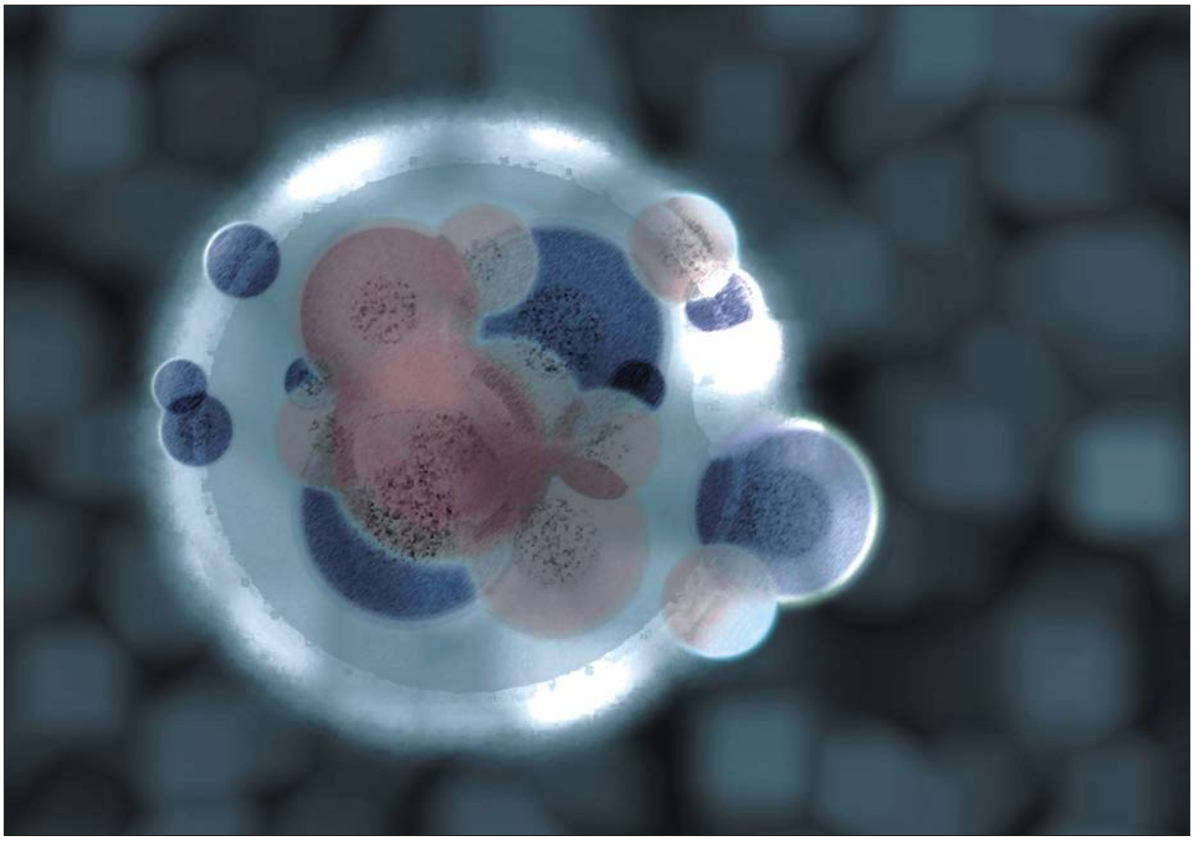

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Evaluation of Effects of Ethanolic Extract from *Platonia insignis* Mart. on Pilocarpine-induced Seizures

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Abstract: The objective of the present study was to evaluate the Central Nervous System (CNS) antioxidant and anticonvulsant activities of Ethanolic Extract (EE) from *P. insignis* in animal models. The EE from *P. insignis* (10 mg kg^{-1}) was tested by intraperitoneal (i.p.) to evaluate effects on lipid peroxidation level, nitrite formation, as well as on locomotor activity by the open-field test and anticonvulsant activity against acute pilocarpine-induced seizures. Wistar rats were treated with, 0.9% saline (i.p., control group), EE (10 mg kg^{-1}, i.p., EE group), pilocarpine (400 mg kg^{-1}, i.p., P400 group), or the combination of EE (10 mg kg^{-1}, i.p.) and pilocarpine (400 mg kg^{-1}, i.p.). After the treatments all groups were observed for 24 h. The lipid peroxidation and nitrite concentrations were measured using spectrophotometric methods. In P400 group rats there was a significant decrease in the motor activity when compared with control group. In EE and pilocarpine co-administered rats was observed a significant increase in motor activity when compared with P400 group, up to 24 h after the administration. In P400 group rats there was a significant increase in lipid peroxidation and nitrite levels. In EE and pilocarpine co-administered rats, antioxidant treatment significantly reduced the lipid peroxidation level and nitrite content after seizures. Present findings strongly support the hypothesis that oxidative stress occurs in striatum during pilocarpine-induced seizures, indicate that brain damage induced by the oxidative process plays a crucial role in seizures pathogenic consequences, and imply that strong protective effect on SNC could be achieved using EE from *P. insignis*.

Key words: Striatum, ethanolic extract, *P. insignis*, lipid peroxidation, nitrite

INTRODUCTION

Epilepsy is one of the most commons serious neurological disorders characterized by recurrent seizures (Nikalje *et al*., 2011; Somani *et al*., 2010). 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-Junior *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 (Boulanger *et al*., 1999). A number of xanthone have been isolated from plants belonging to this family (De Moura *et al*., 2008; Peres *et al*., 2000).

Many new polyisoprenylated benzophenones with a bicyclo (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 anti-inflammatory activities (Acuna *et al*., 2009). The

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polycyclic polyprenylated acylphloroglucinols exhibit a wide variety of biological activities such as antimicrobial, antidepressant, antioxidant, cytotoxic, and antiviral activities (Ciochina and Grossman, 2006).

The fats form the *P. insignis* seeds (bacuri fat) are yellowish solid rich in triacylglycerols and fatty acid (Monteiro *et al*., 1997). 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 (PUFAs) are dietary fatty acids that are involved in a myriad of physiologic processes in the brain. There is some evidence suggesting that PUFAs- and particularly omega-3 PUFAs-may have anticonvulsant effects, both in humans and in animals (Taha *et al*., 2010).

Natural xanthones have been reported in genera *Calophyllum, Platonia, Symphonia,* and *Kielmeyera* (Harborne *et al*., 1999; Peres *et al*., 2000). A series of derivatives of xanthone produced pronounced anticonvulsant activity (Marona *et al*., 2008; Marona *et al*., 2001). The aim of present study was to examine the effects of Ethanolic Extract (EE) from *P. insignis* in evaluate effects 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 were pilocarpine hydrochloride and polyoxyethylene-sorbitan monolated (Tween 80) purchased from Sigma (USA) and Diazepam (DZP) from Cristália (Brazil). Agents were administrated by intraperitoneally (i.p.) route at a dose volume of 0.1 mL 10 g^{-1}.

Plant material and preparation of ethanolic extract of *P. insignis*: The fruits of *P. insignis* were collected at Barras, Piaui State, Brazil, in March 2009. The seeds collected from the fruits of *P. insignis* were dried at 55°C under shade and powdered mechanically. Eight hundred and forty eight grams of crush yielded of seeds was extracted with hexane (63%, w/w), followed by ethanol (EtOH) (5.8%, w/w) in a Soxhlet apparatus (8 h for each solvent). The extract was concentrated in a vacuum evaporator.The concentrated extract was finally freeze-dried to get the yield of 1.8% of ethanolic extract. The dried extract was kept at 4°C in refrigerator in the air tight bottles until use.

Animals and treatment protocols: Adult male Wistar rats (250-280 g) were maintained in a temperature controlled room (26+1°C) with a 12 h light/dark cycle and food and water *ad libitum* (Nutrilabor, Campinas, Brazil). All experiments were performed according to the guide for the care and use of laboratory the US Department of Health and Human Services, Washington, DC from 2009 to 2010. A total of 96 rats were treated with either 10 mg kg^{-1} Ethanolic Extract (EE) from *P. insignis* (i.p., EE) or vehicle (saline/Tween 80 0.5%, i.p.). 30 min after the treatments rats from each above group were randomized to pilocarpine hybrochloride administration (400 mg kg^{-1}, i.p., P400). Thus there are 4 groups of rats in this set of experiments: group 1, EE and P400 co-administration (n = 24); group 2, P400 plus saline treatment (n = 24); group 3, EE alone administration (n = 24); and group 4, vehicle treatment serves as control (n = 24). After the treatments, the animals were recorded in 30×30 cm chambers with: latency to first seizure (any one of the behavioral indices typically observed after pilocarpine administration: wild running, clonuses, tonus, clonic-tonic seizures), number of animals that died after pilocarpine administration. Previous work has shown that convulsions and deaths occurred within 1 and 24 h respectively post pilocarpine injection, so we decided to record the phenotypes of the animals for 24 h after pilocarpine administration. At the end of observations, the survivors were killed by decapitation and their brains were dissected on ice to remove striatum for determinations of lipid peroxidation level and nitrite content. The pilocarpine administration rat group was constituted by those presented seizures, Status Epilepticus (SE) for over 30 min and non-phenotype survivors.

The drug dosages of pilocarpine (400 mg kg^{-1}) and EE (10 mg kg^{-1}) were determined by previous study in our lab (Barros *et al*., 2007; Xavier *et al*., 2007) and the present study (data not shown). The drug doses used in this present study are not equivalent to those used by humans because rats have different metabolic rates.

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 EE of *P. insignis* (10 mg kg^{-1}, i.p.). During 24 h were observed the occurrence of the following general signs of toxicity: piloerection, prostration, writhing, increased evacuation, grooming, discrete groups, dyspnea, sedation, analgesia and palpebral ptosis.

Rats were divided into four groups of 7 animals each. Vehicle received saline/Tween 80 0.5% (control group) and the tested groups were administered with EE (10 mg kg^{-1}, i.p.). The spontaneous locomotor activities of the animals such as ambulation (number of crossings) and rearing were assessed in a cage activity (50×50 ×50 cm) after 24 h of treatment (Asakura *et al*., 1993).

Determinations of lipid peroxidation level in striatum of adult rats pretreated with Ethanolic Extract (EE) 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 EE plus P400 group (n = 6), P400 group (n = 6), EE group (n = 6) and control animal (n = 9) were analyzed by measuring the thiobarbituric-acid-reacting substances in homogenates (Draper and 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 (800x g, 5 min), TBARS determinations were performed spectrophotometrically at 535 nm and expressed as nmol of malondialdehyde (MDA)/g wet tissue.

Determinations of nitrite content in striatum of adult rats pretreated with Ethanolic Extract (EE) from *P. insignis* prior to pilocarpine-induced seizures: To determine nitrite contents of control group (n = 9), EE plus P400 group (n = 6), P400 group (n = 6) and EE group (n = 6), the 10% (w/v) homogenates were centrifuged (800x 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. A550 was measured using a microplate reader. Nitrite concentration was determined from a standard nitrite curve generated using $NaNO_2$. The results above were 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 (χ^2).

RESULTS

Behavioral alterations after pretreatment with Ethanolic Extract (EE) 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 Status Epilepticus (SE), and 30% survived the seizures. All animals pretreated with Ethanolic Extract (EE) 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 (30%) developed progressively within 1-2 h into a long-lasting SE (30%). Table 1 shows that EE (10 mg kg^{-1}) 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 ethanolic extract) showed seizures (Table 1).

Locomotor activity after pretreatment with Ethanolic Extract (EE) from *P. insignis* prior to pilocarpine-induced seizures: In P400 group was observed significant decreases of 19 and 22% of ambulation (number of crossings) and rearing at 24 h after administration, when compared to control group, respectively. In dose of 10 mg kg^{-1} of ethanolic extract caused significant increase of 21 and 35% of ambulation and rearing at 24 h after administration, when compared to P400 group, respectively (Fig. 1).

Lipid peroxidation level and nitrite content in striatum of adult rats pretreated with Ethanolic Extract (EE) from *P. insignis* prior to pilocarpine-induced seizures: Effects of Ethanolic Extract (EE) from *P. insignis* in lipid peroxidation and nitrite concentrations during seizures induced by pilocarpine are presented in Fig. 2. Lipid peroxidation was markedly increased in pilocarpine group in comparison with the corresponding values of the saline group. During acute phase of seizures induced

Table 1: Effect of pretreatment with Ethanolic Extract (EE) from *Platonia insignis* prior to pilocarpine-induced seizures and lethality in adult rats

Groups	Latency to first seizures	(min)Percentage seizures	Percentage survival	Number of animals/group
Pilocarpine	35.00+0.70	100	30	12
EE plus pilocarpine	154.21+1.54[b]	30[a]	80[a]	12
EE	00	00	100[a]	12

Male rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg kg^{-1}, intraperitoneal, i.p., n = 14), EE group with Ethanolic Extract (EE) from *P. insignis* (10 mg kg^{-1}, i.p., n = 14) and the control animals with 0.9% saline (i.p., n = 14). The EE plus pilocarpine group was treated with EE (10 mg kg^{-1}, i.p.) and 30 min before of administration received pilocarpine (400 mg kg^{-1}, i.p., n = 14). 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. The differences in experimental groups were determined by analysis of variance. [a]$p<0.0001$ as compared with pilocarpine group (χ^2-test). [b]$p<0.0001$ as compared with EE plus pilocarpine group (χ^2-test). [c]$p<0.0001$ as compared with pilocarpine group (ANOVA and Student-Newman-Keuls test).

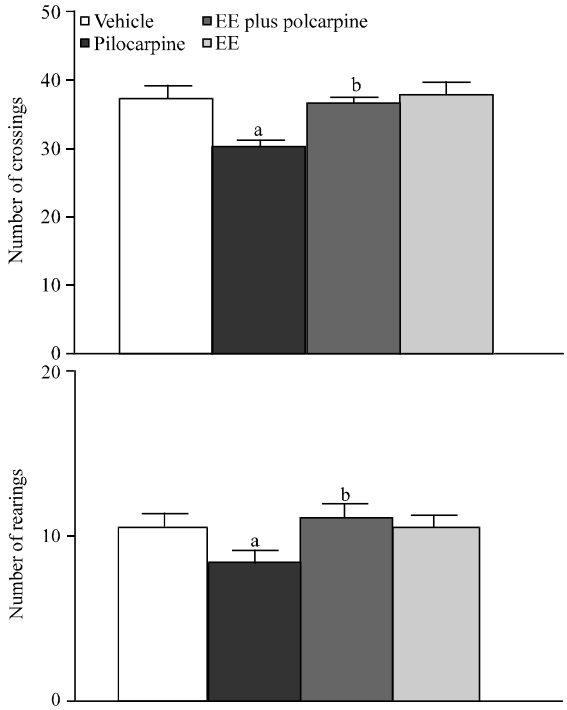

Fig. 1: Effect of Ethanolic Extract (EE) from *P. insignis* in adult rats prior to pilocarpine-induced seizures on performance (number of crossings) in the open field task. Male rats (250–280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg kg^{-1}, intraperitoneal, i.p., n = 12), EE group with Ethanolic Extract (EE) from *P. insignis* (10 mg kg^{-1}, i.p., n = 6) and the control animals with 0.9% saline (i.p., n = 6). The EE plus pilocarpine group was treated with EE (10 mg kg^{-1}, i.p.) and 30 min before of administration received pilocarpine (400 mg kg^{-1}, i.p., n = 12). Results for latency to first seizure are expressed as Mean±SEM 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.The differences in experimental groups were determined by analysis of variance. $^{a}p<0.0001$ as compared with control group (ANOVA and Student–Newman–Keuls test). $^{b}p<0.0001$ as compared with pilocarpine group (ANOVA and Student–Newman–Keuls test)

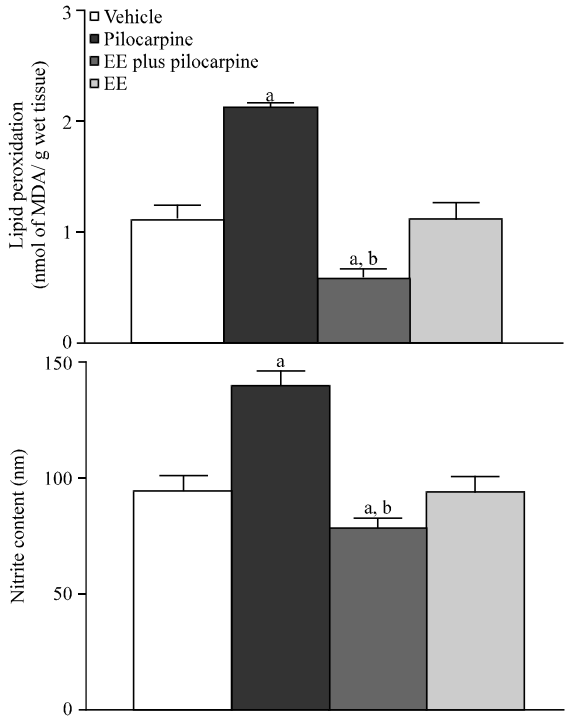

Fig. 2: Effects of Ethanolic Extract (EE) from *P. insignis* on status of lipid peroxidation level and nitrite content in striatum of adult rats prior to seizures induced by pilocarpine. Male rats (250-280 g, 2 months old) were treated with a single dose of pilocarpine (400 mg kg^{-1}, intraperitoneal, i.p., n = 6, P400), EE group with ethanolic extract from *P. insignis* (10 mg kg^{-1}, i.p., n = 6, EE group) and the control animals with vehicle (i.p., n = 9, Control). The TP plus pilocarpine group was treated with EE from *P. insignis* (10 mg kg^{-1}, i.p.) for 30 min prior to pilocarpine injection (400 mg kg^{-1}, i.p., n = 6, EE plus P400). Results are expressed as Means±SEM for the number of animals shown inside in parenthesis. Differences in experimental groups were determined by two-tailed analysis of variance. $^{a}p<0.05$ as compared to control animals (t-Student-Neuman-Keuls test); $^{b}p<0.05$ as compared to P400 group (t-Student-Neuman-Keuls test)

by pilocarpine a significant increase (25%) in thiobarbituric-acid-reacting substances ($p<0.0001$) was observed. Seizures induced by pilocarpine produced a significant increase in striatal nitrite content (48%, $p<0.0001$, Fig. 1). Rats pretreated with EE showed decrease in lipid peroxidation level (72%, $p<0.0001$) and nitrite content (54%, $p<0.0001$) when to compared with the pilocarpine group (Fig. 1). In addition, the pretreatment with EE, 30 min before administration of pilocarpine also

reduced lipid peroxidation level (48%, $p<0.0001$) and nitrite content (32%, $p<0.005$) when compared to the control group (Fig. 1). On the other hand, none of the control animals (vehicle or ethanolic extract) showed alterations in lipid peroxidation level and nitrite content (Fig. 2).

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 EE of *Platonia insignis* showed increase of response to touches and increasing of motor activity. These data are indicative of stimulator activity of the CNS (Almeida *et al*., 1999).

The possible CNS antioxidant and anticonvulsant activities of EE from *P. insignis* were investigated in animal models. The mice treated with EE presented behavioral alterations, such increased of the 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).

EE 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 10 mg kg^{-1}, corroborating with the hypothesis that EE of *P. insignis* did not reduce the CNS activity. Our data suggest that EE 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 *et al*., 2008b).

The increase of the locomotor activity was observed and it can be due to either through a stimulatory effect of the EE of *P. insignis* on CNS or by absence of muscular relaxant activity in the periphery system. Our results indicate that EE 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 (Gustafson *et al*., 1992; Iinuma *et al*., 1996; Wu *et al*., 2008, 2005). The effects of EE 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 EE from *P. insignis* against lipid peroxidation and nitrite formation produced by pilocarpine-induced seizures.

In the present study we investigated the influence of ethanolic extract 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 and Gutteridge, 1999) which are the targets of free radical damage (Gottlieb *et al*., 2006; Halliwell and 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 ethanolic extract 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 ethanolic extract from *P. insignis* decreased the frequency of pilocarpine-induced seizures and increased the survival rate. In our knowledge, these effects of ethanolic extract 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. Ethanolic extract 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 ethanolic extracts of *Hypericum perforatum* in mice belonging to the same genus of plant evaluated in this

study (Hosseinzadeh *et al*., 2005; Vyawahare *et al*., 2007). Further investigations of effects of ethanolic extract 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.

CONCLUSION

Ethanolic extract from *P. insignis* pretreatment significantly reduced the lipid peroxidation level and nitrite content after pilocarpine-induced seizures. Our findings strongly support the hypothesis that oxidative stress occurs in striatum during pilocarpine-induced seizures, indicate that brain damage induced by the oxidative process plays a crucial role in seizures pathogenic consequences, and imply that strong protective effect on SNC could be achieved using ethanolic extract from *P. insignis*.

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