SUPPLEMENTARY MATERIAL

Neuroprotective effects of *Tilia americana* var. *mexicana* on damage induced by cerebral ischemia in mice.

Angeles-López Guadalupe E^a, González-Trujano Ma. Eva^b, Gómez Claudia^a, Chánez-Cárdenas Ma. Elena^c and Ventura-Martínez Rosa^{a*}.

^a*Departamento de Farmacología, Facultad de Medicina, Universidad Nacional Autónoma de México, México;*

^b*Laboratorio de Neurofarmacología de Productos Naturales, Dirección de Investigaciones en Neurociencias, Instituto Nacional de Psiquiatría “Ramon de la Fuente Muñiz”, México;* ^c*Laboratorio de Patología Vascular Cerebral, Instituto Nacional de Neurología y Neurocirugía “Manuel Velasco Suarez”, México.*

*Corresponding autor: Phone: (+5255) 5623-2162; E-mail: rventuram7@hotmail.com

Abstract

Tilia americana var. *mexicana* (*T. americana*) is a plant widely used in Mexico for its medicinal properties on the central nervous system. In the present study, we designed a protocol to investigate the neuroprotective effects of non-polar and polar extracts of *T. americana* on damage induced by cerebral ischemia in mice. Vehicle or extracts were administered immediately after ischemia. Functional neurological deficit, survival percentage and infarct area were determined in each experimental group. Results showed that groups treated with non-polar or polar extracts of *T. americana* had increased survival rate, improved neurological deficits and diminished the infarct area in relation to the ischemic group. In conclusion, this study confirms the neuroprotective activity of *T. americana*, suggests a possible synergism between non-polar and polar constituents and supports its potential as a useful aid in the clinical management of stroke.

Keywords: *Tilia americana* var. *mexicana* (*T. americana*); neuroprotective activity; damage induced by ischemia; infarct area; neurological deficit

Experimental

Plant material and extraction procedure

Inflorescences and leaves of *Tilia americana* L. var. *mexicana* (Schltdl.) Hardin (Tiliaceae) were collected in Tenango de Doria, in the state of Hidalgo, Mexico in June 2007. Plant was identified by Abigail Aguilar, M.Sc. and a voucher specimen was deposited in the herbarium of the Instituto Mexicano del Seguro Social, in Mexico City (IMSS M-15070). A polar extract (aqueous) of the plant was obtained by boiling 24 g of dried and powdered aerial parts in 500 ml of water for 10 min. The resulting extract was separated from residues by gravity filtration; samples were then frozen in liquid nitrogen and later lyophilized for 12 h in a Model Heto lyophilizer (FD3 Lab). For the preparation of the non-polar extract, the air-dried powdered aerial parts (1.86 kg) were successively extracted with hexane (4 L X 3) by maceration at room temperature (22°C). The solvent was separated from the residue by gravity filtration and then evaporated in vacuum. The non-polar

extract was resuspended in 5% Tween 80 in saline solution for administration to animals. This solution was considered as vehicle in all experiments.

Procedure to induced cerebral ischemia

To induce cerebral ischemia we used a sequential carotid artery sectioning model (SCAS, Rodriguez et al., 2000). Adult male CFW mice weighing 30 g and aged 40 weeks were obtained from our breeding facilities and housed in a temperature-controlled room ($22 \pm 2^{\circ}\text{C}$, relative humidity $55 \pm 3\%$) with an automatically timed 12 h light/dark cycle. According to the SCAS model, mice were lightly anesthetized with ether, and the left common carotid artery was exposed through a midline neck incision, separated from the associated vagus and sympathetic nerves, and sectioned between ligatures. After surgery, the incision was closed and mice were kept in a recovery environmentally controlled room and weighed daily. Thirty two days later, the right common carotid artery was sectioned as described above.

Experimental groups

Animals were randomly assigned to four groups of 26 animals each, three of them with cerebral ischemia (ischemic groups) and the other without cerebral ischemia (sham-group); in which, mice were anesthetized and full surgical procedures were done, except for artery section. Fifteen minutes after the second surgery, one group of ischemic animals was administered intraperitoneally with vehicle (5% Tween 80 in saline solution) (ISCH-VEH), another with 300 mg/kg of aqueous (ISCH-AQUO) extract; and another with 300 mg/kg of hexane extract (ISCH-HEX) of *Tilia americana* var. *mexicana*. Doses of the extracts were selected based on a study in which 300 mg/kg, i.p. in rats showed analgesic activity in the pain-induced functional impairment model (PIFIR) without inducing neurological alterations (Martínez et al., 2009).

This study was carried out in accordance with the Declaration of Helsinki, in compliance with the Official Mexican Standard animal care and management, and approved by the corresponding local ethics committees under projects numbers 022-2012 (Facultad de Medicina, UNAM) and NC093280.2 (Instituto Nacional de Psiquiatría).

Determination of neurological deficit and mortality induced by cerebral ischemia

After the second surgery, the resulting neurological deficit was evaluated in all groups at 24, 48 and 72 h. Neurobehavioral alterations were graded using the scale denominated neurological disability status scale (NDSS), which depicts the presence, severity, and progression of functional impairment after brain ischemia. This scale has 10 progressive grades, from 0 to 10, where zero indicates no neurological dysfunction; 2 represents a slight decrease in motility and the presence of passivity; 4 represents moderate neurological dysfunction and includes findings such as moderate hypomotility, flattened posture, hunched back, lateralized posture, ataxic gait, tremors, decreased body tone, muscle weakness, and slight motor incoordination; 6 corresponds to animals with more incapacity but still able to walk, with marked hypomotility, circling jerks or convulsions, front limb flexion, and moderate motor incoordination; 8 corresponds to animals with respiratory distress and severe or total motor incoordination; finally, 10 corresponds to death. At each time, six animals were sacrificed, and their brains were obtained to determine the infarct area. Also, the survival percentage was scored continuously.

Determination of infarct area

To obtain the infarct area, animals were anesthetized and sacrificed by decapitation. Their brains were quickly removed and cut into coronal slices of 2 mm. To reveal the infarct area, the brain slices were immersed in a 2% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) in phosphate buffer at 37°C for 30 min. They were then fixed with 8% paraformaldehyde (pH 7.4) and the unstained area in slices 3 and 4 was measured using Gel Doc™ System Bio Rad (program Quantity One of Bio Rad, version 4. 2. 1) by outlining the margins of this area. Total infarct area was determined by adding the infarct areas of two slices (3 and 4) for 6 to 12 animals per group and was expressed as percent of the infarct area.

Data analysis

Neurological scores for the different treatment groups at the various times were compared to the vehicle-treated group using two-way ANOVA followed by Bonferroni's test. Survival curves were calculated for each group with the Kaplan-Meier method and compared using the log-rank test. Significant differences between infarct areas were determined using one-way ANOVA followed by Dunnett's test. In all cases, a probability value less than 0.05 ($P < 0.05$) indicated statistical significance. Analyses were carried out using Graph Pad Prism version 3.02 for Windows (San Diego, CA).

Reference

Martínez AL, González-Trujano ME, Aguirre-Hernández E, Moreno J, Soto-Hernández M, López-Muñoz FJ. 2009. Antinociceptive activity of *Tilia americana* var. *mexicana* inflorescences and quercetin in the formalin test and in arthritic pain model in rats. *Neuropharmacol.* 56:564-571.

Figures

Figure S1. a. Degree of neurological dysfunction (NDSS score) in SHAM animals and mice subjected to cerebral ischemia with vehicle (VEH); aqueous (AQUO); or hexane (HEX) extracts of *T. americana* (n=20). Calculations as described in the supplementary material. Points represent means $\pm$ s.e.m. * $P < 0.05$, significant difference from ISCH-VEH group after two-way ANOVA followed by Bonferroni's test. b. Survival curves calculated by the Kaplan-Meier method and compared using the logrank test.

Figure S2. a. Percentage of infarct area in SHAM animals and in mice subjected to cerebral ischemia after administration of vehicle (VEH), aqueous (AQUO) or hexane (HEX) extracts of *T. americana* (n=6-12). Bars represent means $\pm$ s.e.m. *P<0.05, significant difference from ISCH-VEH group after one-way ANOVA followed by Dunnett's test. b. Representative photographs of the coronal section showing the motor cortex and striatum, vulnerable regions to damage induced by cerebral ischemia in the SCAS model.