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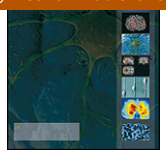

Anticonvulsant effect of phytol in a pilocarpine model in mice

J.P. Costa[a,b], P.B. Ferreira[a], D.P. De Sousa[b], J. Jordan[c], R.M. Freitas[a,*]

[a] Laboratory of Research in Experimental Neurochemistry of Post-Graduation Program in Pharmaceutics Science, Federal University of Piauí, CEP 64.049-550, Teresina, Piauí, Brazil
[b] Department of Pharmacy, Federal University of Sergipe, CEP 49.100-000, São Cristóvão, Sergipe, Brazil
[c] Department of Medical Science, Medical School of Albacete, University of Castlilla-La Mancha, Albacete 02006, Spain

HIGHLIGHTS

- Phytol protected mice against pilocarpine-induced seizures.
- Phytol reduces mortality rate caused by seizures.
- Phytol exhibits anticonvulsant activity in pilocarpine model.

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ABSTRACT

The present study investigated the effects of phytol in pilocarpine-induced seizures. The latency for development of convulsions and mortality rate was recorded in this model using mice. The results revealed that phytol (25, 50 and 75 mg/kg, i.p.) increased latency to first seizure and decreased percentage of these seizures. Moreover, phytol also protected the animals against status epilepticus induced by pilocarpine, and decreased the mortality rate. Mice treated with pilocarpine ($n = 24$) presented 100% of mortality during the first hour of observation. In turn, phytol-pretreated animals within 30 min before the administration of pilocarpine (400 mg/kg) remained alive during the first hour of observation. On the other hand, 6–8 h after administration of pilocarpine it was observed that 10 (41.66%), 8 (33.33%) and 4 (16.66%) animals died (respectively). Thus, the pretreatment with phytol was able to block mortality rate during the first hour in acute phase of seizures, and significantly reduced this rate in a dose-dependent manner ($p < 0.05$), suggesting an anticonvulsant effect. In addition, none of the phytol effects was blocked by pre-treatment with flumazenil, an antagonist of benzodiazepine receptors. In conclusion, phytol exhibits anticonvulsant activity by modulating of neurotransmitter systems, but further investigations are in progress to confirm this pharmacological property.

Epilepsy is a common chronic neurological disorder that affects 1–2% of the world population [22]. Although seizures in two-thirds of the patients can be successfully controlled with anticonvulsant medications, the remaining one-third remains refractory to medical therapy [21]. This fact has stimulated a considerable research for new antiepileptic drugs with higher safety and efficacy than the currently available anticonvulsants. In this regard, medicinal plants have been an important source in the development of new drugs with anticonvulsant activity [7].

Interestingly, in the last years, a large number of evidences has demonstrated that natural products from folk therapies have contributed significantly in the discovery of modern drugs. Additionally, numerous herbal medicines are active on central nervous system (CNS), and they have at least a hypothetical potential to affect chronic conditions that do not respond well to conventional treatments [6–8].

Development and introduction of new drugs with greater safety and efficacy is essential to enhance treatment of epilepsy. The search for less toxic alternatives resulted in decreased use of combinations of medicinal plants and the introduction of synthetic substances. This notion is supported by previous studies showing that some monoterpenes present in several essential oils, such as thymoquinone [18], citronellol [26], α,β-epoxy-carvone [11] and isopulegol [25], possess anticonvulsant activity.

Among the diterpenes, phytol is chemically defined as 3,7,11,15-tetramethylhexadec-2-en-1-ol. Phytol is an aromatic ingredient used in many fragrance compounds. It may be found in cosmetics as well as in non-cosmetic products [19]. Phytol is a member of branched-chain unsaturated alcohols whose common characteristic structural elements are one hydroxyl group per molecule, a

* Corresponding author at: Programa de Pós-graduação em Ciências Farmacêuticas/Universidade Federal do Piauí, Ininga, Teresina, CEP 64.049-550, Piauí, Brazil. Tel.: +55 86 3215 5870; fax: +55 86 3215 5870.
E-mail addresses: rivelilson@pq.cnpq.br, rivmendes@hotmail.com (R.M. Freitas).

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twenty and one double bond carbon atoms can be observed in their chemical structure [3].

Phytol is a constituent of chlorophyll [1,2]. Studies showed that considerable amounts of phytanic acid accumulated in lipids of liver, kidney, muscle and urine of a patient with Refsum's disease [20,28]. However, the effects of this diterpene on central nervous system have not yet been studied. Thus, the objective of this study was to evaluate the anticonvulsant activity of phytol and characterize its mechanism of action on pilocarpine-induced seizures.

For this study, the drugs flumazenil, phytol, pilocarpine hydrochloride, polyoxyethylene-sorbitan monolated (Tween 80) were purchased from Sigma Chemical Co., St. Louis, MO (USA) and diazepam (DZP) from Cristália (Brazil). The dosage of all drugs was expressed as milligrams per kilogram of body weight.

Adult male Swiss mice (25–30 g; 2 months old) were maintained in a temperature-controlled room (26 ± 1 °C) with a 12-h light/dark cycle, with food and water *ad libitum* (Nutrilabor, Campinas, Brazil). All experiments were approved by the Ethics Committee on Animal Experiments at Federal University of Piauí (CEEA/UFPI # 013/11). Mice were divided into five random groups ($n = 24$). The first group represented control group and received placebo vehicle (0.05% Tween 80 dissolved in 0.9% saline); the second group was treated with pilocarpine hydrochloride (400 mg/kg, i.p.). The remaining groups received an injection of phytol (PHY) at doses of 25, 50 and 75 mg/kg emulsified in vehicle. Thirty minutes after drug administration, mice were treated with pilocarpine (i.p.) at a dose of 400 mg/kg. Then, they were observed for 1 h to monitor latency to the first seizure (tonic–clonic seizures with or without rearing), the latency to status epilepticus (intermittent seizures for up to 30 min), occurrences of wild running, clonuses, tonus, clonic–tonic seizures (with or without rearing), the number of animals that seized, and death after P400 administration [13,15].

Previous work has shown that convulsions and deaths occurring always within 1–24 h following postinjection. It was decided to observe animals for 1 h whereas pilocarpine-induced convulsions start within 30–60 min and deaths within 1–24 h after pilocarpine injection. Survivors were sacrificed by decapitation and their brains were dissected and put in ice to remove hippocampus for determinations of neurochemical alterations in future studies. The P400 group was constituted by those rats that presented seizures; SE for over 30 min and did not die within 1 h.

After this experimental procedure, possible participation of the benzodiazepine site of $GABA_A$ receptors of GABAergic receptors in modulating the effect of phytol on anticonvulsant activity was evaluated. For this purpose, FLU, a selective $GABA_A$-BZD receptor antagonist [4,5,12], was administered in two groups of mice, 15 min before either diazepam or PHY treatments. Thirty minutes after the last treatment each animal was injected with pilocarpine. The latency to the first seizure, the number of animals showing convulsions, latency to installation of the status epilepticus, and number of animals that died were the parameters recorded.

In another series of experiments, 30 min after treatment with vehicle or 75 mg/kg of PHY or 5 mg/kg of diazepam or 25 mg/kg of flumazenil (FLU), mice ($n = 12$) received pilocarpine (i.p., 400 mg/kg) they were and observed for 1 h to detect the latency to the first seizure, the latency to status epilepticus, the occurrence of wild running, clonuses, tonus, clonic–tonic seizures, the number of animals that seized and number of animals that died after pilocarpine administration. Diazepam and FLU were used as reference drugs.

Since the results of latency to first seizure and status epilepticus show a parametric distribution, they were compared by one-way analysis of variance (ANOVA) followed by *t*-Student–Newman–Keuls. The number of animals that seized and those that survived were calculated as percentages, and compared with a nonparametric test (χ^2). In both situations, statistical significance was considered when $p < 0.05$. The statistical analyses were performed with the software GraphPad Prism, Version 5.00 for Windows, GraphPad Software (San Diego, CA, USA).The results show that, in general, intraperitoneal administration of phytol did not produce toxic effects on animals' behavior, whereas it was not seen clinical or behavioral signs toxicity during 30 min observation period prior to treatment with pilocarpine. After treatment with doses of 25, 50 and 75 mg/kg, no changes were detected in response to the touch and ambulation.

After 3–5 min following pilocarpine administration, all animals showed peripheral cholinergic signs (miosis, piloerection, chromodacriorrhea, diarrhea and masticatory). They also showed stereotyped movements (continuous sniffing, paw licking and rearing) followed by motor limbic seizures ($p < 0.001$). The convulsive process persisted and built up to a status epilepticus in all mice, leading to death ($p < 0.001$) (Table 1). All animals pre-treated with phytol in this experiment were observed for 1 h after pilocarpine injection and manifested alterations in behavior, such as peripheral cholinergic signs, tremors, staring spells, facial automatisms, wet dog shakes and rearing.

Pre-administration of phytol caused a dose-dependent protection against pilocarpine-induced seizures and status epilepticus and reduced mortality rate induced by pilocarpine ($p < 0.05$, Table 1). Similar outcomes were also observed with diazepam (5 mg/kg) used as reference drug (Table 2).

Pilocarpine induced the first seizure at 7.90 ± 1.68 min and status epilepticus at 14.95 ± 1.57 min. As detailed in Table 1, pretreatment with phytol caused a dose-dependent delay of latency to the first seizure when compared to the pilocarpine group. In addition, phytol caused increasing in latency to installation of status epilepticus.

With 75 mg/kg 83.34% of the animals were protected against seizures; occurrence of status epilepticus was reduced by 83.34%,

Table 1
Effects of phytol and cholinergic drugs on pilocarpine-induced seizures and lethality in adult mice.

Groups	Latency to first seizures (min)	% Seizures	Latency to status epilepticus (min)	% Survival	Number of animals/group
P400	7.90 ± 1.68	100	14.95 ± 1.57	0	24
PHY 25 + P400	19.10 ± 3.83[a]	41.66[d]	29.20 ± 3.75[a]	58.34[d]	24
PHY 50 + P400	24.25 ± 5.17[a,b]	33.33[d,e]	34.10 ± 3.46[a,b]	66.67[d,e]	24
PHY 75 + P400	27.50 ± 6.36[a,b,c]	16.66[d,e,f]	37.50 ± 4.50[a,b,c]	83.34[d,e,f]	24

Results for latency to first seizure and latency to status epilepticus were expressed as mean ± S.E.M. of the number of experiments shown in the table. Result for percentage seizures and percentage survival were expressed as percentages of the number of animals from each experimental group.

[a] $p < 0.05$ as compared with P400 group.
[b] $p < 0.05$ as compared with PHY 25 + P400 group.
[c] $p < 0.05$ as compared with PHY 50 + P400 group (ANOVA and *t*-Student–Newman–Keuls as *post hoc* test).
[d] $p < 0.05$ as compared with P400 group.
[e] $p < 0.05$ as compared with PHY 25 + P400.
[f] $p < 0.05$ as compared with PHY 50 + P400 group (χ^2-test).

Table 2
Effect of pretreatment with phytol and GABAergic drugs on pilocarpine-induced seizures and lethality in adult mice.

Groups	Latency to first seizures (min)	% Seizures	Latency to status epilepticus (min)	% Survival	Number of animals/group
P400	7.90 ± 1.68	100	14.95 ± 1.57	0	24
DZP 5	0	0	0	100	24
DZP 5 + P400	15.40 ± 3.50[a]	25[d]	23.33 ± 0.58[a]	75[c]	24
PHY 75 + P400	27.50 ± 6.36[a]	16.66[c]	37.50 ± 4.50[a]	83.33[c]	24
FLU 25	0	0	0	100	24
FLU 25 + P400	7.92 ± 0.57	100	14.91 ± 0.57	0	24
FLU 25 + DZP 5 + P400	7.89 ± 0.59[b]	100[d]	14.88 ± 0.43[b]	0[d]	24
FLU 25 + PHY 75 + P400	27.42 ± 1.94	16.66	37.45 ± 0.85	83.33	24

Results for latency to first seizure and latency to status epilepticus were expressed as mean ± S.E.M of the number of experiments shown in the table. Result for percentage seizures and percentage survival were expressed as percentages of the number of animals from each experimental group.

[a] $p < 0.05$ as compared with P400 group.
[b] $p < 0.05$ as compared with DZP 5 + P400 group (ANOVA and *t*-Student–Newman–Keuls as *post hoc* test).
[c] $p < 0.05$ as compared with P400 group.
[d] $p < 0.05$ as compared with DZP 5 + P400 (χ^2-test).

latency of status epileptic increased up to 151%, and 83.34% of animals maintained alive in comparison with control group (Table 1). Since this dose of phytol showed a stronger effect ($p < 0.001$), it was selected for follow-up studies. In our experiments, Phytol-treated mice showed no death during the first hour of observation. Deaths have occurred in between 6 and 8 h in the groups treated with phytol after administration of pilocarpine, suggesting that phytol can produce a neuroprotective effects.

The anticonvulsant effects of phytol are more potent than those shown by diazepam (Table 2). Nevertheless, to determine whether the anticonvulsant effects of phytol are exerted *via* GABAergic systems, phytol-treated mice were subjected to a co-treatment with flumazenil, a benzodiazepine receptor antagonist. However, flumazenil did not revert the anticonvulsant effect of phytol, but blocked the diazepam action (Table 2). None of the animals that received injections of 0.05% Tween dissolved in 0.9% saline (control), flumazenil, diazepam and phytol alone presented seizure activity.

In this study, we have used the model of epilepsy induced by a high dose (400 mg/kg) of pilocarpine since our research group has standardized this model. In previous studies, we have already documented data of the acute phase of this model, which facilitates interpretation of results with natural compounds during this model's phase [9,10].

The pilocarpine model is a useful experimental procedure to investigate the development of neuropathology of seizures [8,14]. In this model, the initial precipitating injury is characterized by a prolonged status epilepticus (SE), which causes neuronal loss, gliosis and vacuolar degeneration in rat hippocampus [23].

Although the mechanism of pilocarpine-induced seizures and SE is not completely understood, it is known that it depends on muscarinic activation and alterations in acetylcholinesterase activity in rat hippocampus [16]. Following the toxicity induced by an initial cholinergic phase, a distinct non-cholinergic phase occurs with an excessive production of free radicals [29].

As a consequence of neuronal connection disruption between brain regions, pilocarpine-induced seizures produce dysfunctions in many brain regions [14]. The behavioral changes during seizures in rats have been widely reported. These behaviors are replicable and reversed by acute administration of antiepileptic and antioxidants compounds [24,29].

In the present work, the anticonvulsant effects of phytol was performed. Phytol was first evaluated in a behavioral study that gives a good indication of protection to mortality and status epilepticus induce by pilocarpine.

The phytol is an acyclic terpenoid, which is part of the chlorophyll molecule [27]. Phytol derivatives may activate nuclear hormone receptors and influence gene expression and cell differentiation [17]. These pharmacological effects attributed to phytol can be responsible for controlling neuronal function, influencing neurotransmitter systems and modulating the release and/or synthesis of inhibitory neurotransmitters related to the seizure process.

Surprisingly, results demonstrate that phytol inhibited the action of pilocarpine, and protected mice against death induced by seizures. These discoveries make phytol a good candidate for drugs designed to produce neuroprotection in response to seizures. The current development in the availability of new anticonvulsant drug require appropriate choice of animal models of epilepsy for the identification of anticonvulsant activity as well as new mechanism of action [21].

Results from the present study show that phytol may be effective in blocking generalized tonic–clonic generalized seizures. On the other hand, the genesis of the seizures originated due to pilocarpine action involves the agonistic effect of this drug in muscarinic receptors, which would reduce the inhibitory synaptic transmission to promote excitatory neurotransmission [30]. As reported here, phytol confers protection against seizures induced by pilocarpine. However, our results suggest that the mechanism of phytol do not involve the GABAergic system, since antagonists of GABAergic receptors did not cause significant differences. Therefore, it is reasonable to suggest that the anticonvulsant activity exerted by phytol may not be associated to modulatory effects on GABAergic system. On the other hand, flumazenil was unable to reverse their anticonvulsant effects, which indicates that phytol may exercise its mechanism of action directly and/or indirectly through interaction with other systems neurotransmitters (serotonin, noradrenergic and glutamatergic) that need to be investigated in future studies.

Although phytol is modestly effective when compared to the standard drugs used in our study (*e.g.* diazepam), the observed effects appear to offer a potential advantage over most other anticonvulsant compounds. For example, diazepam is especially effective in preventing the generalized clonic–tonic convulsions induced by pilocarpine.

Herein, we clearly showed that phytol decreased the frequency of pilocarpine-induced seizures and increased the survival rate. In our knowledge, these effects of phytol on mortality rate observed during acute phases of pilocarpine-induced seizures have not been reported before. Thus, these findings may have important implications for understanding the mechanism of epilepsy to promote new advances in the development of selective and targeted-antiepileptic drugs.

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