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Research Article
Evaluation of the Antinociceptive Activity of Calein D and Exploration of the Possible Mechanism of Action

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Abstract

Background and Objective: Pain is an unpleasant sensation related to actual or potential tissue damage. The ongoing search for antinociceptive compounds owes itself to the adverse effects of current analgesics. This study aimed to evaluate the antinociceptive effect of Calein D in the rat formalin model. **Materials and Methods:** Antinociceptive activity was tested for Calein D (at various doses) and a reference drug (piroxicam) in the rat formalin model. To investigate whether muscarinic receptors, serotonin receptors, opioid receptors and nitric oxide (NO) participate in the antinociceptive effect found for Calein D, rats were pretreated with atropine, methiothepin, naloxone or N^G-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME), respectively. To assess the possible role of μ, δ and κ opioid receptor subtypes, animals were pretreated with the selective antagonists CTOP (a selective μ opioid receptor antagonist), SDM-25N (a selective δ opioid receptor antagonist) or nor-binaltorphimine (nor-BNI, a selective κ opioid receptor antagonist), respectively. **Results:** Calein D showed its maximum antinociceptive effect at 56 mg/kg (57.03±2.85%), compared to 35-60% for non-steroidal anti-inflammatory drugs (NSAIDs) in the formalin model. It exhibited significantly greater activity than piroxicam (both at 32 mg/kg; 49.21±4.76% vs. 32.24±8.73%; $p<0.05$). The mechanism of action of Calein D involves muscarinic receptors as well as μ and δ opioid receptors, but not serotonin receptors, κ opioid receptors or NO. **Conclusion:** This was the first report on the antinociceptive activity of D-calein. Muscarinic and opioid receptors are involved in its mechanism of action.

Key words: Calein D, *Calea urticifolia*, antinociception, sesquiterpene lactones, medicinal plants

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

According to the International Association for the Study of Pain (IASP), pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage. It has various causes, the main one being injury. Given that pain is based on noxious stimulation of sensory nerve endings[1], it represents a protective perception of a nociceptive stimulus indicative of bodily damage. However, it becomes a pathological mechanism when occurring outside the window of usefulness. For example, in 2015 pain was the leading cause of disability in most countries[2]. Today, it is a global health problem affecting over 1.5 billion people. Burns, long colds or chemical injuries can cause inflammation and pain. During injuries and subsequent events, damaged cells release diverse range of compounds. Some of these substances can directly stimulate nociceptors, whereas others, such as prostaglandins, can sensitize them. In pain and inflammation, prostaglandins and the cyclooxygenase enzymes (COX-1 and COX-2) responsible for their synthesis have well-established functions. This is demonstrated by the analgesic efficacy of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), the main mechanism of action of which is the inhibition of COX isoforms. The analgesic activity of non-selective COX inhibitors cannot be explained solely by their ability to inhibit peripheral prostaglandin production[3]. The COX-prostaglandin pathway is now known to play an important role in spinal nociceptive processing[4]. The down-regulation of spinal nociception in the central nervous system substantially limits the expression of pain[5].

The progress that has been made in treating chronic pain and inflammatory diseases includes the development of new pharmacological treatments. Nevertheless, pain continues to be a burden worldwide[6,7]. Unfortunately, the drugs used to ameliorate inflammation and induce analgesia have adverse effects. On the one hand, the prolonged consumption of traditional NSAIDs (non-selective COX inhibitors such as aspirin, ibuprofen and naproxen) can generate gastric ulcers. On the other hand, newer NSAIDS that act as selective COX-2 inhibitors (e.g., celecoxib) are related to cardiovascular problems[8] and opioid analgesics lead to issues of tolerance and dependence[9]. Hence, there is a need to seek new substances capable of producing analgesic activity with minimal adverse reactions. Medicinal plants may be an important source of new antinociceptive compounds.

Calea urticifolia (Miller) DC. is a plant utilized in Mexican traditional medicine for the treatment of gastric ulcers[10], diabetes and inflammatory processes[11]. Among the different kinds of compounds isolated from *Calea urticifolia* are germacranolides, including calealactones A-C, 2,3-epoxycalein D and Calein D[12-14]. Sesquiterpene lactones with a germacrene skeleton have shown the following types of activity: Cytotoxic, anti-bacterial, anti-fungal[12], antioxidant, suppression of adipocyte differentiation, anti-inflammatory[11] and analgesic[15]. Current group described a gastroprotective effect for Calein D, a sesquiterpene lactone with a germacrene skeleton[10]. To the best of our knowledge, no other biological activity has yet been reported for this compound. Although, an analgesic effect has been found for some gastroprotective compounds[16-18], there is as yet no published study of the possible antinociceptive activity of Calein D. The current study aimed to evaluate the antinociceptive effect of Calein D in the rat formalin model.

MATERIALS AND METHODS

Study area: This phytochemistry study was performed at Superior Medicine School, National Polytechnic Institute and experimental experiments at the National Institute of Respiratory Diseases. All the studies were performed between July, 2023 to May, 2024.

Animals: All behavioral assays were carried out on male Wistar rats (180-220 g), which were acquired from the animal facilities of the National Institute of Respiratory Diseases (INER, according to the initials in Spanish) in Mexico City. They were housed under a 12 hrs light/dark cycle at a temperature of 22-25°C with free access to food and drinking water. The exception was the 12 hrs overnight fast before behavioral testing. The number of animals employed was 8 animals for the group. All possible efforts were made to avoid unnecessary pain and suffering by the lab animals. At the end of the experimental procedures, rats were submitted to euthanasia in a chamber containing carbon dioxide.

All experiments comply with the relevant guidelines.

Ethical consideration: The Technical Specifications for the Production, Care and Use of Laboratory Animals (NOM-062-ZOO-1999, Mexico) of the Mexican Secretary of Agriculture (SAGARPA), the Committee on the Update of the Guide for the Care and Use of Laboratory Animals of the U.S. National Research Council and the Ethical Standards for Investigation of Experimental Pain in Animals[19,20]. In addition, the present protocol was approved by the Internal Committee for the Care and Use of Laboratory Animals (CICUAL) of the Higher School of Medicine of the National Polytechnic Institute in Mexico City, Mexico (ESM.CICUAL-01/14-03-2018).

Plant material: In July, 2022 the leaves of *Calea urticifolia* were collected in Nandayalud, a community in the municipality of Suchiapa, the State of Chiapas, Mexico. The plant was identified and registered by Manuel de Jesús Gutiérrez Morales from the Flora Department of the Chip Herbarium, which is part of the Botanical Garden of the Secretary of Environmental Protection, Housing and Natural History of the State of Chiapas, Mexico. A specimen of the original collection can be found with the voucher number 39871.

Calein D was obtained from 8 kg of the leaves of *Calea urticifolia*. The leaves were macerated at room temperature (22°C) for three days, first with hexane (20 L×3) and then with dichloromethane (20 L×3). The solvents were evaporated and 445 g (5.56%) of the dichloromethane extract was obtained. Subsequently, 430 g were subjected to column chromatography, then eluted with hexane and a hexane/dichloromethane mixture at a 4:6 ratio to afford Calein D (2.8 g, 0.65%). The compound was verified by comparing its ^{1}H and ^{13}C-NMR spectra to those reported by Sánchez-Mendoza *et al.*[10].

Drugs: Atropine sulfate, methiothepin mesylate salt, N^{G}-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME), naloxone hydrochloride dihydrate and nor-binaltorphimine (nor-BNI) dihydrochloride were purchased from Sigma Aldrich (St. Louis, Missouri, USA). The CTOP and SDM-25 N hydrochloride were acquired from Tocris Bioscience (Bristol, UK). Each drug was prepared at the desired concentration by dissolving it in a normal saline solution (10% Tween 20 and 0.5% carboxymethylcellulose) immediately before each experiment. Drugs for the pretreatments were chosen based on their selectivity for the target receptors as well as their accessibility. Atropine is a non-selective muscarinic receptor antagonist[21], methiothepin a non-selective serotonin (5-HT) receptor antagonist that acts on the 5-HT_1, 5-HT_2, 5-HT_5 and 5-HT_7 subtypes[22], L-NAME a non-selective nitric oxide synthase (NOS) enzyme inhibitor[23], naloxone a non-selective opioid receptor antagonist[24], CTOP a selective μ opioid receptor antagonist[25] (pKi 9.7), SDM-25N a selective δ opioid receptor antagonist[26] (pKi 8.3) and nor-BNI a selective κ opioid receptor antagonist[27] (pKi 9.6-10.7).

Formalin test: Formalin-induced inflammatory pain in rats was assessed by the automated nociception analyzer described in a previous report by Yaksh *et al.*[28]. Briefly, the rats were placed in clear plastic observation chambers for 30 min to allow them to acclimate to their surroundings. Subsequently, they were removed and injected subcutaneously with 50 μL formalin (1%) in the dorsum of the right hind paw, then identified with a ring-shaped metal band placed on the right hind leg. Immediately afterward, the animals were returned to their respective chambers and the nociceptive behavior was counted as the number of flinches. Formalin-induced flinching behavior was counted by video recordings of periods of 1 min every 5 min for 1 hrs. Formalin injection elicits a two-phase response, the first lasting around 10 min and the second from about 10 to 60 min post-injection. In this model, when a compound administered to animals causes a decrease in the number of flinches, it is considered to have antinociceptive activity.

Study design: Thirty independent groups of animals (n = 8) were used for each experimental condition. After a 12 hrs overnight fast, the following compounds were given orally: The vehicle (a saline solution containing 10% of Tween 20 and 1% carboxymethylcellulose, serving as the negative control), piroxicam (32 mg/kg, the positive control) or Calein D (at doses of 1-56 mg/kg). The 20 min later, the formalin test was carried out to assess the possible antinociceptive effect of Calein D.

To investigate whether muscarinic receptors, serotonin receptors, opioid receptors and/or nitric oxide (NO) participate in the antinociceptive activity found for Calein D, the rats were pretreated with atropine (1 mg/kg, i.p.), methiothepin (1 mg/kg, i.p.), naloxone (1 mg/kg, i.p.) or L-NAME (10 mg/kg, i.p.), respectively. To explore the possible role of μ, δ and κ opioid receptors in the antinociceptive effect, the animals were pre-treated with an intraperitoneal injection of one of the following selective antagonists: CTOP (0.1 mg/kg), SDM-25N (0.1 mg/kg) or nor-BNI (0.1 mg/kg), respectively. All pretreatments (antagonists and/or inhibitors) were applied 30 min before administering Calein D, the reference compound or the vehicle. Calein D was given to the animals at the 56 mg/kg dose to examine its mechanism of action because at this dose it showed the highest efficacy in the formalin test. All doses were chosen based on previous reports or our pilot assays[29]. The evaluator was blinded to the treatment received by each animal.

Statistical analysis: Data are expressed as the Mean±Standard Error (SEM) for eight animals per group. Graphs were constructed by plotting the number of flinches as a function of time. In bar plots, data for each treatment are represented as the percentage of the antinociceptive effect,

which was calculated based on the total flinches evoked during phase II, by the following equation[29]:

$$\text{Antinociception (\%)} = \frac{\text{Control flinches - Test flinches}}{\text{Control flinches}} \times 100$$

One-way Analysis of Variance (ANOVA) followed by Tukey's or Dunnett's test was used to compare differences between treatments. Statistical significance was considered at *p≤0.05.

RESULTS

Antinociceptive activity of Calein D: The subcutaneous injection of formalin (1%) in the right hind paw of rats caused flinching behavior. The oral administration of Calein D reduced the number of flinches compared to the vehicle-treated group (Fig. 1a). The effect of Calein D was dose-dependent (Fig. 1b), reaching its maximum (57.04±2.85% antinociceptive effect) at the highest dose of 56 mg/kg. The lesser doses of 32, 10 and 5.6 mg/kg produced 49.21±4.76, 42.61±2.53 and 32.27±10.46% antinociceptive effect, respectively. At 32 mg/kg, the antinociceptive effects of Calein D and piroxicam (the reference drug) were 49.21±4.76 and 32.24±8.73%, respectively, revealing a significantly greater activity for the test compound at that dose. Similarly, the effect achieved was significantly different when comparing the dose of 56 mg/kg (57.04+2.85%) of Calein D to 32 mg/kg of piroxicam.

Possible mechanisms of the antinociceptive effect of Calein D: The antinociceptive effect of Calein D at 56 mg/kg was reversed by pretreatment with atropine (1 mg/kg, i.p.), a muscarinic receptor antagonist (Fig. 2a) and with naloxone (1 mg/kg, i.p.), an opioid receptor antagonist (Fig. 2b). Thus, muscarinic and opioid receptors are involved in the mechanism of action of Calein D. Contrarily, the antinociceptive effect was not reversed by pretreatment with either methiothepin, a 5HT receptor antagonist or L-NAME, a NOS inhibitor (Fig. 2c-d). Therefore, 5HT receptors and NO do not participate in the mechanism of action of Calein D.

Regarding opioid receptors, pretreatment with a selective antagonist of μ receptors (CTOP) and δ receptors (SDM-25N) reversed the antinociceptive effect of Calein D (Fig. 3a-b). Hence, these opioid receptor subtypes play a role in the mechanism of action of Calein D. Since pretreatment with nor-BNI (a selective k receptor antagonist) did not modify the antinociceptive effect of Calein D (Fig. 3c), κ receptors are not involved in its mechanism of action.

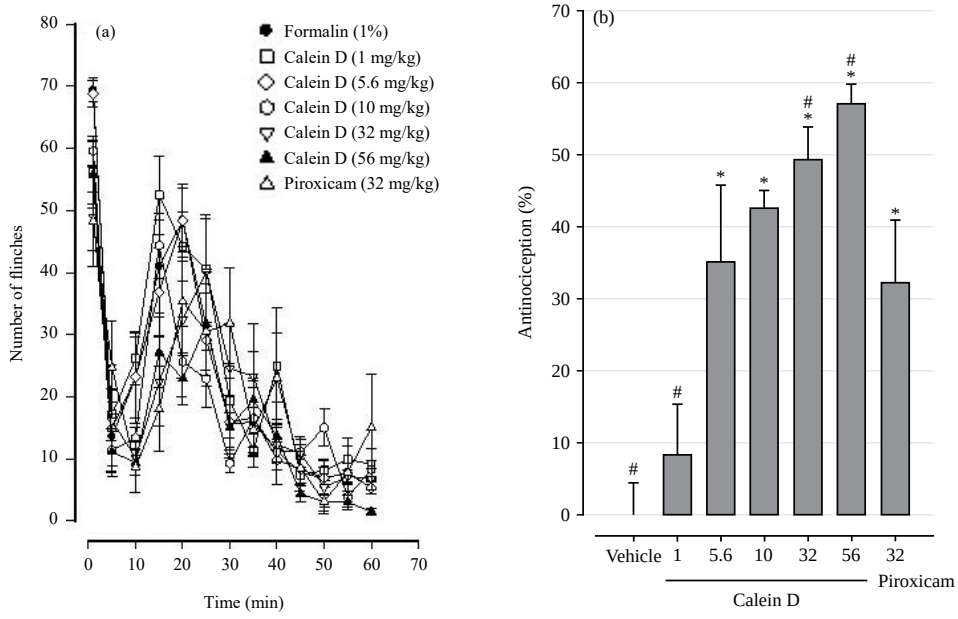

Fig. 1(a-b): (a) Time course of the antinociceptive effect in rats treated with Calein D (1-56 mg/kg) or piroxicam (32 mg/kg, respectively) and (b) Bars represent the mean percentage (for 8 animals) of antinociception ±SEM produced by the administration of Calein D (1-56 mg/kg) and piroxicam (32 mg/kg)

A dose-response effect for Calein D, data corresponds to phase II of the formalin test, *p<0.05 vs the vehicle group and #p<0.05 vs the piroxicam group, in each case based on One-way Analysis of Variance (ANOVA) followed by Tukey's test

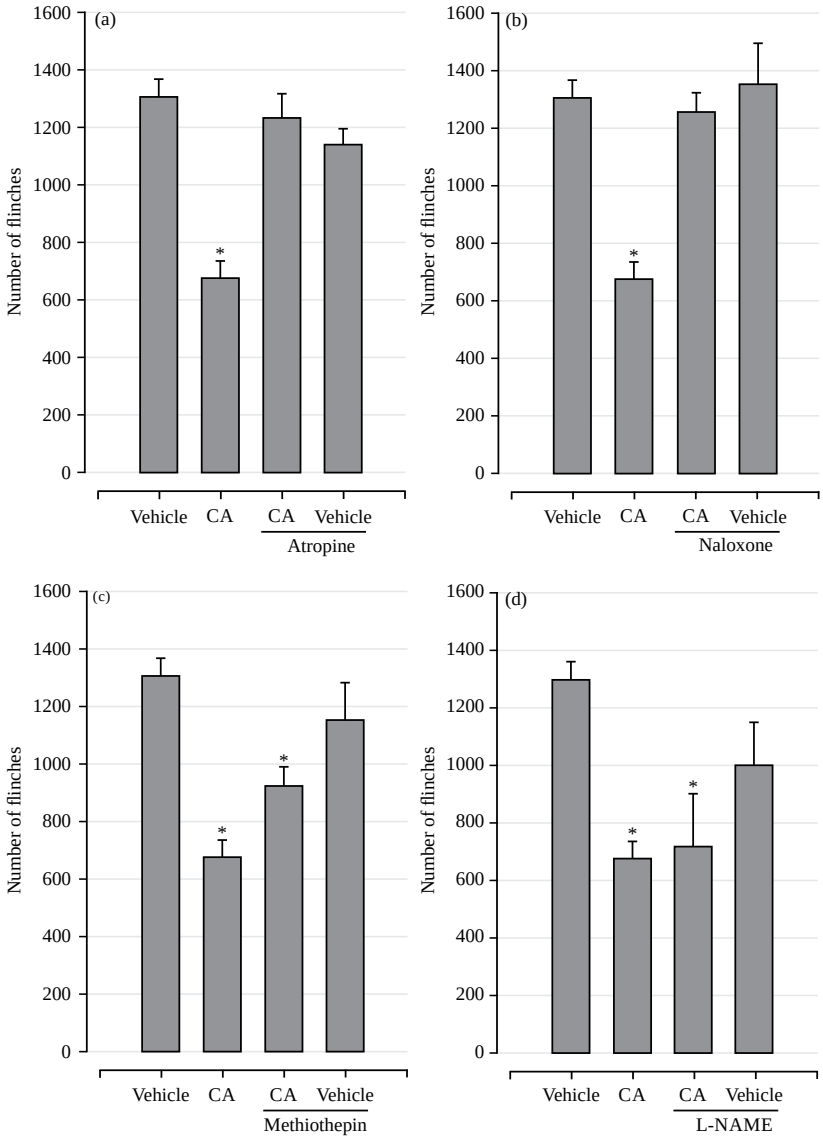

Fig. 2(a-d): Evaluation of the possible participation of (a) Muscarinic receptors, (b) Opioid receptors, (c) Serotonin receptors and (d) Nitric oxide in the antinociceptive effect of Calein D (56 mg/kg; CA)

Data corresponds to phase II of the formalin test, bars represent the mean percentage (for 8 animals) of antinociception ±SEM, *p<0.05 vs vehicle group, as established by One-way Analysis of Variance (ANOVA) followed by Tukey's test

DISCUSSION

The formalin model is very useful for evaluating the effect and mechanism of action of natural products with potential antinociceptive and analgesic activity. This *in vivo* test resembles clinical models of pain associated with tissue injury[30].

The effects of a drug on the formalin test are observed in two phases. The initial phase is distinguished by neurogenic pain, which is initiated by the activation of the Transient Receptor Potential of Calcium Channels (TRPA1) and the release of glutamate, substance P, a related peptide (neurokinin A) and the Calcitonin Gene-Related Peptide (CGRP). These compounds promote hyperalgesia via intracellular messengers and sensitization of the dorsal root ganglia[30]. The second phase is characterized by pain from inflammation, which is caused by formalin-induced tissue damage. The release of various proinflammatory agents alters the cellular environment and interferes with the process of nociception[31]. Additionally, intraplantar injection of formalin into rodents is known to increase spinal levels of excitatory amino acids, PGE_2, NO, tachykinin, kinins and other peptides.

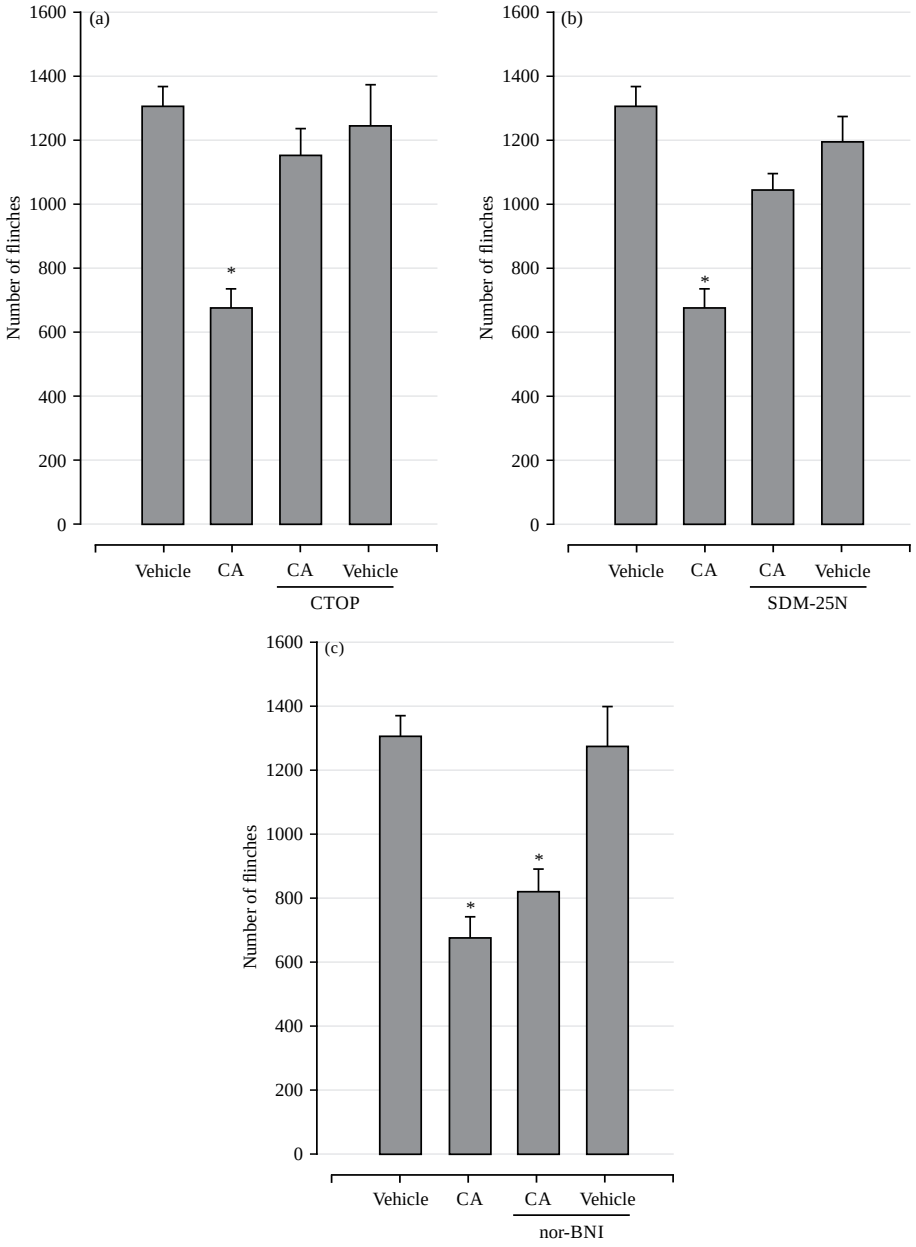

Fig. 3(a-c): Evaluation of the involvement of, (a) μ, (b) δ and (c) κ opioid receptors in the antinociceptive effect of Calein D (56 mg/kg; CA)

Data corresponds to phase II of the formalin test, Bars represent the mean percentage (for 8 animals) of antinociception ±SEM, *p<0.05 vs control (vehicle) group, according to One-way Analysis of Variance (ANOVA) followed by Tukey's test

The NSAIDs have an effect in phase II of the formalin test because their antinociceptive effect is mainly through the inhibition of the biosynthesis of prostaglandins, which are involved in inflammation[31]. Since these drugs do not show any effect in phase I of the formalin test, they do not affect the aforementioned compounds related to this phase. Like NSAIDs, Calein D did not show any activity in phase I. It produced a dose-dependent antinociceptive effect in phase II, revealing that proinflammatory agents are involved in its mechanism of action. However, it does not target prostaglandins, as previously evidenced by Sánchez-Mendoza *et al.*[10] on the gastroprotective effect of Calein D, which is not inhibited by indomethacin.

The current study provides the first evidence of an antinociceptive effect for Calein D. To our knowledge, the only other reported biological activity of this compound is

gastroprotection[10]. The combination of antinociceptive and gastroprotective activity is interesting because gastric acid inhibitors are normally taken with NSAIDs to counteract the risk of gastric damage. The dual function of Calein D may reduce the doses of the analgesic agent.

The antinociceptive effect of NSAIDs in the formalin model is from 35 to 60%[32,33]. Hence, the present finding of 49.21±4.76% antinociception for the 32 mg/kg dose of Calein D indicates that it could be clinically relevant. Indeed, this was significantly greater than the percentage of protection obtained with the reference drug piroxicam at the same dose. The mechanism of action of Calein D was explored by pretreatment with atropine, methiothepin, naloxone and L-NAME. The reversal of the antinociceptive effect of Calein D with atropine pretreatment revealed the participation of muscarinic receptors (mAChRs), which regulate a broad range of physiological processes and are expressed throughout the central and peripheral nervous systems[34]. The mAChRs have been considered an important molecular target in the development of new analgesic drugs due to their involvement in the regulation of chronic pain.

The five types of mAChR receptors (M_1 to M_5) are all related to heterotrimeric G Protein-Coupled Receptors (GPCRs)[35]. The modulation of nociceptive transduction is mediated by the M_2 and M_4 mAChR subtypes. These receptors are coupled to Gi/o proteins and mediate a rapid inhibition of Voltage-Gated Ca^{2+} Channels (VGCCs) to activate βγ subunits. Synaptic transmission is dependent on calcium entry through Ca^{2+} channels in neurons. The VGCCs are fundamental for presynaptic neurotransmitter release. The activity of N-type and P/Q-type Ca^{2+} channels is what determines the synaptic release of glutamate.

The mAChR agonists can activate G Protein-Coupled Inwardly Rectifying K^+ (GIRK) channels in sympathetic and hippocampal CA1 neurons, which causes hyperpolarization. The antinociceptive effects induced by mAChR agonists may be attributed to their combined effects of augmenting the release of inhibitory neurotransmitters GABA and glycine and decreasing the release of excitatory neurotransmitter glutamate in the spinal dorsal region[34]. Thus, Calein D acts as an agonist on one or more of the muscarinic receptors participating in antinociceptive activity. The particular receptor subtypes involved were not presently determined because the available muscarinic receptor antagonists are not fully selective[36]. Future research is needed to define the muscarinic receptor subtypes implicated in nociception by the test compound.

On the other hand, numerous studies have shown a crucial contribution of 5-HT in the regulation or inhibition of nociception pathways[37]. Since 5-HT has pronociceptive activity at the periphery and pro and/or antinociceptive activity at the spinal level, its role in the mechanisms of nociception is complex[37]. The effect of 5-HT reportedly depends on the kind of pain (acute or chronic) and the type of receptor-activated[38]. To date, seven classes of 5-HT receptors have been identified that comprise at least 15 subtypes. All 5-HT receptors are GPCRs except the $5\text{-}HT_3$ receptor, which is ionotropic[39].

According to Bardin[38], peripheral $5\text{-}HT_{2A}$, $5\text{-}HT_3$ and $5\text{-}HT_7$ receptors contribute significantly to peripheral nociceptive transmission during inflammation. The $5\text{-}HT_2$ receptors are coupled to the G q/11 protein and its activation leads to higher levels of IP_3 and DG, generating an antinociceptive effect. The $5\text{-}HT_3$ is a ligand-gated cation channel. When activated, it can depolarize the neuronal membrane and cause antinociception, although its role in the maintenance of the painful stimulus has also been described. The $5\text{-}HT_7$ is coupled to the Gs protein and its activation engenders greater cAMP levels, resulting in pro-and antinociceptive effects[40].

Another possible mechanism of action of nociception is linked to the serotonin system and its relation to the adrenergic system. The activation of 5-HT receptors may increase the release of norepinephrine, which triggers the postsynaptic alpha 2 receptor and leads to an inhibition of sensory nerve conduction of pain impulses[37]. In the current study, the antinociceptive effect of Calein D was not inhibited by pretreatment with methiothepin, a non-specific serotonin receptor antagonist that acts on $5\text{-}HT_1$, $5\text{-}HT_2$, $5\text{-}HT_5$ and $5\text{-}HT_7$ receptor subtypes. Hence, 5-HT receptors do not participate in the mechanism of action of Calein D[37].

In contrast, naloxone pretreatment inhibited the antinociceptive effect of Calein D, revealing a contribution of opioid receptors to its mechanism of action. The activation of such receptors inhibits neurons and thus limits pain transmission from the spinal cord. Opioids produce their analgesic activity by interacting with μ, δ and/or κ opioid receptor subtypes[41], which are all GPCRs[34]. Opioid receptor agonists limit neuronal activity by inhibiting VGCCs in dorsal root ganglion (DRG) neurons and suppressing neuronal excitability us of the activation of GIRK channels in the postsynaptic neurons of the spinal cord. They also inhibit N-type Ca^{2+} channels through voltage-dependent and voltage-independent pathways. The voltage-dependent inhibition is mediated by Gβγ released from Gi/o proteins. Regarding the voltage-independent mechanism, the stimulation of Gq/11 proteins can downregulate Ca^{2+} channel activity, thus inducing internalization. Furthermore, some of the analgesic activity of μ-opioids may stem from modulation

of the descending pathways and the consequent reduction of nociceptive transmission in the spinal dorsal horn[34].

Since the present pretreatment with L-NAME did not modify the antinociceptive effect of Calein D, NO does not contribute to its mechanism of action. This result is in agreement with a previous study of Sánchez-Mendoza *et al*.[10]. on its gastroprotective effect, finding that NO is not involved. The NO, a small gas molecule generated from L-arginine by the NOS enzyme, is important in peripheral and central synaptic transmission. Considering the reports on the pro-and antinociceptive functions of NO, its effects are still controversial. The classic sGC/cGMP/PKG signaling pathway participates in its antinociceptive effects. Briefly, NO activates soluble guanylate cyclase, leading to the production of cGMP, which activates cGMP-dependent protein kinase (PKG) and triggers ATP sensitive K^+ channels. The intracellular K^+ extrusion results in hyperpolarization and a direct blockade of acute and persistent hyperalgesia[42,43]. Additionally, NO decreases the activity of spinal NMDA receptors, attenuating spinal nociceptive transmission. However, the mechanisms in the spinal cord and dorsal root ganglion (DRG) are not fully understood[43]. It is also known that peripheral nerve injury stimulates NO production and this molecule can react with superoxide (released from spine) to generate peroxynitrite, which appears to mediate neuropathic pain. Therefore, NO can promote the maintenance of neuropathic pain[43]. In the formalin test, L-arginine enhances the synthesis of NO[44], thus modulating the excitability of both pre- and post-synaptic neurons in the first synapse of the ascending pathway acting on NMDA receptors[45]. As can be appreciated, the underlying mechanisms of NO are not fully understood[42].

Based on the finding that opioid receptors participate in the mechanism of action of Calein D, specific antagonists of μ, δ and κ receptors were used to identify the receptor subtype. The antinociceptive effect of Calein D was reversed by the pretreatments with CTOP and SDM-25N, revealing the role of the test compound as an agonist for μ and δ opioid receptors. Hence, future research should be carried out on the possible effect of Calein D in combination with opioid agonists to reduce the side effects of the latter drugs.

One study described the activation of the Nuclear Factor Erythroid 2-Related Factor-2 (Nrf2) system by some sesquiterpene lactones isolated from *Calea urticifolia*. Such activation is triggered by the α and β-unsaturated carbonyl groups in the structure of these lactones, given their susceptibility to a Michael-type reaction[46,47] with the sulfhydryl groups of cysteine residues. It has been suggested that Nrf2 and its effectors are implicated in anti-inflammatory pain and neuropathic pain[48,49]. Consequently, the activation of Nrf2 is another plausible factor in the mechanism through which Calein D exerts its antinociceptive effect.

Under normal physiological conditions, Nrf2 is predominately bound to Keap1 in the cytoplasm. Under stress conditions (e.g., chronic pain), Nrf2 is released from Keap1 and translocated into the nucleus, where it heterodimerizes with small Maf proteins and binds to antioxidant response elements[48], ultimately leading to the transcription of antioxidant enzymes[50]. There are two distinct aspects of the anti-inflammatory mechanism of Nrf2. First, it repairs the redox imbalance by controlling the expression of antioxidants. Furthermore, it indirectly decreases the frequency of inflammatory cascade reactions and their subsequent harm by inhibiting and neutralizing reactive oxygen species[50]. This is done by activating many transcription factors, including NF-kB, AP-1, MAPKs and PI3K[49]. Second, Nrf2 regulates the expression of downstream inflammation-associated proteins, such as pro-inflammatory cytokines and chemokines[50]. Among other factors, Heme Oxygenase-1 (HO-1) and NAD(P)H Quinone Oxidoreductase 1 (NQO1) are involved[48]. Indeed, it has been shown that several substances exert antioxidant and analgesic effects by means of the latter two compounds[49].

There is growing evidence of the capacity of Nrf2-activators to synergize with opioids to achieve better analgesic effects, as found in studies conducted with sulforaphane (SFN), butorphanol and other activators. Combining SFN with morphine increases the expression of μ-opioid receptors and increases the production of HO-1 and NQO1. Butorphanol in combination with fentanyl (an opioid drug) triggers Nrf2-ARE signaling via the kappa-opioid receptor. This pathway increases the expression of the downstream genes NQO1 and HO-1 when activated. As can be appreciated, antiallodynic and antihyperalgesic effects seem to result from a mechanism mediated by Nrf2. Hence, the use of Nrf2 activators may be beneficial to lower the doses of opioids and thus reduce their side effects. Overall, Nrf2-based therapy for chronic pain is a very promising area, but more research is needed on the mechanisms involved[48]. Likewise, more studies are necessary to determine the possible relation between Calein D and the Nrf2 system.

CONCLUSION

The current results provide the first scientific evidence of antinociceptive activity by Calein D, a metabolite isolated from the medicinal plant *Calea urticifolia*. The analysis of the mechanism of action demonstrated that muscarinic and opioid receptors (μ and δ but not κ opioid receptor subtypes)

participate in the antinociceptive effect of the test compound. The evaluation of the specific muscarinic receptor subtypes involved is beyond the scope of the present study.

SIGNIFICANCE STATEMENT

Some sesquiterpene lactones with a germacrene skeleton have been shown to help with pain. Calein D is a sesquiterpene lactone with a germacrene skeleton. It has already been shown to have gastroprotective activity. The current study provides the first evidence of an antinociceptive effect for Calein D. The combination of antinociceptive and gastroprotective activity is interesting because NSAIDs are usually taken with gastric acid inhibitors to counteract the risk of damage to the stomach. The dual function of Calein D may reduce the doses of the analgesic agent. However, in future studies, it would be important to determine the proinflammatory cells and muscarinic receptor subtypes that are involved in the nociceptive mechanism of Calein D.

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