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EXPLORATION BY MOLECULAR NETWORKING OF STRYCHNOS ALKALOIDS REVEALS THE UNEXPECTED OCCURRENCE OF STRYCHNINE IN SEVEN STRYCHNOS SPECIES

Olivier Bonnet [a,*], Mehdi A. Beniddir[b], Pierre Champy[b], Vedaste Kagisha [a,c], Alain Nyirimigabo[a, c], Carla Hamann [a], Giorgi Jgerenaia[a, d], Allison Ledoux[a], Alembert Tiabou Tchinda[a,e], Luc Angenot[a], Michel Frederich[a]

[a] *Laboratory of Pharmacognosy, Center of Interdisciplinary Research on Medicines (CIRM), University of Li'ege, B36, 4000, Li'ege, Belgium*
[b] *Equipe "Chimie des Substances Naturelles " BioCIS, CNRS, Universite Paris-Saclay, 5 Rue J.-B. Clement, 92290, Chatenay-Malabry, France*
[c] *School of Medicine and Pharmacy, College of Medicine and Health Sciences, University of Rwanda, Kigali, P.O. Box 3286, Rwanda*
[d] *Department of Pharmaceutical Technology, Faculty of Pharmacy, Tbilisi State Medical University, 33, Vazha Pshavela Ave., Tbilisi, 0177, Georgia*
[e] *Institute of Medical Research and Medicinal Plants Studies (IMPM), PO Box 13033, Yaounde, Cameroon*

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ABSTRACT

Introduction: Plants of the *Strychnos* genus, which include about 200 species, are used for multiple traditional purposes as hunting poison, for example, and have shown interesting pharmacological properties, especially curarizing and tetanizing, but also against malaria. Many monoterpene indole alkaloids have already been isolated and identified. Among them, there is strychnine, a famous alkaloid that can cause death by asphyxiation. *Objective:* Investigate alkaloidic molecular diversity from *Strychnos* genus using molecular networking technique and study the *Strychnos* genus from a chemotaxonomic point of view.

Material and methods: Twenty-eight different species and different plant parts were ground into powder using a grinder. The methanolic extracts were carried out using a pressurized solvent extraction and the alkaloid extract was performed manually with a separating funnel. The extracts were analyzed by HPLC-ESI(+)-Q/TOF. The data were processed using MZmine 2 software and the molecular network was generated on the GNPS platform. The study of the generated molecular network allowed the detection of various alkaloids. Among these is the famous strychnine which has been detected in 7 new *Strychnos* species not yet described as strychnine producers. This identification was investigated using orthogonal approaches, namely TLC, NMR, HPLC-UV and UHPLC-ESI (+)-Q/TOF analyses. The LOD by HPLC-UV of strychnine was also determined.

Results: Further analyses allowed to confirm the presence of strychnine in *S. densiflora* trunk barks but also to show the presence of strychnine with high probability in the trunk barks of *S.*

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camptoneura, *S. congolana*, *S. boonei*, and *S. tchibangensis*, and in the leaves of *S. usambarensis*. About the trunk barks of *S. tricalyisoides*, the probability of a strychnine content remains low.

Conclusion: This work exemplified the efficiency of molecular networking in identifying known metabolites (major and minor alkaloids) involved in the chemotaxonomic study of plants from *Strychnos* genus.

1. Introduction

The pantropical plants of the Strychnos genus from the Loganiaceae family include about 200 species (235 species in World Flora Online) (World Flora Online, 2022) divided into three groups based on geographical areas: one in Central and South America (at least 73 spe¬cies), one in Africa (75 species), and one in Asia and Oceania (about 44 species). These species can be presented in the form of erect or climbing shrubs, lianas, or trees (Bisset, N.G., 1970; Krukoff, B.A., 1972; Bisset, N. G., 1974; Setubal, R.B. et al., 2021).

Plants of the Strychnos genus are well known for their multiple traditional uses (against snakebites, worms, parasites, fevers, ulcers, and many others) and pharmacological properties, particularly the prom¬ising in vitro antiplasmodial and in vivo antimalarial activities that continue to be the subject of research in the fight against malaria and the growing resistance to actual antimalarial treatments of parasites of Plasmodium genus (Frederich, M. et al., 1999; Frederich, M. et al., 2003a, 2003b; Philippe, G. et al., 2005; Philippe, G. et al., 2007; Fentahun, S. et al., 2017). Besides these therapeutic potentials, the plants of Strychnos genus are also known for their toxicity, used during hunts (for example, the roots and leaves of Strychnos usambarensis Gilg), and ordeals (for instance, the roots of Strychnos icaja Baill.). During these ordeals, the person ate or drank a preparation of the plant, the survival of which proved his innocence (Philippe, G. et al., 2004).

Two main types of toxic mechanisms were described for Strychnos plants: tetanizing and curarizing activities. Initially, the hypothesis that the activity depends on the geographical regions was established. Indeed, it was said that tetanizing and curarizing activities were found in Asia and America, respectively. Many years later, this hypothesis was proven wrong, and finally, the activity was closely linked to the alkaloid content. Some tertiary monoterpene indole alkaloids exhibit a tetanizing activity (the ones that present a strychnane-type skeleton, for example), while the bis-quaternary monoterpene indole alkaloids have a strong curarizing activity (Philippe, G. et al., 2004).

Among the tetanizing alkaloids, strychnine (Fig. 1) is the most famous poison. It is a tertiary monoterpene indole alkaloid isolated for the first time in 1818–1819 by Pelletier and Caventou from the seeds of Strychnos nux-vomica L., an Asian species of Strychnos. Its chemical structure was elucidated in 1947 thanks to the works of H. Leuchs and Sir Robert Robinson. Strychnine was then

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unambiguously identified in five other species of Strychnos: S. ignatii P.J. Bergius, S. wallichiana Steud ex. A. DC (Asia), S. lucida R. Br. (Australia), S. icaja Baillon (Africa), and S. panamensis Seem. (Central and South America). Strychnine could be present in seeds of S. tabascana Sprague & Sandwith according to color reactions, but this method of identification is poorly specific, and the investigation should be realized again using modern methods. (Que- tin-Leclercq, J. et al., 1990; Philippe, G. et al., 2004). Strychnine acts by inhibiting the postsynaptic receptor of glycine, an inhibitory neuro¬transmitter necessary for neuronal repolarization (Philippe, G. et al., 2004; Makarovsky, I. et al., 2008). At first, the molecule was used as a stimulant due to its medullary and bulbar stimulating effects. However, because of the significant risks of overdose and death by asphyxiation as a result of the contracture of the diaphragm, strychnine is no longer used today in western medicine, but it is still used sometimes as a doping agent in sports. Strychnine is also used primarily as a pesticide, partic¬ularly to kill rats. Because of the high toxicity of strychnine, its use was prohibited in many countries, such as those of the European Union. At present, it seems that this ban is widespread throughout the world (Patocka, J., 2015; Center For Disease Control and Prevention, 2018).

Despite a large number of studies on the Strychnos genus, it is still interesting today to study their alkaloids, both for their important therapeutic potential described earlier but also for a chemotaxonomic interest. Indeed, an arrangement of classification of plants based on their chemical constituents is called chemotaxonomy or chemical-based tax¬onomy. The classification of Strychnos species, proposed by Dr.

Leeuwenberg in 1969, consists of a subdivision into 12 sections based on botanical aspects (corolla tube/lobe length ratio) (Leeuwenberg, A.J.M., 1969). Interestingly, this classification appears inconsistent with the knowledge about alkaloid content. Nevertheless, in a recent taxonomic revision based on phylogenetic analysis, Setubal et al. (2021) showed most of Leeuwenberg's sections to be non-monophyletic and circum¬scribed most of the strychnine-producing species within one clade (Setubal, R.B. et al., 2021). For the purpose of clarifying these re¬lationships, molecular networking was utilized herein for the stream¬lined exploration of all the alkaloids of those plants. Nevertheless, it is necessary not only to identify chemotaxonomic biomarkers but also to evaluate their relative abundances because these values are important in phylogenic studies.

Chemoinformatics is constantly enhancing the field of metabolomics (Beniddir, M.A. et al., 2021). These last years, molecular networking has begun to be implemented in the field of medicinal plants (Yang, J.Y. et al., 2013; Wang, M. et al., 2016; Nothias, L.F. et al., 2018; Fox Ramos, A.E. et al., 2019a, 2019b). Previous studies directed toward the rein¬vestigation of already investigated Gentianales plants taking advantage of the recently implemented monoterpene indole alkaloids database (MIADB) (Fox Ramos, A.E. et al., 2019a, 2019b) showed that many unique structures remain to be elucidated (Fox Ramos, A.E. et al., 2017; Cauchie, G. et al., 2020; Fox Alcover, C. et al., 2020; Fouotsa, H. et al., 2021; Kouame, T. et al., 2021). Ultimately, this strategy allowed quick discrimination between known and unknown metabolites in order to target the unknown ones. In addition, the molecular network also offered the possibility of detecting known metabolites in species where their presence had never been reported before.

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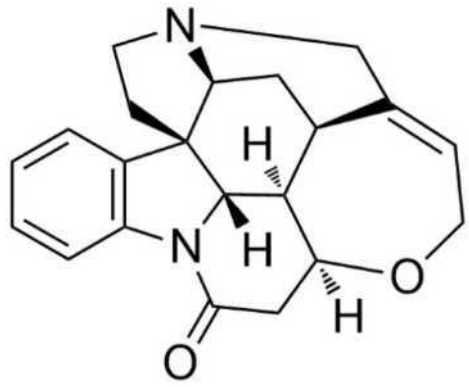
The main objective of this study is, therefore, to explore the Strychnos alkaloids by HPLC-MS/MS and by molecular networking in order to detect new alkaloids with potential use as therapeutic agents, especially against malaria. Moreover, the different identifications provided by the molecular network are also studied and compared with the literature.

Fig. 1. *Structure of strychnine (C21H22O2; Molecular weight: 334.2 g/mol).*



2. Materials and methods

2.1 MATERIALS, CHEMICALS, AND REAGENTS

Twenty-eight different species of *Strychnos* (*S. usambarensis* Gilg ex Engl., *S. variabilis* De Wild., *S. gossweileri* Exell, *S. mellodora* S. Moore, *S. phaeotricha* Gilg, *S. brasiliensis* (Spreng.) Mart., *S. innocua* Delile, *S. henningsii* Gilg, *S. angolensis* Gilg, *S. scheffleri* Gilg, *S. tricalysioides* Hutch. & M.B.Moss, *S. spinosa* Lam., *S. longicaudata* Gilg, *S. malchairi* De Wild., *S. mattogrossensis* S. Moore, *S. icaja* Baill., *S. nux-vomica* L., *S. ignatii* P.J. Bergius, *S. potatorum* L. f., *S. malacoclados* C.H. Wright, *S. camptoneura* Gilg & Busse, *S. congolana* Gilg, *S. boonei* De Wild., *S. staudtii* Gilg, *S. elaeocarpa* Gilg ex Leeuwenberg, *S. densiflora* Baill., *S. tchibangensis* Pellegr. and *S. johnsonii* Hutch. & M.B.Moss.) came from the collections of the Laboratory of Pharmacognosy of the University of Liege (Belgium). These samples were collected within different years in many countries: Rwanda, Congo, Zimbabwe, Tanzania, Cameroon, India, Cambodia, and Brazil. For more details, all the information about the samples used is presented in the Supporting Information (Table S1). After drying at 40 °C, the samples were pulverized in fine powders.

Methanol and dichloromethane came from VWR Chemicals BDH (Leuven, Belgium). Methanol of HPLC grade, trifluoroacetic acid, and ammonia 25% were obtained from Merck (Darmstadt, Germany). The solvents of UHPLC-MS grade (methanol, acetonitrile, and formic acid) were purchased from Sigma-Aldrich (Overijse, Belgium). As for the Milli-Q water, it was supplied by two systems: at the University of Liege, it was a Milli-Q reference A+ system® from Merck (Darmstadt, Germany) and, at Universite Paris-Saclay, it was MILLIPORE Synergy UV® from Merck (Darmstadt,

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Germany). Deuterated methanol and trifluoroacetic acid used for NMR analyses came from Eurisotop (Saint- Aubin, France) and Merck (Darmstadt, Germany), respectively. Maleic acid, used as an internal standard for quantitative analysis of strychnine by NMR, was Sigma-Aldrich's brand (Fluka and Supelco included) (Buchs, Switzerland). Triethylamine and ammonium formate used for the HPLC analyses were purchased from Acros Organics (Geel, Belgium) and Honeywell (Charlotte, North Carolina, USA), respectively. Ethyl acetate, toluene, diethylamine, and 2-propanol, obtained from VWR Chemicals BDH (Leuven, Belgium), and ammonia at 25%, which came from Merck (Darmstadt, Germany), constituted the mobile phase during the TLC analyses. TLC silica gel 60 F_{254} of the Merck brand (Darmstadt, Germany), size 20 × 20 cm, presented a particle size of 5-40 μm. The strychnine reference was obtained from Serva (Heidelberg, Germany).

2.2 SAMPLE PREPARATION

Forty-three samples (as shown in Table S1 in Supporting Information) were first ground using a crusher IKA A10 (Staufen, Germany) to obtain 10 g of powder. Extractions were performed in methanol with the Speed Extractor E-914® (Buchi, Hendrik-Ido-Ambacht, Netherlands). This device allowed the extractions of metabolites from four samples at the same time by pressurized solvent. The cells were filled with the sample and sand. Three cycles of extractions were performed. The pressure was at 100 bars, and the temperature was at 30 °C. Every cycle included 1 min of heat-up time, 15 min of hold time, and 2 min of discharge time. Then, the system was washed with solvent for 2 min and with nitrogen for 3 min. Crude extracts were then evaporated using Rotavapor® and Multivapor® (Buchi) and were dried in the vacuum oven (Heraeus, Hanau, Germany) for one night at room temperature.

In order to compare the alkaloid content of two different types of extracts, one classical alkaloidic extract was prepared. This extract was obtained from the leaves of *S. usambarensis* (November 2007). From 2 g of powder, 20 mL of ammonia solution at 10% w/w was added. A maceration of 30 min was carried out. 100 mL of dichloromethane was added, and the whole was mixed under magnetic stirring for 1 h. The solution was filtered through glass wool and rinsed twice with 50 mL dichloromethane. This organic phase was placed in a separating funnel with 20 mL of sulfuric acid at 2% m/V. After extraction, the aqueous phase was separated, and a second extraction was carried out with another 20 mL sulfuric acid at 2% m/V. The two aqueous phases were put together and brought to a pH of 8-10 with ammonia at 10% w/w. Two other extractions were achieved in a separating funnel with the basified aqueous phase and 50 mL of dichloromethane. The two organic phases were put together. To remove the last traces of water, the organic phase was dried on anhydrous sodium sulfate. After filtration, the solution was evaporated under reduced pressure, and the residue was dried in the vacuum oven for one night at room temperature.

2.3 HPLC-ESI(+)-Q/TOF

The forty-four extracts (43 methanolic extracts and 1 alkaloidic extract) were dissolved and ultrasonicated in methanol of HPLC grade (high-performance liquid chromatography) to obtain a

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concentration of 1 mg/mL. The solutions were then transferred in HPLC vials for injection in the HPLC-MS/MS system.

The Agilent HPLC-MS system (Agilent Technologies, Massy, France), used to analyze the samples, was composed of an Agilent 1260 Infinity HPLC coupled to an Agilent 6530 ESI-Q/TOF-MS (ElectroSpray Ionization Quadrupole Time of Flight Mass Spectrometry) operating in positive mode. The models were: G1367E for HiP sample, G1311B for quaternary pump, G1316A for column compartment, G6530A for TOF/ QTOF mass spectrometer, and G4212B for DAD. The analytical column, a SunFire® C_{18} from Waters (150 × 2.1 mm, 3.5 µm), was used with a flow rate of 250 µL/min. The gradient was linear and variated from 5% B to 100% B in 30 min (A = Water + 0.1% formic acid; B = Methanol). The DAD detector was set at 210, 254, and 280 nm. About the ESI conditions, the settings were the following: a capillary temperature at 320 °C, a source voltage at 3.5 kV, and a sheath gas flow rate of 10 L/min. The injection volume was set at 5 µL. The mass spectrometer was operated in Extended Dynamic Range mode (2 GHz). The divert valve was set to waste for the first 3 min. The scans took place in 6 events: 1 MS scan in positive mode (between *m/z* 100 and 1200), and then MS/MS scans were achieved for the 5 most intense peaks in the MS scan.

The different MS/MS settings are the following: three fixed collision energies (30, 50, and 70 eV), default charge of 1, isolation width of *m/z* 1.3, and minimum intensity of 3000 counts. Purine $C_5H_4N_4$ $[M + H]^+$ ion (*m/z* 121.050873) and hexakis (*1H*, *1H*, *3H*-tetrafluoropropoxy)- phosphazene $C_{18}H_{18}F_{24}N_3O_6P_3$ $[M + H]^+$ ion (*m/z* 922.009798) were used as internal lock masses. To prevent oversampling of the internal calibrant, a permanent MS/MS list exclusion criterion that contains the *m/z* values of the two internal calibrants was set.

2.4 FEATURE-BASED MOLECULAR NETWORKING (NOTHIAS ET AL., 2020)

Before the creation of the molecular network, the files were converted into ".mzXML" (eXtensible Markup Language) by using the MS Convert software edited by ProteoWizard (Chambers, M.C. et al., 2012). The filter used was peak picking, and the algorithm was a vendor. The MS levels 1 and 2 were selected.

Then, the data processing was carried out on the MZmine 2 software (version 2.53) (Pluskal, T. et al., 2010).

- suppression of noise (settings: the intensities were 5.8E3 for MS^1 and 5.0E1 for MS^2);
- creation of peak lists (settings: a minimum of 4 points were necessary to build a peak. The intensity threshold was 5.8E3, and the *m/z* tolerance was *m/z* 0.02 and 10.0 ppm);
- deconvolution (settings: the algorithm was wavelets (ADAP) (Myers, O.D. et al., 2017), the *m/z* center calculation was auto, the retention time and *m/z* ranges for MS^2 scan pairing were 1 min and 0.03 Da, respectively, the S/N threshold was 1, the S/N estimator was intensity window SN, the minimal feature height was 3000, the coef- ficient/area threshold was 2, the peak duration range was 0.02-1.5 min, and the retention time wavelet range was 0.02 and 0.2 min);

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- suppression of isotopes (settings: the *m/z* and retention time tolerances were *m/z* 0.005, 15.0 ppm, and 0.5 min, respectively, the maximum charge was 1, and the representative isotope was the most intense peak);
- to align the data and to have only one data set, an alignment was done (setting: the *m/z* and retention time tolerances were *m/z* 0.02, 15.0 ppm, and 0.8 min, respectively, and the weights for *m/z* and retention time were 100 for both);
- to try to detect a maximum of peaks and so, to propose a maximum of identifications, a gap-filling was carried out (setting: the *m/z* tolerance was *m/z* 0.005 and 15.0 ppm). This processing allows the detection of very minor, indeed even traces of alkaloids;
- the gap-filled peak list was further filtered by deleting specific retention time windows (between 0-2.50 min and 45.59-49.83 min) and by keeping MS/MS data necessary to generate the molecular network.

The processed data were finally exported into the ".mgf" (Mascot Generic Format) and the ".CSV" (Comma-separated values) format to be imported into the GNPS platform (Global Natural Products Social Molecular Networking).

On the GNPS platform, the precursor and fragment ion mass tolerances were 0.02 Da for both. Moreover, the minimal cosine score was 0.65, and the minimal cosine score for matching with the library was 0.7. No filtration was applied, and the analogs were not searched.

2.5 TLC

The extracts of ten *Strychnos* species (Table 1 in the "Results and Discussion" section) and strychnine were deposited on a TLC plate (deposit of 10 μL of solutions at 5 mg/mL for extracts and at 1 mg/mL for strychnine reference). Two mobile phases were prepared: the first one contained toluene-ethyl acetate-diethylamine (7:2:1 V/V/V), and the second one was constituted of ethyl acetate-2-propanol-ammonia 25% (60:25:15 V/V/V). The pulverization solvent was iodoplatinate reagent. In order to prepare this solution, 3 mL of chloroplatinic acid at 100 g/L, 97 mL of water, and 100 mL of potassium iodide at 60 g/L were added. The strychnine spot appeared as a purple color.

2.6 NMR

For the NMR analyses, the method applied was similar to that formerly reported (Frederich, M. et al., 2003a, 2003b). 100 mg of extracts were dissolved in 1 mL of deuterated methanol (CD_3OD, d_4 + 0.03% TMS). Besides these samples, 100 mg of extracts of the ten *Strychnos* species were weighed again in which 500 μg of strychnine was added. The addition of strychnine in the extracts had the objective of visualizing the signals provided by strychnine and comparing them with the samples without strychnine addition. ^1H and HMBC spectra were carried out with 256 and 32 scans, respectively.

2.7 HPLC-UV

The HPLC system was from Agilent (Machelen, Belgium). The models were: G1313A for the samples compartment (ALS), G1311A for the quaternary pump, G1322A for degasser, G1316A for column

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compartment (TCC), and G7117B for DAD. A Luna® PFP from Phenomenex (250 × 4.6 mm, 5 µm) (Utrecht, Netherlands) was used as an analytical column with a flow rate of 1 mL/min. The gradient variated from 5% B to 25.4% B between 2 and 3 min, from 25.4% to 83.2% B between 3 and 37 min, and from 83.2% B to 100% B between 37 and 38 min. After maintaining 100% B for 1 min, the gradient was returned to the initial stage (Solution A was water + 0.05% TFA, and solution B was methanol). The concentrations of injected solutions were at 10 mg/mL and 175 µg/mL for extracts of the ten *Strychnos* species and strychnine reference, respectively. The injected volume was 10 µL, and the DAD detector was set at 254 nm.

2.8 DETERMINATION OF THE LIMIT OF DETECTION (LOD) OF STRYCHNINE BY HPLC- UV

The protocol described thereafter is similar to that reported in the European Pharmacopoeia, version 10 (EDQM, 2019). Strychnine was injected at different concentrations into the same HPLC system and analyzed with the same method described in the previous section. A total of 20 strychnine reference methanolic solutions at different concentrations, obtained by successive two-fold dilutions and ranging from 1 mg/mL to 0.0019 µg/mL, were therefore injected. The injected volume was 10 µL, and the DAD detector was set at 254 nm. The noise level was determined by injecting 10 µL of methanol. Based on the smallest observable peak of strychnine, the signal to noise ratio (S/N ratio) is determined with this formula $\frac{2H}{h}$ where "*H*" is the signal height of strychnine and h is the noise level. The limit of detection (LOD) of strychnine corresponds to the concentration, which presents an S/N ratio value equal to or close to 3.

2.9 QUANTITATIVE ANALYSIS OF STRYCHNINE BY NMR

The method applied was the same as that described in the article of Frederich, M. et al. (2003a, 2003b). 10 mg of methanolic crude extracts of *S. densiflora* trunk barks was weighted. The same amount of methanolic crude extracts of well-known species for their strychnine content was analyzed. The extracts were dissolved into 490 µL of deuterated methanol (CD_3OD, d_4 + 0.03% TMS), to which 10 µL of trifluoroacetic acid was added. The internal standard used was maleic acid. 2.5 mg were dissolved into 5 mL of deuterated methanol (CD_3OD, d_4 + 0.03% TMS), and 500 µL of this solution was added to each crude extract solution. ^1H spectra were carried out with 256 scans. Thanks to the integrations of the maleic acid ethylenic protons and strychnine H-12 proton peaks, the mass concentration of strychnine was determined. Moreover, based on extract yields, the w/w percentages in plant powders were calculated.

2.10 UHPLC-ESI(+)-Q/TOF

UHPLC-ESI(+)-HRMS analyses were achieved by coupling the UHPLC system to a hybrid quadrupole time of flight mass spectrometer Agilent 6546 (Agilent Technologies, Massy, France) equipped with an ESI source, operating in positive ion mode. An analytical column Acq- uity BEH® C_{18} from Waters (100 × 2.1 mm, 1.7 µm) was used. Another method was applied for better visualization of strychnine.

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The gradient was linear and varied from 5% B to 100% B in 12 min at 500 μL/min. After maintaining 100% B at 600 μL/min for 4 min, the gradient returned to the initial stage (A = Water + 0.1% formic acid; B=Acetonitrile). The injection volume of extract of the ten *Strychnos* species and reference solutions was set at 1 μL, and the DAD detector was set at 210, 254, and 280 nm. Source parameters were set as follows: capillary temperature at 320 °C, source voltage at 3500, sheath gas flow rate at 11 L min^{-1}. The divert valve was set to waste for the first 3 min. MS scans were operated in full scan mode from *m/z* 100 to 1200 (0.1 s of scan time) with a mass resolution of 67,000 at *m/z* 922. An MS^1 scan was followed by MS^2 scans of the five most intense ions above an absolute threshold of 3000 counts. Selected parent ions were fragmented at collision energy fixed at 45 eV and an approximate isolation window of 1.3 amu. In the positive ion mode, purine $C_5H_4N_4$ $[M + H]^+$ ion (*m/z* 121.050873) and the hexakis (1*H*,1*H*,3*H*-Tetrafluoropropoxy)-phos- phazene $C_{18}H_{18}F_{24}N_3O_6P_3$ $[M + H]^+$ ion (*m/z* 922.009798) were used as internal lock masses. A permanent MS/MS exclusion list criterion was set to prevent oversampling of the internal calibrant. The results obtained were interpreted with MassHunter (Version B.07.00), a mass spectrometry application software from Agilent.

3. Results and discussion

3.1 EXTRACTIONS YIELDS

The weights and yields of the forty-three methanolic extracts and one alkaloidic extract obtained with the twenty-eight different species are presented in the Supporting Information (Table S2). The yields ranged from 0.55% m/m to 25.92% m/m.

3.2 DEREPLICATION OF STRYCHNOS SPP. METHANOLIC AND ALKALOIDIC EXTRACTS USING FEATURE-BASED MOLECULAR NETWORKING

Based on the data collected during the HPLC-ESI(+)-Q/TOF analyses, the molecular network generated (Fig. 2) contains 8090 nodes, including 217 annotations, inclusive of 63 annotated alkaloids, using, in particular, the MIADB (Monoterpene Indole Alkaloid Database) as part of the GNPS spectral libraries (To access the data, the job link is the following: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=da555 ca03b4048259b2199b4026775de).

Most of the identifications were already reported and described in the literature for *Strychnos* spp. Nonetheless, some annotations were unexpected for some *Strychnos* species. Strychnine $[M+H]^+$ (*m/z* 335.1515, $C_{21}H_{23}O_2$; Retention time: 11.632 min), for instance, was detected in ten *Strychnos* species with a cosine score ranging between 0.71 and 0.89 (Fig. 2 and Table S1).

The three species, *S. icaja*, *S. nux-vomica*, and *S. ignatii*, well-known for their strychnine content, are part of these ten species. Remarkably, strychnine had never been identified for the seven other species, namely in the trunk barks of *S. tricalysioides*, *S. camptoneura*, *S. congolana*, *S. boonei*, *S. densiflora*, *S. tchibangensis* and in the leaves of *S. usambarensis*.

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To confirm the different identifications of strychnine, complementary and orthogonal analyses were performed. The results obtained are described below.

Fig. 2. *Full molecular network from the 44 extracts of Strychnos (43 methanolic extracts and 1 alkaloidic extract) annotated by databases in the GNPS platform. The green nodes correspond to the annotated metabolites. The nodes corresponding to strychnine were highlighted by the blue color. The red color corresponds to unknown metabolites. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)*

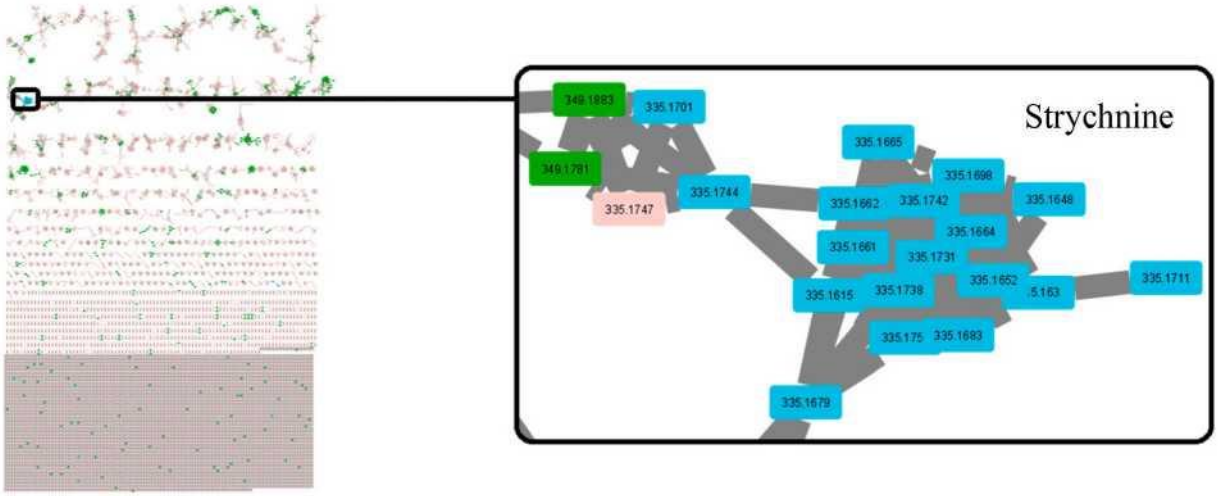
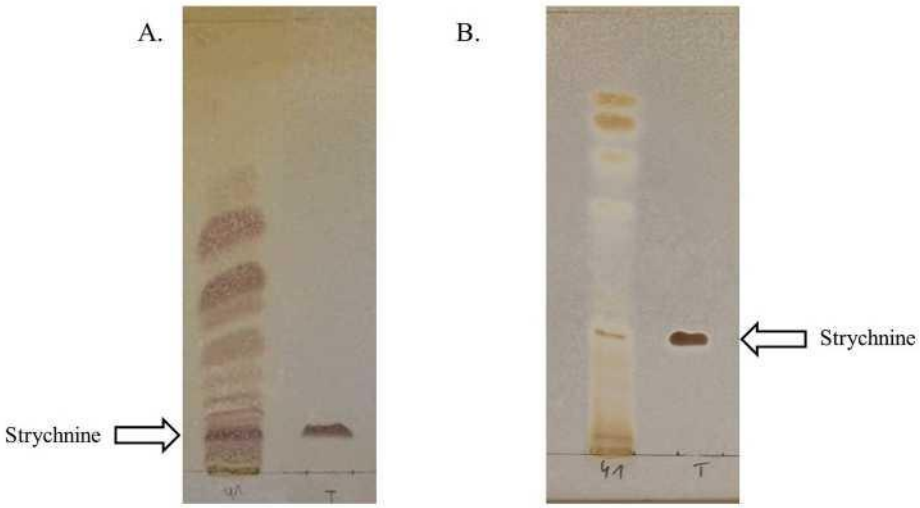


Fig. 3. *Research of strychnine (T) by TLC in the methanolic extract of S. densiflora trunk barks (41). Revelation: iodoplatinate reagent. Observation: under visible light.*
A. Mobile phase: Toluene-ethyl acetate-diethylamine (7:2:1 V/V/V)
B. Mobile phase: Ethyl acetate-2-propanol-ammonia 25% (60:25:15 V/V/V).



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3.3. TLC

Among the species for which the occurrence of strychnine was unknown, the retention factor and the color of spots obtained with iodoplatinate reagent are compatible with strychnine in the trunk barks of *S. densiflora*. The strychnine spot shows a red-brown color (T). The other samples that are not shown in Fig. 3 below do not contain a spot that can be identified with strychnine. This absence of the spot could be related to a very low quantity of strychnine in the studied species. Therefore, it is necessary to confirm the detection of strychnine with a more efficient analysis method such as HPLC or NMR.

Table 1. *Studied Strychnos species that contain strychnine according to our results.*

Trunk barks of *S. tricalysioides* (MeOH extract)	Trunk barks of *S. congolana* (MeOH extract)
Roots and collar barks of *S. icaja* (MeOH extract)	Trunk barks of *S. boonei* (MeOH extract)
Trunk barks, root barks, and seeds of *S. nux- vomica* (MeOH extract)	Trunk barks of *S. densiflora* (MeOH extract)
Seeds of *S. ignatii* (MeOH extract)	Trunk barks of *S. tchibangensis* (MeOH extract)
Trunk barks of *S. camptoneura* (MeOH extract)	Leaves of *S. usambarensis* (Alkaloidic extract)

3.4. NMR

According to the article of Frederich, M. et al. (2003a, 2003b), the H-12 proton of strychnine (Fig. 4) resonates in an uncrowded region of the NMR spectrum as a doublet at $\delta = 8.0$ ppm ($J = 7.896$ Hz) due to the proximity with an aromatic ring and an amide. Once again, this signal was observed only in the methanolic extract of *S. densiflora* trunk barks (Fig. 5).

3.4.1. HPLC-UV

Only the methanolic extract of *S. densiflora* trunk barks showed a peak that has the same retention time and UV spectrum as strychnine (Fig. 6 and Fig. S1 in Supporting Information). Following this result, the same sample was injected in co-elution with the same strychnine reference (175 µg/mL). The increase in peak intensity at 19 min confirms the presence of strychnine, which confirms the results described above.

Given that strychnine was detected with a UV detector in only one sample, whereas it was detected in the molecular network of six other samples, the potential amount of strychnine in the extracts was estimated based on the determination of the LOD (Limit of Detection) using a UV detector. For this purpose, the formula described in the Materials and Methods section was applied. With a strychnine concentration of 0.03 µg/mL in methanol, the value for "*H*" was 0.07233 mAU (signal height), and for "*h*", it was 0.05363 mAU (noise level). "*H*" is the signal height of strychnine, and "*h*" is the noise level.

S/N = 2H/h = 2*0.07233/0.05363 = 0.14276/0.05356 = 2.7

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The LOD of strychnine with our HPLC-UV method was therefore 0.03 µg/mL in the injected extract solutions and corresponding to 0.0003% (w/w) in the plant powders. This result allowed us to conclude that except for the methanolic extract of *S. densiflora* trunk barks that showed a strychnine peak and thus a higher concentration of strychnine, the strychnine content of the six other extracts was lower than 0.03 µg/ mL. This explains why the results were negative for TLC, NMR, and HPLC analyses.

Fig. 4. *Position of the H-12 proton on strychnine.*

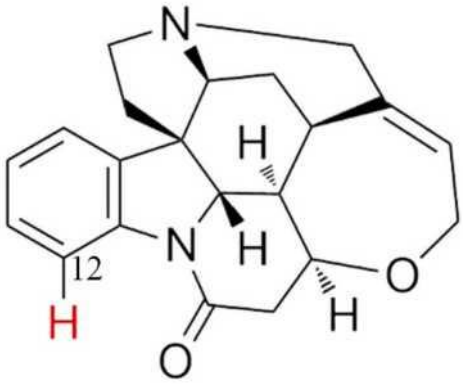
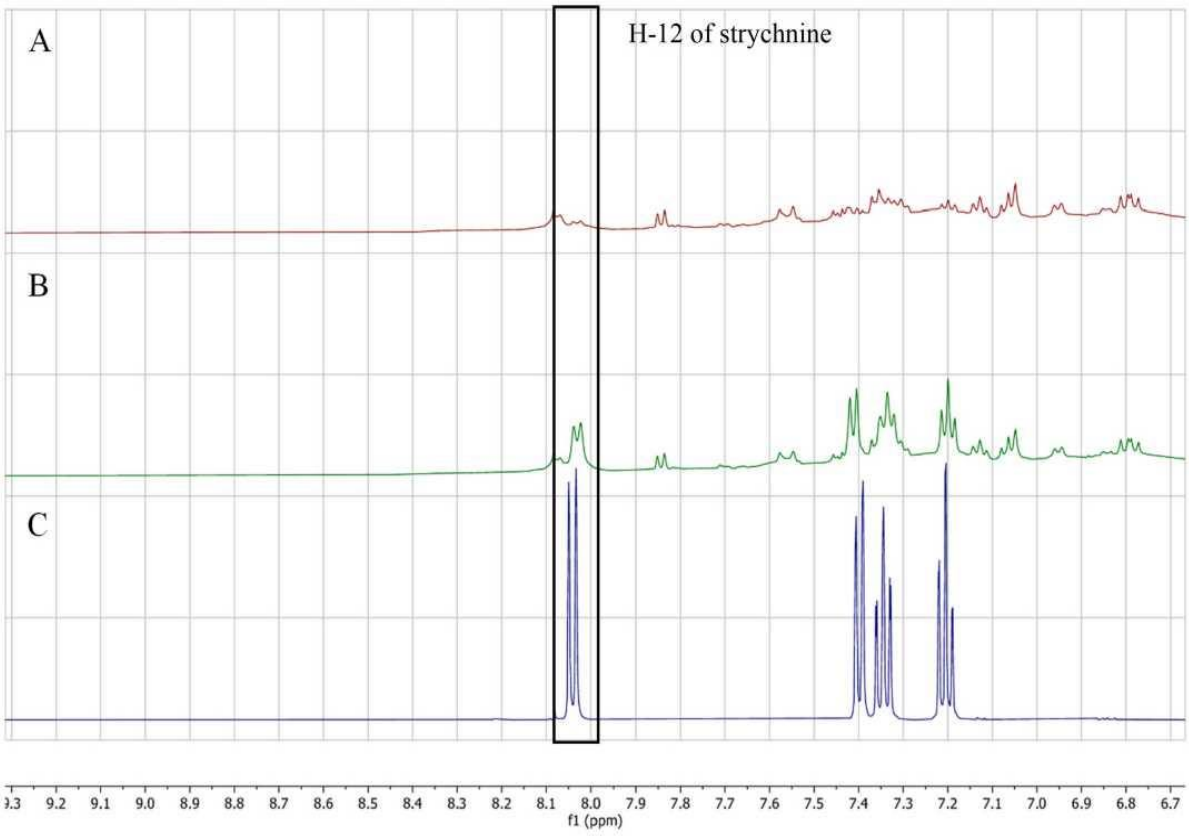


Fig. 5. *Presence of a doublet signal (J = 7.896 Hz) in the region of 8.0 ppm coming from the H-12 proton.*
A. *Proton spectrum of the crude methanolic extract of Strychnos densiflora trunk barks.*
B. *Proton spectrum of the same sample as A with strychnine added.*
C. *Proton spectrum of strychnine reference.*



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3.5. QUANTITATIVE ANALYSIS OF STRYCHNINE BY NMR

During previous research, the amount of strychnine was determined in the well-known poisonous species (*S. nux-vomica*, *S. ignatii* and *S. icaja*) using colorimetric methods, HPLC-UV and NMR analyses (Quetin-Leclercq, J. et al., 1990; Gadi Biala, R. et al., 1996; Frederich, M. et al., 2003a, 2003b). The w/w percentages reported ranged from 0.07 to 2% of strychnine, according to the species. Based on the previously described quantitative NMR method, the aims of these analyses were both to evaluate the strychnine content in the trunk barks of *S. densiflora* in comparison with the content of the well-known poisonous species but also to compare our experimental values of strychnine contents with those reported in the literature (Fig. 7 and Table 2).

The values obtained, presented in Table 2, show that the w/w concentration of strychnine is lower in the trunk barks of *S. densiflora* than in the other species. Until now, strychnine had never been described in *S. densiflora* trunk barks. Previous investigations were conducted on leaves and twigs of this species, but no alkaloid was detected (Bisset, N. G. & Phillipson, J.D., 1971). The different orthogonal analyses finally prove that this species contains alkaloids. Moreover, the estimated strychnine content for *S. densiflora* is consistent with the ethnographic data reported in the literature. In East Cameroun, the root barks of *S. densiflora* ("ilond" in the vernacular) are used as an ordeal poison by Bobilis ethnic group (Bisset, N.G. and Leeuwenberg, A.J.M., 1968). Moreover, this species is also used to brew a toxic mixture for ordeals in Nigeria by the Ibo ethnic group (Neuwinger, H.D., 1994). The need to add other toxic species could be a confirmation that *S. densiflora* has lower toxicity than *S. ignatii*, for example, which is partly related to the difference in strychnine content.

About the strychnine contents for the well-known poisonous species (*S. nux-vomica*, *S. ignatii* and *S. icaja*), the values obtained are slightly lower than those reported for the same species in the literature. For instance, the strychnine content of *S. ignatii* seeds was reported to be 1.24% in the literature (Gadi Biala, R. et al., 1996) compared to our experimental value of 0.79%. This difference could be due to the origin of species (environmental conditions) or the extraction method, as is the case of *S. usambarensis* leaves by comparison of its methanolic crude extract with its alkaloidic extract.

Fig. 6. *A. Chromatogram of methanolic extract of S. densiflora trunk barks (λ = 254.16 nm). B. Chromatogram of strychnine reference (λ = 254.16 nm). C: Comparison of chromatogram A (in blue) with the chromatogram of methanolic extract of S. densiflora trunk barks co-eluted with strychnine reference (in red) (λ = 254.16 nm). . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)*

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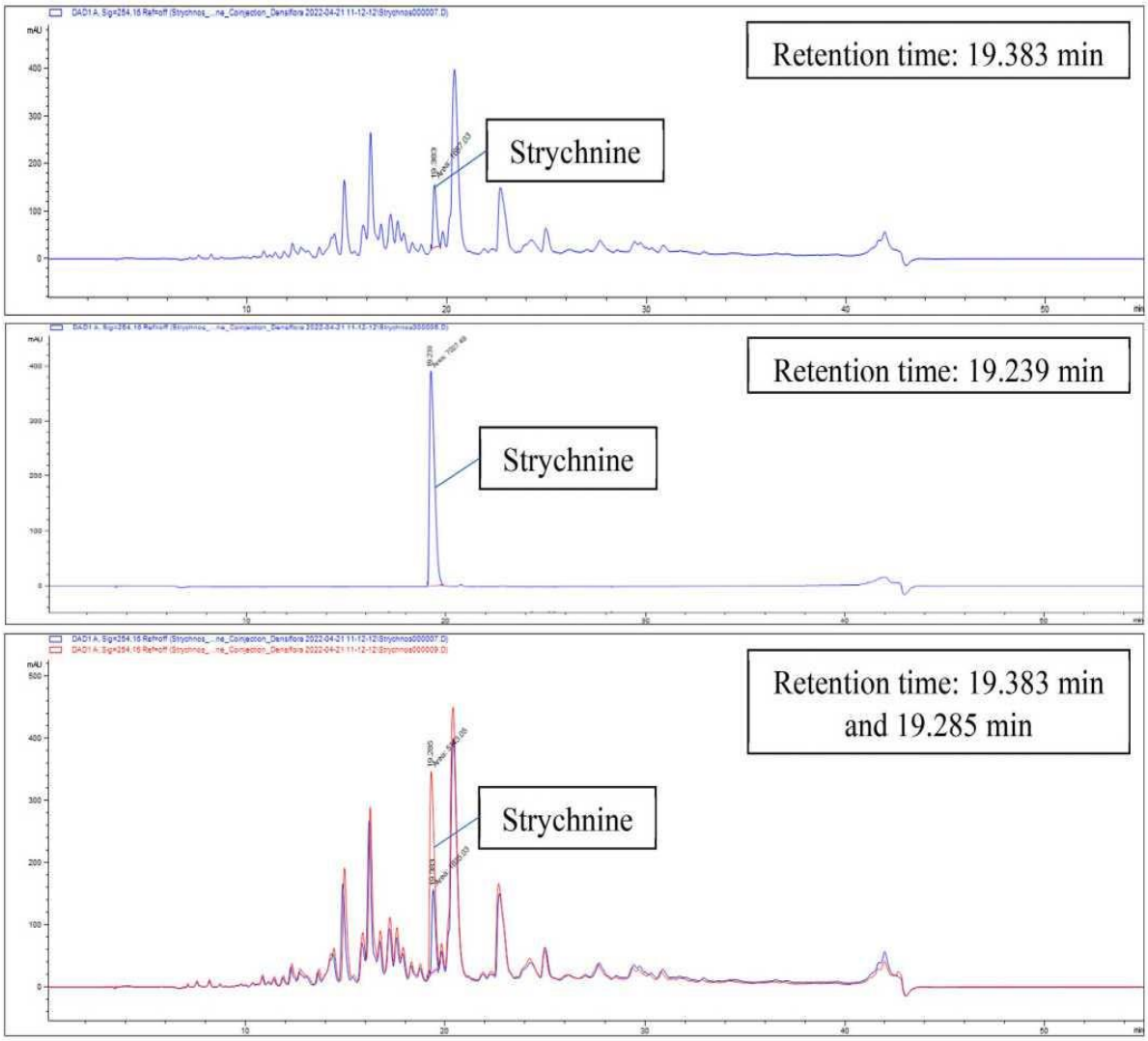

***Table 2**. Strychnine contents in well-known poisonous Strychnos species and in S. densiflora trunk barks using NMR.*

Species	Strychnine contents in plant powders (% w/w)
Roots of *S. icaja* (MeOH extract)	0.41%
Collar barks of *S. icaja* (MeOH extract) Trunk barks of S. *nux-vomica* (MeOH extract)	0.47%
Root barks of S. *nux-vomica* (MeOH extract)	0.40%
Seeds of S. *nux-vomica* (MeOH extract)	0.32%
Seeds of *S. ignatii* (MeOH extract) Trunk barks of *S. densiflora* (MeOH extract)	0.33%
	0.79%
	0.07%

***Fig. 7.** Comparison of doublets signals at 8.02–8.06 ppm generated by H-12 proton of strychnine.*

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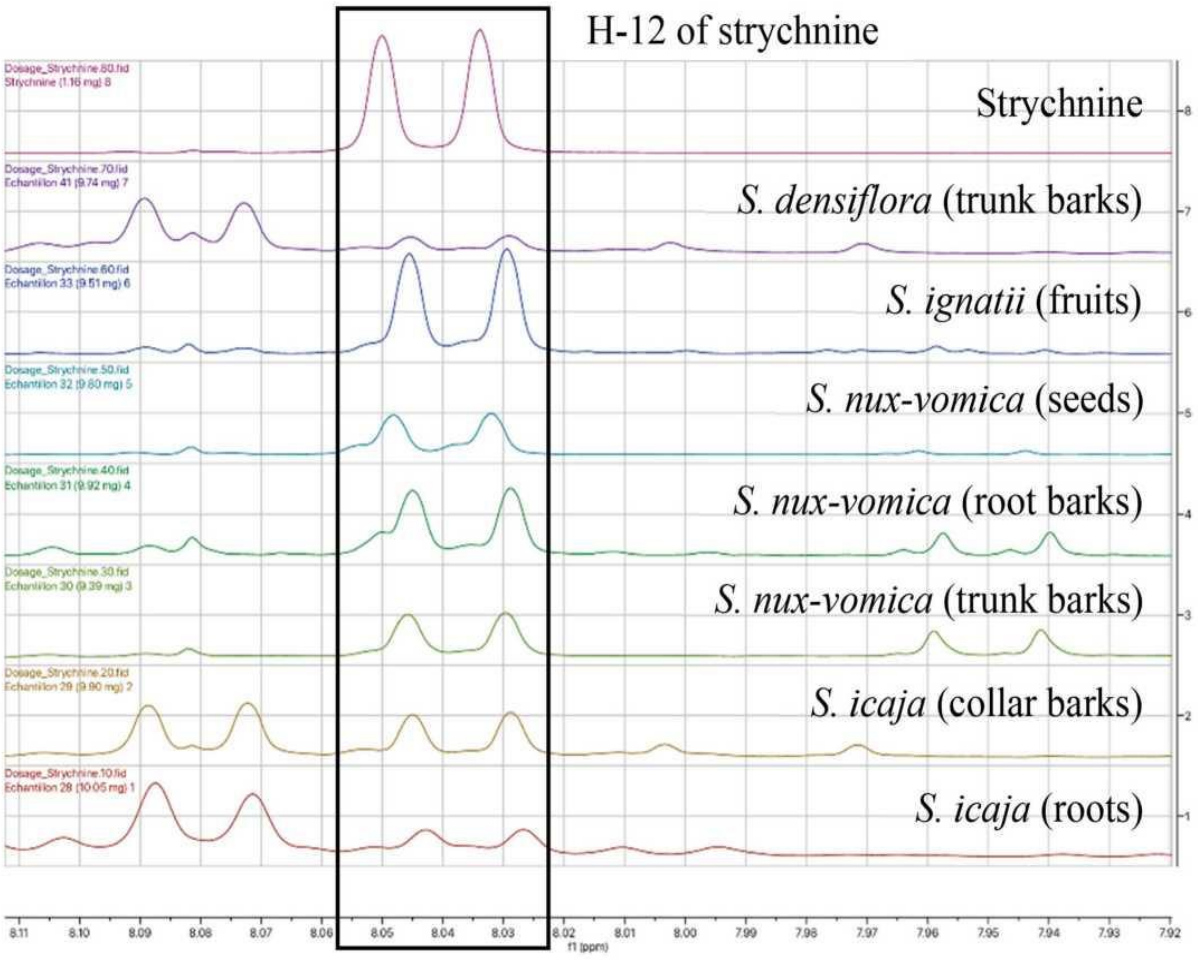

3.6 UHPLC-ESI(+)-Q/TOF

Given that methanolic extracts of *S. tricalysioides*, *S. camptoneura*, *S. congolana*, *S. boonei*, and *S. tchibangensis* trunk barks and alkaloidic extract of *S. usambarensis* leaves could contain very low amounts of strychnine, complementary UHPLC-ESI(+)-Q/TOF analyses were performed with a different column and method, more adequate for strychnine analysis. As shown in Fig. 8, the peak of strychnine $[M+H]^+$ was observed at 2.567 min in all of the analyzed extracts of *Strychnos* species and plant parts.

The MS/MS spectra of extracts and the strychnine reference were compared manually, specifically by targeting the important diagnostic product ions. The important diagnostic product ions consist of the ions at *m/z* 264.1017, 234.0915, 222.0916, 194.0966, 184.0758, 156.0807, 144.0810, and 129.0699. The identifications of the different product ions from strychnine are described in the article by Tian et al. (2013), where each step of strychnine degradation is described (Tian et al., 2013). Comparisons revealed a significant presence of the diagnostic product ions from strychnine in each of the extracts from the studied *Strychnos* species, except for *S. tricalysioides* trunk barks. Indeed, in this extract, the ions of interest are less marked because of the important presence of noise, which can be explained by the significant difference in intensity between the MS/MS spectra from the

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extract and the strychnine reference. The intensity is barely 10^2 in the MS/MS spectrum from methanolic crude extract of *S. tricalysioides* trunk barks, while it is at 10^5 for strychnine reference, which corresponds to a 1000-fold difference of intensity (Fig. S2 in Supporting Information). The diagnostic product ions were also observed in species already known as strychnine producers. The results of mass spectra comparisons are presented in the Supporting Information (Fig. S3-S15).

Table 3, presented below, resumes all the results obtained by molecular networking, TLC, HPLC-DAD, NMR, and UPLC-MS/MS during the study of strychnine. For the trunk barks of *S. tricalysioides*, at the level of UPLC-MS/MS results, the cross is in parentheses because, due to an important presence of noise, it is very difficult to detect strychnine with certainty.

Fig. 8. *Observation of the strychnine peak $[M+H]^+$ (retention time: 2.567 min) in the extracted ion chromatograms (EIC) of all the extracts studied. The extracted mass is m/z 335.1750 (±m/z 0.1000). The last chromatogram corresponds to the strychnine reference.*

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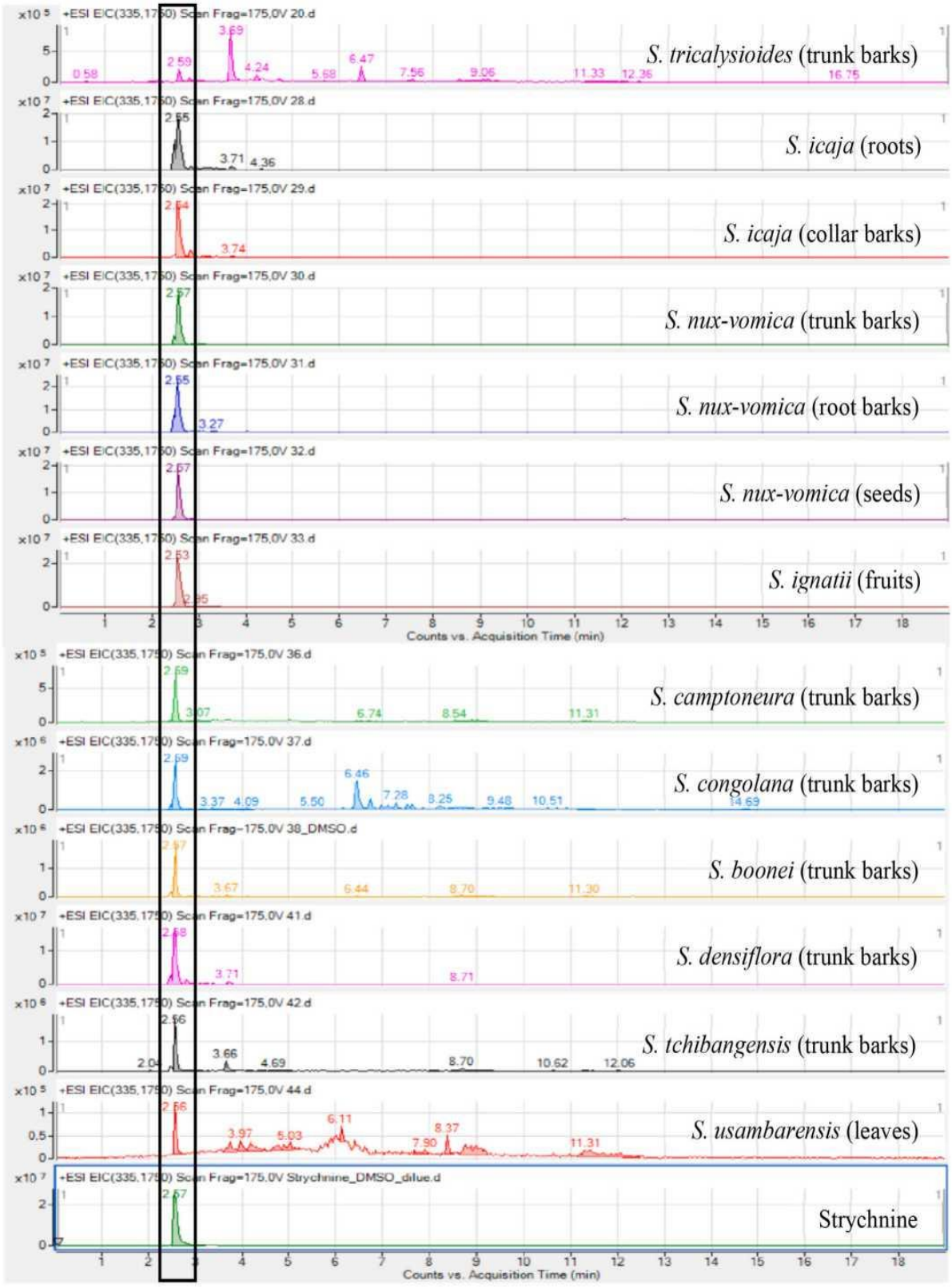

Table 3 *Summary of the results from the strychnine study.*

Strychnos species	Molecular	TLC	HPLC-	NMR	UPLC-MS/MS

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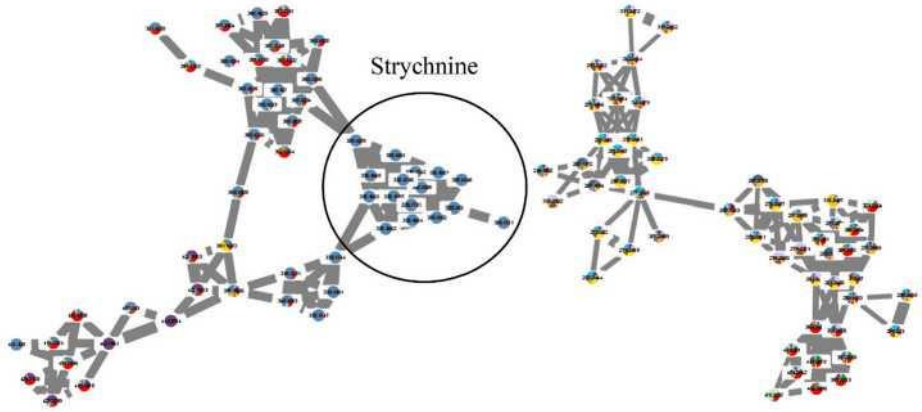
	networking		DAD		
S. tricalysioides (Trunk barks)					
S. icaja (Roots and collar barks)	X				(X)
S. nux-vomica (Trunk barks, root barks	X				X
and seeds)	X				X
S. ignatii (Seeds)	X	X	X	X	X
S. camptoneura (Trunk barks)	X	X	X	X	X
S. congolana (Trunk barks)	X	X	X	X	X
S. boonei (Trunk barks)	X	X	X	X	X
S. densiflora (Trunk barks)	X				X
S. tchibangensis (Trunk barks)	X				X
S. usambarensis (Leaves)	X				X

Fig. 9. *Molecular network highlighting the classification of 28 Strychnos species based on the phylogenetic study. In each node, there is a chart in which each color represents a phylogenetic clade from Setubal et al. (2021) study. The proportionality of colors depends on the quantity of the studied metabolite within the clade. A variety of colors and, therefore, clades can be observed in some clusters (groups of metabolites that have common points). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)*



Strychnine could also be present in trace amounts in other species of *Strychnos*. However, the targeted peaks being lower, the selection of the five most intense metabolites for MS/MS fragmentations did not take these peaks into account. Another MS/MS method should be performed to confirm this hypothesis.

From a taxonomic point of view, the discovery of strychnine in different species, which are not reported as strychnine-containing species in the literature, should be taken into consideration to clarify the taxonomy of the *Strychnos* genus. Indeed, in the current classification proposed by Dr. Leeuwenberg, strychnine-containing species are classified in different sections. However, in the recent phylogenetic study by Setubal et al. (2021), these same species are grouped in the same clade.

The molecular networking technique, which allows a chemotaxo- nomic approach based on alkaloids from *Strychnos* species, does not fully correspond to the classifications proposed by Leeuwenberg and Setubal. For example, the molecular network of *Strychnos* alkaloids with annotations based on the clades of the Setubal study contains some clusters with a mixture of different

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sections which, in the current classifications, are not related to each other (Fig. 9). In order to implement a well- established classification, it would be necessary to perform further studies.

4. Conclusions

The development of metabolomic techniques, particularly molecular networking, in the field of natural products has paved the way for new discoveries, which were not possible with the conventional methods developed in the past years, especially when the quantities of metabolites in the plants were too low. The molecular networking technique makes it possible to obtain a global visualization of *Strychnos* alkaloids and to easily target the interesting species and their known and unknown metabolites, as it was illustrated in this study. Indeed, all the different results allowed us to identify strychnine at a concentration observable by different orthogonal techniques (TLC, HPLC, NMR, and MS) in *S. densiflora* trunk barks and to detect it at lower concentrations only by mass spectrometry in five further *Strychnos* species, namely the trunk barks of *S. camptoneura*, *S. congolana*, *S. boonei*, and *S. tchibangensis*, and the leaves of *S. usambarensis*. For these last 5 species, the observation of a peak with similar MS/MS spectra, retention times, and parent ions to the peak of reference leads to conclude the presence of strychnine with high confidence (level 1 according to the MSI (Metab- olomics Standard Initiative)). This result led to the hypothesis that strychnine could be present in trace amounts in other *Strychnos* species. In the future, to confirm this hypothesis, another method of analysis with UHPLC-ESI(+)-Q/TOF will have to be applied to fragment low- intensity ions.

About the trunk barks of *S. tricalysioides*, the potential very low amount of strychnine, as well as the important presence of noise in the MS/MS spectrum, makes the interpretation difficult. As there is a small peak at the same retention time and with the same *m/z* ratio as strychnine, it is possible that this peak corresponds to strychnine. Therefore, an in-depth study of trunk barks of *S. tricalysioides* should be performed in the future to clarify this hypothesis.

The study conducted in this article is also a contribution to the chemotaxonomic understanding of the species belonging to the *Strych- nos* genus. The discovery of strychnine in species not yet described as producing strychnine must be considered in the development of a future well-established classification of the *Strychnos* genus. Currently, in the study by Setubal et al. (2021), the various strychnine-producing species are present in clade 4. Except for *S. tricalysioides*, where the presence of strychnine remains highly speculative, *S. densiflora* should be added in clade 4. It could also be considered to add the 5 other species in this clade, namely *S. camptoneura*, *S. congolana*, *S. boonei*, *S. tchibangensis*, and *S. usambarensis*.

Molecular networking is an innovative strategy and offers new prospects, especially in the discovery of new natural substances and in the field of chemotaxonomy. The study conducted in this article allowed us to detect for the first time with certainty the presence of strychnine in the trunk barks of *Strychnos densiflora*, a species that is also cited in the ethnopharmacological literature as being used

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as a poison. About the other *Strychnos* species, we were also able to detect strychnine in smaller quantities, which explains why these plants have no use as a poison.

Credit author statement

Olivier Bonnet: Conceptualization, Methodology, Investigation, Data Curation, Writing-Original Draft, Visualization. **Mehdi A. Benid- dir**: Conceptualization, Methodology, Writing-Review and editing. **Pierre Champy**: Conceptualization, Methodology, Writing-Review and editing. **Vedaste Kagisha**: Conceptualization, Investigation, WritingReview and editing. **Alain Nyirimigabo**: Conceptualization, Investigation, Writing-Review and editing. **Carla Hamann**: Conceptualization, Investigation, Writing-Review and editing. **Giorgi Jgerenaia**: Conceptualization, Investigation, Writing-Review and editing. **Allison Ledoux**: Conceptualization, Writing-Review and editing. **Alembert Tiabou Tchinda**: Resources, Writing-Review and editing. **Luc Angenot**: Conceptualization, Resources, Writing-Review and editing. **Michel Frederich**: Conceptualization, Methodology, Writing-Review and editing, Supervision.

Ethical statement

The authors declare that this research article does not involve the use of any human or animal.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.toxicon.2022.06.002.

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