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Original research paper

Chrysin enhances serotonergic and noradrenergic neurotransmission associated with antidepressant effects: A pharmacological study

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ABSTRACT

The aim of this study was to investigate the potential antidepressant-like effect of combined subthreshold and effective doses of chrysin and fluoxetine in adult male Wistar rats and their potential effects on the serotonergic and noradrenergic systems. Seventy rats were divided into seven experimental groups: vehicle (10 % dimethyl sulfoxide solution, DMSO), chrysin (4 or 20 μmol kg^{-1}), fluoxetine (1.6 and 3.2 μmol kg^{-1}), and their combinations. The treatments were administered for 28 consecutive days, and the effects were evaluated in the locomotor activity test (LAT) and forced swim test (FST). The results showed that the treatments did not significantly affect crossings in the LAT. Chrysin, alone or combined, reduced immobility time, increased latency to first immobility and prolonged swimming in the FST, similar to fluoxetine. However, only chrysin (20 μmol kg^{-1}) and its combination with fluoxetine (1.6 μmol kg^{-1}) enhanced climbing behaviour in the FST. Chrysin showed an antidepressant effect, possibly related to enhanced serotonergic and noradrenergic neurotransmission, by increasing climbing and swimming time in the FST. This dual effect suggests a promising antidepressant prototype with different mechanisms of action, allowing the use of subthreshold doses, which could reduce side effects.

Keywords: flavonoid therapy, depressive-like behaviour, behavioural pharmacology, neurotransmitter modulation, chrysin

INTRODUCTION

The combination of antidepressants can have positive or negative consequences, depending on the interaction between the drugs and the individual reaction of the patient. In some cases, combination therapy can improve the efficacy of treatment, especially in patients with resistant depression (1). However, concomitant use of antidepressants with-

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out medical supervision may increase the risk of serious side effects such as serotonin syndrome, hypertensive crises or cardiovascular disorders (2). In search of improving the therapeutic effect, some people resort to self-medication or combine treatments without knowing the possible interactions, which can worsen their clinical condition (3). Therefore, it is important to identify and evaluate the potential benefits of these combinations to optimise the safety and efficacy of antidepressant treatment.

Currently, there are a variety of antidepressants available for the treatment of depression, including monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), and serotonin, dopamine, and norepinephrine reuptake inhibitors (4). Despite their effectiveness, all antidepressants have side effects ranging from drowsiness to sexual dysfunction, limiting their long-term use (5). In addition, a percentage of patients (25–50 %) do not respond to treatment (6), and there is a long latency period (4–6 weeks) before therapeutic effects are observed (7). These limitations have led patients to seek alternative therapies based on natural products (8–10). In this way, some patients, with the aim of improving their pharmacological treatment, simultaneously consume natural products that have been reported to have antidepressant-like properties. However, it is not known whether the combination of natural products such as chrysin and the antidepressant fluoxetine could have a positive effect or side effects on reducing symptoms of depression.

Among the most important compounds that have been studied clinically for their antidepressant properties are flavonoids (11). These include the flavonoid chrysin (5,7-dihydroxyflavone), which is found in propolis, honey and certain plants such as *Matricaria chamomilla, Passiflora incarnata,* and *P. coerulea* (12). Various studies have demonstrated the diverse biological activities of chrysin, including anti-inflammatory, antineoplastic, antioxidant, lipid-lowering, anxiolytic-, and antidepressant-like effects (13–15). Preclinical studies show that 20 and 40 μmol kg^{-1} (5 and 10 mg kg^{-1}) chrysin has an antidepressant-like effect in subjects exposed to chronic unpredictable mild stress (CUMS) for 28 days. This effect is thought to be mediated by the activation of brain-derived neurotrophic factor (BDNF) and neurotrophic growth factor (NGF) signalling pathways in the hippocampus and cerebral cortex, which facilitates the restoration of neurotransmission and reverses the effects induced by chronic stress (16, 17). In addition, serotonergic and dopaminergic neurotransmission systems have been implicated in these effects (18). In this sense, the antidepressant-like effects of this flavonoid are associated with an increase in the expression of 5-HT_{1A} receptors in the hippocampus and a decrease in these receptors in the raphe nucleus (10).

Flavonoids have shown synergistic effects with selective serotonin reuptake inhibitors (SSRIs) and enhance their antidepressant effects through mechanisms that go beyond serotonergic modulation. The combination of naringenin and sertraline reduced oxidative stress and protected mitochondrial signalling pathways, while resveratrol in combination with sertraline regulated the expression of serotonin transporters (SERT) and monoamine oxidase A (MAO-A), enhancing the anxiolytic effect (11, 19). Similarly, pomegranate (*Punica granatum*) extracts in combination with citalopram have shown antidepressant-like effects comparable to those produced by estradiol (20). However, the effects of chrysin in combination with fluoxetine on despair-like behaviour have not yet been studied. Considering that flavonoids can modulate several neurotransmitter systems, it is crucial to assess whether this combination produces a superior or different antidepressant effect compared to independent treatments. The aim of this study was therefore to investigate the effect of chronic

treatment with chrysin in combination with fluoxetine, a first-line antidepressant in the treatment of depression symptoms. Additionally, the effect of this combination was compared with the individual administration of each drug and the possible involvement of the noradrenergic and serotonergic systems was determined by behavioural analysis in the FST.

EXPERIMENTAL

Ethics

All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (21), and the *Norma Official Mexicana para el Uso y Cuidado de Animales de Laboratorio* (22). All efforts were made to minimise animal discomfort and the number of animals in accordance with the 3R (Reduce, Refine and Replace) principles of preclinical research (23). During the experiment, the animal welfare was monitored by using the grimace scale for rats (24).

Animals

Seventy male Wistar rats (two-month-old; 250–300 g at the beginning of the experiments) were housed in Plexiglas cages, 4 rats per cage (44 × 33 × 20 cm), with a 12 h/12 h light/dark cycle (lights on at 07:00 h), average room temperature of 25 ± 1 °C and free access to purified water and food (Purina Pellets, Agribrands Purina Mexico, Mexico) during the study. All experimental sessions were conducted between 09:00 h and 12:00 h.

Drugs

The drug doses were selected according to prior studies reporting antidepressant-like effects of chrysin at 4 and 20 μmol kg^{-1} (corresponding to 1 and 5 mg kg^{-1}) and fluoxetine at 1.6 and 3.2 μmol kg^{-1} (corresponding to 0.5 and 1 mg kg^{-1}) in rats (10, 25). Chrysin (purity ≥ 97 %) was purchased from Sigma-Aldrich (USA), while fluoxetine (Prozac) was obtained from Eli-Lilly Laboratories (Mexico). All compounds were administered orally once per day for 28 days at a volume of 1 mL kg^{-1}. The vehicle used was a 10 % DMSO solution. Administration was performed using a curved stainless-steel oral gavage needle (18G × 3.000 with 2.5 mm ball tip, Cadence, Inc., USA), connected to a 1-mL disposable syringe (Terumo Medical de México, Mexico). This route was selected to resemble human oral drug intake, allowing for gastrointestinal processing that may affect drug metabolism and therapeutic response.

Experimental groups

A cross-sectional study was conducted with seven independent groups. Each group included a total of ten rats. The groups formed were a control group that received vehicle, chrysin 4 μmol kg^{-1}, chrysin 20 μmol kg^{-1}, fluoxetine 1.6 μmol kg^{-1}, fluoxetine 3.2 μmol kg^{-1}, a combination of chrysin 4 μmol kg^{-1} and fluoxetine 1.6 μmol kg^{-1} (I-1), and a combination of chrysin 20 μmol kg^{-1} and fluoxetine 3.2 μmol kg^{-1} (I-2). Before any drug administration, all rats were subjected to a 5-minute pretest in LAT and a 15-minute pretest in FST. The pretest session was discarded for the statistical analysis, considering that it was a

habituation session in the LAT and induction of despair in the FST (26). Twenty-four hours later, the drug treatments were started, and their effect was evaluated on day 28 of treatment, 1 hour after the corresponding administration.

Behavioural test

Locomotor activity test (LAT). – Spontaneous locomotor activity was assessed by placing the rats individually into an opaque Plexiglas cage (44 × 33 × 20 cm), with the floor marked into 12 squares (11 × 11 cm). The number of crossings, as well as the time spent on grooming and rearing behaviours, were measured. Crossing was recorded when the hind legs of the rat crossed the boundary between two squares. Grooming, measured in seconds, involved all self-cleaning behaviours, including cleaning of the head, ears, limbs, and anal-genital area; this behaviour can be significantly influenced by various stressors (27). Rearing, which was also measured in seconds, occurred when the rats stood on their rear legs to explore the vertical space within the cage. This behaviour is related to exploration and can be notably impacted by stressors (28), while anxiolytic and antidepressant drugs may mitigate these effects and either maintain or increase this behaviour relative to control groups (9, 29). After each trial, the cage was thoroughly cleaned with a 10 % alcohol solution to eliminate any residual odour from the previous rat. Following the locomotor activity test, the rats were subjected to the forced swimming test, with a brief 2-minute interval between the tests.

Forced swim test (FST). – Rats were individually placed in a rectangular tank (50 × 30 × 60 cm) filled with water to a depth of 30 cm at a temperature of 25 ± 1 °C, which has been validated for detecting substances with potential antidepressant-like effects (30). The following variables were assessed: (i) latency to the first immobility episode, measured as the time (in seconds) from the rat's introduction into the water until it first became immobile, (ii) total immobility duration (in seconds), defined as the time the rat floated for over 2 seconds without making vigorous movements, (iii) swimming behaviour (in seconds), characterized by active front limb movements for horizontal motion, such as crossing quadrants and turning, and (iv) climbing behaviour (in seconds), recorded when the rat moved its front limbs upward along the walls of the tank. All sessions were video recorded for 5 minutes using a Nikon D3300 camera with an 18–55 mm lens, and behavioural variables were measured by two blinded observers using specialised software, achieving a 95 % concordance rate.

Statistical analysis

Data were analysed using one-way ANOVA to evaluate the effect of treatments (vehicle, chrysin, fluoxetine and the combinations) on behaviour in the LAT and FST. To determine the normality of the data, the Normality Test (Shapiro-Wilk) was used, followed by the Student-Newman-Keuls *post hoc* test. Values of $p \leq 0.05$ were considered statistically significant. The data are expressed as the mean ± SEM. F denotes the statistics for an ANOVA test (analysis of variance). $_{6,63}$ are the degrees of freedom associated with the test. $_{6}$ corresponds to the degrees of freedom in the numerator, *i.e.* those associated with the factors or treatments (between groups). $_{63}$ corresponds to the degrees of freedom in the denominator, which represent the variability within groups or the error (within groups).

RESULTS AND DISCUSSION

Locomotor activity test

The exploratory behaviour was assessed in the LAT to exclude a possible influence of locomotor activity in the interpretation of the antidepressant-like effects in the FST. The number of crossings, time of grooming, and rearing in the LAT are shown in Table I. No significant differences were found between treatments in the number of crossings ($F_{6,63}$= 1.388, p = 0.233, NS) and time spent rearing ($F_{6,63}$= 0.764, p = 0.601, NS). Significant differences were found in the time spent grooming ($F_{6,63}$= 3.450, p = 0.005). The *post hoc* test showed that grooming was higher in the I-1 group compared to the vehicle group. The results showed that although chrysin, fluoxetine, and their combinations did not alter locomotor activity or rearing behaviour, they significantly influenced grooming behaviour.

No significant changes were observed in the LAT, evaluated by crossing and rearing, in any treatment. This suggests that the changes in the FST are not due to general motor changes, but rather to specific antidepressant-like effects. Accordingly, previous studies have shown that prolonged administration of substances with potential antidepressant-like effects does not alter these behaviours (31), reinforcing that they are not sensitive to such interventions. Grooming behaviour reflects animal motivation, increasing with mild stress but drastically decreasing under severe stress (32). Although a statistically significant increase in grooming behaviour was observed only in the I-1 interaction group, other groups treated with chrysin, fluoxetine, and the I-2 combination also showed increased grooming (however, these changes were not statistically significant). This may reflect enhanced emotional regulation or behavioural adaptation rather than anxiety-like behaviour. According to previous literature, grooming under stress can also occur as part of a normal coping strategy or exploratory state. Therefore, the observed increase may not indicate a negative effect of the treatments but rather suggest an active behavioural adjustment (33). Nevertheless, we acknowledge the need for complementary tests (*e.g.*, elevated plus maze) to further clarify the functional significance of this response.

The values are expressed as the mean ± SEM of each evaluated variable. * $p < 0.05$ *vs.* vehicle. One-way ANOVA, *post hoc* Student-Newman-Keuls. I-1 = Combination of chrysin 4 μmol kg^{-1} + fluoxetine 1.6 μmol kg^{-1}, I-2 = combination of chrysin 20 μmol kg^{-1} + fluoxetine 3.2 μmol kg^{-1}.

Table I. Effect of the interaction of chrysin and fluoxetine on crossing, rearing, and grooming in the LAT in the male Wistar rat

Treatment	Crossing (*n*)	Grooming (s)	Rearing (s)
Vehicle	40.200 ± 3.147	42.694 ± 7.658	48.487 ± 6.513
Fluoxetine 1.6 μmol kg^{-1}	44.400 ± 5.704	65.381 ± 8.725	56.178 ± 8.756
Fluoxetine 3.2 μmol kg^{-1}	37.700 ± 7.044	42.847 ± 9.418	39.738 ± 7.412
Chrysin 4 μmol kg^{-1}	44.200 ± 3.941	76.434 ± 14.695	49.790 ± 7.206
Chrysin 20 μmol kg^{-1}	35.100 ± 4.701	72.444 ± 10.205	44.424 ± 5.970
I-1	47.400 ± 3.487	88.582 ± 9.922*	43.562 ± 5.284
I-2	51.100 ± 3.869	83.822 ± 6.922	53.760 ± 4.716

Forced swim test

The present study investigated the antidepressant-like effects of the flavonoid chrysin, both independent and in combination with fluoxetine, using the FST as a validated behavioural paradigm. This test is widely used to evaluate potential antidepressant-like effects in mice and rats, as it measures behavioural despair and the efficacy of pharmacological interventions (26). The analysis of latency to the first immobility revealed significant differences between treatments ($F_{6,63} = 19.229$, $p < 0.001$). The *post hoc* test showed that the fluoxetine 3.2 μmol kg^{-1}, chrysin 4 and 20 μmol kg^{-1}, I-1 and I-2 groups increased this variable compared to the vehicle group (Fig. 1). A short latency to the first immobility is considered an additional indicator of despair-like behaviour (34). In contrast, a prolonged latency is associated with an antidepressant-like effect (35–38) showing the motivation of the rat to escape the stressful situation induced by the swimming test. Our results show that the latency to the first immobility was prolonged after treatment with fluoxetine (3.2 μmol kg^{-1}), chrysin (4 and 20 μmol kg^{-1}), and the two combination groups (I-1 and I-2), supporting the antidepressant-like effect of these treatments (Fig. 1).

Total time of immobility revealed significant statistical differences according to the treatments ($F_{6,63} = 17.300$, $p < 0.001$). *Post hoc* test revealed that fluoxetine 3.2 μmol kg^{-1}, chrysin 4 and 20 μmol kg^{-1}, I-1, and I-2 groups decreased the total immobility time in the FST compared with vehicle (Fig. 2). In this study, fluoxetine (3.2 μmol kg^{-1}), chrysin (4 and 20 μmol kg^{-1}), and their combinations (I-1 and I-2) significantly reduced immobility time, which is considered an indicator of behavioural despair, contrarily, lower values in this variable suggest an antidepressant-like effect (36–40). Notably, the reduction in immobility time in the combination groups shows that chrysin may enhance the effect of fluoxetine

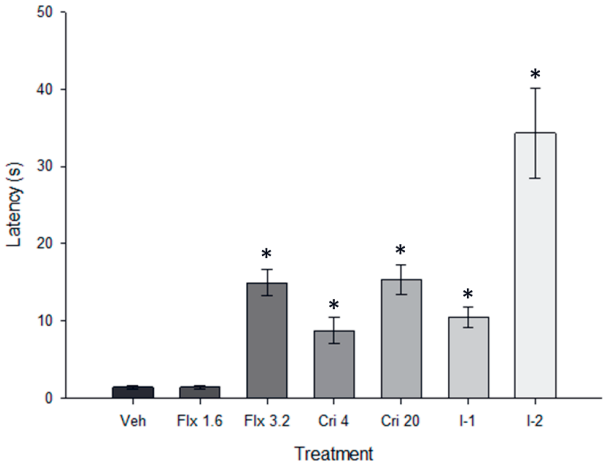

Fig. 1. Latency to the first immobility. Veh = vehicle; Flx 1.6 = fluoxetine 1.6 μmol kg^{-1}, Flx 3.2 = fluoxetine 3.2 μmol kg^{-1}, Cri 4 = chrysin 4 μmol kg^{-1}, Cris 20 = chrysin 20 μmol kg^{-1}; I-1 = combination of chrysin 4 μmol kg^{-1} + fluoxetine 1.6 μmol kg^{-1}; I-2 = combination of chrysin 20 μmol kg^{-1} + fluoxetine 3.2 μmol kg^{-1}. The values are expressed as the mean ± SEM of each evaluated variable. *$p < 0.001$ *vs.* vehicle. One Way ANOVA, *post hoc* Student-Newman-Keuls.

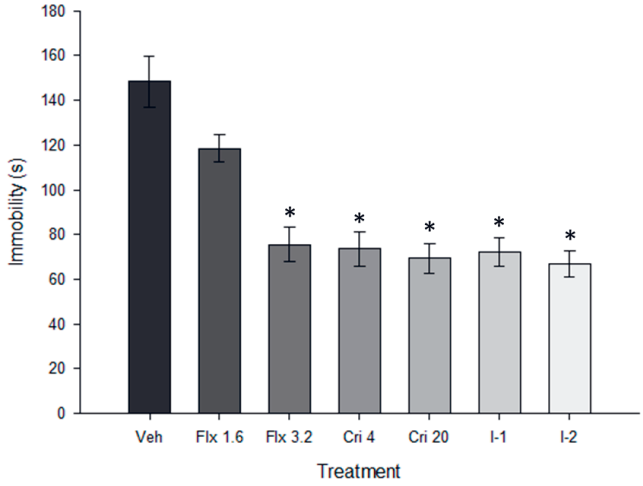

Fig. 2. Total immobility time. Veh = vehicle; Flx 1.6 = fluoxetine 1.6 μmol kg^{-1}, Flx 3.2 = fluoxetine 3.2 μmol kg^{-1}; Cri 4 = chrysin 4 μmol kg^{-1}; Cris 20 = chrysin 20 μmol kg^{-1}; I-1 = combination of chrysin 4 μmol kg^{-1} + fluoxetine 1.6 μmol kg^{-1}; I-2 = combination of chrysin 20 μmol kg^{-1} + fluoxetine 3.2 μmol kg^{-1}. The values are expressed as the mean ± SEM of each evaluated variable. * $p < 0.001$ *vs.* vehicle. One Way ANOVA, *post hoc* Student-Newman-Keuls.

or exert its own antidepressant properties. Additionally, no significant changes in locomotor activity were observed during the LAT, supporting that the observed effects of chrysin and combinations of treatments in the FST were not due to motor changes, if not to motivational effects.

We assessed swimming time, a parameter associated with serotonergic activity. Drugs that enhance serotonergic neurotransmission generally increase swimming behaviour in the FST (38). The statistical analysis revealed significant differences between treatments in the swimming time ($F_{6,63} = 7.425$, $p < 0.001$). *Post hoc* test revealed that fluoxetine 3.2 μmol kg^{-1}, chrysin 4 and 20 μmol kg^{-1}, and I-1 and I-2 groups increased the time spent in swimming compared with the vehicle group (Fig. 3). In this regard, the results showed that all treatments improved swimming behaviour compared to the vehicle, which may suggest that both chrysin and its combination with fluoxetine could modulate serotonergic activity. This is consistent with previous studies indicating that chrysin may exert its effects through serotonergic pathways by increasing serotonin levels in regions such as the hippocampus and prefrontal cortex (17), and by modulating 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in the raphe nuclei, cortex, and hippocampus (10, 41). However, further studies including specific neurochemical assessments are needed to clarify the involvement of the serotonergic system in chrysin's antidepressant-like effects.

We also evaluated climbing behaviour, which has been reported to increase in the FST, particularly by antidepressants that act on the noradrenergic system, such as desipramine and maprotiline (38). The analysis of time spent climbing revealed significant differences by treatments ($F_{6,63} = 5.589$, $p < 0.001$). *Post hoc* test revealed that time spent climbing was increased in the chrysin 20 μmol kg^{-1} and I-2 groups compared to the vehicle group (Fig. 4).

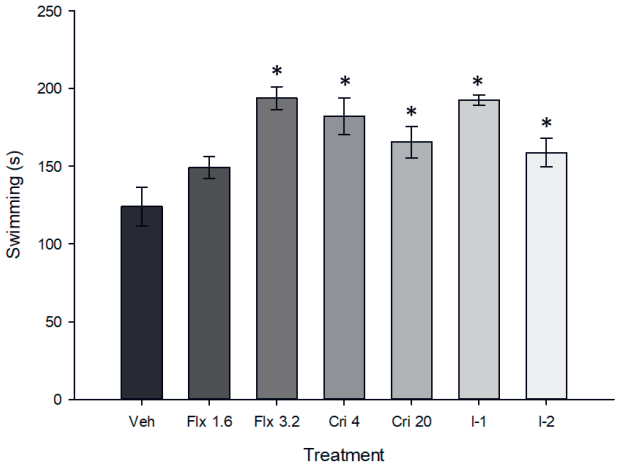

Fig. 3. Swimming time. Veh = vehicle; Flx 1.6 = fluoxetine 1.6 μmol kg^{-1}, Flx 3.2 = fluoxetine 3.2 μmol kg^{-1}; Cri 4 = chrysin 4 μmol kg^{-1}; Cris 20 = chrysin 20 μmol kg^{-1}; I-1 = combination of chrysin 4 μmol kg^{-1} + fluoxetine 1.6 μmol kg^{-1}; I-2 = combination of chrysin 20 μmol kg^{-1} + fluoxetine 3.2 μmol kg^{-1}. The values are expressed as the mean ± SEM of each evaluated variable. * $p < 0.001$ *vs.* vehicle. One-way ANOVA, *post hoc* Student-Newman-Keuls.

The results suggest that the significant increase in climbing time observed in the chrysin 20 μmol kg^{-1} group and the I-2 group may indicate a possible noradrenergic component in the mechanism of action of chrysin. This effect may be due not only to higher doses of chrysin

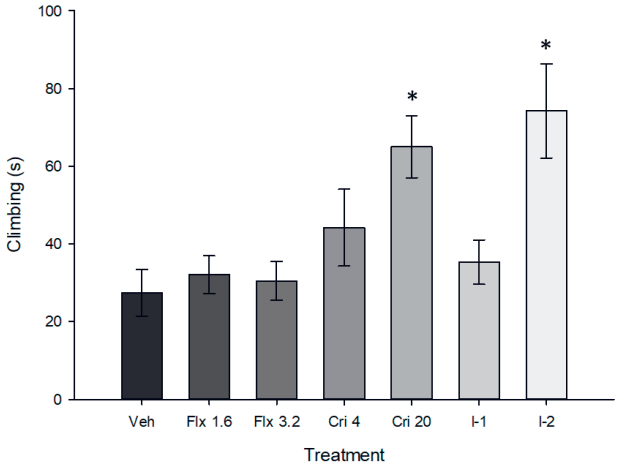

Fig. 4. Climbing time. Veh = vehicle; Flx 1.6 = fluoxetine 1.6 μmol kg^{-1}, Flx 3.2 = fluoxetine 3.2 μmol kg^{-1}; Cri 4 = chrysin 4 μmol kg^{-1}; Cris 20 = chrysin 20 μmol kg^{-1}; I-1 = combination of chrysin 4 μmol kg^{-1} + fluoxetine 1.6 μmol kg^{-1}; I-2 = combination of chrysin 4 μmol kg^{-1} + fluoxetine 3.2 μmol kg^{-1}. The values are expressed as the mean ± SEM of each evaluated variable. *$p < 0.001$ *vs.* vehicle. One-Way ANOVA, *post hoc* Student-Newman-Keuls.

but also to co-administration with fluoxetine, possibly enhancing the noradrenergic activity. Previous studies have reported different mechanisms of action for the antidepressant-like effects of chrysin, including its GABAergic properties (32), which may explain its rapid action, as well as its dopaminergic activity (18), in addition to its effects on the serotonergic system (10). However, this study supports the observations by Farkhondeh and collaborators (42) that treatment with chrysin activates the noradrenergic system, which correlates with the increase in climbing behaviour in the FST. These results highlight the complex mechanism of action of chrysin, suggesting that it may act not only through benzodiazepine-like activity or serotonergic modulation, but also involves noradrenergic activation at higher doses or when combined with fluoxetine. However, further neurochemical analyses are needed to confirm this possibility through the evaluation of specific biomarkers. This could expand the pharmacological prototypes that have been proposed in the preclinical studies to explore new antidepressant alternatives involving combinations of treatments even at subthreshold doses. Furthermore, the results highlight the interactions between chrysin and fluoxetine, as the combination also enhanced noradrenergic neurotransmission, an effect that was not promoted by fluoxetine alone. This could potentially impact certain aspects of chrysin's therapeutic effect, such as shortening the latency to onset of antidepressant effect or attenuating side effects – two of the major drawbacks of conventional antidepressants (5, 7). However, this still needs to be investigated further.

Our study presents some limitations that should be considered. First, no biochemical analyses were performed to confirm the potential involvement of the serotonergic and noradrenergic systems in the antidepressant effect of chrysin, as suggested by the behavioural results. Second, the dosing range used was limited, and future studies should expand it to more precisely characterise its effects. Third, although the forced swim test is widely used due to its predictive validity, the absence of additional models that more comprehensively represent the features of depression limits the translational relevance of the findings. Fourth, the study was conducted exclusively in male subjects, without considering the potential modulatory role of sex steroid hormones in antidepressant response, which should be addressed in future research. Finally, although preclinical evidence supports the hepatoprotective and cardioprotective effects of chrysin, more comprehensive safety assessments are necessary before considering its use in combination therapies.

CONCLUSIONS

The findings of this study contribute to understanding the antidepressant potential of chrysin, suggesting that, at certain doses, it may modulate both the serotonergic and noradrenergic systems. However, further neurochemical studies using specific biomarkers are needed to confirm the involvement of these neurotransmission systems in the observed effect.

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