

Anti-Inflammatory Activity of Hydroxydihydrocarvone

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Hydroxydihydrocarvone (HC) is a synthetic intermediate obtained by hydration of the natural compound (*R*)-(-)-carvone. The aim of the present study was to investigate the possible anti-inflammatory activity of orally administered HC. Toxicity, motor coordination, tail immersion test, as well as carrageenan-induced paw edema and myeloperoxidase (MPO) activity or peritonitis were all evaluated in rodents. HC was force-fed to the animals 1 h before the stimulus. The lethal dose 50% (LD₅₀) of orally administered HC was 1259 mg/kg. No changes in motor coordination were recorded in HC-treated mice in the rotarod test. The time of response to the thermoceptive stimulus in the tail immersion test was longer in HC-treated animals (50, 100, and 200 mg/kg) than in the vehicle-treated group. HC also significantly decreased the area under curve of carrageenan-induced rat paw edema at 100 and 200 mg/kg and MPO activity at 200 mg/kg. Carrageenan-induced neutrophil recruitment to the peritoneal cavity was significantly reduced by HC at doses of 100 or 200 mg/kg, but not 50 mg/kg. These findings demonstrate that orally administered HC exerts antinociceptive and anti-inflammatory activities in rats and mice.

Key words: Terpene, Essential Oils, Inflammation

Introduction

Medicinal plants represent an abounding source of compounds that have potential to treat a number of pathological conditions, such as inflammatory and/or painful conditions (Corson and Crews, 2007). Terpenoids are a large family of these compounds, found mainly in essential oils and have a range of applications, particularly in health. Plant-derived essential oils, have a variety of biological properties attributed to their chemical components, predominantly the monoterpenes (De Sousa *et al.*, 2007a). Previous studies have shown that some monoterpenes present in many essential oils can exert antinociceptive (Abdel-Fattah *et al.*, 2000; Gonçalves *et al.*, 2008), anticonvulsant (De Sousa *et al.*, 2007b, c), sedative (De Sousa *et al.*, 2007d), and anti-inflammatory (Salminen *et al.*, 2008) effects. Monoterpene derivatives also contain several pharmacological properties such as antinociceptive (Almeida *et al.*, 1996; De Sousa *et al.*, 2004) and antidepressant effects (De Sousa *et al.*, 2006a), however, little information is available concerning their anti-inflammatory activity. It is worth mentioning

that this class of compounds possesses an interesting therapeutic potential to treat inflammation, since many of them are shown to inhibit nuclear factor- κ B (NF- κ B) signalling, a pleiotropic pathway involved in the inflammatory and immune responses (Salminen *et al.*, 2008).

Hydroxydihydrocarvone (HC, Fig. 1) is a synthetic intermediate prepared by hydration of the monoterpene (*R*)-(-)-carvone (Büchi and Wüest, 1979). This compound has functional groups and a structure similar to those of some monoterpenes that exert a depressant effect on the central nervous system (CNS) (Silva *et al.*, 2007; Peana *et al.*, 2003; Amaral *et al.*, 2007). A previous study on the pharmacological activities of HC has reported its antinociceptive effect in formalin and

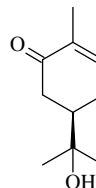


Fig. 1. Chemical structure of hydroxydihydrocarvone.

hot-plate tests, following intraperitoneal (i.p.) administration to mice (Oliveira *et al.*, 2008).

Based on the biological activity of terpenes from essential oils, we hypothesized that HC may have anti-inflammatory activity, along with the antinociceptive effect, following oral administration to mice or rats.

Material and Methods

Chemical compound

HC was prepared in our laboratory as previously described by Büchi and Wüest (1979). It was dissolved in 5% Tween 80 and used as an emulsion.

Animals

Male Swiss mice (25–35 g) or male Wistar rats (150–200 g) were obtained from the Prof. Dr. Thomas George Laboratory of the Federal University of Paraíba or the Animal House of the Federal University of Sergipe, respectively. The animals were kept under standard environmental temperature conditions [$(21 \pm 1)^\circ\text{C}$] with 12 h light/dark periods, light beginning at 06:00 a.m.. Animals were maintained in fast for at least 6 h, and water was provided *ad libitum* until 1 h prior to the experimental procedures. The animals were acclimatized to the laboratory for 1 h prior to the experiments. Animals received humane care in compliance with institutional guidelines.

Determination of lethal dose 50% (LD_{50})

LD_{50} was determined by giving different, orally administered doses of HC (500–2000 mg/kg) to groups of mice ($n = 10$), thereafter mortality was recorded for 7 d. The control group received only the vehicle. LD_{50} was determined by log-probit analysis.

Rotarod test

This technique has been previously described by Dunham and Miya (1957). Mice were placed on a rotating rod (2.5 cm in diameter, rotating at 7 rpm) for a preselection test, and those able to remain on the rod for 3 min in three successive trials were selected for testing. 24 h after the preselection, four groups of mice were treated orally with HC (50, 100, and 200 mg/kg) and vehicle. At 30, 60, 120, and 180 min after treatment, the animals were placed on a rotating bar of the rotarod apparatus for a maximum of 3 min, and

the time spent by each animal on the rotarod was recorded.

Tail immersion test

The lower two-thirds of the tail was immersed in a beaker containing water kept at $(50 \pm 0.5)^\circ\text{C}$ (Janssen *et al.*, 1963). The time in seconds until tail withdrawal from the water was considered as the reaction time. Mice that had a reaction time less than 4 s were selected. The reaction time was then measured 30, 60, 120, and 180 min after i.p. administration of HC (50, 100, and 200 mg/kg), vehicle (control) and morphine (10 mg/kg). The mice were exposed to hot water for no longer than 12 s to avoid tissue injury (Lira *et al.*, 2002).

Rat paw edema

Wistar rats were orally pretreated with 50, 100, or 200 mg/kg of HC (5 mL/kg) or vehicle (Tween 80, 5%). A positive control group of animals received dexamethasone [2 mg/kg, subcutaneous (s.c.)]. 1 h later, animals were anaesthetized by inhalation of halothane and received a subplantar injection of carrageenan (0.5%) or sterile saline in the right paw in a final volume of 0.1 mL. The paw volume was assessed immediately before carrageenan or saline injection, for the basal measurement, and 1, 2, 3 and 4 h thereafter, using a hydroplethysmometer (model 7150, Ugo Basile, Comerio, Italy). The results are expressed as the increase in paw volume (mL) calculated by subtracting the basal volume from each time point. The area under curve ($AUC_{0-4\text{h}}$) was also calculated using the trapezoidal rule.

Myeloperoxidase (MPO) activity in rat paw

4 h after injection of carrageenan or saline in rat paws, animals were sacrificed under halothane anaesthesia and posterior cervical dislocation. The tissues of the paws were immediately collected and placed in a test tube in the presence of 0.5% hexadecyltrimethylammonium bromide in 50 mmol/L potassium phosphate buffer, pH 6.0. Each tissue sample was homogenized, submitted to incubation at 60°C to inactivate catalase, and the homogenate was centrifuged at $12000 \times g$ for 5 min. The supernatants were collected and the MPO assay was performed using a microliter plate scanner. This consisted of mixing 20 μL of sample with 200 μL of *o*-dianisidine solution

(0.167 mg/mL of *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide) before reading the plate. The changes in absorbance were measured at 460 nm every 15 s over a period of 5 min. The MPO activity was expressed as MPO units (U MPO) per milligram of tissue. One unit of MPO activity degrades 1 μ mol of peroxide per minute at 25 °C (Camargo *et al.*, 2008).

Leukocyte migration to peritoneal cavity

Swiss mice (20–30 g) were orally pretreated with 50, 100, or 200 mg/kg of HC (5 mL/kg) or vehicle (Tween 80, 5%). 1 h later, animals were submitted to an injection of 0.25 mL of carrageenan (1%) or sterile saline. Leukocyte counts were measured 4 h after carrageenan injection. The mice were sacrificed under halothane anaesthesia, and 2 mL of a phosphate-buffered saline (PBS) solution containing heparin (5 UI/mL) were injected into the peritoneal cavity. The abdomen was carefully massaged, and the fluid was withdrawn, placed in polypropylene centrifuge tubes, and centrifuged at $1000 \times g$ for 10 min. The resulting cell pellet was gently resuspended in 1.0 mL of PBS/heparin solution, and the total and differential cell counts were assayed. Total cell counts were done using a Neubauer chamber while differential counts were carried out on a minimum of 200 cells using cytopspin preparation stained with Diff-Quick. The cells were classified as polymorphonuclear (neutrophils and eosinophils) or mononuclear (macrophages, mast cells, and lymphocytes) based on normal morphological criteria.

Statistical analysis

The data obtained in the various experiments were evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The results obtained were considered significant when $P < 0.05$.

Results

Determination of lethal dose 50%

Orally administered HC was found to have an LD₅₀ value of 1259 mg/kg with a confidence interval of 1000–1585 mg/kg.

Effect of HC on motor coordination

At the doses evaluated in this study, HC had no significant effect on the motor coordination in the

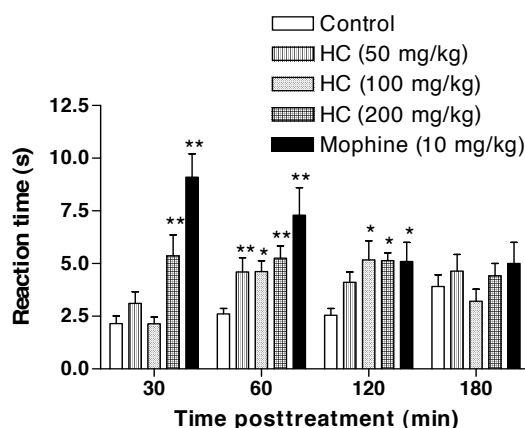


Fig. 2. Effect of HC treatment on the tail immersion test in mice. The values represent means \pm S.E.M. ($n = 8$); * $P < 0.05$, ** $P < 0.01$ significantly different from control.

rotarod test of treated animals at the time points used following treatment.

Effect of HC on nociception in the tail immersion test

Oral administration of HC at the doses of 50, 100, and 200 mg/kg led to a significant reduction in the nociceptive response to tail immersion in hot water, and this effect was dose-dependent (Fig. 2). This effect of HC was observed at the three evaluation time points (30, 60, and 120 min); however, the effect of HC was greater at 60 min post treatment. The decrease in time until tail withdrawal was similar to that achieved with 10 mg/kg of morphine.

Effect of HC on rat paw edema and MPO activity

Carrageenan-induced rat paw inflammation characterized by the increase in paw volume peaked 3 h after injection ($n = 6$), when compared with the injection of saline ($n = 5$). Prior oral treatment of rats with HC the paw edema induced by carrageenan decreased dose-dependently as indicated in Fig. 3. The analysis of area under curves clearly showed the anti-inflammatory effect of HC in the rat paw edema model. HC significantly decreased the area under curve of carrageenan-injected rats [$(P < 0.05$ for 50 mg/kg HC ($n = 6$) or $P < 0.001$ for 100 ($n = 6$) or 200 mg/kg HC ($n = 6$); Fig. 3B]. As expected, treatment with the glucocorticoid dexamethasone (2 mg/kg,

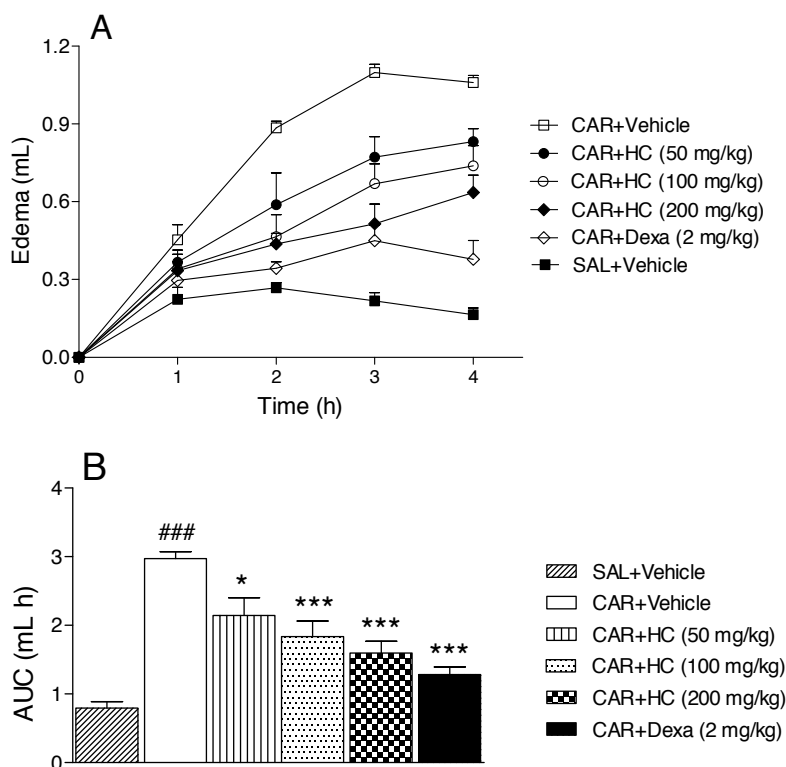


Fig. 3. Effect of HC treatment on carrageenan (CAR)-induced rat paw edema. (A) Time course of rat paw edema in animals injected with saline (SAL) or CAR and previously treated with vehicle, HC or dexamethasone (Dexa) at the indicated doses. (B) Resulting area under curve (AUC) of data from (A). Data are shown as means \pm S.E.M. of $n = 5-7$ rats. ### $P < 0.001$ compared with saline + vehicle group or * $P < 0.05$ and *** $P < 0.001$ compared with carrageenan + vehicle group.

s.c., $n = 5$) significantly inhibited the carrageenan-induced rat paw edema.

Four h after injection of carrageenan in rat paws we also found an increase ($P < 0.001$) in MPO activity, as a marker of neutrophil infiltration in paw tissue when compared with the saline-injected group, which exhibited very low MPO activity in paws [(0.08 ± 0.03) U MPO/mg of paw tissue]. This effect was significantly inhibited by HC at 200 mg/kg ($P < 0.01$, $n = 6$), but not by the other doses of this compound. Dexamethasone-treated animals also showed a significant reduction of paw MPO activity ($P < 0.01$; Fig. 4).

Effect of HC on leukocyte migration in mice peritoneal cavity

As shown in Table I, the i.p. injection of carrageenan induced a marked leukocyte migration (mainly neutrophils) into the peritoneal cavity of

mice ($n = 8$, $P < 0.001$), when compared with injection of saline ($n = 5$). Eosinophils were virtually absent in all groups analysed. Although the pretreatment with HC at 50, 100, or 200 mg/kg ($n = 6$ each) did not change the total leukocyte counts in the peritoneal cavity, it did significantly decrease the polymorphonuclear (neutrophil) cell counts at 100 ($P < 0.001$) or 200 mg/kg ($P < 0.05$), which was not observed at 50 mg/kg. As expected, mice treated with dexamethasone (2 mg/kg, s.c., $n = 5$) showed a significant decrease in the total and polymorphonuclear counts. Mononuclear counts were affected neither by carrageenan injection nor by any doses of HC treatment.

Discussion

In the present study, the effect of orally administered HC was evaluated to investigate its effects on toxicity, motor behaviour/neurotoxicity, and

Table I. Total and differential leukocyte counts in the peritoneal cavity of mice pretreated with vehicle, hydroxydihydrocarvone (HC) or dexamethasone (Dexa) and injected with saline or carrageenan.

Group	Cells ($\cdot 10^6$ /peritoneal cavity)		
	Total	Mononuclear	Polymorphonuclear
Saline + vehicle	3.1 ± 0.5	3.0 ± 0.5	0.10 ± 0.05
Carrageenan + vehicle	$11.8 \pm 0.7^{***}$	3.4 ± 0.4	$8.4 \pm 0.8^{***}$
Carrageenan + HC (50 mg/kg)	10.9 ± 1.5	4.0 ± 0.6	6.9 ± 1.5
Carrageenan + HC (100 mg/kg)	9.1 ± 0.9	5.1 ± 0.6	$4.0 \pm 0.5^{***}$
Carrageenan + HC (200 mg/kg)	9.7 ± 0.7	4.8 ± 0.7	$4.9 \pm 0.6^*$
Carrageenan + Dexa (2 mg/kg)	$5.0 \pm 1.1^{***}$	2.4 ± 0.4	$2.6 \pm 0.6^{***}$

Results are presented as means \pm S.E.M. of cells ($\cdot 10^6$ /peritoneal cavity) for $n = 5-8$ mice.

*** $P < 0.001$ compared with saline + vehicle group or * $P < 0.05$ and *** $P < 0.001$ compared with carrageenan + vehicle group.

antinociceptive action. Additionally, the effects of HC on standard models of inflammation induced by carrageenan were also investigated, and we have demonstrated that HC possesses a considerable anti-inflammatory activity in these models both in rats and mice.

Initially we determined the LD₅₀ value of orally administrated HC and found that high doses of HC resulted in an LD₅₀ of 1259 mg/kg, which is higher than that found when the intraperitoneal route was used (800 mg/kg), indicating the lower toxicity of HC when administered orally (De

Sousa *et al.*, 2006b). That may be due to either lower absorption or higher first-step metabolism. The highest dose used in the following tests was more than 6 times lower than the LD₅₀ observed.

No changes were found in the motor coordination of HC-treated mice. The animals remained on the rotarod for more than 180 s, suggesting that the inhibitory effect of HC may be achieved through central mechanisms and may not be the result of a blockade of the neuromuscular system (Perez *et al.*, 1998; Amos *et al.*, 2001). In the tail immersion test, in which a thermal stimulus is used, an increase in the reaction time is generally considered an important parameter of central antinociceptive activity (Rujjanawate *et al.*, 2003). This test differentiates central opioid-like analgesics from peripheral analgesics (Asongalem *et al.*, 2004). The tail flick response is believed to be a spinally mediated reflex (Chapman *et al.*, 1985). Moreover, Grumbach (1966) has shown that the effectiveness of analgesic agents in the tail flick pain model is strongly correlated with the relief of human pain. In this test, the antinociceptive activity of HC was observed to be dose-dependent, presenting a central antinociceptive-like effect. This effect was observed up to 120 min post treatment as shown in the tail immersion test. This result showed that HC is also effective by the oral route and it is in agreement with data from studies carried out using the intraperitoneal route (De Sousa *et al.*, 2006b).

Besides the antinociceptive effect of HC, possible anti-inflammatory effects were also evaluated. Monoterpenes, as well as other terpenoids, are believed to exert anti-inflammatory activities, and this may take part in the inhibitory effects of many plant essential oils on experimental models

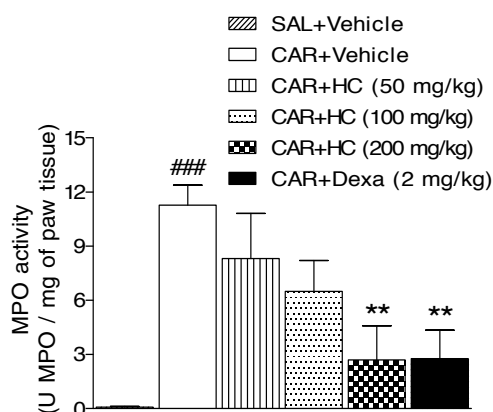


Fig. 4. Effect of HC treatment on carrageenan (CAR)-induced neutrophil influx to rat paws. Myeloperoxidase (MPO) activity was assayed in rat paw from animals injected with saline (SAL) or CAR and previously treated with vehicle, HC or dexamethasone (Dexa) at the indicated doses. Data are shown as means \pm S.E.M. of $n = 5-7$ rats. ### $P < 0.001$ compared with saline + vehicle group or ** $P < 0.01$ compared with carrageenan + vehicle group.

of inflammation, as suggested for *Salvia* or *Phlomis* species (Kamatou *et al.*, 2008; Limem-Ben *et al.*, 2009). In this way, we hypothesized that HC, a synthetic monoterpene derivative, might have such activity, like the natural compounds extracted from plants. As expected, HC decreased the carrageenan-induced edema in rat paws in a dose-dependent manner, suggesting that HC may reduce the vascular permeability, thus decreasing the plasma leakage in rat paws. In addition, this compound reduced the carrageenan-induced neutrophil migration into rat paws, as expressed by the decrease of the MPO activity, a widely used biomarker of neutrophil content in tissues (Bradley *et al.*, 1982; Camargo *et al.*, 2008; Yshii *et al.*, 2009). The latter result suggests that HC affects the neutrophil chemotaxis to the inflammatory focus, most probably through the inhibition of any chemoattractant mediator, as the cell recruitment induced by carrageenan involves production of many mediators, such as prostaglandin E₂, nitric oxide, IL-1 β , and TNF- α (Salvemini *et al.*, 1996; Loram *et al.*, 2007). Of interest, the monoterpene carvacrol was recently shown to reduce the MPO activity in a model of periodontitis in rats (Botelho *et al.*, 2008). In order to further investigate the negative modulation of HC on leukocyte chemotaxis, mice pretreated with HC were submitted to peritoneal injection of carrageenan. As shown previously by others (Salvemini *et al.*, 1996; Alves *et al.*, 2009), carrageenan injection increased the total leukocyte counts in the peritoneal cavity, which was mainly due to recruitment of neutrophils. Although HC treatment did not affect significantly the total or mononuclear (lymphocyte, monocyte, and macrophage) cell counts,

it decreased the neutrophil counts in the peritoneal cavity of mice. This is in agreement with the data showing reduced MPO activity in the paw of rats submitted to treatment with HC, showing that this compound is active in both rats and mice.

The anti-inflammatory effects of HC might take place by various mechanisms, since terpenoids were demonstrated to exert inhibitory activity on the inflammatory signalling cascade of NF- κ B by different interactions in this pathway (Salminen *et al.*, 2008). This is the case for the monoterpenes aucubin (Jeong *et al.*, 2002), catalposide (Kim *et al.*, 2004), genipin (Koo *et al.*, 2004), α -pinene (Zhou *et al.*, 2004), and some other terpenoids, such as lupeol (Fernandez *et al.*, 2001; Saleem, 2009) and ginkgolide B (Li *et al.*, 2009). This mechanism could account for decreased expression of cyclo-oxygenase-2, inducible nitric oxide synthase, and inflammatory cytokine, along with other mediators and effects induced by translocation of NF- κ B to its DNA binding site (Karin and Greten, 2005; Perkins, 2007).

In summary, we have shown here that oral treatment with HC induces anti-inflammatory effects in rodents, characterized by the reduction of paw edema and neutrophil migration. Further studies are necessary to elucidate the mechanisms underlying the anti-inflammatory actions of this monoterpene.

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