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Central Nervous System effects of *Dioclea grandiflora* pods on mice

[Efectos en el sistema nervioso central de la vaina de *Dioclea grandiflora* en camundongos]

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Abstract
Many plant substances are known for their interference with the central nervous system (CNS). *Dioclea grandiflora* Mart. Ex. Benth (Fabaceae) is a plant used in folk medicine to treat prostate disorders and kidney stones whose extracts from its seeds and root barks were reported to have a significant activity on the CNS and analgesic effect in rodents. In this study, the psychopharmacological activities of *D. grandiflora* were investigated, using the pods of this plant. Swiss mice were submitted to acute treatments with ethanol extract from the pods of *D. grandiflora* (EDgP) at doses of 75, 150 and 300 mg/kg by intraperitoneal administration followed by the evaluation of anxiety, depressant and anticonvulsant-related responses. The treatment with EDgP produced a depressant activity on the CNS and a sedative effect in mice. These findings suggest that EDgP has a central activity in mice, indicating an anxiogenic effect.

Keywords: CNS depressant, Sedation, Swiss Mice

Resumen
Varias sustancias de plantas son conocidas por su acción en el sistema nervioso central (SNC). La *Dioclea grandiflora Mart. Ex. Benth (Fabaceae)* es una planta utilizada en la medicina popular para tratar enfermedades en la próstata y piedras en los riñones, cuyos extractos de sus semillas y de las cáscaras de sus raíces presentan una actividad significativa sobre el SNC y efecto analgésico en roedores. En este estudio, las actividades psicofarmacológicas de *D. grandiflora* fueron investigadas, utilizando la vaina de la planta. *Camudongos Swiss* fueron sometidos a tratamientos agudos por la administración intraperitoneal del extracto etanólico de la vaina de *D. grandiflora (EDgP)* en dosis de 75, 150 y 300 mg/kg administrados intraperitonealmente seguida por la evaluación de respuestas relacionadas con la ansiedad, depresión y anticonvulsivo. El tratamiento con EDgP produjo una actividad depresora sobre el sistema nervioso central y un efecto sedante en camundongos. Estos resultados sugieren que EDgP tiene una actividad central en camundongos, indicando un efecto ansiogénico.

Palabras Clave: SNC depresor, sedación, camundongo Swiss

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INTRODUCTION

The clinical use of a large number of medicines such as those employed in the treatment of the central nervous system (CNS) disorders is generally limited by the occurrence of undesirable side effects. Therefore, the search and development of new medications possessing effective therapeutical properties without adverse effects on the organism would be of great interest in the scientific community worldwide. Medicinal plants are a well-recognized source of substances with potential to provide alternative remedies for our ailments. However, a proper evaluation of their actual efficacy in the treatment of diseases or the occurrence of unwanted effects must be addressed. For instance, accumulating pharmacological data indicate that certain substances present in plants are capable of interfering with the normal functioning of the CNS, producing psychomotor impairment or depressant activity (Emamghoreishi *et al*., 2005; Habib *et al.,* 2012).

Dioclea grandiflora Mart. Ex. Benth (Fabaceae), popularly known as "mucunã", "mucunã-de-caroço" and "olho-de-boi" or bullseye, is a vine that grows in the "caatinga" and "cerrado" regions of Northeastern Brazil. According to popular tradition, the seed and root bark of this plant have been widely used to treat prostate disorders and kidney stones (Lima, 1989). Phytochemical studies showed the presence of various substances in *D. grandiflora*, such as the flavanones paraibanol, agrandol, and diosalol (Jenkins *et al*., 1999), dioclein (Bhattacharyya *et al*., 1995) and dioflorin (Bhattacharyya *et al*., 1998), and the dihydroflavonol, dioclenol (Bhattacharyya *et al*., 1997), isolated from the chloroformic ($CHCl_3$) soluble portion of the ethanol root bark extract of this plant. Previous investigations evidenced a significant CNS activity of the $CHCl_3$ and the ethanol extracts obtained from the dried root bark (Batista *et al*., 1995), and the hydroalcoholic extract from the seeds of *D. grandiflora* (Almeida *et al*., 2003). Its major constituent, dioclein, was reported to have significant analgesic effect in rodents (Batista *et al*., 1995) and a potent vasorelaxant endothelium dependent effect in the rat aorta (Lemos *et al*., 1999). A pharmacological screening also revealed the antinociceptive activity of dioclenol and dioflorin in mice (Almeida *et al*., 2000).

In addition to the seeds and root bark of *D. grandiflora*, a study was carried out using another part of this plant, namely the pods, and reported an effective antinociceptive activity in mice (Sá *et al*., 2010). Therefore, the aim of this study was to further investigate the psychopharmacological activities of *D. grandiflora* pods, in a short-term treatment protocol in mice.

MATERIAL AND METHODS

Plant material and preparation of extract

Aerial parts of *D. grandiflora* were collected in Santa Rita, Paraíba, Brazil and authenticated by Dr. Fatima Agra by means of morphological analysis of stem, leaves, seeds and pods. A voucher specimen registered under the number 4440-JPB, MO is deposited in the Lauro Pires Xavier (JPB) Herbarium of the Federal University of Paraíba (UFPB). The pods was dried in an oven at 40°C, and subsequently powdered (135.14 g) and extracted with 70% ethanol/water (v/v%) for 72 h 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%.

Animals

Male Swiss mice (*Mus musculus*) weighing about 30 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- h light/12- h dark photoperiod, with the light period beginning at 06:00 h. 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 h. The experimental protocol was approved by the Ethics Committee of the Laboratory of Pharmaceutical Technology of UFPB (protocol number 0404/08). 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 h prior to the experiments and were not tested more than once. The number of animals used, as well as the intensity of noxious procedures, was kept to a minimum considered necessary to demonstrate the effects of the treatments.

Pentobarbital-induced sleep time

The animals were injected i.p. with sodium pentobarbital (40 mg/kg) after 30 min of i.p. administration of EDgP at the dose level of 300 mg/kg of body weight or distilled water (10 ml/kg), used as the control group. After administration of sodium pentobarbital, the latency (in seconds), which is the

interval between the pentobarbital administration and the beginning of the sleeping time, and the time (in seconds) between loss and recovery of the righting reflex were recorded (Speroni and Minghetti, 1988; Franco *et al*., 2005).

Elevated plus-maze test

The elevated plus-maze apparatus consisted of four arms (30 cm) elevated 39 cm above the floor. Each arm was positioned at 90º relative to the adjacent arms and all arms were connected through a central area (5 x 5 cm), forming a plus sign. The animals were injected i.p. with diazepam (1 mg/kg), used as positive control, or EDgP at the dose levels of 75, 150 and 300 mg/kg of body weight while animals injected i.p. with the same volume of distilled water (10 ml/kg) were used as the control group. Thirty minutes after treatment, each mouse was placed at the center of the maze facing one of the open arms. The number of entries into and time spent (in seconds) on the open and closed arms were recorded for 5 min. Entry into an arm was defined as the animal having crossed with all four paws the dividing line between the central area and the arm (Herrera-Ruiz *et al*., 2008; Han *et al*., 2009; Grundmann *et al*., 2009). After each trial, the plus-maze was carefully cleaned with 10% ethanol solution.

Hole-board test

The hole-board apparatus (UGO Basile, Italy) consisted of a gray panel (40 x 40 cm; 2.2 cm thick) containing 16 equidistant holes of 3 cm in diameter. The animals were injected i.p. with diazepam (1 mg/kg), used as positive control, or EDgP at the dose levels of 75, 150 and 300 mg/kg of body weight, or distilled water (10 ml/kg), used as the control group. Thirty minutes after treatment, each mouse was placed at the center of the hole-board facing away from the observer and the number of head dips was counted for 5 min by photocells placed below the surface of the hole (Han *et al*., 2009). In addition, ambulation and time spent on the board without moving (immobility time) were also measured. After each trial, the hole-board was carefully cleaned with 10% ethanol solution.

Open field test

The open field apparatus consisted of a white painted area measuring 55 cm in diameter with a 100 W lamp attached and used as the main source of illumination. The floor of the open field was divided into several units by black painted lines. The animals were injected i.p. with diazepam (1 mg/kg), used as positive control, or EDgP at the dose levels of 75, 150 and 300 mg/kg of body weight, or distilled water (10 ml/kg), used as the control group. Thirty minutes after treatment, each mouse was placed at the center of the apparatus and ambulation, rearing and grooming were measured for 5 min (Cícero *et al*., 2000; Franco *et al*., 2005). After each trial, the open field was carefully cleaned with 10% ethanol solution.

Pentylenetetrazole test

Pentylenetetrazole (PTZ) was used to induce clonic-tonic convulsion. The animals were injected i.p. with 60 mg/kg of PTZ after 30 min of i.p. administration of diazepam (4 mg/kg), used as positive control, or EDgP at the dose levels of 75, 150 and 300 mg/kg of body weight, or distilled water (10 ml/kg), used as the control group. After PTZ administration, mice were individually placed in boxes and observed for 15 min to detect the occurrence of convulsion. Tonic PTZ convulsion was characterized as a tonic ventroflexion followed by a full extension of forelimb and hind limb. The latency (in seconds), which is the time elapsed until the occurrence of the first episode of clonic PTZ convulsion, was recorded (N`gouemo *et al*., 1996). The animals were kept under observation for a period of 24 h after PTZ administration and the incidence of mortality was noted.

Electroshock test

Electroconvulsive shock (5 mA) was delivered through an electrode attached to the right ear lobe of mice to induce tonic hind limb extension. The animals were injected i.p. with phenytoin (25 mg/kg), used as positive control, or EDgP at the dose levels of 75, 150 and 300 mg/kg of body weight, or distilled water (10 ml/kg), used as the control group. Thirty minutes after treatment, each mouse received the electroshock and the occurrence and duration (in seconds) of tonic convulsion were noted (N`gouemo *et al*., 1996). An animal was considered protected if no convulsion occurred.

Statistical analysis

The data were expressed by mean ± standard error mean (S.E.M.) 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, www.graphpad.com. The difference between groups were considered significant when $P < 0.05$.

RESULTS

Pentobarbital-induced sleep time

The induction of sleep with pentobarbital is a classic pharmacological model for the screening of sedative-hypnotic drugs. As shown in Table 1, the treatment with EDgP at a dose level of 300 mg/kg significantly reduced the latency but did not produce potentiation of pentobarbital sleeping time in the treated animals in comparison with the results obtained in the control group.

Table 1
Effect of pentobarbital-induced sleep time in mice.

Treatment	Latency (s)	Sleep time (s)
Control[a]	345.1 ± 43.7	4633 ± 676.5
EDgP (300 mg/kg)	221.7 ± 25.4*	4980 ± 564.9

Values are mean ± S.E.M. (n = 10). * P < 0.05 vs control.
a = 10 ml/kg of distilled water. s = seconds

Elevated plus-maze test

One-way analysis of variance showed a significant increase in the amount of time spent on the open arms after the administration of positive control diazepam (1 mg/kg) when compared to control. The animals treated with EDgP at 75, 150 and 300 mg/kg also spent more time on the open arms, but the values obtained were not statistically significant. However, a significant decrease in the number of entries into the closed arms was observed at all dose levels (Table 2). No significant variations were observed between the groups.

Table 2
Effect of EDgP on the elevated plus-maze in mice.

Treatment	Dose (mg/kg)	Time spent in the arms (s)		Number of entries	
		Open	Closed	Open	Closed
Control[a]		49.8 ± 9.4	164.2 ± 10.0	4.6 ± 0.7	10.1 ± 0.8
EDgP	75	88.1 ± 21.7	141.1 ± 22.3	4.0 ± 0.9	5.4 ± 0.4**
	150	63.0 ± 23.3	178.1 ± 25.5	3.5 ± 0.9	4.5 ± 0.7**
	300	95.8 ± 25.0	145.5 ± 25.2	3.5 ± 0.9	3.9 ± 0.7**
Diazepam	1	130.6 ± 16.7*	124.2 ± 17.8	7.0 ± 1.0	8.4 ± 1.4

Values are mean ± S.E.M. (n = 10). * P < 0.05 vs control;
**** P < 0.001 vs control, Bonferroni test. a = 10 ml/kg of distilled water. s = seconds.**

Hole-board test

The sedative effect of EDgP was confirmed in the hole-board test as the extract at doses of 75, 150 and 300 mg/kg, as well as diazepam (1 mg/kg i.p.), significantly decreased the number of head dips (Figure 1) and significantly increased the immobility time relative to the control group (Figure 2). EDgP also reduced ambulation, but only the higher dose (300 mg/kg) produced a significant decrease in this parameter (Table 3). The doses were not significantly different between them.

Open field test

EDgP at doses of 75, 150 and 300 mg/kg and diazepam (1 mg/kg i.p.) significantly reduced the spontaneous motor activities (ambulation) (Figure 3) and rearing (Figure 4) in mice in the open field. At doses of 75 and 150, EDgP reduced the grooming activity of the animals, whereas the higher dose of EDgP and diazepam did not decrease this activity at a significant level (Figure 5). No significant difference was observed between the doses in any of the parameters analyzed in this test.

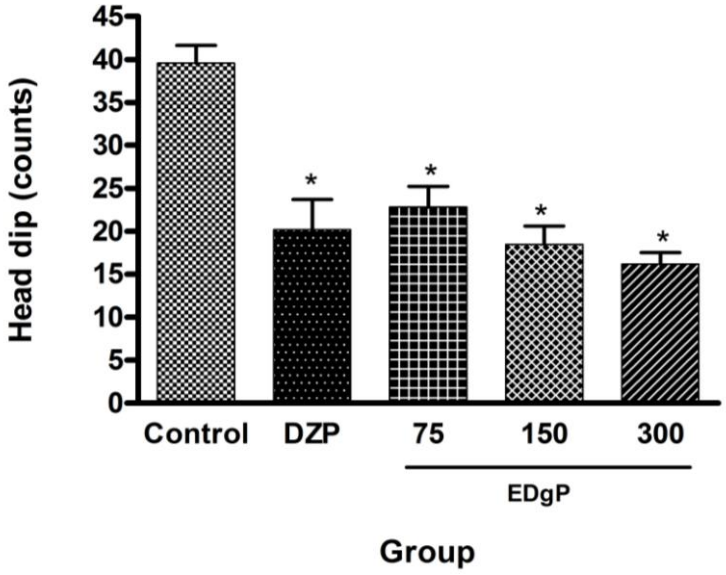

Figure 1
Effect of EDgP on head dips in the hole-board test in mice.
Values are mean ± S.E.M. (n = 10). *P < 0.001 vs control. DZP – Diazepam.

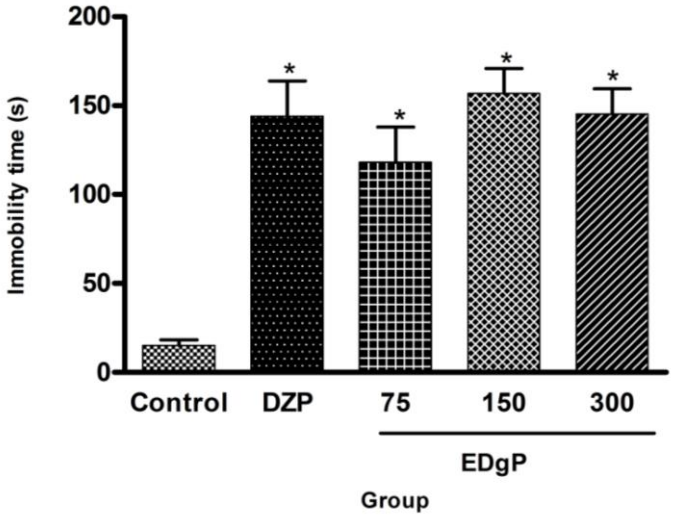

Figure 2
Effect of EDgP on immobility time in the hole-board test in mice.
Values are mean ± S.E.M. (n = 10). *P < 0.001 vs control. DZP – Diazepam.

Table 3
Effect of EDgP on ambulation in the hole-board test in mice

Treatment	Dose (mg/kg, i.p.)	Latency (s)
Control[a]		16.7 ± 1.9
EDgP	75	10.1 ± 2.2
	150	8.1 ± 2.5
	300	6.8 ± 2.1*
Diazepam	1	14.9 ± 2.8

Values are mean ± S.E.M. (n = 10). * P < 0.05 vs control.
a = 10 ml/kg of distilled water. s = seconds.

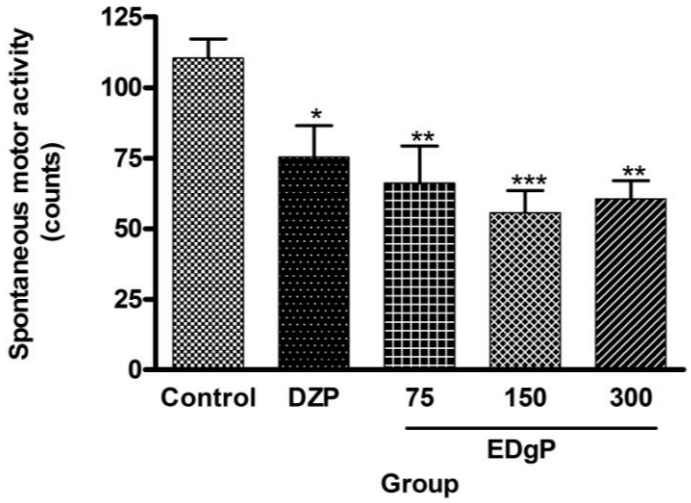

Figure 3
Effect of EDgP on spontaneous locomotion in the open field test in mice.
Values are mean ± S.E.M. (n = 10). *P < 0.05, **P < 0.01, ***P < 0.001 vs control. DZP – Diazepam.

Pentylenetetrazole test

PTZ (60 mg/kg) induction of clonic convulsion in control and EDgP-treated (75, 150 and 300 mg/kg) animals revealed that EDgP neither blocked nor significantly delayed the onset of clonic convulsion (Table 4). Tonic PTZ convulsion occurred in 22% of mice and mortality was low (one death in the 150 mg/kg-treated and three deaths in the 300 mg/kg-treated group).

Electroshock test

In the control and EDgP-treated groups, all the animals exhibited tonic hind limb extension after electroshocks. However, pretreatment with tonic EDgP did not prevent the occurrence of tonic convulsion, but surprisingly increased the duration of the seizure activity. As expected, the phenytoin-treated animals (25 mg/kg) did not exhibit any tonic convulsions (Figure 6).

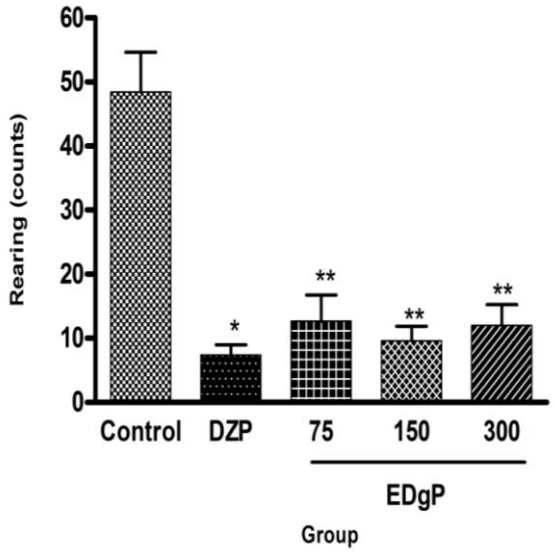

Figure 4
Effect of EDgP on rearing in the open field test in mice.
Values are mean ± S.E.M. (n = 10). *P < 0.05, **P < 0.001 vs control. DZP – Diazepam.

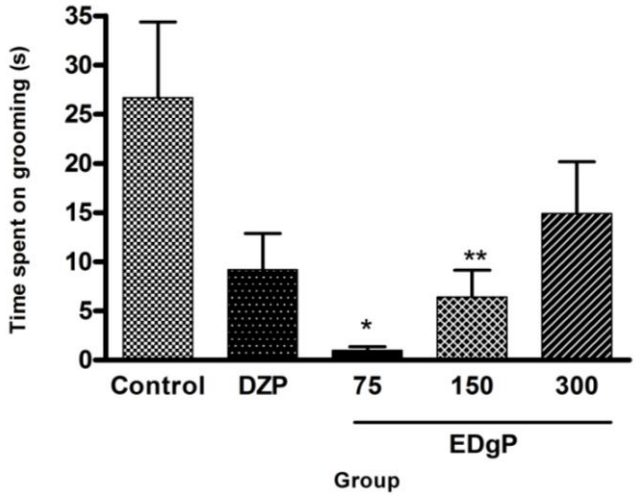

Figure 5
Effect of EDgP on grooming in the open field test in mice.
Values are mean ± S.E.M. (n = 10). *P < 0.01, **P < 0.05 vs control. DZP – Diazepam.

DISCUSSION

D. grandiflora seeds and root bark extracts were shown to produce significant activity on the CNS (Batista *et al*., 1995). They are reported to have flavanones such as dioclein (Figure 7a), dioclenol (Figure 7b) and dioflorin (Figure 7c), which have been shown to have antinociceptive activity in rodents (Bhattacharyya *et al*., 1995; Bhattacharyya *et al*., 1997; Bhattacharyya *et al*., 1998). In this study, an extract obtained from the pods of *D. grandiflora* was used in mice models to further assess its psychopharmacological activities. The doses employed were based on acute toxicity studies and corresponded to 1/10 (75 mg/kg), 1/5 (150 mg/kg) and 1/2.5 (300 mg/kg) of the extract LD50 (753 mg/kg i.p). These doses were lower than the doses used previously in studies with *D. grandiflora* seeds and root bark (Almeida *et al*., 2000). The results obtained

demonstrate that the systemic administration of EDgP to mice displayed a depressant activity on the CNS but not a clear anti-anxiety response, according to the elevated plus-maze, hole-board, open field and pentobarbital-induced sleep tests.

Table 4
Effect of pentylenetetrazole in mice.

Treatment	Dose (mg/kg, i.p.)	Latency (s)
Control[a]		**208.4 ± 78.2**
EDgP	**75**	**157.6 ± 20.0**
	150	**388.6 ± 106.4**
	300	**169.1 ± 29.5**
Diazepam	**4**	**900.0 ± 0***

Values are mean ± S.E.M. (n = 10). * P < 0.05 vs control.
a = 10 ml/kg of distilled water. s = seconds.

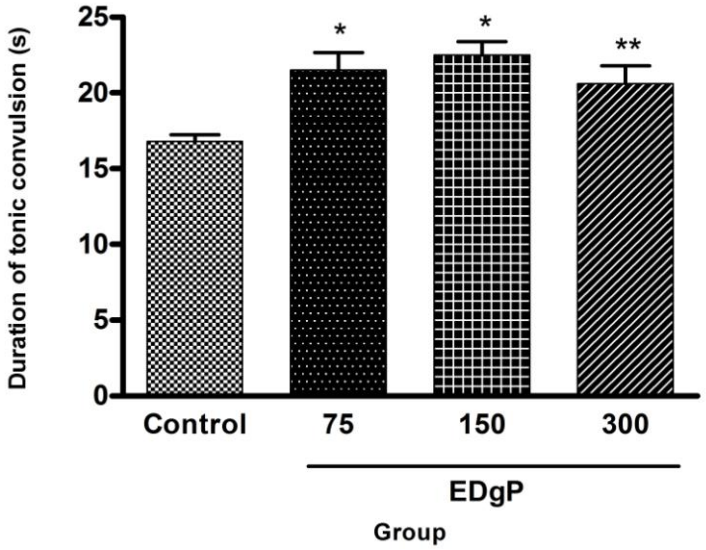

Figure 6
Effect of EDgP on duration of tonic convulsion in the electroshock test in mice.
Values are mean ± S.E.M. (n = 10). *P < 0.01, **P < 0.05 vs control.

It is known that some flavonoids possess anxyolitic, sedative, pro-convulsant, and anti-convulsant effect on the CNS (Fernandez *et al*., 2004; Estrada-Reys *et al*., 2010). In addition, many flavonoids and their derivatives have been shown to bind to the benzodiazepine site of the $GABA_A$ receptor with resulting depressant actions in mice (Marder and Paladini, 2002; Fernandez *et al*., 2006; Aguirre-Hernandez *et al*., 2007; Wang *et al*., 2010). *D. grandiflora* is a plant rich in flavonoids, therefore it is possible that the presence of these substances could account for its effects on the CNS. For instance, *D. grandiflora* was shown to exert ansiolitic-like effects on mice after the administration of alcoholic fraction obtained from the stem bark of this species by use of various behavioral laboratory-based research models, including marble-burying, hole-board and elevated plus maze tests. The effect of the flavonoid dioclenol was also evidenced in the elevated plus maze by displaying significant action in the time spent in open arms; in the hole-boar, showing significant increase in the time spent in the head-dip, and by exhibiting increase marble-burying behavior (De Almeida *et al*., 2009).

The elevated plus-maze test is the most widely used model for rodent anxiety evaluation of novel

substances (Pellow *et al*., 1985; Lister, 1987), in which natural stimuli such as fear of unfamiliar open area and heights are used to induce anxiety in mice and rats (Grundmann *et al*., 2007). The parameters of anxiety analyzed in this test, such as frequency of entries into the open arms and the time spent in open arms are generally increased by anxiolytic substances, which are believed to act via the $GABA_A$ receptor complex. This signaling pathway justifies the use of diazepam as a positive control because this drug is used as a standard anxiolytic employed in behavioral pharmacology (Han *et al*., 2009) and is known to increase the frequency of open arm entries and time spent in the open arms (Moser, 1989; Emamghoreishi *et al*., 2005). In this study, EDgP did not exhibit an anxiolytic activity similar to that observed at 1 mg/kg of diazepam as it did not increase the time spent in the open arms at a significant level in any of the doses analyzed (75, 150 and 300 mg/kg). However, in the hole-board test, the ansiolitic effect of EDgP became more evident since EDgP significantly decreased the number of head dips and increased immobility time displayed by the animals, indicating a depressant activity of the seed pod extract on the CNS. The hole-board is another method used to measure the animal`s response to a novel environment and to assess emotionality, anxiety and/or responses to stress (Nolan and Parkes, 1973; Han *et al*., 2009). In this test, head-dipping behavior may change in response to the emotional state of the animal and an increase in this behavior could reflect the expression of an anxiolytic reaction of the animal (Takeda *et al*., 1998, Han *et al*., 2009).

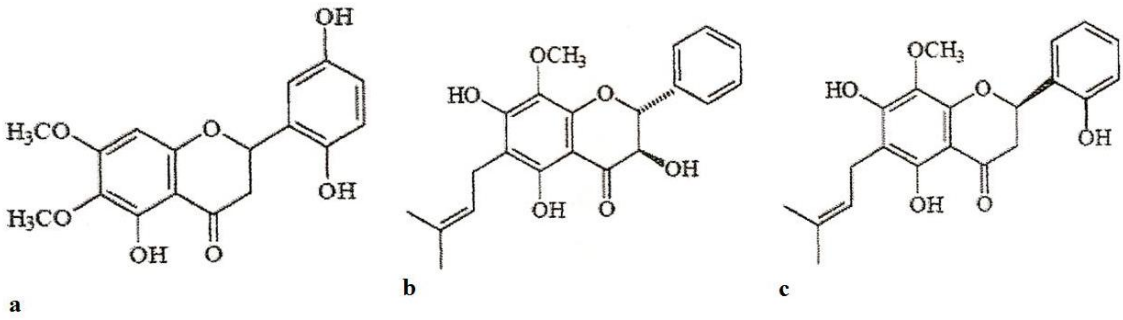

Figure 7
***Dioclea grandiflora* major constituents. (a) dioclein; (b) dioclenol; (c) dioflorin.**

The open field test was used to evaluate the exploratory and emotionality activity of animals (Carlini *et al*., 1986). EDgP decreased ambulation, grooming and rearing, which is indicative of a depressive activity on the CNS. These parameters were taken as responses to the levels of excitability of CNS (Masur *et al*., 1971) and the behavioral effects observed in this study are in agreement with those obtained in previous investigations of depressive drugs of CNS (Almeida *et al*., 2001; Franco *et al*., 2005). The reduction of 50 to 60% in the spontaneous activity caused by EDgP in mice strongly suggests the presence of a sedative component in the activity on the CNS of *D. grandiflora*. However, other factors such as motor impairment or muscle relaxation may also be responsible for the reduction in the ambulatory activity. In fact, a previous study reported a transient impairment of motility in mice treated with the highest dose (300 mg/kg) of EDgP as a significant reduction on motor coordination was observed in animals submitted to the motor performance test (rota-rod) (Sá *et al*., 2010).

The pentobarbital-induced sleep time is a classic pharmacological model used for the screening of sedative-hypnotic drugs. Therefore, this experimental protocol was employed in this study as another tool to assess the depressant activity of EDgP. In a previous work, the treatment of mice with the alcoholic fraction of the stem bark of *D. grandiflora* and dioclenol increased the duration of the sleeping time induced by pentobarbital and reduced latency to sleep (De Almeida *et al*., 2009). In the present investigation, although the extract did not significantly increase the sleeping time, it reduced latency, i.e. it decreased the amount of time taken for the animals to lose their righting reflex at a significant level, providing further evidence for the depressant effect of EDgP on the CNS.

In order to evaluate a possible anti-convulsant action of EDgP in mice, two convulsing tests,

electroshock and PTZ, were performed in this study. The electroshock produces tonic convulsion characterized by tonic hind limb extension whereas PTZ induces clonic-tonic convulsions (Swinyard *et al*., 1989; N`gouemo *et al*., 1996), being a good model to study the effects of anticonvulsant drugs on the propagation of seizure activity (Goodman *et al*., 1953; N`gouemo *et al*., 1996). EDgP did not block the PTZ-induced seizures nor did prevent the occurrence of tonic convulsions. In particular, it was observed that EDgP at all dose levels increased the duration of tonic convulsions in the electroshock test, contrasting with the effects of the alcoholic fraction of the stem bark of *D. grandiflora* and dioclenol that played a protective role against PTZ convulsion (De Almeida *et al*., 2009).

In conclusion, the investigation of some of the neuropharmacological activities of *D. grandiflora* pods revealed that EDgP possess a depressant activity and a sedative effect in mice, but did not protect the animals against the occurrence of convulsions. The main constituents of *D. grandiflora* and the mechanisms involved in these pharmacological effects need to be investigated in further experiments.

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