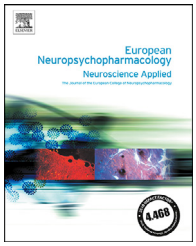
European Neuropsychopharmacology (2020) 000, 1–11



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Anxiolytic-like activity of 5-methoxyflavone in mice with involvement of GABAergic and serotonergic systems - *in vivo* and *in silico* evidences

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Received 15 July 2019; received in revised form 7 May 2020; accepted 24 May 2020
Available online xxx

KEYWORDS
Anxiolytic-like activity;
5-methoxyflavone;
Serotonergic (5-HT_{1A}) receptor;
$GABA_A$ (α_2 subunit-containing) receptor;
Docking

Abstract
Anxiety disorders are common worldwide and novel compounds are investigated for anxiolytic effect. A few studies have demonstrated the anxiolytic-like activity of natural and synthetic flavonoids. 5-methoxyflavone, a synthetic flavone derivative, has been reported to exhibit central nervous system depressant (sedative-hypnotic) effect in an earlier study. The present study was designed to investigate whether 5-methoxyflavone possesses anxiolytic-like activity in mice by employing two unconditioned models of anxiety such as elevated plus maze and light-dark box test. The possible role played by GABAergic ($GABA_A$) and serotonergic ($5HT_{1A}$) systems in the anxiolytic-like effect of 5-methoxyflavone was also investigated in the elevated plus maze test. Molecular docking studies were performed to ascertain the interaction of 5-methoxyflavone with $GABA_A$ (α_2 subunit-containing) and $5HT_{1A}$ receptors. 5-methoxyflavone treatment in mice (10, 20 or 40 mg/kg, i.p) increased the number of entries and time spent in the open arms in an elevated plus maze ($p < 0.001$). In the light-dark box test a significant increase in the time spent in light compartment ($p < 0.001$) and prolonged latency to enter the

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https://doi.org/10.1016/j.euroneuro.2020.05.009

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dark compartment ($p < 0.01$) were also observed. Pretreatment of mice with $5HT_{1A}$ antagonist pindolol (10 mg/kg, i.p) or $GABA_A$ antagonist bicuculline (2 mg/kg, i.p) significantly attenuated the effect of 5-methoxyflavone in the elevated plus maze test. *In silico* studies provided evidences for good binding affinity of 5-methoxyflavone towards $GABA_A$ (α_2 subunit-containing) and serotonergic ($5HT_{1A}$) receptors by H-bond interactions. In conclusion, the present study identified a novel anxiolytic-like effect of 5-methoxyflavone involving GABAergic and serotonergic mechanisms.

1. Introduction

Anxiety disorders including generalized anxiety, panic disorder and social anxiety are common and disabling diseases worldwide. The primary treatment for anxiety related disorders include benzodiazepines, selective serotonin reuptake inhibitors (SSRIs), azapirones and β-adrenoceptor antagonists. However, their usefulness is limited by their side effects like sedation, cognitive impairment and dependence in case of benzodiazepines and sexual dysfunction with respect to SSRI's. β-adrenoceptor antagonists like propranolol and nadolol are occasionally used for performance anxiety, but their use is associated with hypotension. Considering the increased prevalence of anxiety disorders, there is a need for identifying new molecules with good safety profile to treat anxiety.

Flavonoids are one of the plant metabolites that have long been investigated for their properties as ligands at γ-aminobutyric acid type A ($GABA_A$) receptors. Both natural and synthetic flavones have been shown to bind to the benzodiazepine site of $GABA_A$ receptor and exert anxiolytic-like effect in rodents (Marder and Paladini, 2002; Wang et al., 2005). Chrysin, a natural flavone isolated from *Passiflora coerulea L.* (Passifloraceae) exhibited anxiolytic effect in mice (Wolfman et al., 1994). Another natural flavonoid viscosine (4',5,7-trihydroxy-3,6-dimethoxyflavone) isolated from the plant *Dodonaea viscosa* demonstrated anxiolytic, sedative and anticonvulsant activity in rodents (Karim et al., 2015). Studies have shown that flavan-3-ol derivatives are positive modulators of $GABA_A$ receptors with selective action at α_2 subunit containing receptors and exert anxiolytic effect in mice (Fernandez et al., 2008). Synthetic flavonoids like 3-hydroxy-2'-methoxy-6-methylflavone (Karim et al., 2011) and 2'-methoxy-6-methylflavone (Karim et al., 2012) exhibit anxiolytic activity by binding to α_2 subunit containing $GABA_A$ receptors. Behavioral studies showed that a synthetic flavone derivative, 6-methoxyflavanone demonstrated anxiolytic activity in mice (Akbar et al., 2017). In a recent study, 5-methoxyflavone was found to exert a potent sedative and hypnotic effect in mice that was attenuated by picrotoxin and bicuculline suggesting a role for $GABA_A$ receptors in this action (Shanmugasundaram et al., 2018). The prominent sedative and hypnotic activity recorded for 5-methoxyflavone involving $GABA_A$ receptors prompted an investigation on its potential anxiolytic-like effect.

Hence, in the present study, 5-methoxyflavone was investigated for its anxiolytic-like effect in mice by two well established test procedures. The possible role of GABAergic mechanism in the action of 5-methoxyflavone was also analysed by employing bicuculline as an antagonist. Previous studies have identified that flavone derivatives exert anxiolytic activity by their selective action at α_2 subunit-containing $GABA_A$ receptors (Karim et al., 2011, 2012). Hence, *in silico* studies were designed to identify any such interaction of 5-methoxyflavone with α_2 subunit-containing $GABA_A$ receptors.

Serotonergic neurotransmission plays an important role in the regulation of emotional and behavioral responses. Serotonergic ($5\text{-}HT_{1A}$) receptors are widely distributed in the brain particularly in the hippocampus, dorsal raphe nucleus and amygdala (Blier et al., 1993). Azapirone group of drugs like buspirone exert their anxiolytic action by acting as partial agonist at $5\text{-}HT_{1A}$ receptors (Ravindran and Stein, 2010). Spinosin, a flavonoid constituent of *Ziziphi Spinosae Semen*, has been demonstrated to exert anxiolytic-like effect in mice involving serotonergic system (Liu et al., 2015). Hence, the possible role of serotonergic system in the anxiolytic-like effect of 5-methoxyflavone was investigated using pindolol ($5HT_{1A\,/\,1B}$ receptor antagonist). *In silico* experiments were also designed to identify the nature of interaction of 5-methoxyflavone with $5HT_{1A}$ receptors.

2. Experimental procedures

2.1. Animals

Swiss albino mice of either sex (25-30 g) were used for anxiolytic activity and mechanism studies. The animals were kept in polypropylene cages (six in each) on 12h / 12h day / night cycle (lights on at 6 a.m) and the room temperature was maintained between 20 - 23°C, with free access to food and water. To avoid circadian variations and to maintain uniformity, animals were subjected to behavioral experiments between 0900 h and 1400 h. Mice were randomly selected and each group consisted of a minimum of six animals. Each mouse was subjected to the experimental procedure only once to ensure novelty and avoid habituation. The experimental protocol was approved by the institutional animal ethics committee (KN/COL/3404/2014). Proper care and handling of animals were followed based on the directions of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), India.

2.2. Drugs and chemicals

5-methoxyflavone (Fig. 1) was purchased from Research organics, Chennai, India. A fine suspension of 5-methoxyflavone in 0.5% carboxy methylcellulose (CMC) was prepared and injected to mice i.p 30 min before experiments. Diazepam (10 mg/ml ampoule, Hindustan Pharmaceuticals, India), diluted in 0.9% physiological saline was used as a positive control in behavioral experiments.

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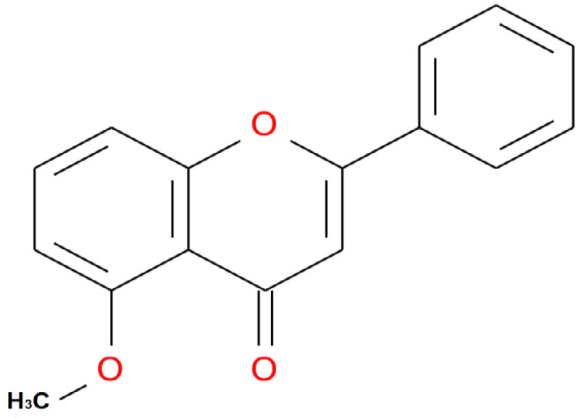

Fig. 1 Chemical structure of 5-methoxyflavone.

(+) Bicuculline (Tokyo Chemical Industry Co Ltd., Tokyo, Japan) $GABA_A$ receptor antagonist and pindolol (Sigma-Aldrich, St Louis, MO, USA) 5-HT_{1A} receptor antagonist prepared as a suspension in 2% Tween-80 were used for anxiolytic mechanism studies. All drugs were prepared freshly on the day of experiment and administered by i.p route in a volume of 10 ml/kg body weight.

2.3. Behavioral tests

2.3.1. Elevated plus maze test

The apparatus consisted of two open arms (30 × 5 cm) and two closed arms (30 × 5 × 15 cm) perpendicular to each other and connected by a central platform (5 × 5 cm). A small ridge of 0.5 cm height covered the edges of both the open arms. The maze was positioned 30 cm above the floor (Lister, 1987). The behavioral experiments were carried out in a quiet room illuminated by a dim light. Different groups of mice were treated with vehicle (CMC), diazepam (1 mg/kg, i.p) or 5-methoxyflavone in doses of 10, 20 or 40 mg/kg, i.p. Thirty minutes later, each mouse was placed in the central platform with its head facing towards an open arm and allowed to explore the maze for 5 min. The parameters observed were; number of entries in the open and closed arms and time spent in the open and closed arms. An arm entry was defined as the animal having all the four paws inside the arm. The percentage of time spent in open arms (PTSOA = stay in open arm (sec)/300 × 100), percentage of entries in the open arm (PEOA = open arm entries/total entries x 100) and the total arm entries (TAE = open arms+closed arms) were calculated for each animal. After each observation, the maze was thoroughly cleaned with a wet cloth using 70% alcohol to remove any residue or odour and allowed to dry. The behavioral parameters were scored manually (in situ) by the observers blinded to the treatment group. An increase in the percentage of time spent and percentage of entries in the open arms indicates anxiolytic activity.

2.3.2. Light - dark box test

An open topped rectangular wooden box measuring 50 × 27 × 25 cm was divided in to a large (27 × 27 cm) compartment and a small (18 × 27 cm) compartment connected through an opening (7 × 7 cm) located in the centre of the partition at the floor level (Bourin and Hascoet, 2003). The floor and inner wall of the small compartment were painted black with the open top covered by a cardboard. The large compartment was painted white and brightly illuminated with a 40 W bulb (100 lux) kept at a height of 40 cm above the box. Different groups of mice were treated with vehicle (CMC), diazepam (1 mg/kg, i.p) or 5-methoxyflavone in doses of 10, 20 or 40 mg/kg, i.p 30 min prior to the procedure. Each mouse was allowed to explore the arena individually for 5 min by placing in the center of the light compartment with its back facing the dark compartment. The following parameters were recorded; (1) latency to the first entry in to the dark compartment, (2) number of transitions between the two compartments and (3) time spent in the light compartment. The behavioral parameters were scored manually by the observers blinded to the treatment group. An increase in the exploration time of light compartment is associated with anxiolytic activity.

2.4. Evaluation of possible mechanisms involved in the anxiolytic-like activity of 5-methoxyflavone using elevated plus maze

A dose of 5-methoxyflavone (40 mg/kg, i.p) that elicited a maximum increase in the percentage of time spent and percentage of entries in the open arms in the elevated plus maze test was selected for the mechanism studies.

2.4.1. Role of GABAergic system

Four groups of mice (n=6) were employed and subjected to two treatment schedules at 15 min interval (1.vehicle + vehicle, 2.(+) bicuculline 2 mg/kg, i.p + vehicle, 3.vehicle + 5-methoxyflavone 40 mg/kg i.p and 4.(+) bicuculline 2 mg/kg, i.p + 5-methoxyflavone 40 mg/kg i.p). These animals were subjected to the test procedure after 30 min. The total arm entries, percentage of time spent and the entries in the open arms of an elevated plus maze were recorded in each animal.

2.4.2. Role of serotonergic system

To investigate the probable role of serotonergic pathway, pindolol (10 mg/kg, i.p) was used (Girish et al., 2013). Four groups of mice (n=6) were employed and subjected to two treatment schedules at 15 min interval (1.vehicle + vehicle, 2.pindolol 10 mg/kg, i.p + vehicle, 3.vehicle + 5-methoxyflavone 40 mg/kg i.p and 4.pindolol 10 mg/kg, i.p + 5-methoxyflavone 40 mg/kg i.p). The total arm entries, percentage of time spent and the entries in the open arms of an elevated plus maze were recorded in each animal 30 min after the above treatment schedule.

2.5. Molecular docking studies

In silico docking of 5-methoxyflavone was carried out with $GABA_A$ (α_2 subunit-containing) and serotonergic (5HT_{1A}) receptors and compared with known ligands. Based on the review of the literature, the gene coded amino acid sequence of the above receptor proteins were retrieved in FASTA format using databases NCBI-Gene database, Ensemble (Aken et al., 2016) and UniProt (https://www.ncbi.nlm.nih.gov/) proteomics database [$GABA_A$ α_2 subunit (P47869) and 5HT_{1A} (P08908)]. The 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/ (Lund et al., 2002; Nielsen et al., 2010). The modelled protein structures were viewed in 3-D form using Accelrys Discovery Studio software (2.5.5 v). The two dimensional structure of the drugs such as 5-methoxyflavone (CID: 94525), diazepam (CID: 3016) and buspirone (CID: 2477) were taken from NCBI Pubchem compound database and converted into three dimensional structure using Online SMILES Translator (https://cactus.nci.nih.gov/index.html). The modelled protein receptor and drug molecule were docked (https://bioinfo3d.cs.tau.ac.il/PatchDock/) using automated Patch Dock server (Duhovny et al., 2002; Schneidman-Duhovny et al., 2005).

2.6. Statistical analysis

The data obtained from elevated plus maze and light-dark box test was statistically analysed with one-way ANOVA followed by

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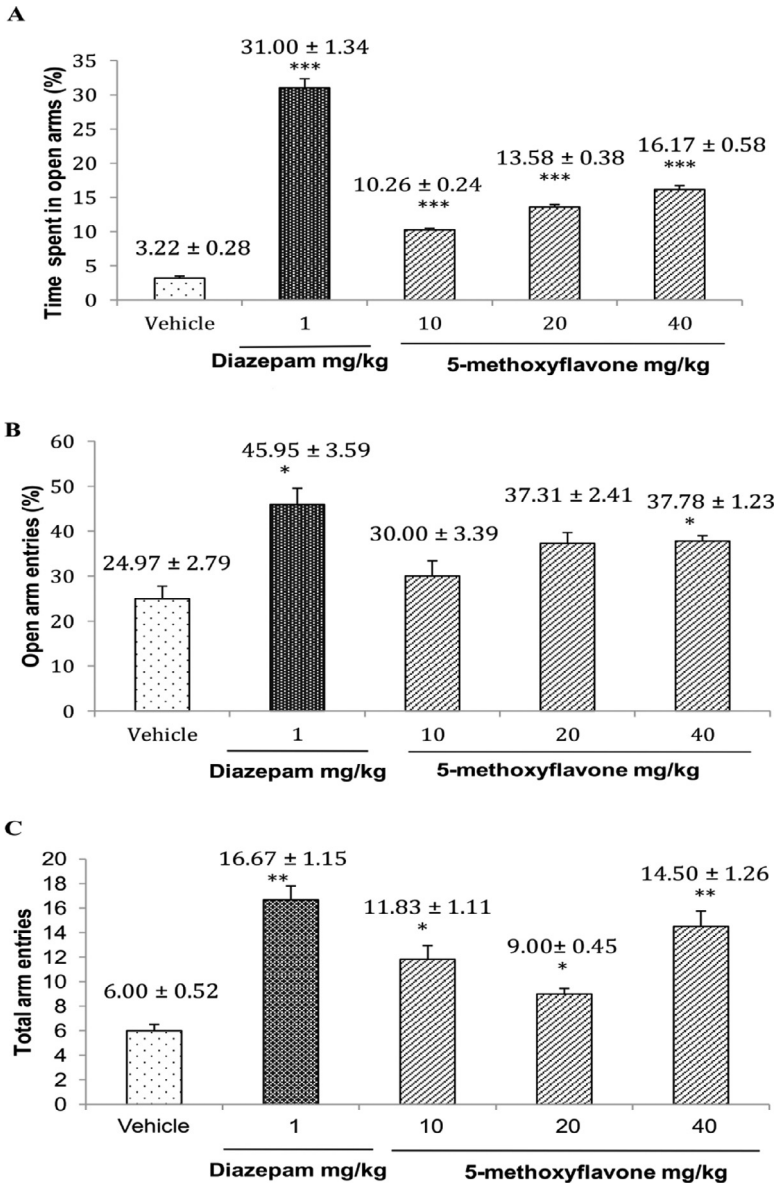

Fig. 2 Effect of 5-methoxyflavone and diazepam on elevated plus maze test. Experiments were carried out in mice 30 min after treatment with diazepam (1 mg/kg) or 5-methoxyflavone (10, 20 & 40 mg/kg). (A) Percentage of time spent in open arms, (B) percentage entries in open arms and (C) total arm entries. Each column represents mean ± S.E.M. ($n = 6$). Statistical analysis was performed by one-way ANOVA followed by Dunnett's *post hoc* test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to vehicle treatment.

post hoc Dunnett's multiple comparison test to compare between multiple groups. For mechanism studies, two-way ANOVA followed by *post hoc* Bonferroni multiple comparison test was used using a software package Sigma Plot version 13 (Systat software, USA). Results are expressed as mean ± S.E.M. Probability values less than 0.05 ($p < 0.05$) were considered as statistically significant.

3. Results

3.1. Elevated plus maze test

5-methoxyflavone demonstrated an anxiolytic-like effect in mice comparable to diazepam in the elevated plus maze (Fig. 2). One-way analysis of variance revealed a significant and dose-dependent increase in the percentage of time spent in the open arms [$F(4, 25) = 216.87$, $p < 0.001$], a significant increase in the percentage of entries in the open arms [$F(4, 25) = 8.18$, $p < 0.001$] and a significant and dose independent increase in the total arm entries [$F(4, 25) = 19.68$, $p < 0.001$] in mice treated with different doses of 5-methoxyflavone. Dunnett's *post hoc* analysis revealed a significant increase in the percentage of time spent in the open arms in diazepam ($p < 0.001$) or different doses of 5-methoxyflavone ($p < 0.001$) treatments (Fig. 2A), a significant increase in the percentage of entries in the open arms in diazepam or 5-methoxyflavone at 40 mg/kg ($p < 0.05$) treatment (Fig. 2B) and a significant increase

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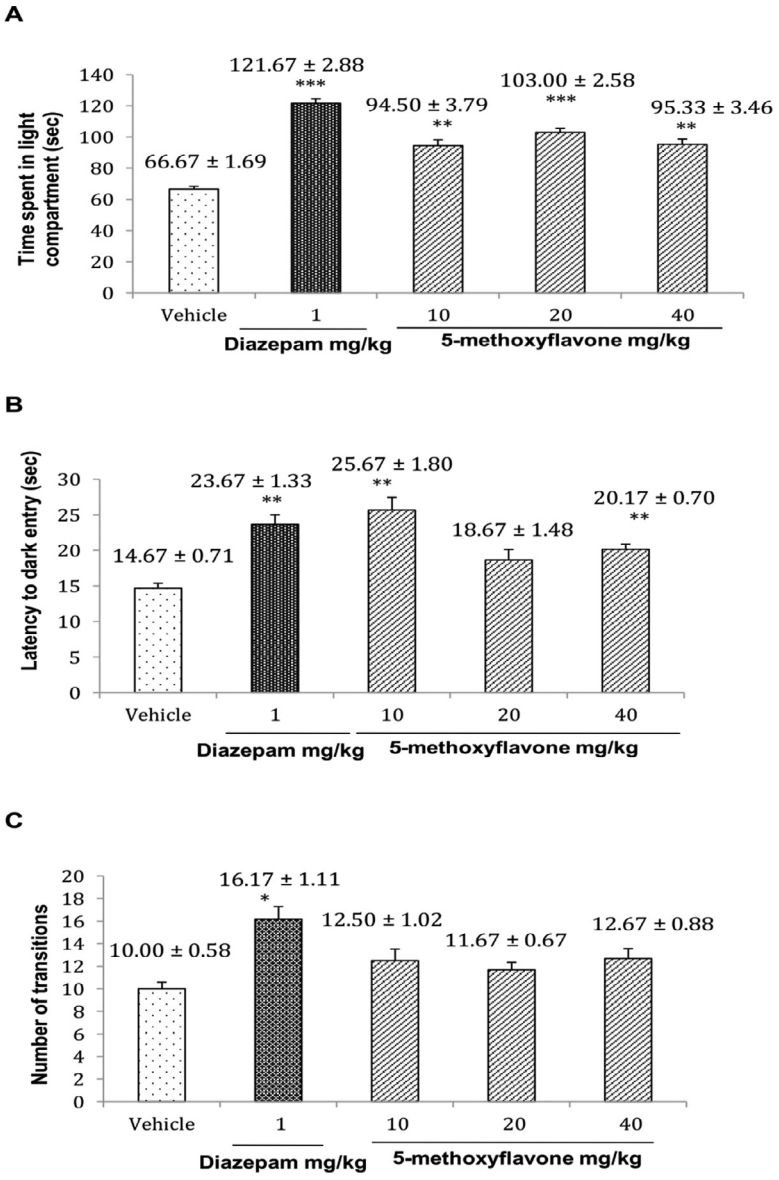

Fig. 3 Effect of 5-methoxyflavone and diazepam in Light-dark box test. Experiments were carried out in mice 30 min after treatment with diazepam (1 mg/kg) or 5-methoxyflavone (10, 20 & 40 mg/kg). (A) Time spent in the light compartment, (B) latency to enter dark compartment and (C) number of transitions. Each column represents mean ± S.E.M. ($n = 6$). Statistical analysis was performed by one-way ANOVA followed by Dunnett's *post hoc* test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to vehicle treatment.

in the total arm entries in diazepam ($p < 0.01$) or 5-methoxyflavone treatments (10, 20 mg/kg, $p < 0.05$ and 40 mg/kg, $p < 0.01$) when compared to vehicle treated animals (Fig. 2C).

3.2. Light - Dark box test

In the light-dark box test, 5-methoxyflavone revealed a significant anxiolytic-like effect in mice comparable to diazepam (Fig. 3). One-way analysis of variance showed a significant but dose independent increase in the time spent in the light compartment [$F(4, 25) = 44.44$, $p < 0.001$], a significant increase in the latency to enter the dark compartment [$F(4, 25) = 11.30$, $p < 0.001$] and a significant increase in the number of transitions [$F(4, 25) = 6.64$, $p = 0.001$]. Dunnett's post hoc test revealed a significant increase in the time spent in the light chamber by diazepam ($p < 0.001$) or 5-methoxyflavone in doses of 10 mg/kg ($p < 0.01$), 20 mg/kg ($p < 0.001$) and 40 mg/kg ($p < 0.01$) compared to vehicle treated animals (Fig. 3A). Similarly, a significant increase in the latency time to enter dark chamber was observed in diazepam ($p < 0.01$) or 5-methoxyflavone in doses of 10 mg/kg ($p < 0.01$) and 40 mg/kg ($p < 0.01$) when compared to vehicle treatment (Fig. 3B). A significant increase in the number of transitions was observed in diazepam ($p < 0.05$) treated animals compared to vehicle treated mice. Eventhough 5-methoxyflavone in various

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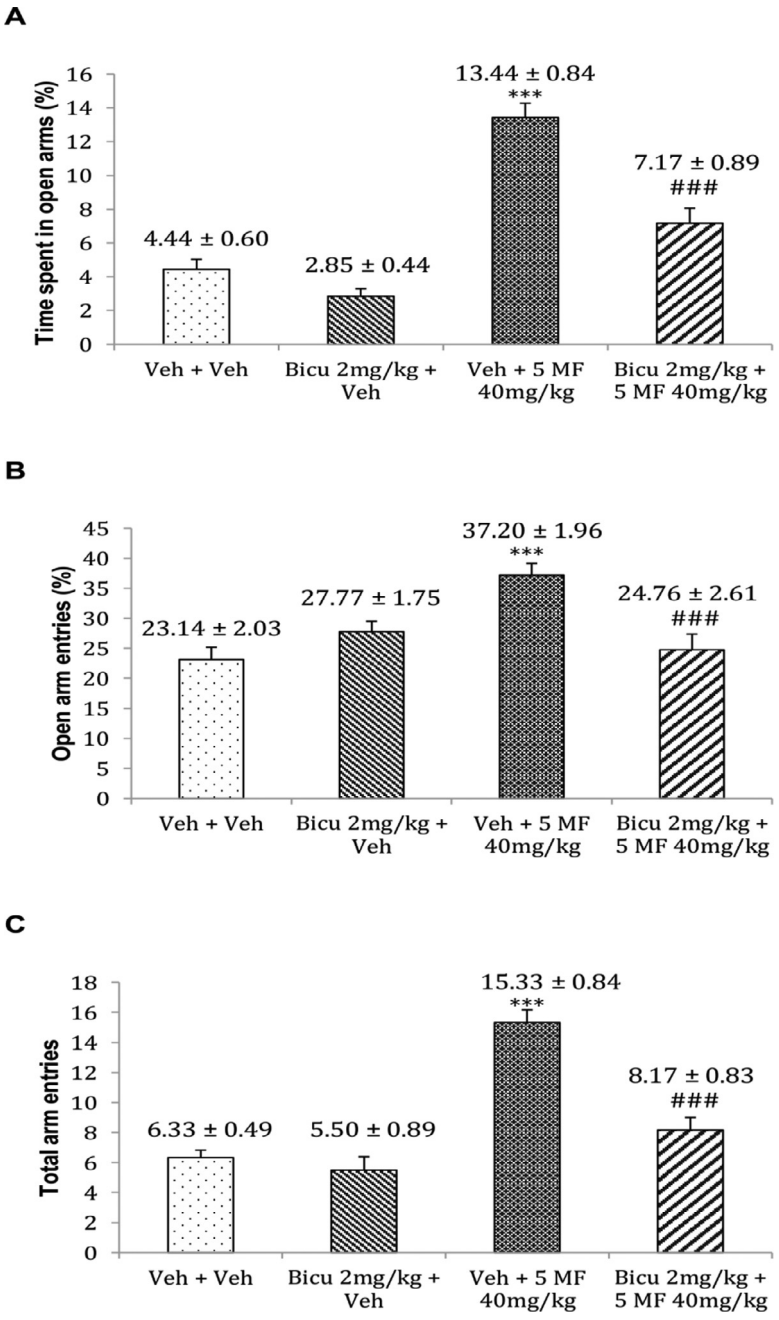

Fig. 4 Effect of bicuculline pre-treatment in mice on elevated plus maze test. (A) Percentage of time spent in open arms (B) percentage entries in open arms and (C) total arm entries. Each column represents mean ± S.E.M. ($n = 6$). Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post hoc* test for multiple comparisons. *** $p < 0.001$ compared to vehicle + vehicle treatment. ### $p < 0.001$ compared to vehicle + 5 MF 40 treated group.

doses increased the number of transitions, the values were not statistically significant (Fig. 3C).

3.3. Effect of bicuculline pre-treatment

The effect of bicuculline pre-treatment on the behavioral responses of 5-methoxyflavone treated mice in elevated plus maze is presented in Fig. 4. Pre-treatment with bicuculline (2 mg/kg) reversed the anxiolytic-like effect of 5-methoxyflavone (40 mg/kg) in the elevated plus maze. In the PTSOA, two-way analysis of variance revealed a significant effect of 5-methoxyflavone treatment [$F(1, 20) = 87.03$, $p < 0.001$], bicuculline treatment [$F(1, 20) = 30.42$, $p < 0.001$] and a significant interaction between bicuculline and 5-methoxyflavone treatments [$F(1, 20) = 10.78$, $p = 0.004$]. Bonferroni *post hoc* analysis revealed a significant increase in the PTSOA by 5-methoxyflavone (40 mg/kg) compared to vehicle + vehicle treatment ($p < 0.001$) and a significant reversal of

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this response by bicuculline pre-treatment ($p < 0.001$ vs vehicle + 5-methoxyflavone treatment, Fig. 4A).

In case of PEOA, two-way ANOVA showed a significant effect of 5-methoxyflavone treatment [$F(1, 20) = 6.84$, $p = 0.017$], bicuculline treatment [$F(1, 20) = 3.42$, $p = 0.079$] and a significant interaction between bicuculline and 5-methoxyflavone treatments [$F(1, 20) = 16.33$, $p < 0.001$]. Bonferroni *post hoc* analysis revealed a significant increase in PEOA by 5-methoxyflavone compared to vehicle+vehicle treatment ($p < 0.001$) and a significant inhibitory effect of bicuculline pre-treatment on the response to 5-methoxyflavone ($p < 0.001$, Fig. 4B).

In total arm entries, two-way ANOVA revealed a significant effect of 5-methoxyflavone treatment [$F(1, 20) = 55.94$, $p < 0.001$], bicuculline treatment [$F(1, 20) = 26.30$, $p < 0.001$] and a significant interaction between bicuculline and 5-methoxyflavone treatments [$F(1, 20) = 16.48$, $p < 0.001$]. Bonferroni *post hoc* analysis showed a significant increase in TAE by 5-methoxyflavone compared to vehicle + vehicle treatment ($p < 0.001$) and a significant attenuation of this response in bicuculline pre-treated animals compared to vehicle + 5-methoxyflavone treatment ($p < 0.001$, Fig. 4C).

3.4. Effect of pindolol pre-treatment

The effect of pindolol pre-treatment on the behavioral responses of 5-methoxyflavone treated mice in elevated plus maze is shown in Fig. 5. Pre-treatment with pindolol (10 mg/kg) completely reversed the anxiolytic-like effect of 5-methoxyflavone. In PTSOA, two-way analysis of variance revealed a significant effect of 5-methoxyflavone treatment [$F(1, 20) = 55.56$, $p < 0.001$], pindolol treatment [$F(1, 20) = 29.18$, $p < 0.001$] and a significant interaction between pindolol and 5-methoxyflavone treatments [$F(1, 20) = 40.08$, $p < 0.001$]. *Post hoc* analysis with Bonferroni test showed a significant increase in the PTSOA by 5-methoxyflavone (40 mg/kg) compared to vehicle + vehicle treatment ($p < 0.001$) and a significant reversal of this response by pindolol pre-treatment ($p < 0.001$, vs vehicle + 5-methoxyflavone treatment, Fig. 5A).

In case of PEOA, two-way ANOVA revealed a significant effect of 5-methoxyflavone treatment [$F(1, 20) = 10.93$, $p = 0.004$], pindolol treatment [$F(1, 20) = 3.51$, $p = 0.076$] and a significant interaction between pindolol and 5-methoxyflavone treatments [$F(1, 20) = 16.53$, $p < 0.001$]. Bonferroni *post hoc* analysis revealed a significant increase in the PEOA by 5-methoxyflavone compared to vehicle + vehicle treated mice ($p < 0.001$) and a significant reduction in this response was produced by pindolol pre-treatment compared to vehicle + 5-methoxyflavone treatment ($p < 0.001$, Fig. 5B).

In total arm entries, two-way ANOVA revealed a significant effect of 5-methoxyflavone treatment [$F(1, 20) = 40.97$, $p < 0.001$], pindolol treatment [$F(1, 20) = 3.80$, $p = 0.066$] and a significant interaction between pindolol and 5-methoxyflavone treatments [$F(1, 20) = 13.09$, $p = 0.002$]. Bonferroni *post hoc* analysis showed a significant increase in TAE by 5-methoxyflavone compared to vehicle + vehicle treatment ($p < 0.001$) and this response was significantly attenuated by pindolol pre-treatment compared to vehicle + 5-methoxyflavone treatment ($p < 0.001$, Fig. 5C).

Table 1 Molecular docking: Binding affinity (Atomic contact energy) score of different agonists at $GABA_A$ (α_2 subunit) and serotonergic ($5\text{-}HT_{1A}$) receptor.

Compound	$GABA_A$ (α_2 subunit) receptor Atomic contact energy (Kcal/Mol)	Serotonergic ($5\text{-}HT_{1A}$) receptor Atomic contact energy (Kcal/Mol)
Diazepam	−205.33	–
GABA	−93.36	–
5-methoxyflavone	−222.82	−224.02
Buspirone	–	−324.25

3.5. Molecular docking studies

The atomic contact energy (ACE) values for ligands such as diazepam, GABA and 5-methoxyflavone at $GABA_A$ (α_2 subunit-containing) receptor are shown in Table 1. 5-methoxyflavone showed a good binding affinity with an ACE value of -222.82 Kcal/Mol at $GABA_A$ (α_2 subunit-containing) receptor. Docking of 5-methoxyflavone at $GABA_A$ (α_2 subunit-containing) receptor predicted a different binding site when compared to the standard ligands diazepam or GABA (Fig. 6).

The ACE value for ligands such as buspirone and 5-methoxyflavone at serotonergic ($5HT_{1A}$) receptor are shown in Table 1. The standard ligand buspirone showed a good binding affinity with an ACE value of -324.25 Kcal/Mol at $5HT_{1A}$ receptor when compared to 5-methoxyflavone -224.02 Kcal/Mol. Docking of 5-methoxyflavone with $5HT_{1A}$ receptor predicted a similar binding pose like the standard ligand buspirone through H-bond interactions (Fig. 7).

4. Discussion

In an earlier study, 5-methoxyflavone demonstrated a significant sedative-hypnotic like effect in mice involving multiple mechanisms including $GABA_A$ (α_1 subunit-containing) receptors (Shanmugasundaram et al., 2018). The aim of the present study was to investigate 5-methoxyflavone for anxiolytic-like effect in two unconditioned models of anxiety namely, elevated plus maze and light - dark box tests. The results of the present study revealed that 5-methoxyflavone treatment in mice significantly increased the percentage of time spent as well as the number of entries in the open arms in the elevated plus maze in a dose-dependent manner (Fig. 2).

In the light - dark box test, the animals treated with 5-methoxyflavone spent more time in the light compartment and the increase was significant but dose independent (Fig. 3). There was a significant increase in the latency to enter the dark compartment. The number of transitions between the two compartments was also increased in 5-

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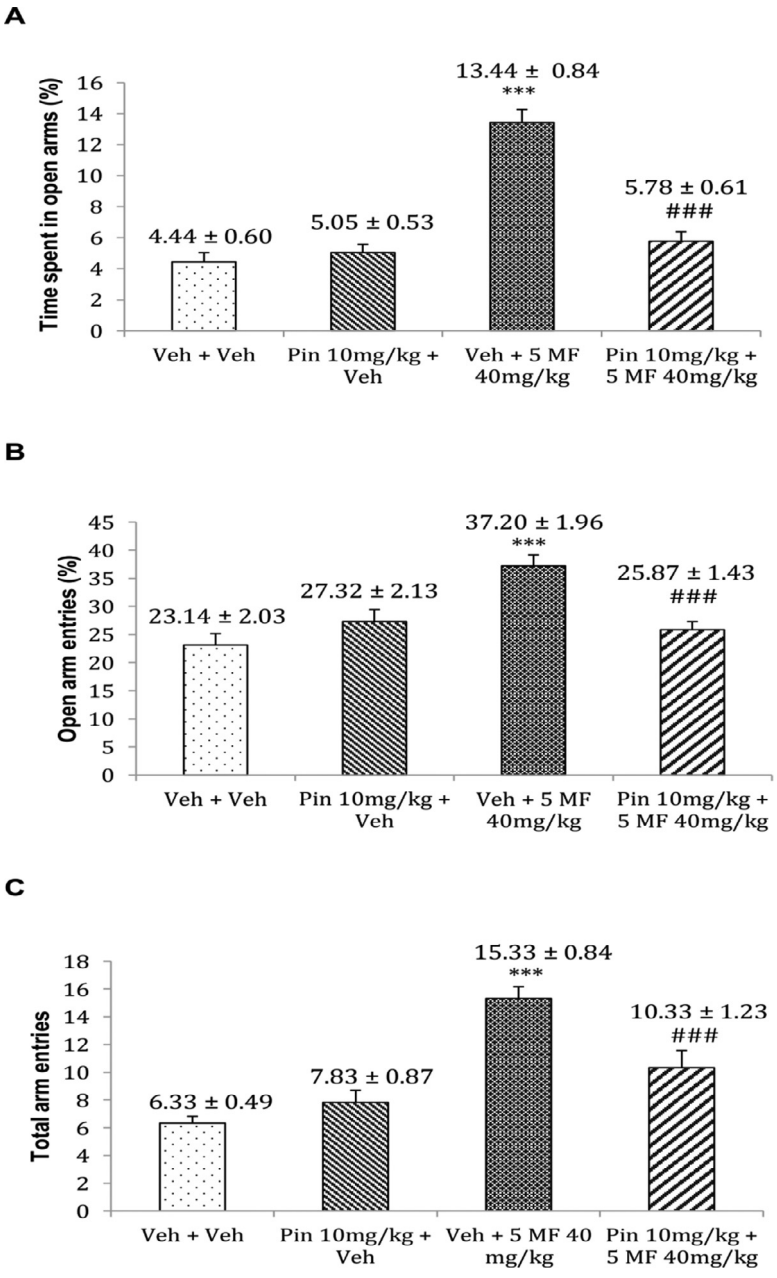

Fig. 5 Effect of pindolol pre-treatment in mice on elevated plus maze test. (A) Percentage of time spent in open arms (B) percentage entries in open arms and (C) total arm entries. Each column represents mean ± S.E.M. ($n = 6$). Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post hoc* test for multiple comparisons. *** $p < 0.001$ compared to vehicle + vehicle treatment. ### $p < 0.001$ compared to vehicle + 5 MF 40 treated group.

methoxyflavone treated animals, however not statistically significant.

Taken together, the findings of the present experiments clearly demonstrated an anxiolytic-like effect of 5-methoxyflavone after i.p administration in mice. The observation of the present study is in agreement with the previously published reports on anxiolytic-like effect of many flavone derivatives such as spinosin (Liu et al., 2015), 6-bromoflavanone (Ognibene et al., 2008) and ellagic acid (Girish et al., 2013).

Generally anxiolytic drugs like diazepam produce sedation and muscle relaxation in higher doses. Behavioral studies have reported the anxiolytic-like activity of flavonoids at lower doses and the effect gets diminished at higher doses indicating the onset of sedative action (Karim et al., 2015; Fernandez et al., 2009). A similar observation has been recorded for 5-methoxyflavone in earlier experiments where sedation and muscle relaxant effects were evident only when doses above 100 mg/kg were employed (Shanmugasundaram et al., 2018). However, an increase in the exploratory activity as revealed by an increase in the total arm entries in the elevated plus maze (Fig. 2) and an increase in the number of transitions in the light-dark box test (Fig. 3) indicates that 5-methoxyflavone does not

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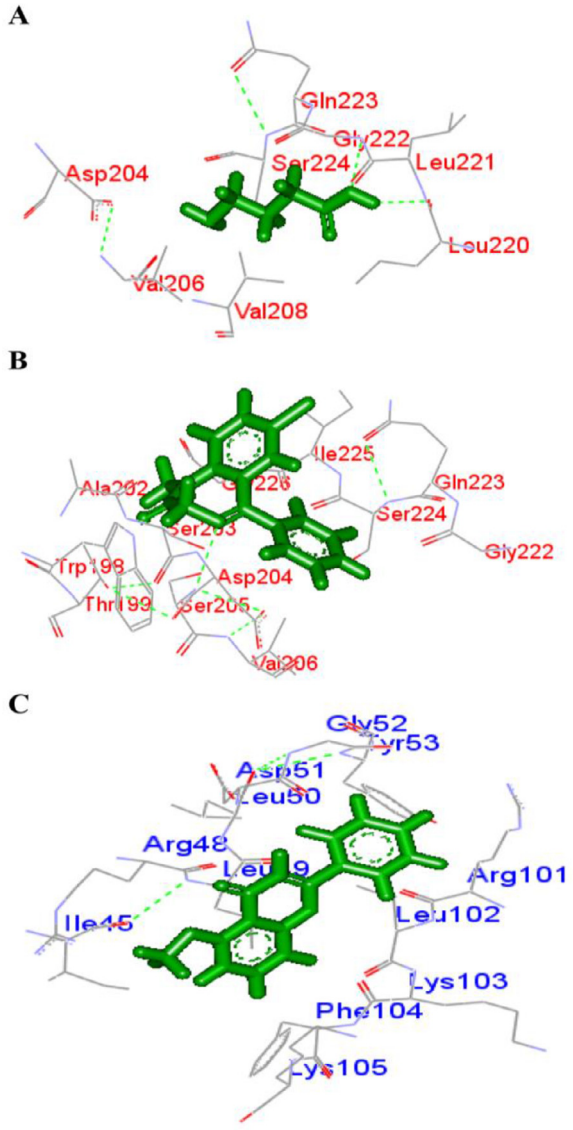

Fig. 6 3D model of docking of different agonists (stick model) at $GABA_A$ (α_2 subunit-containing) receptor (wire frame model). (A) $GABA_A$ receptor + GABA, (B) $GABA_A$ receptor + Diazepam and (C) $GABA_A$ receptor + 5-methoxyflavone. The hydrogen bond interactions of the agonists at $GABA_A$ (α_2 subunit-containing) receptor are shown as green dotted lines. The hydrophobic interactions established by these compounds in the $GABA_A$ (α_2 subunit-containing) receptor binding pocket are also shown.

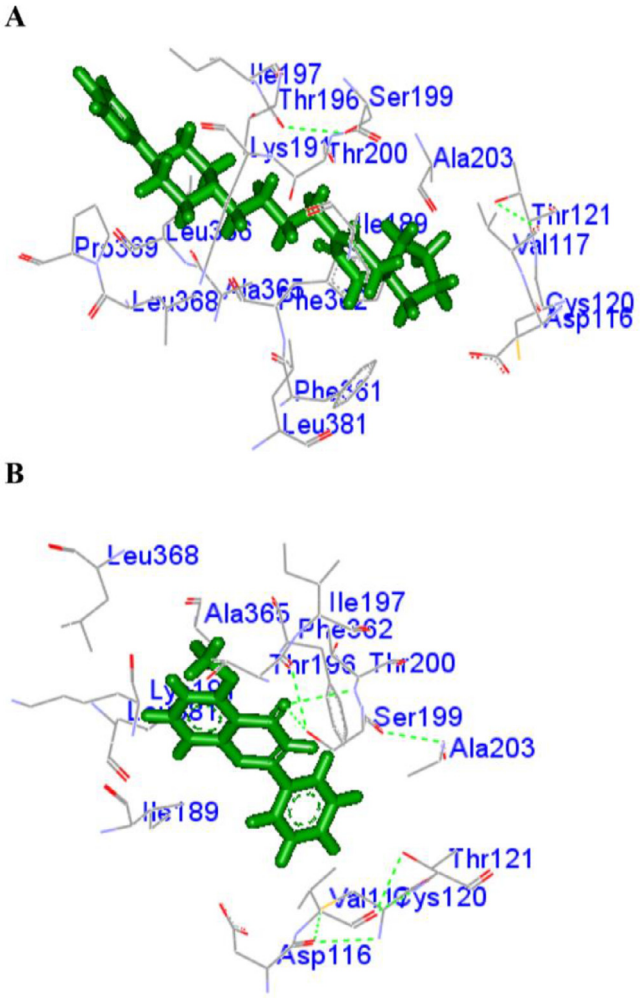

Fig. 7 3D model showing binding site of ligands (stick model) at serotonergic ($5\text{-}HT_{1A}$) receptor (wire frame model). (A) Serotonergic ($5\text{-}HT_{1A}$) receptor + Buspirone and (B) Serotonergic ($5\text{-}HT_{1A}$) receptor + 5-methoxyflavone. The hydrogen bond interactions of the ligands at serotonergic receptor are shown as green dotted lines. The hydrophobic interactions established by these compounds at serotonergic receptor are also shown.

produce any sedation in the doses employed. Hence, it can be stated that the anxiolytic-like effect of 5-methoxyflavone is recorded at much lower doses than that are expected to produce sedation.

After recording an anxiolytic-like effect of 5-methoxyflavone, it was considered interesting to investigate the possible mechanisms involved in this response in mice. A dose of 5-methoxyflavone that produced maximal anxiolytic-like effect (40mg/kg) in the elevated plus maze was selected for the mechanism studies. Natural and synthetic flavonoids appear to act as ligands at the flumazenil dependent (Girish et al., 2013; Liu et al., 2015) and flumazenil independent sites (de Carvalho et al., 2011; Hall et al., 2004) of $GABA_A$ receptors to exert an anxiolytic-like effect. Also, in an earlier study, the sedative-hypnotic effect of 5-methoxyflavone was attenuated by pre-treatment with bicuculline and picrotoxin suggesting a role of $GABA_A$ receptors in this action (Shanmugasundaram et al., 2018). Hence, in the present study, the role of GABAergic neurotransmission in the anxiolytic-like effect of 5-methoxyflavone was investigated in mice.

The effect of 5-methoxyflavone was significantly attenuated by pre-treatment with bicuculline ($GABA_A$ antagonist) as observed by a decrease in the percentage of time spent as well as the number of entries in the open arms by mice (Fig. 4). This observation suggests that the anxiolytic-like effect of 5-methoxyflavone may be due

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to its interaction with $GABA_A$ receptors. It is known that flavonoids modulate a large number of $GABA_A$ allosteric non-benzodiazepine sites (Hanrahan et al., 2011). Drugs binding to α_1 subunit-containing $GABA_A$ receptors exert sedative effect (McKernan et al., 2000) while binding to α_2 subunit-containing $GABA_A$ receptors is associated with anxiolytic effect (Fernandez et al., 2008; Karim et al., 2011). In an earlier *in silico* study, 5-methoxyflavone expressed good binding affinity towards $GABA_A$ (α_1 subunit-containing) receptors similar to diazepam (Shanmugasundaram et al., 2018). To validate the *in vivo* anxiolytic-like effect of 5-methoxyflavone, molecular docking studies were carried out in the present study and the interaction of 5-methoxyflavone with the binding sites on human α_2 subunit-containing $GABA_A$ receptors was analysed mechanistically. The *in silico* studies on $GABA_A$ (α_2 subunit-containing) receptor binding with 5-methoxyflavone, diazepam and GABA identified very close ACE values (Table 1). Moreover, the predicted binding sites for 5-methoxyflavone at $GABA_A$ (α_2 subunit-containing) receptors (Fig. 6) through H-bond interactions were different from ligands such as GABA or diazepam. Hence, 5-methoxyflavone may be predicted to exert an anxiolytic-like effect by binding to an allosteric site on $GABA_A$ (α_2 subunit-containing) receptors. The *in silico* observation supports the present results observed *in vivo* and conclusively suggests a role for $GABA_A$ (α_2 subunit-containing) receptors in the anxiolytic-like activity of 5-methoxyflavone. This proposal is strengthened by earlier electrophysiological studies that reported the involvement of specific subtypes of $GABA_A$ receptors in the anxiolytic-like activity of various flavonoids (Ren et al., 2010; Wang et al., 2008; Hall et al., 2005).

It has been established that alteration in the serotonergic system also plays a role in anxiety disorders. Buspirone, a partial agonist at $5HT_{1A}$ receptor, has shown efficacy in generalised anxiety disorder (Graeff et al., 1996) and inhibits the serotonergic system by acting on somatodendritic auto-receptors (Sharp et al., 1989). Behavioral studies on flavone derivatives such as spinosin (Liu et al., 2015) and gallic acid (Mansouri et al., 2014) have demonstrated the anxiolytic-like effect mediated through serotonergic ($5HT_{1A}$) receptors. Hence, the possible involvement of serotonergic ($5HT_{1A}$) receptors in the anxiolytic-like effect of 5-methoxyflavone was studied using pindolol (β-blocker / 5-$HT_{1A/1B}$ antagonist). The effect of 5-methoxyflavone was significantly attenuated by pre-treatment with pindolol as observed by a reduction in the percentage of time spent as well as the number of entries in the open arms by mice (Fig. 5). These findings suggest a role for serotonergic ($5HT_{1A}$) receptors also in the anxiolytic-like effect of 5-methoxyflavone in mice. Future studies may reveal the location and nature of interaction of 5-methoxyflavone with $5HT_{1A}$ receptors in the central nervous system. Molecular docking studies indicated good binding affinity of 5-methoxyflavone at human $5HT_{1A}$ receptors with an ACE value of -224.02 Kcal/Mol (Table 1) comparable to the standard ligand buspirone ($5HT_{1A}$ partial agonist). Docking of 5-methoxyflavone with $5HT_{1A}$ receptor predicted a similar binding pose like buspirone through H-bond interactions (Fig. 7). These results confirm the *in vivo* experiments and indicate a role for serotonergic system in the anxiolytic-like effect of 5-methoxyflavone.

5. Conclusion

The present study has identified a potential anxiolytic-like effect of 5-methoxyflavone after i.p administration in mice tested on two unconditioned models of anxiety. The anxiolytic activity was mediated by its interaction with $GABA_A$ (α_2 subunit-containing) and serotonergic ($5HT_{1A}$) receptors and the findings have been corroborated by molecular docking studies.

CRediT authorship contribution statement

Jaikumar Shanmugasundaram: Project administration, Writing - review & editing. **Viswanathan Subramanian:** Methodology, Writing - review & editing. **Jagan Nadipelly:** Data curation, Formal analysis. **Parimala Kathirvelu:** Writing - review & editing. **Vijaykumar Sayeli:** Data curation, Formal analysis. **Binoy Varghese Cheriyan:** Data curation, Formal analysis.

Conflict of interest

All authors declare that they have no conflict of interest.

Acknowledgement

The authors acknowledge the help extended by Dr. Balaji Munivelan, CEO & Senior Bioinformatician (bioinfobalaji@gmail.com) ABS Geno-informatics, Chennai, and Dr.R.Vijayaraghavan, Director research, Saveetha Institute of Medical and Technical Sciences, Chennai for their contribution towards *In silico* drug docking studies and statistical analysis of the data respectively.

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