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Research Paper

Antinociceptive and anti-inflammatory properties of 7-hydroxycoumarin in experimental animal models: potential therapeutic for the control of inflammatory chronic pain

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Abstract

Objectives In the present study we investigated the antinociceptive, anti-inflammatory and antipyretic effects of 7-hydroxycoumarin (7-HC) in animal models.

Methods The effects of oral 7-HC were tested against acetic acid-induced writhing, formalin test, tail flick test, complete Freund's adjuvant (CFA)-induced hypernociception, carrageenan-induced paw oedema, lipopolysaccharide-induced fever and the rota rod test.

Key findings 7-HC (3–60 mg/kg) produced a dose-related antinociception against acetic acid-induced writhing in mice and in the formalin test. In contrast, treatment with 7-HC did not prevent thermal nociception in the tail flick test. A single treatment with 7-HC, 60 mg/kg, produced a long-lasting antinociceptive effect against CFA-induced hypernociception, a chronic inflammatory pain stimulus. Notably, at 60 mg/kg per day over 4 days the administration of 7-HC produced a continuous antinociceptive effect against CFA-induced hypernociception. 7-HC (30–120 mg/kg) produced anti-inflammatory and antipyretic effects against carrageenan-induced inflammation and lipopolysaccharide-induced fever, respectively. Moreover, 7-HC was found to be safe with respect to ulcer induction. In the rota rod test, 7-HC-treated mice did not show any motor performance alterations.

Conclusions The prolonged antinociceptive and anti-inflammatory effects of 7-HC, in association with its low ulcerogenic activity, indicate that this molecule might be a good candidate for development of new drugs for the control of chronic inflammatory pain and fever.

Keywords 7-hydroxycoumarin; antiinflammatory; antinociception; antipyretic; chronic pain; gastric toxicity

Introduction

Acute pain serves as a warning device that indicates imminent tissue damage. Chronic pain lacks such a protective function as it persists for months or years after injury without reflecting the severity of a lesion or disease, nor does chronic pain necessarily respond to treatment of the underlying disease.[1] Chronic pain induced by inflammatory processes is a major clinical problem worldwide, Steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used treatments in these chronic pain states.[2] NSAIDs, such as aspirin and indometacin, block the biosynthesis pathway of prostaglandins by inhibiting the cyclooxygenase (COX) enzymes, producing anti-inflammatory, analgesic and antipyretic effects.[3]

The side effects of currently available NSAIDs pose a major problem in their clinical use. For example, NSAIDs can cause gastric injury, including ulceration, due to their non-selective inhibition of both isoforms of the COX enzyme: the constitutive (COX-1) and the inducible (COX-2) isoforms.[4] In addition, the use of steroidal drugs is becoming highly controversial because of their multiple side effects.[5] With high doses of medication and

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prolonged treatment frequently associated with inflammatory disease and chronic syndromes of pain, it is of great interest to find less harmful analgesic–anti-inflammatory drugs.

Coumarins comprise a very large class of compounds that is found throughout the plant kingdom. They are secondary metabolites, occurring naturally in different parts of plants, such as the roots, flowers and fruits.[6–8] The search for useful pharmaceuticals has led to a resurgence of interest in coumarins because these substances display relevant structure-dependent pharmacological activities, while at the same time appearing to be non-toxic in mammalian systems.[6,7] The main metabolite of coumarin (1,2-benzopyrone), a molecule present in a variety of edible fruits and plants, is 7-hydroxycoumarin (7-HC) or umbeliferone.[9,10] Several studies have demonstrated the pharmacological properties of 7-HC, such as immunomodulatory,[11] antioxidant,[12] antitumoural[13,14] and antihyperglycaemic[15,16] activities. In addition, it has also been demonstrated that 7-HC produces antinociceptive effects in acute models of pain.[17]

Chronic pain differs from acute pain, not only in its onset and duration but, more importantly, in its underlying mechanisms,[18] and it therefore often responds poorly to conventional analgesics.[19] Taking into account the biological activities of 7-HC it is possible that this molecule could exhibit antinociceptive effects against chronic pain conditions. Thus, the aim of the current study was to investigate the therapeutic potential of 7-HC against chronic inflammatory pain. To this end, we evaluated the antinociceptive effects of 7-HC in acute and chronic inflammatory pain models, which were induced by formalin and complete Freund's adjuvant (CFA), respectively. The anti-inflammatory and antipyretic effects of 7-HC were also investigated, using carrageenan-induced oedema and lipopolysaccharide (LPS)-induced fever as the models. In addition, we evaluated motor performance alterations and gastric injury associated with therapeutic doses of 7-HC.

Materials and Methods

Animals

Experiments were performed on male Wistar rats (180–200 g) or Swiss mice (30–35 g) from the animal facilities of the Gonçalo Moniz Research Center. Animals were individually housed at 24 ± 1°C, under a 12:12 h light–dark cycle (lights on at 06:00 a.m.), with free access to food and tap water until the day of the experiment. Animal care and handling procedures were in accordance with the guidelines of the International Association for the Study of Pain for the use of animals in pain research[20] and the Institutional Animal Care and Use Committee: FIOCRUZ 26/2009-1. All efforts were made to minimize the number of animals used and any discomfort.

Drugs and administration

Nimesulide, ibuprofen, indometacin, dexamethasone, CFA, carrageenan and LPS (*E. coli* 0111:B4) were obtained from Sigma Chemical Company (St Louis, MO, USA). The stain reagent, haematoxylin–eosin, was purchased from Vetec (Rio de Janeiro, Brazil). Diazepam was obtained from Cristália (Itapira, São Paulo, Brazil). Indometacin was dissolved in Tris HCl 0.1 M pH 8.0 plus saline. Dexamethasone was dissolved in ethanol (1 mg/ml) plus saline. 7-HC was dissolved in DMSO 2% plus saline. The remaining drugs were dissolved directly in saline. Isolation of 7-HC from *Typha domingensis* (thyphaceae) was performed at the Federal University of Paraíba. The chemical purity of 7-HC (more than 98%) was determined by GC/HPLC.[11] Drugs were administrated by oral (p.o.), intraplantar, subcutaneous (s.c.) or intraperitoneal (i.p.) methods. The oral administration of 7-HC was performed by gavage and the control group received the vehicle only.

Writhing test

Mice were treated with 7-HC (1, 3, 15, 30 and 60 mg/kg) or vehicle (saline with DMSO 2%; control group) by oral administration 40 min before acetic acid administration (0.8%, injected at time zero). Indometacin (10 mg/kg i.p.) and nimesulide (5 mg/kg p.o.) were the reference drugs. The acetic acid (0.8% v/v, 10 ml/kg) was injected into the peritoneal cavities of mice, and the animals were then placed in a large glass cylinder. The intensity of nociceptive behaviour was quantified by counting the total number of writhes occurring between 0 and 30 min after stimulus injection.[21] In the present study we used the term 'hypernociception' rather than hyperalgesia or allodynia to define the decrease in the nociceptive withdrawal threshold, since the pain perception in animals is not obvious.

Acute inflammatory pain induced by formalin

Rats were placed in an open Plexiglas observation chamber for 30 min to acclimatize to their surroundings, and were then removed for formalin administration. Acute inflammatory pain was induced by subcutaneous intraplantar injection of formalin 1% (50 μl). Rats were treated with 7-HC (3, 15, 30 and 60 mg/kg) or vehicle (saline with DMSO 2%; control group) by oral administration 40 min before formalin injection at time zero. Indometacin (5 mg/kg i.p.) and nimesulide (5 mg/kg p.o.) were the reference drugs. Rats were gently restrained while the dorsum of the hind paw was subcutaneously injected with 50 μl of formalin 1% (1 : 100 dilution of stock formalin solution, 37% formaldehyde in 0.9% saline). Following injection, each rat was returned to the observation chamber for a 60-min observation period. Rats were observed from 0 to 10 min (early phase) and from 10 to 60 min (late phase) and a nociception score was determined for each period by counting the number of flinches of the injected limb during the observation time.[22]

Chronic inflammatory pain induced by complete Freund's adjuvant

Mechanical allodynia was induced by intraplantar injection of complete Freund's adjuvant (CFA, 20 μl) in mice. Mice were lightly anaesthetized with halothane and received 20 μl of CFA (1 mg/ml of heat-killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monoleate) subcutaneously in the plantar region of the right hind paw, according to a previously reported method.[23]

One group of mice received oral administration of 7-HC (60 mg/kg) or vehicle (saline with DMSO 2%; control group) 40 min before CFA (injected at time zero). A second group received oral administration of 7- HC (60 mg/kg) or vehicle daily, 24 h after the CFA injection (zero time) and again over the following four days. The nociceptive threshold was evaluated before and 2 h after the daily administration. On the 2nd day after the disruption of the daily treatment (day 6 after CFA injection), an acute administration of 7-HC (60 mg/kg) was made.

The mechanical hypernociception was measured, as described previously,[24] as the force (in grams) for a 50% paw withdrawal threshold in response to application of different von Frey filaments (Stoelting, Chicago, USA). A positive response was characterized by the removal of the paw followed by clear flinching movements.

Tail flick test

The tail flick test (Analgesiometer, Insight, Brazil) in rats was conducted as described elsewhere.[25] Each animal was placed in a ventilated tube with the tail laid across a wire coil which was at room temperature (23 ± 2°C). The coil temperature was then raised by the passage of electric current and the latency for the tail withdrawal reflex was measured. Each trial was terminated after 6 s to minimize the possibility of skin damage.

Febrile response induced by lipopolysaccharide

Body temperature (Tb) was measured in conscious and non-restrained rats using battery-operated biotelemetry transmitters (Data Science, St Paul, MN, USA) implanted in the peritoneal cavity. Experimental measurements were conducted in rats housed individually in a room maintained at 28 ± 1°C, within the thermoneutral range for rats.[26] The baseline temperature of the rats was determined prior to any injection. 7-HC (30, 60 and 120 mg/kg p.o.) or vehicle (saline with DMSO 2%; control group) was orally administered 40 min before fever was induced at time zero by an intravenous (through the tail vein) injection of LPS (*E. coli* 0111:B4) at a dose of 5 μg/kg.

Tb was measured for 6 h after the injection of LPS. Nimesulide (1 mg/kg i.p.) was the reference drug.

Paw oedema induced by carrageenan

Oedema was induced by intraplantar injection of carrageenan in mice. 7-HC (30 and 60 mg/kg) or vehicle (saline with DMSO 2%; control group) was orally administered 40 min before carrageenan (injected at time zero). The volume of the paw of each mouse was measured with a plesthysmometer (Ugo Basile, Comerio, Italy) before the administration of carrageenan (200 μg) (Vb, baseline) and 2, 3, 4 and 24 h afterward (Vt), as described previously.[27] The amount of paw swelling was determined for each mouse and the difference between Vt and Vb was taken as the oedema value. Dexamethasone (0.7 mg/kg s.c.) and nimesulide (5 mg/kg p.o.) were the reference drugs.

Ulcerogenic effect

For histopathological analysis, animals were subdivided into four groups (n = 6) as follows: 1, control (vehicle); 2 and 3, 7-HC (60 mg/kg, 4 h or 12 h after the oral administration); 4, indometacin (100 mg/kg, 4 h after oral administration). Mice were killed using deep CO_2 anaesthesia. After removal the stomachs were opened through the greater curvature, washed with saline, fixed in 10% formalin solution, rinsed, dehydrated and embedded in paraffin. Tissue blocks were sectioned at 3 μm thickness (Cryostat RM2145, Leica, Germany), stained with haematoxylin–eosin and observed by light microscopy (40×) to examine morphological alterations, inflammatory cell infiltrate, oedema, epithelial cell loss and necrosis. The histopathological alterations were assessed using the following score:[28] 0, absence; 1, discrete; 2, moderate; 3, moderate to intense; 4, intense. For each stomach, 10 fields (40×) were analysed per section and the result was expressed as mean ± SEM. Morphological analyses were done without knowing to which experimental group each mouse belonged. In order to illustrate severe inflammatory events and high ulcer incidence in mice gastric tissue, we used the indometacin-induced gastric ulcer model.[29]

Motor function assay: rota rod

To evaluate the possible nonspecific muscle-relaxant or sedative effects of 7-HC, mice were submitted to the rota rod task (Insight, Brazil).[30] The animals were selected 24 h previously by eliminating those mice that did not remain on the bar rotated at a constant speed of 5 rpm for two consecutive periods of 120 s. Animals were treated with diazepan (10 mg/kg i.p.), 7-HC (120 mg/kg p.o.) or vehicle and 40 min afterwards were placed on a rotating rod. The latency to falling was measured up to 120 s. The results are expressed as the average time (s) the animals remained on the rota rod.

Data analysis

Data are presented as means ± standard error of the mean (SEM) of measurements made on six to eight animals in each group. Comparisons across three or more treatments were made using one-way ANOVA with Tukey's post-hoc test or, for repeated measures, two-way ANOVA with Bonferroni's post-hoc test, as appropriate. The results for ulcerogenic effects were analysed as medians with their corresponding confidence limits (95%) and compared by the non-parametric Kruskal–Wallis test followed by the Dunn's post-hoc test. All data were analysed using the Prism 4 computer software (GraphPad, San Diego, USA). Statistical differences were considered to be significant at $P < 0.05$. All variations in core body were expressed as changes from the mean basal value (i.e. as ΔT, in °C). Mean baseline temperatures were not statistically different among the groups included in any particular set of experiments.

Results

Antinociceptive effect of 7-HC

Initially the antinociceptive dose of 7-HC in mice was determined using the writhing test. In mice, oral administration of 7-HC (1–60 mg/kg, 40 min before acid injection) produced a significant ($P < 0.05$) dose-related inhibition of the abdominal constrictions induced by the acetic acid (Figure 1). Indometacin (10 mg/kg) and nimesulide (5 mg/kg), which are

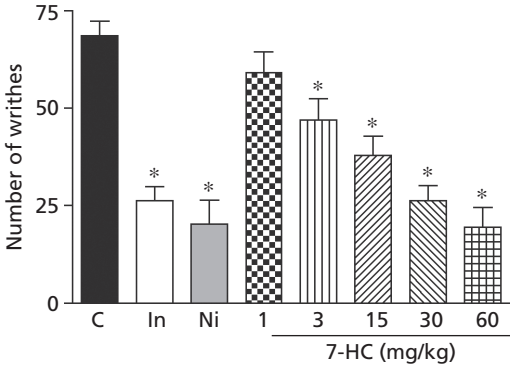

Figure 1 Effect of oral administration of 7-hydroxycoumarin on acetic acid-induced writhing in mice. 7-HC, 7-hydroxycoumarin; C, control (vehicle); In, indometacin; Ni, nimesulide. Data are expressed as means ± SEM; $n = 8$ mice per group. $^{*}P < 0.05$ vs. control group, ANOVA followed by Tukey test.

non-selective and selective COX-2 inhibitors respectively, and which were used as positive controls, also produced significant inhibition of the response to acetic acid-induced writhing.

Figure 2 shows the effect of 7-HC on formalin-induced hypernociception in rats. Injection of formalin in control animals induced a biphasic flinching response, with the early phase ranging from 0 to 10 min (a) and the late phase from 10 to 60 min (b) after the injection. Treatment with 7-HC (15, 30 and 60 mg/kg) by the oral route, 40 min before the administration of formalin, caused a similar antinociceptive effect ($P < 0.05$) as indometacin (5 mg/kg) and nimesulide (5 mg/kg) in both the early and late phases. On the other hand, the oral administration of 7-HC (60 mg/kg) did not alter the latency response to the tail-flick test, in contrast to morphine (5 mg/kg s.c.), which caused a significant increase in response (data not shown).

In another set of experiments, the effect of 7-HC against chronic inflammatory stimuli, was evaluated. The intraplantar injection of CFA (zero time) produced a profound and long-lasting mechanical hypernociception on the ipsilateral paw (Figure 3). To assess the effects of acute pre-treatment with 7-HC on CFA-induced chronic inflammatory pain, animals received 7-HC at 60 mg/kg 40 min before the CFA injection, a dose selected based on the results provided by the writhing test (Figure 3a). Administration of 7-HC significantly decreased mechanical hypernociception 2 h after treatment, and its effect remained significant until 24 h later. The acute treatment with 7-HC did not affect the basal nociceptive threshold (data not shown).

To investigate the effects of long-term treatment, 7-HC (60 mg/kg) was orally administered to mice, once a day, 24 h after the CFA injection and for four consecutive days (Figure 3b). Mechanical hypernociception was evaluated before and 2 h after the treatment because at this time the inhibitory activity of 7-HC on acute experiments was observed. Daily treatment with the 7-HC markedly decreased the paw withdrawal response. Importantly, the antinociceptive effect was maintained throughout the period of treatment, only returning to the control level 2 days after

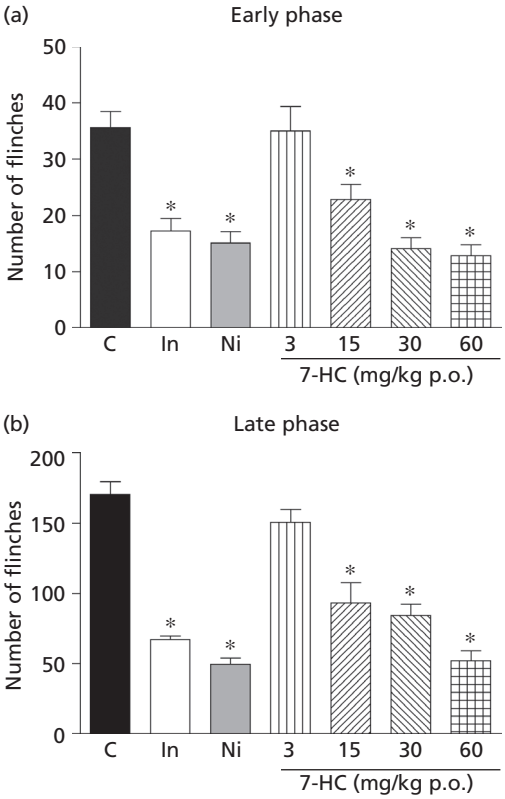

Figure 2 Effect of 7-hydroxycoumarin treatment on acute inflammatory pain induced by formalin in rats. Panels show the effects of 7-HC on the (a) early and (b) late phases of formalin-induced flinches in rats. 7-HC, 7-hydroxycoumarin; C, control (vehicle); In, indometacin; Ni, nimesulide. Data are expressed as means ± SEM; $n = 8$ rats per group. $^{*}P < 0.05$ vs. control group, ANOVA followed by Tukey test.

discontinuation of the daily treatment. On the 6th day, when mechanical hypernociception was re-established, an acute administration of 7-HC (60 mg/kg) produced an antinociceptive effect similar to that observed after the first administration of the daily treatment, indicating that 7-HC does not induce tolerance.

Anti-inflammatory effect of 7-HC

The anti-inflammatory effect of 7-HC was evaluated with the paw oedema model in mice. The results in Figure 4 indicate that oral administration of 7-HC 40 min before carrageenan administration significantly reduces ($P < 0.05$) the oedema. Treatment with 7-HC at 60 mg/kg produced a similar antioedematogenic effect to nimesulide (5 mg/kg). The administration of 7-HC at 30 mg/kg produced a more discrete reduction. This effect was statistically significant at 2 and 4 h after the carrageenan injection. In addition, the oedema was strongly inhibited by pre-treatment (4 h prior) with dexamethasone (0.7 mg/kg).

Antipyretic effect of 7-HC

Figure 5 shows the effect of 7-HC on LPS-induced fever in rats. In the control animals (vehicle), Tb started to increase 1 h after the LPS injection, reached its maximum value

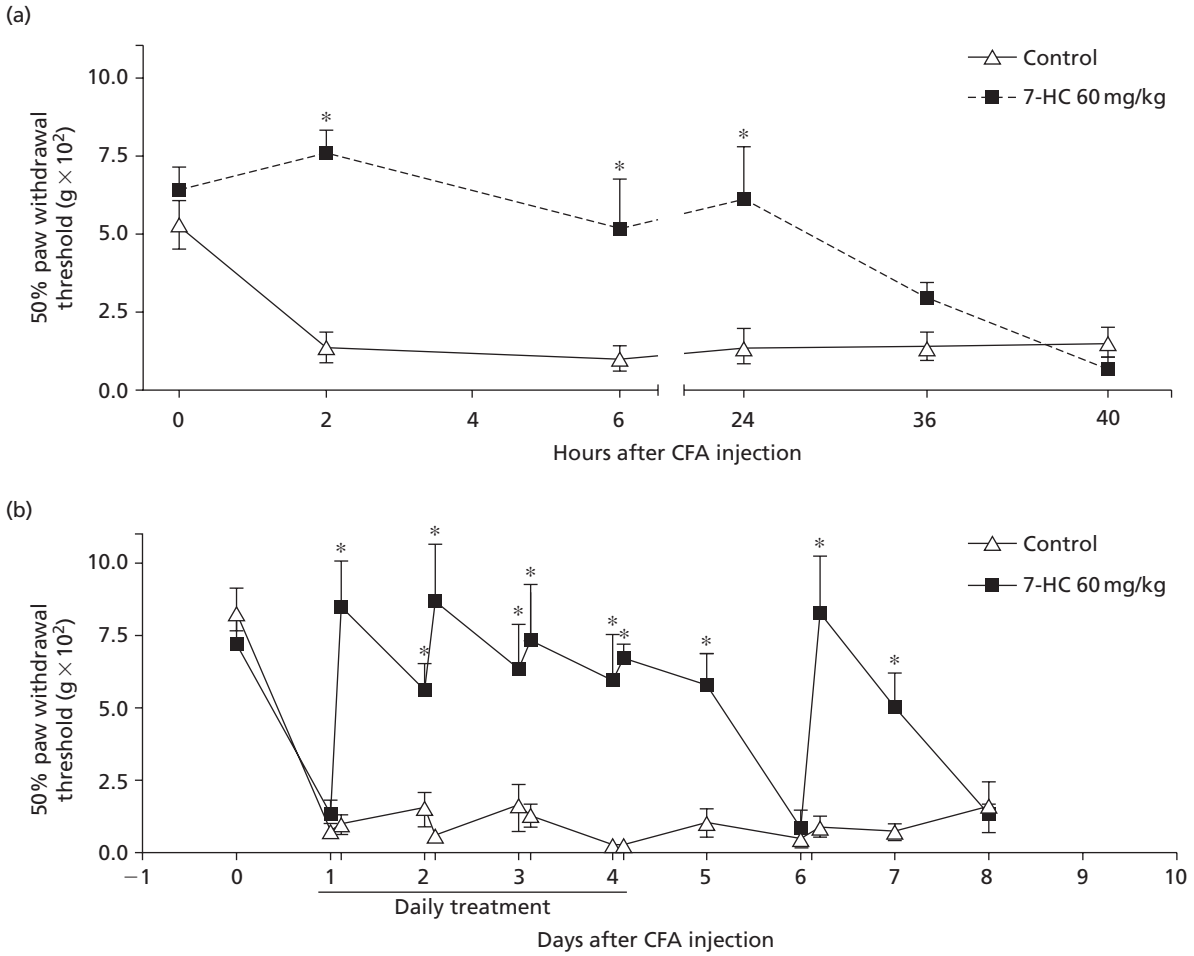

Figure 3 Effect of 7-hydroxycoumarin on mechanical allodynia induced by complete Freund's adjuvant in mice. 7-HC, 7-hydroxycoumarin; CFA, complete Freund's adjuvant. Data are expressed as means ± SEM; $n = 6$ mice per group. $^{*}P < 0.05$ vs. control group, two-way ANOVA followed by Bonferroni test.

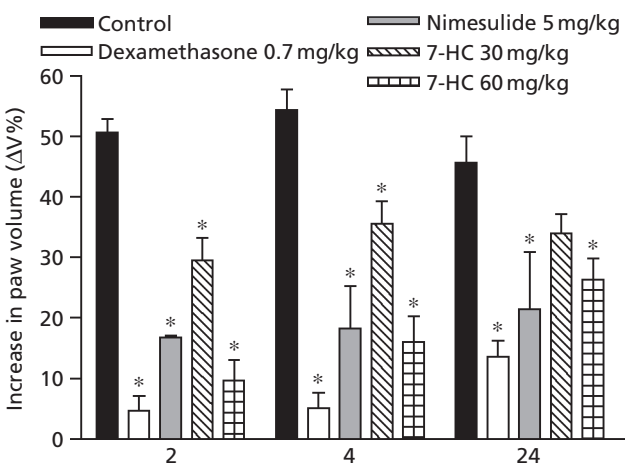

Figure 4 Effect of 7-hydroxycoumarin treatment on carrageenan-induced oedema. 7-HC, 7-hydroxycoumarin. Data are expressed as means ± SEM; $n = 8$ mice per group. $^{*}P < 0.05$ vs. control group, two-way ANOVA followed by Bonferroni test.

around 2 h after injection (an increase of around 2°C from basal values) and remained elevated until the end of the experiment. All doses of 7-HC – 30, 60 and 120 mg/kg – were able to reduce the febrile response to LPS in comparison to control animals, but this reduction was statistically significant ($P < 0.05$) only at the highest dose tested. Nimesulide (1 mg/kg) was used as the reference drug and produced significant antipyretic activity throughout the experimental period.

Gastric injury and motor performance

Table 1 and Figure 6 show the effects of oral administration of 7-HC on the gastric mucosa. In order to illustrate severe inflammatory events and high ulcer incidence in gastric tissue of mice, we used an elevated dose of indometacin. 7-HC was found to be safe from the viewpoint of acute ulcer induction. Four or 12 h after the oral administration of 7-HC (30 and 60 mg/kg) no inflammatory cell infiltrate, oedema, epithelial cell loss or necrosis in the gastric tissue were observed, as opposed to the control (saline with DMSO 2%)

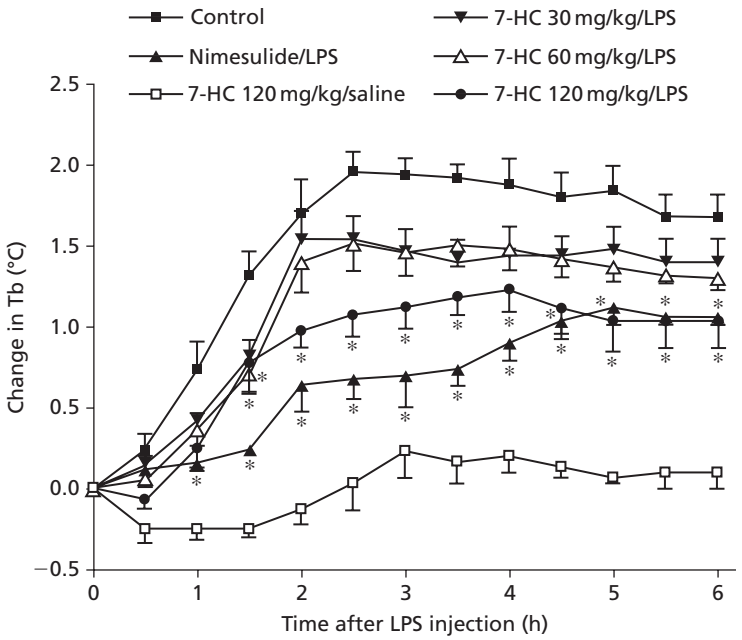

Figure 5 Effect of the 7-hydroxycoumarin treatment on lipopolysaccharide fever. 7-HC, 7-hydroxycoumarin; LPS, lipopolysaccharide; Tb, body temperature. Data are expressed as means ± SEM; n = 8 rats per group. $^{*}P < 0.05$ vs. control group, two-way ANOVA followed by Bonferroni test.

Table 1 Absence of acute gastric damage after oral administration of 7-hydroxycoumarin in mice

Treatment	Dose (mg/kg)	Time (h)	*n*	Inflammatory cells	Epithelial cell loss	Oedema	Necrosis
Control		4	5	0.2 ± 0.4	0	0.4 ± 0.5	0
Indometacin	100	4	5	2.2 ± 0.8*	2.4 ± 0.8*	2.6 ± 0.8*	1.2 ± 1.6
7-HC	30	4	5	0.2 ± 0.4	0.2 ± 0.4	0.4 ± 0.5	0
7-HC	30	12	5	0.4 ± 0.4	0.4 ± 0.8	0.6 ± 0.8	0
7-HC	60	4	5	0.2 ± 0.4	0	0.4 ± 0.5	0
7-HC	60	12	5	0.4 ± 0.5	0.2 ± 0.4	0.6 ± 0.5	0

7-HC, 7-hydroxycoumarin. Values are expressed as means ± SEM, n, number of animals in each group. $^{*}P < 0.05$ compared with vehicle-treated mice (control group). Kruskal–Wallis test with Dunn's post hoc test. For each stomach 10 fields were analysed per section.

group. As expected, a high gastric ulcer and inflammatory event frequency was observed in animals treated with indometacin (100 mg/kg). In the rota rod test, 7-HC-treated mice did not show any significant motor performance alterations at a dose of 120 mg/kg p.o. (100.7 ± 8.2 s) when compared to the control group (97.7 ± 9.1 s). The central nervous system depressant diazepam (10 mg/kg i.p.) reduced the time of mice on the rota rod after 30 min of treatment with this standard drug (7.7 ± 3.9 s; data not shown).

Discussion

In the present study we demonstrate, for the first time, that 7-HC, given orally at a dose that did not induce an acute gastric ulcerogenic effect or motor performance alteration, produces a consistent and long lasting antinociception in a chronic inflammatory pain model. In addition, daily administration of 7-HC markedly decreases the mechanical hypernociception throughout the period of treatment and even 2 days after discontinuation. The antinociceptive effect of 7-HC was also observed in acetic acid-induced writhing and the formalin test, but not in the tail-flick test. Moreover, 7-HC has anti-inflammatory and antipyretic effects against carrageenan-induced oedema and LPS-induced fever.

Oral administration of 7-HC produces a dose-related antinociception when assessed in acetic acid-induced writhing in mice. This test has long been used as a screening tool for the assessment of the analgesic or anti-inflammatory properties of new substances.[21] This method has good sensitivity but shows poor specificity. To avoid misinterpretation of the results, in the present study we confirmed the antinociceptive effect of 7-HC in the formalin test, a model of inflammatory pain that has two distinctive phases which may possibly indicate different types of pain.[31] The early and late phases of the formalin test have obvious differential properties, and therefore this test is useful not only for assessing analgesic substances but also for elucidating the mechanism of analgesia.[32] The early phase, known as

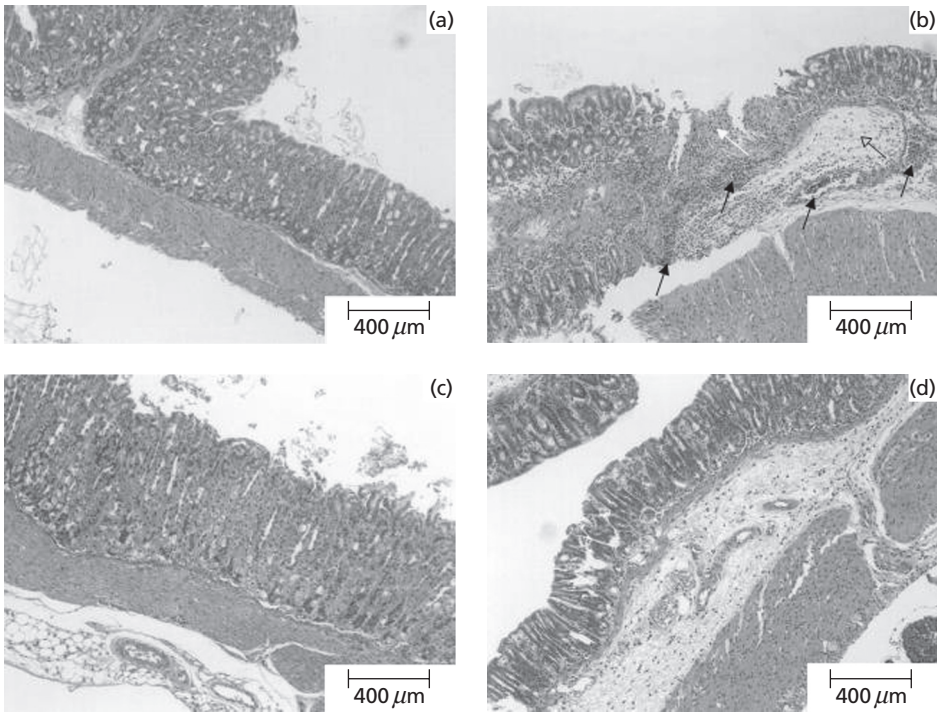

Figure 6 Histopathological analysis of mice stomachs after oral administration of 7-hydroxycoumarin. (a) Absence of inflammatory events and tissue damage in stomach of control mice, 4 h after the oral administration of vehicle (DMSO 2% in saline). (b) Inflammatory cell infiltrate (black arrows), oedema (open arrow), epithelial cell loss and necrosis (white arrow) in stomach of mice, 4 h after the oral administration of indometacin (100 mg/kg). (c and d) Gastric sections of mice 4 and 12 h, respectively, after the oral administration of 7-HC (60 mg/kg). Treatment with 7-HC did not induce significant inflammatory events and tissue damage in gastric mucosa. Gastric sections stained with haematoxylin and eosin. For each stomach 10 fields (40×) were analysed per section.

non-inflammatory pain, is a result of direct stimulation of the nociceptors and reflects centrally-mediated pain. The late phase, known as inflammatory pain, is caused by local inflammation with a release of inflammatory and hyperalgesic mediators.[31]

In the present study we found that 7-HC had an antinociceptive effect both in the early and late phases of the formalin test, an observation that is in line with previous results.[17]. The inhibitory property of 7-HC on the late phase of the formalin test suggests that its antinociceptive activity is due, at least in part, to an anti-inflammatory action. This idea is confirmed by our observation that 7-HC is able to reduce carrageenan-induced oedema in mice. In fact, an anti-inflammatory action of 7-HC has previously been reported.[17]

Although we have demonstrated antinociceptive properties of 7-HC in a range of pain models, the oral administration of 7-HC did not prevent nociception in the tail-flick test. The thermal model of the tail-flick test is considered to be a spinal reflex, but could also involve higher neural structures and this method therefore mainly identifies central analgesics.[33,34] The fact that 7-HC produced antinociception in all models tested except the tail-flick test suggests that it does not block the neural transmission of pain in the way that morphine does. These data reinforce our hypothesis that 7-HC-induced antinociception is related to its anti-inflammatory activity. Moreover, 7-HC treatment at high therapeutic doses did not affect the motor performance of the mice as tested in the rota rod test, disproving any possibility of a relaxation or motor-deficit effect. This result therefore corroborates the antinociceptive effect of 7-HC suggested by the nociceptive tests.

In addition, in the present work we demonstrate for the first time that 7-HC is able to prevent the febrile response induced by LPS. Several studies sustain the hypothesis that COX-2 seems to be the enzyme responsible for the biosynthesis of prostanoids during inflammatory and febrile responses.[35–40] Thus, it is possible that 7-HC exerts antinociceptive, anti-inflammatory, and antipyretic effects by blocking this enzyme. Supporting this hypothesis, Kim *et al*. reported that 7-HC has COX-2 inhibitory activity, since it reduces the production of prostaglandin D2 in mouse bone marrow-derived mast cells.[41] In the same way, 7-HC was found to inhibit the biosynthetic conversion of arachidonic acid to prostaglandin products. The interruption of the synthesis occurred during the initial cyclooxygenation reaction.[42]

Although chronic pain results in enormous morbidity and social cost, it is not yet successfully managed and pharmacologically controlled. To assess the effects of 7-HC in a chronic pain model, we used the model of CFA-induced inflammatory hypernociception. CFA consists of heat-killed mycobacteria suspended in mineral oil, which produces a chronic inflammatory response in rodents. Moreover, the pain stimulus promoted by CFA is persistent rather than a

transient one and may thus resemble some kinds of clinical inflammatory pain states.[43] Our results show that 7-HC produces a marked and long-lasting antinociceptive effect on CFA-induced hypernociception. Interestingly, the antinociception action of 7-HC extended up to 24 h after the treatment, an effect that is rarely reached for clinically used analgesics. Moreover, daily treatment with 7-HC induced an antinociceptive state persisting throughout the treatment period. The antinociception effect of 7-HC was not diminished by the onset of tolerance, since an acute administration of 7-HC 2 days after the daily treatment produced a similar and marked antinociceptive effect. These results reinforce the idea that the antinociceptive effect of 7-HC is associated with its anti-inflammatory action.

It is well recognized that the intraplantar injection of CFA produces persistent inflammatory hypernociception initiated by peripheral nociceptor activation and local release of inflammatory and hyperalgesic mediators, such as cytokines and prostanoids, which are involved in sensitization of nociceptive pathways.[43] Inflammation causes the induction of COX-2,[3] leading to the release of prostanoids, which contribute to the development of peripheral sensitization through phosphorylation of ion channels in nociceptor terminals, increasing excitability and reducing the pain threshold.[44] While the sensitization of nociceptors does not by itself provoke overt pain, it is common to all types of inflammatory pain and is associated with chronic pain.[45] Considering that the antinociceptive effect of 7-HC is associated with an anti-inflammatory action, its ongoing antinociceptive effect in the chronic pain model could be a reflex of reduced prostanoid release and, consequently, of a reduced sensitization of the nociceptors. On the other hand, since the antinociceptive effects of conventional NSAIDs are usually not long-lasting, additional mechanisms may be involved in the 7-HC-induced antinociception.

Recently, Tomaya *et al.* demonstrated that 7-HC interacts with phospholipase A2 and causes a structural modification that inhibits the oedematogenic activity of this enzyme.[46] This result suggests that the anti-inflammatory effect of 7-HC involves the inhibition of phospholipase A2. On the other hand, up-regulation of the expression of COX-2, but not of COX-1, by phospholipase A2 was demonstrated. Hence, COX-2 is the major isoform involved in the synthesis of prostanoids and in the inflammatory response induced by phospholipase A2.[47] Therefore, a COX-2/phospholipase A2 dual inhibitory activity of 7-HC could be responsible for its anti-inflammatory effect. In the same way, the contribution of phospholipase A2 to inflammatory pain is suggested. Phospholipase A2 stimulates the production and release of inflammatory mediators, such as IL-1β, IL-6, TNF-α and PGE2, that are involved with nociceptive sensitization and inflammatory pain.[47,48] In line with this idea, the antinociceptive effect of 7-HC may also be associated with phospholipase A2 inhibition. However, further studies are necessary to corroborate this hypothesis.

Most available anti-inflammatory-analgesic drugs reduce pain and inflammation by blocking the metabolism of arachidonic acid and thereby the production of prostaglandins.[49] In the stomach the prostaglandins are formed via COX-1, the constitutive isoform, and exert cytoprotective effects. Thus, long-term administration of these compounds may lead to development of threatening gastrointestinal ulcers and bleeding.[50–52] Taking this fact into consideration, the acute ulcerogenic effect of oral administration of 7-HC was studied. The 7-HC acute treatment was found safe from the viewpoint of inflammatory events and ulcer induction at therapeutic dose levels. Besides this, 7-HC is a major metabolite of coumarin in humans[9] and no adverse effects of coumarin have been reported in humans using doses up to 7 g daily after 2 weeks of continued treatment.[7,53] The low toxicity in humans and absence of acute gastric side effects reported here reinforce the potential of 7-HC for pharmacological development.

Conclusions

Taken together, these results strongly suggest that 7-HC possesses anti-inflammatory, analgesic and antipyretic effects, which seem to be related to COX-2 inhibition. The inhibition of COX-2 instead of COX-1 is reinforced by the fact that 7-HC does not cause acute stomach lesions, a common feature of nonselective NSAIDs. Thus, 7-HC is a promising substance for the development of new drugs for the control of chronic inflammatory pain and other conditions of inflammation and fever.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Dubner R, Ruda MA. Activity-dependent neuronal plasticity following tissue injury and inflammation. *Trends Neurosci* 1992; 15: 96–103.
2. Strassels SA *et al*. Pharmacotherapy of pain in older adults. *Clin Geriatr Med* 2008; 24: 275–298.
3. Vane JR *et al*. Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol* 1998; 38: 97–120.
4. Tapiero H *et al*. Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. *Biomed Pharmacother* 2002; 56: 215–222.
5. Rainsford KD. Anti-inflammatory drugs in the 21st century. *Subcell Biochem* 2007; 42: 3–27.
6. Murray RD. Coumarins. *Nat Prod Rep* 1989; 6: 591–624.
7. Egan D *et al*. The pharmacology, metabolism, analysis and applications of coumarin and coumarin-related compounds. *Drug Metabolism Reviews* 1990; 22: 503–529.
8. Razavi SM *et al*. Coumarins from the aerial parts of *Prangos uloptera* (Apiaceae). *Braz J Pharmacogn* 2008; 18: 1–5.

9. Lake BG. Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment. *Food Chem Toxicol* 1999; 37: 423–453.
10. Wu FJ, Sheu SJ. Analysis and processing of Chinese herbal drugs: the study of *Fructus aurantii immaturus* (Chin.). *Chin Pharmaceut J* 1992; 44: 257–263.
11. Vasconcelos JF *et al*. Effects of umbelliferone in a murine model of allergic airway inflammation. *Eur J Pharmacol* 2009; 609: 126–131.
12. Hoult JRS, Paya M. Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential. *Gen Pharmacol* 1996; 27: 713–722.
13. Kofinas C *et al*. Cytotoxic coumarins from the aqueous parts of *Terdyliim apulum* and their effects on non small cell bronchial carcinoma cell line. *Planta Med* 1998; 64: 174–176.
14. Lopez-Gonzalez JS *et al*. Apoptosis and cell cycle disturbances induced by coumarin and 7-hydroxycoumarin on human lung carcinoma cell lines. *Lung Cancer* 2004; 43: 275–283.
15. Ramesh B, Pugalendi KV. Antihyperglycemic effect of umbelliferone in streptozotocin-diabetic rats. *J Med Food* 2006; 9: 562–566.
16. Marles RJ, Farnsworth N. Antidiabetic plants and their active constituents: an update. *Prof J Bot Med* 1996; 3: 85–135.
17. Chen YF *et al*. Anti-inflammatory and analgesic activities from roots of *Angelica pubescens*. *Planta Med* 1995; 61: 2–8.
18. Besson JM. The neurobiology of pain. *Lancet* 1999; 353: 1610–1615.
19. Wang LX, Wang ZJ. Animal and cellular models of chronic pain. *Ad Drug Delivery Rev* 2003; 55: 949–965.
20. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983; 16: 109–110.
21. Collier HOJ *et al*. The abdominal constriction response and its suppression by analgesic drugs in the mouse. *Br J Pharmacol Chemother* 1968; 32: 295–310.
22. Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine and brain-stem stimulation in rats and cats. *Pain* 1977; 4: 161–174.
23. Kassuya CA *et al*. Anti-allodynic and anti-oedematogenic properties of the extract and lignans from *Phyllanthus amarus* in models of persistent inflammatory and neuropathic pain. *Eur J Pharmacol* 2003; 478: 145–153.
24. Chaplan SR *et al*. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994; 53: 55–63.
25. D'Amour FE, Smith DL. A method for determining loss of pain sensation. *J Pharmacol Exp Ther* 1941; 72: 74–79.
26. Gordon CJ. Thermal biology of the laboratory rat. *Physiol Behav* 1990; 47: 963–991.
27. Winter CA *et al*. Carrageenan-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. *Proc Soc Exp Biol Med* 1962; 111: 544–547.
28. Laine L, Weinstein WM. Histology of alcoholic hemorrhagic-gastritis: a prospective evaluation. *Gastroenterology* 1988; 94: 1254–1262.
29. Trevethick MA *et al.* Non-steroidal anti-inflammatory drug-induced gastric damage in experimental animals: underlying pathological mechanisms. *Gen Pharmacol* 1995; 26: 1455–1459.
30. Vaz ZR *et al*. Antinociceptive action of 2-(4-bromobenzoyl)-3-methyl-4,6-dimethoxy benzofuran, a novel xanthoxyline derivative on chemical and thermal models of nociception in mice. *J Pharmacol Exp Ther* 1996; 278: 304–312.
31. Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* 1987; 30: 103–114.
32. Shibata M *et al*. Modified formalin test: characteristic biphasic pain response. *Pain* 1989; 38: 347–352.
33. Jensen TS, Yaksh TL. Comparison of antinociceptive action of morphine in the periaqueductal gray, medial and paramedial in rat. *Brain Res* 1986; 363: 99–113.
34. Le Bars D *et al*. Animal models of nociception. *Pharmacol Rev* 2001; 53: 597–652.
35. Bakhle YS, Botting RM. Cyclooxygenase-2 and its regulation in inflammation. *Med Inflamm* 1996; 5: 305–323.
36. Cao C *et al*. Induction by lipopolysaccharide of COX-2 mRNA in the brain: its possible role in the febrile response. *Brain Res* 1995; 697: 187–196.
37. Cao C *et al*. Endothelial cells of the rat brain vasculature express cyclooxygenase-2 mRNA in response to systemic interleukin-1β: a possible site of prostaglandin synthesis responsible for fever. *Brain Res* 1996; 733: 263–272.
38. Lugarini F *et al*. A role for cyclooxygenase-2 in lipopolysaccharide-induced anorexia in rats. *Am J Physiol Regul Integr Comp Physiol* 2002; 283: 862–868.
39. Fabricio ASC *et al.* The effects of selective and non-selective cyclooxygenase inhibitors on endothelin-1-induced fever in rats. *Am J Physiol Regul Integr Comp Physiol* 2005; 288: 671–677.
40. Machado RR *et al*. CCR1 and CCR5 chemokine receptors are involved in fever induced by LPS (*E. coli*) and RANTES in rats. *Brain Res* 2007; 1161: 21–31.
41. Kim JS *et al*. Chemical constituents of the root of *Dystaenia takeshimana* and their anti-inflammatory activity. *Arch Pharm Res* 2006; 29: 617–623.
42. Lee RE *et al*. Inhibition of prostaglandin biosynthesis by coumarin, 4-hydroxycoumarin, and 7-hydroxycoumarin. *Arzneimittelforschung* 1981; 31: 640–642.
43. Larson AA *et al*. Pain threshold changes in adjuvant-induced inflammation: a possible model of chronic pain in the mouse. *Pharmacol Biochem Behav* 1985; 24: 49–53.
44. McCleskey EW, Gold MS. Ion channels of nociception. *Annu Rev Physiol* 1999; 61: 835–856.
45. Basbaum AI. Distinct neurochemical features of acute and persistent pain. *Proc Natl Acad Sci USA* 1999; 96: 7739–7743.
46. Tomaya DO *et al*. Effect of umbelliferone (7-hydroxycoumarin, 7-HOC) on the enzymatic, edematogenic and necrotic activities of secretory phospholipase A2 (sPLA2) isolated from *Crotalus durissus collilineatus* venom. *Toxicon* 2009; 53: 417–426.
47. Moreira V *et al*. Secretory phospholipases A2 isolated from *Bothrops asper* and from *Crotalus durissus terrificus* snake venoms induce distinct mechanisms for biosynthesis of prostaglandins E(2) and D(2) and expression of cyclooxygenases. *Toxicon* 2008; 52: 428–439.
48. Zuliani JP *et al*. Inflammatory events induced by Lys-49 and Asp-49 phospholipasesA2 isolated from Bothrops asper snake venom: role of catalytic activity. *Toxicon* 2005; 45: 335–346.
49. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 1971; 231: 232–235.
50. Robert A. Antisecretory, antiulcer, cytoprotective and diarrheogenic properties of prostaglandins. *Adv Prostaglandin Thromboxane Res* 1976; 2: 507–520.
51. Peskar BM. On the synthesis of prostaglandins by human gastric mucosa and its modification by drugs. *Biochim Biophys Acta* 1977; 487: 307–314.
52. Allison MC *et al*. Gastrointestinal damage associated with the use of nonsteroidal antiinflammatory drugs. *N Engl J Med* 1992; 327: 749–754.
53. Sharifi S *et al*. Pharmacokinetics of coumarin and its metabolites. Preliminary results in three healthy volunteers. *J Ir Coll Phys Surg* 1993; 22: 29–32.