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Effects of isopulegol on pentylenetetrazol-induced convulsions in mice: Possible involvement of GABAergic system and antioxidant activity

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ABSTRACT

The present study investigated the effects of isopulegol, a monoterpene alcohol, in PTZ-induced convulsions and verified possible involved mechanisms. Saline, isopulegol or diazepam were intraperitonealy injected 30 min before PTZ. The latency for development of convulsions and mortality, as well as the mortality protection percentage was recorded. For investigating the involvement of GABAergic system, flumazenil was utilized. The activity of antioxidant enzyme catalase as well as the levels of reduced glutathione and lipid peroxidation were measured in brain hippocampus. Similarly to diazepam, isopulegol significantly prolonged the latency for convulsions and mortality of mice. All animals were protected against mortality at higher dose of isopulegol. Flumazenil pretreatment decreased the prolongation of seizure latency induced by both diazepam and isopulegol, although it was not able to reverse the latency and protection percent for mortality. Isopulegol also significantly prevented PTZ-induced increase in lipid peroxidation, preserved catalase activity in normal levels, and prevented the PTZ-induced loss of GSH in hippocampus of mice. These results suggest that the anticonvulsant and bioprotective effects of isopulegol against PTZ-induced convulsions are possibly related to positive modulation of benzodiazepine-sensitive $GABA_A$ receptors and to antioxidant properties.

1. Introduction

In the last years, an increasing number of studies have demonstrated that natural products from folk remedies have contributed significantly in the discovery of modern drugs worldwide. In Brazil, information from ethnic groups on indigenous traditional medicine has played a vital role in the discovery of novel products from plants as chemotherapeutic agents, which have resulted in a significant body of publications in this area [1–5].

Essential oils are concentrated volatile aromatic compounds produced by aromatic plants that have been found to exhibit a variety of biological properties, such as analgesic [4], spasmolytic [6] and anticonvulsant [3] activities. Monoterpenes are the primary components of these essential oils and the effects of many medicinal herbs have been attributed to them [7–10]. Isopulegol (*p*-menth-8-en-3-ol) (Fig. 1A) is a monoterpene alcohol of *p*-menthane family, intermediate in the preparation of (−)-menthol [11] (Fig. 1B), and it is present in the essential oils of various plants species, such as *Eucalyptus citriodora* Hook [12] and *Zanthoxylum schinifolium* [13]. Isopulegol has been used in the manufacture of fragrances with blossom compositions [14], however, reports with reference to its therapeutic effects, as described in the present work, are scarce in literature.

Epilepsy is one of the most common serious neurological conditions, affecting more than 50 million people worldwide

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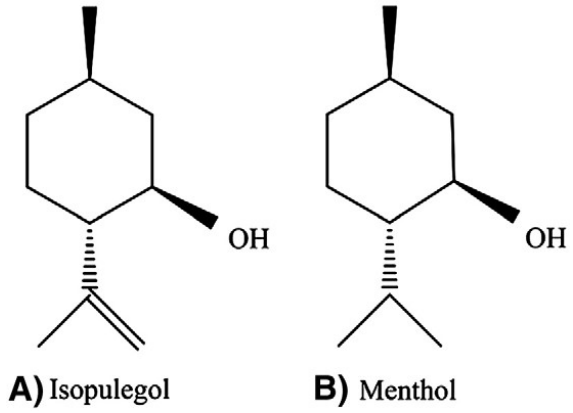

Fig. 1. Structures of isopulegol (A) and menthol (B).

[15]. The current clinically available antiepileptic drugs are associated with a variety of side-effects and chronic toxicity [16]. In this regard, great efforts have been made in search of new antiepileptic drugs with enhanced efficacy and minimal side-effects, including studies of active constituents obtained from medicinal plants [15,17].

Disturbances of the naturally existing balance between the concentrations of inhibitory and excitatory neurotransmitters in the central nervous system (CNS) are assumed to be the main cause of convulsive episodes. Thus, a deficiency in the γ-aminobutyric acid (GABA) concentration may result in many pathological changes in the CNS that can be further implicated in epilepsy. In this context, pentylenetetrazole (PTZ), a selective blocker of GABA transmission, is the most commonly used convulsant chemical agent in animal models to screen drugs for their potential anticonvulsant properties [18–20].

Currently, several monoterpenes, in general, volatile terpenoids, have been reported to have neuroactive properties. Their actions in experimental animal models have been mainly linked to protection against anxiety [21], insomnia [8] and seizures [5,17]. Such disorders are considered likely to involve $GABA_A$ receptors [22], which are the predominant ionotropic receptors for fast inhibitory neurotransmission in the mammalian central nervous system (CNS) [23]. Therefore, stimulation of $GABA_A$ receptors by GABA or added positive modulators (such as benzodiazepines and barbiturates) produces anxiolysis, sedation, anaesthesia, myorelaxation and anticonvulsant actions [24], effects similar to that evoked by several monoterpenes [7,25].

Thus, the blockade of the convulsions chemically induced by PTZ, in rodents, is a characteristic of drugs with depressant effects on the CNS [17], presumably by increasing the inhibitory effects of GABA. Currently, a considerable number of moterpenoids are of interest for their actions as positive modulators of $GABA_A$ receptors. For instance, menthol and structural analogs, including isopulegol and isomenthol, were described as potent positive modulators of these receptors [26]. Corroborating these data, in recent works, our group [21] and collaborators [27] showed that isopulegol presented anxiolitic- and depressant-like pharmacological actions on the CNS in mice. Such effects were reversed by Flumazenil, a recognized competitive antagonist at the central benzodiazepine receptors, suggesting a benzodiazepine-like modulation from isopulegol. These data led us to verify in the present study whether isopulegol would be able to exert any protector effect in PTZ-induced convulsions in mice.

A growing body of evidence has also suggested that reactive oxygen species (ROS) generation may underlie the convulsant and neurotoxic effects of PTZ [28,29]. In fact, several studies have demonstrated an increase in reactive species formation in CNS of animals exposed to PTZ-induced convulsions model, and the treatment with antioxidants seems to attenuate convulsions and/or ROS-induced damage [18,19]. In a recent study conducted by our group [30], we detected an antioxidant action from isopulegol in ethanol-induced ulcer model in mice. Taking these data in account, in the present study, we aimed also to evaluate whether the antioxidant properties from isopulegol would be related to its possible anticonvulsant effect. For that, the activity of antioxidant enzyme catalase, as well as the levels of reduced glutathione (GSH) and lipid peroxidation were measured in brain hippocampus of mice.

2. Materials and methods

2.1. Animals

Male *Swiss* mice (20–30 g) were maintained in standard cages, at a controlled temperature (23 ± 1 °C) with a 12 h dark/light cycle, and food and water *ad libitum*. All animals were treated in accordance with the current law and the NIH Guide for Care and Use of Laboratory Animals. The studies were performed under the consent and surveillance of the Committee of Ethics in Animal Research, Department of Physiology and Pharmacology, Faculty of Medicine, Federal University of Ceará.

2.2. Drugs and dosage

Isopulegol was isolated of the technical grade isopulegol (Dierberger-Brazil) by column chromatography. It was emulsified with 0.2% Tween 80 (Sigma-USA), dissolved in distilled water and intraperitoneally administered at doses of 100 (64.88 mM/kg) and 200 mg/kg (129.64 mM/kg). Flumazenil 2.0 mg/kg (0.54 mM/kg) (União Química/Brazil) and Diazepam (DZP) 1 mg/kg (0.35 mM/kg) (Geigy), were intraperitoneally injected after being dissolved in distilled water. Pentylenetetrazole (PTZ) 99 mg/kg (71.65 mM/kg) (União Química/Brazil) was dissolved in distilled water and subcutaneously administered. Controls received vehicle at the same volume (10 ml/kg) and through the same route as the other treated groups.

2.3. Pentylenetetrazole-induced convulsions

Mice were divided into four groups of 8–10 animals each and treated as follows. The control group received vehicle (saline 0.9% in 3% of Tween 80, i.p.) and the second group was treated with DZP 1 mg/kg (0.35 mM/kg), i.p.. After a pilot test, we identified the more effective doses of isopulegol in this model. So, the two remaining groups were injected with isopulegol at doses of 100 (64.88 mM/kg) or 200 mg/kg (129.64 mM/kg), i.p.. After 30 min, convulsions were induced in all groups by PTZ at 99 mg/kg (71.65 mM/kg), s.c., being its CD97 value, i.e. the dose of PTZ necessary to evoke clonic

seizures in 97% of animals tested [20]. Mice were further observed during 20 min. The latencies to the first clonic seizures, as well as to lethality were recorded. The protection percentage against mortality was also calculated.

2.4. Effects of flumazenil on the anticonvulsant activity of isopulegol

For this assay, six groups of 8–10 mice each were selected. In the first group, mice were given flumazenil 2 mg/kg (0.54 mM/kg), i.p., 15 min before the administration of isopulegol 200 mg/kg (129.64 mM/kg), i.p.. Thirty minutes after treatment with isopulegol, this group received PTZ 99 mg/kg (71.65 mM/kg), s.c.. In the second group, the animals received flumazenil 2 mg/kg (0.54 mM/kg), i.p., 15 min before the administration of diazepam 1 mg/kg (0.35 mM/kg), i.p., and 30 min later they received PTZ 99 mg/kg (71.65 mM/kg), s.c.. Three groups were injected diazepam 1 mg/kg (0.35 mM/kg, i.p.), flumazenil 2 mg/kg (0.54 mM/kg, i.p.), and vehicle (10 ml/kg, i.p.) 30 min before the administration of PTZ 99 mg/kg (71.65 mM/kg, s.c.). The anticonvulsant activity of isopulegol and diazepam in mice pretreated or not with flumazenil was assessed and compared with the control (vehicle) group [31,32].

2.5. Biochemical evaluation (enzymatic assays)

After PTZ-induced convulsions experiment, animals were sacrificed by cervical dislocation, their brains were quickly removed and the hippocampus was dissected for preparation of homogenates 10% (w/v) in 0.05 M phosphate buffer, pH 7.4 or EDTA 0.02 M (GSH measurements) for the enzymatic assays. The protein concentration was measured according to the method described by Lowry et al. [33].

2.5.1. Lipid peroxidation assay

Lipid peroxides formation was analyzed by measuring the thiobarbituric-acidreacting substances (TBARS) in the homogenates, as previously described by Huong et al. [34]. Lipid peroxidation was determined by the absorbance at 532 nm and was expressed as µmol of malondialdehyde (MDA)/mg of protein.

2.5.2. Catalase activity determination

Catalase activity was measured by the method that employs hydrogen peroxide to generate H2O and O2 as described by Maehly and Chance [35]. The activity was measured by the degree of this reaction. The reaction was followed at 230 nm. Standard curve was established using purified catalase (Sigma, MO, USA) under identical conditions. All samples were diluted with 0.1 mmol/L phosphate buffer (pH 7.0) to provoke an inhibition of 50% diluent rate (i.e., the uninhibited reaction) and results were expressed as µM/min/µg protein.

2.5.3. Measurements of glutathione (GSH) levels

GSH levels were evaluated to estimate endogenous defenses against oxidative stress. The method was based on Ellman's reagent (DTNB) reaction with free thiol groups. Production levels of GSH were determined in hippocampus homogenates as described by Sedlak and Lindsay [36]. GSH level was determined by the absorbance at 412 nm and was expressed as ng of GSH/g wet tissue.

2.6. Statistical analyses

Results are presented as mean ± S.E.M. Data were analyzed by ANOVA followed by Student–Newman–Keuls's *post hoc* test or by Fisher's exact test. Results were considered significant at $p<0.05$.

3. Results

3.1. Effects of isopulegol on pentylenetetrazole-induced convulsions

As illustrated in Fig. 2A, similar to diazepam, isopulegol 200 mg/kg (129.64 mM/kg) caused a significant increase of latency (s) for development of PTZ-induced convulsions, as

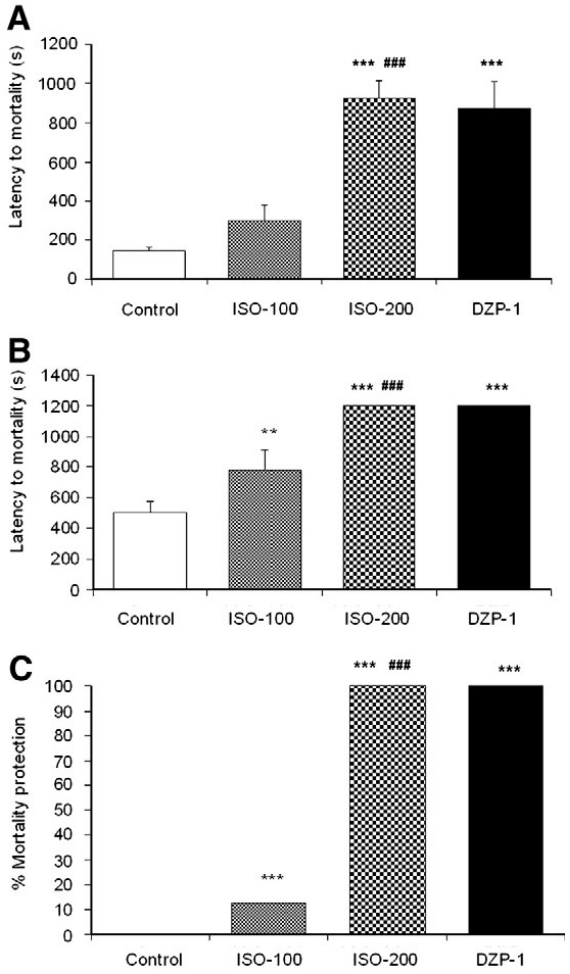

Fig. 2. Effects of isopulegol 100 (64.88 mM/kg, i.p.) and 200 mg/kg, (129.64 mM/kg, i.p.) and diazepam 1 mg/kg (0.35 mM/kg, i.p.) on PTZ-induced seizure in mice. All groups received PTZ 99 mg/kg (71.65 mM/kg, s.c.) 30 min after the treatments. The latency for development of convulsions is shown in A and for mortality in B. The percentage of protection against mortality is shown in C. Values represent the mean ± S.E.M. One-way ANOVA followed by Student–Newman–Keuls's as the *post hoc* and Fisher's exact test were used. $^{**}p<0.01$, $^{***}p<0.001$, as compared with control values; $\#\#\#p<0.001$, as compared with ISO-100 group.

compared with control group treated only with vehicle (control: 144.2 ± 20.1 s; ISO-200: 919.3 ± 90.7 s; DZP-1: 869.9 ± 121.2 s, $p<0.05$). The increase of latency detected with minor dose (100 mg/kg/64.88 mM/kg) was not statistically significant. Fig. 2B shows that isopulegol caused a dose-dependent increase of latency for mortality (control: 500.6 ± 69.6 s; ISO-100: 782.0 ± 128.8 s; ISO-200: 1200.0 ± 0.0 s; DZP-1: 1200.0 ± 0.0 s; $p<0.05$). Interestingly, at the dose of 200 mg/kg (129.64 mM/kg), similar to diazepam, isopulegol gave 100% protection against mortality, while at the dose of 100 mg/kg (64.88 mM/kg) there was a 12.5% protection (Fig. 2C).

3.2. Effects of flumazenil on the anticonvulsant activity of isopulegol

The flumazenil 2 mg/kg (0.54 mM/kg) pretreatment reversed the effects of both diazepam and isopulegol in prolonging convulsion latency, as compared to the control (vehicle) group (control: 139.1 ± 18.1 2; ISO-200: 959.4 ± 86.5; FLU-2 + ISO-200: 318.7 ± 75.9; DZP-1: 911.6 ± 66.61; FLU-2 + DZP-1: 186.3 ± 44.5; $p<0.05$), as shown in Fig. 3A. However, flumazenil was not able to reverse the latency and protection percent for mortality in both isopulegol and diazepam groups (Fig. 3B and C). Animals treated only with flumazenil had shown no alteration on observed parameters, as compared to control groups.

3.3. Lipid peroxidation assay

TBARS levels, the indicator of lipid peroxidation, were significantly increased in the hippocampus of mice treated only with PTZ 99 mg/kg (71.65 mM/kg), when compared with the control group (vehicle) that had not received the stressor agent (saline: 8.2 ± 0.3; PTZ: 9.9 ± 0.3; $p<0.05$). However, the pretreatment with 100 (64.88 mM/kg) and 200 mg/kg (129.64 mM/kg) doses of isopulegol significantly prevented the PTZ-induced increased TBARS levels (ISO-100 + PTZ: 8.0 ± 0.4; ISO-200 + PTZ: 8.1 ± 0.3; $p<0.05$). Animals treated only with isopulegol, at both doses, presented no alteration in TBARS levels (Fig. 4A).

3.4. Catalase activity determination

In the PTZ group, a significant decrease of the catalase activity in hippocampus was observed as compared to the control group (vehicle) (saline: 40.8 ± 3.2; PTZ: 26.4 ± 3.0; $p<0.05$). Pretreatment with isopulegol 100 (64.88 mM/kg) and 200 mg/kg (129.64 mM/kg) was able to keep catalase activity to normal levels, similar to that observed in control group (ISO-100 + PTZ: 41.1 ± 2.8; ISO-200 + PTZ: 35.4 ± 2.8; $p<0.05$). The catalase activity on mice treated only with isopulegol (at both doses) was not significantly different in control values (Fig. 4B).

3.5. Measurements of glutathione (GSH) levels

Fig. 4C shows that the treatment of the mice with PTZ resulted in decreased levels of GSH in hippocampus when compared to the control group (saline: 390.0 ± 18.1; PTZ: 264.2 ± 23.3; $p<0.05$). Interestingly, isopulegol pretreatment not only significantly restored the GSH levels (at 100 mg/kg/64.88 mM/kg dose) (ISO-100 + PTZ: 359.6 ± 14.7; $p<0.05$) but also promoted a significant increase of GSH in mice pretreated with the higher dose of isopulegol (ISO-200 + PTZ: 537.2 ± 57.5; $p<0.05$), as compared to control. GSH levels were also increased in groups treated with isopulegol and not exposed to stressor agent (ISO-100: 469.1 ± 46.7; ISO-200: 535.2 ± 35.1; $p<0.05$), as compared to the saline group.

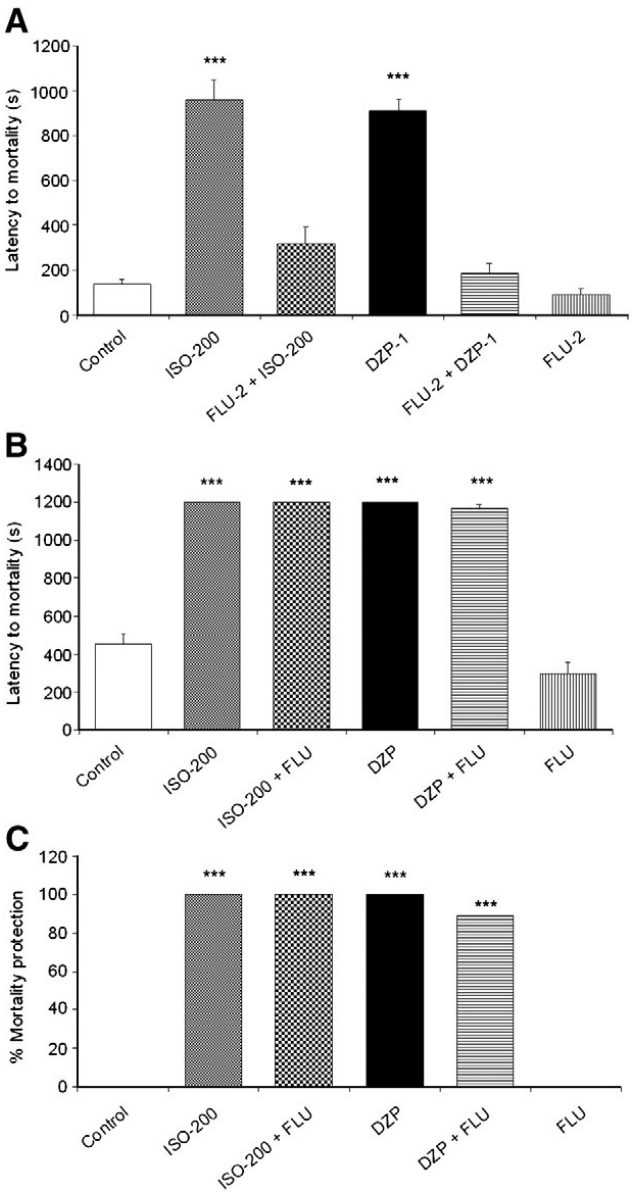

Fig. 3. Effects of flumazenil 2 mg/kg (0.54 mM/kg, i.p.) on the anticonvulsant activity of isopulegol 200 mg/kg (129.64 mM/kg, i.p.) and diazepam 1 mg/kg (0.35 mM/kg, i.p.). All groups received PTZ 99 mg/kg (71.65 mM/kg), s.c.. Latency for development of convulsions is shown in A and for mortality in B. The percentage of protection against mortality is shown in C. Values represent the mean ± S.E.M. One-way ANOVA followed by Student–Newman–Keuls's as the *post hoc* and Fisher's exact test were used. ***$p<0.001$, as compared with control values.

4. Discussion

Currently, several moterpenoids have been shown as positive modulators of $GABA_A$ receptors, including menthol

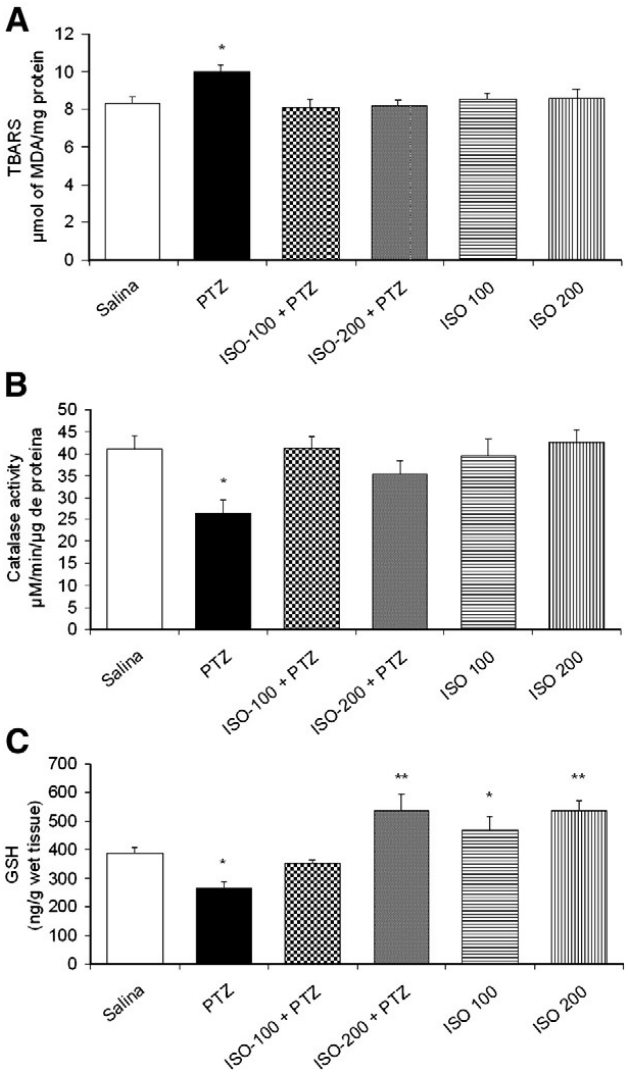

Fig. 4. Effects of isopulegol in lipid peroxidation level (A), activity of antioxidant enzyme catalase (B) and levels of GSH (C) in brain hippocampus of mice. Animals were treated or not with isopulegol 100 (64.88 mM/kg, i.p.) and 200 mg/kg, (129.64 mM/kg, i.p.) and administered or not with PTZ 99 mg/kg (71.65 mM/kg, s.c.), s.c.. Values represent the mean ± SEM. One-way ANOVA followed by Student–Newman–Keuls's as the *post hoc* was used. $^{*}p<0.05$, $^{**}p<0.01$ as compared to the control group (saline 0.9%). TBARS: tiobarbituric-acid-reacting substances; MDA: malondialdehyde; GSH: glutathione.

and related compounds such as isopulegol, isomenthol, borneol and others [7,22,25,26]. Corroborating these data, in recent works, our group [21] and collaborators [27] showed that isopulegol presented anxiolytic- and depressant-like pharmacological actions on CNS in mice. Considering that the blockade of the PTZ-induced convulsions, in rodents, is a characteristic frequently found in drugs with depressant effects on CNS [37], the present study verified whether isopulegol would be able to protect PTZ-induced convulsions in mice, as well as investigated possible mechanisms involved in this action.

Our present results showed that isopulegol significantly prolonged the latency for development of PTZ-induced convulsions (at higher dose) and death (at both doses) of animals. Pretreatment with isopulegol promoted a dose related protection against mortality and, interestingly, all animals survived at the higher dose of isopulegol tested. Similarly, in the group that received DZP the latencies significantly were prolonged and absence of mortality was also observed. These findings suggest that isopulegol presents a possible anticonvulsant and bioprotective profile similar to that seen with diazepam and several other monoterpenoids [17,31,38].

The $GABA_A$ receptor mediates the majority of inhibitory neurotransmission in the mammalian CNS and therefore it is a major focus in epilepsy research [39]. It is widely recognized that PTZ leads to tonic clonic seizures by suppressing the inhibitory effects of GABAergic transmission [40,41]. Similar to several monoterpenes such as menthol, isomenthol [26], borneol [7,22] and thymol [42], isopulegol was recently described as a positive allosteric modulator of $GABA_A$ receptors [26]. Thus, in order to investigate whether the anticonvulsant actions of isopulegol would be GABA-dependent, especially on the classical benzodiazepine sites, flumazenil, a benzodiazepine receptor ($GABA_A$) antagonist, was used. Our results showed that flumazenil pretreatment decreased the prolongation of convulsant latency induced by both diazepam and isopulegol, suggesting a possible involvement of direct activation of benzodiazepine site of $GABA_A$ receptor. These results are in agreement with the previous study conducted by our group [21], in which we also found that the anxiolytic- and depressor-like effects of isopulegol were possibly related to benzodiazepine receptors activation.

An increasing number of natural agents have been found to modulate ionotropic GABA receptors independently of benzodiazepine sites, including the monoterpenes (+)- and (−)-borneol [7], thymol [43] and α,β-epoxy-carvone [17]. As mentioned before in the present study, isopulegol (Fig. 1A) is a structural analog of menthol (Fig. 1B) and both monoterpenes act as positive allosteric modulator of $GABA_A$ receptors. In competition studies, the benzodiazepine antagonist flumazenil had no effect on menthol actions, suggesting that the effects from this terpenoid do not involve classical flumazenil-sensitive benzodiazepine sites. By contrast, the possible depressant effects on CNS exhibited by isopulegol in our previous [21] and in the present study seem be related to flumazenil-sensitive benzodiazepine sites. In the similar way, the depressant and anticonvulsant effects of other monoterpenes were also reversed by flumazenil, such as Epinepetalactone [38] and thymoquinone [31], suggesting a possible common binding site or interaction between theses monoterpenoids and benzodiazepines on the receptor.

Previous studies showed a structure–activity relationship for GABA receptor modulation by several monoterpenoids [44]. By comparing the structures, menthol and isopulegol are cyclohexanol-based analogs and differ only in a double bond in aliphatic chain of isopulegol (Fig. 1A and B), however, studies have evidenced different pharmacological effects from these monoterpenes. When administered to rodents by injection, menthol promoted ambulation [45], while its (−)-enantiomer is endowed with analgesic properties [46]. In our previous work [21] isopulegol exhibited a depressant effect in the pentobarbital-induced sleep test, while our collaborators [27] showed that (−)- menthol was ineffective in this test. Thus, variation in activities observed for both (−)- menthol

and isopulegol analogs suggests that structural differences between these monoterpenoids may also be taking place at the binding site on $GABA_A$ receptors. In fact, the therapeutically useful properties of different benzodiazepines may result from actions on different $GABA_A$ receptor subtypes. For instance, the α1-$GABA_A$ subunit has been related to sedative properties of benzodiazepines, while the α2-$GABA_A$ subunit is responsible for the anxiolytic properties [47]. In this context, we will intend continue to investigate the precise nature of the isopulegol-$GABA_A$ receptor interaction as an avenue of future research.

On the other hand, our results in the present work showed that flumazenil was not able to reverse the increased latency for mortality and consequent death protection percent induced by diazepam and isopulegol. These results agree with those reported by Nassiri-Asl et al. [15]. The authors found that the mortality protection offered by diazepam was poorly reversed by flumazenil pretreatment, suggesting that other mechanisms may to be involved specifically in bioprotective action from both diazepam and isopulegol.

A growing body of evidence has suggested that ROS generation may underlie the convulsant and neurotoxic effects of PTZ [29,48]. Several plant derived products that exhibit direct antioxidant activity are also being shown to have anticonvulsant activity [49]. Recently, our group detected antioxidant properties from isopulegol in ethanol-induced gastric ulcer model in mice [30]. In the light of these data, in the present study, we also investigated whether the isopulegol antioxidant actions would be involved in its anticonvulsant effect in PTZ model. For that, the activity of antioxidant enzyme catalase, as well as the levels of GSH and lipid peroxidation were measured in brain hippocampus of mice.

PTZ has been found to trigger the activation of membrane phospholipases, proteases and nucleases, which cause degradation of membrane phospholipids, proteolysis of cytoskeletone proteins, and protein phosphorylation [29,48]. In fact, in accordance to these studies, the present work showed that acute PTZ-induced seizures lead to an increase in lipid peroxidation in hippocampus of mice. Pretreatment of animals with both doses of isopulegol significantly prevented PTZ-induced elevations in lipid peroxidation. It is also known that convulsions followed by an increase in lipid peroxidation in brain tissue might be diminished by substances with antioxidant properties [28]. Thus, our results suggest that, at least in part, the isopulegol anticonvulsant and bioprotective activities are probably also related to its antioxidant effects against PTZ-induced lipid peroxidation in hippocampus of mice.

Reactive oxygen species, such as superoxide, hydroxyl radical, and hydrogen peroxide (H2O2), are normally produced in the brain. The conversion of H2O2 to H2O can be made by catalase and glutathione peroxidase (that involves the reduced glutathione, a cofactor of this enzyme). When produced in excess, ROS can also cause enzyme inactivation [50]. In fact, the current study showed that catalase activity in the PTZ group was significantly lower than in the control group, demonstrating an alteration in antioxidative brain defenses after stressor agent administration. Isopulegol pretreatment preserved catalase activity in normal levels. These findings suggest that isopulegol could help the brain cells to counteract the PTZ-induced ROS overproduction and the oxidative damage.

GSH is an essential tripeptide and endogenous antioxidant found in all animal cells. It reacts with the free radicals and can protect from singlet oxygen, hydroxide radicals, and superoxide radical damage [28]. Our results showed that the treatment of the mice with PTZ resulted in decreased levels of GSH in hippocampus. Interestingly, isopulegol not only prevented the loss of PTZ-induced GSH, but also promoted increased levels in groups without stress, suggesting that isopulegol could be possibly inducing an increase in GSH levels in hippocampus of mice. In a similar way, in previous studies conducted by our group [30], we found that isopulegol in itself was able to induce an increased GSH level also in hepatic and gastric tissues. Nevertheless, whether isopulegol increases GSH levels preventing depletion or inducing its synthesis requires further investigation.

Thus, our results are in agreement with previous studies that indicate increased oxidative stress during epileptic seizures [29], but they contrast with assays that show increased oxidative stress and decreased antioxidant defense during anticonvulsant therapy [51,52].

In a previous study conducted by our group (Silva et al. [21]), we have reported pharmacological activity from isopulegol at doses of 25 (16.20 mM/kg) and 50 mg/kg (32.41 mM/kg). In the present study, higher doses of isopulegol were needed for effective anticonvulsant and bioprotective effects against PTZ-induced convulsions. The acute oral toxicity of isopulegol was already evaluated in rats [53]. The results obtained and the LD_{50} ($\sim 1030 \pm 100$ mg/kg) value represent a relative low toxicity from isopulegol, which was particularly related to their possible central nervous system depressor proprieties. Despite the knowledge that monoterpenes are often considered to have low toxicity [5,54], we assume that additional studies are recommended to further ascertain the safety of isopulegol use.

In summary, the results of the present study demonstrate that isopulegol presents anticonvulsant and bioprotective effects against PTZ-induced convulsions. Such actions are possibly related to positive modulation of $GABA_A$ receptors and to antioxidant properties. These findings indicate that isopulegol could be a new useful natural neuroprotection and anticonvulsant tool. However, further studies are required in order to evaluate the exact mechanism involved in such action, as well as to better investigate the safety profile from isopulegol use.

Acknowledgments

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References

[1] Sousa FCF, Melo CT, Monteiro AP, Lima VT, Gutierrez SJ, Pereira BA, Barbosa-Filho JM, Vasconcelos SM, Fonteles MF, Viana GS. Antianxiety and antidepressant effects of riparin III from *Aniba riparia* (Nees) Mez (Lauraceae) in mice. Pharmacol Biochem Behav 2004;78(1):27–33.
[2] Sousa FCF, Monteiro AP, Melo CT, Oliveira GR, Vasconcelos SMM, França Fonteles MM, Gutierrez SMM, Barbosa-Filho JM, Viana GSB. Antianxiety effects of riparin I from *Aniba riparia* (Nees) Mez (Lauraceae) in mice. Phytother Res 2005;19(12):1005–8.

[3] Sousa DP, Gonçalves JCR, Quintans-Júnior L, Cruz JS, Araújo DAM, Almeida RN. Study of anticonvulsant effect of citronellol, a monoterpene alcohol, in rodents. Neurosci Lett 2006;401:231–5.
[4] Amaral JF, Silva MIG, Aquino Neto MR, Teixeira Neto PF, Moura BA, Melo CTM, Araújo FLO, Sousa DP, Vasconcelos PF, Vasconcelos SMM, Sousa FCF. Antinociceptive effect of the monoterpene R-(+)-limonene in mice. Biol Pharm Bull 2007;30(7):1217–20.
[5] De Sousa DP, Quintans JL, Almeida RN. Evaluation of the anticonvulsant activity of alfa-terpineol. Pharm Biol 2007;45:69–70.
[6] Lis-Balchin M, Hart S. Studies on the mode of action of the essential oil of lavender (*Lavandula angustifolia* P. Miller). Phytother Res 1999;13:540–2.
[7] Granger RE, Campbell EL, Johnston GAR. (+)- and (−)-borneol: efficacious positive modulators of GABA action at human recombinant $\alpha_1\beta_2\gamma_{2L}$ GABA(A) receptors. Biochem Pharmacol 2005;69:1101–11.
[8] Buchbauer G, Jäger W, Jirovetz L, Meyer F, Dietrich H. Effects of valerian root oil, borneol, isoborneol, bornyl acetate and isobornyl acetate on the motility of laboratory animals (mice) after inhalation. Pharmazie 1992;47(8):620–2.
[9] Santos FA, Rao VSN. Antiinflammatory and antinociceptive effects of 1, 8-cineole a terpenoid oxide present in many plant essential oils. Phytother Res 2000;14:240–4.
[10] Gherlardini C, Galeotti N, Mazzanti G. Local anaesthetic activity of monoterpenes and phenylpropanes of essential oils. Planta Med 2001;67:564–6.
[11] Serra S, Brenna E, Fuganti C, Maggioni F. Lipase-catalyzed resolution of p-menthan-3-ols monoterpenes: preparation of the enantiomer-enriched forms of menthol, isopulegol, trans- and cis-piperitol, and cis-isopiperitenol. Tetrahedron: Asymmetry 2004;14:3313–9.
[12] Rao BRR, Kaul PN, Syamasundar KY, Ramesh S. Comparative composition of decanted and recovered essential oils of *Eucalyptus citriodora* Hook. Flavor Frag J 2003;18:133–5.
[13] Paik SY, Kok KH, Beak SM, Paek SH, Kim JA. The essential oils from *Zanthoxylum schinifolium* Pericarp induce apoptosis of HepG2 human hepatoma cells through increased production of reactive oxygen species. Biol Pharm Bull 2005;28(5):802–7.
[14] Chuah GK, Liu SH, Jaenicke S, Harrison LJ. Cyclisation of citronellal to isopulegol catalysed by hydrous zirconia and other solid acids. J Catalasys 2001;200:352–9.
[15] Nassiri-Asl M, Shariati-Rad S, Zamansoltani F. Anticonvulsant effects of aerial parts of *Passiflora incarnata* extract in mice: involvement of benzodiazepine and opioid receptors. BMC Compl Alternative Med 2007;7:26.
[16] Samren EB, Van Duijn CM, Koch S, Hiilesma VK, Klepel H, Bardy AH, Mannagetta GB, Deichl AW, Gaily E, Granstrom ML, Meinardi H, Grobbee DE, Hofman A, Janz D, Lindhout D. Maternal use of antiepileptic drugs and the risk of major congenital malfor malformations: a joint European prospective study of human teratogenesis associated with maternal epilepsy. Epilepsia 1997;38(9):981–90.
[17] De Almeida RN, De Sousa DP, Nóbrega FFF, Claudino FS, Araújo DAM, Leite JR, Matte R. Anticonvulsant effect of a natural compound α, β-epoxy-carvone and its action on the nerve excitability. Neurosci Lett 2008;443:51–5.
[18] Patsoukis N, Zervoudakis G, Panagopoulos NT, Georgiou CD, Angelatou F, Matsokis NA. Thiol redox state (TRS) and oxidative stress in the mouse hippocampus after pentylenetetrazol-induced epileptic seizure. Neurosci Lett 2004;357:83–6.
[19] Oliveira MC, Furia AF, Royes LFF, Figueira MR, Myskiw JC, Fiorenza NG, Mello CF. Ascorbute modulates pentylenetetrazol-induced convulsions biphasically. Neuroscience 2004;128:721–8.
[20] Swiader MJ, Porêbiak J, Swiader K, Wielosz M, Czuczwar SJ. Influence of cimetidine on the anticonvulsant activity of conventional antiepileptic drugs against pentilenetetrazole-induced seizures in mice. Pharmacol Rep 2006;58:131–4.
[21] Silva MIG, Aquino Neto MR, Teixeira Neto PF, Moura BA, Amaral JF, De Sousa DP, Vasconcelos SMM, Sousa FCF. Central nervous system activity of acute administration of isopulegol in mice. Pharmacol Biochem Behav 2007;88(2):141–7.
[22] Johnston GAR, Chebib M, Duke RK, Fernandez SP, Hanrahan JR, Hinton T, Mewett KN. Herbal Products and GABA Receptors. Encycl Neurosci 2009;4:1095–101.
[23] Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, Langer SZ. Subtypes of γ-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. Pharmacol Rev 1998;50:291–313.
[24] Chebib M, Johnston GAR. GABA-activated ligand gated ion channels: medicinal chemistry and molecular biology. J Med Chem 2000;43:1427–47.
[25] Johnston GAR, Hanrahan JR, Chebib M, Duke RK, Mewett KN. Modulation of ionotropic GABA receptors by natural products of plant origin. Adv Pharmacol 2006;54:285–316.
[26] Watt EE, Betts BA, Kotey FO, Humbert DJ, Griffith TN, Kelly EW, Veneskey KC, Gill N, Rowan KC, Jenkins A, Hall AC. Menthol shares general anesthetic activity and sites of action on the GABAA receptor with the intravenous agent, propofol. Eur J Pharmacol 2008;590:120–6.
[27] De Sousa DP, Raphael E, Brocksom U, Brocksom TJ. Sedative effect of monoterpene alcohols in mice, a preliminary screening. Z Naturforsch [C] 2007;62(7–8):563–6.
[28] Oliveira AA, Almeida JPC, Freitas RM, Nascimento VS, Aguiar LMV, Júnior HVN, Fonseca FN, Viana GSB, Sousa FCF, Fonteles MMF. Effects of levetiracetam in lipid peroxidation level, nitrite–nitrate formation and antioxidant enzymatic activity in mice brain after pilocarpine-induced seizures. Cell Mol Neurobiol 2006;27(3):395–406.
[29] Obay BD, Ezel Tas D, Tümer C, Bilgin HM, Atmaca M. Dose dependent effects of ghrelin on pentylenetetrazole induced oxidative stress in a rat seizure model. Peptides 2008;29:448–55.
[30] M.I.G. Silva, B.A. Moura, M.R. Aquino Neto, A.R. Tomé, N.F.M. Rocha, A.M.R. Carvalho, D.S. Macedo, S.M.M. Vasconcelos, D.P. de Sousa, G.S.B. Viana, F.C.F. Sousa, Gastroprotective activity of isopulegol on experimentally induced gastric lesions in mice: investigation of possible mechanisms of action, Naunyn Schmiedebergs Arch. Pharmacol. doi:10.1007/s00210-009-0429-5.
[31] Hosseinzadeh H, Parvardeh S, Nassiri-Asl M, Mansouri MT. Intracerebroventricular administration of thymoquinone, the major constituent of Nigella sativa seeds, suppress epileptic seizures in rats. Med Sci Monit 2005;11(4):106–10.
[32] Nassiri-Asl M, Zamansoltani F, Shariati-Rad S. Possible role of GABAA-benzodiazepine receptor in anticonvulsant effects of Pasipay in rats. JCIM 2008;6(11):1170–3.
[33] Lowry H, Rosebrough NJ, Farr AL, Randall RJ. Protein measurements with the folin phenol reagent. J Biol Chem 1951;193:265–75.
[34] Huong NT, Matsumoto K, Kasai R, Yamasaki K, Watanabe H. In vitro antioxidant activity of Vietnamese ginseng saponin and its components. Biol Pharm Bull 1998;21:978–81.
[35] Maehly AC, Chance B. The assay catalases and peroxidases. Methods Biochem Anal 1954;1:357–9.
[36] Sedlak J, Lindsay RH. Estimation of total protein bound and nonprotein sulfhydril groups in tissues with Ellman reagents. Anal Biochem 1998;25:192–205.
[37] Anca JM, Lamela M, Calleja JM. Activity on the central nervous system of *Himanthalia* elongate. Planta Med 1993;59:218–20.
[38] Galati EM, Miceli N, Galluzzo M, Taviano MF, Tzakou O. Neuropharmacological effects of epinepetalactone from *Nepeta sibthorpii*. Behavioral and anticonvulsant activity. Pharm Biol 2004;42(6):391–5.
[39] Groves JO, Guscott MR, Hallett DJ, Rosahl TW, Pike A, Davies A, Wafford KA, Reynolds DS. The role of GABAA b2 subunit-containing receptors in mediating the anticonvulsant and sedative effects of loreclezole. Eur J Neurosci 2006;24:167–74.
[40] Fradley RL, Guscott MR, Bull S, Hallett DJ, Goodacre SC, Wafford KA, Garrett EM, Newman RJ, O'Meara GF, Whiting PJ, Rosahl TW, Dawson GR, Reynolds DS, Atack JR. Differential contribution of GABAA receptor subtypes to the anticonvulsant efficacy of benzodiazepine site ligands. J Psychopharm 2007;21(4):384–91.
[41] Crestani F, Assandri R, Tauber M, Martin JR, Rudolph U. Contribution of the α-1-GABAA receptor subtype to the pharmacological actions of benzodiazepine site inverse agonists. Neuropharmacology 1993;43:679–84.
[42] Priestley CM, Williamson EM, Wafford K, Sattelle DB. Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human GABAA receptors and a homooligomeric GABA receptor from *Drosophila melanogaster*. Br J Pharmacol 2003;140:1363–72.
[43] Johnston GA. GABA(A) receptor channel pharmacology. Curr Pharm Des 2005;11(15):1867–85.
[44] Hall AC, Turcottea CM, Bettsa BA, Yeunga WY, Agyemana AS, Burkb LA. Modulation of human GABAA and glycine receptor currents by menthol and related monoterpenoids. Eur J Pharmacol 2004;506:9–16.
[45] Umezu T, Sakata A, Ito H. Ambulation-promoting effect of peppermint oil and identification of its active constituents. Pharmacol Biochem Behav 2001;69:383–90.
[46] Galeotti N, Mannelli LDC, Mazzanti G, Bartolini A, Ghelardini C. Menthol: a natural analgesic compound. Neurosci Lett 2002;322:145–8.
[47] McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P, Garrett L, Bristow L. Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABAA receptor alpha(1) subtype. Nat Neurosci 2000;3:587–92.
[48] Akbasa SH, Yeginb A, Ozbenc T. Effect of pentylenetetrazol-induced epileptic seizure on the antioxidant enzyme activities, glutathione and lipid peroxidation levels in rat erythrocytes and liver tissues. Clin Biochem 2005;38:1009–14.
[49] Hsieh CL, Tang NY, Chiang SY, Hsieh CT, Lin JG. Anticonvulsive and free radical scavenging actions of two herbs, *Uncaria rhynchophylla* (MIQ) Jack and *Gastrodia elata* BL, in kainic acid-treated rats. Life Sci 1999;65:2071–82.

[50] Sharma AB, Sun J, Howard LL, Williams Jr AG, Mallet RT. Oxidative stress reversibly inactivates myocardial enzymes during cardiac arrest. Am J Physiol Heart Circ Physiol 2007;292(1):198–206.
[51] Bhattacharya SK, Ghosh P, Bose R. Pentylenetetrazol induced clonic convulsions in rat. Role of brain monoamines. Mater Med Pol 1978;10 (3):184–7.
[52] Ono H, Sakamoto A, Sakura N. Plasma total glutathione concentration in epileptic patients taking anticonvulsants. Clin Chm Acta 2000;298:135–43.
[53] Bhatia SP, McGinty D, Letizia CS, Api AM. Fragrance material review on isopulegol. Food Chem Toxicol 2008;46:185–9.
[54] Sun J. D-limonene: safety and clinical applications. Altern Med Rev 2007;12(3):259–63.