



ANXIOLYTIC EFFECT OF MORIN IN MICE

MANGAIARKKARASI A.*¹, VISWANATHAN S², RAMASWAMY S³ AND THARANI CB⁴

¹ Department of Pharmacology, Sri Manakula Vinayagar Medical college, Puducherry- 605010.

² Department of Pharmacology, Meenakshi Medical College & Research Institute, Kanchipuram, (Meenakshi Academy of Higher Education & Research, Chennai 600078).

³ Department of Pharmacology, Sri Lakshminarayana Institute of Medical Sciences, Puducherry-605502, India

⁴ Department of Pharmacology, Saveetha Medical College, Saveetha University, Chennai - 600077, India

ABSTRACT

The usage of benzodiazepines, the major class of anxiolytic drugs is invariably accompanied by many side effects like sedation and myorelaxation leading to incoordination of movements. Search for novel anxiolytic agents have identified flavonoids as potential compounds devoid of these adverse effects. In the present study, morin, a pentahydroxy flavone has been investigated in detail for its anxiolytic and other effects on central nervous system in mice. Morin employed in doses of 10, 25 and 50 mg/kg(s.c) was screened for its effect on anxiety using two well validated methods namely elevated plus maze and staircase test. It's influence on other parameters of central nervous system functions was studied by using rotarod, openfield apparatus and pentobarbitone induced sleeping time. Morin displayed a dose-dependent anxiolytic effect similar to diazepam in the animal models of anxiety as revealed by a significant increase in the time spent in open arms of the elevated plus maze and significant reduction in the number of rearing responses in staircase test. In the openfield test morin treatment did not significantly change the number of squares crossed by mice compared to vehicle treatment. Similarly the balancing time of mice when placed on a rotarod was also not altered by morin treatment. These observations indicated that, morin neither altered the motor activity nor produced any myorelaxation in mice. Further, morin pretreatment did not significantly influence the sleeping time of mice after pentobarbitone administration. Taken together, the results of the present study have identified the novel anxiolytic effect of morin without producing sedation, myorelaxation or alteration of motor activity.

Key words: Anxiety, flavone, morin, elevated plus maze, staircase test, rotarod, openfield apparatus.

INTRODUCTION

Anxiety is an emotional state caused by the perception of real or perceived danger, that threatens the security of an individual. Anxiety disorders are considered the most common mental illness present in 15-20% of medical clinic patients (Reus 2008). Drugs like benzodiazepines, buspirone and propranolol are often used as first line approach in the management of anxiety related disorders (O' Donnell and Shelton 2011). These medications have many undesirable side

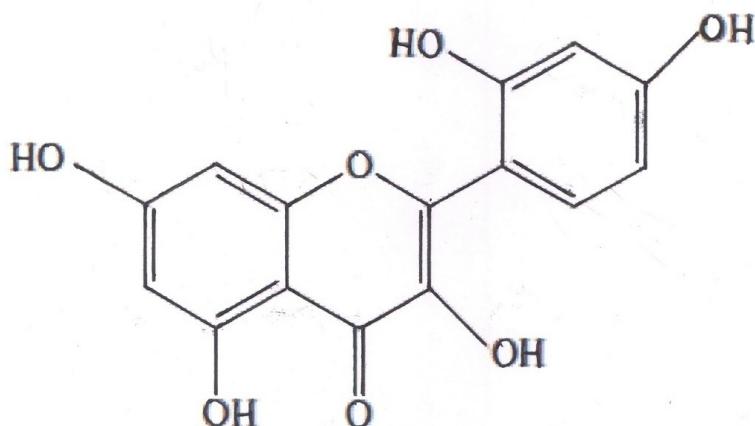
effects and a significant number of patients are resistant to these drugs (Fernandez et al. 2009). Hence there is a need for robust anxiolytic compounds that have lesser side effects.

Flavonoids are an important group of polyphenolic compounds derived from nature. These compounds have been shown to possess many useful biological activities like antioxidant, anti-inflammatory, cytotoxic (Havsteen 2002) and anti-nociceptive properties (Girija et al. 2002;

Uma Maheswari et al. 2006; Vidyalakshmi et al. 2010). Some of the naturally occurring flavonoids and their synthetic derivatives have been reported to selectively bind to the central benzodiazepine receptors and to exert anxiolytic and other benzodiazepine like effects in animals (Medina et al. 1997). The central nervous system depressant effects of many flavonoids have been reported by Fernandez et al (2006). Studies have shown the existence of some flavonoid compounds that

possess anxiolytic effect not associated with myorelaxant, amnestic or sedative actions (Marder and Paladini 2002). Recently the flavonoid glycosides myricitrin, gossypin and naringin have been documented to possess anxiolytic effect in mice (Fernandez et al. 2009). In the present study morin (3, 5, 7, 2', 4' – pentahydroxy flavone (Fig. 1) has been investigated for its effect on anxiety, motor co-ordination and sedation in mice.

Figure – 1. Morin – 3, 5, 7, 2', 4' – pentahydroxy flavone



MATERIALS AND METHODS

Adult male Swiss albino mice weighing 20 – 25g were used in this study. They were maintained at normal room temperature (24 – 30°C) and a 12h : 12h light : dark cycle. They had free access to food and water. The experiments were conducted between 9A.M. to 1P.M. The experimental protocol was approved by the institutional animal ethical committee.

Chemicals

Morin (NIPA Fine Chemicals and organic Intermediates UK) was prepared as a suspension in 1% carboxyl methyl cellulose (Glaxo). Diazepam (Ranbaxy) and pentobarbitone sodium (BDH) were the other chemicals used.

Elevated plus maze

The elevated plus maze (EPM) consists of two open arms, 25 x 25 cm crossed with two closed arms of the same dimension having 25cm high

walls. The arms are connected with a central square 5 x 5 cm giving the apparatus the shape of a plus sign. The maze is kept in a dimly lit room and elevated 25cm above the floor (Pellow & File 1986). Each mouse was placed on the central square facing an open arm and allowed to freely explore the apparatus for five minutes (Fernandez et al. 2009).

The duration of time spent by the mouse in the open arm was carefully recorded using a digital stop watch. The percentage of time spent in open arms was calculated from the total duration of exposure (5 min). Different groups of mice (n=6) were treated with vehicle (0.2ml of 1% carboxy methyl cellulose), diazepam (2mg/kg. s.c) or morin (10, 25 or 50mg/kg. s.c) 30 minutes prior to the experiment. The doses of morin were selected based on a previous study on its antinociceptive effect (Thirugnanasambantham et al. 1985). The apparatus was carefully cleaned after every use to remove any residue or odor.

Staircase test

The staircase test was carried out by the method described by Simiand et al. (1984). The staircase was made of wood and consisted of five identical steps 2.5cm high, 10cm wide, 7.5cm deep surrounded by walls, the height of which (10cm) was constant along the whole length of the staircase. A wooden box (15 x 10 x 10 cm) with one side open was placed facing the staircase. The mouse was gently placed on the floor of the box with its back to the staircase. During a 3min period, the number of steps climbed and the number of rearings made were recorded. A step was considered climbed when all four paws were placed on the step. Different groups of mice were administered with vehicle, diazepam (2mg./kg, s.c.) or morin (10, 25, or 50mg/kg, s.c.) 30min prior to the experiment. The number of steps climbed and the rearing responses were recorded for each mouse. The apparatus was cleaned thoroughly between the recordings.

Open field apparatus

The open field apparatus consists of a large wooden box (96 x 96 x 45 cm) and the floor is divided into 16 equal squares by white lines (Battacharya & Sathyan 1997). The mouse was placed in one corner of the apparatus and the number of squares crossed and rearing responses were counted over a period of five minutes. The apparatus was cleaned after every use. Different groups of animals received the vehicle, diazepam (2mg/kg, s.c.) or morin (10, 25 or 50mg/kg, s.c.) 30 minutes prior to the experiment.

Rotarod test

The effect of morin on muscle coordination was evaluated in mice using a rotarod apparatus (Dunham & Miya 1957). The speed was kept at 12 revolutions per minute and a cut off time of 3minutes was maintained throughout the

experiment. Each animal was placed on this rotating rod and the balancing time was recorded. By a prior screening test mice which had an ability to remain on the rotating rod for more than 3 minutes were selected for the study. Different groups of mice received the vehicle, diazepam (2mg/kg, s.c) or morin (10, 25 or 50mg/kg, s.c) 30 minutes prior to the experiment.

Pentobarbitone sleeping time

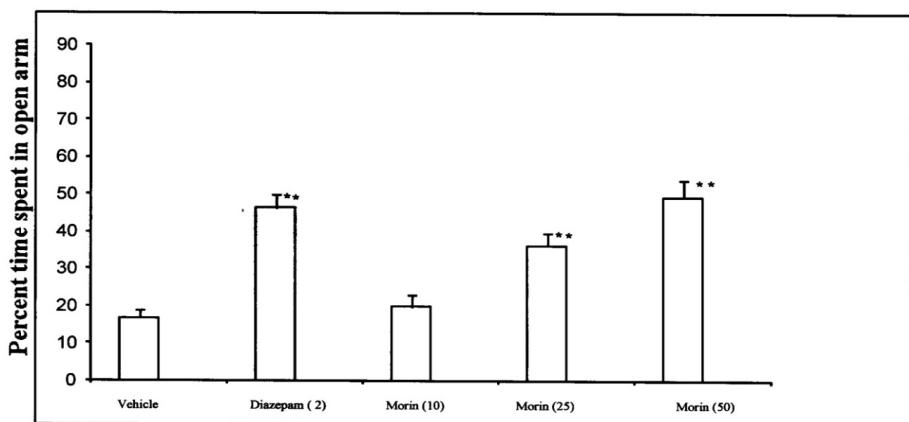
A group of control mice was treated with 1% CMC and other three groups of mice received morin in doses of 10, 25 or 50mg/kg, s.c. After 30 minutes these animals were treated with pentobarbitone sodium (35mg/kg i.p). The time latency for the onset of sleep and duration of sleep were determined by recording the time for the loss and regain of the righting reflex (Anca et al. 1993).

The results were subjected to analysis of variance (ANOVA) followed by Dunnett's 't' test. A p value of < 0.05 was considered statistically significant.

RESULTS

Elevated plus maze test

The vehicle treated control animals spent $16.86 \pm 1.8\%$ time in the open arm of the elevated plus maze. Treatment with diazepam significantly increased the percentage of time spent in open arm to $46.3 \pm 3.55\%$ (fig. 2). A dose dependent increase in the percent time spent in the open arm was observed in morin treated mice. The increase was statistically significant in mice after 25 & 50 mg/kg of morin treatment (36.38 ± 3.07 and $49.45 \pm 4.3\%$) when compared with vehicle treated group. The response noted with 50 mg/kg of morin was comparable to diazepam (2 mg/kg) treatment.

Figure – 2. Effect of morin on elevated plus maze in mice

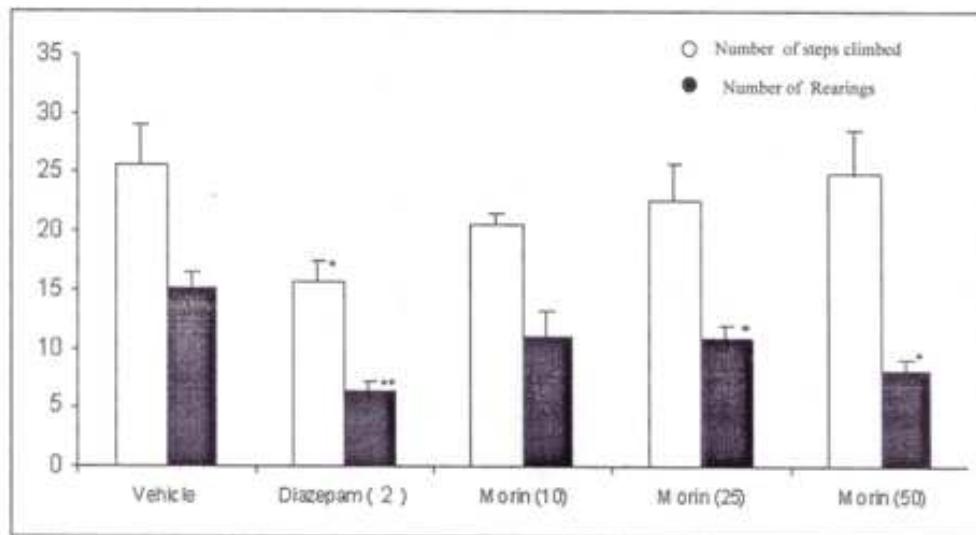
** $P < 0.01$ compared to vehicle treatment.

Each value represents the mean \pm SEM of six observations.

Staircase test

The number of steps climbed by vehicle treated mice was 25.6 ± 3.4 and the rearing responses were 15.2 ± 1.3 (fig.3). Diazepam treatment significantly reduced the number of steps climbed (15.8 ± 1.6) and rearing responses (6.4 ± 0.9) compared to vehicle group. Morin treatment in

different doses did not show any statistically significant change in the number of steps climbed by mice. However, the rearing responses were reduced by morin in all the doses and statistically significant reduction was observed in doses of 25 & 50 mg/kg (10.8 ± 1.1 and 8.2 ± 0.9) compared to vehicle treatment (fig. 3).

Figure- 3. Effect of morin on the responses of mice in a staircase apparatus

* $P < 0.05$ and ** $P < 0.01$ Compared to vehicle treatment

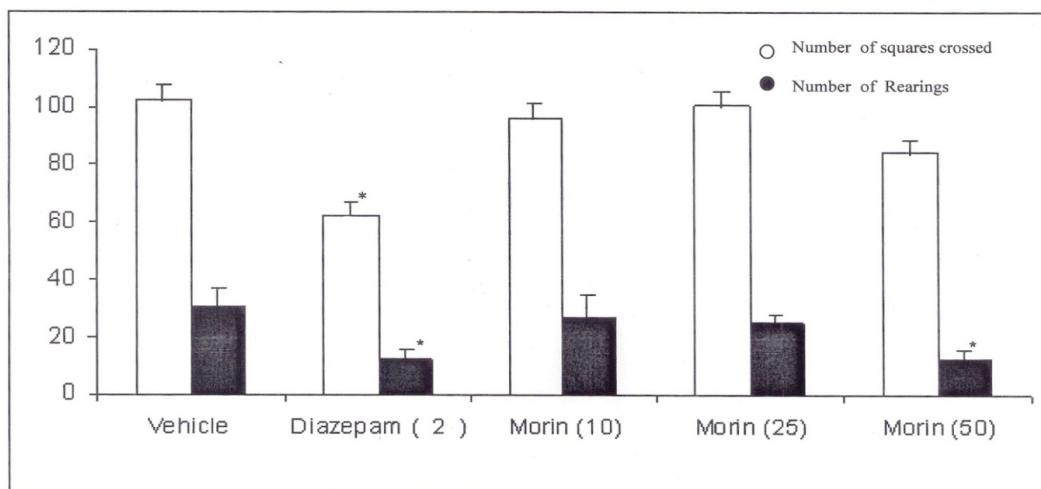
Each value represents the mean \pm SEM of six observations.

Effect on locomotion

In vehicle treated mice the number of squares crossed in 5 min was 102.3 ± 16.6 and the number of rearings was 30.6 ± 6.5 (Fig.4). The mice that received diazepam exhibited a significant decrease in the number of squares crossed (62.2 ± 4.9) and rearing responses (12.8 ± 3.03) in the open field apparatus. Morin in doses of 10 & 25mg/kg did not show any significant change in

the number of squares crossed (96.2 ± 14.7 and 100.6 ± 16.5 or rearing response (26.6 ± 8.5 and 25.3 ± 3.0) compared to vehicle treatment. However, in a dose of 50 mg/kg morin significantly reduced the rearing responses compared to vehicle treatment (12.6 ± 3.02). Though there was a reduction in the number of squares traversed after morin 50 mg/kg treatment (84.1 ± 9.5), it was not statistically significant.

Figure -4. Effect of morin on locomotor activity in mice



* $P < 0.05$ Compared to vehicle treatment

Each value represents the mean \pm SEM of six observations.

Effect on muscle coordination

The balancing time on a rotarod (Table – 1) was significantly reduced in diazepam treated animals (100.2 ± 4.8 sec) compared to vehicle treatment(180 ± 0 sec). In contrast, morin (10, 25

or 50 mg/kg) treated animals were able to balance on the rotarod for the whole period of observation(3min) similar to vehicle treated control animals.

Table – 1. Effect of morin on the balancing time of mice on a rotarod

Treatment(mg/kg, s.c.)	Balancing time (sec.)
Vehicle	180 ± 0
Diazepam (2)	$100.2 \pm 4.8^{**}$
Morin (10)	180 ± 0
Morin (25)	180 ± 0
Morin (50)	180 ± 0

** $P < 0.01$ vs vehicle treated group.

Each value represents the mean \pm SEM of six observations.

Effect on pentobarbitone sleep time

In vehicle treated control animals, the latency time for the onset of sleep after pentobarbitone treatment was 3.1 ± 0.8 min and the duration of sleep was 25.0 ± 1.64 min (Table-2). The

administration of morin in different doses (10, 25 or 50 mg/kg) did not significantly alter the sleep latency or the duration of sleep compared to vehicle treated mice (Table -2).

Table – 2. Effect of morin on pentobarbitone induced sleep time in mice

Treatment (mg/kg, s.c.)	Lateney to sleep (min)	Duration of sleep (min)
Vehicle	3.1 ± 0.8	25 ± 1.64
Morin (10)	2.9 ± 1.2	23.5 ± 1.92
Morin (25)	4.4 ± 1.8	29.8 ± 3.66
Morin (50)	3.6 ± 0.7	22.17 ± 2.47

Each value represents mean \pm SEM of six observations

DISCUSSION

The recent literature is abundant with many prominent central nervous system effects of flavanoid compounds. The anti nociceptive activity of gossypin (Viswanathan et al. 1984), many mono and di-substituted flavones (Thirugnanasambantham et al. 1990,1993) and many dihydroxy flavones derivatives (Girija et al. 2002; Uma Maheswari et al. 2006; Vidhyalakshmi et al. 2010) has been documented. Morin belongs to the family of 2' – hydroxy flavonols which are rare in nature and present in *Morus tinctoria* and few other species (Harborne 1967). An earlier study has revealed a significant antinociceptive action of morin in mice (Thirugnanasamantham et al 1985).

Many studies indicate flavones as ligands for the GABA_A benzodiazepine binding site. Both naturally occurring and synthetic flavones have been shown to bind to this site with high affinity and to exert anxiolytic – like effects in rodents (Marder and Paladini 2002; Wang et al. 2005). In particular, many hydroxy substituted flavones have been shown to exhibit high binding affinity to benzodiazepine receptors (Medina et al 1990 and Paladini et al. 1999). Some of these compounds also exhibited anxiolytic effect in animals with relatively minor sedative or myorelaxant effect. Hence, in the present study, morin, a pentahydroxy flavone has been investigated in detail for the above properties.

The effect of morin was tested on the widely used animal models of anxiety viz; elevated plus maze and staircase test. These tests

are based on unconditioned behavior relying on natural behavioral reactions and do not require specific training of the animals (Bhattacharya and Satyan 1997). The elevated plus maze test has been regarded as an 'approach-avoidance' model because it reveals the conflicting tendencies of a rodent to naturally explore novel environment (approach) versus their innate aversion for potentially dangerous open spaces (avoidance) (Crayan and Holmes 2005). Naturally, rodents spend the majority of the test session in the closed arms of the maze. But anxiolytic drugs increase exploration to the open arms. Such an effect has been revealed for morin in the present study. The percent time spent in open arm by morin (50 mg/kg) treated mice was comparable to that of a standard anxiolytic drug diazepam (fig.2).

Similarly, the staircase test is considered a simple, rapid and sensitive test and clinically active anxiolytics reduced the rearings at doses which did not reduce the number of steps climbed (Bhattacharya and Satyan 1997). Morin treatment significantly reduced the number of rearings in mice compared to vehicle treatment without much change in the number of steps climbed (Fig.3). Diazepam treatment also resulted in a significant reduction in the number of rearing responses. The reduction in the number of steps climbed in diazepam treated mice could be attributed to its sedative and myorelaxant effects.

The results of the above two experiments clearly indicate the anxiolytic property of morin. Even though benzodiazepines are the major class of drugs used in anxiety disorders, their associated sedative and myorelaxant properties necessitate

the search for better drugs. In the present study also diazepam treatment revealed prominent sedative and muscle relaxant effects as indicated by the results of open field test and rotarod test (Fig. 4, Table.1). In diazepam treated animals, a significant decrease in the number of squares crossed and rearings was observed in open field apparatus and the balancing time on a rotarod was also significantly reduced.

However, morin in the doses studied did not alter the locomotor activity compared to control animals in the open field test. Additionally, morin treatment did not alter the sleep latency or duration of sleep in mice after pentobarbitone administration (Table 2). The above observations indicate that morin is devoid of central nervous system depressant effect in general and sedative property in particular.

Another significant observation of the present steady is that morin did not alter the balancing time of mice on a rotarod (Tab -1). This indicates that morin treatment neither affected the muscle co-ordination nor it produced any myorelaxant effect in mice.

Anxiolytic effect without central nervous system depression and myorelaxation will be an advantage since it may not impair the motor activity of the individual. Morin appears to have a favorable effect in this regard.

Possible mechanism of action

The classical anxiolytic benzodiazepines are well known to interact with GABA_A receptors in an allosteric fashion and modulate the effects of GABA (Johnston 2005). Interestingly flavones and many of its derivatives have been identified as high affinity ligands for benzodiazepine receptors (Fernandez et al. 2006). Chrysin and apigenin were initially reported in this series and many semi-synthetic derivatives of flavones containing halogen or nitro groups in its molecule have been also identified as ligands for benzodiazepine receptors (Medina et al. 1990). Most of the compounds exhibiting high affinity to benzodiazepine receptors also produced sedation and myorelaxation except 6, 3' dinitroflavone.

The report of Paladini et al.(1999) also includes morin which has been found to have only a very low affinity (100 times lesser than flavone) for benzodiazepine receptors. Probably because of its low affinity to benzodiazepine receptors this compound has not been screened by the earlier workers for anxiolytic property. The results of the present study clearly indicate the anxiolytic property of morin in two well established animal models of anxiety. Despite the report Paladini et al. (1999) indicating low affinity of morin for benzodiazepine receptor binding, it exhibited significant anxiolytic effect. Additionally the absence of sedation and myorelaxation observed in the present study indicates the likely hood of poor interaction of morin with benzodiazepine receptors.

While describing the possible mechanism of anxiolytic effect observed for a few flavonoid glycosides, Fernandez et al. (2009) suggested that a variety of receptor systems may be responsible for the behavioural responses recorded in their study. Many flavone derivatives were found to be positive modulators of the gamma aminobutyric acid type A (GABA_A) receptors in the central nervous system (Marder and Paladini 2002). However, the CNS depressant action of certain flavonoid glycosides like 2S – hesperidin did not involve classical GABA_A receptor at least not directly (Fernandez et al. 2006). Modulation of potassium channels, nicotinic cholinergic system and inhibition of calcium influx etc. have been suggested as possible relevant targets of flavones to induce CNS depressant effect (Fernandez et al. 2009). The methodology adopted in the present study has not analysed these possibilities and hence the present results do not offer any clear indication of the possible mode of anxiolytic action of morin. At the same time it may be suggested that a strong interaction of morin with benzodiazepine receptors is unlikely (Paladini et al. 1999). Thus the exact mechanism of anxiolytic effect of morin is a subject of future investigation.

In conclusion the present study reveals a selective anxiolytic effect of morin without sedative or myorelaxant properties.

REFERENCES

1. Anca JM, Lamela M, Gato MA, Cadavid I and Callega JM. Activity on CNS of *Himanthalia elongata*; Part II *Planta Medica*, 1993; 59(3): 101 - 105.
2. Bhattacharya SK and Satyan KS. Experimental methods for evaluation of psychotropic agents in rodents: I-Anti-anxiety agents. Indian J. Exp. Biol, 1997; 35: 565-575.
3. Cryan JF, Holmes A The ascent of mouse: advances in modelling human depression and anxiety. Nat Rev Drug Discov, 2005; 4:775-790.
4. Dunham NM and Miya TS. A note on simple apparatus for detecting neurological deficit in rat and mice. J. Am. Pharm. Ass, 1957; 46: 208- 209 .
5. Fernandez SP, Wasowski C, Loscalzo LM et al. Central nervous system depressant action of flavonoid glycosides. Eur J Pharmacol, 2006;539:168-176.
6. Fernandez SP, Michael Nguyen, Yo TT, Chu C, Johnston G A R, Hanrahan J R. and Mary Chebib. The Flavonoid Glycosides, Myricitrin, Gossypin and Naringin Exert Anxiolytic Action in Mice. Neurochem Res, 2009; 34:1867-1875.
7. Girija K, Kannappa Reddy M and Viswanathan S. Antinociceptive effect of synthesised dihydroxy flavones, possible mechanism. Indian J Exptl. Biol, 2002;20:4013-15.
8. Harborne JB. In Comparative Biochemistry of the Flavonoids, Academic Press, New York, 1967.
9. Havsteen BH. In The biochemistry and medical significance of the flavonoids. Pharmacol.Ther, 2002; 96: 67 -202.
10. Johnston GAR . GABA_A receptor channel pharmacology. Curr. Pharm. Res, 2005; 11: 1867-1885.
11. Marder M and Paladini AC, GABA-A-receptor ligands of flavonoid structure, Current Topics in Medicinal Chemistry, vol. 2(8), 2002; pp. 853-867.
12. Medina JH, Paladini AC, Wolfman C, Levi de Stein M, Calvo D, Diaz L and PenaC. Chrysin (5, 7 dihydroxyflavone), a naturally occurring ligand for Benzodiazepine receptors, with anti-convulsant properties. Biochem Pharmacol, 1990; 40 : 2227 - 2232.
13. Medina JH, Viola H, Wolfman C, Marder M, Wasowski C, Calvo D and Paladini AC. Overview – Flavonoids a new family of benzodiazepine receptor ligands. Neurochem, Res. 1997; 22:419-25.
14. O' Donnell J M and Shelton R C. Drug therapy of Depression and Anxiety Disorders. In Goodman & Gillman's. The Pharmacological Basis of Therapeutics, 12th edn. Brunton LL, Chabner BA and Knollmann BC McGraw - Hill Pub. 2011: 399 - 415.
15. Paladini AC, Marder M, Viola H, Wolfman C, Wasowski C and Medina JH. Flavonoids and the CNS : from forgotten factors to potent anxiolytic compounds. J. Pharm Pharmacol, 1999; 51(5) : 519 - 26.
16. Pellow S & File SE. Pharmacol Biochem Behav, 1986; 24 : 524-529.
17. Reus V I. Mental disorders : In Harrison's Principles of Internal Medicine, 17th edition. Fauci AS, Braunwald E, Kasper D L, Hauser SL, Longo D L, Jameson J L and Loscalzo J. McGraw – Hill, 2008; Vol.2: p.2710 - 2723.
18. Simiand J, Keane PE, More M. The staircase test in the mice: a simple and efficient procedure for primary screening of anxiolytic agents. Psychopharmacology, 1984; 84: 48 – 53.
19. Thirugnanasambantham P, Viswanathan S, Reddy MK, Santa Ramachadran and Kameswaran L. Analgesic activity of certain bioflavonoids. Indian J. Pharm. Sci, 1985; 47(6): 230 - 231.
20. Thirugnanasambantham P, Viswanathan S, Krishnamoorthy V, Mythirayee C Ramachandran S and Kameswaran L. Analgesic activity of certain flavone derivatives : A structure - activity study. J. Ethnopharmacol, 1990; 28 : 207 - 214.
21. Thirugnanasambantham P, Viswanathan S, Ramaswamy S, Krishnamoorthy V. Mythirayee C and Kameswaran L. Analgesic activity of certain flavone derivatives-a structure activity study. Cli. Exp. Pharmacol. Phys, 1993; 20(1): 59 - 63.

22. Umamaheswari S, Viswanathan S, Sathiyasekaran BWC, Parvathavarthini S and Ramaswamy S. Antinociceptive activity of certain dihydroxy flavones. *Indian J Pharm Sciences*, 2006; Nov-Dec:749-753.

23. Vidyalakshmi K, Kamalakannan P, Viswanathan S and Ramaswamy S. Antinociceptive effect of certain dihydroxy flavones in mice. *Pharmacology Biochemistry and Behaviour*, 2010; 96:1-6.

24. Viswanathan S, Thirugnanasambantham P, Reddy MK and Lalitha K. Gossypin induced analgesia in mice. *European J Pharmacology*, 1984; 98:289-291.

25. Wang F, Shing M, Huen Y, Tsang SY, and Xue H. Neuroactive flavonoids interacting with GABAA receptor complex. *Curr. Drug Targets CNS Neurol. Disord.*, 2005; 4(5): 575-585.