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Original Article

Psychopharmacological assessment of antidepressant-like, anxiolytic, and sedative-hypnotic effects of *Tilia platyphyllos* Scop. extract using experimental animal models

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Abstract

The prevalence of psychiatric disorders namely depression, anxiety, and sleep disturbances has been increased worldwide, particularly during the COVID-19 pandemic. In this regard, the interest of recent investigations is moved toward phytomedicines and bioactive substances derived from natural sources. Although *Tilia platyphyllos* Scop. contains high amounts of phenolic compounds such as quercetin, kaempferol, and catechin, there is no study on the possible effects of its extract on psychological disorders. The present study was carried out to determine the antidepressant-like, anxiolytic, and sedative-hypnotic effects of the hydroethanolic extract of *T. platyphyllos* leaves using forced swimming test (FST), tail suspension test (TST), elevated plus maze test (EPMT), pentobarbital-induced loss of righting reflex test and open field test (OFT). Following the ethanolic extraction of *T. platyphyllos* leaves, the extraction yield was 14% and the total phenolic and total flavonoid contents were found to be 135.23 ± 0.14 mg gallic acid equivalent/g dry extract and 19.02 ± 0.03 mg rutin equivalent/g dry extract, respectively. Both FTS and TST revealed a significant antidepressant-like activity for the tested extract at 400 mg/kg compared to the control group. In addition, the anxiolytic activity of the extract was proven through OFT and EPMT in the same dose. Finally, *T. platyphyllos* extract at 200 mg/kg and 400 mg/kg significantly increased the sleeping time when compared to the control group reflecting its potential hypnotic activity. Co-administration of *T. platyphyllos* extract at 400 mg/kg and flumazenil as the GABA-A receptor antagonist decreased the sleeping time but the observed effect was not statistically significant. Therefore, we cannot completely rule out the GABA-A receptor's involvement in the hypnotic activity of the extract. The biological results presented here led us to conclude that *T. platyphyllos* extract can be a prominent source of antidepressant, anxiolytic and hypnotic agents. Probably, the main phenolic compounds of *T. platyphyllos* such as quercetin, kaempferol, and catechin are involved in the observed effects. However, there is still a great need for additional investigations on the exact mechanisms.

Keywords: Anxiety, Depression, Flavonoid, Insomnia, Mice, Phenolic content, *T. platyphyllos*.

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1. Introduction

Mental health disorders also called mental illnesses or psychiatric disorders can alter people's moods and potentially can affect a person's regular activity. There are many

different conditions such as depression, anxiety, insomnia, etc., that are recognized as mental illnesses [1]. Depression is characterized by loss of interest, sadness, fatigue, disturbed sleep, a low mood, and worthlessness. Therefore, it can negatively impact the quality of life and the ability to work [2]. Recently, it is estimated that about 300 million individuals are suffering from depression all over the world [3]. Chronic and/or severe anxiety is another prevalent psychological disorder in the world that impacts multiple areas of an individual's life. More than 20% of the adult population have experienced some type of anxiety during their lives [4]. Anxiety is a risk factor for depression and sleep problems such as insomnia. Besides, insomnia is considered one of the key symptoms of depression [5]. On a global scale, the number of people suffering from insomnia is increasing [6]. Although psychiatric disorders are particularly common in high-income countries, their prevalence is also rising in low- or middle-income countries. In addition, the prevalence of psychiatric disorders namely depression, anxiety, and sleep disturbances has been increased during the COVID-19 outbreak [7].

Currently, the treatments for mental health disorders mainly include behavioral therapies and pharmacological treatments. Several types of antidepressant, anxiolytic and hypnotic drugs are often used in the treatment of depression, anxiety, and insomnia, respectively. For instance, tricyclic antidepressants and serotonin reuptake inhibitors are proposed treatments for depression while benzodiazepines could be used for the treatment of anxiety and insomnia [8]. However, these medications are associated with different side effects such as orthostatic hypotension, weight gain, tachycardia, sexual disorders, digestive disorders, dependence, tolerance, and cognitive impairment which frequently cause people not to comply with their treatments [9]. In addition, a long-term use of antidepressant and anxiolytic medicines is compulsory in the treatment of depression and anxiety [10]. In this regard, the interest of recent investigations is moved toward phytomedicines and bioactive substances obtained from natural sources with similar or even higher efficacy but fewer adverse effects to combat these unwanted circumstances demonstrated by conventional treatments. Furthermore, in recent years, medicinal plants have gained more attention for their economic value.

Tilia platyphyllos Scop, also known as large-leaved lime or large-leaved linden, belongs to the *Tilia* genus in the family of *Malvaceae* [11]. In folk medicine, *T. platyphyllos* is used as a diuretic, stomachic, antispasmodic, and sedative agent [12]. In addition, interesting biological activities such as antioxidant, hepatoprotective and anti-neuralgic properties have been previously reported for *T. platyphyllos* [13]. *T. platyphyllos* contains high amounts of phenolic compounds such as quercetin, kaempferol, and catechin which could be responsible for the mentioned therapeutic effect of this plant [13]. The chemical structures of the main phenolic compounds of *T. platyphyllos* are presented in Figure 1.

The therapeutic effects of quercetin, kaempferol, and catechin isolated from different medicinal plants on animal and human

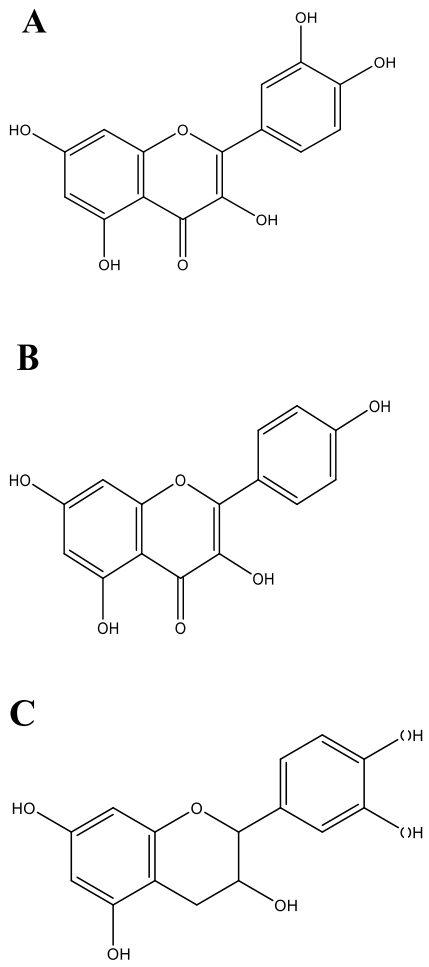


Figure 1. The chemical structure of the main phenolic compounds of *T. platyphyllos.* A) Quercetin, B) Kaempferol and C) Catechin.

mental disorders have been previously reported [14-16]. However, there is no information about the effects of *T. platyphyllos* on mental health disorders. Therefore, in view of this and given bioactive compounds of *T. platyphyllos*, the present study was carried out to determine the antidepressant-like, anxiolytic, and sedative-hypnotic effects of the hydroethanolic extract of *T. platyphyllos* through experimental animal models.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and reagents had appropriate purity and were purchased from Sigma-Aldrich (Germany). The extract, flumazenil, and diazepam were dissolved in dimethyl sulfoxide (DMSO) while pentobarbital, fluoxetine, and imipramine were dissolved in normal saline. For chemicals dissolved in normal saline and DMSO, the injection volume was adjusted at 10 ml/kg and 5 ml/kg, respectively.

2.2. Plant Extraction

The leaves of *T. platyphyllos* were collected in July from Ramsar Forest which is located in the western parts of Mazandaran province in the north of Iran. The identification and authentication of samples were assisted by a botanist at the Herbarium section of Shahid Beheshti University of Medical Sciences, Tehran, Iran where the voucher specimen was kept. Samples were air-dried under shade at room temperature for ten days and then crushed to pass through a sieve with 40 meshes to obtain a fine powder. The powder was extracted with 80% ethanol for 3 days using the maceration method [17]. Following the extraction, the solvent was removed using a rotary evaporator (Heidolph, Germany) under reduced pressure at 22°C. Finally, the extraction yield was calculated.

2.3. Total Phenolic Content and Total Flavonoids Assay

Total phenolic content (TPC) was determined using Folin–Ciocalteu reagent [18]. In brief, 0.5 ml of the sample (400µg/ml) was mixed with Folin–Ciocalteu reagent (2.5 mL).

Five minutes later, 2 mL of sodium carbonate solution (Na2CO3; 75 mg/mL; in water) was added to the mixture and left for 2 h at room temperature in a dark place. Then, the absorbance was measured at 765 nm. Gallic acid standard solution (0 to 200 µg/mL; in methanol) was used to prepare a linear calibration curve and the TPC of the sample was expressed as gallic acid equivalents (mg) per g of dried sample.

Total flavonoid content (TFC) was determined using the aluminum chloride reagent [19]. For this purpose, 2.5 mL of the sample was mixed with 2.5 mL of Aluminum chloride reagent (AlCl3; 20 mg/mL; in methanol) and left at room temperature for 40 min. The absorbance was measured at 415 nm. Rutin standard solution (0 to 150 µg/mL; in methanol) was used to prepare a linear calibration curve and the TFC of the sample was calculated as rutin equivalents expressed in mg per g of dried sample.

2.4. Animals and Treatments

Male Swiss mice (n = 10 in each group) were used in the forced swimming test while male NMRI mice (n = 10 in each group) were used in the other experiments. Mice weighing 18–25 g were obtained from the animal house of Shahid Beheshti University of Medical Sciences. Animals were housed in clean cages in groups of ten in a controlled environment (22 ± 2 ºC temperature, 50-60% humidity, and 12 h. light/dark cycle) with food and water supplied *ad libitum*. Three days before behavioral experiments, mice were moved to the laboratory once a day and allowed to acclimatize. All tests were performed between 9 am and 3 pm in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals and all the protocols were approved by the Animal Care Use Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.AEC.1401.004). In all experiments, animals were treated intraperitoneally (i.p.).

2.5. Evaluation of Antidepressant-like Activity

2.5.1. Forced Swimming Test

Forced swimming test (FST) was performed in the same manner described by Porsolt et al. to evaluate the antidepressant-like activity of *T. platyphyllos* extract [20]. Animals received vehicle, different doses of the extract (100, 200, and 400 mg/kg), fluoxetine (32 mg/kg) [21], and imipramine (32 mg/kg) [22]. Thirty minutes following each treatment, animals were individually placed in a Plexiglass cylindrical container (12 cm diameter × 21 cm height) filled with 14 cm of water. The water temperature was adjusted between 22 - 25 °C. Mice were forced to swim for 6 min and the immobility time was reported in the last 4 min. The total immobility time was defined as the period that animals stopped swimming and tried to float on water without struggling.

2.5.2. Tail Suspension Test

The antidepressant-like activity of *T. platyphyllos* extract was further screened through tail suspension test (TST) [20]. For this purpose, Animals were treated with vehicle, different doses of the extract (100, 200, and 400 mg/kg), fluoxetine (32 mg/kg) [21], and

imipramine (32 mg/kg) [23]. Thirty minutes later, mice were individually dangled by their tail with the adhesive tape positioned 1 cm from the tip of the tail. The experiment lasted for 6 min and immobility time in the last 4 min of the experiment was reported. In this test, immobility time was defined as animals stopped struggling and suspended passively.

2.6. Evaluation of Anxiolytic Activity

2.6.1. Elevated Plus Maze Test

The anxiolytic activity of *T. platyphyllos* extract was determined using the elevated plus maze (EPM) apparatus that consisted of two open arms (length 50 cm, width 10 cm) and two closed arms (length 50 cm, width 10 cm, and 10 cm wall surrounding) arranged into a plus shape. In order to increase anxiety in the open arms, the plus-maze was located one meter above the floor level. Animals were treated with the vehicle or different doses of the extract (100, 200, and 400 mg/kg) 30 min before starting the experiment. Diazepam (1 mg/kg) was used as the positive control [24]. To start the test, each mouse was individually placed on the central platform of the EPM apparatus and allowed to explore for 10 min. The movement of the animal was recorded using a digital camera placed directly overhead and videos were analyzed by Ethovision XT software (Noldus, The Netherlands). The time animal spent in the open arms was reported by the software [25]. The apparatus environment was completely cleaned with 70 % ethanol after each trial to prevent olfactory clues from the previous mouse.

2.6.2. Open Field Test

The anxiolytic activity and psychomotor stimulant activity of *T. platyphyllos* extract were further investigated in the open field test [26]. Animals were treated with vehicle or different doses of the extract (100, 200, and 400 mg/kg) and 30 min later were exposed to the open field test. Diazepam (1 mg/kg) was used as the positive control [24]. Animals were placed individually in the center of a Plexiglas box (40×40×40 cm) to explore freely for 10 min while their activity was recorded using a digital camera placed overhead. Videos were analyzed using Ethovision XT software (Noldus, The Netherlands) and the total distance moved and time spent in the central zone were analyzed. The apparatus environment was completely cleaned with 70 % ethanol after each trial to prevent olfactory clues from the previous mouse.

2.7. Evaluation of Hypnotic Activity

The hypnotic activity and possible involved mechanism of action of *T. platyphyllos* extract were evaluated using the pentobarbital-induced loss of righting reflex method as described previously [27]. Animals were treated with vehicle; the extract (100, 200, and 400 mg/kg), diazepam (2 mg/kg) [27], 30 min before the administration of 40 mg/kg of pentobarbital sodium. One more group of mice was treated with 400 mg/kg of the extract in combination with flumazenil (10 mg/kg) as a known antagonist of benzodiazepine receptors. After the administration of pentobarbital sodium as a sleep-inducing agent, mice were observed for the duration of sleep that was

considered as the time from the loss of righting reflex to its recovery.

2.8. Estimation Maximum Non-Fatal Dose and Median Lethal Dose

The (LD_{50}; the dose that causes death in 50% of animals) was calculated to estimate the possible acute toxicity of the *T. platyphyllos* extract. In this regard, animals in separate groups of five were treated with different doses of the extract and were observed for 48 h. Induction of any mortality was considered as the maximum non-fatal dose and the LD_{50} value with the corresponding confidence limits (95% confidence limit) was determined by the probit test in SPSS software [28].

2.9. Statistical Analysis

The data were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to test for mean differences among different groups. In all experiments, $p < 0.05$ was considered the statistically significant level. All statistical analyses were carried out using Graph Pad Prism software, version 5.0 (San Diego, CA; USA).

3. Results and Discussion

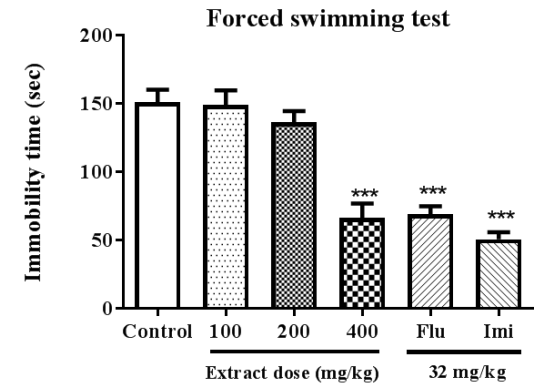
3.1. Extraction Yield, Phenolic Content and Flavonoid Content

Following the ethanolic extraction of *T. platyphyllos* leaves, the extraction yield (g extract/100 g of *T. platyphyllos* leaves) was found to be 14%. The calibration curves obtained by gallic acid and rutin standard solutions were respectively used for the determination of TPC and TFC. The regression equation, correlation coefficient (r^2), TPC, and TFC are presented in Table 1. TPC and TFC reported in our study were significantly higher than what was reported by Selvi et al. (p-value<0.0001) [29]. However various factors such as ecological factors, growth stage, harvesting time, planting condition, and the organ used have a significant effect on the phytochemical constituents and biological activities of plants [30].

3.2. Antidepressant-Like Activity of T. Platyphyllos

FST and TST show different sensitivity to the antidepressant-like activity of various antidepressants with different neurochemical pathways. In the current study, we employed both tests to evaluate the antidepressant-like activity of *T. platyphyllos* extract. FST is the most extensively used paradigm to measure the antidepressant-like activity of novel substances which is based on the observation of animal movement in an inescapable cylinder filled with water. The effectiveness of the treatment will result in an increase in mobility time and escape-directed behaviors in subjects compared to the vehicle [31]. As shown in Figure 2A, the extract at doses of 100 and 200 mg/kg did not show any immobility-reducing activity compared to the control group while following the administration of the extract at the dose of 400 mg/kg, the immobility time was decreased significantly (66.2 ± 10.6 sec) compared to the control group (150.9 ± 9.4 sec). The observed immobility-reducing activity of the extract at the dose of 400 mg/kg was similar to fluoxetine

A



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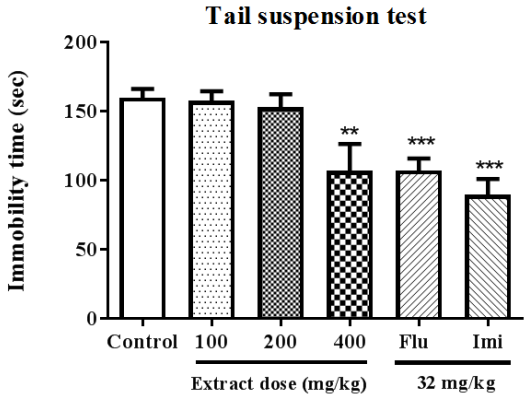


Figure 2. Effects of *T. platyphyllos* extracts as well as fluoxetine (Flu; 32 mg/kg) and imipramine (Imi; 32 mg/kg) on the duration of immobility time in the forced swimming test (A) and tail suspension test (B); Values are presented as mean±SEM; **P<0.01, ***P<0.001 indicate significant differences compared to the control group.

(69.1 ± 5.9 sec) and imipramine (50.5 ± 5.s sec) as positive controls.

Similar to the FST, the TST is primarily based on the observation of animal motion in an unavoidable stressful situation which is the hemodynamic stress of being hung in an uncontrollable position by their tail. If given antidepressant treatment is effective, the duration of immobility will significantly decrease compared to the control group [31]. As illustrated in Figure 2B, following the administration of the extract at the dose of 400 mg/kg, the immobility time (106.9 ± 19.6 sec) was significantly decreased compared to the control group (159.9 ± 6.3 sec) while treatment with the doses of 100 and 200 mg/kg of the extract did not show any significant antidepressant-like activity. Similarly, following the treatment of animals with positive controls including fluoxetine and imipramine, the immobility time decreased to 107.3 ± 8.6 sec and 89.75 ± 11.3 sec, respectively.

Tilia is a genus of about 30 species. The antidepressant activity of *Tilia Americana* L., leaves was previously reported [32]. However, there is no study on the antidepressant activity of *Tilia platyphyllos*. In a study conducted by Martinez-Hernandez et al. (2021), flavonoids such as quercetin glycosides and kaempferol derivatives were introduced as the bioactive component of the *Tilia Americana* var. extract in the observed antidepressant effects [32]. Quercetin can cause antidepressant-like effects due to its neuroprotective, anti-inflammatory, and antioxidant properties. In another study, it was revealed that quercetin involves in the regulation of serotonergic functions and produces antidepressant and anxiolytic effects [33] and in many studies, it has been reported that quercetin attenuates depressive-like behaviors [34-36]. In addition, Hosseinzadeh et al., reported the antidepressant activity of kaempferol using FST in mice and rats [37]. In many other papers, this feature of kaempferol has been reported [38, 39]. Kaempferol prevents pro-inflammatory cytokine levels related to depression and possibly increases the AKT/β-catenin cascade activity in the

Table 1: Total phenolic and total flavonoid contents of *T. platyphyllos* extract.

	Regression equation	r^2	Content
Total phenolic content	y = 0.0087x + 0.3204	0.9981	135.23 ± 0.14 mg GAE/g dry extract
Total flavonoid content	y = 0.017x + 0.0054	0.9990	19.02 ± 0.03 mg RE/g dry extract

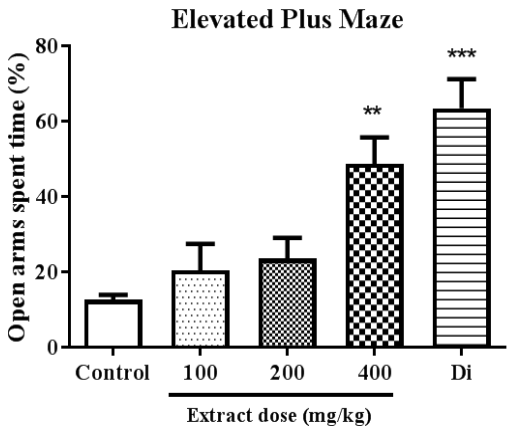
GAE: gallic acid equivalent; RE: Rutin equivalent; r^2: correlation coefficients

prefrontal cortex [40]. Finally, Lee et al. reported that catechin can decrease depression and anxiety-like behaviors [41]. Since *Tilia platyphyllos* contains high amounts of phenolic compounds such as quercetin, kaempferol, and catechin, we can consider these bioactive components responsible for the observed effect in the current study.

3.3. Anxiolytic Activity of T. Platyphyllos

The EPM test is a validated simple behavioral method to assess the anti-anxiety effects of novel substances. In this test, agents with anxiolytic activity will increase the time spent in open-arm and/or open-arm entries. In addition, the anxiety-like behavior could be measured using an open-field apparatus. In the current study, a significant increase in the percentage of time spent in the open arms was observed following the administration of the *T. platyphyllos* extract at the dose of 400 mg/kg (47.7 ± 7.1%) and diazepam as an anxiolytic drug at the dose of 1 mg/kg (63.5 ± 7.7%) comparing to the control group (12.7 ± 1.2%) suggesting anxiolytic activity (Figure 3A). Agents with anxiolytic activity will increase the time spent in the central zone of the open-field apparatus by each animal [42]. Similarly, in the open-field test (Figure 3B), a significant difference in the percentage of time spent in the central zone between the extract at the dose of

A



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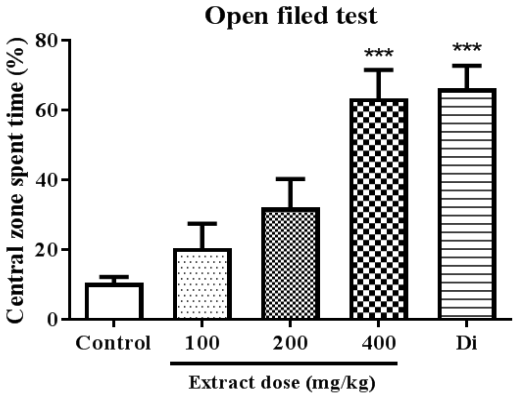

Figure 3. Effects of *T. platyphyllos* extracts, as well as diazepam (Di; 1 mg/kg) on the percentage of open arms spent time and percentage of central zone spent time in the elevated plus-maze test (A) and open field test (B), respectively; Values are presented as mean±SEM; **P<0.01, ***P < 0.001 indicate significant differences compared with the control group.

400 mg/kg (63.4 ± 8.1%) and the control group (10.5 ± 1.6%) was seen. In addition, diazepam as an anxiolytic drug revealed a significant anti-anxiety activity by increasing the percentage of

time spent in the central zone of the apparatus (66.2 ± 6.5%). The observed effects could be related to the main phenolic compounds of *T. platyphyllos* extract such as quercetin, kaempferol, and catechin. Previously, Grundmann et al. demonstrated that kaempferol possesses anxiolytic-like activity that was partially antagonized by concomitant administration of flumazenil as a benzodiazepine receptor antagonist [43]. In another study, the anxiolytic effect of quercetin was reported by Jung et al. It was reported that the observed effect could be mediated through the gamma-aminobutyric acid (GABA-ergic) system [44]. Although kaempferol and quercetin contribute to the anxiolytic activity of *T. platyphyllos*, we cannot rule out the fact that other compounds may have a significant role in the observed effect. Besides, the synergistic effect of other substances also cannot be ruled out which requires further investigation.

3.4. Hypnotic Activity

Since the interaction of kaempferol and quercetin with GABA receptors has already been proven, the hypnotic effect of the extract was further evaluated through the pentobarbital-induced loss of righting reflex method [43, 44]. The ability of the extract to potentiate pentobarbital-induced sleeping time was considered its hypnotic activity [28]. As shown in Figure 4, *T. platyphyllos* extract at the dose of 200 mg/kg and 400 mg/kg and diazepam (2 mg/kg) significantly increased the sleeping time (29.4 ±2.9 min, 34.4 ± 3.4 min, and 83.6 ± 4.1 min, respectively) when compared to the control group (19.2 ± 1.1 min) reflecting its potential hypnotic activity. Co-

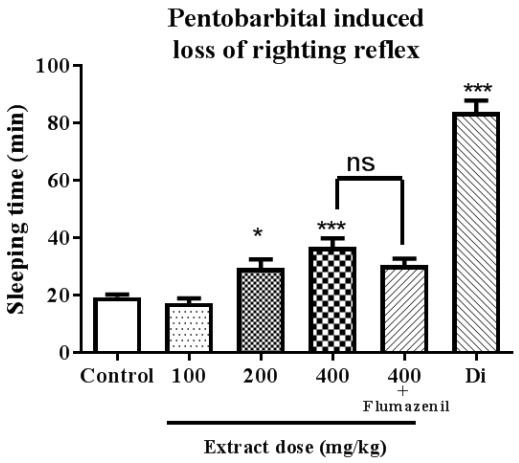

Figure 4. Effect of *T. platyphyllos* extracts as well as diazepam (Di; 2 mg/kg) on the sleep duration in the pentobarbital-induced loss of righting reflex test; Flumazenil was used at dose of 10 mg/kg; Values are expressed as mean ± SEM. * $P < 0.05$, *** $P < 0.001$ indicate significant differences compared to the control group.

administration of *T. platyphyllos* extract at 400 mg/kg and flumazenil, as the GABA-A receptor antagonist, decreased the sleeping time (Figure 4). However, the observed effect was not statistically significant. Therefore, we cannot completely rule out the involvement of GABA-A receptors in the hypnotic activity of the extract. In addition, the results of a study conducted by Aguirre-Hernández et al. suggested that the sedative and hypnotic effect of *T. americana* extract could be due to the presence of quercetin, rutin, isoquercitrin, and through the involvement of serotonergic receptors [45]. Hence, we can propose that the declared sedative and anxiolytic activities of *T. platyphyllos* extract in our study could also be partly related to the activation of serotonergic receptors which is in accordance with its antidepressant-like property.

3.5. Total Locomotor Activity

The effect of the extract on the locomotor activity of mice was measured in the open field test to rule out the hypothesis that the observed reduction in the immobility of animals in the FST and TST could be due to the psycho-stimulant effects of the extracts. The results of the open-field test are demonstrated in Figure 5. As shown in the graph, *T. platyphyllos* extract at all tested doses did not change the locomotor activity of the animals. Therefore, we can conclude that the observed anti-depressant-like activity in FST and TST is specific and not related to the stimulation of the general motor activity of animals. However, diazepam as a positive control significantly decreased the total movement of animals compared to the control group.

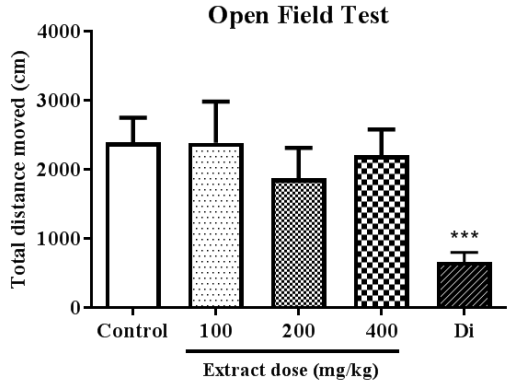

Figure 5. Effect of *T. platyphyllos* extract as well as diazepam (Di; 1 mg/kg) on the total distance moved in the open field test; Values are expressed as mean ± SEM. *** $P < 0.001$ indicate significant differences compared with the control group.

3.6. Estimation Maximum Non-Fatal Dose and Median Lethal Dose

Following intraperitoneal injection of *T. platyphyllos* extract in various doses, the LD_{50} value and maximum non-fatal dose were 4.8 (4.6-5.1) g/kg and 2 g/kg, respectively. The calculated LD_{50} value and maximum non-fatal dose are far from the antidepressant, anxiolytic and hypnotic doses of the extract (400 mg/kg) suggesting that *T. platyphyllos* extract is apparently safe when administered as an antidepressant, anxiolytic, and hypnotic agent. The estimated estimation of LD_{50} value and maximum non-fatal dose could be also used for the selection of the right doses when conducting other behavioral experiments. However, more studies are required for a comprehensive evaluation of *T. platyphyllos* extract toxicity.

4. Conclusion

This study, for the first time, evaluated the antidepressant-like, anxiolytic, and hypnotic effects of the hydroethanolic extract of *T. platyphyllos* through experimental animal models. The biological results presented here led us to conclude that the leaves of *T. platyphyllos* can be a prominent source of antidepressant, anxiolytic and hypnotic agents. Probably, some phytochemicals compounds of *T. platyphyllos* such as quercetin, kaempferol, and catechin can regulate the same pathways targeted by antidepressant, anxiolytic and hypnotic medicines. However, there is still a great need for additional investigations on the mechanisms involved in the observed effects and possible side effects to consider *T. platyphyllos* as a novel treatment for depression, anxiety, and insomnia. In addition, isolation and purification of the components of *T. platyphyllos* extract may yield novel bioactive agents.

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Conflict of interest

The authors declare to have no conflict of interest.

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