Exploring the chemodiversity from the *Strychnos* genus using molecular networking to unveil and identify novel antiplasmodial compounds

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Supervisor: Pr. Michel Frédérich

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To my family, my partner, and my friends,

who have always believed in me, supported me, and encouraged me

throughout the realization of this fascinating research project.

"*Glory is not in never falling, but in getting up every time you fall*."

Confucius

"*Success is the ability to go from failure to failure without losing enthusiasm*."

Winston Churchill

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pleasure, even though this isn't their area of expertise. I couldn’t have done it without you. Thank you for everything, Mom and Dad!

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Abstract

This thesis builds upon previous research into the antiplasmodial properties of plants from the *Strychnos* genus (Loganiaceae family), focusing on several species that have shown promising potential, including *Strychnos icaja* Baill., *Strychnos usambarensis* Gilg ex Engl., and *Strychnos variabilis* De Wild. The initial phase of this study involved an *in vitro* screening of 43 methanolic crude extracts from 28 *Strychnos* species for their antiplasmodial activity against the 3D7 strain of *Plasmodium falciparum*. This screening was complemented by an exploration of the metabolites from these extracts, as well as an alkaloidic extract, using molecular networking, a chemometric method that maps metabolites and visualizes their relationships based on MS/MS spectra. The application of spectral databases allowed for the rapid differentiation of known and unknown compounds, thereby facilitating the bio- and mass-guided fractionation process. This study initially highlighted the importance of further exploring the *Strychnos* genus, revealing clusters of previously unidentified metabolites that may exhibit antiplasmodial properties. Consequently, several species with promising ($IC_{50} \leq 5$ µg/mL) or good (IC_{50} between 5 and 15 µg/mL) antiplasmodial activity were selected, notably the leaves of *S. usambarensis* Gilg ex Engl. and *S. phaeotricha* Gilg, as well as the trunk barks of *S. longicaudata* Gilg.

The second phase of the study focused on validating one of the database identifications, strychnine, which was detected in seven species not previously recognized as producers of this compound. Various analyses confirmed its presence, providing proof of concept for the molecular networking methodology.

The final part of the study centered on isolating and identifying novel antiplasmodial metabolites through bio- and mass-guided fractionation. Analyses of *S. usambarensis* leaves revealed unknown compounds with masses above *m/z* 900. One metabolite, with a mass of *m/z* 944, was isolated. It could be a dimeric or trimeric alkaloid containing a glycosidic moiety. However, its antiplasmodial activity could not be evaluated due to its limited quantity and

structural fragility. In the case of *S. longicaudata* trunk barks, fractionation led to the isolation of alstonine and seven unknown metabolites, five of which demonstrated promising antiplasmodial activities and three of which were subjected to structural elucidation. Lastly, a preliminary study on *S. phaeotricha* leaves revealed good antiplasmodial activity despite the low quantity of alkaloids it contains. The results suggest that the activity may be attributed to compounds from either the alkaloid or terpene classes. Bio- and mass-guided fractionation has been initiated, and further enrichment of the fractions is necessary to progress this research.

Résumé

Cette thèse s'inscrit dans la continuité des recherches sur les activités antiplasmodiales des plantes du genre *Strychnos* (famille des Loganiaceae), dont certaines espèces ont montré un potentiel prometteur comme *Strychnos icaja* Baill., *Strychnos usambarensis* Gilg ex Engl. et *Strychnos variabilis* De Wild. La première partie du travail a impliqué un screening *in vitro* des activités antiplasmodiales de 43 extraits bruts méthanoliques provenant de 28 espèces sur la souche 3D7 de *Plasmodium falciparum*. Ce screening a été complété par l'exploration des métabolites de ces extraits ainsi que d'un extrait alcaloïdique au moyen du réseautage moléculaire, une méthode chimiométrique qui permet de cartographier les métabolites et de visualiser leurs ressemblances au moyen de spectres MS/MS. L'utilisation de bases de données spectrales a permis de distinguer rapidement les composés connus des inconnus, facilitant ainsi des fractionnements bio- et mass-guidés. Cette étude a montré dans un premier temps l'intérêt d'explorer davantage le genre *Strychnos*, mettant en évidence des clusters de métabolites non identifiés qui pourraient avoir des propriétés antiplasmodiales. Dans un second temps, l'ensemble des résultats ont amené à la sélection de plusieurs espèces avec une activité antiplasmodiale prometteuse (CI_{50} ≤ 5 µg/mL) ou bonne (CI_{50} entre 5 et 15 µg/mL), notamment les feuilles de *S. usambarensis* Gilg ex Engl. et de *S. phaeotricha* Gilg ainsi que les écorces de troncs de *S. longicaudata* Gilg.

La deuxième partie de l'étude s'est concentrée sur l'examen d'une des identifications fournies par les bases de données, la strychnine, découverte dans sept espèces qui n'avaient jamais été décrites comme productrices de cette substance. Les différentes analyses ont confirmé sa présence, constituant ainsi une preuve de concept de la méthodologie du réseautage moléculaire.

Enfin, la troisième partie a abordé l'isolement et l'identification de nouveaux métabolites antiplasmodiaux par fractionnement guidé par l'activité biologique et la masse. Des analyses des feuilles de *S. usambarensis* ont révélé des composés inconnus avec des masses supérieures à *m/z* 900. Un métabolite de masse *m/z* 944, potentiellement un alcaloïde dimérique ou trimérique avec

une partie glycosidique, a été isolé. Malheureusement, en raison de la faible quantité isolée et de la fragilité de la structure, l'activité antiplasmodiale n'a pas pu être évaluée. Concernant les écorces de troncs de *S. longicaudata*, le fractionnement a permis d'isoler sept métabolites inconnus montrant des activités antiplasmodiales prometteuses, avec un essai d'élucidation structurale pour trois d'entre eux. Pour terminer, une étude préliminaire sur les feuilles de *S. phaeotricha* a révélé de bonnes activités antiplasmodiales, malgré une faible quantité d'alcaloïdes. Les résultats suggèrent que l'activité pourrait être attribuée à la classe phytochimique des alcaloïdes ou des terpènes. Un fractionnement guidé par l'activité biologique et la masse a été amorcé, nécessitant un enrichissement des fractions pour approfondir cette recherche.

Abbreviations

ACT	Artemisinin Combination Therapy
ADAP	Automated Data Analysis Pipeline
BB	Branch bark
B.C.E.	Before the Common Era
BEH	Bridged Ethyl Hybrid
°C	Degree Celsius
^{13}C APT	^{13}C Attached Proton Test
Chloroform-d	Deuterated Chloroform
COSY	Correlation Spectroscopy
CQR	Chloroquine-Resistant
CQS	Chloroquine-Sensitive
.CSV	Comma-Separated Values (file format)
Da	Dalton (atomic mass unit)
DAD	Diode Array Detector
DB	Database
DEPT-90	Distortionless Enhancement by Polarization Transfer 90 (for NMR)
DEPT-135	Distortionless Enhancement by Polarization Transfer 135 (for NMR)
DNA	Deoxyribonucleic Acid
DPBAE	Diphenylborate of aminoethanol
EDTA	Ethylenediaminetetraacetic Acid
e.g.	*exempli gratia*
EIC	Extracted Ion Chromatogram
ESI(+)	Electrospray (in positive mode)
eV	Electronvolt
F_{254}	Fluorescence 254 nm (TLC plate)
FBMN	Feature-Based Molecular Networking
FTN	Flow-Through Needle
Fr	Fruit
g	Gram
GHz	Gigahertz

GNPS	Global Natural Products Social Molecular Networking
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (buffer)
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High-Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
i.e.	*id est*
kV	Kilovolt
L	Leave
L	Liter
Methanol-d4	Deuterated Methanol
mg	Milligram
.mgf	Mascot Generic Format (file format for mass spectrometry data)
µm	Micrometer
µM	Micromolar
mL	Milliliter
mm	Millimeter
MoNA	MassBank of North America
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
m/z	mass-to-charge ratio
.mzXML	Mass Spectrometry Markup Language XML (file format)
NMR	Nuclear Magnetic Resonance
nm	Nanometer
PDA	Photodiode Array Detector
PEG	Polyethylene Glycol
PFP	Pentafluorophenyl
pH	Potential of Hydrogen (measure of acidity/alkalinity)
ppm	Parts Per Million
Q	Quadrupole
QDa	Quadrupole Detector Array
RB	Root bark
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute (cell culture medium)

RT	Root
S	Seed
S.	*Strychnos*
SB	Stem bark
SIR	Selected Ion Recording
S/N	Signal-to-Noise Ratio
SPE	Solid Phase Extraction
TB	Trunk bark
TIC	Total Ion Current
TLC	Thin-Layer Chromatography
TMS	Tetramethylsilane
TOCSY	Total Correlation Spectroscopy
TOF	Time-Of-Flight
TRIS	Tris(hydroxymethyl)aminomethane (buffer)
UHPLC	Ultra-High-Performance Liquid Chromatography
UV	Ultraviolet
US	Unspecified
V	Volt
% V/V	Volume/Volume percent
% w/w	Weight/Weight percent
% w/V	Weight/Volume percent

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CHAPTER 1

General introduction

1.1. Nature, a rich source of therapeutic compounds

For thousands of years, people have known that plants contain bioactive compounds. For instance, in the 5th century B.C.E., the Greek philosopher Socrates took his own life by consuming poison derived from hemlock. Medicinal plants have been used for millennia. Mesopotamian clay tablets, written around 2600 B.C.E. describe the use of medicinal plants (Newman, D. J. *et al.*, 2003). The Egyptologist Georges Ebers discovered the first known compendium on medicinal plants, which dates back to around 1500 B.C.E. (Aboelsoud, N. H., 2010). Some of the plants described in these ancient texts are still in use today to treat a variety of ailments, from coughs and colds to parasitic infections and inflammation. Over time, pharmacopoeias were written and published, with the first national pharmacopoeia, the *Pharmacopoeia Londinensis*, appearing in 1618 (Gaddum, J. H., 1946; Newman, D. J. *et al.*, 2003).

The use of pure natural compounds as medicines began with the isolation of various metabolites, such as quinine, strychnine, atropine, colchicine, and morphine, in the 1800s. From 1981 to 2019, 42% of marketed drugs (787 out of 1881) contained one or more active ingredient(s) of natural or semisynthetic origin, or were inspired by natural molecules (Newman, D. J., and Cragg, G. M., 2020; Beniddir, M. A., and Poupon, E., 2023). Between 1981 and 2020, approximately 32% (441 out of 1394) of small molecules marketed were of natural origin (Newman, D. J., and Cragg, G. M., 2020).

In the field of anticancer drugs, from 1981 to 2019, 62 out of 185 small molecules marketed (33.5%) were natural products or derived from natural sources. Natural molecules and derivatives are also employed in other therapeutic areas. For instance, 32% (129 out of 401) of antimicrobial, antifungal, antiparasitic, and antiviral treatments are either natural or derived from natural products. Specifically, 57% (93 out of 162) are antimicrobial, 12% (4 out of 34) are antifungal, 45% (9 out of 20) are antiparasitic, and 12% (23 out of 185) are antiviral. These findings highlight that natural products are particularly prevalent in antibacterial and antiparasitic classes (Newman, D. J., and Cragg, G. M., 2020).

Compared to synthetic molecules, studying natural products from plants, fungi, and marine organisms is complex and time-consuming. Isolating and

purifying a sufficient quantity of a natural compound through bioguided fractionation can take from several days to several months. In contrast, dozens or even hundreds of synthetic molecules can be rapidly synthesized using combinatorial chemistry. However, exploring the metabolite content of natural sources facilitates the discovery of new biologically active pharmacophores and original modes of action, leading to highly selective and specific biological activities (Newman, D. J. *et al.*, 2003). To further expand the search for new natural treatments, molecules produced by bacteria or resulting from interactions between bacteria and their hosts are increasingly being studied, which has led to the development of new therapies (Newman, D. J., and Cragg, G. M., 2016).

These data clearly demonstrate the significance of natural products and their derivatives in the medical field. Today, it is crucial to discover new active metabolites, especially in light of the rising resistance to current treatments. This is particularly true for malaria parasites and bacteria, which have developed resistance to antimalarial drugs and antibiotics, respectively (World Health Organization, 2023a; World Health Organization, 2023b). Section 1.3.3. (pages 20-22) discusses in detail the resistance of *Plasmodium* genus parasites to antimalarial treatments. Antibiotic resistance, however, will not be addressed in this thesis.

One phytochemical class that stands out for its structural diversity and promising biological activities against malaria is alkaloids. This class is particularly abundant in plants such as *Cinchona* and those from the *Strychnos* genus (Philippe, G. *et al.*, 2004). The *Strychnos* genus is rich in monoterpene indole alkaloids, a subclass of alkaloids comprising both an indole and a monoterpene part, some of which have shown promising antiplasmodial and antimalarial activities in previous research (Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2002).

1.2. The *Strychnos* genus: A promising source for novel antimalarial treatments

1.2.1. Origins

The *Strychnos* genus was first described in 1753 by Carl Linnaeus (or Carl von Linné) during his study of *Strychnos nux-vomica* L., an Asian species that is a significant source of strychnine. This substance is a well-known poison, historically used as a rat killer due to its tetanizing effects. Additionally, in Europe, strychnine gained some popularity in medicine as a stimulant and tonic in small and controlled doses until the early 20th century. Strychnine is a tertiary monoterpene indole alkaloid, first isolated from its seeds by Pelletier and Caventou in 1818–1819. Its structure was not fully elucidated and confirmed until many years later, thanks to the work of H. Leuchs and Sir Robert Robinson between 1910 and 1947. The total synthesis of strychnine was achieved by Woodward in 1954, and its absolute configuration was determined by X-ray crystallography of its hydrobromide form in 1956 (Woodward, R. *et al.*, 1954; Peerdeman, A. F., 1956; Slater, L. B., 2001; Philippe, G. *et al.*, 2004; Seeman, J. I., and Tantillo, D. J., 2020; Setubal, R. B. *et al.*, 2021). The presence of this potent toxin within the genus led to the term "solanaceous poisonous plants". The name *Strychnos* itself is derived from this term, which has Greek origins (Setubal, R. B. *et al.*, 2021). Historically, these plants were categorized as part of the Solanaceae family, a group of toxic plants known for producing various effects, including narcotic ones.

Today, species of the *Strychnos* genus are no longer associated with the Solanaceae family. They currently belong to the Loganiaceae family, with approximately 200 species (235 species according to World Flora Online (World Flora Online, 2024)). These species are pantropical, meaning they grow in tropical regions of both hemispheres. They can be found at altitudes ranging from sea level to 2,000 meters, thriving in both dry and humid forests (whether flooded or not), as well as in savannahs and grasslands (Setubal, R. B. *et al.*, 2021).

The species are divided into three main groups based on their geographical distribution (Leeuwenberg, A. J. M., 1969; Philippe, G. *et al.*, 2004):

- America, from Southern Mexico to Argentina (85 species);
- Tropical Africa and Madagascar (75 species);
- South and Southeast Asia (46 species), as well as Northern and Northeastern Australia (4 species).

Chemically, alkaloids are the predominant chemical compounds in the *Strychnos* genus. The term "alkaloid," introduced by Meisner in the early 19th century, refers to a group of basic and heterocyclic natural compounds that contain at least one nitrogen atom (Friedrich, F., and von Domarus, C., 1998).

In this section, the genus will be explored in detail, with a focus on its botanical characteristics, taxonomy, toxicity, and alkaloid content. In the next chapter, the traditional uses and pharmacological properties will be investigated.

1.2.2. Botanical aspects

Strychnos plants can be lianas, shrubs, or small trees. The lianas and climbing shrubs can reach heights ranging from 1.5 to 45 m, lengths from 4 to 120 m, and diameters up to 50 cm. Shrubs and subshrubs typically grow to heights of 0.3 to 2 m. Trees, on the other hand, can reach heights from 2 to 40 m, with a maximum diameter of 100 cm (Setubal, R. B. *et al.*, 2021).

The leaves of *Strychnos* plants are simple, entire, and opposite. The leaf venation is acrodromous, meaning that the secondary veins curve in an arc and converge at the apex of the leaf blade. Additionally, the petiole bases are thick and woody, providing support for axillary branches by forming a nodal leaf platform. The wood is further supported by an interxylary phloem. Some leaves are replaced by either herbaceous or lignified tendrils, which can appear solitary, in pairs, or in a series of pairs along the stems. Interpetiolar stipules typically form a straight and ciliated line connecting the bases of the petioles. The flowers are small and light-colored, with petals that are fused along the edges to form a tubular shape (sympetalous). Finally, the ovaries contain two independent cells. The berries range from small to large (1 to 10 cm, and

occasionally up to 20 cm). The seeds are large (0.5–5 cm in length), exhibiting variable shapes, generally disc-shaped to subglobose, slightly flattened or saucer-shaped, and outlined as circular to elliptic. Their seed coat, which can be silky, felty, or scabrous, encloses a spatulate embryo, a horny endosperm, and leaf-like cotyledons (Setubal, R. B. *et al.*, 2021; World Flora Online, 2024).

The presence of lateral veins on the leaves makes *Strychnos* plants easily identifiable. Regardless of the origin of the species, all have these characteristic veins (Leeuwenberg, A. J. M., 1969). The photo below (Figure 1) shows the leaves of *Strychnos usambarensis* Gilg ex Engl., highlighting its lateral veins.

Figure 1: Leaves of *Strychnos usambarensis* and its lateral veins.

1.2.3. Taxonomy

The Loganiaceae is a large family, comprising 460 pantropical species across 16 genera. These species are grouped into four tribes: Antonieae Endl. (14 species, 4 genera), Loganieae Endl. (approximately 150 species, 8 genera), Spigelieae Dumort. (60 to 85 species, 1 genus), and Strychneae Dumort. (about 220 species, 3 genera). The *Strychnos* genus belongs to the Strychneae tribe and is the largest genus in the family, with approximately 200 species.

Over the years, numerous classifications of *Strychnos* species have been proposed (Figure 2) (Setubal, R. B. *et al.*, 2021):

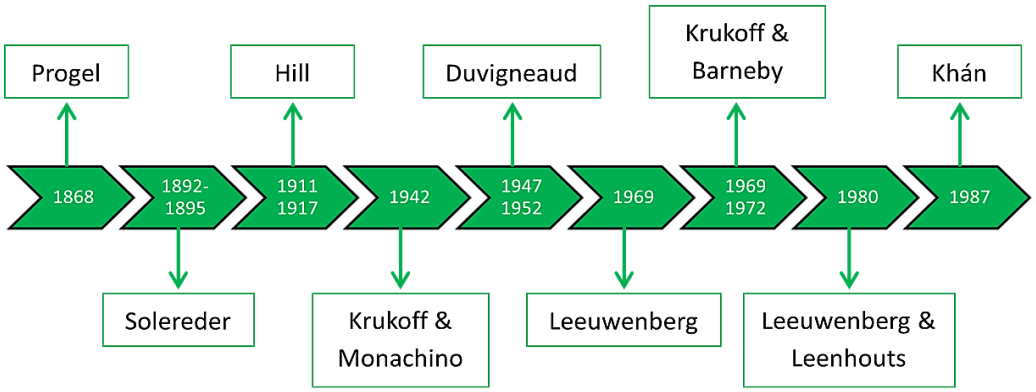


Figure 2: The timeline of *Strychnos* classifications.

In **1868**, Progel proposed a classification of American species based on the ratio of corolla tube length to corolla lobe length.

Between 1892 and 1895, Solereder expanded Progel's system by adding 65 species from various continents.

In **1911**, Hill classified Asian species into two groups based on the ratio established by Progel and Solereder, later modifying this classification in **1917** by introducing four sections.

In **1942**, Krukoff and Monachino adopted the Progel-Solereder system, making a few modifications by placing American species into different sections.

In **1947**, Duvigneaud created the *Malacocladae* section for eight African species with short corolla tubes. In **1952**, he made major revisions to the *Strychnos* classification, introducing 16 new sections and bringing the total to 21. He also criticized the use of the corolla tube-to-lobe length ratio, proposing instead a classification based on tendril and spine morphology, as well as flower and fruit structure.

In **1969**, Leeuwenberg introduced a new classification system with 12 sections, still based on the corolla tube-to-lobe length ratio (Figure 3).

In the same year and in 1972, Krukoff and Barneby added two subsections to Leeuwenberg's classification of the *Breviflorae* section.

In 1980, Leeuwenberg and Leenhouts revised the classification, omitting the two subsections suggested by Krukoff and Barneby.

The most recent modifications were made by Khán in **1987**, when he reorganized 19 species within the existing sections, as well as in the new *Angustiflorae* section, and two subsections derived from *Strychnos*: *Strychnos subsect. Nitidae* and *Strychnos subsect. Wallichianae*.

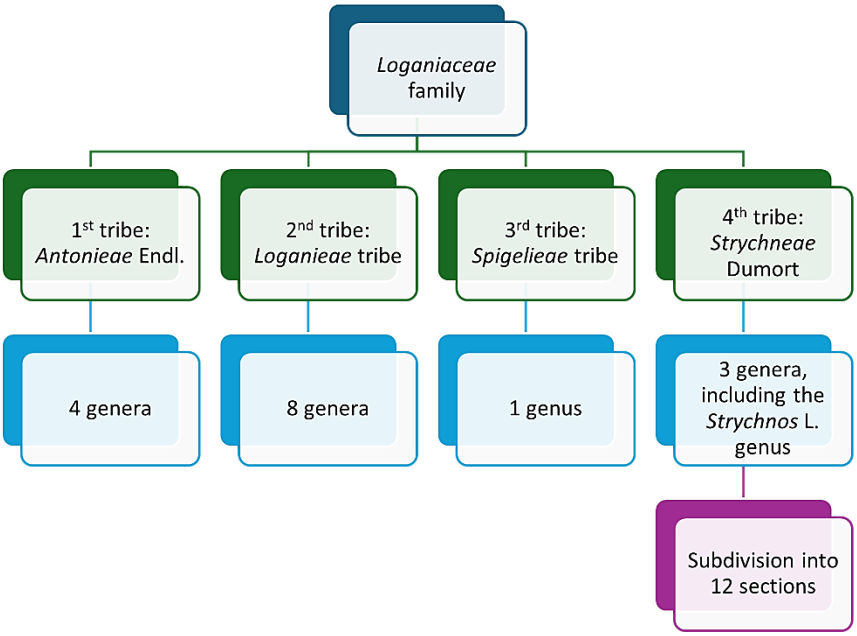


Figure 3: Family portrait of Loganiaceae suggested by Leeuwenberg in 1969.

Among all the classifications, *Strychnos* species are generally classified according to the system proposed by Leeuwenberg. However, a phylogenetic study conducted by Setubal, R. B. *et al.* (2021) revealed that Leeuwenberg's classification requires revision. In addition, chemotaxonomic studies, which involve establishing taxonomy based on metabolite content, further support this conclusion. Indeed, the numerous metabolites isolated from the genus, along with the study we conducted on strychnine as part of this thesis (to be discussed in Chapter 5, pages 253 to 286), confirm the same findings (Bonnet, O. *et al.*, 2022).

However, in these chemotaxonomic studies, the botanical form of *Strychnos* species also plays an important role. For example, in the case of *S. usambarensis*, the chemical composition varies depending on whether the plant appears as a liana (in West, East, and Central Africa) or as a tree (in East Africa). Specifically, only the C-11 and C-12 substituted alkaloids (such as strychnopentamine and strychnofoline), as well as quaternary alkaloids, are found in the tree form. This difference in composition is related to the presence of tendrils in the liana form (Quetin-Leclercq, J. *et al.*, 1991).

Therefore, it is evident that establishing an official classification for the *Strychnos* genus is a complex task, and future investigations will be necessary. The results obtained in this thesis will provide valuable support in achieving this goal.

1.2.4. Toxicology

There are two main types of toxicity associated with plants in the *Strychnos* genus: tetanizing and paralyzing effects (Philippe, G. *et al.*, 2004).

Initially, Asian species of *Strychnos* were described as tetanizing, while American species were thought to cause paralyzing effects. African species were grouped with the Asian ones, and thus considered tetanizing. However, this distinction was later refuted, as it was found that African, Asian, and American species exhibit both paralyzing and tetanizing activities. This led to the conclusion that toxicity is more closely related to the metabolite content of the plant (Philippe, G. *et al.*, 2004).

Tetanizing activity is mainly attributed to the presence of strychnine and its derivatives. As detailed in Section 1.2.1 (Pages 5-6), strychnine was first isolated by Pelletier and Caventou in 1818-1819 from the seeds of *Strychnos nux-vomica* L., an Asian species. This monoterpene indole alkaloid has since been identified in five other species: *S. ignatii* P.J.Bergius, *S. wallichiana* Steud. ex A.DC. (Asia), *S. lucida* R.Br. (Australia), *S. icaja* Baill. (Africa), and *S. panamensis* Seem. (Central and South America). It is also possible that *S. tabascana* Sprague & Sandwith contains strychnine, but further investigation is needed to confirm this. The link between *Strychnos* and strychnine is reflected

in the genus name (see Section 1.2.1, Pages 5-6). However, only a few species contain strychnine. Our research on strychnine led us to detect this alkaloid in seven species that had not previously been described as producers. Except for one, the other six species contained only trace amounts of strychnine (Bonnet, O. *et al.*, 2022). This research is described in detail in Chapter 5 (Pages 253-286).

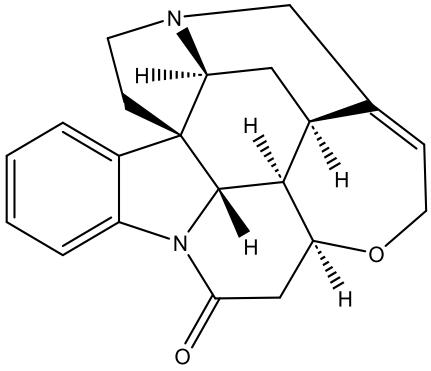
Strychnine is typically accompanied by derivatives. There are three main series of related alkaloids: the strychnine series (e.g., 12-hydroxystrychnine, colubrine, and brucine), the pseudostrychnine series (e.g., pseudostrychnine), and the N-methyl-sec-pseudostrychnine series (e.g., icajine, vomicine, and novacine). These alkaloids generally have weaker tetanizing effects than strychnine itself. However, their presence, in conjunction with strychnine, contributes to the overall tetanizing activity of species in the *Strychnos* genus (Philippe, G. *et al.*, 2004).

At higher doses, strychnine is a powerful convulsant of the spinal cord. It acts on the spinal cord and motoneurons by inhibiting post-synaptic glycine receptors, preventing neuronal repolarization. When glycine binds to its receptors, chloride channels open, allowing Cl^- ions to pass through, leading to hyperpolarization of the neurons. Strychnine may also inhibit glycine release at the pre-synaptic level. As a result, in the presence of strychnine, neurons cannot return to their resting potential, receiving excessive stimulation that causes muscle contractions throughout the body. Individuals intoxicated with strychnine assume a distinctive posture called "opisthotonos", characterized by generalized muscle contractures, backward curvature of the body, and limb extension. These intense contractions can be fatal if untreated. The continuous contraction of the diaphragm severely impairs respiratory function, potentially leading to death by asphyxiation (Figure 4) (Philippe, G. *et al.*, 2004).

At low doses, however, strychnine acts as an analeptic by stimulating some functions of the central nervous system, particularly in the medulla oblongata. This is why strychnine was originally used as a stimulant for the medullary and bulbar centers. It was also employed at higher doses as a rodenticide, particularly for killing rats (hence its nickname "rat poison"), as well as for poisoning pets like dogs and cats (Philippe, G. *et al.*, 2004).

At medicinal doses of 1 to 3 mg, strychnine primarily produces tonic effects by enhancing spinal reflexes. At this dosage, muscle tension increases, but circulatory and respiratory systems remain unaffected. At doses between 5 and 10 mg, the enhancement of spinal reflexes and muscle tension becomes more pronounced, resulting in such intense tension and pain that the body becomes immobile. Above 10 mg, symptoms such as hyperthermia from tonic convulsions, acidosis, rhabdomyolysis, anxiety, muscular paralysis, and dyspnea are observed (Philippe, G. *et al.*, 2004).

Today, the use of strychnine is banned in the European Union due to its high toxicity and the cruelty involved in poisoning rodents and pets. This ban has been implemented in many other countries as well. However, strychnine is still sometimes used as a doping agent in sports (Philippe, G. *et al.*, 2004).



Strychnine

Figure 4: “Opisthotonos" posture caused by strychnine poisoning (Medical News Today, 2017).

The second major type of toxicity associated with *Strychnos* plants is their paralyzing effect. Curare, a well-known hunting poison used by indigenous tribes in the tropical forests of the Amazon, is the most notable example of this toxicity (Figure 5). The toxic action of curare is attributed to the presence of quaternary alkaloids in the crude extract applied to arrowheads. Two quaternary ammonium groups are necessary to produce the curarizing effect, which involves blocking nicotinic acetylcholine receptors and causing muscle relaxation. The muscle relaxant activity decreases sharply as the number of quaternary ammoniums is reduced. With only one quaternary ammonium,

the activity is weak, and it is absent without any (Bisset, N. G., 1988). Moreover, for maximum efficacy, the distance between the two ammonium groups must be about 0.85–0.90 nm. While American species of *Strychnos* contain large quantities of curare, other species, such as the African species *S. usambarensis*, also produce curarizing compounds. Indeed, Professor Luc Angenot discovered the use of *S. usambarensis* root barks as an arrow poison in the Akagera region and identified curarizing bisquaternary alkaloids such as C-dihydrotoxiferine, calebassine, C-curarine, and afrocurarine (Angenot, L., 1971b; Krukoff, B. A., and Monachino, J., 1972; Angenot, L. *et al.*, 1975a; Philippe, G. *et al.*, 2004).

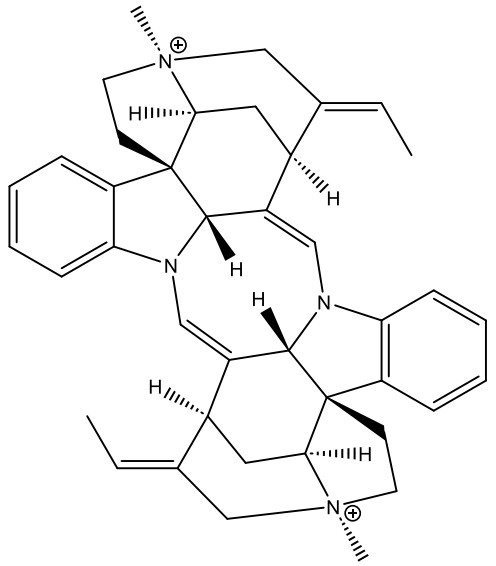


Figure 5: Structure of C-dihydrotoxiferine, a curarizing indole monoterpene alkaloid isolated from the *Strychnos* genus.

Curare was first discovered in 1548 during Alonso Pérez de Tolosa's exploration of the Lake Maracaibo region in Colombia, but it was not until 1596 that the term "ourari", later adapted to "curare", was introduced by Lawrence Keymis during his exploration of the Orinoco. This term referred to a group of poisons used by South American Indians to paralyze animals during hunting. Curare acts at the neuromuscular junction, blocking nicotinic acetylcholine receptors. This binding is competitive with acetylcholine but does not induce muscle depolarization. The paralytic effects of curare can be reversed with

the administration of a cholinesterase inhibitor, such as neostigmine (Philippe, G. *et al.*, 2004).

Curare's curarizing activity is described both in traditional uses, such as hunting and fishing poisons, and in hospitals, where anesthetists use it to relax the patient's muscles during operations (for this purpose, tubocurarine is generally used as a muscle relaxant) (Philippe, G. *et al.*, 2004).

The production of curare and its derivatives has been linked primarily to two plant families: Loganiaceae and Menispermaceae. A total of 25 *Strychnos* species are used in curare preparation, with only two species, *S. ignatii* (from Malaysia) and *S. usambarensis* (from Rwanda), being non-American. Additionally, *S. nux-vomica* (Asian species) and *S. icaja* (African species) contain small amounts of curarizing alkaloids in their root barks. Interestingly, both *S. nux-vomica* and *S. icaja* exhibit both tetanizing and paralyzing properties, as shown by pharmacological studies of different alkaloid fractions (Bisset, N. G. *et al.*, 1977; Bisset, N. G. 1989; Philippe, G. *et al.*, 2004).

Curare can be classified into three groups based on storage methods:

1) Tubocurare is preserved in bamboo tubes;

2) Calabash curare is stored in emptied and dried fruit;

3) Potted curare is placed in terracotta pots.

Tubocurare is typically produced from species of the Menispermaceae family, while calabash curare is generally derived from Loganiaceae species. Potted curare can involve species from both families (Philippe, G. *et al.*, 2004).

As previously discussed, the two primary toxic effects of *Strychnos* plants have significant traditional uses. The literature describes four different types of traditional uses: poisoned arrows for hunting, ordeals, fishing poison, and oracular poison. *S. usambarensis* and *S. icaja* are used to prepare poisoned arrows: *S. usambarensis* gives a curarizing effect, while *S. icaja* produces a tetanizing effect. These arrows are intended to paralyze or kill the prey, preventing escape. In African traditional rituals, *S. icaja* is often used in ordeals, where poison from the root is given to a person suspected of guilt. Survival from the poison is considered proof of innocence. Species such as *S. aculeata* and

S. camptoneura produce fruits that are used in fishing, due to its curarizing effect which paralyzes fish, facilitating their capture. Finally, *S. icaja* is also used as an oracular poison for divination. When a question is asked, poison is administered to a chicken. If the chicken survives, the answer is considered negative. However, a second test is required to confirm this response. If the animal survives again, the previous answer is invalidated, and a new ritual must be performed with a different animal, in a different place, and with a new poison (Bisset, N. G., 1970; Neuwinger, H. D.,1996; Philippe, G. *et al.*, 2004). The traditional uses of *Strychnos* species, including some recently identified applications, will be further explored in the next section.

1.2.5. Alkaloids content

The alkaloids of plants from the *Strychnos* genus are mainly of the monoterpene indole type. From a biosynthetic perspective, they are produced from the key precursor strictosidine, which is formed by the condensation of the amino acid L-tryptophan (providing the indole moiety) and secologanin, an iridoid monoterpene (providing the terpene moiety). Subsequently, various enzymatic transformations have led to the establishment of two groups: the indoline group (specific to the *Strychnos* genus) and the indole group (not specific to the *Strychnos* genus). The indoline group comprises six structural types (Figure 6), while the indole group includes seven (Figure 7) (Bosly, J., 1951; Hesse, M., 1968; Koch, M. *et al.*, 1966; Sandberg, F. *et al.*, 1969; Sarfati, R. *et al.*, 1970; Angenot, L., and Bisset, N. G., 1971a; Koch, M., and Plat, M., 1971; Verpoorte, R., and Sandberg, F., 1971; Angenot, L. *et al.*, 1973a; Angenot, L., and Denoël, A., 1973b; Au, T. Y., 1973; Koch, M. *et al.*, 1973; Bisset, N. G., and Phillipson, J. D., 1974; Phillipson, J. D. *et al.*, 1974; Angenot, L. *et al.*, 1975a; Angenot, L., 1975b; Singh, H. *et al.*, 1975).

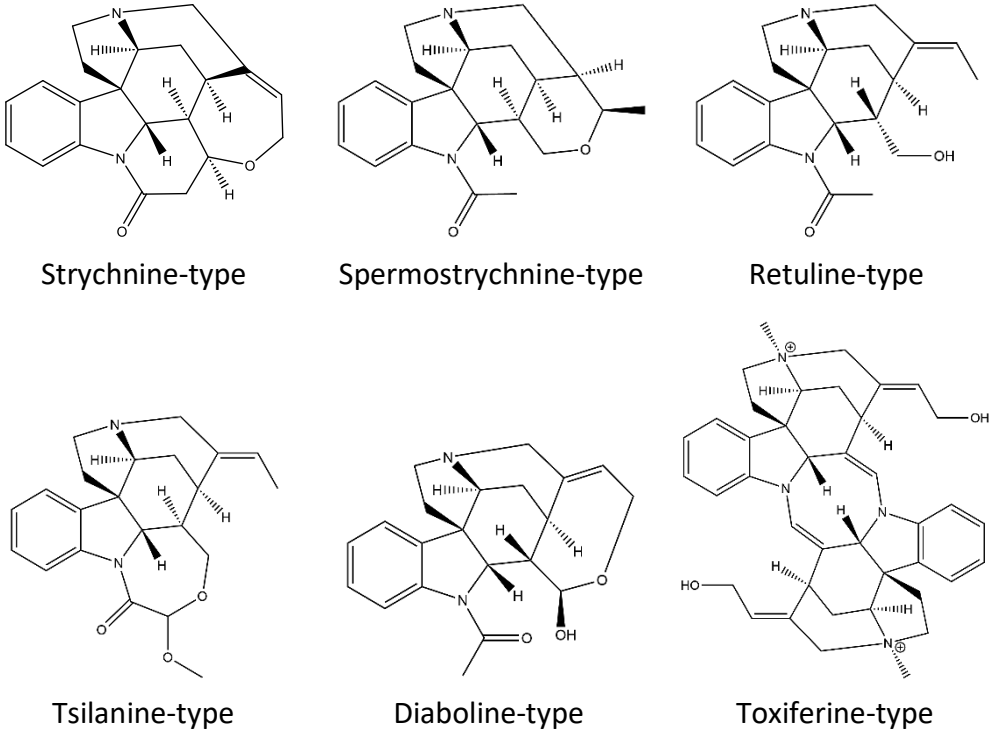
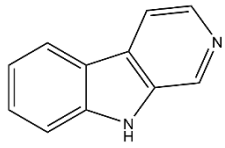
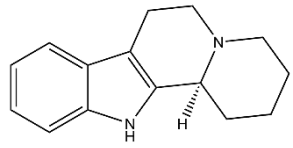


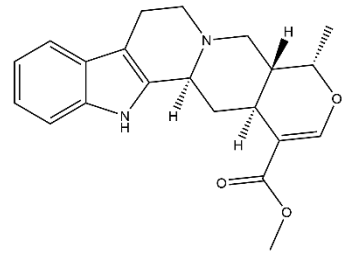
Figure 6: Types of indoline alkaloids from the *Strychnos* genus.



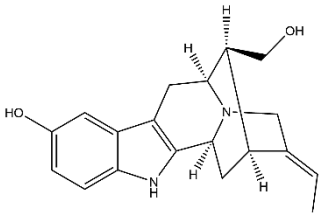
β-carboline-type



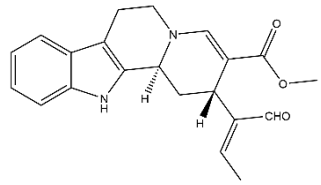
Indoloquinolizine-type



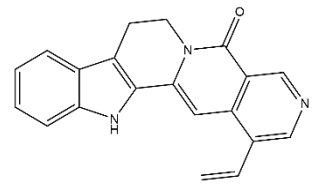
Ajmalicine-type



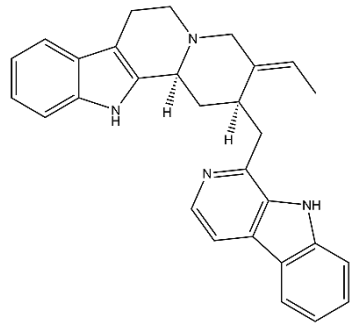
Sarpagine-type



Vallesiachotamine type



Angustine-type



Usambarensine-type

Figure 7: Types of indole alkaloids from the *Strychnos* genus.

1.3. Malaria, a persistent pathology

1.3.1. An ancient disease known for centuries

Malaria has been a well-known disease for thousands of years. Indeed, many ancient texts describe its symptoms. Hindu texts from the 6th century B.C.E. refer to malaria, as do Chinese records from around 2700 B.C.E., Mesopotamian clay tablets from around 2000 B.C.E., and Egyptian papyri from 1570 B.C.E. The Greeks, including Homer and Hippocrates, noted health problems associated with malaria in people living near swamps. Studies on mummies detected antigens from *Plasmodium falciparum*, one of the parasites responsible for malaria in humans, confirming the presence of this disease for millennia (Miller, R. L. *et al.*, 1994; Cox, F. E. G., 2010).

For over 2,500 years, it was believed that miasma from swamps was the cause of fevers. This assumption gave rise to the name malaria, as the Italian word *mal'aria* means "bad air." The discovery of bacteria by Antoni van Leeuwenhoek in 1676 led Louis Pasteur and Robert Koch to suggest, in 1878-1879, that fever attacks could be linked to infections. In 1880, Charles Louis Alphonse Laveran, a French army surgeon, discovered the parasites responsible for malaria. Then, in 1897, Ronald Ross, a young doctor in the Indian Medical Service, determined that mosquitoes from the *Anopheles* genus play a crucial role as vectors in the transmission of avian malaria parasites. The Italian scientists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi, and Ettore Marchiafava demonstrated between 1898-1900 that mosquitoes are also vectors for human malaria. Eighty-six years later, in 1986, Trager and Jensen developed a protocol, still in use today, to maintain parasites in *in vitro* culture. This breakthrough was a major advancement in the understanding and control of malaria, as it enabled scientists to test the sensitivity of parasites to various therapeutic agents (Trager, W., and Jensen, J. B., 1976; Cox, F. E. G., 2010; Barnett, R., 2016).

1.3.2. A parasitic infection spread by mosquitoes

The *Plasmodium* genus includes over 200 species that infect mammals, birds, and reptiles. Six species within this genus are responsible for malaria in humans,

namely *P. falciparum*, *P. ovale*, *P. vivax*, *P. knowlesi*, *P. malariae*, and *P. simium*, a newly emerging species (Cox, F. E. G., 2010; Brasil, P. *et al.*, 2017; Fikadu, M., and Ashenafi, E., 2023).

In the human body, malaria parasites initially develop as sporozoites within liver cells. During the hepatocyte phase of *P. vivax* and *P. ovale* infections, some parasites become hypnozoites, i.e., dormant forms that can remain in the liver for months to years before reactivating to cause a new episode of infection. In red blood cells, the parasites grow as merozoites, transitioning through trophozoite and schizont stages until they cause the red blood cells to burst, releasing new merozoites and triggering fever. This stage is known as the blood stage. A characteristic symptom of malaria is the recurring and regular onset of fever when red blood cells rupture. After several cycles, whose duration varies depending on the *Plasmodium* species, gametocytes are produced. These mature into male and female gametocytes, which can fuse to form a zygote when taken up by a mosquito during its blood meal. In the mosquito, the zygote develops into an oocyst, which releases sporozoites. Once the sporozoites migrate to the mosquito's salivary glands, they can infect a new human host during the next blood meal (Figure 8) (Cox, F. E. G., 2010; Fikadu, M., and Ashenafi, E., 2023).

Therefore, mosquitoes play a critical role in the spread and development of malaria parasites. Of the 400 species in the *Anopheles* genus, only 30 are known to transmit malaria. The primary line of prevention is to protect oneself from mosquito bites. To this end, insecticide-treated nets and indoor residual spraying are the two methods currently recommended by the World Health Organization (WHO). Pyrethroids such as permethrin and deltamethrin are the main pesticides used to treat mosquito nets. Unfortunately, *Anopheles* mosquitoes have developed resistance to pyrethroids. By combining these insecticides with piperonyl butoxide, a substance that inhibits metabolic enzymes like oxidases that break down insecticides, mosquito resistance can be countered. Additional combinations include pyrethroid-chlorfenapyr and pyrethroid-pyriproxyfen. For indoor residual spraying, walls and floors are coated with pesticides. The WHO recommends five classes of pesticides, which have been validated for their efficacy and quality: pyrethroids, organochlorines, carbamates, organophosphates, and neonicotinoids. DDT, an organochlorine

insecticide, is no longer on the WHO's approved list (Fikadu, M., and Ashenafi, E., 2023; World Health Organization, 2023c).

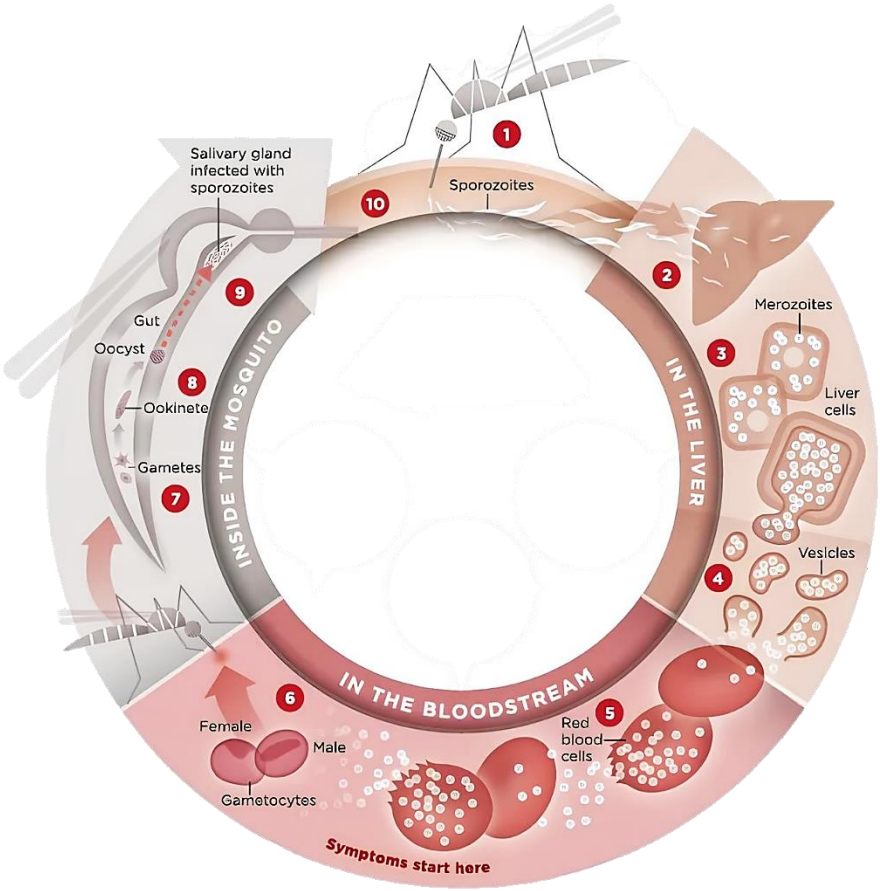


Figure 8: Life cycle of *Plasmodium* (Malaria Vaccine Initiative, 2024).

1.3.3. An extensive therapeutic arsenal but currently powerless

The first record of malaria treatment dates back to the 2nd century B.C.E., when, in China, *Qinghao* (*Artemisia annua*) was administered to a person suffering from fever and chills. In the 16th century, a second treatment was identified: Spanish invaders in Peru discovered that *Cinchona* barks could be used to treat malaria. The active compound, quinine, was isolated from *Cinchona* barks in 1820 by Pelletier and Caventou and was used for many years as a chemoprophylaxis and treatment for malaria. In 1970, Dr. Youyou Tu isolated artemisinin from *Artemisia annua*, a compound highly effective against

malaria. Artemisinin continues to play an important role in antimalarial treatments and serves as the reference compound in antiplasmodial assays.

Quinine and artemisinin derivatives have since been developed. Quinine derivatives include chloroquine, mefloquine, lumefantrine, halofantrine, amodiaquine, primaquine, and piperaquine, while artemisinin derivatives include artemether, arteether, dihydroartemisinin, and sodium artesunate (Fougere, E., and Buxeraud, J., 2018).

Although artemisinin and its derivatives are powerful antimalarials, resistance to these treatments has gradually emerged in parasites. To slow this development, the WHO recommended combining artemisinin with another treatment in what is known as Artemisinin-based Combination Therapy (ACT). This combination has shown high efficacy, especially against *P. falciparum*, the most virulent of the six species infecting humans (World Health Organization, 2023a).

Unfortunately, resistance continues to spread in many countries, reducing the effectiveness of current antimalarial treatments. This trend is reflected in annual malaria reports published by the World Health Organization. In 2022, a total of 249 million cases of malaria were reported across 85 endemic countries, compared to 231 million cases in 2015, an increase of 18 million over seven years. There were 608,000 deaths in 2022, up from 586,000 in 2015, with most cases and deaths occurring in Africa (World Health Organization, 2023a).

In response, extensive research is underway globally to develop new antimalarial drugs aimed at slowing the spread and growth of *Plasmodium* and ultimately eradicating malaria. Plants are a promising source of new therapeutic compounds, and the metabolite content of numerous species is being explored. This is particularly true for species in the *Strychnos* genus, which have shown significant antiplasmodial activity and contain unique active alkaloids (Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2002).

Recently, antimalarial vaccines have been developed: RTS,S/AS01 and R-21/Matrix-M vaccines.

In 2019, 1.5 million children received the first dose of the RTS,S/AS01 vaccine, which was subsequently recommended by the World Health Organization in

2021. The RTS,S/AS01 vaccine contains a fusion antigen, called CSP-antigen, combining the hepatitis B surface antigen (HBsAg) and the CSP epitope. The HBsAg component acts as a matrix, while the CSP epitope induces antibodies against the circumsporozoite protein (CSP) found on the surface of sporozoites from all *Plasmodium* species. Four injections of the RTS,S/AS01 malaria vaccine are required in young children to achieve optimal efficacy.

The R-21/Matrix-M vaccine, recommended by the WHO in October 2023, differs from RTS,S/AS01 in the amount of HBsAg. Since it contains less HBsAg, the number of CSP epitopes available is higher, resulting in a stronger immune response. This second vaccine helps meet the high demand for RTS,S/AS01. For optimal efficacy, R-21/Matrix-M requires also four doses: three at short intervals and a fourth one year later.

Both vaccines are currently in clinical trials. By providing protection against malaria infection, these vaccines could help contain the spread of the disease and advance efforts toward eradication (Collins, K. A. *et al.*, 2021; World Health Organization, 2023a; World Health Organization, 2023d; World Health Organization, 2023e; World Health Organization, 2023f).

1.4. Metabolomics as a tool for uncovering novel natural compounds

1.4.1. Definition and principles

"Omics" refers to scientific fields focused on the high-throughput measurement of biological molecules. There are several categories of omics: proteomics, transcriptomics, genomics, metabolomics, lipidomics, and epigenomics. These fields involve the comprehensive study of proteins and their functions, RNA profiling (gene expression), genomes and DNA sequences, metabolites (or low molecular weight molecules), lipids and their roles, as well as epigenetic changes affecting DNA (methylated DNA or modified histone proteins in chromosomes), respectively (Figure 9) (Clish, C. B., 2015).

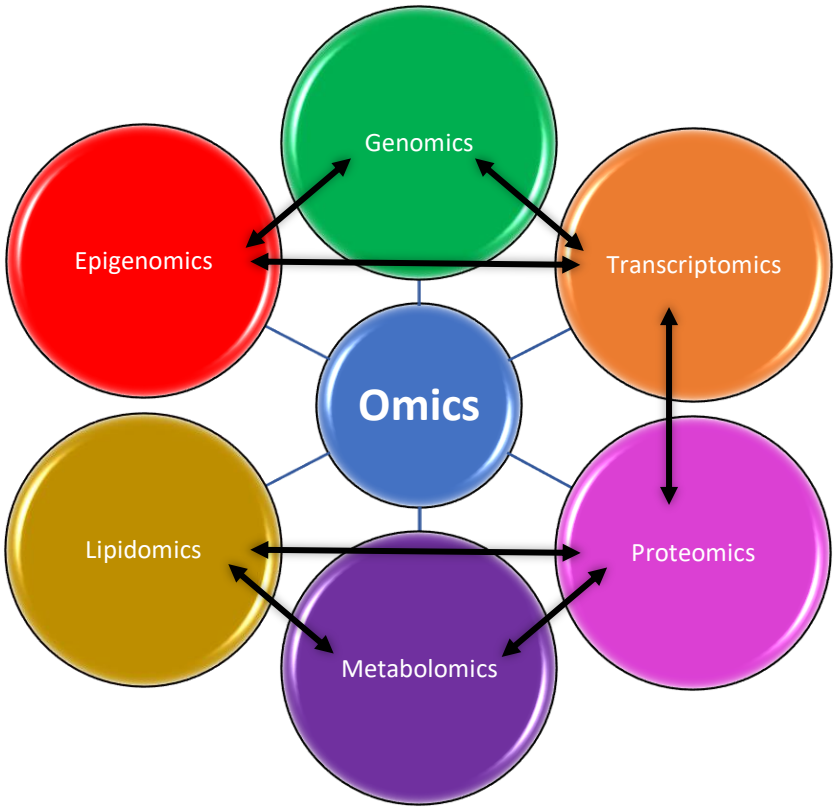


Figure 9: The different categories of omics.

The various levels of “omics” are interconnected, reflecting the hierarchical and dynamic nature of biological processes. Genomics provides the foundation by encoding genes, which are transcribed into mRNA (transcriptomics) and subsequently translated into proteins (proteomics). These proteins, particularly enzymes, drive metabolic pathways, leading to the production of metabolites (metabolomics). Lipidomics, a subset of metabolomics, focuses specifically on lipid molecules, which serve as structural components of membranes, energy reservoirs, and signaling molecules. The relationship between proteomics and lipidomics is particularly significant, as proteins regulate lipid metabolism and signaling, while lipids influence protein function through modifications and interactions, particularly in membranes. Regulatory mechanisms, such as epigenetic modifications, post-transcriptional and post-translational processes, and environmental factors, create feedback loops and modulate these interactions.

Integrating these layers provides a comprehensive understanding of cellular functions, where variations at one level, such as genetic mutations or transcriptional changes, propagate through the system to impact the proteome, metabolome, and lipidome. This multi-omics approach is essential for identifying biomarkers, exploring metabolic pathways, and understanding complex diseases.

In this thesis, we will focus exclusively on metabolomics. This approach has been applied for the past twenty years (Alseekh, S. *et al.*, 2021), but it has only recently emerged in the study of natural metabolites in plants, fungi, and marine organisms. The improved sensitivity of current analytical methods, such as nuclear magnetic resonance (NMR) and mass spectrometry (MS), combined with powerful chemometric tools, provides the ability to explore a wide range of metabolite contents, including both major and minor metabolites (Idle, J. R., and Gonzalez, F. J., 2007; Fox Ramos, A. E. *et al.*, 2019a; Wolfender, J.-L. *et al.*, 2019; Bruguière, A. *et al.*, 2020). In the search for new bioactive molecules, having a comprehensive overview of the compounds involved enables researchers to identify unknown compounds and target them for isolation.

1.4.2. Plant metabolomics

Metabolomics, the study of metabolites or low molecular weight molecules, is a growing field in plant research. It provides insights into the biochemical processes of plants by identifying and quantifying metabolites under specific conditions. With the diversity of primary and secondary metabolites produced by plants, metabolomics is an essential tool for exploring their roles, applications in medicine and agriculture, as well as ecological interactions. Indeed, metabolomics has several applications:

- It enables the identification of bioactive compounds. It has been instrumental in discovering new alkaloids, flavonoids, and terpenoids with therapeutic applications. By analyzing plant metabolic profiles, researchers can identify key compounds linked to traits or activities;
- It plays crucial roles in plant-environment interactions, such as defense mechanisms, pollinator attraction, and competition with

neighboring plants. These studies have provided insights into how metabolic diversity contributes to plant adaptation and evolution;

- It helps in agriculture to identify metabolites associated with desirable traits like stress tolerance, enhanced nutrition, and yield. Integrated with genomics and transcriptomics, it aids breeders in developing tailored crops. Metabolic profiling reveals biomarkers for drought resistance or disease susceptibility, streamlining breeding;
- It differentiates species based on chemical fingerprints, supporting taxonomic studies;
- It ensures product quality for medicinal plants by detecting variations in chemical composition and verifying the presence of active ingredients in herbal medicines.

Advanced analytical techniques like GC-MS and LC-MS are widely used for metabolite detection due to their sensitivity and ability to analyze complex mixtures. NMR analyses are also applied in metabolomics, even though they are less sensitive than MS and MS/MS analyses. However, NMR provides detailed structural information about metabolites. Bioinformatics tools and databases like GNPS and MIADB assist in metabolite annotation and pathway analysis.

However, plant metabolomics faces challenges such as the complexity of plant extracts, lack of standardized methods, and high instrumentation costs. Despite these barriers, it has revolutionized the study of plant biology by offering tools to explore chemical diversity, improve crop traits, and discover bioactive compounds. Integrating metabolomics with other "-omics" approaches provides holistic insights into plant biology and creates new opportunities in agriculture, ecology, and medicine (Hall, R. D., 2006; Bhatt, B. *et al.*, 2022).

1.4.3. A variety of metabolomics tools

Some metabolomics tools rely on MS/MS data, which are highly sensitive, allowing the exploration of both major and minor metabolites. Others depend on NMR data, which, due to lower sensitivity, are limited to the analysis of major metabolites. These two types of data are complementary. Indeed, the annotations provided by the software are based on comparisons between

theoretical and experimental MS/MS fragments or NMR chemical shifts. Such annotations are considered probable identifications and correspond to confidence level 2 according to the Metabolomics Standards Initiative (MSI). By merging the annotations obtained from both types of software, it is possible to achieve confidence level 1. This level corresponds to identifications made using at least two orthogonal techniques, as achieved in this approach. At this level, a 2D structure can be elucidated for each identified metabolite. Increasing attention is being placed on the relevance and accuracy of metabolite identifications. Moreover, advances in metabolomics now enable the integration of these data with chemometric models, such as partial least squares (PLS), which help determine a bioactivity score and thus highlight potentially active metabolites (Sumner, L. W. *et al.*, 2007; Meunier, M. *et al.*, 2024).

Metabolomics is a rapidly evolving field. Numerous metabolomics tools have already been implemented, and new ones are being developed, with more expected to become available in the coming years. Some of these tools are discussed and explained in this section.

1.4.3.1. Tools requiring MS/MS data

The first tool is molecular networking, a method used to map the metabolites of one or more crude extracts or fractions based on their similarities. Global Natural Products Social Molecular Networking (GNPS)[1] is an online platform where high-resolution MS/MS data are processed and organized to generate the molecular network (Wang, M. *et al.*, 2016). Several software programs are available for data processing, including MSConvert[2], MZmine 3[3], OpenMS[4], and XCMS[5] (Smith, C. A. *et al.*, 2006; Sturm, M. *et al.*, 2008; Chambers, M. C. *et al.*, 2012; Schmid, R. *et al.*, 2023). Additionally, numerous mass spectra databases, contributed by various platform users, are available on GNPS, allowing for the discrimination of known metabolites from unknown ones. By comparing

[1] GNPS: https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp.
[2] MSConvert: https://proteowizard.sourceforge.io/download.html.
[3] MZmine 3: http://mzmine.github.io/download.html.
[4] OpenMS: https://openms.de.
[5] XCMS: https://bioconductor.org/packages/3.19/bioc/html/xcms.html.

experimental mass spectra with those in the databases, the software suggests possible identifications, which should then be verified with data from the literature. One such database is MIADB (Monoterpene Indole Alkaloids DataBase), which contains mass spectra of 422 monoterpene indole alkaloids. This database is available on both the GNPS platform and the MetaboLights website and was implemented by Université Paris-Saclay (Fox Ramos, A. E. *et al.*, 2019b).

Once the molecular network is created, it can be visualized using the "Cytoscape" software, where external data, known as "metadata," can be incorporated. Metadata such as plant parts, traditional uses, and antiplasmodial activities can provide valuable insights into the molecular network. For example, questions like "Are there clusters of metabolites from the same plant part?" or "Are these clusters associated with promising antiplasmodial activities?" can be addressed by incorporating metadata into the network (GNPS Documentation, 2022).

Figure 10 illustrates a cluster from a molecular network. A cluster is a group of metabolites with similarities. Each compound in a cluster is represented by a "node," and the links between them are called "edges." The thicker the bond between two molecules, the more similar their structures are. These links are based on "cosine" scores, a mathematical measure of spectral similarity between two MS/MS spectra. A score of 1 indicates complete similarity, while a score of 0 indicates no similarity. Additionally, nodes can contain various information such as *m/z* masses or suggested identifications, and their size can be adjusted based on parameters like peak intensity.

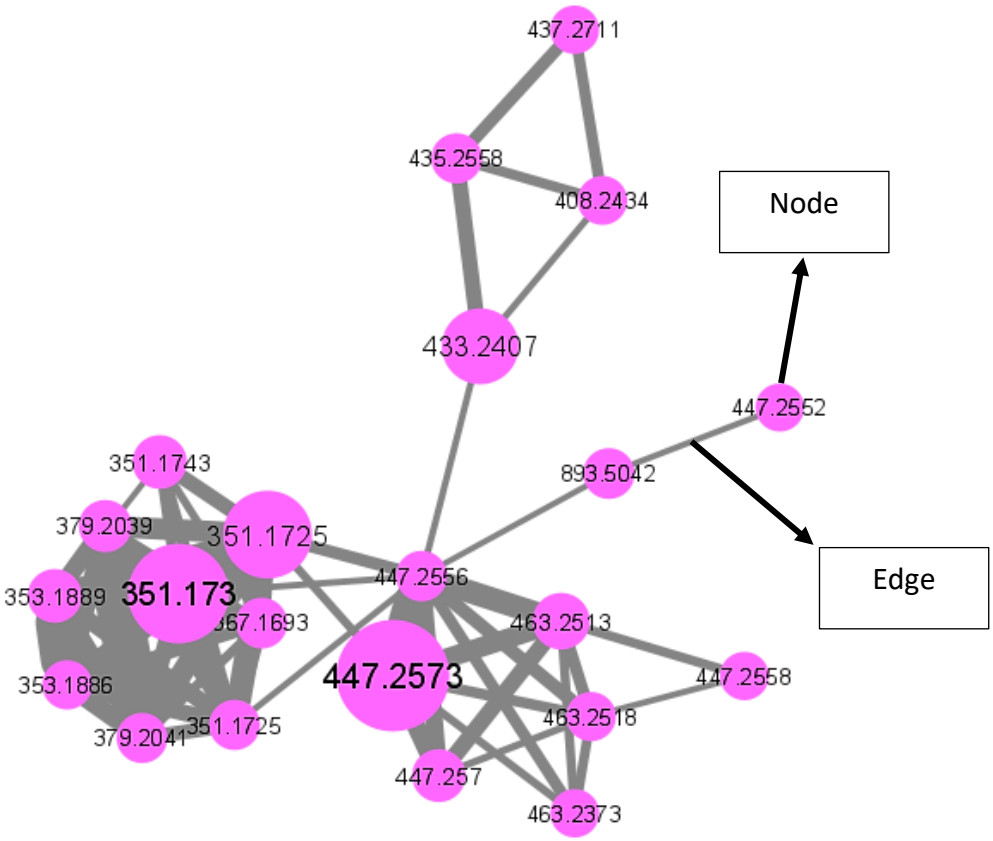


Figure 10: Cluster from a molecular network.

The next software tool is the powerful “SIRIUS”[6]. From processed MS and MS/MS data, SIRIUS can suggest identifications by querying databases, provide molecular formulas for precursor ions and their fragments, predict their fragmentation trees, and, based on molecular fingerprints, predict the compound classes (ranging from phytochemical classifications to structure-based classifications) (Dührkop, K. *et al.*, 2019).

The third and final tool presented in this section is "Inventa"[7]. To highlight the structural novelty potential within various crude extracts, Inventa calculates a multiple representative score based on literature reports, untargeted mass spectrometry data, and MS/MS spectra annotations. By identifying the extracts

[6] SIRIUS: https://bio.informatik.uni-jena.de/software/sirius.
[7] Inventa: https://github.com/luigiquiros/inventa.

with the highest scores, and thus the most structural novelties, Inventa accelerates the discovery of new natural molecules (Quiros-Guerrero, L.-M. *et al.*, 2022).

1.4.3.2. Tools requiring NMR data

"MixONat"[8] is a software that requires carbon spectra (^{13}C APT, DEPT-135, and DEPT-90) of a crude extract or fraction to provide identification suggestions. These identifications are based on comparisons of ^{13}C chemical shifts (in ppm) between experimental data and database entries. The probabilities obtained range from 0 to 1, where a score of 1 indicates that all carbon signals have been matched, and a score of 0 means no match was found. As of the writing of this thesis, no shared database exists for NMR data, although this is expected to change in the coming years. Therefore, it is necessary to generate custom databases to submit to MixONat. By using the "LOTUS"[9] database of metabolites and the "C+H NMR Predictors" software from ACD/Labs, a variety of databases can be created. Additionally, as the LOTUS database is continuously enriched by users, it increases the database options available for MixONat (Bruguière, A. *et al.*, 2020). Unfortunately, because ^{13}C spectra are less sensitive, this software is mainly useful for identifying the major metabolites.

Another tool utilizing NMR data is "MadByte"[10]. It requires two types of two-dimensional data: HSQC (Heteronuclear Single Quantum Coherence) and TOCSY (Total Correlation Spectroscopy). Based on the characteristics of the spin systems, MadByte generates a network that highlights structural motifs common across multiple samples. In this way, common and distinct features between several samples are quickly identified (Egan, J. M. *et al.*, 2021; Flores-Bocanegra, L. *et al.*, 2022).

The final software, presented in this section, which utilizes HSQC data to explore the structures and substructures of metabolites, is SMART. Using artificial intelligence and a database of 100,000 natural products, SMART provides structural hypotheses, along with SMILES, cosine similarities,

[8] MixONat: https://sourceforge.net/projects/mixonat.
[9] LOTUS: https://lotus.naturalproducts.net.
[10] MadByte: https://www.madbyte.org.

and molecular masses of these structures. The software is also capable of providing external links, notably to GNPS, to incorporate additional data (Reher, R. *et al.*, 2020).

This thesis focuses on the molecular networking method. Since MixONat is a complementary tool for molecular networking, it was also used to ensure the highest possible relevance in the identifications.

1.5. Context and objectives of the work

This thesis was conducted within the framework of the search for new natural antimalarial compounds to reduce the spread of malaria, which remains a major public health issue. Despite existing preventive measures and treatments, malaria continues to be the deadliest parasitic disease, particularly in Africa, due to the growing resistance of parasites to these treatments, including chloroquine and artemisinin-based combination therapies (ACTs). Therefore, there is an urgent need to develop new treatments.

The plant kingdom is rich in bioactive substances. For instance, 64% of antimalarial drugs currently on the market are derived from natural sources. Similarly, other categories of treatments, such as antimicrobials and antivirals, also have a significant proportion of natural products. Among the plant species studied, those from the *Strychnos* genus have attracted attention due to their promising antiplasmodial properties. These include the leaves and roots of *Strychnos usambarensis*, the roots of *S. icaja*, and the root barks of *S. variabilis* and *S. nux-vomica*.

The *Strychnos* genus has been studied for over two centuries, and its exploration has been a longstanding focus of the team from the Laboratory of Pharmacognosy at the University of Liège. Indeed, research on this genus has been ongoing for more than three decades, aiming to investigate its alkaloidic content and to study its biological and toxic activities. Over the course of these two centuries of investigation, numerous alkaloids have been isolated and identified, some of which have demonstrated promising antiplasmodial activity, such as isostrychnopentamine and strychnogucine B. As a result of

this intensive global research effort, many major compounds have already been discovered, which poses a growing challenge for identifying new antiplasmodial compounds. This underscores the importance of innovative strategies in natural product research. The application of dereplication tools from metabolomics has made it possible to explore the full range of metabolites, quickly detect those that are unidentifiable, and isolate and identify them. Furthermore, since some metabolomics tools rely on MS/MS data, which are more sensitive than traditional methods like TLC and HPLC, they provide a powerful approach to identifying minor metabolites.

This research project, described in this thesis, is set in this context. Specifically, we use dereplication tools, primarily the molecular networking method, to isolate novel antiplasmodial compounds. We integrate this with the conventional method of bio-guided fractionation, adding a new dimension: mass-guided fractionation. During the purification steps, fractions containing both metabolites active against malaria and a significant number of unidentifiable metabolites are selected for further purification.

The research project is structured around three main objectives:

1) To explore the alkaloid chemodiversity of several *Strychnos* species using molecular networking;
2) To quickly differentiate known from unknown metabolites using shared spectral databases;
3) To identify new alkaloids with potential antimalarial activity.

To address the first two objectives and select species for identifying new antiplasmodial compounds, a total of forty-three methanolic and one alkaloidic crude extracts from various plant parts of 28 *Strychnos* species were analyzed using molecular networking and tested on a *Plasmodium falciparum* strain in *in vitro* assays. The study of these crude extracts and the selection of species are detailed in Chapters 3 and 4.

An additional study was conducted to evaluate the effectiveness of the molecular networking method. Strychnine, a well-known monoterpene indole alkaloid from *Strychnos*, was used as a case study. This study revealed the presence of strychnine in seven species not previously known to produce it. The complete study is described in Chapter 5.

The species selected for purification include *S. usambarensis* Gilg ex Engl. leaves, *S. longicaudata* Gilg trunk barks, and *S. phaeotricha* Gilg leaves. The various purification steps and structural elucidations of the isolated metabolites are detailed in Chapters 6, 7, and 8.

1.6. References

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CHAPTER 2

Comprehensive study of traditional uses and pharmacological properties of African *Strychnos*, alongside a brief overview of the traditional uses of American and Asian *Strychnos*

2.1. Traditional uses and pharmacological properties

In the European Union, a plant is considered to have traditional use if it has been utilized for at least 30 years without scientific evidence of its efficacy. Pharmacological studies have subsequently been conducted in this area, and once its effectiveness is confirmed through research, the plant's use is classified as well-established (Commission européenne, 2011).

The plants of the *Strychnos* genus are documented for their traditional applications in the management of a wide range of ailments, including infections (microbial, fungal, and parasitic), cancers, gastrointestinal disorders, diabetes, pain, sexual disorders, wounds, and more. However, the therapeutic potential of *Strychnos* species must be carefully balanced against their inherent risks. Without proper control, their significant toxicity may outweigh the potential benefits. Nevertheless, within traditional medicinal practices or under medical supervision, these plants represent a valuable resource for managing a wide range of health conditions. To ensure safety and maximize therapeutic efficacy, isolating active metabolites is the most reliable approach, as it enables targeted therapeutic effects while minimizing toxic side effects.

In this thesis, 28 *Strychnos* species were studied. Of these, only 4 are not of African origin: *Strychnos brasiliensis* (Spreng.) Mart., *Strychnos mattogrossensis* S.Moore, *Strychnos nux-vomica* L., and *Strychnos ignatii* P.J.Bergius. Therefore, we decided to describe in detail the traditional uses and pharmacological properties of African species, and, more briefly, those of American and Asian species.

2.1.1. African *Strychnos*

This section presents a comprehensive review of the traditional uses and pharmacological properties of African *Strychnos*, which will be submitted to the *Journal of Ethnopharmacology*. The article is entitled “The African Plants of the *Strychnos* Genus: A Review of their Traditional Uses and Pharmacological Properties” (Figure 11). As part of this thesis, the presentation of the article has been slightly modified to make the chapter more readable.

The African Plants of the *Strychnos* Genus: A Review of their Traditional Uses and Pharmacological Properties

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Figure 11: Screenshot of the review on the traditional uses and pharmacological properties of African *Strychnos*.

ABSTRACT

Ethnopharmacological relevance: African plants of the *Strychnos* genus, belonging to the Loganiaceae family, are extensively utilized by indigenous communities for treating various ailments and for other purposes, such as hunting, fishing, and ordeals. Traditional uses include remedies for malaria and other parasitic infections, microbial and fungal infections, pain of various origins, tuberculosis, gastrointestinal issues, and sexual dysfunctions. Furthermore, these plants have been the subject of extensive research over many years, focusing on their therapeutic properties and their use in preparing poisons, both tetanizing and paralyzing (curarizing). To investigate these effects, *in vitro* and *in vivo* tests on crude extracts, as well as alkaloids isolated from these extracts, have been conducted. The results of these studies have validated the effectiveness of some of the traditional uses.

Aims of the review: There is no comprehensive review in the literature that discusses the traditional uses and pharmacological properties of African species from the *Strychnos* genus. Therefore, the objective of this review is to provide an updated and detailed overview of these uses and properties, and to compare them in order to confirm the effectiveness of some traditional applications.

This paper serves as a valuable source of information that can be used in future studies and investigations of plants from the *Strychnos* genus.

Materials and methods: Articles published between 1946 and 2024 from various scientific journals were gathered using scientific search engines, including Scinfinder, Medline OvidSP, PubMed, and Scopus. Only those articles focused on the traditional uses and pharmacological properties of African *Strychnos* species were selected. The majority of these articles were written in English or French. Articles in other languages were translated using translation software such as DeepL.

Results: The traditional uses and pharmacological properties of African *Strychnos* species have shown that these plants are effective against parasitic infections, particularly malaria, as well as microbial and fungal infections, cancer cells, snake and scorpion venoms. They also affect various biological systems in the human body, including the digestive, respiratory, urinary, and reproductive systems. These activities have been confirmed through *in vitro* and *in vivo* assays of crude extracts and alkaloids isolated from the genus. Additionally, the toxic effects described in traditional uses have also been validated in *in vivo* tests.

Conclusion: The exploration of the diverse traditional uses and pharmacological properties reported in the literature reveals that antiplasmodial/antimalarial activities are the most extensively studied in African *Strychnos* species, followed by cytotoxic/anticancer and antimicrobial activities. Furthermore, the plant parts used, as well as the preparation and administration methods, vary across indigenous populations based on their cultural practices. Among the seventy-five African *Strychnos* species, twenty-seven remain poorly known, primarily due to their low alkaloid content and rarity. Investigating these neglected species could lead to the discovery of new therapeutically valuable metabolites. The advancement of more sensitive techniques for metabolite exploration and isolation presents a unique opportunity to study and identify previously overlooked minor metabolites.

I. INTRODUCTION

For centuries, medicinal plants have been used to treat a wide range of ailments. As an example, the first known compendium on medicinal plants was written by the Egyptologist Georges Ebers in 1500 B.C.E. (Aboelsoud, N. H., 2010). Today, many modern medicines come from natural sources or are inspired by natural compounds. However, a vast portion of the plant world remains unexplored. Further research is essential to uncover the therapeutic molecules hidden within the fascinating world of plants.

This review focuses on pantropical plants of the *Strychnos* genus, a member of the Loganiaceae family, which includes approximately 200 species across Africa, America, Asia, and Oceania (Ohiri, F. C. *et al.*, 1983a; Rasoanaivo, P. *et al.*, 2002; Philippe, G. *et al.*, 2004). For over two centuries, *Strychnos* species have captured the attention of researchers due to both their toxic properties (tetanizing and paralyzing) and their therapeutic potential. During these studies, a wide array of metabolites, primarily monoterpene indole alkaloids, have been isolated and identified from *Strychnos* plants. Notable alkaloids include usambarensine, strychnobiline, sungucine, and strychnogucine A, B, and C (Angenot, L., and Bisset, N. G., 1971a; Tits, M., and Angenot, L., 1978; Lamotte, J. *et al.*, 1979; Frédérich, M. *et al.*, 2001b; Philippe, G. *et al.*, 2003). Additionally, a variety of traditional uses and pharmacological properties have been documented for these plants. For instance, antiplasmodial, antimicrobial, antidiabetic, and antitubercular activities have been attributed to the genus (Wright, C. W. *et al.*, 1991; Wright, C. W. *et al.*, 1994; Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2002; Philippe, G. *et al.*, 2005; Oyedemi, S. O. *et al.*, 2012; Madikizela, B. *et al.*, 2017; Ampa, R. *et al.*, 2018; Oryema, C. *et al.*, 2021; Maroyi, A., 2022; Uttu, A. J. *et al.*, 2022).

Focusing on African species, the objective of this review is to compile and summarize all the documented traditional uses and pharmacological properties of the seventy-five African *Strychnos* species in both therapeutic and toxic contexts (Ohiri, F. C. *et al.*, 1983a; Rasoanaivo, P. *et al.*, 2002; Philippe, G. *et al.*, 2004). This comprehensive study will centralize the various uses and properties of these plants and highlight traditional uses that have been confirmed through pharmacological studies.

II. REVIEWING METHODOLOGY

Using scientific search engines, namely Scinfinder, Medline OvidSP, PubMed and Scopus, the articles from 1946 to 2014 dealing with the traditional uses and pharmacological properties of the seventy-five African *Strychnos* were selected. The table below (Table 1) lists the full names of the seventy-five species.

Table 1: The full names of the seventy-five African *Strychnos* species.

S. aculeata Soler.	*S. gnetifolia* Gilg ex Onochie & Hepper	*S. odorata* A.Chev.
S. afzelii Gilg	*S. gossweileri* Exell	*S. panganensis* Gilg
S. angolensis Gilg	S*. henningsii* Gilg	*S. penninervis* A.Chev.
S. asterantha Leeuwenb.	*S. icaja* Baill.	*S. pentantha* Leeuwenb.
S. barteri Soler.	*S. innocua* Delile	*S. phaeotricha* Gilg
S. bifurcata Leeuwenb.	*S. johnsonii* Hutch. & M.B.Moss	*S. potatorum* L.f.
S. boonei De Wild.	*S. kasengaensis* De Wild.	*S. pungens* Soler.
S. campicola Gilg ex Leeuwenberg	*S. longicaudata* Gilg	*S. retinervis* Leeuwenb.
S. camptoneura Gilg & Busse	*S. lucens* Baker	*S. samba* P.A.Duvign.
S. canthioides Leeuwenberg	*S. madagascariensis* Poir.	*S. scheffleri* Gilg
S. chromatoxylon Leeuwenb.	*S. malacoclados* C.H.Wright	*S. soubrensis* Hutch. & Dalziel
S. chrysophylla Gilg	*S. malchairi* De Wild.	*S. spinosa* Lam.

S. cocculoides Baker	*S. matopensis* S.Moore	*S. splendens* Gilg
S. congolana Gilg	*S. melastomatoides* Gilg	*S. staudtii* Gilg
S. cuminodora Leeuwenb.	*S. mellodora* S.Moore	*S. talbotiae* S.Moore
S. cuniculina Leeuwenb.	*S. memecycloides* S.Moore	*S. tchibangensis* Pellegr.
S. dale De Wild.	*S. millepunctata* Leeuwenb.	*S. ternata* Gilg ex Leeuwenb.
S. decussata (Pappe) Gilg	*S. mimfiensis* Gilg ex Leeuwenb.	*S. tricalysioides* Hutch. & M.B.Moss
S. densiflora Baill.	*S. mitis* S.Moore	*S. trichoneura* Leeuwenb.
S. dinklagei Gilg	*S. moandaensis* De Wild.	*S. urceolata* Leeuwenb.
S. diplotricha Leeuwenb.	*S. mostueoides* Leeuwenb.	*S. usambarensis* Gilg ex Engl.
S. dolichothyrsa Gilg ex Onochie & Hepper	*S. myrtoides* Gilg & Busse	*S. variabilis* De Wild.
S. elaeocarpa Gilg ex Leeuwenb.	*S. ndengensis* Pellegr.	*S. xantha* Leeuwenb.
S. fallax Leeuwenb.	*S. ngouniensis* Pellegr.	*S. xylophylla* Gilg
S. floribunda Gilg	*S. nigritana* Baker	*S. zenkeri* Gilg ex Baker

In terms of language, most articles were in English or French. A minority was written in another language. However, thanks to translation software (DeepL and Google Lens), these articles were translated and read.

III. TRADITIONAL USES AND PHARMACOLOGICAL PROPERTIES

III.1. Infections

III.1.1. Parasitic infections

III.1.1.1. Traditional uses

Parasitic infections include parasites of the *Plasmodium* genus, which are responsible for malaria, parasitic worms such as helminths, protozoa, and trypanosomes. Plants of the *Strychnos* genus are mainly known for their antimalarial properties.

Malaria is a blood-borne disease transmitted by mosquitoes of the *Anopheles* genus, which serve as vectors. The main symptom of malaria is recurring fever, depending on the parasite's life cycle. During the blood stage, also known as the asexual cycle, parasites rupture red blood cells, resulting in fever. Several species of the *Plasmodium* genus are responsible for malaria in humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi*, and *P. simium*. Unfortunately, despite the development of various antimalarial treatments, the parasites have developed resistance (World Health Organization, 2023). The disease remains prevalent today, particularly in Africa. For both preventive and curative purposes, many plant species are traditionally used by local populations. The table below lists these species (Table 2).

Table 2: African species of the *Strychnos* genus traditionally used in the event of malaria infection.

African species	Plant parts	Countries	Administration methods	References
Strychnos camptoneura	Stem barks	Congo	Aqueous extract, *per os*	Morabandza, C. J. *et al.*, 2016
S. dale	Roots	Cameroon	Unspecified	Rolfsen, W. *et al.*, 1978
S. decussata	Leaves	Madagascar	Infusion, *per os*	Rasoanaivo, P. *et al.*, 2002 Maroyi, A., 2022

African species	Plant parts	Countries	Administration methods	References
S. diplotricha	Stem barks	Madagascar	Infusion, *per os* In association with chloroquine	Rasoanaivo, P. *et al.*, 1996 Rasoanaivo, P. *et al.*, 2002
S. henningsii	Leaves	Kenya	Tea, *per os*	Rasoanaivo, P. *et al.*, 2002 Kirira, P. G. *et al.*, 2006
	Stem barks		Unspecified	
S. icaja	Roots	Cameroon	Unspecified	Neuwinger, H. D., 1996 Tchinda, A. T. *et al.*, 2012a
S. innocua	Leaves	Ghana	Solution obtained by boiling leaves, *per os*	Asase, A. *et al.*, 2005
S. madagascariensis	Leaves	Unspecified	Infusion, *per os*	Bisset, N. G., 1970 Rasoanaivo, P. *et al.*, 2002
	Stem barks			
S. matopensis	Unspecified	Unspecified	Unspecified	Francis, P., and Suseem, S. R., 2016
S. mitis	Leaves	Unspecified	Unspecified	Adamu, M. *et al.*, 2013
S. mostueoides	Stems	Madagascar, and Congo	Infusion, *per os* In association with chloroquine	Rasoanaivo, P. *et al.*, 1996 Rasoanaivo, P. *et al.*, 2002
S. myrtoides	Leaves	Madagascar	Infusion, *per os* In association with chloroquine	Rasoanaivo, P. *et al.*, 2002
	Stems			
S. potatorum	Roots	Zimbabwe	Decoction, *per os*	Ngarivhume, T. *et al.*, 2015
	Stems			

African species	Plant parts	Countries	Administration methods	References
S. spinosa	Leaves	Ghana, Guinea	Solution obtained by boiling leaves, *per os*	Bisset, N. G., 1970 Asase, A. *et al.*, 2005 Aremu, A. O., and Moyo, M., 2022 Baldé, A. M. *et al.*, 2021
	Twigs		Apply an ointment of twig powders to the body, external use	
	Roots		Unspecified	
S. usambarensis	Leaves	Unspecified	Unspecified	Francis, P., and Suseem, S. R., 2016 Frédérich, M., 2024 (Internal communication)

As for parasitic infections caused by worms, protozoa or trypanosomes, few traditional uses are described. Only *S. aculeata*, *S. decussata*, *S. henningsii*, *S. innocua*, and *S. spinosa* are used as anthelmintics. In Mozambique, a root decoction of *S. decussata* is taken orally (Maroyi, A., 2022). A decoction of *S. henningsii* barks is traditionally used against gastrointestinal parasites in goats (Francis, P., and Suseem, S. R., 2016). This species is also used in humans. However, no information is available in the literature regarding the method of administration (Bisset, N. G., 1970; Ogeto, J. O. *et al.*, 1984; Tits, M. *et al.*, 1991). A decoction of *S. boonei* barks and the roots of *S. innocua* are used as vermifuges. No further details are provided in the literature (Bisset, N. G., 1970; Angenot, L., and Wauters, J. N., 1974). Regarding *S. aculeata* and *S. spinosa*, their fruits and roots, respectively, are used in cases of intestinal worms (Bisset,

N. G., 1970; Neuwinger, H. D., 1996). An article by Morabandza, C. J. *et al.* mentions the use of an aqueous extract of *S. camptoneura* stem barks for antiparasitic infections other than malaria. However, since no specific details are provided, *S. camptoneura* is regarded as a potential anthelmintic plant.

III.1.1.2. Pharmacological properties

Given the rise in malaria cases and deaths, many researchers have sought new therapeutic avenues, particularly in plants. Among these are plants of the *Strychnos* genus. For this reason, numerous antiplasmodial (*in vitro*) and antimalarial (*in vivo*) tests have been conducted on various *Strychnos* species and their metabolites. The tables below (Tables 3 and 4) list the *in vitro* active species, as well as the *in vitro* and *in vivo* active metabolites isolated from these species, respectively.

Table 3: African *Strychnos* species active *in vitro* against *Plasmodium* parasites.

Species	Plant parts, and extract types	Antiplasmodial activities	Strains of *P. falciparum*	References
Strychnos angolensis	Leaves (Methanol)	Moderate activity	3D7	Frédérich, M. *et al.*, 1999 Philippe, G. *et al.*, 2005 Bonnet, O. *et al.*, 2022a
	Leaves (Ethanol)		FCA 20 Ghana	
	Roots (Ethyl acetate)	Moderate activity	FCA 20 Ghana	
	Roots (Methanol)	Good activity	3D7	
	Roots (Ethanol)	Moderate activity	FCA 20 Ghana	
S. camptoneura	Trunk barks (Methanol)	Moderate activity	3D7	Bonnet, O. *et al.*, 2022a
S. cocculoides	Leaves (Ethyl acetate)	Moderate activity	FCA 20 Ghana	Philippe, G. *et al.*, 2005
S. congolana	Trunk barks (Methanol)	Weak activity	3D7	Bonnet, O. *et al.*, 2022a

Species	Plant parts, and extract types	Antiplasmodial activities	Strains of *P. falciparum*	References
S. decussata	Stem barks (Alkaloidic extract)	Moderate activity	Unspecified (Chloroquine-resistant strain)	Rasoanaivo, P. *et al.*, 2002 Maroyi, A., 2022
S. densiflora	Trunk barks (Methanol)	Moderate activity	3D7	Bonnet, O. *et al.*, 2022a
S. gossweileri	Roots (Ethyl acetate)	Good activity	FCA 20 Ghana	Frédérich, M. *et al.*, 1999 Philippe, G. *et al.*, 2005
	Roots (Ethanol)	Weak activity		
S. henningsii	Leaves (Ethyl acetate)	Moderate activity	FCA 20 Ghana	Philippe, G. *et al.*, 2005 Bonnet, O. *et al.*, 2022a
	Leaves (Methanol)	Weak activity	3D7	
	Leaves (Ethanol)		FCA 20 Ghana	
S. icaja	Collar barks (Methanol)	Good activity	3D7	Frédérich, M. *et al.*, 1999 Philippe, G. *et al.*, 2005 Lusakibanza, M. *et al.*, 2010 Bonnet, O. *et al.*, 2022a
	Leaves (Methanol)	Weak activity	FCA 20 Ghana	
	Roots (Ethyl acetate)	Promising activity	FCA 20 Ghana, and W2 Indochina	
	Roots (Ethanol)			
	Roots (Methanol)		3D7, and W2 Indochina	
	Trunk barks (Methanol)	Moderate activity	3D7	
S. johnsonii	Stems (Ethyl acetate)	Moderate activity	FCA 20 Ghana	Philippe, G. *et al.*, 2005
S. madagascariensis	Stem barks (Alkaloidic extract)	Good activity	Unspecified	Delaude, C. *et al.*, 1992 Rasoanaivo, P. *et al.*, 2002

Species	Plant parts, and extract types	Antiplasmodial activities	Strains of *P. falciparum*	References
S. malacoclados	Root barks (Methanol)	Moderate activity	3D7	Bonnet, O. *et al.*, 2022a
S. malchairi	Leaves (Methanol)	Good activity	3D7	Bonnet, O. *et al.*, 2022a
S. mellodora	Leaves (Ethyl acetate)	Good activity	FCA 20 Ghana	Philippe, G. *et al.*, 2005
	Leaves (Methanol)	Moderate activity		
	Stems (Ethyl acetate)	Good activity		
	Roots (Ethyl acetate)	Good activity		
	Roots (Methanol)	Moderate activity		
S. memecyloides	Leaves (Ethyl acetate)	Good activity	FCA 20 Ghana	Frédérich, M. *et al.*, 1999
S. mostueoides	Stem barks (Alkaloidic extract)	Good activity	Unspecified	Rasoanaivo, P. *et al.*, 1996 Rasoanaivo, P. *et al.*, 2002
S. myrtoides	Stem barks (Alkaloidic extract)	Potentiator of choroquine's antiplasmodial activity	FCM 29 Cameroon	Rasoanaivo, P. *et al.*, 1994
S. phaeotricha	Leaves (Methanol)	Good activity	3D7	Bonnet, O. *et al.*, 2022a
S. scheffleri	Leaves (Ethyl acetate)	Moderate activity	FCA 20 Ghana	Philippe, G. *et al.*, 2005
S. spinosa	Leaves (Dichloro-methane)	Moderate activity	3D7	Bero, J. *et al.*, 2009
		Good activity	W2 Indochina	

Species	Plant parts, and extract types	Antiplasmodial activities	Strains of *P. falciparum*	References
S. tchibangensis	Trunk barks (Methanol)	Weak activity	3D7	Bonnet, O. *et al.*, 2022a
S. usambarensis	Barks (Liana) (Ethyl acetate)	Promising activity	FCA 20 Ghana, and W2 Indochina	Frédérich, M. *et al.*, 1999 Philippe, G. *et al.*, 2005 Bonnet, O. *et al.*, 2022a
	Barks (Tree) (Ethanol)	Moderate activity		
	Leaves (Ethanol)	Promising activity	FCA 20 Ghana, and 3D7	
	Leaves (Methanol)			
	Roots (Ethyl acetate)			
	Roots (Methanol)			
S. variabilis	Roots (Ethyl acetate)	Promising activity	FCA 20 Ghana, and 3D7	Frédérich, M. *et al.*, 1999 Philippe, G. *et al.*, 2005 Bonnet, O. *et al.*, 2022a
	Roots and root barks (Methanol)			
	Barks (Ethanol)	Moderate activity	FCA 20 Ghana	

Interpretation of antiplasmodial activities: promising activity ($IC_{50} \leq 5$ µg/mL), good activity (IC_{50} between 5 and 15 µg/mL), moderate activity (IC_{50} between 15 and 30 µg/mL), weak activity (IC_{50} between 30 and 50 µg/mL), and no activity ($IC_{50} > 50$ µg/mL). 3D7 and FCA 20 Ghana strains are sensible to chloroquine. W2 Indochina and FCM 29 Cameroon strains are resistant to chloroquine.

Regarding *in vivo* tests of crude extracts, an alkaloidic extract from *S. myrtoides* stem barks was tested for its chloroquine potentiating activity. A significant increase in chloroquine efficacy was observed against chloroquine-resistant *Plasmodium yoelii* at a dose of 100 mg/kg/day, reducing parasitemia by 81.10% after five days (Rasoanaivo, P. *et al.*, 1994; Benelli, G. *et al.*, 2017).

Moreover, hydromethanolic, hexane, chloroform, and aqueous crude extracts from *S. mitis* leaves significantly inhibited the growth of *Plasmodium berghei* in infected mice. At the highest dose of 600 mg/kg/day, the aqueous and hydro-methanolic extracts were most effective, suppressing parasitemia by 95.5% and 93.97%, respectively, after four days. A dose-dependent prolongation of survival times was also observed in infected mice (Fentahun, S. *et al.*, 2017).

Table 4: Alkaloids active *in vitro* and/or *in vivo* against *Plasmodium* parasites.

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Akagerine (β-carboline-type)	*S. barteri* (RT, SB) *S. camptoneura* (SB) *S. decussata* (SB) *S. elaeocarpa* (SB) *S. floribunda* (SB) *S. johnsonii* (RB) *S. nigritana* (L, RB) *S. phaeotricha* (RB, SB) *S. spinosa* (L, SB) *S. usambarensis* (Fr, RB)	*In vitro*	Weak activity (K1)	Angenot, L. *et al.*, 1975a Verpoorte, R. *et al.*, 1975 Rolfsen, W. *et al.*, 1978 Oguakwa, J. U. *et al.*, 1980 Rolfsen, W. *et al.*, 1980b Verpoorte, R. *et al.*, 1981 Ohiri, F. C. *et al.*, 1983a Quetin-Leclercq, J. *et al.*, 1986 Massiot, G. *et al.*, 1987 Wright, C. W. *et al.*, 1991 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Hoet, S. *et al.*, 2007 Cao, M. *et al.*, 2012 Casciaro, B. *et al.*, 2020 Maroyi, A., 2022 Tchangou Njiemou, A. F. *et al.*, 2022

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Akagerine lactone (β-carboline-type)	*S. decussata* (SB) *S. johnsonii* (RT, SB)	*In vitro*	Weak activity (FCA 20 Ghana, FCB1-R Colombia, and W2 Indochina)	Olaniyi, A. A., and Rolfsen, W. N. A., 1980 Massiot, G. *et al.*, 1987 Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002 Rasoanaivo, P. *et al.*, 2002
Bisnordihydrotoxiferine (Toxiferine-type)	*S. afzelii* (SB) *S. decussata* (SB) *S. dolichothyrsa* (SB) *S. elaeocarpa* (SB) *S. floribunda* (SB) *S. icaja* (RT, SB) *S. kasengaensis* (SB) *S. longicaudata* (RB, SB) *S. malacoclados* (SB) *S. matopensis* (RB) *S. potatorum* (RB) *S. scheffleri* (SB) *S. variabilis* (RB)	*In vitro*	Moderate activity (FCA 20 Ghana and W2 Indochina)	Massiot, G. *et al.*, 1983a Ohiri, F. C. *et al.*, 1983a Thepenier, P. *et al.*, 1984 Massiot, G. *et al.*, 1988 Massiot, G. *et al.*, 1992 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 1999 Rasoanaivo, P. *et al.*, 2002 Philippe, G. *et al.*, 2003 Tchinda, A. T. *et al.*, 2012b
Chrysopentamine (Usambarensine-type)	*S. usambarensis* (L)	*In vitro*	Promising activity (FCA 20 Ghana, FCB1-R Colombia, and W2 Indochina)	Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2004a

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
17-O-acetyl-10-hydroxycorynantheol (Indoloquinolizine-type)	*S. usambarensis* (L)	*In vitro*	Moderate activity (3D7) Weak activity (W2 Indochina) *High selectivity towards Plasmodium strains (SI close to 1000)*	Cao, M. *et al.*, 2011
Divarine (Toxiferine-type)	*S. malacoclados* (SB)	*In vitro*	Moderate activity (3D7) Promising activity (W2 Indochina)	Tchinda, A. T. *et al.*, 2012b
5,6-Dihydroflavopereirine (Indoloquinolizine-type)	*S. usambarensis* (RB)	*In vitro*	Moderate activity (K1)	Caprasse, M. *et al.*, 1983a Ohiri, F. C. *et al.*, 1983a Wright, C. W. *et al.*, 1991 Wright, C. W. *et al.*, 1994 Wright, C. W. *et al.*, 1996 Delaude, C., and Delaude, L., 1997
Demethoxycarbonyl-3,14-dihydrogambirtannine = Descarbomethoxydihydrogambirtannine (Ajmalicine-type)	*S. johnsonii* (RB, SB)	*In vitro*	Moderate activity (FcM29) Weak activity (FCA 20 Ghana, FCB1-R Colombia, and W2 Indochina)	Angenot, L. *et al.*, 1978c Massiot, G. *et al.*, 1987 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002 Passemar, C. *et al.*, 2011
	S. usambarensis (Fr)			

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Holstiine (Tsilanine-type)	*S. henningsii* (SB)	*In vitro*	Weak activity (FCA 20 Ghana, K1, and W2 Indochina)	Ohiri, F. C. *et al.*, 1983a Massiot, G. *et al.*, 1991 Wright, C. W. *et al.*, 1991 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 1999 Rasoanaivo, P. *et al.*, 2002
Holstiline (Tsilanine-type)	*S. henningsii* (SB)	*In vitro*	Weak activity (K1)	Ohiri, F. C. *et al.*, 1983a Massiot, G. *et al.*, 1991 Wright, C. W. *et al.*, 1991 Wright, C. W. *et al.*, 1994 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002
Icajine (Strychnine-type)	*S. icaja* (Fr, L, RB, SB)	*In vitro*	No activity but it reverses chloroquine and mefloquine resistance (W2 Indochina)	Bisset, N. G., 1965 Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 1999 Frédérich, M. *et al.*, 2001a Rasoanaivo, P. *et al.*, 2002 Rasoanaivo, P. *et al.*, 2011 Tchinda, A. T. *et al.*, 2012a

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Janussine A (Usambarensine-type)	*S. johnsonii* (RB, SB)	*In vitro*	Moderate activity (FCA 20 Ghana) Weak activity (FCB1-R Colombia and W2 Indochina)	Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002
Janussine B (Usambarensine-type)	*S. johnsonii* (RB, SB)	*In vitro*	Weak activity (FCA 20 Ghana and FCB1-R Colombia) Moderate activity (W2 Indochina)	Massiot, G. *et al.*, 1987 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002
Longicaudatine (Indoloquinolizine-type)	*S. afzelii* (SB) *S. chrysophylla* (SB) *S. dolichothyrsa* (SB) *S. longicaudata* (RB, SB) *S. malacoclados* (SB) *S. matopensis* (RB) *S. mimfiensis* (RB) *S. ngouniensis* (RB, TB) *S. urceolata* (SB)	*In vitro*	Promising activity (3D7, FCA 20 Ghana, FCB1-R Colombia, and W2 Indochina)	Massiot, G. *et al.*, 1983a Massiot, G. *et al.*, 1983b Ohiri, F. C. *et al.*, 1983a Verpoorte, R. *et al.*, 1984 Delaude, C. *et al.*, 1992 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002 Tchinda, A. T. *et al.*, 2012b
Longicaudatine F (Indoloquinolizine-type)	*S. longicaudata* (RB, SB) *S. malacoclados* (SB) *S. matopensis* (RB) *S. mimfiensis* (RB)	*In vitro*	Moderate activity (3D7, FCA 20 Ghana, and W2 Indochina) Weak activity (FCB1-R Colombia)	Delaude, C. *et al.*, 1992 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002 Tchinda, A. T. *et al.*, 2012b

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Longicaudatine Y (Indoloquinolizine-type)	*S. longicaudata* (RB, SB) *S. malacoclados* (SB) *S. matopensis* (RB)	*In vitro*	Moderate activity (3D7) Promising activity (W2 Indochina)	Delaude, C., and Delaude, L., 1997 Tchinda, A. T. *et al.*, 2012b
3-Hydroxylongicaudatine Y (Indoloquinolizine-type)	*S. malacoclados* (SB)	*In vitro*	Moderate activity (3D7) Weak activity (W2 Indochina)	Tchinda, A. T. *et al.*, 2012b
Tetrahydrolongicaudatine Y (Indoloquinolizine-type)	*S. usambarensis* (SB)	*In vitro*	Promising activity (W2 Indochina) Moderate activity (FCA 20 Ghana)	Frédérich, M. *et al.*, 1998a Frédérich, M. *et al.*, 2002

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Malagashanine (Spermostrychnine-type)	*S. diplotricha* (SB) *S. mostueoides* (SB) *S. myrtoides* (SB)	*In vitro*	Weak activity but it reverses chloroquine and mefloquine resistance (W2 Indochina)	Rasoanaivo, P. *et al.*, 2001 Rasoanaivo, P. *et al.*, 2002 Frédérich, M. *et al.*, 2008
		In vivo	15% inhibition of parasitemia with a single dose at 10 ppm, and 78% inhibition with the same dose combined with 0.75 ppm of chloroquine (Assay on performed on *Plasmodium yoelii* N67, a chloroquine-resistant strain)	
Matopensine (Toxiferine-type)	*S. kasengaensis* (RB, SB) *S. matopensis* (RB) *S. mimfiensis* (RB) *S. panganensis* (RB)	*In vitro*	Moderate activity (FCA 20 Ghana) Promising activity (FCB1-R Colombia and W2 Indochina)	Massiot, G. *et al.*, 1983c Massiot, G. *et al.*, 1988 Delaude, C. *et al.*, 1992 Nuzillard, J.-M. *et al.*, 1996 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002 Rasoanaivo, P. *et al.*, 2002
Matopensine N-oxide (Toxiferine-type)	*S. kasengaensis* (SB) *S. matopensis* (RB)	*In vitro*	Weak activity (FCA 20 Ghana) Moderate activity (W2 Indochina)	Massiot, G. *et al.*, 1988 Frédérich, M. *et al.*, 2002

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
18-Hydroxymatopensine (Toxiferine-type)	*S. matopensis* (RB)	*In vitro*	Moderate activity (FCA 20 Ghana and FCB1-R Colombia) Promising activity (W2 Indochina)	Massiot, G. *et al.*, 1988 Frédérich, M. *et al.*, 2002
16-Methoxyisomatopensine (Toxiferine-type)	*S. matopensis* (RB)	*In vitro*	Promising activity (FCA 20 Ghana and W2 Indochina) Moderate activity (FCB1-R Colombia) *Good selectivity towards Plasmodium strains (SI value not provided)*	Massiot, G. *et al.*, 1988 Frédérich, M. *et al.*, 2002
Moandaensine (Indoloquinolizine-type)	*S. moandaensis* (RB)	*In vitro*	Weak activity (FCA 20 Ghana) Moderate activity (W2 Indochina)	Verpoorte, R. *et al.*, 2010
Ngouniensine (β-carboline-type)	*S. ngouniensis* (RB)	*In vitro*	Weak activity (FCA 20 Ghana and W2 Indochina)	Massiot, G. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002
Epingouniensine (β-carboline-type)	*S. ngouniensis* (RB)	*In vitro*	Moderate activity (FCA 20 Ghana) Weak activity (W2 Indochina)	Massiot, G. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Ochrolifuanine A (Usambarensine-type)	*S. potatorum* (RB)	*In vitro*	Promising activity (FCA 20 Ghana, FCB1-R Colombia, and W2 Indochina)	Massiot, G. *et al.*, 1992 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002
Ochrolifuanine E (Usambarensine-type)	*S. potatorum* (RB)	*In vitro*	Promising activity (FCA 20 Ghana, FCB1-R Colombia, and W2 Indochina)	Massiot, G. *et al.*, 1992 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002
S-Panganensine (Retuline-type)	*S. panganensis* (RB)	*In vitro*	Moderate activity (FCA 20 Ghana and FCB1-R Colombia) Weak activity (W2 Indochina)	Nuzillard, J.-M. *et al.*, 1996 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002 Rasoanaivo, P. *et al.*, 2002
Panganensine X (Retuline-type)	*S. panganensis* (RB)	*In vitro*	Weak activity (FCA 20 Ghana, FCB1-R Colombia, and W2 Indochina)	Nuzillard, J.-M. *et al.*, 1996 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002 Rasoanaivo, P. *et al.*, 2002
Panganensine Y (Retuline-type)	*S. panganensis* (RB)	*In vitro*	Weak activity (FCA 20 Ghana, FCB1-R Colombia, and W2 Indochina)	Nuzillard, J.-M. *et al.*, 1996 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002 Rasoanaivo, P. *et al.*, 2002

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Retuline (Retuline-type)	*S. camptoneura* (SB) *S. henningsii* (RB, SB) *S. kasengaensis* (L, RB) *S. variabilis* (L, RB, S, SB)	*In vitro*	Weak activity (K1)	Tits, M., and Angenot, L., 1980a Ohiri, F. C. *et al.*, 1983a Thepenier, P. *et al.*, 1984 Thepenier, P. *et al.*, 1990 Wright, C. W. *et al.*, 1991 Wright, C. W. *et al.*, 1994 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002
Isoretuline (Retuline-type)	*S. kasengaensis* (SB) *S. variabilis* (L, RB)	*In vitro*	No activity (K1) but it reverses chloroquine and mefloquine resistance (W2 Indochina)	Tits, M., and Angenot, L., 1980a Thepenier, P. *et al.*, 1984 Thepenier, P. *et al.*, 1990 Wright, C. W. *et al.*, 1991 Wright, C. W. *et al.*, 1994 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2001a
O-Acetylisoretuline (Retuline-type)	*S. kasengaensis* (L, SB) *S. variabilis* (L, RB, S)	*In vitro*	Weak activity (K1)	Ohiri, F. C. *et al.*, 1983a Thepenier, P. *et al.*, 1984 Thepenier, P. *et al.*, 1990 Wright, C. W. *et al.*, 1991 Wright, C. W. *et al.*, 1994

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Retulinal (Retuline-type)	*S. variabilis* (RB)	*In vitro*	Weak activity (K1)	Tits, M. *et al.*, 1980b Wright, C. W. *et al.*, 1991 Wright, C. W. *et al.*, 1994
Isoretulinal (Retuline-type)	*S. kasengaensis* (RB) *S. variabilis* (RB)	*In vitro*	Weak activity (K1)	Tits, M. *et al.*, 1980b Wright, C. W. *et al.*, 1991 Wright, C. W. *et al.*, 1994
9-Methoxy-(16R)-*E*-isositsirikine (Indoloquinolizine-type)	*S. lucens* (RB, SB) *S. madagascariensis* (L, SB)	*In vitro*	Weak activity (FCA 20 Ghana) Moderate activity (W2 Indochina)	Delaude, C. *et al.*, 1992 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002 Rasoanaivo, P. *et al.*, 2002
Strychnine (Strychnine-type)	*S. boonei* (TB) *S. camptoneura* (TB) *S. congolana* (TB) *S. densiflora* (TB) *S. icaja* (B, L, RB, SB) *S. tchibangensis* (TB) *S. usambarensis* (L)	*In vitro*	Inactive (FCA 20 Ghana) Weak activity (W2 Indochina)	Sandberg, F. *et al.*, 1969 Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 1999 Tchinda, A. T. *et al.*, 2012a Bonnet, O. *et al.*, 2022b
N-methyl-sec-isopseudostrychnine (Strychnine-type)	*S. icaja* (SB)	*In vitro*	Weak activity (3D7)	Delaude, C., and Delaude, L., 1997 Tchinda, A. T. *et al.*, 2012a
Strychnobiline (Strychnine-type)	*S. variabilis* (RB)	*In vitro*	Moderate activity (K1)	Tits, M., and Angenot, L., 1978 Wright, C. W. *et al.*, 1991 Wright, C. W. *et al.*, 1994

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Isostrychnobiline (Strychnine-type)	*S. variabilis* (RB)	*In vitro*	Moderate activity (K1)	Tits, M., and Angenot, L., 1978 Wright, C. W. *et al.*, 1991 Wright, C. W. *et al.*, 1994
16,17-Dehydroisostrychnobiline (Strychnine-type)	*S. kasengaensis* (RB) *S. variabilis* (RB)	*In vitro*	Moderate activity (FCA 20 Ghana, K1, and W2 Indochina)	Thepenier, P. *et al.*, 1984 Wright, C. W. *et al.*, 1994 Frédérich, M. *et al.*, 2002
12'-Hydroxyisostrychnobiline (Strychnine-type)	*S. kasengaensis* (RB) *S. variabilis* (RB)	*In vitro*	Moderate activity (K1)	Tits, M. *et al.*, 1979 Thepenier, P. *et al.*, 1984 Wright, C. W. *et al.*, 1994
Strychnobrasiline (Spermostrychnine-type)	*S. diplotricha* (SB) *S. mostueoides* (SB) *S. myrtoides* (L, SB) *S. scheffleri* (L) *S. soubrensis* (SB)	*In vitro*	Weak activity but it reverses chloroquine and mefloquine resistance (W2 Indochina)	Caprasse, M., and Angenot, L., 1981a Ohiri, F. C. *et al.*, 1983a Rasoanaivo, P. *et al.*, 1994 Rasoanaivo, P. *et al.*, 1996 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2001a Rasoanaivo, P. *et al.*, 2002 Frédérich, M. *et al.*, 2008 Rasoanaivo, P. *et al.*, 2011 Benelli, G. *et al.*, 2017
		In vivo	8% inhibition of parasitemia with a dose of 10 ppm and antagonistic effect on chloroquine activity (Assay performed on *Plasmodium yoelii* N67, a chloroquine-resistant strain)	

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Strychnochromine (Spermostrychnine-type)	*S. gossweileri* (RB) *S. henningsii* (RB)	*In vitro*	Weak activity (FCA 20 Ghana and W2 Indochina)	Quetin-Leclercq, J. *et al.*, 1991a Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 1999 Muthaura, C. N. *et al.*, 2015
Strychnofoline (β-carboline-type)	*S. usambarensis* (L, SB)	*In vitro*	Weak activity (K1)	Angenot, L., 1978a Quetin-Leclercq, J. *et al.*, 1991b Wright, C. W. *et al.*, 1991 Wright, C. W. *et al.*, 1994
Isostrychnofoline (β-carboline-type)	*S. usambarensis* (L, SB)	*In vitro*	Moderate activity (FCA 20 Ghana and W2 Indochina)	Angenot, L., 1978a Quetin-Leclercq, J. *et al.*, 1991b Wright, C. W. *et al.*, 1994 Frédérich, M. *et al.*, 1999

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Strychnofurarine (Indoloquinolizine-type)	*S. matopensis* (RB)	*In vitro*	Moderate activity (FCA 20 Ghana, F32 Tanzania, W2 Indochina, and PFB Brazil) Weak activity (FCB1-R Colombia)	Massiot, G. *et al.*, 1988 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002
Strychnogucine A (Strychnine-type)	*S. icaja* (RB)	*In vitro*	Moderate activity (FCA 20 Ghana)	Frédérich, M. *et al.*, 2001b Philippe, G. *et al.*, 2003

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Strychnogucine B (Strychnine-type)	*S. icaja* (RB)	*In vitro*	Promising activity (FCA 20 Ghana and FCB1-R Colombia) *Relatively selective against Plasmodium, being selective against chloroquine-resistant strains with an SI of 176 (determined on the W2 Indochina strain)*	Frédérich, M. *et al.*, 2001b Frédérich, M. *et al.*, 2002 Philippe, G. *et al.*, 2003 Beaufay, C. *et al.*, 2018
		In vivo	Dose of 30 mg/kg reduced parasitemia by 60.3% after 7 days (Assay performed on *Plasmodium berghei*, a chloroquine-sensitive strain)	
Strychnogucine C (Strychnine-type)	*S. icaja* (RB, SB)	*In vitro*	Weak activity (FCA 20 Ghana)	Philippe, G. *et al.*, 2003 Tchinda, A. T. *et al.*, 2012a

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Strychnohexamine (Toxiferine-type)	*S. icaja* (RB)	*In vitro*	Moderate activity (FCA 20 Ghana)	Philippe, G. *et al.*, 2002 Philippe, G. *et al.*, 2003
Strychnopentamine (Usambarensine-type)	*S. usambarensis* (L, SB)	*In vitro*	Promising activity (FCA 20 Ghana, K1, and W2 Indochina)	Angenot, L. *et al.*, 1978b Ohiri, F. C. *et al.*, 1983a Wright, C. W. *et al.*, 1991 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Frédérich, M. *et al.*, 2004a
		In vivo	Inactive against *Plasmodium berghei*, a chloroquine-sensitive strain	

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Isostrychnopentamine (Usambarensine-type)	*S. usambarensis* (L)	*In vitro*	Promising activity (FCA 20 Ghana, F32 Tanzania, FCB1-R Colombia, W2 Indochina, K1, and PFB Brazil) *SI HCT-116, HCT-15, WI-38, KB/Plasmodium: 41-145, 83-295, 15-50, and 119-421 (SI ranges according to Plasmodium strains)*	Angenot, L. *et al.*, 1978b Wright, C. W. *et al.*, 1991 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002 Frédérich, M. *et al.*, 2004b Cao, M. *et al.*, 2011
		In vivo	Dose of 30 mg/kg/day reduced parasitemia by 46% after 5 days (Assay performed on *Plasmodium berghei* NK173, a chloroquine-sensitive strain) Dose of 30 mg/kg/day reduced parasitemia by 61.8% after 5 days (Assay performed on *Plasmodium vinckei petteri*, a chloroquine-sensitive strain)	

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Sungucine (Strychnine-type)	*S. icaja* (L, SB)	*In vitro*	Moderate activity (FCA 20 Ghana) *SI HeLa/FCA 20 Ghana: 2.30* *SI KB/FCA 20 Ghana: 0.79* *SI WI38/FCA 20 Ghana: 0.77* Weak activity (W2 Indochina) *SI HeLa/W2 Indochina: 1.78* *SI KB/W2 Indochina: 0.61* *SI WI38/W2 Indochina: 0.59*	Lamotte, J. *et al.*, 1979 Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 1999 Frédérich, M. *et al.*, 2000 Frédérich, M. *et al.*, 2001b Frédérich, M. *et al.*, 2002 Tchinda, A. T. *et al.*, 2012a
18-Hydroxysungucine (Strychnine-type)	*S. icaja* (RT)	*In vitro*	Promising activity (FCA 20 Ghana, FCB1-R Colombia, and W2 Indochina)	Frédérich, M. *et al.*, 2000 Frédérich, M. *et al.*, 2002

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Isosungucine (Strychnine-type)	*S. icaja* (SB)	*In vitro*	Moderate activity (FCA 20 Ghana) *SI HeLa/FCA 20 Ghana: 12.6* *SI KB/FCA 20 Ghana: 6.84* *SI WI38/FCA 20 Ghana: 6.99* Promising activity (W2 Indochina) *SI HeLa/W2 Indochina: 62.6* *SI KB/W2 Indochina: 34.0* *SI WI38/W2 Indochina: 34.7*	Frédérich, M. *et al.*, 2001b Frédérich, M. *et al.*, 2002 Philippe, G. *et al.*, 2003 Philippe, G. *et al.*, 2007 Tchinda, A. T. *et al.*, 2012a
		In vivo	Dose of 30 mg/kg/day reduced parasitemia by 47% after 4 days (Assay performed on *Plasmodium vinckei* petteri, a chloroquine-sensible strain)	

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
18-Hydroxyisosungucine (Strychnine-type)	*S. icaja* (RB)	*In vitro*	Promising activity (FCA 20 Ghana, W2 Indochina, and PFB Brazil) *SI HeLa/FCA 20 Ghana: 17.7* *SI HeLa/W2 Indochina: 107* *SI KB/FCA 20 Ghana: 19.1* *SI KB/W2 Indochina: 116* *SI WI38/FCA 20 Ghana: 19.8* *SI WI38/W2 Indochina: 120* Moderate activity (F32 Tanzania)	Frédérich, M. *et al.*, 2000 Frédérich, M. *et al.*, 2001b
Usambarensine (Usambarensine-type)	*S. dale* (US) *S. memecyloides* (SB) *S. usambarensis* (RB, SB)	*In vitro*	Moderate activity (FCA 20 Ghana) *SI HeLa/FCA 20 Ghana: 5.40* *SI KB/FCA 20 Ghana: 6.40* *SI WI38/FCA 20 Ghana: 3.05* Promising activity (W2 Indochina, K1, and FcM29) *SI HeLa/W2 Indochina: 13.7* *SI KB/W2 Indochina: 16.3* *SI WI38/W2 Indochina: 7.77*	Angenot, L., and Bisset, N. G., 1971a Ohiri, F. C. *et al.*, 1983a Quetin-Leclercq, J. *et al.*, 1991b Wright, C. W. *et al.*, 1991 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 1998b Frédérich, M. *et al.*, 2002 Passemar, C. *et al.*, 2011

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
5',6'-Dihydrousambarensine (Usambarensine-type)	*S. dale* (US) *S. potatorum* (RB) *S. tchibangensis* (RB, TB) *S. usambarensis* (RB, SB)	*In vitro*	Promising activity (FCA 20 Ghana, K1, and W2 Indochina) *Good selectivity towards Plasmodium strains (SI value not provided)*	Angenot, L., and Bisset, N. G., 1971a Richard, C. *et al.*, 1978 Ohiri, F. C. *et al.*, 1983a Quetin-Leclercq, J. *et al.*, 1991b Wright, C. W. *et al.*, 1991 Massiot, G. *et al.*, 1992 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2002 Rasoanaivo, P. *et al.*, 2002
		In vivo	Inactive against *Plasmodium berghei*, a chloroquine-sensitive strain	
N_b-Methylusambarensine (Usambarensine-type)	*S. usambarensis* (RB)	*In vitro*	Moderate activity (FCA 20 Ghana, K1, and W2 Indochina)	Angenot, L., and Bisset, N. G., 1971a Wright, C. W. *et al.*, 1991 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 1999
17,4',5',6'-Tetrahydrousambarensine (17S) = 17,4',5',6'-Tetrahydro-17α-usambarensine (Usambarensine-type)	*S. ngouniensis* (SB)	*In vitro*	Promising activity (FcM29)	Massiot, G. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Passemar, C. *et al.*, 2011

Alkaloids	Origins	Types of test	Antiplasmodial and antimalarial activities	References
Usambarine (Usambarensine-type)	*S. barteri* (L) *S. nigritana* (U) *S. usambarensis* (L, SB)	*In vitro*	Moderate activity (FCA 20 Ghana, K1, and W2 Indochina)	Koch, M., and Plat, M., 1971 Koch, M. *et al.*, 1973 Angenot, L. *et al.*, 1978d Quetin-Leclercq, J. *et al.*, 1991b Wright, C. W. *et al.*, 1991 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 1999 Frédérich, M. *et al.*, 2002
18,19-Dihydrousambarine (Usambarensine-type)	*S. barteri* (L, S) *S. nigritana* (L, S, SB) *S. usambarensis* (L, SB)	*In vitro*	Moderate activity (FCA 20 Ghana, K1, and W2 Indochina)	Angenot, L. *et al.*, 1978b Nicoletti, M. *et al.*, 1980 Quetin-Leclercq, J. *et al.*, 1991b Wright, C. W. *et al.*, 1991 Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 1999

BB= Branch bark; Fr= Fruit; L= Leave; RB= Root bark; RT= Root; S=Seed; SB=Stem bark; TB= Trunk bark; US= Unspecified.

Interpretation of antiplasmodial activities: promising activity ($IC_{50} \leq 1$ µM), moderate activity (IC_{50} between 1 and 10 µM), and weak activity ($IC_{50} > 10$ µM). 3D7, FCA 20 Ghana, and F32 Tanzania strains are sensible to chloroquine. W2 Indochina, FCB1-R Colombia, K1, PFB Brazil, and FcM29 strains are resistant to chloroquine.

The three main subclasses of alkaloids exhibiting antiplasmodial activities are the strychnine-type alkaloids (14 out of 64 (21.9%)), the usambarensine-type alkaloids (13 out of 64 (20.3%)), and the indoloquinolizine-type alkaloids (10 out of 64 (15.6%)). Antiplasmodial activities are thus present in both indoline-type and indole-type alkaloids. However, there is a predominance in indoline alkaloids, a class specific to the genus. Strychnine, the flagship molecule of *Strychnos* genus, has a large number of derivatives active against malaria, making it the top subclass. Lastly, usambarensine and its derivatives are nearly on the same level as strychnine-type metabolites. This demonstrates that antiplasmodial activities are also significantly present among dimeric indole alkaloids.

As discussed in the previous section, *Strychnos* plants are also used for other parasitic infections, and the results of *in vitro* and *in vivo* tests confirm these activities.

Several metabolites isolated from *S. usambarensis* have been tested against various parasites. Isostrychnopentamine, strychnopentamine, usambarensine, 3',4'-dihydrousambarensine (also referred to as 5',6'-dihydrousambarensine, depending on the numbering system used), and usambarine showed *in vitro* activity against *Entamoeba histolytica* and *Giardia intestinalis*. Additional metabolites, namely 5,6-dihydroflavopereirine, 18,19-dihydrousambarine, N_b-methylusambarensine, and tubulosine (an alkaloid of β-carboline-type), showed antiparasitic activities towards *Entamoeba histolytica* (Wright, C. W. *et al.*, 1991; Wright, C. W. *et al.*, 1994). Usambarensine, usambarine, and 18,19-dihydrousambarine were found to be highly active *in vitro* against *Entamoeba histolytica*, while 5',6'-dihydrousambarensine was the most selective against *Giardia intestinalis* (Rasoanaivo, P. *et al.*, 2002). A quaternary alkaloid, diploceline, isolated from the root barks of *S. gossweileri*, was found to be active against *Entameoba histolytica* and *Trichomonas vaginalis*. The mode of action of this metabolite is distinct from that of metronidazole (Gasquet, M. *et al.*, 1992).

Parasitic infections also include trypanosomes, which cause trypanosomiasis. Strychnogucine B, an alkaloid isolated from the root barks of *S. icaja* that exhibits promising antiplasmodial activity, demonstrated moderate activity *in vitro* and *in vivo* against *Trypanosoma brucei* (Beaufay, C. *et al.*, 2018).

S. spinosa leaves showed good activity against trypanosomes (Hoet, S. *et al.*, 2004; Tittikpina, N. K. *et al.*, 2020). From the leaves, two sterols with *in vitro* antitrypanosomal activities were identified: saringosterol and 24-hydroperoxy-24-vinylcholesterol. Their activities are considered moderate because their IC_{50} are between 1 and 10 µM (Hoet, S. *et al.*, 2007). *S. spinosa* stems also exhibited antitrypanosomal activity although at a lower level (Hoet, S. *et al.*, 2004). Additionally, the essential oil from *S. spinosa* leaves showed a good activity against *Trypanosoma brucei* with very good selectivity towards this parasite (SI of 4.4). Two metabolites from the essential oil, (E)-nerolidol and linalool, were identified as the most active though their activity was classified as moderate. Their respective selectivity indices are 35.7 and greater than 40 (Hoet, S. *et al.*, 2006).

An activity against *Leishmania mexicana* has been observed in the leaves and stems of *S. spinosa*. However, this activity is less potent compared to that against *Trypanosoma brucei* (Hoet, S. *et al.*, 2004; Tittikpina, N. K. *et al.*, 2020).

To conclude the section on parasitic diseases, a few pharmacological activities that are poorly described in the literature are worth mentioning:

- *S. madagascariensis*: larvicide (Maroyi, A., 2021);
- *S. boonei*, *S. henningsii*, *S. mitis*, and *S. potatorum*: anthelmintics (Sandberg, F. *et al.*, 1971; Mallikharjuna, P. B. *et al.*, 2010; Waterman, C. *et al.*, 2010; Francis, P., and Suseem, S. R., 2016; Selogatwe, K. M. *et al.*, 2021).
- *S. spinosa*: acaricide (*in vivo*) and active *in vivo* against *S. mansoni* NTS (parasitic worms causing schistosomiasis) (Madzimure, J. *et al.*, 2013; Muya, K. *et al.*, 2023).

III.1.2. Microbial infections

III.1.2.1. Traditional uses

The aqueous extract of *S. camptoneura* stem barks is traditionally used in Congo in cases of various microbial infections, including skin infections (Morabandza, C. J. *et al.*, 2016; Moulari, B. *et al.*, 2023).

Concerning *S. decussata*, it is used in form of bark decoction taken orally in Kenya, Madagascar, Mozambique, and South Africa for respiratory and venereal infections. Among respiratory infections, *S. decussata* barks, either alone or mixed with other plants, could treat tuberculosis. The barks are also good for sore throats, vaginal infections, and abscesses (Rasoanaivo, P. *et al.*, 2002; Samie, A. *et al.*, 2010; Molander, M. *et al.*, 2014; Maroyi, A., 2022). Sore throats could also be treated by the barks of *S. cocculoides* (Neuwinger, H. D., 1996).

Stem and trunk barks of *S. henningsii*, administered orally in form of infusions or chewed, are used in the eastern region of the O.R. Tambo district. Additionally, a decoction of *S. innocua* roots and stems, taken orally twice a day for one month in the Acholi sub-region of northern Uganda, are traditional treatments for tuberculosis (Madikizela, B. *et al.*, 2017; Oryema, C. *et al.*, 2021). Other infections that could be treated with *S. henningsii* include dysentery (fruit uses) and gynecological or oral infections (Rasoanaivo, P. *et al.*, 2002; Oyedemi, S. O. *et al.*, 2009). As for *S. spinosa*, in addition to its action against tuberculosis, it is used in cases of abscess and three sexually transmitted diseases: songeya, doroba, and bola-bola, which correspond respectively to syphilis, gonorrhea, and lymphogranuloma venereum (Ndubani, P., and Höjer, B., 1999; Bero, J. *et al.*, 2009).

As a traditional remedy for leprosy, *S. diplotricha* stem barks, as well as *S. spinosa* leaves, root barks, and stem barks, are used (Neuwinger, H. D., 1996; Rasoanaivo, P. *et al.*, 1996).

Barks of *S. aculeata*, decoctions of *S. innocua* roots, *S. potatorum* seeds, *S. spinosa* root barks, infusions of *S. cocculoides* roots, and teas made from *S. spinosa* leaves and stems are administered in cases of gonorrhea. In Tanzania's Urambo district (Tabora region), *S. potatorum* is also described as a traditional remedy for syphilis. For the same purpose, *S. spinosa* leaves are traditionally used (Bisset, N. G., 1970; Neuwinger, H. D., 1996; Rasoanaivo, P. *et al.*, 2002; Satish Kumar, B. N. *et al.*, 2010; Mohana Lakshimi, S. *et al.*, 2012; Francis, P., and Suseem, S. R., 2016; Amuri, B. *et al.*, 2017; Ayo, R. G. *et al.*, 2022; Kacholi, D. S., and Amir, H. M., 2022).

In Malawi, meningitis could be treated with infusions of *S. spinosa* leaves. The leaf juice and the root decoction of *S. madagascariensis* are also traditional remedies for meningitis (Bisset, N. G., 1970; Neuwinger, H. D., 1996).

The root decoction of *S. aculeata* is widely used in Zambia for gonorrhea and pneumonia. The latter could also be treated with *S. cocculoides* roots (Bisset, N. G., 1970; Neuwinger, H. D., 1996).

For buccal infections, a decoction of *S. dinklagei* root barks is used as a mouthwash or inhalation (Bisset, N. G., 1970).

To conclude the section on traditional uses for bacterial infections, *S. icaja* is used for ear infections in Gabon, *S. mitis* and *S. potatorum* for urinary tract infections, and *S. spinosa* for dermal and respiratory infections (Francis, P., and Suseem, S. R., 2016; Cock, I. E. *et al.*, 2021a; Samseny, R. R. R. A. *et al.*, 2021).

III.1.2.2. Pharmacological properties

Numerous assays were carried out to evaluate the antimicrobial activities of African *Strychnos* species and their alkaloids. In the ranking of pharmacological study occurrences, these types of studies rank third, just after antiplasmodial/antimalarial and cytotoxic/anticancer activities. For ease of reading, the results are presented in two tables: one showing the antimicrobial activities of African *Strychnos* species, and the other, those of the alkaloids isolated from these species (Tables 5 and 6).

Table 5: Antimicrobial activities of African *Strychnos* species.

Species	Plant parts	Types of test	Antimicrobial activities	References
Strychnos aculeata	Twigs (Ethanolic extract)	*In vitro*	*Pseudomonas aeruginosa* (Inhibition at 5 and 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. afzelii	Stem barks (Alkaline chloroform extract, in particular)	*In vitro*	*Streptococcus sp.*, *Bacillus subtilis*, and *Staphylococcus aureus* (Unspecified dose)	Verpoorte, R. *et al.*, 1978b
S. boonei	Stem barks (Ethanolic extract)	*In vitro*	*Bacillus subtilis* and *Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. camptoneura	Stem barks (Aqueous extract)	*In vitro*	*Streptococcus sp.*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter sp.*, and *Streptococcus β hemolytic* (MIC: 5, 5, 6.25, and 12.25 mg/mL, respectively)	Shah, Z. *et al.*, 1986 Morabandza, C. J *et al.*, 2016
	Seeds (Ethanolic extract)	*In vitro*	*Staphylococcus intermedius*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Micrococcus luteus*, and *Pseudomonas aeruginosa* (MIC : 500 and 750 µg/mL, and for the last three species, 1 mg/mL, respectively)	Gombé Assoungou, H. *et al.*, 2022

Species	Plant parts	Types of test	Antimicrobial activities	References
S. canthioides	Leaves (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. chrysophylla	Stem barks (Unspecified type of extract)	*In vitro*	*Staphylococcus aureus* and *Escherichia coli* (Unspecified dose)	Verpoorte, R. *et al.*, 1984
S. cocculoides	Stem barks (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
	Twigs (Ethanolic extract)		*Bacillus subtilis* and *Staphylococcus aureus* (Inhibition at 50 mg/mL)	
S. cuminodora	Twigs (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 5 and 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. dale	Leaves and twigs (Ethanolic extract)	*In vitro*	*Bacillus subtilis* and *Staphylococcus aureus* (Inhibition at 5 and 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. decussata	Stem barks (Unspecified type of extract)	Unspe-cified	Unspecified	Rolfsen, W. *et al.*, 1980b Maroyi, A., 2022
S. dinklagei	Stem barks (Ethanolic extract)	*In vitro*	*Bacillus subtilis* and *Staphylococcus aureus* (Inhibition at 5 and 50 mg/mL)	Verpoorte, R. *et al.*, 1983b

Species	Plant parts	Types of test	Antimicrobial activities	References
S. dolichothyrsa	Root barks (Ethanolic extract)	*In vitro*	*Bacillus subtilis* and *Staphylococcus aureus* (Inhibition at 5 and 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
	Stem barks (Ethanolic extract)		*Bacillus subtilis* and *Staphylococcus aureus* (Inhibition at 50 mg/mL)	
S. elaeocarpa	Leaves (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. gossweileri	Roots (Unspecified type of extract)	*In vitro*	Gram + bacteria (Unspecified dose)	Ohiri, F. C. *et al.*, 1983a
S. floribunda	Twigs (Ethanolic extract)	*In vitro*	*Bacillus subtilis* and *Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. ignatii	Stem barks (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 5 and 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. innocua	Stem barks (Methanolic extract)	*In vitro*	*Escherichia coli* (MIC: 500 µg/mL)	Abdoulahi, M. I. I. *et al.*, 2023 Achika, J. I. *et al.*, 2023
	Twigs (Ethanolic extract)		*Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b

Species	Plant parts	Types of test	Antimicrobial activities	References
S. johnsonii	Stem barks (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (No growth at 0.04 g/mL)	Ebongue, C. O. *et al.*, 2015
S. longicaudata	Stem barks (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. lucens	Leaves (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. madagascariensis	Leaves and stem barks (Methanolic, dichloromethane, and aqueous extracts)	*In vitro*	*Escherichia coli*, *Brevibacterium agri*, *Brevibacterium linens*, *Staphylococcus aureus* (without resistance, methicillin-resistant, and gentamycin-methicillin resistant), *Staphylococcus epidermis*, *Propionibacterium acnes*, and *Pseudomonas aeruginosa* (MIC values vary between 500 and 8000 µg/mL depending on the pathogen (best activity against *Brevibacterium agri*))	Nciki, S. *et al.*, 2016 Cock, I. E. *et al.*, 2021b
	Fruit pulp (Methanolic extract)	*In vitro*	*Salmonella typhimurium*, *Streptococcus pyogenes*, *Bacillus cereus*, *Klebsiella pneumoniae*, and *Privotella intermedia* (MIC values vary between 6.3 and over 25 mg/mL depending on the pathogen (best activity against *Bacillus cereus*))	Tshikalange, T. E. *et al.*, 2017 Uttu, A. J. *et al.*, 2022

Species	Plant parts	Types of test	Antimicrobial activities	References
S. millepunctata	Leaves and twigs (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
	Stem barks (Ethanolic extract)		*Staphylococcus aureus* (Inhibition at 50 mg/mL)	
S. mitis	Unspecified	*In vitro*	*Escherichia coli* and *Pseudomonas aeruginosa* (Active at 40 µg/mL against *Escherichia coli* and at 160 µg/mL against *Pseudomonas aeruginosa*)	Cock, I. E. *et al.*, 2021b
S. nigritana	Twigs (Ethanolic extract)	*In vitro*	*Bacillus subtilis* and *Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. odorata	Leaves (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b

Species	Plant parts	Types of test	Antimicrobial activities	References
S. potatorum	Epicarp (Ethanolic extract)	*In vitro*	*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Enteroccocus faecalis*, and *Klebsiella pneumoniae* (Activity in the presence of 100, 200, and 300 µg of product)	Murugan, P. K., and Venkatachalam, K., 2019
	Seeds (Alkaloidic extract)	*In vitro*	*Proteus vulgaris*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Vibrio cholerae*, and *Mycobacterium tuberculosis* (Unspecified dose)	Mallikharjuna, P., and Seetharam, Y. N., 2009
S. pungens	Fruit pulp (Methanolic extract)	*In vitro*	*Salmonella typhimurium*, *Streptococcus pyogenes*, *Bacillus cereus*, *Klebsiella pneumoniae*, *Privotella intermedia*, *Salmonella typhimurium* (MIC values vary between 12.5 and over 25 mg/mL depending on the pathogen (best activity against *Salmonella typhimurium*, *Streptococcus pyogenes*))	Tshikalange, T. E. *et al.*, 2017 Uttu, A. J. *et al.*, 2022
S. scheffleri	Stem barks (Ethanolic extract)	*In vitro*	*Bacillus subtilis* and *Staphylococcus aureus* (Inhibition at 5 and 50 mg/mL)	Verpoorte, R. *et al.*, 1983b

Species	Plant parts	Types of test	Antimicrobial activities	References
S. soubrensis	Stem barks (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. spinosa	Fruit pulp (Methanolic extract)	*In vitro*	*Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Privotella intermedia* (MIC values vary between 1.6 and over 25 mg/mL depending on the pathogen (best activity against *Bacillus cereus*))	Tshikalange, T. E. *et al.*, 2017 Uttu, A. J. *et al.*, 2022
	Stem barks (Acetone, alkaloidic, methanolic, and dichloromethane extracts)	*In vitro*	*Escherichia coli*, *Enteroccocus faecalis*, *Staphylococcus aureus*, *Bacillus cereus* (MIC values vary between 0.16 and over 1.25 mg/mL depending on the pathogen (best activity against *Escherichia coli*, *Enteroccocus faecalis*, and *Staphylococcus aureus*))	Isa, A. I. *et al.*, 2014
S. staudtii	Stem barks (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b

Species	Plant parts	Types of test	Antimicrobial activities	References
S. usambarensis	Stem barks (Ethanolic extract)	*In vitro*	*Bacillus subtilis* (Inhibition at 50 mg/mL) *Staphylococcus aureus* (Inhibition at 5 and 50 mg/mL)	Verpoorte, R. *et al.*, 1983b
S. variabilis	Twigs (Ethanolic extract)	*In vitro*	*Staphylococcus aureus* (Inhibition at 50 mg/mL)	Verpoorte, R. *et al.*, 1983b

Table 6: Antimicrobial activities of metabolites isolated from African *Strychnos* species.

Metabolites	Origins	Types of test	Antimicrobial activities	References
Dihydrocycloakagerine (β-carboline-type)	*S. johnsonii* (RB, SB) *S. phaeotricha* (RB, SB)	*In vitro*	*Mycobacterium smegmatis* (Activity in the presence of 100 µg of product)	Massiot, G. *et al.*, 1987 Tchangou Njiemou, A. F. *et al.*, 2022
17-O-Ethylakagerine (β-carboline-type)	*S. johnsonii* (RB) *S. phaeotricha* (RB, SB)	*In vitro*	*Mycobacterium smegmatis* (Activity in the presence of 100 µg of product)	Massiot, G. *et al.*, 1987 Tchangou Njiemou, A. F. *et al.*, 2022
Alstonine (Ajmalicine-type)	*S. camptoneura* (RB, SB) *S. gossweileri* (RB)	Unspecified	Unspecified	Verpoorte, R., and Sandberg, F., 1971 Angenot, L., and Denoël, A., 1972 Ohiri, F. C. *et al.*, 1983a Shah, Z. *et al.*, 1986 Delaude, C., and Delaude, L., 1997
Bisnor-C-alkaloid H (Toxiferine-type)	*S. afzelii* (SB)	*In vitro*	Unspecified	Verpoorte, R. *et al.*, 1978b

Metabolites	Origins	Types of test	Antimicrobial activities	References
Bisnordihydrotoxiferine (Toxiferine-type)	*S. afzelii* (SB) *S. decussata* (SB) *S. dolichothyrsa* (SB) *S. elaeocarpa* (SB) *S. floribunda* (SB) *S. icaja* (RT, SB) *S. kasengaensis* (SB) *S. longicaudata* (RB, SB) *S. malacoclados* (SB) *S. matopensis* (RB) *S. potatorum* (RB) *S. scheffleri* (SB) *S. variabilis* (RB)	*In vitro*	*Bacillus subtilis* (Active at 2 mg/mL)	Verpoorte, R. *et al.*, 1978b Massiot, G. *et al.*, 1983a Ohiri, F. C. *et al.*, 1983a Thepenier, P. *et al.*, 1984 Massiot, G. *et al.*, 1988 Massiot, G. *et al.*, 1992 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Philippe, G. *et al.*, 2003 Tchinda, A. T. *et al.*, 2012b
Campesterol	*S. afzelii* (SB) *S. innocua* (RB)	*In vitro*	Meticillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* (Active at 10 µg/mL)	Verpoorte, R. *et al.*, 1980 Ohiri, F. C. *et al.*, 1983a Uttu, A. J. *et al.*, 2023b

Metabolites	Origins	Types of test	Antimicrobial activities	References
Caracurine V (Toxiferine-type)	*S. afzelii* (SB) *S. chrysophylla* (SB)	*In vitro*	*Bacillus subtilis* (Active at 4 mg/mL)	Verpoorte, R. *et al.*, 1978b Verpoorte, R. *et al.,* 1984 Delaude, C., and Delaude, L., 1997
Diploceline (Indoloquinolizine-type)	*S. gossweileri* (RB)	*In vitro*	*Staphylococcus aureus* and *Streptococcus haemolyticus* (Active at 2 and 0.5 mg/mL, respectively)	Ohiri, F. C. *et al.*, 1983a
Linalool	*S. innocua* (RB)	*In vitro*	Meticillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* (Active at 10 µg/mL)	Uttu, A. J. *et al.*, 2023b

Metabolites	Origins	Types of test	Antimicrobial activities	References
Nerolidol	*S. innocua* (RB)	*In vitro*	Meticillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* (Active at 10 µg/mL)	Uttu, A. J. *et al.*, 2023b
2,13-Octadecadien-1-ol	*S. innocua* (RB)	*In vitro*	Meticillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* (Active at 10 µg/mL)	Uttu, A. J. *et al.*, 2023b
Serpentine (Ajmalicine-type)	*S. camptoneura* (RB, SB)	Unspecified	Unspecified	Verpoorte, R., and Sandberg, F., 1971 Angenot, L., and Denoël, A., 1972 Shah, Z. *et al.*, 1986

Metabolites	Origins	Types of test	Antimicrobial activities	References
β-Sitosterol	*S. afzelii* (SB) *S. innocua* (RB)	*In vitro*	Meticillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* (Active at 10 µg/mL)	Verpoorte, R. *et al.*, 1980 Ohiri, F. C. *et al.*, 1983a Uttu, A. J. *et al.*, 2023b
Umbelliferone	*S. innocua* (RB)	*In vitro*	Meticillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* (Active at 10 µg/mL)	Uttu, A. J. *et al.*, 2023b

Metabolites	Origins	Types of test	Antimicrobial activities	References
Usambarensine (Usambarensine-type)	*S. usambarensis* (RB)	*In vitro* (without testing for cytotoxicity[11])	*Staphylococcus aureus*, *Bacillus subtilis*, *Mycobacterium smegmatis*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Activity in the presence of 100 µg of product)	Caron, C. *et al.*, 1988 Neuwinger, H. D., 1996
5',6'-Dihydrousambarensine (Usambarensine-type)	*S. usambarensis* (RB)	*In vitro* (without testing for cytotoxicity[11])	*Staphylococcus aureus*, *Bacillus subtilis*, *Mycobacterium smegmatis*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Activity in the presence of 100 µg of product)	Caron, C. *et al.*, 1988 Neuwinger, H. D., 1996
18,19-dihydrousambarine (Nigritanine) (Usambarensine-type)	*S. barteri* (L, S, SB) *S. nigritana* (B, L, S)	*In vitro* (without testing for cytotoxicity[11])	*Staphylococcus aureus* (Unspecified dose)	Oguakwa, J. U. *et al.*, 1978 Nicoletti, M. *et al.*, 1980 Casciaro, B. *et al.*, 2020

B= Bark; L= Leave; RB= Root bark; S=Seed; SB=Stem bark.

[11] Many bisindole alkaloids are cytotoxic at a dose between 1 and 10 µg/mL. The antimicrobial activities observed may therefore be linked to this activity. Unfortunately, no cytotoxicity tests were carried out simultaneously.

Among alkaloids, two subclasses stand out for their antimicrobial activities: the usambarensine- and toxiferine-types, each representing 3 out of 11 compounds (27.3%). These subclasses are predominantly bisindole alkaloids, encompassing both indoline- and indole-types.

III.1.3. Fungal infections

III.1.3.1. Traditional uses

For fungal infections, three species are described in the literature as traditional uses: *S. madagascariensis*, *S. potatorum*, and *S. spinosa*. The leaves of the first are used for ringworm infections and the stem barks for *Candida* infections (Masevhe, N. A. *et al.*, 2015; Lall, N. *et al.*, 2019). Concerning the second, it could treat candidiasis (Masevhe, N. A. *et al.*, 2015). As for the third, in Tanzania, it is administered in cases of fungal infections in the broad sense. The plant part was not reported in the literature (Mourice, V. N. M. *et al.*, 2019).

III.1.3.2. Pharmacological properties

Nine species, tested *in vitro* for their antifungal properties, confirmed their activity (Table 7).

Table 7: Antifungal activities of African *Strychnos* species.

Species	Plant parts	Types of test	Antifungal activities	References
Strychnos decussata	Stem barks (Hexane extract)	*In vitro*	*Candida* albicans, Candida *krusei*, and *Fusarium proliferatum* (MIC values vary between 1.88 and 3.75 mg/mL)	Samie, A. *et al.*,2010 Samie, A., and Mashau, F., 2013 Maroyi, A., 2022
S. gossweileri	Roots (Alkaloidic extract)	*In vitro*	*Candida albicans* (Active at 10 mg/mL)	Ohiri, F. C. *et al.*, 1983a
S. innocua	Root barks (Methanolic extract)	*In vitro*	*Candida krusei* (MIC: 5 mg/mL)	Agbeke Iyun, O. R. *et al.*, 2022
S. madagascariensis	Leaves and stem barks (Dichloromethane/ methanol and aqueous extracts)	*In vitro*	*Candida albicans*, *Microsporum canis*, and *Trichophyton mentagrophytes* (MIC values vary between 130 and over 8000 µg/mL depending on species and extract type (best activity against *Microsporum canis*))	Nciki, S. *et al.*, 2016

Species	Plant parts	Types of test	Antifungal activities	References
S. mellodora	Stem barks (Ethanolic extract)	*In vitro*	*Candida albicans*, *Candida glabrata*, and *Aspergillus niger* (MIC values vary between 0.38 and 2.28 mg/mL (best activity against *Candida glabrata*))	Brandt, V. *et al.*, 2001
S. mitis	Leaves (Acetone extract)	*In vitro*	*Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus neoformans* (MIC values vary between 0.31 and 0.63 mg/mL (best activity against *Aspergillus fumigatus* and *Candida albicans*))	Adamu, M. *et al.*, 2012
	Unspecified (Dichloromethane/ methanol extract)	*In vitro*	*Furasium verticilloides*, *Furasium graminareum*, *Furasium oxysporum*, *Aspergillus parasiticus*, *Aspergillus flavus*, and *Aspergillus ochraceus* (MIC values vary between 0.01 and 0.78 mg/mL (best activity against the three species of *Fusarium*))	Molele, P. K. *et al.*, 2023
S. potatorum	Seeds (Alkaloidic extract)	*In vitro*	*Asperillus niger* and *Candida albicans* (Active at 100 and 200 µg/mL)	Mallikharjuna, P, and Seetharam, Y. N., 2009

Species	Plant parts	Types of test	Antifungal activities	References
S. pungens	Unspecified	Unspecified	Unspecified	Tshikalange, T. E. *et al.*, 2017
S. spinosa	Fruits (Dichloromethane/ methanol and aqueous extracts)	*In vitro*	*Candida albicans*, *Microsporum canis*, and *Trichophyton mentagrophytes* (MIC values vary between 250 and over 8000 µg/mL depending on species and extract type (best activity against *Trichophyton mentagrophytes*))	Nciki, S. *et al.*, 2016
	Leaves (Ethanolic, dichloromethane, and petroleum ether extracts)	*In vitro*	*Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus niger* (MIC values vary between 0.313 and 3.75 mg/mL depending on species (best activity against *Candida albicans* and *Aspergillus niger*))	Mourice, V. N. M. *et al.*, 2019
	Leaves (Acetone, alkaloidic, dichloromethane/ methanol, and methanolic extracts)	*In vitro*	*Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (MIC values vary between 0.04 and over 1.25 mg/mL depending on species and extract type (best activity against *Cryptococcus neoformans*))	Isa, A. I. *et al.*, 2014

Two alkaloids of the β-carboline-type have attracted attention for their significant antimycotic activities against human pathogens in the presence of specific glucosidase: dolichantoside and palicoside (MIC between 0.14 and 1.48 mg/mL) (Brandt, V. *et al.*, 2001). Dolichantoside was isolated from *S. gossweileri* roots, *S. mellodora* stem barks, and *S. tricalysioides* stem barks. (Leclercq, J., and Angenot, L., 1984; Tits, M. *et al.*, 1996; Delaude, C., and Delaude, L., 1997; Brandt, V. *et al.*, 1999; Philippe, G. *et al.*, 2005). Palicoside was obtained from *S. mellodora* stem barks and *S. usambarensis* fruits (Tits, M. *et al.*, 1996; Delaude, C., and Delaude, L., 1997; Brandt, V. *et al.*, 1999; Philippe, G. *et al.*, 2005; Cao, M. *et al.*, 2012).

Other metabolites isolated from African *Strychnos* demonstrated *in vitro* antifungal activity. Umbelliferone, linalool, nerolidol, campesterol, β-sitosterol, and 2,13-octadecadien-1-ol, isolated from *S. innocua* root barks, are active against *Candida albicans*, *Candida krusei*, *Aspergillus fumigatus*, and *Aspergillus nigre* (MIC between 5 and 20 µg/mL) (Uttu, A. J. *et al.*, 2023b). In addition, stigmasterol (stigmast-5-en-3β-ol) and campesterol (campest-5-en-3β-ol), both obtained from *S. innocua* root barks, showed promising interactions with the SAP2 protein binding sites of *Candida albicans* (Uttu, A. J. *et al.*, 2023a).

III.1.4. Viral infections

III.1.4.1. Pharmacological properties

None of the publications reviewed for this study described traditional uses for viral infections. However, following the emergence of SARS-CoV-2 (COVID-19) in 2020, pharmacological studies on the antiviral activities of *Strychnos* plants were conducted. The methanolic crude extracts of *S. congolana* trunk barks, *S. densiflora* trunk barks, *S. icaja* roots, *S. johnsonii* leaves, and *S. mellodora* leaves showed antiviral activities during biological assays. Among these, the two most active extracts are *S. icaja* and *S. mellodora* with doses of 20 µg/mL and below 20 µg/mL, respectively. Moreover, no cytotoxicity was observed for all the active extracts (Ledoux, A. *et al.*, 2022). It was also demonstrated that 10'-hydroxyusambarensine, obtained from *S. usambarensis* roots, is potentially active *in vitro* against SARS-CoV-2 (Shahali, A. *et al.*, 2024).

Additionally, the methanolic crude extract from *S. potatorum* seeds inhibited laboratory-adapted HIV-1 strains in *in vitro* assays, leading to the conclusion that *S. potatorum* seeds have antiviral activity against HIV (Palshetkar, A. *et al.*, 2020).

These two studies therefore demonstrate the antiviral potential of African *Strychnos* species, which, to our knowledge, have remained largely unexplored until now. For example, they provide an interesting lead in the fight against SARS-CoV-2.

III.2. Cancer

III.2.1. Traditional uses

Only one article describes *S. usambarensis* as a traditional anticancer agent, which is the only species of the genus with such a use. Unfortunately, this article does not provide additional information concerning this use (Francis, P., and Suseem, S. R., 2016).

III.2.2. Pharmacological properties

The cytotoxic and anticancer activities of crude extracts from African *Strychnos* species and the metabolites isolated from this genus are well-documented. These studies constitute the second most frequently encountered type of activity in the literature, with a total of 3 species and 34 metabolites tested.

The methanolic crude extract from the fruit tegument of *S. madagascariensis* showed a moderate cytotoxicity *in vitro* (LC_{50} value of 39.2 µg/mL) against the HeLa cell line (cervical cancer). This activity was dose-dependent (Oboh, M. *et al.*, 2023).

S. spinosa leaves also demonstrated *in vitro* low cytotoxicity, with LC_{50} values ranging from 30.56 to 689.39 µg/mL. However, when compared to the positive control (doxorubicin), this activity is considered negligible, and *S. spinosa* is therefore classified as a safe plant. Cytotoxic assays were conducted primarily

on the human normal fibroblast cell line WI38 and the BALB/c murine reticulum cell sarcoma J774 (Aremu, A. O., and Moyo, M., 2022).

The last species described is *S. usambarensis*. The dichloromethane and methanol crude extract (50:50 V/V) of its leaves exhibited an IC_{50} value of 11.09 µg/mL against leukemia cells, making *S. usambarensis* a promising candidate for the treatment of leukemia (Omosa, L. K. *et al.*, 2016).

The cytotoxic and anticancer activities of alkaloids isolated from African *Strychnos* plants, as reported in the literature, are summarized in the table below (Table 8).

Table 8: Cytotoxic and anticancer assays of metabolites isolated from African *Strychnos* species.

Metabolites	Origins	Types of test	Cytotoxic and anticancer activities	References
Akagerine (β-carboline-type)	*S. barteri* (RT, SB) *S. camptoneura* (SB) *S. decussata* (SB) *S. elaeocarpa* (SB) *S. floribunda* (SB) *S. johnsonii* (RB) *S. nigritana* (L, RB) *S. phaeotricha* (RB, SB) *S. spinosa* (L, SB) *S. usambarensis* (Fr, RB)	*In vitro*	Melanoma B16 cells (from C57BL mouse melanoma) Cultured Flow 2002 cells (from normal embryonic human lungs) L1210 cells (from DB A/2 mouse ascites tumor) HeLa cells (from human carcinoma) (Active at doses between 1 and 10 µg/mL)	Angenot, L. *et al.*, 1975a Verpoorte, R. *et al.*, 1975 Rolfsen, W. *et al.*, 1978 Oguakwa, J. U. *et al.*, 1980 Rolfsen, W. *et al.*, 1980b Verpoorte, R. *et al.*, 1981 Ohiri, F. C. *et al.*, 1983a Quetin-Leclercq, J. *et al.*, 1986 Massiot, G. *et al.*, 1987 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Hoet, S. *et al.*, 2007 Cao, M. *et al.*, 2012 Casciaro, B. *et al.*, 2020 Maroyi, A., 2022 Tchangou Njiemou, A. F. *et al.*, 2022

Metabolites	Origins	Types of test	Cytotoxic and anticancer activities	References
Alstonine (Ajmalicine-type)	*S. camptoneura* (RB, SB) *S. gossweileri* (RB)	Unspecified	Unspecified	Verpoorte, R., and Sandberg, F., 1971 Angenot, L., and Denoël, A., 1972 Verpoorte, R. *et al.*, 1975 Coune, C., and Angenot, L., 1980 Ohiri, F. C. *et al.*, 1983a Shah, Z. *et al.*, 1986 Delaude, C., and Delaude, L., 1997
N_4-Methylantirhine (Indoloquinolizine-type)	*S. usambarensis* (RT)	*In vitro*	L1210 cells (Active at 25 µg/mL)	Caprasse, M. *et al*., 1984 Quetin-Leclercq, J. *et al.*, 1986 Delaude, C., and Delaude, L., 1997
Betulinic acid	*S. spinosa* (S)	Unspecified	Unspecified	Rabeharitsara, A. T. *et al.*, 2023
Caracurine V (Toxiferine-type)	*S. afzelii* (SB) *S. angolensis* (RB, SB) *S. chrysophylla* (SB) *S. dolichothyrsa* (SB) *S. malacoclados* (US) *S. urceolata* (SB)	*In vitro*	Formation of stable complexes with the DSL and EGF-2 domains of DLL3, blocking the NOTCH signalling pathway and causing cell death (No dose due to virtual screening)	Verpoorte, R. *et al.*, 1978a Verpoorte, R. *et al.*, 1978b Bohlin, L. *et al.*, 1979 Verpoorte, R. *et al.*, 1980 Ohiri, F. C. *et al.*, 1983a Verpoorte, R. *et al.*, 1984 Delaude, C., and Delaude, L., 1997 Joshi, B. P. *et al.*, 2023

Metabolites	Origins	Types of test	Cytotoxic and anticancer activities	References
Dolichantoside (β-carboline-type)	*S. gossweileri* (RT) *S. mellodora* (SB) *S. tricalysioides* (SB)	*In vitro*	Melanoma B16 cells (Active at 10 µg/mL)	Coune, C., and Angenot, L., 1978a Ohiri, F. C. *et al.*, 1983a Leclercq, J., and Angenot, L., 1984 Quetin-Leclercq, J. *et al.*, 1986 Tits, M. *et al.*, 1996 Delaude, C., and Delaude, L., 1997 Brandt, V. *et al.*, 1999 Philippe, G. *et al.*, 2005
Isodolichantoside (β-carboline-type)	*S. gossweileri* (RT)	*In vitro*	L1210 cells (Active at 10 µg/mL)	Coune, C., and Angenot, L., 1980 Ohiri, F. C. *et al.*, 1983a Quetin-Leclercq, J. *et al.*, 1986 Delaude, C., and Delaude, L., 1997
Ellipticine (β-carboline-type)	*S. dinklagei* (SB)	*In vitro*	L1210 leukemia, sarcoma 180, and adenosarcoma 755 by inhibiting DNA, RNA and protein synthesis (No specified dose)	Michel, S. *et al.*, 1980 Ohiri, F. C. *et al.*, 1983a
Eriocitrine	*S. spinosa* (S)	Unspecified	Unspecified	Rabeharitsara, A. T. *et al.*, 2023
Flavopereirine (Indoloquinolizine-type)	*S. angolensis* (US) *S. longicaudata* (SB)	Unspecified	Unspecified	Massiot, G. *et al.*, 1983a Delaude, C., and Delaude, L., 1997

Metabolites	Origins	Types of test	Cytotoxic and anticancer activities	References
5,6-Dihydroflavopereirine (Indoloquinolizine-type)	*S. usambarensis* (RB)	*In vitro*	Active on mouse Ehrlich tumor cells ELT (from a mouse mammary gland carcinoma and transplanted into C57BL mouse peritoneal cavity) and B16 mouse melanoma cells (Active at 50 µg/mL)	Bassleer, R. *et al.*, 1985 Delaude, C., and Delaude, L., 1997
2,7-Dihydroxyapogeissoschizine (Indoloquinolizine-type)	*S. gossweileri* (RB)	*In vitro*	Melanoma B16 cells (Low toxicity at a dose above 75 µg/mL)	Quetin-Leclercq, J. *et al.*, 1994 Delaude, C., and Delaude, L., 1997
Matadine (Indoloquinolizine-type)	*S. gossweileri* (RB)	*In vitro*	DNA intercalator (It stabilizes the covalent DNA-Topoisomerase II complex and stimulates topoisomerase-mediated DNA cleavage) Active on melanoma B16 cells (Active in the range 50 to 200 µg/mL)	Quetin-Leclercq, J. *et al.*,1990 Quetin-Leclercq, J. *et al.*, 1991c Delaude, C., and Delaude, L., 1997 Dassonneville, L. *et al.*, 1999 Rasoanaivo, P. *et al.*, 2002
Melinonine F (β-carboline-type)	*S. usambarensis* (RB)	*In vitro*	It would not act as a DNA intercalator. Active on mouse Ehrlich tumor cells ELT and B16 mouse melanoma cells (Active at 50 µg/mL)	Bassleer, R. *et al.*, 1982 Caprasse, M., and Angenot, L., 1982 Caprasse, M. *et al.*, 1983a Caprasse, M., and Houssier, C., 1983b Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997

Metabolites	Origins	Types of test	Cytotoxic and anticancer activities	References
Normelinonine F (β-carboline-type)	*S. usambarensis* (RB)	Unspecified	It would not act as a DNA intercalator. The cytotoxic activity is unspecified.	Caprasse, M. *et al.*, 1983a Caprasse, M., and Houssier, C., 1983b Delaude, C., and Delaude, L., 1997
Sarracenin	*S. spinosa* (RB)	Unspecified	Unspecified	Uttu, A. J. *et al.*, 2022
Serpentine (Ajmalicine-type)	*S. camptoneura* (RB, SB)	*In vitro*	DNA intercalator Active on melanoma B16 cells (Active in the range 50 to 200 µg/mL)	Verpoorte, R., and Sandberg, F., 1971 Ohiri, F. C. *et al.*, 1983a Shah, Z. *et al.*, 1986 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002
Strychnofoline (β-carboline-type)	*S. usambarensis* (L, SB)	*In vitro*	L1210 cells (Active at 10 µg/mL)	Angenot, L., 1978a Quetin-Leclercq, J. *et al.*, 1986 Quetin-Leclercq, J. *et al.*, 1991b

Metabolites	Origins	Types of test	Cytotoxic and anticancer activities	References
Strychnopentamine (Usambarensine-type)	*S. usambarensis* (L, SB)	*In vitro*	Melanoma B16 cells Cultured Flow 2002 cells L1210 cells HeLa cells Non-cancer human fibroblasts Hepatoma cells (from HW165 hepatoma of Wistar rats) Ehrlich ascites cells (line ELT) It would act by inhibiting RNA synthesis. (Active between 1 and 10 µg/mL)	Angenot, L. *et al.*, 1978b Ohiri, F. C. *et al.*, 1983a Tits, M. *et al.*, 1984 Quetin-Leclercq, J. *et al.*, 1986 Neuwinger, H. D., 1996 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002
		In vivo	Ehrlich ascites tumor growing in the mouse. It would act by inhibiting RNA synthesis. (Active by subcutaneous injections of 1.5 mg for four successive days)	

Metabolites	Origins	Types of test	Cytotoxic and anticancer activities	References
Isostrychnopentamine (Usambarensine-type)	*S. usambarensis* (L)	*In vitro*	Induction of apoptosis in HCT-116 colon cancer cells. Moreover, It would act against apoptosis-resistant cancer cells through a p53-independent pathway. It does not affect the catalytic activity of human topoisomerases I and II. (Active at a concentration of 15 µM)	Angenot, L. *et al.*, 1978b Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2003b Frédérich, M. *et al.*, 2003c Frédérich, M. *et al.*, 2004a Cao, M. *et al.*, 2011
Strychnophylline (β-carboline-type)	*S. usambarensis* (L)	*In vitro*	Melanoma B16 cells Cultured Flow 2002 cells L1210 cells HeLa cells (Active at 10 µg/mL)	Angenot, L., 1978a Ohiri, F. C. *et al.*, 1983a Quetin-Leclercq, J. *et al.*, 1986 Delaude, C., and Delaude, L., 1997
Strychnoxanthine (β-carboline-type)	*S. gossweileri* (RB)	*In vitro*	HeLa cells (Active at 25 µg/mL)	Coune, C. *et al.*, 1984 Quetin-Leclercq, J. *et al.*, 1986 Delaude, C., and Delaude, L., 1997
Sungucine (Strychnine-type)	*S. icaja* (L, RT, SB)	*In vitro*	HL-60 leukemia cells (by apoptosis and necrosis) (GI_{50} value of 0.18 µM) HeLa and KB cancer cell lines WI38 fibroblast non-cancer cell line (IC_{50} values between 6 and 18 µM)	Lamotte, J. *et al.*, 1979 Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2000 Lansiaux, A. *et al.*, 2002 Frédérich, M. *et al.*, 2003a Frédérich, M. *et al.*, 2003b Tchinda, A. T. *et al.*, 2012a

Metabolites	Origins	Types of test	Cytotoxic and anticancer activities	References
Isosungucine (Strychnine-type)	*S. icaja* (RT, SB)	*In vitro*	HL-60 leukemia cells (by apoptosis and necrosis) (GI_{50} value of 1.03 µM) HeLa and KB cancer cell lines WI38 fibroblast non-cancer cell line (IC_{50} values between 9 and 16.6 µM)	Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2000 Lansiaux, A. *et al.*, 2002 Philippe, G. *et al.*, 2003 Tchinda, A. T. *et al.*, 2012a
18-Hydroxyisosungucine (Strychnine-type)	*S. icaja* (RT)	*In vitro*	HeLa and KB cancer cell lines WI38 fibroblast non-cancer cell line (IC_{50} values between 15 and 16.8 µM)	Frédérich, M. *et al.*, 2000
Usambarensine (Usambarensine-type)	*S. dale* (US) *S. memecyloides* (SB) *S. usambarensis* (RB, SB)	*In vitro*	Melanoma B16 cells Cultured Flow 2002 cells L1210 cells HeLa cells (Active at 10 µg/mL)	Angenot, L., and Bisset, N. G., 1971a Ohiri, F. C. *et al.*, 1983a Quetin-Leclercq, J. *et al.*, 1986 Quetin-Leclercq, J. *et al.*, 1991b Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 1998b
5',6'- Dihydrousambarensine (Usambarensine-type)	*S. dale* (US) *S. potatorum* (RB) *S. tchibangensis* (RB, TB) *S. usambarensis* (RB, SB)	*In vitro*	Melanoma B16 cells Cultured Flow 2002 cells L1210 cells HeLa cells (Active between 1 and 10 µg/mL)	Angenot, L., and Bisset, N. G., 1971a Richard, C. *et al.*, 1978 Ohiri, F. C. *et al.*, 1983a Quetin-Leclercq, J. *et al.*, 1986 Quetin-Leclercq, J. *et al.*, 1991b Massiot, G. *et al.*, 1992 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002

Metabolites	Origins	Types of test	Cytotoxic and anticancer activities	References
N_b-Methylusambarensine (Usambarensine-type)	*S. usambarensis* (RB)	*In vitro*	Melanoma B16 cells L1210 cells (Active at 25 µg/mL)	Angenot, L., and Bisset, N. G., 1971a Quetin-Leclercq, J. *et al.*, 1986 Delaude, C., and Delaude, L., 1997
Usambarine (Usambarensine-type)	*S. barteri* (L) *S. nigritana* (US) *S. usambarensis* (L, SB)	*In vitro*	Melanoma B16 cells Cultured Flow 2002 cells L1210 cells HeLa cells (Active between 1 and 10 µg/mL)	Koch, M., and Plat, M., 1971 Koch, M. *et al.*, 1973 Angenot, L. *et al* 1978d Ohiri, F. C. *et al.*, 1983a Quetin-Leclercq, J. *et al.*, 1986 Quetin-Leclercq, J. *et al.*, 1991b Delaude, C., and Delaude, L., 1997
18,19-Dihydrousambarine (= Nigritanine) (Usambarensine-type)	*S. barteri* (L, S) *S. nigritana* (L, S, SB) *S. usambarensis* (L, SB)	*In vitro*	Melanoma B16 cells Ehrlich ascites cells (line ELT) (Active at 50 µg/mL and between 1 and 50 µg/mL, respectively)	Angenot, L. *et al.*, 1978b Nicoletti, M. *et al.*, 1980 Ohiri, F. C. *et al.*, 1983a Tits, M. *et al.*, 1984 Quetin-Leclercq, J. *et al.*, 1991b Delaude, C., and Delaude, L., 1997
10-Hydroxyusambarine (Usambarensine-type)	*S. barteri* (US) *S. nigritana* (US) *S. usambarensis* (L, SB)	*In vitro*	Melanoma B16 cells Cultured Flow 2002 cells L1210 cells HeLa cells (Active at 10 µg/mL)	Angenot, L., and Denoël, A., 1973 Angenot, L. *et al.*, 1978b Angenot, L. *et al.*, 1978c Ohiri, F. C. *et al.*, 1983a Quetin-Leclercq, J. *et al.*, 1986 Quetin Leclercq, J. *et al.*, 1991b Delaude, C., and Delaude, L., 1997

Metabolites	Origins	Types of test	Cytotoxic and anticancer activities	References
N_b-Methyl-10-hydroxyusambarine (Usambarensine-type)	*S. usambarensis* (L, SB)	*In vitro*	Melanoma B16 cells L1210 cells (Active at 25 µg/mL)	Angenot, L. *et al.*, 1978b Angenot, L. *et al.*, 1978c Caprasse, M. *et al.*, 1983c Quetin-Leclercq, J. *et al.*, 1986 Quetin Leclercq, J. *et al.*, 1991b Delaude, C., and Delaude, L., 1997
11-Hydroxyusambarine (Usambarensine-type)	*S. usambarensis* (L, SB)	*In vitro*	Melanoma B16 cells Cultured Flow 2002 cells L1210 cells HeLa cells (Active at 10 µg/mL)	Angenot, L. *et al.*, 1978b Angenot, L. *et al.*, 1978c Ohiri, F. C. *et al.*, 1983a Quetin-Leclercq, J. *et al.*, 1986 Quetin Leclercq, J. *et al.*, 1991b Delaude, C., and Delaude, L., 1997
N_b-Methyl-11-hydroxyusambarine (Usambarensine-type)	*S. usambarensis* (L)	*In vitro*	Melanoma B16 cells L1210 cells (Active at 25 µg/mL)	Caprasse, M. *et al.*, 1983c Quetin-Leclercq, J. *et al.*, 1986 Delaude, C., and Delaude, L., 1997

Fr= Fruit; L= Leave; RB= Root bark; RT= Root; S=Seed; SB=Stem bark; US= Unspecified.

Regarding cytotoxic and anticancer activities, the usambarensine-type and β-carboline-type alkaloids are the most representative. These activities are therefore predominantly found in monomeric and dimeric indolic alkaloids.

III.3. Immune system

III.3.1. Traditional uses

To relieve fever, six species are traditionally used: *S. camptoneura* stem barks (aqueous extract), *S. cocculoides* root and stem barks, *S. decussata* stem barks (infusion or decoction), barks, leaves, and root barks, *S. innocua* stem barks, *S. mitis* leaves, and *S. spinosa* roots and green fruits. This is particularly the case in Tanzania (Bisset, N. G., 1970; Neuwinger, H. D., 1996; Sunghwa, F. *et al.*, 2009; Samie, A. *et al.*, 2010; Adamu, M. *et al.*, 2013; Morabandza, C. J. *et al.*, 2016; Maroyi, A., 2022; Abdoulahi, M. I. I. *et al.*, 2023; Cock, I. E. *et al.*, 2023). As for *S. madagascariensis*, it is used in Tanzania to prevent fever (Bisset, N. G., 1970)

The aqueous extract of *S. camptoneura* stem barks, as well as its barks, leaves, and seeds, are administered to reduce inflammation (Morabandza, C. J. *et al.*, 2016; Moulari, B. *et al.*, 2023). Several other plants are administered for this purpose. *S. decussata* roots are applied externally to reduce inflammation caused by wounds or abscesses (Rasoanaivo, P. *et al.*, 2002). The ripe fruits and seeds of *S. potatorum* could act against inflammation. Moreover, *S. potatorum* could treat scleritis (Sanmugapriya, E., and Venkataraman, S., 2010a; Mohana Lakshimi, S. *et al.*, 2012; Yadav, K. *et al.*, 2014). Another anti-inflammatory species used is *S. spinosa* (leaves and root barks) (Francis, P., and Suseem, S. R., 2016; Aremu, A. O., and Moyo, M., 2022). *S. henningsii* (branches), *S. icaja* (root barks), and *S. madagascariensis* (leaf sap and root decoction) are also administered in cases of one or two inflammatory joint pathology(ies): arthritis and/or rheumatism (Tits *et al.*, 1991; Oyedemi, S. O. *et al.*, 2009; Muthee, J. K. *et al.*, 2011; Francis, P., and Suseem, S. R., 2016; Samseny, R. R. R. A. *et al.*, 2021). These uses will be discussed in the sections III.10.1 and III.10.2 dedicated to the musculoskeletal system (Page 131).

III.3.2. Pharmacological properties

In this section, we find three types of pharmacological properties: anti-inflammatory, immunostimulant, and antipyretic.

The hydroalcoholic crude extract of *S. camptoneura* stem bark exhibits immunostimulant properties: in *in vivo* tests, it increased the number of white blood cells at doses between 100 and 250 mg/kg/day (Akassa, H. *et al.*, 2022). Moreover, the *in vitro* and *in vivo* assays of ethanolic crude extract from *S. potatorum* seeds demonstrated anti-inflammatory activity at doses between 1.56 and 250 µg/mL, as well as 150 mg/kg, respectively. Its *in vivo* effects showed stimulation of immune cells, namely CD3 and NK cells, along with cytokines (IL-10 and IL-4). This anti-inflammatory activity is associated with the presence of polyphenols in its seeds (Morabandza, C. J. *et al.*, 2022).

Total alkaloids from *S. icaja* roots showed remarkable anti-inflammatory activities at a concentration of 0.1 mg/mL in *in vitro* studies (Samseny, R. R. R. A. *et al.*, 2021).

Antipyretic activities were observed *in vivo* at 100 and 200 mg/kg for *S. potatorum* seeds powder and its aqueous extract. This activity is dose-dependent (Sanmugapriya, E., and Venkataraman, S., 2010a; Yadav, K. *et al.*, 2014). In addition, ethanolic and aqueous crude extracts of its seeds were tested *in vivo* for their anti-inflammatory activities. To assess this, both types of extract were administered intraperitoneally to rats at doses of 100 and 200 mg/kg. A moderate to significant anti-inflammatory activity was observed, with effects reported to be long-lasting (Sanmugapriya, E., and Venkataraman, S., 2007a; Mallikharjuna, P. B. *et al.*, 2010; Rahman, S. S. A. *et al.*, 2023).

The fruit seeds of *S. spinosa* contain betulinic acid and eriocitrine, whose anti-inflammatory, antioxidant, and anti-cancer activities are well-documented in the literature (Rabeharitsara, A. T. *et al.*, 2023). The leaves of *S. spinosa* also exhibit promising anti-inflammatory activity and could potentially be used to treat inflammation-related conditions. The IC_{50} values ranged from 88.4 to 325.1 µg/mL, depending on the type of extract. (Isa, A. I. *et al.*, 2016; Tittikpina, N. K. *et al.*, 2020).

The last species is *S. variabilis*. Its root barks contain monomeric alkaloids, such as retuline, some of which exhibit anti-inflammatory activity (Tits, M. *et al.*, 1980b; Tits, M. *et al.*, 1980c; Frédérich, M. *et al.*, 2003b).

III.4. Nervous system

III.4.1. Traditional uses

The primary activity sought in the nervous system is an analgesic effect. Seven species are traditionally used for their analgesic properties: *S. camptoneura*, *S. decussata*, *S. henningsii*, *S. innocua*, *S. panganensis*, *S. potatorum*, and *S. spinosa*. *S. camptoneura* root barks are administered in cases of abdominal pain, while infusions of *S. decussata* barks can be used for headaches. (Amuri, B. *et al.*, 2017; Maroyi, A., 2022). Several traditional uses for the leaves and barks of *S. spinosa* are also described for acting against headaches (Bisset, N. G., 1970; Neuwinger, H. D., 1996). Decoction or infusion of leaves, roots, and root barks from *S. decussata* could also relieve stomach pains (Rasoanaivo, P. *et al.*, 2002). The roots of *S. henningsii* are also traditionally used for stomach pains (Oyedemi, S. O. *et al.*, 2009; Muthee, J. K. *et al.*, 2011). The leaves and stem barks of this species could act on joints (leaves and stem barks) and against chest pain (decoction of leaves and roots) (Wambugu, S. N. *et al.*, 2011). Three other species are traditionally used for chest complaints: *S. afzelii*, *S. longicaudata*, and *S. panganensis* (Bisset, N. G., 1970). The latter is believed to treat chest pain, although the part used is not specified in the articles included in this study (Nuzillard, J.-M. *et al.*, 1996; Francis, P., and Suseem, S. R., 2016). *S. potatorum* seeds are also described in traditional medicine as an analgesic. It could treat all types of moderate to severe pain. (Sanmugapriya, E., and Venkataraman, S., 2010a; Yadav, K. *et al.*, 2014). The roots of *S. innocua* would also be analgesics (Angenot, L., and Wauters, J. N., 1974). Finally, *S. spinosa* is used to relieve earache (fruits) and abdominal pain (root and stem barks) (Bisset, N. G., 1970; Chifundera, K., 2001; Bero, J. *et al.*, 2009; Francis, P., and Suseem, S. R., 2016; Amuri, B. *et al.*, 2017; Aremu, A. O., and Moyo, M., 2022).

Some other traditional uses that could act on the nervous system are described in the literature:

- Roots of *S. cocculoides* (bath administration but no information about the form of administration) are used for cerebral ischemia (Novotna, B. *et al.*, 2020);
- *S. henningsii* could act on dizziness. The plant part is not specified (Francis, P., and Suseem, S. R., 2016);
- *S. potatorum* is used for depression and other nervous system disorders. The plant part is also not specified (Francis, P., and Suseem, S. R., 2016). Moreover, their fruits could relieve thirst (Mohana Lakshimi, S. *et al.*, 2012; Yadav, K. *et al.*, 2014);
- Fruit pulp of *S. aculeata* and leaves of *S. spinosa* are administered in cases of insanity (Bisset, N. G., 1970; Neuwinger, H. D., 1996).

According to the JSTOR Global Plants website, the roots and fruit pulp of *S. dinklagei* also have beneficial effects on the brain and nervous system. However, no further details are provided about these effects (JSTOR, 2024).

III.4.2. Pharmacological properties

The analgesic effects of ethanolic and aqueous crude extracts of *S. potatorum* seeds were evaluated *in vivo*. A dose of 500 mg/kg was injected intraperitoneally. An analgesic effect was observed for the ethanolic crude extract of seeds, with peak activity after 120 minutes, which confirms the analgesic activity of *S. potatorum* seeds. The convulsive activities of *S. potatorum* were also confirmed. (Mallikharjuna, P. B. *et al.*, 2010). Moreover, its seeds demonstrated antinociceptive effects at oral doses of 100 and 200 mg/kg by acting on the central and peripheral nervous systems (Sanmugapriya, E., and Venkataraman, S., 2010a).

III.5. Respiratory system

III.5.1. Traditional uses

As explained in the section III.1.2.1 about traditional uses for microbial infections (Pages 80-82), five African species from the *Strychnos* genus are administered for tuberculosis, a microbial infection caused by *Mycobacterium tuberculosis*. These five species are *S. aculeata* (leaves), *S. cocculoides* (leaves), *S. decussata* (barks), *S. henningsii* (stem and trunk barks), and *S. innocua* (roots and stems). They are administered alone or in combination with other plants in the form of decoctions or chewable products (Bisset, N. G., 1970; Neuwinger, H. D., 1996; Madikizela, B. *et al.*, 2017; Oryema, C. *et al.*, 2021; Maroyi, A., 2022). The roots of *S. aculeata* could also act against pneumonia. (Neuwinger, H. D., 1996).

In addition, the seeds of *S. potatorum* are traditionally administered in cases of bronchitis (Sanmugapriya, E., and Venkataraman, S., 2010a; Gangwar, U., and Choubey, A., 2019).

Finally, a decoction of roots and root barks of *S. spinosa* is used for asthma (Bisset, N. G., 1970; Neuwinger, H. D., 1996).

III.5.2. Pharmacological properties

Studies undertaken by Famewo *et al.* (2017) on *S. decussata* confirmed the antituberculosis activity of mixtures containing *S. decussata* barks at a concentration of less than 25 µg/mL.

S. potatorum seeds demonstrated significant inhibitory activity against *Mycobacterium tuberculosis* at concentrations ranging from 12.5 to 100 µg/mL, further confirming their antituberculosis potential (Mallikharjuna, P, and Seetharam, Y. N., 2009).

The dichloromethane crude extract of *S. spinosa* root barks is active against *Mycobacterium tuberculosis* with a MIC of 125 µg/mL. This study justifies its use as an antituberculosis agent (Ballo, M. *et al.*, 2020).

III.6. Digestive system

III.6.1. Traditional uses

A variety of traditional uses could act on the digestive system. The therapeutic effects in cases of, for example, stomach pains, gastric problems, and metabolic disorders, are sought in African plants of the *Strychnos* genus. These different activities are listed in the table below (Table 9).

Table 9: Traditional uses of African *Strychnos* for the digestive system.

Traditional uses	Species	Plant parts	References
Gastrointestinal problems (Gastropathy, diarrhoea, and nausea)	*S. cocculoides*	Unripe fruit (juice)	Neuwinger, H. D., 1996
	S. decussata	Decoction or infusion of leaves, roots, root barks, and stem barks	Maroyi, A., 2022
	S. henningsii	Barks	Bisset, N. G., 1970 Tits, M. *et al.*, 1991 Randrianarivony, T. N., 2017
	S. innocua	Stem barks	Abdoulahi, M. I. I. *et al.*, 2023
	S. madagascariensis	Barks, roots, and leaves (Decoction)	de Wet, H. *et al.*, 2010 Mallikharjuna, P. B. *et al.*, 2010 Lall, N. *et al.*, 2019 Maroyi, A., 2021
	S. potatorum	Seeds	Sanmugapriya, E., and Venkataraman, S, 2007b Sanmugapriya, E., and Venkataraman, S., 2010a Satish Kumar, B. N. *et al.*, 2010 Gangwar, U., and Choubey, A., 2019
	S. spinosa	Barks, leaves, and roots	Bisset, N. G., 1970 Neuwinger, H. D., 1996

Traditional uses	Species	Plant parts	References
Colic	*S. cocculoides*	Roots (Decoction)	Novotna, B. *et al.*, 2020
	S. henningsii	Barks	Bisset, N. G., 1970 Tits, M. *et al.*, 1991
	S. potatorum	Seeds	Mohana Lakshimi, S. *et al.*, 2012
	S. spinosa	Leaves	Bero, J. *et al.*, 2009
Purgative	*S. aculeata*	Seeds	Bisset, N. G., 1970
	S. henningsii	Unspecified	Francis, P., and Suseem, S. R., 2016
	S. spinosa	Leaves and root barks	Bisset, N. G., 1970 Neuwinger, H. D., 1996
Dysentery	*S. madagascariensis*	Fruits (Freed of the seeds)	Bisset, N. G., 1970
	S. potatorum	Seeds	Sanmugapriya, E., and Venkataraman, S., 2010a
	S. spinosa	Fruits (Pericarp), and root barks	Bisset, N. G., 1970 Amuri, B. *et al.*, 2017

Traditional uses	Species	Plant parts	References
Gastrointestinal tract pain (Stomach aches and cramps)	*S. afzelii*	Seeds	Verpoorte, R. *et al.*, 1978b
	S. camptoneura	Stem barks (Aqueous extract)	Morabandza, C. J. *et al.*, 2016
	S. cocculoides	Root and stem barks	Sunghwa, F. *et al.*, 2009
	S. decussata	Decoction or infusion of leaves, roots, root barks, and stem barks	Maroyi, A., 2022
	S. henningsii	Roots	Tits, M. *et al.*, 1991 Oyedemi, S. O. *et al.*, 2009 Muthee, J. K. *et al.*, 2011 Francis, P., and Suseem, S. R., 2016 Randrianarivony, T. N., 2017
	S. icaja	Maceration of roots in palm wine	Neuwinger, H. D., 1996
	S. pungens	Roots (Decoction)	Bisset, N. G., 1970
	S. spinosa	Leaves and root and stem barks	Bisset, N. G., 1970 Neuwinger, H. D., 1996 Chifundera, K., 2001 Bero, J. *et al.*, 2009 Amuri, B. *et al.*, 2017
Emetic (in cases of intoxication, for example)	*S. aculeata*	Seeds	Bisset, N. G., 1970
	S. cocculoides	Unripe fruits	Neuwinger, H. D., 1996 Ndubani, P., and Höjer, B., 1999
	S. dinklagei	Fruit pulp	JSTOR, 2024
	S. innocua	Seeds	Bisset, N. G., 1970
	S. madagascariensis	Roots (Decoction or infusion)	Lall, N. *et al.*, 2019
	S. potatorum	Ripe fruits	Mohana Lakshimi, S. *et al.*, 2012
	S. spinosa	Fruits, leaves, roots, and seeds	Neuwinger, H. D., 1996

Traditional uses	Species	Plant parts	References
Stomachic	*S. potatorum*	Seeds	Satish Kumar, B. N. *et al.*, 2010
Hepatoprotective	*S. potatorum*	Seeds	Sanmugapriya, E. and Venkataraman, S., 2006 Mohana Lakshimi, S. *et al.*, 2012
Jaundice	*S. innocua*	Stem barks	Abdoulahi, M. I. I. *et al.*, 2023
	S. potatorum	Rape fruits and seeds	Sanmugapriya, E., and Venkataraman, S., 2010a Mohana Lakshimi, S. *et al.*, 2012
Bladder stones	*S. potatorum*	Seeds	Gangwar, U., and Choubey, A., 2019
Diabete and reduction of blood glucose	*S. camptoneura*	Seeds	Ampa, R. *et al.*, 2018
	S. henningsii	Leaves and stem barks	Oyedemi, S. O. *et al.*, 2009 Mohammed, A. *et al.*, 2014
	S. icaja	Leaves	Samseny, R. R. R. A. *et al.*, 2021
	S. potatorum	Seeds	Sanmugapriya, E., and Venkataraman, S., 2010a
	S. spinosa	Root barks	Amuri, B. *et al.*, 2017 Aremu, A. O., and Moyo, M., 2022
Obesity management	*S. spinosa*	Fruits	Aumeeruddy, M. Z., and Mahomoodally, M. F., 2021

III.6.2. Pharmacological properties

Pharmacological assays have confirmed some traditional uses described in the previous section, notably antihyperglycemic and antidiabetic activities. Additionally, antilipidemic, hypolipidemic, antidiarrheal, and hepatoprotective properties have also been evaluated.

In *in vivo* tests, at doses ranging from 200 to 800 mg/kg, the aqueous extract of *S. camptoneura* seeds showed a significant reduction in blood glucose levels in both healthy and diabetic rats, and slowed the peak of blood glucose levels in rats previously injected with an overdose of glucose (Ampa, R. *et al.*, 2018).

This is also true for the aqueous extract of *S. henningsii* stem barks, which reduces blood glucose levels, water consumption, and triacylglycerol levels. The best results were obtained at 250 mg/kg. In addition, at a dose of 500 mg/kg, it significantly reduces cholesterol levels. In the studies, the body weight of diabetic rats was normalized at all tested doses, and their glucose tolerance was effectively restored to near-normal levels after 90 minutes at doses of 250 mg/kg and 500 mg/kg. These results therefore confirm both the antidiabetic and antilipidemic potential of the aqueous extract of *S. henningsii* stem barks (Oyedemi, S. O. *et al.*, 2012). Henningsiin is potentially responsible for the antidiabetic activity observed by regulating insulin and influencing glucose and lipid metabolism. It acts on several targets to produce the antidiabetic effect (Pereira, A. S. P. *et al.*, 2019).

Aqueous and methanolic crude extracts of *S. madagascariensis* fruit tegument and pulp inhibit several enzymes, namely α-amylase, α-glucosidase, and pancreatic lipase. IC_{50} values ranged from 0.055 to 0.10 mg/mL. They could therefore help in the treatment of diabetes (Oboh, M. O. *et al.*, 2020).

In vivo tests were also carried out on *S. potatorum*. Firstly, it was confirmed that *S. potatorum* seeds are both antidiabetic and antidiarrhoeal at doses ranging from 100 to 400 mg/kg (Biswas, S. *et al.*, 2002; Biswas, A. *et al.*, 2012). At doses at 100 and 200 mg/kg, the seeds significantly reduced serum levels of total cholesterol, triglycerides, low-density lipoprotein cholesterol, and very-low-density lipoprotein cholesterol in rats in a dose- and time-dependent manner. Therefore, its seeds have an antihyperlipidemic activity (Shah, U. S., and Patel, K. N., 2012). Finally, the seeds, at doses of 100 and 200 mg/kg, and

the polysaccharides they contain are hepatoprotective agents, reducing serum marker enzymes such as glutamate oxaloacetate transaminase (SGOT) and glutamate pyruvate transaminase (SGPT) (Sanmugapriya, E. and Venkataraman, S., 2006; Rahman, S. S. A. *et al.*, 2023).

To conclude this section, *S. spinosa* is notable for its antidiabetic properties. *In vitro* assays revealed that its leaves inhibit the α-glucosidase enzyme through an uncompetitive mechanism. Additionally, the decoction of its root bark exhibited a promising *in vivo* antihyperglycemic effect in guinea pigs at a dose of 500 mg/kg. These activities were also evaluated and confirmed by injecting methanolic crude extract of leaves in rats. At doses of 150, 300, and 500 mg/kg, a reduction in glucose, cholesterol, and triacylglycerol was demonstrated. In conclusion, it has been suggested that *S. spinosa* can both activate insulin and inhibit the synthesis of triacylglycerols and cholesterol (Amuri, B. *et al.*, 2017; Adinortey, M. B. *et al.*, 2018; Waziri, P. M. *et al.*, 2020; Aremu, A. O., and Moyo, M., 2022).

III.7. Urinary system

III.7.1. Traditional uses

In the literature, only five African species from the *Strychnos* genus are described as traditional uses for the urinary system: *S. aculeata*, *S. dinklagei*, *S. icaja*, *S. madagascariensis*, and *S. potatorum*. The decoction of *S. dinklagei* root or stem barks is used as diuretic, which helps reduce oedema and dropsy. The root decoction and leaf sap of *S. madagascariensis* is administered in cases of unspecified kidney disorders (Bisset, N. G., 1970; JSTOR, 2024). The barks of *S. aculeata*, and the root decoction of *S. spinosa* also could reduce dropsy (Neuwinger, H. D., 1996). A maceration of *S. icaja* roots in palm wine administered at low doses (unspecified precise doses) could have diuretic effects (Neuwinger, H. D., 1996). As for *S. potatorum*, its seeds could be nephroprotective and diuretic, and are traditionally used for strangury and kidney stones (Satish Kumar, B. N. *et al.*, 2010; Mohana Lakshimi, S. *et al.*, 2012; Gangwar, U., and Choubey, A., 2019).

III.7.2. Pharmacological properties

The ethanolic crude extract of *S. potatorum* seeds demonstrated significant nephroprotective activity *in vivo* and could potentially help treat acute kidney damage induced by nephrotoxins (Varghese, R. *et al.*, 2011; Mohana Lakshimi, S. *et al.*, 2012). Moreover, *in vivo*, the methanolic crude extract of its seeds has significant diuretic activity. This activity is dose-dependent, with notable hypernatremic, hyperchloremic, and hyperkalemic effects. The effective dose is 600 mg/kg body weight (Satish Kumar, B. N. *et al.*, 2010).

III.8. Reproductive system

III.8.1. Traditional uses

Aphrodisiac effects, treatment of menstrual and erectile disorders, *postpartum* bleeding, sterility, gynecological complaints, and sexually transmitted infections are all sought-after activities in the traditional use of *Strychnos*. Barks of *S. aculeata* (aphrodisiac), leaves and stem barks of *S. afzelii* (aphrodisiac), stem barks of *S. camptoneura* (erectile disorders), root barks of *S. cocculoides* (sexually transmitted infections, menstrual disorders, and *postpartum* bleeding), barks of *S. decussata* (sexually transmitted infections and gynecological complaints), stem barks of *S. madagascariensis* (menstrual disorders), seeds of *S. potatorum* (erectile disorders), and roots of *S. spinosa* (erectile disorders and sterility) are used for these sexual disorders and infections (Bisset, N. G., 1970; Verpoorte, R. *et al.*, 1978b; Neuwinger, H. D., 1996; Abdillahi, H. S., and Van Staden, J., 2012; Mohana Lakshimi, S. *et al.*, 2012; Francis, P., and Suseem, S. R., 2016; Morabandza, C. J. *et al.*, 2017).

For *postpartum* bleeding, a decoction of *S. cocculoides* roots is taken orally, whereas for sexually transmitted infections, either the roots or the fruit are used. In Zambia, three sexually transmitted infections, called *songeya*, *doroba*, and *bola-bola*, which correspond respectively to syphilis, gonorrhea, and lymphogranuloma venereum, are described. The first is characterized by genital wounds, sometimes accompanied by skin eruptions. The symptoms of the second are pus discharges, accompanied by backache and difficulty in urinating. The last infection causes fever. In addition, sufferers of *bola-bola*

experience difficulty walking and swelling in the groin area, with bumps resembling tennis balls. It has even been reported that, at times, the swollen lymph nodes burst and release pus (Ndubani, P., and Höjer, B., 1999; Novotna, B. *et al.*, 2020). A decoction of *S. cocculoides* roots can also be used for uterine pain and menorrhagia, while the barks are administered for the prevention of miscarriage (Neuwinger, H. D., 1996).

Other species were described as traditional uses. For example, decoctions of *S. decussata* barks, *S. potatorum* leaves and roots, and *S. spinosa* root barks, or tea made from *S. madagascariensis* stem barks, are administered for sexually transmitted infections such as *songeya*, *doroba*, and *bola-bola* (Bisset, N. G., 1970; Delaude, C. *et al.*, 1992; Neuwinger, H. D., 1996; Ndubani, P., and Höjer, B., 1999; Rasoanaivo, P. *et al.*, 2002; Lawal, F. *et al.*, 2019; Aremu, A. O., and Moyo, M., 2022; Kacholi, D. S., and Amir, H. M., 2022; Maroyi, A., 2022).

For disorders of sexual function, aqueous extracts of *S. camptoneura* stem barks are administered. The species *S. potatorum* and *S. spinosa* are also traditionally used for this purpose. Unfortunately, the specific parts of *S. potatorum* and *S. spinosa* used were not reported in the articles included in this study. These three *Strychnos* species can be used for erectile dysfunction in men (Francis, P., and Suseem, S. R., 2016; Morabandza, C. J. *et al.*, 2016).

To conclude this section, seven traditional uses still need to be described:

- *S. decussata* (barks) and S. *henningsii* (unspecified plant part) in cases of gynecological complaints and infections (Oyedemi, S. O. *et al.*, 2009; Samie, A. *et al.*, 2010);
- *S. cocculoides* (roots) and *S. madagascariensis* (unspecified plant part) for menstrual problems (Novotna, B. *et al.*, 2020; Maroyi, A., 2021);
- Both *S. cocculoides* and S. *spinosa* (infusion of roots) could treat infertility (Neuwinger, H. D., 1996; Bero, J. *et al.*, 2009; Tittikpina, N. K. *et al.*, 2020);
- *S. aculeata* (fruit pulp) for its abortifacient effects (Bisset, N. G., 1970; Neuwinger, H. D., 1996);
- *S. aculeata* (decoction of barks) and *S. spinosa* (decoction of leaves) in cases of scrotal elephantiasis (Bisset, N. G., 1970; Neuwinger, H. D., 1996);

- *S. innocua* (bathing in an infusion of leaves) could act against uterine fibroma (Bisset, N. G., 1970).
- *S. madagascariensis* (barks and twigs) could facilitate birth (Bisset, N. G., 1970).

III.8.2. Pharmacological properties

Very few pharmacological assays were carried out to investigate the reproductive activities of African *Strychnos*. Only the activities of *S. camptoneura* and *S. potatorum* were confirmed.

In vivo studies in rats demonstrated that aqueous and hydroalcoholic extracts of *S. camptoneura* stem barks, administered at doses of 100 and 250 mg/kg, significantly affected sexual parameters, such as sexual mounting, frequency of erections, number of ejaculations, and latency time. These aphrodisiac effects, potentially linked to the presence of flavonoids and sterols, modulate dopaminergic and cholinergic pathways (Morabandza, C. J. *et al.*, 2017). Moreover, at doses of 100 and 250 mg/kg, the hydroethanolic extract of *S. camptoneura* stem barks showed spermatogenic activity, significantly increasing sperm count, motility, and vitality (Akassa, H. *et al.*, 2023).

As described in section III.1.4.1 about pharmacological properties for treating antiviral infections (Pages 101-102), the methanolic crude extract of *S. potatorum* seeds exhibited antiviral activities *in vivo* against laboratory-adapted HIV-1 strains. Its seeds could therefore help in managing AIDS infections (Palshetkar, A. *et al.*, 2020). Additionally, a 70% methanolic crude extract of *S. potatorum* seeds demonstrated contraceptive activity in male rats in *in vivo* tests (Yadav, K. *et al.*, 2014).

III.9. Circulatory system

III.9.1. Traditional uses

Few traditional uses targeting the circulatory system were reported for African *Strychnos* species. The roots of *S. dinklagei* are traditionally used for oedema. Additionally, a decoction of its leaves, root barks, and stem barks in

palm oil is believed to affect the heart, particularly in cases of palpitations and tachycardia (Bisset, N. G., 1970; JSTOR, 2024). Concerning *S. potatorum*, it exhibits several potential actions on the circulatory system: actions against Raynaud's disease (unspecified plant part), diaphoretic effects (ripe fruit), effects on anemia (seeds), and tonic properties (seeds) (Mohana Lakshimi, S. *et al.*, 2012; Francis, P., and Suseem, S. R., 2016). *S. henningsii* could also act as a tonic agent. Unfortunately, the plant part used and the method of administration are not described in the article (Francis, P., and Suseem, S. R., 2016). Leaf decoction of *S. spinosa* could treat anemia (Neuwinger, H. D., 1996).

In Gabon, *S. icaja* is traditionally used in cases of hemorrhoids. No information is given on the plant part used or the method of administration (Francis, P., and Suseem, S. R., 2016; Samseny, R. R. R. A. *et al.*, 2021).

In Togo, sickle cell anemia, a disease that causes deformation and degradation of red blood cells, could be treated with *S. innocua* roots in combination with *Raphia sudanica* leaves and *Elaeis guineensis* fruit shells. This mixture is administered orally as a decoction. This use was subsequently confirmed in a study (Brukum, M. F. *et al.*, 2023).

For hypertension, the administration of *S. madagascariensis* could help reduce blood pressure (Maroyi, A., 2021).

Infusion of *S. decussata* barks could purify and detoxify the blood. It is administered orally and is primarily used for this purpose in South Africa (Maroyi, A., 2022).

The roots of *S. spinosa*, in powdered form to be chewed, are administered for bilious hemoglobinuria (Bisset, N. G., 1970; Neuwinger, H. D., 1996).

III.9.2. Pharmacological properties

The use of *S. innocua* roots, in combination with *Raphia sudanica* leaves and *Elaeis guineensis* fruit shells, to treat sickle cell anemia was confirmed in a study involving *in vitro* tests. Indeed, the ethanolic crude extract of stem barks and the hydroethanolic crude extract of root barks are both rich in polyphenolic compounds and flavonoids. Using these two extracts at a concentration of

2.5 mg/mL, the sickling of red blood cells was reduced at a rate of 35% for stem barks and 22% for root barks. Moreover, both extracts exhibited weak antioxidant activity (Brukum, M. F. *et al.*, 2023).

Chloroform, petroleum ether and methanolic crude extracts of *S. potatorum* seeds at doses ranging from 200 to 800 mg/kg are powerful anti-anaphylactic by inhibiting histamine and nitric oxide release. The level of activity was assessed in *in vivo* tests in rats (Patil, U. J. *et al.*, 2011). Moreover, the seeds have hypotensive activities at doses 3 and 4 mg/kg. Research has demonstrated that this activity is linked to the presence of various phytochemical classes of metabolites, with indole alkaloids being the primary contributors. For example, diaboline, a monoterpene indole alkaloid isolated from the root barks of *S. potatorum*, has hypotensive effect (Singh, H., and Kapoor, V. K., 1976; Singh, H., and Kapoor, V. K., 1980; Mallikharjuna, P. B. *et al.*, 2010; Wambugu, S. N. *et al.*, 2011).

The ethanolic crude extract of *S. camptoneura* trunk barks exhibited hemolytic activity at doses of 100 and 250 mg/kg. This activity may be attributed to the presence of saponins and tannins (Akassa, H. *et al.*, 2022).

Many species showed antioxidant and antiradicals properties:

- *S. camptoneura* (hydroethanolic crude extract of trunk barks (*in vivo*) at doses between 2.5 and 40 mg/mL);
- *S. henningsii* (aqueous crude extract of stem barks (*in vitro* and *in vivo*) at doses ranging from 250 to 1000 mg/kg);
- *S. icaja* (alkaloidic crude extracts of stems, as well as dichloromethane, ethyl acetate, butanolic, and aqueous crude extract of roots (*in vitro*), at a concentration of 0.1 mg/mL);
- *S. innocua* (hexane, chloroform, ethyl acetate, and methanolic crude extracts of stem barks (*in vitro*) at doses between 31.25 and 500 µg/mL. Hydroethanolic and ethanolic crude extracts of stem barks (*in vitro*) with IC_{50} values of 718.8 and 1038 µg/mL. Hydroethanolic and ethanolic crude extracts of root barks (*in vitro*) with IC_{50} values of 1783 and 1907.5 µg/mL, respectively. Hexane, chloroform, ethyl acetate, and methanolic crude extracts of leaves (*in vitro*) at doses between 50 and 250 µg/mL);

- *S. madagascariensis* (ethanolic crude extract of root barks (*in vitro*) with an IC_{50} value of 9.60 µg/mL);
- *S. mitis* (acetone crude extract of leaves (*in vitro*) with an EC_{50} of 3.5 mg/mL)
- *S. potatorum* (methanolic crude extract of seeds (*in vitro* and *in vivo*) at a concentration of 1 mg/mL and a dose of 200 mg/kg, respectively, as well as ethanolic crude extract of fruit epicarp (*in vitro*) with an IC_{50} value of 54.46 µg/mL)

(Tits, M. *et al.*, 1991; Oyedemi, S. O. *et al.*, 2010; Sanmugapriya, E. *et al.*, 2011; Adamu, M. *et al.*, 2014; Yadav, K. *et al.*, 2014; Murugan, P. K., and Venkatachalam, K., 2018; Lall, N. *et al.*, 2019; Maroyi, A., 2021; Samseny, R. R. R. A. *et al.*, 2021; Selogatwe, K. M. *et al.*, 2021; Abirami, A. *et al.*, 2022; Ayo, R. G. *et al.*, 2022; Achika, J. I. *et al.*, 2023; Akassa, H. *et al.*, 2023; Brukum, M. F. *et al.*, 2023).

Moreover, polysaccharide fractions isolated from *S. innocua*, *S. nux-vomica*, and *S. potatorum* seeds also exhibited anticoagulant properties at doses ranging from 0.5 to 5 mg/L (Adinolfi, M. *et al.*, 1994).

S. cocculoides fruits contain a juice rich in phenolic antioxidant compounds. These include kaempferol, quercetin, caffeic acid, protocatechuic acid, and iridoids (Ngadze, R. T. *et al.*, 2018).

Betulic acid and eriocitrine, the main compounds found in *S. spinosa* seeds, are well documented in the literature for their antioxidant properties (Rabeharitsara, A. T. *et al.*, 2023). There is also loganic acid, isolated particularly from *S. potatorum* seeds, which demonstrated *in vitro* antioxidant activity by scavenging free radicals with an IC_{50} value of 149 mg/mL. It also protects cells from heavy metal-mediated toxicity at doses ranging from 0.04 to 100 mg/mL (Abirami, A. *et al.*, 2022).

III.10. Musculoskeletal system

III.10.1. Traditional uses

For the musculoskeletal system, few traditional uses of African *Strychnos* species are described in the literature. The roots of *S. dinklagei* could treat gouty arthritis, while *S. henningsii* roots could act against arthritis (Muthee, J. K. *et al.*, 2011; JSTOR, 2024). Finally, another inflammatory pathology, rheumatism, could be treated with *S. henningsii* branches, *S. icaja* root barks, as well as *S. madagascariensis* leaf sap and root decoction (Bisset, N. G., 1970; Tits, M. *et al.*, 1991; Oyedemi, S. O. *et al.*, 2009; Francis, P., and Suseem, S. R., 2016; Samseny, R. R. R. A. *et al.*, 2021).

III.10.2. Pharmacological properties

The anti-arthritic activity of *S. potatorum* seeds was evaluated *in vivo* at the dose of 200 mg/kg. The seed powder and its aqueous extract demonstrated anti-arthritic activity by significantly normalizing hematological and biochemical abnormalities in arthritic rats (Sanmugapriya, E. *et al.*, 2010b).

III.11. Integumentary system

III.11.1. Traditional uses

In this section, we will describe traditional uses in cases of ulcers, wounds, burns, and various other skin problems such as allergies, infections, pimples, warts, *etc*.

To begin this section, ulcers could be treated with various African species, namely *S. camptoneura*, *S. decussata*, *S. diplotricha*, *S. potatorum*, and *S. spinosa*. The decoction of *S. camptoneura* leaves and barks are applied on ulcers and wounds (Bisset, N. G., 1970; Neuwinger, H.D., 1996; Morabandza, C. J. *et al.*, 2016). In Kenya, ulcers could be treated using *S. diplotricha* stem barks. The leaves of *S. potatorum* are used as a poultice on maggot-infected ulcers (Yadav, K. *et al.*, 2014).

Next, *S. madagascariensis* leaves, barks and fruits are traditionally used to alleviate and act against burns and wounds. For example, this is the case in a rural community in northern Maputaland (de Wet, H. *et al.*, 2013; Nciki, S. *et al.*, 2016; Lall, N. *et al.*, 2019; Shai, K. N. *et al.*, 2020). Concerning the wounds, other species are also used for this purpose: *S. boonei* barks, *S. camptoneura* leaves (decoction to apply on the skin), *S. cocculoides* roots (orally administration to stop bleeding from a wound), *S. decussata* roots (external application to reduce inflammation due to a wound), *S. diplotricha* (unspecified plant part and administration), *S. henningsii* (unspecified plant part and administration), *S. melastomatoides* young leaves, *S. pungens* leaves, and *S. spinosa* leaves (Bisset, N. G., 1970; Neuwinger, H. D., 1996; Rasoanaivo, P. *et al.*, 1996; Oyedemi, S. O. *et al.*, 2009; Rasoanaivo, P. *et al.*, 2002; Morabandza, C. J. *et al.*, 2016; Novotna, B. *et al.*, 2020).

Finally, other skin problems are described in the literature:

- *S. camptoneura* could help prevent, reduce, or treat various skin diseases. The plant part and the method of administration are unspecified (Morabandza, C. J. *et al.*, 2022);
- *S. cocculoides* roots are used for boils on the legs by administering a decoction in bath water (Novotna, B. *et al.*, 2020);
- *S. icaja* is traditionally used in cases of skin allergy. The plant part and the method of administration are unspecified (Francis, P., and Suseem, S. R., 2016);
- The decoction of *S. innocua* root barks is applied to skin infections (Sallau, M. S. *et al.*, 2022);
- On the one hand, in the presence of leukoderma, roots of *S. potatorum* are used. On the other hand, the pimples could be treated using their barks (Mohana Lakshimi, S. *et al.*, 2012; Loganathan, S., and Selvam, K., 2018);
- The last uses concern *S. spinosa*. The infusion of their leaves is administered externally by applying on scabies and other skin diseases such as infections. Warts, wound, and acne could also be treated with its fruits and fruit sap (Neuwinger, H. D., 1996; de Wet, H. *et al.*, 2013; Rasoanaivo, P. *et al.*, 2002; Nciki, S. *et al.*, 2016; Aremu, A. O., and Moyo, M., 2022).

III.11.2. Pharmacological properties

The anti-ulcer property of *S. potatorum* seeds was confirmed in *in vivo* tests. At doses of 100 mg/kg and 200 mg/kg, the seed powder and its aqueous extract reduced acid secretion and increased the protective activity of mucin in rats. Therefore, antisecretory and mucoprotective actions were observed. Histological studies of rat mucosa further confirmed the anti-ulcer activity. It is likely that the mucoprotective activity is due to the presence of polysaccharides in the seeds (Sanmugapriya, E., and Venkataraman, S, 2007b).

An additional assay was conducted to evaluate the healing potential of the ethanolic crude extract of *S. camptoneura* seeds. At a concentration of 1.56 µg/mL, a significant increase in growth factors was observed, enhancing the healing capacity. Phytochemical analysis identified a substantial presence of compounds that promote healing in the seeds. However, a low level of toxicity was detected at this concentration. Despite this, cell viability remained optimal, exceeding 70% (Moulari, B. *et al.*, 2023).

III.12. Others

Other traditional uses of African *Strychnos* that could not be classified in the previous sections are listed in this section and described in the table below (Table 10).

Table 10: List of other traditional uses.

Species	Plant parts	Traditional uses	References
Strychnos afzelii	Stems	Chewing sticks (Bitter taste)	Bisset, N. G., 1970 Verpoorte, R. *et al.*, 1978b
	Leaves	Scent	
S. camptoneura	Stem barks (Aqueous extract)	Hernias	Morabandza, C. J. *et al.*, 2016

Species	Plant parts	Traditional uses	References
S. cocculoides	Unripe fruits and leaves (Infusion)	Sore eyes	Neuwinger, H. D., 1996
	Fruits	Food source	Mapunda, E. P. *et al.*, 2019 Mbiyangandu, K., 1985 Ngadze, R. T. *et al.*, 2017b Ngadze, R. T. *et al.*, 2018 Saka, J. *et al.*, 2007
	Unspecified	Ethnoveterinary remedy	Jambwa, P. *et al.*, 2022
S. dale	Roots	Fetish	Rolfsen, W. *et al.*, 1978
S. decussata	Fruits	Food source	Maroyi, A., 2022
	Barks and roots (Infusion or decoction)	Charm and ritual	
	Barks, leaves, roots, root barks, and stem barks (Infusion or decoction)	Ethnoveterinary remedy	
	Root barks	Tobacco	

Species	Plant parts	Traditional uses	References
S. dinklagei	Roots	Swelling	JSTOR, 2024
S. henningsii	Unspecified (Decoction)	Appetizer (To stimulate the appetite)	Bisset, N. G., 1970 Ogeto, J. O., and Maitai, C. K., 1983 Ogeto, J. O. *et al.*, 1984
	Barks and resins	Ethnoveterinary remedy	Bisset, N. G., 1970 Selogatwe, K. M. *et al.*, 2021
S. icaja	Unspecified	Hernias	Francis, P., and Suseem, S. R., 2016
S. innocua	Fruits	Food source	Bisset, N. G., 1970 Ngadze, R. T. *et al.*, 2017a
	Roots	Ethnoveterinary remedy (East coast fever, known as "makebe")	Tabuti, J. R. S. *et al.*, 2003
		Sore eyes in children	Bisset, N. G., 1970
	Twigs	Toothbrush	Bisset, N. G., 1970
S. madagascariensis	Leaves (Infusion)	Sore eyes	Lall, N. *et al.*, 2019
	Unspecified	Charm and rituals	Maroyi, A., 2021
	Unspecified	Insecticide	Maroyi, A., 2021
	Unspecified	Ethnoveterinary remedy	Maroyi, A., 2021
	Fruits, leaves, and roots	Food source	Ngadze, R. T. *et al.*, 2017a Shai, K. N. *et al.*, 2020
S. melastomatoides	Stems	Chew sticks	Bisset, N. G., 1970

Species	Plant parts	Traditional uses	References
S. mitis	Leaves	Ethnoveterinary remedy	Selogatwe, K. M. *et al.*, 2021
S. potatorum	Fruits	Eye disease	Mohana Lakshimi, S. *et al.*, 2012
	Seeds	Demulsifying agent	Satish Kumar, B. N. *et al.*, 2010
	Fruits	Relieving hallucinations	Mohana Lakshimi, S. *et al.*, 2012 Francis, P., and Suseem, S. R., 2016
S. scheffleri	Stem barks	Used by nursing mothers to clean their uterus	Tchinda, A. T. *et al.*, 2007
	Fruits Sap + Other unspecified parts	Domestic use: making baskets, eating fruit, and washing children's mouths	Caprasse, M., and Angenot, L., 1981a
S. spinosa	Leaves and twigs	Ear and eye inflammations	Bisset, N. G., 1970 Neuwinger, H. D., 1996 Francis, P., and Suseem, S. R., 2016
	Fruits	Charm or sorcery	Kinda, P. T. et *al.*, 2017
	Unspecified	Ethnoveterinary remedy (Sleeping sickness in cattle)	Aremu, A. O., and Moyo, M., 2022
	Fruits	Food source	Aremu, A. O., and Moyo, M., 2022 Asase, A. *et al.*, 2005 Hassan, H. *et al.*, 2015 Khan, M. A. *et al.*, 2016
	Roots (Decoction)	Hernias	Bisset, N. G., 1970 Neuwinger, H. D., 1996

III.13. Toxicity and antidote

As explained in the introduction to this review, *Strychnos* is known as much for its therapeutic properties as for its toxic effects, which are used in hunting and ordeals. Initially, it was hypothesized that the tetanizing and curarizing activities were linked to the species' origin. Thus, Asian *Strychnos* species are more tetanizing, while American *Strychnos* species are more paralyzing. As for African *Strychnos*, they are either considered similar to Asian species or regarded as alkaloid-free. Subsequently, this hypothesis was concluded to be erroneous and, therefore, was abandoned, replaced by the suggestion that activity depends on metabolite content (Delaude, C., and Delaude, L., 1997; Philippe, G. *et al.*, 2004).

The two main toxicities of plants from the *Strychnos* genus are also described in traditional uses. Moreover, various pharmacological tests confirmed these toxicities.

In contrast, some species serve as antidotes for snake bites and scorpion stings. These uses are also discussed in this section.

III.13.1. Traditional uses

Four types of traditional uses related to the toxicity of African *Strychnos* are reported in the literature: fishing poison, poisoned arrows for hunting, ordeals, and oracular poison. This section discusses these uses.

The fruits of *S. aculeata* and *S. camptoneura*, the leaves and fruits of *S. potatorum*, the leaves of *S. samba* and *S. spinosa*, as well as all the parts of *S. icaja*, are used to poison fish (Bisset, N. G., 1970; Neuwinger, H. D., 1996; Neuwinger, H. D., 2004; Philippe, G. *et al.*, 2004).

S. angolensis (unspecified plant part), *S. densiflora* root barks, *S. icaja* roots, *S. samba* root barks, and *S. spinosa* barks are used for ordeals. The general principle of these tests was to determine a person's innocence or guilt by having them consume poison. If the person survived, their innocence was affirmed. These ordalies were used until the latter part of the 19th century (Bisset, N. G.,

and Leeuwenberg, A. J. M., 1968; Bisset, N. G., 1970; Neuwinger, H. D., 1996; De Smet, P. A. G. M., 1998; Frédérich, M. *et al.*, 2003b; Philippe, G. *et al.*, 2004).

The roots of *S. icaja* serve as an adjuvant in arrow poison. Similarly, *S. usambarensis* is known for its curarizing effect, which induces muscle relaxation at the neuromuscular junction through competitive blockade of post-synaptic nicotinic acetylcholine receptors, leading to paralysis in the prey's limbs, with the respiratory muscles being the last to be affected. Arrows are coated with poison prepared from the roots and leaves of *S. usambarensis* (Bisset, N. G., and Leeuwenberg, A. J. M., 1968; Angenot, L., 1971b; Bisset, N. G., 1989; Neuwinger, H. D., 1996; Frédérich, M. *et al.*, 2003b; Philippe, G. *et al.*, 2004; Cao, M. *et al.*, 2011; Francis, P., and Suseem, S. R., 2016). In a mixture of arrow poison from the Central African Republic, the fruits of *S. spinosa* are described. However, due to its low toxicity, it is highly probable that it does not significantly contribute to the poison's overall toxicity (Bisset, N. G., and Leeuwenberg, A. J. M., 1968; Bisset, N. G., 1970; Neuwinger, H. D., 1996). Finally, the last arrow poisons to report are the root and stem barks of *S. camptoneura*, as well as the fruits of *S. cocculoides* (Neuwinger, H. D., 1996).

S. icaja is also reported to be used as an oracular poison. The process involves asking a question and then administering a mixture of water and grated barks to a chicken. If the chicken survives, the answer to the question is negative, while, if it succumbs, the answer is positive. A second test is conducted on another chicken to confirm the answer. If the answer matches the first, the ritual is considered invalid. In this case, a new session must be conducted using a different poison, location, and animals. This practice is carried out in northern Congo (formerly Zaire) (De Smet, P. A. G. M., 1998).

Finally, according to the article by Shai, K. N. *et al.*, published in 2020, *S. madagascariensis* is also used as a poison. However, no details of this use are provided.

Other toxicities are described in the literature, but these are not associated with traditional use. This is particularly true for the barks of *S. afzelii*, which are toxic and may potentially cause respiratory and circulatory problems (Sandberg, F. *et al.*, 1971). There is also *S. variabilis*, whose root barks are reputed to be a potent poison (Angenot, L. *et al.*, 1975b; Frédérich, M. *et al.*, 2003b).

Interestingly, the *Strychnos* genus is also a source of antidotes, particularly for snake bites and scorpion stings. The root and stem barks of *S. cocculoides*, the leaves of *S. congolana*, the barks and roots of *S. decussata*, the stem barks of *S. henningsii*, the leaves and fresh roots of *S. innocua*, the barks of *S. potatorum* (mixed with the barks of *S. colubrina* and cow urine), and the roots of *S. spinosa* are traditionally used in cases of snake bites (Bisset, N. G., 1970; Neuwinger, H. D., 1996; Auta, A. *et al.*, 2012; Naturu, S. *et al.*, 2013; Molander, M. *et al.*, 2014; Tittikpina, N. K. *et al.*, 2020; Mokua, S. K. *et al.*, 2021; Maroyi, A., 2022; Mogha, N. G. *et al.*, 2022). As for scorpion stings, two species are described in the literature: *S. decussata* (decoction or infusion of roots administered orally or root powder applied externally) and *S. potatorum* (seeds) (Maroyi, A., 2022; Nath, S., and Mukherjee, A. K., 2023).

III.13.2. Pharmacological properties

A significant amount of research was conducted on the *Strychnos* genus to investigate its paralyzing and curarizing properties. The table below (Table 11) lists all the African *Strychnos* tested in pharmacological assays, classifying them according to whether they are tetanizing (convulsive), paralyzing, or have no effect. The doses administered vary up to 1000 mg/kg.

Concerning the paralyzing properties, two main categories are differentiated in the context of this work: namely curarizing and muscle relaxant compounds. The first category includes all compounds that competitively antagonize nicotinic acetylcholine receptors in striated skeletal muscle. Mainly belonging to the curare class, these compounds have powerful effects and are therefore dangerous. The second category, muscle relaxants, refers to compounds with other forms of muscle-relaxing activity, such as papaverine, an isoquinoline alkaloid isolated from *Papaver somniferum*, which has spasmolytic effects on smooth muscles.

Table 11: African *Strychnos* species investigated for tetanizing and paralyzing activities.

Species	Tetanizing effects	Paralyzing effects	No effect	References
Strychnos aculeata	/	Fruits, seeds, and stem barks	/	Sandberg, F. *et al.*, 1971 Rolfsen, W. *et al.*, 1979 Ohiri, F. C. *et al.*, 1983a
S. afzelii	/	Stem barks	/	Verpoorte, R. *et al.*, 1978b
S. angolensis	Root and stem barks	Root and stem barks	/	Angenot, L., 1971b Sandberg, F. *et al.*, 1971 Bohlin, L. *et al.*, 1979 Rolfsen, W. *et al.*, 1979 Ohiri, F. C. *et al.*, 1983a Verpoorte, R. *et al.*, 1983a
S. barteri	Barks	Barks	/	Sandberg, F. *et al.*, 1971 Verpoorte, R., and Bohlin, L., 1976 Ohiri, F. C. *et al.*, 1983a
S. boonei	Stem barks	Barks and stem barks	/	Sandberg, F. *et al.*, 1971 Ohiri, F. C. *et al.*, 1983a
S. camptoneura	Stem barks	Root and stem barks	/	Sandberg, F. *et al.*, 1971 Verpoorte, R., and Sandberg, F., 1971 Angenot, L., and Denoël, A., 1972 Rolfsen, W. *et al.*, 1979 Ohiri, F. C. *et al.*, 1983a
S. chrysophylla	Stem barks	Stem barks	/	Sandberg, F. *et al.*, 1971 Ohiri, F. C. *et al.*, 1983a Verpoorte, R. *et al.*, 1984
S. cocculoides	Branches and leaves	Barks (T), root barks, and stem barks	Barks (K)	Geevaratne, M. *et al.*, 1977 Ohiri, F. C. *et al.*, 1983a
S. congolana	/	Stem barks (G)	Stem barks (CAR)	Rolfsen, W. *et al.*, 1979 Ohiri, F. C. *et al.*, 1983a
S. cuminodora	/	Root and stem barks	/	Ohiri, F. C. *et al.*, 1983a

Species	Tetanizing effects	Paralyzing effects	No effect	References
S. dale	Barks and stem barks	Barks and stem barks	/	Sandberg, F. *et al.*, 1971 Rolfsen, W. *et al.*, 1978
S. decussata	/	Barks	/	Geevaratne, M. *et al.*, 1977
S. densiflora	/	/	Barks	Sandberg, F. *et al.*, 1971
S. dinklagei	Leaves, root barks, and stem barks	Leaves, root barks, and stem barks	/	Sandberg, F. *et al.*, 1971 Ohiri, F. C. *et al.*, 1983a
S. dolichothyrsa	/	Stem barks	/	Sandberg, F. *et al.*, 1971 Verpoorte, R., and Baerheim Svendsen, A., 1978c Verpoorte, R., 1978d Ohiri, F. C. *et al.*, 1983a
S. elaeocarpa	Stem barks	Leaves and stem barks	/	Sandberg, F. *et al.*, 1971 Rolfsen, W. *et al.*, 1978 Ohiri, F. C. *et al.*, 1983a
S. fallax	/	Stem barks	/	Rolfsen, W. *et al.*, 1979 Ohiri, F. C. *et al.*, 1983a
S. floribunda	Root and stem barks	Root and stem barks	/	Sandberg, F. *et al.*, 1971 Ohiri, F. C. *et al.*, 1983a
S. henningsii	Alkaloids (Unspecified plant part)	/	Branches and stem barks	Ogeto, J. O., and Maitai, C. K., 1983 Ohiri, F. C. *et al.*, 1983a Ogeto, J. O. *et al.*, 1984
S. icaja	Leaves and stem barks	Root barks	/	Rolfsen, W. *et al.*, 1979 Kambu, K. *et al.*, 1980 Ohiri, F. C. *et al.*, 1983a Neuwinger, H. D., 1996 Philippe, G. *et al.*, 2004
S. innocua	/	Root barks	Branches and leaves	Ohiri, F. C. *et al.*, 1983a
S. johnsonii	Root and stem barks	Stem barks	/	Rolfsen, W. *et al.*, 1979 Ohiri, F. C. *et al.*, 1983a
S. longicaudata	Root and stem barks	Root and stem barks	Leaves	Ohiri, F. C. *et al.*, 1983a
S. lucens	/	Leaves and barks	Unspecified	Geevaratne, M. *et al.*, 1977 Ohiri, F. C. *et al.*, 1983a
S. madagascariensis	/	/	Root and stem barks	Ohiri, F. C. *et al.*, 1983a

Species	Tetanizing effects	Paralyzing effects	No effect	References
S. malacoclados	Barks	Root barks	/	Verpoorte, R., and Baerheim Svendsen, A., 1974 Neuwinger, H. D., 1996
S. memecyloides	/	Stem barks	Root and stem barks	Rolfsen, W. *et al.*, 1979 Ohiri, F. C. *et al.*, 1983a
S. millepunctata	/	Root and stem barks	/	Ohiri, F. C. *et al.*, 1983a
S. mimfiensis	/	Barks	/	Sandberg, F. *et al.*, 1971
S. ngouniensis	/	Stem barks	/	Ohiri, F. C. *et al.*, 1983a
S. nigritana	/	/	Root and stem barks	Ohiri, F. C. *et al.*, 1983a
S. panganensis	/	Barks	/	Geevaratne, M. *et al.*, 1977
S. phaeotricha	/	Stem barks	/	Rolfsen, W. *et al.*, 1979
S. potatorum	Root barks	Root and stem barks	/	Ohiri, F. C. *et al.*, 1983a
S. pungens	/	/	Branches and stem barks	Ohiri, F. C. *et al.*, 1983a
S. samba	Root barks	Leaves, root barks, and stem barks	/	Ohiri, F. C. *et al.*, 1983a
S. scheffleri	/	Root and stem barks	/	Geevaratne, M. *et al.*, 1977 Ohiri, F. C. *et al.*, 1983a
S. soubrensis	Stem barks	Stem barks	/	Ohiri, F. C. *et al.*, 1983a Ohiri, F. C. *et al.*, 1983b
S. spinosa	/	Leaves, root barks, stems, and stem barks	Barks	Verpoorte, R., and Bohlin, L., 1976 Ohiri, F. C. *et al.*, 1983a
S. splendens	/	Barks	/	Sandberg, F. *et al.*, 1971 Ohiri, F. C. *et al.*, 1983a
S. staudtii	Barks	Barks, root barks, and stem barks	/	Sandberg, F. *et al.*, 1971 Ohiri, F. C. *et al.*, 1983a
S. ternata	/	Root and stem barks	/	Ohiri, F. C. *et al.*, 1983a

Species	Tetanizing effects	Paralyzing effects	No effect	References
S. tricalysioides	/	Stem barks	/	Leclercq, J., and Angenot, L., 1984
S. urceolata	/	Root and stem barks	/	Ohiri, F. C. *et al.*, 1983a
S. usambarensis	Barks and leaves	Barks and leaves	/	Sandberg, F. *et al.*, 1971 Geevaratne, M. *et al.*, 1977 Neuwinger, H. D., 1996
	/	Root barks (Cura)	/	Angenot, L. *et al.*, 1975c
S. xantha	Root and stem barks	Branches	Leaves	Ohiri, F. C. *et al.*, 1983a

CAR= Central African Republic; G= Gabon; K= Kenya; T= Tanzania; Cura= curarizing activity.

Some alkaloids, purified from plants of the *Strychnos* genus, exhibited either tetanizing or curarizing properties during pharmacological assays.

In the literature, ten alkaloids were described as curarizing, nine as muscle relaxant, and thirteen as tetanizing (Tables 12, 13, and 14).

Table 12: Curarizing alkaloids isolated from African *Strychnos* species.

Alkaloids	Origins	Plant parts	References
Afrocurarine (Indoloquinolizine-type) (*In vitro*: IC_{50} value of 3.2 ± 0.9 µM) (*In vivo*: ED_{50} value of 3 µM/kg)	*S. usambarensis*	Root barks	Angenot, L. *et al.*, 1975c Delaude, C., and Delaude, L., 1997 Wins, P. *et al.*, 2003
C-calebassine (Toxiferine-type) (*In vivo*: ED_{50} value of 0.481 ± 0.13 µM/kg)	*S. usambarensis*	Root barks	Angenot, L. *et al.*, 1975c Delaude, C., and Delaude, L., 1997

Alkaloids	Origins	Plant parts	References
C-curarine (Toxiferine-type) (*In vivo*: ED_{50} value of 0.153 µM/kg)	*S. usambarensis*	Root barks	Angenot, L. *et al.*, 1975c Delaude, C., and Delaude, L., 1997
Diploceline (Indoloquinolizine-type) (*In vitro*: IC_{50} value of 60 ± 20 µM)	*S. gossweileri*	Roots	Coune, C., and Angenot, L., 1978b Wins, P. *et al.*, 2003
5,6-Dihydroflavopereirine (Indoloquinolizine-type) (*In vitro*: IC_{50} value of 40 ± 5 µM)	*S. usambarensis*	Root barks	Caprasse, M. *et al.*, 1983a Wins, P. *et al.*, 2003
Fluorocurarine (Toxiferine-type) (*In vitro*: IC_{50} value of 11 ± 5 µM)	*S. usambarensis*	Root and stem barks	Waser, P., 1967 Caprasse, M. *et al.*, 1981b Wins, P. *et al.*, 2003
Fluorocurine (Toxiferine-type) (*In vitro*: IC_{50} value of 28 ± 6 µM)	*S. scheffleri*	Stem barks	Caprasse, M., and Angenot, L., 1981a Tits, M. *et al.*, 1981
	S. variabilis	Root barks	
Macusine B (Sarpagine-type) (*In vitro*: IC_{50} value of 8.5 ± 2.2 µM)	*S. decussata*	Stem barks	Angenot, L., 1975d Olaniyi, A. A. *et al.*, 1981 Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Wins, P. *et al.*, 2003
	S. usambarensis	Root barks	

Alkaloids	Origins	Plant parts	References
Mavacurine (Indoloquinolizine-type) (*In vitro*: IC_{50} value of 50 ± 20 µM)	*S. scheffleri*	Stem barks	Caprasse, M., and Angenot, L., 1981a Tits, M. *et al.*, 1981
	S. variabilis	Root barks	
C-dihydrotoxiferine (Toxiferine-type) (*In vivo*: ED_{50} value of 0.382 ± 0.34 µM/kg)	*S. usambarensis*	Root barks	Angenot, L. *et al.*, 1975c Delaude, C., and Delaude, L., 1997

Table 13: Muscle relaxant alkaloids isolated from African *Strychnos* species.

Alkaloids	Origins	Plant parts	References
Caracurine V (Toxiferine-type) (Effective dose: 10-13 mg/kg)	*S. afzelii*	Stem barks	Verpoorte, R. *et al.*, 1978a Verpoorte, R. *et al.*, 1978b Bohlin, L. *et al.*, 1979 Verpoorte, R. *et al.*, 1982 Delaude, C., and Delaude, L., 1997 Ohiri, F. C. *et al.*, 1983a Verpoorte, R. *et al.*, 1984
	S. angolensis	Root and stem barks	
	S. chrysophylla	Stem barks	
	S. dolichothyrsa	Stem barks	
	S. malacoclados	Unspecified	
	S. urceolata	Leaves and stem barks	

Alkaloids	Origins	Plant parts	References
Decussine (β-carboline-type) (Effective dose: between 50 and 100 mg/kg)	*S. dale*	Stem barks	Rolfsen, W. *et al.*, 1980c Rolfsen, W. *et al.*, 1981a Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002
	S. elaeocarpa	Stem barks	
Isosplendine (Spermostrychnine-type) (Unspecified effective dose)	*S. aculeata*	Stem barks	Koch, M. *et al.*, 1969 Ohiri, F. C. *et al.*, 1983a Ohiri, F. C. *et al.*, 1983b Weeratunga, G. *et al.*, 1984 Delaude, C., and Delaude, L., 1997
	S. soubrensis	Stem barks	
	S. splendens	Fruits	
11-Methoxy-macusine A (Sarpagine-type) (Effective dose: 75 mg/kg)	*S. angolensis*	Root and stem barks	Verpoorte, R. *et al.*, 1983a
Malindine (Angustine-type) (Effective dose: 75 mg/kg)	*S. decussata*	Stem barks	Rolfsen, W. *et al.*, 1980b Olaniyi, A. A. *et al.*, 1981 Rolfsen, W. *et al.*, 1981b Neuwinger, H. D., 1996 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Maroyi, A., 2022
	S. usambarensis	Roots	

Alkaloids	Origins	Plant parts	References
Melinonine A (Ajmalicine-type) (Unspecified effective dose)	*S. camptoneura*	Stem barks	Sandberg, F. *et al.*, 1971
Strychnocarpine (β-carboline-type) (Effective dose: 125 mg/kg)	*S. elaeocarpa*	Stem barks	Rolfsen, W. *et al.*, 1980a Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002
	S. floribunda	Stem barks	
N_1-Acetyl-O-methylstrychnosplendine (Spermostrychnine-type) (Effective dose: 50 mg/kg)	*S. aculeata*	Stem barks	Caprasse, M., and Angenot, L., 1981a Weeratunga, G. *et al.*, 1984 Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997
	S. scheffleri	Leaves	
Usambarensine (Usambarensine-type) (Effective dose: From a concentration of 3.10^{-6} M)	*S. dale*	Unspecified	Angenot, L. *et al.*, 1975c Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997
	S. memecyloides	Stem barks	
	S. usambarensis	Root barks	

Effective doses correspond to those obtained in the screen grip test on mice[12].

[12] The screen grip test involves observing when the mouse falls off the screen after tilting and shaking it.

Table 14: Convulsive alkaloids isolated from African *Strychnos* species.

Alkaloids	Origins	Plant parts	References
Akagerine (β-carboline-type) (CD_{50}[13] at 50 mg/kg)	*S. barteri*	Root and stem barks	Rolfsen, W. *et al.*, 1980b Ohiri, F. C. *et al.*, 1983a Massiot, G. *et al.*, 1987 Neuwinger, H. D., 1996 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Maroyi, A., 2022 Tchangou Njiemou, A. F. *et al.*, 2022
	S. camptoneura	Stem barks	
	S. dale	Stem barks	
	S. decussata	Stem barks	
	S. elaeocarpa	Stem barks	
	S. floribunda	Stem barks	
	S. johnsonii	Roots	
	S. nigritana	Roots	
	S. phaeotricha	Root and stem barks	
	S. spinosa	Leaves and stem barks	
	S. usambarensis	Fruits and root barks	
17-O-Methylakagerine (β-carboline-type) (CD_{50} at 45.3 mg/kg)	*S. dale*	Stem barks	Rolfsen, W. *et al.*, 1980b Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Maroyi, A., 2022
	S. decussata	Stem barks	
	S. elaeocarpa	Stem barks	

[13] CD_{50} refers to the dose that causes convulsions in 50% of mice.

Alkaloids	Origins	Plant parts	References
10-Hydroxy-17-O-methylakagerine (β-carboline-type) (CD_{50} at 75 mg/kg)	*S. decussata*	Stem barks	Rolfsen, W. *et al.*, 1980b Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Maroyi, A., 2022
Diaboline (Diaboline-type) (No convulsions up to 250 mg/kg)	*S. afzelii*	Stem barks	Massiot, G. *et al.*, 1983a Ohiri, F. C. *et al.*, 1983a Delaude, C. *et al.*, 1992 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Philippe, G. *et al.*, 2004
	S. henningsii	Root and stem barks	
	S. longicaudata	Stem barks	
	S. matopensis	Root barks	
	S. mimfiensis	Root barks	
	S. potatorum	Leaves, seeds, root barks, and stem barks	
	S. pungens	Leaves, root barks, and stem barks	
Icajine (CD_{50} at 46.5 mg/kg)	*S. icaja*	Fruits, leaves, root barks, and stem barks	Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Tchinda, A. T. *et al.*, 2012b

Alkaloids	Origins	Plant parts	References
10-Hydroxy-21-O-methylkribine (β-carboline-type) (CD_{50} at 80 mg/kg)	*S. decussata*	Stem barks	Rolfsen, W. *et al.*, 1980b Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Maroyi, A., 2022
10-Hydroxy-epi-21-O-methylkribine (β-carboline-type) (CD_{50} at 84 mg/kg)	*S. decussata*	Leaves, fruits, root barks, and stem barks	Rolfsen, W. *et al.*, 1980b Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Maroyi, A., 2022
Macusine B (Sarpagine-type) (Active at a dose of 50 mg/kg)	*S. decussata*	Stem barks	Leonard, B. E., 1965 Angenot, L., 1975d Olaniyi, A. A. *et al.*, 1981 Ohiri, F. C. *et al.*, 1983a Verpoorte, R. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002
	S. usambarensis	Root barks	
Spermostrychnine (Spermostrychnine-type) (Clonic convulsions at 250 mg/kg)	*S. aculeata*	Stem barks	Weeratunga, G. *et al.*, 1984 Neuwinger, H. D., 1996 Delaude, C., and Delaude, L., 1997

Alkaloids	Origins	Plant parts	References
Strychnine (Strychnine-type) (CD_{50} at 0.427 mg/kg)	*S. boonei*	Trunk barks	Kambu, K. *et al.*, 1980 Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002 Philippe, G. *et al.*, 2004 Bonnet, O. *et al.*, 2022b
	S. camptoneura	Trunk barks	
	S. congolana	Trunk barks	
	S. densiflora	Trunk barks	
	S. icaja	Root and stem barks	
	S. potatorum	Unspecified	
	S. tchibangensis	Trunk barks	
	S. usambarensis	Leaves	
12-Hydroxystrychnine (Strychnine-type) (CD_{50} at 0.545 mg/kg)	*S. icaja*	Root and stem barks	Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Tchinda, A. T. *et al.*, 2012b
(+)-Tubotaiwine (Retuline-type) (Clonic convulsions at 200 mg/kg)	*S. angolensis*	Root and stem barks	Bohlin, L. *et al.*, 1979 Massiot, G. *et al.*, 1983a Ohiri, F. C. *et al.*, 1983a Delaude, C. *et al.*, 1992 Delaude, C., and Delaude, L., 1997 Rasoanaivo, P. *et al.*, 2002
	S. dolichothyrsa	Stem barks	
	S. mimfiensis	Root barks	
	S. mitis	Leaves, root barks, and stem barks	
	S. ngouniensis	Root and stem barks	

Alkaloids	Origins	Plant parts	References
Vomicine (Strychnine-type) (CD_{50} at 42.6 mg/kg)	*S. icaja*	Leaves, fruits, root barks, and stem barks	Ohiri, F. C. *et al.*, 1983a Delaude, C., and Delaude, L., 1997 Frédérich, M. *et al.*, 2000 Tchinda, A. T. *et al.*, 2012a Tchinda, A. T. *et al.*, 2012b
	S. malacoclados	Stem barks	

The “antidote” effects of African *Strychnos* were also tested, specifically regarding the treatment of snake bites. At a concentration of 0.01 mg/mL, the aqueous extract from *S. innocua* leaves inhibited the hyaluronidase and protease enzymes from venom of the snakes *Bitis arietans* et *Naja nigricollis*. The anti-venom activities of *S. decussata* roots and *S. spinosa* barks and roots were also confirmed. For *S. decussata* roots, the maximum inhibition observed was 48% with the aqueous crude extract against the proteolytic activity of *Bitis arietans* venom (Dose tested: 2.5 mg/mL). As for *S. spinosa* barks and roots, maximum inhibition was observed at 29% with the crude ethanolic extract from the roots against the hyaluronidase enzyme in *Naja nigricollis* venom (Dose tested: 5 mg/mL) (Molander, M. *et al.*, 2014; Aremu, A. O., and Moyo, M., 2022).

III.14. Species still unknown today

There are still many African species of the *Strychnos* genus that remain poorly known, with their alkaloid content either scarcely explored or entirely unstudied. Out of 75 African species, 27 are currently considered unknown. The purpose of this section is to list these species: *S. asterantha*, *S. bifurcata*, *S. campicola*, *S. canthioides*, *S. chromatoxylon*, *S. congolana*, *S. cuminodora*, *S. cuniculina*, *S. densiflora*, *S. fallax*, *S. gnetifolia*, *S. malchairi*, *S. melastomatoides*, *S. millepunctata*, *S. moandaensis*, *S. mostueoides*, *S. ndengensis*, *S. odorata*, *S. penninervis*, *S. penthanta*, *S. phaeotricha*, *S. retinervis*, *S. talbotiae*, *S. tchibangensis*, *S. ternata*,

S. xylophylla, and *S. zenkeri*. There are two main reasons why these species are so poorly known. The first is that the alkaloid content is low. In various studies, researchers focused on species rich in alkaloids. The second reason is that these species are not widely distributed globally. As they are rare, they are either not traditionally used or their use is not easily documented. Additionally, it is difficult to collect enough plant material to study the metabolites they contain (Delaude, C., and Delaude, L., 1997).

IV. CONCLUSIONS AND PROSPECTS

This review, which covers the traditional uses and pharmacological properties of the 75 African *Strychnos* species, highlights the importance of this genus in the therapeutic field. Indeed, numerous traditional uses and pharmacological properties are described in the literature, addressing a variety of diseases that affect different biological systems of the human body, including the digestive, nervous, respiratory, and urinary systems.

Plants of the *Strychnos* genus are frequently used in the traditional practices of African populations. A wide variety of traditional uses, both therapeutic and toxic, were described in the literature. However, the plant parts used, as well as the methods of preparation and administration, can vary from one population to another.

The pharmacological properties most frequently mentioned in the literature, in descending order, are antiplasmodial/antimalarial activities (23 species and 62 metabolites), cytotoxic/anticancer activities (3 species and 34 metabolites) and antimicrobial activities (12 species and 15 metabolites). Antiplasmodial and antimalarial activities predominate, with 31.9% of species showing crude extract(s) active against malaria. Moreover, the number of antiplasmodial and antimalarial metabolites is over twice that of cytotoxic and anticancer metabolites, and four times greater than that of antimicrobial metabolites.

The 130 alkaloids cited in the review were categorized into various subclasses of indoline and indole alkaloids. From the perspective of these subclasses, based on data for the three most represented biological activities in the literature, the top four positions are occupied by β-carboline-type alkaloids (23 out of 130,

17.7%), followed by those of the usambarensine and indoloquinolizine types (each 17 out of 130, 13.1%), the strychnine type (16 out of 130, 12.3%), and finally the toxiferine type (14 out of 130, 10.8%). Furthermore, both monomeric and dimeric active alkaloids were identified, with a predominance of the monomeric form. However, this observsation should be interpreted cautiously, as some dimers could not be classified within the two dimeric bisindole subclasses defined in this study, namely the toxiferine and usambarensine types. For example, longicaudatine, a hybrid of the strychnine and indoloquinolizine types, was categorized as an indoloquinolizine-type monomeric alkaloid, despite being structurally a dimeric bisindole alkaloid. Based on the structures described in the Supplementary Material, from a structural perspective, there are approximately as many monomeric alkaloids as dimeric ones.

The same species are frequently discussed in the literature, including *S. potatorum*, *S. spinosa*, and *S. usambarensis*. However, out of 75 species, 27 remain poorly known to this day, representing 36% of the total. These species have been overlooked for various reasons, including low alkaloid content and rarity.

Given the promising activities of *Strychnos* species highlighted in this review, it would be valuable to explore the overlooked species. These may contain new therapeutically interesting molecules yet to be discovered. The development of new and more sensitive techniques for metabolite exploration and isolation now makes it possible to study the minor compounds.

V. SUPPLEMENTARY MATERIAL

As the supplementary material for this article is substantial and the article has not yet been submitted or published, the data can be accessed through the following Orbi link: https://orbi.uliege.be/handle/2268/323782.

They can also be accessed by scanning this QR code:

2.1.2. American *Strychnos*

Many American *Strychnos* species produce fruit with sweet flesh and a pleasant fragrance. However, the seeds of these fruits are often poisonous. In addition, the poison from American *Strychnos* is commonly extracted to make poisoned darts, which contains curare, a set of compounds that cause paralyzing effects.

Traditional uses associated with American *Strychnos* include stomachic, tonic, febrifuge and antimalarial, fishing poison, poison for killing dogs or other animals, treatment of menstrual disorders and venereal diseases, abortifacient, treatment of diseases of the liver, spleen, stomach and intestine, aphrodisiac, dental analgesic, rheumatism, and treatment of convulsive and spasmodic disorders (Quetin-Leclercq, J. *et al.*, 1990).

2.1.3. Asian *Strychnos*

A variety of traditional uses are described for Asian *Strychnos* species, addressing a range of ailments affecting one or more of the body's biological systems. Among Asian *Strychnos* species, four are particularly well-documented in the literature: *S. ignatii* P.J.Bergius, *S. minor* Dennst., *S. nux-vomica* L., and *S. potatorum* L.f.

Various traditional uses are described in the literature: emetic, purgative, stomach, intestinal and abdominal complaints, stimulant and tonic, treatment of snakebite and poisoning, worm and parasite infections, febrifuge and antimalarial, analgesic, rheumatism, ulcers and wounds, eye disorders, skin disorders and leprosy, epilepsy and mental illnesses, aphrodisiac, venereal diseases, abortifacient, urinary diseases, and many others (Bisset, N. G., 1974).

2.2. References

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CHAPTER 3

Exploration of the chemical space from twenty-eight *Strychnos* species

3.1. Forewords

Chapters 3 and 4 form the first major part of this thesis. Before proceeding with the purification of compounds active against malaria, it is essential to carefully select the *Strychnos* species for purification. The choice of these species will significantly influence the subsequent work.

To select the appropriate *Strychnos* species, the metabolite content from a diverse collection of crude extracts from twenty-eight different species was analyzed according to various parameters and by specifically targeting alkaloids as the phytochemical class of interest. Among the collection, forty-three are methanolic crude extracts, and one is an alkaloidic crude extract. The latter was derived from the same plant material as one of the methanolic extracts, specifically from *Strychnos usambarensis* leaves, with the goal of comparing the metabolite content between two different extraction methods. Notable differences were observed, particularly in the detection of strychnine. This aspect will be further discussed in Chapter 5, which addresses the study of this monoterpene indole alkaloid through molecular networking.

This chapter covers the selection of the species collection, their origin, preparation of plant material, extractions, HPLC-MS/MS analyses, data processing, as well as generation, annotation, and interpretation of the molecular network.

3.2. Collection of *Strychnos* species and their origins

The Laboratory of Pharmacognosy at the Université de Liège houses an extensive collection of *Strychnos* species, which has been gradually built up over the years through various research projects on the genus. The collection includes over 100 samples from species across several continents, with a variety of plant parts. Ultimately, forty-three samples from various plant parts of twenty-eight *Strychnos* species were selected for extraction, based on the quantity and the condition of the samples, specifically whether they could be easily ground if grinding was required.

Most of the samples are from African species and were collected in different years. Before being deposited in one or more herbaria, they were identified by experts. These references serve the purpose of quickly identifying the species in future collections. Each sample was dried at 40°C, stored in a dry and moderate room temperature environment, and shielded from light. The samples were then ground into a 10 g powder using an IKA A10 mill (Staufen, Germany).

Sample numbers, species names, corresponding plant parts, harvest dates, origins, reference specimen numbers, and storage locations are outlined in the table below (Table 15). The oldest sample studied was collected 78 years ago, raising concerns about the stability of alkaloids over time. However, studies on *Strychnos* alkaloids have shown that these compounds remain stable even after many years. To assess this, the phytochemical profiles of older samples were compared to those of the same species collected a few years earlier (Phillipson, D. J., 1982; Biala, R. G. *et al.*, 1998; Frédérich, M. *et al.*, 1998; Eloff, J. N., 1999; Soto-Sobenis, A. *et al.*, 2001; Harborne, J. B., 2012; Yilmaz, A. *et al.*, 2012; Jonville, M.-C. *et al.*, 2013). Although oxidation, hydrolysis, and heat-induced degradation could not be entirely ruled out, no significant degradation was observed in the studies. Additionally, the potential presence of degradation products may offer new avenues for the hemisynthesis of novel antimalarial drugs. If any isolated alkaloids are confirmed to result from degradation, hemisynthesis studies could be conducted to develop new biologically promising compounds.

Table 15: Origins of the forty-three *Strychnos* samples.

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
1	*Strychnos usambarensis* Gilg ex Engl.	Leaves	November 2007	Rwanda (Akagera National Park, locality of Ndego)	Ulg20070608 (Herbarium of the Pharmaceutical Institute in Liège)
2			August 2008	Rwanda (Akagera National Park, locality of Ndego)	Ulg20080816 (Herbarium of the Pharmaceutical Institute in Liège)
3		Whole yellow fruits	November 2007	Rwanda (Akagera National Park, locality of Ndego)	Ulg20070608 (Herbarium of the Pharmaceutical Institute in Liège)
4		Root barks	October 1988	Congo (Locality of Luki)	Delaude HB3377 (Herbarium of Botanical Garden of Belgium at Meise)
5		Stem and twig barks	August 1948	Congo (Forest gallery in Kasenga)	Duvigneaud H1397 (Herbarium of Botanical Garden of Belgium at Meise and herbarium of the Pharmaceutical Institute in Liège)

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
6	*Strychnos variabilis* De Wild.	Root barks	1970	Congo (Kinshasa, along the roads)	Evrard 6592 (Herbarium of Botanical Garden of Belgium at Meise)
7		Trunk barks	1971	Congo (Kinshasa, Funa district)	Evrard 6592 (Herbarium of Botanical Garden of Belgium at Meise)
8	*Strychnos gossweileri* Exell	Root barks	1994	Congo (near Matadi)	Delaude HB5690 (Herbarium of Botanical Garden of Belgium at Meise)

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
9	*Strychnos mellodora* S.Moore	Roots	August 1989	Zimbabwe (Chirinda Forest)	Delaude 7831 (Herbarium of Botanical Garden of Belgium at Meise)
10		Trunk barks	July 1997	Congo	Delaude 7831 (Herbarium of Botanical Garden of Belgium at Meise)
11		Leaves	August 1989	Zimbabwe (Chirinda Forest)	Delaude 7831 (Herbarium of Botanical Garden of Belgium at Meise)
12	*Strychnos phaeotricha* Gilg	Leaves	Mai 1948	Congo (City of Kikwit)	Duvigneaud H914 (Herbarium of Botanical Garden of Belgium at Meise and herbarium of the Pharmaceutical Institute in Liège)
13	*Strychnos brasiliensis* (Spreng.) Mart.	Trunk barks	September 1997	Brazil (State of Parana, locality of Curitiba)	Cervi 6268 (Herbarium of the department of Botany from the Federal University of Parana

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
14	*Strychnos innocua* Delile	Leaves	August 2014	Tanzania (Kaole village of Bagamoyo district)	The reference specimen was deposited in the Herbarium unit of the department of Botany in the University of Dar es Salaam
15		Stem barks	July 1948	Congo (Locality of Keyberg)	Duvigneaud H1267 (Herbarium of Botanical Garden of Belgium at Meise and herbarium of the Pharmaceutical Institute in Liège)
16	*Strychnos henningsii* Gilg	Leaves	July 1948	Congo (Province of Katanga, between Tenge and Kolwezi)	Duvigneaud H1147 (Herbarium of Botanical Garden of Belgium at Meise and herbarium of the Pharmaceutical Institute in Liège)
17	*Strychnos angolensis* Gilg	Root barks	1948	Congo	Duvigneaud 1952 (Herbarium of Botanical Garden of Belgium at Meise)
18		Leaves	1948	Congo	Duvigneaud 1952 (Herbarium of Botanical Garden of Belgium at Meise)

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
19	*Strychnos scheffleri* Gilg	Leaves	June 1946	Congo (Forest in the north of Chikapa)	Duvigneaud H1088A (Herbarium of Botanical Garden of Belgium at Meise and herbarium of the Pharmaceutical Institute in Liège)
20	*Strychnos tricalysioides* Hutch. & M.B.Moss	Trunk barks	November 1972	Cameroon (Near bridge in Loum-Kumba road, right bank of Mungo Creek)	Leeuwenberg 10604 WAG.1067021 (Herbarium Vadense at Wageningen*)
21	*Strychnos spinosa* Lam.	Leaves	August 2014	Tanzania (Kaole village of Bagamoyo district)	The reference specimen was deposited in the Herbarium unit of the department of Botany in the University of Dar es Salaam

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
22	*Strychnos longicaudata* Gilg	Roots and stems	April 1948	Congo (Locality of Kasongo-Lunda)	Duvigneaud H786b (Herbarium of Botanical Garden of Belgium at Meise and herbarium of the Pharmaceutical Institute in Liège)
23		Trunk barks	August 2009	Cameroon	Breteler F.J. 2135 Identified by comparison with the specimen of collection Herbarium n°9940 SRFCam at the National Herbarium of Cameroon Kemeuze 7B (Herbarium of Botanical Garden of Belgium at Meise)

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
24	*Strychnos malchairi* De Wild.	Leaves	June 1948	Congo (City of Panzi)	Duvigneaud H988 (Herbarium of the Belgian National Botanical Garden in Meise and herbarium of the Pharmaceutical Institute in Liège)
25		Trunk barks	June 1948	Congo (City of Panzi)	Duvigneaud H988 (Herbarium of the Belgian National Botanical Garden in Meise and herbarium of the Pharmaceutical Institute in Liège)
26	*Strychnos mattogrossensis* S.Moore	Leaves	June 1987	Brazil (near Manaus, Municipio do Careiro, Igarapé Grande do Lago do Rei)	COELHO, D. Herb. no. 142800 (Herbarium of INPA at Manaus, herbarium in the Department of Plant Taxonomy, Agricultural University, Wageningen (Netherlands) and herbarium of the Pharmaceutical Institute in Liège)

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
27	*Strychnos icaja* Baill.	Trunk barks	August 2009	Cameroon (6 km of Bertoua, on the road between Bertoua and Garoua Boulaï)	Breteler F.J. 1419 Identified by comparison with the specimen of collection Herbarium n°9934 SRFCam at the National Herbarium of Cameroon Kemeuze 11B (Herbarium of the Belgian National Botanical Garden in Meise)
28		Roots	June 2014	Congo (Province of Tshuapa, territory of Befale, locality of Lileko, in the equatorial forest)	Recolted by Phaacuen José Bayolo Identified by chemical fingerprinting
29		Collar barks	May 1948	Congo (17 km from Kikwit to Leverville)	Duvigneaud H900 (Herbarium of Botanical Garden of Belgium at Meise and herbarium of the Pharmaceutical Institute in Liège)

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
30	*Strychnos nux-vomica* L.	Trunk barks	February 1999	India (State of Tamil Nadu, city of Chennai)	NV01 (Herbarium of the Pharmaceutical Institute in Liège)
31		Root barks	July 2007	Cambodia (Kampong Spoe Province)	SNVET.CaKS.07 Herbarium of the Faculty of Pharmacy in Marseille Identified by Pr. Sun Kaing Cheng (botanist in the Faculty of Pharmacy of Phnom Penh, Cambodia)
32		Seeds	October 2012	Commercial sample	Commercial sample obtained from Denolin (Braine l'Alleud, Belgium) and Longeval (Deux Acres, Belgium) Identified according to the description in the Swiss and French Pharmacopoeia

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
33	*Strychnos ignatii* P.J.Bergius	Fruits	November 1958	Commercial samples	Commercial sample obtained from Denolin (Braine l'Alleud, Belgium) and Longeval (Deux Acres, Belgium) Identified according to the microscopic description in Perrot and Gathercoal (Gathercoal and Wirth, 1947; Perrot 1943)
34	*Strychnos potatorum* L.f.	Trunk barks	July 1948	Congo (Keyberg)	Duvigneaud H1202 (Herbarium of the Belgian National Botanical Garden in Meise and herbarium of the Pharmaceutical Institute in Liège)
35	*Strychnos malacoclados* C.H.Wright	Root barks	August 2009	Cameroon (6 km of Bertoua, on the road between Bertoua and Garoua Boulaï)	Kemeuze 14B (Herbarium of the Belgian National Botanical Garden in Meise) Identified at the National Herbarium of Cameroon

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
36	*Strychnos camptoneura* Gilg & Busse	Trunk barks	August 2009	Cameroon	Breteler FJ 2572 Identified by comparison with the specimen of collection Herbarium n°9923 SRFCam at the National Herbarium of Cameroon
37	*Strychnos congolana* Gilg	Trunk barks	August 2009	Cameroon	Letouzey 10555 Identified by comparison with the specimen of collection Herbarium n°23418 SRFCam at the National Herbarium of Cameroon
38	*Strychnos boonei* De Wild.	Trunk barks	August 2009	Cameroon	Letouzey 1181 Identified by comparison with the specimen of collection Herbarium n°3427 SRFCam at the National Herbarium of Cameroon
39	*Strychnos staudtii* Gilg	Trunk barks	August 2009	Cameroon	Nos 6105 Identified by comparison with the specimen of collection Herbarium n°31205 SRFCam at the National Herbarium of Cameroon

Sample numbers	Names	Parts	Dates	Origins	Reference specimens
40	*Strychnos elaeocarpa* Gilg ex Leeuwenb.	Trunk barks	August 2009	Cameroon	Bos 5405 Identified by comparison with the specimen of collection Herbarium n°31198 SRFCam at the National Herbarium of Cameroon
41	*Strychnos densiflora* Baill.	Trunk barks	August 2009	Cameroon	Thomas D.W. 4286 Identified by comparison with the specimen of collection Herbarium n°53634 HNC at the National Herbarium of Cameroon
42	*Strychnos tchibangensis* Pellegr.	Trunk barks	August 2009	Cameroon	Biholong M. 432 Identified by comparison with the specimen of collection Herbarium n°42104 SRFCam at the National Herbarium of Cameroon
43	*Strychnos johnsonii* Hutch. & M.B.Moss	Leaves	June 1948 (Identified by Leeuwenberg in 1976)	Congo (Panzi)	Duvigneaud H998 (Herbarium Vadense at Wageningen* and herbarium of the Pharmaceutical Institute in Liège)

*The WAG herbarium moved to Leiden and is now part of the Naturalis Herbarium.

According to the IUCN Red List of Threatened Species, the majority of these twenty-eight species are not considered threatened in the wild. Indeed, only *S. staudtii* and *S. elaeocarpa* are currently classified as vulnerable, which means there is a high risk of extinction in the wild if no measures are taken to mitigate their significant population decline. It is therefore recommended to avoid collecting new samples of these two species for the time being (International Union for Conservation of Nature and Natural Resources, 2024).

3.3. Pressurized solvent extractions using SpeedExtractor E-914®

The forty-three crude methanolic extracts were obtained using SpeedExtractor E-914® (Büchi, Hendrik-Ido-Ambacht, Netherlands), which utilizes pressurized solvent extraction. This device not only automates the extraction process but also provides higher yields and reduced solvent consumption compared to manual methods. It has four parallel extraction positions, allowing for simultaneous extraction of up to four samples. As a result, 11 extraction runs were required to obtain all the crude methanolic extracts.

For each extraction, powdered samples, mixed with sand in a 1:5 ratio, were placed in a mortar. The mixture was then homogenized using a spatula or paper to prevent breakage of the sand particles. The sand's role was to enhance the flow of solvent into the extraction cells, minimizing the risk of blockages. After placing a metal filter and a paper filter in the extraction cell, the sample-sand mixture was transferred into the cell. A final paper filter was placed on top before the extraction began.

Each extraction cycle consists of three steps: heat-up, hold, and discharge, with specific time settings for each. For the extracts reported in the table above, the times for heat-up, hold, and discharge were set at 1 minute, 15 minutes, and 2 minutes, respectively. A total of three cycles were performed for each sample. After completing each cycle, the extractor was briefly cleaned with the extraction solvent. At the end of the third cycle, the extractor was

washed with the same solvent for 2 minutes, followed by drying using nitrogen for 3 minutes. The set pressure during the extraction was 100 bar, and the temperature was maintained at 30°C (Figure 12).

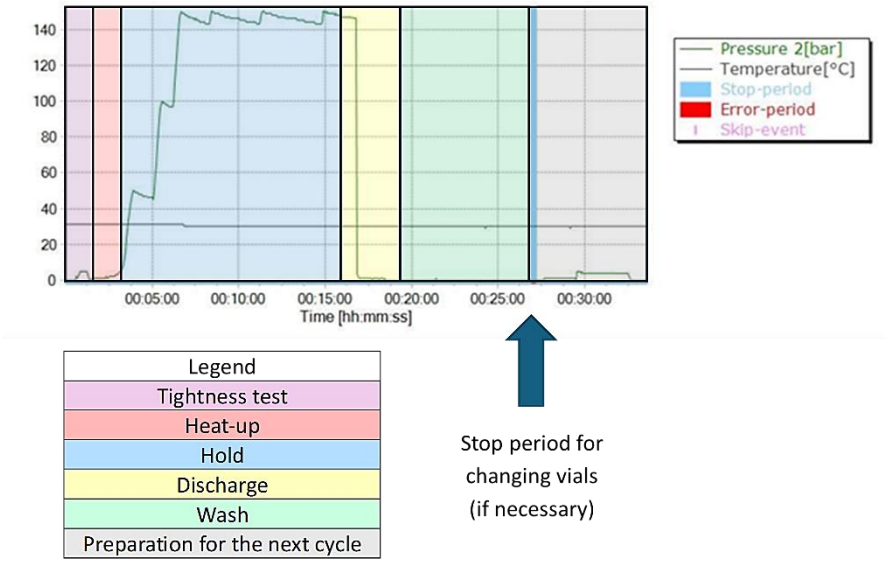


Figure 12: The various extraction stages of SpeedExtractor E-914®.

Before starting the extractions, a repeatability study was conducted using the root barks of *S. gossweileri*. Three independent extraction series (n=3) were performed, spaced one week apart. In each series, all four extraction positions were used, resulting in a total of four extracts per series. In the end, 12 extracts were obtained. These extracts were subsequently evaporated using a Rotavapor® and a Multivapor® (Büchi), followed by storage in a vacuum oven overnight at room temperature (Heraeus, Hanau, Germany). After evaporation, the average yield per series was determined and compared. The results of the three experiments were very similar, confirming the repeatability of the extraction process using the SpeedExtractor E-914® (Figure 13).

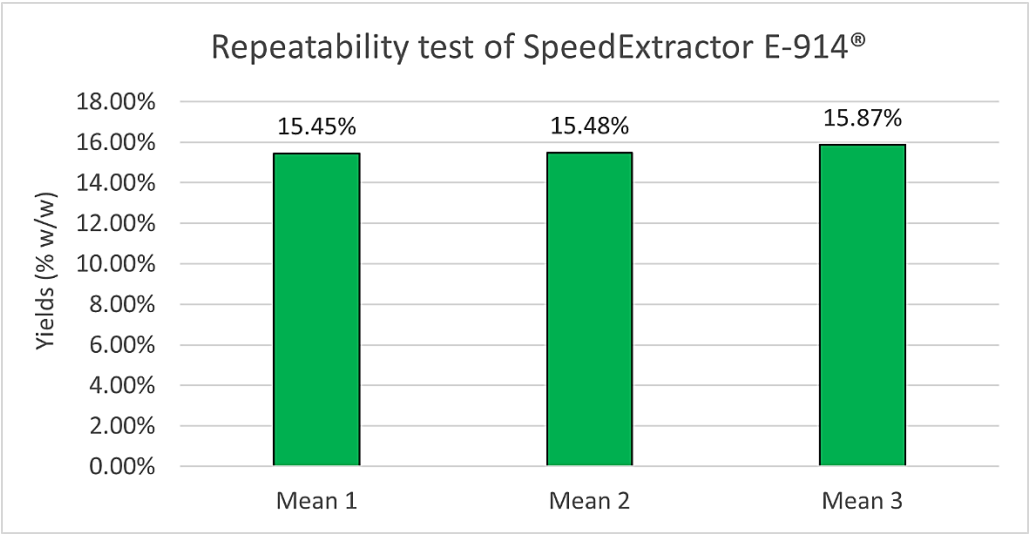


Figure 13: Results from the SpeedExtractor E-914® repeatability study (n=3; SD: 0.00234; CV: 1.50%).

Once repeatability was confirmed, extractions were conducted, and the yields were determined. The yields obtained for each extraction are presented in the table below (Table 16). The yields ranged from 0.55% w/w to 25.92% w/w.

Table 16: Weights and yields during the extraction of plant powders from twenty-eight *Strychnos* species.

Names	Parts and reference specimens	Weights	Types of extract	Yields (% w/w)
Strychnos usambarensis	Leaves (Ulg20070608)	5.44 g	MeOH	15.6 %
		2.00 g	Alkaloidic	4.00 %
	Leaves (Ulg20080816)	5.44 g	MeOH	25.9 %
	Fruits (Ulg20070608)	5.44 g	MeOH	13.1 %
	Root barks (Delaude HB3377)	5.44 g	MeOH	6.80 %
	Stem and twig barks (Duvigneaud H1397)	5.44 g	MeOH	15.8 %
Strychnos variabilis	Root barks (Evrard 6592)	5.44 g	MeOH	0.550 %
	Trunk barks (Evrard 6592)	5.44 g	MeOH	3.31 %
Strychnos gossweileri	Root barks (Delaude HB5690)	5.44 g	MeOH	14.0 %
Strychnos mellodora	Roots (Delaude 7831)	5.44 g	MeOH	9.19 %
	Trunk barks (Delaude 7831)	5.44 g	MeOH	2.57 %
	Leaves (Delaude 7831)	5.44 g	MeOH	6.61 %
Strychnos phaeotricha	Leaves (Duvigneaud H914)	5.44 g	MeOH	6.62 %
Strychnos brasiliensis	Trunk barks (Cervi 6268)	5.44 g	MeOH	11.8 %

Names	Parts and reference specimens	Weights	Types of extract	Yields (% w/w)
Strychnos innocua	Leaves (Deposited in the Herbarium unit of the department of Botany in the University of Dar es Salaam)	5.44 g	MeOH	23.2 %
	Stem barks (Duvigneaud H1267)	5.44 g	MeOH	7.72 %
Strychnos henningsii	Leaves (Duvigneaud H1147)	5.44 g	MeOH	14.0 %
Strychnos angolensis	Root barks (Duvigneaud 1952)	5.44 g	MeOH	9.01 %
	Leaves (Duvigneaud 1952)	5.44 g	MeOH	5.33 %
Strychnos scheffleri	Leaves (Duvigneaud H1088A)	5.44 g	MeOH	8.64 %
Strychnos tricalysioides	Trunk barks (Leeuwenberg 10604)	5.44 g	MeOH	0.740 %
Strychnos spinosa	Leaves (Deposited in the Herbarium unit of the department of Botany in the University of Dar es Salaam)	5.44 g	MeOH	25.0 %
Strychnos longicaudata	Roots and stems (Duvigneaud H786b)	5.44 g	MeOH	3.86 %
	Trunk barks (Breteler F.J. 2135)	5.44 g	MeOH	5.51 %

Names	Parts and reference specimens	Weights	Types of extract	Yields (% w/w)
Strychnos malchairi	Leaves (Duvigneaud H988)	5.44 g	MeOH	6.62 %
	Trunk barks (Duvigneaud H988)	5.44 g	MeOH	6.07 %
Strychnos mattogrossensis	Leaves (COELHO, D. Herb. no. 142800)	5.44 g	MeOH	0.920 %
Strychnos icaja	Trunk barks (Breteler F.J. 1419)	5.44 g	MeOH	9.19 %
	Roots (Recolted by Phaacuen José Bayolo)	5.44 g	MeOH	9.93 %
	Collar barks (Duvigneaud H900)	5.44 g	MeOH	9.19 %
Strychnos nux-vomica	Trunk barks (NV01)	5.44 g	MeOH	8.27 %
	Root barks (SNVET.CaKS.07)	5.44 g	MeOH	4.23 %
	Seeds (Commercial sample)	5.44 g	MeOH	6.80 %
Strychnos ignatii	Fruits (Commercial sample)	5.44 g	MeOH	7.17 %
Strychnos potatorum	Trunk barks (Duvigneaud H1202)	5.44 g	MeOH	8.46 %
Strychnos malacoclados	Root barks (Kemeuze 14B)	5.44 g	MeOH	1.65 %
Strychnos camptoneura	Trunk barks (Breteler FJ 2572)	5.44 g	MeOH	0.740 %
Strychnos congolana	Trunk barks (Letouzey 10555)	5.44 g	MeOH	3.49 %

Names	Parts and reference specimens	Weights	Types of extract	Yields (% w/w)
Strychnos boonei	Trunk barks (Letouzey 1181)	5.44 g	MeOH	0.550 %
Strychnos staudtii	Trunk barks (Nos 6105)	5.44 g	MeOH	4.23 %
Strychnos elaeocarpa	Trunk barks (Bos 5405)	5.44 g	MeOH	2.87 %
Strychnos densiflora	Trunk barks (Thomas D.W. 4286)	5.44 g	MeOH	2.76 %
Strychnos tchibangensis	Trunk barks (Biholong M. 432)	5.44 g	MeOH	2.57 %
Strychnos johnsonii	Leaves (Duvigneaud H998)	5.44 g	MeOH	13.4 %

3.4. Alkaloidic extractions of *Strychnos usambarensis* leaves

From a sample of *S. usambarensis* leaves harvested during the wet season in November 2007 (sample 1), an alkaloidic extract was performed manually.

To start, 20 mL of a 10% w/w ammonia solution was added to 2 g of powdered leaves. This mixture was macerated for 30 minutes, followed by the addition of 100 mL of dichloromethane. The mixture was stirred for 1 hour under magnetic stirring. The solution was then filtered through glass wool, which was rinsed twice with 50 mL of dichloromethane. This organic phase was transferred to a separating funnel, where 20 mL of 2% w/V sulfuric acid was added. After extraction, the aqueous phase was separated, and a second extraction was conducted with another 20 mL of 2% w/V sulfuric acid. The two aqueous phases were combined and adjusted to a pH of 8–10 using 10% w/w ammonia solution. Two additional extractions were carried out by adding 50 mL of dichloromethane to the basified aqueous phase in a separating funnel. The two organic phases were pooled together. To remove residual water, the organic phase was dried with anhydrous sodium sulfate. After filtration, the solution was evaporated under reduced pressure, and the residue was dried in a vacuum oven overnight at room temperature.

Following this process, sections 3.5, 3.6, and 3.7 describe the detailed procedure for generating the molecular network of forty-four crude extracts from twenty-eight *Strychnos* species. Both this procedure and the one described above for obtaining the crude extracts are also comprehensively outlined in two published articles in *Frontiers in Molecular Biosciences* and *Toxicon* journals. These are further discussed later in this thesis, specifically in section 3.9 (pages 214 to 233) and in Chapter 5 (section 5.2, pages 256 to 281) (Bonnet, O. *et al.*, 2022a; Bonnet, O. et al., 2022b).

3.5. MS/MS analyses

The forty-four crude extracts obtained in the previous steps were dissolved in HPLC grade methanol to achieve a concentration of 1 mg/mL. After filtration

through 0.2 µm pore size filters, the solutions were transferred to HPLC vials for injection into the HPLC-MS/MS system. These analyses were conducted at the Université Paris-Saclay.

The Agilent HPLC-MS system (Agilent Technologies, Massy, France), used to analyze the samples, was composed of an Agilent 1,260 Infinity HPLC coupled to an Agilent 6,530 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 at a flow rate of 250 µL/min. The gradient was linear and varied 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 1,200), 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 3,000 counts. Purine $C_5H_4N_4$ $[M+H]^+$ ion (*m/z* 121.050873) and hexakis (1*H*, 1*H*, 3*H*-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 calibrants, a permanent MS/MS exclusion criterion was set, containing the *m/z* values of the two internal calibrants.

Each data item received has a .d extension and can be displayed using MassHunter software (Version B.07.00). Before generating the molecular network, these data must be converted to .mzXML (eXtensible Markup Language) format and processed. To accomplish this, we used two software packages: MSConvert and MZmine 2 (the previous version of the current MZmine 3).

3.6. Data processing on MZmine 2

The MSConvert software, developed by ProteoWizard, converts the .d extension file into the .mzXML (eXtensible Markup Language) format, which can then be imported into MZmine 2 for processing (Chambers, M. C. *et al.*, 2012). The filter used was peak picking, with the vendor algorithm applied. MS levels 1 and 2 were selected.

The various .mzXML files were imported into MZmine 2 (version 2.53) and processed in multiple stages (Pluskal, T. *et al.*, 2010):

1) **Noise suppression**: *settings*: the intensities were 5.8E3 for MS^1 and 5.0E1 for MS^2. Noise suppression is crucial to prevent interference signals in the molecular network;
2) **Creation of peak lists**: *settings*: a minimum of 4 points were required to build a peak. The intensity threshold was 5.8E3, and the *m/z* tolerance was set to *m/z* 0.02 and 10.0 ppm. The data were processed from the peak lists;
3) **Deconvolution**: *settings*: the algorithm used was wavelets (ADAP) (Myers, O. D. *et al.*, 2017), with auto-calculation for the *m/z* center. 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 set to 1, with the S/N estimator as intensity window SN. The minimal feature height was 3,000, the coefficient/area threshold was 2, the peak duration range was 0.02-1.5 min, and the retention time wavelet range was 0.02-0.2 min. The purpose of the deconvolution step is to clean up the data by removing unwanted peaks;
4) **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 set to 1, and the most intense peak was chosen as the representative isotope. Isotopes were removed at this stage to prevent overloading the molecular network with redundant data;
5) **Alignment**: *settings*: the *m/z* and retention time tolerances were *m/z* 0.02, 15.0 ppm, and 0.8 min, respectively. The weights for *m/z* and retention time were set to 100 each. Aligning the data allows for

the creation of a single dataset. Alignment is performed only when multiple datasets (and therefore multiple peak lists) are present;

6) **Gap-filling**: *settings*: the *m/z* tolerance was *m/z* 0.005 and 15.0 ppm. Gap-filling was performed to detect as many peaks as possible and therefore maximize the number of potential identifications. This step helps identify minor or trace alkaloids;
7) **Filtration**: the gap-filled peak list was further filtered by removing specific retention time windows (between 0-2.50 min and 45.59-49.83 min) and retaining the MS/MS data necessary for generating the molecular network.

The processed data were finally exported into the ".mgf" (Mascot Generic Format) and ".CSV" (Comma-separated values) formats for import into the GNPS platform (Global Natural Products Social Molecular Networking) (Wang, M. *et al.*, 2016).

3.7. Feature-based molecular networking on the GNPS community platform

The GNPS website[14] is an online community platform launched in 2016 by Wang, M. *et al.* It enables users to generate molecular networks, annotate them using shared databases of MS/MS spectra, expand these databases by importing MS/MS spectra of purified compounds, and utilize various annotation tools, such as MolNetEnhancer, which categorizes metabolite groupings (referred to as clusters) based on their chemical class. Additionally, databases can be used to distinguish between known and unknown metabolites within the molecular network and to suggest identifications for the known metabolites by comparing experimental MS/MS spectra with database entries. Among the databases available, MIADB (Monoterpene Indole Alkaloids DataBase) is particularly noteworthy, containing 422 monoterpene indole alkaloids as of the time of

[14] The GNPS website is available at this link: https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp.

writing (Fox Ramos, A. E. *et al.*, 2019a). This makes it an indispensable resource for studying the metabolite content of *Strychnos* species.

The analysis was conducted using the "Feature Networking" workflow (Nothias, L.-F. *et al.*, 2020). The .mgf and .CSV files containing the necessary data to generate the molecular network, along with additional data such as retention time and intensity, were imported into the platform. The precursor and fragment ion mass tolerances were set at 0.02 Da for both. The minimal cosine score was set at 0.65, and the minimal cosine score for matching with the library was 0.7. No filtration was applied, and analogs were not searched.

The molecular network was automatically generated, and the results, along with suggested identifications, can be accessed through the following link: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=da555ca03b4048259b2199b4026775de. The next section explores the generated network and the identifications provided by the platform (Figure 14).

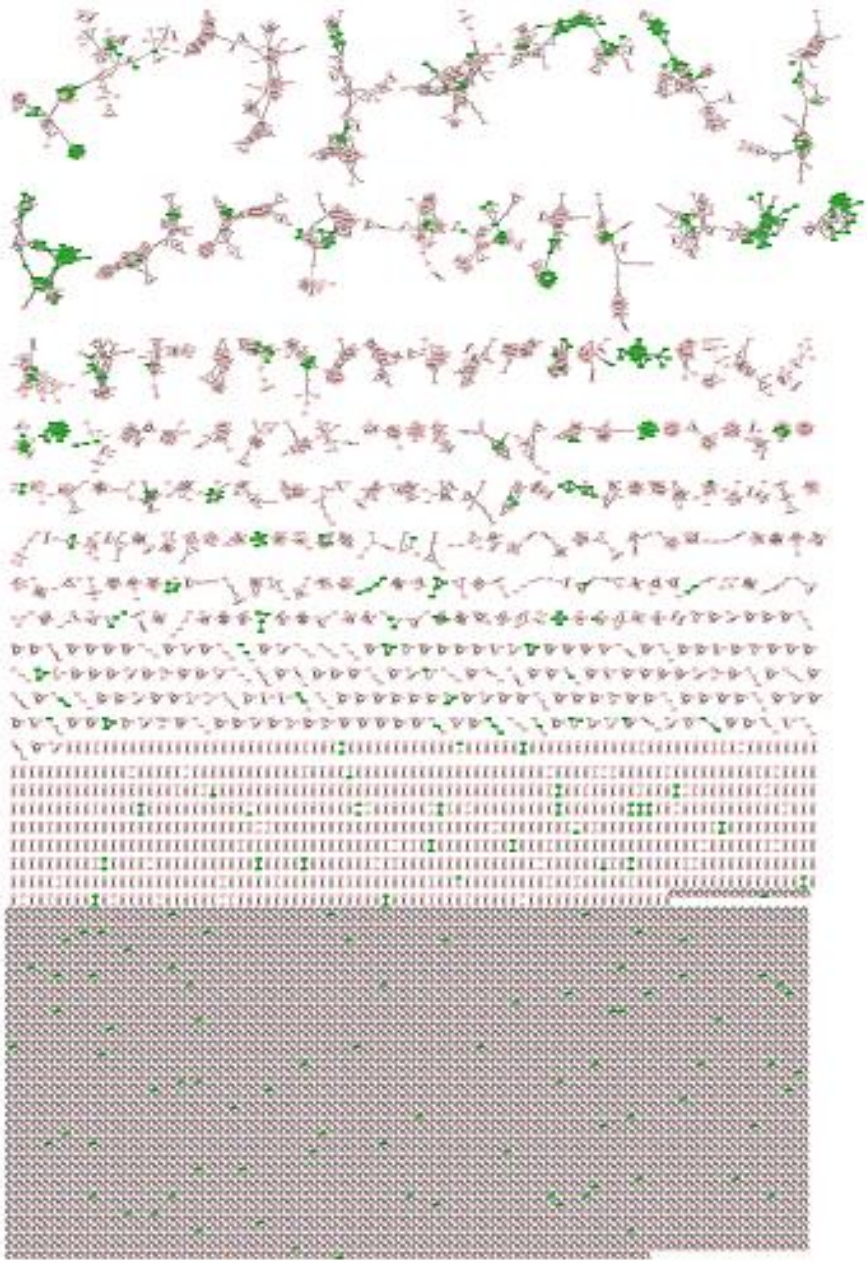


Figure 14: Feature-based molecular network from forty-four crude extracts of twenty-eight *Strychnos* species. The nodes in green are the identified metabolites, while those in red are the unidentified ones.

3.8. Highlighting known metabolites using shared spectral databases

Thanks to the shared spectra data, known compounds were quickly identified and highlighted in the molecular network of the forty-four *Strychnos* crude extracts. These suggested identifications were explored by manually comparing experimental MS/MS spectra with those derived from databases and literature to confirm them. We searched both the GNPS databases and MassBank of North America (MoNA) (MassBank of North America, 2024) for theoretical spectra. Additionally, we focused exclusively on alkaloids. Several identifications were confirmed (Figure 15).

Some of the identifications are well-known in the literature. These include usambarensine, 19,20-dihydrousambarensine, usambarine and strychnofoline, all of which were isolated from *S. usambarensis*. We also identified sungucine, which was isolated from *S. icaja*. However, there were also some identifications that caught our attention because, based on our research, they had never been described in the literature before. This is particularly true of strychnine, detected in seven species not previously described as producers of this alkaloid, which will be further explored in Chapter 5 (pages 253 to 286) of this thesis.

Akagerine

- *S. usambarensis* (Fr+RB+STB)
- *S. elaeocarpa* (TB)

Isodolichantoside

- *S. usambarensis* (Fr+RB)
- *S. variabilis* (TB)
- *S. gossweileri* (RB)
- *S. mellodora* (R+TB+L)
- *S. angolensis* (L)
- *S. tricalysioides* (TB)
- *S. elaeocarpa* (TB)
- *S. johnsonii* (L)

N_b-methylusambarensine

- *S. usambarensis* (RB)

Isomalindine

- *S. usambarensis* (L+Fr+RB+STB)
- *S. mellodora* (TB)
- *S. elaeocarpa* (TB)

Sungucine

- *S. icaja* (R+CB)
- *S. densiflora* (TB)

Brucine

- *S. mattogrossensis* (L)
- *S. nux-vomica* (TB+RB+S)
- *S. ignatii* (Fr)

α-Colubrine

- *S. icaja* (CB)
- *S. nux-vomica* (TB+RB+S)
- *S. ignatii* (Fr)

2,7-Dihydroxyapogeissoschizine

- *S. gossweileri* (RB)

C-fluorocurarine

- *S. icaja* (TB)
- *S. malacoclados* (RB)

Holstiline

- *S. henningsii* (L)

Protostrychnine

- *S. icaja* (R+CB)
- *S. nux-vomica* (RB)
- *S. densiflora* (TB)

Strychnine N-oxide

- *S. nux-vomica* (TB+RB+S)
- *S. ignatii* (Fr)

N_4-methylantirhine

- *S. usambarensis* (Fr+RB)
- *S. gossweileri* (RB)
- *S. mellodora* (R+TB+L)
- *S. tricalysioides* (TB)

Antirhine

- *S. gossweileri* (RB)
- *S. mellodora* (R)
- *S. icaja* (CB)
- *S. nux-vomica* (TB+RB)
- *S. johnsonii* (L)

Panarine

- *S. tricalysioides* (TB)
- *S. longicaudata* (TB)
- *S. icaja* (TB)
- *S. nux-vomica* (TB)
- *S. potatorum* (TB)
- *S. malacoclados* (RB)
- *S. camptoneura* (TB)
- *S. congolana* (TB)
- *S. boonei* (TB)
- *S. staudtii* (TB)
- *S. elaeocarpa* (TB)
- *S. tchibangensis* (TB)

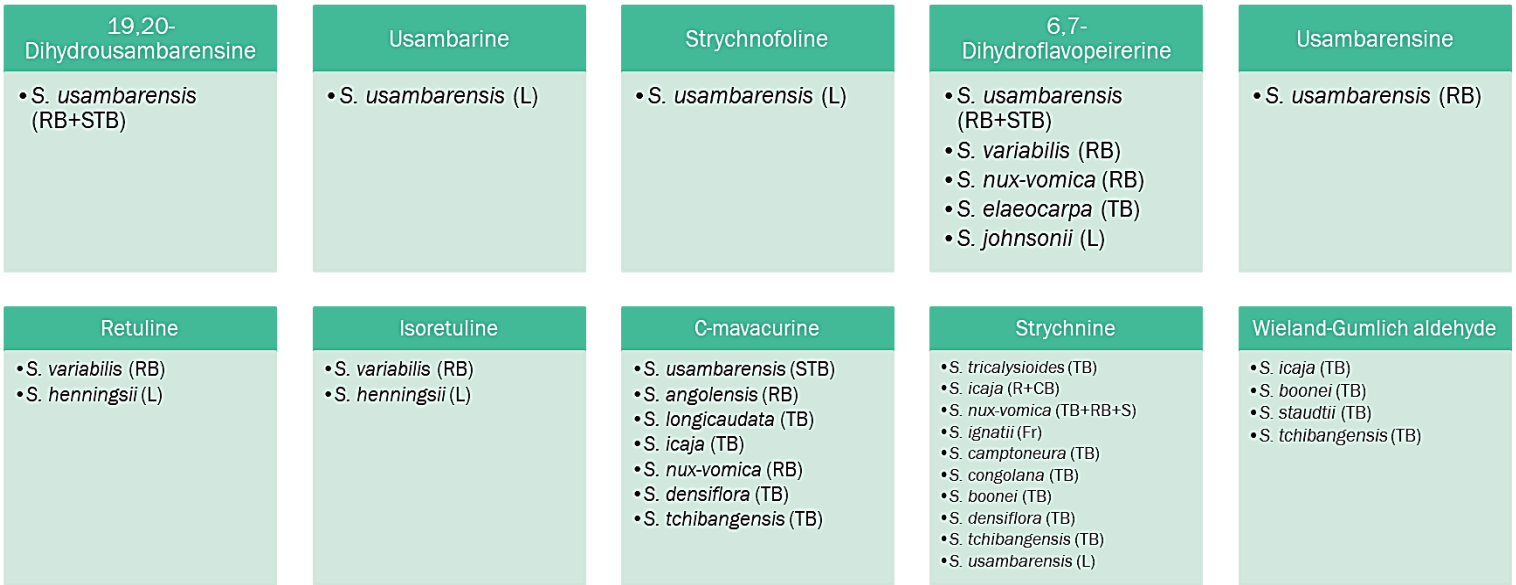
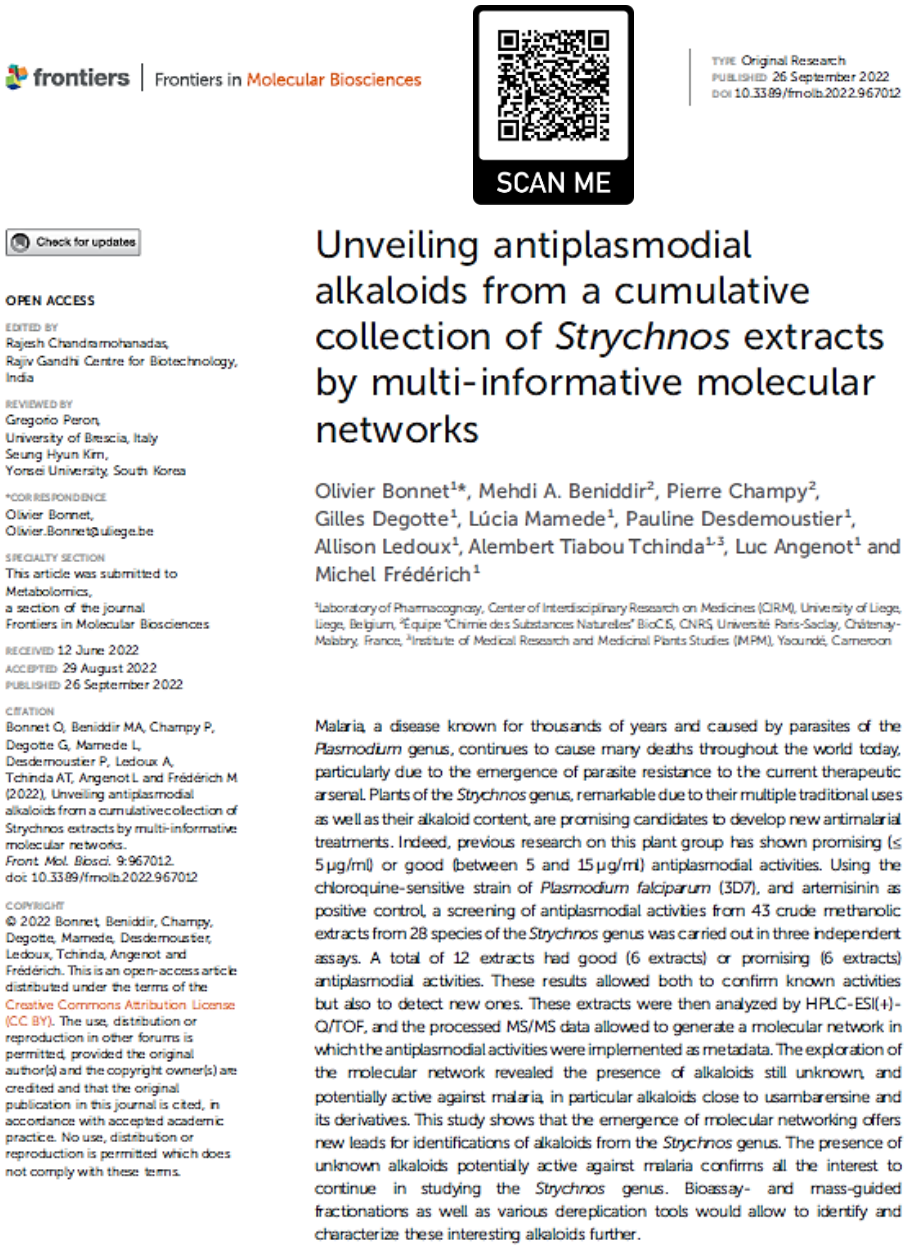


Figure 15: Identifications confirmed within the molecular network of the forty-four crude extracts of *Strychnos* species (CB= Collar barks; Fr= Fruits; L= Leaves; R= Roots; RB= Root barks; S= Seeds; STB= Stem and twig barks; TB= Trunk barks).

The following section discusses the use of antiplasmodial activities from the *Strychnos* crude extracts as metadata. These activities are incorporated into the molecular network to add a biological dimension to the annotations, thereby highlighting potentially novel alkaloids active against malaria.

This work was published in a paper titled "Unveiling Antiplasmodial Alkaloids from a Cumulative Collection of *Strychnos* Extracts by Multi-informative Approaches" in the journal *Frontiers in Molecular Biosciences* (2022), 9:967012. The article's presentation has been slightly modified to ensure consistency within the thesis and to enhance readability. Additionally, small typographical errors identified in the article have been corrected in the following section. Unfortunately, one interpretative error, which we overlooked during the publication process, was noticed while writing this thesis. Specifically, the methanolic crude extract of *S. malacoclados* root barks was noted to exhibit good antiplasmodial activity, when in fact, its activity was moderate. With an antiplasmodial activity of 18.4 µg/mL ± 4.19, it falls within the moderate range (between 15 and 30 µg/mL). This error has also been rectified in the thesis.

3.9. Article: “Unveiling antiplasmodial alkaloids from a cumulative collection of *Strychnos* extracts by multi-informative molecular networks”



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Unveiling antiplasmodial alkaloids from a cumulative collection of *Strychnos* extracts by multi-informative molecular networks

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Malaria, a disease known for thousands of years and caused by parasites of the *Plasmodium* genus, continues to cause many deaths throughout the world today, particularly due to the emergence of parasite resistance to the current therapeutic arsenal. Plants of the *Strychnos* genus, remarkable due to their multiple traditional uses as well as their alkaloid content, are promising candidates to develop new antimalarial treatments. Indeed, previous research on this plant group has shown promising (≤ 5 µg/ml) or good (between 5 and 15 µg/ml) antiplasmodial activities. Using the chloroquine-sensitive strain of *Plasmodium falciparum* (3D7), and artemisinin as positive control, a screening of antiplasmodial activities from 43 crude methanolic extracts from 28 species of the *Strychnos* genus was carried out in three independent assays. A total of 12 extracts had good (6 extracts) or promising (6 extracts) antiplasmodial activities. These results allowed both to confirm known activities but also to detect new ones. These extracts were then analyzed by HPLC-ESI(+)-Q/TOF, and the processed MS/MS data allowed to generate a molecular network in which the antiplasmodial activities were implemented as metadata. The exploration of the molecular network revealed the presence of alkaloids still unknown, and potentially active against malaria, in particular alkaloids close to usambarensine and its derivatives. This study shows that the emergence of molecular networking offers new leads for identifications of alkaloids from the *Strychnos* genus. The presence of unknown alkaloids potentially active against malaria confirms all the interest to continue in studying the *Strychnos* genus. Bioassay- and mass-guided fractionations as well as various dereplication tools would allow to identify and characterize these interesting alkaloids further.

Figure 16: Screenshot of our article describing the screening of antiplasmodial activities from the *Strychnos* methanolic crude extracts and the annotation of the molecular network derived from these extracts (Bonnet, O. *et al.*, 2022a).

I. INTRODUCTION

The pantropical family of Loganiaceae is divided into four tribes: Antonieae Endl., Loganieae Endl., Spigelieae Dumort, and Strychneae Dumort. A total of 16 genera, including 460 species, are distributed in these different tribes. Nearly half of these species (approximately 200) are in the *Strychnos* genus, that belongs to the tribe Strychneae Dumort. The plants of the *Strychnos* genus are distributed in different continents, namely Africa (75 species), Asia and Oceania (about 44 species), and Central and South America (at least 73 species) (Bisset, N. G., 1970; Krukoff, B. A., 1972; Bisset, N. G., 1974; Setubal, R. B. *et al.*, 2021; World Flora Online, 2022).

Regarding the pharmacology of the *Strychnos* genus, emphasis in the past was on the investigation of the alkaloids present in tetanizing or curarizing species. Indeed, many *Strychnos* species are the classic base poisons of South American as well as South-East Asian arrow and blowpipe poisons (Krukoff, B. A., 1972; Bisset, N. G., 1974). The place of *Strychnos* in the hierarchy of African hunting poisons is secondary and their use limited very locally. The Banyambo, a small tribe in Tanzania, produce a complex curarizing poison. of which the roots of *Strychnos usambarensis* play an important part (Angenot, L., 1971). On the contrary, the importance of *Strychnos icaja* as a trial by ordeal poison is higher and known a long time ago in several countries of West and central Africa (Gabon, Congo, Democratic Republic of the Congo, Central African Republic) (Philippe, G. *et al.*, 2004).

However, it is evident that the plants of many African *Strychnos* species are also well known for their multiple traditional uses (Bisset, N. G., 1970). Indeed, they are used to treat snakebites, arthritis, rheumatism, asthma, bronchitis, diarrhea, hemorrhoids, dyspepsia, fever, and many others (Dr. Duke's Phytochemical and Ethnobotanical Databases, 2021). All these traditional uses make these plants interesting candidates in the development of new drug treatments. That is why the *Strychnos* genus was the subject of numerous research works in our laboratory for more than three decades, especially for its promising antiplasmodial (*in vitro*) and antimalarial (*in vivo*) properties. During these investigations, many isolated monoterpene indole alkaloids showed promising activities on various chloroquine-sensitive (CQS) strains, as well as on some chloroquine-resistant (CQR) strains (Wright, C. W. *et al.*,

1994; Wright, C. W. *et al.*, 1996; Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2000; Frédérich, M. *et al.*, 2001; Frédérich, M. *et al.*, 2002; Frédérich, M. *et al.*, 2003; Tchinda, A. T. *et al.*, 2012; Tchinda, A. T. *et al.*, 2014). Among the most active compounds on CQS strains, we can cite strychnogucine B (0.6170 µM ± 0.067) and strychnohexamine (1.097 µM ± 0.099), both alkaloids isolated from the roots of *S. icaja*. Against CQR strains, we could give as examples 3',4'-dihydrousambarensine (0.032 µM ± 0.002), and isostrychnopentamine, both isolated respectively from roots and leaves of *S. usambarensis* without forgetting strychnogucine B (0.085 µM ± 0.01), and 18-hydroxyisosungucine (0.14 µM ± 0.046) from *S. icaja* roots (Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2001; Philippe, G. *et al.*, 2003). Other papers described *in vitro* and *in vivo* screenings with crude extracts of various *Strychnos* species (e.g., Philippe, G. *et al.*, 2005; Philippe, G. *et al.*, 2007; Fentahun, S. *et al.*, 2017).

Malaria (from the Italian mal'aria meaning "bad air") is a widespread disease in the world that is known for thousands of years. In fact, Indian texts dating back to the sixth century B.C.E. described the symptoms of malaria infection. This disease, transmitted by the bites of female mosquitoes of the *Anopheles* genus, is caused by a protozoan parasite of the Apicomplexa phylum, and more precisely from the *Plasmodium* genus. Five species are capable of infecting humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi* (Cox, F. E. G., 2010). To fight this disease, different hygiene and dietary advice are applied such as wearing long clothes or using insecticide impregnated mosquito nets. In addition, a variety of antimalarial treatments were developed such as artemisinin-based treatments. These treatments led to a reduction in the number of cases. However, the parasites began to show increasing resistance to these drugs, preventing the goal of total eradication of this disease. Malaria remains a major public health problem today, especially in Africa where it is devastating. Indeed, in Africa, between 2019 and 2020, malaria cases increased from 213 million to 228 million, and deaths caused by malaria increased from 534,000 to 602,000 (World Health Organization, 2021a; World Health Organization, 2021b; World Health Organization, 2022).

In the face of this uncontrollable situation, researchers are conducting important research to find new molecules to develop new antimalarial

treatments. Expanding the therapeutic arsenal would allow to counter the growing resistance of the parasites and potentially eradicate the pathology. In view of their promising antiplasmodial and antimalarial potential, plants of the *Strychnos* genus are therefore very interesting candidates.

In recent years, the field of natural products research has evolved significantly with the development of various dereplication tools, including molecular networking, which allows the quick discrimination of known and unknown metabolites based on comparisons with databases of mass spectra (Yang, J. Y. *et al.*, 2013; Wang, M. *et al.*, 2016; Nothias, L.-F. *et al.*, 2018; Fox Ramos, A. E. *et al.*, 2019a; Fox Ramos, A. E. *et al.*, 2019b; Beniddir, M. A. *et al.*, 2021). Inspired by these advances, we envisioned that the use of molecular networks would be an efficient approach to both explore the chemodiversity of alkaloids in plants of the *Strychnos* genus but also to target metabolites that have not yet been identified in the previous studies.

In this context, the objectives of this study were first to screen the antiplasmodial activities of methanolic crude extracts from a large number of *Strychnos* species, to explore then by molecular networking the chemodiversity of monoterpene indole alkaloids, and to finally apply as metadata in the molecular network the antiplasmodial activities obtained with the aim of detecting and targeting molecular families containing unknown alkaloids potentially active against malaria.

II. MATERIALS AND METHODS

II.1. Materials, chemicals, and reagents

The twenty-eight species of *Strychnos*, preserved in the collection of the University of Liege (Belgium), were collected in several countries: Rwanda, Congo, Zimbabwe, Tanzania, Cameroon, India, Cambodia, and Brazil. These species were *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 and 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. All information about the samples used in this study is presented in the Supplementary Table S1 in this article, as well as in the section 3.2 of this chapter (see pages 183-197). Each sample was dried at 40°C, stored dry at moderate room temperature, and protected from light. Some samples were collected several years ago. The question of the stability of alkaloids was therefore raised. Numerous previous studies of alkaloids showed a high stability (Phillipson, J. D., 1982; Frédérich, M. *et al.*, 1998; Eloff, J. N., 1999; Soto-Sobenis, A. *et al.*, 2001; Harborne, J. B., 2012; Yilmaz, A. *et al.*, 2012). Oxidation reactions cannot be excluded, but no examples were highlighted in the different studies.

Methanol and DMSO were purchased from VWR Chemicals BDH (Leuven, Belgium). Methanol of HPLC grade was obtained from Merck (Darmstadt, Germany). The solvents of UHPLC-MS grade (methanol and formic acid) came from Sigma-Aldrich (Machelen, Belgium). About milli-Q water, two systems were used: a milli-Q reference A+ system® at the University of Liège and a MILLIPORE Synergy UV® at Université Paris-Saclay. These two systems were purchased from Merck (Darmstadt, Germany).

II.2. Sample preparation

A total of forty-four samples, described in the Supplementary Table S1 in this article, and in the section 3.2 (see pages 183-197), were ground using an IKA A10 mill (Staufen, Germany) to obtain 10 g powder. Extractions were then performed in methanol using the SpeedExtractor E-914® (Büchi, Hendrik-Ido-Ambacht, Netherlands). This device allows extractions with pressurized solvents, which offers a better extraction. Moreover, by means of four cells each containing plant powder and sand, four samples were extracted at the same time during three extraction cycles. These cycles were composed of 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 was dried with nitrogen for 3 min. The crude extracts collected were evaporated using Rotavapor® and

Multivapor® (Büchi). In order to dry the extracts, they are placed in a vacuum oven (Heraeus, Hanau, Germany) for one night at room temperature.

II.3. Antiplasmodial assays

In vitro cultures of *P. falciparum* in the asexual erythrocyte stage were maintained following the procedure of Trager and Jensen (1976). The 3D7 strain of parasites is a chloroquine-sensitive strain that was obtained from the Malaria Research and Reference Reagent Resource Center, MR4. The culture medium was composed of RPMI 1640 (Gibco, Fisher Scientific, Merelbeke, Belgium) containing NaHCO3 (32 mM), HEPES (25 mM), and L-glutamine. The host cells were human red blood cells (A+ or O+). The medium was supplemented with 1.76 g/L of glucose (Sigma-Aldrich, Machelen, Belgium), 44 mg/mL of hypoxanthin (Sigma-Aldrich, Machelen, Belgium), 100 mg/L of gentamicin (Gibco, Fisher Scientific, Merelbeke, Belgium), and 10% human pooled serum (A+ or O+), as previously described.

Each crude extract was dissolved in DMSO at a concentration of 10 mg/mL. The solutions of crude extracts were then diluted in the culture medium: for each solution to test, two-fold dilutions were performed eight times on a 96-well plate. With this method, the highest concentration tested was 100 µg/ml. Moreover, each sample was tested in duplicate. As positive control for all assays, artemisinin (Sigma-Aldrich, Machelen, Belgium) was used at an initial concentration of 100 ng/ml. After leaving the parasites in incubation with the diluted solutions of the crude extracts for 48 h, the impact on parasite growth was revealed using SYBR Green, a DNA intercalating compound. The procedure was adapted from the method described in the article of Dery, V. *et al.* (2015). The SYBR Green solution was diluted in a lysis buffer composed of TRIS buffer (Sigma-Aldrich, Machelen, Belgium), EDTA (Merck, Darmstadt, Germany), saponin (Alfa Aesar, Karlsruhe, Germany), and triton (Merck, Darmstadt, Germany). Thus, 500 ml of lysis buffer contained 1.20 g TRIS buffer, 0.73 g EDTA, 40 mg saponin, and 0.4 ml triton. In order to reveal a plate, 2 µl of SYBR Green solution was diluted in 10 ml of lysis buffer. In new 96-well plates, 100 µl of solutions from assays were placed and 75 µL of SYBR Green were added. After 2 h of incubation, the plates were read with the FlexStation®

(Molecular Devices, Winnersh, United Kingdom) at 490 nm excitation wavelength and 530 nm emission wavelength.

The half maximal inhibitory concentration (IC_{50}) values were calculated from graphs. Averages of three IC_{50} values from three independent experiments (n = 3) performed on different days were calculated.

II.4. Mass spectrometry analyses

A total of forty-three methanolic crude extracts were dissolved and ultrasonicated in methanol of HPLC grade (high-performance liquid chromatography). The concentration obtained was at 1 mg/mL. After transferring all the solutions into HPLC vials, they were injected into the HPLC-MS/MS system.

The Agilent HPLC-MS system (Agilent Technologies, Massy, France) was composed of two modules: an Agilent 1,260 Infinity HPLC coupled to an Agilent 6,530 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 used was a SunFire® C_{18} purchased from Waters (150 mm × 2.1 mm, 3.5 µm). The flow rate was at 250 µL/min. About the gradient, it was linear and variated from 5% B to 100% B in 30 min (A = Water + 0.1% formic acid; B = Methanol). The injection volume was 5 µL. The DAD detector was set at 210, 254, and 280 nm. For ESI conditions, the capillary temperature, the source voltage, and the sheath gas flow rate were set at 320°C, 3.5 kV, and 10 L/min, respectively. The mass spectrometer worked with the Extended Dynamic Range mode (2 GHz). Thanks to the divert valve, the first 3 min were eliminated. For every scan, one MS scan in positive mode was performed between *m/z* 100 and 1,200, and the five most intense ions were fragmented. Three fixed collision energies were applied at 30, 50, and 70 eV. The default charge was 1. The isolation width and minimum intensity were set at *m/z* 1.3 and 3,000 counts, respectively. Purine $C_5H_4N_4$ $[M+H]^+$ ion (*m/z* 121.0509) and hexakis (1*H*, 1*H*, 3*H*-tetrafluoropropoxy)-phosphazene

$C_{18}H_{18}F_{24}N_3O_6P_3$ $[M+H]^+$ ion (*m/z* 922.0098) were constituted the internal lock masses. The *m/z* values of the two internal calibrants were implemented in a permanent MS/MS exclusion criterion in order to prevent the signals from the internal lock masses oversample the signals from samples analyzed.

II.5. Feature-based molecular networking

The method of feature-based molecular networking is described in the article of Nothias, L.-F. *et al.* (2020).

First, the data obtained from the mass spectrometry analyses are converted into an ".mzXML" file (eXtensible Markup Language). The conversion was performed using MSConvert software edited by ProteoWizard (Chambers, M. C. *et al.*, 2012). The filter and the algorithm used were peak picking and vendor, respectively. The MS levels 1 and 2 were selected.

Then, the ".mzXML" files were processed using MZmine 2 software (version 2.53) (Pluskal, T. *et al.*, 2010). Different stages of processing were carried out: suppression of noise (Method: Mass detection), creation of peak lists (Method: ADAP Chromatogram builder), deconvolution (Method: Chromatogram deconvolution), grouping of isotopes (Method: Isotopic peaks grouper), alignment (Method: Join aligner), gap-filling (Method: Same RT and *m/z* range gap filler), filtering (Method: Feature list rows filter), and export (Methods: Export to CSV file and Export/Submit to GNPS/FBMN).

The intensities applied for the suppression of noises were the 5.8E3 and 5.0E1 for MS^1 and MS^2, respectively. For the creation of peak lists, a minimum of four points were necessary to build a peak. The intensity threshold was set at 5.8E3, and the *m/z* tolerance was *m/z* 0.02 and 10.0 ppm. About the deconvolution, the different settings applied were the following: 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 3,000, the coefficient/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.

About the suppression of isotopes, 0.005, 15.0 ppm, and 0.5 min were the values for *m/z* retention time tolerances. The maximum charge was set at 1. The most intense peak corresponded to the representative isotope. All the peak lists were gathered according to the following criteria: 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 the two settings. During the filtration step, only the MS/MS data were kept because they are essential to generate the molecular network. Moreover, during this step, the retention windows between 0-2.50 min and 45.59-49.83 min were deleted. Finally, the processed files are exported into ".mgf" without merging MS/MS spectra and ".CSV" formats to be imported into the GNPS platform (Global Natural Products Social Molecular Networking).

On the GNPS platform, the tolerances for precursor and fragment ions were set at 0.02 Da. The minimal cosine score for binding two metabolites was 0.65. For matching with the library, the threshold for the cosine score was 0.7. No filtration was applied, and the analogues were not searched. To highlight the active nodes against malaria, as metadata, a ".txt" file listing the antiplasmodial activities of the forty-three methanolic crude extracts was also imported.

II.6. MolNetEnhancer annotation

The MS/MS data of the global molecular network were further annotated using MolNetEnhancer workflow exploiting exclusively the GNPS experimental annotations. MolNetEnhancer is a workflow that allow to have a more comprehensive chemical overview of metabolomics and to highlight structural details for each MS/MS spectra. Using the automated chemical classification through ClassyFire, the classifications provided by MolNetEnhancer were of different levels: from kingdoms to subclasses (Ernst, M. *et al.*, 2019).

III. RESULTS AND DISCUSSION

III.1. Methanolic extractions and yields

Forty-three methanolic crude extracts were obtained from different parts of twenty-eight *Strychnos* species. All the weightings and yields are presented in Supplementary Table S2 in this article, as well as in section 3.3 of this chapter (see pages 197-203). The yields were between 0.55% w/w and 25.92% w/w.

III.2. Antiplasmodial assays of methanolic crude extracts from *Strychnos* spp.

The half maximal inhibitory concentration (IC_{50}) values for the forty-three methanolic crude extracts are described in Figure 17. Sample number identifications are described in Supplementary Table S1 in this article, as well as in section 3.2 (see pages 183-197). Results are averages expressed in µg/mL ± standard deviation (S.D.) of IC_{50} values of three independent experiments. For artemisinin, the mean IC_{50} value was 3.09 ± 1.57 ng/mL, which means that the IC_{50} values obtained were valid (Ledoux, A. *et al.*, 2017). To interpret the antiplasmodial activity levels, we have implemented the following classification, which is inspired by the World Health Organization's guidelines, with a few modifications (Jansen, O. *et al.*, 2012): an activity ≤ 5 µg/mL was considered as a promising antiplasmodial activity, between 5 µg/mL and 15 µg/mL as a good activity, between 15 µg/mL and 30 µg/mL as a moderate activity, between 30 µg/mL and 50 µg/mL as a weak activity, and ≥ 50 µg/mL as a lack of activity. Among the forty-three methanolic crude extracts tested, 14% (6 out of 43) showed a promising antiplasmodial activity, 12% (5 out of 43) a good activity, 16% (7 out of 43) a moderate activity, 7% (3 out of 43) a weak activity, and 51% (22 out of 43) a lack of activity. *Strychnos* species with promising and good antiplasmodial activities are the following: *S. usambarensis* Gilg ex Engl. leaves (November 2007 and August 2008), *S. usambarensis* Gilg ex Engl. root barks, *S. variabilis* De Wild. root barks, *S. phaeotricha* Gilg leaves, *S. angolensis* Gilg root barks, *S. longicaudata* Gilg trunk barks, *S. malchairi* De Wild. leaves, *S. icaja* Baill. roots, *S. icaja* Baill. collar barks, and *S. nux-vomica* L. root barks. While antiplasmodial activities from trunk barks of *S. longicaudata* and from leaves of *S. phaeotricha* and *S.malchairi* were never

reported in the literature, the other results confirmed the reported activities in previous studies (Frédérich, M. *et al.*, 1999; Philippe, G. *et al.*, 2005).

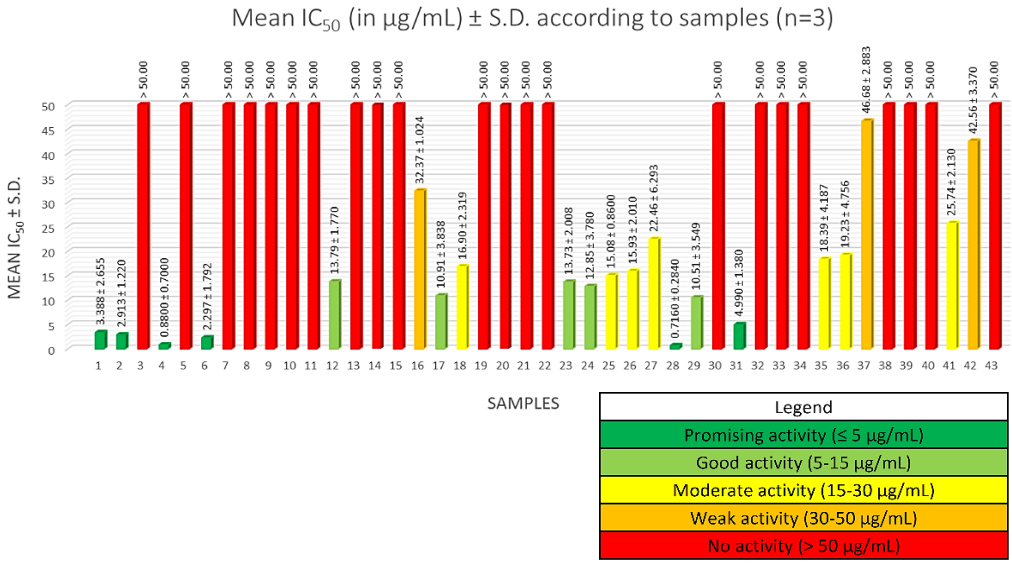

Figure 17: Mean IC_{50} values of forty-three methanolic crude extracts from twenty-eight *Strychnos* species.

III.3. Feature-based molecular networking of *Strychnos* spp. methanolic crude extracts and exploration of their alkaloidic content

All processed data have been deposited on the GNPS platform by using the Feature-Based Molecular Networking (FBMN) workflow. The molecular network obtained contained 5,904 nodes, including 105 unique annotations (Figure 18). The following link provides access to the job and the molecular network:
https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=880ec1b92b6d4cd1b73a225f2ab3dcdb.

Different monoterpene indole alkaloids, such as 3',4'-dihydrousambarensine, strychnine, icajine, strychnofoline, sungucine, were annotated thanks to the GNPS libraries.

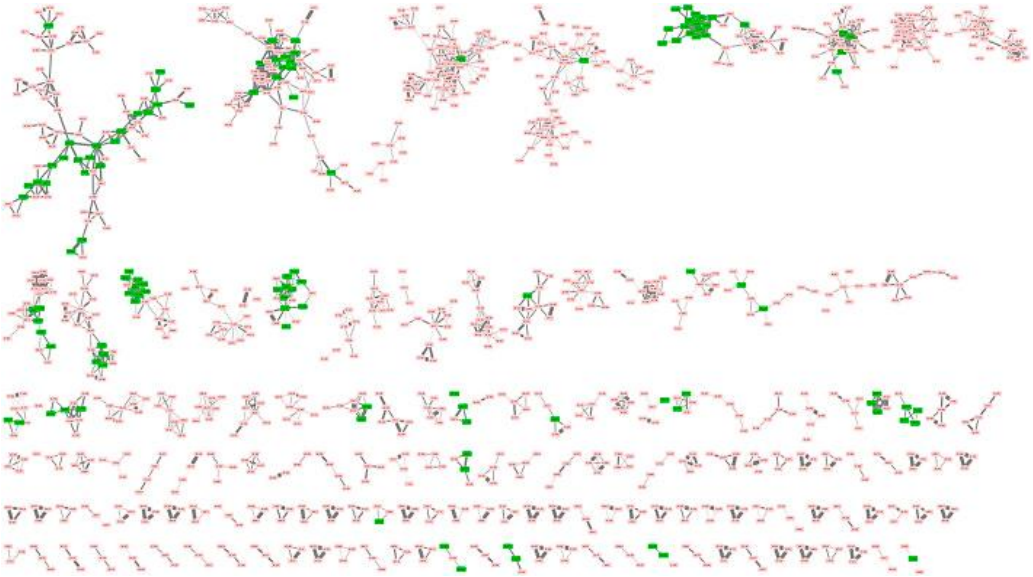

Figure 18: Part of molecular network from twenty-eight *Strychnos* species (Clusters with less than 3 nodes are not shown). The green nodes correspond to the annotated metabolites, while the red nodes are the unannotated ones.

The results provided by the MolNetEnhancer workflow are shown in Figure 19. In this study, we interpreted the molecular network considering the class level. As in Figure 18, clusters with less than three ions have not been included in the figure for image size reasons. Moreover, in order to add a global quantitative aspect in the molecular network, the size of the nodes is related to the sum of precursor intensities. The larger the node, the greater the intensity of the precursor ion in question. A total of 11 clusters were categorized into the phytochemical class of alkaloids. These clusters were framed in Figure 19.

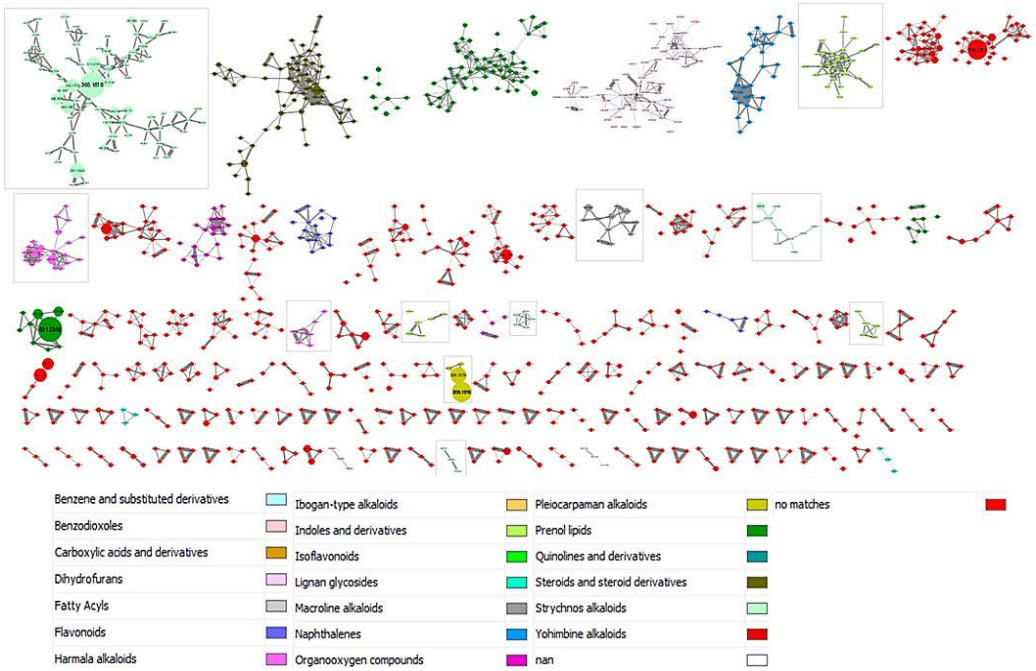

Figure 19: MolNetEnhancer-based annotation of the molecular network from twenty-eight *Strychnos* species (Figure 18). The color legend is described below the molecular network. The framed clusters contain the phytochemical class of alkaloids. The size of nodes is related to the sum of precursor intensities.

Nevertheless, some classifications of metabolites in clusters, proposed by the workflow, may be imprecise, or even faulty. This may be due to a misinterpretation of the workflow or to the presence of artifacts in the data. For example, we observed that strychnofoline, at *m/z* 483.2684, which is an oxindole monoterpenoid alkaloid, was assigned to the harmala alkaloids, presented in the Supplementary Figure S4 in this article. It is likely that this misclassification is related to the absence of the precise chemical class of strychnofoline in ClassyFire, a web-based application for automated structural classification of chemical entities (Djoumbou Feunang, Y. *et al.*, 2016). For this reason, the application suggested the closest chemical class to strychnofoline, namely harmala alkaloids. Despite these inaccuracies, the classifications obtained are close to the expected result, and are therefore good indications for identifying metabolites.

The antiplasmodial activities from the forty-three methanolic crude extracts of *Strychnos*, described in point III.2, were added as metadata to the global molecular network. These data, presented as pie charts within the nodes, allow to annotate the molecular network based on antiplasmodial activities and, therefore, to point to clusters and nodes with promising and good antiplasmodial activities. The proportions of the slices in the pie charts represent the total intensities of the metabolite corresponding to the node of interest across different groups based on antiplasmodial activity levels for the forty-three methanolic crude extracts, using the following color codes: dark green for promising, light green for good, yellow for intermediate, orange for poor, and red for absent antiplasmodial activity (Figure 20).

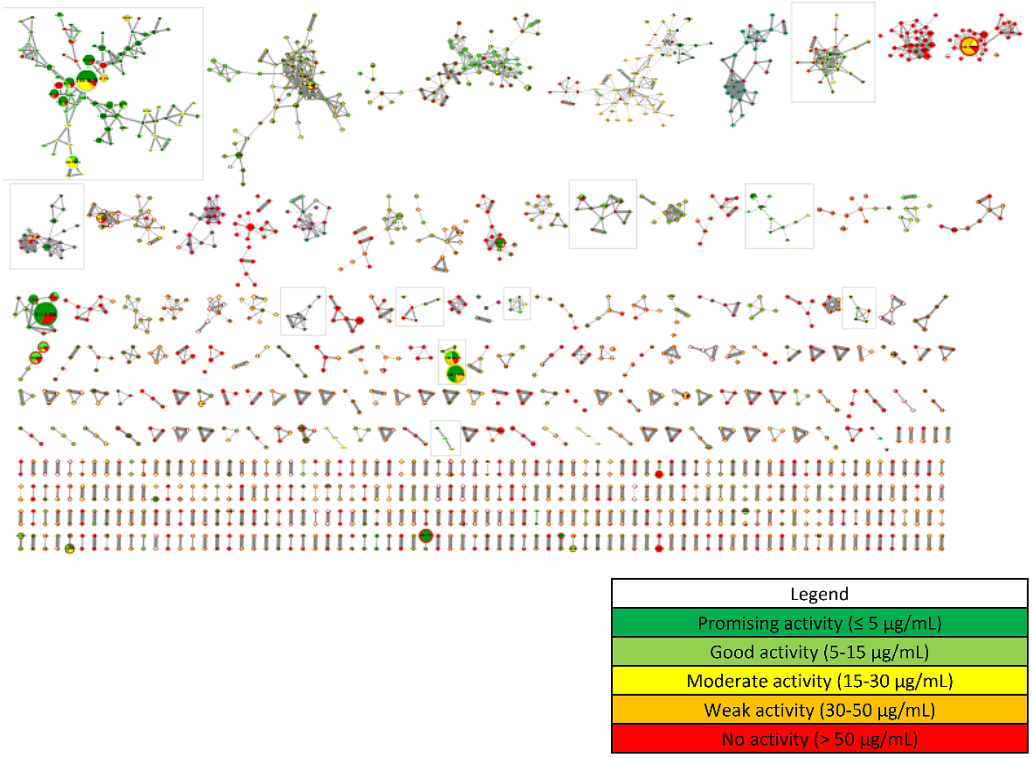

Figure 20: Implementation of antiplasmodial activities as metadata in the molecular network from twenty-eight *Strychnos* species (Figure 18).

Each of the eleven framed clusters contain a variety of known alkaloids, namely akuammicine, N_4-methylantirhine, brucine, α-colubrine,

corynantheidal, 6,7-dihydroflavopeirerine, icajine, malindine, isomalindine, C-mavacurine, naucleidinal, olivacine, panarine, retuline, strychnine, strychnofoline, strychnogucine C, sungucine, tubotaiwine, usambarensine, 3',4'-dihydrousambarensine, N_b-methylusambarensine, vincosamide, and vomicine. Moreover, some of these alkaloids, present mainly in extracts with promising or good activity, are related to ions of different masses still unknown to date and also present mainly in extracts with the same levels of antiplasmodial activity. However, it is important to note that an alkaloid, not identified in the molecular network, is not necessarily unknown in the literature. Indeed, identifications depend on databases that are not yet complete today. Enriching these databases would allow better identifications and even more efficient targeting of metabolites that were never reported in the literature.

As an example, the third framed cluster includes usambarensine, 3',4'-dihydrousambarensine, and N_b-methylusambarensine (Figure 21; Supplementary Table S3 in this article). The class assigned to this group is harmala alkaloids, which is confirmed because usambarensine and its derivatives include a harmane group in their structure (Figure 22).

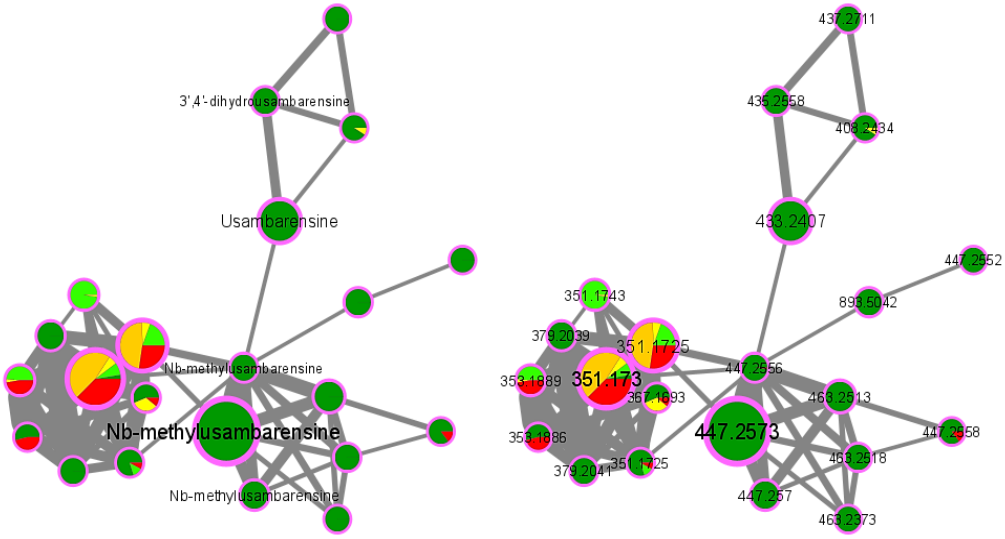

Figure 21: Alkaloids cluster n°3: Cluster of usambarensine and its derivatives.

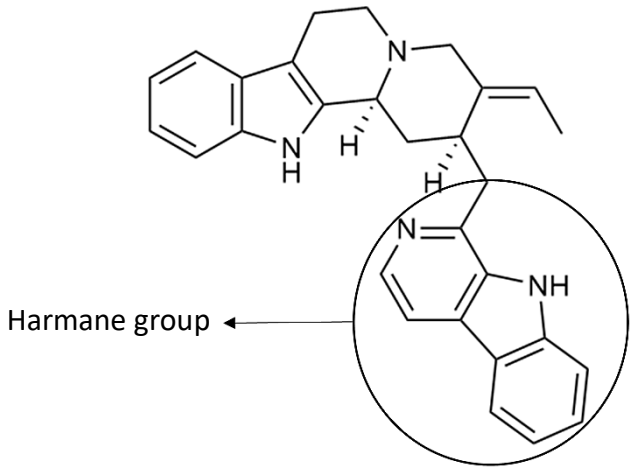

Figure 22: Structure of usambarensine.

Well-known in the *Strychnos* genus, especially for their promising antiplasmodial property against CQS and CQR strains of *P. falciparum*, usambarensine (CQS: 1.516 µM ± 0.031; CQR: 0.594 µM ± 0.052) and its derivatives are related to many non-identified ions, such as those at *m/z* 408.2434, 437.2711, 447.2552, 463.2373, 893.5042. A possible identification for the mass at *m/z* 437.2711 would be 1′,2′,3′,4′-tetrahydrousambarensine (Dictionary of Natural Products, 2022). Regarding the other masses, no identification could be proposed. For more information, the Supplementary Table S4 in this article includes all the different *m/z* values mentioned above, the generated molecular formulas using the mass spectrometry application software MassHunter (Version B.07.00), all suggested identifications provided by the Dictionary of Natural Products (Version 31.1) (Dictionary of Natural Products, 2022), the resulted interpretations, and the applied tolerances. Moreover, the nodes of all these ions are relatively small, which means that, based on the whole metabolites' contents of the forty-three methanolic crude extracts, the abundance of these ions is rather low. This observation would explain why the old methods did not allow us to visualize and identify these molecules of interest in previous research, especially on the leaves and roots of *S. usambarensis*.

As another example, we can cite the fifth framed cluster that belongs to the group of *Strychnos* alkaloids and proposes strychnogucine C and sungucine as annotations (Figure 23; Supplementary Table S3 in this article).

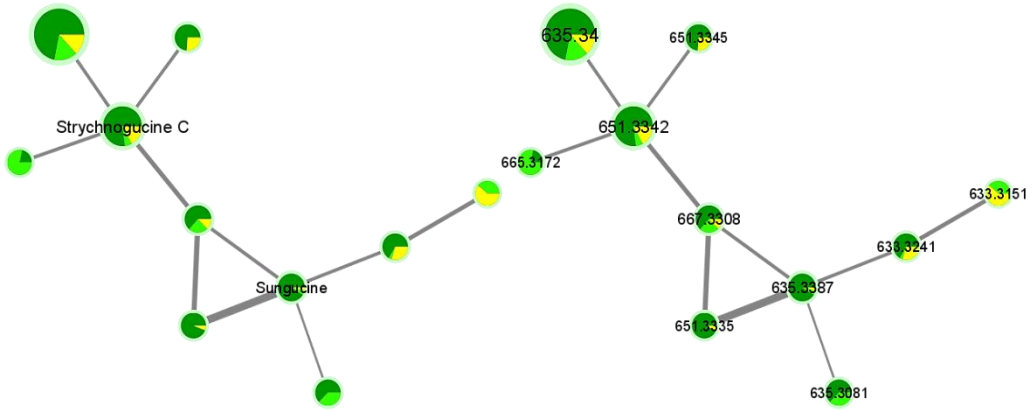

Figure 23: Alkaloids cluster n°5: Cluster of sungucine and strychnogucine C.

These two alkaloids are also well known in the *Strychnos* genus, especially sungucine, isolated for the first time from the roots of *S. icaja*, and showed a promising antiplasmodial activity (CQS: 2.292 µM ± 0.049; CQR: 1.659 µM ± 0.089). Associated with these two alkaloids, different non-identified ions with masses at *m/z* 633.3241, 633.3151, 665.3172 and, 667.3308 are observed. The mass at *m/z* 667.3308 could correspond to strychnogucine B, which is a derivative of strychnogucine C. Moreover, several masses at *m/z* 635 and 651, corresponding to the masses of sungucine and strychnogucine C, respectively, are also present in the cluster. The differences in retention time and the low cosine values (from 0.67 to 0.70) lead rather to the hypothesis that they are different from each other, or even that they are isomers of sungucine and strychnogucine C. The ions at *m/z* 651.3335 and 651.3345 could correspond to either strychnogucine A, which is also a derivative of strychnogucine C, or 18-hydroxysungucine, or 16,17-didehydro-17,23-dihydro-18-hydroxysungucine. All other masses, present in the fifth framed cluster, remain unknown (Supplementary Table S4 in this article).

Further investigation is needed to identify all the unknown alkaloids within the cluster. In addition, the sizes of the nodes show us that the abundances of the unknown ions are relatively low, except for the one with mass

at *m/z* 635.3400 where the size is significantly larger. Indeed, its intensity is 10 times more important (Intensity at E8 compared to the other unknown ions that are at intensity E7).

For the other framed clusters, they are presented in the Supplementary Figures S1–S8 in this article. Among these clusters, one contains strychnofoline, which also shows interesting antiplasmodial activities and is linked to three unidentified alkaloids (*m/z* 422.2317, 424.2404, and 440.2273) potentially active against malaria (Supplementary Tables S3 and S4 in this article). A total of about 90 unknown alkaloids potentially active against malaria were detected using molecular networking. The advantage of this working method is therefore to target directly the unknown alkaloids, and, thus, to avoid wasting time to isolate already known alkaloids using the classical bio-guided fractionation method.

About the clusters in red, i.e., those where the MolNetEnhancer workflow did not provide a classification, it is important not to ignore them. Indeed, there may be a significant number of unknown ions that show interesting antiplasmodial activities. A closer look at these groups showed the presence of many masses above *m/z* 400. These masses could notably correspond to dimeric alkaloids that, in previous studies, demonstrated very good activities against *P. falciparum*.

Therefore, the development of mass spectrometry and chemoinformatic methods, such as molecular networking, allows to have an overview of the metabolites' content of the extracts studied, to quickly identify known ones, and to detect new potentially active ones, even with low quantities. The use of other dereplication tools such as ISDB (*In-Silico* spectral DataBase) (Allard, P.-M. *et al.*, 2016), MS2LDA (Wandy, J. *et al.*, 2018), MixONat (Bruguière, A. *et al.*, 2020), and MADByTE (Egan, J. M. *et al.*, 2021), and the realization of various bio- and mass-guided fractionations would allow to characterize and identify the new active metabolites observed in this study.

IV. CONCLUSION

Plants of *Strychnos* genus have fascinated researchers for more than two centuries because of their multiple traditional uses and their richness in metabolites, and more particularly in alkaloids, promising in the therapeutic field. Indeed, previous studies have highlighted various pharmacological activities of some species from the genus, especially against *Plasmodium* parasites, responsible for malaria, which continues to cause many deaths throughout the world, especially in Africa. In the face of growing parasite resistance, the current therapeutic arsenal is no longer sufficient to stop infections.

Following the implementation of innovative chemoinformatic methods such as molecular networking, an exploration of the alkaloid content of forty-three methanolic crude extracts from *Strychnos* species was performed. In addition, these extracts were tested against the chloroquine-sensitive *P. falciparum* strain (3D7) in three independent test series. A total of 26% (11 extracts out of 43) showed promising (≤ 5 µg/ml) and good (between 5 and 15 µg/ml) antiplasmodial activities. The active extracts were obtained from leaves (November 2007 and August 2008) and root barks of *S. usambarensis*, root barks of *S. variabilis*, leaves of *S. phaeotricha*, root barks of *S. angolensis*, trunk barks of *S. longicaudata*, leaves of *S. malchairi*, roots and collar barks of *S. icaja*, and root barks of *S. nux-vomica*. Some of these activities were never reported in the literature, namely these of trunk barks of *S. longicaudata*, as well as leaves of *S. phaeotricha* and *S. malchairi*.

These results, implemented as metadata in the molecular network of the forty-three methanolic crude extracts, allowed us to highlight the presence of many alkaloids still unknown and potentially active against malaria. This is notably the case of alkaloids whose structures are close to those of usambarensine (at *m/z* 408.2434, 447.2552, 463.2373, 893.5042, ...), sungucine and strychnogucine C, (at *m/z* 633.3241, 633.3151, 665.3172, 667.3308, ...), strychnofoline (at *m/z* 422.2317, 424.2404, and 440.2273), and their corresponding derivatives, well known for their promising antiplasmodial properties.

In the future, it would be interesting to investigate further these still unknown alkaloids and to isolate them using bioassay- and mass-guided fractionations. The use of dereplication tools is also a way to obtain leads for the characterization and identification of these metabolites, even if they are present in small quantities.

Thus, this study demonstrates that the *Strychnos* genus still constitutes a significant therapeutic source, and that the new approaches of dereplication offer new identification leads.

V. SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.967012/full#supplementary-material. They can also be accessed via the QR code below:

3.10. References

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CHAPTER 4

Selection of *Strychnos* species for further study

4.1. Forewords

Now that the molecular network of the forty-four *Strychnos* crude extracts has been generated and annotated, it is essential to establish selection criteria for wisely choosing the species to prioritize during the purification stages. This selection is critical for the subsequent phases of the work, as it will influence the discovery of new antiplasmodial metabolites. Prioritizing the right species will increase the likelihood of identifying novel active compounds.

In total, five criteria were established: antiplasmodial activities, presence of alkaloids in clusters from the molecular network, quantities of plant material, presence of multi-charged compounds, and the number of references in the literature. These different criteria do not carry the same weight. Indeed, the first criterion, which concerns the antiplasmodial activities of the crude extracts, is crucial for identifying new antiplasmodial compounds. On the other hand, the fifth criterion, which reports the number of references for each of the previously selected species, carries less weight in the decision-making process, and may even be considered optional. Indeed, the fact that a species is extensively described in the literature does not imply that there is nothing more to discover about it. Older methods were less sensitive, which prevented the exploration of minor metabolites. Today, thanks to metabolomics, this is no longer the case, opening new perspectives for all species.

Each of these criteria is thoroughly discussed in the following section, and the chapter ends with the selection of the species.

4.2. Selection criteria

4.2.1. Criterion 1: Antiplasmodial activities

As the primary objective of this thesis is to isolate new antiplasmodial alkaloids, the first criterion is the antiplasmodial activities of the forty-four *Strychnos* crude extracts. In the previous chapter, section 3.9 (see pages 214-233), all the results from the screening of the antiplasmodial activities of the methanolic crude extracts were presented in a graph (see Figure 17).

Based on slightly modified standards from the World Health Organization (Jansen, O. *et al.*, 2012), antiplasmodial activity below 5 µg/mL, between 5 and 15 µg/mL, between 15 and 30 µg/mL, between 30 and 50 µg/mL, and above 50 µg/mL correspond, respectively, to promising, good, moderate, low, and no antiplasmodial activity. The results show that 6 out of the 43 methanolic crude extracts (14%) exhibit promising antiplasmodial activity, while 5 extracts (12%) demonstrate good activity. The species corresponding to these extracts include *Strychnos usambarensis* Gilg ex Engl. (leaves and root barks), *S. variabilis* De Wild. (root barks), *S. phaeotricha* Gilg (leaves), *S. angolensis* Gilg (root barks), *S. longicaudata* Gilg (trunk barks), *S. malchairi* De Wild. (leaves), *S. icaja* Baill. (roots, and collar barks), and *S. nux-vomica* L. (root barks) (Bonnet, O. *et al.*, 2022).

In the case of the alkaloidic extract of *S. usambarensis* leaves, moderate antiplasmodial activity was observed, with a mean IC_{50} of 18.5 ± 2.66 µg/mL.

All antiplasmodial assays were carried out on the 3D7 strain (chloroquine-sensitive strain, CQS) of *Plasmodium falciparum* at a starting concentration of 10 mg/mL. Mean IC_{50} values were obtained from three independent assays (n=3). The complete methodologies for maintaining the parasite culture and conducting the tests are described in Chapter 3 (see section 3.9, pages 214-233).

Only crude extracts showing promising and good antiplasmodial activities were selected for further processing. A total of 11 extracts were therefore selected. The alkaloidic extract of *S. usambarensis* leaves is an exception to this selection. Despite its moderate activity against malaria, this sample was chosen for purification due to a specific reason, which will be discussed later in the chapter.

4.2.2. Criterion 2: Identification of alkaloid clusters in the molecular network of crude extracts using the MolNetEnhancer tool

For this second criterion, we explored the molecular network of the forty-four *Strychnos* crude extracts, and the MS/MS data were annotated according to chemical classes using the MolNetEnhancer workflow. Based exclusively on the experimental annotations provided by the GNPS platform, this workflow

offers automated chemical classifications at different levels using ClassyFire (Ernst, M. *et al.*, 2019). This annotation allowed us to quickly identify the clusters containing the majority of alkaloid phytochemical classes (Figure 24).

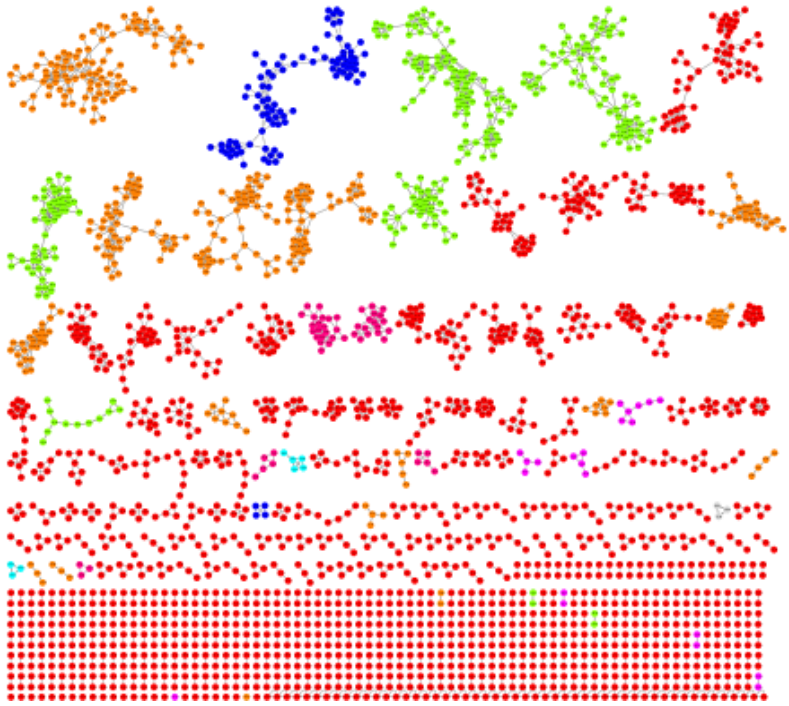

Figure 24: MolNetEnhancer-based annotation of the molecular network from forty-four *Strychnos* crude extracts. The orange color corresponds to alkaloids.

The presence or absence of alkaloids within alkaloid clusters was assessed for each sample, and a graph illustrating their occurrences was generated (Figure 25).

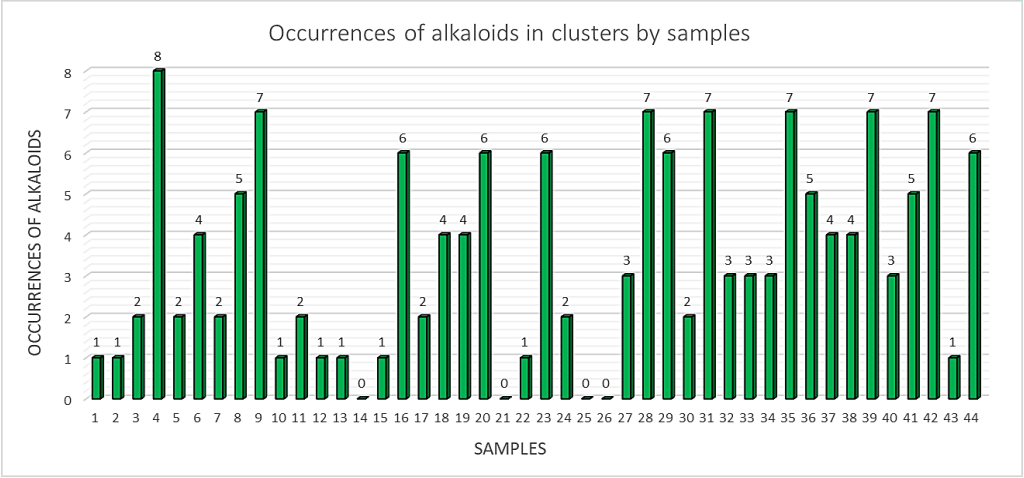

Figure 25: Occurrence graph of alkaloids in alkaloid clusters according to the forty-four crude extracts. Number identifications are listed in the table of origins in Chapter 3 (see section 3.2, pages 183-197).

Among the analyzed samples, the methanolic crude extract of *S. usambarensis* root barks was the most abundant in alkaloid clusters. In four extracts, the MolNetEnhancer workflow failed to identify alkaloid clusters. These extracts were from *S. innocua* leaves, *S. spinosa* leaves, *S. malchairi* trunk barks, and *S. mattogrossensis* leaves. Since these extracts had already been excluded based on the antiplasmodial activity criterion, there are still 11 extracts to prioritize at the end of this second criterion.

4.2.3. Criterion 3: Quantities of plant material

To isolate a compound in sufficient quantity for identification, it is crucial to start with a large amount of plant material. For this work, the minimum amount was set at 50 g of powdered plant material. Of the 11 remaining extracts, *S. malchairi* leaves, *S. icaja* roots and collar barks, and *S. nux-vomica* root barks were discarded due to their insufficient quantities available in the laboratory. As a result, only 7 samples remain for the next criterion.

4.2.4. Criterion 4: Presence of multi-charged metabolites

In the literature, bisindole alkaloids are primarily known for their high activity against malaria. Notable examples include usambarensine, strychnobiline, sungucine, and strychnogucine A and B (Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2001). Therefore, for this fourth criterion, the number of dimeric compounds was estimated by manually analyzing the MS and MS/MS spectra to identify and assess the occurrence of multi-charged compounds in the seven crude extracts. A multi-charged metabolite can be easily recognized by two characteristics:

- When it is monocharged, the *m/z* difference between isotopes is 1. In this *m/z* ratio, "m" corresponds to mass and "z" to charge. When doubly charged, for instance, the *m/z* ratio is divided by 2, resulting in an *m/z* difference of 0.5 between the isotopes;

- For the same compound, when it can be ionized multiple times, there will be a different *m/z* ratio for each ionization step. For example, a monocharged compound with an *m/z* ratio of 301 and two ionizable sites will also have an *m/z* ratio of 151 (Figure 26).

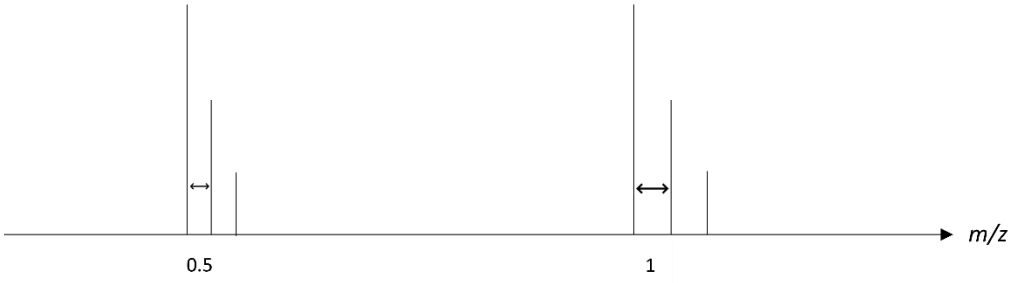

Figure 26: Characteristics of MS spectra of multi-charged compounds.

It should be noted that there are also dimeric compounds with only one ionization site. These were therefore not included in the estimated values, as only multi-charged compounds were considered in this study.

The graph below shows the estimated number of multi-charged compounds in the seven remaining crude extracts (Figure 27).

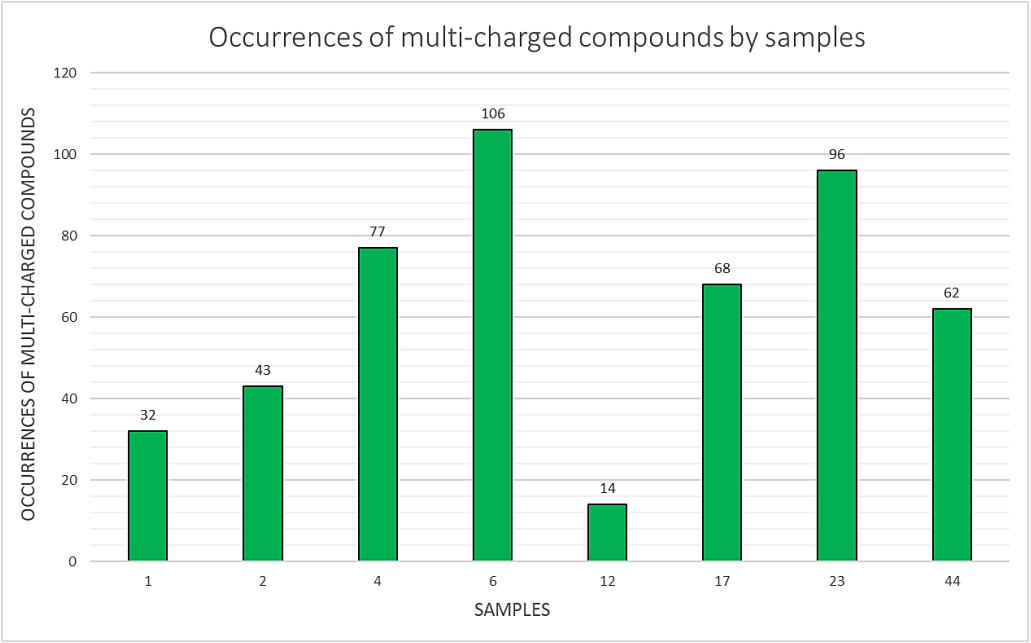

Figure 27: Occurrence graph of multi-charged compounds according to seven methanolic crude extracts from *Strychnos* species.

1: Leaves of *S. usambarensis* (November 2007); 2: Leaves of *S. usambarensis* (August 2008); 4: Root barks of *S. usambarensis*; 6: Root barks of *S. variabilis*; 12: Leaves of *S. phaeotricha*; 17: Root barks of *S. angolensis*; 23: Trunk barks of *S. longicaudata*.

S. variabilis root barks (sample 6) and *S. longicaudata* trunk barks (sample 23) have the highest number of multi-charged compounds, estimated at 106 and 96, respectively. Conversely, one sample stands out: sample 12, corresponding to the leaves of *S. phaeotricha*. This sample contains relatively few multi-charged compounds, and its molecular network shows a low alkaloid content, with only one alkaloid cluster. However, the methanolic crude extract of its leaves displayed good antiplasmodial activity against the 3D7 strain (CQS strain) of *Plasmodium falciparum* (13.8 ± 1.77 µg/mL). This suggests that the activity may be attributed to a phytochemical class other than alkaloids,

which is uncommon in the *Strychnos* genus. This is the reason we selected this species for further study.

Samples 1 and 2 both correspond to *S. usambarensis* leaves, but they were harvested at different times. Sample 1 was collected during the wet season (November 2007), while sample 2 was collected during the dry season (August 2008). A slight difference between the two samples can be observed in the graph, highlighting the influence of seasonal weather on metabolite content.

Finally, it is evident that leaves (samples 1, 2, and 12) have a lower alkaloid content compared to root and trunk barks, with root barks from samples 4, 6, and 17, and trunk barks from sample 23, showing higher alkaloid levels.

4.2.5. Criterion 5: Number of references in the literature

The final criterion considers the number of references available for the seven remaining crude extracts: *S. usambarensis*, *S. variabilis*, *S. phaeotricha*, *S. angolensis*, and *S. longicaudata*. A greater number of references generally suggests that a substantial number of new compounds have already been identified, thereby highlighting the increasing challenge of discovering additional novel compounds. This was assessed by determining the number of references listed in SciFinder for each species, as shown in Figure 28.

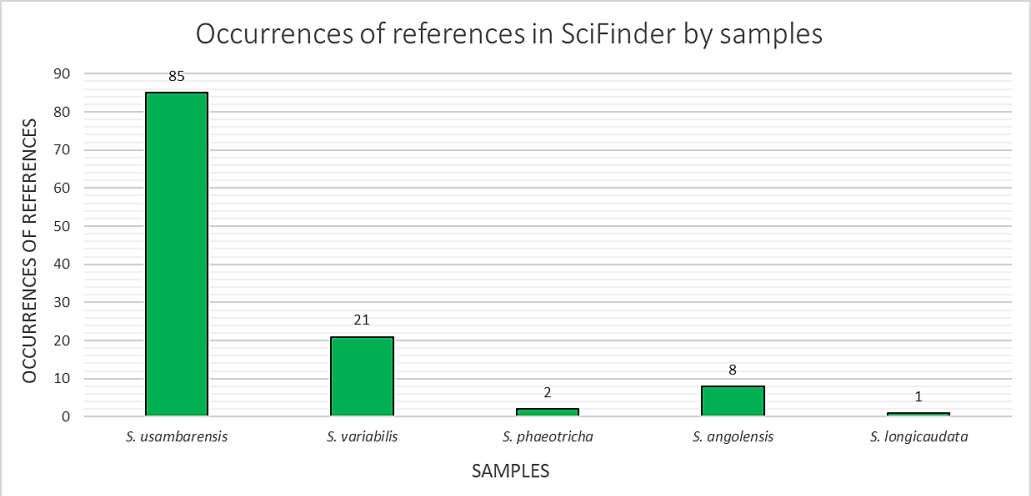

Figure 28: Occurrence graph of references provided by Scifinder according to five *Strychnos* species.

S. usambarensis is the most referenced species in the literature, while *S. phaeotricha*, *S. angolensis*, and *S. longicaudata* are the least referenced, and therefore, the least studied.

4.3. Selected species for further studies

Through the five criteria, four species caught our attention: *S. variabilis* root barks (sample 6), *S. phaeotricha* leaves (sample 12), *S. angolensis* root barks (sample 17), and *S. longicaudata* trunk barks (sample 23).

Despite the large number of references and its moderate antiplasmodial activity against the 3D7 strain (CQS strain) of *Plasmodium falciparum*, the alkaloidic extract of *S. usambarensis* leaves was selected for purification, as it revealed metabolites with high masses (above *m/z* 900) in its MS and MS/MS spectra and molecular network. Although *S. usambarensis* has been extensively studied, such metabolites have never been detected or studied before, likely due to the limited sensitivity of previous methods in detecting minor metabolites. Therefore, this extract was selected in an attempt to identify these metabolites.

In conclusion, five species are prioritized for the rest of the project. Unfortunately, due to time constraints, the study of *S. variabilis* and *S. angolensis* root barks will not be discussed further in this thesis. Chapters 6, 7, and 8 are each devoted to the study of a single species.

4.4. References

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CHAPTER 5

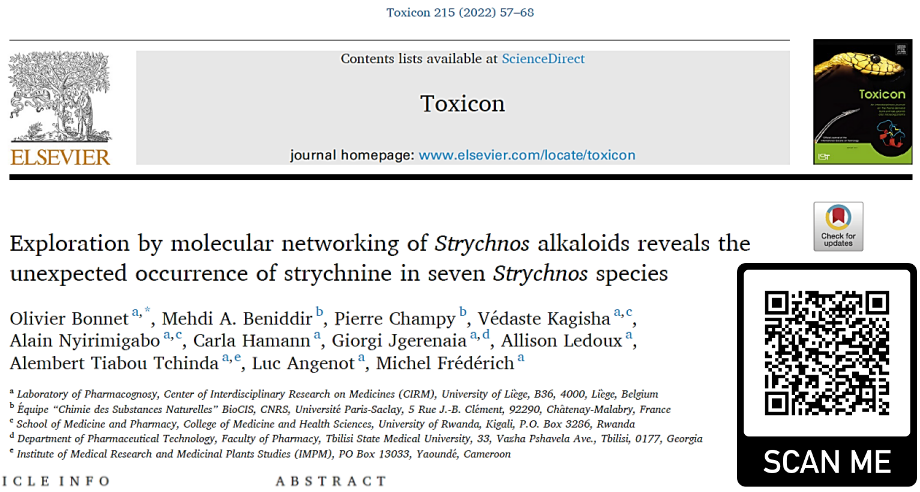
Study of strychnine: An innovative proof of concept for using molecular networking to discover natural compounds

5.1. Forewords

Samples for purification have been selected. To isolate new compounds, mass-guided fractionation will be performed using the molecular networking method. However, before proceeding, it is essential to evaluate this workflow by studying a known compound within the *Strychnos* genus. The choice was made to focus on a well-known molecule, strychnine, which was first isolated from *Strychnos nux-vomica* L. Notably, the molecular network annotations of the forty-four crude extracts revealed the unexpected presence of strychnine in seven species: *S. tricalysioides* Hutch. & M.B.Moss, *S. camptoneura* Gilg & Busse, *S. congolana* Gilg, *S. boonei* De Wild., *S. densiflora* Baill., *S. tchibangensis* Pellegr., and *S. usambarensis* Gilg ex Engl.

This chapter, based on the article "Exploration by molecular networking of *Strychnos* alkaloids reveals the unexpected occurrence of strychnine in seven *Strychnos* species," published in *Toxicon* (2022, 215, 57-68), details the methodology and results of the study of strychnine (Bonnet, O. *et al.*, 2022). The article has been slightly modified for continuity within the thesis and to improve the chapter's readability. Additionally, a few minor typographical errors have been corrected.

5.2. Article: "Exploration by molecular networking of *Strychnos* alkaloids reveals the unexpected occurrence of strychnine in seven *Strychnos* species"



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Exploration by molecular networking of *Strychnos* alkaloids reveals the unexpected occurrence of strychnine in seven *Strychnos* species

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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. 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.

Figure 29: Screenshot of our article about the study of strychnine, published in *Toxicon* journal (Bonnet, O. *et al.*, 2022).

I. 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 species), 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, fever, ulcers, and many others) and pharmacological properties, particularly the promising *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 (Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2003a; Frédérich, M. *et al.*, 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*) and ordeals (for instance, the roots of *Strychnos icaja*). During these ordeals, the person consumed a preparation made from the plant, and their survival was seen as proof of their 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 (Figure 30) 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 unambiguously identified in five other species of *Strychnos*: *S. ignatii*, *S. wallichiana* (Asia), *S. lucida* (Australia), *S. icaja* (Africa), and *S. panamensis* (Central and South America). Strychnine could be present in seeds of *S. tabascana* according to color reactions, but this method of identification is

poorly specific, and the investigation should be realized again using modern methods. (Quetin-Leclercq, J. *et al.*, 1990; Philippe, G. *et al.*, 2004). Strychnine acts by inhibiting the postsynaptic receptor of glycine, an inhibitory neurotransmitter 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, particularly 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).

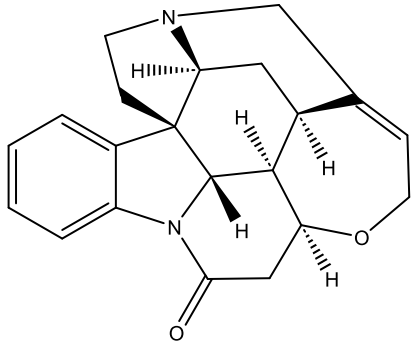

Figure 30: Structure of strychnine ($C_{21}H_{22}N_2O_2$; Molecular weight: 334.2 g/mol).

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 taxonomy. 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

circumscribed most of the strychnine-producing species within one clade (Setubal, R. B. *et al.*, 2021). For the purpose of clarifying these relationships, molecular networking was utilized herein for the streamlined 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; Fox Ramos, A. E. *et al.*, 2019b). Previous studies directed toward the reinvestigation of already investigated *Gentianales* plants taking advantage of the recently implemented monoterpene indole alkaloids database (MIADB) (Fox Ramos, A. E. *et al.*, 2019a; Fox Ramos, A. E. *et al.*, 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; Kouamé, 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.

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.

II. MATERIALS AND METHODS

II.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 Leeuwenb., *S. densiflora* Baill., *S. tchibangensis* Pellegr., and *S. johnsonii* Hutch. & M.B.Moss.) came from the collection of the Laboratory of Pharmacognosy of the University of Liège (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 previously in Chapter 3, section 3.2 (see pages 183-197). 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, sulfuric acid 96 %, 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 Liège, it was a milli-Q reference A+ system® from Merck (Darmstadt, Germany), and at Université Paris-Saclay, it was MILLIPORE Synergy UV® from Merck (Darmstadt, Germany). Deuterated methanol (CD_3OD, d_4 + 0.03% TMS) and trifluoroacetic acid used for NMR analyses came from Euriso-top (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 20x20 cm, presented a particle size of 5-40 µm. The strychnine reference was obtained from Serva (Heidelberg, Germany).

II.2. Sample preparation

Forty-three samples (see Chapter 3, section 3.2, pages 183-197) were first ground using a crusher IKA A10 (Staufen, Germany) to obtain 10 g of powder. Extractions were performed in methanol with the SpeedExtractor E-914® (Büchi, 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 bar, and the temperature was at 30°C. Every cycle included 1 minute of heat-up time, 15 minutes of hold time, and 2 minutes of discharge time. Then, the system was washed with solvent for 2 minutes and with nitrogen for 3 minutes. Crude extracts were then evaporated using Rotavapor® and Multivapor® (Büchi), and were dried in the vacuum oven (Heraeus, Hanau, Germany) for one night at room temperature.

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 *Strychnos usambarensis* (November 2007). From 2 g of powder, 20 mL of ammonia solution at 10% w/w was added. A maceration of 30 minutes was carried out. 100 mL of dichloromethane was added, and the whole was mixed under magnetic stirring for 1 hour. The solution was filtered through glass wool, which was rinsed twice with 50 mL dichloromethane. This organic phase was placed in a separating funnel with 20 mL of sulfuric acid at 2% w/V. After extraction, the aqueous phase was separated, and a second extraction was carried out with another 20 mL of sulfuric acid at 2% w/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.

II.3. HPLC-ESI(+)-Q/TOF

The forty-four extracts (forty-three methanolic extracts and one alkaloidic extract) were dissolved and ultrasonicated in methanol of HPLC grade (high-performance liquid chromatography) to obtain a concentration of 1 mg/mL. The solutions were then transferred to 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 1,260 Infinity HPLC coupled to an Agilent 6,530 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 x 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 minutes (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 minutes. The scans took place in six events: one MS scan in positive mode (between *m/z* 100 and 1,200), and then MS/MS scans were achieved for the five 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 3,000 counts. Purine $C_5H_4N_4$ $[M+H]^+$ ion (*m/z* 121.050873) and hexakis (1*H*, 1*H*, 3*H*-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 calibrants, a permanent MS/MS exclusion criterion that contains the *m/z* values of the two internal calibrants was set.

II.4. Feature-based molecular networking (Nothias, L. F. *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 minimal 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 minute and 0.03 Da, respectively, the S/N threshold was 1, the S/N estimator was intensity window SN, the minimal feature height was 3,000, the coefficient/area threshold was 2, the peak duration range was 0.02-1.5 minutes, and the retention time wavelet range was 0.02 and 0.2 minutes);
- Suppression of isotopes (settings: the *m/z* and retention time tolerances were *m/z* 0.005, 15.0 ppm, and 0.5 minute, 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 minute, 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 alkaloids, indeed even trace amounts;
- The gap-filled peak list was further filtered by deleting specific retention time windows (between 0-2.50 minutes and 45.59-49.83 minutes) 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 ".CSV" (Comma-separated values) formats 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.

II.5. TLC

The extracts of ten *Strychnos* species (Table 17 in the "Results and Discussion" section, see page 268) 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 an 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.

II.6. NMR

For the NMR analyses, the method applied was similar to that formerly reported (Frédérich, M. *et al.*, 2003a). 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 to the extracts aimed to visualize the signals produced by strychnine and to compare them with those of samples without added strychnine. ^1H and HMBC spectra were carried out with 256 and 32 scans, respectively.

II.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 compartment (TCC), and G7117B for DAD. A Luna® PFP from Phenomenex (250 x 4.6 mm, 5 µm) (Utrecht, Netherlands) was used as an analytical column with a flow rate of 1 mL/min. The gradient varied from 5% B to 25.4% B between 2 and 3 minutes, from 25.4% to 83.2% B between 3 and 37 minutes, and from 83.2% B to 100% B between 37 and 38 minutes. After maintaining 100% B for 1 minute, 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.

II.8. Determination of the limit of detection (LOD) of strychnine by HPLC-UV

The following protocol is similar to that outlined in version 10 of the European Pharmacopoeia (European Pharmacopoeia, 2019).

Strychnine was injected at different concentrations into the same HPLC system and analyzed with the same method described in the previous section. Twenty methanolic reference solutions of strychnine, prepared through successive twofold dilutions and with concentrations ranging from 1 mg/mL to 0.0019 µg/mL, were subsequently 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) for strychnine is defined as the concentration at which the signal-to-noise (S/N) ratio is equal to or close to 3.

II.9. Quantitative analysis of strychnine by NMR

The method applied was the same as that described in the article of Frédérich, M. *et al.* (2003a). 10 mg of the methanolic crude extract of *S. densiflora* trunk barks was weighed. 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.

II.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 6,546 (Agilent Technologies, Massy, France) equipped with an ESI source, operating in positive ion mode. An analytical column Acquity BEH® C_{18} from Waters (100 x 2.1 mm, 1.7 µm) was used. Another method was applied for better visualization of strychnine. The gradient was linear and varied from 5% B to 100% B in 12 minutes at 500 µL/min. After maintaining 100% B at 600 µL/min for 4 minutes, the gradient returned to the initial stage (A= Water + 0.1% formic acid; B=Acetonitrile). The injection volume for the extracts from the ten *Strychnos* species and the 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 3,500 V, sheath gas flow rate at 11 L/min. The divert valve was set to waste for the first 3 minutes. MS scans were operated in full scan mode from *m/z* 100 to 1,200 (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 3,000 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)-phosphazene $C_{18}H_{18}F_{24}N_3O_6P_3$ $[M+H]^+$ ion (*m/z* 922.009798) were used as internal lock masses. A permanent MS/MS exclusion criterion was set to prevent oversampling of the internal calibrants. The results obtained were interpreted with MassHunter (Version B.07.00), a mass spectrometry application software from Agilent.

III. RESULTS AND DISCUSSION

III.1. Extractions yields

The weights and yields of the forty-three methanolic extracts and one alkaloidic extract obtained from the twenty-eight different species are presented in the table 16 in Chapter 3 (see section 3.3, pages 197-203). The yields ranged from 0.55% w/w to 25.92% w/w.

III.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 (Figure 31) contains 8,090 nodes, with 217 annotations, among which 63 are annotated alkaloids, particularly identified using 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=da555ca03b4048259b2199b4026775de).

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, $C_{21}H_{23}N_2O_2$; Retention time: 11.63 minutes), for instance, was detected in ten *Strychnos* species with a cosine score ranging between 0.71 and 0.89 (Figure 31 and Table 17).

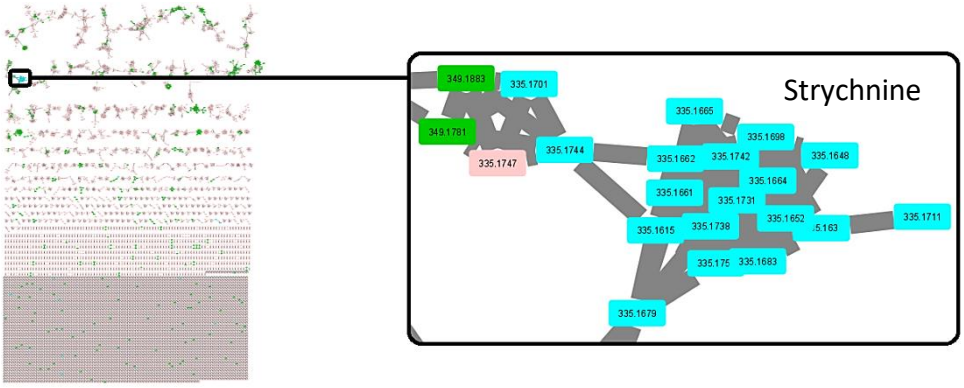

Figure 31: Full molecular network from the forty-four extracts of *Strychnos* (forty-three methanolic extracts and one alkaloidic extract) annotated by databases in the GNPS platform. The green nodes correspond to the annotated metabolites. The nodes corresponding to strychnine were highlighted by the blue color. The red color corresponds to unknown metabolites.

Table 17: Studied *Strychnos* species containing 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)

The three species, *S. icaja*, *S. nux-vomica*, and *S. ignatii*, well-known for their strychnine content, are among 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*.

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

III.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 Figure 32 below do not contain a spot identifiable as 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 using a more accurate analytical method such as HPLC or NMR.

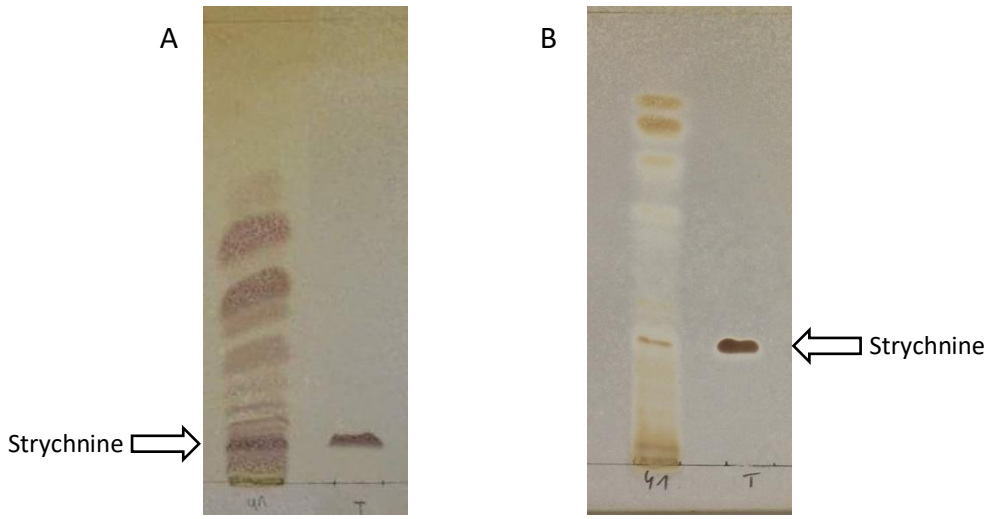

Figure 32: Research of strychnine (T) by TLC in the methanolic extract of *S. densiflora* trunk barks (41).

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).

III.4. NMR

According to the article of Frédérich *et al.* (2003a), the H-12 proton of strychnine resonates in an uncrowded region of the proton spectrum as a doublet at δ=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 (Figures 33 and 34).

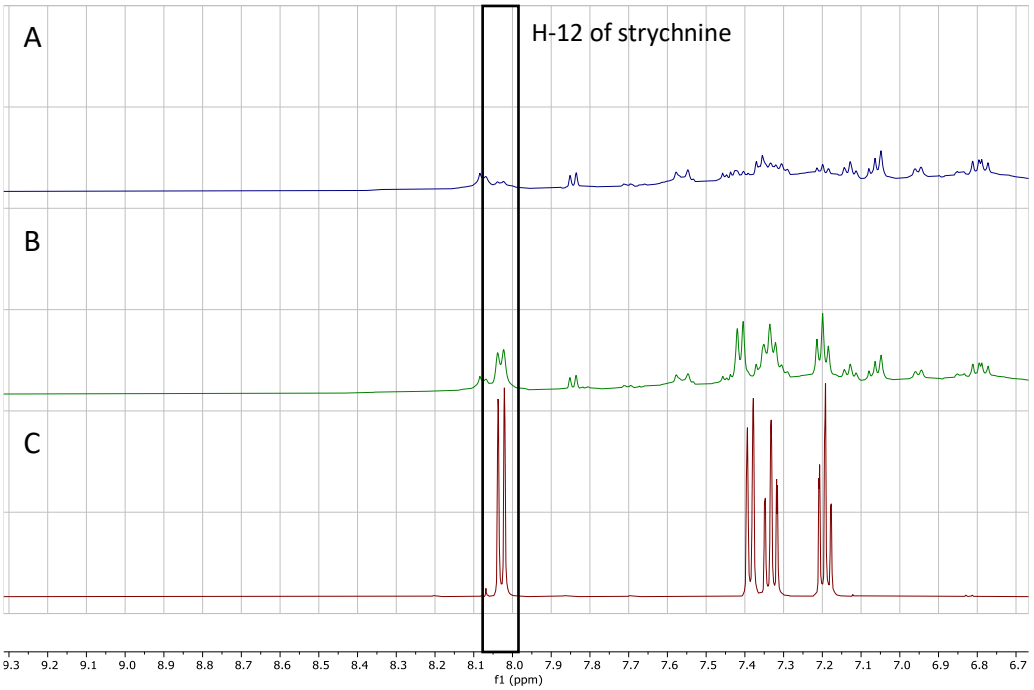

Figure 33: Presence of a doublet signal (J=7.896 Hz) in the region of 8.0 ppm coming from the H-12 proton.

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 the strychnine reference.

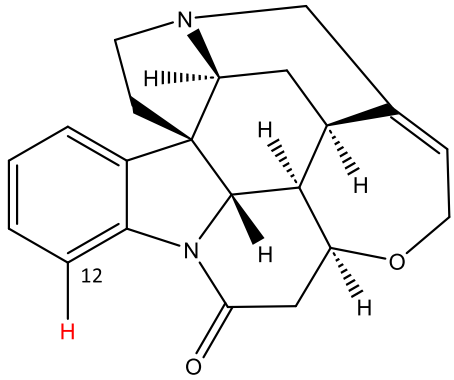

Figure 34: Position of the H-12 proton on strychnine.

III.5. HPLC-UV

Only the methanolic extract of *S. densiflora* trunk barks showed a peak that has the same UV spectrum as strychnine and retention time (Figures 35 and 36). 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 minutes confirms the presence of strychnine and supports the results described above.

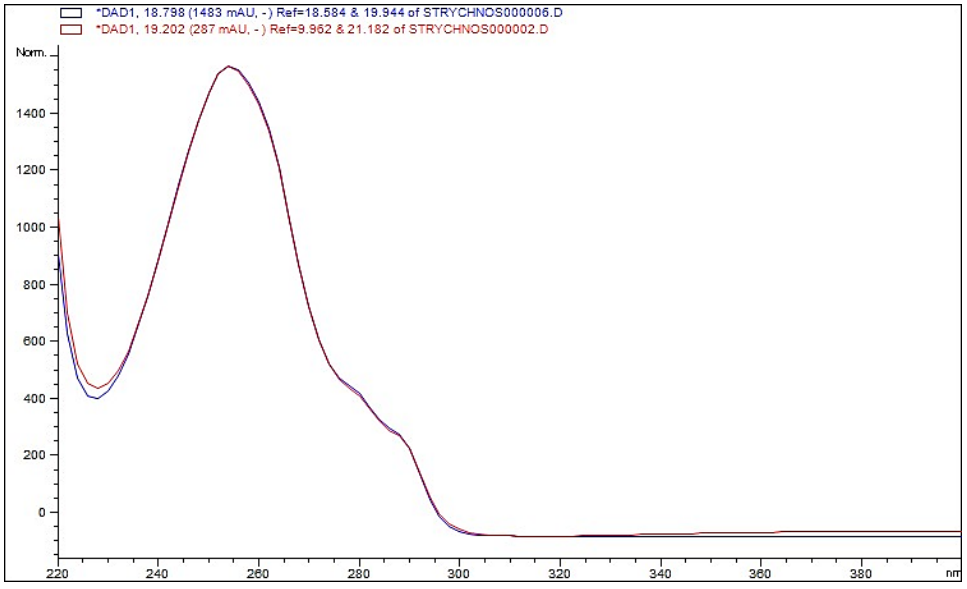

Figure 35: Superposition of the UV spectra of the strychnine reference and the peak at 19.20 min in the methanolic crude extract of *Strychnos densiflora* trunk barks.

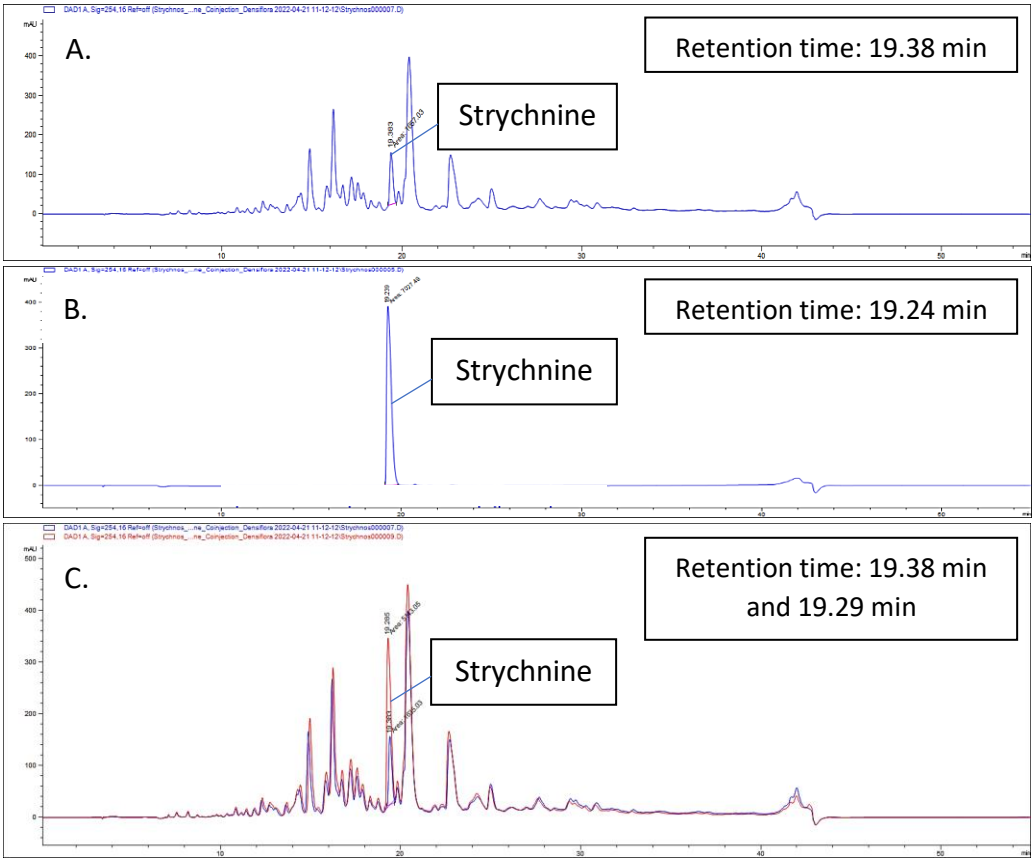

Figure 36: Chromatograms of the methanolic extract of *S. densiflora* trunk barks and the strychnine reference.

A. Chromatogram of the methanolic extract of *S. densiflora* trunk barks (λ=254.16 nm).

B. Chromatogram of the strychnine reference (λ=254.16 nm).

C. Comparison of the chromatogram A (in blue) with the chromatogram of the methanolic extract of *S. densiflora* trunk barks, co-eluted with the strychnine reference (in red) (λ=254.16 nm).

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.

$$\frac{S}{N} = \frac{2H}{h} = \frac{2 * 0.07233}{0.05363} = \frac{0.14276}{0.05356} = 2.7$$

The LOD of strychnine using our HPLC-UV method was 0.03 µg/mL in the injected extract solutions, corresponding to 0.0003% (w/w) in the plant powders. Based on this result, we concluded that, except for the methanolic extract of *S. densiflora* trunk barks, which exhibited a strychnine peak indicating a higher concentration of strychnine, the strychnine content in the six other extracts was below 0.03 µg/mL. This explains the negative results obtained with TLC, NMR, and HPLC analyses.

III.6. 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; Frédérich, M. *et al.*, 2003a). 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 objectives of these analyses were twofold: to evaluate the strychnine content in the trunk barks of *S. densiflora* in comparison with that of well-known poisonous species, and to compare our experimental values of strychnine content with those reported in the literature (Figure 37 and Table 18).

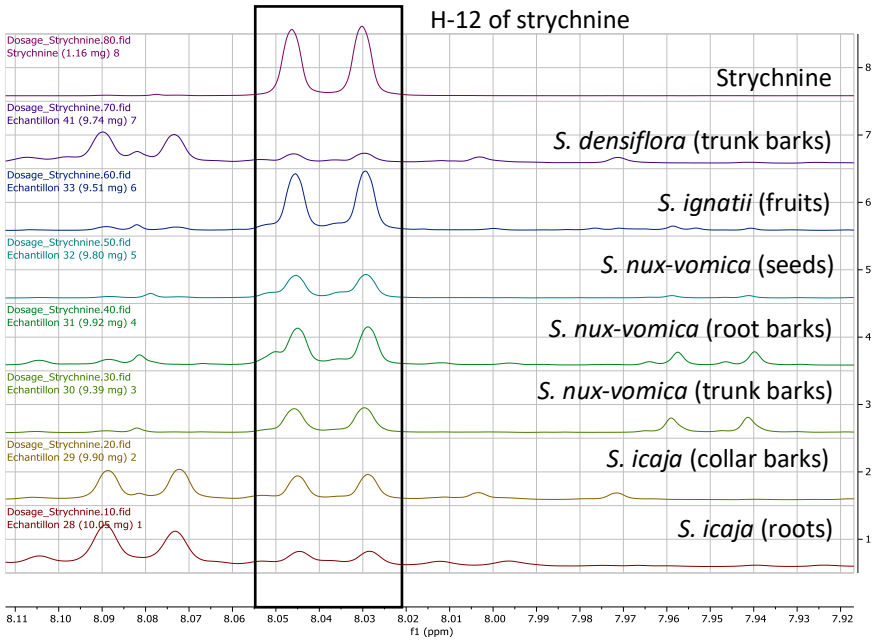

Figure 37: Comparison of doublets signals at 8.02-8.06 ppm generated by H-12 proton of strychnine.

Table 18: 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)	0.47%
Trunk barks of S. *nux-vomica* (MeOH extract)	0.40%
Root barks of S. *nux-vomica* (MeOH extract)	0.32%
Seeds of S. *nux-vomica* (MeOH extract)	0.33%
Seeds of *S. ignatii* (MeOH extract)	0.79%
Trunk barks of *S. densiflora* (MeOH extract)	0.07%

The values obtained, presented in Table 18, 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., and 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 attributed to the species' origin (environmental conditions) or the extraction method, as observed with *S. usambarensis* leaves, where differences were noted between the methanolic crude extract and the alkaloidic extract.

III.7. 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 Figure 38, the peak of strychnine $[M+H]^+$ was observed between 2.53 and 2.59 minutes in all analyzed extracts of *Strychnos* species and plant parts.

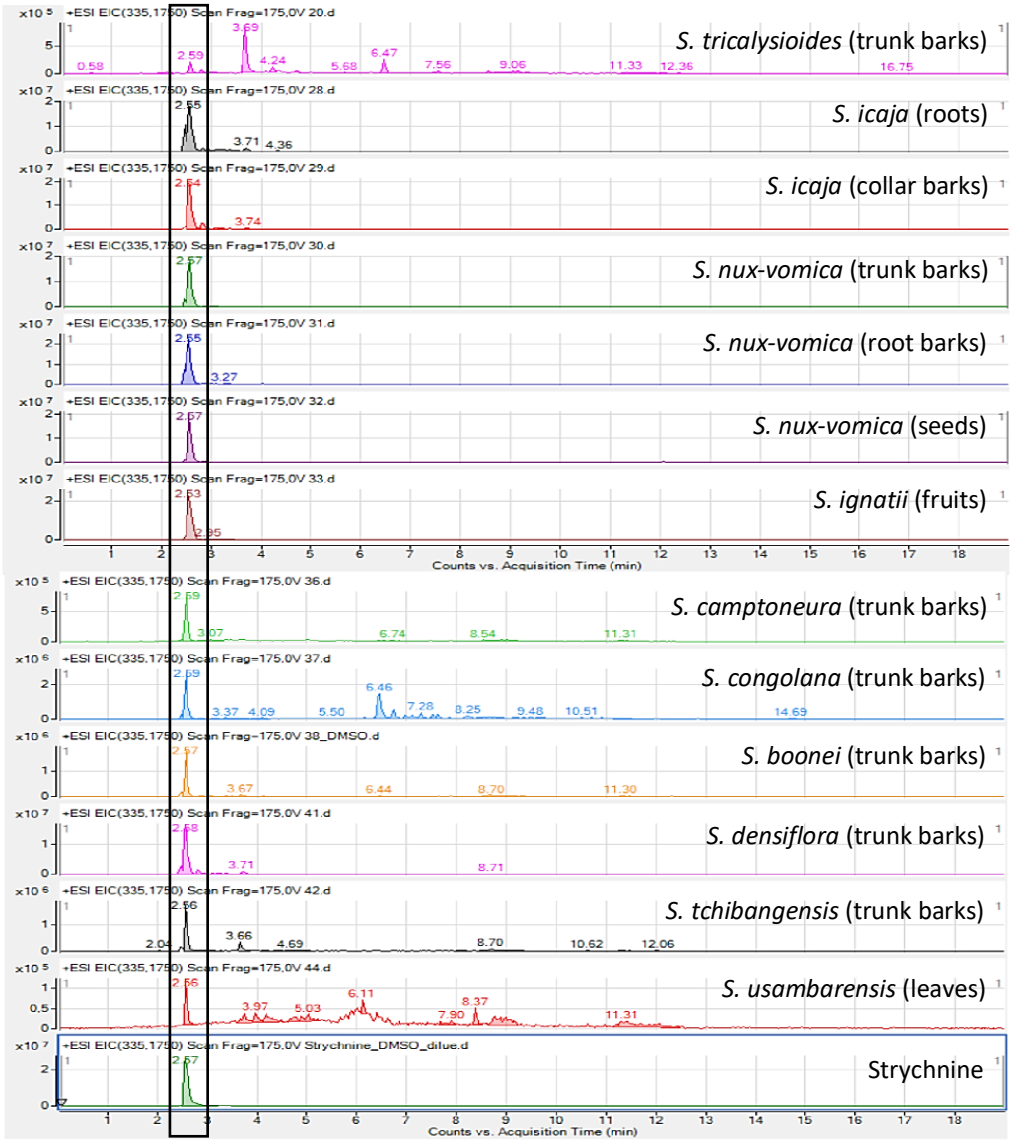

Figure 38: Observation of the strychnine peak $[M+H]^+$ (retention time between 2.53 and 2.59 minutes) in the extracted ion chromatograms (EIC) of all the extracts studied. The extracted mass is *m/z* 335.1750 (± *m/z* 0.1000). The last chromatogram corresponds to the strychnine reference.

The MS/MS spectra of the extracts and the strychnine reference were compared manually, with a specific focus on key diagnostic product ions. The key 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, J.-X. *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 extract and the strychnine reference. The intensity is barely 10^2 in the MS/MS spectrum from the methanolic crude extract of *S. tricalysioides* trunk barks, while it is at 10^5 for the strychnine reference, which corresponds to a 1000-fold difference of intensity (Figure 39). 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 (Figures from S3 to S15 in the article's additional information)

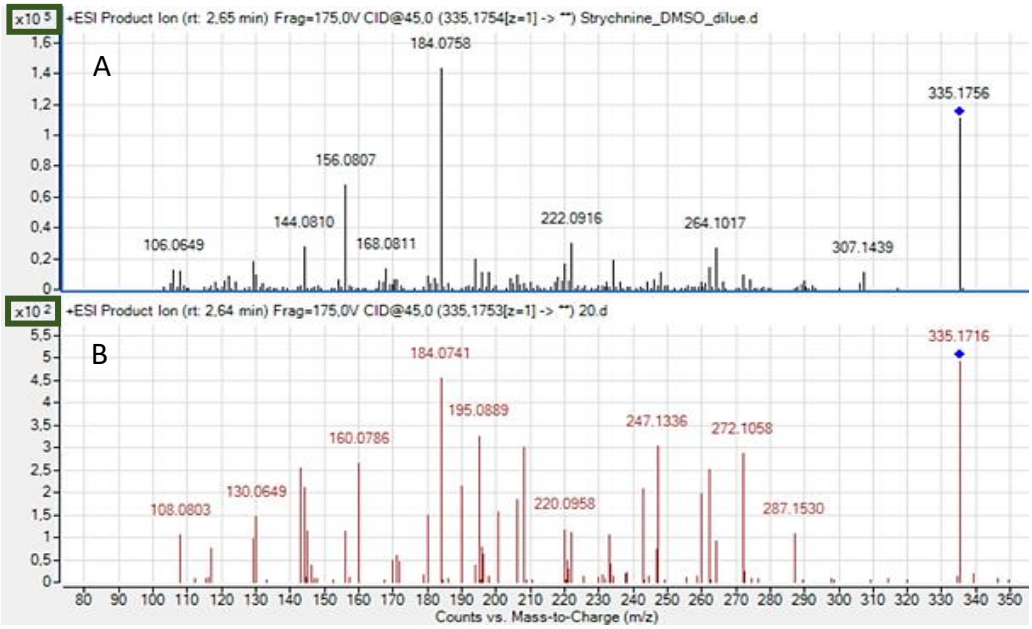

Figure 39: Comparison of MS/MS spectra between the strychnine reference and the methanolic crude extract of *Strychnos tricalysioides* trunk barks.

A. MS/MS spectrum from the strychnine reference.
B. MS/MS spectrum from the methanolic crude extract of *S. tricalysioides* trunk barks (Intensity difference compared to MS/MS spectrum A: x1000).

Table 19, 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.

Table 19: Summary of the results from the strychnine study.

***Strychnos* species**	**Molecular networking**	**TLC**	**HPLC-DAD**	**NMR**	**UPLC-MS/MS**
S. tricalysioides (Trunk barks)	X				(X)
S. icaja (Roots and collar barks)	X	X	X	X	X
S. nux-vomica (Trunk barks, root barks, and seeds)	X	X	X	X	X
S. ignatii (Seeds)	X	X	X	X	X
S. camptoneura (Trunk barks)	X				X
S. congolana (Trunk barks)	X				X
S. boonei (Trunk barks)	X				X
S. densiflora (Trunk barks)	X	X	X	X	X
S. tchibangensis (Trunk barks)	X				X
S. usambarensis (Leaves)	X				X

Strychnine could also be present in trace amounts in other species of *Strychnos*. However, the targeted peaks being lower in intensity, 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 method, which provides a chemotaxonomic approach based on alkaloids from *Strychnos* species, does not fully align with the classifications proposed by Leeuwenberg and Setubal. For example, the molecular network of *Strychnos* alkaloids, with annotations based on the clades from the Setubal study, contains clusters that mix different sections that are not related in the current classifications (Figure 40). To establish a more reliable classification, further studies would be needed.

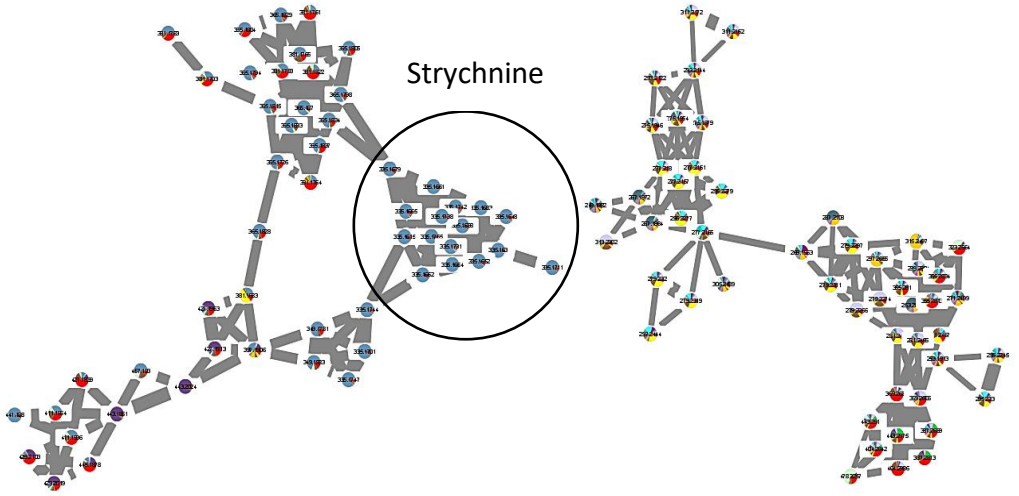

Figure 40: Molecular network highlighting the classification of twenty-eight *Strychnos* species based on the phylogenetic study. In each node, there is a chart in which each colour represents a phylogenetic clade from Setubal *et al.* (2021) study. The proportionality of colours depends on the quantity of the studied metabolite within the clade. A variety of colours and, therefore, clades can be observed in some clusters (groups of metabolites that have common points).

IV. CONCLUSIONS

The development of metabolomic methods, 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 (Metabolomics 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 using 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.

This study also contributes to advancing the chemotaxonomic understanding of the species belonging to the *Strychnos* genus. The discovery of strychnine in several 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 to 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 also mentioned in ethnopharmacological literature as being used 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.

V. SUPPLEMENTARY MATERIAL

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2022.06.002. They can also be accessed via the QR code below:

5.3. References

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CHAPTER 6

Isolation of minor metabolites using mass-guided fractionation: Investigating the leaves of *Strychnos usambarensis*

6.1. Forewords

Chapters 6, 7, and 8 focus on the purification of novel metabolites with antiplasmodial activity. This work follows both the selection of species for purification and the demonstration of the molecular networking method's effectiveness in exploring and identifying metabolites.

This chapter focuses on the leaves of *Strychnos usambarensis* Gilg ex Engl. Chapter 7 presents our work on the trunk barks of *Strychnos longicaudata* Gilg, and we will conclude this section with preliminary studies on the leaves of *Strychnos phaeotricha* Gilg.

6.2. Article: "Isolation of original high molecular weight metabolites from the leaves of *Strychnos usambarensis* by mass-guided fractionation "

Exploration of the molecular network from the crude alkaloidic extract of *S. usambarensis* leaves revealed the presence of metabolites with *m/z* values above 900. Since such masses had not previously been described in this species, and given the significant antiplasmodial activity associated with dimeric alkaloids, mass-guided fractionation was employed to purify one of these compounds. The results presented below describe this study from detection to purification and structural elucidation testing. Unfortunately, due to the low quantities of the isolated compound, its final structure and its antiplasmodial activity could not be determined.

The results have been presented in the form of an article that will potentially be submitted in the future (Figure 41). As part of the thesis, minor modifications were made to the text of the article to maintain continuity with the other chapters.

Isolation of Original High Molecular Weight Metabolites from the Leaves of *Strychnos usambarensis* by Mass-Guided Fractionation

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Figure 41: Screenshot of the article on the study of *S. usambarensis* leaves.

ABSTRACT

Introduction: Numerous studies have focused on the toxic and therapeutic properties of plants from the *Strychnos* genus, belonging to the Loganiaceae family. Among these is *Strychnos usambarensis*, an African species extensively studied for its antiplasmodial properties. In fact, it represents a promising source of novel antimalarial compounds, essential for combating the rise in malaria cases and deaths due to the increasing resistance of malaria parasites to current antimalarial treatments.

Objectives: To isolate and identify novel high molecular weight compounds potentially active against malaria by exploring the metabolite content of various parts of *Strychnos usambarensis*.

Materials and methods: The metabolite contents of crude extracts from the leaves, stem and twig barks, fruits, and root barks of *Strychnos usambarensis* were explored using molecular networking. This methodology allowed for the detection of novel compounds with *m/z* values above 900 in the leaves. To isolate and identify one of these compounds, mass-guided fractionation was employed, followed by a series of purification methods.

Results: A compound with a mass of *m/z* 944.2848 was purified and analyzed by MS/MS and NMR to elucidate its structure. The data identified it as a novel dimeric or trimeric alkaloid with a glycosidic moiety. Unfortunately, its final structure could not be elucidated. Moreover, due to its low structural stability, rapid degradation occurred, preventing the determination of its antiplasmodial activity.

Conclusions and prospects: A novel molecule was isolated for the first time in the leaves of *Strychnos usambarensis*. Assessing its *in vitro* and *in vivo* activities against malaria requires a larger quantity, which could be obtained through faster and less labor-intensive methods, such as hemisynthesis or total synthesis.

I. INTRODUCTION

The Loganiaceae family comprises pantropical plants of the *Strychnos* genus, which are found across Africa, Central and South America, Asia, and Oceania. This genus includes approximately 200 species, with 75 species native to Africa, at least 73 to the Americas, and around 44 to Asia and Oceania. Depending on their habitat, these plants take the form of lianas, shrubs, or small trees (Bisset, N. G., 1970; Krukoff, B. A., 1972; Bisset, N. G., 1974; Setubal, R. B. *et al.*, 2021).

The *Strychnos* genus has been extensively studied for its tetanizing, curarizing, and therapeutic properties (Angenot, L., 1971a; Sandberg, F. *et al.*, 1971; Angenot, L. *et al.*, 1975; Geevaratne, M. *et al.*, 1977; Ohiri, F. C. *et al.*, 1983; Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2002; Philippe, G. *et al.*, 2004; Philippe, G. *et al.*, 2005; Oyedemi, S. O. *et al.*, 2012; Madikizela, B. *et al.*, 2017; Ampa, R. *et al.*, 2018; Oryema, C. *et al.*, 2021; Bonnet, O. *et al.*, 2022a; Bonnet, O. *et al.*, 2022b; Maroyi, A., 2022; Uttu, A. J. *et al.*, 2022). This study focuses specifically on the genus's antimalarial properties. Several *Strychnos* species, along with alkaloids isolated from them, have demonstrated promising activities against malaria in *in vitro* assays, with some alkaloids also showing efficacy in *in vivo* studies (Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2002; Philippe, G. *et al.*, 2005; Philippe, G. *et al.*, 2007; Lusakibanza, M. *et al.*, 2010; Fentahun, S. *et al.*, 2017). Among the active species is *Strychnos usambarensis*, an African plant whose root barks have traditionally been used as hunting

poison. Arrows coated with this poison paralyze prey (due to curarizing properties), preventing them from escaping (Bisset, N. G., and Leeuwenberg, A. J. M., 1968; Angenot, L., 1971a). Previous research has demonstrated promising antiplasmodial activities (IC_{50} values ≤ 5 µg/mL) in the barks, leaves, and roots of *S. usambarensis* against both chloroquine-sensitive (CQS) and chloroquine-resistant (CQR) strains of *Plasmodium falciparum*, one of the parasites responsible for malaria. The IC_{50} values vary depending on the plant part, extract type, and strain, with ranges as follows (Frédérich, M. *et al.*, 1999; Bonnet, O. *et al.*, 2022a):

- CQS: 0.287 to 23.5 µg/mL (FCA 20 Ghana), and 0.880 to superior to 50.0 µg/mL (3D7);
- CQR: 0.0390 to 0.970 µg/mL (W2 Indochina).

Numerous active alkaloids have been isolated from *S. usambarensis*, including usambarensine and derivatives (e.g., 5',6'-dihydrousambarensine, N_b-methylusambarensine), usambarine and derivatives (e.g., 18,19-dihydro-usambarine, 10-hydroxyusambarine), strychnofoline, isostrychnofoline, and strychnopentamine. Many of these alkaloids exhibit strong antiplasmodial activities (Angenot, L., and Bisset, N. G., 1971b; Koch, M., and Plat, M., 1971; Angenot, L., and Denoël, A., 1972; Koch, M. *et al.*, 1973; Angenot, L. *et al.*, 1978a; Angenot, L. *et al.*, 1978b; Caprasse, M. *et al.*, 1983; Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2002).

These findings underscore *S. usambarensis* as a valuable source of potential antimalarial compounds, offering hope in the fight against malaria, a significant global public health issue. A large number of cases and deaths are reported every year in the annual reports published by the World Health Organization (World Health Organization, 2023). Thanks to the commercialization of antimalarial treatments, the spread of malaria parasites by *Anopheles* mosquitoes was slowed for some years. However, resistance to these treatments is rising, rendering many of them less effective or even ineffective. As a result, malaria infections and fatalities are once again increasing (World Health Organization, 2023)

This study investigated *S. usambarensis* to address the resurgence of malaria by identifying novel natural compounds for the development of new antimalarial drugs.

The metabolite content of crude extracts from *S. usambarensis* leaves, stem and twig barks, root barks, and fruits was explored using mass spectrometry and molecular networking to highlight unknown compounds. Original metabolites with *m/z* values above 900 were initially detected in the crude alkaloidic extract of leaves. Such high molecular weights had never been previously reported in this species. Based on the literature, high *m/z* masses are likely to correspond to dimeric or trimeric compounds. Several bisindole compounds have also been reported to exhibit strong antiplasmodial activities (Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2001; Frédérich, M. *et al.*, 2002). Therefore, these unknown compounds could also be active against malaria. Moreover, since these metabolites were observed in an alkaloidic extract, they are presumed to belong to the alkaloid class.

To isolate these compounds, mass-guided fractionation was performed, employing a combination of purification methods, including preparative and analytical HPLC and open-column chromatography. Each purification step specifically targeted these high molecular weight metabolites, with monitoring conducted using mass spectrometry and molecular networking analyses (Yang, J. Y. *et al.*, 2013; Wang, M. *et al.*, 2016; Nothias, L.-F. *et al.*, 2018; Fox Ramos, A. E. *et al.*, 2019).

II. MATERIALS AND METHODS

II.1. Materials, chemicals, and reagents

The samples of leaves (November 2007 and August 2008), stem and twig barks (August 1948), root barks (October 1988), and fruits (November 2007) of *S. usambarensis* Gilg ex Engl. came from the collection of the Laboratory of Pharmacognosy of the University of Liège (Belgium). The leaves and fruits were collected in Rwanda (in Akagera National Park) while the stem, twig, and root barks are from Congo (in the Forest gallery in Kasenga for the stem and twig barks, and in the locality of Luki for the root barks). Reference specimens are

preserved in the Herbarium of the Pharmaceutical Institute in Liège for leaves and fruits (Ulg20070608 and Ulg20080816), as well as in the Herbarium of Botanical Garden of Belgium at Meise for the stem, twig, and root barks (Delaude HB3377 and Duvigneaud H1397).

Methanol and dichloromethane were purchased from VWR Chemicals BDH (Leuven, Belgium). Methanol of HPLC grade, sulfuric acid 96 %, and ammonia 25% were from Merck (Darmstadt, Germany). Formic acid, used in mobile phases, was purchased from Biosolve BV®. The solvents of UHPLC-MS grade (methanol, acetonitrile, and formic acid) came from Sigma-Aldrich (Machelen, Belgium). The Milli-Q water was supplied using two systems: a Milli-Q reference A+ system® from Merck (Darmstadt, Germany) at the University of Liège, and a MILLIPORE Synergy UV® from Merck (Darmstadt, Germany) at Université Paris-Saclay during (U)HPLC-ESI(+)-Q/TOF analyses. Concerning NMR analyses, deuterated methanol (CD_3OD, d_4 + 0.03% TMS) was purchased from Euriso-top (Saint-Aubin, France).

II.2. Sample grinding and extraction

To grind the five dried samples and obtain 10 g of powder, an IKA A10® crusher (Staufen, Germany) was used. For the rest of the study, the entire sample of leaves collected in November 2007 was ground using a Retsch ZM100 grinder (Aartselaar, Belgium). These five powders were mixed with sand at a 1:5 ratio, respectively, and then extracted with methanol using the SpeedExtractor E-914® (Büchi, Hendrik-Ido-Ambacht, Netherlands). With its four metal cells, this unit can perform four extractions simultaneously, optimizing extraction yields by pressurizing solvents while reducing solvent consumption.

During the extraction process, six cycles were applied, each divided into three stages: heat-up, hold, and discharge. The first stage involved heating the cells (heat-up). The subsequent steps consisted of filling the cells with solvent and holding it there (hold), followed by discharging the liquid extracts into the collection bottles (discharge). For each step, specific durations were defined: 1 minute for heat-up, 10 minutes for hold, and 8 minutes for discharge. Once the cycles were completed, the SpeedExtractor E-914® was rinsed with the extraction solvent for 2 minutes and dried by passing nitrogen through it for

3 minutes. The set pressure was 150 bar, and the temperature was maintained at 30°C. The five crude extracts obtained were evaporated using a Rotavapor® and Multivapor® (Büchi) and dried overnight at room temperature in a vacuum oven (Heraeus, Hanau, Germany, and Memmert, Brussels, Belgium). The next day, the dried extracts were weighed to determine their yields.

In addition to these extractions, an alkaloidic extract of *S. usambarensis* leaves (collected in November 2007) was prepared to compare the metabolite content between two different types of extract from the same sample. The methodology, previously described in another publication (Bonnet, O. *et al.*, 2022b), is as follows: 20 mL of a 10% w/w ammonia solution was added to 2 g of powdered leaves. After 30 minutes of maceration, 100 mL of dichloromethane was added, and the mixture was stirred magnetically for 1 hour. The solution was then filtered through glass wool, which was rinsed twice with 50 mL of dichloromethane. In a separating funnel, the organic phase was brought into contact with 20 mL of 2% w/v sulfuric acid. After separating the aqueous and organic phases, a second extraction was performed with another 20 mL of 2% w/v sulfuric acid. The two aqueous phases were combined, and the pH of the aqueous solution was adjusted to 8–10 using 10% w/w ammonia. The basified aqueous phase was then subjected to two additional extractions with 50 mL of dichloromethane in a separating funnel. At this stage, the organic phase was recovered. The final traces of water were removed from the organic phase using anhydrous sodium sulfate (Merck, Darmstadt, Germany). Finally, the organic extract was evaporated under reduced pressure using a Rotavapor® (Büchi) and dried overnight at room temperature in a vacuum oven (Heraeus, Hanau, Germany, and Memmert, Brussels, Belgium).

II.3. HPLC-ESI(+)-Q/TOF

The six crude extracts were analyzed by HPLC-ESI(+)-Q/TOF at Université Paris-Saclay to generate MS/MS mass spectra, a crucial step for developing molecular networks. These networks enable the exploration of the metabolite content of the extracts and facilitate the rapid detection of unidentified metabolites. The spectral databases available on the GNPS platform are used to suggest potential identifications within the molecular networks, facilitating

the rapid detection of unknown compounds (Wang, M. *et al.*, 2016). The methodology for performing mass spectrometry analyses, processing the data, and generating the molecular networks employed in this study is also described in one of our publications released in 2022 (Bonnet, O. *et al.*, 2022b).

Extracts were weighed, dissolved in methanol, filtered through a 0.2 µm pore size filter, and transferred to HPLC vials for injection. The system used to analyze the extracts was a HPLC-ESI(+)-Q/TOF from Agilent (Agilent Technologies, Massy, France). It was composed of an Agilent 1,260 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 used was a SunFire C_{18} (150 × 2.1 mm, 3.5 µm) from Waters. The flow rate applied was 250 µL/min, with a linear gradient varying from 5% B to 100% B over 30 minutes (A = Water + 0.1% formic acid; B = Methanol). The wavelengths applied for the DAD detector were 210, 254, and 280 nm. About the ESI conditions, the capillary temperature, the source voltage, and the sheath gas flow rate were set at 320 °C, 3.5 kV, and 10 L/min, respectively. The volume injection for each sample was 5 µL. The mass spectrometer was operated in Extended Dynamic Range mode (2 GHz). All peaks detected during the first 3 min were excluded using the divert valve. Each scan contains six results: one MS scan in positive mode (in the range of *m/z* 100 and 1,200), and MS/MS scans for the five most intense peaks in the MS scan.

The settings applied to acquire MS/MS spectra were 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 3,000 counts. Purine $C_5H_4N_4$ $[M+H]^+$ ion (*m/z* 121.050873) and hexakis (1*H*, 1*H*, 3*H*-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. With the purpose to prevent oversampling with the internal calibrants, a permanent MS/MS exclusion criterion was applied to exclude all signals coming from the two internal calibrants. The MassHunter software (Version B.07.00), a mass spectrometry application software from Agilent, was used to display MS and MS/MS spectra acquired.

II.4. Feature-based molecular networking

The article of Nothias, L.-F. *et al.* (2020) reports the methodology to generate a feature-based molecular network of crude extracts or fractions. Before data processing begins, the files generated by the mass spectrometer are converted to ".mzXML" extension files (eXtensible Markup Language) using MSConvert software edited by ProteoWizard (Chambers, M. C. *et al.*, 2012). The filter and the algorithm used were peak picking and vendor, respectively. The .mzXML files were then imported into MZmine 2 (version 2.53) (an older version of the current MZmine 3) (Pluskal, T. *et al.*, 2010). Noise removal, peak list creation, deconvolution (i.e., removal of unwanted peaks), isotope grouping, and filtration are the necessary steps to properly process the data and generate the molecular network. The following parameters were applied:

- Noise removal (method: mass detection): The intensities applied for the suppression of noises were 5.8E3 and 5.0E1 for MS^1 and MS^2, respectively;

- Peak list creation (method: ADAP chromatogram builder): A minimum of four points was required to build a peak. The intensity threshold was set at 5.8E3, and the *m/z* tolerance was *m/z* 0.02 and 10.0 ppm;

- Deconvolution (method: Chromatogram deconvolution):
    - The algorithm was wavelets (ADAP) (Myers, O. D. *et al.*, 2017);
    - The *m/z* center calculation was set to 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 3,000;
    - The coefficient/area threshold was 2;
    - The peak duration range was 0.02-1.5 min;
    - The retention time wavelet range was 0.02 and 0.2 min;

- Isotope grouping (method: Isotopic peaks grouper): *m/z* 0.005, 15.0 ppm, and 0.5 min were, respectively, the *m/z* and retention time tolerances. The maximum charge was set at 1, and the representative isotope was the most intense peak;

- Filtration (method: Feature list rows filter): In the processed peak list, the peaks in the time range from 0 to 2.50 minutes and from 45.59 to 49.83 minutes were deleted. Moreover, only MS/MS spectra were retained for generating the molecular network.

Using the “Export to CSV file” and “Export/Submit to GNPS/FBMN” methods, “.mgf" (Mascot Generic Format) files, containing the essential data for building the molecular network and ".CSV" (Comma-separated values) files, reporting other information, such as retention times, were exported.

These two files were then uploaded to the GNPS (Global Natural Products Social Molecular Networking) online platform, which automatically generates the molecular network (Wang, M. *et al.*, 2016). The parameters applied on this platform are as follows: 0.02 Da for precursor and fragment ion mass tolerances, 0.65 for the minimal cosine score, 0.7 for the minimal cosine score for matching with the library, no filtration was applied, and the analogs were not searched.

II.5. Preparative HPLC-DAD

Metabolites with masses greater than *m/z* 900 were purified, particularly by preparative HPLC. The system used was a Varian ProStar preparative HPLC (Prep-HPLC) equipped with a diode array detector and a Büchi fraction collector C-660 unit. Compounds were separated using a Luna® PFP column (250 mm x 21.20 mm, 5 µm) purchased from Phenomenex (Utrecht, Netherlands). The DAD detector was set to 270 nm and 350 nm, with peaks primarily observed at 350 nm. The mobile phase consisted of water with 0.1% formic acid (pH around 2.60) (A) and methanol (B). A gradient was applied: from 5% B to 25.4% B between 2.12 and 3.19 minutes, from 25.4% B to 83.2% B between 3.19 and 39.29 minutes, and from 83.2% B to 100% B between 39.29 and 40.36 minutes. After maintaining 100% B for 1.06 minutes, the gradient was

returned to the initial conditions. The flow rate was set at 20 mL/min. Prior to injection, samples were dissolved in 90% A and 10% B, and filtered through a 0.45 µm pore size filter. Injections were carried out manually, with a maximum injection volume of 10 mL, corresponding to the maximum capacity of the injection loop.

II.6. Open columns

To optimize the separation of metabolites, a long and narrow column was selected. The stationary phase used was Sephadex™ LH-20, purchased from GE Healthcare and Cytiva, which performs better in such columns. This liquid chromatography medium, made from hydroxypropylated dextran beads, is a polysaccharide network that separates compounds based on molecular size. Larger molecules are less retained and elute faster from the column.

First, Sephadex™ LH-20 was weighed, with approximately 1 g of sample requiring 100 g of stationary phase. After weighing, the stationary phase was transformed into a gel by mixing it with bidistilled methanol to minimize contamination. Absorbent cotton was placed at the bottom of the empty column, and the column was washed with methanol. The gel was then carefully added to the column. Once the stationary phase was secured in place, excess solvent was drained by opening the tap. Before applying the sample for purification, a thin layer of sand (UCB, Leuven, Belgium) was added on top of the stationary phase to protect it from potential damage. Under a gentle flow of solvent, the sample migrated through the stationary phase, where the various compounds were separated by size exclusion. The fractions were collected in test tubes as they exited the column. The collected samples were evaporated using Rotavapor® and Multivapor® (Büchi), then dried at room temperature in a vacuum oven (Heraeus, Hanau, Germany, and Memmert, Bruxelles, Belgium) for two days. To monitor the targeted metabolites, the fractions were analyzed using UPLC-QDa (Waters, Antwerp, Belgium).

II.7. Purification by analytical HPLC-DAD

Due to insufficient purity, fractions were further purified using an Agilent analytical HPLC system (Machelen, Belgium). The purification involved collecting the peak(s) of interest directly at the DAD detector outlet into test tubes. The HPLC system components included the G1313A for the autosampler (ALS), G1311A for the quaternary pump, G1322A for the degasser, G1316A for the column compartment (TCC), and G7117B for the DAD detector. The analytical column used for metabolite separation was the Luna® PFP (250 mm x 4.6 mm, 5 µm), purchased from Phenomenex (Utrecht, Netherlands). The mobile phase consisted of Milli-Q water with 0.1% formic acid (A) and methanol (B). The gradient program was as follows: from 5% B to 25.4% B between 2 and 3 minutes, from 25.4% B to 83.2% B between 3 and 37 minutes, and from 83.2% B to 100% B between 37 and 38 minutes. After maintaining 100% B for 1 minute, the gradient was returned to its initial conditions. Fractions were dissolved in a 50:50 mixture of A and B to achieve an approximate concentration of 10 mg/mL. Following centrifugation at 2000 rpm for 5 minutes in Eppendorf tubes pre-cleaned with the same 50:50 solvent mixture, the supernatant was collected and transferred into HPLC vials. DAD detection wavelengths were set at 210 nm, 254 nm, 280 nm, and 300 nm. The flow rate was maintained at 1 mL/min, and the injection volume was 50 µL.

II.8. UPLC-QDa

The Acquity UPLC-QDa (Quadrupole) system, used to monitor the targeted compounds (*m/z* > 900), was purchased from Waters (Antwerp, Belgium). The system components included the Sample Manager FTN (SN M19FTP099G, Ver 1.71.395), Quaternary Solvent Manager (SN A20QSP242A, Ver 1.72.415), column compartment, QDa mass spectrometer (SN KBD5552, Ver V), and PDA detector (SN C20UPD080A, Ver 1.70.63.67). The column utilized for separation was a Kinetex F5, obtained from Phenomenex (Utrecht, Netherlands). The mobile phase consisted of Milli-Q water with 0.1% formic acid (A) and UPLC-MS/MS grade methanol (B). The gradient was as follows: 5% B to 25.4% B from 1.25 to 1.88 minutes, 25.4% B to 83.2% B from 1.88 to 23.13 minutes, and 83.2% B to 100% B from 23.13 to 23.76 minutes. After holding at 100% B

for 0.62 minutes, the gradient returned to the initial conditions. The flow rate was 0.2 mL/min, and detection wavelengths were set at 210, 254, 280, and 300 nm. The injection volume was 5 µL. The system was cleaned using 50:50 and 10:90 V/V mixtures of acetonitrile and water. After the analyses, the column was rinsed with a 65:35 methanol-water mixture for 1 hour at a flow rate of 0.2 mL/min.

For QDa analyses, both MS Scan (full scan, where all masses passing through the detector generate a signal in the TIC chromatogram) and SIR (Selected Ion Recording, where specific masses are detected as they pass through the detector) modes were used. Seven specific masses were selected for SIR analysis, namely *m/z* 927, 932, 943, 947, 995, 1032, and 1101, all of which had been observed in previous MS and MS/MS analyses. Masses were acquired from 0.15 to 35 minutes over a range of *m/z* 100-1,200, in positive ionization mode. The cone voltage was set at 30 V, while source and probe temperatures were maintained at 120°C and 74°C, respectively.

II.9. NMR and MS analyses

The various purification steps led to the isolation of one targeted metabolite with an *m/z* value of 944. This compound was subsequently analyzed using NMR and mass spectrometry to elucidate its structure.

NMR spectra were acquired using a Bruker Avance III HD 700 MHz spectrometer equipped with a helium-cooled probe (cryoprobe) (Bruker, BioSpin GmbH, Germany). The whole mass isolated was dissolved in 200 µL of deuterated methanol (CD_3OD, d_4 + 0.03% TMS) and placed in 3 mm diameter NMR tubes from CortecNet (Les Ulis, France). A minimal volume of solvent was added to concentrate the fraction as much as possible. The following spectra were recorded with respective scan numbers: ^{1}H (256 scans), COSY (8 scans), ^{13}C (5120 scans), HSQC (32 scans), HMBC (128 scans), and TOCSY (8 scans), using standard Bruker parameters. NMR spectra were visualized and interpreted using MestReNova software (Version 15.0.1).

The UHPLC-ESI(+)-HRMS system consisted of a UHPLC coupled to a hybrid quadrupole time-of-flight mass spectrometer (Agilent 6,546, Agilent

Technologies, Massy, France) equipped with an ESI source operating in positive ion mode. The analytical column used was an Acquity BEH® C_{18} (100 × 2.1 mm, 1.7 µm) purchased from Waters. A linear gradient was applied: 5% B to 100% B in 12 minutes at a flow rate of 500 µL/min, followed by holding at 100% B for 4 minutes at 600 µL/min, and then returning to initial conditions (A = water + 0.1% formic acid; B = acetonitrile). The injection volume was 1 µL, and the DAD detector wavelengths were set to 210, 254, and 280 nm. Source parameters were set as follows: capillary temperature at 320 °C, source voltage at 3500 V, and sheath gas flow rate at 11 L/min. All signals detected during the first 3 min were discarded using the divert valve. MS scans were operated in full scan mode from *m/z* 100 to 1,200 (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 3,000 counts. Selected parent ions were fragmented using a fixed collision energy of 45 eV and an isolation window of approximately 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)-phosphazene $C_{18}H_{18}F_{24}N_3O_6P_3$ $[M+H]^+$ ion (*m/z* 922.009798) were used as internal lock masses. With the purpose to prevent oversampling with the internal calibrants, a permanent MS/MS exclusion criterion was applied to exclude all signals coming from the two internal calibrants. To display MS and MS/MS spectra acquired, the MassHunter software (Version B.07.00) was used.

III. RESULTS AND DISCUSSION

III.1. Extraction yields

The yields obtained varied from 4% w/w to 26% w/w, depending on the plant part of *S. usambarensis* and the type of extract. The detailed yields were as follows: 15.6% w/w for the methanolic extract and 4.0% w/w for the alkaloidic extract of the leaves collected in November 2007, 25.9% w/w for the leaves collected in August 2008, 13.1% w/w for the fruits, 6.8% w/w for the root barks, and 15.81% w/w for the stem and twig barks.

III.2. Highlighting high molecular weight metabolites using feature-based molecular networking of crude extracts (methanolic and alkaloidic) from various parts of *S. usambarensis*

In the initial phase, the exploration of the molecular networks from the six crude extracts revealed metabolites that are well-known in *S. usambarensis*, as identified through shared MS/MS spectral databases on the GNPS platform. These metabolites include usambarine, usambarensine, 19,20-dihydrousambarensine, N_b-methylusambarensine, strychnofoline, and akagerine. By rapidly identifying these known compounds, they can be excluded from further focus, allowing attention to shift toward the unknown metabolites. The molecular network generated from MS/MS data of forty-four crude extracts from twenty-eight *Strychnos* species, including the six extracts from *S. usambarensis* in this study, can be accessed through the following link: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=da555ca03b4048259b2199b4026775de.

The MS and MS/MS spectra, along with the molecular network of the alkaloidic crude extract from *S. usambarensis* leaves (November 2007), revealed previously unidentified molecules with *m/z* values above 900 (Figure 42). These *m/z* values, notably 932.5437, 947.5331, 995.4763, 1032.4717, and 1101.4623, had not been observed in this species before. The molecular network of the alkaloidic crude extract from the leaves of *S. usambarensis* (sample 44) can be accessed on GNPS through the following job link:
https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=1507f353dec24df6a9f973dd1d1c9ac1.

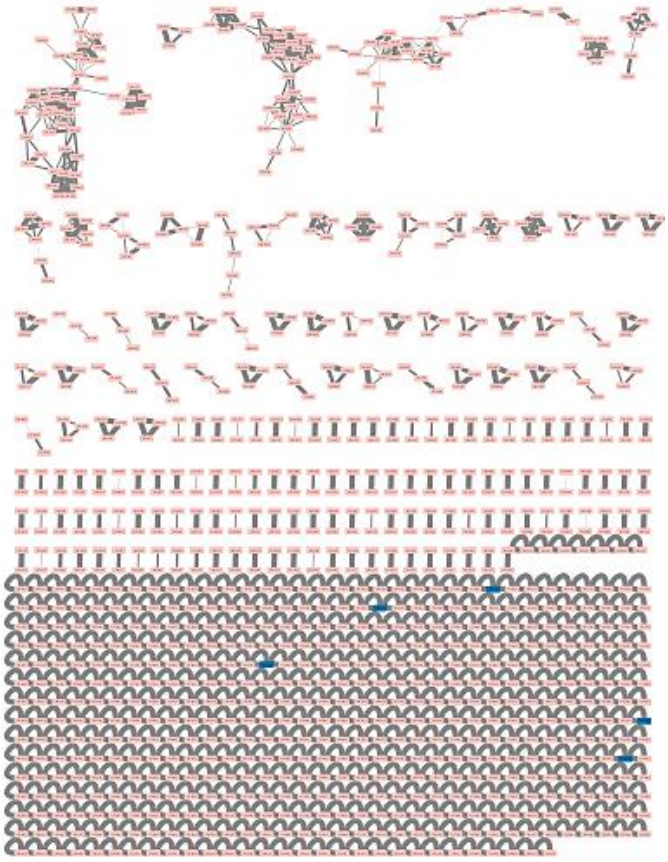

Figure 42: Feature-based molecular network from alkaloidic crude extract of *S. usambarensis* leaves. Blue nodes correspond to metabolites with *m/z* above 900.

As these molecules were found in an alkaloidic crude extract, it is likely that they belong to the phytochemical class of alkaloids. Given their high molecular weight, they are probably dimers or even trimers. In the literature, several bisindole alkaloids, such as usambarensine, strychnobiline, sungucine, and strychnogucine B, have demonstrated strong antiplasmodial activities (Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2001; Frédérich, M. *et al.*, 2002). Considering the well-known antiplasmodial properties of *S. usambarensis*, these compounds are likely to exhibit activity against malaria. To investigate these molecules further, isolation using mass-guided fractionation was performed, specifically targeting these high-molecular-weight compounds.

III.3. Comparison of the metabolite composition from crude methanolic and alkaloidic extracts of *S. usambarensis* leaves

Only the molecular network of the alkaloidic extract from the leaves revealed the compounds we were targeting. However, when comparing the metabolite composition of the crude methanolic and alkaloidic extracts from the same sample using MS spectra (obtained using UPLC-QDa), the targeted metabolites were detected in both extracts. For instance, the mass at *m/z* 942.7 was observed in both extracts (Figure 43).

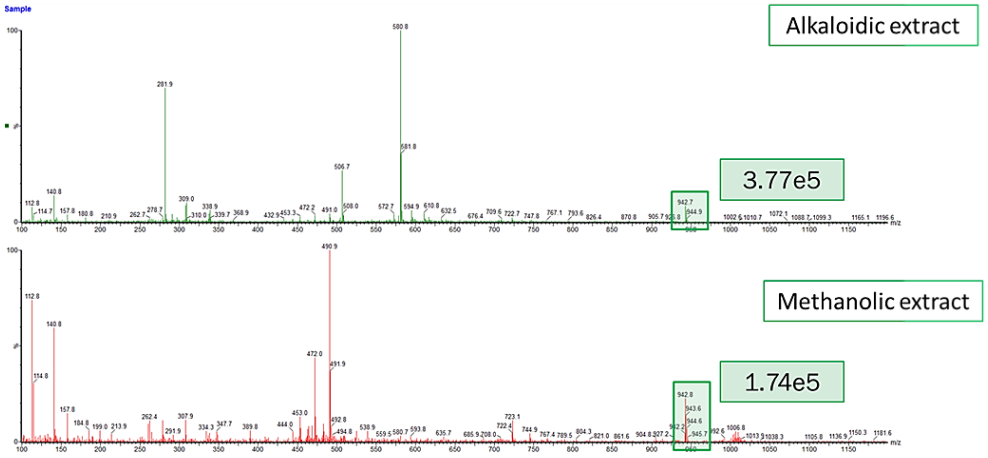

Figure 43: Comparison of the metabolite composition of methanolic and alkaloidic crude extracts from *S. usambarensis* leaves (November 2007).

In terms of intensities, the mass at *m/z* 942.7 is slightly higher in the alkaloidic extract, which is expected given that this type of extract is designed to concentrate alkaloids. However, the crude methanolic extract was selected for isolation for two reasons.

First, the extraction yields of alkaloidic extracts are generally lower than those of methanolic extracts. Consequently, to obtain a sufficient quantity of extract for purifying a sufficient mass of these minor compounds, a large quantity of plant material would be required. In contrast, methanolic extracts, which typically offer higher yields, allow for obtaining more extract from less material.

The second reason is that alkaloidic extractions are time-consuming and tedious. Therefore, to save time, purifications were carried out on the crude methanolic extracts.

III.4. Isolation of a compound with an *m/z* mass above 900 from methanolic crude extracts of *S. usambarensis* leaves

III.4.1. Preparative HPLC

The first purification step involves preparative HPLC. Unfortunately, we had no prior knowledge of the retention times of the targeted metabolites, as their UV signals were too weak to be detected due to their low quantities. To address this issue, several test injections were performed beforehand, during which fractions were collected according to a defined time interval. By analyzing these fractions using UPLC-QDa, the metabolites were tracked. Ultimately, a 5-minute time window was identified during which the molecules eluted from the column and passed through the DAD detector, specifically from 19.5 minutes to 24.5 minutes (Figure 44).

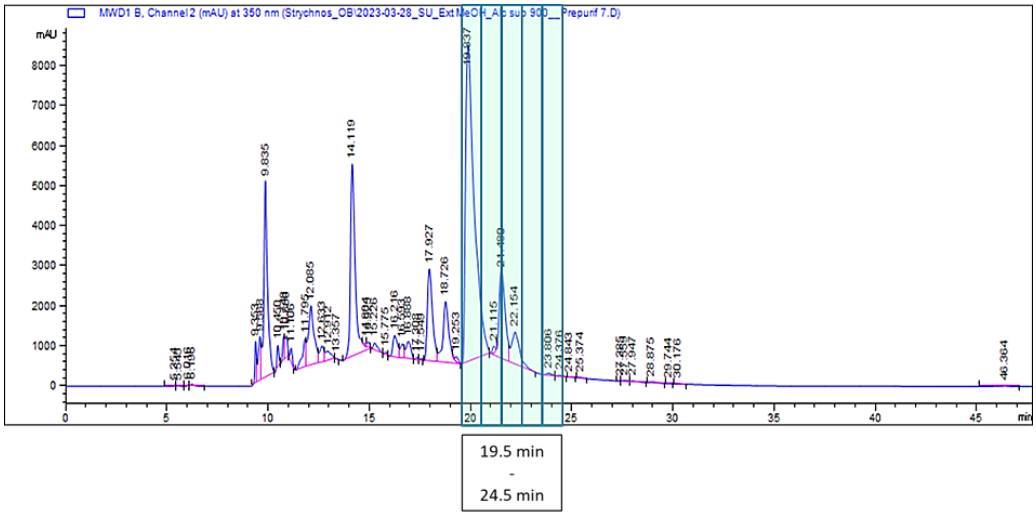

Figure 44: Chromatogram and purification strategy for the methanolic crude extract of *S. usambarensis* leaves by preparative HPLC.

In total, twenty injections of crude methanolic extracts from leaves, with a total mass of 6.79 g, were performed, and the fractions were collected into five different flasks, each corresponding to a 1-minute interval within the set time frame. After transferring the fractions to pre-weighed test tubes, the masses were determined. Fraction 1 (labeled '2.1 S2') had the highest weight at 121.5 mg, while the other fractions ranged in weight from 43.2 to 121.5 mg (Figures 45 and 46).

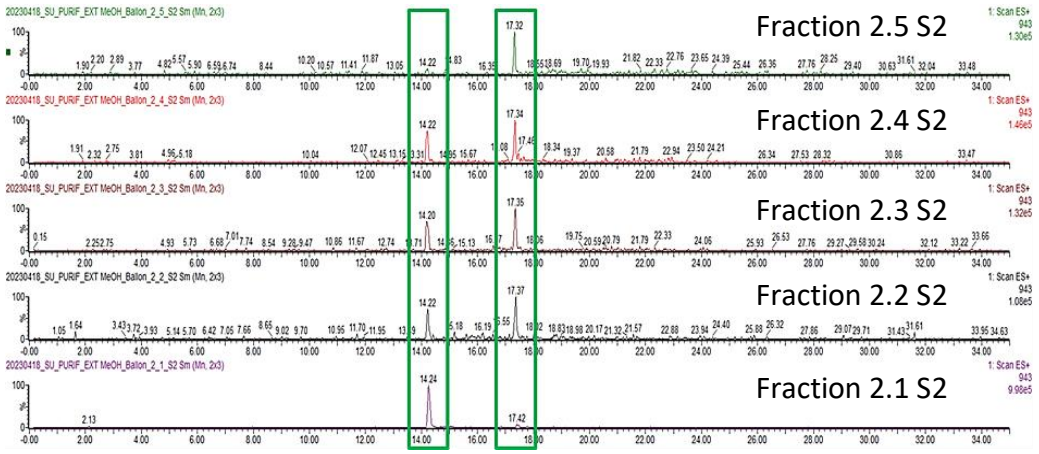

Figure 45: Extracted-Ion Chromatograms (EIC) of the five fractions by highlighting the mass at *m/z* 943.

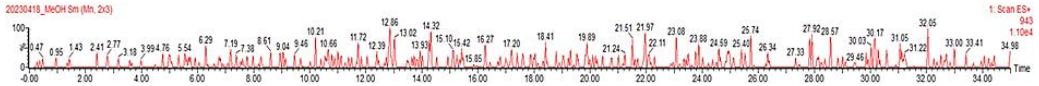

Figure 46: Extracted-Ion Chromatograms (EIC) of the blank (injection of methanol) by highlighting the mass at *m/z* 943.

The chromatograms in Figures 45 and 46 reveal two peaks: one at 14 minutes and the other at 17 minutes of retention time. As these peaks are absent in the blank chromatogram, it can be concluded that these compounds are present in the fractions obtained by preparative HPLC. The peak at 14 minutes corresponds to *m/z* 943.4, while the peak at 17 minutes corresponds to *m/z* 942.7. Given the slight mass difference, these metabolites are likely structurally related. However, the three-minute difference in retention time suggests they are not identical. Alternatively, one compound might result

from the other as an artifact, possibly formed during the preparative HPLC purification process. This artifact formation could explain both the observed mass difference and the variation in retention time.

Since fraction 1 (2.1 S2) had the highest weight and contained the most intense peak (at 14 minutes), it was purified again by preparative HPLC using the same methodology, except that fractions were collected in tared test tubes. A total of 59 fractions were collected. UPLC-QDa analyses revealed that fractions 37, 38, and 39 contained the compound with an *m/z* of 943.4. The combined weight of these three fractions was 37.82 mg. The next step involved purifying these fractions using Sephadex™ LH-20, a polymer that separates compounds by size exclusion.

III.4.2. Open columns

Fractions 37, 38, and 39 were purified independently, resulting in a total of 150 subfractions, which were grouped into 31 groups. Nine of these groups were found to contain the target compound based on UPLC-QDa analyses, with a total mass of 6.23 mg. Unfortunately, the purity was insufficient for structure identification. Furthermore, the small quantity of the nine subfractions made large-scale purification methods, such as preparative HPLC, unfeasible. As a result, it was decided to proceed with small-scale purification using injections into analytical HPLC.

III.4.3. Analytical HPLC

The nine subfractions collected from the first open column were injected one or two times into the HPLC-DAD system at a concentration of approximately 10 mg/mL. Fractions were collected in tared test tubes at the exit of the DAD detector.

The fractions were analyzed using UPLC-QDa and categorized into sets numbered 1 to 15 based on their metabolite content. Some groups were further subdivided due to slight variations among fractions within the same set. Notably, group 8B (designated as 'SU-HPLC-8B') contained the target

compound with good purity. However, mass spectrometry analyses revealed a mass of *m/z* 944.4 instead of *m/z* 943.3, suggesting that the compound underwent a structural modification during the purification process, resulting in the addition of one mass unit. This mass difference could be attributed to the conversion of an amide into a carboxylic acid.

The subfraction "SU-HPLC-8B" was analyzed by NMR for compound identification. Unfortunately, the presence of numerous interfering impurities made it difficult to distinguish the signals from the target molecule and those from the impurities.

To improve the purity of the subfraction "SU-HPLC-8B", another open column containing Sephadex™ LH-20 was used. The resulting fractions were pooled based on their metabolite content, evaporated, and dried. The NMR analysis of the new subfraction "SU-HPLC-8B" showed improved purity, which was sufficient to attempt identifying the compound with an *m/z* mass of 944.4. The total mass of the subfraction "SU-HPLC-8B" was 2.11 mg.

III.5. Structural elucidation of the metabolite with a mass at *m/z* 944.4

III.5.1. MS/MS analyses

During UHPLC-ESI(+)-HRMS analyses, the *m/z* mass at 944.2848 was fragmented. Unfortunately, due to its low intensity in the MS spectrum (intensity of 10^3), the MS/MS fragments blended with the noise, making spectrum interpretation difficult. However, the more intense peak corresponding to *m/z* 928.3119 was also fragmented. With an intensity between 10^5 and 10^6, its fragments were more easily observed. Upon comparing the MS/MS fragments of the two ions, a similar mass difference was noted between the two initial masses. This led to the conclusion that *m/z* 928.3119 is a fragment of *m/z* 944.2848, likely due to its fragility during passage through the source, resulting in a loss of 15.99 Da, which could correspond to an oxygen atom. It is important to note that in the MS/MS spectrum of *m/z* 928.3119, fragmentation occurred on its isotope with *m/z* 929.3138, as no MS/MS fragmentation of *m/z* 928.3119 itself was observed. Finally, the presence of

a fragment at *m/z* 179 suggests a glycosidic component in the structure, making it likely that at least one glucose unit is present (Figure 47).

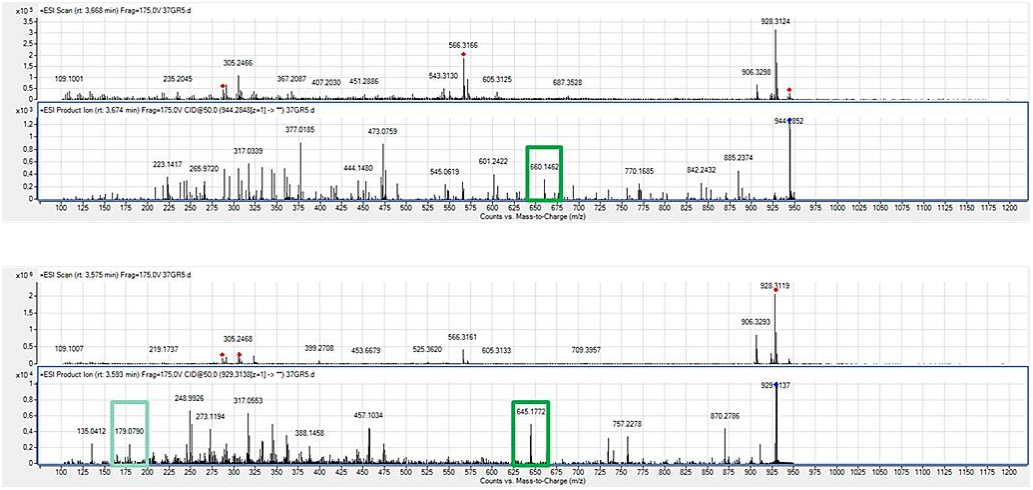

Figure 47: MS/MS spectra of masses at *m/z* 928.3119 (bottom spectrum, with a fragmentation of *m/z* 929.3138) and *m/z* 944.2848 (top spectrum).

III.5.2. NMR analyses

The various NMR spectra acquired, including the proton, HSQC, and HMBC spectra, are presented in Figures 48, 49, and 50, respectively. Additional spectra can be found in the Supplementary material, Figures S1 to S3.

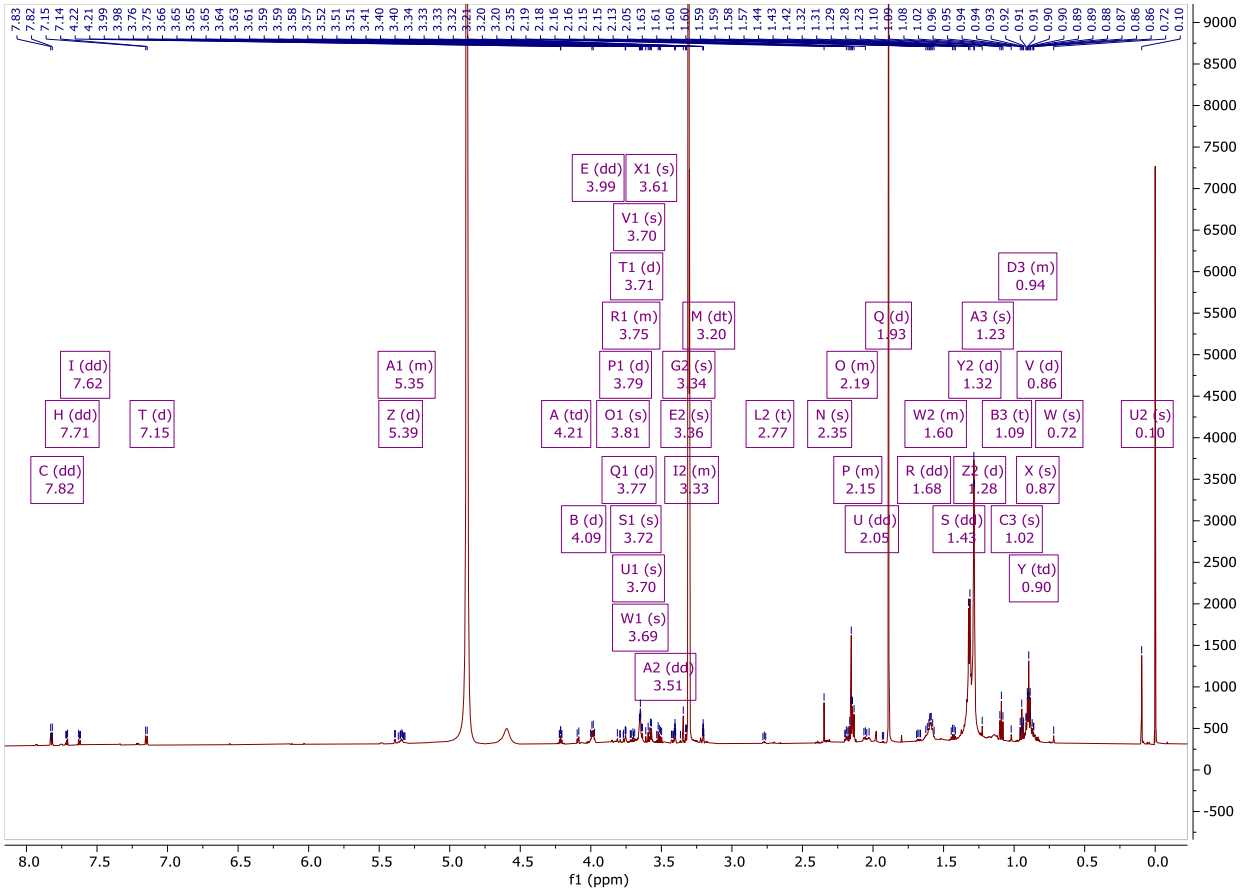

Figure 48: ^{1}H spectrum of the compound with *m/z* 944.2848.

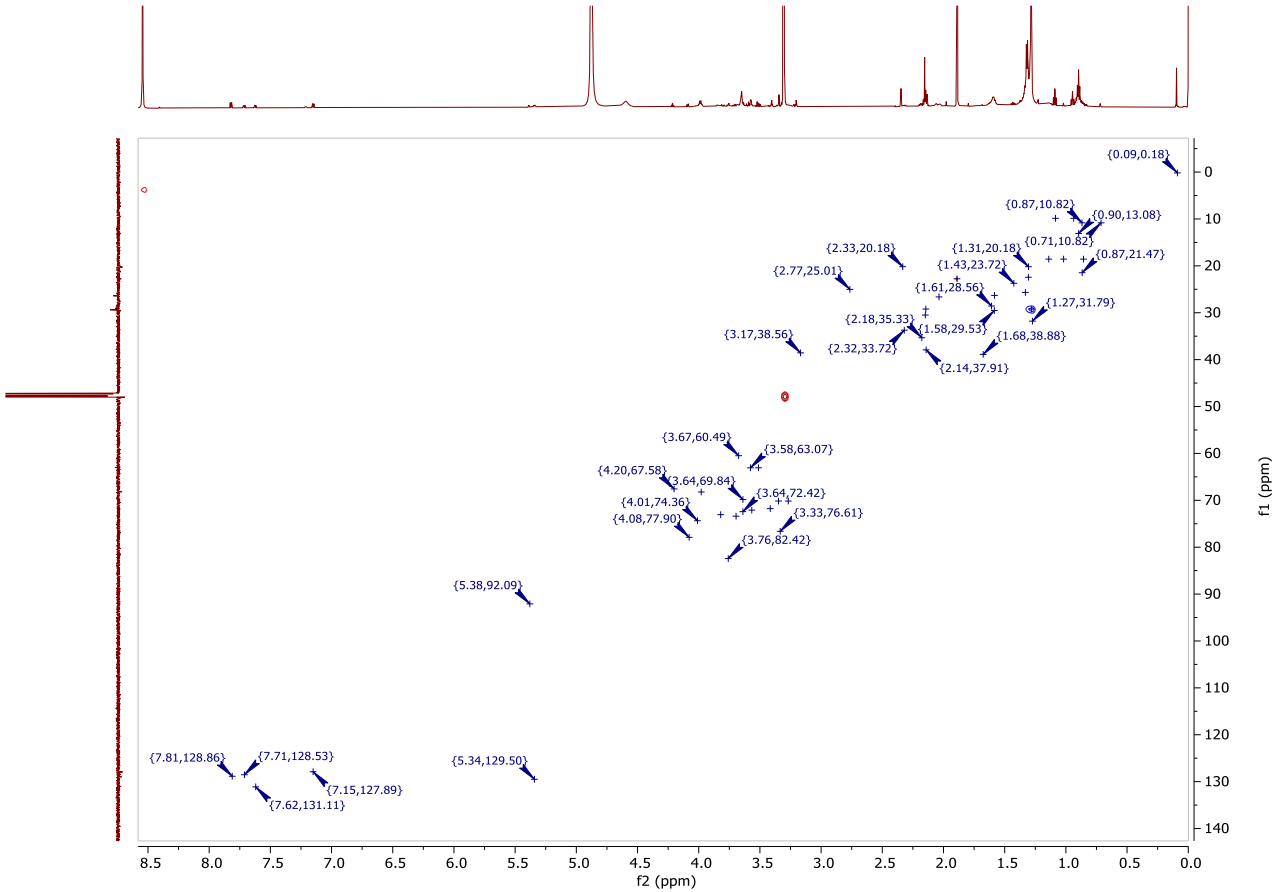

Figure 49: HSQC spectrum of the compound with *m/z* 944.2848.

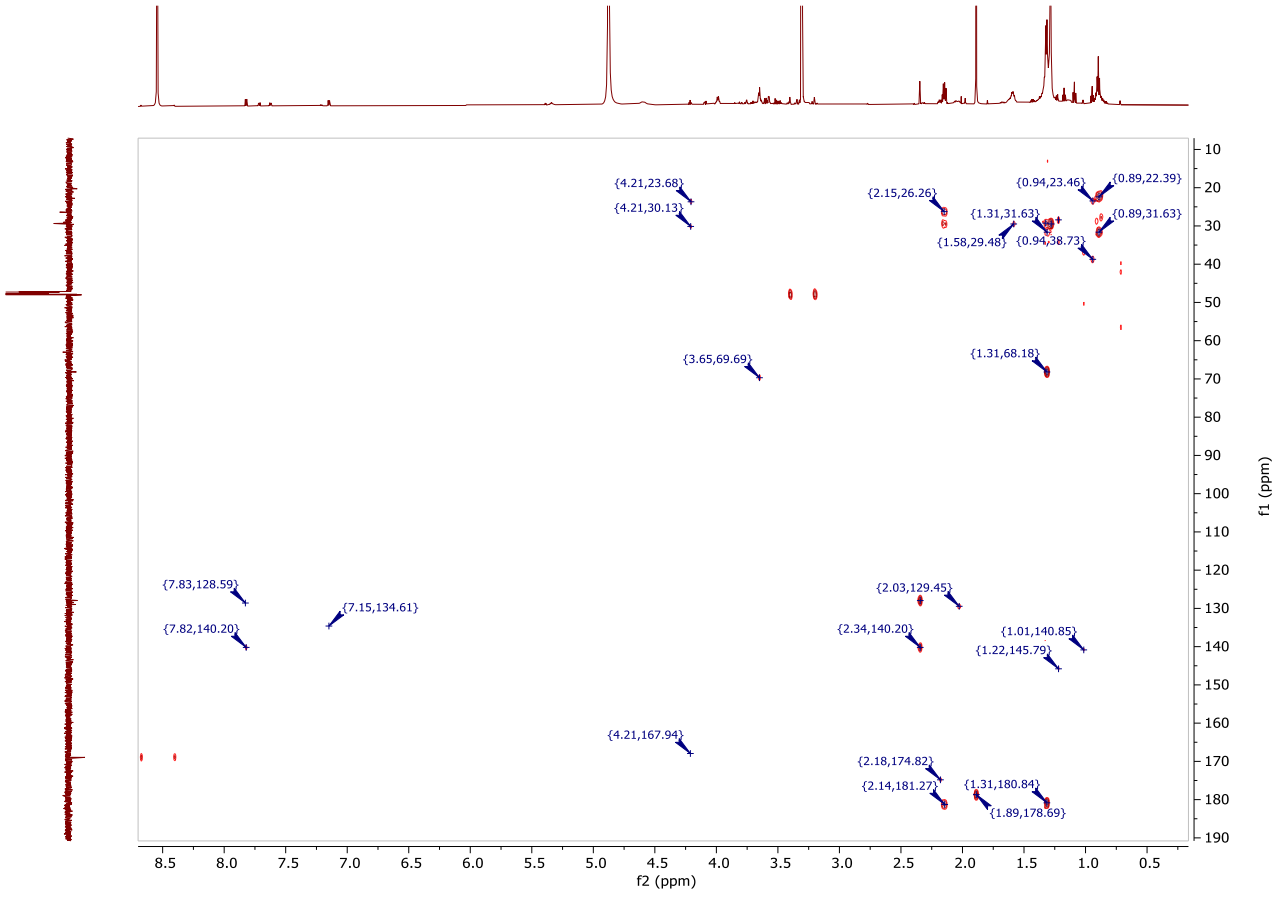

Figure 50: HMBC spectrum of the compound with *m/z* 944.2848.

The presence of a quaternary carbon at 168.96 ppm suggests a carboxylic acid or ester functional group. Additionally, a hemiacetal group is identified in the structure due to the presence of a CH carbon at 92.09 ppm, which corresponds to the anomeric carbon of a glucose unit, confirming the presence of a glycosidic moiety. Deshielded carbons observed between 60 and 70 ppm are likely in close proximity to nitrogen, supporting the hypothesis that the compound belongs to the phytochemical class of alkaloids. In line with this, four strongly deshielded protons in the region of 7.15-7.82 ppm are observed, coupled to carbons between 125 and 135 ppm, suggesting they are part of a benzene ring. HMBC correlations with two quaternary carbons at 134.83 and 140.20 ppm (which show no coupling with protons) further suggest the presence of two quaternary carbons that may link the benzene ring to a potential indole structure. If this hypothesis is confirmed, and considering the even molecular mass, the compound likely contains an even number of nitrogen atoms. The relatively low carbon count also points to a symmetrical structure, consisting of approximately 60 carbon atoms forming two identical halves.

Unfortunately, due to the low quantity of the isolated compound and the presence of impurities, the structural elucidation remains incomplete. To resolve this, further isolation of the compound with minimized impurities is required. One potential method for achieving this is HPLC-SPE (solid-phase extraction), which could isolate and concentrate the target compounds selectively in capsules, followed by metabolite recovery through nitrogen purging.

Finally, the antiplasmodial activity of the isolated compound could not be determined due to rapid degradation resulting from its low structural stability.

IV. CONCLUSIONS AND PROPSECTS

Plants of the *Strychnos* genus, belonging to the Loganiaceae family, have been the subject of extensive research due to their tetanizing, curarizing, and therapeutic properties. This is particularly true for *Strychnos usambarensis*, which has been extensively studied for its antiplasmodial activity. As such, this species represents a promising source of new antimalarial compounds that

could potentially lead to the development of novel drugs. The development of new antimalarial treatments is crucial in light of the rising cases of malaria and the increasing resistance of malaria parasites to current treatments.

To achieve this objective, a study focused on the leaves of *S. usambarensis*. Molecular networking analysis of their metabolites uncovered previously unreported compounds with *m/z* values exceeding 900, which had not been documented in this species before. After purifying the crude methanolic leaf extract using preparative HPLC, five fractions were collected, containing *m/z* masses at 943.4 and 942.7, corresponding to retention times of 14 minutes and 17 minutes, respectively. The fraction with the highest weight, predominantly containing the mass at *m/z* 943.4, was selected for further purification. The subfractions of interest were run successively through an open column, an analytical HPLC column, and again through an open column.

MS/MS analysis revealed the presence of a mass at *m/z* 944.2848, suggesting that the compound underwent a structural modification during the mass-guided fractionation. The observed mass difference may be due to the conversion of an amide into a carboxylic acid. NMR analysis of the compound showed the presence of a carboxylic acid or ester group, at least two nitrogen atoms, and an anomeric carbon from a glucose unit, indicating the presence of a glycosidic moiety. The structure also appears to be symmetrical. Unfortunately, the full structure could not be determined due to low compound quantities, the presence of impurities, and insufficient signal clarity. Additionally, the fragility of the structure prevented the assessment of its antiplasmodial activity.

In the future, isolating a larger quantity of the metabolite with an *m/z* mass of 944.2848, while minimizing impurities, will be crucial for further elucidating its structure and assessing its antiplasmodial activity *in vitro*, and potentially its antimalarial activity *in vivo*. If these activities are promising and the compound is free of toxicity, it will be essential to develop a faster and more efficient method for obtaining larger quantities to continue studies and potentially move toward commercialization. One promising approach could involve hemisynthesis or total synthesis.

V. SUPPLEMENTARY MATERIAL

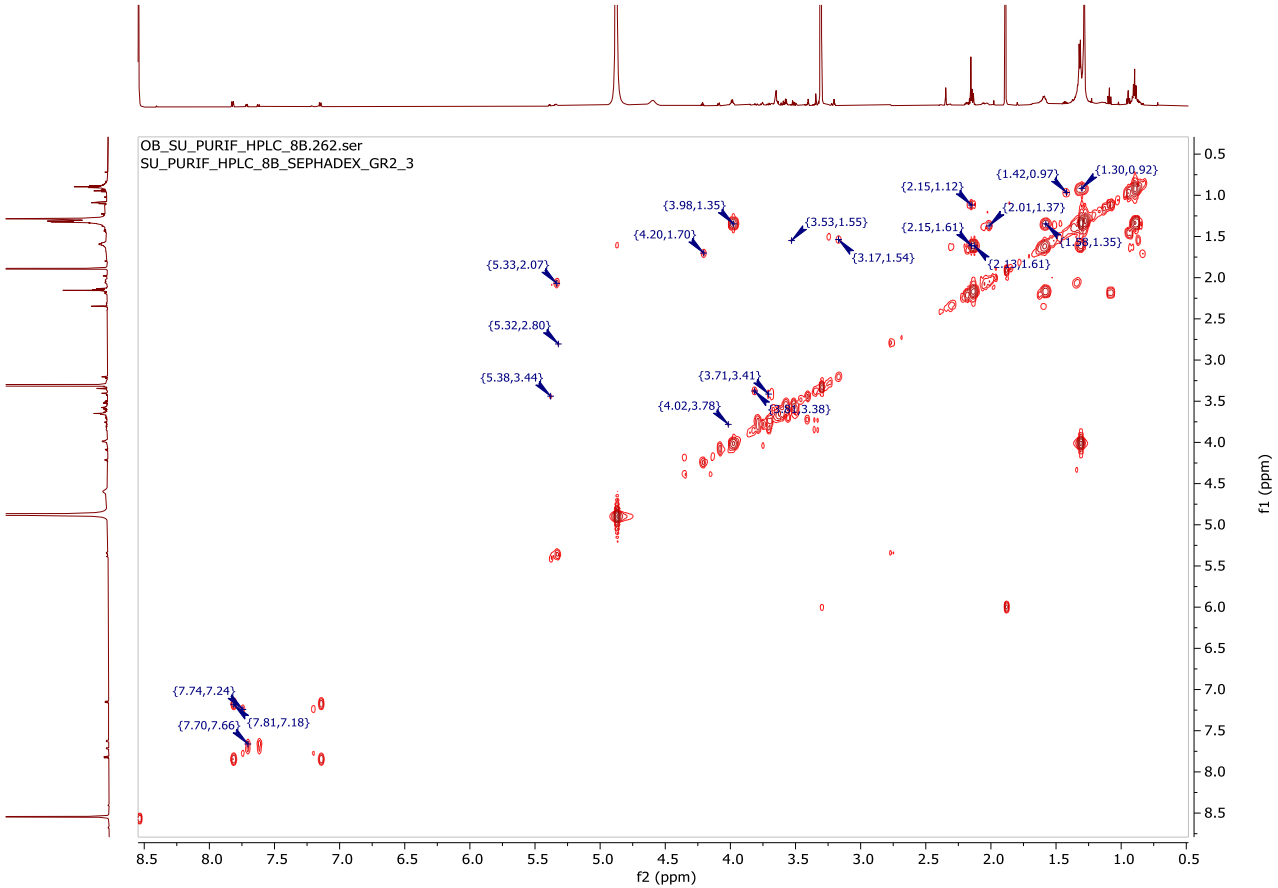

Figure 51: COSY spectrum of the compound with *m/z* 944.2848.

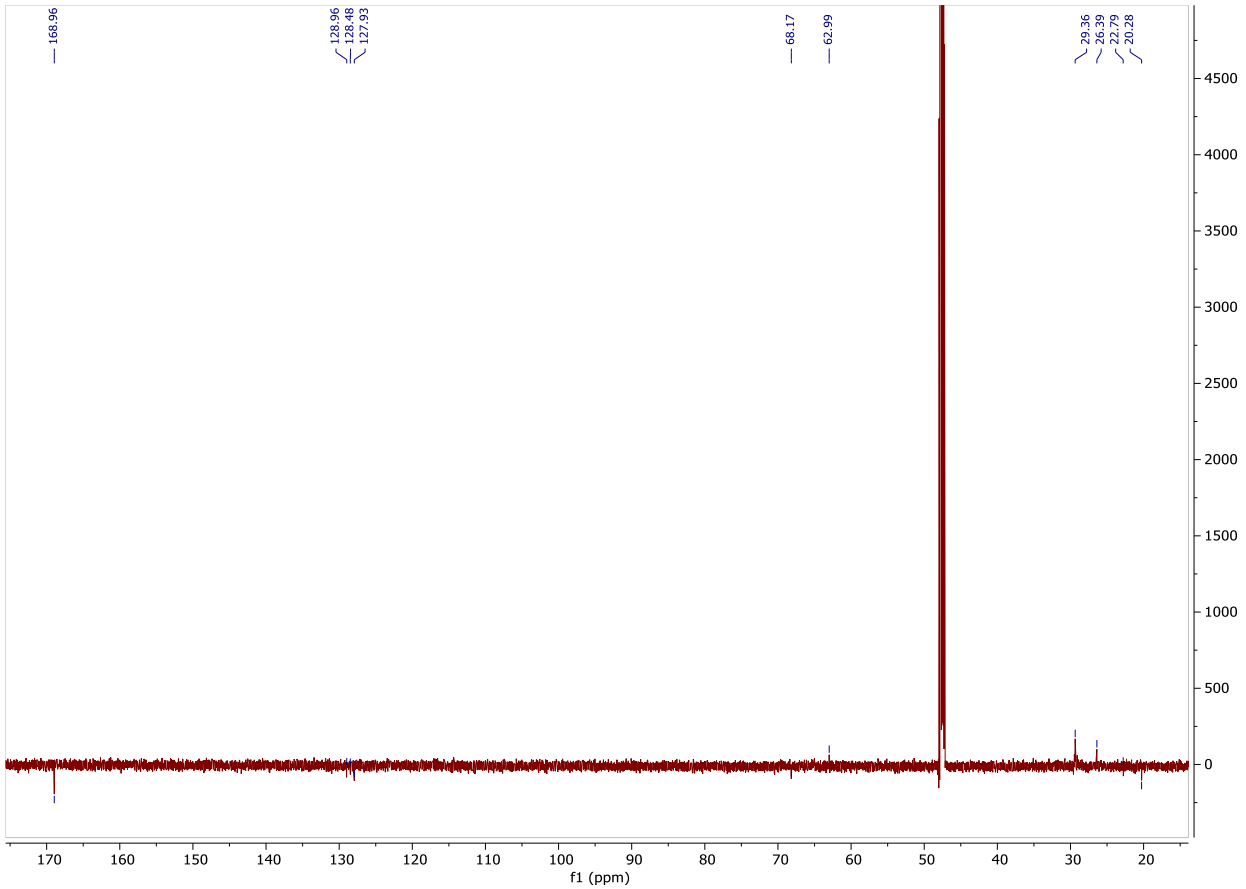

Figure 52: ^{13}C APT spectrum of the compound with *m/z* 944.2848.

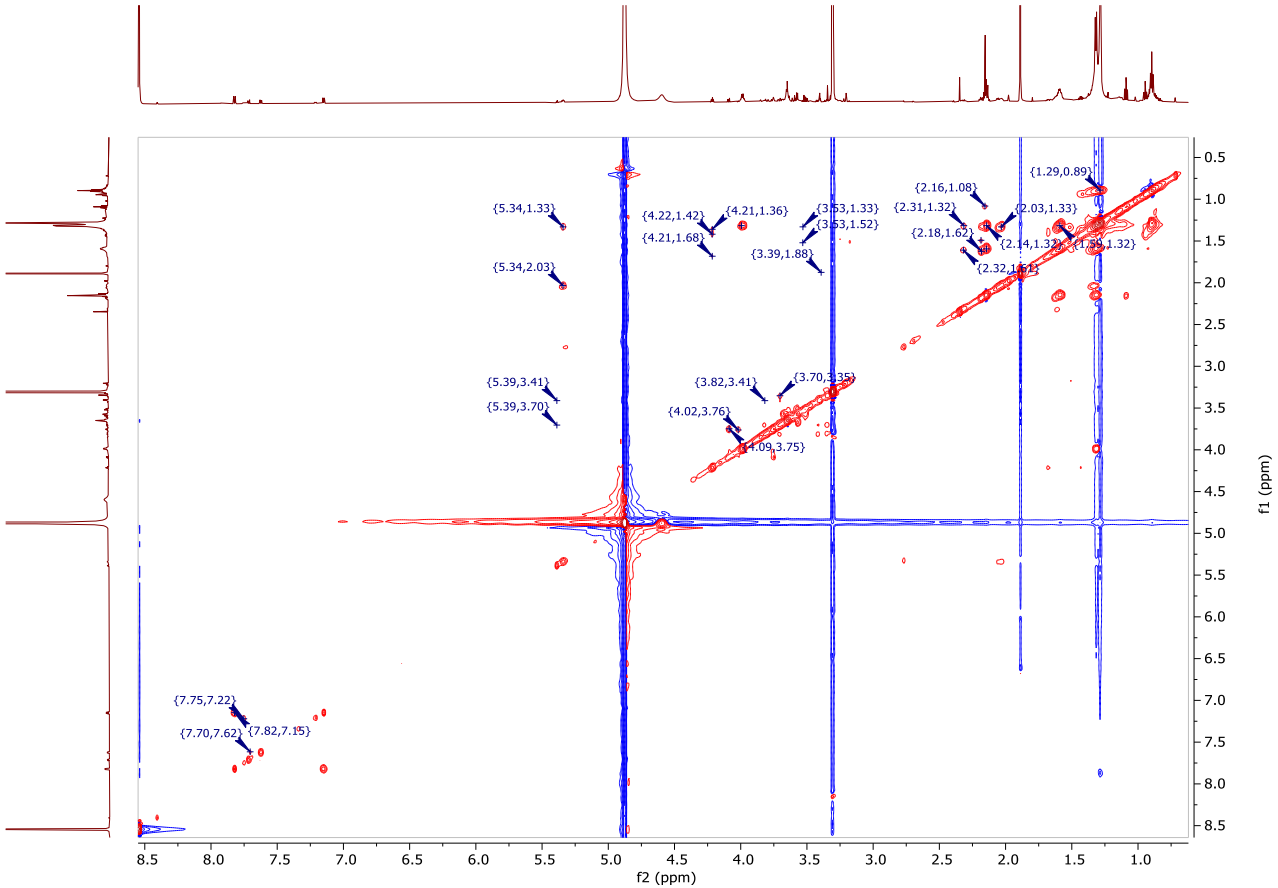

Figure 53: TOCSY spectrum of the compound with *m/z* 944.2848.

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CHAPTER 7

Exploring the metabolite content of *Strychnos longicaudata* trunk barks through bio- and mass-guided fractionation

7.1. Forewords

A sample of *Strychnos longicaudata* Gilg trunk barks was chosen for purification purposes (see Chapter 4, pages 241-251). The aim of this chapter is twofold: to explore the metabolite content of *S. longicaudata* trunk barks and to isolate new antiplasmodial compounds through bio- and mass-guided fractionation. This methodology, applied at every stage of purification, integrates both a biological dimension, through the *in vitro* testing of antiplasmodial activities of fractions, and a chemical dimension, by tracking unknown mass(es) using mass spectrometry analyses and the molecular networking method. Ultimately, original metabolites will be isolated and analyzed using mass spectrometry (MS/MS) and nuclear magnetic resonance spectroscopy (NMR) to elucidate their chemical structures.

7.2. Article: "In-depth study of alkaloids from *Strychnos longicaudata* trunk barks to discover original antiplasmodial compounds"

Methanolic, dichloromethane, and alkaloidic crude extracts obtained from the trunk barks of *Strychnos longicaudata* exhibited significant antiplasmodial activity against the 3D7 strain of *Plasmodium falciparum*. Metabolites from the dichloromethane and alkaloidic extracts were analyzed using molecular networking based on MS/MS data, while carbon spectra were examined with the MixONat software. Additionally, the dichloromethane extract was subjected to various purification methods during bio- and mass-guided fractionation. This methodology involved testing each fraction for antiplasmodial activity and employing molecular networking to explore their metabolite content at each stage of purification. Ultimately, novel active compounds were isolated and their structures elucidated using MS/MS and NMR analyses.

The article presented below is intended for potential submission in the future (Figure 54). As part of the thesis, minor modifications were made to the article's text to ensure continuity with the other chapters.

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In-Depth Study of Alkaloids from *Strychnos longicaudata* Trunk Barks to Discover Original Antiplasmodial Compounds

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Figure 54: Screenshot of the article on the study of *S. longicaudata* trunk barks.

ABSTRACT

Introduction: The search for new antimalarial compounds in plants is crucial to combat the parasites responsible for malaria, which are increasingly resistant to current antimalarial treatments. The *Strychnos* genus, from the Loganiaceae family, is a promising candidate due to its wealth of antiplasmodial and antimalarial metabolites. Among the approximately 200 species in the genus, the metabolite content of the African species *Strychnos longicaudata* remains largely unexplored.

Objectives: To explore and isolate metabolites from *Strychnos longicaudata* trunk barks in order to uncover new antiplasmodial compounds.

Materials and methods: Two distinct methodologies were applied to achieve this objective:

- Exploration of crude extracts: Using two complementary metabolomics tools, molecular networking and MixONat, to quickly identify known compounds and uncover new ones in the species.

- Isolation of targeted metabolites: Applying bio- and mass-guided fractionation to the dichloromethane crude extract to target unidentified metabolites active against malaria throughout all purification steps, ultimately elucidating their structure.

Results: Molecular networking and MixONat analysis of dichloromethane and alkaloidic crude extracts revealed several known metabolites in *Strychnos longicaudata*, including bisnordihydrotoxiferine, longicaudatine, longicaudatine Y, longicaudatine F, normavacurine, and Wieland-Gumlich aldehyde. New compounds never before detected in this species were also identified, such as geissoschizol, normacusine B, and tubotaiwinal. During bio- and mass-guided fractionation of the dichloromethane crude extract, which exhibited promising antiplasmodial activity against the 3D7 strain (chloroquine-sensitive) of *Plasmodium falciparum* (IC_{50} of 4.94 ± 2.51 µg/mL), eight compounds were isolated. Structural elucidation was attempted for only three of the eight metabolites, using MS, MS/MS, and NMR data. Alstonine, identified for the first time in *S. longicaudata*, was among these metabolites. Additionally, a structure was proposed for the subfraction SL23 GR3, and structural hints were observed for the subfraction SL16 GR5.5. Both SL23 GR3 and SL16 GR5.5 were original alkaloids that demonstrated promising antiplasmodial activity against 3D7 strain (IC_{50} of 0.432 ± 0.240 µg/mL for SL23 GR3 and 0.163 ± 0.0708 µg/mL for SL16 GR5.5), while alstonine showed weaker activity (IC_{50} of 19.0 ± 5.28 µM).

Conclusions and prospects: The use of molecular networking and MixONat facilitated the rapid identification of known metabolites in a complex mixture, enabling the targeted exploration of unidentified compounds during fractionation. After bio- and mass-guided fractionation, eight metabolites were isolated, including two novel ones with promising antiplasmodial activity. In the future, isolating larger quantities of these two compounds would be valuable in order to elucidate their structures, as well as further purifying the five other metabolites that could not undergo structural elucidation in this study. Additionally, some active fractions still require further exploration and purification, as numerous original metabolites remain to be discovered and characterized in this species.

I. INTRODUCTION

Strychnos longicaudata is an African species of the *Strychnos* genus, belonging to the Loganiaceae family. Of the approximately 200 species in the genus, 75 are native to Africa. The other species originate from South America (at least 73), as well as Asia and Oceania (around 44). Depending on their habitat, *Strychnos* species can be shrubs, trees, or lianas (Bisset, N. G., 1970; Krukoff, B. A., 1972; Bisset, N. G., 1974; Setubal, R. B. *et al.*, 2021).

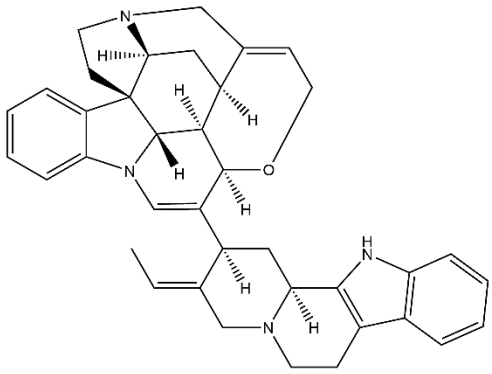
The *Strychnos* genus is well known for its toxic properties, characterized by tetanizing and/or curarizing effects, which vary by species, and its wide range of therapeutic activities. These species have numerous traditional uses and have demonstrated various pharmacological effects in both *in vitro* and *in vivo* assays (Angenot, L., 1971; Sandberg, F. *et al.*, 1971; Angenot, L. *et al.*, 1975; Geevaratne, M. *et al.*, 1977; Ohiri, F. C. *et al.*, 1983; Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2002; Philippe, G. *et al.*, 2004; Philippe, G. *et al.*, 2005; Oyedemi, S. O. *et al.*, 2012; Madikizela, B. *et al.*, 2017; Ampa, R. *et al.*, 2018; Oryema, C. *et al.*, 2021; Bonnet, O. *et al.*, 2022a; Bonnet, O. *et al.*, 2022b; Maroyi, A., 2022; Uttu, A. J. *et al.*, 2022). Therefore, *Strychnos* represents a promising source for discovering new therapeutic compounds.

Developing new treatments has become essential for tackling some diseases, particularly malaria. The parasites responsible for malaria, belonging to the *Plasmodium* genus, have shown increasing resistance to existing treatments, reducing their effectiveness (World Health Organization, 2023). Given the promising *in vitro* and *in vivo* activities against malaria of some *Strychnos* species and their alkaloids, the genus has attracted significant attention for the discovery of new antimalarial drugs (Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2002; Philippe, G. *et al.*, 2005; Philippe, G. *et al.*, 2007; Lusakibanza, M. *et al.*, 2010; Fentahun, S. *et al.*, 2017).

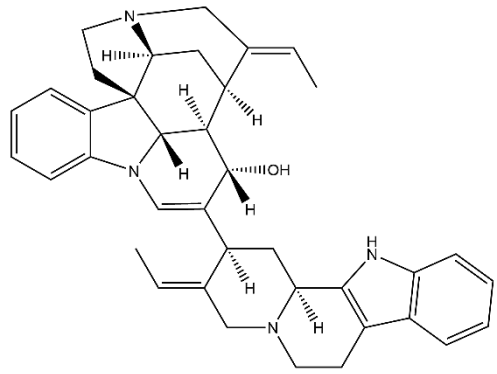
This study is set in this context. During an *in vitro* screening for antiplasmodial activity against *Plasmodium falciparum* (3D7, a chloroquine-sensitive strain), the crude methanolic extract of *Strychnos longicaudata* trunk barks exhibited good antiplasmodial activity (between 5 and 15 µg/mL), with an IC_{50} value of 13.7 ± 2.01 µg/mL (Bonnet, O. *et al.*, 2022a). Further results in this article

reveal even stronger activity (below 5 µg/mL) for the crude dichloromethane extract of *S. longicaudata* trunk barks. Moreover, the limited literature on this species suggests it remains largely unexplored to date.

Several dimeric alkaloids have been reported in *S. longicaudata*, including longicaudatine, longicaudatine F, longicaudatine Y, bisnordihydrotoxiferine, and bisnor-C-alkaloid H. The species also contains monomeric alkaloids such as cantleyine, tetrahydrocantleyine, diaboline, flavopeirerine, normavacurine, 1,2-dehydrodesacetylretuline, 23-hydroxy-2,16-dehydroretuline, N_1-desacetyl-18-hydroxyisoretuline, N_1-desacetyl-18-acetoxyisoretuline, strychnovoline, and Wieland-Gumlich aldehyde. Among these, longicaudatine and longicaudatine F have shown promising antiplasmodial activities (Figure 55). The IC_{50} values for longicaudatine are 0.986 µM (FCA 20 Ghana, chloroquine-sensitive), 0.560 µM (FCB1-R Colombia, chloroquine-resistant), and 0.569 µM ± 0.228 (W2 Indochina, chloroquine-resistant), while for longicaudatine F, the IC_{50} values are 7.70 µM (FCA 20 Ghana), 11.7 µM (FCB1-R Colombia), and 9.58 µM (W2 Indochina) (Massiot, G. *et al.*, 1983a; Massiot, G. *et al.*, 1983b; Massiot, G. *et al.*, 1989; Thepenier, P. *et al.*, 1990; Delaude, C., and Delaude, L., 1997; Frédérich, M. *et al.*, 2002).



Longicaudatine



Longicaudatine Y

Figure 55: Structures of longicaudatine and longicaudatine Y.

In this study, the first step involved manually producing different types of crude extracts from the trunk barks of *Strychnos longicaudata*. Three crude extracts, methanolic, dichloromethane, and alkaloidic, were tested against

the chloroquine-sensitive 3D7 strain of *Plasmodium falciparum* to identify the extract(s) with the strongest antiplasmodial activity.

In the second step, the metabolite content of the selected extract, the dichloromethane crude extract, was analyzed using MS and MS/MS data, combined with the molecular networking method (Yang, J. Y. *et al.*, 2013; Wang, M. *et al.*, 2016; Nothias, L.-F. *et al.*, 2018; Fox Ramos, A. E. *et al.*, 2019). Additionally, both the dichloromethane and alkaloidic extracts were analyzed using MixONat software, which proposes metabolite identifications based on carbon spectra data (^{13}C APT, DEPT-135, and DEPT-90) from crude extracts or fractions (Bruguière, A. *et al.*, 2020).

The bio- and mass-guided fractionation process began with the purification of the dichloromethane crude extract using preparative HPLC. The resulting fractions were grouped, tested *in vitro* against malaria, and analyzed by mass spectrometry and molecular networking to identify active fractions containing major unidentified metabolites. These fractions were subsequently purified using either open-column chromatography or preparative TLC to isolate individual compounds. *In vitro* antiplasmodial assays revealed that most isolated compounds exhibited promising activity against the 3D7 strain. Furthermore, structural elucidation attempts were conducted using MS, MS/MS, and NMR data.

II. MATERIALS AND METHODS

II.1. Materials, chemicals, and reagents

The trunk barks of *Strychnos longicaudata* Gilg were collected in August 2009 in Cameroon. Reference specimens are preserved at the National Herbarium of Cameroon (Breteler F.J. 2135) and the Herbarium of the Botanical Garden of Belgium in Meise (Kemeuze 7B).

Acetone, methanol, dichloromethane, and sodium acetate were purchased from VWR Chemicals BDH (Leuven, Belgium). Methanol of HPLC grade, trifluoroacetic acid, sulfuric acid, and ammonia 25% came from Merck (Darmstadt, Germany). The solvents of UHPLC-MS grade (methanol, acetonitrile, and formic acid) were purchased from Sigma-Aldrich (Machelen,

Belgium). The Milli-Q water was supplied using two systems: a Milli-Q reference A+ system® from Merck (Darmstadt, Germany) at the University of Liège, and a MILLIPORE Synergy UV® from Merck (Darmstadt, Germany) at Université Paris-Saclay during (U)HPLC-ESI(+)-Q/TOF. To perform NMR analyses, deuterated methanol (CD_3OD, d_4 + 0.03% TMS) and deuterated chloroform ($CDCl_3$ + 0.03% TMS) used to dissolve the isolated compounds came from Euriso-top (Saint-Aubin, France).

II.2. Sample grinding and extraction

The entire dried sample was ground using a Retsch ZM100 grinder (Aartselaar, Belgium). After weighing the powder, it was soaked for 5 minutes in 25% ammonia before adding methanol or dichloromethane, depending on the type of crude extract. The mixture was subjected to an ultrasonic bath (EMAG Technologies®) for 1 hour, then filtered through paper, minimizing residue accumulation on the filter paper. The filter paper was subsequently rinsed with the extraction solvent. This process was repeated five more times, resulting in six liquid extracts, which were pooled together in a flask. The pooled contents were partially evaporated using a Rotavapor® (Büchi) before being transferred to a pre-weighed glass test tube for complete evaporation. The resulting dry extract was left to dry overnight at room temperature in a vacuum oven (Heraeus, Hanau, Germany, and Memmert, Brussels, Belgium). Finally, the extract was weighed, and the yield was determined.

Additionally, an alkaloidic extract of *S. longicaudata* trunk barks was prepared using a method previously reported (Bonnet, O. *et al.*, 2022b). First, 20 mL of 10% w/w ammonia solution was added to 2 g of powder and left to macerate for 30 minutes. Subsequently, 100 mL of dichloromethane was added, and the mixture was stirred magnetically for 1 hour. The solution was filtered through glass wool, which was rinsed twice with 50 mL dichloromethane. The organic phase was extracted with 20 mL of 2% w/V sulfuric acid in a separating funnel, and a second extraction was performed with another 20 mL of 2% w/V sulfuric acid. The two aqueous phases were combined, and the pH was adjusted to 8-10 by adding 10% w/w ammonia. The basified aqueous phase was subjected to two extractions with 50 mL of dichloromethane

in a separating funnel. The two organic phases were combined, and residual water was removed by adding anhydrous sodium sulfate (Merck, Darmstadt, Germany). Finally, the liquid extract was evaporated under reduced pressure using a Rotavapor® (Büchi) and dried overnight at room temperature in a vacuum oven (Heraeus, Hanau, Germany, and Memmert, Brussels, Belgium).

II.3. HPLC-ESI(+)-Q/TOF

The analyses by HPLC-ESI(+)-Q/TOF of the two crude extracts provided the data necessary to build the molecular networks. These analyses were performed at the Université Paris-Saclay. Exploring the metabolite content of the two extracts will help identify any unknown metabolites by using the spectral databases available on the GNPS platform, allowing them to be targeted during the purification steps (Wang, M. *et al.*, 2016). The methodology used for mass spectrometry analyses, data processing, and molecular network generation is also described in one of our publications from 2022 (Bonnet, O. *et al.*, 2022b).

After weighing, dissolving in methanol, filtering through a 0.2 µm pore size filter, and transferring to HPLC vials, the extracts were injected into an HPLC-ESI(+)-Q/TOF from Agilent (Agilent Technologies, Massy, France). This setup included an Agilent 1,260 Infinity HPLC coupled with an Agilent 6,530 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 was a SunFire C_{18} (150 × 2.1 mm, 3.5 µm) from Waters. The flow rate was set at 250 µL/min, and the mobile phase composition varied linearly with a gradient from 5% B to 100% B in 30 min (A = Water + 0.1% formic acid; B = Methanol). For the DAD, the applied wavelengths were 210, 254, and 280 nm. Regarding the ESI conditions, the capillary temperature, source voltage, and sheath gas flow rate were set at 320 °C, 3.5 kV, and 10 L/min, respectively. The volume injected for each sample was 5 µL. The mass spectrometer operated in Extended Dynamic Range mode (2 GHz). During the first 3 minutes, the divert valve excluded all detected peaks. Each scan recorded six results: one MS scan in positive mode

(in the range of *m/z* 100-1,200), and MS/MS scans for the five most intense peaks in the MS scan.

To acquire MS/MS spectra, many settings were applied, i.e., three fixed collision energies (30, 50, and 70 eV), default charge of 1, isolation width of *m/z* 1.3, and minimum intensity of 3,000 counts. Purine $C_5H_4N_4$ $[M+H]^+$ ion (*m/z* 121.050873) and hexakis (1*H*, 1*H*, 3*H*-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 with the internal calibrants, a permanent MS/MS exclusion criterion was set, containing the *m/z* values of the two internal calibrants. To display MS and MS/MS spectra acquired, the MassHunter software (Version B.07.00), a mass spectrometry application software from Agilent, was used.

II.4. Feature-based molecular networking

The article by Nothias, L.-F. *et al.* (2020) provides a detailed methodology for constructing a feature-based molecular network of crude extracts or fractions on the GNPS platform. The first step involved converting the files generated by the mass spectrometer into ".mzXML" extension files (eXtensible Markup Language) using MSConvert software, developed by ProteoWizard (Chambers, M. C. *et al.*, 2012). The filtering and algorithm applied were peak picking and vendor, respectively. The second step consisted of importing the .mzXML files into MZmine 2 (version 2.53) (an older version of the current MZmine 3) (Pluskal, T. *et al.*, 2010) for processing. This included removing noise, creating peak lists, performing deconvolution (i.e., removing unwanted peaks), grouping isotopes, and filtering to retain only MS^2 data. The specific parameters applied during each processing step are described below:

- Noise removal (method: mass detection): The intensities applied for the suppression of noises were 5.8E3 and 5.0E1 for MS^1 and MS^2, respectively;
- Peak list creation (method: ADAP chromatogram builder): A minimum of four points were required to build a peak. The intensity threshold was set at 5.8E3, and the *m/z* tolerance was *m/z* 0.02 and 10.0 ppm;
- Deconvolution (method: Chromatogram deconvolution):
    - The algorithm was wavelets (ADAP) (Myers, O. D. *et al.*, 2017);

- The *m/z* center calculation was set to 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 3,000;
- The coefficient/area threshold was 2;
- The peak duration range was 0.02-1.5 min;
- The retention time wavelet range was 0.02 and 0.2 min;

- Isotope grouping (method: Isotopic peaks grouper): *m/z* 0.005, 15.0 ppm, and 0.5 min were, respectively, the *m/z* and retention time tolerances. The maximum charge was set at 1, and the representative isotope was the most intense peak;
- Filtration (method: Feature list rows filter): In the processed peak list, the peaks within the time ranges from 0 to 2.50 minutes, and from 45.59 to 49.83 minutes, were deleted. Additionally, only MS/MS spectra were retained for generating the molecular network.

The third step was to export the data in the ".mgf" (Mascot Generic Format) and ".CSV" (Comma-separated values) formats. The "Export to CSV file" and the "Export/Submit to GNPS/FBMN" methods in MZmine 2 were used to export the data in these two required formats. The ".mgf" files contain the essential data for generating the molecular network, while the ".CSV" files provide additional information, such as retention times.

In the final step, the ".mgf" and ".CSV" files were uploaded to GNPS (Global Natural Products Social Molecular Networking), an online community platform that generates feature-based molecular networks and suggests identifications based on shared spectral databases (Wang, M. *et al.*, 2016). The parameters applied to build the molecular networks were as follows:

- 0.02 Da for precursor and fragment ion mass tolerances;
- 0.65 for the minimal cosine score;
- 0.7 for the minimal cosine score for matching with the library;
- No filtration was applied;
- The analogs were not searched.

II.5. MixONat analyses

Dichloromethane and alkaloidic crude extracts were dissolved in deuterated chloroform ($CDCl_3$ + 0.03% TMS) at a concentration of 30 mg/mL. This concentration was chosen based on the work reported by Bruguière, A. *et al.* (2020). Specifically, 30 mg of crude extracts were weighed and dissolved in the minimum amount of solvent possible to maximize their concentration, thereby ensuring the highest number of signals in the NMR spectra. Furthermore, by highly concentrating the samples, fewer scans were required, reducing the overall analysis time.

After dissolution, the solutions were transferred into 5 mm diameter NMR tubes. Three types of carbon spectra, ^{13}C APT, DEPT-135, and DEPT-90, were recorded, with a total of 6144 scans.

The databases used in MixONat are derived from both a structure database ("SDF" file) and theoretical chemical shifts. The “C+H NMR Predictors” software from ACD/Labs, along with an algorithm developed by Nuzillard J.-M. (2021), were employed to predict the chemical shifts of structures from the SDF file. As a result, a c-type database was generated. For the analyses described in this article, the *Strychnos* genus database was used.

For the carbon spectra, after processing the data and selecting the peaks of interest, their intensities and chemical shifts were copied and pasted into an Excel file. Once the table titles were removed, the file was saved in .CSV format. In Notepad++ (version 8.6.8), semicolons were replaced by commas, and minus signs in the ^{13}C APT data were removed.

Finally, the c-type database and the three .CSV files were imported into MixONat to launch the analysis. Equivalent carbons were accepted during the analyses, meaning that the chemical shifts in the carbon spectra could be matched twice.

II.6. UHPLC-ESI(+)-HRMS

During mass-guided fractionation, at each purification step, the different masses present in the fractions were analyzed. In this way, unknown masses

were targeted throughout the purification process. For this purpose, a UHPLC-ESI(+)-HRMS system, consisting of a UHPLC system on one side and a hybrid quadrupole time-of-flight mass spectrometer (Agilent 6,546, Agilent Technologies, Massy, France) equipped with an ESI source on the other, was used. The MS/MS spectra obtained were interpreted both manually and through molecular networking. Additionally, to elucidate their chemical structure, the isolated metabolites were analyzed using the same system.

Analyses were performed in positive mode with an Acquity BEH® C_{18} analytical column. The gradient was linear, varying from 5% B to 100% B over 12 minutes at a flow rate of 500 µL/min. For the next 4 minutes, the gradient maintained 100% B with a flow rate of 600 µL/min. After this step, the gradient returned to the initial conditions (A = Water + 0.1% formic acid; B = Acetonitrile). The wavelengths used in the DAD detector were 210, 254, and 280 nm. A volume of 1 µL was injected for each sample.

Source parameters were set as follows: capillary temperature at 320°C, source voltage at 3500 V, and sheath gas flow rate at 11 L/min. All signals detected during the first 3 min were discarded using the divert valve. MS scans were operated in full scan mode from *m/z* 100 to 1,200 (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 3,000 counts. Selected parent ions were fragmented at collision energy fixed at 45 eV, and at 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)-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 with the internal calibrants, a permanent MS/MS exclusion criterion was set, containing the *m/z* values of the two internal calibrants. MS and MS/MS spectra were displayed on the MassHunter software (Version B.07.00).

II.7. Analytical HPLC-DAD

The metabolite composition of methanolic and dichloromethane crude extracts was analyzed using an Agilent analytical HPLC system (Machelen, Belgium). The HPLC system was composed of the following components:

G1313A for the samples compartment (ALS), G1311A for the quaternary pump, G1322A for degasser, G1316A for column compartment (TCC), and G7117B for DAD. The mobile phase consisted of Milli-Q water with 0.05% trifluoroacetic acid (A) and methanol (B), with the following gradient: from 5% B to 25.4% B between 2 and 3 minutes, from 25.4% to 83.2% B between 3 and 37 minutes, and from 83.2% B to 100% B between 37 and 38 minutes. After maintaining 100% B for 1 minute, the gradient returned to the initial conditions. The analytical column used was Luna® PFP (250 mm x 4.6 mm, 5 µm) from Phenomenex (Utrecht, Netherlands).

The two types of crude extracts were dissolved in methanol to obtain a concentration of approximately 10 mg/mL. After filtration through 0.45 µm pore size filters, the solutions were transferred to HPLC vials for injection. The wavelengths applied to the DAD detector were 210 nm, 254 nm, 280 nm, and 300 nm. The flow rate was set at 1 mL/min, and the injection volume was 10 µL.

II.8. Preparative HPLC-DAD

Purification of the dichloromethane crude extract of *Strychnos longicaudata* trunk barks was initiated by performing four injections on a Varian ProStar preparative HPLC (Prep-HPLC) system, equipped with a diode array detector (DAD) and a Büchi fraction collector C-660 unit. The preparative column used was a Luna® PFP (250 mm x 21.20 mm, 5 µm), purchased from Phenomenex (Utrecht, Netherlands).

For sample preparation, the extracts were dissolved in a mixture of 90% water (A) and 10% methanol (B), then filtered through a 0.45 µm pore size filter before being manually injected. The maximum injection volume was 10 mL, corresponding to the maximum capacity of the injection loop. The wavelengths applied in the DAD detector were 254 and 280 nm.

The mobile phase was composed of water with 0.05% trifluoroacetic acid (pH around 2.12) (A) and methanol (B). A gradient was applied as follows: from 5% B to 25.4% B between 2.12 and 3.19 minutes, from 25.4% B to 83.2% B between 3.19 and 39.29 minutes, and from 83.2% B to 100% B between 39.29

and 40.36 minutes. After maintaining 100% B for 1.06 minutes, the gradient was returned to the initial conditions. The flow rate was set at 20 mL/min.

II.9. Open columns

Open column chromatography was performed using silica gel 60 (0.040-0.063 mm) for column chromatography, purchased from Merck (Darmstadt, Germany). Approximately 100 g of silica gel was used to separate compounds from 1 g of sample. After weighing the sample, it was mixed with the initial mobile phase, consisting of dichloromethane and methanol (14:1 V/V), to form a gel. An absorbent cotton wad was added to the lower part of the empty column. The column was then washed with the initial mobile phase before slowly adding the gel, allowing it to settle and fix in the column. After this stage, the solvent was removed by opening the tap, and a thin layer of sand (UCB, Leuven, Belgium) was added to protect the stationary phase from damage. With a gentle flow of solvent, the sample gradually migrated through the stationary phase, and the compounds separated according to their affinity for the stationary phase and, therefore, their polarity.

Three different mobile phase mixtures were used during purification: dichloromethane and methanol in ratios of 14:1 V/V, 12:3 V/V, and 10:5 V/V, respectively. Initially, the mobile phase was composed of the 14:1 V/V mixture. After the sample had migrated with the first mobile phase, the second mobile phase (12:3 V/V mixture) was added, and the final mobile phase (10:5 V/V mixture) was used to complete the purification. These gradients were optimized in preliminary tests using silica gel 60 F_{254} TLC plates (Merck, Darmstadt, Germany).

UV monitoring at 366 nm was used to track compound separation within the column. Fractions were collected in test tubes at the column exit, and their contents were analyzed on silica gel 60 F_{254} TLC plates (0.2 mm) to determine their purity and group similar fractions together. Two different mobile phases were used for the TLC analysis: the first was a mixture of methanol, 25% sodium acetate, and acetone (6.5:3.5:2 V/V/V), and the second was a mixture of dichloromethane and methanol (12:3 V/V). The fractions were then evaporated using a Rotavapor® and Multivapor® (Büchi) and dried at room

temperature in a vacuum oven (Heraeus, Hanau, Germany, and Memmert, Bruxelles, Belgium) for 2 days. The mass of the fractions was determined after leaving the vacuum oven.

II.10. Preparative TLC

Another method of purifying the compounds involved preparative TLC. These TLCs were performed either on plates coated with silica gel 60 F_{254} (0.5 mm) for preparative TLC (PLC) or on plates coated with silica gel 60 F_{254} (0.2 mm) for small-scale purification. The sample was applied in a continuous line, 1 cm from the bottom of the plate, using 10 µL capillaries. Migration occurred over a distance of 18 cm. The mobile phase used was a mixture of methanol, 25% sodium acetate, and acetone (6.5:3.5:2 V/V/V).

After migration was complete, the spots were visualized under UV at 366 nm and 254 nm. They were carefully scraped off with a spatula and collected into glass test tubes. To wash the silica, a mixture of bidistilled methanol and acetone (50:50 V/V) was used. If this solution was not effective in fully extracting the metabolites from the silica, a few drops of ammonia were added to adjust the pH to around 8, and the compounds were then extracted using ethyl acetate. If any residual compounds remained, the solution was acidified with a few drops of hydrochloric acid and methanol was added to facilitate extraction.

An ultrasonic bath from EMAG Technologies® was used to aid in dissolving the metabolites and detaching them from the silica. Each fraction was centrifuged using an Eppendorf Centrifuge 5804 model at 5,000 rpm for 5 minutes, and the supernatant was collected in tared test tubes to ensure the removal of silica traces from the solution. The purity of the fractions was checked using silica gel 60 F_{254} TLCs (0.2 mm), and similar fractions were grouped together. Two mobile phases were used for TLC analysis: the first was a mixture of methanol, 25% sodium acetate, and acetone (6.5:3.5:2 V/V/V), and the second was a mixture of dichloromethane and methanol (12:3 V/V).

Evaporation was carried out using a Multivapor® (Büchi), and the fractions were dried in a vacuum oven (Heraeus, Hanau, Germany; Memmert, Bruxelles,

Belgium) at room temperature for two nights. The mass of each fraction was determined after drying in the vacuum oven.

II.11. NMR analyses

To elucidate the structure of isolated metabolites, in addition to MS and MS/MS analyses described above, NMR spectra were recorded using a Bruker Avance III HD 700 MHz, equipped with a helium-cooled probe (cryoprobe) (Bruker, BioSpin GmbH, Germany). A volume of 200 µL of deuterated methanol (CD_3OD, d_4 + 0.03% TMS) was added to the whole fraction to dissolve it. A minimal volume of solvent was added to concentrate the fraction as much as possible. The solution was placed in 3 mm diameter NMR tubes, which came from CortecNet (Les Ulis, France). ^{1}H, COSY, ^{13}C, HSQC, HMBC, and TOCSY spectra were acquired with 128, 4, 5120, 8, 64, and 8 scan numbers, respectively, using standard Bruker parameters. NMR spectra were displayed and interpreted using MestReNova software (Version 15.0.1).

II.12. Antiplasmodial assays

The methodology for maintaining *in vitro* cultures of *Plasmodium falciparum* in the asexual erythrocyte stage follows the procedure described by Trager and Jensen (1976). The 3D7 strain of *P. falciparum*, which is chloroquine-sensitive, was obtained from the Malaria Research and Reference Reagent Resource Center (MR4). The culture medium was RPMI 1640 (Gibco, Fisher Scientific, Merelbeke, Belgium) supplemented with $NaHCO_3$ (32 mM), HEPES (25 mM), and L-glutamine. The host cells used were human red blood cells (A+ or O+). The medium was further supplemented with 1.76 g/L glucose (Sigma-Aldrich, Machelen, Belgium), 44 mg/mL hypoxanthine (Sigma-Aldrich, Machelen, Belgium), 100 mg/L gentamycin (Gibco, Fisher Scientific, Loughborough, U.K.), and 10% human pooled serum (A+ or O+), as previously described.

Crude extracts were dissolved in DMSO at a concentration of 10 mg/mL, while fractions and isolated metabolites were dissolved in ethanol at concentrations of 10 mg/mL and 1 mg/mL, respectively. These solutions were then diluted in the culture medium. Two-fold dilutions were performed

eight times on a 96-well plate, resulting in a maximum concentration of 100 µg/mL for crude extracts and fractions, and 10 µg/mL for isolated compounds. Each sample was tested in duplicate. Artemisinin (Sigma-Aldrich, Machelen, Belgium) was used as a positive control at an initial concentration of 100 ng/mL for all assays.

After exposing the parasites to the diluted samples and incubating for 48 hours, the impact on parasite growth was assessed using SYBR Green, a DNA intercalating compound Dery *et al.* (2015). To prepare the SYBR Green solution, it was mixed with a lysis buffer composed of TRIS buffer (Sigma-Aldrich, Machelen, Belgium), EDTA (Merck, Darmstadt, Germany), saponin (Alfa Aesar, Karlsruhe, Germany), and Triton (Merck, Darmstadt, Germany). For the lysis buffer, 500 mL contained 1.20 g TRIS buffer, 0.73 g EDTA, 40 mg saponin, and 0.4 mL Triton.

To reveal the antiplasmodial activities, 2 µL of SYBR Green solution were diluted in 10 mL of lysis buffer. Then, 100 µL of assay solutions were placed in 96-well plates, and 75 µL of the SYBR Green solution were added. The plates were incubated for 2 hours and then read using the FlexStation® device (Molecular Devices, Winnersh, United Kingdom) at wavelengths of 490 nm (excitation) and 530 nm (emission).

The half-maximal inhibitory concentration (IC_{50}) values were calculated from graphs. The averages of three IC_{50} values from three independent experiments (n = 3) conducted on different days were calculated. To interpret the antiplasmodial activity levels of extracts or fractions, we have implemented the following classification, which is inspired by the World Health Organization's guidelines, with a few modifications (Jansen, O. *et al.*, 2012): an activity ≤ 5 µg/mL was considered as a promising antiplasmodial activity, between 5 µg/mL and 15 µg/mL as a good activity, between 15 µg/mL and 30 µg/mL as a moderate activity, between 30 µg/mL and 50 µg/mL as a weak activity, and ≥ 50 µg/mL as a lack of activity. For isolated compounds, IC_{50} values ≤ 1 µM, between 1 and 10 µM, and > 10 µM indicate promising, moderate, and weak activity, respectively. For compounds whose molar mass could not be determined, the same reference values, expressed in µg/mL, were applied.

III. RESULTS AND DISCUSSION

III.1. Extraction yields

The yields obtained from manual methanolic, dichloromethane, and alkaloidic extractions were 6.9% w/w, 2.3% w/w, and 0.2% w/w, respectively.

III.2. Antiplasmodial activities of crude extracts from *S. longicaudata* trunk barks

For artemisinin, the mean IC_{50} value was 3.092 ± 1.570 ng/mL, confirming the validity of the IC_{50} values obtained (Ledoux, A. *et al.*, 2017). The IC_{50} values from the *in vitro* antiplasmodial assays of the methanolic, dichloromethane, and alkaloidic crude extracts were 13.7 ± 2.01 µg/mL, 4.94 ± 2.51 µg/mL, and 1.77 ± 0.489 µg/mL, respectively. While the methanolic crude extract exhibited good antiplasmodial activity (5-15 µg/mL), the dichloromethane and alkaloidic crude extracts demonstrated promising activity (≤ 5 µg/mL). Since alkaloidic crude extracts generally have lower yields than dichloromethane crude extracts, and their extraction process is lengthy and tedious, the dichloromethane crude extract was chosen for further purification.

III.3. Feature-based molecular network of dichloromethane crude extract

The molecular network of the dichloromethane crude extract from the trunk barks of *Strychnos longicaudata* comprises 1,339 nodes (each representing a metabolite) and 1,609 edges (indicating connections between nodes). Only five suggested identifications were proposed based on shared spectral databases: longicaudatine, Wieland-Gumlich aldehyde, venecurine, strychnine, and an unidentified metabolite with the molecular formula $C_{16}H_{24}O_3$ (Figure 56). The molecular network presented in this study is accessible on GNPS using the following link: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=f77f06b158614c49bbe1d44358ed6dd2.

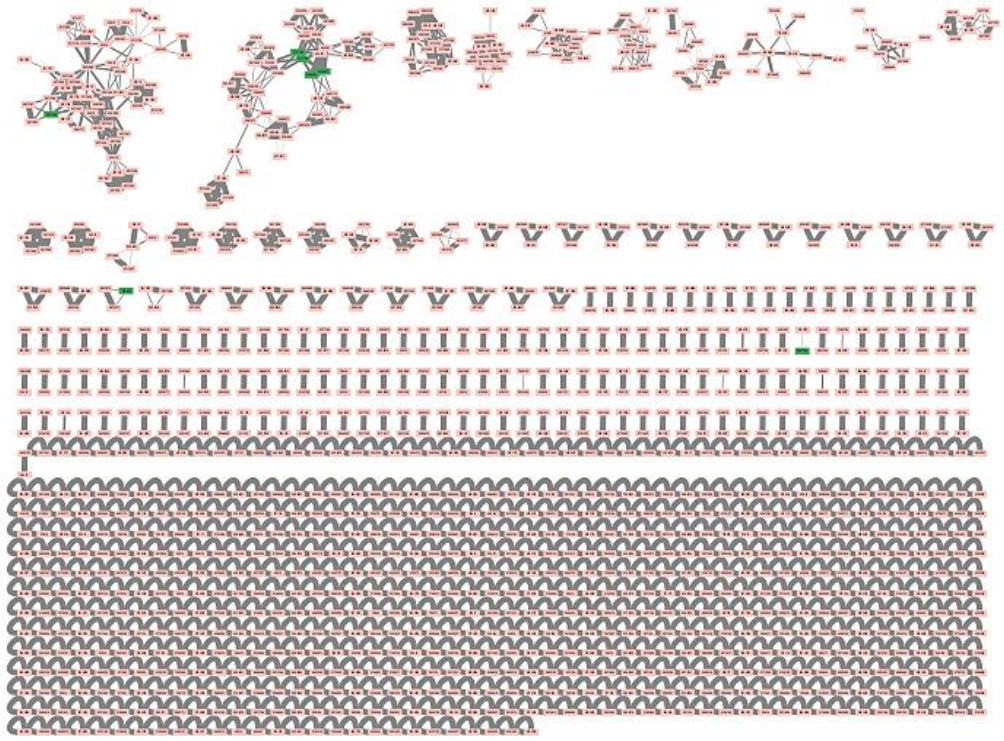


Figure 56: Feature-based molecular network of the dichloromethane crude extract from *S. longicaudata* trunk barks. The nodes in green are the annotated metabolites, while those in red are the unidentified ones.

Among the metabolites suggested by the molecular network are longicaudatine and Wieland-Gumlich aldehyde, two compounds previously reported in *Strychnos longicaudata* as well as other *Strychnos* species. In contrast, identifications such as venecurine and strychnine have never been reported within this species. The limited number of identified compounds may be attributed to the low representation of MS/MS spectra from *S. longicaudata* compounds in GNPS databases.

III.4. MixONat analyses of dichloromethane and alkaloidic crude extracts

First, a MixONat analysis was conducted on the carbon spectra of the crude extract obtained using dichloromethane from the trunk bark of *S. longicaudata*. Unfortunately, most of the metabolites suggested by the software belonged to

the phytochemical class of sterols. Among these, a sitosterol derivative was identified as the most probable candidate. Sitosterols are well-known compounds in the plant kingdom. For instance, β-sitosterol has been identified in various species of the *Strychnos* genus, including the stem barks of *S. afzelii*, *S. dolichothyrsa*, and *S. floribunda*, the root barks of *S. innocua*, the seeds of *S. potatorum*, and in the leaves of *S. spinosa* (Singh, H. *et al.*, 1975; Siwon, J. *et al.*, 1977; Ohiri, F. C. *et al.*, 1983; Verpoorte, R. *et al.*, 1980; Verpoorte, R. *et al.*, 1981; Hoet, S. *et al.*, 2007; Uttu, A. J. *et al.*, 2023).

To focus on the phytochemical class of interest, namely alkaloids, the metabolite content of the alkaloidic extract was also analyzed using MixONat. Among the suggested identifications, 28 showed probabilities exceeding 70%. This probability reflects the percentage of carbons in the database spectra that matched those in the experimental spectra. Identifications with probabilities below 70% were excluded from further analysis. Among the 28 suggested identifications, five metabolites (bisnordihydrotoxiferine, normavacurine, longicaudatine, longicaudatine Y, and longicaudatine F) have been previously reported in *S. longicaudata*, and they presented the following probabilities: 95% for bisnordihydrotoxiferine, 79% for normavacurine, 71% for longicaudatine, 74% for longicaudatine Y, and 71% for longicaudatine F (Massiot, G. *et al.*, 1983a; Massiot, G. *et al.*, 1989; Delaude, C., and Delaude, L., 1997).

Other suggested metabolites included compounds that have been described in the *Strychnos* genus but not specifically in *S. longicaudata*. These include geissoschizol, tubotaiwinal, yohimb-19-ene, normacusine B, retuline and/or isoretuline, N-desacetylretuline and/or N-desacetylisoretuline, 4',17-dihydro-17α-tchibangensine and/or 4',17-dihydro-17β-tchibangensine, among others. Manual comparisons of the ^{13}C APT carbon spectra from databases with those of isolated compounds revealed significant similarities for some metabolites, including geissoschizol, tubotaiwinal, and normacusine B. This suggests that these compounds are either present in *S. longicaudata* or that the species contains structurally similar metabolites. Additionally, masses corresponding to these compounds were observed in the molecular network described earlier. However, while geissoschizol and normacusine B are listed in the Monoterpene

Indole Alkaloid Database (MIADB), the software was unable to confidently associate these compounds with the spectra.

To conclude, it is crucial to emphasize that these results should be interpreted with caution. Neither molecular networking nor MixONat analyses are definitive for metabolite identification. Further analyses, or bio- and mass-guided fractionation, are required to validate these hypotheses.

All results and suggested identifications are presented in Supplementary Figure S1 and Table S1, included in the supplementary material accompanying this article.

III.5. Bio- and mass-guided fractionation of dichloromethane crude extract (Figure 57)

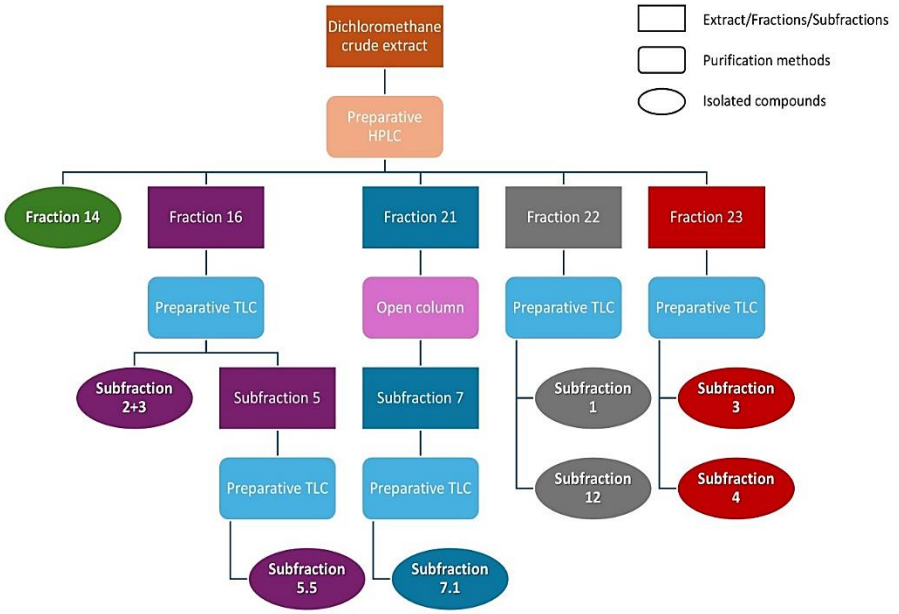


Figure 57: Overview of purification stages.

III.5.1. Preparative HPLC

The dichloromethane crude extract of *S. longicaudata* stem barks was first purified by preparative HPLC. A total of four injections, with a total mass of

1.42 g, were performed, and the collected fractions were grouped into thirty groups. Among these, fraction 14 exhibited good purity in TLC and HPLC analyses and was therefore directly analyzed by MS/MS and NMR to determine its chemical structure. This compound is the primary component of the dichloromethane crude extract from *S. longicaudata* trunk barks.

The remaining fractions were analyzed using MS and MS/MS to generate a molecular network. Using this methodology, the metabolite content was explored, leading to the selection of fractions rich in unidentified compounds. In addition to the identifications provided by the molecular networks, the major masses were further analyzed using CHEMnetBase's Natural Product Dictionary (CHEMnetBASE, 2023). A biological criterion was also incorporated into this study. Specifically, the fractions were tested against the 3D7 strain of *P. falciparum*. Only those fractions that showed promising or good antiplasmodial activity were selected for further purification. At the end of this process, a total of twenty-three fractions were chosen for further analysis.

III.5.2. Open columns

Fraction group 21, which demonstrated good activity (IC_{50} between 5 and 15 µg/mL) against the 3D7 strain of *P. falciparum* (IC_{50} = 9.42 µg/mL), contained a compound that exhibited a clearly visible pink coloration under UV light at 366 nm and a very light pink coloration under visible light. An example of the TLC analysis of the pink compound after purification, shown under UV at 366 nm, is provided in Supplementary Figure S2 (Supplementary material). To identify this metabolite, the fraction was purified using an open column previously packed with silica. Fifty-eight subfractions were collected and grouped into twenty-nine fractions. TLC analysis revealed that the pink compound was present in the subfraction 7 (SL21 GR7). However, the purity was insufficient for structural determination, so further purification was carried out using preparative TLC.

III.5.3. Preparative TLC

The purification by preparative TLC of several fractions allowed the isolation of compounds with sufficient purity to elucidate their chemical structure. Fractions 16, 22, and 23 exhibited promising or good antiplasmodial activities (IC_{50} ≤ 5 µg/mL, and between 5 and 15 µg/mL, respectively) against the 3D7 strain of *P. falciparum*, with IC_{50} values of 1.88 µg/mL, 5.50 µg/mL, and 3.06 µg/mL, respectively. Furthermore, the analysis of the metabolite content of these three fractions revealed the presence of the following major metabolites:

- Fraction 16:
    - *m/z* 349.1548 (Potential identification: Alstonine);
    - *m/z* 565.2958 (No identification).
- Fraction 22:
    - *m/z* 442.3378 (No identification);
    - *m/z* 547.2498 (No identification).
- Fraction 23:
    - *m/z* 339.2072
        - Many potential identifications:
            - Akagerine; Me ether;
            - Alstonerine; 20α,21-Dihydro;
            - Geissoschizol; O-Ac;
            - Quinicine; Demethoxy, 9R-alcohol, N1-Ac;
            - Quinine; O-De-Me, O6'-Et;
            - Retuline;
            - Retuline; 16-Epimer;
            - Sarpagine; 10-Me ether, N1-Me;
            - Secodine;
            - Spermostrychnine;
            - Spermostrychnine; 19-Epimer.
    - *m/z* 500.3796 (No identification).

Finally, concerning fraction 16, it contained an intriguing metabolite that turns fluorescent yellow-orange under UV light at 366 nm. An example of TLC analysis of the yellow-orange compound under UV at 366 nm is shown in

Supplementary Figure S3 in the Supplementary material. Such compounds have never been detected or identified in *S. longicaudata*, prompting the decision to target this compound during the purification of fraction 16.

Preparative TLC (0.5 mm) of fractions 16, 22, and 23 produced 18, 12, and 12 subfractions, respectively. The subfractions 2 and 3 from fraction 16 (SL16 GR2+3), 1 and 12 from fraction 22 (SL22 GR1 and SL22 GR12), as well as 3 and 4 from fraction 23 (SL23 GR3 and SL23 GR4), were selected for further study to elucidate their chemical structures. While their purity was not optimal, the TLC, NMR, and MS analyses suggested that it could still be sufficient to identify the compounds in these subfractions.

Two additional preparative TLC runs were performed on "conventional" TLC plates (0.2 mm) to minimize metabolite losses due to silica retention. These runs targeted the subfraction 5 from fraction 16 (SL16 GR5, characterized by the yellow-orange spots) and the subfraction 7 from fraction 21 (SL21 GR7, characterized by the pink spot). While the subfraction SL16 GR5 was obtained through preparative TLC, the subfraction SL21 GR7 was isolated using an open column. Sufficient purity was eventually achieved for both target compounds during this additional purification step. The subfractions SL16 GR5.5 and SL21 GR7.1 were selected for MS/MS and NMR analyses.

III.6. Structural elucidation of the isolated metabolites

III.6.1. MS/MS analyses

MS and MS/MS spectra of the isolated metabolites not shown in the article can be found in the Supplementary material (Figures S6, S9, S11, S13, and S16). The MS and MS/MS spectra of the fraction 14, as well as the subfractions SL23 GR3 and SL16 GR5.5, discussed in detail later in the article, are shown in Figures 58, 59, and 60. Additionally, the table below summarizes the interpretations of the mass spectra for all isolated metabolites (Table 20).

Table 20: Interpretation of mass spectra of the isolated compounds.

Fractions (from preparative HPLC)	Subfractions (from open column and/or preparative TLC)	Masses observed
14 (Weighed mass: 1.10 mg)	/	*m/z* 349.1551
16 (Weighed mass: 7.85 mg)	2 and 3 (Weighed mass: 35.1 mg, with silica contamination)	*m/z* 563.2820 (Dimer)
	5.5 (Weighed mass: 28.0 mg, with silica contamination)	*m/z* 340.2613 *m/z* 565.2592 (Dimer) *m/z* 663.4564
21 (Weighed mass: 10.9 mg)	7.1 (Weighed mass: 3.08 mg, with minor silica contamination)	*m/z* 219.1753 *m/z* 234.2071 *m/z* 258.2800 *m/z* 445.3848 *m/z* 566.4284
22 (Weighed mass: 15.6 mg)	1 (Weighed mass: 22.3 mg, with silica contamination)	*m/z* 580.2458 (Dimer) *m/z* 582.2616 (Dimer)
	12 (Weighed mass: 5.07 mg, with minor silica contamination)	*m/z* 442.3397
23 (Weighed mass: 18.8 mg)	3 (Weighed mass: 14.3 mg, with silica contamination)	*m/z* 339.2086 *m/z* 615.3352 (Dimer)
	4 (Weighed mass: 8.10 mg, with silica contamination)	*m/z* 585.3242 (Dimer) *m/z* 615.3348 (Dimer)

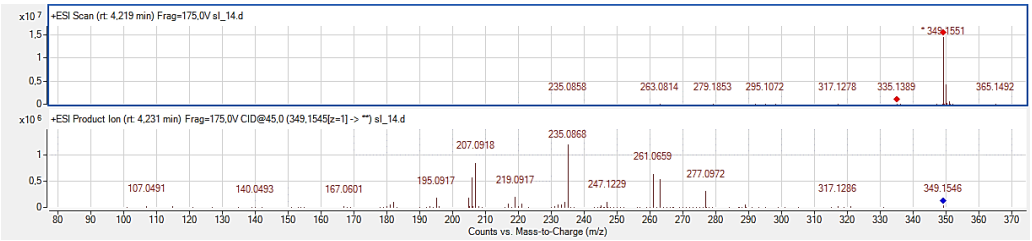


Figure 58: Mass spectra of the fraction 14 (*m/z* 349.1551).

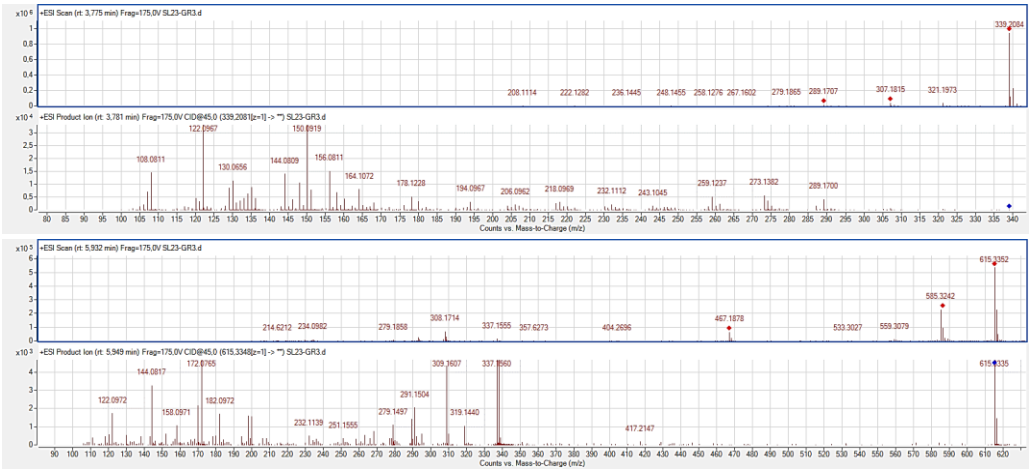


Figure 59:

Top part: Mass spectra of the subfraction SL23 GR3 (*m/z* 339.2086).
Bottom part: Mass spectra of the subfraction SL23 GR3 (*m/z* 615.3352).

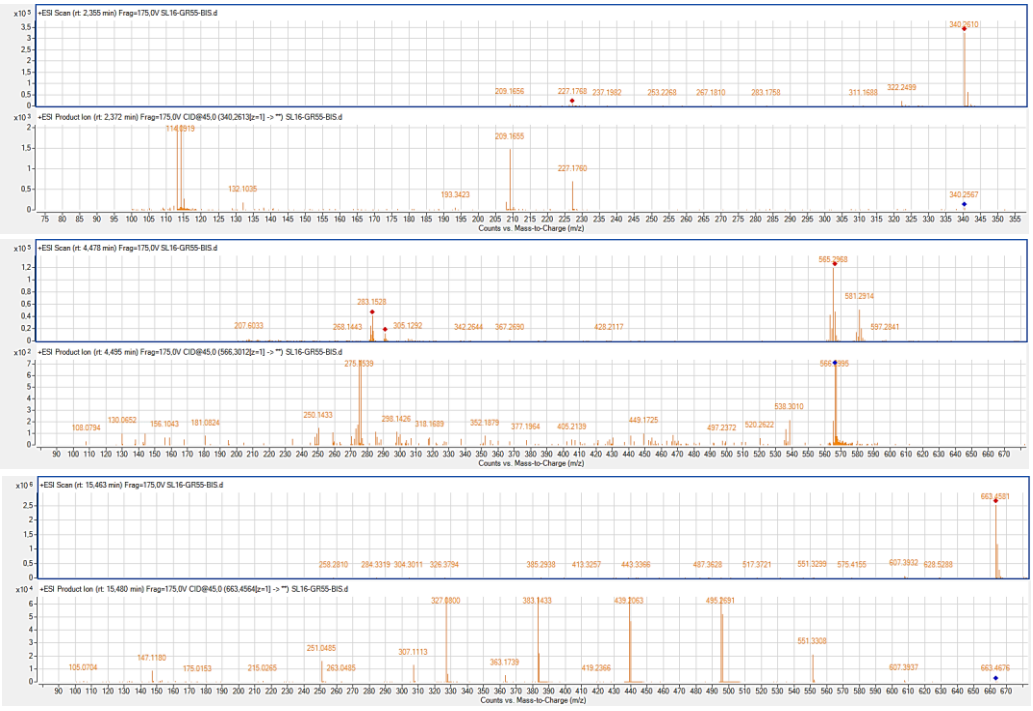


Figure 60:

Top part: Mass spectra of the subfraction SL16 GR5.5 (*m/z* 340.2613).
Center part: Mass spectra of the subfraction SL16 GR5.5 (*m/z* 566.3012).
Bottom part: Mass spectra of the subfraction SL16 GR5.5 (*m/z* 663.4564).

Interpretations revealed that the isolated compounds are predominantly dimeric alkaloids. Additionally, the fraction 14 was assigned the molecular formula $C_{21}H_{21}N_2O_3$ with a confidence score of 100% (Figure 61). This specific mass could not be identified in the available databases, suggesting that it may correspond to a novel metabolite. Consequently, NMR spectra are essential for elucidating their chemical structure. Regarding the subfraction SL21 GR7.1, the structural elucidation process is likely to be challenging due to the number of masses observed and its lower-than-expected purity.

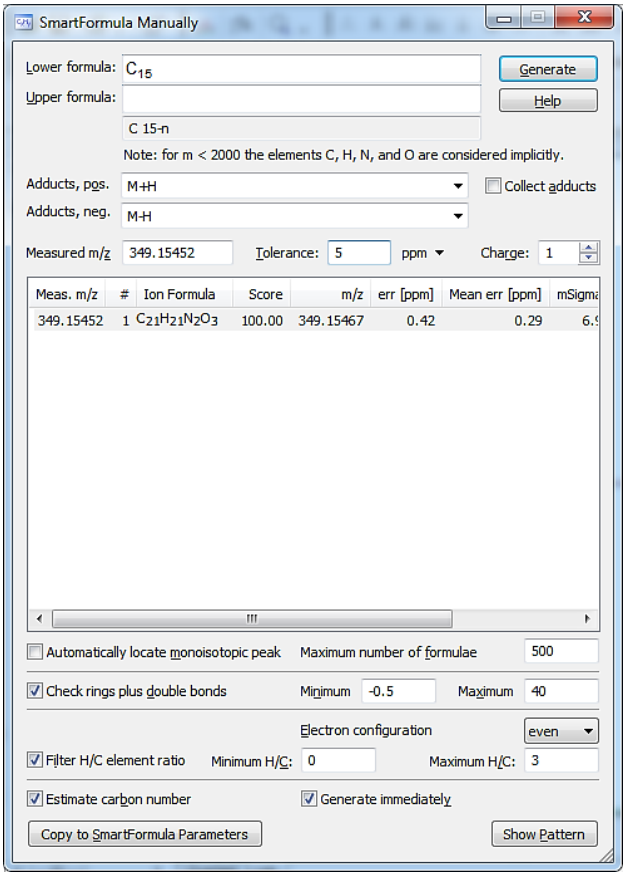


Figure 61: Proposed molecular formula for the fraction 14.

III.6.2. NMR analyses

NMR, MS, and MS/MS spectra not shown in the article can be found in the Supplementary Material of this article. For several isolated compounds,

unfortunately, none or very few signals were observable in the ^{13}C APT carbon spectrum due to insufficient quantities. To address this issue, the chemical shifts of the carbons were determined using HSQC and HMBC spectra. Consequently, in the Supplementary Material, the HSQC and HMBC spectra are presented instead of the ^{13}C APT carbon spectra.

Fraction 14:

The table below (Table 21) lists all observed carbon and proton chemical shifts, multiplicities, carbon types, as well as H/H (COSY) and C/H (HMBC) correlations in the NMR spectra of the fraction 14 (Figures 62, 63, 64, 65, and 66).

Table 21: Interpretation of NMR spectra of the fraction 14.

Positions	^{1}H (ppm)	H/H correlations (COSY)	^{13}C (ppm)	C/H correlations (HMBC)	^{13}C Attached-Proton-Test (APT)
2	/	/	132.15	6, 14	Quaternary
3	/	/	134.02	5, 15, 21	Quaternary
5	8.07 (t)	6	131.03	21	CH
6	8.10 (d)	5	115.44	/	CH
7	/	/	140.10	5, 9	Quaternary
8	/	/	131.64	6, 10, 12	Quaternary
9	7.69 (t)	10	119.56	11	CH
10	7.37 (t)	9, 11	122.14	12	CH
11	8.06 (t)	10, 12	122.32	9	CH

12	7.77 (d)	11	114.07	10	CH
13	/	/	144.64	9, 11	Quaternary
14	3.26 (dd) 4.20 (dd)	15	28.89	20	CH_2
15	3.03 (q)	14, 20	26.16	17, 19, 21	CH
16	/	/	107.60	14, 20	Quaternary
17	7.60 (s)	/	155.41	15, 19	CH
18	1.44 (d)	19	18.20	20	CH_3
19	3.83 (m)	18, 20	70.70	15, 17, 21	CH
20	2.43 (m)	15, 19, 21	35.74	14, 16, 18	CH
21	4.39 (dd), 4.84 (dd)	20	54.81	5, 15, 19	CH_2
22	/	/	166.84	15, 17, 23	Quaternary
23	3.79 (s)	/	51.68	/	CH_3

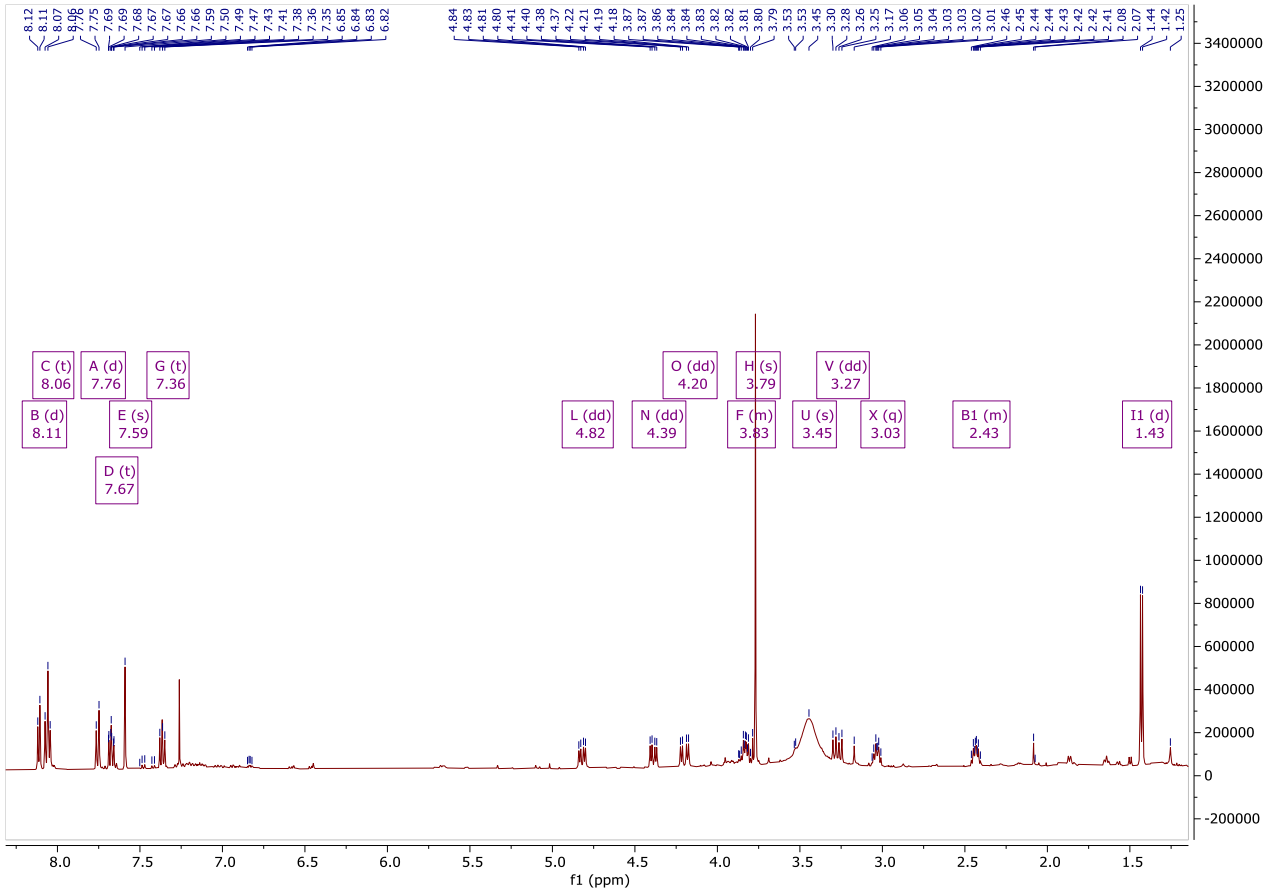


Figure 62: ^{1}H spectrum of the fraction 14.

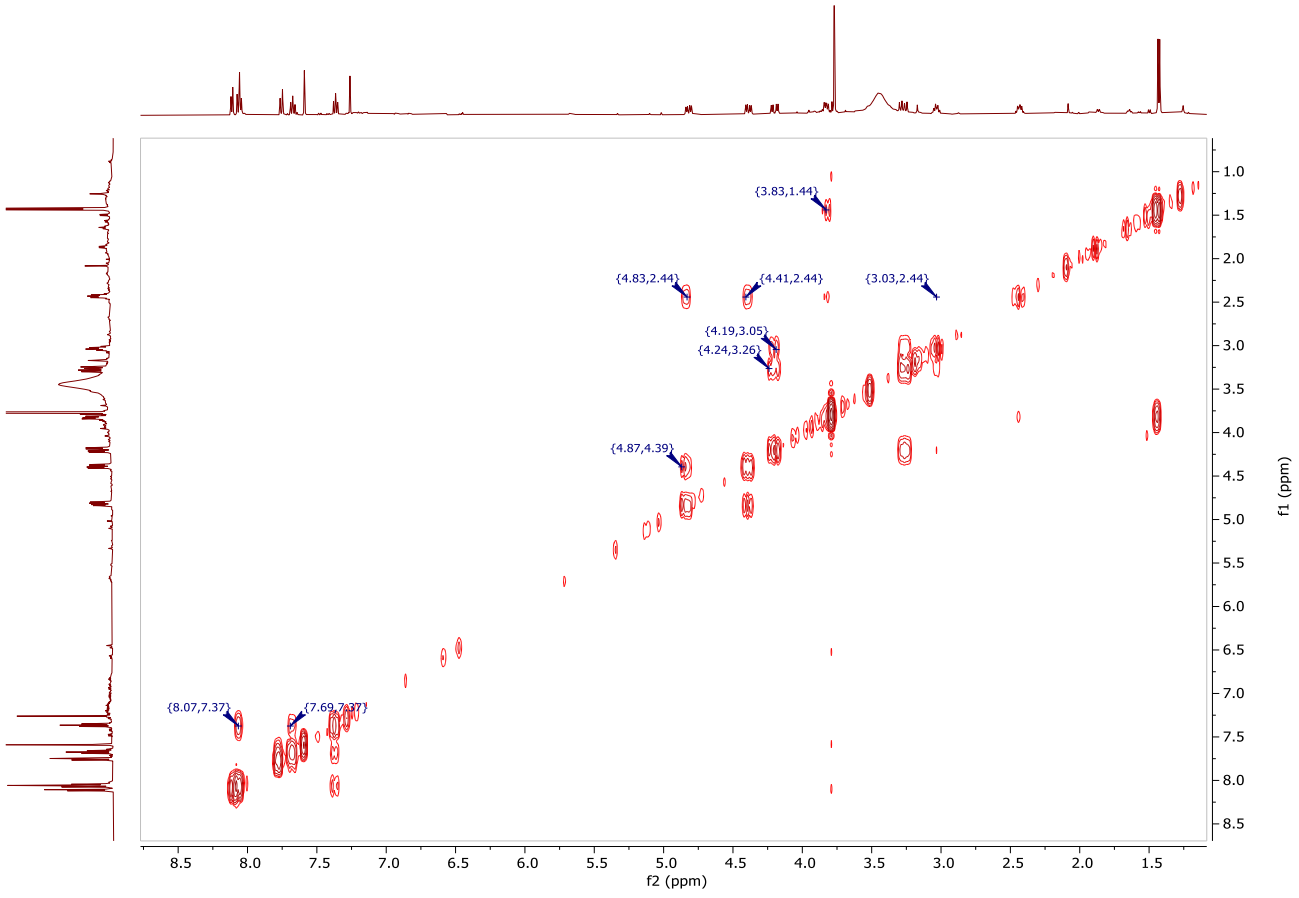


Figure 63: COSY spectrum of the fraction 14.

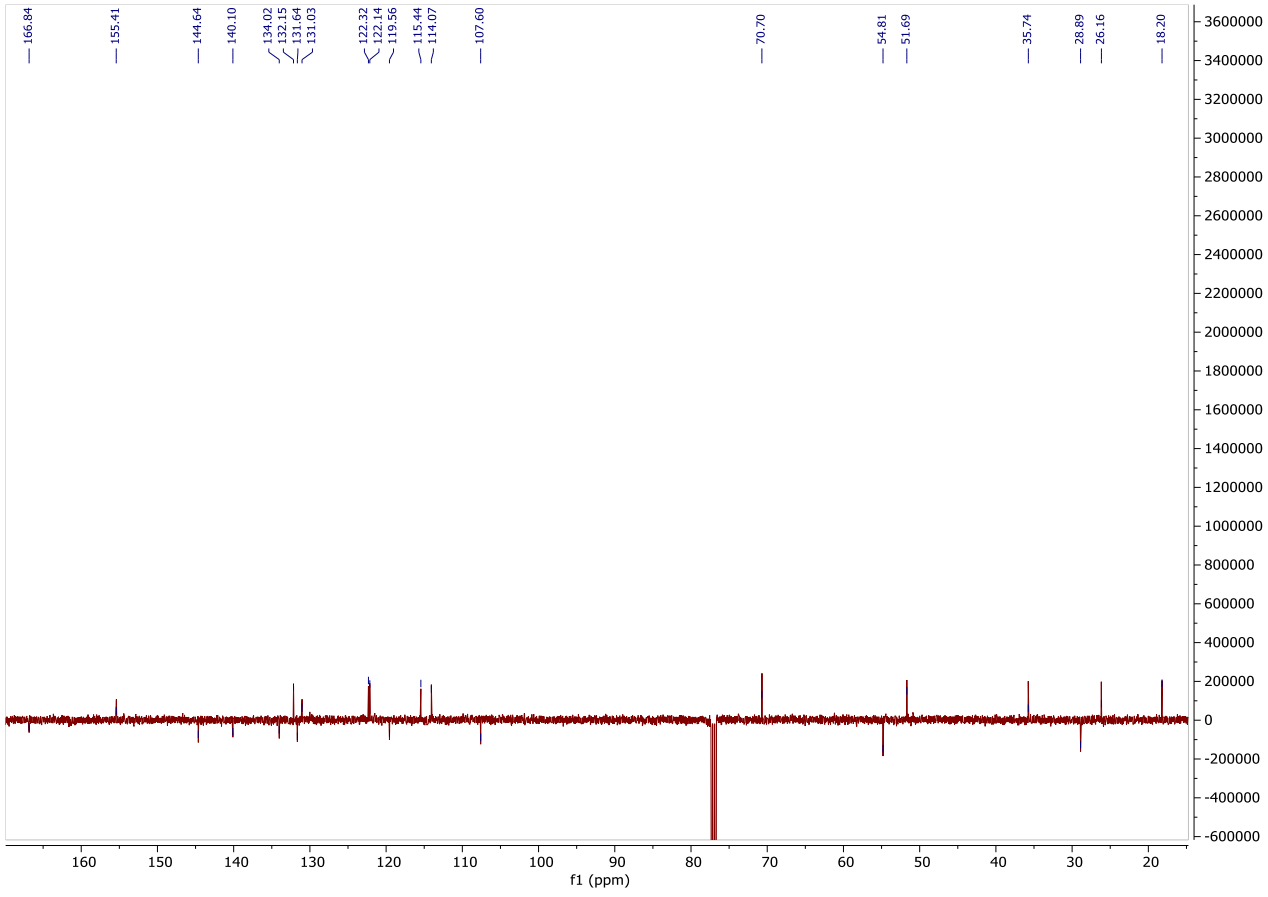


Figure 64: ^{13}C APT spectrum of the fraction 14.

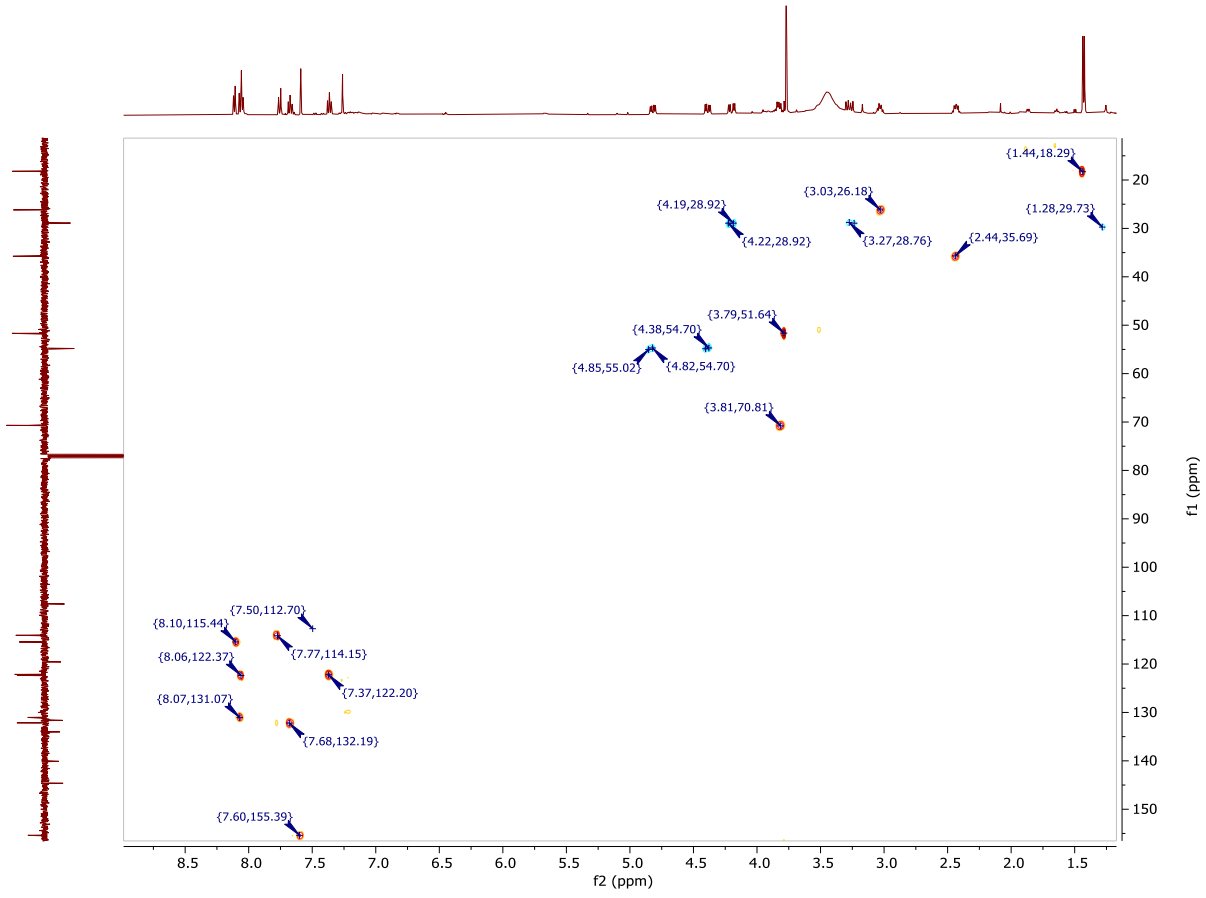


Figure 65: HSQC spectrum of the fraction 14.

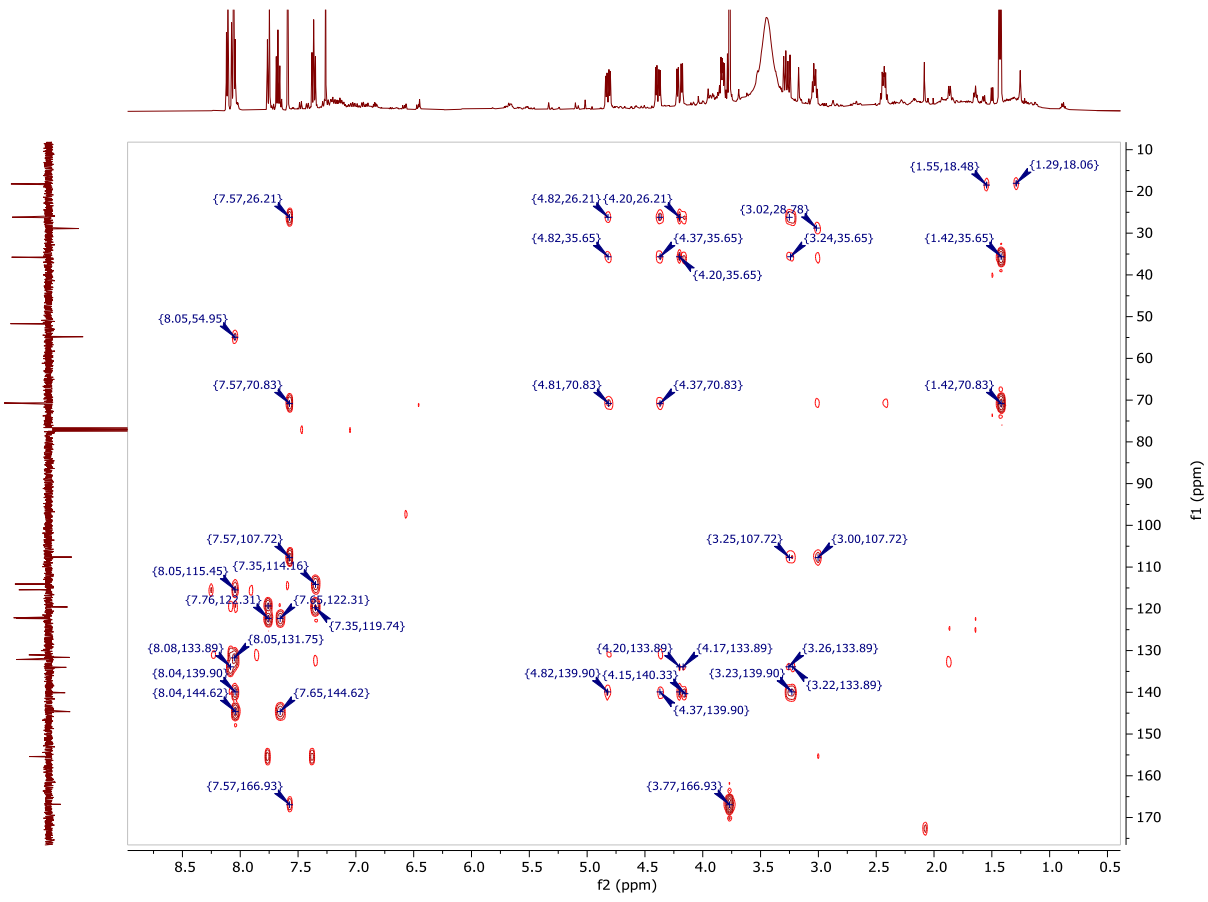


Figure 66: HMBC spectrum of the fraction 14.

Thanks to MS, MS/MS, and NMR data, the compound in the fraction 14 was identified as alstonine (Figure 67). Since the NMR spectra of alstonine were not available in the literature, the identification was performed using predicted spectra from MestReNova, C+H NMR Predictors by ACD/Labs, as well as the NP-MRD (Natural Products Magnetic Resonance Database) (Wishart, D. S. *et al.*, 2022). This alkaloid, already reported in other *Strychnos* species, such as *S. camptoneura* (stem and root barks) and *S. gossweileri* (roots), had never been described in *S. longicaudata* until now (Verpoorte, R., and Sandberg, F., 1971; Coune, C., and Angenot, L., 1978; Ohiri, F. C. *et al.*, 1983; Delaude, C., and Delaude, L., 1997).

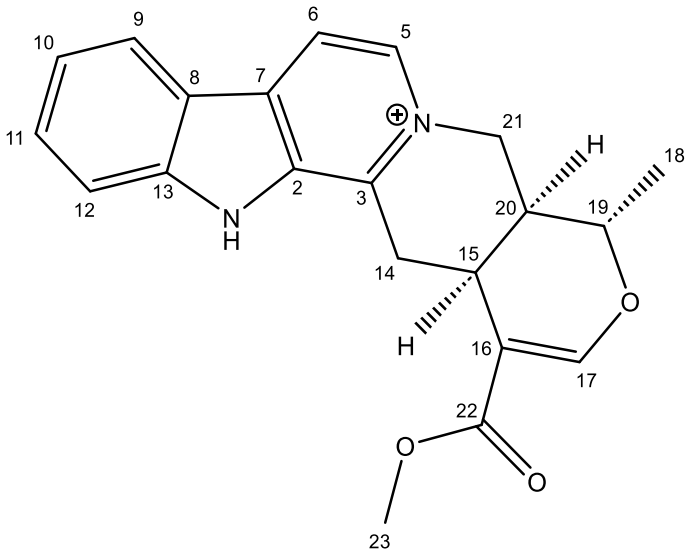


Figure 67: Structure of alstonine.

Returning to the molecular networking study, alstonine had not been identified initially because its MS/MS spectrum was not included in the GNPS databases. After its mass spectrum was added to the databases, alstonine was successfully detected and identified within the molecular network, further confirming its presence in the dichloromethane crude extract.

Subfraction SL23 GR3:

Concerning the subfraction SL23 GR3, two masses were observed: *m/z* 339.2086 and *m/z* 615.3352. Notably, the mass at *m/z* 615.3352 corresponds to a dimer, which could potentially exhibit symmetry. Table 22 summarizes all the chemical shifts, multiplicities, carbon types, as well as H/H (COSY) and C/H (HMBC) correlations in the NMR spectra of the subfraction SL23 GR3 (Figures 68, 69, 70, 71, and 72). However, due to the low quantity of the isolated compound and the presence of impurities, some signals are missing. As a result, some correlations have been inferred from the spectra. These inferred correlations are highlighted in orange.

Table 22: Interpretation of NMR spectra of the subfraction SL23 GR3.

Positions	^{1}H (ppm)	H/H correlations (COSY)	^{13}C (ppm)	C/H correlations (HMBC)	^{13}C Attached-Proton-Test (APT)
C13	/	/	137.45	2, 9, 11, 17’	Quaternary
C13		/		2’, 9’, 11’, 17	
C20	/	/	127.73	14, 16, 18	Quaternary
C20		/		14’, 16’, 18’	
C8	/	/	126.01	2, 3, 6, 10, 12	Quaternary
C8’		/		2’, 3’, 6’, 10’, 12’	
C11	7.19 (td)	10, 12	122.26	9	CH
C11’		10’, 12’		9’	
C19	5.63 (q)	18	120.17	15, 21	CH
C19’		18’		15’, 21’	
C10	7.09 (td)	9, 11	119.42	12	CH
C10’		9’, 11’		12’	
C9	7.5 (dd)	10	117.92	11	CH
C9’		10’		11’	
C12	7.41 (dd)	11	111.32	10	CH
C12’		11’		10’	
C17	4.41 (d)	16	98.22	2, 2’, 15	CH
C17’		16’		2’, 2, 15’	
C2	3.76 (dd)	16	64.49	3, 6, 15, 17	CH
C2’		16’		3’, 6’, 15’, 17’	

C21	4.29 (dd)	/	64.28	3, 5, 15, 19, 27	CH_2
C21’	4.44 (dd)	/		3’, 5’, 15’, 19’, 27’	
C3	4.94 (t)	14	60.66	2, 5, 15, 21, 27	CH
C3’		14’		2’, 5’, 15’, 21’, 27’	
C7	/	/	52.49	5, 9, 13, 14, 16, 17	Quaternary
C7’		/		5’, 9’, 13’, 14’, 16’, 17’	
C22	3.32 (s)	/	47.91	3, 5, 21	CH_3
C22’		/		3’, 5’, 21’	
C16	2.00 (q)	2, 15, 17	47.27	14	CH
C16’		2’, 15’, 17’		14’	
C14	2.14 (td), 2.54 (td)	3, 15	32.09	16	CH_2
C14’		3, 15		16’	
C15	3.23 (q)	14, 16	25.66	2, 3, 17, 19, 21	CH
C15’		14’, 16’		2’, 3’, 17’, 19’, 21’	
C18	1.77 (d)	19	11.79	/	CH_3
C18’		19’		/	
C5	?	6	?	3, 21, 27	CH_2
C5’		6’		3’, 21’, 27’	
C6	?	5	?	2, 3	CH_2
C6’		5’		2’, 3’	

In orange: Missing/inferred signals in the NMR spectra.

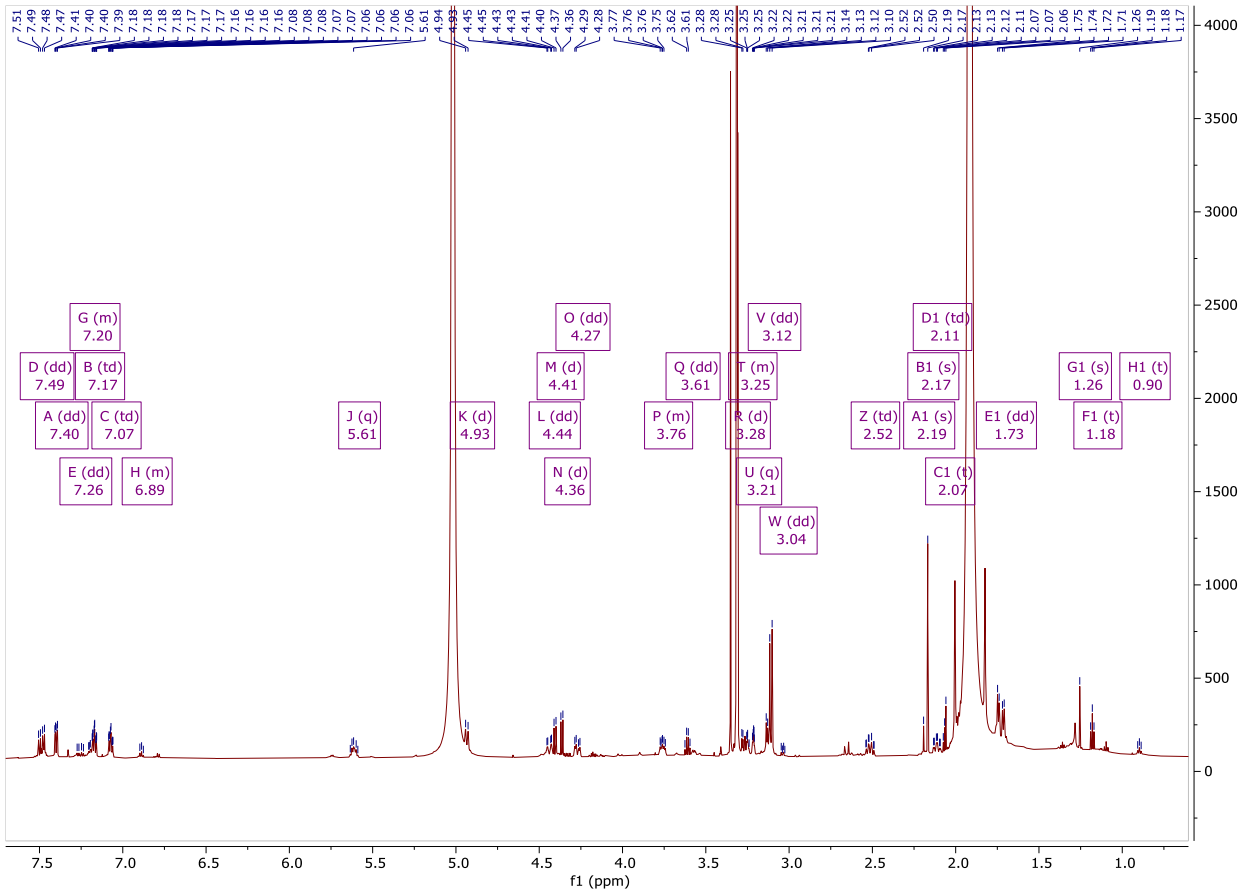


Figure 68: ^{1}H spectrum of the subfraction SL23 GR3.

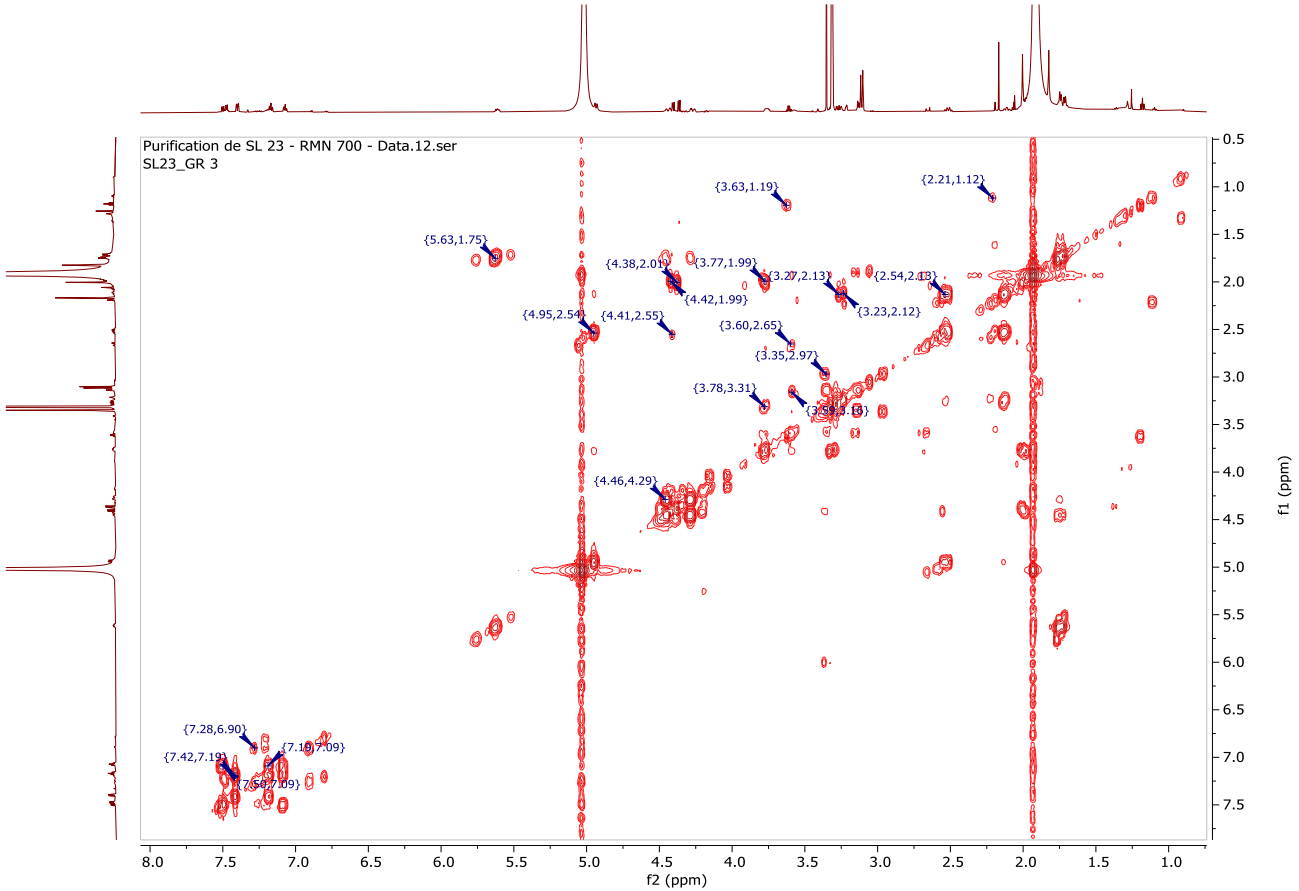


Figure 69: COSY spectrum of the subfraction SL23 GR3.

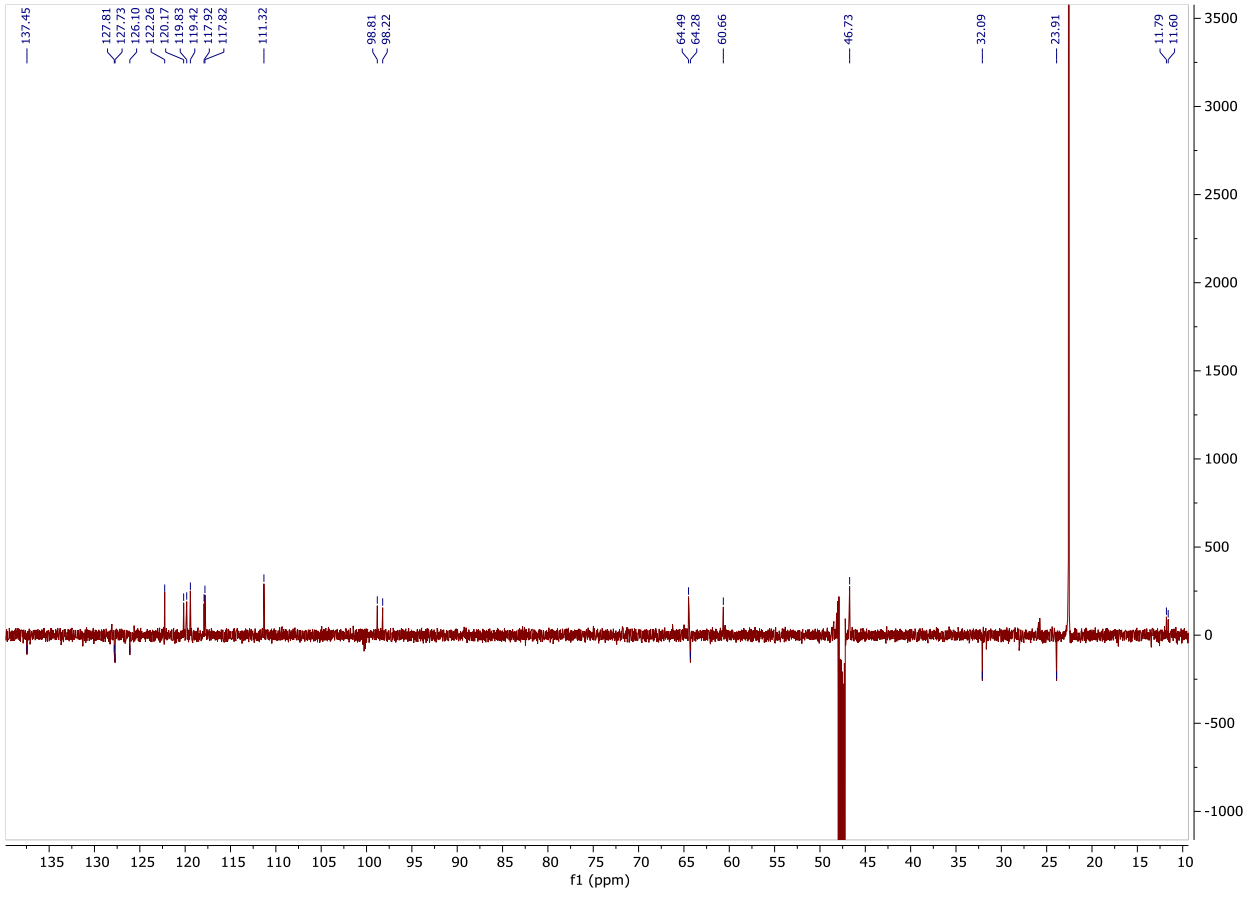


Figure 70: ^{13}C APT spectrum of the subfraction SL23 GR3.

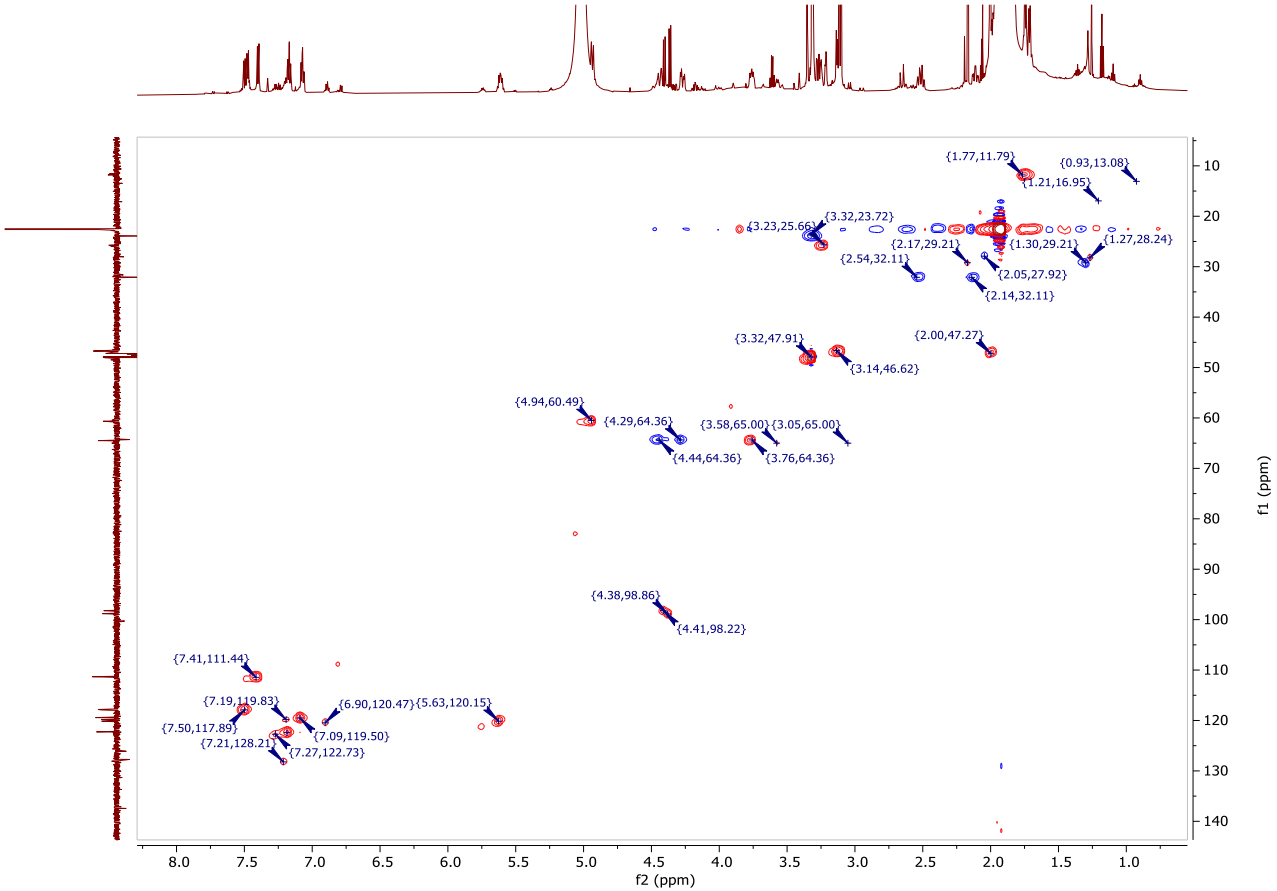


Figure 71: HSQC spectrum of the subfraction SL23 GR3.

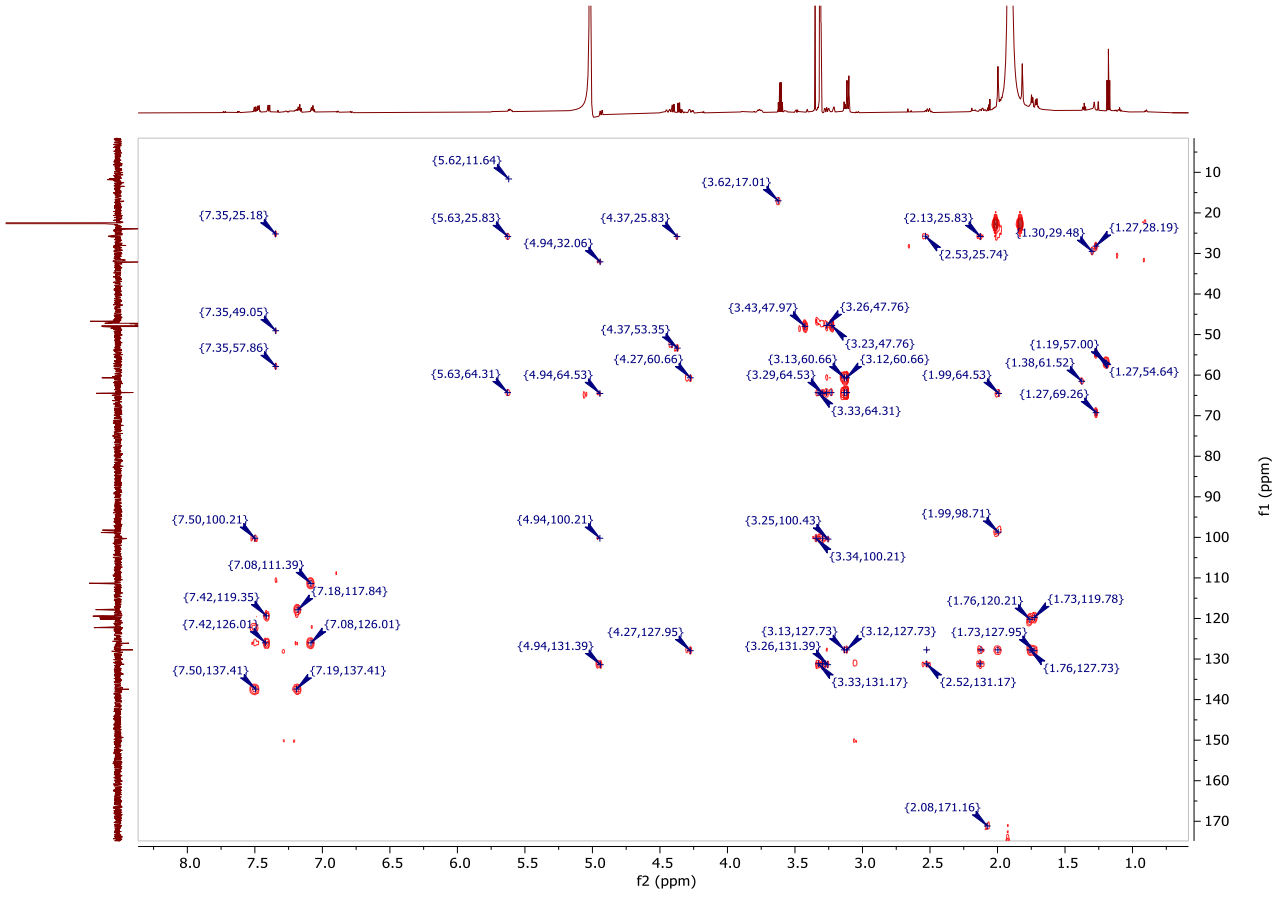


Figure 72: HMBC spectrum of the subfraction SL23 GR3.

The table indicates the presence of symmetry in the structure, suggesting that the metabolite is a dimer with a mass of *m/z* 615.3352. The mass at *m/z* 339.2086 is likely a fragment of this metabolite, generated in the source during analysis.

Although the signals for carbons 5/5' and 6/6' could not be observed in the NMR spectra, the overall data support the hypothesis that the structure is similar to that of matopensine, a symmetrical dimeric alkaloid isolated from *S. matopensis* (Massiot, G. *et al.*, 1983c; Massiot, G. *et al.*, 1988). This hypothesis was developed based on literature data (Massiot, G. *et al.*, 1988). However, one distinctive feature stands out: the presence of a hemiacetal function at the 17/17' carbons (98.22 ppm). Unfortunately, incorporating this function into the structure creates challenges, as it both to obtain the desired mass as well as to maintain the symmetry and the two CH groups at these positions. To resolve this, alternative functional groups were explored. By adding a peroxide function between the two carbons, simulations yielded a chemical shift very close to 98 ppm. This suggests the presence of a peroxide function at the center of the structure, between carbons 17 and 17' (Figure 73).

Lastly, a methyl group (47.91 ppm) is present at the two nitrogens in positions 4 and 4', indicating that the compound is likely a bisquaternary alkaloid (Figure 73).

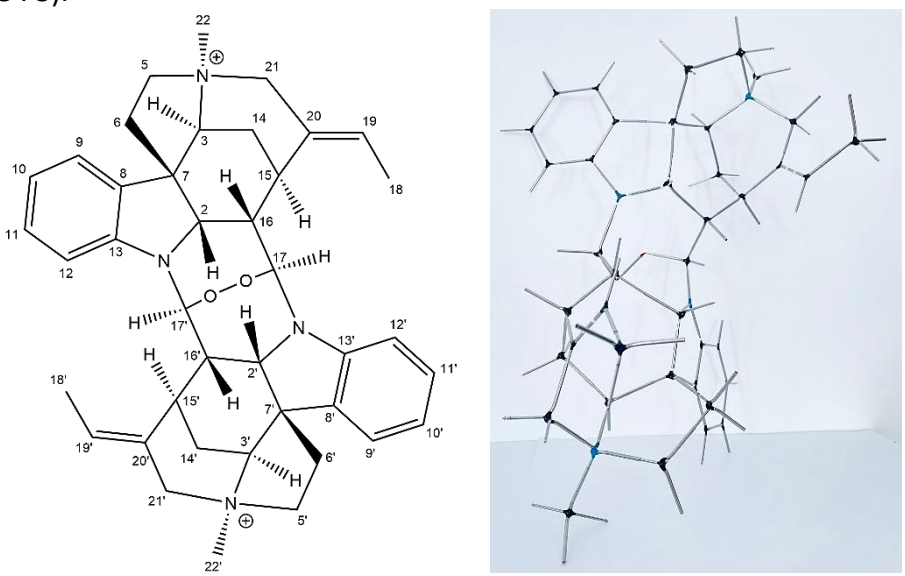


Figure 73: Suggested structure of the subfraction SL23 GR3, derived from matopensine.

Unfortunately, there are missing correlations in the proposed structure, particularly between a carbon at 52–53 ppm (likely corresponding to C7) and carbon 17, which is associated with the peroxide function. Additionally, the absence of signals from carbons 5/5' and 6/6' may be attributed to the low quantity of the metabolite isolated, as well as impurities and solvent interference that obscured the signals, complicating the structural elucidation.

From a mass spectrometry perspective, the MS/MS fragments of matopensine obtained using an ESI source have unfortunately not yet been documented in the literature. Consequently, it is not possible to compare the experimental MS/MS fragments with those of matopensine to confirm the presence of a derivative of this compound.

The structure shows a molar mass of 616.8498 g/mol and is expected to appear at *m/z* 308.1883 as $[M]^{2+}$ during mass spectrometry analysis. However, a peak at *m/z* 615.3352 is observed, which could correspond to a monocharged form $[M]^{+}$ of the structure. Comparing the expected mass of the monocharged form at *m/z* 616.3766 $[M]^{+}$ with the observed mass reveals a discrepancy of one unit. This difference could be due to either degradation of the structure during the mass spectrometry study or an error in the proposed structure. A likely explanation is a Hofmann degradation at the bond between the quaternary nitrogen and carbon 5 during the analysis, resulting in the loss of one hydrogen. This hypothesis is supported by the observation that the ion $[M\text{-}H]^{+}$ appears with higher intensity than the ion $[M]^{+}$ in the mass spectrum of subfraction SL23 GR3. Such degradation is often seen with quaternary alkaloids. For instance, Massiot, G. *et al.* (1988) observed this type of degradation when studying the roots of *Strychnos matopensis*. Additional investigations, particularly involving further isolation and purification, are required to validate this hypothesis and the proposed structure.

Subfraction SL16 GR5.5:

Regarding the subfraction SL16 GR5.5, no structural proposal could be made from the experimental data due to the presence of impurities and the low amount of compound, which complicated structural elucidation. However, some information could still be extracted from the data (Figures 74, 75, and 76).

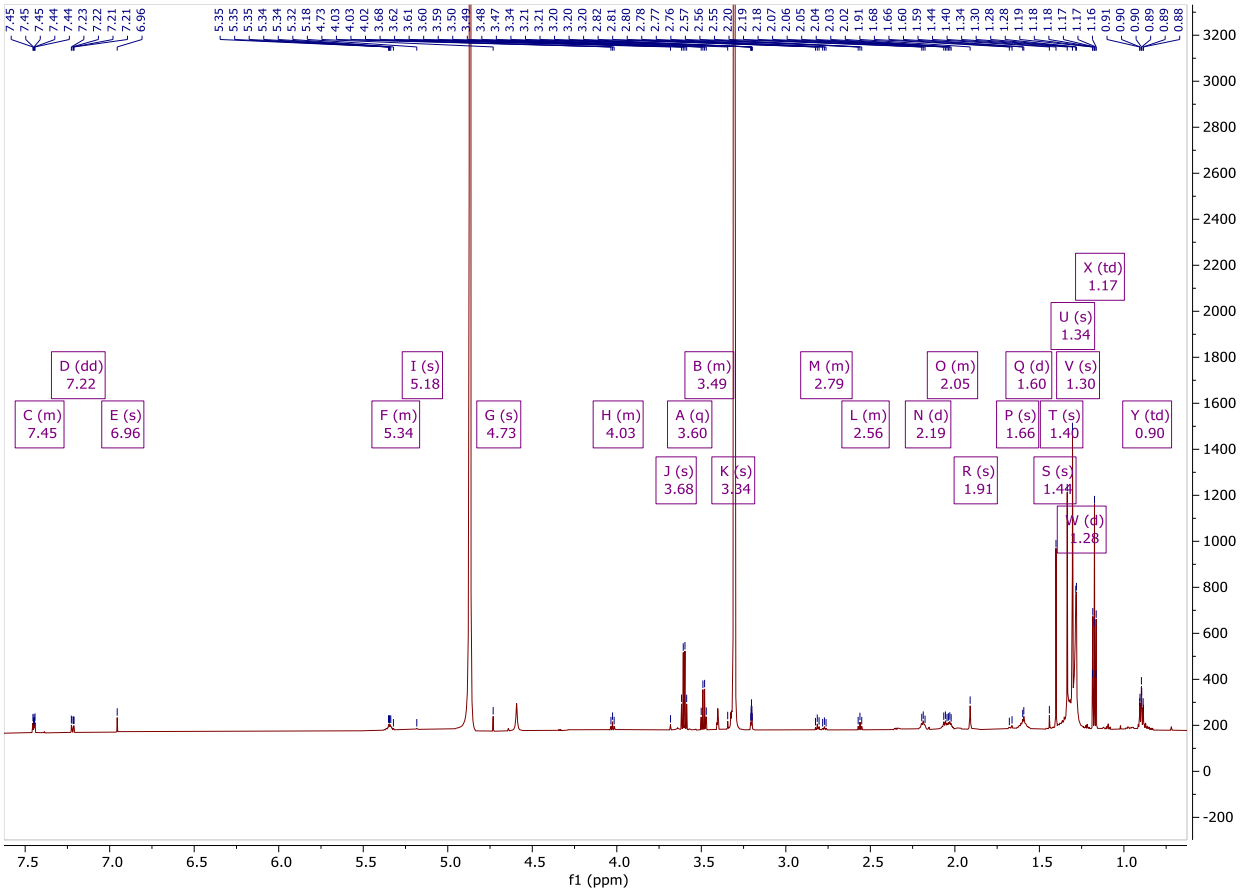


Figure 74: ^{1}H spectrum of the subfraction SL16 GR5.5.

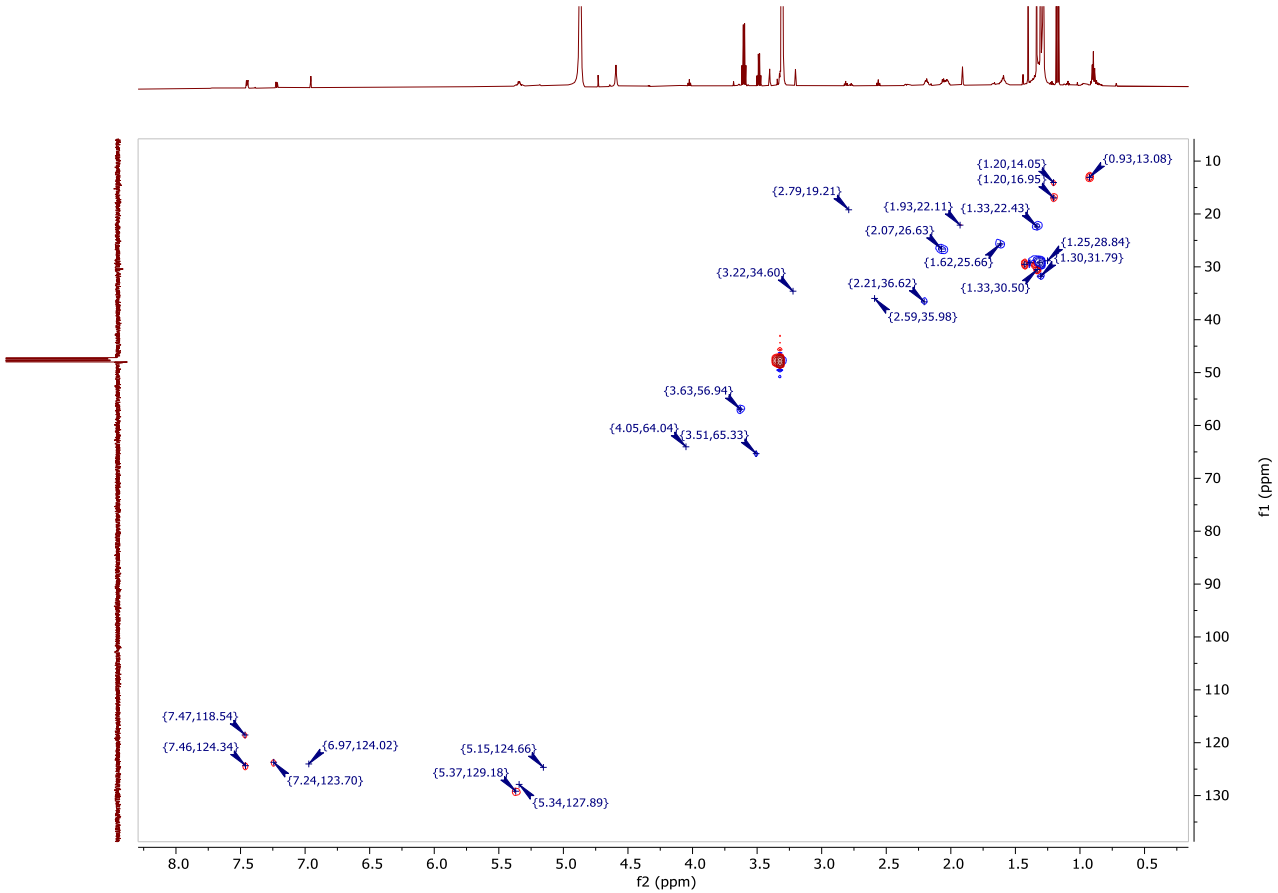


Figure 75: HSQC spectrum of the subfraction SL16 GR5.5.

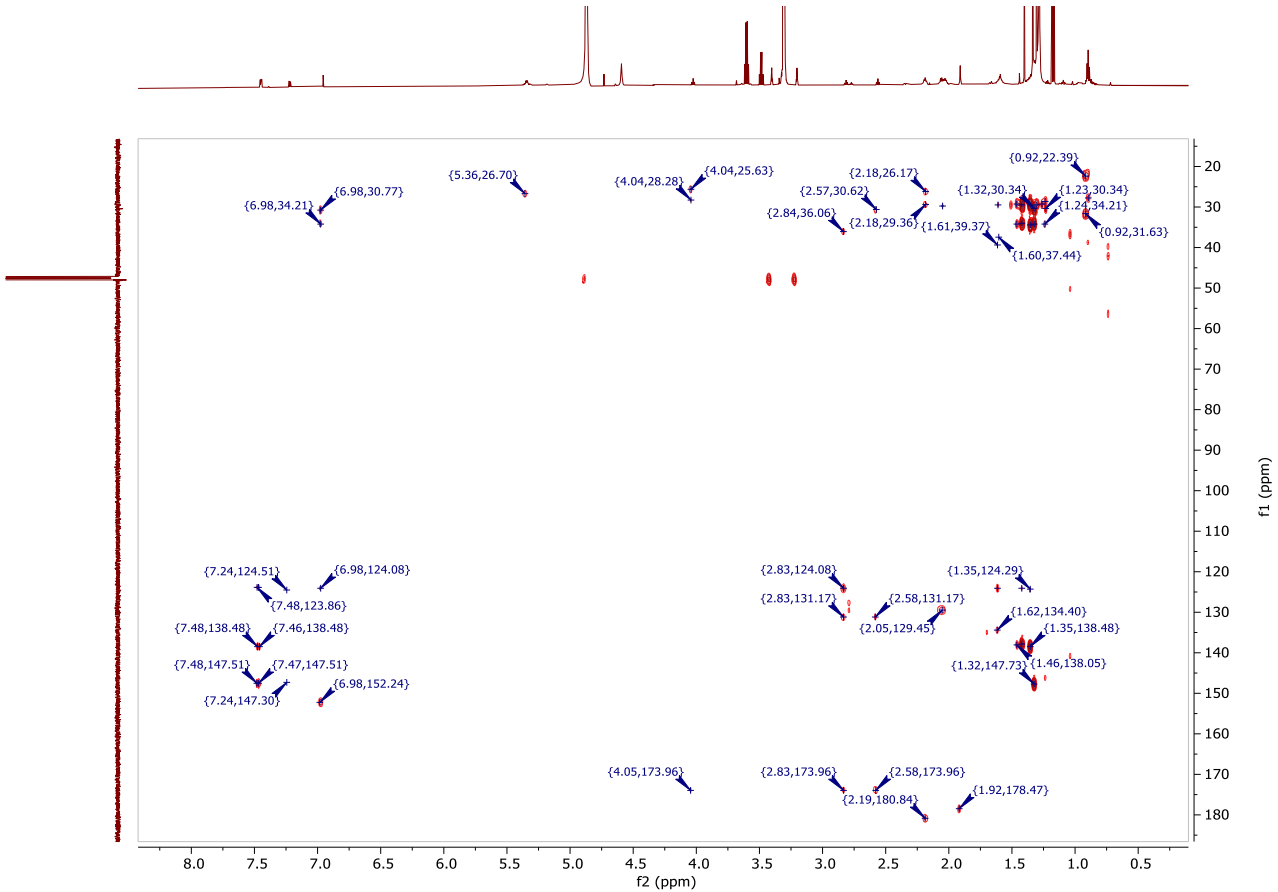


Figure 76: HMBC spectrum of the subfraction SL16 GR5.5.

Some chemical shifts correspond to those of N_b-methyl-longicaudatine, as observed using spectra simulated with MestReNova, along with C+H NMR Predictors software from ACD/Labs. Unfortunately, the match is not sufficient to definitively conclude that the isolated compound is structurally similar to N_b-methyl-longicaudatine. However, the presence of an ethylidene indoloquinolizine moiety is highly probable, as suggested by the MS/MS fragments at *m/z* 251.0485 and 250.1433. This suggests the compound belongs to the phytochemical class of alkaloids and further supports the hypothesis that the structure is related to longicaudatine (Massiot, G. *et al.*, 1988). Similar to the subfraction SL23 GR3, it is likely that the observed mass corresponds to the ion $[M-H]^+$, due to the potential Hofmann degradation of the quinolizidine portion between the nitrogen and carbon 5 (Massiot, G. *et al.*, 1988). Due to the lack of signals and the presence of impurities, further interpretations are challenging. Consequently, the subfraction SL16 GR5.5 requires additional concentration and purification.

Other isolated metabolites:

Unfortunately, the insufficient quantity and purity of the remaining isolated metabolites hindered their identification.

III.7. Antiplasmodial assays of isolated compounds

All isolated compounds were tested in three independent experiments against the 3D7 strain (CQS strain) of *Plasmodium falciparum*, and alstonine was additionally tested on the W2 and Fcb1 strains (CQR strains). The mean IC_{50} value obtained for artemisinin was 3.89 ± 1.64 ng/mL (13.8 ± 5.80 nM). Alstonine, the major compound of the dichloromethane crude extract contained in the fraction 14, showed weak activity, with IC_{50} values of 6.62 ± 1.84 µg/mL (18.9 ± 5.27 µM), 19.4 ± 5.86 µg/mL (55.5 ± 16.8 µM), and 8.24 ± 2.41 µg/mL (23.6 ± 6.91 µM) against 3D7, W2, and Fcb1, respectively. Therefore, it does not contribute on its own to the promising antiplasmodial activity of the dichloromethane crude extract.

The subfractions SL16 GR2+3 and SL16 GR5.5 exhibited IC_{50} values of 0.503 ± 0.124 µg/mL and 0.163 ± 0.0708 µg/mL, respectively. For the subfractions SL22 GR1 and SL22 GR12, the IC_{50} values obtained were 0.0984 ± 0.0871 µg/mL and 0.165 ± 0.0741 µg/mL, respectively. As for subfraction SL23 GR3, it was also highly active, with an IC_{50} of 0.433 ± 0.240 µg/mL (0.702 ± 0.389 µM). Unfortunately, subfraction SL23 GR4 was considered weakly active because its IC_{50} exceeded 10 µg/mL.

Observation of blood smears after antiplasmodial assays with the isolated compounds at concentrations corresponding to the IC_{50} values confirmed these results. Indeed, for the active fractions, 50% of the parasites were eliminated compared to the negative control, while for the non-active fractions, few parasites were eliminated.

In conclusion, except for alstonine and the subfraction SL23 GR4, all isolated compounds demonstrated promising antiplasmodial activities with very low IC_{50} values, down to 0.0984 µg/mL.

IV. CONCLUSIONS AND PROSPECTS

The trunk barks of *Strychnos longicaudata*, an African species belonging to the Loganiaceae family, showed good antiplasmodial activity for its methanolic crude extract and promising antiplasmodial activity for its dichloromethane and alkaloidic crude extracts. Despite these activities, this species has been largely understudied. In response to the growing resistance to current malaria treatments, its metabolite content was explored using molecular networking, MixONat analyses, and bio- and mass-guided fractionation as part of efforts to combat the disease.

Molecular network analysis of the dichloromethane crude extract revealed two compounds already reported in this species: longicaudatine and Wieland-Gumlich aldehyde. On the other hand, other compounds suggested by the molecular network, such as venecurine and strychnine, had never been described in this species. Further analyses will confirm these hypotheses.

Another approach for studying metabolite content involved analyses using the MixONat software. These were carried out with the dichloromethane and

alkaloidic crude extracts. Most sterols were identified in the dichloromethane extract. As for the alkaloidic extract, five compounds identified by the software with probabilities between 71% and 95% are already known in *S. longicaudata*, namely bisnordihydrotoxiferine, normavacurine, longicaudatine, longicaudatine Y, and longicaudatine F. Additionally, there are suggestions of metabolites never reported in the species, such as geissoschizol, tubotaiwinal, and normacusine B, with probabilities above 70%.

During bio- and mass-guided fractionation of the dichloromethane crude extract from *S. longicaudata* trunk barks, using various purification methods (preparative HPLC, TLC, and open column chromatography), eight molecules were isolated. Structural elucidation of three compounds was undertaken using MS, MS/MS, and NMR data. The first is alstonine (the major metabolite in the trunk barks of *S. longicaudata*), which showed weak antiplasmodial activity, not explaining the promising activity observed in the dichloromethane crude extract. The structures of two other compounds were studied: the subfractions SL23 GR3 and SL16 GR5.5. The first appears to be a derivative of matopensine containing a peroxide function at its center and two quaternary nitrogens, while the second could be of the longicaudatine type due to observed similarities and the probable presence of an ethylidene indoloquinolizine moiety. Both of these compounds showed promising antiplasmodial activities.

In addition, antiplasmodial assays on *Plasmodium falciparum* strain 3D7 showed promising antiplasmodial activities for three other isolated compounds: the subfractions SL16 GR2+3, SL22 GR1, and SL22 GR12. Unfortunately, structural elucidation of these was too complicated due to insufficient purity and very low quantities. Concerning the subfraction SL23 GR4, its antiplasmodial activity was weak.

For the continuation of this study, it would first be interesting to isolate larger quantities of the metabolites whose quantities were too low to be identified by NMR, and to remove any remaining impurities. Secondly, there are still many active metabolites to be isolated and identified in *S. longicaudata* trunk barks. Molecular networking and MixONat analyses revealed some compounds that have never been described in this species. Further studies will confirm or refute these suggested identifications. Additionally, during bio- and mass-guided fractionation, other fractions with promising antiplasmodial activities were

obtained. Further exploration of metabolite content through fractionation will make it possible to isolate additional original compounds of potential interest in the fight against malaria.

V. SUPPLEMENTARY MATERIAL

As the supplementary material for this article is substantial and the article has not yet been submitted or published, the data can be accessed through the following Orbi link: https://orbi.uliege.be/handle/2268/323782. They can also be accessed by scanning the QR code below.

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CHAPTER 8

Preliminary study of alkaloidic content in *Strychnos phaeotricha* leaves using dereplication methods

8.1. Forewords

This final chapter, which concludes the in-depth investigation of *Strychnos* alkaloids in this thesis, is dedicated to the study of *Strychnos phaeotricha* Gilg leaves. Although these leaves are low in alkaloids, their crude methanolic and dichloromethane extracts demonstrated good antiplasmodial activity. Using TLC analysis, molecular networking, and MixONat software, metabolites from the leaves were analyzed to identify the source of the antiplasmodial activity.

8.2. *Strychnos phaeotricha,* one of the few *Strychnos* species with a low alkaloid content

Strychnos phaeotricha is an African species that takes the form of a liana. This species has been little studied to date because it was evaluated as low in alkaloids and thus has not been prioritized by researchers studying *Strychnos* alkaloids. However, a few alkaloids have been identified in its roots and stem barks, namely akagerine and its derivatives (akagerine lactone, 17-O-ethylakagerine, tetrahydroakagerine, dihydrocycloakagerine), ajmalicine, tetrahydroalstonine, and dihydrocorynantheol. The presence of these alkaloids confers antimicrobial properties to *S. phaeotricha*. Moreover, its stem barks also exhibit muscle-relaxing properties (Rolfsen, W. *et al.*, 1979; Delaude, C., and Delaude, L., 1997; Tchangou Njiemou, A. F. *et al.*, 2022).

8.3. Methanolic and dichloromethane extractions using pressurized solvents with SpeedExtractor E-914®

The procedures for crushing leaves and performing extractions using the SpeedExtractor E-914® are the same as those described in Chapters 6 and 7 (cf sections 6.2. (subsection II.2., pages 294-295), and 7.2. (subsection II.2., pages 333-334). After drying, the extracts were weighed to determine the yields, which were 6.62% w/w for the methanolic crude extract and 4.88% w/w for the dichloromethane crude extract.

8.4. Testing of antiplasmodial activities of the methanolic and dichloromethane crude extracts from *S. phaeotricha* leaves

Both crude extracts were tested against *Plasmodium falciparum* strain 3D7 (chloroquine-sensitive strain). The full methodology used is detailed in Chapter 7, section 7.2 (subsection II.12., pages 342-343). During these assays, the mean IC_{50} value for artemisinin was 3.09 ± 1.57 ng/mL, which indicates that the IC_{50} values obtained for both extracts are valid (Ledoux, A. *et al.*, 2017). With IC_{50} values of 13.8 ± 1.77 µg/mL for the methanolic crude extract and 13.6 ± 0.796 µg/mL for its dichloromethane counterpart, both demonstrated good antiplasmodial activity. Moreover, the activities are very similar, suggesting that the metabolite(s) responsible was (were) extracted by both solvents.

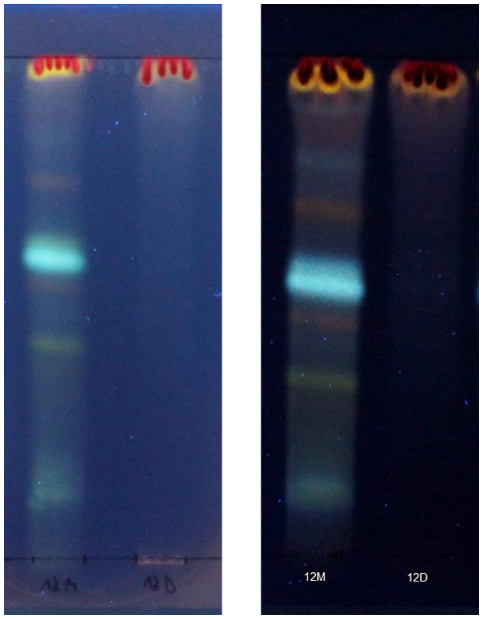
The contents of these extracts were further analyzed by phytochemical screening to determine the phytochemical class(es) present.

8.5. Phytochemical screening by TLC analyses

All analyses were performed on silica gel 60 F_{254} TLC plates (0.2 mm). The mobile phases varied depending on the phytochemical class being studied. Deposition volumes were either 10 µL or 50 µL.

8.5.1. Flavonoids

The mobile phase consisted of ethyl acetate, anhydrous formic acid, glacial acetic acid, and water (100:11:11:26 V/V/V/V). The revelation solvent was a mixture (1:1) of a 10 g/L solution of aminoethanol diphenylborate (DPBAE) in methanol and a 50 g/L solution of macrogol 400R (PEG) in methanol. After spraying, the plate was left to rest for 30 minutes before being read under UV light at 366 nm (Figure 77).



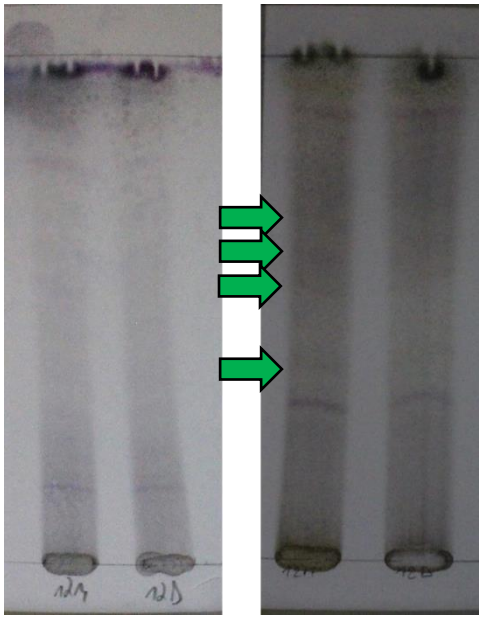
10 µL 50 µL

Figure 77: TLC analysis of flavonoids from the methanolic (left part of TLCs, “12M”) and dichloromethane (right part of TLCs, “12D”) crude extracts of *S. phaeotricha* leaves.

Only the methanolic extract contains fluorescent spots of various colors, corresponding to flavonoids. The blue fluorescence is attributed to the presence of phenolic acids.

8.5.2. Terpenes

To study terpenes, a mixture of dichloromethane and methanol (18:2 V/V) was used as the mobile phase. Vanillin in sulfuric acid, prepared by dissolving 1% (w/v) vanillin in ethanol and adding 2 mL of concentrated sulfuric acid to produce 100 mL, is sprayed onto the plate to reveal the terpenes. Heating the plate for 10 minutes at 100°C is essential for the vanillin-sulfuric acid reagent to reveal the compounds. If spots appear under visible light, this indicates the presence of terpenes in the extract (Figure 78). However, caution must be exercised when using vanillin in sulfuric acid, as this reagent is not specific to terpenes and can reveal other phytochemical classes.



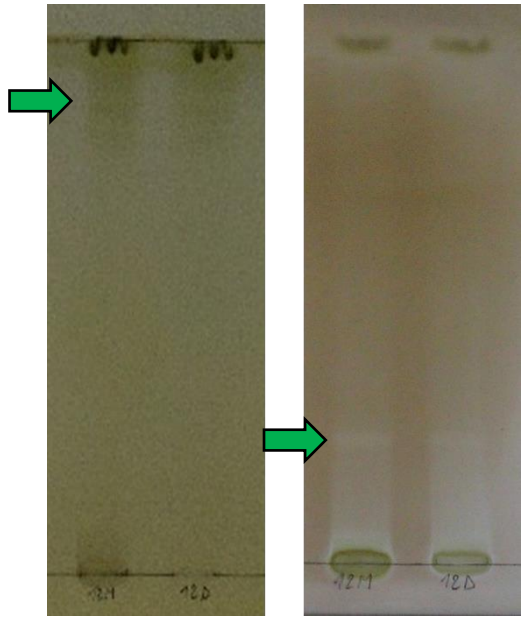
10 µL 50 µL

Figure 78: TLC analysis of terpenes from the methanolic (left part of TLCs, “12M”) and dichloromethane (right part of TLCs, “12D”) crude extracts of *S. phaeotricha* leaves.

Two violet spots are clearly visible in both extracts. Since these spots migrate the same distance, it is highly probable that they originate from the same compound. Additionally, other faint violet spots, highlighted by green arrows, can be observed, identical in both extracts. Based on these observations, it can be concluded that the two extracts likely share the same terpene composition.

8.5.3. Alkaloids

The mobile phase that allows good migration of alkaloids consists of dichloromethane, methanol, and 25% ammonia in a proportion of 8:2:0.5 (V/V/V). Two different reagents were used to reveal the alkaloids: Dragendorff's and iodoplatinate reagents (Figure 79). For both reagents, the TLC plate was directly observed under visible light after spraying.



50 µL 50 µL

Figure 79: TLC analysis of alkaloids from the methanolic (left part of TLCs, “12M”) and dichloromethane (right part of TLCs, “12D”) crude extracts of *S. phaeotricha* leaves.
Left TLC: Dragendorff reagent; **Right TLC**: iodoplatinate reagent.

To prepare 80 mL of Dragendorff reagent, 1.7 g of bismuth reagent and 20 g of tartaric acid were dissolved in 40 mL of water. To this suspension, 40 mL of a 40% (w/v) potassium iodide solution was added. The mixture was stirred for 1 hour before being filtered. This solution must be protected from light. For spraying, it should be diluted by mixing 5 mL of the solution with 10 mL of water.

For the iodoplatinate reagent, a volume of 200 mL was prepared by mixing 3 mL of a 100 g/L chloroplatinic acid solution, 97 mL of water, and 100 mL of a 60 g/L potassium iodide solution.

Slight spots were observed with both reagents in both extracts, confirming the low alkaloid content in *Strychnos phaeotricha* leaves.

8.5.4. Saponins

For the study of saponins, the mobile phase consisted of a mixture of four solvents: dichloromethane, glacial acetic acid, methanol, and distilled water (64:32:12:8 V/V/V/V). To reveal the saponins, the vanillin in sulfuric acid reagent was sprayed onto the TLC plate (cf 8.5.2. Terpenes) (Figure 80).

50 µL

Figure 80: TLC analysis of saponins from the methanolic (left part of TLCs, “12M”) and dichloromethane (right part of TLCs, “12D”) crude extracts of *S. phaeotricha* leaves.

Only dark spots were observed in the methanolic crude extract during the study of saponins. Therefore, it appears that the methanolic extract is the only one containing saponins.

8.5.5. Conclusion of the phytochemical screening

As the antiplasmodial activities were very similar in both crude extracts, one hypothesis is that the activity derives from the same phytochemical class present in both extracts. Therefore, based on the results of the phytochemical

screening, flavonoids and saponins, which were only present in the methanolic crude extract, were ruled out as potential sources of activity. Moreover, it was suggested that alkaloids and terpenes are likely responsible for the observed antiplasmodial activity in the leaves.

8.6. Liquid-liquid extraction of methanolic crude extract

To further investigate the origin of the antiplasmodial activity, a liquid-liquid extraction was performed on the methanolic crude extract of *S. phaeotricha* leaves. Dichloromethane was used as the organic phase, and distilled water as the aqueous phase. Three extractions were carried out, yielding three phases: the organic phase, the interphase, and the aqueous phase.

These phases were then tested against the 3D7 strain of *P. falciparum*. The IC_{50} value for artemisinin was 4.62 ng/mL. The aqueous phase showed moderate activity with an IC_{50} value of 20.5 µg/mL, while the organic phase and interphase exhibited promising activities with IC_{50} values of 1.19 µg/mL and 3.16 µg/mL, respectively.

In conclusion, this study demonstrated that:

- *S. phaeotricha* is a promising species in the fight against malaria, given the observed antiplasmodial activity.

- The antiplasmodial activity was present in both the organic phase and the interphase, indicating that the active metabolites are either apolar (organic phase) or amphiphilic (interphase). These observations are consistent with the results of the phytochemical screening, which identified terpenes and alkaloids as potential sources of activity. Both classes exhibit a strong affinity for the organic phase (except for quaternary alkaloids, which were excluded), supporting the hypothesis formed during the screening.

8.7. Metabolomic analyses using molecular networking and MixONat

To further explore the metabolites from *S. phaeotricha* leaves, molecular networking and MixONat analyses were conducted. The procedures for data acquisition, processing, generating the molecular networks, and analysis using MixONat are described in detail in Chapter 7, which is dedicated to the study of *S. longicaudata* (see section 7.2, subsections II.3., II.4. and II.5., pages 334 to 337). The molecular network of the methanolic extract of *S. phaeotricha* leaves can be accessed using the following link: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=28dbde4c03f94572922912ecd48dacca.

The molecular network of the methanolic crude extract consists of 458 nodes and 474 edges (Figure 81). Unfortunately, only 10 nodes were annotated, and only 8 different compounds were identified, one of which is a phthalate (potentially related to plastic contamination):

- Cycloart-23-ene-3β, 25 diol;
- Oxybutynin;
- Dibutyl phthalate;
- Kaempferol;
- Kaempferol 3-O-rutinoside;
- Luteolin;
- (4aS, 6aS, 6bR, 9R, 10R, 11R, 12aR)-10, 11-dihydroxy-9-(hydroxymethyl)-2, 2, 6a, 6b, 9, 12a-hexamethyl-1, 3, 4, 5, 6, 6a, 7, 8, 8a, 10, 11, 12, 13, 14b-tetradecahydropicene-4a-carboxylic acid.

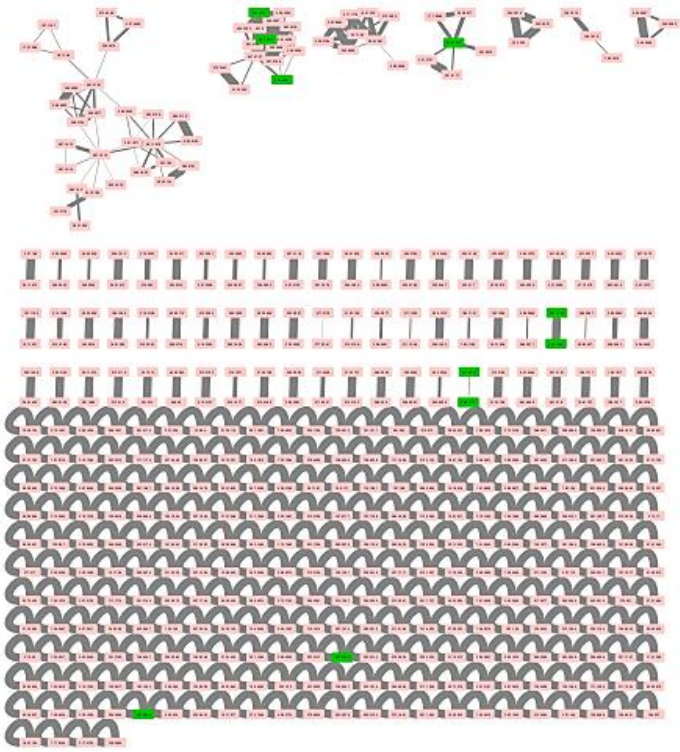


Figure 81: Feature-based molecular network from the methanolic crude extract of *S. phaeotricha* leaves. The nodes in green are the annotated metabolites, while those in red are the unidentified ones.

The molecular network was also analyzed using the MolNetEnhancer workflow, which was introduced earlier in the thesis in Chapter 3, section 3.9 (cf subsection II.6., page 222). Two phytochemical classes were suggested: lipids and lipid-like molecules, as well as phenylpropanoids and polyketides (Figure 82). The job link is provided below: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=61440ce841134fd5b81189f587f4ae79.

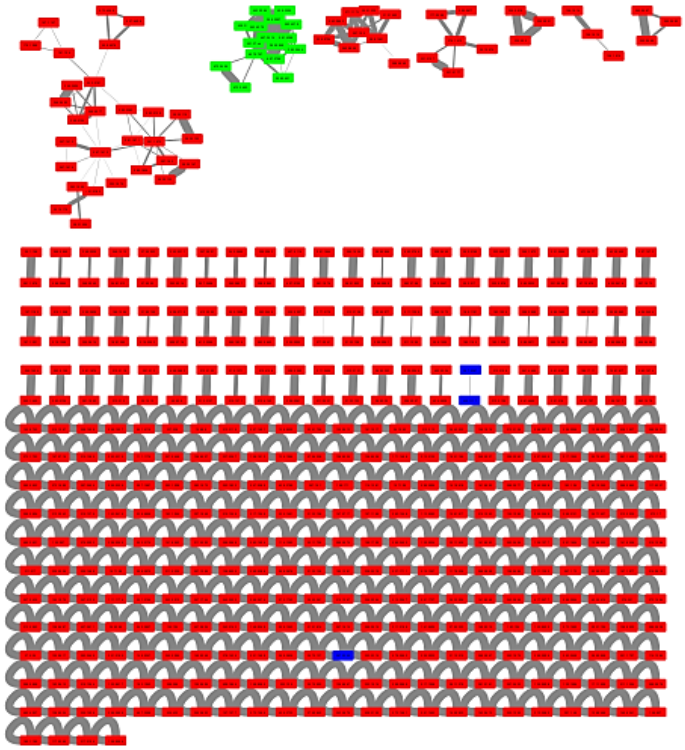


Figure 82: MolNetEnhancer annotation of the molecular network from the methanolic crude extract of *S. phaeotricha* leaves. Green= lipids and lipid-like molecules; Blue= phenylpropanoids and polyketides; Red= No matches.

The organic phase, which exhibited promising antiplasmodial activity, was also analyzed using molecular networking. The network consisted of 1129 nodes, 7 of which were annotated, and 1097 edges (Figure 83). The molecular network and data can be accessed via the following link: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=ad462140d4cd4879a79d81d918006533. Regrettably, apart from codeine, none of the proposed identifications turned out to be terpenes or alkaloids:

- Benzenepropanamide, N-[2-(acetyloxy)-1-(phenylmethyl)ethyl]-alpha-(benzoylamino)-;
- (S,S)-asperphenamate;
- Triphenylphosphine oxide|diphenylphosphorylbenzene;
- Codeine;
- Tricin.

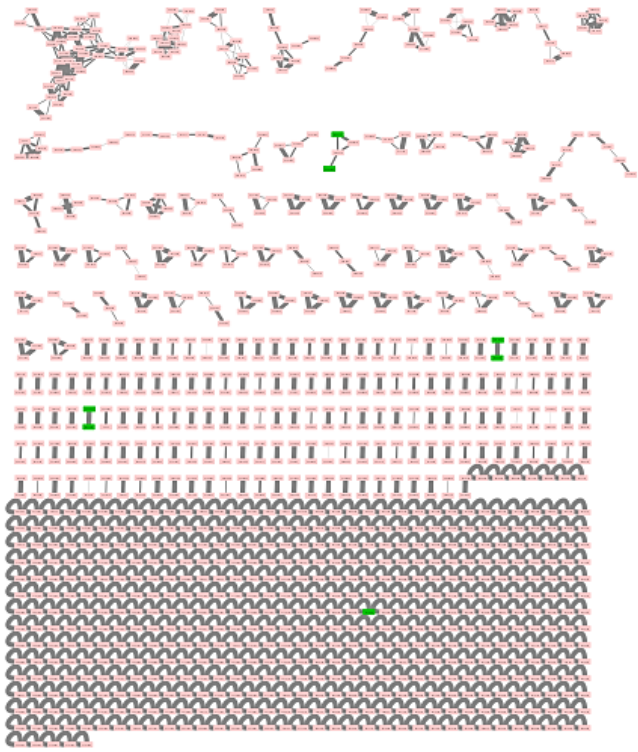


Figure 83: Feature-based molecular network from the organic phase. The nodes in green are the annotated metabolites, while those in red are the unidentified ones.

Unfortunately, the results obtained from molecular networking were not very conclusive. Except for cycloart-23-ene-3β, 25-diol, a sterol, and codeine, an alkaloid, no other compounds from the terpene or alkaloid families were identified by the databases or detected using the MolNetEnhancer workflow.

To further investigate, an alternative approach was applied using MixONat, another metabolomics tool that relies on the chemical shifts of carbon spectra. This method would allow for additional identifications, particularly of terpenes or alkaloids, which were previously difficult to detect. Since MixONat relies on less sensitive data (carbon spectra) compared to molecular networking (MS/MS), the dichloromethane crude extract was selected due to the absence of flavonoids and saponosides, as shown by the screening results, which increases the likelihood of identifying terpenes and/or alkaloids.

For the analysis, the *Strychnos* database was used, and equivalent carbons were allowed (Figure 84).

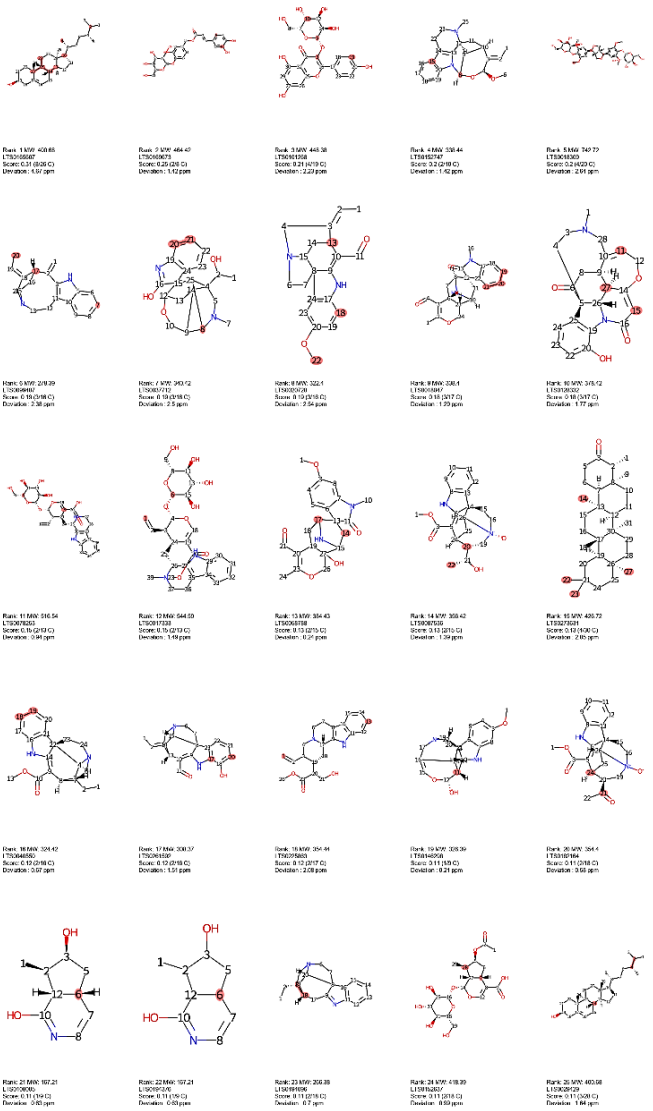


Figure 84: Part of the MixONat results from the dichloromethane crude extract of *S. phaeotricha* leaves.

The results remain inconclusive: the highest probability among the suggested structures is only 31%, which is too low to consider them as potential identifications. However, some indole alkaloids were suggested. To improve

these results, an alkaloidic extraction was performed following the procedure described previously, particularly in Chapter 7, section 7.2 (see subsection II.2., pages 333-334). The weight of the alkaloidic extract was 16.64 mg, corresponding to a yield of 0.17%. This extraction confirmed the presence of alkaloids in *S. phaeotricha* leaves, suggesting that the initial difficulty in detecting them was due to their low concentration. A new MixONat analysis was subsequently carried out using this alkaloidic extract (Figure 85).

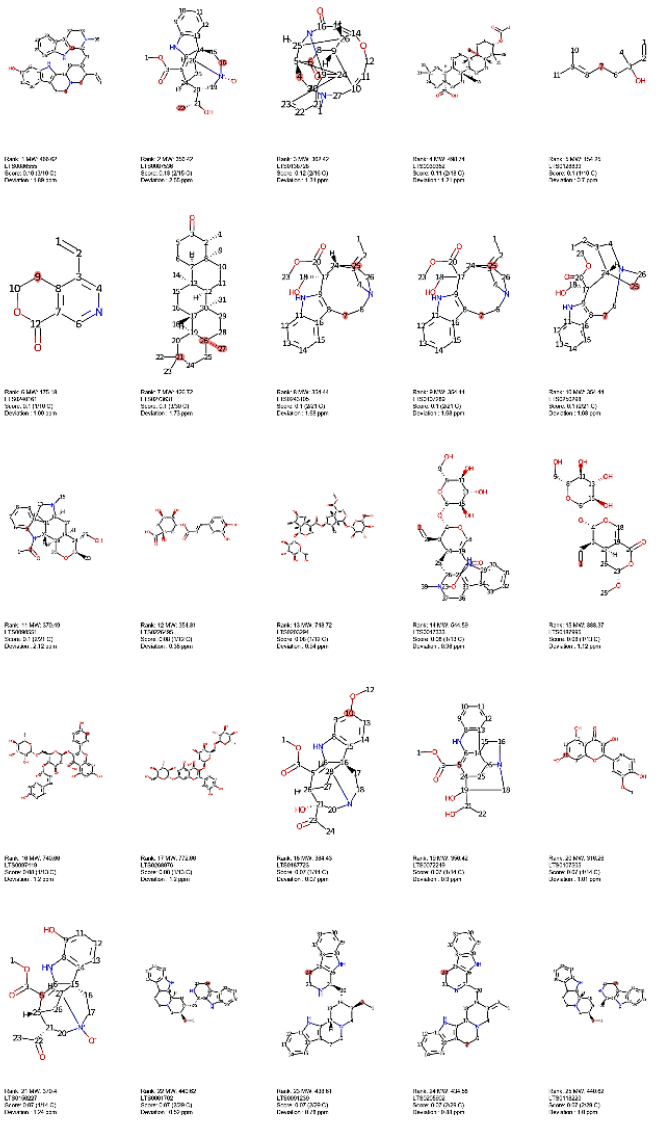


Figure 85: Part of the MixONat results from the alkaloidic crude extract of *S. phaeotricha* leaves.

Unfortunately, the results obtained with the alkaloidic extract were no better, with the highest probability reaching only 16%. As a result, none of the identifications suggested by the software can be considered reliable. To improve the results, one crude extract was fractionated, which should reduce the complexity of the composition and potentially lead to better results with the software. Following the same approach as before, the dichloromethane extract was selected for purification. Fractionation was performed using preparative HPLC.

8.8. Fractionation of dichloromethane crude extract by preparative HPLC

The column used was a C_{18} type (270 x 25 mm, 10 µm). The mobile phase consisted of methanol (Channel A) and water + 0.1% formic acid (Channel B). The gradient varied as follows: for the first 3.19 minutes, the mobile phase was 5% A and 95% B. From 3.19 to 4.78 minutes, the gradient changed to 25.4% A and 74.6% B. From 4.78 to 59.01 minutes, the mobile phase became 83.2% A and 16.8% B. From 59.01 to 95.70 minutes, the mobile phase reached 100% A and remained at this level until 97.29 minutes. Finally, from 97.29 to 98.89 minutes, the mobile phase returned to initial conditions and remained there until 122.81 minutes to ensure complete elution of the sample before the next injection. The flow rate was set to 20 mL/min, and the wavelengths applied were 254 nm and 280 nm. The dichloromethane crude extract was dissolved in 90% A and 10% B and filtered through a 0.45 µm pore size filter before manual injection. The maximum injection volume was 10 mL, corresponding to the maximum capacity of the injection loop.

During fractionation, 65 fractions were collected and grouped into 18 groups based on chromatographic data. Each group was weighed. Except for groups 16 (2.50 mg) and 17 (1.98 mg), the remaining groups weighed less than 1 mg, which was insufficient for MixONat analysis. To obtain reliable results, a concentration of 30 mg/mL is required for NMR analysis (cf Chapter 7, section II.5, page 337). Therefore, further purification by preparative HPLC is needed. However, due to time constraints, fraction enrichment and MixONat analysis were not completed within the scope of this thesis.

8.9. Conclusions and prospects

This preliminary study demonstrated that *S. phaeotricha* leaves exhibited promising or good antiplasmodial activities. Moreover, these activities were very similar between the methanolic and dichloromethane crude extracts, which could be attributed to the presence of the same phytochemical class in both extracts. TLC analysis of their metabolite content revealed the presence of terpenes and a small amount of alkaloids in both extracts. However, molecular networking and MixONat analyses did not provide conclusive identifications of these metabolites. Despite this, the alkaloidic extract confirmed the presence of alkaloids in the leaves. Although the probabilities in the MixONat analyses of the dichloromethane and alkaloidic extracts were low, several indole alkaloids were suggested for identification.

To explore the leaves further, the dichloromethane crude extract was fractionated using preparative HPLC. However, the quantities of the fraction groups were too small to be analyzed by MixONat. In the future, it would be valuable to study these groups by mass spectrometry to determine their purity and the masses of the compounds they contain. Additionally, further purification through additional preparative HPLC could help concentrate these fractions for more detailed analysis.

8.10. References

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CHAPTER 9

Discussion, conclusions, and prospects

Numerous species from the *Strychnos* genus, belonging to the Loganiaceae family and native to Africa, America, Asia, and Oceania, have been extensively studied for their therapeutic potential. Indeed, these plants have been associated with a wide range of traditional uses (Leeuwenberg, A. J. M., 1969; Angenot, L., Denoël, A., 1972; Philippe, G. *et al.*, 2004; Bonnet, O. *et al.*, 2022a; Bonnet, O. *et al.*, 2022b), including treatments for malaria, cancer, microbial infections, tuberculosis, gastrointestinal issues, diabetes, sexual disorders, venereal diseases, and oxidative stress, among others (cf review "The African plants of the *Strychnos* genus: A review of their traditional uses and pharmacological properties").

Despite their therapeutic potential, the use of *Strychnos* species requires a careful benefit-risk assessment. When used in an uncontrolled manner, their significant tetanizing and curarizing properties can outweigh the benefits. However, under medical supervision or in traditional medicinal practices, these plants offer promising options for managing diverse pathologies. The most reliable approach is to isolate active metabolites, which maximizes therapeutic efficacy while mitigating toxicity.

For over two centuries, research on the *Strychnos* genus has focused on identifying the metabolites in various plant parts from different species and studying their pharmacological properties. The alkaloid phytochemical class has been particularly studied due to its significant presence in many species of the genus. For example, 5',6'-dihydrousambarensine, strychnogucine B, and strychnobiline are indole alkaloids isolated from *Strychnos usambarensis* Gilg ex Engl., *Strychnos icaja* Baill., and *Strychnos variabilis* De Wild. (Angenot, L., and Bisset, N. G., 1971; Tits, M., and Angenot, L., 1978; Lamotte, J. *et al.*, 1979; Frédérich, M. *et al.*, 2001; Philippe, G. *et al.*, 2003). All three compounds demonstrated significant antiplasmodial activities in *in vitro* tests. Additionally, the *in vivo* antimalarial activity of strychnogucine B was found to be moderate (Tits, M., and Angenot, L., 1978; Frédérich, M. *et al.*, 1999; Frédérich, M. *et al.*, 2002; Beaufay, C. *et al.*, 2018). These results underscore the potential of *Strychnos* plants as a promising source for discovering new antimalarial compounds. Research on this genus, both in the past and within the context of this thesis, contributes to the ongoing fight against malaria.

Malaria is a parasitic disease that has been known for thousands of years. It is transmitted to humans through the bite of mosquitoes from the *Anopheles* genus during their blood meal. The disease is caused by parasites from the *Plasmodium* genus, with six species responsible for malaria in humans: *P. falciparum*, *P. ovale*, *P. vivax*, *P. knowlesi*, *P. malariae*, and *P. simium*, a newly emerging species (Cox, F. E. G., 2010; Brasil, P. *et al.*, 2017; Fikadu, M., and Ashenafi, E., 2023).

A variety of antimalarial treatments have been marketed, including artemisinin and its derivatives (such as artemether), and quinine and its derivatives (such as chloroquine) (Fougere, E., and Buxeraud, J., 2018). In addition, preventive measures like insecticide-treated nets and indoor residual spraying have been implemented (Fikadu, M., and Ashenafi, E., 2023; World Health Organization, 2023b). Despite these efforts, malaria continues to be a significant global health issue, particularly in Africa. Resistance to existing antimalarial treatments has increased, rendering some drugs less effective or even ineffective. In 2022, 249 million malaria cases were reported across 85 endemic countries, resulting in 608,000 deaths. In response to growing resistance, the World Health Organization (WHO) recommended Artemisinin-based Combination Therapy (ACT), which pairs artemisinin with another treatment. These combinations help slow the development of resistance while maintaining high efficacy (World Health Organization, 2023a).

However, this solution is temporary, as ACTs will eventually lose their effectiveness. Thus, discovering new antimalarial compounds is crucial for the development of future treatments.

Recently, two antimalarial vaccines, RTS,S/AS01 and R-21/Matrix-M, have been developed and recommended by the WHO. These vaccines are currently in clinical trials and may provide a promising preventive measure (Collins, K. A. *et al.*, 2021; World Health Organization, 2023a; World Health Organization, 2023c; World Health Organization, 2023d; World Health Organization, 2023e). Furthermore, there is ongoing intensive research into new antimalarial molecules. Many researchers are focusing on the discovery of natural compounds, given that 45% of antiparasitic treatments originate from natural sources (Newman, D. J., Cragg, G. M., 2020). The work presented in this thesis contributes to this effort by exploring the metabolite content of several

Strychnos species and using metabolomics tools, primarily molecular networking and MixONat, to identify novel antiplasmodial compounds.

Metabolomics is the study of all metabolites or low molecular weight molecules in a high-throughput manner (Clish, C. B., 2015). While this methodology has been in use for around two decades, it has only more recently been applied to the study of natural molecules in plants, fungi, and marine organisms (Idle, J. R., and Gonzalez, F. J., 2007; Alseekh, S. *et al.*, 2021). Molecular networking is a methodology for mapping the metabolite content of a complex mixture (such as a crude extract or fraction) using MS/MS data. It groups metabolites based on similarities and suggests possible identifications by comparing theoretical and experimental MS/MS spectra using shared spectral databases (Nothias, L.-F. *et al.*, 2020). In contrast, MixONat is a tool that proposes metabolite identifications based on the chemical shifts of carbon spectra from crude extracts or fractions (Bruguière, A. *et al.*, 2020). While molecular networking is more sensitive and can identify minor metabolites, MixONat is typically used to identify major compounds. Both approaches enable the rapid identification of known metabolites, helping to target unidentified ones more efficiently. By adding a mass dimension to bio-guided fractionation, unknown metabolites can be specifically tracked throughout the process, opening up new opportunities for the discovery of novel antiplasmodial compounds. In the second part of this thesis, a proof of concept for molecular networking and a trial of the mass-guided fractionation approach were presented in Chapters 5 and 6, followed by bio- and mass-guided fractionation in Chapter 7.

Before beginning the purification process, it was essential to establish criteria for selecting the species to be purified. This work started with forty-four crude extracts (forty-three methanolic and one alkaloidic), representing twenty-eight species from the *Strychnos* genus. These samples were stored in the extensive collection of the Pharmacognosy laboratory. Although some of the samples were quite old (the oldest being collected 78 years ago, in 1946), studies have shown that alkaloids in plants are highly stable, with minimal degradation over time. However, oxidation, hydrolysis, and heat-induced processes remain possible, potentially causing compound breakdown. Such degradation could open new avenues for the hemisynthesis of novel antimalarial drugs.

Based on antiplasmodial activities, the presence of alkaloid-containing clusters, the quantity of plant material, the occurrence of multi-charged compounds (as antiplasmodial activity is often linked to dimers), and the number of references in the literature, four species were selected: *S. variabilis* De Wild. root barks (sample 6), *S. phaeotricha* Gilg leaves (sample 12), *S. angolensis* Gilg root barks (sample 17), and *S. longicaudata* Gilg trunk barks (sample 23). However, an additional sample was included, the alkaloidic extract from the leaves of *S. usambarensis* Gilg ex Engl. (sample 44), due to its MS and MS/MS spectra as well as its molecular network, which detected compounds with *m/z* values above 900.

In the first instance, strychnine, a well-known monoterpene indole alkaloid, was studied to evaluate the molecular networking methodology. During the exploration of forty-four crude extracts, the GNPS platform software suggested numerous alkaloid identifications, specifically sixty-three alkaloids, including usambarensine, 19,20-dihydrousambarensine, usambarine, strychnofoline, sungucine, and strychnine. The latter was detected in ten species, seven of which had never previously been reported as producers of strychnine. These species included the trunk barks of *S. tricalysioides* Hutch & M.B.Moss, *S. camptoneura* Gilg & Busse, *S. congolana* Gilg, *S. boonei* De Wild., *S. densiflora* Baill., *S. tchibangensis* Pellegr., and the leaves of *S. usambarensis* Gilg ex Engl. Further analyses by TLC, NMR, HPLC-DAD, and UPLC-MS/MS, as well as comparison of the MS/MS spectra with that of strychnine, confirmed its presence in the seven *Strychnos* species with high confidence (level 1 according to the MSI, Metabolomics Standard Initiative). Except for the trunk barks of *S. densiflora*, where it was more abundant, strychnine was found in trace amounts in the other species. This explains why it had never been detected in these species using conventional methods, which are less sensitive. Therefore, this study serves as a proof of concept for the molecular networking methodology, demonstrating its effectiveness in identifying both major and minor compounds.

The following study focused on the leaves of *Strychnos usambarensis* Gilg ex Engl., well-known for its antiplasmodial activity in the literature. The aim was to isolate one of the metabolites with a mass superior to *m/z* 900, using mass-guided fractionation. The masses *m/z* 927, *m/z* 932, *m/z* 943, *m/z* 947, *m/z* 995,

m/z 1032, and *m/z* 1101 are examples of the masses observed. Such masses had never previously been detected in the *Strychnos* genus. It will also make it possible to implement and evaluate a mass-guided fractionation methodology. The latter involves studying the content of the various fractions at each step of purification in order to track the masses of interest. Therefore, the biological aspect was not addressed in this study.

After purifying the methanolic crude extract through preparative and analytical HPLC-DAD, as well as open column chromatography, a compound with a mass m/z of 944.2848 was isolated. This compound was analyzed by NMR, MS, and MS/MS to elucidate its chemical structure. The observed signals suggested that the compound is a dimeric or trimeric alkaloid, possibly symmetrical, containing a glycosidic moiety and a carboxylic acid or ester functional group. Moreover, since the mass is even, the structure is likely to contain an even number of nitrogen atoms. Unfortunately, the determination of its full structure and antiplasmodial activity could not be achieved due to the presence of impurities, missing signals, and rapid degradation caused by its low structural stability.

Following the implementation of molecular networking and mass-guided fractionation methodologies, bio- and mass-guided fractionation was conducted to further investigate the metabolites of the trunk barks of *Strychnos longicaudata* Gilg, an African species that has been relatively underexplored. The dichloromethane and alkaloidic extracts of this species exhibited promising antiplasmodial activities, with IC_{50} values of 4.94 ± 2.51 µg/mL and 1.77 ± 0.489 µg/mL, respectively. Furthermore, molecular networking and MixONat analysis identified six metabolites previously reported in *S. longicaudata*: bisnordihydrotoxiferine, longicaudatine, longicaudatine F, longicaudatine Y, normavacurine, and Wieland-Gumlich aldehyde. Given the large number of unidentified metabolites still present and their promising antiplasmodial activities, *Strychnos longicaudata* trunk barks are a promising source of novel antiplasmodial compounds.

The first purification step was carried out on the dichloromethane crude extract using preparative HPLC, which led to the isolation of alstonine, a well-known compound within the *Strychnos* genus, though it had never been described in *Strychnos longicaudata* before. Fractions were selected based on

their molecular network and antiplasmodial activity and subsequently purified by open column chromatography or preparative TLC. Five subfractions exhibiting promising antiplasmodial activities were analyzed by NMR, MS, and MS/MS in an attempt to elucidate their chemical structures. Unfortunately, only one structure could be proposed for the subfraction SL23 GR3, which was identified as a derivative of matopensine, featuring two quaternary nitrogens and a peroxide function at the center. Additionally, a possible structural interpretation was discussed for the subfraction SL16 GR5.5, which showed chemical shifts resembling those of longicaudatine-type metabolites, particularly N_b-methyl-longicaudatine, and suggested the potential presence of an ethylidene indoloquinolizine moiety.

Finally, the leaves of *Strychnos phaeotricha* Gilg were investigated in a preliminary study, as their crude methanolic and dichloromethane extracts exhibited good antiplasmodial activities, despite having a low alkaloidic content based on molecular network and TLC analyses. The primary objective was to determine the origin of this activity, which could potentially arise from similar types of molecules, as the two extracts showed very similar activities (IC_{50} values of 13.8 ± 1.77 µg/mL for the methanolic extract and 13.6 ± 0.796 µg/mL for the dichloromethane extract).

Phytochemical screening revealed the presence of terpenes and a low level of alkaloids in both extracts. Furthermore, the organic and aqueous phases obtained through liquid-liquid extraction from the methanolic extract demonstrated promising and moderate antiplasmodial activities, respectively, with IC_{50} values of 4.62 µg/mL for the organic phase and 20.5 µg/mL for the aqueous phase. Thus, the hypothesis that terpenes and/or alkaloids might be responsible for the antiplasmodial activity remains plausible, as the organic phase, which was obtained with dichloromethane, contained both terpenes and tertiary alkaloids.

The investigation of the metabolite content in the crude extracts of *S. phaeotricha* leaves through molecular networking and MixONat analyses produced inconclusive findings. Only eight metabolites were identified in the molecular network, including a single sterol and no alkaloids, while the identifications suggested by MixONat had probabilities too low to be considered reliable (below 31%). Consequently, fractionation by preparative

HPLC was conducted on the dichloromethane extract to further investigate the composition of fractions using MixONat, with the hope of achieving better identification probabilities due to the less complex composition of the fractions. Unfortunately, the initial fractions obtained were too small in quantity to be analyzed by MixONat and will require further enrichment before analysis.

In conclusion, these results highlight the ongoing significance of studying plants from the *Strychnos* genus, even though they have been studied for over two centuries. Furthermore, the application of metabolomic tools, such as molecular networking and MixONat, in the field of natural product discovery opens up new opportunities for identifying novel antiplasmodial metabolites, including both major and minor compounds. This was evident in the fractionation of *S. usambarensis* and *S. longicaudata* (Chapters 6 and 7). However, this purification methodology has a limitation that must be addressed, particularly in the purification of minor metabolites for structural elucidation. While MS/MS analyses are highly sensitive and allow for the identification of compounds through molecular networking, isolating these compounds in sufficient purity and quantity during fractionation proves challenging. This issue was clearly illustrated in Chapters 6 and 7 of this thesis. For instance, numerous purification steps were required to isolate a small quantity of the dimeric or trimeric glycoalkaloid with a mass of *m/z* 944.2848 from *S. usambarensis* leaves. In the case of *S. longicaudata* trunk barks, only the structures of three out of eight isolated compounds could be investigated due to their low quantities and, in some cases, insufficient purity. Therefore, this purification methodology has its constraints. To address this, one potential solution would be to enrich fractions as much as possible through repeated fractionation. However, this approach would necessitate the use of a large amount of plant material, which is not always readily available.

As another application, these metabolomics tools can also be utilized to investigate known metabolites, as demonstrated in the study of strychnine in Chapter 5. Several potential future directions are outlined for this project. Some prospects are specific to individual chapters of this thesis, while others concern the project as a whole.

Regarding specific prospects, it would be valuable to explore the additional identifications suggested by the molecular network of the forty-four crude

extracts from the twenty-eight *Strychnos* species. Notably, strychnine was identified in seven previously unreported species, suggesting that further identifications may be possible using the molecular network.

About the study of *S. usambarensis* leaves, the antiplasmodial activity of the compound with an *m/z* mass of 944.2848 could not be determined. Isolating this compound again in larger quantities would provide the opportunity to assess its antiplasmodial potential. Additionally, other compounds with masses above *m/z* 900 have yet to be identified.

For the trunk barks of *S. longicaudata*, three key research directions are proposed. First, using the same purification methods, the five active isolated compounds that could not be identified can be further enriched. This will allow their final purification, addressing the issue of insufficient purity in certain cases. Moreover, it will increase the chances of elucidating their chemical structure by intensifying signals in NMR spectra. Second, molecular networking and MixONat analyses have revealed metabolites that have not been previously described in the literature for this species, and further analysis would be valuable to confirm these findings. Lastly, several active fractions containing unidentified metabolites were not purified within the scope of this thesis due to time constraints. Searching for identifications through MixONat analyses, followed by purification using preparative HPLC, open column chromatography, or preparative TLC, are potential avenues for further advancing this study.

The final specific prospects involve the study of *S. phaeotricha* leaves, which resulted in the isolation of fractions through preparative HPLC. However, their analysis could not be performed due to the limited quantities obtained. To continue this investigation, it will be crucial to perform several rounds of fractionation to sufficiently concentrate the fractions for MixONat analysis. This will help elucidate the origin of the antiplasmodial activity in the leaves.

There are also broader prospects for this research project. Three out of the five selected species were studied in this thesis, leaving the root barks of *S. angolensis* and *S. variabilis* yet to be explored. The selection of the five species was performed manually based on various defined criteria. Today, in the field of metabolomics, new software tools have emerged to improve the efficiency of interpreting large datasets. Among these is the “Inventa”

software, introduced in Chapter 1 and developed in 2022 at the University of Geneva. This tool identifies structural novelties within complex mixtures of metabolites (Quiros-Guerrero, L.-M. *et al.*, 2022). Additionally, chemometric models could be employed to identify the metabolites potentially responsible for the antiplasmodial activities observed during the screening of the forty-four crude extracts (Meunier, M. *et al.*, 2024). The combination of "Inventa" software and chemometric models offers a more automated and efficient approach to species selection within the *Strychnos* genus. Applying this method to the dataset of forty-four crude extracts could help confirm whether the same species would be prioritized and potentially identify additional candidates worth exploring.

Additionally, only twenty-eight out of approximately 200 *Strychnos* species were investigated in this study. With around 172 species remaining unexplored, there is significant potential for further research on these plants. The "Inventa" software could be utilized to identify which of the remaining species warrant further investigation. If plant material from these species can be obtained, this large-scale study could uncover many more promising antiplasmodial compounds.

Another application of molecular networking would be the exploration of metabolite profiles based on the vegetative forms of plants from the *Strychnos* genus. As demonstrated in the study by Quetin-Leclercq, J. *et al.* (1991), the chemical composition varies depending on whether the plant takes the form of a liana or a tree. Comparing the metabolite compositions between two distinct vegetative forms within the same species could therefore be highly insightful. Such research might reveal antiplasmodial alkaloids specific to one form, guiding future investigations to prioritize the more promising morphology.

Finally, the methodology developed in this thesis could be applied not only to other species of *Strychnos*, but also to the study of other plant genera, fungi, and marine organisms.

The future of *Strychnos* plants looks bright, given the promising results from this research and the vast array of metabolites yet to be discovered. With malaria cases and deaths still on the rise, it is crucial to continue our efforts

to combat this devastating disease. Every researcher's contribution plays a vital role in this fight. Through collaboration, we can improve our understanding of the disease, counteract parasite resistance, reduce cases and deaths, and ultimately work toward eradication. Sharing our results with the scientific community is essential to disseminate knowledge, provide new insights, and inspire further ideas and discoveries. As the famous African proverb says, "Alone, we go faster. Together, we go further". Through collective intelligence, there are no limits to what we can achieve.

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CHAPTER 10

Appendices

10.1. Publications as first author associated with this work

Bonnet, O., Beniddir, M. A., Champy, P., Kagisha, V., Nyirimigabo, A., Hamann, C., Jgerenaia, G., Ledoux, A., Tiabou Tchinda, A., Angenot, L., Frédérich, M., 2022. Exploration by molecular networking of *Strychnos* alkaloids reveals the unexpected occurrence of strychnine in seven *Strychnos* species. *Toxicon*, 215, 57–68. https://doi.org/10.1016/j.toxicon.2022.06.002.

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10.2. Publications as co-author

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10.4. List of posters

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Abstract

This thesis expands upon earlier studies investigating the antiplasmodial properties of plants within the *Strychnos* genus (Loganiaceae family), highlighting several species that have demonstrated promising potential, including *Strychnos usambarensis*, *Strychnos icaja*, and *Strychnos variabilis*. The first part of the work involved an *in vitro* screening of the antiplasmodial activities of 43 methanolic crude extracts from 28 species against the 3D7 strain of *Plasmodium falciparum*. This screening was complemented by the exploration of the metabolites from these extracts, as well as from an alkaloidic extract using molecular networking, a chemometric method that maps metabolites and visualizes their similarities through MS/MS spectra. The use of spectral databases allowed for the rapid distinction between known and unknown compounds, thereby facilitating bio- and mass-guided fractionations. This study initially highlighted the importance of further exploring the *Strychnos* genus, revealing clusters of unidentified metabolites that may possess antiplasmodial properties. Subsequently, the results led to the selection of several species with promising (IC_{50} < 5 µg/mL) or good (IC_{50} between 5 and 15 µg/mL) antiplasmodial activity, notably the leaves of *S. usambarensis*, *S. phaeotricha*, and the trunk barks of *S. longicaudata*.

The second part of the study concentrated on examining one of the identifications supplied by the databases, specifically strychnine, which was discovered in seven species that had not been previously recognized as producers of this compound. The various analyses confirmed its presence, thus providing proof of concept for the molecular networking methodology.

Finally, the third part addressed the isolation and identification of new antiplasmodial metabolites through bio- and mass-guided fractionations. Analyses of the leaves of *S. usambarensis* revealed unknown compounds with masses greater than 900 *m/z*. A metabolite with a mass of 944 *m/z*, potentially a dimeric or trimeric alkaloid with a glycosidic moiety, was isolated. Unfortunately, due to the low quantity isolated and the fragility of the structure, the antiplasmodial activity could not be evaluated. Regarding the trunk barks of *S. longicaudata*, fractionation identified seven unknown metabolites showing promising antiplasmodial activities, with structural elucidation attempted for three of them. Lastly, a preliminary study on the leaves of *S. phaeotricha* revealed good antiplasmodial activities despite a low quantity of alkaloids. The results suggest that the activity could be attributed to phytochemical classes such as alkaloids and terpenes. A bio- and mass-guided fractionation has been initiated, requiring further enrichment of the fractions to deepen this research.