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## Investigación

# Anti-inflammatory Active Compounds from the *n*-Hexane Extract of *Euphorbia hirta*

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*Dedicated to the memory of Dr. Lydia Rodríguez-Hahn*

**Resumen.** El extracto hexánico de las partes aéreas de *Euphorbia hirta* L. (Euphorbiaceae), así como sus principales constituyentes triterpénicos;  $\beta$ -amirina (**1**), 24-metilencicloartenol (**2**), y  $\beta$ -sitosterol (**3**) fueron evaluados como agentes anti-inflamatorios en el modelo del TPA. Tanto el extracto como los triterpenos aislados mostraron actividad anti-inflamatoria significativa y dependiente de la dosis. Con el fin de investigar si los compuestos aislados poseen efectos aditivos en sus propiedades antiflogistas, se realizaron evaluaciones de algunas combinaciones duales y triples de los triterpenos. Los resultados obtenidos mostraron que algunas de estas combinaciones presentaron valores mas altos de inhibición del edema que aquellos correspondientes a cada uno de los triterpenos por separado.

**Palabras clave.** *Euphorbia hirta*, Euphorbiaceae, triterpenos,  $\beta$ -amirina, 24-metilencicloartenol,  $\beta$ -sitosterol, actividad anti-inflamatoria.

**Abstract.** The *n*-hexane extract of the aerial parts of *Euphorbia hirta* L. (Euphorbiaceae) and its main triterpenes,  $\beta$ -amyrin (**1**), 24-methylencycloartenol (**2**), and  $\beta$ -sitosterol (**3**) were evaluated for anti-inflammatory effects in mice. Both the extract and the triterpenes exerted significant and dose-dependent anti-inflammatory activity in the TPA-induced ear model. Some dual and triplet combinations of the triterpenes were tested as anti-inflammatory agents. The results showed that the combinations were higher in magnitude as anti-inflammatory agents than those produced by each triterpene alone.

**Keywords.** *Euphorbia hirta*, Euphorbiaceae, triterpenes, 24-methylencycloartenol,  $\beta$ -amyrin,  $\beta$ -sitosterol, anti-inflammatory activity.

## Introduction

*Euphorbia hirta* (Euphorbiaceae) is a pantropic herbaceous wild plant which has been widely used in several countries as antidiarrheic, antidiuretic and expectorant, and also as a remedy for bronchitis, asthma, intestinal ailments of children and for the treatment of various skin diseases [1]. In México, it grows in the Sierra Norte de Puebla region, where is used by the native people under the names of hierba de la golondrina or sabañonxihuit for the treatment of cancer and skin diseases [2]. It has been published the analgesic, antipyretic and anti-inflammatory activities of a lyophilized aqueous extract of *E. hirta* [3]. In this report we describe the anti-inflammatory activity of a *n*-hexane extract of *E. hirta* as well as the isolation and identification of the compounds responsible of the anti-inflammatory effect on the 12-*O*-tetradecanoyl phorbol acetate (TPA)-induced mouse ear edema test [11]. Furthermore, in order to investigate a possible addition of their effects, several combinations of the active principles isolated also were tested.

## Discussion

The three triterpenes:  $\beta$ -amyrin (**1**), 24-methylencycloartenol (**2**), and  $\beta$ -sitosterol (**3**), isolated from *E. hirta* were examined for their inhibitory effects on TPA-induced inflammation in mice. The inhibitory effects were compared with those of the commercially available anti-inflammatory drug indomethacin (table 1). All of the triterpene alcohols markedly inhibited the TPA-induced inflammation, within a range of 0.1-0.3 mg per ear at the 50% inhibitory dose, being  $\beta$ -amyrin (**1**) the most potent, since it exhibited the strongest inhibitory effect (0.12 mg per ear). These data are in complete agreement with those published for the anti-inflammatory activities of **1**, **2** and **3**, tested in other models, such as carageenin- and ethyl phenyl propiolate-induced edema in rats [4-6]. Based on these results, it was shown that the skeleton of the tested compounds has no influence on the anti-inflammatory activity, since the three compounds tested were active.

The presence of **1**, **2** and **3** in *E. hirta* leads to the question if their affect could be additive. Obviously, in the *n*-hexane

**Table 1.** Inhibitory effects by *E. hirta* extract, compounds **1-3** and indomethacin.

Compound	ED <sub>50</sub> (mg/ear)	V <sub>max</sub>	K <sub>m</sub>
<i>n</i> -hexane extract	0.70 ( <i>r</i> = 0.96)		
β-amyirin ( <b>1</b> )	0.12 ( <i>r</i> = 0.998)	13.64	1.23 × 10 <sup>-1</sup>
24-methylen-cycloartenol ( <b>2</b> )	0.26 ( <i>r</i> = 0.987)	9.56	8.72 × 10 <sup>-2</sup>
β-sitosterol ( <b>3</b> )	0.14 ( <i>r</i> = 0.989)	8.33	5.07 × 10 <sup>-3</sup>
indomethacin	0.20 ( <i>r</i> = 0.989)		

*r*: correlation coefficient

extract their activities are diminished, since it showed the higher ED<sub>50</sub> value. Probably this behavior could be due to the presence of other components in the extract. Then, in order to answer the formulated question, several dual and triplet combinations of pure triterpenes were tested. The results are shown in table 2.

Taking into account that the triterpenes tested exert their anti-inflammatory effects by interacting reversibly with one population of receptors, and that their affect is proportional to the number of receptors occupied, we used the Seegers's methodology [7] to estimate the activities of the different combinations (V calculated), and compare these values with those experimentally observed (V experimental).

The results shown in table 2 clearly indicate that almost all the dual combinations except one were additive, at least on the concentrations used; among the two triplet combinations only one showed an additive profile. On the other hand, all the dual combinations at 0.25 mg per ear, presented higher values than a simple addition of effects of each triterpene. Since inhibitors of TPA-induced inflammation have demonstrated their inhibitory activities against tumor promotion, these triterpenoids, specially **1**, and the dual combinations described here are expected to be potent anti-tumor agents [6].

## Experimental section

**Materials and Methods. Animals.** Groups of six male CD-1 mice weighing 25-30 g were used. The animals were provided by Facultad de Medicina, Universidad Nacional Autónoma de México. All animals were maintained in suitable nutritional and environmental conditions through the experiments.

**Plant material.** *E. hirta* was collected in San Nicolás de los Ranchos Puebla, in September of 1994. A voucher has been deposited at the Herbario Nacional, Universidad Nacional Autónoma de México. (MEXU 1516) Air dried and powdered whole plant was extracted successively with hexane and MeOH at room temperature. The solvents were removed under reduced pressure.

Chromatography of the bioactive *n*-hexane extract (17.4 g) on Si gel (250 g) using *n*-hexane with increasing propor-

tions of EtOAc gave a mixture of β-amyirin and 24-methylen-cycloartenol, which was separated by fractional crystallization from acetone and methanol, affording 232 mg of β-amyirin and 59 mg of 24-methylen cycloartenol. From the subsequent fractions 525 mg of β-sitosterol were obtained.

The identities of β-amyirin, 24-methylen-cycloartenol and β-sitosterol were confirmed by comparison of their physical and spectroscopic properties (IR, MS, <sup>1</sup>H and <sup>13</sup>C-NMR) with the data reported in the literature [8-10].

**Anti-inflammatory activity.** The 12-*O*-tetradecanoyl phorbol acetate (TPA) ear was performed as already described [11]. Groups of 6 male CD-1 mice (25-39 g) were anaesthetized with Ketalar® and 10 μl of an ethanolic solution containing the irritant (25 μg of TPA, Sigma) and the appropriate amount of the substances under testing were applied to the inner surface of the right ear of mice (surface: about 1 cm<sup>2</sup>), the left ear remaining untreated. Control animals received only the irritant and vehicle. Four hours later the animals were killed by cervical dislocation and plug (9 mm in diameter) was removed from both the treated and the untreated ear. The difference in weight between the two plugs was taken as a measure of the edematous response [12].

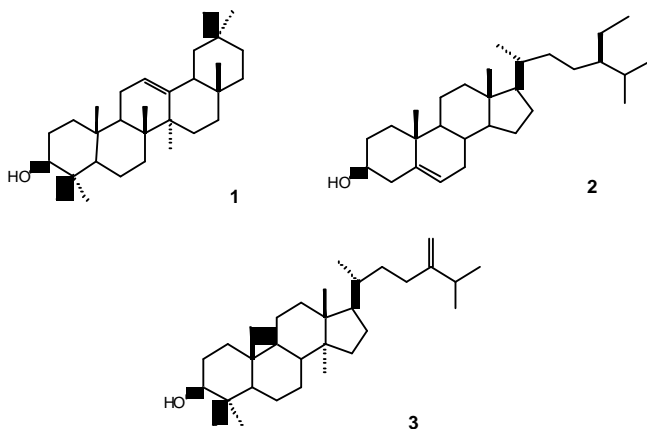
**Statistical analysis.** All data are represented as mean ± standard error mean (SEM) or as percentages. The analysis of variance (ANOVA) and the Dunnet's test were used to compare several groups with a control. P values of 0.05 or less were considered significant. ED<sub>50</sub> values were evaluated according to a linear regression model, plotting log dose versus percentage or activity. Assuming that the drugs tested all exert their anti-inflammatory effects by interacting reversibly with one population of receptors, and that their effects is proportional to the number of receptors occupied, the relationships between the effect observed, V, and the dose of the drug given, A, is presented by equation 1

$$V = V_{\max} \cdot A / K + A \quad (1).$$

**Table 2.** Data of inhibition for the combinations of compounds **1-3**.

Combination	dose (mg/ear)	Inhibition (%)	V (calculated)	V (experimental)
<b>2 + 3</b>	0.025	28.13*	6.61	4.14
	0.25	82.93**	8.25	12.20
<b>1 + 2</b>	0.025	26.69*	3.6	3.88
	0.25	87.53**	9.39	12.88
<b>1 + 3</b>	0.025	20.05	0.76	2.92
	0.25	65.04**	8.56	9.56
<b>1 + 2 + 3</b>	0.017	21.14	7.28	3.12
	0.17	56.64	6.45	8.36

\* p < 0.05; \*\* p < 0.01

**Figura 1.**

The parameters which characterize this equation, and which can be estimated from the observations are  $V_{\max}$ , the maximum effect which is theoretically attained when all receptors are occupied by an infinite dose of the drugs, and  $K$ , the dissociation constant for the drug-receptor complex.

In the present study estimates of  $V_{\max}$  and  $K$  were determined by a regression linear transformation of equation 1. In order to obtain response metameters, ( $V_{\text{experimental}}$ ), which increase with increasing doses of the drugs, the difference between the mean response observed in the vehicle-treated mice and the response observed in the drug-treated mice was taken.

Under the assumptions stated earlier and under the assumption that the drugs act additively, the activities ( $V_{\text{calculated}}$ ) of dual and triple drug combinations may be predicted by the following equations.

$$V_{ab} = A \cdot V_{\max} \cdot a \cdot K_b + B \cdot V_{\max} \cdot b \cdot K_a / K_a \cdot K_b + A \cdot K_b + B \cdot K_a$$

$$V_{abc} = A \cdot V_{\max} \cdot a \cdot K_b \cdot K_c + B \cdot V_{\max} \cdot b \cdot K_a \cdot K_c + C \cdot V_{\max} \cdot c \cdot K_a \cdot K_b / K_a \cdot K_b \cdot K_c + A \cdot K_b \cdot K_c + B \cdot K_a \cdot K_c + C \cdot K_a \cdot K_b$$

In which for drugs a, b and c respectively:  $K_a$ ,  $K_b$  and  $K_c$  = the dissociation constant of the drug-receptor complex.  $V_{\max} \cdot a$ ,  $V_{\max} \cdot b$  and  $V_{\max} \cdot c$  = the maximum effect. A, B and C = the dose.

$V_{ab}$  and  $V_{abc}$  are the effects of the dual and triple combinations, respectively. The differences between the measured effects of each combination and the expected effects were tested at the 5% level of significance using the estimate of the standard error of the mean response observed. The effect of a combination is classified as additive when the mean response observed does not differ significantly from its expectation.

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