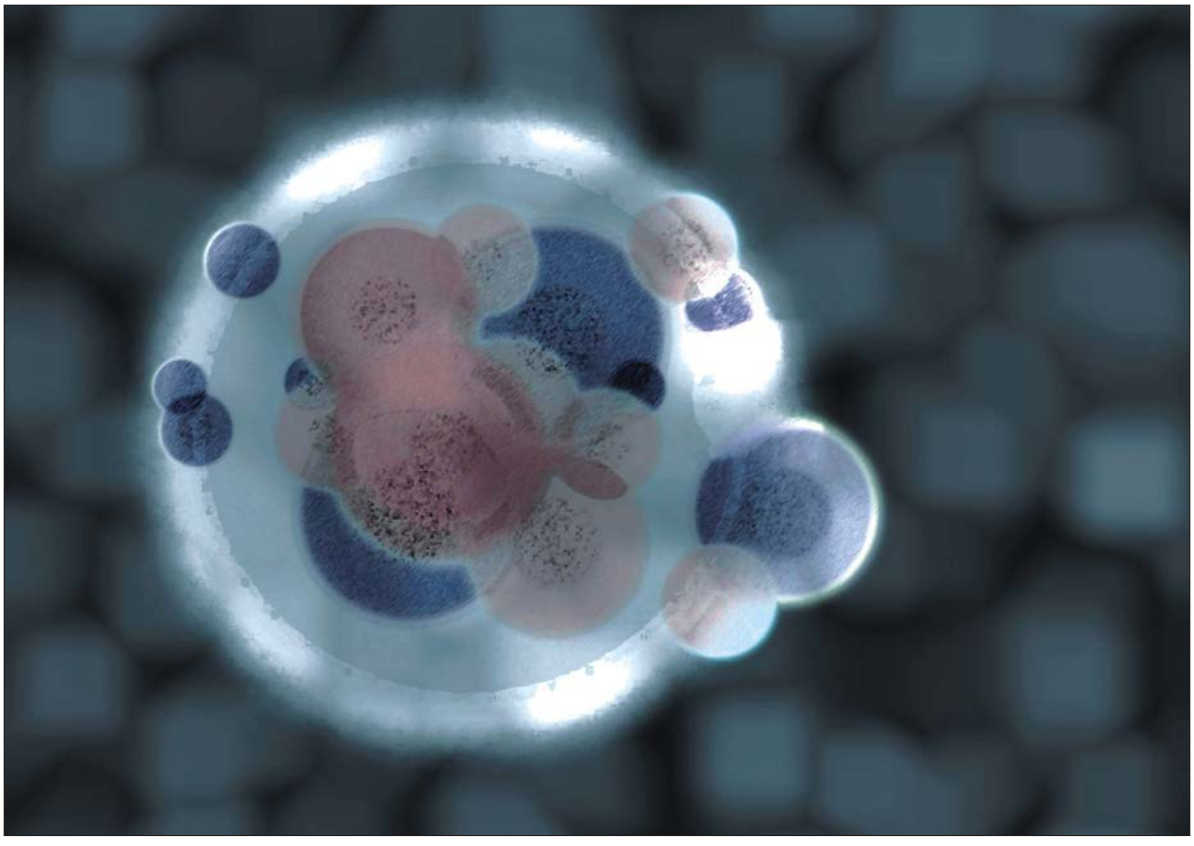

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Polyphenolic Content, Free Radical-scavenging Activity and Isolation of Tiliroside from *Heliocarpus terebinthinaceus* (Tiliaceae) Seeds

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Abstract: *Heliocarpus terebinthinaceus* (Tiliaceae) is a plant native to Central America with a potential as an herbal medicine. The aim of this work was to study the chemical composition of the plant seeds in terms of polyphenolic and flavonoid compounds and the free radical-scavenging capacity of the seed extracts. *H. terebinthinaceus* seeds can be considered as a good source of Total Polyphenols (TPP) with a content of 1053±18 mg of gallic acid equivalents 100 g^{-1} of dry matter. The half maximum scavenging concentrations (SC50) of the polar extracts ranged from 1308±23 to 1970±13 μg mL^{-1}. For the chemical elucidation, the ethyl acetate extract was purified using two types of chromatography: silica gel flash column and preparative thin layer. The structural elucidation of the main compound was achieved by 1D and 2D Nuclear Magnetic Resonance (NMR), Infrared (IR) and High Resolution Mass Spectrometry (HRMS). From the structural analyses, the isolated compound was identified as tiliroside. This is the first report on the isolation of tiliroside from plants belonging to the *Heliocarpus* genus.

Key words: *Heliocarpus terebinthinaceus*, total polyphenols, total flavonoids, free radical-scavenging capacity, tiliroside

INTRODUCTION

Heliocarpus terebinthinaceus (Tiliaceae) is commonly known as cuetla and is native from southern Mexico and Central America. According to the local medicinal knowledge, the aerial parts of this plant possess properties against acne and gastrointestinal disorders. Moreover, it is well known the therapeutic use of the flowers of the *Tilia* (Tiliaceae) genus to control cough and bronchitis (Gruenwald *et al*., 2000) and for their anti-inflammatory, antinociceptive (Toker *et al*., 2004a) and anxiolytic activity (Herrera-Ruiz *et al*., 2008).

Since plants of *Tilia* (Tiliaceae) genus have shown both antioxidant (Dimitrios, 2006) and antimicrobial (Nohynek *et al*., 2006) activities, it is possible that such functional properties may be associated with polyphenolic compounds, including flavonoids. It has been reported that the aerial parts of plants belonging to the Tiliaceae family contain secondary metabolites with a flavonoid structure. For example, kaempferol-3, 7-*O*-α-L-dirhamnoside and quercetin-3, 7-O-α-L-dirhamnoside have been isolated from *Tilia argentea* (Toker *et al*., 2004b).

Consequently, the study of natural products of Tiliaceae plants is very relevant to relate the antioxidant activity to the specific flavonoids present in these plants. This study describes the quantification of total polyphenols (TPP) and flavonoids (TF), free radical-scavenging capacity, as well as the isolation of tiliroside from *H. terebinthinaceus* seeds. This is the first phytochemicals study of *H. terebinthinaceus*.

Experimental

Materials and equipment: Folin-Ciocalteu reagent, gallic acid, rutin, 2,6-di-t-butyl-4-methylphenol (BHT), 2,2-diphenyl-1-picrylhidracyl (DPPH), 2-aminoethyl diphenylborinate, KBr, HCO_2H, Et_2O, DMSO, DMSO-d_6 and D_2O were purchased from Sigma-Aldrich Inc. (USA) Na_2CO_3, hexane, $CHCl_3$, EtOAc, acetone and MeOH, were purchased from J.T. Baker (USA) and EtOH from Reasol (Mexico).

Absorbance readings were obtained with a microplate reader ELx808 (BioTek Instruments Inc., USA). Solvent degassing was performed in an 8510 sonicator (Branson Ultrasonics Co., USA). Solvent evaporation was carried out in a LABOROTA 4000-efficient rotary evaporator

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Table 1: TPP, TF and free radical-scavenging capacity in seeds of *H. terebinthinaceus* extracted by Soxhlet

Extract	TPP (GAE mg 100g^{-1} seed)[a] (DM)	TF (RE mg 100g^{-1} seed)[b] (DM)	Free radical-scavenging SC_{50} (μg mL^{-1})
Hexane	8.1±0.2	1.04±0.02	-
Et_2O	98.6±1.8	16.8±0.2	-
$CHCl_3$	120.1±0.8	21.5±0.2	-
EtOAc	301.6±12.3	41.6±1.4	1970±13
Acetone	348.4±12.5	59.3±1.0	1308±23
MeOH	176.2±3.5	59.9±1.8	1810±24
BHT[†]	-	-	26.0±1.0

[a]GAE: Gallic acid equivalents. [b]RE: Rutin equivalents. [†]BHT: Positive control. Each value is the Mean±S.D of at least three replicates, SC_{50} is the final concentration of extract in the well required to decrease the initial DPPH concentration by 50%. DM: Dry matter. Calibration equation for TPP, y = 0.0029x-0.0278 (R^2 = 0.999). Calibration equation for TF, y = 0.0189x-0.0721 (R^2 = 0.995)

(Heidolph, Germany), equipped with a MZ2C vacuum pump (Vacuubrand, Germany) and an ECO-SEV-10 cold-water recirculating bath (SEV, Mexico). Chromatographic purification was performed using flash column chromatography with silica gel packing (230-400 mesh, 60 Å) followed by preparative thin layer chromatography (TLC) employing Techware Analtech plates of 20×20 cm and 500 μm of thickness. The tiliroside IR spectrum was obtained using a Spectrum GX instrument (Perkin Elmer, USA) with KBr plates. The high resolution mass spectrum (HRMS) was obtained using an LC/MSD TOF instrument (Agilent Technologies, USA) equipped with an APCI source. TLC analyses were performed with silica gel UVG-54 plates (Merck, USA). TLC spots were visualized at 254 nm. The melting point was measured with a Fisher-Johns apparatus and is uncorrected. NMR data were recorded on a GSX-DELTA ECLIPSE+400 spectrometer (Jeol, Japan), operating at 399.78 and 100.53 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts were referenced to tetramethyl silane (TMS). The tiliroside structure was assigned by analyzing the 1D, 2D (HETCOR and COSY) data using ^{1}H and ^{13}C NMR experiments.

Plant material: Seeds of *Heliocarpus terebinthinaceus* were collected at the Universidad Technologica de la Mixeca Campus (1751 m of altitude, 17°49'32" N, -97°48'4" W) in Huajuapan de León, Oaxaca, Mixteca. The voucher specimen was deposited in the SERBO Herbarium, San Sebastián Tutla, Oaxaca, Mexico (http://serboax.org/ herbario/). The seeds were selected, dried and crushed in a domestic worm mill and stored in amber vials at -20°C previously to sample pretreatment.

Preparation of extracts: Crushed seeds of *H. terebinthinaceus* (81 g) were successively extracted in a Soxhlet apparatus during 5 h using hexane, Et_2O, $CHCl_3$, EtOAc, acetone and MeOH (180 mL of each solvent). The corresponding extracts were obtained by evaporation under vacuum in a rotary evaporator and stored at -20°C prior to analysis. Extracts yields are given in Table 1.

Total polyphenols assay: The assay method reported by Santos-Sanchez *et al.* (2012) was used. The TPP content was expressed as mg of gallic acid equivalents (GAE) per 100 g of dry matter (DM).

Total flavonoids assay: The spectrophotometer assay for the quantitative determination of flavonoid content was adapted to a microplate reader instrument from the work of El Hariri *et al.* (1991). Briefly, 100 mu: μL of extract or standard were mixed with 50 mu: μL of 2-aminoethyl diphenylborinate (1%in MeOH). After 30 min settling, the sample absorbance was determined at 405 nm. The TF content was expressed as mg of rutin equivalents (RE) per 100 g DM. All measurements were performed in triplicate.

Free radical-scavenging capacity assay: The assay reported by Julian-Loaeza *et al.* (2011) was adapted to a microplate reader instrument. Briefly, 10 μL of extract were mixed with 100 μL of DPPH• solution (125 mM in DMSO). The reaction mixture was covered and kept in the dark at room temperature for 30 min, followed by absorbance measuring at 545 nm. BHT was used as control. The DPPH• scavenging was calculated using the equation: S (%) = 100 x (A_{DPPH} - A_s)/A_{DPPH}, where A_{DPPH} is the absorbance value of the DPPH$^•$ blank and A_s is the absorbance value of the tested sample or control. Sample SC_{50} data were obtained by plotting the decrease in DPPH• absorbance, S(%), as a function of extract concentration. The SC_{50} value indicates the concentration of the extract that causes 50% of radical scavenging. All measurements were performed in triplicate.

Purification: The EtOAc extract (700 mg) was redissolved in acetone and was introduced into a flash column packed with silica gel. A solvent gradient was performed as follows: hexane, the initial eluent, was swapped to $CHCl_3$ by employing $CHCl_3$ 20% composition changes until the eluent was $CHCl_3$ 100%. Then $CHCl_3$:EtOAc (1:1) was used as eluent and was swapped to EtOAc using composition changes with EtOAc 20%. The tiliroside was obtained in the $CHCl_3$:EtOAc (3:7) fraction, which was further purified by preparative TLC using

acetone:$CHCl_3$:formic acid (HCO_2H) (3:2:0.1) as eluent. Isolation was achieved by crystallization with a mixture of acetone:hexane at 4°C to obtain 2.7 mg of tiliroside, which accounted for 0.39% of the EtOAc extract.

Structure elucidation of kaempferol-3-O-(6''-O-E-p-coumaroyl)-β-D-gluco-pyranoside (tiliroside): Light yellow solid, mp 255-258°C (lit. 257-260°C, Bajaj *et al*., 1986), R_f (AcOEt) 0.27. FT-IR (KBr, $_{max}$, cm^{-1}) 3251 (OH), 1684 (C = O), 1608 (C = C), 1183 (C-O). $HRMS^+$ m/z 595.1430 $[M+H]^+$ (calcd for $C_{30}H_{27}O_{13}$, 595.1452), 617.1242 $[M + Na]^+$ (calcd for $C_{30}H_{26}O_{13}Na$, 617.1271). ^1H NMR (DMSO-d_6, δ, ppm, J/Hz): 10.2 (1H, s, OH), 7.99 (2H, d, J = 8.6, H-2', H-6'), 7.36 (2H, d, J = 8.6, H-2''', H-6'''), 7.34 (1H, d, J = 15.9, H-7'''), 6.86 (2H, d, J = 8.6, H-3', H-5'), 6.79 (2H, d, J = 8.6, H-3''', H-5'''), 6.38 (1H, d, J = 1.7, H-8), 6.15 (1H, d, J = 1.7, H-6), 6.11 (1H, d, J = 15.9, H-8'''), 5.45 (1H, d, J = 7.3, H-1"), 5.20 (1H, br.s, OH), 5.15 (1H, br.s, OH), 4.28 (1H, dd, J = 11.8, 1.4, H-6"A), 4.03 (1H, dd, J = 11.8, 6.5, H-6"B), 3.38 (1H, ddd, J = 9.1, 6.5, 1.4, H-5"), 3.25 (1H, t, J = 8.6, H-3"), 3.21 (1H, dd, J = 8.6, 7.3, H-2"), 3.16 (1H, dd, *J* = 9.1, 8.6, H-4"). ^{13}C NMR (DMSO-d_6, δ, ppm): 177.9 (s, C-4), 166.7 (s, C-9'''), 164.8 (s, C-7), 161.7 (s, C-5), 160.5 (s, C-4'), 160.3 (s, C-4'''), 157.0 (s, C-8a), 156.9 (s, C-2), 145.1 (d, C-7'''), 133.7 (s, C-3), 131.4 (d, C-2', C-6'), 130.7 (d, C-2''', C-6'''), 125.5 (s, C-1'), 121.4 (s, C-1'''), 116.3 (d, C-3', C-5'), 115.6 (d, C-3''', C-5'''), 114.2 (d, C-8'''), 104.4 (s, C-4a), 101.6 (d, C-1"), 99.3 (d, C-6), 94.2 (d, C-8), 76.8 (d, C-3"), 74.8 (d, C-5"), 74.7 (d, C-2"), 70.6 (d, C-4"), 63.5 (t, C-6").

Statistical analysis: Data obtained from experiments were expressed as mean±standard deviation.

RESULTS AND DISCUSSION

Traditionally, the extraction of natural products has been performed by maceration, which is a considerably lengthy process. For this reason a Soxhlet method was employed for polyphenolic compound extraction. The dry matter yields of the organic solvent extracts of *H. terebinthinaceus* seeds diminished with solvent polarity. The hexane extract resulted in the highest yield (1.77%) because of the seeds' high oil content.

Plant-derived polyphenolic compounds can be considered as high-value fine chemicals since they have a broad range of biological activity, including antioxidant properties (Qiao *et al*., 2011). In this regard, the concentration of total polyphenols (TPP) in *H. terebinthinaceus* seeds was determined. The TPP and TF values were obtained using a spectrophotometric method with the Folin-Ciocalteu reagent and the 2-aminoethyl diphenylborinate-flavonoid complex, respectively. The acetone extracts had the highest TPP content (348.4±12.5 mg GAE 100 g^{-1} DM), followed by the EtOAc extracts (301.6±12.3 mg GAE 100^{-1} g DM), Table 1; these extracts had the highest TPP concentrations. The total amount of TPP was 1053±18 mg GAE 100 g^{-1} DM. This result shows that *H. terebinthinaceus* seeds are a good source of TPP. With respect to TF content, the highest concentrations were obtained in the EtOAc (41.6 ± 1.4 mg RE 100 g^{-1} DM), acetone (59.3±1.0 mg RE 100 g^{-1} DM) and MeOH (59.9±1.8 mg RE 100 g^{-1} DM) extracts.

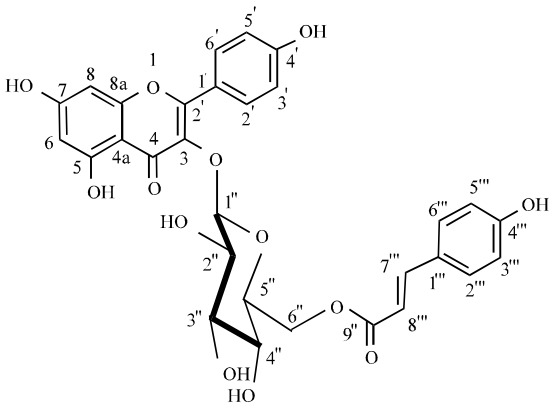

Fig. 1: Chemical structure of tiliroside

The DPPH• scavenging assay has been widely used to evaluate the antioxidant activity of different plant extracts (Alothman *et al*., 2009; Prior *et al*., 2005). The antiradical activity of the extracts at different concentrations was tested by the DPPH• assay, the lower number indicating the higher antioxidant quality, Table 1. The SC_{50} values of the polar extracts ranged from 1308±23 to 1970±13 μg mL^{-1}. The tests for the hexane, Et_2O and $CHCl_3$ extracts did not achieved the SC_{50} concentration, due to their low content of polyphenolic compounds. The percentage of maximum antiradical activity of hexane, Et_2O and $CHCl_3$ extracts were 25.63±0.29, 27.54±0.56 and 36.86±0.59%, respectively.

In the last years there has been an increasing interest in the discovery of new natural sources of flavonoids because of their beneficial effects on human health (Qiao *et al*., 2011). The EtOAc extract was selected since it has an intermediate polarity and thus allows an easy and cheap separation with silica gel, a normal phase commonly used in chromatography. The EtOAc extract of *H. terebinthinaceus* seeds contained tiliroside as the main flavonoid, Fig. 1. The isolation of the main flavonoid from an extract was performed. The EtOAc extract was selected since it has an intermediate polarity and thus allows an easy and inexpensive separation with silica gel, a normal phase commonly used in chromatography.

The unambiguous structure assignment of the isolated compound was made by comparing the spectroscopic data of this compound with that reported for kaempferol-3-O-(6''-O-E-p-coumaroyl)-β-D-glucopyranoside (tiliroside) (Kaouadji *et al*., 1993). According to the literature, this compound has anti-complement (Ferreres *et al*., 2009), antinflammatory (Sala *et al*., 2003), antioxidant (Sala *et al*., 2003), antidiabetic (Zhu *et al*., 2010), hepatoprotective (Matsuda *et al*., 2002) and anticancer (Tsimplouli *et al*., 2011) activity. Sala *et al*. (2003) reported that the tiliroside has a very high antioxidant activity measured by the DPPH• test, with an SC_{50} of 6 μM (3.57 μg mL^{-1}).

CONCLUSIONS

To conclude, *H. terebinthinaceus* seeds have a high total polyphenol content (1053±18 mg GAE 100 g^{-1} DM). The main flavonoid in the EtOAc extract was isolated and characterized as tiliroside, a flavonoid with a broad range of relevant biological activities. Therefore, these seeds can be used as a source of antioxidants in functional foods. This is the first report on the isolation of tiliroside from plants belonging to the *Heliocarpus* genus.

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