SHORT COMMUNICATION

Antidepressant-like effects of a methanol extract of Leonotis nepetifolia in mice

Daniel Arrieta-Báez, Mayra Beatriz Gómez-Patiño, Noé Jurado Hernández, Lilian Mayagoitia-Novales, Ana María Dorantes-Barrón and Rosa Estrada-Reyes

Instituto Politécnico Nacional CNMN, Luis Enrique Erro s/n, Unidad Profesor Adolfo López Mateos, Gustavo A. Madero, México Ciudad de México; Laboratorio de Fitofarmacología, Dirección de Investigaciones en Neurociencias, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz, Tlalpan, Ciudad de México, México; Departamento de Etopología, Dirección de Investigaciones en Neurociencias, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz, Tlalpan, Ciudad de México, México

ABSTRACT
This study evaluated the antidepressant-like effects of a methanol extract of Leonotis nepetifolia in behavioural tests in mice. Our results showed that a single administration of the extract significantly reduced immobility behaviour in the tail suspension test, while three administrations were necessary to diminish immobility behaviour in the forced swimming test. A daily dose of the extract for 28 days improved body weight gain and significantly reduced corticosterone levels of mice exposed to chronic unpredictable mild stress. Metabolic profiling of the extract revealed that nepetaefolin, methoxynepataefolin, and 7-O-β-glucoside luteolin were the main products. Acute and repeated administration of the extract produced antidepressant-like effects in animals subjected to chronic stress. Our results suggest the hypothalamus-hypophysis-adrenal axis participates in the antidepressant actions of the extract. These results show that alterations in behaviour elicited by stress can be prevented with L. nepetifolia treatment.

ARTICLE HISTORY
Received 18 June 2021
Accepted 22 March 2022

KEYWORDS
Leonotis nepetifolia; antidepressant; chronic stress; nepetaefolin; methoxynepataefolin

CONTACT Rosa Estrada-Reyes restrada@imp.edu.mx; estrarosa@hotmail.com

Supplemental data for this article can be accessed online at https://dx.doi.org/10.1080/14786419.2022.2058939.

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1. Introduction

*Leonotis nepetifolia* is native to Africa but is currently found worldwide. *L. nepetifolia* is commonly known as Christmas candlestick, Klip dagga, or Lion’s ear and tail. *L. nepetifolia* is employed in folk medicine to alleviate asthma, rheumatism, headache, and nervousness as a tranquilizer agent. Many studies have shown its antibacterial, antioxidant, anti-inflammatory, and antimalarial activities, among other activities. The interest in this species continues to be high due to its numerous reported biological activities; thus, recently, the antimicrobial and antileishmanial effects of hydroalcoholic extracts of leaves and roots of *L. nepetifolia* were reported. These effects were related to aglycone and glucoside flavonoids, such as apigenin, crisiliol, and glycosides of luteolin (Pinto de Oliveira et al. 2019). The anticonvulsant effect of *Leonotis nepetifolia* polar extracts was reported in chicken and mouse experiments (Ayanwuyi et al. 2009). In mice, a methanol extract of *L. nepetifolia* produced sedative and anxiolytic-like effects (Ayanwuyi et al. 2016). However, the antidepressant effects of *L. nepetifolia* have not been explored. This study aimed to investigate the antidepressant-like effects of a methanol extract of *Leonotis nepetifolia* in two depression predictive paradigms in mice, forced swimming and tail suspension tests, as well as in a chronic unpredictable mild stress model. We explored the involvement of the hypothalamic-hypophysis-adrenal axis in the antidepressant activity of the extract. The metabolic profile of the extract was analysed by ultra-high-performance liquid chromatography-electrospray-mass spectrometry (UPLC-ESI-MS). The effects of the methanol extract on ambulatory activity in the open field and rotarod tests were assessed.

2. Results and discussion

Literature data show that *Leonotis nepetifolia* has a great variety of pharmacological activities; however, its effects on the central nervous system have little been studied.

We evaluated the antidepressant-like effects of a methanol extract of *L. nepetifolia* administered as a single or triple oral dose in two predictive behaviour mouse paradigms, the tail suspension test (TST) and the forced swimming test (FST).
The passive immobility behaviour is elicited in both the forced swimming and the tail suspension trials. Nevertheless, the TST is less stressful than the FST (Petit-Demouliere et al. 2005). These tests present variability in response to certain antidepressants, indicating potentially different substrates and neurochemical pathways mediating performance in these tests. For example, previous reports indicate that the FST fails to detect the acute effects of SSRIs (Cryan et al. 2005), while the TST is sensitive to most antidepressants.

As shown in Figure S1A in the TST, both the extract (10, 100, and 200 mg/kg) and imipramine (25 mg/kg), non-selective monoamines and serotonin reuptake inhibitor, significantly decreased the duration of immobility compared to that in the group treated with saline ($H = 30.19$, $df = 5$, $p < 0.001$). On the other hand, neither treatment with a single dose of the methanol extract nor fluoxetine, a serotonin reuptake inhibitor (SSRI), was enough to reduce immobility behaviour in the FST (data not shown). However, three administrations of the extract or the inhibitor of serotonin reuptake caused a significant decrease in the duration of immobility (Figure S1B); therefore, the extract showed an increasing antidepressant-like effect from 1 to 100 mg/kg, similar to FLX at 10 and 15 mg/kg ($H = 45.95$, $df = 6$, $p < 0.001$), while at 200 mg/kg, it diminished this effect. The extract showed a biphasic dose-response pattern, with a stimulatory effect at low doses and a high dose inhibitory effect (Son et al. 2008). This hormetic pattern is often observed in extracts, active constituents’ plants, and drugs with actions on the central nervous (CNS), which may activate one or more adaptive cellular stress response pathways (Mattson and Cheng 2006). The selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine also show a dose-response hormetic pattern. SSRIs interact with the system under complex homeostatic control, and a long-term may return the system to equilibrium and restorative synaptic homeostasis. These hormetic responses are commonly triggered when a drug interacts with homeostatic mechanisms as an adaptive compensatory process due to an initial disruption in homeostasis (Andrews and Amsterdam 2020).

Interestingly, the methanol extract (repeated doses) showed the same dose-response pattern described by FLX. It allows us to suggest that the serotonergic system could be participating in the L. nepetifolia methanol extract antidepressant-like effect. However, specific experiments are necessary to clarify this issue.

The dose-response profile of the methanol extract is critical to establish the dose range in which hemesis pathways are activated without the adverse effects that could be at high doses. The non-linear regression analysis of the dose-response pattern indicated that the extract has a mean effective dose ($ED_{50}$) of 90.0 mg/kg ($r = 0.986$), and the ratio between lethal dose ($LD_{50}$) and $ED_{50}$ was higher than 30, indicating that the methanol extract has a wide therapeutic safety range (Figure S4).

Besides, mice ambulatory activity was measured in the open-field test (OFT) (Table S1) to exclude possible unspecific side effects of treatments on locomotor activity. These data showed that the antidepressant-like actions of the extract were not associated with ambulatory activity changes. The hypothalamic-pituitary-adrenal (HPA) axis integrates and regulates the neuroendocrine-immune responses to stress necessary to maintain homeostasis in mammals. However, the long-term activation of this stress regulation system can have harmful effects on health. The imbalance of this system is
also observed in patients with depression, showing learning and cognition impairment (Bao and Swaab 2019).

The FST was also used to assess whether repeated doses of the methanol extract of *L. nepetifolia* produced antidepressant-like effects in animals subjected to mild stress stimuli for 28 days (CUMS). In a first experiment, two independent groups were used to confirm the CUMS effect on the passive immobility behaviour, the group I: unstressed group receiving a daily injection of vehicle and group II: CUMS group also receiving placebo, but was subjected to CUMS test, the results showed that in chronically stressed animals was significantly higher than that in unstressed animals (*t* = 3.41, *p* = 0.004) the duration of immobility measured in the FST (Figure S1C). This result confirmed that mild chronic stress induces depression-like behaviour, which significantly increases the duration of immobility time in the FST.

The immobility behaviour of stressed animals treated with a daily dose of the extract was compared with the immobility of stressed mice that receive only the vehicle. The results of Kruskal-Wallis analyses showed that both the extract (1, 10, 100, and 200 mg/kg/day) and FLX (1 mg/kg/day) significantly reduced the duration of immobility (*H* = 40.56, *fd* = 5, *p* ≤ 0.001). Thus, the mean passive behaviour of mice treated with the extract decreased up to 75% compared with mice in the saline-treated group (Figure S1D).

The reduction in immobility time in the FST and TST was not associated with ambulatory activity changes, indicating that it is due to a specific antidepressant-like effect. Table S2 shows that there were no significant changes in the number of counts (*H* = 8.46, *fd* = 8, *p* = 0.38) or rearing number (*H* = 10.0, *fd* = 8, *p* = 0.26) in the OFT compared to the control group. Finally, the motor coordination of mice subjected to CUMS was evaluated 30 min after the last administration on the rotarod test, showing that the extract did not impair motor coordination or cause muscle tone loss at either dose (data not shown).

Prolonged stress can also induce anhedonia-like behaviour in mice, such as the lack of appetite and low water consumption, as reflected by slow growth and body weight loss (Monteiro et al. 2015). The extract reduced stress-related behaviours in the chronic mild unpredictable stress test (Figure S2). Thus, growth measured by an increase in body weight in the mice subjected to CUMS began to diminish compared with unstressed mice from the initiation of the adverse stimuli and during the 28 days of treatment. Two-way repeated-measures ANOVA revealed a significant effect of the main factors: treatment (*F*<sub>(5,702)</sub> = 15.12, *p* < 0.001), day (*F*<sub>(26,702)</sub> = 119.57, *p* < 0.001) and a significant treatment × day interaction (*F*<sub>(130,702)</sub> = 4.31, *p* < 0.001). Paired comparisons revealed that on the 6th day, untreated mice showed a reduction in weight gain compared to mice treated with FLX and the extract at 100 mg/kg (*p* = 0.004 and *p* = 0.015, respectively); this difference was also observed throughout the 23 remaining days. Starting on the 18th day, treatment with both saline and 200 mg/kg extract showed significant differences compared to treatment with saline (*p* = 0.003 and *p* = 0.014, respectively). Thus, at the end of the 28 days of treatment, we obtained three homogeneous groups: saline and 10 mg/kg/day of extract (*p* = 0.997), saline and 200 mg/kg (*p* = 0.164), and 100 mg/kg/day and fluoxetine (1 mg/kg/day) (*p* = 0.277), while the extract at 10 mg/kg/day did not show any ability to counteract the effect of stress (Figure S2). These results suggest that *L. nepetifolia* extract can reduce anhedonia-like behaviour, such as low growth, in animals exposed to prolonged stress
conditions. Prolonged stress also causes hyperactivity of the hypothalamus-hypophysis-adrenal (HHA) axis. This loss of homeostasis is characterised by the secretion of stress mediators, such as glucocorticoids, e.g., corticotropin hormone is released in response to stressful stimuli and overactivates the HHA axis, which induces an exacerbated secretion of cortisol or corticosterone into the circulation (Pariante and Miller 2001). In this sense, the effect of antidepressant drugs is associated with a reduction in HHA axis activity, suggesting that depression symptoms are partly related to the rise in circulating levels of corticosterone (Kim and Haller 2007). We found that stressed animals’ corticosterone levels significantly decreased with the L. nepetifolia and FLX treatments. Thus, in animals that received the extract at 100 and 200 mg/kg/day or FLX (1 mg/kg/day), corticosterone levels were decreased by 50% compared to animals that did not receive treatment \( (F_{(5,35)}= 5.71, p < 0.001) \) but did not differ between 100 and 200 mg/kg/day (Table S2). These results suggest that the methanol extract of L. nepetifolia restores homeostasis, which does not eliminate the possibility of responding to other types of stimuli, such as hunger or the challenge of social coexistence. The catecholamine hypothesis for mood disorders proposes that depression is associated with a deficiency in biogenic amines, especially catecholamines and serotonin in the brain, which act as neurotransmitters. Thus, the monoaminergic and serotonin systems play a critical role in the pathophysiology of depression diseases. Monoamine oxidase enzymes participate in the degradation of these neurotransmitters, and the monoamine oxidases inhibitors increase their brain concentration, thereby producing antidepressant effects (Stafford et al., 2006; Pesarico et al. 2014). The HHA axis and the aminergic systems work together to restore homeostasis during the stress response.

In contrast, the mismatch between these systems leads to changes in mood and depression (Bao and Swaab 2019). The effects of MAOA’s activity assay showed that the methanol extract at 0.1 and 1 mg/mL selectively inhibited MAO-A activity but did not alter MAO-B activity compared with clorgyline and deprenyl used as the positive control (Table S3). MAO-A shows a greater affinity for noradrenaline and serotonin, whereas MAO-B shows a greater affinity for beta-phenylethylamine. These results add more support to the idea that the methanol extract interacts with the monoamines system to induce its antidepressant-like effects.

Although both behavioural and neurochemical evaluations point towards a mechanism of action mediated by monoamines, specifically serotonin, we cannot rule out the mediation of other neurotransmitter systems in the antidepressant-like effect of L. nepetifolia methanol extract.

Metabolic profiling with UPLC-ESI-MS is the most reliable and unequivocal technique to characterise active extracts’ chemical composition (obs.: observed, calc.: calculated). So, UPLC-ESI-MS positive-mode analysis of the methanol extract led to the identification of different compounds (Table S4). Peak observed at 7.6 min showed an ion peak, \([M+H]^+\), at \( m/z \) 437.1808, which correspond to methoxynepetaefolin [1] according to the molecular composition \( C_{23}H_{32}O_{8} \) (calc. for \( C_{23}H_{33}O_{8}^{+}, 437.2007 \), as well as an unidentified isomeric form observed at 8.7 min, with the same ion peak \([M+H]^+\) at \( m/z \) 437.1808 \( C_{23}H_{33}O_{8}^{+} \) corresponding to the molecular formula \( C_{23}H_{32}O_{8} \). Under the same conditions Nepetaefolin \( (C_{22}H_{28}O_{7}) \) [2] at \( RT = 9.2 \) min (ion
peak [M + H]^+ observed at m/z 405.1873 (C_{22}H_{29}O_{7}^+) , m/z_{calc.} 405.1835), leunorone C (C_{22}H_{34}O_{5}) [3] at 6.3 min (ion peak [M + H]^+; observed at m/z 379.1907 (C_{22}H_{35}O_{5}^+) ; m/z_{calc.} 379.1907), nepetaefuranol (C_{22}H_{30}O_{8}) [4] at RT 6.5 min (ion peak [M + H]^+; observed at m/z 423.2028 (C_{22}H_{31}O_{6}^+); m/z_{calc.} 423.1941), and crotoninsularin diterpene (C_{20}H_{28}O_{4}), (ion peak [M + H]^+; observed at m/z 333.2052 (C_{20}H_{29}O_{4}^+); m/z_{calc.} 333.2060) were identified. Figure S3 shows the structure of the main metabolites labdane-type diterpene, which are chemotaxonomic markers of the *Leonotis* genus. The flavonoids 7-O-β-glucoside luteolin (C_{21}H_{20}O_{11}) at RT = 6.0 min (ion peak [M + H]^+; observed at m/z 449.1097 (C_{21}H_{20}O_{11}^+); m/z_{calc} 449.1078) and quercetin 3-rhamnoside were also detected; these last molecules could be decomposition products of the aromatic amine leucorin, as reported in several *Leonotis* species, including *Leonotis leonurus*. The chemical composition of the antidepressant extract allows us to suggest that the labdane diterpene derivatives are the main active principles for the antidepressant actions of *L. nepetifolia* without excluding the participation of flavonoids, characteristics of the Lamiaceae family which have shown a wide range of activities on CNS (Estrada-Reyes et al. 2014; Venditti et al. 2016; Frezza et al. 2021).

3. Conclusion

Our results showed that acute or triple administration of the methanol extract of *L. nepetifolia* caused antidepressant-like effects without altering locomotor activity. Repeated extract administration produced a robust antidepressant-like effect in animals exposed to prolonged stress. Additionally, *L. nepetifolia* reversed weight loss and high corticosterone levels. These results show that alterations in behaviour elicited by stress can be prevented with *L. nepetifolia* treatment.

Acknowledgments

The authors are indebted to botanist Beatriz González Hidalgo, M. Sc., for plant identification, Francisco Ruiz for providing the experimental animals.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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