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Toxicological and Sedative Effects of Chipilin (*Crotalaria longirostrata*) Leaf Extracts Obtained by Maceration and Supercritical Fluid Extraction

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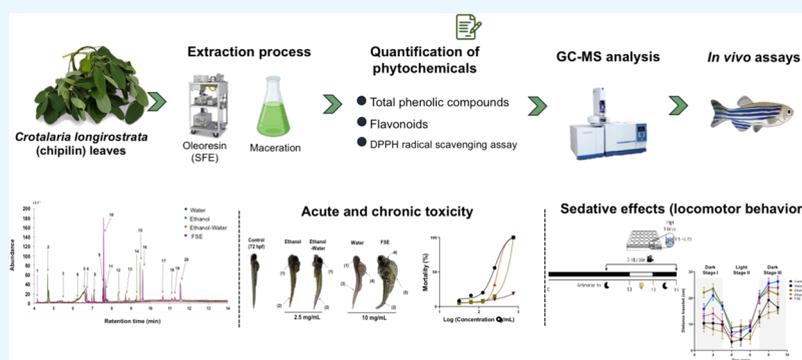
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ABSTRACT: Chipilin (*Crotalaria longirostrata*) is consumed as a vegetable in the preparation of traditional dishes. As a folk medicine, Chipilin extracts are used as a hypnotic and sedative agent; however, there are few reports that support these uses. This study aimed to characterize the compounds present in Chipilin leaf extracts and to investigate their sedative effect using zebrafish as an *in vivo* model. Extracts were obtained by maceration with water (H_2O), ethanol (EtOH), and EtOH-H_2O, while oleoresin was obtained by supercritical fluid extraction (SFE). Total phenolic and flavonoid contents were quantified by colorimetric methods. Phytochemical constituents were identified by gas chromatography−mass spectrometry (GC-MS) analysis. The chronic and acute toxicities of Chipilin extracts were tested in zebrafish embryos and larvae, respectively. Chipilin sedative effect was tested by the larvae response to dark−light−dark transitions. EtOH-H_2O extracts had the highest value of total phenolics (5345 ± 5.1 μg GAE/g), followed by water and oleoresin (1815 ± 5.1 and 394 ± 5.1 μg GAE/g, respectively). In water extracts were identified the alkaloid trachelanthamidine, 1,2β-epoxy- and the alkyl ketone 7,9-di-*tert*-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, while oleamide, α-monostearin, and erucamide were detected in all samples except in water extracts. Oleoresin extract had the lowest embryotoxicity ($LC_{50} = 4.99$ μg/mL) and the highest sedative effects. SFE is a green alternative to obtain Chipilin extracts rich in erucamide, an endocannabinoid analogue, which plays an important role in the development of the central nervous system and in modulating neurotransmitter release.

1. INTRODUCTION

Crotalaria longirostrata Hook. & Arn. (*Fabaceae*), known as Chipilin, is a leguminous plant native to Central America and Southern Mexico. Chipilin leaves and shoots have been used for thousands of years as vegetables in the preparation of traditional meals. Local empirical knowledge about the medicinal uses of *Crotalaria* sp. has been registered,[1] but despite the fact that *C. longirostrata* is considered one of the 16 most important edible leaf species worldwide, scarce literature is reported to support its use.[2]

As a folk medicine, Chipilin extracts are used to treat several health conditions.[1,3,4] The antimicrobial, antifungal, insecticidal, and anticancer activities of acetonic and methanolic extracts of Chipilin leaves and branches have been reported.[5−8]

As an ethnomedicine, Chipilin is recommended as an evening meal to treat insomnia and anxiety.[1,9−11] However, its pharmacological activity as a sedative agent is not well-known; in addition, little is known about the compounds that are present in Chipilin extracts that could be related to this action. Thus, further research is needed.[1,12] On the other hand, the selection of an efficient extraction method is decisive for
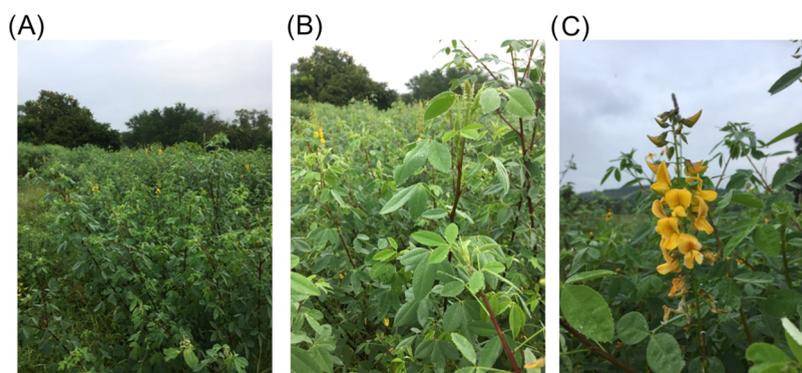
Figure 1. *Crotalaria longirostrata* plants. (A) Plants growing in traditional fields; (B) *C. longirostrata* leaves; (C) *C. longirostrata* flowers.

the identification of novel phytochemicals. Indeed, the identification of new sources of bioactive compounds and their functions has a growing demand in the healthcare, pharmaceutical, food, and beverage industries.[13]

Traditional methods such as maceration, digestion, percolation, decoction, infusion, countercurrent extraction, and continuous hot extraction have been commonly used for the extraction of bioactive compounds. These processes have some disadvantages, such as the use of a large number of solvents, low extraction yields, and high extraction temperatures. Supercritical fluid extraction (SFE) using carbon dioxide (CO_2) as a solvent is considered a new green technology classified as generally recognized as safe (GRAS), which has motivated its application for the extraction of plant bioactive compounds.[14,15]

For all of the above, this study aimed to characterize Chipilin leaf extracts obtained by the traditional maceration process using water, ethanol, and ethanol−water as solvents, as well as the oleoresin obtained by the SFE process. The total phenolic compound and flavonoid contents as well as the antioxidant activity of Chipilin extracts were evaluated by colorimetric methods. Phytochemicals in Chipilin extracts were identified by gas chromatography−mass spectrometry (GC-MS), and their toxicological effects were tested in zebrafish embryos, while their sedative activity was evaluated in larvae using the swimming test in a dark−light−dark transition assay.

2. MATERIALS AND METHODS

2.1. Plant Material. *C. longirostrata* leaves were collected in September 2021 from plants grown in open fields in the municipality of Chiapa de Corzo, Chiapas, Mexico (16°39′16″N, 93°0′36″W), at an altitude of about 400 m and characterized by their humid and warm tropical climate (Figure 1). A voucher specimen of *C. longirostrata* was taxonomically identified and deposited in the Herbarium of the National Institute of Forestry and Agricultural Research (INIFAP-Mexico) under the number INI23000126. The leaves were cleaned and left to dry at 34 °C. Clean and dry leaves were ground in a knife mill to obtain a fine powder, which was passed through a 100 mesh to obtain a uniform particle size.

2.2. Preparation of Extracts. *2.2.1. Maceration Process.* Extracts were prepared by maceration of Chipilin leaf powder in a ratio of 1:20 (w/v) in distilled water (H_2O), ethanol (EtOH), and EtOH-H_2O (1:1, v/v), for 24 h at room temperature under constant agitation. Extracts were centrifuged at 12,000*g* for 1 min, and the supernatants were filtered through 0.22 μm sterile syringe filters (Sigma-Aldrich, St.

Louis, MI). The supernatants were recovered and stored in an amber flask at 4 °C until needed. All extractions were carried out in triplicates.

2.2.2. Supercritical Fluid Extraction (SFE). Chipilin leaf powder (100 g) was subjected to a dynamic extraction using an FSC BioBotanical Extraction System (Waters Co., Milford, MA). The extraction conditions using ethanol as the cosolvent are shown in Supporting Table S1. The obtained oleoresin was stored in amber flasks at 4 °C until needed. Oleoresins were obtained in triplicate.

2.3. Phytochemical Screening. *2.3.1. Total Phenolic Compound Content.* Total phenolic compounds were quantified using the Folin−Ciocalteu method[16] with some modifications. Briefly, the extracts (20 μL) were mixed with 780 μL of Milli-Q water and 50 μL of Folin−Ciocalteu reagent. The mixture was incubated for 10 min in the dark, and then, 150 μL of 20% Na_2CO_3 solution was added, mixed, and incubated for 2 h at room temperature in the dark. The absorbance of the samples was measured at 765 nm by using a microplate reader (Multiskan GO spectrophotometer, Thermo-Fisher Scientific Inc., Waltham, MA). A calibration curve was prepared using gallic acid (GA) at different concentrations (0−500 μg/mL). The total phenolic content was expressed as milligrams of GA equivalents/g of sample (GAE/g). All measurements were carried out in triplicates.

2.3.2. Total Flavonoid Content. Flavonoid quantification was carried out using 200 μL of the extract mixed with 800 μL of 2% $AlCl_3$ ethanolic solution.[17] Samples were then incubated for 10 min in the dark. Absorbance was read at 430 nm using a microplate reader (Multiskan GO, Thermo-Fisher). A calibration curve was prepared using quercetin (Q) as the standard at concentrations from 0 to 100 μg/mL. The results were expressed as Q equivalents/g sample (QE/g).[18] All determinations were carried out in triplicates.

2.3.3. DPPH Radical Scavenging Test. Chipilin leaf extracts were assayed for their antioxidant capacity using the DPPH (2,2-difenil-1-picrylhydrazyl) method.[18] Extracts (100 μL) were mixed with 100 μL of a 400 μM DPPH ethanolic solution. The samples were incubated in the dark for 30 min, and the absorbance was measured at 517 nm. The results were expressed as the percentage of reduced DPPH using the following formula

$$\% \text{ DPPH radical scavenging activity} = \frac{(A_o - A_m)}{A_o} \times 100$$

where A_m is the absorbance of the testing sample and A_o is the DPPH initial absorbance.

2.4. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. Chromatography analyses were carried out using an Agilent 7820A/5977E GC-MS system (Agilent Technologies, Inc., Willington, DE) provided with an autosampler 7863 (Agilent Technologies) and a capillary column HP5-MS (30 m × 0.25 μm i.d., ×0.25 mm film thickness, Agilent Technologies); the injector temperature was settled at 220 °C. The initial oven temperature was 50 °C for 1 min, followed by 30 °C/min until 280 °C for 10 min, and then at 15 °C/min until reaching 300 °C for 4 min. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The sample (5 μL) was injected (20:1 split ratio), the ionization energy was 70 eV, and the mass range evaluated ranged from 45 to 550 m/z. The GC-MS system was calibrated automatically by the system verification Tune (detector optimization) using the Perfluoro-compound FC-43 (Thermo-Fisher) as the standard. Compounds were identified by comparing their retention times and mass spectra against the National Institute of Standards and Technology Mass spectra (NIST MS v2.2.).

2.5. Evaluation of Toxic and Sedative Effects.
2.5.1. Zebrafish Maintenance. Adult zebrafish of the AB-Tu/-Wik hybrid line (>6 months of age) were maintained in tanks with a recirculating water system under standard rearing conditions (pH 7.5, conductivity 500–750 μS, 28 °C, and 14/10 h light/dark cycle). Zebrafish were fed daily with a brine shrimp culture (Brine Shrimp Direct, pellets, Zeigler Bros, Inc.). The nitrate, nitrite, and ammonia contents were monitored every week to maintain the water quality. Egg production was carried out by natural spawning in a breeding tank at a 1:2 (male/female) ratio. The experimental protocol was approved by the Animal Care and Use Committee of the Institute of Neurobiology of the National Autonomous University of Mexico (protocol number 95A).

Chipilin extracts at different concentrations were prepared daily in an E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$, 0.33 mM MgSO$_4$, and 1% DMSO). The control group for each treatment was maintained in the E3 buffer. For the chronic toxicity test, the extract concentrations were 31.25, 62.5, 125, 250, and 750 μg/mL. For the acute toxicity test, extract concentrations tested were 0.5, 1, 2.5, 5, 7, 10, and 50 mg/mL.

2.5.2. Zebrafish Embryo Chronic Toxicity Test (FET). The toxic effect of the Chipilin extracts was evaluated as a chronic fish embryotoxicity (FET) test under the Guidelines for the Testing of Chemicals No. 236 of the Organization for Economic Co-operation and Development.[19] The embryotoxicity test evaluates lethality, sublethality, and teratogenic parameters within the first 120 h post fertilization (hpf).[20]

Fertilized embryos ($n = 20$) at 2 hpf were transferred to Petri dishes (60 mm × 15 mm) and incubated with different extract concentrations (31.25, 62.5, 125, 250, and 750 μg/mL). Embryos were exposed to the extract solutions (8 mL) since 2 hpf and continued until 5 days post fertilization (dpf), with daily replacement of the solutions (Supporting Figure S1). The plates were incubated at 28 °C with a 14/10 h light/dark cycle, and the evaluation criteria for embryos and larvae from 24 to 120 hpf were carried out.[21] Egg coagulation, somite formation, and tail detachment were monitored from 24 hpf; edema formation, pigmentation, and heart rate from 48 hpf; and embryo hatching and scoliosis from 72 hpf. The mean lethal concentrations (LC$_{50}$) were obtained from the percentage of accumulated mortality at different hours of

evaluation, compared to the logarithm of the concentrations. All experiments were carried out in triplicate.

2.5.3. Zebrafish Larvae Acute Toxicity Test. To assess the acute toxicity of the Chipilin extracts, larvae ($n = 10$) of 72 hpf were individually transferred to a 96-well plate and incubated with a single dose of 200 μL of different working solutions (0.5, 1, 2.5, 5, 7, 10, and 50 mg/mL). Changes in morphology, blood circulation, and heartbeats were monitored at 0, 1, 3, 5, 7, 12, and 24 h. The plates were incubated at 28 °C with a 14/10 h light/dark cycle. The experiments were carried out in triplicate.

2.5.4. Sedative Activity of Chipilin Extracts. The response of the larvae to dark–light–dark transitions was evaluated according to a reported protocol.[22] Larvae at 7 days post fertilization ($n = 16$) that were exposed to working solutions with daily replacement from 2 hpf were individually transferred to a 24-well plate with 1.5 mL of E3 buffer. The plate was set above a light chamber and covered with black conical plastic that held a video camera on top. The larvae were habituated to darkness for 10 min, and recording began during the last 3 min of this period, after which the light was turned on and recorded for a further 3 min. Finally, the light was turned off, and the recording continued for another 3 min.

Velocity and swimming trajectories were analyzed using EthoVisionXT software (Noldus IT, Wageningen, The Netherlands). The total locomotion of the individual larvae was summarized by three response variables: the distance traveled, speed, and latency at the moment of starting the movement. Experiments were carried out in triplicate in a fish room at 28 °C.

2.6. Statistical Analysis. Statistical analyses were carried out using GraphPad Prism 8 software (La Joya, CA). The comparison between groups was performed using one-way ANOVA followed by Tukey's test with $p < 0.05$ for statistically significant differences.

3. RESULTS AND DISCUSSION

3.1. Phytochemical Screening and Antioxidant Activity. The highest total phenolic compound contents (5345 ± 5.1 μg GAE/g) were observed in ethanol–water extracts (EtOH-H$_2$O), followed by water extract (1815 ± 5.1 μg GAE/g), oleoresin (394 ± 5.1 μg GAE/g), and EtOH extracts (153 μg ± 2.7 μg GAE/g) (Table 1). The Chipilin water extract showed a similar total phenolic content to the values reported in the literature.[4] In relation to flavonoid content, the EtOH extract showed the highest content (4841

Table 1. Total Phenolic, Flavonoids, and Antioxidant Capacities of Different Extracts Obtained from *C. longirostrata* (Chipilin) Leaves[a]

extract	total phenolic[b]	flavonoids[c]	antioxidant[d] capacity
water	1815 ± 4.7[b]	234.5 ± 4.3[d]	21.3 ± 1.1[c]
ethanol	153.0 ± 2.7[d]	4841.3 ± 3.5[a]	48.3 ± 1.4[b]
ethanol–water	5345 ± 5.1[a]	1630 ± 57[c]	64.3 ± 2.0[a]
SFE	393.9 ± 5.1[c]	1870 ± 5.1[b]	24.3 ± 2.1[c]

[a]Data shows the mean ± standard deviation ($n = 3$). [b]Reported as μg gallic acid equivalents/g of sample on dry matter. [c]Reported as μg of quercetin equivalents/g of sample on dry matter. [d]Reported as % of DPPH radical scavenging activity. Different superscript letters indicate significant differences among means at $p \leq 0.05$. SFE, supercritical fluid extraction.

Table 2. Identification of Volatile and Semi-volatile Metabolites by GC-MS from *C. longirostrata* (Chipilin) Extracts[a]

Peak	CAS #	RT	Name*	Relative abundance (%)				Chemical structure	Common name
				Water (H₂O)	EtOH	EtOH-H₂O	SFE		
Organic heterocyclic compounds									
1	496-16-2	4.17	Benzofuran, 2,3-dihydro	ND	ND	**0.48**	ND		Coumaran
2	15211-03-7	4.69	Trachelanthamidine, 1,2β-epoxy-	1.58	**2.02**	1.87	ND		Pyrrolizidine alkaloid
4	73410-02-3	6.49	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one	ND	ND	ND	**0.06**		Benzofuran
5	97-30-3	6.58	α-D-Glucopyranoside, methyl	1.92	0.19	1.4	ND		Derived from the reaction with methanol
7	82304-66-3	6.99	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	**0.72**	ND	0.01	ND		Diketone (alkyl-ketone)
Terpenes									
6	504-96-1	6.68	1-Hexadecene, 7,11,15-trimethyl-3-methylene-	ND	0.32	ND	0.73		Neophytadiene (Sesquiterpenoid)
10	150-86-7	7.58	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]]	ND	3.1	1.17	5.61		Phytol (Acyclic diterpenoid)
16	111-02-4	9.60	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)	ND	0.07	ND	2.82		Squalene (Triterpenoid)
Lipids									
9	112-63-0	7.53	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	ND	ND	ND	**0.37**		Linoleic acid, methyl ester
8	57103	7.08	n-Hexadecanoic acid	0.02	0.75	0.25	0.41		Palmitic acid
11	301-00-8	7.67	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	ND	1.56	0.91	0.74		Linolenic acid, methyl ester
12	301-02-0	8.33	9-Octadecenamide, (Z)-	ND	0.81	ND	0.06		Oleic acid amide; Oleamide
13	23470-00-0	8.71	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	ND	0.4	0.26	0.06		2-Monopalmitin
14	123-94-4	9.28	Octadecanoic acid, 2,3-dihydroxypropyl ester	ND	0.3	0.05	0.11		α-Monostearin
15	112845	9.468	13-Docosenamide, (Z)-	ND	2.56	0.02	0.22		Erucic acid amide, erucamide
Phytosterols									
17	59-02-9	10.64	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,5,7,8-tetramethyl-2(4,8,12-trimethyltridecyl), [2R-[2R*(4R*,8R*)]]	ND	0.19	ND	0.57		α-tocopherol (Vitamin E)
18	474-62-4	11.11	Ergost-5-en-3-ol, (3β,24R)-	ND	**0.02**	ND	**0.33**		Campesterol (Ergoesterol)
19	83-48-7	11.26	Stigmasta-5,22-dien-3-ol, (3β,22E)-	ND	**0.07**	ND	**0.44**		Stigmasterol (Phytosterol)
20	83-47-6	11.55	Stigmasta-5-en-3-ol, (3β,24S)-	ND	**0.78**	ND	**2.98**		β-Sitosterol (Stigmastane)

[a]RT, retention time (min); SFE, supercritical fluid extraction; ND, not detected. *, name according to report at NIST Chemistry WebBook.
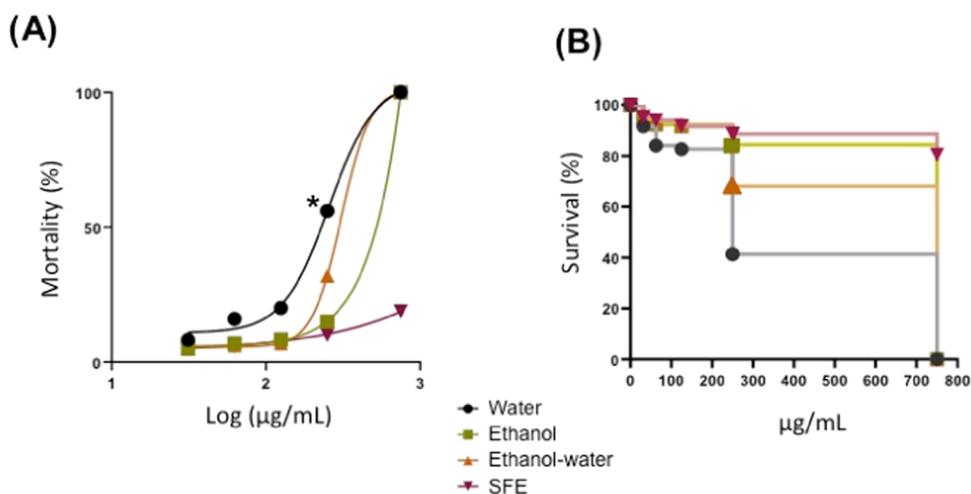
Figure 2. (A) Accumulated mortality of zebrafish embryos at 120 h post fertilization (hpf) in the presence of Chipilin extracts. Concentrations of Chipilin extracts were 31.25, 62.5, 125, 250, and 750 μg/mL. Each point represents the medium ($n = 20$), and asterisk means a mean with significant difference at $p < 0.05$. (B) Accumulated toxicity on zebrafish embryos of Chipilin extracts at 120 hpf. Concentration of Chipilin tested ranged from 0 to 800 μg/mL. SFE, supercritical fluid extraction.

$\pm$ 3.5 μg QE/g) followed by oleoresin and EtOH-H$_2$O extract (1870 $\pm$ 5.1 and 1630 $\pm$ 5.7 μg QE/g, respectively). The lowest flavonoid content was obtained with water (234 $\pm$ 4.3 μg QE/g) (Table 1). Supercritical carbon dioxide is a nonpolar solvent where low-polarity compounds are easily dissolved (isoflavones, flavanones), but the addition of a cosolvent, such as ethanol, can enhance the extraction of polar compounds.[14]

The EtOH-H$_2$O extract showed the highest antioxidant activity with 64.3% inhibition of DPPH, followed by the EtOH extract, oleoresin, and water extract (48.3, 24.3, and 21.3%, respectively) (Table 1). The antioxidant activity was in relation to the amounts of total phenolic compounds and flavonoid content.

3.2. GC-MS Analysis. Chipilin extracts were analyzed by GC-MS, and representative chromatograms are shown in Supporting Figure S2. The relative abundance of the extracted compounds among processes varies qualitatively and quantitatively. Four compounds were identified in the water extract (peaks 2, 7, 5, and 8), 15 in the EtOH extract (peaks 2, 5, 6, 8 10−20), 10 in the EtOH-H$_2$O extract (peaks 1, 2, 5, 7, 8, 10, 11, and 13−15), and 16 compounds in oleoresin (peaks 3, 4, 6, 8−20).

Among heterocyclic compounds, benzofuran, 2,3-dihydrobenzofuran (peak 1) was detected only in the EtOH-water extract (Table 2). Benzofurans and their derivatives can be used to design and develop novel therapeutics.[23] Trachelanthamidine, 1,2β-epoxy- (peak 2) was identified in all samples except in oleoresin, and 7,9-di-*tert*-butyl-1-oxaspiro-(4,5)deca-6,9-diene-2,8-dione (peak 7) was identified in water and EtOH-H$_2$O extracts (Table 2). Trachelanthamidine is a pyrrolizidine alkaloid (PA) that has been detected in *Boraginaceae*, *Fabaceae*, *Asteraceae*, and *Heliotropium transalpinum*. PAs from plants are toxic compounds that can cause mutations in DNA in herbivores and humans.[24] The identification of one alkaloid belonging to the iminosugar group was reported and related to Chipilin's insecticidal action.[7] The oxaspiro compound, reported as a diketone or akyl-ketone, has been detected by GC-MS in *Brasiliorchis schunkeana* and *Ficus religiosa* seeds.[25,26] Several activities such as steroidal antimineralocorticoid, antiandrogen, and glucocorticoid have been reported for this compound.[27] Oleoresin

obtained by SFE was characterized by the presence of 6-hydroxy-4,4,7α-trimethyl-5,6,7,7α-tetrahydrobenzofuran-2(4H)-one (peak 4), a volatile compound isolated from *Sargassum horneri* with tested anti-inflammatory activity showing antitumor, antibacterial, antioxidative, and antiviral activities.[28]

Neophytadiene (peak 6), a sesquiterpene, was detected only in EtOH and oleoresin extracts, whereas phytol (a diterpene) was detected in all samples, except in the water extracts (Table 2). Neophytadiene is known for its anti-inflammatory properties,[29] and antimicrobial activity against *Salmonella typhimurium* and *Staphylococcus aureus* has been reported.[30] Phytol is an acyclic diterpene alcohol with antioxidant properties, and its anti-inflammatory activity was demonstrated by a reduction in IL-1ß and TNF-α levels. Phytol is also associated with the prevention of metabolic diseases, and its anticonvulsant activity has been reported.[31,32]

The only lipid detected in water extracts was palmitic acid, which was also detected in all samples (Table 2). Linoleic acid methyl ester (9,12-octadecadienoic acid (Z,Z)-, methyl ester), a fatty acid methyl ester of linoleic acid, was detected only in oleoresin. This compound acts as a key regulator of gut homeostasis by suppressing NF-κB-mediated pro-inflammatory responses.[33]

The presence of oleamide, α-monostearin (an emulsifier), and erucamide was detected in all samples, except in water extracts (Table 2). It has been reported that oleamide, the sleep hormone, has hypnotic properties, which have been demonstrated in rats by reduction of distance traveled.[34,35] In mice, oleamide significantly reduced sleep latency and wake time while increasing nonrapid eye movement and total sleep time.[35] Erucamide is an endocannabinoid analogue, a compound that plays important roles in central nervous system development and several physiological processes such as modulation of neurotransmitter liberation, regulation of pain perception, and cardiovascular, gastrointestinal, and hepatic functions.[36,37]

Squalene (peak 16), a natural triterpene, is a metabolite intermediate of the sterol biosynthetic pathway that possesses antioxidant properties.[38] Some sterols, α-tocopherol (vitamin E), campesterol, stigmasterol, and γ-sitosterol, were detected
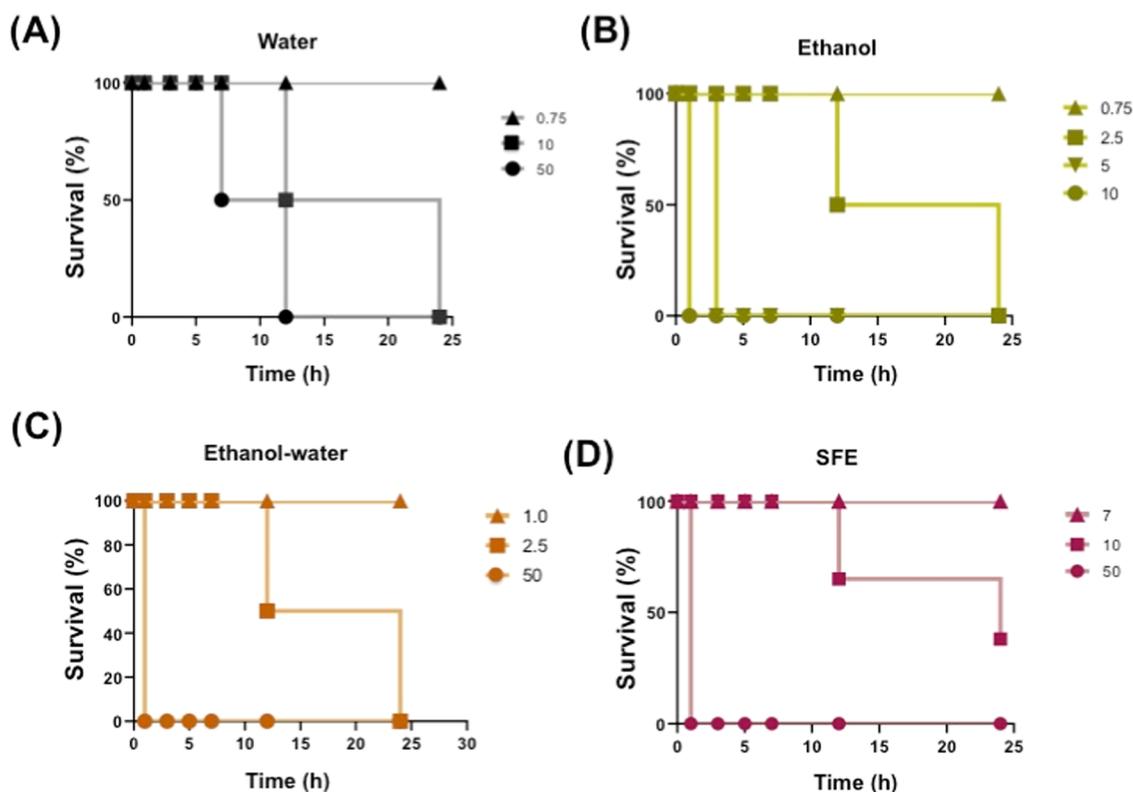
Figure 3. Survival of zebrafish larvae at 72 hpf (hours post fertilization) in the presence of different Chipilin extracts extracted with different solvents: (A) water (0.75, 10, and 50 mg/mL); (B) ethanol (0.75, 2.5, 5, and 10 mg/mL); (C) ethanol−water (10, 2.5, and 50 mg/mL); and (D) SFE (supercritical fluid extraction) at 7, 10, and 50 mg/mL.

only in EtOH and oleoresins; however, higher abundances of these compounds were observed in oleoresins (Table 2).

3.3. Evaluation of Chronic and Acute Toxicity in Zebrafish Embryos. Zebrafish (*Danio rerio*) provides multiple advantages as a model organism[39,40] because it easily absorbs small molecules; its use has increased as a model to evaluate toxicological studies and behavioral analysis such as stress, fear, and anxiety.[40] In addition, zebrafish shares similarities with the structure and function of the human nervous system and provides a good platform for the efficient detection of psychoactive and sleep-modulating molecules.[41−44] Chipilin extracts and oleoresin were tested in fish embryos, and their toxicity was evaluated by continuous exposure to different extract concentrations from 2 hpf to 5 dpf, with daily replacement of the solutions and constant monitoring. Toxicity at concentrations of 31.25, 62.5, and 125 μg/mL was not significantly different from that of the control (E3 buffer). However, abnormalities in zebrafish development were observed at concentrations of 250 μg/mL and above (Supporting Table S2). The highest toxicity of Chipilin extracts, measured by egg coagulation or mortality, was observed in the water extract (56%), followed by EtOH-water (32%) and EtOH (10%), whereas oleoresin showed no significant toxicity compared to the control (Supporting Table S2). The water and EtOH extracts induced some malformations in embryonic development after 48 hpf such as observed by delayed tail detachment, edema formation, decreased pigmentation, and decreased heart frequency. The water extract causes scoliosis (skeletal deformities), and the EtOH-water extract delayed the hatching of the embryo (Supporting Table S3).

According to the dose−response curves for mortality of zebrafish embryos accumulated up to 120 hpf, the water extract showed the highest degree of toxicity with an LC_{50} of 2.41 μg/mL, followed by the EtOH-water extract with an LC_{50} of 2.49 μg/mL, and the EtOH extract with an LC_{50} of 3.41 μg/mL, and oleoresin presented the lowest degree of toxicity with an LC_{50} of 4.99 μg/mL (Figure 2A). At a maximum concentration evaluated of 750 μg/mL, most of the extracts caused 100% mortality within the first 72 hpf, highlighting again that oleoresins had the lowest mortality, 18.8% (Figure 2B).

The acute toxicity or exposure to a single dose of Chipilin extracts, at different concentrations (0.5−50 mg/mL), was evaluated in 72 hpf larvae for 24 h. Results showed that the maximum concentration tolerated by larvae of water and EtOH extracts indicated by a 100% survival was 0.75 mg/mL. A mortality of 100% was observed with water extract (at 50 mg/mL) within the first 12 h of exposure, while 10 mg/mL EtOH extract was sufficient to obtain 100% mortality in 2 h (Figure 3A,B). For the EtOH-water extract, the maximum concentration tolerated by larvae was 1 mg/mL, and 100% mortality was achieved with 50 mg/mL after 2 h of exposure (Figure 3C). The highest concentration of oleoresin tolerated by larvae with 100% survival was 7 mg/mL, which was 10 times higher than that of the other extracts; however, 100% mortality was also obtained at 50 mg/mL within 2 h (Figure 3D). Some of the alterations observed in the larvae when exposed at concentrations of 2.5 mg/mL for EtOH and EtOH-water extracts and 10 mg/mL for water and oleoresin are shown in Supporting Figure S3.

The toxicity of the extracts was dose-dependent, and the water extract showed higher toxic effects at lower concen-

trations. This could be attributed to the synergistic effect of metabolites present in this sample, where the alkaloid trachelanthamidine was the most abundant, and this has been attributed to carcinogenic and hepatotoxic effects;[24] however, it is also considered a harmless metabolite.[45]

3.4. Evaluation of Sedative Effects in Zebrafish Larvae.

Zebrafish is an important model for psychoactive substance research because its brain structure, neurochemistry, and sleep/wake pattern are similar to those of humans.[42] Then, behavioral tests with zebrafish larvae exposed to light–dark transitions were proposed to assess the effect of psychoactive molecules. During the light–dark transition, the locomotor activity increases and is attributed to an increased stress/anxiety level, while the dark–light transition decreases locomotion.[46] To evaluate the sedative properties of Chipilin, the larvae locomotor behavior was evaluated in dark–light–dark transitions in the presence of Chipilin extracts (125 μg/mL) from 2 hpf to 7 dpf (Figure 4A). Zebrafish larvae respond
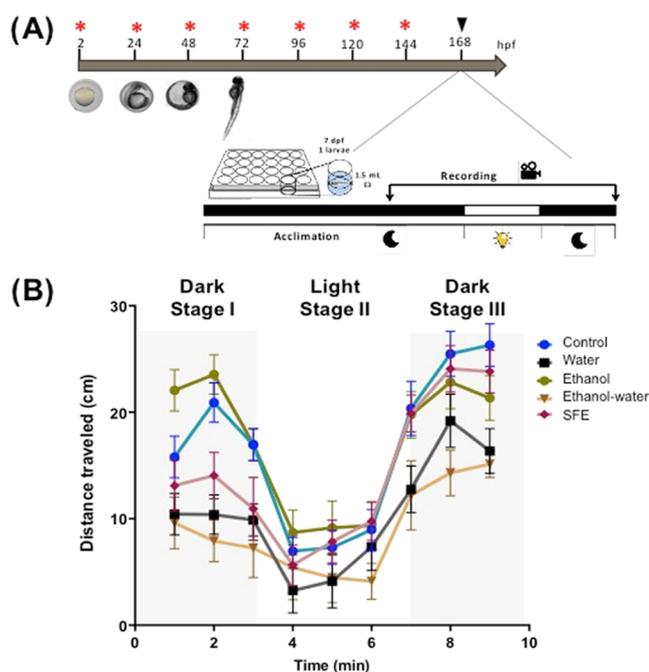

Figure 4. (A) Design for the assay for *D. rerio* larvae locomotion. In each well were added 1.5 mL of E3 buffer and one larva of 7 dpf (days post fertilization). Red asterisks indicate the time of addition of Chipilin extracts. Black triangle indicates the time for recording the fish movement. (B) Distance traveled by zebrafish embryos over time on the dark–light–dark transitions. SFE, supercritical fluid extract.

to changes in lighting, and their motor activity decreases in the presence of light and increases during periods of dark. Behavioral parameters that allow the assessment of fish motor activity include swimming speed, distance traveled, angular velocity, thigmotaxis preference, and feeding performance. Variations in these criteria are related to external stress, anxiety level, and neurotoxicity.[47]

Under control conditions, zebrafish showed a normal pattern of behavior with a higher distance traveled during the dark (stages I and III) and lesser movement during illumination (stage II). When Chipilin extracts were added, all treatments induced a decrease in movement in stage I, except for the EtOH extract, which seemed to accelerate the zebrafish movement. In stage II, when light was turned on (phase III),

zebrafish treated with oleoresin showed no significant differences from the control, EtOH showed a significant decrease in movement compared to stage I, but zebrafish treated with water and EtOH-water extracts showed a significant decrease in movement (Figure 4B).

In addition to the distance traveled, the swimming speed of the larvae was also analyzed (Figure 5). In stage I, it was observed that the swimming speed decreased significantly in the presence of water and EtOH-water extracts as well as oleoresin, but no effect on the speed of swimming was observed when the EtOH extract was administered. This behavior was similar in stage II, but in stage III, larvae treated with water and EtOH-water extracts showed a significant decrease in swimming speed compared to the control, and the EtOH extract showed a tendency to increase the speed of swimming (Figure 5A). In the case of oleoresin, the swimming speed during stage III was similar to that of the control, which could indicate recovery from sedation. In contrast, for the water and EtOH-water extracts, the sedative effect persisted despite the deprivation of the light stimulus. The movement pattern of the larvae (Figure 5B) also showed that the control and oleoresin have a similar pattern of swimming, and zebrafish subjected to water extract induces a lower exploration.

The present results showed that the Chipilin oleoresin has a sedative effect without causing toxic effects since after exposure to the light stimulus (stage III) its behavior was similar to that of the control. The total sedative or toxic phenotype of the aqueous and EtOH-water extracts remained throughout the entire experiment, where there was a decrease in motor activity and swimming speed. The anticonvulsant activities of numerous phyto-constituents identified in plant extracts have been described, such as phytol, oleamide, erucamide, phenolic acids, and *p*-coumaric acid,[48] which selectively bind to the GABAA receptor and are likely involved in the sedative effect of the extracts. Zebrafish have many conserved neurotransmitter pathways compared with humans, where GABA receptors are the most abundant and, therefore, the most studied against bioactive compounds. However, we are not unaware that other neuroreceptors may be involved in the total sedative effect observed.[49,50] These metabolites can act as anticonvulsants because of their high lipophilicity, which facilitates their penetration through the blood–brain barrier.[51] However, despite the fact that our results coincide with the ethnopharmacological use of this medicinal plant in the popular tradition, studies must be carried out to elucidate the mechanisms of action of this property. In addition, it is necessary to establish whether the synergy between the phytochemicals could be, in part, responsible for the sedative effect or if it is attributed to a particular compound.

4. CONCLUSIONS

Chipilin leaves have been used as a vegetable to prepare traditional dishes, and in folk medicine, Chipilin leaf extracts are used as a hypnotic and sedative agent; however, there is scarce literature to support these uses. The present results showed that the maceration process with water, EtOH, and EtOH-H$_2$O showed the highest content of total phenolic and flavonoid compounds, and the oleoresin obtained by SFE contained more diversity of compounds identified by GC-MS. SFE also had the lowest toxicity for zebrafish embryos. The locomotion assay of zebrafish larvae exposed to oleoresin and the aqueous and ethanolic extracts showed a sedation phenotype; however, the SFE oleoresin showed a complete
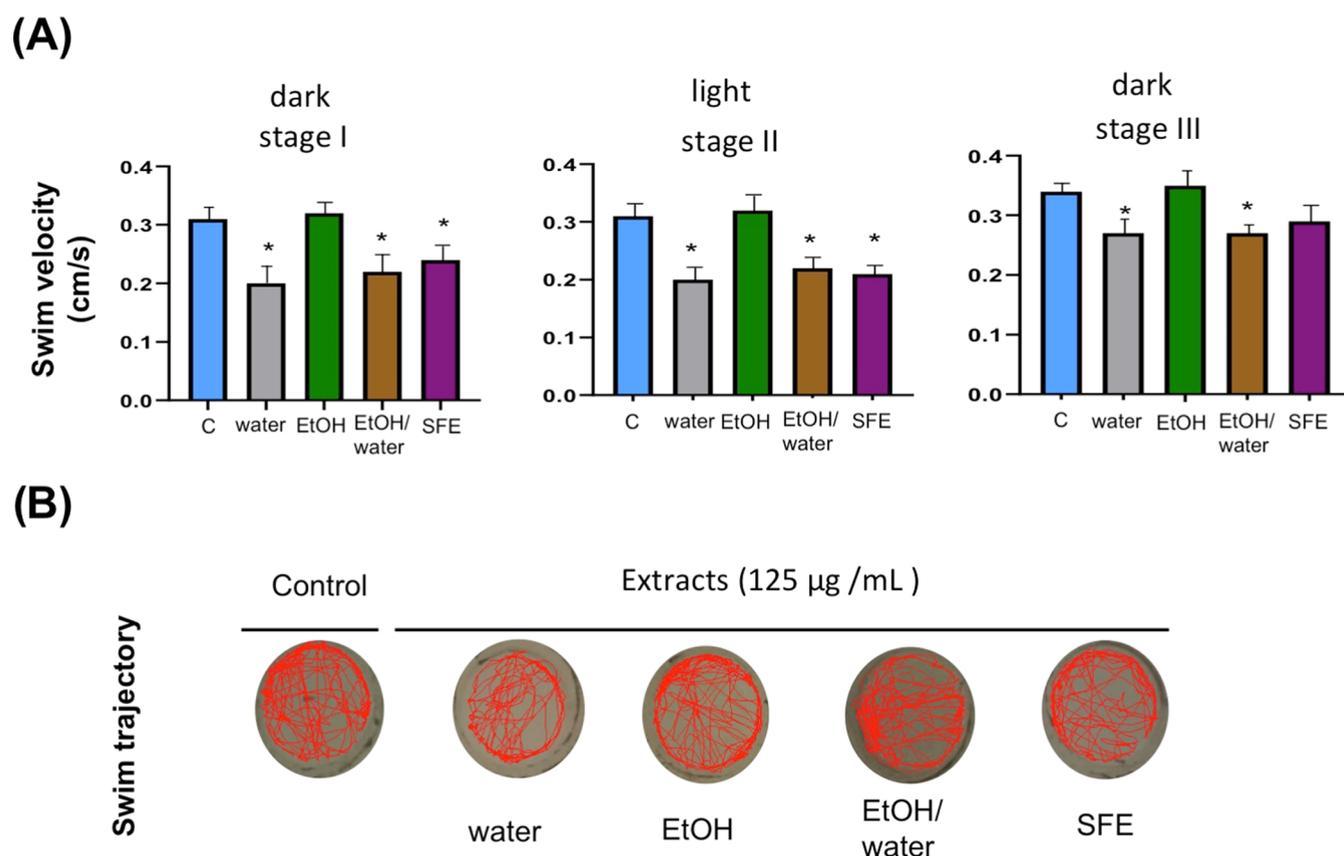
(A)

(B)

Figure 5. (A) Swim velocity and (B) swim trajectory of zebrafish larvae at 7 dpf (days post fertilization) exposed to 125 μg/mL Chipilin extract during the dark−light−dark transitions. C, control; SFE, supercritical fluid extract.

sedation phenotype with high recovery as the control after a dark−light−dark transition test. The present results represent a new perspective on the Chipilin crop as a source for the production of high-value products with novel uses such as a sedative or hypnotic agent.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c08290.

Dynamic conditions for oleoresin extraction from *C. longirostrata* (Chipilin) leaves using CO_2-supercritical fluids (Table S1); lethal and sublethal end points of the chronic embryotoxicity test in zebrafish (*D. rerio*) (Table S2); cumulative embryotoxicity up to 120 hpf of *C. longirostrata* (Chipilin) extracts in zebrafish (*D. rerio*) (Table S3); measurement of *D. rerio* embryotoxicity by chronic and acute administration of Chipilin extracts (Figure S1); representative GC-MS chromatogram profiles of Chipilin leaf extracts obtained with water, ethanol, ethanol−water, and supercritical fluid extraction (Figure S2); and zebrafish morphological alterations at 12 h after larvae exposition with Chipilin extracts (Figure S3) (PDF)

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A.H.-R.: Investigation, methodology, writing. A.D.L.-R.: GC-MS analysis. V.M.R.-V. and J.M.G.-A.: Chipilin leaf recollection and leaf drying, writing—review. J.M.G.-A.: Author of Chipilin photos in Figure 1. L.R.R.-O.: Supervision, resources, and writing—review. A.P.B.d.l.R.: Funding acquisition, con-

ceptualization, resources, supervision, writing—review and editing.

Notes

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