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Anti-inflammatory and Antinociceptive Effects of the Aqueous Extract of the Bark of *Chrysobalanus icaco Linnaeus*

Tatiane Bezerra de Oliveira[1*], Carlson Helder Reis de Carvalho Júnior[1], Fernanda Virgínia Barreto Mota[1], Larissa Cardoso Corrêa de Araújo[1], Maria Bernadete Sousa Maia[2], Karina Perrelli Randau[3], Silene Carneiro do Nascimento[1] and Teresinha Gonçalves da Silva[1]

[1]Department of Antibiotics, Federal University of Pernambuco, Recife, Pernambuco, Brazil.
[2]Department of Physiology and Pharmacology, Federal University of Pernambuco, Recife, Pernambuco, Brazil.
[3]Department of Pharmaceutical Science, Federal University of Pernambuco, Recife, Pernambuco, Brazil.

Authors' contributions

This work was carried out in collaboration between all authors. Authors TGS and SCN designed the study. Authors KPR, TBO, FVBM, CHRCJ and LCCA performed the experiments and managed the analyses of the study. Authors TBO, TGS and MBSM wrote the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: This paper describes the anti-inflammatory and antinociceptive effects of the aqueous extract of *Chrysobalanus icaco*.
Study Design: Study the anti-inflammatory and antinociceptive activity of the aqueous extract of *C. icaco* using *in vivo* models.
Place and Duration of Study: Department of Antibiotics and Department of Pharmaceutical Science, Federal University of Pernambuco, Recife, Pernambuco, Brazil, between March 2010 and March 2012.
Methodology: The anti-inflammatory activity was evaluated by carrageenan/dextran-induced paw edema, vascular permeability induced by acetic acid and subcutaneous air pouch models, with measurements of cell migration, nitric oxide and cytokines TNF-α and

**Corresponding author: Email: tacapitu@yahoo.com.br;*

IL-6. Acetic acid-induced abdominal writhing, formalin and hot plate tests were performed to investigate the antinociceptive effects.
Results: The aqueous extract of *C. icaco* (100, 200 and 400 mg/kg) reduced dextran/carrageenan-induced rat paw edema, the vascular permeability, cell migration, and nitric oxide concentration. However, it did not affect the levels of TNF-α and IL-6 produced in the pouch. The aqueous extract also demonstrated antinociceptive activity by acetic acid-induced abdominal writhing and formalin test, and was able to prolong the reaction time in the hot plate model in the first hour with activity similar to morphine (2.5 mg/kg, s.c.). There was no acute toxicity in mice after oral administration of the aqueous extract at doses of 2 g/kg.
Conclusion: Our results indicate that the aqueous extract of *C. icaco* demonstrated anti-inflammatory activity through the reduction of vascular permeability, inhibition of cellular migration and nitric oxide production and that the antinociceptive activity seems to be mediated by central mechanisms.

Keywords: Pain; anti-inflammatory activity; cytokines; herbal medicines nitric oxide.

1. INTRODUCTION

Chrysobalanus icaco Linnaeus is a shrub species belonging to the family *Chrysobalanaceae* with a distribution in the coastal areas in the Caribbean, South America and West Africa [1]. *C. icaco* has several uses, including food, medicine, handicrafts, and ornamental uses. In Brazil, the consumption of fruits of *C. icaco* in natura is much appreciated by coastal communities [2]. The leaves, bark and roots of this plant are used in traditional medicine in Brazil to control various diseases, such as diabetes, diarrhea and inflammation [3-5]. According to Alves et al. [6], the methanol extract obtained from the leaves has a potential anti-angiogenic effect and reduces the formation of new vessels by approximately 44%. The presence of diterpenes and triterpenes in the leaves of *C. icaco* has also been reported. Diterpenoids isolated from the leaves of *C. icaco* have been shown to act as inhibitors of human immunodeficiency virus (HIV) [7]. Pomolic acid, a triterpene isolated from the leaves of *C. icaco*, induced apoptosis in a multi-drug resistant leukemia cell line [8].

Phytochemical investigations report the presence of myricetin in *C. icaco* L. leaves [9]. Myricetin is considered a primitive flavonoid character, and its presence has been used as a chemotaxonomic marker in the *Chrysobalanaceae* family [10,11]. Studies in the literature have reported the trienoic and tetraenoic acids and their oxo derivatives [12] as well as catechol tannins in the seed oil of *C. icaco* [13].

Inflammation is an essential response to tissue injuries that are induced by physical, chemical or biological insults. Acute and chronic inflammation are essential to the restoration of homeostasis [14,15]. During the inflammation of peripheral tissues, numerous mediators are produced by endothelial cells, resident cells, and leukocytes that are recruited to the site of injury. Many of these mediators (e.g., protons, cytokines, and nerve growth factors) are known to elicit pain through the activation of specialized primary afferent neurons called nociceptors. The inflammatory response is amplified by migration of leukocytes into the inflamed tissue, by the production of cytokines, chemokines, and growth factors (e.g., nerve growth factor), and by tissue acidification [16,17].

Pain is an indication of inflammation, and inflammatory pain is a major clinical problem in many disorders, including arthritis. Inflammatory pain is characterized by increased

sensitivity of the affected tissue to mechanical or heat stimuli. Following the tissue injury, invading or resident immune cells release inflammatory mediators, including cytokines, chemokines, nerve growth factor, ATP and prostaglandins. Inflammatory mediators also arise from cell destruction (e.g., hydrogen ions) or are delivered by the circulation (e.g., bradykinin) [18]. Various inflammatory mediators as well as tissue acidification act synergistically to increase the activity of the nociceptive primary afferent neurons and to lower the threshold of nociceptors, which induces and maintains the development of pain and hyperalgesia [19,20]. Inflammatory diseases and pain are some of the most important health problems in the world. Currently, few drugs are effective in treating these disorders, and many of them cause severe side effects. Therefore, the search for new therapies is extremely important [21].

Despite its popular use for the treatment of infectious and inflammatory diseases, there are no published studies that evaluate the anti-inflammatory activities of the bark of *C. icaco*. Therefore, this study investigated the anti-inflammatory and antinociceptive properties of the aqueous extract of the bark of *C. icaco* to evaluate the anti-inflammatory effects of the plant that support its use in folk medicine.

2. MATERIALS AND METHODS

2.1 Experimental Animals

We used male wister rats (190-200 g) and male Swiss albino mice (30-35 g) for all pharmacological experiments, except for acute toxicity (both sexes of mice). The animals were obtained from the animal house of the Antibiotics Department of the Federal University of Pernambuco (UFPE), which is registered in the Brazilian College of Animal Experimentation (COBEA) under nº 18. The animals were kept in polypropylene boxes at a temperature of 22 ± 3°C with a light-dark cycle of 12 hours and received balanced feed and water *ad libitum*. The animals were fasted for 4 hours before each experiment.

2.2 Drugs and Reagents

Indomethacin and acetylsalicylic acid (ASA) were purchased from Sigma–Aldrich (St. Louis, MO, USA), and acetic acid was purchased from Merck (Rio de Janeiro, Brazil). EDTA was purchased from Labtest (São Paulo, Brazil). Mouse TNF-α and IL-1ß ELISA kits were purchased from *e*Bioscience (San Diego, CA, USA). Morphine sulfate was purchased from União Química (São Paulo, Brazil). All drugs were dissolved in 0.9% saline solution immediately prior to use. The negative control group was composed of animals treated with vehicle.

2.3 Collection of Plant Samples and Preparation of Extract

The bark of *C. icaco* was collected in the municipality of Japaratinga, Alagoas (Brazil) in April 2009. A voucher specimen is deposited in the herbarium of the Agricultural Research Institute of Pernambuco (Pernambuco, Brazil) under No. 83131. The bark was dried in an oven at 37°C and crushed for the preparation of the aqueous extract of *C. icaco* (AECI). The decoction was prepared using 200 g of powder in 1000 mL of water and was heated to 100°C for 30 minutes. The decoction was filtered, and the excess solvent was removed from the filtrate using a rotary evaporator at 60°C under reduced pressure. The residue was

subsequently lyophilized and stored at -20°C until use. Immediately prior to use, the dry extract was suspended in saline (0.9% NaCl).

2.4 Phytochemical Analysis of the Plant Extract

The chromatographic analyses were made by TLC on Si gel (MERCK-Germany, 105553) developed by different solvent systems [22,23] with little modification. It was verified the presence or absence of alkaloids, terpenes/sterols, saponins, flavonoids, phenylpropanoids, coumarins, and condensed proanthocyanidins, leucoanthocyanidins.

2.5 Acute Toxicity

The adopted parameters were determined according to the National Agency of Sanitary Surveillance of Brazil (ANVISA) [24]. Groups of 10 mice (five males and five females) received the oral vehicle (saline 0.9% NaCl) or AECI (2 g/kg). This dose was chosen following the recommendations of ANVISA for the welfare of the animals. The behavioral parameters observed were: attention, analgesia, spontaneous motor activity, locomotion, lack of appetite, apathy, response to touch, nasal discharge, piloerection, ptosis, respiratory, cyanosis, stereotypy, writhing, aggressiveness, ataxia and posture, urination, diarrhea and convulsions [25]. The animals were observed during one hour and 24 hours after the administration of the extract. The signs of toxicity and other parameters (weight gain, food and water consumption and mortality) were recorded during 14 days. On the last day of observation, the animals were anesthetized with a terminal overdose of xylazine/ketamine for the collection of blood samples by cardiac puncture for hematological analysis and for the observation of macroscopic changes of the viscera.

2.6 Carrageenan/dextran-induced Paw Edema

The model of rat paw edema was performed according to the procedure of Winter et al. [26]. Groups of 6 rats each were treated orally with AECI (100, 200 or 400 mg/kg), indomethacin (standard drug, 10 mg/kg), cyproheptadine (antihistamine, 10 mg/kg) or saline solution (NaCl 0.9%). One hour after receiving the drug(s), each animal received a subcutaneous injection of 0.1 mL of 1% carrageenan or 0.1% dextran in the right hind paw. The edema was measured immediately prior to the carrageenan injection and 1, 2, 3, and 4 hour later or immediately after dextran injection and 30, 60, 90 and 120 minutes. The paw edema volume was determined in milliliters as the difference between the final and initial volumes and was assessed with a plethysmometer from Ugo Basile, Italy.

2.7 Acetic Acid-induced Increase in Vascular Permeability

The test was carried out using the technique described by Whittle [27]. Mice were treated orally with saline, AECI (100, 200 and 400 mg/kg) or indomethacin (10 mg/kg). Thirty minutes after these treatments, each animal received an intravenous injection of 2% Evan's blue solution (w/v) in 0.9% of saline. Thirty minutes later, each mouse received intraperitoneally 0.5 mL of 0.1% acetic acid solution. Twenty minutes after acetic acid injection, the animals were euthanized in a CO_2 chamber, the peritoneal fluid was collected and the concentration of Evan's blue was measured by absorbance at 630 nm in a spectrophotometer.

2.8 Subcutaneous Air Pouch (SAP)

The anti-inflammatory activities of AECI were tested by the formation of air pouches on the dorsal cervical region of mice via a subcutaneous injection of 2.5 mL of sterile air on day 0, which was followed by a second injection of 2.5 mL of sterile air 3 days later. On day six, the mice orally (p.o) received AECI (100, 200 or 400 mg/kg), indomethacin (10 mg/kg) or vehicle. The doses were chosen according to the results of the acute toxicity test. One hour after administration of the drug, inflammation was induced by injecting 1 mL of carrageenan suspension (1% in saline solution) into the air pouch. After 6 h, the animals were euthanized in a CO_2 chamber, and the pouches were washed with 3 mL of saline solution containing 3 μM EDTA. Polymorphonuclear leukocytes were counted using an ABX Micros 60 hematology analyzer [28]. The average number of leukocytes from the treated groups was compared with the number of leukocytes of the control group, which was defined as 100%.

The exudates collected from the air pouch were stored in a freezer at −20°C for determination of cytokine and nitric oxide levels. The quantification of these mediators was performed with doses that showed better inhibition of the migration of polymorphonuclear cells in the air pouch experiment (over 50 % of inhibition).

2.9 Quantification of TNF-α and IL-6 Levels

Quantification of the TNF-α and IL-6 levels in the supernatants were performed by sandwich ELISA using kits that were specific for mice according to the manufacturer's instructions (*e*Bioscience, San Diego, California, USA). The lower detection limit of the assays was 10 pg/mL.

2.10 Nitrate Measurement

To evaluate nitric oxide (NO) production, nitrate (the stable metabolite of NO) concentrations in the supernatants were measured according to the protocol described previously [29]. An aliquot of 100 μL of sample was transferred to a microplate and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 10% H_3PO_4) for 10 min at room temperature. The absorbance was measured at 540 nm using a microplate reader, and the nitrate concentration was calculated using a standard curve of sodium nitrate.

2.11 Acetic Acid-induced Abdominal Writhing

The model of abdominal constrictions induced by acetic acid was performed according to the procedure of Koster et al. [30] with minor modifications. Mice received AECI (100, 200 or 400 mg/kg, p.o.), acetylsalicylic acid (ASA) (200 mg/kg, p.o.) or saline (p.o.) 60 minutes before the administration of acetic acid. After one hour, 1% acetic acid was injected (0.1 mL/10 g) into the peritoneal cavity of the animals. Ten minutes later, the mice were observed, and the level of writhing during a 20 minutes period was recorded. The percentage inhibition of writhing was calculated by comparing the average contortions of the treated and control groups.

2.12 Formalin Test

The mice received an injection of 20 µL formalin (2.5%, v/v) into the dorsal surface of the left hind paw. Immediately after injection, we recorded the time that the mice spent licking the injected paw [31]. The nociceptive and inflammatory response developed in two phases: the first occurred 5 min after the formalin injection (first phase, neurogenic pain response), and the second occurred 15–30 min after the formalin injection (second phase, inflammatory pain response). The animals were pre-treated with oral doses of AECI (200 mg/kg), ASA (200 mg/kg) or saline 60 min before injection of the formalin. A dose of 200 mg/kg AECI was chosen as the best dose for the acetic acid-induced abdominal writhing test. The morphine sulfate (2.5 mg/kg, s.c.) was administered 45 minutes before the induction of nociception.

2.13 Hot Plate

This test followed the procedure described by Adzu et al. [32] with minor modifications. The mice were placed on a hot plate (Insight equipments, Ribeirão Preto, SP, Brazil) set at 55±1°C. Latency of the discomfort reactions (i.e., paw licking or jumping) was determined before and after the administration of the drug. The cut-off time was 30 seconds. Reaction times were recorded when the animals licked their fore- and hindpaws and jumped at several intervals before and 30, 60, 120, 180 and 240 minutes following the administration of AECI (200 mg/kg, p.o.), morphine (2.5 mg/kg, s.c.) or saline (p.o.).

2.14 Statistical Analysis

All of the experimental groups consisted of 6 animals. The results are presented as the means±S.D. Differences between the groups were determined by analysis of variance (ANOVA – one way), followed by Bonferroni's post hoc test. The results were considered statistically significant when $*P < 0.05$, $** P < 0.01$, $*** P < 0.001$ and $**** P < 0.0001$. Analyses were performed using GraphPad Prism 5.0.

3. RESULTS

The phytochemical profile from the aqueous extract of stem bark of *C. icaco* detected the presence of triterpenes, steroids and flavonoids. On the other hand, it wasn't verified the presence of alkaloids, coumarins, condensed proanthocyanidins, leucoanthocyanidins and saponins. Studies in the literature have reported the presence of terpenes and flavonoids in fruits and leaves of *Chrysobalanaceae* [7-9,33,34].

In the acute toxicity study, all the animals treated orally with AECI at 2 g/kg remained alive and did not manifest any significant visible signs of toxicity at the doses evaluated. There were no changes in physiological parameters (weight gain, feed and water intake) and no occurrence of death throughout the experiment. The hematological parameters were evaluated 14 days after the oral administration of extract. It was evaluated red blood cells, hemoglobin, hematocrit, mean corpuscular hemoglobin concentration, mean cell volume, mean corpuscular hemoglobin, white blood cells and platelets. There were no changes in hematological parameters evaluated when compared to control (data not shown).

The treatment with AECI at doses of 100, 200 and 400 mg/kg were able to significantly reduce the paw edema induced by carrageenan and dextran in all time measured. The

maximum inhibition of edema swelling was noted with dose of 400 mg/kg (53%) for both types of edema Table 1.

The oral administrations of AECI in all doses reduced the vascular permeability induced by acetic acid Table 2. AECI in the dose of 400 mg/kg showed effects similar to indomethacin (10 mg/kg).

The treatment with the AECI demonstrated anti-inflammatory activity at all doses tested in the subcutaneous air pouch model, reducing cell migration Table 3. The dose of 400 mg/kg showed anti-inflammatory activity similar to indomethacin (10 mg/kg). The groups treated with AECI at doses of 200 and 400 mg/kg significantly decreased the NO levels when compared to the control group Fig. 1.

Table 1. Effect of AECI on carrageenan- and dextran-induced paw edema in rats

Group	Dose (mg/kg)	Carrageenan induced paw edema	% Inhibition	Dextran induced paw edema	% Inhibition
Control	-	1.65 ± 0.08	-	0.64 ± 0.05	-
AECI	100	0.86 ± 0.02****	48	0.38 ± 0.06***	41
	200	0.80 ± 0.05****	51	0.32 ± 0.07***	50
	400	0.78 ± 0.05****	53	0.30 ± 0.04***	53
Indomethacin	10	0.51± 0.04****	69		
Cyproheptadine	10			0.23 ± 0.08***	64

*Data represent the means ± SD volume of edema (mL) at times 3 hours after injection of 1% carrageenan and 30 minutes after injection of 0.1% dextran. (N=6); ***P < 0.001 ****P< 0.0001 vs. control. Significance was determined using ANOVA followed by Bonferroni's post hoc test*

Table 2. Effects of AECI on vascular permeability induced by acetic acid

Group	Dose (mg/kg)	Dye leakage (µg/mL)	% Inhibition
Control	-	50.6 ± 1.77	-
AECI	100	31.2 ± 1.64****	39
	200	26.6 ± 2.30****	45
	400	21.5 ± 1.73****	59
Indomethacin	10	19.4 ± 1.82****	63

*Values are expressed as mean ± S.D.; (N = 6). ****P< 0.0001 v.s Control. Significance was determined using ANOVA followed by Bonferroni's post hoc test*

Table 3. Effect of EACI on cell migration in a subcutaneous air pouch

Group	Dose (mg/kg)	Celullar migration (10^3 / mm^3)	Inhibition %)
Control carrageenan	-	11.3 ± 1.62	-
Control saline	-	0.26 ± 0.05***	-
AECI	100	6.93 ± 0.80***	36.1
	200	5.60 ± 0.41***	50.4
	400	4.72 ± 0.94***#	58.3
Indomethacin	10	3.88 ± 0.69***	65.6

*Values are expressed as mean ± S.D.; (N = 6). ***P<0.001 v.s Control carrageenan. # - Not significant v.s indomethacin. Significance was determined using ANOVA followed by Bonferroni's post hoc test*

The groups treated with AECI (200 or 400 mg/kg) showed no reduction in the levels of TNF-α (629±27 and 578±26 pg/mL, respectively) when compared to the control group (613±25 pg/mL) Fig. 2A. The levels of IL-6 were not reducing in the groups treated with the AECI at doses 200 and 400 mg/kg (2704±54 and 2783±10 pg/mL, respectively) when compared to the control group (2655±83 pg/mL) Fig. 2B.

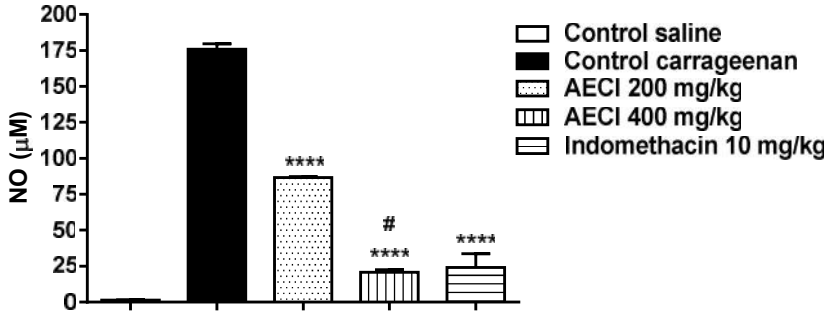

Fig. 1. Effect of AECI on the level of nitric oxide in the inflammatory exudate
*Values are expressed as the means ± SD (n = 6), **** P <0.0001 vs. control carrageenan. # - Not significant vs. indomethacin. Significance was determined using ANOVA followed by Bonferroni's post hoc test*

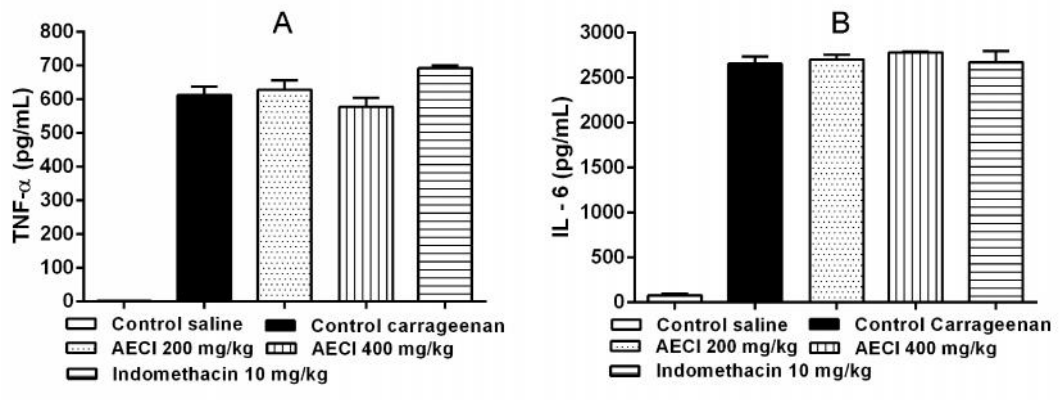

Fig. 2. Effect of AECI on the level of cytokines in the inflammatory exudate
Values are expressed as the means ± SD (n = 6). Significance was determined using ANOVA followed by Bonferroni's post hoc test

Treatment with AECI significantly reduced the number of writhing induced by acetic acid Fig. 3. Doses of 200 and 400 mg/kg showed an antinociceptive effect similar to those obtained with ASA (200 mg/kg, orally). The other tests of antinociception were performed at a dose of 200 mg/kg because this dose had an antinociceptive effect similar to the dose of 400 mg/kg.

AECI (200 mg/kg) significantly reduced the licking time induced by 2.5% formalin in first phase Fig. 4A and second phase Fig. 4B. In the first phase, AECI had antinociceptive activity similar to that of morphine Fig. 4A.

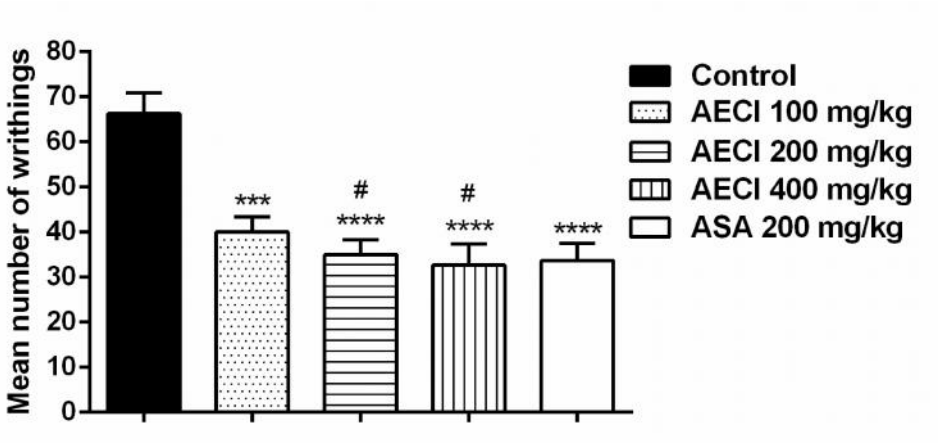

Fig. 3. Effect of AECI on abdominal writhing induced by acetic acid

*Values are expressed as the means ± SD and represent the number of writhings induced by 1% acetic acid (n = 6), *** P <0.001, **** P <0.0001 vs. control. # - Not significant vs. ASA. Significance was determined using ANOVA followed by Bonferroni's post hoc test*

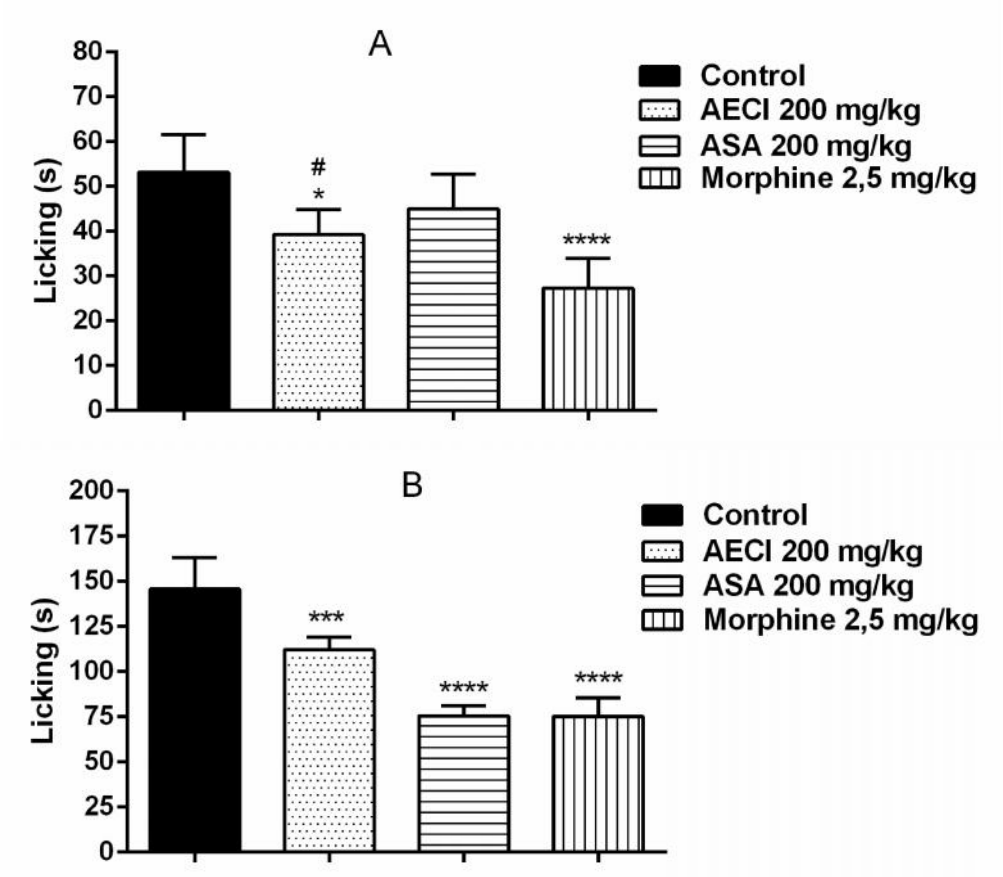

Fig. 4. Effect of AECI on nociception induced by 2.5% formalin

*A - First phase B - Second phase. Values are expressed as the means ± SD (n = 6), * P <0.001 *** P <0.001 **** P<0.0001 vs. control. # - Not significant vs. ASA. Significance was determined using ANOVA followed by Bonferroni's post hoc test*

AECI at a dose of 200 mg/kg significantly prolonged the reaction time in the hot plate test (55 ± 1°C), when compared with the control only in the first hour after administration of the extract Table 4, and this activity was similar to that of morphine.

Table 4. Effect of AECI on the response to temperature-induced nociception

Group	Dose (mg/ kg)	Latency time (s)					
		0	30	60	120	180	240
Control	-	9.0±0.3	9.4±0.3	10.2±1.3	9.0±0.5	9.5±1.3	10.7±1.2
AECI	200	9.3±1.3	10.6±0.9	13.6±1.2*#	9.6±0.5	10.2±0.9	8.8±0.3
Morphine	2,5	9.4±0.5	11.7±1.5*	12.5±1.8*	15.4±1.1**	16.7±1.7**	16.3±1.4**

*Data represent the means ± SD of the latency time(s) for paw licking or jumping at a temperature of 55 ± 1 °C at 0, 30, 60, 120, 180 and 240 minutes after treatment (n = 6). *P <0.05, **P <0.01 vs. Control. #not significant vs. morphine. Significance was determined using ANOVA followed by Bonferroni's post hoc test*

4. DISCUSSION

This study investigated the use in traditional medicine of the bark of *C. icaco* by studying its activity in acute inflammation and antinoceptive models. The rat paw edema model has been widely used for research on new drugs. Inflammation induced by carrageenan is an acute process that is highly reproducible and well-studied. The development of edema induced by carrageenan is a biphasic model with multiple mediators acting in sequence to produce the inflammatory response [35].

In the early phase (1-2 h), the release of histamine, serotonin, bradykinin and cytokines occurs, and this release promotes vasodilation and increased vascular permeability. In addition to triggering the formation of edema, these mediators contribute significantly to cell migration, especially the migration of neutrophils to the inflammatory site. The later phase (3-5 h) is characterized by the elevated production of prostaglandins, the activation of COX-2 and NO release [36,37]. AECI at all doses showed significant inhibition of edema in both phase of the inflammatory response. Based on this observation, the anti-inflammatory effect shown by AECI may be attributed to the inhibition of the release of proinflammatory mediators of acute inflammation, such as histamine, prostaglandins and NO.

Dextran is a polysaccharide which stimulates the degranulation of macrophages, promoting the release of vasoactive amines. The main mediators in this model histamine and serotonin are important vasodilator responsible for increased vascular permeability and edema formation [38]. In this study, it was shown that AECI inhibited the edema induced by dextran in rats; these results suggest that this extract intervenes in inflammatory cell degranulation and release of vasoactive amines such as histamine, serotonin and bradykinin.

The increased vascular permeability induced by acetic acid is known to induce inflammation exudative leads to initial release of chemical mediators such as prostaglandin E2 (PGE2), histamine and serotonin peritoneal fluid, resulting in increased vascular permeability [39]. The results suggest that the AECI inhibits the release of vasoactive amines and prostaglandins. These results corroborate the data presented in the model of dextran paw edema.

The air pouch model is commonly used for screening new drugs for the treatment of arthritis. This model mimics the inflamed synovial membrane of patients with rheumatoid arthritis, specifically through the infiltration of the polymorphonuclear cells and the release of proinflammatory mediators [40]. The treatment of animals with AECI at dose of 400 mg/kg

inhibited the migration of polymorphonuclear leukocytes to the inflammatory site, similarly to the inhibition produced by indomethacin, and indicated that AECI has a potential anti-inflammatory effect.

To elucidate the molecular mechanism of action, were evaluated the anti-inflammatory and analgesic properties of AECI and subsequently determined the molecular mechanisms of the properties by focusing on the amounts of the proinflammatory cytokines TNF-α, IL-6 and NO in the air pouch exudates. It was observed that carrageenan dramatically increased the TNF-α and IL-6 concentrations in the exudate of the air pouch and was not significantly reduced by treatment with AECI. However, the level of NO was reduced significantly by this treatment. NO is an important regulatory molecule in a variety of physiological functions, such as vasodilatation, that leads to vascular leakage [41]. Induction and activation of nitric oxide synthases (NOS) and excessive production of NO are common features of almost all diseases associated with infection and acute or chronic inflammation, although the contribution of NO to the pathophysiology of these diseases is highly multifactorial and still a matter of controversy [42]. Vasodilatation, a feature of acute inflammation, is dependent on NO release for the production of inflammatory mediators, such as bradykinin, histamine, and substance P, and could increase edema formation and facilitate leukocyte migration [43,44]. Therefore, we suggest that the inhibition of edema and cell migration caused by treatment with AECI is dependent on the signaling pathway of nitric oxide.

An analgesic effect usually accompanies an anti-inflammatory effect. Therefore, the analgesic effect of AEC was examined in three nociceptive animal models: acetic acid-induced abdominal writhing, formalin and the hot plate test. Among the models of nociception used in this study, the test of writhing induced by acetic acid is a typical model to assess pain of an inflammatory origin. This test is not specific but has good sensitivity and is a screening tool used to evaluate the analgesic and anti-inflammatory activity of new agents [45]. Acetic acid acts by releasing endogenous mediators that stimulate the nociceptive neurons [35]. In this study, AECI was able to reduce the contortions at doses of 100, 200 and 400 mg/kg. The model of writhing induced by acetic acid is not a specific model because different classes of compounds also inhibit writhing, such as hypotensive agents, depressants and stimulants of the nervous system, and antihistamines [45]. Therefore, the interpretation of pain reduction through stimulation by acetic acid should be conducted cautiously and considered along with the results of other tests.

The advantage of the formalin model of nociception is that it can be used to discriminate pain in the central and peripheral components [46,47]. The test consists of two different phases separated in time. The first phase (0-5 min) corresponds to neurogenic pain, and the second phase (15-30 min) corresponds to inflammatory pain. Morphine, a typical opioid drug, inhibits nociception in both phases, but drugs with peripheral action, such as corticosteroids and indomethacin, inhibit only the second phase. Moreover, drugs such as acetylsalicylic acid, which inhibit prostaglandin synthesis, block only the second phase of the formalin test [48]. AECI was able to decrease the time that animals spent licking the injected paw during both phases. The effect produced in the first stage was similar to that presented by morphine. These results suggest that the antinociceptive effect presented by AECI involves central mechanisms. To confirm this hypothesis, we performed the hot plate test, in which AECI demonstrated nociceptive effects during the first 60 minutes. This result indicates that the extract has a central analgesic activity. This activity was shown to have short duration. The cause for this short duration is not known, but may involve the metabolism or pharmacokinetics of bioactive compounds in the extract.

Although the elucidation of the molecular mechanisms of action requires further study, AECI attenuated carrageenan-induced inflammation by reducing cell migration and the levels of NO in the exudate of the air pocket and additionally showed no significant effect on the concentrations of TNF-α and IL-6. AECI showed an antinociceptive effect on both inflammatory and neurogenic pain.

Phytochemical studies of *Chrysobalanaceae* family show a predominance of terpenes, including core kaurene diterpene, flavonoids and their glycosides.

In this study, we confirmed the presence of the triterpene, steroids and flavonoids. Triterpenoids are a large class of plant secondary metabolites that have been demonstrated to exhibit a variety of biological activities including anti-inflammatory and antinociceptive activities [49-51]. Lupeol, a pentacyclic triterpene, is a biologically active constituent that has anti-inflammatory effects [50]. The carnosol (diterpene) and a mixture of ursolic acid /oleanolic acid (triterpenes) isolated from *Salvia officinalis* reduced the inflammatory phase of formalin test and cinnamaldehyde-induced nociception [51]. It was reported the antinociceptive effect of oleanolic acid against abdominal constrictions induced by acetic acid and nociceptive behavior induced by capsaicin involves the participation of opiod system [52,53].

A variety of biological effects have been ascribed to flavonoids. These polyphenolic compounds and their derivatives display a remarkable spectrum of biological activities including anti-inflammatory. Special attention has been given to their antioxidant and anti-inflammatory properties, *in vitro* and *in vivo* [54-56]. The molecular mechanisms involved in the anti-inflammatory activities of flavonoids have been suggested to include inhibition of pro-inflammatory enzymes, such as cyclooxygenase-2, lipoxygenase, inducible NO synthase, and inhibition of NF-kB [57]. Therefore, the anti-inflammatory and antinociceptive activities shown by aqueous extracts of *C. icaco* may be attributed to the presence of flavonoids and terpenes, but studies for the isolation of chemical constituents are being conducted to confirm this hypothesis.

5. CONCLUSION

This study investigated the acute toxicity and the pharmacological basis for the use of the bark of *C. icaco* in folk medicine by studying its activity in models of acute inflammation. In addition, the antinociceptive activity and toxicity of the aqueous extract of C. icacowere investigated. The AECI not shown to be toxic because it does not alter physiological and behavioral parameters. The results obtained in this study showed that AECI possesses anti-inflammatory, antinociceptive properties and support the traditional use of this plant to treat inflammatory conditions.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Animal Studies Committee of the Federal University of Pernambuco approved the experimental protocols (number 23076.050728/2010-84). The experimental protocols were

conducted following the technical and ethical principles recommended by the norms of the National Institute of Health Guide for Care and Use of Laboratory Animals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Smith N, Mori SA, Henderson A, Stevenson DW, Heald SV. Flowering plants of the neotropics. 1st ed. Princeton and Oxford published in association with The New York Botanical Garden; 2004.
2. Fonseca-Kruel VS, Peixoto AL. Etnobotânica na reserva extrativista marinha de arraial do Cabo, RJ, Brasil. Acta Bot Brasilica. 2004;18:177-190. Portuguese
3. Costa OA. Brazilian plants with hypoglycaemic effects. Leandra. 1977;7:63-75.
4. Barbosa-Filho JM, Vasconcelos THC, Alencar AA, Batista LM, Oliveira RAG, Guedes ND, et al. Plants and their active constituents from South, Central, and North America with hypoglycemic activity. Rev Bras Farmacogn. 2005;15:392-413.
5. Agra MF, Freitas PF, Barbosa-Filho JM. Synopsis of the plants known as medicinal and poisonous in Northeast of Brazil. Rev Bras Farmacogn. 2007;17:114-140.
6. Alves SP, Teruszkin BI, Henriques NS, Castilho RO, Kaplan MAC, Cabral MC, et al. *Chrysobalanus icaco* L. extract for antiangiogenic Potential observation. Int J Mol Cell Med. 2000;5:667-669.
7. Gustafson KR, Munro MHG, Blunt JW, Cardellina JH, Mcmahon JB, Gulakowski RJ, et al. HIV inhibitory natural products. 3. Diterpenes from *Homalanthus acuminatus* and *Chrysobalanus icaco*. Tetrahedron. 1991;47(26):4547- 4554.
8. Fernandes J, Castilho RO, da Costa MR, Wagner KS, Kaplan MAC, Gattass CR. Pentacyclic triterpenes from *Chrysobalanaceae* species: cytotoxicity on multidrug resistant and sensitive leukemia cell lines. Cancer Lett. 2003;190:165-169.
9. Chadefaud M, Emberger L. Chrysobalanacées. In: Traite' de Botanique Systematique, Vol. 2, Published Masson, Paris; 1960. French
10. Coradin L, Giannasi DE, Prance AT. Chemosystematic studies in the Chrysobalanaceae. Flavonoids in *Parinari*. Brittonia. 1985;37:169-178.
11. Mendez J, Bilia AR, Morelli I. Phytochemical investigations of *Licania genus*. Flavonoids and triterpenoids from *Licania pittieri*. Pharm Acta Helv. 1995;70(1):223-226.
12. Gunstone FD, Subbarao R. New tropical seed oils. Part I. conjugated trienoic and tetraenoic acids and their oxoderivatives in the seed oils of *Chrysobalanus icaco* and *Parinarium laurinum*. Chem Phys Lipids. 1967;1:349-359.
13. Verma VS, Raychaudhuri SP. Effect of catechol tannins isolated from a medicinal plant *Chrysobalanus icaco* on the infectivity of potato virus X. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg. 1972;127:178-179.
14. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. Science. 2010;327:291–295.
15. Cain D, Kondo M, Chen H, Kelsoe G. Effects of acute and chronic inflammation on B-cell development and differentiation. J Investig Dermatol Symp Proc. 2009;129:266–277.
16. Cabot PJ, Carter L, Gaiddon C, Zhang Q, Schäfer M, Loeffler JP, et al. Immune cell-derived beta-endorphin. Production, release, and control of inflammatory pain in rats. J Clin Invest. 1997;100:142–148.

17. Cao YQ, Mantyh PW, Carlson EJ, Gillespie AM, Epstein CJ, Basbaum AI. Primary afferent tachykinins are required to experience moderate to intense pain. Nature. 1998; 392:390–394.
18. Schaible HG. Peripheral and central mechanisms of pain generation. Handb Exp Pharmacol. 2007;177:3–28
19. Coutaux A, Adam F, Willer JC, Le Bars D. Hyperalgesia and allodynia: peripheral mechanisms. Joint Bone Spine. 2005;72:359–371.
20. Hua S, Cabot PJ. Mechanisms of peripheral immune-cell-mediated analgesia in inflammation: clinical and therapeutic implications. Trends Pharmacol Science. 2010;31:427-433.
21. Marinho DG, Alviano DS, Matheus ME, Alviano CS, Fernandes PD. The latex obtained from *Hancornia speciosa* Gomes possesses anti-inflammatory activity. J Ethnopharmacol. 2011;135:530–537.
22. Harbone JB. Phytochemical methods. A guide to modern techniques of plant analysis. Chapman and Hill, 3rd ed., London; 1998.
23. Wagner H, Bladt S. Plant Drug Analysis. A Thin Layer Chromatography Atlas, third ed., Springer, Berlim; 2001.
24. National Health Surveillance Agency (ANVISA): Guia para a condução de Estudos não clínicos de Segurança necessários ao Desenvolvimento de Medicamentos; 2010. Accessed Jan 2011. Available:http://portal.anvisa.gov.br/wps/wcm/connect/30dd7a0047457fa68b53df3fbc4c6735/guia+para+a+condu%c3%87%c3%83o+de+estudos+n%c3%83o+cl%c3%8dnicos+de+seguran%c3%87a+necess%c3%81rios+ao+desenvolvimento+de+medicamentos.pdf?mod=ajperes. Portuguese
25. Malone MH, Robichaud RC. A Hippocratic screen for pure and crude drug materials. Lloydia. 1962;25:320–332.
26. Winter CA, Risley EQ, Nuss GW. Carrageenan-induced edema in hind paw of rat as an assay for anti-inflammatory drugs. Proc Soc Exp Biol Med. 1962;11:544–547.
27. Whittle BA. The use of changes in capillary permeability in mice to distinguish between narcotic and nonnarcotic analgesics. Br Pharmacol Chemother. 1964;22: 246-53.
28. Guerra ASHS, Malta DJN, Laranjeira LPM, Maia MBS, Colaço NC, Lima MCA, et al. Anti-inflammatory and antinociceptive activities of indole-imidazolidine derivatives. Int Immunopharmacol. 2011;11(11):1816-1822.
29. Xu J, Xu X, Verstraete W. Adaptation of *E. coli* cell method for microscale nitrate measurement with the Griess reaction in culture media. J Microbiol Methods. 2000;41:23–33.
30. Koster R, Anderson M, De Debeer EJ. Acetic acid for analgesic screening. Federation Proceeding. 1959;18:412-418.
31. Gomes NM, Rezende CM, Fontes SP, Matheus ME, Fernandes PD. Antinociceptive activity of Amazonian Copaíba oils. J Ethnopharmacol. 2007;109:486–492.
32. Adzu B, Amos S, Kapu SD, Gamaniel KS. Anti-inflammatory and anti-nociceptive effects of *Sphaeranthus senegalensis*. J Ethnopharmacol. 2003;84:169–173.
33. Castilho RO, Oliveira RR, Kaplan MAC. Licanolide, a new triterpene lactone from *Licania tomentosa*. Fitoterapia. 2005;76:562–566.
34. Castilho RO, Kaplan MAC. Constituintes químicos de *Licania tomentosa* BENTH. (CHRYSOBALANACEAE). Química Nova. 2008;31:66-69.
35. Guzzo LS, Saúde-Guimarães DAS, Silva ACA, Lombardi JA, Guimarães HN, Grabe-Guimarães A. Antinociceptive and anti-inflammatory activities of ethanolic extracts of *Lychnophora* species. J Ethnopharmacol. 2008;116:120–124.

36. Posadas I, Bucci M, Roviezzo F, Rossi A, Parente L, Sautebin L, et al. Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression. Br J Pharmacol. 2004;142:331–338.
37. Morris CJ. Carrageenan-induced paw edema in the rat and mouse. In: Methods in Molecular Biology, 1st ed. Totowa, Humana Press Inc.; 2003.
38. Lo TN, Almeida AP, Beaven MA. Dextran and carrageenan evoke different inflammatory responses in rat with respect to composition of infiltrates and effect of indomethacin. J Pharmacol Exper Therap. 1982;221(1): 261-267.
39. Choi JH, Jung BH, Kang OH, Choi HJ, Parques PS, Cho SH, et al. The Anti-inflammatory and Anti-nociceptive Effects of Ethyl Acetate Fraction of *Cynanchi Paniculati Radix*. Biol Pharm Bull. 2006;29(5):971-975.
40. Sedgwick AD, Lees P. A comparison of air pouch, sponge and pleurisy models of acute carrageenan inflammation in the rat. Agents Actions. 1986;18(3-4):439.
41. Shin S, Jeon JH, Park D, Jang JY, Joo SS, Hwang BY, et al. Anti-inflammatory effects of an ethanol extract of *Angelica gigas* in a Carrageenan-air pouch inflammation model. Exp Anim. 2009;58:431-436.
42. Hamza M, Wang XM, Wu T, Brahim JS, Rowan JS, Dionne RA. Nitric oxide is negatively correlated to pain during acute inflammation. Mol Pain. 2010;15(6):55-65.
43. Wright CD, Mülsch A, Busse R, Osswald H. Generation of nitric oxide by human neutrophils. Biochem Biophys Res Commun. 1989;160:813-819.
44. Sautebin L, Ialenti A, Ianaro A, Di Rosa M. Endogenous nitric oxide increases prostaglandin biosynthesis in carrageenin rat paw oedema. Eur J Pharmacol. 1995;286:219-222.
45. Le Bars D, Gozariu M, Cadden SW. Animals models of nociception. Pharmacol Rev 2001;53:597-652.
46. Belvisi MG, Chung KF, Jackson DM, Barnes PJ. Opioid modulation of noncholinergic neural bronchoconstriction in guinea-pig *in vivo.* Br J Pharmacol. 1998;95:413–418.
47. Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. Pain. 1992;51(1):5–17.
48. Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain. 1987;30(1):103-114.
49. Ryu SY, Oak MH, Yoon SK, Cho DI, Yoo GS, Kim TS, et al. Anti-allergic and anti-inflammatory triterpenes from herb of *Prunella vulgaris*. Planta Med. 2000;66:358–360.
50. Geetha T, Varalakshmi P. Anti-inflammatory activity of lupeol and lupeol linoleate in rats. J Ethnopharmacol. 2001;76:77–80.
51. Rodrigues MR, Kanazawa LK, das Neves TL, Silva CF, Horst H, Pizzolatti MG, et al. Antinociceptive and anti-inflammatory potential of extract and isolated compounds from the leaves of *Salvia officinalis* in mice. J Ethnopharmacol. 2012;139(2):519-26.
52. Maia JL, Lima-Junior RC, Melo CM, David JM, Campos AR, Santos FA, et al. Oleanolic acid, a pentacyclic triterpene attenuates capsaicin-induced nociception in mice: possible mechanisms. Pharmacol Res. 2006;54:282–286.
53. Freitas CS, Baggio CH, Dos Santos AC, Mayer B, Twardowschy A, Luiz AP, et al. Antinociceptive properties of the hydroalcoholic extract, fractions and compounds obtained from the aerial parts of *Baccharis illinita* DC in mice. Basic Clin Pharmacol Toxicol. 2009;104:285–292.
54. Meotti FC, Luiz AP, Pizzolatti MG, Kassuya CAL, Calixto JB, Santos AR. Analysis of the Antinociceptive Effect of the Flavonoid Myricitrin: Evidence for a Role of the L-Arginine-Nitric Oxide and Protein Kinase C Pathways. J Pharmacol Exp Ther. 2006;316:789–796.

55. Calixto JB, Otuki MF, Santos ARS. Anti-inflammatory compounds of plant origin: I. Action on arachidonic acid pathway, nitric oxide and nuclear factor κB (NFκB). Planta Med. 2003;69:973–983.
56. Calixto JB, Campos MM, Otuki MF, Santos ARS. Anti-inflammatory compounds of plant origin: II. Modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. Planta Med. 2004;70:93–103.
57. Serafini M, Peluso I, Raguzzini A. Antioxidants and the immune system Flavonoids as anti-inflammatory agents. Proc Nut Society. 2010;69:273–278.

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