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Antinociceptive properties of caffeic acid derivatives in mice

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ABSTRACT

Ten ester derivatives from caffeic acid were synthesized, and their antinociceptive properties are evaluated in mice. The most active compound, dodecyl ester derivative, exhibited potent and dose-related activity against the writhing test, with a calculated ID_{50} value of 15.1 (11.9–19.1) μmol/kg and MI of 78.8% being several times more active than reference drugs. It was also effective in other experimental models, such as formalin, capsaicin and glutamate-induced pain tests, but was inactive in the hot-plate test. Although the mechanism of action has still not been elucidated, these results appear to support its therapeutic potential against painful diseases.

1. Introduction

Caffeic acid (**1**) and its derivatives are widely distributed in medicinal plant (fruits, vegetables, wine, and olive oil, among others) and are therefore present in human plasma in a diet dependent concentration [1,2]. Indeed, their esters and amide derivatives exhibit a broad spectrum of biological activities, including anti-oxidative properties, and they have been shown to scavenge a number of reactive species, including DPPH radicals, and peroxyl and hydroxyl radicals [3–6]. Furthermore, caffeic acid has been shown to possess anti-inflammatory and protective effects against Ni induced oxidative liver damage [7–9]. Studies carried out with COX enzyme inhibitory assays have demonstrated that some caffeate derivatives inhibit the cyclo-oxygenases COX-1 and COX-2 enzymes [10]. We have previously demonstrated that some of its derivative esters present promising anti-inflammatory effects [11]. This evidence prompted a search of antinociceptive agents from natural sources, and related synthetic derivatives, such as caffeoyl esters. The present report deals with the evaluation of different caffeic acid ester derivatives against some models of pain in mice. Some structural aspects are also discussed. In addition, some reference drugs, acetyl salicylic acid (ASA), acetaminophen (ACE) and morphine (MOR) were included for the purpose of comparison.

2. Chemistry

A scheme of the synthesis of derivatives (**2**–**11**) is illustrated in Fig. 1. All the compounds were synthesized using the esterification procedure proposed by Fischer with some modifications [12]. In the present work, acetyl chloride was used as the source of HCl catalyst *in situ* (Fig. 1A). Subsequently occurs the nucleophilic attack of alcohol on the carbonyl group of caffeic acid, protonated by HCl, giving the respective ester and water (Fig. 1B). In all the syntheses was used alcohol in excess looking for better yields, accelerate the reaction time, and solubilize the caffeic acid. When caffeic acid was not soluble in alcohol, acetone was used as dissolvent. Initially the alcohol in excess was added to acetyl chloride drop by drop, under stirring in an ice bath. Caffeic acid was added to this mixture, which was refluxed until the end of the reaction, monitored by TLC.

3. Pharmacology

All the compounds (**1**–**11**, Table 1) were initially administered intraperitoneally (i.p) at 10 mg/kg, 30 min before of writhing test, in an attempt to select the most active compound. Thus, the dodecyl caffeate (**6**) was then tested in more specific models using different doses to evaluate the dose-response effect, calculate the maximum inhibition and ID_{50}.

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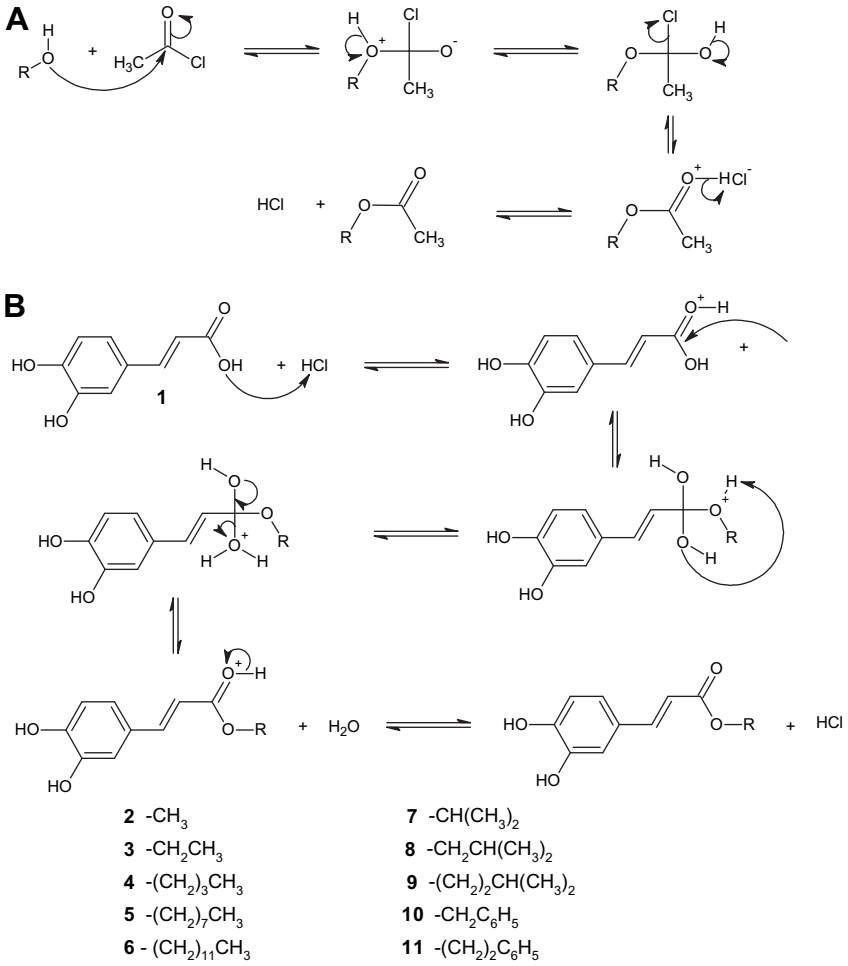


Fig. 1. Scheme of synthesis of caffeic acid derivatives.

4. Results and discussion

Recently, natural products have attracted much attention as sources of new antinociceptive agents [13–17]. Although caffeic acid and some derivatives have been pharmacologically investigated against several experimental models, few papers have reported their antinociceptive activities. We therefore synthesized ten caffeic acid derivatives, first evaluating them against the writhing test in mice.

Table 1 shows that all the derivatives with aliphatic chain caused a similar or higher antinociceptive effect than acetyl salicylic acid and acetaminophen, two well-known drugs used as reference, while the two compounds with aromatic chain demonstrated lower activity when analyzed against the writhing test in mice, at 10 mg/kg. Considering that the compound **6** showed

Table 1
Antinociceptive activity of caffeic acid derivatives and reference drugs against acetic acid-induced abdominal constriction in mice at 10 mg/kg, given intraperitoneally.

Compounds	Inhibition (%)
1	34.6 (±3.9)*
2	54.5 (±2.6)**
3	58.1 (±4.4)**
4	45.0 (±2.2)**
5	46.2 (±2.1)**
6	78.8 (±1.5)**
7	31.0 (±5.5)*
8	52.0 (±2.5)**
9	75.8 (±4.5)**
10	8.8 (±4.8)
11	27.5 (±2.8)**
ACE	38.0 (±1.0)**
ASA	35.0 (±2.0)*

Each group represents the mean ± SEM of 6 experiments. *$p < 0.05$ and **$p < 0.01$ compared with the respective control values.

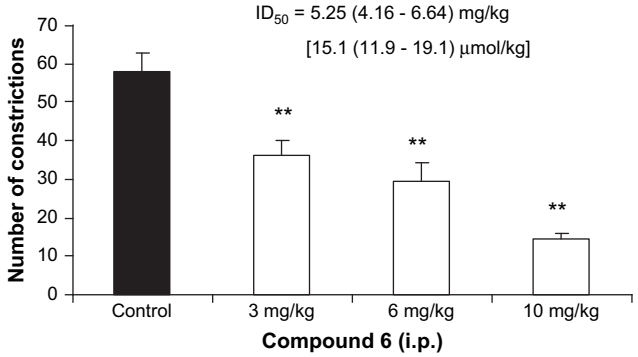


Fig. 2. Effect of **6** (3–10 mg/kg, i.p.) against acetic acid-induced abdominal constrictions in mice. Each column represents mean ± S.E.M. of six experimental values. *Significance levels, when compared with the control group *$p < 0.05$; **$p < 0.01$.

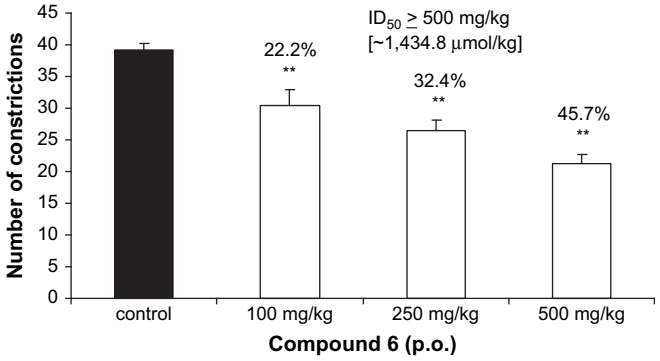


Fig. 3. Effect of **6** (100–500 mg/kg, i.p.) against acetic acid-induced abdominal constrictions in mice by oral route. Each column represents mean ± S.E.M. of six experimental values. *Significance levels, when compared with the control group. $^{*}p < 0.05$; $^{**}p < 0.01$.

a more pronounced effect and a better yield, it was evaluated in other specific models, and compared with the standard drugs used as reference, such as acetyl salicylic acid (ASA), acetaminophen (ACE) and morphine (MOR). As can be observed in Fig. 2, the compound **6** caused a dose-dependent antinociceptive effect with an ID_{50} calculated value of 15.1 (11.9–19.1) μmol/kg and maximal inhibition of 78.8%, being about 9-fold more active than acetyl salicylic acid and acetaminophen whose values of ID_{50} were 133 (73–243) μmol/kg and 125 (104–250) μmol/kg, respectively [17].

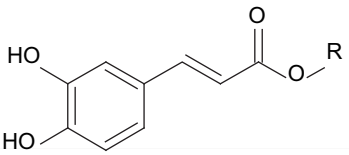
Local peritoneal receptors are believed to be partly involved in the abdominal constriction response. The method has been associated with prostanoids in general, i.e., increased levels of PGE_2 and $PGF_{2\alpha}$ in peritoneal fluids, as well as lipoxygenase products, by some researchers. Therefore, the results of the acetic acid-induced writhing strongly suggest that its mechanism of action may be partly linked to lipoxygenases and/or cyclo-oxygenases [18]. We have also evaluated this compound by the oral route at 500 mg/kg. In this model, **6** was effective in dose-dependent form, with an estimated ID_{50} value of 1434.8 μmol/kg (Fig. 3), being about two times less potent than acetyl salicylic acid and equipotent to acetaminophen, which presented ID_{50} values of 605 (516–705) and 1145 (708–1846) μmol/kg [17] respectively, in the same experimental model. These results suggest that it is absorbed by the gastrointestinal tract. However, the effect was not as representative as when administered by the intraperitoneal route.

In an attempt to verify this difference, some molecular properties and structural factors were analyzed for caffeic acid derivatives, according to the method proposed by Lipinski [19,20], who evaluates the oral bioavailability *in silico* (Table 2).

Recently, Leeson and Springthorpe [21] demonstrated the influence of drug-like concepts on decision-making in medicinal chemistry, comparing the physicochemical profiles of recently discovered oral drugs from four large multinational organizations: AstraZeneca, GlaxoSmithKline, Merck and Co., and Pflizer. They demonstrated that the drug lipophilicity is changing less over time than other physical properties, which suggests that this is an especially important drug-like property, and its control is important for ultimate success in drug development. This is not surprising, since the role of log *P* in influencing drug potency, pharmacokinetics, and toxicity has been established for many years. This property essentially reflects the key event of molecular desolvation in the transfer from the aqueous phases to the cell membranes and protein binding sites, which are mostly hydrophobic in nature.

However, if the lipophilicity is too high, there is an increased likelihood of binding to multiple targets and resultant pharmacologically based toxicology, as well as poor solubility and metabolic clearance. Therefore, the difference in activity between the two routes evaluated for compound **6** can be explained in part by its high lipophilicity (log $P = 7.04$), since this value is higher than those established by Lipinski, whose value for good oral bioavailability must not exceed 5. An extension of the "rule of five" is a rotatable bond count that is now a widely used filter following the finding that rotatable bond greater than 10 are correlated with decreased oral bioavailability. The mechanistic basis for the rotatable bond

Table 2
Theoretical studies of solubility and permeability of caffeic acid derivatives by Lipinski's rule of five.



No	R	No. atom	log *P*[a]	MW	No. ON[b]	No. OHNH[c]	No. rotb.[d]	Volume	TPSA[e]	No. viol.[f]
1	–H	13	0.94	180.1	4	3	2	154.5	77.75	0
2	$-CH_3$	14	1.56	194.2	4	2	3	172.0	66.76	0
3	$-CH_2CH_3$	15	1.93	208,2	4	2	4	188.8	66,76	0
4	$-(CH_2)_3CH_3$	17	2.99	236.3	4	2	6	222.4	66.76	0
5	$-(CH_2)_7CH_3$	21	5.02	292.4	4	2	10	289.6	66.76	1
6	$-(CH_2)_{11}CH_3$	25	7.04	348.5	4	2	14	356.8	66.76	1
7	$-CH(CH_3)_2$	16	2.30	222.2	4	2	4	205.4	66.76	0
8	$-CH_2CH(CH_3)_2$	17	2.68	236.3	4	2	5	222.2	66.76	0
9	$-(CH_2)_2CH(CH_3)_2$	18	3.21	250.3	4	2	6	239.0	66.76	0
10	$-CH_2C_6H_5$	20	3.15	270.3	4	2	5	243.7	66.76	0
11	$-(CH_2)_2C_6H_5$	21	3.36	284.3	4	2	6	260.5	66.76	0
ASA	–	13	1.43	180.2	4	1	3	155.6	63.60	0
ACE	–	11	0.68	151.2	3	2	1	140.0	49.33	0
MOR		21	1.16	285.3	4	2	0	256.7	52.9	0

[a] Method for log *P* prediction developed at Molinspiration (mi log *P*2.2 – November 2005) is based on group contributions.
[b] Sum of N and O H-bond acceptors.
[c] Sum of NH and OH H-bond donors.
[d] Number of rotatable bonds.
[e] Topological polar surface area.
[f] Number of violations.

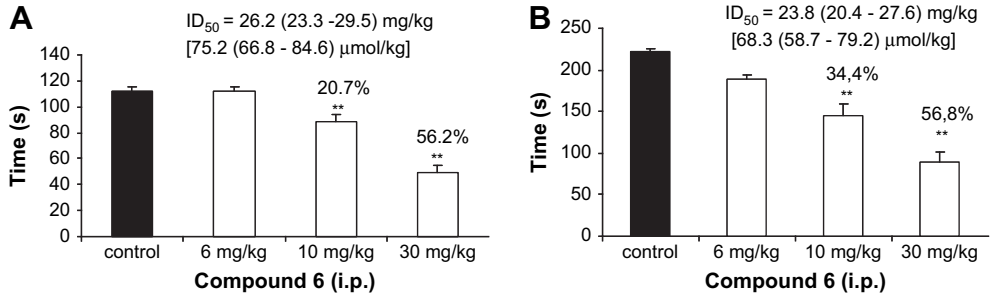


Fig. 4. Effect of **6** (6–30 mg/kg, i.p.) against formalin-induced pain in mice. A = the first phase (0–5 min) and B = second phase (15–30 min). Each column represents mean ± S.E.M. of six experimental values. *Significance levels, when compared with the control group. $^{*}p < 0.05$; $^{**}p < 0.01$.

filter is unclear, because the rotatable bond count does not correlate with the *in vivo* clearance rate, but the filter is reasonable from an *in vitro* screening viewpoint because ligand affinity decreases, on average, by 0.5 kcal for each two rotatable bonds [21]. Thus, this is another important property in evaluating the results found by the oral route, since compound **6** once again goes beyond the threshold, showing 14 rotatable bonds.

With respect to the formalin test, **6** caused marked, dose-dependant inhibition, particularly in the second phase by the systemic route. The calculated ID_{50} values were 75.2 (66.8–84.6) and 68.3 (58.7–79.2) µmol/kg, with maximum inhibition of 56.2 and 56.8%, respectively (Fig. 4). It is important to note that the reference drugs practically prevented only the inflammatory effects (second phase) with ID_{50} values of 123.0 (77.0–209.0) and 120.0 (90–161) µmol/kg [22], respectively to ASA and ACE and that the compound **6** was 3-fold more active in this pain model. Drugs that act primarily on the central nervous system inhibit both phases equally, while peripherally acting drugs inhibit the late phase. The early phase is probably a direct result of the stimulation of nociceptors in the paw, and reflects centrally mediated pain, while the late phase is due to inflammation with a release of serotonin, histamine, bradylkinin and prostaglandins, and to a certain extent, the sensitization of the central nociceptive neurons [23–25].

When evaluated against the capsaicin test, which provided more direct evidence of the antinociceptive effect of this compound on neurogenic pain, compound **6** reduced the licking/biting response to intraplantar capsaicin in a dose-dependent manner, presenting a maximum inhibition of 58.6% at 60 mg/kg, with ID_{50} calculated of 133.7 (122.7–145.5) µmol/kg (Fig. 5). This suggests its involvement with the antagonism of the vanilloid receptor (VR1) [26]. The facts that compound **6** exhibits significant antinociception when assessed against the neurogenic (first phase of formalin test and capsaicin-induced algesic response), seems to be relevant. It has been well-documented that the majority of the nonsteroidal anti-inflammatory drugs analyzed so far are usually ineffective in preventing formalin or capsaicin-induced neurogenic pain [27].

When analyzed by the glutamate test (Fig. 6), **6** showed maximum inhibition of 65.2% with calculated ID_{50} of 110.2 (98.7–123.1) µmol/kg, suggesting that this compound could be inhibiting the liberation of neuropeptides from sensory fibers, namely NKs and kinins involved in the peripheral role of controlling the nociceptive process [27].

In the hot-plate test, **6** did not induce any antinociceptive effect. Since supraspinal and spinal opioid receptors play an important role in this assay, it is possible that **6** do not act on central opioid receptors or produce a release of endogenous opioid peptides [28] (Fig. 7). Since the activity of the central nervous system (CNS) involves blood–brain barrier (BBB) permeation, various kinds of *in silico* prediction methods have been developed [29].

Norinder and Haeberlein [29] proposed two very simple rules to a compound that could have a high chance of entering in the brain. The sum of nitrogen and oxygen (N + O) should be five or less and log BBB $(C\log P - (\mathrm{N}+\mathrm{O})) > 0$. Analyzing these parameters, the compound **6** demonstrated a sum (N + O) of four and the log BBB of 3.03, suggesting that could be active on the CNS. Although lipophilicity was the first of the descriptors to be identified as important for CNS permeation, and *C* log *P* correlates nicely with log BBB and increasing lipophilicity, increasing brain penetration. Meanwhile, the mean value for *C* log *P* for marketed CNS drugs is 2.5 [29]. Other important descriptors are polar surface area (PSA) and rotatable bonds, which has been used as a predictor for blood–brain barrier (BBB) penetration by many researchers [30,31]. In general,

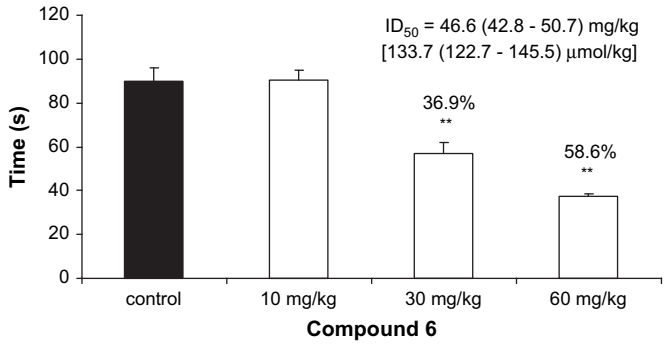


Fig. 5. Effect of **6** (10–60 mg/kg, i.p.) against capsaicin-induced pain in mice. Each column represents mean ± S.E.M. of six experimental values. *Significance levels, when compared with the control group. $^{*}p < 0.05$; $^{**}p < 0.01$.

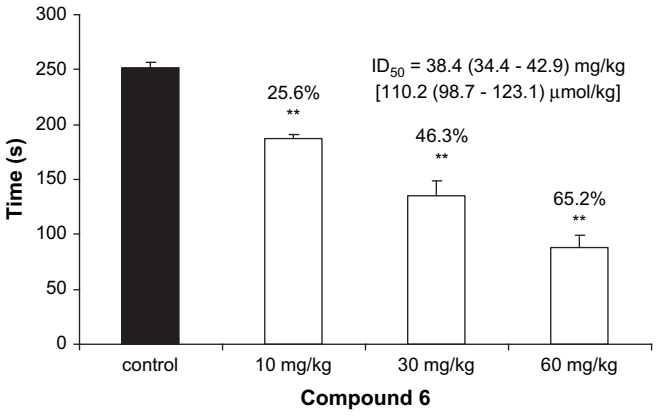


Fig. 6. Effect of **6** (10–60 mg/kg, i.p.) against glutamate-induced pain in mice. Each column represents mean ± S.E.M. of six experimental values. *Significance levels, when compared with the control group. $^{*}p < 0.05$; $^{**}p < 0.01$.

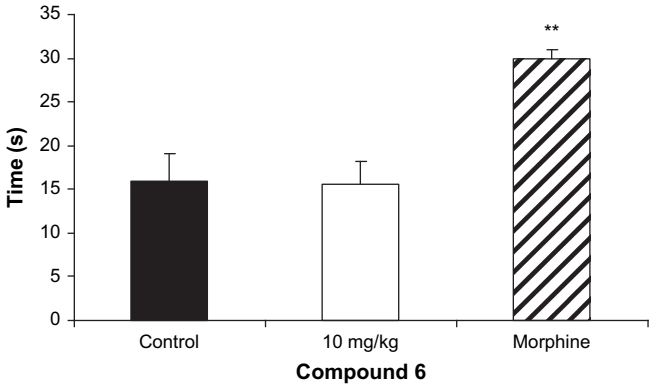


Fig. 7. Effect of **6** (10 mg/kg, i.p.) against hot-plate induced pain in mice. Each column represents mean ± S.E.M. of six experimental values. *Significance levels, when compared with the control group. $^{*}p < 0.05$; $^{**}p < 0.01$.

drugs aimed at the central nervous system (CNS) tend to have lower polar surface areas than those of other classes. For molecules to penetrate the BBB and thus act on receptors in the CNS, the PSA should preferably be less than 60 Å^2. Morphine is the agonist with maximal intrinsic activity for the opioid system, and has 52.90 Å^2 of PSA, compared with compound **6**, for which PSA is 66.76. This is a negative point against the activity in the hot-plate test. In relation to the rotatable bonds, CNS drugs have significantly fewer than other drug classes. Most centrally acting compounds have a rotatable count of five or less [29]. Compared with morphine, a standard drug, it has zero rotatable bonds, while compound **6** has fourteen, making it unfavorable for action on the CNS.

5. Conclusions

We have shown that all the caffeic acid derivatives with aliphatic chain caused a similar or higher antinociceptive effect than the drugs used as reference, and the two compounds with aromatic chain demonstrate lower activity when analyzed against the writhing test at 10 mg/kg in mice. Compound **6** showed a more pronounced effect and was then selected for more detailed analysis, exhibiting an antinociceptive profile in other experimental models as well. However, the results indicate that it is only slightly absorbed by the oral route. This difference may be attributed to certain molecular properties and structural factors, according to the method proposed by Lipinski, which evaluates oral bioavailability *in silico* and can be explained in part by its high lipophilicity.

Although the mechanisms underlying these effects have not been elucidated, the findings are of interest because they support, at least partly, the notion that caffeic acid, a natural compound widely distributed in plants, may be useful for the development of new and effective peripheral analgesics against painful diseases. Studies are currently in progress to determine the possible action mechanisms responsible for the antinociceptive properties of the most active compound (**6**).

6. Experimental protocols

6.1. Chemistry

Caffeic acid (3,4-dihydroxy-cinnamic acid, 97%) and all alcohols were purchased from Aldrich Chemical Co. (Milwaukee, WI). The chromatography column was carried out using 70–230 mesh silica gel (Aldrich). Silica gel Merck pre-coated aluminum plates with 200 μm layer thickness were used for the thin layer chromatography. Melting points were determined on a Micro-Química PF-300 apparatus with a digital thermometer, and are uncorrected. ^{1}H NMR and ^{13}C spectra were obtained using a Bruker 400 spectrometer. NMR samples were prepared using deuterated chloroform ($CDCl_3$) purchased from Aldrich Chemical Co. FT-IR spectra were obtained using a BOMEM spectrophotometer, and the samples were prepared as pressed KBr plates.

6.1.1. General synthesis of caffeic acid (CA) derivatives

The derivatives were obtained by reaction of caffeic acid with the appropriate alcohol and acetyl chloride as catalyst under reflux (2–4 h; 60–70 °C), according to the methodology previously described [12,32]. The respective products were purified by recrystallization or chromatographic column over silica gel eluted with a mixture of hexane:acetone with increasing polarity. The reaction was considered complete, and the purity of all synthesized compounds was examined by thin layer chromatography (TLC) using Merck silica gel pre-coated aluminum plates with 200 μm layer thickness, with several solvent systems of different polarities. The structural characterization was performed by conventional spectroscopic data (IR, ^{1}H and ^{13}C NMR) and compared directly with the literature data [33]. Considering that all the compounds studied are well-known, we describe below only the synthesis of the most active compound (**6**).

6.1.2. Synthesis of dodecyl-3,4-dihydroxycinnamate (dodecyl caffeate) (**6**)

Caffeic acid (1.8 g, 9.9 mmol), acetyl chloride (0.10 g, 0.5 mmol), dodecyl alcohol (50 ml), and benzene (50 ml), were placed in a 250 ml 3-neck round bottomed flask equipped with a magnetic stirring bar. The mixture was stirred, heated to reflux, and monitored by thin layer chromatography. TLC considered the reaction complete after about 4 h of refluxing. The reaction mixture was concentrated on a rotatory evaporator, and purified by chromatographic column over silica gel eluted with a mixture of hexane:acetone with increasing polarity. The purity of the compound was examined by thin layer chromatography (TLC) using Merck silica gel pre-coated aluminum plates with 200 μm layer thickness. The final product was recovered as a white crystalline solid. Yield: 64%; mp: 161–163 °C. IR (KBr plate) ν_{max} 3495, 3312, 1684, 1635, 1602. ^{1}H NMR ($CDCl_3$) δ 7.52 (d, $J = 16$ Hz, 1H), 7.15 (d, $J = 2$ Hz, 1H), 7.04, 7.01 (dd, $J = 2.0$, 2.1 and 8.2 Hz, 1H), 6.85 (d, $J = 8.2$ Hz, 1H), 6.27 (d, $J = 15.9$ Hz, 1H), 3.70 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 167.9, 148.6, 146.1, 145.7, 127.5, 122.5, 116.3, 115.2, 115.1, 115.1, 51.5.

6.1.3. Solubility and permeability estimate: Lipinski's rule of five

Computational approaches were used to estimate the solubility and permeability of the synthesized compounds, using the "rule of 5" proposed by Lipinski and its extensions [19,20]. This rule predicts that poor absorption or permeation is more likely when there are more than 5 H-bond donors and, more than 10 H-bond acceptors; when the molecular weight (MW) is greater than 500; the calculated $C\log P$ is greater than 5 (or $M\log P > 4.15$); and its extension parameters polar surface area (PSA) more than 140 Å^2 or the sum of the H-bond donors and acceptors is more than 12 and the rotatable bond more than 10. These physicochemical parameters are associated with acceptable aqueous solubility and intestinal permeability, and comprise the first steps in oral bioavailability. The values for MW, $\log P$, number of H-bond acceptors and donors, PSA and rotatable bond were obtained from the on-line program free molinspiration, by JME Editor, courtesy of Peter Ertl of Novartis, available on the website: http://www.molinspiration.com/cgi-bin/properties.

6.2. Pharmacological evaluation

6.2.1. Animals

Swiss mice (25–35 g), housed at 22 ± 2 °C under a 12-h light/12 h dark cycle and with access to food and water *ad libitum*, were acclimatized to the laboratory for at least 1 h before testing. The experiments reported here were carried out in accordance with the current ethical and care guidelines for the care of laboratory animals and the investigation of experimental pain in conscious animals [34]. The experiments were approved by the local Ethics Committee (113/2005-03 UNIVALI). The number of animals (6–8 for group of treatment) and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the treatments.

6.2.2. Drugs

The following drugs were used: Acetyl salicylic acid, acetaminophen, capsaicin and glutamate hydrochloride (Sigma–Aldrich), acetic acid and formaldehyde (Merck) and morphine hydrochloride (Cristália). All the compounds were dissolved in Tween 80 (E. Merck), plus 0.9% of NaCl solution, with exception of capsaicin, which was dissolved in absolute ethanol. The final concentration of Tween 80 and ethanol did not exceed 5% and did not cause any effect *per se*.

6.2.3. Acetic acid-induced writhing

Abdominal constriction induced by intraperitoneal injection of acetic acid (0.6%) was carried out according to the procedures described previously by Collier and co-workers [35] with minor modifications. Male Swiss mice (25–30 g) were pretreated with derivatives (10 mg/kg, i.p.) or compound **6** (3, 6 and 10 mg/kg, i.p. or 100, 250 and 500 mg/kg, p.o.) 30 min before acetic acid injection. The control animals received a similar volume of vehicle (0.9% NaCl and Tween 80, 10 ml/kg, i.p.). All the experiments were carried out at 23 ± 2 °C. After the challenge, pairs of mice were placed in separate boxes and the number of constrictions of the abdominal muscles, together with stretching, were counted cumulatively over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of abdominal contractions between the control animals and the pretreated animals.

6.2.4. Formalin-induced nociception

The procedure used was essentially similar to that described previously [24]. Animals from the same strain were used and 20 μl of 2.5% formalin solution (0.92% formaldehyde), which was injected intraplantarly into the right hindpaw. After injection, the time spent licking the injected paw was timed and considered as indicative of pain. The initial nociceptive scores normally peaked 5 min after formalin injection (first phase) and 15–30 min after formalin injection (second phase), representing neurogenic and inflammatory pain, respectively. In order to investigate the possible antinociceptive action 6, 10 and 30 mg/kg, i.p. of compound **6** was used.

6.2.5. Capsaicin-induced nociception

The procedure used was similar to that described previously [36]. After the adaptation period, 20 ml of capsaicin (1.6 μg/paw) was injected intraplantarly into the right hindpaw. The animals were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. The animals were treated with compound **6** via i.p. (10, 30 and 60 mg/kg) 30 min before capsaicin injection, respectively. The control animals received a similar volume of saline, intraperitoneally.

6.2.6. Glutamate-induced nociception

The animals were treated with compound **6** via i.p. (10, 30 and 60 mg/kg) 30 min before the glutamate injection. A volume of 20 μl of glutamate solution (30 μmol\paw) was injected intraplantarly under the surface of the right hindpaw, as described by Beirith and co-workers [27]. After injection, the animals were observed from 0 to 15 min. The time spent licking or biting the injected paw was timed with a chronometer and considered as indicative of pain.

6.2.7. Hot-plate test

The hot-plate test was used to measure response latencies, according to the method described by Eddy and Leimback [28]. The mice were treated with saline solution, morphine (10 mg/kg, sc) or compound **6** (10 mg/kg, i.p.), and placed individually on a hot plate maintained at 56 ± 1 °C. The time between placing the animal on the hot-plate and the occurrence of either the licking of the hindpaws, shaking the paw or jumping off the surface was recorded as response latency. Mice with baseline latencies of more than 20 s were eliminated from the study and the cut-off time for the hot-plate latencies was set at 30 s. The animals were treated 30 min before the assay.

6.3. Statistical analysis

The results are represented as a mean ± SEM, except for the ID_{50} values (i.e., the dose that reduced responses by 50% relative to the control values), which are presented as geometric means accompanied by their respective 95% confidence limits. The ID_{50} values were determined by linear regression GraphPad. Statistical significance between the groups was calculated by means of analysis of variance followed by the Newman–Keuls multiple comparison tests. *P*-values less than 0.05 ($P < 0.05$) were considered as indicative of significance.

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