



Original article

Chrysin (5,7-dihydroxyflavone) exerts anxiolytic-like effects through GABA_A receptors in a surgical menopause model in rats

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ABSTRACT

The present study investigated the effects of the flavonoid chrysin (5,7-dihydroxyflavone) on anxiety-like behavior in rats in a model of surgical menopause and evaluated the participation of γ -aminobutyric acid-A (GABA_A) receptors in these actions. At 12 weeks post-ovariectomy, the effects of different doses of chrysin (0.5, 1, 2, and 4 mg/kg) were evaluated in the elevated plus maze, light/dark test, and locomotor activity test, and comparisons were made with the clinically effective anxiolytic diazepam. The participation of GABA_A receptors in the actions of chrysin was explored by pretreating the rats with the noncompetitive GABA_A chloride ion channel antagonist picrotoxin (1 mg/kg). The results showed that chrysin (2 and 4 mg/kg) reduced anxiety-like behavior in both the elevated plus maze and light/dark test, and these effects were similar to diazepam. Pretreatment with picrotoxin had no effects on its own but prevented the anxiolytic-like effects of chrysin in both tests. Chrysin also increased rearing and grooming, without significantly altering the number of crossings in the locomotor activity test; these effects were also similar to diazepam. In conclusion, the flavonoid chrysin produced anxiolytic-like effects through actions on GABA_A receptors in a model of surgical menopause in rats. These findings support the hypothesis that this flavonoid could be a future natural alternative for ameliorating symptoms of anxiety after surgical menopause in women.

1. Introduction

Menopause occurs through natural aging or surgical procedures, such as bilateral oophorectomy [1], consequently reducing the levels of some hormones, such as 17 β -estradiol and progesterone [2]. This reduction is associated with vasomotor symptoms, hot flashes, vaginal dryness, osteoporosis, and cognitive deterioration [3,4]. It also results in several changes in emotional and affective states, including periods of irritability, anxiety, and mood swings [5,6]. Interestingly, these latter effects are also detected in ovariectomized rats [7,8]. Such preclinical research may help elucidate the mechanisms of mood disorders that are typically observed in women who undergo surgical menopause and may be useful for screening potential therapeutic substances to ameliorate such symptoms [9].

Hormone replacement therapy is frequently used to ameliorate physical, emotional, and affective alterations in menopausal women [10,11]. However, long-term hormone replacement therapy may also produce severe side effects (e.g., a greater risk of heart attacks, venous thromboembolic events, cerebrovascular accidents, endometrial hyperplasia, breast cancer, and stroke, among others), which restricts its long-term use in some women [12,13]. Additionally, the pharmacological response to antidepressants drugs (including selective serotonin reuptake inhibitors and tricyclic antidepressants) in menopausal women may be affected by variations in the levels of ovarian hormones that can limit their use in particular cases [14]. The use of benzodiazepines, such as diazepam, is effective for the treatment of anxiety symptoms in menopausal women, but potential side effects (e.g., physical and psychological dependence, sedation, and anterograde

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amnesia, among others) can limit their use in the long-term [15].

Several preclinical reports suggest that flavonoids may be a potential alternative to benzodiazepines for reducing anxiety. These natural compounds exert anxiolytic-like effects similar to benzodiazepines [16] but without producing the usual anterograde amnesia, sedation, and muscle relaxation that are produced by this class of drugs [17,18]. The flavonoid chrysin (5,7-dihydroxyflavone), isolated from *Passiflora caerulea* L. (Passifloraceae), produces anxiolytic-like activity in male mice and rats through actions on γ -aminobutyric acid-A (GABA_A) receptors. These actions are similar to the effects of diazepam but without significant sedative effects [16,17,19–21]. However, the potential anxiolytic-like effects of chrysin have not been tested in females that have lower concentrations of ovarian hormones that are caused by ovariectomy in the long-term. If such beneficial effects of chrysin are found, then this flavonoid may be a promising nonsteroidal and nonbenzodiazepine therapeutic alternative for the treatment of anxiety symptoms that are associated with natural or surgical menopause (i.e., lower concentrations of ovarian hormones), thus potentially avoiding the side effects of current therapeutic agents [22,23].

The present study evaluated the effects of different doses of the flavonoid chrysin in a model of surgical menopause in rats. Anxiety-like behavior was tested in the elevated plus maze and light/dark test, and the effects were compared with the clinically effective anxiolytic diazepam. Finally, the mechanism of the behavioral effects of chrysin was explored by pretreating the animals with the noncompetitive GABA_A chloride ion channel antagonist picrotoxin.

2. Materials and methods

2.1. Ethics

The experimental procedures were performed in accordance with national and international ethical recommendations based on the *Especificaciones Técnicas para la Producción, Cuidado y Uso de Animales de Laboratorio NOM-062-ZOO-1999* [24] and the National Institutes of Health Guide for the Care and Use of Laboratory Animals [25]. All efforts were made to minimize animal discomfort during the study.

2.2. Animals

Adult female Wistar rats (3 months old), weighing 200–250 g, were used. They were housed in Plexiglas cages (five rats per cage) under a 12 h/12 h light/dark cycle (lights on at 7:00 AM) at 25 °C ± 1 °C with ad libitum access to food and water.

2.3. Ovariectomy

The surgical procedure was performed in rats at 3 months age. An abdominal ventral incision was made under deep anesthesia with sodium pentobarbital (60 mg/kg, i.p., Cheminova de México, Ciudad de México, México Reg. SAGARPA Q-7048-044) and atropine sulfate (0.05 mg/kg, i.p., Sigma-Aldrich, St. Louis, MO, USA) as previously described [26]. Both ovaries were removed, and the surgical area was carefully cleaned with saline solution and benzalkonium chloride (Medipharm, San Luis Río Colorado, Sonora, México). The muscle and skin were sutured separately. To minimize postsurgical pain, the analgesic and antipyretic medication Dipirona50° (50 mg/kg, i.m., Virbac Animal Health, Guadalajara, México) was administered for 4 days after surgery. The rats were returned to the housing facilities for 12 weeks to ensure the long-term absence of ovarian hormones and the expression of high anxiety-like behavior [7]. After this time (i.e., at 6 months of age), the rats were randomly assigned to each experimental group, received their respective treatments, and were then subjected to the behavioral tests.

2.4. Behavioral tests

The rats were separately evaluated in the dark/light test or elevated plus maze, followed by the locomotor activity test. On the testing day, the rats were brought to the experimental room at 9:00 AM (i.e., 2 h after the light phase began) and left for 1 h to acclimate to the novel surroundings. The behavioral tests began at 10:00 AM (i.e., 3 h after the light phase began).

2.4.1. Light/dark test

The light/dark test is a broadly validated and useful tool for studying anxiety-like behavior and screening anxiolytic and anxiogenic drugs [27]. The light/dark box had a 80 cm × 40 cm base with 40 cm high walls. The box was divided into two equal chambers (40 cm × 40 cm × 40 cm) by a divider that had a doorway (10 cm × 10 cm) that allowed the rats to cross freely from one chamber to the other [28]. The light compartment was completely illuminated by a 40 W white light. The dark compartment was not illuminated.

On the testing day, the rats were individually placed in the middle of the dark compartment facing the doorway, and activity was recorded for 5 min. The following variables were evaluated: (a) latency to the first entry into the light compartment (i.e., the time from initially placing the rat in the dark compartment until it crossed completely to the light compartment), (b) total time spent in the light compartment, and (c) total number of entries into the light compartment. These variables were selected because they provide a reliable measure of experimental anxiety [28,29]. The frequency and time spent exploring the light compartment were also evaluated. Exploration was assumed when the rat leaned out of the dark compartment until its head and half of its body were in the light compartment, without crossing completely to the light compartment, and returned to the dark compartment [8]. After the light/dark test, the rat was evaluated in the locomotor activity test. Approximately 2 min elapsed between tests.

2.4.2. Elevated plus maze

The apparatus consisted of two opposite open and closed arms set in a plus configuration and was situated in an illuminated room at 40 lx. The dimensions of the open arms were 50 cm length × 10 cm width. The dimensions of the closed arms were 50 cm length × 10 cm width with 40 cm high walls. The entire maze was elevated 50 cm above the floor.

To evaluate the effects of the treatments, the rats were individually placed in the center of the maze, facing an open arm. The following variables were evaluated: (a) time spent on the open arms, (b) number of entries into the open arms, (c) total number of entries (open arms + closed arms), and (d) percentage of open arm entries ([open arm entries] / [total arm entries] × 100) [30,31]. After the elevated plus maze test, the rats underwent the locomotor activity test. Approximately 2 min elapsed between tests.

2.4.3. Locomotor activity test

The rats were individually placed in the locomotor activity cage (44 cm length × 33 cm width × 20 cm height). The floor of the cage was delineated into twelve 11 cm × 11 cm squares to evaluate spontaneous locomotor activity, grooming, and rearing for 5 min. At the beginning of the test, the rat was gently placed in one of the corners of the cage. The following variables were measured: (a) number of squares crossed (crossings; a crossing was considered when the rat passed from one square to another with its hind legs), (b) time (in seconds) spent rearing (rearing was considered when the rat acquired a vertical posture relative to the cage floor), and (c) time (in seconds) spent grooming, including paw licking, nose/face grooming, head washing, body grooming/scratching, leg licking, and tail/genital grooming [32].

Digital video cameras (Sony DCR-SR42, 40 × optical zoom, Carl Zeiss lens) were installed above each apparatus (light/dark test, elevated plus maze, and locomotor activity test) to record activity. Two

independent observers measured the behavioral variables using *ex profeso* software to record the number and time (in seconds) of each evaluated behavioral variable until > 95% agreement was reached among the observers. After each test session, the apparatus was carefully cleaned with a 10% ethanol solution to remove the scent of the previous rat, which can influence spontaneous behavior of the subsequent rat.

2.5. Experimental procedures

2.5.1. Experiment 1. Effect of chrysanthemum on anxiety-like behavior compared with diazepam

Forty-two rats at 12 weeks postovariectomy were assigned to six independent groups ($n = 7$ /group). The vehicle group received 5% dimethylsulfoxide (DMSO) solution (Golden Bell Reactivos, México City, México) in which chrysanthemum and diazepam were prepared. Four groups received different doses of chrysanthemum (0.5, 1, 2, and 4 mg/kg; Sigma-Aldrich, St. Louis, MO, USA). One group received diazepam (2 mg/kg; Laboratorios Cryopharma S.A. de C.V., México City, México). Diazepam was used as the reference anxiolytic drug at a dose that reduces anxiety-like behavior in rats [31,33]. The doses of chrysanthemum were based on Wolfman et al. 1994 [17], in which 1 mg/kg produces anxiolytic-like effects in male mice. Considering that the present experiments used ovariectomized female rats, three additional doses of chrysanthemum (0.5, 2, and 4 mg/kg) were included in the doses-response curve to have a wider range of possible anxiolytic-like effects. All of the treatments were administered intraperitoneally in a volume of 1 ml/kg. Sixty minutes after the injection, the rats were evaluated in the light/dark test and then in the locomotor activity test.

Another 48 rats at 12 weeks postovariectomy were assigned to six experimental groups ($n = 8$ /group) and received the identical treatment schedule described above, with the exception that these rats were evaluated in the elevated plus maze and then in the locomotor activity test.

2.5.2. Experiment 2. Influence of GABA_A receptor antagonism on anxiolytic-like effect of chrysanthemum

The light/dark test and elevated plus maze were used to explore the participation of GABA_A receptors in the anxiolytic-like effects of the minimum effective dose of chrysanthemum. Thirty-two rats at 12 weeks postovariectomy were assigned to four independent groups ($n = 8$ /group). The vehicle group (vehicle-1) received 0.9% saline in which picrotoxin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved plus vehicle-2 in which chrysanthemum was dissolved (5% DMSO solution, Golden Bell Reactivos, México City, Mexico). A second group received vehicle-1 plus 2 mg/kg chrysanthemum. A third group received 1 mg/kg picrotoxin plus vehicle-2. A fourth group received 1 mg/kg picrotoxin plus 2 mg/kg chrysanthemum. Vehicle-1 or picrotoxin was administered 90 min before the behavioral test, and vehicle-2 or chrysanthemum was administered 60 min before the behavioral test. The administration schedules were based on previous studies, in which 1 mg/kg picrotoxin antagonized the anxiolytic-like effects of GABAergic compounds in behavioral models of anxiety [34,35]. All of the treatments were administered intraperitoneally in a volume of 1 ml/kg. The effects of the treatments were evaluated in the light/dark test and then in the locomotor activity test.

Another 32 rats at 12 weeks postovariectomy were assigned to four experimental groups ($n = 8$ /group) that received identical treatment schedules as described above for the GABA_A antagonist, with the exception that the effects of the treatments were evaluated in the elevated plus maze and then in the locomotor activity test.

2.6. Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA), with treatment as the independent factor. Values of $p \leq 0.05$ in the ANOVA were followed by the Student-Newman-Keuls *post hoc*

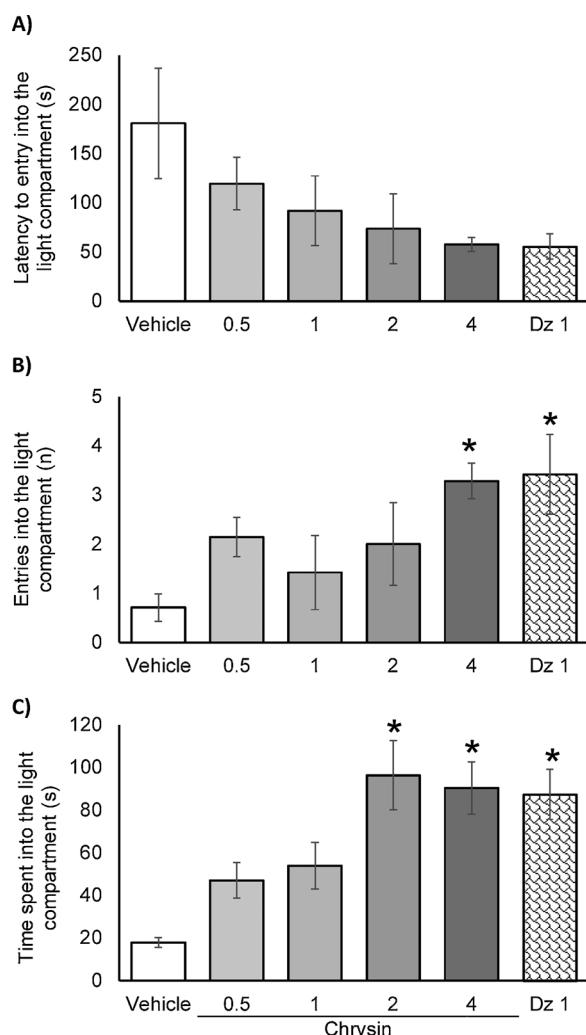


Fig. 1. Experiment 1. Light/dark test. (A) Chrysanthemum and diazepam (Dz) did not exert significant effects on the latency to the first entry into the light compartment. (B) Chrysanthemum (4 mg/kg) and diazepam significantly increased the number of entries into the light compartment compared with the vehicle group. (C) Chrysanthemum (2 and 4 mg/kg) produced the longest time spent in the light compartment, similar to diazepam, compared with the vehicle group. * $p < 0.05$, vs. vehicle group (Student-Newman-Keuls *post hoc* test).

test. The results are expressed as mean \pm standard error.

3. Results

3.1. Experiment 1. Effect of chrysanthemum on anxiety-like behavior compared with diazepam

3.1.1. Light/dark test

In the light/dark test, the statistical analysis indicated no significant effect of treatment on the latency to the first entry into the light compartment ($F_{5,36} = 2.06$, $p = 0.0938$). Although the latency decreased as the dose of chrysanthemum increased, these changes were not significantly different from the vehicle group (Fig. 1A). The statistical analysis indicated a significant effect of treatment on the number of entries into the light compartment ($F_{5,36} = 2.87$, $p = 0.0280$). The *post hoc* test revealed that rats that were treated with chrysanthemum (4 mg/kg) made the most entries into the light compartment compared with the vehicle group, and this effect was similar to diazepam (Fig. 1B). The analysis of the time spent in the light compartment indicated a significant effect of treatment ($F_{5,36} = 7.690$, $p < 0.001$). Rats that were treated with

chrysanthemum (2 or 4 mg/kg) spent significantly more time in the light compartment compared with the vehicle group, and this effect was similar to diazepam (Fig. 1C).

No significant effect of treatment on the latency to explore the light compartment was found ($F_{5,36} = 0.595$, $p = 0.703$): vehicle, 9.24 ± 1.73 s; 0.5 mg/kg chrysanthemum, 8.58 ± 0.91 s; 1 mg/kg chrysanthemum, 7.31 ± 1.47 s; 2 mg/kg chrysanthemum, 12.87 ± 6.57 s; 4 mg/kg chrysanthemum, 13.24 ± 2.15 s; diazepam, 8.80 ± 2.60 s. Significant effects of treatment on the number of explorations of the light compartment were observed ($F_{5,36} = 4.130$, $p < 0.004$): vehicle, 5.00 ± 0.95 ; 0.5 mg/kg chrysanthemum, 8.00 ± 0.65 ; 1 mg/kg chrysanthemum, 8.86 ± 0.67 ; 2 mg/kg chrysanthemum, 12.00 ± 1.49 ; 4 mg/kg chrysanthemum, 10.00 ± 1.83 ; diazepam, 11.57 ± 1.51 . Significant effects of treatment on the time spent exploring the light compartment were observed ($F_{5,36} = 4.640$, $p < 0.002$): vehicle, 10.80 ± 1.30 s; 0.5 mg/kg chrysanthemum, 23.9 ± 6.43 s; 1 mg/kg chrysanthemum, 33.90 ± 6.62 s; 2 mg/kg chrysanthemum, 49.6 ± 5.77 s; 4 mg/kg chrysanthemum, 41.10 ± 9.39 s; diazepam, 50.80 ± 10.19 s. The *post hoc* test revealed that chrysanthemum (2 or 4 mg/kg) significantly increased the number of explorations of the light compartment and time spent exploring the light compartment compared with the vehicle group, and these effects were similar to diazepam.

3.1.2. Locomotor activity

The locomotor activity test was conducted after the light/dark test. The statistical analysis indicated no significant effect of treatment on the number of crossings ($F_{5,36} = 0.994$, $p = 0.435$). However, significant effects of treatment were observed on the time spent rearing ($F_{5,36} = 5.430$, $p < 0.001$) and time spent grooming ($F_{5,36} = 8.210$, $p < 0.001$). The *post hoc* test revealed that chrysanthemum (2 and 4 mg/kg) significantly increased the time spent rearing and the time spent grooming compared with the vehicle group, and these effects were similar to diazepam (Table 1).

3.1.3. Elevated plus maze

The statistical analysis revealed significant effects of treatment on the time spent on the open arms ($F_{5,42} = 45.962$, $p < 0.001$), number of entries into the open arms ($F_{5,42} = 12.326$, $p < 0.001$), and percentage of entries into the open arms ($F_{5,42} = 17.059$, $p < 0.001$). The *post hoc* test showed that rats that were treated with chrysanthemum (1, 2, or 4 mg/kg) exhibited significant increases in the aforementioned variables compared with the vehicle group, and these effects were similar to diazepam (Fig. 2). The analysis of the total number of arm entries (open arms + closed arms) revealed a significant effect of treatment ($F_{5,42} = 2.788$, $p < 0.029$). The *post hoc* test indicated a significant difference between the 0.5 and 2 mg/kg doses of chrysanthemum ($p < 0.045$) but not between any dose of chrysanthemum or diazepam and the vehicle group (vehicle, 8.25 ± 0.84 ; 0.05 mg/kg chrysanthemum, 7.75 ± 0.56 ; 1 mg/kg chrysanthemum, 10.12 ± 0.59 ; 2 mg/kg chrysanthemum, 11.75 ± 1.35 ; 4 mg/kg chrysanthemum, 10.50 ± 1.07 ; diazepam, 10.87 ± 0.95).

Table 1

Effects of chrysanthemum and diazepam on crossing, rearing, and grooming in locomotor activity test after light/dark test in experiment 1.

Groups	Crossing (n)	Time spent in rearing (s)	Time spent in grooming (s)
Vehicle	31.7 ± 6.21	17.7 ± 2.65	10.5 ± 1.80
Chrysanthemum (0.5 mg/kg)	32.4 ± 3.77	21.1 ± 2.36	11.7 ± 1.66
Chrysanthemum (1 mg/kg)	25.9 ± 3.38	21.8 ± 2.77	16.8 ± 1.78
Chrysanthemum (2 mg/kg)	33.0 ± 3.75	$32.2 \pm 3.09^*$	$27.2 \pm 1.95^*$
Chrysanthemum (4 mg/kg)	30.3 ± 3.91	$30.8 \pm 2.98^*$	$30.4 \pm 4.28^*$
Diazepam (2 mg/kg)	39.1 ± 4.32	$31.0 \pm 2.22^*$	$28.0 \pm 5.04^*$

* $p < 0.002$ vs control, Student-Newman-Keuls.

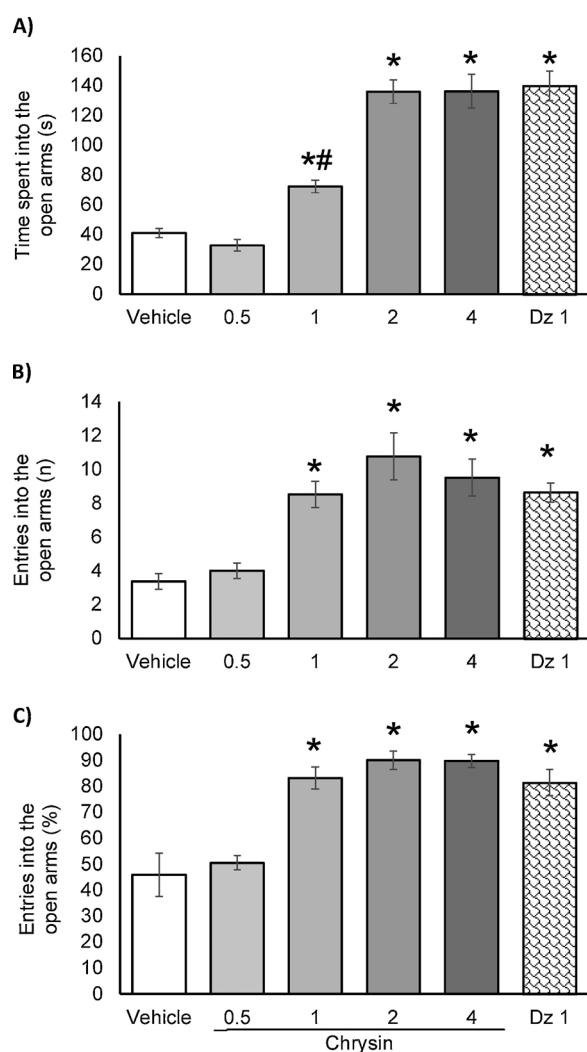


Fig. 2. Experiment 1. Elevated plus maze. Chrysanthemum (1, 2, and 4 mg) increased the time spent on the open arms (A), increased the number of entries into the open arms (B), and increased the percentage of entries into the open arms (C), similar to diazepam (Dz), compared with the vehicle group. * $p < 0.05$, vs. vehicle group (Student-Newman-Keuls *post hoc* test).

3.1.4. Locomotor activity

The locomotor activity test was conducted after the elevated plus maze. The statistical analysis did not indicate a significant effect of treatment on the number of crossings ($F_{5,42} = 2.293$, $p = 0.063$). Nonetheless, significant effects of treatment on the time spent rearing ($F_{5,42} = 7.130$, $p < 0.001$) and time spent grooming ($F_{5,42} = 15.712$, $p < 0.001$) were detected. The *post hoc* test revealed that chrysanthemum (2 and 4 mg/kg) significantly ($p < 0.002$) increased the time spent rearing and the time spent grooming compared with vehicle and chrysanthemum (0.5 mg/kg), and these effects were similar to diazepam. No significant differences were found between the different doses of chrysanthemum (1, 2, or 4 mg/kg) and diazepam (Table 2).

3.2. Experiment 2. Influence of GABA_A receptor antagonism on the anxiolytic-like effects of chrysanthemum

3.2.1. Light/dark test

Significant effects of treatment were detected on the latency to the first entry into the light compartment ($F_{3,28} = 19.918$, $p < 0.001$), number of entries into the light compartment ($F_{3,28} = 4.469$, $p < 0.011$), and time spent in the light compartment ($F_{3,28} = 66.956$, $p < 0.001$). The *post hoc* test revealed that chrysanthemum (2 mg/kg) significantly

Table 2

Effects of chrysanthemum and diazepam on crossing, rearing, and grooming in locomotor activity test after elevated plus maze in experiment 1.

Groups	Crossing (n)	Time spent in rearing (s)	Time spent in grooming (s)
Control	53.1 ± 3.90	22.2 ± 2.43	19.8 ± 3.00
Chrysanthemum (0.5 mg/kg)	62.6 ± 2.39	24.4 ± 1.82	13.4 ± 0.95
Chrysanthemum (1 mg/kg)	63.6 ± 3.66	26.7 ± 2.01	23.9 ± 3.92
Chrysanthemum (2 mg/kg)	56.7 ± 3.82	33.9 ± 1.84*	31.8 ± 1.35*
Chrysanthemum (4 mg/kg)	52.5 ± 1.57	34.4 ± 2.14*	34.8 ± 1.52*
Diazepam (2 mg/kg)	63.5 ± 5.11	34.5 ± 2.22*	35.9 ± 1.38*

* p < 0.002 vs control, Student-Newman-Keuls.

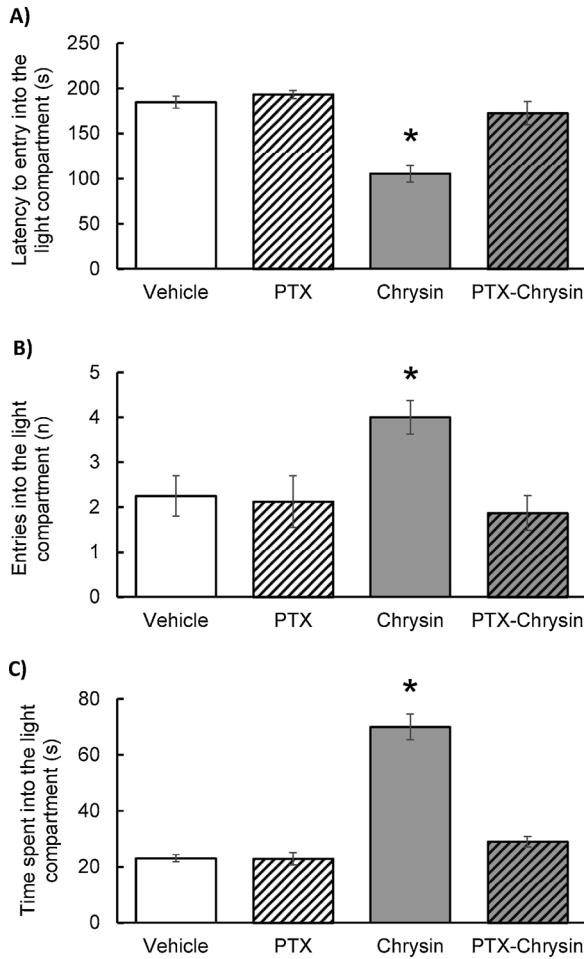


Fig. 3. Experiment 2. Light/dark test. Chrysanthemum (2 mg/kg) decreased the latency to the first entry into the light compartment (A), increased the number of entries into the light compartment (B), and increased the time spent in the light compartment (C) compared with the vehicle group. Picrotoxin (PTX) did not produce any effect on its own but antagonized the effects of chrysanthemum on each of these variables. *p < 0.05, vs. vehicle group (Student-Newman-Keuls post hoc test).

(p < 0.001) reduced the latency to the first entry into the light compartment and increased the number of entries into and time spent in the light compartment compared with the vehicle and picrotoxin groups (Fig. 3). These effects were prevented by pretreatment with picrotoxin, which alone did not produce significant effects on these variables.

No significant effect of treatment on the latency to explore the light compartment was found ($F_{3,28} = 0.846$, $p = 0.480$): vehicle: 14.57 ± 2.20 s; picrotoxin, 18.25 ± 4.55 s; chrysanthemum, 13.86 ± 1.54 s;

Table 3

Effects of picrotoxin, chrysanthemum, and combination on crossing, rearing, and grooming in locomotor activity test after light/dark test in experiment 2.

Groups	Crossing (n)	Time spent in rearing (s)	Time spent in grooming (s)
Vehicle	29.8 ± 1.39	15.2 ± 1.10	8.6 ± 0.94
Picrotoxin (1 mg/kg)	34.6 ± 2.63	16.1 ± 1.25	10.5 ± 0.62
Chrysanthemum (2 mg/kg)	25.6 ± 2.58	28.1 ± 1.08*	30.5 ± 1.91*
Picrotoxin-Chrysanthemum	30.0 ± 2.46	14.7 ± 2.02	11.9 ± 1.17

* p < 0.002 vs vehicle, Student-Newman-Keuls.

picrotoxin + chrysanthemum, 12.31 ± 1.45 s. Significant effects of treatment on the number of explorations were observed ($F_{3,28} = 8.833$, $p < 0.001$): vehicle, 3.50 ± 0.63 ; picrotoxin, 3.00 ± 0.62 ; chrysanthemum, 9.00 ± 1.41 ; picrotoxin + chrysanthemum, 4.00 ± 0.86 . Significant effects of treatment on the time spent exploring the light compartment were found ($F_{3,28} = 69.736$, $p < 0.001$): vehicle, 10.97 ± 1.44 s; picrotoxin, 8.14 ± 2.28 s; chrysanthemum, 39.49 ± 2.03 s; picrotoxin + chrysanthemum, 13.56 ± 0.76 s. The post hoc test revealed that chrysanthemum (2 mg/kg) significantly ($p < 0.001$) increased the number of entries into the light compartment and time spent exploring the light compartment compared with the vehicle and picrotoxin groups, and these effects were blocked by pretreatment with picrotoxin.

3.2.2. Locomotor activity

The locomotor activity test was conducted after the light/dark test. No significant effect of treatment on the number of crossings was found ($F_{3,28} = 2.509$, $p = 0.079$). A significant effect of treatment on the time spent grooming was observed ($F_{3,28} = 66.413$, $p < 0.001$). The post hoc test showed that chrysanthemum-treated rats exhibited a significant ($p < 0.001$) increase in the time spent grooming compared with the vehicle group (Table 3). The analysis also indicated a significant effect of treatment on the time spent rearing ($F_{3,28} = 7.329$, $p < 0.001$). The post hoc test showed that chrysanthemum (2 mg/kg) significantly ($p < 0.001$) increased the time spent rearing compared with the control group (Table 3), and this effect was prevented by pretreatment with picrotoxin, which alone did not produce significant effects in the locomotor activity test.

3.2.3. Elevated plus maze

In the elevated plus maze, the statistical analysis indicated significant effects of treatment on the time spent on the open arms ($F_{3,28} = 52.991$, $p < 0.001$), number of entries into the open arms ($F_{3,28} = 8.541$, $p < 0.001$), and percentage of entries into the open arms ($F_{3,28} = 13.768$, $p < 0.001$). The post hoc test showed that chrysanthemum significantly ($p < 0.001$) increased the time spent on the open arms, number of entries into the open arms, and percentage of entries into the open arms compared with the vehicle and picrotoxin groups. These effects of chrysanthemum were blocked by pretreatment with picrotoxin (Fig. 4). The analysis of the total number of arm entries (open arms + closed arms) did not indicate a significant effect of treatment ($F_{3,28} = 1.013$, $p < 0.402$; vehicle, 9.13 ± 0.58 ; picrotoxin, 10.13 ± 1.04 ; chrysanthemum, 11.63 ± 1.87 ; picrotoxin + chrysanthemum, 12.87 ± 2.44).

3.2.4. Locomotor activity

The locomotor activity test was conducted after the elevated plus maze. The statistical analysis did not indicate a significant effect of treatment on the number of crossings ($F_{3,28} = 2.729$, $p = 0.063$). The analysis revealed significant effects of treatment on the time spent rearing ($F_{3,28} = 15.06$, $p < 0.001$) and the time spent grooming ($F_{3,28} = 14.334$, $p < 0.001$). The post hoc test revealed that chrysanthemum significantly ($p < 0.001$) increased the time spent rearing and the time

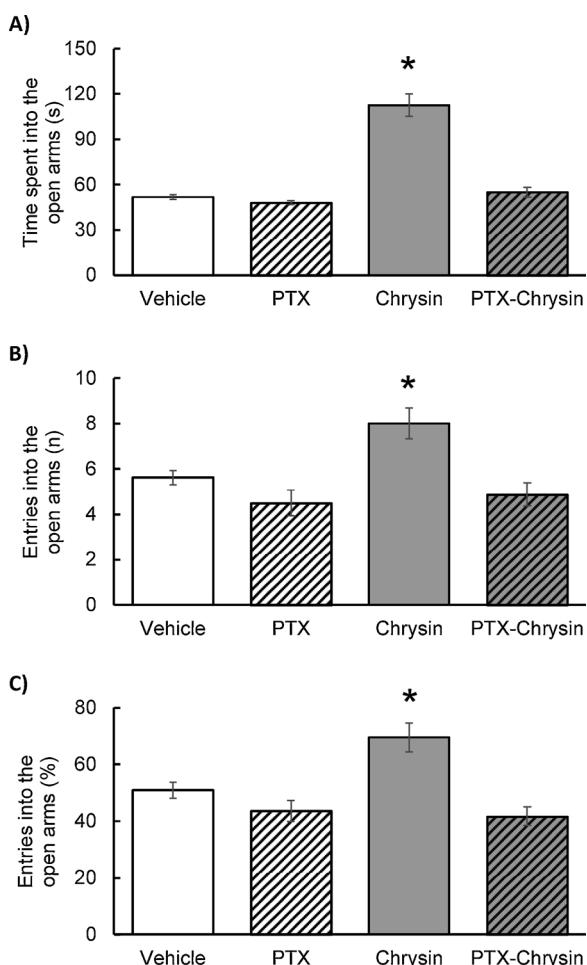


Fig. 4. Experiment 2. Elevated plus maze. Chrysins (2 mg/kg) increased the time spent on the open arms (A), increased the number of entries into the open arms (B), and increased the percentage of entries into the open arms (C) compared with the vehicle group. Picrotoxin (PTX) did not produce any effect on its own but antagonized the effects of chrysins on each of these variables. * $p < 0.05$, vs. vehicle (Student-Newman-Keuls post hoc test).

Table 4
Effects of picrotoxin, chrysins, and combination on crossing, rearing, and grooming in locomotor activity test after elevated plus maze in experiment 2.

Groups	Crossing (n)	Time spent in rearing (s)	Time spent in grooming (s)
Vehicle	45.6 ± 3.59	19.5 ± 1.48	18.4 ± 2.32
Picrotoxin (1 mg/kg)	43.0 ± 2.02	24.8 ± 1.99	19.9 ± 1.60
Chrysins (2 mg/kg)	50.0 ± 4.71	33.8 ± 1.61*	30.5 ± 0.99
Picrotoxin-Chrysins	55.6 ± 2.37	22.3 ± 1.20	17.1 ± 1.23

* $p < 0.002$ vs vehicle, Student-Newman-Keuls.

spent grooming compared with the vehicle and picrotoxin groups, and this effect was prevented by pretreatment with picrotoxin (Table 4), which alone did not produce significant effects on the evaluated variables in the locomotor activity test.

4. Discussion

The present study explored the effects of different doses of chrysins in a rat model of surgical menopause using two experimentally validated models of anxiety. We also explored the participation of GABA_A

receptors in the anxiolytic-like effects of chrysins by pretreating the animals with the noncompetitive GABA_A receptor antagonist picrotoxin. In the elevated plus maze and light/dark test, chrysins (2 and 4 mg/kg) produced anxiolytic-like effects, and these effects were similar to diazepam. Pretreatment with picrotoxin (1 mg/kg) had no effects on its own but prevented the anxiolytic-like effects of chrysins in both the elevated plus maze and light/dark test. Chrysins increased the time spent rearing and grooming, without altering the number of crossings in the locomotor activity test, and these effects were similar to diazepam. Altogether, these findings indicate that the flavonoid chrysins produces anxiolytic-like effects in rats with surgical menopause by acting at least partially on GABA_A receptors.

The light/dark test is a valid model for exploring the anxiolytic- and anxiogenic-like effects of drugs by measuring the amount of time rats spend in the light compartment [36]. Animals that are treated with clinically effective anxiolytic drugs (e.g., diazepam, alprazolam, and buspirone [37]), some steroid hormones [38], and some natural products (e.g., xanthines [39] and the phytoestrogen genistein [29]) spend more time in the light compartment, the principal variable that is used as an indicator of anxiolytic-like activity [8,28,29]. In the present study, the flavonoid chrysins (2 and 4 mg/kg) increased the total time spent in the light compartment, indicating an anxiolytic-like effect in rats with the long-term absence of ovarian hormones. A greater time spent exploring the light compartment in the light/dark test is used as an additional indicator of anxiolytic-like activity [8], and this effect was also identified when ovariectomized rats were injected with chrysins or diazepam, thus supporting the anxiolytic activity of this flavonoid. In Experiment 1, the effect of 2 mg/kg chrysins on the time spent in the light compartment was significantly different from vehicle, with no significant difference in the number of entries into the light compartment or latency to enter the light compartment (Fig. 1). In Experiment 2, the three variables were significantly different from the vehicle group. These differences in the significance of the effects of 2 mg/kg chrysins (Figs. 1, 3) on the variables could be related to the number of groups and the number of rats that were included in each experiment, but increasing the number of replicates was unnecessary because the principal variable for detecting an anxiolytic effect in the light/dark test (i.e., the time spent in the light compartment) significantly increased compared with the vehicle group. This anxiolytic-like effect of 2 mg/kg chrysins was confirmed in the elevated plus maze (Figs. 2, 4). Finally, the relatively low number of animals that were included in the present study is consistent with the 3Rs (refine, reduce, replace) of ethical recommendations for preclinical research [40], which has also been reported in previous studies that used the light/dark test [8,28,29].

The anxiolytic-like effects that were observed in the light/dark test were replicated in the elevated plus maze, another validated model for measuring anxiogenic- and anxiolytic-like effects in rats [30]. This test explores rats' innate aversion to open and illuminated spaces and heights. Anxiolytic drugs usually increase the number or percentage of entries into the open arms and time spent on the open arms [30]. These same effects were produced by chrysins in the elevated plus maze, even at a dose of 1 mg/kg, which is half the effective dose of chrysins that exerted anxiolytic-like effects in the light/dark test. Additionally, the total number of arm entries in this test is used as an index of general motor activity in rats to discard possible influences on the time spent on the open arms [26]. In the present study, the total number of arm entries was not influenced by the treatments. The lack of an effect on this variable is consistent with the results of the locomotor activity test, thus discarding any possible motor influence on the effects of the treatments in the elevated plus maze and supporting the anxiolytic-like effect of chrysins.

Chrysins at doses of 2 and 4 mg/kg was as effective as diazepam in reducing anxiety-like behavior. Diazepam is commonly used as a reference drug to evaluate anxiolytic activity, and it exerts its effects by acting on GABA_A receptors [41]. Picrotoxin is a noncompetitive

antagonist of GABA_A receptor chloride ion channels and was used to explore the partial mechanism of action of the anxiolytic-like effect of the lowest effective dose of chrysanthemum (2 mg/kg) in both behavioral tests. The effects of anxiolytic drugs that exert their actions on GABA_A receptors are blocked by picrotoxin. In the present study, pretreatment with picrotoxin did not produce intrinsic effects in either model of anxiety but prevented the effects of the effective dose of chrysanthemum (2 mg/kg). Therefore, the observed anxiolytic effects of chrysanthemum in the present study appeared to be mediated at least partially by GABAergic actions. This assertion is supported by previous studies in which bicuculline, a competitive antagonist of the GABA binding site in the GABA_A receptor [42], and flumazenil, a selective antagonist of the binding site for benzodiazepines in the GABA_A receptor [20], blocked the anxiolytic-like effects of chrysanthemum. Previous studies have shown that chrysanthemum has high affinity for GABA_A receptors [44,45]. When GABAergic agonists activate these receptors, the conductance of chloride ions increases, thus hyperpolarizing the neuron and consequently inhibiting neuronal activity [43]. This neurophysiological effect that occurs through GABA_A receptor chloride ion channels is associated with the anxiolytic effects of several substances, including benzodiazepines, barbiturates, psychoactive drugs, some neurosteroids, and flavonoids [41,46].

Picrotoxin, in addition to blocking GABA_A receptors and the anxiolytic-like effects of different substances [34,35], has also been reported to inhibit Cl⁻ flux in glycine, GABA_C, Glu-Cl, and 5-HT₃ receptors in studies *in vitro* [47–49] using retinal and hippocampal cells from rats at postnatal day 12. However, the participation of these receptors in the regulation of anxiety and the effect of anxiolytic drugs has not been sufficiently explored. In the present study, we can discard the possible participation of these receptors in the effects that were detected in Experiment 2. The group that was treated only with picrotoxin did not present any significant differences in the behavioral variables in the light/dark test, elevated plus maze, and locomotor activity test, but picrotoxin effectively blocked the anxiolytic-like effect of chrysanthemum. The possibility that the dose of picrotoxin (1 mg/kg) was toxic may also be discarded because no side effects that are usually associated with picrotoxin (e.g., ataxia, convulsions, salivation, and emesis, among others) were detected in the present study. In fact, the toxic dose of picrotoxin in rodents has been reported to be > 4 mg/kg [50,51]. Therefore, the present data suggest that the actions of chrysanthemum are at least partially associated with GABA_A receptor activation, without discarding the possible participation of other neurotransmitter systems.

Considering that the effects of chrysanthemum reported herein appeared to be associated with the activation of GABA_A receptors, the anxiolytic-like effects of chrysanthemum in the behavioral tests could be influenced by changes in nociception, in which previous studies reported that chrysanthemum produced hyperalgesia via GABA_A receptor activation [52]. The activation of GABA_A receptors induces hyperalgesia but also antinociceptive effects [53,54]. Hyperalgesia has been reported in mice that were subjected to the tail immersion test using chrysanthemum doses between 20 and 100 mg/kg, and the most significant effect was detected at 100 mg/kg [52]. In contrast, an antinociceptive effect of chrysanthemum was reported at a dose of 50 mg/kg in rats in the formalin test, and the most significant effect was observed at 150 mg/kg [55]. Nonetheless, in the present study, we can discard the possible influence of nociceptive effects of chrysanthemum in ovariectomized rats, given that 25 mg/kg chrysanthemum is the minimal dose that has been reported to exert a nociceptive effect [52], which is six-times higher than the highest dose that was used in the present study (4 mg/kg), which exerted anxiolytic-like effects.

General locomotor activity was evaluated to identify or discard possible motor effects (i.e., hypoactivity or hyperactivity) that could interfere with behavioral activity in the light/dark test and elevated plus maze. The behavioral effects of chrysanthemum in both experimental models were not associated with any alterations of the motor component, thus supporting a typical anxiolytic-like effect [30]. Additionally, rearing and grooming were assessed because both of these behaviors

have been proposed to be emotional indicators in rats when they are exposed to novel environments [56]. In the present study, the vehicle group exhibited the lowest level of grooming, possibly associated with a state of anxiety [8]. The reduction of grooming in “anxious” rats can be prevented by diazepam and other substances (e.g., alprazolam) with well-characterized anxiolytic actions [8,57,58]. Chrysanthemum prevented the decrease in grooming, suggesting a decrease in anxiety-like behavior. Similarly, rearing is considered a behavioral component of anxiety. Anxious animals spend more time quiet and alert to their surroundings instead of actively exploring, so they spend less time rearing [56]. Rats in the vehicle group spent less time rearing, and chrysanthemum and diazepam increased this variable. Therefore, the present findings support the anxiolytic-like effects that were observed in the elevated plus maze and light/dark test in ovariectomized rats.

Finally, flavonoids, including chrysanthemum, have been reported to exert anxiolytic- and antidepressant-like effects in rats [16,17,21,59]. To our knowledge, however, no study has reported the anxiolytic-like effects of chrysanthemum in rats with the long-term absence of ovarian hormones. Our findings support the hypothesis that chrysanthemum may be a therapeutic alternative to ameliorate anxiety that is associated with surgical or natural menopause in women. Chrysanthemum may be an alternative to the use of estrogens or benzodiazepines to avoid the typical side effects that are produced by these agents [12,15].

5. Conclusion

The present results provide evidence that the flavonoid chrysanthemum produces anxiolytic-like effects through partial actions on GABA_A receptors in rats in a model of surgical menopause. These findings support the hypothesis that this flavonoid may be a complementary alternative to ameliorate symptoms of anxiety in women during natural or surgical menopause. Further studies are needed to investigate the effects of chrysanthemum in rats with the natural long-term absence of ovarian hormones (i.e., in aged female rats) to mimic natural menopause in women. The possible side effects of long-term chrysanthemum treatment should also be investigated before it can be recommended as a safe and effective alternative for the treatment of anxiety symptoms in women with natural or surgical menopause.

Author contributions

JFRL, FHL, and JCE conceived the project, developed the experimental design, and wrote the protocol. FHL, JCE, EVHH, and ERA performed the experiments and measured the behavioral variables. JFRL, FHL, JCE, BBM, and ERD performed the statistical analysis and interpreted the results. JFR, JCE, and BBM wrote the manuscript. All of the authors reviewed, discussed, and approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest with regard to the research, authorship, and publication of this article.

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