SUPPLEMENTARY MATERIAL

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

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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 a triple administration was necessary to diminish the immobility behaviour in the forced swimming test. A daily dose for 28 days of the extract improved the body weight gain and significantly reduced the corticosterone levels of mice exposed to chronic unpredictable mild stress. The extract's metabolic profile revealed the presence of nepetaefolin, methoxynepataefolin, and 7-*O*-*β* glucoside luteolin as the main products. The extract's acute and repeated administration produced antidepressant-like effects in animals subjected to chronic stress. Our results suggest the hypothalamus hypophysis adrenal participates in the extract antidepressant actions. These results show that alterations in behaviour elicited by stress can be prevented with *L. nepetifolia* treatment.

Keywords: *Leonotis nepetifolia*, antidepressant, chronic stress, nepetaefolin, methoxynepataefolin.

1. Experimental

1.1 Vegetal material

Leonotis nepetifolia (L.) R. Br. (synonyms: *Leonotis kwebensis* N.E. Br., *Leonotis nepetifolia* var. *nepetifolia*, *Leonotis ovata* Bojer, *Leonurus marrubiastrum* Lour., *Leonurus nepetifolius* (L.) Mill., *Phlomis nepetifolia* L., *Stachys mediterranea* Vell, according to The Plant List at www.theplantlist.org).

Botanist Beatriz González Hidalgo from the Herbarium of the Universidad Autónoma Metropolitana identified the species. A specimen was deposited (voucher no. 13052) in the Herbarium of Universidad Autónoma Metropolitana Unidad Xochimilco, Mexico.

Air-dried and finely ground aerial parts of L. nepetifolia (500 g) were extracted by maceration in MeOH (5 L) for three days; this procedure was repeated once. Next, the solvent was eliminated by distillation under reduced pressure at 50 °C, which led to 56 g of methanol extract (11.9% dried vegetal material weight).

1.2 Metabolic profiling of the methanol extract of L. nepetifolia.

Metabolic profiling by UPLC-DIESI–MS of the methanol extract of *L. nepetifolia*

Samples of the methanolic extract were dissolved in methanol and analysed by ultra-high-performance liquid chromatography-mass spectroscopy (UPLC-DIESI-MS). An Ultimate 3000 ultra-high-performance liquid chromatography (UPLC) system (Dionex corp., CA, USA) with photodiode array detection (PAD) was coupled to a Bruker MicrOTOF-Q II system by an electrospray ionization (ESI) interface (Bruker Daltonics, Billerica, USA), Electrospray Ionization (ESI) analysis was done on a Bruker micrOTOF-Q II, the

compound-related peaks were found in positive ion mode (ESI^+). The capillary potential was -4.5 kV, the dry gas temperature was 200 °C, and the drying gas flow 4 L/min—total ion chromatograms from m/z 100 to 1000. The obtained patterns were analysed by a Bruker Compass Data Analysis 4.0 (Bruker Daltonics, Technical Note 008, 2004, Bruker Daltonics, Billerica, MA, USA), which provided a list of elemental formulas using Generate Molecular Formula (Bruker technical Note008, 2004). Bruker Daltonics Technical Note 008, 2004. Molecular Formula Determination Under automation. https://www.bruker.com/fileadmin/user_upload/3-Service/Support/Separations_MassSpectrometry/Software/ESI-Compass/Release-Notes_DataAnalysis_4-1.pdf**.**

1.3 Animals

All experiments were performed with male Swiss Webster mice (20-30 g body weight) obtained from the vivarium of the Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz. Mice remained housed with eight animals per box (37 × 27 × 15 cm) under a 12 h light/dark inverted cycle (lights on at 20:00 h) and temperature- and humidity-controlled conditions. All experiments were performed following the Mexican official norm (NOM-062-ZOO-1999) and the general principles of laboratory animal care (NIH publication # 85-23, revised in 1990). The local ethical committee approved this project (NC190127.0).

1.3.1 Drugs

All drugs were dissolved in saline solution (0.9% NaCl in water) and administered at a constant 10 ml/kg volume. Imipramine (IMI; 25 mg/kg) and fluoxetine (FLX; 10 and

15 mg/kg) administrated by intraperitoneal route (i.p.; -30 min before to test) served as the positive controls in the TST, or the FST, and the OFT. To CUMS test FLX (i.p.; 1 mg/kg/day) was used as a positive control. The control groups were administered with saline solution (CTL). The oral route was used for the methanol extract administration. Doses of the extract are expressed as mg of dried extract per kilogram of body weight (mg/kg).

1.4 Behavioural evaluation

1.4.1 Antidepressant-like effect in the tail suspension test (TST)

Six independent groups (eight mice per group) were assigned as follows: a CTL group, an IMI group, and four groups that received 1, 10, 100, or 200 mg/kg methanol extract, respectively. Acoustically and visually isolated mice were fastened by the tail and suspended 50 cm above the surface of a wooden box by adhesive tape placed approximately 1 cm from the tip of the tail. Each mouse has hovered for 6 min, and long-lasting immobility was recorded during the final 4 min of the test. The immobility behaviour was measured only when the mouse remained hanging wholly and passively motionless. The 5-min test was videotaped and later scored by two observers blinded to the treatments applied (Estrada-Reyes et al., 2018).

1.4.2 Antidepressant-like effect in the forced swimming test (FST)

Mice were individually placed into glass cylinders (height: 21 cm, diameter: 14.5 cm) containing 15 cm of water at 23±2 °C. Two swimming sessions were conducted: a 15-min pre-test session, followed by a 5-min session (test) 24 h later. At the end of each session, the animals were removed, gently drained, placed in a warm cage to recover (23±2

°C) for 15 minutes, and returned to their home cages. The session test was videotaped and later scored by two observers blinded to the treatments applied.

For the single administration test, six independent groups (eight mice per group) received: saline (CTL), IMI, or the methanol extract at 1, 10, 100, or 200 mg/kg.

For the triple administration test, immediately after the swimming pre-test, six independent groups of mice received the first administration of FLX, the methanol extract (10, 100, or 200 mg/kg) or vehicle, a second dose after 17 h and a third dose 30 min before starting the swimming session (Martínez-Vázquez et al., 2012).

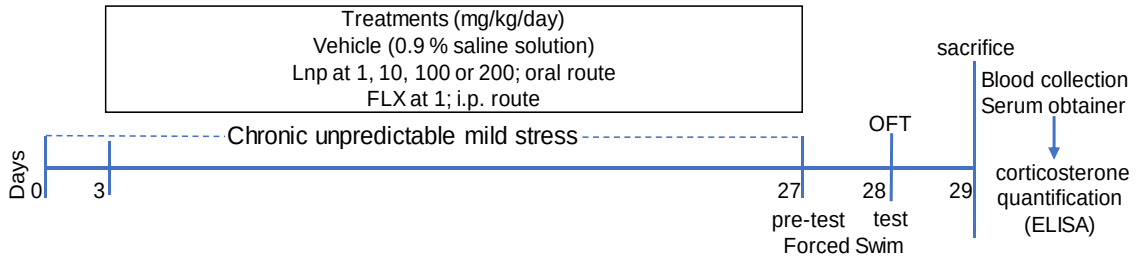
1.4.3 Evaluation of the antidepressant-like effect of the methanol extract of L. nepetifolia in animals subjected to the chronic unpredictable mild stress (CUMS) test

The CUMS test consisted of various unpredictable mild stressors once a day for 28 consecutive days. Mice received different adverse stimuli chosen randomly, and they could not predict them, and these stimuli were not employed more than two times at the same day/night cycle time.

The stressors included two hours of water or food deprivation (not more than two hours), a 1 min tail suspension, overnight illumination, filthy cage, white noise for two minutes, a wet box, shaking the mice (for 5 min), and constraining mice in a narrow cell (2 h). (Wu et al., 2017; Willner, 2016). To corroborate the chronic unpredictable mild stress (CUMS) effects on the immobility time in the FST, one vehicle-treated group was subjected to CUMS for 28 days. On day 27, the mice were subjected to the pre-test session, and 24 h later, they were subjected to the FST.

The antidepressant-like effects of the methanol extract of L. nepetifolia and fluoxetine

(FLX) in the FST in animals subjected to the chronic unpredictable stress (CUMS) test.



For the CUMS test, a total of 48 mice were randomly divided into six groups as follows:

Group I: an unstressed control group that received daily vehicle administration (CUMS-non-CTL).

Group II: CUMS control group that received daily vehicle administration (CUMS-CTL).

Groups III, IV, and V: CUMS groups receiving the extract at 1, 10, 100, or 200 mg/kg/day, respectively. Group VI: CUMS group receiving FLX (1 mg/kg/day). All groups were dosed 30 min before the stressful stimulus.

1.4.4 Effects of L. nepetifolia methanol extract on ambulatory activity

To detect possible unspecific effects of the treatments, we measured the ambulatory activity of experimental mice in the open field test (OFT). OFT consists of an opaque-plexiglass box (40 × 30 × 20 cm), the surface was divided into 12 equal squares (11× 11 cm). The mouse was placed in any corner of the cage; the number of times the animal crossed a square (counts number) and the number of times that stands on its hind legs (rearing number) were registered for a 5-min period (Estrada-Reyes et al., 2018).

1.5 Evaluation of the Effect of the methanol extract of L. nepetifolia on corticosterone serum levels in mice subjected to the CUMS test

Corticosterone serum levels of mice subjected to the CUMS test and treated for 28 days with a single oral dose daily of the extract at 1, 10, 100, or 200 mg/kg/day, FLX (1 mg/kg/day), or vehicle (CUMS-CTL) and mice in the untreated group (CUMS-non-CTL) were measured in the serum obtained on the 29th day. Serum corticosterone was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Inc., UK).

1.6 MAOs Inhibition Assays

Mitochondrial preparations from brain mice were used to evaluate the methanol extract effect on MAO-A and MAO-B activities

Briefly, MAO was obtained from the mitochondrial fraction from brain mice (Stafford et al., 2007; Porras-Ramírez et al., 2020). The brain fresh got was washed in ice-cold PBS buffer (2 mL, pH 7.6) and homogenized 1:40 (w/v) in 0.3 M sucrose (30 s). Then, the homogenate was centrifuged at 1000 x g for 15 min, and the supernatant was centrifuged again at 12000 x g for 30 min. The pellet was resuspended in 2 mL of 0.3 M sucrose and 30 mL of 1.2 M sucrose added; a mitochondrial button was obtained by centrifugation to 50000 x g for two hours. The mitochondrial pellets were reconstituted in a PBS buffer and stored in aliquots. Aliquots of samples were incubated at 37 °C for 5 min in a medium containing buffer solution (Na_2PO_4/ KH_2PO_4 isotonic with KCl, pH 7.4), 4 mM 5HT or 2 nM β-PEA as specific substrates for MAO-A and B, respectively. An inhibition experiment of MAO activities was performed by adding clorgyline (MAO-A selective inhibitor) or deprenyl (MAO-B selective inhibitor) and the methanol extract (substrate). Protein

concentration was adjusted to 1 mg/mL, and it was measured by Bradford's method using bovine serum albumin as standard.

1.7 Acute toxicity of Lnp was evaluated, according to the Organization for Economic Co-operation and Development (OECD) guidelines.

Groups of three animals were used for each step. It was selected at 300 and 2000 mg/kg t as starting doses. These doses are above doses that produced antidepressants effects (1 to 200 mg/kg), which did not elicit apparent side effects or toxicity. Animals were observed individually after dosing, and along the first 30 minutes, at least once within the first four h, periodically during the early 24 hours, and daily for a total of 14 days, recording apparent signs of toxicity, such as lethargy, tremors, convulsions, changes in skin or mucous membranes or locomotor behaviour.

Results: there was no mortality or morbidity observed in mice through fourteen days following single oral administration of Lnp. Animals did not show any changes in general appearance during this period. Morphological characteristics such as skin colour, fur, or eyes appeared normal; tremors, convulsions, rash, diarrhoea, or apparent pain signs were not observed. According to OECD's globally harmonized system, this classifies it at the fifth level, which shows that the extract has relatively low toxicity (OECD's procedure 423*).

Additionally, during the 28-day repeat administration in animals subjected to chronic unpredictable mild stress (CUMS) test, we did not observe apparent toxicity signs at any of the extract doses tested (1 to 200 mg/kg/day) in the CUMS.

*OECD (2002), *Test No. 423: Acute Oral toxicity - Acute Toxic Class Method*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264071001-en.

1.8 Statistical analysis

Data were analysed with Kruskal-Wallis's ANOVA test, followed by the Mann-Whitney U test for paired comparisons when the nonparametric ANOVA showed a statistical significance ($p<0.05$).

Corticosterone levels were analysed by ANOVA followed by Bonferroni's test for multiple comparisons *vs.* control group. CUMS protocol differences, body weight changes, days, and treatments were analysed using two-way repeated-measures ANOVA (one-factor repetition; treatment and body weight change were the variation sources), followed by the Holm-Sidak test. Significant differences were considered at $p\leq0.05$. All statistical analyses and plots were performed with SigmaPlot software version 12.3 and GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

Table S1. Effect of acute treatment with *L. nepetifolia* (Lnp) and imipramine (IMI) on ambulatory behaviour in the Open Field Test.

Treatment (mg/kg)	Count number	Rearings number
CTL	48.7± 4.4	33.0±3.4
IMI 25	44.8±1.1	34.6±.4.7
FLX1	46±3.2.63	27.3±3.35
FLX 10	43.7±2.2	25.62±3.1
FLX 15	49.0±1.8	28.3±0.7
Lnp 1	51.5±3.2	36.1±2.5
Lnp 10	50.70±3.2	29.1±5.1
Lnp 100	46.0±3.2	25.85±1.2
Lnp 200	46.3±3.5	30.71±3.9
	H= 8.46, fd=8, p=0.38	H=10.05, fd=8, p=0.26

Effect of control (CTL; saline solution), imipramine (IMI; 25 mg/kg), fluoxetine (FLX; 10 and 15 mg/kg), and the methanol extract of *Leonotis nepetifolia* (Lnp) on the ambulatory activity. Data represents the mean ± standard error of the mean (SEM). The differences between treatments were analysed using the nonparametric analysis of variance based on the rank of Kruskal-Willi's treatments versus the control group (CTL).

Table S2. Serum corticosterone levels in serum of mice exposure to CUMS

Treatment (mg/kg/day)	Corticosterone (pg/mL)	Bonferroni t-test *vs* CTRL
CTRL-CUMS	52.94±6.96	
CTRL non-CUMS	15.83±2.91***	t=4.91, p≤ 0.001
FLX 1	24.00±4.58**	t=3.83, p<0.002
Lnp10	29.80±6.17*	t=3.06, p=0.023
Lnp100	22.60±4.36**	t=4.02, p=0.002
Lnp 200	27.89±5.96*	t=3.31, p=0.012
	$F_{(5,35)}$=5.71, p<0.001	

R^2= 0.98754

Effect of single-dose daily for 28 days of fluoxetine (FLX) and the methanol extract of *Leonotis nepetifolia* (Lnp) on serum corticosterone levels of animals subjected to chronic unpredictable mild stress (CUMS) Differences between treatments were made using one-way variance analysis, followed by Bonferroni's t-test. Significantly different from vehicle-treated group (CTL), *, p<0.05, **, p<001, ***, p ≤ 0.001.

Table S3. Effects of the methanol extract of *Leonotis nepetifolia* (Lnp), clorgyline, and deprenyl on monoamine oxidases A and B activity in mitochondrial preparations

Substrate (4 mM)	Inhibitor (mg/mL)	MAO-A (IU/mg protein)	MAO-B (IU/mg protein)
5HT		22.06±0.01507	
5HT	Lnp 0.1	4.53±0.065***	
	Lnp 1.0	12.43±0.092**	
5HT	clorgyline	2.53±0.087***	
PEA		----	17.69±7.02
PEA	Lnp 0.1	----	27.9±0.975
	Lnp1.0	----	22.3±2.34
PEA	deprenyl	----	12.4±1.24

The effects of the MAOs activity assay showed that the methanol extract and clorgyline inhibited the MAO-A activity compared with clorgyline and deprenyl used as the positive controls. In contrast, the extract did not affect the MAO-B activity. Results depicted the mean ± standard error of the mean of three experiments. Significantly different when compared with control groups **p<001, ***, $p \leq 0.001$.

Table S4 Main constituents of the *Leonotis nepetifolia* methanol extract.

RT [min]	Name	$[M+H]^+_{obs}$	$[M+H]^+_{calc.}$	Formula	Error (ppm)	mSigma	%RA
0.8	N, N-Dimethyllevulinamide	144.0991	144.1019	$C_7H_{13}NO_2$	-21.5	8.7	15.6
0.9	N,2,3,4,5-pentahydroxypentana-mide	182.0551	182.0659	$C_5H_{11}NO_6$	6.5	21.3	18.6
	[2-(3-Acetyloxy-4-methoxyphenyl)-7-hydroxy-4-oxochromen-5-yl] acetate	385.0903	385.0917	$C_{20}H_{16}O_8$	4.0	42.3	1.0
4.7	Methyl 8-hydroxyoctanoate	175.1463	175.1328	$C_9H_{18}O_3$	-10.3	13.2	0.8
5.2	Methyl tri-O-methyl gallate	227.0900	227.0914	$C_{11}H_{14}O_5$	-6.2	21.5	8.4
5.9	Quercetin 3-rhamnoside	449.1099	449.1078	$C_{21}H_{20}O_{11}$	3.8	35.9	6.8
6.0	7-*O*-*β*- glucoside luteoline	449.1097	449.1078	$C_{21}H_{20}O_{11}$	10.3	25.7	6.2
	Crotinsularin	333.2052	333.2060	$C_{20}H_{28}O_4$	0.4	25.8	10.2
6.9	nepetaefuranol	423.2028	423.2013	$C_{22}H_{30}O_8$	3.8	26.9	1.8
7.6	methoxynepetaefolin	437.1808	437.2169	$C_{23}H_{32}O_8$	-25.8	9.1	0.4
8.7	Methoxynepetaefolin isiomer	437.2185	437.2169	$C_{23}H_{32}O_8$	-13.4	36.9	1.4

9.2	Nepetaefolin	405.1873	405.1907	$C_{22}H_{28}O_7$	8.6	39.5	5.4
9.9	Leonurenone C	379.2411	379.2479	$C_{22}H_{34}O_5$	-2.2	7.7	13.2
10.7	Dehidro-leonotin	351.2504	351.2166	$C_{20}H_{30}O_5$	-7.6	17.2	2.4
10.8	Undecyl benzoate	277.2141	277.2162	$C_{18}H_{28}O_2$	7.7	47.2	3.2
11.8	*p*-Coumaric Acid 4-*O*-|A-D-Glucuronide	341.0910	341.0867	$C_{15}H_{16}O_9$	12.2	28.2	3.8

$[M+H]^+_{exact}$: Molecular Weight exact, $[M+H]^+_{obs}$: Molecular Weight observed, Molecular Weight calc.: calculated % RA: % Relative Area. Error [ppm]: Absolute value of the deviation between measured mass and theoretical mass of the selected peak [ppm].

mSigma: Combined value for the standard deviation of the masses and intensities for all peaks, given in [milliSigma].

Figure S1 Effect of the methanol extract of *Leonotis nepetifolia*, imipramine, fluoxetine in the TST, FST, and FST-CUMS.

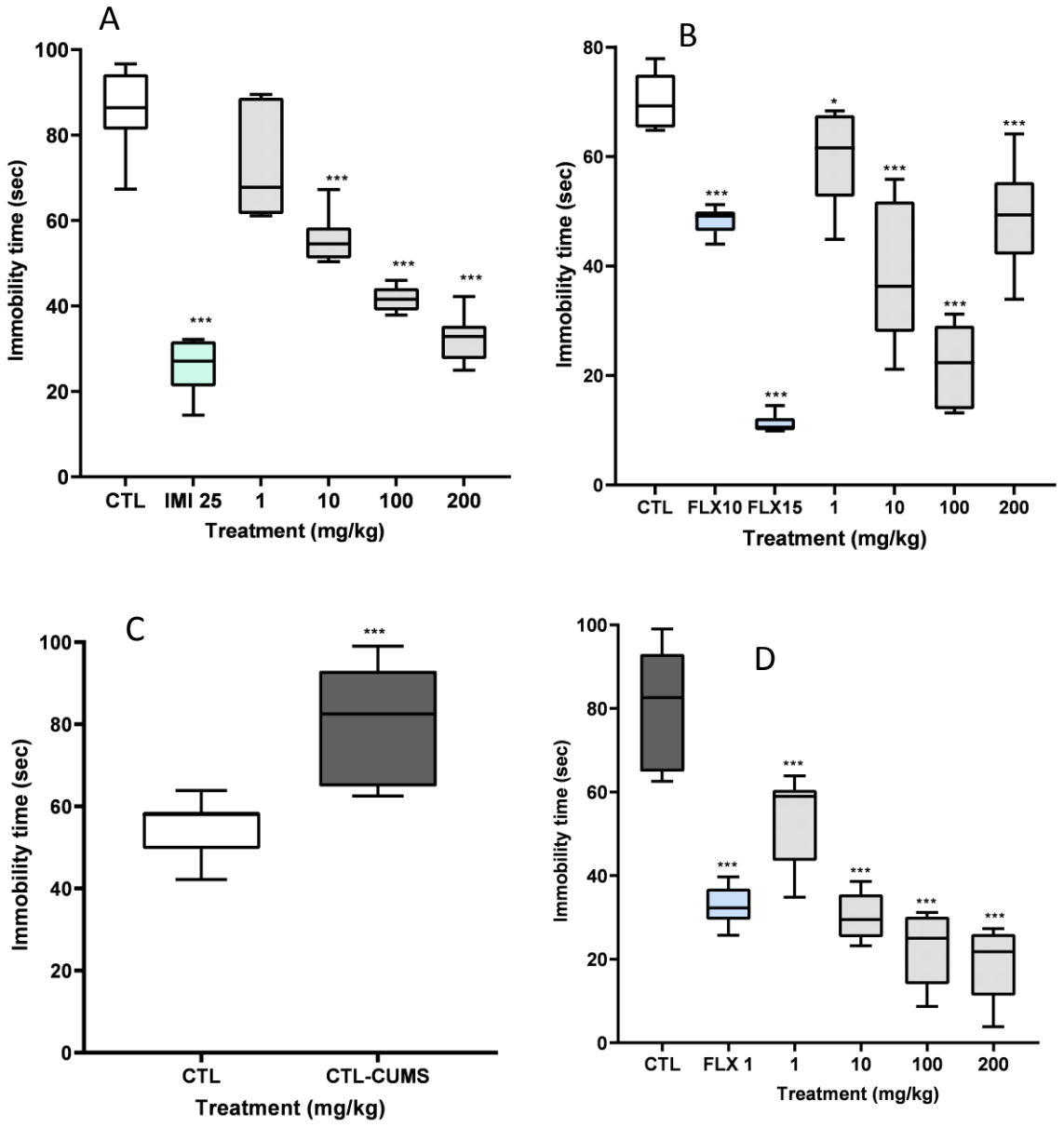


Figure S1A Effect of the methanol extract of *L. nepetifolia* and imipramine (IMI) in the Tail Suspension Test.

Figure **S1B** Effect of triple administration with fluoxetine (FLX; 10 and 15 mg/kg) and the methanol extract of *Leonotis nepetifolia* (Lnp) on the immobility time in mice in the Forced Swimming Test.

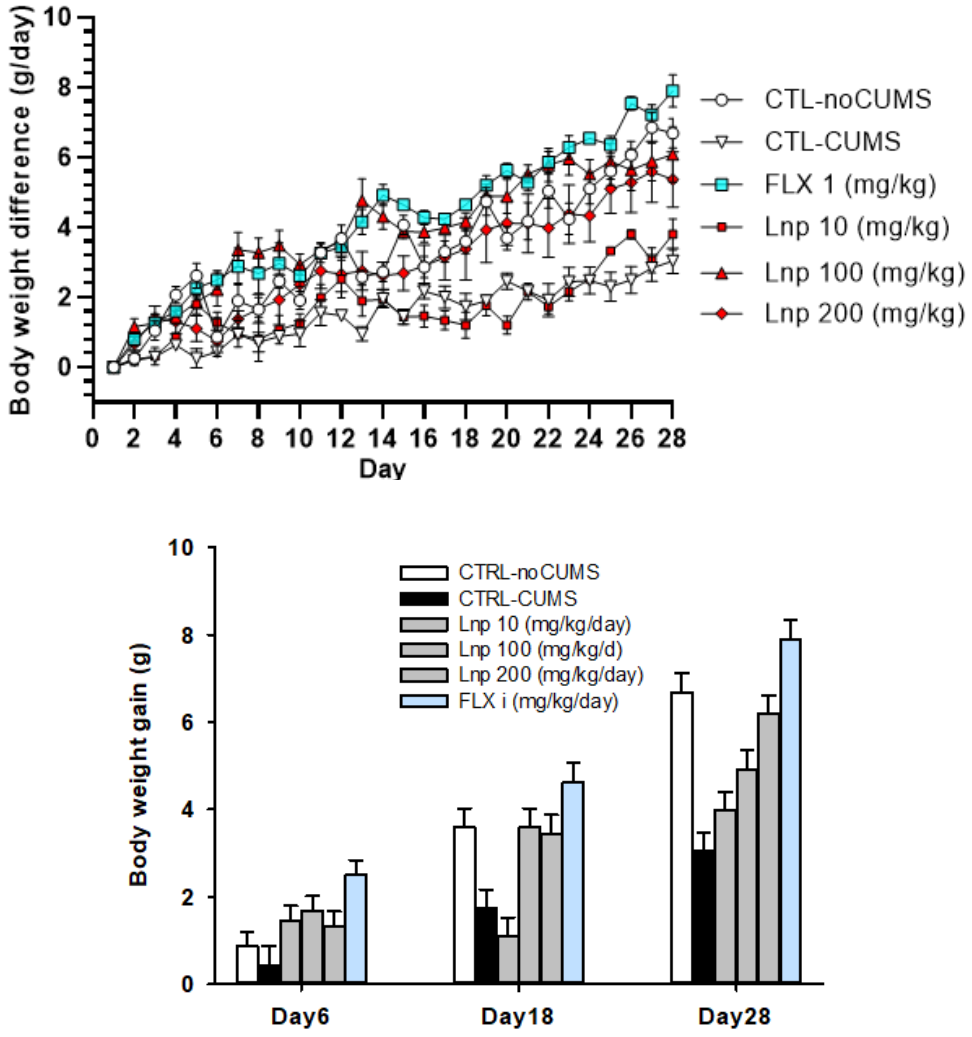
Data represent the mean ± standard error of the mean (SEM). The differences between treatments were analysed using the nonparametric analysis of variance based on rank of Kruskal-Wallis, followed by the Man Whitney paired test. * $p < 0.05$ ***, $p<0.001$ versus the control group (CTL).

Figure S1C Effects of Chronic Unpredictable Mild Stress (CUMS) in animals treated with saline solution, and animals without CUMS.

Figure S1D Effects of the methanol extract of *Leonotis nepetifolia* (Lnp) and fluoxetine (FLX) in Forced Swimming Test (FST) of animals subjected to Chronic Unpredictable Mild Stress (CUMS).

Control group (CTL, saline solution), fluoxetine (FLX; 1 mg/kg/day), and Lnp at 1, 10, 100, and 200 mg/kg/day. Differences between treatments were analysed using the Kruskal-Wallis' analysis of variance based on rank, followed by the Mann-Whitney-U-test. Significantly different from vehicle-treated group (CTL) ***, $p \leq 0.001$.

Figure S2 Chronic Unpredictable Mild Stress on the bodyweight of mice treated with methanol extract of *Leonotis nepetifolia* (Lnp) or fluoxetine (FLX)

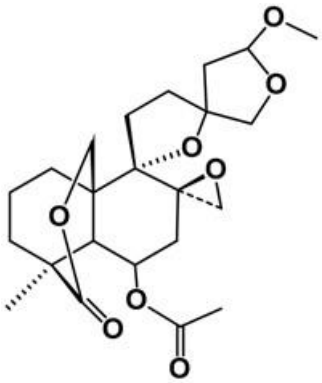


CTL-noCUMS: control group of mice without stress treated with saline solution. CTL-CUMS: control group of mice subjected to chronic unpredictable mild stress treated with saline solution. FLX: fluoxetine, 1mg/kg/day. Lnp: methanol extract of *Leonotis nepetifolia* (mg/kg/day). Data represent the mean ± standard error of the mean. CUMS protocol differences, body weight changes, days, and treatments were analysed using two-way

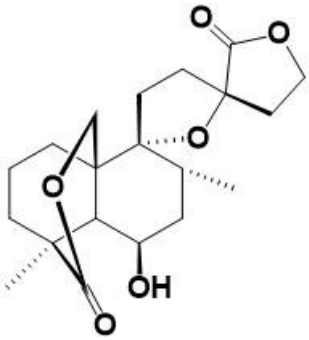
repeated-measures ANOVA (one-factor repetition; treatment and body weight change were the variation sources), followed by the Holm-Sidak test. Significant differences were considered at $p \leq 0.05$. The bar graph shows the days on which significant changes were observed in the body weight.

Figure S3 Representative constituents of methanol extract of *Leonotis nepetifolia*

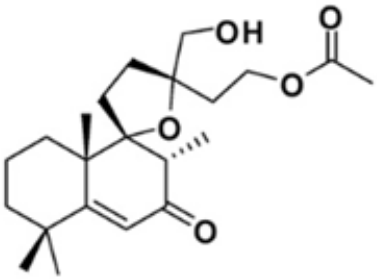
Methoxynepetaefolin [1]



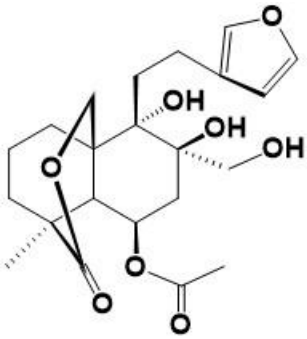
Nepetaefolin [2]



Leonurenone C [3]



Nepetaefuranol [4]



Analysis of the methanol extract of *L. nepetifolia* by positive UPLC-DIESI-MS analysis MALDI-TOF MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry).

Figure S4 The non-linear regression analysis of the dose-response pattern of the treatment with a triple administration of the methanol extract of *L. nepetifolia*.