

Full Length Research Paper

Methanolic extracts from roots and cell suspension cultures of *Waltheria americana* Linn induce GABA release in cerebral slices of mouse brain

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This study evaluates and compares the release of the neurotransmitter γ -aminobutyric acid (GABA) induced by methanolic extracts obtained from roots of wild plants and from cell suspension cultures of *W. americana* Linn. The release of this neurotransmitter was evoked by crude and fractioned extracts derived from both roots of wild plant and cell suspension cultures, tested at three concentrations: 10, 50 and 100 $\mu\text{g/ml}$ by means of *in vitro* incubation of slices of mouse cerebral cortex. Firstly, crude extracts from wild plant roots obtained by applying solvents of different polarities (n-hexane, dichloromethane and methanol) were tested at a concentration of 10 $\mu\text{g/ml}$ with no observed effect on the level of GABA release, but when the same extracts at a concentration of 100 $\mu\text{g/ml}$ was evaluated, methanol extract (MeOH) was determined to be the most effective in terms of the release of GABA. The results suggest that wild plant and cell suspension cultures of *W. americana* produce some compounds that cause an increase in the release of GABA in *in vitro* experiments, corroborating with the ethnomedical applications associated with this species, and indicates high potential for research and development of new active drugs for the treatment of several central nervous system (CNS) disorders related to the GABA system.

Key words: Calli, cell suspension cultures, GABAergic system, neurological disorder, *Waltheria indica*.

INTRODUCTION

Neurological disorders have become a serious health problem, affecting millions of people worldwide (WHO, 2013). Thus modern pharmacology has been directed towards the search for active compounds, especially those that affect brain neurochemistry by increasing neuronal inhibition. Enhancing GABAergic activity is

considered as one of the main drug action mechanisms for treating these types of diseases. The inhibitory effect produced by the release of γ -amin butyric acid (GABA) is critical to maintaining neuronal homeostasis, which is why changes in the activity of this neurotransmitter system have been associated with several neuropsychiatric

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disorders (Yogeeswari et al., 2007). At present, although different medications are offered by the pharmaceutical industry worldwide, these have limitations and require improvement. The need for chronic administration of antidepressants, together with their limited efficacy (about 65%) and adverse side effects (changes in body weight, nausea, sexual dysfunction, diarrhea) make them inadequate and unsatisfactory (Kirsch, 2008; Turner and Rosenthal, 2008). Furthermore, typical anxiety related drugs such as benzodiazepines though effective, also produce side effects such as sedation, amnesia, dangerous interactions with alcohol, myorelaxation and development of tolerance or dependence following chronic administration (Vinkers, 2012; Dell'osso and Lader, 2013). In the last few years, great interest in the study of natural products has emerged, due to their potential pharmacological application. Research and pursuit of new alternatives to conventional psychotropic drugs, such as phytomedication based on standardized extracts of plant species, which are considered to manifest some physiological effects, but which may act more effectively and harmlessly are currently in progress (Leon-Rivera et al., 2011; Huerta-Reyes et al., 2013). *Waltheria americana* Linn (syn. *Waltheria indica*) (Zongo et al., 2013) is a plant that has attracted medical interest because of its application in traditional medicine to treat various central nervous system-related conditions, such as pain, neuralgia (Saunders, 2007; Borokini and Omotayo, 2012), headache (Mexico and Hawaii), vertigo (Hawaii), seizures, and sleep problems (Leonard, 1998; Hamidu et al., 2008). A previous pharmacological study, focussing on aqueous extracts from the root and stem of *W. americana*, suggested its analgesic potential (Mohammed et al., 2007). Moreover, the ethanol or aqueous extracts from the entire plant proved to have sedative and anticonvulsant properties, as well as causing an increase in sleep time induced by amylobarbitone, and decreased motor activity and pentylentetrazole-induced convulsions, thereby either preventing or delaying death among animals (Hamidu et al., 2008). Likewise, the sedative effect of the cyclo-peptide, adouetin Z isolated from *W. americana* was evaluated by measuring the reduction in spontaneous motor activity among mice (Blanpin et al., 1963). These characteristics recommend the possible application of *W. americana* for the treatment of neurological diseases, as they act on neuronal excitability, ameliorating mood disorders, among other afflictions. The objective of this study was thus to evaluate the effect of extracts derived from wild plant roots and cell suspensions cultures of *W. americana*, as affecting GABA release in cerebral slices of mouse brain.

MATERIALS AND METHODS

Plant

Mature seeds and roots from *W. americana* were collected during April 2011 (dry season) in the town of La Tigra, located in the

Municipality of Puente de Ixtla, Morelos, Mexico, with an average elevation of 1015 m above sea level. A copy of the voucher specimen was authenticated and deposited at the HUMO Herbarium of the Autonomous University of the State of Morelos (voucher specimen number 9940).

Establishment of cell suspension culture

Callus was induced from an *in vitro* cultured seedling from *W. americana* at half MS strength (Murashige and Skoog, 1962) and then gelled with 3.0 g/L Phytigel® (Sigma) medium, supplemented with 30 g/L sucrose (w/v), 2.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg/L 6-benzylaminopurine (BAP). Cultures were incubated in growth chambers at $25 \pm 1^\circ\text{C}$, during a 16 h photoperiod. Cell suspension cultures were initiated from soft friable callus, inoculated in 250 ml Erlenmeyer flasks containing 50 ml of MS liquid medium, supplemented with 4.5% sucrose, 100 mg/L myo-inositol and 2 mg/L 2,4-D. Cultures were maintained under continuous agitation at 110 rpm in an orbital shaker (Labtech, India) and incubated in darkness at $25 \pm 1^\circ\text{C}$.

Preparation of extracts and bioassay directed fractioning

Dried and ground roots (100 g) of *W. americana* were extracted three times at room temperature by maceration with 400 ml of *n*-hexane (C_6H_{14}) over a 72 h period, followed by three extractions using 400 ml of dichloromethane (CH_2Cl_2), under the same conditions. Subsequently, residual material was extracted using methanol (400 ml $\times$ 3), to provide 0.1088, 1.166 and 10.624 g/kg dry weight of each extract, respectively, later solvents were removed by vacuum. For fractioning, the methanol-soluble extract (5 g) was successfully re-extracted using CH_2Cl_2 (20 ml $\times$ 4) and later with H_2O (20 ml $\times$ 4), yielding 4 g of a CH_2Cl_2 of insoluble material. The solid was subjected to gravity column chromatography during reverse phase (C_{18}) silica gel (20 g), using gradients of CH_3OH in H_2O (0:100 to 100:0). A total of 7 fractions (20 ml each) were collected and combined resulting in the (F-2EMRWA) fraction.

Because the best result in drug trials was obtained using methanol extracted from roots, it was decided to use the same solvent for the preparation of dry biomass and liquid phase (culture medium) extracts, derived from cell suspension cultures. Dried biomass (20 g) and lyophilized culture medium (20 g) were subjected to maceration with methanol (200 ml), following the same extraction procedure.

Animals

Work was carried out on female albino mice (*Mus musculus*) CD-1 strain (20 to 25 g weight). Mice were provided by the animal facility at the Institute of Biotechnology of the Autonomous University of Mexico (IBT-UNAM). All animals were housed at 5 per cage, maintained under laboratory conditions at 25°C , 12 h light/12 h dark cycle, with lights turned on at 07:00 a.m. and free access to water and standard food pellets (Harlan Teklad Global Diet, Blackthorn, UK). Mice were adapted to the laboratory environment over a three week period, prior to experiments. Experiments for determining activity on CNS-related conditions were carried out between 8:00 a.m. and 12:00 p.m. in an adjacent special noise-free room, with controlled illumination. All experimental procedures were implemented, following protocol approved by the Institutional Research Committee, in compliance with the Official Mexican Norm (NOM-062-ZOO-1999). A minimum number of animals and a certain time period for observation was implemented, in order to obtain consistent data.

Table 1. GABA release evoked by the different treatments on cerebral cortex slice at 30 and 180 s.

Treatment	GABA release 30 s (pmol/mg protein)	GABA release 180 s (pmol/mg protein)
Negative control	0.5 ± 0.5	1.7 ± 1.7
Positive control	37.3 ± 6.8	95.3 ± 9.2
MeOH extract roots (100 µg/ml)	24.6 ± 2.6	35.4 ± 2.8
F-2EMRWA Fraction (50 µg/ml)	110.4 ± 13.4	153.9 ± 18.4
F-2EMRWA Fraction (100 µg/ml)	49.5 ± 2.4	58.6 ± 3.19
MeOH extract cellular suspension (50 µg/ml)	263.0 ± 25.7	332.1 ± 47.7
MeOH extract cellular suspension (100 µg/ml)	181.9 ± 32.9	236.6 ± 22.9

Experiments in the absence of extracts were taken as negative control and in the presence of a high concentration of K⁺ (47 mM KCl) as positive control. Values are expressed as pmol of GABA released/mg protein and are mean ± standard error of mean (SEM). N = 5.

Experiments for release and quantification of GABA in brain cortical slices

In vitro experiments for GABA release were performed by applying an incubation method as follows: mice were sacrificed by cervical dislocation and brain dissection was immediately carried out on a cold plate. Subsequently, middle cortex slices (250-300 µm) were obtained manually, using a razor blade and a cover glass guide (Gutiérrez and Delgado-Coello, 1989). Slices were placed at 4°C in 2 ml of a modified Krebs-Ringer medium (basal medium) (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.18 mM MgSO₄, 1.18 mM KH₂PO₄, 20 mM C₄H₁₁NO₃, 25 mM Tris-HCl buffer, 5.6 mM glucose), pH adjusted to 7.4 in continuous oxygenation with O₂ bubbles for 5 min. 100 µM of amino-oxyacetic acid (AOAA) was added to the medium to prevent GABA metabolism. Subsequently, tissue slices were incubated in a vial with 2 ml of the same basal medium, during 10 min at 37°C with constant aeration and then each one was placed in a vial containing 2 ml of Krebs-Ringer medium, under the same conditions. Thereafter, crude extracts of different polarity were added at a final concentration of 10, 50 and 100 µg/ml, so they could be tested. Aliquots of 200 µl were collected at different times (0, 30, 60, 90, 120 and 180 s). At the end of the experiment, the content of GABA in each collected aliquot was determined by applying high performance liquid chromatography (HPLC), previous derivation with O-phthalaldehyde (Herrera-Ruiz et al., 2007). In order to standardize the variation in the size of the cerebral cortex slices, the protein content of each one was quantified using the Lowry method (Lowry et al., 1951), 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 a high concentration of K⁺ (47 mM KCl) as positive control. GABA concentration in each aliquot was corrected for changes in volume and results were expressed in pmol of GABA released into the incubation medium/mg protein.

Statistical analysis

Statistical analysis of results was carried out using the software GraphPad Prism Version 6.01, using analysis of variance test (ANOVA) for two-way, and a post hoc test was subsequently performed, applying the Tukey test with a level of significance of p < 0.01. Values are expressed as the mean ± standard error from 5 different independent experiments.

RESULTS AND DISCUSSION

The physiological and clinical result of a decrease in the GABAergic neurotransmission in the CNS has been implicated in the occurrence of serious neuronal disorders such as sleep disorders, neuropathic pain, epilepsy, anxiety, depression, Parkinson's disease, schizophrenia and dementia. As a first approximation, it appears that any increase in the accumulation of extracellular GABA would have a positive effect for the treatment of these afflictions (Beleboni et al., 2004; Conti et al., 2004; Yogeewari et al., 2007).

Modulator substances for the release of GABA from medicinal plants have been considered to offer important sources for the production of new drugs. The effect caused on GABA by secondary metabolites present in the different extracts tested, for example extracellular increase in its concentration, maybe due to modifying some of the processes involved in neurotransmission, such as the release, transport and/or removal of the neurotransmitter from the synaptic cleft (Conti et al., 2004; Beleboni et al., 2004). When analyzing the *W. americana* extracts of different polarity (C₆H₁₄, CH₂Cl₂ and MeOH) prepared from wild plant roots at a concentration of 10 µg/ml, no effect on the levels of GABA released was observed (data not shown). However, when evaluating the different extracts at a concentration of 100 µg/ml, it was observed that the MeOH extract induced a continuous increment of GABA, up to 35.445 pmol/mg protein at 180 s, with significant release of GABA compared to baseline (negative control) (1.772 pmol/mg protein at 180 s), however, showing a lesser effect than observed in the presence of a release stimulus (positive control) (95.3 pmol/mg protein at 180 s) (Figure 1 and Table 1). For *n*-hexane and dichloromethane extracts, the release of GABA levels was recorded as similar to that observed in the negative control which was almost zero; thus it was determined that these extracts have no effect on the release of GABA (data not shown).

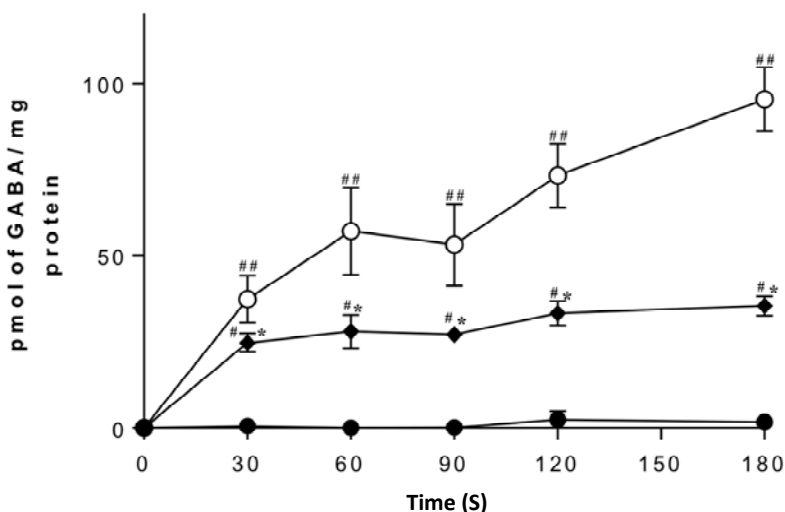


Figure 1. GABA release evoked by 100 µg/ml MeOH roots extract (◆) on cerebral cortex slice for 3 min. Experiments in the absence of extracts were taken as negative control (●) and in the presence of a high concentration of K⁺ (47 mM KCl) as positive control (○). ANOVA: $F(2,8) = 37.47$, $p < 0.0001$; (#) $p < 0.01$ and (##) $p < 0.001$ versus negative control (Tukey test); (*) $p < 0.01$ and (**) $p < 0.001$ versus positive control (Tukey test).

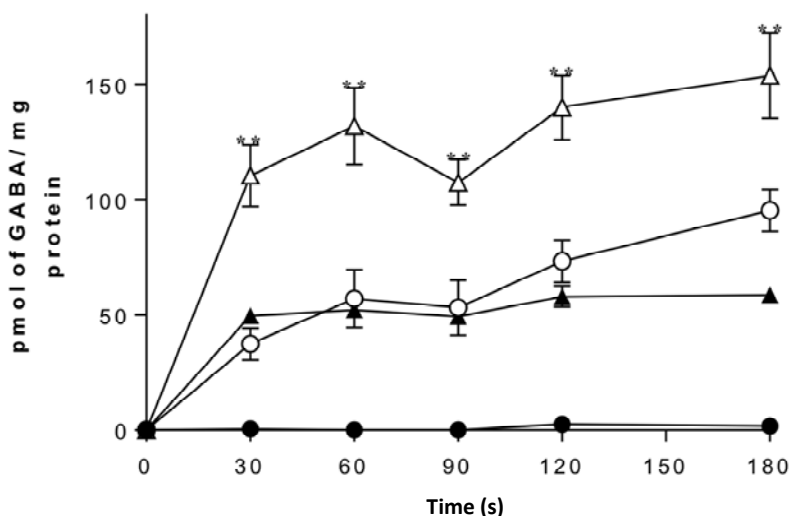


Figure 2. GABA release evoked by F-2EMRWA fraction at two different concentrations, 50 µg/ml (Δ) and 100 µg/ml (▲) on cerebral cortex slice for 3 min. Experiments in the absence of extracts were taken as negative control (●) and in the presence of a high concentration of K⁺ (47 mM KCl) as positive control (○). ANOVA: $F(3,12) = 112.3$, $p < 0.0001$; (**) $p < 0.001$ versus positive control (Tukey test).

The fraction analyzed (F-2EMRWA) from the MeOH extract, at a concentration of 50 and 100 µg/ml, manifested a significant increase on the basal release of GABA, compared to whole crude extract and greater effect on the release of GABA was observed, when analyzing the lesser concentration of 50 µg/ml (153.911 pmol/mg protein at 180 s), as compared to the

concentration of 100 µg/ml (58.699 pmol/mg protein at 180 s), attributing possible hormetic effect on the GABAergic system, showing that the lower dose of extract caused greater liberation of the neuro transmitter than the higher dose (Figure 2 and Table 1). Similar results were observed when examining MeOH extracts prepared from the cultured cells in suspension at

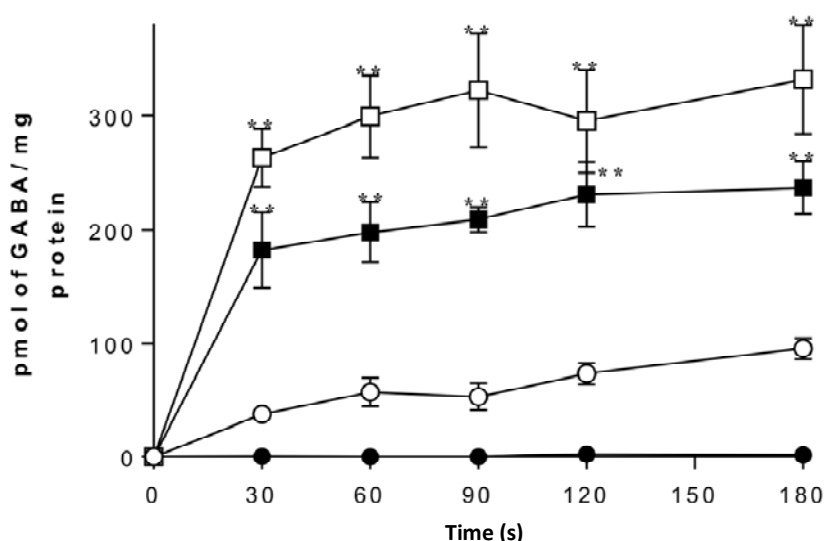


Figure 3. GABA release evoked by extract of the biomass of suspension cultures at two different concentrations, 50 µg/ml (□) and 100 µg/ml (■) on cerebral cortex slice for 3 min. Experiments in the absence of extracts were taken as negative control (●) and in the presence of a high concentration of K⁺ (47 mM KCl) as positive control (○). ANOVA: $F(3,12) = 465.3$, $p < 0.0001$; (**) $p < 0.001$ versus positive control (Tukey test).

a concentration of 50 and 100 µg/ml. These were considered to have a positive effect on the GABAergic system, when using only the extract produced from biomass and not that from the culture medium (data not shown), observing a GABA release significantly higher than that caused by the MeOH crude extract and the fraction prepared from the root of wild plant, at both concentrations tested, and greater effect on the release of GABA when analyzing the lesser concentration of 50 µg/ml (332.108 pmol/mg protein at 180 s) compared to the concentration of 100 µg/ml (236.697 pmol/mg protein at 180 s), determining this extract as the most effective, in terms of the release of GABA *in vitro* (Figure 3 and Table 1).

The results suggest that both the wild plant roots and cell suspension cultures of *W. americana* produce compounds that enhance the *in vitro* extracellular GABA concentration in cerebral brain slices, with a possible neuropharmacological effect on the GABA-ergic system. These findings therefore justify the traditional medicinal use of the plant. The isolation of active chemicals from roots of this plant and from cell suspension cultures may serve as a prototype or “indicate compounds” for the synthesis of more effective drugs with potential application for the management of nervous disorders.

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Abbreviations: CNS, Central nervous system; 2,4-D, 2,4-dichlorophenoxyacetic acid; GABA, γ-amino butyric acid; MS, Murashige and Skoog.

Conflicts of interest

The authors declare no conflict of interest

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