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Full Length Research Paper

Carvacrol, (–)-borneol and citral reduce convulsant activity in rodents

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Carvacrol, a monoterpenic phenol present in essential oils of the Labiatae family, has been used through the ages as a source of flavor in food and for medicinal purposes. Borneol is a monoterpene found in several species of *Artemisia* and *Dipterocarpaceae,* used for anxiety, pain and anesthesia in traditional Chinese. Citral, a mixture of two geometrical isomers (neral and geranial), is one of the most important compounds in some citrus oils and has central nervous system (CNS) properties. The anticonvulsant effect of carvacrol (CARV), (–)-borneol (BOR) and citral (CIT) was investigated in two animal models of epilepsy. Mice were pretreated with CARV, BOR or CIT (50, 100, and 200 mg/kg, i.p.) 30 min before pentylenetetrazole (PTZ) or maximal electroshock (MES) tests, the two most important animal epilepsy tests. The latency for development of convulsions and protection percentage was recorded. In order to investigate the involvement of GABAergic system, flumazenil was utilized. These monoterpenes, CARV in a higher, but not in a lower dose ($p < 0.001$), BOR and CIT in all doses ($p < 0.05$ or $p < 0.001$), were capable of promoting an increase of latency for the development of convulsions induced by PTZ. Additionally, these compounds were efficient in preventing the tonic convulsions ($p < 0.05$) induced by MES. However, the GABAergic neurotransmitter system might be involved, at least in BOR effects. Henceforth, our results suggest that CARV, BOR and CIT possess anticonvulsant activity effect against PTZ-induced convulsions and MES.

Key words: Carvacrol, (–)-borneol, citral, anticonvulsant activity.

INTRODUCTION

Epilepsy is the most frequent neurologic infection characterized by excessive temporary neuronal discharge. The overall prevalence of the disease is 1.0% of the population and up to 50 million people worldwide (McCagh et al., 2009). Despite the development of several new anticonvulsants, the treatment of epilepsy still remains inadequate. About one third of patients do not respond well to currently available treatment, even if multiple drugs with complementary activities are used (WHO, 2001). Furthermore, more than 50% of epilepsy patients experience undesired side effects of drug treatment and even life-threatening conditions (Löscher and Schmidt, 2006). However, numerous therapeutic approaches are being used to better control the widespread clinical

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problem of convulsive attacks that affect a significant proportion of the human population (Araújo et al., 2003). One approach is to discover new anticonvulsive drugs from natural sources with enhanced efficacy and minimal side effects (Nsour et al., 2000; Schachter, 2009).

In this regard, essential oils are volatile compounds produced by aromatic plants that have been found to exhibit a variety of biological properties, such as analgesic (Melo et al., 2010), cardiovascular (Menezes et al., 2010) and anticonvulsant (De Sousa et al., 2006; De Sousa et al., 2007a; De Sousa et al., 2007b; Silva et al., 2009). Monoterpenes are the primary components of these essential oils and the effects of many medicinal herbs have been attributed to them (Carlini, 2003; Quintans-Júnior et al., 2008a).

Carvacrol (CARV), the predominant monoterpenic phenol present in many essential oils of the Labiatae family including *Origanum*, *Satureja*, *Thymbra*, *Thymus* and *Corydothymus* species, has been used through the ages as a source of flavor in food and for medicinal purposes (Kirimer et al., 1995). Some biological effects, such as analgesic (Aydin et al., 1996) and antioxidant (Aeschbach et al., 1994) activities have also been reported.

Borneol (BOR), a bicyclic monoterpenoid alcohol, present in the essential oils of numerous medicinal plants of the Dipterocarpaceae (e.g. *Dipterocarpus turbinatus* tree), Lamiaceae (e.g. *Rosmarinus officinalis* or *Salvia officinalis*), Valerianaceae (e.g. *Valeriana officinalis*) or Asteraceae (e.g. *Matricaria chamomilla*) families, has been used as a therapeutic agent in China for over 1500 years (Hattori, 2000; Xiao-fei et al., 2008; Horváthová et al., 2009). According to the pharmacopoeia of People's Republic of China, BOR is an ingredient in about 63 herbal products (SPC, 2005). In folk remedies, BOR is used also for several other purposes, such as treatment of abdominal pain, particularly stomachache, for application on injuries, burns, to relieve rheumatic pains, haemorrhoids, skin diseases, ulcerations of the mouth, ear, eye or nose and in aromatherapy (Wang et al., 2006; Horváthová et al., 2009). Likewise, citral (CIT) is one of the most important flavor compounds in some citrus oils. Citral consists of a mixture of two geometrical isomers, neral and geranial, and is highly susceptible to acid-promoted and oxidative degradation (Kimura et al., 1983). Gurgel do Vale et al. (2002) demonstrated central nervous system (CNS) properties of citral, limonene and myrcene in rodents, such as hypnotic and anxiolytic. However, little is known about the CNS effects of CARV, (–)-BOR and citral.

Since several monoterpenes demonstrate CNS activity, such as anticonvulsant and anxiolytic, and since there is no literature reports of studies about the anticonvulsant activity of CARV, BOR and CIT, we decided to further explore these effects using the two most important animal models of epilepsy (Smith et al, 2007): pentylenetetrazole (PTZ)-induced convulsion and maximal electroshock test (MES).

MATERIALS AND METHODS

Drugs

The drugs used were PTZ, phenytoin (PHE), polyoxyethylene-sorbitan monolated (Tween 80), purchased from Sigma (St Louis, MO., USA); Diazepam (DZP) and flumazenil (FLU), from Cristália (Brazil). Carvacrol (CARV, with 99.9% purity), BOR with 95.0% purity and CIT with 96.0% purity, (mixture of *cis* and *trans*) were purchased from Sigma (St Louis, MO., USA). Agents were administered intraperitoneally (i.p.) at a dose volume of 0.1 ml/10 g.

Experimental animals and research protocol approval

Male Swiss mice (30 - 35 g), 2 - 3 months of age, were used throughout this study. They were kept in the departmental animal house inside a well cross ventilated room at 25 ± 2°C on a 12 h light/dark cycle (lights on 06:00 - 18:00 h) with free access to food (purina) and water. They were used in groups of 10 animals each. All experiments were carried out between 09:00 am and 04:00 pm in a quiet room. Experimental protocols and procedures were approved by the Animal Care and Use Committee (CEPA/UFS # 26/09) at the Federal University of Sergipe, Brazil.

PTZ convulsion test

The inhibitory effect of CARV, BOR and CIT were tested on PTZ-induced convulsion. Mice were divided into groups (n = 10, each), the first group worked as control and received vehicle (2 drops of tween 80 0.2% in distilled water, the solvent for monoterpenes), whereas the second group was treated with diazepam (DZP, 2 mg/kg, i.p.). A pilot study showed that the vehicle did not possess anticonvulsant effect (data not shown). The remaining groups received an injection of CARV, BOR or CIT (50, 100, or 200 mg/kg, i.p.). After 30 min of drug administration, the mice were treated with PTZ (i.p.) at a dose of 60 mg/kg to induce clonic convulsions (Smith et al., 2007). The latency and percentage of clonic convulsions inhibition were registered. The incidence of deaths was registered during 48 h after the injection of PTZ.

Effects of flumazenil on PTZ-induced convulsion

The effects of selective γ-amino-butyric acid-benzodiazepine ($GABA_A$-BZD) receptor antagonist, flumazenil (File and Pellow, 1986), on the anticonvulsant activity of CARV, BOR and CIT were investigated. In the experimental groups; mice were given flumazenil (FLU) (10 mg/kg, i.p.) 20 min before the administration of monoterpenes (CARV, BOR or CIT, 200 mg/kg, i.p.) (50 min before the injection of PTZ). In the standard group, the animals received FLU 20 min before the administration of diazepam (DZP, 2 mg/kg, i.p.) (50 min before the injection of PTZ). The anticonvulsant activity of CARV, BOR, CIT or DZP in mice pretreated with FLU was assessed.

Maximal electroshock test

MES produces reproducible tonic convulsions characterized by tonic hindlimb extension (THE) (Oliveira et al., 2001). In this experiment, electroconvulsive shock (130 V, 150 pulses/s, 0.5 s) was delivered through auricular electrodes (ECT UNIT 7801- Ugo Basile) to induce THE. Mice were divided into groups (n = 10, per group), the first group was used as control and received vehicle, while the second group was treated with PHE (25 mg/kg, i.p.). The other groups received an injection of monoterpenes, in a similar

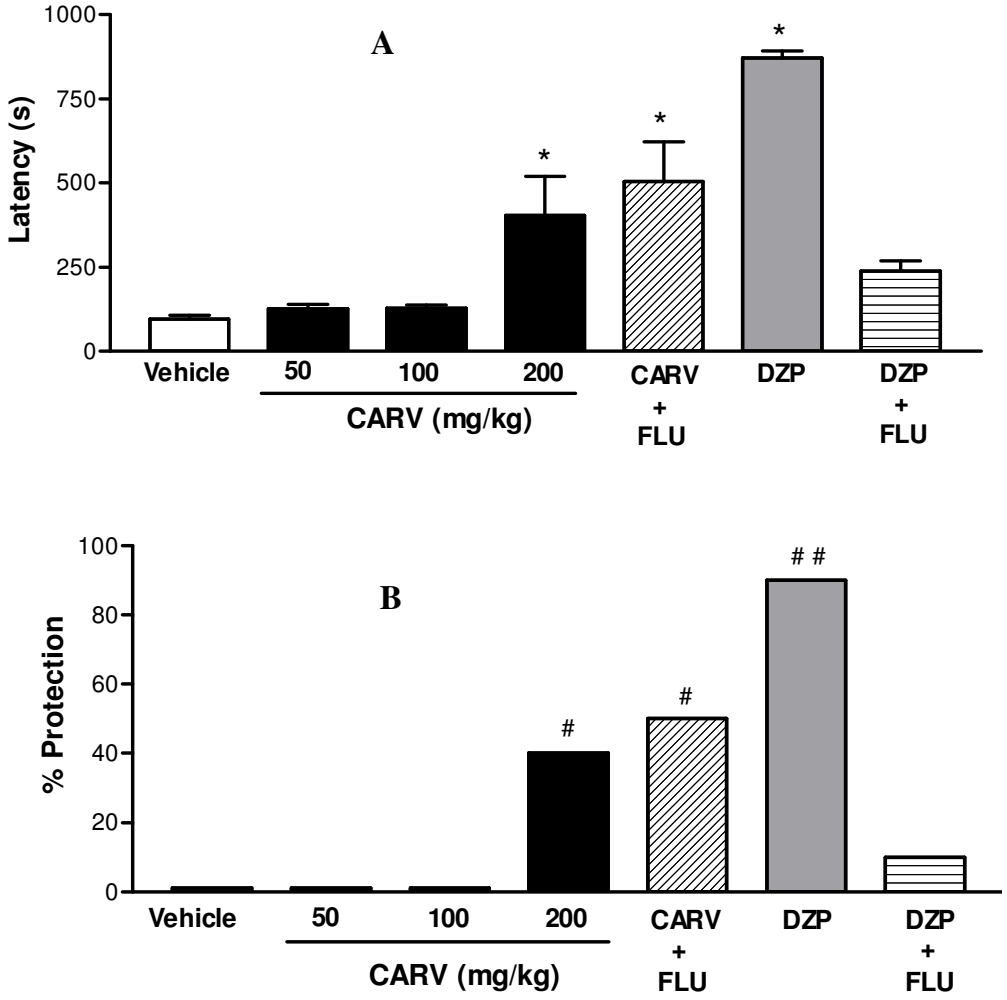

Figure 1. Protective effect of carvacrol (CARV, 50, 100 or 200 mg/kg, i.p.) against convulsion produced after PTZ (60 mg/kg, i.p.) injection in mice. The effects of CARV on latency (A) and percentage (%) protection (B). Results are expressed as mean ± SEM, n = 10 in each group. *, **Significant at $p < 0.05$ and 0.001, respectively, as compared to control (vehicle), one way ANOVA followed by Dunnet's test. #, ##significant at $p < 0.01$ and 0.001, respectively, compared with control (no protection), Fisher`s exact test.

way, before the experiment. After 30 min, all groups received electroconvulsive shock. The animals that did not exhibit THE were considered protected during the time of tonic convulsion (Tortoriello and Ortega, 1993).

Statistical analysis

The data obtained were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett`s t test. The incidence (%) of clonic or tonic-clonic convulsions, as well as the mortality, were evaluated by Fisher`s exact test. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

The effects of CARV, BOR or CIT on PTZ-induced convulsions are shown in Figure 1, 2 and 3, respectively. Single dose i.p. administration of PTZ (60 mg/kg, i.p.) caused clonic convulsions as well as lethality in mice.

As shown in Figure 1 (A and B), pretreatment with the CARV at higher dose (200 mg/kg, i.p.) significantly increased time onset of clonic convulsions (latency) ($p < 0.001$) and protected animals from PTZ-induced convulsion ($p < 0.01$) in mice as compared to control group. However, mortality did not alter through CARV administration (data not shown). The pretreatment with DZP (2 mg/kg, i.p.) significantly prolonged the latencies and was effective in preventing clonic seizures induced by PTZ in 90% of the animals ($p < 0.001$).

In the control group, PTZ consistently induced clonic seizures in 100% of mice. Pretreatment with the BOR at all doses significantly reduced ($p < 0.05$ or $p < 0.001$) in a dose-dependent manner; the incidence of clonic PTZ convulsion, significantly increased the latency of clonic

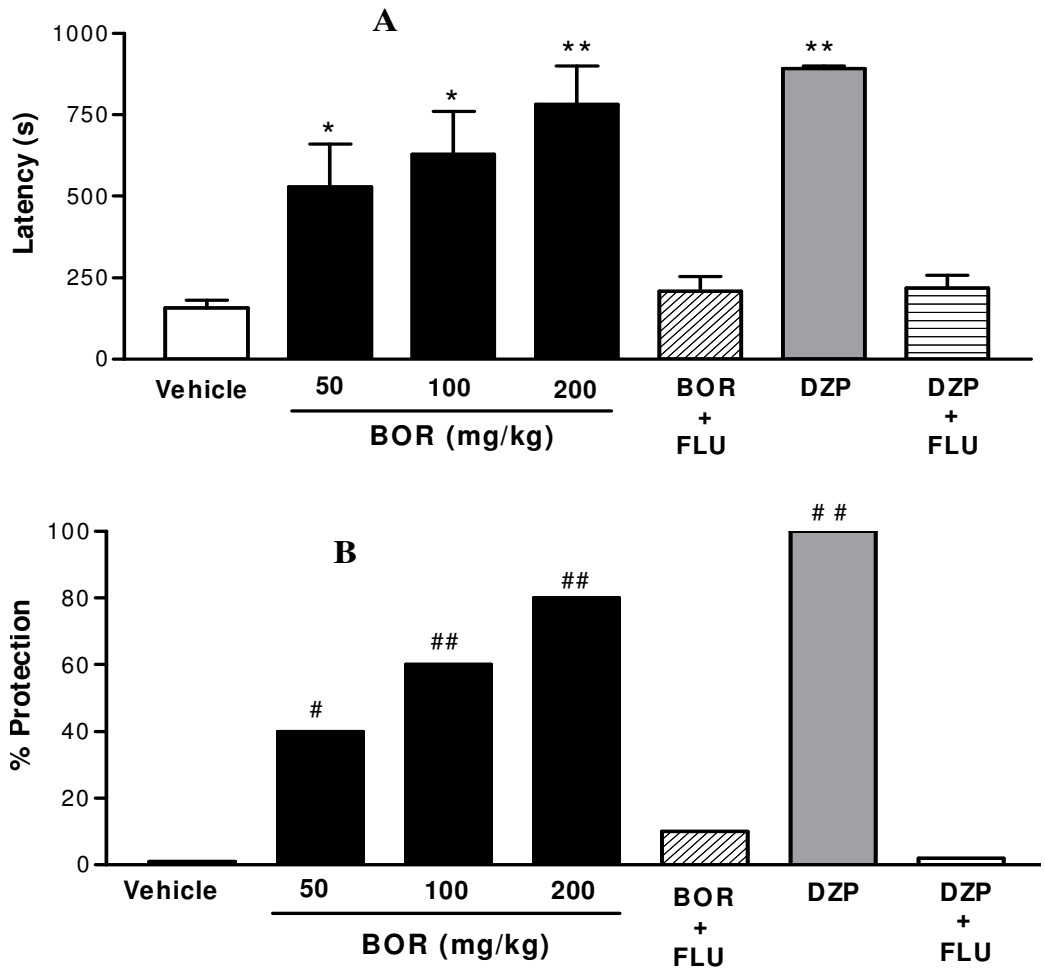

Figure 2. Protective effect of (–)-borneol (BOR, 50, 100 or 200 mg/kg, i.p.) against convulsion produced after PTZ (60 mg/kg, i.p.) injection in mice. The effects of BOR on latency (A) and percentage (%) protection (B). Results are expressed as mean ± SEM, n = 10 in each group. *, **Significant at $p < 0.05$ and 0.001, respectively, as compared to control (vehicle), one way ANOVA followed by Dunnet's test. #, ##significant at $p < 0.01$ and 0.001, respectively, compared with control (no protection), Fisher`s exact test.

convulsion at the tested doses. DZP (2 mg/kg) blocked the clonic convulsions and mortality (data not shown) induced by PTZ in mice. Nevertheless, the administration of FLU (10 mg/kg, i.p.) antagonized the effect of BOR or DZP in the prolongation of convulsion latency (Figure 2).

Mice pretreated with CIT, at all doses, showed significant ($p < 0.05$ or $p < 0.001$) increase in time onset of clonic convulsions (latency) which significantly ($p < 0.05$) protected animals from PTZ-induced convulsion in mice. These results were not antagonized by FLU, as shown in Figure 3 (A and B). Additionally, mortality was significantly reduced ($p < 0.05$) by CIT (100 and 200 mg/kg) administration (data not shown).

As shown in Table 1, CARV (200 mg/kg), BOR (100 and 200 mg/kg) and CIT (100 and 200 mg/kg) were effective in preventing tonic convulsions induced by MES. However, the highest dose of CIT (40% of protection) was less effective as compared to 100 mg/kg (60% of protection). Standard group (PHE, 25 mg/kg) completely protected the animals from tonic convulsion ($p < 0.001$).

DISCUSSION

The main goal of this study is to evaluate the effects of CARV, BOR or CIT on the anticonvulsant activity using two animal tests of convulsion: PTZ-induced convulsions and MES.

The new drugs with antiepileptic activity (AEDs) are identified and developed as a result of their 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 for the discovery of

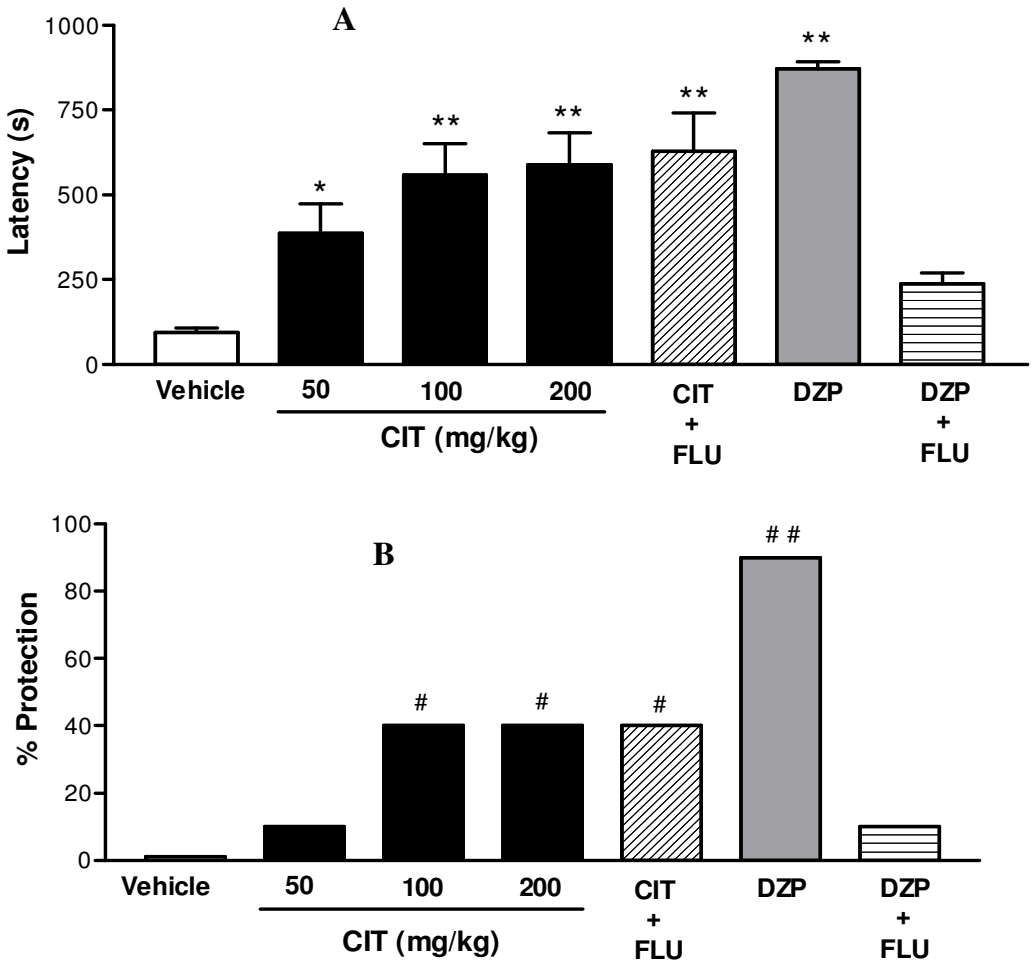

Figure 3. Protective effect of citral (CIT, 50, 100 or 200 mg/kg, i.p.) against convulsion produced after PTZ (60 mg/kg, i.p.) injection in mice. The effects of CIT on latency (A) and percentage (%) protection (B). Results are expressed as mean ± SEM, n = 10 in each group. *, **Significant at $p < 0.05$ and 0.001, respectively, as compared to control (vehicle), one way ANOVA followed by Dunnet's test. #, ##significant at $p < 0.01$ and 0.001, respectively, compared with control (no protection), Fisher`s exact test.

Table 1. Effects of CARV, BOR or CIT on maximal electroshock test in mice.

Treatment	Dose (mg/kg)	% Inhibition of convulsion	Time spent of convulsioning (s)
Vehicle	-	0	16.0 ± 0.3
PHE	25	100##	0.0 ± 0.0**
CARV	50	0	15.8 ± 1.2
CARV	100	20	19.7 ± 4.2
CARV	200	40#	9.0 ± 3.8*
BOR	50	0	17.3 ± 1.9
BOR	100	40#	8.5 ± 1.6*
BOR	200	60##	7.1 ± 2.3**
CIT	50	0	15.0 ± 0.9
CIT	100	60##	8.0 ± 3.6*
CIT	200	40#	7.0 ± 1.1*

*, **Significant at $p < 0.05$ and 0.001, respectively, as compared to vehicle (control group), one way ANOVA followed by Dunnet's test. #, ##significant at $p < 0.01$ and 0.001, respectively, compared with control (no protection), Fisher`s exact test.

new antiepileptic drugs (White et al., 1998; Löscher and Schmidt, 2006; Smith et al., 2007). PTZ is considered as an experimental model for the "generalized absence convulsions" (Oliveira et al., 2001). PTZ has been reported to produce convulsions by inhibiting GABA neurotransmission (Löscher and Schmidt, 2006). GABA is the main inhibitory neurotransmitter substance in the brain, and is widely implicated in epilepsy. Enhancement of GABAergic neurotransmission has been shown to inhibit or attenuate convulsion, while inhibition of GABAergic neurotransmission or activity is known to promote and facilitate convulsion (Haruna, 2000; Smith et al., 2007). BOR and CIT were more effective than CARV for all doses and increased time onset of clonic convulsions, while CARV was effective only at the highest dose. Nevertheless, antagonism of PTZ-induced convulsion suggests that the CARV, BOR and CIT might have effects on GABAergic neurotransmission.

Generally, compounds that possess anticonvulsant activity in the epilepsy of the lesser types are effective in inhibiting the convulsions in the model of the PTZ (Vida, 1995). Therefore, the capacity of these monoterpenes (BOR and CIT with better effects than CARV) to promote protection against PTZ-induced convulsions suggests the profile of an anticonvulsant drug that could be useful in these types of crises. In order to determine the role of $GABA_A$-BZD receptors participation in the monoterpenes (CARV, BOR or CIT) induced anticonvulsant effects, FLU, a specific antagonist of the BZD site in the $GABA_A$-BZD receptor complex, was used (File and Pellow, 1986). However, the presence of FLU was not capable of reverting the anticonvulsant effect of monoterpenes (CARV and CIT), suggesting that the mechanism of action does not involve the direct activation of the BZD site of the $GABA_A$-BZD receptor. In contrast, when the effect of BOR was evaluated using FLU, it produced significant antagonism. Interestingly, this result was different from that found by Granger et al. (2005) that showed a positive modulation on the channel which is unrelated to the $GABA_A$-BZD receptor.

An increasing number of natural agents have been found to modulate ionotropic GABA receptors independently of benzodiazepine sites, including the monoterpenes (+)- and (–)-borneol (Granger et al., 2005), thymol (Johnston, 2005), citronellol (De Sousa et al., 2006) and α,β-epoxy-carvone (Almeida et al., 2008). Granger et al. (2005) demonstrate that (+)- and (-)-borneol are highly efficacious as positive modulators of the effects of low GABA concentrations at human recombinant $\alpha_1\beta_{2\gamma 2L}$ $GABA_A$ receptors.

MES test is the most frequently used animal model for the identification of anticonvulsant activity of drugs for the "grand mal" (Oliveira et al., 2001; Smith et al., 2007). All the currently available antiepileptic activity, which are clinically effective in the treatment of generalized tonic-clonic convulsions, PHE, phenobarbital, lamotrigine and carbamazepine, are effective in the MES test (Löscher, 1998). Our results suggest that monotepenes (CARV, BOR or CIT) may prove to be important chemical leads for future antiepileptic activity.

Some essential oils of plants have been used in folk medicine as an antiepileptic remedy (Quintans-Júnior et al., 2008b). Pharmacological studies have demonstrated the anticonvulsant properties of these essential oils in animal models and their biological effects have been attributed to the monoterpenes (Nsour et al., 2000; Schachter, 2009). The protective effects of the essential oils can occur through different mechanisms due to the diversity of chemical constituents. Several terpenes of essential oils present sedative and hypnotic effects (De Sousa et al., 2007c). Recently, our group demonstrated that citronellal, α-terpineol and citronellol and other monoterpenes, possess significant CNS depressant activity, such as sedative, hypnotic, anticonvulsant and central analgesic properties (De Sousa et al., 2006; De Sousa et al., 2007a; Melo et al., 2010; Quintans-Júnior et al., 2010). These effects may be associated with GAB Aergic system and decreased peripheral nerve excitability.

Conclusion

Summarizing our data, the results suggest a possible anticonvulsant effect of CARV, BOR and CIT. The precise mechanisms of possible behavioral effects of these compounds are not yet fully understood. However, regarding BOR, these effects are probably observed through the modulation of GABAergic system through enhancement of $GABA_A$-BZD receptor. Nevertheless, more studies will be required for the elucidation of the relationship between this effect and the neuronal mechanisms.

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Abbreviations:

CNS, Central nervous system; **CARV,** carvacrol; **BOR,** (–)-borneol; **CIT,** citral; **PTZ,** pentylenetetrazole; **MES,** maximal electroshock; PHE, phenytoin; **DZP,** diazepam; **FLU,** flumazenil; **THE,** tonic hindlimb extension; $GABA_A$-**BZD,** γ-amino-butyric acid-benzodiazepine.

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