

## Research Article

# Anti-Inflammatory Activity of Triterpenes Isolated from *Protium paniculatum* Oil-Resins

Patrícia D. O. de Almeida,<sup>1</sup> Ana Paula de A. Boleti,<sup>1</sup> André Luis Rüdiger,<sup>2</sup>  
Geane A. Lourenço,<sup>3</sup> Valdir Florêncio da Veiga Junior,<sup>2</sup> and Emerson S. Lima<sup>1</sup>

<sup>1</sup>Laboratório de Atividade Biológica, Faculdade de Ciências Farmacêuticas, Universidade Federal do Amazonas (UFAM), Avenida Gen. Rodrigo Otavio, No. 6200, 69077-000 Manaus, AM, Brazil

<sup>2</sup>Instituto de Ciências Exatas, Departamento de Química, Universidade Federal do Amazonas, Avenida Gen. Rodrigo Otavio, No. 6200, 69077-000 Manaus, AM, Brazil

<sup>3</sup>Laboratório de Farmacologia, Departamento de Ciências Fisiológicas, Instituto de Ciências Biológicas, Universidade Federal do Amazonas, Avenida Gen. Rodrigo Otavio, No. 6200, 69077-000 Manaus, AM, Brazil

Correspondence should be addressed to Emerson S. Lima; [eslima@ufam.edu.br](mailto:eslima@ufam.edu.br)

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*Protium* is the main genus of the Burseraceae family and one of the most common genera in South America, with an important species called “breu.” Gum and oil-resins of this species are used as tonic and stimulant and for the treatment of ulcers and inflammation. The present study aims to isolate and investigate the anti-inflammatory activity of triterpene compounds isolated from oil-resin of *Protium paniculatum*. The pentacyclic triterpenes  $\alpha,\beta$ -amyrin, acetylated  $\alpha,\beta$ -amyrin,  $\alpha,\beta$ -amyrone, and brein/maniladiol did not alter the viability of murine J774 macrophages ( $IC_{50} > 20 \mu\text{g/mL}$ ), with the exception of mixture of brein/maniladiol which showed moderate cytotoxic activity. Also it was observed that compounds at  $10 \mu\text{g/mL}$  inhibited more than 80% of production of  $\text{NO}^*$ , although only  $\alpha,\beta$ -amyrin was able to inhibit the production of  $\text{TNF-}\alpha$  ( $52.03 \pm 2.4\%$ ). The compounds inhibited the production of IL-6 and induced the production of IL-10 in murine J774 macrophages stimulated by LPS.  $\alpha,\beta$ -Amyrone inhibited the expression of COX-2 and also inhibited the formation of paw or ear edema in rats and mice, having a quick and immediate effect. This study may provide the basis for future investigations on the therapeutic role of  $\alpha,\beta$ -amyrone in treating inflammation.

## 1. Introduction

Inflammation is a defense reaction of the body, and a local response of living tissues to injury in mammals aimed at eliminating or limiting the spread of an injurious agent [1]. The use of medicinal plants or their active components is becoming an increasingly attractive approach for treating various inflammatory disorders [2]. The origin of the anti-inflammatory properties of various phytomedicines can be explained by the presence of substances such as flavonoids, alkaloids, tannins, saponins, anthraquinones, triterpenoids, and other constituents which act as inhibitors of molecular targets and proinflammatory mediators in inflammatory responses [3].

Triterpenoids are constituents that have aroused great interest in recent years due to their pharmacological potential, with numerous therapeutic activities, such as anticancer, anti-inflammatory, antiviral, antibacterial, antifungal, antidiuretic, giardicidal, and acetylcholinesterase inhibitors [4–6].  $\alpha,\beta$ -Amyrin is a pentacyclic triterpene and constitutes the main component of the resin *Protium* sp. Furthermore, other compounds have been isolated from the resin *Protium* sp., and little is known about its anti-inflammatory properties [7].

In the last 10 years, studies have shown systemic anti-inflammatory action of  $\alpha,\beta$ -amyrin associated with inhibition of the transcription factor  $\text{NF-}\kappa\text{B}$ , inhibition of COX-2, and the production of proinflammatory cytokines [7, 8]. It was recently shown that  $\delta$ -amyrone, a constituent which is

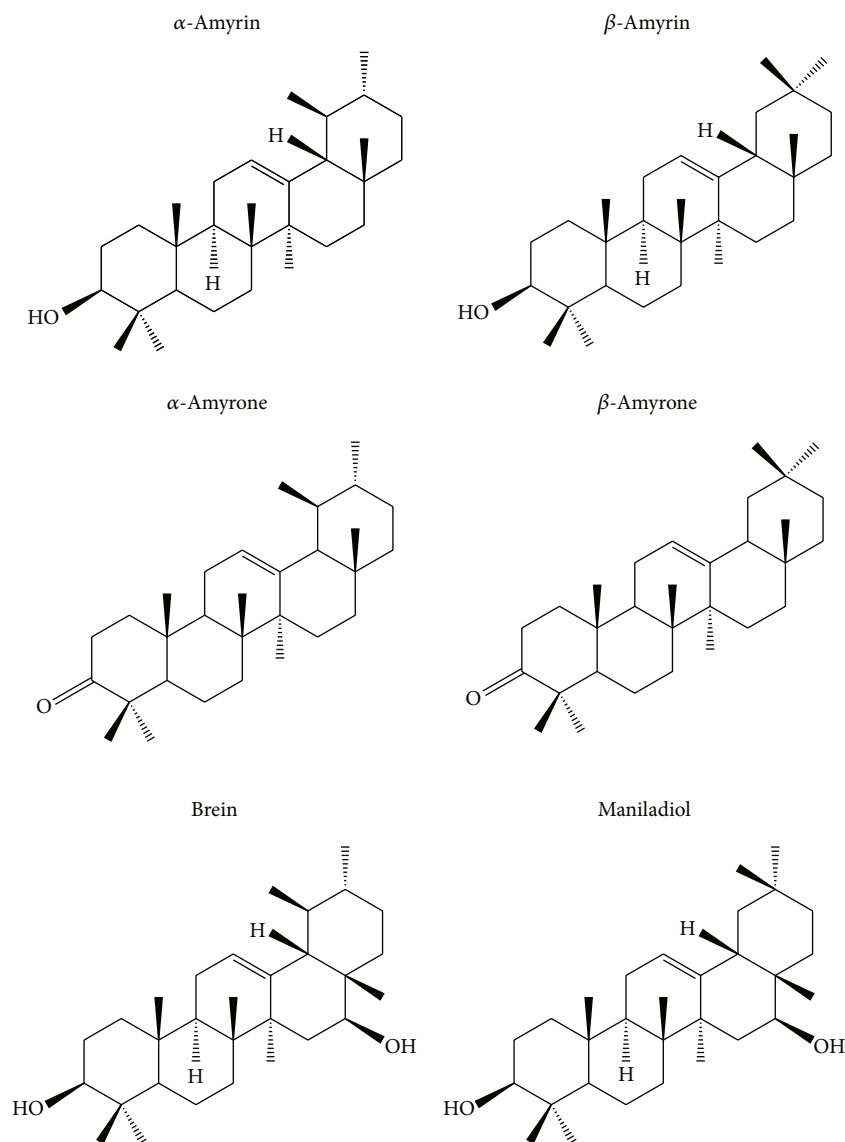


FIGURE 1: Chemical structure of the compounds isolated from *Protium* spp. resin.

extracted and separated from of *Sedum lineare* Thunb., inhibited the ear edema in xylene-induced mouse ear edema and also decreased the level of nitric oxide (NO), prostaglandin E2 (PGE2), interleukin-6 (IL-6), and leukocyte numbers in acetic acid-induced peritonitis *in vivo* [9].

Based on evidence that *Protium* species accumulate, mainly tetracyclic and pentacyclic triterpenoids were isolated from *Protium paniculatum* mixtures of triterpenoids brein/maniladiol and  $\alpha,\beta$ -amyrin [10]. Synthetic derivatives, acetylated amyrin and  $\alpha,\beta$ -amyrone, were obtained from  $\alpha,\beta$ -amyrin. This study aims to evaluate the anti-inflammatory activity of triterpenoids cited, considering that there are few studies in the literature showing possible biological activity.

## 2. Methods

**2.1. Plant Material.** Oleoresin of *Protium paniculatum* var. *modestum* (PPM) was collected in Ducke Forest Reserve, 26,

Highway AM-010, Km 26, Manaus, AM, Brazil. The species was catalogued by the Flora Project of Ducke Reserve of the National Institute of Amazonian Research (Instituto Nacional de Pesquisas da Amazônia, INPA) and it was identified by Burseraceae taxonomists: Ph.D. Douglas C. Daly and Ph.D. José Eduardo L. S. Ribeiro. Voucher was deposited in the New York Botanical Garden (1413737) and the INPA herbarium (191303).

**2.2. Extraction and Isolation.** Mono- and dihydroxylated triterpenes were isolated from the insoluble material which resulted from PPM oleoresin hexanic extraction (Figure 1). Samples were solubilized with ethyl acetate (1008.9 mg); this material was submitted to gravity chromatography over silica gel (mesh: 70–230,  $\varnothing_{\text{column}}$ : 2.5 cm, and  $m_{(\text{SiO}_2)}$ : 40 g) using dichloromethane (DCM) and ethyl acetate with gradient polarity. Ketones and acetyl derivatives were obtained from the amyirin mixture by chemical reactions. The data relating

to isolations, identification, and reactions are described in Supplementary Material available online at <http://dx.doi.org/10.1155/2015/293768>.

**2.3. Cell Culture.** The murine macrophage cell line J774 was kindly provided by Dr. Leda Quercia Vieira (Laboratory of Gnotobiology and Immunology, UFMG, MG, Brazil) and was cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub> in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin (Invitrogen). Lipopolysaccharide (LPS) was prepared as a 1 mg/mL stock solution in sterile water and stored at -20°C. The triterpene compounds were added along with treatment with LPS.

**2.4. Animals.** Female Wistar rats (200 g each) and Swiss mice (25–35 g) were previously housed in standard polypropylene cages under controlled conditions of temperature (22 ± 2°C) and 12 h light/dark cycle, with free access to diet and water. Mice were allowed to adapt to laboratory for at least 1 h before testing. All experimental procedures using animals were performed following international guidelines and approved by the Institutional Animal Ethics Committee (number 002/2013 CEEA/UFAM).

**2.5. Cell Viability Assay.** The cytotoxicity of triterpenes compounds to the murine macrophage cell line J774 was determined by the Alamar Blue method as described by Nakayama and coworkers [11]. Briefly, adherent cells (5 × 10<sup>3</sup> cells/well) were grown in 96-well tissue culture plates and exposed to the triterpenes: α,β-amyrin, acetylated α,β-amyrin, α,β-amyrone, and brein/maniladiol (2.5; 5; and 10 µg/mL) for 24, 48, and 72 h. After incubation, the Alamar Blue solution (10 µL of 0.4% Alamar Blue (resazurin) in PBS) was added and the cells were incubated for 3 h at 37°C. Fluorescence was measured (excitation at 545 nm and emission at 595 nm) and expressed as a percentage of the cells in the control after background fluorescence was subtracted. Doxorubicin (5 µg/mL) was used as a positive control of cell death. The assays were performed in triplicate.

**2.6. NO<sup>•</sup> Production Assay.** Nitric oxide (NO<sup>•</sup>) production by J774 cells was assayed by measuring the accumulation of nitrite in the culture medium using Griess reaction [12]. Briefly, after incubation of the cells (1 × 10<sup>6</sup> cells/mL) with triterpenes compounds in different concentrations of 2.5; 5; and 10 µg/mL, cells were incubated for 24 h with LPS (1 µg/mL), at 37°C in a 5% CO<sub>2</sub> incubator. Nitric oxide was measured as NO<sub>2</sub><sup>-</sup> in culture supernatant by reaction with Griess reagent. Absorbance of the reaction product was determined at 560 nm using a microplate reader (DTX 800, Beckman). Sodium nitrite was used as a standard to calculate nitrite.

**2.7. Measurement of Cytokines.** Macrophage cells (1 × 10<sup>6</sup> cells/mL) were incubated with the triterpenes compounds in a concentration of 10 µg/mL and then stimulated with 1 µg/mL of LPS. The culture supernatants were collected after

24 h of LPS stimulation. The levels of cytokines in the culture media were measured by flow cytometry (BD Cytometric Bead Array, CBA, Mouse Inflammation kit) according to the manufacturer's instructions.

**2.8. Western Blot Analysis.** J774 cells were cultured in 96-well plates (1 × 10<sup>6</sup> cells per well) and incubated with α,β-amyrone in concentrations of 2.5; 5; and 10 µg/mL. Cells were stimulated with LPS (1 µg/mL) and incubated for 24 hours. After incubation, cells were washed with phosphate buffered saline and lysed with lysis buffer (Tris-HCl [50 mM, pH 7.5]), 150 mM NaCl, 0.5% nonidet P-40, 1 mM EGTA, 1 mM MgCl<sub>2</sub>, 10% glycerol, and proteases inhibitors (cocktail of protease inhibitors EDTA-free, Roche; 1 mM PMSF). After 1 hour at 4°C, cell lysates were obtained by centrifugation at 10,000 g for 10 minutes. The total protein concentration in the lysates was measured by Bradford method [13], protein assay using bovine serum albumin as the standard.

Samples containing equal amounts of protein concentration were separated by 12% of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Nonspecific binding was blocked with Tris-buffered saline with Tween 20 (1M Tris-HCl [pH 7.5], 2.5 M NaCl, and 0.5% Tween 20) containing 5% nonfat milk for 2 hours at room temperature. The membranes were incubated overnight with the primary antibody [COX-2 and β-actin (abcam, ab52237, and ab8227, resp.)] diluted in Tris-buffered saline with Tween 20 (1:1.000 and 1:2.000, resp.) and then washed with Tris-buffered saline with Tween 20 and incubated with horseradish peroxidase-conjugated anti-immunoglobulin G antibody (goat anti-rabbit immunoglobulin G) as secondary antibody for 1 hour at room temperature. The immunoblots were visualized with a chemiluminescence detection kit, used according to the manufacturer's recommendations (SuperSignal West Pico Chemiluminescent Substrate, Prod # 34080, Thermo Scientific).

**2.9. Carrageenan Induced Paw Edema Assay.** Paw edema was induced by intraplantar injection of 100 µL of 1% carrageenan into the right hind paw of rats as previously described [14]. Animal groups were treated with α,β-amyrone (10 and 5 mg/kg, v.o.) and indomethacin (10 mg/kg, v.o.) and the control animals received identical treatments with the vehicle, which was 3% Tween 80 (10 mg/kg) in saline in this study. After sixty minutes, the animals received an intraplantar injection of carrageenan. The paw volume was measured thereafter at "0 hours" and then at 1, 2, 3, 4, and 5 hours after carrageenan injection using a hydroplethysmometer (Panlab, SLU). The results are expressed as the increase in paw volume (mL) calculated by subtracting basal volume.

**2.10. Ear Phenol-Induced Edema.** Inflammation was induced in Balb C mice (*n* = 5/group) by local administration of 20 µL of a solution of phenol diluted in acetone (10%) (group 1), administered after 20 µL α,β-amyrone solution at concentrations of 0.6 mg, 0.3 mg, and 0.1 mg or dexamethasone of 0.1 mg dissolved in acetone. Sixty minutes after application, mice were euthanized and both ears were removed. Circular

TABLE 1: Cell viability of J774 cells treated with 5, 10, and 20  $\mu\text{g}/\text{mL}$  of isolated triterpenes for 24, 48, and 72 hours.

Concentration ( $\mu\text{g}/\text{mL}$ )	24 hours			48 hours			72 hours		
	20	10	5	20	10	5	20	10	5
$\alpha,\beta$ -Amyrin	57.3 $\pm$ 1.9	86.4 $\pm$ 0.8	105.8 $\pm$ 2.3	36.4 $\pm$ 3.1	88.5 $\pm$ 2.8	110.0 $\pm$ 2.5	37.1 $\pm$ 3.5	88.7 $\pm$ 1.2	97.2 $\pm$ 1.8
Acetylated $\alpha,\beta$ -amyrin	87.1 $\pm$ 0.5	152.9 $\pm$ 0.7	124.4 $\pm$ 1.9	32.1 $\pm$ 2.4	99.5 $\pm$ 3.6	125.0 $\pm$ 1.7	47.7 $\pm$ 1.9	98.0 $\pm$ 2.9	103.5 $\pm$ 2.7
$\alpha,\beta$ -Amyrone	119.4 $\pm$ 0.5	153.8 $\pm$ 1.8	144.0 $\pm$ 0.78	81.9 $\pm$ 2.9	126.7 $\pm$ 0.7	133.5 $\pm$ 1.5	65.3 $\pm$ 1.7	102.0 $\pm$ 0.7	105.2 $\pm$ 3.5
Brein/maniladiol	40.6 $\pm$ 1.1	82.4 $\pm$ 1.9	97.2 $\pm$ 3.5	13.3 $\pm$ 2.0	47.6 $\pm$ 2.9	90.0 $\pm$ 1.2	5.4 $\pm$ 0.6	23.8 $\pm$ 3.1	80.2 $\pm$ 1.6
Indomethacin	100.1 $\pm$ 3.2	123.6 $\pm$ 2.9	117.5 $\pm$ 3.7	50.5 $\pm$ 2.8	100.8 $\pm$ 1.3	105.9 $\pm$ 0.8	57.9 $\pm$ 1.1	102.3 $\pm$ 1.7	103.8 $\pm$ 2.9
Doxorubicin	26.0 $\pm$ 0.4	27.3 $\pm$ 1.2	25.7 $\pm$ 2.6	9.3 $\pm$ 1.4	10.0 $\pm$ 0.1	10.1 $\pm$ 0.2	3.8 $\pm$ 0.7	3.9 $\pm$ 0.07	3.9 $\pm$ 0.1
DMSO	70.8 $\pm$ 0.7	108.6 $\pm$ 0.6	113.3 $\pm$ 0.6	38.9 $\pm$ 0.2	98.9 $\pm$ 2.7	109.5 $\pm$ 2.9	33.4 $\pm$ 2.3	98.6 $\pm$ 1.1	99.0 $\pm$ 1.3
Medium	106.1 $\pm$ 4.0	100.0 $\pm$ 9.7	101.30 $\pm$ 1.5	106.7 $\pm$ 1.8	101.9 $\pm$ 11.0	103.4 $\pm$ 2.1	99.9 $\pm$ 4.8	98.9 $\pm$ 3.4	95.4 $\pm$ 4.2

Notes. Data are presented as % mean  $\pm$  standard error ( $n = 3$ ). SE: standard error; DMSO: dimethyl sulfoxide.

sections were removed, using a biopsy punch with a diameter of 5 mm, and weight of the inflamed ears was compared with weight of the ear against-lateral not treated with the phlogistic agent. The increase in weight caused by the irritant was measured by subtracting the weight of the untreated left ear section from that of the treated right ear sections [15].

**2.11. Statistical Analysis.** Results are expressed as the means and standard deviations of triplicate measurements. Each experiment was performed at least three times. Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test. A value of  $P < 0.05$  indicated significance. Western blots are representative of 3 independent experiments.

Data obtained from animal experiments were expressed as the mean  $\pm$  standard error of the mean ( $\pm$ SEM). Statistical differences between the treated and the control groups were analyzed statistically by analysis of variance (ANOVA) followed by Dunnett's test, in the tutorial Prisma 3.0. Results with  $*P < 0.05$  and  $**P < 0.01$  were considered significant.

### 3. Results

Before evaluating the anti-inflammatory effects of triterpenes isolated from *Protium paniculatum* on LPS-stimulated J774 macrophages, first the cytotoxic effects were investigated. Triterpenes did not exhibit a significant reduction in viability of macrophages compared with the positive control, showing  $\text{IC}_{50} > 20 \mu\text{g}/\text{mL}$ , except that triterpene brein/maniladiol showed  $\text{IC}_{50} = 16.02 \mu\text{g}/\text{mL}$  after 24 hours of treatment (Table 1).

Because  $\text{NO}^*$  is known to be a proinflammatory mediator in inflammatory disorders [16], we investigated whether triterpenes inhibit  $\text{NO}^*$  production in LPS-induced J774 cells. We measured the accumulation of nitrite in the culture media and found that triterpenes concentration-dependently inhibited nitrite levels in the conditioned media of LPS-induced cells. Figure 2 shows the inhibitory effect of triterpenes,  $\alpha,\beta$ -amyrin, acetylated  $\alpha,\beta$ -amyrin,  $\alpha,\beta$ -amyrone, and brein/maniladiol, on  $\text{NO}^*$  production at concentration of 1.25–10  $\mu\text{g}/\text{mL}$ . The triterpenes inhibited the production of  $\text{NO}^*$  at 98.34  $\pm$  0.9%; 96.05  $\pm$  0.8%; 99.86  $\pm$  1.1%; and 75.43  $\pm$

2.8%, at 10  $\mu\text{g}/\text{mL}$ , respectively, and showed  $\text{IC}_{50}$  at 4.96  $\pm$  0.2; 5.04  $\pm$  0.12; 4.61  $\pm$  0.08; and 6.49  $\pm$  0.02  $\mu\text{g}/\text{mL}$  at 10  $\mu\text{g}/\text{mL}$ , respectively (Figures 2(a), 2(b), 2(c), and 2(d)). Indomethacin was used with the positive control of anti-inflammatory effect showing an inhibition of 86.31  $\pm$  1.2% in  $\text{NO}^*$  production at 10  $\mu\text{g}/\text{mL}$  (Figure 2(e)).

LPS induce production of proinflammatory cytokines such as the tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-1 and IL-6 in cells. As shown in Figure 3, among the triterpenes evaluated, only the  $\alpha,\beta$ -amyrin led to a significant decrease in TNF- $\alpha$  levels (52.03  $\pm$  2.4%) at a concentration of 10  $\mu\text{g}/\text{mL}$  (Figure 3(a)). However, the other triterpenes, with exception of acetylated  $\alpha,\beta$ -amyrin, inhibited the production of IL-6. Figure 3(b) shows that  $\alpha,\beta$ -amyrin,  $\alpha,\beta$ -amyrone, brein/maniladiol, and indomethacin at a concentration of 10  $\mu\text{g}/\text{mL}$  inhibited the IL-6 levels at 67.81  $\pm$  2.8%; 61.43  $\pm$  3.2%; 61.27  $\pm$  5.1%; and 64.24  $\pm$  2.8%, respectively. Furthermore only  $\alpha,\beta$ -amyrone showed an inhibition in IL-10 level; an anti-inflammatory cytokine is secreted under different conditions of immune activation by a variety of cell types, including T cells, B cells, and monocytes/macrophages (Figure 3(c)).

Due to acetylation,  $\alpha,\beta$ -amyrin did increase inhibition of  $\text{NO}^*$  and TNF- $\alpha$ , and despite the fact that brein/maniladiol showed potential anti-inflammatory activity, it exhibited moderate cytotoxicity activity in J774 murine macrophage cells. Moreover, it is a mixture that needs to be further isolated and characterized. For these reasons we evaluated only triterpene  $\alpha,\beta$ -amyrone. Thus, the protein expression levels of the COX-2 in LPS-challenged cells with and without the treatment of  $\alpha,\beta$ -amyrone were evaluated by western blotting (Figures 4(a) and 4(b)). Treatment with  $\alpha,\beta$ -amyrone showed inhibited COX-2 expression in a concentration-dependent manner, reduced by approximately 90%, at concentrations of 5 or 10  $\mu\text{g}/\text{mL}$ .

Figure 5 shows that oral administration of  $\alpha,\beta$ -amyrone (5 and 10 mg/kg,  $n = 5$ ) induced dose-dependent rat paw edema compared with animals receiving only saline. The edema was of rapid onset and relatively short duration (36.3  $\pm$  1.6% and 54.5  $\pm$  1.1%, resp.), after 1 hour of treatment compared to the edema after 3 hours (72.2  $\pm$  1.3% and 79.1  $\pm$  0.4%, resp.). In phenol-induced ear edema in a murine model, it was found that the triterpene  $\alpha,\beta$ -amyrone exhibited significant

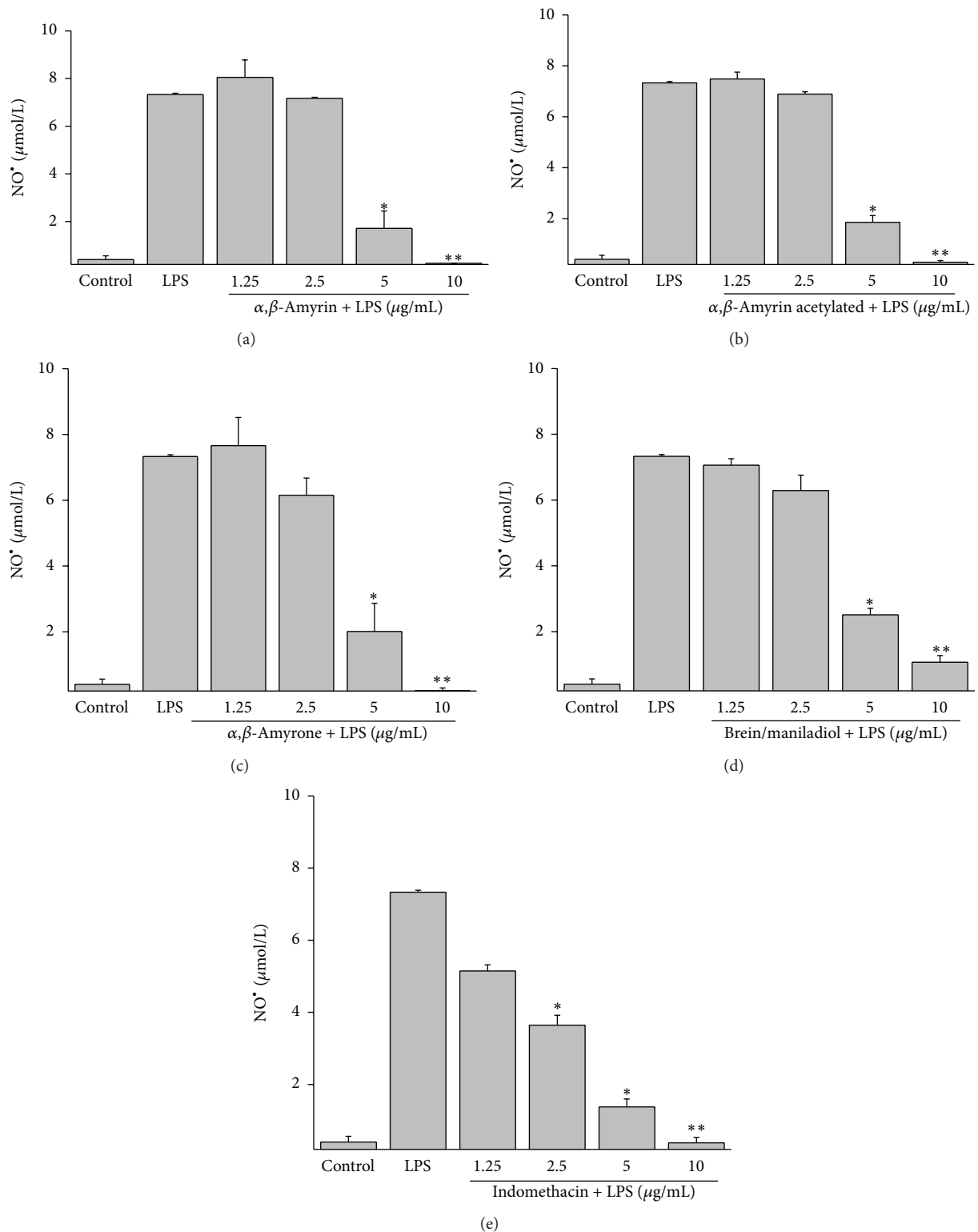


FIGURE 2: Effect of the isolated triterpenes on NO\* production in LPS-stimulated J774 cells. (a)  $\alpha,\beta$ -amyrin (b) acetylated  $\alpha,\beta$ -amyrin (c)  $\alpha,\beta$ -amyrone (d) brein/maniladiol, and (e) indomethacin. Production of NO\* was assayed in culture supernatants of macrophages stimulated with LPS (1 µg/mL) for 24 h in the presence of the four compounds (1.25–10 µg/mL). The nitrite values are the mean  $\pm$  SD from three independent experiments. Significance was determined using Student's *t*-test (\*  $P < 0.05$ ; \*\*  $P < 0.01$  compared to LPS).

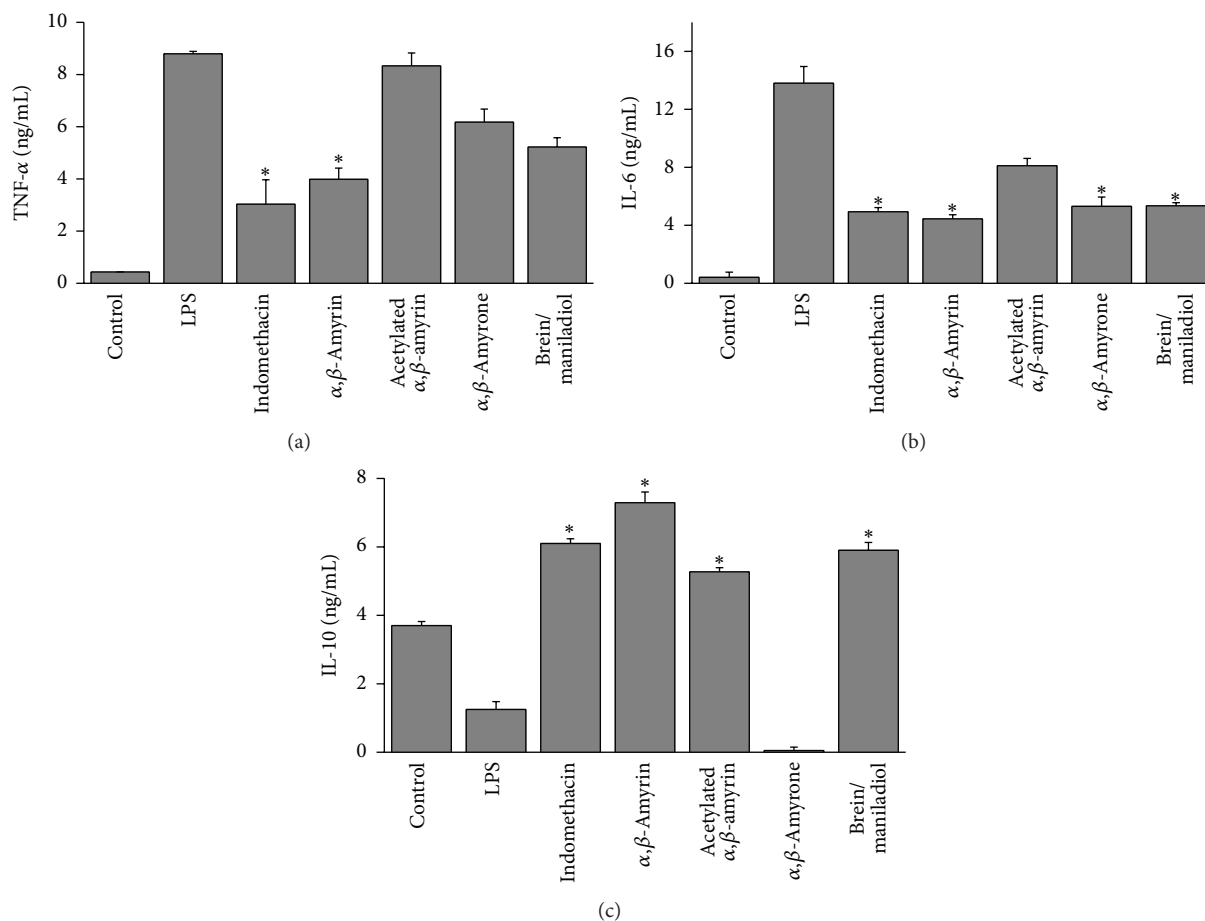


FIGURE 3: Effect of the isolated triterpenes on cytokine production in LPS-stimulated J774 cells. (a) TNF- $\alpha$ , (b) IL-6, and (c) IL-10. Indomethacin (10  $\mu\text{g/mL}$ ) was used as a standard. The production of cytokines was assayed in the culture supernatants of macrophages stimulated with LPS (1  $\mu\text{g/mL}$ ) for 24 h in the presence of the four compounds (10  $\mu\text{g/mL}$ ). Each value was the mean  $\pm$  SD from three independent experiments. The significance was determined using Student's  $t$ -test (\*  $P < 0.05$  compared to LPS).

inhibition in ear edema formation in a dose-related manner. It caused 47% inhibition at the dose of 0.6 mg/kg body weight, respectively, compared with the standard drug dexamethasone where the inhibition was 36% at the dose of 0.1 mg/kg body weight Figure 5(b).

#### 4. Discussion

The use of natural products, especially those derived from medicinal plants, is a traditional form of providing relief from illness. Over the years, natural products have contributed enormously to the development of important therapeutic drugs used currently in modern medicine [17, 18]. Recent studies have shown that the resin of *Protium* sp. displays marked anti-inflammatory activity, in different models of inflammation, with hepatoprotective potential, topical anti-inflammatory action, pancreatic injury, and colitis [5, 6, 18, 19].

Our study demonstrated for the first time the cytotoxic and anti-inflammatory effects of natural triterpenes  $\alpha, \beta$ -amyrin, brein/maniladiol, and synthetic triterpenes acetylated  $\alpha, \beta$ -amyrin and  $\alpha, \beta$ -amyrone on LPS-stimulated J774

macrophages. The triterpenes did not exhibit a significant reduction in macrophage viability compared with the positive control, showing  $\text{IC}_{50} > 20 \mu\text{g/mL}$ , except triterpene brein/maniladiol which showed  $\text{IC}_{50} = 16.02 \mu\text{g/mL}$  in 24 hours of treatment. Similar results were observed by Siani et al. [20] who verified that essential oil obtained by steam distillation (leaves and resin) from *Protium* species at 100  $\mu\text{g/mL}$  inhibited the proliferation of different cell lines, with 76–89% inhibition of J774 cells after 72 h of treatment. As noted, brein/maniladiol showed cytotoxic effects on J774 macrophages. Likewise, Ukiya et al. [7] showed that maniladiol isolated from the nonsaponifiable lipid fraction of the edible flower extract of *Chrysanthemum morifolium* exhibited moderate cytotoxicity in kidney cancer cell lines and accentuated activity in breast cancer.

$\text{NO}^{\bullet}$  plays an important role in various inflammatory conditions where it is produced by the inducible form of nitric oxide synthase (iNOS) from the amino acid L-arginine [21, 22].  $\text{NO}^{\bullet}$  in tissue is susceptible to manipulation by proinflammatory cytokines [23].  $\text{NO}^{\bullet}$  has important immune, cardiovascular, and neurological second messenger functions implicated in sepsis, cancer, and inflammation. A variety of

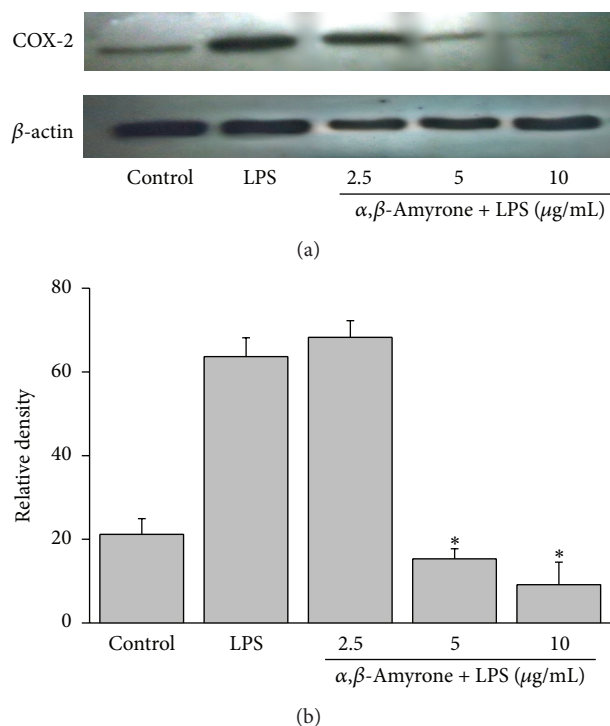


FIGURE 4: Effect of the triterpene  $\alpha,\beta$ -amyrone isolated from *Protium* ssp. on COX-2 expression in LPS-stimulated J774 cells. (a) J774 cells were pretreated with concentrations of 2.5, 5, and 10  $\mu\text{g/mL}$  of  $\alpha,\beta$ -amyrone and LPS (1  $\mu\text{g/mL}$ ) for 24 h. The cells were lysed, and the lysates were analyzed by immunoblotting with an anti-COX-2 antibody. The blot was stripped and re probed with an anti-actin antibody to confirm equal loading. (b) Relative density of COX-2 protein was performed using the ImageJ Software. The significance was determined using ANOVA (\* $P < 0.05$  compared to LPS).

stimuli, such as with LPS, TNF- $\alpha$ , and IFN- $\gamma$ , can result in the production of a massive amount of NO $\cdot$  by the activated macrophages which can participate in the pathological processes in several acute and chronic inflammatory disorders [24]. Our results suggest that triterpenes from *P. paniculatum* have dose-dependent anti-inflammatory activities related to their inhibition of NO $\cdot$  in macrophages without affecting the viability of these cells.

Our results were better than those obtained by Siani et al. [20] who demonstrated that essential oil obtained from leaves and resin from *Protium* species at 100  $\mu\text{g/well}$ , changed the NO $\cdot$  production from stimulated mouse macrophage after 24 hours of pleurisy induction, in which the resin of *P. heptaphyllum* inhibited 74% and *P. strumosum* inhibited 46% of the NO $\cdot$  production. In contrast, the triterpenes isolated from *Protium paniculatum*,  $\alpha,\beta$ -amyrin, acetylated  $\alpha,\beta$ -amyrin,  $\alpha,\beta$ -amyrone, and brein/maniladiol, at a concentration of 10  $\mu\text{g/mL}$  inhibited the production of NO $\cdot$  at  $98.34 \pm 0.9\%$ ;  $96.05 \pm 0.8\%$ ;  $99.86 \pm 1.1\%$ ; and  $75.43 \pm 2.8\%$ , respectively.

Furthermore, the media of IC $_{50}$  of triterpenes were similar with the media observed by Niu et al. [25] who evaluated their potential to inhibit the NO $\cdot$  production induced by LPS

stimulation in RAW 264.7 macrophages of one new olean-13(18)-ene-3,12,19-trione, and two known oleanene triterpenes  $\delta$ -amyrone and  $\delta$ -amyrin acetate isolated from a petroleum ether fraction from an alcohol extract of the whole plant of *Sedum linear* Thunb., which exhibited values of IC $_{50}$  at 9.91  $\mu\text{M}$ , 12.24  $\mu\text{M}$ , and 43.34  $\mu\text{M}$ , respectively.

Monocytes and macrophages are key players in inflammatory responses and are also major sources of proinflammatory cytokines and enzymes including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukins (ILs), cyclooxygenase (COX), and nitric oxide synthase (NOS) [24, 26]. These genes of proinflammatory mediators are strongly induced during inflammation and are responsible for its initiation and persistence. TNF- $\alpha$  are cytokines that act as signaling molecules for immune cells and coordinate the inflammatory response [24]. In this study, among the triterpenes tested, only  $\alpha,\beta$ -amyrin inhibited TNF- $\alpha$  production. This result corroborates with several studies of inhibitory effects of  $\alpha,\beta$ -amyrin on TNF- $\alpha$  production in different models of inflammation [5, 8, 18, 27].

Interleukin-6 (IL-6) is one of the earliest and most important proinflammatory cytokines produced in response to inflammatory stimuli [28]. The presence of IL-6 in tissues is not an unusual occurrence, but its production can lead to uncontrolled exposure and subsequent chronic inflammation, and they are strongly associated with many types of cancer [29]. As our continuing research on anti-inflammatory agents, a number of plant extracts and natural products have been discovered to suppress the secretion of IL-6 in LPS-stimulated macrophages *in vitro* [28, 30]. Interestingly, all the triterpenes tested exerted inhibitory effects on IL-6 production at 10  $\mu\text{g/mL}$ , except acetylated  $\alpha,\beta$ -amyrin. Similar results were demonstrated by Lee et al. [31] who isolated seven flavonoids from the methanol extracts of *Psoralea corylifolia* (bakuchiol, bavachinin, neobavaisoflavone, corylifol A, corylin, isobavachalcone, and bavachin) and found that these compounds were able of inhibit IL-6 production by action of STAT3 promoter activity in Hep3B cells. These compounds also inhibited STAT3 phosphorylation induced by IL-6 in Hep3B cells.

Interleukin-10 (IL-10) is produced by activated macrophages and T cells and plays an important role in anti-inflammatory responses, including the inhibition of cytokine production (tumor necrosis factor- $\alpha$ , IL-6, and IL-12) in macrophages induced by lipopolysaccharide [31]. This study verified that triterpenes, except  $\alpha,\beta$ -amyrone, showed induced IL-10 production. So, the decline of TNF- $\alpha$  accumulation in our study was consistent with findings in several studies demonstrating that IL-10 can suppress TNF- $\alpha$  production in human monocytes and macrophages or even cause diminished levels of TNF- $\alpha$  and IL-6 [32, 33].

Similar results were observed by Zdzisińska et al. [34] who evaluated the immunomodulatory properties of triterpene betulin and its oxidized form, betulinic acid, as agents inducing cytokines examining human whole blood stimulated by mitogens (PHA). It was observed that triterpene betulin induced TNF- $\alpha$  production in a dose-dependent manner but did not induce the production of IL-10 and IFN- $\gamma$ ; these results suggest that secretion of IFN- $\gamma$ , IL-10, and TNF- $\alpha$  can be regulated by different mechanisms or various

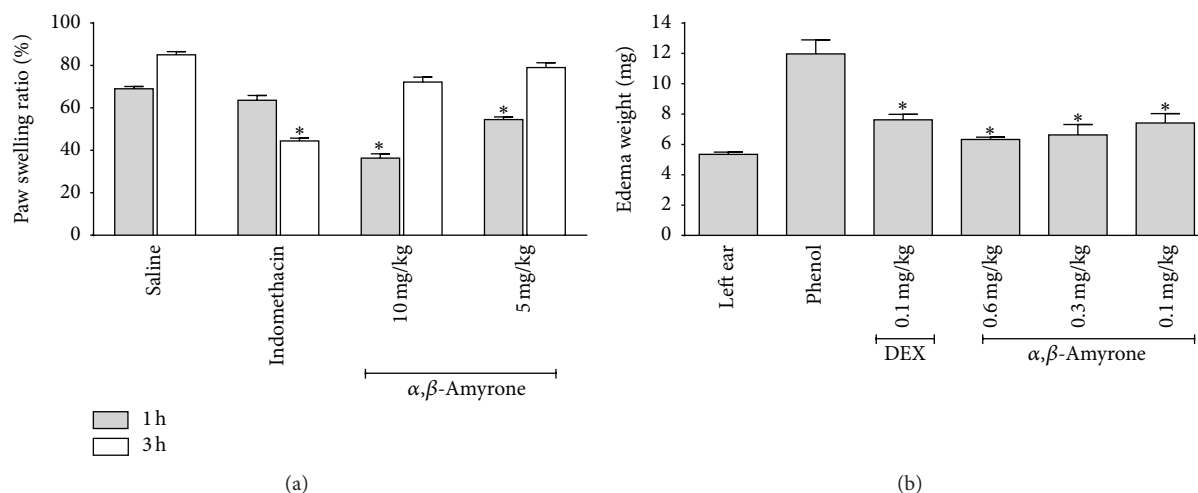


FIGURE 5: Effect of triterpene  $\alpha,\beta$ -amyrone on rats paw edema induced by 1% of solution of carrageenan into the intraplantar surface of right hind paw and the effects of  $\alpha,\beta$ -amyrone on mice ear edema induced by a phenol model. (a)  $\alpha,\beta$ -amyrone was administered at concentrations of 5 and 10 mg/kg and the edema was measured at the indicated times. The effect of saline injected in the control group is also shown. (b)  $\alpha,\beta$ -amyrone was administered at concentrations of 0.6, 0.3, and 0.1 mg/kg and dexamethasone of 0.1 mg/kg on mice ear edema induced by a phenol model. Data are expressed as mean  $\pm$  standard error of five animals per group. The significance was determined using ANOVA and Dunnett's test (\*  $P < 0.05$  compared with control group).

types of leukocytes in whole blood differ in their sensitivity to betulin, unlike, betulinic acid, which did not influence the TNF- $\alpha$  production but inhibited the production of IFN- $\gamma$  and increased production of IL-10.

Cyclooxygenases are inducible enzymes that catalyze the production of prostaglandins, which contribute to the inflammatory process and tissue damage. It has been reported that COX-2 can also be activated by high concentrations of nitric oxide, contributing towards more intense inflammatory responses as seen in many chronic inflammatory disorders [22]. In the current study, we verified only Cox-2 expression of triterpene  $\alpha,\beta$ -amyrone that was able to inhibit COX-2 in a concentration-dependent manner. Several natural products of plant origin have been shown to transmit their anti-inflammatory activities through suppression of COX-2; however, suppression of nitric oxide production is critical for this [22, 35]. In previous studies the ability of triterpene  $\alpha,\beta$ -amyrin to inhibit COX-2 expression using a different model of inflammation is shown, as in the case of topical inflammation in rats and a colitis model [2, 18]. In accordance with the prepreliminary results  $\alpha,\beta$ -amyrone was able to inhibit the production of NO $^*$ , IL-6, and COX-2 expression, suggesting that the mechanism by which  $\alpha,\beta$ -amyrone exerts its anti-inflammatory activity is the same mechanism by which  $\alpha,\beta$ -amyrin acts, that is, by inhibiting the nuclear factor-kappa B (NF- $\kappa$ B).

The model of paw edema induced by carrageenan is an appropriate test and widely used for evaluating anti-inflammatory activity of different compounds [1]. Carrageenan induced hind paw edema is the standard experimental model of acute inflammation. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic

effects [36]. The present study of anti-inflammatory activity of  $\alpha,\beta$ -amyrone against carrageenan induced paw edema shows that triterpenes have a significant effect on inflammation and markedly reduced the swelling at 10  $\mu$ g/mL after 3 hours of treatment.

$\alpha,\beta$ -Amyrone showed the activity at concentrations of 10 and 5 mg/kg and showed a different effect of indomethacin, whereas  $\alpha,\beta$ -amyrone showed a maximum effect in the first hour after administration of carrageenan with a decrease with respect to time, and indomethacin at a dose of 10 mg/kg showed increased activity after three hours of induction of inflammation, when the carrageenan starts to show its greatest inflammatory effect, decreasing effect in five hours, which indicates an effect faster than  $\alpha,\beta$ -amyrone.

In the present study, the significant anti-inflammatory effect of topical application of triterpene  $\alpha,\beta$ -amyrone in phenol-induced mouse ear edema was shown for the first time. Phenol is an irritant agent for stimulating contact dermatitis in mice [37, 38]. Skin keratinocyte membranes are ruptured upon direct contact with phenol, resulting in protein kinase C mediating release of inflammatory mediators such as IL-1 $\alpha$ , TNF- $\alpha$ , and IL-8 [38–40]. The topical anti-inflammatory activity of  $\alpha,\beta$ -amyrone was demonstrated by results showing that  $\alpha,\beta$ -amyrone dose-dependently attenuated the phenol-induced ear edema with an effect as potent as dexamethasone and showing the property of this substance to penetrate the skin and exert its activity in deeper layers which could be indicator of its potential use in pharmaceutical formulations with anti-inflammatory properties. In addition, the anti-inflammatory activity of  $\alpha,\beta$ -amyrone needs the additional studies which will provide clinical evidences in context of specific inflammatory inductions and/or microbial infection activity.



## 5. Conclusion

The triterpenes  $\alpha,\beta$ -amyirin, acetylated  $\alpha,\beta$ -amyirin,  $\alpha,\beta$ -amyrone, and brein/maniladiol are capable of modulating an immune response. In particular, the triterpene  $\alpha,\beta$ -amyrone showed no cytotoxic potential in J774 macrophages and exerted immunomodulatory activity at low concentrations, characterized by its inhibitory effects on the production of proinflammatory mediators such as NO<sup>\*</sup>, IL-6, and COX-2 expression and inducing the production of anti-inflammatory cytokine IL-10, and reduced paw edema induced by carrageenan in rats, as well as reducing ear edema in mice.

## Abbreviations

COX <sub>2</sub> :	Cyclooxygenase 2
Dexa:	Dexamethasone
DMSO:	Dimethyl sulfoxide
EDTA:	Ethylenediaminetetraacetic acid
EGTA:	Ethylene glycol tetraacetic acid
IL6:	Interleukin-6
IL10:	Interleukin-10
LPS:	Lipopolysaccharide
MgCl <sub>2</sub> :	Magnesium chloride
NaCl:	Sodium chloride
NF- $\kappa$ B:	Nuclear factor kappa B
NO:	Nitric oxide
PBS:	Phosphate buffered saline
PMSF:	Phenylmethanesulfonyl fluoride
TNF $\alpha$ :	Tumor necrosis factor- $\alpha$ .

## Disclosure

This study is part of the project of Regional Scientific Development/FAPEAM (DCR) of Professor Dr. Ana Paula de A. Boleti, developed in the laboratory of biological activities, Faculty of Pharmaceutical Sciences, Federal University of Amazonas, under the supervision of Professor Dr. Emerson S. Lima.

## Conflict of Interests

Authors declare no conflict of interests.

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