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Cyane-carvone, a Synthetic Derivative of Carvone, Inhibits Inflammatory Response by Reducing Cytokine Production and Oxidative Stress and Shows Antinociceptive Effect in Mice

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Abstract—Cyane-carvone (CC) was studied to elucidate its anti-inflammatory, antinociceptive, and antioxidant effects in *Mus musculus*. Anti-inflammatory (bradykinin, histamine, prostaglandin E_2, serotonin, and carrageenan) and antinociceptive (acetic acid and formalin) models were utilized. Myeloperoxidase activity, interleukin (IL)-1β, tumor necrosis factor alpha (TNF-α), and glutathione (GSH) levels were evaluated. Analysis of variance followed by Student-Newman-Keuls' test was done. Results were compared with control groups (significantly when $p<0.05$). In bradykinin, histamine, prostaglandin E_2, and serotonin tests, 75 mg/kg CC decreased significantly paw edema (t=30, 60, 90, and/or 120 min). In carrageenan test, 50 and 75 mg/kg CC (t=3 h and t=4 h) and 25 mg/kg CC (t=4 h) decreased significantly paw edema. CC (75 mg/kg) inhibited significantly mieloperoxidase activity and decreased IL-1β and TNF-α, and all doses increased GSH levels. CC (75 mg/kg) decreased significantly the number of contortions of animals and time of licking (phase 2). CC showed anti-inflammatory, antinociceptive, and antioxidant effects in mice.

KEY WORDS: anti-inflammatory; antinociceptive; cyane-carvone; mice.

INTRODUCTION

Inflammation is a response of the body to injury that occurs in an organism, detecting threats and damages [1]. This process occurs as a defensive response, which induces profound physiological adaptations triggered in an attempt to limit tissue damage and remove the pathogenic insult. Mechanisms of inflammation involve dilatation of arterioles, venules, and capillaries; exudation of fluids; and leukocyte migration into the inflammatory area [2].

Myeloperoxidase (MPO) is stored in azurophilic granules of polymorphonuclear neutrophils and macrophages, and this enzyme is involved in the oxidative stress and inflammation [3]. Cytokines are proteins that control inflammatory responses. Two cytokines, tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1, are involved in rheumatoid diseases, such as arthritis. The most studied of the IL-1 proteins are IL-1α and IL-1β, regulated by transcriptional and posttranslational mechanisms or natural competitive antagonist [4].

Several studies associate brain and chronic pain. Neuroimaging modified knowledge of how pain affects the brain. The new notion is that chronic pain as a very complex state in which patterns of sensory system activation are integrated aberrantly with activity in

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other brain systems, including emotional, cognitive, and modulatory processes. Chronic pain is a direct result of neurological disease or may even be considered an integral part of underlying disease [5].

This work investigates a synthetic drug as a possible treatment for diseases involving pain and/or inflammation. The carvone (*p*-mentha-6,8-dien-2-one) is a monoterpene ketone found as the major active component in various essential oils of medicinal plants [6]. It has antimicrobial [7], fungicidal, and fungistatic [8] properties. Cyane-carvone (CC), a synthetic derivative of carvone, possesses anticonvulsant activity probably due to modulation of cholinergic system and reduction of neuronal oxidative stress mainly through the free radicals scavenger [9]. Cyane-carvone also had no acute toxicity and suggested a possible anxiolytic effect that needs to be further investigated in order to elucidate its mechanism of action [10].

The potential pharmacological activity of CC, a synthetic derivative of carvone, was studied in experimental models to elucidate its anti-inflammatory, antinociceptive, and antioxidant effects in mice.

MATERIALS AND METHODS

Reagents

λ-Carrageenan, serotonin, histamine, bradykinin, prostaglandin E_2 (PGE_2), indomethacin, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical (St. Louis, MO, USA). Heparin and morphine were provided by Merck (Brazil). All drugs were dissolved in physiological saline (0.9 %). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Cyane-carvone preparation

Cyane-carvone was prepared via reaction of (*R*)-(−)-carvone with potassium cyanide and acetic acid in ethanol under the published conditions [11]. Figure 1 shows the chemical structure of cyane-carvone.

Animals

Swiss male mice (25–30 g, 2 months old) were randomly housed in appropriate cages at 25±2 °C under a light/dark cycle of 12 h with free access to food (Purina®, Brazil) and water. Protocols and procedures were approved by the Ethic Committee in Experimentation with Animals at UFPI (CEEA/UFPI Number 016/2011). The experiments were performed according with the Guide for Care and Use of Laboratory of the US Department of Health and Human Services, Washington, DC (1985) [13]. All doses were expressed in milligrams per kilogram and were orally administrated (p.o.).

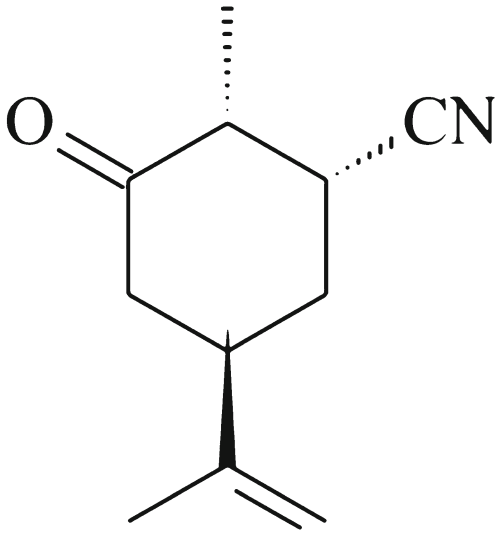

Fig. 1. Chemical structure of cyane-carvone. Taken from reference [12].

Effect of Cyane-carvone on Bradykinin-Induced Paw Edema in Mice

Animals were divided into four groups of five animals per group. Group I served as a negative control (0.1 mL/10 g of 0.9 % saline, p.o., SAL group). Group II was given no pretreatment (BRAD group). Group III was given 75 mg/kg CC, p.o. (CC 75 group) according to a previous toxicology work [9]. Group IV was given a reference standard (10 mg/kg indomethacin, p.o., INDO group). Animals of different groups were treated with respective drugs, and subsequently 1 h after treatment, 50 μL of bradykinin (6 nmol/paw) was injected subcutaneously into the plantar region of the right hind paw to induce edema. The paw volume was measured initially and at 30, 60, 90, and 120 min after bradykinin injection using the plethysmographic method of Hargreaves [14] in order to observe CC's effect on inflammation induced by bradykinin. Percentage of increase in paw volume from baseline was calculated and compared with control.

Effect of Cyane-carvone on Histamine-Induced Paw Edema in Mice

Animals were divided into four groups of five animals per group. Group I served as a negative control (0.1 mL/10 g of 0.9 % saline, p.o., SAL group). Group II was given no pretreatment (HIST group). Group III was given 75 mg/kg CC, p.o. (CC 75 group). Group IV was given a reference standard (10 mg/kg indomethacin, p.o., INDO group). Animals of different groups were treated with respective drugs, and subsequently 1 h after treatment, 50 μL of histamine (100 μg/paw) was injected subcutaneously into the plantar region of the right hind paw to induce edema. The paw volume was measured initially and at 30, 60, 90, and 120 min after histamine injection using the plethysmographic method of Winter *et al.* [15] in order to observe CC's effect on inflammation induced by histamine. Percentages of increase in paw volume from baseline were calculated and compared with control.

Effect of Cyane-carvone on Prostaglandin E_2-Induced Paw Edema in Mice

Animals were divided into four groups of five animals per group. Group I served as a negative control (0.1 mL/10 g of 0.9 % saline, p.o., SAL group). Group II was given no pretreatment (PGE_2 group). Group III was given 75 mg/kg CC, p.o. (CC 75 group). Group IV was given a reference standard (10 mg/kg indomethacin, p.o., INDO group). Animals of different groups were treated with respective drugs, and subsequently 1 h after treatment, 50 μL of freshly prepared suspension of PGE_2 (3 nmol/paw) was injected subcutaneously into the plantar region of the right hind paw to induce edema. The paw volume was measured initially and at 30, 60, 90, and 120 min after PGE_2 injection using plethysmographic, according to a previous work [16] in order to observe CC's effect on inflammation induced by PGE_2. Percentage of increase in paw volume from baseline was calculated and compared with control.

Effect of Cyane-carvone on Serotonin-Induced Paw Edema in Mice

Animals were divided into four groups of five animals per group. Group I served as a negative control (0.1 mL/10 g of 0.9 % saline, p.o., SAL group). Group II was given no pretreatment (SEROT group). Group III was given 75 mg/kg CC, p.o. (CC 75 group). Group IV was given a reference standard (10 mg/kg indomethacin, p.o., INDO group). Animals of different groups were treated with respective drugs, and subsequently 1 h after treatment, 50 μL of 1 % (*w*/*v*) serotonin was injected subcutaneously into the plantar region of the right hind paw to induce edema. The paw volume was measured initially and at 30, 60, 90, and 120 min after serotonin injection using a plethysmographic method, according to a previous work [17] in order to observe CC's effect on inflammation induced by serotonin. Percentage of increase in paw volume from baseline was calculated and compared with control.

Effect of Cyane-carvone on Carrageenan-Induced Paw Edema in Mice

Mice were divided into six groups of five animals per group. Group I served as a negative control (dimethyl sulfoxide, DMSO group). Group II was given no pretreatment (CG group). Group III was given a reference standard (10 mg/kg indomethacin, p.o., INDO group). Groups IV, V, and VI were given, respectively, 25 mg/kg CC, p.o. (CC 25 group), 50 mg/kg CC, p.o. (CC 50 group), and 75 mg/kg CC, p.o. (CC 75 group). Animals of different groups were treated with respective drugs, and subsequently 1 h after treatment, 1 % carrageenan 0.1 mL was injected subcutaneously into the plantar region of the right hind paw to induce edema. The paw volume was measured initially and at 1, 2, 3, and 4 h after carrageenan injection using the plethysmographic method of Yesilada and Küpeli [18] in order to observe CC's effect on inflammation produced by carrageenan. Percentage of increase in paw volume from baseline was calculated and compared with control.

Myeloperoxidase Activity on Carrageenan Model of Induced Paw Edema

MPO activity was done on carrageenan-induced paw edema according to a previous work [19].

Briefly, 50–100 mg of paw tissue was homogenized in potassium buffer containing 0.5 % of hexadecyltrimethylammonium bromide (HTAB). The homogenate was centrifuged at 4,500 rpm for 15 min at 4 °C. The pellet was resuspended, and MPO activity was assayed by measuring the change in absorbance at 450 nm using *o*-dianisidine dihydrochloride and 1 % hydrogen peroxide. MPO activity was reported as units per milligram of tissue. A unit of MPO activity was defined as that converting 1 μmol of hydrogen peroxide to water in 1 min at 22 °C.

Evaluation of Neutrophil Migration

Evaluation of neutrophil migration was done according to a previous work [20]. Mice were injected intraperitoneally with 2.0 % DMSO, 75 mg/kg cyane-carvone, or 10 mg/kg indomethacin. Thirty minutes later, the animals were injected with carrageenan (500 μg/cavity, 250 μL). After 4 h, mice were sacrificed and peritoneal cavities were washed with 1.5 mL of heparinized phosphate-buffered saline (PBS). The volumes recovered were similar in all experimental groups and were equivalent to ~95 % of the injected volume. Total cell counts were performed in a Neubauer chamber, and differential cell counts (100 cells) were carried out on cytocentrifuge slides stained with hematoxylin and eosin. The results are presented as the number of neutrophils per milliliter of peritoneal exudate. Aliquots of the peritoneal exudates were stored at −70 °C for later analysis of cytokine content and glutathione (GSH) levels.

Cytokine Measurements

The levels TNF-α and IL-1β were evaluated using sandwich ELISA as described previously [21]. Briefly, microliter plates were coated overnight at 4 °C with an antibody against mice TNF-α or IL-1β (2 μg/mL). Blocking of nonspecific binding sites was accomplished by incubating plates with PBS containing 2 % bovine serum albumin (BSA) for 90 min at 37 °C. After blocking the plates, the test samples and each standard at various dilutions were added in duplicate and incubated at 4 °C for 24 h. The plates were washed three times with buffer. After washing the plates, 50 μL of biotinylated sheep polyclonal anti-TNF-α and anti-IL-1β (diluted 1:1,000 with assay buffer 1 % BSA) was added to the wells. After further incubation at room temperature for 1 h, the plates were washed, and 50 μL of streptavidin-HRP diluted 1:5,000 was added to all wells. The reagent *o*-phenylenediamine dihydrochloride (50 μL) was added 15 min later, and the plates were incubated in the dark at 37 °C for 15–20 min. After color development, the reaction was stopped with the addition of sulfuric acid (1 M), and absorbance was measured at 490 nm. Results are expressed as picograms per milligram of protein and reported as mean ± SD.

GSH Levels

The concentration of glutathione in the peritoneal exudates was estimated according to method previously described [22]. Aliquots (600 μL) of the peritoneal exudates were centrifuged at 3,000 rpm for 15 min at 4 °C. Next, 400 μL of each supernatant was mixed with 800 μL of Tris buffer (0.4 M, pH 8.9) and 20 μL of 0.01 M 5,5-dithio-bis (2-nitrobenzoic acid). Subsequently, the samples were stirred for 3 min and read on a spectrophotometer at 412 nm. GSH concentration was determined via a reduced GSH standard curve, which was generated in parallel. GSH levels are expressed as grams per milliliter of exudates.

Acetic acid-Induced Writhing Test

The writhing test was performed as described previously [23]. Mice were administered with SAL (0.1 mL/10 g of 0.9 % saline, p.o., SAL group), 75 mg/kg CC, p.o. (CC 75 group), and standard analgesic drug morphine (10 mg/kg) 30 min prior to administration of acetic acid (0.6 %; AA group). Animals were observed individually, and the number of writhes (abdominal constriction) was counted for 20 min commencing 5 min after injection of acetic acid. The number of writhes of treated group was compared to control group and represented as percent inhibition.

Formalin Test

Mice were injected with 20 μL of 1 % formalin in 0.9 % saline into sub-plantar space of the hind paw, and observations were made as described by Hunskaar and Hole [24]. The duration of paw licking was determined between 0–5 min (first phase) and 15–30 (second phase) after formalin injection (2.5 %; FOR group). Mice were administered with SAL (0.1 mL/10 g of 0.9 % saline, p.o., SAL group), 75 mg/kg CC, p.o. (CC 75 group), and standard analgesic drug morphine (10 mg/kg) 30 min prior to formalin injection. The paw licking time of treated animals was compared to control group and represented as percent inhibition.

Statistical Analysis

Data were expressed as mean ± SEM. Data obtained were evaluated by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls' post hoc test by GraphPad Prism version 5.00 for Windows (GraphPad Software; San Diego, CA, USA). Differences were considered statistically significant when $p<0.05$.

RESULTS

Figure 2 shows the influence of 75 mg/kg CC in bradykinin-, histamine-, PGE_2- and serotonin-induced paw edema tests in mice. In Fig. 2a, CC 75 group significantly showed decreased paw edema by 47.37 % (t= 30 min, $p<0.05$) and 67.86 % (t=60 min, $p<0.05$) when

compared with BRAD group. In Fig. 2b, CC 75 group significantly showed decreased paw edema by 57.45 % (t=30 min, p<0.05), 82.14 % (t=60 min, p<0.05), 91.11 % (t=90 min, p<0.05), and 71.43 % (t=120 min, p<0.05)

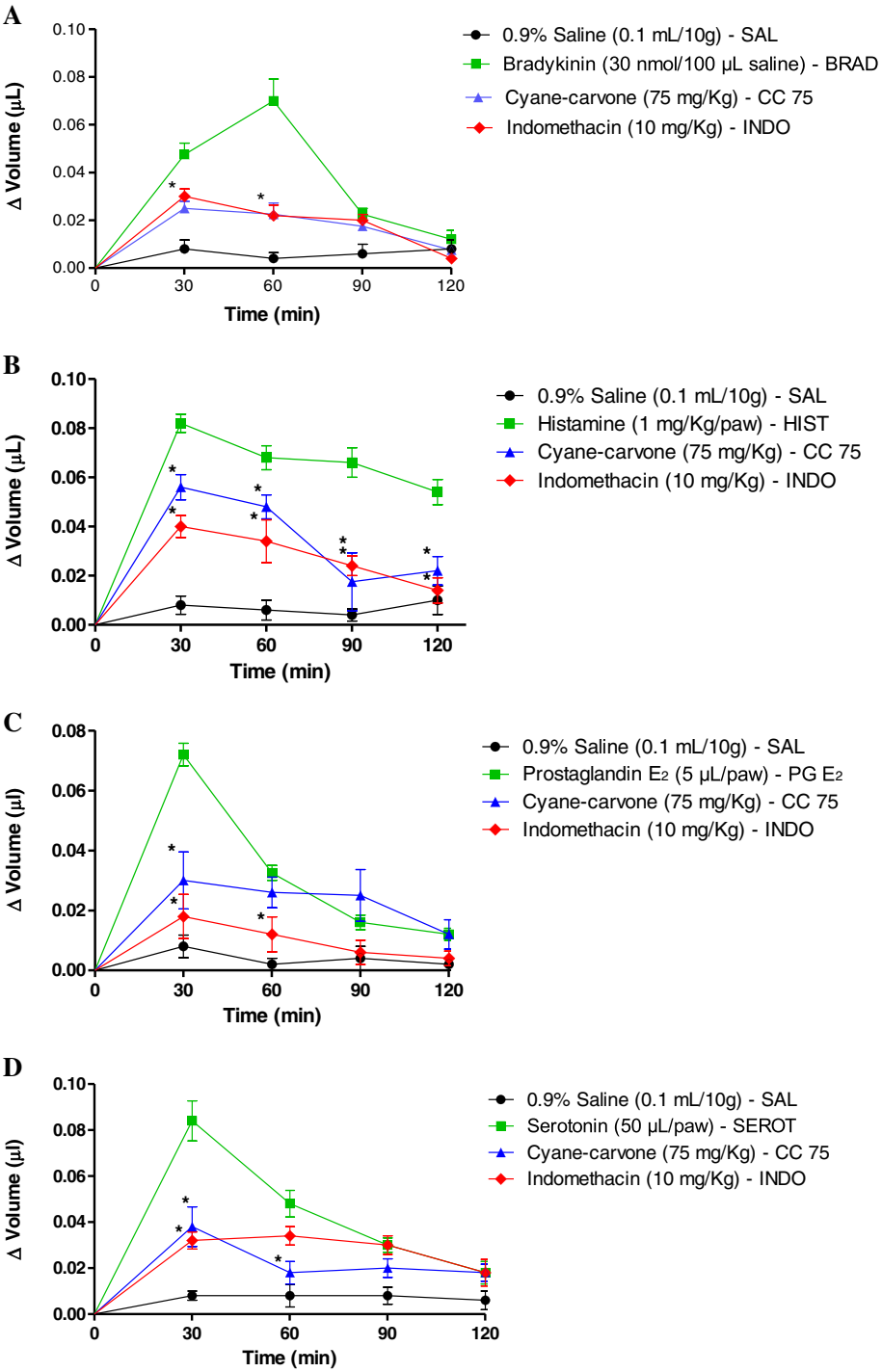

Fig. 2. Effects of cyane-carvone on bradykinin-, histamine-, prostaglandin E_2-, and serotonin-induced paw edema tests in mice. Values of paw edema are expressed in mean ± SEM. *p<0.05 compared with BRAD, HIST, PGE_2 or SEROT groups (one-way ANOVA followed by Newman-Keuls' test).

Table 1. Effects of Cyane-carvone on Carrageenan-Induced Paw Edema in Mice

Treatment	Doses (mg/kg)	Paw edema (mL)			
		1 h	2 h	3 h	4 h
DMSO		0.006±0.004	0.004±0.002	0.004±0.002	0.002±0.002
Control (CG)		0.016±0.004	0.022±0.002	0.038±0.003	0.054±0.006
Indomethacin	10	0.012±0.002 (25 %)	0.008±0.002* (63.64 %)	0.012±0.002* (68.42 %)	0.022±0.003* (59.26 %)
Cyane-carvone	25	0.015±0.003 (6.25 %)	0.020±0.004 (9.09 %)	0.030±0.003 (21.05 %)	0.028±0.008* (48.15 %)
	50	0.012±0.004 (25 %)	0.020±0.005 (9.09 %)	0.025±0.001* (34.21 %)	0.021±0.005* (61.11 %)
	75	0.012±0.002 (25 %)	0.018±0.004 (18.18 %)	0.020±0.003* (47.37 %)	0.020±0.005* (62.96 %)

Values of paw edema are expressed in mean ± SEM (n=5). Percentage of inhibition of paw edema is indicated in parentheses
*p<0.05 (compared with the control group, one-way ANOVA followed by Newman-Keuls' test)

when compared with HIST group. In Fig. 2c, CC 75 significantly decreased paw edema by 31.7 % (t=30 min, p<0.05) when compared with PGE_2 group. In serotonin model (Fig. 2d), CC 75 significantly decreased paw edema by 54.8 % (t=30 min, p<0.05) and 62.5 % (t=60 min, p< 0.05) when compared with SEROT group. Indomethacin (10 mg/kg, p.o.) used as a positive reference standard also showed a significant reduction of mice paw edema as compared with BRAD, HIST, PGE_2, and SEROT groups (Fig. 2a–d).

Table 1 shows the influence of CC in carrageenan-induced paw edema in mice. CC groups showed no change in paw edema when compared with control group in the first and second hour. However, CC 50 group decreased significantly paw edema by 34.21 % (t=3 h) and 61.11 % (t=6 h) and CC 75 group significantly decreased paw edema by 47.37 % (t=3 h) and 62.96 %, when compared with control group. CC 25 group also decreased paw edema by 48.15 % (t=4 h) when compared with control group. The group treated with indomethacin decreased significantly paw edema by 63.64 % (t=2 h) and 68.42 % (t=3 h) when compared with control group.

Figure 3 shows the influence of 75 mg/kg CC in MPO activity on carrageenan-induced paw edema in mice. CC 75 plus carrageenan group significantly showed a decrease in enzyme activity (47.02 %) when compared with DMSO plus carrageenan group. The group treated with indomethacin also significantly decreased MPO activity in 55.63 % when compared with DMSO plus carrageenan group.

Figure 4 shows the influence of 75 mg/kg CC in IL-1β and TNF-α levels in the peritoneal fluid of mice treated with carrageenan. CC 75 group significantly showed a decrease in IL-1β and TNF-α levels of 62.83 and 60.09 %, respectively, when compared with CG group. Figure 4 also shows the influence of 25, 50, and 75 mg/kg CC in GSH levels in the peritoneal fluid of mice treated with carrageenan. CC 25, CC 50, and CC 75 groups significantly showed an increase in GSH levels of 35.06, 52.79, and 64.64 %, respectively, when compared with SAL group.

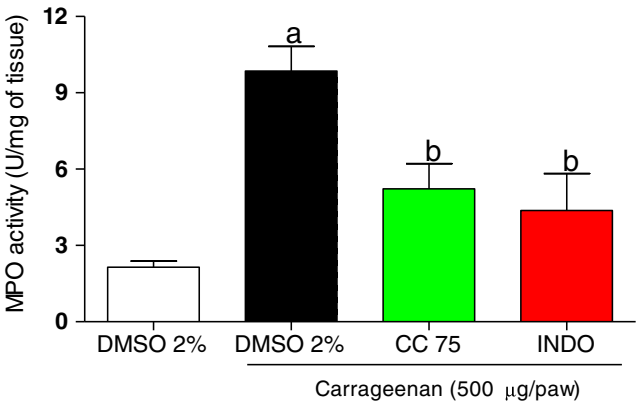

Fig. 3. Effects of cyane-carvone on myeloperoxidase (MPO) activity on carrageenan-induced paw edema. Values of MPO are expressed in mean ± SEM. *DMSO* dimethyl sulfoxide, *CC 75* cyane-carvone (75 mg/kg), *INDO* indomethacin (10 mg/kg). [a]p<0.05 compared with DMSO (one-way ANOVA followed by Newman-Keuls' test). [b]p<0.05 compared with DMSO+carrageenan (one-way ANOVA followed by Newman-Keuls' test).

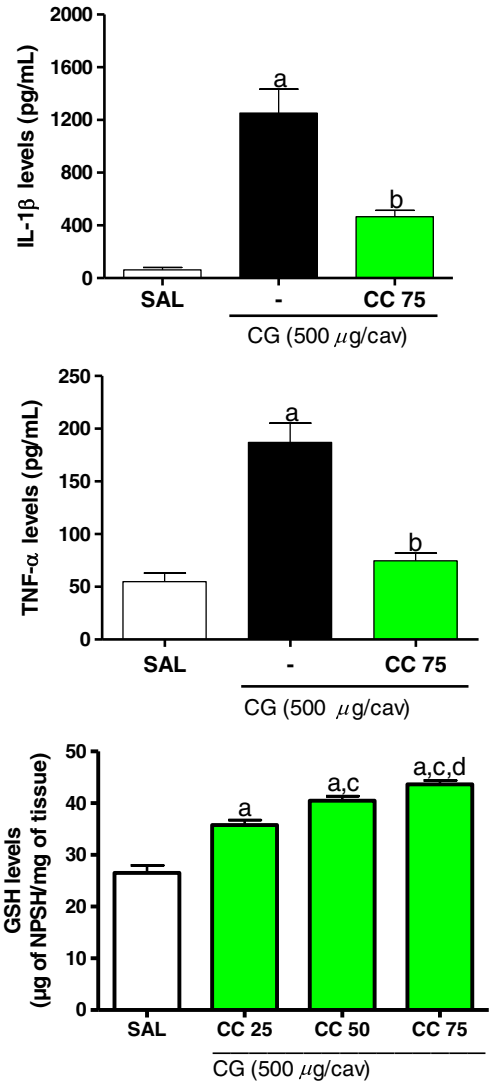

Fig. 4. Effects of cyane-carvone on IL-1β and TNF-α levels and glutathione levels in the peritoneal fluid of mice pretreated with carrageenan. Values of IL-1β, TNF-α, and GSH levels are expressed as mean ± SEM. *SAL* 0.9 % saline (0.1 mL/10 g), *CG* carrageenan (500 μg/cav), *CC* cyane-carvone (25, 50 or 75 mg/kg). [a]$p<0.05$ compared with SAL group (one-way ANOVA followed by Newman-Keuls' test). [b]$p<0.05$ compared with CG group (one-way ANOVA followed by Newman-Keuls' test). [c]$p<0.05$ compared with CC 25 group (one-way ANOVA followed by Newman-Keuls' test). [d]$p<0.05$ compared with CC 50 group (one-way ANOVA followed by Newman-Keuls' test).

In acid acetic writhing test (Fig. 5), CC 75 group significantly showed a decrease in the number of contortions of animals (96 %) compared to AA group.

In formalin test in mice (Fig. 6), the time of licking was not significantly different in CC 75 group as compared to FOR group in phase 1, but was significantly decreased by 97.14 % in phase 2.

DISCUSSION

The present study establishes the anti-inflammatory and antinociceptive activities of CC in different experimental animal models. Pain and inflammation process are associated with diseases such as arthritis, cancer, and vascular diseases. Inflammation is not only the response of tissues to infection and injury, but it can contribute to disease physiology, such as asthma, multiple sclerosis, colitis, inflammatory bowel disease, and atherosclerosis [25].

According to Campos and Calixto [26], B1 and B2 kinin receptors can trigger edema in rat paw. The B2 receptors are constitutive and can interact in a synergistic manner with several inflammatory mediators, but these receptors induce desensitization of B receptor expression which may involve cytokine production. Bradykinin can stimulate prostanoid synthesis and activate sensory neurons to release the proinflammatory neuropeptide substance P and calcitonin gene-related peptide. It had important implications on the manifestation of acute and chronic inflammatory process [26]. In bradykinin-induced paw edema test in mice, CC showed a significant reduction of paw edema when compared with the group treated only with bradykinin.

Histamine-induced paw edema is premature in chemically induced inflammation. The edema occurs primarily due to the action of histamine. Histamine is released following the mast cell degranulation by inflammatory mediators. This phenomenon causes the release of neuropeptide and prostaglandins from endothelial cell, leading to hyperalgesia and other proinflammatory effects [25]. CC and indomethacin showed a significant inhibition in histamine-induced paw edema in mice.

Acute inflammation causes an increase in vascular permeability and cellular infiltration, leading to edema. This swelling is a result of extravasation of fluid and proteins as well as accumulation of leukocytes at the inflammatory site [27]. Among the most important inflammatory mediators responsible for the sensitization of nociceptive neurons are primary prostaglandins (PGs), especially the series E_2 (PGE_2), known for its key role in induction and maintenance mechanisms responsible for the sensitization of primary nociceptors [28]. In the prostaglandin E_2-induced paw edema test in mice, CC significantly showed a reduction as compared with the group treated with only PGE_2.

Serotonin, like histamine, is an important inflammation mediator, and it is a potent vasodilator substance and can increase the vascular permeability [29–31]. CC

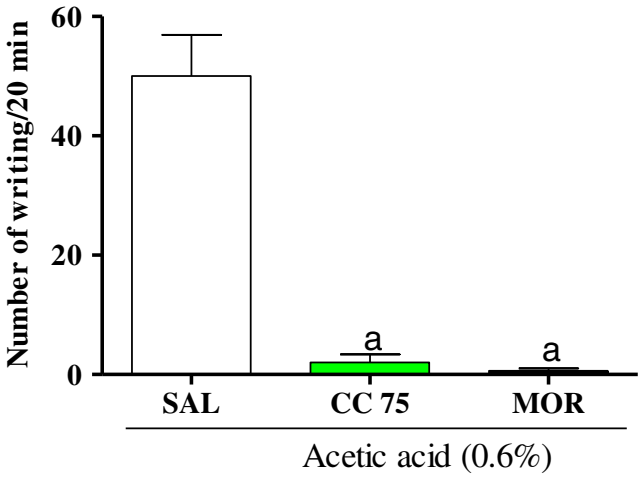

Fig. 5. Effects of cyane-carvone on acid acetic writhing test in mice. The results of the acid acetic writhing test are expressed in mean ± SEM. *SAL* 0.9 % saline (0.1 mL/10 g), *CC 75* cyane-carvone (75 mg/kg), *MOR* morphine (10 mg/kg). [a]$p<0.05$ compared with SAL group (one-way ANOVA followed by Newman-Keuls' test).

significantly showed decreased paw edema in mice submitted to serotonin test, leading to the possibility of action of the monoterpene as a serotonergic antagonist.

Carrageenan-induced rat paw edema is a widely used test to determine the anti-inflammatory activity, and it has been fully characterized [32–35]. It has been shown that COX-2 reaches maximal expression 1 h from carrageenan local injection [36]. Mouse paw edema has been increasingly used to test new anti-inflammatory drugs as well as to study the mechanisms involved in inflammation. In 1987, Henriques and coworkers [37] showed that carrageenan injection into the mouse paw induces a biphasic edema. The first phase is characterized by an edema of little intensity and unrelated to dose of carrageenan used, while the second phase develops after 24 h, displaying a more pronounced edema with a maximum effect between 48 and 72 h.

Carrageenin, from the Irish word "carraigin" meaning Irish moss, refers not only to species of red alga *Chondrus crispus* found along the rocky areas of the Atlantic coast of the British Isles, Europe, and North America but also refers to its mucopolysaccharide extract, discovered by the British pharmacist Stanford in 1862. The name was later changed to carrageenan so as to comply with the "an" suffix for polysaccharides. Structurally, the carrageenan is a complex group of polysaccharides made up of repeating galactose-related monomers and are of three main types: lambda, kappa, and iota. Each has their own gel characteristics which are all thermally reversible. The lambda form does not gel strongly at room temperature and it is injectable to induce an inflammatory response. Inflammation induced by carrageenan is acute, nonimmune, well-researched, and highly reproducible. Cardinal signs of inflammation (edema, hyperalgesia, and erythema) develop immediately following subcutaneous injection, resulting from action of proinflammatory agents—bradykinin, histamine, tachikinins, complement and reactive oxygen, and nitrogen species. Such agents can be generated in situ at the site of insult or by infiltrating cells. Neutrophils readily migrate to the sites of inflammation and can generate proinflammatory reactive oxygen and other species. The inflammatory response is usually quantified by an increase of paw size (edema) which is maximal at around 5-h post-carrageenan injection and is modulated by inhibitors of specific molecules with the inflammatory cascade. The nonsteroidal noninflammatory drug indomethacin is a clinically useful example [38].

Carrageenan causes neutrophil infiltration that is followed by NADPH oxidase activation and an oxygen-respiratory burst. Then, oxygen-derived free radicals are generated, which cause lipid peroxidation, increase vascular permeability, elicit cellular recruitment, and produce tissue damage [39]. In order to explore the effects of antioxidant defenses on acute inflammation process, MPO activity was evaluated in a carrageenan model in the paw of adult mice. CC 75 plus carrageenan group significantly showed a decrease in enzyme activity (47.02 %) when compared with DMSO plus carrageenan group. The group treated with indomethacin and carrageenan also significantly decreased MPO activity (55.63 %) when compared with DMSO plus carrageenan group.

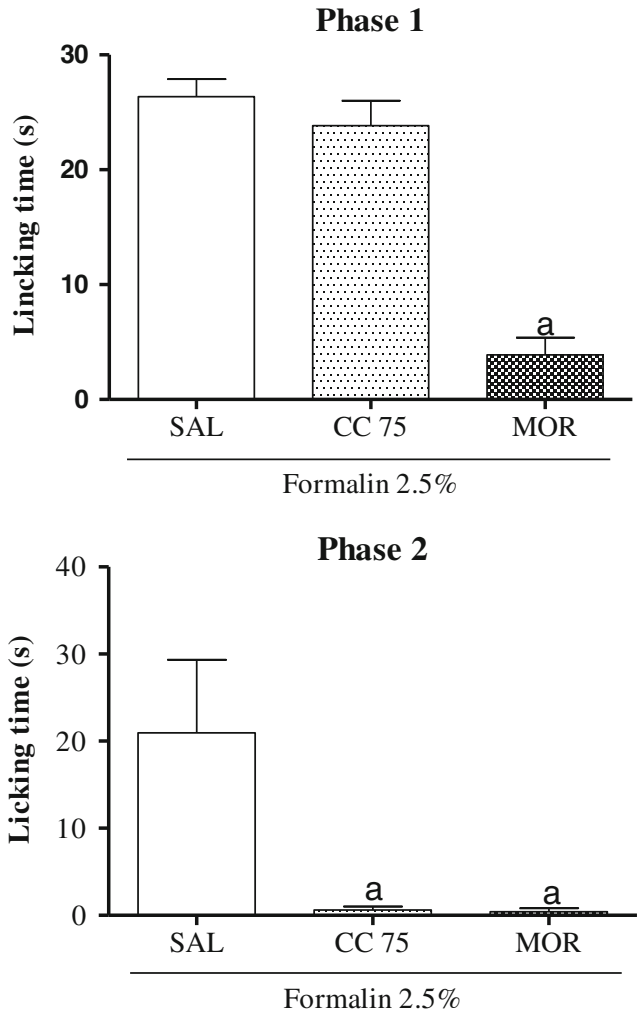

Fig. 6. Effects of cyane-carvone on improved formalin test in mice. The results of the formalin test are expressed in mean ± SEM. *SAL* 0.9 % saline (0.1 mL/10 g), *CC 75* cyane-carvone (75 mg/kg), *MOR* morphine (10 mg/kg). $^{a}p<0.05$ compared with SAL group (one-way ANOVA followed by Newman-Keuls' test).

The intraplantar injection of carrageenan into the mice hind paw induced an inflammation (swelling and erythema) that was maximal by the fourth hour following carrageenan administration and produced a time-dependent polymorphonuclear leukocyte (PMN) accumulation into the paw tissue [40]. A marked reduction of MPO activity indicates reduced neutrophil accumulation in the inflamed paw. MPO inibition may be involved in mechanism of anti-inflammatory action of CC.

IL-1 was first cloned in the 1980s and rapidly emerged as a key player in the regulation of inflammatory processes. The term IL-1 refers to two cytokines, IL-1α and IL-1β, which are encoded by two separate genes. The effects of IL-1 are tightly controlled by several naturally occurring inhibitors, such as IL-1 receptor antagonist (IL-1Ra), IL-1 receptor type II (IL-1RII), and other soluble receptors. Numerous IL-1 inhibitors have been developed and tested primarily in rheumatoid arthritis, with only modest effects. By contrast, the use of IL-1 antagonists has been uniformly associated with beneficial effects in patients with hereditary autoinflammatory conditions associated with excessive IL-1 signaling, such as cryopyrinopathies and IL-1Ra deficiency. Successful treatment with IL-1 blockers has also been reported in other hereditary autoinflammatory diseases, as well as in nonhereditary inflammatory diseases, such as Schnizler's syndrome, systemic-onset juvenile idiopathic arthritis, and adult Still's disease. The role of microcrystals in the

regulation of IL-1β processing and release has provided the rationale for the use of IL-1 inhibitors in crystal-induced arthritis [41]. After carrageenan-induced paw edema test in mice, 75 mg/kg CC significantly decreased IL-1β as compared with the group treated only with carrageenan, possibly indicating that the inhibition of IL-1β is one of the anti-inflammatory mechanisms of action of CC.

TNF-α is a central regulator of inflammation, and TNF-α antagonists may be effective in treating inflammatory disorders in which TNF-α plays an important pathogenetic role [42]. After carrageenan-induced paw edema test in mice, 75 mg/kg CC significantly decreased TNF-α as compared with the group treated only with carrageenan, possibly indicating that the inhibition of TNF-α is one of the anti-inflammatory mechanisms of action of CC.

GSH is a free radical scavenger and has been suggested to play an important role against carrageenan-induced local inflammation, by promoting hydrogen transference and acting as a cofactor for the enzyme GSH peroxidase [43, 44]. CC significantly increased GSH levels in mice, possibly indicating that this is one of the anti-inflammatory mechanisms of action of CC. Therefore, we could infer that the protective effect of CC might be explained, at least in part, by an increase in GSH concentration. An alternative possibility is that the increase in GSH levels could be secondary to a decrease in free radical production.

Pain is a pervasive public health problem, and analgesic drugs play an important role in its treatment. The most widely used analgesics have included μ-opioid agonists, anti-inflammatory steroids, and nonsteroidal anti-inflammatory drugs. These drugs are not uniformly effective, and side effects may occur. Consequently, one long-standing focus of drug discovery has been the search for novel analgesics [45].

The nociceptive system is essential to protect body cells from damage, but under pathological conditions, pain becomes a disease [46]. The writhing test is a model of visceral pain [47]. CC group and the group administered with morphine analgesia were able to significantly reduce the number of writhes of the animals as compared to saline group. This result suggests that CC shows an analgesic effect in mice.

Formalin-induced nociception consists of two different nociceptive states, and distinct mechanisms underlie the two phases of behavioral response. Acute nociception (phase 1) is followed by the facilitated state (phase 2). The phase 1 response appears to result from the immediate and intensive increase in activity of the primary afferent fibers induced by formalin, reflecting an acute pain. On the other hand, the phase 2 response may result from the activation of a wide dynamic range of neurons in the dorsal horn, with a continuous low level of activity in the primary afferent fibers [48]. Therefore, phase 2 represents a facilitated state which appears to be a prominent and intensified pain state in spite of a decreased level of afferent input. This pain model has been utilized as a tool for observing the effects of various antinociceptive agents on these two types of pain at once [49]. The time of licking was not significantly different in CC 75 group as compared to SAL group in phase 1, but it was significantly different (97.14 %) in phase 2. This result also reinforces the possible analgesic effect of CC.

CONCLUSION

In conclusion, the present study demonstrates that CC exerts an antinociceptive action against formalin- and acetic acid-induced writhing tests in mice, and it shows an anti-inflammatory effect against bradykinin-, histamine-, PGE_2-, serotonin-, and carraageenin-induced paw edema tests in mice. The mechanisms of action of anti-inflammatory effect should involve the inhibition of IL-1β, TNF-α, and MPO and the increase of GSH levels. So, these findings support the knowledge that CC can be used, after more studies, in a new pharmaceutical formulation as a promising alternative to treat pain or inflammation diseases.

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