Original article

**Neuropharmacological and neuroprotective activities of some metabolites produced by cell suspension culture of *Waltheria americana* Linn.**

Jorge Mundo¹, Juana Villeda-Hernández¹, Maribel Herrera-Ruíz², María del Carmen Gutiérrez³, Jesús Arellano-García⁴, Ismael León-Rivera⁵,*, Irene Perea-Arango⁶,*

¹Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, Col. Champlía, 62209 Cuernavaca, Morelos, Mexico
²Centro de Investigaciones Químicas, IICBA, Universidad Autónoma del Estado de Morelos, Avenida Universidad 1001, Col. Champlía, 62209 Cuernavaca, Morelos, Mexico
³Centro de Investigación Biomédica del Sur, IMSS, Argentina 1, Col. Centro, Xochitepec, Morelos, Mexico
⁴Instituto Nacional de Neurología y Neurocirugía “Manuel Velasco Suárez”, insurgentes Sur No. 3877, Tlalpan, la Fama, 14269 Ciudad de México, Mexico

**A R T I C L E   I N F O**

Article history:
Received 8 May 2017
Received in revised form 26 June 2017
Accepted 6 July 2017

Keywords:
Waltheria americana
Cell culture
Neuroprotective
GABA

**A B S T R A C T**

*Waltheria americana* is a plant used in Mexican traditional medicine to treat some nervous system disorders. The aims of the present study were to isolate and determine the neuropharmacological and neuroprotective activities of metabolites produced by a cell suspension culture of *Waltheria americana*. Submerged cultivation of *W. americana* cells provided biomass. A methanol-soluble extract (WAsc) was obtained from biomass. WAsc was fractionated yielding the chromatographic fractions 4WAsc-H₂O and WAsc-CH₂Cl₂. For the determination of anticonvulsant activity in vivo, seizures were induced in mice by pentylenetetrazol (PTZ). Neuropharmacological activities (release of gamma amino butyric acid (GABA) and neuroprotection) of chromatographic fractions were determined by in vitro histological analysis of brain sections of mice post mortem. Fraction 4WAsc-H₂O (containing saccharides) did not produce neuronal damage, neurodegeneration, interstitial tissue edema, astrocytic activation, nor cell death. Pretreatment of animals with 4WAsc-H₂O and WAsc-CH₂Cl₂ from *W. americana* cell suspensions induced an increase in: GABA release, seizure latency, survival time, neuroprotection, and a decrease in the degree of severity of tonic/tonic-clonic convulsions, preventing PTZ-induced death of up to 100% of animals of study. Bioactive compounds produced in suspension cell culture of *W. americana* produce neuroprotective and neuropharmacological activities associated with the GABAergic neurotransmission system.

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1. Introduction

Epilepsy is a neurological disorder characterized by a long-term predisposition to recurrent episodes of unexpected seizures [1], due to changes that excite and inhibit several neural circuits, being the motor cortex one of the main areas involved [2]. This neurological disorder is considered to be the second most common sudden seizures after stroke [3], and the third cause of neurological disability [4]. It has been estimated that the number of people all around the world suffering epilepsy is approximately 50 million [5,6], and in Mexico 1–2 million people [7]. A high percentage of this people did not receive adequate medical treatment due to financial problems [8]. Antiepileptic drugs have an efficacy less than 70%, besides side effects as a result of a long-term therapy, without presenting neuroprotective benefits. This fact has promoted the search for new therapeutic alternatives that have greater efficacy [9–11].

Traditional medicine and plants have been used as an alternative method of health care for people suffering epilepsy [12]. It is estimated that 90% of patients suffering epilepsy receive treatment from traditional medicine including herbal remedies [13]. By this reason, many studies have been focused to isolate...
natural products and characterizing their pharmacological mode of action [8,14,15].

The above situation has encouraged the use of biotechnological strategies, such as the in vitro culture of medicinal plants for the controlled production of metabolites. The implementation of in vitro cultures such as callus induction and cell proliferation in suspension cultures with high yields of secondary metabolites and biomass, has been recognized as a potential methodology for the production of new molecules of industrial importance in a wide range of fields such as pharmacology and medicine. An advantage of this in vitro technique is not being subject to any seasonal restriction or geographical conditions [15–17]. Clinical trial results in bioactive extracts from plant tissue cultures, along with its use in the pharmaceutical industry have shown similar or superior results compared to wild plants. Plant tissue culture represents a promising area in present and future research [18–20].

Waltheria americana Linn. is commonly named in Mexico as "guasimilla, manrubio, escobillo blanco, hierba del soldado, malva del monte, or tapacola" [21]. This plant is used in Mexican traditional medicine to treat nervous disorders such as pain, neuralgia, headache, dizziness, sleep problems, and seizures [22]. An aqueous root extract of aerial parts of W. americana exhibited analgesic potential [23,24]. Hamidu et al. reported that an ethanol/aqueous extract showed sedative and anticonvulsant properties [25]. The cyclo-peptide, adoetin Z isolated from W. americana, showed a sedative effect [26]. In a previous report, we have showed that a methanol-soluble extract obtained from cell suspension culture of W. americana has an effect on the GABAergic system by inducing an increase in GABA release, higher than that produced by crude extracts or fractions of wild plants roots [27]. The GABA neurotransmission system is one of the main mechanisms that decrease neuronal hyperexcitability, and the main site of action of drugs used to treat epilepsy [28,29].

The animal model of PTZ-seizure induction (a GABA A receptor antagonist) has been an acute model widely used to carry out the screening of active compounds in the research phase and have played a fundamental role in the discovery of novel antiepileptic drugs, being a standard procedure with high predictive value of anticonvulsive and neuroprotective activity. PTZ induces convulsive seizures by imbalance between neural inhibition and excitation when interacting with the GABAergic system [30–32].

In order to determine the components of W. americana responsible of the activity on CNS, we decided to isolate and evaluate chromatographic fractions from the methanol-soluble extract of cell culture suspensions on brain cortex of mice post mortem after receiving pentylenetetrazol.

2. Materials and methods

2.1. Plant material

Mature seeds of Waltheria americana Linn. were obtained from plants collected in April 2011 in the locality of La Tigra, Puente de Ixtla, Morelos State, Mexico at 1015 m altitude. Plant material was identified and authenticated by Biol. Gabriel Flores Franco of the Herbarium HUMO of Centro de Investigación en Biodiversidad y Conservación, Universidad Autónoma del Estado de Morelos (Voucher specimen 9940).

2.2. Establishment of cell suspension culture

Callus was induced from W. americana seedlings germinated in vitro on MS medium [33] at 50% of salts and vitamins, supplemented with sucrose 30 g/L (w/v), 2,4-dichlorophenoxyacetic acid (2,4-D) 2.5 mg/L, 6-benzylaminopurine (BAP) 1.0 mg/L and gelled with 3.0 g/L Phytagel (Sigma). The cultures were incubated in a growth chamber at 25 ± 1 °C, with a photoperiod of 16 h light/8 h darkness. Cell suspension cultures were generated from friable calli inoculated in 250 ml Erlenmeyer flasks containing 50 ml of liquid MS medium at 50% of salts and vitamins, supplemented with sucrose 2.5%, myo-inositol 100 mg/L, 2,4-D 2.5 mg/L and BAP 1.0 mg/L. Cultures were maintained under continuous stirring at 110 rpm on an orbital shaker (Labtech, India) and incubated in the dark at 25 ± 1 °C.

2.3. Isolation of chromatographic fractions

Biomass from a W. americana suspension cell culture was separated, dried, and powdered. Dried biomass (30 g) was subjected three times to extraction by maceration with 120 ml of methanol at room temperature for 72 h each time. After filtration, methyl alcohol was removed by evaporation under reduced pressure, and the residue was dried by lyophilization, providing 6.975 g of dry crude extract (WAsc). This extract was subjected to fractionation by column chromatography on reverse phase C18 silica gel (20 g), using a gradient continuous of H2O in CH3OH/CH3Cl2 (100:0 to 1:100) yielding 20 fractions. The most polar fraction (4WAsc–H2O) and the less polar fraction (WAsc–CH2Cl2) were the most abundant, and these fractions were used for the biological experiments.

2.3.1. Characterization of chromatographic fractions

Fraction 4WAsc–H2O (20 mg) was dissolved in D2O (0.6 ml) and fraction 4WAsc–CH2Cl2 (20 mg) was dissolved in CDCl3 (0.6 ml), and then both samples were analyzed by NMR using a Varian INNOVA UNITY 400 spectrometer. One-dimensional (1H and 13C) and two-dimensional NMR (COSY, TOCSY, HSQC, and HMBC) experiments were obtained with a 5-mm inverse detection pulse field gradient probe at 25 °C. Proton chemical shifts were referenced to the residual signal for D2O at δ 4.90 and TMS for CDCl3 solution.

WAsc–CH2Cl2 fraction (20 mg) was dissolved in CHCl3, and then analyzed by gas chromatography (Agilent 6890) coupled to a mass detector (Agilent 3760), equipped with an automatic injector. A 25 m HP-5 capillary column was employed in the analysis. The temperature of the injector block was set to 240 °C, and the detector block was set to 250 °C. The column temperature was initially set to 50 °C for 2 min, and later increased to 260 °C at a rate of 10 °C per min, column was held at this final temperature for 5 min. Helium flow was set to 1 ml/min.

2.4. Laboratory animals

Female adult albino (Mus musculus) mice (CD-1 strain) from 20 to 25 g provided by the animal facility of Instituto de Biotecnología, Universidad Nacional Autónoma de México, were used for in vitro release analyzes of GABA and male adult albino mice (ICR strain) of 25 to 30 g obtained by ENVIGO RMS S.A. de C.V. (Mexico) were used for in vivo analyzes of anticonvulsive activity. Animals were housed in acrylic boxes in groups of five, fed with standard meal pellets (ENVIGO Mexico), water ad libitum and maintained under laboratory conditions (temperature 24–25 °C, under a 12 h light-dark cycle) at least 3 weeks to adapt to the laboratory environment prior to experiments, which were carried out between 8:00 am and 12:00 pm in a special room without noise and controlled lighting. The ethical authorization for this research was obtained through the protocol approved by the Institutional Research Committee, conducting animal experiments in accordance with the Mexican Official Norm (NOM-062-ZOO-1999). A minimum number of animals and a certain period of time for observation were implemented in order to obtain consistent data.
2.5. Drugs

Diazepam (DZP, Cryopharma Laboratory, Mexico) was used as anticonvulsant drug, pentylentetrazol (PTZ, Sigma-Aldrich Chemical) as a seizure-inducing drug and 1% Tween 20 solution (TW, Merck) as a solvent for drugs. All reagents were of analytical grade.

2.6. Release and quantification of GABA in brain cortical slices

In vitro experiments for GABA release were performed using the following incubation method: mice were sacrificed by cervical dislocation and brain dissection was performed immediately on a cold plate. Subsequently, slices of medium bark (250–300 μm) were manually obtained using a razor blade and a glass coverslip as a guide [27,34]. The slices were placed at 4°C in 2 ml Krebs-Ringer modified medium (basal medium) (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.18 mM MgSO₄, 1.18 mM KH₂PO₄, 20 mM C₆H₁₂NO₃, Tris–HCl, 5.6 mM glucose), pH 7.4 with constant oxygenation by O₂ bubbles for 5 min. 100 μM of amino-oxyacetic acid was added to the medium to prevent the GABA metabolism. Subsequently, tissue slices were incubated in a vial with 2 ml of the same basal medium for 10 min at 37°C with constant aeration, then each one was placed in a vial containing 2 ml of Krebs-Ringer medium under the same conditions. Wasc (10, 25, 50 and 100 μg/ml), 4Wasc-H₂O (5, 10, 25, 50 and 100 μg/ml) and Wasc-CH₂Cl₂ (50 μg/ml) fractions were evaluated. Aliquots (200 μl) were collected at different times (0, 30, 60, 90, 120 and 180 s). At the end of the experiments, GABA content in each aliquot was determined by high performance liquid chromatography (HPLC) prior to derivatization with O-phthalaldehyde [35]. In order to standardize the variation in the size of the cerebral cortex slices, the concentrated protein of each slice was quantified using the Lowry method [36], previously homogenizing the tissue in 1 ml of distilled water. Experiments in the absence of extracts (only solvents used) were taken as negative control and in the presence of 47 mM KCl as positive control. The concentration of GABA in each aliquot was corrected for changes in volume and the results were expressed as pmol of GABA released in the incubation medium/mg protein.

2.7. Pentylentetrazol-induced seizures

Mice were divided into five groups. Group A was treated with WASC extract (12.5, 18.5, 25, 37.5, 50, 100, 200, 300 and 600 mg/kg) per oralis (p.o.); Group B received 4WASC-H₂O (5, 10, 20, 40 and 80 mg/kg p.o.); Group C received WASC-CH₂Cl₂ (80 mg/kg p.o.); Group D received DZP (1.0 mg/kg) intraperitoneally (i.p.) as a positive control; Group E received the vehicle solution of 1% Tween 20 (100 μl/10 g p.o.) as a negative control. Previous results have shown that PTZ at a dose 80 mg/kg administrated i.p. (sixty minutes after receiving their respective treatment), is the minimum dose to produce seizures [29]. After injection of PTZ, mice were placed separately in transparent plexiglass cages (25 cm × 15 cm × 10 cm) and observed for 30 min. The time elapsed prior to the onset of the chronic seizures, the occurrence of the seizures, the survival time and the percent of mortality protection were recorded [37]. The severity of seizures was assessed on a revised Racine’s scale as follows: Stage 0, No seizure observed; Stage 1, Sudden behavioral arrest and/or motionless staring; Stage 2, Facial jerking with muddle or muzzle and eye; Stage 3, Neck jerks; Stage 4, Clonic seizure in a sitting position; Stage 5, Convulsions including clonic and/or tonic–clonic seizures while lying on the belly and/or pure tonic seizures; Stage 6: Convulsions including clonic and/or tonic–clonic seizures while lying on the side and/or wild jumping.

2.8. Assessment of neuronal damage

The experimental animals treated with 4WASC-H₂O fraction were immediately submitted to dissection of the brain tissue, fixation in 4% formaldehyde at 4°C for further processing. The samples of brain from all animal were embedded in liquid paraffin, and thin sagittal sections of 5 μm thickness were cut and placed on slides obtained in a manual rotation microtome (RM2255, Leica Microsystems, Germany) and mounted on glass slides with a pretreatment with silane (Trimethoxysilane, Sigma Aldrich®). Tissues were evaluated by histologic technique using hematoxylin & eosin (H & E) as well as Aminocupric (A-Cu-Ag) to determine cell damage and/or neurodegeneration.

2.9. Immunohistochemistry analysis in cortex

An immunohistochemical analysis was performed with the following panel of antibodies: GFAP (giall fibrillary acidic protein, clone 6F2, Dako, Glostrup, Denmark) dilution 1:100 to determine astrogliosis and Neu-N (neuronal nuclear antigen, monoclonal Mouse, A-60 clone, Merck, Millipore, Billerica, MA, USA) 1:100 dilution to determine cell viability. Plates were dried at 37°C using an incubator. Sections containing cortex from each group were washed in phosphate buffered saline (PBS) (two times for 5 min), incubated in 0.1 M sodium citrate (pH 6.0) plus triton X-100% (Sigma-Aldrich USA) in a water bath for 30 min, rinsed three times in PBS buffer for 5 min, to block endogenous peroxidase activity was incubated in hydrogen peroxide 3% for 30 min, washed in PBS (two times for 5 min) and incubated in 1% bovine serum albumin (BSA). After two consecutive PBS washes, sections were incubated overnight at 4°C in PBS–BSA containing the primary antibodies. Slides were incubated with the avidi-biotin-peroxidase complex (ABC Elite, Vector Burlingame, CA). After washing, some of the markers were developed with the chromogen 3,3 diaminobenzidine (LSAB kit, DAKO, USA). Nuclear counterstaining with H & E immunohistochemically stained was mounted and covers. All immunohistochemical stains were performed with a sealed wet chamber (Nexus; Ventana, Illkirch, France) and following a staining system according to the manufacturer’s protocol. Random visual fields were observed within the cortical layers using an optical microscope (DM500, Leica Microsystems, Wetslar, Germany) and performing the qualitative and semi-quantitative analysis with a microcomputational imaging system (Leica Application Suite LAS EZ Version 3.11, Switzerland). Brain tissue samples from untreated mice were used as negative control, obtaining 30 images per group for evaluation at 40× amplification. The mean and standard deviation were calculated for each parameter. All measurements were performed in triplicate.

2.10. Statistical analysis

Statistical analysis of the results was performed using GraphPad Prism Version 6.01 software. For GABA release a two-way analysis of variance (ANOVA) and the post-hoc Tukey multiple comparison test with a significance level of p < 0.01 was applied. Values are expressed as the mean ± standard error of 3 independent experiments. The anticonvulsive activity was performed by one-way ANOVA and was applied the post hoc Dunnett test with a significance level of p < 0.05. Values are expressed as the mean ± S.E.M. of 5 independent experiments. The evaluation of the neuroprotective effect, was performed by unidirectional ANOVA and was applied the post-hoc Tukey multiple comparison test with a significance level of p < 0.01. Values are expressed as the mean ± S.E.M. of 3 independent experiments.
3. Results and discussion

3.1. Biomass production

*W. americana* suspension cultures in liquid MS medium by placing friable callus formed from exposure of axenic seedlings to the regulators 2,4-D acid and BAP, growth and proliferation of free cells were observed, obtaining the best biomass yield in dry weight after 20 days of cultivation with 0.84 ± 0.007 g/50 ml of medium, representing an increase of 2 times the size of the initial inoculum (0.42 g ± 0.004 g). The cell suspensions were filtered, separating the biomass from the liquid phase (broth). The dry biomass (30 g) was subjected to extraction by maceration with methanol providing 6.975 g of dry crude extract (Wasc) having a yield of 23.25%.

3.2. Characterization of fractions

The diversity of bioactive secondary metabolites obtained from plant cell culture systems is very wide. This biotechnology strategy has become a viable and renewable source for the production of new molecules of pharmacological interest [16,38]. NMR data indicated that the signals in the 1H and 13C NMR spectrum of 4WAsc-H2O, correspond to saccharide compounds as the most abundant metabolites in this fraction. Anomeric signals were identified at 5.22 and 4.41 ppm because of the multiplicity for the signals as doublets. The integrated peak areas for these signals were 1.0: 1.10 from low to high field. In the HSQC NMR spectrum it was possible to correlate the anomeric proton signals with two anomeric carbon signals at 92.7 and 94.6 ppm. The first anomeric

<table>
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<th>Treatment</th>
<th>Concentration (μg/ml)</th>
<th>GABA release (pmol/mg protein)</th>
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<tr>
<td></td>
<td></td>
<td>30 s</td>
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<tr>
<td>Negative control</td>
<td>12.24 ± 2.13</td>
<td>17.16 ± 2.84</td>
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<tr>
<td>Positive control</td>
<td>49.57 ± 2.69</td>
<td>109.33 ± 5.77</td>
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<tr>
<td>MeOH extract WAsc</td>
<td>92.76 ± 18.28</td>
<td>154.42 ± 28.85</td>
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<tr>
<td>MeOH extract WAsc</td>
<td>99.33 ± 12.13</td>
<td>172.28 ± 6.72</td>
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<tr>
<td>MeOH extract WAsc</td>
<td>304.42 ± 39.66</td>
<td>327.45 ± 0.24</td>
</tr>
<tr>
<td>MeOH extract WAsc</td>
<td>146.76 ± 16.50</td>
<td>173.37 ± 20.13</td>
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<tr>
<td>Fraction WAsc-CH2Cl2</td>
<td>142.74 ± 11.44</td>
<td>273.95 ± 32.37</td>
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<tr>
<td>Fraction 4WAsc-H2O</td>
<td>106.04 ± 16.03</td>
<td>223.37 ± 4.16</td>
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<tr>
<td>Fraction 4WAsc-H2O</td>
<td>156.04 ± 13.98</td>
<td>239.53 ± 15.49</td>
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<td>Fraction 4WAsc-H2O</td>
<td>271.11 ± 34.22</td>
<td>249.63 ± 38.01</td>
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<tr>
<td>Fraction 4WAsc-H2O</td>
<td>273.49 ± 23.43</td>
<td>346.64 ± 43.84</td>
</tr>
<tr>
<td>Fraction 4WAsc-H2O</td>
<td>430.16 ± 13.22</td>
<td>509.88 ± 30.73</td>
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Experiments in the absence of extracts were taken as negative control and in the presence of 47 mM KCl as positive control. Values are expressed as pmol of released GABA/mg protein as the mean ± S.E.M. (n = 3). *p < 0.001 against the positive control (Tukey’s test).
signal was assigned to glucose units, with an anomeric configuration of α from their JH1-H2 of 3.8 Hz in the 1H NMR spectrum. The methylene signal detected at 65.2 ppm in the HSQC spectrum, suggests that the hexose units is 6-O-substituted. Within its structural diversity, there are molecules that present anticonvulsive and neuroprotective activity, mediated by mechanisms such as decreased astrocytic activity, anti-inflammatory effect, antioxidant, inhibition of Na⁺ and Ca²⁺ T-type channels, GABA_A receptor
modulation, and protection of hippocampal cells and cerebral cortex. Simple saccharides or analogs that exhibit changes in their primary structure show these biological activities on the CNS. A clear example is the compound 2-deoxy-D-glucose (an analogue of glucose), which is in preclinical development for the treatment of epilepsy. Several carbohydrate-based formulations with important pharmaceutical applications are currently on the market, for example topiramate, a simple sugar with strong anticonvulsive and neuroprotective activity [39,40,41].

Meanwhile, the \(^{1}H\) and \(^{13}C\) NMR spectrum of WAsc-CH\(_{2}\)Cl\(_{2}\) showed that fatty acids are the most abundant compounds in this fraction. Saturated (SFA) and unsaturated fatty acids (UFA) of W. americana were analyzed by gas chromatography-mass spectrometry. A total of 7 fatty acids were established. These fatty acids varied in the length from C10 to C24. The largest component of the total fatty acid was identified as C18:1 w 9 (oleic acid). In addition, monounsaturated fatty acids (MUFA) measured as 59.07% of the total fatty acid composition, were more abundant than SFA (21.46%) and polyunsaturated fatty acids PUFAs (19.48%). The most abundant fatty acid recorded in W. americana was oleic acid (58.6%), followed by linoleic acid (19.02%), stearic acid (6.09%) and palmitic acid (5%). These are the four most abundant fatty acids, which constituted 88.7% of the total fatty acid pool. Fatty acids stems are important constituents of plants with recognized roles as storage material, and components of plasmalemma and other cellular organelle membranes. In plants, fatty acids are more abundant in leaves. Clinical and experimental data have shown that this group of metabolites present anticonvulsive and neuroprotective activity, proposing some possible mechanisms that mediate the function as antioxidant, anti-inflammatory, stabilization of neuronal receptors, modulation of Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\)-T-type in GABAergic interneurons and the induction of glutamate decarboxylase (GAD), an enzyme that facilitates the conversion of glutamate to GABA, decreasing the duration and frequency of seizures, preventing the epileptic status associated with neuropathological changes, besides preserving and stimulating the formation of GABAergic interneurons in the hippocampus and cerebral cortex. Compounds such as oleic, linoleic, \(\alpha\)-linolenic acid...

Fig. 5. Effect of different doses of 4WAsc-H\(_{2}\)O (5, 10, 20, 40 and 80 mg/kg, p.o) and WAsc-CH\(_{2}\)Cl\(_{2}\) (80 mg/kg, p.o) on PTZ-induced seizures (80 mg/kg i.p) in mice. (A) Onset of tonic seizures; (B) number of tonic/tonic-clonic seizures; (C) survival time; (D) percentage of protection against death. Values are expressed as the mean ± S.E.M. (n = 5) for the onset of tonic seizures, number of tonic/tonic-clonic seizures and survival time. One-way ANOVA p < 0.0001; (*) p < 0.05, (**) p < 0.01, (***) p < 0.001 compared to vehicle (Dunnett’s test). DZP (diazepam), VEH (1% tween 20).
from the omegas group, PUFA, MUFAs, short chain fatty acids (SCFAs), medium chain fatty acids (MCFAs) or more complex triglyceride triheptanoin probably play a role in neurotransmission. The SCFAs 2-propylpentanoic acid or valproic acid (VPA) is a commonly used broad spectrum antiepileptic drug which potentiates GABAergic neurotransmission by stimulating GABA synthesis and inhibiting its degradation. Fatty acids with VPA-related structures have also been associated with anticonvulsive and neuroprotective properties [42–44]. Chemical and pharmacological analyzes suggest that W. americana suspension cultures produce compounds that may have the ability to facilitate GABAergic transmission.

### 3.3. GABA release evoked by Wasc, 4WAsc-H2O, and WAsc-CH2Cl2

Monitoring of GABA levels is an effective tool to determine activity on the central nervous system (CNS), since it is considered the amino acid that acts as the main inhibitory neurotransmitter of the CNS [45,46]. In assessing lower concentrations than the previously reported (50 and 100 µg/ml) for WAsc, a significant increase in free neurotransmitter concentration (p < 0.001) was induced by 10 and 25 µg/ml of WAsc, as compared with values observed in the presence of high potassium, however the degree of release did not show a significant difference when compared to the concentration of 100 µg/ml, attributing the greatest effect at the concentration of 50 µg/ml, which corroborates the possible hermetic effect of the complete extract on the GABAergic system previously published (Fig. 1 and Table 1) [27]. When analyzing fractions derived from WAsc, an effect on the GABA release was observed for the WAsc-CH2Cl2 at concentration of 50 µg/ml and 4WAsc-H2O in the range of concentrations of 10 to 100 µg/ml, describing an effect dose response significantly higher (p < 0.001) as compared to values observed in the positive control (Figs. 2 and 3 and Table 1).

It could be probable that the fractionation favors this effect since, the diversity of metabolites present in crude extracts could interact synergistically, however, the interaction sometimes can diminish the desired effect due to chemical interactions, low concentrations of the active metabolites or saturation of the system, but another possibility is that the inducer metabolite could

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**Fig. 6.** Histological sections stained with H & E and percentage of conserved cells from the cortical region of brains of mice: (A) treated with 1% tween 20 p.o.; (B) pretreatment with 1% tween 20 p.o. and subsequent administration of PTZ 80 mg/kg i.p. (negative control); (C) pretreatment with DZP 1.0 mg/kg i.p. and subsequent administration of PTZ 80 mg/kg i.p. (positive control); (D) pretreatment with 4WAsc-H2O 40 mg/kg p.o. and subsequent administration of PTZ 80 mg/kg i.p.; (E) pretreatment with 4WAsc-H2O 80 mg/kg p.o. and subsequent administration of PTZ 80 mg/kg i.p. Values are expressed as the mean ± S.E.M. (n = 3). One-way ANOVA: F (4,10) = 351.7, p < 0.0001; * (p < 0.001) against the negative control, and b (p < 0.001) against the positive control (Tukey's test). Preserved cells (arrows), damage cells (arrowheads) and interstitial edema (asterisks).
be absent from the fraction. Understanding the role of GABA as an inhibitory neurotransmitter in the brain has led to the investigation of compounds obtained from medicinal plants that modify the arrangement of GABA in order to increase its inhibitory activity and thus protect against seizures [3,46,47].

3.4. Anticonvulsant effect of WAsc, 4WAsc-H2O, and WAsc-CH2Cl2 on PTZ-induced seizures

There is a correlation between the different types of seizures and the dose of PTZ used, in the present study it was used at a dosage of 80 mg/kg i.p., producing convulsions similar to the generalized tonic/clonic in humans, having 100% mortality of the experimental animals and showing a clear pattern of neurodegeneration in hippocampal, amygdala, and cerebral cortex regions [30,48,49]. The pretreatment of the experimental animals with WAsc caused a bi-phasic response in the lower doses (12.5 to 37.5 mg/kg o.p., and even at 50 mg/kg p.o.), causing an increase in latency and survival time, decreasing the severity of tonic-clonic seizures and preventing death of up to 80% of mice after PTZ-induced seizures; while high doses (100 to 600 mg/kg p.o.) induced opposite behavior. As observed in Fig. 4, Table 2 in each range of doses lower and higher, there is no sign of behavior dose dependency. In the case of lower doses, each parameter analyzed was statistically different from the group study that received a treatment with tween 20 at 1%, with exception to the number of seizures in which only the dose of 18.5 mg/kg o.p. induced a significant reduction respect to negative control (p < 0.005). When 4WAsc-H2O was administered, induced an effect dose-dependent (Fig. 5, Table 2), causing a rise in latency in the groups treated with 40 and 80 mg/kg p.o., which presented a significantly difference (p < 0.01) compared to the group treated with the vehicle. It was not observed statistically a significant protection against tonic-clonic seizures but the number of seizures was maintained in a range of 1.6 to 2.6 decreasing its severity. WAsc-CH2Cl2 at a dose of 80 mg/kg p.o. did not induce a statistically change in latency or on the number of seizures when compared both parameters with group treated with vehicle (p > 0.05), even when the average of seizures was 1.6. These treatments induced a significant increment (p < 0.001) in time of survival of animals.
with respect to negative control, thus protecting against death in 100% of animals of study.

These results suggest that WAsc, 4WAsc-H2O, and WAsc-CH2Cl2 have an anticonvulsant effect mediated by GABAergic potentiation mechanisms. PTZ acts as a non-competitive antagonist, probably through an allosteric interaction with the picrotoxin-binding site in GABA\textsubscript{A}-like receptors, inhibiting the activation of Cl\textsuperscript{-} channels, resulting in an alteration in the balance of neuronal inhibition and excitation. Molecules acting through GABAergic mechanisms are effective on this model [31,32,50], suggesting that the compounds present in WAsc and their fractions may have penetrated the blood-brain barrier, exerting an effect by preventing the death of the mice after the seizures, showing a probable inhibitory potential for neurotoxic death caused by the chemical convulsant [51].
3.5. Neuroprotective effect of 4WAsc-H₂O on neuronal damage in cerebral cortex

Deficiencies in GABAergic transmission are closely related to hyperexcitability and impairment of normal neuronal function, which triggers structural and molecular changes in various brain regions leading to epileptogenesis, favoring neurodegeneration and cell death [3, 46, 52]. When determining the effect against PTZ-induced structural damage on neuronal cells in the cerebral cortex of mice pretreated with 40 mg/kg p.o. of 4WAsc-H₂O in H&E-stained tissue, a higher percentage of preserved cells was observed (74.88 ± 1.43%), compared to the percentage after PTZ injection in mice pretreated with the vehicle (18.33 ± 3.39%) (p < 0.001). When administering 80 mg/kg p.o. of 4WAsc-H₂O, the effect on cell preservation was better (92.39 ± 0.72%), diminished structural damage [Fig. 6]. A similar effect was observed when assessing PTZ-induced neurodegeneration in A-Cu-Ag-stained tissue, when 4WAsc-H₂O was given a dosage of 40 mg/kg p.o, the percentage of preserved cells (79.03 ± 0.69%) was higher (p < 0.001), compared to the percentage after injection of PTZ in mice pretreated with the vehicle (31.78 ± 2.42%). When administering 80 mg/kg p.o. of 4WAsc-H₂O, the protective effect was higher, having a percentage of preserved cells of 89.39 ± 0.46%, diminished the neurodegeneration [Fig. 7]. The protective effect to structural damage and neurodegeneration caused by 4WAsc-H₂O (80 mg/kg p.o.) is statistically equal to that induced by diazepam (1.0 mg/kg), used as an anticonvulsant and neuroprotective drug. These results suggest that the protective effect of 4WAsc-H₂O on neurological impairment may be associated with improved PTZ-induced morphological damage.

When 80 mg/kg i.p. of PTZ was administrated, an increase in glial activation was detected, observing greater GFAP immunoreactivity and a significant decrease in cell viability, observing lower Neu-N immunoreactivity. Pretreatment with 40 and 80 mg/kg p.o. of 4WAsc-H₂O decreased the formation of interstitial tissue edema, astrocytic activation (lower GFAP immunoreactivity) and cell death (greater Neu-N immunoreactivity). Both parameters were observed in normal levels of cellular reactivity, showing similar morphology to that of rest, comparable with group pre-treated with DZP [Fig. 8]. These findings could indicate a possible participation of anti-inflammatory mechanisms that contribute to the neuroprotective effect of 4WAsc-H₂O. Although normal astrocytes are distributed along the cerebral cortex, they increase in number and reactivity by inducing neuronal damage after addition of a neurotoxin, being able to participate in the epileptogenic process by damaging mitochondrial membranes, synthesizing, and releasing mediators of inflammation such as TNF-α and IL-1β, participating as mediators of neurodegeneration. Glial activation is generally considered a key mechanism in the regulation of neuronal cell death [3, 53].

In the neurobiology of the epileptic process is well known that reducing neurodegeneration and other alterations, diminishes the consequences of behavior after epileptic status. Therefore developing neuroprotective treatments has an important role in the prevention of epileptogenesis and recovery of damage in patients, who if they are not under treatment, could develop drug-resistant epilepsy [54, 55, 56]. The observed anticonvulsive and neuroprotective action is probably due to the inhibition of the effect of PTZ on the GABA receptors, acting on factors involved in GABAergic neurotransmission, such as modulation of GABA receptors, inhibition of GABA-transaminase or the transporter recapture of GABA GAT-1, modulating the synaptic levels of GABA by modifying the mechanisms of release, transport and/or elimination of the neurotransmitter of the synaptic cleft [52] (Fig. 9).

4. Conclusions

The present study showed that compounds present in the methanol-soluble extract of W. americana cell suspensions, besides exerting a potential anticonvulsive activity, also act as neuro-protective agents by reducing morphological alterations, neurodegeneration, interstitial edema, astrocytic reactivity, and death neuronal caused by the intense seizure activity induced by PTZ administration in the cerebral cortex of mice. Further studies should be carried out to isolate and identify the pure compounds from this extract and explore the mechanism of action at the molecular level.

Conflict of interests

The authors declare that they have no competing interests. All authors have read and approved the final version of the manuscript.

Acknowledgments

Authors are thankful to Lucero Valladares, for the technical support during the pharmacological experiments. J. Mundo is grateful to CONACYT for postgraduate scholarship (254148).

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