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ORIGINAL ARTICLE

Antidepressant-Like Effects of Methanol Extract and Fractions of *Hypericum juniperinum* Kunth in the Forced Swimming Test

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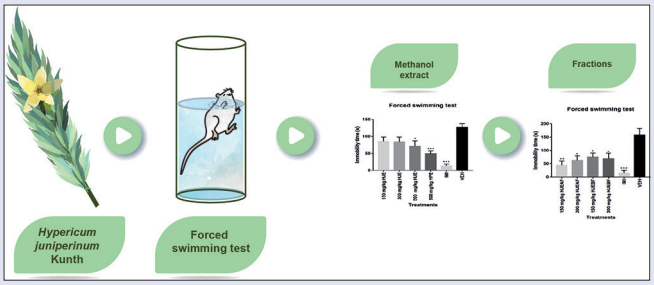
ABSTRACT

Background: Some members of the genus *Hypericum* have been shown to demonstrate antidepressant-like effects. In Colombia, approximately 54 species of *Hypericum* have been reported, and only a few have been investigated chemical and pharmacologically. **Objective:** The aim of this study was to evaluate the antidepressant-like effects of a methanol extract, as well as ethyl acetate and butanol fractions, obtained from aerial parts of *Hypericum juniperinum* K. **Materials and Methods:** Behavioral and locomotor activities were evaluated in the open field test (OFT). Antidepressant-like activity was measured in the forced swimming test (FST) in male Swiss albino mice. Preliminary phytochemical screening as well as a high-performance liquid chromatography (HPLC) profile of the active fractions of *H. juniperinum* was performed. **Results:** Treatment with the methanol extract at 500 mg/kg and the ethyl acetate and butanol fractions at 150 and 300 mg/kg resulted in a decrease in the immobility times in FST. In the OFT, none of the treatments presented altered behavior or locomotor activity of mice. Preliminary phytochemical screening identified terpenes/steroids, flavonoids, phenol derivatives, tannins, and saponins. In the HPLC analysis of the fractions, rutin, quercitrin, and quercetin were identified with the help of coinjection of standards. **Conclusion:** This is the first report of the antidepressant-like activity of extracts and fractions obtained from *H. juniperinum* in the FST model of depression. Flavonoids may be responsible for the antidepressant-like action of *H. juniperinum*.

Key words: Antidepressant-like properties, forced swimming test, *Hypericum juniperinum*, open field test, section *Brathys*

SUMMARY

- The aim of this research was to evaluate the antidepressant like activity of the methanol extract; and ethyl acetate and butanol fractions from aerial parts of *Hypericum juniperinum* K. The crude methanol extract and fractions showed significant effects in the forced swimming test animal model of depression. By means of HPLC coinjection with standards we identified three flavonoids in the *H. juniperinum* fractions. This kind of compounds may be the responsible for the activity displayed by *H. juniperinum* samples.



Abbreviations used: FST: Forced swimming test; HJE: Methanol extract of *Hypericum juniperinum*; HJEAF: Ethyl acetate fraction obtained from the methanol extract of *Hypericum juniperinum*; HJEBF: Butanol fraction obtained from the methanol extract of *Hypericum juniperinum*; HPE: Methanol extract of *Hypericum perforatum*; HPLC: High-performance liquid chromatography; IMI: Imipramine; MeOH: Methanol; OFT: Open field test; t_R: Retention time; UV: Ultra violet; VEH: Vehicle.

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INTRODUCTION

Depression is a widespread mental disorder and is considered a primary cause of disability.[1] It is estimated that approximately 350 million people suffer from this illness.[2] According to the duration, severity, and frequency of the symptoms, depression can be classified as mild, moderate, or severe.[3] Depression is characterized by a lack of interest and pleasure and commonly presents as symptoms of weakness, guilt, helplessness, and hopelessness, as well as sleep, eating, and concentration disorders, which can affect lifestyle and social relationships.[4] When depression is severe, it is strongly associated with suicide.[5] Depending on the type of depression diagnosed, psycho and pharmacotherapy approaches are employed for the treatment and management of this disorder.[6] Usually, antidepressant medications are used in moderate-to-severe depression despite the fact that they present marked adverse effects and are not always effective or accessible.[7,8] To overcome these problems, the use of natural products has increased as a promising cost-effective alternative to help treat or prevent depression.[9] Herbal products such as *Crocus sativus* L., *Rhodiola rosea* L., *Lavandula angustifolia* Mill, and *Hypericum perforatum* L. have demonstrated significant therapeutic effects.[10] The latter, known as St John's wort, is a species of European origin indicated to treat mild and moderate depression with less adverse effects.[11] The antidepressant action of *H. perforatum* has mainly been attributed to

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metabolites such as naphthodianthrones,[12] acylphloroglucinols,[13] and flavonoids.[14]

On the other hand, an increasing number of studies have reported the antidepressant-like activity of different species of *Hypericum*.[15-18] In Colombia, approximately 54 species of *Hypericum* have been described;[19] analysis of the chemical constituents and/or evaluation of biological and pharmacological activities have only been performed in a limited number of these species. We are interested in evaluating the antidepressant-like effects of *Hypericum* species from Colombian paramos to identify alternative sources with antidepressant activity.

Taking into consideration the botanical relationship between *H. perforatum* and other *Hypericum* species, a similar metabolic profile might be found and analogous activities could be expected. Therefore, the aim of this study was to assess the antidepressant-like activity of the methanol extract, as well as ethyl acetate and butanol fractions, obtained from aerial parts of *Hypericum juniperinum* K., a native species of Colombia with no reports of antidepressant action to date.

MATERIALS AND METHODS

Animals

Animals were supplied by the Department of Pharmacy Bioterium, Universidad Nacional de Colombia-Bogotá. Adult male Swiss albino mice weighing 25–30 g and aged 7–9 weeks were used in the present study. Mice were housed in plastic cages with a 12 h light/dark cycle with free access to food and tap water. All of the experiments conducted in this study were approved by the Ethics Committee for Animal Experimentation of the Faculty of Sciences, Universidad Nacional de Colombia-Bogotá.

Plant material

Aerial parts (stems, leaves, and flowers) of *H. juniperinum* K. (Hypericaceae) were collected during the dry season in the Cuitiva municipality (Vereda Arbolocos), Boyacá, Colombia. Plant material was identified by Orlando Rivera Díaz Herbario Nacional Colombiano de la Universidad Nacional de Colombia where a voucher specimen was deposited (COL589611).

Ethics statement

The Ministerio de Ambiente y Desarrollo Sostenible granted permission to collect samples and perform this research (Contrato de Acceso a Recurso Genético No 121 Otrosí Nº 3).

Preparation of plant extract and fractionation

Dried and milled plant material (1.24 kg) was extracted three times by percolation with 80% methanol (MeOH) based on the description provided by Gaedcke.[20] The extract was concentrated under reduced pressure in a rotary evaporator and dried in a water bath at 50°C. In total, 395.58 g of methanol extract was obtained, and 100 g of this extract was dissolved in an H_2O: MeOH (9:1) mixture and submitted to liquid–liquid extraction with solvents of increasing polarity as previously described.[21] Finally, the extracts of hexane, chloroform, ethyl acetate, butanol, and water were obtained with weights of 1.0, 6.7, 20.1, 34.0, and 13.7 g, respectively. Based on the yields obtained and the metabolites presumably present in each extract, ethyl acetate and butanol fractions were evaluated in animal models.

Phytochemical screening

Detection of plant secondary metabolites was carried out using the crude extract as previously described.[22] Tube tests and thin-layer chromatography were used to evaluate the presence of common plant secondary metabolites, including terpenes/steroids, alkaloids, coumarins, anthraquinones, flavonoids, saponins, tannins, and cardiac glycosides.

Drugs

Imipramine, which was used as a positive control in the antidepressant test, was obtained from Sigma-Aldrich (St. Louis, MO, USA). Behavioral mice studies were carried out with commercially available clonazepam (Coquan®). Tablets of a standardized extract of *Hypericum perforatum* (OKEY®) were acquired from a supermarket, its coating was removed with water, and then, it was dried, powdered, and weighted (500 mg). Finally, the powder was dissolved in 10 ml of the vehicle used to solubilize the samples (see pharmacological treatment section). The obtained extract was included in the initial forced swimming test (FST) as a positive control.

Pharmacological treatment

All extracts and fractions of *H. juniperinum*, as well as the standardized commercial extract of *H. perforatum*, clonazepam, and imipramine, were solubilized in a vehicle of propylene glycol (10%), glycerin (10%), polysorbate 80 (2%), and distilled water (78%), and sonicated. All treatments and solutions were prepared the day of the tests and administered orally 0.1 ml/10 g body weight.

Pharmacological evaluation was performed as follows. Before the FST, all animals were subjected to an open field test (OFT) to assess their behavioral and locomotor activity. Clonazepam was used as a positive control. All mice were distributed randomly into groups (n = 6 animals per group). Initially, three doses of the methanol extract of *H. juniperinum* (150, 300, and 500 mg/kg; p.o.) and one dose of *H. perforatum* (500 mg/kg; p.o.) were administered in the FST. Based on the results obtained in the evaluation of the methanol extract of *H. juniperinum*, a liquid–liquid fractionation of this extract was performed. Then, the obtained ethyl acetate and butanol fractions were orally administered at doses of 150 and 300 mg/kg and evaluated using the same antidepressant test. Imipramine was used as a positive control in the evaluation of the extract and fractions. Mice received a daily dose of each treatment in the 2 days before the tests, as a multiple-dose administration. The day of the test, animals were administered 1 h before the experiment. Behavioral and antidepressant tests were recorded on video.

Open field test

The OFT was employed to evaluate the exploratory behavior of mice and was performed as previously described,[23] with minor modifications. Briefly, each mouse was located in the center of a box (30 cm × 30 cm × 15 cm) with the floor divided in 16 squares. Then, over a period of 5 min, the total number of squares crossed (number of counts in 5 min), both in the center and in the periphery, as well as the stay time in every zone, was recorded. Clonazepam was used as a positive control (0.3 mg/kg; p.o).

Forced swimming test

The FST was conducted to evaluate the antidepressant-like activity of the extract and fractions. This test was performed as previously described.[24] In the FST, mice were individually forced to swim for 6 min in a vertical plastic cylinder (40 cm height × 18 cm diameter) containing 15 cm of water (25°C) and the immobility time was measured, which was defined as the minimum movement necessary to keep its head above water. Imipramine was used as a positive control (35 mg/kg; p.o).

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) analysis was carried out using an Agilent 1260 infinity system equipped with diode array

detector, quaternary pump, on-line degasser, and autosampler. The chromatographic analysis was performed using a Luna C18 column (4, 6 mm × 250 mm, 5 µm). The detection wavelengths were set at 254 and 340 nm. The flow rate and injection volume were 0.5 mL/min and 10 uL, respectively. The mobile phase consisted of solvent A (water containing 0.5% formic acid) and solvent B (acetonitrile), using the following gradient elution: 0–8 min, 90%–10%; 8–15 min, 65%–35%; 15–30 min, 25%–75%; and 30–35 min, 90%–10%. All determinations were carried out in triplicate. Peak identification was performed by coinjection with standards; 10 ul of each extract and 5 ul of the standard were used.

Sample preparation

Twenty-five milligrams of powdered extract (fractions of *H. juniperinum* and the extract of *Hypericum perforatum*) was added to a volumetric flask, diluted in 25 ml of a mixture of methanol and water (9:1), and sonicated. Previous to the injection, each extract was filtered through a 0.45-mm membrane. All samples were injected three times.

Preparation of flavonoid standard solutions

Stock solutions of quercetin, rutin, and quercitrin were used at the same concentration (1 mg/ml). All standards were prepared separately and dissolved in a MeOH: H_2O (9:1) mixture and then sonicated. Quercetin (purity ≥95%), quercitrin (purity ≥97.0%), and rutin hydrate (purity ≥94%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analysis

The results are expressed as the mean ± standard error of the mean. One-way analysis of variance followed by Dunnett's multiple comparison tests was used to compare differences between groups. Statistical analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, California, USA). Differences between data were considered statistically significant when $P < 0.05$ compared to the control group (Vehicle).

RESULTS

Preliminary phytochemical screening revealed the presence of terpenes/steroids, flavonoids, tannins, saponins, and phenolic compounds. The OFT was employed to evaluate the exploratory behavior of mice. In this model, there were no statistically significant differences between the samples tested and the negative control (methanol extracts of *H. juniperinum* and *H. perforatum* vs. vehicle).

Antidepressant-like activity was assessed with the FST. The results obtained with the methanol extracts of *H. juniperinum*, *H. perforatum*, and imipramine in the FST are shown in Figure 1. The extract of *H. perforatum* at a dose of 500 mg/kg clearly resulted in significantly decreased immobility times compared with the control group ($P < 0.001$). Likewise, the methanol extract of *H. juniperinum* at the maximum dose tested (500 mg/kg) showed an antidepressant-like effect following p.o. administration ($P < 0.05$). In this experiment, imipramine (35 mg/kg) was used as a positive control, and as expected, exhibited statistically significant activity ($P < 0.001$) compared with the control group.

Considering the aforementioned results, the ethyl acetate and butanol fractions obtained from partitioning of the methanol extract of *H. juniperinum* were evaluated in the same mouse model of antidepressant activity. Similar to tests performed with the methanol extract of *H. juniperinum*, the OFT was performed. None of the extracts analyzed showed any significant alteration with respect to behavioral or locomotor activities in mice with the OFT.

In the FST, the ethyl acetate and butanol fractions at 150 and 300 mg/kg showed a reduction in immobility times, which were statistically significant compared with the control values. At both doses, the ethyl acetate extract of *H. juniperinum* exhibited better activity compared with the butanol extract. In addition, the positive control group (imipramine, 35 mg/kg) resulted in a statistically significant reduction in immobility time ($P < 0.001$) [Figure 2].

The HPLC analysis of both active fractions allowed us to determine the presence of flavonoids. Three of them were identified by means of coinjection with reference standards. Specifically, in the HPLC profile

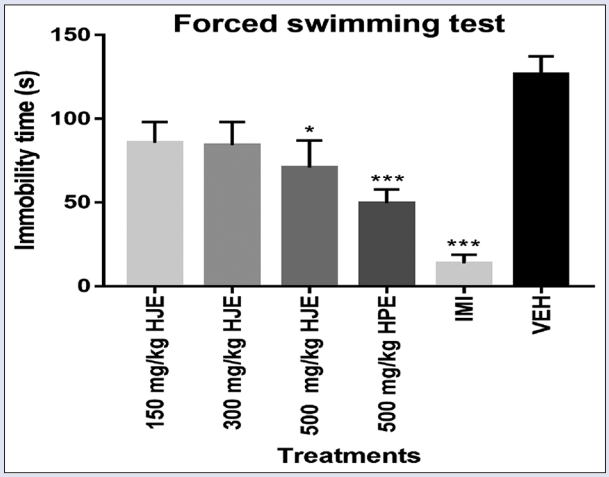

Figure 1: Effects of the methanol extract of *Hypericum juniperinum* (HJE) at 150, 300, and 500 mg/kg and the methanol extract of *Hypericum perforatum* (HPE) at 500 mg/kg in the forced swimming test. Imipramine (35 mg/kg) was used as a positive control. Vehicle (VEH), propylene glycol: glycerin: polysorbate-80:water (10:10:2:78). Data are expressed as the mean ± standard error of the mean. *$P < 0.05$ and ***$P < 0.001$ compared with the control vehicle

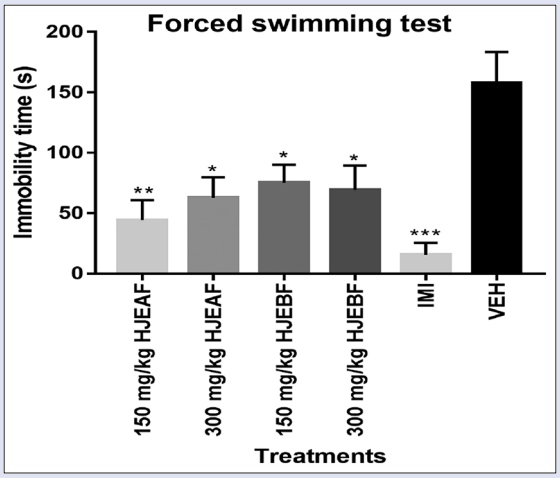

Figure 2: Effects of the ethyl acetate (HJEAF) and butanol (HJEBF) fractions obtained from the methanol extract of *Hypericum juniperinum* at 150 and 300 mg/kg in the forced swimming test. Imipramine (35 mg/kg) was used as a positive control. VEH, propylene glycol: glycerin: polysorbate-80:water (10:10:2:78). Data are expressed as the mean ± standard error of the mean. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the control vehicle

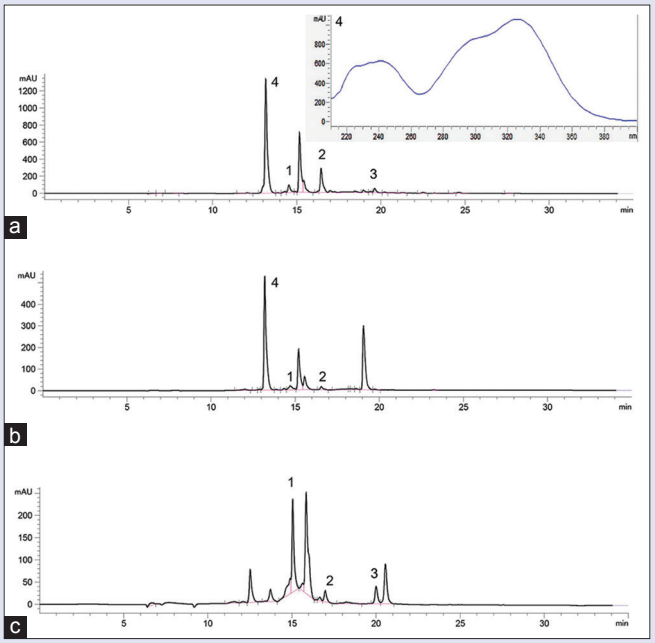

Figure 3: High-performance liquid chromatography chromatograms of (a) ethyl acetate fraction, (b) butanol fraction, and (c) standardized extract of *Hypericum perforatum* with diode array detection at 340 nm. (1) Rutin, (2) quercitrin (quercetin 3-*O*-rhamnoside), (3) quercetin, and (4) unidentified flavonoid. For chromatographic conditions, see Materials and Methods section

of the ethyl acetate fraction, rutin(1) and quercitrin (2), as well the flavone quercetin (3), were identified. In the HPLC profile of the butanol fraction, only the glycosylated flavonoids were present. In both fractions, a major peak (4) with a t_R of 12.9 min was detected. In general terms, the ethyl acetate and butanol fractions of *H. juniperinum* showed a similar chromatographic profile to that of *H. perforatum* reference extract [Figure 3].

DISCUSSION

H. juniperinum K. (sect. *Brathys*) is a native bushy plant, 0.2–0.5 m tall, which can be found in paramo and subparamo regions of Colombia. This species, conventionally known as "chite," is commonly used as a broom, and the flowers are used in cough treatment.[25] To the best of our knowledge, assessment of the pharmacological properties of *H. juniperinum* on the nervous central system has not been performed.

The OFT was carried out before the FST to avoid false-positive or negative outcomes induced by stimulatory effects of treatments in the antidepressant evaluation.[26] The OFT is a model commonly employed to measure exploratory behavior and general activity in rodents.[27] Based on the OFT, no significant alterations in behavior or locomotor activity of the extract or fractions of *H. juniperinum* were observed compared with the control. These results discard the possibility that the antidepressant-like action of the extract and fractions of *H. juniperinum* could be due to a stimulatory effect. On the other hand, the FST is considered an adequate protocol for assessing antidepressant-like effects of compounds and extracts.[28] In this test, mice are submitted to stress conditions when they are submerged in a tank filled with water without possibility of escape. Initially, animals are active as they try to overcome the adversity; then, the same situation drives them to immobility, which reflects behavioral despair, similar to depression in humans.[29] Most of the drugs available to treat depression reduce immobility time in the FST.[30,31] These findings demonstrated, for the first time, that orally administered methanol crude extract of *H. juniperinum* at a dose of 500 mg/kg, as well as ethyl acetate and butanol fractions at doses of 150 and 300 mg/kg, resulted in significant antidepressant-like effects in mice in the FST.

Various studies have been published recently in which the antidepressant-like activity of *Hypericum* species from different sections has been demonstrated. For example, *H. enshiense* (section *Hypericum*),[32] *H. foliosum* (section *Androsaemum*),[33] *H. reflexum* (section *Adenosepalum*),[34] *H. caprifoliatum* (section *Trigynobrathys*),[17] *H. andinum*, and *H. laricifolium* (section *Brathys*)[35] have been shown to exhibit antidepressant-like effects in mouse models of depression. Antidepressant effects of *H. perforatum* are commonly attributed to hypericin and pseudohypericin, as well as to hyperforin,[36] although some authors have reported that extracts of *H. perforatum* without hypericin and hyperforin maintain antidepressant-like activity.[37] This confirms that other types of metabolites, in addition to hypericin and hyperforin, could be responsible for antidepressant activity.

In 2011, Crockett and Robson performed a complete description of the chemotaxonomy of the *Hypericum* genus. They described that in species belonging to the section *Hypericum* (i.e., *H. perforatum*), there is a concomitant presence of hypericin, hyperforin, and flavonoids. However, in other sections such as *Tryginobrathys* and *Brathys*, hypericin and hyperforin are absent. Specifically, in the section *Brathys*, to which *H. juniperinum* belongs, aglycones of flavonoids such as quercetin, glycosidic flavonoids (rutin, hyperoside, quercitrin, and isoquercitrin), and xanthones are mainly reported.[38] In addition, the presence of dimeric acylphloroglucinols is considered a chemotaxonomic marker in the *Brathys* and *Thrigynobrathys* sections.[35,39] As mentioned previously, there is no evidence of the presence of hypericin and hyperforin in the *Brathys* section. Therefore, we cannot attribute the antidepressant-like activity of *H. juniperinum* to these metabolites. In addition, despite the fact that there is no previous information regarding the chemical constituents of *H. juniperinum*, we detected flavonoids and phenol derivatives in our preliminary phytochemical screening and identified three flavonoids in the HPLC analysis. Rutin, hyperoside, isoquercitrin, quercitrin, quercetin, amentoflavone, and I3, II8-biapigenin are described as the most common flavonoids in the genus *Hypericum*.[40] In the present study, the flavonoids rutin, quercitrin, and quercetin were identified by coinjection with authentic samples. The main compound (4; t_R: 12.9) could not be identified, but according to the UV spectrum, this metabolite has two maximum peaks at 240 and 320 nm, suggesting the presence of a flavonoid. Hence, it is likely that flavonoids (aglycone and glycosides) account, at least in part, for the biological activity of the methanol extract and ethyl acetate and butanol fractions of this plant. This is feasible since the antidepressant activity of pure flavonoids and extracts enriched in these types of compounds has been previously demonstrated.[14,41]

The antidepressant-like effects of *Hypericum perforatum* flavonoid extracts, and flavonoids in general, are probably explained by an increasing of the levels of neurotransmitters in the serotonergic, noradrenergic, and dopaminergic systems.[42] Particularly, some flavonoids present in *H. perforatum* extracts have been investigated to determine their mechanisms of action. For example, rutin, a glycosylated flavonoid identified also in *H. juniperinum* ethyl acetate and butanol fractions, increases the availability of 5-HT and NE in the synaptic cleft.[41] On the other hand, antidepressant-like effect of hyperoside is attributed to a cytoprotective action.[43] Furthermore, some flavonoids isolated from *H. perforatum* demonstrated regulation of the hypothalamic–pituitary–adrenal axis evidenced with a reduction of circulating plasma levels of adrenocorticotropic

hormone and cortisol.[44] With respect to *H. juniperinum*, further studies are needed to determine the bioactive compounds and the mechanism implied in the antidepressant effect of extracts from this Andean species.

CONCLUSIONS

The results of this study demonstrate, for the first time, the antidepressant-like effects of the crude methanol extract, and ethyl acetate and butanol fractions, of *H. juniperinum* in the FST model of depression by means of reduction of immobility time. In addition, no behavioral or locomotor alteration was shown following treatment with *H. juniperinum*. Our findings indicate that flavonoids may be responsible for the antidepressant-like action of *H. juniperinum*. Further studies with *H. juniperinum* regarding the underlying mechanism of action and the identification of bioactive metabolites are necessary.

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Conflicts of interest

There are no conflicts of interest.

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