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Anti-inflammatory effect of triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene obtained from *Combretum leprosum* Mart & Eich in mice

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ABSTRACT

Ethnopharmacological relevance: The 3β, 6β, 16β-trihydroxylup-20(29)-ene (TTHL) is a pentacyclic triterpene obtained from a medicinal plant named *Combretum leprosum*. In folk medicine, this plant is used to treat several diseases associated with inflammation and pain. We previously demonstrated that TTHL presents a significant antinociceptive effect, suggesting the involvement of the glutamatergic system.

Aim of the study: This study was designed to investigate the effect of TTHL on nociception and vascular permeability induced by acetic acid. We also evaluated the effect of TTHL on carrageenan-induced peritonitis and the levels of cytokines (interleukin 1-β [IL-1β], tumor necrosis factor α [TNF-α] and interleukin 10 [IL-10]) on peritoneal fluid.

Materials and methods: TTHL was administered orally by intra-gastric gavage (i.g.) 60 min prior to experimentation. Abdominal contractions and vascular permeability were induced by an intraperitoneal (i.p.) injection of acetic acid (0.6%). We also investigated whether TTHL decreases carrageenan-induced peritonitis (750 μg/cavity) by measuring leukocyte migration and vascular permeability. In addition, we evaluated the effects of TTHL on TNF-α, IL-1β and IL-10 release induced by carrageenan on peritoneal fluid. The levels of these cytokines were measured by ELISA.

Results: TTHL (0.01–10 mg/kg) administered by intra-gastric (i.g.) gavage inhibited (69 ± 3%) acetic acid-induced abdominal constrictions, with an ID_{50} of 0.15 (0.03–0.8) mg/kg. TTHL (10 mg/kg) also reduced the leukocyte infiltration induced by acetic acid, with an inhibition of 59 ± 9 but had no effect on abdominal vascular permeability. In addition, indomethacin (10 mg/kg, i.p.) reduced the nociceptive behavior (92 ± 1%), total leukocyte migration (29 ± 3%) and capillary permeability (71 ± 3%) induced by acetic acid. While the glucocorticoid dexamethasone (2 mg/kg, s.c.) reduced partially but significantly the nociception (31 ± 1%), besides to promote a marked reduction on total leukocyte migration (60 ± 2%) to the peritoneal cavity caused by acetic acid. In a model of peritonitis induced by carrageenan, TTHL also reduced total leukocyte migration, mainly neutrophils (inhibition of 84 ± 3% and 85 ± 2% at 30 mg/kg and 100 mg/kg, respectively). Likewise, dexamethasone (0.5 mg/kg, i.p.) resulted in an inhibition of 93 ± 3%. Nevertheless, carrageenan-induced abdominal vascular permeability was reduced by dexamethasone but was not altered by TTHL. Furthermore, dexamethasone and TTHL significantly reduced the TNF-α and IL-1β levels in peritoneal fluid, whereas the IL-10 levels were unchanged.

Conclusions: Altogether, our data confirm the antinociceptive effect of TTHL and demonstrate its effect in inflammatory animal models, providing novel data about this compound, which could be useful as an anti-inflammatory drug.

1. Introduction

Inflammation is a crucial and necessary function of the innate immune system, protecting the host against pathogens and initiating specific immunity responses. Hence, inflammation is a

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complex process that is frequently associated with pain. Since ancient times, people have been relying on plants as a prophylactic or therapeutic to restore and maintain health. Plants are well known to be an important source of many biologically active compounds (Andrade et al., 2007), especially for the treatment of pain and inflammatory diseases.

Certain species of *Combretum* (Combretacea) have been used in traditional medicine to treat diseases such as pain (McGaw et al., 2001). *Combretum leprosum* Mart & Eich, found in the northeastern region of Brazil, has been widely used in folk medicine. Popularly known as mufumbo, cipoaba or mofumbo, *Combretum leprosum* infusions are prepared with the aerial parts (stems, leaves and flowers) and roots and used as hemostatics and sedatives to heal wounds during the treatment of uterine bleeding (Facundo et al., 2005; Lira et al., 2002; Lopes et al., 2010). Chemical investigation of this plant led to the isolation of several compounds, such as flavonoids and triterpenes (Facundo et al., 1993).

In a previous study, we demonstrated that the ethanolic extract obtained from the flowers of *Combretum leprosum* evoked antinociception in several models of chemical and thermal pain in mice (Pietrovski et al., 2006). Furthermore, we reported that the pentacyclic triterpene 3β, 6β, 16β-trihydroxylup-20(29)-ene (TTHL), obtained from *Combretum leprosum*, presents antinociceptive effects that involve the spinal glutamatergic system, certain endogenous pain inhibitory systems, such as the opioidergic (through μ, κ and δ receptors) and serotonergic systems (5-HT_{1A} and 5-HT_{2A} receptors), as well as inhibitory signaling pathways including Gi/o protein activation and the opening of ATP dependent-, voltage- and large-conductance Ca^{2+}-gated potassium channels (Longhi-Balbinot et al., 2009, 2011).

Considering the antinociceptive effect of TTHL and the fact that triterpenes have significant anti-inflammatory activity (Andrade et al., 2007; Costa et al., 2003; Geetha and Varalakshmi, 2001; Hasmeda et al., 1999; Lucetti et al., 2010; Safayhi and Sailer, 1997), this study evaluated the effect of the triterpene TTHL on nociception and vascular permeability induced by acetic acid. We also investigated the effect of TTHL on carrageenan-induced peritonitis and the levels of cytokines (interleukin 1-β [IL-1β], tumor necrosis factor α [TNF-α] and interleukin 10 [IL-10]).

2. Materials and methods

2.1. Animals

All experiments were performed after approval of the protocol by the Ethics Committee for Animal Research of the Federal University of Santa Catarina and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983). The experiments were conducted using male Swiss mice (25–35 g) that were obtained from the Federal University of Santa Catarina's animal facility and housed at 22 ± 2 °C under a 12 h light/12 h dark cycle (lights on at 06:00 a.m.) with access to food and water ad libitum. The mice were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. The numbers of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.2. Abdominal constriction, peritoneal capillary permeability and leukocyte infiltration caused by intraperitoneal injection of acetic acid

Abdominal constrictions were induced according to procedures described previously (Collier et al., 1968; Lucena et al., 2007) and resulted in the contraction of the abdominal muscle along with a stretching of the hind limbs in response to an intraperitoneal (i.p.) injection of acetic acid (0.6%, 0.45 ml/mouse). Mice were anesthetized by isoflurane inhalation (1–2%) and pre-treated intravenously (i.v., 200 μl) with 2.5% Evans Blue Dye Solution, which was used as a marker of capillary permeability. One hour later, the mice received TTHL (dose range: 0.01–10 mg/kg) by intra-gastric gavage (i.g.) 60 min prior to the acid acetic injection. In a separate series of experiments, was investigated the effect of indomethacin (10 mg/kg, i.p.) or dexamethasone (2 mg/kg s.c.), used as positive controls, 30 min and 4 h before the acetic acid injection, respectively. The control animals received the same volume of vehicle (10 ml/kg, i.g.). After the challenge, the mice were placed in separate boxes, and the number of abdominal constrictions was cumulatively counted over a period of 20 min. Immediately after the test, the animals were sacrificed by CO_2 asphyxiation according to the guidelines for the care and use of experimental animals (Heine, 1965), and their peritoneal cavity was washed with 1 ml of cold sterile saline (NaCl 0.9%) plus heparin (25 UI/ml). After 30 s of gentle manual massage, the exudate was retrieved. Total cell counts were performed with a Neubauer chamber via optical microscopy, after diluting a sample of the peritoneal fluid with Türk solution (1:20). The absorbance of the supernatant was read at 620 nm with an ELISA analyzer. The peritoneal capillary permeability is expressed in terms of the dye (μg/ml) that leaked into the peritoneal cavity, according to the standard curve of the Evans blue dye (Lucena et al., 2007; Mazzardo-Martins et al., 2010).

2.3. Carrageenan-induced peritonitis (peritoneal leukocyte counts and peritoneal capillary permeability)

Carrageenan-induced peritonitis was validated according to procedures described previously (Montanher et al., 2007; Pagano et al., 2002). The animals received an i.p. injection of 0.5 ml of carrageenan (750 μg per cavity) diluted in sterile saline. At the beginning of the experiment, the mice were anesthetized by isoflurane inhalation (1–2%), and Evans blue dye solution (25 mg/kg) was injected intravenously. One hour later, the mice were pre-treated with TTHL (10–100 mg/kg, i.g.) or dexamethasone (DEXA; 0.5 mg/kg, i.p., positive control) 60 min or 30 min before carrageenan injection, respectively. The control animals received the same volume of vehicle (10 ml/kg, i.g.). The dose of dexamethasone was chosen according to data in the literature (Montanher et al., 2007) as well as based on previous studies in our laboratory (Silva et al., 2011). Four hours after peritonitis induction, the mice were sacrificed by CO_2 asphyxiation and their peritoneal cavity was washed with 1 ml of cold sterile saline (NaCl 0.9%) plus heparin (25 UI/ml). The peritoneal fluid was collected for further analysis. Total leukocyte count and capillary permeability were determined as described above (for more details see Section 2.3). To determine the differential leukocyte count, the peritoneal cells were cytocentrifuged onto slides using a Cytospin (Tharmac, Germany) and stained with May–Grüwald–Giemsa (Montanher et al., 2007; Silva et al., 2011).

2.3.1. Determination of cytokines levels in the peritoneal fluid

Four hours after carrageenan-induced peritonitis, peritoneal fluid samples was collected as described above and was used to estimate the cytokine levels by enzyme-linked immunosorbent assay (ELISA) (Mizgerd et al., 2001). Peritoneal fluid aliquots of 100 μl were used to measure tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-10 levels using mouse cytokine ELISA kits from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions. The absorbances for all cytokines