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3. Anticonvulsant effects of *p*-cymene in mice after pilocarpine-induced seizures

Talita Mendes de Oliveira[1], Rusbene Bruno Fonseca de Carvalho[1,2]
Adriana da Rocha Tomé[3], Thiago Henrique Costa Marques[1,4]
Rivelilson Mendes de Freitas[1,4] and Sidney Gonçalo de Lima[1,2]

[1]Laboratório de Pesquisa em Neuroquímica Experimental do Programa de Pós-graduação em Farmacologia do Centro de Ciências da Saúde da UFPI. Campus Ministro Petrônio Portela 64.049-550, Teresina, Piauí, Brasil; [2]Departamento de Química, Campus Ministro Petrônio Portela, 64.049-550, Teresina, Piauí, Brasil; [3]Universidade Estadual do Ceará, Campus Itaperi Av Paranjana 1700 Itaperi, Fortaleza, 60740-000, Ceará, Brasil; [4]Programa de Pós-graduação em Biotecnologia/RENORBIO. Campus Ministro Petrônio Portela, 64.049-550, Teresina, Piauí Brasil

Abstract. This experiment was performed to investigate the anticonvulsant-like effects of *p*-cymene (CYM), a natural hydrocarbon that is a component of essential oils, ie, natural products extracted from vegetable materials, by using experimental paradigms of convulsions in comparison with a known anticonvulsant, phenobarbital. CYM at doses of 50, 100 or 150 mg/kg promoted a reduction of 25, 50 and 75%, respectively, against pilocarpine-induced seizures, and it was efficacious in increasing both the latency to first seizures and the survival percentage, resulting in 25, 50 and 87.5% of protection against death induced by seizures, respectively. The reference drug phenobarbital (50 mg/kg) also produced a significant protection (50%), respectively. Its monoterpene, at 50, 100 or 150 mg/kg, was

Correspondence/Reprint request: Dr. Rivelilson Mendes de Freitas, Laboratório de Pesquisa em Neuroquímica Experimental/Campus Ministro Petrônio Portela da Universidade Federal do Piauío 64.049550 Teresina, Piauí, Brasil. E-mail: rivelilson@pq.cnpq.br

also capable to increase the latency for installation of status epilepticus induced by pilocarpine. Additionally, it was observed that the CYM pretreatment increased the acetylcholinesterase activity in mice hippocampus after pilocarpine-induced seizures. The present results clearly indicate the anticonvulsant ability of CYM, which can be, at least in part, explained by the increased activity of the acetylcholinesterase enzyme. It is concluded that *p*-cymene may have anticonvulsant-like effects in the pilocarpine-induced seizures, and these effects may be mediated by GABAergic transmission.

Introduction

The aim of this chapter is to describe several studies which have attempted to measure/detect effects of compound natural (*p*-cymene) on behavioral alterations and neuronal damage in hippocampus and striatum of rodents in epilepsy model induced by pilocarpine. Epilepsy is a disease characterized by a wide range of symptoms resulting from a variety of cerebral disorders. Semiologically, it is classified as partial or generalized. The clinical manifestations can arise through sensitive, sensory, psychological, vegetative and motor signs and symptoms of simple or complex nature, depending on the neural system implicated in the genesis of the disease. Epilepsy crises have a recurrent nature and tend to always present with the same characteristics over long periods. Epilepsy is not a single disease but represents a variety of diseases with underlying cerebral dysfunctions that may have different causes. Thus, a priori, it comprises a heterogenous clinical entity [1].

It has been estimated that 50 million people worldwide suffer from epilepsy. Partial epilepsy is the most common form, occurring in around 60% of epilepsy patients. Up to 30% of epilepsy patients will not achieve adequate control over the disease [2].

It is important to differentiate convulsions from epileptic seizures. Convulsions signify transitory occurrences of signs and symptoms resulting from synchronic or excessively abnormal neuronal activity in the brain, triggered by convulsogenic factors. These include metabolic disorders involving glucose, electrolytes, increased temperature or cranial-encephalic trauma, among others. On the other hand, epilepsy is a cerebral disorder with long-lasting predisposition towards generating epileptic crises, which lead to neurobiological, cognitive, psychological and social consequences. Status epilepticus is a severe form of clinical presentation of epileptic crises and is characterized by long duration. Depending on the type of crisis, it may have high morbidity-mortality [1].

Antiepileptic drugs are the initial treatment for the great majority of epilepsy patients. For 150 years, physicians have been prescribing

antiepileptic drugs to patients with recent diagnoses of epilepsy, without any formal scientific evaluation regarding the efficacy, safety and tolerability of such drugs. For example, phenobarbital and phenytoin were registered and put on the market without any randomized clinical trial having been conducted to evaluate their efficacy and safety [3]. Indication of a specific drug for a particular condition of epilepsy is based on clinical studies with varying levels of evidence, many without sufficient methodological quality. Drugs are chosen with regard not only to the data on the clinical trials available, but also to variables such as the type of epilepsy, the patient's age and the supposed mechanism of action of the drug.

The action of antiepileptic drugs occurs through several mechanisms. Although a considerable number of antiepileptic drugs are available for the treatment of epilepsy there is still an urgent need for development of new drugs as alternatives [4].

The essential oils are natural products that exhibit a variety of biological properties, such as analgesic [5], anticonvulsant [6] and anxiolytic [7] and [8]. Those effects are attributed to the monoterpenes which are the major chemical components of these essential oils. For instance, the monoterpene cyano-carvone, has been reported to have anticonvulsant activity in mice. Similarly, cyano-carvone presented significant increases in the latency of pilocarpine-induced seizures [9].

p-Cymene (1-isopropyl-4-methylbenzene; Figure 1) is a natural hydrocarbon that is a component of essential oils, ie, natural products extracted from vegetable materials. For example, the essential oils obtained from oregano and thyme, traditional Mediterranean spices, are characterized by a very high content of monoterpenes such as *p*-cymene (trace to 52%), γ-terpinene (2-52%), thymol (trace to 64%), and carvacrol (1-80%) [10,11].

However, *p*-cymene occurs in the oils of many other gymnospermic and angiospermic plants. It is metabolized by the liver and the major *p*-cymene metabolite is cuminyl alcohol [12]. Furthermore, formation of *p*-cymene as a

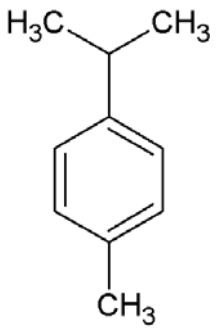

Figure 1. Chemical structure of *p*-cymene (1-isopropyl-4-methylbenzene).

dead-end metabolite from certain nonaromatic monoterpenes has been observed in anoxic enrichment cultures [13,14] and degradation has been also demonstrated with anaerobic and aerobic bacteria [15].

Some of the pharmacological actions of *p*-cymene have been studied [16-18]. According to previous studies, the antimicrobial effect of monoterpenes such *p*-cymene may result, partially at least, from a gross perturbation of the lipid fraction of the plasmic membrane of the microorganism [19,20].

Thus, the objective of the present study was to determine the anticonvulsant activity of *p*-cymene, as well as their effects on neuronal damage in hippocampus and striatum of mice in epilepsy model induced by pilocarpine.

1. Behavioral alterations in epilepsy model

Status epilepticus is a medical and neurological emergency. Overall, mortality is 17 to 26% [21-23]. An additional 10 to 23% of patients who survive status epilepticus are left with new or disabling neurological deficits [22,24]. Traditionally, status epilepticus is defined as continuous or repetitive seizure activity persisting for at least 30 minutes without recovery of consciousness between attacks. More recently, authors have suggested that seizures exceeding 5 to 11 minutes should be considered status epilepticus [25,26]. For all practical purposes, a patient should be considered to be in status epilepticus if a seizure persists for more than 5 minutes, as very few single seizures will last this long.

Several types of status epilepticus exist. Clinically, the most important distinction to make is between convulsive and nonconvulsive status epilepticus, based on whether or not rhythmic jerking of the extremities is observed. Typically, patients who present with generalized convulsive status epilepticus are expected to awaken gradually after the motor features of seizures disappear. If the level of consciousness does not improve by 20 minutes after cessation of movements, or the mental status remains abnormal 30 to 60 minutes after the convulsions cease, nonconvulsive status epilepticus must be considered and urgent electroencephalogram is advised.

In a study by De Lorenzo and colleagues [27], 14% of patients treated successfully for convulsive status epilepticus were in nonconvulsive status epilepticus when electroencephalogram was begun; of the patients who underwent continuous electroencephalogram monitoring after convulsive status epilepticus was controlled, 48% had nonconvulsive seizures.

Status epilepticus is an emergency situation which requires prompt medical treatment. If it is severe permanent brain damage or death has to be

prevented with pretreatment with natural compounds. Status epilepticus often occurs in individuals with a history of seizures, in whom there are neuronal substrates already predisposed towards supporting seizure activity.

The pilocarpine model is an useful animal experimental to investigate the development of acute, silent and chronic phases [28]. Immediately after pilocarpine administration, all animals persistently presented behavioral changes, including initial akinesia, ataxic lurching, peripheral cholinergic signs (miosis, piloerection, chromodacryorrhea, diarrhea and masticatory automatisms), stereotyped movements (continuous sniffing, paw licking, rearing and wet dog shakes that persisted for 10-15 min), clonic movements of forelimbs, head bobbing and tremors.

These behavioral changes progressed to motor limbic seizures as previously described by Tursky and colleagues [29]. Limbic seizures lasted for 7-15 min evolving to status epilepticus for a period longer than 30 min. During 1 h of acute phase of seizures, no case of fatality was observed between the adult mice. However, during the 24 h observation of this phase, 100% of adult animals died [9,30-32]. Similarly, these results for the behavioral alterations in pilocarpine model were described in previously studies [9,33].

According to our previous studies [9,32], few minutes after pilocarpine administration, the animals exhibited stereotyped oral and masticatory movements, hypokinesia, salivation, tremor and partial or generalized limbic seizures. Approximately 30 min after pilocarpine injection, the seizures evolved to status epilepticus lasting 12-18 h. During this period, 80% of animals died due to status epilepticus. This acute phase was followed by a silent period varying from 4 to 44 days (mean of 15 days) during which the animals displayed normal behavior.

A chronic period of spontaneous and recurrent seizures (SRS) (3-4 seizures/week) was also observed and all animals which survived status epilepticus, displayed the chronic phase. During the interictal period, there were no behavioral alterations in the animals.

In epilepsy model, pilocarpine induced the first seizure to occur at 7.83 ± 0.27 min. All the mice that received pilocarpine injection (at a dose 400 mg/kg, i.p.) presented generalized tonic-clonic convulsions with status epilepticus, and no animal survived the seizures [9]. In the present study, we have demonstrated that the systemic administration of *p*-cymene can completely abolish pilocarpine-induced seizures. This was evident from concurrent behavioral changes consistent with anticonvulsant state. This was also reflected by elicited alterations in the oxidative stress and acetylcholinesterase activity after pilocarpine-induced seizures. Considering

that, *p*-cymene was effective in preventing tonic convulsions induced by pilocarpine this compound could be useful in grand mal epilepsy.

2. *p*-Cymene effects on behavioral alterations in epilepsy model

Status epilepticus is a neurological emergency that requires prompt diagnosis and treatment, as delay is associated with a higher likelihood of poor response to treatment and worse outcome. Generalized convulsive status epilepticus is one of the most common medical emergencies in clinical practice. Status epilepticus occurs not only in people with epilepsy but also in the context of other neurological disorders and systemic illness. The annual incidence in the UK is about 9000-14000 new cases per year [35].

Status epilepticus accounts for 3.5% of admissions to emergency departments in the developed nations and for 11% in a developing country [36]. Prompt recognition and treatment are required to prevent associated complications. However, prospective randomised trials regarding the treatment are few. Formulation of newer anticonvulsants is limited to the oral route and hence could not be widely used in the initial management of status epilepticus. Hence, there is a substantial need to improve measures for both prevention and effective management of this life-threatening condition.

Searching the plant kingdom for a treatment or cure for convulsions or seizures is not an unreasonable approach to the problem; particularly, when one considers that no pharmacological prototype has been found outside either the plant or animal kingdom. The history of herbal treatments for convulsions is coincidental with the history of medicine as well as with the history of civilization itself. Anticonvulsants are used to control convulsions occurring in epilepsy, tetanus, cerebral hemorrhage, eclampsia and in poisoning with convulsants [37].

According to Cheymol [38], convulsions arise due to a sudden excessive and rapid discharge in the grey matter of the brain. Convulsions have the focal origin, the form of seizures depending on the site of focus in the brain, the regions to which discharge spreads and the effects of post ictal paralysis of these regions.

The nervous system contains some antioxidant enzymes, including superoxide dismutase and glutathione peroxidase that are expressed in higher quantities than catalase [39]. This spectrum of enzymatic defense suggests that the brain may efficiently metabolize superoxide, but it may have difficulties in eliminating the hydrogen peroxide produced by this reaction. The accumulation of hydrogen peroxide is of major concern since the brain contains large quantity of iron and copper, which may catalyze the formation of hydroxil radical, which, in turn, can induce lipid peroxidation [40].

The glutathione peroxidase is presented in large amounts during the Central Nervous System (CNS) development, but decreases in aged rats [41]. Nevertheless, other scavengers such as ascorbic acid and alpha-tocopherol also decrease the propagation of radical chain reaction. For these reasons, free radicals have been pointed as important molecules involved in the nervous system pathologies such as Huntington disease, Alzheimer, ischaemia and epilepsy [42].

In epilepsy model induced by pilocarpine administration, we found that superoxide dismutase and catalase activities in the hippocampus are not altered during the acute phase of seizures. On the other hand, according to several authors, the augment of these enzymatic activities could decrease the O2- and H_2O_2 levels. Taken together, these results show that during the acute phase, the hippocampus of the adult animals in pilocarpine model after seizures is more vulnerable to oxidative stress.

In addition, high-levels of hydroperoxides were also observed in the same group of animals, which indicated that the lipid peroxidation could be dependent of disability of the antioxidant enzymatic (superoxide dismutase and catalase) activities. As the hydroperoxides are a class of compounds produced as the result of phospholipid peroxidation, its high concentration in the tissue suggests that the hippocampal cells are more vulnerable to damage during the acute period of seizures. Thus, the results described in literature about pilocarpine model suggest that the beneficial effects of antioxidants compounds in reducing the behavioral changes caused by seizures may be partly explained by their ability to remove free radicals, and prevent the formation of hydroperoxides in hippocampus of seized rats.

Adesina [43] carried out phytochemical and pharmacological studies on 50 plants which were used as anti-convulsants, viz. anti-leptazol and anti-strychrine tests. Chang [44] reported the characterization of biologically active compounds from the plant-kingdom. This chapter is restricted only to p-cymene which possess either anticonvulsant activity and the results are summarized in Table 1.

The need for animal models of epilepsy is driven by the constraints of studying human epileptic brain. Although a great deal has been learned through the study of human epileptic brain tissue throughout the past 100 years, and particularly based in recent experiments with pilocarpine model, our work was aimed at investigating the antioxidant effects of *p*-cymene in adult mice under pilocarpine-induced seizures. Our studies have demonstrated that all animals pretreated with the *p*-cymene at the dose (50, 100 or 150 mg/kg) during the first 3-5 minutes of acute phase of seizures induced by pilocarpine injection also manifested behavior alterations, such as peripheral cholinergic signs, tremors, staring spells,

facial automatisms, wet dog shakes, rearing and motor seizures, which develop progressively within 10 min into a long-lasting status epilepticus. However, these behavioral changes occur at lower rates (Table 1). The findings also suggest that when administered 30 min before pilocarpine, *p*-cymente reduces the percentage of animals that seized, increases latency to the first seizure and the survival percentage (Table 1).

In preclinical practice, the animals pretreated with *p*-cymene (50 mg/kg) in pilocarpine model developed cholinergic reactions, 75% had seizures, 75% built up to status epilepticus and 25% of the animal died (Table 1). *p*-Cymene administration, 30 min before pilocarpine injections, increased the latency to the onset of the first seizure in 129% and latency of the status epilepticus in 96% (Table 1). In pilocarpine model, it is also shown that when administered at the smaller dose (100 mg/kg), 30 min before pilocarpine injection, *p*-cymene can be decrease by 50% the percentage of animals that seized, increased (213%) latency to the first seizure, and increased survival (41%) (Table 1).

The pretreatment with *p*-cymene at doses 50, 100 or 150 mg/kg was not able to revert peripheral cholinergic signs, tremors and stereotyped movements induced by pilocarpine, but the compound caused a dose-dependent reduction in pilocarpine-induced seizures, at the doses of 50 (25%), 100 (50%) and 150 mg/kg (75%). A clear protector effect was also observed with atropine (25 mg/kg), phenobarbital (50 mg/kg) and diazepam (5 mg/kg), which were used as a reference drugs (Table 1). As verified in our study, *p*-cymene was dose-dependently effective in increasing the latency to first seizures induced by pilocarpine at the doses of 25, 50 and 75 mg/kg.

According to Table 1, *p*-cymene caused an increase of latency for installation of status epilepticus induced by pilocarpine (400 mg/kg) at doses of 25 (96%), 50 (158%) and 75 mg/kg (481%). Table 1 shows that *p*-cymene significantly increased the survival rate after pilocarpine-induced seizures at doses of 25 (25%), 50 (50%) and 75 mg/kg (87.5%). The reference drugs atropine (25 mg/kg), phenobarbital (50 mg/kg) and diazepam (5 mg/kg) also produced a significant protection (100, 50 and 75%), respectively. None of the control animals (vehicle, atropine, phenobarbital, diazepam or *p*-cymene) presented behavioral changes.

Mice were acutely treated with the drug doses shown in the table above and 30 min afterwards they received pilocarpine (400 mg/kg). Following, the animals were observed for 24 h for assessment of cholinergic reactions, motor seizures which develop progressively within 10-12 min into a long-lasting

Table 1. Effect of pretreatment with *p*-cymene on behavioral alterations in pilocarpine-induced seizures and lethality in adult mice.

Drugs	Dose (mg/kg)	Cholinergic Reactions (%)	Latency to first seizure (min)	Seizures (%)	SE (%)	Survival rate (%)
Pilocarpine	400	100	7.83 ± 0.27	100	100	00
***p*-cymene**	50	100	18.0 ± 0.27[a]	75[d]	75[d]	25[d]
	100	100	24.5 ± 0.82[a,b]	50[d,e]	50[d,e]	50[d,e]
	150	100	52.0 ± 5.0[a,b,c]	25[d,e,f]	25[d,e,f]	87.5[d,e,f,]
Diazepam	5	100	9.0 ± 0.98[a]	100	100	25[d]
Phenobarbital	50	100	45.3 ± 1.70[a]	50[d]	50[d]	50[d]
Atropine	25	00	-	00[d]	00[d]	100[d]

status epilepticus (SE) and survival rate. Results of latency to first seizure are expressed in minutes (min) as mean ± S.E.M of the number of experiments shown in the experimental groups and the others in percentages. [a]$p<0.05$, vs pilocarpine; [b]$p<0.05$ vs *p*-cymene 50; [c]$p<0.05$ vs *p*-cymene 100 (ANOVA and *t*-Student Newman Keuls *post hoc* test); [d]$p<0.05$, vs pilocarpine; [e]$p<0.05$ vs *p*-cymene 50; [f]$p<0.05$ vs *p*-cymene 100 (χ^2 test).

Hence, it could be expected that natural compounds such as *p*-cymene, can be used as scavengers of free radicals, reducing brain injury induced by pilocarpine. In previous histopathological analyses, *p*-cymene protected animals against seizures, status epilepticus and brain damage induced by pilocarpine (Figure 1) by decreasing the percentage of seizures, status epilepticus and death in relation to three doses tested.

3. *p*-Cymene effects on histopathological alterations in epilepsy model

A wide variety of lesions have been reported in the different studies of epilepsy in the literature; though the question is still raised if these observed lesions are the cause or the consequence of seizures. The lesions that might play a role in the onset, maintenance and progression of pharmacoresistance of epileptic seizures, as well as the lesions that might results in are not yet well predicted and comprehensible [45].

Over the last few decades, the refinement and advancement of neurobiological investigative tools along with the proliferation of epilepsy surgery have offered the possibility to study in detail some network, cellular and molecular properties of the human brain [46-48]. Such analyses, which have been most often performed on brain tissue obtained from epileptic patients, not only have revealed some basic principles of network and cellular physiology of human nerve cells but also have provided direct information on the pathophysiology underlying this disease.

Early necropsy reports have demonstrated two etiological forms of temporal lobe epilepsy. This pathology could be due either to specific structural epileptogenic lesions or to discrete histological abnormalities in the hippocampus which have been given the generic name of hippocampal sclerosis [49-53]. Hippocampal sclerosis is characterized by extensive loss of pyramidal cell bodies particularly in CA1, reactive gliosis, granule cell dispersion in the dentate gyrus, and mossy fibre reorganization [54-59].

Epileptic patients with marked hippocampal sclerosis have a characteristic clinical history, well-localized electroencephalographic abnormalities, neuropsychological deficits and metabolic patterns that individualize a syndrome referred to as mesial temporal lobe epilepsy. Mesial temporal lobe epilepsy is by far the most common form of temporal lobe epilepsy [60,61]. It usually appears between five and 10 years, although sometimes with a later onset, after complicated febrile convulsions, status epilepticus, encephalitis or head trauma [62-63]. Recurrent spontaneous mesial temporal lobe epilepsy seizures are associated with alterations of consciousness and orofacial automatisms; secondarily generalized motor seizures are rare.

Preclinical studies have demonstrated that rodents sustain prolonged behavioral deficits following epileptic brain injury, in some cases culminating in the cognitive and histopathological hallmarks of human epilepsy. However, few studies have examined the long-term consequences and compound natural effects on experimental epileptic brain injury.

Some remarks should to be made at this point. For instance, it must be emphasized that the majority of human data have resulted to date from in vivo methodological approaches with rodents, and in particular the brain slice preparation; therefore, these findings do reflect the activity of a ‘‘reduced’’ system that does not possess the capabilities of an intact brain [64].

In addition, the majority of these experiments have been performed in rodents presenting with partial epileptic disorders that are often pharmacoresistant and thus amenable for in-depth investigations leading to neurosurgical interventions. It is also important to note that the interpretation of data obtained from human slice experiments is hampered by the absence of ‘‘normal’’ human controls. Such a problem has been partially worked out by employing ‘‘non-epileptic’’ cortical samples (for instance, tissue resected during removal of a tumor located deep in the brain). However, even in such cases, it is unlikely that this brain tissue was ‘‘normal’’. Throughout this chapter we will also compare the findings obtained by studying the *p*-cymene effects on mice brain tissue with evidence gathered in cerebral histophatological analyses in epilepsy model pilocarpine-induced.

A variety of epilepsy models reflect the effects of lipoic acid, ubiquinone, cyano-carvone, acid ascorbic and alpha-tocopherol and specify their action [65-70]. Previously, it had been demonstrated that natural compounds reduced the frequency of epileptiform activity [71-72]. In recent years, many of the effects of *p*-cymene have been discovered, including their actions on mitochondrial functions.

Some studies have reported that *p*-cymene did not changed the oxygen consumption by respiratory chain (state 2 respiration). However, *p*-cymene decreased the mitochondrial membrane potential ($\Delta\psi$), depressed the rate of ADP phosphorylation (state 3), and stimulated the oxygen consumption after phosphorylation of ADP (state 4). The respiratory control ratio (state 3/state 4) was decreased as a consequence of the inhibition of state 3 and stimulation of state 4 respiration but the ADP/O index remained unaltered as well as the mitochondrial Ca^{2+} fluxes. Moreover, *p*-cymene did not induced mitochondrial membrane disruption but depressed the $\Delta\psi$, and the stimulatory effect observed on state 4, similar to the effect observed on state 2 respiration plus ATP, was inhibited by oligomycin. These effects suggest that *p*-cymene allows a proton leak through the Fo fraction of the phosphorylative system, changing the mitochondrial proton motive force and ATP synthesis capacity [18]. Therefore, these data suggest mitochondria as a target for *p*-cymene action mechanisms.

Our results demonstrated that seizure pattern and brain damage observed in pilocarpine-treated animals differ from those pretreated with *p*-cymene (100 or mg/kg) plus pilocarpine (400 mg/kg). The latter reproduced the syndrome with lower intensity of histopathological changes and mortality rate, in comparison with the *p*-cymene (100 or 150 mg/kg) plus pilocarpine, corroborating the outcomes obtained by Ayyildiz and collaborators [73], Costa and collaborators [9] and Freitas [74]. The percentage of status epilepticus (100%) that was found further corroborated prior investigations [9,75].

The precise target of the anticonvulsant action of *p*-cymene and derivatives has not yet been fully established but it has been reported that the mode of action involves several targets in the cell. *p*-Cymene has been shown to have neuroprotective properties. In fact, it has been reported that the anticonvulsant effects of *p*-cymene and derivatives may result, partially at least, from a reduction of the perturbation of the lipid fraction of the plasmic membrane, acting by protection the cytoplasmic membrane against free radicals. The membrane integrity favors its functions not only as a barrier but also as a matrix for enzymes and as an energy transducer.

Changes in the fatty acid composition of neuronal cell membranes (an increase in unsaturated fatty acids) and changes in membrane fluidity have

been observed during pilocarpine-induced seizures [9,76]. Therefore, the hydrophobicity enables them to partition in the lipids of the cell membrane and mitochondria, rendering them permeable and leading to leakage of cell contents [77]. In addition to the effects on the membrane integrity and transmembrane potential, treatment with this monoterpenes can induced increases of the intracellular ATP levels when neuronal cells were exposed to epileptic activity.

Brain tissue examinations of the animals pretreated with *p*-cymene (50, 100 or 150 mg/kg; **Figures 1 C, D and E**), did not reveal hippocampal histopathological changes. Then again, pilocarpine-treated animals presented neuronal loss, gliosis, and typical vacuolar degeneration in hippocampus region (**Figure 1 B**). Histopathological damage in hippocampus was observed only in 37.5% of the animals co-administered with p-cymene (50 mg/kg), and that 30 min after pretreatment received pilocarpine (400 mg/kg), respectively (**Table 2 and Figure 1 F**).

In addition, the analyses of histopathological damage in hippocampus of mice pretreated with *p*-cymene (100 or 150 mg/kg), and that 30 min after pretreatment received pilocarpine (400 mg/kg) did not reveal hippocampal histopathological changes (**Table 2 and Figures 1 G and H**). Moreover, the analyses of histopathological damage in hippocampus of mice pretreated with vehicle (control group) revealed no neuronal damage (**Figure 1 A**).

[A] Control group; **[B]** Pilocarpine group; **[C]** *p*-cymene 50 group; **[D]** *p*-cymene 50 plus pilocarpine groups was treated with *p*-cymene (50 mg/kg) and 30 min before pilocarpine; **[E]** *p*-cymene 100 group; **[F**] *p*-cymene 100 plus pilocarpine group was treated with *p*-cymene (100 mg/kg) and 30 min before pilocarpine; [G] *p*-cymene 100 group; [H] p-cymene 150 plus pilocarpine group was treated with p-cymene (150 mg/kg) and 30 min before pilocarpine. Severity of lesion was expressed as mean ± S.E.M. of scores of damage based in a scale from zero (none) to 100 (total) percent of structural involvement. Brain damage was considered positive if there was at least 50% hippocampal involvement. Hematoxylin & Eosin staining (H&E). Magnification, 100 X. One representative experiment with n=8 is shown.

Severity of lesion in hippocampus was reduced in 51.2% of the animals co-administered with *p*-cymene (50 mg/kg), and that 30 min after pretreatment received pilocarpine (400 mg/kg), respectively (Table 2).

Pilocarpine was administered in a single dose (400 mg/kg, pilocarpine, n=8), and *p*-cymene groups with *p*-cymene (50, 100 or 150 mg/kg; n=8). The p-cymene plus pilocarpine groups were treated with *p*-cymene (50, 100 or 150 mg/kg, n=8) and 30 min before pilocarpine. Severity of lesion was expressed as mean ± S.E.M. of scores of damage based on a scale from zero (none) to 100 (total) percent of structural involvement. Brain damage was

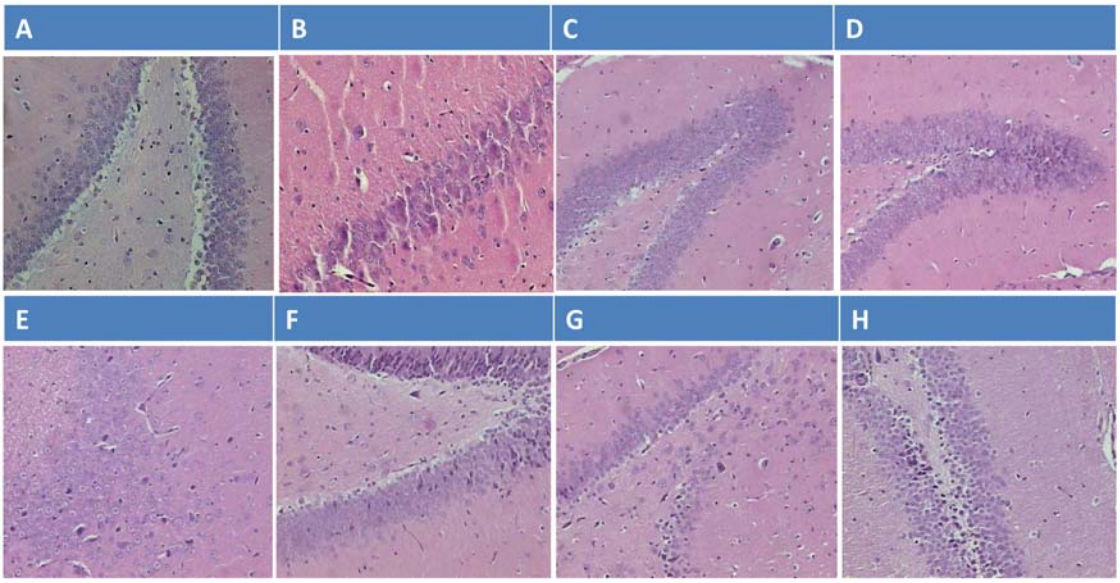

Figure 1. Histopathological alterations in mice hippocampus treated with pilocarpine, *p*-cymene or their combinations.

Table 2. Histopathological alterations in hippocampus of mice pretreated with *p*-cymene after pilocarpine-induced seizures.

Drugs	Dose (mg/kg)	Mice with lesion (%)	Severity of lesion (%)	Number of animals with lesion per group
Pilocarpine	400	87.5	63.92 ± 0.3	7
***p*-cymene**	50	00	00	0
	100	00	00	0
	150	00	00	0
***p*-cymene plus pilocarpine**	50	37.5[a]	12.9 ± 0.2[b]	3[a]
	100	00	00	0
	150	00	00	0

defined as present if there was at least 50% hippocampal involvement. Results for % mice with brain lesion and % severity of lesion are expressed as percentages of the number of animals inside in parenthesis. [a]$p<0.05$ compared with pilocarpine group (χ^2 test). [b]$p<0.05$ compared with pilocarpine group (ANOVA and t-Student Newman Keuls *post hoc* test).

Brain tissue examinations of the animals pretreated with *p*-cymene (50, 100 or 150 mg/kg; **Figures 2 C, D and E**), did not reveal striatal histopathological changes. Then again, pilocarpine-treated animals presented neuronal loss and typical vacuolar degeneration in striatum region (**Figure 2 B**). Histopathological damage in striatum was observed only in

25% of the animals co-administered with *p*-cymene (50 mg/kg), and that 30 min after pretreatment received pilocarpine (400 mg/kg) (**Table 3 and Figure 2 F**). In addition, the analyses of histopathological damage in striatum of mice pretreated with *p*-cymene (100 or 150 mg/kg), and that 30 min after pretreatment received pilocarpine (400 mg/kg) did not reveal striatal histopathological changes (**Table 3 and Figures 2 G and H**). Moreover, the analyses of histopathological damage in striatum of mice pretreated with vehicle (control group) revealed no neuronal damage (**Figure 2 A**).

Severity of lesion was expressed as a mean ± S.E.M. of scores of damage based in a scale from zero (none) to 100 (total) percentage of striatum involvement. Brain damage was considered positive if there was at least 50% striatal involvement showed by Hematoxylin & Eosin staining (HE). Pictures (100 X) shown are from one representative experiment of n=8. **[A]** Control group; **[B]** Pilocarpine group; **[C]** *p*-cymene 50 group; **[D]** *p*-cymene 50 plus pilocarpine groups was treated with *p*-cymene (50 mg/kg) and 30 min before pilocarpine; **[E]** *p*-cymene 100 group; **[F]** *p*-cymene 100 plus pilocarpine group was treated with p-cymene (100 mg/kg) and 30 min before pilocarpine; **[G]** *p*-cymene 100 group; **[H]** *p*-cymene 150 plus pilocarpine group was treated with p-cymene (150 mg/kg) and 30 min before pilocarpine.

Severity of lesion in hippocampus was reduced in 44.23% of the animals co-administered with p-cymene (50 mg/kg), and that 30 min after pretreatment received pilocarpine (400 mg/kg), respectively (Table 3).

The outcomes confirm that p-cymene (50, 100 or 150 mg/kg) decreased the frequency of pilocarpine-induced seizures, status epilepticus and brain lesions in mice. In addition, *p*-cymene decreases the severity of hippocampal

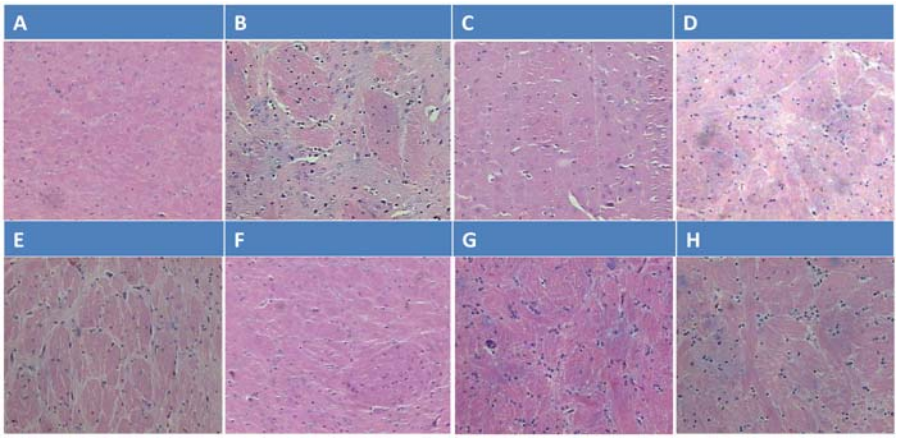

Figure 2. Histopathological alterations in mice striatum treated with pilocarpine, *p*-cymene or their combinations.

Table 3. Histopathological alterations in striatum of mice pretreated with *p*-cymene after pilocarpine-induced seizures.

Drugs	Dose (mg/kg)	Mice with lesion (%)	Severity of lesion (%)	Number of animals with lesion per group
Pilocarpine	400	62.5	56.19 ± 0.34	5
***p*-cymene**	50	00	00	0
	100	00	00	0
	150	00	00	0
***p*-cymene plus pilocarpine**	50	25[a]	11.96 ± 0.57[a]	2
	100	00	00	0
	150	00	00	0

and striatal lesions and mortality rate caused by pilocarpine. Costa and collaborators [9] demonstrated that the injection of cyano-carvone, 30 min before pilocarpine administration, prevented the occurrence of epileptic discharges. Since there are wide variations of p-cymene and cyano-carvone doses used in different models of seizure, more detailed investigations are necessary before an ultimate conclusion on the effects of those compounds on pilocarpine-induced seizures can be achieved.

Pilocarpine was administered in a single dose (400 mg/kg, pilocarpine, n=8), and *p*-cymene groups with *p*-cymene (50, 100 or 150 mg/kg; n=8). The p-cymene plus pilocarpine groups were treated with p-cymene (50, 100 or 150 mg/kg, n=8) and 30 min before pilocarpine. Severity of lesion was expressed as mean ± S.E.M. of scores of damage based on a scale from zero (none) to 100 (total) percent of structural involvement. Brain damage was defined as present if there was at least 50% striatal involvement. Results for % mice with brain lesion and % severity of lesion are expressed as percentages of the number of animals inside in parenthesis. [a]$p<0.05$ compared with pilocarpine group ($\chi 2$ test); b$p<0.05$ compared with pilocarpine group (ANOVA and *t*-Student Newman Keuls *post hoc* test).

In conclusion, there is an accumulation of free radicals after status epilepticus induced by pilocarpine, and oxidative changes in other parameters during the acute phase. This finding suggests that seizures, status epilepticus and deaths induced by pilocarpine have a large participation in brain oxidative stress, which is closely related to the mechanism of propagation and/or maintenance of the epileptic focus by pilocarpine. These results suggest that free radicals as well as the muscarinic receptor activation seem to be involved in the genesis of seizures and brain damage obtained with pilocarpine. On the other hand, the muscarinic activation seems to play a major role in the neuronal damage produced by pilocarpine. *p*-Cymene can

exert neuroprotective function during acute phase of seizures, thereby decreasing the severity of hippocampal and striatal lesions. All these outcomes indicate the promising therapeutic potential of *p*-cymene in treatments for neurodegenerative diseases.

Conclusion

Our results have shown that *p*-cymene possesses anticonvulsant activity probably due to the modulation of cholinergic system. *p*-Cymene may be helpful to produce neuronal protection and may be considered as a potential natural anticonvulsant. However, additional studies should be conducted in order to determine its clinical use.

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