, 8, 3' - trihydroxy flavone on rat paw oedema

Treatment mg/Kg, sc	Paw Diameter (cm)					
	0 Hour	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Vehicle	0.38 ± 0.01	0.86 ± 0.02	0.97 ± 0.05	1.18 ± 0.06	1.33 ± 0.06	1.38 ± 0.02
Diclofenac – (10)	0.34 ± 0.01 (0)	0.87 ± 0.01 (0)	0.66 ± 0.02* (31.96)	0.47 ± 0.02* (60.17)	0.39 ± 0.01* (70.68)	0.38 ± 0.01* (72.46)
7,8,3'-THF (25)	0.43 ± 0.02 (0)	0.86 ± 0.01 (0)	0.87 ± 0.01 (10.31)	0.69 ± 0.02* (41.53)	0.59 ± 0.01* (55.64)	0.52 ± 0.01* (62.32)
7,8,3'-THF (50)	0.42 ± 0.01 (0)	0.90 ± 0.01 (0)	0.86 ± 0.02 (11.34)	0.63 ± 0.02* (46.61)	0.60 ± 0.01* (54.89)	0.50 ± 0.02* (63.76)
7,8,3'-THF (100)	0.43 ± 0.02 (0)	0.97 ± 0.01 (0)	0.65 ± 0.01* (32.99)	0.53 ± 0.01* (55.10)	0.50 ± 0.01* (62.41)	0.44 ± 0.01* (68.11)
7,8,3'-THF (200)	0.44 ± 0.01 (0)	0.86 ± 0.01 (0)	0.70 ± 0.01* (27.8)	0.50 ± 0.01* (57.63)	0.45 ± 0.01* (66.17)	0.40 ± 0.01* (71.02)

Each value represents the mean ± SEM of six observations.
The values in parenthesis indicate the percentage inhibition.
*$p < 0.05$ compared with respective vehicle treatment.

Effect of combination of diclofenac sodium and 3,3',4'-Trihydroxy flavone on rat paw oedema

Diclofenac (1 mg/kg) produced a mild but significant reduction in oedema as indicated by the reduction in the paw diameter. 3, 3'4' – THF (25 mg/kg) *per se* elicited an inhibition of 53% of paw oedema. When diclofenac was administered in combination with 3,3',4'-THF the reduction in paw diameter was enhanced to 61.2%. This is significantly different compared to the individual reduction in paw diameter observed for diclofenac or 3,3',4'-THF (Table 24).

Effect of combination of diclofenac sodium and 3,6,3'- Trihydroxy flavone on rat paw oedema.

Consecutive administration of diclofenac and 3,6,3'-THF offered a significant reduction in paw diameter from the third hour onwards. The reduction in paw diameter observed at 4^{th} and 5^{th} hour were significant compared to the individual treatment value of either diclofenac or 3,6, 3'- THF. A maximum reduction of 47% and 55.8% were recorded at 5^{th} hour for diclofenac and 3,6,3'- THF respectively. However, the combination resulted in an enhanced inhibition of 68.84% (Table 25).

TABLE - 24

Effect of combination of 3, 3', 4'- trihydroxy flavone and diclofenac on rat paw oedema

Treatment mg/Kg, sc	Paw Diameter (cm)					
	0 Hour	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Vehicle	0.38 ± 0.01	0.86 ± 0.02	0.97 ± 0.05	1.18 ± 0.06	1.33 ± 0.06	1.38 ± 0.02
Diclofenac – 1.0	0.38 ± 0.02 (0)	0.89 ± 0.02 (0)	0.87 ± 0.02 (10.31)	0.83 ± 0.02* (29.66)	0.78 ± 0.02* (41.3)	0.73 ± 0.02* (47.1)
3,3',4'-THF (25)	0.39 ± 0.01 (0)	0.94 ± 0.02 (0)	0.87 ± 0.02 (10.31)	0.80 ± 0.02* (32.20)	0.69 ± 0.03* (48.12)	0.64 ± 0.03* (53.62)
Diclofenac (1.0) + 3,3',4'-THF (25)	0.43 ± 0.02 (0)	0.89 ± 0.01 (0)	0.83 ± 0.01* (14.43)	0.83 ± 0.01* (29.66)	0.66 ± 0.02* (50.37)	0.54 ± 0.01*≠ (61.12)

Each value represents the Mean ± SEM of six observations.

The values in parenthesis indicate the percentage inhibition.

*$p < 0.05$ compared with respective vehicle treatment.

≠$p < 0.05$ compared with either diclofenac or 3, 3',4' - THF.

TABLE - 25

Effect of combination of 3, 6, 3' - trihydroxy flavone and diclofenac on rat paw oedema

Treatment mg/Kg, sc	Paw Diameter (cm)					
	0 Hour	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Vehicle	0.38 ± 0.01	0.86 ± 0.02	0.97 ± 0.03	1.18 ± 0.06	1.33 ± 0.06	1.38 ± 0.02
Diclofenac (1.0)	0.38 ± 0.02 (0)	0.89 ± 0.02 (0)	0.87 ± 0.02 (10.31)	0.83 ± 0.02* (29.66)	0.78 ± 0.02* (41.3)	0.73 ± 0.02* (47.10)
3,6,3'-THF (25)	0.44 ± 0.01 (0)	0.89 ± 0.01 (0)	0.84 ± 0.01 (13.40)	0.80 ± 0.02* (32.20)	0.69 ± 0.02* (48.12)	0.61 ± 0.01* (55.79)
Diclofenac (1.0) + 3,6,3'-THF (25)	0.38 ± 0.02 (0)	0.88 ± 0.1 (0)	0.81 ± 0.02* (16.49)	0.75 ± 0.02* (36.44)	0.58 ± 0.01*≠ (56.39)	0.43 ± 0.01*≠ (68.84)

Each value represents the mean ± SEM of six observations

The values in parenthesis indicate the percentage inhibition

*$p < 0.05$ compared with respective vehicle treatment

≠$p < 0.05$ compared with either diclofenac or 3, 6, 3' – THF

Effect of combination of diclofenac sodium and 6,3',4'-Trihydroxy flavone on rat paw oedema

Consecutive treatment of diclofeanc (1 mg) with 6,3',4'- THF offered a greater reduction in paw diameter compared to either treatment alone during the observation period from 3rd to 5th hour. The inhibition of paw oedema recorded at 5th hour for the combined treatment (63%) was higher than that recorded for individual treatments (Table 26).

Effect of combination of diclofenac and 7,8,3'- Trihydroxy flavone on rat paw oedema

The effect of combination of diclofenac (1mg/kg) and 7,8,3'-THF (25mg/kg) on the reduction in the paw diameter was not significantly different from that of 7,8,3'- THF. Almost similar percentage of inhibition of oedema was recorded for this combination as well as 7,8,3'- THF from 3rd to 5th hour of observation (Table 27).

Effect of combination of trihydroxy flavones and diclofenac sodium on acetic acid induced abdominal constriction

Different trihydroxy flavones in a dose of 25 mg/kg produced a mild but significant reduction in the number of abdominal constrictions, which ranged between 21.8 and 23.8. (table 28). Diclofenac in a dose of 1 mg/kg also reduced the number of abdominal constriction to a similar degree. A marked and highly significant reduction in abdominal constrictions was noticed when diclofenac was combined with all trihydroxy flavones except 3, 6,3'- THF (Table 28).

TABLE - 26

Effect of combination of 6, 3', 4' - trihydroxy flavone and diclofenac on rat paw oedema

Treatment mg/Kg, sc	Paw Diameter (cm)					
	0 Hour	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Vehicle	0.38 ± 0.01	0.86 ± 0.02	0.97 ± 0.03	1.18 ± 0.06	1.33 ± 0.06	1.38 ± 0.02
Diclofenac – (1.0)	0.38 ± 0.02 (0)	0.89 ± 0.02 (0)	0.87 ± 0.02 (10.31)	0.83 ± 0.02* (29.68)	0.78 ± 0.02* (41.3)	0.73 ± 0.02* (47.1)
6,3',4'-THF (25)	0.43 ± 0.01 (0)	0.86 ± 0.01 (0)	0.79 ± 0.01 (18.56)	0.76 ± 0.01* (35.60)	0.66 ± 0.01* (50.38)	0.59 ± 0.01* (57.24)
Diclofenac (1.0) + 6,3',4'-THF (25)	0.42 ± 0.01 (0)	0.87 ± 0.02 (0)	0.75 ± 0.021≠ (22.68)	0.69 ± 0.01*≠ (41.53)	0.56 ± 0.01*≠ (57.89)	0.51 ± 0.01*≠ (63.04)

Each value represents the mean ± SEM of six observations.

The values in parenthesis indicate the percentage inhibition.

*$p < 0.05$ compared with respective vehicle treatment.

≠$p < 0.05$ compared with either diclofenac or 6, 3',4' - THF

TABLE - 27

Effect of combination of 7, 8, 3' - trihydroxy flavone and diclofenac on rat paw oedema

Treatment mg/Kg, sc	Paw Diameter (cm)					
	0 Hour	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Vehicle	0.38 ± 0.01	0.86 ± 0.02	0.97 ± 0.03	1.18 ± 0.06	1.33 ± 0.06	1.38 ± 0.02
Diclofenac (1)	0.38 ± 0.02 (0)	0.89 ± 0.02 (0)	0.87 ± 0.02* (10.31)	0.83 ± 0.02* (29.66)	0.78 ± 0.02* (41.3)	0.73 ± 0.02* (47.10)
7,8,3'-THF (25)	0.43 ± 0.02 (0)	0.86 ± 0.01 (0)	0.87 ± 0.01 (10.31)	0.69 ± 0.02* (41.53)	0.59 ± 0.01* (55.64)	0.52 ± 0.01* (62.32)
Diclofenac (1.0) + 7,8,3'-THF (25)	0.42 ± 0.02 (0)	0.86 ± 0.01 (0)	0.75 ± 0.02* (22.7)	0.70 ± 0.03* (40.68)	0.62 ± 0.01* (53.39)	0.51 ± 0.02* (63.04)

Each value represents the mean ± SEM of six observations.

The values in parenthesis indicate the percentage inhibition.

*$p < 0.05$ compared with respective vehicle treatment

Table - 28

Effect of trihydroxy flavones on diclofenac induced antinociception

Treatment mg/Kg, sc	Number of abdominal constrictions	
	Without Diclofenac	With Diclofenac$ 1 mg / kg, sc
Vehicle	32.0 ± 0.89	23.33 ± 1.75*
3,3',4'-THF (25)	23.80 ± 0.87*	16.17 ± 1.19* ≠
3,6,3'-THF (25)	21.8 ± 1.45*	21.3 ± 1.45*
6,3',4'-THF (25)	23.83 ± 0.40*	16.50 ± 1.28*≠
7,8,3'-THF (25)	23.67 ± 1.15*	18.0 ± 1.41*≠

Each value represents the mean ± SEM of six observations.

*p < 0.05 compared to vehicle treatment.

≠p < 0.05 compared with Trihydroxy flovone / Diclofenac treatment

$ The animals received diclofenac 15 min before THF treatment and were challenged with acetic acid at 60 min.

Effect of trihydroxy flavones on cyclooxygenase – 1

The investigated trihydroxy flavones inhibited cyclo oxygenase-1 activity in a concentration dependent manner. Among the tested compounds, 3,6,3'- trihydroxy flavone produced a maximum inhibition of 84.5% in 50 μM concentration. In a similar concentration 6,3'4'- THF and 7,8,3'- THF produced an inhibition of 76%, While 3,3',4'- THF produced an inhibition of 58.8% (table 29). The IC_{50} values recorded for these compounds in increasing order are as follows:

3,6,3' – THF = 22.35 μM

6,3',4' – THF = 27.61 μM

7,8,3' – THF = 27.97 μM

3,3',4' – THF = 39.40 μM

The IC_{50} value of ibuprofen in this assay procedure was found to be 14.68 μM.

Effect of trihydroxy flavones on cyclooxygenase 2

A concentration dependent inhibition of cyclooxygenase – 2 was clearly eivdent for all the tested trihydroxy flavones. A maximum of 81% inhibition was recorded for 3,6,3'- THF in a concentration of 50 μM. In the same concentration maximum inhibition of 59%, 66% and 63% was recorded for 3, 3',4' – THF, 6,3',4'- THF and 7,8,3'- THF respectively (Table 30). The IC_{50} values recorded for the above compounds to inhibit cyclo oxygenase – 2 are as follows:

TABLE – 29

Effect of trihydroxy flavones on Cyclooxygenase – 1

Treatment mg/Kg, sc	Concentration (μM)					
	10	20	30	40	50	IC_{50} (μM)
	% Inhibition					
3,3'4' – Trihydroxy flavones	18.36	32.41	41.55	51.24	58.79	39.40
3,6'3'- Trihydroxy flavone	28.5	46.13	67.97	77.87	84.56	22.35
6,3',4'- Trihydroxy flavone	22.5	39.13	59.72	68.89	76.14	27.61
7, 8, 3'- Trihydroxy flavones	25.3	37.23	56.68	67.87	76.4	27.97

Each value represents the mean of 3 observations

* IC_{50} value calculated by linear regression analysis

TABLE – 30

Effect of trihydroxy flavones on Cyclo oxygenase –2

Treatment mg/Kg, sc	Concentration (μM)					
	10	20	30	40	50	IC 50* (μM)
	% Inhibition					
3,3'4' – Trihydroxy flavone	17.48	30.97	45.97	50.48	59.18	40.34
3,6'3' -Trihydroxy flavone	39.52	57.15	67.23	75.46	81.23	16.97
6,3',4' -Trihydroxy flavone	25.52	39.91	49.76	58.48	66.18	31.98
7,8,3'- Trihydroxy flavone	27.36	38.54	45.43	54.68	63.9	28.46

Each value represents the mean of 3 observations

* IC_{50} value calculated by linear regression analysis

3,6,3' – THF = 16.97 μM

7,8,3' – THF = 28.46 μM

6,3',4' – THF = 31.98 μM

3,3',4' – THF = 40.34 μM

The IC_{50} value for celecoxib in the same assay procedure was found to be 2.24 μM.

It is pertinent to point out that all the investigated trihydroxyflavones almost equally inhibited both cyclo oxygenase - 1 and cyclo oxygenase – 2. The IC_{50} values for inhibition of cyclooxygenase 1 and 2 for these compounds are also almost identical.

Effect of trihydroxy flavones on interlleukin – 1β

A concentration dependent inhibition of interleukin - 1β activity was recorded for all the tested trihydroxy flavones. Nearly 70% inhibition was recorded for 3,3',4'- THF and 6,3',4'- THF in a concentration of 50 μM. 3,6,3'- THF exhibited a maximum inhibition of 78.6% in the same concentration while that recorded for 7,8,3'- THF was 76% The IC_{50} values recorded for the above trihydroxy flavones are as follows

3,6,3' – THF = 109.97 μM

7, 8, 3' – THF = 121.46 μM

6, 3', 4' – THF = 129.98 μM

3, 3', 4' – THF = 142.3 μM

The IC_{50} value of dexamethasone to inhibit interleukin 1β in the same experimental procedure was found to be 101.23 μM. It can be appreciated that the IC_{50} value of 3,6,3'- THF is almost similar to that of dexamethasone, whereas the other compounds possessed a slightly higher IC_{50} value (Table 31).

Effect of trihydroxy flavones on Tumor necrosis factor – α

The investigated trihydroxy flavones exerted a potent inhibition of activity of tumor necrosis factor α. The inhibition was concentration dependent (Tables 32 – 35). A fairly high degree of inhibition was recorded for 3,6,3'- THF and 7,8,3',-THF in concentrations ranging from 10 – 50 μM. A maximum of 73.28% inhibition was recorded for 15 μM concentration of 3,6,3'- THF (Table 33). In a similar concentration, 7,8,3'- THF produced a maximum inhibition of 63.23% (Table 35). The inhibitory action of 3,3'4'- THF and 6,3', 4'- THF on tumor necrosis factor α was revealed only in higher concentrations. A maximum of 59.32% inhibition was observed for 3,3',4'- THF (Table 32) and 62.77% inhibition was observed for 6,3',4'- THF in a concentration of 500 μM (Table 34). The IC_{50} values calculated for the above compounds are as follows:

3,6,3' – THF = 28.66 μM

7,8,3' – THF = 35.48 μM

6,3',4' – THF = 393.14 μM

3,3',4' – THF = 430.68 μM

TABLE – 31

Effect of trihydroxy flavones on Interleukin – 1β

Treatment mg/Kg, sc	Concentration (μM)					
	10	20	30	40	50	IC 50* (μM)
	% Inhibition					
3,3'4' – Trihydroxy flavone	19.08	29.32	38.12	55.66	69.76	142.34
3,6,3'- Trihydroxy flavone	27.89	34.23	44.98	62.12	78.65	109.97
6,3',4'- Trihydroxy flavone	20.82	30.24	40.45	61.96	71.76	129.98
7, 8, 3'- Trihydroxy flavone	19.21	30.46	41.98	65.09	75.87	121.46

Each value represents the mean of 3 observations

* IC_{50} value calculated by linear regression analysis

Table – 32

Effect of 3, 3', 4' - trihydroxy flavone

on Tumor necrosis factor – Alpha

3,3',4' – THF Concentration (μM)	**% Inhibition**
100	9.45
200	17.66
300	31.48
400	47.55
500	59.32
IC – 50*	430.68μM

Each value represents the mean of three observations.

*IC – 50 value calculated by linear regression analysis.

Table – 33

Effect of 3, 6, 3' - trihydroxy flavone on

Tumor necrosis factor – Alpha

3,6,3' – THF Concentration μM	**% Inhibition**
10	24.16
20	39.16
30	56.13
40	65.15
50	73.28
IC-50*	28.66 (μM)

Each value represents the mean of three observations.

*IC – 50 value calculated by linear regression analysis.

Table – 34

Effect of 6, 3', 4' - Trihydroxy flavone

on Tumor necrosis factor - Alpha

6,3',4' – THF Concentration (μM)	**% Inhibition**
100	11.56
200	23.45
300	37.68
400	52.97
500	62.77
IC – 50*	393.14 (μM)

Each value represents the mean of three observations.

*IC – 50 value calculated by linear regression analysis.

Table – 35

Effect of 7, 8, 3' - trihydroxy flavone

on Tumor necrosis factor - Alpha

7,8,3' – THF Concentration (μM)	**% Inhibition**
10	22.66
20	32.14
30	45.16
40	57.46
50	63.23
IC – 50	35.48(μM)

Each value represents the mean of three observations.

*IC – 50 value calculated by linear regression analysis.

The IC_{50} value for dexamethasone to inhibit tumor necrosis factor α was recorded to be 25.33 μM. It can be appreciated that the IC_{50} values of 3,6,3'-THF (28.66 μM) and 7,8,3'- THF (35.48 μM) are similar to dexamethasone value. The IC_{50} values recorded for 3,3',4'- THF (430.68) and 6,3',4'- THF (393.14) are many fold higher than dexamethasone value.

Effect of trihydrody flavones on nitric oxide scavenging activity.

Vitamin – C was found to produce a concentration dependent inhibition of nitrogen derived free radicals. A maximum of 87.88% inhibition was recorded for vitamin C in a concentration of 200 μg/ml. The different trihydroxy flavones also exhibited a significant concentration dependent inhibition of nitrogen derived free radical generation.

A similar degree of inhibition was recorded for all the investigated trihydroxy flavones in a concentration of 40 μg/ml. All the trihydroxy flavones produced an inhibition ranging from 71-77% (Table 36). Increasing the concentration to 200 μg/ml enhanced the inhibition activity marginally. In the highest concentration tested (200 μg/ ml) all the trihydroxy flavones showed an inhibition ranging from 77 – 82% compared to 88.0% observed with vitamin C

Effect Trihydroxy flavones on DPPH scavenging activity.

Standard antioxidant vitamin – C produced a concentration dependent inhibition of DPPH activity (Table – 37). Almost 97% inhibition was recordd in a concentration of 200 μg/ml. The investigated trihydroxy flavones produced varying degree of inhibition of DPPH activity. Even though a concentration dependent inhibition could be recorded, the response was not uniform for all the

Table – 36

Effect of trihydroxy flavones on nitric oxide scavenging activity

Percentage inhibition by					
Concentration μg/ml	**3,3',4'-THF**	**3,6,3'-THF**	**6,3',4'-THF**	**7,8,3'-THF**	**Vitamin-C**
1	38.76	28.9	30.94	20.81	15.01
5	45.96	45.56	43.39	40.0	28.0
10	55.21	61.87	45.34	49.26	38.6
20	66.62	76.37	66.22	66.73	51.18
40	73.0	76.86	70.64	71.11	54.09
80	77.2	77.0	71.20	74.14	59.69
100	77.3	78.0	71.26	79.0	66.21
200	82.19	78.3	77.14	79.20	87.88

Each value represents the mean percentage inhibition of 3 observations.

Table 37

Effect of trihydroxy flavones on DPPH scavenging activity

Percentage inhibition by					
Concentration μg/ml	**3,3',4'-THF**	**3,6,3'-THF**	**6,3',4'-THF**	**7,8,3'-THF**	**Vitamin-C**
1	34.05	13.89	18.10	0.01	24.43
5	42.27	16.48	30.95	4.5	24.58
10	46.91	17.04	54.34	34.4	24.69
20	48.14	23.28	58.07	36.7	24.91
40	49.49	26.44	58.34	46.8	25.65
80	53.89	26.5	59.82	53.25	66.07
100	54.20	28.95	61.03	65.25	80.11
200	59.70	39.63	64.16	66.84	97.25

Each value represents the mean percentage inhibition of 3 observations

tryhydroxy flavones. A maximum of 39.63% inhibition was recorded for 3-6'-3'-THF in a concentration of 200 μg/ml. A higher degree of inhibition was evident for all the other tri hydroxy flavones in the same concentration. A maximum of 60 to 67% of inhibition was recorded for 3, 3'4'-THF, 6,3',4'- THF and 7,8,3'–THF; compared to 97% inhibition produced by the vitamin C (Table 37).

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 (Thirugnana sambantham *et al.,*1985) and many hydroxy and methoxy derivatives of flavone (Thirugnana sambantham *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), Venkataramanan *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 effect of flavones

A few isolated reports indicated the antinociceptive effect of certain flavonol glycosides like hydroxy ethylrutoside (Ramaswamy, 1980), gossypin (Viswanathan *et al.,* 1984**)** and rutin (Thirugnana sambantham *et al.,* 1985). However, a detailed structure activity study was reported by Thirugnana sambantham *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 potential antinociceptive action of flavones; Kaempferol 3, 7, - O-α dimethyl rhamnoside (Toker et al 2004), quercetin (Kaur et al 2005), myricitrin (Meotti et al 2006), naringenin derivative (Orhan et al 2006) and a few dihydroxy flavones (Uma Maheswari et al 2006) also support the need for a focussed investigation on this beneficial effect of flavone derivatives. Hence, in the present study four new trihydroxy flavone derivatives, hitherto not subjected to any biological evaluation were selected and the compounds were screened by a battery of tests to identify their pharmacological profile.

STUDY PATTERN

The pharmocological profile of selected trihydroxy flavones with close structural relation was carried out in the following stages.

1. Since all the chosen trihydroxy flavones were new compounds for biological screening they were subjected to preliminary screening and acute toxicity testing in mice.

2. The effect of trihydroxy flavones on motor co-ordination and locomotor activity was tested in mice since the subsequent experiments on antinociception mainly involve an assessment of some kind of motor activity of animals.

3. In the next stage, the antinociceptive potential was screened by selecting three different well established procedures, viz.,

   a. Acetic acid induced abdominal constriction.

   b. Formalin induced nociception

   c. Hot water tail immersion method.

4. The participation of cation channels and other neuronal pathways in the antinociceptive action of trihydroxy flavones was examined by employing suitable interacting drugs. The roles of opioid system, GABAergic, adrenergic pathways and potassium channel were considered, in these experiments.

5. The potent antinociceptive effect observed for these compounds widened the scope of study to screen these compounds for possible anti

inflammatory activity. Carrageenan induced paw oedema in rats was employed for this purpose.

6. In a subsequent study, the effect of tridydroxy flavones on some mediators of pain and inflammation was investigated. The action of trihydroxy flavones on prostaglandins, interleukin – 1β, tumor necrosis factor - α and reactive oxygen species / free radicals was studied by employing suitable in vitro experiments.

7. A possible synergistic effect of trihydroxy flavones with a standard NSAID was examined in the management of pain and inflammation.

8. The above set of experiments yielded interesting results and are discussed below in relation to the existing literature in this area of research.

Preliminary screening and acute toxicity

The safe nature of trihydroxy flavones was revealed by the results of acute toxicity test in which no mortality was observed in mice with any of the test compounds even in a dose of 2g/kg.

This observation is in accordance with many previous reports on flavonoid compounds. Thirugnanasambantham (1987) reported that flavone and many of its monohydroxy and mono methoxy compounds did not exhibit any toxicity up to 2g/kg in mice. Similar observations were reported by Girija (2000) and Uma Maheswari (2008) for many dihydroxy flavone derivatives. The above findings

confirm the postulations of Havsteen (2002) that, “the margin of safety for therapeutic use of flavonoid in human is considered to be very large, probably much superior to any other drug in current use”.

Effect on motor activity

The available methods to screen any drug for antinociceptive property 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 tail from hot water and biting response to the intra-plantar injection of formalin, all 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 trihydroxy flavones on the spontaneous motor activity in mice was tested using an open field apparatus and the effect on muscle co-ordination was studied using a rota rod.

Treatment with trihydroxy flavones did not alter either the spontaneous motor activity or balancing time on a rotarod in mice. Thus the test compounds have not influenced the motor activity of animals even in the highest dose tested. The findings of the present results are similar to the previous reports on many other flavone compounds like, gossypin (Viswanathan et al 1984), flavone and its monohydroxy derivatives (Thirugnanasambantham 1987) and many dihydroxy flavone derivatives (Girija 2000 and Umamaheswari 2008). It can also be presumed that any response recorded in the antinociceptive assay procedure may be considered as a true response.

Methods for studying antinociception

The selected tridydroxy flavones were subjected to three different assay procedures to assess the antinociceptive 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. This is also considered as a model of visceral pain. Hot water tail immersion (Sewell and Spencer 1976) is also a well established procedure for antinociceptive assay which employs a high degree of thermal nociception. Hence the compounds exhibiting good antinociceptive effect in this method may be considered as potent analgesics.

In the present study formalin induced nociception was also employed to investigate the antinociceptive action of trihydroxy flavones. 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 et al, 1992). Intraplantar injection of formalin results in licking and biting of the injected paw. This nociceptive behaviour appears in two distinct phases. The first phase starts immediately after injection of formalin and lasts for about five minutes. It is probably due to direct chemical stimulation of nociceptors (Dubuission and Dennis 1979). This is followed by a quiscent period of about 10 minutes. 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, NSAID and monoamines (Calcagnetti et al, 1988, Hunskaar and Hole 1989, Rosland et al 1990). Formalin test was employed by Rajendran et al (2000) to investigate the antinociceptive activity of flavone and by Umamaheswari et al (2006) to investigate the antinociceptive action of dihydroxy flavones. Hence, in the present study formalin test was also included to assess the antinociceptive activity of trihydroxy flavones.

Thus 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 and Hole, 1997).

Effect of trihydroxy flavones on various nociceptive procedures:

The findings of the present study indicate that, subcutaneous administration of trihydroxy 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 trihydroxy flavones exhibited nearly 54 to 66% inhibition of nociceptive response (Tables 3 to 6). The ED_{50} Values observed for 3,3',4'- THF (69.18mg) and 3,6,3' - THF (67.6) are similar indicating equipotent antinocieptive activity in this assay model. The ED_{50} Values of 6,3',4'- THF (104.7 mg) and 7,8,3' - THF (79.43 mg) indicate that,

these two compounds appear to be less potent than the former two compounds in inhibiting acetic acid induced nociception. It is important to note that morphine in a dose of 5mg/kg was able to produce 100% inhibition of abdominal constrictions. The present results indicate that the trihydroxy flavones in the maximally tested dose (200mg) are less effective than morphine.

The results obtained from formalin test also substantiate the antinociceptive effect of trihydroxy flavones. A significant and dose related antinociception was clearly evident for all the tested trihydroxy flavones against both neurogenic (early phase) and inflammatory (late phase) pain responses caused by formalin injection in mice. The degree of inhibition of late phase of nociception was higher when compared to early phase for all the trihydroxyflavones. This finding indicates much more favourable effectiveness of trihydroxy flavones on the inflammatory phase of formalin nociception. The antinociceptive efficacy of 3,3',4'- THF and 3,6,3'- THF appear to be similar in fomalin assay as recorded by their maximal inhibitory response in a dose of 200mg/kg (Tables 7 and 8). However, the maximum inhibitory response recorded for 6,3',4'- THF and 7,8,3'- THF were comparatively less in the same assay procedure.

Morphine in a dose of 5 mg produced nearly 95% inhibition of acute phase and 90% inhibition chronic phase of formalin pain response. It is pertinent to point out that 3,3',4'- THF and 3,6,3'- THF could produce more than 90% inhibition of late phase of formalin nociception. The present results are in agreement with the earlier reports on many flavonoids. 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 (Rajendran et al 2000). Several dihydroxy flavones also produced a similar pattern of inhibition of formalin nocception. 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 for dihydroxy flavone derivatives (Umamaheswari et al 2006). Significant inhibition of pain response by trihydroxy flavones in both the phases of formalin nociception indicate the effectiveness of these compounds against both neurogenic and inflammatory pain. Moreover a higher degree of inhibition on the late phase of formalin nociception may suggest a more preferential and predominant effect of trihydroxy flavones on inflammatory pain.

The results of thermal model of nociception provide further support to the antinociceptive potential of trihydroxy flavones. The investigational compounds produced a significant increase in reaction time in the tested doses (table 11 to 14). Morphine (5mg) treatment provided nearly 74% inhibition of thermal nociception. 3,3',4'- THF produced a maximum of 14.6% inhibition and 7,8,3'-THF produced 28% maximum inhibition in a dose of 200mg/kg. The responses for 3,6,3'- THF and 6,3,4'- THF were lying in between. Since none of the trihydroxy flavones could produce more than 50% inhibition of thermal nociception, the ED_{50} values could not be computed.

Comparison of antinociceptive efficacy among flavones

Perusal of literature indicates that the flavone nucleus *per se* has inherent antinociceptive action which was modified to varying degrees by different substitutions (Thirugnanasambantham et al 1990, 1993).

In another 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, viz acetic acid induced abdominal constriction assay. The present study also identified certain trityhdroxy flavones that exhibited nearly 55 to 66% inhibition of nociception by this method. However, in a recent report umamaheswari et al (2006) reported certain dihydroxy flavones to possess nearly 100% inhibition of nociception in this method.

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

Structure activity relationship of flavonoids

The basic flavone nucleus, many of the monomethoxy, two dihydroxy flavones and two flavone glucosides were synthesised by Thirugnana sambantham (1987) and were investigated for thieir antinociceptive activity. In another study, Girija (2000) screened six dihydroxy flavone derivatives for their antinociceptive and other pharmacological actions. In addition to these reports several polyhydroxy flavones and flavonol glucosides like hydroxy ethyl rutoside (Ramaswamy et al 1980), gossypin (Viswanathan et al 1984) 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 action 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 substitutents in different positions. After elaborately studying, several flavone derivatives, Thirugnanasambantham (1987) suggested certain structural determinants for the antinociceptive activity of flavones. These can be stated as follows.

1. Flavone nucelus *per se* possesses anti nociceptive action which was found to involve opioid mechanism.

2. This antinociceptive activity was modified to varying degrees by hydroxylation, methoxylation or glucosylation at various positions of flavone nucleus.

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

4. In a subsequent study, Girija (2000) reported that dihydroxylation of flavone nucleus did not uniformly enhance the antinociceptive potency of monohydroxy flavones.

5. Umamaheswari (2008) reported that, introduction of a hydroxyl group at 3'-position has improved the antinociceptive efficacy of many mono hydroxy flavones.

Scrupulous analysis of the existing literature on the structure activity relationship of various flavone compounds suggests that, polyhydroxylation of the flavone nucleus and presence of hydroxyl group in the 3'-position of the flavone ring may bring out more potent analgesic compounds.

The studies carried out by Umamaheswari (2008) indicated that hydroxylation at 3'-position of the flavone nucleus enhanced the antinociceptive efficacy of certain monohydroxy flavones. A few dihydroxy flavones thus prepared (2',3'-DHF, 5,3'-DHF and 7,3'-DHF) exhibited potent antinociceptive activity compared to their respective mono hydroxy compounds (2'-hydroxy flavone, 5-hydroxy flavone and 7-hydroxy flavone). This observation suggested that hydroxylation at 3'- position of the flavone nucleus may enhance the analgesic efficacy of flavone compounds. This concept requires confirmation and hence in the present study a few trihydroxy flavone compounds were selected

with a primary criterion that these compounds possess a hydroxyl group at 3'-position.

In an earlier study, Girija (2000) reported that 3, 6-dihydroxyflavone could produce a maximum of 72% inhibition (200 mg/kg) of abdominal constrictions after acetic acid injection in mice. The ED_{50} of this compound was reported to be 79.43 mg/kg, s.c. In the present study, another hydroxyl group was incorporated at 3'- position of the same molecule and the resulted compound (3, 6, 3'-THF) was investigated. This trihydroxy flavone produced a maximum of 58.34% inhibition of abdominal constriction in a dose of 200 mg/kg and the ED_{50} value being 67.6 mg/kg.

The compound of 3, 4'-dihydroxy flavone in a dose of 400 mg/kg produced a maximum inhibition of 66% of acetic induced abdominal constriction in mice (Girija 2000). In the present study 3,3',4'- trihydroxy flavones could produce a maximum inhibition of 60% in a dose of 200 mg/kg. The ED_{50} value of 3,4-dihydroy flavone was 114.5 mg/kg in mice and that of 3,3',4'- THF in the present study was found to be 69.15 mg/kg.

The antinociceptive activity of 7,8 dihydroxy flavone was investigated by Thirugnanasambantham (1987). The percentage inhibition of abdominal constriction reported for this compound in doses of 100, 200, 400, and 800mg/kg were found to be 15.6, 44.1, 71.9 and 78.5% respectively. The ED_{50} of the compound was found to be 200mg/kg. In the present study 7,8,3'- trihydroxy flavone in doses of 25, 50,100 and 200 mg/kg produced an inhibiton of 26, 39.6,

54, and 53.7% inhibition of abdominal constriction respectively. The ED_{50} value of 7,8,3'-THF was found to be 79.4 mg/kg.

The antinociceptive study of 3'4'-dihydroxy flavone (Girija 2000) indicated that a maximum of 65.4% inhibition of abdominal constriction could be achieved in a dose of 400mg/kg, the ED_{50} value being 117.5 mg/kg. In the present study a compound with yet another hydroxyl group (6,3',4'-THF) has been investigated. 6,3',4'- THF could produce a maximum of 66% inhibition of acetic acid induced abdominal constriction even in a dose of 200mg/kg with an ED_{50} value of 79.43mg/kg.

Correlating the structure and activity it can be stated that trihydroxylation of the flavone nucleus and in particular compounds with ortho dihydroxylation in the B-ring (3,3',4'-THF and 6,3',4'-THF) exhibited enhanced antinciceptive efficacy. The suggestion put forth by Thirugnana Sambantham (1987) and later corroborated by Umamaheswari (2008) that introduction of additional hydroxyl groups in the flavone ring may result in more efficacious compounds, has been substantiated in the present study.

However, same conclusions could not be drawn for trihydroxy flavone with ortho dihydroxylation in the A-ring (7, 8, 3'-THF). The antinociceptive efficacy of 7,8 – dihydroxy flavone was not substantially increased by introduction of an additional hydroxyl group at 3'- position.

Similarly, introduction of an additional hydroxyl group at 3' position in case of 3,6-dihydroxy flavone (3,6,3'-THF) failed to improve the antinociceptive efficacy of the parent compound.

In the present study only four trihydroxy flavones have been studied for their antinociceptive action and an attempt made to correlate the activity with chemical structure. The presently available limited data may not be adequate to suggest a detalied structure activity relationship. Further expanded studies with more poly hydroxy flavones may help us to understand this aspect in a better manner.

Mechanisms involved in the antinociceptive action of trihydroxy flavones.

Opioid mechanism: Many previous studies on the antinociceptive effect of flavone derivatives described the participation of opioid mechanism in mediating their antinociceptive action. Compounds like epicatechin and gossypin were first reported to mediate their antinociceptive action through opioid mechanism (Viswanathan 1984). In subsequent stuides many mono substituted flavones were also reported to possess opioid mediated antinociceptive activity (Thirugnana sambantham et al, 1990, 1993). Several dihydroxy flavones (Grija et al 2002, Umamaheswari et al 2006) and quercetin, a penta hydroxyflavone (Naidu et al 2003 b) were also found to utilise opioid pathways in mediating their antinociceptive effect. The results of the present study are in agreement with the above presented earlier evidences. Naloxone was able to significantly attenuate the antinociceptive activity of the test compounds. This observation confirms the

earlier reports and conclusively suggests a role for opioid mechanism in the antinociceptive action of trithydroxy flavones.

Investigation on other neuronal pathways and cation channels

The binding of opioid agonist with specific receptors triggers a series of events in cellular function. These include alterations in the neurotransmitter functions such as cholinergic, adrenergic, dopaminergic, serotonergic, and GABA ergic systems. Further, a role for many ion channels in particular calcium, potassium and chloride channels in mediating opioid antinociception has also been documented. Since the investigated trihydroxy flavones exhibited opioid mediated anti nociception, it was considered interesting to further investigate the role played by various neuronal pathways / potassium channel in the action of these compounds.

Role of adrenergic system

Drugs acting on adrenergic system have been reported to possess antinociceptive effect by interacting with opioid system or acting independently. The potent α_2 adrenergic agoinsts like clonidine and xylazine have been reported to possess potent analgesic effect acting independent of opioid system (Bentley et al 1977, Browning et al 1982). The antinociceptive effect of morphine was antagonised by naloxone and yohimbine whereas that of clonidine and xylazine was antagonised only by yohimbine. The role of α adrenergic system in opioid action was also indicated by the finding that clonidine pretreatment

effectively antagonised the acute and chronic tolerance development to morphine analgesia (Ramaswamy et al 1981 and 1983).

In the present study, yohimbine, a relatively specific α_2 antagonist was used to find out the involvement of α-2 adrenergic system in the antinociceptive action of trihydroxy flavones. Yohimbine pretreatment significantly attenuated the antinociceptive effect of 3,6,3'-THF. However, the antinociceptive effect of other investigated trihydroxy flavones was not altered by yohimbine. This observation suggests a role for α_2 adrenergic system in the antinociceptive action of 3,6,3'-THF. Quercetin was reported to primarily involve the modulation of α_2 adrenergic pathways in its antinociceptive action (Kaur et al., 2005). This report also supports the involvement of α adrenergic system in the actions of flaovone compounds.

Role of GABAergic system

The inhibitory GABAergic system has been found to play a major role at many sites in the neuronal pathway mediating nociception. GABA receptor agonist 4,5,6,7-tetra hydro-isoxazole (5,4) pyridine 3-ol (THIP) and muscimol were found to exhibit antinociceptive activity *Per se* and also potentiated opioid analgesia (Hill et al 1981). GABAergic system has been found to contribute to opioid antinociception and the subsequent tolerance development (Srinivasan et al 1990). Prior to this Ramaswamy et al (1989) have shown that prolactin, which acts similar to morphine through opioid pathway also involves GABAergic mechanism in its antinociceptive response. Further, a flavonol glucoside,

gossypin was found to recruit GABAergic pathways in mediating its antinociceptive response (Viswanathan et al 1993). In addition, gabapentin, a dervative of GABA is currently one of the most sought after drugs in the management of neuropathic pain. All the above evidences prompted an investigation on the role of GABAergic system in the antinociceptive action of trihydroxy flavones.

The results indicated that bicuculline, a GABA antagonist effectively suppressed the antinociceptive effect of 6,3'4'- THF and 7,8,3'-THF revealing that GABAergic system is definitely involved in the action of these two compounds. This observation is similar to that recorded for gossypin a flavonol glucoside (Viswanathan et al 1993). However, the antinociceptive effect of 3,3',4' - THF and 3,6,3'-THF remained unaffected by bicuculline pretreatment, thus ruling out GABAergic involvement in their antinociceptive action.

Role of potassium channels in antinociception

A role for ATP sensitive potassium channels in opioid induced antinociception has been documented (Ocana et al 1990, Wild et al1991). Activation of opioid receptors may result in opening of potassium channels in the central nervous system (Amoroso et al 1990). Further confirmatory evidence is also available to suggest that morphine and some of the μ and delta receptor agoinsts act by opening ATP sensitive potassium channels to elicit analgesia, as glibenclamide a blocker of this channel antagonizes the analgesic response (Amorosa et al 1990, Ocana et al 1990, Wild et al 1991). Prolactin which exhibits

opioid mediated antinociceptive action also has been shown to involve such mechanism (Shiwade and Ramaswamy 1995). In a previous study the antinociceptive effect of 7-hydroxy flavone was attenuated by glibenclamide thus establishing a role for ATP sensitive potasium channel in the antinociceptive action of 7^{-} hydroxyflavone (Venkataramanan 1998).

In the present study, the antinociceptive effect of 7,8,3'-THF was attenuated by glibenclamide pre-treatment suggesting that potassium channels may be involved in the action of 7,8,3'-THF (Table 17).

However, the antinociceptive effect of other investigated compounds was not alltered by prior treatment with glibenclamide and thus excluding the participation of potassium channel in their antinociceptive response.

Peripheral mechanisms

The action of both endogenous as well as exogenous opioid analgesics at many sites outside the central nervous system to produce the analgesic effect is under active consideration. Pain due to 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 the 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). In a recent study the analgesic activity of a new opioid agonist N-[1-phenlyl pyrazoyl) N-[1-2phenethyl)

4-piperidyl propenamide (IQMF-4) has been shown to predominantly involve peripheral mechanisms (Goicoechea et al 2008).

It is now well accepted that, opioid analgesia can be peripherally modulated (Rusell et al 1987, Wenk et al 2006). There are several examples of μ opioid agonists that can produce analgesia by a peripheral mechanism (Al-Khrasani et al 2007, Bileviciute –Ljungar et al 2006, Ji et al 2006). In addition k opioid receptor agonist may also exhibit peripherally mediated antinociception sans the classical side effect like dysphoria, or sedation associated with central kappa receptor (Kumar et al 2005).

The results of the present study revealed significant antinociceptive action of trihydroxy flavones especially on inflammatory models of pain. Blockade of this response by naloxone reveals the involvement of opioid receptor in the above action. It may be suggested that the investigated trihydroxy flavones may preferentially act in the periphery (including the inflammatory sites) and recruit opioid receptors to mediate their effect. This suggestion stems from the fact that, administration of trihydroxy flavones did not produce any behavioural alteration in the experimental animals indicating lack of central effects. Moreover, several monohydroxy flavones and dihydroxy flavones which were reported to possess potent opioid mediated antinociceptive effect did not exhibit any tolerance upon repeated administration (Thirugnanasambantham 1987, Viswanathan et al 1985, Umamahesvari 2008). The peripheral opioid like action of flavonoids has been further confirmed by their effect in delaying the small intestinal transit (Viswanathan et al 1984 a). These evidences suggest a possibility that,

flavonoid group of compounds may be considered as peripherally acting analgesics utilizing opioid pathways. However, this aspect requires further detailed investigation.

Anti inflammatory effect of trihydroxy flavones

One of the earliest therapeutic applications of flavonoids is in the treatment of some inflammatory diseases. The benefical effects of flavonoids in rheumatoid arthiritis (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 a significant antiinflammatory activity in several animal models of acute and chronic inflammation; Taxifolin (Gupta et al 1971), gossypin (Parmar and Ghosh 1978), hesperedin (Shahidi 1998), titonine and its derivatives (Carvalho et al 1999), Sylimarin (Gupta et al 2000) and naringen (Havsteen 2002).

In two detailed studies, the anti inflammatory activities of monomethoxy flavones (Muthiah et al 1993) and monohydroxy flavones (Arivudainambi 1996) were investigated. It was reported that monohydroxy derivatives of flavone were potent anti inflammatory agents than their corresponding mono methoxy derivatives. A recent study conducted by Umamaheswari (2008) corroborated this and potent antiflammatory activity of several dihydroxy flavone derivatives was evident. The marked antiinflammatory activity observed for mono hydroxy flavones and dihydroxy flavones necessitates an investigation on the antiinflammatory activity of the higher homologus series, namely trihydroxy

flavones. Moreover in the present study all the trihydroxy flavones were found to exert potent antinociceptive activity especially in inflammatory models of pain like acetic acid writhing and late phase of formalin nociception. Hence an investigation on their antiinflammatory activity was considered imminent.

The compounds were screened for their effect on acute inflammation using carrageenan induced paw oedema, a well established animal model. This 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 oedema have been therapeutically correlated to possess good antiinflammatory activity.

The results of the present study indicate a dose and time dependent antiinflammatory activity for all the tested trihydroxy flavones. A marked reduction in paw diameter was clearly evident in all the doses tested with peak effect at 5^{th} hour of observation; nearly 60 to 72% of maximum inhibition of inflammation was recorded for these compounds in a dose of 200mg/kg.

The potent antiinflammatory activity of trihydroxy flavones, in addition to consolidating the earlier findings on the said effect of flavonoid compounds further reinforces the hypothesis of Arivudainambi et al (1996) that poly hydroxy flavones may exert potent antiinflammatory activity.

The novel antiinflammatory activity of trihydroxy flavones coupled with their antinociceptive property has enormous therapeutic applications.

The currently available drugs to treat inflammation mainly belong to two important groups; glucocorticoids and NSAID. Glucocorticoids although very potent in anti inflammatory effect are also endowed with serious adverse effects.

Long term use of steriods may affect almost all systems of the body and may result in metabolic derangement, immunosuppression, osteoporosis, myopathy, catract, peptic ulceration and Cushing syndrome (Schimmer and Parker 2006). The other group of drugs namely NSAID also on regular use produce many introlerable side effects. Similar to corticosteriods, NSAID also can produce extensive gastric mucosal damage and ulceration. Induction of gastric or intestinal ulcer is known to affect 15-30% of NSAID users. The cuncurrent use of corticosteriods or alcohol consumption further aggrevates the above risk.

Eventhough COX-2 selective inhibitors have much less propensity to induce gastric ulcer, cardiovascular complications resulting from the use of COX-2 inhibitors are major limitations to their chronic usage (Bresallier et al 2005). Regular usage of NSAID may result in salt and water retention, oedema and decreased efficacy of anti-hypertensive drugs and diuretics.

The inhibitory effect of NSAID on platelet function increases the risk of hermorrhage. 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 afore mentinoed adverse effects of conventional antiinflammatory drugs necessitate the search for newer and safer antiinflammatory agents. Flavonoid group of drugs appear to be promising in this regard. This proposition arises from the fact that, potent antiinflammatory 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 rutoside and (+) cyanidanol-3 has been established in many experimental animal models. The above flavonoids protected the animals from uelcers induced by pyloric ligation, restraint and drugs (Parmar 1977, Parmar and Parmar 1998).

The potent anticular 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 antiinflammatory activity (Jang et al 2002). Thus many flavonoid compounds while exhibiting potent antiinflammatory activity do not show any evidence of ulcer induction. Such an ulcer - sparing effect of trihydroxy flavones employed in the present study requires confirmation by future studies.

Effect of trihydroxy 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 trihydroxy flavones exhibited potent antinociceptive and antiinflammatory activities, 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 IL1$_\beta$ mediate various reactions in the cascade of inflammation and in sensitization of pain fibres. Reactive oxygen species / free radicals have also been found to play a crucial role in many types of inflammatory arthritis. Hence in the present study the effect of trihydroxy flvones on the above mediatrors was studied employing suitable in vitro experiments.

Effect of trihydroxy flavones on cyclo oxygenase

Cyclooxygenase (COX) is the first enzyme in the synthetic pathway to form prostaglandin from arachidonic acid. A good correlation has been reported between the potency of an agent as COX inhibitor and antiinflmmatory activity. Among the two well - understood isoforms of cyclooxygenase, COX-1 is primarily

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.

The results of the present experiments revealed marked inhibitory activity of COX enzyme by the tested trihydroxy flavones.

Both the isoforms of cyclo oxygenase, COX-1 and COX–2 were inhibited to varying degrees by the tested tryhydroxy flavones. The inhibition of COX -1 ranged between 59 and 85% for all the different trihydroxyflavones. The inhibition of COX -2 ranged from 59 to 81% for the above compounds in the same concentration. 3,6,3'- trihydroxy flavone showed a maximum activity in inhibiting both COX-1 and COX -2. Almost similar degree of inhibition of COX -1 and COX-2 could be recorded for these compounds. Thus it is very obvious that the trihydroxy flavones exert a significant inhibitory action on cyclooxygenase responsible for the generation of prostaglandins which is an important mediator of inflammation and pain.

The present observation is in accordance with many earlier reports indicating the COX inhibitory activity of flavone compounds. Luteolin and chrysin (Harris et al 2006), 5,7- dihydroxy, 7-methoxy flavone (Daott et al 2003), and wogon in (Park et al 2001 and Chi et al 2005) are widely reported for their

inhibitory effect on prostaglandin synthesis. The present study has also identified a homologous series of trihydroxy flavones with potent inhibitory action on cyclooxygenase. This finding brings out the important mechanism by which the trihydroxy flavones exert their potent antinociceptive and antiinflammatory actions.

Effect of trihydroxy flavones on certain cytokines (TNF α and IL-1β)

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, tumor necrosis factor and various growth factors. In a complex co-ordinated network they act on leukocytes, vascular endothelial cells and osteoclasts (Rang et al 2003). Some cytokines have pro inflammatory actions and some other cytokines have anti inflammatory action. The primary proinflammatory cytokines are tumor 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 includes IL-4, IL-10, IL-13 and tumor growth factor β. The concentration of many inflammatory cytokines are increased in the synovium of patients with inflammatory arthritis. At the site of inflammtion peptides like subsance 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 1L-1 or TNF-α (Burke et al 2006).

Interleukins

Two important cytokines have been identified to play an essential role in co-ordinating the inflammatory process, especially interleukin–1 (IL-1) and tumor necrosis factor. These are derieved from mononuclear cells and macrophages. IL-1 and TNF are considered as the principal mediators of biological responses to endotoxins and many other infectious stimuli. IL-1 and TNF appear to work in concert with each other and with other growth factors and cytokines.

IL-1 comprises of two distinct poly peptides (IL-1α, IL-1β) that bind to the same cell surface receptor and produce similar biological responses. Plasma IL-1 levels are increased in patients with certain inflammatory diseaes like reheumatoid arthritis. IL-1 and TNF produce many proinflammatory responses. IL-1 and TNF may release bradykinin which may be responsible for the pain during inflammation.

In the present study, the investigated trihdyroxy flavones produced a concentration dependent inhibition of interleukin Iβ. In a maximum concentration of 50 µM these compounds produced an inhibition ranging from 69 to 78%. Similar to the action in COX, 3,6,3'- trihydroxy flavone exerted a slighty higher response (78%) then the other compounds (Table 31). In a recent study fisetin has been shown to decrease the production of a variety of interleukins (IL-1β, IL-4, IL-6, and IL-8) in stimulated human mast cells (Park et al 2007).

Tumor necrosis factor- α

Tumor necrosis factor - α (TNF -α) is a pro inflammatory cytokine that is involved in the pathophysiology of number of disorders including Crohn's disease, rheumatoid arthiritis, ankylosing spondylitis and psoriatic arthritis. Monoclonal antibodies like infliximab and soluble TNF-receptor, etanercept directed against TNF-α are used in treating rhematoid arthiritis and ankylosing spondylitis (Singh and Suruchi 2007). TNF α is a polypeptide primarily produced by activated monocytes and macrophages. This polypeptide mediates many immune and inflammatory responses including activation and differentiation of monocytes and macrophages and expression of adhesion molecule on the endothelial cells. This can stimulate PGE_2 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.

In the present study, the investigational trihydroxy flavones inhibited the activity of TNF-α to varying degrees. The compounds 3,6,3'-THF and 7,8, 3'-THF exhibited potent inhibition in concentrations ranging from 10-50 µM. The IC-50 values recorded for 3,6,3'- THF was 28.66 µM and that of 7,8,3'- THF was 35.48 µM compared to 25.23 µm for dexamethasone. The compounds 3,3',4'-THF and 6,3',4'-THF exerted an inhibitory action in higher concentration ranging from 100-500 µM. The IC-50 of 3,3'4'- THF was 431 µM and that of 6,3',4'-THF was 393 µM. Thus significant inhibition of an important mediator of inflammation was clearly evident for trihydroxy flavone derivatives.

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 (Manjeeth and Ghosh 1999). Luteolin has been shown to inhibit TNF--α production in vitro and in vivo and to exert good antiinflammtory activity. (Ueda et al 2004). Luteolin has further been shown to possess inhibitory effect on TNF-α induced. IL-8 production in intestinal epithelial cells (Kim et al 2005 a).

The presence of hydroxyl groups in the flavone nucleus has been suggested as an important determinant in this activity. Many flavones like apigenin, luteolin, chrysin, kaempferol, quercetin and bicalaein have been shown to inhibit TNF α induced upregulation of inter cellular adhesion molecule-1 which has been implicated in inflammation and carcinogensis. 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).

The results of the present study are in agreement with the earlier reports revealing an inhibitory effect on two of the proinflammatory cytokines (TNF-α and IL-1 β). The inhibitory action exerted on the above cytokines may effectively contribute to the antiinflammatory action of trihydroxy flavones.

Effect of trihydroxy flavones or free radical generation

Several types of reactive oxygen species are generated in the body in the form of free radicals as a result of metabolic reactions. These may be either oxygen derived or nitrogen derived and called pro oxidants. They attack macromolecules including proteins, DNA, and lipids etc. causing cellular/tissue damage.

To counter this effect, the body is endowed with another category of compounds called antioxidants. The antioxidants are produced either endogenously or obtained from exogenous sources.

Endogenous antioxidants include enzymes like superoxide dismutase catalase, glutathione peroxidase and glutathione reductase, minerals like Se, Mn, Cu and Zn and vitamins like A, C & E. Other compounds with anti oxidant activity include gluathione, 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 disease, neurological disease, malignancies, renal diseses, 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 rectivity and damaging activity towards macromolecules. The oxygen derived species include super oxides (O_2^-), hydoxy (OH^-), hydroperoxide HO_2, peroxyl (ROO^-), alkoxyl (RO^-) as free radicals and hydrogen peroixde (H_2O_2),

hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen (O°) as non radicals. Similarly nitrogen derived oxidant species are mainly nitric oxide (NO), Peroxinitite ($ONOO^-$) nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3).

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

Many drugs used in rheumatoid arthritis mediate their therapeutic action by multiple mechanisms; An important effect being reduction of oxidative damage at inflammatory sites either by inhibiting reactive oxygen production or by scavanging such noxious molecules (Aruoma 1996).

Flavonoids which are abundant in fruits, vegetables and medicinal plants are effective free radicals svavengers. Many beneficial effects of flavonoids have been attributed to the antioxidant / free radical scavanging properites (Burda and Oleszek 2001). In the present study the investigated trihydroxy flavones exhibited potent antinociceptive and antinflammatory activities. The effect of

these compounds on free radical generation/scavanging was investigated against DPPH free radical and nitric oxide-radical by in-vitro methods. A dose dependent inhibition of DPPH free radicals generation activity was recorded for all the trihydroxy flavones. Nearly 66% inhibition of DPPH activity was recorded for 3,3',4 THF, 6, 3',4' THF and 7,8,3' THF while 40% inhibition could be recorded for 3,6,3' THF (Table 37). The free radical seavanging activity was further corroborated by the ability of the tested trihydroxy flavones in scavanging nitric oxide radicals. Almost 80% inhibition could be recorded for all the trihydroxy flavones tested (Table 36).

Many earlier reports suggest that dihydroxylation or poly hydroxylation of flavone nucleus favours high antioxidant activity (Harborne and Williams 2000; Heim et al 2002, Furosawa et al 2005).

The results of the present study are in agreement with the above proposal. All the trihydroxy flavones investigated in the present study exhibited potent antioxidant / free radical scavanging activity suggesting that, this may be one of the mechanisms in mediating their biological actions.

Anti nociceptive synergism of trihydroxy flavones with diclofenac

Combination of drugs is employed in many diseases to achieve maximum therapeutic benefit and at the same time to reduce the adverse effect of individual drugs. In chronic pain situation NSAID are usually combined with opioids to reduce the tolerance development. In the present study various trihydroxy flavones have been proved to exert significant antinociceptive and

antiinflammatory activities utilising multiple pathways. It may be suggested that combination of trihydroxy flavone with currently used NSAID may enhance their efficacy. This has been attempted by studying the combination of minimally effective doses of diclofenac with a minimum dose of trihydroxy flavone.

Diclofenac in a dose of 1mg/kg produced a mild but significant reduction in number of abdominal constrictions (23) compared to vehicle treatment (32).

All the tested trihydroxy flavones in a dose of 25 mg / kg also produced a similar degree of reduction of number of abdominal constrictions (21.8 to 23.8).

However, in diclofenac pre treated animals administration of trihydroxy flavones resulted in further significant reduction in the number of abdominal constrictions (16 to 18) table 28. This potentiated antinociceptive response was recorded with all the trihydroxy flavones except 3,6,3' - trihydroxy favone. Thus the present results reveal a synergistic antinociceptive effect of trihydroxy flavones with a NSAID.

Anti inflammatory synergism of trihydroxy flavones with diclofenac:

Since the trihydroxy flavones potentiated the antinociceptive activity of diclofenac it was considered interesting to study the antiinflammatory efficacy of such a combination. The reduction in paw diameter with diclofenac (1mg/kg) was 47% and that of other trihydroxy flavones in a dose of 25 mg/kg ranges from 53-62%. (Tables 24-27). However, in combination, further significant reduction in

paw oedema (61-68%) could be observed with all the trihydroxy flavone barring 7,8,3' trihydroxy flavone.

Thus the present results indicate a synergistic effect of trihydroxyflavones on the antinociceptive and antiinflammatory effect of a NSAID. This synergism suggests that a combination of above classes of agents may help to achieve maximum therapeutic effect with minimal side effects.

The results of many previous studies have revealed the gastric mucosal protection offered by flavonoid compounds (Martin et al 1994, 1998, Parmar 1977, Parmar 1988 Goel et al 1988, Guerro et al 1994, Viswanathan et al 1981). Such an ulcer protective effect for trihydroxy flavones remains to be established. Never the less, it can be suggested that combination of a flavonoid compound may protect against the gastric mucosal damage resulting from NSAID usage which is a major side effect that precludes regular use of such drugs. In addition, the reduced requirement of NSAID may also help to avoid other adverse effects too.

SUMMARY

The main objective of the present study was to conduct a systematic screening of structurally related trihydroxy flavone compounds (3,3',4'-trihydroxy flavone, 3,6,3'-trihydroxy flavone, 6,3'4'-trihydroxy flavone and 7,8,3'-trihydroxy flavones) for their antinociceptive and antiinflammatory potential. These trihydroxy flavone compounds have hitherto not been subjected to any biological screening.

Antinociceptive Screening

Followed by preliminary screening and acute toxicity testing, the trihydroxy flavones were investigated for their antinociceptive potential in mice by following well established screening methods

1. Acetic acid induced abdominal constriction
2. Formalin induced nociception
3. Hot water tail immersion method

The participation of opioid system, GABAergic, adrenergic system and potassium channel in the mediation of antinociceptive effect of the trihydroxy flavones was also investigated.

Antiinflammatory study

The potent antinociceptive effect observed for these compounds broadened the scope of the study and a possible antinflammatory effect was also examined. For this purpose, a well established method namely carrageenan induced rat paw oedema technique was adopted.

Effect on mediators of pain and inflamamtion

In a subsequent stage, the effect of trihydroxy flavones on some important mediators of pain and inflammation was investigated. The action of these compounds on prostaglandins, interleukin - 1β, tumor necrosis factor - α and reactive oxygen species / free radicals was studied by employing suitable in-vitro experiments.

A possible synergistic effect of trihydroxyflavones with a standard NSAID (diclofenac) was also examined both in antinociceptive and anti inflammatory effects.

Safety

The results of preliminary screening and acute toxicity testing indicated the safe nature of these trihydroxy flavones which was revealed by the results of acute toxicity test in which no mortality was observed in mice with any of the test compounds even in a dose of 2g/kg body weight. The above finding confirms the postulations of Havsteen (2002) that, “The margin of safety for the therapeutic use of flavonoid in human is considered to be very large, probably much superior to any other drug in current use”.

The spontaneous motor activity or balancing time on a rotarod of mice was not altered by treatment with trihydroxy flavones. The test compounds have not influenced the motor activity of animals even in the highest dose tested. These findings indicate that any response recorded in the antinoceptive assay procedures will be a true response and not due to an inherent modification of motor activity by the test compounds.

Antinociceptive effect

Administration of trihydroxy flavones in mice produced a consistent and dose related antinociception in acetic acid induced visceral nociception. The tested trihydroxy flavones exhibited significant inhibition of nociceptive response. In the formalin induced nociception both the early phase and late phase of nociceptive response were significantly attenuated by these trihydroxy flavones. In the thermal model of nociception, the investigational compounds produced a mild but significant inhibition of nociceptive response. The acetic acid induced nociception and late phase of formalin nociception are considered to represent the inflammatory pain response (Tjolsen and Hole 1997). Significant attenuation of both the above responses by the trihydroxy flavones suggest that these compounds may be more effective in inflammatory pain.

Structure activity relationship

An attempt has been made to correlate the structure of the trihydroxy flavones and their antinociceptive activity in relation with the existing literature on this area. A salient point arising from the above analysis is that, introduction of

another hydroxyl group in a B ring of ortho-di-hydroxylated flavone attributed enhanced antinociceptive efficacy to the dihydroxy flavone.

Mechanisms involved in the antinociceptive action of trihydroxy flavones

Opioid mechanism: The opioid antagonist naloxone significantly attenuated the antinociceptive activity of the test compounds. This observation provides evidence for the involvement of opioid mechanism in the antinociceptive action of these compounds. This observation confirms the earlier reports on the opiod-mediated antinociceptive activity of flavone compounds.

Adrenergic involvement: α-2 adrenergic antagonist yohimbine antagonised the antinociceptive effect of 3,6,3' - trihydroxy flavone suggesting a role for α-2 adrenergic system in the action of 3,6,3'- trihydroxy flavone. However, the antinociceptive action of other tested trihydroxy flavones was not altered by yohimbine.

GABAergic system

The present results indicated that bicuculline, a GABA antagonist effecively suppressed the antinociceptive effect of 6,3'4' - THF and 7,8,3'-THF revealing that GABAergic system is involved in the action of these two compounds. The results excluded the role of GABAergic system in the antinociceptive action of 3,3',4' - and 3,6,3'-tri hydroxy flavone.

Role of potassium channel

The antinociceptive effect of 7,8,3' - trihydroxy flavone was attenuated by glibenclamide pre treatment suggesting a role for potassium channel involvement in the action of the above compound. The results did not reveal the participation of potassium channel in the action of other three tested compounds.

Anti inflammatory effect of trihydroxy flavones

The results of the present study indicated a dose and time dependent antinflammatory activity of all the tested trihydroxy flavones. A marked reduction in the paw diameter of rat was clearly evident in all the doses employed with a peak effect at fifth hour of observation. Nearly 60-72% of maximum inhibition of inflammation was recorded for these compounds.

Effect of trihydroxy flavones on certain mediators of inflammation and pain

Cyclooxygenase

The in vitro experiments carried out indicated a marked inhibitory activity of COX enzyme by the tested trihydroxy flavones. Both the isoforms of cyclooxygenase (COX-1 and COX-2) were inhibited to varying degree by the tested trihydroxy flavones.

Tumor necrosis factor -α

The activity of tumor necrosis factor-α was significantly inhibited by the investigated trihydroxy flavones.

Interleukin - 1β

The results of present study also revealed a potent inhibitory action of trihydroxy flavones on interleukin -1β.

Free radical scavenging

A high degree of free radical scavenging / antioxidant activity was revealed for the investigated trihydroxy flavones.

Antinociceptive and anti inflammatory synergism

A synergistic effect for most of the trihydroxy flavones with diclofenac could be demonstrated in the antinoceptive as well as antiinflammatory effects.

The results of the present study have identified the novel antinociceptive and antinflammatory actions of few trihydroxy flavones. The findings of this work provide additional support to the existing literature on the biological significance of flavonoid group of compounds. Perhaps, this may be first report on the above biological actions of a homologous series of trihydroxy flavones. The study has helped us to identify the multiple pathways that may be involved in the antinociceptive actions of trihydroxy flavones. The role played by several endogenous mediators in the antiinflammatory effect of trihydroxy flavones was also brought to limelight from the experiments conducted. An indication for a

possible synergistic effect with an established NSAID diclofenac was also available from the present results. Further exploitation of the findings reported in the present study will definitely help to identify safe and effective compounds to treat pain and inflammation.

CONCLUSION

Pain and inflammation are complex processes involving multitude of endogenous chemicals and neurotransmitters. Significant advancement in understanding the patho-physiology of pain and inflammation have identified multiple targets to alleviate them. Morphine and many of its synthetic derivatives as well as a wide variety of NSAID are used for many decades for this purpose. Eventhough, these drugs offer considerable relief, their inherent side effects limit their regular use in chronic pain or inflammatory diseases. Recent investigations on flavone compounds have indicated them as novel therapeutic agents in many diseases.

Identification of an unique combination of analgesic and anti-inflammatory activities without provoking gastric ulceration makes flavones to be highly preferred candidates to treat pain and inflammation. The present study has revealed significant antinociceptive and anti-inflammatory activities of a few trihydroxy flavones and also has added a few more compounds to the arena of biologically effective flavone compounds for therapeutic exploitation. The multiple pathways through which flavones may bring about their beneficial effects also have been identified in the present study. Future organised clinical investigations shall reveal the utility of these compounds either alone or as adjuncts to currently employed analgesic and antiinfllammtory drugs.

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