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Central nervous system activity of acute administration of isopulegol in mice

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Abstract

Isopulegol is a monoterpene alcohol intermediate in the preparation of (−)-menthol and it is present in the essential oils of various plants. This work presents behavioral effects of isopulegol in animal models of open field, elevated plus maze (EPM), rota rod, hole board, barbiturate-induced sleeping time, tail suspension and forced swimming tests in mice. Isopulegol was administered intraperitoneally to male mice at single doses of 25 and 50 mg/kg, while diazepam 1 or 2 mg/kg and imipramine 10 or 30 mg/kg were used as standard drugs. The results showed that, similar to diazepam (1 mg/kg), both doses of isopulegol significantly modified all the observed parameters in the EPM test, without alter the general motor activity in the open field test. In the same way, both doses of isopulegol increased the number of head dips in the hole-board test. Forced swimming and tail suspension tests showed that isopulegol (25 and 50 mg/kg) was able to induce a significant increase in the immobility time, in opposite to imipramine, a recognized antidepressant drug. There was a decrease in the sleep latency time and prolongation of the pentobarbital-induced sleeping time with both doses of Isopulegol. Different from diazepam (2 mg/kg), isopulegol (25 e 50 mg/kg) had no effect on the motor coordination of animals in the rota rod test. These results showed that isopulegol presented depressant- and anxiolytic-like effects.

Keywords: Isopulegol; Anxiolytic and depressant effects; Essential oils; Monoterpene

1. Introduction

Data from the World Health report (WHO, 2001) have demonstrated that approximately 450 million people suffer from a mental or behavioral disorder. The prevalence of anxiety mental condition has risen in recent years (Andrews et al., 2000) and depressive disorders are common and often disabling (Pan et al., 2005). Therefore, the search for new compounds as therapeutic alternatives for such disorders has progressed constantly (Irie et al., 2004; Klodzinska et al., 2004), including the studies realized by our group (Melo et al., 2006; Sousa et al., 2005a; Sousa et al., 2005b; Sousa et al., 2004).

An increasing number of studies have demonstrated that plant derived essential oils exhibit a variety of biological properties, such as anticonvulsant (De Sousa et al., 2006), analgesic (Almeida et al., 2001) and central activities (Gurgel do Vale et al., 2002). Several of these described effects are frequently attributed to monoterpenes, which are the major chemical components of those essential oils. Isopulegol (p-menth-8-en-3-ol) (Fig. 1), the substance used in this work, is a monoterpene alcohol, which is present in the essential oils of various plants, such as *Corymbia citriodora* Hook (Vernin et al., 2004), *Eucalyptus citriodora* Hook (Rao et al., 2003), *Zanthoxylum schinifolium* (Paik et al., 2005) and in other aromatic plant species (Mastelic et al., 1998). It is a 3-oxygenated monoterpene of *p*-menthane family, intermediate in the preparation of (−)-menthol (Serra et al., 2003), and have been used in the manufacture of fragrances with blossom compositions (Chuah et al., 2001). Recently, it was described that essential oils from *Zanthoxylum schinifolium* pericarp, which contained isopulegol, were able to induce apoptosis of HepG2 Human Hepatoma Cells, suggesting a plausible utilization of those oils as an anti-tumor agent in Hepatocellular carcinoma

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therapy (Paik et al., 2005). However, as far as we know, reports with reference to the therapeutic effects from isolated isopulegol, as we used in the present work, are scarce in literature.

As referred previously, isopulegol is an intermediate in the synthesis of (−)-menthol and therefore both compounds present similar structures (Serra et al., 2003). Previous studies in the literature demonstrated that menthol was able to promote ambulation in mice (Umezu and Morita, 2003; Umezu et al., 2001), an effect similar to that presented by psychostimulants. Such considerations led us to investigate whether isopulegol would be able to present comparable actions to that presented by menthol. For that, in this study we evaluated the behavior effects of isopulegol on animal models of central nervous system actions.

2. Materials and methods

2.1. Animals

Male Swiss mice (30 g) were used in each experiment and maintained at a controlled temperature (23±1 °C) with a 12 h dark/light cycle and free access to water and food. Animals were treated in accordance with the current law and the NIH Guide for Care and Use of Laboratory Animals.

2.2. Drugs

Isopulegol was emulsified with 0.2% Tween 80 (Sigma-USA) and dissolved in distilled water. Animals were treated with the substance at doses of 25 and 50 mg/kg, intraperitoneally, 30 min before the experiments. Controls received vehicle at the same volume (10 ml/kg) and were administered in the same route as the treated groups. Diazepam (DZP) 1 or 2 mg/kg (União Química/Brazil), Flumazenil (FLU) 2.5 mg/kg (União Química/Brazil) and Imipramine (IMP) 10 or 30 mg/kg (Geigy), used as standards, were intraperitoneally injected after dissolved in distilled water. Isopulegol was separated and obtained by column chromatography of the technical grade isopulegol (Dierberger — Brazil).

2.3. Experimental protocol

The animals were tested during the light period and observed in a closed room with constant temperature (23±1 °C) and poorly illuminated with a 15-V red light, except in the forced swimming test which was illuminated with normal light. All tests were performed in different days with distinct groups of animals.

2.4. Elevated plus maze test (EPM)

The elevated plus maze for mice (Lister, 1987) consisted of two perpendicular open arms (30×5 cm) and two closed arms (30×5×25 cm) also in perpendicular position. The open and closed arms were connected by a central platform (5×5 cm).

The platform and the lateral walls of the closed arms were made of transparent acrylic and the floor of black acrylic. The maze was 45 cm above the floor. After treatment, the animal was placed at the center of the plus maze with its nose in the direction of one of the closed arms, and observed for 5 min, according to the following parameters: number of entries in the open and closed arms, and time of permanence in each of them. The time of permanence measures the time spent by the animal in the open and closed arms. Anxiolytic compounds reduce the animal's aversion to the open arms and promotes the exploration thereof. The animals were divided into four groups with 10–15 per group.

2.5. Open-field test

The open-field area was made of acrylic (transparent walls and black floor, 30×30×15 cm) divided into nine squares of equal area. This apparatus was used to evaluate the exploratory activity of the animal (Archer, 1973). The observed parameters were as follows: number of squares crossed (with the four paws) and number of grooming and rearing. The animals were divided into four groups with 8−15 animals per group.

2.6. Hole-board test

The hole-board test for exploratory behavior in mice was used as described previously by Clark et al. (1971). The apparatus used was an Ugo Basile of 60×30 cm with 16 evenly spaced holes with built-in infrared sensors. In brief, adult male mice were randomly divided into four groups with 8−15 mice per group. Two groups received graded doses of isopulegol (25 and 50 mg/kg ip). One group received DZP (1 mg/kg ip) as standard and the remaining group named control received vehicle. Thirty minutes after the administration of respective drugs, the number of head dips into the holes was counted for each animal for 5 min.

2.7. Forced swimming test

The Porsolt et al. (1978) swimming test includes two exposures to a water tank, spaced 1 day apart. For these experiments the tank size was 22 cm in diameter and 40 cm in height. The tank had a rounded lid and contained 20 cm high fresh water at 25 °C. During the first exposure, mice were placed in the tank and left

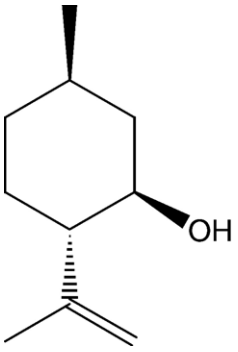

Fig. 1. Structure of isopulegol.

there for 5 min during which immobility time was registered. A mouse was considered immobile when it remained floating in the water, without struggling, making only very slight movements necessary to keep its head above water. The animals were divided into four groups with 10–13 animals per group. Each animal was used only once.

2.8. Tail suspension test.

The tail suspension test has been described by Steru et al. (1985). Male Swiss mice were housed in plastic cages in a 12 h light cycle with food and water freely available. Animals were transported from the housing room to the testing area in their own cages and allowed to adapt to the new environment for 1 h before testing. For the test, the animals were divided into four groups with 8–15 animals per group. They were suspended on the edge of a shelf 58 cm above a table top by adhesive tape placed approximately 1 cm from the tip of the tail. The duration of immobility is recorded for a period of 6 min.

2.9. Pentobarbital sleeping time

Thirty minutes after intraperitoneal administration of isopulegol at both doses or vehicle, all groups received sodium pentobarbital (40 mg/kg, intraperitoneally, i.p.). The time elapsed between the administration of pentobarbital until the loss of the righting reflex was recorded as the sleep latency. The time since the injection up to the loss of the righting reflex is recorded as sleep latency and the time elapsed between the loss and voluntary recovery of the righting reflex is recorded as sleeping time. (Wambebe, 1985; Rolland et al., 1991). For the test, the animals were divided into four groups with 8-15 animals per group.

2.10. Rota rod

Motor coordination was measured using the rota-rod test, adapted from Egashira et al. (2004). Animals were trained to the rota-rod test before the pharmacological test. Mice, divided into 12 groups, with 8 per group, were placed with the four paws on a 2.5 cm diameter bar, 25 cm above the floor and the time of permanence on the bar was measured for 2 min, for each animal. The rotating speeds were of 5, 15 or 40 rpm and different groups were used at all rotating speeds.

2.11. Statistical analyses

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

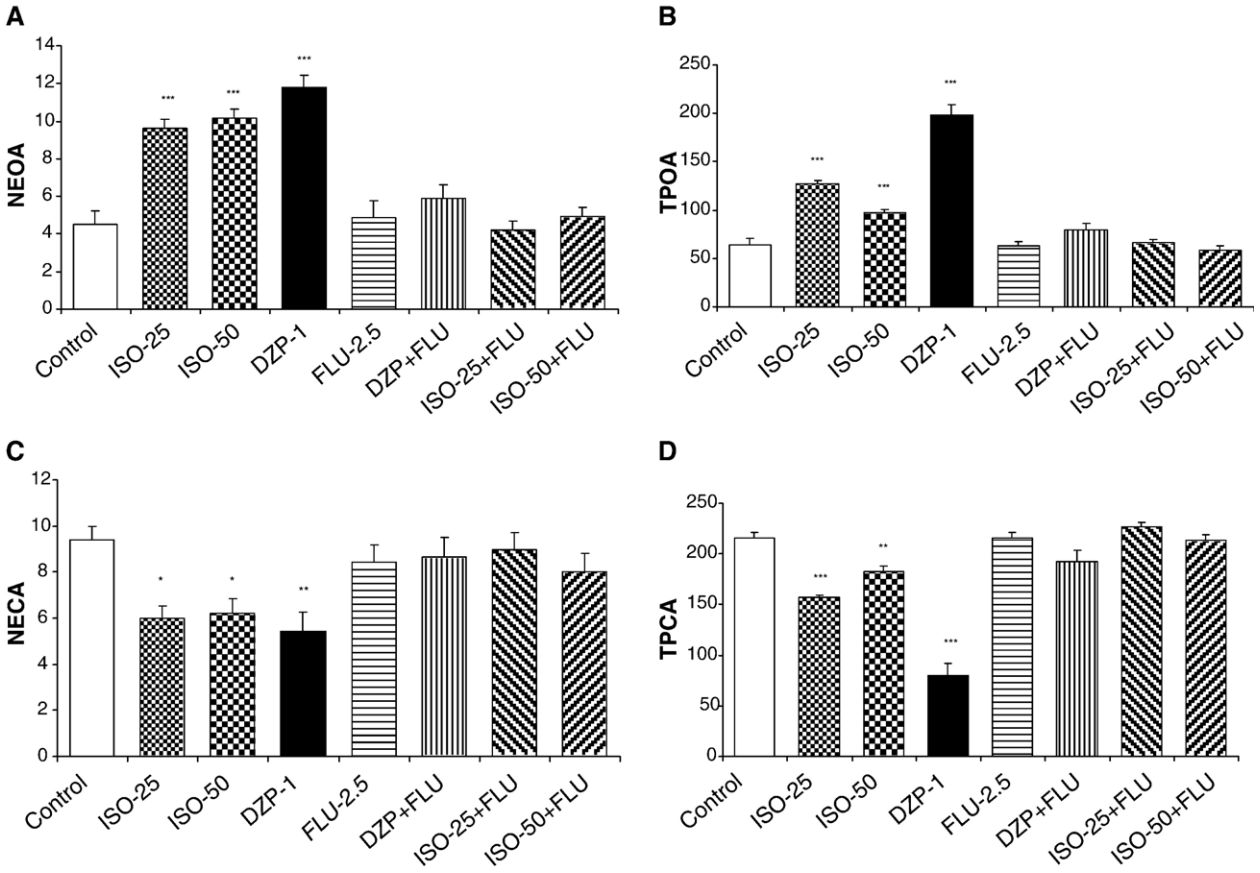

Fig. 2. Plus-maze test of groups of mice which received vehicle, isopulegol (25 and 50 mg/kg), DZP (1 mg/kg) or FLU (2.5 mg/kg). (A) NEOA: number of entries in the open arms; (B) TPOA (s): time of permanence in the open arms; (C) NECA: number of entries in the closed arms; (D) TPCA (s): time of permanence in the closed arms. The results are presented as mean ± S.E.M. Significant difference compared with control (*$P<.05$; **$P<.01$; *** $P<.001$). ANOVA and Student-Newman-Keuls's as the *post hoc* test.

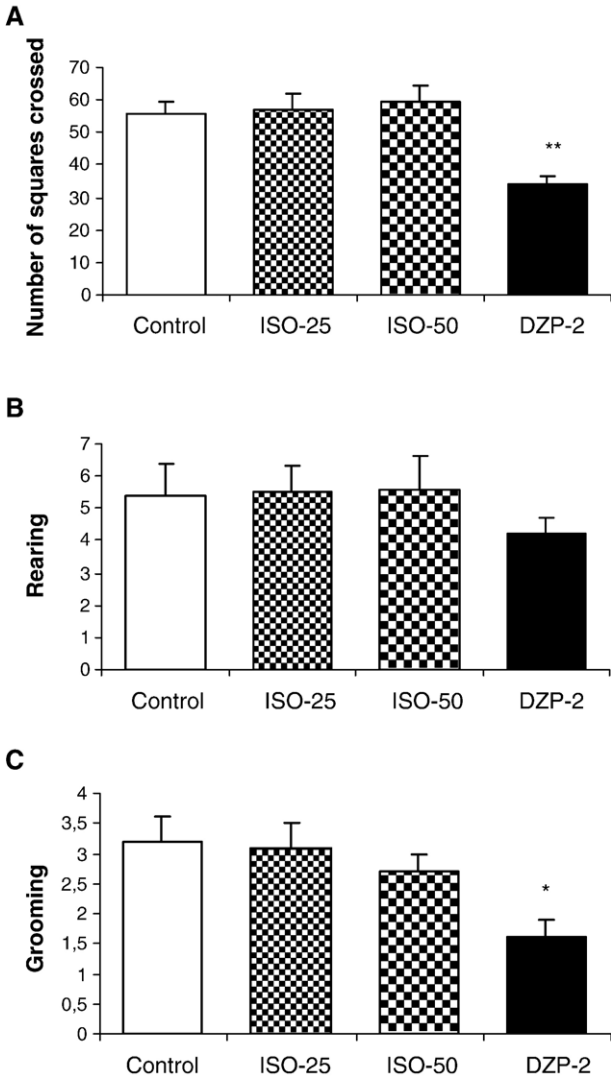

Fig. 3. Open-field test of groups of mice which received vehicle, isopulegol (25 and 50 mg/kg) and DZP (2 mg/kg). (A) Number of squares crossed. (B) Rearing. (C) Grooming. The results are presented as mean±S.E.M. Significant difference compared with control (*P<.05; **P<.01). ANOVA and Student–Newman–Keuls's as the *post hoc* test.

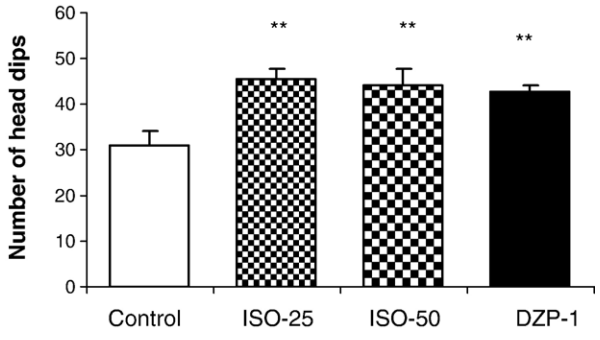

Fig. 4. Hole-board test of groups of mice which received vehicle, isopulegol (25 and 50 mg/kg), and DZP (1 mg/kg). The figure shows the number of head dips. The results are presented as mean±S.E.M. Significant difference compared with control (**P<.01). ANOVA and Student–Newman–Keuls's as the *post hoc* test.

3. Results

3.1. Elevated plus maze test (EPM)

Effects of isopulegol (25 and 50 mg/kg) or diazepam (1 mg/kg) in the elevated plus maze test are presented in Fig. 2. In this test, the groups treated with isopulegol at both doses and diazepam significantly modified all the observed parameters: the number of entries in the open arms (NEOA) [ISO-25, ISO-50, DZP-1: $F(7,89)=25.37$, $P<.05$] and time of permanence in the open arms (TPOA/s) [ISO-25, ISO-50, DZP-1: $F(7,86)=73.62$, $P<.05$], as well as the number of entries in the closed arms (NECA) [ISO-25, ISO-50, DZP-1: $F(7,87)=4.37$, $P<.05$] and time of permanence in the closed arms (TPCA/s) [ISO-25, ISO-50, DZP-1: $F(7,89)=43.63$, $P<.05$]. The results are presented as mean±S.E.M.

3.2. Open-field test

Fig. 3 shows that isopulegol (25 and 50 mg/kg) did not alter the number of crossings, rearing, and grooming, as compared to controls. The animals treated with diazepam (2 mg/kg) decreased the number of crossings [DZP-2: $F(3,57)=6.26$, $P<.05$] and grooming [DZP-2: $F(3,57)=3.29$, $P<.05$] but did not alter the number of rearing, as compared to the control group.

3.3. Hole-board test

Similar to DZP (1 mg/kg), isopulegol at both doses (Fig. 4) increased the number of head dips [ISO-25, ISO-50, DZP-1: $F(3,36)=6.13$, $P<.05$] as compared to controls.

3.4. Forced swimming test

In this test, isopulegol at both doses (25 and 50 mg/kg i.p.) induced a significant increase in the immobility time in mice, as compared to control. On the other hand, the animals treated with imipramine 10 mg/kg, as expected of an antidepressive drug, was able to decrease that parameter [ISO-25, ISO-50, IMP: $F(3,57)=35.87$, $P<.05$] (Fig. 5).

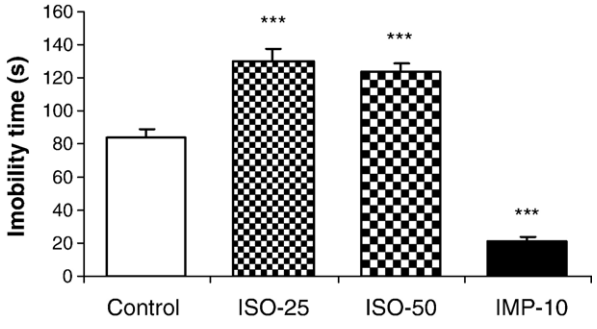

Fig. 5. Forced swimming of groups of mice which received vehicle, isopulegol (25 and 50 mg/kg) and imipramine (10 mg/kg). The figure shows immobility time (s). The results are presented as mean±S.E.M. Significant difference compared with control (*** P<.001). ANOVA and Student–Newman–Keuls's as the *post hoc* test.

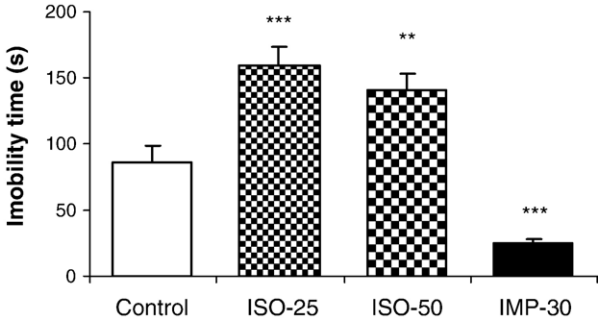

Fig. 6. Tail suspension test of groups of mice which received vehicle, isopulegol (25 and 50 mg/kg) and imipramine (30 mg/kg). The figure shows immobility time (s). The results are presented as mean ± S.E.M. Significant difference compared with control (** $P<.01$; *** $P<.001$). Student–Newman–Keuls's as the *post hoc* test.

3.5. Tail suspension test

Similar to those results observed in forced swimming test, Fig. 6 shows that isopulegol (25 and 50 mg/kg) significantly increased the immobility time in animals, while imipramine 30 mg/kg produced opposite effect [ISO-25, ISO-50, IMP: $F(3,38)=28.45$, $P<.05$] as compared to the control group.

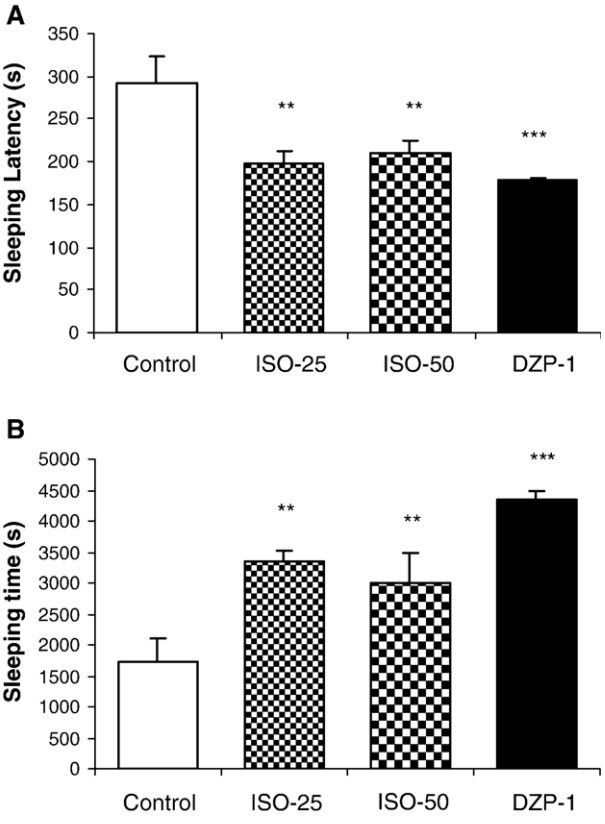

Fig. 7. Effects of mice treatment with isopulegol (25 and 50 mg/kg ip) and diazepam (1 mg/kg) on sleep latency time (A) and sleeping time (B) caused by pentobarbital (40 mg/kg). The results are presented as mean ± S.E.M. Significant difference compared with control (** $P<.01$; *** $P<.001$). Student–Newman–Keuls's as the *post hoc* test.

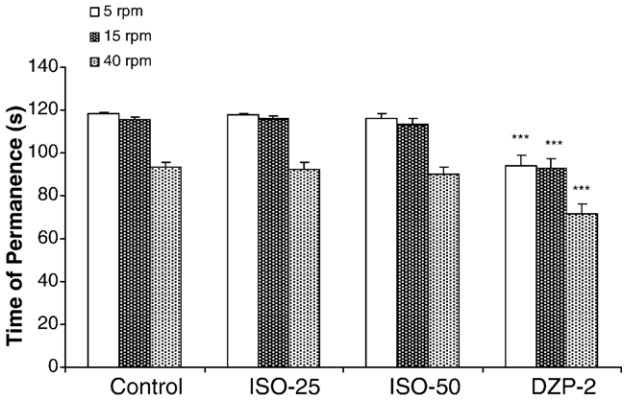

Fig. 8. Rota rod test of groups of mice which received vehicle, isopulegol (25 and 50 mg/kg) and DZP (2 mg/kg). The figure shows time of permanence (s). The results are presented as mean ± S.E.M. Significant difference compared with control (***$P<.001$). ANOVA followed by Student–Newman–Keuls's as the *post hoc* test.

3.6. Pentobarbital sleeping time.

The absolute values of sleep latency and sleeping time showed in Fig. 7 demonstrate that animals treated with isopulegol (25 and 50 mg/kg) or diazepam (1 mg/kg), 30 min before injection of pentobarbital, presented a decrease in the sleep latency [ISO-25, ISO-50, DZP-1: $F(3,38)=7.54$, $P<.05$] and prolongation of pentobarbital-induced sleeping time [ISO-25, ISO-50, DZP-1: $F(3,38)=13.92$, $P<.05$].

3.7. Rota rod

No alteration was observed on the rota rod test (Fig. 8) at 5, 15 and 40 rpm after treatment with isopulegol (25 and 50 mg/kg) as compared to control, while diazepam (2 mg/kg) in a relaxant muscular dose, as expected, decreased this parameter [5 rpm: DZP-1: $F(3,31)=19.02$, $P<.05$; 15 rpm: DZP-1: $F(3,31)=19.70$, $P<.05$; 40 rpm: DZP-1: $F(3,31)=8.48$, $P<.05$].

4. Discussion

Isopulegol is a monoterpene alcohol intermediate in the preparation of (−)-menthol and it is present in the essential oils of various plants. Previous studies have demonstrated that menthol administered intraperitoneal or subcutaneously promotes effect similar to that of psychostimulants (Umezu and Morita, 2003; Umezu et al., 2001). Based in these considerations, we assumed to investigate whether the isopulegol would be also involved in the ability to act on the central nervous system, since, as far as we know, there are no studies in the literature on the central actions of this substance.

In the present work, the effects of isopulegol were studied in several behavior animal models, such as EPM, open-field, hole board, forced swimming, tail suspension, barbiturate-induced sleeping time and rota rod tests, to investigate its possible central activity. These tests are classical models for screening central nervous system actions providing information about

psychomotor performance, anxiety, myorelaxant activity and depression. It is well known that benzodiazepines act as anxiolytics (at low doses), anticonvulsants, and also produce sedation and a myorelaxant effect at higher doses (Melo et al., 2006). Thereby, our group has used diazepam at 1 mg/kg in EPM and hole board tests and 2 mg/kg in open field and rota rod tests, as standard drug.

The present study showed that administration of different doses of isopulegol in mice was able to induce anxiolytic-like effects in the EPM and hole board tests. The EPM test is considered one of the most widely validated tests for assaying new benzodiazepine-like anxiolytic agents (Pellow et al., 1985; Rodgers et al., 1997). The benzodiazepines are the most widely prescribed central nervous system depressants, with selective activity at the inhibitory $GABA_A$ receptor complex. By enhancing the frequency of Cl^- channel opening and thus Cl^- flux through the $GABA_A$ receptor, benzodiazepines potentiate the inhibitory effect of GABA (Lilly and Tietz, 2000). At both doses and similar to DZP, in the EPM test isopulegol significantly reduced the animal's aversion to the open arms and promoted the exploration thereof, indicating anxiolytic effect. Flumazenil is a recognized competitive antagonist at the central benzodiazepine receptor and was used to elucidate the possible mechanism by which isopulegol is actuating in this model. The results showed that flumazenil reversed not only the diazepam effect but also the isopulegol effect, indicating that both drugs might present a similar mechanism of action.

In further to corroborate the anxiolitic activity observed in the EPM test, we decided to use the hole board model, in which it is also observed that the exploration is gradually inhibited by anxiety (Crawley, 1985). In this way, similar to EMP, this test is also useful for modeling anxiety and anxiolytics agents have been shown to increase the number of head dips (Takeda et al., 1998). Our results showed that isopulegol at both doses increased the number of head dips, indicating anxiolytic-like effect.

Drugs that increase general motor activity may provide false-positive results in the number of entries into the open arms and number of head dips in the EPM and hole board tests, respectively. In this way, we decided to study the effects of isopulegol in the open-field test, a classical animal model used to evaluate autonomic effects of drugs and general activity of animals (Novas et al., 1988). In this test, the groups treated intraperitoneally with isopulegol (25 and 50 mg/kg), at doses which produced anxiolytic-like effects, did not significantly change motor activity in mice, differently from DZP (2 mg/kg), which decreased this parameter. Therefore, it is unlikely that the effects produced by isopulegol observed in the plus-maze and hole board tests are based on the stimulation of general motor activity.

Taking into consideration the anxiolytic-like effects of isopulegol observed in the above cited tests, we decided, in addition, to investigate the role of isopulegol in depressant animal models. For this, we realized the forced swimming and tail suspension tests, which have been useful experimental models for screening antidepressant activity. Drugs with established antidepressant activity, as imipramine, reduce the time during which the animals remain immobile (Porsolt et al., 1977; Willner, 1990). Our results showed that isopulegol, at both doses, was able to increase the total time spent in immobility of mice exposed to those tests, indicating depressant activity, in opposite to the psychostimulants effects presented by menthol in previous studies (Umezu and Morita, 2003; Umezu et al., 2001).

A deficit in motor coordination would very likely affect performance in the forced swimming and tail suspension tests. In this way, we aimed to investigate the effects of isopulegol in the rota rod test, a classical animal model used to evaluate peripheral neuromuscular blockage. Our findings showed that isopulegol (25 to 50 mg/kg), different from diazepam (2 mg/kg), had no significant effect on the motor coordination of the animals on rota rod test. Thus, the observed increase in the immobility time probably is not related to peripheral neuromuscular blockage, but may involve neurons that control central depressant activity (Adzu et al., 2002).

Pentobarbital sleeping time test was also used to confirm or not the possible depressive-like effects observed with isopulegol in the previous tests of this study. Decrease in sleep latency and increase in sleeping time are classically related to central nervous system (CNS) depressant drugs (Willianson et al., 1996). Earlier studies have related prolongation of barbital hypnosis to pentobarbital metabolic inhibition or action on the CNS involved in the regulation of sleep (Kaul and Kulkarni, 1978). Ours findings showed that isopulegol, at both doses, decreased the sleep latency time and increased the duration of sleeping, which possibly confirm the depressant activity of CNS detected before. These results corroborate those of Fujimori and Cobb (1995), who proposed that the enhancement of barbital hypnosis is a good index of CNS depressant activity.

In summary, taken together the results in plus maze and hole board tests suggest antianxiety effects of isopulegol. Parameters observed in the forced swimming, tail suspension and pentobarbital sleeping time tests support the idea that isopulegol possibly presents depressor activity on the central nervous system, in opposite to the previous psychostimulants effects presented by menthol. These results are similar to that found by Aguirre-Hernández et al. (2007), who demonstrated that hexane and methanol extracts of *Tilia americana* var. *mexicana* inflorescences exert both depressant and anxiolytic profiles on the CNS. An explanation for the inconsistency between the present anxiolytic and depressant effects of isopulegol and the previously reported psychostimulant actions of menthol is difficult to be formulated, once reports with reference to the behavioral or pharmacological effects from isopulegol are scarce in literature. However, it is possible to speculate that such discrepant finds could be related to the tenuous structural differences among both compounds. Therefore, further studies need to be performed in order to elucidate the antianxiety and depression mechanisms of isopulegol.

Acknowledgments

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