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Anxiolytic-, antidepressant- and anticonvulsant-like effects of the alkaloid montanine isolated from *Hippeastrum vittatum*

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Abstract

Compounds isolated from different members of the Amaryllidaceae family are becoming relevant options for the treatment of neurological disorders and neurodegenerative diseases. In particular, species of the *Hippeastrum* genus are important source of alkaloids with a wide profile of putative therapeutical applications. Here, we report on the behavioral and pharmaco-toxicological characterization of montanine, an isoquinoline alkaloid isolated from *Hippeastrum vittatum*, an ornamental plant found throughout the world. In mice, montanine showed a LD_{50} of 64.7 mg/kg and 67.6 mg/kg for male and female, respectively. When given i.p., montanine dose-dependently decreased sodium pentobarbital-induced sleep, protected against pentylenetetrazole-provoked convulsions, increased the number of entries and the time spent in the open arms of an elevated plus maze and augmented the time spent struggling during a forced swimming test. When given immediately after inhibitory avoidance training, montanine did not affect avoidance memory retention in rats. Our results suggest that montanine, as other alkaloids isolated from Amaryllidaceae species, has psychopharmacological activities including anxiolytic, antidepressive and anticonvulsive effects.

Keywords: Montanine; Alkaloid; Amaryllidaceae; *Hippeastrum*; Anxiety; Memory; Depression; Convulsion

1. Introduction

Several alkaloids isolated from different members of the Amaryllidaceae family possess a wide range of pharmacological properties, particularly antiviral (Lopez et al., 2003a; Lee et al., 2003; Szlavik et al., 2004), antitumoral (Liu et al., 2004; Jiang et al., 2005; McLachlan et al., 2005) and psychopharmacological (Houghton et al., 2004; Elgorashi et al., 2004, 2006) activities. *Hippeastrum vittatum* Herbert (Amaryllidaceae) is found throughout South-America and other continents. In Brazil, hybrids of this species are used for ornamental purposes. Previous studies with extracts prepared from the bulbs and flowers of several members of this family have led to the isolation and pharmacological characterization of a variety of alkaloids including lycorine (Fennell et al., 2003; Onofri et al., 2003), pancracine (Labrana et al., 2002; Evidente et al., 2004) and galanthamine (Lopez et al., 2003b; Kaya and Gozler, 2005). Some of these compounds have shown to be active at the central nervous system level. Galanthamine, for example, is a very potent and specific acetylcholinesterase inhibitor (Thomsen and Kewitz, 1990; Thomsen et al., 1990), and this activity has been exploited therapeutically (Wilkinson et al., 2004) to increase the low levels of brain acetylcholine associated with Alzheimer's disease (Raskind et al., 2000; Heinrich and Lee Teoh, 2004).

Our interest to investigate biologically active alkaloids synthesized by members of the *Hippeastrum* genus led us to isolate the isoquinoline montanine from bulbs of *H. vittatum* and to

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Table 1
Acute toxicity–mortality induced by montanine (i.p.) as determined 24 h after its administration

Montanine (mg/kg)	*N*	Death/total	Death (%)
Male			
10	10	0/10	0
30	10	0/10	0
60	10	2/10	20
70	10	1/10	10
100	10	10/10	100
Female			
10	10	0/10	0
30	10	0/10	0
60	10	0/10	0
70	10	0/10	0
85	10	7/10	70

characterize behaviorally and toxicologically this compound in the search for putative therapeutical applications. Therefore, here we present, to the best of our knowledge for the first time, a psychopharmacological screening of montanine.

2. Material and methods

2.1. Plant material

Fresh bulbs of *H. vittatum* were collected in Rio Grande do Sul, Brazil, during the flowering season of 2002. The material was identified and authenticated by M. Sobral from the Faculty of Pharmacy, UFRGS. A voucher specimen (No. 9998; Silva and Sobral) has been deposited in the UFRGS Herbarium for future reference.

2.2. Preparation of H. vittatum extracts and isolation of montanine

Fresh bulbs (2.29 kg) were triturated and macerated with EtOH for 2 days. The procedure was repeated until the eluate was negative to Bertrand reagent. The EtOH extracts were pooled and dried under vacuum and the residue was partitioned in light petroleum and HCl (10%). The HCl phase was washed with CH_2Cl_2 and the acid phase thus obtained basified with NH_4OH (pH 9) and extracted first with CH_2Cl_2 and subsequently with *n*-butanol. The residues obtained by drying under vacuum yielded 3.73 g and 8.0 g of the CH_2Cl_2 and *n*-butanol fractions, respectively. The CH_2Cl_2 fraction was resuspended and chromatographed on silica-gel using the circular centrifuge technique using an increasing gradient of CH_2Cl_2–MeOH (volume rate = 100–0, 95–5, 90–10, 85–15, 80–20, 75–25, 70–30, 100 ml each). Sixty aliquots were collected. Aliquots with similar TLC behavior were combined into two major fractions. Fraction 1 resulted in 2.0 g of pure montanine (PM = 301) identified using spectroscopic methods.

2.3. Animals

Swiss albino mice (25–30 g) and Wistar rats (250–300 g) of both sexes were used. Animals were housed four to a cage and maintained at 22–23 °C under a 12 h light/dark cycle (lights on at 7:00 AM) with free access to food and water. All experiments were conducted blind to the treatment condition of the animals and following the USA *National Institute of Health Guidelines for Animal Care and Use* and were approved by the Animal Care and Ethical Committees of the Universidade Federal de Rio Grande do Sul. Each animal was utilized only once.

2.4. Drugs

Imipramine, diazepan, pentylenetetrazole and sodium pentobarbital were obtained from Sigma (USA). Imipramine and pentylenetetrazole were dissolved in distilled water while diazepan was prepared in 20% propylene glycol. Pentobarbital was dissolved in 20% v/v Tween-80. Montanine was diluted in 10% DMSO in saline. Drugs were prepared before use.

2.5. Preliminary acute toxicity

To determine the acute toxicity of montanine, mice were given different doses of this alkaloid (10–100 mg/kg; 10 μl/g of body weight, i.p.). The animals were observed during 1 h for symptoms of toxicity and the number of deaths within 24 h was recorded. LD_{50} was determined as previously described (Litchfield and Wilcoxon, 1949).

2.6. Open field and elevated plus maze

To analyze their exploratory and locomotor activities, animals were placed on the left rear quadrant of a 50 × 50 × 39 cm open field with black plywood walls and a brown floor divided into 12 equal squares, as previously described (Kerr et al., 2005). The number of line crossings and the number of rearings were measured over 5 min and taken as an indicator of locomotor and exploratory activity, respectively. To evaluate their anxiety state, animals were exposed to an elevated plus maze (Chopin et al., 1985; Da Silva et al., 2006). The total number of entries into the four arms, the number of entries and the time spent in the open arms were recorded over a 5 min session. Thirty minutes before exposure to the open field arena or to the plus maze, mice received different doses of montanine or vehicle (10 μl/g of body weight, i.p.).

2.7. Forced swimming test

Behavioral despair was assessed using a procedure similar to that described by Porsolt and coworkers (Porsolt et al., 1978a).

Table 2
Effect of montanine (i.p.) on locomotor and exploratory activity in the plus maze

Treatment	Dose (mg/kg)	Number of rearings	Number of crossings
Vehicle	–	27.5±3.2	67.9±5.8
Montanine	10	31.4±2.8	65.8±3.6
Montanine	30	25.0±2.4	57.9±4.0
Montanine	60	3.6±1.2**	27.8±6.3**

Data are presented as mean±SEM. $N = 10–15$. $^{**}p < 0.01$ vs. vehicle-treated mice in Newman–Keuls comparison after ANOVA.

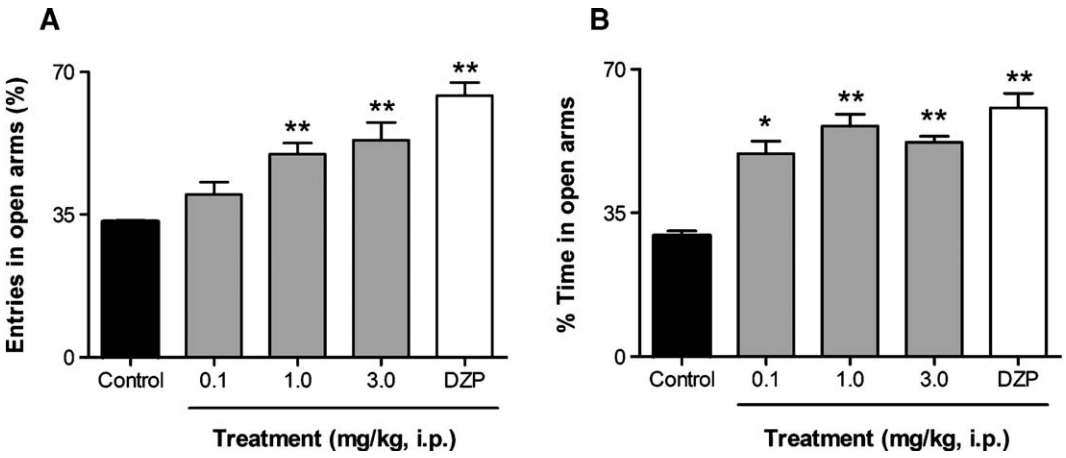

Fig. 1. Effect of montanine (i.p.) and diazepam (DZP) on the % of entries (A) and time spent (B) in the open arms of a plus maze during a 5 min behavioral session. Date are reported as mean±SEM. $N=10–15$. *$p<0.05$ and **$p<0.01$ vs. control group in Newman–Keuls comparison after ANOVA.

Briefly, mice were individually placed in a circular tank (46 cm tall × 20 cm in diameter) filled with tap water (22 °C) to a depth of 20 cm and left there for 6 min. During this period the behavior of the animals was recorded by an observer. Mice were considered immobile when remained floating without struggling and making only slight movements necessary to maintain the head above the water. Montanine (0.1–3 mg/kg, i.p.), imipramine (5 mg/kg, i.p.) and vehicle were given 30 min before the swimming session. The dose of imipramine was determined from previous studies (Campos et al., 2004; Poleszak et al., 2005; Hinojosa et al., 2006; Wesolowska et al., 2006) and pilot experiments.

2.8. Sodium pentobarbital-induced sleeping time

Thirty minutes after the intraperitoneal injection of montanine (10 µl/g of body weight; 0.1–10 mg/kg) or 10% DMSO in saline, mice received sodium pentobarbital (50 mg/kg, i.p.). The time elapsed from pentobarbital injection to the loss of the righting reflex was taken as sleeping latency. The time elapsed between the loss and voluntary recovery of the righting reflex was considered as the total sleeping time (Wolfman et al., 1996).

2.9. PTZ-induced seizures

The potential anticonvulsivant action of montanine was analyzed using the pentylenetetrazole (PTZ) method. Briefly, mice were immobilized using a transparent plastic restrainer and given vehicle or different doses of montanine (10–60 mg/kg, i.p.) 30 min before receiving 75 mg/kg of PTZ i.p.; animals were observed for 60 min thereafter. The latency to the first clonic or tonic episode was recorded for each animal (Malawska, 2003).

2.10. Inhibitory avoidance training

Rats were trained in a one-trial, step-down inhibitory avoidance paradigm (IA), a highly validated learning task in which stepping-down from a platform present in a given context is associated with a footshock resulting in an increase in step-down latency (Izquierdo et al., 2004; Cammarota et al., 1998, 2000, 2003, 2005; Bevilaqua et al., 2005). The IA training apparatus was a 50 × 25 × 25 cm Plexiglas box with a 5 cm high, 8 cm wide, and 25 cm long platform on the left end

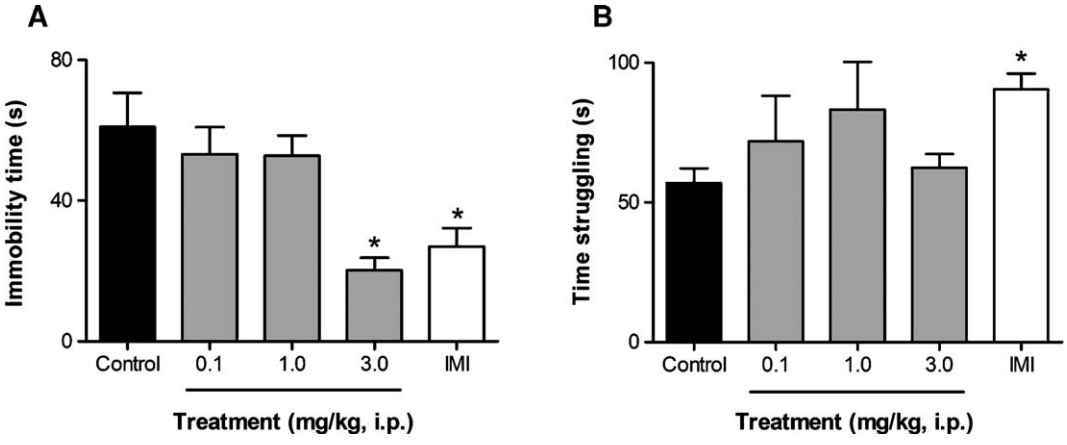

Fig. 2. Effect of montanine and imipramine (IMI) in the immobility time (A) and in the time spent struggling (B) for mice submitted to a forced swimming test. Data are presented as mean±SEM. $N=10–13$. *$p<0.05$ vs. control group Newman–Keuls comparison after ANOVA.

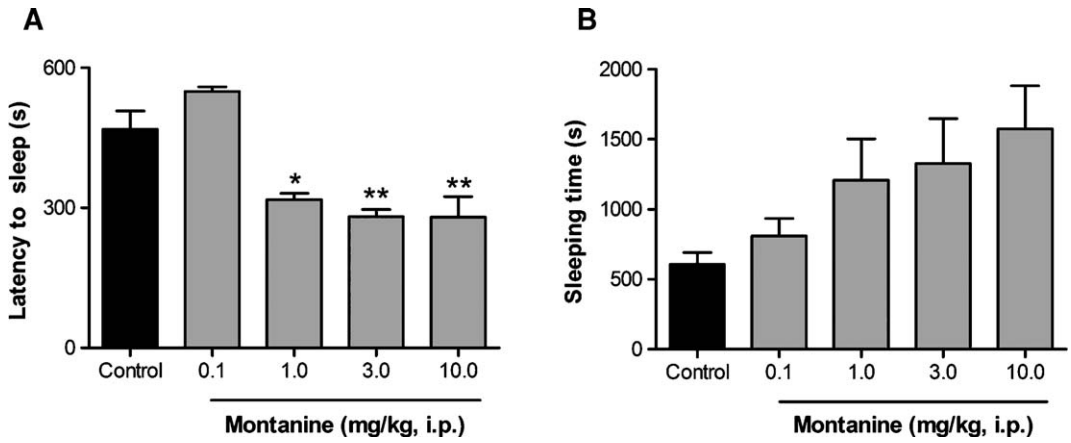

Fig. 3. Effect of montanine (i.p.) on sleep latency (A) and in the total sleeping time (B) induced by pentobarbital (50 mg/kg) in mice. Data are presented as mean ± SEM. N=8–10. *p<0.05, **p<0.01 vs. control group in Newman–Keuls comparison after ANOVA.

of a series of bronze bars that constitutes the floor of the box. During training, animals were gently placed on the platform facing the left rear corner of the training box. When they stepped down and placed their four paws on the grid, received a 2-s, 0.5 mA scrambled footshock. After that, they were immediately withdrawn from the training box. Memory retention was evaluated in a test session carried out 24 h after training. At test, trained animals were placed back on the training box platform until they eventually stepped down to the grid. The latency to step-down during the test session was taken as an indicator of memory retention. A ceiling of 180 s was imposed to step-down latencies during the retention test. Montanine (3–10 mg/kg) or vehicle were given i.p. immediately after IA training.

2.11. Statistical analysis

Data are presented as mean ± SEM except for IA step-down latencies which are presented as median ± interquartile range. Parametric data were analyzed by one-way ANOVA followed by Newman–Keuls test. IA data were analyzed using the Kruskal–Wallis non-parametric test followed by Dunn's comparisons.

3. Results

3.1. Acute toxicity

When given to male mice at 60, 70 or 100 mg/kg i.p. montanine altered motor activity, decreased the respiratory rate and induced violent body tremors and clonic convulsions, ultimately causing death in 20, 10 and 100% of the cases, respectively (Table 1). Lower doses of montanine (10 and 30 mg/kg) did not produce any noticeably motor or behavioral alteration and did not cause death within 24 h of its administration. Female mice seem to be more resistant to the adverse effects of montanine administration since only doses of 85 mg/kg (Table 1) or higher (not shown) produced death. The calculated LD_{50} for montanine was 64.7 mg/kg for male and 67.6 mg/kg for female mice.

3.2. Open field and elevated plus maze

As can be seen in Table 2, when given i.p. montanine (60 mg/kg) reduced the number of crossings ($F13.18=5.67$, $p<0.01$) and rearings ($F19.12=6.18$, $p<0.01$) in the open field test. No effect was observed when montanine was given at 10 and 30 mg/kg. Montanine increased, in a dose-dependent manner, the number of entries (1.0 and 3.0 mg/kg; $F27.15=3.77$, $p<0.01$ vs diazepam; Fig. 1A) and the time spent in the open arms (0.1, 1.0 and 3.0 mg/kg; $F10.94=3.70$, $p<0.01$ vs. diazepam; Fig. 1B) during a 5-min behavioral session in an elevated plus maze.

3.3. Forced swimming

When given i.p. at 3 but not at 0.1 or 1.0 mg/kg montanine reduced the immobility time in the forced swimming test ($F3.16=2.47$, $p<0.05$; Fig. 2A) but had no effect at all on the time spent in struggling (Fig. 2B). Imipramine was used as a reference drug for these studies (Campos et al., 2004; Poleszak

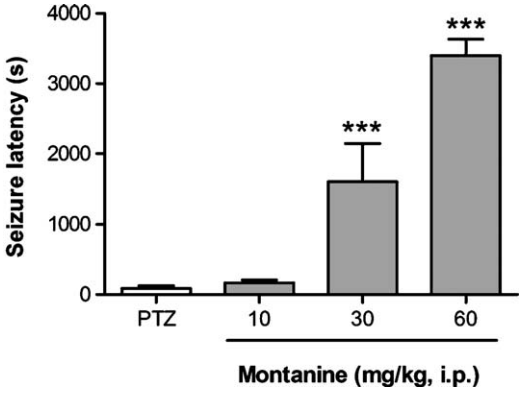

Fig. 4. Effect of montanine on the seizures induced by pentylenetetrazole (PTZ). The latency to the first observable seizure activity was measured and averaged across animals in each treatment group. Data are reported as mean ± SEM. N=10–15. ***p<0.001 vs. PTZ-treated mice in Newman–Keuls comparison after ANOVA.

et al., 2005; Hinojosa et al., 2006; Wesolowska et al., 2006). As expected, when given i.p. imipramine ($F3.16=2.47$, $p<0.05$) reduced both the immobility time and the time spent struggling (Fig. 2A and B).

3.4. Pentobarbital-induced sleeping time

As can be seen in Fig. 3A, pretreatment with montanine (1.0, 3.0 and 10.0 mg/kg, i.p.) reduced the sleeping latency ($F9.72=3.54$, $p<0.01$) but did not significantly affect the total sleeping time induced by pentobarbital (Fig. 3B).

3.5. Pentylenetetrazole-induced convulsions

When given i.p. at 30 or 60 but not at 10 mg/kg montanine suppressed the clonic seizures induced by PTZ (75 mg/kg; $F21.04=4.16$, $p<0.01$; Fig. 4).

3.6. Inhibitory avoidance memory

When given i.p. immediately after inhibitory avoidance training, montanine (3–10 mg/kg) did not affect memory retention as tested 24 h post-training (not shown).

4. Discussion

After the discovery that the alkaloid galanthamine is a potent acethylcholinesterase inhibitor and, consequently, very important for the symptomatic treatment of Alzheimer's disease (Liu et al., 2004), the interest in the isolation and characterization of alkaloids from Amaryllidaceae has increased exponentially (Elgorashi et al., 2004). In this respect, it has been reported that more than 23 different alkaloids isolated from species of the Amaryllidaceae family, including lycorine, homolycorine and hipeastrine, are biologically active. Most of them exhibit a prominent anticholinesterase activity (Elgorashi et al., 2004).

In this paper, we report on the isolation of the alkaloid montanine from *H. vittatum*. To do that, we utilized the method previously described by Hofmann and coworkers (Hofmann et al., 2003) and analyzed some of the neuropharmacological properties of this isoquinoline. Since the determination of preliminary acute toxicity indicated LD_{50} values of 64.7 and 67.6 mg/kg for male and female mice respectively, we evaluated the behavioral effects of montanine using lower doses.

We found that montanine reduces locomotor activity and has sedative, anxiolytic, anticonvulsant and antidepressant effects in mice. The general depressant activity of montanine was confirmed by the decrease in the latency to sleep and its tendency to increase the pentobarbital-induced sleeping time, which may be attributed to an inhibition of pentobarbital metabolism or to an action in the regulation of sleep (Morais et al., 1998). The reduction in the number or rearings and crossings in the open field test confirms the central activity of montanine, since it is conceded that rearing is a function of the excitability level of the central nervous system (Masur et al., 1971).

Montanine also showed anxiolytic-like effects when evaluated in the elevated plus maze. Anxiety, a symptom accompanying various central nervous system disorders and a disorder by itself, is characterized in humans by a tense and exhaustive physical alertness (Jackson and Turkington, 2005). Other species display a variety of defensive reactions in response to predators, some understood as correlated states of anxiety (Rodgers et al., 1995). Rodents demonstrate anxiety, fear and curiosity when placed in a new environment, and an overall assessment of behavior could be determined through the observation of freezing, grooming (fear), rearing, head-dips (curiosity) and the number of fecal boluses (Takeda et al., 1998; Nic et al., 2003; Costa-Campos et al., 2004). The elevated plus maze has been frequently used to detect and evaluate anxiolytic/anxiogenic properties of drugs (Takeda et al., 1998; Pellow and File, 1986; Pellow and File, 1987). The frequency and time spent in the open arms is the major index of the anxiety in the plus-maze model, given the fact that an open area is extremely aversive to rodents (Pellow and File, 1986). Montanine dose-dependently increased the percentage of open-arms entries and the time spent in those arms. These results suggest that, when given i.p., this alkaloid has an anxiolytic-like effect. We also showed that montanine has a dose-dependent anticonvulsant activity in the PTZ-induced seizure model. The PTZ test represents a valid model for human generalized myoclonic seizures (Malawska, 2003). PTZ induces seizures in rodents by blocking the Cl^- channel of $GABA_A$ receptors. It has been reported that GABAergic neurotransmission plays an important role in stress, anxiety (Zwanzger and Rupprecht, 2005), pain (Rode et al., 2005) and epilepsy (Ito et al., 2005; Perucca, 2005). In fact, several drugs such as benzodiazepines, carbamazepine, and some others are therapeutically used for treatment of such disorders (Zwanzger and Rupprecht, 2005). Our results indicate that montanine may act on the BDZ site of the GABA receptor in the mouse's brain. Thus, the anxiolytic, hypnotic effects of montanine could be caused by its combined action on several neurotransmitter receptor systems, including $GABA_A$ receptors.

The forced swimming test has been validated as a suitable tool to evaluate drugs with putative antidepressant effects (Porsolt et al., 1978a; Anisman and Matheson, 2005; Matthews et al., 2005). When rodents are forced to swim in a confined space, they tend to become immobile after vigorous activity (struggling). This inescapable stressful situation can be evaluated by assessing different behavioral strategies (Porsolt et al., 1978b). Administration of montanine prior to the test reduced total immobility time and enhanced struggling behavior. Several authors have proposed that immobility during the test could be an efficient adaptive response to this stress (Porsolt et al., 1978a; Galea et al., 2001; Martijena et al., 1998). Montanine affects the normal pattern of behavior during the test, suggesting an antidepressant effect in response to inescapable stress. The effect of montanine on inhibitory avoidance memory consolidation was also examined. In contrast with other Amaryllidaceae alkaloids, montanine did not affect long-term memory retention when given i.p. immediately post-training sessions, at least at the doses employed here. Chronic toxicity studies are in due course to assess the real toxicological profile of this alkaloid.

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