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Investigación

Isolation and Chemical Transformations of Some Anti-inflammatory Triterpenes from *Salvia mexicana* L. var. *minor* Benth.

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Dedicated to Dr. Alfonso Romo de Vivar

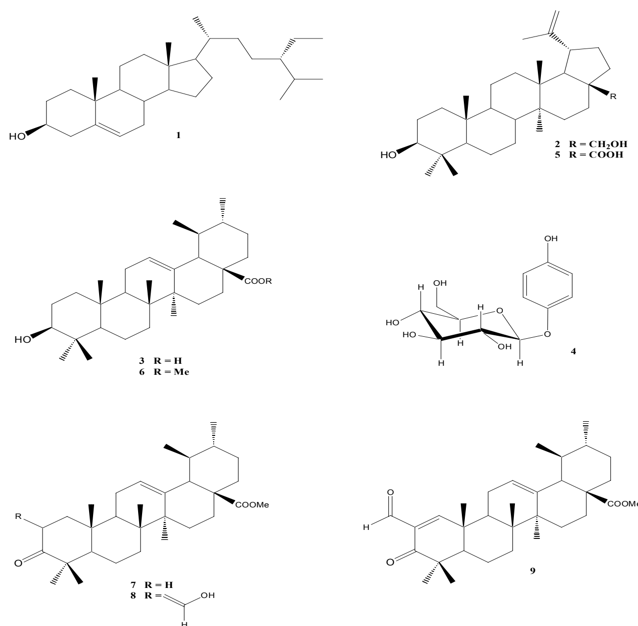
Abstract. The acetone and methanol extracts of aerial parts of *Salvia mexicana* L. var. *minor* showed anti-inflammatory and antioxidant properties in the TPA y DPPH models respectively. The chromatography of these extracts led the isolation of β-sitosterol, betulinol, betulinic acid, ursolic acid and arbutin. The presence of these triterpenes is in agreement with previos phytochemical studies of *Salvia*, however this is the first time that arbutin is isolated from a species of this genus. On the other hand, since none or the isolated compound showed antioxidant properties in the DPPH model, it can be inferred that minor compounds not isolated or synergism effects could account for the antioxidant properties of the extracts. It is known that some pentacyclic triterpene derivatives with an α,β-unsaturated carbonyl in the ring A showed a better nitric oxide synthase inhibition activity that the natural triterpenes. It was decided to synthesize the methyl ester of 2-formyl-3-oxo-urs-28-oic and 2-formil-3-oxo-urs-1-en-28-oic acids from ursolic acid and evaluate them using the DPPH and TPA models. The results showed that both compounds have anti-inflammatory activity, but only the 2-formyl-3-oxo-ursol-28-oic acid methyl ester was active in DPPH assay, which is in agreement with the proposed mechanism of this test. This is the first chemical study of *Salvia mexicana* L. var. *minor* (Benth).
Keywords: Triterpenos, *Salvia mexicana* var. *minor*, antiinflamatory activity, chemical transformations.

Resumen. Los extractos acetónico y metanólico de las partes aéreas de *Salvia mexicana* L. var. *minor*, mostraron poseer propiedades antiinflamatorias y antioxidantes en los modelos de TPA y DPPH, respectivamente. La cromatografía de estos extractos permitió el aislamiento de β-sitosterol, betulinol, ácido betulínico, ácido ursólico y arbutina. La presencia de estos triterpenos está de acuerdo con estudios previos de *Salvia*, sin embargo, es la primera vez que se aísla arbutina de una especie de éste género. Posiblemente las propiedades antiinflamatorias de los extractos se deban a la presencia del β-sitosterol y ácido ursólico, compuestos con probadas actividades antiinflamatorias. Por otro lado, es probable que las propiedades antioxidantes de estos extractos, se deban a la presencia de compuestos minoritarios o a efectos sinérgicos, ya que ninguno de los compuestos aislados fue activo en el modelo de DPPH. Datos recientes en la literatura señalan que algunos derivados de triterpenos pentacíclicos con una cetona α,β-insaturada en el anillo A, presentan una mayor inhibición de la enzima óxido nítrico sintetasa que los triterpenos naturales, por lo que se decidió obtener los ésteres metílicos de los ácidos 2-formil-3-oxo-urs-28-oico y 2-formil-3-oxo-urs-1-en-28-oico a partir del ácido ursólico, y evaluarlos en los modelos de DPPH y TPA. Los resultados muestran que ambos tienen propiedades antiinflamatorias, pero solo el éster metílico del ácido 2-formil-3-oxo-ursol-28-oico fue activo en el modelo de DPPH, resultado que está de acuerdo con el mecanismo asociado a esta prueba. Este es el primer estudio químico de la especie *Salvia mexicana* L. var. *minor* (Benth).
Palabras clave: Triterpenos, *Salvia mexicana* var. *minor*, actividad anti-inflamatoria, transformaciones químicas.

Introduction

Salvia is an important genus consisting of ca 900 species in the family Lamiaceae (formerly Labiatae). Some species of Salvia have been cultivated worldwide to be used in folk medicine and for culinary purposes [1]. The dried leaves of *S. officinalis* (sage) L., for example, is well known for their antioxidative properties used in the food processing industry but applicable also to the area of human health [2]. Studies on the chemical constituents of *Salvia* have been mainly confined to the diterpenoids and the tanshinones [3,4], and several reviews of these components have already been published [5, 6]. In addition, there are several reports on the biological activities of some species of this genus [7-9].

Most of the 500 species of Salvia found in Mexico, Central and South America belong to the Subgenus Jungia (formerly Calosphace) [10]. The species *Salvia mexicana* has been divide in two varieties: *S. mexicana* var. *major* Benth. and *S. mexicana* L. var. *minor* Benth. Acetone extract of the aerial parts from the former afforded narigenine and a *cis*-languidulane diterpenoid named salvimexicanolide [10]. In addition, from the chloroform extract of the aerial parts of this species β-sitosterol, betulinic acid and a triterpenic lactone called salviolide were isolated [11]. As a part of our ongoing systematic studies looking for bioactive compounds from Mexican species [12], we report in this paper the chemical study, the free radical scavenging and the anti-inflammatory activities of some extracts and isolates from *S. mexicana* L.



Scheme 1

var. *minor*. To our knowledge this is the first report on the chemical constituents as well as the free radical scavenging and anti-inflammatory activities of this species.

Results and Discussion

Flowers and leaves of this species were studied separately. The hexane, acetone and methanol extracts of each limb were obtained.

The method of DPPH free radical can be used to evaluate the antioxidant activity of specific compounds or extracts in a short time. It is based on the transformation of the stable free radical 1,1-diphenyl-2-picryl hydrazyl (DPPH) to α,α-diphenyl-β-picryl hydrazine by means of putative antioxidant compounds [13].

On the other hand, the TPA-induced edema test is a screening method to evaluate the ability of test compounds or extracts to prevent an inflammatory reaction in response to the edemogen.

The values of the anti-oxidative evaluation, by DPPH method of some extracts of *S. mexicana var. minor* are shown in Table 1. Those of the anti-inflammatory evaluation assessed by TPA-induced edema in mice are shown in Table 2.

According to these results, the acetone and methanol extracts from flowers, as well as the methanol from leaves were active in the DPPH assay. A different pattern is observed in the TPA assay where only the hexane and acetone extracts from the flowers were active.

In order to isolate the possibly involved components, all the active extracts were chromatographed. Then, from the flower hexane extract, β-sitosterol (**1**; 157 mg; 0.034 %) and betulinol (**2**; 10 mg; 0.002 %) were isolated, while ursolic acid (**3**; 3.612 g; 0.8 %) and arbutin (**4**; 563 mg; 0.12 %) were isolated from the flower acetone extract. Arbutin (**4**) was the only compound isolated from the flower and leave methanol extracts, 3.180 g (0.69 %) and 5.103 g (0.96 %) respectively. The presence of **1**, **2** and **3** in *S. mexicana* var. *minor* are in agreement with previous phytochemical reports of this genus. To our knowledge this is the first time that arbutin is isolated from species of Salvia genus.

All the isolated compounds were inactive in DPPH assay, thus indicating that activity of the extracts is due to the minor constituents not isolated or to a synergic effect. These results are in agreement with the assumed mechanism of this reaction, which postulate that the free radical scavenging activity of a compound in DPPH assay is attributed to their hydrogen donating ability [14].

On the other hand, the anti-inflammatory activities of the β-sitosterol (**1**) and ursolic acid (**3**) are well documented [15, 16] then the presence of **1** and **3** in this species could account for its anti-inflammatory activity (Table 2).

It is known that phorbol esters, such as TPA, induce skin inflammation and a hyperproliferative response with an infiltration of neutrofils [17]. It is also known that TPA stimulates PLA_2, and that consequently a release of arachidonic acid and prostaglandins occurs [18]. Although the mechanism by which TPA causes inflammation is not completely clear, it seems to be related in part to the release of eicosanoid mediators. Then inhibitors of cyclooxygenase and lipoxygenase, as ursolic acid, have proven activity in the TPA model [19, 20]. The high output of nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS), which is expressed in activated macrophages, plays an important role in host defense. However, excessive production of NO can also destroy functional normal tissues during acute and chronic inflammation. From a structure-activity study between 80 ursolic and oleanolic derivatives, the 2-cyano-3,12-dioxooleana-1,9-dien-28-oic, is

Table 1. Free radical scavenging activities of some extracts of *S. mexicana* L. var. *minor*.

Extracts	Concentration (ppm)	Reduction of DPPH (%)
Acetone (flowers)	10	12.64*
	100	84.47*
	1000	95.35*
Methanol (flowers)	10	17.74*
	100	92.86*
	1000	91.55*
Methanol (leaves)	10	13.55*
	100	90.76*
	1000	92.52*
Nordihydroguaiaretic acid (positive control)	7.17	94.69*

The results were analyzed by ANOVA. Statistical comparison were made between control group and the experimental groups using a Dunnet's test. $*p \leqslant 0.05$.

the product with the highest inhibitory activity against production of nitric oxide (NO) induced by interferon γ (IFN-γ) in mouse macrophages. In general, it was found that oleanolic and ursolic derivatives with a 1-en-3-one functionality in ring A have significant inhibitory activity against production of NO. Also it is known that ursolic acid up regulate iNOS and TNF-α expression through NF-κB transactivation in the resting macrophages [21]. Taking this information into account, it was decided to evaluate the free radical scavenging as well as the anti-inflammatory properties of both **8** and **9**. Compounds **8** and **9** were synthesized from **3**, according to the route illustrated in Scheme 1.

The results showed that only **8** was active as free radical scavenger (Table 3). However, both **8** and **9** showed almost the same activity as anti-inflammatory agents as ursolic acid (Table 4). These findings clearly indicate that the free radical scavenger activity of **8** is due to its hydrogen donating ability. On the other hand, in contrast to their inhibitory activity against the production of nitric oxide, the presence of unsaturated moieties in **8** and **9** are not relevant in terms of their anti-inflammatory activity, since both of them showed almost the same activity as ursolic acid.

Materials and Methods

General. The melting points (uncorrected) were determined on a Fisher-Johns apparatus. IR spectra were recorded as KBr pellets or liquid film on a Nicolet spectrophotometer model Magna 750. Mass spectra were recorded at 70 eV on a Jeol JMS-AX505HA mass spectrometer. NMR spectra were measured using Varian-Gemini 200 and Varian VXR-300 (^{1}H, 200 or 300 MHz, ^{13}C, 75 MHz) spectrometers in $CDCl_3$ or DMSO-d_6 with TMS as internal standard.

Plant material. Aerial parts of *S. mexicana* L. var. *Minor* Benth. were obtained from an orchard localized in Xahuen street in San Miguel Tlaixpan (Texcoco, Edo. de Mexico, Mexico) in 2002. A voucher specimen was deposited in the Herbario Nacional (MEXU-1054424).

Flowers (456 g) and leaves (531 g) were separately treated. Then, plant material was exhaustively extracted with *n*-hexane, acetone and MeOH, successively. From the flowers, 24.62 g (5.39 %, dry weight) of the hexane extract, 24.47 g (5.36 %, dry weight) of acetone extract and 73.26 g (16.06 %, dry weight) of methanol extract were obtained, while from the leaves, 13.19 g (2.48 %, dry weight), 29.33 g (5.52 %, dry weight) and 179.55 g (33.77 %, dry weight) were obtained respectively.

All the extracts were cromatographed using an open column packed with Si-gel (G- Altech, 0.2-0.5 mm, ASTM) in a 1:30 proportion to the extract and eluted with solvent mixtures of increasing polarity starting with hexane and ending with methanol.

Chromatography of hexane extract of flowers. From the hexane extract of the flowers a total of 65 fractions of 200 mL each, were collected. Fractions showing similar TLC data were combined, affording eight pools (F1-F8): F3 (fractions 24-26, eluted with hexane-EtOAc, 9:1), F4 (fractions 27-35, eluted with hexane-EtOAc, 8:2). β-sitosterol (**1**, 157 mg) was isolated from F3 and betulinol (**2**; 10 mg) from F4.

Chromatography of acetone extract of flowers. From the acetone extract of the flowers a total of 265 fractions of 200 mL each were collected. Fractions showing similar TLC data were combined, affording six pools (F1-F6): F3 (fractions 36-161, eluted with hexane-EtOAc, 7:3), F4 (fractions 162-237, eluted with hexane-EtOAc, 1:1). Ursolic acid (3; 3.682 g) was isolated from F3 and arbutin (4; 563 mg) from F4.

Chromatography of MeOH extract of flowers. From the MeOH extract of the flowers a total of 111 fractions of 200 mL each were collected. Fractions showing similar TLC data were combined, affording nine pools (F1-F9): F5 (fractions 50-77, eluted with EtOAc), F6 (fractions 78-89, eluted with EtOAc-MeOH, 9:1), F7 (fractions 90-93, eluted with EtOAc-MeOH, 7:3), F8 (fractions 94-102, eluted with EtOAc-MeOH, 1:1) and F9 (fractions 103-111, eluted with MeOH). Arbutin (4; 3.180 g) was isolated from F5-F8 pools.

Chromatography of hexane extract of leaves. When the extract was concentrated, a yellowish solid precipitate (235 mg), which was filtered and chromatographed. A total of 16 fractions of 50 mL each were collected. Fractions showing similar TLC data were combined, affording three pools (F1-F3): F1 (fractions 1-4, eluted with hexane), F2 (fractions 5-8, eluted with hexane-EtOAc, 9:1) and F3 (fractions 9-16, eluted with hexane-EtOAc, 8:2). Betulinic acid (**5**; 84 mg; 36%) was isolated from F3. The remanent extract (13.19 g) afforded a total of 22 fractions of 200 ml each. Fractions showing similar TLC data were combined, affording six pools (F1-F6): F3 (fractions 7-12, eluted with hexane-EtOAc, 7:3), F4 (fractions

Table 2. Anti-inflammatory activities of some extracts of *S. mexicana* L. var. *minor*.

Extracts	Edema (mg, average SE)	Inhibition (%)
Control (methanol)	15.47 ± 0.32	—
Hexane (flowers)	4.77 ± 0.50	69.17*
Acetone (flowers)	5.60 ± 0.91	63.79*
Methanol (flowers)	13.70 ± 0.31	11.42
Methanol (leaves)	13.10 ± 1.07	15.0
Indomethacin (positive control)	1.07 ± 0.03	91.35*

All the extracts were tested at 1 mg / ear doses. The results were analyzed by ANOVA. Statistical comparison were made between control group and the experimental groups using a t student test. $*p \leqslant 0.01$

13-14, eluted with hexane-EtOAc, 1:1), F5 (fractions 15-19, eluted with hexane-EtOAc, 3:7) and F6 (fractions 20-22, eluted with EtOAc). β-sitosterol (**1**; 686 mg) was isolated from F3-F6 pools.

Chromatography of acetone extract of leaves. From the acetone extract of the leaves a total of 33 fractions of 200 mL each, were collected. Fractions showing similar TLC data were combined, affording nine pools (F1-F9): F3 (fractions 8-10, eluted with hexane-EtOAc, 7:3), F4 (fractions 11-15, eluted with hexane-EtOAc, 1:1), F5 (fractions 16-18, eluted with hexane-EtOAc, 3:7), F6 (fractions 19-21, eluted with EtOAc), F7 (fractions 22-25, eluted with EtOAc-MeOH, 7:3), F8 (fractions 26-29, eluted with EtOAc-MeOH, 1:1) and F9 (fractions 30-33, eluted with MeOH). Ursolic acid (3; 4.210 g; 14.35%) was isolated from F3-F5 pools and arbutin (**4**; 2.103 g; 7.168%) was isolated from F5-F8 pools.

Chromatography of MeOH extract of leaves. The MeOH extract was partitioned between *n*-butanol and CH_2Cl_2. It afforded the *n*-butanol extract (46.16 g), which was chromatographed yielding a total of 22 fractions of 200 mL each. Fractions showing similar TLC data were combined, affording nine pools (F1-F9): F6 (fractions 9-13, eluted with EtOAc-MeOH, 9:1), F7 (fractions 14-15, eluted with EtOAc-MeOH, 7:3), F8 (fractions 16-18, eluted with EtOAc-MeOH, 1:1) and F9 (fractions 19-22, eluted with MeOH). Arbutin (**4**; 5.103 g, 4.3 %) was isolated from F6-F9 pools.

Ursolic acid methyl ester (6). A solution of ursolic acid (1 g, 2.19 mmol) in a mixture of ether / MeOH (50 mL) was cooled down to 0 °C in an ice-bath. Ethereal diazomethane was added until permanent yellow color was obtained. After 24 h the solvent was removed by distillation under low pressure to give ursol-28-oic acid methyl ester (**6**; 758 mg; 1.61 mmol; 73.5 % yield).

3-oxo-urs-28-oic acid methyl ester (7). To a solution of **6** (750 mg, 1.6 mmol) in acetone (10 ml) was added an excess of Jones's reagent at 0 °C while stirring. The reaction course was followed by TLC. After 55 min, the excess of Jones's was destroyed by addition of MeOH, and then the reaction mixture was diluted with H_2O (30 mL). Extraction with CH_2Cl_2 (4 × 10 mL), drying (Na_2SO_4), filtration and evaporation of the solvent gave a residue, which by crystallization from hexane-EtOAc afforded **7** (321 mg; 0.7 mmol; 43.7 % yield). Mp 182-184°C, IR (KBr) ν_{max}: 2935, 2867, 1726, 1695, 1459, 1380 and 1142 cm^{-1}. EIMS 70eV m/z: 468 (M^+, $C_{31}H_{48}O_3$), 453, 419, 407, 262, 249, 203 and 189. ^1H NMR 200 MHz $CDCl_3$ δ: 5.27 (1H, m, H-12), 3.61 (3H, s, OMe), 2.60 (1H, d, H-18), 2.25 (2H, m, H-2), 1.08 (3H, s), 1.04 (6H, s), 1.06 (3H, d, J=8 Hz), 0.95 (3H, s), 0.85 (3H, d, J=7Hz), 0.79 (3H, s).

2- Formyl-3-oxo-urs-28-oic acid methyl ester (8). To a solution of 0.7 mmol of **7** in 7 mL of dry pyridine, held under nitrogen, was added 1.5 mL (18.7 mmol) of ethyl formate (distilled from phosphorus pentoxide) followed by 1 mL of a solution of 294 mg (13.3 mmol) of sodium in 6 mL of absolute methyl alcohol. The resulting solution was then kept at room temperature under nitrogen overnight. The reaction was evidenced by the appearance of a deep color and / or the formation of an insoluble precipitate. The mixture was poured into a cold solution of 16 mL of glacial acetic acid in 150 mL of water, and the resulting precipitate was extracted with CH_2Cl_2. The organic layer was washed with water and then extracted with 3 × 100 mL of 2 % potassium hydroxide solution. The combined basic extract were washed with ether and acidified with 10 mL of glacial acetic acid. Extraction of the aqueous layer with CH_2Cl_2 in the usual manner, afforded 2-formyl-3-oxo-urs-28-oic methyl ester (**8**; 226 mg; 0.46 mmol; 70 % yield).

Reddish viscous liq. IR ($CHCl_3$) ν_{max}: 2925, 2869, 1725, 1636, 1587, 1455, 1360 and 1147 cm^{-1}. EIMS 70eV m/z: 496 (M^+, $C_{32}H_{48}O_4$), 481, 478, 437, 421, 262, 249, 233, 203 and 189. ^1H NMR 200 MHz $CDCl_3$ δ: 14.91 (1H, s, OH chelated), 8.57 (1H, s, H-23), 5.29 (1H, m, H-12), 3.61 (3H, s, OMe), 2.32 (1H, d, H-18), 1.25, 1.19, 1.11, 1.09, 0.8 (3H, s, each), 0.93 (3H, d, J=8 Hz), 0.86 (3H, d, J=7Hz).

Table 3. Free radical scavenging activities of **8** and **9**.

Compound	Concentration (ppm)	Reduction of DPPH (%)
2- Formyl-3-oxo-	10	12.69*
urs-28-oic acid	100	40.87*
methyl ester (8)	1000	79.43*
2- Formyl-3-oxo-	10	N. A.
urs-1-en-28-oic	100	N. A.
acid methyl ester (9)	1000	N. A.

The results were analyzed by ANOVA. Statistical comparison were made between control group and the experimental groups using a Dunnet's test. $*p \leqslant 0.05$, N. A. = Not active.

Table 4. Anti-inflammatory activities of **8** and **9**.

Compound	Edema (mg, average SE)	Inhibition (%)
Control (EtOAc)	11.80 ± 0.045	-
2- Formyl-3-oxo-urs-28-oic acid methyl ester (**8**)	3.03 ± 0.86	74.29*
2- Formyl-3-oxo-urs-1-en-28-oic acid methyl ester (**9**)	3.03 ± 0.86	74.29*

All the compounds were tested at 1 mg / ear doses. The results were analyzed by ANOVA. Statistical comparison were made between control group and the experimental groups using a t student test. *$p \leq 0.01$. The reported % of inhibition of ursolic acid is 74.4 % at 1 mg/ear doses [16].

2- Formyl-3-oxo-urs-1-en-28-oic acid methyl ester (**9**). PhSeCl (120 mg) was dissolved in 12 mL of CH_2Cl_2 and cooled to 0 °C and 0.06 g (40 µl) of pyridine was added. After 15 min, 0.2 g of **8** in 3 mL of CH_2Cl_2 was added and the mixture was stirred for 15 min more. The CH_2Cl_2 solution was extracted with two 5 mL portions of 10 % HCl and cooled back to 0 °C, at which time 0.1 mL of 30 % H_2O_2 was added. An additional 0.1 mL of 30 % H_2O_2 was added after 10 min and again after 20 min. After an additional 10 min, 0.5 mL of H_2O was added and the CH_2Cl_2 layer was separated and washed with 5 mL of saturated $NaHCO_3$. After being dried over Na_2SO_4, the solution was filtered and the solvent evaporated under vacuum to yield **9** (53 mg; 0.11 mmol; 24 % yield). Reddish viscous liq. IR ($CHCl_3$) ν (cm^{-1}): 2921, 2858, 2721, 1719, 1672, 1604, 1455, 1379, 1224 and 1110. EIMS 70eV m/z: 494 (M^+, $C_{32}H_{46}O_4$), 479, 476, 435, 419, 314, 262, 249, 233, 203, 189, 158, 133 and 117. ^1H NMR (200 MHz, $CDCl_3$): 10.01 (1H, s, COH), 7.80 (1H, s, H-1), 5.38 (1H, m, H-12), 3.62 (3H, s, OMe), 2.28 (1H, d, H-18), 1.25, 1.18, 1.09, 0.96, 0.87 (3H, s, each), 1.17 (3H, d, J = 7 Hz), 0.87 (3H, d, J = 8Hz).

Free radical scavenging Activity. DPPH assay was performed essentially according to the modified method of Cottele. Reaction mixture containing different concentrations of test samples in DMSO and 100 mM DPPH ethanol solution in 96-well microliter plates, were incubated at 37 °C for 30 min, and subsequently the absorbencies were measured at 515 nm in a microplate reader Elx 808. Measurements were performed in triplicate in at least three independent experiments. The % inhibition of each compound was determined by comparison with a DPPH ethanol blank solution [22]. The results were analyzed by ANOVA. Statistical comparisons were made between control group and the experimental groups using Dunnet's test.

Animals. Male CD-1 mice, weighing 20-25 g each were used. Instituto de Fisiología Celular, Universidad Nacional Autónoma de México provided the experimental animals. All animals were held under standard laboratory conditions in the animal house (temperature 27 ± 1 °C). They were fed laboratory diet and water *ad libitum*. All experiments were carried out using 4-8 animals per group.

TPA-induced edema model. Effects of the test substances on TPA-induced ear edema in mice were studied as described by De Young [17] with slight modifications. The substances (1 mg / ear) were applied topically. A solution of TPA (2.5 µg) in EtOH (10 µL) was applied topically to both faces (5 µL each face) of the right ear of the mice, 10 min after the test substances were applied (10 µL each face). The left ear received ethanol (10 µL) first, and 20 µL of the respective solvent subsequently.

Four hours later the mice were killed by cervical dislocation. A 7-mm diameter plug was removed from each ear. The swelling was assessed as the difference in weight between right and left ear plugs [19]. Inhibition of edema (EI, %) was calculated by the equation:

EI (%) = 100 – [B × 100 / A], with A = edema induced by TPA alone, and B = edema induced by TPA plus sample.

Data were expressed as the mean SEM of 4-8 mice. All the extracts and compounds were tested at 1 mg / ear doses. The results were analyzed by ANOVA. Statistical comparisons were made between control group and the experimental groups using a t student test. *$p < 0.01$.

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