Anxiolytic Effects of Erythrinian Alkaloids from *Erythrina mulungu*

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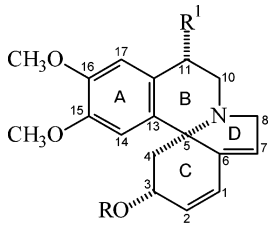
One new erythrinian alkaloid derivative, (+)-11α-hydroxyerythravine (**1**), and the known (+)-erythravine (**2**) and (+)-α-hydroxyerysotrine (**3**) were isolated from the flowers of *Erythrina mulungu*. Their structures were determined by spectroscopic/spectrometric data interpretation of ^{1}H, ^{13}C, and 2D NMR and MS experiments. The relative configuration was established by NOESY analysis, while the conformation adopted by these molecules was evaluated through molecular modeling studies and coupling constants obtained by NMR analysis. Furthermore, the anxiolytic effects of the *E. mulungu* aqueous alcoholic crude extract and of the purified alkaloids were evaluated using the elevated T-maze test.

There are about 115 known species of *Erythrina* Mart. (Leguminoseae, Papilionaceae) in the tropics and subtropics, as well as in some temperate regions of the world,[1] and approximately half of them have been studied.[2] Phytochemical analyses have demonstrated that these plants accumulate erythrinian alkaloids, flavonoids, and terpenes.[3−5] *Erythrina* plant species are widely used in folk medicine to treat different health problems such as agitation, insomnia, and inflammatory processes.[2] Previous studies have demonstrated the analgesic and anti-inflammatory effects of the crude extracts of *E. senegalensis*,[6] *E. velutina*, and *E. mulungu*.[7] It has been demonstrated that alkaloids isolated from *E. americana* have anticonvulsant, hypotensive, hypnotic, and analgesic effects.[8] The tranquilizer effects of crude alkaloid fractions from *E. americana* were investigated in a model that provokes aggressiveness in rats by visual isolation.[9] It was observed that crude alkaloids diminished the aggressive behavior, and their effects were similar to the benzodiazepine compound diazepam.[9]

E. mulungu, popularly known as mulungu, is a medium well-branched tree native to Southern Brazil, where it is appreciated for its beautiful reddish flowers. Since its flowers have the same color of corals, sometimes this species is also called coral tree.[10] In popular medicine, a tincture prepared from the leaf or bark decoction from *E. mulungu* is used to calm agitation and other disorders of the nervous system, i.e., insomnia and depression.[11] It has been demonstrated that acute and chronic treatments with aqueous alcoholic extracts from *E. mulungu* produce anxiolytic-like effects on a specific subset of defensive behavior in rats exposed in the elevated T-maze (ETM) and in the light−dark transition model (LDTM).[12,13] These antianxiety effects were similar to that provoked by the well-known anxiolytic compound diazepam.[12,13]

As part of our research project focusing on phytochemical investigation of medicinal plants with central nervous system activity, we now report the isolation of a new (**1**) as well as two known (**2**, **3**) erythrinian alkaloids and their anxiolytic properties.



	R	R^1
1	H	OH
2	H	H
3	CH_3	OH

Results and Discussion

(+)-11α-Hydroxyerythravine (**1**) was obtained as an amorphous powder and showed a molecular ion $[M + H]^+$ at *m*/*z* 316.3681 in its HRESIMS-MS, consistent with the molecular formula $C_{18}H_{21}NO_4$. The UV spectrum of **1** suggested that it contained a typical aromatic chromophore with maximum absorption at 283.6, 234.7, and 204.7 nm. IR absorption bands at 1615 and 3260 cm^{-1} indicated the presence of a conjugated system and a hydroxyl group, respectively. The aromatic part of the ^{1}H NMR spectrum exhibited two singlets at δ 6.91 and 6.77, which in conjuction with two *O*-methyl groups at δ 3.90 and 3.71 indicated similarity to that of (+)-11β-hydroxyerysotrine,[3,14] except for the exchange of an *O*-methyl for a hydroxyl group in **1**. Additionally, the spectrum of **1** showed signals for one trisubstituted double bond at δ 5.66 br s and for one disubstituted double bond at δ 6.96 dd and 5.96 dd, evidencing the erythrinian skeleton for **1** (Table 1). Two oxymethine protons, H-3 at δ 4.37 (m) and H-11 at δ 4.74 (dd; J = 3.60, 4.64 Hz), were identified by HMQC spectrum, and using these protons as a starting point, the COSY spectrum permitted the assignment of the H-4 and H-10 methylenes, respectively. The relative configuration of C-11 was determined by the coupling constant of H-11 (δ 4.74 dd; 3.60, 4.64), which is in good agreement with an α-oriented 11-OH, as was also observed for compound **3**. Taking into account that most ^{1}H NMR data published for several 11-hydroxyerythrinian derivatives were recorded at 360 MHz, 100 MHz, or under, the relative configuration of the stereogenic center at C-11 was analyzed by NMR experiments and also by molecular modeling studies. The ^{1}H NMR data recorded in pyridine-d_6 clearly

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Table 1. ^{1}H and ^{13}C NMR Data for Alkaloids **1** in Pyridine-d_5 and **3** in CD_3Cl

	1			**3**					
							theoretical J_{HH} (Hz)[a]		
position	δ_C	δ_H (mult.)	J_{HH} (Hz)	δ_C	δ_H (mult.)	J_{HH} (Hz)	conf. **3a**	conf. **3b**	conf. **3c**
1	135.0	5.96 (dd)	10.5, 2.10	131.5	5.94 (dd)	10.5	n.d.[b]	n.d.	n.d.
2	124.5	6.47 (dd)	10.5, 3.2	125.5	6.52 (dd)	10.5, 2.5	n.d.	n.d.	n.d.
3	67.2	4.37 (m)		76.0	3.98 (m)		n.d.	n.d.	n.d.
4	43.9	2.40 (dd)	5.0, 11.7	40.5	2.34 (dd)	5.5, 11.5	6.6, 9.8	6.2, 9.9	5.5, 10.7
		1.01 (dt)	11.7, 10.1		1.75 (t)	11.5	9.8	9.9	10.7
5	66.3			66.3					
6	141.3			142.0					
7	124.4	5.66 (br, s)		123.5	5.67 (br, s)		n.d.	n.d.	n.d.
8	58.8	3.96 (dd)	16.0, 3.2	58.7	3.78 (dd)	14.5, 3.0	n.d.	n.d.	n.d.
		3.93 (br, d)	16.0		3.88 (br, d)	14.5			
9									
10	50.8	3.60 (dd)	10.0, 3.60	50.9	3.55 (dd)	14.0, 4.0	9.5	10.2	2.8
		3.16 (br, d)	16.0		3.07 (dd)	10.0, 3.5	7.1	6.2	2.7
11	63.7	4.74 (dd)	3.60, 4.64	64.5	4.64 (dd)	3.5, 4.0	7.1, 9.5	6.2, 10.2	2.7, 2.8
12	128.0			128.3					
13	129.0			129.7					
14	108.4	6.91 (s)		108.7	6.91 (s)				
15	148.3			148.3					
16	148.8			148.5					
17	110.4	6.77 (s)		110.3	7.77 (s)				
OCH_3-3				76.0	3.30 (s)				
OCH_3-15	56.0	3.90 (s)		56.2	3.83 (s)				
OCH_3-16	56.2	3.71 (s)		56.3	3.57 (s)				

[a] Values for the H−C−C−H dihedral angle, in degrees, obtained from the PM5 minimized conformation in $CDCl_3$. [b] Not determined.

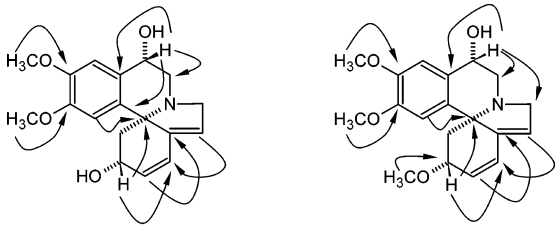

Figure 1. C−H long-range correlations in the HMBC spectra of **1** and **3**.

demonstrate all coupling constants of H-11 with H_{ax},H_{eq}-10 (Table 1), which is completely compatible with an α-OH position at C-11. The ^{13}C NMR (Table 1) data also were comparable with those of the alkaloid **3**, which possesses C-11 with the same configuration. When we analyzed the ^{13}C NMR spectra of **1** and **3**, the signal due to C-11 in both was observed at higher fields, $\Delta\delta \approx 10$ ppm, when comparable to the same carbon of (+)-11β-hydroxyerysotrine (also named (+)-erythrartine), which is a C-11 epimer.[3,14] The total assignment of **1** was done by examination of the COSY, HMQC, NOESY, and HMBC (Figure 1) data of this compound. To confirm the relative configuration of C-3 and C-11 and to establish the conformation of **1**, the structure of (+)-α-hydroxyerysotrine (**3**) was used as a model for a conformational analyses approach with the introduction of dihedral angle constraints obtained from the coupling constants between H11−H10 and H3−H4. On the basis of ^{1}H NMR data, the structure of compound **3** was build with an α-OH at C-11 (similar to compound **1**) and submitted to conformational analysis using the semiempirical methods AM1 and PM5 in the gas phase, with $CHCl_3$ and pyridine solvents, as previously described.[15,16] The semiempirical methods were applied to compound **3** since it was previously identified as the 11α-epimer,[14] and our studies demonstrated that **3** has an 11α-hydroxyl, similar to that of the new derivative **1**. Four conformers were evaluated for this compound, obtained through inversion of ring B (two half-chairs) and of ring C (two envelopes). One of the half-chairs of ring B was not stable and changed to a twist-boat-like conformation (Figure 2C). This twist-boat, together with the conformational restraining induced by ring D, avoided the inversion of the envelope in ring C, allowing us to obtain only three minimum energy conformations for compound **3**, named **3a**, **3b**, and **3c** (Figure 2). The Haasnot−Altona parametrization of the Karplus equation[18] estimates the $^3J_{H,H}$ coupling constants from the electronegativity of the substituents and from the H−C−C−H dihedral angle geometry. The obtained theoretical coupling constants for conformations **3a**, **3b**, and **3c** are presented in Table 1, and their energies are shown in Table 2. The comparison between theoretical and experimental coupling constants allowed characterization of the conformation of the erythrinian alkaloids as represented by conformation **3c** in Table 1 and Figure 2. As already described by us,[16] this Hamiltonian presents better agreement with experimental data when compared to AM1.

Compound **3** was reported as the 11α-epimer in *E. lysistemon*.[14] However, the authors presented for epimeric compounds the same coupling constants, for the 11α- and 11β-epimers. In the previous and related studies, we did not find detailed ^{1}H NMR information that could confirm the relative configuration of each epimer, and thus an intensive ^{1}H NMR and molecular modeling analyses were performed in this paper. Alkaloid **2** was identified as (+)-erythravine (**2**).[17]

To investigate the anxiolytic effects of the alkaloids, behavioral tests were conducted using the ETM. As shown in Figure 3 at doses of 200 and 400 mg/kg crude extract (CE) impaired inhibitory avoidance acquisition was observed, similar to that observed with diazepam (DZP, 2 mg/kg; ip), a classical anxiolytic drug. Two-way ANOVA showed a significant effect of treatment ($F(5,49) = 5.4$; $P < 0.0001$) and trials ($F(4,196) = 27.37$; $P < 0.0001$), but not of treatment versus trial interaction ($F(20,196) = 0.87$, $P = 0.626$). Post hoc comparisons revealed that both CE (100−400 mg/kg) and DZP reduced significantly the latency to leave the enclosed arm when compared with the control group ($P < 0.05$, Duncan's test). Additionally, one-way ANOVA (followed by Duncan's test) revealed that the latency to leave the open arm was significantly lower for CE (400 mg/kg) treated animals when compared with the control group ($F(5,49) = 3.47$, $P = 0.009$).

Figure 3B illustrates the effects of compound **1** on inhibitory avoidance and escape latencies. Two-way ANOVA showed significant effect of treatment ($F(3,33) = 8.30$; $P < 0.001$), trials ($F(4,132 = 14.75$; $P < 0.0001$), and treatment versus trials interaction

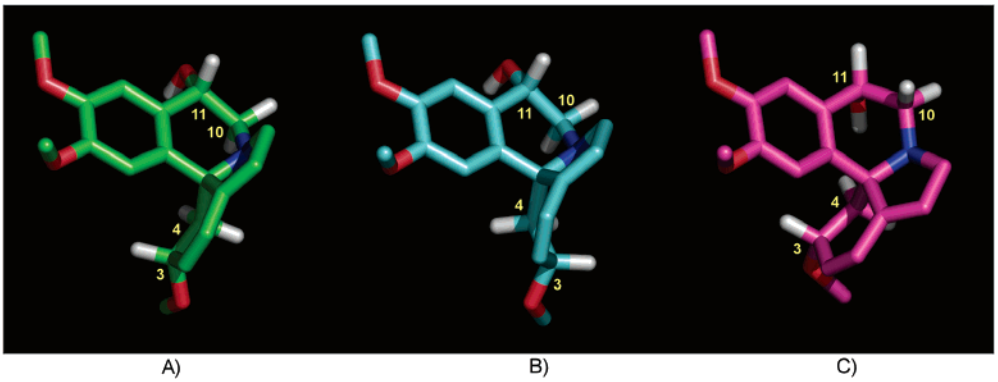

Figure 2. Structure of the obtained conformations for compound **3**: (A) conformation **3a**, (B) conformation **3b**, and (C) conformation **3c**. With the exception of hydrogen atoms attached to carbon atoms C-3, C-4, C10, and C-11 the nonpolar hydrogen atoms were omitted in order to clarify the image. This computer-generated 3D interpretation also can be applied to compound **1**, since the difference between these structures does not change significantly the values calculated for energy minimization.

Table 2. Heats of Formation (kcal/mol) for the Conformations Obtained from Conformational Analysis Using the AM1 and PM5 Semiempirical Methods

	vacuum		$CDCl_3$		pyridine	
conformation	AM1	PM5	AM1	PM5	AM1	PM5
3a	−81.4	−99.8	−89.5	−112.4	−91.6	−115.4
3b	−81.0	−98.6	−89.3	−111.7	−91.8	−114.7
3c	−81.2	−102.0	−89.7	−114.2	−91.7	−117.5

($F(12,132) = 2.42$; $P < 0.01$). Individual comparisons revealed that latencies to leave the enclosed arm were decreased with both **1** (10 mg/kg) and DZP treatments. Different from CE, one-way ANOVA did not reveal any effect of **1** or DZP on the latency to leave the open arms ($F(3,33) = 0.71$; $P = 0.54$).

The effects of **2** in the ETM measurements are shown in Figure 3 C. Two-way ANOVA showed significant effect of treatment ($F(3,55) = 13.40$, $P < 0.0001$), trials ($F(4,220) = 44.23$; $P < 0.0001$), and treatment versus trials interaction ($F(12,220) = 6.33$; $P < 0.0001$) in the inhibitory avoidance measurements. Duncan's post hoc test revealed that animals treated with **2** (3 and 10 mg/kg) or DZP exhibited lower latencies to leave the enclosed arm of the ETM when compared with the control group ($P < 0.05$). Neither **2** nor DZP significantly changed escape latencies (one-way ANOVA: $F(3,55) = 0.61$, $P < 0.60$).

Figure 3D shows the effects of **3** on the inhibitory avoidance and escape latencies in the ETM test. Two-way ANOVA showed effects of treatment ($F(3,31) = 7.65$; $P < 0.0001$), trials ($F(4,124) = 18.38$; $P < 0.0001$), and treatment versus trials interaction ($F(12,124) = 2.14$; $P < 0.001$) on inhibitory avoidance acquisition. Duncan's post hoc test revealed that the doses of 3 and 10 mg/kg of **3** significantly impaired the inhibitory avoidance task, an effect also shown with DZP ($P < 0.05$). One-way ANOVA showed that neither **3** nor DZP produced significant effects on latency to leave the open arm ($F(3,31) = 0.24$; $P = 0.86$).

Additionally, one-way ANOVA revealed no treatment effects of CE, **1**, **2**, **3**, or DZP on the number of rearing and crossings (locomotor activity) in the arena test (see Table 3).

The present study demonstrated that acute po treatment with CE attenuated anxiety-related responses in mice submitted to an animal model of anxiety, i.e., the ETM. These results corroborate a previous study,[12] in which the aqueous alcoholic extract of *E. mulungu*, at a dose of 200 mg/kg, produced anxiolytic-like effects in the inhibitory avoidance in the ETM in rats. These authors also reported an antianxiety effect with a lower dose of CE (100 mg/kg) in the light−dark transition model. In that study CE increased both the number of transitions between light and dark compartments and the time spent in the illuminated area, measures considered as anxiety indices.[12] Similar effects have been reported with chronic treatment with an equivalent range of doses (50−200 mg/kg) of CE on inhibitory avoidance in the ETM as well as on the number of transitions between compartments, and in the time spent in the illuminated compartment in the light−dark transition model.[13]

This study demonstrated that the erythrinian alkaloids isolated from *E. mulungu* provoked anxiolytic-like effects in the ETM. The new erythrinian alkaloid **1** and the two known alkaloids **2** and **3** impaired the inhibitory avoidance task, and these effects were similar to that produced by DZP, a classical anxiolytic drug.

The underlying mechanisms involved in the anxiolytic effects of the erythrinian alkaloids were not in the scope of the present study and remain to be determined. However, it has been demonstrated that the dihydro-β-erythroidine, an erythrinian alkaloid, antagonizes the excitatory effects of serotonin (5-HT) at the 5-HT_3 receptor.[24] Thus, it is likely that the anxiolytic effects of the erythrinian alkaloids from *E. mulungu* involve antagonism at 5-HT3 receptors, as shown for dihydro-β-erythroidine.[24] Although attractive, this hypothesis remains to be empirically tested. It was demonstrated that the crude extract of *E. vespertilio* inhibited platelet 5-HT release.[23] Platelets have been used as a useful model for studying Ca^{2+}-dependent 5-HT release,[23] the main action of serotonin at 5-HT_3 receptors.

It is interesting to note that the highest dose (400 mg/kg) of the crude extract decreased escape latency (i.e., the latency to leave the open arm) in the ETM. Similar effects obtained with other classes of drugs have been previously interpreted as anxiolytic, instead of anxiogenic.[19] It has been suggested that drugs that diminish risk-assessment behavior, such as buspirone and the serotonin releaser fenfluramine, also decrease escape latencies in the ETM.[19] Curiously, the pure alkaloids did not alter escape latencies. It is likely that synergistic interactions with other compounds of the crude extract are important to provoke the anxiolytic effects observed in the escape task.

Taken together, these results suggests that the new erythrinian alkaloid (+)-11α-hydroxy-erythravine (**1**) and the known (+)-erythravine (**2**) and (+)-α-hydroxyerysotrine (**3**) are, in part, responsible for the anxiolytic effects observed with the crude extract of *E. mulungu*.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter using a quartz curvette (length 1 cm). UV spectra were recorded on a HPLC system Varian PRO STAR 240, UV-diodo-array 330 detector. The IR spectrum was recorded on a Perkin-Elmer 1600 or Nicolet EMACT-40 FTIR spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA 500 MHz NMR spectrometer operating at 500 MHz for ^{1}H and 125 for ^{13}C nuclei, respectively. Solvent peaks were used as a reference standard. For chromatographic procedures, silica gel PF 254 and silica gel (230−

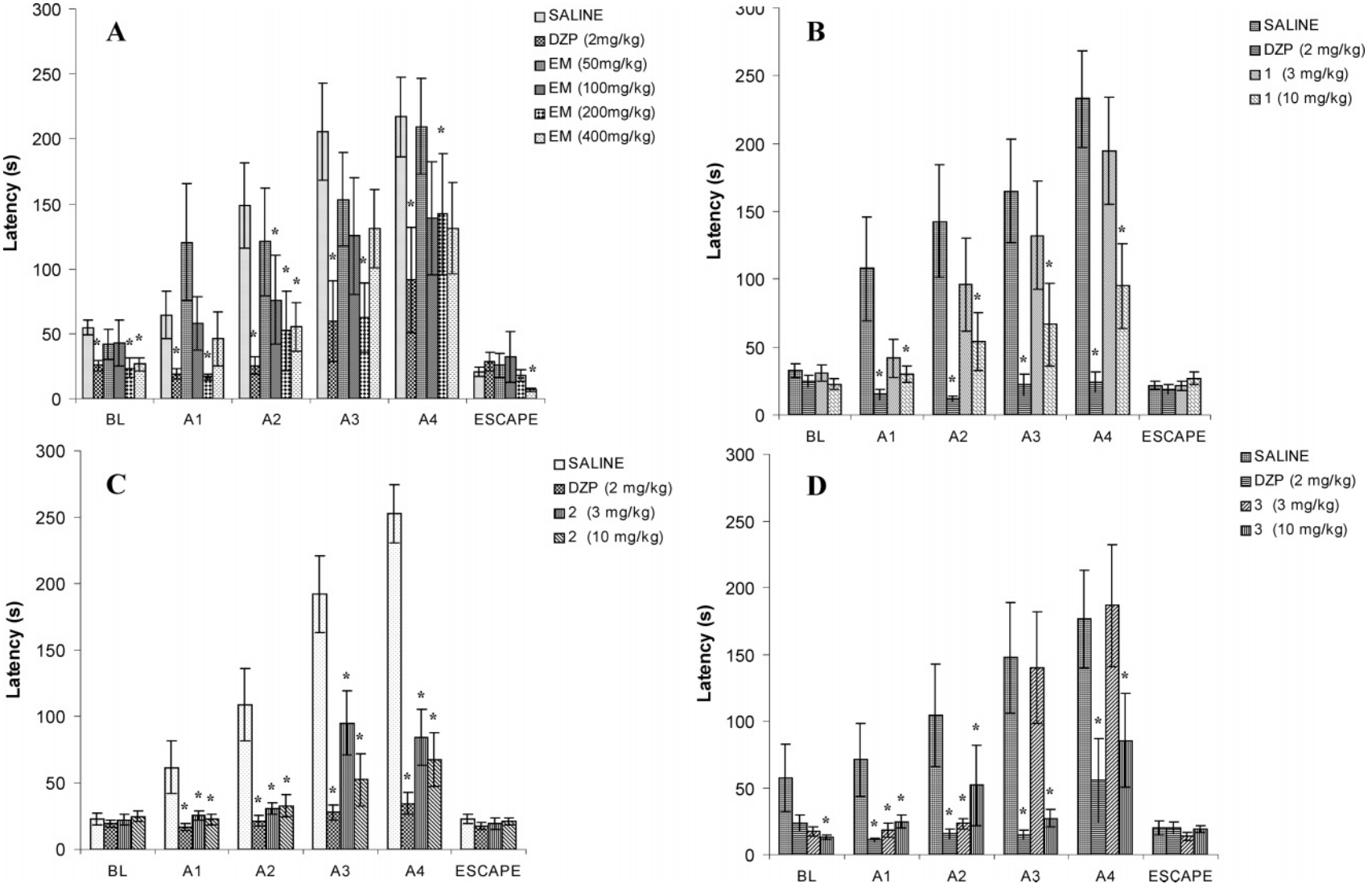

Figure 3. Effects (mean ± SEM) of CE (A), **1** (B), **2** (C), and **3** (D) ($n = 9-17$) on the behavior of mice submitted to the inhibitory avoidance and escape tasks in the elevated T-maze. *$P \leq 0.05$ vs control group (Duncan post hoc test).

Table 3. Effects (mean ± SEM) of the Crude Extract (CE) and Alkaloids (**1**−**3**) in Mice Submitted to the Locomotor Activity Test in the Arena

	crossing	rearing
Crude Extract		
saline	159.00 ± 14.80	32.50 ± 3.70
DZP (2 mg/kg)	167.44 ± 23.68	24.00 ± 2.29
CE (50 mg/kg)	178.89 ± 8.71	40.44 ± 3.12
CE (100 mg/kg)	166.10 ± 4.40	37.80 ± 2.52
CE (200 mg/kg)	199.44 ± 19.50	40.67 ± 4.25
CE (400 mg/kg)	167.30 ± 18.31	38.40 ± 5.37
	($F(5,49) = 0.78$; $P = 0.56$)	($F(5,49) = 1.90$; $P = 0.11$)
(+)-11α-OH-Eritravine		
saline	153.20 ± 22.66	25.20 ± 3.76
DZP (2 mg/kg)	154.63 ± 19.48	19.13 ± 2.03
1 (3 mg/kg)	158.50 ± 7.12	27.70 ± 3.20
1 (10 mg/kg)	126.56 ± 13.59	22.44 ± 3.50
	($F(3,33) = 0.76$; $P = 0.51$)	($F(3,33) = 1.20$; $P = 0.32$)
Eritravine		
saline	156.36 ± 10.00	30.73 ± 3.69
DZP (2 mg/kg)	143.71 ± 17.85	26.79 ± 2.14
2 (3 mg/kg)	157.33 ± 10.98	37.07 ± 2.90
2 (10 m/kg)	157.93 ± 12.43	31.71 ± 3.10
	($F(3,55) = 1.12$; $P = 0.34$)	($F(3,55) = 3.17$; $P = 0.10$)
(+)-α-OH-Erisotrine		
saline	164.56 ± 16.04	30.67 ± 3.50
DZP (2 mg/kg)	146.00 ± 17.15	20.33 ± 3.76
3 (3 mg/kg)	135.30 ± 16.61	29.60 ± 2.64
3 (10 mg/kg)	159.00 ± 18.74	29.63 ± 4.14
	($F(3,31) = 0.63$; $P = 0.59$)	($F(3,31) = 1.54$; $P = 0.22$)

400 mesh or 60−230 mesh) (Merck) were used. Molecular modeling calculations were performed using the BioMedCAChe program version 6.1.2 (BioMedCAChe, 2001).

Plant Material. Influorescences of *E. mulungu* were collected in September 2002, at Rifania, São Paulo, Brazil. A voucher specimen is deposited in the herbarium of the Departament of Vegetal Biotechnology, at the University of Ribeirão Preto, Brazil, under the reference number HPM-0032.

Extraction and Isolation. Fresh influorescences (3 kg) were macerated with EtOH/H_2O (70:30) at 30−50 °C over a period of 10 days. The solvent was removed under reduced pressure to afford a humid crude extract (CE) (120 g) that was acidified with a 10% HOAc solution. This solution was extracted with $CHCl_3$ and was then made alkaline (pH 9−10) with NH_4OH and re-extracted with $CHCl_3$ to afford a mixture of crude alkaloids (670 mg).

The alkaloidal fraction was separated by flash chromatography on Si gel, eluting with $CHCl_3/CH_3OH$ (100:0 → 70:30), to yield 60 fractions of 20 mL each, grouped into five (1−2 = A; 3−5 = B; 6−20 = C; 21−30 = D; 31−60 = E). Additional preparative TLC on Si gel of the fractions B, D, and E using Et_2O/acetone/EtOH/NH_4OH (50:43:4:3) afforded (+)-11-α-hydroxyerythravine (**1**) (48 mg), (+)-erythravine (**2**) (95 mg), and (+)-11-α-hydroxyerysotrine (**3**) (36 mg).

(+)-11α-Hydroxyerytravine (1): amorphous powder; $[\alpha]_D^{25}$ +8.7 (*c* 1.0, MeOH); UV ($CDCl_3$) λ_{max} (log ϵ) 283 (3.45), 234 (4.3) nm; IR (KBr) ν_{max} 3640, 1650, 1500, 1465, 1250 cm^{-1}; ^1H NMR, Table 1; ^{13}C NMR, Table 1; HRSIMS-MS *m*/*z* 316.3681 (calcd for $C_{18}H_{21}NO_4$, 316.3741).

(+)-Erythravine (2): white powder; ^1H and ^{13}C NMR data were consistent with those previously reported.[17]

(+)-α-Hydroxyerysotrine (3): white, amorphous powder; $[\alpha]_D^{25}$ +8.8 (*c* 1.0, MeOH); ^1H and^{13} NMR data were in agreement with those previously reported.[3,14]

Molecular Modeling. The system composed by rings A−D was constructed and submitted to conformational analysis. Each of the obtained conformations was further submitted to full geometry optimization using AM1 and PM5 semiempirical methods in both gas and solvent phases. For representation of $CDCl_3$ and pyridine solvents the COSMO solvation model was used. The relative permittivity value for $CDCl_3$ was adjusted to $\epsilon = 4.9$ and for pyridine to $\epsilon = 12.5$, while the effective VDW radius of the solvent molecules was adjusted to 2.81 Å for $CDCl_3$ and to 2.9 Å for pyridine. Hessian matrix analyses were employed to unequivocally characterize the obtained conformations as true potential energy surface minima. All calculations were performed using BioMedCAChe program version 6.1.2.[25] In order to obtain theoretical $^3J_{H,H}$ coupling constants for the minimum energy conformations produced in conformational analysis, we applied the Haasnot−Altona parametrization of the Karplus equation[18] to the endocyclic angle geometries.

Behavioral Studies. Animals. Male Swiss mice (São Paulo State University/UNESP, SP, Brazil), weighing 25−35 g, were housed in groups of 10 per cage (41 cm × 34 cm × 16 cm) and maintained under a normal 12 h light cycle (lights on 07:00 h) in a temperature/humidity controlled environment (23 ± 1 °C/55 ± 5%). Food and water were freely available. All mice were experimentally naive. Experimental procedures were in compliance with the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Local Ethical Committee (no. 35/2004).

Compound Administration. CE and alkaloids were suspended in saline (NaCl 0.9%) and diazepam (DZP) in saline + 2% Tween 80. Animals were orally treated with CE (50, 100, 200, and 400 mg/kg) and with a range of doses (3−10 mg/kg) of each isolated alkaloid, or vehicle (saline), and intraperitoneally with DZP (2 mg/kg). All compounds were administered 30 min before the experimental session.

Apparatus. Elevated T-Maze (ETM). The ETM was positioned 38.5 cm above the floor with three arms of equal dimensions (30 cm × 5 cm). One arm was enclosed by transparent glass walls (15 cm) and stood perpendicular to two open arms. To avoid falls, the open arms were surrounded by a Plexiglas rim 0.25 cm high.

Locomotor Activity Test (LAT). To assess locomotor activity, animals were exposed to a plastic rectangular box (40 cm × 48 cm × 30 cm) with its floor divided into 30 squares (8 cm^2).

A standard procedure was used for both tests.[19]

Statistics. All results were initially submitted to Levene's test for homogeneity of variance. Where this test yielded significance, results were log transformed and again submitted to Levene's test, before being submitted to ANOVA. The inhibitory avoidance measures obtained in the ETM were submitted to two-way ANOVA with treatment as the independent factor and trial as the dependent factor. Where significant main effects or interactions were obtained, data were further analyzed by Duncan's post hoc test. The escape latency in the ETM and LAT results were submitted to one-way ANOVA, followed by Duncan's post hoc test when significant. In all cases a P value < 0.05 was considered significant.

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