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Anticonvulsant activity of β-caryophyllene against pentylenetetrazol-induced seizures

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ABSTRACT

Increasing evidence suggests that plant-derived extracts and their isolated components are useful for treatment of seizures and, hence, constitute a valuable source of new antiepileptic drugs with improved efficacy and better adverse effect profile. β-Caryophyllene is a natural bicyclic sesquiterpene that occurs in a wide range of plant species and displays a number of biological actions, including neuroprotective activity. In the present study, we tested the hypothesis that β-caryophyllene displays anticonvulsant effects. In addition, we investigated the effect of β-caryophyllene on behavioral parameters and on seizure-induced oxidative stress. Adult C57BL/6 mice received increasing doses of β-caryophyllene (0, 10, 30, or 100 mg/kg). After 60 min, we measured the latencies to myoclonic and generalized seizures induced by pentylenetetrazole (PTZ, 60 mg/kg). We found that β-caryophyllene increased the latency to myoclonic jerks induced by PTZ. This result was confirmed by electroencephalographic analysis. In a separate set of experiments, we found that mice treated with an anticonvulsant dose of β-caryophyllene (100 mg/kg) displayed an improved recognition index in the object recognition test. This effect was not accompanied by behavioral changes in the open-field, rotarod, or forced swim tests. Administration of an anticonvulsant dose of β-caryophyllene (100 mg/kg) did not prevent PTZ-induced oxidative stress (i.e., increase in the levels of thiobarbituric acid-reactive substances or the decrease in nonprotein thiols content). Altogether, the present data suggest that β-caryophyllene displays anticonvulsant activity against seizures induced by PTZ in mice. Since no adverse effects were observed in the same dose range of the anticonvulsant effect, β-caryophyllene should be further evaluated in future development of new anticonvulsant drugs.

1. Introduction

Epilepsy is a common neurological disease, which affects [1] and has been considered a major worldwide public health problem [2]. Recurrent epileptic seizures and behavioral comorbidities such as depression, anxiety, psychosis, and cognitive deficits largely affect the quality of life of the patients with epilepsy and their families [3]. There is the further complication that seizures in a significant percentage of patients remain inadequately controlled by currently available pharmacological treatments [4]. In addition, most anticonvulsant drugs display adverse effects such as ataxia, sedation, and cognitive dysfunction at serum concentrations within the therapeutic range for epileptic seizures [5]. Accordingly, discovery of a new anticonvulsant with better efficacy and improved safety profile is of fundamental importance [4]. In this context, several plant extracts and products may be useful for the treatment of convulsions or seizures, and therefore, natural products constitute a promising source of new antiepileptic drugs [6].

β-Caryophyllene is a natural bicyclic sesquiterpene that is a constituent of many plants [7]. Several biological activities have been reported for β-caryophyllene, including anti-inflammatory [7], anti-alcoholism [8], antinociceptive [9], anxiolytic, and antidepressant [10] properties. Interestingly, recent accumulating evidence indicates that β-caryophyllene is neuroprotective in several experimental paradigms [11–14]. For instance, administration of β-caryophyllene protects against cerebral ischemic injury in rats [11,14] and reduces astrogliosis and microglial activation in a transgenic mouse model of Alzheimer's disease [12]. Since neuroprotective compounds may display anticonvulsant activity

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and vice versa [15], and in order to further evaluate the potential therapeutic applications of β-caryophyllene, our present study aimed to test the hypothesis that this natural compound displays anticonvulsant effects. In addition, we investigated the effect of β-caryophyllene on selected behavioral parameters and on seizure-induced oxidative stress.

2. Materials and methods

2.1. Animals and reagents

Adult C57BL/6 mice (25–35 g, 60–90 day-old) of both genders were used. Animals were maintained under controlled light and environment (12:12 h light–dark cycle, 24 ± 1 °C, 55% relative humidity) with free access to water and food (Purotrato, Santa Maria, RS, Brazil). All experimental protocols aimed to keep the number of animals used to a minimum, as well as their suffering. These were conducted in accordance with national and international legislation (guidelines of Brazilian Council of Animal Experimentation — CONCEA — and of U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals — PHS Policy), and with the approval of the Ethics Committee for Animal Research of the Federal University of Santa Maria (process 016/2014). Pentylenetetrazol (PTZ) and β-caryophyllene were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). All other chemicals were reagent grade and purchased from local suppliers.

2.2. Behavioral seizure evaluation

Animals were individually placed in glass boxes and injected with increasing doses of β-caryophyllene (10, 30, or 100 mg/kg; i.p.) or its vehicle (0.9% NaCl containing 0.05% Tween 80). Sixty minutes thereafter, PTZ (60 mg/kg, i.p.) was injected, and the animals were observed for 15 min. During this time, we recorded the latency to myoclonic jerks, latency to generalized seizure, and the duration of the first generalized seizure. All solutions were administered at 10 mL solution per kg of body weight. Doses and schedules for drug injections were selected based on the literature [11,14] and on pilot experiments.

2.3. Electroencephalographic (EEG) recordings

Seizure activity and the effect of β-caryophyllene were evaluated in a subset of animals (n = 3–4) by EEG recordings. For recording electrode implantation, animals were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, two stainless steel screw electrodes were placed over the parietal cortex, along with a ground lead positioned over the nasal sinus. The electrodes were connected to the multipin socket and were fixed to the skull. Meloxicam (200 mg/kg, s.c.) and metamizole (100 mg/kg, s.c.) were administrated immediately before and for three days after the surgical procedure.

Six days after the surgery, each animal was transferred to a Plexiglas cage (25 × 25 × 40 cm) and habituated for 20 min before EEG recordings. The mouse was then connected to a 100× headstage preamplifier (model #8202-DSE3) in a low-torque swivel (Pinnacle Technology Inc., Lawrence, KS, USA), and the EEG was recorded using a PowerLab 16/30 data acquisition system running LabChart 7.2 software (AD Instruments, Castle Hill, Australia). Routinely, a 30-min baseline recording was obtained to establish an adequate control period. After the baseline recording, mice were injected with β-caryophyllene (100 mg/kg) or vehicle, 60 min before the injection of PTZ. Following convulsant injection, the EEG signals were recorded for 15 min. Electroencephalographic signals were amplified, filtered (0.1 to 50.0 Hz, bandpass), digitalized (sampling rate 1024 Hz), and stored in a PC for off-line analysis.

2.4. Behavioral tests

In order to investigate the effects of an anticonvulsant dose of β-caryophyllene (100 mg/kg) on exploratory behavior and motor skills of the mice, we evaluated performance in the open-field, object recognition, rotarod, and forced swim tests. Independent groups of mice were used in each test, and each animal was used only once.

2.4.1. Open-field test

Animals were placed in the central area of a round open field (56 cm in diameter), which had its floor divided into 10 equal areas. Five areas of the apparatus had their borders limited by the walls of the arena and were considered as peripheral areas. The remaining five areas that had no contact with the walls of the apparatus were considered as central areas. β-Caryophyllene (100 mg/kg) or its vehicle were injected 60 min before the beginning of the test, and the number of crossed areas (crossings) as well as the number of rearing responses (animal stands on its hind legs) were recorded for 5 min.

2.4.2. Object recognition test

The object recognition test consisted of three sessions, namely, habituation #1 (first training session), habituation #2 (second training session, 4 h after training), and memory evaluation (test session, 24 h after habituation #1). During the first training session, two identical objects (transparent cylindrical plastic bottles) were equidistantly placed in the center of the same open-field arena described above, and the time spent in exploration of each object was recorded for 10 min. Four hours thereafter (habituation #2), one of the bottles was replaced for a new object (plastic red apple), and the time spent in exploration of each object was measured for 10 min. Finally, 24 h after training, the plastic red apple was replaced with another new object (triangular plastic cup), and the time spent in exploration of each object was recorded for 10 min (test session). Any subjects that failed to complete a minimum of 10-second exploration time in the test trial (three vehicle-treated and three β-caryophyllene-treated animals) were excluded from the analysis. The object recognition index was calculated with the following formula: recognition index = (time spent in new object) / (time spent in the new object + time spent in the familiar object). β-Caryophyllene (100 mg/kg) or its vehicle were injected 60 min before the beginning of the test session.

2.4.3. Rotarod test

Fine motor coordination was assessed by using the rotarod test. The task consisted of one training session and one testing session, carried out 24 h apart. Trial starts with the mouse being placed in the apparatus (3.7 cm rod diameter, 8 rpm constant speed) and ends when the mouse falls off the rod or after reaching the cutoff time of 60 s two consecutive times. During the training session, the maximum number of attempts was 10. A resting time of 60 s was allowed between each trial. In the test session, mice were observed for 4 min, and the latency to the first fall was recorded. β-Caryophyllene (100 mg/kg) or its vehicle were injected 60 min before the beginning of the test session.

2.4.4. Forced swim test

Mice were placed in individual, clear polyvinyl chloride (PVC) cylinders (30 cm tall × 10 cm diameter) containing 23–25 °C water (20 cm-deep to prevent the mouse's tail from touching the cylinder bottom). Water was changed between subjects. The immobility time during the 5 min of test was recorded. Immobility was assigned when no additional activity was observed other than that required to keep the mouse's head above water. β-Caryophyllene (100 mg/kg) or its vehicle were injected 60 min before the beginning of the test session.

2.5. Neurochemical assays

In order to investigate whether an anticonvulsant dose of β-caryophyllene (100 mg/kg) would protect against seizure-induced oxidative stress, we measured thiobarbituric acid-reactive substances (TBARS) and nonprotein thiols (NPSH) in an independent group of mice treated with β-caryophyllene and PTZ. The experimental design consisted of four groups: vehicle + NaCl 0.9%, vehicle + PTZ, β-caryophyllene + NaCl 0.9%, and β-caryophyllene + PTZ. β-Caryophyllene or its vehicle were injected 60 min before the injection of PTZ or NaCl 0.9%, and after 15 min of behavioral seizure evaluation, the cerebral cortex and hippocampus of the animals were homogenized in 30 mM Tris–HCl buffer (pH 7.4).

2.5.1. TBARS content

Lipid peroxidation was estimated by measuring TBARS and was expressed in terms of malondialdehyde (MDA) content, according to the method described by Boeira et al. [16]. Thiobarbituric acid-reactive substance content was measured in a medium containing 100 μL of tissue homogenate, 15 μL of 8.1% SDS, 60 μL of acetic acid buffer (2.5 M, pH 3.4), and 115 μL of 0.81% thiobarbituric acid. The mixture was heated at 95 °C for 120 min in a water bath. After cooling to room temperature, absorbance was measured in the supernatant at 532 nm. The results were calculated as nmol MDA/mg of protein.

2.5.2. NPSH content

Nonprotein thiol levels were determined according to the method described by Boeira et al. [16]. Homogenates were precipitated with TCA (10%) and subsequently centrifuged at 3000 ×g at 4 °C for 10 min. After the centrifugation, the supernatant fraction (100 μL) was added to a reaction medium containing potassium phosphate buffer (1 M, pH 7.4) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, 10 mM). The NPSH levels were measured spectrophotometrically at 412 nm. The results were calculated using a standard curve constructed with reduced glutathione (GSH) and expressed as nmol NPSH/mg of protein.

2.6. Statistical analyses

Kolmogorov–Smirnov test was used to verify data normality, and Bartlett's test was used to verify homogeneity of variances. Seizure latencies were analyzed by the Kruskal–Wallis test followed by post hoc analyses with the Mann–Whitney test with sequential Holm–Bonferroni correction. Dose–response relationships were tested with the Spearman's rank correlation test. Seizure duration was analyzed by one-way ANOVA. Data from behavioral testing were analyzed by Student's t-test. The TBARS and NPSH contents were analyzed by a two-way ANOVA. A probability of $P < 0.05$ was considered significant.

3. Results

Fig. 1 shows the effect of increasing doses of β-caryophyllene on the seizures induced by PTZ. Statistical analysis revealed that the dose of 100 mg/kg increased the latency for the first myoclonic jerk (167% average increase versus vehicle-treated mice) [$H(4) = 8.069$, $P < 0.05$ — Fig. 1A]. Spearman's analysis revealed a significant positive correlation between the doses of β-caryophyllene and the latency to PTZ-induced myoclonic seizures ($rs = 0.4237$, $P < 0.005$). Onset latency [$H(4) = 1.542$, $P > 0.05$ — Fig. 1B] and duration [$F(3,41) = 0.5221$, $P > 0.05$ — Fig. 1C] of the first PTZ-induced generalized tonic–clonic seizure did not significantly change. Mortality rate was zero since no mouse died

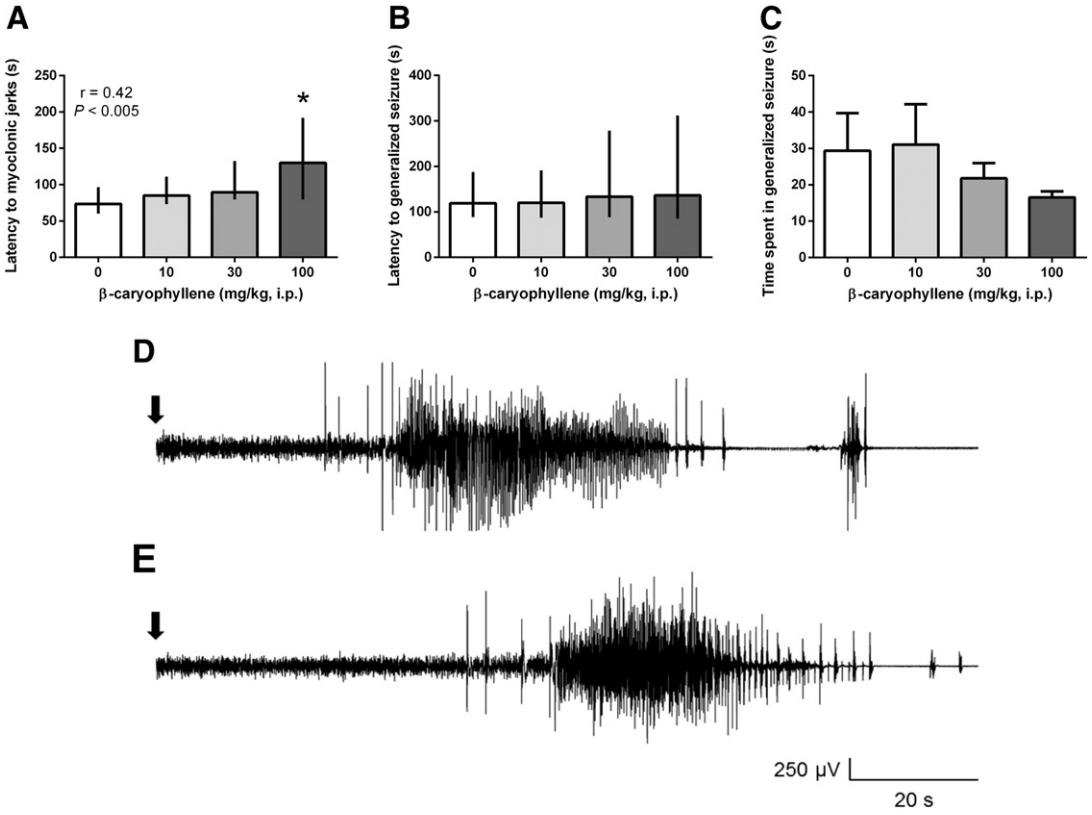

Fig. 1. Effect of β-caryophyllene on seizure behavior. Latency to (A) myoclonic jerks or (B) generalized tonic–clonic seizures and (C) duration of generalized seizures induced by PTZ were measured after the intraperitoneal administration of β-caryophyllene. Data are presented as median and interquartile range for n = 8–14 each group. The Spearman's correlation coefficient and its respective *P* appear as the inset in A. The asterisk indicates a statistically significant difference from control group ($P < 0.05$). Representative electroencephalograms observed in the cerebral cortex of mice after administration of (D) vehicle plus PTZ or (E) β-caryophyllene (100 mg/kg) plus PTZ are also shown. The arrow indicates the administration of PTZ.

Table 1
Effect of β-caryophyllene (100 mg/kg) on selected behavioral parameters. Independent groups of mice (n = 9 per group) were used in each test. Data are presented as mean ± S.E.M. Statistical analyses were performed with two-tailed unpaired Student's t.

Behavioral parameter	Treatment	
	Vehicle	β-Caryophyllene
Crossings	70.56 ± 5.85	59.00 ± 7.14
Rearing responses	7.00 ± 1.87	6.67 ± 2.16
Time spent in center (%)	9.83 ± 1.46	6.17 ± 1.37
Latency to fall (s)	194.4 ± 30.13	149.1 ± 27.34
Immobility time (s)	79.67 ± 19.63	50.78 ± 13.38

during the post-PTZ 15-min evaluation period. Electroencephalographic recordings confirmed and extended the data related to the effect of the β-caryophyllene on the seizures induced by PTZ (Fig. 1D–E). Treatment with β-caryophyllene (100 mg/kg) delayed the appearance on the EEG of PTZ-induced myoclonic jerks but did not alter the duration or the generalized seizure-associated wave patterns in the EEG recordings, which appeared as a combination of multispike plus slow waves, multiple sharp waves, and major seizure activity.

In order to investigate whether effects on behavior accompanied the anticonvulsant effect of β-caryophyllene, we tested an independent group of mice in the open-field, rotarod, forced swim, and object recognition tests. No significant differences were found in the number of crossings [$t(16) = 1.252$, $P > 0.05$ — Table 1], rearings [$t(16) = 0.1164$, $P > 0.05$ — Table 1], or time spent in the center [$t(16) = 1.823$, $P > 0.05$ — Table 1] in the open-field test. In addition, the latency to fall off the rod was not statistically different between vehicle-treated and β-caryophyllene-treated animals [$t(16) = 1.114$, $P > 0.05$ — Table 1]. Moreover, no significant differences were found in the immobility time in the forced swim test [$t(16) = 1.216$, $P > 0.05$ — Table 1]. Interestingly, a significant difference between vehicle-treated and β-caryophyllene-treated animals was found in the object recognition test. Animals treated with β-caryophyllene displayed higher values of object recognition index than their vehicle-treated counterparts [$t(14) = 4.204$, $P < 0.05$ — Fig. 2A]. The total time spent in object exploration during the test trial was not significantly different between β-caryophyllene-treated and vehicle-treated animals ($t(14) = 0.5874$, $P > 0.05$ — Fig. 2B).

In the last set of experiments, we investigated the effect of β-caryophyllene on seizure-elicited oxidative stress. Seizures induced by PTZ increased TBARS levels in the cerebral cortex [$F(1,24) = 4.45$, $P < 0.05$ — Fig. 3A] but not in the hippocampus [$F(1,24) = 0.0981$, $P > 0.05$ — Fig. 3B]. On the other hand, NPSH content was unaltered in the cerebral cortex [$F(1,24) = 0.2889$, $P > 0.05$ — Fig. 3C] but decreased in the hippocampus [$F(1,24) = 7.186$, $P < 0.05$ — Fig. 3D] after PTZ-induced seizures. Treatment with β-caryophyllene did not significantly alter these seizure-induced neurochemical changes.

4. Discussion

Increasing evidence suggests that β-caryophyllene displays neuroprotective actions. For instance, β-caryophyllene significantly attenuated morphological deterioration and lactate dehydrogenase release in mixed rat cortical neurons/glia cultures submitted to oxygen–glucose deprivation [11]. Moreover, incubation of C6 glioma cells with β-caryophyllene prevented the cytotoxicity induced by the excitatory amino acid glutamate [17]. Importantly, these in vitro findings are corroborated by results obtained from experiments in intact animals. In fact, administration of β-caryophyllene decreased cerebral infarct size and edema [11,14] and prevented the decline of neurological deficit scoring [14] after middle cerebral artery occlusion in rats. In addition, β-caryophyllene prevented cognitive impairment, β-amyloid burden, astrogliosis, and microglial activation in the APP/PS1 transgenic mice model of Alzheimer's disease [12].

Despite these compelling lines of evidence that β-caryophyllene is neuroprotective, to the best of our knowledge, only one study has investigated the effect of this natural product on seizures [13]. In the study by Liu et al. [13], mice received a 2-day treatment with β-caryophyllene (30 or 60 mg/kg, i.p.) before a single systemic dose of kainate. Animals pretreated with β-caryophyllene exhibited lower seizure scores when compared with the vehicle–kainate group, suggesting that treatment with β-caryophyllene attenuates the seizure activity induced by administration of this excitotoxin [13]. Taking into consideration our present results, systemic administration of β-caryophyllene increased latency to the myoclonic seizures induced by PTZ, a convulsant that has been widely used in the study of mechanisms of seizure generation, spreading, and termination, as well as development and screening of new compounds with anticonvulsant activity [18]. Importantly, EEG experiments confirmed the results from behavioral seizure analysis, demonstrating that the β-caryophyllene-induced delay of seizure onset also occurs at the electrophysiological level. Altogether, these results suggest that β-caryophyllene is anticonvulsant in seizure models with substantial predictive value for discovery of new anticonvulsants [18]. In this context, future studies shall evaluate the anticonvulsant potential of β-caryophyllene in other seizure models. Moreover, other treatment schedules (e.g., chronic administration) should be tested, since repeated administration may change anticonvulsant efficacy [19]. In fact, the efficacy of some clinically useful anticonvulsants, including primidone, valproate, and vigabatrin, may increase during prolonged treatment [19].

The present results are particularly interesting in light of the recognized low toxicity of β-caryophyllene. In fact, the acute administration of doses up to 5 g/kg in mice did not cause mortality or signs of toxicity [20], and the LD50 in rats or rabbits exceed 5 g/kg [21]. Moreover, β-caryophyllene was not mutagenic in *Salmonella typhimurium* strains or in an unscheduled DNA synthesis assay at concentrations up to 150 mg/plate and 10 mg/mL [22]. In this context, it is important to

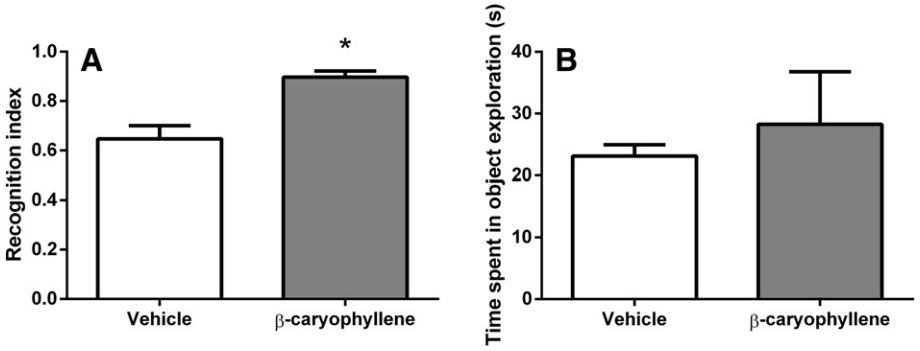

Fig. 2. Effect of β-caryophyllene (100 mg/kg) on mice performance in the object recognition test. (A) Recognition index and (B) total time spent in object exploration during test trial. β-Caryophyllene (100 mg/kg) or its vehicle were injected 60 min before the beginning of the test session. Data are mean + standard error of the mean for n = 8 per group. The asterisk indicates a statistically significant difference from control group ($P < 0.05$).

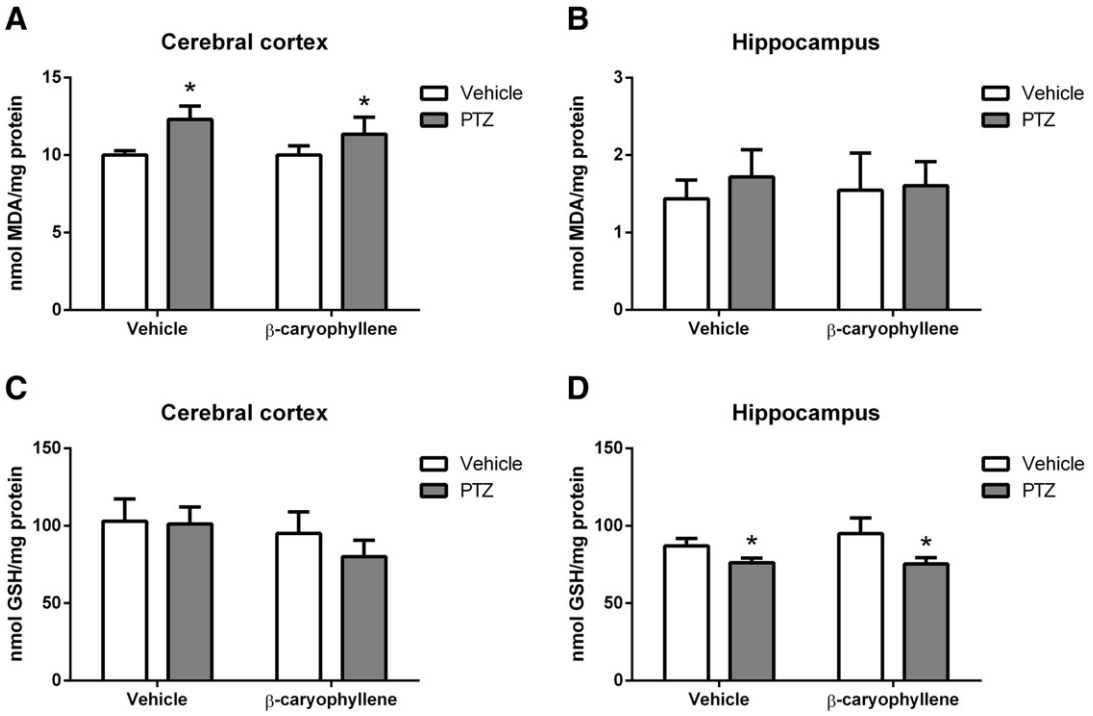

Fig. 3. Lack of effect of β-caryophyllene (100 mg/kg) on seizure-induced oxidative stress. TBARS content in the (A) cerebral cortex and (B) hippocampus and NPSH content in the (C) cerebral cortex and (D) hippocampus. Data are mean + standard error of the mean for n = 6–8 each group. The asterisk indicates a statistically significant difference from control group ($P < 0.05$).

note that the presently reported anticonvulsant effect of a single dose of β-caryophyllene was not accompanied by adverse effects related to spontaneous (open-field exploration) or forced (swimming test) locomotor activity and on motor coordination performance (rotarod), further suggesting the low toxicity of this natural product.

Interestingly, we found that animals treated with β-caryophyllene displayed higher values of object recognition index than their vehicle-treated counterparts. Since the schedule for β-caryophyllene administration in this set of experiments was planned to match that used in the anticonvulsant test (i.e., injection of the compound 60 min before PTZ), β-caryophyllene was administered 60 min before the beginning of the test session (24 h after habituation). Regarding this point, this finding may be suggestive of improved memory recall ability after administration of β-caryophyllene. Notwithstanding, it should be noted that the object recognition test has become a widely used model for the investigation into learning and memory alterations, but results from this test may also reflect changes in behavioral parameters of attention, anxiety, and preference for novelty [23]. Therefore, additional studies are needed to investigate the potential clinical implications of these findings as well as its underlying mechanisms.

We also found that administration of an anticonvulsant dose of β-caryophyllene did not protect against seizure-induced increase in TBARS content and decrease in NPSH levels, suggesting that β-caryophyllene did not affect PTZ-induced oxidative stress. In this context, Liu et al. [13] reported that a two-day pretreatment with β-caryophyllene attenuated the kainic acid-induced increase in TBARS levels and decrease of superoxide dismutase, catalase, and glutathione peroxidase activities. Although these findings appear somewhat conflicting with our present results, in the study by Liu et al. [13], the protective effect of β-caryophyllene against seizure-induced oxidative stress accompanied a significant decrease of seizure severity (lower seizure scores of β-caryophyllene-treated animals). Conversely, in the present study, β-caryophyllene did not have an effect on the duration of generalized seizures, suggesting that PTZ-induced seizure severity was not altered. In this context, since it has been demonstrated that kainic acid-induced seizure scores correlate positively with MDA or protein carbonyl contents and negatively with glutathione peroxidase activity or reduced/oxidized glutathione ratio [24], it is possible that improvement of seizure-induced oxidative stress depends on a concomitant decrease of seizure severity. Alternatively, important methodological differences between the present study and that by Liu et al. [13] should be noted, including the convulsant (kainic acid versus PTZ) and the schedule of β-caryophyllene administration (one versus two injections).

5. Conclusion

β-Caryophyllene displayed anticonvulsant activity against seizures induced by PTZ and improved recognition index in the object recognition test. No adverse effects on motor functions were detected in the open-field, rotarod, or forced swim tests. More studies are needed to evaluate the mechanisms underlying the anticonvulsant effects of β-caryophyllene as well as its potential as a safer, better tolerated, new anticonvulsant compound.

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Conflict of interest

The authors declare no conflict of interest.

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