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Anticonvulsant effect of a natural compound α,β-epoxy-carvone and its action on the nerve excitability

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ABSTRACT

The anticonvulsant effect of α,β-epoxy-carvone (EC), a monoterpene monocyclic, was investigated in three animal models. EC at 300 or 400 mg/kg promoted protection of 75% and 87.5%, respectively, against convulsions induced chemically by pentylenetetrazole (PTZ) and it was efficient in prevents the tonic convulsions induced by maximal electroshock (MES) in doses of 200, 300 or 400 mg/kg, resulting in 25%, 25% and 100% of protection, respectively. This monoterpene was also capable to promote an increase of latency for development of convulsions induced by picrotoxin (PIC) at 300 or 400 mg/kg and presented a significant protection against convulsions at doses of 200, 300 or 400 mg/kg, resulting in 12.5%, 12.5% and 100% of protection, respectively. On the other hand, the anticonvulsant effect of EC, was not affected by pretreatment with flumazenil (FLU), a selective antagonist of benzodiazepine site of $GABA_A$ receptor. Additionally was observed that EC treatment reduced the levels of in vitro lipoperoxidation and decreased (21.2%) the amplitude of compound action potential after 30 min of incubation. The present results clearly indicate the ability of EC to modulate the anticonvulsant and antioxidant effects. However, our data suggests that the action mechanisms are not due a direct activation of the $GABA_A$ benzodiazepine receptors, but could be associated with the reduction of isolated nerve excitability, possibly involving a voltage-gated Na^+ channels blockade.

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Epilepsy is the term used for a group of disorders characterized by recurrent spontaneous seizures [18] and up to 5% of the world population develops epilepsy in their lifetime [40]. Current epidemiologic studies show a prevalence rate for active epilepsy in approximately 1% of the population [48]. Significant seizure control can be achieved in 70–80% of persons with epilepsy, and complete control can be obtained in 60% with the drugs available [34]. Interesting fact, of the 877 new drugs that had been developed in period 1981–2002, 6% were natural products, 27% were derived from natural products, and 16% synthetics developed in the model of a natural product [32], demonstrating that the nature is a source important to lead the new anticonvulsant agents. In this context are present the essential oils, that are natural products with different applications, especially in the area of the medicine and cosmetic. They contribute to the pleasures of natural flavors and fragrances. In addition, many of them are found to exhibit varied biological properties [9], such as spasmolytic [27], anxiolytic [37], antinociceptive [41], and anticonvulsant [2] activity. These effects are probably due to great structural diversity of the essential oils constituents. This notion is supported by previous studies which showed that some monoterpenes present in many essential oils possess anticonvulsant activity in animal experiments [10,11,14]. Moreover, it was related that monoterpenes exhibited several types of pharmacological properties, for instance antinociceptive [16], sedative [13] and antidepressant [17]. EC is a monoterpene that can be found in the essential oils of *Carum carvi* [23], *Kaempferia galangal* [24], and other plants [25], but also obtained through organic synthesis [26]. In earlier studies, the antimicrobial effect of EC was investigated against *Staphylococcus aureus* and *Candida albicans* [4], and De Sousa et al. showed that EC presents pharmacological effects in central nervous system (CNS) [12]. These facts led us to verify the anticonvulsant potential of EC and to characterize its action mechanism, in eletrophysiologic level, verifying the participation of ionic channels in the effect of EC through studies using the technique of single sucrose gap.

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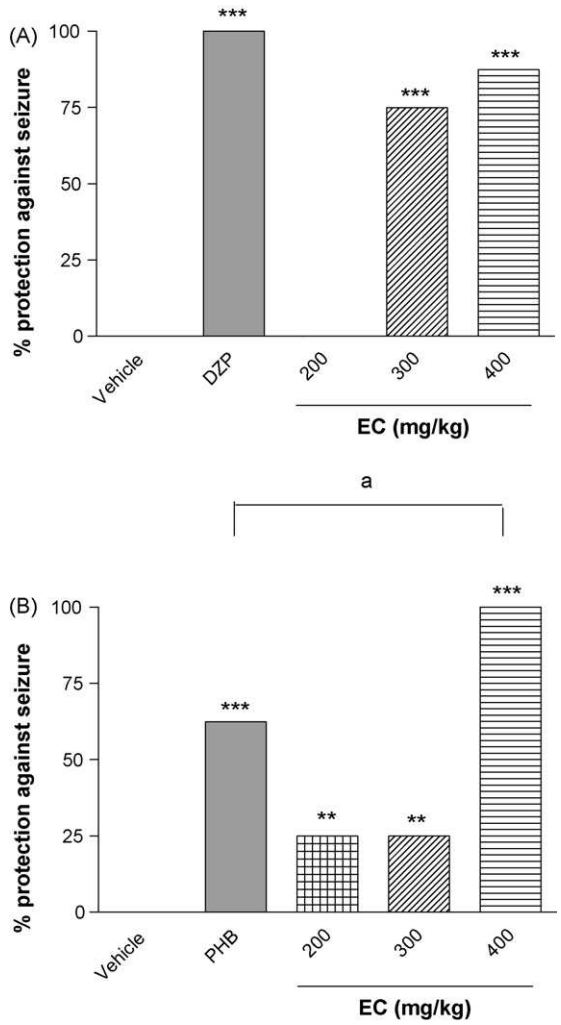

Fig. 1. Effects of EC on seizure induced by PTZ (A) and MES (B) in mice. Values are the percentage of protection against seizure; $^{**}p < 0.05$; $^{***}p < 0.001$, as compared to vehicle (control), $^{a}p < 0.001$ (PHB vs. EC), Fisher's exact test.

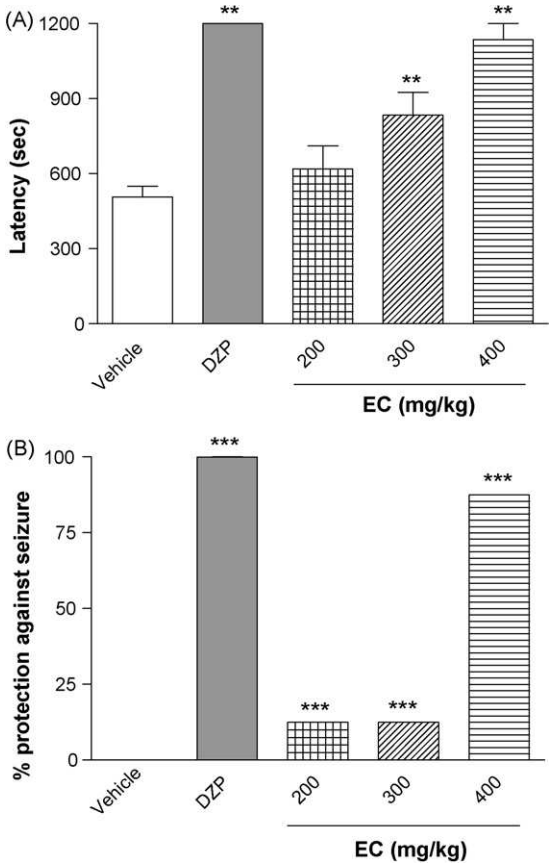

Fig. 2. Effects of EC on PIC-induced seizure in mice, the latency for development of convulsions (A) and the percentage of protection against seizure (B). Values represents the mean ± S.E.M. one-way ANOVA/Dunnett's test, $^{**}p < 0.01$ and Fisher's exact test, $^{***}p < 0.001$, as compared with control values.

The EC was obtained at Laboratory of Pharmaceutical Technology, Federal University of Paraíba as already described [26], and was prepared in 5% polyoxyethylene-sorbitan monolate (Tween 80) as an emulsion (vehicle). Phenobarbital (PHB), diazepam (DZP), PTZ, PIC, FLU, and (Tween 80) were purchased from Sigma (USA). The drugs were administered via intraperitoneal route.

Male mice of Swiss strain (3 months of age), weighing 28–35 g and male Wistar rats weighing 230–350 g (3 months of age), were obtained from our research animal house. Animals were maintained at constant room temperature (21 ± 2 °C) with a 12L:12D light schedule (lights on from 6:00 a.m. to 6:00 p.m.) and free access to food and water. All experiments were performed between 7:00 a.m. and 1:00 p.m. and carried out in accordance with ethical committee acts (CEPA No. 0503/05).

Mice were divided into five groups ($n = 8$). The control group received vehicle and the second group was treated with DZP (4 mg/kg). The remaining groups were injected with EC at doses of 200, 300 or 400 mg/kg. After 30 min, all the mice were treated with PTZ (60 mg/kg) and observed for at least 15 min to detect the occurrence of the first episode of forelimb clonus [45].

In another series of experiments, 30 min after treatment with vehicle, 200, 300 or 400 mg/kg of EC, mice ($n = 8$) received an electroconvulsive shock applied via ear-clip electrodes (0,5 mA and 15 pulses/s for 0.5 s) by a constant current stimulation (eletroconvulsor ECT unit model 7801) to induce tonic hindlimb extension (THE). PHB (25 mg/kg) was used as reference drug. The percentage of animals showing tonic convulsions, characterized by the presence of THE, was observed [33].

In PIC-induced convulsions, mice were randomly divided into five groups ($n = 8$). The control group received vehicle, and the positive control group was treated with DZP 4 mg/kg. The remaining groups received an EC treatment at doses of 200, 300 or 400 mg/kg. After 30 min of drug administration, the mice were treated with PIC (8 mg/kg) and observed for at least 20 min to detect the occurrence of the first episode of forelimb clonus.

The combined administration of FLU with EC or DZP, were performed using mice that were divided into six groups of eight each which treated with vehicle; FLU (20 mg/kg), DZP (4 mg/kg), FLU (20 mg/kg) + DZP (4 mg/kg); EC (300 mg/kg) and FLU (20 mg/kg) + EC (300 mg/kg), respectively.

FLU, a selective $GABA_A$-BZD receptor antagonist [7,8,19,20], was administered in two groups of mice each, 15 min before DZP or EC treatments. Forty-five minutes after the last treatment each animal was injected with PTZ (60 mg/kg). The latency and the number of animals showing convulsions were the parameters recorded.

Antioxidant activity in vitro was assessed by measuring the inhibition of spontaneous lipoperoxidation of homogenates from the brains of rats in the presence of different concentrations of EC (1.66; 16.6; 41.5; 83 and 166 μg/mL). Products of spontaneous lipoperoxidation react with thiobarbituric acid to form a colored compound, the intensity of which is measured at 535 nm in a spectrophotome-

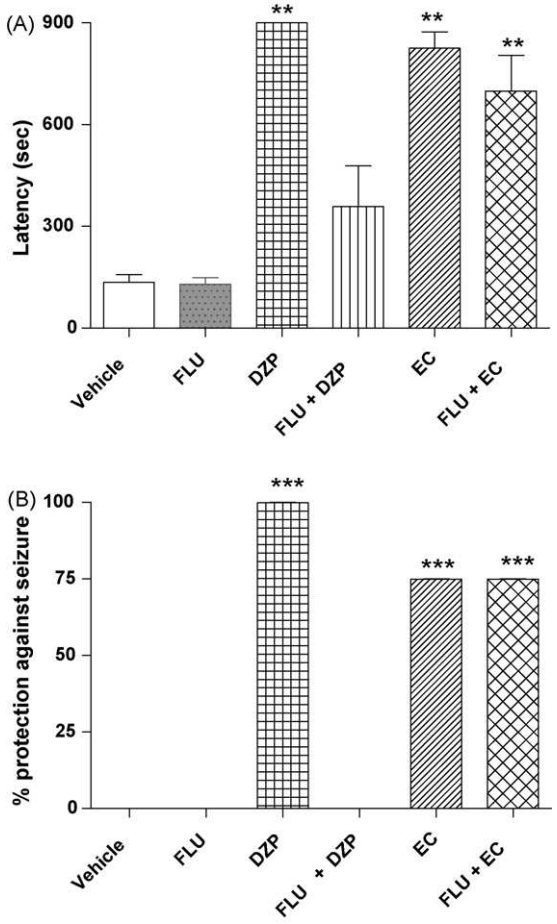

Fig. 3. Effects of EC (300 mg/kg), on PTZ-induced seizure in mice. Values represents the latency for development of convulsions (A) and the percentage of protection against seizure (B). Data indicate the mean ± S.E.M.; one-way ANOVA/Dunnett's test, $^{**}p < 0.01$ and Fisher's exact test, $^{***}p < 0.001$, as compared with corresponding control values.

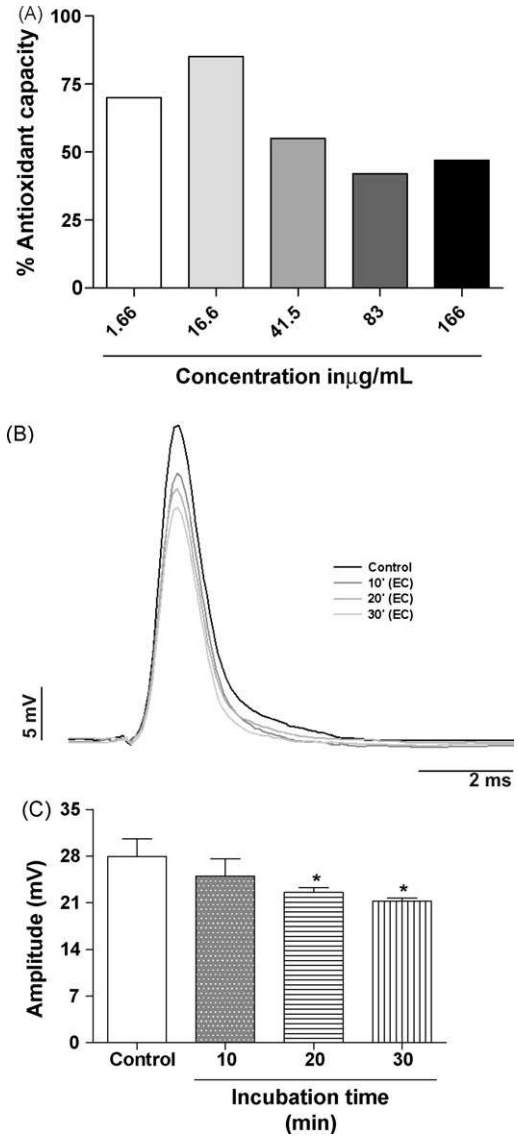

Fig. 4. Antioxidant capacity at different concentrations of EC. $Q_{1/2} = 50$ μg/mL (A). Effects of EC on the amplitude of compound action potential (CAP). (B) It is shown the time course of CAP recordings during EC incubation and (C) has shown the amplitude of CAP in presence of EC (5 mM). Each point represents the mean ± S.E.M. of four nerve bundles taken at 30 min. One way ANOVA and Dunnett's test, $^{*}p < 0.05$, as compared to control.

ter. After 1 h of incubation of brain homogenate at 37 °C, antioxidant capacity is calculated for each concentration of the EC. The concentration of the EC that inhibits 50% of peroxidation is referred to as $Q_{1/2}$ [30,44].

Procedures for effect of EC on nerve excitability experiments were similar as described in previous papers [21]. Briefly, the sciatic nerves from rats were carefully removed and desheathed. One nerve bundle was positioned across the sectioned chamber, which contained vaseline at the partitions to electrically isolate these sections and some of them, were used to apply supramaximal stimulation, which consisted of 100 μs isolated rectangular voltage pulses, delivered by a stimulator (CF Palmer, Model 8048, UK), triggered manually. All sections were filled with physiological solution with the following composition (in mM): NaCl 150; KCl 4.0; $CaCl_2$ 2.0; $MgCl_2$ 1.0; [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid] (HEPES) 10, adjusted to pH 7.4 with NaOH, except for one section, which was filled with isotonic (280 mM) sucrose solution that was continuously renewed, EC, at different concentrations, was introduced into the test section. The potential difference between the test and sucrose section was recorded every 10 min. Data were converted to digital form by a microcomputer-based 12-bit A/D converter at a rate of 10.5 kHz and later analyzed using a suite of programs (Lynx, São Paulo, Brazil). To quantify the effects of EC we used the amplitude (which is the potential difference between the baseline and the maximal voltage of the compound action potential, CAP), and the time constant of repolarization (τ_{rep}) that was calculated by the equation $V = V_0 \exp(t/\tau)$ using non-linear regression analysis applied to the repolarization phase of the CAP.

The results were expressed as mean ± S.E.M. and tested with Fisher's exact test or one-way analysis of variance (ANOVA) followed by Dunnet's test. A probability level of 0.05 was accepted as significant. All data were analyzed with software package GraphPad Prism Version 4.0 (GraphPad Sotware Incorporated, San Diego, USA).

EC significantly caused a dose-dependent protection against PTZ-induced convulsions, 300 (75%; $p < 0.001$) and 400 mg/kg (87.5%; $p < 0.001$). A clear protector effect also observed with DZP (4 mg/kg), used as a reference drug (Fig. 1A). However, EC (200 mg/kg) was not effective.

As illustrated in Fig. 1B, EC was effective to prevent tonic convulsions induced by MES at the dose of 200 (25%; $p < 0.01$), 300 (25%;

$p < 0.01$) and 400 mg/kg (100%; $p < 0.001$). The reference drug PHB (25 mg/kg) also produced a significant protection (62.5%; $p < 0.001$).

According Fig. 2A, EC caused an increase of latency for development of convulsions induced (PIC 8 mg/kg) at doses of 300 (833.6 ± 91.2 s) or 400 mg/kg (1136.0 ± 64.2 s) compared with control group treated with vehicle (507.1 ± 42.7) but no effect was observed with minor dose. Fig. 2B shows that EC promoted significant protection against convulsions at doses of 200 mg/kg (12.5%), 300 mg/kg (12.5%) or 400 mg/kg (87.5% of-protection).

The presence of FLU was not capable to revert the anticonvulsant effect of EC, but blocked the effect of DZP (Fig. 3).

The antioxidant effect *in vitro* of different concentrations of EC is indicated by increasing inhibition of spontaneous lipid peroxidation levels. The inhibition of 50% of the process being calculated at 50 μg/mL (Fig. 4A).

EC reduced in a significant manner the amplitude of CAP after 20 or 30 min of incubation of EC in concentration of 5 mM (Fig. 4B and 4C). There was no significant change in τ_{rep} (data not shown, $n = 4$).

The new drugs with antiepileptic activity are identified and developed as resulted of its ability to block induced acute convulsions in animal models of epilepsy. Amongst the several models used in the discovery of new drugs, MES, the test of PTZ and the kindling in rats are used in programs of discovery of new antiepileptic drugs [1,28,29,45,47].

The inhibition of the induced convulsions for the PTZ is a methodology considered as an experimental model for "convulsive crises generalized of the clonic type" [33,43]. Therefore, is known that the blockade of the induced convulsions chemically for the PTZ, in rodents, is a characteristic of drugs with depressant effects on the CNS with anticonvulsant actions [3].

Generally compounds that possess anticonvulsant activity in the epilepsy of the small type are effective in inhibiting the convulsions in the model of the PTZ [46]. So, the capacity of EC to promote protection against the induced convulsions for the PTZ, suggests a profile of anticonvulsant drug that could be useful in these types of crises.

The second anticonvulsant model used was the MES seizures test. This model has shown adjusted in the reproduction of the ictal phenomenon of the focal epilepsy [31]. Also is very used in the identification of efficient anticonvulsant drugs in the treatment of the generalized tonic–clonic epilepsies or the grand mal epilepsy [5,33,43]. Considering that EC was effective to prevent tonic convulsions induced by MES, this compound could be useful in grand mal epilepsy.

Some essential oils of plants have been used in folk medicine as an antiepileptic remedy [35,36]. Pharmacological studies have demonstrated the anticonvulsant properties of these essential oils in animal models [2]. The protective effects of the essential oils can occur for different mechanisms due to diversity of chemical constituents. Several terpenes of essential oils present sedative effects [11,15]. Some are effective in inhibiting experimental convulsions, such as the (+)-carvone [11].

Concerning the effects of EC on convulsions, it is known that the efficacy of the GABA system plays an essential inhibitory role on activity of the CNS and we also investigated this effect on PIC-induced convulsions that is a stimulatory substance of CNS and was utilized for chemical induction by inhibition of GABA receptors [22,38]. EC caused an increase of latency for development of convulsions induced by PIC and promoted significant protection against convulsions, suggesting involvement of GABAergic system on anticonvulsant effect.

The $GABA_A$ receptors are target for many anticonvulsant drugs [45,46]. Therefore, to evaluate the participation of benzodiazepine site of $GABA_A$ receptor in anticonvulsant effect of EC we tested this compound on presence of FLU. The presence of FLU was not capable to revert the anticonvulsant effect of EC suggesting not involvement of direct activation of benzodiazepine site of $GABA_A$ receptor [7,8]. Recent studies showed that seizures and status epilepticus can be associated with oxidative stress [6]. The results of this study indicated that EC reduced the levels of in vitro lipid peroxidation. The membrane lipid peroxidation, which is due to an increase in free radical and decrease in antioxidant defense mechanisms has been suggested to be accidentally involved in same forms of epilepsy [42]. The anticonvulsant protection induced by EC may be mediated by antioxidant effect.

It has related that the molecular targets of some antiepileptic drugs is voltage-dependent ionic channels, including sodium, calcium and the potassium channels and that cerebral sodium channels are modulated by some anticonvulsants agents [39,46]. In this way, to better characterize the anticonvulsant action of EC on ionic channels, we performed studies using the single sucrose gap technique. EC depressed the CAP amplitude leading to the suggestion that the anticonvulsant effect observed in the experimental models used in this work is likely to occur, at least, as a result of the voltage-dependent Na^+ channel blockade.

In conclusion, our results showed that EC possesses anticonvulsant activity probably due to the modulation of GABAergic system and reduction of neuronal excitability mainly through the voltage-dependent Na^+ channels. On the other hand, EC can alter oxidative stress by antioxidant effect. EC may be helpful to produce neuronal protection and may be considered as a potential natural anticonvulsant. However, additional studies are needed for clinical use.

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