CHAPTER VII

Pharmacological Investigations on some of the

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INTRODUCTION

Algesia (pain) is a protective reflex response, usually evoked by an external or internal noxious stimulus, that signals either the presence of an injury or some tissue damage. Some refer to it as an unpleasant sensory and emotional experience normally associated with actual or potential tissue damage. The exact mechanism of pain perception has not been clearly elucidated. There seem to be many different mechanisms for pain modulation. Pain itself may be classified into referred (when felt in areas remote from the injured site) and visceral (when felt in the actual site of damage). In contrast to other sensory receptors, the pain receptors adapt very little and sometimes get excited after stimulus so that the pain steadily increases, resulting in hyperalgesia. This is, very often, the reason for the success of placebotherapy in the management of pain. Ideally, pain can be relieved by removing its cause. Since the causes of pain are not always understood or can be rectified immediately, several conditions warrant the use of analgesics to ameliorate the sensation of pain. Analgesics are drugs that selectively relieve pain by acting in the CNS or on peripheral pain mechanisms, without significantly altering consciousness.

Analgesics may be grouped into either of the two classes: opioids or non-steroidal anti-inflammatory drugs (NSAID). Opioids act by blocking impulses at specific opioid receptor sites while the NSAID analgesics work by preventing the
production of prostaglandin, one of the chemical mediators that trigger the transmission of pain signals to the brain. Though both classes are effective, they do possess serious untoward effects like addiction liability and gastritis.

Despite the synthesis of several surrogates, morphine, the opium alkaloid, which Serturner first isolated in 1803, still claims the superiority. Even today, it is the most potent pain killer used clinically and is the standard analgesic against which all newer compounds are compared. However, the opioids display strong analgesic and addictive properties and the mechanisms underlying opioid addiction are still poorly understood. Though the past two decades have witnessed the development of many novel potent opioids by the pharmaceutical industry, the ideal analgesic is still eagerly awaited. In this pursuit, several other classes of compounds, especially from natural sources are being simultaneously screened to explore the possibility of potential analgesic property contained in them.

Flavonoids, originally considered as dietary constituents have regained research significance especially because they exhibit promising antinociceptive response besides being anti-inflammatory (possibly due to 5-lipoxygenase, 12-lipoxigenase and cyclo-oxygenase inhibition). Surprisingly they also possess antiulcer (mainly because of histidine decarboxylase, catechol-O-methyl transferase and dihydroxy-phenylalanine decarboxylase inhibition) activity which is uncommon with the prevailing NSAIDs. A study of the structure-activity relationship has led to the suggestion that the flavonoids may utilise different mechanisms in eliciting analgesia. The position of the substituents in the flavonoid skeleton appears to effect significant changes in analgesic potency.
PHARMACODYNAMICS OF OPIOID ANTINOCICEPTION

Physiological actions of opiates are stereospecific and structural modifications alter their potency. Several decades of pharmacology has led to the proposal that opiates exert their biological action at the level of the CNS and specifically activate membrane receptors, thus interfering with a complex endogenous neurotransmitter system.

Neurotransmitters

Opioid peptides and their receptors control autonomic functions and modulate immune responses. Hence, the binding of opioid agonists with specific receptors triggers a series of events in cellular functions, that include alterations in the neurotransmitter functions such as cholinergic, dopaminergic, serotonergic and gamma aminobutyric acid systems.

Opioid receptors

The opioid system is composed of three receptor types known as μ, δ- and κ-receptors, which are activated by a family of structurally related endogenous peptides (Table VII 1). The relative involvement of these three receptors in opiate mediated antinociceptive mechanisms in mice has been assessed. Genes encoding three families of opioid peptides: pro-enkephalin, pro-dynorphin and pro-opiomelanocortin and their receptors: μ-opioid receptor, δ-opioid receptor and κ-opioid receptor have been cloned and characterised. This neuromodulatory
system has been implicated in the control of behaviours that are essential for self and species survival, including responses to noxious information and stress, reward and motivation.66-69

**Molecular mechanisms**

Possibly an alternative or a rather parallel pathway, altering the functions of second messenger systems like cyclic AMP has also been implicated. A role for voltage gated ion channels, mainly, Ca\(^{2+}\), K\(^{+}\) and Cl\(^{-}\) channels in mediating opioid antinociception has also been documented.

**Endocrine function**

Endogenous opioid peptides have also been shown to modulate the release of hormones, such as follicular stimulating hormones leutinizing hormone and vasopressin. The antinociceptive activity of morphine has been found to be influenced by the endocrine status of the adrenal and thyroid.70 It has been established that vasopressin,\(^{71}\) thyroid stimulating hormone,\(^{72}\) prolactin,\(^{73}\) insulin\(^{74}\) and histamine\(^{75}\) possess an inherent antinociceptive response.

**Molecular pharmacology of opioid receptors**

Opioid receptors represent the primary targets for opioid drugs and each of these is distributed differently throughout the CNS.\(^{75}\) Certain regions (striatum and dorsal horn of the spinal cord) express all three receptors, while others exhibit abundant sites for one receptor type only (thalamic nuclei for the \(\mu\)-receptor or the
claustrum for the k-receptor). Moreover, each receptor is implicated in opioid function in a distinct manner and to a different extent. In addition to spinal and supraspinal antinociceptive sites of action, there are evidences suggestive of a peripheral analgesic effect for exogenous opioids associated with inflammation.

Until recently, based on pharmacological approaches (agonists and antagonists tools), the relative antinociceptive activity was adjudged as: MOR being the best antinociceptive but also exhibiting the highest abuse liability, while DOR being poor analgesic and less addictive. Due to the strong dysphoric properties of KOR-agonists, their use in pain treatment needs to be restricted to periphery.

Today, germline mutation of opioid receptor genes have enabled the production of transgenic mice which lack specific opioid receptor. These cloned models are now being used to evaluate the role of opioid receptors in physiological and pathological functions. Studies using MOR-deficient mice led to the conclusion that MOR is necessary to mediate morphine action on pain pathways and suggested that DOR and KOR do not participate in morphine analgesia under standard experimental conditions. However, compensatory mechanisms do appear to play a role in mutant (MOR-deficient) strains, since studies have also indicated, at least, in inflammatory algesia, the release of endogenous DOR activation and increased sensitivity to δ-specific ligands. Subsequent investigation has revealed that the expression of MOR is not required for DOR-mediated antinociception or stimulation of intracellular second messenger pathways and that DOR directly mediate supraspinal and spinal antinociception. This has been derived based on the experimental data which indicated that the DOR remained
functionally coupled to G-proteins and produced antinociceptive actions in MOR-knockout mice.87

**Relationship between glycaemic and algesic states**

The idea that changes in the blood glucose level and pain threshold may be related has evolved out of the clinical study wherein poorly treated diabetic subjects tend to develop painful neuropathy that diminishes on their attaining euglycaemic status with insulin therapy.88 It is also observed that manoeuvres to elicit antinociception through opioid mechanisms like swim exercise or restraint stress have also been shown to modify the glycaemic state.89-93 Nevertheless, the results of extensive research employing experimentally induced diabetic animal and clinical models are varied. Some are well agreeing while others are disagreeing with the relation between the two parameters. A few observations are suggestive of totally no relationship between the two.94-109 The reasons for the conflicting results may be many. But evidences suggest that the probable causes may be (1) chemical destruction of the β-islets of pancreas in the chemically induced diabetic subjects also causes structural and functional damage to the nervous system,110-113 (2) the choice of the assay procedure adopted to investigate the mechanism involved in the antinociception elicited by various agents114 and (3) irreversible, permanent damage of the nerve endings in chronic diabetes or poorly treated subjects. Though no direct association has been established so far between glycaemic and algesic status, evidences could be derived for antinociception associated with hyperinsulinaemia.115-118
The present investigation also envisages an enquiry into the possible relationship between the glycaemic and algesic states besides testing the antinociceptive potential of some of the flavonol glycosides, isolated from natural sources*.

* This work has been presented in the Southern Regional conference of the Indian Pharmacological Society, held at St. John's Medical College, Bangalore, India in June 2000 (8th to 10th) Abstr. No. F 1
MATERIALS AND METHODS

Drugs and Chemicals used

Six flavonol glycosides, kaempferol 3-0-β-D-rutinoside, K 3-0-β-D-rutinoside-7-0-β-D-glucoside, K 3-0-β-D(6"-p-coumaroyl) glucoside, isomeric mixture of K 3-0-β-D-(6"-p-coumaroyl) galactoside and K 3-0-β-D-(2"-p-coumaroyl) galactoside, quercetin 3-0-β-D-neohesperidoside, and Q 3-0-α-L-rhamnoside isolated as described in the previous chapters. Glacial acetic acid (AR, Sarabhai, Wadodara, India). Naloxone hydrochloride (Endo Laboratories, USA) and Carboxymethyl cellulose (Glaxo Laboratories, Mumbai, India).

Animals used

Randomly bred Swiss male albino mice (25-30 g) were used. They were maintained in the animal house at room temperature (35-36°C) with 12 h light and 12 h dark cycles. Food and water were available ad libitum. The experiments were carried out during light cycle.

Assessment of antinociception

This was done by two methods, viz. chemical and mechanical. In the chemical method, animals received 10 ml/kg of 0.6% acetic acid intraperitoneally at the 60th min after each dose of the flavonoid and observed for the following 15 min to quantify the number of abdominal constrictions.
Any significant decrease ($P < 0.05$) in this when compared with vehicle treatment was considered as antinociceptive response. In the mechanical assay $^{120}$, a bull-dog clip with thin rubber sleeves was applied at the root of the tail and the time taken by the animal to make an effort to dislodge the clip was recorded. Any significant increase ($P < 0.05$) in this reaction time compared with the control was considered antinociceptive response. In both the assay procedures, each animal was used only once.

**Measurement of blood glucose**

Blood glucose was measured using AMES glucometer with appropriate glucostix (Bayer Diagnostics, Wadodra, India). Drops of blood were collected by puncturing the tail vein of the mouse prior to any exposure of drug and just before acetic acid challenge and blood glucose measured. The values were expressed as percentage change from the initial pre-treatment value of that mouse which was presumed as 100%.

**Drug treatment**

The animals received intraperitoneally the six flavonol glycosides isolated as suspension in Tween 80 sixty min prior to antinociceptive testing, either chemical or mechanical. They received either 25, 50 or 100 mg/kg, which was arrived at based on our pilot studies. The changes induced by these substances on the blood glucose or induced nociception (both chemical and mechanical) were recorded.
Mechanism of antinociception

The role of opioid system on the possible changes induced by the flavonol glycosides on noxious stimulus was investigated by pretreating the animals with naloxone (1 mg/kg., i.p.), a specific opioid antagonist, 15 min prior to flavonol glycoside injection.

Analysis of data

The data were subjected to statistical analysis by the analysis of variance using ANOVA followed by Dunnet's "t" test. A probability value of less than 0.05% was considered significant.
RESULTS

Study of antinociception by noxious chemical induction method

All the six flavonol glycosides did not modify significantly the motor activity of the mice as tested by rota rod method (data not shown). A dose related inhibitory effect on the number of abdominal constrictions elicited by acetic acid was noticed. This effect was noticed for 25 mg/kg which increased after 50 or 100 mg/kg. An approximate 48 to 85% inhibition was recorded (Table VII.2) and (Fig VII.1).

Study of antinociception by mechanical noxious stimulus method

In the tail clip assay procedure, a significant increase in the reaction time after 25 mg and 50 mg/kg dosage was recorded for the six compounds tested. However, this increase was reduced after the 100 mg/kg dose for compounds 3 and 4 and in fact the increase in the reaction time recorded was not statistically significant (Table VII.3) and (Fig VII.2).

Role of opioid system

Naloxone per se at the dose used attenuated the antinociceptive response of all the six flavonol glycosides in both the methods studied (Table VII.4).

Effect on blood glucose levels

The compounds employed in the present study in the doses tested (25/50/100 mg/kg) did not significantly modify the blood glucose levels, when
compared with the pre-treated ones. However, there was a mild hypoglycemia observed after 100 mg/kg dose (Table VII.5) and (Fig VII.3). This decrease in the blood glucose level was statistically insignificant.
DISCUSSION

More than 3% of the entire world population is victim of Diabetes mellitus and the number is ever increasing. There is every reason to suppose that diabetes will remain a threat to public health in this millennium also. The prevalence and incidence of diabetes are steadily increasing in India. Other epidemiological data suggest that Indians as a race are genetically more prone to develop type 2 diabetes; they develop diabetes about a decade earlier than most other population groups, and are especially prone to the microvascular complications, viz. ischemic heart disease and stroke. According to WHO report, prevention of diabetes and its consequences are not only a major challenge for the future, but also essential, if “Health For All” is to be an attainable target. It has also strongly emphasized the need for basic research to evaluate the efficacy of traditional medicines for Diabetes mellitus.

As mentioned earlier, flavonoids possess the unique property of being anti-inflammatory, antinociceptive and antiulcerogenic. Consequently, they have been subjected to elaborate pharmacological investigations for assessing these properties as well as their antidiabetic activities. Gossypin has been investigated, in detail, for its antinociceptive activity. The results indicate that they utilise the opioid pathways in eliciting antinociception. The special character of gossypin, which is a combination of anti-inflammatory, analgesic and antiulcer properties when found to induce antidiabetic activity triggered the possible utility of flavonoids in the management of pain, especially in patients suffering from painful
diabetic neuropathy. In accordance with the earlier reports on the flavonoid antinociception properties, the present study also provide additional supporting information that the flavonol glycosides investigated, exhibited a significant antinociceptive response. It is to be noted here that this response was observed in all the doses administered in the noxious chemical induction procedure but not that significant in the tail clip assay. The notion that the chemical assay may be ideal for peripheral analgesia and tail clip assay may be suitable for central origin is put to challenge today. Hyashi and Takemori have reported \(^{124}\) that morphine produced potent antinociceptive response with minimal \(ED_{50}\) of 0.33 mg/kg in the chemical assay compared to the \(ED_{50}\) of 10 mg/kg in the thermal and mechanical assays. They, therefore, described the chemical assay to be the most sensitive and precise method involving minimal nociception. This may possibly be the reason for the compounds in the present study to produce marked effect in the chemical assay procedure. The failure to produce sufficient antinociception in the tail clip assay procedure after the 100 mg/kg dosage of compounds 3 and 4 is, however, difficult to explain with the available data.

The 6 compounds, investigated also exhibited mild hypoglycaemia, which is in accordance with earlier observations. \(^{122,125,128}\)

Previous reports have presented varied views on the possible association between the glycaemic and algesic states. \(^{85,129,130}\) However, recent detailed investigations excluded a possible cause-effect relationship between changes in the two states \(^{115-118}\). The findings of the present study also support this view since all the compounds elicited significant antinociceptive response sans significant effect of the glycaemic status. In accordance with the earlier observations \(^{10}\) the
antinociceptive activity of the six compounds clearly indicate the involvement of an opioid-like mechanism since their analgesic activity was attenuated by naloxone.

In summary, the six flavonol glycosides, isolated and characterised using chemical and spectral methods from the plant materials for the first time were found to possess significant antinociceptive response and can be considered along with other flavonoid group of substances for inclusion in diabetic diet or as a prophylactic for susceptible (vulnerable) group. They may also be considered as possible candidates for clinical trials, especially in situations like painful diabetic neuropathy. Further studies on the effect of these compounds on the serum insulin level may shed more light on the possible hypoglycaemic activity, especially at higher doses, since intra-cerebroventricular administration of insulin has been reported to have produced antinociceptive response independent of its action on the glycaemic state.⁷⁴
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208


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210
TABLE VII 1

Nomenclature of opioid receptors
(IUPHAR recommendation)

<table>
<thead>
<tr>
<th>Preferential endogenous opioid ligands</th>
<th>Opioid Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IUPHAR Recommendation</td>
</tr>
<tr>
<td>Enkephalins</td>
<td>OP1</td>
</tr>
<tr>
<td>Dynorphins</td>
<td>OP2</td>
</tr>
<tr>
<td>B-endorphins</td>
<td>OP3</td>
</tr>
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TABLE VII 2

Antinociceptive effects of the flavonol glycosides by acetic acid induced abdominal constrictions assay in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of abdominal constrictions / 15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 mg</td>
</tr>
<tr>
<td>Kaempferol 3-0-rutinoside</td>
<td>9.50 ± 3.46*</td>
</tr>
<tr>
<td>K 3-0-rutinoside-7-0-glucoside</td>
<td>18.34 ± 4.05*</td>
</tr>
<tr>
<td>K 3-0-(p-coumaryl) glucoside</td>
<td>17.58 ± 2.76*</td>
</tr>
<tr>
<td>K 3-0-(p-coumaryl) galactosides</td>
<td>19.84 ± 4.83*</td>
</tr>
<tr>
<td>Quercetin 3-0-neohesperidoside</td>
<td>16.33 ± 2.88*</td>
</tr>
<tr>
<td>Q 3-rutinoside</td>
<td>15.60 ± 1.21*</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM
In vehicle treated animals the number of abdominal constrictions is 38.00 ± 0.97
*p < 0.001 when compared with vehicle treatment
## TABLE VII 3

Antinociceptive effects of the flavonol glycosides by the tail clip assay in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reaction time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 mg</td>
</tr>
<tr>
<td>Kaempferol 3-O-rutinoside</td>
<td>3.42 ± 2.82**</td>
</tr>
<tr>
<td>K 3-O-rutinoside-7-O-glucoside</td>
<td>2.98 ± 4.74**</td>
</tr>
<tr>
<td>K 3-O-(p-coumaryl) glucoside</td>
<td>-4.43 ± 2.89**</td>
</tr>
<tr>
<td>K 3-O-(p-coumaryl) galactosides</td>
<td>-4.24 ± 1.88**</td>
</tr>
<tr>
<td>Quercetin 3-O-neohesperidoside</td>
<td>11.67 ± 3.74**</td>
</tr>
<tr>
<td>Q 3-rutinoside</td>
<td>7.28 ± 4.80**</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM of 6 experiments
In vehicle treated animals the reaction time is 1.88 ± 0.59
**p > 0.05 when compared with vehicle treatment
### TABLE VII 4

**Influence of naloxone pretreatment on flavonol glycoside induced antinociception and vehicle treatment**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Assay Procedures</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No.of Abdominal</td>
<td>Reaction Time (s)</td>
</tr>
<tr>
<td></td>
<td>Constrictions</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>38.00 ± 0.97</td>
<td>1.88 ± 0.59</td>
</tr>
<tr>
<td>Kaempferol 3-0-rutinoside</td>
<td>25.72 ± 1.83*</td>
<td>2.04 ± 1.01*</td>
</tr>
<tr>
<td>K 3-0-rutinoside-7-0-glucoside</td>
<td>26.80 ± 1.40*</td>
<td>2.58 ± 1.44*</td>
</tr>
<tr>
<td>K 3-0-(p-coumaryl) glucoside</td>
<td>24.33 ± 1.73*</td>
<td>3.42 ± 0.48**</td>
</tr>
<tr>
<td>K 3-0-(p-coumaryl) galactosides</td>
<td>30.67 ± 1.67*</td>
<td>3.92 ± 1.34**</td>
</tr>
<tr>
<td>Quercetin 3-0-neohesperidoside</td>
<td>28.83 ± 2.04*</td>
<td>3.90 ± 0.15*</td>
</tr>
<tr>
<td>Q 3-rutinoside</td>
<td>27.30 ± 2.41*</td>
<td>2.34 ± 1.20*</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM  
Naloxone (1 mg/kg, i.p.) was administered 15 min prior to acetic acid injection;  
*p < 0.01; **p > 0.05
**TABLE VII 5**

Effect of flavonol glycosides on the blood glucose of the mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood Glucose (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 mg</td>
</tr>
<tr>
<td>Kaempferol 3-0-rutinoside</td>
<td>98.42 ± 8.52**</td>
</tr>
<tr>
<td>K 3-0-rutinoside-7-0-glucoside</td>
<td>98.86 ± 10.25**</td>
</tr>
<tr>
<td>K 3-0-(p-coumaryl) glucoside</td>
<td>96.22 ± 12.51**</td>
</tr>
<tr>
<td>K 3-0-(p-coumaryl) galactosides</td>
<td>94.92 ± 6.21**</td>
</tr>
<tr>
<td>Quercetin 3-0-neohesperidoside</td>
<td>95.98 ± 12.61**</td>
</tr>
<tr>
<td>Q 3-rutinoside</td>
<td>96.49 ± 15.33**</td>
</tr>
</tbody>
</table>

*Values are expressed as percentage considering the vehicle treated value (100.90 ± 5.27) as 100%

**p > 0.05
Fig. VII.2. Relative increase in the reaction times caused by the flavonol glycosides compared to that of naloxone pretreatment (assumed unity)

**Compounds**
- 1: Kaempferol 3-O-rutinoside
- 2: K 3-O-rutinoside-7-O-glucoside
- 3: K 3-O-(p-coumaryl) glucoside
- 4: K 3-O-(p-coumaryl) galactosides
- 5: Quercetin 3-O-neohesperidoside
- 6: Q 3-O-rutinoside

**Legend**
- □ 25 mg
- ■ 50 mg
- □ 100 mg/kg of Flavonol glycosides
Fig. VII. 3. Percentage decrease in blood glucose levels caused by the flavonol glycosides

KEY: 1-Kaempferol 3-0-rutinoside; 2-K 3-0-rutinoside-7-0-glucoside; 3-K 3-0-(p-coumaryl) glucoside; 4-K 3-0-(p-coumaryl) galactosides; 5-Quercetin 3-0-neohesperidoside; 6-Q 3-0-rutinoside

- 25 mg/kg □ 50 mg/kg □ 100 mg/kg of Flavonol glycosides