Research Article

Mehreen Arif, Naeem Ur Rehman, Irfan Anjum, Khalid Rauf*, Amal Alotaibi, Ghala Alhmidani

6-Methoxyflavone improves anxiety, depression, and memory by increasing monoamines in mice brain: HPLC analysis and *in silico* studies

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Abstract: 6-Methoxyflavone (6-MOF) is a flavonoid that has been reported to be a GABA-A receptor agonist and reverses cisplatin-induced hyperalgesia and allodynia. Considering the varied neuropharmacological profile of 6-MOF, this study was intended to determine the pharmacological effects of 6-MOF on locomotion, anxiety, novel object recognition (NOR), depression, spatial memory, socialization behavior, nest-building behavior, and depression in various groups of mice. Selected groups of mice were injected with 25, 50, and 75 mg/kg 6-MOF. Using HPLC-UV, the frontal cortex, striatum, and hippocampus of the sacrificed mice were analyzed for the levels of vitamin C, dopamine, serotonin, noradrenaline, adenosine, and its metabolites. Statistical analysis showed significant results in socialization behavior and elevated plus maze with 75 mg/kg. In Y-maze, NOR 6-MOF showed significant results at all three doses, while in tail suspension test (TST), 50 and 75 mg/kg showed significant results; however, no statistical significance was observed in nest-building behavior; 50 and 75 mg/kg 6-MOF showed significant results in the Morris water maze. 6-MOF raised vitamin C levels in the frontal cortex and hippocampus. Serotonin, dopamine, and nor-adrenaline levels were raised in the hippocampus and striatum. It has also imparted region-specific neuroprotection by improving adenosine and its metabolite levels. *In silico* studies performed using PyRx have shown that the minimum binding energy of 6-MOF with antioxidant enzyme is −7.1 k/cal/mol. The binding energy showed that 6-MOF was successfully docked with an anti-oxidant enzyme. In conclusion, *in silico* and behavioral studies showed that 6-MOF can be a potential candidate for the treatment of cognitive decline, anxiety, and depression.

Keywords: serotonin, dopamine, noradrenaline, 6-methoxyflavone, adenosine

1 Introduction

Flavonoids are secondary metabolites found in the organs and tissues of plants. These naturally occurring metabolites are the most common and exhibit several advantageous physiological effects, making them frequently enticing in several scientific domains [1]. Flavonoids are TrkB receptor agonists as well as acetylcholine esterase inhibitors [2]. Flavonoids have varied pharmacological properties like antioxidants [3,4], antidepressants, memory enhancers [5], antiviral [6], anti-inflammatory [4,7], anticancerous [6], anxiolytic [8], and neuroprotective because of which they have been used in the treatment of several ailments [9] and antiapoptotic activities [10,11]. They have an effective role in traumatic brain injury [12] and have significant effects on methamphetamine and glutamate-induced neurotoxicity [13,14]. By interrelating with serotonin, γ-aminobutyric acid, dopamine, and glycine, they regulate the functioning of neurons [15]. Even though flavonoids improve cognitive abilities in humans and animals, the mechanism by which flavonoids

*** Corresponding author: Khalid Rauf,** Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus, Abbottabad, Khyber Pakhtoonkhwa, 22060, Pakistan,
e-mail: khalidrauf@cuiatd.edu.pk
Mehreen Arif: Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus, Abbottabad, Khyber Pakhtoonkhwa, 22060, Pakistan; Department of Basic Medical Sciences, Shifa College of Pharmaceutical Sciences, Shifa Tameer-e-Millat University, Islamabad 44000, Pakistan, e-mail: meher.adnan@gmail.com, dr.mehreen.scps@stmu.edu.pk
Naeem Ur Rehman: Faculty of Pharmacy, Gomal University, Dera Ismail Khan, Khyber Pakhtoonkhwa, 29220 Pakistan,
e-mail: pharmacistnaeem@hotmail.com
Irfan Anjum: Department of Basic Medical Sciences, Shifa College of Pharmaceutical Sciences, Shifa Tameer-e-Millat University, Islamabad 44000, Pakistan, e-mail: anjuum95@yahoo.com
Amal Alotaibi: Department of Basic Science, College of Medicine, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia, e-mail: amaalotaibi@pnu.edu.sa
Ghala Alhmidani: College of Medicine, Imam Mohammad Ibn Saud Islamic University, Riyadh, Saudi Arabia,
e-mail: ghala.al.2020@gmail.com

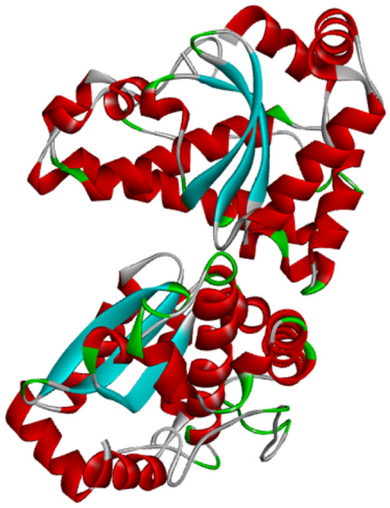

Figure 1: Three-dimensional structure of the antioxidant SOD spike receptor-binding domain with PDB ID: 1gv3.

influence cognitive function has not yet been fully understood [16].

6-Methoxyflavone (6-MOF; Figure 2) is isolated from *Anvillea garcini* leaves [17], *P. decora* leaf [18], *Pimelea simplex* and *F. Muell* [19], and ray flower of *Helianthus* series *Corona-Solis*, and is a flavonoid [20]. In the aerial parts of *Centaurea bruguierana*, 6-MOF derivatives, cirsimaritin, cirsilineol, and hispidulin, have been identified [21]. It has reported activity against ethanol-induced cognitive decline and has been shown to improve brain neurochemistry, including dopamine, noradrenaline, and serotonin. 6-MOF has also imparted antioxidant effect by improving vitamin-C levels [22]. It has been reported to alleviate cisplatin-induced neuropathies [23]. 6-MOF has been reported to act as a GABA modulator at the $GABA_A$ receptor, which is a flumazenil-insensitive allosteric modulator of human recombinant α1β2γ2L and α2β2γ2L that are expressed in the oocytes of *Xenopus laevis* [24,25]. It has been reported that 6-MOF has immunomodulatory effects because of the suppression of NFAT-mediated T-cell activation [26]. It has antioxidant and anti-inflammatory activities [27]. Studies have also reported the anti-apoptotic mechanism of 6-MOF in HeLa cells [28]. 6-MOF imparts neuroprotection in brain areas by improving the levels of adenosine, inosine, and hypoxanthine [22]. 6-MOF extracted from the leaves of *Stevia rebaudiana* has antidiabetic activity [29]. It also has antifungal activity against oral *Candida albicans* infection [30]. Although 6-MOF has poor water solubility [31,32], still upon parenteral administration, stable serum levels above 1 μg persist with higher levels found in key brain areas [33]. 6-MOF (6-methoxy-2-phenylchromen-4-one) is a colorless white powdered solid and has a molecular weight of 252.26, non-water soluble, soluble in chloroform, dichloromethane, ethyl acetate, dimethylsulfoxide (DMSO), acetone [34] and crosses the blood–brain barrier, prevents neuroinflammation, blocks nitric oxide centrally [27], antiallodynic [25] and has an LD50 >4 g/kg [35].

This study was intended to determine the pharmacological effects of 6-MOF on locomotion, anxiety via elevated plus maze (EPM), novel object recognition (NOR), depression via tail suspension test (TST), spatial memory, socialization behavior, nest-building behavior, and depression.

2 Methods

2.1 *In silico* screening of 6-MOF

2.1.1 Selection of target proteins

The 3-D structure of the antioxidant enzyme superoxide dismutase (SOD) (Figure 1) was downloaded as PDB files from the protein data bank (http://www.rcsb.org/pdb/home/home.do). In the 3-D structure of PDB, all water molecules that were not involved in ligand interaction were removed and all the missing atoms and valences were corrected (Figure 2).

2.1.2 Selection of ligands

The chemical structure of 6-MOF was downloaded from PubChem compound database in SDF format (Figure 2) (https://pubchem.ncbi.nlm.nih.gov/). It was then converted to a PDBQT file using the PyRx tool to generate atomic coordinates.

2.1.3 Target and ligand optimization

Drug Discovery Studio version 3.0 software was used to optimize the PDB coordinates of the target proteins and 6-MOF for docking studies (missing residues were added). These coordinates exhibited stable conformation and the least amount of energy.

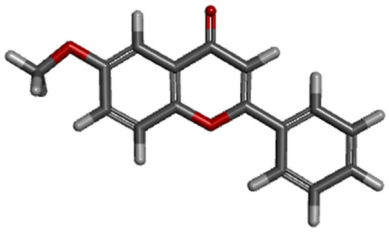

Figure 2: Ligand 6-MOF from PubChem.

2.1.4 Analysis of target active binding sites

The target protein's active binding sites were examined using the Biovia Drug Discovery Studio version 3.0. The active sites are the ligand's coordinates in the initial target protein grids (https://discover.3ds.com/discovery-studio-visualizer).

2.1.5 Molecular docking studies

The ligand- and protein-binding affinities, as well as the binding of pharmacological targets, protein receptors, or enzymes for interactions, are studied through virtual screening. We have studied molecular docking using PyRx, a free version of the program. The coordinates X = 119.202, Y = 1.0971, and Z = 8.1660 were all established in the grids that were placed in the active site pocket center, and the lowest binding energies were the most appropriate for interactions. All default docking techniques were employed [36].

2.2 Behavioral evaluation studies

2.2.1 Animals

Male BALB/c mice (22–28 g) were procured from the Veterinary Research Institute Peshawar, Pakistan (n = 6/group). The temperature in the animals' housing was kept at 22 ± 2.0°C; they had freely available food and water and maintained a circadian rhythm. Experimental protocols were approved under the ethical number **PHM.Eth/CS-M03-015-1106**, and the ethical committee of the COMSATS University Islamabad, Abbottabad campus, provided approval for all experiments that follow the UK's Animals Scientific Procedure Act (1986).

2.3 Experimental protocol

Animals were distributed into four groups. A vehicle containing DMSO, Tween 80, and normal saline (5:1:94) was used to dissolve 6-MOF [23,37]. Group 1 was administered normal saline/vehicle (10 mL/kg p.o.), and Groups 2, 3, and 4 were administered 25, 50, and 75 mg/kg of 6-MOF, respectively, orally [37]. Group 6 received fluoxetine 5 mg/kg [38] as a positive control for depression (TST), and Group 7 received diazepam 1 mg/kg [39] as a positive control for anxiety (EPM). Then, 1-h post-drug/vehicle administration, behavioral tests were performed, and after the completion of the behavioral section, animals were killed by cervical decapitation. The frontal cortex, hippocampus, and striatum were excised and stored at −80°C for neurotransmitter quantification.

2.4 Behavioral activity tests

2.4.1 Spontaneous alternation Y-maze

Three equal-length arms positioned at a 120° angle (21 cm long, 8.5 cm wide, 40 cm tall) were used. A total of 5 min was given for each animal to freely investigate each arm after being placed in the center. The camera was used to record how much time was spent on each arm. The algorithm for calculating alternations, entries in each arm, and alternation numbers was used to investigate spontaneous Y-maze activity [22].

$$\%\ \text{alternations} = \frac{\text{Total alternations}}{\text{Number of arm entries}} \times 100.$$

2.4.2 NOR

An open arena (60 cm × 50 cm × 40 cm) was used for a 3-day protocol. On day 1, the animals spent 10 min examining the test boxes (acclimatization phase). The animals were familiarized with two objects for 10 min on day 2 (training phase). On day 3, a familiar object was swapped with a new one, and each animal spent 10 min analyzing it. An overhead camera recorded the duration of each [22].

2.4.3 Socialization test

The procedure used a male juvenile mouse as the presenter animal, and the mice were accustomed for 30 min. The investigational animal was first introduced to a presenter and allowed for a 5-min interaction (sampling phase). The novel male adolescent mouse was introduced for a 5-min encounter after an hour in the recognition phase, and sniffing time was recorded [22].

2.4.4 Nest building behavior

Animals were individually given a piece of cotton nesting (5 cm × 5 cm) and then left for the night. Every single nest was evaluated according to the standards set forth by Kraeuter et al. [22].

2.4.5 Measurement of Morris water maze (MWM)

For this test, an opaque circular pool measuring 120 cm in circumference ×60 cm in height was employed. In addition to being separated into four quadrants, the pool's walls were covered with geometric shapes that functioned as special cues. Mice were trained for 5 days in the first phase (training) to locate a platform position that was 1 cm below the water's surface (13 cm wide and 34 cm high). During every training session, the platform was placed in the unchanged quadrant. In Phase 2 (Trial), there were five trials per day, with a 10-min break in between each. In each trial, one of the quadrants and a random starting point were selected, with the mice facing the wall. A secret platform had to be located and occupied by each animal within 90 s. When the animals could not locate the hidden platform, they were left there for 20 s (Phase 3). On the probe trial day, the platform and mice were permitted to delve freely for 90 s. Platform location crossings, time spent, and the number of entries in the target quadrant were recorded using a video camera [22].

2.4.6 TST

To assess the test compound's anti-depressant properties, the tail suspension method was used. With the aid of adhesive tape, each animal was held for 6 min while being suspended from its tail to a wooden apparatus that was 59 cm above the ground. A camera was used to record mobility time. Following a minute of acclimation, mobility time was recorded [40,41].

2.4.7 EPM

Anxiety-like behavior was performed using EPM. Two open arms and two closed arms (16 × 5) made up the apparatus, and 12 cm high, black-painted walls encircled closed arms. The maze was raised around 25 cm from the ground. Using a video camera placed at a height of 100 cm, the behavior was captured for 5 min. The total number of entries in the closed and open arms for each mouse was calculated. When all four of the mouse's limbs were in the arm, it was deemed legitimate [42,43].

2.4.8 Locomotor activity test (open field)

Boxes (45 cm × 45 cm) with internal line markings that divided the field into four quadrants were used to measure the locomotor activity (22.5 cm × 22.5 cm). A video camera was positioned 230 cm above each animal's locomotor box to record the event as it transpired. Each animal was then placed inside. The equipment was thoroughly cleaned with 70% ethanol in between animal examinations, and the times the lines crossed at a rate of 30 per minute were recorded [22].

2.5 Neurotransmitter and vitamin C quantification using HPLC

2.5.1 Sample and standard preparation

Following the behavioral assessment, the mice were decapitated, frontal cortical, striatal, and hippocampal tissues were excised, precisely weighed, and maintained at −80°C. Homogenization of the tissues was carried out on chilled perchloric acid (0.2%) following cold centrifugation (4°C; 12,000 rpm), and the supernatant was separated. The standard stock solutions were made by dissolving 1 mg of each of the following substances in 10 mL of HPLC-grade water. The standard solution of all neurotransmitters was then diluted to provide various concentrations ranging from 100 to 500 ng/mL [22].

2.5.2 Chromatographic conditions

For the chromatographic analysis, a Waters Alliance 2690 separation module with a UV detector was utilized (USA). A 5 µm particle size (250 × 4.6 mm) C18 stainless steel column was utilized. HPLC grade water and 20 mM monobasic sodium phosphate (5:95, v/v) were employed with isocratic elution at 280 nm and a column temperature of 35°C, and the elution rate of 0.5 mL/min for vitamin C, dopamine, noradrenaline, and vitamin C methanol. HPLC grade water and 0.01 M monobasic sodium phosphate (5:95 v/v) were employed for the measurement of adenosine, inosine, hypoxanthine, acetonitrile, and isocratic elution at 260 nm, the column was at room temperature, and a flow rate was 1 mL/min [22].

2.6 Statistical analysis

Graph Pad Prism-8 was used, and the results are presented as ±SEM. ANOVA (one-way) and a *post hoc* Dunnett's test were used. Data significance was as follows: $*p > 0.05$, $**p > 0.01$ and $***p > 0.001$.

The target proteins were inserted using the PyRx program, and the imaging was done with Discovery Studio (Accelrys, San Diego, CA, USA), a potent simulation tool.

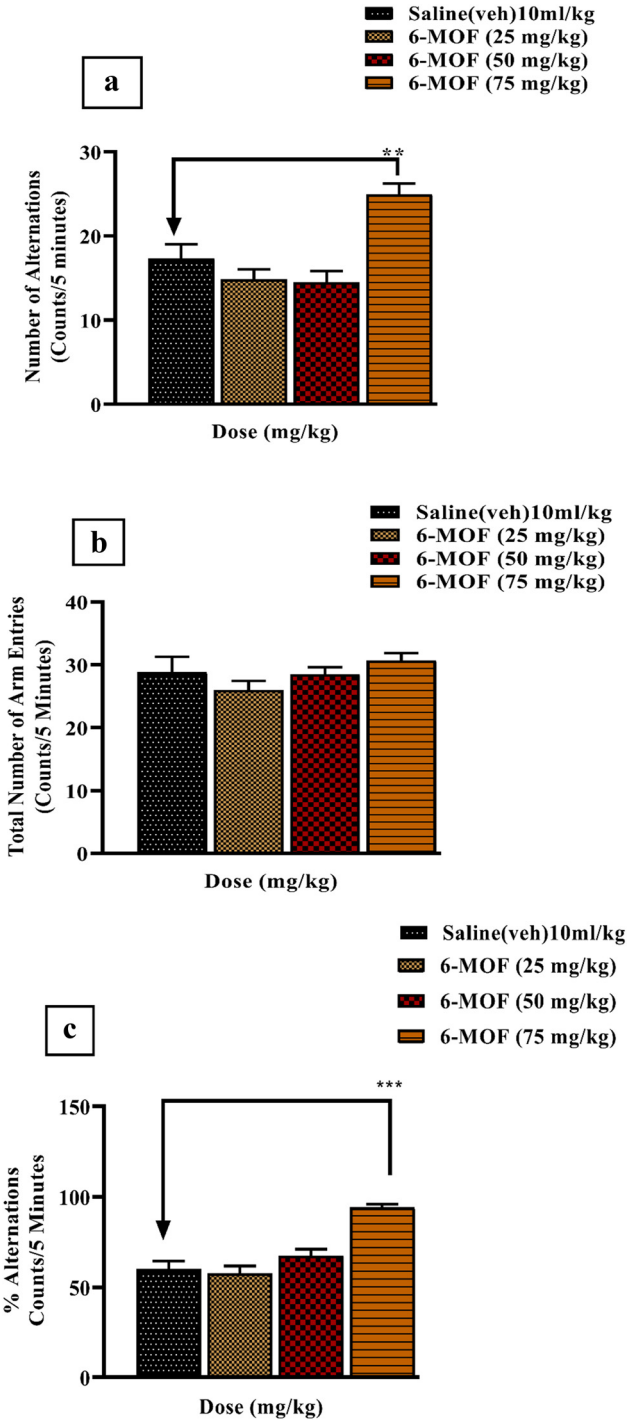

Figure 3: Effect of 6-MOF on Y-Maze: (a) number of alternations, (b) number of entries in each arm, and (c) percentage of alternations. $^{**}p < 0.01$ and $^{***}p < 0.01$.

3 Results

3.1 Effect of 6-MOF on the spontaneous alternation of Y-maze

6-MOF showed considerable improvement in spontaneous alternations with the highest dose ($^{**}p < 0.01$) (Figure 3a). However, no substantial disparity in the number of entries was observed (Figure 3b). A considerable improvement in the percentage of alternations was observed with the two highest tested doses of 6-MOF ($^{***}p < 0.01$) (Figure 3c) relative to the saline group.

3.2 Effect of 6-MOF on NOR

Following the administration of 6-MOF, a notable increase in the amount of time spent investigating novel objects relative to a familiar one was noticed with 25 mg/kg ($^{*}p < 0.05$), 50, and 75 mg/kg ($^{***}p < 0.001$) relative to the saline group (Figure 4).

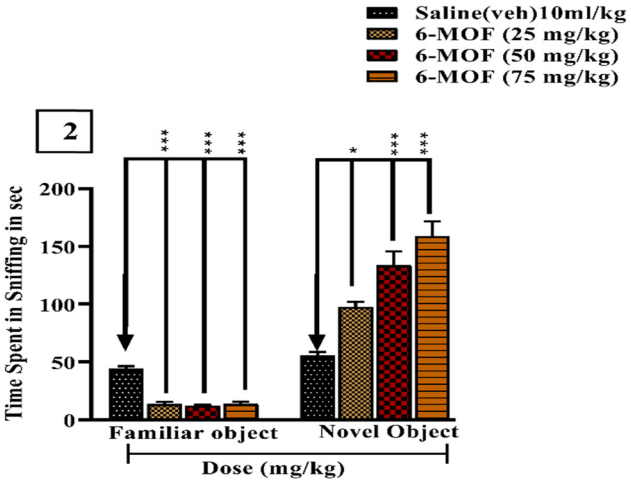

Figure 4: Effect of 6-MOF on NOR test. $^{*}p < 0.05$ and $^{***}p < 0.001$.

3.3 Effect of 6-MOF on socialization behavior

6-MOF showed significant improvement in the socialization behavior, as the treatment showed improved recognition of previously presented mice but results are not statistically significant. However, compared to a novel mouse, significantly enhanced socialization was observed with the highest dose of 6-MOF, i.e., 75 mg/kg ($^{*}p < 0.05$) of 6-MOF compared to the saline group (Figure 5).

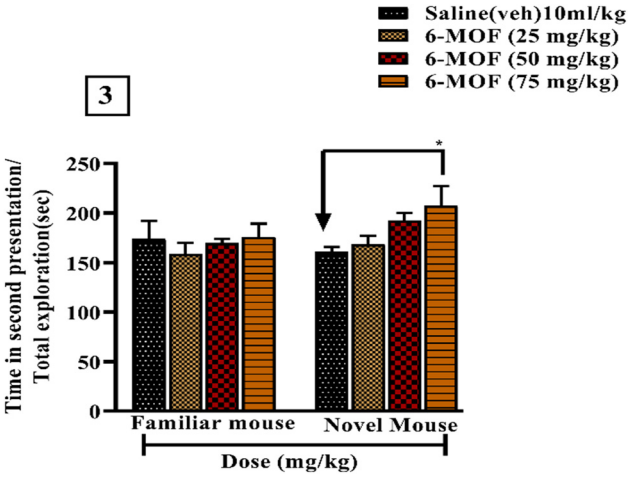

Figure 5: Effect of 6-MOF on socialization behavior; *$p < 0.05$.

3.4 Effect of 6-MOF on nest-building behavior

Treatment with 6-MOF showed a marked positive emotional state and improved skilled-based learning as the height, width, and quality of the nest built by treated mice were significant relative to the saline group. However, the results are statistically not significant (Figure 6a–c).

3.5 Effect of 6-MOF on the MWM

The highest dose of 6-MOF, 75 mg/kg, significantly augmented the amount of time spent in the target quadrant (**$p < 0.01$) (Figure 7a). The two highest doses have shown considerable improvement in the overall number of entries in the target quadrant (**$p < 0.01$) (Figure 7b), as well as the number of platform location crossings (***$p < 0.001$) (Figure 7c) compared to the saline group.

3.6 Effect of 6-MOF on the TST

A momentous intensification in mobility time was observed with the two highest doses of 50 mg/kg (**$p < 0.01$) and 75 mg/kg (***$p < 0.001$) as well as fluoxetine 5 mg/kg (***$p < 0.001$) relative to the saline group (Figure 8).

3.7 Effect of 6-MOF on the EPM

6-MOF showed a noteworthy anxiolytic effect as animals showed significant improvement in time spent in open arms with the highest tested dose (*$p < 0.05$); diazepam also showed a significant anxiolytic effect (***$p < 0.001$) relative to the saline group (Figure 9).

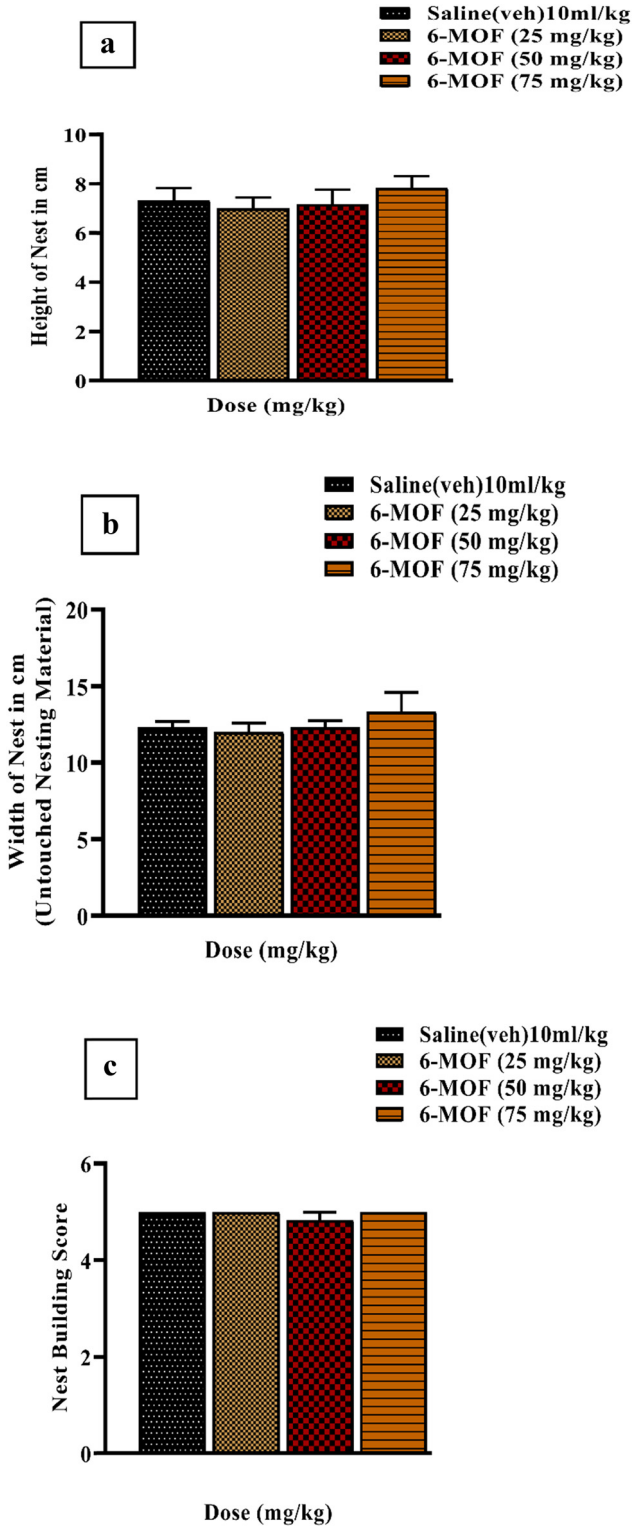

Figure 6: Effect of 6-MOF on the nest building behavior: (a) height of the nest, (b) width of the nest, and (c) nest building score. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and (d–g) represents the nest builds.

Figure 6: (*Continued*)

3.8 Effect of 6-MOF on the locomotor activity (open field test)

6-MOF showed significant improvement in locomotion as a momentous hyperlocomotion was observed at all three doses (**$p < 0.01$, ***$p < 0.001$) relative to the saline group (Figure 10).

3.9 Effect of 6-MOF on the frontal cortex tissue levels of dopamine, noradrenaline, serotonin, and vitamin C

A substantial increase in frontal cortical noradrenaline levels was observed with 75 mg/kg (***$p < 0.001$) 6-MOF. Frontal cortical vitamin C levels were also boosted at all three doses (*$p < 0.05$). However, no effect was observed on dopamine and serotonin relative to the saline group (Table 1).

3.10 Effect of 6-methoxyflavone on the hippocampal tissue levels of dopamine, noradrenaline, and vitamin C

6-MOF has remarkably improved hippocampal dopamine and noradrenaline levels with the highest tested dose (***$p < 0.001$ and **$p < 0.01$). An increase in serotonin levels was observed at all three doses (*$p < 0.05$ and ***$p < 0.001$), respectively. However, all three doses have shown marked improvement in the hippocampal vitamin C levels (**$p < 0.01$ and ***$p < 0.001$) relative to the saline group (Table 2).

3.11 Effect of 6-MOF on the striatal tissue levels of dopamine, noradrenaline, serotonin, and vitamin C

Considerable improvement in the striatal dopamine levels was observed at 75 mg/kg (***$p < 0.001$). All three administered doses showed improvement in the noradrenaline levels (*$p < 0.05$ and ***$p < 0.001$). The highest dose of 6-MOF improved striatal vitamin C and serotonin levels (*$p < 0.05$ and ***$p < 0.001$), respectively, relative to the saline group (Table 3).

3.12 Effect of 6-MOF on the frontal cortex tissue levels of adenosine, inosine, and hypoxanthine

A notable frontal cortical neuroprotection was imparted at all three doses of 6-MOF, as a significant decrease in adenosine and hypoxanthine levels was observed T all three doses of 6-MOF (***$p < 0.001$). Inosine levels were also significantly decreased AT all three doses (*$p < 0.05$ and ***$p < 0.001$) as well as hypoxanthine levels were also decreased AT all three doses (***$p < 0.001$) relative to the saline group (Table 4).

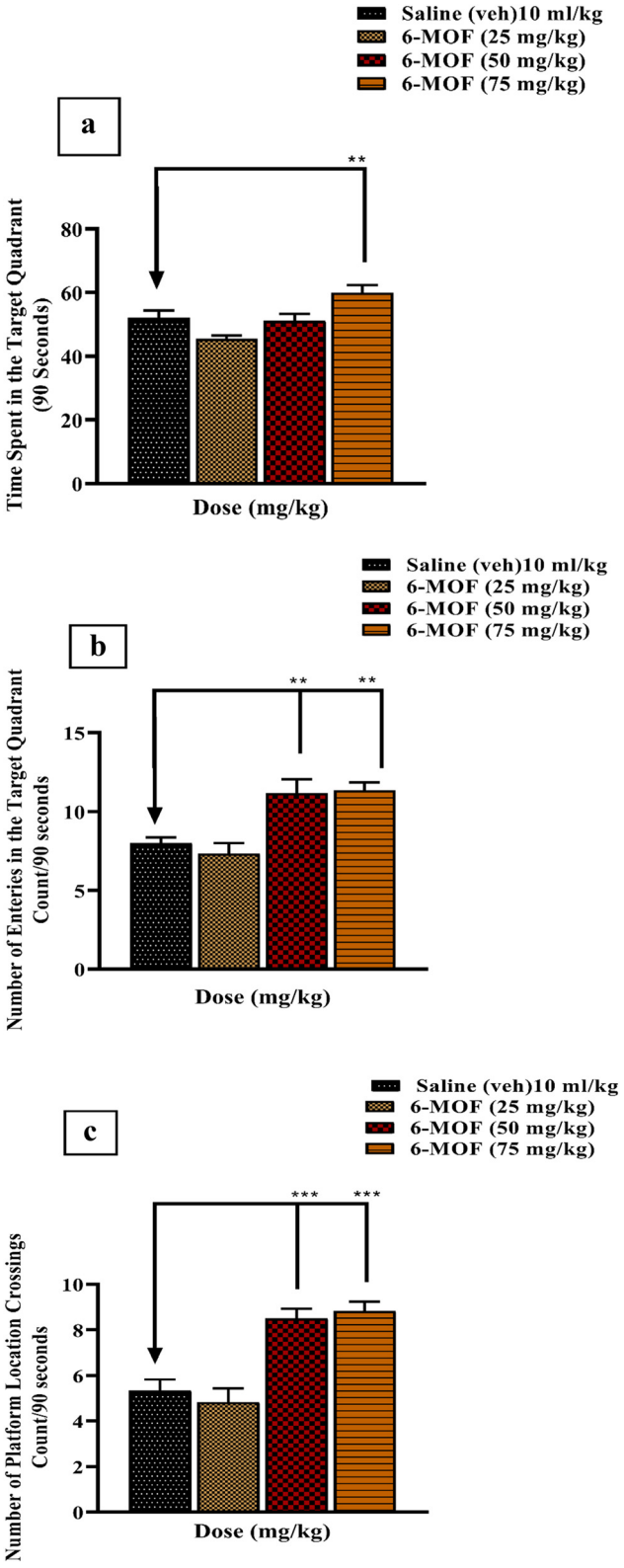

Figure 7: Effect of 6-MOF on MWM. (a) Time spent in the target quadrant, (b) the number of entries in the target quadrant, and (c) the number of platform location crossings. *p < 0.05, **p < 0.01, and ***p < 0.001.

3.13 Effect of 6-MOF on the hippocampal tissue levels of adenosine, inosine, and hypoxanthine

6-MOF imparted considerable neuroprotection in the hippocampus as all three doses have considerably decreased hippocampal adenosine, inosine, and hypoxanthine (***p < 0.001) relative to the saline group (Table 5).

3.14 Effect of 6-MOF on the striatal tissue levels of adenosine, inosine, and hypoxanthine

Striatal neuroprotection was imparted at all three doses of 6-MOF, as adenosine inosine and hypoxanthine levels were significantly decreased at all three doses (***p < 0.001) relative to the saline group (Table 6).

3.15 *In silico* binding affinity of 6-MOF with SOD

The minimum binding energy indicated that the SOD protein (target enzyme) was successfully docked with 6-MOF flavonoid (Table 7 and Figure 11).

4 Discussion

The therapeutic potential of flavonoids in the management and prevention of cancer, cerebrovascular illnesses, cardiovascular diseases, and neurodegenerative diseases is supported by an enormous amount of scientific evidence [44]. Decision-making ability, short-term working memory, and learning have all been assessed using Y-Maze [45]. In the current study, our findings have shown that 6-MOF has significant results in the spontaneous alternations task (Figure 3a–c). The processes of memory retrieval, consolidation, and acquisition include a variety of neurotransmitters, such as dopamine, acetylcholine, glutamate, noradrenaline, and serotonin [46]. The prefrontal cortex and hippocampal regions are largely involved in the establishment of working memory. The cholinergic neural system influences, and the glutamatergic circuit mediates this entire process [47,48]. In the hippocampus, 6-MOF was found to have significantly

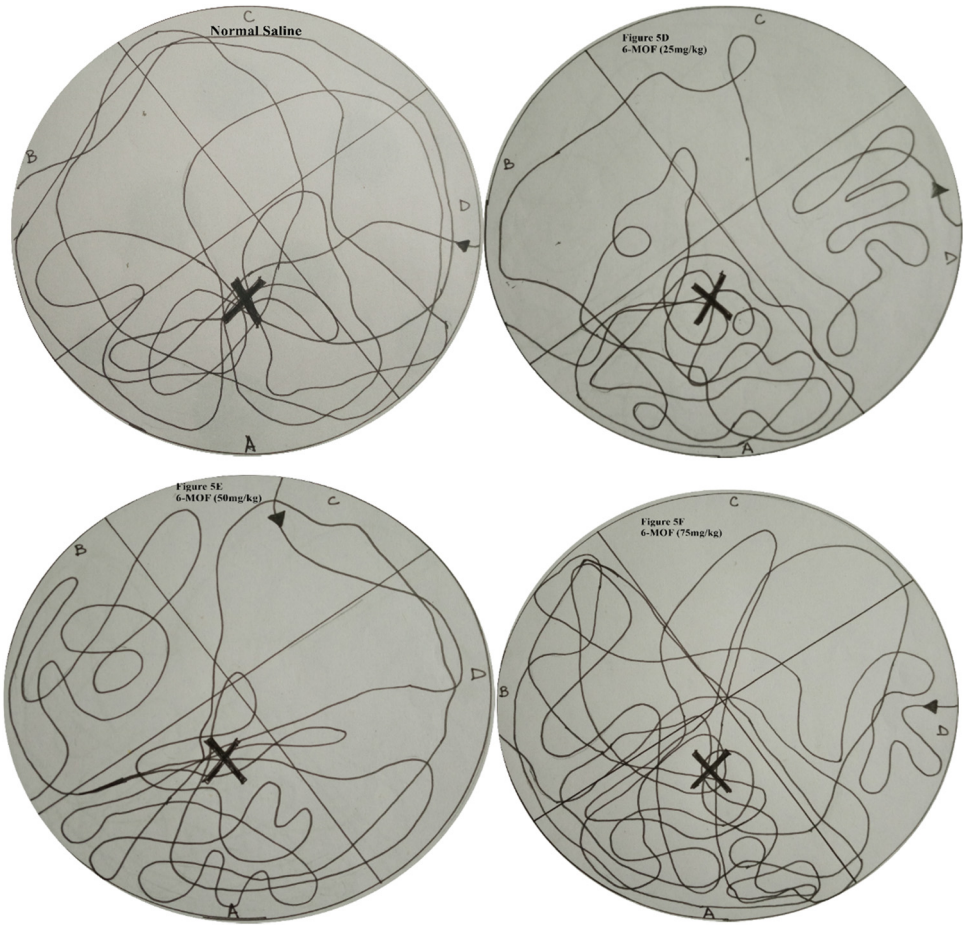

Figure 7: (*Continued*)

increased levels of dopamine, noradrenaline, serotonin, and vitamin C (Table 2). Previous research has indicated that activating dopamine D1-like receptors in the hippocampus enhances memory formation in that area, and inhibitors of monoamine oxidase-B were found to increase dopamine levels outside of hippocampus cells [49]. 6-MOF had an inhibitory effect on MAO-B [50] that could indicate higher

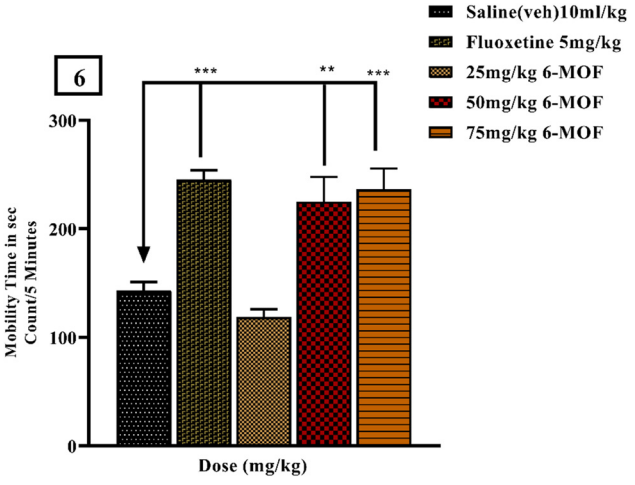

Figure 8: Effect of 6-MOF on the TST. $^{**}p < 0.01$ and $^{***}p < 0.001$.

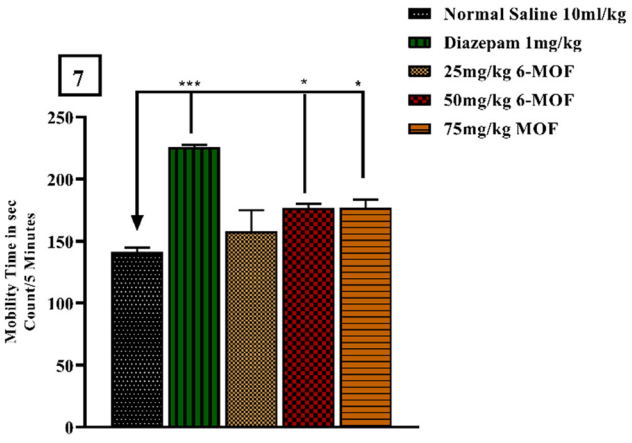

Figure 9: Effect of 6-MOF on the elevated plus-maze; $^{*}p < 0.05$ and $^{***}p < 0.001$.

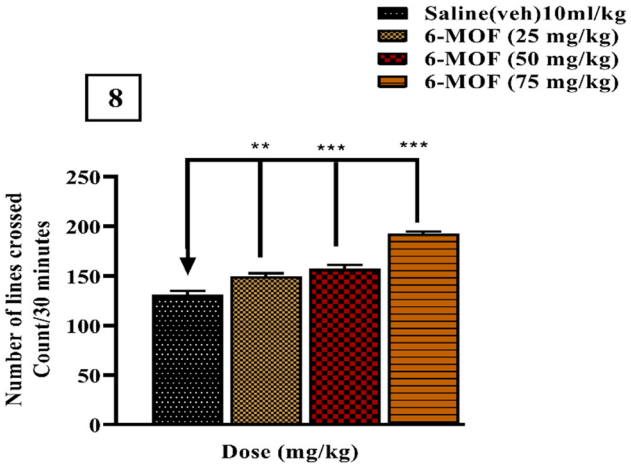

Figure 10: Effect of 6-MOF on the locomotor activity. $^{**}p < 0.01$ and $^{***}p < 0.001$.

dopamine levels in the hippocampal regions, confirming the importance of D1 dopamine receptors for improved spatial memory, as assessed by the Y-maze. By protecting the hippocampal antioxidant system, which was demonstrated to have an anti-oxidant effect against memory-related deficits in numerous disorders, vitamin C improves memory [51], which indicates that 6-MOF's antioxidant action also contributes to neuroprotection. Additionally, it has been noted that medications that increase noradrenaline levels in the brain enhance cognition [52]. 6-MOF considerably increased the hippocampal noradrenaline levels (Table 2), which is one of the reasons for improving cognitive abilities. It is generally known that adenosine has a modulatory influence on the hippocampus and striatum, two important brain regions involved in memory [53]. Adenosine has been documented to play an important role in neuronal activity inhibition [54], axon formation [55], and neuroprotection [56]. Adenosine receptors A_1 are widely distributed hippocampus, cerebellum, and cerebral cortex [57]. Studies have shown that A_{2A} receptor antagonists improve spatial memory in Y-maze [53]. Flavonoids are reported to have an antagonist effect on A_{2A} and A1 receptors [58] and have been shown to improve Y-maze performance [59]. 6-MOF improved Y-maze performance by modulating adenosine and its metabolites in the hippocampus, frontal cortex, and striatum (Tables 4–6), which may have contributed toward significant Y-maze performance. An NOR task was used to evaluate object recognition memory; it is a non-spatial and non-aversive test [60]. 6-MOF improved the time spent with the novel object (Figure 4). Relational, episodic, and contextual memory formation are all critical functions of the middle prefrontal cortex [61]. The prefrontal cortex and perirhinal cortex are neurologically connected to the hippocampus, which allows it to function as a functional component in memory task recognition by activating the executive center [62]. The frontal cortex and hippocampal levels of vitamin C increased (Tables 1 and 2). Vitamin C enhances cognition and memory by protecting against free radical damage, preserving oxidative processes and stored in the frontal cortex [51,63]. The hippocampus is involved in learning, memory formation, consolidation, and retrieval through the release

Table 1: Levels of neurotransmitters and vitamin C expressed in the frontal cortex tissue

Treatments	Dopamine (ng/mg of wet tissue)	Noradrenaline (ng/mg of wet tissue)	Vitamin C (ng/mg of wet tissue)	Serotonin (ng/mg of wet tissue)
Saline (vehicle)	23.1 ± 1.6	74.2 ± 3.1	18.6 ± 2.7	32.3 ± 4.4
6-MOF (25 mg/kg)	14.4 ± 1.4	45.8 ± 2.3	27.7 ± 2.8*	9.2 ± 0.9
6-MOF (50 mg/kg)	19.3 ± 1.8	50.4 ± 2.3	28.6 ± 3.1*	17.4 ± 2.6
6-MOF (75 mg/kg	20.96 ± 1.6	96.0 ± 0.4***	31.1 ± 2.2*	26.0 ± 3.9

$^{*}p < 0.05$ and $^{***}p < 0.001$.

Table 2: Levels of neurotransmitters and vitamin C expressed in the hippocampal tissue

Treatments	Dopamine (ng/mg of wet tissue)	Noradrenaline (ng/mg of wet tissue)	Vitamin C (ng/mg of wet tissue)	Serotonin (ng/mg of wet tissue)
Saline (10 mL/kg)	3.3 ± 0.1	264.5 ± 8.0	33.4 ± 5.4	8.7 ± 1.1
6-MOF (25 mg/kg)	3.6 ± 0.2	211.5 ± 3.0	67.8 ± 6.6**	17.9 ± 1.1*
6-MOF (50 mg/kg)	3.8 ± 0.1	237.8 ± 10.8	86.4 ± 5.5***	18.6 ± 1.1*
6-MOF (75 mg/kg)	14.6 ± 0.2***	308.8 ± 10.2**	110.5 ± 5.0***	35.2 ± 5.8***

$^{**}p < 0.01$ and $^{***}p < 0.001$.

Table 3: Levels of neurotransmitters and vitamin C expressed in the striatal tissue

Treatments	Dopamine (ng/mg of wet tissue)	Noradrenaline (ng/mg of wet tissue)	Vitamin C (ng/mg of wet tissue)	Serotonin (ng/mg of wet tissue)
Saline (10 mL/kg)	3.4 ± 0.2	219.5 ± 4	65.2 ± 4.1	59.7 ± 2.3
6-MOF (25 mg/kg)	3.4 ± 0.2	225.2 ± 6.5*	23.3 ± 1.6	16.5 ± 2.6
6-MOF (50 mg/kg)	3.7 ± 0.1	238.4 ± 4.1***	49.8 ± 6.5	21.0 ± 3.8
6-MOF (75 mg/kg)	63.8 ± 0.1***	240.6 ± 8.6***	74.8 ± 11.1*	88.1 ± 3.4***

*$p < 0.05$ and ***$p < 0.001$.

Table 4: Levels of adenosine, inosine, and hypoxanthine expressed in the frontal cortex tissue

Treatments	Adenosine (ng/mg of wet tissue)	Inosine (ng/mg of wet tissue)	Hypoxanthine (ng/mg of wet tissue)
Saline (10 mL/kg)	40.1 ± 2.9	3.3 ± 0.4	7.4 ± 0.7
6-MOF (25 mg/kg)	28.9 ± 1.6***	2.1 ± 0.1*	4.1 ± 0.1***
6-MOF (50 mg/kg)	26.4 ± 0.5***	1.9 ± 0.1*	2.6 ± 0.4***
6-MOF (75 mg/kg)	26.9 ± 1.0***	1.3 ± 0.4***	2.4 ± 0.4***

*$p < 0.05$ and ***$p < 0.001$.

of dopamine, serotonin, and noradrenaline [46]. The hippocampus has higher amounts of serotonin, dopamine, and noradrenaline (Table 2), which is explained by the increased time spent interacting with new objects and enhanced object recognition memory. Research has demonstrated that long-term cannabis usage causes memory impairments in the detection of novel objects, which are counteracted by antagonistic interactions with the adenosine A2AR receptor [64]. Adenosine receptor antagonism plays a key role spectrum of biological activities of reported flavonoids [65], and 6-MOF has a regulatory effect on adenosine and its metabolites (Tables 4–6), which may have contributed to enhanced object recognition memory. Survival, social cooperation, reproduction, and social behavior adaption all depend on socialization. In rodents, this behavior is the natural animal tendency to spend more time exploring unfamiliar subjects rather than familiar ones [66]. In our study, 6-MOF has improved socialization behavior (Figure 5) as well as enhanced dopamine and noradrenaline in the hippocampus

Table 5: Levels of adenosine, inosine, and hypoxanthine expressed in the hippocampal tissue

Treatments	Adenosine (ng/mg of wet tissue)	Inosine (ng/mg of wet tissue)	Hypoxanthine (ng/mg of wet tissue)
Saline (10 mL/kg)	17.0 ± 2.2	2.7 ± 0.4	1.4 ± 0.1
6-MOF (25 mg/kg)	5.1 ± 0.2***	0.5 ± 0.1***	0.3 ± 0.4***
6-MOF (50 mg/kg)	8.9 ± 0.4***	0.4 ± 0.8***	0.3 ± 0.7***
6-MOF (75 mg/kg)	7.2 ± 0.5***	0.3 ± 0.0***	0.1 ± 0.1***

***$p < 0.001$.

Table 6: Levels of adenosine, inosine, and hypoxanthine expressed in the striatal tissue

Treatments	Adenosine (ng/mg of wet tissue)	Inosine (ng/mg of wet tissue)	Hypoxanthine (ng/mg of wet tissue)
Saline (10 mL/kg)	20.1 ± 3.8	10.7 ± 2.2	11.0 ± 1.4
6-MOF (25 mg/kg)	9.0 ± 0.8***	8.3 ± 0.5***	6.8 ± 1.3***
6-MOF (50 mg/kg)	5.8 ± 0.3***	6.5 ± 1.1***	4.9 ± 0.6***
6-MOF (75 mg/kg)	1.7 ± 0.2***	5.2 ± 0.4***	3.4 ± 1.8***

***$p < 0.001$.

Table 7: Binding affinity of 6-MOF with SOD

Docking pose	Protein–ligand complex	Binding affinity	rmsd/ub[a]	rmsd/lb[b]
1	1gv3–protein-6-MOF	−7.1	0	0
2	1gv3–protein-6-MOF	−7.1	2.358	1.737
3	1gv3–protein-6-MOF	−6.8	23.524	21.578
4	1gv3–protein-6-MOF	−6.7	6.649	1.832
5	1gv3–protein-6-MOF	−6.6	30.708	27.896
6	1gv3–protein-6-MOF	−6.4	2.603	1.997
7	1gv3–protein-6-MOF	−6.4	23.886	21.23
8	1gv3–protein-6-MOF	−6.4	6.72	2.079
9	1gv3–protein-6-MOF	−6.4	7.616	5.026

[a]rmsd/ub: root mean square deviation/upper bond; [b]rmsd/lb: root mean square deviation/lower bond: 1gv3: superoxide dismutase (SOD); 6-MOF: 6-methoxyflavone.

(Table 2). The basolateral amygdala and the dorsal hippocampus CA1 area are of extreme importance for the control of social behavior [67]. Nonetheless, dopamine works on D1/D5 receptors, and noradrenaline acts on β-receptors in these regions, both of which are essential for social recognition [66]. This may be one of the reasons that socialization behavior is improved with 6-MOF. Adenosine is a reported neuromodulator and plays a noteworthy role in neuronal excitability and synaptic transmission [68,69]. A_1 and A_{2A} receptors are widely distributed in the brain primarily striatum hippocampus and olfactory bulb [69]; these brain areas are involved in the regulation of motivation [70] and the process of social behaviors [71]. A_1 receptors are abundantly distributed in the hippocampus [72]. It has been reported that antagonism at A_1 and A_{2A} receptors improves social memory [73]. Flavonoids are reported as A_{2A} and A1 receptor antagonists [58]. 6-MOF improved social recognition, possibly by modulating adenosine, inosine, and hypoxanthine levels in the frontal cortex, hippocampus, and striatum (Tables 4–6). To assess thermoregulatory and affective behavior, positive emotional states nest-building behavior test was performed. Dopaminergic and noradrenergic systems play a significant role in nest-building behavior [74]. Adenosine receptors are thickly articulated in the striatum and have a modulatory impact on dopamine neurotransmission. There is substantial confirmation of antagonistic interaction among A_{2A} and D2 and also A_1 and D_1 receptors in the striatum [75]. 6-MOF has shown an increase in the levels of noradrenaline and dopamine (Table 1) and also a decrease in adenosine and its metabolite levels (Table 6), which may have contributed toward no alternation in the executive functions. All three doses of 6-MOF have not shown any alternation in nest-building behavior (Figure 6). Literature shreds of evidence specified that the hippocampus is important for the acquisition and consolidation of spatial memory, and spatial performance, in general and specifically in MWM, is dependent on the functional integrity of the hippocampus. Abrasions in the forebrain, hippocampus, striatum, cerebral cortex, and cerebellum were reported to impair MWM performance [76]. It has been reported that a spur in the noradrenergic system augments MWM acquisition [77]. Previous studies have reported that l-deprenyl, an MAO-B inhibitor, alleviated scopolamine-induced deficits in MWM [76,77]. According to studies in rats, lesions in the mesohippocampal dopaminergic system cause impairments in spatial memory [78]. The extracellular levels of dopamine in the hippocampus have been reported to be elevated in response to the use of monoamine oxidase-B inhibitors [49]. 6-MOF, being a flavonoid and an inhibitor of MAO-B [50], increased dopamine levels in the hippocampus (Table 2), thereby contributing toward the significant MWM performance (Figure 7a–c). Adenosine receptors A_{2A} are extensively distributed in the striatum. It has also been reported that A1 receptor activation in the hippocampus inhibits the release of acetylcholine, and consumption of A1 antagonist caffeine has a modulatory effect on spatial memory [79]. Literature has already reported that fluoxetine improves the levels of noradrenaline and dopamine in the frontal cortex, thereby imparting an antidepressant effect [80]. Swertisin, an isolated flavonoid, has shown improvement in the MWM task by blocking the A1 receptor [59]. 6-MOF decreased adenosine levels in the hippocampus and striatum (Tables 5 and 6), which may be the contributing factor in spatial memory enhancement. Activation of the $GABA_A$ receptor has shown anxiolytic effects [81]. 6-MOF has been reported to have a $GABA_A$ agonist effect [25], so it can be an aspect toward anxiolytic effect of 6-MOF (Figure 8). Additionally, 6-MOF has improved monoamine levels in the frontal cortex and hippocampus (Tables 1 and 2), which can be a contributing factor in the anxiolytic effect. 6-MOF has shown an increase in the levels of vitamin C in the frontal cortex (Table 1). Dihydroxyascorabte is metabolized to vitamin C in CNS cells. Among the brain areas, the hippocampus and prefrontal cortex are the main areas that have

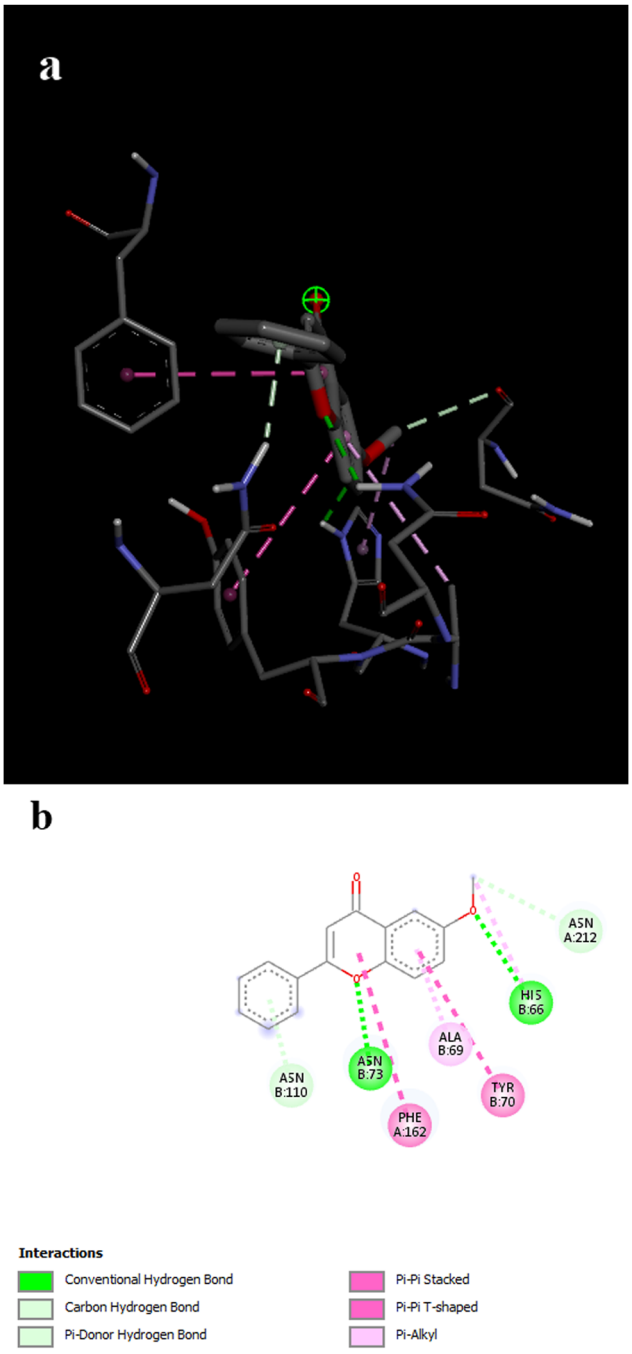

Figure 11: Conformational changes observed due to the binding of 6-MOF with PDB ID: 1gv3: (a) surface area interactions of 6-MOF with the receptor binding domain of SOD and (b) 2D interaction between 6-MOF and the receptor protein.

maximum stores of ascorbic acid, thereby imparting the antioxidant effect [63]. Studies have shown that the antioxidant effect of vitamin C improves mood and cognition by modulating monoaminergic and glutamatergic neurotransmitter systems that play a pivotal role in antidepressant and anxiolytic effects, which is attributed to the frontal cortex's ability to cope with stress and fear [82]. Adenosine has a direct relationship with anxiety, and the anxiogenic effect of adenosine receptor antagonists, caffeine, has been accredited to adenosine A_1 receptor blockade [83]. 6-MOF considerably increased mobility time and decreased immobility time to elicit antidepressant effects (Figure 9). Literature has reported that the inhibitory impact that GABA typically has on some dopaminergic neurons will be lowered by diazepam and increase dopamine release. Therefore, using benzodiazepines will raise the release of dopamine, which enhances the feeling of pleasure and reward using diazepam [84]. Serotonin reuptake inhibitors, tricyclic antidepressants, and serotonin-norepinephrine reuptake inhibitors are recommended for the treatment of depression and anxiety-like behavior [85]. The amygdala, hippocampus, and frontal cortex are some of the brain regions that are largely involved in regulating and expressing anxiety-like behavior [86]. Anxiety is mediated by D1 and D2 receptors, and studies have linked anxiety to dopaminergic systems [87]. Literature studies have also shown that postsynaptic 5-HT_{1A} receptors located at the dorsal hippocampus arbitrate anxiogenic effects, while endogenous dorsal hippocampal cholinergic neurons arbitrate anxiolytic effects [88]. 6-MOF significantly increased the levels of dopamine, noradrenaline, and serotonin in the hippocampus (Table 2), which might be involved in the antidepressant effect of 6-MOF. In the current study, our findings showed that 6-MOF produced significant hyper-locomotion (Figure 10). Elevated extracellular noradrenaline levels in the hippocampal region due to postsynaptic activation of 5-HT1A receptors induce hyperlocomotion and hyperexcitation [89]. Our study showed an increase in noradrenaline levels in the hippocampus (Table 2), which can be attributed to hyperlocomotion caused by 6-MOF. Earlier, the same compound has been reported to significantly reverse cisplatin-induced allodynia in the same doses orally [23]. The three most significant natural antioxidants that aid in scavenging these free radicals are glutathione (GSH), catalase (CAT), and SOD. Superoxide radicals and NO can react to form the highly reactive peroxynitrite anions (ONOO−). This highly reactive radical is created when NO and superoxide anion react, and it can cause cytotoxicity, neurotoxicity, and apoptotic cells [90]. Furthermore, flavonoids activate enzymes that scavenge free radicals, such as CAT and SOD, which lower the concentrations of free radical oxygen species, such as singlet oxygen, hydroxyl radical, and superoxide radical [91]. *An in silico* study was performed to determine the binding affinity of 6-MOF with SOD enzyme. Results have shown that the minimum binding energy of 6-MOF with antioxidant enzyme is −7.1 k/cal/mol (Table 7). The binding energy showed that 6-MOF was successfully docked with an antioxidant enzyme (Figure 10a and b), thereby showing the antioxidant effect of 6-MOF.

5 Conclusion

6-MOF showed notable effects in improving the cognitive aspects, modulating decision making, short-term memory, long-term memory, spatial memory, motor and skilled-based memory, anxiety, and depression, which was evaluated via behavioral evaluation. Neurochemically, 6-MOF showed profound results in modulating key neurotransmitters in the frontal cortex, hippocampus, and striatum, as well as imparted generalized neuroprotection by modulating adenosine and its metabolite levels. Thus, 6-MOF would be a potential novel candidate for managing cognitive decline, anxiety, and depression.

6 Limitations

More significant molecular-level studies are required to gain insight into the molecular mechanisms by which 6-MOF exerts significant pharmacological effects.

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