ABSTRACT

Results: The THOA showed effect with 5 and 10 mg/kg in the acute pain induced by acetic acid (49% and 62% of the control group) and formalin (150% and 188% increase in mechanical threshold) and formalin (36% and 60% of the control group), respectively. These results indicate an inhibition of hyper nociception, while the reduction in the production of cytokines (TNF-α inhibition–64% and 88%; IL-1β inhibition–48% and 55%, respectively) confirmed the inflammatory inhibition by carageenan. The THOA did not induce motor impairment. The THOA was not toxic after oral administration (LD50>50 mg/kg).

Conclusion: These data provide initial evidence that THOA decreases the inflammatory hyper nociception probably by inhibition of IL-1β and TNF-α production, proving to be effective in reducing pain and inflammation.

Keywords: Chrysobalanus icaco, Cytokines, Mechanical hyperalgesia, Mice

INTRODUCTION

Inflammation is a natural defence mechanism of the body against cells or tissues injured either due to chemical, mechanical or thermal stimuli or infections [1]. The inflammatory response must be approximately terminated to prevent tissue damage, and failure to terminate inflammation results in chronic inflammation and cellular destruction. Inflammation can lead to a variety of diseases, such as atherosclerosis, rheumatoid arthritis, cancer, and allergies. During the inflammatory process, many kinds of cells are activated, and these cells secrete various pro-inflammatory molecules, including cytokines and nitric oxide (NO) [2, 3].

Inflammatory hyperalgesia results from the sensitization of primary afferent neurons which is better described as hyper nociception in animal models. It is induced by inflammatory mediators, such as prostaglandins and sympathetic amines, which directly sensitize peripheral nociceptive neurons [4]. The release of these direct-acting hyper nociceptive mediators is generally preceded by a cascade of cytokines [5]. In addition, it is broadly accepted that the cytokines, produced by either immune or central nervous system cells, might directly sensitize the peripheral nociceptors [6]. The NSAIDs are the most commonly prescribed agents for the management of inflammatory disorders. Currently, there is great concern about adverse effects these drugs. Therefore, new approaches are now considered for the development of new anti-inflammatory agents, with less adverse effects than the current ones [7].

Consumption of C. icaco as a tea prepared with various parts of the plant has been thoroughly used, in folk medicine, for the control of several pathologies such as leucorrhoea, haemorrhages and chronic diarrhea. This species is known in Brazil as abajurú, abajuru, abajeru, guajuarú, among other popular names appear on the Brazilian coast [8]. The species is known to produce hypoglycemic, antiangiogenic, anti-inflammatory and antirheumatic effects [9-11]. The 2α-3β-6β-23-tetrahydro-olean-12-en-28-oic acid (THOA) was isolated from the methanolic extract of Chrysobalanus icaco leaves. Thus, the aim of the study was to evaluate the antinociceptive and anti-inflammatory activities of THOA.

MATERIALS AND METHODS

Plant material

The leaves of Chrysobalanus icaco were collected in the city of Rio de Janeiro, Brazil. The plant was classified by Rosa Fux (Botanical Garden of Rio de Janeiro, Brazil) and a voucher specimen was deposited in the Herbarium of the National Museum under the number R195941.

Instruments and reagents

NMR spectra were recorded on a Bruker Avance 500 MHz instrument with TMS as an internal standard. Chemical shifts are expressed in δ values. Separations were carried out with VETEC silica gel (230–400 mesh size) for column chromatography.
Extraction and isolation of 2α-3β-6β-23-tetrahydro-olean-12-en-28-oic acid (THOA)

The dried and powdered leaves of *C. icaco* (2.50g) were extracted exhaustively by maceration at room temperature with MeOH. The part of the extract was concentrated under reduced pressure to a year of crude extract (220g). The crude extract (60g) was suspended in MeOH/H2O (9:1) and successively partitioned with hexane, ethyl acetate and butanol. The ethyl acetate fraction (5.0g), was separated using on a silica gel column and then eluted with binary mixtures of hexane-ethyl acetate-methanol in increasing polarity gradient. The 20 collected fractions were analysed by thin-layer-chromatography (TLC) and reunited in groups. Sub-fractions 9-12 (170 mg) were separated again on silica gel column and then eluted using CHCl3/MeOH (9:1) to yield (100 mg) one amorphous white solid. It was characterised as a 2α-3β-6β-23-tetrahydro-olean-12-en-28-oic acid by using NMR (H 13C (1D and 2D) spectral data and comparing with literature data. [12-14].

**Animals**

Male Swiss mice (20–22 g) were obtained from our animal facility. The animals were maintained in a room with a controlled temperature (22±2°C) and a 12 h light/dark cycle with free access to food and water. Twelve hours (h) before each experiment, the animals received only water, to avoid possible interference of food with the absorption of the drug. The experimental protocol was approved by the Ethics Committee for Animal Research of the Federal Rural University of Rio de Janeiro (CONEP-UFRJ) under number 23083.004813/2012-16.

**Chemicals**

The following substances were used: acetic acid (Vetec, Rio de Janeiro, Brazil), formaldehyde (Merck, Darmstadt, Germany), dexamethasone (purity-99%), L-NAME (purity-98%), L-Arginine (purity-99%), acetylsalicylic acid (purity-99%), l-carrageenan, and dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA), and morphine (purity-97%) (Cristália, São Paulo, Brazil).

**Treatments**

Increasing doses of the THOA were administered orally (1, 5 and 10 mg/kg-p.o.). Morphine, acetylsalicylic acid and dexamethasone were used as positive controls. The doses of morphine (5.01 [2.47–8.68] mg/kg-p.o.-opioid analgesic drug) and dexamethasone (2.25 [1.82-2.79] mg/kg subcutaneous administration, s. c-steroidal anti-inflammatory) were obtained by calculating the ED50 (confidence limits) in acetic acid-induced abdominal writhing and air pouch tests that were performed beforehand. The ED50 values (the dose producing 50% of the maximal effect) for the anti-nociceptive and anti-inflammatory actions were obtained by fitting the data points representing the anti-nociceptive and anti-inflammatory effects demonstrated in these models by nonlinear regression (sigmoidal function) using GraphPad Prism software version 5.0 (San Diego, California, USA) (data not shown). The dose of the acetylsalicylic acid was 100 mg/kg administered-p.o., according to Guilhon et al. [15].

Distilled water mixed with dimethyl sulfoxide, a solubilizing agent, was used as a vehicle (2.5%). For the preparation of different THOA doses, the control group consisted of mice that received only distilled water.

**Acetic acid-induced abdominal writhing test**

In order to evaluate the antinociceptive effect of the THOA, different groups were treated orally with vehicle, distilled water, morpshine, acetylsalicylic acid or the THOA [1–10 mg/kg] 60 min before the subplantar injection of formalin. Formalin-induced behaviour was assessed as previously described by Hunskar et al. [17]. Mice received a subplantar injection of 0.02 ml of formalin (2.5% v/v) into the dorsal surface of the left hind paw. The mice were immediately placed into an individual observation chamber and the number of writhes after the i. p. administration of 1.2% (v/v) acetic acid in MeOH/H2O immediately after the injection.

**The formalin test**

Animals previously described by Koster et al. [16]. In brief, the total number of writhes after the i. p. administration of 1.2% (v/v) acetic acid (0.01 ml/g) was recorded over a period of 30 min, beginning immediately after the injection.

**The formalin test**

In order to discriminate between inflammatory and non-inflammatory activities of the THOA, different groups were treated orally with vehicle, distilled water, morphine, acetylsalicylic acid or the THOA [1–10 mg/kg] 60 min before the subplantar injection of formalin. Formalin-induced behaviour was assessed as previously described by Hunskar et al. [17]. Mice received a subplantar injection of 0.02 ml of formalin (2.5% v/v) into the dorsal surface of the left hind paw. The mice were immediately placed into an individual observation chamber and the number of writhes after the i. p. administration of 1.2% (v/v) acetic acid in MeOH/H2O immediately after the injection. The second phase was the inflammatory response, recorded 35-50 min after the formalin injection. To evaluate the participation of particular systems on the effect shown by THOA, L-NAME (1, 2 and 3 mg/kg) and L-arginine (1, 3 and 5 mg/kg) were administered intraperitoneally (i.p.) 15 min before the oral administration of the THOA. The L-NAME and L-arginine doses were chosen by comparing increasing doses of these substances against a single THOA dose.

**The von frey test**

In order to evaluate the antinociceptive effect of the THOA on mechanical hyperalgesia, different groups were treated orally with vehicle, distilled water, THOA [1–10 mg/kg] or the dexamethasone (s.c.) 60 min before the subplantar injection of carrageenan. The test consisted of evoking a hind paw flexion reflex with a hand held force transducer (Insight Scientific Equipment, SP, Brazil) adapted with a 0.5 mm2 polypropylene tip [18]. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The endpoint was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an averaging of 3 measurements. The test was performed three hours after the administration of carrageenan.

**Air-pouch test**

In order to evaluate the effect of the THOA on the production of cytokines, different groups were treated orally with vehicle, distilled water, THOA [1–10 mg/kg] or the dexamethasone (s.c.) 60 min before the carrageenan injection. Air pouches were generated as previously described by Vigil et al. [19]. An area of dorsal skin (3 cm×2.5 cm) was disinfected with iodophor and shaved to provide the pouch site. Seven millilitres of sterile air was injected subcutaneously in a single site with a 16-gauge needle and a 10 ml syringe. The air pouches were injected with sterile air on alternate days for 3 d. During this period, redness, swelling, exudation and air leak were not observed, which suggested that the air pouch model was successfully established. On the fourth day, the animals received carrageenan (0.1% w/v) administered by the subcutaneous route (s.c) and the animals were euthanised 4 h later with an overdose of pentobarbital. The animals were fixed on a surgical table and an incision into the dorsal skin was made to perforate the air pouch. The cavity was then washed with 1.0 ml of sterile phosphate buffered saline (PBS, pH 7.6, containing NaCl [1.30 mmol], Na2PO4 [5 mmol] and KH2PO4 [1 mmol] and heparin (20 IU/ml) in distilled water). Samples of fluid were then collected from each mouse’s air pouch cavity.

**Enzyme-linked immunosorbent assay (ELISA)**

After the Von Frey test, the TNF-α and IL-1β levels were estimated [20]. The animals were euthanized, and the subcutaneous tissue of the paws was collected and homogenized, three hours after the administration of carrageenan on the Von Frey test, in phosphate buffered saline (PBS) containing 0.4 M NaCl, 0.05% Tween 20, 0.5% bovine serum albumin (BSA), 0.1 mmol phenylmethylsulfonyl fluoride, 0.1 mmol benzethonium chloride, 10 mmol EDTA and 0.001% aprotinin (37.6 mg per 100 ml of PBS with EDTA). Then, the samples were centrifuged at 3000 rpm for 15 min at 4°C. The TNF-α and IL-1β levels were estimated using an ELISA kit (enzyme-linked immune sorbent assay), following the manufacturer’s recommendations (Peprotech).

After the air pouch test, the supernatants from exudates collected in the air pouch cavity were used to measure TNF-α and IL-1β. TNF-α
and IL-1β were estimated by enzyme-linked immune-sorbent assay (ELISA), using the protocol supplied by the manufacturer (Peprotech).

**The open-field test**

In order to evaluate the motor impairment induced by the THOA, different groups were treated orally with vehicle, distilled water, morphine or the THOA (1-10 mg/kg). Five days before behavioural testing, each animal was handled daily for a few minutes.

The procedure followed was similar to the method described by Barros et al. [21]. The mice received the oral administration and were placed individually in an observation chamber (60 min after oral administration), in which the floor was divided into 50 squares (5 x 5 cm). The total number of squares covered by the animals in 5 min was counted.

**In vivo toxicological evaluation**

A acute toxicity test was performed according to the WHO guidelines [22] and the Organization of Economic Co-operation and Development guidelines for testing of chemicals [23]. Acute toxicity was determined following the experimental model described previously by Lorke [24]. A single oral dose of THOA (50 mg/kg) was administered to a group of ten mice. Behavioural parameters observed over a period of 7 d included convulsion, hyperactivity, grooming, loss of righting reflex, increased or decreased respiration, and sedation.

After this period animal were killed by cervical dislocation, stomachs were removed and an incision along the greater curvature was made. The number of ulcers (single or multiple erosion, ulcer or perforation) and hyperemia were measured.

**Statistical analysis**

All experimental groups consisted of 7–10 animals. The results are presented as the mean±standard error of the mean (SEM). Statistical significance between the groups was determined using one-way analysis of variance (ANOVA) followed by Bonferroni’s test for the significance between the groups. The results are expressed as the mean±SEM (n=7-10). The statistical significance was calculated by One-way ANOVA followed by Bonferroni’s test. *p<0.05, **p<0.01 and ***p<0.001 when comparing the THOA- and vehicle-treated groups.

**RESULTS**

**Effect of THOA on the acetic acid-induced writhing test**

The intraperitoneal injection of acetic acid (1.2%) induced an average of 57.8±5.2 writhes in a period of 30 min. Doses of 1, 5 and 10 mg/kg inhibited writhing by 27% (42.2±9.8 writhes), 49% (29.7±5.0) and 62% (22.2±9.8 writhes), respectively. Morphine (5.01 mg/kg) inhibited the number of writhes by approximately 50% compared with the control group (fig. 1B).

**Effect of THOA on the formalin test**

Pre-treatment with the THOA significantly reduced the time that the mice spent licking their injected paws after formalin injection, only in the second phase. In the second phase, the inhibitory effect was observed only with the highest doses (5 and 10 mg/kg) (fig. 2A).

In the second phase, the THOA showed 36 and 60% inhibition at doses of 5 and 10 mg/kg, respectively (fig. 2A). Morphine (5.01 mg/kg) inhibited the number of licks by approximately 50% compared with the control group in both the 1st and 2nd phases. Acetylsalicylic acid (200 mg/kg) inhibited the number of licks by approximately 60% compared with the control group in the 2nd phase. The previous administration of L-NAME and L-arginine did not reduce the antinociceptive effect produced by the THOA in the 2nd phase on the formalin test (fig. 2B and 2C).

**Effect of THOA on the von frey test**

The orally administered THOA induced an increase in mechanical threshold with the highest doses (5 and 10 mg/kg). The THOA showed 150% and 188% increase in the doses of 5 and 10 mg/kg, respectively (fig. 3A).

THOA significantly reduced levels of TNF-α and IL-1β in paws administered with carrageenan. Doses of 5 and 10 mg/kg inhibited TNF-α production by 64% and 88%, respectively and doses of 5 and 10 mg/kg inhibited IL-1β production by 48% and 55% (fig. 3B and 3C).

**Effect of THOA on the open field test**

Pre-treatment of mice with the THOA significantly suppressed TNF-α and IL-1β production on the air pouch test (fig. 4A and 4B). Doses of 5 and 10 mg/kg inhibited TNF-α production by 50% and 83%, respectively and doses of 5 and 10 mg/kg inhibited IL-1β production by 30% and 70%, respectively.

**Effect of THOA on the air pouch test**

Pre-treatment of mice with the THOA significantly suppressed TNF-α and IL-1β production on the air pouch test (fig. 4A and 4B). Doses of 5 and 10 mg/kg inhibited TNF-α production by 50% and 83%, respectively and doses of 5 and 10 mg/kg inhibited IL-1β production by 30% and 70%, respectively.

**Effect of THOA on the open field test**

In the open-field test, the THOA had no significant effect on locomotor activity compared with the control and vehicle groups at the 10 mg/kg dose or another dose tested (data not shown). By contrast, morphine significantly decreased locomotor activity (fig. 4C).
Fig. 2: Evaluation of increasing doses of THOA (A) and evaluation of the dose of antagonists used concurrently with oral administration of THOA in the formalin test (B and C). Note: In B, the mice were pre-treated i.p. with L-arginine (1, 3, and 5 mg/kg) 15 min before the oral administration of THOA or L-NAME. In C, mice were pre-treated i.p. with L-NAME (1, 2, and 3 mg/kg) 15 min before injection of THOA. The dose of THOA used was 10 mg/kg, and that of L-NAME was 5 mg/kg. In A, the mice were pretreated with water, vehicle, morphine (5.01 mg/kg), Acetylsalicylic acid (ASA–200 mg/kg) or THOA (1, 5 and 10 mg/kg) 60 min before the formalin injection. The results are expressed as the mean±SEM (n=7-10). The statistical significance was calculated by One-way ANOVA followed by Bonferroni’s test. * p<0.05, ** p<0.01 and *** p<0.001 when comparing the THOA-, vehicle-and morphine-treated groups with the control group. In B, * p<0.05, ** p<0.01 and *** p<0.001 when comparing the group administered THOA alone with the THOA+antagonist-treated groups. * p<0.05, ** p<0.01 and *** p<0.001 when comparing the group administered L-NAME alone with the L-NAME+L-arginine-treated group. In C, ** p<0.01 and *** p<0.001 when comparing the group administered THOA alone with the THOA+antagonist-treated groups.

Fig. 3: The effects of orally administered THOA in evaluation of the hyper nociception inflammatory (A) and TNF-α (B) and IL-1β (C) production in the Von Frey test. Note: The mice were treated with water, vehicle, dexamethasone (2.25 mg/kg; s. c.) or the THOA (1, 5 and 10 mg/kg). PBS or carrageenan was applied in the paws of the animals. The results are expressed as the mean±SEM (n=7-10). The statistical significance was calculated by One-way ANOVA followed by Bonferroni’s test. * p<0.05, ** p<0.01 and *** p<0.001 when comparing the THOA-, vehicle-and dexamethasone-treated groups with the carrageenan-control group. * p<0.05, ** p<0.01 and *** p<0.001 when comparing carrageenan-control group with the PBS-control group.

In vivo toxicological evaluation

The THOA described in this paper was evaluated for acute toxicity in mice. No intoxication symptoms (convulsion, hyperactivity, grooming, loss of righting reflex, increased or decreased respiration, and sedation) were observed in the animals. The THOA was not toxic after oral administration (50 mg/kg). No lesions and hyperemia were observed in the gastric mucosa observed.
The present study aimed to evaluate the inflammatory hyper nociception induced by carrageenan in mice and the production of NO during inflammation, and this is related to the involvement of NO in peripheral nociception [38]. Previous administration of L-NAME (an NO synthase inhibitor) or L-arginine (a substrate of NO synthase) in the formalin test did not alter the antinociceptive effect of THOA. These results show that the production and release of NO is not important to the effect of the compound in the formalin test.

The effect of THOA was also evaluated in a model of mechanical inflammatory hyper nociception induced by carrageenan in mice and the production of IL-1β and TNF-α. Carrageenan is an inflammatory agent that is largely used as a pharmacological tool for investigating inflammatory hyper nociception in rats and mice. When injected intraplantarly in animal’s hind paw, it induces an inflammatory process associated with hyper nociception [18]. Tissue injury originated after the injection of carrageenan involves the release of different chemical mediators such as PGE2, mast cells products histamine and serotonin, neuropeptides, and pro-inflammatory cytokines among others [39, 40]. Thus, the obtained results show a relationship between inhibition of mechanical hyperalgesia and inhibition of the production/release of TNF-α and IL-1β.

The activation or overproduction of cytokines has related the onset of pain and hyperalgesia [41, 42]. Among the cytokines, TNF-α has the key role in the inflammatory and nociceptive process because it is able to stimulate its own production and the release of several other cytokines such as IL-6, IL-8 and IL-1β [39, 43]. The pro-inflammatory cytokine IL-1β is produced and secreted under pathological conditions by multiple cell types such as fibroblasts, lymphocytes and endothelial cells [44]. Its pro-nociceptive actions are mediated by production of a signaling cascade, involving the production of nitric oxide, bradykinin and prostaglandins [44].

In the air pouch model was confirmed the inhibitory effect on production and release of cytokines. This model is certainly suitable for investigating the inflammatory process which occurs during sterile inflammation and is suitable for determining the role of several molecules in inflammation, including cytokines and other inflammatory mediators.
chemokines because it consists of a sterile cavity without living cells and therefore free of pre-existing inflammatory conditions [45].

To exclude the possibility that the anti-nociceptive action of the THOA could be related to nonspecific disturbances in the locomotor activity of the animals, the open field test was used. We observed that at the doses that have anti-nociceptive action, the THOA did not alter the motor performance of the mice.

CONCLUSION

In conclusion, this study demonstrated the acute antinociceptive activity on inflammatory hyperalgesia in mice of the THOA. The compound inhibited the inflammatory mechanical hyperalgesia probably by inhibition of cytokines (TNF-α and IL-1β production). However, the levels of nitric oxide peripherals were not crucial to its ability to reduce the production of cytokines. These data provide initial evidence that THOA has potential to be developed as a drug for inflammatory and nociceptive events, but more studies are required to elucidate its mechanism of action further.

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CONFLICT OF INTERESTS

The authors declare that there is not conflict of interest.

REFERENCES


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