



Active compounds and anti-inflammatory activity of the methanolic extracts of the leaves and callus from *Tilia americana* var. *mexicana* propagated plants

Karen Flores-Sánchez^{1,2} · Francisco Cruz-Sosa² · Alejandro Zamilpa-Alvarez¹ · Pilar Nicasio-Torres¹

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Abstract

Tilia americana var. *mexicana* is used in Mexican traditional medicine to treat anxiety and inflammatory processes. Several glycosides derived from quercetin and kaempferol, including tiliroside, isoquercetin, and quercetin-3- β -D-glucoside, were reported as the main anxiolytic compounds in this species; to our knowledge, compounds with anti-inflammatory effects have not been previously described. In this study, whole plants were obtained from rooted cuttings with indole-3-butyric acid (IBA) under greenhouse conditions. Multiple shoots and callus cultures were established from apical and axillary buds from *T. americana* var. *mexicana* cuttings. The apical buds (75%) were the best explant for shoot induction (2–3 shoots per explant) on Murashige and Skoog (MS) medium supplemented with 2.0 mg L⁻¹ of 6-benzyl aminopurine plus 0.25 mg L⁻¹ α -naphthaleneacetic acid. Callogenesis occurred in both types of buds in the treatments constituted by thidiazuron with 0.1 mg L⁻¹ IBA. High-performance liquid chromatography analysis of leaves and callus methanolic extracts allowed the identification of quercetin-3- β -D-glucoside and tiliroside anxiolytic compounds, and of the scopoletin anti-inflammatory compound. The methanolic leaf and callus extracts showed anti-inflammatory activities in a 12-*O*-tetradecanoylphorbol-13-acetate-induced ear edema model with median effective doses (ED₅₀) of 0.38 and 1.73 mg per ear for the leaf and callus extracts, respectively.

Keywords Anti-inflammatories · Anxiolytics · Propagation · Quercetin-3- β -D-glucoside · Scopoletin · Tiliroside

Abbreviations

BAP 6-Benzyl
amino-9-(2-tetrahydropyranyl)-9H-purine
IBA Indole-3-butyric acid
BA 6-Benzyl aminopurine
KIN Kinetin

MS Murashige and Skoog
NAA α -Naphthaleneacetic acid
PVPP Polyvinylpyrrolidone
TDZ Thidiazuron
TPA 12-*O*-Tetradecanoylphorbol-13-acetate
2,4-D 2,4-Dichlorophenoxyacetic acid

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Key message *Tilia americana* var. *mexicana* plants were propagated and callus tissues were generated from apical buds; leaf and callus methanolic extracts have quercetin-3- β -D-glucoside, tiliroside and scopoletin compounds, and were active in the ear edema mouse model.

✉ Pilar Nicasio-Torres
pisaliva@yahoo.com.mx

¹ Centro de Investigación Biomédica del Sur (CIBIS), Instituto Mexicano del Seguro Social (IMSS), Argentina #1, 62790 Xochitepec, Morelos, Mexico

² Departamento de Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa, Ciudad de México, Mexico

Introduction

Tilia americana var. *mexicana* (Schltdl.) Hardin is known in Mexico as *cirimo*, *tila*, and *tilia* (Martínez and Matuda 1979; Martínez 1969; Flores-Olvera and Lindig 2005). Aerial tissues of this species are used in traditional Mexican medicine to treat several central nervous system-related illnesses such as anxiety, headache, and insomnia. Infusions of flowers and fruits are also used to treat colon spasms, menstrual irregularities, rheumatism, and hypertension (Martínez 1969; Pavón and Rico 2000; Monroy-Ortiz and Castillo-España 2007). Pharmacological reports demonstrated that methanolic extracts from *T. americana* var. *mexicana*

inflorescences induced an anxiolytic effect after oral and intraperitoneal administration in mice (Aguirre-Hernández et al. 2007, 2010; Herrera-Ruiz et al. 2008). The methanolic extracts of flowers and bracts contain several glycosides that are mainly derived from quercetin and kaempferol such as tiliroside, isoquercetin, and quercetin-3- β -D-glucoside as shown in Fig. 1 (Herrera-Ruiz et al. 2008; Aguirre-Hernández et al. 2007, 2010; Nogueroń-Merino et al. 2015). The sedative activities of inflorescence and leaf methanolic extracts were attributed to quercetin, rutin, and isoquercetin (Cárdenas-Rodríguez et al. 2014; Aguirre-Hernández et al. 2016). Additionally, the anti-nociceptive effects of inflorescence infusions were demonstrated and attributed to quercetin flavonoids (Martínez et al. 2009). However, the use of *T. americana* var. *mexicana* in the traditional medicine to treat illnesses that involve an inflammatory process has not been evaluated to our knowledge. Despite its important pharmacological properties, the collection of *T. americana* var. *mexicana* inflorescences from trees in their natural habitats has been restricted by the Mexican Ministry of the Environment and Natural Resources (SEMARNAT, NOM-059-ECOL-2010) because the species is considered at risk of extinction (SEMARNAT 2010) even if it is not recorded on the red list of the International Union for Conservation of Nature (IUCN). In addition, this species presents sexual propagation problems because seeds have two types of dormancy: (1) an exogenous dormancy, during which the seed coat is impermeable to water and (2) an endogenous dormancy, during which the embryo is immature (Zurita-Valencia et al. 2014).

Micropropagation studies have already been applied for *Tilia* species, including *Tilia amurensis* shoot recovery from secondary somatic embryos from cotyledon and hypocotyl explants grown on Murashige and Skoog (MS)

medium (Murashige and Skoog 1962) with 1.0 mg L⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D) (Kim et al. 2006). In-vitro shoots of *T. platyphyllos* have been obtained from nodal segments cultured on MS medium with low concentrations of 6-benzyl amino-9-(2-tetrahydropyranyl)-9H-purine (BAP), and thidiazuron (TDZ) with indole-3-butyric acid (IBA). Also, somatic embryogenesis was obtained from zygotic embryos grown on MS supplemented with 2,4-D (Chalupa 2003). *T. mexicana* has been propagated through axillary buds. Shoots were generated in MS medium with 0.25 mg L⁻¹ of α -naphthaleneacetic acid (NAA) and 1.0 mg L⁻¹ of 6-benzyl aminopurine (BA). Root formation was achieved with IBA after 45 days and the resulting plants had well-developed leaves and roots (Zurita-Valencia et al. 2014). Similarly, *T. mexicana* was propagated through the rooting of cuttings by applying Radix[®] rooter powder and 10,000 ppm of IBA in which 51.1% of stakes were rooted (Muñoz-Flores et al. 2011). The search for alternatives in preserving *T. americana* var. *mexicana* plants is mandatory, and despite the advances reported on the propagation of this species, to our knowledge, there are no reports on the acclimatization and growth in greenhouse and environmental conditions of these plants or on the production of active compounds by means of in-vitro callus cultures. Thus, this research focused on the propagation of *T. americana* var. *mexicana* by means of cuttings and their acclimatization; in parallel, the effects of growth regulators in apical and axillary buds on the generation of multiple buds and callus development was examined. Furthermore, the traditional use of the methanolic leaf and callus extracts as anti-inflammatory agents was evaluated using a model of acute inflammation in addition to the capacity of calluses to produce active compounds.

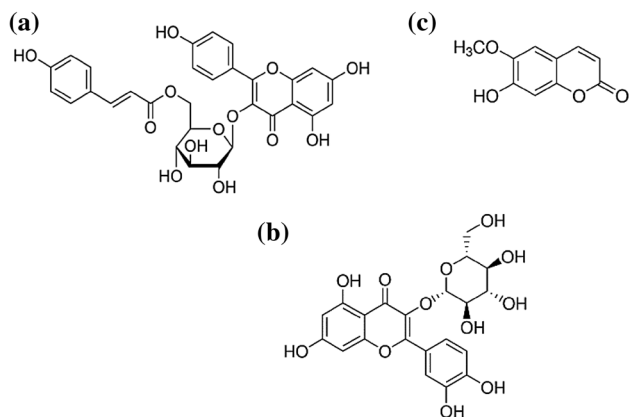


Fig. 1 Tiliroside (a) and quercetin-3- β -D-glucoside (b) active compounds isolated from inflorescences of wild trees, and scopoletin (c) detected in leaves of wild trees and callus of *Tilia americana* var. *mexicana*

Materials and methods

Plant material

Branches of *T. americana* var. *mexicana* were collected in Mexicapa, Mexico State, Mexico in October and November 2015. Tree samples were authenticated by Abigail Aguilar, M.Sc., Head of the medicinal herbarium at the Instituto Mexicano del Seguro Social in Mexico City [IMSSM], and vouchers were stored for reference under #IMSSM-5099.

Vegetative propagation

Branches were cut in lengths of 20 cm comprising three or four nodes. A horizontal cut was made in the upper end and a vertical one in the lower end of the cuttings; these were immediately submerged in water at the basal end. Later, the cutting were spliced with Radix[®] 10,000 rooting

powder (IBA, 10,000 ppm) and placed in plastic boxes (60×50×20 cm) containing a commercial substrate (Sunshine Fine mixture [70–80%] Canadian peat moss, vermiculite, ground lime [chalk], and a moisturizing agent) and a garden-soil mixture (2:1). The boxes were placed in a greenhouse under semi-shade mesh (90%) with irrigation every other day, and these were supplied with the mineral nutrients of MS medium at 25% every 15 days (Murashige and Skoog 1962; Muñoz-Flores et al. 2011). The numbers of dead and rooted cuttings were registered after 2 months. After a period of 7 months, the rooted cuttings were individualized, transferred into plastic pots (27×32 cm), and preserved under greenhouse conditions. Well-developed plants (17 months) were transferred to larger pots (55×86 cm) and exposed to environmental conditions in the CIBIS Garden at Xochitepec, Morelos, Mexico.

Induction of in-vitro cultures

In order to obtain pathogen-free buds, young apical and axillary buds from *T. americana* var. *mexicana* cuttings were removed and disinfected by a serial process of Extran® (0.5%, 1.0% v/v) and sodium hypochlorite commercial solutions (0.35%, and 0.7%, v/v) at exposure times of 2, 3, and 5 min for Extran® and of 5, 10, and 15 min for sodium hypochlorite after which time they were immediately rinsed with sterile distilled water (Borges et al. 2009). The explants were then transferred into glass containers with 40 mL of MS medium with the antibiotics chloramphenicol (50.0 mg L⁻¹) and amphotericin B (5.0 mg L⁻¹) for microbial growth inhibition (Nicasio-Torres et al. 2012). The MS medium was supplied with 30 g L⁻¹ of sucrose, 100 mg L⁻¹ of casein hydrolysate, 100 mg L⁻¹ of glutamine (Sigma–Aldrich, México), and different treatments resulting from combinations of growth regulators: (1) BA (0, 0.5, 1.0, or 2.0 mg L⁻¹) mixed with NAA at 0.25 mg L⁻¹ and/or (2) TDZ (0, 0.005, 0.01, or 0.02 mg L⁻¹) mixed with IBA at 0.1 mg L⁻¹ (Chalupa 2003; Zurita-Valencia et al. 2014). Media were adjusted to pH 5.7, 3.0 g L⁻¹ PhytaGel (Sigma–Aldrich, México), and 1.0 g L⁻¹ of polyvinylpyrrolidone (PVPP) were added and autoclaved at 1.0 kg cm⁻² for 18 min at 120 °C.

One apical or axillary bud per glass container with 20 explants per hormonal treatment were incubated at 26±2 °C during a light:dark (16-h:8-h) photoperiod under 50 μM m⁻² s⁻¹ warm white-fluorescent light intensity and relative humidity of 60%. Explants were transferred onto fresh medium every 4 weeks. The percentage of explants with evidence of shoot and/or callus development were scored for all treatments after 60 and 90 days in culture; during each period, the shoot number and leaf number per shoot were also registered. Results were compared at 60 and 90 culture days by means of factorial analysis of variance

(ANOVA) AXB (BA X TDZ) followed by a Tukey multiple-range test. Values of $p \leq 0.05$ were considered statistically significant (SAS ver. 9.1 statistical software; SAS Institute, Inc.). After 10 months in culture, callus biomasses from TDZ (0.005 mg L⁻¹) in combination with IBA (0.1 mg L⁻¹) were dried for extraction and chemical analyses.

Scopoletin, quercetin-3-β-D-glucoside, and tiliroside analyses

Three samples of dry callus biomasses and leaves from the branches were separately extracted with reagent-grade methanol (Merck, México) at 1:20 (w/v) by maceration at room temperature three times (24 h for each procedure). The methanol extracts obtained for each sample were filtered through filter paper (Whatman No. 1), pooled, and concentrated to dryness under reduced pressure. Dry callus biomasses previously obtained with 1.0 mg L⁻¹ of 2,4-D in combination with 0.5 mg L⁻¹ of kinetin (KIN) (data not shown) were also extracted for high-performance liquid chromatography (HPLC) profile comparison (Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2016). The scopoletin, quercetin-3-β-D-glucoside, and tiliroside contents in each tissue were compared by ANOVA followed by Tukey multiple-range tests. Values of $p \leq 0.05$ were considered statistically significant (SAS ver. 9.1 statistical software; SAS Institute, Inc.).

Analyses of HPLC

Methanolic extract analyses were carried using a Waters system (2695 Separation Module) coupled to a diode array detector (2996) with a 190–600 nm detection range and operated by the Manager Millennium software system (Empower ver. 1; Waters Corp., Boston, MA, USA). Separation of compounds was performed in a Spherisorb® RP-18 column (250×4.6 mm, 5 μm; Waters) employing a constant temperature of 25 °C during analyses. Samples (20 μL) were eluted at a 1.0 mL min⁻¹ flow rate with (A) high-purity H₂O (with CF₃COOH to 0.5% v/v; TFA, Sigma–Aldrich, México) and (B) high-purity CH₃CN-gradient (Merck) mobile phases and detection at λ = 343 nm for scopoletin (Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2016), λ = 355 nm for quercetin-3-β-D-glucoside, and λ = 314 nm for tiliroside. Analyses of scopoletin (99%, Sigma–Aldrich, México), quercetin-3-β-D-glucoside (90%), and tiliroside (98%, Sigma–Aldrich, México) were performed by comparing their retention times (quercetin-3-β-D-glucoside, 9.22 min; scopoletin, 11.15 min, and tiliroside, 17.33 min) and absorbance spectra. Calibration curves were constructed with standard solutions of 2.5, 5, 10, and 20 μg mL⁻¹ and fitted using a linear-squares model $(y) = m(x) + b$ using Microsoft Office Excel software 2010 with correlation values of ≥ 0.9995 . Scopoletin presented a regression equation of $Y = 164,224$

(X) – 34,923 with an $R^2 = 0.9968$. For quercetin-3- β -D-glucoside, $Y = 97,701 (X) - 29,361$, and $R^2 = 0.9997$, and for tiliroside, $Y = 51,931 (X) - 136,524$ with an $R^2 = 0.9984$.

TPA-induced mouse ear edema

Male ICR mice (weight, 28 g) were used and experiments were performed according to Official Mexican Regulation NOM-062-ZOO-1999 Guidelines (Technical Specifications for the Production, Care, and Use of Laboratory Animals) and international ethical guidelines for the care and use of experimental animals. The mice were maintained at a temperature of 22 ± 3 °C, a humidity of $70 \pm 5\%$, with 12-h/12-h light/dark cycles and food/water *ad libitum*. The experimental protocol was authorized by the Local IMSS Health Research Committee (Registry number R-2016-1702-4).

The mice were assigned to groups of six each, and 12-*O*-tetradecanoylphorbol-13-acetate ([TPA]; 99% purity; Sigma–Aldrich, Mexico City, Mexico) dissolved in 20 μ L (2.5 μ g) of acetone was applied on the right ear's internal and external surfaces to generate edema. The left ear remained untreated. Doses of 0.125, 0.25, 0.5, and 0.75 mg per ear of leaves and 0.75, 1.0, 1.5, 1.75, and 2.0 mg per ear of callus (TDZ/IBA) methanolic extracts, and indomethacin as positive control (99% purity; Sigma–Aldrich) were dissolved in acetone and applied topically to both ears immediately after TPA administration.

Four hours after inflammatory agent administration, the animals were sacrificed by cervical dislocation, and circular sections (6 mm in diameter) were taken from both treated and non-treated ears. The ear sections were immediately weighed to determine ear edema by difference in weight. Percentage of inhibition was obtained utilizing the following expression: $\text{Inhibition \%} = ([\text{control} - \text{treatment}/\text{control}] \times 100)$ (Murugananthan and Shivalinge Gowda 2012). A curve of the doses of leaves or callus methanolic extracts versus ear-edema inhibition were fit using a linear least-squares regression model $(y) = m(x) + b$ utilizing Microsoft Office Excel 2010 software. The resulting *m* and *b* parameters were employed in order to determine the median effective dose (ED_{50}) of leaf and callus methanolic extracts.

Results and discussion

Obtaining plants of *T. americana* var. *mexicana* by means of cuttings

Cuttings of *T. americana* var. *mexicana* were used as source of shoots. A total of 60% of the cuttings produced buds after 8 days of cultivation; their first leaves were



Fig. 2 Propagation process of *Tilia americana* var. *mexicana*: cuts (a), cuttings with shoots after 25 days at greenhouse (b), plants after 7-months (c), and plants in environmental conditions cultivated in plastic pots with commercial substrate and garden-soil mixture after 17-months (d)

heart-shaped and pink and green in color (Fig. 2a). After 25 days, the shoots turned green and developed a large number of apical and axillary buds (Fig. 2b). Only 3.12% of the viable cuttings showed resistance when we tried to remove them, suggesting that they were rooting. After 7 months, whole plants of *T. americana* var. *mexicana* with new stems and green leaves were obtained under greenhouse conditions. Vigorous plants (35–93 cm with 3–5 branches) of 17 months were exposed to environmental conditions (Fig. 2c, d). The rooting of a cutting is influenced by the type of hormone used, the physiology of the branch, and the geographical region in which it grows, among other factors. Whole plants of *T. americana* var. *mexicana* were obtained from cuttings collected during the months of October and November after the period of fructification and rooting with Radix® 10,000 and IBA (Muñoz-Flores et al. 2011; Pavón and Rico 2000).

In-vitro culture induction

The most effective disinfection process of viable apical and axillary buds (95%) occurred with 0.5% Extran[®] for 5 min and 0.7% NaOCl for 15 min and was applied in all of the following experiments. The resulting explants were green and exhibited a slight brown color in the cut areas (Fig. 3b, f). Extran[®] is a neutral liquid solution of anionic and non-ionic surfactants and phosphates used as an excellent disinfectant in various laboratory processes; in addition, its use is safe for the environment and humans.

The apical and axillary buds of *T. americana* var. *mexicana* cultured in hormone-free MS medium (control) did not develop callus or shoots. Twenty percent of both bud types were elongated, but their elongation was lower than that observed in buds grown on MS medium supplemented with TDZ and/or BA (Fig. 3c, g). After 30 days of culture, the buds did not form shoots or callus in any of the treatments evaluated (Fig. 3b, f), they remained green and the leaf primordia began to open. By day 60, shoot formation and callogenesis varied according to the type and concentration of the cytokin used. Based on ANOVA and the Tukey *post-test*_{0.05} (Table 1), it was determined that in apical buds, shoot formation was similar when these were grown on MS medium supplemented with BA or TDZ ($p > 0.05$); however, after 90 days, the apical bud number with shoots increased

only on MS medium supplemented with the highest concentration of BA (2.0 mg L⁻¹) in combination with 0.25 mg L⁻¹ of NAA. Instead, the apical-bud mainly developed calluses (Table 1) at 60 days in culture in the MS medium supplemented with TDZ plus 0.1 mg L⁻¹ of IBA and increased further in correlation with TDZ concentration after 90 days in culture.

When axillary buds were used as the explant, bud elongation and shoot formation depended on the selected hormone. After 60 days, maximal responses were obtained with the reduction of BA concentration (Table 1), and this response increased by 90 days. Conversely, with TDZ the axillary buds mainly developed calluses (Table 1) at 60 days in culture, and callusing increased with TDZ concentration and culture time.

In general, the shoots obtained were green with an average height of 2.0 cm and green and dentate leaves (Fig. 3c, g). The generated calluses were all beige and semi-hard with a brown hue with BA (Fig. 3d), while those obtained with TDZ (Fig. 3h) were friable with a pink hue. In both treatments, shoot development and rhizogenesis were not observed.

These results differ from those reported by Zurita-Valencia et al. (2014), who obtained the best induction of shoot in axillary buds of *T. mexicana* on MS medium with 1.0 mg L⁻¹ BA plus 0.25 mg L⁻¹ NAA, while the best response in our study

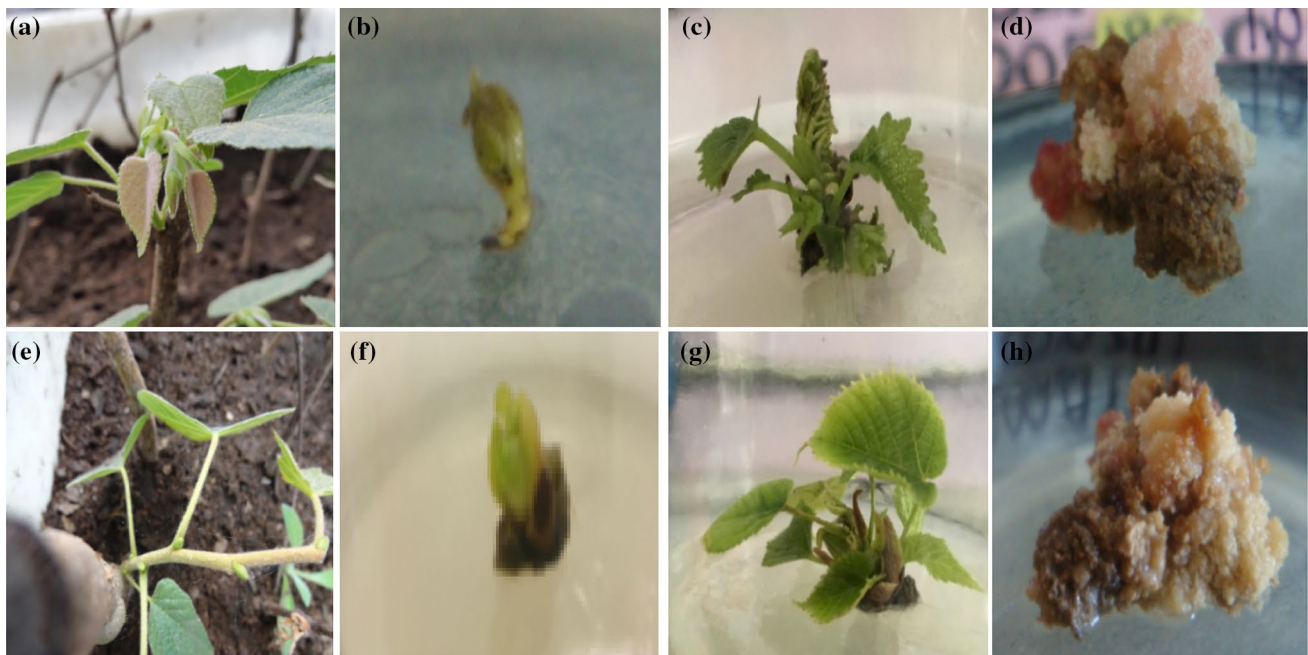


Fig. 3 Apical buds **a** from *Tilia americana* var. *mexicana* cuttings, **b** cultivated on Murashige and Skoog (MS) medium, **c** shoots after 90 days cultivated with 2.0 mg L⁻¹ 6-benzylaminopurine (BA) with 0.25 mg L⁻¹ α -naphthaleneacetic acid (NAA), and callus, **d** with 0.005 mg L⁻¹ thidiazuron (TDZ) with 0.1 mg L⁻¹ indole-

3-butyric acid (IBA). Axillary buds from cuttings (**e**), cultivated on MS medium (**f**) and axillary bud with shoots after 90 days (**g**), with 0.5 mg L⁻¹ of BA with 0.25 mg L⁻¹ NAA, and callus (**h**) with 0.02 mg L⁻¹ TDZ with 0.1 mg L⁻¹ IBA

Table 1 Effect of 6-benzylaminopurine (BA) and thidiazuron (TDZ) concentrations on survivor explants and the development of callus and shoots on apical and axillary buds of *Tilia americana* var. *mexicana*

Explant	Time (days)	Treatment (mg L ⁻¹)		Explants with shoots (%)	F	p	Explant (%) / shoot number	Explants with callus (%)	F	p		
		BA	TDZ									
Apical bud	60	0.5	–	50	NS	10 (2)	15	26.06	0.0001	60		
		1.0	–	50			–				15	
		2.0	–	40			–				5	
		–	0.005	45			–				20 (2–4)	50**
		–	0.01	50			–				50**	
	–	0.02	35	–	55**							
	90	0.5	–	55	2.7	0.1	15 (2)	15	58.11	0.0001		
		1.0	–	50	6		–	20				
		2.0	–	75**	–		35 (2–3)	5				
		–	0.005	45	–		45 (2–4)	60**				
–		0.01	50	–	–		70**					
Axillary bud	60	–	0.02	14	5.12	0.008	4 (2)	80**	11.75	0.0008		
		0.5	–	55**			–	25 (2)			20	
		1.0	–	25			–	–			25	
		2.0	–	10			–	–			45	
		–	0.005	20			–	–			65**	
	–	0.01	0	–	–	60**						
	–	0.02	20	–	–	55**						
	90	0.5	–	60**	5.66	0.005	30 (2–3)	20	12.56	0.0006		
		1.0	–	25	–		–	40				
		2.0	–	10	–		–	65				
–		0.005	20	–	–		70					
–		0.01	0	–	–		65					
–	0.02	25	–	–	80**							

BA in combination with 0.25 mg L⁻¹ α -naphthaleneacetic acid (NAA)

TDZ in combination with 0.1 mg L⁻¹ indole-3-butyric acid (IBA)

NS, not significant; values with ** are different statistically according to Tukey_{0.05} test ($p \leq 0.05$)

was observed with 0.005 mg L⁻¹ TDZ plus 0.1 mg L⁻¹ IBA at 90 days of culture in which 2–4 shoots per explant developed (Table 1). In explants of axillary buds in *T. platyphyllos*, the highest response was obtained using lower BA concentrations (0.6 mg L⁻¹) and TDZ (0.005 mg L⁻¹) in combination with 0.1 mg L⁻¹ IBA (Chalupa 2003). Several factors can influence cellular differentiation such as type of explant, the type and concentration of selected plant growth regulators, and the culture medium, among others (Coste et al. 2012; Martínez et al. 2017). Thus, in explants of *T. cordata* and *T. amurensis*, 1.0–5.0 mg L⁻¹ BA promoted the formation of 3–5 shoots/explant (Zurita-Valencia et al. 2014).

Scopoletin, quercetin-3- β -D-glucoside, and tiliroside contents

HPLC analysis of the methanolic extracts of *T. americana* var. *mexicana* from leaves of wild trees and calluses

from apical-buds developed with 0.005 mg L⁻¹ TDZ plus 0.1 mg L⁻¹ IBA (Fig. 4), revealed the presence of quercetin-3- β -D-glucoside (tr = 9.220 min), scopoletin (tr = 11.155 min), and tiliroside (tr = 17.481 min). The ANOVA and Tukey_{0.05} *post*-test indicated that wild tree leaves had higher content of scopoletin, while the quercetin-3- β -D-glucoside content was superior in callus, and the tiliroside content was similar in both (Table 2). These differences are possibly due to the time of plant collection because the physiological conditions of the plants change with the seasons and different environmental factors such as light, temperature, water, soil type, and salinity (Figueiredo et al. 2008; Yang et al. 2018). Scopoletin content in callus was similar, and quercetin-3- β -D-glucoside content was higher than that detected in the callus previously produced (data not shown) from leaf explants on MS medium with 2.0 mg L⁻¹ 2,4-D and 0.5 mg L⁻¹ KIN. In addition, in those calluses, tiliroside

Fig. 4 Chromatograms for quercetin-3- β -D-glucoside ($\lambda = 355$ nm; 9.2 min), scopoletin ($\lambda = 343$ nm; 11.1 min) and tiliroside ($\lambda = 314$ nm; 17.3 min) detected in methanolic extracts of leaves (a), and leaf (b) and apical bud (c) calluses of *Tilia americana* var. *mexicana* cultivated on Murashige and Skoog (MS) medium with 2.0 mg L⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D) with 0.5 mg L⁻¹ of kinetin (KIN), and 0.005 mg L⁻¹ thidiazuron (TDZ) with 0.1 mg L⁻¹ indole-3-butyric acid (IBA), respectively

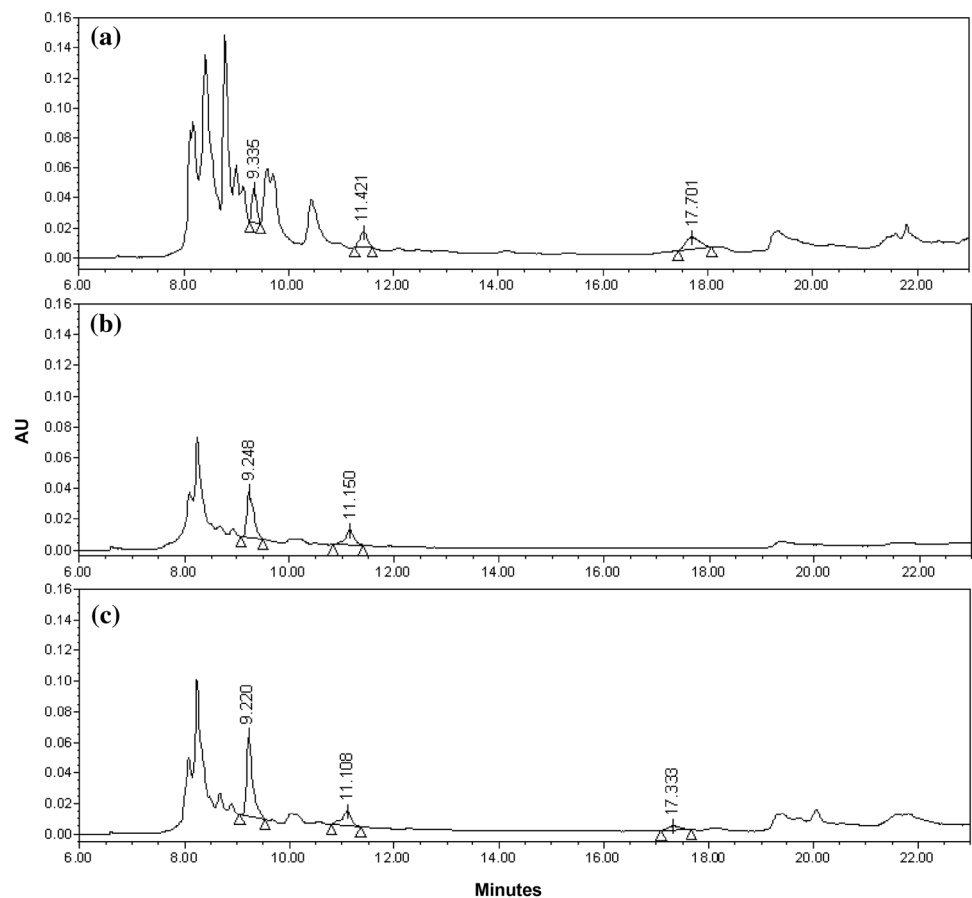


Table 2 Contents of active compounds in methanolic extracts from leaves and callus of *Tilia americana* var. *mexicana*

Vegetal tissues	Concentration ($\mu\text{g g}^{-1}$ biomass)		
	Scopoletin	Quercetin-3- β -D-glucoside	Tiliroside
Cutting leaves	11.30 \pm 0.42**	23.55 \pm 2.74	97.38 \pm 9.62
Callus from apical buds ^a	7.64 \pm 2.30	115.86 \pm 4.02**	88.24 \pm 1.01
Callus from leaf explant ^b	4.47 \pm 6.01	55.10 \pm 5.00*	ND
<i>p</i>	<0.0001	<0.0001	<0.0001

Values with ** and * are statistically different according to the Tukey_{0.05} test ($p \leq 0.05$)

ND, non detected

^aCallus from apical bud cultivated on MS medium with 0.005 mg L⁻¹ thidiazuron (TDZ) in combination with 0.1 mg L⁻¹ indole-3-butyric acid (IBA)

^bCallus from leaf explants cultivated on MS medium with 2.0 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 0.5 mg L⁻¹ kinetin (KIN)

was not detected. This difference in the chemical profile could be due to the growth regulators employed for callus generation in addition to the origin of the explant, implying the expression of genetic, ontogenetic, and morphogenetic factors (Figueiredo et al. 2008; Muñoz-Flores et al. 2011; Yang et al. 2018). These callus tissues could be an alternative for cell-suspension development for active compound production (Verpoorte et al. 2002; Murthy et al. 2014).

Anti-inflammatory activity

TPA-induced auricular edema in mice was employed as a substance capable of causing local inflammation characterized by vasodilatation, cellular infiltration, and erythema during the first 3 or 4 h after irritant application (Muruganathan and Shivalinge Gowda 2012). In the negative control group, the maximal inflammation level, evaluated as the increase in the weight of the auricular edema, was 10.31 mg

Table 3 Effect of leaves and callus methanolic extracts of *Tilia americana* var. *mexicana* on auricular edema induced with 12-*O*-tetradecanoylphorbol-13-acetate (TPA)

Treatment	Doses (mg/ear)	Ear weight (mg)	Edema inhibition (%)
TPA	–	10.31 ± 0.74	–
Indomethacin	0.50	3.46 ± 0.34	65.23 ± 3.42
Leaves methanolic extract	0.125	10.38 ± 0.15	5.50 ± 1.99
	0.25	7.52 ± 0.90	32.00 ± 8.17
	0.50	4.23 ± 0.55	57.45 ± 5.49
	0.75	1.70 ± 0.89	82.91 ± 8.92
	1.00	9.30 ± 0.21	12.85 ± 1.93
Callus methanolic extract from apical buds	1.50	7.90 ± 0.48	28.16 ± 4.62
	1.75	5.58 ± 0.34	47.71 ± 3.14
	2.00	3.28 ± 0.38	70.15 ± 1.60

Mean ± standard deviation of the mean (n = 5)

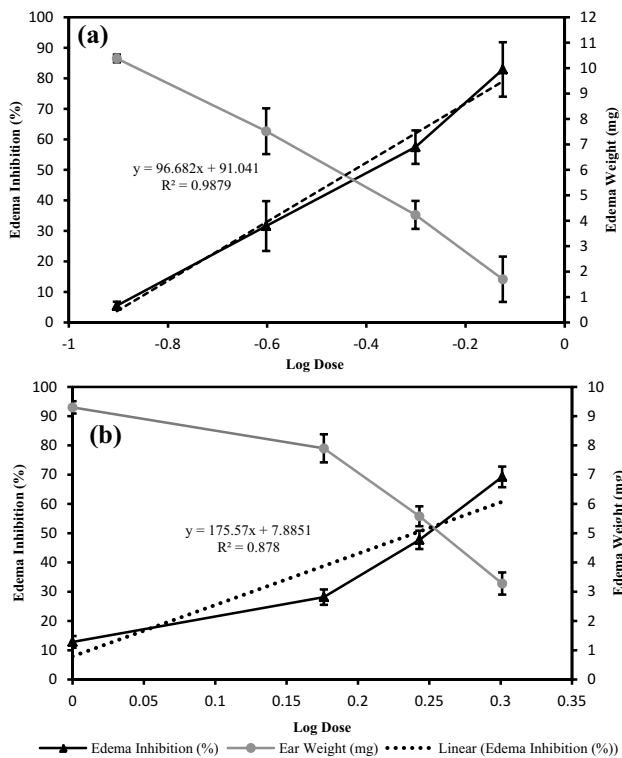


Fig. 5 Relationship between the doses of leaves (a) and callus (b) methanolic extracts from *Tilia americana* var. *mexicana* and ear edema inhibition, as well as versus 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema weight. The “best-fit” line shown was generated by linear regression of the data (n = 5); squares of correlation coefficient (R^2) regression equations is reported. Vertical bars represent the standard deviation of the means (n = 5)

(100%) after 4 h (Table 3). The ear-edema weights in the groups treated with leaves and callus methanolic extracts in addition to indomethacin were lower than those produced in the negative control group (Table 3). The methanolic leaf extract at 0.5 mg per ear exerted a similar effect to that of indomethacin, which was dose-dependent with a median effective dose (ED_{50}) of 0.38 mg per ear (Fig. 5).

The ED_{50} of 1.73 mg per ear of the methanolic callus extract was higher than that determined for the leaf methanolic extract, possibly because the scopoletin content in the callus is lower than that in the leaves. The anti-inflammatory and anti-arthritic effects of scopoletin from plants have already been reported (Pan et al. 2010; García-Rodríguez et al. 2012). It will be important to isolate and identify the anti-inflammatory compounds produced in callus tissues and leaves of wild plants other than scopoletin. It will also be important to evaluate elicitors to increase the production of compounds with anxiolytic activity and in addition, to test other systems for the in-vitro propagation of *Tilia americana* var. *mexicana*.

Conclusions

Tilia americana var. *mexicana* plants were obtained through the rooting of cuttings that currently grow in an experimental land parcel. In the methanolic extract of the leaves, the content of compounds with anxiolytic activity such as quercetin-3- β -D-glucoside and tiliroside was determined; this extract was also active in the model of a TPA-induced ear edema. In parallel, callus cultures were obtained from the axillary buds from cuttings in MS medium complemented with TDZ/IBA; these tissues have also anti-inflammatory activity and the capacity to produce some of the active compounds detected in the leaves.

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Author contributions As a Ph.D. student, KF-S participated in all of the experimental work, in the collection, analysis, and interpretation of data, and in the writing of the manuscript. FC-S supervised the establishment of the factorial design experiments, provided the scopoletin, quercetin-3- β -D-glucoside, and tiliroside standards, and was

the Thesis Co-Director of KF-S. A-Z participated in the extraction and in establishment of analytical methods for the quantification of compounds. PN-T performed the supervision of the establishment of in-vitro cultures and the evaluation of anti-inflammatory activity, and also was the Thesis Co-director of KF-S, participating in the writing of the manuscript and approving the final version of the manuscript to be submitted.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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