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Original Article

Anxiolytic and anticonvulsant effects of dioclenol flavonoid isolated from stem bark of *dioclea grandiflora* on mice

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Summary: The aim of the present study is to demonstrate the anxiolitic and anticonvulsant effect of fraction (alcoholic) obtained from the stem bark of *Dioclea grandiflora* (Dg) and Dioclenol on mice using several behavioural assays. Groups of mice treated by the intraperitoneal (i.p.) route with doses of 15, 30, and 60 mg/kg (i.p.) of the fraction and Dioclenol with a dose of 10 mg / kg showed significant action in the Elevated Plus-maze (EPM) (time spent in open arms and time in spent in the closed arms). The Hole-board Test also showed a significant increase in the time spent in the Head-dip and Marble-Burying Tests. The same treatment increased the duration of the sleeping time induced by Sodium Pentobarbital, and showed a significant increase in protection against Pentylenotetrazole induced convulsion. These results indicate an anxiolitic-like and anticonvulsant-like effect of the fraction of stem bark of Dg and Dioclenol in mice. The phytochemical analysis suggests that the alcoholic fraction has higher concentration of flavonoid active (Dioclenol) and deserves further analysis. The studies conducted with the *Dioclea grandiflora*, can contribute, in the long term, in the field of its action in the CNS this flavonoid.

Industrial relevance: The studies conducted with the *Dioclea grandiflora*, can contribute, in the long term, in the field of its action in the CNS.

Keywords: Dioclea grandiflora; Dioclenol; Flavonoid; Behavioural Effects.

Introduction

Dioclea grandiflora Mart. ex. Benth (Leguminosae) (Dg). This plant popularly known in Brazil as "mucunã-de-caroço," "olho-de-boi or bull's eye" and "mucunã," which thrives in the semi-arid regions of Northeast Brazil, where the Dg roots are frequently used in popular medicine in treating kidney and prostate diseases, as a sedative and tonic and it is also known to have antiepileptic properties (Batista, Almeida and Bhattacharyya 1995; Mattei, Leite and Tufik, 1995). Based on this FHA, Dg root and seed extracts were exhaustively studied yielding the following results: isolation of Dioclein, a Flavanone and Dioclenol (Diocl), (Fig 1) (Bhattacharyya *et al* 1997)**.** The study conducted in 1995 with alcoholic extract of the seeds had a depressing action in the Central Nervous System (Mattei, Leite and Tufik 1995), followed by the isolation of Dioflorin (Bhattacharyya *et al* 1998).

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The isolation of a new bioactive flavanone, Dioclein (Bhattacharyya, Batista and Almeida, 1995)**,** resulted from this tissue. More detailed separation of the earlier chromatographic fractions in the isolation of Dioclein revealed the presence of others metabolites from which Dioclenol, a minor constituent, was subsequently isolated, and in the following year, from Dioflorin, a minor flavonoid. The agrandol (5,7-dihydroxy-6-methoxy-8-prenylflavanone), paraibanol (3,5,7,2′,5′ pentahydroxy-6 methoxyflavanone), and diosalol (3,5,7,2′,5′-pentahydroxy-6-methoxy-8-prenylflavanone). β-amyrin and 5, 7, 2′, 5′-tetrahydroxy-6-methoxy-8-prenylflavanone were also identified. All structural assignments were based solely on spectral data (Jenkins *et al.*, 1999). A pharmacological effect with the Dioclein was identified through the vasorelaxant action held in the aorta of rats (Lemos *et al*., 1999). In 2003, a central antinociceptive effect of alcoholic fraction of seeds in several experimental models was demonstrated (Almeida *et al.,* 2003).

The purpose of this study was to evaluate the possible anxiolitic FHA action of Dg and Diocl, using different experimental models of behaviour, based on the broad spectrum of the flavonoids present in their pharmacological actions. According to the citation of flavonoids on the C.N.S, this is often mentioned in literature, with special reference to anxiety (Marder and Paladini, 2002; Coleta et al., 2006; Elisa *et al.*, 2008; Miguel *et al.*, 2008).

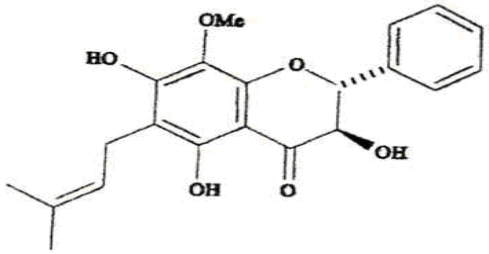

Figure 1: Dioclenol [3, 5, 7-trihydroxy-8-methoxi-6-prenylflavanone]

Material and Methods

Plant Material: *Dioclea grandiflora* Stem bark (Dg) was collected in the region around the town of Santa Rita, State of Paraíba, Brazil, in December 2007. The plant was identified by Prof. Maria de Fatima Agra of the Federal University of Paraíba, and a voucher specimen was deposited at the Lauro Pires Xavier Herbarium and registration n° JPB -44404 JPB, MO.

Extraction and Isolation: 10 kg of plant material was extracted as described previously (Bhattacharyya et al., 197) The $CHCl_3$-soluble portion of the crude ethanolic extract was chromatographer on silica gel (E. Merck, 230-400 mesh) and eluted with $CHCl_3$, and 70 mL fractions were collected. Evaporation of the $CHCl_3$ fractions gave 12,5 g of residue. Sephadex-LH 20 CC using as eluent MeOH achieved final purification. The yields were 77 mg of Dioclenol.

Animals: The animals used in the experiments were Swiss male albino mice (25-30 g), obtained from the vivarium of the Department of Antibiotics of the Federal University of Pernambuco. 10 animals were used per group. The animals were kept at a controlled temperature of 22 ± 2 °C, with a light/dark period of 12 h, with the light period beginning at 06:00 am, and they were given food and water *ad libitum*. All experiments were conducted between 10:00 am and 04:00 pm.

The animals were carefully monitored and maintained in accordance with the recommendation of the Brazilian College of Animal Experimentation (COBEA) and the National Institute of Health Guide for Care and use of Laboratory animals. Therefore, subsequently, the Universities Ethics Committee on Animal Experiments (ECAE) UFPE, protocol number 008196/2005-29, adopted these recommendations as criteria for evaluation.

Drugs: Diazepam (DPZ 2.5 mg/kg, i.p.) was used as the standard anxiolitic drug; Pentobarbital sodium (PBS, 55 mg/kg, i.p.) All drugs were obtained from Sigma® (U.S.A.). The control group was composed of saline + cremophor 0.1% utilized as an emulsifier in the preparation of the suspension of alcoholic fraction of Dg (FHA 30 and 60 mg/kg, i.p.) and Dioclenol (Diocl 10 mg/kg, i.p.) All substances were applied to the animals (mice) at a volume of 0.1 mL/10g.

Experimental groups: Groups of ten male albino mice (25-30g) were conditioned to the laboratory environment (12 h light for 12 h dark), with free access to water and food. Doses of Diocl (10 mg/kg, i.p.) and FHA 30 and 60 mg/kg (i.p.); Diazepam (DPZ (2.5 mg/kg, i.p.) were administered 30-min before of the test.

Pentobarbital sleeping time: The test was performed in six groups of mice (n= 10, each group). Six groups received the Diocl at a dose of 60 mg/kg, i.p., while the control group received normal saline (0.1 mL/kg, i.p. +

PBS 55 mg/kg, i.p.). The 3nd group was given Diazepam (2.5 mg/kg, i.p.), the 4rd group was given Diocl (10 mg/kg, i.p.) and FHA in the 5th and 6th. Thirty minutes later, Pentobarbital Sodium (55 mg/kg, i.p.) was administered to each mouse to induce sleep. The interval between loss and recovery of righting reflex was used as an index of hypnotic effect. Fig 2a and 2b (Speroni and Minghetti, 1988).

Marble-burying Test: Twenty-five glass beads (20 mm diameter) were used for each test. Polyethylene cages (30 × 36 × 13 cm) were constructed with a vinyl roof with holes for air-intake, with a 5-centimetre layer of sawdust on the floor of the cage. Each mouse in each group was placed in individual cages with sawdust for 15 min (habituation) and then the animal was returned to its cage of origin. Twenty-five glass beads were placed randomly spaced 3 cm from each other, with a layer of 5 inches of sawdust in the habituation cage.

The animals were reintroduced (one animal at a time) into the cage (In each test the mice were returned to the same cage in which it had been accustomed to). Ten animals were used in each group. The test group received the Diocl at dose of 10 mg / kg (i.p) and FHA at doses of 30 and 60 mg/kg. After 15 minutes the test was terminated by the withdrawal of the mouse and the number of balls that were more than two-thirds covered with sawdust was counted. After each test, the sawdust was replaced and the glass beads were washed with water and with 70% alcohol/water (v/v) (Njung'e and Handley, 1991).

Hole-board Test: The exploratory behaviour was evaluated using the Hole-board test. The device consisted of a square wooden plate (40 cm x 40 cm), 2 cm thick, with 16 holes (2 cm in diameter), regularly spaced on the surface 3 cm from the edge. The unit was raised to the height of 50 cm from the floor in a lit room. Each mouse in each group was placed in the centre of the plate and the number of depressions in the head opening was immediately counted for three consecutive periods of 5 minutes each. All experiments were conducted between 10:00 am and 04:00 pm. After each test, the equipment was cleaned with 70% ethanol, with a paper towel (Treit; Pinel and Fibiger, 1981).

Elevated Plus-maze Test: A device consisting of two open arms (30 x 5 x 0.25 cm) and two closed arms (30 x 5 x 15 cm) of a common central platform (5 x 5 cm) evaluated the elevated plus-maze (EPM). Two pairs of identical arms were opposite each other. The entire apparatus was elevated to a height of 40 cm from the floor. At the beginning of the session, the mouse was placed in the centre of the maze, its head facing an open arm and allowed to explore the labyrinth for 5 min. The following parameters were observed: the time spent on each arm.

The apparatus was thoroughly cleaned with a wet towel with 70% alcohol after each animal. The mice were divided into 4 groups. DPZ (2.5 mg / kg, i.p) was used as positive control and the Diocl doses of 10 mg/kg, FHA at doses of 30 and 60 mg/kg. All experiments were conducted between 10:00 am and 04:00 pm. After each test, the equipment was cleaned with 70% ethanol, with a paper towel (Lister, 1987).

Locomotor activity: The system used to assess the locomotor activity consisted of a wooden box (36 x 15 x 20 cm) with squares painted in white on the floor with black stripes, divisions between the squares (2.65 cm). Each mouse was placed in the center of the box. The motility was assessed as to whether the four legs of the animal were within each square. The locomotor activity was measured after 10-min of the administration of Diocl (10 mg/kg) and FHA (30 and 60 mg/kg). The time was recorded with a stopwatch and the number of squares crossed was recorded. The assessment period was 10-min carried out 30-min after the administration of the drugs (Adapted from Wolfman et al, 1994).

Statistics analysis: Statistical analysis was performed using one-way analysis of variance (ANOVA) with *post hoc* Duncan's test. $P<0.05$ was considered significant. All data are express as mean ± S.E.M. All statistical analysis was carried out in the Program Graph Pad Prism.

RESULTS

Sodium Pentobarbital-induced sleep time.

Saline, DPZ, Diocl and FHA were given 30-min prior to Pentobarbital injection (i.p.) and the latency to loss of righting reflex was measured. In the saline treated control animals the righting reflex was lost after 68±09s of Pentobarbital injection (Fig 1) Injection of FHA at dose 30, and 60 mg/kg, significantly suppressed the latency to sleep * $p<0.01$ Similar to Dioclenol (10 mg/kg, i.p.) and DPZ (2.5 mg/kg, i.p.) 30-min prior to Pentobarbital reduced the latency to sleep *$p<0.01$ (Fig. 2.)

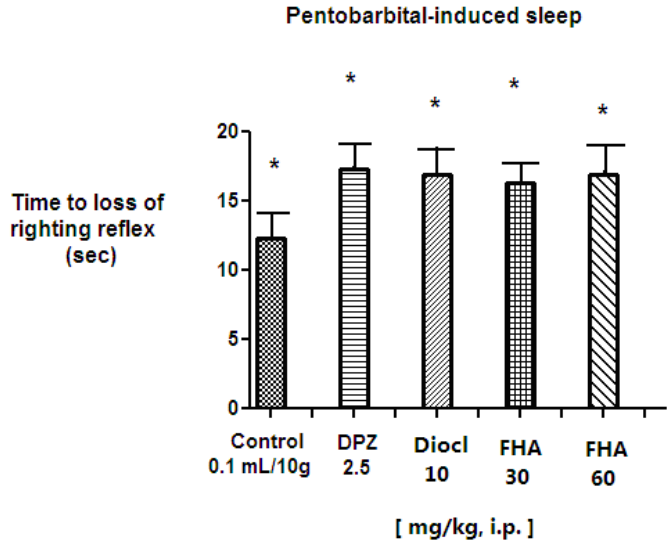

Figure. 1. n=10. Effect of Control, DPZ, Diocl and FHA on time to loss of righting reflex. The data wereevaluated by analysis of variance (ANOVA) with post hoc Duncan's *t*-test, **P* <0.01.

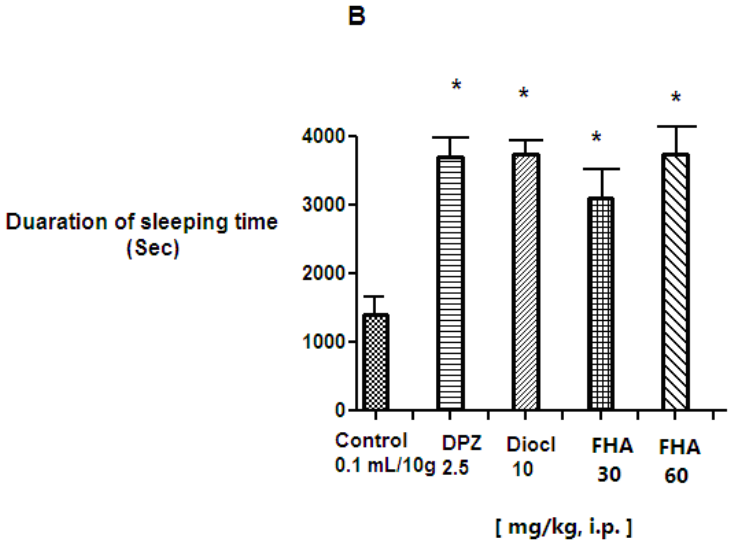

Figure 2. Effect of Control, DPZ, Diocl and FHA on time sleep induced by Pentobarbital sodium. The data were evaluated by analysis of variance (ANOVA) with post hoc Duncan's *t*-test, *p<0.001.

Marble-burying Test: To examine this premise, we studied the effect of the representative of Diazepam; Dioclenol, and fractions on burying behaviour. As expected, control exhibited a significant decrease in the Marble burying behaviour. However, DPZ, Diocl and FHA prompted an increase in marble burying at doses 2.5 mg/kg., 10 mg/kg and 30, and 60 mg/kg, i.p., respectively *p<0.004 (Fig. 3).

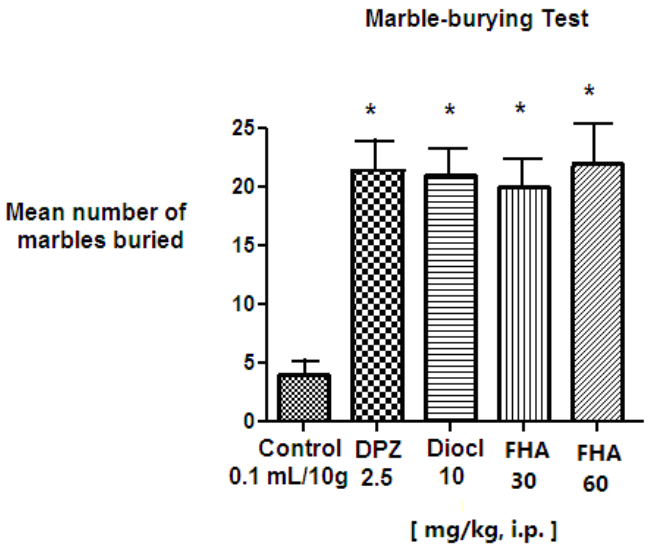

Figure 3: Effect of Control, DPZ, Diocl and FHA on Marble-burying test. The data were evaluated by analysis of variance (ANOVA) with Post hoc Duncan's *t*-test, *p<0.004, n=10.

Hole-board Test: The effect of DPZ, Diocl, and FHA on the Hole-board test is presented in Figure 3. In the same doses there was a significant increase for behaviour, except the control group *p<0.01 (Fig. 4).

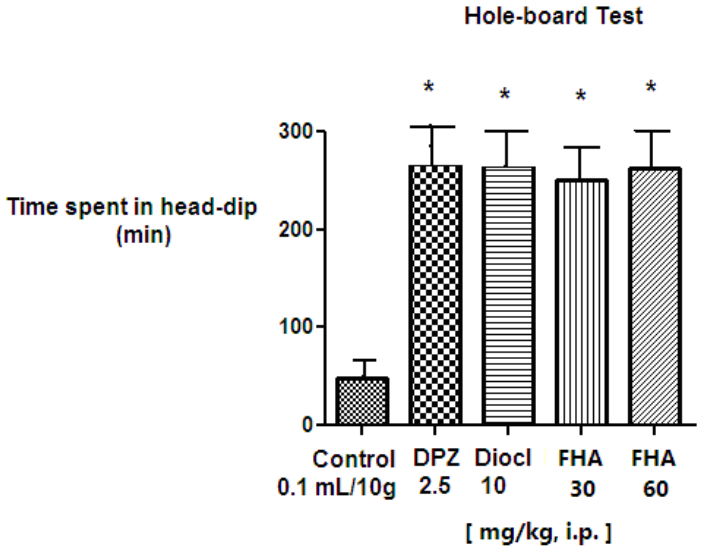

Figure 4. Effects of Diocl and FHA in Hole-board Test, the fraction were evaluated in relation to Diazepam and control. All data cited are the mean ± S.D. 10 animals were used in each group. The data were evaluated by a one-way analysis of variance (ANOVA) with post hoc Duncan's *t*-test, *p<0.01.

Elevated plus-maze: In this model Diocl (10 mg/kg, i.p.), DPZ (2.5 mg/kg, i.p.), and FHA at all doses (30 and 60 mg / kg, i.p.) had their anxiolitic effect determined by time spent in open arms p <0.01. Conversely, the time spent in closed arms was reduced during the evaluation, p >0.05). The number of entries in closed arms was not significant (Data not shown).

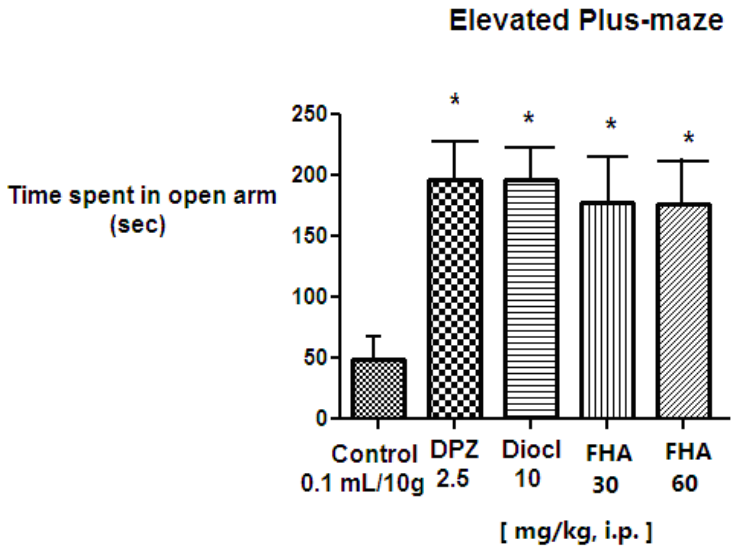

Figure 5: Effect of Diocl and FHA were evaluated in relation to the control and DPZ. All data cited are the mean ± S.D. 10 animals were used in each group ($p < 0.05$). The data were evaluated by analysis of variance (ANOVA) with post hoc Duncan's *t*-test, *$p<0.01$.

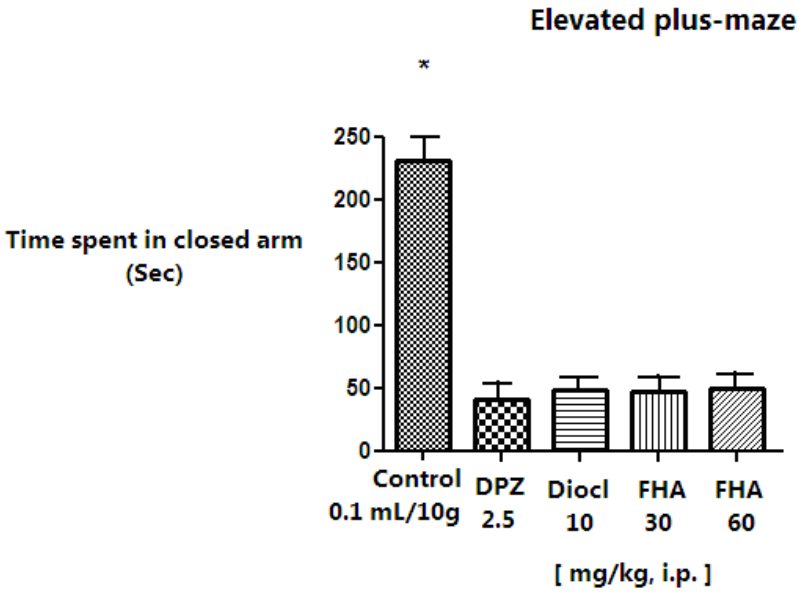

Figure 6. Effect of Diocl and FHA were evaluated in relation to the control and DPZ. All data cited are the mean ± S.E.M. 10 animals were used in each group. The data were evaluated by analysis of variance (ANOVA) with post hoc Duncan's *t*-test, * $p>0.05$.

Locomotor activity: The effect of DPZ, Diocl, and FHA on locomotor activity was measured 30-min after the administration of drugs**.** Locomotor activity was significantly decreased in animals injected with DPZ (2.5 mg/kg), Diocl 10 mg/kg), and FHA (30, and 60. mg/kg), Diocl (10 mg/kg, i.p.), compared with control group (saline 0.85%, i.p.). The reduction in locomotor activity was evident within the time of 10-min. *P <0.02 (Fig 5).

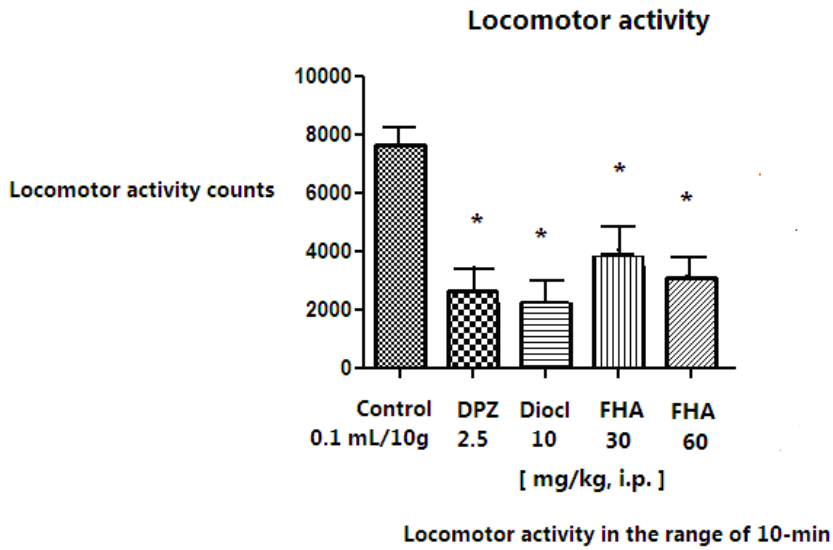

Figure 7: Effect of DPZ, Diocl, and FHA on spontaneous locomotor activity in the range of 10-min. The locomotor activity counts (mean ± S.E.M.) were measured over the range of 10-min. The assessment was 10-min, 30-min after the administration of drugs. **P*<0.02 compared with vehicle-treated controls.

Discussion and Conclusion

The aim of this study was to identify in the Diocl and stem bark anticonvulsant and anxiolytic effect through the following behavioral tests: sodium pentobarbital-induced sleep time, Pentylenotetrazole-induced convulsion (PTZ), Marble-burying Test, Hole-board test, and Elevated plus-maze (EPM). Actually, many flavonoids were found to be ligands for the γ-aminobutyric acid type A ($GABA_A$) receptors in the Central Nervous System; which led to the hypothesis that they act as benzodiazepine-like molecules, probably in place of specific action on the receptor $GABA_A$ (Goutman et al., 2003; Kavvadias et al., 2004; Hall et al., 2005). Their behavioural effects of anxiety and sedation in animal models (Marder and Paladini, 2002) support this. Due to the increased knowledge of the diversity of $GABA_A$ receptor subtypes, the number of studies with cloned receptors of defined subunit composition has recently risen, and experiments with some natural and/or synthetic flavones and flavanones have shown that they can modulate γ-aminobutyric acid ($GABA_A$) generated chloride currents, either positively or negatively (Goutman *et al*., 2003). However, none of these studies evaluated the action of flavonoids, despite the act that they are the main form of these compounds found in nature. The $GABA_A$ receptor complex comprises a Cl^- channel and binding sites for several compounds, such as benzodiazepines, barbiturates and neuroactive steroids (Korpi 2002).

Furthermore, a site for convulsant drugs is also present in the complex. All these binding sites are allosterically coupled, resulting in a network of interactions that ultimately regulate the permeability of the Cl^- channel (Johnston, 2005). Many flavones derivatives were found to be ligands for the $GABA_A$ receptors in the CNS; and to bind to the benzodiazepine binding site with resulting depressant actions in mice (Mader and Paladini, 2002; Johnston, 2005; De-Paris *et al*., 20000)**.** In this study, the authors demonstrate an anxiolitic-like action using various experimental models for assessment of anxiety using the FHA and Diocl of the Dg. In FHA induced sleep time shows a significant effect at doses of 30, and 60 mg/kg respectively. In the Marble burying test FHA and Diocl all doses showed a statistically significant effect ($p<0.01$). In the assessment with the Hole-board test, the FHA showed a significant effect at doses of 30 mg/kg and 60 mg/kg ($p<0.01$). The Diocl in both tests of the EPM (time spent in the open arm) showed a statistically significant effect $p<0.01$. The FHA in both doses demonstrated the same effect. The results obtained in this experiment, can suggest a possible participation of flavonoids present in the stem bark of *Dioclea grandiflora* (FHA) as responsible for this pharmacological action together with Diocl. It is clear from the results obtained in this study that *D. grandiflora* is a plant with multiple active compounds (Flavonoids, amino acids, etc) which may explain the activity of various actions including the C. N.S. as demonstrated in this study (Carlini, 2003). The phytochemical analysis suggests that the alcohol fraction has higher concentrations of flavonoids actives (Dioclenol) and deserves further analysis.

The results obtained with the FHA and Dioclenol may suggest that the anxiolitic and anticonvulsant effect probably occurs in the fraction of the benzodiazepine site of GABA receptors. The data that Dioclenol is a flavonoid (a minor flavanonol) where the pharmacological actions of this chemical group most often occur at the level of the CNS is very relevant to this conclusion.

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References

Almeida, E. R.; Almeida, R. N.; Navarro, D. S.; Bhattacharyya, J.; Silva, B. A.; Birnbaum, J. S. P. Central antinociceptive effect a alcoholic extract of Dioclea grandiflora seeds in rodents. *J. Ethnopharmacol*. **2003,** 88, 1-4.

Batista, J. S.; Almeida, R. N.; Bhattacharyya; J. Analgesic effects of *Dioclea grandiflora* constituents in rodents. *J. Ethnopharmacol.* **1995**, 45, 207-210.

Bhattacharyya, J.; Batista, J. S.; Almeida, R. N. Dioclein a flavanone from the roots of *Dioclea grandiflora. Phytochemistry*. 1995, 38 (1), 277-278.

Bhattacharyya, J.; Majetich, G.; Jenkins, T. M.; Almeida, R.N. Dioflorin a minor flavanoid from *Dioclea grandiflora. J. Nat. Prod*. **1998**, 61, 413-414.

Bhattacharyya, J.; Majetich, G.; Spearing, P.; Almeida, R. N. Dioclenol, a minor flavanonol from the root-bark of *Dioclea grandiflora*. *Phytochemistry*. **1997**, 46 (2), 385-387.

Carlini, E. A.; Plants and Central Nervous System. *Pharmacol. Biochem. Behav.* **2003**, 75, 501-512.

Coleta, M.; Batista, M. T.; Graca Campos, M.; Carvalho, R.; Dulce Cotrim, M.; DE Lima, T. C. M.; Proenca Da Cunha, A. Neuropharmacological evaluation of the putative anxiolytic effects of *Passiflora edulis*, its sub-fractions and flavonoid constituents. *Phytother Res*. **2006**, 20 (12), 1067-1073.

De-Paris, F.; Neves, G.; Salgueiro, J. B.; Quevedo, J.; Izquierdo I.; Rates, S. M. K. Psychopharmacological screening of *PFHAffia glomerata* Spreng. (Amarathanceae) in rodents. *J Ethnopharmacol*. **2000**, 73 (1-2), 261-269.

Elisa, O.; Paolo, B.; Walter, A.; Luciano, S.; Giovanni L. Behavioral effects of 6-bromoflavanone and 5-methoxy-6,8-dibromoflavanone as anxiolytic compounds. *Prog Neuropsychopharmacol Biol Psychiatry.* **2008**, 32 (1), 128-134.

Goutman, J. D.; Maximiliano, D.; Doñate-Oliver, W. F.; Pomata, P. E.; Daniel J. C. Flavonoid modulation of ionic currents mediated by GABAA and GABAC receptors. *Euro J Pharmacol*. **2003**, 461 (2-3), 79-87.

Hall, B. J.; Chebib, M.; Hanrahan, J. R.; Graham, J. A. R. 6-Methylflavanone, a more efficacious positive allosteric modulator of γ-aminobutyric acid (GABA) action at human recombinant α2β2γ2L than at α1β2γ2L and α1β2 GABAA receptors expressed in *Xenopus* oocytes. *Euro J Pharmacol*. **2005**, 512 (2-3), 97-104.

Jenkins, T.; Bhattacharyya. J.; Teng, Q.; Agra. M. F.; Almeida, R. N. Flavonoids from root-bark of *Dioclea grandiflora*. *Phytochemistry*. 1999, 52, 723 – 730.

Johnston G. A. R. GABAA Receptor Channel. *Curr Pharm Des*. **2005**, 11, 1867-1885.

Kavvadias, D.; Sand, P.; Youdim, K. A.; Rice-Evans, C.; Baur, R.; Sigel, E.; Riederer, P.; Schreier, P. The flavone hispidulin, a benzodiazepine receptor ligand with positive allosteric properties, traverses the blood–brain barrier and exhibits anti-convulsive effects. *Br J Pharmacol*. **2004,** 142, 811–820.

Korpi, E. Drug interactions at GABAA receptors. *Prog Neurobiol*. **2002**, 67(2), 113-117.

Lemos, V. S.; Freitas, M. R.; Muller, B.; Lino, Y. D.; Queiroga, C. E. G.; Cortes, S.. F. Dioclein a new nitric oxide and endothelium dependent vasodilator flavonoid. *Euro J Pharmacol*. **1999**, 386, 41-46.

Lister, R. G. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology.* **1987**, 92,180-185.

Marder, M.; Paladini, A. C. $GABA_A$-Receptor Ligands of Flavonoid Structure. *Curr Top Med Chem*. **2002**, 2, 853- 867.

Mattei, R.; Leite, J. R.; Tufik, S. A study of the pharmacological actions of *Dioclea grandiflora* Martius ex. Bentham. *Sao Paulo Med. J.* **1995**, 113 (1), 687-691.

Miguel, C.; Maria, G. C.; Maria D. C.; Thereza C. M. L.; António, P. C. Assessment of luteolin (3′,4′,5,7-tetrahydroxyflavone) neuropharmacological activity. *Behav Brain Res*. **2008,** 189 (1), 75-82.

Njung'e, K.; Handley, S. L. Evaluation of marble-burying behavior as a model of anxiety. *Pharmacol. Biochem. Behav*. **1991,** 38, 63-67.

Speroni, E.; Minghetti, A. Neuropharmacological activity of extract from *Passiflora incarnate. Planta Med*. **1988,** 54, 488-491.

Treit, D.; Pinel, J. P.; Fibiger, H. C. Conditioned defensive burying: a new paradigm for the study of anxiolitic agents. *Pharmacol. Biochem. Behav*. **1981**, 15 (4), 619– 626.

Wolfman, C.; Viola, H.; Paladini, A; Dajas, F.; Medina, J.H. Possible anxiolitic effects of Chrysin, a Central benzodiazepine Receptor Ligand isolated from *Passiflora coerulea*. *Pharmacol. Biochem. Behav*. **1994**, 47, 1-4.