Evaluation of effects of dichloromethane fraction from *Platonia insignis* on pilocarpine-induced seizures

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**Abstract:** The objective of present study was to evaluate the antioxidant and anticonvulsant activities of dichloromethane fraction (DMF) from *Platonia insignis* Mart., Clusiaceae. The DMF from *P. insignis* (2 mg/kg) was tested by intraperitoneal (i.p.) to evaluate effects on lipid peroxidation level, nitrite formation, as well as on locomotor and anticonvulsant activities. Wistar rats were treated with, (saline/Tween 80 0.5%, i.p., control group), DMF (2 mg/kg, i.p., DMF group), pilocarpine (400 mg/kg, i.p., P400 group), or the combination of DMF (2 mg/kg, i.p.) and pilocarpine (400 mg/kg, i.p., DMF plus P400). After the treatments all groups were observed for 24 h. In P400 group rats there was a decrease in the motor activity when compared with control group. In DMF plus P400 co-administered rats was observed an increase in motor activity when compared with P400 group. In P400 group rats there was a significant increase in lipid peroxidation and nitrite levels. In DMF plus P400 co-administered rats, antioxidant treatment significantly reduced the lipid peroxidation level and nitrite content after seizures. Previous findings strongly support the hypothesis that oxidative stress occurs in rat striatum during pilocarpine-induced seizures, and our results imply that strong neuroprotective effect on this brain region could be achieved using DMF from *P. insignis*.

**Keywords:** lipid peroxidation, nitrite, pilocarpine, *P. insignis*, seizures

**Introduction**

Medicinal plants play a key role not only in self-medication but also in medical practice of the wild world. The power of the chemical and biochemical techniques in studying such plants has led to discovery of important agents for treatment of long-last diseases. These discoveries emphasize the pharmaceutical potential of the medicinal plants (Bezerra et al., 2008).

The term epilepsy is collectively designated for a group of chronic central nervous system disorders characterized by spontaneous occurrence of seizures generally associated with the loss of consciousness and body movements of convulsions (Chauhan et al., 1988). The search for antiepileptic compounds with more selective activity and lower toxicity continues to be an area of intensive investigation in medicinal chemistry (Malawska, 2005). Various phytochemical and pharmacological studies have been carried out on these anticonvulsant plants (Quintans Júnior et al., 2008a).

*Platonia insignis* Mart., Clusiaceae, commonly known as “bacuri”, is a thick-skinned fruit, with approximate dimension of an orange, which contains a large quantity of resins. The pulp enclosing the seeds is white, bittersweet, with a pleasant smell and taste. The fruit can be consumed raw or in the form of juice, ice-cream or jam (Alves & Jennings, 1979). A number of xanthone have been isolated from plants belonging to this family (Ollis et al., 1965).

Many new polyisoprenylated benzophenones with a bicycle-[3.3.1]-nonane-2,4,9-trione core structure have been isolated from plants in the Clusiaceae family, and their potent biological properties have been the subject of several studies. This review summarizes the biological activities reported for these secondary metabolites including cytotoxic, antimicrobial, antioxidant, and...
fractionated using polarity increasing solvents. The extract was added 100 mL of water, which was then Soxhlet apparatus (8 h for each solvent). In the ethanol (63%, w/w), followed by 95% ethanol (5.8%, w/w) in a 55 °C under shade and powdered mechanically. Crushed fruits were collected from the fruits of *Platonia insignis* (voucher No.: ICN TEPB 27.164). The seeds of Biology Department of Federal University of Piauí, and deposited at the “Graziela Barroso”, Herbarium March 2009. A voucher specimen has been identified fruits were collected at Barras, Piauí State, Brazil, in the month of March 2009. The fats form the *P. insignis* seeds (bacuri fat) are yellowish solid rich in triacylglycerols and fatty acid (Bentes et al., 1986). The composition of bacuri fat, the seed fat of *P. insignis* and found that its chief component acids are palmitic and oleic acid, with smaller proportions of stearic and palmitoleic acids and probably traces of myristic, arachidic, and linoleic acids.

Omega-3 and omega-6 polyunsaturated fatty acids (PUFA) are dietary fatty acids that are involved in a myriad of physiologic processes in the brain. There is some evidence suggesting that PUFA and particularly omega-3 PUFA may have anticonvulsant effects, both in humans and animals (Taha et al., 2010).

Natural xanthones have been reported in genera *Calophyllum*, *Platonia*, *Symphonia*, and *Kielmeyera* (Jackson et al., 1966). A series of xanthone derivatives have shown pronounced anticonvulsant activity (Marona et al., 2001; Marona et al., 2008). The aim of present study was to examine the effects of DMF from *P. insignis* on lipid peroxidation level and nitrite formation in rat striatum, as well as research their anticonvulsant activity in adult rats prior to pilocarpine-induced seizures.

**Materials and methods**

**Drugs**

The drugs used pilocarpine hydrochloride, trichloroacetic acid, thiobarbituric acid, sodium nitrite, and polyoxyethylene-sorbitan monolated (Twee 80) were purchased from Sigma (USA). Agents were administrated by intraperitoneally (*i.p.*) route at a dose volume of 0.1 mL/10 g.

**Plant material and preparation of DMF from *P. insignis***

The *Platonia insignis* Mart., Clusiaceae, fruits were collected at Barras, Piauí State, Brazil, in March 2009. A voucher specimen has been identified and deposited at the “Graziela Barroso”, Herbarium of Biology Department of Federal University of Piauí, Brazil (Voucher No.: ICN TEPB 27.164). The seeds collected from the fruits of *P. insignis* were dried at 55 °C under shade and powdered mechanically. Crushed seeds yielded 848 g was extracted with n-hexane (63%, w/w), followed by 95% ethanol (5.8%, w/w) in a Soxhlet apparatus (8 h for each solvent). In the ethanol extract it was added 100 mL of water, which was then fractionated using polarity increasing solvents. The ethanol extract was fractionated with dichloromethane (8 x 100 mL) to obtain a dichloromethane soluble fraction. The fraction was concentrated in a vacuum evaporator. The concentrated extract was finally freeze-dried to get the yield of 3.4% of DMF. The dried extract was kept at 4 °C in refrigerator in the air tight bottles until use.

**Behavioral effects and locomotor activity**

Behavioral screening of the rats was performed following parameters described by Almeida et al. (1999) and animals were observed at 24 h after *i.p.* administration of DMF of *P. insignis* (2 mg/kg, *i.p.*). During 24 h were...
observed the occurrence of the following general signs (piloerection, prostration, writhing, evacuation, grooming, dyspnea, sedation, analgesia and palpebral ptosis).

Rats were divided into four groups of seven animals each. Vehicle received saline/Tween 80 0.5% (control group) and the tested groups were administered with DMF (2 mg/kg, i.p.). The spontaneous locomotor activity of the animals was assessed in a cage activity (50 cm × 50 cm × 50 cm) after 24 h of treatment (Asakura et al., 1993).

**Determination of lipid peroxidation level in striatum of adult rats pretreated with DMF from P. insignis prior to pilocarpine-induced seizures**

For all experimental procedures, 10% (w/v) homogenates of the area of the brain investigated were prepared for all groups. Lipid peroxidation levels in the DMF plus P400 group (n=6), P400 group (n=6), DMF group (n=6) and control animal (n=9) were analyzed by measuring the thiobarbituric-acid-reacting substances in homogenates (Draper & Hadley, 1990). Briefly, the homogenates were mixed with 1 mL 10% trichloroacetic acid and 1 mL 0.67% thiobarbituric acid, and were heated in a boiling water bath for 15 min, and then butanol (2:1, v/v) was added to the solution. After centrifugation (800 x g, 5 min), TBARS determinations were performed spectrophotometrically at 535 nm and expressed as nmol of malondialdehyde (MDA)/g wet tissue.

**Determination of nitrite content in striatum of adult rats pretreated with DMF from P. insignis prior to pilocarpine-induced seizures**

To determine nitrite contents of control group (n=9), DMF plus P400 group (n=6), P400 group (n=6) and DMF group (n=6), the 10% (w/v) homogenates were centrifuged (800 x g, 10 min). The supernatants were collected, and nitric oxide production was determined based on the Griess reaction (Green et al., 1981). Briefly, 100 µL supernatant was incubated with 100 µL of the Griess reagent at room temperature for 10 min. Nitrite concentration was determined from a standard nitrite curve generated using NaNO₂. Nitrite determinations were performed spectrophotometrically at 550 nm using a microplate reader and expressed as nM.

**Statistical analysis**

Results of latency to first seizure, locomotor activity and neurochemical alterations were compared by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test (p<0.05) (Graphpad program Intuitive, Software for Science, San Diego, CA). The number of animals that seized and the number that survived were calculated as percentages (seizures percentage and survival percentage, respectively), and compared with a nonparametric test (χ²).

**Results**

**Behavioral alterations after pretreatment with DMF from P. insignis prior to pilocarpine-induced seizures**

Pilocarpine induced the first seizure at 35.00±0.70 min. All the animals studied showed generalized tonic-clonic convulsions with SE, and 30% survived the seizures. All animals pretreated with DMF from P. insignis were observed for 24 h before pilocarpine injection and their manifested alterations in behavior, such as peripheral cholinergic signs (100%), tremors (50%), staring spells, facial automatisms, wet dog shakes, rearing and motor seizures (100%) developed progressively within 1-2 h into a long-lasting SE (100%). Table 1 shows that DMF (2 mg/kg) administration before pilocarpine treatment reduced by 70% the percentage of animals that seized (p<0.0001), increased latency (341%) to the first seizure (154.21±1.54 min) (p<0.0001) and increased (50%) the survival (p<0.0001), when compared to the pilocarpine only group. None of the control animals (vehicle or dichloromethane fraction) showed seizures (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Latency to first seizures (min)</th>
<th>Percentage seizures</th>
<th>Percentage survival</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>00</td>
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<tr>
<td>P400</td>
<td>35.00±0.70</td>
<td>100</td>
<td>30</td>
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<tr>
<td>DMF + P400</td>
<td>154.21±1.54</td>
<td>30°</td>
<td>80°</td>
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<tr>
<td>DMF</td>
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Male rats (250-280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg, i.p., n=12, P400), DMF group with DMF from P. insignis (2 mg/kg, i.p., n=12) and the control animals with 0.9% saline/Tween 0.5% (i.p., n=12). The DMF plus pilocarpine group was treated with DMF (2 mg/kg, i.p.) and 30 min before of administration received pilocarpine (400 mg/kg, i.p., n=12, DMF + P400). Results for latency to first seizure are expressed as mean ± S.E.M of the number of experiments shown in the table. Result for percentage seizures and percentage survival are expressed as percentages of the number of animals from each experimental group (n=12 per group). The differences in experimental groups were determined by Analysis of Variance. *p<0.0001 as compared with P400 group (χ²-test). *p<0.0001 as compared with DMF+P400 group (χ²-test). *p<0.0001 as compared with P400 group (ANOVA and Student–Newman–Keuls as post hoc test).
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**Locomotor activity after pretreatment with DMF from *P. insignis* prior to pilocarpine-induced seizures**

In P400 group was observed significant decreases of 9 and 21% of number of crossings and rearing, when compared to vehicle group, respectively. In dose of 2 mg/kg of DMF caused significant increase of 11 and 7% of ambulation and rearing, when compared to P400 group, respectively. In addition, the pretreatment with DMF, 30 min before administration of pilocarpine increased number of crossings (48%, *p*<0.001) and rearing (49%, *p*<0.001) when compared to the P400 group (Figure 1). On the other hand, none of the control animals (vehicle or dichloromethane fraction) showed alterations in number of crossings and rearing (Figure 1).

**Figure 1.** Effect of DMF from *P. insignis* on number of crossings and rearing of adult rats prior to pilocarpine-induced seizures. Male rats (250-280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg, *i.p.*, *n*=6, P400), DMF group with DMF from *P. insignis* (2 mg/kg, *i.p.*, *n*=6, DMF group) and the control animals with vehicle (saline/Tween 80 0.5%, *i.p.*, *n*=9, control group). The DMF + P400 group was treated with DMF (2 mg/kg, *i.p.*) and 30 min before of administration received pilocarpine (400 mg/kg, *i.p.*, *n*=6, DMF + P400). Results are expressed as mean ± S.E.M of the number of experiments shown in parenthesis. The differences in experimental groups were determined by Analysis of Variance (ANOVA). *a* *p*<0.0001 as compared with vehicle group (ANOVA and Student-Newman-Keuls as post hoc test). *b* *p*<0.0001 as compared with P400 group (ANOVA and Student-Newman-Keuls as post hoc test).

**Lipid peroxidation level and nitrite content in striatum of adult rats pretreated with DMF from *P. insignis* prior to pilocarpine-induced seizures**

Effects of DMF from *P. insignis* in lipid peroxidation and nitrite concentrations during seizures induced by pilocarpine are presented in Figure 2. Lipid peroxidation was markedly increased of in pilocarpine group in comparison with the corresponding values of the vehicle group. During acute phase of seizures induced by pilocarpine a significant increase (89%) in thiobarbituric-acid-reacting substances (*p*<0.0001) was observed. Seizures induced by pilocarpine produced a significant increase in striatal nitrite content (94%, *p*<0.0001, Figure. 2). Rats pretreated with DMF showed decrease in lipid peroxidation level (48%, *p*<0.001) and nitrite content (49%, *p*<0.001) when compared with the pilocarpine group (Figure 2). In addition, the pretreatment with DMF, 30 min before administration of pilocarpine also reduced lipid peroxidation level (94%, *p*<0.0001) and nitrite content (51%, *p*<0.05) when compared to the vehicle group (Figure 2). On the other hand, none of the control animals (vehicle or dichloromethane fraction) showed changes in lipid peroxidation level and nitrite content (Figure 2).

**Figure 2.** Effects of DMF on status of lipid peroxidation level and nitrite content in striatum of adult rats after seizures induced by pilocarpine. Male rats (250-280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg/kg, *i.p.*, *n*=6, P400), DMF group with DMF from *P. insignis* (2 mg/kg, *i.p.*, *n*=6, DMF group) and the control animals with vehicle (saline/Tween 80 0.5%, *i.p.*, *n*=9, control group). The DMF + P400 group was treated with DMF (2 mg/kg, *i.p.*) and 30 min before of administration received pilocarpine (400 mg/kg, *i.p.*, *n*=6, DMF + P400). Results are expressed as mean ± S.E.M of the number of experiments shown in parenthesis. The differences in experimental groups were determined by Analysis of Variance (ANOVA). *a* *p*<0.0001 as compared with vehicle (ANOVA and Student-Newman-Keuls as post hoc test). *b* *p*<0.0001 as compared with P400 group (ANOVA and Student-Newman-Keuls as post hoc test).
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Discussion

In folk medicine of the Brazilian Northeast P. insignis seed oil’s is used for treatment of eczemas, herpes, and diarrheas (Agra et al., 2007). In pharmacological behavioral screening, the animals treated with DMF of P. insignis showed increase of response to touches and increasing of motor activity. These data are indicative of stimulatory activity of the CNS (Almeida et al., 1999).

The possible CNS antioxidant and anticonvulsant activities of ethanol extract (Costa Júnior et al., 2010) and garcinielliptone FC (Costa Júnior et al., 2011) from P. insignis were investigated in vitro assays and in animal models. In present study we investigated effects of DMF from P. insignis in animal models. The mice treated with DMF presented behavioral alterations, such increased ambulation, palpebral ptosis, and stimulation. These behavioral changes suggest a possible effect on CNS; however, they are different to drugs that reduce the CNS activity (Morais et al., 2004; Netto et al., 2009).

Previous studies using low doses of ethanol extract and fractions from P. insignis demonstrated pharmacological activity in central nervous system, our experiments only with one dose of DMF present results anticonvulsant and antioxidant for this fraction. However, we need to investigate the same dose of DMF, as has central activity in other tests in vitro and in animal models of seizures (pentylenetetrazol and picrotoxin) to clarify the possible action mechanism of this fraction.

Dichloromethane fraction of P. insignis at the highest dose caused a significant increase of ambulation in the test of spontaneous movement after 24 h in the dose of 2 mg/kg, corroborating with the hypothesis that DMF of P. insignis did not reduce the CNS activity. Our data suggest that DMF may be a stimulatory to the CNS, since studies shows that reduction of the ambulation of the animals is characteristic of depressant drugs (Carlini, 2003; Freire et al., 2006; Leite et al., 2008; Quintans Júnior et al., 2008b).

The increase of the locomotor activity was observed and it can be due to either through a stimulatory effect of the DMF of P. insignis on CNS or by absence of muscular relaxant activity in the periphery system. Our results indicate that DMF could possess a stimulator activity.

The molecular observations of epilepsy include the temporal correlation between free radical generation and the development of seizures in some pathological conditions, and the protective efficacy of antioxidant treatments against some types of seizures. P. insignis, one of the effective antioxidant, not only has antioxidant functions, but also has functions in pro-oxidant (Hosni et al., 2010; Lenta et al., 2007). Previous studies indicated that P. insignis has antioxidant activity in several animal models (Wu et al., 2005; Wu et al., 2008; Iinuma et al., 1996; Gustafson et al., 1992). The effects of DMF of P. insignis leaves in CNS have not yet been determined, therefore, it would be important to conduct these studies to clarify its brain action mechanism in pilocarpine-induced seizures. In this study, we demonstrated a role of DMF from P. insignis against lipid peroxidation and nitrite formation produced by pilocarpine-induced seizures.

In the present study we investigated the influence of DMF from P. insignis on the level of lipid peroxidation and nitrite content in the rat striatum during pilocarpine-induced seizures. Generation of reactive oxygen species is currently viewed as one of the process through which epileptic activity exert their deleterious effects on brain (Rauca et al., 2004). These reactive oxygen species in the absence of an efficient defense mechanism cause peroxidation of membrane polyunsaturated fatty acids (Castagne et al., 1999). Brain is particularly susceptible to peroxidation due to simultaneous presence of high levels of polyunsaturated fatty acids and iron (Halliwell & Gutteridge, 1999) which are the targets of free radical damage (Gottlieb et al., 2006; Halliwell & Gutteridge, 1989). We showed the lipid peroxidation was rising in striatum homogenate of rats after 24 h of acute phase of seizures. The increase of lipid peroxidation was reflected by the rise of thiobarbituric-acid-reacting substances level which may be related to its intermediate free radicals formed during pilocarpine-induced seizures.

Literature has shown that pilocarpine-induced seizures led to changes in nitric oxide metabolism, and increased the production of its metabolites (nitrite and nitrate). The increased metabolites may interact with glutamatergic receptors to produce part of its stimulatory action on the central nervous system (Maczurek et al., 2008; Michiels et al., 1994). The reduction in nitrite content, after pretreatment with DMF from P. insignis, is most readily explained as a consequences of radical formation inhibiting, scavenges reactive oxygen species and lipid peroxidation products (Tejada et al., 2006).

Herein, we clearly showed that DMF from P. insignis decreased the frequency of pilocarpine-induced seizures and increased the survival rate. In our knowledge, these effects of DMF on lipid peroxidation and nitrite formation observed during acute phases of pilocarpine-induced seizures have not been reported before. Thus, these findings might have important implications for understanding the mechanism of epilepsy to promote new advances in the development of selective and targeted antiepileptic drugs. DMF from P. insignis might protect the striatum against neuronal damages regularly observed during seizures.

Our results confirm data previously reported in the literature that demonstrate anticonvulsant activity of ethanol extract of Hypericum perforatum in mice belonging to the same family of plant evaluated in this study (Hosseinzadeh et al., 2005; Vyawahare et al.,...
2007). Further investigations of effects of DMF from *P. insignis* against necrosis, apoptosis and/or autophagy observed during the acute phase of this epilepsy model are in progress to confirm its neuroprotective effects.

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