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Characterization of 6-methoxyflavanone as a novel anxiolytic agent: A behavioral and pharmacokinetic approach.

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Abstract

Benzodiazepines are regularly prescribed for the treatment of anxiety though there are side effects. Flavonoids have selective affinity for $GABA_A$ receptors implicated in anxiolytic-like activity in rodents, but are devoid of the unwanted side effects of benzodiazepines. In this study, 6-methoxyflavanone (6-MeOF), a positive allosteric modulator of γ-amino butyric acid (GABA) responses at human recombinant $GABA_A$ receptors, was evaluated for its behavioral profile in the elevated plus-maze, as well as the staircase- and open-field tests in mice. In addition, the distribution of 6-MeOF in selected brain areas involved in anxiety (amygdala and cerebral cortex) was also examined using a validated high performance liquid chromatography/ultraviolet detection (HPLC/UV) method. 6-MeOF (10, 30 and 50 mg/kg) exerted an anxiolytic-like effect, increasing entries and time spent in the open arm and the central platform, as well as head-dipping frequency in the mouse elevated plus-maze assay. It also decreased rearing incidence without suppressing the number of steps ascended in the staircase test. Whereas, in the open-field anxiety test, 6-MeOF had no effect on locomotor activity at lower doses, a decrease was observed at the highest dose (100 mg/kg). 6-MeOF additionally produced an anxiolytic-like increase in the time spent at the center of the open-field apparatus. These effects were preferentially antagonized by pentylenetetrazole (15 mg/kg). Furthermore, pharmacokinetic studies disclosed a rapid appearance of 6-MeOF in the plasma and discrete brain areas. Taken together, our findings suggest that 6-MeOF readily crosses the blood brain barrier (BBB) generating anxiolytic activity, mediated through the GABAergic system.

Keywords
Flavonoid; 6-Methoxyflavanone; anxiety; $GABA_A$ receptors; elevated plus-maze; staircase anxiety test.

Chemical compounds
6-Methoxyflavanone (PubChem CID: 97860)

1. Introduction

Generalized anxiety disorder, social anxiety and panic disorder have long been recognized as being amongst the most commonplace disabling conditions in society (Association, 1994). Although selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) are considered as primary treatments for anxiety, benzodiazepines are still quite regularly prescribed despite having unwanted side effects (Davidson, 2009; Paladini et al., 1999). Benzodiazepines act as positive allosteric modulators via a subpopulation of γ-amino butyric acid receptors ($GABA_A$) increasing the frequency of chloride channel opening (Chebib and Johnston, 2000). In the brain, GABA tends to be the most abundant inhibitory neurotransmitter regulating different physiological phenomena including sleep, anxiety, memory formation and reward (Zeilhofer et al., 2009) and $GABA_A$ receptors are implicated as a major inhibitory element (Macdonald and Olsen, 1994). Accordingly, GABA inhibitory interneurons function via $GABA_A$ receptor subtypes that are involved in mediating behavior (Mohler et al., 2004).

A few years ago, a new family of ligands possessing a flavonoid nucleus, was unveiled by a research group in a quest for safer $GABA_A$ receptor modulators (Ognibene et al., 2008). These polyphenolic compounds (phenyl benzopyrones) have been found not only in plants, but also in dietary components and they have been produced synthetically as well (Kempuraj et al., 2005). Flavonoids have been extensively examined for their peripheral actions, however, a selective affinity for $GABA_A$ receptors has been reported in studies using rat and bovine brain membrane binding assays (Hong and Hopfinger, 2003). In conjunction with binding studies, behavioral investigations have also disclosed anxiolytic-like activity of flavonoids in rodents without

unwanted benzodiazepine side effects (Griebel et al., 19**9**9). Furthermore, a number of synthetic derivatives of natural flavonoids are known for their potent anxiolytic properties (Karim et al., 2011; Fernandez et al., 2008; Griebel et al., 1999).

Interestingly, a recent radioligand binding study of 6-MeOF (a synthetic flavanone, Fig. 1) has revealed that its molecular binding site is different from other known flavonoid modulators of $GABA_A$ receptors. Thus, it binds to a novel allosteric site independent of both high and low affinity benzodiazepine binding sites (Hall et al., 2014). Consequently, it acts upon γ-subunit containing $GABA_A$ receptors like diazepam, but is insensitive to flumazenil antagonism whilst inhibiting [^{3}H]-flunitrazepam binding at the same time. Hence, 6-MeOF as a $GABA_A$ receptor ligand, may conceivably exhibit an anxiolytic profile. Therefore, this study was devised to investigate such a propensity of activity *in vivo* utilizing different anxiety models. We also investigated the possible contribution of GABAergic mechanisms to the pharmacological activity of 6-MeOF by pretreatment with a designated $GABA_A$ antagonist, pentylenetetrazole (Rattka et al., 2011). Additionally, high performance liquid chromatography (HPLC) coupled with UV detection was employed to determine the pharmacokinetic profile as well as the distribution pattern of 6-MeOF both in plasma and the brain areas (cerebral cortex and amygdala) involved in anxiety.

2. Materials and methods

2.1. Drugs and chemicals

6-Methoxyflavanone (>95%, Sigma-Aldrich, USA), HPLC grade acetonitrile (99.9%) as well as methanol (99.9%) (Fisher Scientific, UK), diazepam (Valium, 10 mg/2 mL, Roche, Pakistan) and pentylenetetrazole (≥98%, Sigma Aldrich, UK) were employed. 6-MeOF was dissolved in a vehicle comprising of Tween 80 (1%), DMSO (5%) and saline (94%).

2.2. Animals

BALB/c mice (18-30 g) of both sex (equal numbers of males and females per group), bred and maintained in a controlled environment (temperature = 22 ± 2º C and relative humidity = 60 ± 10%) on a 12:12 h day/night cycle (lights on at 7:00 am) in the animal house (Department of Pharmacy, University of Peshawar) were used. Food and water were provided *ad libitum* and both males as well females were included experimentally because previous studies have verified that mice of either sex may be employed in measures of anxiety (File, 2001). All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and according to the rules and ethics set forth by the Ethical Committee of the Department of Pharmacy, University of Peshawar. Approval for this study was granted with the registration number: 08/EC-15/Pharm (dated: April 10, 2015).

2.3. Behavioral analysis

Mice were housed in polycarbonate cages in groups of three per cage (dimensions 42.5 × 26.6 × 15.5 cm). All the behavioral tests were performed during the light phase between 8:00 am to 2:00 pm. On the test day, the mice were transferred to a dimly illuminated room (red light, 40 lux) 1 h prior to experimentation. Each experiment was performed on separate groups of animals (n = 6) according to the guidelines of Animal (Scientific Procedures) Act 1986. The sample size was calculated according to the resource equation method (Festing and Altman, 2002). There are no previous reports on the pharmacological activity of 6-MeOF, so the dose range selected (10–100 mg/kg) was based on those of other synthetic flavonoids known to exert anxiolytic activity (Karim et al., 2011; Ognibene et al., 2008). Diazepam is widely used in the dose range 1.0–3.0 mg/kg as a standard in different anxiety tests, therefore 2.0 mg/kg was selected as mid-range (Karim et al., 2011; Fernandez et al., 2009). PTZ is considered non-convulsive and anxiogenic at

doses lower than 30 mg/kg, so a 15 mg/kg dose was selected for the purpose of the current study (Hoeller et al., 2013; Rodgers et al., 1995).

2.4. Elevated plus-maze

The apparatus was comprised of two open arms (27 × 5 × 0.25 cm) and two closed arms (27 × 5 × 15 cm) extended from a central platform (5 × 5 cm) at a height of 40 cm above floor level (Macri et al., 2002). The animals were habituated in a dimly illuminated laboratory (red light, 40 lux) 1 h prior to testing. The mice were then placed on the intersection of the open and closed arms facing the open arm, 20 min post-treatment with vehicle, diazepam (2.0 mg/kg; i.p.) or 6-MeOF (10, 30, 50 and 100 mg/kg; i.p.). They were allowed to freely explore the maze for 5 min. All the experimental sessions were recorded with a digital camera (Cat's Eye IR IP Camera, Taiwan). Parameters including the percentage of entries and time spent in open arms (entries/time spent in open arms/ total entries/time × 100), time spent at the intersection/center of the apparatus (s) and closed arm entries (animal entry with all the four paws into either closed arm) were considered as spatio-temporal. Other parameters incorporating head-dipping frequency (depressing head-shoulders at the edges of maze) and the frequency of rearing were considered under ethological measures (Rodgers et al., 1995; Pellow et al., 1985). In the drug combination study, PTZ (15 mg/kg; i.p.) was administered 30 min prior to drug administration. After each trial the apparatus was thoroughly swabbed with a wet paper towel (soaked in a mixture of ethanol, detergent and water) in order to remove any odor or residues (Karim et al., 2011).

2.5. Staircase test

Mice (18-24 g) were administered vehicle, 6-MeOF (10, 30, 50 and 100 mg/kg; i.p.) or diazepam (2.0 mg/kg; i.p.). In the drug combination experiments, PTZ (15 mg/kg; i.p.) was administered

30 min prior to drug treatment. Thirty min later, the number of steps ascended and rears by each animal were observed for 3 min using the staircase apparatus according to the methods described by Simiand and coworkers (1984). A step was considered to be ascended only if the criterion was met whereby an animal placed all four paws on the step.

2.6. Open-field test

Locomotor activity was evaluated in a box with dimensions of 50 × 40 cm, and divided into four equal quadrants by lines. Mice (22 ± 2 g) were administered 6-MeOF (10, 30, 50 and 100 mg/kg; i.p.) or diazepam (2.0 mg/kg; i.p.). In the drug combination experiments, PTZ (15 mg/kg; i.p.) was administered 30 min prior to drug treatment. Thirty min later, the animals were placed at the center of the recording apparatus and the number of lines crossed and time spent at the center of the apparatus by each animal was recorded for 5 min using a digital camera (Cat's Eye IR IP Camera, Taiwan) (Hall, 1934).

2.7. Pharmacokinetic profile of 6-methoxyflavanone

6-MeOF was analyzed in plasma and selected brain areas including cerebral cortex and amygdala. The cerebral cortex and amygdala were selected because of their evident role in modulating anxiety (LeDoux, 2003; Davis, 1992). Mice (25-30 g) were administered an i.p. dose of 6-MeOF (30 mg/kg) and the plasma along with selected brain areas were collected after 15, 30, 45, 60 and 120 min post-administration (n = 6 each for different time periods). The HPLC/UV method used for the determination of 6-MeOF in the plasma and brain was highly reproducible and validated with respect to accuracy, precision, linearity, sensitivity and stability (Akbar et al., 2016).

2.7.1. Extraction procedure

For plasma samples, the collected blood was centrifuged (K240R, Centurion Scientific, UK) for 10 min and the supernatant obtained was mixed with 200 µl of HPLC grade methanol (extraction solvent) and vortex mixed (Gyromixer, Parkland Scientific Production, Pakistan) for 20 s. The sample was then centrifuged at 16,000 × g for 5 min followed by drying of the supernatant with nitrogen. The analyte was reconstituted with the mobile phase and 20 µl of sample was then injected directly into the HPLC/UV system (Xie et al., 2004). Selected brain areas were excised, weighed and homogenized (Wise stir HS 30E) in HPLC grade methanol (200 µl) at 2012.4 × g. The samples were then centrifuged at 12000 × g for 20 min and filtered through a 0.45 µm filter. The analyte obtained was dried by nitrogen evaporation and reconstituted with 200 µl mobile phase. The resulting sample was injected (20 µl) into the HPLC/UV system (de Rijke et al., 2006).

2.7.2. Analytical method

The HPLC system was operated at 30º C and consisted of double pumps (LC-20AT Shimadzu, Japan) coupled to a UV detector (SPD-20A Shimadzu, Japan) and column (Purospher C18, 250 mm × 4.6 mm × 5 µm particle size). The mobile phase was prepared by mixing few drops of 1% TEA (triethylamine) in methanol and water at a ratio of 75:25 v/v, sonicated for 15 min and filtered under vacuum through a 0.45 µm filter paper. The system flow rate was adjusted to 1.0 ml/min and the wavelength of the detector set at 240 nm, the peak of 6-MeOF being obtained within a runtime of 9 min. The peaks for different samples were confirmed by spiking them with standard 6-MeOF.

The pharmacokinetic parameters were estimated by a non-compartmental method using pharmacokinetic solutions 2.0 software package (Summit research services, Pharmacokinetics

and metabolism software 68911 open field Dr. Mantrose, CO 81401, USA).

2.8. Statistical analysis

Data were expressed as mean ± S.E.M. or S.D. One way ANOVA with *post hoc* Dunnett's test was employed, while two-factor independent ANOVA was applied in the drug combination studies using GraphPad Prism 5 (GraphPad Software Inc. San Diego CA, USA).

3. Results

3.1. Elevated plus-maze

3.1.1. Spatio-temporal parameters

Interestingly, the profile of activity exhibited by diazepam (2.0 mg/kg, $P < 0.001$) with regard to open arm entries by mice was comparable to that of 6-MeOF. Consequently, 6-MeOF [(10 mg/kg, $P < 0.05$), (30 mg/kg, $P < 0.001$) and (50 mg/kg, $P < 0.001$)] significantly increased the open arm entries in a dose-dependent manner, as compared to the vehicle [$F_{(5,30)} = 20.98$, $P < 0.0001$]. However, this parameter was decreased by the higher dose of 100 mg/kg (Fig. 2A). Similarly, the percentage of time spent in the open arms was substantially increased by 6-MeOF (30 mg/kg, $P < 0.001$ and 50 mg/kg, $P < 0.001$) [$F_{(5,30)} = 16.94$, $P < 0.0001$] (Fig. 2B). Likewise, the time spent on the central platform was also increased by 6-MeOF in a dose-dependent manner, in comparison with the vehicle treated group (30 mg/kg, $P < 0.01$ and 50 mg/kg, $P < 0.001$) [$F_{(5,30)} = 26.21$, $P < 0.0001$] (Fig. 2D). Moreover, the number of closed arm entries was not affected by 6-MeOF, suggesting that there was no effect on general locomotor activity (Fig. 2C) [$F_{(5,30)} = 1.055$, $P < 0.404$]. In the drug combination studies, pretreatment with PTZ (15 mg/kg) significantly prevented the increase promoted by 6-MeOF (30 mg/kg) in the entries [$F_{(1,30)} = 76.60$, $P < 0.0001$ for pretreatment; $F_{(5,30)} = 20.98$, $P < 0.0001$ for treatment and $F_{(1,30)} =$

10.69, $P < 0.0001$ for interaction] and time spent in the open arms [$F_{(1,30)} = 88.64$, $P < 0.0001$ for pretreatment; $F_{(5,30)} = 16.94$, $P < 0.0001$ for treatment and $F_{(1,30)} = 2.21$, $P < 0.0001$ for interaction] as shown in Fig. 5A.

3.1.2. Ethological parameters

Diazepam (2.0 mg/kg, $P < 0.001$) and 6-MeOF (at dose of 50 mg/kg, $P < 0.001$) significantly increased the frequency of wall-rearing in mice as compared to the vehicle control (Fig. 3A) [$F_{(5,30)} = 11.66$, $P < 0.0001$]. Similarly, a significant [$F_{(5,30)} = 29.25$, $P < 0.0001$] increase in the frequency of unprotected head-dipping was displayed by mice treated with either diazepam (2.0 mg/kg, $P < 0.001$) or 6-MeOF [(30 mg/kg, $P < 0.001$) and (50 mg/kg, $P < 0.001$)] as compared to the vehicle treated group (Fig. 3B).

3.2. Staircase test

As shown in Fig. 4B, 6-MeOF dose-dependently (10 mg/kg, $P < 0.05$; 30 mg/kg, $P < 0.001$; 50 mg/kg, $P < 0.001$ and 100 mg/kg, $P < 0.001$) decreased the number of rearing episodes compared to the vehicle control group [$F_{(5,30)} = 16.01$, $P < 0.0001$]. In contrast, there was biphasic response in the staircase test. Thus, 6-MeOF augmented (10 mg/kg, $P < 0.05$ and 30 mg/kg, $P < 0.001$) the number of steps ascended when compared to vehicle control (Fig. 4A) [$F_{(5,30)} = 32.09$, $P < 0.0001$] but decreased ascended step numbers at higher doses, attaining statistical significance ($P < 0.001$) at 100 mg/kg. In the drug combination studies, pretreatment with PTZ (15 mg/kg) significantly prevented the decrease induced by 6-MeOF (30 mg/kg) in the number of rears [$F_{(1,30)} = 28.50$, $P < 0.0001$ for pretreatment; $F_{(5,30)} = 16.01$, $P < 0.0001$ for treatment and $F_{(1,30)} = 9.96$, $P = 0.0949$ for interaction] and the increase in number of steps ascended [$F_{(1,30)} = 49.78$, $P < 0.0001$ for pretreatment; $F_{(5,30)} = 32.09$, $P < 0.0001$ for treatment and $F_{(1,30)} = 19.52$, $P < 0.0001$ for interaction] as shown in Fig. 5B.

3.3. Open-field test

The locomotor activity of the animal group treated with diazepam (2.0 mg/kg) or 6-MeOF (10, 30, 50 and 100 mg/kg) was compared with those administered vehicle in the open-field test. 6-MeOF at lower doses (10, 30 and 50 mg/kg) had no significant effect on locomotion, however, it dose-dependently [$F_{(5.30)}$= 37.69, P< 0.0001] increased the time spent at the center of the apparatus (10 mg/kg, P < 0.01; 30 mg/kg, P < 0.001 and 50 mg/kg, P < 0.001), when compared to vehicle control. In contrast, diazepam (2.0 mg/kg, P < 0.001) and highest dose of 6-MeOF (100 mg/kg, P < 0.001) [$F_{(5,30)}$ = 12.61, P< 0.0001] notably decreased locomotor activity and the time spent at center of the apparatus (Fig. 6). Pretreatment with PTZ (15 mg/kg) did not modify the locomotor activity but significantly decreased the promoted time spent at the center of the apparatus by 6-MeOF (30 mg/kg, P < 0.05) [$F_{(1,30)}$ = 72.71, P < 0.0001 for pretreatment; $F_{(5,30)}$ = 37.69, P < 0.0001 for treatment and $F_{(1,30)}$ = 5.97, P = 0.0014 for interaction] (Fig. 7).

3.4. Pharmacokinetic profile of 6-methoxyflavanone

The area-specific distribution of 6-MeOF in plasma and selected brain areas including the cerebral cortex and amygdala were analyzed. The highest concentration of 6-MeOF was observed in plasma (2.51 ± 1.65 µg/ml) followed by the amygdala (0.73 ± 0.07 µg/g) and cerebral cortex (0.70 ± 0.04 µg/g) 30 min after administration. These concentrations declined rapidly with time, falling below the limit of detection after 120 min in plasma and 60 min in the discrete brain areas as shown in Fig. 8. The pharmacokinetic parameters in Table 1 further verify that 6-MeOF quickly appeared in the systemic circulation with a T_{max} of 30 min and the $T_{1/2}$, C_{max} and AUC values being 46.23 ± 16.2 min, 2.7233 ± 1.5812 µg/ml and 62.982 ± 25.272 µg ml/min respectively. This was in contrast to the maximal level of 6-MeOF observed in the amygdala (AUC of 30 ± 0.083 µg g/min) compared to other brain areas.

4. Discussion

Anxiety-related disorders frequently require a pharmacological approach as a first-line treatment, although a number of unwanted side effects accompany the currently available therapeutic options. Accordingly, the demand for substitute pharmacological agents is mounting. The aim of the present study was to evaluate the behavioral profile of 6-MeOF in mice utilizing different anxiety tests. The elevated plus-maze is a validated test for the assessment of anxiety in rodents, especially while addressing benzodiazepine-induced anxiolysis. This method may be employed not only for evaluating anxiolytic but also anxiogenic effects of pharmacological agents in rodents (Pellow et al., 1985). Mice invariably prefer location within the closed arms avoiding the unprotected open arms due to fear related anxiety associated with exposure plus the elevation of the apparatus above the floor (Fernandes and File, 1996). With regard to spatio-temporal measures, the treated mice (6-MeOF and diazepam) spent appreciably longer in the open arms and on the central platform of the apparatus. The number of open arm entries was raised at the same time, implicating an anxiolytic-like effect of both 6-MeOF and diazepam. The vehicle treated mice, by way of contrast, showed preference for the closed arms and spent less time on the central platform of the apparatus. Usually, less time spent at the intersection/central platform is thought to be an index of more anxious behavior (Carola et al., 2002). Whereas, the mice treated with 6-MeOF (10, 30 and 50 mg/kg) exhibited an anxiolytic profile analogous to that of the diazepam treated mice. A novel environment is often coupled with a notable degree of anxiety that is evaluated by measuring the entries and time spent in the open arms (unprotected). Agents that augment these measures are considered to be anxiolytic, while those that reduce them are regarded as anxiogenic in nature (Macri et al., 2002; Rodgers et al., 1995; Pellow et al.,

1985). Similarly, the ethological parameters representing the anxiolytic-like behavior of 6-MeOF, in raising the frequency of unprotected head-dipping and wall-rearing were comparable to diazepam. In the context of this finding, an increased head-dipping frequency in mice is associated with low anxiety levels (Rodgers et al., 1995).

6-MeOF significantly reduced the number of rears in a dose-dependent manner without reducing the number of steps ascended in the staircase test. In light of this, $GABA_A$ receptors mediate rearing in the staircase test and anxiolytic agents are reported to decrease rearing without altering the number of steps ascended (Emmanouil and Quock, 1990). This effect was blocked by flumazenil that proved to be inactive alone. Non-benzodiazepines (such as buspirone and tricyclic antidepressants) are known to suppress both the rearing and number of steps ascended, while $GABA_A$-benzodiazepine receptor agonists display a dissociation between these two measures i.e. they suppress rearing but do not affect the number of steps ascended (Belzung et al., 1988; Pollard and Howard, 1986). This dissociated effect was also observed in our study. In the staircase test, diazepam and 6-MeOF (10 and 30 mg/kg) both reduced the incidence of rearing without decreasing the number of steps ascended. However at the higher dose of 100 mg/kg, 6-MeOF significantly reduced both measures and this high dose effect was probably derived from an α_1-subunit containing $GABA_A$ receptor mediated sedative action of the flavanone (Hall et al., 2014; Rudolph et al., 1999).

Ambulatory locomotor activity was assessed in the open-field test which has been designated as a model of normal anxiety since it is susceptible to the anxiolytic-like activity of benzodiazepines in rodents (Prut and Belzung, 2003). Mice with elevated alertness exhibit increased locomotion and conversely, a mobility decline is associated with the onset of sedation (Yadav et al., 2008). 6-MeOF did not modify locomotor activity at an anxiolytic dose level.

However, at the highest dose (100 mg/kg), it markedly reduced locomotor activity in a similar fashion to diazepam. Furthermore, an increase in the time spent in the central location of the open field arena was observed in the 6-MeOF (10, 30 and 50 mg/kg) treated as opposed to the vehicle treated animals. This is an anxiolytic-like response since mice typically display thigmotaxis by preferring peripheral arena positioning (Prut and Belzung, 2003). It is important to note that 6-MeOF exhibited sedative-depressant actions only at doses at least two to three fold higher than those producing anxiolytic-like effects. Hence, in comparison to diazepam, which has an implicit overlap of doses producing therapeutic and unwanted side effects, 6-MeOF ameliorates anxiety at doses well-below those inducing sedation.

6-MeOF induced anxiolytic-like effects at lower doses (30 and 50 mg/kg) in the unconditioned models of anxiety. In these paradigms, an increase in dose was matched by an increase in anxiolytic action. However, at the highest dose employed (i.e. 100 mg/kg), there was a loss of anxiolytic activity. In this instance, a depressant effect may well have been associated with α_1-subunit GABA mediated sedative effects of 6-MeOF due to the fact that α_1- and α_2-subunit containing $GABA_A$ receptors mediate sedative and anxiolytic activities respectively (Hall et al., 2014; Rudolph et al., 1999). Various flavonoids have been reported to act either upon the benzodiazepine site of $GABA_A$ receptors (Möhler et al., 2004; Viola et al., 1995) causing sedation or to have minimal depressant activity on the central nervous system (Fernandez et al., 2008; Viola et al., 1995; Wolfman et al., 1994) without actually inducing any quantifiable sedative effects. The low dose (up to 50 mg/kg) anxiolytic-like effect might explain the fact that 6-MeOF exhibits a much greater efficacy in modulating α_2-subunit containing $GABA_A$ receptor activity, which would appear to induce an anxiolytic action at low receptor occupancy. Thus, the selectivity of 6-MeOF for α_2-subunit containing $GABA_A$ receptors (Hall et al., 2014) along with

its *in vivo* behavioral effects in mice support its application as an anxiolytic agent.

The position 6 of the flavone nucleus is considered active even without any substitution (Huang et al., 2001) and is known for conferring anxiolytic properties to this group, whereas, the flavanone nucleus itself is thought to be inactive with regard to anxiolysis (Wang et al., 1999). However, recent studies have established that substitution at position 6 on the flavanone nucleus augments the anxiolytic properties of this group (Ognibene et al., 2008).

The biodistribution of 6-MeOF was evaluated in plasma as well as the cerebral cortex and amygdala. The pharmacokinetic profile revealed that 6-MeOF had penetrated the blood-brain barrier (BBB) because an appreciable concentration level was achieved in these brain regions 30 min after administration. Various studies on rodents and humans have reported a role of the amygdala in processing fear and anxiety-related behaviors (LeDoux, 2003; Gonzalez et al., 1996; Sananes and Davis, 1992) and intra-amygdaloid benzodiazepine injections initiate the onset of anxiolytic effects (Hodges et al., 1987). Moreover, the amygdala is extensively connected to different cerebral cortical regions, especially the medial and orbital zones of the prefrontal cortex. In respect of this, GABA afferents of the cerebral cortex synapse in the amygdala, providing a crucial inhibitory input to this region (Davis, 1992). Analysis of the pharmacokinetic data revealed an increased brain to plasma ratio in the amygdala (K_p= 0.47) followed by the cerebral cortex (K_p= 0.37) suggesting that 6-MeOF is readily distributed into these brain areas. The i*n vivo* brain to plasma ratio has been extensively utilized as a gold standard method for determining the brain uptake of drugs. The normal K_p ratio ranges from -2.00 to +1.00 and drugs having a value greater than 0.3 are considered to be readily distributed in the brain (Di et al., 2008). Thus, 6-MeOF expressed K_P ratios in the amygdala and cerebral cortex of an order indicating that they not only cross the BBB but also distribute in these brain areas which have

been implicated in anxiety.

PTZ, a non-selective $GABA_A$ receptor modulator known to act upon the picrotoxin site and reduce chloride influx (de Carvalho et al., 2011; Fraser et al., 2010), significantly reversed the anxiolytic-like effects induced by 6-MeOF in all the anxiety tests employed. However, it lacked anxiolytic activity per se in the current study. Moreover, diazepam acts upon the benzodiazepine site on the $GABA_A$ receptor complex and flumazenil is a selective antagonist at this site which is distinct from the picrotoxin site and so PTZ did not antagonize the effects of diazepam (Huang et al., 2001). *In vitro* studies have also shown that flumazenil does not reverse the $GABA_A$ receptor modulatory effects of 6-MeOF (Hall et al., 2014) suggesting a different site of action for this particular flavanone. PTZ significantly attenuated the anxiolytic effects produced by 6-MeOF, further implicating an involvement of the GABAergic system. This accords with radioligand binding studies that ascertained 6-MeOF as a positive allosteric modulator at $GABA_A$ receptors with a unique binding site (Hall et al., 2014). Hence, our findings correlate with *in vitro* binding studies and the *in vivo* behavioral profile of 6-MeOF manifests an anxiolytic proclivity.

5. Conclusion

The outcome of this study suggests that 6-MeOF penetrates the BBB generating *in vivo* anxiolytic activity, most probably mediated via flumazenil-insensitive PTZ binding sites. Therefore 6-MeOF might be employed as a therapeutic option in the treatment of anxiety, though clinical and further molecular studies are required to clarify the exact underlying protective mechanisms.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

References

Akbar, S., Subhan, F., Karim, N., Shahid, M., Ahmad, N., Ali, G., Mahmood, W., Fawad, K., 2016. 6-Methoxyflavanone attenuates mechanical allodynia and vulvodynia in the streptozotocin-induced diabetic neuropathic pain. Biomed. & Pharmacother. 84, 962-971.

Association, A.P., 1994. Diagnostic and statistical manual of mental disorders (DSM). Washington, DC: Am. Psychiatric Association 143-147.

Belzung, C., Vogel, E., Misslin, R., 1988. Benzodiazepine antagonist RO 15-1788 partly reverses some anxiolytic effects of ethanol in the mouse. Psychopharmacol. 95, 516-519.

Carola, V., D'Olimpio, F., Brunamonti, E., Mangia, F., Renzi, P., 2002. Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. Behav. Brain Res. 134, 49-57.

Chebib, M., Johnston, G.A., 2000. GABA-activated ligand gated ion channels: medicinal

chemistry and molecular biology. J. Med. Chem. 43, 1427-1447.

Davidson, J.R., 2009. First-line pharmacotherapy approaches for generalized anxiety disorder. J. Clin. Psychiat. 70, 25-31.

Davis, M., 1992. The role of the amygdala in fear and anxiety. Ann. Rev. Neurosci. 15, 353-375.

de Carvalho, R.S.M., Duarte, F.S., de Lima, T.C.M., 2011. Involvement of GABAergic non-benzodiazepine sites in the anxiolytic-like and sedative effects of the flavonoid baicalein in mice. Behav. Brain Res. 221, 75-82.

de Rijke, E., Out, P., Niessen, W., Ariese, F., Gooijer, C., Brinkman, U.A.T., 2006. Analytical separation and detection methods for flavonoids. J. Chromatogr. A. 1112, 31-63.

Di, L., Kerns, E.H., Carter, G.T., 2008.Strategies to assess blood-brain barrier penetration. Expert Opin. Drug Discov. 3, 677-687.

Emmanouil, D.E., Quock, R.M., 1990. Effects of benzodiazepine agonist, inverse agonist and antagonist drugs in the mouse staircase test. Psychopharmacol. 102, 95-97.

Fernandes, C., File, S.E., 1996. The influence of open arm ledges and maze experience in the elevated plus-maze. Pharmacol.Biochem. Behav. 54, 31-40.

Fernandez, S.P., Mewett, K.N., Hanrahan, J.R., Chebib, M., Johnston, G.A., 2008. Flavan-3-ol derivatives are positive modulators of $GABA_A$ receptors with higher efficacy for the α 2 subtype and anxiolytic action in mice. Neuropharmacol. 55, 900-907.

Fernandez, S.P., Nguyen, M., Yow, T.T., Chu, C., Jonhnston G.A.R., Hanrahan, J.R., Chebib, M., 2009. The flavonoid glycosides, myricitrin, gossypin and naringin exert anxiolytic action in mice. Neurochem. Res. 34, 1867-1875.

Festing, M.F., Altman, D.G., 2002. Guidelines for the design and statistical analysis of experiments using laboratory animals. ILAR J. 43, 244-258.

File, S.E.. 2001. Factors controlling measures of anxiety and responses to novelty in the mouse. Behav. Brain Res. 125, 151-157.

Fraser, L.M., Brown, R.E., Hussin, A., Fantana, M., Wittaker, A., O'Leary, T.P., Lederle, L., Holmes, A., Ramos, A., 2010. Measuring anxiety-and locomotion-related behaviours in mice: a new way of using old tests. Psychopharmacol. (Berl). 211, 99-112.

Gonzalez, L.E., Andrews, N., File, S.E., 1996. 5-HT1A and benzodiazepine receptors in the basolateral amygdala modulate anxiety in the social interaction test, but not in the elevated plus-maze. Brain Res. 732, 145-153.

Griebel, G., Perrault, G., Tan, S., Schoemaker, H., Sanger, D.J., 1999. Pharmacological studies on synthetic flavonoids: comparison with diazepam. Neuropharmacol. 38, 965-977.

Hall, B.J., Karim, N., Chebib, M., Johnston, G.A., Hanrahan, J.R., 2014.Modulation of ionotropic GABA receptors by 6-Methoxyflavanone and 6-Methoxyflavone. Neurochem. Res. 39, 1068-1078.

Hall, C.S., 1934. Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. J. Comp. Psychol. 18, 385-403.

Hodges, H., Green, S., Glenn, B.,1987. Evidence that the amygdala is involved in benzodiazepine and serotonergic effects on punished responding but not on discrimination. Psychopharmacol. 92, 491-504.

Hoeller, A.A., Duzzioni, M., Duarte, F.S., Leme, L.R., Costa, A.P.R., Santos, E.C.S., de Pieri, C.H., dos Santos, A.A., Naime, A.A., Farina, M., 2013. GABA-A receptor modulators alter emotionality and hippocampal theta rhythm in an animal model of long-lasting anxiety. Brain

Res. 1532, 21-31.

Hong, X., Hopfinger, A.J., 2003. 3D-pharmacophores of flavonoid binding at the benzodiazepine GABAA receptor site using 4D-QSAR analysis. J. Chem. Info. Model. Sci. 43, 324-336.

Huang, R.Q., Bell-Horner, C.L., Dibas, M.I., Covey, D.F., Drewe, J.A., Dillon, G.H., 2001. Pentylenetetrazole-induced inhibition of recombinant γ-aminobutyric acid type A ($GABA_A$) receptors: mechanism and site of action. J. Pharm.Exp. Ther. 298, 986-995.

Karim, N., Gavande, N., Wellendorph, P., Johnston, G.A.R., Hanrahan, J.R., Chebib, M., 2011. 3-Hydroxy-2′-methoxy-6-methylflavone: A potent anxiolytic with a unique selectivity profile at $GABA_A$ receptor subtypes. Biochem. Pharmacol. 82, 1971-1983.

Kempuraj D, Madhappan, B., Christodoulou, S., Boucher, W., Cao, J.,Papadopoulou, N., Cetrulo, C.L., 2005. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. Br. J. Pharmacol. 145, 934-944.

LeDoux, J., 2003. The emotional brain, fear, and the amygdala. Cell. Mol. Neurobiol. 23, 727-738.

Macdonald, R.L., Olsen, R.W., 1994. $GABA_A$ receptor channels. Annu. Rev. Neurosci. 17, 569-602.

Macrì, S., Adriani, W., Chiarotti, F., Laviola, G., 2002. Risk taking during exploration of a plus-maze is greater in adolescent than in juvenile or adult mice. Anim. Behav. 64, 541-546.

Mohler, H., Fritschy, J.M., Crestani, F., Hensch, T., Rudolph, U., 2004. Specific $GABA_A$ circuits in brain development and therapy. Biochem. Pharmacol. 68, 1685-1690.

Ognibene, E., Bovicelli, P., Adriani, W., Saso, L., Laviola, G., 2008. Behavioral effects of 6-bromoflavanone and 5-methoxy-6,8-dibromoflavanone as anxiolytic compounds. Prog. Neuro-Psychopharmacol. Biol. Psychiat. 32, 128-134.

Paladini, A., Marder, M., Viola, H., Wolfman, C., Wasowski, C., Medina, J., 1999. Flavonoids and the central nervous system: from forgotten factors to potent anxiolytic compounds. J. Pharm. Pharmacol. 51, 519-526.

Pellow, S., Chopin, P., File, S.E., Briley, M., 1985. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Meth. 14, 149-167.

Pellow, S., File, S.E., 1984. Multiple sites of action for anxiogenic drugs: behavioural,

electrophysiological and biochemical correlations. Psychopharmacol. (Berl). 83, 304-315.

Pollard, G.T., Howard, J.L., 1986. The staircase test: some evidence of nonspecificity for anxiolytics. Psychopharmacol. (Berl). 89, 14-19.

Prut, L., Belzung, C.,2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur. J. Pharmacol. 463, 3-33.

Rattka, M., Brandt, C., Bankstahl, M., Bröer, S., Löscher, W., 2011. Enhanced susceptibility to the GABA antagonist pentylenetetrazole during the latent period following a pilocarpine-induced status epilepticus in rats. Neuropharmacol. 60, 505-512.

Rodgers, R., Cole, J., Aboualfa, K., Stephenson, L., 1995. Ethopharmacological analysis of the effects of putative 'anxiogenic'agents in the mouse elevated plus-maze. Pharmacol. Biochem. Behav. 52, 805-813.

Rudolph, U., Crestani, F., Benke, D., Brunig, I., Benson, J.A., Fritschy, J-M, Martin, J.R., Bluethmann, H., Mohler, H., 1999. Benzodiazepine actions mediated by specific γ-aminobutyric acid(A) receptor subtypes. Nature 401, 796-800.

Sananes, C., Davis, M., 1992. NMDA lesions of the lateral and basolateral nuclei of the

amygdala block and shock of sensitization of fear-potentiated startle. Behav. Neurosci. 106, 72-80.

Simiand, J., Keane, P., Morre, M., 1984. The staircase test in mice: a simple and efficient procedure for primary screening of anxiolytic agents. Psychopharmacol. (Berl). 84, 48-53.

Viola, H., Wasowski, C., Levi da Stein, M., Wolfman, C., Silvers, R., Dajas, F., Madina, J.H., Paladini, A.C., 1995. Apigenin, a component of Matricaria recutita flowers, is a central benzodiazepine receptors-ligand with anxiolytic effects. Planta Med. 61, 213-216.

Wang, Q., Han, Y., Xue, H., 1999. Ligands of the $GABA_A$ receptor benzodiazepine binding site. CNS Drug Rev. 5, 125-144.

Wolfman, C., Viola, H., Paladini, A., Dajas, F., Medina, J.H., 1994.Possible anxiolytic effects of chrysin, a central benzodiazepine receptor ligand isolated from Passiflora coerulea. Pharmacol. Biochem. Behav. 47, 1-4.

Xie, F., Wulster-Radcliffe, M., Hilt, R., Kissinger, C.B., Kissinger, P.T., 2004.Determination of naringenin in rat plasma with the Culex® automated blood sampler coupled with liquid chromatography/electrochemistry. Asian J.Drug Metab. Pharmacokin. 4, 29-33.

Yadav, A., Kawale, L., Nade, V., 2008. Effect of Morus alba L.(mulberry) leaves on anxiety in mice. Indian J. Pharmacol. 40, 32-36.

Zeilhofer, H.U., Möhler, H., Di Lio, A., 2009. GABAergic analgesia: new insights from mutant mice and subtype-selective agonists. Trends Pharmacol. Sci. 30, 397-402.

FIGURE LEGENDS

Fig. 1. Chemical structure of 6-methoxyflavanone (6-MeOF).

Fig. 2. Effect of 6-methoxyflavanone (6-MeOF; 10, 30, 50, and 100 mg/kg) and diazepam (DZ; 2.0 mg/kg) in mice on (A) the % open arm entries, (B) the % time spent in open arms, (C) the number of closed arm entries and (D) the time spent in the center of the apparatus in the elevated plus-maze test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle control (Veh) (ANOVA with *post hoc* Dunnett's test) ($n = 6$).

Fig. 3. Effect of 6-methoxyflavanone (6-MeOF; 10, 30, 50, and 100 mg/kg) and diazepam (DZ; 2.0 mg/kg) in mice on (A) the wall-rearing frequency and (B) the head-dipping frequency in the elevated plus-maze test. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle control (Veh) (ANOVA with *post hoc* Dunnett's test) ($n = 6$).

Fig. 4. Effect of 6-methoxyflavanone (6-MeOF; 10, 30, 50, and 100 mg/kg) and diazepam (DZ; 2.0 mg/kg) in mice on (A) the number of steps ascended and (B) the number of rears in the staircase test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle control (Veh) (ANOVA with *post hoc* Dunnett's test) ($n = 6$).

Fig. 5. Effect in mice on the (A) % entries and time spent in the open arms in the elevated plus-maze and (B) number of rears and number of steps ascended in the staircase test, 30 min after i.p. treatment with diazepam (DZ-2.0 mg/kg), 6-methoxyflavanone (6-MeOF-30 mg/kg) pretreated with pentylenetetrazole (PTZ-15 mg/kg), pentylenetetrazole (PTZ-15 mg/kg) alone or vehicle (Veh). Bars represent mean ± S.E.M. Results were analyzed by performing 2-factor independent ANOVA among the groups. $^{**}P < 0.01$, $^{***}P< 0.001$ compared to diazepam (2.0 mg/kg) alone or 6-MeOF (30 mg/kg) alone ($n = 6$).

Fig. 6. Effect of 6-methoxyflavanone (6-MeOF; 10, 30, 50, and 100 mg/kg) and diazepam (DZ; 2.0 mg/kg) in mice on (A) locomotor activity (number of line crossings) and (B) the time spent at the center of apparatus in the open-field test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle control (Veh) (ANOVA with *post hoc* Dunnett's test) ($n = 6$).

Fig.7. Crossings and time spent at the center of the apparatus 30 min after i.p. treatment with 6-methoxyflavanone (6-MeOF-30 mg/kg) and diazepam (DZ-2.0 mg/kg) in animals pretreated with pentylenetetrazole (PTZ-15 mg/kg); pentylenetetrazole (PTZ-15 mg/kg) alone or vehicle (Veh) in the open-field test. Bars represent mean ± S.E.M. $^{*}P < 0.05$ compared to diazepam (2.0 mg/kg) alone or 6-MeOF (30 mg/kg) alone (two-way ANOVA) ($n = 6$).

Fig. 8. Area-specific distribution of 6-MeOF post administration (30 mg/kg; i.p.) at 15, 30, 45, 60 and 120 min.

Fig.9. Chromatograms showing (A) mobile phase spiked with standard 6-MeOF (1 µg/ml), and *in vivo* analyte taken 30 min post administration (30 mg/kg; i.p.) from mice (B) plasma, and (C) brain

TABLES

Table 1. Pharmacokinetic parameters of 6-methoxyflavanone (30 mg/kg; i.p.) observed in the plasma and selected brain areas of mice.

Sample	**Pharmacokinetic parameter**			
	$T_{1/2}$ **(min)**	T_{max}**(min)**	C_{max}**(µg/ml, µg/g)**	**AUC (µg ml/min, µg g/min)**
Plasma	46.23 ± 16.2	30 ± 10.7	2.7233 ± 1.5812	62.982 ± 25.272
Cerebral cortex	36.15 ± 3.06	30 ± 10.01	0.7 ± 0.008	23.23 ± 0.30
Amygdala	23.2 ± 0.16	30 ± 0.04	0.7 ± 0.012	30 ± 0.083

Values are expressed as mean ± S.D. $n = 6$.

FIGURES

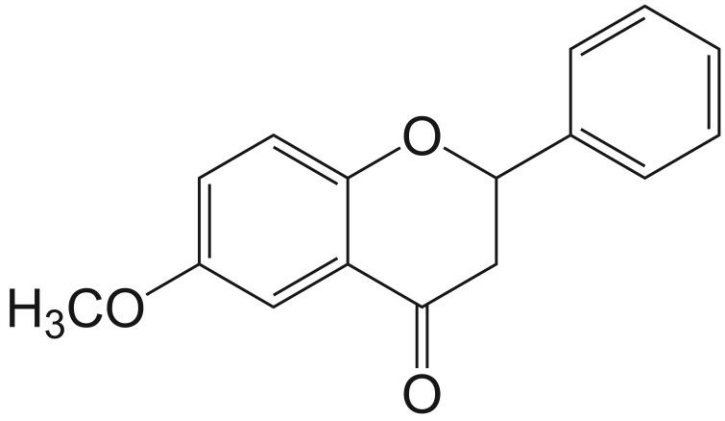


Fig. 1

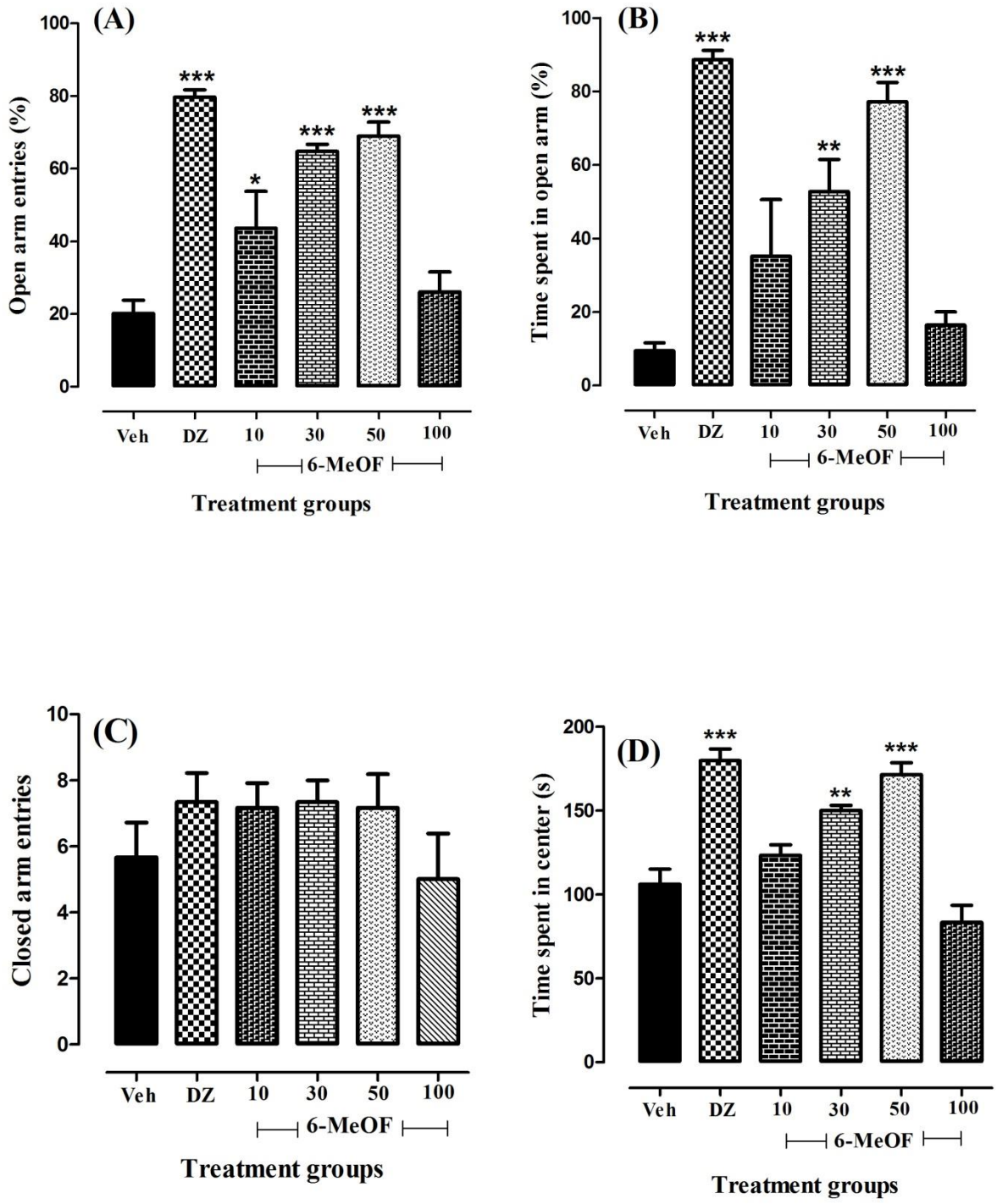
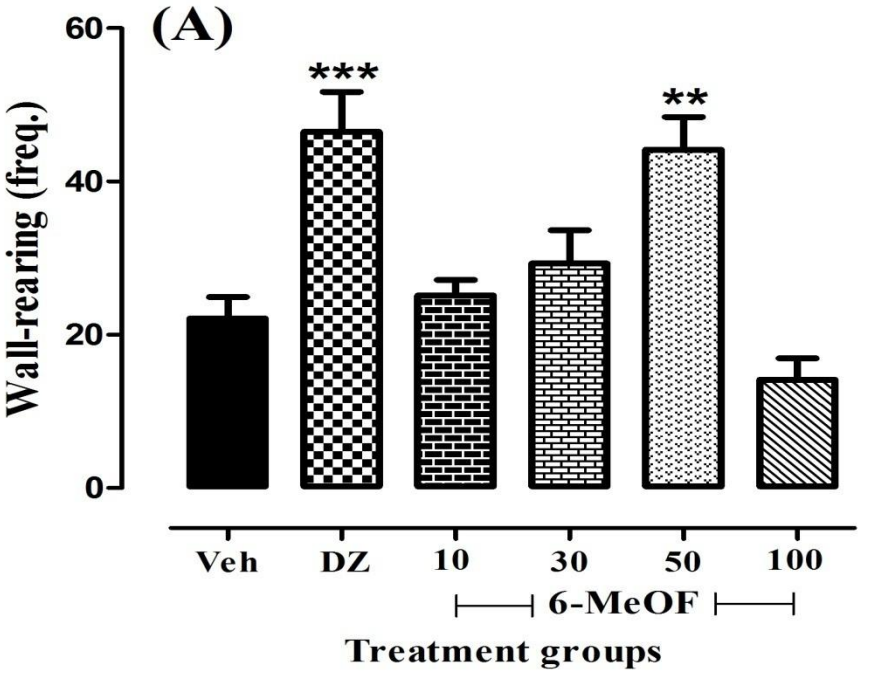

(A)
Open arm entries (%)
100
80
60
40
20
0
***
*
***
***
Veh
DZ
10
30
50
100
6-MeOF
Treatment groups
(B)
Time spent in open arm (%)
100
80
60
40
20
0
***
**
***
Veh
DZ
10
30
50
100
6-MeOF
Treatment groups
(C)
Closed arm entries
10
8
6
4
2
0
Veh
DZ
10
30
50
100
6-MeOF
Treatment groups
(D)
Time spent in center (s)
200
150
100
50
0
***
**
***
Veh
DZ
10
30
50
100
6-MeOF
Treatment groups

Fig. 2


(A)
Wall-rearing (freq.)
60
40
20
0
***
**
Veh
DZ
10
30
50
100
6-MeOF
Treatment groups

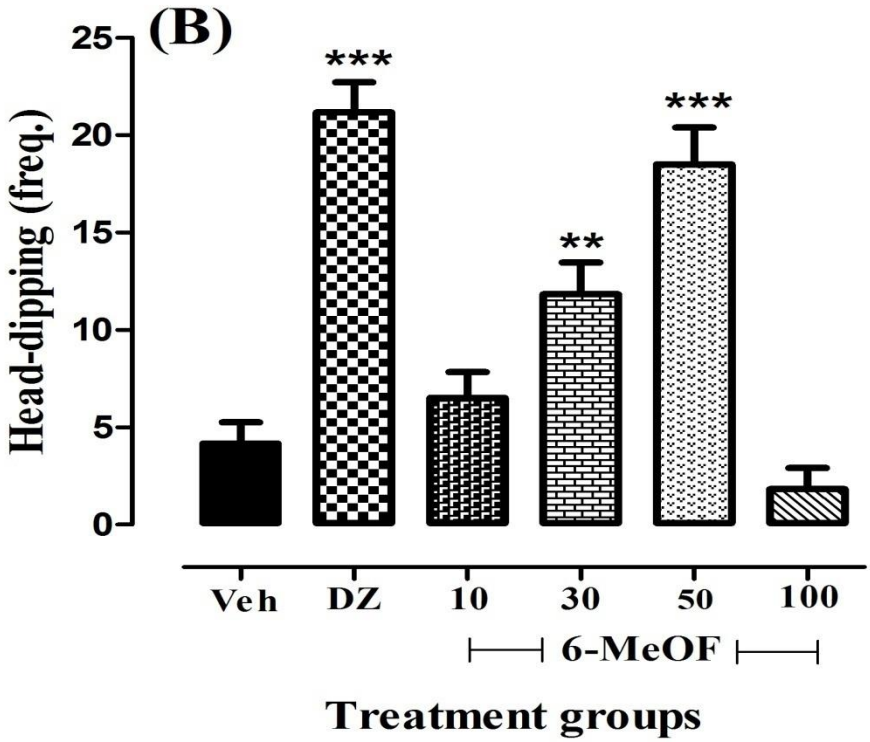

(B)
Head-dipping (freq.)
25
20
15
10
5
0
***
**
***
Veh
DZ
10
30
50
100
6-MeOF
Treatment groups

Fig. 3

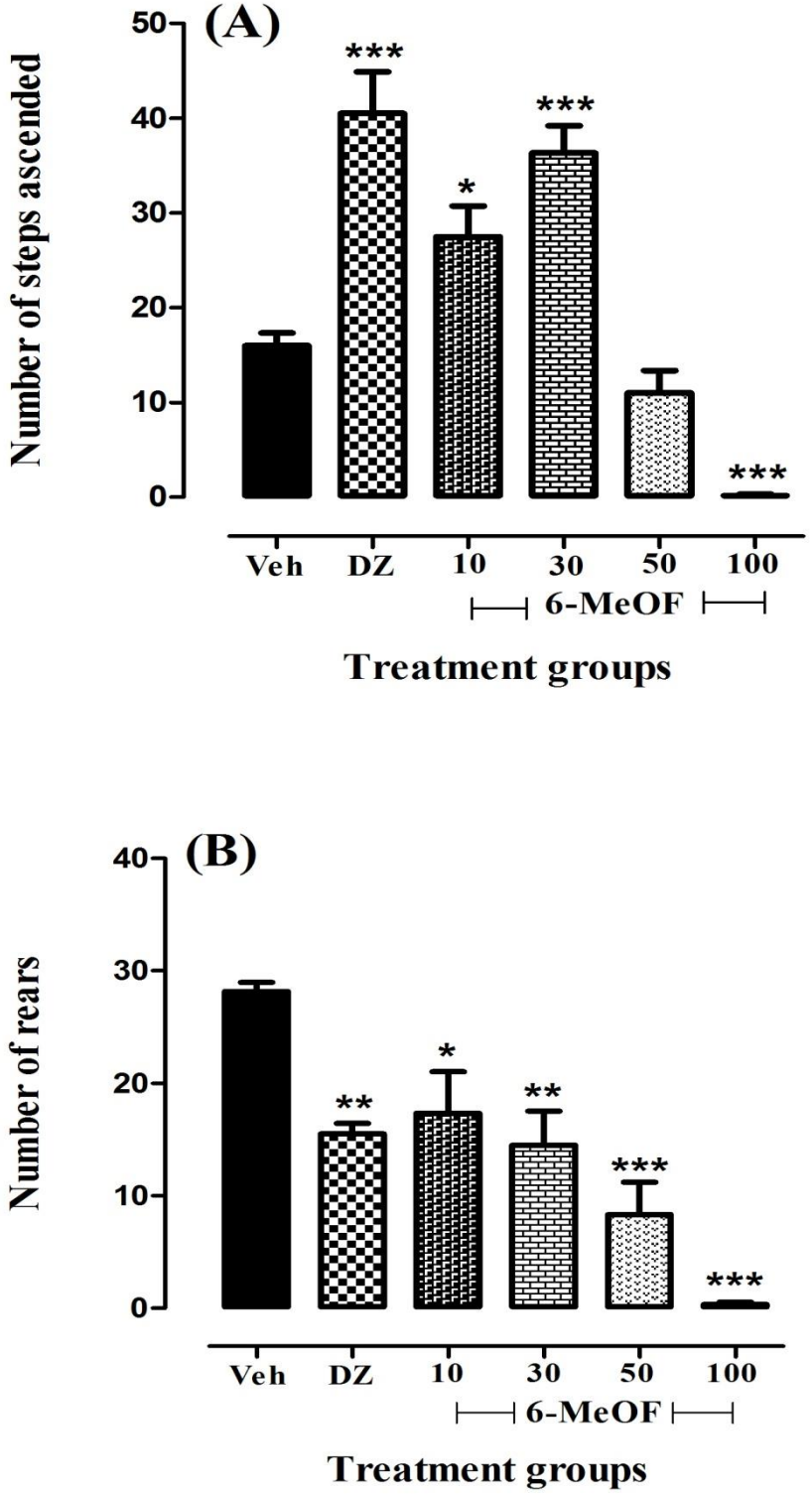

(A)
Number of steps ascended
50
40
30
20
10
0
***
*
***
***
Veh
DZ
10
30
50
100
6-MeOF
Treatment groups
(B)
Number of rears
40
30
20
10
0
**
*
**
***
***
Veh
DZ
10
30
50
100
6-MeOF
Treatment groups

Fig. 4

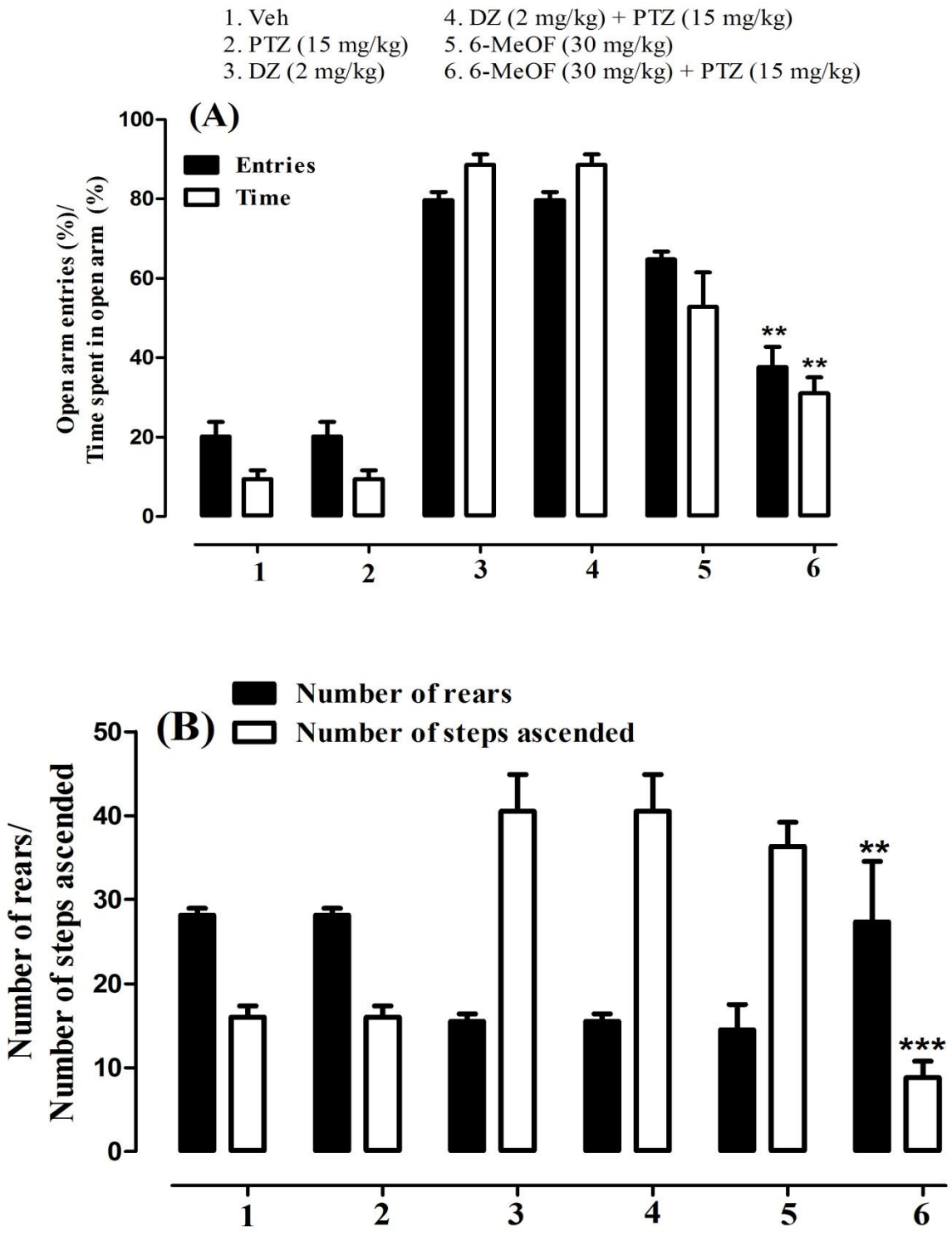

1. Veh
2. PTZ (15 mg/kg)
3. DZ (2 mg/kg)
4. DZ (2 mg/kg) + PTZ (15 mg/kg)
5. 6-MeOF (30 mg/kg)
6. 6-MeOF (30 mg/kg) + PTZ (15 mg/kg)
(A)
Entries
Time
Open arm entries (%)/
Time spent in open arm (%)
100
80
60
40
20
0
**
**
1
2
3
4
5
6
(B)
Number of rears
Number of steps ascended
Number of rears/
Number of steps ascended
50
40
30
20
10
0
**
***
1
2
3
4
5
6

Fig. 5

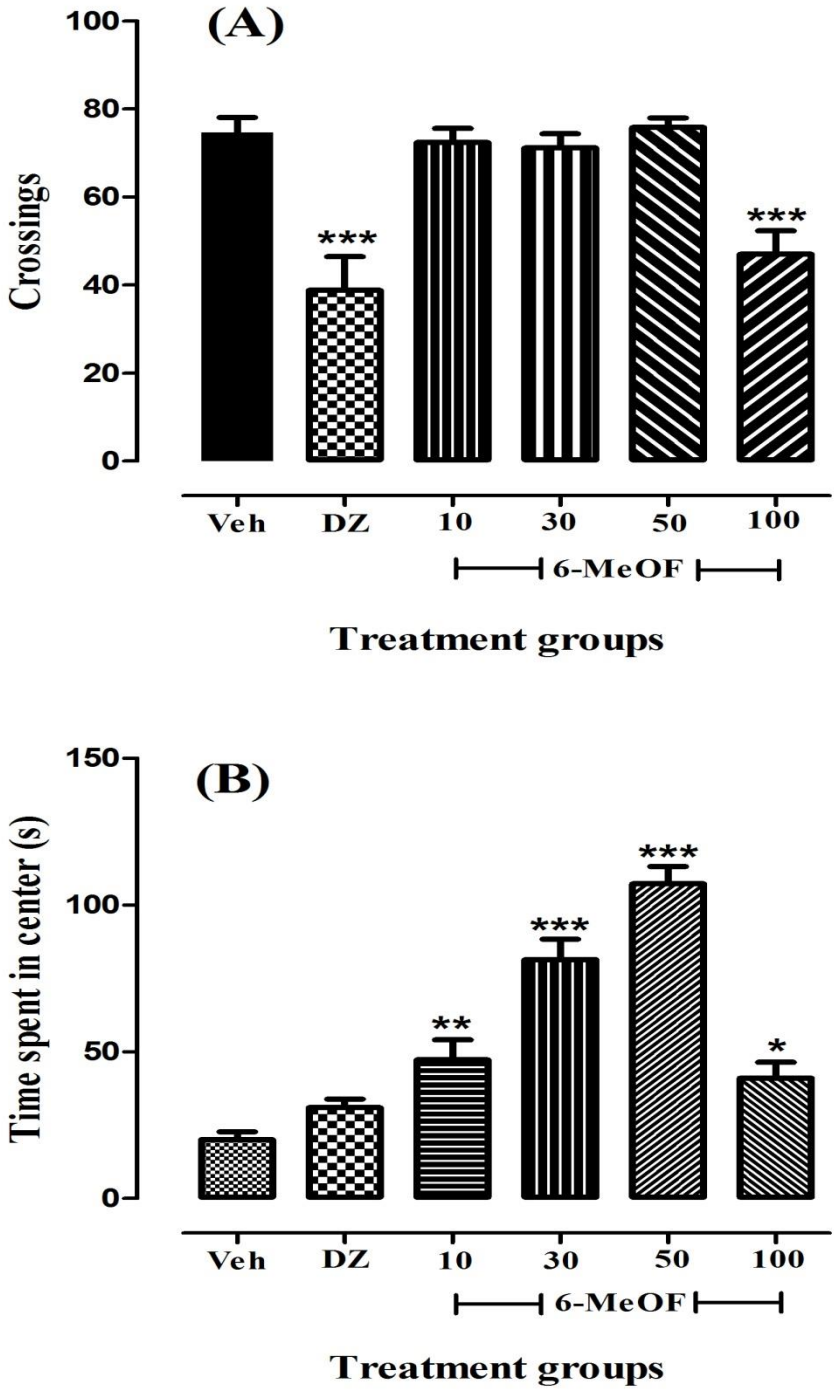

(A)
Crossings
100
80
60
40
20
0
***
***
Veh
DZ
10
30
50
100
6-MeOF
Treatment groups
(B)
Time spent in center (s)
150
100
50
0
**
***
***
*
Veh
DZ
10
30
50
100
6-MeOF
Treatment groups

Fig. 6

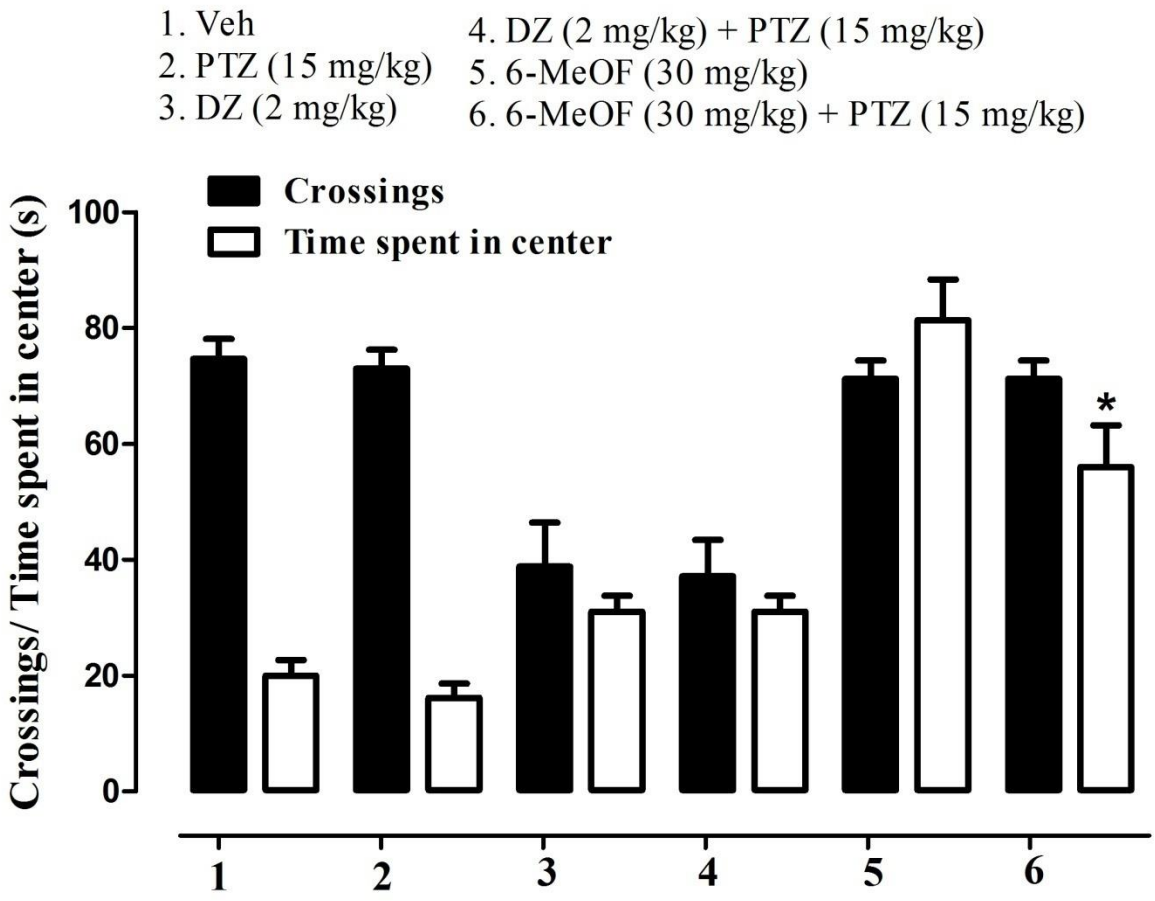

1. Veh
2. PTZ (15 mg/kg)
3. DZ (2 mg/kg)
4. DZ (2 mg/kg) + PTZ (15 mg/kg)
5. 6-MeOF (30 mg/kg)
6. 6-MeOF (30 mg/kg) + PTZ (15 mg/kg)
Crossings
Time spent in center
Crossings/ Time spent in center (s)
100
80
60
40
20
0
*
1
2
3
4
5
6

Fig. 7

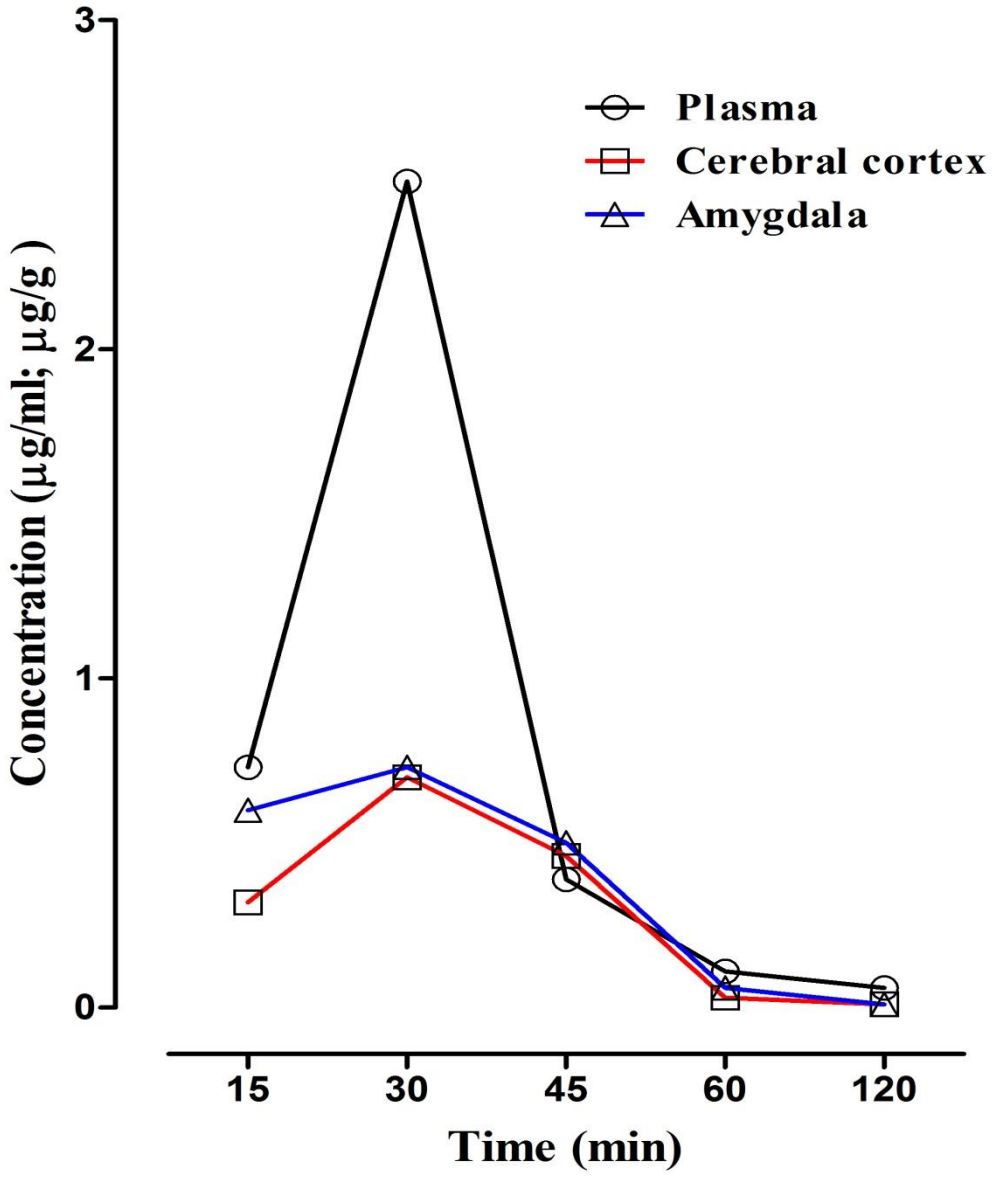

Plasma
Cerebral cortex
Amygdala
Concentration (µg/ml; µg/g)
3
2
1
0
15
30
45
60
120
Time (min)

Fig. 8

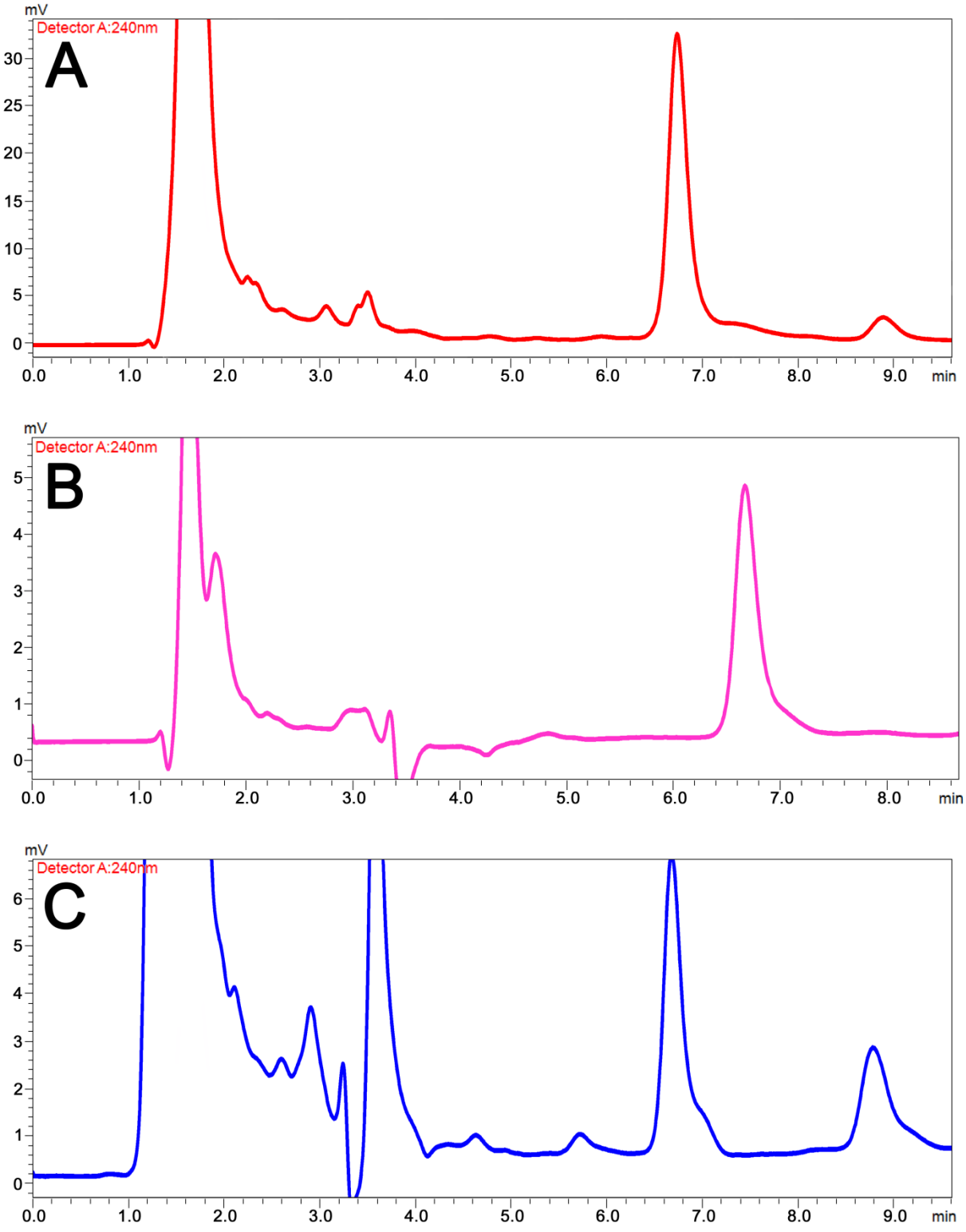

mV
Detector A:240nm
A
30
25
20
15
10
5
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
min
mV
Detector A:240nm
B
5
4
3
2
1
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
min
mV
Detector A:240nm
C
6
5
4
3
2
1
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
min

Fig. 9