Erythroxylum pungens Tropane Alkaloids: GC-MS Analysis and the Bioactive Potential of 3-(2-methylbutyryloxy)tropan-6,7-diol in Zebrafish (*Danio rerio*)

Authors

Letícia Gondim Lambert Moreira[1], Maria Elisa Leite Ferreira[2], Fernanda Priscila Santos Reginaldo[1], Estela Mariana Guimarães Lourenço[3], José Angelo Silveira Zuanazzi[4], Euzébio Guimarães Barbosa[1], Leandro de Santis Ferreira[1], Arthur Germano Fett-Neto[5], Alberto José Cavalheiro[6], Ana Carolina Luchiari[2], Raquel Brandt Giordani[1]

Affiliations

1 Department of Pharmacy, Federal University of Rio Grande do Norte, Natal, RN, Brazil
2 Department of Physiology and Behavior, Federal University of Rio Grande do Norte, Natal, RN, Brazil
3 Faculty of Pharmaceutical Sciences, Food and Nutrition, Federal University of Mato Grosso do Sul, Campo Grande, MS, Brazil
4 Faculty of Pharmacy, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
5 Center for Biotechnology and Department of Botany, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil
6 Chemistry Institute, São Paulo State University, Araraquara, SP, Brazil

Key words

Erythroxylum pungens, Erythroxylaceae, tropane alkaloids, *Danio rerio*, Cyprinidae, Caatinga

received March 28, 2020
accepted after revision September 16, 2020
published online

Bibliography

Planta Med 2020
DOI 10.1055/a-1264-4302
ISSN 0032-0943

Georg Thieme Verlag KG, Rüdigerstraße 14,
70469 Stuttgart, Germany

Correspondence

Prof. Dr. Raquel Brandt Giordani
Department of Pharmacy, Federal University of Rio Grande do Norte
Gal. Gustavo Cordeiro de Farias Street, s/n, 59012-570, Natal, RN, Brazil
Phone: + 55 84 32 15 98 18, Fax: + 55 84 33 42 98 33
ufrn01156@ufrn.edu.br

Supplementary material is available under https://doi.org/10.1055/a-1264-4302

ABSTRACT

Tropane alkaloids are specialized plant metabolites mostly found in the Erythroxylaceae and Solanaceae families. Although tropane alkaloids have a high degree of structural similarity because of the tropane ring, their pharmacological actions are quite distinct. Brazil is one of the main hotspots of *Erythroxylum* spp. diversity with 123 species (almost 66% of the species catalogued in tropical America). *Erythroxylum pungens* occurs in the Caatinga, a promising biome that provides bioactive compounds, including tropane alkaloids. As part of our efforts to investigate this species, 15 alkaloids in specimens harvested under different environmental conditions are presented herein. The occurrence of 3-(2-methylbutyryloxy)tropan-6,7-diol in the stem bark of plants growing in their natural habitat, greenhouse controlled conditions, and after a period of water restriction, suggests that it is a potential chemical marker for the species. This alkaloid was evaluated for several parameters in zebrafish (*Danio rerio*) as a model organism. Regarding toxicity, teratogenic effects were observed at 19.5 µM and the lethal dose for embryos was 18.4 µM. No mortality was observed in adults, but a behavioral screen showed psychostimulatory action at 116.7 µM. Overall, the alkaloid was able to cause zebrafish behavioral changes, prompting further investigation of its potential as a new molecule in the treatment of depression-like symptoms. *In silico*, targets involved in antidepressant pathways were identified by docking.

Introduction

Erythroxylum P. Browne is the largest genus in the Erytroxylaceae family, and its center of endemism is South America, notably Brazil, Bolivia, and Venezuela. In Brazil, 123 species of *Erythroxylum* were described among a catalogue of 187 species in tropical America [1]. *Erythroxylum* spp. produce tropane alkaloids with important therapeutic activities such as stimulants, anticholinergics, antiemetics, anesthetics, and antidepressants [2,3]. The best-known species of this genus are cocaine producers such as *Erythroxylum coca* and *Erythroxylum novogranantense*. The coca plant is an integral part of South American native culture, especially in high altitude places such as Peru and Colombia. As a historical tradition in these regions, the plant is consumed as a tea or chewed mainly to help fight mountain sickness [2,3].

Erythroxylum pungens O. E. Shulz (Erythroxylaceae), popularly known as "Rompe-gibão", is a shrub or small tree endemic in the Caatinga, a Brazilian exclusive biome that covers almost all of the northeast region [4]. Eleven tropane alkaloids, among them 3-(2-methylbutyryloxy)tropan-6,7-diol, were reported in *E. pungens*, as well as the unprecedented occurrence of *N,N*-dimethyltryptamine in Erythroxylaceae [5]. These findings raised a number of questions regarding chemodiversity and alkaloid accumulation profile, both in natural habitats and in growth-controlled conditions. In addition, the biological potential of tropane alkaloids *in vivo* should also be addressed.

The search for biologically active compounds from biological screenings has been successfully applied in the discovery of several types of drugs [6]. In this context, zebrafish (*Danio rerio* Hamilton, 1822) has been established as an integrative experimental model that closes the gap between *in vitro* and *in vivo* testing [7]. Zebrafish has also attracted scientific attention in recent years due to the relatively high genetic similarity with humans (70% homologous genes), small size, high fecundity, and short developmental cycle, all of which are practical advantages of this vertebrate as a model [8]. Since its reproduction is external and the eggs are transparent, the toxicology of diverse compounds at specific embryonic stages can be easily determined without further developmental interference. Moreover, the phylogenetic conservation of significant neurotransmitters, hormones, and receptors between the zebrafish and mammals [9,10] confers substantial translational relevance to this model.

Herein, the biological effect of 3-(2-methylbutyryloxy)tropan-6,7-diol) on zebrafish embryos and adults is described. The occurrence of this in the stem bark of plants growing in their natural habitat, in greenhouse controlled conditions, and after a period of water restriction suggest its potential as a reliable chemical marker for the species. Furthermore, a phytochemical analysis of *E. pungens* by GC-MS yielded 15 tropane alkaloids.

Results and Discussion

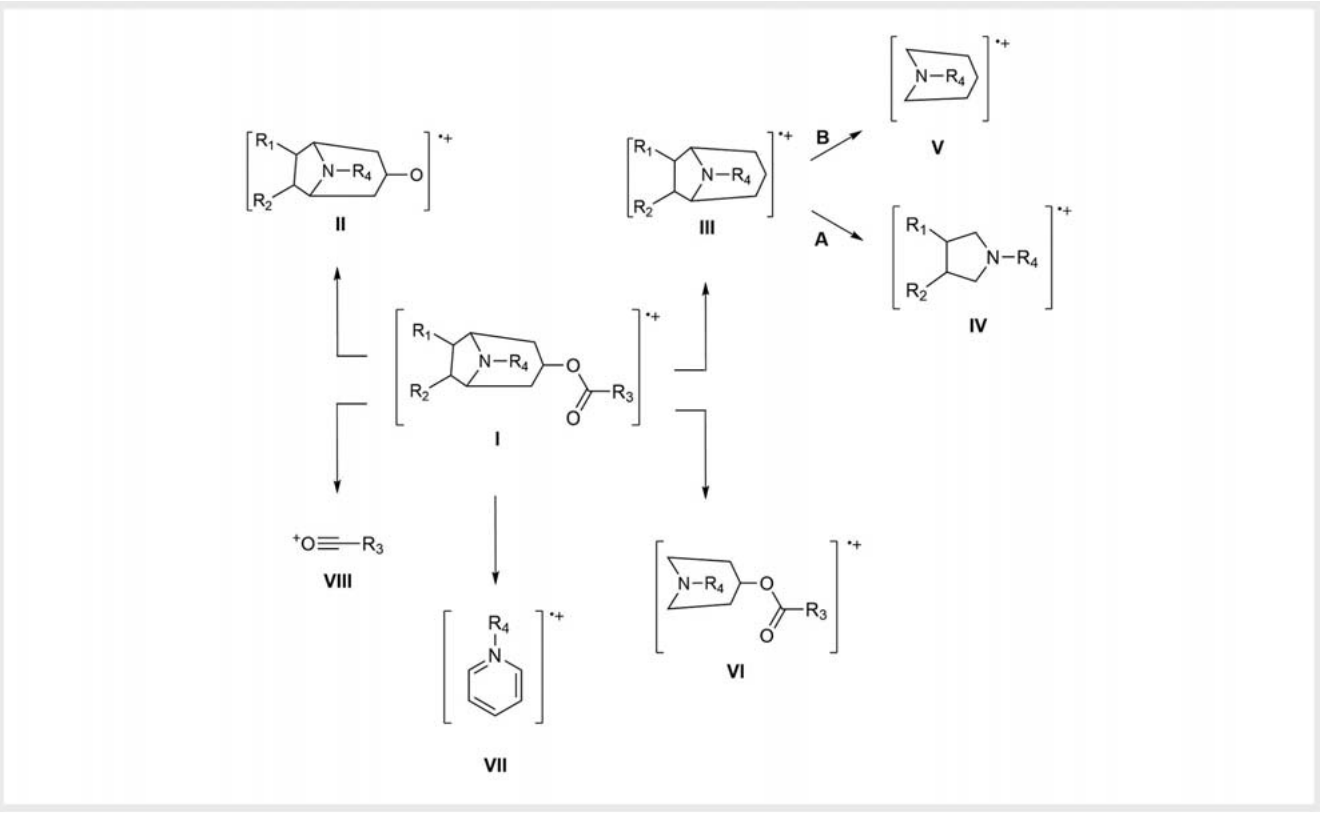
The cytotoxic potential and the structure-based docking results [5] pointed at 3-(2-methylbutyryloxy)tropan-6,7-diol being an aliphatic bioactive tropane alkaloid. In order to better understand this occurrence, some alkaloid-enriched fractions from plants grown in different conditions were evaluated by GC-MS: (i) plants harvested in their natural habitat, (ii) plants growth in a greenhouse with regular irrigation, and (iii) plants growth in a greenhouse without water for 20 days. In all experimental conditions, 3-(2-methylbutyryloxy)tropan-6,7-diol was detected in the stem bark (**Table 1S**, Supporting Information). Tropane alkaloids were produced by 12-month-old plants under greenhouse control conditions and up to 20 days without irrigation in both leaves and stem barks. However, some alkaloids occurred in an organ specific way regarding T3, T10, and T20 experimental times (**Fig. 20S**, Supporting Information). The plant growth is slow, therefore no important change in this parameter was observed during the experiment, but in T10 and T20, the leaves were withered with color alterations. The phytochemical profile was similar to that of adult plants harvested in a semiarid natural environment (**Table 1S**, Supporting Information), with discrete structural differences in the tropane alkaloids identified.

The differences in alkaloid profiles between adult field-grown plants and those cultivated in the greenhouse may be the result of a number of factors. Developmental aspects related to plant age (i.e., 12-month-old juveniles versus adult phase with reproductive activity) may regulate specialized metabolites profiles both quantitively and qualitatively, particularly in perennial species. This has been observed in a number of plants, including alkaloid accumulating ones such as *Delphinium ajacis* [11]. On the other hand, differences in environmental conditions may also impact alkaloid composition involving variables such as irradiance, temperature, water and mineral relations, and pathogen and herbivore attack, among others [12]. The differences in environmental factors, both biotic and abiotic, may have driven different alkaloid signatures in the field versus greenhouse grown *E. pungens*. The adaptive value of the differences in tropane alkaloid composition observed in *E. pungens* is not clear at present but may be relevant for overall fitness.

The absence of overt differences in the alkaloid profile and growth (**Fig. 20S**, Supporting Information) among plants cultivated under the different irrigation regimens may reflect the fact that *E. pungens* is highly adapted to water restriction, being able to efficiently balance growth and alkaloid metabolism in a wide range of irrigation conditions. However, differences in the profile may change only quantitatively facing the tested conditions.

Considering all samples analyzed, six tropane alkaloids (**3, 7, 9, 11, 14**, and **15**), in addition to nine alkaloids previously reported in *E. pungens* (**1, 2, 4–6, 8, 10, 12**, and **13**), were identified [5]. The tentative characterization was based on literature reports on alkaloids in Erythroxylaceae [13–17], a GC-MS library search (NIST), and mass spectra analysis (▶ **Fig. 1** and **Figs. 1S–15S**, Supporting Information). The mass spectrum of the tropane alkaloids obtained by eletronic ionization (EI, 70 eV) afforded typical fragmentation patterns, which have historically proven useful in the characterization of unknown members of this class [18–21].

Some diagnostic fragments and described metabolites to the genus have been used to support the structural assignment of tropane alkaloids, mainly because of a shortage of biomass that prevented the isolation and application of other analytical techniques to confirm the orientation of the substituents attached to tropane nucleus. Even with the analytical tool limitation, a general fragmentation pathway in tropane alkaloids by EI is shown in



▶ **Fig. 1** Overview of the fragmentation pathway used in the structural proposition of the tropane alkaloids present in *E. pungens* species.

▶ **Fig. 1**. In order to substantiate this way, the detailed EI fragmentation of the alkaloid 3-(2-methylbutyryloxy)tropan-6,7-diol, further identified by NMR, is presented in the Supporting Information. ▶ **Table 1** lists the detailed relative abundance of each ion for alkaloids tentatively identified in *E. pungens* extracts.

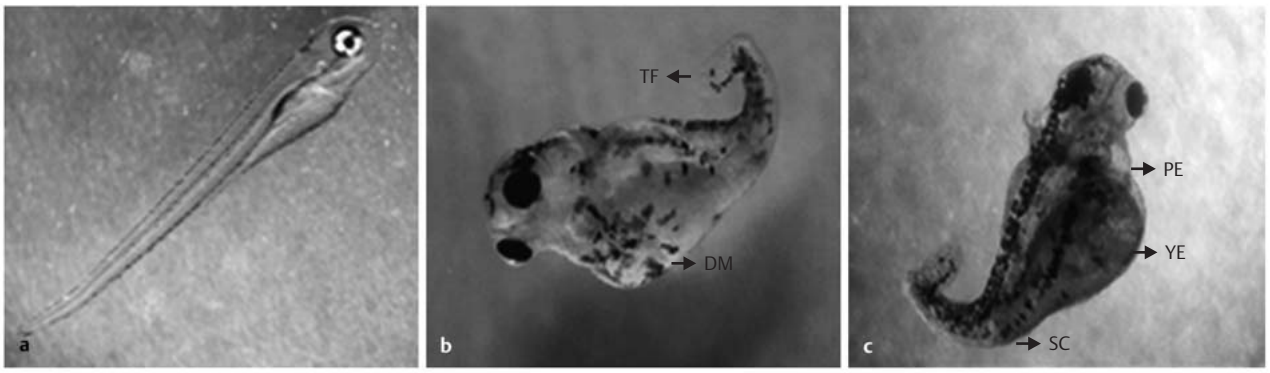
The presence of ions *m/z* 110 and 124 are classic in distinguishing nortropane and tropane alkaloids [5]. Besides, some notable fragments are related to the elimination of C6–C7 and its substituents, when present, as well as the substituents in the tropane nucleus, especially at C-2 and C-3 [19–21]. The structure of substituents may afford some characteristic fragments such as tropylium for aromatics and a MacLafferty rearrangement for aliphatic chains larger than four carbons [22–25]. One of these fragmentation mechanisms may influence the presence of other characteristic ions. Usually, the substituent at C-3 is an ester, and some characteristics fragments, i.e., alpha cleavage, allow for recognizing the substituent [26, 27]. However, CO_2 elimination, which is common to esters, may not be observed after the McLafferty rearrangement [28], and the 44 amu loss could be related to C6–C7 elimination if there is a hydroxyl group at this position. The loss for C6–C7 can have different mass losses, as mentioned before, and may be 60 amu for two hydroxyl groups or higher if there is a substituent attached to a hydroxyl [5]. The attribution of the hydroxylation at C-6 and/or C-7 and the substitution pattern will require further detailing. However, considering that in Erythroxylaceae the occurrence of C-6 being substituted in tropane alkaloids is more frequent than C-7 as well as the hypothesis that esterification in C-7 occurs after the stereospecific reduction of the ketone at C-3 [14], in this study, the results were discussed regarding C-6 being preferentially substituted for both oxygenation and esterification. Even though this issue requires experimental support regarding the biosynthesis of tropane alkaloids in *Erythroxylum*, the mass spectra show a lot of important information about structure and disclose the GC-MS analysis as a relevant tool to characterize tropane alkaloids.

The mass spectrum of alkaloid **3** showed $[M]^{+\cdot}$ at *m/z* 243, corresponding to the molecular formula $C_{12}H_{21}NO_4$. The base peak at *m/z* 94 and the occurrence of a peak at *m/z* 183 $[M\text{-}60]^{+\cdot}$ is characteristic of a monoester of 3,6,7-trihydroxytropane. Also, this fragment indicates an attachment of two hydroxyl groups to C-6 and C-7 [5, 13, 18]. The esterifying acid was tentatively identified as isobutyric acid by the presence of *m/z* 156 $[M - C_4H_7O_2]$ and *m/z* 43 (C_3H_7) as well as the absence of a peak at *m/z* 211 [M – 28], which would be produced as the McLafferty rearrangement of *n*-butyric acid [14]. Therefore, alkaloid **3** is an undescribed alkaloid tentatively identified as 3-isobutyryloxytropan-6,7-diol and has the same substituent observed for alkaloid **1** at C-3.

Alkaloid **14** was tentatively identified as 3-phenylacetoxytropan-6-propionyl-7-ol, an unprecedented tropane alkaloid. The $[M]^{+\cdot}$ at *m/z* 347 corresponds to the molecular formula $C_{19}H_{25}NO_5$ and the base peak at *m/z* 94 indicated an ester function attached to C-3 of the di/trisubstituted tropane nucleus. The occurrence of *m/z* 212 and 228 suggested the attachment of

▶ **Table 1** Tropane alkaloids tentatively identified in *E. pungens*. Numbers I–X represent the fragments afforded by GC-MS regarding the general scheme presented in ▶ **Fig. 1**.

Alkaloid	Part plant	R_1	R_2	R_3	R_4	CF	*m/z* [M] +/ base peak	Ions relative intensity									
								I (%)	II (%)	III (%)	IV (%)	V (%)	VI (%)	VII (%)	VIII (%)	IX (%)	X (%)
(1) 3-isobutyryloxytropan-6-ol	Leaves, stem bark	H	OH	$CH(CH3)_2$	CH_3	$C_{12}H_{21}NO_3$	227/94	227 (6)	156 (2)	140 (20)	99 (2)	97 (3)	183 (4)	94 (100)	71 (3)	43 (13)	42 (16)
(2) 3-isovaleryloxytropan-6-ol	Leaves, stem bark	H	OH	$CH_2CH(CH_3)_2$	CH_3	$C_{13}H_{23}NO_3$	241/94	241 (2)	156 (1)	140 (21)	99 (3)	97 (2)	197 (2)	94 (100)	85 (2)	57 (10)	
(3) 3-isobutyryloxytropan-6,7-diol	Stem bark	OH	OH	$CH(CH_3)_2$	CH_3	$C_{12}H_{21}NO_4$	243/94	243 (3)	172 (1)	156 (9)	115 (1)	97 (1)	183 (4)	94 (100)	71 (3)	43 (17)	
(4) 3-benzoyloxytropane	Leaves	H	H	(C_6H_5)	CH_3	$C_{15}H_{19}NO_2$	245/124	245 (8)	140 (7)	124 (100)	83 (43)	97 (1)	217 (1)	94 (36)	105 (13)	77 (18)	
(5) 3-(2-methylbutyryloxy) tropan-6,7-diol	Stem bark	OH	OH	$CH(CH_3)CH_2CH_3$	CH_3	$C_{13}H_{23}NO_4$	257/94	257 (1)	172 (1)	156 (7)	115 (1)	97 (1)	197 (4)	94 (100)	85 (2)	57 (14)	
(6) 3-phenylacetoxytropane	Leaves, stem bark	H	H	CH_2-(C_6H_5)	CH_3	$C_{16}H_{21}NO_2$	259/124	259 (11)	140 (11)	124 (100)	83 (38)	97 (9)	231 (1)	94 (21)	119 (1)	91 (15)	
(7) 3-benzoyloxytropan-6-ol	Leaves	H	OH	(C_6H_5)	CH_3	$C_{15}H_{19}NO_3$	261/94	261 (1)	156 (1)	140 (13)	99 (2)	97 (5)	217 (2)	94 (100)	105 (15)	77 (18)	76 (1)
(8) 3-(4′-methoxy) benzoyloxytropane	Stem bark	H	H	(C_6H_4)-*p* OCH_3	CH_3	$C_{16}H_{21}NO_3$	275/124	275 (11)	140 (8)	124 (100)	83 (26)	97 (3)	247 (1)	94 (21)	135 (3)	107 (13)	
(9) 3-benzoyloxytropan-6,7-diol	Stem bark	OH	OH	(C_6H_5)	CH_3	$C_{15}H_{19}NO4$	277/94	277 (1)	172 (1)	156 (2)	115 (2)	97 (1)	217 (2)	94 (100)	105 (20)	77 (23)	76 (2)
(10) 3-(2-methylbutyryloxy) tropan-6-acetyl-7-ol	Leaves, stem bark	OH	$OCOCH_3$	$CH(CH_3)CH_2CH_3$	CH_3	$C_{14}H_{25}NO_5$	299/94	299 (4)	214 (1)	198 (1)	157 (1)	97 (1)	197 (1)	94 (21)	85 (1)	57 (6)	
(11) 3-(4′-hydroxy-2′,5′-dimethoxy) benzoyloxynortropane	Leaves	H	H	(C_6H_2)-3,5$(OCH_3)_2$-4-OH	H	$C_{16}H_{21}NO_5$	307/110	307 (1)	126 (5)	110 (100)	69 (5)	83 (3)	279 (1)	80 (30)	181 (5)	153 (3)	152 (1)
(12) 3-(2-methylbutyryloxy) tropan-6-propionyl-7-ol	Leaves, stem bark	OH	$OCOCH_2CH_3$	$CH(CH_3)CH_2CH_3$	CH_3	$C_{15}H_{27}NO_5$	313/94	313 (4)	228 (1)	212 (8)	171 (4)	97 (1)	197 (4)	94 (100)	85 (2)	57 (8)	
(13) 3-(3′,5′-dimethoxy-4′-hydroxy) benzoyloxytropane	Leaves, stem bark	H	H	(C_6H_2)-3,5$(OCH_3)_2$-4-OH	CH_3	$C_{17}H_{23}NO_5$	321/124	321 (8)	140 (14)	124 (100)	83 (32)	97 (1)	293 (1)	94 (24)	181 (7)	153 (4)	
(14) 3-phenylacetoxytropan-6-propionyl-7-ol	Stem bark	OH	$OCOCH_2CH_3$	CH_2-(C_6H_5)	CH_3	$C_{19}H_{25}NO_5$	347/94	347 (4)	228 (1)	212 (5)	170 (1)	97 (1)	231 (7)	94 (100)	119 (1)	91 (12)	
(15) 3-benzoyloxytropan-6-propionyl-7-ol	Leaves, stem bark	OH	$OCOCH_2CH_3$	(C_6H_5)	CH_3	$C_{18}H_{23}NO_5$	333/94	333 (3)	228 (2)	212 (4)	171 (1)	97 (1)	217 (4)	94 (100)	105 (10) VIII-CO	77 (11) VIII-CO-H	



▶ **Fig. 2** **a** Zebrafish embryo control individual. **b** and **c** Embryo malformations observed at the concentration of 19.5 µM. Teratogenic effects: pericardial edema (PE), yolk sac edema (YE), tail flexion (TB), dispersion of melanin (DM), and spinal curvature (SC).

one hydroxyl group and a propionyl group at C-6 and C-7 [5, 13, 14]. The esterifying acid was identified as phenylacetic acid by the presence of ions corresponding to $[M\text{-}C_8H_7O_2]^{+\cdot}$ in addition to *m/z* 91 (C_7H_7) [14, 15, 29–31].

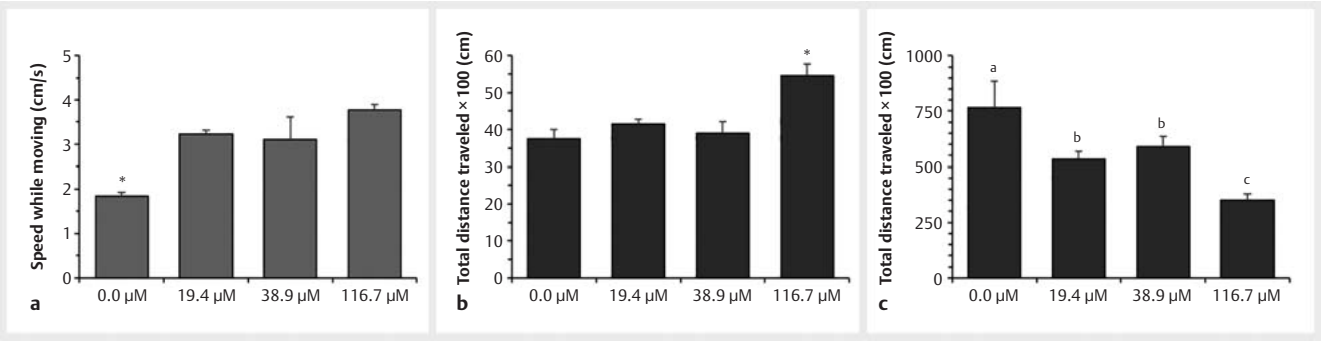
The EI mass spectral fragmentations of alkaloids **7** and **15** were consistent with those of a benzoic acid ester (*m/z* 105, 77). The same fragmentation route was observed for **11**, with the substituents at aromatic ring (*m/z* 181 and 153). The occurrence of fragment ion peaks of $[M - C_6H_5CO]^{+\cdot}$ and $[M - C_6H_5CO_2]^{+\cdot}$ confirmed the presence of benzoic acid as the esterifying acid attached to C-3 [5, 13, 14]. The differences among these alkaloids are located at the C-6 and C-7 substitution. The mass spectrum of alkaloid **15** showed the base peak and molecular ion at *m/z* 94 and $[M]^{+\cdot}$ and *m/z* 333, respectively, corresponding to the elemental composition $C_{18}H_{23}NO_5$ [13, 31]. The occurrence of a peak at $[M - 116]^{+\cdot}$, besides *m/z* 212, and 228, suggested the attachment of one hydroxyl group and a propionyl group at C-6 and C-7 [5, 13, 14] in the same way that explained alkaloid **14**. Therefore, **15** was tentatively identified as 3-benzoyloxytropan-6-propionyl-7-ol. To our knowledge, this alkaloid is reported for the first time in *Erythroxylum*. The occurrence of peaks at $[M - 60]^{+\cdot}$ suggested the attachment of two hydroxyl groups at C-6 and C-7 in alkaloid **9**, with a molecular ion at *m/z* 277 and a base peak at *m/z* 94, consistent with $C_{15}H_{19}NO_4$. Therefore, alkaloid **9** was tentatively identified as 3-benzoyloxytropane-6,7-diol.

Alkaloid **7** gave a mass spectrum consistent with the molecular formula $C_{15}H_{19}ON_3$, $[M]^{+\cdot}$ *m/z* 261 and the base peak at *m/z* 94. The peak at $[M - 44]^{+\cdot}$ indicated the attachment of the hydroxyl group at C-6 or C-7 and, thus, alkaloid **7** would be 3-benzoyloxytropan-6-ol.

Finally, alkaloid **11** showed the same fragmentation pattern of a previously described alkaloid, 3-(3′,5′-dimethoxy-4′-hydroxy) benzoyloxytropane (**13**) [5]. However, important differential features are ions *m/z* 307 (molecular ion) and *m/z* 110 (base peak) and the absence of *m/z* 124 in the mass spectrum of alkaloid **11** (see Supporting Information). Therefore, alkaloid **11** is 3-(3′,5′-dimethoxy-4′-hydroxy)benzoyloxynortropane, a tropane alkaloid without the *N*-methyl substitution that afforded the characteristic ion *m/z* 110.

E. pungens stem bark afforded 3-(2-methylbutyryloxy)tropan-6,7-diol (**5**), which was isolated from the alkaloid-enriched fraction, as previously described [5], and was used in biological assays. The toxicity test on zebrafish embryos indicated that morphological changes were observed after 48 hpf (hours post-fertilization). Following 96 h of exposure, the alkaloid lethal toxic concentration (LC_{50}) was estimated at 18.4 µM with a significative dose-response curve [LC_{50} = 18.4 ± 1.7 µM, curve b: (Intercept) t = − 6.03, $p < 0.01$; e: (Intercept) t = 10.54, $p < 0.01$] (**Fig. 22S**, Supporting Information). The results showed that at 19.5 µM, a variety of malformations in embryos was induced, including yolk sac edema (YE), pericardial edema (PE), tail bending (TB), spine curvature (SC), and dispersion of melanin (DM), suggesting that this alkaloid could affect the heart and central nervous system development in zebrafish embryos (▶ **Fig. 2 a, c**). PE of zebrafish was the typical cardiac malformation. Melanin cell dispersion and caudal curvature observed in this study may be indicative of the central nervous system/backbone malformation [32]. At a concentration of 19.5 µM, the percentage of malformations was 41 % while the higher concentration of 38.9 µM caused the mortality of all embryos.

The embryonic stage is a more sensitive phase of the fish life cycle, as is the case in all vertebrates. Metabolic detoxification at this stage is very slow compared to adult individuals [32], and the developing nervous system is a major target to external factors that affect its proper formation. The zebrafish embryo presents fast development, as neural induction initiates at the early embryonic phase and the neural tube is formed at 24 hpf [33]. These features have made zebrafish a suitable model for neurotoxicity research. Embryos exposed to ethanol develop malformations similar to fetal alcohol spectrum disorder in humans [34] and early contact with contaminants such as rotenone and MPTP (1-methyl-4-phenyl-5-tetrahydropyridine) lead to the loss of dopaminergic neurons, similar to what takes place in Parkinson's disease [35]. Not only the encephalon but also the heart has its formation during initial zebrafish development. Heart formation initiates as



▶ **Fig. 3** Locomotor and anxiety-like behavior of adult zebrafish treated acutely with tropane alkaloid 3-(2-methylbutyryloxy)tropan-6,7-diol. Bars indicate average zebrafish behavior for 30 min in the novel tank test. **a** Average speed, **b** total distance traveled, **c** freezing. *Statistical significance (one-way ANOVA; $p < 0.05$). Different letters indicate statistical differences between groups (one-way ANOVA; $p < 0.05$).

early as 5 hpf and the heart is functional at 24 hpf when circulation is started [33]. From this point on, the presence of external disruptors with cholinergic activity, such as 3-(2-methylbutyryloxy)tropan-6,7- diol, can affect heart function and growth, as well as over stimulate the brain. The apoptosis that is commonly observed during the first developmental phase may be augmented in the presence of exogenous stimulants.

In adult zebrafish, organs are already formed, and apoptosis plays specific functions in normal physiology. However, apoptosis is profoundly affected by external environmental compounds [36]. For the adult, tropane alkaloid (**5**) exposure for 96 h did not promote a lethal response in any of the concentrations (19.5, 38.9, and 116.7 µM. On the other hand, the higher concentrations caused behavioral changes in adult fish.

One-way ANOVA showed statistical significance between the groups' swimming speed ($F = 3.40$, $p = 0.0188$). The Tukey test indicated that all concentration of the tropane alkaloid increased zebrafish speed compared to the control ($p < 0.05$) (▶ **Fig. 3a**). No statistical significance was observed for maximum swimming speed (one-way ANOVA, $F = 2.45$, $p = 0.0647$). The last locomotor parameter, total distance traveled, was also evaluated and one-way ANOVA showed a statistical significance between groups ($F = 3.28$, $p = 0.022$). The Tukey test revealed that 116.7 µM increased zebrafish distance traveled compared to the other concentrations ($p < 0.05$) (▶ **Fig. 3b**).

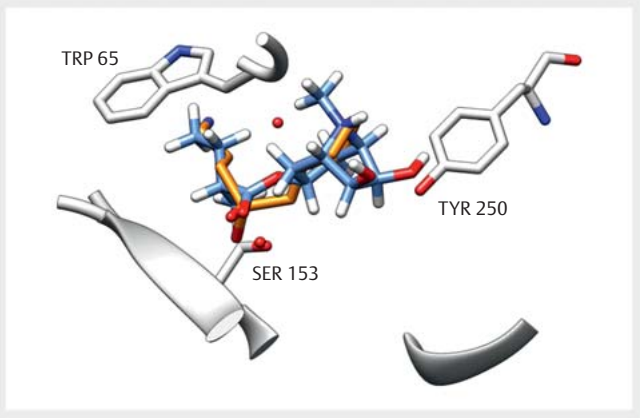
For the two parameters that are used to indicate fear/anxiety-like responses, one-way ANOVA showed a statistical significance between groups regarding freezing behavior ($F = 2.65$, $p = 0.044$), but no statistical significance for distance from the bottom of a tank ($F = 2.35$, $p = 0.0739$). The Tukey test indicated that all concentration of the tropane alkaloid decreased freezing, and 116.7 µM reduced this behavior the most ($p < 0.05$) (▶ **Fig. 3c**).

The increase in swimming velocity observed in zebrafish exposed to the tropane alkaloid 3-(2-methylbutyryloxy)tropan-6,7-diol was associated with a reduction in the behavioral parameter related to fear/anxiety; the freezing behavior is indicative of the stimulant effect of this alkaloid. It is known that anxiolytic agents generally decrease anxiety-like behaviors [37], which was corroborated by our data. Zebrafish exposed to the tropane alkaloid 3-(2-methylbutyryloxy)tropan-6,7-diol showed decreased freezing/immobility compared to control fish in the paradigm of the novel tank.

The novel tank test is a commonly used protocol to obtain behavioral data related to animal locomotion and anxiety. This kind of task helps to identify pharmacological effects of drugs applied to various species such as rodents [38, 39], primates [40, 41], and fish [37, 42–44]. When previously applied to zebrafish, the novel tank protocol disclosed similarities between animal models regarding strategies adopted in the exploration of the novel environment.

Freezing, that is the complete absence of movements (except gills and eyes), is a common indicator of anxiety [43, 44] and usually the result of high anxiety/stress when associated with hypoactivity. On the contrary, hyperactivity has been observed in zebrafish undergoing cocaine withdrawal, as well as other psychostimulants drugs [45]. Thus, it seems that the tropane alkaloid used in this study presents psychostimulant activity in zebrafish, leading to a condition of high agitation (observed by the locomotor parameters) and reduced fear/anxiety-like response (observed by the reduced freezing). Furthermore, we observed a concentration-dependent response. That is, the higher the concentration, the higher the increase in animal excitation. In this sense, it is important to continue investigations on the biological properties of 3-(2-methylbutyryloxy)tropan-6,7-diol, including future studies on the effects of the alkaloid on the neurotransmitter system in order to better understand its activity and long-term exposure. In this regard, new and specific therapies need to be devised for specific interventions, and 3-(2-methylbutyryloxy)tropan-6,7-diol may be beneficial as in cases of treatment-resistant depression.

In order to contribute to the investigation of the pharmacological potential of (**5**), an *in silico* assay indicated that the alkaloid showed a better theoretical interaction with targets from *Homo sapiens*, mainly in anti-inflammatory and antidepressant pathways, using the structure-based strategy (**Table 2S**, Supporting Information), and in the antiproliferative pathway according to the ligand-based *in silico* approach (**Table 3S,** Supporting Information). A ranking with the best 25 results in a Reverse Virtual Screening was performed (**Fig. 21S**, Supporting Information). Among the targets pointed out by both inverse virtual screening methodologies, the similarity between the tropane alkaloid (**5**)



▶ **Fig. 4** Binding mode of the tropane alkaloid (blue) at the binding site of the GABA receptor (PDB ID: 4MS1), and tridimensional comparison with the inhibitor (orange).

and an inhibitor of the GABA receptor stands out. This receptor plays an important role in the neurotransmission in the brain, and its inhibition is related with antidepressant and anxiolytic actions [46]. In order to better investigate this particular bioactive potential, the intermolecular interactions between the alkaloid and the amino acid residues of GABA were investigated. In fact, the high similarity with the GABA inhibitor was reflected by the promising occupation at the active site of the receptor, and important hydrogen bonds were observed involving SER 153 and TYR 250 (▶ **Fig. 4**).

Overall, the results highlight the chemodiversity of *E. pungens* harvested from its natural semiarid environment of the Caatinga as well as harvested from greenhouse culture conditions. The aliphatic tropane alkaloid 3-(2-methylbutyryloxy)tropan-6,7-diol, displayed a teratogenic/lethal effect on zebrafish embryos, but it was not lethal in adults. The novel tank assay performed to evaluate locomotor/anxiety responses in adult fish indicated that the alkaloid had psychostimulant activity. The results show that zebrafish present ontogenetic and concentration-dependent behavioral alterations when in contact with the tropane alkaloid 3-(2-methylbutyryloxy)tropan-6,7-diol. Neurological effects should be evaluated to confirm the toxicity of the alkaloid in this animal model, such as the possibility of hallucinogenic effects.

Materials and Methods

Plant material

Fresh stems bark (200 g) and leaves (250 g) of *E. pungens* O. E. Shulz (Erythroxylaceae), as well as seeds from ripe fruits, were harvested at Estação Ecológica do Seridó (ESEC), [6°34′48.15′′S; 37° 15′16.69′′W], Serra Negra do Norte, Rio Grande do Norte, Brazil, in May 2018 (vegetative organs) and in January 2014 (seeds) at the end of the rainy season. Authorization for harvesting the plant material was granted by SISBIO (32749-2) and permission to access the Brazilian genetic patrimony was provided by SISGEN (A4D2E0B). The species was identified by botanist Msc. Alan de Araújo Roque and voucher number UFRN 21666 was deposited at the Herbarium of Federal University of Rio Grande do Norte, Brazil.

The seeds were immersed in water for 24 h, then disinfected with 0.2% sodium hypochlorite (v/v) for 5 min under occasional agitation and washed four times in distilled water [5]. Sowing was done using sand and bovine manure in the proportion of 3 : 1 (w/w) as the substrate. After emergence and expansion of the cotyledons, the seedlings were transplanted into pots using the same substrate. After 12 months of growth in a greenhouse at the Plant Biotechnology Laboratory of the Federal University of Rio Grande do Norte – UFRN, plants were selected with a similar growth stage (considering plant height, stem thickness, number of leaves) and randomly distributed into control and water restriction treatment groups. The plants were separated into 7 groups with 7 individuals (n = 7), totaling 49 specimens of *E. pungens*: growth control, T3-treated (irrigation was suspended for 3 days), T3-control (watered on alternate days for 3 days), T10-treated (irrigation was suspended for 10 days), T10-control (watered on alternate days for 10 days), T20-treated (irrigation was suspended for 20 days), and T20-control (watered on alternate days for 20 days. After days of exposure to each treatment, plant material was collected, fast frozen in liquid nitrogen, and then stored in a freezer at − 80 °C. The seven individuals in each group were separated by organs, and the biomass obtained was pooled, constituting an analytical sample that was extracted to obtain the alkaloid-enriched fraction and was further analyzed by GC-MS.

Extraction and isolation

The extraction of fresh stem bark and fresh leaves of *E. pungens* harvested in their natural habitat was initiated by a process of turbolysis by using ethanol 99.6% in 1 : 10 (w/v). That means 1 g of plant material for 10 mL of extractor solvent. After that, the maceration method was used at room temperature (23 ± 2 °C) with renewal of the solvent every 24 h. The samples harvested from the growth-controlled greenhouse were ground with a pestle and mortar in liquid nitrogen and the ethanolic extraction was carried out in the same way for the field-harvested material. The maceration method used was repeated 4 times at room temperature (23 ± 2 °C) for 24 h. The crude extracts were concentrated under reduced pressure and submitted to filtration using Celite to remove chlorophyll, other pigments, and waxes. The post-filtration extract was acidified with 10% HCl and then subjected to acid-base extraction using *n*-hexane at pH 2. Then, the extract was basified up to pH 11 with NH_4OH and extracted with $CHCl_3$ in order to afford an alkaloid-enriched fraction. The isolation of 3-(2-methylbutyryloxy)tropan-6,7-diol was carried out through TLC (10 × 20 cm and thickness of 0.5 mm with silica gel 60 GF_{254}; Macherey-Nagel). In order to carry out the biological assay with zebrafish, the alkaloid was dissolved in water at pH 6.0 adjusted with HCl.

Spectroscopic analysis

The GC-MS data were acquired on a Shimadzu GC-2010 gas chromatograph coupled with a mass spectrometer with an electron impact ionization source (EI 70 eV; Shimadzu). A Durabond-DB5 (30 m × 0.25 mm × 0.25 µm) column was used with the injector at 250 °C, 1 : 20 split, temperature range from 90 to 300 °C at a

rate of 6 °C/min, and helium as the carrier gas. Samples were analyzed at 10 mg/mL after dissolving in $CHCl_3$. The ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a Bruker Advance DRX spectrometer (Bruker). All spectra were measured at 25 °C and samples were dissolved in $CDCl_3$. The chemical shifts are given on the δ (ppm) scale and were referenced to residual $CHCl_3$ (δ_H 7.24 and δ_C 77.23 ppm).

Structure and ligand-based inverse virtual screening of alkaloid 3-(2-methylbutyryloxy)tropan-6,7-diol

The conformers of alkaloid 3-(2-methylbutyryloxy)tropan-6,7-diol were drawn using the program MarvinSketch 16.9.5 (ChemAxon Ltd.). The 3D structures were geometrically optimized using the semi-empirical method PM7 incorporated in the software MOPAC2016 [47].

The target library used for the structure-based inverse virtual screening was constructed from the RCSB PDB Protein Data Bank [48] and comprised more than 22 000 structures with bound ligands. Molecular docking simulations were performed using AutoDockVINA [49] automated ad hoc by shell scripting and the bounded ligands were deleted before each simulation. The grid boxes were constructed individually considering the size of the binding site of each protein. The lowest score values were used to rank the results and only the values < − 7.5 were considered as potential targets.

In order to search for other possible targets for the alkaloid, a ligand-based inverse virtual screening was carried out. For this purpose, the ligand library encompassing approximately 26 000 crystallographic ligands also from the RCSB PDB Protein Data Bank was used. The process of similarity calculation between the alkaloid and the ligands of the library was made automatically with a shell script developed ad hoc. Every 3D score of similarity was calculated based on the ShaEP algorithm after the alignment through the pharmacophore [50] algorithm. To rank the results, only values with a similarity score > 0.5 were considered as a promising result.

Zebrafish maintenance

Adult zebrafish (n = 32, both sexes, wild-type short fins) were obtained from a local farm (Natal, Rio Grande do Norte state) and acclimated in 50 L tanks with a multistage filtration system. Ambient and water temperature were kept at 28 °C, water pH at 7.2, oxygen at 6 mg/L, and illumination set at a 12-h light-dark cycle. The animals were fed twice a day with commercial pelleted food and brine shrimp (*Artemia salina*). All procedures were approved by the Animal Ethics Committee of the Federal University of Rio Grande do Norte (approved the protocol under license number CEUA 021/2019, June 6, 2019).

Reproductive males and females were chosen for breeding following a previously established protocol. Two females and one male were set up in each breeding tank and left overnight for spawning at the first hour of morning light. Eggs were collected, counted, and placed in petri dishes for alkaloid exposure.

At 24 hpf, eggs were placed in 24-microtitulation plates and exposed to one out of four previously prepared concentrations of the alkaloid 3-(2-methylbutyryloxy)tropan-6,7-diol. The alkaloid was dissolved in system water to achieve the following concentrations: 0.0 (control), 5.8, 7.8, 19.5, and 38.9 µM. A total of 28 eggs were used for each concentration. Eggs were distributed randomly through 24-well microtitulation plates, 4 eggs/well, so that each alkaloid concentration was distributed to 7 wells. Eggs were left in the alkaloid solution for 2 h, and then washed with system water and placed in another microtitulation plate containing only system water. Embryo development was followed for 96 h and malformation and death events were recorded.

To evaluate the effects of the alkaloid in adult fish, we performed a toxicity test for 96 h and used the novel tank test paradigm. Adult zebrafish of both sexes were used for this assay (n = 32), and were randomly selected and divided into 4 groups to be exposed to 4 concentrations of the isolated alkaloid: control 0.0 µM (n = 8), 19.5 µM (n = 8), 38.9 µM (n = 8), and 116.7 µM (n = 8). The concentrations were prepared in 2 L tanks (20 × 15 × 10 cm) and fish were individually placed in each tank. Fish behavior was recorded by a video camera (Sony Digital Video Camera Recorder DCR-SX45) positioned in front and 50 cm away from the tank for 30 min. Behavior was analyzed by the tracking software ZebTrack/UFRN, (developed in the MATLAB, version R2014a, platform). The parameters evaluated were total distance traveled, average swimming speed, distance from the bottom of the tank, maximum swimming speed, and total time spent immobile.

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences Software – SPSS. Results are expressed as the mean ± SEM. The experimental results were based on one-way ANOVA (factor: concentration), and the Tukey test was used for mean comparisons; p values > 0.05 were considered significant. The embryo mortality rate assessment was performed from the dose-response curve using R software (version x64 3.5.3, R Core Team) with the command for the "drc" package.

3-(2-methylbutyryloxy)tropan-6,7-diol

White crystals, chemical formula: $C_{13}H_{23}NO_4$, NMR; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.90 (3H, t, J = 7.4, H-4′), 1.13 (3H, d, J = 7.0, H-5′), 1.43–1.50 (3H, m, H-2endo, H-4endo, H-3′a), 1.67 (1H, doublet of quintet, J = 13.6; 7.4, H-3′b), 2.21 (2H, dt, J = 16.0, 4.8, H-2exo, H-4exo), 2.49 (3H, s, H-8), 3.05 (2H, br s, H-1, H-5), 4.42 (2H, s, H-6endo, H-7endo), 5.02 (1H, t, J = 4.8, H-3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.8 (C4′), 16.6 (C5′), 26.2 (C3′), 26.8 (C2, C4), 34.0 (C8), 41.5 (C2′), 65.5 (C1/C5), 66.4 (C3), 74.6 (C6/C7), 175.6 (C1′). EI, 70 eV, m/z (%): 257 (1), 197 (4), 156 (7), 94 (100), 57 (14) [5].

Supporting information

Mass spectra of alkaloids (**Figs. 1S–15S**) tentatively identified in *E. pungens* in their natural habitat and cultivated in a greenhouse (**Figs. 16S** and **17S**), GC-MS profile of *E. pungens* cultivated (**Fig. 19S**) and wild species (**Fig. 18S**), a ranking of the top 25 *in silico* results regarding biological targets (**Fig. 20S**), tables with a structure-based strategy (**Table 1S**) and ligand-based *in silico* approach (**Table 2S**) are available in the Supporting Information.

Contributors' Statement

L. G. L. M., M. E. L. F., F. P. S. R., E. M. G. L. carried out the experimental procedures. L. G. L. M., F. P. S. R., L. S. F., J. A. S. Z., A. G. F. N., A. J. C. and R. B. G. analyzed and discussed chemical data as well as contribute to write the manuscript. E. M. G. L. and E. G. B. analyzed and discussed the in silico data as well contribute to write the manuscript. L. G. L. M., M. E. L. F. and A. C. L. analyzed and discussed biological data and contributed to write the manuscript. L. G. L. M., A. C. L. and R. B. G. planned the study and all authors discussed together about the major conclusions.

Acknowledgements

L. G. L. M. is the recipient of fellowships from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil. J. A. S. Z. acknowledges CNPQ for a research fellowship. This work was supported by a CNPq (Brazil) grant [445 149/2014–0] awarded to R. B. G. and INCT BioNat (grant # 465 637/2014–0) as well as Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) – (Finance Code 001) and São Paulo Research Foundation (FAPESP grant INCT-BioNat 2014/50 926–0). The authors acknowledge George Stephenson Batista, manager of the Ecological Station of Seridó, Dr. Gabrielle Macedo Pereira for valuable efforts in plant culture at the greenhouse, Professor Cristiane Elisabeth Costa de Macedo of the Plant Biotechnology Laboratory of the Federal University of Rio Grande do Norte – UFRN for lending the greenhouse, Professor Mauro Vieira de Almeida and Professor Richard Michael Grazul of the Chemistry Institute of the Federal University of Juiz de Fora – UFJF for several preliminary GC-MS analysis, and Serrapilheira Institute.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Costa-Lima JL, Loiola MIB, Jardim JG. Erythroxylaceae no Rio Grande do Norte, Brasil. Rodriguésia 2014; 65: 659–671

[2] Restrepo DA, Saenz E, Jara-Muñoz OA, Calixto-Botía IF, Rodríguez-Suárez S, Zuleta P, Chavez BG, Sanchez JA, D'Auria JC. *Erythroxylum* in focus: an interdisciplinary review of an overlooked genus. Molecules 2019; 24: 1–27

[3] Grynkiewicz G, Gadzikowska M. Tropane alkaloids as medicinally useful natural products and their synthetic derivatives as new drugs. Pharmacol Rep 2008; 60: 439–463

[4] Loiola MIBB, Agra MDF, Sidney GS, Queiroz RT. Flora da Paraíba, Brasil: Erythroxylaceae Kunth. Acta Bot Bras 2007; 21: 473–487

[5] Macedo Pereira G, Moreira LGL, Neto TDSN, Moreira de Almeida WA, Almeida-Lima J, Rocha HAO, Barbosa EG, Zuanazzi JAS, de Almeida MV, Grazul RM, Navarro-Vázquez A, Hallwass F, Ferreira LS, Fernandes-Pedrosa MF, Giordani RB. Isolation, spectral characterization, molecular docking, and cytotoxic activity of alkaloids from *Erythroxylum pungens* O. E. Shulz. Phytochemistry 2018; 155: 12–18

[6] Thomford NE, Senthebane DA, Rowe A, Munro D, Seele P, Maroyi A, Dzobo K. Natural products for drug discovery in the 21st Century: innovations for novel drug discovery. Int J Mol Sci 2018; 19: 1–29

[7] Pitchai A, Rajaretinam RK, Freeman JL. Zebrafish as an emerging model for bioassay-guided natural product drug discovery for neurological disorders. Medicines 2019; 6: 61

[8] Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, McLaren S, Sealy I, Caccamo M, Churcher C, Scott C, Barrett JC, Koch R, Rauch GJ, White S, Chow W, Kilian B, Quintais LT, Guerra-Assunção JA, Zhou Y, Gu Y, Yen J, Vogel JH, Eyre T, Redmond S, Banerjee R, Chi J, Fu B, Langley E, Maguire SF, Laird GK, Lloyd D, Kenyon E, Donaldson S, Sehra H, Almeida-King J, Loveland J, Trevanion S, Jones M, Quail M, Willey D, Hunt A, Burton J, Sims S, McLay K, Plumb B, Davis J, Clee C, Oliver K, Clark R, Riddle C, Elliot D, Threadgold G, Harden G, Ware D, Begum S, Mortimore B, Kerry G, Heath P, Phillimore B, Tracey A, Corby N, Dunn M, Johnson C, Wood J, Clark S, Pelan S, Griffiths G, Smith M, Glithero R, Howden P, Barker N, Lloyd C, Stevens C, Harley J, Holt K, Panagiotidis G, Lovell J, Beasley H, Henderson C, Gordon D, Auger K, Wright D, Collins J, Raisen C, Dyer L, Leung K, Robertson L, Ambridge K, Leongamornlert D, McGuire S, Gilderthorp R, Griffiths C, Manthravadi D, Nichol S, Barker G, Whitehead S, Kay M, Brown J, Murnane C, Gray E, Humphries M, Sycamore N, Barker D, Saunders D, Wallis J, Babbage A, Hammond S, Mashreghi-Mohammadi M, Barr L, Martin S, Wray P, Ellington A, Matthews N, Ellwood M, Woodmansey R, Clark G, Cooper J, Tromans A, Grafham D, Skuce C, Pandian R, Andrews R, Harrison E, Kimberley A, Garnett J, Fosker N, Hall R, Garner P, Kelly D, Bird C, Palmer S, Gehring I, Berger A, Dooley CM, Ersan-Ürün Z, Eser C, Geiger H, Geisler M, Karotki L, Kirn A, Konantz J, Konantz M, Oberländer M, Rudolph-Geiger S, Teucke M, Lanz C, Raddatz G, Osoegawa K, Zhu B, Rapp A, Widaa S, Langford C, Yang F, Schuster SC, Carter NP, Harrow J, Ning Z, Herrero J, Searle SM, Enright A, Geisler R, Plasterk RH, Lee C, Westerfield M, de Jong PJ, Zon LI, Postlethwait JH, Nüsslein-Volhard C, Hubbard TJ, Roest Crollius H, Rogers J, Stemple DL. The zebrafish reference genome sequence and its relationship to the human genome. Nature 2013; 496: 498–503

[9] Rinkwitz S, Mourrain P, Becker TS. Zebrafish: an integrative system for neurogenomics and neurosciences. Prog Neurobiol 2011; 93: 231–243

[10] Kalueff AV, Stewart AM, Gerlai R. Zebrafish as an emerging model for studying complex brain disorders. Trends Pharmacol Sci 2014; 35: 63–75

[11] Waller GR, Nowacki EK. Metabolic (catabolic) Modifications of Alkaloids by Plants. In: Waller GR, Nowacki EK, eds. Alkaloid Biology and Metabolism in Plants. Boston: Springer US; 1978: 183–249

[12] Magedans YMS, Rodrigues-Corrêa K, Costa C, Matsuura H, Fett-Neto AG. Sustainable production of bioactive alkaloids in *Psychotria* L. of southern Brazil: propagation and elicitation strategies. Acta Bot Bras 2019; 33: 607–617

[13] Christen P, Roberts MF, Phillipson JD, Evans WC. Alkaloids of *Erythroxylum zambesiacum* stem-bark. Phytochemistry 1993; 34: 1147–1151

[14] Christen P, Roberts MF, Phillipson JD, Evans WC. Alkaloids of *Erythroxylum monogynum* root-bark. Phytochemistry 1995; 38: 1053–1056

[15] Al-Said MS, Evans WC, Grout RJ. Alkaloids of *Erythroxylum hipericifolium* stem bark. Phytochemistry 1989; 28: 671–673

[16] Zanolari B, Guilet D, Marston A, Queiroz EF, Paulo MQ, Hostettmann K. Methylpyrrole tropane alkaloids from the bark of *Erythroxylum vacciniifolium*. J Nat Prod 2005; 68: 1153–1158

[17] Oliveira SL, Da Silva MS, Tavares JF, Sena-Filho JG, Lucena HFS, Romero MAV, Barbosa-Filho JM. Tropane alkaloids from *Erythroxylum* genus: distribution and compilation of 13C-NMR spectral data. Chem Biodivers 2010; 7: 302–326

[18] Berkov S, Pavlov A, Kovatcheva P, Stanimirova P, Philipov S. Alkaloid spectrum in diploid and tetraploid hairy root cultures of *Datura stramonium*. Z Naturforsch C Biosci 2003; 58: 42–46

[19] Kan-Fan C, Lounasmaa M. Sur les alcaloïdes de *Knightia deplanchei* Vieill. ex Brongn. et Gris (Protéacées). Acta Chem Scand 1973; 27: 1039–1052

[20] Witte L, Muller K, Arfmann HA. Investigation of the alkaloid pattern of *Datura innoxia* plants by capillary gas-liquid-chromatography-mass spectrometry. Planta Med 1987; 53: 192–197

[21] Blossey EC, Budzikiewicz H, Ohashi M, Fodor G, Djerassi C. Mass spectrometry in structural and stereochemical problems – XXXIX: Tropane alkaloids. Tetrahedron 1964; 20: 585–595

[22] Dohmeier-Fischer S, Krämer N, Grützmacher HF. Rearrangement by intermediate ion/neutral complexes during the McLafferty fragmentation of unsaturated ketones. Eur J Mass Spectrom 1995; 1: 310

[23] MacLeod JK, Djerassi C. Mass spectrometry in structural and stereochemical problems. CXXXVI. Primary hydrogen isotope effects in the McLafferty rearrangement. J Am Chem Soc 1967; 89: 5182–5190

[24] Kingston DGI, Bursey JT, Bursey MM. Intramolecular Hydrogen transfer in mass spectra. II. The McLafferty rearrangement and related reactions. Chem Rev 1974; 74: 215–242

[25] Bursey JT, Bursey MM, Kingston DGI. Intramolecular hydrogen transfer in mass spectra. I. Rearrangements in aliphatic hydrocarbons and aromatic compounds. Chem Rev 1973; 73: 191–234

[26] Basas-Jaumandreu J, de las Heras FXC. Allelochemicals and esters from leaves and inflorescences of *Sambucus nigra* L. Phytochem Lett 2019; 30: 107–115

[27] Nicolescu TO. Interpretation of Mass Spectra. In: Aliofkhazraei M, ed. Mass Spectrometry. London: Intech Open Limited; 2017: 24–72

[28] Heringa MF, Slowik JG, Goldmann M, Signorell R, Hemberger P, Bodi A. The distant double bond determines the fate of the carboxylic group in the dissociative photoionization of oleic acid. Chemphyschem 2006; 18: 12–19

[29] Philipov S, Berkov S. GC-MS Investigation of tropane alkaloids in *Datura stramonium*. Z Naturforsch C 2002; 57: 559–561

[30] Ionkova I, Witte L, Alfermann HA. Spectrum of tropane alkaloids in transformed roots of *Datura innoxia* and *Hyoscyamus gyorffyi* cultivated *in vitro*. Planta Med 1994; 60: 382–384

[31] El Bazaoui AE, Bellimam MA, Soulayman A. Nine new tropane alkaloids from *Datura stramonium* L. identified by GC/MS. Fitoterapia 2011; 82: 193–197

[32] Liu H, Sheng N, Zhang W, Dai J. Toxic effects of perfluorononanoic acid on the development of zebrafish (*Danio rerio*) embryos. J Environ Sci 2015; 32: 26–34

[33] Schmidt R, Strähle U, Scholpp S. Neurogenesis in zebrafish – from embryo to adult. Neural Dev 2013; 8: 1–13

[34] Bilotta J, Barnett JA, Hancock L, Saszik S. Ethanol exposure alters zebrafish development: a novel model of fetal alcohol syndrome. Neurotoxicol Teratol 2004; 26: 737–743

[35] Bretaud S, Lee S, Guo S. Sensitivity of zebrafish to environmental toxins implicated in Parkinson's disease. Neurotoxicol Teratol 2004; 26: 857–864

[36] Hannun YA, Luberto C. Ceramide in the eukaryotic stress response. Trends Cell Biol 2000; 10: 73–80

[37] Blaser RE, Chadwick L, McGinnis GC. Behavioral measures of anxiety in zebrafish (*Danio rerio*). Behav Brain Res 2010; 208: 56–62

[38] Karl T, Pabst R, von Hörsten S. Behavioral phenotyping of mice in pharmacological and toxicological research. Exp Toxicol Pathol 2003; 55: 69–83

[39] Gould TD, Dao DT, Kovacsics CE. The open Field Test. In: Gould TD, ed. Mood and Anxiety related Phenotypes in Mice. Baltimore: Springer; 2009: 1–20

[40] Ferguson SA, Bowman RE. A nonhuman primate version of the open field test for use in behavioral toxicology and teratology. Neurotoxicol Teratol 1990; 12: 477–481

[41] Dal-Pan A, Pifferi F, Marchal J, Picq JL, Aujard F, Restrikal C. Cognitive performances are selectively enhanced during chronic caloric restriction or resveratrol supplementation in a primate. PLoS One 2011; 6: 1–9

[42] Champagne DL, Hoefnagels CCM, de Kloet RE, Richardson MK. Translating rodent behavioral repertoire to zebrafish (*Danio rerio*): relevance for stress research. Behav Brain Res 2010; 214: 332–342

[43] Rosemberg DB, Rico EP, Mussulini BH, Piato AL, Calcagnotto ME, Bonan CD, Dias RD, Blaser RE, Souza DO, Oliveira DL. Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments. PLoS One 2011; 6: 1–11

[44] Stewart AM, Gaikwad S, Kyzar E, Kalueff AV. Understanding spatio-temporal strategies of adult zebrafish exploration in the open field test. Brain Res 2012; 1451: 44–52

[45] Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff AV. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res 2009; 205: 38–44

[46] Luscher B, Shen Q, Sahir N. The GABAergic deficit hypothesis of major depressive disorder. Mol Psychiatry 2011; 16: 383–406

[47] Stewart JJP. MOPAC2016. Stewart Computational Chemistry. Accessed August 16, 2019 at: http://OpenMOPAC.net

[48] Bernstein FC, Koetzle TF, Williams GJ, Meyer EF jr., Brice MD, Rodgers JR, Kennard O, Shimanouchi TM, Tasumi M. The protein data bank: a computer-based archival file for macromolecular structures. J Mol Biol 1977; 112: 535–542

[49] Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem 2010; 31: 455–461

[50] Korb O, Monecke P, Hessler G, Stützle T, Exner TE. PharmACOphore: multiple flexible ligand alignment based on ant colony optimization. J Chem Inf Model 2010; 50: 1669–1681