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## New insights in the mode of action of (+)-erythravine and (+)-11 $\alpha$ -hydroxy-erythravine alkaloids

Erica A Gelfuso, Suelen L Reis, Daiane S R Aguiar, Silmara A Faggion, Flávia M M Gomes, Diogo T Galan, Steve Peigneur, Ana M S Pereira, Márcia R Mortari, Alexandra O S Cunha, et al.

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1 **Title: New insights in the mode of action of (+)-erythravine and (+)-11 $\alpha$ -hydroxy-**  
2 **erythravine alkaloids**

3

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19

1 **Abstract**

2 Erythrinian alkaloids ((+)-erythravine and (+)-11- $\alpha$ -hydroxy-erythravine) have been pointed as  
3 the main responsible agents for the anticonvulsant and anxiolytic properties of *Erythrina*  
4 *mulungu* Mart ex Benth. The present work provides a new set of information about the mode  
5 of action of these alkaloids by the use of a complementary approach of neurochemical and  
6 electrophysiological assays. We propose here that the antiepileptic and anxiolytic properties  
7 exhibited by both alkaloids appear not to be related to the inhibition of glutamate binding or  
8 GABA uptake, or even to the increase of glutamate uptake or GABA binding, as investigated  
9 here by the use of rat cortical synaptosomes. Similarly, and even in a high concentration, (+)-  
10 erythravine and (+)-11- $\alpha$ -hydroxy-erythravine did not modulate the main sodium and potassium  
11 channel isoforms checked by the use of voltage-clamp studies on *Xenopus laevis* oocytes.  
12 However, unlike (+)-11- $\alpha$ -hydroxy-erythravine, which presented a little effect, it was possible  
13 to observe that the (+)-erythravine alkaloid produced a significant inhibitory modulation on  
14  $\alpha_4\beta_2$ ,  $\alpha_4\beta_4$  and  $\alpha_7$  isoforms of nicotinic acetylcholine receptors also checked by the use of  
15 voltage-clamp studies, which could explain at least partially its anxiolytic and anticonvulsant  
16 properties. Since (+)-11- $\alpha$ -hydroxy-erythravine and erythravine modulated nicotinic  
17 acetylcholine receptors to different extents, it is possible to reinforce that small differences  
18 between the chemical structure of these alkaloids can affect the selectivity and affinity of target-  
19 ligand interactions, conferring distinct potency and/or pharmacological properties to them, as  
20 previously suggested by differential experimental comparison between different erythrinian  
21 alkaloids.

22 **Keywords:** Electrophysiology; *E. mulungu*; Erythrinian alkaloids; GABA; Glutamate; Ion  
23 Channels.

24

## 1 **1 Introduction**

2 In the last decades, the gradual increase of studies searching for new antiepileptic and  
3 anxiolytic drugs has pointed *Erythrina mulungu* (Mart. ex Benth.) (syn. *E. verna* Velloso, 1825)  
4 as a special source of active compounds (Fahmy et al., 2019). Flowerings of this plant have  
5 been used as raw material for a large set of industrialized herbal preparations in different  
6 countries. Several investigations have validated the antidepressant, anxiolytic, anticonvulsant  
7 and sedative properties popularly acclaimed to *E. mulungu* (Bezerra Carvalho et al., 2014; De  
8 Oliveira et al., 2012; Durigan et al., 2004; Lorenzi and Matos, 2008; Rodrigues et al., 2008;  
9 Valli et al., 2013).

10 *E. mulungu* has been considered as a great source of erythrinian alkaloids. Indeed, the  
11 plant flowerings reserve a huge collection of alkaloids with different chemical structures and  
12 pharmacological activities, attending as a natural library for a chemical diversity of compounds  
13 for which structure-activity relationship deserves strong pharmaceutical attention (Feitosa et  
14 al., 2012; Majinda, 2018). Previous studies have demonstrated that (+)-erythravine and (+)-11-  
15  $\alpha$ -hydroxy-erythravine alkaloids are among those responsible for antiepileptic and anxiolytic  
16 properties exhibited by *E. mulungu* flowerings (Faggion et al., 2011; Flausino et al., 2007;  
17 Gelfuso et al., 2020; Santos Rosa et al., 2012). However, scarce information is presented about  
18 the mechanism of action of these alkaloids both at molecular and cellular levels (Flausino et al.,  
19 2007; Setti-Perdigão et al., 2013).

20 Around 50 million people worldwide are diagnosed with different types of epilepsy  
21 (World Health Organization, 2019). The high prevalence and/or incidence of this neurological  
22 disorder negatively impacts on social, health and economic demands from different countries  
23 (Bell et al., 2014). Similarly, anxiety disorders affect different segments of our modern society  
24 and usually appears as a comorbid condition in many epileptic patients (de la Loge et al., 2016).  
25 Despite significant differences in the molecular and cellular bases involved on the development

1 of epilepsy and anxiety, the sharing of some pathophysiological mechanisms and neural  
2 pathways appears as overlapping explanations for both disorders (Hamid et al., 2011). In this  
3 context, an imbalance between GABA and glutamate neurotransmissions and/or between  
4 different ion channels in the onset and the development of epilepsy and anxiety has been  
5 proposed (Hamid et al., 2011; Reddy and Kuruba, 2013; Zubareva et al., 2018). Moreover,  
6 studies with nicotinic receptors demonstrate the relevance of this receptor-channel for both  
7 disorders, as well as its relationship with glutamatergic plasticity and cognitive functions such  
8 as memory and learning, suggesting an important connection with epilepsy or anxiety and a  
9 new avenue for development novel drugs acting on new or diversified pharmacological targets  
10 (Dani and Bertrand, 2007; Ghasemi and Hadipour-Niktarash, 2015; Zarrindast and Khakpai,  
11 2019).

12 Considering this last statement and also the physiopathology underlying both epilepsy  
13 and anxiety, it is chiefly appropriate to investigate the potential effects of (+)-erythravine and  
14 (+)-11- $\alpha$ -hydroxy-erythravine alkaloids on synaptic events such as of GABA and glutamate  
15 binding and uptake as well as on different sodium and potassium channels isoforms or also on  
16 different isoforms of cholinergic receptors. This approach may bring a broad body of  
17 information useful for a better understanding of different pharmacological aspects related to  
18 erythrinian alkaloids and to the ethnopharmacology regarding use of *E. mulungu* herbal  
19 preparations.

## 20 21 **2 Material and Methods**

### 22 **2.1 Alkaloids**

23 Flowers of *E. mulungu* were collected in Rifaina, São Paulo, Brazil (20°07'41,7";  
24 47°28'1'54,5"; 1037m; CGEN-Brazil: 02001.005109/2011-10). The alkaloids chromatographic  
25 isolation was carried out according to Flausino and colleagues (Flausino et al., 2007). Nuclear

1 magnetic resonance (Varian Inc., USA) was used to confirm the chemical identity and purity  
2 degree of (+)-erythravine and (+)-11- $\alpha$ -hydroxy-erythravine.

3

#### 4 **2.2 Ethics statement animal experimentation**

5 The procedures performed with rats were approved by the University of Ribeirão Preto  
6 Ethic Committee (06/2008). All procedures were conducted in accordance with the Guide for  
7 the Care and Use of Laboratory Animals (National Research Council (US) Committee for the  
8 Update of the Guide for the Care and Use of Laboratory Animals, (2011) and all efforts were  
9 made to minimize animal suffering and to reduce the number of animals used.

10 The use of the frogs was in accordance with the license number LA1210239 of the  
11 Laboratory of Toxicology & Pharmacology, University of Leuven. The use of *Xenopus laevis*  
12 was approved by the Ethical Committee for animal experiments of the University of Leuven  
13 (P186/2019). All animal care and experimental procedures agreed with the guidelines of  
14 'European convention for the protection of vertebrate animals used for experimental and other  
15 scientific purposes (Strasbourg, 18.III.1986).

16

#### 17 **2.3 Neurochemical assays: $^3\text{H}$ -Glutamate and $^3\text{H}$ -GABA uptake and binding**

18 Synaptosomal preparation was performed according to the method described by Gray  
19 & Whittaker, Coutinho-Netto et al. and Coutinho-Netto et al. (Gray and Whittaker, 1962;  
20 Coutinho-Netto et al., 1981; Coutinho-Netto et al., 2009). Cerebral cortices from healthy male  
21 Wistar rats (200-250 g) were rapidly removed and homogenized in ice-cold 0.32 M sucrose  
22 using Potter-Elvehjem Labo Stirrer LS-50 Yamato-type equipment. The sample was then  
23 centrifuged for 10 minutes at 1700 g (4 °C). The supernatant was collected and centrifuged for  
24 20 minutes at 21.200 g (4 °C). The pellet was resuspended in Krebs-phosphate buffer (5 mM,  
25 pH 7.4). The synaptosome fraction obtained was used in the glutamate and GABA uptake

1 assays. For the binding assay, the synaptosome fraction was further homogenized in 50 mM  
2 Tris-HCl buffer (pH 7.4) and then centrifuged at 3000 g (4 °C) (5 min). The pellet was washed  
3 in 50 mM Tris-HCl buffer (pH 7.4) and resuspended in the same buffer. Finally, the obtained  
4 synaptic membranes were centrifuged (3000 g/5 min/4 °C) and stored at -20 °C for at least 18  
5 h. Protein content for both synaptosome fraction and the synaptic membrane was determined  
6 by the Lowry method modified by Hartree (Hartree, 1972).

7 Uptake assays were started by adding 5 nM of radio-labelled [<sup>3</sup>H]-GABA (GE  
8 Healthcare; 90 Ci/mmol) or 10 nM of radio-labelled [<sup>3</sup>H]-Glutamate (GE Healthcare; 52  
9 Ci/mmol) to the synaptosome fraction (0.25 mg of protein/ml at final concentration). Binding  
10 assays were started by adding of 5 nM of [<sup>3</sup>H]-GABA or 25 nM of [<sup>3</sup>H]-Glutamate to the  
11 synaptic membranes (1.0 mg of protein/ml at final concentration). Both assays were performed  
12 in the presence or absence of increasing final concentrations (0.001-10 µg/mL) of (+)-  
13 erythravine or (+)-11 $\alpha$ -hydroxy-erythravine completed to the final volume with 50 mM Tris-  
14 HCl buffer (0.3 ml for binding assays) or Krebs-phosphate buffer (0.5 ml for uptake assays).  
15 Samples were incubated for 3 min at 25°C or 37°C respectively for [<sup>3</sup>H]-GABA and [<sup>3</sup>H]-  
16 Glutamate uptake assays. Incubation was performed for 30 min at temperatures of 25°C or 37°C  
17 for [<sup>3</sup>H]-GABA and [<sup>3</sup>H]-Glutamate binding assays, respectively.

18 All reactions were carried out in triplicate and stopped by centrifugation (3000 g, 3 min,  
19 4°C). Pellets were washed twice with ice-cold distilled water and homogenized in absolute  
20 methanol for uptake experiments or in 50 mM Tris-HCl buffer for binding assays. Samples of  
21 supernatants (uptake experiments) or whole tube content (binding experiments) were  
22 transferred to scintillation vials containing 1 ml of the biodegradable scintillation cocktail  
23 ScintiVerse (Fisher Scientific, USA) and quantified in a scintillation counter with a counting  
24 efficiency of 30-40% for <sup>3</sup>H. Non-specific GABA/Glutamate uptake and GABA/Glutamate  
25 binding were respectively estimated in parallel probes with non-radiolabelled GABA and

1 Glutamate (1 mM, final concentration). These values were subtracted to give the total specific  
2 uptake or binding. Results were expressed as % of inhibition of GABA/Glutamate uptake or  
3 GABA/Glutamate binding in relation to the control.

4

#### 5 **2.4 Electrophysiological Recordings – Voltage-clamp**

6 Oocytes were obtained through partial ovariectomy from sedated female frogs (*Xenopus*  
7 *laevis*) by using a solution containing tricaine mesylate and sodium carbonate (1g/L). After  
8 collection recovery, oocytes were injected with 20–70 nL of cRNA coding for Na<sub>v</sub>, K<sub>v</sub> channels  
9 or nicotinic receptors (nAChR) using a micro-injector (Drummond Scientific, EUA). cRNAs  
10 were prepared with mMESSAGING mMACHINE transcription kit T7 or SP6 (Ambion, USA).  
11 Then, the oocytes were incubated in a ND-96 solution (96 NaCl, 2 KCl, 1.8 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>,  
12 and 5 HEPES-pH 7.4; in mM) supplemented with gentamicin sulfate (50 mg/L; Rotexmedica,  
13 Trittau, Germany). Theophylline (90 mg/L; ABC chemicals, Wauthier Braine, Belgium) was  
14 also added in the solution for oocytes injected with cRNA encoding Na<sub>v</sub> channels. Whole-cell  
15 currents were collected under controlled temperature (18-22°C) using the two-electrode  
16 voltage-clamp technique (GeneClamp 500 amplifier; Axon Instruments, Foster City, CA,  
17 USA), driven by a pClamp data acquisition system (Molecular Devices, Sunnyvale, CA, USA).  
18 The final concentration (10 μM) of each alkaloid was added to the camera containing the oocyte  
19 in a bath solution ND-96; in case of action on the channel or receptor tested, a sufficient number  
20 of measurements were obtained using additional and serial concentrations to attain statistical  
21 significance. Voltage and current electrodes were filled with 3M KCl, and the resistances of  
22 both electrodes were maintained as low as possible (0.5 to 1.5 MΩ). The alkaloids-induced shift  
23 in the current-voltage relationship was obtained by averaging the peak amplitude of at least  
24 three control responses performed in triplicate. Whole-cell current traces were evoked from a  
25 holding potential of –90 mV. For Nav 1.3 and Nav 1.6 channels, whole-cell current traces were



1 evoked every 5s for 100ms to 0mV, which corresponds to the maximal activation of the Nav-  
2 subtype in control conditions. The elicited currents were sampled at 20 kHz and filtered at 2  
3 kHz using a four-pole, low-pass Bessel filter. Kv1.1, Kv1.2, Kv1.4 and Kv4.2 currents were  
4 evoked by 500ms depolarizations to 0 mV followed by a 500ms pulse to -50 mV, from a  
5 holding potential of -90 mV. hERG or Kv11.1 peak and tail currents were generated by a 2.5s  
6 prepulse from -90 mV, depolarized to +40 mV followed by a 2.5s pulse to -120 mV and then  
7 held at -90 mV for 7.4 seconds. For measuring nAChR currents, current traces were evoked  
8 from an initial potential of -90 mV to -70 mV for 400 seconds then returned to -90 mV. The  
9 receptor was activated by the addition of 300  $\mu$ M of ACh.

10

## 11 **2.5 Statistical analysis**

12 Electrophysiological data were analyzed using Origin 7.5 software (Originlab,  
13 Northampton, MA, USA) and presented as the result of at least 3 independent experiments ( $n$   
14  $\geq 3$ ), the effective dose 50% was calculated through the nonlinear regression with the  
15 normalized response. Neurochemical assays were evaluated by one-way ANOVA followed  
16 Newman-Keuls using GraphPad Prism 7 (GraphPad Software), considering  $p < 0.05$  as  
17 statistically significant.

18

## 19 **3 Results**

### 20 **3.1 Neurochemical assays: $^3\text{H}$ -Glutamate and $^3\text{H}$ -GABA uptake and binding**

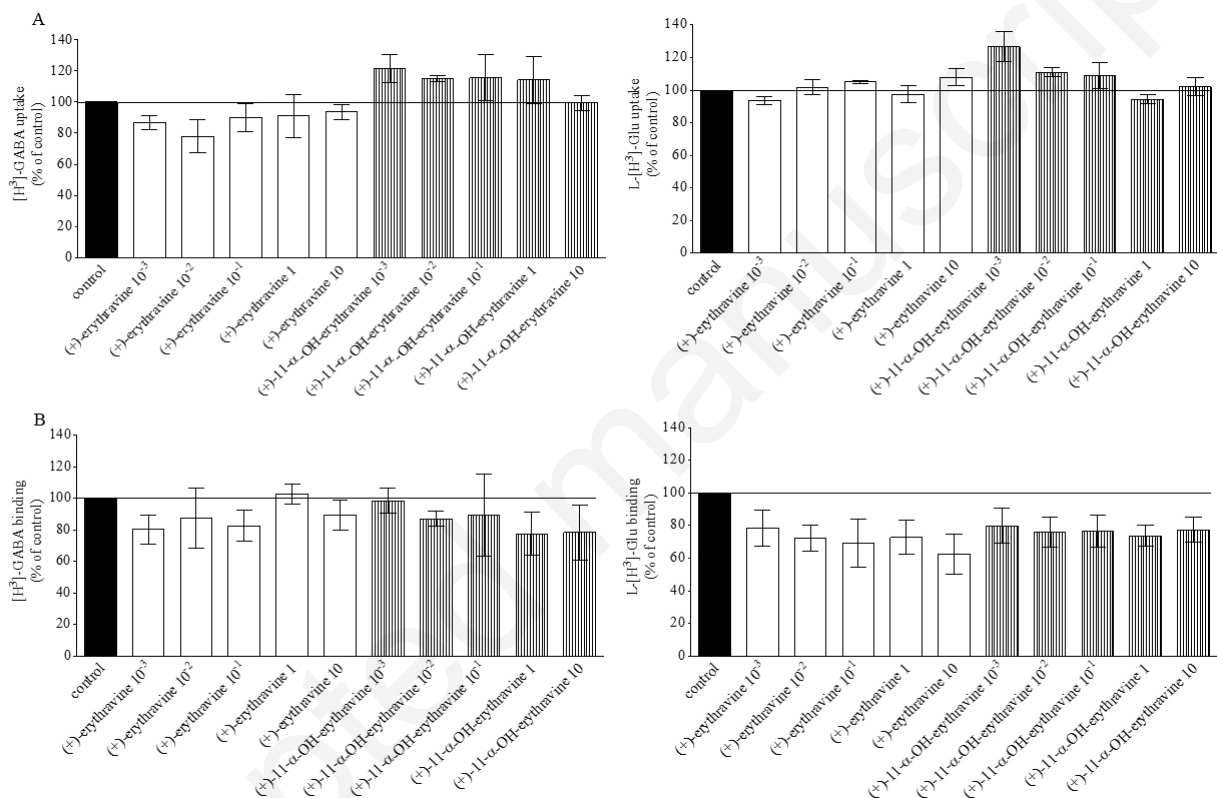
21 We investigated whether the alkaloids (+)-erythravine and (+)-11- $\alpha$ -hydroxy-  
22 erythravine would act by modifying  $\text{H}^3$ -glutamate and  $\text{H}^3$ -GABA uptake and/or binding.

23 Figures 1A and 1B present data obtained from  $\text{H}^3$ -GABA and  $\text{H}^3$ -Glutamate synaptosomal  
24 uptake and binding, respectively, in presence or absence of different concentrations of both  
25 alkaloids. It can be noticed that both (+)-11- $\alpha$ -hydroxy-erythravine and (+)-erythravine acted

1 neither on the uptake nor on the binding of the referred neurotransmitters according to our  
 2 experimental conditions. The alkaloid concentrations ranged from 0.001 to 10  $\mu\text{g/mL}$  and were  
 3 broad enough to be pharmacologically relevant and representative.

4

5 Figure 1 Analysis of the potential effects of erythrinian alkaloids on binding and reuptake of  
 6  $[^3\text{H}]\text{GABA}$  and  $\text{L-}[^3\text{H}]\text{Glutamate}$



7

8 Representative graph of erythrinian alkaloids concentrations from 0.001 to 10  $\mu\text{g/mL}$  on (A)  
 9 uptake and (B) binding of  $[^3\text{H}]\text{GABA}$  (left) and  $\text{L-}[^3\text{H}]\text{Glutamate}$  (right) in cerebrocortical  
 10 synaptosomes of rats. The data presented are representative of three independent experiments  
 11 carried out in triplicate. Data were analyzed using the one-way ANOVA (error bars represent  
 12 SEM).

13

### 14 3.2 Electrophysiology

15

16 Next, we checked if the alkaloids would act by modulating of sodium and potassium  
 17 channels and/or nicotinic receptors. Data showed that both alkaloids, (+)-erythravine and (+)-  
 18 11- $\alpha$ -hydroxy-erythravine, at a final concentration of 10  $\mu\text{M}$ , did not promote significant  
 changes at the activation and/or inactivation of the potassium channels (isoforms  $\text{Kv}1.1$ ,  $\text{Kv}1.2$ ,

1 K<sub>v</sub> 1.4, K<sub>v</sub> 4.2 e K<sub>v</sub>11.1) and sodium channels (isoform Nav1.3 and Nav1.6) (Table 1) (Figure  
2 2).

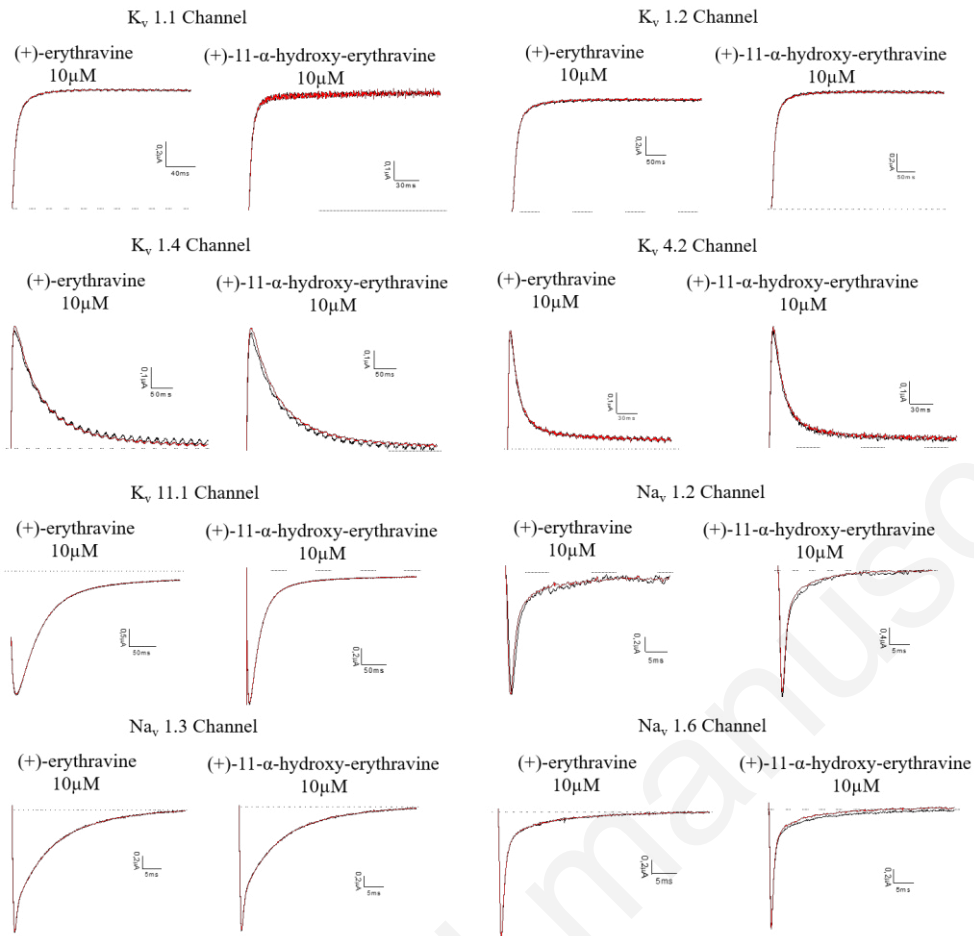
3

4 Table 1

<b>Isoform</b>	<b>Control</b>	<b>(+)-erythravine 10<math>\mu</math>M</b>	<b>(+)-<math>\alpha</math>-OH-erythravine 10<math>\mu</math>M</b>
K <sub>v</sub> 1.1	0.598 $\pm$ 0.076	0.599 $\pm$ 0.099	0.659 $\pm$ 0.085
K <sub>v</sub> 1.2	0.073 $\pm$ 0.067	0.093 $\pm$ 0.068	0.066 $\pm$ 0.066
K <sub>v</sub> 1.4	1.966 $\pm$ 1.908	1.989 $\pm$ 1.904	2.001 $\pm$ 1.895
K <sub>v</sub> 4.2	0.413 $\pm$ 0.030	0.417 $\pm$ 0.067	0.415 $\pm$ 0.104
K <sub>v</sub> 11.1	-1.063 $\pm$ 0.247	-0.981 $\pm$ 0.150	-1.038 $\pm$ 0.242
Nav1.3	-0.585 $\pm$ 0.037	-0.575 $\pm$ 0.033	-0.535 $\pm$ 0.035
Nav1.6	-0.546 $\pm$ 0.085	-0.546 $\pm$ 0.075	-0.547 $\pm$ 0.063

5 Analysis of the potential effects of erythrinian alkaloids at a final concentration of 10  $\mu$ M in  
6 sodium and potassium channels using oocytes of *X. laevis* performed in triplicate. The data  
7 were analyzed using the Originlab and they are presented means  $\pm$  SEM.  
8

9 Figure 2 Current-Voltage relation for the potassium and sodium channels



1

2 Example of a recording from oocytes illustrating electrophysiological screening of (+)-  
 3 erythravine and (+)-11- $\alpha$ -hydroxy-erythravine at a final concentration of 10  $\mu$ M on  $K_v$  channels  
 4 and  $Na_v$  channels expressed in *Xenopus* oocytes. \*Represents traces after alkaloids application  
 5 and overlapping control traces before alkaloid application.

6

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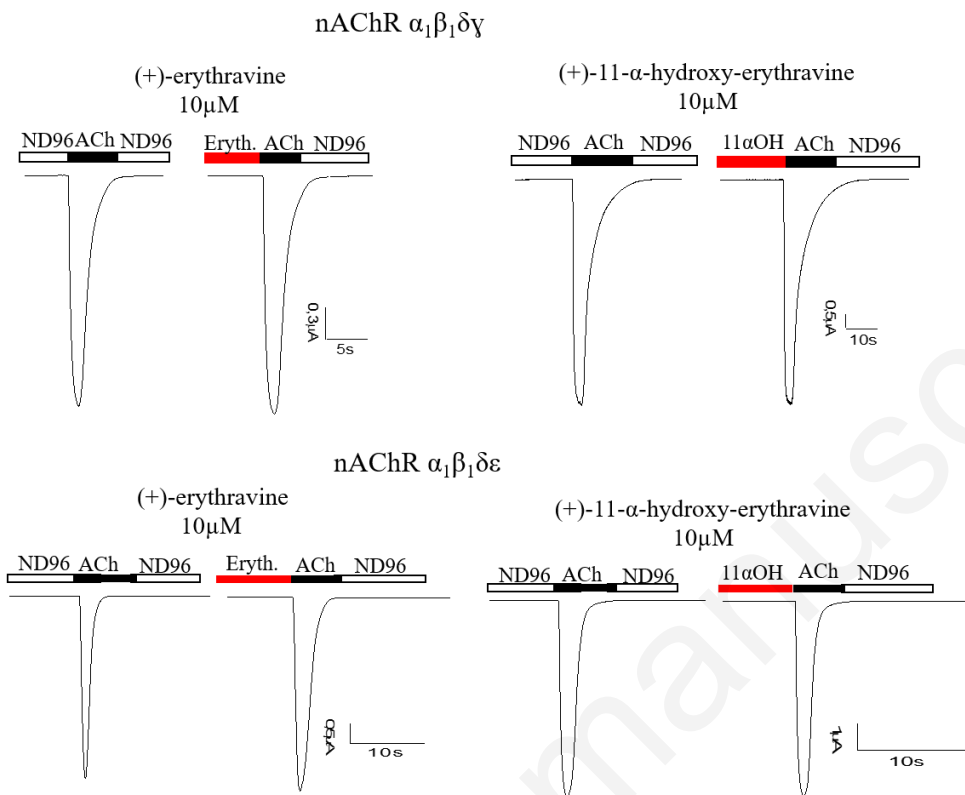
14

15

However, these alkaloids seem to selectively act on specific isoforms of the nicotinic  
 receptors (Figure 3A and 3B). (+)-erythravine and (+)-11- $\alpha$ -hydroxy-erythravine (both at 10  
 $\mu$ M) did not promote any significant modulation on ACh-evoked current amplitude mediated  
 by  $\alpha_1\beta_1\delta\gamma$  and  $\alpha_1\beta_1\delta\epsilon$  nAChRs. However, differences in the magnitude of action were observed  
 for (+)-erythravine and (+)-11- $\alpha$ -hydroxy-erythravine when tested for others nAChR types.  
 Indeed, while the (+)-11- $\alpha$ -hydroxy-erythravine (10  $\mu$ M) presented a lesser and very discrete  
 action for the nAChRs  $\alpha_4\beta_4$  (23.52%),  $\alpha_4\beta_2$  (10.42%) and  $\alpha_7$  (3.56%), on the other hand, (+)-  
 erythravine (10  $\mu$ M) elicited a very significant inhibition of the electric currents mediated by  $\alpha_7$   
 (87.78%),  $\alpha_4\beta_2$  (83.65%) and  $\alpha_4\beta_4$  (55.73%) nAChR isoforms (Table 2).

1

2 Figure 3A Current-Voltage relation for the nAChR

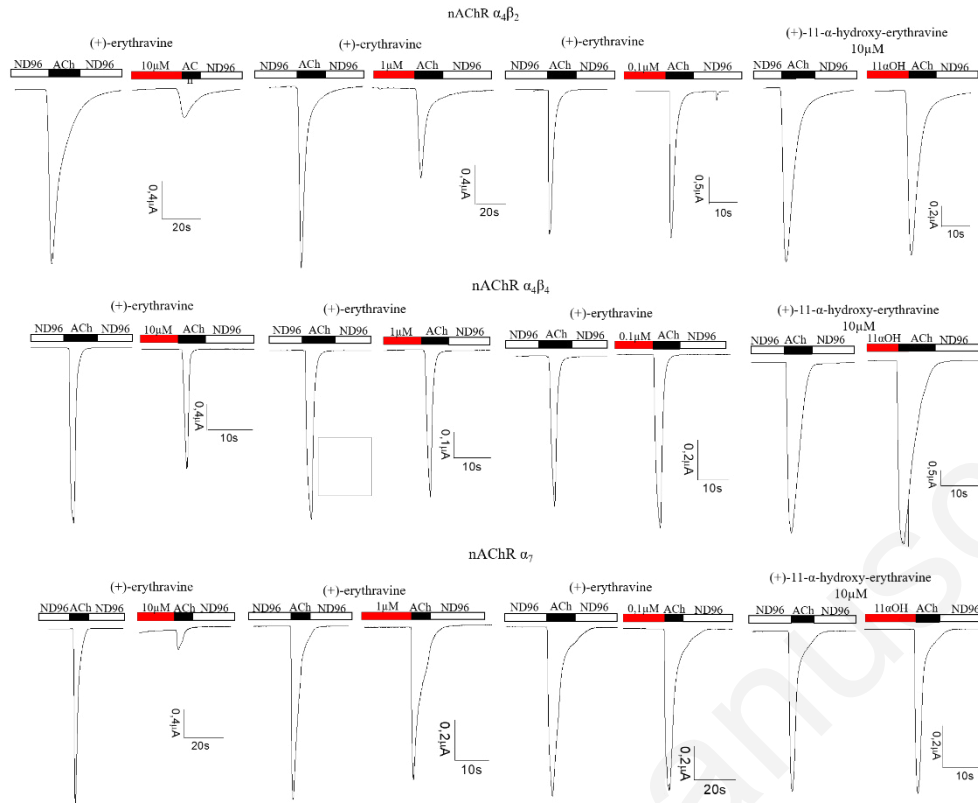


3

4 Example of a recording from oocytes illustrating electrophysiological screening of (+)-  
 5 erythravine and (+)-11- $\alpha$ -hydroxy-erythravine at a final concentration of 10  $\mu$ M on  $\alpha_1\beta_1\delta\gamma$  and  
 6  $\alpha_1\beta_1\delta\epsilon$  nicotinic acetylcholine receptors expressed in *Xenopus* oocytes.

7

8 Figure 3B Current-Voltage relation for the nAChR



1  
2 Example of a recording from oocytes illustrating the blockade screening of (+)-erythravine and  
3 (+)-11- $\alpha$ -hydroxy-erythravine using concentrations from 10 to 0.1  $\mu$ M on  $\alpha_4\beta_2$ ,  $\alpha_4\beta_4$  and  $\alpha_7$   
4 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes.

5  
6 Table 2

Isoform	% inhibition (+)-erythravine 10 $\mu$ M	% inhibition (+)- $\alpha$ -OH-erythravine 10 $\mu$ M
$\alpha_1\beta_1\delta\gamma$	2.861 $\pm$ 1.409	1.457 $\pm$ 2.609
$\alpha_1\beta_1\delta\epsilon$	0.065 $\pm$ 0.328	3.462 $\pm$ 2.760
$\alpha_4\beta_2$	83.651 $\pm$ 1.945	10.424 $\pm$ 0.845
$\alpha_4\beta_4$	55.731 $\pm$ 5.033	23.516 $\pm$ 1.999
$\alpha_7$	87.783 $\pm$ 0.744	3.563 $\pm$ 1.140

7 Effects of alkaloids (+)-erythravine and (+)-11- $\alpha$ -hydroxy-erythravine isolated of the plant *E.*  
8 *mulungu* in nicotinic acetylcholine receptors using oocytes of *X. laevis* performed in triplicate.  
9 The data were analyzed using the Originlab and they are presented means  $\pm$  SEM.

10  
11 Considering the higher blocking activity performance of (+)-erythravine for the  
12 nicotinic isoforms  $\alpha_7$ ,  $\alpha_4\beta_2$ ,  $\alpha_4\beta_4$ , the assayed concentrations were extended to 1 and 0.1  $\mu$ M,  
13 allowing to estimate the EC<sub>50</sub>. The EC<sub>50</sub> values (please see Table 3) confirm the high affinity

1 of the alkaloid (+)-erythravine for the  $\alpha_4\beta_2$ ,  $\alpha_4\beta_4$  and  $\alpha_7$  nAChR isoforms, especially for the case  
 2 of  $\alpha_4\beta_2$  and  $\alpha_7$  receptors in which the  $EC_{50}$  values were lower (1.04 and 2.84  $\mu$ M, respectively).

3

4 Table 3

Nicotinic Receptor	Concentration for 50% inhibition
$\alpha_4\beta_2$	1.04 $\mu$ M
$\alpha_4\beta_4$	9.98 $\mu$ M
$\alpha_7$	2.84 $\mu$ M

5 Concentration of alkaloid (+)-erythravine for 50% inhibition in different nicotinic acetylcholine  
 6 receptors considering the higher blocking activity performance electrophysiological  
 7 experiments previously demonstrated. The data were analyzed using the Originlab.  
 8

#### 9 4 Discussion

10 The aim of the present work was to investigate the possible mechanisms of action of the  
 11 alkaloids (+)-erythravine and (+)-11- $\alpha$ -hydroxy-erythravine. Our results bring forward  
 12 substantial evidence regarding the mechanisms by which these alkaloids promote their  
 13 pharmacological effects, especially in the case of (+)-erythravine. In this regard, it is important  
 14 to notice that erythrinian alkaloids, including (+)-erythravine and (+)-11- $\alpha$ -hydroxy-  
 15 erythravine, have been reported to have anticonvulsant and anxiolytic-like effects in different  
 16 sets of animal models (Faggion et al., 2011; Gelfuso et al., 2020; Santos Rosa et al., 2012).  
 17 Epilepsy and anxiety are commonly observed as comorbid disorders (Keezer et al., 2016).  
 18 Moreover, both diseases shares some neurobiological basis, in particular related to the  
 19 imbalance between excitatory and inhibitory neurotransmitters (Meldrum, 2000; Zarcone and  
 20 Corbetta, 2017). This is specially reinforced by the well-evidenced use of GABA and/or  
 21 glutamate-modulating agents for pharmacological treatment of different types of anxiety and  
 22 epilepsy disorders (Averill et al., 2017; Mula, 2016). Despite the importance of GABA and  
 23 glutamate in epilepsy and anxiety, and the fact that the alkaloids inhibit convulsive seizures  
 24 triggered by GABAergic antagonists and glutamatergic agonists in the acute seizure models

1 (Faggion et al., 2011), (+)-erythravine and (+)-11- $\alpha$ -hydroxy-erythravine show no significant  
2 modifications on uptake and binding of these neurotransmitters.

3 Although alterations on GABA and glutamate neurotransmitter synaptic events like in  
4 case of binding and uptake have been clearly related to the physiopathology and treatment of  
5 epilepsy and anxiety, dysfunctions in other structures or synaptic events involving even GABA  
6 and glutamate indirectly or other neurotransmitters also play an important role in the  
7 development of these diseases, including the sodium and potassium ionic channels and the  
8 nicotinic cholinergic receptors (Hamid et al., 2011; Mula, 2016; Zarrindast and Khakpai, 2019).  
9 The concentration of 10  $\mu$ M of each alkaloid on voltage-clamp tests is considered relatively  
10 high at the pharmacological point of view and is used in the context of eliminatory prospective  
11 assays.

12 Sodium channels are responsible for the depolarization of the membrane and conduction  
13 of action potentials. It is commonly known that the inhibition of sodium channels can explain  
14 the antiepileptic and/or anxiolytic action of several drugs, such as carbamazepine, lamotrigine  
15 and phenytoin (Catterall, 2012). Among the isoforms of sodium channels related to  
16 pharmacological scope/interest of this work, are  $Na_v1.1$ ,  $Na_v1.2$ ,  $Na_v1.3$ ,  $Na_v1.6$ ,  $Na_v\beta$   
17 (Dussaule and Boulleret, 2018; Musto et al., 2019).

18 Different from sodium channels, an increase in the conductance of  $K^+$  ions results in  
19 neuronal hyperpolarization, exerting an inhibitory effect on the neuronal pathway function  
20 (Barrese et al., 2010). Thus, studies with isoforms of potassium channels are of great value,  
21 especially those in which mutations in the genes responsible for their expression are already  
22 described in the literature being related to epilepsy, for example,  $K_v1.1$  (gene *KCNA1*/ gene  
23 *LGI1*),  $K_v1.2$  (*KCNA2*) e  $K_v4.2$  (gene *KCND2*) (Barrese et al., 2010; Errington et al., 2005;  
24 Meldrum and Rogawski, 2007). However and despite the absence of some important sodium  
25 channels in our representative experimental panel (such as  $Na_v1.1$  and  $Na_v\beta$ ), (+)-erythravine



1 and (+)-11- $\alpha$ -hydroxy-erythravine did not work through a negative or positive modulation of  
2 sodium and/or potassium channels, respectively, at least not for the isoforms and alkaloids at  
3 the concentrations here studied.

4 Finally, we investigated the participation of nicotinic receptors on the mode of action of  
5 both alkaloids. These receptors are functionally linked to different ion channels and GABAergic  
6 and glutamatergic neurotransmission pathways, thus being importantly related to the  
7 pathophysiology and treatment of different neurological disorders, including epilepsy and  
8 anxiety (Mula, 2016; Zarrindast and Khakpai, 2019). Indeed, the nicotinic cholinergic receptors  
9 can modulate the release of neurotransmitters, being activated via  $\text{Ca}^{2+}$  influx and are described  
10 as important structures in several physiological processes such as learning and memory and  
11 motor control (Dineley et al., 2015; Zarrindast and Khakpai, 2019). The modulation of  $\alpha_4\beta_2$  is  
12 suggested to play an important role in genetic epileptic syndromes as well as in the autosomal  
13 dominant nocturnal frontal lobe epilepsy (Garibotto et al., 2019). On the other hand, the  $\alpha_7$   
14 isoform is a major nicotinic cholinergic receptor that acts presynaptically by modulating the  
15 release of neurotransmitters, such as glutamate and GABA (Dineley et al., 2015; Zarrindast and  
16 Khakpai, 2019). It is demonstrated that the increase in the glutamate clearance elicited by  
17 nicotine action on  $\alpha_7$  has a neuroprotective effect against neuroinflammation and  
18 neurodegenerative disorders (Dineley et al., 2015).

19 The most important result presented on this work is the selective inhibitory action of  
20 (+)-erythravine on the nicotinic receptors  $\alpha_4\beta_4$  and especially on  $\alpha_4\beta_2$  and  $\alpha_7$  isoforms to the  
21 detriment of others evaluated in this study. The action of (+)-erythravine in nicotinic receptors,  
22 can at least partially, explain its anxiolytic and anticonvulsant properties as previously  
23 demonstrated (Faggion et al., 2011; Santos Rosa et al., 2012; Setti-Perdigão et al., 2013). The  
24 results from this study are added up and are in accordance to Setti-Perdigão and colleagues  
25 (2013) data, which showed an important selectivity of (+)-erythravine for nicotinic receptors

1 specifically expressed in the CNS ( $\alpha_4\beta_2$  e  $\alpha_7$ ) (Setti-Perdigão et al., 2013) to the detriment of  
2 others studied isoforms expressed in skeletal neuromuscular junctions ( $\alpha_1\beta_1\delta\epsilon$ ), ganglionic  
3 nicotinic receptors ( $\alpha_7$ ,  $\alpha_3\beta_4$ ) (Fagerlund et al., 2016; Lebbe et al., 2014). This reinforces the  
4 probable participation of the inhibitory mechanism of specific nicotinic receptors (particularly  
5  $\alpha_4\beta_2$  e  $\alpha_7$ ) in the anxiolytic and anticonvulsant actions of (+)-erythravine. However, it is  
6 important to note that (+)-11- $\alpha$ -hydroxy-erythravine showed an almost null or very discrete  
7 effect on the same type of receptors. Thus, since (+)-11- $\alpha$ -hydroxy-erythravine and erythravine  
8 modulated  $\alpha_4\beta_2$ ,  $\alpha_4\beta_2$  and  $\alpha_7$  nicotinic receptors at different extents and they have a very similar  
9 pharmacological performance as anxiolytic and anticonvulsant as previously demonstrated  
10 (Faggion et al., 2011; O. Flausino et al., 2007), it is possible to assume that other neuronal and  
11 synaptic structures or neurotransmission pathways are involved on the mode of action of the  
12 erythrinian alkaloids besides the nicotinic receptors. Moreover, it cannot be excluded that small  
13 differences between the chemical structure of these alkaloids can affect the selectivity and  
14 affinity of target-ligand interactions on nicotinic receptors composed by the  $\alpha_4$ ,  $\alpha_7$ ,  $\beta_2$ ,  $\beta_3$  and  $\beta_4$   
15 subunits. Thus, the erythrinian alkaloids represent a phytochemical class for which the study of  
16 structure-activity relationship along their chemical diversity deserves a strong pharmaceutical  
17 attention, important for the rational development of new drugs with action on the CNS,  
18 including those useful against anxiety and epilepsy.

19

## 20 **5 Conclusion**

21 In this work we have described the mechanism of action of alkaloids, unlike most of the  
22 commercially available anxiolytic and anticonvulsant drugs, does not involve modulation of  
23 the synaptic events related to GABA and glutamate binding or uptake, neither involves sodium  
24 or potassium channels. Particularly in the case of (+)-erythravine, an inhibitory modulation on

1 nicotinic receptors with a selective action for the isoforms of  $\alpha_4\beta_4$ ,  $\alpha_4\beta_2$  and  $\alpha_7$  was observed,  
2 which may explain at least partially its mode of action.

3

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