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Publication Date

2019-04-01

DOI

10.1016/j.jep.2018.12.037

Journal of Ethnopharmacology 233 (2019) 131–140

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Tabebuia aurea decreases hyperalgesia and neuronal injury induced by snake venom

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ARTICLE INFO

Keywords:
ATF-3
Bothrops
Pain
Iridoids
Specioside

ABSTRACT

Ethnopharmacological relevance: *Tabebuia aurea* (Silva Manso) Benth. & Hook. f. ex S. Moore is used as anti-inflammatory, analgesic and antiophidic in traditional medicine, though its pharmacological proprieties are still underexplored. In the bothropic envenoming, pain is a key symptom drove by an intense local inflammatory and neurotoxic event. The antivenom serum therapy is still the main treatment despite its poor local effects against pain and tissue injury. Furthermore, it is limited to ambulatorial niches, giving space for the search of new and more inclusive pharmacological approaches.

Aim of the study: evaluation of *Tabebuia aurea* hydroethanolic extract (HEETa) in hyperalgesia and neuronal injury induced by *Bothrops mattogrossensis* venom (VBm).

Materials and methods: Stem barks from *Tabebuia aurea* were extracted with ethanol and water (7:3, v/v) to yield the extract HEETa. Then, HEETa was analyzed by LC-DAD-MS and its constituents were identified. Snake venoms were extracted from adult specimens of *Bothrops mattogrossensis*, lyophilized and kept at −20 °C until use. Male *Swiss* mice, weighting 20–25 g, were used to hyperalgesia (electronic von Frey), motor impairment (Rotarod test) and tissue injury evaluation (histopatology and ATF-3 immunohistochemistry). Therefore, three experimental groups were formed: VBm (1 pg, 1 ng, 0.3 µg, 1 µg, 3 and 6 µg/paw), HEETa orally (180, 540, 720, 810 or 1080 mg/kg; 10 mL/kg, 30 min prior VBm inoculation) and VBm neutralized (VBm: HEETa, 1:100 parts, respectively). In all set of experiments a control (saline group) was used. First, we made a dose-time-response course curve of VBm's induced hyperalgesia. Next, VBm maximum hyperalgesic dose was employed to perform HEETa orally dose-time-response course curve and analyses of VBm neutralized. Paw tissues for histopathology and DRGs were collected from animals inoculated with VBm maximum dose and treated with HEETa anti-hyperalgesic effective dose or neutralized VBm. Paws were extract two or 72 h after VBm inoculation and DRGs, in the maximum expected time expression of ATF-3 (72 h).

Results: From HEETa extract, glycosylated iridoids were identified, such as catalpol, minecoside, verminoside and specioside. VBm induced a time and dose dependent hyperalgesia with its highest effect seen with 3 µg/paw, 2 h after venom inoculation. HEETa effective dose (720 mg/kg) decreased significantly VBm induced hyperalgesia (3 µg/paw) with no motor impairment and signs of acute toxicity. HEETa antihyperalgesic action starts 1.5 h after VBm inoculation and lasted up until 2 h after VBm. Hyperalgesia wasn't reduced by V*Bm:* HEETa neutralization. Histopathology revealed a large hemorragic field 2 h after VBm inoculation and an intense inflammatory infiltrate of polymorphonuclear cells at 72 h. Both HEETa orally and VBm: HEETa groups had a reduced inflammation at 72 h after VBm. Also, the venom significantly induced ATF-3 expression (35.37 ± 3.25%) compared with saline group (4.18 ± 0.68%) which was reduced in HEETa orally

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https://doi.org/10.1016/j.jep.2018.12.037
Received 24 August 2018; Received in revised form 21 December 2018; Accepted 23 December 2018
Available online 24 December 2018

(25.87 ± 2.57%) and VBm: HEETa (19.84 ± 2.15%) groups.
Conclusion: HEETa reduced the hyperalgesia and neuronal injury induced by VBm. These effects could be related to iridoid glycosides detected in HEETa and their intrinsic reported mechanism.

1. Introduction

Through seasonal savannas or wet-savanna grasslands in the Neotropics, *Tabebuia aurea* (Bignoniaceae) has a wide distribution, occurring from the Midwest region to Northeast of Brazil and presenting an consolidate genetic diversity (Bueno et al., 2014; Collevatti et al., 2014, 2015). In the traditional medicine, *Tabebuia aurea* is a medicinal plant that serves as an antiophidic due to its applicability in the treatment of snakebites, commonly by chewing, infusing, macerating with alcohol or topical application of its stem bark to treat the excruciating pain evoked by venom inoculation (Hadju and Holmman, 2012; Reis et al., 2014).

In South America, snakebites represent a public health problem mainly caused by *Bothrops* snakes (Cruz et al., 2009; Harrison et al., 2009) where in Brazil, *Bothrops mattogrossensis* represents an endemic snake in several states (Machado et al., 2014; Silva and Rodrigues, 2008).

During bothropic envenomation, pain is an important local symptom, consequence of tissue injury evoked by phospholipases A_2, metalloproteinases and serineproteases present in the venom (Gutiérrez and Ownby, 2003; Gutiérrez and Rucavado, 2000; Serrano and Maroun, 2005). In most cases, the injury drives to compartment syndrome, characterized by intensive pain, followed by loss of sensory nerve function and leading to amputation of limbs, culminating to a permanent disability of individuals (Gutiérrez et al., 2006; Jorge et al., 1999).

The polyvalent or specific antivenom stills the main therapy, but it is limited to ambulatorial niches and poorly neutralizes pain, hemorrhage and myotoxicity present in the envenoming (Picolo et al., 2002; Gutiérrez and Ownby, 2003). Thus, these appointments stimulate the research of complementary therapies and more inclusive pharmacological approaches.

Previous studies of Reis et al. (2014) demonstrated that *Tabebuia aurea* has anti-inflammatory and antimyotoxic activity against the venom of *Bothrops neuwiedi*, namely today as *Bothrops mattogrossensis* (Silva and Rodrigues, 2008). However, its analgesic effect remained unclear, despite the ethnopharmacological potential of this plant to treat snakebites (Hadju and Holmman, 2012; Reis et al., 2014).

Therefore, the aim of this study was to investigate whether the hydroethanolic extract of *Tabeuia aurea* (HEETa) attenuate pain and tissue injury in mice poisoned with *Bothrops mattogrossensis* venom as well as to determinate the chemical composition of HEETa by LC-DAD-MS.

2. Materials and methods

2.1. Snake venom

A pool of snake venom was extracted from adult specimens of *Bothrops mattogrossensis* (VBm), which were kept under housing and feeding controlled conditions. Only one batch of venom was used. The pool of venom was lyophilized and kept at −20 °C until use. The protein concentration in the lyophilized venom was measured by Bradford method (Bradford, 1976).

2.2. Animals

Male Swiss mice, weighting 20–25 g, were obtained from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB – State University of Campinas – Unicamp, Campinas, SP, Brazil). Experiments were conducted in accordance with the guidelines of the Committee for Research and Ethical Issues of IASP on using laboratory animals (Zimmermann, 1983). All animal experimental procedures and protocols were approved by the Committee on Animal Research of the State University of Campinas (UNICAMP), protocol 3903-1A. Mices were housed in plastic cages with soft bedding (five/cage) on a 12:12 light/dark cycle with food and water ad libitum. Prior to the tests they were randomly divided in groups. For almost all the behavior experiments, the groups consisted of six mice: five received the dose to be tested and one, saline (control group). For the rotarod tests, eight mice were used, half of them as control group.

2.3. Nociceptive paw electronic pressure-meter test for mice

The mechanic nociception was evaluated according to the method described by Cunha et al. (2004). In a quiet room, 60 min before all the tests, the animals were placed in acrylic boxes (23 cm × 20 cm × 18 cm) to adaptation. All tests were performed when mice were awake and freely moving in the acrylic boxes.

The test consisted of provoking paw withdrawal reflex using a hand-held force transducer with a 0.5 mm^2 polypropylene tip (Electronic Analgesimeter, Insight®, Brazil) applied to metatarsal footpad. The measurement of the pressure was calibrated in grams and automatically recorded at instant of paw withdrawn. The stimulus was repeated three times to obtain three measures. They arithmetic mean was considered the mechanical withdrawal threshold (g) of the animal. For all experiments, baseline and post-treatment measurements were performed. The intensity of hyperalgesia was reported as the Δ (delta) of the mechanical withdrawal threshold (g), which was calculated by subtracting the baseline value from that measured after treatments.

2.4. Intraplantar subcutaneous injection

VBm or saline (NaCl 0.9% solution) were injected (30 μL) subcutaneously in the mice left metatarsal footpad (intraplantar) with an insulin syringe (30 G needle, 30 units syringe, BD *Ultra-Fine* ®). Mice were awake and gently contained.

2.5. VBm maximum effective dose and its peak of action: a dose-response-time analysis of VBm induced hyperalgesia

A dose-response-time curve for the hyperalgesia induced by the venom of *Bothrops mattogrossensis* (VBm) was made. The doses of 1 pg, 1 ng, 0.3 μg, 1 μg, 3 and 6 μg/paw of VBm were tested. The hyperalgesia was evaluated in the ipsi and contralateral injection paws at 0.5, 1, 1.5, 2, 3, 4, 24 and 72 h after inoculation. Animals presenting spontaneous pain behavior or signs of paw necrosis were immediately discharged. VBm maximum hyperalgesic dose at its peak of time action was then selected and used in tests with the extract.

2.6. Plant material and extract preparation

The stem barks of *Tabebuia aurea* were collected in the region located in Abobral (19°34'12.54"S, 57°00'44.15"W), Passo do Lontra, Miranda, MS, Brazil at July of 2015. A voucher was deposited in the herbarium of Federal University of Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil (Number 29578), and identified by Flavio Macedo Alves. This study was approved for the acquisition of samples for genetic access of components – 010273/2013.

HEETa was obtained by extracting stem barks (200 g) with percolation (EtOH:H_2O – 7:3, v/v) followed by concentration under reduced

pressure by rotatory evaporator, as described by Reis et al. (2014).

2.7. LC-DAD-MS analyses

An Ultra-Fast Liquid Chromatograph (UFLC) system (Shimadzu, Kyoto, Japan) coupled to a diode array detector (DAD) and a high resolution time-of-flight (TOF) mass spectrometer micrOTOF Q-III (Bruker Daltonics, Billerica, USA) were used in the analyses. The spray voltage was 4.5–3.5 kV, using nitrogen as nebulizer (4 bar), drying (9.0 L/min) and collision gas. LC-DAD-MS analysis were performed in negative and positive ion modes (m/z 120–1200).

The chromatograph column was a C-18 Kinetex (2.6 μ, 150 × 2.2 mm, Phenomenex®, USA), which was protected with a pre-column from the same material. The mobile phase was deionized water (solvent A) and acetonitrile (solvent B), both added formic acid 0.1% (v/v), applying a gradient elution profile as described by Dembogurski et al. (2018). HEETa was prepared at concentration 1 mg/mL (methanol and water, 7:3 v,v), filtered on Millex® filter (PTFE, 0.22 μm × 3.0 mm, Millipore), and 1 μL was injected on the chromatographic system.

2.8. HEETa orally effective dose: dose-response curve for oral treatment

Five doses of HEETa orally (180; 540; 720; 810 or 1080 mg/kg) were evaluated. HEETa was diluted with saline solution till a gavage volume of 10 mL/kg. After 30 min, VBm maximum hyperalgesic dose was inoculated. The hyperalgesia was evaluated at the time of maximum venom hyperalgesia.

2.9. Determination of motor impairment: Rotarod test

Initially, mice were trained at velocity of 14 rpm and cut-off time of 180 s. In the day after, half of the animals received HEETa orally (effective dose; 10 mL/kg) and the others, saline orally (10 mL/kg). After 60 min, each mouse was tested in rotarod for one time (14 rpm during 180 s).

2.10. Time course analysis of the antihyperalgesic effect of HEETa orally

Mice were treated with the effective dose of HEETa previously obtained. After 30 min, VBm was inoculated. Then, hyperalgesia was evaluated at 0.5, 1, 1.5, 2, 3, 4, 24 and 72 h. Further, we tested the ability of the effective dose of HEETa orally attenuate the previously hyperalgesia established by VBm. We did an extra set of experiments treating animals 30 min after the venom inoculation. Next, the hyperalgesia was measured at 1, 1.5, 2, 3, 4, 24 and 72 h.

2.11. Neutralization of the venom

We performed an experimental approach to assess the neutralization of the venom by HEETa, as previously described by Gutierrez et al. (1990). The maximum hyperalgesic dose of VBm was incubated with HEETa to obtain venom/extract ratio (1/100; w/w; 30 μL), carried out at 37 °C for 30 min. Control was performed using saline incubated with venom at the same conditions used in the experiment with venom/extract.

2.12. Time course analysis of the antihyperalgesic effect from VBm neutralized

A time course analysis of the antihyperalgesic effect from VBm neutralized was performed. In this step, mice were treated with neutralized venom (VBm: HEETa; 1:100; w/w; 30 μL; subcutaneously; left paw) or saline (control). Then, hyperalgesia was evaluated at 0.5, 1, 1.5, 2, 3, 4, 24 and 72 h after inoculation of treatments.

2.13. Evaluation of treatments with VBm, HEETa and VBm: HEETa in paw tissue and dorsal root ganglion

2.13.1. Euthanasia and tissue harvest

Initially, 2 or 72 h after treatments, animals were terminally anesthetized with ketamine (120 mg/kg) and xylazine (16 mg/kg) administered together intraperitoneally (0.4 mL) and perfused through the ascending aorta with saline follow by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (4 °C). After the perfusion, the subcutaneous tissue of the left hind paw and L4, L5 and L6 dorsal root ganglions (DRGs) were collected.

2.13.2. Histopathology of paw tissue

Histopathological analysis of subcutaneous tissue from left hind paw were performed in animals euthanized 2 or 72 h after treatments. Tissues were embedded in paraffin and sections of 5 μm were performed. The sections were stained with hematoxylin and eosin (HE) and placed at optical microscope for histopathological analysis.

2.13.3. Dorsal root ganglion Immunohistochemistry

DRGs (L4, L5 and L6) were collected from animals euthanized 72 h after treatments. Next, were fixed in 4% PFA for 24 h and put in 30% sucrose solution for 48 h. Each DRG were embedded in OCT compound (Sakura Finetek, Torrance, CA, USA) and cut in optimum temperature into 14 μm sections using a cryostat. These sections were put on gelatinized slides and stored at −20 °C.

For Immunohistochemistry detection of ATF-3-labeled neurons, the slides were preincubated 30 min in PBS containing glycine (0.1 M). Tissues were blocked with 2% bovine serum albumin (BSA) and triton X-100 (0.1%) for 1 h. After washing with 0.1 M PBS, the slides were incubated overnight with the anti-ATF-3 primary antibody (1: 200, rabbit polyclonal; sc-188, Santa Cruz®) in PBS solution containing 0.1% triton X-100% and 2% BSA. The slides were washed once with the same solution used for incubation of the primary antibody and then washed 3 times for 5 min with 0.1 M PBS. Next, they were incubated with secondary antibody (donkey anti-rabbit Alexa 594, 1: 1000) for 1 h. The slides were then washed 3 times for 5 min in 0.1 M PBS and then incubated overnight with the conjugated primary antibody anti-Fox-3 protein (NeuN, rabbit polyclonal, 1:200, bs-1613R-A488; Bioss®) in PBS solution containing 0.1% triton X-100% and 2% BSA. After that, they were washed 3 times for 5 min in 0.1 M PBS and, finally, they were being left for 10 min with DAPI (0.25 μg/mL; SIGMA®) for nuclei staining.

2.13.3.1. Cell counting. The expression of ATF-3 was assessed by cell counting in neurons on the DRG of saline, VBm, HEETa orally and VBm: HEETa neutralized groups. Three animals per group were adopted, one slide for each animal and six sections per slide analyzed, totaling an approximate mean of 1600 cells/group analyzed. Data are represented as normalized arithmetic mean of the percentage expression of ATF-3 in the analyzed groups, which was calculated by number of ATF-3 marked neurons divided per number of neurons in field plus 100.

2.14. Acute toxicity evaluation for the effective dose of HEETa

The acute toxicity for the oral treatment with HEETa was evaluate taking the guidelines from the Society of Toxicologic Pathology (Sellers et al., 2007).

In this experiment, the effective dose of HEETa were given orally by two protocols: in the first, mice received daily HEETa (10 mL/kg; orally) or saline (control; 10 mL/kg; orally) during seven consecutive days and then were euthanized 24 h after the last dose. In the second protocol, a single dose of HEETa (10 mL/kg; orally) or saline (control; 10 mL/kg; orally) was given to the animals and they were euthanized 24 h after.

For euthanasia, animals were terminally anesthetized with ketamine

(120 mg/kg) and xylazine (16 mg/kg) administered together intraperitoneally (0.4 mL). Next, the blood collection was performed and sequentially, the liver, spleen, kidney, lung, heart, duodenum and jejunum were collected, weighed and embedded separately in 4% paraformaldehyde (PFA) solution supplemented with 0.1 M of phosphate buffer (pH 7.4) for tissue processing and histopathological analysis of organs.

2.14.1. Blood collection and serum biomarkers analysis

After deep anesthesia, animals were submitted to thoracotomy and a sample of 1 mL from the peripheral blood was collected through the supra-hepatic branch of the vena cava and put in to eppendorf tubes. Next, tubes were put in water bath at 37 °C for 30 min and then submitted to centrifugation at 1000 rpm during 30 min at 4 °C. The serum was collected and destined to biochemical measure of Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Albumin (ALB) and Creatine.

2.14.2. Histopathology of organs

For histopathological analyses of organs, the tissues were processed and embedded in paraffin. Sections of 5 µm were performed, stained with hematoxylin and eosin (HE) and the slides were placed at optical microscope to visualization of morphological patterns from animals submitted to the two treatment protocols adopted in the acute toxicity assay.

2.15. Statistical analysis

Statistical analysis was performed using Graphpad Prism 5 (Graphpad Software Inc., USA) for determination of mean, standard deviation and error. The comparison between the groups to determine the levels of statistical significance was performed using either one-way or two-way analysis of variance (One-way or Two-way ANOVA) followed by the Bonferroni test for multiple comparisons. The dose-response curve was constructed by means of non-linear regression of values obtained for the doses used [Log (dose)]. Particularly, for the Rotaroad test, the T-student test was performed to determine statistical difference between the analyzed groups. The level of statistical significance was $p < 0.05$.

3. Results

3.1. Hyperalgesia induced by VBm is a local, dose and time dependent effect

VBm induced a time and dose dependent hyperalgesia, with maximum effect at 2 h after inoculation, decaying after this time and returning to the baseline at 72 h (Fig. 1A). The maximum hyperalgesic effect without side effects was reached with the 3 µg/paw dose. No hyperalgesia was seen in the contralateral (right) paw (Fig. 1B).

Mice inoculated with 6 µg/paw presented spontaneous pain behavior, licking copiously the affected paw. An intense vascular impairment, 30 min after inoculation, followed by signs of possible necrosis (dark blue skin), were also observed (Supplementary Figure 1A). These animals were immediately euthanized.

The amount of protein in the lyophilized venom was 0.79 µg for 1.67 mg of crude venom. For the maximum hyperalgesic dose (3 µg) a value of 1.5 ng of protein was obtained.

3.2. Identification of the constituents from HEETa by LC-DAD-MS

The identification of components was based on spectral data of UV, MS, and MS/MS, compared with published data on literature, and some components was confirmed by co-injection of authentic standards. Twelve metabolites were detected from HEETa (Table 1, Fig. 2), including iridoids and phenolic derivatives.

The compounds **2** and **6–11** were identified as glycosylated iridoids. Intense ions at *m/z* 523.1469 and 537.1620 $[M-H]^-$ were observed for **6** and **10**, and characterized the molecular formulae $C_{24}H_{28}O_{13}$ and $C_{25}H_{30}O_{13}$, respectively. These compounds revealed UV spectra compatible with a presence of a caffeic acid group (λ_{max} = 299 and 327 nm). In addition, the compound 6 showed fragments ions at *m/z* 179 and 161, which correspond to caffeic acid followed by a loss of a water molecule, and the product ion *m/z* 361 $[M-H\text{-hexose}]^-$ confirmed the presence of a hexose. The metabolite **10** exhibited fragment ions at *m/z* 261, 193 and 161, which are relative to the cleavage from the cyclopentane ring, ferulic acid and a hexose, respectively. Thus, the compounds **6** and **10** were identified as verminoside and minecoside, both glycosylated iridoids linked to phenylpropenic acids. This fragmentation profile is consistent with from them by Amessis-Ouchemoukh et al. (2014), and they were reported from *T. aurea* (Santos et al., 2017).

From the UV spectrum, the compound **8** exhibited bands at 299 and 313 nm, suggesting the presence of a coumaric acid substituent. In addition, this compound exhibited intense ions at *m/z* 509.1595 $[M+H]^+$ and 507.1449.1469 $[M-H]^-$, which are relative to molecular formula $C_{24}H_{28}O_{12}$. The fragment ion at *m/z* 345 was yielded from a loss of 162 *u* and confirmed the presence of hexose. The fragment ions *m/z* 163 and 145 are relative to coumaric acid and a subsequent loss of a water molecule. While, the product ion *m/z* 231, yielded by the cleavage of the pentane ring, suggests the iridoid group (Hong et al., 2010; Amessis-Ouchemoukh et al., 2014; Santos et al., 2017). Thus, compound **8** was identified as the glycosylated iridoid specioside, as reported by Santos et al. (2017) from *T. aurea*, and confirmed by the injection of authentic standard. For the other iridoids, the identification

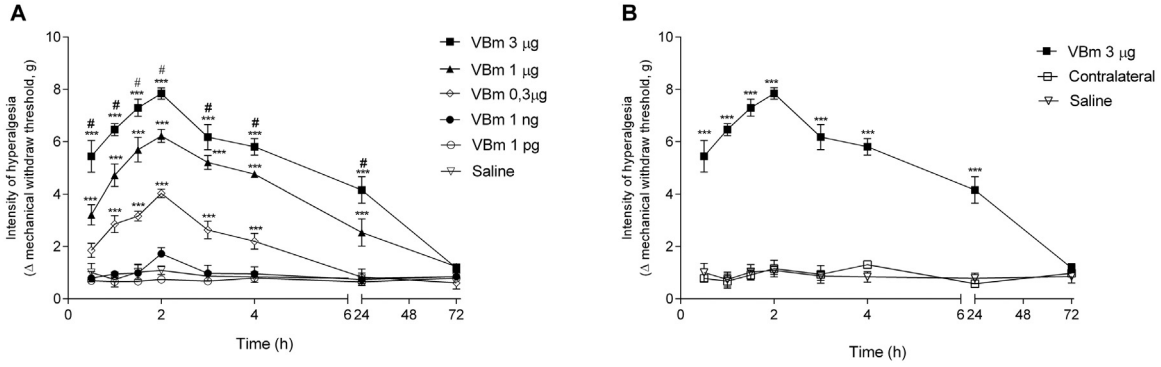

Fig. 1. Hyperalgesia induced by VBm is a local, dose and time dependent effect. Animals received VBm (1 pg, 1 ng, 0.3 µg, 1 µg or 3 µg/animal, 30 µL, subcutaneously, left paw) or saline solution (30 µL, subcutaneously, left paw). VBm (0.3 µg, 1 µg or 3 µg/animal) induced mechanical hyperalgesia at 0.5, 1, 1.5, 2, 3, 4 and 24 h after inoculation (A). VBm (3 µg/animal) don't induced hyperalgesia in the contralateral paw (B). Results expressed as mean ± SEM. Symbols * and # indicate $p < 0.001$ for VBm vs Saline and $p < 0.05$ for VBm 3 µg vs VBm 1 µg, respectively. Two-way ANOVA, Bonferroni post-test.

Table 1
Compounds identified from *Tabebuia aurea* hydroethanolic extract (HEETa) by LC-DAD-MS.

Peak	RT (min)	Compound	UV (nm)	MF	*m/z* [M + H] $^{+}$	*m/z* [M-H] $^{-}$	*m/z* - MS/MS (negative mode)
1	1.1	Di-*O*-hexoside	–	$C_{12}H_{22}O_{11}$	343,1255	341,1089	–
		Tetra-*O*-hexoside	–	$C_{24}H_{42}O_{21}$	667,2356	665,2183	383, 179
2	2.2	Catalpol	–	$C_{15}H_{22}O_{10}$	385,1129^{Na}	361,1167	169
3	6.5	*O*-pentosyl-hexosyl 3,4-dimethoxyphenol	280	$C_{19}H_{28}O_{12}$	471,1480^{Na}	447,1547	315, 153
4	7.9	NI	280	$C_{19}H_{28}O_{12}$	471,1496^{Na}	447,1522	191
5	10.2	NI	280	$C_{19}H_{28}O_{11}$	455,1526^{Na}	431,1556	191
6	19.0	Verminoside	299, 327	$C_{24}H_{28}O_{13}$	525,1603	523,1469	361, 179, 161
7	20.1	*O*-Coumaroyl-harpagide	295, 315	$C_{24}H_{30}O_{12}$	511,1806	509,1660	329, 163
8	21.5	Specioside	299, 313	$C_{24}H_{28}O_{12}$	509,1595	507,1449	345, 327, 231, 187, 163, 145
9	23.1	*O*-Coumaroyl-asystasioside	299, 313	$C_{24}H_{29}ClO_{12}$	–	543,1251	507, 345
10	23.3	Minecoside	299, 327	$C_{25}H_{30}O_{13}$	539,1759	537,1620	375, 261, 193, 175, 161
11	23.7	O-Veratroylcatalpol	260, 290	$C_{24}H_{30}O_{13}$	527,1781	525,1604	249, 221, 181, 163

RT: retention time; MF: molecular formula; NI: not identified; ^{Na}: [M + Na] + . All molecular formula presented errors lower than 8 ppm.

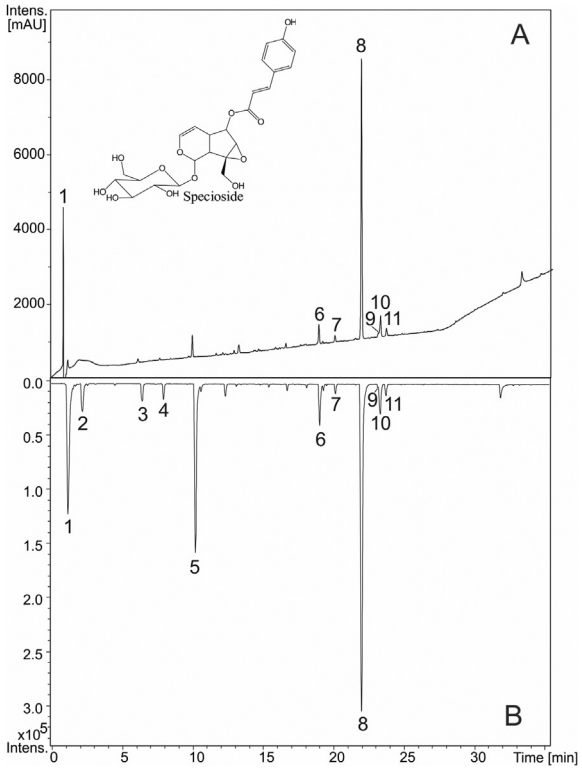

Fig. 2. Chromatograms of *Tabebuia aurea* hydroethanolic extract (HEETa) at 220–350 nm (A) and base peak chromagram in negative ion mode (B).

was based on the spectral characteristics described herein, and so the metabolites **2**, **7**, **9** and **11** were identified as catalpol, *O*-cumaroyl-harpagide, *O*-cumaroyl-asistasioside and *O*-veratroilcatalpol (Hong et al., 2010; Amessis-Ouchemoukh et al., 2014), respectively. These compounds were identified from T. aurea, and they showed similar spectral data reported by Santos et al. (2017).

3.3. HEETa decreases the hyperalgesia induced by VBm without motor impairment

HEETa given orally reduced the hyperalgesia of VBm in a dose-dependent manner (Fig. 3A, B). A significative reduction in the hyperalgesia evoked by VBm was seen for the dose of 720 mg/kg. Subsequent doses (810 or 1080 mg/kg) did not show statistical differences with regard to 720 mg/kg dose (Fig. 3B). In this way, 720 mg/kg was considered the effective dose of HEETa. In the rotarod test, it didn't evoke motor impairment (Fig. 3C) as no statistical difference was observed between saline (177.2 ± 0.86 s) and animals treated with 720 mg/kg dose (178.4 ± 1.07 s).

3.4. Time course analysis performed with HEETa orally or VBm neutralized reveals an early action and different effects from HEETa in the hyperalgesia induced by VBm

In the time course analysis with HEETa orally (720 mg/kg), HEETa started its antihyperalgesic effect in 1.5 h after VBm. This effect lasted until 2 h after inoculation of venom (Fig. 3D, E). Pre and post-treatment with HEETa orally reduced the hyperalgesia induced by the venom. However, in the experiment performed with VBm neutralized, HEETa failed in attenuate venom's hyperalgesia. Animals treated with VBm: HEETa (1:100) mixture showed the same pain behavior as control group (Fig. 3F).

3.5. HEETa reduced inflammatory infiltrate without affect the hemorrhage evoked by VBm

The histopathological analysis of subcutaneous tissues revealed hemorrhage field 2 h after VBm inoculation (control group, Fig. 4A) similar to both HEETa orally (Fig. 4C) and VBm neutralized (VBm: HEETa, 1:100) (Fig. 4E) groups. Nevertheless, inflammatory infiltrate was lower in HEETa orally and VBm: HEETa treated mice (Fig. 4B and D). Additionally, at 72 h, no signs of hemorrhage were seen in all groups analyzed.

3.6. HEETa reduced ATF-3 expression

Almost 40% (35.37 ± 3.25%) of DRGs neurons nuclei expressed ATF-3 at 72 h after VBm inoculation (Fig. 5A, B). HEETa significantly reduced ATF-3 expression to 25.87 ± 2.57% in oral treatment and also, in VBm neutralized group (19.84 ± 2.15%). There was no difference in ATF-3 expression between oral HEETa and neutralized VBm (Fig. 5B).

3.7. HEETa shown no toxicity in the acute toxicity assay

The treatment with effective dose of HEETa (720 mg/kg) during seven consecutive days or with a single dose didn't shown any toxicity. No statistical difference was observed on organs weight (Table 2) as well as on the histopathological or biochemical patterns obtained for HEETa in comparison with control groups (Fig. 6A-D; Table 3).

In the histopathological analysis of organs (Fig. 6A-D), the spleen presented white and red pulp well delimited and no hemosiderin deposition was observed. The small intestine, segment of duodenum and jejunum, presented intestinal villi and glands preserved, without loss of coating epithelium and without stroma edema. The liver, kidney, lung and heart were well organized, maintaining their characteristic morphologies. No necrosis, hemorrhage or leukocyte infiltrate was

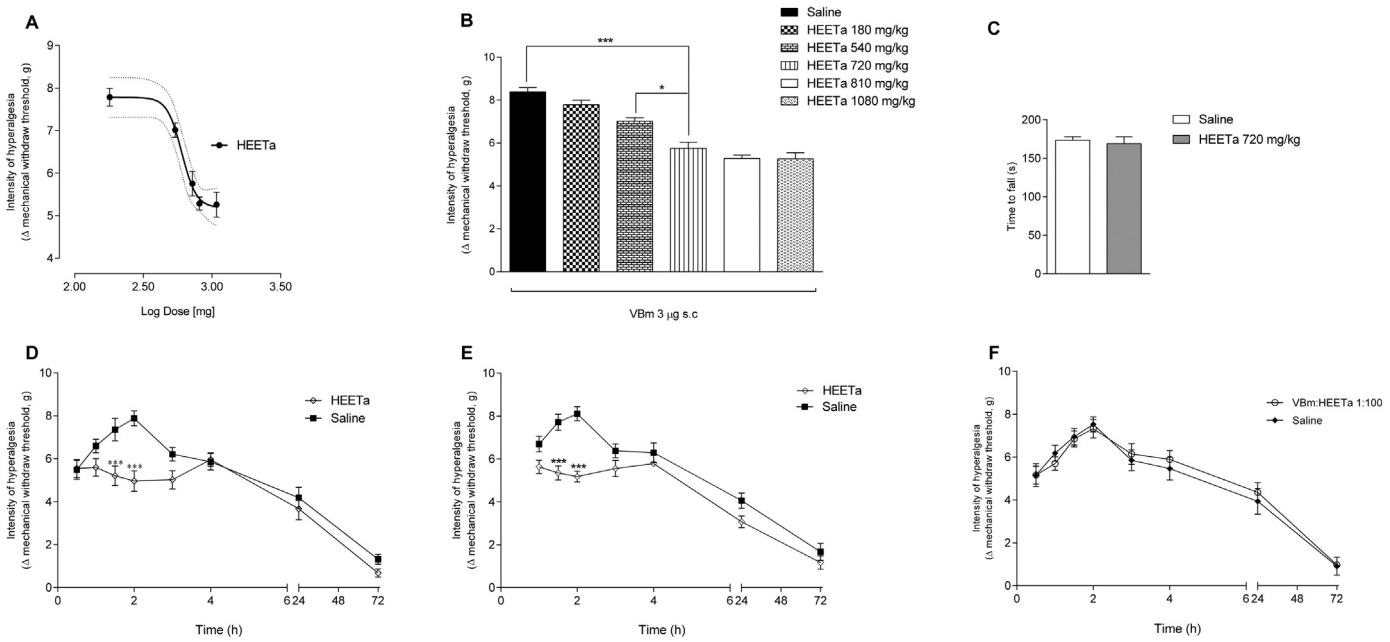

Fig. 3. Dose-dependent decrease of VBm hyperalgesia by HEETa (A, B) without motor impairment with effective dose (720 mg/kg) (C). HEETa (720 mg/kg, orally) given before or after VBm (3 µg/animal, 30 µL, subcutaneously, leftpaw) reduce the hyprelgesia induced by the venom in 1.5 and 2.0 h after it's inoculation (D, E). HEETa fail to reduce the hyperalgesia of VBm in the experiment performed with VBm neutralized (VBm: HEETa; 1:100) (F). (A) Doses (mg) expressed by the mean ± SEM (R^2 = 0.8257, DF = 21, SS = 5.355; SS = absolute sum of squares; DF = degrees of freedom). (B, C, D, E and F) Results expressed as mean ± SEM. Symbols * and *** indicate $p < 0.05$ and $p < 0.001$, respectively, for comparison between groups. T – student test (C); One-way (B) or Two-way ANOVA (D, E and F) follow by Bonferroni post-test.

observed in any of the analyzed organs. However, in the lung of all groups, was possible observed some areas that indicated signs of post-death atelectasis. This alteration probably is due to the use of high anesthetic concentration in the euthanasia method, which leads to cardiorespiratory depression and hypoxia, and also, it's a classical side effect observed for ketamine (Cui et al., 2011) and xylazine (Amouzadeh et al., 1991).

With regard to biochemical patterns analyzed (Table 3), animals treated with a single dose of HEETa or for seven consecutive days shown similar values for ALP, ALT, ALB and Creatinine. No statistical difference was seen when compared the two groups treated with HEETa with their respective controls.

4. Discussion

Considering more inclusive and ubiquitous pharmacological strategies, ethnopharmacology shown to be a potential tool, since plant extracts due to their wide molecular composition, can present different pharmacological targets leading to different therapeutic activities, which in turn, culminate for a single objective in specific, the relief of pathological symptoms and a better quality in the clinical condition of individuals (Patwardhan, 2005; Leonti and Casu, 2013). Taking in specific snakebites, in Brazil, a potential field can be explored in plant medicines (Dutra et al., 2016; Marcussi et al., 2007).

The antiophidic activity of genus *Tabebuia sp*. against the bothropic

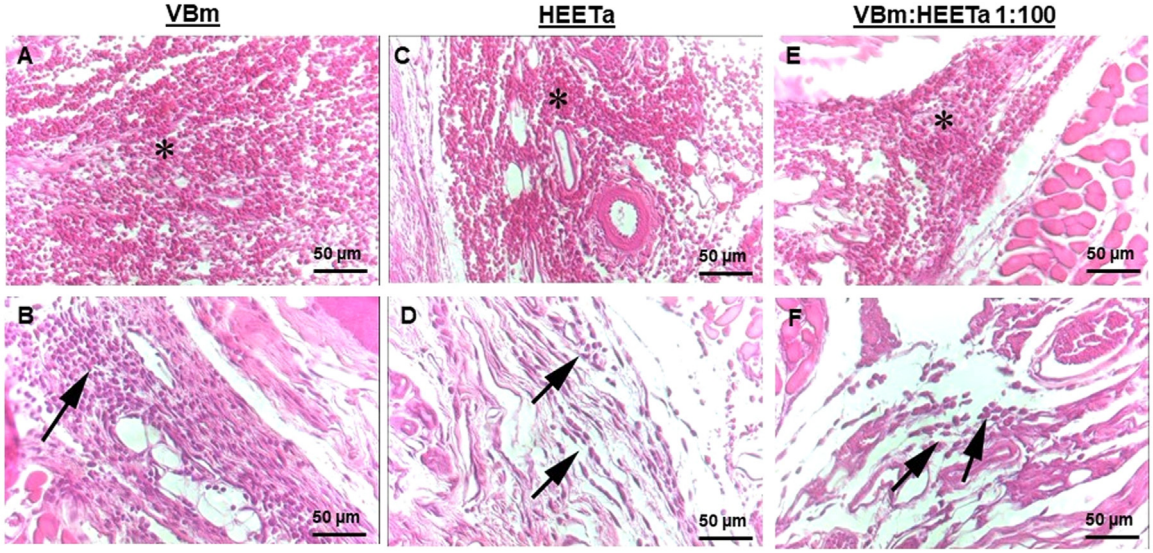

Fig. 4. Histopathological observations two and 72 hours after inoculation of VBm in animals treated with HEETa or VBm:HEETa 1:100. Symbol * indicate hemorrhagic field and arrows indicate inflammatory infiltrate.

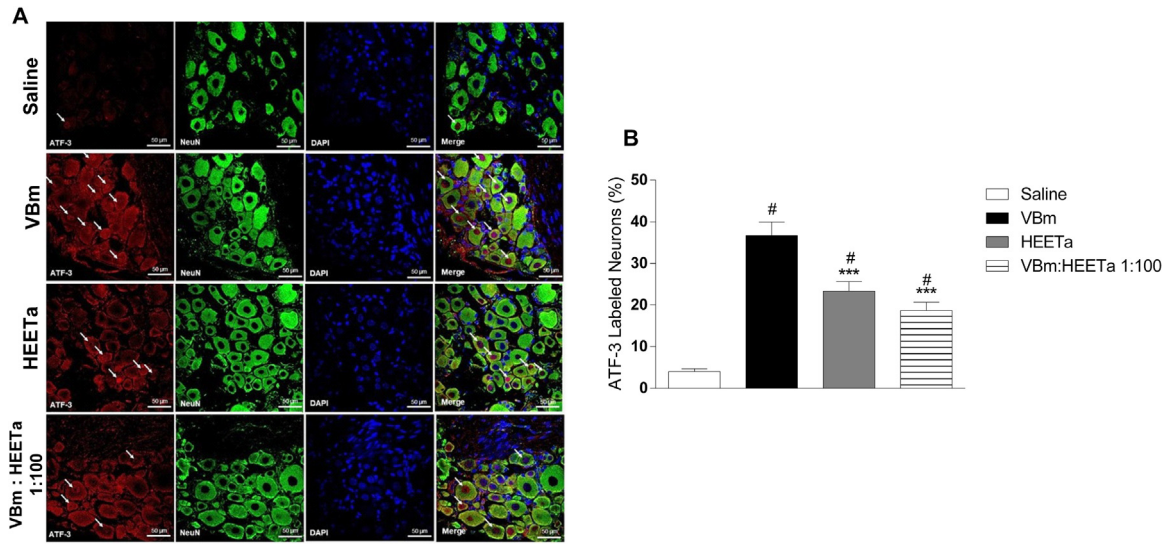

Fig. 5. VBm induced a neuronal injury through expression of ATF-3 on dorsal root ganglia (A, B). The pretreatment with HEETa (720 mg/kg; orally) or with the mixture VBm: HEETa 1:100 decrease the expression of ATF-3 and neuronal injury induced by VBm (A, B). Results expressed as mean ± SEM. Symbol # and *** indicate $p < 0.05$ for groups vs saline group and $p < 0.01$ for VBm vs HEETa or VBm: HEETa 1:100 group, respectively. One-way ANOVA, Bonferroni post-test.

envenoming has been previous related (Otero et al., 2000a,b; Reis et al., 2014). Particularly for *Tabebuia aurea,* the antimyotoxic, antihemorrhagic and anti-inflammatory actions front the bothropic envenoming was early reported by Reis et al. (2014). In this research, we consolidate the therapeutic potential of *T. aurea* adding its ability to decrease pain and neuronal injury which are two important local events of bothropic venom activity (Chacur et al., 2002, 2003; Zamuner et al., 2004; Zhang et al., 2017a).

Initially, our standard investigation made with VBm shown that the venom evoked hyperalgesia is a dose-time response and local event. The maximum effect occurs at two hours after inoculation and lasts 24 h for the highest doses (1 and 3 μg/paw) declining, but not disappearing, at least, until 72 h after inoculation. The absence of hyperalgesia in the contralateral (right) uninoculated paw indicated that the systemic absorption of the venom has no influence in local pain neither there is a venom neuronal transport mechanism to spinal cord, at least for 72 h, that could elicit a contralateral pain effect. Also, we assume that we have reached the suboptimal non-necrotic hyperalgesic dose (3 μg/paw) with VBm, since a higher one (6 μg/paw) produced an intense vascular impairment 30 min after inoculation, followed by signs of possible severe tissue damage.

The inflammatory and hyperalgesic events triggered by bothropic venoms has been shown to lead a high inflammatory and hyperalgesic response mediated by polymorphonuclear cells, release of cytokines (IL-1β, TNF-α, IL-6), bradykinin, cyclooxygenase II expression and synthesis of prostaglandin E_2 (Wanderley et al., 2014; Gutiérrez and Ownby, 2003; Gutierrez et al., 2000; Serrano and Maroun, 2005). The inflammatory and hyperalgesic event is proportional to the amount of venom inoculated (Chaves et al., 1995; Teixeira et al., 1994; Trebien and Calixto, 1989). Thus, the increasing hyperalgesia for different doses of VBm in our time course analysis are probably related to venom concentrations used and their toxic effects in local tissue, triggering a minor or major inflammatory, hyperalgesic or toxic event as the amount of toxins are changed in the site of inoculation.

Bothropic venoms can had great variability among species (Campos et al., 2013; Furtado et al., 1991). This are linked with the expression and activity of proteolytic enzymes such as metalloproteinases, serineproteases or phospholipases A_2 (Guércio et al., 2006; Serrano et al., 2005; Zelanis et al., 2012), which implies in different noxious activities between venoms samples (Campos et al., 2013; Furtado et al., 1991; Zelanis et al., 2012). Taking these data, is reasonable consider that corresponding proteolysis, hemorrhage and inflammation made by venom enzymes can change between venom samples, leading to different tissue damages and, consequently, changes in the time course sensitization of peripheral nociceptive fibers (Hucho and Levine, 2007; Chacur et al., 2002, 2003; Zhang et al., 2017a). Thus, this could explain and reinforce the divergence observed in our time course analysis of the hyperalgesia performed with VBm samples and previous works executed with different venoms from *Bothrops* snakes. Besides, it is important to say that our work, for the first time, made a standard investigation to show the hyperalgesic and tissue damage responses to *B. mattogrossensis* venom.

Twelve metabolites were identified on our HEETa phytochemical analysis, including glycosylated iridoids such as catalpol, minecoside, verminoside and specioside.

From in vitro studies, catalpol, minecoside and verminoside have

Table 2
Measures of organ weight in animals submitted to treatment with HEETa (single dose or during seven consecutive days) and their respective controls. Two-way ANOVA, Bonferroni post-test.

Organ	Group				
	Saline Single Dose Treatment	HEETa Single Dose Treatment	Saline seven days treatment	HEETa seven days treatment	*P* value
Liver	1.634 ± 0.122	1.630 ± 0.068	1.574 ± 0.091	1.478 ± 0.118	0.6845
Kidney	0.222 ± 0.008	0.226 ± 0.011	0.186 ± 0.009	0.196 ± 0.018	0.0930
Spleen	0.136 ± 0.016	0.132 ± 0.011	0.092 ± 0.013	0.094 ± 0.008	0.6185
Lung	0.168 ± 0.008	0.192 ± 0.011	0.176 ± 0.017	0.198 ± 0.012	0.3634
Heart	0.142 ± 0.007	0.146 ± 0.007	0.126 ± 0.008	0.128 ± 0.010	0.2864
Duodenum	0.120 ± 0.018	0.154 ± 0.018	0.110 ± 0.012	0.126 ± 0.016	0.3261
Jejunum	0.204 ± 0.011	0.184 ± 0.021	0.168 ± 0.010	0.170 ± 0.027	0.5258

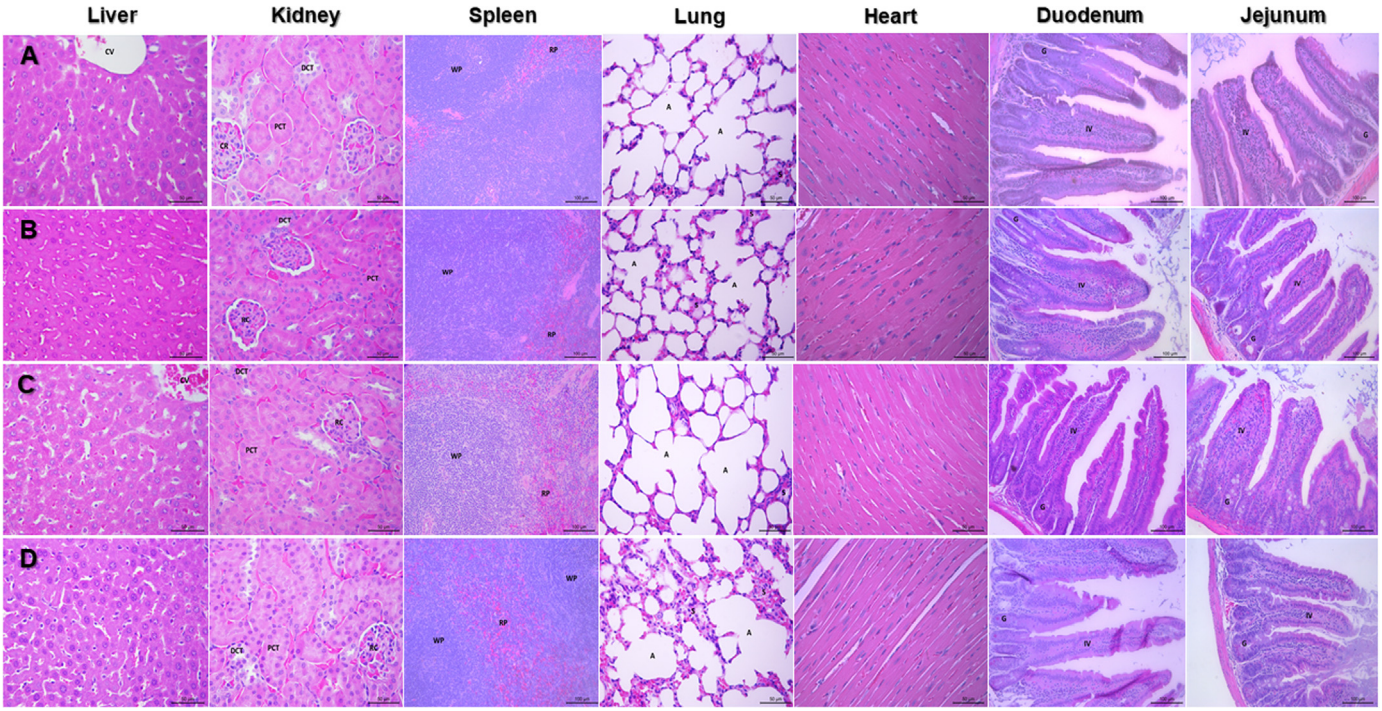

Fig. 6. Histopathological observations in mice treated orally with HEETa by a single dose (B), during seven consecutive days (D) or with their respective controls: saline by a single dose (A) or during seven consecutive days (C). CV: Central vein, DCT: distal convoluted tubule, PCT: proximal convoluted tubule, RC: renal corpuscle, RP: red pulp, WP: white pulp, A: alveolus, S: stroma, G: gland, IV: Intestinal villi. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

shown anti-inflammatory effects, and mechanisms like inhibition of pro-inflammatory cytokine synthesis such as IL-1β and TNF-α, inhibition of COX-II expression and synthesis of PGE_2 (Park et al., 2010; Viljoen et al., 2012). While specioside have shown antioxidant properties by inhibition of reactive oxygen species production and up-regulation of genes related with superoxide dismutase activity and expression, such as Sod-1, Sod-2 and Sod-3 (Asthana et al., 2015).

We find the HEETa antihyperalgesic effective dose by dose-response-time curves analysis. Established the effective dose of HEETa, we assessed the acute toxicity of this dose, and both the kinetic and dynamic of effect from extract in the hyperalgesia evoked by VBm. In our behavior tests, HEETa given orally reduced the hyperalgesia evoked by VBm in a dose-dependent manner, with absence of motor impairment for the effective dose (720 mg/kg), reinforcing its anti-hyperalgesic effect as no motor function was lost. HEETa given orally before or after the venom reduced its hyperalgesia. Particularly, the use of HEETa after venom inoculation and absence of toxic effects by both protocols tested in the toxicity assay (single dose or seven consecutive days) indicate the clinical applicability of the extract.

In this sense, is possible that the mechanisms reported above to iridoids are probable the responsible for the antihyperalgesic effect of HEETa, where the extract components would be antagonizing priory the inflammatory and hyperalgesic events triggered by venom inoculation.

VBm evoked significant ATF-3 expression on DRGs. Thereby, these data show that VBm is responsible for neuronal injury. ATF-3 has its pick of expression 72 h after nerve injury (Tsujino et al., 2000). Based in venom compounds, it is due to a direct chemical action in peripheral nerve endings of the affected tissue and/or to classical inflammatory molecules such IL-1β or TNF-α that can also promote ATF-3 expression (Lu et al., 2007; Zhang et al., 2017b). The ATF-3 expression was substantially reduced to 25.87 ± 2.57% and 19.84 ± 2.15% in oral HEETa and VBm: HEETa groups, respectively, with no significant difference between them. It shows that HEETa attenuated nerve injury, which contributes to reduce venom morbidity.

The histopathological analysis of paw tissue detected hemorrhagic fields two hours and a great amount of polymorphonuclear cells 72 h after VBm inoculation. In HEETa oral and VBm: HEETa groups the inflammatory cell infiltrate was discrete at 72 h. Those actions probably contributed to the antihyperalgesic effect of HEETa orally. However, hyperalgesia was not affect with the venom neutralization assay performed with VBm: HEETa. In an initial analysis, it proves the absence of chemical antagonism for this effect.

In addition, we have to consider that the direct effect of the venom in nociceptors and through hyperalgesic pathways was sufficient to sustain hyperalgesia even with a less inflammatory infiltrate. The cell

Table 3
Measures of biochemical markers Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Albumin (ALB) and Creatine in treatment with HEETa (single dose or during seven consecutive days) and their respective controls. Two-way ANOVA, Bonferroni post-test.

Marker	Group				
	Saline Single Dose Treatment	HEETa Single Dose Treatment	Saline seven days treatment	HEETa seven days treatment	*P* value
ALP (U/L)	133.4 ± 14.41	122.1 ± 15.980	87.73 ± 9.086	124.4 ± 14.340	0.1731
ALT (U/L)	46.97 ± 4.276	48.47 ± 7.943	44.00 ± 2.501	44.27 ± 5.023	0.9167
ALB (g/L)	4.133 ± 0.2906	3.467 ± 0.5207	3.133 ± 0.6960	3.333 ± 0.3528	0.5381
Creatinine (mg/dL)	0.0666 ± 0.0333	0.1333 ± 0.0333	0.1667 ± 0.0666	0.0666 ± 0.0333	0.3437

membrane disruption leads to prostaglandins (PGs) synthesis (Ricciotti et al., 2011). Both E_1 and E_2 PGs evoke hyperalgesia (Khasar et al., 1994) with a long last duration (Villarreal et al., 2013) but the E_2 isoform has a preponderant role (Ito et al., 2001) acting as a final inflammatory mediator and direct sensitizing the nociceptors. Besides what, after noxius stimuli, the release of P substance in spinal cord contributes to inflammation and immune cells activation with consequent release of lysosomal enzymes, prostaglandins E_1 and E_2 and interleukins 1 and 6 (Su et al., 2014; Zhang and An, 2007). In sum, not only the cell inflammatory infiltrate is responsible for PGE_2 release, but also the membrane cells disruption and the noxius stimuli itself.

Another possibility is that HEETa depends of enteral administration to gain antihyperalgesic effect. This hypothesis could explain the discrepancy between antihyperalgesic effect of oral HEETa and subcutaneously inject neutralized VBm: HEETa.

It is possible to propose that HEETa decreased VBm's ATF-3 expression (neuronal injury) by two pathways. One is iridoids anti-inflammatory and antioxidant activities as VBm's cytotoxicity is induced by oxygen reactive species and cytokines. The other, HEETa can exerted a chemical antagonism in the VBm toxins responsible for inflammation and neuronal injury. In this case, the chelating property of iridoids (Yamane et al., 2010) are neutralizing the ions like calcium or zinc present in the active site of phospholipase A_2 and metalloproteases (Gutiérrez and Ownby, 2003; Gutiérrez and Rucavado, 2000). This would justify both the lower inflammatory infiltrate and decreased ATF-3 expression obtained for VBm: HEETa (1:100) group 72 h after the venom inoculation.

Taking together what we found in our work and the large information about bothropic venom's tissue injury, we can say that VBm toxins induced hyperalgesia by a direct noxius action in soft tissue and peripheral nerve terminals (Cintra-Francischenelli et al., 2010; Zamuner et al., 2004; Zhang et al., 2017a) and also, by the inflammatory pathway where hyperalgesic molecules (bradykinin, PGE_2, IL-1β and TNF-α) are synthetized (Chacur et al., 2002, 2004; Chaves et al., 1995; Serrano and Maroun, 2005; Teixeira et al., 2009).

In summary, our results sustaining the ethnopharmacological use of *Tabebuia aurea*, showing that it can be an accessible and adjunctive therapy for bothropic envenoming, given its ubiquitous distribution and therapeutic effects against noxious, inflammatory and neuronal injury events elicited during snakebite.

5. Conclusions

The hyperalgesia evoked by *Bothrops mattogrossensis* envenoming is a local event which lasts, at least, 72 h and occurs concomitantly with neuronal injury.

The hydroethanolic extract of *Tabebuia aurea* given orally can be used to relieve pain, inflammation and nerve injury induced by chemical stimuli.

The data obtained in this work corroborates with popular use of *Tabebuia aurea* for medicinal purposes and highlight its therapeutic potential.

Acknowledgments

The authors want to thank for the technical support provided by the Laboratory of Pain Studies, National Institute of Science and Technology on Photonics Applied to Cell Biology (INFABIC), and the Laboratory of Natural Products and Mass Spectrometry from the State University of Campinas (UNICAMP) and Federal University of Mato Grosso do Sul (UFMS), respectively. Thanks are also given to Brazilian research funding agencies: Coordination for the Improvement of Higher Level -or Education- Personnel (CAPES), National Council for Scientific and Technological Development (CNPQ), São Paulo Research Foundation (FAPESP) and Foundation of Support to the development of Education, Science and Technology of Mato Grosso do Sul (FUNDECT), for the grants awarded.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2018.12.037.

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