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Effects of extracts, flavonoids and iridoids from *Penstemon gentianoides* (Plantaginaceae) on inhibition of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) in LPS-Activated RAW 264.7 macrophage cells and their antioxidant activity[¥].

[Efectos de extractos, flavonoides e iridoides de *Penstemon gentianoides* (Plantaginaceae) sobre la inhibición de oxido nitrico sintasa inducible (iNOS), ciclooxigenasa-2 (COX-2) celulas de macrofagos murinos RAW 264.7 activadas con LPS y sus actividades antioxidantes.]

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Abstract

Penstemon gentianoides (HBK) (Kunth) Poir (Plantaginaceae) is an evergreen shrub that grows the throughout high mountains from Guatemala, Mexico and Southern states of US. Its leaves and roots have been used therapeutically for inflammation-related conditions from Aztec times, but systematic studies of its anti-inflammatory activity are lacking and no specific active components have been identified. In this study, methanol, n-hexane, CH_2Cl_2, ethyl acetate and methanol/water (6:4) extracts, luteolin, diosmetin, verbascoside, martynoside, pensteminoside, globularisicin and plantarenaloside isolated from this plant were evaluated by determining their inhibitory effects on the production of proinflammatory mediators in lipopolysaccharide (LPS)-activated RAW 264.7 murine macrophage cells. Ethyl acetate extract, luteolin, and diosmetin exhibited potent anti-inflammatory and antioxidant activities. The results indicated that luteolin and diosmetin suppressed the LPS induced production of nitric oxide (NO), through the down-regulation of inducible nitric oxide synthases (iNOS) and cyclo-oxygenase 2 (COX-2) protein expressions and showed a potent antioxidant activity against DPPH, TBARS and DCFH. The inhibition of enzymes and NO production by selected extracts and compounds was dose-dependent with significant effects seen at concentration as low as 50 µM. Thus, luteolin and diosmetin may provide a potential therapeutic approach for inflammation associated disorders.

Keywords: *Penstemon gentianoides*, anti-inflammatory activity, antioxidant activity, COX-2 Inhibitors, NO inhibitors, Plantaginaceae.

Resumen

Penstemon gentianoides (HBK) (Kunth) Poir (Plantaginaceae) es un arbusto perenne que crece a lo largo de las montañas altas de Guatemala, México y los estados del sur de los EE.UU.. Sus hojas y raíces se han utilizado terapéuticamente para afecciones relacionadas con inflamación desde la época de los aztecas, pero no existen estudios sistemáticos de su actividad anti-inflamatoria y ninguno de los metabolitos activos específicos han sido identificados. En este estudio, los extractos de metanol, n-hexano, CH_2Cl_2, acetato de etilo y metanol/agua (6:4), junto con, luteolina, diosmetina, verbascósido, martynoside, pensteminoside, globularisicin y plantarenaloside, aislados desde esta planta se evaluaron mediante la determinación de sus efectos inhibitorios sobre la producción de mediadores proinflamatorios en macrófagos murinos activados con lipopolisacárido (LPS)-RAW 264,7. El extracto de acetato de etilo, luteolina y diosmetina exhibieron una potente actividad anti-inflamatoria y antioxidante. Los resultados indican que luteolina y diosmetina suprimen la producción de óxido nítrico (NO), a través de la regulación de óxido nítrico sintasa-inducible (iNOS) y la ciclooxigenasa-2 (COX-2) ambas expresiones de proteínas. Ademas mostró una potente actividad antioxidante contra DPPH, TBARS y DCFH. La inhibición de las enzimas y la producción de NO por los extractos seleccionados y compuestos es dependiente de la dosis con efectos significativos visto en una concentración tan baja como 50 mM. Por lo tanto, luteolina y diosmetina puede proporcionar un enfoque terapéutico potencial para transtornos asociados a los procesos de inflamación.

Palabras Clave: *Penstemon gentianoides*, anti-inflammatory activity, antioxidant activity, COX-2 Inhibitors, NO inhibitors, Plantaginaceae.

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Introduction

The use of traditional medicine is widespread and plants can provide a large source of novel active biological compounds with different activities: antiinflammatory, anti-cancer, anti-viral, anti-bacterial, antioxidant, and other activities. Continuing with our systematic search of biological activities of natural products from the Mexican flora, now we wish to report the isolation and the inhibitory activities of inflammatory mediators of extracts and fractions, including flavonoids, iridoids and phenylpropanoids from *Penstemon gentianoides*. This species of *Penstemon* (Plantaginaceae) is a shrub of 1 to 3 m of high, growing from Guatemala, Mexico and US mountains, this plant is endemic from Mexico, where is used by the indigenous people under the common names of “jarritos”, “parrito”, and “campanitas” as anti-inflammatory, emollient, balsamic, laxative and against rheumatic pains (Dominguez et al., 2005). Additionally, *P. gentianoides* is used in traditional medicine in Mexico, in the ethno-pharmacology treatment of diverse inflammatory pathologies (Dominguez et al., 2005).
Previous studies on these plants (*P. campanulatus* and *P. gentianoides*) reported the presence of iridoids (Jimenez-Estrada et al., 2006) known as catalpol, aucubin, and penstemide. Extracts of these plants had showed antioxidant activity (Dominguez et al., 2005), penstemide showed antitumor activity and laxative effect (Jimenez-Estrada et al., 2006), among others biological activities as antimicrobial activity (Li et al., 2009) and as feeding stimulant of butterfly larvae against its predator birds (Bowers, 1983; Seigler, 1998) have been found. The huge foliar concentrations of iridoids may provide specialist herbivores with compounds that they either sequester for their own defense or use as a means of avoiding competition for food from generalist herbivores (Peñuelas et al., 2006).

A body of that nitric oxide (NO) is involved in various pathophysiological processes including inflammation and carcinogenesis, and inducible isoforms of nitric oxide synthases (iNOS) is mainly responsible for the production of large amounts of this mediator (Schmidt and Walter, 1994). At the same time, it is known that ROS (reactive oxygen species) are involved in inflammatory response (Babior et al., 1993). ROS can act like messenger increasing the response, or giving the peroxidation of cellular membranes increasing the damage. Nitric oxide synthase (NOS) is an important enzyme involved in regulation of inflammation, vascular tone, neurotransmission, tumor cells and other homeostasis of human body. Nitric oxide (NO) is a radical generated via oxidation of the terminal guanidine nitrogen atom of L-arginine by NOS and also an important cellular second messenger. NO is released during a variety of pathophysiological responses including circulatory shock, inflammation and carcinogenesis (Ohshima and Bartsch, 1994; Mordan et al., 1993).

NO plays a dual role in some cases as beneficial and in others as detrimental molecule in inflammation process. There are three main types of NOS isoforms (Marletta, 1993). Both neuronal (nNOS) and endothelial (eNOS) are constitutively expressed, whereas iNOS is inducible in response to interferon-g, lipopolysaccharide (LPS) and a variety of proinflammatory cytokines (Szabo, 1995).

The induced iNOS catalyzes the formation and release of a large amount of NO, which plays a key role in the pathophysiological of a variety of diseases including septic shock. Therefore, NO production catalyzed by iNOS may reflect the degree of inflammation and provides a measure by which effects of drugs on the inflammatory process can be assessed. Thus, along with selective inhibitors of iNOS enzyme activity, a rational enable to address to develop more selective agents for suppression of these genes that might be over expressed during the inflammation or carcinogenic process.

Cyclooxygenase (COX) which has two isoforms, COX-1 and COX-2, is the enzyme that catalyzes the rate-limiting step in prostaglandin synthesis, converting arachidonic acid into prostaglandin H2, which is then further metabolized to prostaglandin E2 (PGE2), PGF2α, PGD2 and other eicosanoids (Funk, 2001; Cao and Prescott, 2002). COX-1 is constitutively expressed in many tissues and plays a role in tissue homeostasis (Amiram et al., 1988). COX-2 which can be expressed in a variety of cells and tissues is an inducible isoform the expression of which is stimulated by growth factors, inflammatory cytokines, carcinogens, and tumor promoters, implying a role for COX-2 in both inflammation and control of cell growth (Alice et al., 1989; Dean et al., 1991; Subbaramaiah et al., 1996). Thus, compounds that inhibit the activity or expression

of COX-2 might be an important target for antiinflammation or cancer chemoprevention.

In the present study, in addition to methanol, hexane, dichloromethane, ethyl acetate, and methanol/water(6:4) extracts, luteolin **1**, diosmetin **2**, pensteminoside **3**, plantarenaloside **4**, globularicisin **5**, verbascoside **6**, and martynoside **7**, isolated from leaves of *Penstemon gentianoides* were screened to discover new lead compounds in suppression of corresponding enzyme expression with the assay system of inhibition of activity on iNOS and NO accumulation, in LPS-stimulated RAW 264.7 cells, a murine macrophage cell line. Additionally, the antioxidant activity of the compounds was studied.

Previously, we have reported the antioxidant activities of extracts (Dominguez et al., 2005), and the anti-inflammatory activity of MeOH, CH_2Cl_2, and AcOEt extracts from roots and aerial parts and catalpol, penstemide, luteolin **1**, diosmetin **2**, pensteminoside **3**, plantarenaloside **4**, globularicisin **5**, verbascoside **6**, and martynoside **7**, isolated from *P. campanulatus* and *P. gentianoides* against the effect assay on the 12-O-tetradecanoyl phorbol acetate (TPA)-induced mouse ear edema test (Dominguez et al., 2010).

Materials and methods

Biological Material.

The sample of plant material was studied based on its popular use as ethnomedicine and previous reports (Dominguez et al., 2005; 2007; Jimenez-Estrada et al., 2006). *P. gentianoides* (HBK) Poiret, Lindl. Don. Plantaginaceae was collected on the highest hills (>3000 m) within the Park “Los Dinamos”, near Mexico City. The plant was identified botanically by Prof. Francisco Ramos (Instituto de Biologia, UNAM), and voucher specimen was deposited at the Herbarium of the Biology Institute at UNAM (MEXU). The collected plant was air-dried and prepared for extraction. The main morphological parts of the sample were separated (flowers, leaves, and roots), were milled and extracted with methanol.

On the basis of their popular use as ethnomedicines, only samples of leaves and flowers were studied. The methanol extract of the leaves (A) was dried and redissolved in methanol/water (6:4) and then partitioned into hexane (C), dichloromethane (D), and ethyl acetate (E), leaving a residue (B), as shown in Scheme 1. Because most of the activity was associated with the ethyl acetate extract, only this extract was evaluated. Furthermore, the ethyl acetate partition of *P. gentianoides* was fractionated by column chromatography with Si-gel as stationary phase.

Chemicals and solvents.

All reagents used were either A.R. grade or chromatographic grade, purchased from Sigma Chemical Co. Methanol, CH_2Cl_2, $CHCl_3$, KCl, $CuSO_4$, silica gel GF254 analytical chromatoplates, silica gel grade 60, (70-230, 60A°) for column chromatography, *n*-hexane, and ethyl acetate were purchased from Merck. Lipopolysaccharide (LPS, *E. coli* 0111: B4), Dulbecco’s modified eagle’s medium (DMEM), penicillin sodium, streptomycin, protease inhibitor cocktail, naphthylethylenediamine, sulfanilamide, H_3PO_4 solution and sodium bicarbonate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), phenylmethyl-sulfonylfluride (PMSF), dithiothreitol (DTT), naphtylethylenedianine, DCFH, L-N6-(1-imino-ethyllysine(L-NiL), *Escherichia coli* lipopolysaccharide (LPS), fetal bovine serum (FBS), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Life Technologies Inc. (Grand Island, NY, USA). 7.5% SDS-polyacrylamide gel from Bio-Rad, COX-2, iNOS, β-actin monoclonal antibodies, radish peroxidase labeled rabbit anti-goat IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). ECL western blotting detection reagent was from Amersham (Buckinghamshire, U.K.).

Apparatus.

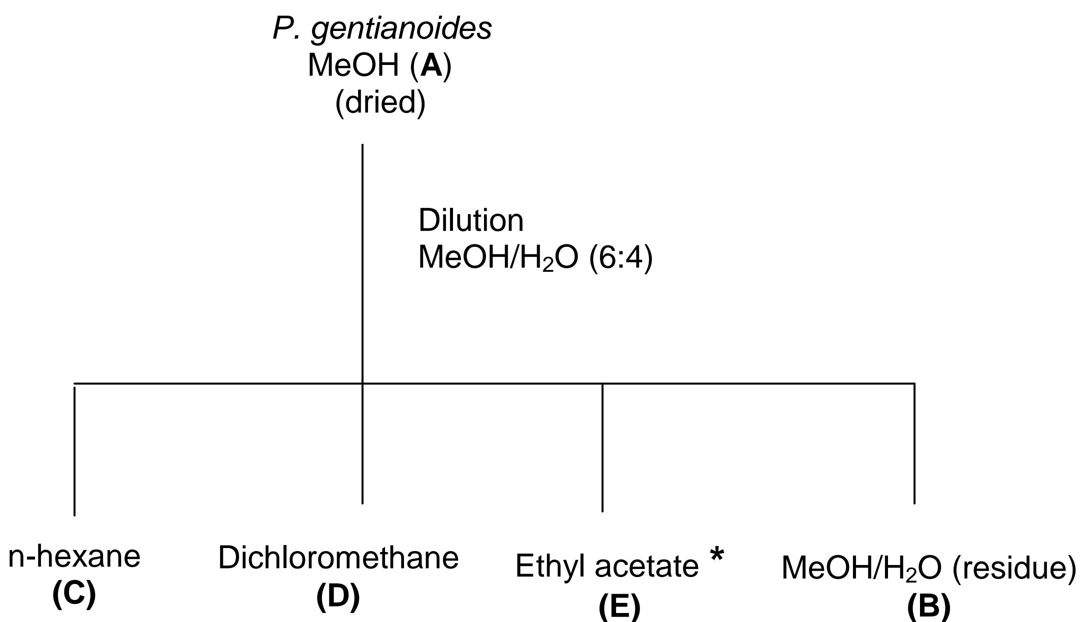
^{1}H-NMR spectra were recorded at 300 MHz, ^{13}C-NMR at 75 at MHz, on Varian VXR-300S spectrometer, chemical shifts (ppm) are related to $(CH_3)_4Si$ as internal reference, $CDCl_3$ and acetone-d6 from Aldrich Chemical Co. were used as solvents, coupling constants are quoted in Hz. IR spectra were obtained in KBr on a Perkin Elmer 283-B and a FT-IR Nicolet Magna 750 spectrophotometers. UV spectra for pure compounds were determined on a Shimadzu UV-160 and Spectronic model Genesys 5 spectrophotometer was used for biological activities. Additionally, were used Biotek ELx800 and Biotek Flx800 UV and Fluorometer lectors, respectively. Melting points were obtained on a Fisher-Johns hot-plate apparatus and remain uncorrected.

General experimental procedures

HPLC was performed on a WATERS Model 600E, equipped with Bondapack RP 18 column, 250 x 8 mm, speed flux 1.5 ml/min, speed paper 0.5 cm/min., UV detector 280 nm, mobile phase MeOH/H20 7:3 v/v. Analytical TLC were performed on silica gel 60 F254 E. Merck plates and the spots were visualized by spraying with a 10% solution of H_2SO_4, followed by heating at 110 °C.

Extraction and isolation of iridoids, flavonoids and phenylpropanoids.

The procedure was carried out in similar form for all samples. Briefly, for example with leaves: this sample (1.2 Kg) was extracted with methanol. The methanol extract (180 g) (A) was dried and redissolved in MeOH-H_2O (6:4), then partitioned with *n*-hexane (C), CH_2Cl_2 (D) and EtOAc (E), leaving a residue (B), see Scheme 1 (Dominguez et al., 2007).



Scheme 1. Method of obtaining of extracts and fractions from leaves of *P. gentianoides*. Extract E* and fraction F-5*, showed highest antioxidant activity against DPPH and TBARS, and the most high content of polyphenols measured by Folin-Ciocalteu method.

The ethyl acetate partition (46.22 g) showed high antioxidant activity and was further fractionated into eight fractions (I-VIII), by open column chromatography using Silica gel (type G, 10-40 μm, Sigma-Aldrich), (Dominguez et al., 2005). The elution of column was carried out with *n*-hexane-EtOAc in different ratios and adding MeOH to increase the polarity of the gradient until 100% MeOH was reached. All fractions were analyzed by TLC using ceric sulfate as the developing system. Additionally, as the ethyl acetate partition (E) showed high antioxidant activity, the elution of all fractions was also followed by TLC analysis as antioxidant bioautographic assay (Cespedes et al., 2008) using a methanol solution of DPPH at 0.2 %. From different fractions were isolated, purified and identified all compounds **1**-**7** (Dominguez et al., 2007). Almost all extracts and fractions yielded similar compounds by conventional phytochemical procedures and these were identified and collected for bioassays (Figure 1).

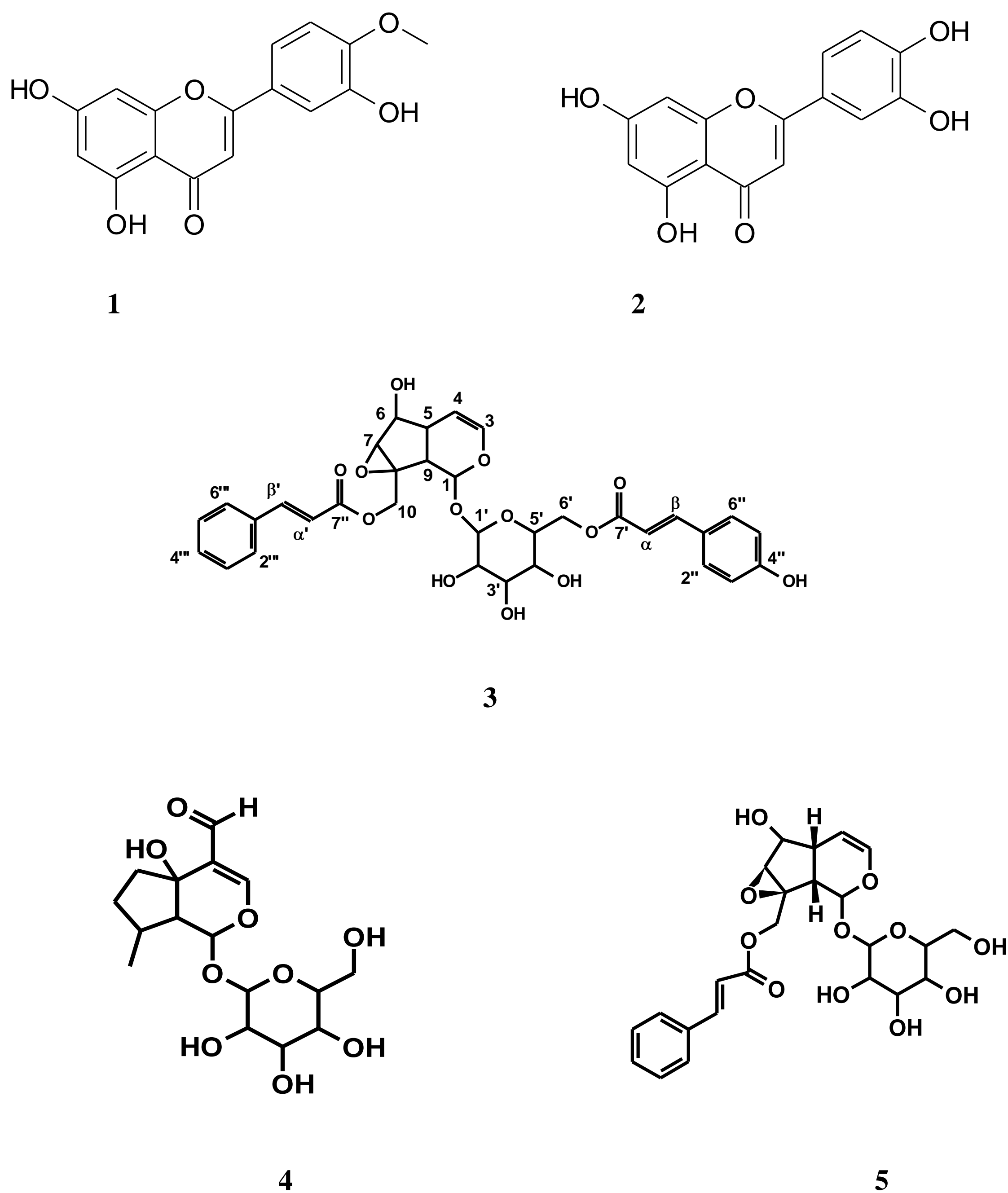


Figure 1. Chemical structures of luteolin 1, diosmetin 2, pensteminoside 3, plantarenaloside 4, globularisicin 5.

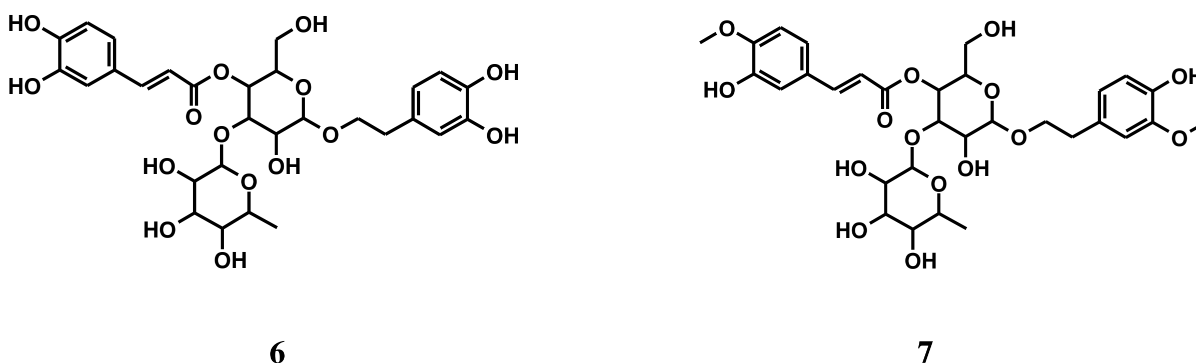


Figure 1 (cont): Chemical structures of verbascoside **6** and martynoside **7**.

Cell Culture

Raw 264.7 murine macrophage cells were obtained form American Type Culture Collection (ATTC, Rockville, MD, USA). Maintained in Dulbecco's modified Eagles Medium (DMEM) containing 100 units/mL penicillin G sodium, 100 units/mL streptomycin, supplemented with 10% heat inactivated FBS under endotoxin-free conditions at 37 ^{o}C in a 5% CO_2 atmosphere.

Cell stimulation

Raw 264.7 cells were plated in T-25 tissue culture flasks (3.0 x 106 cells/flask). Cells were incubated in DMEM for 24 h. The cells were replaced with new media DMEM 10% FBS with or without LPS (1µg/mL, from Escherichia coli, serotype 0111:B4), plus the plant extracts, fractions, or pure compounds and incubated for 12 h. Cells were then washed with PBS and lysed with NaCl 250 mM, HEPES 50 mM pH 7.9, EDTA 5 mM, Nonidet p-40 0.1%, DTT 0.5 mM, PMSF 1 mM, Na-orthovanadate 0.5mM, NaF 3 mM and Protease inhibitor cocktail 1µL by 1-3 X 10^7 cells. Protein was determined by the Bio-Rad method.

***Western blotting analysis*.**

Total protein (40 µg/lane) was on a 7.5% SDS polyacrylamide gel under standard conditions and electro-blotted to a NTT membrane in 15% methanol, 25 mM Tris and 192 mM glycine. The membrane was blocked with nonfat milk in TTBS saline 1 h at 37 °C or overnight at 4 °C before incubation with primary antibody (1:500 for iNOS, 1:1000 dilutions for COX-2) in 5% milk in TTBS for 1 h at 37 °C. After thorough washing, the membrane was incubated with a secondary antibody radish peroxidase (1:25,000) for 1 h at 37 °C. The immunoreactive bands were visualized using an enhanced chemoluminescence system Amersham.

Toxicity assay

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). 80,000 cells/well were plated under the same conditions as cell stimulation. After 12 h incubation, MTT (20 µL, 5 mg/ml in PBS) was added to each well and incubated for 1.5 h in a CO_2 incubator at 37 ºC. The medium was removed and DMSO (200 µL) added to dissolve the absorbed MTT crystals. The plate (96 wells) was incubated for another 15 min before measuring the absorbance at 550 nm (Biotek ELx800).

Measurement of nitric oxide formation by iNOS activity in cultured LPS-induced RAW 264.7 cells

Macrophage cells were maintained in DMEM supplemented with penicillin/streptomycin and 10% FBS at 37 °C, 5% CO_2 in humidified air.

For evaluating the inhibitory activity of test materials on iNOS, the cells in 10% FBS_/DMEM without were plated in 96-well plates (500,000 cells/well), and then incubated for 24 h. The cells were replaced with new media, and then incubated in the medium with 1 μg/mL of LPS and test samples. After additional 12 h incubation, the media were removed and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction. Briefly, 50 μL of Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4 solution) were added to 50 μL of each supernatant from LPS, or sample-treated cells in triplicate. The plates (96 wells) were incubated for 5 min, and then were read at 570 nm against a standard curve of sodium nitrite (Biotek ELx800).

DCFH assay.

80,000 cells/well were plated under the same conditions as cell stimulation at room temperature. After the 12 h incubation, medium was added 200 μL plus 100 μM DCFH per 30 min, the medium was removed and the cells were washed and added with 200 μL PBS pH 7.5 and the plate was placed in the reader and the fluorescence recorded every minute for 120 min, using an excitation l = 485/20 and emission l = 582/20 (Biotek FLx800).

Estimation of lipid peroxidation.

As an index of lipid peroxidation, TBARS levels were measured using rat brain homogenates according to the method described by Ng with some modifications (Ng, Liu, & Wang, 2000), and as is described in Dominguez et al., 2005. Results are expressed as nanomols of TBARS per milligram of protein, with percent inhibition after 30 min calculated as the inhibition ratio (IR), where *C)* absorbance of the control and *E)* absorbance of the test sample. These values were plotted against the log of the concentrations of individual extracts and fractions, and a decrease of 50% in peroxidation was defined as the EC_{50} (Dominguez, et al., 2005).

$$IR\ (\%) = [(C - E)/C] \times 100$$

Reduction of the 2,2-diphenyl-1-picrylhydrazyl radical.

Extracts and partitions were chromatographed on TLC and examined for antioxidant effects by spraying the TLC plates with DPPH reagent. Specifically, the plates were sprayed with 0.2% DPPH in methanol (Cespedes, Lemus, Salazar, Cabrera, & Sharma, 2003). Plates were examined 30 min after spraying, and active compounds appear as yellow spots against a purple background. In addition, TLC plates were sprayed with 0.05% β-carotene solution in chloroform and then held under UV254 light until the background bleached. Active components appeared as pale yellow spots against a white background (Cespedes, Uchoa, Salazar, Perich, & Pardo, 2002). Furthermore, each fraction and compounds were analyzed with DPPH in microplates of 96 wells as follows: extracts, partitions, and fractions (50 μL) were added to 150 μL of DPPH (100 μM, final concentration) in methanol (the microtiter plate was immediately placed in a Biotek model ELx808) and their absorbances read at 515 nm after 30 min. Quercetin and α-tocopherol were used as standards (Dominguez, et al., 2005).

Estimation of total polyphenol content

The total phenolic content of extracts was determined using the Folin- Ciocalteu reagent: 10 μL sample or standard (10-100 μM Catechin) plus 150 μL diluted Folin-Ciocalteu reagent (1:4 reagent: water) was placed in each well of a 96 well plate, and incubated at RT for 3 min. Following addition of 50 μL sodium carbonate (2:3 saturated sodium carbonate: water) and a further incubation of 2 h at RT, absorbance was read at 725 nm. Results are expressed as μmol Cat E per gram. All tests were conducted in triplicate (Singleton, Orthofer, & Lamuela- Raventos, 1999).

Statistical evaluations

Data are expressed as mean ± SD of results obtained from number (n) of experiments. Differences between data sets were assed by one way analysis of variance (ANOVA) followed by

Dunnett's test. The results are given in the text as probability values, with $p < 0.05$ adopted as the criterion of significance, differences between treatments means were established with a Dunnett's test. The EC50 values for each activity were calculated by Probit analysis on the basis of the percentage of inhibition obtained at each concentration of the samples. EC50 is the concentration producing 50% inhibition. Completely statistical analysis was performed by means of the MicroCal Origin 8.0 statistical and graphs PC program.

RESULTS AND DISCUSION

Phytochemical analysis of extracts and structural determination of compounds 1 - 7.

The identification of major compounds was achieved by comparison of their spectroscopic and physical data with those published previously reported by Dominguez et al., 2007 (Figure 1). All compounds were obtained by column chromatography from ethyl acetate extract (scheme 1). From the ethyl acetate extract, compounds were purified with Sephadex LH-20: *n*-hexane / CH_2Cl_2 / MeOH 2:1:1 and with CH_2Cl_2 / MeOH (8:2), mainly. Compounds **4** (121 mg) and 5 (200 mg) were isolated from fraction F-3 using *n*-hexane/ethyl acetate (1:1) and compound 1 (148.5 mg) using ethyl acetate / MeOH (8:2) as eluting system. Compound **3** (110 mg) was isolated from fraction F-4 using *n*-hexane/CH_2Cl_2/MeOH (2:1:1) mixture. From fraction F-5 with CH_2Cl_2-MeOH (8:2) was isolated compound **6** (60 mg) and with CH_2Cl_2-MeOH (9:1) compound **7** (40 mg). Compound **2** (200 mg) was isolated from the MeOH crude extract by cc using ethyl acetate/MeOH (8:2) as elutant. The structural elucidation of all compounds was previously reported (Dominguez et al., 2007). All data were compared with those of literature, our data base and with authentic samples.

Anti-inflammatory activity of extracts and secondary metabolites from P. gentianoides. NO Production.

Nitric oxide (NO), which is derived from the oxidation of L-arginine, is recognized as a mediator and regulator in pathological reactions, especially in acute inflammatory response (Surh et al., 2001). i-NOS is expressed in macrophages by stimulation with LPS among others, increasing NO production. In this study, NO inhibitory activity of extracts and major compounds from aerial parts of *P. gentianoides* was evaluated by using a LPS-stimulated RAW 264.7 cell assay. In our previous studies, the ethyl acetate and CH_2Cl_2 extracts from the aerial parts of *P. gentianoides* exhibited excellent inhibitory activity against reactive oxygen species (ROS) (Dominguez et al., 2005) and a strong anti-inflammatory activity against TPA-induced inflammation in mouse ear edema model (Dominguez et al., 2010).

To determine further the effects of those extracts and phenolic compounds on NO production, different concentrations of test samples were incubated with LPS-activated RAW 264.7 cell macrophages. As shown in Figure 2, the nitrite level produced in cultured supernatant of RAW 264.7 cells was markedly elevated for extracts A, B, these extracts do not shows a significant inhibition at 100 μg/mL, after 24h of treatment with LPS. However, extract C and D showed an inhibition very near to 50% (15.1 and 14.6 μM of nitrite, respectively) and extract E was the strongest inhibitor with 80% of activity (5.3 μM of nitrite concentration). Thus, the CH_2Cl_2 (D) and ethyl acetate (E) extracts of *P. gentianoides* leaves significantly inhibited LPS-induced NO production in a dose-dependent manner (Figure 2-A). Relative to LPS treatment only 21 % of NO production was inhibited in the ethyl acetate extract treated cells at the concentrations of 50 μg/mL (data not shown).

Figure 2

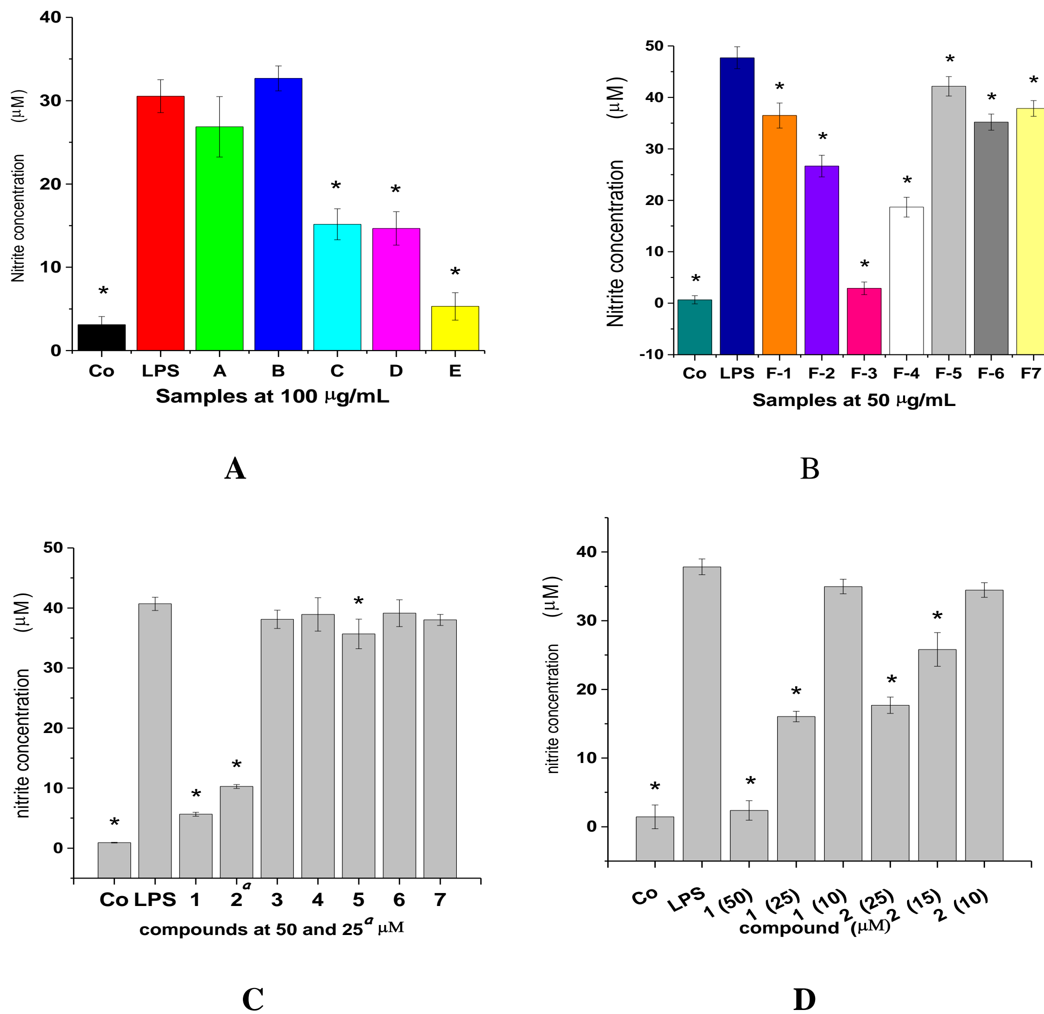


Figure 2. Nitric oxide production in macrophage RAW 264.7 cells measured whit Griess reagents. Cells stimulated for 24 hr with LPS (1 μg/mL) only, or LPS, extracts and pure compound from *P. gentianoides*: A) (at 100 μg/mL) A: leaves MeOH; B: MeOH/H_2O; C: n-Hexane; D: CH_2Cl_2; E: Ethyl acetate. B) 1) luteolin, [a]2) diosmetin at 25 μM, 3) globularisicine, 4) pensteminoside, 5) plantarenaloside 6) verbascoside and 7) martinoside. (50, [a]25 μM). C) dose response experiment1) luteolin (50, 25 and 10 μM) 2) diosmetin (25, 15 and 10 μM). At the end of incubation, 100 μL of the medium was removed for measuring nitrite production. Control values were obtained in the absence of LPS. Data were derived from three independent experiments and expressed as means SE. *P<0.05 indicate significant differences from the LPS-treated group.

In relation to the fractions, F-3 was the strongest inhibitor with an activity greater than 95% at 50.0 μg/mL, this is in agree with the inhibition of iNOS enzyme by this fraction, other fraction with a significant activity was F-4 with a 60% of inhibition of NO production, but not show the same activity against iNOS expression (Fig. 2-B).

The most active compounds inhibiting the NO production were **1** and **2** (Fig. 2-C). Quantifying the production of nitrite is a technique used to determine the indirect production of NO in

macrophages, which are known to be capable of reaching produce 4 x 106 NO molecules per cell from the iNOS enzyme (Dedon et al., 2004). This determination was commonly made using the test Griess. In conducting the trial with the pure compounds was observed that the compounds luteolin **1** and diosmetin **2** were which had a higher inhibition in the production of nitrites used to the maximum concentrations (25 and 50 μM, respectively) (Figure 2- C) and that the other compounds showed no effect which coincides with the results obtained in the expression of iNOS enzyme (Figure 4).

To check the effect of the compounds **1** and **2** an experiment was conducted at different concentrations (10, 25 and 50 μM for both compounds, respectively) (Figure 2-D) as in the expression of the enzyme iNOS, there was a dose response kinetics in which luteolin **1** showed greatest inhibitory activity on the production of nitrite, with respect to diosmetin **2**, these data are consistent with inhibition of the expression of the enzyme COX-2, and iNOS enzyme, because it is known that the production of nitrite is closely linked to the expression of COX-2.

Cytotoxicity

To evaluate whether the inhibition of NO production was possibly caused by the cytotoxicity effect of test extracts or compounds, the viability of test cells was determined by the MTT assay. Ethyl acetate, CH_2Cl_2 extracts and compounds **1** to **7** show no significant cytotoxicity to RAW 264.7 cells at test used concentrations (Figure 3 A and B, respectively). Thus, the inhibition of NO production (from 10 to 50 μM for compounds and at 50 and 100 μg/mL for extracts) in LPS-stimulated RAW 264.7 cells by extracts (Figure 3-A) and compounds (Figure 3-B) of *P. gentianoides* was not due to cytotoxicity.

Mechanism elucidation for anti-inflammatory activity of extracts and compounds from P. gentianoides.

Effects on iNOS and COX-2 levels in LPS-activated RAW 264.7 macrophages.

As mentioned in the background, macrophages are cells that are closely related to the inflammatory response, these cells arrive at the site of inflammation, and initiates a series of events and signals triggered in the first place by neutrophils, it also knows that macrophages can secrete protease, eicosanoids, cytokines, ERO and EPM (Nathan, 2002). It is well known that NOS activity is induced by cytokines such as TNF-α and IL-1β that plays an essential role in many inflammatory lesions (De Nardin, 2001). NOS catalyzes NO synthesis, there are three isoforms including a neuronal (nNOS), and endothelial (eNOS) and an endotoxin or cytokine-inducible (iNOS) form (Rosen et al., 2002). Both nNOS and eNOS, often grouped together as constitutive NOS (cNOS), iNOS is usually not detectable in healthy tissues but is expressed after immunological challenge or injury. The expression of iNOS and its enzymatic activity have been observed (Thomsen et al., 1994; 1995; Vane et al., 1994; MacMicking et al., 1997). In this study, the effect of MeOH (A), MeOH/H_2O (6:4) (B), n-hexane (C), CH_2Cl_2 (D), ethyl acetate (E) extracts and compounds 1 – 7 on iNOS and COX-2 expression was also investigated. As shown in Figure 4, unstimulated RAW 264.7 cells (control) showed barely detectable iNOS. In contrast 12 h incubation with LPS resulted in a large increase in iNOS expression. However, at 50 μM compounds **1** and **2** completely suppressed the iNOS expression. The proposed mechanism associated with the reduction of NO production are scavenging of NO, suppression of iNOS enzyme activity, inhibition of iNOS gene expression and/or down-regulation of iNOS enzyme by modulation of enzyme activities related to signal transduction, etc. (Park et al., 2000; Sheu et al., 2001; Chiang et al., 2005; Kim et al., 1999; Paul et al., 1995). In our study, our samples inhibited NO production in macrophages via one or more of these mechanisms. Cyclooxygenase (COX) is the rate-limiting enzyme in PG synthesis and exists as two isoforms: constitutive (COX-1) and inducible (COX-2) (Sheu et al., 2001). Like iNOS, COX-2 is an important

enzyme that mediates inflammatory processes. Multiple lines of compelling evidence support COX-2 playing a role in the development of tumors (MacMicking et al., 1997). Thus, aberrant or excessive expression of iNOS and COX-2 is implicated in inflammatory disorders and the pathogenesis of cancer. Luteolin **1** and diosmetin **2** and ethyl acetate extract not only showed strong inhibitory activity on iNOS expression, but also significantly inhibited the COX-2 expression in LPS-stimulated macrophages. Figure 4-A and 4-C shows that Luteolin **1**, Diosmetin **2** and ethyl acetate extract suppressed the LPS-induced COX-2 expression in a dose dependent manner.

Figure 4-D shows that ethyl acetate extract suppressed the LPS-induced COX-2 expression in a dose-dependent manner. Approximately, 40 and 90% reduction were observed at 50 and 100 µg/mL, respectively, as determined by densitometry analysis. At concentrations up to 100 µg/mL ethyl acetate extract can completely inhibit the expression of COX-2 in LPS-stimulated cells. On the other hand, this extract inhibits around 40% and completely (100%) the expression of iNOS at 50 and 100 µg/mL, respectively. Thus, one of the mechanisms of ethyl acetate inhibition of NO production in LPS-stimulated macrophages is mediated by the down-regulation of iNOS and COX-2 expressions. Further studies on intracellular signaling cascades leading to COX-2 and iNOS reduction by ethyl acetate extract of this plant are of interest. Additionally, in vivo pharmacological research on the anti-inflammatory activity of ethyl acetate extract should also be addressed.

In figure 4-A is observed that the compound luteolin (1) had an inhibitory activity of the enzyme expression of iNOS, introducing the increased activity to 50 µM and losing almost entirely activity to 10 µM (figure 4-C). In the case of the expression of the enzyme COX-2 was observed a similar effect though minor, this result is consistent with that reported in other articles, showing how the inhibitory activity of this compound (Matsuda 2003), therefore it was considered to the luteolin as a positive control of this trial.

The compound diosmetin (**2**), introduced a similar pattern, the expression of iNOS enzyme to the maximum concentration 25 µM, but in the case of COX-2 effect was less than that found the luteolin at the same concentration of 10 µM. The same experiment was carried out with the other compounds globularisicin (**3**), plantarenaloside (**4**) pensteminoside (**5**) martinoside (**6**) and verbascoside (**7**) to the same 25 µM) without observing significant effect on concentrations (50 and 10 µM) the expression of both enzymes (Figure 4-B).

To corroborate the effect of the compounds luteolin (**1**) and diosmetin (**2**) an experiment was conducted to dose response (10, 25 and 50 µM respectively). In Figure 4-C the flavonoid luteolin presented major inhibitory activity of iNOS expression of the enzyme with respect to the diosmetin. In the case of the COX-2 enzyme was observed with a similar behavior luteolin although the effect was smaller than in the expression of iNOS, diosmetin did not present a significant effect on the expression of COX-2 and the lowest concentration 10 µM there was a slight stimulation.

On the other hand, according to a test conducted by Kim et al., (2004) these flavonoids (of flavones type) comply with the structural characteristics necessary to inhibit the production of nitrites, which are: a ring A, C-5, 7 dihydroxy replaced and a ring B C-2', 3' dihydroxy replaced in the case of luteolin and a ring-2'-hydroxy B C C 3'-metoxy substituted for diosmetin whose replacement metoxylated form provides lower activity as shown in conducted tests.

Antioxidant activity

In Figure 5 shown that the compound luteolin **1** presented protective activity against oxidative stress (DCFH), which led us to conclude that the inhibitory activity of the expression of iNOS and in the production of nitrites was favored by the antioxidant activity.

Figure 3

A

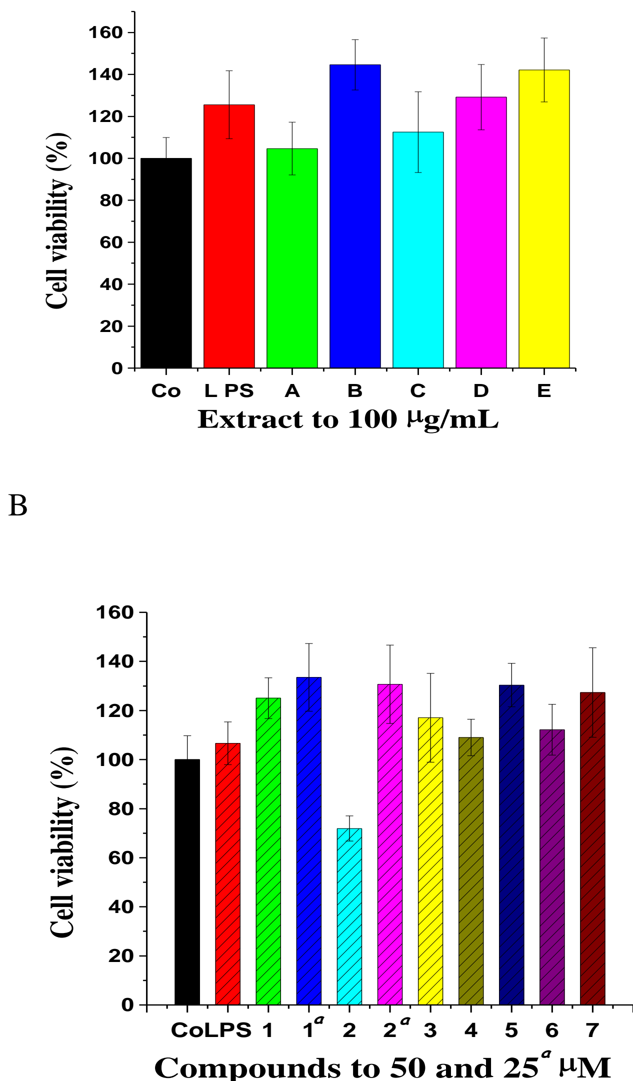


Figure 3. Viability of macrophages RAW 264.7 treated with LPS (1 µg/mL) with and without the assayed compounds and extracts. Co= Control, LPS= lipopolysaccharide. Data are expressed as the mean ± SE at least of three independent experiments. The viability of cells without LPS was the 100%. *P < 0.05 represent a significant difference compared with values obtained with cells without LPS.

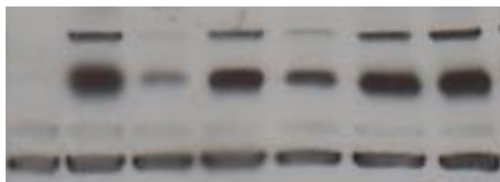
According to the background oxidative stress is involved at different levels in inflammatory response by stimulating the activity of NF- and production of different mediators such as IL-1, IL-6, TNF-α, IFN-γ in the cellular signaling, thus it is possible that luteolin through its antioxidant activity has decreased the studied factors which are involved in the inflammatory response. With regard to the present diosmetin despite a slight prooxidant activity also inhibited the expression of iNOS and COX-2 as well as in the production of nitrites, which suggest that the mechanism of action is separate from the production of reactive species.

In the case of the other compounds these did not show antioxidant activity, including pensteminoside and verbascoside, showed an apparent increase of oxidative stress, like the diosmetin. The result obtained with the verbascoside disagrees with the antioxidant activities reported in the literature (Aldini et al., 2006; Xiong et al., 2000). The table 1 shows the antioxidant activity of the compounds isolated from *P. gentianoides* at a concentration of 50 µM. It is noted that the compound verbascoside showed a larger reduction DPPH radical activity with a 87.25%, followed by luteolin activity with a 66.2%. The compounds martinoside and diosmetin showed less reduction DPPH radical activity with a 58.13% and 22.48%, respectively. Globularicisin, plantarenaloside and pensteminoside did not show activity to this concentration. Similar activity was observed in the trial against TBARS where luteolin and verbascoside showed the greatest inhibition on lipid peroxidation (93.28% and 87.53% respectively), followed by diosmetin (34.29%) and martynoside (28.52%). The antioxidant activity of the extracts and fractions were published previously in Dominguez et al., 2005.

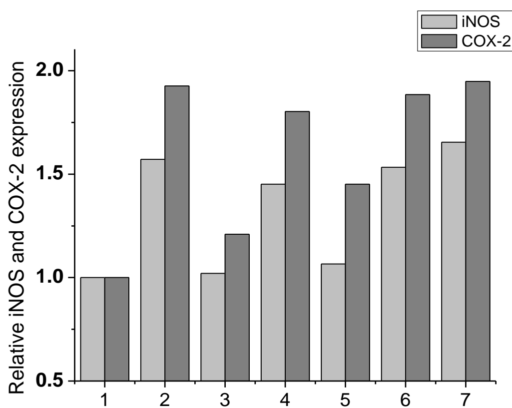
Table 1. Antioxidant activity of pure compounds from *P. gentianoides* against DPPH and Lipid peroxidation at 50 µM of concentration. The data are expressed as mean of three independent experiments ± SE. Globularisicin **3**, plantarenaloside **4** and penstemide **5** not show activity at the used concentration.

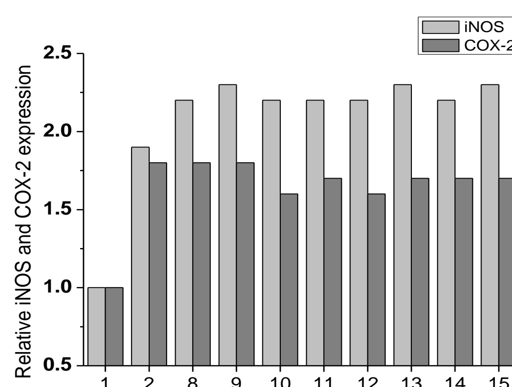
Compound (50 µM)	% scavenging DPPH$^{\bullet}$	% inhibition of lipid peroxidation
luteolin **1**	66.2 ± 10.34	93.28 ± 0.82
Diosmetin **2**	22.48 ± 0.87	34.29 ± 6.87
martinoside **6**	58.13 ± 1.24	28.52 ± 3.28
verbascoside **7**	87.25 ± 1.34	87.53 ± 1.4

A B



LPS - + + + + + + - + + + + + + + + +
CP - - **1 1 2 2 3** - + **4 4 5 5 6 6 7 7**
[µM] - - 50 10 25 10 50 - - 50 10 50 10 50 10 50 10





C **D**

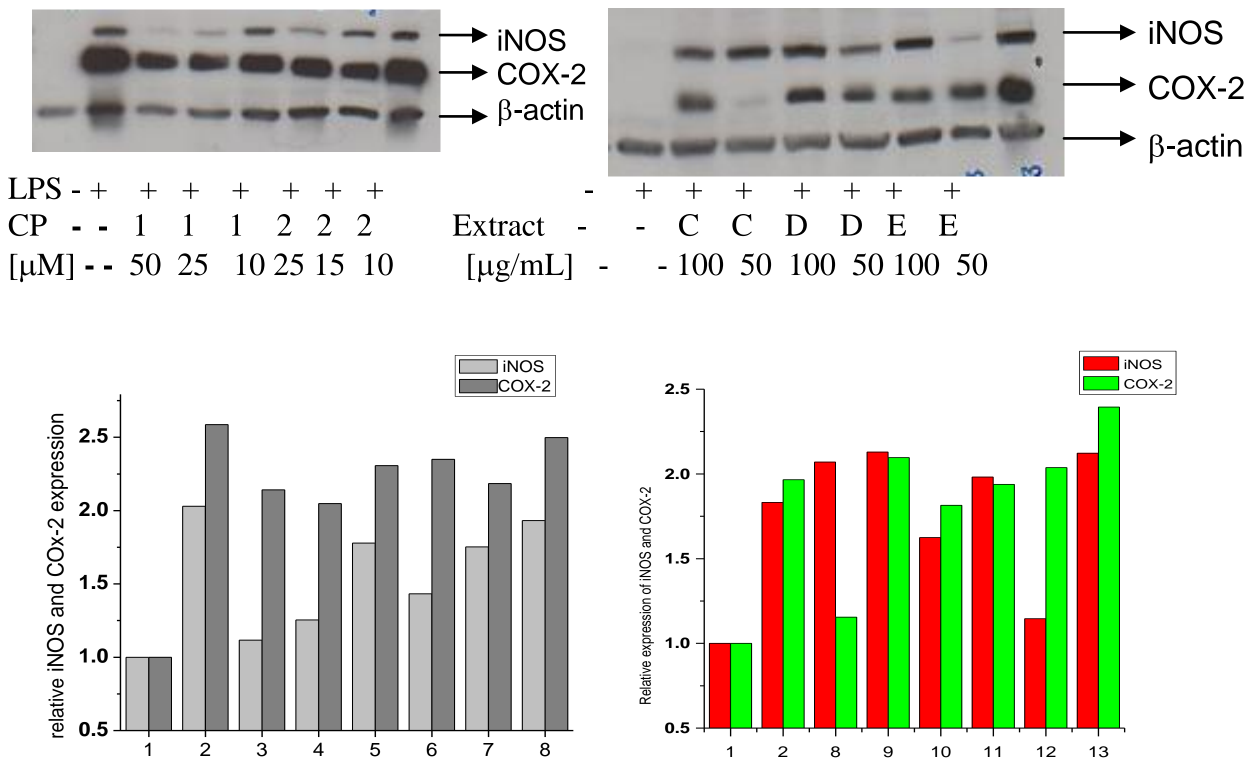


Figure 4.- Representative Western blots. iNOS and COX-2 protein expression in macrophage RAW 264.7 cells stimulated for 24 hr with LPS (1 μg/mL) only, or LPS and pure compound from *P. gentianoides*: **A**) luteolin **1**, diosmetin **2**, and globularisicine **3**, **B**) pensteminoside **4**, plantarenaloside **5**, verbascoside **6** and martynoside **7**. **C**) Dose response experiment luteolin **1** (50, 25 and 10 μM), and diosmetin **2** (25, 15 and 10 μM). Equal amounts of total proteins (40 μg/line) were subjected into 10% SDS ± PAGE, and expression of iNOS, COX-2, and β-actin protein was detected by western blotting using specific antibodies. β-actin protein here was used as an internal control. Data was described as means ± SE of iNOS/β-actin or COX-2/β-actin. The densitometry evaluation of the blot is depicted below. One of three representative experiments is shown. **D**) Dose response experiment with extracts **D** and **E**.

CONCLUDING REMARKS: In the particular case of verbascoside, it is known that this compound has a strong antioxidant activity which was reflected in the essays against DPPH, but not in the essay of antioxidant activity in the RAW 264.7 macrophages, this could be due to the hydrophilic properties upon the hydroxyl groups of both the aromatic rings as sugars and not allow it to cross the lipid membranes and exercise their antioxidant effect for the same reasons it may not have shown effect in inhibiting the expression of COX enzymes -2 and iNOS. With these data highlights the variability of the results of different trials designed to measure the same effect.

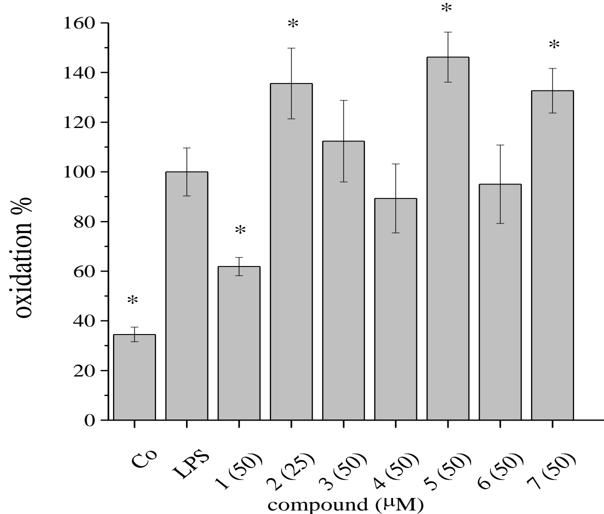


Figure 5.- Inhibition of oxidative stress in macrophage RAW 264.7 cells evaluated with DCFH Cells stimulated for 24 hr with LPS (1 μg/mL) only, or LPS and pure compound from *P. gentianoides*: 1) luteolin, [a]2) diosmetin, 3) globularisicin, 4) pensteminoside, 5) plantarenaloside 6) verbascoside and 7) martynoside. (50, [a]25 μM). Data were derived from three independent experiments and expressed as means SE. *P<0.05 indicate significant differences from the LPS-treated group.

On the other hand, the compounds luteolin and diosmetin showed antioxidant activity in DPPH test and TBARS. The luteolin, is a flavonoid with antioxidant activity reported by its replacement ortho dihydroxy in the ring B. The inhibiting the expression of COX-2 enzymes and iNOS by luteolin and diosmetin, as well as the production of nitrite is not new, since it is known that the luteolin interferes with cascades of signals in which stimulated with LPS is the RAW 264.7 cell line, blocking the main (Xagorari et al., 2002), pro-inflammatory molecules such as TNF-α however, does not take reports of the antioxidant activity of these compounds in RAW 264.7 macrophages using DCFH.

Accordingly to these results it is suggested that not all compounds with antioxidant activity in the chemical models presented in biological models which are present in a greater number of factors, which may affect activity, and that the antioxidant activity can be a factor that promotes the inhibition of the expression of certain enzymes.

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