Research Article

Antinociceptive Activity of the Chloroform Fraction of *Dioclea virgata* (Rich.) Amshoff (Fabaceae) in Mice

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Acute treatment with the chloroform fraction of *Dioclea virgata* (Rich.) Amshoff (CFDv) in mice produced decreased ambulation and sedation in the behavioral pharmacological screening. Doses of 125 and 250 mg/kg CFDv decreased latency of sleep onset in the test of sleeping time potentiation. In the open field, animals treated with CFDv reduced ambulation and rearing (250 mg/kg), as well as defecation (125; 250 mg/kg). Regarding the antinociceptive activity, CFDv (125, 250, 500 mg/kg) increased latency to first writhing and decreased the number of writhings induced by acetic acid. In the formalin test, CFDv (250 mg/kg) decreased paw licking time in the first and second phases indicating antinociceptive activity that can be mediated both peripherally and at the central level. CFDv did not affect motor coordination until 120 minutes after treatment. CFDv shows psychopharmacological effects suggestive of CNS-depressant drugs with promising antinociceptive activity.

1. Introduction

Natural products, including medicinal plants, have been the primary source for obtaining new drugs with therapeutic potential throughout history. It is estimated that approximately half of the drugs in use are derived from natural products. According to the World Health Organization, poverty and lack of access to modern medicine leads from 65% to 80% of the world population in developing countries to critically depend on plants for primary health care [1].

*Dioclea virgata* (Rich.) Amshoff, commonly known as “cipó-pixuma” or “feijão-de-boi,” is an extremely hairy woody vine and member of the family Fabaceae (Leguminosae) [2]. Its leaves are popularly used in decoction to treat fever and malaria [3].

Although the popular uses are reported, the species is poorly scientifically investigated, being found only in one immunological study [4] with seeds of *Dioclea virgata* (Rich) Amshoff. Furthermore, Almeida et al. (1999) [5] performed a preliminary behavior assessment in mice treated intraperitoneally with ethanolic crude extract of *Dioclea virgata* suggesting a possible depressant action of this plant.

Other members of the family Fabaceae have already demonstrated activity on the central nervous system (CNS), such as *Clitoria ternatea*, which showed nootropic, anxiolytic, antidepressant, and anticonvulsant activities in mice and rats [6]; *Desmodium gangeticum* showed promising activity to improve memory and potential for treatment of dementia and Alzheimer’s [7]; *Erythrina velutina* and *Erythrina mulungu*, popularly used in Brazil, showed CNS-depressant profile and anticonvulsant activity, possibly acting on the glycinergic system [8]; *Dioclea grandiflora* Mart ex Benth demonstrated CNS-depressant effects such as: anti-Parkinsonian [9] and antinociceptive activity in mice [10, 11].

The aim of this study was to investigate the possible psychopharmacological effects of the chloroform fraction of *Dioclea virgata* using investigative methodologies on the CNS activity in mice, in order to expand scientific knowledge about the species.
2. Material and Methods

2.1. Animals. Male and female (nulliparous and nonpregnant) 3-month-old Swiss mice (Mus musculus) (30–40 g body weight) 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 21 ± 1°C and all experiments were conducted between 12:00 and 17:00 hour.

All experiments were approved by the Ethics Committee on Animal Research of LTF/UFPB, under the opinion no. 0503/07.

For each test, animals were randomly selected and equally divided into groups of 10 animals (five males and five females). All animals were taken to the testing environment at least one hour before the experiments and not tested more than once. The behavioral testing protocols were conducted under blind conditions.

2.2. Botanical Material. Leaves and branches of Dioclea virgata (Rich.) Amshoff were collected on 04/26/2006 in Santa Rita, Paraíba, Brazil, being the botanical identification and morphological description performed by Prof. Dr. Maria de Fátima Agra from the botany sector, Federal University of Paraíba (UFPB), and authenticated in the Herbarium Lauro Pires Xavier (JPB) UFPB, where a voucher specimen is deposited under the code AGRA 5993 JPB.

2.3. Preparation of the Chloroform Fraction of Dioclea Virgata. Preparation of the chloroform fraction of Dioclea virgata was performed by Prof. Dr. Bhattacharyya’s team—LTF/UFPB. Leaves and branches of Dioclea virgata were dried, powdered, and extracted with methanol. The extract was concentrated in a Rotavapor apparatus, obtaining the crude methanol extract. Subsequently, it was partitioned with CHCl₃/H₂O (1:1), obtaining the water and chloroform phases. A vacuum filtration of the latter was performed to obtain the chloroform extract free of inorganic particles. It was placed on a silica gel column and eluted with different solvents following an increasing polarity order: hexane, chloroform, ethyl acetate, and methanol. The chloroform fraction obtained by this column was used in the experiments.

2.4. Substances. Glacial acetic acid (Synth-USA), distilled water (LTF/UFPB-Brazil), sodium chloride (Merck-USA), morphine chloride (Merck-USA), ethanol (LTF/UFPB-Brazil), 37% formaldehyde (Vetec-Brazil), 2.5% formalin (LTF/UFPB-Brazil), sodium pentobarbital (Sigma-Aldrich-USA), Tween 80 (Merck-USA), and Salicylic acid (Sigma-Aldrich, USA).

Drugs were administered intraperitoneally (i.p.) at 0.1 mL/10 g of mice. Doses were prepared minutes before use, dissolved in distilled water or 0.9% saline solution. CDFv was dissolved with 5% Tween 80.

2.5. Pharmacological Evaluation

2.5.1. Behavioral Pharmacological Screening and LD₅₀ Calculation. Animals were divided into groups of 10 mice and treated with different CFDv doses (between 125 and 1000 mg/kg) or vehicle intraperitoneally. Groups were observed at 30, 60, 120, 180, and 240 minutes after the respective treatments; behaviors or changes indicating pharmacological activity on the central nervous system were recorded according to the method described by Almeida et al. (1999) [5].

Assessment of possible toxic effects was carried out with the same animals used in the behavioral screening under 72 h observation to record the deaths occurrence and LD₅₀ determination.

2.5.2. Pentobarbital-Induced Sleeping Time. This test evaluates the substance-induced depressive activity, since the latency reduction to sleep onset and/or additional increase in sleep time induced by pentobarbital from treatment with the test substance is indicative of CNS-depressant activity [12].

Thirty minutes after the respective treatments, animals received 40 mg/kg pentobarbital sodium (intraperitoneally) and were placed in individual boxes to record the hypnotic effect of latency and sleep time [13, 14].

2.5.3. Rota Rod Test. The method proposed by Dunham and Miya (1957) [15] involves placing mice on a rotating bar and measuring the effect of muscle relaxation or motor incoordination produced by the drug under study [16].

Animals were preselected without substance administration through a criterion of permanence in the Rota Rod machine’s rotary bar (Rota-Rod Ugo Basile mod. 7750) at a constant speed of 7 revolutions per minute (7 rpm) for at least three minutes [17].

Twenty-four hours after preselection, mice deemed suitable were divided into four groups of 10 animals. Motor performance was measured as time spent walking on a rotating rod (7 rpm) during three minute trials evaluated at 30, 60, and 120 minutes after i.p. injection of CFDv (125, 250, and 500 mg/kg) or vehicle [11, 18].

2.5.4. Open-Field Test. This test evaluates the exploratory activity of animals, since their natural tendency is exploring the new environment, despite the stress and conflict that is caused [19].

The animals were submitted individually for a period of 5 minutes to an open-field test (Insight mod. EP 154C), 30 minutes after pretreatments. The parameters observed included: ambulation (recorded by the number of segments crossed by the animal with four legs), grooming, number of rearing occurrences and number of fecal masses [20]. The parameters observed were performed live.

2.5.5. Acetic Acid-Induced Abdominal Writhing. A solution of 0.8% acetic acid intraperitoneal injection in mice causes a local irritation, characterized by writhing followed by hind limbs extensions due to the nociceptor stimulation [21].
### Table 1: Summary of multiple tests conducted with CFDv and their results.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>General tests</td>
<td></td>
</tr>
<tr>
<td>Behavioral pharmacological screening</td>
<td>CNS-depressant activity</td>
</tr>
<tr>
<td>Pentobarbital-induced sleeping time</td>
<td>There was no effect on total sleeping time</td>
</tr>
<tr>
<td>Rota Rod test</td>
<td>Absence motor abnormality or neurotoxicity</td>
</tr>
<tr>
<td>Open-field test</td>
<td>Sedative action</td>
</tr>
<tr>
<td>Specific tests (antinociceptive activity)</td>
<td>Antinociceptive activity</td>
</tr>
<tr>
<td>Acetic acid-induced abdominal writhing</td>
<td>Activity may probably be mediated in the CNS level</td>
</tr>
<tr>
<td>Formalin test</td>
<td></td>
</tr>
</tbody>
</table>

![Figure 1: Effect of CFDv on the latency to pentobarbital-induced sleep onset in mice. Values are expressed as mean ± S.E.M. (n = 10), *P < 0.05 versus control group. ANOVA followed by Dunnett’s Multiple Comparison Test.](image)

Usually, drugs with analgesic properties reduce or inhibit this behavior [22].

Five groups of 10 mice received the following pretreatments by i.p. route: vehicle, 6 mg/kg morphine or CFDv (125, 250, and 500 mg/kg). Thirty minutes after initial pretreatment, animals received a solution of 0.8% acetic acid in distilled water (0.1 mL/10 g) injected i.p. and were placed in individual boxes for 20 minutes to record the latency to the first writhing and number of writhings [23].

#### 2.5.6. Formalin Test

In this test, formalin solution is injected into the mouse’s subplantar region leading to the stimulation of nociceptors [24]. The response produced by formalin is biphasic: the first phase, usually within the first 5 minutes after formalin injection, the response is neurally mediated; then there is an interphase of about 10 minutes characterized by inhibitory pain mechanisms and the second phase (15–30 minutes), and the response follows the release of inflammatory mediators [25].

Four groups of 10 mice received the following pretreatments by i.p. injection: vehicle, 250 mg/kg of CFDv, 6 mg/kg morphine, or 100 mg/kg acetylsalicylic acid (ASA). After

30 minutes, 20 μL of 2.5% formalin solution was injected into the subplantar region of the mice’s right hind paw. The parameter recorded was total time spent paw licking after formalin injection during both pain phases: first 5 minutes (neurogenic pain) and between 15 to 30 minutes (inflammatory pain) [25].

#### 2.6. Statistical Analysis

Data were expressed by mean ± standard error (S.E.M.) or percentage and analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test for parametric measures or Kruskal-Wallis test followed by Dunn’s multiple comparison test for nonparametric measures. Tests were performed using the GraphPad Prism, version 4.0 (GraphPad Software Incorporated, San Diego, Calif, USA). The difference between groups was considered significant at $P < 0.05$.

### 3. Results

Table 1 shows a summary of multiple tests conducted with CFDv and their results.

#### 3.1. Behavioral Pharmacological Screening and LD$_{50}$

At the dose of 125 mg/kg behavioral effects resulting from the treatment with chloroform fraction were not verified.

Animals treated with 250 and 500 mg/kg CFDv showed decreased ambulation at 30 and 60 minutes, and those receiving 500 mg/kg also showed diminished touch response.

After treatment with 1000 mg/kg mice showed decreased ambulation and reduced touch response, sedation, presence of writhing, and increased defecation up to 30 minutes. At 60 minutes of observation, animals showed reduced touch response, sedation, and diminished ambulation. Doses of 125, 250, and 500 mg/kg CFDv did not cause death of any mice. There were 10% deaths in the group of animals treated with 1000 mg/kg. From 1000 mg/kg, it was not possible to solubilize the fraction under study and the LD$_{50}$ could not be calculated.

#### 3.2. Pentobarbital-Induced Sleeping Time

As illustrated in Figure 1, sleep latency decreased significantly ($P < 0.05$) after CFDv administration at 125 (193.2 ± 8.2 s) and 250 mg/kg (190.1 ± 7.1 s) compared to the control group (236.6 ± 8.5 s). The dose 500 mg/kg (206.5 ± 19.7 s) did not affect significantly this parameter.
None of the CFDv doses (125, 250, and 500 mg/kg) was able to modify significantly the sleeping time of animals compared with the control group (Figure 2).

3.3. Rota Rod Test. None of the CFDv doses was able to reduce the animals’ permanence time on the revolving bar at 30 minutes after treatment (125 mg/kg: 175.2 ± 3.6 s; 250 mg/kg: 179.9 ± 0.1 s; 500 mg/kg: 163.8 ± 13.2 s) compared with the control group (179.8 ± 0.2 s). No significant impairment in motor activity was detected at 60 minutes (Control: 177.4 ± 1.7 s; 125, 250, and 500 mg/kg CFDv, 175.3 ± 3.4 s; 178.9 ± 1.1 s; 176.0 ± 3.7 s resp.) and at 120 minutes (125 mg/kg: 177.5 ± 1.9 s; 250 mg/kg: 171.8 ± 7.3; 500 mg/kg: 162.3 ± 14.7 s; Control: 178.5 ± 1.0 s) after treatment with CFDv (Figures 3, 4, and 5).

3.4. Open-Field Test. Animals receiving 250 mg/kg CFDv showed reduction in ambulation (94.6 ± 9.1) compared to the control group (140.8 ± 14.0) (Figure 6).

Grooming behavior in the groups treated with CFDv (125, 250 and 500 mg/kg) was not changed (15.3 ± 5.8, 7.8 ± 2.4 and 15.1 ± 5.6 seconds, resp.) compared to the control group (3.1 ± 1.4 seconds).
Only animals treated with 250 mg/kg CFDv (25.8 ± 4.2) decreased significantly the number of rearing occurrences compared to the control (43.4 ± 4.7) (Figure 7). Furthermore, the doses of 125 mg/kg (0.3 ± 0.1) or 250 mg/kg (0.2 ± 0.1) significantly reduced the number of fecal masses compared to the control (1.4 ± 0.2) whilst the one of 500 mg/kg (0.8 ± 0.3) did not (Figure 8).

3.5. Acetic Acid-Induced Abdominal Writhing. In all groups treated with CFDv (125 mg/kg; 722.5 ± 131.0 s; 250 mg/kg: 999.6 ± 106.1 s; 500 mg/kg: 890.3 ± 79.47 s), there was an increased latency to the first writhing appearance compared to the control group (278.2 ± 25.4 s), similar to morphine (1127.0 ± 73.5 s) (Figure 9).

As shown in Figure 10, there was a decrease in the number of writhings in all groups for which the CFDv was administered (125 mg/kg: 8.7 ± 2.7; 250 mg/kg: 3.6 ± 1.8; 500 mg/kg: 5.3 ± 1.7) compared to control (24.3 ± 2.8).
Figure 10: Effect of CFDv on the number of writhings on acetic acid-induced abdominal writhings in mice. Values are expressed as mean ± S.E.M. (n = 10), *P < 0.05, ***P < 0.001 versus control group. Kruskal-Wallis test followed by Dunn’s Multiple Comparison Test.

Figure 11: Effect of CFDv on 1st phase on formalin-induced licking in mice. Values are expressed as mean ± S.E.M. (n = 10), **P < 0.01 versus control group. ANOVA followed by Dunnett’s Multiple Comparison Test.

Figure 12: Effect of CFDv on 2nd phase on formalin-induced licking in mice. Values are expressed as mean ± S.E.M. (n = 10), **P < 0.01 versus control group. ANOVA followed by Dunnett’s Multiple Comparison Test.

3.6. Formalin Test. Mice treated with 250 mg/kg CFDv showed significant reduction of paw licking time in the first phase, with 60.9 ± 9.8 seconds compared to control 91.1 ± 6.8 (Figure 11). Thus, we obtained a result similar to that of the standard group treated with morphine (37.5 ± 4.8). In the group treated with 100 mg/kg ASA, there was no significant reduction in the parameter evaluated (81.7 ± 1.5).

As the result shown in Figure 12, CFDv at 250 mg/kg (12.3 ± 10.4 s) reduced the paw licking time during the second phase of the formalin test when compared with the control group (213.3 ± 51.6 s), morphine (33.7 ± 8.1 s) and ASA (89.7 ± 11.5 s).

4. Discussion

This work consisted of investigating the CFDv antinociceptive activity. To observe the suggestive effect on the central nervous system (CNS) and/or autonomic nervous system (ANS) and possible toxic effects, we performed a behavioral pharmacological screening with CFDv at the doses of 125, 250, 500 and 1000 mg/kg, administered intraperitoneally in mice [26].

Efects observed on the doses of 250, 500, and 1000 mg/kg suggested a possible CNS-depressant activity, for example, reduced ambulation, impaired touch response and sedation [5, 27, 28].

These results are similar to the ones reported by Almeida et al. [5] who observed CNS-depressant activity in mice subjected to behavioral screening treated with crude ethanol extract of Dioclea virgata (Rich.) Amshoff.

Pentobarbital-induced sleeping time, Rota Rod, and open-field tests were chosen as general tests to investigate the possible CFDv’s psychodepressant activity observed in the screening.

Through the test for sleeping time, potentiation induced by sodium pentobarbital is possible to assess whether the tested substance has neurosedative action or a hypnotic profile [29].

Pentobarbital, as a barbiturate, acts primarily at the synapses where the neurotransmission is mediated by GABA in type-A GABAergic receptors. These receptors are characterized by being ion channels permeable to chloride, in which
barbiturates act by increasing conductance to this ion and thus enhancing the inhibitory GABA effect that is the main central inhibitory neurotransmitter [30].

It is worth mentioning that the test for pentobarbital-induced sleeping potentiation is not a specific test, since drugs devoid of central action, such as those that decrease oxygen uptake by tissues or even those that cause vasodilation or vasoconstriction, potentiate the sleeping time by interfering with the pentobarbital biotransformation in the cytochrome P450 complex, and thus may produce the same actions of CNS-depressant drugs [31, 32].

Latency decrease was observed in this test period considered between the pentobarbital administration and the hypnotic effect onset [33], being characterized by loss of righting reflex in animals treated with 125 and 250 mg/kg CFDv. However, the fraction studied did not affect the sleeping time when compared to the control group.

A possible myorelaxing activity as well as the animal’s motor coordination was evaluated in the test using the Rota Rod through the total time of permanence in the rotating bar [34]. The lack of motor coordination in the Rota Rod test is a characteristic of pharmacological agents, such as skeletal muscle relaxants or drugs that reduce the CNS activity, such as neuroleptics, anxiolytics, sedatives, and hypnotics [35, 36].

It is noteworthy, however, that the Rota Rod test is a nonspecific method, since it measures neurological effects, stimulants, and depressants on motor coordination indiscriminately to which is also assigned the term neurotoxicity [37]. As no significant reduction in animals’ permanence time in the revolving bar at 30, 60, and 120 minutes after the three doses studied (125, 250, and 500 mg/kg CFDv) has occurred, it may indicate that the CFDv treatment does not interfere with motor coordination, thus ruling out a muscle relaxant effect or even a neurotoxicity that is common to some drugs with CNS-depressant profile.

The open-field test, originally described by Hall in 1934 for studying emotionality in rats, is a method used to assess exploratory behavior, reaction to the new, anxiety, memory, and stimulating activity, in addition to sedation and locomotor activity [20, 38]. Animals subjected to open-field test showed decrease in ambulation as well as in the number of rearing times when receiving 250 mg/kg CFDv. There was also defecation reduction for animals treated with 125 and 250 mg/kg CFDv, but there was no effect with the 500 mg/kg dose. Grooming was the only parameter that did not change with the three doses tested.

The inhibition of ambulation and rearing is related to drugs with sedative action [20, 39]. Research shows that a high level of emotionality is related to increases in defecation; anxiolytic drugs reduce defecation [40, 41]. However, further studies to investigate whether CDFv caused changes in smooth muscle activity of the gastrointestinal system would be needed. It cannot be concluded that CDFv reduced defecation by reducing anxiety. According to Shaw et al. 2007 [41], grooming usually increases in situations of fear or anxiety in rodents being an adaptation index to a stressful situation. Anxiolytic drugs reduce this behavior in the open field test.

To analyze the possible CFDv antinociceptive activity, two behavioral methods were used to induce nociception: the acetic acid-induced writhing test, which produces chemical nocuous stimulation in the periphery system with a medullar component, and the formalin test, animal model with nociceptors stimulation that results in a pain-indicative behavior biphasic model.

The acetic acid-induced abdominal writhing test, an animal model for nociceptor stimulation to screening drugs with analgesic activity based on irritation caused after intraperitoneal injection of acetic acid solution. This injection can produce a peritoneal inflammation characterized by contractions of abdominal muscles followed by hind limbs extension [21, 23].

Although simple, fast, and reliable to assess the antinociceptive activity of substances [42], is a low specific method since it is sensitive to nonsteroidal anti-inflammatory drugs, narcotics and other centrally acting drugs, anticholinergics, and antihistamines [43, 44].

Treatment with CFDv caused increased latency to the first writhing in mice and reduction in the number of writhings in the three experimental groups, similar to the standard group treated with morphine; being observed that changes in these parameters were not dose-dependent.

These results are similar to those reported by Batista et al. (1995) [10] with aqueous fraction and flavonoid dioclein obtained from the ethanol extract of *Dioclea grandiflora*, as well as those of Sá et al. [11] conducted with *Dioclea grandiflora* seed pod. Among other plant species of the family Fabaceae evaluated in the acetic acid methodology, it may be mentioned that the extract of *Erythrina velutina* and *Erythrina mulungu*, the aqueous extract of *Desmodium gangeticum* DC, as well as the extract and some fractions from *Erythrina crista-galli*, reduced the number of writhings in rodent studies [8, 45, 46].

In an attempt to better characterize the CFDv activity found in the acetic acid-induced writhing test, the formalin model was employed and CFDv injected at a dose of 250 mg/kg. Formalin produced a different biphasic response where analgesics may act differently in the first and second trial phases [47]. The first phase of the formalin model lasts a few minutes and begins immediately after the injection of formalin in the plantar surface of the animal and is due to release of the substance P and direct chemical stimulation of afferents, especially C fibers [48, 49]. It reflects the neurogenic component of nociception being sensitive to drugs that act primarily on the central system, such as opioids [50].

Between the first and second phases of the formalin test, there is a rest period called the “interface” which occurs due to an activation of inhibitory processes GABA-mediated mechanisms, since the type-A GABAergic agonists inhibits the decrease of pain manifestations during this period [51, 52].

The inflammatory component of the nociceptive response (second phase) starts after 10 to 15 minutes of “interface” and is the result of inflammatory mediator release such as bradykinin, histamine, sympathomimetic amines, tumoral-α necrosis factor, and interleukins [50] or a facilitation of spinal synaptic transmission [53, 54].
It is interesting to consider that centrally acting drugs such as opioids inhibit both formalin test phases. Nevertheless, peripheral acting drugs such as anti-inflammatory drugs are effective only in the second phase [49, 55]. According to Hunskaar and Hole [25], the second phase is sensitive to both NSAIDs and corticosteroids.

Similar to the results of Sá et al. (2010) [11] with Dioclea grandiflora, CFDv produced nociceptive response reduction in both formalin test phases, similarly to morphine and other centrally acting analgesic drugs, which are widely effective in preventing the pain formalin-induced pain in both testing phases [44], thus indicating that the CFDv antinociceptive effect presents a central component and a possible anti-inflammatory activity. As expected, the analgesic effect produced by ASA was evident only in the second phase of this test [56].

5. Conclusions

Data presented in this study showed that CFDv exerts current antinociceptive activity with little or no effects and no dose-response relationships in tests for CNS depression or sedation. The reduction in pain responsiveness seemed no dose-response relationships in tests for CNS depression and the CNS. However, further studies are necessary to elucidate the mechanism behind the observed effects.

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