

## Anti-Inflammatory and Cytotoxic Activities of Chichipegenin, Peniocerol, and Macdougallin Isolated from *Myrtillocactus geometrizans* (Mart. ex Pfeiff.) Con.

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The oleanane-type triterpene chichipegenin and the sterols peniocerol and macdougallin, isolated from *Myrtillocactus geometrizans*, showed anti-inflammatory activities in both the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema model and the carrageenan-induced rat paw edema model. All tested compounds inhibited the TPA-induced edema in a dose-dependent manner, with ED<sub>50</sub> values less than or equal to that shown by indomethacin. Among them, peniocerol was the most active compound. However, only peniocerol and macdougallin reduced carrageenan-induced rat paw edema. On the other hand, peniocerol and macdougallin showed cytotoxicity against several human cancer cell lines. These results indicate that compounds isolated from *M. geometrizans* possess anti-inflammatory and cytotoxic properties, and the presence of chichipegenin in the aerial parts could justify the medicinal uses attributed to the plant.

**Key words:** *Myrtillocactus geometrizans*, Anti-Inflammatory, Cytotoxicity

### Introduction

Cancer has been associated with the inflammation process since 1863, when Rudolf Virchow discovered leukocytes in neoplastic tissue. Since then, there is increasing evidence that chronic inflammation in damaged tissues contributes as a significant risk factor to tumour promotion, progression, and metastasis (Mantovani *et al.*, 2008). Furthermore, in the microenvironments of various tumour types, elevated levels of pro-inflammatory cells have been found, and the transcription nuclear factor kappa-B (NF- $\kappa$ B), which is involved in the inflammatory process, is expressed. Also, high levels of the pro-inflammatory enzymes cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) have been reported (Kundu and Surh, 2008).

Therefore, as an alternative to traditional treatments that are losing their effectiveness or are

nonspecific and highly toxic, some of the specific components of the chronic inflammatory response have recently become potential therapeutic targets for achieving chemoprevention of cancer or chemotherapy (Dolcet *et al.*, 2005; Fitzpatrick *et al.*, 2008; Wang and Lin, 2008; Wink *et al.*, 2008; de Souza Pereira, 2009).

Taking into account the above, our group has made considerable efforts to identify phytochemicals that show both anti-inflammatory activity as well as cytotoxic properties (Oviedo-Chavez *et al.*, 2004, 2005; Flores-Rosete and Martínez-Vázquez, 2008). Our results and those of other groups have shown that triterpenes and sterols are promising groups of natural compounds, not only for their anti-inflammatory properties, but also for their cytotoxicity against several human cancer cell lines. These properties make these compounds attractive to develop new antitumour drugs (Akihisa and Yasukawa, 2001; Parra-Delgado *et al.*, 2006).

As part of our systematic search for bioactive secondary metabolites from plants, we decided to begin a series of phytochemical and biological studies using extracts and compounds isolated from *Myrtillocactus geometrizans* (Mart. ex Pfeiff.) Con. (Cactaceae), which is commonly known in Central Mexico as “garambullo” and is used as anti-inflammatory remedy in the Mixteco (Oaxaca State) and Otomie (Hidalgo State) folk medicines (Luna-Morales and Aguirre, 2001; Sanchez-Gonzalez *et al.*, 2008).

In a previous work we reported the isolation of chichipegenin (**1**), peniocerol (**2**), and macdougallin (**3**) from this species (Cespedes *et al.*, 2005). Although these compounds have been isolated in previous studies from several species of the Cactaceae family (Sandoval *et al.*, 1957; Djerassi *et al.*, 1957, 1965; Knight *et al.*, 1966; Knight and Petit, 1969; Khong and Lewis, 1975; Kircher and Bird, 1982), their biological activities, which could explain the traditional use of *M. geometrizans*, have not been studied.

The aim of the present research was to evaluate the anti-inflammatory activity of compounds **1**, **2**, and **3** in both the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema model and the carrageenan-induced rat paw edema test, as well as to assess their cytotoxic activities against a set of human cancer cell lines in the sulforhodamine B test.

## Material and Methods

### General experimental procedures

All solvents, sulforhodamine B (SRB), RPMI-1640 medium, dimethyl sulfoxide (DMSO), doxorubicin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), indomethacin, Tween 80, carrageenan  $\lambda$  type IV, trichloroacetic acid, tris[hydroxymethyl]aminomethane (Tris), trypsin-EDTA, sodium pentobarbital, streptomycin, L-glutamine, and penicillin were from Sigma Chemical Co., St. Louis, MO, USA. Phosphate buffered-saline (PBS), Dulbecco's modified essential medium (DMEM), and fetal bovine serum were from GIBCO, Grand Island, NY, USA. Colon carcinoma (HCT-15), breast carcinoma (MCF-7), leukemia (K-562 CML), central nervous system (CNS) carcinoma (U-251 Glioblastoma), and prostate carcinoma (PC-3) cell lines were supplied by the National Cancer Institute, USA.

### Isolation

3 $\beta$ ,16 $\beta$ ,22 $\alpha$ ,28-Tetrol-olean-12-ene (chichipegenin, **1**), 3 $\beta$ ,6 $\alpha$ -diol-cholest-8-ene (peniocerol, **2**), and 14 $\alpha$ -methyl-3 $\beta$ ,6 $\alpha$ -diol-cholest-8-ene (macdougallin, **3**) were isolated and purified as previously described (Cespedes *et al.*, 2005). Copies of the original spectra are obtainable from the author for correspondence.

### Animals

Male CD-1 mice, weighing 25–30 g, were provided from Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México. Male Wistar rats, weighing 190–210 g were provided from Instituto Nacional de Enfermedades Respiratorias (INER), México, México. They were treated as approved by the Animal Care and Use Committee (PROY-NOM-087-ECOL-SSA1-2000). All animals were held under standard laboratory conditions in the animal house at (27  $\pm$  1) °C with a 12 h/12 h light-dark cycle and were fed with laboratory diet and water *ad libitum*. All experiments were carried out using a minimum of six animals per group.

### TPA-induced ear edema test

Evaluation of anti-inflammatory effects of compounds **1–3** was performed according to the TPA-induced mouse ear edema test previously described (Oviedo-Chavez *et al.*, 2004). Briefly, groups of six male CD-1 mice were anesthetized with sodium pentobarbital [3.5 mg/kg, intraperitoneal (i.p.) injection], and a solution of 2.5  $\mu$ g TPA dissolved in 10  $\mu$ L of ethanol was topically applied to both sides of the right ear of the mice (5  $\mu$ L each side). The left ear received only ethanol (5  $\mu$ L each side). After 10 min of TPA treatment, compounds **1–3** were separately applied in a 0.01–0.47 mg/ear dose range, dissolved in ethanol. Indomethacin (**4**) as reference drug was applied in a 0.04–0.46 mg/ear dose range, dissolved in 1:1 ethanol/acetone. Control animals received only the respective solvent mixture. 4 h later the animals were sacrificed by cervical dislocation and a plug (7 mm in diameter) was removed from each ear. The swelling was assessed as the difference in weight between right and left ear plugs. The percentage of edema inhibition (EI, %) was calculated by the equation: EI (%) = 100 – [B · 100/A], where A is the edema induced by TPA alone, and B is the edema induced by TPA plus sample.

Data were expressed as the mean  $\pm$  SME of six mice. The effective dose 50 (ED<sub>50</sub>) values were estimated from linear regression equations calculated with significant data.

#### Carrageenan-induced rat paw edema test

The carrageenan-induced rat paw edema was performed according to the method described previously (Oviedo-Chavez *et al.*, 2004), with slight modifications. Briefly, the basal volume of the right paw of each rat was measured with a plethysmometer (model 7150, UGO, Basile, Varese, Italy). Immediately thereafter, compounds **1–3** were administered i.p. in a solvent mixture of Tween 80 (5%) in water and DMSO (9:1 v/v), at doses of 45, 60, and 100 mg/kg body weight. Indomethacin (**4**) was administered at doses of 2.5, 5, 7.5, and 10 mg/kg i.p. in the same solvent mixture. The control group received i.p. only the solvent mixture. 1 h later, paw edema was induced by subplantar injection of 0.1 mL of carrageenan  $\lambda$  (0.1% in saline) into the plantar surface of the right hind paw of all animals. The paw volume was measured 1, 2, 3, 4, and 5 h after the carrageenan injection. The anti-inflammatory activity was measured as the area under the curve (AUC). Total inhibition (TI, %) was obtained for each group and at each record using the following ratio: TI (%) = [AUC<sub>control</sub> – AUC<sub>treat</sub>] · 100/AUC<sub>control</sub>, where AUC<sub>control</sub> is the area under the curve of the control group, and AUC<sub>treat</sub> is the area under the curve of the treated group. Data were expressed as the mean  $\pm$  SME.

#### Sulforhodamine B (SRB) cytotoxicity assay

The cytotoxic effects of compounds **1–3** were determined following protocols previously described (Oviedo-Chavez *et al.*, 2005). The human prostate carcinoma (PC-3), leukemia (K-562), central nervous system carcinoma (U-251), breast carcinoma (MCF-7), and colon carcinoma (HCT-15) cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100  $\mu$ g/mL streptomycin, and 1% nonessential amino acids. They were maintained at 37 °C in a 5% CO<sub>2</sub> atmosphere with 95% humidity. Adherent cells were detached with 0.1% trypsin-EDTA to make single-cell suspensions. Viable cells were counted using a hemacytometer. Cells (5,000–10,000 cells/well) were seeded in 96-well microtiter

plates and incubated at 37 °C. After 24 h, cells were treated with seven different concentrations (1–50  $\mu$ M) of the test compounds initially dissolved in DMSO (20 mM) and further diluted in medium to produce the desired concentrations. The plates were incubated for another 48 h at 37 °C. Doxorubicin was used at five different concentrations (0.01–5  $\mu$ M) as a positive control. After 48 h, adherent cell cultures were fixed *in situ* by adding 50  $\mu$ L of cold 50% (w/v) trichloroacetic acid, and the mixture was incubated for 30 min at room temperature with 0.4% SRB. Unbound SRB solution was removed washing three times with 1% acetic acid. Plates were air-dried, protein-bound SRB was dissolved with Tris buffer, and optical densities were read on an automated spectrophotometric plate reader at a wavelength of 515 nm. The concentrations required to inhibit cell growth by 50% (IC<sub>50</sub>) were calculated.

#### Statistics

The one-way analysis of variance (ANOVA) and Dunnett's test were used to compare several groups with the respective control. Values of \**p* < 0.05 or \*\**p* < 0.01 were considered significant.

#### Results

The triterpene chichipegenin (**1**) together with the sterols peniocerol (**2**) and maddockallin (**3**) (Fig. 1) were tested for their anti-inflammatory activities using the TPA-induced ear edema in mice and carrageenan-induced rat paw edema model, as well as for their *in vitro* cytotoxic properties.

#### TPA-induced ear edema test

All tested compounds showed anti-inflammatory activity in a dose-dependent manner, with

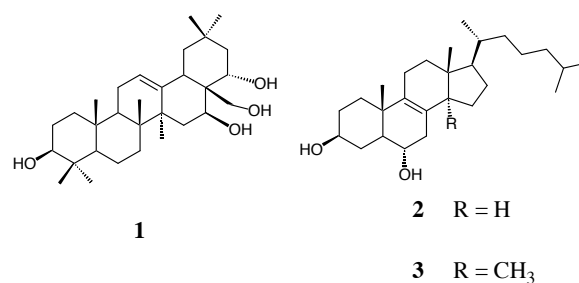


Fig. 1. Chemical structures of chichipegenin (**1**), peniocerol (**2**), and maddockallin (**3**) isolated from *Myrtillocactus geometrizans*.

Table I. Effect of topical administration of chichipegenin (**1**), peniocerol (**2**), macedougallin (**3**), and indomethacin (**4**) on TPA-induced ear edema. The data represents the mean of 6 animals  $\pm$  standard mean error (mean  $\pm$  SME). All data were analysed by ANOVA followed by Dunett's test, and the values of \*  $p \leq 0.05$  and \*\* $p \leq 0.01$  are considered as statistically different with respect to the control.

Compound	Dose [ $\mu\text{mol}/\text{ear}$ ]	Edema [mg]	Inhibition (%)	ED <sub>50</sub> [ $\mu\text{mol}/\text{ear}$ ]
<b>1</b>	0	12.55 $\pm$ 0.38	-	0.172 $r = 0.99$
	0.031	11.57 $\pm$ 0.35	7.77	
	0.1	7.60 $\pm$ 1.96 **	39.44	
	0.31	4.70 $\pm$ 0.60 **	62.55	
	1	2.57 $\pm$ 0.41 **	79.48	
<b>2</b>	0	14.46 $\pm$ 0.70	-	0.091 $r = 0.95$
	0.031	10.22 $\pm$ 0.79 *	28.20	
	0.1	5.88 $\pm$ 1.18 **	58.71	
	0.31	4.66 $\pm$ 1.44 **	67.23	
	1	4.35 $\pm$ 0.53 **	69.45	
<b>3</b>	0	15.21 $\pm$ 0.78	-	0.27 $r = 0.97$
	0.1	11.83 $\pm$ 1.34	16.73	
	0.18	10.65 $\pm$ 1.00 **	35.71	
	0.31	5.38 $\pm$ 1.25 **	62.15	
	1	2.63 $\pm$ 0.71 **	81.5	
<b>4</b>	0	16.24 $\pm$ 0.86	-	0.272 $r = 0.97$
	0.13	10.53 $\pm$ 1.04 **	35.15	
	0.24	8.18 $\pm$ 0.34 **	48.18	
	0.42	7.10 $\pm$ 1.34 **	56.29	
	0.75	4.97 $\pm$ 1.70 **	69.42	
	1.3	1.57 $\pm$ 0.33 **	89.19	

ED<sub>50</sub> values between 0.09 and 0.27  $\mu\text{mol}/\text{ear}$ . The results are summarized in Table I. All compounds showed activity comparable to indomethacin (ED<sub>50</sub> = 0.272  $\mu\text{mol}/\text{ear}$ ). Among them, sterol **2** had a strong inhibitory effect (ED<sub>50</sub> = 0.091  $\mu\text{mol}/\text{ear}$ ), while the tripterpene **1** (ED<sub>50</sub> = 0.172  $\mu\text{mol}/\text{ear}$ ) and compound **3** (ED<sub>50</sub> = 0.27  $\mu\text{mol}/\text{ear}$ ) had almost the same order of potency as indomethacin.

#### Carrageenan-induced rat paw edema test

Compounds **1**, **2**, and **3** were evaluated in the carrageenan-induced rat paw edema test, and indomethacin was included as a reference drug. The *in vivo* data are summarized in Table II. Subplantar injection of carrageenan induced edema which reached a maximum 4 h after administration. The value of the area under the curve (AUC) was used to quantify the temporal evolution of the inflammation produced by carrageenan, and low AUC values indicated anti-inflammatory activity.

Both compounds **2** and **3**, in doses of 45, 60, and 100 mg/kg *i.p.*, showed significant dose-dependent inhibition of the AUC with ED<sub>50</sub> values of 31.88 mg/kg and 53.25 mg/kg, respectively. Al-

though compound **1** also showed anti-inflammatory activity, its effect did not reach 50% inhibition, even at a dose of 100 mg/kg.

Unlike in the TPA test, indomethacin (ED<sub>50</sub> = 6.68 mg/kg) was more potent than compounds **1**–**3**. However, compounds **2** and **3** at a dose of 100 mg/kg showed inhibition of the AUC equivalent to that produced by indomethacin at 10 mg/kg.

#### Sulforhodamine B (SRB) cytotoxicity assay

The cytotoxic activity of compounds **1**, **2**, **3**, and doxorubicin was evaluated against central nervous system carcinoma (U-251), prostate carcinoma (PC-3), leukemia (K-562), colon carcinoma (HCT-15), and breast cancer (MCF-7) human cell lines. The values of 50% inhibitory concentration (IC<sub>50</sub>) are shown in Table III. Among the compounds, **1** did not inhibit the growth of cancer cells by more than 50% at a dose of 200  $\mu\text{M}$ . On the other hand, **2** and **3** showed moderate cytotoxicity against all cancer cell lines with IC<sub>50</sub> values of 7.50 to 24.73  $\mu\text{M}$ . **2** was more active against all the human cancer lines tested except against the K-562 line where **3** was more active. Never-

Table II. Effects of chichiopenin (**1**), peniocerol (**2**), macedougallin (**3**), and indomethacin (**4**) in the carrageenan-induced rat paw edema model. The data represents the mean of 6–9 animals  $\pm$  standard mean error (mean  $\pm$  SME). All data were analysed by ANOVA followed by Dunett's test, and the values of  $*p \leq 0.05$  and  $**p \leq 0.01$  are considered as statistically different with respect to the control. ND, not determined.

Compound	Dose [mg/kg]	Edema [mL/h]					AUC	AUC inhibition (%)	ED <sub>50</sub> [mg/kg]
		1	2	3	4	5			
Control	-	0.42 $\pm$ 0.05	0.85 $\pm$ 0.08	1.01 $\pm$ 0.06	1.06 $\pm$ 0.04	1.06 $\pm$ 0.04	3.65 $\pm$ 0.1	-	-
<b>1</b>	45	0.31 $\pm$ 0.10	0.68 $\pm$ 0.09	0.66 $\pm$ 0.04	0.67 $\pm$ 0.08	0.57 $\pm$ 0.08	2.44 $\pm$ 0.25*	33.15	ND
	100	0.34 $\pm$ 0.05	0.47 $\pm$ 0.1	0.66 $\pm$ 0.2	0.70 $\pm$ 0.2	0.74 $\pm$ 0.2	2.37 $\pm$ 0.20**	35.07	
<b>2</b>	30	0.20 $\pm$ 0.03	0.43 $\pm$ 0.04	0.50 $\pm$ 0.06	0.65 $\pm$ 0.06	0.63 $\pm$ 0.06	1.98 $\pm$ 0.18**	45.75	31.88
	45	0.11 $\pm$ 0.02	0.27 $\pm$ 0.04	0.46 $\pm$ 0.02	0.66 $\pm$ 0.03	0.68 $\pm$ 0.04	1.80 $\pm$ 0.07**	50.68	
	60	0.15 $\pm$ 0.01	0.27 $\pm$ 0.04	0.24 $\pm$ 0.05	0.33 $\pm$ 0.09	0.35 $\pm$ 0.06	1.09 $\pm$ 0.20**	70.10	
	100	0.34 $\pm$ 0.02	0.27 $\pm$ 0.02	0.19 $\pm$ 0.01	0.16 $\pm$ 0.04	0.19 $\pm$ 0.03	0.89 $\pm$ 0.01**	75.70	
<b>3</b>	45	0.27 $\pm$ 0.06	0.61 $\pm$ 0.05	0.63 $\pm$ 0.05	0.66 $\pm$ 0.06	0.62 $\pm$ 0.07	2.35 $\pm$ 0.24*	35.62	53.25
	60	0.33 $\pm$ 0.02	0.33 $\pm$ 0.03	0.33 $\pm$ 0.06	0.35 $\pm$ 0.06	0.40 $\pm$ 0.05	1.38 $\pm$ 0.12**	62.33	
	100	0.28 $\pm$ 0.01	0.18 $\pm$ 0.01	0.23 $\pm$ 0.01	0.23 $\pm$ 0.05	0.28 $\pm$ 0.05	0.90 $\pm$ 0.09**	75.30	
<b>4</b>	2.5	0.36 $\pm$ 0.04	0.79 $\pm$ 0.07	0.90 $\pm$ 0.05	0.90 $\pm$ 0.06	0.93 $\pm$ 0.05	3.24 $\pm$ 0.20*	11.23	6.68
	5.0	0.28 $\pm$ 0.02	0.62 $\pm$ 0.07	0.79 $\pm$ 0.08	0.82 $\pm$ 0.07	0.81 $\pm$ 0.07	2.78 $\pm$ 0.25**	23.80	
	7.5	0.18 $\pm$ 0.02	0.36 $\pm$ 0.05	0.49 $\pm$ 0.08	0.53 $\pm$ 0.09	0.63 $\pm$ 0.07	1.78 $\pm$ 0.26**	51.23	
	10.0	0.16 $\pm$ 0.05	0.26 $\pm$ 0.08	0.21 $\pm$ 0.04	0.19 $\pm$ 0.06	0.15 $\pm$ 0.04	0.81 $\pm$ 0.02**	77.67	

theless, the IC<sub>50</sub> values are an order of magnitude higher than those exhibited by doxorubicin.

## Discussion

Both TPA- and carrageenan-induced inflammation models have been frequently used to identify anti-inflammatory activity both of extracts of medicinal plants and of substances isolated from them.

Topical application of TPA induces a prolonged biphasic inflammatory response, with a first phase characterized by edema of the dermis and increased levels of TNF- $\alpha$ , followed by a secondary phase in which the enzyme COX-2 is induced, accompanied by the accumulation of pro-inflammatory cells and production of eicosanoids such as leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), among others (Sánchez and

Moreno, 1999; Murakawa *et al.*, 2006). Topical administration of compounds **1**, **2**, and **3** strongly inhibited TPA-induced edema in the same, or even higher, magnitude as the cyclooxygenase inhibitor indomethacin. Our results suggest that these compounds could interfere with the biosynthesis or activity of eicosanoids, since it has been demonstrated that 3 h after application of TPA, eicosanoids levels began to increase significantly in treated tissues and correlated with the magnitude of the inflammatory response (Murakawa *et al.*, 2006).

On the other hand, the subplantar injection of carrageenan induced an acute rat paw edema, which develops in three phases: an initial phase mediated by both histamine and 5-hydroxytryptamine, followed by a second kinin-mediated phase, notably the endogenous nonapeptide bradykinin produced by kallikrein (Di Rosa,

Table III. IC<sub>50</sub> values ( $\mu$ M) of chichiopenin (**1**), peniocerol (**2**), macedougallin (**3**), and doxorubicin (DOX) on human cancer cell lines. The data represents the mean  $\pm$  standard mean error (mean  $\pm$  SME) of three independent experiments. The IC<sub>50</sub> values were obtained by interpolation of plots (activity *vs.* log [ $\mu$ M]) from statistically significant data. ND, not determined.

Compound	IC <sub>50</sub> [ $\mu$ M]				
	U-251	PC-3	K-562	HCT-15	MCF-7
<b>1</b>	>200	>200	>200	>200	>200
<b>2</b>	24.73 $\pm$ 3.9	19.35 $\pm$ 0.45	10.37 $\pm$ 0.73	10.87 $\pm$ 2.36	10.17 $\pm$ 0.79
<b>3</b>	ND	20.78 $\pm$ 0.79	7.50 $\pm$ 0.1	17.30 $\pm$ 0.22	23.28 $\pm$ 0.17
DOX	0.09 $\pm$ 0.02	0.32 $\pm$ 0.02	0.28 $\pm$ 0.01	0.23 $\pm$ 0.01	0.14 $\pm$ 0.01

1972), and a final phase attributed to local production of prostaglandins (PG), whose synthesis is mediated mainly by COX-2 (Seibert *et al.*, 1994).

Although **2** and **3** at a dose of 100 mg/kg body weight significantly inhibited rat paw edema in all phases, suggesting a nonselective inhibitory effect against the mediators implied in carrageenan-induced edema, their anti-inflammatory effects were significantly more pronounced and prolonged against the third phase of the inflammation model, in which eicosanoids are implicated. Our results suggest that the anti-inflammatory effects observed with both compounds **2** and **3** are due to interference with eicosanoid mediators. But additional experiments are necessary to support this proposal.

On the other hand, only sterols **2** and **3** showed moderate cytotoxic activities against human cancer cell lines. Both compounds have the same  $3\beta,6\alpha$ -diol-cholest-8-ene core and are similar to oxysterols. Oxidized derivatives of cholesterol and phytosterols (especially diols and triols) have been reported to be strongly toxic to a number of cultured human tumoural and normal cell lines. This toxicity was previously demonstrated to occur via the induction of apoptosis in cells (Lordana

*et al.*, 2009; Koschutnig *et al.*, 2009; Hovenkamp *et al.*, 2008). Due to the structural similarity of sterols **2** and **3** with oxidized phytosterols, it can be supposed that the mechanism of their cytotoxicity is similar.

In conclusion, our study has demonstrated that a triterpene and two sterols isolated from *M. geometrizans* possess *in vivo* and *in vitro* activities such as suppressing inflammation and the viability of cancer cell lines. These novel bioactivities would provide greater insight into their medicinal value and contribute to the knowledge of garambullo. The anti-inflammatory activity displayed by compound **1**, and its presence in aerial parts of *M. geometrizans* (Cespedes *et al.*, 2005), could explain the use of the aerial parts of this species in traditional medicine.

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