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Anxiolytic- and antidepressant-like effects of an aqueous extract of *Tanacetum parthenium* L. Schultz-Bip (Asteraceae) in mice

Jorge Cárdenas[a], Valeria Reyes-Pérez[a,b,c], María Dolores Hernández-Navarro[b], Ana María Dorantes-Barrón[c], Salvador Almazán[d], Rosa Estrada-Reyes[c,*]

[a] *Instituto de Química, Universidad Nacional Autónoma de México (UNAM), Coyoacán, 04510 Ciudad de México (CDMX), México*
[b] *Facultad de Química, Universidad Autónoma del Estado de México (UAEM), Toluca, Estado de México, México*
[c] *Laboratorio de Fitofarmacología, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz (INPRFM), Calzada México-Xochimilco 101, Col. San Lorenzo Huipulco, Del. Tlalpan, 14370 CDMX, México*
[d] *Departamento de Bioelectrónica, Dirección de Investigaciones en Neurociencias, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz (INPRFM), Calzada México-Xochimilco 101, Col. San Lorenzo Huipulco, Del. Tlalpan, 14370 CDMX, México*

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ABSTRACT

Aim: *Tanacetum parthenium* L. Schultz-Bip (Asteraceae) is widely used worldwide in traditional medicine for the treatment of convulsions and culture-bound syndromes such as *susto* (fear). The aim of this work was to evaluate the anxiolytic- and antidepressant-like effects of an aqueous extract of *T. parthenium* in behavioral paradigms in mice. The effects of *T. parthenium* were compared with those produced by anxiolytic and antidepressant drugs. We carried out the chemical characterization of the main constituents of *T. parthenium*. The involvement with the GABAergic and serotoninergic neurotransmitter systems were explored be means of synergic and antagonist experiments.

Materials and methods: The anxiolytic-like effect was evaluated using the Burying Behavior Test (BBT) and the Elevated Plus-Maze Test (PMT). The antidepressant-like effect was evaluated in the Forced Swimming Test (FST), and ambulatory activity was assessed in the Open Field Test (OFT). Employing the behavioral tests, synergism and antagonism experiments with Alprazolam, Muscimol, and Picrotoxin were carried out in the PMT. In a series of independent experiments, concomitant administration of *T. parthenium* and Alprazolam, Fluoxetine, or *p*-chlorophenylalanine were conducted in the FST.

For chemical characterization, High-Performance Liquid Chromatography-Electro Spray Ionization-Mass Spectrometry (HPLC-ESI-MS) analysis was performed.

Results: *T. parthenium* exerts clear anxiolytic- and antidepressant-like effects in mice, without affecting the ambulatory activity of the experimental subjects.

Conclusions: Anxiolytic- and antidepressant-like *T. parthenium* effects result, at least part from the involvement of the GABAergic system. Our results support the use of *Tanacetum parthenium* in traditional medicine and suggest its therapeutic potential in the comorbid anxiety and depression.

1. Introduction

Tanacetum parthenium L. Schultz-Bip (Asteraceae) is a bushy aromatic plant with feathery leaves and daisy like flowers, due resemblance to *matricaria*, it is known as feverfew, is a perennial species native to Eurasia, specifically the Balkan Peninsula, Anatolia and the Caucasus. However, cultivation has spread it around the world, and it is now also found in the remainder of Europe, in North America, and in Chile (Jeffrey, 2001; Pareek et al., 2011). It has adapted to Mexico, where it is known by several popular synonyms, including *altamiza* (feverfew), and *hierba de Santa María* (St. Mary's grass), *manzanilla grande* (large feverfew), manzanilla romana (Roman *chamomile*), *mastranzo*, *matlali*, and *yerba santa*. At the State of México; Otomí ethnia recognizes it as dhata manzanilla. The Náhuatlacas' native culture of Tlaxcala state is also known as caltemesha, and it is found distributed throughout the entire country, (Villaseñor and Espinosa, 1998). In traditional medicine in Denmark, *T. parthenium* is utilized in the prophylactic treatment of migraine, epilepsy, and convulsions, as well as a sedative and a sleep inducer (Jäger et al., 2006, 2009).

* Corresponding author.
E-mail address: estrarosa@hotmail.com (R. Estrada-Reyes).

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In Mexican traditional medicine, *T. parthenium* is used to relieve ailments, such as fevers, migraine, headache, rheumatoid arthritis, stomachache, toothache, and as an analgesic, anti-inflammatory, and antispasmodic agent. This species is also employed to treat culture-bound syndromes such as *susto* (roughly translated as fear), *espanto* (nervousness), or *quemados* (burns) (Diccionario enciclopédico de la Medicina Tradicional Mexicana, 2009; Bourbonnais-Spear et al., 2007; Estrada-Reyes et al., 2014). Within this cross-cultural construction of illness, there is an association between beliefs of *susto* and depression. *Susto* has also been associated with social *role* stress and depressive symptoms (Weller et al., 2008) and *espanto* has been related with *nervousness or nerves*, to communicate anxiety and emotional distress (Mendenhall et al., 2012). Furthermore, although its etiology is an area of active research, American Psychiatry Association (DSMV, 2013) associates *susto* with mental disorders, including a number of anxiety and fear-related conditions (i.e., Post-Traumatic Stress Disorder [PTSD], anxiety disorder, major depressive disorders, somatoform disorders, and other specified neurotic disorders).

Phytochemical studies of this species have demonstrated a large number of secondary metabolites including sesquiterpene lactones, such as parthenolide, santamarin, and other potentially active constituents including flavonols such as santin, and flavonoid glycosides such as 6-hydroxykaempferol derivatives, quercetagetin, apigenin and apigenin 7-glucuronide, and luteolin, among others (Majdi et al., 2011; Long et al., 2003; Chávez and Chávez, 1999; Williams et al., 1999a and 1999b).

On the other hand, in humans, there are high levels of comorbidity between anxiety and depression disorders (Kessler et al., 2010; Kelsey and Collimore, 2014), that share neural pathways; thus, numerous neurotransmitters systems are involved in the underlying mechanisms of anxiolytic and antidepressant drugs.

GABA is the principal inhibitory neurotransmitter in the mammalian Central Nervous System (CNS) and plays an important role in epilepsy and anxiety (Olsen and DeLorey, 1999; Möhler, 2006; Treiman, 2001). Therefore, it is not surprising that modulation of GABAergic transmission comprises one of the main mechanisms for controlling these disorders (Morimoto et al., 2004; Sherif and Ahmed, 1995; Beleboni et al., 2004). Is widely known that anxiolytic drugs such as the Benzodiazepines (BDZ) act to enhance the effect of GABA at the $GABA_A$ receptor, even more so, some antidepressant derivatives of BDZ, such as Alprazolam, not only induce anxiolytic actions, but also have antidepressant effects; thus, they are useful in the treatment of comorbid anxiety and depression.

The aim of this work was to investigate the anxiolytic- and antidepressant-like effects of an aqueous extract of *T. parthenium* in behavioral paradigms in mice. The main constituents of *T. parthenium* were determined by High-Performance Liquid Chromatography-ElectroSpray Ionization-Mass Spectrometry (HPLC-ESI-MS) analysis.

The anxiolytic effect of *T. parthenium* was evaluated utilizing the Burying Behavior Test (BBT) and the Plus-Maze Test (PMT), and the results compared with the effects of Diazepam (DZ), and Alprazolam (ALP). The antidepressant-like effect of *T. parthenium* was evaluated with the Forced Swimming Test (FST) and compared with the effects of Fluoxetine (FLX) and ALP. The effects of *T. parthenium* on locomotor activity were evaluated in the Open Field Test (OFT).

We also explored the possible neurotransmission pathways that underlie *T. parthenium* actions using synergism with sub-effective doses of the GABAergic agonist, muscimol, with non-effective doses of *T. parthenium*. *T. parthenium* anxiolytic actions were blocked by co-administration of *T. parthenium* at 5 mg/kg with Picrotoxin, a GABAergic antagonist in the PMT. Finally, in independents experiments, *T. parthenium* antidepressant effects included enhancement with the co-administration of a *T. parthenium* non-effective dose in combination with a non-effective FLX or ALP dose in the FST, and their actions were blocked by the combination of *T. parthenium* effective doses with *p*-chlorophenylalanine (PCPA), a serotoninergic full antagonist.

2. Materials and methods

2.1. Plant material

Leaves of *Tanacetum parthenium* Asteraceae (before; *Chrysanthemum parthenium*) were collected in July 2014 in the Estado de México Municipality of Zinacantepec, Contadero de Matamoros (San José) México, in the yard of a private home 19°03′47″N 99°54′47″W altitude, 3073 m above sea level.

Botanical identification was carried out by Óscar Hinojosa-Espinosa, M. SC., and a sample was deposited in the Universidad Nacional Autónoma de México (UNAM) National Herbarium (voucher specimen number: MEXU1392990).

The aerial parts of *Tanacetum parthenium* were air-dried and finely ground and aqueous extracts were prepared at a ratio of 10 g of vegetal material per 90 mL of boiling distilled water and heated for 10 min. The resulting extract was allowed to cool to room temperature, filtered, and dried in a Heto FD3 freezer dryer at −50 °C and 0.01 mBar, yielding 15.1% (1.51 g) of dried aqueous extract. The extract was stored − 4 °C until pharmacological assays or HPLC analyses were performed. To facilitate HPLC analysis, 20-mg aliquots of the aqueous extract were filtered through an OASIS Waters HLB 1 cc pre-column, eluted with H_2O, H_2O-MeOH mixtures (80:20, 50:50, and 20:80), MeOH, and MeCN prior to HPLC analysis.

2.1.1. Aqueous extract analysis by HPLC-ESI-MS

The aqueous extract was analyzed by Aligent Technologies 1200 Series HPLC, utilizing a Millennium Chromatography Manager (464) system, with an Ultraviolet (UV) Detector of Diode (DAD) rearrangement (Waters 2996). Reverse-phase separation was conducted at room temperature, employing a Synergi Polar-RP 80A 150×2.00 mm I.D., with a 4-μm particle size. The column was eluted at a flow-rate of 0.2 mL/min, in gradient mode, with mixtures of MeOH/H_2O (3:97; 5 min), MeOH/H_2O (80:20; 40 min), and H_2O (5 min), for a total runtime of 50 min. Elution was monitored at 220 and 254 nm. HPLC was coupled to a Bruker Daltonics Esquire 6000 mass spectrometer.

An aliquot of the aqueous extract was filtered through C18 Waters Sep-Pak® cartridges. Three fractions were obtained and injected into the HPLC system. Santin [**1**] and santamarin [**2**] were previously isolated from organic extracts of *Tanacetum parthenium* and served as standards. HPLC peaks were not corrected for response factors and are reported as relative percentage of the area (Estrada-Reyes et al., 2010). The identity and purity of the standards were confirmed by their physical and chemical properties and their spectral data (^1H and ^{13}C Nuclear Magnetic Resonance [NMR] and MS), by means of comparison with results described in the literature.

2.2. Pharmacological evaluations

2.2.1. Animals

Adult male Swiss Webster mice (weighing 20–30 g) were obtained from the Vivarium at the Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz (INPRFM) Vivarium. All animals were housed eight per cage in a temperature-controlled (20–21 °C) room under inverted light:dark conditions (12 h:12 h, lights on at 22:00 h). All behavioral evaluations were performed between 10:00 and 14:00 h. Animals had *ad libitum* access to Purina rodent chow and water. The animals were managed in agreement with The General Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985) and the 'Norma Oficial Mexicana' (NOM-062-ZOO-1999); furthermore, the experimental protocol was approved by the local ethical committee (NC093620.0).

2.2.2. Drugs and dosage

Doses are expressed as milligrams of drug per kilogram of Body Weight (BW) *per* mouse. *T. parthenium* (TP) (0.5, 1.0, 5, 10, 20, and

40 mg/kg) was dissolved in saline (0.9% NaCl) and administered orally (p.o.) by gastric gavage. Diazepam (DZ; 0.25, 0.5, 1, or 2 mg/kg), Muscimol (MUS; 0.5 mg/kg),

Fluoxetine (FLX; 10 mg/kg), *p*-chlorophenylalanine (PCPA; 100 mg/kg), Picrotoxin (PTX; 0.5 mg/kg), and Alprazolam (ALP; 0.062, 1.25, 0.25, and 0.5 mg/kg) were purchased from Sigma-Aldrich, (Mexico City, Mexico). These were dissolved in saline and administered Intraperitoneally (IP). Control animals received the same volume of the vehicle (saline, 0.9% NaCl). For habituation, all mice received a daily p.o. or IP administration of saline for 5 days prior to treatment. Each treatment had its own control group, which was treated with the corresponding vehicle. Drugs were administered 30 min before the test, and all experiments were performed with independent groups of eight mice each.

2.2.2.1. Burying Behavior Test (BBT). Mice were individually tested in a cage with exactly the same dimensions as the home cages (15×24×11 cm), but with an electrified prod (7 cm long) emerging from one of its walls, 2 cm above the bedding material of fine sawdust. Each time the animal touched the prod, the former received an electric shock of 0.3 mA. The source of the shock was a constant current shocker (direct current apparatus, manufactured by Eng. Salvador Almanza, Bioengineering Department, INPRFM). The prod remained electrified throughout the test. Immediately after the placement of the animal in the cage, its behavior was recorded for 10 min. Once the animal received the first shock, it typically moved toward the prod, recognizing it as an aversive stimulus. The animal then sprayed and pushed a pile of bedding material ahead with rapid alternating movements of its forepaws. The parameters registered in this anxiety test were burying behavior latency (time, in sec, from the first shock to the display of the burying behavior) and cumulative burying behavior (cumulative time, in sec, that the animals spend burying the prod). In this test, a decrease in cumulative burying behavior is interpreted as a reduction in anxiety (Pinel and Treit, 1978; Treit, 1985; De Boer and Koolhaas, 2003; López-Rubalcava et al., 2006). An increase in burying behavior latency is considered to reflect decreased reactivity (the readiness of the animal to respond to a certain condition).

2.2.2.2. Elevated Plus-Maze Test (PMT). This model has been widely validated for measuring anxiolytic-like effects in rodents (Lister, 1987). The apparatus consisted of two opposite open arms (30×8 cm), intersected (center platform) by two closed arms of the same dimensions, with 19-cm-high walls. The arms were connected to an 8×8 cm central square. The apparatus was elevated 55 cm above the floor, in a dimly illuminated room. Mice were placed individually in the center of the plus-maze, facing an open arm. During the 5-min test, the time spent in each arm was measured. The conventional spatial-temporal measurements comprised the number of entries (all paws on open or closed arms and expressed as percentage of total entries), Time spent in the Open Arms (TOA) was expressed as a percentage of the Time spent in the Open Arms (TOA), % TOA=TOA/TCA×100 (Time spent in Closed Arms; TCA), % Open Arm Entries (% OAE), OAE=Open Arm Entries/[Closed Arm Entries+Open Arm Entries]×100 and time on the Central Platform (TC)=[TOA+TBC] −300; (300 s=test time, in such a way that TOA+TCA+TC=300 s). Percentage of time and number of entries in the open arms are considered indices of anxiety-level behavior (Lister, 1987; Rocha et al., 2002). Ethological behaviors, such as time spent on the Central Platform (CP) and number of head dipping was also registered.

2.2.2.3. Forced Swimming Test (FST). Mice were individually placed into glass cylinders (height: 21 cm, diameter; 14.5 cm) containing 15 cm of water at 23 ± 1 °C. All animals were forced to swim for a 15-min period (pre-test), followed by a 3-min swimming session (test) 24 h later. Total immobility time was measured in sec. Immobility behavior was scored when the mouse remained floating and treading water just sufficiently to keep its nose above water. After the swimming sessions, the mice were removed from the cylinder and carefully dried, placed in heated cages for 20 min, and then returned to their home cages. All experimental sessions were videotaped and later scored by an observer who was unaware of the pharmacological treatments (Porsolt et al., 1977a, 1977b; Martínez-Vázquez et al., 2012).

2.2.2.4. Open Field Test (OFT). To discard possible unwanted or nonspecific side effects of drug treatments on locomotor activity, all treatments studied in the anxiety and depression paradigms were analyzed in the Open Field Test (OFT). The OFT system (LE8825, Panlab InfraRed (IR) Harvard apparatus) consisted of Perspex panels (45×45 cm, 2.2-cm thick) in a 2-Dimensional (2D) (X and Y axes) square frame, a frame support, and a control unit. Each frame counted employed 16×16 infrared beams for optimal subject detection. The frames were controlled by independent control units. Acti Track software was utilized to analyze the animals' trajectories. Data were exported to a computer for analysis. The number of times the animal entered each square (counts/10 min) was recorded. A change in counts was considered an alteration in the locomotor activity of the experimental subject, produced by the drugs (Estrada-Reyes et al., 2014).

To explore the participation of the GABAergic system in the anxiolytic- and antidepressant-like actions of T. parthenium, the following experiments were performed.

Experiment I: PTX at 0.5 mg/kg was administered simultaneously with *T. parthenium* (5 mg/kg; −30 min) to mice, followed by testing in the PMT.

Experiment II: One group was treated with a non-effective dose of the $GABA_A$ agonist muscimol (MUS; 0.5 mg/kg) jointly with a threshold dose of *T. parthenium* (0.5 mg/kg), insufficient to induce anxiolytic effects. After 30 min, the mice were subjected to the PMT to determine the possible synergic effect.

Experiment III: Sub-threshold doses of alprazolam (ALP, 0.03 mg/kg), an anxiolytic, triazolobenzodiazepine (thiazolo-BDZ), and *T. parthenium* (0.5 mg/kg) were administered together to mice that, after 30 min, were subjected to the PMT.

To determine whether antidepressant drugs are able to block or enhance the antidepressant-like effects of T. parthenium in the forced swimming paradigm, experiments to antagonize and synergize the drug were designed.

Experiment I: A group was treated with T. parthenium at a single sub-effective dose (5 mg/kg) in combination with a sub-threshold dose of Fluoxetine (FLX; 10 mg/kg), a serotonin reuptake inhibitor. Thirty min later the mice were tested in the FST.

Experiment II: Two independent groups of eight animals each were pretreated with PCPA (100 mg/kg, an inhibitor of serotonin synthesis), and a third group was given only the vehicle, once a day, for 4 consecutive days (Cassani et al., 2014). After the last administration of PCPA, one group was treated with *T. parthenium* (20 mg/kg, p.o.) and the remaining groups only received vehicle. Then, all mice were tested in the FST 30 min later.

2.2.2.5. Statistical analysis. Results are present as the mean ± Standard Error of the Mean (SEM). Data were analyzed with Kruskal–Wallis ANalysis Of VAriance (ANOVA) on ranks ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$), followed by the Mann–Whitney rank sum test. The SigmaPlot ver. 12.5 statistical software program was used to make the graphics and to conduct the analysis.

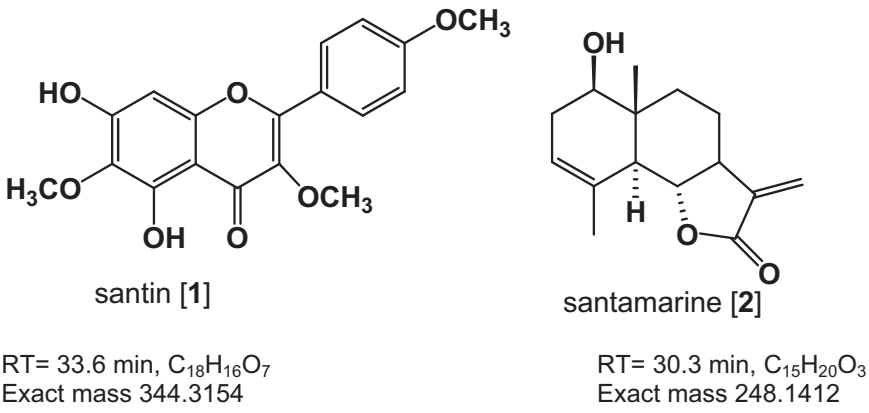

Fig. 1. HPLC and structural characteristics of santin [1] and santamarine [2], secondary metabolites present in the aqueous extract of *Tanacetum parthenium*. RT=33.6 min, $C_{18}H_{16}O_7$ RT=30.3 min, $C_{15}H_{20}O_3$ Exact mass 344.3154 Exact mass 248.1412.

3. Results

3.1. Chemical Analysis of aqueous extract of T. parthenium

Santin and santamarine were isolated from the organic extracts and utilized as standards in the HPLC-MS analysis. Sesquiterpene lactones, such as parthenolide or canin, were not isolated from the organic extract and were not identified in the aqueous extract of *T. parthenium*. The aqueous extract of *T. parthenium* was found to contain the flavone santin [**1**] at 7.4% (relative abundance), and santamarine [**2**] (an isomeric form of parthenolid), a sesquiterpene lactone, was found at 4% (Fig. 1). It has been described that parthenolid is present only in trace in Mexican samples of feverfew (Hausen, 1981).

The BBT was employed to measure the anxiolytic effects of different doses of *T. parthenium* aqueous extract. As presented in Table 1, *T. parthenium* at 5, 10, and 20 mg/kg decreased the cumulative time that animals spent burying the prod and increased burying behavior latency in a dose-dependent fashion; this effect was similar to the results produced by DZ at 0.5 mg/kg. The one-way ANOVA on ranks revealed that for the two parameters analyzed (cumulative burying behavior and burying behavior latency), both *T. parthenium* and DZ exhibited statistically significant differences with respect to the control group (H=29.420; df=5; $p \leq 0.001$).

3.2. Effects of increasing doses of T. parthenium on the PMT are depicted in Fig. 2

The acute treatment with *T. parthenium* at 0.5, 1, 5, and 10 mg/kg produced a significant increase in both % of time spent in the open arms (H=30.22; df=6; $p \leq 0.001$), and percentage of open-arm entries (H=32.75; df=6; $p \leq 00.1$) compared with the vehicle-treated group. Similarly, mice treated with DZ at doses ranging from 0.25 to 2.0 mg/kg produced a significant increase in % of time spent in the open arms in a dose-dependent manner (H=32.292; df=4; $p \leq 0.001$) and percentage of open-arm entries (H=28.530; df=4; $p \leq 0.001$), and mice treated with ALP at 0.25 and 0.5 mg/kg also spent significantly more time on the open arms and presented an increased percentage of entries on the open arms. At higher doses (20 and 40 mg/kg), *T. parthenium* did not produce changes compared with the vehicle-treated group.

Other parameters measured in the PMT are illustrated in Table 2. Animals treated with *T. parthenium* (1, 5, 10, 20, and 40 mg/kg) also demonstrated increases in time spent in head dipping (H=20.410; df=6; $p \leq 0.01$), while time spent on the CP only increased significantly with *T. parthenium* at 40 mg/kg (H=13.6; df=6; $p \leq 0.03$). Likewise, total arm entries differed only at major *T. parthenium* doses (20 and 40 mg/kg) from those of the vehicle-treated group.

In the FST, as presented in Fig. 3, *T. parthenium* at 10-, 20-, and 40-mg/kg doses significantly reduced immobility time with respect to the control group (H=33.590; df=5; $p \leq 0.001$). In a similar manner, ALP at 0.03 and 0.062 mg/kg significantly reduced immobility time compared with the control group (H=22.10; df=2; $p \leq 0.001$). In contrast with *T. parthenium* and ALP, acute FLX administration was not able to modify the immobility behavior, this fact in concordance with those previously reported (Martínez-Vázquez et al., 2012).

All of the drugs were tested in the OFT, and the effects of *T. parthenium* on the locomotor and exploratory activity of the experimental animals are exhibited in Table 3. *T. parthenium* did not modify the count number (H=19.18; df=6; $p=0.51$) or alter the rearing number (H=12, df=6, p=0.06) compared with the control group, *T. parthenium* did not alter any exploratory parameters in the OFT. In addition, no drug modified the ambulatory behavior of the animals tested (data not shown).

Table 1
Effect of TP and DZ (0.5 mg/kg) on the burying latency time and burying cumulative time in the Burying Behavior Test.

Treatment (mg/kg)	Burying latency time (s)	Burying cumulative time (s)
Control	32.90 ± 5.99	76.44 ± 10.92
DZ 0.5	35.97 ± 4.12	36.79 ± 5.68***
TP 1	28.90 ± 6.36	70.62 ± 11.05
TP 5	26.43 ± 12.56	31.12 ± 4.32**
TP 10	38.43 ± 11.45	9.28 ± 4.32***
TP 20	34.44 ± 7.06	6.71 ± 2.51***
	H=10.1, df=5, p=0.072	H=29.4, df=5, p≤0.001

Effect of treatment with single dose (p.o.) of TP at 1, 5, 10, 10, and 20 mg/kg and single treatment with diazepam (DZ 0.5 mg/kg), by intraperitoneally via in the BBT in mice. All results are expressed as the mean ± SEM of 8 animals. Comparisons were made using the Kruskal-Wallis analysis of variance based on rank, followed by the Mann-Whitney-*U*-test.

*** $p < 0.001$.

** $p < 0.01$.

3.3. Effects of co-administration of T. parthenium plus PTX and plus ALP in the PMT

To determine whether the GABAergic antagonist Picrotoxin could block the anxiolytic actions of *T. parthenium*, an anxiolytic dose of *T. parthenium* (5 mg/kg) was jointly administered with PTX at 0.5 mg/kg; after 30 min, the experimental animals were subjected to the PMT. As can be observed in Table 4, treatment with PTX alone at 0.5 mg/kg did not produce any effect in comparison with the control group, while this same PTX dose was able to block the anxiolytic effect produced by *T. parthenium* when the two were jointly administered. In addition, the *T. parthenium* /PTX combination reduced the % of time spent in open arms by 70% with respect to the control group.

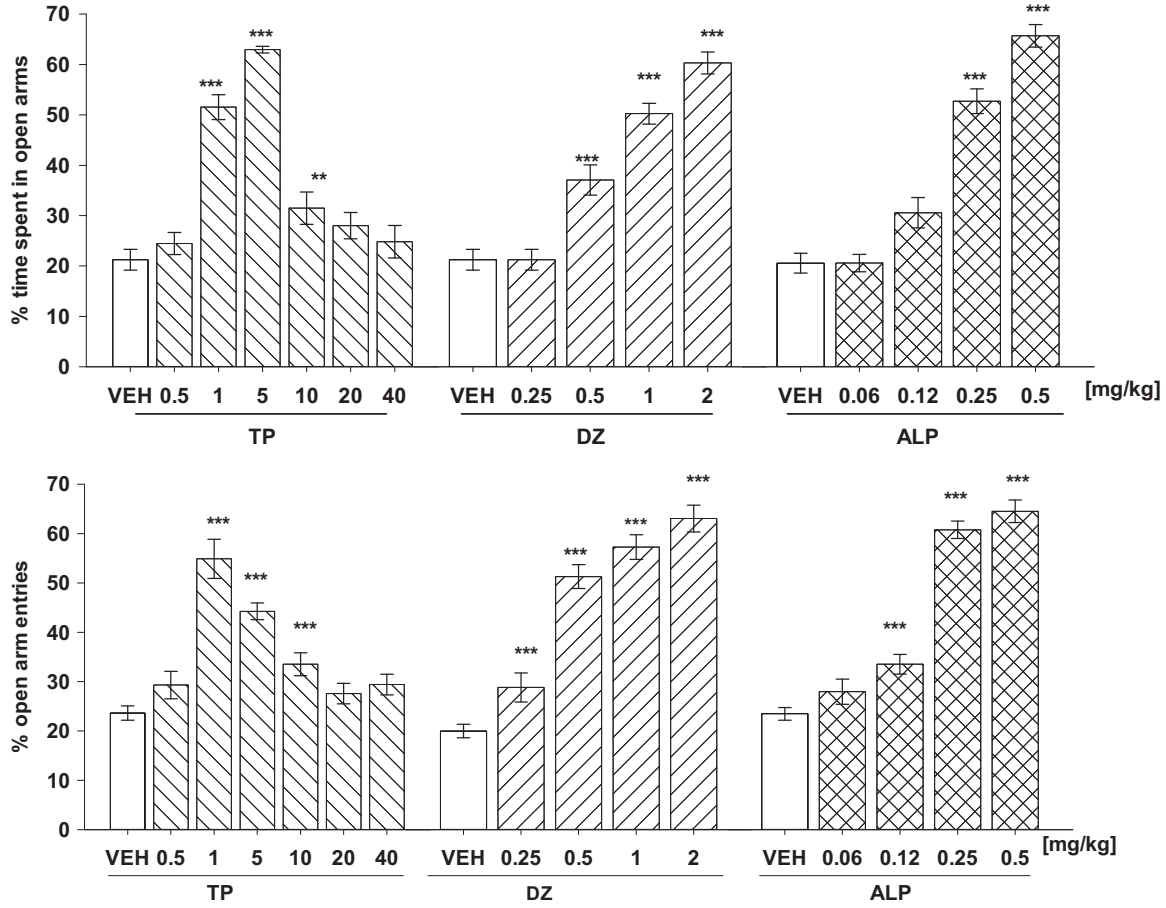

Fig. 2. Effects of a single-dose oral with the TP and effect of a single-dose intraperitoneally of DZ and ALP on the elevated plus-maze test in mice. Effects of TP via orally dose, and DZ and ALP intraperitoneally dose on the elevated plus-maze test in mice. Percentage of entries and time spent in open arms (open arms/total×100) is shown. TP (aqueous extract of *Tanacetum parthenium*) was administered *per os*. DZ (diazepam) and ALP (alprazolam) were administered intraperitoneally. Data are expressed as the mean ± SEM. of groups of 8–9 animals. Comparisons were made using the Kruskal-Wallis analysis of variance based on rank, followed by the Mann-Whitney-*U*-test. Significantly different from vehicle treated group *p≤0.05, **p≤.01, and ***p≤0.001.

Table 2
Effects of a single-dose treatment with the TP, DZ, and ALP upon the behavioral parameters recorded in the elevated plus-maze test in mice.

Treatment (mg/kg)	TCA	%TCA	Total arm entries	central platform time (s)	time spent in head dipping (s)
VEHICLE	176.4 ± 11.5	75.5	17.37 ± 1.1	66.2 ± 10.9	13 ± 0.8
TP0.5	138.9 ± 10.5**	70	19.25 ± 1.5	61.5 ± 10.8	14.1 ± 1.1
TP1	106 ± 11.6**	49	18.6 ± 1.6	83.5 ± 11.2	27.7 ± 3.5***
TP 5	127 ± 6***	40	20.8 ± 1.3	79.28 ± 8.9	23.3 ± 2.2***
TP10	162.2 ± 11.2	69	19.3 ± 0.7	63.2 ± 6.9	17 ± 3.3**
TP20	143.4 ± 7.7	71	24.2 ± 1.2***	99.1 ± 10.8	18 ± 2.3**
TP40	152.9 ± 8.7	76	22.2 ± 1.65**	98.8 ± 5.6*	16 ± 2.3**
	H=18.36, df=6. P=0.005		H=17.78, df=6, p=0.007	H=13.60, df=6, p≤0.03	H=20.41, df=6, p=0.002
VEHICLE	216.4 ± 10.9	79	16.3 ± 1.2	66.3 ± 6.9	10.25 ± 1
DZ 0.25	143 ± 8***	45	15.1 ± 1.3	117.6 ± 6.2***	11.87 ± 1
DZ 0.5	129.3 ± 5***	63	19.3 ± 0.8	93.9 ± 6.5*	14.3 ± 1
DZ 1	110 ± 5.7**	58	19 ± 2.1	77.31 ± 16	17.5 ± 1.8
DZ 2	89.3 ± 7.1***	39	19.2 ± 2	72.2 ± 11.7	22 ± 2.1
	30.06, df=4, p≤0.001		H=6.41, df=4, p≤0.17	H=21.8, df=4, p≤0.001	H=18.35, df= 4, p≤0.001
VEHICLE	178 ± 10.4	76	16.7 ± 1	65.4 ± 9.6	13. ± 0.7
ALP 0.06	173.3 ± 5.1	79	14.5 ± 1.3	80.9 ± 7.4	10.2 ± 1.4
ALP 0.12	188.2 ± 11.9	80	14.1 ± 2.1	63.9 ± 7.5	12.6 ± 1.7
ALP 0.25	66.7 ± 8.9***	56	21 ± 1.9	98.9 ± 10.7	25.2 ± 1.8
ALP 0.5	116.1 ± 7.11***	67	31.6 ± 2.3***	54.3 ± 9.	29.8 ± 2.5
	H=32.32, df=4, p≤0.001		H=24.1, df=4, p≤0.001	H=6.4, df=4, p≤0.17	H=28.34, df=4, p=≤0.001

Effects of a single-dose treatment with the TP (5–40 mg/kg), DZ (0.25–2 mg/kg), and ALP (0.06–0.5 mg/kg) upon time spent in close arms (TCA), % TCA, total arm entries, central time (s), and time spent in head dipping (s) the in the elevated plus-maze test in mice. All results are expressed as the mean ± s.e.m. of 8 animals. Comparisons were made using the Kruskal-Wallis analysis of variance based on rank, followed by the Mann-Whitney-*U*-test.

** $p < 0.01$.

*** $p < 0.001$.

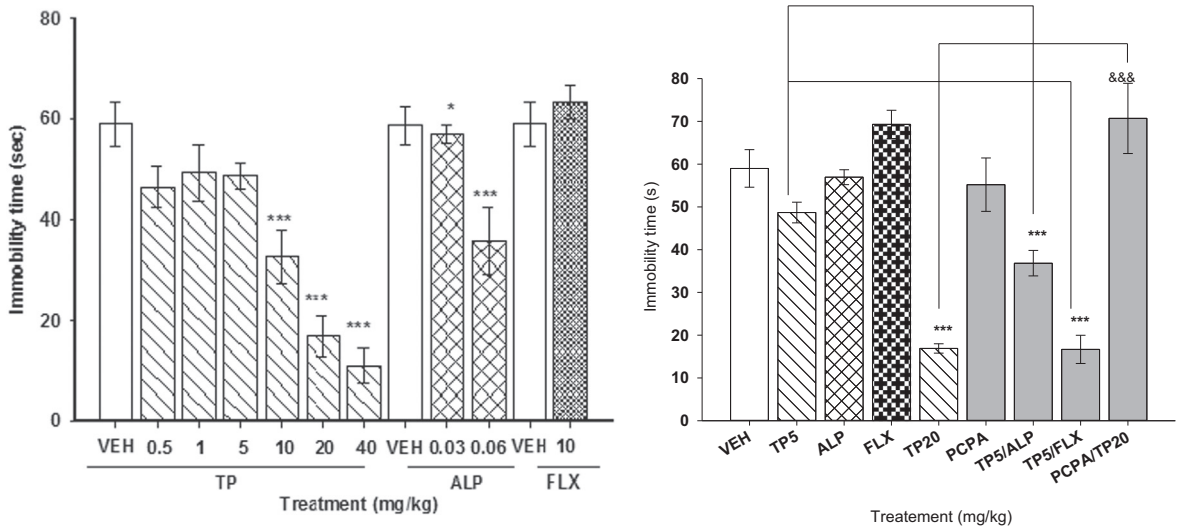

Fig. 3. Effects of TP, ALP, and FLX on immobility time in the Forced Swimming Test. Antidepressant-like effects of an aqueous extract of *T. parthenium* (TP), alprazolam (ALP), and fluoxetine (FLX) on the forced swimming test. Data are expressed as the mean ± SEM of groups of eight animals; Comparisons were made using the Kruskal-Wallis analysis of variance based on rank, followed by the Mann-Whitney-*U*-test. Significantly different from vehicle treated group (VEH) *p≤0.05 and ***p≤0.001.

Table 3
Effect of aqueous extract of leaves of *Tanacetum parthenium* (TP) on the ambulatory activity of mice in the OFT.

Treatment (mg/kg)	n	Count number/5 min mean ± s.e.m.
control	7	42.85 ± 4.96
TP 0.5	8	33.00 ± 2.98
TP 1	8	55.62 ± 4.53
TP 5	8	47.71 ± 3.57
TP 10	8	37.12 ± 3.29
TP 20	8	36.62 ± 1.99
TP 40	8	39.00 ± 1.75
		H=2.87, df=6, p=0.579
control	8	50.75 ± 2.78
DZ 0.25	8	58.12 ± 5.85
DZ 0.5	8	59.50 ± 5.64
DZ 1.0	8	46.87 ± 5.91
DZ 2.0	8	44.25 ± 6.16*
		H=19.18, df=4, p=0.04

Results are expressed as mean ± SEM statistical comparisons were made between the saline-treated group and the experimental groups using a Kruskal-Wallis One Way Analysis of Variance on Ranks, followed by the Mann-Whitney Rank Sum Test (p* < 0.05).

Table 4
Effects of the combination of TP (0.5 mg/kg) *plus* PTX (0.5 mg/kg), TP (0.5 mg/kg) *plus* ALP (0.12 mg/kg), and TP (0.5 mg/kg) plus MUS (0.5 mg/kg) in plus-maze test.

Treatment (mg/kg)	Time spent in open arms (s)	Time spent in close arms (s)	% Time spent in open arms (s)
control	57.2 ± 4.3	176.4 ± 11.5	21.2 ± 2.0
TP 0.5	59.5 ± 3.9	138.9 ± 10.5	30.4 ± 2.2
PTX 0.5	47.3 ± 2.6	211.1 ± 7.3^{++}	18.4 ± 1.3
ALP 0.12	43.3 ± 6.0	194.1 ± 11.7	18.5 ± 2.8
TP 5/PTX 0.5	13.0 ± 2.0^{++}	231.9 ± 8.1^{+++}	5.3 ± 0.7^{+++}
TP 0.5/ALP 0.12	90.7 ± 9.28	102.3 ± 7.8***	46.7 ± 2.9***
MUS 0.5	66.1 ± 4.30	144.0 ± 9.1	31.5 ± 3.6
MUS 0.5/TP 0.5	124.8 ± 12.9***	107.0 ± 17.9***	54.8 ± 5.6***
	H=61.51, df=9, p≤0.001	H=49.02, df=9, p≤0.001	H=59.11, df=9, p≤0.001

Effect of co-administration of TP at 0.5 mg/kg (p.o.) with picrotoxin (PTX; 5 mg/kg, IP), TP (0.5 mg/kg) with alprazolam (ALP; 0.12 mg/kg, IP), and TP (0.5 mg/kg, p.o.) with muscimol (MUS, 0.5 mg/kg, IP). All results are expressed as the mean ± SEM of 8 animals. Comparisons were made using the Kruskal-Wallis analysis of variance based on rank, followed by the Mann-Whitney-U-test: ** p < 0.01 (*significant increase with respect to control group), (+ significant decrease with respect to control group).
$^{++}$ p < 0.01.
$^{+++}$ p < 0.001.
*** p < 0.001.

In this same paradigm, the combination of a sub-threshold dose of *T. parthenium* (0.5 mg/kg) and an ineffective dose of ALP (0.12 mg/kg) were evaluated. Our results revealed that the combination of *T. parthenium* /ALP at a non-effective dose, significantly increased time and percentage spent in open arms and reduced time spent in closed arms in the PMT, compared with the control group (Table 4).

3.4. *Effects of co-administration of* T. parthenium *plus ALP, plus FLX, and plus PCPA in the FST*

To determine whether ALP could enhance the antidepressant-like actions of *T. parthenium*, a combination of non-effective doses of *T. parthenium* (5 mg/kg) and ALP (0.03 mg/kg) were co-administered and the effect was evaluated in the FST. As demonstrated in Fig. 3, ALP, a benzodiazepine-type antidepressant drug at 0.03 mg/kg, in combination with 5 mg/kg of *T. parthenium*, providing an antidepressant-like effect with a statistically significant difference in the Mann–Whitney test: T=97; *n*=8, and *p*≤0.001. Likewise, the combination of threshold doses of *T. parthenium* with a single, non-effective dose of FLX (10 mg/kg) facilitated the antidepressant actions of *T. parthenium* (T=89; *n*=8; *p*=0.001) compared with the control-treated group.

We explored the involvement of the 5-HT system in the antidepressant-like effect of *T. parthenium*. For this purpose, 5-HT was depleted by means of the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (PCPA, 100 mg/kg, i.p., 4 days) prior to treatment with the *T. parthenium* effective dose (20 mg/kg). The consequent results showed that PCPA blocked the anti-immobility effect of *T. parthenium*, because the group treated with *T. parthenium plus* PCPA did not exhibit a significant difference with respect to the control group (Mann–Whitney test: T=51; *n*=8; *p*=0.08).

4. Discussion

Anxiety and depression disorders are highly prevalent conditions and entertain high levels of comorbidity (Kessler et al., 2010; Kelsey and Collimore, 2014). Individuals affected by both anxiety and

depressive disorders concurrently have generally exhibited greater levels of functional impairment, reduced quality of life, and poorer treatment outcomes compared with individuals with only one of these disorders (Lamers et al., 2011).

As mentioned previously, *Tanacetum parhtenium* is employed by several medical conditions, although to date, few *in vivo* experiments have been carried out. In the present work and, to our knowledge for the first time, we studied the effects of the aqueous extract of *T. parhtenium* on the Central Nervous System (CNS).

One of main purposes of the present study was to analyze the anxiolytic-like effects of the aqueous extract of *T. parthenium*. To allow for this, the effects of *T. parthenium* were evaluated in two predictive animal models of anxiety. In the first assay, experimental anxiety values were determined employing the defensive burying behavior test; this is a conflict model largely validated for the study of both anxiolytic and anxiogenic drugs (Treit, 1985; López-Rubalcava et al., 2006). Our results revealed that the aqueous extract of *T. parthenium* at 5, 10, and 20 mg/kg produced a significant dose-dependent reduction compared with the control group in burying accumulative time, similar to the effects of Diazepam (DZ). It has been reported that compounds with anxiolytic actions decrease burying behavior, conjointly with burying-time latency. Some authors have considered that this behavior is the animals' reactivity in an inverse manner and indicate that classic anxiolytics such as Benzodiazepines (BDZ) decrease reactivity, (López-Rubalcava et al., 2000). It is noteworthy that, herein, the decrease in burying behavior was not accompanied by an increase in burying behavior latency; thus, unlike DZ, *T. parthenium* clearly produced an anxiolytic-like effect without affecting animal reactivity. The anxiolytic effect of *T. parthenium* was also evaluated in the elevated Plus-Maze Test (PMT) (Lister, 1987; López-Rubalcava et al., 2006). The anxiolytic-like effect of TP was indicated by a significant increase in both entry number and time spent in open arms. These responses were observed at lower doses of 0.5–10 mg/kg, achieving best effect at 5- and 10-mg/kg doses. Frequency of entries is the natural spatiotemporal index of anxiety; it is reduced by anxiolytic drugs such as DZ and can be increased by anxiogenic drugs such as picrotoxin (PTX) (López-Rubalcava et al., 2000). In agreement with these findings, we found that *T. parthenium* demonstrated an anxiolytic profile similar to that of this antianxiety drug. Reduction of time spent on the Central Platform (CP) comprises another index of a reduced “decision-making” (hesitant behavior) behavior. Both parameters are accepted as reliable indicators of anxiety and fearfulness (Rocha et al., 2002). *T. parthenium* at any dose did not alter time spent on CP or in total arm entries. These observations also indicate that the anxiolytic-like effect of *T. parthenium* is selective and is not associated with a general stimulation of locomotor activity as a consequence of exposure to a novel environment. These facts were also confirmed by Open Field Test (OFT) results, which did not show any change in the locomotor activity of mice treated with *T. parthenium*. The anxiolytic effects induced by in the PMT were similar to those produced by DZ, an anxiolytic and sedative BDZ that acts as a positive allosteric modulator of the GABA at the BDZ site. Additionally, *T. parthenium* actions were also compared with those induced with alprazolam (ALP), a full agonist to the $GABA_A$ receptor (Rang et al., 1996; Hascoet and Bourin, 1997) that, in addition to its anxiolytic effects produces antidepressant effects. Thus, the three drugs demonstrated a similar anxiolytic effect, with ALP that of greatest pharmacological potency.

To explore the possible involvement of the GABAergic neurotransmitter system in the anxiolytic actions of *T. parthenium*, we conducted independent synergic experiments in which non-effective doses of *T. parthenium* (0.5 mg/kg) and sub-threshold doses of MUS (0.5 mg/kg) or ALP (0.12 mg/kg) were jointly administered. Our results revealed that the anxiolytic-like effect of *T. parthenium* in the PMT was enhanced with both concomitant administration of *T. parthenium* with MUS as well as with ALP. Furthermore, PTX is a non-competitive antagonist of the $GABA_A$ receptor that specifically acts at a site associated with the chloride channel preventing the inhibitory action of GABA, which increase anxiety levels (Petty et al., 1995). In this study, this drug was able to completely block the actions of *T. parthenium* in the PMT. It could be concluded that *T. parthenium* interacts with this neurotransmitter system to mediate its anxiolytic-like actions or *T. parthenium* contains compounds that facilitate GABAergic transmission. In this regard, the anxiolytic actions of numerous flavonoid compounds have been described (Estrada-Reyes et al., 2012; 2014); thus, it is likely that santin [**1**] could be contributing to the actions of *T. parthenium*. In addition, these results are consistent with those previously reported in an *in vitro* assay (Jäger et al., 2009). Taken together, these results suggest that *T. parthenium* interacts with the $GABA_A$ receptor, probably at $GABA_A$ receptor subtypes, in binding sites different from those at classical BDZ effects, produce anxiolytic effects. However, it is necessary to conduct specific studies to determine the specific target.

On the basis of *T. parthenium* in traditional medicine, another purpose was evaluated in this study of its antidepressant-like action in the Forced Swimming Test (FST) in mice. The FST induces a state of immobility in animals facing an inescapable situation of swimming; such immobility behavior has been hypothesized to reflect hopelessness, which may in turn reflect an aspect observed in depressive disorders in humans. Thus, the antidepressant-like activity of a drug is expressed by a decrease in the immobility of animals subjected to the FST. Our results demonstrated that *T. parthenium* induced a dose-dependent reduction of immobility time in the FST at 10, 20, and 40 mg/kg: this antidepressant effect was similar to that produced by ALP (Rang et al., 1996; Hascoet and Bourin, 1997; Hascoet and Bourin, 1997).

Monoaminergic, serotoninergic, and noradrenergic systems are known to play major roles in major depression (Perona et al., 2008). However, the GABA system also participates in the antidepressant actions of thiazole-BDZ such as ALP that, in addition to enhancing the release of serotonin (5-HT) in the hippocampus to produce antidepressant actions, may act by means of a GABAergic mechanism that is independent of the BDZ-site receptor (Jonas and Cohon, 1993; Kalueff and Nutt, 2007; Al-Tubuly et al., 2008). Thus, as may be observed in Fig. 3, fluoxetine (FLX) was not able to reduce immobility time in the FST, as might be expected. This fact agrees with previous reports on Selective Serotonin Reuptake Inhibitor (SSRI) antidepressant drugs, such as FLX at an acute dose, which does not exert an antidepressant-like effect on the FST (Cassani et al., 2014, 2015).

Interestingly, the combination of a sub-effective dose of FLX (10 mg/kg) in combination with a non-effective dose of *T. parthenium* (5 mg/kg) revealed to evident antidepressant activity in the FST.

The synergistic antidepressant-like effect produced by a sub-effective dose of *T. parthenium* and FLX comprised an interesting result that may derive from the effect of *T. parthenium* in the FST and an interaction with the serotoninergic system. In accordance with our earlier findings, we depleted 5-HT by means of the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (PCPA) before treatment with TP. PCPA blocked the antidepressant actions of *T. parthenium* in the FST. These findings support the involvement of the serotoninergic neurotransmitter system in the antidepressant action of *T. parthenium*. In general, a large number of evidences indicate that the serotonin neurotransmitter system is strongly involved in the regulation of mood and anxiety disorders (Millan, 2003). Several pieces of evidence have been implicated in abnormalities in serotonergic transmission in the pathology of depressive disorders. However, we think that it is necessary to perform experiments aimed at determining the specific mechanism underlying the anxiolytic and antidepressant actions of *T. parthenium*.

Previous studies have shown that many psychostimulant drugs can decrease immobility behavior in the FST. These drugs also cause marked increases in the ambulatory activity of the experimental animals. Therefore, to avoid a false positive antidepressant effect, the

locomotor activity of animals subjected to the FST was evaluated employing the OFT. Our results showed that *T. parthenium* significantly reduced immobility time in the FST without increasing ambulatory activity, which confirmed that *T. parthenium* induces a clear, antidepressant-like effect in the FST.

In summary, numerous neurotransmitter systems underlie the states of anxiety and depression. It is accepted that BDZ-type anxiolytics act enhancing the effect of GABA at the $GABA_A$ receptor. Although these classical BDZ are effective anxiolytics, they lack the antidepressant effects. In this study, we demonstrated that *T. parthenium* induced both anxiolytic and antidepressant actions that are mediated, at least in part, by its interaction with $GABA_A$ receptors. Additionally, our findings also show the involvement of the serotonergic neurotransmitter system in the antidepressant actions of *T. parthenium*.

5. Conclusions

This study showed that a single oral treatment of *T. parthenium* induces anxiolytic- and antidepressant-like effects in several behavioral paradigms in mice. *T. parthenium* did not induce alterations in locomotor activity. These results led to evidence of the participation of the GABAergic and serotonergic systems in the actions of *T. parthenium*. Our results support the use of *Tanacetum parthenium* in traditional medicine and reveal its utility for the treatment of comorbid disorders of depression and anxiety.

Competing interest

The authors declare no conflict of interest.
The authors declare that they have no competing interests.

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