Pharmacological Activity of (*R*)-(+)-Pulegone, a Chemical Constituent of Essential Oils

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Z. Naturforsch. **66c**, 353–359 (2011); received April 24, 2010/March 10, 2011

(*R*)-(+)-Pulegone is a monoterpene found in essential oils from plants of the Labiatae family. This compound is a major constituent of *Agastache formosanum* oil. In this study, the effect of (*R*)-(+)-pulegone on the central nervous system was evaluated. (*R*)-(+)-Pulegone caused a significant decrease in ambulation and an increase in pentobarbital-induced sleeping time in mice, indicating a central depressant effect. (+)-Pulegone also significantly increased the latency of convulsions as assessed by the pentylenetetrazole (PTZ) method. The antinociceptive properties of this monoterpene were studied in chemical and thermal models of nociception. Chemical nociception induced in the first and second phase of the subplantar formalin test was significantly inhibited by (*R*)-(+)-pulegone and was not blocked by naloxone. Thermal nociception was also significantly inhibited while (*R*)-(+)-pulegone increased the reaction latency of the mice in the hot plate test. These results suggest that (*R*)-(+)-pulegone is a psychoactive compound and has the profile of an analgesic drug.

Key words: Essential Oils, Antinociceptive Activity, Analgesic

Introduction

Medicinal plants contain a diversity of biologically active compounds that comprise several chemical classes, including terpenes, saponinic glycosides, steroids, alkaloids, and flavonoids (Liang and Fang, 2006; Da Silva *et al*., 2006; De Sousa and De Almeida, 2005). Some of these compounds, primarily monoterpenes, are found in essential oils extracted from plants. Essential oils have distinctive fragrances and/or flavours and are used in cosmetic as well as medical applications. To this point, many of these oils exhibit biological properties (Craveiro *et al*., 1981), such as spasmolytic (Lis-Balchin and Hart, 1999), anxiolytic (Pultrini *et al*., 2006), antinociceptive (Santos *et al*., 2005), and anticonvulsant (Almeida *et al*., 2003; De Almeida *et al*., 2011) activities.

Monoterpenes and other chemical compounds found in essential oils are structurally simple molecules, but the recently reported studies of their pharmacological properties (De Sousa *et al*., 2007a, b, c; De Sousa, 2011; Amaral *et al*., 2007; Silva *et al*., 2007; De Almeida *et al*., 2008) indicate that they have the complex profile of psychoactive drugs. (*R*)-(+)-Pulegone is a monoterpene found in essential oils from plants of the Labiatae family. In nature, pulegone occurs in both (+) and (–) forms. Dextrorotatory pulegone is obtained from oils from *Mentha pulegium* (pennyroyal), *M. longifolia* (horsemint), and others. Levorotatory pulegone is the major constituent of *Agastache formosanum* (hummingbird mint) oil (Kocovský *et al*., 1986). Pulegone is also present in essential oils that are known to be bioactive, such as the analgesic oil extracted from the Chinese herb *Shizonepeta tenuifolia* Briq. (Yamahara *et al*., 1980).

In our earlier studies, the structure-activity relationship of the analogues of rotundifolone, a monoterpene isolated from the essential oil of the leaves of *Mentha* x *villosa* (mojito mint), was investigated. In preliminary investigations, the monoterpene (*R*)-(+)-pulegone presented significant antinociceptive activity in the acetic acid-induced writhing test (De Sousa *et al*., 2007c). This observation was further studied in the current work by evaluating the impact of (*R*)-(+)-pulegone on the central nervous system (CNS)

via a variety of experimental behavioural models in mice.

Material and Methods

Chemicals

(*R*)-(+)-Pulegone was purchased from Aldrich Chemical Co. (Jacksonville, FL, USA). Sodium pentobarbital, pentylenetetrazole (PTZ), diazepam (DZP), morphine, and polyoxyethylene-sorbitan monooleate (Tween 80) were purchased from Sigma (St. Louis, MO, USA). (*R*)-(+)-Pulegone was mixed with 5% Tween 80 to produce an emulsion.

Animals

Male Swiss mice (28–34 g) were obtained from the animal research facility at the Federal University of Sergipe, Aracaju, Brazil. The animals were maintained at constant room temperature [(23 ± 1) °C] with a 12 h/12 h light-dark cycle (light provided from 6:00 am to 6:00 pm) and free access to food and water. All behavioural tests were conducted between 1:00 and 5:00 pm and were approved by the Institutional Ethics Committee for the Care and Use of Animals (approval #0503/05).

Behavioural effects

The behavioral screening of the mice was performed at 0.5, 1, and 2 h after intraperitoneal (ip) injection of (*R*)-(+)-pulegone, as described previously (De Almeida and De Oliveira, 2006).

Locomotor activity

Mice were divided into two groups of eight animals each and injected with vehicle (control) or (*R*)-(+)-pulegone (200 mg/kg ip). The spontaneous motor activity of the animals was assessed for an observation period of 5 min in an activity cage (controller model 7441 and grid-floor detecting arrangement cage model 7432; Ugo Basile, Comerio, VA, Italy) 30, 60, and 120 min after injection (De Sousa *et al*., 2007b).

Pentobarbital-induced sleeping time

Sodium pentobarbital at a hypnotic dose of 40 mg/kg ip was injected into three groups (n = 8) of mice 30 min after pretreatment with vehicle (ip, control), (*R*)-(+)-pulegone at a dose of 100 mg/kg ip, or (*R*)-(+)-pulegone at a dose of 200 mg/kg ip. The duration of sleep as assessed by the loss and recovery of the righting reflex was recorded (De Sousa *et al*., 2007a).

Pentylenetetrazole-induced convulsions

Mice were divided into five groups (n = 8). The control and positive control groups received 5% Tween 80 ip or DZP (4 mg/kg ip), respectively. The remaining groups received an injection of (*R*)-(+)-pulegone at doses of 100, 200, or 300 mg/kg ip. Thirty min after drug administration, the mice were injected with PTZ (60 mg/kg ip) and observed for at least 15 min to detect the occurrence of the first episode of forelimb clonus (Swinyard *et al*., 1989).

Formalin test

The formalin test is used to clarify possible mechanisms of the antinociceptive effect of a compound of interest (Vida, 1995). Animals were injected with (*R*)-(+)-pulegone (31.3–125 mg/kg ip), vehicle (ip, control), or morphine (10 mg/kg ip) 30 min prior to the injection of formalin (Wheeler-Aceto *et al*., 1990). They were then injected with 20 μl of 2.5% formalin (0.92% formaldehyde diluted in saline) in the subplantar area of the right hind paw. The duration of paw licking was measured 1–5 min (first phase) and 15–30 min (second phase) after the formalin injection. The amount of time spent licking the injected paw was considered as the nociceptive response.

Hot plate test

Animals were placed on a hot plate maintained at (47 ± 0.5) °C. The time elapsed between placing the animals on the hot plate and the animals either licking their fore or hind paws or jumping off the surface was considered to be the response latency. Mice with baseline latencies of more than 15 s were excluded from the study. Response latency testing was measured prior to ip administration (baseline) of (*R*)-(+)-pulegone (31.3–125 mg/kg), vehicle (control), or morphine (10 mg/kg) 30 and 60 min after each treatment. The cut-off time for the hot plate test latency was set at 30 s to avoid tissue injury (Woolfe and Macdonald, 1944).

Possible antagonism of the antinociceptive effect of (R)-(+)-pulegone by pretreatment with naloxone

Naloxone (NLX) was administered subcutaneously (sc) to all experimental animals at a dose of 5 mg/kg. After 15 min, the test group received 125 mg/kg ip of (*R*)-(+)-pulegone, while the control group received ip vehicle and the standard group received morphine (10 mg/kg ip). The evaluations were made by submitting the animals to the formalin test.

Statistical analysis

Statistical analyses were performed using the analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test. A probability level of 0.05 was regarded as significant.

Results and Discussion

(*R*)-(+)-Pulegone is a monoterpene ketone. Its chemical structure is shown in Fig. 1. (*R*)-(+)-Pulegone demonstrated a central depressant effect in mice at a dose of 200 mg/kg ip, as observed by decreased locomotor activity, increased passivity, and sedation 0.5 h after administration. Administration of this compound also caused palpebral ptosis (not shown) and a significant decrease in spontaneous motor activity 0.5 and 1 h after administration (Fig. 2). The CNS-depressant effect of (*R*)-(+)-pulegone was confirmed by an increase of the pentobarbital-induced sleeping time and was observed at both 100 and 200 mg/kg ip (Fig. 3).

Interestingly, Umezu (2010) showed that pulegone promoted ambulation, a CNS-stimulant action, in imprinting control region (ICR) mice via the dopaminergic system. However, it is important to consider that different experimental conditions, including the type and age of the animals employed and the purity of the compound used in the study, may lead to different experimental results. In the evaluation of the anticonvulsant profile, (*R*)-(+)-pulegone (300 mg/kg ip) significantly increased the latency of PTZ-induced convulsions and had an effect similar to that of DZP, a standard anticonvulsant drug (Fig. 4).

PTZ is the prototype pharmacological agent in the class of systemic convulsants. This drug is used in screening tests for anticonvulsants in part because the antiabsence drug ethosuximide, which is effective against PTZ-induced seizures, fails to alter maximal electroshock (MES) thresholds. Therefore, it has become common practice to presume that drugs that are effective against PTZ seizures have the potential to serve as thera-

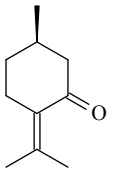

Fig. 1. Chemical structure of (*R*)-(+)-pulegone.

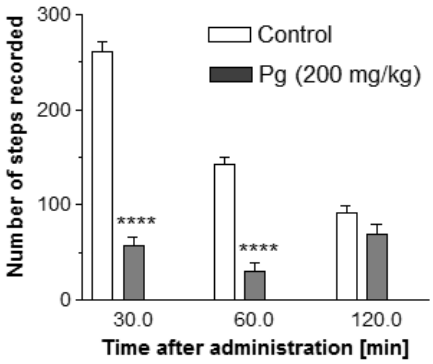

Fig. 2. Effect of (*R*)-(+)-pulegone (Pg) on the locomotor activity in mice. The parameter evaluated was the total number of pulses measured in an activity cage. Values represent the mean ± the standard error of the mean (S.E.M.) ($n = 8$). ****$p < 0.0001$, significantly different from the vehicle control.

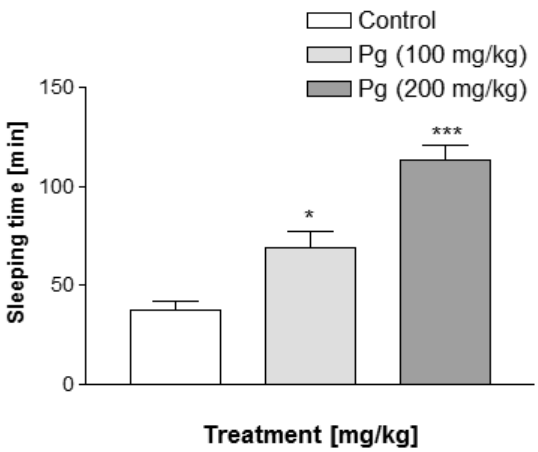

Fig. 3. Effect of (*R*)-(+)-pulegone (Pg) on pentobarbital-induced hypnosis in mice. Values represent the mean ± S.E.M. ($n = 8$). *$p < 0.05$, ***$p < 0.001$, significantly different from the vehicle control.

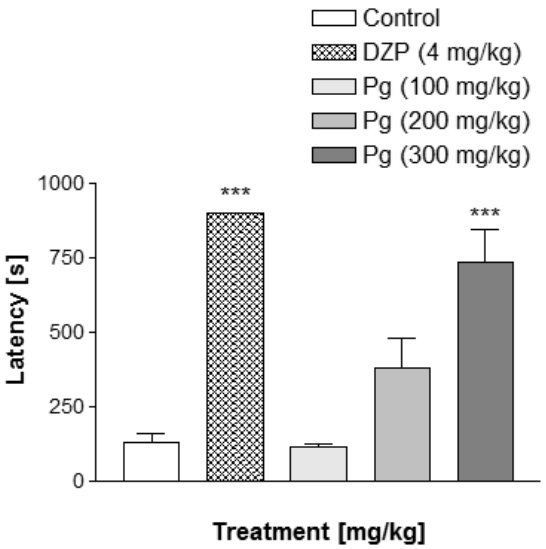

Fig. 4. Effect of (*R*)-(+)-pulegone (Pg) on the latency of the first post-injection convulsion induced by pentylenetetrazol. Values represent the mean ± S.E.M. (n = 8). ***$p < 0.001$, significantly different from the control.

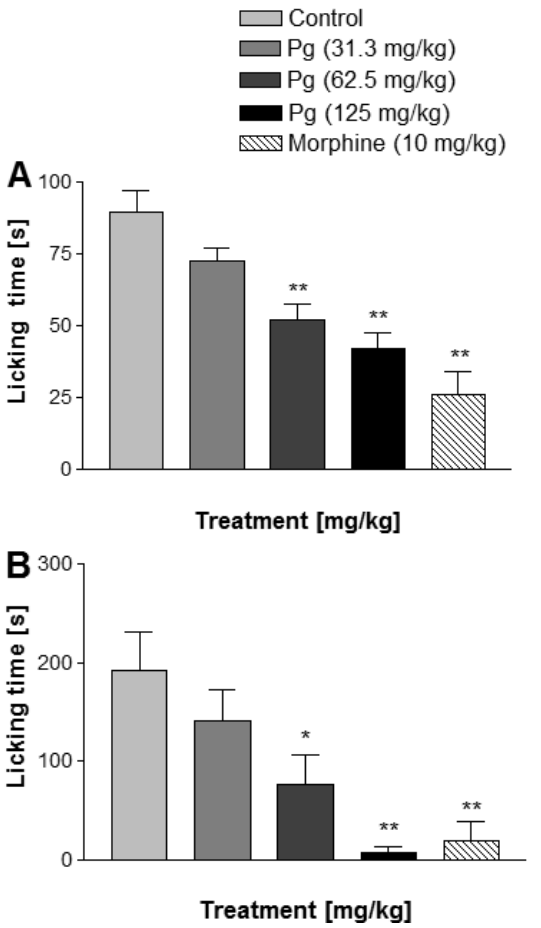

Fig. 5. Effect of (*R*)-(+)-pulegone (Pg) on the formalin test in mice. (A) First phase and (B) second phase. Values represent the mean ± S.E.M. (n = 8). *$p < 0.05$, **$p < 0.01$, significantly different from the control.

peutic agents for antiabsence seizures. However, the mechanism of action of PTZ is only partially understood. At the synaptic level, PTZ appears to interact with the GABA receptor benzodiazepine chloride ionophore complex, decreasing the potency of GABA inhibition and leading to seizures (Fisher, 1989). Conversely, the enhancement of neural inhibition by GABA is a common therapeutic strategy for treating seizures and other CNS disorders such as sleep disturbances and muscle spasms (Chebib and Johnston, 2000). Generally, compounds with anticonvulsant activity against petit mal epilepsy are effective in PTZ-induced seizure models (Vida, 1995). Hence, (*R*)-(+)-pulegone may be useful for the treatment of petit mal epilepsy.

The antinociceptive activity of (*R*)-(+)-pulegone was assessed using several pain models. The formalin test is a model of acute and tonic pain and is considered a more valid model for clinical pain than tests with mechanical or thermal stimulation (Amaral *et al.*, 2007). (*R*)-(+)-Pulegone (31.3–125 mg/kg ip) dose-dependently inhibited both phases of the formalin test in a manner similar to that of morphine (Figs. 5A, B). The first phase results from the direct chemical stimulation of the nociceptive afferent fibers, mainly C fibers, and leads to the release of substance P (Heapy *et al.*, 1987). This release can be inhibited by centrally acting analgesics such as morphine. The second phase results from the action of inflammatory mediators (*e.g.*, prostaglandins, serotonin, histamine, and bradykinin) that are released locally (Murray *et al.*, 1988; Rujjanawate *et al.*, 2003), as well as from enhanced synaptic transmission in the spinal cord (Vida, 1995; França *et al.*, 2001). Therefore, the results suggest that (*R*)-(+)-pulegone has a central antinociceptive effect.

This pharmacological property was confirmed by the hot plate test, which specifically measures central thermal nociceptive responses (Parkhouse and Pleuvry, 1979). Animals were treated with (*R*)-(+)-pulegone at doses of 31.3, 62.5, and

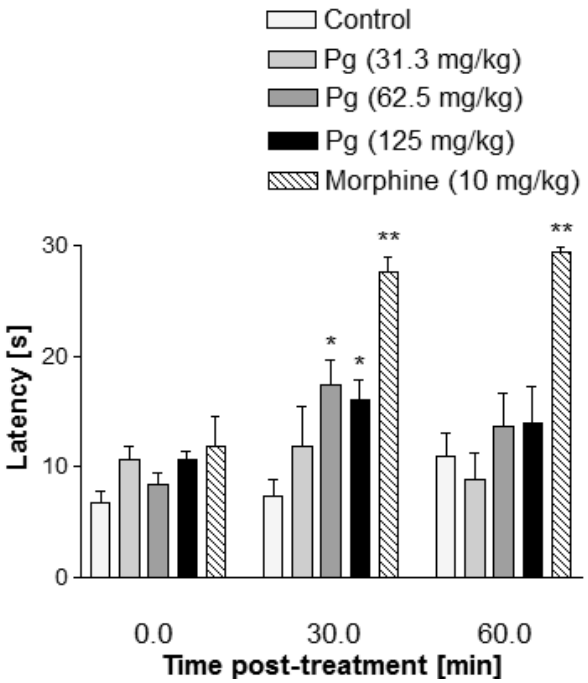

Fig. 6. Effect of (*R*)-(+)-pulegone (Pg) on the hot plate test in mice. Values represent the mean ± S.E.M. (n = 8). *$p < 0.05$, **$p < 0.01$, significantly different from the control.

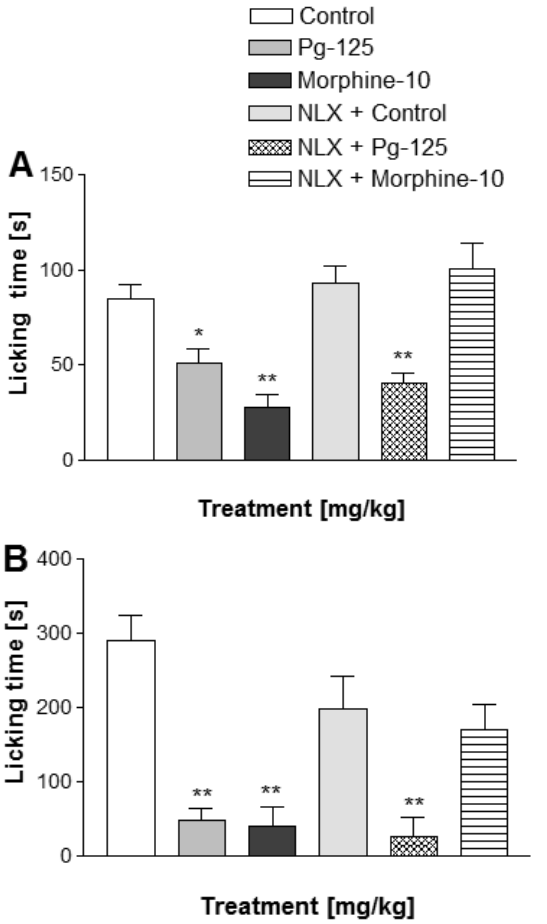

Fig. 7. Influence of pretreatment with naloxone (NLX, 5 mg/kg sc) on the antinociceptive effect induced by (*R*)-(+)-pulegone (Pg). Results were assessed by the formalin test in mice. (A) First phase and (B) second phase. Values represent the mean ± S.E.M. (n = 8). *$p < 0.05$, **$p < 0.01$, significantly different from control.

125 mg/kg or vehicle. The latency measurements were performed before all treatments (basal), 30 and 60 min after administration. The antinociceptive effect of the compound was observed by an increase in the reaction time of the mice subjected to the hot plate, as shown in Fig. 6. The observed reaction time latency indicates that (*R*)-(+)-pulegone exerts its actions via a supraspinal component (Yaksh and Ruby, 1976). Hence, this monoterpene contributes to the analgesic effect of essential oils that contain pulegone, such as those derived from *Shizonepeta tenuifolia* Briq. (Yamahara *et al.*, 1980).

Several essential oils are reported to exhibit CNS-depressant activity (Almeida *et al.*, 2003). Monoterpenes are the major components of these oils. The central activity of other monoterpenes that have a ketone group as part of their structure [*e.g.*, carvone (De Sousa *et al.*, 2007a) and *α*,*β*-epoxy-carvone (De Almeida *et al.*, 2008)] has been demonstrated. Compounds derived from monoterpene ketones, such as hydroxydihydrocarvone, also have antinociceptive effects (De Sousa *et al.*, 2006). Therefore, the results observed with (*R*)-(+)-pulegone are consistent with those reported for other monoterpene ketones that belong to the same chemical class.

Animals were next pretreated with naloxone, an opioid antagonist that opposes the effects of opioid agonists such as morphine. The result of this test showed that naloxone was unable to cancel the antinociceptive effect of (*R*)-(+)-pulegone in the formalin test (Figs. 7A, B). On the other hand, the effect of morphine was blocked, suggesting nonparticipation of the opioid system in the modulation of pain by (*R*)-(+)-pulegone. These results are consistent with those found for another monoterpene ketone, (–)-carvone.

This monoterpene also has antinociceptive actions with nonparticipation of the opioid system. However, the antinociceptive activity is associated with decreased peripheral nerve excitability (Gonçalves *et al*., 2008), suggesting that (*R*)-(+)-pulegone may potentially act through a similar mechanism.

The present study demonstrated the psychopharmacological profile of (*R*)-(+)-pulegone in several behavioural models and indicated that this compound has psychoactive properties. The study also indicated that the antinociceptive actions of (*R*)-(+)-pulegone were most likely unrelated to classical opioid receptor stimulation.

Acknowledgements

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Apoio a Pesquisa e Inovação Tecnológica do Estado de Sergipe (FAPITEC) for providing financial support.

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