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Behavioral and neurochemical studies in mice pretreated with garcinielliptone FC in pilocarpine-induced seizures

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ABSTRACT

Garcinielliptone FC (GFC) isolated from hexanic fraction seed extract of species *Platonia insignis* Mart. It is widely used in folk medicine to treat skin diseases in both humans and animals as well as the seed decoction has been used to treat diarrheas and inflammatory diseases. However, there is no research on GFC effects in the central nervous system of rodents. The present study aimed to evaluate the GFC effects at doses of 25, 50 or 75 mg/kg on seizure parameters to determine their anticonvulsant activity and its effects on amino acid (γ-aminobutyric acid (GABA), glutamine, aspartate and glutathione) levels as well as on acetylcholinesterase (AChE) activity in mice hippocampus after seizures. GFC produced an increased latency to first seizure, at doses 25 mg/kg (20.12 ± 2.20 min), 50 mg/kg (20.95 ± 2.21 min) or 75 mg/kg (23.43 ± 1.99 min) when compared with seized mice. In addition, GABA content of mice hippocampus treated with GFC75 plus P400 showed an increase of 46.90% when compared with seized mice. In aspartate, glutamine and glutamate levels detected a decrease of 5.21%, 13.55% and 21.80%, respectively in mice hippocampus treated with GFC75 plus P400 when compared with seized mice. Hippocampus mice treated with GFC75 plus P400 showed an increase in AChE activity (63.30%) when compared with seized mice. The results indicate that GFC can exert anticonvulsant activity and reduce the frequency of installation of pilocarpine-induced *status epilepticus*, as demonstrated by increase in latency to first seizure and decrease in mortality rate of animals. In conclusion, our data suggest that GFC may influence in epileptogenesis and promote anticonvulsant actions in pilocarpine model by modulating the GABA and glutamate contents and of AChE activity in seized mice hippocampus. This compound may be useful to produce neuronal protection and it can be considered as an anticonvulsant agent.

1. Introduction

Epilepsy is a disorder in brain function characterized by recurrent and unpredictable seizures. The prevalence in general population is approximately 1% (Stahl, 2010), affecting at least 50 million people worldwide (Goldenberg, 2010). The central nervous system (CNS) has high amino acid concentrations that bind to the postsynaptic receptors, receptors γ-amino butyric acid (GABA), which is involved in excitatory action and inhibitory action, and glutamate, which is involved in excitatory action, are considered the most important neuroactive amino acids (Golan et al., 2009; Goodman and Gilman, 2012). In current context, it is important to evaluate the effects of natural products on participation of these and other amino acids in neurotransmission systems in epilepsy models.

In recent years, evidence showed that natural products used as folk remedies have contributed significantly for the discovery of modern medicines worldwide (Costa Junior et al., 2011). In particular, studies with species *Platonia insignis* Mart have demonstrated special interest in evaluating regional flora and its safety and efficacy in preclinical trials (Santos et al., 2013). In addition, many herbal medicines can be active on CNS, and have at least a hypothetical potential for chronic conditions such as epilepsy that does not respond well to conventional treatments (Quintans et al., 2007; Campelo et al., 2010; Marques et al., 2011).

This work proposes to study a compound isolated from species *P. insignis*, popularly known in Brazil as "bacuri". Belonging to the family Clusiaceae, *P. insignis* is popularly used as antidepressants (Bilia et al., 2002; Viana et al., 2005), being equivalent to fluoxetine and imipramine,

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with reduced adverse effects (De Vry et al., 1999). This compound corresponds to a polyprenylated acylphloroglucinol benzophenone which has a core (diphenyl methanone) substituted groups isoprenylates (3-methyl-2-butenila) and 2-isopropenyl-hex-5-enyl. It shows a pattern of triple one oxygenation of aromatic rings of core diphenylmethanone accompanied by additional intramolecular cyclization, which yields a bicyclic system of nine carbon atoms. The compound is termed garcinielliptone FC [C38H50O6; (8,8-dimethyl-1-(3,4-dihidroxibenzoil)-2-hydroxy-3,5-di(γ,γ-dimethylallyl)-7-(2-isopropenilhex-5-enyl)-7α-H-trans-bicyclo [3.3.1]nona-2-en-4,9-dione)] (Fig. 1).

In the study of pilocarpine-induced seizures in rodents, several plants have shown promising results. Therefore, there is a growing need to research for possible pharmacological properties of plants in search of new therapies showing efficacy, no serious adverse effects and low cost. Within this perspective, several research groups have been studying the anticonvulsant potential of natural compounds in pilocarpine-induce seizures. Over the years, the pilocarpine model has been widely used since the 80s as it replicates the phenomenological features of human temporal lobe epilepsy (Turski et al., 1983, 1989).

Therefore, the present study aimed to evaluate the GFC effects on seized parameters to investigate anticonvulsant activity and its effects on amino acids (γ-aminobutyric acid (GABA), glutamine, aspartate and glutathione) levels and of acetylcholinesterase enzyme (AChE) activity in mice hippocampus after pilocarpine-induced seizures, to provide literature data, particularly in area of epilepsy.

2. Materials and methods

2.1. Extraction and isolation

The seeds were dried at 55 °C and powdered. After crush 848.2 was extracted with hexane (63%, w/w). The hexane extract was subjected to silica gel and eluted with *n*-hexane containing increased amounts of ethanol extract (EtOAc) and washed with methanol at process end. The resultant hexane extract yields 51 subfractions. The fraction 33 was further purified on thin layer chromatography (TLC) plates and eluted with chloroform–methanol ($CHCl_3$–MeOH) (9:1) to yield GFC (22 mg) thus being identified by spectroscopic methods (Costa Junior et al., 2011).

2.2. Animals

Mice (*Mus musculus*) of *Swiss* strain, males and females, weighing between 25 and 35 g, from Central Animal Facility of Agricultural Sciences Center, Federal University of Piaui, were used in experiments. The animals received water and diet ad libitum and were maintained under controlled lighting (cycle 12 h light/dark) and temperature (25 ± 2 °C). The project was approved by Ethics Committee in Animal Experimentation of Federal University of Piaui (072/2012).

2.3. Drug treatment

The GFC was emulsified with 0.05% Tween 80 (Sigma, USA) and dissolved in 0.9% saline vehicle. The animals were pretreated with pilocarpine at dose 400 mg/kg intraperitoneally (i.p.) emulsified in vehicle, with GFC at doses 25, 50 and 75 mg/kg orally (o.r.) during the evaluation of anticonvulsant activity and 30 min later, GFC pretreated animals received pilocarpine at dose 400 mg/kg. The dose of 75 mg/kg was selected for analysis of action mechanism, therefore, showed a greater increase in seizure latency when compared to other doses ($p < 0.05$).

Various toxicity studies were performed to ensure the use of this compound. With regard to acute toxicity, the 50% lethal dose (LD_{50}) was determined orally in mice and rats, which resulted in deaths at doses greater than 5.0 g/kg, ensuring the use of doses 25, 50 and 75 mg/kg (data not shown).

2.4. Experimental protocol

Mice were divided into five groups (n = 10, per groups). The first group was used as control and received the vehicle (0.05% Tween 80 dissolved in 0.9% saline; vehicle group) and the second group was treated with pilocarpine hydrochloride (400 mg/kg, i.p.; P400 groups). The remaining groups were treated with GFC at doses of 25, 50 and 75 mg/kg (GFC25, GFC50 and GFC75, groups, respectively). Thirty minutes after GFC administration, mice were treated with pilocarpine (i.p.) at dose of 400 mg/kg (GFC25 plus P400, GFC50 plus P400 and GFC75 plus P400 groups, respectively). Behavioral changes were observed during 1 h. The observed parameters were a number of peripheral cholinergic, tremors, stereotyped movements (SM), seizures, status epileptic (SE), and mortality rate. The SE was defined as continuous seizures during a

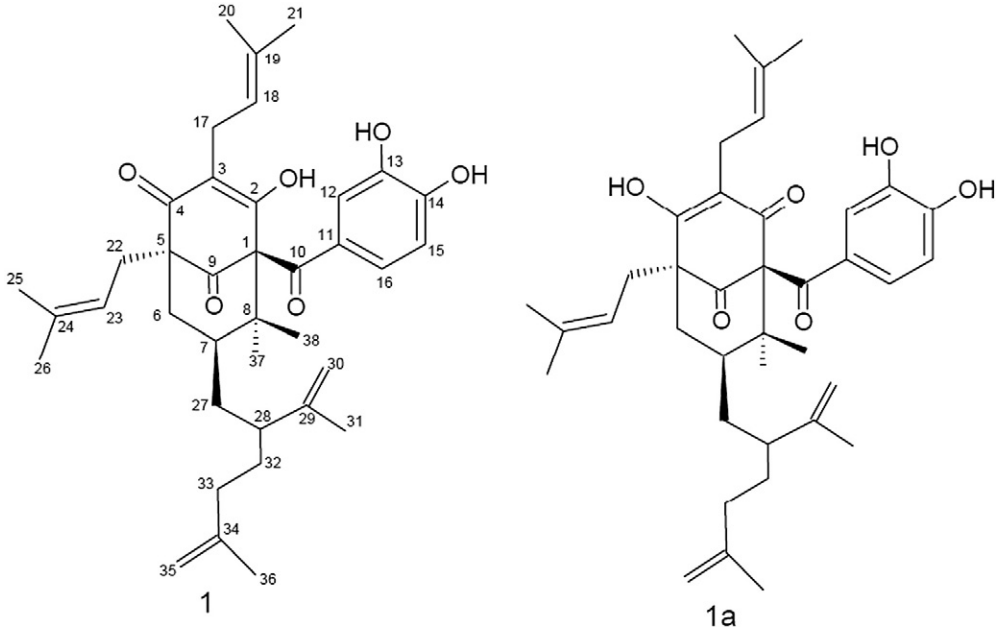

Fig. 1. Garcinielliptone FC (1/1a) isolated from seeds of *P. insignis* (8,8-dimethyl-1-(3,4-dihydroxybenzoyl)-4-hydroxy-3,5-di(δ,δ-dimethylallyl)-7-(2-isopropenylhex-5-enyl)-7α-H-trans-bicyclo [3.3.1]nona-3-en-2,9-dione (1a)).

Table 1
Behavioral alterations in mice treated with garcinielliptone FC (GFC) in pilocarpine-induced seizures.

Groups	Cholinergic reactions (%)	Tremors (%)	Stereotyped movements (%)	*Status epilepticus* (%)	Survival rate (%)
P400	100	100	100	100	00
DZP 5 plus P400	100	100	100	50*	50*
GFC25 plus P400	100	100	13.75*	32.5*	32.5*
GFC50 plus P400	100	100	18.75*	63.75*,**	65*,**
GFC75 plus P400	100	100	15.75*	85*,**,***	90.1*,**,***

Mice (25–30 g, 2 months-old) were treated with a single dose of pilocarpine (400 mg/kg, i.p., n = 10). Animals were acutely pretreated, with garcinielliptone FC (GFC) at doses of 25, 50 or 75 mg/kg (o.r.) and 30 min after receiving pilocarpine 400 mg/kg (P400, i.p.; n = 10). Diazepam 5 mg/kg (DZP, positive control, o.r.) and 30 min after receiving P400 (i.p.; n = 10). Results for cholinergic reaction, tremors, stereotyped movements, *status epilepticus* and survival rat were expressed as percentage of number of animals in each experimental group.
* $p < 0.05$, when compared with P400 group;
** $p < 0.05$, when compared with GFC25 plus P400 group;
*** $p < 0.05$ when compared with GFC50 plus P400 group.

period longer than 30 min. SE was induced by method of Turski et al. (1983). Mortality rate was determined 1 h after pilocarpine-induced SE.

In another step of experiment, 30 min after treatment with vehicle, GFC75 mg/kg and GFC75 plus P400, mice (n = 10, per groups) were observed for at least 1 h to detect installation latency of first seizure, number of animals that had status epileptic and number of animals that died after administration of pilocarpine (P400).

After 24 h of observation, animals were euthanized and their brains removed for dissection of hippocampus to measure the neurochemical changes in amino acid levels and AChE activity to evaluate the GFC effect on these neurotransmitter systems. γ-Aminobutyric acid (GABA), glutamine, glutathione and aspartate levels as well as acetylcholinesterase (AChE) enzymatic activity in mice hippocampus were studied in 10% homogenates prepared in sodium phosphate buffer (150 mmol/L, pH 7.4). The protein concentration was measured according to the method described by Lowry et al. (1951) as well as acetylcholinesterase (AChE) activity was measured by the method of Ellman et al. (1961). Results were expressed as μmol per g tissue for amino acid levels and the AChE activity was expressed in μmol acetylcholine hydrolyzed/mg protein/min.

2.5. Behavioral study

Previously, another study showed that *status epileptic* and deaths occurred between 1 and 24 h, respectively, after injection of pilocarpine (Turski et al., 1983). All experimental groups of GFC were observed after treatment, according to experimental protocols, during 24 h observation.

The following parameters observed were a number of peripheral cholinergic signs, tremors, stereotyped (miosis, piloerection, chromodacryorrhea, diarrhea and masticatory automatisms), continuous sniffing, paw licking, rearing and wet dog shakes that persisted for 10–15 min, clonic movements of forelimb, head bobbing and tremors. These behavioral changes progressed to motor limbic *status epilepticus* as previously described by Turski et al. (1983). Limbic seizures persisted

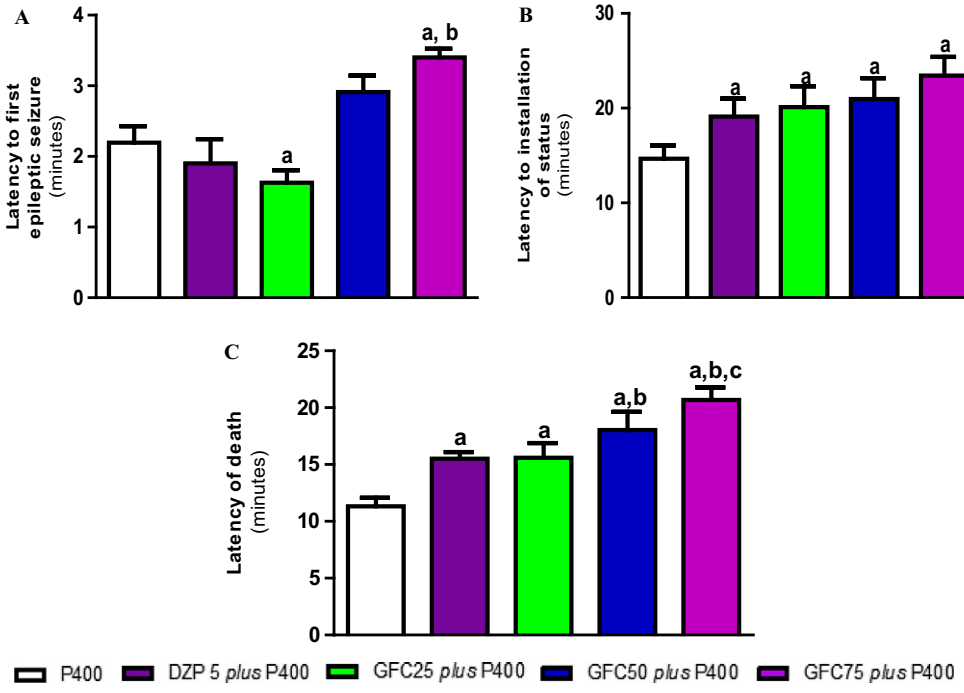

Fig. 2. Latency to first seizure (2A), latency to installation of *status epilepticus* (2B), and latency of death (2C) in mice pretreated with garcinielliptone FC (GFC) after pilocarpine-induced seizures. The animals were pre-treated with pilocarpine 400 mg/kg, (n = 10, i.p., P400 group), or GFC at doses 25, 50 and 75 mg/kg, (n = 10, o.r.) and 30 min after received pilocarpine 400 mg/kg (P400, i.p.; n = 10), or diazepam 5 mg/kg (DZP 5, positive control, o.r.) and 30 min after receiving P400 (i.p.; n = 10) and or vehicle (0.05% Tween 80 dissolved in 0.9% saline, i.p., n = 10, control group). The group GFC25, 50 or 75 mg/kg plus P400 group were pre-treated with GFC at doses 25, 50 and 75 mg/kg for 30 min before with pilocarpine (400 mg/kg). The differences in experimental groups were determined by analysis of variance (ANOVA) followed by *t*-Student–Newman–Keuls post hoc test. [a]$p < 0.05$ when compared with P400 group, [b]$p < 0.05$ when compared with DZP 5 plus P400 group, [c]$p < 0.05$ when compared with GFC25 plus P400 group. GFC = garcinielliptone FC; P400 = pilocarpine.

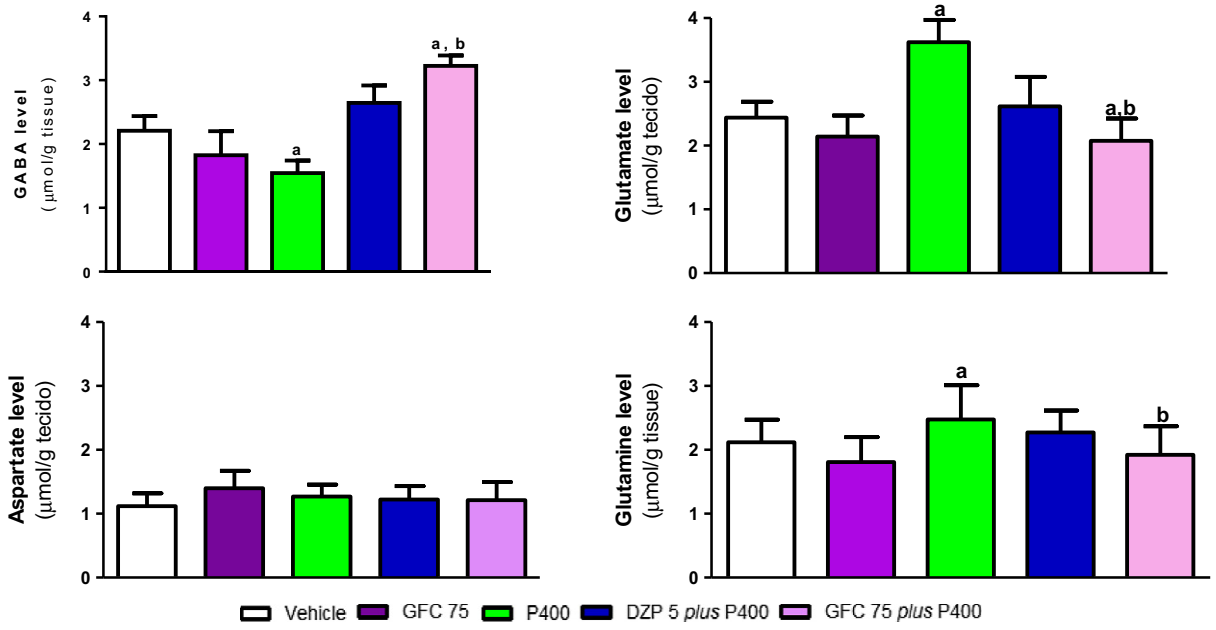

Fig. 3. Garcinielliptone FC (GFC) effects on amino acids level in mice hippocampus after pilocarpine-induced seizures. The animals were pretreated with pilocarpine 400 mg/kg, (n = 8, i.p., P400 group), or GFC at dose 75 mg/kg, (n = 8, o.r.) and 30 min after received pilocarpine 400 mg/kg (P400, i.p.; n = 8), or diazepam 5 mg/kg (DZP 5, positive control, o.r.) and 30 min after received P400 (i.p.; n = 8) and or vehicle (0.05% Tween 80 dissolved in 0.9% saline, i.p., n = 8, control group). The group GFC75 plus group were pre-treated with GFC75 mg/kg, 30 min before with P400. The differences in experimental groups were determined by analysis of variance (ANOVA) followed by *t*-Student–Newman–Keuls post hoc test. [a]p < 0.05 when compared with vehicle group; [b]p < 0.05, when compared with P400 group. GFC = Garcinielliptone FC; P400 = Pilocarpine.

for 30–50 min evolving to SE in all mice. During the behavioral study, all animals died.

2.6. Statistical analysis

Results are expressed as means ± SD for the number of experiments. The *t*-Student–Newman–Keuls test was used for multiple comparisons of means of two groups of data whose differences were considered statistically significant at $p < 0.05$. Differences in experimental groups were determined by analysis of variance (ANOVA).

3. Results

3.1. Behavioral study of mice pretreated with garcinielliptone FC and pilocarpine

All animals treated with pilocarpine (400 mg/kg, i.p., n = 10; P400 groups), which presented after 3–10 min the peripheral cholinergic signs (PCS) (miosis, piloerection, chromodacryorrhea, diarrhea and masticatory automatisms), stereotyped movements (SM) (increased activity of biting, scratching, chewing, rearing and/or wet-dog shakes) and tremors that progressed to seizures and *status epilepticus* (SE). The mortality rate was 100% in this group (Table 1).

Pilocarpine induced the first seizure after 2.2 ± 0.24 min. According to Fig. 2, GFC caused an increase in latency to the first epileptic seizure induced by P400, at doses 25 mg/kg (1.63 ± 0.17 s), 50 mg/kg (2.92 ± 0.23 s) and 75 mg/kg (3.40 ± 0.13 s), of 25.90%, 43.37% and 87.95% when compared with P400 group, ($p < 0.05$), respectively. GFC produced an increased latency for development of pilocarpine-induced *status epilepticus* at doses 25 mg/kg (20.12 ± 2.20 min), 50 mg/kg (20.95 ± 2,21 min) and 75 mg/kg (23.43 ± 1.99 min), of 37.34%, 43.00% and 59.93% when compared with P400 group ($p < 0.05$), respectively. Furthermore, the GFC produced an increased latency to death induced by pilocarpine at doses 25 mg/kg (15.60 ± 1.28 min), 50 mg/kg (18.04 ± 1.60 min) and 75 mg/kg (20.67 ± 1.10 min) of 37.68%, 59.22% and 82.43% when compared with P400 group ($p < 0.05$), respectively (Fig. 2). Similarly, DZP 5 plus P400 showed an increase latency for development of pilocarpine-induced *status epilepticus* as well as in latency to death induced by pilocarpine when compared with vehicle. On the other hand, GFC75 plus 75 detected an increase in latency to death induced by pilocarpine when compared with DZP 5 plus P400 ($p < 0.05$).

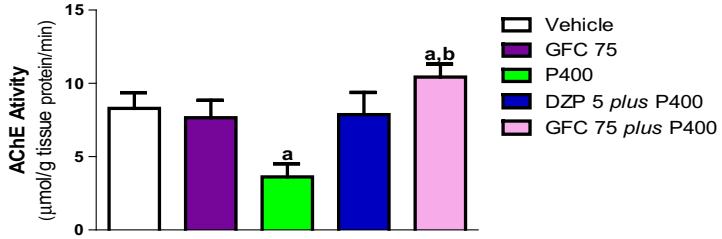

Fig. 4. GFC effects on acetylcholinesterase enzyme (AChE) activity in mice hippocampus after pilocarpine-induced seizures. The animals were pretreated with pilocarpine 400 mg/kg, (n = 8, i.p., P400 group), or GFC at dose 75 mg/kg, (n = 8, o.r.) and 30 min after received pilocarpine 400 mg/kg (P400, i.p.; n = 8), or diazepam 5 mg/kg (DZP 5, positive control, o.r.) and 30 min after received P400 (i.p.; n = 8) and or vehicle (0.05% Tween 80 dissolved in 0.9% saline, i.p., n = 8, control group). The group GFC75 plus group were pre-treated with GFC75 mg/kg, 30 min before with P400. The differences in experimental groups were determined by analysis of variance (ANOVA) followed by *t*-Student–Newman–Keuls post hoc test. [a]p < 0.05 when compared with vehicle group; [b]p < 0.05, when compared with P400 group. GFC = Garcinielliptone FC; P400 = Pilocarpine.

The GABA level in mice hippocampus during the acute phase of seizures, which is related to regulation of inhibitory neurotransmission in seizures and *status epilepticus*, was altered in GFC plus P400 groups when compared with P400 group ($p < 0.05$) as there was an increase in GABA concentration 52.01% in mice pretreated with GFC at dose of 75 mg/kg (GFC75 plus P400 group) when compared with P400 group ($p < 0.05$) (Fig. 3).

In amino acid levels (glutamate and glutamine), during the acute phase of seizures, there were decreases of 13.55% and 21.80% in glutamine and glutamate levels in mice hippocampus pretreated with GFC at dose 75 mg/kg and after 30 min from receiving pilocarpine 400 mg/kg (GFC75 plus P400) when compared with P400 group, respectively ($p < 0.05$) (Fig. 3). On the other hand, there were no changes in aspartate level in mice hippocampus of all groups when compared with vehicle ($p > 0.05$). In addition, none of amino acid studies verified changes in GFC75 and DZP 5 plus P400 ($p > 0.05$).

Mice pretreated with GFC75 and received pilocarpine 400 mg/kg (GFC75 plus P400) 30 min after showed an increase of 65.3% in AChE activity when compared with P400 group ($p < 0.05$, Fig. 4). On the other hand, there were no changes in enzymatic activity in DZP 5 plus P400 and GFC75 groups when compared with vehicle ($p > 0.05$). In addition, the group treated with pilocarpine 400 mg/kg (P400) showed a decrease in AChE activity when compared with vehicle ($p < 0.05$, Fig. 4).

4. Discussion

In animal epilepsy models, several plants have shown anticonvulsant effects. Among these studies in plants that can be highlighted, *Hypericum perforatum* L. (Rego et al., 2007), *Hypericum grandifolium* Choisy (Sánchez-Mateo et al., 2009), *Hypericum caprifoliatum* Cham. & Schltdl (Viana et al., 2005), *Abrus precatorius* L., *Clausena anisata* Willd and *Hoslundia opposita* Vahl (Moshi et al., 2005), *Citrus limon* (Campelo et al., 2011) demonstrated satisfactory results in epilepsy model by pilocarpine-induced seizures. It is important to mention that many medicinal plants have activities on CNS and they have at least a hypothetical potential to affect chronic conditions such as anxiety, depression, headaches or epilepsy that does not respond well to conventional treatments (Rego et al., 2007; Pedersen et al., 2009). Studies have shown that the use of plants help to expand the therapeutic arsenal in this area and reduces costs with available treatments (Sarmento et al., 2014; Marques et al., 2014), reinforcing the need for completion of this study and justifying the importance of the evaluation of new compounds isolated from natural products.

Our results suggest that GFC can exert anticonvulsant activity against and reduce the number of animal presented pilocarpine-induced *status epilepticus*, as demonstrated by an increase in the latency of onset of seizures and decrease in mortality rate of animals. Previous data showed that increase in free radicals content during seizures leads to an increase in glutamate concentration and consequently there is the facilitation to the spread of seizures and neuronal damages in various brain regions (Barros et al., 2007; Kersante et al., 2013). Recently, our research group demonstrated that GFC has antioxidant effect against nitric oxide (NO) and hydroxyl radicals *generated* in vitro and that the ethanolic extract of seeds of *P. insignis*, species from which GFC was isolated, was able to promote an increased latency for development of picrotoxin- and pentylenetetrazol-induced seizures (Costa Junior et al., 2011).

The data found regarding behavioral changes in rodents after pilocarpine-induced seizures, are in accordance with observations previously described in other studies (Marinho et al., 1998; Santos et al., 2008; Freitas, 2009; Santos et al., 2011). In the present study, it was demonstrated that oral administration at GFC at doses 25, 50 and 75 mg/kg reduced the number of animals that showed seizures and *status epilepticus* pilocarpine-induced, and also reduced the mortality rate. The results suggest that GFC may have an important anticonvulsant action in pilocarpine-induced seizures, probably by potentiation or increase of hippocampal inhibitory neurotransmission.

Once associated with pilocarpine-induced seizures, it has been reported previously that the an increase in GABA concentration after *status epilepticus* and a decrease after 24 h of acute phase of seizures (Freitas et al., 2011) can occur in this epilepsy model. Benzodiazepines such as diazepam and clonazepam do not interfere with any of behavioral parameters observed in mice, suggesting that the GABAergic system does not interact with peripheral effects mediated by pilocarpine. In turn, the GFC demonstrated efficacy in reducing the number of animals that presented seizures and evolved to *status epilepticus*, and also decreased the mortality rate in mice treated with GFC. Reinforcing the hypothesis about the possible action mechanism of GFC can be directly related with GABAergic system.

Excitatory amino acids such as glutamate and aspartate are involved in generation and expression of epileptic seizures in mammalian brain (Kersante et al., 2013). After its interaction with the NMDA-subtype of glutamate receptor, glutamate induces the Ca^{2+} influx, increasing the neuronal nitric oxide synthase (nNOS) activity and nitric oxide (NO) production which may contribute to neuronal damage (Tome et al., 2010). As noted previously GFC was able to reduce NO levels in in vitro experimental models, which may contribute to the anticonvulsant effect observed in vivo in the present study. On the other hand, inhibitory amino acids such as GABA and glycine counteract the neuronal excitation. GABA, acting through the $GABA_A$ receptor, increases the chloride influx, leading to hyperpolarization of cell membrane and antagonism of epileptic seizures. Thus, our results suggest that the anticonvulsant effects of the GFC may be mediated by modulation of the levels correlated with mediators of excitatory and inhibitory neurotransmitter systems.

The involvement of cholinergic mechanisms of pilocarpine is well-established in epilepsy (Turski et al., 1989). Acetylcholinesterase (AChE) activity has a pivotal role in cholinergic neurotransmission and hydrolysis of neurotransmitter acetylcholine to terminate the transmission of nerve impulses. The lack of results in AChE activity probably owing abnormally high of acetylcholine levels at cholinergic synapses can induce seizures. The accumulation of acetylcholine results in excessive stimulation of muscarinic and nicotinic receptors. Signs of poisoning are seen as increased salivation, difficulty breathing, tremors, seizures and death. Increased cholinergic activity in brain is probably related to the initial phase of *status epilepticus* (McDonough and Shih, 1997; Fernandes, 2013). The cholinergic activity produced by GFC is supposedly supported by reduction in seizures and mortality rates. In addition, in correspondence with this hypothesis, the GFC increases the latency to the first seizure and installation latency to *status epilepticus* which may enhance its probable mechanism of action anticonvulsant.

5. Conclusion

This study is extremely relevant since the data presented in this report with respect to effects on the central nervous system in the pilocarpine-induced seizures in mice model is being presented for the first time in the literature regarding the subject of this study.

The results show that GFC decreases the frequency of pilocarpine-induced seizures and increases survival rate. According to a literature review, these GFC effects in mortality rate observed during the acute phase of pilocarpine-induced seizures were not previously registered. Thus, these findings may have important implications for understanding the pathophysiological epilepsy mechanisms in order to promote new advances in the development of antiepileptic drugs. The GFC also protected mice against *status epilepticus* observed during pilocarpine-induced seizures. Due to its prominent neuropharmacological effects observed in our experiments during the acute phase of seizures, new neurochemical studies with GFC are underway to elucidate the action mechanism and justify the use of this natural compound as a potential therapeutic agent.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.pbb.2014.05.021.

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