Original Article

In vivo anti-arthritic and antioxidant effects from the standardized ethanolic extract of *Moussonia deppeana*

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ABSTRACT

Moussonia deppeana (Schltdl. & Cham.) Klotzsch ex Hanst., Gesneriaceae, known as tlachichinole, is a Mexican medicinal plant used for treatment of chronic inflammation-related diseases such as arthritis. In this paper, the main metabolite verbascoside was quantified in ethanolic extract; anti-arthritic and antioxidant activities were also evaluated in Complete Freund's Adjuvant induced arthritis in mice, with complete hematological evaluation, and oxidative stress measure in edema and ganglionic tissues on day 28. In popliteal ganglion, CD4^{+} lymphocytes and tumor necrosis factor alpha concentration were measured in addition to histological analysis. Ethanolic extract contained 79.2 mg of verbascoside/g extract, and this extract at 450 mg/kg generated an inhibition of 24% over paw edema development and increased body weight gain on final day. For hematological parameters, same dose decreased total leukocytes and lymphocytes, as well as decreased oxidation rate over biomolecules in edema and ganglionic tissues, and increased antioxidant enzyme activity. In ganglionic tissue, CD4^{+} lymphocytes and tumor necrosis factor alpha level showed no differences at any tested dose compared to complete Freund's adjuvant untreated group. Histological analysis of popliteal ganglion revealed moderate reduction of follicular hyperplasia, leukocyte infiltration and lipid inclusions at 450 mg/kg dose. Ethanolic extract of *M. deppeana* possesses anti-edematous activity associated to a moderate reduction in follicular hyperplasia, with immune-modulatory and antioxidant effects during experimental arthritis in mice.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that affects the synovial membrane and leads to the degeneration of articular tissue and later to bone erosion, as a consequence of chronic inflammation (Smolen et al., 2016). It affects 2–4% of the world population (Wong et al., 2010), and its frequency varies depending on the ethnic group. In 2012, Mexico reported a prevalence of 59% in women and 41% in men (ratio, 3:1) and an incidence of 0.3% in Mexican population (González-López et al., 2013). When this disease progresses, the patient exhibits mobility disabilities; finally, 15–20% of patients will require surgery within a period of 5 years, giving this illness a high impact on the patients' quality of life. Cardiel et al. (2014) reported in Mexico an annual treatment cost approaching US$6000, of which US$2000 are provided by the Federal Government, and the remainder by the patient's relatives (representing 15% of their income). RA is characterized by increased levels of immune cells, such as macrophages and lymphocytes in the synovial space and a high concentration of free radicals (FR), mostly reactive oxygen species (ROS), which could irreversibly affect articular tissue through the oxidation of its biomolecules, contributing to progression of the disease (Lonkar and Dedon, 2011).

Anti-arthritic treatment is long and is focused on the inhibition of key mediators of the chronic inflammation process, such as interleukins (tumor necrosis factor alpha, TNF-α), cells (lymphocytes T), and enzymes (induced cyclooxygenase), with the aim of regulating these or of decreasing their degenerative impact on articular tis-

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sue (Ventura-Ríos et al., 2012). Co-administration of non-steroidal anti-inflammatory drugs, gluco-corticosteroids, disease-modifying anti-rheumatic drugs, and biological therapies such as rituximab and infliximab (Covelli et al., 2010), is mostly prescribed. However, these prolonged therapies cause several adverse effects, such as gastrointestinal bleeding, cyto- and hepatotoxicity, immunosuppression or osteoporosis; and recurrent infectious disease (Scott et al., 2010; Toscano et al., 2010; Harboe et al., 2012; Ventura-Ríos et al., 2012).

Currently, in Mexican traditional herbal medicine, there are few plants with preclinical studies that showed anti-arthritic activity and a protective effect on chronic inflammation course. These include *Yucca schidigera*, *Dodonaea viscosa*, and *Sphaeralcea angustifolia*, this latter species the only one featuring a clinical trial for the treatment of osteoarthritis (García-Rodríguez et al., 2012; Salinas-Sánchez et al., 2012; Romero-Cerecero et al., 2013; Cheeke et al., 2016).

Moussonia deppeanna (Schltdl. & Cham.) Klotzsch ex Hanst., Gesneriaceae (commonly known as *tlachichinole*) is used in Mexican traditional medicine to treat several inflammatory diseases, kidney failure, rheumatic pain, and gastrointestinal diseases (Domínguez-Ortiz et al., 2010; Gutiérrez-Rebolledo et al., 2016). Recently, it has been described that its ethanolic (EtOH) extract from aerial parts showed a good anti-inflammatory activity in 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and carrageenan models, showing median effective dose (ED$_{50}$) = 1.5 mg/ear and 450 mg/kg, respectively, with a median lethal dose (LD$_{50}$) of >2 mg/kg by intragastric (*i.g.*) route. In the sub-acute toxicity test in healthy mice, the EtOH extract did not cause lethality and any alteration on biochemical and hematological parameters; also, histological analysis demonstrated no damage in liver, kidneys, and spleen. Phytochemical analyses revealed that the EtOH extract contains verbascoside (main compound), ursolic and oleanolic acids, apigenin, and hesperetin; these were identified by spectroscopic and spectrometric methods and high performance liquid chromatography (HPLC) (Gutiérrez-Rebolledo et al., 2016).

Verbascoside has been isolated from some medicinal plants (*Verbascum*, *Buddleja*, *Striga* genus, and from species of the *Gesneriaceae* family) (Filho et al., 2012; Huang et al., 2013; Alipieva et al., 2014). The metabolite has anti-inflammatory properties: inhibition of paw edema formation in rat (Akdemir et al., 2011), inhibition of the induced nitric oxide synthase (NOS) enzyme (Marzocco et al., 2007), decreased ear edema formation in mice (Sánchez et al., 2013), inhibition of myeloperoxidase and nuclear factor kappa B (NF-κB) (Paola et al., 2011), decreased levels of interferon gamma (IFN-γ), interleukin 1β, TNF-α, and an increase in superoxide dismutase (SOD) activity (Hausmann et al., 2007; Mazzon et al., 2009; Esposito et al. 2010; Lenoir et al., 2011).

In this work, the anti-arthritic and antioxidant activities *in vivo* from the *M. deppeana* EtOH extract were evaluated in experimental arthritis induced by complete Freund's adjuvant (CFA) in mice; also, HPLC quantification of verbascoside was measured.

Materials and methods

Collection of plant material and crude extract preparation

Aerial parts of *Moussonia deppeana* (Schltdl. & Cham.) Klotzsch ex Hanst., Gesneriaceae, were collected in Coatepec, Veracruz, Mexico, in February, 2013 (GPS coordinates 19°32′34.0″ N 96°51′58.4″ W, voucher number 17139, deposited in FEZA UNAM Herbarium). EtOH extract was prepared following the procedure previously described (Gutiérrez-Rebolledo et al., 2016).

HPLC analysis of the EtOH extract

Verbascoside content in EtOH extract was quantified by HPLC utilizing a Waters 2695 separation module HPLC system equipped with a Waters 996 photodiode array detector and Empower Pro software (Waters Corporation, USA), as previously described (Cárdenas-Sandoval et al., 2015; Gómez-Aguirre et al., 2012). The following analytical conditions were employed: column ZORBAX Eclipse XDB-C18 (5 mm, 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$_3$CN (eluent B), starting from 95% A at 50% in 20 min, returning to 95% for 20–25 min; this was maintained for 35 min; the flow rate was 0.7 ml/min and the injection volume was 20 ml; peaks were detected at 280 nm. Retention time for verbascoside was 7.9 min (λ_{max} = 218, 247, 290, and 330 nm) and quantity was estimated by interpolation of peak areas in a calibration curve (y = 22,144x − 16,1443; R^2 = 0.999). *M. deppeana* EtOH extract and pure verbascoside standard were analyzed three times (triplicate), and results were expressed in mg of verbascoside/g dry extract weight. All chemical reagents were purchased from Sigma–Aldrich.

Animals' in vivo assay

Adult Balb/C male mice (25 ± 3 g) were obtained from Animal Vivarium of National Medical Center XXI Century, Mexican Social Security Institute, Mexico City, and were maintained under laboratory conditions. Project was approved by National Commission of Scientific Investigation and Bioethics with code CNIC-IMSS R-2013-785-053.

Monoarthritis induced with CFA

This experiment was carried out according to Rasool et al. (2006), with modifications. All groups (n = 7) were injected subcutaneously with 25 μl of CFA in the right hind paw on days zero and 14 (re-injection). Treatment groups were administered by *i.g.* route with phenylbutazone (PBZ, 100 mg/kg), and EtOH extract (200, 450, and 900 mg/kg) daily from day 7 to 27. All samples were solubilized in Tween 80:water (1:9); groups of healthy and un-treated arthritic mice received only vehicle. Paw edema was measured at different times (days 1, 4, 7, 14, 15, 21, and 28) (*Et*) with a digital micrometer (Mitutoyo model 293-831) and the value of day zero (*Eo*) was determined as baseline. Body weight (BW) gain was also registered on the same days compared to day zero. Inhibition percentage of edema development in each group was calculated from days 14 to 28 by comparison with CFA un-treated group as follows:

$$\% \text{inhibition} = \frac{(Et - Eo)_{\text{CFA group}} - (Et - Eo)_{\text{Treated group}}}{(Et - Eo)_{\text{CFA group}}} \times 100$$

Hematological and oxidative stress (OS) parameters

On day 28, blood samples were collected from each mouse for complete hematological analysis, which was performed in a Beckman Coulter Cell Counter. Later, mice were euthanized, hind paw with chronic edema and nearest popliteal ganglion tissues were obtained in cold to determine OS biomarkers. Tissue samples (500 mg) were homogenized in cold phosphate-buffered saline solution (2 ml at pH 7.4), and one ml of each homogenate was centrifuged at 10,500 × g and 4 °C/15 min; activity of SOD, catalase (CAT), and glutathione peroxidase (GSH-Px) was determined from the supernatants, while lipid peroxidation (LPO) and protein carbonyl content (PCC) were evaluated from un-centrifuged

homogenates samples. All absorbance was measured in Shimadzu UV-1700 Double Beam Scanning UV-Vis Spectrophotometer.

LPO quantification

Thiobarbituric acid-reactive substances (TBARS) reagent (2 ml) (16% trichloroacetic acid – TCA, 0.5% thiobarbituric acid – TBA, and 0.3 N hydrochloric acid – HCl) were added to 500 μl of un-centrifuged homogenate. Samples were processed according to Büege and Aüst (1978) and Gutiérrez-Rebolledo et al. (2015). Absorbance was read at 535 nm. Malondialdehyde (MDA) content was determined using Molar extinction coefficient (MEC) of $1.56 \times 10^5 \, M^{-1} \, cm^{-1}$ and results were expressed as μmol MDA/g tissue.

PCC quantification

TCA 20% (300 μl) were added to 150 μl of un-centrifuged homogenates and these were processed as described by Parvez and Raisuddin (2005) and Gutiérrez-Rebolledo et al. (2015). Absorbance was read at 360 nm, PCC was determined employing MEC of $21,000 \, M^{-1} \, cm^{-1}$, and results were expressed as μmol of reactive carbonyls/g tissue.

Antioxidant enzymes

SOD was determined as described by Misra and Fridovich (1972), measuring absorbance at 30 s and at 5 min, and the difference between these two values was used to calculate adrenochrome concentration with MEC of $4020 \, M^{-1} \, cm^{-1}$ at 480 nm. Data was extrapolated in a calibration curve ($y = -0.004x + 0.0518$; $R^2 = 0.9536$), and results were expressed as SOD International Units (IU)/g tissue. CAT activity was evaluated as described by Radi et al. (1991), measuring absorbance at time zero and at 1 min, and difference was employed to calculate hydrogen peroxide (H_2O_2) denaturalization with MEC of $0.043 \, mM^{-1} \, cm^{-1}$ at 240 nm; results were expressed as mmol H_2O_2 consumed/min/g tissue. According to this method, one CAT IU/g tissue represents 1 μmol of H_2O_2 consumed/min/g tissue. GSH-Px activity was determined according to Plagia and Valentine (1967), measuring absorbance at time zero and at 1 min with MEC of $6.2 \, mM^{-1} \, cm^{-1}$ for 360 nm, results were expressed as mmol NADPH/min/g tissue.

Interleukin and CD4⁺ lymphocyte quantification

Popliteal ganglion near the right hind paw of healthy mice, arthritic un-treated mice, those treated with PBZ (100 mg/kg) and with EtOH extract at 450 mg/kg (dose that showed best antioxidant activity in vivo) were obtained and disrupted in cold ISS, filtered, and free cells were isolated for extra-cellular (CD4⁺) and intra-cellular (interleukins) stain according to Brüstle et al. (2007), Heizmann et al. (2013), and Paul et al. (2016). The following antibodies were used: anti-CD4-FITC clone:GK1.5 (Miltenyi Biotec), and anti-TNF α-APC (eBioscience). Samples were measured in a flow cytometer MACS Quant analyzer (Miltenyi Biotec); results were analyzed using FlowJo version 10 statistical software and reported as percent of CD4⁺ cells (%) and Median Fluorescence Intensity (MFI) for TNF-α concentration.

Histological analysis

Popliteal ganglions were extracted from euthanized mice and tissue biopsies were fixed in 10% formalin, and later embedded in paraffin; these blocks were cut into 4–5 μm slices with a rotary microtome and stained with hematoxylin and eosin. Stained slices were examined under a light microscope (Leika White MP32).

Statistical analysis

SigmaPlot ver. 12.0 statistical software (2011–2012) was utilized for analysis of results and graphic elaboration. Data is presented as standard error of the mean (SEM). BW gain and development of paw edema were analyzed with bifactorial analysis of variance (ANOVA) and with a post hoc Student–Newman–Keuls (SNK) test; $p < 0.05$ was considered statistically significant. For hematological analysis parameters and oxidative damage parameters in tissues, one-way ANOVA was employed with a post hoc SNK test; $p < 0.05$ was considered significant. Finally, for TNF-α, MFI, and CD4⁺ cells percentage values, 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 $p < 0.05$.

Results and discussion

Quantification of verbascoside in active EtOH extract

Maceration process produced 60 g of EtOH extract with a yield of ≈17% with respect to plant material's dry weight. Content of verbascoside in EtOH extract was 79.2 mg/g of dry extract ($R_t = 9.21$ min) (Fig. 1).

Previously, the presence of verbascoside as the main secondary metabolite in M. deppeana EtOH extract was described (Gutiérrez-Rebolledo et al., 2016); in addition, the presence of other minor secondary metabolites such as ursolic and oleanolic acids (triterpenes) in the lipophilic fractions obtained from the EtOH extract, were also described, as well as apigenin and hesperetin, the minor compounds was identified by HPLC in the polar fraction. The importance of these results is that verbascoside, apigenin and hesperetin had not been reported for M. deppeana.

In other medicinal plants, verbascoside has been quantified by HPLC. For example, in the Euphrasia rostkoviana methanolic (MeOH) extract, 25.6 mg/g of dry plant material was described, and the presence of this compound may explain, in part, the beneficial effect of Euphrasia related to eye diseases (Blazics et al., 2011). M. deppeana EtOH extract contains 79.2 mg/g of dry extract (3-fold more) and the anti-inflammatory activity exhibited by this extract in acute inflammation mice models is due to this metabolite (Gutiérrez-Rebolledo et al., 2016). Verbascoside exerts several beneficial effects on the immune process (Akdemir et al., 2011), including a chemo-preventive effect against skin cancer, intracellular radical scavenging, and antioxidant and antitumor activity (Chen et al., 2013; Estrada-Zúñiga et al., 2016; Ramírez et al., 2016). Another plant is Buddleja cordata; micro-propagated plantlets obtained by in vitro culture yielded 1.0 mg of verbascoside/g of dry biomass (Estrada-Zúñiga et al., 2016).

Experimental arthritis

Ethanolic extract at 450 and 900 mg/kg generated similar statistical values in paw edema inhibition; ≈15%, ≈24%, and ≈25% for days 15, 21, and 28, respectively, compared to CFA untreated group. Lower dose (200 mg/kg) showed a poor anti-inflammatory effect (≈12–19% inhibition) from days 15 to 28. Anti-inflammatory effect of the extract at medium and high doses was similar to PBZ group (24%) only for day 21; meanwhile, PBZ demonstrated an anti-inflammatory effect from day 14 to 28, and the inhibition effect was greater than ≈34%, compared to CFA untreated group. It is noteworthy that EtOH extract at 450 and 900 mg/kg achieved a similar effect to that PBZ after two weeks of treatment (day 21),
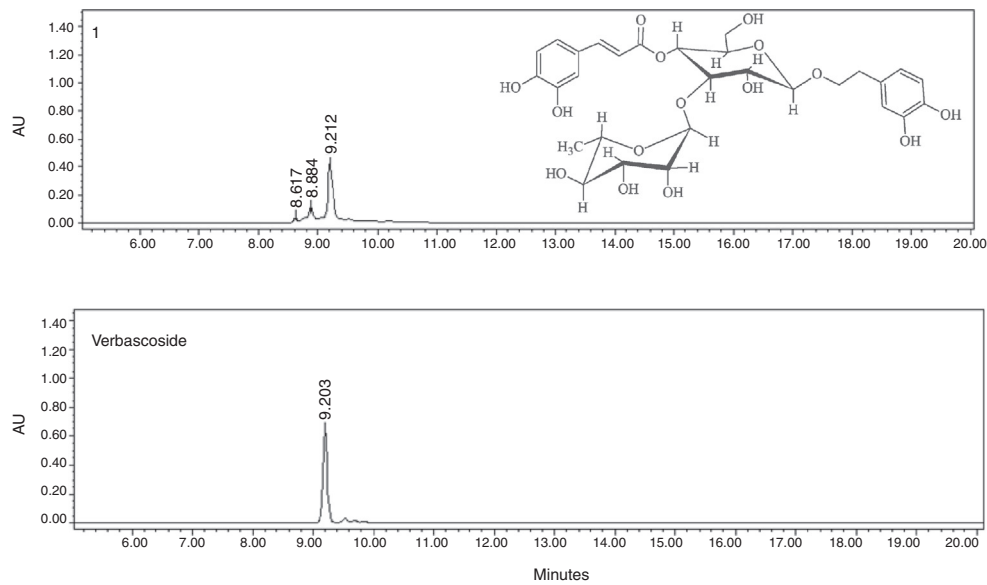
Fig. 1. Chromatogram of the ethanolic extract from *Moussonia deppeana* aerial parts (1) and verbascoside standard.
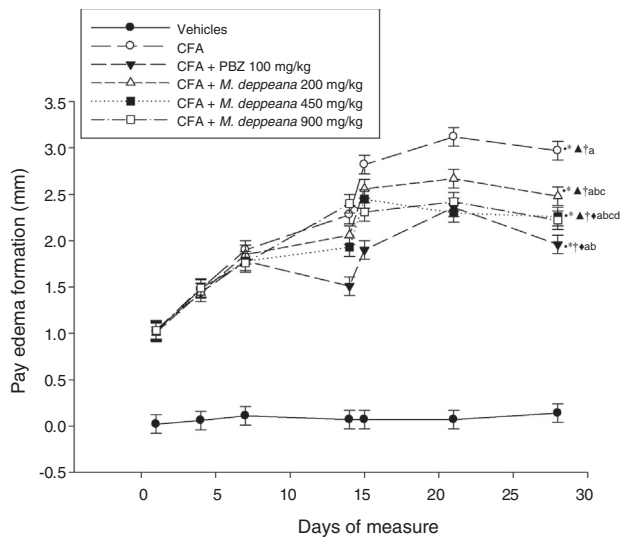

Fig. 2. Anti-arthritic activity of *Moussonia deppeana* ethanolic extract on the edema development during CFA-induced arthritis in mice. Data showed as mean ± standard error. Treatments were administered daily by oral via from day 7 to 28. Two-way analysis of variance (ANOVA) of repeated measures (RM), post hoc Student Newman Keuls (SNK) ($p \leq 0.05$). [a] vs vehicles; [b] vs CFA control; [c] vs CFA + PBZ; [d] vs CFA + EtOH 200 mg/kg; [e] vs CFA + EtOH 450 mg/kg; ● vs day 1; * vs day 4; ▲ vs day 7; † vs day 14; ■ vs day 15; ♦ vs day 21. CFA, complete Freund's adjuvant; PBZ, phenylbutazone; $n = 7$.

Fig. 3. Anti-arthritic activity of *Moussonia deppeana* ethanolic extract on the body weight gain during CFA-induced arthritis in mice. Data showed as mean ± standard error. Treatments were administered daily by oral via from day 7 to 28. Two-way analysis of variance (ANOVA), post hoc Student Newman Keuls (SNK) ($p \leq 0.05$). [a] vs vehicles; [b] vs CFA control; [c] vs CFA + PBZ; [d] vs CFA + EtOH 200 mg/kg; [e] vs CFA + EtOH 450 mg/kg; ● vs day 1; * vs day 4; ▲ vs day 7; † vs day 14; ■ vs day 15; ♦ vs day 21. CFA, complete Freund's adjuvant; PBZ, phenylbutazone; $n = 7$.

while PBZ maintained a constant inhibitory effect on paw edema development 1 day after re-inoculation (day 15) (Fig. 2).

In terms of BW gain during adjuvant-induced arthritis, EtOH extract-treated arthritic group at 450 mg/kg demonstrated similar values to those of healthy mice, while CFA un-treated group and EtOH extract groups at 200 and 900 mg/kg exhibited the lowest BW gain from days 15 to 28 compared to groups of healthy animals. PBZ treated group exhibited a statistically significant increase in BW gain on days 21 and 28 (2.13 and 3 g, respectively), compared to CFA un-treated group (0.88 and 2.50 g), but these values were lower than those observed in healthy animals and in EtOH extract-treated mice at 450 mg/kg (≈2.80 and ≈3.70 g, respectively) (Fig. 3).

Results described for experimental arthritis model revealed that the most effective EtOH extract dose was 450 mg/kg related to edema development, being no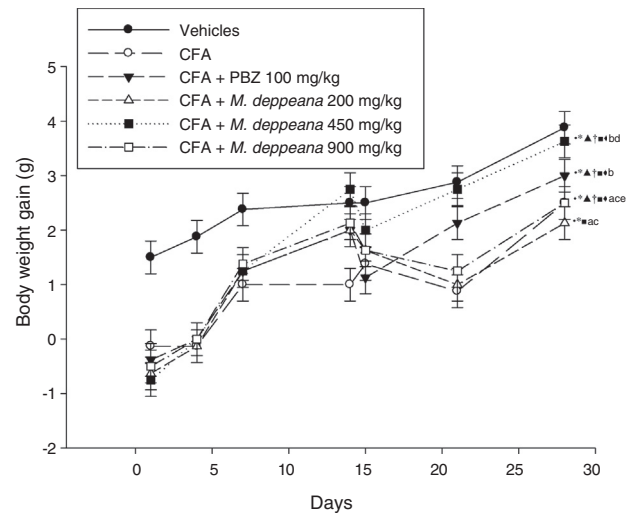teworthy that this dose corresponds to the ED$_{50}$ value observed in acute inflammation phase (carrageenan model) (Gutiérrez-Rebolledo et al., 2016). CFA-induced experimental arthritis was previously described for mice (Rasool et al., 2006), and for rats (García-Rodríguez et al., 2012), and these authors maintained that edema development was related to an increase in neutrophils, lymphocyte CD4$^+$ infiltration, and to the increased production of pro-inflammatory mediators (TNF-α, IL-1β, IL-6, and INF-γ).

Body weight gain during experimental arthritis, in rats and mice, has been used both in past and recent works as an indirect parameter as a measure of the establishment and development of a chronic inflammatory process (Pan et al., 2017). Authors described that BW decrease or slow increase during experimental arthritis is due to muscle mass loss caused by poor food consumption, because of increased sensitivity through the nociceptors, generating the
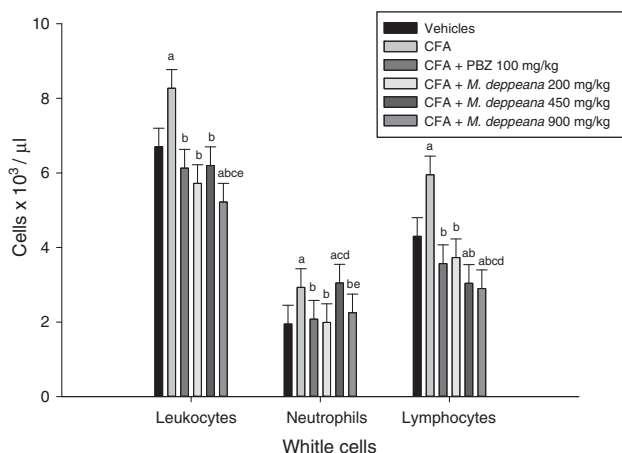
Fig. 4. Total leukocyte count and differential (neutrophils and lymphocytes) count in mice with CFA-induced arthritis treated with *Moussonia deppeana*. Data showed as mean ± standard error. One-way ANOVA, post hoc SNK ($p \leq 0.05$). [a]vs vehicles; [b]vs CFA control; [c]vs CFA + PBZ; [d]vs CFA + EtOH 200 mg/kg; [e]vs CFA + EOH 450 mg/kg. CFA, complete Freund's adjuvant; PBZ, phenylbutazone; $n = 7$.

appearance of hyperalgesia, allodynia and cachexia in the arthritic animals (Mbiantcha et al., 2017; Taksande et al., 2017).

Other studies have described that the pro-inflammatory cytokines, such as TNF-α, are inhibitors of neuropeptide Y/leptin axis, decreasing food intake and modifying BW gain through an anorexigenic effect (González-Hita et al., 2006; Rasool et al., 2006; Taksande et al., 2017).

Hematological, interleukin, and CD4+ lymphocyte profile

On day 28, arthritic groups treated with EtOH extract at 200, 450 and 900 mg/kg showed no significant differences between them; however, they produced statistically significant decrease in total leukocyte count of 30.83, 25.03 and 36.88%, respectively, compared to CFA un-treated group (8.27 cells $\times 10^3/\mu$l), with total values statically similar to healthy mice (6.70 cells $\times 10^3/\mu$l) on day 28. On the other hand, PBZ-treated arthritic mice showed a statistically significant decrease of 25.88% compared to CFA un-treated mice (Fig. 4).

Neutrophil values in EtOH extract-treated arthritic group at 450 mg/kg demonstrated a statistically significant increase of 4.10% on day 28 compared to CFA un-treated group (2.93 cells $\times 10^3/\mu$l); however, animals that were administered with EtOH extract at doses of 200 and 900 mg/kg showed a statistically significant decrease of nearly 30% compared to CFA control group; meanwhile, groups treated with PBZ showed a statistically significant decrease of nearly $\approx$29% compared to un-treated CFA mice (Fig. 4).

Lymphocyte count in arthritic treated with EtOH extract groups at 200, 450 and 900 mg/kg showed a statistically significant decrease of 39.04%, 48.91% and 51.26%, respectively, compared to un-treated CFA group (5.95 cells $\times 10^3/\mu$l), similar even to values shown by healthy animals (4.30 cells $\times 10^3/\mu$l). PBZ treated group generated statistically significant decrease in lymphocytes of $\approx$39.50% compared to un-treated CFA mice, and were statistically similar to healthy mice (Fig. 4).

CD4+ cell quantification and TNF-α concentration are described in Figs. 5 and 6, respectively. No statistical differences between un-treated CFA group (56.42%, and 84 MFI, respectively) and those mice treated with PBZ or EtOH extract at 450 mg/kg ($\approx$55% and $\approx$75 MFI, respectively) were observed; however, all of these groups demonstrated a statistically significant increase in these two parameters when compared with healthy mice (32.68% and
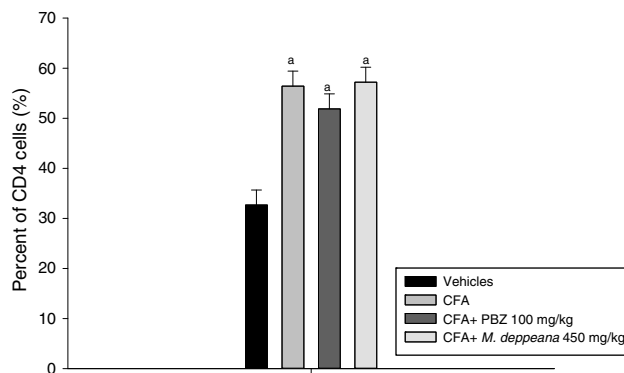
Fig. 5. Effect of *Moussonia deppeana* ethanolic extract on tumor necrosis factor alpha secreting CD4+ lymphocytes content in ganglionic tissue during experimental arthritis. Data showed as mean ± with its standard error (s.e.). Statistical analysis ANOVA on ranks ($p \leq 0.05$). [a]vs vehicle; CFA, complete Freund adjuvant; PBZ, phenylbutazone; $n = 7$.
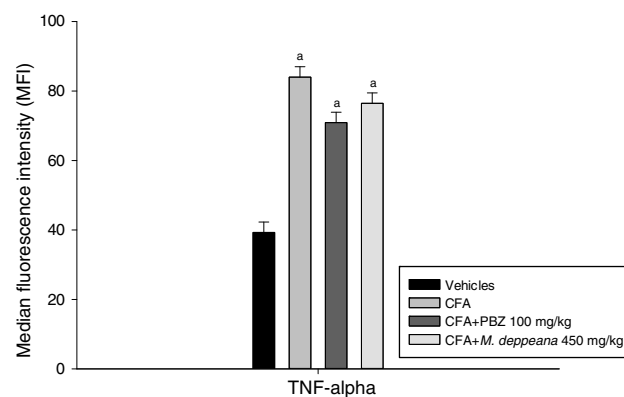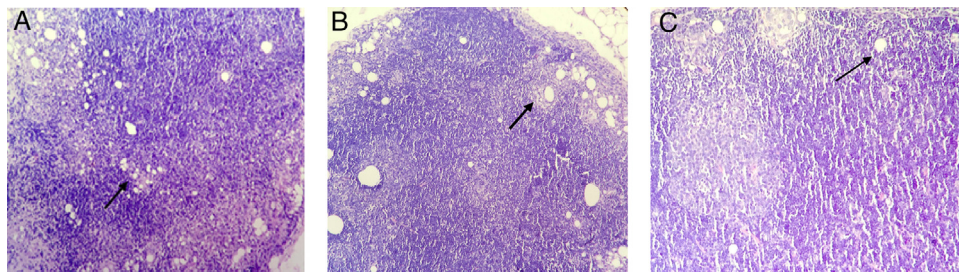

Fig. 6. Effect of *Moussonia deppeana* ethanolic extract on tumor necrosis factor alpha concentration in ganglionic tissue during experimental arthritis. Data showed as mean ± with its standard error (s.e.). Statistical analysis ANOVA on ranks ($p \leq 0.05$). [a]vs vehicle; CFA, complete Freund adjuvant; PBZ, phenylbutazone; MFI, median fluorescence intensity; TNF-α, tumor necrosis factor-alpha; $n = 7$.

39.30 MFI, respectively), which demonstrated the establishment of the experimental arthritis.

Interleukin values and lymphocyte sub-population results are important because these are related to the mechanism by which CFA-induced experimental arthritis is established (Franch et al., 1994). The inhibition exerted by verbascoside on these pro-inflammatory mediators is well-known and previously described (Hausmann et al., 2007; Esposito et al., 2010). White-cell differential results suggest a possible mechanism by which secondary metabolites contained in EtOH extract generate their anti-arthritic effect, similar to PBZ, because both showed lower leukocyte and lymphocyte values, decreasing the production and action of pro-inflammatory mediators aside from TNF-α, such as Il-1β and INF-γ (García-Rodríguez et al., 2012; Gutiérrez-Rebolledo et al., 2015). Also, EtOH extract exhibited an increase in neutrophils, these cells being related to the auto-regulation of the immune process and first but non-effective phagocytosis (Kolaczkowska and Kubes, 2013).

Histological analysis

In popliteal ganglion, un-treated CFA group showed intense follicular hyperplasia with formation of lipid inclusions, while arthritic mice treated with EtOH extract at 450 mg/kg or PBZ exhibited moderate lipid inclusions; however, follicular hyperplasia was lower in both groups than in CFA un-treated mice, even lower in PBZ-administered animals (Fig. 7).

Fig. 7. Effect of *Moussonia deppeana* ethanolic extract on histologic findings in popliteal ganglion of Complete Freund's Adjuvant-induced arthritic mice (*n* = 7). (A) Histologic popliteal ganglion of arthritis untreated mice on day 28, where tissue showed an increase in follicular hyperplasia, with leukocyte infiltration as well as lipid inclusions. Stained with hematoxylin and eosin 16×. (B) Histologic ganglionic tissue of arthritis mice treated with *M. deppeana* Ethanolic extract at 450 mg/kg on day 28, where there is also a moderate follicular hyperplasia, with a lesser leukocyte infiltration and lipid inclusions compared to complete Freund's adjuvant control group (A). Stained with hematoxylin and eosin 16×. (C) Histologic popliteal ganglion tissue from arthritis mice treated with phenylbutazone (100 mg/kg) on day 28, where a greater decrease in follicular hyperplasia with lower leukocyte infiltration and lipid inclusions was observed. Stained with Hematoxylin and eosin 16×.

In previous works the beneficial effect of secondary metabolites isolated from medicinal plant extracts have been shown on the damage generated in the joint tissue of laboratory animals with experimental arthritis, decreasing joint swelling, bone resorption and follicular hyperplasia through the inhibition of some chemokines and cytokines (Kumar et al., 2016; Morinobu et al., 2008).

In this context, the reduction of follicular hyperplasia in ganglionic tissue observed in arthritic mice treated with *M. deppeana* EtOH extract may be due to its main metabolite verbascoside, which is a well-known suppressor of the accumulation of CD4$^+$ cells during inflammatory process in rats (Hayashi et al., 1994), through the inhibition of the oxidative degradation and later phosphorylation of the IkB-α protein complex of TNF-α-activated endothelial cells by its antioxidant effect suppressing the codification of adhesion proteins such as intercellular adhesion molecule-1 (ICAM-1) and P-selectin, decreasing leukocyte infiltration toward tissue (Hayashi et al., 1996; Mazzon et al., 2009; Niu et al., 2017; Paola et al., 2011).

On the other hand, it has also known that minor metabolites contained in EtOH extract, such as apigenin, suppress leukocyte infiltration by the inhibition of genic expression of the vascular cell adhesion molecule-1 (VCAM-1) and E-selectin in the membrane of TNF-α-activated endothelial cells, through the prevention in the formation of DNA-protein complexes (Choi et al., 2004). As well, triterpenes identified in EtOH extract of *M. deppeana* showed the same effect in previous works, while ursolic acid reduced the genic expression of ICAM-1 in endothelial cells by the suppression of Na$^+$/K$^+$-ATPase and amino acid transport (Mitsuda et al., 2014); oleanolic acid has shown that it reduces the infiltration of immune cells to the tissue mainly CD4$^+$ T lymphocytes (Nataraju et al., 2009).

All these secondary metabolites are well-known potent inhibitors of the NF-kB pathway, this being closely related to leukocyte infiltration, prostaglandin biosynthesis, and edema formation (Pesce et al., 2015); however, this cannot be the mechanism of action of the EtOH extract, since TNF-α levels were found to be high during arthritis, this transcription factor being the one that codes for this pro-inflammatory cytokine.

OS microenvironment

PCC and LPO oxidation rates in edema tissue revealed that EtOH extract at 450 mg/kg decreased both in 52.18% and 45.53%, respectively, compared to un-treated CFA. In arthritic mice treated with EtOH extract at 200 and 900 mg/kg there was a slight decrease (13% and 21% for PCC, respectively), and only 900 mg/kg dose showed a statistically significant decrease in LPO values of 20% in edema homogenate, compared to un-treated CFA group. Finally, PBZ treated group, PCC and LPO values decreased by 53.83% and 60.91%, respectively, with respect to un-treated CFA mice. (Table 1).

For ganglionic tissue, the same effect was observed; EtOH extract at 450 mg/kg decreased PCC and LPO levels, followed by PBZ and EtOH extract at 900 and 200 mg/kg. In both tissues, oxidative damage over proteins and lipids caused by experimental arthritis had the same rate in un-treated CFA mice; also, the protective effect of EtOH extract against OS was similar in edema and in ganglionic tissues; in this case, the 450-mg/kg dose was the most effective (Table 1).

Values for antioxidant enzymatic activity on edema homogenate are described in Table 2. Arthritic mice treated with EtOH extract at 200, 450 and 900 mg/kg, were statically similar between themselves and un-treated CFA group, while PBZ generated a statistically significant decrease in SOD activity of 22.32% compared to un-treated CFA mice and all the other study groups. For CAT activity, only arthritic mice treated with PBZ and EtOH extract at 450 and 900 mg/kg demonstrated a statistically significant decrease of ≈61% compared to un-treated CFA group. Finally, for GSH-Px activity in edema tissue, EtOH extract at 200, 450 and 900 mg/kg showed a statistically significant decrease of 26.05, 45, 65.26%, respectively, followed by PBZ (≈45%), compared to un-treated CFA mice value (Table 2).

Results of antioxidant enzyme activity for popliteal ganglionic tissue are described in Table 2, in which values of SOD activity were statistically similar among all groups and showed a similar effect compared to edema tissue, because all treatments exhibited statistically similar values to those of un-treated CFA mice; however, these values were higher than those shown by healthy animals. For CAT and GSH-Px activities, only EtOH extract at 450 and 900 mg/kg showed a significant decrease (≈40% and ≈64%, respectively) compared to un-treated CFA mice. PBZ and EtOH extract (200 mg/kg) groups did not show important activity compared to un-treated CFA group (Table 2).

PCC and LPO values for ganglionic and edema tissues are related to the previously described histological analysis, because one of the features of the LPO process is the formation of lipid inclusions or micro-vesicles generated by the denaturalization of cellular membrane phospholipids into free fatty acids, which accumulate in the cytoplasm (Ayala et al., 2014); in this case, those from ganglionic tissue, due to the onset of OS during experimental arthritis. To date, there is, to our knowledge, no published work that mentions the indirect *in vivo* antioxidant effect during experimental arthritis in mice generated by the EtOH extract of *M. deppeana* or by its main metabolites. CFA-induced experimental arthritis has been previously related to deregulation of the oxide/reduction microenvironment balance, due to the high activity of antioxidant enzymes measured in tissue; however, there is also a greater oxidation process in the biomolecules (proteins and lipids) (García-Rodríguez et al., 2012; Gutiérrez-Rebolledo et al., 2015; Kumar et al., 2016).

Table 1
Oxidative stress biomarkers in edema and ganglionic tissue of mice with CFA-induced arthritis treated with *Moussonia deppeana* ($n = 7$).

Treatments	Edema		Ganglionic tissue	
	PCC (μm carbonyl/g)	LPO (μm MDA/g)	PCC (μm carbonyl/g)	LPO (μm MDA/g)
Vehicles	5.30 ± 0.20	0.16 ± 0.04	5.63 ± 0.20	0.21 ± 0.02
CFA	22.66 ± 1.21[a]	4.81 ± 0.30[a]	63.37 ± 4.38[a]	6.99 ± 0.50[a]
PBZ 100 mg/kg	10.46 ± 0.45[ab]	1.88 ± 0.04[ab]	16.47 ± 0.99[ab]	3.70 ± 0.18[ab]
EtOH extract				
200 mg/kg	19.38 ± 1.09[abc]	4.70 ± 0.34[ac]	35.68 ± 1.51[abc]	3.90 ± 0.13[ab]
450 mg/kg	11.07 ± 0.16[abd]	2.62 ± 0.09[abcd]	11.36 ± 0.67[abcd]	1.36 ± 0.13[abcd]
900 mg/kg	17.31 ± 1.40[abce]	3.88 ± 0.27[abcde]	19.75 ± 1.63[abde]	1.93 ± 0.15[abcd]

Data showed as mean ± with standard error. One-way ANOVA, post hoc SNK ($p \leq 0.05$). [a] vs vehicles; [b] vs CFA control; [c] vs CFA + PBZ; [d] vs CFA + EtOH 200 mg/kg; [e] vs CFA + EtOH 450 mg/kg. CFA, complete Freund's adjuvant; PBZ, phenylbutazone; PCC, protein carbonyl content; LPO, lipid peroxidation; MDA, malondialdehyde.

Table 2
The effect of *Moussonia deppeana* on antioxidant enzymatic activity in edema and ganglion tissues of mice with CFA-induced arthritis ($n = 7$).

Tissue	Antioxidant enzymes	Vehicles	CFA	PBZ 100 mg/kg	EtOH extract		
					200 mg/kg	450 mg/kg	900 mg/kg
Edema	SOD (IU/g)	76.58 ± 5.90	112 ± 3.16[a]	87 ± 7.25[b]	94.50 ± 8.24[ab]	109.08 ± 8.69[acd]	117.83 ± 4.82[acd]
	CAT (IU/g)	290 ± 0.06	3430 ± 240[a]	1280 ± 120[ab]	1360 ± 70[ab]	1400 ± 100[ab]	1320 ± 150[ab]
	GSH-Px (mmol NADPH/g)	1.02 ± 0.27	7.14 ± 1.49[a]	3.19 ± 0.68[ab]	5.28 ± 1.40[a]	4.29 ± 1.16[ab]	2.48 ± 0.87[bde]
Ganglion	SOD (IU/g)	50.33 ± 3.40	99.92 ± 4.25[a]	92.83 ± 3.14[a]	107.83 ± 5.54[ab]	82.42 ± 7.20[abcd]	88.25 ± 8.58[abcd]
	CAT (IU/g)	370 ± 70	2410 ± 280[a]	1800 ± 280[ab]	850 ± 130[bc]	1770 ± 230[abd]	1150 ± 180[abce]
	GSH-Px (mmol NADPH/g)	1.34 ± 0.16	1.57 ± 0.13	1.09 ± 0.22[b]	1.59 ± 0.13[ac]	0.65 ± 0.05[abcd]	0.47 ± 0.09[abcd]

Data showed as mean ± with standard error. One-way ANOVA, post hoc SNK ($p \leq 0.05$). [a] vs vehicles; [b] vs CFA control; [c] vs CFA + PBZ; [d] vs CFA + EtOH 200 mg/kg. CFA, complete Freund's adjuvant; PBZ, phenylbutazone; IU, international units; NADPH, reduced nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase.

Other minor secondary metabolites in the *M. deppeana* EtOH extract, such as ursolic and oleanolic acids (triterpenes), and polyphenols such as apigenin and hesperetin have been identified in this medicinal plant, and it is well-known that these are potent inhibitors of the oxidative pathway of acute and chronic inflammation, decreasing free radical concentration and aiding in the regulation of the immune response through their indirect antioxidant effect *in vivo* (Recio et al., 2000; García-Rodríguez et al., 2012; Gutiérrez-Rebolledo et al., 2015; Kumar et al., 2016). In addition, verbascoside has been described as a compound that decreases LPO values and that also increases SOD activity during dextran sulfate sodium-induced colitis in rats (Lenoir et al., 2011). Other main metabolites identified in the EtOH extract included ursolic and oleanolic acids possess important anti-inflammatory and immune-modulatory activities (López-García et al., 2015; Tsao and Yin, 2015).

M. deppeana, medicinal plant of Mexican traditional medicine, is a good candidate for further investigation as a possible phytomedication. To date, its ethno-medicinal use to treat arthritis as an anti-inflammatory agent has been demonstrated in acute and chronic inflammation models, as well as its safety (acute and sub-acute toxicity) in preclinical studies. This work, being the first one in which the anti-arthritic effect of this medicinal plant was evaluated, is of great interest, since it establishes the bases of future investigations which will be focused on continuing the search for new molecules with biological activity and little or no toxicity for the treatment of not only arthritis, but as a possible remedy for other auto-immune diseases such as erythematous lupus, ankylosing spondylitis and atherosclerosis, because current allopathic treatments are long, expensive, and cause severe adverse effects. However more studies need to be done, such as chronic toxicity and the use of another experimental model other than murine.

Conclusion

Moussonia deppeana EtOH extract contains 79.2 mg verbascoside/g of dry extract, and generates a moderate anti-edematous effect during experimental arthritis at 450 mg/kg dose, a similar activity to reference drug phenylbutazone. This dose showed a beneficial effect on BW gain in arthritic mice and also an immune-modulating effect on leukocytes and lymphocytes in peripheral blood; however, in the ganglionic tissue, CD4+ T-cell sub-populations, as well as the concentration of TNF-α, remained unaltered by treatment with the EtOH extract at 450 mg/kg dose. Finally, 450-mg/kg dose also exerted a moderate decrease in follicular hyperplasia in the paw edema of arthritic animals, and appears to protect against oxidative damage to proteins and lipids, decreasing the endogenous antioxidant enzymatic response in both edema and ganglionic tissues, antioxidant activity being one of its action mechanisms that generate a beneficial effect during experimental arthritis. The study served to lay the groundwork for future research that supports the ethno-medicinal use of *M. deppeana* as an herbal remedy in the treatment of arthritis in Mexican traditional medicine.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

GAGR contributed running all the laboratory work related to experimental arthritis establishment, taking tissues and blood samples, evaluation of antioxidant effect, interleukins concentra-

tion, extract preparation, statistical analysis and writing the final manuscript. LGS contributed to biological studies by giving all OS reagents and to facilitate the use of the spectrophotometer. AKCR contributed to biological studies by giving part of interleukins quantification reagents as well the laboratory for the establishment of these molecular biology assays, and for help in the interpretation of the results from flow cytometer. AGSR contributed with the histological analysis of sub-plantar edema and popliteal ganglion tissues, as well the interpretations after the stain. AZ contributed to chromatographic analysis and quantification of verbascoside from Ethanolic extract.

MAJA supervised the laboratory work and contributed to critical reading of the manuscript, besides obtaining the economic funds to be able to carry out the project.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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