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Health risk associated with the oral consumption of “Chiniy-tref”, a traditional medicinal preparation used in Martinique (French West Indies): Qualitative and quantitative analyses of aristolochic acids contained therein.

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Abstract

“Chiniy-tref” (CT) is a traditional preparation used in folk medicine in Martinique Island (French West Indies) that is nowadays mainly taken orally to prevent or act against any “manifestation of evil”. CT is easily prepared at home by macerating larvae of the endemic swallowtail *Battus polydamas* (ssp.) *cebriones* (Dalman, 1823), sometimes accompanied by a leaf of its host-plant *Aristolochia trilobata* L., in commercial rum. We have previously reported the detection of nephrotoxic and carcinogenic aristolochic acids (AAs) I and II in CT, leading the Regional Health Agency (ARS) of Martinique to issue an alert regarding the potential risks associated with its consumption in 2015. In order to complete the toxicity risk assessment for oral consumption of CT, a full qualitative analysis of AAs and their analogues (AAAs) was performed, as well as a quantitative determination of the major AAs, namely AAs I and II. The phytochemical profiling of AAAs present in CT, that also corresponds to that of *B. polydamas cebriones* larvae feeding on *A. trilobata*, has been established for the first time by ultra-high performance liquid chromatography/electrospray ionization quadrupole time of-flight tandem mass spectrometry. AAs I and II were quantified in a small panel of tinctures

by using a validated UHPLC/UV method, allowing us to estimate the probable daily intakes of these toxins by CT consumers. The results proved the existence of a real risk of renal toxicity associated with the chronic oral consumption of CT in Martinique, and more generally of similar “snake bottles” throughout the Caribbean.

Keywords

"Chiniy-tref"; traditional medicine; aristolochic acids; phytochemical profile; probable daily intakes; aristolochic acid nephropathy.

1. Introduction

“Chiniy-tref” (CT) is a popular traditional medicinal preparation used in Martinique Island (French West Indies), which is nowadays essentially consumed orally for its medico-magical properties for preventing or acting against any “manifestation of evil” (Nossin, 2006). Literally translated from French-based Caribbean Creole, “Chiniy-tref” (CT) means “caterpillar of tref”, “Tref” being the vernacular name of *Aristolochia trilobata* L. (*Aristolochiaceae*). Indeed, this preparation is obtained by maceration in alcohol, mostly commercial rum, of living larvae (*i.e.* caterpillars) of the endemic swallowtail *Battus polydamas* (*ssp.*) *cebriones* (Dalman, 1823) (*syn. xenodamas* (Hübner, 1825)) (Papilionidae: Troidini), feeding on the young leaves of the quasi-exclusive host-plant *A. trilobata*. Larvae of swallowtails belonging to the tribe Troidini feed exclusively on *Aristolochia* plants (Hall, et al., 2017) and are known to sequester aristolochic acids (AAs) present in plant tissues, preventing them from predation due to their extreme bitterness. They also act as feeding and oviposition stimulants for larvae or adults, respectively (Hall, et al., 2017; Priestap, et al., 2012). CT may also contain leaves of *A. trilobata* as additional ingredient and thus fits the definition of a “snake bottle”. This term refers to a remedy prepared from plant(s) and/or insects and used in the Lesser Antilles to prevent or treat snakebites. Typically, according to C. Lans *et al.* ingredients are macerated in alcohol or olive oil and kept in 150 ml flasks (Lans, et al., 2001). A preparation similar to CT is also reported on the Island of Trinidad (Lans, et al., 2001; Boos, 1986). This remedy is also consumed in St Lucia, St Vincent and the Grenadines and French Guyana (Nossin & Weniger, 2009).



Fig. 1. Pictures of “Chiniy-tref” showing floating components: a leaf of *Aristolochia trilobata* L. (left) and larvae of *Battus polydamas cebriones* (left and right) (Credits: X.C.).

The current popularity of CT is directly originated from the complex mix of knowledges, skills and practices from Carib, European, African and -to a lesser extent- Asian and Indian populations that constitute the traditional Creole medicine (Moretti, 1991).

It all started with a venomous snake, particularly the endemic Martinique lancehead snake *Bothrops lanceolatus* (Bonnaterre, 1790) (Rufz, 1859). One of the most popular remedy for the treatment of snakebites in Martinique and throughout the Caribbean Basin was, and remains, *A. trilobata* (leave, root and the whole plant), and this use was probably already known to native Caribs (Bocquillon-Limousin, 1891; Heinrich, et al., 2009). The “doctrine of signatures” which evokes similarity concept between plant and disease may probably explain the selection of this plant in the past. Indeed, the three-lobed triangular plant leaf has a very similar shape to that of the snake's head, whereas its pipe-shaped blossom may look like a snake ready to bite (Bocquillon-Limousin, 1891; Nossin, 2017) (Fig. 2).



Fig. 2. Pictures of *Aristolochia trilobata* L. (left and center) and *Battus polydamas cebriones* (right) (Credits: X.C. & T.G., resp.).

The use of CT in this indication is later and comes from the fact that *B. polydamas* larvae develop a bitter taste by feeding on the bitter leaves of *A. trilobata* and hence gains the associated properties. Indeed, bitterness is perceived in the Creole culture and particularly in the traditional Martinican medicine as a major feature of antidotes for bites and poison, and more generally to prevent any influence of evil (Nossin, 2006). Other traditional medicinal uses of CT included treatment of digestive disorders, hypertension, diabetes, pain, particularly abdominal and gynecological pain. It may also be given for facilitating delivery during childbirth and removal of the placenta (Lans, et al., 2001; Nossin, 2006; Boos, 1986). Some magical properties have also been attributed to the preparation since it contains sacrificed caterpillars able to safely grow and feed on the snake head-shaped and highly toxic leaves of *A. trilobata*. *Bothrops lanceolatus* is considered in Martinique as the “supreme predator” being at the top of the food chain. Everything that acts against him must be able to act on any other form of evil. In a symbolic way, caterpillars are uncommon creatures endowed with power and incommensurable force (Nossin & Weniger, 2009; Nossin, 2017).

Finally, CT became a renowned medico-magical remedy, able for instance to protect a person from a spell, to avoid any poisoning when dining with strangers or supposed counteract the effects of potions given by women to men (locally called “stan home” or “stay home”) to ensure marriage (Boos, 1986; Nossin & Weniger, 2009).

During a preliminary work, our team have highlighted the presence of aristolochic acids I and II in CT (Cachet, et al., 2016). These compounds are considered as group 1 carcinogens for human by the International Agency of Research on Cancer (IARC, 2012) and responsible of urothelial cancers. The Regional Health Agency (ARS) of Martinique published a press release and a warning letter for health care professionals in August 2015 reminding that the sale of AAs-containing herbs and preparations is strictly prohibited and that their consumption must be avoided (ARS Martinique, 2015).

However, CT remains readily available and can be prepared by consumers themselves or bought on the black market.

In continuation of our previous work, we sought to assess the risk associated with the oral consumption of CT. Thus, a complete qualitative analysis of aristolochic acids and their analogues (AAAs), as well as a quantitative determination of the most abundant AAs (*i.e.* AA I and AA II) by UHPLC-UV were performed on CT and results are reported here. On the basis

of the measured contents, probable average daily doses of AA I and II were estimated, and the results are discussed.

2. Materials and Methods

2.1. Materials and standards

Herbal material. Dried *A. trilobata* leaves were purchased locally either from a commercial supplier (Sud-Botanik, Le Marin) in 2015 (Voucher number: C48829-21), or from the Fort-de-France market in 2016 (Voucher number: C48793-111), then stored at -20°C. Samples were authenticated in comparison with a reference sample N°8898 of *A. trilobata* (aerial parts) harvested in 2015 from the botanical garden of the Museum National d'Histoire Naturelle de Paris (M.N.H.N.) (Voucher number: C48793-63). *Insect material.* Caterpillars of *B. polydamas cebriones* growing on *A. trilobata* leaves were collected and identified in Martinique by Dr A. Bateau (Regional Health Agency of Martinique) the 3 November 2016, then killed by freezing at -20°C and stored at this temperature (Voucher number: C48793-113). *Samples of "Chiniy-tref"*: Bottles of CT were locally purchased on the illegal market and registered under numbers: CT_2016, CT_2017-50, CT_2017-55 and CT_2017-62. When available, recipes were reported in Table 1. Sample CT_2016 was used as reference tincture to establish phytochemical fingerprinting of CT as well as for the validation of the UHPLC-UV method developed for the quantification of AA I and AA II (validation standard). A self-made tincture (CT_2017-44) was prepared in order to study the kinetic of extraction of AA I and AA II from caterpillars, by macerating four defrosted caterpillars (corresponding to a mass of 4.8 g of fresh insect) with 250 ml of commercial rum (44° GL), then kept at room temperature (Temperature records are reported in Supplementary data S1) in an amber colored glass bottle. AA I and AA II contents in the tincture N°CT_2017-44 were measured after 3 (T3: May 12, 2017), 45 (T45: June 26, 2017) and 52 (T52: July 3, 2017) days of maceration. *Reference standards.* AA I (European Pharmacopoeia CRS, purity > 97.0% by HPLC), AA II (Sigma-Aldrich, > 97.0% purity), AA C or IIIa (Phytolab, 78.9% purity), AA D or IVa (Phytolab, 96.6% purity), Aristolactam I (Phytolab, 98.9% purity). AA Ia was prepared from AA I according to the procedure described by Priestap *et al.* (Priestap, et al., 2012).

Table 1. Details on the mode of preparation of CT samples analyzed in the study.

Registration number	CT_2017-44 ^a	CT_2016 ^b	CT_2017-50 ^b	CT_2017-55 ^b	CT_2017-62 ^b
Starting date for maceration (T0)	May 9, 2017	Not precisely known: 2016	Nov 25, 2016	Feb 04, 2017	Apr 15, 2017
Mode of preparation	4 caterpillars were added at the same time to rum (250 ml)	Unknown	3 (Nov 25) +5 (Nov 27) +1 (Nov 28) +5 (Dec 02)	9 (Feb 04) +3 (Feb 05) +3 (Feb 06) +3 (Feb 07)	6+7+7 Unknown frequency
Alcoholic degree of rum (GL) ^c	44°	50°	50°	55°	62°
Number of caterpillars per liter	16	Unknown	14	18	20
Other AAs-containing ingredient	No	No	No	1 leaf of <i>A. trilobata</i>	1 leaf of <i>A. trilobata</i>

^aself-made preparation, ^bpurchased preparations, ^cGL = degrees Gay-Lussac

2.2 Phytochemical profiling of AAAs in CT and *A. trilobata* leaves by UHPLC-RP18-HRMS²/MS².

Sample preparation:

Reference standards solutions were prepared from stock solutions (0.1 mg/ml) and diluted at the concentration of 10 µg/ml with methanol. "CT_2016" tincture was diluted with 30% aqueous acetonitrile up to 1:4 (V/V). Oven dried (for 5 days at 60 °C) powdered herbal drugs (leaves from samples N° C48829-21 and N° C48793-63: 0.90 and 0.89 g, respectively) were extracted with methanol (50.0 ml) in an ultrasonic water bath (Branson Bransonic 2800, 40 kHz, 245 W, Emerson Electric Co., St. Louis, USA). The solution was decanted, and 500 µl of the supernatant was diluted with 30% aqueous acetonitrile up to 1:2 (V/V) to obtain a final concentration of herbal sample around 8.5 mg/ml. All solutions were finally filtered through a 0.2 µm nylon filter disk prior the injection.

Chromatographic conditions:

Separations were performed using an Ultimate 3000 RSLC system equipped with a binary pump, an autosampler and a thermostated column compartment, equipped with a diode array detector (DAD) (195–800 nm) (Dionex, Germering, Germany). Components were separated on a C18 Luna Omega column of 150 mm × 2.1 mm with a particle size of 1.6 μm (Phenomenex, Le Pecq, France). The mobile phase was made up of 0.1% formic acid in water (phase A), and 0.08% formic acid in acetonitrile (phase B). A solvent gradient was applied as follows: 0–0.1 min: 3% B, 0.1–26 min: 3–80% B, 26–26.5 min: 80–95% B, 26.5–29.5 min: 95% B, 29.5–30 min: 95–3% B, and finally 30–33 min: 3% B. The column was introduced in a thermostated compartment heated at 40 °C. All solutions were prepared in HPLC grade solvent. The injection volume was set at 1 μL and the flow rate was set at 500 μL/min. MS experiments were carried out on a maXis Ultra High Resolution-Quadrupole-Time-of-Flight (UHR-Q-TOF) mass spectrometer (Bruker, Bremen, Germany) in positive electrospray ionization (ESI) mode. Capillary voltage was set at +4.5 kV. The flows of nebulizing and drying gas (nitrogen) were respectively set at 2.0 bar and 9.0 L/min and drying gas was heated at 200 °C. Mass spectra were recorded in the range 50–1650 *m/z*. MS/MS experiments were conducted using data dependent acquisition (DDA) mode (auto-MS/MS) in a mass window from *m/z* 150–1200. Three precursor ions with intensities higher than 400 au were selected per fragmentation cycle among the most intense ions to be fragmented. These three precursor ions were allowed to be selected for two consecutive cycles and were then placed on an exclusion list for 0.05 min. The collision energy was set at 35 eV and was applied as follows: 88% of the collision energy was applied during half of the fragmentation cycle, and 117% of the collision energy was applied during the half remaining cycle time. Raw data were processed using DataAnalysis 4.0 software (Bruker, Bremen, Germany).

2.3 Assay of AA I and AA II in CT and validation thereof.

Method validation - Calibration curve for the standards:

The UHPLC-UV method for the assay of AA I and AA II was validated according to the International Conference on Harmonization (ICH) Q2 (R1) guideline (Validation of analytical procedures: text and methodology Q2 (R1), 2005). Linearity, accuracy, precision (repeatability and intermediate precision) parameters were studied in the concentration range from 40% to 160% of the theoretical concentration (100%) of AA I and AA II in the test

solution prepared with the tincture CT_2016 (*i.e.* 0.056 mg/ml and 0.0050 mg/ml for AA I and AA II, respectively). Other parameters such as specificity, limit of detection (LOD) and limit of detection (LOQ) were also investigated. Specificity was defined as the absence of peak overlap. Peak purity was checked by both DAD and mass detection. LOD and LOQ were defined as the concentration of component providing a signal-to-noise ratio (S/N) of three and ten, respectively. These parameters were thus estimated by serial dilution of standard solutions.

Three calibration standard solutions were prepared containing AA I at nominal concentrations between 0.022-0.090 mg/mL (*i.e.* 40, 100 and 160%) by diluting a stock solution of AA I (0.11 mg/ml in 30% aqueous acetonitrile) with the same solvent. Three calibration standard solutions were prepared containing AA II at nominal concentrations between 0.0020-0.0080 mg/mL (*i.e.* 40, 100 and 160%) by diluting a stock solution of AA II (0.10 mg/ml in 30% aqueous acetonitrile) with the same solvent. Calibration standards were analyzed in duplicates. Validation standards were prepared at five concentration levels (*i.e.* 40, 70, 100, 130 and 160%) by diluting the tincture CT_2016 with 30% aqueous acetonitrile and were analyzed in triplicates. All solutions were prepared using HPLC grade solvent and filtered through a 0.2 µm nylon filter disk prior the injection.

Sample preparation:

Test solutions of CT were prepared by diluting tincture with 30% aqueous acetonitrile up to 1:4 (V/V) and analyzed in triplicate. A methanolic extract of *A. trilobata* leaves was prepared by extracting the dried powdered material (1.0 g from sample C48793-111, in duplicate) with methanol (80 ml) using Soxhlet apparatus for 8 h. The resulting solution was diluted to 100.0 ml in a volumetric flask and analyzed in duplicate in the same manner as CT. A methanolic extract of fresh caterpillars of *B. polydamas* was prepared as follows: Six defrosted caterpillars were individually cut in half and pieces were grouped into two “homogeneous” pools A and B of 3.6 and 3.9 g, respectively. Each sample was frozen with liquid nitrogen, coarsely crushed, and Soxhlet extracted in methanol for 8 h. The resulting extract was transferred in a 100.0 ml volumetric flask, the volume was made up to with methanol. Analysis was performed in duplicate in the same manner as that of CT.

Determination of moisture content:

Moisture content of the dried herbal sample was measured by loss in weight on drying at 100-105°C and was performed in duplicate on 1 g of material.

Chromatographic conditions:

Separation were performed using an Ultimate 3000 UHPLC-System (Thermo Fisher Scientific, Courtaboeuf, France) equipped with a RS binary pump, an autosampler, a thermostated column compartment and a UV-Visible detector ($\lambda_1 = 250$ and $\lambda_2 = 310$ nm) and controlled by the Chromeleon software 6.80.15 from Thermo Fisher Scientific. Components were separated on a C18 Hypersil Gold column of 100 mm x 2.1 mm with a particle size of 1.9 μm (Thermo Fisher Scientific). The mobile phase was composed of 0.1% formic acid in water (phase A), and 0.1 % formic acid in acetonitrile (phase B). A solvent gradient was applied as follows: 0–2 min: 30 % B, 2–2.5 min: 30–33 % B, 2.5–8 min: 33 % B, 8–8.5 min: 33-100 % B, 8.5–10 min: 100 % B, 10–10.5 min: 100–30 % B and finally 10.5–17 min: 30% B. The column oven temperature, the injection volume and the flow rate were set at 40 °C, 2 μL and 600 $\mu\text{L}/\text{min}$, respectively.

To check the specificity of the method, another UHPLC system was used. Analyses were carried out in the “Service HPLC-MASSE” of the Institut de Chimie des Substances Naturelles, CNRS, 91198, Gif sur Yvette, France on an Acquity UPLC system (Waters, Mildford, MA, USA), driven by a MassLynx 4.1 software, coupled with a Acquity DAD 996 UV/Visible detector (200–400 nm) and a triple quadrupole (QqQ) Acquity mass spectrometer (Micromass-Waters Manchester, England) (m/z mass ranging from 50 to 800), with an ESI source operated in positive ionization mode at 3.2 kV capillary voltage.

3. Results and discussion

3.1. Phytochemical profiling of aristolochic acids and aristolochic acid analogues (AAAs) in “Chiniy-tref” and *A. trilobata* leaves by UHPLC-DAD-HRMS/MS.

The qualitative analysis of AAAs present in CT was carried out by UHPLC-DAD-HRMS/MS on sample CT_2016 that was considered representative. Anyway, all samples of CT showed similar chromatographic profiles. Compounds detected by reverse-phase chromatography with UV detection at the characteristic band of 310 nm were further characterized by tandem mass spectrometry. Some of them were unambiguously identified in the analyzed

sample by comparison of chromatographic and spectral data (*i.e.* Retention time (t_R), wavelengths of maximum absorption (λ_{max}), m/z values of the pseudomolecular ions and of the MS or MS² fragment ions) (Table 2) with those of reference compounds, namely AA I, AA Ia, AA II, AA IIIa, AA IVa and AL I (Fig. 3). Five additional AAs and AAAs were also detected and tentatively characterized by comparison of their data with literature (Priestap, et al., 2012). Compound eluting at $t_R = 16.8$ min exhibited the same pseudomolecular ion at m/z 342 (342.0607, calculated for C₁₇H₁₂NO₇: 342.0608, $\Delta ppm = 0.3$) as that of AA I, as well as the same fragmentation pattern and could be tentatively assigned to AA III. Compounds eluted at 15.8 and 17.7 min showed a protonated ion at 264.0655 (calculated for C₁₆H₁₀NO₃: 264.0655, $\Delta ppm = -0.1$), and m/z 372.0712 (calculated for C₁₈H₁₄NO₈: 372.0714, $\Delta ppm = 0.5$) respectively, and were putatively identified as AL II and AA IV, respectively. Finally, two more polar AAs ($t_R = 8.7$ and 10.4 min) had a molecular formula of C₂₂H₁₉NO₁₂ deduced from the pseudomolecular ion observed at the same m/z 490 (490.0987/490.0968, respectively, calculated for C₂₂H₂₀NO₁₂: 490.0980, $\Delta ppm = -1.4/3.6$). Both compounds exhibited a characteristic fragment ion at m/z 310 (310.0350/310.0344, respectively, calculated for C₁₆H₈NO₆: 310.0346, $\Delta ppm = -1.2/0.6$) also observed in the MS spectra of AA Ia and AA IIIa, that corresponds to the loss of a hexose moiety (162 uma) and of a water molecule ([M-162-H₂O]⁺, acylium ion). Both compounds were reasonably characterized as the glycosyl derivatives of AA Ia and AA IIIa.

Table 2. Identification of aristolochic acids and their analogues in “Chiniy-tref” (sample CT_2016) by UHPLC-ESI-QTOF-MS/MS.

Peak number	RT (min)	m/z [M+H] ⁺	Molecular formula	Δmass error (ppm)	Other detected m/z on MS scan ^a	ESI-MS ² data (m/z)	Putative identification (**confirmed by comparing with reference standard) ^b	Relative content in Chinyi tref ^c	Relative content in A. t. leaf ^c / MNHN
1	8.7	490.0987	C ₂₂ H ₁₉ NO ₁₂	-1.4	507.1244 ([M+NH ₄] ⁺), 512.0802 ([M+Na] ⁺)	328 (M-hexose), 310 (M-hexose-H ₂ O), 284 (M-hexose-CO ₂) [*] [*] in source fragmentation	AA Ia hexoside or AA IIIa hexoside or isomer	+	++/++
2	10.4	490.0968	C ₂₂ H ₁₉ NO ₁₂	3.6	507.1250 ([M+NH ₄] ⁺), 512.0802 ([M+Na] ⁺)	328 (M-hexose), 310 (M-hexose-H ₂ O), 284 (M-hexose-CO ₂) [*] [*] in source fragmentation	AA Ia hexoside or AA IIIa hexoside or isomer	+	++/++
3	12.9	328.0450	C ₁₆ H ₉ NO ₇	0.4	310.0344 ([M-H ₂ O+H] ⁺), 350.0270 ([M+Na] ⁺)	266, 238, 208, 180	AA IIIa = AA C ^{**}	+++	++/++
4	13.9	358.0560	C ₁₇ H ₁₁ NO ₈	-0.7	340.0454 ([M-H ₂ O+H] ⁺), 380.0377 ([M+Na] ⁺)	296, 281, 253	AA IVa = AA D ^{**}	++	+/+
5	14.0	328.0446	C ₁₆ H ₉ NO ₇	1.8	310.0345 ([M-H ₂ O+H] ⁺), 350.0270 ([M+Na] ⁺)	266, 180	AA Ia	++	+++ /+++
6	15.8	264.0655	C ₁₆ H ₉ NO ₃	-0.1	286.0468 ([M+Na] ⁺)	206	AL II	++	nd/nd
7	16.5	312.0497	C ₁₆ H ₉ NO ₆	1.8	294.0396 ([M-H ₂ O+H] ⁺), 334.0322 ([M+Na] ⁺)	250, 222, 194, 192, 164, 139	AA II ^{**}	+++	++/++
8	16.6	294.0770	C ₁₇ H ₁₁ NO ₄	-3.2	316.0568 ([M+Na] ⁺)	279, 251	AL I ^{**}	++	+/+
9	16.8	342.0607	C ₁₇ H ₁₁ NO ₇	0.3	324.0501 ([M-H ₂ O+H] ⁺), 364.0428 ([M+Na] ⁺)	236	AA III or isomer	++	+/+
10	17.3	342.0607	C ₁₇ H ₁₁ NO ₇	0.5	324.0502 ([M-H ₂ O+H] ⁺), 364.0427 ([M+Na] ⁺)	280, 265, 237	AA I ^{**}	+++	++ /+++
11	17.7	372.0712	C ₁₈ H ₁₃ NO ₈	0.5	354.0607 ([M-H ₂ O+H] ⁺), 394.0530 ([M+Na] ⁺)	x	AA IV or isomer	++	nd/nd

^aBolded values correspond to base peaks; ^bData for standards are given in Supplementary data S2; ^cEstimation based on absolute peak intensities: +++ (> 1.10⁵) > ++ (1.10⁴ – 1.10⁵) > + (< 1.10⁴) > nd (not detected).

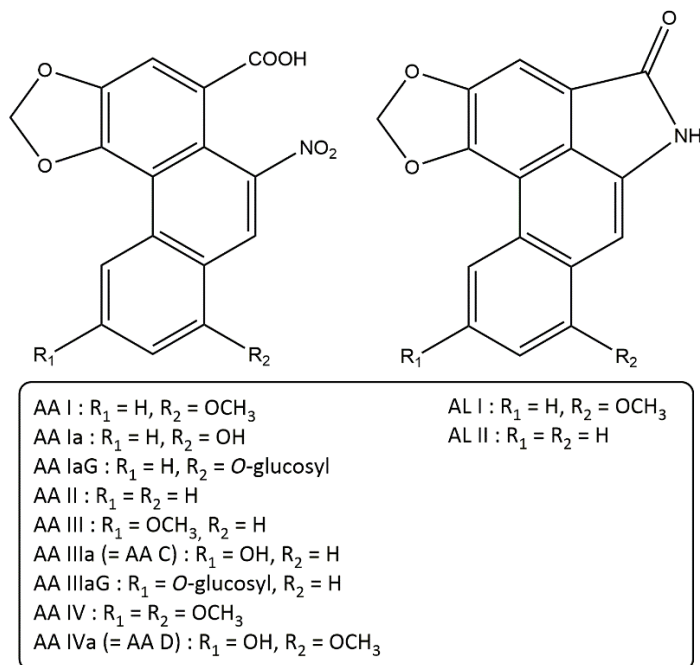


Fig. 3. Chemical structures of aristolochic acids (AA) and aristolactams (ALs) identified or putatively identified in *A. trilobata* leaves and CT tinctures.

Typical Base Peak Chromatogram (BPC), as well as superimposed ion extracted chromatograms of all m/z corresponding to the most intense base peak ions of AAAs detected in CT_2016, and traced with numbered peaks (No. 1–11), are depicted in Fig. 4.

The relative amounts of AAAs were estimated from the absolute intensities of base peaks observed on BPC chromatograms and are given in Table 2. These results could also be deduced from the chromatographic peak areas measured at 250 or 310 nm for UHPLC-UV, since the profiles obtained by both detectors appeared similar. The major AAAs found in CT were AA I, AA II, and to a lesser extent AA IIIa.

With the exception of AL II and the compound eluting at 17.7 min (peak 11, tentatively identified as AA IV), the same AAAs were detected in the leaf samples analyzed under the same conditions (See supplementary data S3 and S4). However, in these cases AAs Ia, I and II were the most abundant compounds. It should be noted that the presence of AA I and II in the leaves of *A. trilobata* has previously been reported (Michl, et al., 2016). Likewise, differences in AAA composition between insect tissues of *Battus* larvae and their *Aristolochia* hostplant have been thoroughly discussed elsewhere for *B. polydamas* larvae feeding on *A. argentina* (Priestap, et al., 2012). However, to the best of our knowledge, this is the first report on the chemical profiling of AAAs sequestered by *B. polydamas* larvae reared on *A. trilobata*.

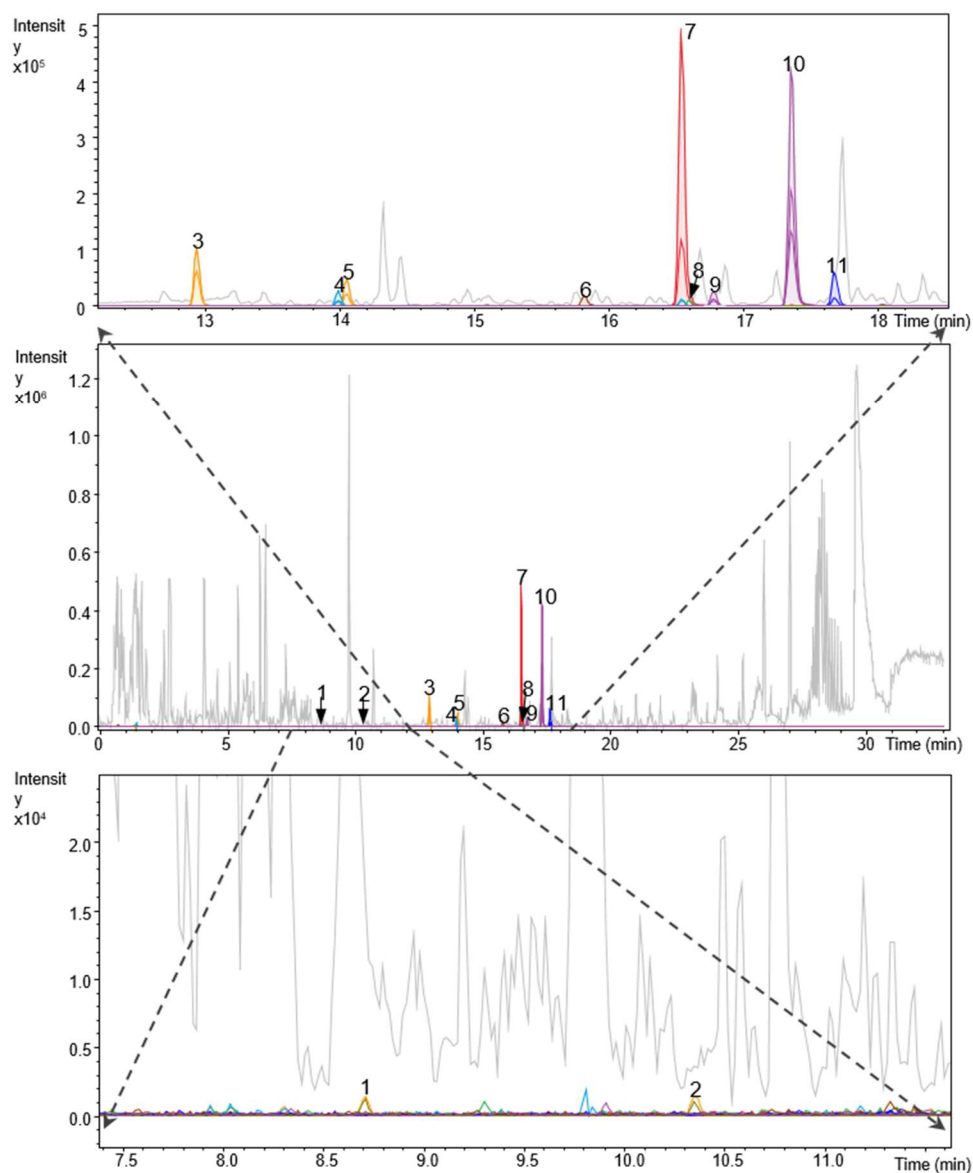


Fig. 4. Typical LC/MS base peak chromatogram (BPC) (grey trace), and superimposed ion extracted chromatograms (colored traces) of all m/z corresponding to the base peak ions of AAAs detected in CT_2016. Peak numbers in the chromatogram refer to compounds listed in Table 2.

3.2 Determination of the AA I and AA II content in herbs, caterpillar and “Chiniy-tref” extracts

The UHPLC-C18-UV method, developed for the quantification of AA I and AA II in CT, was validated according to the ICH recommendations and found to be specific, linear, precise, accurate and sensitive. Results are given in Table 3, as well as other calibration parameters.

Table 3. Validation parameters: specificity, linearity, accuracy, precision, LOD and LOQ of the proposed UHPLC method, and other calibration parameters (calibration range and linear regression).

	AA I (RT # 9 min)	AA II (RT # 7 min)	Acceptance criteria
Specificity	No co-elution at 250 nm.	No co-elution at 310 nm.	Peak purity checked with both DAD and mass detectors.
Linearity^a	D ₁ R ² = 0.99386 D ₂ R ² = 0.99205 D ₃ R ² = 0.99323 Mean R ² = 0.97798	D ₁ R ² = 0.99368 D ₂ R ² = 0.99238 D ₃ R ² = 0.99211 Mean R ² = 0.96533	R ² >0.99
Calibration range	0.022-0.090 mg/ml [40-160%]	0.0019-0.0077 mg/ml [40-160%]	/
Linear regression ^b	$y = 26.2913x + 0.0074$	$y' = 139.1747x' - 0.0253$	/
Accuracy	Recovery (%): [100.99-101.97] Relative bias (%): [0.99-1.97]	Recovery (%): [100.38-104.20] Relative bias (%): [-1.33-0.38]	Relative bias < 2%
Precision: intra-day	CV ^c (%): [1.29-2.37]	CV (%): [1.02-2.47]	Mean CV < 2%
Precision: inter-day	CV (%): [2.41-3.32]	CV (%): [1.49-2.58]	Mean CV < 3%
LOD	33 µg/100 ml	1 µg/100 ml	/
LOQ	93 µg/100 ml	5 µg/100 ml	/

^aD: Day; ^by and y': peak area; x and x': respective concentration of AA I and II expressed in mg/ml; ^cCV: coefficient of variation

The method was then applied to the determination of AA I and AA II contents in CT, *A. trilobata* and *Battus polydamas* samples and the results are depicted in Table 4.

Table 4. AA I and AA II contents in CT, *A. trilobata* and *B. polydamas* samples.

Sample (Time of analysis)	AA	Content in mg/ml	Content in mg/g	AA I/AA II ratio
CT_2016 (May 10, 2017)	AAI	0.23	/	92/8
	AAII	0.021	/	
CT_2017 50° (T ₀ +7 months, July 03, 2017)	AAI	0.036	/	88/12
	AAII	0.0047	/	
CT_2017 55° (T ₀ +5 months, July 03, 2017)	AAI	0.039	/	86/14
	AAII	0.0066	/	
CT_2017 62° (T ₀ +8 months, Dec 12, 2017)	AAI	0.053	/	89/11
	AAII	0.0064	/	
CT_2017 44° (T ₀ +3 days, May 15, 2017)	AAI	0.023	/	88/12
	AAII	0.0030	/	
CT_2017 44° (T ₀ +45 days, June 26, 2017)	AAI	0.036	/	90/10
	AAII	0.0040	/	
CT_2017 44° (T ₀ +52 days, July 03, 2017)	AAI	0.037	/	90/10
	AAII	0.0040	/	
<i>A. trilobata</i> leaves (Martinique, N°C48793-111)	AAI	/	0.35 ^a	65/35
	AAII	/	0.19 ^a	
<i>B. polydamas cebriones</i> (Whole larvae)	AAI	/	1.82	91/9
	AAII	/	0.17	

^a Results are expressed in dry material, based on the mean value of the measured moisture content (Loss on drying), namely 5.9%.

Values given for CT samples CT_2016, CT_2017 50°, CT_2017 55° and CT_2017 62° correspond to the maximum contents reached after 3 months of monitoring and ranged from 36 to 230 µg/ml and from 4 to 21 µg/ml for AA I and AA II, respectively. However, AA I and II contents were found significantly higher in tincture CT_2016 whose recipe is unknown, and quite similar in others. These significant differences more likely reflect a difference in the mode of preparation of CT than in the inherent variability of biological materials. This has been demonstrated by performing assays on the model tincture CT_2017 44°. Results showed that the concentration in AA I and AA II increased over time and reached an equilibrium concentration. Furthermore, the amount of AAs present in the tincture corresponds to an exhaustive extraction of these compounds from caterpillar tissues during maceration. Indeed, the theoretical mounts of AA I and II were calculated from the recipe followed for the sample preparation (*i.e.* 4,8 g of fresh caterpillars macerated in 250 ml of 44° rum) and from the assay results obtained for whole fresh caterpillars, namely 1.82 and 0.17 mg/g of AA I and AA II, respectively. Theoretical amounts were then compared with the measured practical amounts and were found to be very close, considering the non-

homogeneity of the caterpillar sample: AA I: 0.035 vs 0.037 mg/ml, resp.; AA II: 0.0040 vs 0.0033 mg/ml, resp. In conclusion, AA I and AA II contents in CT depend on both the recipe used and the original content in the insect.

Furthermore, a common practice involves that people who possess a CT tincture at home maintain it by adding additional rum and/or caterpillars directly into the initial preparation (Nossin & Weniger, 2009; Hall, 2017). This may explain the high levels of AA I and AA II found in sample CT_2016.

Interestingly, from another point of view, the ratio of AA I to AA II of each sample was calculated and values reported in Table 3. Except for *A. trilobata* leaves (AA I / AA II = 65/35), ratios were found to be around 90/10. This corroborates previous observations by Priestap et al. (Priestap et al., 2012 and references cited therein) reporting that AA I was the major AA present in *B. polydamas* tissues whatever the host-plant concerned (As mentioned earlier, none of these studies dealt with *B. polydamas* feeding on *A. trilobata* leaves). According the authors, such a phenomenon might be explained by repressed degradation of AA I, rather than selective sequestration.

3.3 Public health risks associated with oral consumption of CT.

It has been clearly established that AAs and particularly AA I and II, are nephrotoxic, genotoxic and carcinogenic. Their intake is responsible for chronic tubulointerstitial renal disease with a rapid progression towards end-stage renal disease, frequently accompanied by upper tract urothelial carcinoma. This toxicity as well as the underlying mechanisms have been extensively studied -and continue to be- in the epidemiological contexts of the “Chinese herbs nephropathy”, AA nephropathy and Balkan endemic nephropathy (Han, *et al.*, 2019; Chan, *et al.*, 2019). Plants containing AAs, particularly those belonging to the genus *Aristolochia* and finally AAs were classified respectively in 2002 and 2008 as carcinogenic to Humans (Group 1) by the International Agency for Research on Cancer (IARC) (IARC, 2012).

Our results clearly demonstrate the presence of significant amount of AA I and II in CT, but also the identification of minor AAAs whose toxicities are not well established (Michl *et al.*, 2016). Nevertheless, there is little information available regarding the consumption habits of CT in terms of frequency and amount consumed. It has been reported, however, that a dose of three tablespoons per day (about 45 ml) of CT may be “recommended” in the case of digestive disorders (Nossin & Weniger, 2009). On this basis, the estimated daily intake (EDIs)

of AA I and II could be calculated using a body weight of 70 kg as a default value, and results presented in Table 5.

Table 5. Estimated daily intakes (EDIs) of AA I and AA II calculated on the basis of a 45 ml daily dose and a body weight of 70 kg.

Sample	AA I + AA II ($\mu\text{g}/\text{ml}$) ^a	EDI ($\mu\text{g}\cdot\text{kg}^{-1}\text{ bw day}^{-1}$)
CT_2016	251	108
CT_2017 50°	41	17
CT_2017 55°	46	20
CT_2017 62°	60	25
CT_2017 44°	41	18

^a Values were rounded off to the nearest whole number.

These values are obviously in the same range of those found in slimming pills containing *Aristolochia fangchi* instead of *Stephania tetranda*, namely 7-31 $\mu\text{g}\cdot\text{kg}^{-1}\text{ bw day}^{-1}$, consumed by patients from Belgium who developed severe renal diseases (Abdullah *et al.*, 2017).

Another major risk factor is of course the chronic exposure to toxins. In the case of nephropathies reported in Belgium, the resulting maximum dose of AAs was estimated at 25 $\mu\text{g}/\text{kg}$ consumed during an average thirteen months. In another case of aristolochic acid nephropathy (AAN) reported in China, the exposure doses were estimated between 0.7 and 1.5 $\text{mg}\cdot\text{day}^{-1}$ of AAs intermittently for one to ten years (Grollman *et al.*, 2009; IARC, 2012). As mentioned in the introduction, CT is a popular remedy deeply anchored in the local medicinal culture, which has drifted from a treatment of snake bites to “a cure for all ills”. It is thus frequently used for widespread medicinal and mostly magical purposes, probably throughout life. This suggests an actual risk of exposure to AAs, and consequently of AAN development for consumers, that needs to be evaluated by further epidemiologic investigations.

Conclusions

The phytochemical profiling of AAAs present in CT, that also corresponds to that of *Battus polydamas cebriones* larvae feeding on *A. trilobata*, has been established for the first time by ultra-high performance liquid chromatography/electrospray ionization quadrupole time of-flight tandem mass spectrometry (UHPLC-ESI-QTOF-MS/MS). AA I, and to a lesser extent AA

It were found to be the major AAs. Both molecules were quantified in a small panel of tinctures by using a validated UHPLC/UV method, allowing us to estimate the probable daily intakes of these toxins by CT consumers. The results proved the existence of a real risk of renal toxicity associated with the chronic oral consumption of CT. To our opinion, the consequences of this practice may be an underestimated major health concern in Martinique, and also in other localities throughout the Caribbean Arc in which people are exposed to venomous snake bites and “snake bottle” prepared and consumed, such as in Trinidad, St Lucia and St Vincent (...). It is noticeable that the absence of venomous snakes in the neighbor island of Guadeloupe is correlated with the non-consumption of CT, that is even absent from the island. Further epidemiologic studies are therefore needed in order to assess and clearly define the risk of AANs and urothelial cancers associated with the oral use of CT. Finally, this study raises the problem of addressing deeply rooted misconceptions of consumers towards traditional remedies that do not really fall under any type of regulation, and how to convince them to modify their risky behavior.

Recent works showed that AAs inhibit the activity of enzymes present in snake venoms (*i.e.* phospholipase A2, L-amino acid oxidase), supporting traditional use of AA-containing preparations as antidote for snake bites (Ebrahimi *et al.*, 2018; Bhattacharjee *et al.*, 2017) and justifying a use of CT restricted to topic administration, and only in this indication.

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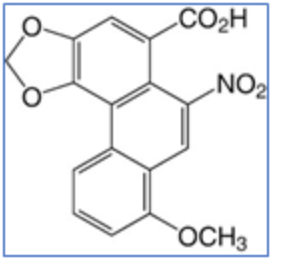
Battus polydamas



Aristolochia trilobata



Chiniy-tref



Aristolochic acid I



A risk of aristolochic acid nephropathy?