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ORIGINAL ARTICLE

Biochemical and molecular modulation of CCl₄-induced peripheral and central damage by *Tilia americana* var. *mexicana* extracts

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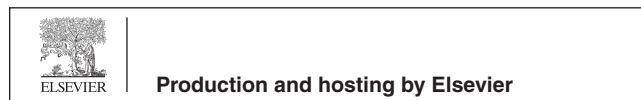
Abbreviations: Ac.E, ethyl acetate extract group; ALB, serum albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; Aq.E, aqueous extract group; Aq.E + CCl₄, aqueous extract-CCl₄ group; AST, aspartate aminotransferase; BACT, β-actin; BB, total bilirubin; bp, base pair; CCl₄, carbon tetrachloride; CCl₃, trichloromethyl; CCl₃OO•, trichloromethylperoxy radical; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; CREA, creatinine; CK, creatine kinase; COX-2, cyclooxygenase; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; EDTA, ethylenediaminetetraacetic acid disodium salt; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; GST, glutathione-S-transferase; HO-1, heme oxygenase-1; H₂O₂, hydrogen peroxide; He.E, hexane extract group; He.E + CCl₄, hexane extract-CCl₄ group; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal administration; MDA, malondialdehyde; Me.E, methanol extract group; Me.E + CCl₄, methanol extract-CCl₄ group; NADPH, nicotinamide adenine dinucleotide phosphate; NBT, nitro blue tetrazolium; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor erythroid-derived 2-like 2; O.O, olive oil group; oxo8-dG, 8-hydroxy-2'-deoxyguanosine; Var., variant; PPARγ, peroxisome proliferator-activated receptor gamma; RNA, ribonucleic acid; ROS, reactive oxygen species; SOD, superoxide dismutase; SOD1, superoxide dismutase-1; SOD2, superoxide dismutase-1; TNF-α, tumor necrosis factor; UK, United Kingdom; USA, United States of America; γ-GLOB, γ-globulin

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KEYWORDS

Tilia americana var. *mexicana*;

Oxidative stress;

Antioxidant;

CCl₄-induced damage;

Neuroprotective effects;

Hepatoprotective effects

Abstract Around the world, species from the genus *Tilia* are commonly used because of their peripheral and central medicinal effects; they are prepared as teas and used as tranquilizing, anti-convulsant, and analgesic agents. In this study, we provide evidence of the protective effects of organic and aqueous extracts (100 mg/kg, i.p.) obtained from the leaves of *Tilia americana* var. *mexicana* on CCl₄-induced liver and brain damage in the rat. Protection was observed in the liver and brain (cerebellum, cortex and cerebral hemispheres) by measuring the activity of antioxidant enzymes and levels of malondialdehyde (MDA) using spectrophotometric methods. Biochemical parameters were also assessed in serum samples from the CCl₄-treated rats. The *T. americana* var. *mexicana* leaf extracts provided significant protection against CCl₄-induced peripheral and central damage by increasing the activity of antioxidant enzymes, diminishing lipid peroxidation, and preventing alterations in biochemical serum parameters, such as the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ -globulin (γ -GLOB), serum albumin (ALB), total bilirubin (BB), creatinine (CREA) and creatine kinase (CK), relative to the control group. Additionally, we correlated gene expression with antioxidant activity in the experimental groups treated with the organic and aqueous *Tilia* extracts and observed a non-statistically significant positive correlation. Our results provide evidence of the underlying biomedical properties of *T. americana* var. *mexicana* that confer its neuro- and hepatoprotective effects.

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1. Introduction

The *Tilia* genus belongs to the Tiliaceae family, which consists of 25–80 species, which are mainly distributed in Europe and Asia, with a few species in North America (Hardin, 1990; Pavón and Rico-Gray, 2000). In Mexico, *Tilia americana* var. *mexicana* is known locally as “cyrimbo”, “flor de tila”, “sirima”, “tirimo”, “jonote” or “tilio”, due to its geographical distribution, which is confined to the lower mountainous forest that covers less than 1% of the Mexican territory (Flores et al., 1971; González-Espinosa et al., 2011). Phytochemical studies have demonstrated that members of the *Tilia* genus synthesize hydrocarbons, esters, aliphatic acids, polyphenols, and terpenoids (Pietta et al., 1993; Buchbauer et al., 1995; Matsuda et al., 2002).

Aguirre-Hernández et al. showed that polar extracts from *Tilia* inflorescences are composed of abundant quercetin and kaempferol glycosides (Aguirre-Hernández et al., 2010). Quercetin and kaempferol are common dietary flavonoids and are of particular interest as antioxidants because they possess a 3-hydroxyl group with a relatively low oxidation potential; this hydroxyl group is oxidized irreversibly, thus avoiding redox cycling (Duthie and Morrice, 2012). Recently, we demonstrated the antioxidant effects of different *Tilia* extracts, particularly the methanol and aqueous extracts, *in vitro* using a spectrophotometric technique (Carmona-Aparicio et al., 2014; Cárdenas-Rodríguez et al., 2014). Antioxidant and radical scavengers have been studied with respect to the mechanism of carbon tetrachloride (CCl₄) toxicity and the protection of liver cells from CCl₄-induced damage by lipid peroxidation (Weber et al., 2003; Manibusan et al., 2007) as well as in the presence of different antioxidant enzymes. Treatment with CCl₄ has been shown to reduce the gene expression of antioxidant enzymes, and many natural products are capable of reversing these effects. As examples, de Oliveira and Silva et al. reported that the phenolic fraction of *Halimeda opuntia* (Linnaeus) Lamourox tetrahydrofuran extract

reversed the gene expression changes and the decrease in catalase (CAT) and superoxide dismutase (SOD) activity induced by CCl₄ in the livers of rats (de Oliveira e Silva et al., 2012). Manubolu et al. (1997) showed that an ethanol extract of *Actinopterys radiata* reversed the effects of CCl₄ on the gene expression of not only CAT and SOD but also glutathione peroxidase (GPx), in the livers of rats (Manubolu et al., 1997). The authors mentioned that rutin is one of eight phenolic derivatives involved. Chen et al. (2014) found that the diterpenoid andrographolide prevented gene expression alterations in Cu/Zn-SOD, glutathione-S-transferase (GST) and hemeoxygenase-1 (HO-1) caused by CCl₄-induced damage in the livers, hearts and kidneys of rats (Chen et al., 2014). Moreover, El-Sayed et al. (2015) showed that a *Cichorium intybus* root extract reversed the decrease in CAT and GPx gene expression in this model of damage (El-Sayed et al., 2015).

This evidence supports the hypothesis that natural products derived from components of vegetal species possess considerable potential as antioxidant and radical scavengers to promote health. Nevertheless, it is important to determine which type of plants displays this activity and to what extent. In the present study, we provide evidence of the potential protective effect of a *T. americana* var. *mexicana* extract in inhibiting CCl₄-induced peripheral and central damage in rats by evaluating the effects of this extract in the livers and brains of rats using various enzymatic markers of damage as well as biochemical and molecular parameters.

2. Materials and methods**2.1. Animals**

Male Wistar rats weighing 180–220 g were used in this study. The experimental protocol (INP, number 04/2013) followed the guidelines of the Norma Oficial Mexicana Guide for the use and care of laboratory animals (NOM-062-ZOO-1999)

and for the disposal of biological residues (NOM-087-ECOL-1995). The housing room was maintained under constant conditions of temperature (21 ± 1 °C), relative humidity (50–60%) and lighting (12-h light/dark cycle). Filtered air (5-mm particle filter) was exchanged 18 times per hour. The animals were provided with a standard commercial rat chow diet (Harlan Teklad Global diet 2018S sterilized, Harlan Teklad, Madison, USA) and reverse osmosis-filtered water.

2.2. Chemicals

Xanthine, nitro blue tetrazolium (NBT), 3,3'-diaminobenzidine, bovine serum albumin, xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH), oxidized glutathione (GSSG), reduced glutathione (GSH), trimethoxypropane, 5,5-dimethyl-1-pyrrolin-N-oxide (DMPO), hypoxanthine, glutathione reductase (GR), and 2,4-dinitrophenylhydrazine, 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma (St. Louis, MO, USA). Ethylenediaminetetraacetic acid disodium salt (EDTA), ammonium sulfate, and copper chloride were purchased from JT Baker (Mexico City, Mexico). Hydrogen peroxide (H₂O₂) formaldehyde, and sodium carbonate were purchased from Mallinckrodt (Paris, KY, USA). Sodium azide was purchased from Merck (Mexico City, Mexico). Trizol reagent, molecular weight markers, agarose, ethidium bromide solution, and synthesized primers (endogenous and antioxidant enzymes) were purchased from Invitrogen (Carlsbad, CA, USA) and the KAPA SYBR FAST qPCR Master Mix kit was purchased from Kappa Biosystems (Kappa Biosystems, London, UK).

2.3. Plant material

T. americana var. *mexicana* (Schltdl.) Hardin (Tiliaceae) was collected in Puebla, Mexico, in June 2010. Susana Valencia-Avalos identified the species, and a voucher specimen of the plant (number 131,613) was deposited in the herbarium of the Faculty of Sciences, National Autonomous University of Mexico, Mexico City, for future reference.

2.4. Preparation of the plant extract

Polarity-specific *T. americana* var. *mexicana* (Tilia) extracts were prepared to examine antioxidant potential as a function of the nature of the constituents (Aguirre-Hernández et al., 2010; Cárdenas-Rodríguez et al., 2014), as follows: *Tilia* leaves were air-dried, powdered (1.8 kg) and successively extracted with hexane (4 L × 3), methanol (4 L × 3), and ethyl acetate (4 L × 3) by maceration at room temperature (22 °C). The solvents were separated from the residues by gravity filtration and then evaporated in a vacuum. The final crude extract yield was calculated as a percentage of the total dry weight (% d.w.) and included the following: 3.0 g of hexane extract (0.95%), 29.8 g of methanol crude extract (9.4%), and 3.01 g of ethyl acetate extract (1.4%). In the case of the aqueous extracts, air-dried, powdered leaves (26 g) were infused in boiling water (500 mL) for 10 min. The resulting extract was separated from its residue by gravity filtration; samples were frozen in liquid nitrogen and freeze-dried for 12 h in a Heto FD3 Lab lyophilizer. The final crude aqueous extract consisted of 3.58 g of extract (13.76% d.w.).

2.5. CCl₄-induced damage in rats

Extract doses were determined according to previous studies that demonstrated biological effects (Cárdenas-Rodríguez et al., 2014). The animals were divided into 10 groups of three rats. Each group received one treatment as follows:

Olive oil group: olive oil 0.1 mL kg⁻¹ orally (p.o.), for 6 days (O.O).

Hexane extract group: hexane extract 100 mg kg⁻¹/olive oil 0.1 mL kg⁻¹ (p.o.), for 6 days (He.E).

Ethyl acetate extract group: ethyl acetate extract 100 mg kg⁻¹/olive oil 0.1 mL kg⁻¹ (p.o.), for 6 days (Ac.E).

Methanol extract group: methanol extract 100 mg kg⁻¹/olive oil 0.1 mL kg⁻¹ (p.o.), for 6 days (Me.E).

Aqueous extract group: aqueous extract 100 mg kg⁻¹/PBS 0.1 mL kg⁻¹ (p.o.), for 6 days (Aq.E).

Carbone tetrachloride group: CCl₄ 1.5 mL kg⁻¹/olive oil 0.1 mL kg⁻¹ (i.p.), the last 3 days of the total treatment (CCl₄).

Hexane extract-CCl₄ group: hexane extract 100 mg kg⁻¹/olive oil 0.1 mL kg⁻¹ (p.o.), for 6 days + CCl₄ 1.5 mL/kg/olive oil 0.1 mL/kg (i.p.), the last 3 days of this treatment (He.E + CCl₄).

Ethyl acetate extract-CCl₄ group: ethyl acetate extract 100 mg kg⁻¹/olive oil 0.1 mL kg⁻¹ (p.o.), for 6 days + CCl₄ 1.5 mL kg⁻¹/olive oil 0.1 mL kg⁻¹ (i.p.), the last 3 days of this treatment (Ac.E + CCl₄).

Methanol extract-CCl₄ group: methanol extract 100 mg kg⁻¹/PBS 0.1 mL kg⁻¹ (p.o.), for 6 days + CCl₄ 1.5 mL kg⁻¹/olive oil 0.1 mL kg⁻¹ (i.p.), the last 3 days of this treatment (Me.E + CCl₄).

Aqueous extract-CCl₄ group: aqueous extract 100 mg kg⁻¹/PBS 0.1 mL kg⁻¹ (p.o.), for 6 days + CCl₄ 1.5 mL kg⁻¹/olive oil 0.1 mL kg⁻¹ (i.p.), the last 3 days of this treatment (Aq.E + CCl₄).

Forty-eight hours after the end of the 6 days of treatment, the animals were anesthetized with pentobarbital (0.6 μL kg⁻¹, i.p.), and blood was collected via heart puncture. Then, the liver and brain (cerebellum, cortex and cerebral hemispheres) were immediately excised and stored at -70 °C until analysis of antioxidant enzymes and lipid peroxidation.

2.6. Preparation of tissue homogenates

Tissues were homogenized in phosphate buffer (0.1 M, pH 7.0) with 0.1% Triton X-100 using a Brinkmann Polytron model PT 2000 (Westbury, USA) and centrifuged at 19,000 × g for 10 min. The supernatant was used to quantify total protein by Lowry's method (Lowry et al., 1951) and evaluation of the activity of antioxidant enzymes, such as SOD, CAT, GPx, GR, and glutathione transferase (GST).

2.7. Serum biochemical markers of tissue injury

Blood samples were incubated for 45 min at room temperature. Blood serum was separated by centrifugation at 600 × g for 15 min and then stored at -20 °C. The activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-globulin (γ-GLOB), serum albumin (ALB), total bilirubin (BB), creatinine (CREA) and creatine kinase (CK) was measured according to the methods recommended by the International Federation of Clinical

Chemistry (Bergmeyer et al., 1978) using an autoanalyzer (Dimension AR, Dade Behring Inc., Newark, USA).

2.8. Lipid peroxidation analysis

The concentration of malondialdehyde (MDA) in the whole-tissue homogenates was measured using a trimethoxypropane standard curve. The reaction mixture consisted of 0.026 M TBA, 0.211 M HCl, 6.66% trichloroacetic acid and 1 mM deferoxamine mesylate. Each of the different homogenates (200 μ L) was added to 1 mL of the reaction mixture, and these mixtures were vortexed vigorously and heated at 100 °C for 10 min. The mixtures were cooled, and 1 mL of n-butanol-pyridine (15:1) was added. After the mixtures were centrifuged at 1200 \times g for 10 min, the organic layers were separated, and the absorbance was measured at 530 nm. MDA is an end product of lipid peroxidation that reacts with TBA to form TBARS. The TBARS level was expressed as nmol of MDA/mg of protein (Coballase-Urrutia et al., 2011).

2.9. Antioxidant enzyme activity

CAT assay: CAT activity was assayed at 25 °C using a method based on the decomposition of H₂O₂ (Coballase-Urrutia et al., 2011). Diluted homogenate (1:40; 5 μ L) was added to 720 μ L of 30 mM H₂O₂, in 10 mM potassium phosphate solution; the reaction was monitored at 240 nm. Under these conditions, the decomposition of H₂O₂ by the CAT contained in the samples exhibits first-order reaction kinetics according to the equation $k = 2.3/t \log(A_0/A)$, where k is the first-order reaction rate constant, t is the time period over which the decrease in H₂O₂ as a result of CAT activity was measured (15 s), and A_0 and A are the optical densities at 0 and 15 s, respectively. The results were expressed as k /mg protein.

SOD assay: SOD activity was assayed using a previously reported method (Coballase-Urrutia et al., 2011). A competitive inhibition assay was performed using the xanthine-xanthine oxidase system to reduce NBT. In a final volume of 166 μ L, the reaction mixture contained the following: 0.122 mM EDTA, 30.6 μ M NBT, 0.122 mM xanthine, 0.006% bovine serum albumin and 49 mM sodium carbonate. The liver homogenate (1:50 dilution; 33 μ L) was added to the reaction mixture, followed by 30 μ L of a xanthine oxidase solution to a final concentration of 2.5 U/L. This reaction mixture was then incubated at room temperature for 30 min. The reaction was stopped with 66 μ L of 0.8 mM cupric chloride, and the optical density was read at 560 nm. Complete NBT reduction (100%) was obtained in a tube in which the sample was replaced by distilled water. The amount of protein that inhibited NBT reduction by 50% was defined as one unit of SOD activity. The results were expressed as U/mg protein.

GPx assay: GPx activity was assayed using a previously described method (Coballase-Urrutia et al., 2011). The reaction mixture consisted of 50 mM potassium phosphate solution (pH 7.0), 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 25 U/mL GR, and 1 mM GSH at 25 °C. Liver homogenate, diluted 1:10 (100 μ L), was added to 800 μ L of the reaction mixture. The reaction mixture was then allowed to incubate for 5 min at room temperature before the reaction was initiated by the addition of 32 μ L of 2.5 mM H₂O₂ solution. The absorbance at 340 nm was recorded for 3 min, and

the activity was calculated from the slope of these recordings as μ mol of NADPH oxidized per min, taking into account that the millimolar absorption coefficient of NADPH is 6.22. The values from blank reactions in which the homogenate was replaced by distilled water were subtracted from the experimental values in each assay. One unit of GPx activity was defined as the amount of enzyme that oxidized 1 μ mol of NADPH per min. The results were expressed as U/mg protein.

GR assay: GR activity was assayed using GSSG as the substrate and by measuring the oxidation of NADPH at 340 nm (Coballase-Urrutia et al., 2011). The reaction mixture consisted of 0.1 M potassium phosphate (pH 7.6), 0.5 mM EDTA, 1.25 mM NADPH, and 0.5 mM GSSG at 25 °C. Diluted homogenates (1:5; 25 μ L) were added to 475 μ L of the reaction mixture. The absorbance at 340 nm was recorded for 3 min, and the activity was calculated from the slope of these recordings as μ mol of NADPH oxidized per min, taking into account that the millimolar absorption coefficient of NADPH is 6.22. One unit of GR activity was defined as the amount of enzyme that oxidized 1 μ mol of NADPH per min. The data were expressed as U/mg protein.

GPx assay: GPx activity was assayed using a previously described method (Coballase-Urrutia et al., 2011). The reaction mixture consisted of 50 mM potassium phosphate solution (pH 7.0), 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 25 U/mL GR, and 1 mM GSH at 25 °C. Liver homogenate, diluted 1:10 (100 μ L), was added to 800 μ L of the reaction mixture. The reaction mixture was then allowed to incubate for 5 min at room temperature before the reaction was initiated by the addition of 32 μ L of 2.5 mM H₂O₂ solution. The absorbance at 340 nm was recorded for 3 min, and the activity was calculated from the slope of these recordings as μ mol of NADPH oxidized per min, taking into account that the millimolar absorption coefficient of NADPH is 6.22. The values from blank reactions in which the homogenate was replaced by distilled water were subtracted from the experimental values in each assay. One unit of GPx activity was defined as the amount of enzyme that oxidized 1 μ mol of NADPH per min. The results were expressed as U/mg protein.

2.10. Gene expression of antioxidant enzymes

2.10.1. Isolation of total RNA from liver and brain tissue samples

Tissue samples (100 mg) from the brain (cortex, cerebral hemispheres and cerebellum) and liver from treated rats were separated and homogenized individually in Trizol reagent. Total cellular RNA was extracted according to the manufacturer's protocol. The concentration of total RNA in each sample was measured by a Nanodrop Spectrophotometer (Delaware, USA) using the ratio of 260 nm/280 nm. The quality of the isolated RNA was assessed based on the integrity of the 28S, 18S, and 5S bands after ethidium bromide electrophoresis of a 1% formaldehyde denaturing gel. The gels were visualized under a UV transilluminator (EDAS 290 KODAK, New Haven). All extracted RNA was stored at -80 °C (Bandala et al., 2012).

2.10.2. Quantitative real-time PCR assay

Specific oligonucleotides were designed and optimized at 59 °C for the SOD1, SOD2 GPx, CAT and GR genes and for the following reference genes: 18S ribosomal subunit, glyceraldehyde-

Table 1 Primer sequences for the endogenous genes and for SOD1, SOD2, GPx, CAT and GR. From left to right: gene name, primer sequence and fragment size.

Symbol	Nucleotide sequence Forward primer Reverse primer	Amplicon size (base pair, bp)	Accession number
18S	GTAACCCGTTTGAACCCCAT CCATCCAATCGGTAGTAGCG	151	NR_003286
GAPDH	AGT TCA ACG GCA CAG TCA AG GGT GAA GAC GCC AGT AGA CTC	175	NM_002046
G6PDH	GATGATCCAGCCTCCTACAAG GGGCTTCTCCACTATGATGC	159	NM_000402
BACT	GGACGATATGGAGAAGATTTGG AGAGGCATACAGGGACAACAC	199	NM_001101
SOD1	AAGAAACATGGCGGTCCAG CCACCATAGTACGGCCAATG	163	NM_000454
SOD2	TGTGTCTGTGGGAGTCCAAG GTGCTCCCACACATCAATCC	155	NM_000636
GPx	CGATACGCCGAGTGTGGTTT GGGCATCGTCCCCATTTAC	158	NM_000581
CAT	GGCACACTTTGACAGAGAGCG AATCGGACGGCAATAGGAGTC	155	NM_001752
GR	AAGCGGGATGCTTACGTGAG CAGGATGTGCGGAGCAGTG	160	NM_000637

Table 2 Effects of the *Tilia americana* var. *mexicana* leaf extracts on biochemical serum parameters after CCl₄-induced peripheral and central damage in Wistar rats.

Group	AST (U/L)	ALT (U/L)	ALP	γ-GLOB	ALB	BB
O.O	120.0 ± 1.32	65.0 ± 5.21	212.1 ± 7.01	7.9 ± 1.10	1.11 ± 0.03	0.15 ± 0.05
He.E	181.2 ± 1.09	76.61 ± 1.10	198.5 ± 1.56	5.9 ± 1.45	0.99 ± 0.01	0.14 ± 0.01
Act.E	125.2 ± 0.65	70.34 ± 1.23	158.3 ± 2.90	7.0 ± 1.14	0.81 ± 0.04	0.11 ± 0.02
Me.E	196.5 ± 1.21	80.11 ± 2.34	200.1 ± 1.32	3.45 ± 1.23 ⁺	0.88 ± 0.05	0.18 ± 0.01
Aq.E	199.78 ± 2.5	90.08 ± 1.56	212.5 ± 2.10	3.01 ± 1.34 ⁺	0.72 ± 0.02 ⁺	0.22 ± 0.03 [■]
He.E + CCl ₄	198.6 ± 6.81	82.6 ± 5.42	207.1 ± 11.3	6.9 ± 3.02	0.98 ± 0.02	0.19 ± 0.05
Act.E + CCl ₄	138.5 ± 5.04	79.0 ± 3.11	161.0 ± 12.4	8.1 ± 1.02	1.01 ± 0.05	0.17 ± 0.01
Me.E + CCl ₄	212.5 ± 9.11	88.7 ± 1.91	218.0 ± 6.13	4.25 ± 1.0 ⁺	0.90 ± 0.05	0.21 ± 0.02
Aq.E + CCl ₄	250.0 ± 12.2	97.0 ± 9.25	232.3 ± 7.71	3.75 ± 1.0 ⁺	0.70 ± 0.02 ⁺	0.26 ± 0.10
CCl ₄	734.6 ± 6.0*	366.0 ± 6.15*	260.9 ± 3.0*	2.03 ± 1.01*	0.63 ± 0.05*	0.31 ± 0.11*

Aspartate aminotransferase (AST; U/L), alanine aminotransferase (ALT; U/L), alkaline phosphatase (ALP; U/L), γ-globulin (γ-GLOB; g/dL), serum albumin (ALB; g/dL), total bilirubin (BB; μmol/L). CCl₄ effect was compared against all groups. Its effect was significantly different in all groups but with different statistical significances in each biochemical serum parameter measured: **p* < 0.0001 vs all groups (in AST); **p* < 0.0001 against vs all groups (in ALT); **p* < 0.0001 vs all groups (in ALP); **p* < 0.0001 vs O.O, He.E, Act.E, He.E + CCl₄ and Act.E + CCl₄ groups and ⁺*p* < 0.05 vs Me.E, Aq.E, Me.E + CCl₄ and Aq.E + CCl₄ groups (in γ-GLOB); **p* < 0.0001 vs O.O, He.E, Act.E, Me.E, He.E + CCl₄, Act.E + CCl₄ and Me.E + CCl₄ groups and ⁺*p* < 0.05 vs Aq.E and Aq.E + CCl₄ groups (in ALB); **p* < 0.0001 vs O.O, He.E, Act.E, Me.E, He.E + CCl₄, Act.E + CCl₄, Me.E + CCl₄ and Aq.E + CCl₄ groups and [■]*p* < 0.01 vs Aq. E group (in BB). Each quantification was performed twice in triplicate on samples from three rats, and the values represent the mean ± SD. O.O, group receiving olive oil; He.E, group receiving hexane extract; Act.E, group receiving ethyl acetate extract; Me.E, group receiving methanol extract; and Aq.E, group receiving aqueous extract.

3 phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PDH) and β-actin (BACT) (Table 1). The sequences were obtained from GenBankTM. Endogenous gene expression (G6PDH) was validated with BestKeeper software (Pfaffl et al., 2002). The RT-PCR conditions were optimized with a thermal cycle (gradient Px2 Thermal Cycler Hybaid, Franklin, MA). The reaction mixture contained 10 μL of KAPA SYBR FAST qPCR Master Mix[®] (2X), 0.4 μL of dUTP (10 mM), 0.4 μL of ROX Low, 0.4 μL of KAPA RT Mix (50X), 4 μL of free water, 0.4 μL of sense primers and 0.4 μL of antisense primers (10 mM) and 4 μL of total RNA

(10 ng/μL). Reverse transcription was performed at 52 °C by 5 min. PCR was performed at 95 °C by 2 min; followed of 35 cycles at 95 °C by 3 s, 60 °C by 10 s, data melting temperature of 60–90 °C; and finally, 30 °C by 2 min. The sizes of the PCR products are shown in Table 1. The parameters of the amplifications, such as temperature, primer concentrations, dNTPs and volumes, were transferred to the amplification protocol in real time with the Rotor Gene 6.0 detection system (Corbett Life Science, Sydney City, Australia). The amplification products of the RT-PCR were separated by electrophoresis on 2% agarose gel and analyzed using the electrophoresis EDAS 290

Table 3 Effects of different extracts of *Tilia americana* var. *mexicana* leaves on antioxidant enzymes in the liver of Wistar rats treated with CCl₄ at a dose of 1.5 mL/kg for three days.

Group	CAT k/mg/protein	SOD U/mg/protein	GPx U/mg/protein	GR U/mg/protein	GST U/mg/protein	MDA nM/mg/protein
O.O	0.80 ± 0.09 [▲]	12.66 ± 0.88 [▲]	0.90 ± 0.004	0.172 ± 0.030	0.158 ± 0.027	1.01 ± 0.16
He.E	0.88 ± 0.09 ⁺	10.98 ± 1.29 [▲]	0.81 ± 0.006	0.163 ± 0.020	0.111 ± 0.033 ⁺	2.48 ± 0.24
Act.E	0.73 ± 0.07	8.29 ± 1.85 ⁺	0.71 ± 0.007	0.159 ± 0.003	0.122 ± 0.017 [▲]	2.53 ± 0.46
Me.E	0.72 ± 0.07	13.05 ± 0.44 [■]	0.77 ± 0.006	0.161 ± 0.018	0.114 ± 0.010 [▲]	2.41 ± 0.20
Aq.E	0.73 ± 0.06	11.20 ± 1.06	0.66 ± 0.007	0.162 ± 0.019	0.151 ± 0.052 ⁺	1.29 ± 0.47
He.E + CCl ₄	0.64 ± 0.08	8.41 ± 1.82 [■]	0.66 ± 0.008	0.150 ± 0.009 ⁺	0.106 ± 0.05 ⁺	2.74 ± 0.08
Act.E + CCl ₄	0.68 ± 0.06 [▲]	10.76 ± 0.88 [■]	0.69 ± 0.010	0.149 ± 0.020 ⁺	0.096 ± 0.021 [▲]	1.10 ± 0.12
Me.E + CCl ₄	0.66 ± 0.06 [▲]	8.01 ± 0.99 [■]	0.64 ± 0.011	0.147 ± 0.013 ⁺	0.089 ± 0.011 [▲]	1.54 ± 0.34
Aq.E + CCl ₄	0.60 ± 0.08 ⁺	7.08 ± 0.057 [■]	0.50 ± 0.009	0.138 ± 0.021 [▲]	0.128 ± 0.027 [▲]	0.68 ± 0.22
CCl ₄	0.30 ± 0.09 [*]	4.41 ± 1.54 [*]	0.37 ± 0.008 [*]	0.095 ± 0.009 [*]	0.078 ± 0.006 [*]	8.08 ± 0.08 [*]

CCl₄ effect was compared against all groups. Its effect was significantly different in all groups but with different statistical significances in each biochemical parameter measured: ^{*}*p* < 0.0001 vs Act.E, Me.E, Aq.E, and He.E + CCl₄ groups, ⁺*p* < 0.001 vs He.E and Aq.E + CCl₄ groups and [▲]*p* < 0.05 vs O.O, Act. E + CCl₄ and Me.E + CCl₄ groups (in CAT); ^{*}*p* < 0.0001 vs Aq.E group, ⁺*p* < 0.001 vs Act.E group, [■]*p* < 0.01 vs Me.E, He.E + CCl₄, Act.E + CCl₄, Me.E + CCl₄ and Aq.E + CCl₄ groups and [▲]*p* < 0.05 vs O.O and He.E groups (in SOD); ^{*}*p* < 0.0001 vs all groups (in GPx); ^{*}*p* < 0.0001 vs O.O, He.E, Act.E, Me.E and Aq.E, groups, ⁺*p* < 0.001 vs He.E + CCl₄, Act.E + CCl₄ and Me.E + CCl₄, groups and [▲]*p* < 0.05 vs Aq.E + CCl₄ group (in GR); ^{*}*p* < 0.0001 vs O.O group, ⁺*p* < 0.001 vs He.E, Aq.E and He.E + CCl₄ groups and [▲]*p* < 0.05 vs Act.E, Me.E, Act.E + CCl₄, Me.E + CCl₄, and Aq.E + CCl₄ groups (in GST); ^{*}*p* < 0.0001 vs all groups (in MDA). Each quantification was performed twice in triplicate on samples from three rats, and the values represent the mean ± SD. O.O, group receiving olive oil; He.E, group receiving hexane extract; Act.E, group receiving ethyl acetate extract; Me.E, group receiving methanol extract; and Aq.E, group receiving aqueous extract.

analysis system (Floriano-Sánchez et al., 2009; Bandala et al., 2012).

2.10.3. Quantification of SOD1, SOD2, GPx, CAT and GR mRNA

The CP values (CP is defined as the number of cycles in which the fluorescence intensity increased above the baseline fluorescence of the sample), of the endogenous candidate genes (GAPDH, G6PDH, BACT and 18S) and the SOD1, SOD2, GPx, CAT and GR genes were exported from Rotor-Gene 6.0 software (Corbett Life Science, Sydney City, Australia) to calculate efficiencies using the REST[®] statistical model (Tinzl et al., 2004; Floriano-Sánchez et al., 2009), and the data were plotted to construct a linear regression to compare the logarithmic concentration (total RNA) against the CP. To correlate the expression of the candidate endogenous genes and to determine the more stably expressed genes, the BestKeeper software was used to export the CP. The Excel tool of the Rotor-Gene 6.0 software was used to show the melting temperature (Tm) of each amplified product. HKG expression was analyzed using the BestKeeper statistical model, and the CP values were analyzed by Pearson correlation (Tinzl et al., 2004).

2.11. Statistics

The biochemical data were expressed as the mean ± SD and analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test (GraphPad Prism 4.0 software, San Diego, USA). *P* < 0.05 was considered statistically significant. For all of the samples, the absolute quantification data were normalized to the expression of G6PDH and were analyzed by Student's *t* test. Spearman's test was used to evaluate the correlations between antioxidant enzyme activity and enzyme gene expression.

3. Results

3.1. Effect of *Tilia* extracts on serum biochemical markers after CCl₄ damage

The administration of CCl₄ significantly increased serum levels of AST, ALT, ALP, CREA, BB, and CK and decreased levels of γ-GLOB and ALB. Pretreatment with non-polar (hexane), medium-polar (ethyl acetate) and polar (ethanol and aqueous) *Tilia* extracts enabled the recovery of all of the serum biochemical markers after CCl₄-induced damage. The medium-polar extract provided the greatest protection against the increase in AST, ALT, ALP, CRE, BB and CK levels and against the decrease in γ-GLOB and ALB levels that were induced by CCl₄; in terms of the effects of the extracts, this extract was followed by the non-polar and polar (ethanol and aqueous) extracts compared with the vehicle.

3.2. Effects of *Tilia* extracts on antioxidant enzymes and lipid peroxidation in liver

To better understand the mechanisms of protection against CCl₄-induced damage in the presence of *Tilia* extracts, we evaluated the cells' natural antioxidant defenses. Antioxidant activity was reduced in rats treated with CCl₄, as observed in the vehicle group. Pretreatment with the extracts prevented this 40–50% reduction in CAT, SOD, GPx and GR activity (Table 3).

The hexane and aqueous extracts provided the strongest protection for GST activity, followed by the ethyl acetate and methanol extracts. We also confirmed that CCl₄ administration increased the concentration of TBARS (expressed as MDA) in the liver by 87.5% compared to administration of vehicle (*p* < 0.0001) (Table 3). Administration of the hexane, ethyl acetate, methanol and aqueous extracts prior to

Table 4 Effect of *Tilia americana* var. *mexicana* leaf extracts on antioxidant enzyme activity in the cerebellum of Wistar rats during CCl₄-induced injury.

Group	SOD	CAT	GPx
O.O	76.31 ± 2.0	0.074 ± 0.005	0.040 ± 0.001
He.E	57.07 ± 1.4	0.064 ± 0.001	0.038 ± 0.002
Act.E	50.33 ± 1.7 [▲]	0.051 ± 0.005	0.030 ± 0.001
Me.E	55.75 ± 1.1	0.052 ± 0.003	0.034 ± 0.001
Aq.E	55.10 ± 1.8	0.059 ± 0.003	0.036 ± 0.001
He.E + CCl ₄	38.40 ± 1.3 ⁺	0.047 ± 0.002	0.035 ± 0.002
Act.E + CCl ₄	37.12 ± 1.3	0.053 ± 0.002	0.037 ± 0.002
Me.E + CCl ₄	50.98 ± 1.4 ⁺	0.060 ± 0.003	0.033 ± 0.002
Aq.E + CCl ₄	34.40 ± 1.5	0.065 ± 0.003	0.034 ± 0.001
CCl ₄	46.88 ± 1.7 [*]	0.028 ± 0.002 [*]	0.015 ± 0.001 [*]

CCl₄ effect was compared against all groups. Its effect was significantly different in all groups but with different statistical significances in each antioxidant activity measured: ^{*}*p* < 0.0001 vs O. O, He.E, Me.E, Aq.E, Act.E + CCl₄ and Aq.E + CCl₄ groups, ⁺*p* < 0.001 vs He.E + CCl₄ and Me.E + CCl₄ groups and [▲]*p* < 0.05 vs Act.E group (in SOD); ^{*}*p* < 0.0001 vs all groups (in CAT) and ^{*}*p* < 0.0001 vs all groups (in GPx). Each quantification was performed twice in triplicate on samples from three rats, and the values represent the mean ± SD. O.O, group receiving olive oil; He.E, group receiving hexane extract; Act.E, group receiving ethyl acetate extract; Me.E, group receiving methanol extract; and Aq.E, group receiving aqueous extract.

CCl₄-induced lipid peroxidation prevented 66.08%, 86.39%, 80.95% and 91.58% of the reduction, respectively.

3.3. Antioxidant enzyme activity in the brain

CCl₄-induced damage in the brain decreased the activity of antioxidant enzymes (*p* < 0.0001) (see Tables 4–6). In the cerebellum (Table 4), the methanol extract significantly protected SOD and GPx activity, whereas the aqueous extract protected CAT. In the cortex (Table 5), the methanol extract provided the greatest protection for SOD, CAT and GPx, with an increase of 23.53%, 23.53% and 5.46%, respectively, compared to the vehicle group. Similarly, in the cerebral hemispheres (Table 6), the methanol extract provided the strongest protection in the presence of SOD and CAT; however, the aqueous extracts also showed protective effects for GPx.

3.4. Brain lipid peroxidation

The hexane and ethyl acetate extracts significantly inhibited the CCl₄-induced brain damage to a similar extent, followed by the methanol and aqueous extracts. Lipid peroxidation in the cerebellum was modulated as follows: He.E + CCl₄, 81.16%; Act.E + CCl₄, 80.98%; Me.E + CCl₄, 64%; and Aq.E + CCl₄, 49.44%. The changes observed in the cerebral cortex were as follows: He.E + CCl₄, 68.59%; Act.E + CCl₄, 55.65%; Me.E + CCl₄, 42.03%; and Aq.E + CCl₄, 28.63%. The changes in the cerebral hemispheres were as follows: He.E + CCl₄, 55.42%; Act.E + CCl₄, 66.97%; Me.E + CCl₄, 46.65%; and Aq.E + CCl₄, 27.48% (see Table 7).

3.5. Correlations between antioxidant enzyme activity and gene expression

(A) In the brain: Gene expression was significantly increased by several folds among the evaluated groups, as described below. In the cerebral cortex: CAT in the O.O (11), He.E (5.6), Act.E (57.5), Me.E (7.8) and Aq.E (11.7) groups; SOD

Table 5 Effects of *Tilia americana* var. *mexicana* leaf extracts on antioxidant enzyme activity in the cortex of Wistar rats after CCl₄-induced damage.

Group	SOD	CAT	GPx
O.O	67.13 ± 1.08	0.017 ± 0.004	0.055 ± 0.006
He.E	57.07 ± 1.4	0.014 ± 0.003	0.053 ± 0.003
Act.E	50.33 ± 1.7	0.011 ± 0.002 [▲]	0.049 ± 0.003
Me.E	55.75 ± 1.6	0.013 ± 0.002	0.051 ± 0.001
Aq.E	35.40 ± 1.5	0.014 ± 0.003	0.055 ± 0.004
He.E + CCl ₄	37.17 ± 1.3	0.010 ± 0.001 [▲]	0.043 ± 0.002
Act.E + CCl ₄	50.98 ± 1.4	0.012 ± 0.003	0.048 ± 0.004
Me.E + CCl ₄	51.34 ± 1.2	0.013 ± 0.002	0.052 ± 0.005
Aq.E + CCl ₄	38.20 ± 1.9	0.010 ± 0.002 [▲]	0.051 ± 0.002
CCl ₄	28.88 ± 1.1 [*]	0.004 ± 0.001 [*]	0.018 ± 0.006 [*]

CCl₄ effect was compared against all groups. Its effect was significantly different in all groups but with different statistical significances in each antioxidant activity measured: ^{*}*p* < 0.0001 vs all groups (in SOD); ^{*}*p* < 0.0001 vs O.O, He.E, Me.E, Aq.E, Act.E + CCl₄ and Me.E + CCl₄ groups and [▲]*p* < 0.05 vs Act.E, He.E + CCl₄ and Aq.E + CCl₄ groups (in CAT); ^{*}*p* < 0.0001 vs all groups (in GPx). Each quantification was performed twice in triplicate on samples from three rats, and the values represent the mean ± SD. O.O, group receiving olive oil; He.E, group receiving hexane extract; Act.E, group receiving ethyl acetate extract; Me.E, group receiving methanol extract; and Aq.E, group receiving aqueous extract.

Table 6 Effects of *Tilia americana* var. *mexicana* leaf extracts on antioxidant enzyme activity in the cerebral hemispheres of Wistar rats with CCl₄-induced damage.

Group	SOD	CAT	GPx
O.O	67.13 ± 1.50	0.013 ± 0.001	0.046 ± 0.004
He.E	60.41 ± 1.02	0.012 ± 0.003	0.045 ± 0.003
Act.E	58.24 ± 1.21	0.010 ± 0.005	0.040 ± 0.002
Me.E	53.83 ± 1.42	0.011 ± 0.001	0.041 ± 0.002
Aq.E	50.96 ± 1.13	0.010 ± 0.001	0.043 ± 0.001
He.E + CCl ₄	53.64 ± 1.05	0.009 ± 0.002	0.039 ± 0.001
Act.E + CCl ₄	50.31 ± 1.72	0.007 ± 0.002	0.040 ± 0.002
Me.E + CCl ₄	49.85 ± 1.61	0.009 ± 0.003	0.042 ± 0.001
Aq.E + CCl ₄	44.43 ± 1.53	0.007 ± 0.001	0.044 ± 0.002
CCl ₄	38.04 ± 1.81 [*]	0.003 ± 0.001 [*]	0.011 ± 0.003 [*]

CCl₄ effect was compared against all groups. Its effect was significantly different in all groups but with different statistical significances in each antioxidant activity measured: ^{*}*p* < 0.0001 vs all groups (in SOD); ^{*}*p* < 0.0001 vs all groups (in CAT) and ^{*}*p* < 0.0001 vs all groups (in GPx). Each quantification was performed twice in triplicate on samples from three rats, and the values represent the mean ± SD. O.O, group receiving olive oil; He.E, group receiving hexane extract; Act.E, group receiving ethyl acetate extract; Me.E, group receiving methanol extract; and Aq.E, group receiving aqueous extract.

Table 7 Effect of *Tilia americana*. var. *mexicana* leaf extracts on MDA (nM/mg/protein) in the cerebellum, cortex and cerebral hemispheres of the brains of Wistar rats after CCl₄-induced brain injury.

Group	Cerebellum	Cortex	Cerebral hemispheres
O.O	0.045 ± 0.003	0.068 ± 0.001	0.108 ± 0.0003
He.E	0.148 ± 0.004	0.175 ± 0.002	0.124 ± 0.0001
Act.E	0.093 ± 0.003	0.174 ± 0.002	0.175 ± 0.0001
Me.E	0.185 ± 0.002	0.237 ± 0.001	0.237 ± 0.0002
Aq.E	0.266 ± 0.001	0.306 ± 0.001	0.306 ± 0.0001 ⁺
He.E + CCl ₄	0.101 ± 0.003	0.136 ± 0.002	0.193 ± 0.0003
Act.E + CCl ₄	0.102 ± 0.002	0.192 ± 0.003	0.143 ± 0.0001
Me.E + CCl ₄	0.193 ± 0.004	0.251 ± 0.001	0.231 ± 0.0001
Aq.E + CCl ₄	0.271 ± 0.001	0.309 ± 0.001	0.314 ± 0.0003
CCl ₄	0.536 ± 0.002*	0.433 ± 0.003*	0.433 ± 0.0003*

CCl₄ effect was compared against all groups. Its effect was significantly different in all groups but with different statistical significances in each lipid peroxidation value measured by brain region: * $p < 0.0001$ vs all groups (in cerebellum); * $p < 0.0001$ vs all groups (in cortex) and * $p < 0.0001$ vs O.O, He.E, Act.E, Me.E, He.E + CCl₄, Act.E + CCl₄, Me.E + CCl₄ and Aq.E + CCl₄ groups and ⁺ $p < 0.001$ vs Aq.E group (in cerebral hemispheres). Each quantification was performed twice in triplicate on samples from three rats, and the values represent the mean ± SD. O.O, group receiving olive oil; He.E, group receiving hexane extract; Act.E, group receiving ethyl acetate extract; Me.E, group receiving methanol extract; and Aq.E, group receiving aqueous extract.

in the O.O (16.4), He.E (17.2), Act.E (21.8), Me.E (16.1) and Aq.E (16.1) groups; GPx in the O.O(3.4), Act.E (8.5) and Me.E (70) groups; and GR in the O.O (1.8), Act.E (1.4), Me.E (2.4), and Aq.E (1.7) groups vs the CCl₄ group ($p < 0.05$). In the cerebral hemispheres: CAT in the O.O (2.6), Act.E (1.8), Me.E (5.7) and Aq.E (3.2) groups; SOD in the O.O (1086), He.E (510.9), Act.E (1280.3), Me.E (1344.1) and Aq.E (1477.1) groups; GPx in the O.O (3.1), He.E (4.0), Act.E (6.5), Me.E (2.5) and Aq.E (6.1) groups; and GR in the O.O (1616), He.E (1945.9), Act.E (410.6), Me.E (2468.5) and Aq.E (730) groups vs the CCl₄ group ($p < 0.05$). In the cerebellum: CAT in the O.O(1.9), He.E (4.6), Act.E (4.2), Me.E (6.4) and Aq.E (4.8) groups; SOD in the O.O (0.3), He.E (11.5), Act.E (16.1), Me.E (26.4) and Aq.E (15.6) groups; GPx in the O.O (3.5), He.E (9.5), Act.E (32.5), Me.E (8.0) and Aq.E (18) groups; and GR in the He.E (4.8) and Aq.E (1.3) groups vs the CCl₄ group ($p < 0.05$).

CAT: in the cortex in the O.O and CCl₄ groups; in the cerebral hemispheres in the O.O, He.E and Me.E groups; and in the cerebellum in the O.O, and Act.E groups. Cu/Zn-SOD: in the cortex in the O.O, Act.E and CCl₄ groups; and in the cerebral hemispheres and cerebellum in the O.O and Act.E groups. Mn-SOD: in the cortex in the Act.E, Me.E and CCl₄ groups; in the cerebral hemispheres in the O.O, He.E, Act.E, Me.E, Aq.E and CCl₄ groups; and in the cerebellum in the O.O, Act.E, Me.E, Aq.E and CCl₄ groups. GPx: in the cortex in the O.O and CCl₄ groups; in the cerebral hemispheres in the O.O and Aq.E groups; and in the cerebellum in O.O group.

(B) In the liver: CAT gene expression was significantly increased by several folds in the O.O, Act.E and Aq.E groups (1.7), as was SOD in the O.O (1.2), He.E (2.2), Me.E (1.9) and Aq.E (1.5) groups; GPx in the O.O (0.3) and Act.E (0.1) groups; and GR in the O.O (1.1), He.E (1.0), Act.E (1.5) and Aq.E (1.3) groups vs the CCl₄ group ($p < 0.05$).

In addition, we observed a non-significant positive correlation between gene expression and antioxidant activity in the control groups, as described below.

CAT: in the He.E group. Cu/Zn-SOD and Mn-SOD: in the CCl₄ group. GPx: in the He.E and Ac.E groups; GR: in the O.O, Ac.E, Me.E, Aq.E and CCl₄ groups.

4. Discussion

In this study, we provided biochemical and molecular experimental evidence of the protective effects of non-polar, medium-polar and polar *T. americana* var. *mexicana* extracts on CCl₄-induced damage in the liver and brain. This evidence was obtained by analyzing the antioxidant properties of the extracts. This study provided the first evidence of the antioxidant properties of this species in an *in vivo* model.

Oxidative stress is considered the major event responsible for CCl₄ toxicity, in which a small lipophilic molecule spreads easily throughout the lipid compartments of the body and is metabolized in the liver. The mechanism of toxicity involves CYP 450-mediated bioactivation, which produces free radicals, such as trichloromethyl (CCl₃·) and/or trichloromethyl peroxy radical (CCl₃OO·). This radical stimulates the production of reactive oxygen species (ROS) by Kupffer cells and induces lipid peroxidation (Weber et al., 2003). The peroxidized lipids then damage organelle membranes in liver cells, causing swelling and necrosis of hepatocytes and the release of cytosolic enzymes such as ALT and AST into the circulation (Sahreen et al., 2011). Treatment with CCl₄ causes a significant increase in the levels of these markers, indicating oxidative injury in the liver. The levels of γ -GLOB and ALB also decrease, indicating that CCl₄ causes oxidative injury and severe damage, as observed in previous studies (Sadigh-Eteghad et al., 2011; Coballase-Urrutia et al., 2013; Chae et al., 2014). Administration of the organic and aqueous extracts of *Tilia* (He.E, Ac.E, Me.E and Aq.E, 100 mg/kg) by 3 days before and 3 days during the CCl₄ treatment prevented the changes in the biomarkers (Table 2), which is an indication of normal liver parenchyma function and the regeneration of hepatocytes (Thabrew et al., 1987).

The lipid solubility of CCl₄ enables it to cross cell membranes, and when it is systemically administered, it is distributed and deposited mainly in the liver and brain. The time course of the elimination of CCl₄ appears to be governed by the blood perfusion rate and the lipid content of the tissue (Sanzgiri et al., 1997). SOD, CAT and GST have been reported

to constitute a mutually supportive defense mechanism against ROS (Valko et al., 2007). Coordinated action of the antioxidant system is essential for the detoxification of free radicals. SOD reduces concentrations of the highly reactive superoxide radical by converting it into H₂O₂. CAT and GPx decompose H₂O₂ and protect tissue from highly reactive hydroxyl radicals (Halliwell and Gutteridge, 2007). Endogenous GSH is synthesized primarily in the liver and plays an important role in the cell defense system; it acts as an antioxidant and as a co-factor for GPx and is involved in the reduction in the peroxides that are formed during lipid peroxidation. Oxidized glutathione (GSSG) is concomitantly formed through this process. Under normal physiological conditions, GSSG is reduced to GSH by GR at the expense of NADPH reduction, thereby forming a redox cycle (Dringen, 2000). Changes in the activity of GPx, GR and GST can affect the concentrations of GSH. Iskusnykh et al. evaluated the damage caused by free radicals, the levels of lipid peroxidation products and the changes in GPx and GR activity caused by CCl₄ toxicity in the livers of rats, and they observed a maximum increase in GR and GPx levels of 2.1- and 2.5-fold, respectively, after exposure to CCl₄. These levels coincided with the maximum level of free radical oxidation. GPx1 and GR transcript levels increased by 2.5- and 3.0-fold, respectively, suggesting that overexpression of these enzymes is involved in the increased tolerance of hepatocytes to oxidative stress (Iskusnykh et al., 2013).

We also showed that CCl₄ significantly decreased the activity of the antioxidant enzymes CAT, SOD, GPx, GR and GST in the liver, likely due to protein inactivation by free radicals. Similar results have also been shown previously for antioxidant enzyme systems in the liver during oxidative stress induced by CCl₄ (Al-Olayan et al., 2014; Al-Sayed et al., 2014; Dai et al., 2014). Administration of the non-polar, medium-polar and polar extracts prevented the decrease in antioxidant enzyme activity in the injured liver tissue. This protective effect was evident in the reduction in lipid peroxidation and the improvement of biochemical markers. Similar results with other extracts have been reported (El-Gazayerly et al., 2014; Ganie et al., 2014; Ghasemi et al., 2014).

Oxidative stress initiates the peroxidation of polyunsaturated fatty acids in cell membranes (Zhou et al., 2013), which is one of the most common reactions produced by free radical attacks on biological structures and the accumulation of oxidized lipids in the cell membrane (Janero, 2014). Our results showed that *Tilia* leaf extracts that differed in polarity reduced MDA production. The administration of a toxic agent such as CCl₄ was shown to significantly ($p < 0.0001$) increase hepatic MDA content, which indicates an increase in lipid peroxidation due to the presence of (CCl₃OO[•]), which is capable of binding to proteins or lipids or the abstraction of hydrogen atoms from unsaturated lipids (Brattin et al., 1985; Recknagel et al., 1989; Williams and Burk, 1990; Marathe et al., 2000). The oxidation of lipids has been implicated in the development of cardiovascular and cerebrovascular disease. MDA and other lipid oxidation products can modify membranes and impair their function in the following ways: (a) Fluidity is decreased; and (b) Membrane-bound enzymes and receptors are inactivated in red blood and endothelial cells. This damage accelerates the formation of foam cells and atherosclerotic plaques and increases the risk of heart disease and aging (Anzai et al., 1999; Yehuda et al., 2002). The significant decrease in the level of hepatic MDA we observed

suggested that the *Tilia* extracts of different polarities provided substantial protection against CCl₄-induced hepatic lipid peroxidation. The *Tilia* extracts preserved the liver from damage by stimulating hepatic regeneration and inhibiting liver lipid peroxidation, leading to membrane stabilization and the normalization of biochemical profiles. Greater protection was achieved by the ethyl acetate and hexane extracts, followed by the methanol and aqueous extracts.

Several studies have shown that the natural antioxidant defense system has a limited capacity for protection in the brain compared to in peripheral tissues, particularly GSH, which is the major intracellular antioxidant in the brain. GSH detoxifies intracellular H₂O₂ to H₂O and O₂ via subsequent oxidation to GSSG by the enzyme GPx. GSSG is recycled to GSH via GR (Smith et al., 1996). Brain tissue has been reported to exhibit low levels of CAT activity, and brain regions exhibit different levels of antioxidant enzymes including GPx and SOD (Gaunt and de Duve, 1976; Cárdenas-Rodríguez et al., 2014).

Our data demonstrated that the potential of *T. americana* var. *mexicana* leaves as an antioxidant depends on which of its different types of constituents is employed. Differential neuroprotection was observed according to the sensitivity of brain regions; the greatest sensitivity was observed in the cerebellum, followed by the cortex and hemispheres. Non-polar and medium-polar extracts do not reduce antioxidant enzyme activity (Baek et al., 1999; Manoli et al., 2000). Our results are consistent with the idea that oxidative stress vulnerability in the brain is region specific and dependent on the Fenton reaction, which is catalyzed by local concentrations of endogenous iron. Other authors have reported that areas such as the cortex, hypothalamus, hippocampus and striatum are more susceptible to oxidative damage than the cerebellum (Mandavilli and Rao, 1996; Arivazhagan et al., 2002). Kang et al. (1999) reported that variations in GSH levels in the brain may have major implications for the susceptibility of various brain regions to oxidative stress (Kang et al., 1999).

Lipoperoxidation has been shown to cause neuronal excitotoxicity (Madrigal et al., 2001; Cattani et al., 2014), which leads to an increase in iron levels, the generation of peroxy/alkoxy radicals and free radicals, and subsequent potentiation of lipid peroxidation (Schipper, 2004; Stankiewicz and Brass, 2009; Li et al., 2012). Cardozo-Pelaez et al. (2000) showed that oxidative stress affects some brain regions more than others and that these regional differences were inversely correlated with the regional capacity to remove 8-hydroxy-2'-deoxyguanosine (8-OH-dG) from DNA; this same relationship has been observed for antioxidant enzymes (Cardozo-Pelaez et al., 2000). CCl₄-induced hepatic coma has also been reported to disrupt GABAergic transmission in most cerebral regions (Díaz-Muñoz and Tapia, 1988). Recently, CCl₄-induced activation of tumor necrosis factor (TNF)- α , inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) in the protein and mRNA levels has been reported, in the liver (Esmaeili and Alilou, 2014). *Tilia* could inhibit TNF- α -mediated activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which is a transcription factor that is regulated by the redox state involved in oxidative stress. The different polarity *Tilia* extracts normalized the activity of antioxidant enzymes in the analyzed brain regions by inhibiting lipid peroxidation; this effect may reduce the risk for cerebrovascular diseases and the adverse effects of brain aging.

Some antioxidant enzymes have been demonstrated to be subject to transcriptional and post-translational regulation (Touati, 1988; Espinosa-Diez et al., 2015). GPx is transcriptionally upregulated as part of the cellular response to oxidative stress, as is p53 (Hussain et al., 2004). Oxidative mechanisms or direct exposure to oxidants may also regulate GPx gene transcription via the NF- κ B and nuclear factor erythroid-derived 2-like 2 (Nrf2) pathways (Brigelius-Flohé and Flohé, 2011). Other studies have shown that CAT gene expression increases in the presence of hydrogen peroxide and lipid peroxides (Onumah et al., 2009). Mn-SOD is regulated via methylation of CpG islands and histone modifications (Hurt et al., 2007; Hitchler et al., 2008). In another study, p53 was found to physically interact with Mn-SOD and to inhibit its superoxide scavenging activity (Zhao et al., 2005). NF- κ B, mTOR, ERK and p38 also regulate Mn-SOD gene expression (Wong, 1995; Takada et al., 2002; Li et al., 2010). In addition, computer-based searches of transcription factor binding sites have suggested the involvement of the peroxisome proliferator-activated receptor gamma (PPAR γ) and NF- κ B pathways in the regulation of the expression of human Cu/Zn-SOD and human CAT (Wong, 1995; Nakamura and Omaye, 2008; Okuno et al., 2010). The mechanisms of action of many plant extracts and natural products in cellular processes are still not clearly understood. However, a series of experiments showed that relatively low concentrations of flavonoids are required to stimulate the transcription of a critical gene for GSH synthesis in cells (Myhrstad et al., 2002). Chemo-preventive agents, such as phenolic antioxidants (e.g., gentisic acid, gallic acid, ferulic acid, p-coumaric acid, quercetin, myricetin and kaempferol), dithiol ethiones and isothiocyanates, have been shown to selectively induce the activation of antioxidant enzymes through the Nrf2 pathway (Hodnick et al., 1986; Tian et al., 1997; Miura et al., 1998; Boots et al., 2003; Lotito and Frei, 2006). Lipophilic compounds, such as α -tocopherol and conjugated linoleic acid isomers, have also been suggested to be possible PPAR γ activators. Isomers of conjugated linoleic acid have been shown to have an affinity for PPAR γ (Belury, 2002; Nakamura and Omaye, 2009). Lipophilic compounds may regulate the gene expression of antioxidant enzymes such as Cu/Zn-SOD and CAT through redox-sensitive transcription factors such as NF- κ B and PPAR γ , depending on the concentrations of the compound. Our antioxidant enzyme gene expression data and correlation analysis also demonstrated that the non-polar, medium-polar and polar constituents of the *T. americana* var. *mexicana* leaf extracts positively modulated the gene expression of CAT, SOD, GPx and GR in the livers and brains of rats after CCl₄-induced damage. In a preliminary study, we reported that the hexane extract of *T. americana* var. *mexicana* contains 0.06% of β -sitosterol as one of its main active metabolites (Aguirre-Hernández et al., 2007). β -sitosterol has been shown to exhibit several types of pharmacological activity, and its potential as an antioxidant has been illustrated by its ability to restore the levels of enzymatic and non-enzymatic antioxidants (Paniagua-Pérez et al., 2008; Baskar et al., 2012). The effects of β -sitosterol on antioxidant enzymes have been considered to depend in part on the estrogen/phosphatidylinositol 3-kinase pathway (Vivanco and Moreno, 2005). On the one hand, characteristic flavonoid derivatives such as kaempferol and quercetin have been reported to be major components in the medium-polar and

polar extracts of *Tilia* species (Pietta et al., 1993; Arteché and Vanaclócha, 1998; Toker et al., 2001). On the other hand, the specific flavonoids identified in *T. americana* var. *mexicana* collected from different regions of Mexico included kaempferol-3,7-O-dirhamnoside (kaempferitrin), quercetin-3-pentosylhexoside, kaempferol-3-pentosylhexoside, isoquercitrin, quercetin-3-O-rutinoside (rutin), astragalín, quercitrin, kaempferol-3-O-rhamnoside and tiliroside (Herrera-Ruiz et al., 2008; Pérez-Ortega et al., 2008; Aguirre-Hernández et al., 2010).

The antioxidant capacity of flavonoids is well known, and they can act as antioxidants in chemical systems due to their extensive conjugated π -electron systems that facilitate the donation of electrons from the hydroxyl moieties to the oxidizing radical. This antioxidant effect is considered poor when individual flavonoids are evaluated (Duthie and Morrice, 2012); however, in plant extracts, this type of antioxidant effect appears to be much more potent due to the synergy that occurs in the presence of several antioxidants at the same time. According to the results of this study, the antioxidant activity depends on the nature of the constituents, as demonstrated by the dependence of the response on the polarity of the extract evaluated. Certainly, the combination of all of the compounds contained in *T. americana* var. *mexicana* supports its potential as an antioxidant vegetal species, suggesting it may be useful for therapies for degenerative diseases.

5. Conclusion

Our results indicate that components of *T. americana* var. *mexicana* leaves that exhibit different characteristics and polarities participate in a synergistic manner to protect against lipid peroxidation by maintaining basal antioxidant activity and normal serum biochemical parameters, demonstrating the free radical scavenging properties of this plant. A significant increase in CAT, SOD, GPx and GR gene expression in the livers and brains of rats exposed to CCl₄-induced injury but not in the presence of the *T. americana* var. *mexicana* leaf extracts suggests that the different metabolites act as activators of nuclear transcription factors such as NF- κ B and Nrf2. Our results provide the first evidence that the protective effects of *T. americana* var. *mexicana* involve antioxidant activity, which may underlie the traditional medicinal effects and biomedical properties of this plant, and also suggest that this species possesses neuro- and hepatoprotective activity, demonstrating its potential in the prevention of age-related deterioration of the brain or liver and its applications for neuro- and hepatopathies.

Authors and contributions

Elvia Coballase-Urrutia, Liliana Carmona-Aparicio and Noemí Cárdenas-Rodríguez designed the study, performed the experiments, analyzed the results and wrote the manuscript; María Carolina González-García, Eithan Núñez-Ramírez, Esaú Floriano-Sánchez, María Eva González-Trujano, Berenice Fernández-Rojas, José Pedraza-Chaverrí, Hortencia Montesinos-Correa, Liliana Rivera-Espinosa, and Aristides III Sampieri performed the experiments, and Liliana Carmona-Aparicio obtained the financial support. All of the authors reviewed the final manuscript.

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