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Biological profiling of piperazinediones for the management of anxiety

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ABSTRACT

The anxiolytic effect of earlier reported piperazinediones was assessed by elevated plus maze (EPM), hole-board and open-field (OFT) tests. The rats were administered pretreatment of three different doses *i.e.* 2, 1 and 0.5 mg/kg of compounds **52**, **53** and **55** for seven consecutive days. Compound **52** and diazepam showed increase in open arm entries, increase in time spent therein and total arm entries at 1 mg/kg dose. The compound also produced increase in the number of head dip, sniffing behavior and total number of squares crossed compared to diazepam. In OFT paradigm grooming behavior, number of central squares crossed and the time spent in central area did not reveal statistical differences for diazepam and compound **52** at 1 mg/kg dose. Flumazenil mediated antagonism experiments of these showed that they were acting through benzodiazepine site on GABAA receptor. The levels of 5HT and 5HIAA were estimated in amygdalar region. Level of 5HT was found to be equivalent in case of compound **52** and diazepam treatment at dose of 1 mg/kg. Interestingly, compound **52** did not display sedative effect at higher dose in both animal models. Thus, present study indicated that compound **52** produced anti-anxiety property through modulation of GABAergic transmission.

1. Introduction

Abnormal and pathological fears, leading to different mental disorders, are related to anxiety (Ernsberger et al., 1993). Further, stress (traumatic and chronic) acts as precipitating factor in psychiatric conditions and deteriorates the quality of life leading to stressful experience (Ströhle and Holsboer, 2003). The dysregulation of serotonergic, γ-amino butyric acid (GABA) and noradrenergic neurotransmitters are considered to be involved in the anxiety disorders (Hale et al., 2012; Kendall et al., 2010). The expression of various $5HT_2$ (Serotonin-2) receptors has been reported in amygdala, which is center for regulation of stress and other emotional responses (Kennett et al., 1996). The subtypes of $5HT_2$ *i.e.* $5HT_{2A}$ and $5HT_{2c}$ are found to be actively involved in anxiety. The $5HT_{2A}$, associated with the release of GABA, acts as mediator that accelerates release of GABA (Goitia et al., 2016). Therefore, $5HT_{2A}$ agonists exhibit anxiolytic effect.

It was found that amygdala $5HT_{2A}$ inhibited anxiety behavior. In amygdalar region, $5HT_{2A}$ is the crucial receptor involved in serotonergic facilitation of GABA secretion (Jiang et al., 2009). It is also established that benzodiazepines modulate the action of GABA through $GABA_A$ receptor and provide anxiolytic effect (Campo-Soria et al., 2006). Thus, any compound which facilitates $5HT_{2A}$ receptor mediated GABAergic synaptic transmission and/or increases the action of GABA at post synaptic membrane in the amygdala region, may exhibit anxiolytic effect (Garabadu and Krishnamurthy, 2014).

Previously, we developed a novel series of piperazinediones through pharmacophore-based data mining. They were found to be dual inhibitors acting through inhibition of AChE and MMP-2 for the treatment of AD (Kumar et al., 2018). Their *in-vivo* biological profiling also indicated that compound **52** had anxiolytic property and decreased neophobia. Further, various reports indicated that piperazine containing compounds showed anti-anxiety effect (Bockaert et al., 1987; Caccia et al., 1982; Sorg, 1988). This study involves screening of three piperazinediones (compounds **52**, **53** and **55**), bearing the same skeleton but varied substitutions, for the anxiolytic effect (Fig. 1).

2. Methods

2.1. Animals

Adult male Wistar rats weighing 200–210 g were used in the study. The animals were kept in polyacrylic cages (22.5 × 37.5 cm) at room temperature (24–27 °C) with 12 h dark and light cycle with food and water *ad libitum*. The food was with held 1 h before behavioral study. The procedure and quantity of animals required for the study were approved by the Institutional animal ethical committee (Protocol No.

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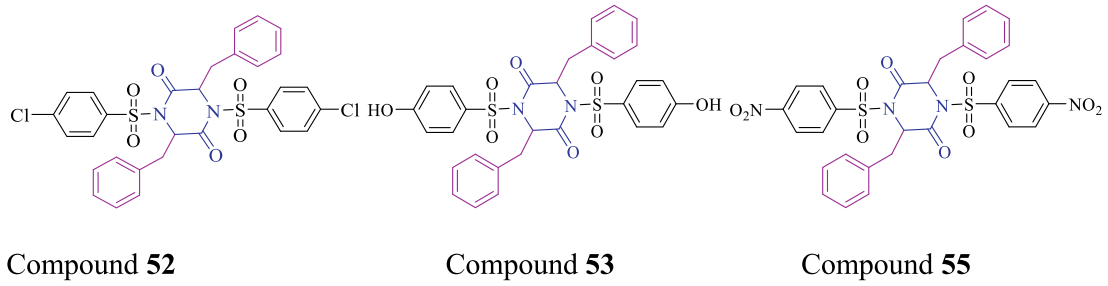

Fig. 1. Compounds used for the anxiolytic activity.

Dean/13e14/CAEC/342).

2.2. Materials

Diazepam was purchased from Sigma-Aldrich and the study was performed on elevated plus maze (EPM), open field test apparatus (OFT) and hole board test apparatus (HBT).

2.3. Experimental design

2.3.1. Experimental protocol and drug administration

Present study was divided in three sets of experiments. In the first set, rats were divided into twelve experimental groups of six animals each. These were: (i) vehicle (1 ml) (ii) Diazepam (1 mg/kg), (iii) compound **52** (0.5 mg/kg), (iv) compound **52** (1 mg/kg), (v) compound **52** (2 mg/kg), (vi) compound **53** (0.5 mg/kg) (vii) compound **53** (1 mg/kg), (viii) compound **53** (2 mg/kg), (ix) compound **55** (0.5 mg/kg) (x) compound **55** (1 mg/kg) (xi) compound **55** (2 mg/kg) and (xii) control (no treatment). The vehicle group received distilled water as vehicle while diazepam was given at dose of 1 mg/kg p.o. LD_{50} of the compounds was determined and reported earlier. Compound **52** produced significant anticholinesterase activity at dose of 10 mg/kg in scopolamine induced amnesia (Kumar et al., 2018). Further, the pilot study was undertaken and doses of 0.5, 1 and 2 mg/kg were selected for detailed study. Diazepam and test compounds **52**, **53** and **55** were freshly dissolved in distilled water before dosing. The route of drug administration was per oral (p.o.) for all the groups. Diazepam, compounds **52**, **53** and **55** were administered once daily in the groups for seven consecutive days and behavior was evaluated on the fifth, sixth and seventh consecutive days. Different sets of animals were used for EPM, OFT and HBT experiments. Amygdalar tissues were collected by the standard protocol and were stored at −80 °C for neurochemical analysis (Palkovits and Brownstein, 1988).

In the next set of experiments, GABA mediated mechanism was elucidated by taking the most active compound *i.e.* compound **52**, of the above experiments, at its effective dose (1 mg/kg). Thirty six male rats were equally divided into six groups *viz.* control (no treatment), vehicle, diazepam, compound **52** (1 mg/kg), diazepam + FZ, compound **52** (1 mg/kg) + FZ. Vehicle was administered to group vehicle (p.o.), diazepam was administered to group diazepam and diazepam + FZ (1 mg/kg, p.o.). Group compound **52** (1 mg/kg) and compound **52** (1 mg/kg) + FZ received compound **52** (1 mg/kg, p.o.). On seventh day, flumazenil (10 mg/kg, i.p.), a competitive antagonist of $GABA_A$, was administered 30 min before the oral administration of diazepam and compound **52** in diazepam + FZ and compound **52** (1 mg/kg) + FZ groups (Foyet et al., 2012).

The third set of experiments was carried out to evaluate sedative effect of compound **52** at dose of 1 mg/kg. Twenty-four male rats were divided in four different groups *viz.* control, vehicle, diazepam (6 mg/kg) and compound **52** (6 mg/kg). Sedative dose of diazepam (6 mg/kg) was administered to diazepam group (You et al., 2012) and 6 mg/kg of the compound **52** was administered to group compound **52** (6 mg/kg) for seven consecutive days.

2.3.2. Elevated plus-maze test

Rats, 30 min after dosing, were kept in previously validated elevated plus-maze apparatus. Plus-shaped wooden apparatus, made-up of four opposing arms of 30 cm × 5 cm each, was elevated at 40 cm from the floor. Two of the opposing arms, known as closed arms, were enclosed by 15 cm-high side and end walls. Whereas, the other two arms were open with no walls. Every rat was placed in the central area of maze facing toward open arm and behavior was recorded for 5 min for each rat. Sodium hypochlorite solution was used to clean up the apparatus before the placing each subject (Bagosi et al., 2018).

2.3.3. Open field test

The instrument used for this experiment was an open field box of dimensions 60 cm × 60 cm with center area of an open field box marked into 10 cm × 10 cm square. A 60 W bulb, at height of 80 cm, was used as the source of illumination. In the open field test, anxiolytic activity was evaluated for 5 min. The ratio of time spent in center and total time, the ratio of distance entries in the center area to total distance and the number of entries in the center area were the parameters of observation during 5 min. Rats were placed at the center area and activity was recorded. Sodium hypochlorite solution was used to clean up the apparatus before placing the subjects (Carola et al., 2002).

2.3.4. Hole board

The hole-board apparatus consisted of 40 × 40 cm dimension and 2.2 cm thickness having 16 equidistant holes of 3 cm diameter. The board was placed 15 cm above the table. The floor was divided into 9 squares of 10 × 10 cm each with gray water resistant marker. Rats were placed at the center of the board. Number of head dips, latency to the first head dip, and number of squares crossed with all four paws were assessed for 5 min (Santos et al., 2017).

2.3.5. Estimation of serotonin

The neurotransmitter level (5-HT) was estimated in amygdala using high performance liquid chromatography (HPLC) (Kubo et al., 1987). Briefly, the amygdala was homogenized in 0.17 M perchloric acid by glass homogenizer. Homogenates were then centrifuged at 33,000 ×*g* (REMI, India) at 4 °C. After centrifugation, 20 µl of the supernatant was injected into a column (Spherisorb, RP C18, 5 mm particle size, 4.6 mm i.d. × 250 mm at 308 C) through HPLC pump (Binary Gradient Pump) connected to an electro chemical detector (Model 2465) at a potential of 0.8 V with glassy carbon working electrode and Ag/AgCl reference electrode. The mobile phase consisted of 32 mM citric acid, 12.5 mM disodium hydrogen orthophosphate, 1.4 mM sodium octyl sulfonate, 0.05 mM EDTA, and 16% (v/v) methanol (pH 4.2). The flow rate was kept at 1.2 ml/min. Protein content was estimated colorimetrically (Lowry et al., 1951).

2.3.6. Statistical analysis

Data are presented as mean ± S.E.M. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by post-hoc Student–Newman–Keuls test. $P < 0.05$ was considered to be statistically significant (N = 6).

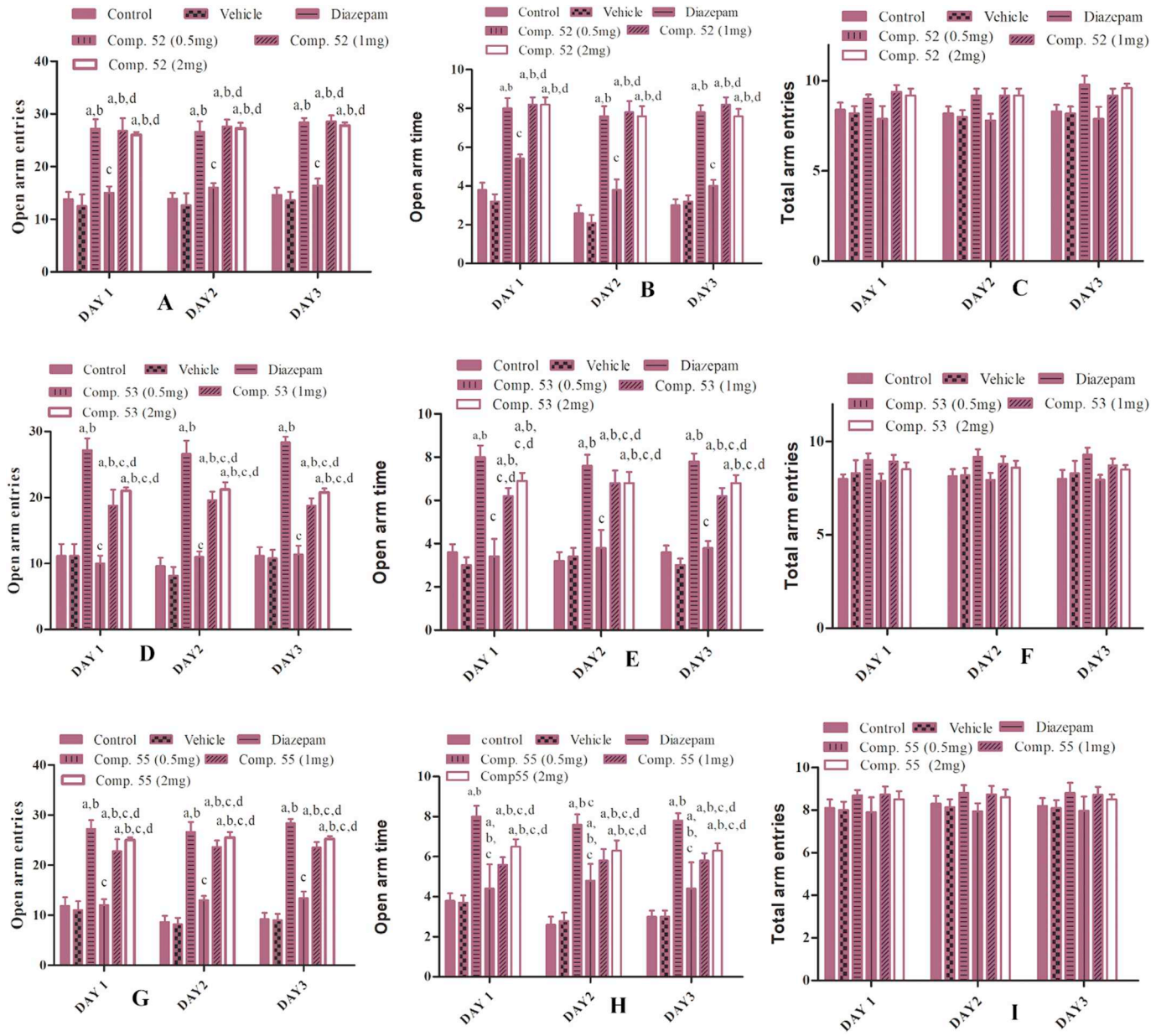

Fig. 2. Elevated plus-maze: two-way ANOVA was applied to demonstrate the results. (A, D, G) Open arm entries of animals on treatment with compounds **52**, **53** and **55** respectively ($F_A = 13.91$, $P_A = 0.0001$, $F_D = 18.64$, $P_D = 0.0001$, $F_G = 20.54$, $P_G = 0.0001$); (B, E, H) time spent in open arm by animals after treatment with compound **52**, **53** and **55** ($F_B = 13.53$, $P_B = 0.0001$, $F_E = 17.42$, $P_E = 0.0001$, $F_H = 13.91$, $P_H = 0.0001$); (C, F, I) total arm entries of animals on treatment with compound **52**, **53** and **55** ($F_c = 0.4562$, $P_c = 0.8783$, $F_F = 2.5241$, $P_F = 0.0000$, $F_I = 17.12$, $P_I = 0.0000$).

3. Results

3.1. Elevated plus-maze

Emotional behavior was evaluated by using EPM. Statistical analysis showed that there was an increase in the open arm entries and open arm time on treatment with diazepam in comparison to control and vehicle groups (Fig. 2). Open arm entry of the animals, on treatment with compound **52**, was found to be increased at dose of 1 mg/kg (Fig. 2A) and was comparable with diazepam. Open arm entry of animals, when treated with compounds **53** and **55**, were found to be less than standard (diazepam) even at the double dose (2 mg/kg, Fig. 2D, G). Open arm time spend by the animals signifies the anxiety level. Compound **52** and diazepam treatments showed low anxiety level at dose of 1 mg/kg for three consecutive days (Fig. 2B). Open arm time for the compounds **53** and **55** were found to be less than diazepam and compound **52** (Fig. 2E, H). The locomotory behavior, which is explained by the total arm entries, indicates that the synthesized compounds **52**, **53** and **55** maintain descent locomotory behavior at 1and 2 mg/kg doses (Fig. 2C, F, I). The results indicate that the synthesized compounds do not affect the locomotory center of the brain.

3.2. Open field (animal test)

Motor activity of the animals was assessed by open field test (OFT). Anxiolytic compounds surge the total time spent by rodents in the open area. The different parameters analyzed in the OFT included rearing, total number of central square crossing and time spent in central square. Rearing behavior, considered as exploratory tendency of subjects, is used as a measure of anxiety (Seibenhener and Wooten, 2015). Diazepam increased grooming, number of central square crossing and time spent in central area in OFT in comparison to control and vehicle

Table 1
Behavioral effect of compounds **52**, **53** and **55** on OFT at 0.5, 1 and 2 mg/kg doses. All values are mean ± SEM (N = 5). One-way ANOVA followed by Student–Newman–Keuls test.

Group	Ambulation (no.)	Rearing (no.)	Grooming (no.)	Number of central squares crossed (no.)	Time spent in the central area (s)
Control	58.70 ± 1.85	16.00 ± 1.83	8.71 ± 1.98	6.27 ± 1.75	6.60 ± 2.22
Vehicle	57.21 ± 1.98	16.60 ± 1.80	7.84 ± 1.24	6.38 ± 1.78	6.42 ± 2.17
Diazepam	58.8 ± 2.14	17.00 ± 2.04	16.25 ± 1.57[a,b]	14.72 ± 2.27[a,b]	14.50 ± 2.01[a,b]
Compound **52** (0.5 mg/kg)	56.24 ± 1.23	15.25 ± 1.75	9.80 ± 2.41[c]	7.58 ± 2.04	7.84 ± 1.77[c]
Compound **52** (1 mg/kg)	58.3 ± 1.92	16.00 ± 1.68	18.59 ± 1.14[a,b,d]	15.41 ± 1.70[a,b,d]	14.47 ± 1.94[a,b,d]
Compound **52** (2 mg/kg)	55.12 ± 2.21	16.04 ± 1.74	18.50 ± 1.80[a,b,d]	15.88 ± 1.75[a,b,d]	14.84 ± 2.47[a,b,d]
Compound **53** (0.5 mg/kg)	56.24 ± 2.37	16.25 ± 2.18	7.90 ± 1.51[c,e,f]	6.32 ± 1.77[c,e,f]	6.31 ± 2.58[c,e,f]
Compound **53** (1 mg/kg)	57.24 ± 2.07	16.89 ± 2.40	14.01 ± 1.52[a,b,d,g]	12.24 ± 2.04[a,b,d,e,f,g]	12.37 ± 2.17[a,b,d,g]
Compound **53** (2 mg/kg)	54.20 ± 2.05	18.01 ± 2.17	16.15 ± 1.71[a,b,d,g]	14.21 ± 1.74[a,b,d,g]	13.94 ± 1.74[a,b,d,g]
Compound **55** (0.5 mg/kg)	58.52 ± 1.68	16.17 ± 1.74	8.88 ± 1.74[c,e,f,h,i]	7.24 ± 2.04[c,e,f,h,i]	7.28 ± 2.45[c,e,f,h,i]
Compound **55** (1 mg/kg)	58.71 ± 2.47	18.01 ± 1.70	17.85 ± 1.78[a,b,d,g,j]	14.88 ± 2.27[a,b,d,g,j]	14.00 ± 2.01[a,b,d,g]
Compound **55** (2 mg/kg)	54.54 ± 2.18	17.89 ± 2.08	15.51 ± 2.08[a,b,d,g,j]	15.00 ± 2.42[a,b,d,g,j]	14.28 ± 2.54[a,b,d,g]

[a] $P < 0.005$ compared to control.
[b] $P < 0.005$ compared to vehicle.
[c] $P < 0.005$ compared to diazepam.
[d] $P < 0.005$ compared to compound **52** (0.5 mg/kg).
[e] $P < 0.005$ compared to compound **52** (1 mg/kg).
[f] $P < 0.005$ compared to compound **52** (2 mg/kg).
[g] $P < 0.005$ compared to compound **53** (0.5 mg/kg).
[h] $P < 0.005$ compared to compound **53** (1 mg/kg).
[i] $P < 0.005$ compared to compound **53** (2 mg/kg).
[j] $P < 0.005$ compared to compound **55** (0.5 mg/kg).

at dose of 1 mg/kg. Compounds **52**, **53** and **55** did not produce significant increase in rearing behavior at selected doses. Compound **52** showed enhanced grooming behavior at 1 and 2 mg/kg doses (18.59 ± 1.14, 18.50 ± 1.0 respectively) compared to diazepam (16.25 ± 1.57). Further, compounds **53** and **55** exhibited significant improvement in grooming behavior (16.15 ± 1.01, 17.85 ± 1.78) at doses of 2 and 1 mg/kg, respectively, in comparsion to control and vehicle groups. Anxiolytic compounds increase the number of central squares crossed and time spent in the central area. Compounds **52** and **55** showed statistically comparable results (compound **52**, 15.41 ± 1.20, 15.88 ± 1.75 and compound **55**, 14.88 ± 1.27, 15.00 ± 1.42 for center squares crossing and compound **52**, 14.47 ± 1.74, 14.84 ± 1.47 and compound **55**, 14.00 ± 1.01, 14.28 ± 1.54 at doses of 1 and 2 mg/kg respectively for time spent in central area) with diazepam.

OFT showed dose dependency at 0.5 and 1 mg/kg (Table 1).

3.3. Hole board

Head dip score was significantly higher in case of diazepam as compared to control and vehicle (Fig. 3A, D, G). Diazepam and compound **52** showed identical head dip score at 1 mg/kg dose for three consecutive days. Compounds **53** and **55** exhibited significantly low head dip scores when compared with diazepam at same and double doses (Fig. 3D, G). Sniffing, grooming, rattling *etc.*, are explorative behavior exhibited by rodents. Sniffing behavior of the compound **52** at 1 and 2 mg/kg doses was comparable to diazepam (Fig. 3B). Compound **53** showed significantly higher sniffing behavior as compared to diazepam at all doses, whereas, it was comparable in case of compound **55** (Fig. 3E, H). There was no significant difference in the number of squares crossed by the rats (Fig. 3C, F, I).

3.4. Amygdalar monoamines and their metabolites

Serotonin (5HT), 5-hydroxyindoleaceticacid (5HIAA) and their ratio were found to be increased in diazepam treated animals when compared with control and vehicle (Table 2). Compounds **52** and **55** treated groups possessed the same amount of 5HT and 5HIAA as diazepam at 1 and 2 mg/kg doses. Norepinephrine (NE) level was found to be approximately same in case of control, vehicle, diazepam, compounds **52**, **53** and **55** treated groups at dose 0.5 mg/kg, but it was increased in case of compounds **52** and **55** at doses of 1 and 2 mg/kg. However, significant difference in the levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), and ratios of DOPAC/DA and HVA/DA among the groups were not observed (Table 2).

3.5. Flumazenil antagonism on anxiolytic activity of compound 52 (1 mg/kg) in EPM

Diazepam and compound **52** treatments significantly increased the open arm entries and open arm time as compared with control and vehicle on seventh day. Flumazenil antagonism reductions were observed in the open arm entries and open arm time in diazepam and compound **52** treatment groups. However, there was no significant difference in flumazenil antagonism in control, vehicle, diazepam, and compound **52** groups (Fig. 4).

3.6. Flumazenil antagonism on anxiolytic activity of compound 52 (1 mg/kg) in OFT

Ambulation and rearing behavior was found to be consistent among different groups. The number of grooming, central squares crossing and time spent in the central areas were increased in case of diazepam and compound **52**, when compared with control and vehicle groups on seventh day. Flumazenil antagonism decreased these behaviors significantly as compared to diazepam and compound **52** treatment groups (Table 3).

3.7. Flumazenil antagonism on anxiolytic activity of compound 52 (1 mg/kg) in hole board

Flumazenil antagonism significantly decreased the number of head dips in compound **52** treated group as compared to diazepam, indicating increase in anxiety level (Fig. 5A). Further, Flumazenil in combination with diazepam and compound **52** showed increase in sniffing behavior which was unfavorable for anxiolytic activity (Fig. 5B). The number of squares crossed by the animals were also unchanged in different treatment groups (Fig. 5C).

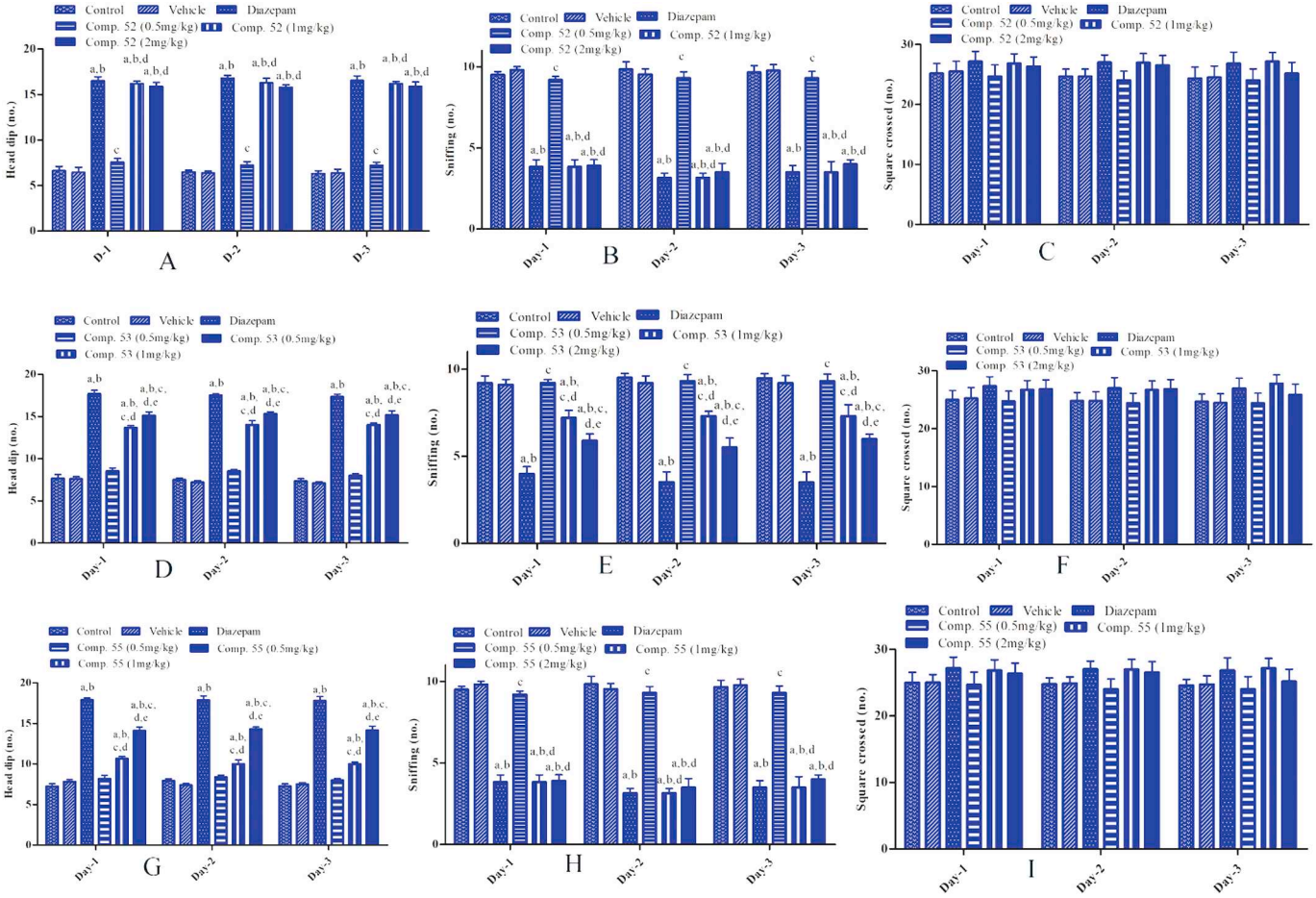

Fig. 3. Compounds **52**, **53** and **55** used for hole board experiment. (A, D, G) Effect of compounds **52**, **53** and **55** on head dip no. at dose of 0.5, 1 and 2 mg/kg. (B, E, H) Sniffing score of mice at three different doses. (C, F, I) Square crossed by the mice in hole board experiment. All values are mean ± SEM ($N = 6$). $^{a}P < 0.05$ compared to control, $^{b}P < 0.05$ compared to vehicle, $^{c}P < 0.05$ compared to diazepam, $^{d}P < 0.05$ compared to compound at dose of 0.5 mg/kg, $^{e}P < 0.05$ compared to compound at dose of 1 mg/kg (one-way ANOVA followed by Student–Newman–Keuls test).

*3.8. Sedative effect of diazepam and compound **52** in OFT and EPM tests*

Sedation is most common side effect of diazepam, which is observed at its higher doses. Behavioral parameters for the sedation in terms of OFT ambulation and EPM total arm entries at dose of 6 mg/kg of diazepam and compound **52** were represented in Fig. 6A, B. The sedative effect of diazepam was found in both animal models when compared with control. Interestingly, compound **52** did not show sedative effect at higher dose also in both animal models (Fig. 6A, B).

4. Discussion

The emotional or environmental stress might produce neuro-chemical changes leading to anxiety. Anxiety is the phenomenon which is usually associated with a specific part of brain *i.e.* limbic system. The amygdala in the limbic system initiates processing of external emotional stimuli and produces adequate response toward the same leading to anxiety (Martin et al., 2009). The amygdala is also referred as center of anxiety (Martin et al., 2009).

Various reports suggested that increased level of acetylcholine (Ach) in the brain produces anti-anxiety effect through nicotinic and muscarinic$_1$ receptors (File et al., 1998). Anxiolytic effect of Ach is mediated through hippocampus. Further, another study indicated that micro-infusion of physostigmine, an AChE inhibitor, in dorsal and ventral hippocampus had increased number of entries in open arm in EPM as well as reduced burying behavior in shock-probe test. Thus, it indicated that AChE inhibitor may have anxiolytic effect (Degroot and Treit, 2002). The hypothesis was further investigated by Degroot et al., and established that increasing hippocampal Ach level along with stimulating the GABAergic system of the medial or the lateral septum reduces anxiety (Degroot et al., 2001). We, therefore, hypothesized that previously designed, synthesized, characterized and biologically evaluated piperazinediones might serve as anti-anxiety agent. Compounds **52**, **53** and **55** were found to possess anxiolytic activity. The activity of these compounds was evaluated in different animal models. 5HT$_2$ mediated serotonergic release was found to facilitate GABA release in amygdala region (Ciranna, 2006). Behavioral and flumazenil induced antagonism showed that compounds **52**, **53** and **55** exhibited anxiolytic effect by GABA$_A$ mediated mechanism. Additionally, the most potent compound **52** was deprived of sedation. The animal model of anxiety is based on the innate general avoidance behavior. It is reported that grooming behavior is significant parameter in OFT to evaluate the anxiety. Normally, rodents avoid to spent time in central area that induces the anxiety (Carter and Shieh, 2015; Gogas et al., 2007). Treatment with diazepam and compounds **52** and **55** (1 mg/kg) showed significant anxiolytic activity. Diazepam and all other compounds at different doses are deprived of sedative effect.

Aversion of rodents for the open space is the basic principal of EPM (Commissaris, 1993). Generalized anxiety, phobia and post-traumatic stress disorder are explored by EPM (Walf and Frye, 2007). In this investigation, diazepam and compound **52** (1 mg/kg) showed maximum activity. Interestingly, none of the above compounds and diazepam exhibited decrease in the total arm entries, which signify the locomotor activity of animals.

Table 2
Pharmacological effects of compounds **52**, **53** and **55** on levels of monoamines, their metabolites and ratio in amygdala. All values are ± SEM (N = 6). One-way ANOVA followed by Student–Newman–Keuls test.

	Monoamine (ng/mg protein)								
	5HT	5HIAA	5HIAA/5HT	NE	DA	DOPAC	DOPAC/DA	HVA	HVA/DA
Control	16.52 ± 0.81	1.96 ± 0.12	0.11 ± 0.15	6.85 ± 0.30	12.24 ± 0.52	2.69 ± 0.14	0.21 ± 0.27	5.01 ± 0.13	0.40 ± 0.25
Vehicle	16.47 ± 0.95	1.90 ± 0.14	0.11 ± 0.15	6.82 ± 0.25	12.18 ± 0.43	2.64 ± 0.10	0.21 ± 0.23	4.98 ± 0.15	0.40 ± 0.35
Diazepam	27.01 ± 1.13[a,b]	5.12 ± 0.25[a,b]	0.18 ± 0.22[a,b]	7.18 ± 0.31	12.72 ± 0.60	2.90 ± 0.16	0.22 ± 0.27	5.88 ± 0.17	0.46 ± 0.28
Compound **52** (0.5 mg/kg)	17.87 ± 1.01[c]	2.24 ± 0.18[c]	0.12 ± 0.18[c]	7.12 ± 0.24	13.54 ± 0.38	2.81 ± 0.24	0.22 ± 0.63	5.74 ± 0.21	0.45 ± 0.55
Compound **52** (1 mg/kg)	27.79 ± 1.51[a,b]	5.82 ± 0.24[a,b,d]	0.19 ± 0.16[a,b,d]	8.74 ± 0.43	13.20 ± 0.66	3.41 ± 0.42	0.26 ± 0.64	5.75 ± 0.46	0.45 ± 0.70
Compound **52** (2 mg/kg)	27.55 ± 1.24[a,b]	5.57 ± 0.41[a,b,d]	0.20 ± 0.33[a,b,d]	8.43 ± 0.83	12.85 ± 1.23	3.71 ± 0.71	0.28 ± 0.58	5.52 ± 0.75	0.42 ± 0.61
Compound **53** (0.5 mg/kg)	16.81 ± 1.08[c,e,f]	2.00 ± 0.61[c,e,f]	0.11 ± 0.56[c,e,f]	6.95 ± 0.51	12.72 ± 1.05	2.70 ± 0.55	0.21 ± 0.52	5.00 ± 0.54	0.39 ± 0.51
Compound **53** (1 mg/kg)	18.72 ± 1.05[c,e,f]	3.71 ± 0.53[c,e,f]	0.19 ± 0.50[a,b,d,g]	7.52 ± 0.42	13.51 ± 0.84	3.24 ± 0.70	0.23 ± 0.83	5.58 ± 0.61	0.41 ± 0.72
Compound **53** (2 mg/kg)	20.00 ± 1.10[a,b,c,d,e,f,g]	4.28 ± 0.51[a,b]	0.24 ± 0.46[a,b,d,g]	8.01 ± 0.63	12.94 ± 0.71	3.52 ± 0.54	0.27 ± 0.76	5.21 ± 0.51	0.40 ± 0.71
Compound **55** (0.5 mg/kg)	17.31 ± 1.51[c,e,f]	2.10 ± 0.64[c,e,f]	0.12 ± 0.42[c,e,f,h,i]	7.00 ± 0.37	12.90 ± 1.04	2.74 ± 0.81	0.21 ± 0.78	4.90 ± 0.43	0.37 ± 0.41
Compound **55** (1 mg/kg)	21.51 ± 1.24[a,b,c,d,e,f,g]	4.10 ± 0.53[a,b]	0.19 ± 0.43[a,b,d,e,f,g,j]	7.88 ± 0.63	13.71 ± 0.94	3.40 ± 0.51	0.24 ± 0.56	5.35 ± 0.52	0.39 ± 0.55
Compound **55** (2 mg/kg)	26.79 ± 1.27[a,b,d,g,h,i,j,k]	5.00 ± 0.81[a,b,d,g,j]	0.18 ± 0.64[a,b,d,e,f,g,j]	8.25 ± 0.56	13.40 ± 1.32	3.80 ± 0.60	0.28 ± 0.45	5.76 ± 0.62	0.42 ± 0.47

[a] $P < 0.05$ compared to control.
[b] $P < 0.05$ compared to vehicle.
[c] $P < 0.05$ compared to diazepam.
[d] $P < 0.05$ compared to compound **52** at dose of 0.5 mg/kg.
[e] $P < 0.05$ compared to compound **52** at dose of 1 mg/kg.
[f] $P < 0.05$ compared to compound **52** at dose of 2 mg/kg.
[g] $P < 0.05$ compared to compound **53** at dose of 0.5 mg/kg.
[h] $P < 0.05$ compared to compound **53** at dose of 1 mg/kg.
[i] $P < 0.05$ compared to compound **53** at dose of 2 mg/kg.
[j] $P < 0.05$ compared to compound **55** at dose of 0.5 mg/kg.
[k] $P < 0.05$ compared to compound **55** at dose of 1 mg/kg.

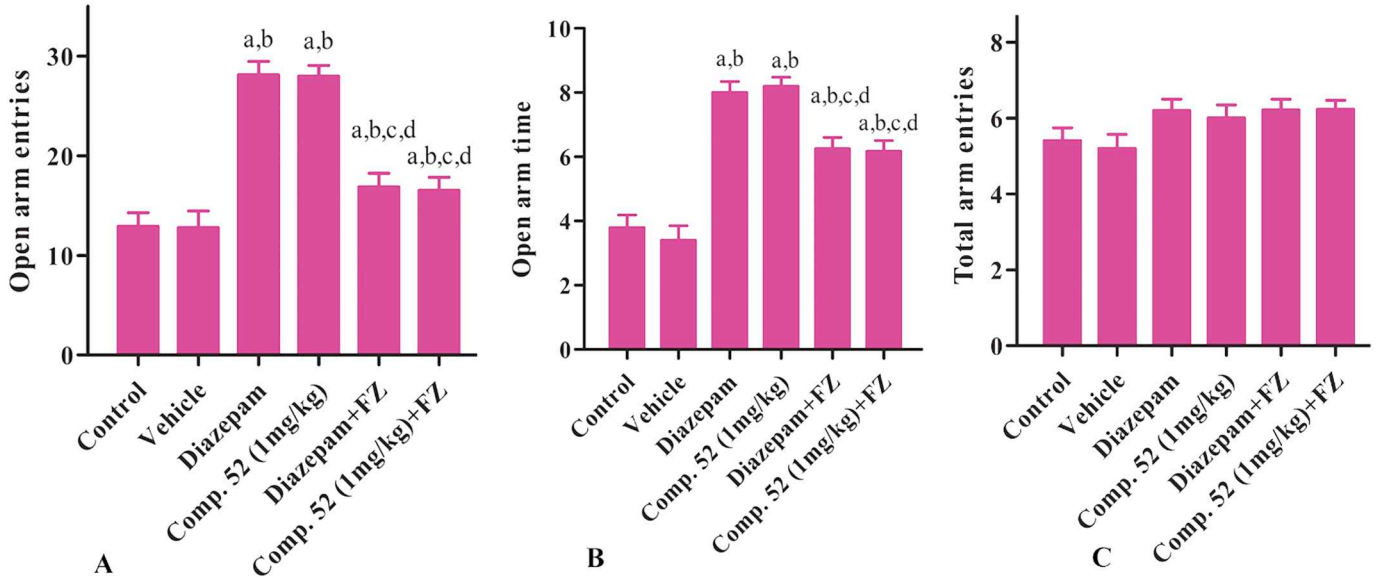

Fig. 4. Flumazenil (FZ) antagonism on the activity of diazepam and compound **52** at dose of 1 mg/kg in EPM experiment. (A) Open arm entries of animals; (B) open arm time; (C) total arm entries. All values are mean ± SEM ($N = 5$). [a]$P < 0.05$ compared to control, [b]$P < 0.05$ compared to vehicle, [c]$P < 0.05$ compared to diazepam, [d]$P < 0.05$ compared to compound **52** (1 mg/kg). (One-way ANOVA followed by Student–Newman–Keuls test).

Table 3
Flumazenil (FZ) antagonism on the activity of diazepam and compound **52** in OFT experiment at dose of 1 mg/kg. All values are mean ± SEM (N = 5). One-way ANOVA followed by Student–Newman–Keuls test.

Group	Ambulation (no.)	Rearing (no.)	Grooming (no.)	Number of central squares crossed (no.)	Time spent in the central area (s)
Control	58.20 ± 2.60	16.52 ± 2.15	8.59 ± 1.71	5.98 ± 1.81	6.84 ± 1.82
Vehicle	58.06 ± 2.34	16.55 ± 2.24	8.34 ± 1.57	6.11 ± 1.94	6.72 ± 1.54
Diazepam	58.27 ± 2.15	18.22 ± 1.84	16.41 ± 2.17[a,b]	14.59 ± 2.14[a,b]	14.74 ± 2.15[a,b]
Compound **52** (1 mg/kg)	58.58 ± 1.94	18.51 ± 2.02	18.71 ± 1.84[a,b]	15.01 ± 2.34[a,b]	14.82 ± 2.35[a,b]
Diazepam + FZ	56.99 ± 2.01	16.54 ± 2.34	9.74 ± 2.21[c,d]	8.41 ± 2.22[c,d]	7.91 ± 2.27[c,d]
Compound **52** (1 mg/kg) + FZ	57.84 ± 1.84	17.36 ± 2.14	8.57 ± 2.14[c,d]	7.41 ± 2.21[c,d]	8.42 ± 2.21[c,d]

[a] $P < 0.005$ compared to control.
[b] $P < 0.005$ compared to vehicle.
[c] $P < 0.005$ compared to diazepam.
[d] $P < 0.005$ compared to compound **52** (1 mg/kg).

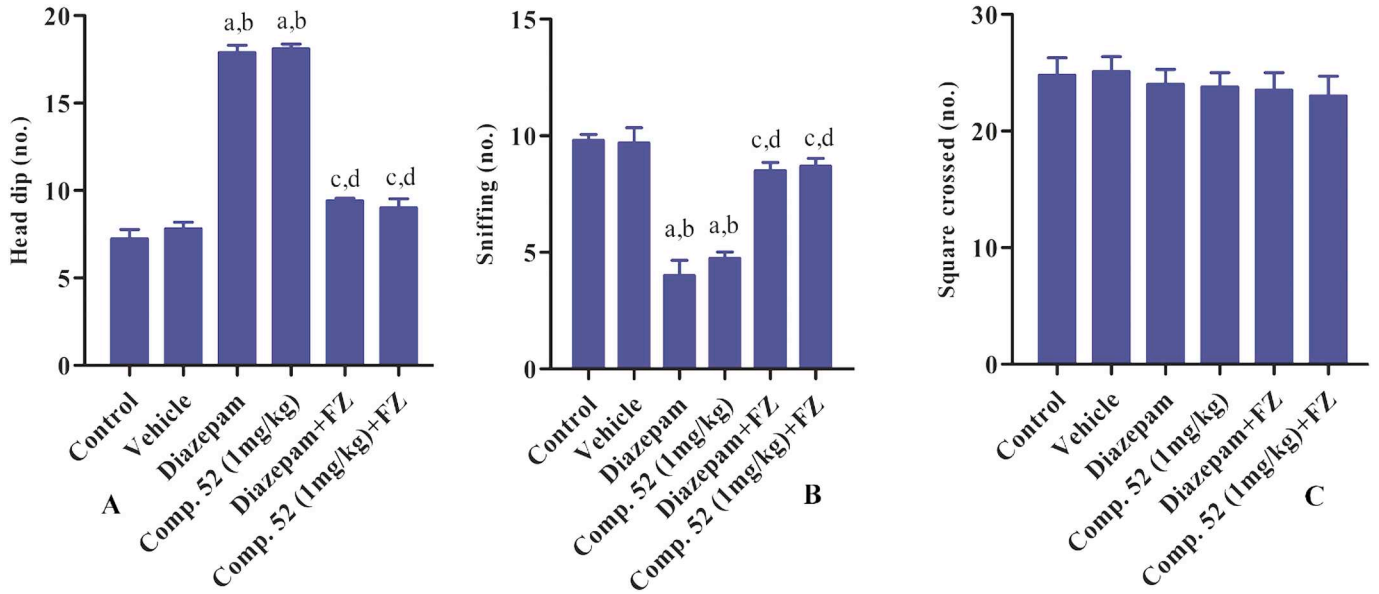

Fig. 5. Flumazenil (FZ) antagonism on the activity of diazepam and compound **52** at dose of 1 mg/kg in hole board experiment. (A) Number of head dip; (B) number of sniffing; (C) square crossed by animals. All values are mean ± SEM (N = 5). [a]$P < 0.05$ compared to control, [b]$P < 0.05$ compared to vehicle, [c]$P < 0.05$ compared to diazepam, [d]$P < 0.05$ compared to compound **52** (1 mg/kg). (One-way ANOVA followed by Student–Newman–Keuls test).

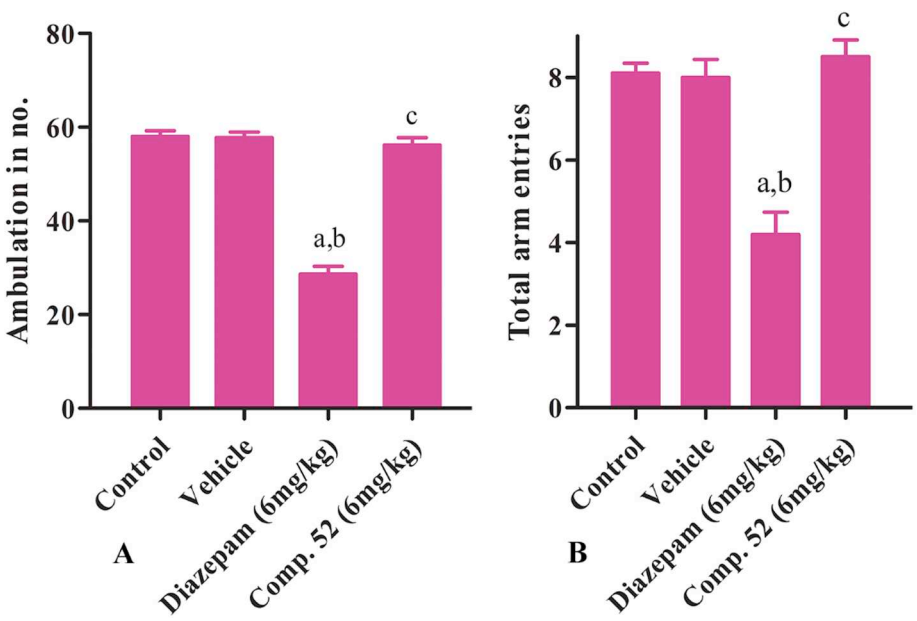

Fig. 6. The effect of diazepam (6 mg/kg, p.o.) and compound **52** (6 mg/kg; p.o.) on ambulation (a) and total arm entries (b) in OFT and EPM, respectively. All values are mean ± SEM (N = 5). [a]$P < 0.05$ compared to control, [b]$P < 0.05$ compared to vehicle, [c]$P < 0.05$ compared to diazepam (6 mg/kg) [one-way ANOVA followed by Student–Newman–Keuls test].

Hole board model specifies the anxiety in rodents. Head-dip and edge-sniff are closely related activities and are also strongly linked to anxiety. Present study showed significant anxiolytic activity in terms of head dip and sniffing behavior. Compound **52**, at dose of 1 mg/kg, was most potent among three compounds. It has been reported that number of square crossings indicated the locomotor activity. Diazepam and compounds **52**, **53** and **55** showed similar locomotor activity as control and vehicle.

The deregulation of noradrenergic, serotonergic or both, mainly in amygdalar tissues, lead to anxiety. The drugs facilitating release of any of the neurotransmitters help to manage anxiety. Our work indicates that levels of 5HT, 5HIAA and their ratio are increased in diazepam as reported earlier (Bailey and Toth, 2004). The levels of 5HT was significantly increased in case of compound **52** (1 mg/kg) and compound **55** (2 mg/kg). The level of 5HT in case of compound **52** at dose of 1 mg/kg was almost equivalent to diazepam. The levels of 5HIAA and ratios of 5HIAA/5HT were increased in diazepam and compound **52** and **55** at doses of 1 and 2 mg/kg respectively. Diazepam exhibited its anxiolytic effect by improving serotonergic release (Bailey and Toth, 2004). Based on earlier reports including diazepam, compound **52** may be postulated as an anxiolytic molecule acting by modulating amygdalar serotonergic and noradrenergic systems, which was further evaluated.

Koyama et al. reported that presynaptic 5-HT3 receptor, through Ca^{+2} influx, modulate the release of GABA in rat amygdala neurons (Koyama et al., 2000). GABA and 5HT are functionally and neuroanatomically reticulated with bidirectional relationship in system mediated activity of brain (Forchetti and Meek, 1981; Nishikawa and Scatton, 1983). Thus, $GABA_A$ mediated anxiolytic action of most active compound **52** (1 mg/kg) was evaluated by co-administration with flumazenil. Anxiolytic activity of diazepam and compound **52** was blocked in EPM, hole board and OFT animal models, without affecting locomotor activity. Flumazenil mediated antagonism of anxiolytic activity was similar to earlier reports (Foyet et al., 2012; You et al., 2012). These findings suggested that the activity of synthesized compounds may involve $GABA_A$ mediated mechanism.

Diazepam is used as a sedative at higher doses and considered its adverse effect when anxiolytic activity is desired. Thus, sedative effect of diazepam and compound **52** was evaluated at dose of 6 mg/kg. Diazepam significantly decreased the number of ambulation and total arm entries in OFT and EPM animal models. Compound **52** did not decrease the ambulation number and total arm entry when compared with control and vehicle. The results signify that the synthesized compound **52** lacks sedative effect.

5. Conclusion

It is evident from the study that compounds **52**, **53** and **55** have anxiolytic activity at different doses. Compound **52** was most active at 1 mg/kg dose. It stimulated amygdalar serotonergic and noradrenergic systems. The activity may be mediated through alterations in amygdalar $5HT_{2A}$ facilitated serotonergic response. Further, the compound exhibited $GABA_A$ mediated anxiolytic response in different animal models and lacked sedative adverse effect. Thus, it may serve as a potential drug candidate for the treatment of anxiety.

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Conflict of interest

Authors declare no conflict of interest.

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