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Research Article

Antinociceptive and Toxicological Effects of *Dioclea grandiflora* Seed Pod in Mice

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The acute treatment of mice with an ethanolic extract from the seed pod of *Dioclea grandiflora* (EDgP) at doses of 75, 150 and 300 mg/kg by intraperitoneal administration produced a significant antinociceptive effect as displayed by the acetic acid-induced writhing test and the formalin test. The antinociception was observed through the first (neurogenic pain) and second (inflammatory pain) phases in the formalin test. The hot plate test did not show an increase in the antinociceptive latency whereas the motor performance was affected by the administration at 300 mg/kg at the beginning (30 minutes) of the observation period but not at later periods (60 and 120 minutes). These results suggest that EDgP has a central antinociceptive action and a possible anti-inflammatory activity in mice.

1. Introduction

Dioclea grandiflora Mart. Ex. Benth (Leguminosae), popularly known as "mucunã", "mucunã-de-caroço", and "olho-de-boi" or bull's eye, is a vine that grows in the "caatinga" and "cerrado" regions of Northeastern Brazil. In folk medicine, the seed and root bark of this plant have been widely used to treat prostate disorders and kidney stones [1]. Previous studies reported a significant central nervous system (CNS) activity of the chloroformic ($CHCl_3$) and ethanolic extract obtained from the dried root bark [2] and the hydroalcoholic extract from the seeds of *D. grandiflora* [3].

Phytochemical investigations have shown the presence of various substances in this plant. For instance, two new flavanones, dioclein [4] and dioflorin [5], and a new dihydroflavonol, dioclenol [6], were isolated from the $CHCl_3$ soluble portion of the ethanolic root bark extract. Subsequently, paraibanol, agrandol, and diosalol were also isolated from the root bark of this plant [7]. Its major constituent, dioclein, was reported to have significant analgesic effect in rodents [2] and a potent vasorelaxant endothelium-dependent effect in the rat aorta [8]. Later, a pharmacological screening revealed the antinociceptive activity of dioclenol and dioflorin in mice [9].

It is evident from the literature and previous investigations that *D. grandiflora* possesses analgesic activity, namely, the seed and root bark. The aim of this study was to assess the antinociceptive activity of a different part of this plant, that is, the seed pod, using chemical and thermal models of acute pain in mice.

2. Material and Methods

2.1. Plant Material and Preparation of Extract. Aerial parts of *D. grandiflora* were collected in Santa Rita, Paraíba, Brazil

and authenticated in the Lauro Pires Xavier (JPB) Herbarium of the Federal University of Paraíba (UFPB), where a voucher specimen registered under the number 4440-JPB, MO is deposited. The seed pod was dried in an oven at 40°C, and subsequently powdered (135.14 g) and extracted with 70% ethanol/water (v/v%) for 72 hours in a Soxhlet apparatus. The extract was then concentrated using a rotary evaporator and a dry solid was obtained, corresponding to a yield of 4.70%.

2.2. Animals. Male Swiss mice (*Mus musculus*) weighing 30–40 g were obtained from the vivarium of the Laboratory of Pharmaceutical Technology of UFPB, where they were born and bred. The animals were housed under standard laboratory conditions, with a 12-hour light/12-hour dark photoperiod, with the light period beginning at 06:00 hour. They were fed on rat chow pellets and received water *ad libitum*. The room temperature was kept at 23 ± 1°C and all experiments were conducted between 10:00 and 16:00 hour. The experimental protocol was approved by the Ethics Committee of the Laboratory of Pharmaceutical Technology of UFPB (protocol number 0404/08). The number of animals used in the tests as well as the intensity of noxious procedures was kept to a minimum considered necessary to demonstrate the effects of the treatments.

2.3. Nociceptive Tests. For each test, the animals were selected at random and divided evenly into five groups of 10 animals to be used as follows. All animals were brought to the test room at least 1 hour prior to the experiments and were not tested more than once.

2.3.1. Writhing Test. The antinociceptive activity was assessed by the acetic acid abdominal constriction test (writhing test), a chemical visceral pain model induced by intraperitoneal (i.p.) injection of acetic acid, that consists of a constriction of the abdominal muscle together with an elongation of the body and a stretching of the hind limbs [10]. The animals were injected i.p. with 10 mL/kg of 0.8% (v/v solution) acetic acid after 30 minutes of i.p. administration of morphine (6 mg/kg), used as positive control, or EDgP at the dose levels of 75, 150, and 300 mg/kg of body weight. Animals injected i.p. with the same volume of distilled water (10 mL/kg) were used as the control group. Ten minutes after the administration of the acetic acid, pairs of mice were placed in separate boxes and the number of abdominal writhes was counted for 15 minutes. The antinociceptive activity was expressed as the reduction in the number of abdominal writhes when compared to the morphine-treated and control animals.

2.3.2. Formalin Test. The method used was similar to that described previously by Hunskaar [11]. This test consists in inducing nociception in mice by an injection with 20 μL of 2.5% formalin solution (0.92% formaldehyde) under the dorsal surface skin of the right hind paw. Following intraplantar injection of formalin, pairs of mice were placed in separate glass recipients provided with mirrors to enable a complete view of the nociceptive behavior. A cumulative scoring of episodes of hind paw licking was monitored and taken as nociceptive response. Two periods of licking activity were considered. The first period, known as early or first phase (neurogenic pain), started immediately after the formalin injection and lasted 5 minutes. The second period, known as late or second phase (inflammatory pain), occurred 15–30 minutes after formalin injection. The animals were injected i.p. with morphine (10 mg/kg), used as positive control, or EDgP at the dose levels of 75, 150, and 300 mg/kg of body weight 30 minutes before the formalin test. Animals injected i.p. with the same volume of distilled water (10 mL/kg) were used as the control group.

To determine the participation of opioid receptors, the effect of the opioid antagonist naloxone on the antinociceptive activity of *D. grandiflora* seed pod was assessed. Naloxone was injected 15 minutes prior to the administration of the test drug at a dose of 300 mg/kg and morphine (10 mg/kg) in two groups of 10 mice.

2.3.3. Hot-Plate Test. This test was used to measure response latencies according to the method described previously [12]. The animals were placed individually on the hot-plate warmed to 55 ± 1°C and the latencies (in seconds), which is the time elapsed until the appearance of a reaction to the thermal stimulus (lifting or licking of the paws), were recorded as an index of nociception. A cut-off time was set at 30 seconds (maximal latency) to minimize injury to the animals' paws. Approximately 1 hour before the experiment, the reaction time(s) for each mouse was determined and mice with baseline latencies of more than 10 seconds were eliminated from the study. The animals were injected i.p. with morphine (10 mg/kg), used as positive control, or EDgP at the dose levels of 75, 150, and 300 mg/kg of body weight. Control animals received i.p. the same volume of distilled water (10 mL/kg). The tests were performed at 30, 60, and 120 minutes after the respective treatments.

2.4. Toxicity Tests

2.4.1. Acute Toxicity Test (LD50). Groups of mice (n = 10) were separately injected i.p. with 250, 500, 1000, and 2000 mg/kg of seed pod extract to find the dose that kills 50% of the animals, which were observed for 14 days after treatment.

2.4.2. Motor Performance Test (rota-rod). The possible occurrence of nonspecific effects of *D. grandiflora*, such as muscle-relaxation or sedation, was assessed in mice submitted to the rota-rod test [13]. Motor performance was measured as time spent walking on a rotating rod (7 rpm) during 3-minute trials (rota-rod Ugo Basile mod. 7750). To this effect, mice were submitted to a preselection 24 hours before testing and only those which remained on the revolving bar of the rota-rod for 5 minutes were selected. Motor performance was evaluated immediately before (basal), 30, 60, and 120 minutes after i.p. injection of *D. grandiflora* ethanolic extract (300 mg/kg) or distilled water (10 mL/kg).

2.5. Statistical Analysis. The data were expressed by mean ± standard error (S.E.M.) (except DL50) and were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test or Student's *t*-test depending on the case. The tests were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, http://www.graphpad.com/. The difference between groups was considered significant when $P < .05$. DL50 was calculated using probit analysis of SPSS statistical program.

3. Results

3.1. Writhing Test. The results obtained with the writhing test are given in Table 1. EDgP inhibited the intensity of acetic acid-induced visceral nociceptive response after the i.p. administration of all three doses (75, 150, and 300 mg/kg i.p.). The animals treated with morphine (6 mg/kg) were used as positive control, which showed significant reduction in the number of abdominal constrictions (writhes). In comparison with the control group, this response was reduced in 91.9%, 87.4%, and 97.8%, in presence of 75, 150, and 300 mg, respectively. The maximal inhibition of acetic acid-induced nociception was produced by the highest dose tested (300 mg/kg), while the morphine-treated group showed a 94.6% reduction in the nociception inhibition response.

3.2. Formalin Test. Table 2 shows the results obtained with the formalin test. The treatment of mice with EDgP (75, 150, and 300 mg/kg i.p.) resulted in a significant inhibition of the formalin-induced licking in the neurogenic (first phase) pain and the inflammatory (second phase) pain of the formalin test. The most significant antinociceptive response was observed on the second phase at 150 mg/kg dose of the extract, which reduced 100% of the formalin-induced licking response, followed by the 300 mg/kg (87.6%) and 75 mg/kg (81.2%) doses and the morphine-treated group (74%). The treatment of animals with morphine (10 mg/kg i.p.) inhibited the induced-licking in the neurogenic pain (61%) more efficiently than the *D. grandiflora* extract (45.7% at 75 mg/kg, 58.6% at 150 mg/kg and 49.3% at 300 mg/kg).

As shown in Table 2, prior treatment with the opioid receptor antagonist naloxone (6 mg/kg i.p.) fully blocked the antinociceptive activity of morphine, but failed to prevent the antinociceptive response in mice treated with the extract at the dose of 300 mg/kg. Instead, the pretreatment with naloxone augmented the antinociception by the extract in both phases of the formalin test.

3.3. Hot Plate Test. The results in Table 3 show that the treatment of mice with morphine (10 mg/kg i.p.) increased the latency response in the hot plate test at 30, 60 and 120 minutes after treatment. On the other hand, EDgP did not significantly influence the reaction time of the animals to the hot plate at doses of 75, 150 and 300 mg/kg in any of the analyzed periods.

3.4. Acute Toxicity Test. The 250 mg/kg dose of the extract did not cause the death of any treated animal, whereas the 500, 1000, and 2000 mg/kg doses killed 10%, 90%, and 100% of the treated animals, respectively. The deaths occurred within five days of the 14-day observation period. Therefore, these data indicate that the LD50 for this extract is 753 mg/kg i.p. (95% confidence limits: 595–929 mg/kg i.p.).

3.5. Motor Performance Test (rota-rod). A significant reduction in motor activity of mice was observed at 30 minutes after treatment with EDgP at a dose of 300 mg/kg i.p. (148.3 ± 11.2 seconds) when compared to control values (176.4 ± 1.9 seconds). No significant impairment in motor activity was detected at 60 minutes (C: 178.6 ± 1.4 seconds; T: 174 ± 2.54 seconds) and at 120 minutes (C: 171.9 ± 7.4 seconds; T: 179.7 ± 0.3 seconds) after treatment with the extract.

4. Discussion

Accumulating pharmacological data indicate that plants are a substantial source of active compounds capable of exerting potential therapeutic activity in the organism. For instance, *D. grandiflora*, a plant traditionally used for treating kidney and prostate diseases [1], is known to have significant activity on the central nervous system [2]. The phytochemical analysis of seeds and root bark of this plant led to the isolation and identification of various substances, namely dioclein, dioclenol, and dioflorin, which have been reported to have antinociceptive activity in rodents [4–6].

The present study demonstrates that EDgP, administered systemically to mice, displayed marked antinociceptive action, according to two different models of nociception (acetic acid-induced writhing reaction and formalin-induced licking), and provides some evidence on the mechanism implicated in this effect.

The acetic acid-induced writhing test in mice is an efficient nociceptive model for the screening of analgesic and anti-inflammatory drugs and is also regarded as a useful procedure to assess visceral inflammatory pain [14, 15]. In this study, EDgP significantly reduced the acetic acid-induced abdominal writhes in mice. However, the effect was not dose dependent as the doses used (75, 150, and 300 mg/kg) produced similar responses in the treated animals (i.e., inhibitory effect). This finding supports the results obtained by previous works that demonstrated the analgesic effect of seeds and root bark of *D. grandiflora* in rodents [2, 9].

Additionally, a significant antinociceptive effect of EDgP was observed in the formalin test, a reliable model of nociception that can be used to investigate the possible mechanism of antinociceptive action of new analgesic drugs. This test comprises two excitatory phases and one inhibitory interphase of the nociceptive response by employment of tonic stimulus [16]. The first phase (neurogenic pain) involves the direct stimulation of sensorial afferent C-fibers by formalin, while the second phase (inflammatory pain) involves a peripheral inflammatory process and is believed to arise from nociceptive spinal neuron hyperactivity [17,

Table 1: Effect of EDgP on acetic acid-induced writhings in mice.

Treatment	Dose (mg/kg, i.p.)	Number of writhings	Inhibition (%)
Control (10 mL/kg, i.p.)		22.3 ± 3.1	
D. grandiflora	75	1.8 ± 1.4*	91.9
	150	2.8 ± 2.2*	87.4
	300	0.5 ± 0.5*	97.8
Morphine	6	1.2 ± 1.2*	94.6

Values are mean ± S.E.M. ($n = 10$). *$P < .001$ versus control, Bonferroni test.

Table 2: Effect of EDgP on formalin-induced licking in mice.

Treatment	Dose	Paw licking (s)			
	(mg/kg, i.p.)	Early phase (0–5 minutes)	Inhibition (%)	Late phase (15–30 minutes)	Inhibition (%)
Control (10 mL/kg i.p.)		85.8 ± 7.6		233.9 ± 23.6	
D. grandiflora	75	46.6 ± 6.5*	45.7	44.0 ± 27.5*	81.2
	150	35.5 ± 5.3*	58.6	0.0 ± 0.0*	100
	300	43.5 ± 5.0*	49.3	29.0 ± 27.9*	87.6
Morphine	10	33.5 ± 4.6*	61.0	60.7 ± 24.8*	74.0
Naloxone + *D. grandiflora*	6 + 300	47.1 ± 4.3*	45.1	4.9 ± 4.9*	97.9
Morphine	10	69.2 ± 8.0	19.3	246.3 ± 30.5	0

Values are mean ± S.E.M. ($n = 10$). *$P < .001$ versus control, Bonferroni test.

18]. Therefore, the biphasic pain-related behaviors, such as licking, seem to be associated with two distinct mechanisms. One mechanism involves the first phase, which starts immediately after formalin injection and lasts only a few minutes; the other mechanism comprises the second phase, starting 15 min after formalin injection and lasting at least for 60 min. The inflammatory process depends on the sequential activation of various mediators and has been correlated with the increase of prostaglandin production, cyclo-oxygenase (COX) induction, and nitric oxide release [18, 19]. It is well established that both phases of the formalin test can be inhibited by centrally acting drugs, whereas peripherally acting drugs, such as aspirin, only inhibit the second phase [20]. The outcome of this research showed that, on the second phase, EDgP produced a higher antinociceptive response than that displayed by morphine, particularly at the dose of 150 mg/kg. Thus the antinociceptive effects of *D. grandiflora* extract in the writhing test and in both phases of the formalin test not only suggest a central (neurogenic) and peripheral (inflammatory) action, but also imply that this extract exerts an anti-inflammatory activity.

It is known that tissue damage is associated with the release of inflammatory nociceptors [21]. One of the main concerns in nociception studies has been the search for alternative opioid-like drugs that can act at opioid receptors outside the CNS with the purpose of avoiding the common side effects, such as tolerance and dependence, of central acting drugs [22, 23]. Opioids, such as morphine, display peripheral antinociceptive responses under conditions of inflammation [24]. In this study, as the antinociception of EDgP was comparable to that of morphine, the participation of opioid receptors was assessed by use of the selective antagonist naloxone in the formalin test. Naloxone (6 mg/kg i.p.) did not reverse the antinociceptive effect of EDgP; instead, it increased it at the dose level of 300 mg/kg while it considerably reduced morphine analgesic effect. Similarly, a higher dose of naloxone (10 mg/kg) has been reported to produce no effect in the formalin test in rats [25]. The data of this research indicate that central or peripheral active endogenous opioids do not seem to be mediating the antinociceptive effect of EDgP in the formalin test.

In the thermal model for nociception (hot plate test), the results show that, although the pain threshold reaction of the animals increased after the administration of EDgP at the doses of 75, 150, and 300 mg/kg in all analyzed periods, the latency responses were not considered statistically significant. On the other hand, morphine (10 mg/kg) produced a significant antinociceptive effect in all observation periods when compared to control values. Similar findings were observed in previous works to the extent that several compounds (i.e., NS 398–COX-2 inhibitor-indomethacin and other non-steroid anti-inflammatory drugs) failed to produce a significant antinociceptive effect on animals submitted to the hot plate test [26]. This suggests that the substances present in EDgP are particularly effective in relieving painful inflammatory states.

One possible mechanism involved in the nociceptive response is the transient receptor potential vanilloid 1 (TRPV1), a nonselective cation channel which is known to play an important role in the transmission and modulation of pain as well as the integration of diverse painful stimuli [27]. The TRPV1 receptor is considered a molecular integrator of various physicochemical noxious stimuli such as the formalin-induced acute nocifensive behavior [28].

Table 3: Effect of EDgP on the hot plate test in mice.

Treatment	Dose (mg/kg, i.p.)	Reaction time (s) 30 minutes	60 minutes	120 minutes
Control (10 mL/kg, i.p.)		7.2 ± 1.3	8.3 ± 1.1	6.5 ± 0.9
D. grandiflora	75	11.8 ± 2.8	10.7 ± 2.2	8.0 ± 1.3
	150	9.2 ± 1.6	11.8 ± 1.8	10.1 ± 1.7
	300	12.2 ± 1.8	10.0 ± 2.0	9.6 ± 1.6
Morphine	10	18.5 ± 2.5*	11.5 ± 0.8*	11.0 ± 1.7*

Values are mean ± S.E.M. ($n = 10$). *$P < .05$ versus control.

Upon tissue damage and the consequent inflammation, inflammatory mediators increase the sensitivity of TRPV1 to the noxious stimuli. Antagonists have been shown to block TRPV1 activity, thus reducing pain. In rats, for instance, TRPV1 antagonists were effective in reducing nociception from inflammatory and neuropathic pain models [29]. In this work, the evidence is suggestive of an antinociceptive effect of EdgP that could be related to TRPV1 activity and its possible role as a TRPV1 antagonist. However, this assumption still needs to be verified.

The LD50 was used as reference for the choice of doses of this work. The doses corresponded to 1/10 (75 mg/kg), 1/5 (150 mg/kg), and 1/2.5 (300 mg/kg) of LD50 and were lower than the doses used on previous studies [3]. In the rota-rod test, EDgP caused a minor, but significant, effect on motor coordination of mice treated with the highest dose (300 mg/kg) at 30 minutes, but not at the two other time points (60 and 120 minutes), pointing to a transient impairment of motility in mice.

In conclusion, the data presented in this study showed that EDgP exerts a nondose-dependent antinociceptive activity, as displayed by different algesiometric tests, and also suggest a possible anti-inflammatory effect. Moreover, this work corroborates the findings of previous researches that demonstrated the analgesic activity of other parts of *D. grandiflora*. However, further studies are necessary to elucidate the mechanism behind the observed effects.

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