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Sedative–hypnotic like effect of 5–methoxyflavone in mice and investigation on possible mechanisms by *in vivo* and *in silico* methods

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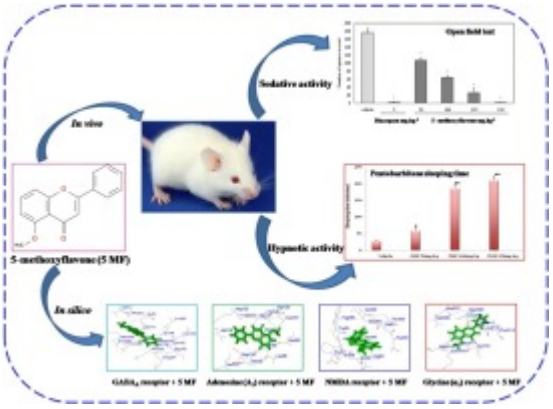
Highlights

- A dose-dependent reduction in spontaneous locomotor activity was observed.
- Duration of pentobarbitone sleeping time and ether anaesthesia were prolonged.
- $GABA_A$, glycine, adenosine and NMDA receptors might play a role in the above action.
- In silico studies supplemented the *in vivo* findings at the above receptor sites.

Abstract

Flavonoids have been shown to possess central nervous system (CNS) depressant effect mediated through the ionotropic $GABA_A$ receptors. In the present study, 5-methoxyflavone was evaluated for sedative-hypnotic like activity in mice and the mechanisms involved by employing a battery of tests including molecular docking studies. In the open field test, 5–methoxyflavone in various doses (50, 100 and 150 mg/kg, i.p) exhibited a significant and dose–dependent reduction in the spontaneous locomotor activity ($F(530) = 87.17$ $P < 0.001$). Pretreatment with 5–methoxyflavone decreased the latency to sleep induction after pentobarbitone or ether administration and also significantly increased the duration of sleep ($p < 0.001$). A significant and dose-dependent myorelaxant effect was observed with 5–methoxyflavone in the inclined plane, horizontal wire test and rota rod test. Pretreatment with picrotoxin, bicuculline, glycine, caffeine or NMDA either decreased or completely abolished the hypnotic effect of 5-methoxyflavone in mice. The above results revealed the involvement of $GABA_A$, adenosine, glycine and NMDA receptors in the hypnotic effect of 5-methoxyflavone. The results of *in silico* studies indicated that, 5–methoxyflavone exhibits good binding affinity towards these receptors by H-bond interactions. In conclusion, the present study identified a novel and potential sedative-hypnotic like effect of 5–methoxyflavone involving multiple mechanisms.

Graphical abstract



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Keywords

Adenosine; Central nervous system; Docking; Gamma-amino butyric acid A; Sedative-hypnotic; 5-methoxyflavone

1. Introduction

Flavonoids are an important constituent of our regular diet and offer many health benefits due to their remarkable antioxidant potential, interaction with many enzymes and modulation of cell signalling pathways [1]. A few reports are also available pertaining to the effect of flavonoids on CNS functions. Flavonoids like gossypin [2] morin and rutin [3] and synthetic hydroxy derivatives [4,5] and methoxy derivatives of flavone [6,7] have been found to exert significant anti-nociceptive effect in various animal models of pain. Among the compounds studied, 5–methoxyflavone was reported to possess a very low ED_{50} for anti–nociceptive action [8] and found to exert a sedative effect in mice but this was not investigated in detail. Many other evidences also are available to indicate the central nervous system (CNS) depressant effect of flavone compounds in experimental animals. Flavonoid glycosides such as hesperidin, naringin, diosmin and gossypin exhibited a CNS depressant effect in mice [9]. Anxiolytic effect was also demonstrated for a few flavonoids such as myricitrin, gossypin and naringin [10]. As a corroborative evidence, many flavonoids have been shown to act as ligands of the $GABA_A$ receptors in the CNS [11,12].

The above reports reveal yet another interesting facet of the therapeutic utility of flavonoid compounds. Central nervous system depressant drugs have wide applications such as sedative, hypnotic, anticonvulsant and general anaesthetic. However, only very few studies have been carried out on flavonoids to explore this potential. Hence, based on a previous report [8] it was considered interesting to investigate in detail the CNS depressant activity of 5–methoxyflavone in various behavioural parameters in mice.

2. Materials and methods

2.1. Drugs and chemicals

5–methoxyflavone (Fig. 1), strychnine and picrotoxin were purchased from Research organics, Chennai, India. Diethyl ether (TKM Pharma, Hyderabad, India), diazepam (Hindustan Pharmaceuticals, India), pentobarbitone sodium (BDH, Mumbai, India), bicuculline methiodide (Sigma-Aldrich, St Louis, MO, USA), caffeine (Merck Specialities Pvt Ltd., Mumbai, India), N-Methyl-DL-aspartic acid (Sigma-Aldrich, St Louis, MO, USA) were the other drugs used in the study. Diazepam was used as a standard drug for comparison in behavioural experiments. 5–methoxyflavone was prepared as a fine suspension in 0.5% carboxy methylcellulose and injected i.p 30 min before any procedure. All drugs were administered by i.p injection in a volume of 10 ml/kg bodyweight.

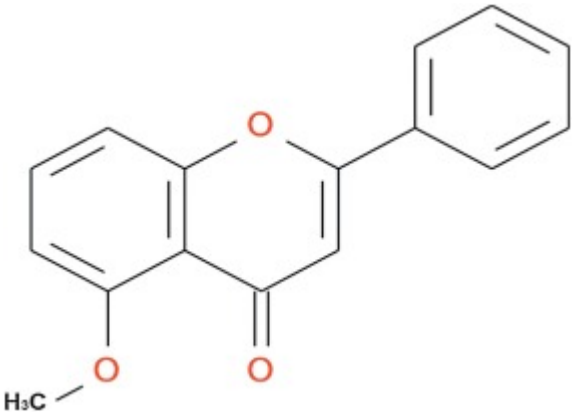

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Fig. 1. 5-methoxyflavone.

2.2. Animals

Adult Swiss albino mice of either sex weighing 20–25 g were used for the experiments. The animals were housed in groups of six in polypropylene cages in a

controlled environment (21–24 °C), with free access to food and water and maintained on 12 h/12 h day/night cycle (lights on at 6 a.m). The behavioural experiments were performed between 0900 and 1400 h to avoid circadian variations and to maintain uniformity. Each test group consisted of a minimum of six mice and were randomly selected. The experimental protocol was approved by the institutional animal ethics committee. Proper care of the animals during the experimental procedures was taken in accordance with the directions of committee for the purpose of control and supervision of experiments on animals (CPCSEA), India.

2.3. Preliminary screening

Different groups of mice were treated with 5–methoxyflavone in doses of 25, 50, 100 or 200 mg/kg, i.p. The above doses were selected based on the anti-nociceptive activity of the compound reported earlier [6]. The animals were placed in a perspex observation chamber and monitored closely for their behavioural parameters like spontaneous activity, aggressiveness, tremors, convulsion, righting reflex, ataxia, catalepsy, Straub's tail and autonomic changes like salivation, urination, defecation, pinna and corneal reflexes for a period of 2 h.

2.4. Acute toxicity studies

Since the test compound in a dose of 200 mg/kg showed a potent CNS depressant activity during the preliminary screening, graded doses were used for the toxicity study. Mice of either sex were randomly allocated to four groups and received 5–methoxyflavone i.p in doses of 400, 500, 600 or 700 mg/kg. Animals were observed closely for abnormal behaviour and mortality during the first 6 h and followed up to 14 days for any further mortality.

2.5. Open field test

The apparatus consists of a wooden box of dimensions 96 × 96 X 30 cm and the arena is divided in to 16 squares of which 4 are central squares and 12 are peripheral squares. The animal was placed in one corner of the wooden box and the following parameters; (i) number of squares crossed (ii) number of rearings and (iii) number of groomings were observed for 5 min [13]. After each observation, the floor of the arena was cleaned with damp cloth and spirit before introducing another animal. 5–methoxyflavone was administered i.p in doses of 50, 100, 125 or 150 mg/kg to different groups of mice 30 min prior to the procedure.

2.6. Pentobarbitone sleeping time

Different groups of mice received 5–methoxyflavone in doses of 50, 100 or 150 mg/kg, i.p 30 min prior to the administration of pentobarbitone (50 mg/kg, i.p). The sleep latency (time between pentobarbitone administration and loss of righting reflex) and duration of sleep (from loss of righting reflex to recovery of the reflex) were recorded in each animal [14]. The control animals were treated with pentobarbitone alone.

2.7. Potentiation of ether anaesthesia

Three ml of ether was measured and poured over 1 g of cotton wool placed in a plastic container (1 L) with lid. The test compound was administered to mice i.p in three different doses (50, 100 &150 mg/kg) 30 min prior to the exposure of ether. The latency for the onset of loss of righting reflex and the total anaesthetic time (the time between the loss and regain of righting reflex) were determined.

2.8. Horizontal wire test

The apparatus consists of a 15 cm long horizontally strung steel wire (1 mm diameter) suspended at a height of 25 cm above the table [15]. Initially the mice were subjected to two training sessions at an interval of 5 min by holding the tail and allowed to grasp the wire with their forepaws and then released. The normal animal will be able to grasp the wire with its hind paws within 5 sec. 5–methoxyflavone in doses of 50, 100 or 150 mg/kg was administered i.p to 10 animals selected in each group and the test was repeated 30 min later. Mice that were unable to grasp the wire with at least one hind paw within 5 s were considered to have failed the test. Diazepam (5 mg/kg, i.p) was used as a standard drug for comparison and administered 30 min prior to the test.

2.9. Rotarod test

The test is used to evaluate the activity of drugs interfering with motor coordination. The apparatus consists of a horizontal metal rod (3 cm in diameter) with a textured surface, attached to a motor with the speed adjusted to 10 rpm [16]. The rod is placed 50 cm above the table. Mice were subjected to a training session and tested for their ability to remain on the revolving rod for 60 sec. 5–methoxyflavone in doses of 50, 100 or 150 mg/kg was administered i.p to 6 animals in each group and the test was repeated 30 min later. The time taken by the animals to fall down from the revolving rod was noted. Diazepam (5 mg/kg, i.p) administered 30 min prior to the test was used as a standard drug for comparison.

2.10. Inclined plane

The apparatus consists of two rectangular plywood boards with one board as the base

and the other as the movable inclined plane connected at one end by a hinge [17]. The degrees of slope are marked on the base plywood and the movable inclined plane is set at 65°. Mice are placed on the upper part of the inclined plane and are given 30 s to hang on or to fall off. Animals that are able to sustain on the inclined plane for more than 30 s are selected for the test. Three different groups of mice (n = 10) received 5–methoxyflavone in doses of 50, 100 or 150 mg/kg, i.p and again placed on the inclined plane after 30 min. The animals that are not able to sustain for more than 5 s are considered to have failed the test. Diazepam 5 mg/kg administered 30 min prior to the test was used as a standard drug for comparison.

2.11. Evaluation of possible mechanisms involved in the hypnotic activity of 5–methoxyflavone

A dose of 5–methoxyflavone (200 mg/kg, i.p) that demonstrated effective hypnotic activity in 100% of the animals during preliminary screening was selected for this purpose. To ascertain the possible mechanisms involved in the hypnotic activity of 5–methoxyflavone, mice were pretreated with various interacting chemicals i.p 15 min prior to the administration of the test compound. The dose of each interacting chemical used was selected based on prior literature or after some pilot experiments. The latency time to loss of righting reflex and the duration of sleep after 5–methoxyflavone treatment were noted in these animals. The following mechanisms were investigated; GABAergic system (bicuculline methiodide 50 mg/kg, i.p and picrotoxin 2 mg/kg, i.p), Adenosinergic system (caffeine 50 mg/kg, i.p), Glutamatergic system (N-Methyl-DL-aspartic acid 100 mg/kg, i.p) and Glycinergic system (strychnine 1 mg/kg, i.p).

2.12. Molecular docking studies

In silico docking of 5–methoxyflavone with different receptors were carried out and compared with known ligands. Based on the literature study, the gene coded amino acid sequence of various receptor proteins were retrieved in FASTA format using databases NCBI-Gene database, Ensemble [18] and UNIPROT (https://www.ncbi.nlm.nih.gov/ ↗) proteomics database [$GABA_A$ α_1 subunit (P14867), adenosine A_1 (P30542), adenosine A_{2a} (P29274), adenosine A_3 (P0DMS8), NMDA 2 A subunit (Q12879) and Glycine α_1 subunit (P23415)] in order to perform three dimensional structure prediction of protein. The various gene coded amino acid sequences were converted into 3-D structure using automated protein modeling server CPH3.0 model server http://www.cbs.dtu.dk/services/CPHmodels/ ↗ [19,20]. The modeled protein structures were viewed in 3-D form using Accelrys Discovery Studio software (2.5.5 v). The two dimensional structure of the drugs were taken from

NCBI Pubchem compound database and converted into three dimensional structure using ONLINE SMILES TRANSLATOR. The modeled protein receptor and drug molecule were docked using automated PATCHDOCK server (https://bioinfo3d.cs.tau.ac.il/PatchDock/ ↗) [21,22].

2.13. Statistical analysis

Data are expressed as mean ± S.E.M. One-way ANOVA followed by *post hoc* Dunnett's multiple comparison test was used to compare the data between vehicle and drug treated groups. One-way ANOVA followed by *post hoc* Kruskal-Wallis test was used in open field test and rota-rod experiments. Chi-square test was used to analyze the data from horizontal wire test and inclined plane. A p value < 0.05 was considered statistically significant (SPSS v.16).

3. Results

3.1. Preliminary screening

Mice treated with 5–methoxyflavone in doses of 25 and 50 mg/kg did not show any change in the behavioural or autonomic responses when compared with control group. A decrease in the spontaneous locomotor activity was observed in mice that received 100 mg/kg but without any change in other behavioural or autonomic responses. Mice treated with 5–methoxyflavone in a dose of 200 mg/kg exhibited deep sedation with loss of righting reflex and decrease in muscle tone. The autonomic activities like defaecation, micturition, pinna reflex were absent during deep sedation but the corneal reflex was still intact. No mortality was observed in all the groups studied.

3.2. Acute toxicity studies

When 5–methoxyflavone was administered to mice in a dose of 400 mg/kg, i.p, there was no immediate or delayed mortality during the observation period of 24 h and all the animals survived up to 14 days. The percentage of mortality observed with 500, 600 and 700 mg/kg, were 50%, 66.6% and 100% respectively. The LD_{50} of 5–methoxyflavone was calculated by the graphical method [23] and found to be 540 mg/kg (i.p) in mice.

3.3. Pentobarbitone sleeping time

In vehicle treated animals, the latency for loss of righting reflex after pentobarbitone administration was 4.11 ± 0.29 min and the duration of sleep was 27.96 ± 2.58 min (

Table 1). Treatment with 5–methoxyflavone in doses of 100 and 150 mg/kg significantly decreased the latency time to loss of righting reflex [$F(320) = 14.01$, $P < 0.05$ and $P < 0.01$ respectively] when compared to vehicle treated group. There was a significant and dose-dependent increase in the duration of sleep observed after treatment with 5–methoxyflavone when compared to vehicle treatment [$F(320) = 105.45$, $P < 0.05$ (50 mg/kg) and $P < 0.001$ (100 and 150 mg/kg)]. Thus, 5–methoxyflavone significantly decreased the latency for sleep induction and also increased the duration of sleep induced by pentobarbitone in a dose dependent manner.

Table 1. Effect of 5-methoxyflavone (5-MF) on pentobarbitone induced[a] sleep in mice.

Treatment (mg/kg, i.p)	Time for loss of Righting reflex (min)	Duration of sleep (min)
Vehicle	4.11 ± 0.29	27.96 ± 2.58
5-MF – 50	4.89 ± 0.62	58.70 ± 6.05*
5-MF – 100	2.77 ± 0.27*	185.56 ± 11.15***
5-MF – 150	1.75 ± 0.14**	209.92 ± 11.99***

a Pentobarbitone (50 mg/kg, i.p) was administered 30 min after treatment with vehicle / 5-methoxyflavone. Each value represents mean ± S.E.M. (n = 6).

* $p < 0.05$.

** $p < 0.01$ and.

*** $p < 0.001$ compared to vehicle treatment, one-way ANOVA followed by Dunnett's *post hoc* multiple comparison test.

3.4. Potentiation of ether anaesthesia

In vehicle treated mice, the latency for loss of righting reflex upon exposure to ether was 75.83 ± 1.87 s and the duration of anaesthesia was 45.00 ± 2.03 s (Table 2). A significant and dose dependant reduction in the latency time to loss of righting reflex [$F(3,20) = 194.98$, $P < 0.001$] was observed after ether exposure in animals treated with various doses of 5–methoxyflavone. A significant and dose dependent increase in

the duration of ether anaesthesia [F(3,20) = 85.76, $P < 0.001$] was also observed in these animals compared to vehicle treatment. Thus, 5–methoxyflavone significantly decreased the time for onset of ether anaesthesia and also prolonged the duration of anaesthesia.

Table 2. Effect of 5-methoxyflavone (5-MF) on ether[a] anaesthesia in mice.

Treatment (mg/kg, i.p)	Time for loss of Righting reflex (sec)	Duration of sleep (sec)
Vehicle	75.83 ± 1.87	45.00 ± 2.03
5-MF – 50	57.00 ± 1.51*	64.17 ± 2.14*
5-MF – 100	45.33 ± 1.54*	202.17 ± 14.18*
5-MF – 150	26.17 ± 0.87*	1217.00 ± 120.29*

a Each mouse was exposed to ether (3 ml) 30 min after treatment with 5–methoxyflavone / vehicle. Each value represents mean ± S.E.M. (n = 6).

* $p < 0.001$ compared to vehicle treatment, one-way ANOVA followed by Dunnett's *post hoc* multiple comparison test.

3.5. Open field test

A significant and dose-dependent decrease was observed in the number of squares crossed by mice treated with different doses of 5–methoxyflavone compared to vehicle treatment [F (5,30) = 87.17 $P < 0.001$] indicating a dose-dependent sedative effect (Fig. 2). The decrease in locomotor activity observed with 5–methoxyflavone in a dose of 150 mg/kg (1.17 ± 0.98) was comparable with the standard drug diazepam (1.67 ± 1.09). A significant reduction in the rearing response was observed in doses of 50 and 100 mg/kg of 5–methoxyflavone treated animals. A complete abolition of rearing response observed in doses of 125 and 150 mg/kg ($P < 0.001$) was similar to diazepam treated animals (data not shown). Mice treated with 5–methoxyflavone in doses of 100, 125 and 150 mg/kg ($P < 0.001$) completely abolished the grooming response similar to diazepam treated animals (data not shown).

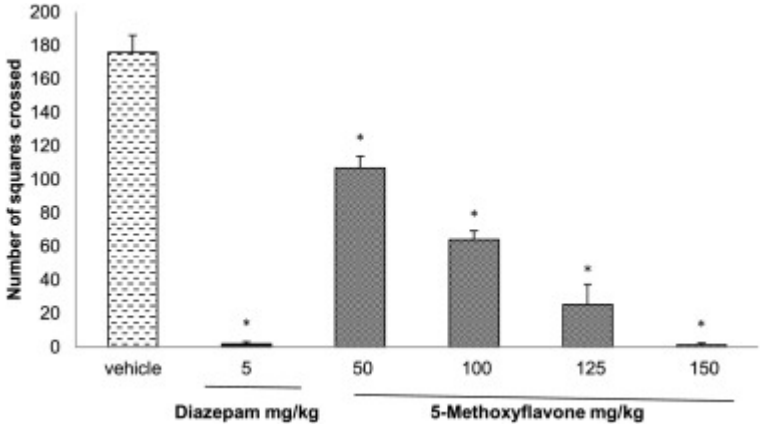

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Fig. 2. Effect of 5-Methoxyflavone (50, 100, 125 & 150 mg/kg) and diazepam (5 mg/kg) on locomotor activity of mice in open field test. Each column represents mean ± S.E.M. (n = 6). * $p < 0.001$ compared to vehicle treatment, one-way ANOVA followed by Kruskal–Wallis test.

3.6. Rotarod test

The mean values of balancing time of mice observed in rota-rod test are shown in Fig. 3. One-way ANOVA showed a significant decrease in the mean balancing time of mice treated with 5–methoxyflavone in doses of 100 and 150 mg/kg [$F(4,25) = 130.37$ $P < 0.001$] when compared with vehicle treated group. 5–methoxyflavone in a dose of 150 mg/kg showed greater reduction in the balancing time when compared to diazepam treated animals.

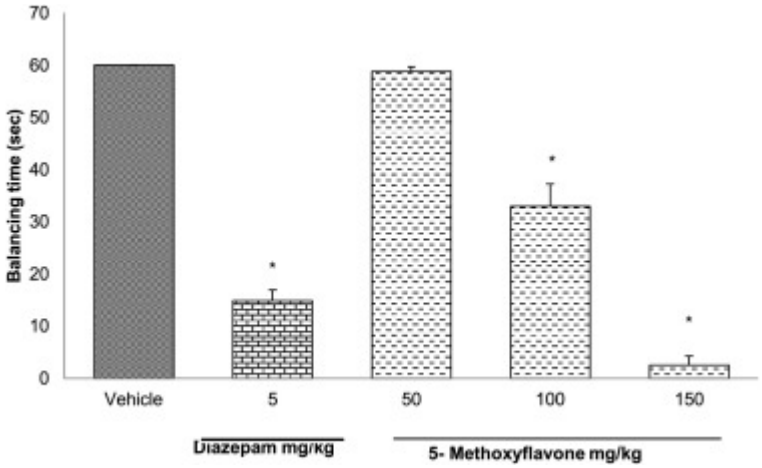

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Fig. 3. Effect of 5-Methoxyflavone (50, 100 & 150 mg/kg) and diazepam (5 mg/kg) on balancing time of mice on a rota rod apparatus. Each column represents mean ± S.E.M.

(n = 6). * p < 0.001 compared to vehicle treatment, one-way ANOVA followed by Kruskal-Wallis test.

3.7. Horizontal wire test

The results of the horizontal wire test are expressed as the percentage of mice able to grasp the wire within 5 s. In vehicle treated control mice, 100% of the animals were able to grasp the wire within 5 s (Table 3). Diazepam (5 mg/kg, i.p) treatment elicited a significant decrease ($P < 0.001$) in the percentage of mice that were able to grasp the wire. 5–methoxyflavone in a dose of 50 mg/kg did not significantly decrease the wire grasping response, where as in a dose of 100 and 150 mg/kg, it significantly reduced ($P < 0.001$, Chi-Square test) the percentage of mice grasping the wire and demonstrated a significant myorelaxant effect.

Table 3. Effect of 5–Methoxyflavone (5-MF) on mice placed on a horizontal wire ∞ and inclined plane ≠.

Treatment (mg/kg, i.p)	% of mice grasping wire	% of mice holding on to inclined plane
Vehicle	100	100
Diazepam – 5	10*	0*
5-MF – 50	90	100
5-MF – 100	50*	80*
5-MF – 150	0*	30*

Chi-square test (inclined plane) value = 24.55 (n = 10). ≠ Percentage of mice that could hold on to the inclined plane for more than 5 s was noted in different treatment groups.

∞Percentage of mice that could grasp the wire with their hind paws within 5 s was noted in different treatment groups.

*

p < 0.001 compared to vehicle treatment. Chi-square test (horizontal wire test) value = 20.01 (n = 10).

3.8. Inclined plane

Diazepam treatment (5 mg/kg) significantly decreased ($P < 0.001$) the percentage of mice that were able to hold on to the inclined plane for more than 5 s (Table 3).

Similarly, a significant reduction ($P < 0.001$, Chi-Square test) was observed in the percentage of mice that were able to hold on to the inclined plane after treatment with 100 and 150 mg/kg of 5–methoxyflavone and thus indicating a significant myorelaxant effect.

3.9. Effect of various interacting chemicals on 5–methoxyflavone induced hypnosis in mice

The time for onset of loss of righting reflex after administration of 5–methoxyflavone (200 mg/kg, i.p) was 7.83 ± 0.30 min. NMDA (100 mg/kg, i.p) pretreatment delayed the onset time to 17.83 ± 0.87 min. Picrotoxin (2 mg/kg, i.p) pretreatment completely abolished the onset of loss of righting reflex and ultimately the hypnotic effect of 5–methoxyflavone. The onset time for loss of righting reflex was not significantly altered by bicuculline methiodide, caffeine or strychnine pretreatment (data not shown).

The duration of sleep observed in mice after i.p administration of 5–methoxyflavone (200 mg/kg, i.p) was 142.00 ± 2.55 min. Picrotoxin pretreatment completely abolished the sleeping time in mice (Fig. 4). Pretreatment with bicuculline methiodide, caffeine, NMDA or strychnine significantly decreased the duration of sleep induced by 5–methoxyflavone.

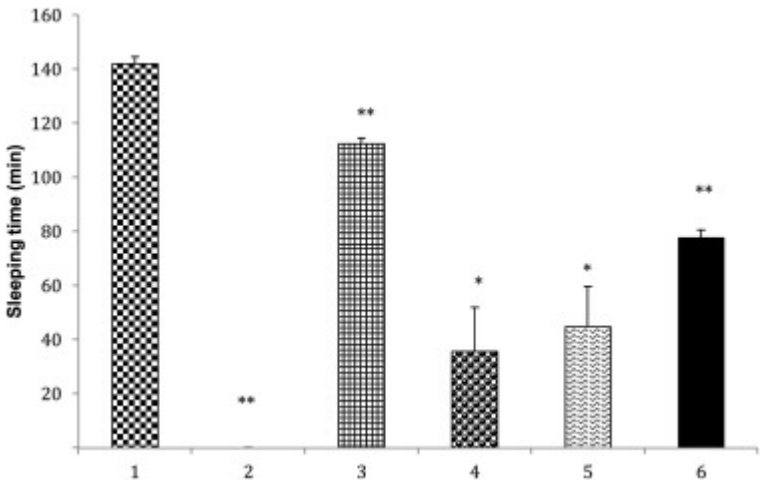

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Fig. 4. Effect of various interacting chemicals on 5-methoxyflavone induced hypnosis in mice. **1.** 5 M F (200 mg/kg); **2**. Picrotoxin (2 mg/kg) + 5 M F (200 mg/kg); **3**. Strychnine (1 mg/kg) + 5 M F (200 mg/kg); **4**. Bicuculline methiodide (50 mg/kg) + 5 M F (200 mg/kg); **5**. Caffeine (50 mg/kg) + 5 M F (200 mg/kg); **6**. NMDA (100 mg/kg) + 5 M F (200 mg/kg). Interacting chemicals were administered i.p 15 min before treatment with 5-methoxyflavone. Each column represents mean ± S.E.M. (n = 6). * $p < 0.05$ and ** $p < 0.001$ compared to 5-MF (200 mg/kg) treatment alone, one-way ANOVA followed

by post hoc Dunnett's *t*-test.

3.10. Molecular docking studies

The Atomic contact energy (ACE) value of 5–methoxyflavone (-346.43 Kcal/Mol) at $GABA_A$ (α_1 subunit) receptor was almost similar to that of diazepam and thiopentone (Table 4). Docking of 5–methoxyflavone with $GABA_A$ (α_1 subunit) receptor binding site predicted an orientation similar to diazepam, thiopentone, propofol and GABA (Fig. 5). The ACE values identified for adenosine and 5–methoxyflavone at adenosine A_1, A_{2a} and A_3 receptors are shown in Table 5. The predicted binding site for 5–methoxyflavone was similar to the endogenous ligand adenosine at A_{2a} and A_3 receptors. However, different binding 3D pose was observed for 5–methoxyflavone on A_1 receptor (Fig. 6). The ACE value of 5–methoxyflavone and the standard ligand ketamine at NMDA (2 A subunit) receptor were −210.15 Kcal/Mol and −237.31 Kcal/Mol respectively. Docking of 5–methoxyflavone with NMDA receptor predicted binding pose similar to ketamine (Fig. 7A & B). A high ACE value for 5–methoxyflavone (−223.57 Kcal/Mol) was observed at glycine (α_1 subunit) receptor compared to the endogenous ligand glycine (−73.40 Kcal/Mol). The predicted binding sites for 5–methoxyflavone and the endogenous ligand glycine were different on glycine (α_1 subunit) receptor (Fig. 7C & D).

Table 4. Molecular docking: Binding affinity (Atomic contact energy, ACE) score of different agonists at $GABA_A$ (α_1 subunit) receptor.

Compound	Atomic contact energy (ACE) value (Kcal/Mol)
Diazepam	−366.66
GABA	−111.13
Propofol	−244.14
Thiopentone	−327.94
5–methoxyflavone	−346.43

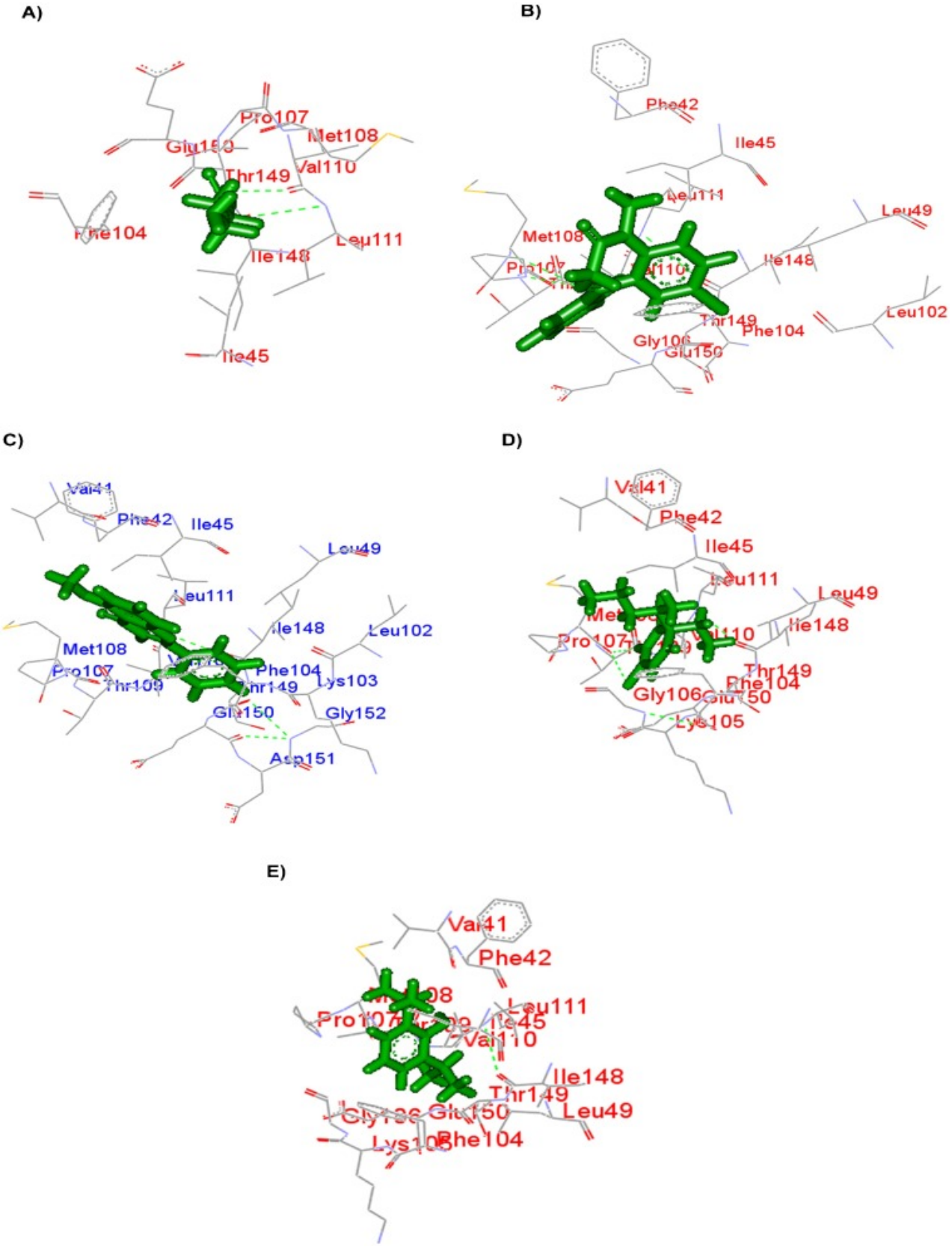

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Fig. 5. 3D model of docking of different agonists (stick model) at $GABA_A$ (α_1 subunit) receptor (wire frame model). (**A**) $GABA_A$ receptor + GABA, (**B**) $GABA_A$ receptor + Diazepam, (**C**) $GABA_A$ receptor + 5-methoxyflavone, (**D**) $GABA_A$ receptor + Thiopentone

and (**E**) $GABA_A$ receptor + Propofol. The hydrogen bond interactions of the agonist at $GABA_A$ (α_1 subunit) receptor are shown as green dotted lines. The hydrophobic interactions established by these compounds in the $GABA_A$ (α_1 subunit) receptor binding pocket are also shown.

Table 5. Molecular docking: Binding affinity (Atomic contact energy, ACE) score of adenosine and 5-methoxyflavone at different subtypes of adenosine receptor.

Compound	**ACE value at Adenosine (A_1) Kcal/ Mol**	**ACE value at Adenosine (A_{2a}) Kcal/ Mol**	**ACE value at Adenosine (A_3) Kcal/ Mol**
Adenosine	−213.29	−261.46	−271.50
5–methoxyflavone	−241.77	−233.83	−274.15

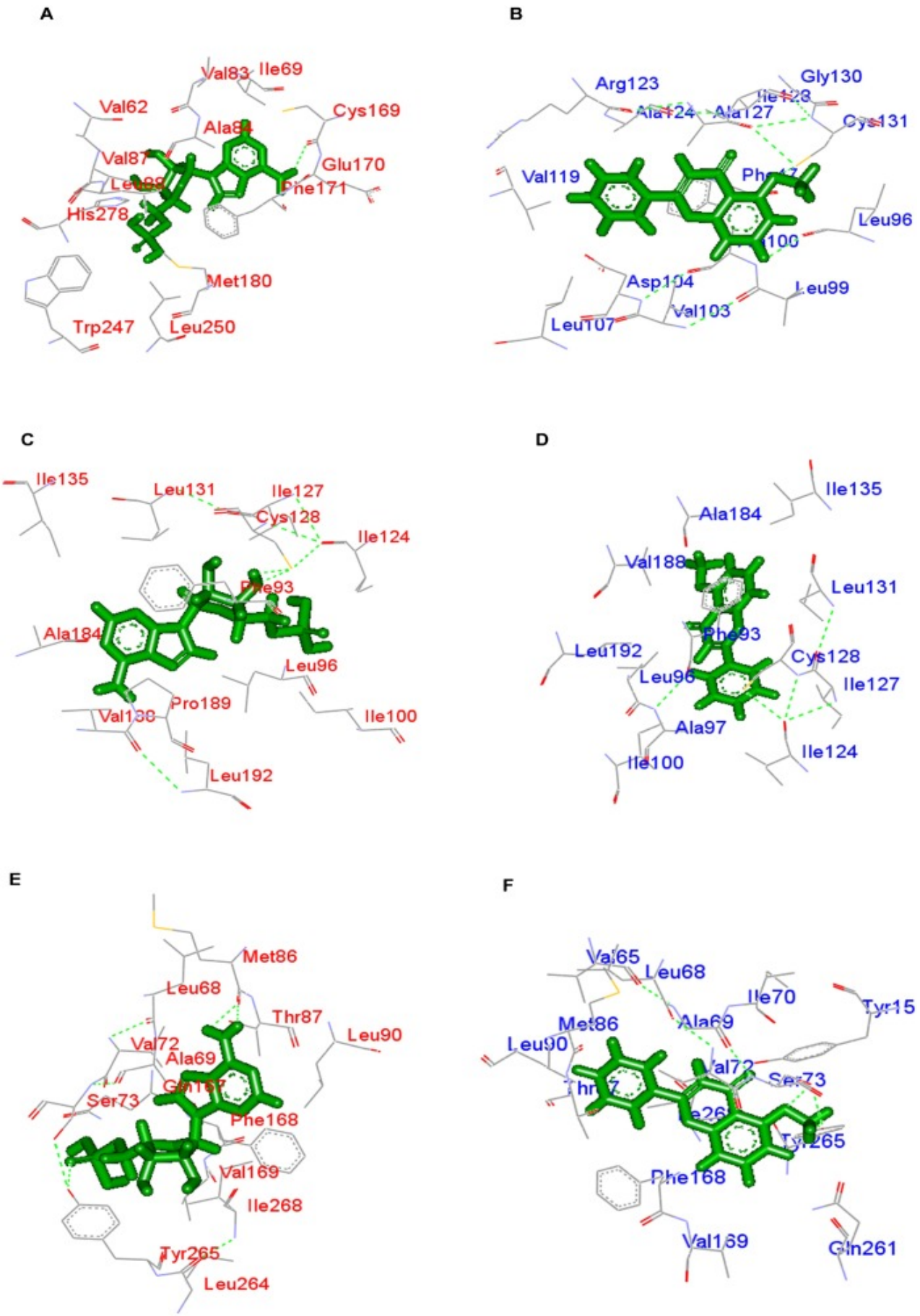

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Fig. 6. 3D model of docking of adenosine and 5-methoxyflavone (stick model) at adenosine A_1, A_2 and A_3 receptors (wire frame model). (**A**) Adenosine A_1 receptor + adenosine; (**B**) Adenosine A_1 receptor + 5-methoxyflavone; (**C**) Adenosine A_{2a} receptor + adenosine; (**D**) Adenosine A_{2a} receptor + 5-methoxyflavone; (**E**) Adenosine A_3 receptor + adenosine; (**F**) Adenosine A_3 receptor + 5-methoxyflavone. The hydrogen bond interactions of the ligands at different subtypes of adenosine receptor are shown as green dotted lines. The hydrophobic interactions established by these compounds in the adenosine receptor binding pocket are also show.

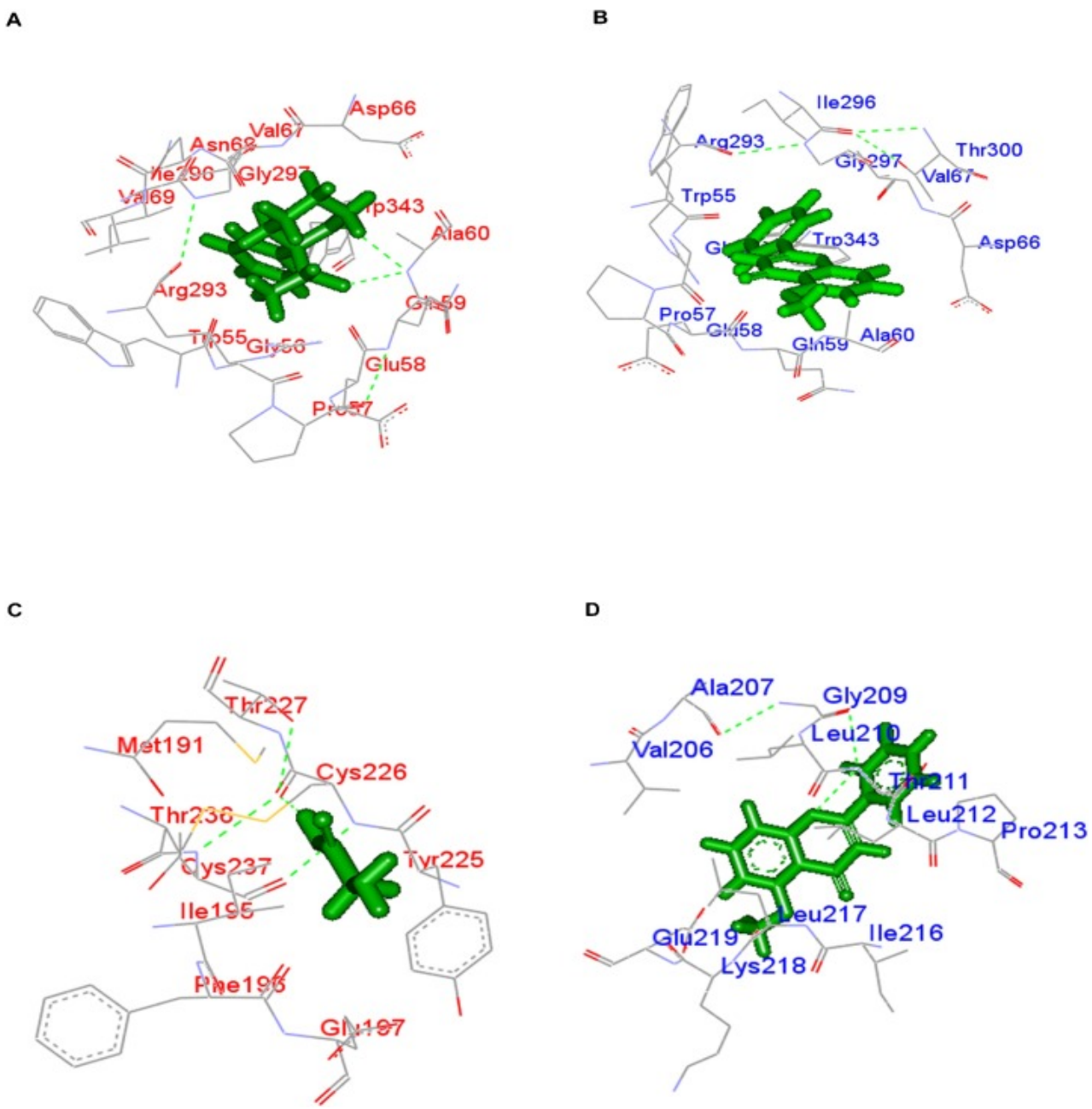

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Fig. 7. 3D model showing binding site of ligands at NMDA (2 A subunit) and glycine (α_1) receptors. (**A**) NMDA (2 A subunit) receptor + Ketamine; (**B**) NMDA (2 A subunit)

receptor + 5-methoxyflavone; (**C**) Glycine (α_1) receptor + Glycine; (**D**) Glycine (α_1) receptor + 5-methoxyflavone. The hydrogen bond interactions of the ligand at NMDA and glycine (α_1) receptors are shown as green dotted lines. The hydrophobic interactions established by these compounds at NMDA and glycine (α_1) receptors are also shown.

4. Discussion

The results of preliminary screening with 5–methoxyflavone indicated a dose-dependent CNS depressant effect on mice. Marked sedation with loss of righting reflex and decreased muscle tone were evident in a dose of 200 mg/kg of 5-methoxyflavone in mice. In acute toxicity testing, 5–methoxyflavone did not produce any mortality over a period of 14 days in a dose of 400 mg/kg. However, significant mortality was evident in doses above 500 mg/kg in mice. The LD_{50} of 5–methoxyflavone was calculated by the graphical method [23] and found to be 540 mg/kg, i.p in mice.

The results of the open field test reveal a significant and dose–dependent reduction in the spontaneous locomotor activity of mice treated with 5–methoxyflavone. The decrease in locomotor activity recorded in a dose of 150 mg/kg of 5–methoxyflavone was comparable to diazepam. The CNS depressant effect of 5–methoxyflavone was further evident from the results of pentobarbitone potentiation and prolongation of ether anaesthesia. The time for the onset of loss of righting reflex after pentobarbitone was significantly decreased by pre-treatment with 5–methoxyflavone. The duration of sleep after pentobarbitone administration in mice was significantly increased in a dose-dependent manner by 5–methoxyflavone pre-treatment (Table 1). A similar potentiation was observed in mice exposed to ether where a significant prolongation in the duration of anaesthesia was recorded (Table 2). The present observations from the open field test, potentiation of pentobarbitone sleeping time and duration of ether anaesthesia clearly demonstrated the sedative-hypnotic like activity of 5–methoxyflavone.

Earlier studies have reported the CNS depressant effect of several flavonoids like 2S–neohesperedin, 2S–naringin, diosmin, gossypin and rutin in mice [9,10]. Apigenin, isolated from Matricaria recutita flowers has been reported to possess sedative and anxiolytic activity in mice [24]. The sedative effect of a naturally occurring flavonoid viscosine was suggested due to actions *via* α_1 subunit containing $GABA_A$ receptors [25]. All the above flavone compounds have complex chemical structure in that they possess multiple substitutions and some are also glycosides. However, the presently investigated compound is a simple flavone with one methoxy substitution in the 5th position. Moreover the earlier reports do not indicate any marked CNS depression for

the investigated compounds *per se*. But 5–methoxyflavone *per se* induced a dose–dependent increasing degree of CNS depression leading to profound hypnosis and this simple methoxyflavone may be considered unique among flavone derivatives.

Another interesting observation in the present study was that, 5–methoxyflavone significantly reduced the balancing time of mice in the rota-rod test (Fig. 3). A similar observation was recorded in horizontal wire test where in 5–methoxyflavone treatment significantly reduced the percentage of mice that could grasp the wire within 5 s (Table 3). Additionally, 5–methoxyflavone significantly reduced the ability of mice to hold on to an inclined plane (Table 3). The above results clearly indicate a loss of muscle tone and motor co-ordination in mice treated with 5–methoxyflavone. $GABA_A$ receptors are implicated in the myorelaxant effect of sedatives like benzodiazepines [26]. It will be interesting to investigate such a mechanism in the myorelaxant effect observed for 5–methoxyflavone. Thus, the results of the present study indicate that 5–methoxyflavone was able to produce different stages of CNS depression and myorelaxant effect in a dose–dependent fashion.

Activation of inhibitory pathways or blockade of excitatory pathways in the central nervous system can lead to different stages of CNS depression. The role of neurotransmitters like GABA, glycine, glutamate and adenosine in this action has been well established. It was considered interesting to investigate the possible role played by these mechanisms in the hypnotic effect of 5–methoxyflavone. GABA is the major inhibitory neurotransmitter present in the CNS and the activation or modulation of $GABA_A$ receptors leads to CNS depression. Many therapeutically used sedative-hypnotics like benzodiazepines, barbiturates, various classes of anticonvulsant drugs and majority of general anaesthetics involve $GABA_A$ receptor in mediating their effect. Incidentally, several flavone derivatives have been reported to interact with $GABA_A$ receptor and exert anxiolytic [[27], [28], [29]] and sedative effect [30].

Pretreatment with bicuculline methiodide, a competitive antagonist at $GABA_A$ receptor significantly reduced the duration of sleep induced by 5–methoxyflavone in mice without altering the latency to sleep onset. In addition, picrotoxin a non-competitive antagonist at $GABA_A$ receptor completely abolished the hypnotic effect of 5–methoxyflavone. These observations clearly indicate a role for $GABA_A$ receptor in the hypnotic like effect of 5–methoxyflavone. Extensive structure activity relationship studies have established flavonoids as ligands for $GABA_A$ receptor [11,12]. The present result is in consonance with the above reports and clearly suggests a role of $GABA_A$ receptor in the hypnotic like effect of 5–methoxyflavone.

Glycine is also proved to be an inhibitory neurotransmitter in the CNS. Propofol has been shown to modulate both $GABA_A$ and glycine receptors to produce the hypnotic

effect [31]. Pretreatment with strychnine, a glycine antagonist, produced a significant reduction in the duration of sleep in 5–methoxyflavone treated mice (Fig. 4). This observation suggests a role for glycine also in mediating the hypnotic like effect of 5–methoxyflavone.

An important role for adenosine system has been identified in the regulation of sleep-wake cycle and this has been suggested as a novel target for sedative–hypnotic drugs [32]. Adenosine binds to adenosine A_1 receptor and A_{2A} receptor in multiple sites of CNS to induce physiological sleep. A predominant role of A_{2A} receptor in sleep regulation has been suggested based on the arousal effect of caffeine by its antagonistic action on A_{2A} receptors [33,34]. The activation of A_{2A} receptors has been suggested to enhance the activity of GABAergic neurons in the ventro lateral pre-optic nucleus to promote sleep [35,36]. Prior administration of caffeine significantly reduced the sleeping time of mice treated with 5–methoxyflavone (Fig. 4).This observation suggests a role for adenosinergic system in the hypnotic like effect of 5–methoxyflavone.

In addition to the activation of inhibitory mechanisms, participation of excitatory pathways also has been suggested and investigated in the hypnotic activity of many CNS depressants. Ketamine is an accredited intravenous general anaesthetic that acts by antagonism of the excitatory NMDA receptors. Another commonly used intravenous anaesthetic thiopental primarily potentiates $GABA_A$ receptor mediated inhibitory neurotransmission [37]. Additionally, the involvement of NMDA receptor has also been suggested to play a role in the hypnotic effect of thiopental [38]. In the present study, the time for onset of loss of righting reflex in 5–methoxyflavone treated mice was significantly extended by NMDA pre-treatment. Similarly, the duration of sleep was also significantly reduced by nearly 45% (Fig. 4).This observation suggests a possible role of NMDA receptor in the hypnotic effect of 5–methoxyflavone. This is not surprising, since other therapeutically used hypnotic or intravenous general anaesthetic drugs also have been shown to interact with the excitatory neurotransmission as described above.

To validate the *in vivo* sedative-hypnotic activity of 5–methoxyflavone, molecular docking studies were carried out and the interaction of 5–methoxyflavone with the binding sites on human $GABA_A$ (α_1 subunit), adenosine (A_1, A_{2a} and A_3), NMDA (2 A subunit) and glycine (α_1) receptors were analysed mechanistically. The In silico studies on $GABA_A$ (α_1 subunit) receptor binding with 5–methoxyflavone and other hypnotics like diazepam, thiopentone and propofol identified very close ACE values. (Table 4). Moreover, the predicted binding sites for 5–methoxyflavone at $GABA_A$ (α_1 subunit) receptor (Fig. 5) through H-bond interactions were almost similar to endogenous

ligand GABA and other hypnotics like diazepam, thiopentone and propofol. The endogenous ligand adenosine and 5–methoxyflavone showed almost similar ACE values at A_1, A_{2a}, and A_3 adenosine receptors (Table 5). The predicted binding site for 5–methoxyflavone was similar to the orientation of endogenous ligand adenosine at A_{2a} and A_3 adenosine receptors (Fig. 6). However, the binding 3D pose was different for adenosine and 5–methoxyflavone at adenosine A_1 receptor. The interaction energy as well as the predicted binding pose observed for standard ligand ketamine and 5–methoxyflavone at NMDA (2 A subunit) receptor (Fig. 7A&7B) were almost similar. The higher interaction energy noted for 5–methoxyflavone at glycine (α_1) receptor suggests a remarkable binding affinity for this flavone than the endogenous ligand glycine. The predicted binding site for 5–methoxyflavone and glycine (Fig. 7C&D) at the above receptor showed different orientation. These findings support the results observed from *in vivo* experiments carried out in mice.

A protein domain analysis tool, Pfam was employed to identify the similarities of amino acid sequence between human and mouse receptors investigated in the present study [39]. The sequence homology of the various receptors studied in the present investigation are similar in mice and humans: $GABA_A$ (99.6%), A_1 adenosine (94.8%), NMDA (99.0%) and glycine (98.9%). Even though the *in vivo* experiments were carried out in mice, which revealed a prominent sedative-hypnotic effect of 5–methoxyflavone, the similar homology of amino acid sequence of mouse and human neuronal receptors ($GABA_A$, adenosine, NMDA and glycine) strongly predict a similar sedative-hypnotic like effect of 5–methoxyflavone in humans as well.

5. Conclusion

The present study has revealed the unique and potent sedative-hypnotic like effect of 5-methoxyflavone in mice. The possible roles of many neuronal mechanisms contributing to this action have also been identified. The therapeutic potentials of CNS depressants are enormous. Based on its interaction with many inhibitory neuronal pathways such as GABA, 5-methoxyflavone may be predicted to be useful as an anxiolytic, anticonvulsant and also as a general anaesthetic. Investigations on these aspects are in progress in our laboratory.

Conflict of interest

All authors declare that there are no conflicts of interests.

Authorship contributions

Contributed in research design: V. Subramanian.

Conducted experiments and docking: JS.

Performed data analysis: BVC, V. Sayeli, JN.

Manuscript preparation and correction: JS, PK, V. Subramanian.

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