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Anti-inflammatory and toxicological evaluation of *Moussonia deppeana* (Schldl. & Cham) Hanst and Verbascoside as a main active metabolite

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ABSTRACT

Ethnopharmacological relevance: Moussonia deppeana, known as Tlachichinole, is a Mexican medicinal plant used for treatment of inflammatory diseases, influenza, diarrhea, gastrointestinal disorders and arthritis.
Aim of the study: In this paper the antioxidant and anti-inflammatory activities as well as the acute and sub-acute toxicological effects were evaluated for the ethanolic extract from aerial parts of M. deppeana, also its phytochemical analysis is described.
Materials and methods: Phytochemical analysis and compound isolation were performed with thin layer chromatography. The chemical identification of the main compound was performed by ^{1}H NMR (COSY, NOESY, HSQC and HMBC) spectra. *In vitro* antioxidant capacity and total phenolic content for the ethanolic extract and its primary fractions was determined by DPPH and Folin-Ciocalteu reagent. Acute and subacute toxicity tests were evaluated on Balb/C mice. Finally acute anti-inflammatory evaluation was tested for a local (TPA) and systemic (carrageenan) murine model.
Results: The main compound isolated from the ethanolic extract of *M. deppeana* was Verbascoside, which was isolated from F3 and was identified by ^{1}H NMR and COSY data. Furthermore oleanolic and ursolic acids were isolated from primary fractions F1 and F2. Ethanolic extract showed IC_{50} = 6.71 mg/mL for DPPH test and 664.12 μg QE/mL for the total phenolic content. The LD_{50} value was > 2 g/kg by i.g. route in male and female mice. Sub-acute administration (28 days) of the ethanolic extract (1 g/kg) did not cause lethality or alter any hematological and biochemical parameters, in addition, histological analysis of the major organs exhibited no structural changes. Anti-inflammatory activity of the ethanolic extract showed an ED_{50} = 1.5 mg/ear and 450 mg/kg for TPA and carrageenan test, respectively. Primary fractions generated moderate local and systemic anti-inflammatory activity.
Conclusion: The ethanolic extract from the aerial parts of *M. deppeana* did not cause any lethality or adverse effect in either of the acute and sub-acute toxicity tests. This exhibited an important local and systemic anti-inflammatory activity and also moderate antioxidant capacity. Moreover, the primary fraction F2 was more active for the TPA model while the primary fraction F3 was most active in the carrageenan model *in vivo.* The main compound isolated from F3 was verbascoside; on the other hand also ursolic and oleanolic acids were isolated from F1 and F2.

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1. Introduction

Remedies and products prepared from herbal extracts are among the most common types of traditional medicine because these have been employed for the treatment of human diseases since ancient times (Zhang et al., 2010). World Health Organization (WHO) estimates that nearly 80% of the population uses traditional medicine because the current allopathic medicine causes several side effects along with the high cost and limited access (Debas et al., 2006; Obomsawin, 2011). Therefore is necessary that government develop policies and programs to regulate their use and the search for new drugs from natural medicinal plants with few adverse effects (Zeng and Jiang, 2010). In this context, today the toxicological studies for medicinal plants are mandatory because are consumed by much of the population, only their natural origin; however, some species produce a serious toxicity in human (Youns et al., 2010).

Mexico has over 3,000 medicinal plants that are widely used for the treatment of several diseases; however, very few species have been studied with a toxicological and pharmacological aim; hence, the risks and true benefits of these plants remain unknown (Rodríguez-Fragoso et al., 2008). Some plants such as nopal (*Opuntia ficus*) and aloe (*Aloe vera*) are employed for the treatment of diabetes; however, they generate many adverse side effects, such as diarrhea and poor platelet aggregation. Other plants, such as peppermint (*Mentha piperita*), dandelion (*Taraxacum officinale*) and chaparral (*Larrea divaricata*) are used for the treatment of gastric inflammatory diseases, but with side effects such as hepato- and nephrotoxicity (Déciga-Campos et al., 2007).

Moussonia deppeana (Schldl. & Cham) Hanst (syn. *Kohleria deppeana*, *Gesneria deppeana, M. elongata*), commonly known as *Tlachichinole*, *Tlalchichinolli*, *Cacahuatillo* and *Clachichinole* or *Valletilla*, is usually used as a medicinal plant in Mexico (Ramírez-Roa and Chiang, 2010). This shrub belongs to the *Gesneriaceae* family which is found from northern Mexico to Panama (Ramirez-Roa and Hernandez-Varela, 2011). On the ancient use of this medicinal plant in Mexico little it is known; however, in some parts of Central and South America it described that was used by American Indian tribes for blood diseases by the color of their flowers and against snake bite (Kvist and Skog, 1992). Currently, it is used for the treatment of inflammation and pain of the stomach, kidney disease, vaginal infection, tumors, gastrointestinal disorders (gastritis and diarrhea), duodenal ulcer, acne, burns, skin wounds, bleeding, cough, flu, arthritis and certain diseases related to inflammation (Kvist and Skog, 1992; Argueta and Gallardo-Vázquez, 1994; Calzada et al., 1998. Castillo-Juárez et al., 2009. Dominguez-Ortiz et al., 2010; Villavicencio Nieto and Pérez Escandón, 2010; Robles-Zepeda et al., 2011; BDMTM, 2016).

It is noteworthy that this plant is widely sold in Mexico as a dietary supplement in several preparations such as capsules, powders, alcoholic solutions and infusions; however, this medicinal species is not registered by Mexican Ministry of Health as a drug. Scarce phytochemical research describes the presence of β-sitosterol, ursolic and oleanolic acids, 2-methyl anthraquinone, chromones and stigmasterol in the aerial parts of *M. deppeana* (Noguera et al., 1994; Reyes-Blas, 1995; Fai and Tao, 2010); furthermore, the antioxidant potential *in vitro* and the acute anti-inflammatory effect *in vivo* of successive organic extracts (Hexanic -Hex-, Ethyl acetate -EtOAc- and Ethanolic -EtOH-) from these aerial parts have been described. The EtOAc extract showed a major antioxidant capacity and the Hex extract exhibited a major topical anti-inflammatory effect (Domínguez-Ortiz et al., 2010). In addition, from these successive extracts only EtOAc extract showed antimycobacterial activity *in vitro* against *Mycobacterium tuberculosis* with minimal inhibitory concentration (MIC) ≤ 25 μg/mL, while it did not show antiprotozoal activity *in vitro* with a median inhibitory concentration (IC_{50}) of >300 μg/mL (Jimenez-Arellanes et al., 2013).

Another study showed that the methanolic (MeOH) extract from the whole plant was active against *E. histolytica* and *G. lamblia*, with $IC_{50} = 120.92$ and 65.71 μg/mL, respectively (Calzada et al., 1998). The MeOH extract from leaves was active against *Helicobacter pylori* (MIC = 15.6 μg/mL) while the aqueous extract was inactive (MIC ≥ 1 mg/mL) (Castillo-Juárez et al., 2009). In addition, the MeOH extract from the whole plant was moderately active against clinically isolated *H. pylori* (MIC $<1{,}600$ μg/mL) (Robles-Zepeda et al., 2011). Finally, a MeOH extract from leaves demonstrated moderate activity against *Trypanosoma cruzi* (MC_{100} = 250–500 μg/mL) (Abe et al., 2005).

Despite the widespread use of *M. deppeana* in Mexican traditional medicine, there are no toxicological studies of this species to this day. In this manuscript, the acute and sub-acute toxicity of the EtOH extract of this medicinal plant is described, as well as its *in vitro* antioxidant capacity and its acute anti-inflammatory effect on two murine models *in vivo*. The isolation and identification of Verbascoside as a major compound is also described for this species for the first time.

2. Material and methods

2.1. Collection of plant material and crude extract preparation

Moussonia deppeana was collected in Veracruz State, Mexico, in February 2013. A specimen was identified by Luis Bojórquez-Galván from the Biological Research Institute of the Universidad Veracruzana and a specimen voucher was registered under reference CIB8987. The aerial parts (1,371 g) were dried at room temperature under conditions of darkness; afterward, these were macerated with EtOH (6 L) for 1 week (3 times). The extract was filtered and concentrated at 40 °C using a rotary evaporator (Buchii RE-111) coupled to a vacuum system (BuchiiVac V-153) and a cooling system (ECO 20). The extract was left under conditions of darkness until its use.

2.2. Liquid/liquid partition and phytochemical analysis

The crude EtOH extract (5 g) was subjected to liquid/liquid partition with acetonitrile (CH_3CN, F1), chloroform ($CHCl_3$, F2) and MeOH (F3). Each solvent was evaporated in a rotaevaporator coupled to a vacuum pump. In F1 and F2 primary fractions, β-sitosterol, stigmasterol, ursolic acid (**3A**) and oleanolic acid (**3B**) were detected by Thin-Layer Chromatography (TLC) using dichloromethane (CH_2Cl_2): MeOH (96:4) or Hex: EtOAc (6:4), as mobile phases, while in the F3 fraction, polyphenolic compounds such as Verbascoside (**3C**) were isolated by preparative TLC as major compound using EtOAc: EtOH: H_2O (100:13.5:10) or EtOAc: Formic acid: Acetic acid: H_20 (10:1.1:1.1:0.3), as mobile phases. Triterpenes were detected with aqueous H_2SO_4 10% and polyphenolic compounds were identified with 2-amine-ethyl ester diphenylboric acid 1% in MeOH with polyethylenglycol 5% in EtOH as a chromogenic agent. The detected and isolated compounds were compared with Sigma pure standards.

2.2.1. General experimental procedures and structural elucidation

Analytical and preparative TLC was carried out on silica gel 60 F_{254} precoated aluminum plates (0.2 mm, Merck). High-Performance Liquid Chromatography (HPLC) analysis was carried out in Waters equipment (Waters, USA) comprising a 600E multisolvent delivery system with a 486 UV detector. Equipment control, data acquisition, processing and management of the HPLC information were performed by Empower 2 software (Waters).

The analytical conditions were employed: column ZORBAX Eclipse XDB-C18 (5 μm, 4.6 × 250 mm i.d.), with pre-column (Agilent Technologies); mobile phase linear gradient of 0.0125 N aqueous-acetic acid (eluent A) and CH_3CN (eluent B), starting from 95% A at 50% in 20 min, returning to 95% for 20 min to 25 min, this was maintained for 35 min; the flow rate was 0.7 mL/min and the injection volume was 20 μl; peaks were detected at 280 nm; this condition was utilized for EtOH extract. Primary fraction F3 was analyzed under linear gradient, started from 100% A reached 60% in 30 min, and returned to 60–100% A from 40 to 45 min, with a flow rate of 0.7 mL/min and a 20-μl injection volume; peaks were detected at 280 nm. Structural characterization of triterpenes (**3A** and **3B**) and the major compound (**3C**) was determined through Protonic Nuclear Magnetic Resonance (^{1}H NMR). ^{1}H NMR was performed in a Varian Inova 500 spectrometer (at 500 MHz, 25 °C), and chemical shifts are expressed in parts per million (ppm) relative to Trimethylsilane as an internal standard in deuterated $CDCl_3$ and deuterated MeOH. Spectroscopic data of each compound were compared with previously reported. The solvents were obtained from Mallinckrodt and the reagents were purchased from Sigma Aldrich Corp. DOSY ^{1}H experiments were performed on a Varian Inova 500 spectrometer equipped with a gradient pulse amplifier and 5 mm indirect detection probe. The Diffusion bi-polar pulse pair stimulated (DBPPSTE) pulse sequence was used to obtain the DOSY spectra, acquired with a diffusion delay of 160 and 190 msec for the primary fraction F3 and the EtOH extract, respectively, with gradient-pulse amplitudes ranging from 0.30 to 32 G cm^{-1} for 3 msec of the gradient of stabilization with a 300-msec delay. The standard Varian protocol was used for processing in VNMRJ (version).

2.3. Antioxidant capacity in vitro

2.3.1. DPPH radical-scavenging capacity

2,2-Diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, D9132–5 G) radical inhibition potential was determined according to the method described by Brand-Williams et al., (1995) and Miliauskas et al., (2004). The EtOH extract was evaluated from 3.15, 6.25, 12.5 and 50 mg/mL (R^2 = 0.96). The experiment was carried out twice and the percentage of inhibition was calculated with the following formula (Domínguez-Ortiz et al., 2010): % Inhibition = [AC-AS/AC] x 100.

AS: Absorbance sample, AC: absorbance DPPH control.

2.3.2. Total phenolic content (TPC)

This assay was determined using the Folin-Ciocalteu reagent with the method previously described by Spanos and Wrosltad (1990) and Sellapan et al., (2002). Each experiment was carried out by triplicate and the results were expressed as μg quercetin equivalents (QE)/mL, using a standard quercetin graph (range 1.56–25 μg/mL): Absorbance = 0.0018 [QE (μg/mL)] − 0.0039 R^2 = 0.9260.

2.4. Animal´s in vivo assay

Adult Balb/C mice (25 ± 5 g) of both sexes were used for acute and sub-acute toxicity studies. The mice were obtained from the Animal Vivarium of CMN-SXXI, IMSS, Mexico City and were maintained in plastic cages during a 7-day conditioning period prior to the experiments under laboratory conditions (12 – h/12 – h light/dark cycles; temperature 25 ± 2 °C; humidity 55–80%) with Rodent Chow food and water *ad libitum*. Experiments were performed following the statutes of the International Committee for the Care and Use of Laboratory Animals (IACUC) and Mexican Official Norm (NOM-062-ZOO-1999) revised in 2015. The project was approved by the National Commission of Scientific Investigation with code registration CNIC-IMSS R-2013-785-053.

2.5. Acute toxicity

This test was performed according to procedure TG 423 described by the Organization for Economic Co-operation and Development and Test Guideline (OECD TG 423, 2008). Groups of three animals per sex were employed and the EtOH extract was administered by an intragastric (i.g.) route after a fasting period of 12 h. Control received only the vehicle (Tween 80-H_2O, 1:9) and the treated group received a single administration of EtOH extract at 0.5, 1 and 2 g/kg body weight (BW) doses, in a volume not exceeding 10 mL/kg BW. The animals were maintained under observation for 14 days and their BW gain was recorded on days 3, 7, 9 and 14. After that, the animals were euthanized and the liver, stomach, spleen and both kidneys were extracted and macroscopic observation was conducted to find gross pathological lesions and relative weight changes. This experiment was performed in two independent tests for each group and sex.

2.6. Sub-acute toxicity study

For this study, EtOH extract was administered by i.g. route daily for 28 days following the method described in OECD TG 407 (2008). Two groups of 20 animals each (10 males and 10 females) were used. Group I, received vehicle while Group II received the EtOH extract at a doses of 1 g/kg BW in a maximal volume of 10 mL/kg of BW. The BW gain was recorded from day zero and every 7 days until day 28. At the end of the study, the animals were maintained under fasting conditions for 12 h prior to blood sampling, which was performed by retro-orbital puncture. A blood sample was collected with EDTA for hematological analysis and the remaining blood was collected to obtain serum for biochemical analysis. Hematological analysis was performed in a Beckman Coulter Cell Counter and the following parameters were measured: total Red blood cell count (RBC); Hemoglobin (Hb); Hematocrit (Hto); Mean corpuscular volume (MCV); Mean corpuscular hemoglobin concentration (MCHC); Mean corpuscular hemoglobin (MCH); platelets; total White blood cell count (WBC) and a differential study of white cells, such as lymphocytes, segmented neutrophils, eosinophils, monocytes and basophils.

For the biochemical analysis the following parameters were measured: Alkaline phosphatase (ALP); Alanine aminotransferase (ALT); Aspartate aminotransferase (AST); urea; creatinine; glucose; total Cholesterol (CHOL); High density lipoprotein (HDL); and Triacylglycerides (TAG), employing the automatic Selectra Analyzer (Model Vitalab 2) and commercial kits (RANDOX). Finally, Low and Very low density lipoprotein (LDL and VLDL, respectively), were calculated according to the formulas described by Wilson et al., (1981):

VLDL = (TAG/5)LDL = (CHOL-HDL) x VLDL.

2.6.1. Histological analysis

The mice were sacrificed by cervical dislocation and a necropsy was performed soon after death for the macroscopic examination of liver, lungs, heart, kidneys and spleen. Tissue biopsies from these organs were fixed in 10% formalin, processed and embedded in paraffin. The paraffin block was cut into 4–5 μm slices with a rotary microtome and stained with Hematoxylin and Eosin (H&E) following the procedure described by Cornejo-Garrido et al., (2012). The samples were examined under a light microscope for the search of microscopic lesions.

2.7. In vivo anti-inflammatory activity

2.7.1. Tetradodecylphorbol-13-acetate (TPA) induced ear edema

This assay was conducted as described by Dominguez-Ortiz et al., (2010) and Garcia-Rodriguez et al., (2014). Control was treated with TPA (2.5 μg) in acetone on the right ear (W's) and then left ear received only 25 μl of acetone (Wo). The experimental groups (male and females; $n = 7$) received TPA and 30 min later were treated with EtOH extract, indomethacin (0.5, 1, and 2 mg/ear) or primary fractions F1, F2 and F3 (2 mg/ear) in the right ear (Ws). Anti-inflammatory activity was calculated according to the weight difference between the ear sections (6 mm) at 6 h, compared with the control group, using the following formula:

% Inhibition = [(Ws-Wo) control – (W´s – Wo) treated/(Ws-Wo) control] X 100.

2.7.2. Carrageenan-induced edema in mice

This model was performed as described by Dominguez-Ortiz et al., (2010) and Garcia-Rodriguez et al., (2014). Treated groups ($n = 7$) received by the i.g. route indomethacin (10 mg/kg), EtOH extract (150, 300, 450 and 600 mg/kg) or primary fractions F1, F2 and F3 (150 mg/kg) 1 h prior to the injection of carrageenan (20 μl, 2%). The sample were solubilized in Tween 80:water 1:9 and the control received only vehicle. The percentage of inhibition was calculated by comparing the measurement of the paw edema at different times (1, 2, 3, 5 and 7 h) (E_t) using a digital micrometer and the value of time zero (baseline) (E_0). The results were analyzed with the formula described by Olajide et al., (1999):

% Inhibition = (Et − Eo) carrageenan − (Et − Eo) treated/(Et − Eo) carrageenan] x 100.

2.7.3. Median effective dose (ED_{50})

ED_{50} was determined by means of the data obtained from the previous experiments and their dose-response curves for TPA and Carrageenan (Perazzo et al., 2013). This method was used to evaluate the effectiveness of the EtOH extract of *M. deppeana* in relation to the inhibition of inflammatory process by comparison with the Indomethacin groups.

2.8. Statistical analysis

Sigma Plot ver. 12.0 statistical software (2011–2012) was utilized for the analysis of the results and graphic elaboration. Data was presented as standard error of the mean (SEM). BW gain values in acute and sub-acute toxicity tests and the development of paw edema in the carrageenan model were analyzed with bifactorial Analysis of variance (ANOVA) and with a post hoc Student Newman Keuls (SNK) test. Results of $p < 0.05$ were considered statistically significant. For sub-acute toxicity, parameters of hematological and biochemical analyses, ear edema weight in TPA, and IC_{50} values for DPPH method, one-way ANOVA was employed with a post hoc SNK test, in which $p < 0.05$ was considered significant. Finally, for the percentage of inhibition of DPPH radical and for hematocrit (Hto) values in the sub-acute toxicity model, a Kruskal-Wallis test (ANOVA by ranks) was carried out, in addition to a post hoc SNK test, in which relevant outcomes were those with a value of $p < 0.05$.

3. Results and discussion

3.1. Phytochemical Analysis

Maceration process produced 60 g of EtOH extract with an average yield of 17% with respect to the material's dry weight. The EtOH extract was analyzed by HPLC, in which apigenin (Retention time -R_t- = 24.68 min, λ_{max} 266.6 and 333.4 nm) and hesperetin (R_t = 25.46 min, λ_{max} 231.1 and 285.6 nm) (Fig. 1) were detected as a minor compounds. This chromatogram was compared with different standards under the same conditions (Fig. 2). By liquid/liquid partition from EtOH extract three fractions were obtained: F1 (CH_3CN, 8.24%); F2 ($CHCl_3$, 4.14%) and F3 (MeOH, 47.21%). The chemical fractionation in open column of F1 and F2 led to the detection of β-sitosterol with a Retention factor (R_f) of 0.43 and stigmasterol R_f = 0.49, using Hex:$CHCl_3$ (7:3) as an elution system; the mixture of ursolic and oleanolic acids showed R_f = 0.47 in Hex:EtOAc (6:4) and R_f = 0.52 in CH_2Cl_2:MeOH (96:4). These compounds were identified by a comparison of the R_f with their commercial reference. The mixture of ursolic acid (Fig. 3(A)) and oleanolic acid (Fig. 3(B)) was isolated by preparative TLC and was identified by comparison with standards and ^{1}H NMR spectra, these data were compared with that previously described (Seebacher et al., 2003; Cornejo-Garrido et al., 2012). These metabolites have been previously reported in *M. deppeana* extracts (Noguera, 1994; Fai and Tao, 2010).

TLC analysis of F3 fraction showed the presence of a major compound with R_f = 0.47 o 0.57 in the elution system EtOAc:EtOH:H_2O (10:1.4:0.5) and EtOAc:CH_2O_2:CH_3COOH: H_20 (10:1.1:1.1:0.3), respectively. F3 was subjected to HPLC (Fig. 4(A)) and ^{1}H NMR analysis (Fig. 5). In the HPLC chromatogram, was observed a main compound with R_t = 27.38 min and with λ_{max} 218 and 330 nm, the R_t was different from chlorogenic acid

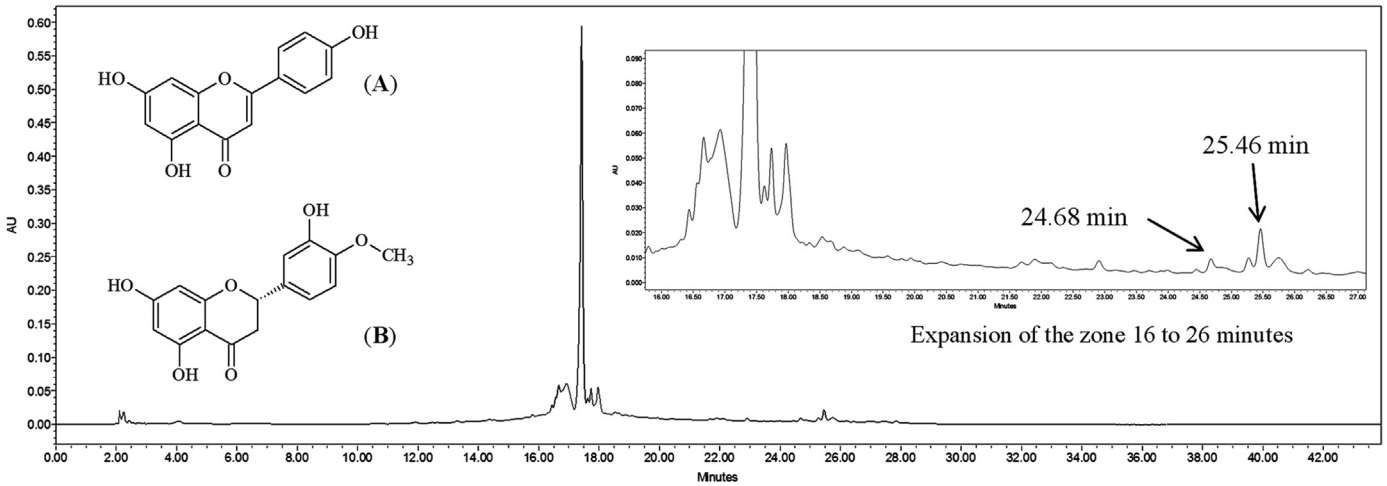

Fig. 1. HPLC chromatogram of the EtOH extract from *Moussonia deppeana* aerial parts.

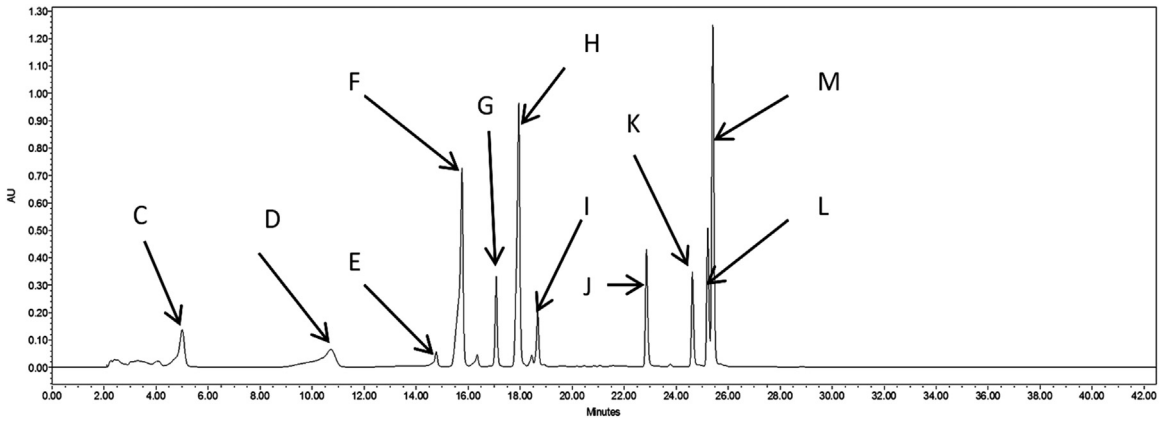

Fig. 2. HPLC chromatogram of different polyphenol standards (Sigma).

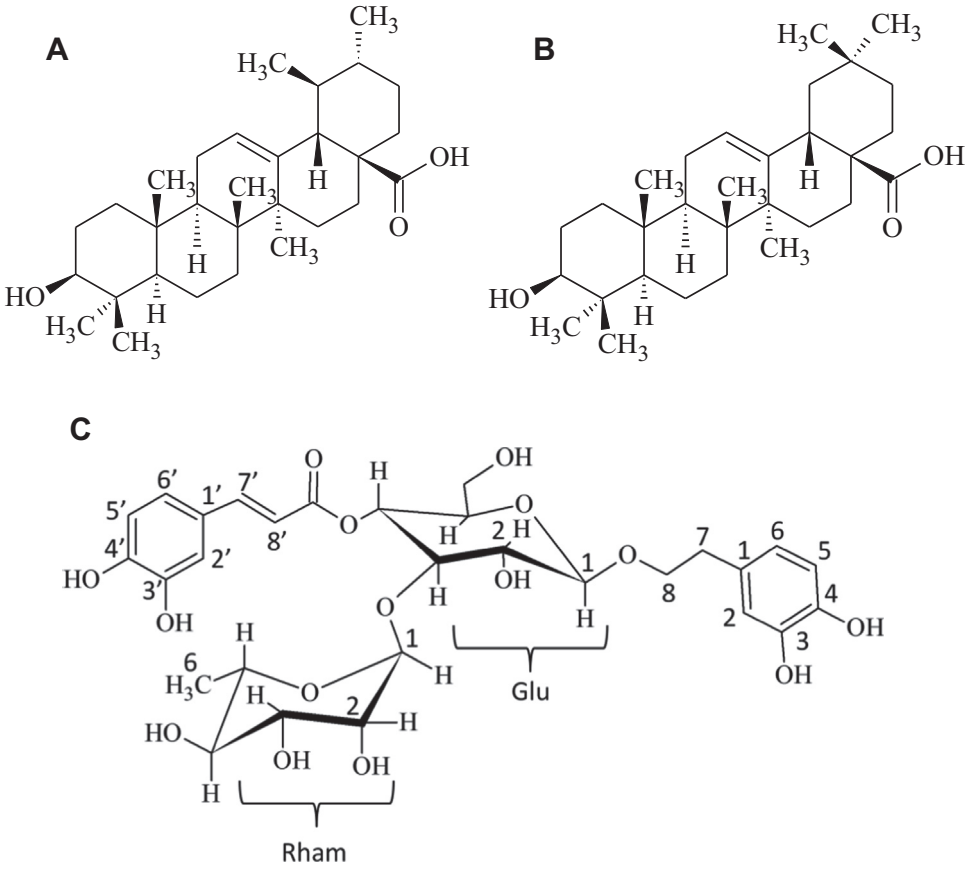

Fig. 3. Chemical structure of identified compounds in the EtOH extract of *Moussonia deppeana*: ursolic acid (**A**), oleanolic acid (**B**) and verbascoside (**C**).

(21.50 min, λ_{max} = 218.1, 240.5, 317.8 nm) or caffeic acid (24.38 min, λ_{max} = 218.1, 239.4, 322.6 nm), these standards were analyzed under the same HPLC conditions (Figs. 4(B) and (C)). In ^{1}H NMR spectra, signals for aromatic proton (between 6.2 and 7.6 ppm) were observed; these signals indicate the presence of chlorogenic acid or caffeic acid derivate; also a signal by the anomeric proton (δ 5.20, d, J = 2 Hz for rhamnose and δ 4.35, d, J = 8 Hz for glucose) was observed. In addition, a signal at δ 1.13, d, J = 6 Hz for the CH_3-rhamnose (Fig. 5) was observed. The main compound was purified by TLC and identified by ^{1}H NMR, the multiplicity of their signals and coupling constants are described in Table 1. The analysis of ^{1}H NMR spectra as well as COSY, NOESY, HSQC and HMBC data were compared with those described in the literature, allowed us to identify the compound as Verbascoside or Acteoside (Fig. 3(C)) (Ghisalberti, 2005; Schlauer et al., 2004; Blazics et al., 2011), with a molecular weight 624. The assignment of the numbers of protons was conducted based on the description of Blazics et al., (2011). The presence of this secondary metabolite has been reported in some species of the *Gesneriaceae* family (Verdan and Stefanello, 2012). To our knowledge, this is the first time that its presence has been reported in *M. deppeana*. Verbascoside as a major compound in both F3 fraction and complete EtOH extract (Fig. 5). It can be used as a fingerprint in these samples and in another polar extract of several commercial preparations of *M. deppeana.* The most important signals that we were allowed us to detect are indicated in the ^{1}H NMR spectrum (Fig. 5). When we compared the ^{1}H NMR profiles of the primary F3 fraction with complete EtOH extract, the spectra are very similar (Fig. 5). To confirm the presence of Verbascoside in EtOH extract and in F3 fraction, the 2D Diffusion ordered spectroscopy (DOSY)

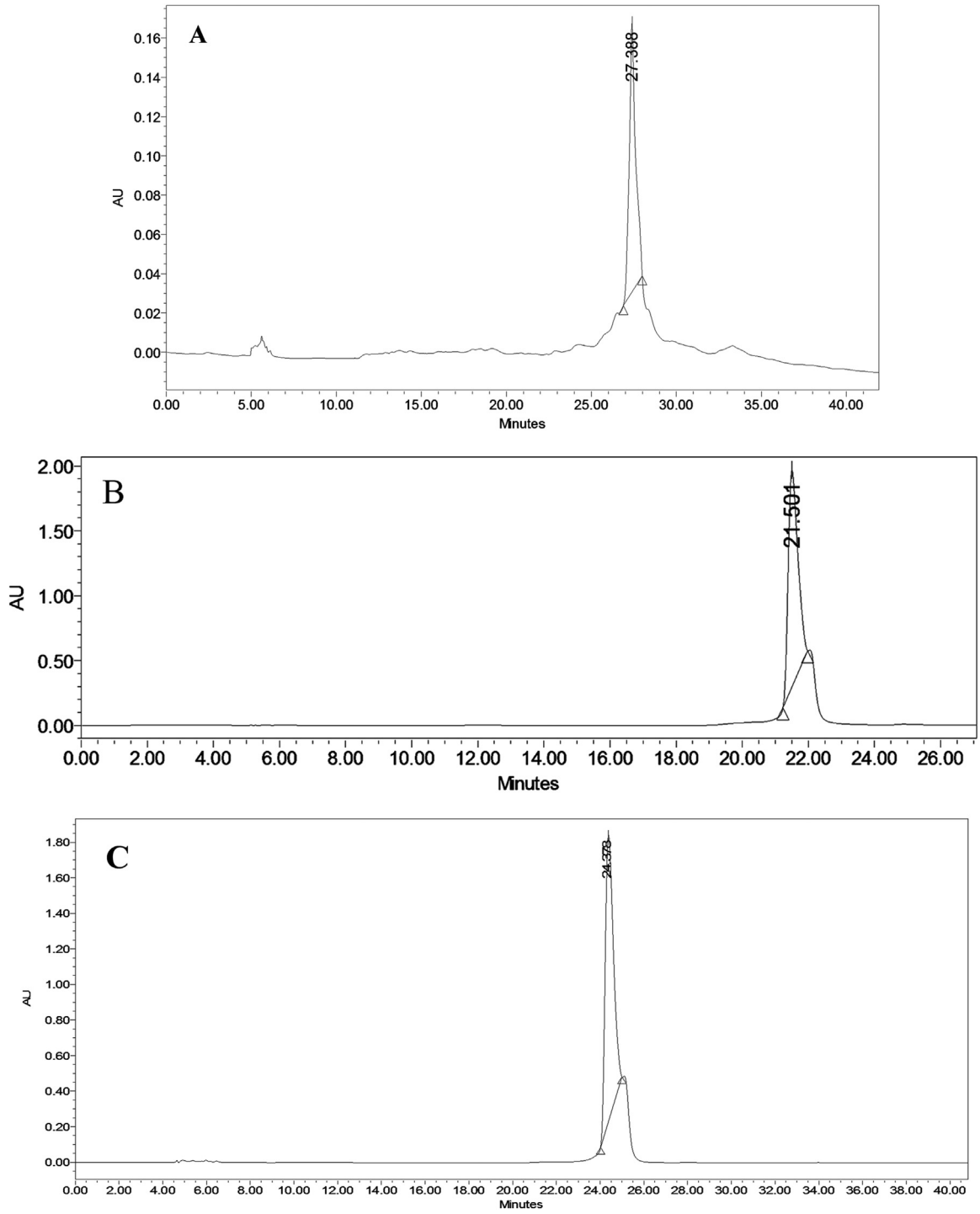

Fig. 4. HPLC chromatogram of primary fraction F3 (A), chlorogenic acid (B) and caffeic acid (C).

^{1}H NMR experiment was performed (Fig. 6). This experiment permits the virtual separation and structural identification of the components from complex matrixes, such as the extract from medicinal plants, without altering the sample. This experiment can also allow us to obtain the metabolic fingerprint of the sample (Politi et al., 2009; Balayssac et al., 2009; León et al., 2011). Through this experiment, the presence of Verbascoside as a main compound in the EtOH extract was corroborated with a diffusion coefficient 1.3×10^{-10} m^2/sec (Fig. 6). The identification and quantitative determination of Verbascoside in several samples can be obtained by using HPLC and DOSY-^{1}H NMR. The quantification by HPLC and Liquid Chromatography-Mass Spectroscopy (LC/MS) of Verbascoside from *Euphrasia rostkoviana* and *Scrophularia scorodonia* has been described (Fernández et al., 2005; Blazics et al., 2011).

3.2. Antioxidant capacity in vitro

EtOH extract at 50 mg/mL showed 91.23% inhibition of the DDPH radical, $IC_{50} = 6.70$ mg/mL. For the TPC test, EtOH extract at 12.5 mg/L exhibited 664.12 μgQE/mL. This extract generated more radical inhibition as compared with those previously described

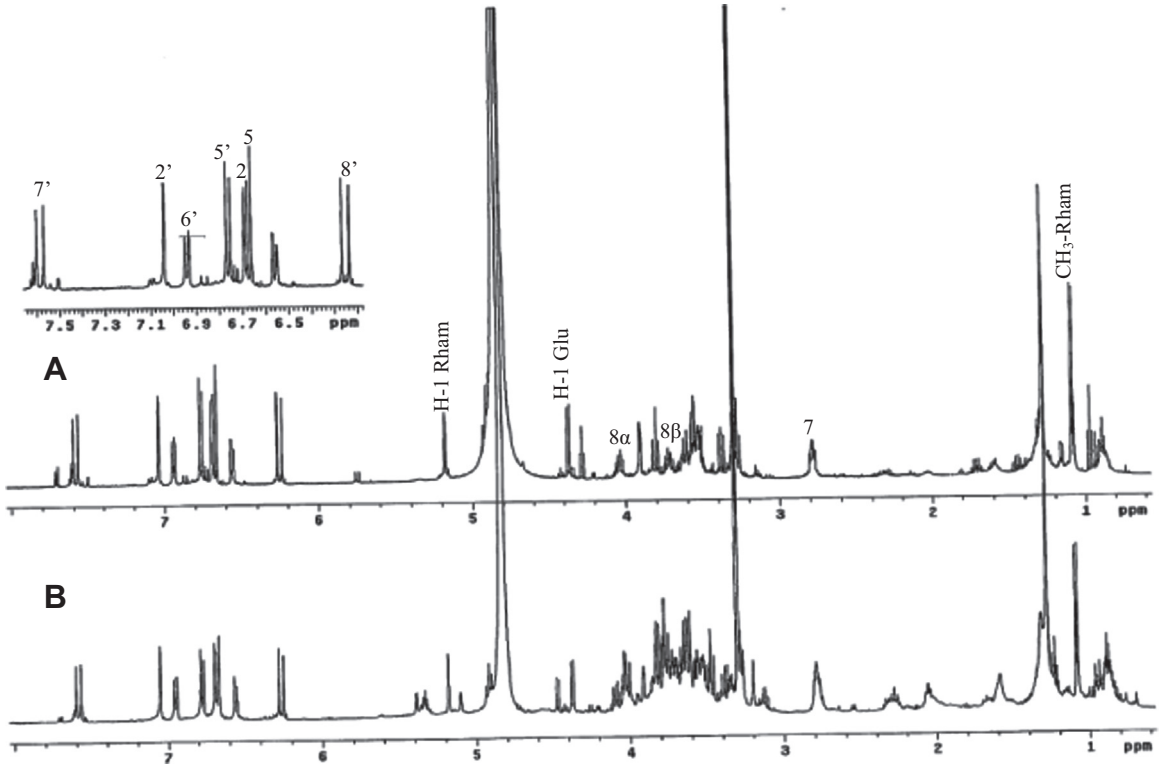

Fig. 5. [1]H NMR spectra of primary fraction F3 (A) and the EtOH extract of *M. deppeana* (B).

Table 1
[1]H NMR spectroscopic data (500 MHz, CDCl3 + CD_3OD) by Verbascoside.

Proton		δ_H (ppm)	*J* (Hz)	δ_H (ppm)*	*J* (Hz)*
Hydroxy-tyrosol	2	6.69 *d*	(2)	6.69 *d*	(2)
	5	6.67 *d*	(8)	6.66 *d*	(8)
	6	6.56 *dd*	(2.5, 8)	6.58 *dd*	(2.5, 8)
	7	2.79 bt	–	2.78 *t*	–
	8	3.75 *m*	–	4.03 *m*	–
		4.07 *m*			
Caffeoyl	2′	7.04 *d*	(2)	7.04 *d*	(2)
	5′	6.76 *d*	(8)	6.77*d*	(8)
	6′	6.94 *dd*	(2, 8)	6.95 *dd*	(2, 8)
	7′	7.59*dd*	(16)	7.58 *d*	(15.9)
	8′	6.25 *dd*	(16)	6.26 *d*	(15.9)
Rhamnose	1	5.19 *d*	(2)	5.18 *d*	(1.6)
	2	3.93 *dd*	(1.8, 3)	3.90 *dd*	(1.6, 3.2)
	5	3.51 *dd*	6, 9.5	3.72 *m*	–
	6	1.09 *dd*	6	1.08 *d*	6.2
Glucose	1	4.38 *d*	(8)	4.37 *d*	(7.9)
	2	3.45 *d*	(9)	3.38 *dd*	(7.9, 9.2)
	6	3.62 *m*		3.62 *m*	–
	6	3.35 *dd*	(6, 11)	3.35 *dd*	(6, 11)

* [1]H NMR data in CD_3OD[26–28]. J values are given in parenthesis.

(Domínguez-Ortiz et al., 2010), in which the successive extracts (Hex, EtOAc and EtOH) showed 41%, 92% and 70%, respectively at 33 μg/mL, with IC_{50} = 41, 18 and 22 μg/mL for each extract. This author also described TPC for these three extracts with values of 21, 329 and 389 μg Gallic Acid (GA)E/g of extract, respectively at an 1 mg/mL concentration (Domínguez-Ortiz et al., 2010), using GA as standard reference.

3.3. Toxicity tests

3.3.1. Acute toxicity

LD_{50} for *M. deppeana* EtOH extract in both sexes of Balb/C mice was >2 g/kg by i.g. route. It is considered a Category 5 substance according to the OECD TG 423 (Arthur et al., 2011). For three doses tested, no deaths or hazardous signs of toxicity were registered for 14-days. Results of BW gain on day 7 (1.26 ± 0.22 g), and day 14 (1.64 ± 0.61 g), demonstrated that male mice administered with 1 g/kg of EtOH extract exhibited statistically a lower BW gain than control. On the other hand, the group treated with 2 g/kg also showed a poor BW gain of 1.38 ± 0.39 g for day 7 and of 1.24 ± 0.32 g for day 14, as compared with those exhibited for the control (2.30 ± 0.17 g and 2.26 ± 0.70 g, by day 7 and 14, respectively) (Fig. 7). Female mice demonstrated a similar behavior on BW gain; in this case, the group treated with the extract (1 g/kg) exhibited a statistically poor increase: 0.90 ± 0.23 g for day 7 and of 1.64 ± 0.39 g for day 14. The group treated with 2 g/kg of the extract also showed a poor BW increase values of 0.72 ± 0.12 g for day 7 and of 1.02 ± 0.38 g for day 14, compared with the control that exhibited on day 7 (2.04 ± 0.42 g), and on day 14 (2.96 ± 0.49 g) (Fig. 8). For male and female mice the BW gain was dose-dependent.

Despite the poor BW gain, the EtOH extract administration did not cause macroscopic alteration in the gastric system, neither cause diarrhea. These observations are very important because when this system is affected, BW gain parameters are also altered. In some cases, plant extracts possess secondary metabolites that induce alteration in food intake and nutrient absorption, or can also modulate the metabolism and storage of lipids and proteins, as well they can cause the inhibition of key digestive enzymes (gastric or pancreatic lipase, amylase or chymotrypsin) (Sahib et al., 2012; Verma and Paraidathathu, 2014).

However further studies are required to elucidated which action mechanism is involved with this poor BW gain, these could be supported through animal models such as hyper caloric diet-

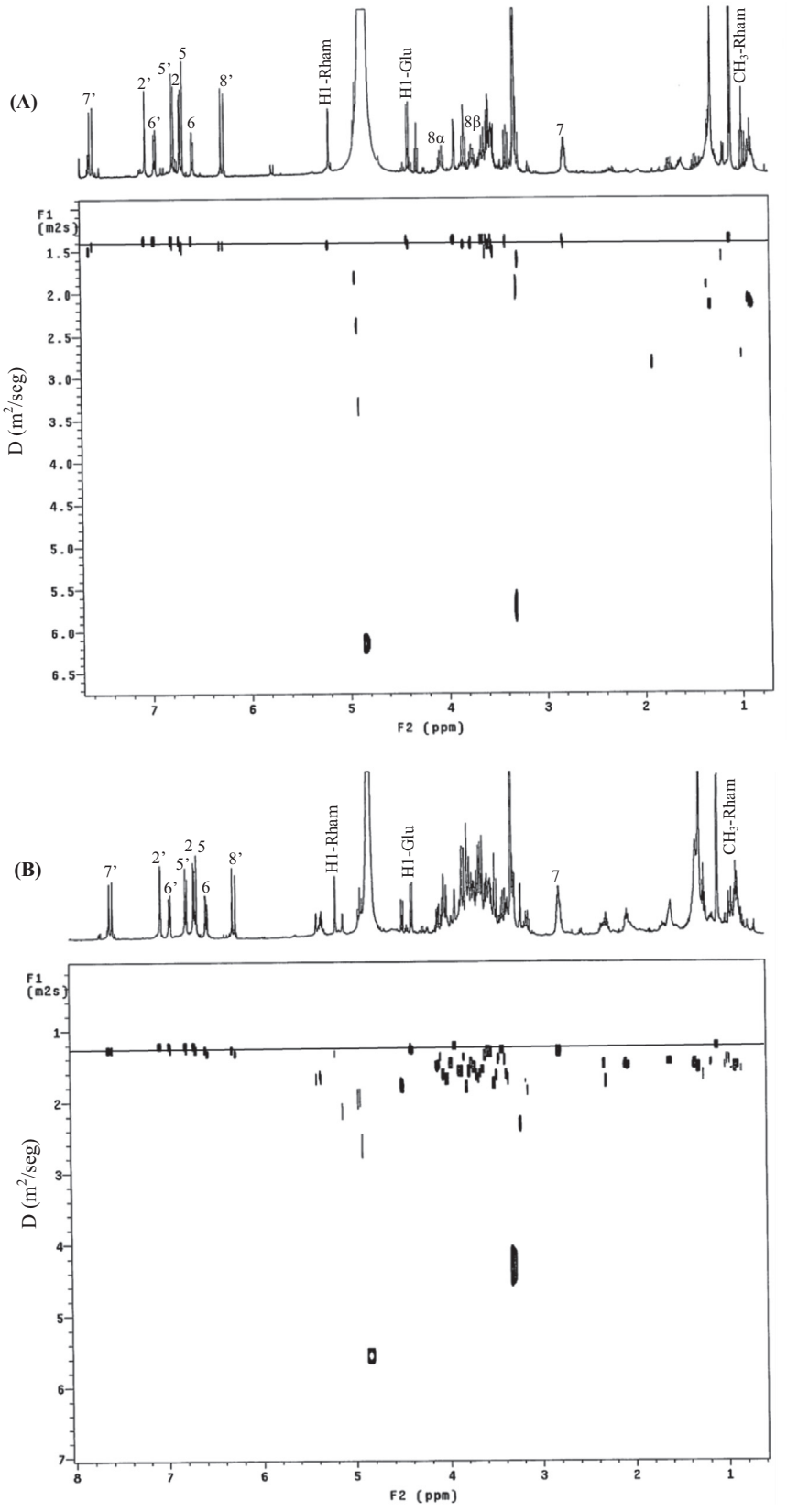

Fig. 6. DOSY ^{1}H NMR experiment of primary fraction F3 (A) and the EtOH extract (B) from *M. deppeana* with coefficient diffusion.

induced obesity in mice and nutrient absorption in small intestine of mice (Nilsson et al., 2012).

It is noteworthy that LD_{50} of Verbascoside (main compound of the *M. deppeana* EtOH extract), has been previously described; this value was 5 g/kg BW by the intraperitoneal (i.p.) route, not causing lethality or any adverse effects in mice (Etemad et al., 2015). Moreover, ursolic and oleanolic acids have been described as non-toxic compounds, this were tested in Balb/C mice (both sexes)

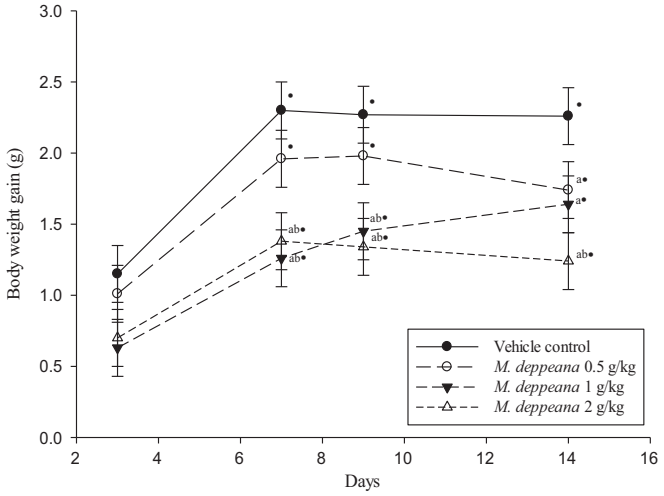

Fig. 7. Effect of the EtOH extract from *M. deppeana* on BW gain a single dose administered via i.g. in Balb/C male mice.

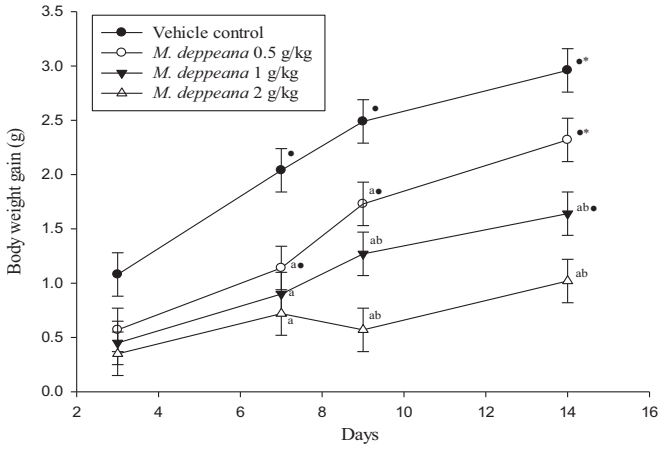

Fig. 8. Effect of EtOH extract from *M. deppeana* on BW gain of a single dose administered via i.g. in Balb/C female mice.

with a LD_{50} > 300 mg/kg by s.c. route (Cornejo-Garrido et al., 2012).

3.3.2. *Sub-acute toxicity*

Administration of the EtOH extract (1 g/kg) by i.g. route for 28 days, showed no significant changes in behavior and locomotion and no mice died. Male mice showed a statistically lower BW gain from day 14–28 (0.73 ± 0.21 g and 0.99 ± 0.16 g, respectively) as compared with the control for the same days (1.16 ± 0.08 g and 1.78 ± 0.14 g, respectively) (Fig. 9). A similar effect was observed in acute toxicity; however, female animals exhibited no statistical alteration in BW gain during the same period.

For hematological parameters (Hb, Hto, platelets, RBC and WBC) no statistical differences were found between the treated groups (1 g/kg) for male and female mice with respect to the control, although differences between sexes were found; these were due to the gender and not produced by the EtOH extract. Also, there were no significant changes among study groups in terms of the leukocyte differential count and other parameters, such as MCV, MCH and MCHC (data not shown).

No changes were observed in any of the biochemical parameters evaluated in any of the study groups (control or treated) for male and female mice on the final day of the study (data not shown). When any of these parameters is altered, such as liver enzymes, creatinine, or urea in serum, the plant extract generates

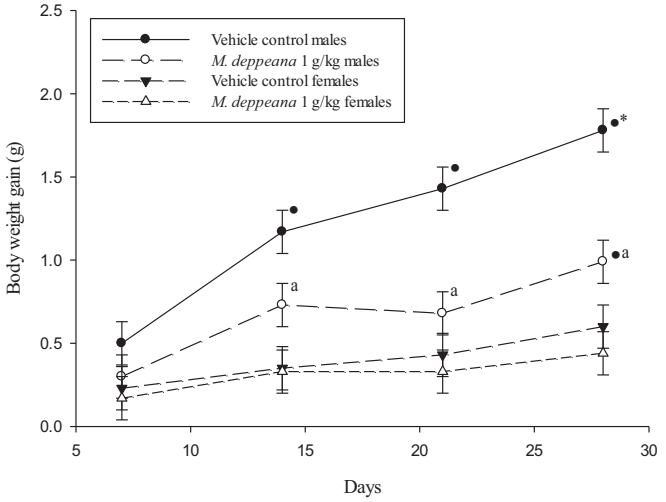

Fig. 9. Effect of EtOH extract from *M. deppeana* on the BW gain in sub-acute toxicity (28 days), administered via i.g. in Balb/C mice of both sexes.

Table 2
Anti-inflammatory activity of the EtOH extract from *M. deppeana* on ear edema induced with TPA in Balb/C mice.

Treatment	Dose (mg/ear)	Auricular edema (mg) and percentage of inhibition	
		Males	Females
TPA control	–	20.73 ± 1.79	15.94 ± 0.88
Indomethacin	0.5	16.02 ± 0.39[a] (22.75%)	13.11 ± 0.83[a] (17.71%)
	1	14.22 ± 0.79●[a] (31.43%)	9.06 ± 0.28●[a] (43.17%)
	2	9.62 ± 0.30●*[a] (53.62%)	7.80 ± 0.33●*[a] (51.06%)
ED_{50}	–	2.05 mg/ear	1.74 mg/ear
EtOH extract	0.5	16.23 ± 0.73[a] (21.70%)	12.94 ± 0.64[a] (18.79%)
	1	12.18 ± 0.73●[a] (41.24%)	9.41 ± 0.36●[a] (40.93%)
	2	8.18 ± 0.14●*[a] (60.53%)	6.93 ± 0.23●*[a] (56.53%)
ED_{50}	–	1.41 mg/ear	1.53 mg/ear

Each group represents the mean ± Standard error of the mean (SEM). Values in parentheses indicate the percentage of inhibition of the edema with respect to the TPA group. One-way Analysis of Variance (ANOVA), post-hoc Student-Newman-Keuls ($p \leq 0.05$), for each sex.

[b] vs. Indomethacin. ED_{50}, median Effective Dose; $n = 7$ for each group.

[a] *vs.* TPA control

adverse effects in the function of liver or kidneys, respectively (Ashafa et al., 2012; Nana et al., 2011). Histological examination performed on spleen, kidney, and liver from animals of both sexes, showed no microscopic structural abnormalities generated by daily i.g. administration of EtOH extract (data not shown).

It is noteworthy that the sub-acute toxicity of Verbascoside was previously reported, this compound did not cause alteration on either biochemical and hematological parameters, or the histological level of the main organs (kidney, heart, lungs, spleen, liver, and brain) in treated animals by 21 days by i.p. route with 60 mg/kg dose (Etemad et al., 2015). Furthermore, the sub-acute toxicity of ursolic and oleanolic acids was previously described (Cornejo-Garrido et al., 2012), reporting that the triterpene mixture at 13 mg/kg BW dose, administered by s.c. route, did not cause any alteration in hematological and biochemical parameters, nor did it provoke histological alteration on liver, spleen and kidney.

Table 3
Anti-inflammatory activity of the primary fractions F1, F2 and F3 on ear edema induced with TPA in Balb/C mice.

Treatment	Auricular edema (mg) and inhibition percent	
	Males	Females
TPA control	18.84 ± 0.85	17.51 ± 0.62
Indomethacin	9.94 ± 0.33[a] (47.23%)	10.46 ± 0.89[a] (40.29%)
F1 2 mg	12.96 ± 0.34[a,b] (31.24%)	12.74 ± 0.84[a,b] (27.24%)
F2 2 mg	8.85 ± 0.26[a] (53.03%)	9.50 ± 0.36[a] (45.76%)
F3 2 mg	16.16 ± 0.59[a,b,c,d] (14.22%)	13.61 ± 0.41[a,b,d] (22.28%)

Each group represents the mean ± sem. The dose for each treatment was 2 mg/ear. Values in parenthesis indicate the percent of inhibition edema with respect to TPA control group. One way ANOVA, post hoc Student Newman Keuls ($p \leq 0.05$), for each sex.

[a] *vs* TPA control.
[b] *vs* Indomethacin.
[c] *vs* F1 CH_3CN.
[d] *vs* $CHCl_3$; n= 7 for each group.

3.4. In vivo anti-inflammatory activity

3.4.1. TPA model

EtOH extract showed a similar anti-inflammatory activity over ear edema formation induced with TPA in both male and female mice. This was a dose-dependent effect with a percentage of inhibition nearly ≈60.53% (Table 2). These results were similar to that of Indomethacin group (control reference). The ED_{50} value for EtOH extract and Indomethacin was 1.41 and 2.05 mg/ear, respectively, for male mice, and ED_{50} value were 1.53 and 1.74 mg/ear, for female mice. For the primary fractions, only F2 showed a notorious effect with 45.76% of ear edema inhibition similar to that of the indomethacin group (Table 3). In a previous paper, the topical anti-inflammatory effect of three successive extracts from *M. deppeana* was described, in which Hex extract was the most active with 39% of inhibition in ear edema formation, followed by the EtOAc extract (28%) and EtOH extract with 4% of inhibition at 2 mg/ear dose (Domínguez-Ortiz, et al., 2010), this anti-inflammatory effect was lower than that generated by the direct EtOH extract (≈ 60.53%) at the same dose. The authors concluded that only Hex and EtOAc showed good local anti-inflammatory activity. The local anti-inflammatory activity observed for the direct EtOH extract is due to the presence of metabolites such as ursolic and oleanolic acids, which have been described as anti-inflammatory agents, being strong inhibitors of Nuclear factor kappa beta (NF-kβ) and the key enzymes COX-2 and lipoxygenase, suppresing the generation of prostaglandins (Giner-Larza et al., 2001; Rathee et al., 2009; Yadav et al., 2010; Sultana and Saify, 2012; Kim et al., 2012). On the other hand, Verbascoside has been described also as an anti-inflammatory compound in TPA model, at 1.6 mg/ear dose showing 40% ear edema inhibition (Sanchez et al., 2013), despite its low lipophilicities, which limits its activities due to skin permeation (Parhiz et al., 2015). Other minor phenolic compounds found in the EtOH extract of *M. deppeana* were apigenin and hesperetin; these have been described as anti-inflammatory compounds in murine models since they interrupt important pathways, such as the generation of prostaglandins through COX-2 inhibition or reduce the NF-kβ expression and transduction (Korkina et al., 2011; Wang et al., 2014).

Table 4
Anti-inflammatory activity of EtOH extract from *M. deppeana* on paw edema induced with Carrageenan in Balb/C mice.

Treatment	Paw edema (mm) Males T_{5h}	Females ED_{50} (mg/kg)	$T_{5\ h}$	ED_{50} (mg/kg)
Carrageenan	1.17 ± 0.05	–	1.23 ± 0.07	–
Indomethacin 10 mg/kg	0.61 ± 0.05[a] (47.80%)	10	0.56 ± 0.04[a] (54.36%)	10
EtOH extract (mg/kg)				
150	0.83 ± 0.04[a,b] (29.67%)	383	0.85 ± 0.02[a,b] (31.19%)	447
300	0.61 ± 0.04[a,c] (47.31%)		0.73 ± 0.06[a,b,c] (40.59%)	
450	0.56 ± 0.05[a,c] (52.22%)		0.61 ± 0.04[a,c,d] (50.69%)	
600	0.87 ± 0.02[a,b,d,e] (25.76%)		0.91 ± 0.05[a,b,d,e] (26.79%)	

Each group represents the mean ± Standard error of the mean (SEM). Values in parentheses indicate the percentage of inhibition of edema with respect to the carrageenan group. Two-way Analysis of Variance (ANOVA), post-hoc Student Newman Keuls ($p \leq 0.05$).

[a] *vs.* Carrageenan control;
[b] *vs.* Indomethacin;
[c] *vs.* M. deppeana 150;
[d] *vs.* M. deppeana 300;
[e] *vs. M. deppeana* 450; EtOH, Ethanolic extract; ED_{50}, median Effective Dose for 5 h; $n = 7$ for each group.

3.4.2. Carrageenan model

EtOH extract at 300 and 450 mg/kg generated 47.31% and 52.22% in paw edema inhibition in male mice, respectively at hour 5; this percentage was similar to the indomethacin group (47.80%) (Table 4). For female mice (Table 4), best doses were also 300 and 450 mg/kg (5 h), they showed 40.59% and 50.59% of inhibition, respectively, similar data to those for indomethacin (54.36%). Doses of 150 and 600 mg/kg generated poor anti-inflammatory activity at the same time (≈31.19 and ≈26.79%, respectively), in both sexes. ED_{50} values for EtOH extract were 383 and 447 mg/kg for male and female mice, respectively, and Indomethacin showed an $ED_{50} = 10$ mg/kg for both genders. F1 and F3 primary fractions showed a moderate anti-inflammatory effect at 150 mg/kg (5 h), with an inhibition percent of 11.31% and 28.87%, respectively for male mice, while for female mice at the same time (5 h), 19.06% and 31.32% inhibition on paw edema formation was observed (Table 5).

Table 5
Anti-inflammatory activity of the primary fractions F1, F2 and F3 on paw edema induced with Carrageenan in Balb/C mice.

Treatment	Paw edema (mm) Males T_{5h}	Females T_{5h}
Carrageenan	1.01 ± 0.02	1.07 ± 0.01
Indomethacin 10 mg/kg	0.58 ± 0.01[a] (42.98%)	0.50 ± 0.01[a] (53.44%)
Primary fractions (150 mg/kg)		
F1	0.90 ± 0.04[a,b] (11.31%)	0.87 ± 0.02[a,b] (19.06%)
F2	0.98 ± 0.03[b,c,e] (3.70%)	0.86 ± 0.02[a,b] (19.84%)
F3	0.72 ± 0.01[a,b,c,d] (28.87%)	0.74 ± 0.01[a,b,c,d,c] (31.32%)

Each group represents the mean ± Standard Error of the Mean (SEM). Values in parentheses indicate the percentage of inhibition of edema with respect to the Carrageenan group. Two-way Analysis of Variance (ANOVA), post-hoc Student Newman Keuls ($p \leq 0.05$).

[a] *vs.* Carrageenan control.
[b] *vs.* Indomethacin.
[c] *vs.* F1 150.
[d] *vs.* F2 150; F1, CH_3CN fraction; F2, $CHCl_3$ fraction; F3, MeOH fraction; $n = 7$ for each group.

Previously, the anti-inflammatory effect was described for the EtOAc extract (obtained by successive procedures); this extract was active at 100 mg/kg dose (administered i.p.) and showed good inhibition (43%) at hour 1 but poor inhibition was observed at hour 5 (17%) (Domínguez-Ortiz et al., 2010).

Systemic anti-inflammatory effect demonstrated by EtOH extract and its primary fractions (F1, F2, and F3) in carrageenan model, was mainly due to its major identified metabolites such as ursolic and oleanolic acid, verbascoside, apigenin and hesperetin, which are well-known COX-2 inhibitors with an effect similar to indomethacin (González et al., 2011; Kashyap et al., 2016), however further studies are required to complete elucidated through which pharmacological mechanisms the anti-inflammatory effect occur (Necas and Bartosikova, 2013).

3.5. Median effective dose

The ED_{50} value is calculated in models of acute inflammation *in vivo* in order to have a basis on which dose to be used *in vivo* models of chronic inflammation can be determined. For example, in the literature, there are scarce descriptions of this value, which generally reports the percentage of inhibition. Some medicinal species, such as *Citrus aurantium* (Karaca et al., 2007), *Euterpe oleracea* (Favacho et al., 2011), and *Washingtonia filifera* (Hemmati et al., 2015), have ED_{50} = 0.079, 1226.8 and 164.4 mg/kg, respectively.

Finally, the minor secondary metabolites detected in the EtOH extract are currently being isolated in order to determine their anti-inflammatory and antioxidant activities *in vivo*, as well as their acute and sub-acute toxicity. Regarding Verbascoside, it is necessary that this compound undergo evaluation in models of local and systemic chronic inflammation in order to determine its possible use in chronic diseases such as arthritis, lupus, ankylosing spondylitis and atherosclerosis, among others. This work helps to know the real potential of *M. deppeana* (a medicinal plant) as a source of anti-inflammatory compounds; however, more research is needed on this medicinal species to know their anti-arthritic, antioxidant and hepatoprotective properties.

4. Conclusions

This study provides important information concerning the support of the ethnopharmacological uses of the EtOH extract from the aerial parts of *M. deppeana* as an anti-inflammatory plant with a scare toxicological effect. Also, the safety of the use of *M. deppeana* at a mid-term period (28 days) was established. By chemical fractionation and preparative TLC, Verbascoside (as a major metabolite) and the mixture of oleanolic and ursolic acids were isolated and identified; these metabolites are mainly responsible for the anti-inflammatory activity exhibited by the EtOH extract and its primary fractions. Verbascoside has not been described for *M. deppeana*, comprises a main anti-inflammatory compound present in the EtOH extract and was identified by HPLC and DOSY ^{1}H NMR. This compound is a fingerprint for EtOH from *M. deppeana* and it can be used as a marker in different commercial preparations. However, further studies, such as subchronic and chronic toxicity, mutagenicity, carcinogenicity and teratogenicity, are required to confirm the ethnomedical use of this plant for a long-term period. Also is necessary evaluate the anti-inflammatory activity of the main compound in models of chronic inflammation (arthitis, lupus) and quantify the content Verbascoside in the EtOH extract of *M. deppeana*.

5. Conflict of interests

The authors declare that they have no competing interests. All authors read and approved the final version of the manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2016.04.033.

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