BIO-ANALYTICAL STUDY OF PLANTS USED IN TRADITIONAL MEDICINE IN TOGO

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Abbreviations

Ara	Arabinose
CI	Chemical ionization
DCM	Dichloromethane
EA	Ethyl acetate
EI	Electro-ionization
ESI	Electrospray ionization
FC	Frequency of Citation
FCSi	Heat-inactivated fetal bovine serum
FCS	Fetal bovine serum
Fig.	Figure
Fl	Fidelity level
FTICR	Fourier Transform Ion Cyclotron Resonnance
g	Grams
GC	Gas Chromatography
Glc	Glucose
h	Hours
HPLC	High-Performance Liquid Chromatography
IC_{50}	Inhibition Concentration at 50%
ICF	Informant Consensus Factor
IUV	Intraspecific Use Value
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate dibasic
LC	Liquid Chromatography
LPLC	Low Pressure Liquid Chromatography
MALDI	Matrix Assisted Laser Desorption/ Ionisation
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MeOH	Methanol
µg	Micrograms
MIC	Minimal Inhibitory Concentration

Min	Minutes
mL	Milliliters
µm	Micrometers
MS	Mass Spectrometry
m. p	Melting point
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
Na_2HPO_4	Sodium phosphate dibasic
OUV	Overall Use Value
PE	Petroleum ether
PI	Percentage of Inhibition
PI_{50}	50% of Percentage of Inhibition
PPV	Plant Part Value
RPMI	Roswell Park Memorial Institute
RU	Reported Use
SU	Specific Use value
TH	Traditional healers
TLC	Thin Layer Chromatography
TOF	Time-of-flight
UV	Use Value
Xyl	Xylose

Zusammenfassung

Die Untersuchung von Pflanzen in Togo ist kompliziert, da moderne Techniken nicht verfügbar sind. Die computerunterstützte Produktidentifikation aus dem traditionellen Gebrauch (CAPITURE) wurde im Rahmen einer ethnobotanischen Umfrage zur traditionellen Behandlung von Pilzerkrankungen im Tchamba-Bezirk (Togo) evaluiert. Diese Methode hat die biologisch aktivsten Pflanzen aus der 43 untersuchten Spezies-Befragung vorhergesagt und identifiziert: *Pterocarpus erinaceus* um gegen Fungi aktiv zu sein und *Daniellia oliveri* gegen Bakterien. Die Pflanzen wurden dann gegen Fungi, Bakterien und Krebszellen getestet. Wie mit CAPITURE vorhergesagt, war *P. erinaceus* aktiver gegen Mykose und *D. oliveri* gegen Bakterien. Interessanterweise präsentierten beide Pflanzen Aktivität auf Krebszellen, ohne toxisch für normale menschliche Zellen zu sein. Ein dritter Schritt wurden mit Hilfe der analytischen Chemie die für die biologischen Aktivitäten verantwortlichen Verbindungen identifiziert. Die meisten dieser Verbindungen wurden in den Pflanzenarten oder in der Natur überhaupt nicht mit biologischer Aktivität im mikro-molaren Bereich berichtet. Schließlich wurde eine pharmazeutische Technologie verwendet: durch Nanopulverisieren der Pflanzenteile wurde eine bessere biologische Aktivität im Vergleich zu dem organischen Extrakt beobachtet. Abschließend führte diese Forschung zur Entdeckung neuer Moleküle mit interessanter biologischer Aktivität, die eine weitere und detailliertere Untersuchung benötigen.

Summary

The investigation of plants used for traditional medicine in Togo is complicated as modern techniques are not available. Computer-aided product identification from traditional usage records (CAPITURE) was evaluated in the context of an ethnobotanical survey on the traditional treatment of fungal diseases in Tchamba District (Togo). This method predicted and identified the most biologically active plants out of the 43 species survey-recorded: *Pterocarpus erinaceus* predicted to be more active against fungi and *Daniellia oliveri* against bacteria. The plants were then tested against fungi, bacteria and cancer cells. As predicted with CAPITURE, *P. erinaceus* was more active against fungi and *D. oliveri* against bacteria. Interestingly, both plants presented activity on cancer cells without being toxic to normal human cells. In a third step, using analytical chemistry, the compounds responsible for the biological activities were identified. Most of those compounds have never been reported in the plant species or in nature at all, with biological activity in the micromolar range. Finally, pharmaceutical technology was used: by nanosizing the powder of the plant organs, a better biological activity was observed in comparison to that of the organic extract. In conclusion, this research led to the discovery of new molecules with an interesting biological activity that will need further and more detailed investigation.

Allgemeine Zusammenfassung

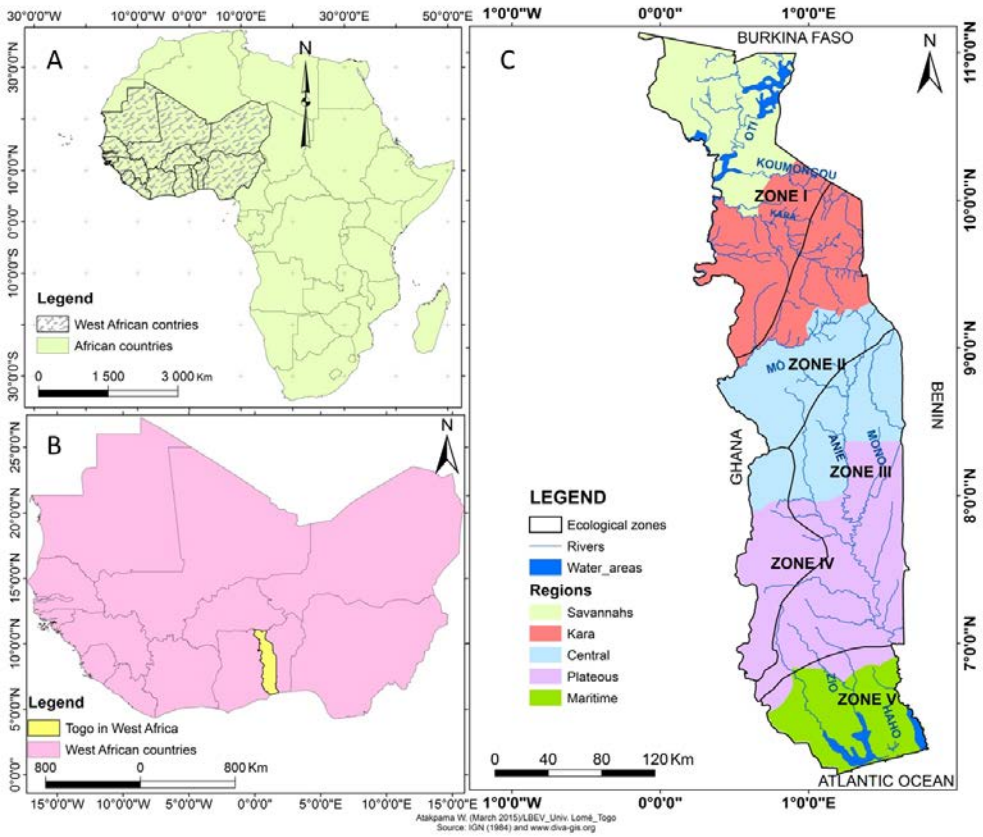
I. Allgemeine Einleitung

TOGO ist ein Land im westlichen Teil Afrikas. Es ist begrenzt durch den Atlantischen Ozean im Süden, Benin im Osten, Ghana im Westen und Burkina Faso im Norden. Das Land liegt zwischen 6 ° und 11 ° Nord Längengrad und 0 ° und 2 ° Ost Breite.

Er streckt sich in der Länge, folglich profitiert es von einem abwechslungsreichen Klima und Vegetation. Es beherbergt zwei Arten von tropischen Klima. Im südlichen Teil des Landes (bis zum 7. Parallele) gibt es ein Guineas subäquatoriales Klima mit zwei regnerischen Jahreszeiten und im nördlichen Teil des Landes (nach dem 7. Parallele) gibt es nur eine Regenzeit, deren Dauer sich verringert Schrittweise von Süden nach Norden. Darüber hinaus, um die 7. Parallele, haben wir die beiden Arten von Klima mit dem Beispiel der Stadt Atakpamé, wo es praktisch keine trockene Jahreszeit gibt (Adjoussi, 2000). Dieser Unterschied in dem Klima, das im Lande vorhanden ist, führt daher zu einer abwechslungsreichen Vegetation. Nach der Topographie, Geomorphologie, Klimatypen und floristischen Affinitäten ist Togo in fünf ökologische Zonen unterteilt (Ern, 1979):

- Zone I auch bekannt als nordliche Ebene ist die Domäne der sudanianischen Savannen dominiert von Combretaceae-Familien und stacheligen Bäumen und einem riesigen Gebiet von Parklandschaften und Schutzgebieten (Adjonou *et al*., 2009). Einige Pfade von Uferwäldern sind am Ufer des Flusses zu finden.
- Zone II oder Nordgebirge, entspricht einem Mosaik von Savannen, offene Wälder, trockene dichte Wälder und weite Gebiete mit Parksorten mit einer breiten Palette von Holzarten, die von *Parkia biglobosa* und *Vitellaria paradoxa* dominiert werden (Kebenzikato *et al*., 2014).
- Zone III oder zentral Ebene ist die Domäne von guineischen bewaldeten Savannen und Trockenwälder, offene Wälder und Uferwälder (Brunel *et al*., 1984). Das Klima hat einige Ähnlichkeiten mit der Zone I, aber im Gegensatz dazu variiert der jährliche Niederschlag zwischen 1200 mm und 1500 mm für eine Gesamtzahl von regen bis 120 Tage.
- Zone IV oder südliche Berge zeichnet sich vor allem durch eine Vegetation von halb-abfallende dichte Wälder und guineische Savannen so genannte Berge-Savannen (Akpagana et Guelly, 1994).
- Zone V oder Küstenebenen ist ein Mosaik aus Gestrüpp, landwirtschaftlich genutzten Flächen, Bricht, Strandwiesen und brechen Waldflecke die manchmal, heiligen Wald sind (Kokou *et al*., 2005).

Togo hat eine Bevölkerung von rund 6,1 Millionen Einwohnern aus 51,4% Frauen und 48,6% Männer. Mehr als die Hälfte (62,3%) der Bevölkerung verlassen sich in ländlichen Gebieten und insgesamt ist die Bevölkerung sehr unterschiedlich ethnisch (mehr als 40 ethnische Gruppen) (Kuevi, 1981, DGSCN, 2011, Goeh-Akue et Gaibor, 2010). Die Hauptaktivitäten der in ländlichen Gebieten lebenden Personen sind Landwirtschaft, Aufzucht und Fischerei. Die Hauptnahrungsmittelkulturen, die im Land angebaut werden, sind: Mais (*Zea mays* L. var), Sorghum (*Sorghum bicolor* var L.), Hirse (*Pennisetu americanum*), *Reis* (Oryza spp), Maniok (*Manihot esculenta* Crantz), Yamswurzel (hauptsächlich *Dioscorea alata* und *D. cayensis*), Cowpeas (*Vigna unguiculata* L.) und Erdnuss (*Arachis hypogaea* var) (Ministère de l'Agriculture, de l'Elevage et de la pêche, 2007, Akpavi *et al*., 2013).



<u>Abbildung 1</u>: Karte von Togo.

A: Westafrika in Afrika; **B:** Togo in Westafrika und **C:** Togo und seine verschiedenen Merkmale (Regionen, ökologische Zonen, Flüsse, etc., ...).

Auf der Seite des Gesundheitswesens wurde im Jahr 2010 ein nationaler Bericht erstellt, dass 61,5% der Togolesen unter der Schwelle der Armut leben. Diese extreme Armut hat einen schlechten Einfluss auf den Gesundheitsversorgungszugang zu Menschen im ganzen Land. Zusätzlich zu dieser finanziellen Grenze gibt es auch eine geografische Grenze, die durch eine

ungleichmäßige Verteilung der Gesundheitsdienstleister im ganzen Land gekennzeichnet ist. In der Tat, ein weiterer Bericht im Jahr 2009, dass es 610 Ärzte und 106 Apotheker registriert für das ganze Land. Davon arbeiten 28% der Ärzte in der Hauptstadt Lomé (10% der Landfläche) allein und 80% der Apotheker arbeiten auch in Lomé allein und die meiste Zeit in privaten Drogerien. Es wurde auch berichtet, dass Infektionskrankheiten unter den Top 10 Krankheiten des Landes waren, d. H. die Krankheiten, die zu mehr Todesfällen und Verletzungen führten, und denen, die das Land mit erheblichen Geldmengen ausgeben, um zu kämpfen. Darüber hinaus werden nur 30% der Togolesen im Allgemeinen berichtet, dass sie in Krankenhäusern gehen, wenn sie sich mit Fragen des Gesundheitswesens befassen (UNDAF, 2007, Ministère de la Santé, 2015, DGSN, 2009, DRSP-C, 2009, Tittikpina, 2012). Die Menschen benutzen folglich die andere Art von Medizin, die ihnen zur Verfügung steht: die traditionelle Medizin. Im Jahr 2010 hat die Weltgesundheitsorganisation (WHO) geschätzt, dass mehr als 80% der Menschen in den Entwicklungsländern traditionelle Medizin verwenden, um die Krankheiten zu heilen, von den sie leiden. Diese Institution definiert die traditionelle Medizin als "die Summe der Kenntnisse, Fähigkeiten und Praktiken auf der Grundlage der Theorien, Überzeugungen und Erfahrungen, die in verschiedenen Kulturen einheimisch sind, ob sie explizit sind oder nicht, die bei der Erhaltung der Gesundheit sowie bei der Prävention eingesetzt werden, Diagnose, Verbesserung oder Behandlung von körperlicher und geistiger Erkrankung "(WHO, 2010). Togo als Entwicklungsland ist keine Ausnahme von dieser Regel, wie durch den schwachen Prozentsatz der Menschen, die in Krankenhäuser gehen, bestätigt wird. Der Gebrauch der traditionellen Medizin und besonders der Pflanzen ist ein kultureller Aspekt für Togoischen Menschen. Zum Beispiel werden Blätter von *Newbouldia laevis*, werden verwendet in der Neujahrszeremonie in der Guin ethnischen Gruppe des südlichen Teils des Landes, um den heiligen Stein zu decken, dessen nachfolgende Farbe von den Einheimischen geglaubt wird, ein Gesamtgefühl des Jahres zu geben (Tittikpina, 2016). In den ethnischen Gruppen des zentralen Teils des Landes wird ein polyherbisches Formulierungsgetränk verwendet, um Kinder nach einer bestimmten Anzahl von Monaten zu ernähren (Edorh *et al*., 2015). Viele Studien haben die Verwendungen der Pflanzen im Land gemeldet (Tittikpina, 2016). Es müssen jedoch noch weitere Studien durchgeführt werden, um eine globale Sicht auf die traditionelle Nutzung der Pflanzen im ganzen Land zu ermöglichen. In der Tat macht die ethnische Vielfalt zu einer vielfältigen Vegetation Togo zu einer idealen Wahl für ethnobotanische und Arznei Studien. Es wird dazu beitragen, jahrhunderte alte Traditionen zu bewahren und eine wissenschaftliche Grundlage

für die Nutzung dieser Pflanzen zu schaffen, die automatisch in die moderne Medizin überführt werden. Mit diesem Ziel, da Infektionskrankheiten zu den vorrangigen Krankheiten gehören, wurde im September 2010 im Tchamba-Landkreis (Tittikpina, 2012) eine ethnobotanische Befragung durchgeführt. Diese Umfrage führte zu 43 Pflanzen, wie sie vor allem von traditionellen Heiler (TH) dieses Teils des Landes verwendet wurden, um Krankheiten zu behandeln, die durch Bakterien und Pilze verursacht wurden. Nach einer vorläufigen biologischen Untersuchung an einigen zufällig ausgewählten Pflanzen, nämlich *Pterocarpus erinaceus* Poir, *Daniellia oliveri* (Rolfe) Hutch. Et Dalz Und *Anchomanes difformis* (Blume) Engler (Tittikpina 2012, Tittikpina *et al*., 2013) ist ein strategischer Forschungsweg geplant:

- Erarbeitung einer Methodik zur objektiven Vorhersage der biologisch aktiven Spezies die aus einer ethnobotanischen übersicht resultieren
- Überprüfen die Genauigkeit dieser Methode, indem die biologischen Untersuchungen an den Pflanzen durchführen
- Identifizierung den Wirkungen der aktivsten Pflanzen vorhandenenchemischen Verbindungen und Fraktionen, die für die möglichen biologischen Aktivitäten verantwortlich sein können.
- eine Alternative zum Extraktionsverfahren vorzuschlagen, indem die biologische Aktivität von nanopartikeln der Pflanzenmaterialien erhalten bleiben.

II. Ethnobotanische Studie

Die Natur bietet eine Schatztruhe aus Naturprodukten mit oft erstaunlichen biologischen Aktivitäten, die seit Jahrhunderten weltweit für landwirtschaftliche und medizinische Anwendungen eingesetzt werden. Die üppige Vegetation in Westafrika ist besonders reich an medizinischen Pflanzen, und die einheimischen Stämme in Ländern wie Togo beschäftigen ein umfangreiches Arsenal an Pflanzen und pflanzlichen Produkten als Teil ihrer traditionellen Medizin. Verschiedene Studien wurden durchgeführt, um Informationen über diese traditionelle Medizin zu sammeln und die meiste Zeit eine riesige Anzahl von Pflanzen nicht nur in Togo, sondern auch in anderen Teilen Westafrikas zu erzeugen. Zum Beispiel wurden in Togo Untersuchungen über Pflanzen zur Bekämpfung von Diabetes und Malaria im südlichen Teil des Landes und an Pflanzen zur Behandlung von Erkrankungen des Zentralnervensystems im ganzen Land durchgeführt (Koudovo *et al*., 2011, Kpodar *et al*., 2015, Kantati *et al*., 2016). In Westafrika, Studien über Pflanzen für die Wundheilung in

Dongonland in Mali verwendet. In der mütterlichen Gesundheitsversorgung im Katsina-Staat in Nigeria und zur Behandlung von Blutung in Benin wurden versuchen durchgeführt (Inngjerdingen *et al*., 2004, Kankara *et al*., 2015, Klotoé *et al*., 2012).

Dennoch, welche Pflanzen, Pflanzenprodukte und Stoffe darin enthalten sind, sind aktiv und können gegen bestimmte Krankheiten angewendet werden, und die aufgrund von (traditionell) bekannten Nebenwirkungen toxisch oder weniger attraktiv sein können, ist bei weitem nicht trivial. Zugegebenermaßen konnten moderne analytische Methoden der Probenanalyse und Substanz-Identifizierung, wie z. B. automatisierte Chromatographie, gepaart mit Massenspektrometrie, spezifische Substanzen aus Rohstoffen innerhalb von Stunden identifizieren. Gleichzeitig können gleichermaßen automatisierte Aktivitätsbildschirme ein Aktivitäts- oder Toxizitätsprofil gegen Mikro-Organismen oder Zellen innerhalb eines Tages oder zwei zeigen. Daher ist unsere Fähigkeit, auf (neue) medizinisch aktive Substanzen im Labor heute zu sehen, Lichtjahre, die im Vergleich zu den umständlichen Studien der Generation vor uns fortgeschritten sind.

Mit anderen Worten, die berühmte Frage "Wo zu beginnen" in unserem Kontext kann man am effizientesten durch eine kurze Konsultation des Medicinmann beantworten. In Togo zum Beispiel können wirtschaftliche Schwierigkeiten und ein unebener Zugang zu Gesundheitseinrichtungen und modernen Medikamenten die Ausbreitung von Infektionskrankheiten ermöglichen, die in diesem Land unter den Top 10 der vorrangigen Krankheiten stehen (Ministère de la Santé 2015, Tittikpina, 2012). Natürliche Heilmittel auf der Grundlage von lokal angebauten Pflanzen und traditionelle Kenntnisse im Zusammenhang mit ihnen oft die einzige Quelle und Ressource, um solche Bakterien-und Pilzinfektionen zu behandeln. Infolgedessen ist es wichtig, solche Pflanzen weiter zu untersuchen, und tatsächlich wurden verschiedene Studien im Land durchgeführt, um den traditionellen therapeutischen Ansatz in den Vordergrund zu rücken (Koudovo *et al*., 2011, Kpodar *et al*., 2015, Kpodar *et al*., 2016 und Hoekou *et al*., 2016).

Dennoch wissen wir, dass die Förderung der traditionellen Medizin allein die Ausbreitung solcher Krankheiten nicht stoppen wird. Es ist auch unmöglich, alle Pflanzen von Togo, die mit einer oder mehreren medizinischen Verwendungen für alle (Arten von) möglichen medizinischen Anwendungen verbunden sind, zu screenen. Hier ist ein strukturierterer und letztlich auch fokussierterer Ansatz eindeutig gerechtfertigt. Wir haben daher eine einfache computergestützte "Vorwahlmethode" entwickelt, die (a) bislang unstrukturiert und mündlich traditionelle Kenntnisse in halbstrukturierten Interviews verabschiedet hat, (b) extrahiert

quantitative Zahlenwerte aus diesen Zeugnissen, (c) nutzt diese Numerische Werte in einem Algorithmus zu (d) Rang, Auswahl und damit identifizieren die vielversprechendsten Pflanzen oder Pflanzenteile für weitere Labor-basierte Untersuchungen im Ausland. "Computergestützte Produktidentifikation aus traditionellen Gebrauchsrekorden" (Computer Aided Products Identification from Traditional Usage Records, CAPITURE) beruht daher auf jahrhundertealten Erfahrungen mit Medikamenten und menschlichen Patienten und dürfte daher das Feld der möglichen Kandidaten einschränken. Ein Anspruch, den wir im Labor für Pflanzen mit Vermutete antimikrobielle (antimykotische) Aktivität, ausgewählt aus dem Tchamba-Bezirk von Togo bewertet haben.

Der Tchamba-Bezirk in Togo, der im zentralen östlichen Teil des Landes, ist einzigartig, da es der einzige Bezirk ist, der Menschen aus neun verschiedenen ethnischen Gruppen zusammenbringt: *Tchamba*, *Koussountou*, *Tem*, *Tem Fulani*, *Kabyè*, *Ana-Ifè*, *Bassar*, *Lamba* und *Logba*. Wir haben deshalb diesen spezifischen, engen, lokalen ethnischen Schmelztiegel von Menschen, ihren Kulturen und Traditionen ausgewählt, da er (a) ein umfangreiches und vielfältiges lokales traditionelles medizinisches Wissen verspricht und (b) mit seiner hohen Konzentration von traditionellen die Heilern in kurzer Zeit effizient befragt werden können.
Darüber hinaus gab es in diesem Bezirk keine vorherige Untersuchung der traditionellen Verwendungen von Pflanzen gegen infektiöse (Pilz-) Krankheiten. Daher wird unsere Studie unvoreingenommen sein und Aufzeichnungen und Daten erzeugen, die in jedem Fall neuartig und originell sein werden. In den Hauptlokalen des Bezirks im September 2010 wurden mit einem Fragebogen, der Informationen über die personenbezogenen Daten über den Befragten suchte, halb strukturierte Einzelgespräche über Pflanzen zur Behandlung von Pilzerkrankungen mit 53 traditionellen Heiler (TH) durchgeführt. Der Name der Erkrankung in der Landessprache und die Symptome, die verwendeten Pflanzenteile und ihre Art der Vorbereitung und Verabreichung sowie andere mit dieser Pflanze behandelte Krankheiten werden registriert. Die Fragebögen wurden in Microsoft Excel-Tabellen eingegeben und analysiert. Die Analyse basierte auf der Berechnung einiger numerischer Indizes, die mit einer bibliographischen Überprüfung unterstützt wurden, die wir in die CAPITURE-Methode umgesetzt haben. Folgende Indizes wurden verwendet:

- die gemeldete Verwendung (Reported Use, **RU**) ist definiert als die Gesamtzahl der für jede Anlage gemeldeten Verwendungen
- der Use Value (**UV**) zeigt die relative Bedeutung der lokal bekannten Arten:

$$UV = \frac{\sum U \text{ or } RU}{n}$$

wo ∑**U** das gleiche wie das **RU** ist, ist die Gesamtzahl der Zitate pro Spezies und **n** die Anzahl der Informanten.

- Plant Part Value (**PPV**) liefert Informationen über den am häufigsten verwendeten Pflanzenteil zur Behandlung die Pilzkrankheiten. Die PPV für einen Anlagenteil ist gleich dem Verhältnis zwischen der Anzahl der gemeldeten Gesamtnutzungen für den Teil der Anlage und der Gesamtzahl der gemeldeten Verwendung für die Anlage **RU**-Anlagenteil / **RU**-Anlage
- der spezifische Gebrauchswert (Specific Use value, **SU**) ist die Anzahl der Male, die eine spezifische Verwendung eines Pflanzenteils gegen eine Pilzerkrankung durch den Befragten gemeldet wird.
- Der Intraspecific Use Value (**IUV**) ist die Ration zwischen dem SU eines Anlagenteils und dem RU für diese Anlage **SU** Anlagenteil / **RU** Anlage.
- Der Informant Consensus Factor (ICF) ist ein Wert zwischen 0 und 1. Die **ICF** wird wie folgt berechnet: Anzahl der Verwendungszwecke für die Behandlung von Pilzerkrankungen (**Nur**) abzüglich der Anzahl der verwendeten Arten (**nt**), geteilt durch **Nur** minus eins. Hohe ICF-Werte werden erhalten, wenn nur eine oder mehrere Pflanzenarten von einem hohen Anteil an Informanten zur Behandlung einer bestimmten Kategorie verwendet werden, während niedrige ICF-Werte darauf hindeuten, dass Informanten nicht übereinstimmen, welche Pflanze zu verwenden ist

$$ICF = \frac{Nur - nt}{Nur - 1}$$

(Gomez-Beloz, 2002; Andrade-Cetto *et al*., 2011, Wouyo Atakpama *et al*., 2012, Gazanneo *et al*., 2005).

Die Analyse wurde wie in den folgenden Zeilen beschrieben durchgeführt.

- **Erst Schritt**

- Zuerst wurden die RU und die UV auf alle Pflanzen berechnet, um eine lange Liste zu erzeugen Pflanzen
- Zweitens wurde ein Cut-off auf Basis eines RU (≥2) oder eines UV (≥ 0,0377) angewendet, um a zu erhalten Kurze Liste der Pflanzen
- Drittens wird eine bibliographische Überprüfung an den Pflanzen aus der kurzen Liste durchgeführt, um zu erhalten eine Liste von Pflanzen, die (a) häufig im Tchamba-

Bezirk verwendet wurden, um (verschiedene) Pilzerkrankungen zu behandeln, (b) wurden unabhängig als medizinische Pflanzen in der Literatur erwähnt und (c) wo noch von ausreichender Neuheit, um weitere Untersuchungen zu rechtfertigen.

- **Letze Schritt**

Eine zweite Charge von Indizes werden auf den Pflanzen berechnet, die auf der Präzedenzliste stehen. Dieser letzte Schritt könnte auf zwei Arten durchgeführt werden:

- erstens können wir die folgenden spezifischen Indizes in dieser Reihenfolge berechnen: die **PPV**, die **SU** und die **IUV**. Hier führt uns die **PPV** zu den interessantesten Pflanzenteilen oder Organen zur Behandlung von Pilzerkrankungen. Die **SU** zeigt auf Pflanzenteile, die speziell zur Behandlung einer bestimmten Pilzerkrankung verwendet werden, und die **IUV** verfeinert den Vergleich weiter, indem sie die **SU** von verschiedenen Teilen derselben Pflanze vergleicht. Die interessantesten Pflanzen sind die mit dem höchsten **SU** und **IUV**.
- zweitens können wir die **ICF**, die **SU** und die **IUV** berechnen. Hier muss das **ICF** berechnet sein für die verschiedenen Arten von Pilzerkrankungen, die von der TH als erster Schritt berichtet wurden. Der zweite Schritt ist, sich auf Pilzerkrankungen zu konzentrieren, die den höchsten **ICF** haben. Und im dritten Schritt können wir die **SU** und die **IUV** für die Pflanzenteile (aus der kurzen Liste) berechnen, die für die Behandlung der Erkrankungen mit dem höchsten **ICF** verwendet wurden. Und auch hier werden die interessantesten Pflanzen diejenigen sein, die den höchsten **SU** und damit die **IUV** aufweisen.

Mit diesem Ansatz konnten wir die interessantesten Pflanzen vorhersagen, die uns führen werden, um die höchsten biologischen Aktivitäten aus den 43 Arten der Umfrage und ihre spezifischen Aktivitäten zu haben: *Pterocarpus erinaceus* saft gegen Scherpilzflechte, *Daniellia oliveri* saft gegen Intertrigo und beziehungsweise, Ihre Wurzeln und Rinden gegen Candidiasis.

Zusammenfassend konnten wir unsere Methode nutzen, um von einer grundlegenden ethnopharmakologischen Untersuchung von 53 TH im Tchamba-Distrikt von Togo zu einer strukturierten, objektiven Einschätzung des vorhandenen Wissens über die Verwendung von natürlichen Pflanzenprodukten gegen ein gemeinsames Spektrum zu gelangen Pilzinfektionen. Während unsere Vorgehensweise immer noch unbeantwortete Fragen hinterlässt (z. B. in Bezug auf Pflanzenarten, die aus der Liste eliminiert werden) und bietet

auch genügend Raum für Verbesserungen (z. B. durch die Einführung weiterer chemischer und ökologischer Parameter), hat es uns dennoch erlaubt, die riesige Anzahl von Medizinische Pflanzen in Togo zu einem ausgewählten wenigen gefunden. Gleichzeitig werden wir die bisherigen wenigen Leads, insbesondere im Zusammenhang mit *P. erinaceus* und *D. oliveri*, genauer untersuchen, wobei zu berücksichtigen ist, dass der CAPITURE-Ansatz nur ein Vor-Screen ist, dem ein volles Laboratorium folgen muss Basierte Analyse der (aktiven) chemischen Inhaltsstoffe, die in den Pflanzen gefunden wurden, eine Bewertung ihres Spektrums an biologischen Aktivitäten und potenziellen Verwendungen und eine vollständige Untersuchung der zugrunde liegenden Wirkungsweise. Wie bei mehreren Gelegenheiten erwähnt, wird die Langzeitliste mit insgesamt 43 Anlagen immer für eine Neubewertung von Pflanzen, Pflanzenteilen und Infektionen sein, falls die nötigen Pflanzen entstehen sollen (zum Beispiel, wenn die ausgewählten Pflanzen zu einer Sackgasse oder Zeit führen und die Finanzierung für weitere Analysen verfügbar ist).

III. Biologischer Teil

Pflanzen sind eine wertvolle Quelle für biologisch aktive Verbindungen: 17.810 Arten werden weltweit als Medizin verwendet (SOTWP, 2016). Im Jahr 2001 stammen etwa 25% der weltweit verschriebenen Medikamente aus Pflanzen. Von den 252 Drogen, die von der Weltgesundheitsorganisation (WHO) als grundlegend und wesentlich angesehen werden, sind 11% ausschließlich pflanzlichen Ursprungs und eine signifikante Anzahl von synthetischen Drogen, die aus natürlichen Vorläufern gewonnen wurden (Preise, 2001). Und diese Zahl hat sich über Jahrzehnte erhöht und wird weiter zunehmen, angesichts der neuen Herausforderungen, denen sich die Gesundheitssysteme der ganzen Welt gegenübersehen: Auftauchen neuer und / oder alter Infektionskrankheiten, Drogenresistenz und Ausbreitung nicht übertragbarer Krankheiten. Gegenwärtig sind sie wegen der antimikrobiellen Resistenz (O'Neill, 2016) auf dem Vormarsch und sind in den Entwicklungsländern häufiger, wo einer von zwei Menschen vor Infektionen vorzeitig im Vergleich zu den entwickelten Ländern (WHO, 2012, Tittikpina, 2012). Der jüngste Ausbruch von 'Ebola' in einigen Ländern Westafrikas ist ein weiteres Beispiel für die Wiederauftauchung von Infektionskrankheiten, wenn der Kampf gegen sie vorbei sein sollte, wie von vielen Experten in den 1970er Jahren behauptet wurde. In der Tat, im Jahr 1970, der Chirurg-General der Vereinigten Staaten von Amerika, zeigte, dass es "Zeit war, das Buch über Infektionskrankheiten zu schließen, erklären den Krieg gegen die Pestilenz gewonnen und verlagern nationale Ressourcen zu

solchen chronischen Problemen wie Krebs und Herzerkrankungen "(WHO, 2000). Nach den 1970er Jahren waren viele Projekte oder Programme auf nur nicht-kommutierbare Krankheiten ausgerichtet, bis vor kurzem, als die Antibiotikaresistenz aufgetreten ist. In der Tat hat ein Bericht über die Zulassungen neuer Antibiotika durch die Food and Drug Administration (FDA) in den USA pro Fünfjahreszeit (von 1983 bis 2012) einen linearen und konstanten Rückgang gezeigt (Boucher *et al*., 2013). Mikroben haben diese Periode der Nicht-Entdeckung oder Forschung auf Infektionskrankheiten verwendet, um sich genetisch zu entwickeln und Arten von Resistenz zu entwickeln. Tuberkulose, zum Beispiel mit Menschen entwickelt, und Antibiotika-Resistenz in *M. tuberculosis* tritt ausschließlich durch spontane Mutation (Davies and Davies, 2010). Außerdem hat die WHO vor kurzem eine Antibiotika-resistente Prioritätsliste veröffentlicht, unter der die kritische Prioritätsliste multiresistente *Acinetobacter*, *Pseudomonas* und verschiedene *Enterobacteriaceae* einschließlich *Escherichia coli* (WHO, 2017) enthält. Neue Leads haben dann gefunden, um diese globalen Gesundheitsprobleme zu lösen. Die Pflanzen sind eine gute Wahl nicht nur, weil sie in der Vergangenheit schon viele gute Ergebnisse erzielt haben, sondern auch wegen des erheblichen Potenzials, das nicht bewertet wurde. Dieser globale Kontext unterscheidet sich daher nicht sehr von dem in den vorherigen Zeilen beschriebenen togoischen Kontext. Die Suche nach neuen Molekülen, die auf traditioneller Medizin basieren, präsentiert sich dann als Lösung nicht nur togoische, sondern auch global. Die beiden Pflanzen, die nach der CAPITURE-Methode ausgewählt wurden und auf ethnobotanischen Untersuchungen zu Infektionskrankheiten basieren, werden daher gegen eine breite Palette von Bakterien, Pilzen und Nematoden getestet. Der globale Aufstieg von nicht übertragbaren Krankheiten wird es auch wert sein, ihre Aktivitäten gegen Krebszellen zu überprüfen, auch wenn ihre Verwendung gegen Krebserkrankungen während der ethnobotanischen Erhebungen von TH nicht gemeldet wurde. In der Tat, Artemisinin zur Behandlung von Malaria, wurde auch berichtet, um Anti-Krebs-Aktivität zu zeigen, obwohl *Artemisia annua* L (die Pflanze kommt es aus), wird in der chinesischen Medizin zur Behandlung von Malaria (Das, 2015) verwendet. Diese biologischen Tests ermöglichen es, die aktivsten Extrakte und Pflanzenteile auszuwählen, auf denen anschließend die phytochemische Analyse durchgeführt wird.

Die beiden Pflanzen wurden gegen die folgenden Bakterien getestet:

- Gram positive Cocci: *Enterococcus faecalis* ABC 3 (ATCC 29212) und *Staphylococcus aureus* ABC 1 (ATCC 29213).

- Gram negative Bazillen: *Escherichia coli* ABC 5 (ATCC 25922), *Klebsiella peumoniae* ABC 42 und *Enterobacter cloacae* ABC 291.
- Nicht-fermentierende Gram negative Bazillen: *Pseudomonas aeruginosa* ABC 4 (ATCC 27853) und *Acinetobacter baumannii* ABC 14.

Tests gegen Mykose mit den Pflanzen wurden gegen zwei Arten von Pilzen (*Aspergillus niger*, *Aspergillus fumigatus*) und eine Hefe (*Candida albicans*) durchgeführt.

Die Methodik, die zur Durchführung der antibakteriellen und antifungalen Tests verwendet wurde, war die Brüdenverdünnungsmethode, wie von EUCAST, 2015 und CLSI, 8. Auflage empfohlen.

Der rohe Extrakt der Rinden von *D. oliveri* stellte die niedrigste MIC (Konzentration, die 100% des Bakterienwachstums hemmt) gegen Bakterien, nämlich *E. faecalis*, bei 16 µg/mL und *D. oliveri* war am weitesten am aktivsten bei Bakterien. Alle seine Teile (Blätter, Rinden und Wurzeln) waren auf allen Bakterien und ihren nachfolgenden Fraktionen aktiv: Wann immer ein MIC nicht mit einem Extrakt oder einer Fraktion erhalten wurde, gibt es fast immer eine Hemmung des Bakterienwachstums bei der höchsten getesteten Konzentration (256 µg/mL). Insgesamt wurden 55 MIC-Werte (im Bereich von 16 µg/mL bis 256 µg/mL) mit den Extrakten und Fraktionen aus dieser Pflanze im Vergleich zu *P. erinaceus* mit nur 34 MIC-Werten (im Bereich von 32 µg/mL bis 256 µg /mL) gemessen. Tatsächlich waren nicht alle Teile von *P. erinaceus* auf Bakterien aktiv, seine Rinde ware am aktivsten und einige MIC-Werte oder Hemmung des Bakterienwachstums bei 256 µg/mL wurden mit einigen der Fraktionen aus den Blättern und Wurzeln erhalten. Im Gegensatz dazu zeigte sich *P. erinaceus* mit den Anti-Mykose-Ergebnissen aktiver. In der Tat war der rohe Extrakt der Wurzeln von *P. erinaceus* gegen *A. fumigatus* mit einem MIC bei 16 µg /mL aktiv. Unsere bisherige Studie (Tittikpina *et al*., 2013) hat die Aktivität der Stammrinde der beiden Pflanzen auf *C. albicans* mit dem aktivsten *P. erinaceus* bestätigt. Eine frühere Studie hat auch die Aktivität von *P. erinaceus* gegen *Trycophyton rubrum* bestätigt (Etuk *et al*., 2008). Diese Ergebnisse haben die Prognosen der CAPITURE-Methode bestätigt. In der Tat, hat CAPITURE vorhergesagt, dass *P. erinaceus*, um gegen *Trycophyton rubrum* (beteiligt in Scherpilzflechte) und *Candida albicans* (beteiligt an Candidiasis) aktiv zu sein. Außerdem hat es für *D. oliveri* gegen *Candida albicans*, die an Candidiasis beteiligt ist, aber auch in Intertrigo, dass zusätzlich durch *P. aeruginosa* und *S. aureus* verursacht werden könnte, aktiv gezeigt. Diese Methode zeigte eindeutig, dass *P. erinaceus* gegen Pilze und *D. oliveri* gegen

Bakterien aktiver war, unter Berücksichtigung der Arten und der Anzahl der Keime, die an den Krankheiten beteiligt waren, die sie heilen sollten.

Außerdem wurden die beiden Pflanzen auch gegen fünf Arten von Krebszellen getestet:

- Hs 683 und U 373: Gliomzellen (Tumorzellen aus Wirbelsäule oder Gehirn)
- SKMEL 28: Melanomzellen (Tumorzellen aus der Haut)
- A 549: Lungenkarzinomzellen (Tumorzellen aus der Lunge).
- MDA-MB 231: Brustkrebszellen

Die Methodik, die verwendet wird, um den Antikrebs-Assay durchzuführen, ist der MTT-Assay, der auf der Fähigkeit des NADPH-abhängigen zellulären Succinat-Dehydrogenase Enzym basiert, das in den Mitochondrien von lebenden Zellen vorhanden ist, um eine chemische Verbindung zu reduzieren [Tetrazolium-Farbstoffes MTT 3- (4, 5-dimethylthiazol-2-yl) -2,5-diphenyltetrazoliumbromid (gelbe Farbe) zu seiner unlöslichen Formazanform (violette Farbe]. Diese Farbe konnte mit einem ELISA-Spektrophotometer gemessen werden und die Extinktion ist proportional zur Anzahl der lebenden Zellen (Mosmann, 1983). Interessante Ergebnisse wurden auch mit den Roh-Extrakten aus den Teilen der beiden Pflanzen mit IC 50 (Konzentration, die 50% der Krebszellen tötet) im Bereich von 28 µg/mL bis 91 µg/mL erhalten. Die aktivste Pflanze war *P. erinaceus* mit all seinen Teilen, die gegen mindestens eine Art von Gliomzellen aktiv waren. Seine Wurzeln waren der aktivste Teil, IC 50 im Bereich von 28 µg/mL bis 54 µg/mL gegen alle fünf Arten von Krebszellen mit dem niedrigsten IC 50, 28 µg/mL gegen U 373. Nur die Blätter von *D. oliveri* war gegen Hs 683 bei 91 µg / mL aktiv (der höchste IC 50, der während des Anti-Krebs-Tests beobachtet wurde). Darüber hinaus wurden die Pflanzen gegen die Nematoden *Steinernema feltiae* getestet, aber ihre rohen Extrakte und Fraktionen waren gegen diesen Parasiten bei Konzentrationen bis zu 10 mg / mL nicht aktiv.

Nach diesen verschiedenen Bio-Assays wurde die Toxizität der aktivsten Extrakte und Fraktionen im Wettbewerb einer normalen menschlichen Zelllinie (MRC-5-Zellen) unter Verwendung des MTT-Assays festgestellt. In den meisten Fällen waren die Extrakte und Fraktionen nicht toxisch für jene normalen menschlichen Zellen in Konzentrationen, bei denen sie gegen Bakterien, Pilze, Hefe und Krebszellen aktiv waren.

Alle diese Bio-Assays haben uns eine globale Sicht auf die beiden biologischen Aktivitäten gegeben. *P. erinaceus* und *D. oliveri* sind gegen Bakterien, Pilze und Krebszellen mit sehr interessanten MIC-Werten von 16 µg/mL bis 256 µg/mL und IC 50 -Werten von 28 µg/mL

bis 91 µg/mL aktiv. Die Ergebnisse bestätigen die Verwendung dieser beiden Pflanzen in der Volksmedizin in Togo zur Behandlung von Infektionskrankheiten. Außerdem haben die Pflanzen auch interessant eine nicht aufgezeichnete traditionelle Aktivität, eine Anti-Krebs-Aktivität. Wichtig ist, dass die erzielten Ergebnisse die Vorhersagen der CAPITURE-Methode bestätigt haben. In der Tat hat diese Methode vorhergesagt, dass *P. erinaceus* besonders gegen Pilze und *D. oliveri* gegen Bakterien und Pilze aktiv sei. Ohne Überraschung präsentierte *D. oliveri* die beste Aktivität auf Bakterien (MIC bei 16 µg/mL) und die größte Anzahl aktiver Extrakte mit zusätzlich einer Aktivität gegen *Candida albicans* (MIC: 1.88-30 mg/mL). *P. erinaceus* stellte die beste Aktivität gegen Pilze (MIC bei 16 µg/mL auf *Aspergillus niger*) mit zusätzlich eine sehr gute Aktivität gegen Bakterien dar. *P. erinaceus* zeigte auch eine Aktivität gegen *C. albicans* (1.88 mg/mL - 30 mg/mL). Der nächste Schritt besteht darin, die aktiven Prinzipien zu finden, die die Aktivitäten auslösen und Bio-Assays auf diesen Verbindungen durchführen.

IV. Analytischer Teil

Analytische Chemie-Werkzeuge lassen sich grob in 2 Hauptgruppen unterteilen: Trennverfahren, deren Ziel die Isolierung der gewünschten aktiven Moleküle und Charakterisierungstechniken ist, die die Bestimmung der chemischen Strukturen der Wirkstoffe ermöglichen. Der analytische Teil dieser Arbeit bestand darin, diese verschiedenen Methoden zu verwenden, um die Verbindungen zu enthüllen, die für die mit den aktivsten Fraktionen und / oder Extrakten beobachteten biologischen Aktivitäten verantwortlich sein können. Vom Rohstoff bis zu den aktiven Molekülen müssen während des analytischen Teils unserer Arbeit verschiedene Schritte eingehalten werden (Abbildung 2):

- extraktion wird auf dem Rohmaterial durchgeführt, um einen Rohextrakt zu erhalten.
- mindestens ein Aufreinigungsverfahren wird auf den Rohextrakten ausgeführt, um gereinigten Extrakten zu erhalten (dh hier raffiniert, nicht unbedingt rein) oder Fraktionen, bei denen eine qualitative Analyse im allgemeinen mit Trennverfahren (Gas- und / oder Flüssigchromatographie) an Massenspektrometrie (GC-MS und / oder LC-MS)
- der letzte Schritt entspricht der Isolierung oder Reinigung der Fraktionen (oder Raffinierte Extrakte) unter Verwendung von chromatographischen Techniken, wie z. B. Niederdruck-Flüssigchromatographie (LCPC); Flash Chromatographie (FC) und Dünnschicht Chromatographie (TLC), um reine Moleküle zu erhalten. Um die

gereinigten Moleküle zu charakterisieren und zu identifizieren, werden dann qualitative Analysen an den isolierten Verbindungen unter Verwendung der Kernmagnetische Resonanz (NMR 1D oder 2D) und Massenspektrometrie (einschließlich hochauflösender Massenspektrometrie HRMS) durchgeführt. Nach der Charakterisierung werden komplementäre Informationen über die Verbindung durch Ultraviolett (UV) und Infrarot (IR) Spektren und Schmelzpunkte erhalten.

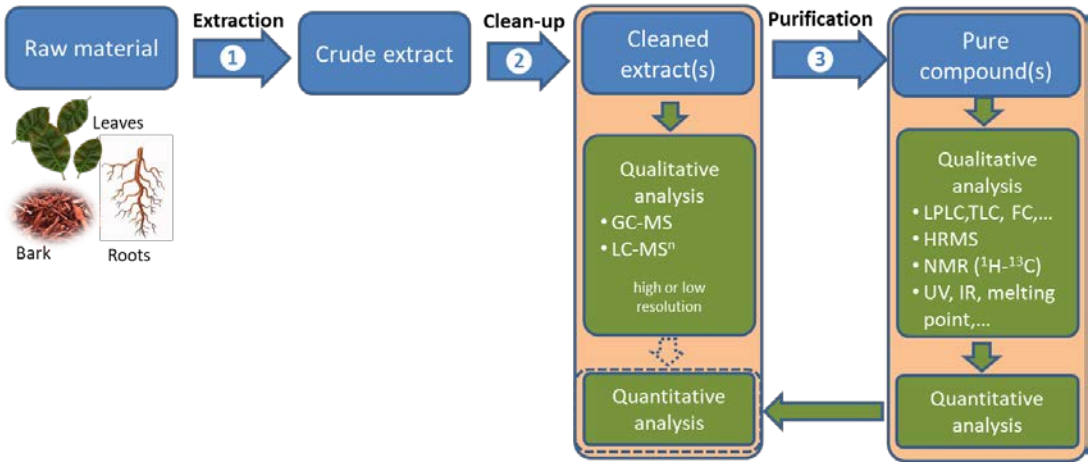


Abbildung 2: Verschiedene Schritte in den analytischen Studien

Der Rohstoff ‚Raw material' könnte entweder die Blätter sein, die Rinde oder die Wurzeln einer Pflanze. Der Roh-Extrakt ‚Crude extract' ist auch der Roh-Extrakt und der gereinigte Extrakt ‚Cleaned extracts' ist auch der Bruchteil. Pure-Moleküle ‚Pure compounds' sind reine Verbindungen, die nach Isolierungs- und Reinigungsprozessen an den Fraktionen oder Rohextrakten erhalten werden. GC-MS und LC-MS sind Gas- und Flüssigkeitschromatographie an Massenspektrometrie gekoppelt. Niederdruck-Flüssigchromatographie (LCPC); Flash Chromatographie (FC), Dünnschicht Chromatographie (TLC).

Die analytischen Studien konzentrierten sich in erster Linie auf *P. erinaceus*, da es die Pflanze mit der breitesten Palette von Aktivitäten, antibakterielle, antimykotische und anti-Krebs ist, im Vergleich zu *D. oliveri*, die mehr oder weniger nur antibakterielle Aktivitäten präsentierten. Die Bio-geführte Isolierung von Verbindungen wurde an der Ethylacetat-Fraktion des Roh-Extrakt der Rinden von *P. erinaceus* und dem Roh-Extrakt seiner Wurzeln unter Verwendung einer normalen Phase-offenen Säulenchromatographie erreicht. Dies führte zur Isolierung von 2,3-Dihydroxypropyloctacosanoat (1); *β*-sitosterol (2); Stigmasterol (3); Campesterol (4), Friedelin (5) und *β*-sitosteryl-*β*-D-glucopyranosid (6) in der Ethylacetat-Fraktion seiner Rinde mit MIC-Werten um 4 µg/mL gegen *Staphylococcus* Bakterien. In dem

Roh-Extrakt seiner Wurzeln wurden mit MIC-Werten von 8 µg/mL bis 256 µg/mL gegen *A. fumigatus* die folgenden Substanzen isoliert: *β*-sitosteryl-*β*-D-glucopyranosid (1), Formononetin (2), Pseudobaptigenin (3), Isoliquiritigenin (4), Muningin (5), Friedelin (6) und eine neue Isoflavone und ein neu Arylpropanoid. Alle diese Verbindungen außer Friedelin wurden in dieser Pflanze noch nicht berichtet und die beiden neuen Verbindungen (einer von ihnen hat eine MIC bei 256 µg / ml auf *A. fumigatus*) wurde bisher nie in der Natur berichtet. Die isolierten Verbindungen wurden unter Verwendung von 1D- und 2D-NMR und Molekularstimmung durch Massenspektrometrie identifiziert. Unter Verwendung nur massenspektrometrischer Methoden, die an chromatographische Methoden gekoppelt sind, wurden in der Butanolfraktion der Rinden dieser Pflanze noch niemals identifiziert Strukturen vorgeschlagen: Pseudobaptigenin-Glc-Xyl (oder Ara); Formononetin-Glc-Xyl (oder Ara); Genistein-Glc-Xyl (oder Ara) und deren Isomere sowie andere Strukturen. Durch die an die Massenspektrometrie (GC-MS) gekoppelte Gaschromatographie an den apolaren Fraktionen von *D. oliveri* und *P. erinaceus* wurden in beiden Pflanzen viele andere Verbindungen identifiziert, die in diesen Pflanzenarten vorher noch nicht bekannt waren, wie zB: Terpene, Phytosetrole, Fett säuren, einschließlich ungesättigter Fettsäuren und Phenole.

Mit der analytischen Chemie, von der Trennung bis zu den Nachweismethoden, konnten wir Verbindungen identifizieren, einige von ihnen isolieren und ihre biologischen Aktivitäten testen. Die GC-MS-Analyse, die auf den apolaren Fraktionen der Blätter, Rinde und Wurzeln realisiert wurde, zeigte die Anwesenheit von Fettsäuren mit Terpenen, Sterolen und Phenole. Die mit diesen apolaren Fraktionen beobachteten antibakteriellen und gegen pilz Aktivitäten sind nicht überraschend, da Terpene, Fettsäuren, Monoglyceride und Phenole weitgehend dokumentiert sind, um solche biologischen Aktivitäten zu besitzen. Einige Verbindungen wurden isoliert und ihre Strukturen wurden unter Verwendung von NMR bewiesen. Andere Moleküle wurden unter Verwendung von LC-MS für eine *in-situ* chemische Profilierung auf einigen Fraktionen charakterisiert. Die meisten der isolierten und oder identifizierten Verbindungen wurden in diesen Arten oder in der Natur überhaupt nicht berichtet. Leider konnten wir in dieser Studie keine Isolierung und Strukturidentifikation von Proben von *D. oliveri* durch NMR durchführen. Eine vorläufige Untersuchung der polaren Fraktionen dieser Pflanze durch LC-MS wurde jedoch durchgeführt. Diese Voruntersuchung hat die Anwesenheit einer beeindruckenden Anzahl von Anthocyanidinen festgestellt. Daneben ist auch bei *P. erinaceus*, bei dem z. B. mehr als 500 Verbindungen, die im Butanol-Extrakt

deiner Rinde vorhanden sind, mehr zu entdecken. Weitere Studien könnten uns helfen, einen globalen Überblick über die chemische Zusammensetzung der beiden Pflanzen zu geben.

V. Pharmazeutische Technologie, Nanopulverisieren von Pflanzen: Nanosized Natural Pflanzenmaterial von *Pterocarpus erinaceus* Poir mit vielversprechenden antibakteriellen Aktivitäten

Extraktion ist die häufig verwendete Methode, um die in den Pflanzen enthaltenen Wirkstoffe freizusetzen. Dies impliziert die Verwendung von organischen Lösungsmitteln, die die meiste Zeit nicht sicherer sind, nicht nur für die Person, die die Extraktion führt, sondern auch nicht für die Umwelt. Von der Extraktion bis zum reinen Wirkstoff müssen mehrere Schritte befolgt werden: Trennung und Isolierung mittels chromatographischer Methoden, Strukturidentifikation mittels fortgeschrittener Techniken wie Nuklearmagnetresonanz (NMR) und Massenspektrometrie (MS), wie in den vorherigen Zeilen dieser Arbeit beschrieben. Die reinen Verbindungen werden dann zu Formularen verarbeitet, die für die medizinische Versorgung geeignet sind, wie Cremes, Sprays, Pillen, usw.... Und hier ist es notwendig, Geräte zu verwenden, die die meiste Zeit nicht in Entwicklungsländern wie Togo verfügbar sind. In solchen Ländern wird das Pflanzenmaterial gewöhnlich nach Mazeration, Abkochen oder Infusion von frischen oder getrockneten Pflanzen in Wasser, in Alkohol oder einer Mischung von beiden angewendet. Es wird daher interessant sein, billige, leicht verfügbare Wege zu finden, um diese bioaktiven Verbindungen aus Pflanzen sicher und umweltfreundlich herzustellen.

Um dieses Problem zu lösen, haben wir unsere Aufmerksamkeit auf nanopulvern, wenig verarbeitete, rohe Pflanzenmaterialien gelenkt (bitte beachten Sie, dass hier der Ausdruck "Nanopulverung" verwendet wird, um das Arsenal der verwendeten Mahl- und Homogenisierungsmethoden zu beschreiben und bedeutet nicht, dass die Materialien, diese Verfahren bestehen notwendigerweise auch aus Partikeln mit Durchmessern im Nanometerbereich). Wir möchten solche Techniken testen und sehen, ob sie in der Lage sind, die aktiven Moleküle in einer Weise zu entschlüsseln, um die gleiche Wirkung zu erzielen wie diejenige, die mit dem organischen Extrakt aus demselben Pflanzenteil erhalten werden kann.

Um dieses Ziel zu erreichen, sollten wir in der Lage sein, von einem einfachen Mahlen mit z. B. eine Kaffee oder Maismühle (wie in vielen afrikanischen Ländern verwendet) zu gehen, um Partikel mit verschiedenen Durchmessern (Millimeter groß) zu Partikeln von kontrollierten Größen (Nanometer groß) mit Durchmessern zu erhalten. Um das letztere zu

erhalten, stellt sich das Nassmehlen als Methode der Wahl dar, da es sehr gute Teilchengrößen (Fahr, 2015) erhalten kann. Unter den heute üblichen Verfahren zum Nassmahlen, dh mahlen mit Kolloidmühlen, Perlenmahlen und Hochdruckhomogenisierung (HPH), ist HPH die Methode die hier diskutierten wird: Der hohe Energieeintrag während des Homogenisierungsprozesses ermöglicht eine effiziente Verminderung des Materials innerhalb kurzer Zeit (Jahnke, 2001). HPH ist auch eine bekannte und damit gut etablierte, unkomplizierte und sichere Technik, die in der Praxis häufig nicht nur in der Pharmaindustrie, sondern auch im Bereich der Kosmetik- und Lebensmittelproduktion angewendet wird. Darüber hinaus ist eine Großserienfertigung möglich (Keck, 2006). Es ist auch anzumerken, dass die grundlegenden HPH-Geräte, von den Regulierungsbehörden akzeptiert werden, sind relativ kostengünstig (ca. 10 000€) und weltweit verfügbar.
Pterocarpus erinaceus Frinde, deren Extrakte sich als sehr aktiv gegen verschiedene Bakterien bewiesen haben, wurden daher als Pflanzenmaterial ausgewählt, auf dem solche Versuche durchgeführt wurden.

Die Nanopulverung der Rinde wurde in drei aufeinanderfolgenden Schritten realisiert: erstens ein Trockenfräsen mit einem FastPrep 24 Instrument (MP Biomedicals, Solon, OH, USA) Precellys Kits (Bertin Technologies, Montigny-le-Bretonneux, Frankreich); zweitens eine Stabilisierung durch Suspension in 1% Plantacare® 2000 UP (Alkyl-Polyglycosid, BASF, Ludwigshafen, Deutschland) in destilliertem Wasser zu 1% Makro-Suspensionen von fein gemahlenen Pflanzenmaterialien und drittens eine Vor-homogenisierung mit einem MICCRA D-9 Homogenisator-Disperser (MICCRA GmbH, Müllheim, Deutschland) in verschiedenen Zyklen, gefolgt von einer sukzessiven Homogenisierung mit einem APV Gaulin LAB 40 (APV GmbH, Mainz, Deutschland) Hochdruck-Homogenisator (Griffin *et al*., 2015). Die 1% Makro-Suspensionen von fein gemahlenen Pflanzenmaterialien wurden verwendet, um die Bio-Assays auf einem Nematoden (*Steinernema feltiae*), einem Bakterium (*Escherichia coli*) und einer Hefe (*Saccharomyces cerevisiae*) mit einem Vergleich der Ergebnisse mit den zuvor erhaltenen durchzuführen mit dem organischen Extrakt. Die Nanopartikeln aus der Rinde von *P. erinaceus* zeigten eine konzentrationsabhängige Aktivität gegen *S. feltiae* und *E. coli* bei einer Konzentration von 1%. Im Gegensatz dazu beeinflusste der Rindenextrakt von *P. erinaceus* nicht die Lebensfähigkeit von *S. feltiae*, sondern verringerte das Bakterienwachstum von einem OD_{593} von 0,8 in der Kontrolle auf nur eine OD_{593} von 0,2 (dh

auf etwa 25% des ursprünglichen Wertes), wenn sie in einer Konzentration von 256 µg/mL eingesetzt werden.

Im wesentliche hat dieser Teil der Studie, untersützung für die Idee, dass die Nanopulverung von pflanzlichen Materialien es uns ermöglicht, von einem rohen getrockneten Pflanzenmaterial zu einem anwendbaren Partikel-basierten Liefer- und Freisetzungssystem, in nur einem oder wenigen Schritte macht. Während es noch viele Fragen gibt, die noch in der Zukunft angesprochen und beantwortet werden sollen, ist keines dieser Probleme unüberwindbar. Abhängig von den verfügbaren Mitteln können die Methoden zur Nanopulverung variiert und verfeinert werden, was letztlich zu definierteren und stabileren Partikeln führt. Freisetzungseigenschaften können auch durch solche Prozesse gesteuert werden, und so können die (physikalischen) Eigenschaften des Teilchens selbst und die biologische Aktivität, die durch die von ihm freigesetzten Stoffe verursacht wird, sein.
Zukünftige Studien konzentrieren sich daher nicht nur auf die Vorbereitung einer breiten Palette von Partikeln aus einer ebenso breiten Palette von lokalen Anlagen und unterschiedlicher anwendungsspezifischer Qualität. Sie werden auch ein viel breiteres Spektrum möglicher Anwendungen in den Bereichen Ernährung und Kosmetik, in der Prävention und Therapie von Krankheiten berücksichtigen. Hier können Herz-Kreislauf-, entzündungshemmende, Anti-Krebs-und Anti-Infektions-Agenten an der Spitze der Untersuchung sein. Partikel von *P. erinaceus* haben zum Beispiel bereits in diesem Zusammenhang eine gewisse Aktivität gezeigt und nach einer Untersuchung eine neue Ära für lokale gewachsene und produzierte Partikel gegen Dysenterie und einfache Hautinfektionen erläutern können.
Letztendlich wird auch bei landwirtschaftlichen Anwendungen ein Schwerpunkt liegen, da die in der Landwirtschaft benötigten Mengen deutlich höher sind als in der Medizin, während das potenzielle Risiko für den Menschen geringer ist.

VI. Schlussfolgerung und Perspektiven

Von der traditionellen Medizin bis zu modernen Untersuchungen, von Pflanzen zu einer Medizin, hat diese Studie noch einmal die Wirksamkeit von Pflanzen unterstrichen, in diesem Fall in der traditionellen Medizin im Tchamba Bezirk von Togo verwendet, um verschiedene Infektionskrankheiten zu behandeln. Die Studie hat dazu beigetragen, die offensichtlichste Frage, die immer nach ethnobotanischer Umfrage aufgeworfen wurde, zu adressieren, wenn

die Pflanzen aktiv sind? Im Rahmen dieser Untersuchung wurde ein Komputer Program vorgeschlagen, um die interessantesten Pflanzen vorherzusagen und anschließend auszuwählen. Auch wenn eine solche Methode Verbesserungen erfordert, ergibt sich interessante Ergebnisse, wie die biologischen Aktivitäten der nach dieser Methode ausgewählten Pflanzen belegen. In Bezug auf den biologischen Teil lohnt es sich, die Aktivität der beiden Pflanzen gegen andere Arten von Gram-positiven Bakterien, insbesondere die *Staphylococcus*, zu untersuchen, da es eine bevorzugte Aktivität gegen diese Art von Keim gibt. Der analytische chemische Teil hat erhebliche strukturelle Informationen über die beiden Pflanzen zur Verfügung gestellt. Weitere Untersuchungen sind jedoch erforderlich, insbesondere bei *D. oliveri*, wo keine Isolierung durchgeführt wurde, sondern auch mit den massenspektrometrischen Studien an *P. erinaceus*-Extrakten und Fraktionen. Die gefundenen neuen Strukturen sollten auch weiter untersucht werden, um ihre gesamte biologische Aktivitäten zu bewerten und zu sehen, ob daraus neue Arzneimittel gewonnen werden könnten. Auf diese Weise wird der Transfer von der traditionellen zur modernen Medizin abgeschlossen sein. Im Allgemeinen werden ausführlichere Studien eine vollständige Karte dieser Pflanzen liefern: biologische Aktivitäten, pharmakologische Eigenschaften, Toxikologie, bioaktive Verbindungen, usw.....

Résumé général de la thèse

1. Introduction générale

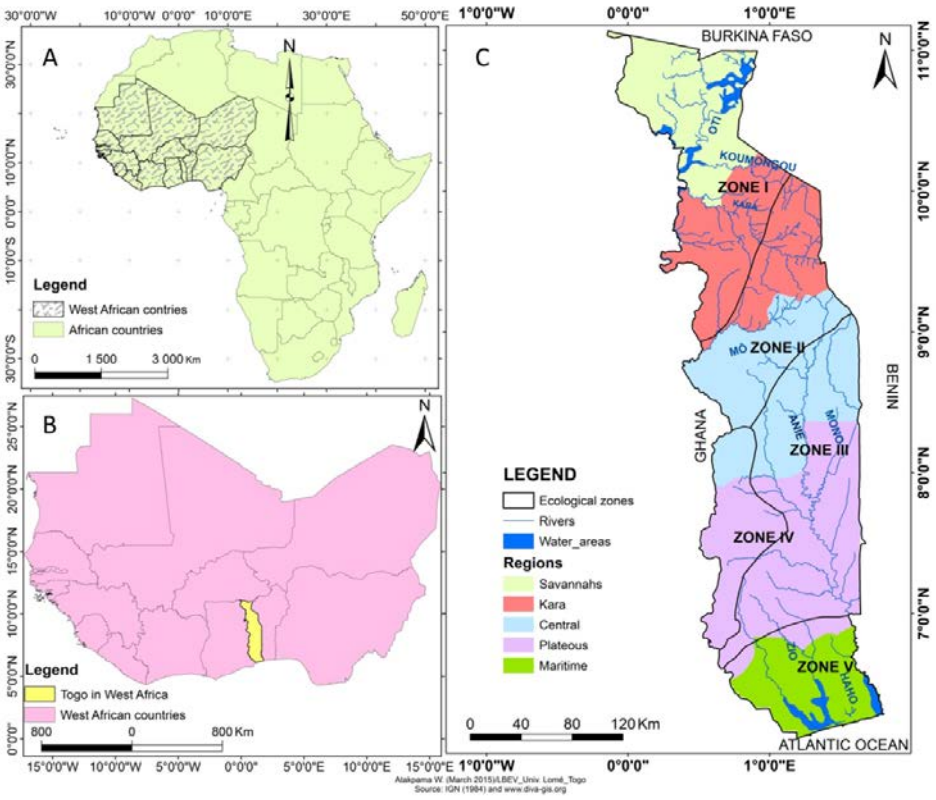
Le TOGO est un pays de l'Afrique de l'Ouest. Il est bordé au Nord par le Burkina Faso, au Sud par l'Océan atlantique, à l'Est par le Bénin et à l'Ouest par le Ghana. Il se situe entre les 6 ° et 11° longitude nord et les 0° and 2° latitude est (Figure 1).

Le pays s'étire longitudinalement et de ce fait, bénéficie de climats et d'une végétation diverses. Le climat est un climat tropical de deux types. Dans la partie méridionale du pays (jusqu'au 7^{e} parallèle), c'est un climat guinéen de type subéquatorial caractérisé par deux saisons de pluie : une grande saison de pluie de Mars à Juillet avec un maximum en Juin et une petite saison des pluies de Septembre à Octobre. Dans la partie septentrionale du pays (après le 7^{e} parallèle), on retrouve une seule saison de pluie dont la durée diminue progressivement du sud au nord. Et autour du 7^{e} parallèle, on retrouve les deux types de climat, avec pour exemple, la ville dénommée Atakpamé où on ne retrouve particulièrement pas de saison sèche (Adjoussi, 2000). Cette diversité de climat donne naissance à une diversité de végétation. En prenant en compte la topographie, la géomorphologie, les types de climat et les affinités floristiques, le Togo peut être subdivisé en cinq zones écologiques (Ern, 1979) : I, II, III, IV, and V (Figure 1).

- Zone I ou la 'zone des plaines du nord' est le domaine des savanes soudaniennes dominées par la famille des *Combretaceae*, des arbres épineux et un vaste domaine de parcs et d'aires protégées (Adjonou *et al*., 2009).
- Zone II ou 'zone des montagnes du nord', correspond à une mosaïque de savanes, de forêts ouvertes, de forêts denses et sèches et, de vastes domaines de parcs avec une grande variété d'arbres ligneux dominés par *Parkia biglobosa* et *Vitellaria paradoxa* (Kebenzikato *et al*., 2014).
- Zone III ou zone des plaines centrales est le domaine de savanes boisées guinéennes ; de forêts sèches, ouvertes et de galerie (Brunel *et al*., 1984).
- Zone IV ou zone des montagnes du sud est caractérisée par une végétation de forêts denses semi-décidues et de savanes guinéennes communément nommées savanes de montagnes (Akpagana et Guelly, 1994).
- Zone V ou zone des plaines côtières : c'est une mosaïque de fourrés, aires agricoles, jachères, prairies côtières, parcelles de forêts jachères qui sont parfois des forêts sacrées (Kokou *et al*., 2005).

Concernant sa population, le Togo a une population de près de 6,1 millions d'habitants dont 51.4% de femmes et 48.6 % d'hommes. Plus de la moitié de cette population (62.3%) vit en

zone rurale et est très diversifié sur le plan ethnique (plus de 40 groupes ethniques) (DGSCN, 2011, Goeh-Akue & Gayibor, 2010 ; Kuevi, 1981). Les activités principales des populations qui vivent en zone rurale sont l'agriculture, l'élevage et la pêche. L'agriculture porte principalement sur les produits vivriers. Les produits vivriers majoritairement cultivés sont : le mais (Zea mays *L*. var), le sorgho (*Sorghum bicolor* var L.), le mil (*Pennisetu americanum*), le riz (*Oryza* spp), le manioc (*Manihot esculenta* Crantz), l'igname (surtout *Dioscorea alata* et *D. cayensis*), le niébé (*Vigna unguiculata* L.), et l'arachide (*Arachis hypogaea* var) (Ministère de l'Agriculture, de l'Elevage et de la pêche, 2007 ; Akpavi *et al*., 2013).



<u>Figure 1 :</u> Carte du Togo.

A : Afrique de l'Ouest en Afrique ; **B** : Togo en Afrique de l'Ouest ; **C** : Togo et ses différentes subdivisions (écologiques montrées par une délimitation en noir sur la carte et administratives révélées par un coloriage). Les zones écologiques sont dénommées : Zones I, II, III, IV et V.

Sur le plan sanitaire, selon un rapport national établi en 2010 ; 61,5% des Togolais vivraient en-dessous du seuil de pauvreté. Cette pauvreté extrême a une mauvaise influence sur la santé de la population dans tout le pays. En plus de cette limite financière, s'ajoute une limite

géographique caractérisée par une inégale répartition des prestataires des soins de santé sur l'étendue du territoire national. En effet, un autre rapport établi en 2009 révèle que pour 610 médecins et 106 pharmaciens enregistrés dans tout le Togo, 28% de ces médecins et 80% de ces pharmaciens travaillaient à Lomé (la capitale qui représente 10% de la surface du Togo). Ce rapport a également établi que les maladies infectieuses se retrouvaient parmi les dix maladies prioritaires, c'est-à-dire, ces maladies qui entraînent plus de pertes en vies humaines et ou d'incapacités, et sur lesquelles l'Etat emploie d'énormes financements. En plus de cela, seulement 30% des Togolais en général, font usage des hôpitaux ou centres de santé lorsqu'ils sont malades (UNDAF, 2007 ; DGSCN, 2009 ; DRSP-C, 2009 ; Tittikpina, 2012 ; Ministère de la Santé, 2015). La majorité de la population utilise la médecine qui leur est facilement accessible : la médecine traditionnelle. En 2010, l'Organisation Mondiale de la Santé (OMS) a estimé que plus de 80% de la population vivant dans les pays en voie de développement utilise la médecine traditionnelle pour le traitement de maladies. Le Togo, faisant partie des pays en voie de développement, ne fait pas exception à cette règle comme le confirme le faible pourcentage des Togolais qui consultent dans les centres de santé en cas de maladie. L'utilisation de la médecine traditionnelle et surtout les plantes, fait partie intrinsèque de la culture au Togo. Par exemple, les feuilles de *Newbouldia laevis* sont utilisées dans les cérémonies de nouvelle année dans l'ethnie Guin (située dans la zone V) pour couvrir une pierre sacrée dont la couleur selon la croyance prédit l'aspect général de la nouvelle année, si celle-ci sera bonne ou mauvaise (Tittikpina *et al*., 2016). Les groupes ethniques du centre du pays utilisent une boisson locale faite à base de plusieurs plantes pour nourir les nouveaux-nés à partir d'un certain nombre de mois après leur naissance (Edorh *et al*., 2015). Beaucoup d'études ont aussi été réalisées dans le pays pour documenter l'utilisation des plantes en médecine traditionnelle (Tittikpina *et al*., 2016). Cependant, un plus grand nombre d'études est nécessaire. En effet, la combinaison d'une diversité d'ethnies avec une végétation diverse, fait du Togo, un endroit privilégié pour réaliser des enquêtes ethnobotaniques et sur les pharmacopées. Cela permettra de préserver ces traditions vieilles de centaines d'années, faire des tests au laboratoire pour donner une base scientifique à l'utilisation de ces plantes qui pourraient ainsi être utilisées en médecine moderne. Dans cet objectif, des enquêtes ethnobotaniques ont été réalisées en Septembre 2010 dans la Préfecture de Tchamba au Togo (Tittikpina, 2012). Ces enquêtes ont permis de relever quarante-trois plantes comme étant les plus utilisées par les tradipraticiens dans cette partie du Togo pour traiter les maladies causées par des bactéries et champignons. Après une première analyse biologique sur trois plantes,

sélectionnées de façon arbitraire, nommément *Pterocarpus erinaceus* Poir, *Daniellia oliveri* (Rolfe) Hutch. et Dalz. et *Anchomanes difformis* (Blume) Engler (Tittikpina 2012 ; Tittikpina *et al*, 2013) ; un projet de recherche a été établi avec les objectifs suivants:

- élaborer une méthodologie objective pour prédire les plantes qui seraient plus actives sur le plan biologique ou pharmacologique après des enquêtes ethnobotaniques.
- vérifier l'exactitude de cette méthode en effectuant les tests biologiques / ou pharmacologiques nécessaires sur les plantes sélectionnées par cette méthode et voir si les plantes présentent des activités biologiques / pharmacologiques.
- identifier les composés chimiques présents dans les extraits et ou fractions de plantes et qui pourraient être responsables des activités biologiques / pharmacologiques observées.
- proposer une alternative écologique à l'extraction par des solvants organiques en testant l'activité de nanoparticules de plantes.

2. Enquête ethnobotanique

La nature fournit un immense trésor de produits naturels avec des activités biologiques souvent étonnantes, qui ont été utilisés en agriculture et en médecine pendant des siècles dans le monde entier. La végétation luxuriante en Afrique de l'Ouest est particulièrement riche en plantes médicales et les populations tribales dans des pays comme le Togo, emploient un vaste arsenal de plantes et de produits dérivés de plantes dans le cadre de leur médecine traditionnelle. Diverses études ont été menées pour recueillir des informations sur cette médecine traditionnelle, générant la plupart du temps un grand nombre de plantes non seulement au Togo, mais aussi dans d'autres parties de l'Afrique de l'Ouest. Par exemple, au Togo, des enquêtes sur les plantes utilisées pour étudier le diabète et le paludisme dans la partie méridionale du pays et sur les plantes utilisées pour traiter les maladies du système nerveux central dans tout le pays ont été menées (Koudovo *et al*., 2011 ; Kpodar *et al*., 2015 ; Kantati *et al*., 2016). Et en Afrique de l'Ouest, les connaissances traditionnelles sur les plantes utilisées pour la cicatrisation chez les Dongon au Mali ; dans les soins de santé maternelle dans l'État de Katsina au Nigéria et pour traiter l'hémorragie au Bénin ont été étudiées (Inngjerdingen *et al*., 2004 ; Klotoé *et al*., 2012 et Kankara *et al*., 2015).

Pourtant, l'identification dans les plantes et les produits végétaux, des substances actives pouvant être administrées contre certaines maladies, substances pouvant aussi être toxiques ou

moins attrayantes en raison des effets secondaires (traditionnellement connus), est loin d'être évidente. En effet, les méthodes analytiques modernes d'analyse des échantillons et d'identification structurale, telles que la chromatographie couplée à la spectrométrie de masse, peuvent permettre l'identification de substances spécifiques à partir de matières brutes en quelques heures. Dans le même temps, des tests d'activité biologique et ou pharmacologique également automatisés, peuvent révéler un profil d'activité ou de toxicité contre les microorganismes cibles ou des cellules dans un temps très court. Par conséquent, notre capacité à identifier des (nouvelles) substances médicalement actives dans le laboratoire est de nos jours très développé par rapport à ce qui pouvait être effectué il y a quelques décennies.

En d'autres termes, la fameuse question « Où commencer ? » dans notre contexte, peut être mieux répondu par une brève consultation de la médecine. Au Togo, par exemple, les difficultés économiques et l'accès irrégulier aux établissements de santé et aux médicaments modernes permettent la propagation de maladies infectieuses qui, dans ce pays, figurent parmi les dix maladies prioritaires (Ministère de la Santé 2015 ; Tittikpina, 2012). Les remèdes naturels basés sur des plantes cultivées localement et les connaissances traditionnelles qui leur sont liées représentent souvent la seule source et ressource pour traiter ces infections bactériennes et fongiques. Par conséquent, il est essentiel d'étudier davantage ces plantes. En effet, certaines études ont été menées dans le pays pour mettre l'approche thérapeutique traditionnelle à l'avant-garde (Koudovo *et al*., 2011 ; Kpodar *et al*., 2015 ; Kpodar *et al*., 2016 et Hoekou *et al*., 2016).

Néanmoins, nous savons que la promotion de la médecine traditionnelle seule ne risque pas d'empêcher la propagation de ces maladies. Il est également impossible d'étudier toutes les plantes du Togo associées à une ou plusieurs utilisations médicales. De ce fait, une approche plus structurée et finalement plus ciblée est clairement justifiée. Nous avons donc développé une simple « méthode de présélection assistée par ordinateur » qui (a) enregistre jusqu'alors non structurées et transmises oralement, les connaissances traditionnelles dans des entretiens semi-structurés, (b) extrait des valeurs numériques quantitatives de ces témoignages, (c) utilise ces valeurs numériques dans un algorithme pour (d) classer, sélectionner et donc identifier les plantes ou les parties de plantes les plus prometteuses sur le plan biologique / ou pharmacologique comme sujets pour des recherches approfondies. L'identification assistée par ordinateur de produits basés sur des utilisations en médecine traditionnelle (Computer Aided Product Identification from Traditional Usage Records, CAPITURE) repose donc sur une

utilisation centenaire et traditionnelle de plantes sur des êtres humains, par conséquent, est susceptible d'affiner le champ des candidats possibles (Tittikpina *et al*., 2017). Cette affirmation, nous l'avons ensuite évaluée au laboratoire pour les plantes avec une activité antimicrobienne suspectée (antifongique) sélectionnée après des enquêtes menées auprès de tradipraticiens dans le district de Tchamba au Togo.

La Préfecture de Tchamba au Togo, qui se trouve au centre-est du pays, est unique car elle est la seule préfecture qui regroupe des personnes de neuf groupes ethniques différents : *Tchamba*, *Koussountou*, *Tem*, *Tem Fulani*, *Kabyè*, *Ana-Ifè*, *Bassar*, *Lamba* et *Logba*. Nous avons donc choisi ce 'melting pot' ethnique car cette préfecture (a) promet des connaissances médicales traditionnelles étendues et variées et (b) peut être étudiée efficacement avec sa forte concentration de guérisseurs traditionnels à courte distance. En outre, il n'y a eu aucune enquête préalable sur les utilisations traditionnelles des plantes contre les maladies infectieuses (fongiques) dans cette préfecture. Ainsi, notre étude ne sera pas biaisée et générera des données qui seront dans tous les cas, nouvelles et originales. Des interviews individuelles semi-structurées sur les plantes utilisées pour le traitement des maladies fongiques ont été réalisées avec 53 guérisseurs traditionnels (GT) dans les principales localités de la préfecture en septembre 2010 en utilisant un questionnaire demandant des informations sur : les données personnelles sur l'interviewé, le type de maladies fongiques, le nom de la maladie dans la langue locale, ses symptômes, les parties de plantes utilisées, leur mode de préparation et d'administration ainsi que d'autres maladies traitées avec cette plante. Les questionnaires ont ensuite été saisis et analysés en utilisant Microsoft Excel. L'analyse était basée sur le calcul de certains indices numériques aidés d'un examen bibliographique pour confectionner la méthode CAPITURE. Les indices suivants ont été utilisés :

- le **Reported Use** (**RU**) ou 'utilisation enregistrée' est définie comme le nombre total d'utilisations déclarées pour chaque plante
- le **Use Value** (**UV**) ou 'valeur d'usage' (Equation 1) démontre l'importance relative des espèces connues localement: où ∑**U** identique à RU, est le nombre total de citations par espèce, **U** est l'utilisation et **n** le nombre d'informateurs**.**

$$UV = \frac{\sum U \text{ or } RU}{n} \qquad \text{(Equation 1)}$$

- le **Plant Part Value** (**PPV**) ou 'Vale**u**r de la partie de plante' donne des informations sur la partie la plus utilisée d'une plante pour traiter les maladies fongiques. Le PPV pour une partie de la plante est égal au rapport entre le nombre total d'utilisations signalées pour cette partie de plante et le nombre total d'utilisations signalées pour la plante : **RU** $_{\text{partie plante}}$/ **RU** $_{\text{plante.}}$
- le **Specific Use value** (**SU**) ou 'valeur d'utilisation spécifique' est le nombre de fois où l'enquêté a signalé une utilisation spécifique d'une partie de plante contre une maladie fongique.
- le *Intraspecific Use Value* (**IUV**) ou 'valeur d'utilisation intraspécifique' est le ratio entre la SU d'une partie de plante et la RU pour cette plante **SU** $_{\text{partie plante}}$/ **RU** $_{\text{plante}}$.
- le **Informant Consensus Factor** (**ICF**) ou 'facteur de consensus des enquêtés' est une valeur comprise entre 0 et 1. L'ICF est calculé comme suit: nombre de citations d'utilisation pour le traitement des maladies fongiques (**Nur**) soustrait du nombre d'espèces utilisées (**nt**), divisé par **Nur** moins l'unité. Les valeurs élevées de l'**ICF** sont obtenues lorsqu'une seule ou quelques espèces de plantes sont utilisées par une proportion élevée d'informateurs pour traiter une catégorie particulière, tandis que les faibles valeurs de l'**ICF** (Equation 2) indiquent que les informateurs ne sont pas d'accord sur la plante à utiliser (Gomez-Beloz, 2002 ; Andrade-Cetto *et al*., 2011 ; Atakpama *et al*., 2012 ; Gazanneo *et al*., 2005).

$$ICF = \frac{Nur - nt}{Nur - 1} \qquad \text{(Equation 2)}$$

L'analyse a été effectuée en suivant deux étapes ci-dessous décrites.

- Première étape

- Tout d'abord, la RU et l'UV ont été calculées sur toutes les plantes pour générer une *longue liste* de plantes.
- Deuxièmement, une sélection basée sur une RU (≥2) ou une UV (≥ 0,0377) a été appliquée pour obtenir une *courte liste* de plantes
- Troisièmement, un examen bibliographique est effectué sur les plantes de la *courte liste* pour obtenir une *liste finale* de plantes qui sont fréquemment utilisées dans le district de Tchamba pour traiter (diverses) maladies fongiques, (b) sont rarement mentionnées dans la littérature et (c) ont encore une nouveauté suffisante pour justifier une enquête plus approfondie.

- Etape finale

Un deuxième lot d'indices est calculé sur les plantes figurant sur la *liste finale*. Cette étape finale pourrait être exécutée de deux manières :

- d'abord, nous pouvons calculer les indices spécifiques suivants dans cet ordre : le PPV, le SU et l'IUV. Ici, le PPV nous conduit à la (les) partie (s) ou aux organes les plus intéressants pour traiter les maladies fongiques. Le SU souligne les parties de plantes spécifiquement utilisées pour traiter une maladie fongique particulière et l'IUV, affine la comparaison en la portant sur les différentes parties d'une même plante. Les plantes les plus intéressantes seront celles avec le SU et l'IUV les plus élevés.
- deuxièmement, nous pouvons calculer l'ICF, le SU et l'IUV. Ici, l'ICF doit être calculé sur les différents types de maladies fongiques rapportés par le GT en première étape. La deuxième étape consiste à se concentrer sur les maladies fongiques qui ont l'ICF le plus élevé. Et dans la troisième étape, nous pouvons calculer la SU et l'IUV pour les parties de plantes (présentes sur la liste finale) utilisées pour le traitement des maladies et ayant l'ICF le plus élevé. Et là encore, les plantes les plus intéressantes seront celles qui présentent le SU le plus élevé et donc l'IUV le plus élevé.

À l'aide de cette approche, nous avons pu prédire les plantes les plus intéressantes qui nous conduiront à avoir les meilleures activités biologiques parmi les 43 espèces recensées et leurs activités spécifiques : la sève de *Pterocarpus erinaceus* contre la teigne, la sève de *Daniellia oliveri* contre l'intertrigo et respectivement leurs racines et leurs écorces du tronc contre la candidose.

En résumé, nous avons pu utiliser notre méthode pour passer d'une enquête ethnopharmacologique de base de 53 GT dans la Préfecture de Tchamba au Togo à une évaluation plus structurée et objective des connaissances existantes sur l'utilisation de produits végétaux naturels contre un éventail de fréquentes infections fongiques. Bien que notre approche laisse encore diverses questions sans réponse (par exemple en ce qui concerne les espèces végétales éliminées de la liste) et offre également suffisamment de place pour l'amélioration (par exemple en introduisant d'autres paramètres chimiques et environnementaux), elle nous a néanmoins permis de réduire le vaste nombre de plantes médicales trouvées au Togo à quelques plantes sélectionnées. Dans le même temps, nous étudierons de plus près les quelques pistes identifiées jusqu'à présent, en particulier dans le contexte de *P. erinaceus* et *D. oliveri*, en gardant à l'esprit que l'approche CAPITURE n'est

qu'un pré-exercice qui se poursuivra par un travail plus complet au laboratoire : évaluation des spectres d'activités biologiques ou pharmacologiques des plantes, une détermination des composés chimiques (actifs) présents dans les plantes, une étude complète des modes d'action sous-jacents de ces composés, etc... Comme mentionné à plusieurs reprises, la longue liste avec un total de 43 plantes sera toujours là pour une réévaluation des plantes, des parties de plantes et des infections en cas de besoin (par exemple, si les plantes sélectionnées conduisent à une impasse ou si un financement devient disponible pour des analyses supplémentaires).

3. Partie biologique

Les plantes sont une source précieuse de composés biologiquement actifs, 17 810 espèces dans le monde sont utilisées comme médicaments (SOTWP, 2016). En 2001, environ 25% des médicaments prescrits dans le monde proviennent de plantes. Sur les 252 médicaments considérés comme fondamentaux et essentiels par l'Organisation mondiale de la santé (OMS), 11% sont exclusivement d'origine végétale et un nombre significatif sont les médicaments synthétiques obtenus à partir de précurseurs naturels (Rates, 2001). Ce nombre a augmenté au cours des décennies et continuera à augmenter compte tenu des nouveaux défis auxquels sont confrontés les systèmes de santé dans le monde : émergence de maladies infectieuses (nouvelles ou anciennes), résistance aux médicaments et propagation de maladies non transmissibles. Les maladies infectieuses sont actuellement en hausse en raison de la résistance aux antimicrobiens (O'Neill, 2016) et sont plus fréquents dans les pays en développement où une personne sur deux meurt prématurément à partir d'infections par rapport aux pays développés (OMS, 2012 ; Tittikpina, 2012). La récente flambée de 'Ebola' dans certains pays d'Afrique de l'Ouest est un autre exemple de la progression de maladies infectieuses alors que la lutte contre ces derniers était supposée terminée selon de nombreux experts dans les années 1970. En effet, en 1970, le chirurgien général des États-Unis d'Amérique a indiqué qu'il était « temps de fermer le livre sur les maladies infectieuses, déclarer que la guerre contre la peste était gagnée et transférer les financements nationaux à des projets de recherches sur des maladies chroniques tels que le cancer et les maladies cardiaques » (OMS, 2000). Après les années 70, les recherches ont été orientées principalement vers les maladies non transmissibles jusqu'à récemment avec l'apparition de résistance aux antibiotiques. En effet, un rapport sur les approbations de nouveaux antibiotiques par la Food and Drug Administration (FDA) aux États-Unis par période de 5 ans

de 1983 à 2012 a montré une diminution linéaire et constante de l'introduction de nouveaux antibiotiques (Boucher *et al*., 2013). Les germes ont utilisé cette période d'abstinence de découverte ou de recherche sur les maladies infectieuses, pour évoluer pour certaines d'entre elles sur le plan génétique et développer des résistances. La tuberculose, par exemple, a évolué avec les humains et la résistance aux antibiotiques chez *Mycobacterium tuberculosis* se produit exclusivement par mutation spontanée (Davies and Davies, 2010). L'OMS a récemment publié une liste prioritaire de germes résistants aux antibiotiques. Dans la sous-liste des germes ayant atteint le point critique présente sur cette liste, on retrouve : l'*Acinetobacter* multirésistant, les *Pseudomonas* et diverses *Enterobacteriaceae* dont *Escherichia coli* (OMS, 2017). De nouvelles pistes doivent ainsi être trouvées pour résoudre ce problème de santé mondial. Les plantes sont alors un bon choix non seulement parce qu'elles ont donné beaucoup de bons résultats dans le passé, mais aussi en raison du grand potentiel qu'elles possèdent. Ce contexte mondial n'est pas par conséquent bien diférent du contexte togolais décrit dans les lignes précédentes. La recherche de nouvelles molécules en se basant sur la médecine traditionnellle se présente alors comme une solution pas seulement togolaise mais aussi mondiale. Les deux plantes sélectionnées dans le chapitre précédent à l'aide de la méthode CAPITURE basée sur des enquêtes ethnobotaniques sur les maladies infectieuses, feront ainsi l'objet de cette recherche de nouvelles molécules. Diférents extraits préparés à partir des parties de ses plantes, seront donc testées contre une large gamme de bactéries, de champignons et de nématodes. Dû au fait de la hausse croissante du taux des maladies non transmissibles, il sera également utile de vérifier leurs activités contre les cellules cancéreuses, même si leur utilisation contre ces maladies cancéreuses n'a pas été rapportée par les tradipraticiens. En effet, l'artémisinine (isolée *d'Artemisia annua* L, utilisée dans la médecine traditionnelle chinoise pour traiter le paludisme) a également été signalée comme ayant une activité anticancéreuse, bien que ce ne soit pas l'utilisation principale de cette plante en médecine traditionnelle chinoise (Das, 2015). Ces tests biologiques permettront de sélectionner les extraits et parties de plantes les plus actives sur lesquels l'analyse phytochimique sera ultérieurement réalisée.

Les deux plantes ont été testées contre les bactéries suivantes :

- Cocci à Gram positif : *Enterococcus faecalis* ABC 3 (ATCC 29212) et *Staphylococcus aureus* ABC 1 (ATCC 29213).

- Bacilles à Gram négatif : *Escherichia coli* ABC 5 (ATCC 25922), *Klebsiella pneumoniae* ABC 42, *Enterobacter cloacae* ABC 291.
- Bacilles à Gram négatif non fermentants : *Pseudomonas aeruginosa* ABC 4 (ATCC 27853) et *Acinetobacter baumannii* ABC 14.

Les tests antifongiques ont été réalisés contre deux types de champignons (*Aspergillus niger*, *Aspergillus fumigatus*) et une levure (*Candida albicans*).
La méthodologie utilisée pour exécuter les tests antibactériens et antifongiques est la méthode de dilution en milieu liquide selon les recommandations de l'EUCAST, 2015 et de la CLSI, 8^{e} édition. Des résultats intéressants ont été obtenus. L'extrait brut des écorces de tronc de *D. oliveri* a présenté la CMI (concentration qui inhibe 100% de la croissance bactérienne) la plus faible sur les bactéries, à savoir *E. faecalis*, à 16 µg / mL. *D. oliveri* est en effet la plante la plus active sur les bactéries. Presque tous les extraits bruts et les fractions de ses parties (feuilles, écorces de tronc et racines) sont actifs sur toutes les bactéries : chaque fois qu'une CMI n'a pas été obtenue avec un extrait ou une fraction, on remarque presque toujours une inhibition de la croissance bactérienne à la plus haute concentration testée (256 µg / mL). Au total, 55 valeurs de CMI ont été obtenus avec les extraits et les fractions de cette plante par rapport à *P. erinaceus* qui a eu seulement 34 valeur de CMI. En effet, toutes les parties de *P. erinaceus* ne sont pas actives sur les bactéries, les écorces de tronc étaient les plus actives et un certain nombre de CMI ou une inhibition de la croissance bactérienne à 256 µg / mL, ont été de temps à autre observées avec certaines des fractions provenant des feuilles et des racines. Le contraire a été observé lors des tests antifongiques. En effet, la CMI la plus intéressante a été obtenue avec l'extrait brut des racines de *P. erinaceus* sur *A. fumigatus*, à 16 µg / mL. *D. oliveri* n'a pas présenté d'activité sur les champignons. Notre étude précédente (Tittikpina *et al*., 2013) a confirmé l'activité des écorces de tronc des deux plantes sur *C. albicans*, la meilleure activité étant observée avec *P. erinaceus*. Une étude antérieure a également confirmé l'activité de cette dernière contre *Trycophyton rubrum* (Etuk *et al*., 2008). Ces résultats confirment les prédictions de la méthode CAPITURE. En effet, CAPITURE a prédit que *P. erinaceus* était actif contre *Trycophyton rubrum* (impliqué dans la teigne) et *Candida albicans* (impliqué dans la candidose). CAPITURE a aussi prédit l'activité de *D. oliveri* contre *C. albicans* impliqué dans la candidose, mais aussi dans l'intertrigo qui pourrait également être causé par *P. aeruginosa* et *S. aureus*. Cette méthode montre clairement que *P. erinaceus* était plus actif sur les champignons et *D. oliveri* sur les bactéries, compte tenu des

types et du nombre de germes impliqués dans les maladies que les deux plantes étaient supposées guérir.

En outre, les deux plantes ont également été testées contre 5 types de cellules cancéreuses :

- Hs 683 et U 373 : cellules de gliome (cellules tumorales de la colonne vertébrale ou du cerveau)
- SKMEL 28 : cellules de mélanome (cellules tumorales de la peau)
- A 549 : cellules de carcinome pulmonaire (cellules tumorales du poumon).
- MDA-MB 231 : cellules cancéreuses du sein.

La méthodologie utilisée pour exécuter les tests anticancéreux est le dosage MTT basé sur la capacité de l'enzyme, succinate déhydrogénase, NADPH dépendante et présente dans les mitochondries des cellules vivantes, de réduire un composé chimique, le bromure de 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium (MTT de couleur jaune) à sa forme Formazan (de couleur pourpre) insoluble. Cette couleur est mesurable par spectrophotométrie et l'absorbance est proportionnelle au nombre de cellules vivantes (Mosmann, 1983). Des résultats intéressants ont également été obtenus avec les extraits bruts des deux plantes avec une CI_{50} (concentration qui tue 50% des cellules cancéreuses) allant de 28 µg / mL à 91 µg / mL. La plante la plus active est *P. erinaceus* avec toutes ses parties actives sur au moins un type de cellules de gliome. Ses racines étaient la partie la plus active, CI_{50} obtenue sur l'ensemble des 5 types de cellules cancéreuses, avec la CI_{50} la plus basse sur U373 à 28 µg / mL. Seules les feuilles de *D. oliveri* se sont révélées actives sur Hs 683 (la CI_{50} la plus élevée observée lors de ces tests anti-cancéreux, à 91 µg/mL).

En outre, les deux plantes ont été testées contre le nématode *Steinernema feltiae* mais elles n'ont pas présenté d'activité contre ce parasite à des concentrations aussi élevées que 10 mg / mL.

Après ces différents tests biologiques, la toxicité des extraits et des fractions les plus actifs a été évaluée sur une lignée cellulaire humaine normale (cellules MRC-5) en utilisant le dosage MTT. Dans la plupart des cas, les extraits et les fractions n'étaient pas toxiques pour ces cellules humaines normales aux concentrations auxquelles elles étaient actives contre les bactéries, les champignons et les cellules cancéreuses.

Tous ces tests ont donné une vision globale du potentiel biologique de ces deux plantes. Ils sont actifs sur les bactéries, les champignons et sur les cellules cancéreuses avec des valeurs de CMI et CI_{50} très intéressantes. Les résultats ont confirmé l'utilisation de ces deux plantes

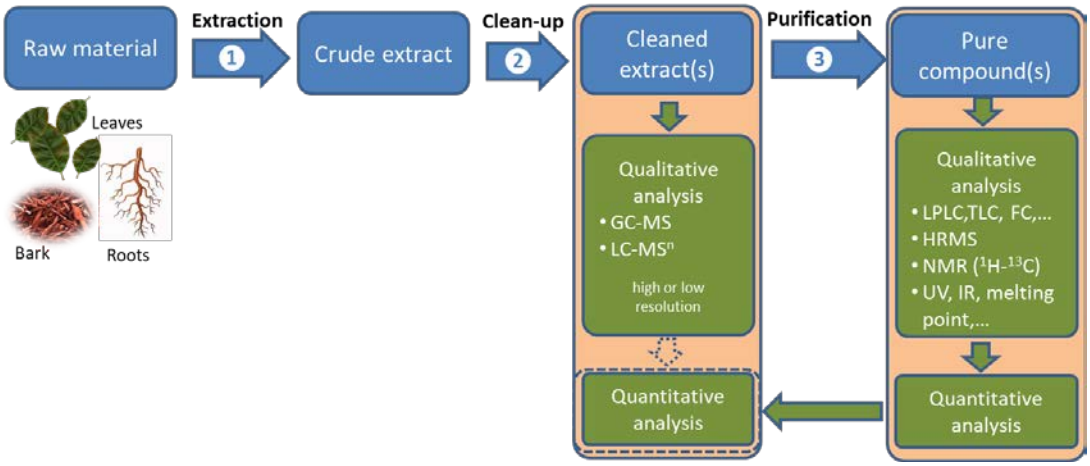
dans la médecine traditionnelle au Togo pour la guérison des maladies cutanées infectieuses. Ils ont aussi révélé le potentiel anticancéreux de ces plantes. Fait important, ces tests ont confirmé les prédictions de la méthode CAPITURE. En effet, cette méthode a prédit que *P. erinaceus* était particulièrement actif sur les champignons et *D. oliveri* sur les bactéries et les champignons. Sans surprise, *D. oliveri* a présenté la meilleure CMI (CMI à 16 µg / mL) et le plus grand nombre d'extraits actifs sur les bactéries avec en plus, une activité sur *Candida albicans* (MIC : 1,88 à 30 mg / mL). *P. erinaceus* a présenté la meilleure activité sur les champignons (CMI à 16 µg / mL sur *Aspergillus fumigatus*) avec en plus, une activité de certains de ces organes sur les bactéries. *P. erinaceus* a également présenté une activité sur *C. albicans* (1,88 mg / mL - 30 mg / mL). La prochaine étape consistera donc à trouver les principes actifs qui sont responsables des activités observées et à tester leurs activités biologiques.

4. Partie analytique

Les outils de chimie analytique peuvent être subdivisés approximativement en 2 groupes principaux : méthodes de séparation dont le but ultime est l'isolement des molécules actives souhaitées et des techniques de caractérisation qui permettent la détermination des structures chimiques des composés actifs. La partie analytique de ce travail a consisté à utiliser ces différentes méthodes pour révéler les composés qui peuvent être responsables des activités biologiques observées avec les fractions et extraits les plus actifs. De la matière première aux molécules actives, différentes étapes doivent être suivies pendant la partie analytique de notre travail (Figure 2) :

- l'extraction est effectuée sur la matière première pour obtenir un extrait brut.
- au moins une procédure de fractionnement est réalisée sur les extraits bruts pour obtenir des extraits purifiés (c'est-à-dire raffinés, pas nécessairement purs) ou des fractions sur lesquelles une analyse qualitative est effectuée généralement en utilisant des méthodes de séparation (chromatographie gazeuse et liquide) couplées à la spectrométrie de masse (GC-MS et / ou LC-MS)
- l'étape finale correspond à l'isolement ou à la purification des fractions (ou extraits raffinés) en utilisant des techniques chromatographiques telles que, par exemple, la Chromatographie liquide à basse pression (CLPC); la Chromatographie Flash (FC) et la Chromatographie à couche mince (TLC), pour obtenir des molécules pures. Afin de caractériser et d'identifier les molécules purifiées, des analyses qualitatives sont ensuite effectuées sur les composés isolés en utilisant la Résonance Magnétique

Nucléaire de première et seconde dimension (RMN ^{1}D ou ^{2}D) et la spectrométrie de masse (y compris la spectrométrie de masse à haute résolution HRMS). Après la caractérisation, des informations complémentaires sur le composé peuvent être obtenues par les spectres Ultra Violet (UV) et infrarouge (IR) et les points de fusion.



<u>Figure 2</u>: Different étapes dans la partie analytique

Le 'Raw material' (matière première) peut être soit les feuilles, les écorces de tronc ou les racines d'une plante. Le '*crude extract'* est l'extrait brut et le '*cleaned extract'* représentent des fractionnés (ou fractions). Les molécules pures sont obtenues après l'application de procédés d'isolement et de purification sur les fractions ou les extraits bruts. La GC-MS et la LC-MS sont la chromatographie en phase gazeuse et en phase liquide couplée à la spectrométrie de masse. Chromatographie liquide à basse pression (LCPC) ; Chromatographie flash (FC), Chromatographie sur couche mince (TLC).

Le travail d'analyse a porté principalement sur *P. erinaceus*, qui a présenté en plus d'activités anti-fongiques, les meilleures activités anticancéreuses par rapport à *D. oliveri*. Une analyse bio-guidée de composés a été effectuée sur la fraction acétate d'éthyle de l'écorce de troncs de *P. erinaceus* et l'extrait brut de ses racines en utilisant une purification par colonne ouverte en phase normale. Cela a conduit à l'isolement de la friedeline (1) ; du 2,3-dihydroxypropyloctacosanoate (2) ; du *β*-sitostérol (3) ; du Stigmastérol (4) ; du campesterol (5) et du *β*-sitosteryl-*β*-*D*-glucopyranoside (6) dans la fraction acétate d'éthyle de l'écorce de tronc avec des CMI autour de 4 µg / mL contre les bactéries du genre *Staphylococcus*. La friedeline (1) ; le *β*-sitosteryl-*β*-*D*-glucopyranoside (2) ; la formononetine (3) ; la pseudobaptigenine (4) ; l'isoliquiritigénine (5) ; la muningine (6) et une nouvelle isoflavone et un nouveau arylpropanoïd ont été isolés de l'extrait brut de ses racines avec une CMI de 8 µg /

mL à 256 µg / mL contre *A. fumigatus*. Tous ces composés, à l'exception de la friedeline, n'ont jamais été rapportés dans cette espèce végétale et les deux nouvelles molécules (l'une d'entre elles a une CMI de 256 µg / mL contre *A. fumigatus*) n'ont jamais été signalées dans la nature. Les composés isolés ont été identifiés en utilisant la RMN (^{1}D et ^{2}D) aidée d'une détermination de la masse moléculaire par spectrométrie de masse. En utilisant uniquement des méthodes de spectrométrie de masse couplées à des méthodes chromatographiques, des structures jamais rapportées dans la nature ont également été proposées dans la fraction butanol des écorces de tronc de cette plante : pseudobaptigenine-Glc-Xyl (ou Ara) ; formononetine-Glc-Xyl (ou Ara) ; genistéine-Glc-Xyl (ou Ara) et leurs isomères en plus d'autres composés. En utilisant la chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC-MS) sur les fractions apolaires des fractions des différents organes de *D. oliveri* et *P. erinaceus*, de nombreux composés, jamais rapportés dans les deux plantes pour la majorité d'entre eux, ont également été identifiés : terpènes, phytosetrols, acides gras dont des acides gras insaturés et polysaturés, des composés phénoliques, etc...

En conclusion, en utilisant la chimie analytique, de la séparation aux méthodes de détection, nous avons pu identifier des composés, isoler certains d'entre eux et tester leurs activités biologiques. L'analyse GC-MS réalisée sur les fractions apolaires des feuilles, des écorces de troncs et des racines a permis de mettre en évidence des acides gras, stérols, terpènes et des composés phénoliques. L'activité antifongique et antibactérienne observée avec ces fractions apolaires ne sont pas surprenantes, beaucoup d'études l'ont déjà révélé (Adi *et al*., 1994 ; Mulyaningsih *et al*., 2011 et Balogun and Zhiqiang, 2014). D'autres molécules ont pu être isolées des extraits et ou fractions actifs et identifiés par RMN. Une identification *in-situ* des extraits a aussi été réalisée en utilisant la spectrométrie de masse couplée à la chromatographie liquide. La plupart des composés isolés et ou identifiés n'ont jamais été décrits dans les deux espèces ou dans la nature. Malheureusement, dans cette étude, nous n'avons pas été en mesure d'exécuter un travail analytique plus approfondi sur *D. oliveri*. Néanmoins, le profil GC-MS de ses fractions apolaires a donné beaucoup d'informations sur la composition de ses feuilles, écorces de tronc et racines. Une identification préliminaire de ses fractions polaires par LC-MS a également conduit à noter un grand nombre d'anthocyanidines. En outre, même avec *P. erinaceus* (avec par exemple plus de 500 composés présents dans la fraction butanol de ses écorces de tronc), il reste encore matière à

découvrir. Des études approfondies permettront de donner une vue générale de la composition chimique de ces deux plantes.

5. Technologie pharmaceutique, nanoparticules de poudres végétales : nanoparticules d'écorce de tronc de *Pterocarpus erinaceus* Poir barks aux activités antibiotiques prometteuses

L'extraction est la méthode habituelle utilisée pour faire apparaître les composés actifs contenus dans les plantes. Cela implique l'utilisation de solvants organiques qui sont la plupart du temps toxiques non seulement pour la personne qui exécute l'extraction mais aussi pour l'environnement. De cette extraction au composé actif pur, plusieurs étapes doivent être suivies : la séparation et l'isolement en utilisant des méthodes chromatographiques, l'identification de la structure à l'aide de techniques avancées telles que la résonance magnétique nucléaire (RMN) et la spectrométrie de masse (MS), comme cela a été fait dans les chapitres précédents de ce travail. Les composés purs sont ensuite transformés en des formes appropriées telles que des crèmes, des pulvérisations, des pilules, etc…. Et là encore, on utilise des équipements perfectionnés qui ne sont pas disponibles dans des pays en développement tels que le Togo. Dans de tels pays, la préparation traditionnelle à base de plantes est utilisée la plupart du temps après la macération, la décoction ou la percolation d'organes de plantes frais ou secs dans l'eau, l'alcool ou un mélange des deux. Il serait donc intéressant de trouver des moyens peu coûteux et facilement accessibles pour libérer ces composés bioactifs des plantes de manière sûre et respectueuse de l'environnement. Pour résoudre ce problème, nous nous intéressons à la réduction sous forme de nanoparticules des matériaux végétaux bruts peu traités. Nous souhaitons tester cette technique et voir si elle est capable de libérer les molécules actives de manière à obtenir le même effet que celui qui peut être obtenu avec l'extrait de la même partie de plante.

Pour atteindre cet objectif, nous devrions pouvoir partir d'un simple broyage, par exemple un broyeur de café ou de maïs (utilisé dans de nombreux pays africains), ce qui permet d'obtenir des particules de différents diamètres (millimètres à peu près) à des particules de taille contrôlée avec des diamètres autour du nanomètre. Pour obtenir ce dernier, le broyage en milieu humide se présente comme une méthode de choix car il permet d'obtenir des granulés très fins (Fahr, 2015). Parmi les différents procédés de broyage en milieu humide disponible aujourd'hui, c'est-à-dire le broyage avec les moulins colloïdaux, le broyage avec des perles et l'homogénéisation à haute pression (HPH), la HPH est la méthode de choix pour les

échantillons abordés ici : l'apport énergétique élevé lors du processus d'homogénéisation permet une réduction efficiente de la taille du matériel dans un court laps de temps (Jahnke, 2001). L'HPH est également une technique bien connue et donc bien établie, simple et sécurisée qui est fréquemment utilisée, non seulement dans l'industrie pharmaceutique, mais aussi en cosmétique et la production alimentaire. En outre, une production à grande échelle est possible (Keck, 2006). Il convient également de noter que l'équipement HPH de base, accepté par les autorités réglementaires, est relativement peu coûteux (environ 10 000 euros) et disponible dans le monde entier.
Les écorces de *Pterocarpus erinaceus* dont les extraits organiques se sont révélés très actifs contre différentes bactéries ont été sélectionnées comme matériau végétal sur lequel de telles expériences se dérouleront.

La réduction de la poudre des écorces de tronc à la taille du nanomètre a été réalisée en 3 étapes consécutives : en premier lieu un broyage à sec à l'aide d'un instrument FastPrep 24 (MP Biomedicals, Solon, OH, USA). Kits Precellys (Bertin Technologies, Montigny-le-Bretonneux, France); en second lieu une stabilisation par suspension dans 1% Plantacare® 2000 UP (alkyl-polyglycoside, BASF, Ludwigshafen, Allemagne) dans de l'eau distillée pour donner 1% de macro-suspensions de matériaux végétaux finement broyés et, finalement, une pré-homogénéisation en utilisant un MICCRA D-9 Homogénizer-Disperser (MICCRA GmbH, Müllheim, Allemagne) à différents cycles, suivie d'une homogénéisation successive en utilisant un homogénéisateur haute pression APV Gaulin LAB 40 (APV GmbH, Mainz, Allemagne) (Griffin *et al*., 2015). Les macro-suspensions à 1% de matières végétales finement broyées obtenues, ont été utilisées pour réaliser les tests biologiques sur un nématode (*Steinernema feltiae*), une bactérie (*Escherichia coli*) et une levure (*Saccharomyces cerevisiae*) en comparant les résultats avec ceux précédemment obtenus avec l'extrait organique brut. Les particules 'nano-taille' de l'écorce de tronc de *P. erinaceus* présentaient une activité dépendant de la concentration contre *S. feltiae* et *E. coli* lorsqu'ils étaient employés à une concentration de 1%. En revanche, l'extrait obtenu à partir du solvant organique de l'écorce de tronc *P. erinaceus* n'a pas affecté la viabilité de *S. feltiae* mais a réduit la croissance bactérienne d'un OD_{593} de 0,8 dans le contrôle à seulement un OD_{593} de 0,2 (soit environ 25% de la croissance initiale), lorsqu'il était employé à une concentration de 256 μg · mL-1.

Essentiellement, notre étude a soutenu l'idée que la ‘nano-taille’ des matériaux végétaux peut nous permettre de passer d'un matériau végétal brut et sec à un système capable de libérer les molécules bioactives en juste quelques étapes. Bien qu'il y ait des problèmes à résoudre et des questions auxquelles il faut trouver des réponses, des approches de solutions peuvent être trouvées. En fonction des fonds disponibles, les méthodes de ‘réduction à la taille du nanomètre’ peuvent être modifiées et affinées pour obtenir des particules dont la taille est homogène et sont plus stables. La libération des molécules bioactives peut être contrôlée par de tels procédés tout comme les propriétés physiques de la particule elle-même et celle des molécules bioactives libérées. Les travaux futurs se concentreront non seulement sur la préparation d'une large gamme de particules provenant d'une large gamme de plantes locales et d'une qualité différente, spécifique à l'application mais aussi, un large éventail d'applications possibles dans les domaines de la nutrition, de la cosmétique, la prévention des maladies et la thérapie. Ici, les agents cardiovasculaires, anti-inflammatoires, anticancéreux et anti-infectieux peuvent constituer la base de tels travaux. Les particules de *P. erinaceus*, par exemple, ont déjà montré une certaine activité dans ce contexte et, après un examen plus approfondi, pourraient annoncer une nouvelle ère pour la production locale de particules ‘nano-taille’ contre la dysenterie et les infections cutanées simples. Enfin, l'accent sera également mis sur les applications agricoles pour proposer des solutions écologiques pour remplacement des pesticides actuellement employés.

6. Conclusion et perspectives

De la médecine traditionnelle à la médecine moderne, des plantes à une molécule, cette étude a permis de prouver l'efficacité des plantes utilisées dans la médecine traditionnelle dans le district de Tchamba au Togo pour traiter les maladies infectieuses. Elle a permis de donner une réponse positive à la première question qui survient toujours après l'enquête ethnobotanique, les plantes sont-elles actives ? Ce travail a été plus loin en proposant également une approche de pré-selection pour aider à prévoir les plantes les plus intéressantes. Même si une telle méthode nécessite des améliorations, elle donne des résultats intéressants comme en témoignent les activités biologiques observées avec les plantes sélectionnées par une telle méthode. En ce qui concerne la partie biologique, il conviendrait d'étudier davantage l'activité des deux plantes contre d'autres types de bactéries à Gram positif, en particulier celles appartenant au genre *Staphylococcus* car il existe une activité

préférentielle contre ce germe. La partie analytique a contribué à donner beaucoup d'informations sur la structure des molécules présentes dans les deux plantes. Cependant il reste encore beaucoup à faire, particulièrement avec *D. oliveri* où aucun isolement de composés actifs n'a été effectué, mais aussi avec les études de spectrométrie de masse sur des extraits et des fractions de *P. erinaceus*. On retrouve quand même une littérature sur des composés isolés dans *D. oliveri* même si cette littérature est faible. Les composés jamais décrits dans la nature et qui ont été découverts, devraient également faire l'objet d'études poussées pour évaluer leur gamme complète d'activités biologiques et aussi pour voir si de nouveaux produits pharmaceutiques pourraient en découler. De cette manière, le transfert à la médecine moderne sera complet. En général, des études plus approfondies fourniront une carte complète de ces plantes : activités biologiques, propriétés pharmacologiques, toxicologie, composés bioactifs, etc...

General Introduction

TOGO is a country located in the Western Part of Africa (Figure 1). It is limited by the Atlantic Ocean to the south, Benin to the east, Ghana to the west, and Burkina Faso to the north. The country is between the 6 ° and the 11° North Longitude and 0° and 2° East Latitude.

1. General characteristics

1.1. Climate and vegetation

There are mainly two types of tropical climate in Togo. In the southern part of the country (until the 7th parallel), there is a Guinean sub-equatorial climate with two raining seasons: the major one from March to July with a maximum in June, and the short one from September to October. In the northern part of the country (after the 7th parallel), there is only one raining season whose duration reduces progressively from south to north. In addition, around the 7th parallel, there are two types of climate with the example of the city named Atakpamé where there is practically no arid season (Adjoussi, 2000). This difference in the climate present in the country gives birth to a diverse vegetation. According to the topography, geomorphology, climate types and floristic affinities, Togo is subdivided into five ecological zones (Ern, 1979): I, II, III, IV and V (Figure 1).

- Zone I, also known as Northern Plains, is the domain of Sudanian savannas dominated by *Combretaceae* families and spiny trees and a vast domain of parklands and protected areas (Adjonou *et al*., 2009). Some paths of riparian forests are found along river banks. This zone is characterized by a long arid season almost six to seven months, marked by an early Harmattan (a dry wind cold and hot, very common in West Africa). The rainfall concentrated between May and October varies from 1000 to 1300 mm per year.
- Zone II, or Northern Mountains zone, corresponds to a mosaic of savannahs, open forests, dry dense forests, and vast areas of parklands with a wide range of woody species dominated by *Parkia biglobosa* and *Vitellaria paradoxa* (Kebenzikato *et al*., 2014). This mountainous region is submitted to two types of seasons: a raining season from April to October and an arid season from November to March. The mean annual rainfall is 1300 mm per year with a maximum in August-September.
- Zone III, or Central Plains, is the domain of Guinean wooded savannas and dry forests, open forests and riparian forests (Brunel *et al*., 1984). The climate has some

similarities with the one of Zone I but, the annual rainfall varies between 1200 mm and 1500 mm for a total number of rain reaching 120 days.

- Zone IV, or Southern Mountain zone, is characterized mainly by a vegetation of semi-deciduous dense forests and Guinean savannahs, so-called mountains savannahs (Akpagana and Guelly, 1994). The climate is a sub-equatorial type with four seasons: a major raining season from March to July followed by a short raining season in August and a major arid season from November to February, preceded by a short raining season from September to October (Trochain, 1980). The total annual rainfall oscillates around 1800 mm.
- Zone V or coastal plains: is a mosaic of thickets, croplands, fallows, coastal grasslands, savannahs, sprinkled sacred forest patches (Kokou *et al*., 2005). The climate is sub-equatorial marked by a rainfall deficit (around 800 mm/year in Lomé).

1.2. Pedology and hydrography

Togo is crossed from northeast to southwest on nearly 400 km in length and 70 km in width by the Atakora mountain chain which reaches its maximum altitude at Mount Agou (986 m). The chain divides the country into two large plains: Oti and Mono Plains (Ministère de l'Agriculture de l'Elevage et de la Pêche, 2007). The hydrographic stature of Togo is subdivided into three main basins:

- In the northern part, the Volta basin that collects water from Koumongou, Kara and Mô rivers through the Oti River.
- In the center, the Mono Basin
- In the southern part of the country, the Coastal basin crossed by the coastal rivers, Haho and Zio, which flow into Togo Lake.

1.3. Population

Togo has a population of around 6.1 million inhabitants comprising 51.4% women and 48.6% men. More than half (62.3%) of its population live in rural areas. The density of inhabitants is unequally distributed on the territory. Indeed, 42% of the people are living in the coastal part of the country representing 10% of the total area of the country. The population is mainly composed of young people, 62% of the people are less than 25 years (DGSCN, 2009). More than 40 ethnic groups exist in Togo which are distributed into 3 main groups (Kuevi, 1981; Goeh-Akue and Gayibor, 2010):

- Adja-Ewé group in the southern part of the country especially zone V formed by Ewé, Ouatchi, and Mina;
- Kabyè-Tem in the central part of the country represented by Kotokoli, Lamba, Kabyè, Nawda, and Ntribou;
- Para-gourma in the north (especially in zone I) and composed of Bassar, Moba, Gourma, Tamberma, Ngangan, Mossi, Marpusi and Natchamba. This group is also represented in the center of the country by the Tchamba ethnic group.
- Other ethnical groups: Bariba, Ife, Akposso, etc, …..

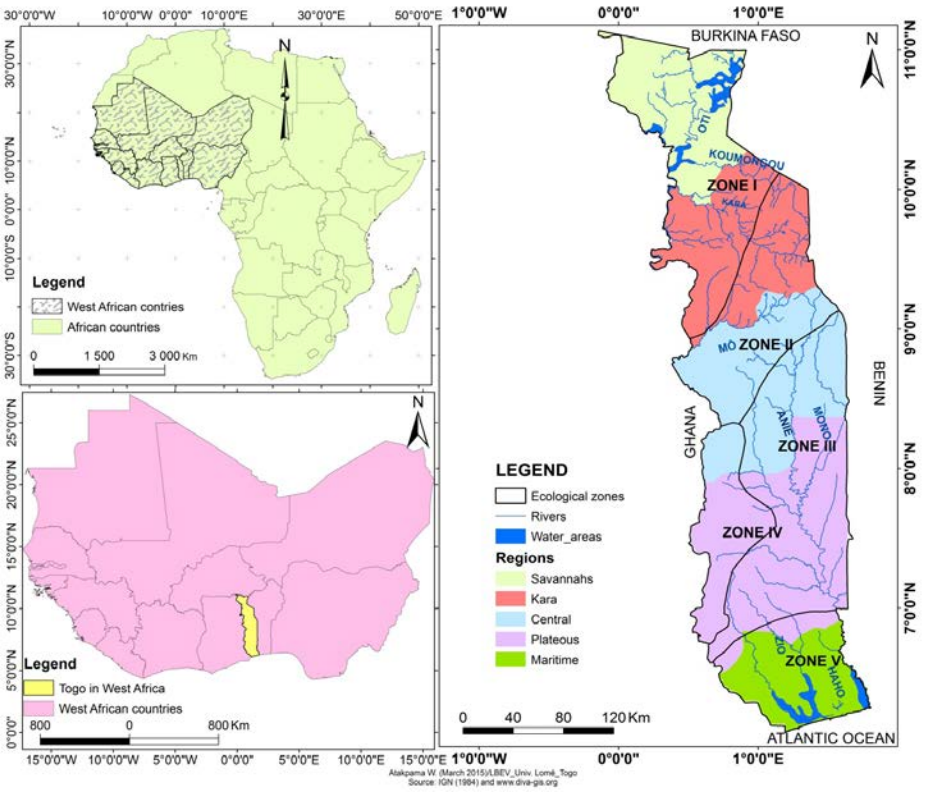


Figure 1: Map of Togo.

A: West Africa in Africa; **B**: Togo in West Africa and **C**: Togo and its various subdivisions (ecological shown by a delimitation in black on the map and administrative revealed by a coloring). The ecological zones are called: Zones I, II, III, IV and V.

As earlier mentioned, majority of the population live in rural areas where their main activities are farming, breeding and or fisheries. Farming as the main activity concerns food crops. The main food crops grown in the country are corn (Zea mays *L*. var), sorghum (*Sorghum bicolor* var L.), millet (*Pennisetu americanum*), rice (*Oryza* spp), cassava (*Manihot esculenta* Crantz),

yams (mainly *Dioscorea alata* and *D. cayensis*), cowpea (*Vigna unguiculata* L.), and groundnut (*Arachis hypogaea* var) (Ministère de l'Agriculture, de l'Elevage et de la pêche, 2007; Akpavi *et al*., 2013). Other plants naturally present in the country are used for alimentation and are not necessarily grown by people. This is the case of *Adansonnia digitata* L., *Vitellaria paradoxa* Gaertn, *Sterculia setigera* Del., *Parkia biglobosa* Jacq. Dong and *Borassus aethiopum*. The plants are sometimes conserved in parklands or harvested directly from natural stands such as forests and savannahs (Folega *et al*., 2011; Padakale *et al*., 2015).

2. Healthcare

A national report published in 2010 established that 61.5% of Togolese were living under the threshold of poverty. This extreme poverty has a bad influence on acess to healthcare by people all over the country. In addition to this financial limit, there is also a geographical limit characterized by an uneven distribution of healthcare providers all over the country. Indeed, another report in 2009 established that there were 610 medical doctors and 106 pharmacists registered in the whole country. Out of this number, 28% of medical doctors and 80% of pharmacists are working in the capital city Lomé (10% of the country surface) alone and most of the time in private drugstores. It has also been reported that infectious diseases were among the top ten diseases of the country, which lead to more deaths and/or injuries and for which the country is spending considerable amounts of money to combat. In addition to this, only 30% of Togolese, in general, are reported to visit hospitals when facing healthcare issues (UNDAF, 2007; DGSN, 2009; DRSP-C, 2009; Tittikpina, 2012). Consequently, people resort to using the other types of medicine that are available to them; mainly the traditional medicine (Table 1).

In 2010, the World Health Organization (WHO) estimated that more than 80% of people in the developing world use traditional medicine to cure ailments affecting them. The organisation defines traditional medicine as ‘the total sum of the knowledge, skills, and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 2010).

Table 1: Plants used in traditional medicine to treat various highly prevalent infectious disease conditions in Togo.

It should be noted that some of the plants seem to possess more than one "activity", which indeed may be due to the presence of rather reactive compounds with already known biological activity.

Method used- D: decoction; P: powder; I: infusion; J: Juice; C: crushed and A: ashes. Plant part- L: leaves; Ap: aerial parts; F: fruits; R: roots; T: thorns; Ep: entire plant; S: seed; B: barks; St: stems. (Retrieved from Tittikpina *et al*., 2016a)

Plant name	Family	Part used	Method used	Disease treated	Reference(s)
Nauclea latifolia Sm.	Rubiaceae	AP	D	Malaria, Intestinal parasites	Guenoukpati, 1994; Agody, 2007
Newbouldia laevis (P. Beauv Seeman ex Bureau).	Bignoniaceae	L/ST	D	Malaria, Intestinal parasites, Dysentery/ Diarrhoea	Guenoukpati, 1994; Gunn, 2011
Ocimum gratissimum L.	Lamiaceae	L	J/A	Malaria, Intestinal parasites, Dysentery/ Diarrhoea	Tchacondo *et al*., 2012; Moukaïla, 2011
Piliostigma thonningii (Schumach.) Milne-Redh.	Phyllantaceae	L/R	I/P	Malaria, Intestinal parasites, Dysentery/ Diarrhoea	Agody, 2007
Securidata longe-pedonculata Fresen.	Polygalaceae	R/L	P/D	Malaria, Intestinal parasites, Dysentery/ Diarrhoea	Tchacondo *et al.,* 2012;
Cissus aralioides (Baker) Planch.	Vitaceae	R	PD	Malaria, intestinal parasites	Tchacondo *et al*., 2012

Togo as a developing country is not an exception to this rule, as confirmed by the feeble percentage of people going to hospitals. The use of traditional medicine and especially plants

is a cultural aspect for Togolese. For example, leaves of *Newbouldia laevis*, are used in the New Year ceremony by the Guin ethnic group of the southern part of the country to cover the sacred stone, whose subsequent colour is believed by the locals to provide an overall feeling of the year to come (Table 2).

Table 2: Examples of plants used for traditional rituals in Togo

Whilst many of these uses fall outside the remit of natural sciences, some of them, such as staining of objects, may provide a clue regarding (active) ingredients. Consequently, those plants should not be dismissed outright from a more pharmaceutical perspective.
Method used- D: decoction; P: powder; I: infusion; J: Juice; C: crushed and A: ashes. Plant part – L: leaves; F: fruits; R: roots; T: thorns; Ep: entire plant; S: seed; B: barks. (Retrieved from Tittikpina *et al*, 2016a)

Plant	Family	Part used	Method used	Purpose	Reference (s)
Carica papaya L.	Caricaceae	R	D/ P	Release from spells	Moukaïla, 2011
Capsicum frutescens L	Solanaceae	F	C	Protection against witchcraft	Gunn, 2011
Dichrostachys cinerea (L.) Wight and Am.	Fabaceae	T	A	Protection against witchcraft	Tchakondo, *et al.* 2012
Gardenia ternifolia Schumach and Thonn	Rubiaceae	L	I	Protection against auto-accidents	Gunn, 2011
Gynandropsis gynandra	Capparidaceae	L	J	Release from spells	Gunn, 2011
*Leptadenia hastata(*Pers.) Decne.	Asclepiadaceae	EP	I	Protection against witchcraft	Agody, 2007, Gunn, 2011
Newbouldia laevis (P. Beauv Seeman ex Bureau).	Bignoniaceae	EP	D	Release from spells, Purifi-cation	Gunn, 2011
Piliostigma thonningii (Schumach.) Milne-Redh.	Phyllantaceae	R	PD	Release from spells	Tchakondo, *et al.* 2012
Securidaca longepe-donculata Fresen.	Polygalaceae	R	D	Repelling evil spirits	Gunn, 2011
Spermacoce stachydea DC.	Rubiaceae	S	D	Repelling evil spirits	Tchakondo, *et al.* 2012
Xeroderris stuhlmannii (Taub.) Mendoça and E. P.	Fabaceae	S, B	D	Protection against witchcraft	Tchakondo, *et al.* 2012

Among the ethnic groups that occupy the central part of the country, a polyherbal formulation drink is used to feed children after a certain number of months (Edorh *et al.*, 2015). Many studies have reported other uses of the plants in the country especially for the treatment of non-infectious diseases (Table 3).

Table 3: Examples of plants used in traditional medicine to treat non-infectious diseases in Togo

M: maceration, Fl: flowers. (Retrieved from Tittikpina *et al*, 2016a)

Plant name	Familly	Part used	Method used	Disease treated	Reference(s)
Aloe buettneri A. Berger	Liliaceae	L	D	Inflammatory and gastric ulcers	Metowogo et al., 2011
Sansevieria liberica hort. ex Gerome & Labroy	Agavaceae	R/ L	D/ M	Gynaeco-logical disorders	Agody, 2007
Zea mays L.	Poaceae	Fl	I	Epilepsy	Sema, 2015;
Bambusa vulgaris Schrad. ex Wendel.	Poaceae	L	I/D	Diabetes	Lawson, 2015
Conyza aegiptiaca (L.) Aiton	Asteraceae	L	I	Skin disease, menstrual disorders	Batawila et al., 2002
Picralima nitida (STAPH)	Apocynaceae	RB	M	Insomnia	Guenoukpati, 1994
Stereospermum kunthianum Cham.	Bignoniaceae	R St B	D/ P D	Stomach, mental illness, abscesses, female infertility, male infertility Female infertility	Tchakondo et al., 2012

More studies, however, need to be conducted to provide a more global view on the traditional use of the plants all over the country. Indeed, the ethnic diversity combined with a diverse vegetation turns Togo into an ideal choice for ethnobotanical and pharmacopeia studies. It will help preserve centuries' old traditions and build a scientific basis for the use of those plants which will automatically be transferred to modern medicine. With that aim in mind, since infectious diseases are among the priority diseases, an ethnobotanical survey was conducted in September 2010 in the Tchamba district of the country (Tittikpina, 2012). The survey revealed 43 plants as the ones primarily used by traditional healers (TH) in this part of the country to treat diseases caused by bacteria and fungi. After a preliminary biological investigation on some randomly selected plants namely *Pterocarpus erinaceus* Poir, *Daniellia oliveri* (Rolfe) Hutch. *et* Dalz. and *Anchomanes difformis* (Blume) Engler (Tittikpina, 2012 ; Tittikpina *et al.,* 2013), a more strategic research wok was designed to:

- Elaborate a methodology to objectively predict the most biologically active species that would result from an ethnobotanical survey.
- Verify the accuracy of this method by performing biological assays on the plants predicted to be the most active ones.
- Identify the chemical compounds present in the most active plants' extracts and fractions, that may be responsible for the possible biological activities observed.
- Propose an alternative to the extraction procedure by testing the biological activity of nano-sized plant materials.

The research work that will present in its different subsequent following chapters has been turning around those four defined objectives.

Chapter 1: Ethnobotanical study of plants used in the Tchamba district of Togo to treat infectious diseases

1. Introduction

Nature provides a treasure chest of natural products with often amazing biological activities, which have been used for agricultural and medical applications for centuries throughout the world. The lush vegetation in Western Africa is particularly rich in medicinal plants, and the native tribes in countries such as Togo employ a vast arsenal of plants and plant-derived products as part of their Traditional Medicine. Various studies have been carried out to gather information on this traditional medicine, generating most of the time a huge number of plant not only in Togo but also in other parts of West Africa. For example, in Togo, surveys on plants used to treat diabetes and malaria in the southern part of the country and on plants used to treat central nervous system diseases in the whole country have been conducted (Koudovo *et al*., 2011; Kpodar *et al*., 2015; Kantati *et al*., 2016). Elsewhere in West Africa, traditional knowledge on plants used for wound healing in the Dogon land in Mali, for maternal healthcare in Katsina State (Nigeria) and for hemorrhage treatment in Benin, has been investigated (Inngjerdingen *et al*., 2004; Kankara *et al*., 2015; Klotoé *et al*., 2012).

Still, identifying which plants, plant products and substances contained therein which are active and may be applied to certain diseases, and which may be toxic or less attractive because of (traditionally) known side effects is far from trivial. Admittedly, modern analytical methods of sample analysis and compound identification, such as automated chromatography coupled with mass spectrometry could identify specific substances from crude materials within hours. At the same time, equally automated activity screens can reveal an activity or toxicity profile against target (micro-)organisms or cells within a day or two. Hence our ability to screen for (new) medically active substances in the laboratory today is light-years more advanced compared to the cumbersome studies of the generation before us.

Notwithstanding the advancement in the toolbox of analysis, compound identification and robotic activity screens cannot still analyze the entire flora of the planet. Even those powerful methods *a priori* require a certain narrowing down of particularly interesting plants, and here often rely heavily on the century-old knowledge of traditional medicine. And indeed, whilst most developing countries, for instance in Africa, Asia or South America can hardly afford the kind of expensive methodology for (bio)analysis and activity screens, those countries, at the same time, are exceptionally rich in medicinal plants and traditional knowledge related to them. The World Health Organization (WHO), for instance, has estimated that 80% of the

population in the developing world still rely on traditional medicine to meet their healthcare needs (WHO, 2010).

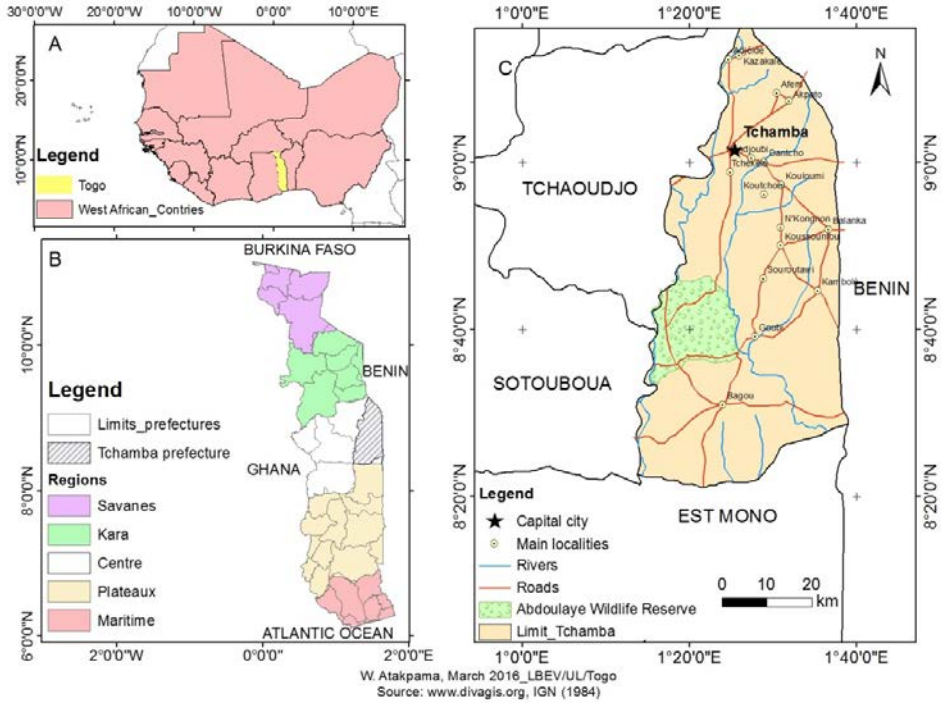
In other words, the famous question "Where to begin" in our context may well be answered most efficiently by a brief consultation of the Medicine (Wo)man. In Togo, for instance, economic hardship and an uneven access to healthcare facilities and modern medication allow the spread of infectious diseases, which in this country rank among the top ten priority diseases (Ministère de la Santé 2015, Tittikpina, 2012). Natural remedies based on locally grown plants and traditional knowledge related to them often represent the only source and resource to treat such bacterial and fungal infections. Consequently, it is essential to investigate such plants further, and indeed various studies have been carried out in Togo to bring the traditional therapeutic approach to the forefront (Koudovo *et al*., 2011; Kpodar *et al*., 2015; Kpodar *et al*., 2016 and Hoekou *et al*., 2016).

Nonetheless, we know that the promotion of traditional medicine alone is unlikely to stop the spread of such diseases. It is also impossible to screen all plants in Togo associated with one or more medical uses for all (kind of) possible medical applications. Hence, a more structured and ultimately also more focused approach is clearly warranted. We have therefore developed a simple computer-aided "pre-selection method" which (a) records hitherto unstructured and orally passed on traditional knowledge in semi-structured interviews, (b) extracts quantitative numerical values from these testimonies, (c) uses these numerical values in an algorithm to (d) rank, select and hence identify the most promising plants or plant parts as leads for further laboratory-based investigations. "Computer-aided product identification from traditional usage records" (CAPITURE) therefore relies on centuries of experience with medicines and human patients, and therefore is likely to narrow down the field of possible candidates - a claim we have subsequently evaluated in the laboratory for plants with suspected antimicrobial (antifungal) activity selected from the Tchamba District of Togo.

2. Selection of a suitable area for an ethnobotanical survey: the Tchamba District of Togo

Togo is a country with a lush and facet-rich vegetation, and home to numerous known and suspected medical plants (Figure 1.1). It is also ethnographically highly diverse and hence features a rich and diverse, often tribal knowledge of traditional medicine, which is still the main (re)source for the treatment of many diseases (Tittikpina, 2012). The Tchamba District

in Togo, which is found in the central eastern part of the country is unique as it is the only district that brings together people from nine different ethnic groups: *Tchamba*, *Koussountou*, *Tem*, *Tem Fulani*, *Kabyè*, *Ana-Ifè*, *Bassar*, *Lamba* and *Logba*. This specific, narrow, local ethnical melting pot of people was therefore selected based on their cultures and traditions as it (a) promises extensive and varied local traditional medical knowledge and (b) can be surveyed efficiently with its high concentration of traditional healers within short distances. Furthermore, there has been no previous investigation of traditional uses of plants against infectious (fungal) diseases in this district, hence our study will be unbiased and generate records and data which in any case will be novel and original.



<u>Figure 1.1:</u> Map of Togo showing the study area.

A: Togo in West Africa. **B**: Togo and its different regions and districts. **C**: Tchamba District and the localities prospected during the survey.

3. Recording of traditional knowledge and data collection

To identify a reasonable number of plants or plant parts for further studies, we have investigated the use of specific plants in the traditional treatment of fungal diseases. Semi-structured individual interviews on plants used for the treatment of fungal diseases have been conducted with 53 traditional healers (TH) in the main localities of the district in September

2010 using a questionnaire (Table 3, Appendix). As can be seen, the questionnaire sought the following information: personal data about the interviewee, the type of fungal disease, the name of the disease in the local language and the symptoms, the plant parts used, and its/ their mode of preparation and administration, as well as other diseases treated with that plant. The interviews were coupled by a brief field observation of plant species described and a collection of specimens of the mentioned plant species for further identification and authentication at the Laboratory of Botany and Plants Ecology of the University of Lomé with a cross-reference to the website www.ipni.org. Voucher specimens were deposited at the Herbarium of the University of Lomé.

It must be mentioned that such interviews may reveal sensitive data, and hence we have implemented a high level of safeguards and informed consent. For instance, to protect the rights of the TH over the information they provide and also to safeguard the biodiversity of the said area, consent from the CERMETRA (the National Association of Traditional Healers in Togo) was obtained prior to the survey. CERMETRA also delegated two representatives who accompanied us throughout the survey. Prior to each interview, an oral informed consent was obtained from the traditional healer being interviewed (Tittikpina, 2012).

3.1. Turning testimonies into data

There are many indices used in ethnobotanical surveys to convert the interviews into quantitative data (Gomez-Beloz, 2002; Gazanneo *et al*., 2005; Hoffman *et al*., 2007; Telefoa *et al*., 2010; Andrade-Cetto *et al*., 2011; Wouyo Atakpama *et al*., 2012; Kantati *et al*., 2016):

- the **Reported Use** (**RU**) is the total number of uses reported for each plant
- the **Frequency of Citation** (**FC**) is is the ratio of number of times a species was mentioned to the total number of times all species were mentioned.
- the **Use Value** (**UV**) demonstrates the relative importance of species known locally (Equation 1.1)

$$UV = \frac{\sum U \text{ or } RU}{n} \qquad \text{(Equation 1.1)}$$

where ∑**U** (same as the **RU**, is the total number of citations per species) and **n** the number of informants.

- the **Informant Consensus Factor** (**ICF**) is a value comprised between 0 and 1. The **ICF** is calculated as follows: the number of use citations for the treatment of fungal diseases (**Nur**) minus the number of species used (**nt**), divided by **Nur** minus one. High Fic values are obtained when only one or a few plant species are reported to be used by a high proportion of informants to treat a category of fungal diseases, whereas low Fic values indicate that informants disagree over which plant to use (Equation 1.2).

$$ICF = \frac{Nur - nt}{Nur - 1} \qquad \text{(Equation 1.2)}$$

- the **Fidelity level** (**Fl**): calculates a ratio between the number of informants who cite the use of a species for the same major purpose and the total number of informants who mentioned any use for the species (Equation 1.3).

$$Fl = \frac{Nt}{N} \times 100 \qquad \text{(Equation 1.3)}$$

where **Nt**: number of citations for the species to treat fungal diseases; **N**: number of informants.

Most of the time after those first indices computation, researchers are capable of giving a global view of the plants used in a certain community to treat certain diseases and to deduce some as the most important because they are the ones having the highest indices. But researchers who would like to run scientific studies on a specific plant, to confirm or infirm its uses and or study its maybe interesting chemistry, are impeded by questions they ask themselves: which plant to choose and which plant part to focus on for a beginning. We are taking the example of this study to propose a guided approach to the choice of the most interesting plant part(s) on which to conduct a deeper research either in pharmacology or natural products chemistry. For this, we propose the computing of some other indices in addition to the previous ones.

To have a better idea on how the plants that have the highest indices (precedently defined) are managed by traditional healers to treat the different fungal diseases and on which the researcher could base the choice, the following parameters could also be computed:

- the **Plant Part Value** (**PPV**). It gives information on the part of a plant most used to treat the fungal diseases. the PPV for a plant part is equal to the ratio between the number of total reported use for the plants part and the total number of reported use for that plant (**RU** $_{\text{plant part}}$/ **RU** $_{\text{plant}}$)
- the **Specific Use value** (**SU**): is the number of times a specific use of a plant part against a fungal disease is reported by the respondent
- the **Intraspecific Use Value** (**IUV**): is the ratio between the SU of a plant part and the RU for this plant (**SU** $_{\text{plant part}}$/ **RU** $_{\text{plant}}$). It allows a comparison within different parts of a plant used to treat fungal diseases (Alfredo Gomez-Beloz, 2002; Wouyo Atakpama, 2012).

Another parameter that could be also computed by the scientist, to have a global idea of the different types of diseases and the use of the plants in each type, is the **Overall Use Value** (**OUV**). This value is computable only in one disease, to make an internal comparison of plants used to treat the same disease. It is the ratio of **SU** of a plant over the total number of **RU** for this same plant (**SU** $_{\text{plant}}$ / $\sum$**RU** $_{\text{plant}}$).

To convert loosely structured interviews that we have recorded into quantitative, numerical data, questionnaire sheets were typed into Microsoft Excel spreadsheets and analyzed. The analysis was based on the computation of some of the indices defined in the precedent lines aided with a bibliographic review that we converted into a method. Within this context, it should once more be emphasized that the method at hand must be robust yet also simple and "doable" with access to limited resources. Microsoft Excel spreadsheets enable simple calculations of parameters and a subsequent graphical output. Instead of Microsoft Excel, free software such as Free Office, Libre Office, WPS Office or any other free software such as Excel that could allow simple calculations could also be used.

3.2. CAPITURE of the most relevant plants based on calculated values

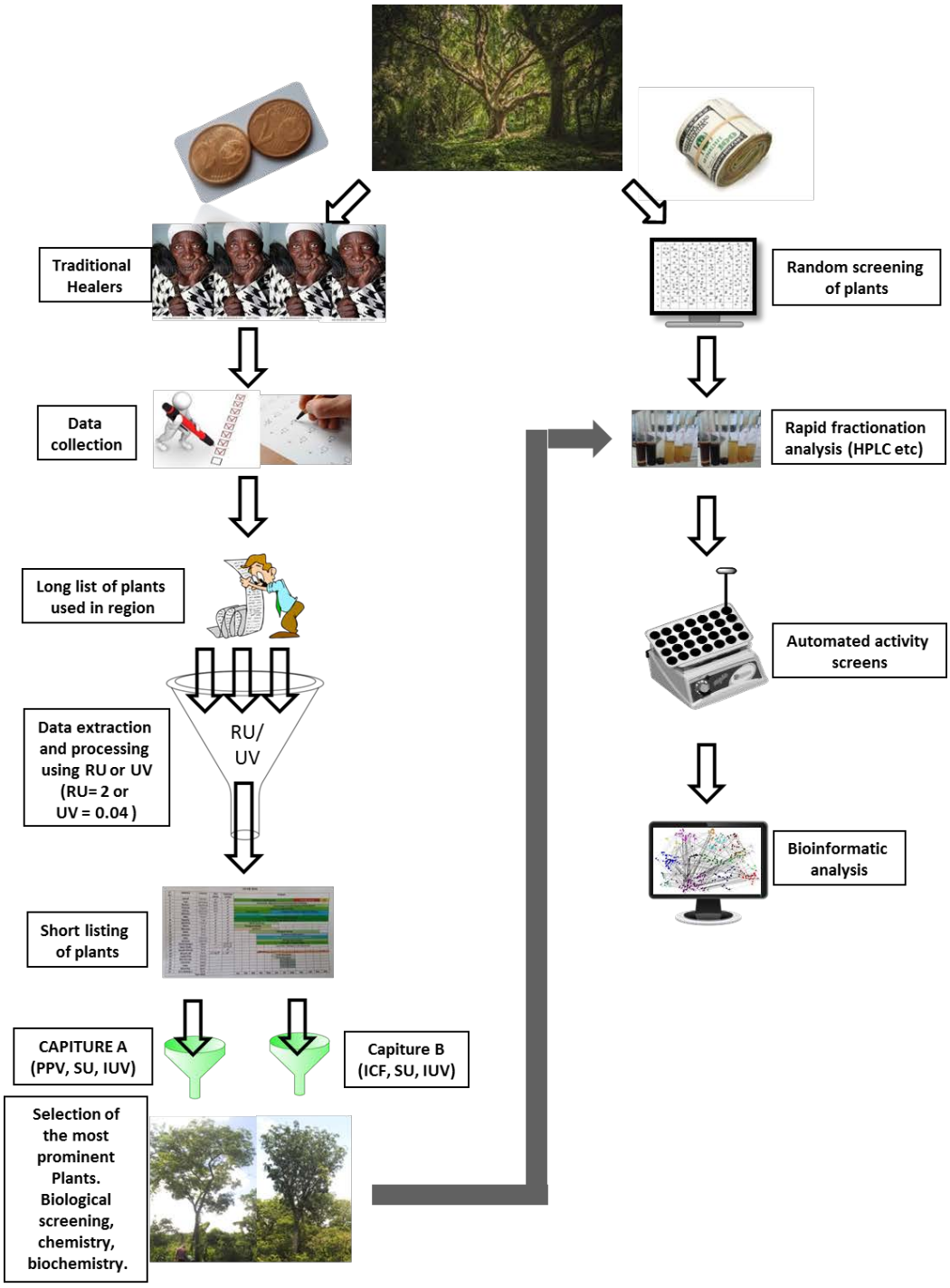
Equipped with the various parameters and values calculated as described above, we have then developed reliable selection criteria to rank the 43 plants put forward by the traditional healers and to select the most promising ones, without, of course, ruling out any of the others (Figure 1.2).

After the RU and the UV for all plants were calculated (the Frequency of Citation or the Fidelity level could also be used instead of the RU), a *long-list list* of likely candidates was obtained. A cut-off based on an RU (≥2) or a UV (≥ 0.0377) was applied to obtain a *short list*.

Subsequently, a bibliographic review on the plants with the highest UV (≥ 0.0377) or RU (≥2) was conducted. This resulted in a *final list* of plants which were (a) frequently used in the Tchamba District to treat (various) fungal diseases, (b) were independently mentioned as medicinal plants in the literature and (c) where still of sufficient novelty to warrant further investigation.

For these plants, we have two alternatives:

- firstly, we can compute the following specific indices in this order: PPV, SU and IUV. Here, the PPV leads us to the most interesting plant part(s) or organs to treat fungal diseases. The SU points to plant parts specifically used to treat one fungal disease and the IUV, as already mentioned, refines the comparison further by comparing the SU of different parts of the same plant. The most interesting plants will be the ones with the highest SU and IUV.
- secondly, we may compute the ICF, SU and IUV. Here, the ICF needs to be calculated for the different types of fungal diseases reported by the TH (Table 3) as a first step. The second step is to focus on fungal diseases that have the highest ICF. And in the third step, we can compute the SU and the IUV for the plant parts (from the *short list*) recorded to be used for the treatment of the diseases with the highest ICF. And here again, the most interesting plants will be the ones which exhibit the highest SU and therefore IUV.



<u>Figure 1.2:</u> A schematic overview of the CAPITURE method.

This method is preferably employed as an ethnobiological recording and pre-screening exercise before more eloquent and expensive analytical and activity screening methods are unleashed.

4. Results

The use value (UV) and the reported use (RU) were computed for all 43 plant species identified as part of the survey. Eventually, *Pterocarpus erinaceus* (UV = 0.28), *Daniellia oliveri* (UV = 0.11), *Ficus virosa* (UV = 0.05) *and Paullinia pinnata* (UV = 0.05) yield the highest values among the 43 species and, based on these values, it appears that they are the preferred plants administered by traditional healers to treat fungal diseases (Table 1.1, see also Table 1 in Appendix where the information on the plants recorded is provided). Since the UV values differ considerably, a cut-off of UV at 0.04 was implemented to consider those plants with an UV $\geq$ 0.04. This gives a *short-list* of plants namely *Allium sativum*, *Anacardium occidentale*, *Calotropis procera, Cochlospermum planchonii*, *Quisqualis indica*, *Ricinus communis, Desmodium gangeticum, Flueggea virosa*, *Daniellia oliveri*, *Pterocarpus erinaceus, Xeroderris stuhlmannii, Milicia excels, Musa sapientum, Piper guineense, Zea mays, Paullinia pinnata, Nicotiana tabacum* and *Vitex doniana*. The bibliographic survey was then performed on plants from the *short list* (See Table 2 in Appendix, the table provides information gathered during the review exercise).

Table 1.1: Long list of plants obtained after the survey.

Indices. ∑RU: the sum of Reported Uses, UV: Use Value. **Local names**. Tch: Tchamba, Te: Tem, La: Lamba, K (Kounssountou), Lo: Logba, Ka: Kabyè. **Type of fungal and related diseases**. R: Ringworm; I: Intertrigo; OC: Oral Candidiasis; SC: Sexual Candidiasis; F: Felon; Ony: Onychomycosis. **Plants parts**. Bu: bulb; B: barks; Fr: fruits; FrS: fruits shell; R: roots; Rhi: rhizome; S: sap; Wp: whole plant; L: leaves; Se: seeds; Tr: Trunk. **Voucher number**. NA: not appropriate.

Plant	Voucher specimen	Used part	Disease	∑ RU	UV
Allium sativum L. [cult.]	NA	Bu	R	2	0.04
Mangifera indica L. [cult.]	TOGO 01797	B	OC and SC	1	0.02
Anacardium occidentale L. [cult.]	TOGO 01768	B, Fr	OC, SC and I	2	0.04
Uvaria chamae P. Beauv.	TOGO 01950	R	SC	1	0.02
Anchomanes difformis (Blume) Engl.	TOGO 09515	Rhi	F	1	0.02
Cocos nucifera L. [cult.]	NA	FrS	F	1	0.02
Calotropis procera (Aiton) R.Br.	TOGO 02209	S	R	2	0.04
Tridax procumbens L.	TOGO 01151	Wp	OC and SC	1	0.02
Cochlospermum planchonii Hook.f.	TOGO 00466	B, R	SC and I	2	0.04
Quisqualis indica L.	TOGO 15085	R	OC and SC	2	0.04
Brysocarpus coccineus Thonn. ex Schumach.	TOGO 15081	R	OC and SC	1	0.02
Dioscorea alata L. [cult.]	TOGO 10414	Rhi	OC and SC	1	0.02
Dioscorea cayenensis Lam. [cult.]	TOGO 10424	Rhi	OC and SC	1	0.02
Bridelia ferruginea Benth.	TOGO 03089	L	R	1	0.02

Jatropha curcas L. [cult.]	TOGO 15092	Fr	OC	1	0.02
Ricinus communis L.	TOGO 03732	Fr, Se	OC, SC and R	2	0.04
Ficus virosa (Roxb. ex Willd.) Voigt	TOGO 03760	R, B	SC and Ony	4	0.08
Desmodium gangeticum (L.) DC	TOGO 05946	R	SC and R	2	0.04
Pterocarpus erinaceus Poir.	TOGO 15077	S, B, R	R, OC and SC	15	0.28
Cassia alata L.	TOGO 15094	L	R	1	0.02
Cassia occidentalis L.	TOGO 15088	Wp	Ony	1	0.02
Detarium microcarpum Guill. & Perr.	TOGO 00176	B	OC and SC	1	0.02
Daniellia oliveri (Rolfe) Hutch. & Dalziel	TOGO 15076	S, B	I, OC and SC	6	0.11
Khaya senegalensis (Desr.) A.Juss.	TOGO 01797	B	OC	1	0.02
Pseudocedrela kotschyi (Schweinf.) Harms	TOGO 12736	B	OC and SC	1	0.02
Xeroderris stuhlmannii (Taub.) Mendonça & E.C. Sousa	TOGO 06769	Wp, B	R	2	0.04
Parkia biglobosa (Jacq.) R.Br. ex G.Don	TOGO 15084	Tr	F	1	0.02
Ficus thonningii Blume	TOGO 05199	L	R	1	0.02
Milicia excelsa (Welw.) C.C.Berg	TOGO 12751	Sa, B	F and SC	2	0.04
Musa sapientum L.	TOGO 15091	Fr	O and SC	3	0.06
Piper guineense (Schum and Thonn.)	TOGO 06862	Fr	F, OC and SC	2	0.04
Zea mays L. [cult.]	TOGO 11532	Fr	S and OC	2	0.04
Gardenia aqualla Stapf & Hutch	TOGO 07309	Tr	OC	1	0.02
Morinda lucida Benth	TOGO 07498	R	OC and SC	1	0.02

Citrus limon (L.) Burm.f. [cult.]	TOGO 15089	Fr, R, B	F, Ony, I, OC and SC	1	0.02
Citrus sinensis (L.) Osbeck	TOGO 15093	Fr	SC	1	0.02
Paullinia pinnata L.	TOGO 15082	R	Ony, F	4	0.08
Nicotiana tabacum L. [cult.]	TOGO O8500	L	R	2	0.04
Cola gigantea A. Chev	TOGO 08587	B	SC	1	0.02
Heliotropium indicum L.	TOGO 02508	L	OC	1	0.02
Vitex doniana Sweet	TOGO 09273	L, B	R	2	0.04
Aframomum melegueta [Roscoe] K. Schum	NA	Fr	OC	1	0.02

Surprisingly, this survey revealed that virtually none of the plant species identified have been previously studied *in vivo* for antifungal activity (except *P. erinaceus*) (Etuk *et al*., 2008). Furthermore, the underlying chemical composition and activity of the materials so far is also not well documented. It also appears that none of them, with the notable exceptions of *P. erinaceus* and *Ricinus communis*, have been cited previously in other ethnopharmacological studies (Etuk *et al*., 2008; Manpreet *et al*., 2012). In contrast, plants such as *Allium sativum* (the common garlic), *Calotropis procera* and *Anacardium occidentale* which have been previously studied extensively did not score highly in the CAPITURE analysis (Khan *et al*., 2000; Sumbul *et al*., 2004; de Freitas *et al*., 2011; Goyal *et al*., 2013). Running this review has helped to identify a *final list* of plants as promising candidates and as the most original plants to be analyzed and studied further considering the feeble data of previous biological or chemical studies on them. The plants are *P. erinaceus*, *D. oliveri*, *Ficus virosa* and *P. pinnata* which also represent the ones mostly used by traditional healers as revealed by the survey. The two options described in the CAPITURE method was then applied to those four plants.

In the case of the first option, after the first two steps above, namely the calculation of Usage Values (UV) and a literature survey for activity and originality, the third step involved the calculation of the more specific and informative PPV, SU and IUV parameters for the plants on the *final list*. The results obtained for the PPV are summarized in Table 1.2. In the case of *P. erinaceus*, the bark (0.4) and the sap (0.53) represent the parts most frequently employed by TH to treat fungal infections. Similarly, in the case of *D. oliveri*, the sap (0.71) and bark (0.28) are most frequently used, whilst for *F. virosa* and *P. pinnata*, the roots are the only parts utilized (Table 1.2).

Table 1.2: RU and PPV of the most valuable plant parts

The PPV parameter indicates which parts of a plant are most frequently utilized by TH, hence narrowing down the plant material to be studied.

	Plant part	RU plant part	PPV
P. erinaceus	Bark	6	0.4
	Sap	8	0.53
	Roots	1	0.066
D. oliveri	Bark	2	0.28
	Sap	5	0.71
P. pinnata	Roots	4	1
F. virosa	Roots	4	1

To address the question which particular fungal infections to consider, the SU and IUV were computed. As this calculation places a very narrow focus, *i.e.* on a specific plant, its specific part(s) and specific infections, it provides highly refined information yet in some cases also fails because of the limited number of records (43) or incomplete or unspecific records available. For instance, a reasonable calculation has not been possible for the roots of *F. virosa* and *P. pinnata* because they were not used against a specific fungal disease. The results obtained for the other plant parts are shown in Table 1.3. It becomes immediately apparent that the sap of *P. erinaceus* is frequently used in the treatment of *ringworm*, whilst the sap of *D. oliveri* is utilized in the context of *intertrigo*.

Table 1.3: SU and IUV of the most valuable plants

The SUV and IUV parameters provide highly refined information as to which parts of a selected plant are used in the treatment of specific fungal infections.

	Plant part	Specific reported use	SU	IUV
P. erinaceus	Sap	Ringworm (8 times)	8	1
	Roots	Candidiasis (1)	1	1
D. oliveri	Sap	Intertrigo (5)	5	1
	Barks	Candidiasis (1)	1	1

The other alternative in the CAPITURE method is to compute the ICF which revealed that ringworm (0.59), intertrigo (0.57) and candidiasis (0.41) are the fungal diseases with the highest ICF (Table 1.4). The high value of ICF means that most of the TH agree on a set of plants on the *short list*, which they use preferably to treat ringworm, intertrigo and candidiasis. Consequently, the SU and IUV of those plants were computed just focusing on their use in the treatment of the two ailments. This way, the results presented in Table 1.3 was obtained.

Table 1.4: The main Symptoms of the fungal diseases and the informant consensus factor (ICF).

Local names. Tch: Tchamba, Te: Tem, La: Lamba, K: Kounssountou, Lo: Logba, Ka: Kabyè.
Indices. Nur: number of uses; nt: number of plants species; ICF: Informant Consensus Factor.

Disease	Nur	nt	ICF	Name in the local language	Symptoms according to traditional healers
Oral candidiasis	31	25	0.2	Magna (Tch, K, Te, La, Lo, Ka) referring to oral one as 'Nomèkan' (K) and 'Koumoponiyo' (Tch)	Itching on women genital parts with sometimes making urinating difficult (sexual candidiasis), weird white bands appearing in the mouth of children (oral candidiasis).
Sexual candidiasis	29	23	0.21		
Ringworm	28	12	0.59	Kidjapem (Tch); Agbélékoukoua (K)	White circles appearing on children's heads with sometimes a disappearing of hair in the middle of the circle.
Intertrigo	8	4	0.57	Tingbonboloti (Tch), Bombolana (K)	White weird things appearing between the toes associated with wounds sometimes.
Onychomycosis	8	7	0.14	Kaka (Tch, K, Te)	White or brown weird things appearing on nails.
Felon	4	4	0	Kissali (Tch), Kassala (K)	Inflammation of one's finger around the nail area with sometimes white or weird things, very painful, forbidding to put one's hand in the water.

Eventually, the present study was able to indicate which parts of the plants are used for the treatment of which specific fungal infections. This has culminated in the understanding that

the sap of ***P. erinaceus*** is the most effective plant material employed against **ringworm**, whilst the roots of this plant seem to be particularly effective against **candidiasis**. The sap of ***D. oliveri*** appears to be effective against **intertrigo**, and the roots of this tree bear promise in the treatment of **candidiasis**.

The identification of these 2 candidates, extracted from 53 testimonies provided by TH from the Tchamba District and narrowed down from 43 individual plant species mentioned in this survey, is the final outcome of the CAPITURE pre-selection exercise. These plants (described in the following lines) materials have therefore been collected and studied in considerably more detail in antimicrobial assays indicative of the relevant biological activity (to be presented in the second chapter).

Description of *Pterocarpus erinaceus* Poir

The Greek-Latin name '*Pterocarpus*' comes from the Greek words *pteron* (wing in English) and *karpos* (fruit in English) (referring to the winged membrane that surrounds the fruit of these species) and the Latin word *erinaceus* means Hedgehog in English (in comparison to the central part of the seed covered with stiff hairs crossed in all directions, like the thorns of the hedgehog). The plant is named *Boutô* in Tchamba and *Barwood*, *Muninga* or *vène* in English. It is a small tree, 10 to 15 m of height. Its leaves are imparipinnate, alternate and distilled (Figure 1.3). The leaves have a rachis of 15-25 cm long with 4 to 5 pairs of leaflets alternate or sub-opposed. The leaflets are elliptic, 5 to 10 cm long, 3 to 6 cm wide, with a rounded base or short cuneiform, generally emarginated. The yellow flowers are numerous, loose and short panicles. Flowering occurs in March-April, before the foliage. The corolla is short, 10-12 mm. The calyx is pubescent, 5 mm long, with short and obtuse triangular teeth. The fruits are winged, suborbicular, broad from 4 to 6 cm and have in the center, above the seed, numerous rigid, spiny bristles, crossing each other in all directions (Berhaut J, 1976; Tittikpina, 2012).

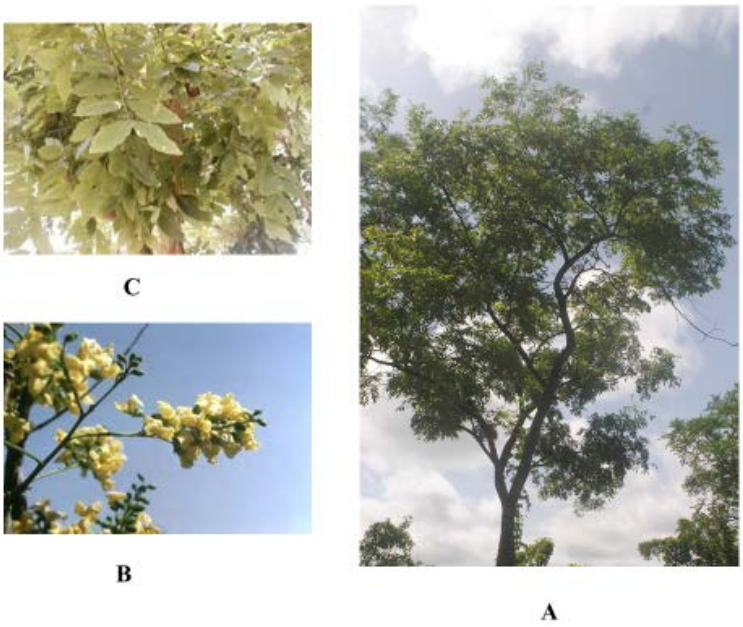


Figure 1.3: ***Pterocarpus erinaceus***

A: Tree**; B:** Flowers and **C:** Leaves (Retrieved from Tittikpina, 2012)

Description of *Daniellia oliveri* (Rolfe) Hutch. et Dalz

Daniellia oliveri plant was named in honor of the English botanist Daniel Olivier, former curator of the Kew Museum, and principal author of 'Flora of Tropical Africa' (1868-1871). It is called *Boussâ* in Tchamba and *Africa copaiba balsam* tree in English. This tree, 15 to 20 m of height, is found in the wooded savannah, where it blooms in February-March. The alternate paripennial leaves have spines of 15 to 30 cm long with 4 to 9 pairs of opposite or sub-opposed leaflets. The leaflets are ovate, elliptic, 9 to 15 cm long, 4 to 7 cm wide at the base and have an obtuse apex. The flowers are white and are arranged in a terminal panicle of 15 to 30 cm long (Fig 1.4). This panicle bears horizontal, distilled small roots of 5 to 15 cm of length. The fruit is a flat, obovate pod with rigid papery valves resembling those of *P. erinaceus*. A long funicule of 15 mm is attached to one of the valves (Berhaut, J, 1976; Tittikpina, 2012).

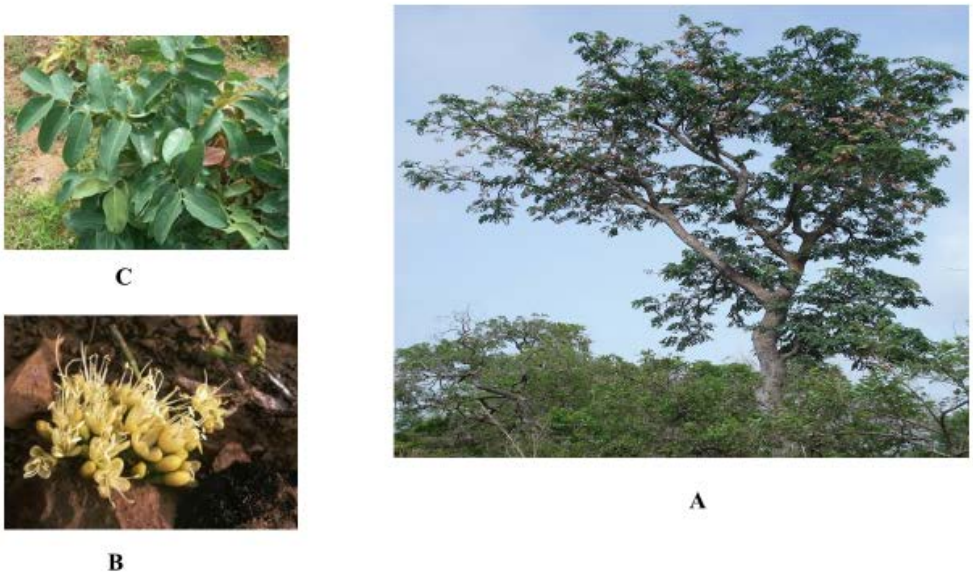


Figure 1.4: ***Daniellia oliveri***

A: Tree; B: Flowers and C: Leaves (Retrieved from Tittikpina, 2012).

5. Discussion

Overall, the results obtained as part of this study corroborate the idea that it is possible to use the vast and diverse knowledge held by traditional medicine and healers in countries such as Togo to identify interesting plants and plant parts used for the treatment of certain diseases. Undeniably, the CAPITURE approach presented in this study appears to have considerable advantages, not only over a "blind" shotgun approach of random analysis of the entire vegetation but also of more conventional tactics which have relied on individual stories and hearsay.

Indeed, the main advantage of the CAPITURE method is clearly that it combines the knowledge of traditional medicine with modern computational analysis. It is, therefore, able to rely on centuries of experience with human patients and this without the need to perform a single clinical trial, animal study or even cell culture experiment. At the same time, CAPITURE does not simply rely on the testimony of individual healers. By skimming the testimonies of tens of TH, and by subsequently assigning numerical values and calculating parameters for selection, it can "level" the errors, superstitions, and beliefs that may be held or harbored by individual TH. The use of parameters and indicators is also superior to, let's call it "intuition", whereby the various testimonies (structured or not) are simply read and the

most promising plants and parts are picked by "intuition". In fact, it is probably fairly challenging to memorize 43 individual questionnaires, and as the number of TH interviewed could be expanded considerably, hence simply looking at their answers will not be sufficient to narrow down the selection.

And finally, the three steps involved in this method, *i.e.* the recording of testimonies, the assignment of numerical values and the selection via these values and the parameters calculated from them, also provide additional benefits "on the side". CAPITURE does, for instance, put on record century-old passed on knowledge of TH for the first time, and before it may get lost. Here, visits, often accompanied by members of the respective guild, are paramount, as many of those THs hardly have access to modern means of (tele-) communication.

The method does, of course, leave space for further improvement. It may be advantageous, for instance, to also record knowledge on side effects or toxicity. At the same time, larger numbers of testimony would ensure a more reliable outcome of some of the assessments which have been complicated due to lack of sufficient, statistically significant numbers.

Whilst bacterial infections have not been the first focus of this study, it is obvious that the CAPITURE method can also be applied to other diseases, including infections by bacteria, plasmodia and parasitic worms (Tittikpina *et al*., 2016a). In fact, as TH are General Practitioners, there is no limit regarding possible ailments, and it would even be possible and straightforward to run such a CAPITURE analysis in Togo for, let's say, squirrel bites (Tittikpina *et al*., 2016a), animal diseases (Zabou, 2014) or central nervous system disorders (Kantati *et al*., 2016). It could be applied not only to plants but to different products used in traditional medicine, for instance when dealing with the healthcare of humans, of animals and the protection of plants. And, of course, the method could also be applied to other domains of traditional uses such as plants used as insect repellents, herbicides or green pesticides. Here, an insect and animal repellent based on plants from the Tchamba District, and inspired by traditional uses, has been developed by a local entrepreneur and is now used by local farmers to protect their crops (http://wire.barza.fm/en/farmer-stories, site consulted as on the 11th July 2016).

Whilst it is simple to expand this method with regard to the number of participants surveyed, the kind of data recorded via the questionnaires and the kind of diseases targeted and applications anticipated, it is also possible to fine-tune, refine or amend the algorithms and selection criteria involved. For instance, it may be useful to add a chemical survey of (known)

active ingredients to the ethnobotanical survey and literature survey on activities. It is known that plants belonging to the same family often feature the same (active) ingredients, and hence an additional selection parameter could be introduced to account for this. Another important aspect which may need to be considered is ecology. There is no deliberation yet on the impact of the environmental threat to conservation if and when such plants may be harvested for medical or even agricultural purposes. Indeed, sap, roots, and barks are the most popular parts of plants used, and a more widespread use of these materials may damage the trees or shrubs in question. Hence an additional parameter reflecting the abundance of a given plant and the need for its conservation may be necessary and could even serve as a knock-out criterion. Other refinements in the sampling or in the algorithm are also possible.

The specific indices (PPV, IUV and SUV) used in the CAPITURE approach was first reported by Gomez-Beloz (2002) where they were used to widen the knowledge about specific pre-defined plants by interviewing 40 members of the Winikina Warao community in Venezuela. CAPITURE, on the other hand, has explicitly avoided a narrow focus on one community because of possible bias, for instance, based on a narrow tradition, superstition or magic. Indeed, the results obtained agree with the information gathered from individual TH. Besides, in CAPITURE, the Disease Consensus Index (DCI) was not computed as proposed by Andrade-Cetto *et al*. (2006). They authors proposed the CDI to be computed for the search of specific plants to treat a single disease within a specific community. In the CAPITURE method, we have rather computed the ICF. Indeed, the survey of TH explicitly did not focus on just one specific disease but on fungal diseases in more general terms, hence representing a broad disease category. Eventually, the ICF was not only easy to compute but was also pivotal in narrowing down this broad disease category to a more focused number of specific fungal infections. Crucially, the latter were defined by the TH themselves, and not by any pre-selection or narrow disease category, hence leaving the door open for relevant input from the testimonies.

Furthermore, the ICF - unlike the CDI - is also not leading directly to a specific plant against a specific disease. It is rather useful to encircle and to narrow down the field. It, therefore, appears that the ICF is a valuable parameter and selection tool when the survey is applied in an ethnicity melting pot such as the Tchamba District and the questionnaire used is not simply based on “yes” or “no” answers.

In any case, the design and subsequent application of semi-structured questionnaires are essential for success. Simply ticking boxes will not capture all relevant information. It will

rather belittle the traditional knowledge held by TH and will also result in inadequate records of that knowledge for future generations. Indeed, using the ICF in addition to the IUV and SUV is guiding us to interesting results that are also in agreement with the information gathered from individual TH.

One hallmark of the method presented has been the rather strict reduction of potential candidates, which at each step has eliminated certain plants, plant products or disease targets. This elimination has been ruthless, and without considering any "intuition", personal taste or discussion on it - otherwise garlic would surely have ended up on the table eventually. It must be emphasized once more, that this kind of elimination is necessary to provide a focus, yet does not belittle the potential use of particular plants which ultimately did not end up in the *short-list*. In principle, all 43 plants mentioned by the TH could be useful, yet a qualified choice and priority boarding are required. It is therefore exciting that *P. erinaceus* and *D. oliveri* have ended up on the (laboratory) table, yet this does not rule out that any of the other plants may be useful as well.

6. Conclusions

In summary, this study was able to employ CAPITURE method to move on from a basic ethnopharmacological survey of 53 TH in the Tchamba District of Togo to a more structured, objective appraisal of existing knowledge on the use of natural plant products against a spectrum of common fungal infections. Whilst the approach still leaves various questions unanswered (*e.g.* with regard to plant species eliminated from the list) and also provides sufficient room for improvement (*e.g.* by introducing further chemical and environmental parameters), it nonetheless has allowed us to wiggle down the vast number of medicinal plants found in Togo to a selected few. In future, the mehod could be expanded to involve more TH and also to consider additional diseases. This way we will not only identify additional leads for promising medical plants, but it will also be able to record, store and safeguard the century-old knowledge of TH which has been passed on through the generations which is always at risk of "getting lost" in the mist of time or the rain-forest. Importantly, this kind of recording is structured and hence can capture tens or even hundreds of testimonies, not in form of traditional "stories from the forest" but as focused, comprehensive yet down to the point questionnaires.

At the same time, we will investigate more closely the few leads identified so far, especially in the context of *P. erinaceus* and *D. oliveri*, bearing in mind that the CAPITURE approach is only a pre-screen to be followed by a full laboratory-based analysis of the (active) chemical ingredients found in the plants, an assessment of their spectrum of biological activities and potential uses and a full investigation of the underlying mode(s) of action. As mentioned on several occasions, the *long-list* with a total of 43 plants will always be around for a reappraisal of plants, plant parts and infections should the need arise (*e.g.* if the selected plants lead to a dead end or time and funding become available for additional analyses).

Eventually, the CAPITURE method, in its present or refined form, will also be applied to other countries, communities and diseases, especially but not exclusively in the developing world, where records on the traditional uses of medicinal plants are still surprisingly rare and where local communities may benefit greatly from such knowledge and the possibilities of treatment which come with it.

Chapter 2: Biological activities of *Pterocarpus erinaceus* and *Daniellia oliveri*

1. Introduction

Plants are a valuable source of biologically active compounds: 17,810 species worldwide are used as medicine (SOTWP, 2016). In 2001, about 25% of the drugs prescribed worldwide originate from plants. Of the 252 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusive of plant origin and a significant number are synthetic drugs obtained from natural precursors (Rates, 2001). This number has increased over the decades and will continue increasing considering the new challenges faced by the health systems of the entire world. These challenges include emergence of new and/ or ancient infectious diseases, drug resistance and the spread of non-communicable diseases. Presently, infectious diseases are on the rise because of antimicrobial resistance (O'Neill, 2016), and are more frequent in developing countries where one out of two people dies prematurely from infections, when compared to developed countries (WHO, 2012; Tittikpina, 2012). The recent outbreak of 'Ebola' in some countries of West Africa is another shocking example of the increase of infectious diseases when the fight against them was supposed to be over as claimed by many experts in the 1970's. Indeed, in 1970, the Surgeon-General of the United States of America, indicated that it was "time to close the book on infectious diseases, declare the war against pestilence won, and shift national resources to such chronic problems as cancer and heart disease" (WHO, 2000). After 1970, many projects or programs have then been oriented on only non-commutable diseases, until recently, when the antibiotic resistance re-appeared.

A report on the approvals of new antibiotics by the Food and Drug Administration (FDA) in the US per five-year period, 1983 to 2012, has shown a linear and constant decrease (Boucher *et al*., 2013). Microbes have used this period of non-discovery or non-research on infectious diseases, to evolve genetically by developing different types of resistance. Tuberculosis, for instance, evolved with humans, and antibiotic resistance in *M. tuberculosis* occurs exclusively by spontaneous mutation (Davies and Davies, 2010). Besides, the WHO has recently published an antibiotic-resistant priority list comprising among others multiresistant *Acinetobacter*, *Pseudomonas* and various *Enterobacteriaceae* including *Escherichia coli* (WHO, 2017). Therefore, new leads must be discovered to resolve those global health issues. Plants are a good choice not only because they have already yielded many good results in the past but also because of the considerable potential that has not been evaluated. This global context is therefore not very different from the togolese context described in the previous lines. The search for new molecules based on traditional medicine then presents itself as a

solution not only togolese but also global. The two plants selected using the CAPITURE method and based on ethnobotanical surveys on infectious diseases will, therefore, be tested against a broad range of bacteria, fungi and nematodes. Giving the global rise of non-communicable diseases, it will also be valuable to check their activities against cancer cells even if their use against cancer was not reported during the ethnobotanical surveys by TH. Indeed, artemisinin used to treat malaria has also been reported to exhibit anti-cancer activity even though *Artemisia annua* L (the plant it comes from), is used in Chinese medicine to treat malaria (Das, 2015). These biological tests will make it possible to select the most active extracts and parts of plants on which the phytochemical analysis will subsequently be carried out.

2. Collection and preparation of plant material

Fresh leaves, trunk barks and roots from *P. erinaceus* and *D. oliveri* were collected in Tchamba District in the central part of Togo. After a formal identification by a botanist, Voucher specimens were deposited at the Herbarium of the University of Lomé (Togo) under the number:

- TOGO 15076 for *Daniellia oliveri* (Rolfe) Hutch. and Dalz (Fabaceae).
- TOGO 15077 for *Pterocarpus erinaceus* Poir (Fabaceae)

The fresh plant materials were brought to the Laboratory of Botany and Plants Ecology where they were dried at 25°C. After drying, they were milled, sealed and brought to Europe by plane for the research work.

3. Extraction and fractionation

This part is the experimental part of the part 'Separation methods' presented in the 3rd Chapter, reserved to the analytical part of this work.

Extraction and fraction were first performed on the plants' parts to obtain extracts and fractions on which the bio-assays were done. The extraction technique employed in this work is maceration. It involves soaking the plant material in a solvent contained in a closed vessel for a certain period (24 h to 72 h) after which the liquid was strained off and the marc is pressed to recover large amounts of occluded solutions. The obtained strain and the press out liquid are mixed and separated from impurities by filtration.

3.1. Check-up

The check-up was carried out on roots of the two plants: 300 mg each of Pterocarpus erinaceus and Daniellia oliveri was separately dissolved in a volume of 10 ml of different solvents or mixtures of solvents as presented in Table 2.1.

Table 2.1: **Preparation of root extracts of the two plants using different solvents**

V: volume in proportion

Solvent	Proportions
Dichloromethane	100
Dichloromethane + Methanol	50v: 50v
Methanol	100
Ethanol	100
Ethanol and water	20 v: 80v and 50v: 50v

Filtration using paper filter and evaporation of the resulting filtrate with rotavapor was carried out for *Pterocarpus erinaceus* after one day following appearance of a colorful and concentrated upper layer. For *Daniellia oliveri*, coloration was obtained after 2 days. Hence, filtration and evaporation was done after 2 days. The dried residues obtained were dissolved in methanol to yield solutions used to prepare TLC plates. Eluents of different polarities were used as summarized in Table 2.2.

Table 2.2: **Solvents used for the elution of the TLC plates prepared**

V: volume in proportion

Elution solvent	Proportions
Ethyl acetate	100
Dichloromethane	100
Dichloromethane + Methanol	90v: 10v
Cyclohexane + Dichloromethane: 50v- 50v	50v: 50v
Cyclohexane + Ethyl Acetate	90v : 10v ; 80v : 20v and 50v : 50v

After elution, TLC plates were monitored under UV to check the presence or absence of spots. Results are presented in Table 2.3 and Figure 2.1.

Table 2.3: **Observations made under UV of the TLC plates on root extracts of *P. erinaceus* after their elution**

+: spots observed; -: spots not observed**.**

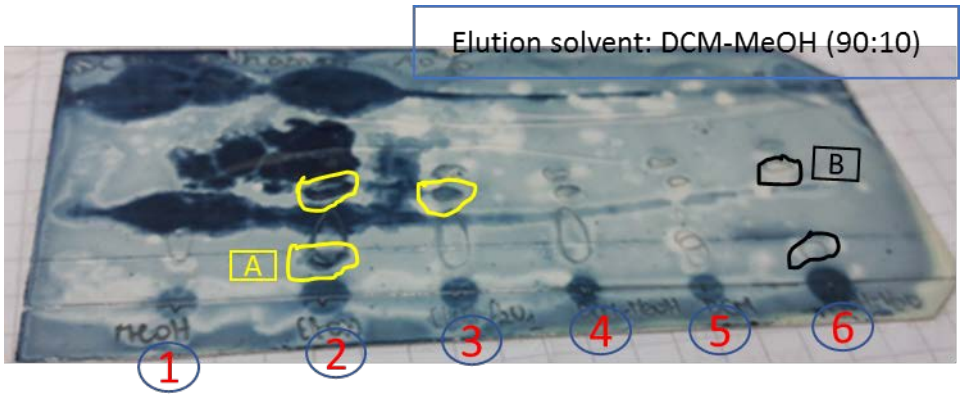
Solvent	Spots
Ethyl acetate	+++
Dichloromethane	+
Dichloromethane + Methanol (90v: 10v)	++
Cyclohexane + Dichloromethane (50v: 50v)	-
Cyclohexane + Ethyl Acetate (90v : 10v)	-
Cyclohexane + Ethyl Acetate (80v : 20v)	-
Cyclohexane + Ethyl Acetate (50v : 50v)	+

After monitoring under UV, TLC plates were soaked in molybdate (composition: 50 g of Ammonium molybdate + 2 g of ceric sulfate + sulphuric acid solution for 1 L) and put at 60 °C to dry in an incubator. New spots were observed as presented in Table 2.4 (Figure 2.1).

Table 2.4: **Observations made on the TLC plates on root extracts of *P. erinaceus* after soaking in molybdate.**

+: spots observed; -: spots not observed**.**

Solvent	Spots
Ethyl acetate	-
Dichloromethane	-
Dichloromethane + Methanol (90v: 10v)	+
Cyclohexane + Dichloromethane (50v: 50v)	-
Cyclohexane + Ethyl Acetate (90v :10v)	-
Cyclohexane + Ethyl Acetate (80v-20v)	-
Cyclohexane + Ethyl Acetate (50v : 50v)	+



<u>Figure 2.1</u>: TLC plate obtained on the root extracts of *P. erinaceus* after elution in DCM-MeOH (90:10)

DCM: Dichloromethane and MeOH: Methanol; 1: Methanol extract of the roots, 2: Ethanol extract, 3: Ethanol + Water (20v: 80v), 4: DCM-MeOH (50v: 50v), 5: DCM: 100%, 6: Ethanol+ Water (20v: 80v); A: Spot observed after soaking in molybdate; **B**: spot observed during monitoring under UV.

By running the different elution, monitoring under UV and soaking in molybdate, the observations made concerning spots on the plates, could be explained by the following:

- cyclohexane-ethyl acetate (90v: 10v), no movement because compounds are polar, they are consequently not moved by the solvent (a)
- cyclohexane-ethyl acetate (50v: 50v), the first movement of components is observed because the eluent is more polar than the precedent one (b)
- ethyl acetate (100%), the compounds are almost on the front line because the polarity of the eluent is high in comparison to that of the compounds (c)
- cyclohexane-dichloromethane (50v: 50v), no movement observed (d)
- dichloromethane (100%), initial movement of the compounds is observed because the eluent is less apolar than the precedent (e)
- with the eluent dichloromethane-methanol (10%), the movement of the compounds observed is more pronounced than the previous ones (f, Figure 2.1)

Based on these observations, it was obvious that the extraction of the roots of *P. erinaceus* will be better enhanced using a mixture of methanol and dichloromethane (50%-50%) to achieve the movements observed between (e) and (f). The same procedure was carried out on the roots of *Daniellia oliveri* (Figure 2.2).

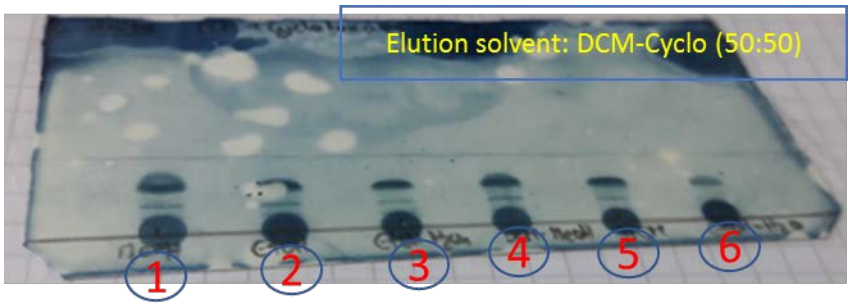


Figure 2.2: **TLC plate obtained on the extracts of *D. Oliveri* roots after elution in DCM-Cyclo (50:50)**

DCM: Dichloromethane and Cyclo: cyclohexane; 1: methanol extract of the roots, 2: ethanol extract, 3: Ethanol+ water (20v: 80v), 4: DCM-MeOH (50v: 50v), 5: DCM: 100%, 6: Ethanol+ water (20v: 80v). All the spots observed on the TLC plate have been observed after soaking in molybdate.

By eluting the TLC plates in the different solvents and mixtures of solvents as described in Table 2.2, spots were obtained with cyclohexane-ethyl acetate (90 v: 10 v) and this movement was pronounced when the solvent of elution was more polar. No spots were visible under UV. TLC plates were then soaked in the molybdate solution where spots were visible in all the elution systems used and described in Table 2.2. It has been consequently decided that the extraction of the roots will be performed using methanol (100%) as it yielded the best separation.

3.2. Extraction

The extraction of *D. oliveri* was done by maceration of 3 kg of plant materials in methanol (MeOH) for 72 h while a mixture of methanol and dichloromethane (DCM) (50v: 50v) was used extract *P. erinaceus* for a period of 48 h. Different MeOH-DCM raw extracts of the leaves, trunk barks and roots of *P. erinaceus* as well as MeOH extracts of the leaves, trunk barks and roots of *D. oliveri* were obtained (Table 2.5).

3.3. Fractionation

Fractionation as the second step of the extraction aimed to separate compounds present in the raw extract according to their polarities and consequently their affinities with a solvent, meaning: polar compounds will be attracted by polar solvents and non-polar ones will also be attracted by non-polar solvents.

The raw extracts (each of the six above mentioned, except for the MeOH of *D. oliveri* which was not soluble in water) is solubilized in water and partitioned successively with solvents of increasing polarity: petroleum ether, dichloromethane, ethyl acetate and butanol. The following fractions were therefore obtained at the end: petroleum ether, dichloromethane, ethyl acetate, butanol and water fractions for each of the plant's parts, *ie* the leaves, barks and roots of *P. erinaceus* and for the leaves and barks of *D. oliveri*. The fractions obtained from the roots of *D. oliveri* were: petroleum ether, dichloromethane, ethyl acetate and the remaining methanolic fraction (Table 2.5).

Table 2.5: Different quantity of material obtained

Qt: quantity; PE: petroleum ether, DCM: dichloromethane; EA: ethyl acetate; But: butanol

Plant	Part	Qt of powder material (in kg)	Qt of raw extract obtained (in g)	Qt of raw extract reserved for biological and chemical tests (in g)	Qt of PE fraction obtained (in g)	Qt of DCM fraction obtained (in g)	Qt of EA fraction obtained (in g)	Qt of But fraction obtained (in g)	Qt of water fraction obtained (in g)
P. erinaceus	Leaves	3	145.5	12.98	100.43	2.05	4.66	18.1	33.5 g
	Trunk barks	3	323.8	33.76	0.19	0.38	18.48	141.8	106.79
	Roots	3	54	6.64	7	7.71	7.15	15.93	11.9
D. oliveri	Leaves	3	419.8	4.5710	6.723	33.3945	7.148	99.95	165.49
	Trunk barks	3	468.9	11.325	0.4235	1.5850	56.6403	49.9	150.88
	Roots	3	126.9	4.77	3.55	8.66	1.01	101.46 (final methanol fraction)	

4. Bio-assays

Antibacterial, antifungal, anti-nematicidal, anti-cancer and cytotoxicity tests have been performed on the different plant's extracts and fractions. Before going deeper, let us start with some definitions:

- a MIC is the Minimal Inhibitory Concentration. It is the minimal concentration of extract that inhibits 100% of bacterial growth. The lower the MIC for an extract is, the better it is, because, a low MIC indicates that the extract is highly active
- a PI (Percentage of Inhibition) is the percentage of bacterial growth inhibition that has been obtained using the highest concentration of extract tested. It is determined when a MIC was not observed at the highest concentration tested (256 µg/mL).

4.1. Antibacterial and antifungal tests

The methodology used to run the antibacterial and antifungal tests is the broth dilution method as recommended by EUCAST, 2015 and CLSI, 8th edition. Tests were carried out on seven different bacterial species and three different fungal species, representing the most frequent bacterial and fungal species encountered respectively in bacterial and fungal infections in humans.

The following bacteria provided by the ABC Platform® Bugs Bank have been used:

- Gram positive Cocci: *Enterococcus faecalis* ABC 3 (ATCC 29212) and *Staphylococcus aureus* ABC 1 (ATCC 29213).
- Gram negative bacilli: *Escherichia coli* ABC 5 (ATCC 25922), *Klebsiella pneumoniae* ABC 42, *Enterobacter cloacae* ABC 291.
- Non-fermenting Gram negative bacilli: *Pseudomonas aeruginosa* ABC 4 (ATCC 27853) and *Acinetobacter baumannii* ABC 14.

The following fungi provided by the ABC Platform® Bugs Bank have been used: *Candida albicans*, *Aspergillus niger* and *Aspergillus fumigatus*.

Tests have been firstly performed on the raw extracts and secondly on the fractions.

4.1.1. Material used for anti-bacterial and anti-fungal tests

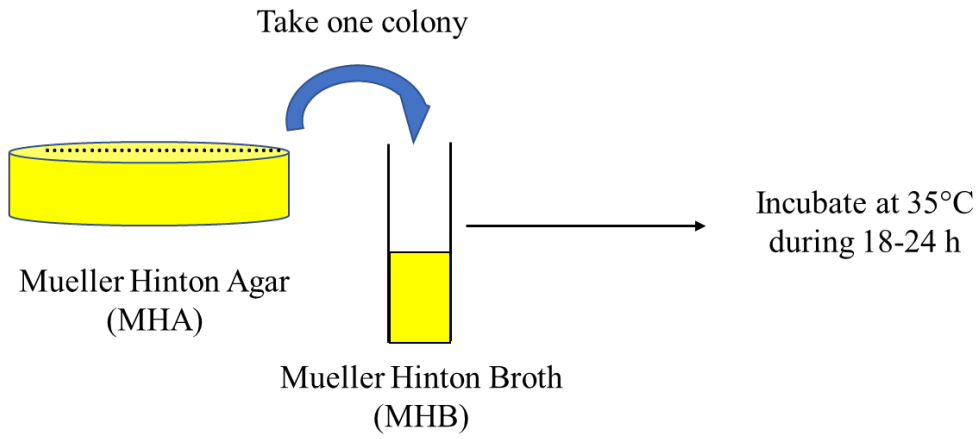
The following elements were part of the materials used: Mueller Hinton Agar (MHA); Mueller-Hinton Broth (MHB); distilled water; bleach; dimethylsulfoxide (DMSO); 96 wells plates; Petri dishes; Sabourhaud agar; Roswell Park Memorial Institute (RPMI)-1640 medium without carbonates and with phosphates; Tween 20

Syringes of 10, 20 or 50 mL; filters 0.22 µm (Filters Millex® GP; 0.22 µm); tubes of 15 and 50 mL; sterile pipettes of 5, 10 and 25 mL; multichannel pipettes of 5-50 µL; Malassez cell; Mac Farland reader; ELISA spectrophotometer.

4.1.2. Preparation of bacteria inoculum

At j0 (one day before the test date), the bacteria were defrosted and inoculated (with a loop) in one MHA-containing Petri dish and one 5 mL MHB-containing tube. The two media were incubated at 35°C for 18-24 h.

At j (On the test day), one colony was taken from the freshly grown bacteria in the Petri dish after which new broth tubes were prepared for the following day. The bacterial Petri dishes were kept in a cool place (5°C) (Figure 2.3).



<u>Figure 2.3:</u> Preparation of bacteria broth on test day (j)

One colony was taken from the Petri dish and incubated for further preparation of bacterium inoculum.

To prepare the bacterium inoculum, the broth tube was centrifuged at 4500 xg for 10 min. The supernatant was thrown in bleach and 1 mL of distilled water was added to the residue. The residue was vortexed to obtain a homogenized solution (Figure 2.4).

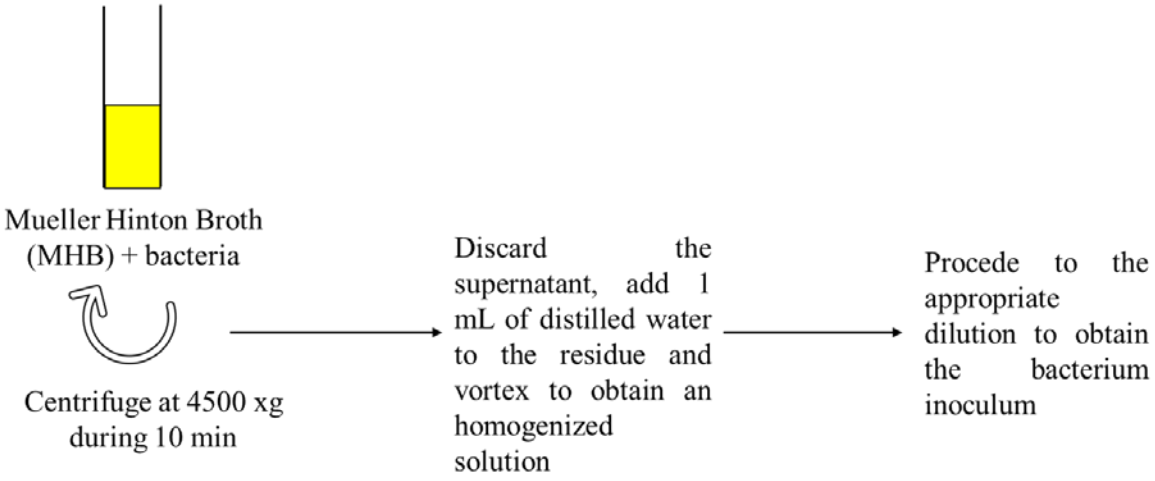


Figure 2.4: Preparation of bacteria inoculum.

The broth tube precedently obtained was vortexed after incubation. The residue obtained was used to prepare the bacterium inoculum.

The bacterium inoculum (50 mL) was prepared by adding distilled water to the precedent solution and performing the dilutions (depending on the bacterium) as presented in Table 2.6.

Table 2.6: Bacteria dilutions

The dilutions are prepared according to the type of bacteria.

Bacterium	Dilution
Enterococcus faecalis ABC 3 (ATCC 29212)	1/6000 (10 µL of Solution 1 in 60 mL of distilled water)
Escherichia coli ABC 5 (ATCC 25922)	1/100 (100 µL of Solution 1 in 10 mL of distilled water)
Other bacteria	1/1000 (10µL of Solution 1 in 10 mL of distilled water)

4.1.3. Preparation of yeast and fungi inoculum

At j0 (one day before the test day), a loop of *Candida albicans* was incubated on Sabourhaud agar at 35°C for 18-24 h.

At j-14 days at least (days before test day), a loop of *Aspergillus niger* and *Aspergillus fumigatus*, was incubated on inclined Sabourhaud agar tubes. The fungi was used only when complete colonization of the agar has been realised. This complete colonization was recognized by the production of black spores in the case of *A. niger* and green ones in the case of *A. fumigatus*.

Preparation of yeast inoculum

At j (on test day), 5 colonies of yeast were cultured in 5 mL of distilled water. The solution obtained was used to run a dilution with the RPMI to obtain a suspension whose concentration was equal to 0.5 McFarland.

Preparation of fungi inoculum

At j (on test day), *A niger* and *A. fumigatus* spores were removed by adding 5 mL of distilled water with 0.1% of Tween 20 to a colonized inclined agar tube (Tween helped to remove and individualize spores more efficiently). The solution obtained was sampled and then diluted to 10. Spores were counted under the microscope using Malassez cell as presented in Figure 2.5. The counting was performed as follows :

- a drop of the diluted solution was introduced under a thin slice and the Malassez slice.
- the cells present in the rectangles (there are 25 rectangles per square) and the two squares of the cell were counted to obtain two numbers (*n1*: the number of spores in the first square and *n2*: the number of spores in the second)
- the number of spores is given by the equation 2.1:

$$\frac{(n1 + n2) * \left(\frac{Df}{2}\right)}{25.10^{-8}} \quad \text{(Equation 2.1)}$$

n1: the number of spores in the first square

n2: the number of spores in the second

D*f*: dilution factor (10 in case of the tenth dilution)

25: number of rectangles

10^{-8}: to have a conversion to liter (L)

After counting, a dilution of the stock solution of spores using RPMI is produced to obtain a suspension of spores containing a number of spores belonging to the range 1-5 x 10^5 spores/mL.

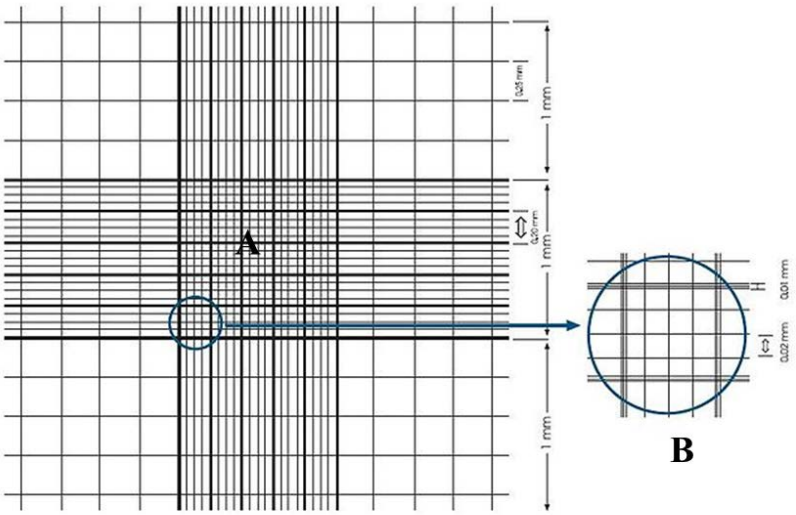


Figure 2.5: Aspect of one square of the Malassez cell under the microscope
A: the 25 rectangles of the square; **B**: one rectangle (only the cells present in the boundaries of every rectangle were counted).

4.1.4. Preparation of the extracts' solutions and plates

All the experiments described in this section were performed in 8 wells and repeated 3 times (Table 2.7 and Figure 2.6).

The positive controls used for the anti-bacterial tests were amoxicillin, ampicillin, oxacillin, penicillin G, ticarcillin and vancomycin while the one used for anti-fungal tests was amphotericin B. To avoid misleading results with the handling of these drugs, the positive control was tested on independent plates.

Tests were run using 96 well plates with each concentration replicated in 8 wells and repeated 3 times. The extract was tested at concentrations ranging from 256 µg/mL (highest) to 1 µg/mL (the lowest). An initial stock of extract (at 1024 µg/mL) was prepared by dissolving 10.24 mg of the extract in 500 µL of DMSO and made up to 10 mL with distilled water.

Antibacterial tests were completed using a robot, the Biomeck 4000 while anti-fungal tests were run by hand, following the general scheme as shown in Figure 2.6 and Table 2.7.

Table 2.7: Composition of each well of the 96 well-plates

Medium: Mueller Hinton Broth for antibacterial tests and RPMI-1640 without carbonates and with phosphates for anti-fungal tests.

Well	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
	Medium control	Extract control	Bacteria control	The concentration of extract ranging from 256 µg/mL the highest to 1 µg/mL the lowest.								
Medium (µL)	100	75	50	50	50	50	50	50	50	50	50	50
Plant extract (1024 µg/mL) (µL)	-	25	-	50	50	50	50	50	50	50	50	50
Cascade dilution												
Bacteria, fungi or yeast inoculum (µL)	**-**	**-**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**

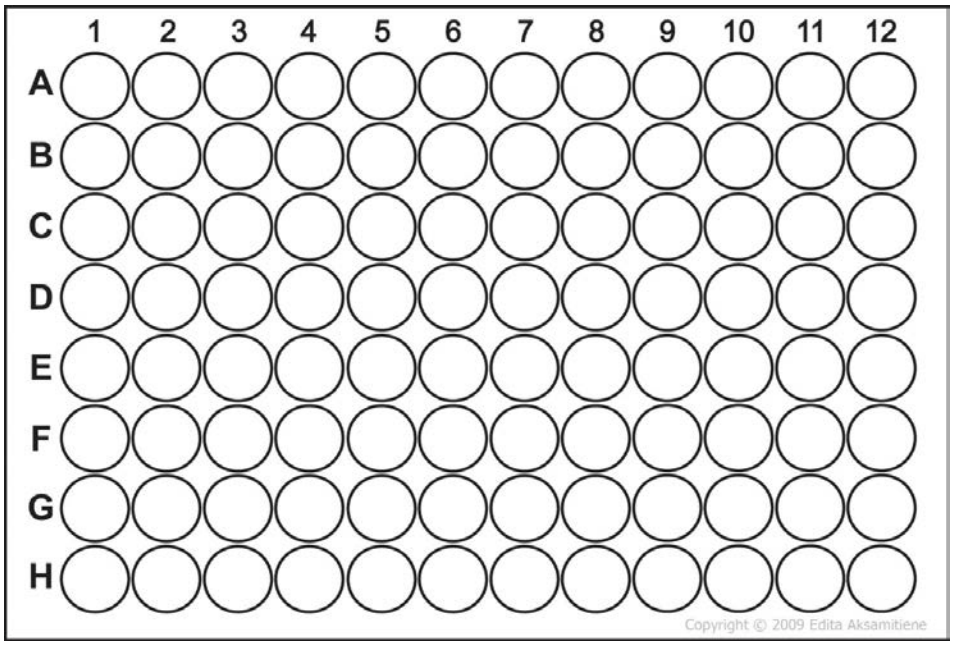


Figure 2.6: Schematic presentation of a 96 wells plate, anti-microbial testing.
Line **1** (from **A** to **H**): Medium control; Line **2** (from **A** to **H**): Bacteria control; Line **3** (from **A** to **H**): extract control; Line **4** to **12** (from **A** to **H**): different concentrations of the extract ranging from 256 µg/mL to 1 µg/mL.

4.1.5. The methodology used to perform the test:

The micro-broth dilution method was employed to run the anti-microbial test coupled with the detection of the activity by a reading of the absorbance at 540 nm. After distribution of the extract, the broth, bacterium inoculum, yeast or fungus inoculum, the plates were incubated at 35°C for 18-24 h for bacteria and yeast and 48-72 h for fungi. At the end of the incubation time, plates were read by an ELISA spectrophotometer at 540 nm and data were automatically computed and generated as Excel tables.

The raw extracts of the two plants were tested followed by those of the fractions to compare their activities and to ascertain whether the activities were kept even with the fractionation process. This was carried out systematically for all the raw extracts and fractions during antibacterial tests.

4.1.6. Computing of the data obtained

Data were computed using Excel 2016 to obtain graphs (mean of 8 wells and the standard deviation). The MIC was obtained from the graph (Figure 2.7) as the value that gives the

closest absorbance value (the absorbance appears as a bar as shown in Figure 2.7) to the ones of the 'Extract control' and 'Medium or Broth control' when those three control absorbances are within a certain range. Indeed, the 'Extract control' and 'Medium or Broth control' are perfect controls when their absorbances are equal and very close to 0 (between 0 and 0.15) as shown in Figure 2.7. The 'bacterium, yeast or fungi control' is perfect for the test when its absorbance is greater than 0.2 (suggesting that the germs have grown in the inoculum prepared and so will be the germs distributed in the wells). Three MIC values are therefore obtained from the three replicates and the final MIC is the mean of the three values. Considering the precision of the method, a difference of one dilution is not significant. For example from the graph shown in Figure 2.7, the MIC is 32 µg/mL. Indeed, this value has generated a bar whose length is closest to the ones of the 'Extract control' and 'Medium or Broth control' with no significant difference between the MIC at 16 µg/mL and that at 32 µg/mL.

Whenever the absorbance observed at the highest concentration (256 µg/mL), is higher than the absorbances observed for the 'Extract control' and 'Medium or Broth control' histograms, the MIC is said to be greater than 256 µg/mL (>256 µg/mL). In addition to this, when the histogram at 256 µg/mL is lower than the 'Bacterium, Yeast or Fungi control', it means that there is a germ inhibition at this concentration and in that case, the percentage of inhibition (PI) is determined (Equation 2.2).

A = [(mean of 8 values corresponding to the 8 wells at 256µg/ml) – (mean of 8 values corresponding to the 8 wells of extract control)]

B = [(mean of 8 values corresponding to the 8 wells of bacterium control – mean of 8 values corresponding to the 8 wells at of medium control)]

$$PI = \left[1 - \frac{A}{B}\right] \times 100 \quad \text{(Equation 2.2)}$$

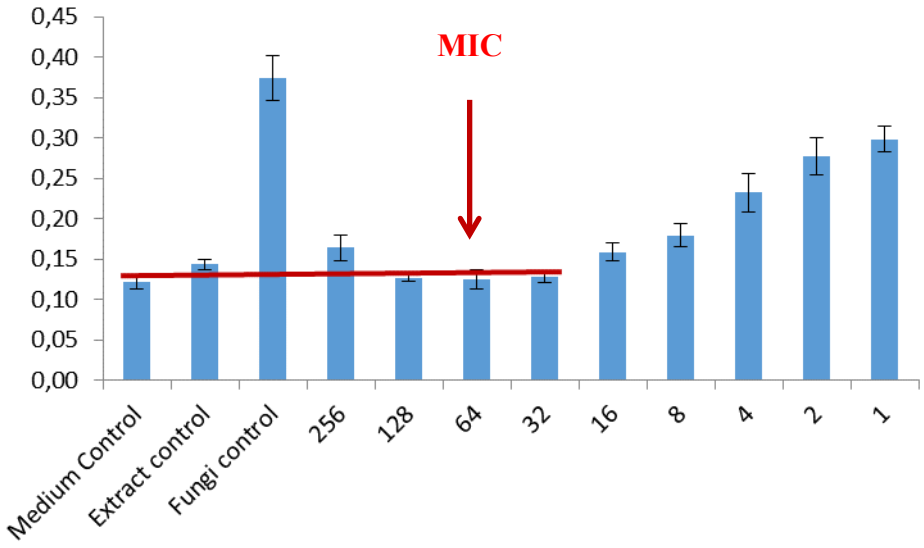


Figure 2.7: Example of data computation, determination of the anti-fungal activity of the DCM-MeOH raw extract of the roots of the roots of *P. erinaceus* on *A. fumigatus*.
The absorbance is illustrated on the graph by the length of the bar. The 'Extract control' and the 'Medium control' absorbances are equal and close to zero (values between 0 and 0.15), the 'Fungi control' absorbance is greater than 0.2: the controls are consequently good and the data computing could continue. The MIC is 32 µg/mL because this value gives an absorbance which is closest to the 'Extract control' and 'Medium control' absorbances, 32 µg/mL in this case.

4.2. Anti-cancer assay

The following cancer cells used for the study were:

- Hs 683 and U 373: glioma cells (tumor cells from the spine or the brain)
- SKMEL 28: melanoma cells (tumor cells from skin)
- A 549: lung carcinoma cells (tumor cells from the lung).
- MDA-MB 231: breast cancer cells

The cancer cells were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA), the European Collection of Cell Culture (ECACC, Salisbury, UK), and the Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ, Braunschweig, Germany).

The methodology used to accomplish the anticancer assay was the MTT (tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay based on the ability of the NAD(P)H-dependent cellular succinate dehydrogenase enzyme present in the mitochondria of living cells, to reduce the tetrazolium dye (yellow color), to its insoluble formazan form

(purple color). This color could be measured with an ELISA spectrophotometer and the absorbance is proportional to the number of living cells (Mosmann, 1983).

4.2.1. Material

The following apparatus, reagents and chemicals were used: RPMI culture medium; heat-inactivated fetal bovine serum (FCSi); 100 µg/mL gentamicin; penicillin−streptomycin (200 units/mL and 200 µg/mL); DMSO; glutamine; Non-Essential Amino Acid (NEAA); sodium pyruvate; trypan blue; 96 well-plates with a flat bottom; distilled water; bleach; sodium chloride (NaCl); potassium chloride (KCl); sodium hydrogen phosphate (Na_2HPO_4); potassium hydrogen phosphate (KH_2PO_4); tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; tubes of 15 and 50 mL; sterile pipettes of 5, 10 and 25 mL; multichannel pipettes of 5-50 µL and TC-20 BIORAD.

4.2.2. Preparation of cells

Days before the test, the U373, U251, Hs683, SKMEL-28 and A549 cell lines were cultured and maintained with a constant control, in RPMI culture medium supplemented with 10% heat-inactivated fetal bovine serum, to obtain good confluent cells. Cell culture media were supplemented with 4 mg glutamine, 100 µg/mL gentamicin; at 37°C in a 5% CO_2 humidified atmosphere.

At j0, the cells and the following solutions were prepared:

- cells were prepared by putting 10,000 cells in 100 µL of complete medium and filling every well of the 96 well-plates except for the wells belonging to the first two columns (Table 2.8). The plates were then incubated for 48 h at 37°C in an incubator and under 5% of carbon dioxide.
- complete medium: RPMI + 10 % FCSi + 1% glutamine + 100 µg/ml gentamicin + penicillin-streptomycin (200 units/ml and 200 µg/ml).
- phosphate buffered saline (PBS): NaCl (8g/L) + KCl (0.2 g/L) + Na_2HPO_4 (1.4 g/L) + KH_2PO_4 (0.2 g/L).

4.2.3. Preparation of extracts

At j2 (on the 2nd day after J0), plants' extracts were prepared by dissolving 2 mg of extract in 200 µL of DMSO. Then 100 µL of the solution (S1) was taken and adjusted it to 10 ml with complete medium to obtain a second solution (S2). S2 had a concentration of 200 µg/mL. The other range of concentrations to be used (100 µg/mL to 0.78 µg/mL) was prepared by realizing cascade dilutions in bottle tubes of 15 mL using a complete medium. The final concentration of DMSO in the extract never exceeds 2% of the final volume (Table 2.8).

Table 2.8: Composition of each well of the 96 wells plate during anti-cancer testing
The medium is a complete medium composed of RPMI culture medium + 10 % FCSi + 1% Glutamine + 100µg/ml Gentamicin + Penicillin-Streptomycin (200 units/ml and 200 µg/ml).

Well	**1 (A to H)** **Medium control**	**2(A to H)** **Extract control**	**3 (A to H)** **Cells control**	**From 3 to 12 (A to H)** **Extracts with concentration from 100 µg/mL to 0.78 µg/mL**				
Medium µL	100	-	100	-	-	-	-	-
Plant extract µL	-	100	-	100	100	100	100	100

4.2.4. Methodology

At j2, the cell plates (prepared at j) were taken from the incubator and emptied in a tub containing bleach. The wells (from 1 to 12 horizontally and A to H vertically) were filled following the scheme as described in Table 2.9. After filling the wells, plates were put in the incubator at 37°C under 5% of carbon dioxide for 24 h. The MTT stock solution was prepared by dissolving 5 mg of MTT in 1 mL of PBS and kept in a cool place (5°C) to be used the following day. The Sodium Dodecyl Sulfate (SDS) solution was also prepared by dissolving 50 g of SDS powder in 500 mL of PBS and adding 445 µL of hydrochloric acid (0.01 mL/L) after shaking and light warming. The SDS solution will be used to dissolve the formazan which will be formed in the cells.

At j5 (72 h after j2), the cells viability was checked by performing the MTT assay. Plates were taken from the incubator and emptied. Each well was filled with 100 µL of MTT solution obtained by dissolving 1 mL of MTT stock solution in 9 mL of EMEM. Plates were incubated again for 3 h after which 100 µL of SDS was added to each well before incubation for another 4 h. Plates were then shaken and their absorbance was read using a spectrophotometer at 540 nm and 690 nm.

4.2.5. Computation of the data obtained

Data were automatically generated as Excel tables. The latter were computed with Excel 2016: absorbance at 540 nm was substracted from absorbances at 690 nm because the MTT absorbs at 540 nm. The IC_{50} was then determined by using the prevision formula in Excel: at which concentration, considering all the absorbance means from 100 µg/mL to 0.78 µg/mL, an absorbance corresponding to 50% of alive cells was obtained. The calculation was automatically achieved using Excel and the IC_{50} was obtained.

4.3. Tests against nematodes

4.3.1. Material

The following materials and reagents were used:

- Nematode: *Steinernema feltiae* provided by Schneckenprofi Ltd (Hennstedt, Germany).
- DMSO; distilled water; Eppendorf tubes; 96 well plates; ethanol 70%; microscope; Eppendorf warming plate.

4.3.2. Preparation of nematode samples

Nematodes upon reception were stored in a cool place (2°C) until use. Suspensions of nematodes were prepared fresh by dissolving 2 g in 250 mL. They were then left at room temperature under agitation for 30 min, to warm the nematodes up. The viability (number of nematodes alive / number of nematodes dead) was checked at the end by counting: 1 mL of the solution is deposited on a slide and the nematodes were counted under the microscope (4-fold magnification). This viability should be greater than 80% for the solution to be used to carry out the test.

4.3.3. Preparation of the extract

The extracts' solutions were prepared by dissolving 20 mg in 200 µL of DMSO adjusted to 2 mL with distilled water. The first concentration to be obtained was 10 mg/mL. Dilutions were performed to obtain two other concentrations: 5 and 2.5 mg/mL. Extracts are prepared in Eppendorf tubes. All the raw extracts and fractions were tested.

4.3.4. Running of the test: methodology

Each concentration of the extract was tested in three wells and repeated three times (on 3 different days). The positive control is ethanol 70%. The plate was prepared as shown in Table 2.9.

Plates were then kept in a dark place for 24-48 h. At the end of this period, the viability of nematodes was checked. Distilled water at 50°C (100 µL) was added to the wells to warm the nematodes up and wells were observed under the microscope. Only the living nematodes were counted. The viability after 24-48 h (V) was evaluated by comparing it to the viability before the addition of the extract solution (Equation 2.3).

Vo: viability at t = 0 h

Vi: viability after 24-48 h

$$\frac{Vi}{Vo} * 100 \quad \text{(in percentage) (Equation 2.3)}$$

Table 2.9: Composition of each well of a plate during anti-nematicidal testing

The positive control is ethanol and the extract control was obtained by dissolving 20 mg in 200µL of DMSO adjusted to 2 mL by distilled water.

Well	1	2	3	4	5	6
	Positive control	Extract control	Nematode control	10 mg/mL	5 mg/mL	2.5 mg/mL
Plant extract	-	100	-	50	50	50
Nematode solution (µL)	-	-	100	50	50	50

4.3.5. Computation of the data obtained

Whenever the Vi was equal to Vo, the viability was no longer computed. This implies that the extract did not have any activity on the nematodes.

4.4. Tests for cytotoxicity

The cytotoxicity tests were performed to see whether the biologically active extracts and/or fractions are toxic to normal human cells. Indeed, a good activity associated with a huge toxicity may not be healthy for human beings.

The cells used for the cytotoxicity activity were MRC-5 cells (ATCC CCL-171, human lung fibroblasts). The methodology used is the MTT assay as previously described in section 3.2.

4.4.1. Material

The material used resembles the one described for the anti-cancer tests in section 4.2.1. with the following new elements: Eagle's Minimal Essential Medium (EMEM), Fetal Calf Serum (FCSi) and Human Fetal Lung Fibroblast Cells (MRC-5 cells).

4.4.2. Preparation of MRC5 cells

The MRC5 cells are prepared like cancer cells (as precedently described in section 4.2.2.) with slight differences.

Days before the test, MRC5 cells were grown in EMEM supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine (G7513-100 mL, Sigma-Aldrich), at 37°C in a 5% CO_2 humidified atmosphere.

At j-2 (2 days before the test), the cells are prepared as described in section 4.2.2. but with using a complete medium composed of: EMEM + 2 % FCSi + 1% Glutamine + 1 % Pyruvate sodium + 1% (Non-Essential Amino Acid). The PBS is also prepared as precedently described.

4.4.3. Preparation of the extract

At j (2 days after the first step J-2, the test date), plant extracts were prepared by dissolving 5.12 g of extract in 200 µL of DMSO to obtain a first solution named s1. 100 µL of s1 was adjusted to 10 mL with complete medium to obtain a second solution (s2). s2 had a concentration of 256 µg/mL. The other range of concentrations to be used (128 µg/mL to 1 µg/mL) was prepared by making cascade dilutions in bottle tubes of 15 mL using the

complete medium. The final concentration of DMSO in the extract never exceeds 2% of the final volume (Table 2.8).

4.4.4. Methodology

The test was carried out following the same guidelines as in section 4.2.4 with slight changes. At j, the plates were taken from the incubator and emptied in a tub containing bleach. The wells were there after filled following the scheme as described in Table 2.8. After filling the wells, plates were put in the incubator at 37°C under 5% of carbon dioxide for 24 h (in the case of the most active extracts or fractions on bacteria, 48 h for the ones which were more active on fungi). The MTT stock solution was also prepared at j (as previously shown in section 4.2.4). At j1 (the following day for active extracts or fractions on bacteria) or at j2 (48 h for the active extracts or fractions on fungi), the cells viability was checked by running the MTT assay as precedently described in section 4.2.4

4.4.5. Computation of the data obtained

The computing of the data obtained is the same as in the case of anti-cancer testing as previously described in section 3.2.5.

5. Results

5.1. Antibacterial tests

5.1.1. *Pterocarpus erinaceus*

The roots of *P. erinaceus* gave the lowest yield (Table 2.5) in terms of the quantity of extract obtained, but with good activity. The raw extract exhibited activity against all the bacteria investigated with a percentage inhibition (PI) ranging from 42% to 74% at 256 µg/mL, the highest concentration tested (Table 2.10).

Table 2.10: Antibacterial activities of the raw extracts (MeOH-DCM, 1:1) of *P. erinaceus* parts.

Raw extract: methanolic-dichloromethane (MeOH-DCM) extract. Antibacterial activity was based on the determination of the relevant MIC (concentration that inhibits 100% of bacterial growth) values.

1: leaves; **2**: trunk barks; **3**: roots.

n.o: no MIC observed at the highest concentration (256 µg/mL) tested.

X%: PI (percentage of inhibition of bacterial growth) observed at 256 µg/mL, the highest concentration under investigation.

MICs are marked in bold. Each experiment has been repeated three times (n=3).

	E. faecalis	*S. aureus*	*P. aeruginosa*	*A. baumannii*	*E. coli*	*K. pneumoniae*	*E. cloacae*
1-	n.o	n.o	*66%*	n.o	n.o	n.o	n.o
2-	**32**	**128**	**256**	**256**	**256**	*54%*	**256**
3-	*74%*	*77%*	*67%*	*59%*	*73%*	*48%*	*42%*

The individual fractions obtained from the whole root extract were also active, with the best results obtained against Gram-positive bacteria (*E. faecalis* and *S. aureus*) and *P. aeruginosa*. The MIC values obtained were ranged from 64 to 256 µg/mL against the three bacteria (Table 2.11).

Besides the roots, the trunk barks could be identified as the most active organ of this plant. The raw extract was active against all the seven bacteria under investigation and MIC values ranged from 32-256 µg/mL on six out of the seven bacterial strains under investigation (*i.e. E. faecalis*, *S. aureus*, *P. aeruginosa*, *A. baumannii*, *E. coli* and *E. cloacae*). The bark also demonstrated the lowest MIC against the bacteria: 32 µg/mL against *E. faecalis* (Table 2.10). For comparison, for a pure compound with a molecular weight of 320 g/mol, this would imply a concentration of just 100 µM. Not surprisingly, the fractions obtained from the barks were also active against all the bacteria under investigation (Table 2.11). Within this context, the butanol and water fractions were the most active: they have shown the same MIC value obtained with the whole extract against four bacteria: 256 µg/mL on *P. aeruginosa*, *A. baumannii*, *E. cloacae* and *E. coli*; 128 µg/mL on *S. aureus* and 32 or 64 µg/mL on *E. faecalis*, respectively. The ethyl acetate fraction also exhibited an interesting activity, with MIC values in the range of 64 - 256 µg/mL observed against three bacteria (*i.e. S. aureus*, *E. faecalis* and *A. baumannii*). In contrast, the more apolar petroleum ether and dichloromethane

fractions appeared to be less active. They were only active against the two Gram-positive bacteria (*E. faecalis* and *S. aureus*). The dichloromethane fraction was active at the highest MIC observed during the tests (256 µg/mL). The petroleum ether fraction was active against *E. faecalis* at a concentration almost equal to that of the whole extract, *i.e.* 64 µg/mL (Tables 2.10 and 2.11).

The leaves were the least active plant of all the organs studied. The raw extract obtained from the leaves inhibited the growth of just one bacterium, namely *P. aeruginosa*, and at 66% when the extract was applied at the highest concentration under investigation (256 µg/mL) (Table 2.10). Surprisingly, fractions from the whole extract were active against all bacteria studied. The petroleum ether, dichloromethane, ethyl acetate and water fractions showed good activity against Gram-positive bacteria (*E. faecalis* and *S. aureus*) and *P. aeruginosa*, with the notable exception of the petroleum ether fraction, which was not active against *E. faecalis*. Out of all those fractions, the ethyl acetate was the most active one with a MIC at 256 µg/mL against *E. faecalis* and *S. aureus* (Table 2.11).

Table 2.11: Antibacterial activities of the fractions obtained from the raw extracts of *Pterocarpus erinaceus*.
1'-; **-2'**-; **-3'**-: fractions derived from the leaves (1); the trunk barks (2) and roots (3), respectively.
n.o: no MIC (concentration that inhibits 100% of bacterial growth) observed at the highest concentration (256 µg/mL) tested.
X%: PI (percentage of inhibition of bacterial growth) observed at 256 µg/mL, the highest concentration under investigation.
MICs are marked in bold. Each experiment has been repeated three times (n=3).

	E. faecalis	*S. aureus*	*P. aeruginosa*	*A. baumannii*	*E. coli*	*K. pneumoniae*	*E. cloacae*
1'-Petroleum ether	n.o	*50%*	*50%*	n.o	*50%*	n.o	n.o
1'-Dichloromethane	*50%*	*50%*	*50%*	n.o	*43.5%*	n.o	n.o
1'-Ethyl acetate	**256**	**256**	n.o	n.o	n.o	n.o	n.o
1'- Butanol	n.o	n.o	n.o	n.o	n.o	n.o	n.o
1'- Water	*18%*	*26%*	*48%*	*13%*	*28%*	*44%*	*33%*
2'- Petroleum ether	**64**	**256**	*47%*	n.o	*70%*	*50%*	n.o
2'- Dichloromethane	**256**	**256**	*37%*	n.o	*69%*	*42%*	*39%*
2'-Ethyl acetate	**128**	**64**	*54%*	**256**	*58.5%*	*48.5%*	*29.5%*
2'-Butanol	**32**	**128**	**256**	**256**	**256**	*74.71%*	**256**
2'-Water	**64**	**128**	**256**	**256**	**256**	*85%*	**256**
3'-Petroleum ether	**256**	**256**	**256**	*55%*	*77.5%*	*73%*	*61%*
3'-Dichloromethane	*84%*	**256**	*78%*	*47%*	*66%*	*46.5%*	*50%*
3'-Ethyl acetate	**64**	*75%*	*81.5%*	**256**	*60.5%*	*74.5%*	*55.5%*
3'-Butanol	**256**	*75%*	*64%*	*67%*	*48%*	*29%*	*42%*
3'- Water	*69%*	*33%*	*65%*	*70%*	*39%*	*37%*	*43%*

As the trunk barks appear to be the most active plant organ with MIC values obtained (from the whole extract to the fractions), a more detailed investigation was conducted on this plant organ. Since the extracts and fractions derived from the trunk barks were more active against Gram-positive bacteria (with MIC values ranging from 32 µg/mL to 256 µg/mL), and were particularly active against *Staphylococcus aureus*, the most promising fractions from these trunk barks (*i.e.* ethyl acetate, butanol and water) were consequently tested against two other *staphylococcal* strains frequently encountered in common bacterial infections in Togo: *S. aureus* (but resistant to methicillin, *MRSA*) and *S. epidermidis* (Kombate *et al*., 2011). The activities against these two pathogenic bacteria were compared to the activities of the three fractions against the other Gram-positive bacteria under investigation. Amazingly, the ethyl acetate fraction exhibited a MIC of 32 µg/mL against *S. epidermidis* (Table 2.12). For the butanol and water fractions, a MIC was observed at 64 µg/mL and 128 µg/mL on these two germs, with a pronounced inhibition of bacterial growth at concentrations lower than 64 µg/mL. We have therefore decided to check whether the inhibition observed could be equal to 50% of bacterial growth inhibition (PI_{50}) at those low concentrations. Interestingly, the PI_{50} (*i.e.* the concentration which inhibits 50% of bacterial growth) of the butanol and water fractions against those two bacteria was in the range of just 1 µg/mL (Table 2.12), pointing towards a considerable activity of these fractions against those pathogenic strains in micromolar concentrations of compounds contained therein.

Table 2.12: Antibacterial activities of the most effective fractions of *P. erinaceus* against *MRSA* and *S. epidermidis*.

PI_{50}: concentration at which a 50% of bacterial growth is inhibited at a concentration lower than 256 µg/mL. *MRSA*: Methicillin-Resistant *Staphylococcus aureus*. *S. epidermidis*: *Staphylococcus epidermidis*. ND: not determined. Tests have been repeated three times (n=3).

	MRSA		*S. epidermidis*	
	MIC	PI_{50}	MIC	PI_{50}
2'- Ethyl acetate	**256**	ND	**32**	**ND**
2'- Butanol	**64**	**1**	**64**	**1**
2'- Water	**128**	**1**	**64**	**1**

5.1.2. *Daniellia oliveri*

In general, the raw extracts of all the parts of this plant are active. Indeed, whenever no MIC was obtained, an inhibition of bacterial growth was always observed at the highest concentration tested, 256 µg/mL (Table 12).

Table 2.13: Antibacterial activities of the raw extracts (MeOH) of *Daniellia oliveri* parts.

Raw extract: methanolic (MeOH) extract. Antibacterial activity is based on the determination of the relevant MIC (concentration that inhibits 100% of bacterial growth) values.
21: leaves; **22**: trunk barks; **23**: roots.
n.o: no MIC observed at the highest concentration (256 µg/mL) tested.
X%: PI (percentage of inhibition of bacterial growth) observed at 256 µg/mL.
MICs are marked in bold. Each experiment has been repeated three times (n=3).

	E. faecalis	*S. aureus*	*P. aeruginosa*	*A. baumannii*	*E. coli*	*K. pneumoniae*	*E. cloacae*
21-	**32**	**256**	*74%*	*87%*	*88%*	*78%*	*75%*
22-	**16**	**128**	**256**	*84%*	**256**	*75%*	*86%*
23-	**64**	**256**	*59%*	*50%*	*44%*	*58%*	*37%*

It is also almost the same situation with all the fractions coming from the raw extracts of all the plant parts, except with the water fraction of the leaves, the dichloromethane fraction and the petroleum ether fraction of the trunk barks, where no activity was observed at 256 µg/mL (the highest concentration tested) respectively against *Pseudomonas aeruginosa* and *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Table 2.14).

The extraction and fractionation achieved on the different parts of the plant yielded good quantities in comparison to *P. erinaceus*, but like this plant, the roots yielded the lowest quantity of extract. The roots are also the least active part of *D. oliveri*. The raw extract (MeOH) inhibited 100% of bacterial growth only in the case of the two Gram positive bacteria tested: a MIC at 64 µg/mL on *Enterococcus faecalis* and 256 µg/mL on *Staphylococcus aureus*. The roots have also inhibited the growth of the other tested bacteria (namely *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) at 256 µg/mL with a percentage of inhibition (PI) ranging from 37% to 79% (Table 2.13).

When tested on those different germs, all its fractions (petroleum ether, dichloromethane, ethyl acetate and the final methanolic fraction) were also active. The least active of its fractions is the petroleum ether one. The latter exhibited a MIC of 32 µg/mL (not significantly different from 16 µg/mL) against *E. faecalis* and inhibited the growth of the other bacteria at 256 µg/mL with a PI ranging from 19% to 75%. The second least active fraction of the roots is the final methanolic fraction with MIC of 128 µg/mL against the two Gram-positive bacteria and a PI at 256 µg/mL, from 38.5 % to 79.5 % against the other bacteria. Like this fraction, the dichloromethane fraction was also active against *E. faecalis* and *S. aureus* with respective MIC at 32 µg/mL and 128 µg/mL. This fraction has partly inhibited the growth of the 5 remaining bacteria at 256 µg/mL with a percentage of inhibition (PI) from 32% to 84 %. The ethyl acetate fraction, the most active fraction of the roots, has shown a MIC on *E. faecalis*, *S. aureus* and *P. aeruginosa* respectively at 64 µg/mL and 128 µg/mL and, a PI against the other germs in the range of 36% to 57%. It could be noticed that no matter the fraction, there is always a high activity against Gram-positive cocci (*E. faecalis* and *S. aureus*). Besides, the petroleum ether and the dichloromethane fraction exhibited the lowest MIC among the roots extract and fractions: 32 µg/mL against *E. faecalis* (almost equal to 16 µg/mL) (Table 2.14).

The leaves are the second most active plant part. The raw extract demonstrated an inhibition with MICs against the same bacteria as the raw extract of the roots; 32 µg/mL against *E. faecalis* and 256 µg/mL against *S. aureus*. The inhibition of the five remaining bacteria was achieved at 256 µg/mL with PI in the range of 75% to 88%. The PI observed against those bacteria were higher compared to the ones observed against them with the raw extract from the roots (Table 2.13). More MIC values were obtained with the fractions obtained from the raw extract of the leaves (18 in total) when compared to the fractions obtained from the raw extract of the roots (8) (Table 2.14). Some fractions were even active against all the bacteria, with 100% of bacterial growth inhibition. It is the case of the butanol fraction of the leaves with MIC values ranging from 64 µg/mL to 256 µg/mL on the 7 bacteria tested. The butanol fraction of the leaves was the only fraction among *D. oliveri* leaves and extracts to have shown MIC against all the bacteria. The petroleum ether fraction of the leaves has also revealed a MIC at 256 µg/mL against all the bacteria except *K. pneumoniae*. The third most active fraction of the leaves, the aqueous fraction inhibited *E. faecalis*, *S. aureus* and *E. coli* with MIC at 64 µg/mL and 256 µg/mL. The dichloromethane and ethyl acetate fraction showed MIC against *S. aureus* only and at the highest concentration tested 256 µg/mL. At this

concentration, the dichloromethane fraction partly inhibited the growth of other bacteria except for *E. faecalis* (where no activity has been observed at this concentration) with PI from 59% to 85%. The ethyl acetate fraction of the leaves has also inhibited the growth of all the other bacteria with PI ranging from 52% to 86% (Table 2.14).

The most active part of *D. oliveri* is surely its trunk barks: 25 MIC values (raw extracts and fractions) in comparison to 20 MIC values with the leaves (raw extract and fractions) and 10 MIC values with the roots (raw extract and fractions) (Tables 2.13 and 2.14). The lowest MIC during the antibacterial tests of this chapter was obtained with the raw extract of the trunk bark against *E. faecalis*: 16 µg/mL. MICs were also observed against *S. aureus*, *P. aeruginosa* and *E. coli*, respectively, at 128 µg/mL and 256 µg/mL. The other bacteria; *A. baumannii*, *K. pneumoniae* and *E. cloacae*; were inhibited with high PI, respectively, at 84%, 75% and 86% (Table 2.13). Consequently, the fractions of the trunk barks were tested on the 7 bacteria under investigation. The most active appeared to be the water fraction, with MIC values obtained against all the bacteria (64 µg/mL to 256 µg/mL) except against *A. baumanii* where, however, a PI of 86% at 256 µg/mL was recovered. The second most active is the butanol fraction with MIC from 64 µg/mL to 256 µg/mL against 5 bacteria and PI respectively at 82.5% and 70.5% against *E. coli* and *K. pneumoniae* (Table 2.14). The ethyl acetate fraction exhibited MIC values at 64 µg/mL and 128 µg/mL against *E. faecalis* and *S. aureus*, at 256 µg/mL against *A.baumannii* and *E. coli* and PI between 52% and 86% against the other germs. The dichloromethane fraction was active with a MIC at 256 µg/mL against *E. faecalis*, *S. aureus* and *E. cloacae*. No activity was noticed at 256 µg/mL against *P. aeruginosa* and against the other bacteria, a PI from 67% to 71% was obtained. The petroleum ether fraction was active against *E. faecalis* at 128 µg/mL, against *S. aureus* and *K. pneumoniae* at 256 µg/mL. On the remaining Gram-negative bacilli (*E. coli* and *E. cloacae*), a partial inhibition was noticed at the highest concentration tested (256 µg/mL) with PI at 74 % and 46 %. No activity was observed on *P. aeruginosa* and *A. baumanii* at 256 µg/mL (Table 2.14).

Table 2.14: **Antibacterial activities of the fractions obtained from the raw extracts of *Daniellia oliveri*.**
21'-; **22'**-; **23'**-: fractions derived from the leaves (21); the trunk barks (22) and roots (23), respectively.
n.o: no MIC (concentration that inhibits 100% of bacterial growth) observed at the highest concentration (256 µg/mL) tested.
X%: PI (percentage of inhibition of bacterial growth) observed at 256 µg/mL, the highest concentration under investigation.
MICs are marked in bold. Each experiment has been repeated on three different occasions (n=3).

	E. faecalis	*S. aureus*	*P. aeruginosa*	*A. baumannii*	*E. coli*	*K. pneumoniae*	*E. cloacae*
21'-Petroleum ether	**256**	**256**	**256**	**256**	**256**	no	**256**
21'- Dichloromethane	no	**256**	*72%*	*82%*	*85%*	*82%*	*59%*
21'-Acetate	*78%*	**256**	*63%*	*71%*	*86%*	*52%*	*69%*
21'-Butanol	**64**	**128**	**128**	**256**	**256**	**256**	**256**
21'-Water	**64**	**256**	no	*83.5%*	**256**	*63%*	*90%*
22'- Petroleum ether	**128**	**256**	no	no	*74%*	**256**	*46%*
22'- Dichloromethane	**256**	**256**	no	*67%*	*71%*	*67.24%*	**256**
22'-Acetate	**64**	**128**	*79.5%*	**256**	**256**	*78%*	76%
22'-Butanol	**64**	**128**	**256**	**256**	*82.5%*	*70.5%*	**256**
22'-Water	**64**	**128**	**128**	*85%*	**256**	**256**	**256**
23'-Petroleum ether	**32**	*70%*	*75%*	*20%*	*68%*	*19%*	*39%*
23'- Dichloromethane	**32**	**128**	*84%*	*15%*	*63%*	*33%*	*32%*
23'- Ethyl acetate	**64**	**64**	**128**	*45%*	*57%*	*36%*	*38.5%*
23-MeOH final	**128**	**128**	*74%*	*79.5%*	*55%*	*75.5%*	*38.5%*

It was noticeable that whenever a MIC was observed with either a raw extract of a fraction from a part of *D. oliveri*, the activity was always present on one of the two Gram-positive cocci (either *E. faecalis* and *S. aureus*). The most active fractions were therefore investigated further by testing their activities on two other types of Gram-positive bacteria that are frequently encountered in infections in Togo: Methicillin-Resistant *Staphylococcus aureus* (*MRSA*) and *Staphylococcus epidermidis* (Table 2.15). The most active fractions of the leaves (petroleum ether, butanol and water) were tested. The petroleum ether fraction did not exhibit activity at the highest concentration tested 256 µg/mL on *MRSA* and *S. epidermidis*. In contrast, the other two fractions demonstrated a MIC value at almost equal concentrations, 64 and 128 µg/mL (no significant difference between 64 µg/mL and 128 µg/mL because of the precision of the method as explained in Section 4.1.6) against the two bacteria with a 50% inhibition of bacterial growth (PI_{50}) at 1 µg/mL. The same result was obtained with the butanol and water fractions of the trunk barks. The petroleum ether and ethyl acetate fractions were active against *MRSA* and *S. epidermidis* with MIC almost equal to 128 µg/mL (no significant difference between 128 µg/mL and 256 µg/mL). The dichloromethane fraction showed only a MIC of 256 µg/mL against *S. epidermidis* but partly inhibited the growth of *MRSA* at 256 µg/mL with a PI at 63.54%. In the case of the roots, its ethyl acetate fraction was not active against the two bacteria at 256 µg/mL. Its dichloromethane fraction was the most active with MIC equal to 128 µg/mL against *MRSA* and *S. epidermidis*. The final methanolic fraction of the roots was only active against MRSA at 64 µg/mL (Table 2.15).

Table 2.15: Antibacterial activities of the most effective fractions of *Daniellia oliveri* against *MRSA* and *S. epidermidis*.

PI_{50}: concentration at which a 50% of bacterial growth is inhibited at a concentration lower than 256 µg/mL. *MRSA*: Methicillin-Resistant *Staphylococcus aureus*. *S. epidermidis*: *Staphylococcus epidermidis*. ND: not determined. n.o: no MIC (concentration that inhibits 100% of bacterial growth) observed at the highest concentration (256 µg/mL) tested.
X%: PI (percentage of inhibition of bacterial growth) observed at 256 µg/mL, the highest concentration under investigation.
MICs are marked in bold. Each experiment has been repeated three times (n=3).

	MRSA		*S. epidermidis*	
	MIC	PI_{50}	MIC	PI_{50}
21'- Petroleum ether	no	ND	no	ND
21'- Butanol	**128**	**1**	**128**	**1**
21'- Water	**128**	**1**	**64**	**1**
22'- Petroleum ether	**256**	ND	**128**	ND
22'- Dichloromethane	*63.54%*	ND	**256**	ND
22'- Ethyl acetate	**128**	ND	**256**	ND
22'- Butanol	**128**	**1**	**64**	**1**
22'- Water	**128**	**1**	**64**	**1**
23'- Dichloromethane	**128**	ND	**256**	ND
23'- Ethyl acetate	no	ND	no	ND
23'- MeOH final	**64**	ND	no	ND

5.2. Anti-fungal tests

The raw extracts coming from the leaves, the trunk barks and roots of the two plants were tested on *Candida albicans*, *Aspergillus fumigatus* and *Aspergillus niger*. *D. oliveri* did not present any activity against yeast and the fungi at the highest concentration tested (256 µg/mL). Activity was observed only with the raw extract of the roots of *P. erinaceus* with a MIC at 16 µg/mL against *A. fumigatus*. Consequently, the fractions obtained from the raw extract of the roots were tested on this fungus. Interestingly, the ethyl acetate fraction of the roots exhibited a MIC almost equal to that of the raw extract: 32 µg/mL (a difference of one dilution is not significant). The dichloromethane fraction was also active with MIC at 64 µg/mL. No MIC was observed with the other fractions at a concentration of 256 µg/mL, the

petroleum ether and water fractions exhibited an inhibition of *A. fumigatus* growth respectively at 32.47% and 41.75%.

5.3. Anti-cancer tests

The raw extracts of the leaves, trunk barks and roots of the two plants were tested on 5 cancer lines: Hs 683, U 373, SKMEL 28, A549 and MDA-MB 231. Among the extracts of *D. oliveri*, only the leaves were active against a type of glioma cells Hs 683 with an IC_{50} at 91 µg/mL, the highest IC_{50} registered during those anti-cancer tests (Table 2.16). The leaves, trunk barks and roots of *P. erinaceus* were all active. The most active part of this plant was the roots, it has exhibited an IC_{50} against all the cancer cells under investigation. The raw extract of the roots of *P. erinaceus* was active against all the 5 cancer lines with IC_{50} ranging from 28 µg/mL to 55 µg/mL. The roots of *P. erinaceus* exhibited an IC_{50} not only at concentrations lower than the ones observed with the other parts of *P. erinaceus* but also with the leaves of *D. oliveri*. The lowest IC_{50} of the whole assay was also obtained with the roots of *P. erinaceus* at 28 µg/mLon a type of glioma cells U 373 (Table 2.16). The raw extract (MeOH-DCM, 1:1) of the leaves of this plant killed 50% of two lines of glioma cells, Hs 683 and U 373, at respective concentrations of 68 µg/mL and 85 µg/mL. The trunk bark was the least active part of *P. erinaceus*: an IC_{50} was obtained only on Hs 683 at 71 µg/mL.

Table 2.16: Anti-cancer activities of the raw extracts (MeOH-DCM) of *P. erinaceus* parts and raw extracts (MeOH) of *D. oliveri* parts

Anti-cancer activity is based on the determination of the relevant IC_{50} (concentration that kills 50% of cancer cells) values in µg/mL.
1, **2** and **3**: methanol-dichloromethane raw extract of respectively the leaves, the trunk barks and the roots of *P. erinaceus*. **22**: methanolic raw extract of the trunk barks of *D. oliveri*. IC_{50} values are in bold.
n.o: no IC_{50} obtained at the highest concentration (100 µg/mL) tested.

	Hs683	U373	SKMEL28	A549	MDA-MB231
1	**68**	**85**	no	no	no
2	**71**	no	no	no	no
3	**54**	**28**	**55**	**51**	**45.93**
22	**91**	no	no	no	no

5.4. Tests against nematodes

No activity was observed with any of the raw extracts and the subsequent fractions at a concentration less or equal to 10 mg/mL. The nematodes were still alive at the tested concentrations with either the raw extracts or the fractions obtained from them.

5.5. Cytotoxicity tests

Unlike the antibacterial and antifungal tests, the determination of the IC_{50} was very difficult as illustrated by the standard deviation obtained for some extracts. In general, no toxicity was observed with most of the raw extracts and fractions at the highest concentration tested (256 µg/mL) (Tables 2.17 and 2.18).

In the case of *P. erinaceus*, an IC_{50} was observed only with the butanol fraction of the trunk barks at 78.06 µg/mL and the dichloromethane fraction of the roots at 190.8 µg/mL against MRC 5 cells (Table 2.17).

Table 2.17: Toxicity of the most effective raw extracts and fractions of *P. erinaceus* parts on MRC 5 cell lines.

1, **2** and **3**: leaves, trunk barks and roots of *P. erinaceus*. Raw extract: methanol-dichloromethane (MeOH-DCM, 1:1). **1'**, **2'**, **3'**: fractions derived from the leaves (1), trunk barks (2) and roots (3), respectively.

Cytotoxicity has been determined as IC_{50} (concentration that kills 50% of cells) expressed in µg/mL. n.t: no toxicity observed at the highest concentration tested (256 µg/mL).

IC_{50} is marked in 'bold'. Tests have repeated three times (n=3)

	IC_{50}
1-MeOH	n.t
2-MeoH	n.t
2'- Ethyl acetate	n.t
2'-Butanol	**78.06 ±0.26**
2'-Water	n.t
3-MeOH	n.t
3'-Dichloromethane	**190.8 ± 0.22**
3'-Acetate	n.t
3'-Butanol	n.t
3'- Water n.t	

More IC_{50} values were obtained with *D. Oliveri* fractions: 5 fractions exhibited some toxicity against MRC 5 cells in comparison to *P. erinaceus* where only 2 fractions were somehow toxic (Tables 2.17 and 2.18). The petroleum ether, butanol and water fractions of the trunk barks of *D. oliveri* showed an IC_{50} respectively at 198.02 µg/mL, 81.18 µg/mL and 76.96 µg/mL. The dichloromethane and final methanolic fraction of the roots also exhibited an IC_{50} respectively at 161.15 µg/mL and 210.67 µg/mL (Table 2.18). To get a better understanding and implication of these toxicity values, some comparison needs to be done with the respective antibacterial, anti-fungal or anti-cancer activity observed with the different active extracts fractions (Tables 2.17 and 2.18).

Table 2.18: Toxicity of the most effective raw extracts and fractions of *D. oliveri* parts on MRC 5 cell lines.

21, **22** and **23**: leaves, trunk barks and roots of *D. oliveri*. Raw extract: methanol (MeOH). **21**', **22'**, **23'**: fractions derived from the leaves (1); trunk barks (2) and roots (3), respectively. Cytotoxicity has been determined as IC_{50} (concentration that kills 50% of cells) expressed in µg/mL. n.t: no toxicity observed at the highest concentration tested (256 µg/mL). **IC_{50} is marked in 'bold'**. Tests have repeated three times (n=3)

	IC_{50}
21-MeOH	n.t
21'-Butanol	n.t
21'-Water	n.t
22-MeOH	n.t
22'- Petroleum ether	**198.02 ± 0.19**
22'- Ethyl acetate	n.t
22'- Butanol	**81.18 ± 0.07**
22'- Water	**76.96 ± 0.06**
23-MeOH	n.t
23'- Dichloromethane	**161.15 ± 0.18**
23-MeOH final	**210.67 ± 0.23**

6. Discussion

6.1. Antibacterial and cytotoxicity tests

6.1.1. *Pterocarpus erinaceus*

The results obtained for the various extracts and fractions of the different parts of *P. erinaceus* confirm our preliminary studies conducted a few years ago (Tittikpina *et al*., 2013). Indeed, a MIC value of 1.88 mg/mL against *S. aureus* was obtained from the ethyl acetate fraction of the trunk barks of *P. erinaceus* as part of that earlier work. This work used the spread on agar method to evaluate the antibacterial activities which probably explained the differences in MIC values obtained.

Following the procedure described in the flow chart (Figure 2.8), toxicity tests were carried out for the most active fractions (*i.e.* ethyl acetate, butanol and water fractions) and on the whole extracts of the trunk barks, with the aim to rule out any major, negative effects on normal human cells. In sharp contrast to the activity observed against bacteria, for most of the extracts and fractions except the butanol fraction of the trunk barks no significant toxicity was found against normal human cells at corresponding active concentrations (Table 2.19). These findings agree well with previously performed intraperitoneal and oral toxicity tests with the ethanolic extract of the trunk barks of this plant in albino Wistar rats (Salawu *et al*., 2008). The trunk barks, therefore, appear to be active against various bacteria without being toxic to normal human cells used or when administrated orally to rats. Therefore, the ethyl acetate and water fractions were rather amenable to further investigation (as described in Figure 2.8). In contrast, the toxicity of the butanol fraction is not negligible, with an IC_{50} of 78 µg/mL in human cells, compared to a MIC varying form 32 µg/mL to 256 µg/mL in bacteria.

In summary, the trunk barks of *P. erinaceus* (especially the ethyl acetate and water fractions of its whole extract) and to a certain extent the butanol fractions, are active against bacteria without being overly toxic to normal human cells. Therefore, these three fractions should be the ones to be considered for any analytical investigation to decipher the constituent compounds.

6.1.2. *Daniellia oliveri*

The activity observed on *S. aureus* with raw extract from different parts and fractions confirmed the preliminary results previously obtained with a MIC mainly in the range of 1.88 mg/mL to 7.5 mg/mL, the difference due to the methodology used to run the tests (Tittikpina *et al*., 2013). Another study has also confirmed part of the results obtained in the present

work. The study investigated the antibacterial activity of the leaves using the agar well diffusion assay. The leaves presented a higher activity against *S. aureus* in comparison to *P. aeruginosa* and *E. coli* at concentrations from 50 mg/mL to 5 mg/mL; the butanol fraction was also the most active of the leave fractions (Ahmadu *et al*., 2004 and Ahmadu *et al*., 2007). Other studies have also investigated the activity of the leaves and trunk barks using the same method as the precedent study. The researchers demonstrated an inhibition of bacterial growth at 60 mg/mL with the ethanolic extract of the leaves and trunk barks of *D. oliveri* (Temitope *et al*., 2012). El-Mahmood *et al*. 2008 have also evaluated the antibacterial activities of *D. oliveri* leaves, barks and roots ethanolic and water extracts. MIC values ranging from 6.25 mg/mL to 100 mg/mL were obtained with the extracts against *E. coli*, *S. aureus*, *K. pneumoniae* and *S. dysenteriae.*

Just like in the case of *P. erinaceus*, following the procedure described in Figure 2.8, toxicity tests were performed on the most effective raw extracts and fractions. In general, no toxicity was observed with the most active raw extracts and fractions at concentrations at which they are active, except with the butanol and water fractions of the trunk barks. Indeed, with a respective IC_{50} of 81.18 and 76.96 µg/mL for those butanol and water fractions and with MIC ranging from 64 µg/mL to 256 µg/mL, the toxicity is not negligible and some more studies are consequently needed. Some other researchers have demonstrated the trunk bark to be not toxic when administrated orally in mice with LD 50 greater than 3.5 g/kg (Kabore *et al*., 2010).

In conclusion with *D. oliveri*, the following fractions are the most active ones, needing further chemical investigation: petroleum ether, butanol and water fractions of the leaves; petroleum ether, dichloromethane, ethyl acetate, butanol and water fractions of the trunk barks; the dichloromethane, the ethyl acetate and the final methanolic fraction of the roots.

6.1.3. General

Overall, the different extracts and their subsequent fractions showed a very good activity against the seven bacteria on which the tests were run. The extracts have MIC ranging from 16 µg/mL (the lowest) to 256 µg/mL (the highest which is also the highest concentration of extract tested). *D oliveri* appears to be the most active plant against bacteria, considering its number of active extracts and fractions, 55 MIC values against 34 MIC values for *P. erinaceus*. Indeed, *D. oliveri* has its leaves, barks, and roots which have shown almost always a MIC on the 7 bacteria: 10 MIC values for the roots, 20 MIC values for the leaves and 25

MIC values with the trunk barks. With *P. erinaceus*, MICs were more concentrated on its barks with 25 MIC values for its trunk barks, 2 MIC values for its leaves and 7 MIC values for its roots. The methanol extract of the trunk barks of *D. oliveri* showed the best MIC of the antibacterial activity recorded: 16 µg/mL on *E. faecalis*. On the same bacteria, the methanol extract of the bark of *P. erinaceus* has a MIC of 32 µg/mL. Nevertheless, a difference of one dilution is not so significant. And consequently, one could conclude that the trunk barks of the two plants bear the same importance.

In conclusion, *D. oliveri* is far more active on bacteria than *P. erinaceus*. This not surprising. Indeed, the CAPITURE method has predicted this species to be active against intertrigo that is caused by *S. aureus*, *P. aeruginosa* and *C. albicans*. The method has also predicted *P. erinaceus* to be active against ringworm (caused by a fungus, *Trycophyton rubrum*) and *C. albicans*. When combining these two predictions, more anti-fungal activity should be obtained with *P. erinaceus* and more anti-bacterial activity with *D. oliveri*. The antibacterial activity preponderance of *D. oliveri* is confirmed through the antibacterial testing while the anti-fungal activity preponderance of *P. erinaceus* is also confirmed through the previously described anti-fungal testing.

6.2. Antifungal and cytotoxicity results

The test was first performed on the raw extract and in the second step, it was run on the fractions of the raw extract that gave a good activity. As observed during the anti-bacterial assays, the fractions from raw extracts that did not present very good activities also did not present good activities. For example, the raw extract of the leaves of *P. erinaceus* did not present a MIC less than 256 µg/mL on the 7 types of bacteria used in this study. Consequently, only the ethyl acetate fraction of the leaves was active on two germs (of the 7) and with a MIC of 256 µg/mL which appears not to be of interest compared to other fractions which have a MIC of 64 µg/mL.

As a global result, the raw extracts did not show a good activity on the yeast and the fungi except for one, the roots of *P. erinaceus*. The methanol-dichloromethane extract of the roots of *P. erinaceus* (3) was less active on the seven bacteria tested (no MIC). However, it showed the highest activity on a fungus, *Aspergillus niger*, with a MIC equal to 16 µg/mL, the best MIC of all the MIC obtained with the two plants extracts on all bacteria and fungi. The fractions obtained from this raw extract were also tested. The dichloromethane and ethyl

acetate fractions also showed very good activities, respectively a MIC of 64 µg/mL and 32 µg/mL that are not so much different from 16 µg/mL considering the high precision and the repeatability of the method used to run the tests. The good activity of the non-polar fractions coming from the methanol extract suggests that the active principles that originate the activity will be more non-polar molecules rather than polar ones.

D. oliveri did not show activity on the fungi at 256 µg/mL and the two plants were not active against *C. albicans* at 256 µg/mL. However, earlier studies have shown activity against fungi and yeast at higher concentrations. According to one of those studies, the methanol extract of the leaves and the methanol and water extracts of the trunk barks were active against *Aspergillus niger, Candida albicans, Candida krusei, Rhizopus stolonifer, Epidermophyton floccosum, Trichophyton floccosum, Trichophyton interdigitale and Trichophyton rubrum* with MIC from 3.125 to 200 mg/mL (Onwukaeme, 1995). Previous study has shown an activity on *C. albicans* of the leaves, trunk barks and roots of *P. erinaceus* with MIC ranging from 1.875 mg/mL to 15 mg/mL with the leaves being the most active. The same study also showed the activity of *D. oliveri* leaves, trunk barks and roots with MIC ranging from 1.875 mg/mL to 30 mg/mL with the most active plant being the trunk barks (MIC: 1.875 mg/mL) (Tittikpina *et al*., 2013).

The active extracts and fractions: raw extract of the roots of *P. erinaceus*, dichloromethane and ethyl acetate fractions of this raw extract were subsequently tested on MRC 5 cells to see whether they were toxic to normal human cells lines. All of them were not toxic at the highest concentration tested 256 µg/mL for a MIC belonging to the range 16 µg/mL to 64 µg/mL, except the dichloromethane fraction that presented an IC_{50} at 190.8 µg/mL. Nevertheless, with an IC_{50} at 190.8 µg/mL compared to a MIC of 64 µg/mL, the toxicity is almost inexistent.

6.3. Anti-cancer and cytotoxicity tests

The roots of *P erinaceus* seem to be the most active on the cancer cell lines with IC_{50} ranging from 28 µg/mL to 55 µg/mL. The leaves were active against two cancer lines and the trunk barks only against one cancer line. Notably, all the parts were always active against the glioma cells (Hs 683 and U 373). Besides, the lowest IC_{50} was observed with the roots against U 373 at 28 µg/mL. It could, therefore, be of interest just like in the case of antifungal, anti-bacterial and anti-nematicidal tests, to also test the fractions coming from the raw extract of

the active parts and investigate further the preferential activity on glioma cells. The raw extracts of the roots, leaves and trunk barks did also not exhibit any toxicity on MRC 5 cells at 256 µg/mL. This plant part is therefore active on cancer cells lines without being toxic to normal human cells. It is also the case with the leaves of *D. oliveri* that was active on Hs 683 at the highest IC_{50} observed (91 µg/mL) without being toxic at the highest concentration tested (256 µg/mL).

6.4. Nematicidal activities

The two plants did not show an activity on the nematode *Steinernema feltiae* at concentrations ≤ 10 mg/mL. The results are not surprising because traditional healers did not suggest the use of the two plants to cure parasitosis. The activity may be present but at concentration higher than 10 mg/mL. This may explain the use of the leaves of these two plants as anti-nematicidal in cattle. Considering the huge amount of leaves eaten by cattle per day, the activity could be present at higher concentrations.

6.5. Cytotoxicity tests

Most of the raw extracts and their subsequent fractions were not toxic at the concentration at which they are active. Based on this result, one may conclude that the two plants are not toxic to human beings. But, a deeper check needs to be run as toxicity is caused and implied by and in different processes.

7. Conclusions

Altogether, these bio-assays have provided a global view on the biological activities of the two plants. *P. erinaceus* and *D. oliveri* are active against bacteria, fungi and cancer cells with very interesting MIC values from 16 µg/mL to 256 µg/mL and IC_{50} values from 28 µg/mL to 91 µg/mL. The results confirm the use of these two plants in folk medicine in Togo for the treatment of infectious cutaneous diseases. Besides, interestingly, the plants have also exhibited a non-recorded traditional activity, an anti-cancer activity. Importantly, the results obtained have confirmed the predictions of the CAPITURE method. Indeed, this method has predicted that *P. erinaceus* was especially active against fungi and *D. oliveri* against bacteria and fungi. Without any surprise, *D. oliveri* presented the best activity on bacteria (MIC at 16

µg/mL) and the greatest number of active extracts in addition to an activity against *Candida albicans* (MIC: 1.88-30 mg/mL). *P. erinaceus* presented the best activity against fungi (MIC at 16 µg/mL on *Aspergillus niger*) in addition to a very good activity against bacteria. *P. erinaceus* also presented an activity against *C. albicans* (1.88 mg/mL – 30 mg/mL). The next step will, therefore, be to find the active principles that elicited the activities with a view to performing bio-assays on those compounds. This methodology involves bio-guided strategy of studying the chemical components of the two plants as summarised in Figure 2.8.

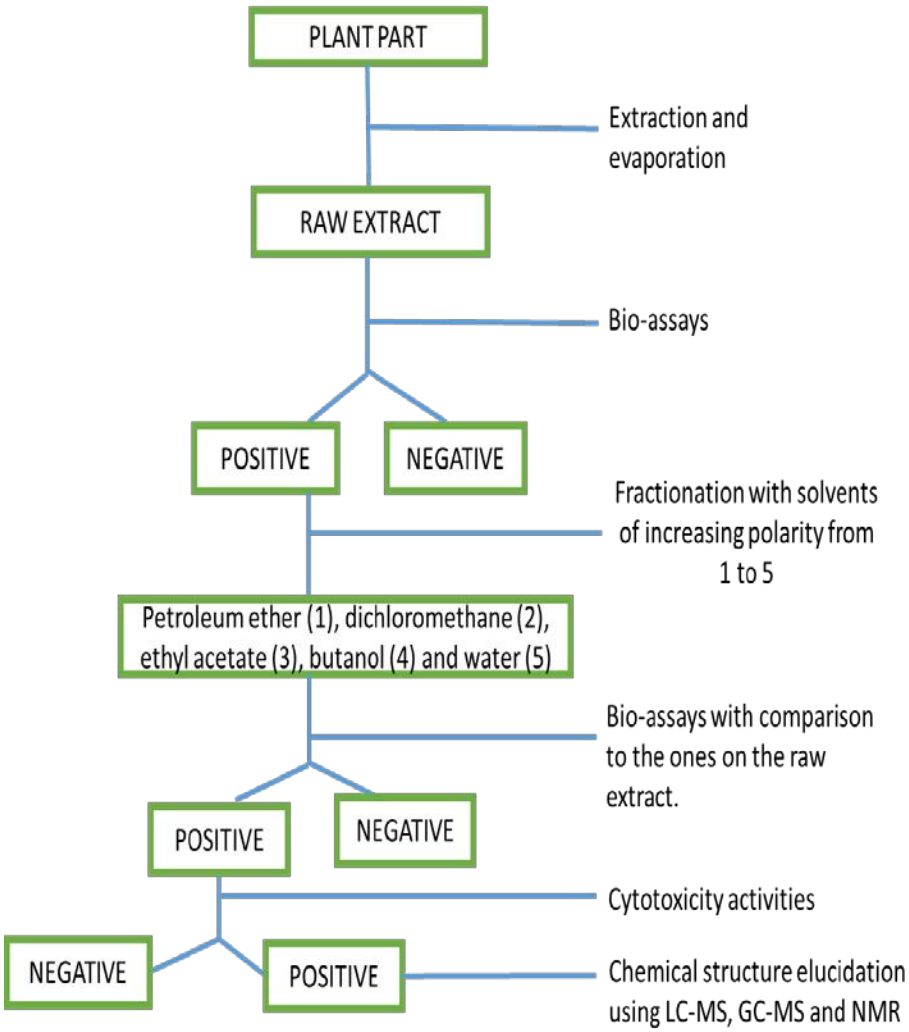


Figure 2.8: Flowchart of the bio-guided isolation to be run on the two plants

Raw extract: methanolic-dichloromethane (MeOH-DCM, 1:1) or methanolic (MeOH) extract. Antibacterial activity - Positive: activity observed against microorganisms; Negative: no activity observed against microorganisms. Cytotoxic activities – Positive: not toxic to normal human MRC-5 cells; Negative: toxic to MRC-5 cells.

Chapter 3: Analytical studies on the most biologically active extracts of *Pterocarpus erinaceus* and *Daniellia oliveri*

1. Introduction

Analytical chemistry tools can be subdivided roughly into two main groups. The first group includes separation methods whose ultimate goal is the isolation of the desired active molecules while the second comprises characterisation techniques which enable the determination of the chemical structures of the active compounds. The analytical part of this work involves using these different methods to unveil the compounds that may be responsible for the biological activities observed with the most active fractions and/or extracts. From the raw materials to the active molecules, different steps were followed during the analytical part of the work (Figure 3.1):

- extraction was performed on the raw material to obtain a crude extract.
- at least one clean-up procedure was completed on the crude extracts to obtain purified extracts (meaning here refined, not necessarily pure) or fractions on which a qualitative analysis was carried out generally using separation methods (gas and/or liquid chromatography) coupled to mass spectrometry (GC-MS and/or LC-MS)
- the ultimate step involved isolation or purification of the fractions (or refined extracts) using chromatographic techniques such as Low-Pressure Liquid Chromatography (LPLC); Flash Chromatography (FC) and Thin Layer Chromatography (TLC), to obtain pure molecules. In order to characterize and identify the purified molecules, qualitative analyses are then performed on the isolated compounds using first and second dimension Nuclear Magnetic Resonance (NMR 1D or 2D) and mass spectrometry (including High-Resolution Mass Spectrometry HRMS). Following the characterization, complementary information on the compound is obtained by Ultra Violet (UV) and Infrared (IR) spectra and melting points.

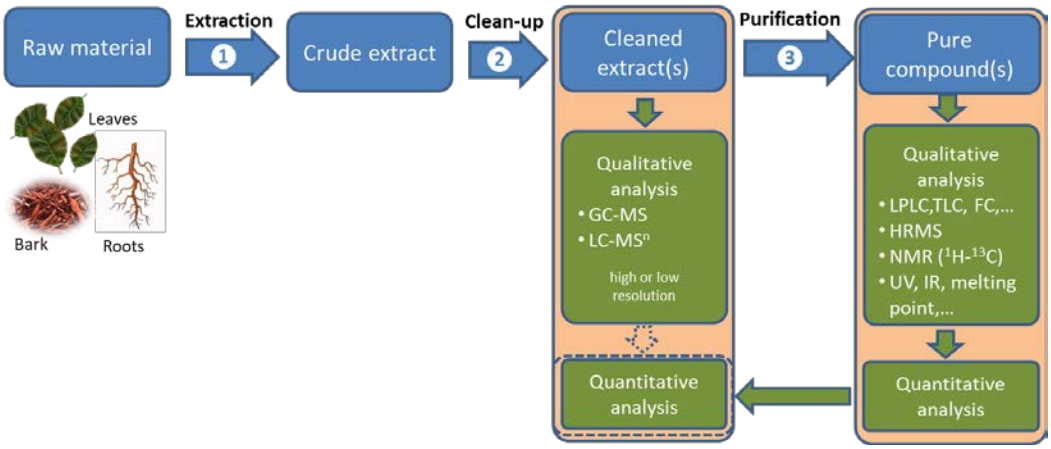


Figure 3.1: Chart of the work performed on the two plants in the analytical part

The raw material could either be the leaves, the trunk barks or the roots of a plant. The crude extract is also the raw extract and the cleaned extract is also the fraction. Pure molecules are pure compounds obtained after isolation and purification processes on the fractions or crude extracts. GC-MS and LC-MS are gas and liquid chromatography coupled to mass spectrometry. Low-Pressure Liquid Chromatography (LPLC); Flash Chromatography (FC), Thin Layer Chromatography (TLC).

Additionally, a quantitative analysis could also be run at each step in case of available standard (commercial or already purified from the plant or another plant known to contain the studied compound). Thus after identification, a formal quantification of some already known compounds can be carried out on the fraction. The pure compounds obtained could also be used to quantify their presence in the extract or the fraction from which they are obtained.

P. erinaceus was extensively studied in comparison to *D. oliveri*, considering the fact that it was the plant with the wider range of activities (antibacterial, anti-fungal and anti-cancer). On *D. oliveri*, an identification work was carried out on the apolar fractions. In the following sections, the different methods used at every step will be described followed by the presentation of the results obtained. A later formal discussion will explain the results obtained.

2. Extraction

Extraction is a technique of separation. It involves transferring components from one phase to another: from a liquid phase to another liquid phase (liquid-liquid extraction) or from a solid phase to a liquid one (solid-liquid extraction). Extraction from plants is very complex considering, the huge number of compounds present in them. There are therefore different

extraction techniques depending on the types of compounds to be extracted and the means of extraction. The extraction phase is the most important when dealing with identification of phytochemicals, not only because it is the first step but also it conditions and influences further works. To extract compounds from plants, there are two types of extraction: conventional extraction methods and modern or improved extraction methods. The conventional methods include maceration, hydrodistillation and Soxhlet extraction while the modern ones include Ultrasound-assisted extraction (UAE), Enzyme-assisted extraction (EAE), Microwave-assisted extraction (MAE) , Pulsed electric field assisted extraction (PEAE), Supercritical fluid extraction (SFE), Pressurized liquid extraction (PLE) or Accelerated Solvent Extraction (ASE) (Delazar *et al*., 2012; Puri *et al*., 2012; Azmir *et al*., 2013; Pico, 2013; Chaimbault, 2014).

The improved extraction methods present more advantages compared to the conventional ones. Indeed, they save time and the quantity of solvent to be used; they give better yields in comparison to conventional methods and finally, they allow to focus on a particular type of compounds. However, their main disadvantages are the high prices of the material and the prior knowledge sometimes needed on the compounds to be extracted. For example in the case of enzymatic extraction, it is important to understand their catalytic property and mode of action, the optimal operational conditions and which enzyme or enzyme combination is appropriate for the plant material selected. It is also compulsory to have a prior knowledge of the cell wall composition of the raw material. The use of modern methods is consequently of help in the case of pre-analytical treatment (removal of some type of compounds, preparation to chromatography, etc) and/or analytical control of herbal products. For example, UAE has been used to extract tirucallane-like triterpenes from *Juliana adstringens* (Anacardiaceae) and glycosylated phenolic antioxidants such as myricitrin-3-rhamnoside from *Acacia confusa* flowers and buds (Puri *et al*., 2012; Delazar *et al*., 2012; Alupui *et al*., 2012; Ibañez *et al*., 2012; Tang *et al*., 2012; Azmir *et al*., 2013; Pico, 2013; Bucar *et al*., 2013).

Based on this fact, modrn methods are not used when dealing with unknown plants from the folk medicine. The conventional methods are the preferably used methods in that case with maceration being the first to be considered. Indeed, a lot of studies have proven that better results are obtained with maceration than with any other methods. For example, the maximum amount of total flavonoids and total phenolics compounds was obtained from the leaves of *Sideritis* spp in comparison to ultra-sound assisted and microwave-assisted extraction methods (Alipieva *et al*., 2010). In another study, the extracts obtained by maceration of the

leaves of *Vernonia amygdalina* D. and *Occimum gratissimum* L. showed a better hypoglycemic activity in rats in comparison to the extracts obtained by MAE (Okoduwa *et al*., 2016). Maceration is also the method used by traditional healers (TH). The most used forms reported during the ethnobotanical survey was maceration in water or a decoction in hot water. Based on these reasons, the maceration procedure was chosen to prepare the crude extracts of the different parts (leaves, trunk barks, and roots) of the two plants.

2.1. Maceration

Maceration is an age long practice in many homes. For example, coffee and tea preparations are types of maceration. Maceration consists of 3 steps: the material to be extracted is dried and grounded into small particles to increase its contact area with the solvent and put in a closed vessel. Secondly, the solvent to be used is added to this vessel and mixed. The vessel is closed for a certain time (depending on the wanted outcome). Thirdly and lastly, the liquid is recovered and the solid residue (marc) is pressed and sometimes washed again with the same solvent to recover, as much as possible, large amounts of the occluded solutions. The recovered solutions are added to the liquid and filtred using filtration paper and or Buchner filter. Occasional shaking in maceration facilitate extraction by two ways; (a) increase diffusion, (b) remove the concentrated solution from the sample surface by bringing new solvent to the menstruum for more extraction yield (Azmir *et al*., 2013).

2.2. Maceration run on *Pterocarpus erinaceus* and *Daniellia oliveri*

To initiate a maceration, one needs to choose the best solvents. The procedure used to choose the solvents is fully described in section 2. Extraction and fractionation of chapter 2 of this work. The extraction was run on *P. erinaceus* parts with a mixture of MeOH and DCM (1:1) and on *D. oliveri* with MeOH (100%) using the liquid-solid procedure (liquid being the solvent and the solid, the raw material). Biological tests were run with those crude extracts as described in Chapter 2.

2.3. Other types of conventional methods that could be easily run in developing countries

As mentioned in the precedent lines, because of their price, modern methods of separation are not most of the time available, especially in developing countries.

- **Soxhlet extraction**

Soxhlet extractor was first proposed by German chemist Franz Ritter Von Soxhlet (1879). It was designed mainly for extraction of lipid but now it is not limited to this only. It is composed of 3 parts: a thimble to contain the sample to be extracted, a percolator where the solvent circulates and a siphon mechanism which empties the thimble periodically. The Soxhlet extraction has widely been used for extracting valuable bioactive compounds from various natural sources. A small amount of dry sample is placed in a thimble. The thimble is then placed in distillation flask which contains the solvent of particular interest. After reaching to an overflow level, the solution of the thimble holder is aspirated by a siphon. Siphon unloads the solution back into the distillation flask. This solution carries extracted solutes into the bulk liquid. The solute remains in the distillation flask and solvent passes back to the solid bed of plant. The process runs repeatedly until the extraction is completed (Azmir *et al*., 2013). Some studies have revealed this method to be the best one for extraction on some plants. For example, the soxhlet was found to yield better extraction with methanol in phenolic compounds in *Osbeckia parvifolia* L., in comparison to the methanolic maceration procedure (Rajan Murugan and Thangaraj Parimelazhagan, 2014). It was the same with the extraction of protocatechuic, p-hydroxy-benzoic, vanillic, and ferulic acids in *Sambucus nigra* L. inflorescence (Waksmundza-Hainos *et al*., 2007).

- **Hydrodistillation**

Distillation is a chemical process during which different liquids present in a mixture could be separated from each other using temperature and based on the differences in their boiling points. Volatile oils from natural products are still mainly obtained by this process although the high temperature could lead to changes in the structure of the chemical components.

In hydrodistillation (using of water), firstly, the plant materials are packed in a still compartment. Secondly, water is added in sufficient amount and then brought to boil. Alternatively, direct steam is injected into the plant sample. Hot water and steam act as the main influential factors to free bioactive compounds of plant tissue. Indirect cooling by water condenses the vapor mixture of water and oil. Condensed mixture flows from condenser to a separator, where oil and bioactive compounds separate automatically from the water (Silva *et al*., 2005; Bucar *et al*., 2013). This method is still of choice because of its low cost even if better results are obtained with some of the modern methods (Gachkar *et al*., 2007; Okoh *et al*., 2010)

2.4. Summarised presentation of some of the modern methods of extraction

- **Ultrasound-assisted extraction (UAE)**

Ultrasound is used to increase contact between solid and solvent due to an increase of pressure which favors penetration and transport, and temperature which improves solubility and diffusivity. However, in UAE, the extraction is still time-consuming. Anthraquinones from the roots of *Morinda citrifolia* L. was realized using UAE and yielded better results in comparison to conventional methods (Surasak *et al*., 2006).

- **Enzyme-assisted extraction (EAE)**

Various enzymes such as cellulases, pectinases, and hemicellulase are used to hydrolyze cell wall components thereby increasing cell wall permeability and enhancing the extraction of the chemical from plants. It consequently results in higher extraction yields of bioactive compounds. Polysaccharides from the fruiting body of *Tricholoma matsutake* L. was extracted using this method (Xiulian *et al*., 2011).

- **Microwave-assisted extraction (MAE)**

Microwaves are used for extraction and they allow the following: the desorption of solutes from the various active sites in the sample matrix under the pressurized and elevated temperature conditions, the diffusion of extraction fluid into the matrix and the partition of the solutes from the sample matrix into the extraction fluid. Extraction of Artemisinin from the leaves of *Artemissia annua* L using this method yielded better results in comparison to Soxhlet method and normal stirring extraction (Jin-yu *et al*., 2002).

- **Pulsed electric field assisted extraction (PEAE)**

The principle is to destroy cell membrane structure by using an electrical field with the aim of increasing extraction. Anthocyanins from the purple-fleshed potatoes were extracted using PEAE (Puértolas *et al*., 2013).

- **Supercritical fluid extraction (SFE)**

Extraction is done with a supercritical fluid (most of the time CO_2 but also subcritical water) to take advantage of the gas-like properties of diffusion, viscosity, and surface tension, and liquid-like density and solvation power of such substance. Better results with the extraction are therefore obtained. For example, the method yielded the best results in the extraction of di-caffeoylquinic acids from a plant in comparison to MAE and PEAE (Gourguillon *et al*., 2016).

- **Pressurized liquid extraction (PLE)**

This extraction allows the use of a high pressure with high temperatures, to avoid boiling of the solvent used for extraction. The high temperature permits a better dissolution of the compounds in the solvent, permitting high rates of extraction. Studies have shown that this method yielded higher values for total oil content, oxysterols in food and carotenoids in plants (Boselli *et al*., 2001 and Denery *et al*., 2004; Mulbry *et al*., 2009).

3. Clean-up: fractionation

The original crude extract is often a very complex sample containing a huge number of different compounds. Thus it is difficult to characterize its composition and moreover, it is impossible to figure out which molecule(s) is (are) responsible of a given therapeutic effect. The clean-up run was done to obtain refined (simplified) extracts from the original crude one. To achieve this goal, fractionation which is a liquid-liquid or a liquid-solid extraction was used. The idea is to separate compounds present in the raw or crude extract according to their polarities and subsequently their affinities with a particular solvent *i.e* polar compounds will be attracted by polar solvents and non-polar ones will also be attracted by non-polar solvents. All the raw extracts obtained were soluble in water except the roots of *D. oliveri*. Consequently, the crude dry extracts were dissolved in water and their fractions obtained by a liquid-liquid partition with petroleum ether, dichloromethane, ethyl acetate, and butanol. At the end of the partition, the remaining water fraction was obtained. With the roots of *D. oliveri*, the different solvents were successively poured on the crude dry extract for 24 to 48 h and recovered after this period. This gave only petroleum ether, dichloromethane, ethyl acetate, butanol and a remaining methanolic fraction.

4. Qualitative analysis of the cleaned-up extracts

The aim of this analysis was to realize an *in-situ* identification of compounds in the cleaned-up extracts or fractions. Primarily the analysis was realized using gas chromatography coupled to mass spectrometry and liquid chromatography coupled to mass spectrometry.

The name ‘chromatography’ comes from 2 Greek words ‘chroma’ which means ‘colour’ and ‘graphein’ meaning ‘to write’ which means separate colors. When chromatography is used in agreement with the definition, it is a separation method. It could also be used as a purification process and in that case, it intends to bring out pure compounds. Additionally, the method can

also be used as an analytical method to aid in compounds identification. Sometimes, it helps in between the purification and identification processes with the running of preparative chromatography, designated to separate an extract in further refined parts.

4.1. Gas chromatography (GC)

The Gas chromatography is a column and an elution chromatography: the stationary phase is coated on a column through which all the separated substances must travel before being detected at its end. It allows the separation of mixtures existing in gaseous forms or which could be converted into gas by elevating the temperature. The GC is run in a way so such the high temperature will not destroy substances as much as possible. A GC apparatus is usually composed of: the carrier gas, the injector chamber, the column in the column incubator and the detector.

- **The carrier gas**

The carrier gas to be used should respect certain conditions: high purity, inert (unable to interact chemically with the sample); very low viscosity, compatible with the detection system. The most used ones are nitrogen; helium and hydrogen. Helium was the carrier gas used in the present work because of the internal diameter of the column (250 μm) and its compatibility with a mass spectrometric detector (MSD).

- **The sample injector and injection**

The injection chamber has 2 functions: instantaneous volatilization of the sample and homogeneous mixture of the formed vapor and the carrier gas. The sample solution to study has to be diluted and the injection is done quickly so that peaks enlargement is avoided. In the present study, the injector was a split/splitless injector which is one of the most common types of injectors. It is perfectly compatible with a screening analysis as it allows the regulation of the amount injected onto the columns (splitless for diluted samples, split with a variable ratio for concentrated samples).

- **The column incubator**

Its aim is to brings or/and maintains the column to the desired temperature. To have a homogenous temperature, the incubator has a great capacity and a ventilation cooling system. A programming system is usually incorporated to allow a time-dependent increase of the temperature during a chromatography, allowing the simultaneous separation of high and low volatile compounds in the same run.

- **The column**

The column is a narrow tube containing the stationary phase. Most of the analytical columns used are made of a long tube (several tens of meters) in fused silica glass, coated with a thin layer of stationary phase. They are named Wall Coated Open Tubular) and are the most used in natural products research.

Concerning the stationary phases, there are lots of them: from very apolar ones like 5%phenyl-95% methyl siloxane to very polar ones like Poly-ethylene-Glycol (PEG). In this work, the first one was used because of its versatility and applicability at very high temperatures to allow the elution of fewer volatiles compounds such as sterols and pentacyclic triterpenes.

- **The detector**

The historic ones are: Flame ionization detector (FID), thermal conductivity detector (TCD), Nitrogen phosphorus detector (NPD), Flame photometric detector (FPD), Photoionization detector (PID), Electrolytic conductivity detector (ELCD), Atomic emission detector (AED). They are supplemented nowadays by the mass spectrometric detectors (which we have used in our work). For the latter ones, the different types of mass spectrometric analyzers that exist could be coupled to a GC, including the hybrid and high-resolution analyzers (see Section 4.3 of this chapter on mass spectrometry) are used. In this work, we were in need of a detector that could give us structural information, consequently mass spectrometry was the best choice. Indeed, they furnish accurate and precise result when coupled with an existing library. A quadrupole mass spectrometric analyzer was the one used in the present work.

4.2. Liquid chromatography (LC)

Liquid chromatography is a column chromatography. The older version requires filling a column with a stationary phase and using the gravity to let the mobile phase (MP) flow through it. Nowadays, the mobile phase could flow through the stationary phase (SP) using various methods. SP with thinner and regular granulometry could also be obtained (some micrometers). This type of SP increases the exchange surface and the resolution of the chromatography. Although there are different types of liquid chromatography, the ones used in this part of the work was the High-Performance Liquid Chromatography (HPLC). The equpment requires a high pressure whch is applied onto the MP when it flows through the column. In comparison to GC, the carrier gas in the HPLC is replaced by a solvent reservoir,

with the presence of a high-pressure pump. Like the GC, HPLC has an injector, a column, and detectors.

The pressure applied by the high-pressure pump is greater than 24 Mpa. The solvent reservoirs contain the same solvent or different solvents that could be delivered to obtain a mixed solvent in the mixing vessel for delivery of the MP, by acting on the gradient valve. The solvent reservoirs are of small capacity (most of the time 1 to 2 L, a small quantity of MP (20 or 50 mL) is also needed for the chromatography. The solvent reservoirs have to be waterproof (to avoid solvent evaporation and or contamination by atmospheric water) and are sometimes equipped with devices (bubbling with nitrogen or magnetic stirring) that are intended to remove dissolved gases, especially oxygen.

The HPLC could either be used as a purification process in which case it is called a preparative HPLC. When it is used to identify compounds as done in this part of the chapter, it is an analytical HPLC.

- **Column**

Columns used in HPLC are usually short and straight. The diameters are 1; 4.6 and 8 mm depending on the type of chromatography used. 1 mm for microflow chromatography; 4.6 mm for classical analytical chromatography and 8 mm for semi-preparative chromatography.

There are different types of SP used in HPLC, the most common are surely the reverse phase. This phase is preferred in phytochemical screening because it is versatile (able to retain polar and apolar compounds) when it is used in elution gradient. And, it could also support the injection of complex samples (mixtures of thousands of compounds) as could be a plant extract. It allows to have a first overview of the polarity of compounds present in an extract and depending on the result observed, another type of phase or column could be used such as hydrophilic interaction chromatography (HILIC) or di-dimensional chromatography.

- **Detection or detectors**

The detection is realized using different methods such as UV-Vis, fluorescence, electrochemical detection, refractive index (RID) or evaporative light detection (ELSD) in the case of absence of chromophore and many other methods among which mass spectrometry. The ones used in this work are the UV absorption and above all the mass spectrometry because of its sensitivity and the structural information provided.

4.3. Mass spectrometry

4.3.1. Definition

A mass spectrometer determines the mass-to-charge (*m/z*) ratio or a property related to *m/z* of a compound. A mass spectrum is a plot of ion abundance (or ion currents) versus *m/z*. The spectrum is presented in terms of Daltons (Da) per unit charge. The relative abundance of isotopologs helps to decide which elements contribute to a formula of a compound and to estimate the number of atoms of a contributing element. Under the conditions of certain mass spectrometric experiments, fragmentation of ions can provide structural information on the compounds of interest. Thus, the MS elucidates the connectivity of atoms within smaller molecules, identifies functional groups, determines the (average) number and eventually the sequence of constituents of macromolecules and in some cases even yields their three-dimensional structure (for proteins for example) (Gross, 2011; Glish and Vachet, 2003). Mass spectrometry as seen in the precedent lines is a detection tool that could be coupled with the various chromatographic methods studied. A spectrometric apparatus is usually composed of the following parts: an ion source composed by an introduction chamber and an ionization chamber; an analyzer and the collector-recorder part (Figure 3.2).

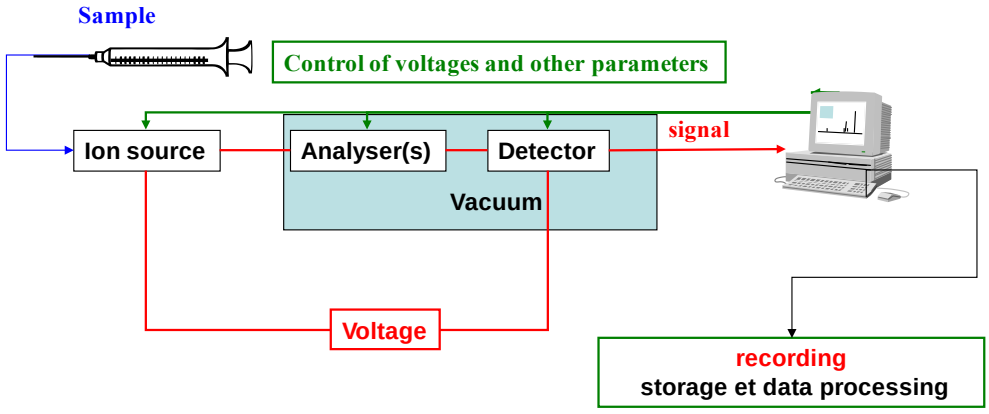


Figure 3.2: Schematic representation of a mass spectrometer.
A voltage is applied between the ion source and the detector. The recording, storage, and procession of data are achieved by a computer. A software installed in this computer allows most of the time the control of voltages and other parameters (Retrieved from Chaimbault, P. 2000)

The control of voltages and other parameters (pressure, gas flow,…) is run through a computer software. The storage and data processing is also run by a computer. The detector

could either be a photographic plate, a Faraday cup, an electron multiplier or an electron-optical ion detector.

4.3.2. Ionization source

In the ionization chamber, molecules contained in the sample are ionized. The mass spectrometer could be run in two modes: a positive mode where molecules lose electrons and give birth to positive ions and a negative mode where they gain electrons. Different types of ionization techniques are run. They could roughly be subdivided into 2 types: hard ionization (electro-ionization) during which a high residual energy is left onto the molecule leading to consecutive fragments and soft ionization techniques which are the opposite (Figure 3.3, Hamon *et al*., 1990).

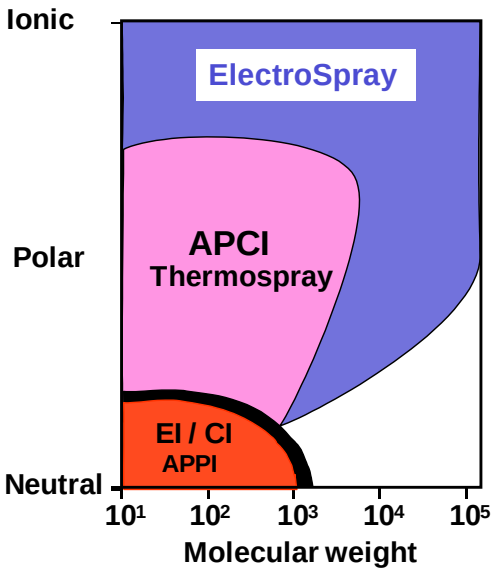


Figure 3.3: Graph showing the type of ionization method used based on the polarity and the molecular weight of the compound to be analyzed.

The ionization method mostly depends on nature, i.e the polarity and the molecular weight of the compounds to be analyzed. EI: electro-ionization, CI: chemical ionization, APPI: Atmospheric Pressure Photoionization and APCI: Atmospheric Pressure Chemical Ionization (Retrieved and adapted from Chaimbault, 2000).

4.3.2.1. Electro-ionization (EI)

During EI, a beam of electrons (negative charges) is formed under vacuum (typically around 10^{-5} torr). from a heated metallic filament (e.g., tungsten). These electrons are then electrically accelerated and directed to collide with a vaporized sample, causing electron expulsion from

the analyst and subsequent formation of positively charged radical cations. These conditions are not suitable for large molecules or many biological materials as the bombarding electron possess a very high energy (70 eV). Consequently, polar molecules are too fragile to be observed as a molecular ion ($M^{\cdot+}$) and the excess of energy leads to their fragmentation. This method is consequently used for small molecules or apolar ones, being, therefore, the method of choice during a mass spectrometry coupled to a gas chromatography (GC-MS) (Figure 3, El-Aneed *et al*., 2009). Except for direct introduction, the GC is often the only way of introducing sample in the source. GC is particularly adapted to MS coupling as the small amount of carrier gas (mobile phase) can be easily sent from the source to maintain the operating vacuum. This method was the one we have used during the GC-MS analysis on our fractions.

4.3.2.2. Chemical ionization (CI)

In contrary to the EI, during CI, the electrons beam impacted a reacting gas (methane, isobutane, ammonia but also in some case vapor of methanol or acetonitrile). This electron of feeble energy first ionizes this reactive gas to produce highly reactive species (most of the time the protonated reacting gas or some protonated clusters). The molecules of interest undergo a collision with these reactive species leading to their ionization by charge transfer (most of the time, the transferred ion is a proton). Similar to EI, this method poses some limitations in terms of mass range (<1000) and requires specific sample characteristics with regard to thermal stability and volatility. CI is a little bit softer than EI with respect to the production of the molecular ion ([M+H]+ in positive ion mode or [M-H]- in negative ion mode). However, the success of the analyses strongly depends on the choice of the reacting gas (very good knowledge of proton affinities). Nevertheless, both EI and CI failed to ionize the most valuable, thermally unstable, polar biological compounds (Figure 3). As for EI, CI is the method of choice for GC-MS equipment (Hamon *et al*., 1990; El-Aneed *et al*., 2009). As the choice of the right reacting gas may be a problem for an exhaustive run, we have only kept the EI to provide results on our samples. Moreover, contrary to EI, CI does not allow the use of the spectrum libraries (NIST) which help or guide the interpretation of MS results.

4.3.2.3. Electrospray ionization (ESI)

In electrospray, the process of ionization occurs at atmospheric pressure. Thus, this technique of ionization is compatible with a liquid introduction of the sample which can be done either directly (infusion and Flow Injection Analysis) or via liquid chromatography (Figure 3.4).

The suitable solvents in ESI are rather polar solvents such as methanol, acetonitrile, water or a mixture of them. The sample is first introduced into the ionization source via a thin needle (which is an electrode). A high difference of potential is applied between the needle and a counter electrode (typically from 3 to 5 kV), resulting in the formation of highly charged droplets, it is nebulization. After nebulization, the droplets are driven electrically toward the analyzer where they are dried with the aid of a warm neutral gas (usually nitrogen) to produce ions in a gas phase (Figure 3.4). ESI is meant to be used to produce ions from macromolecules such as proteins. However, the may be adapted to small and more or less polar metabolites. Indeed, this particularly mild ion source (it is actually the mildest in mass spectrometry) reduce the propensity of small and more or less polar metabolites to fragment during the ionization process. A molecular ion (mainly protonated in the positive ion mode or deprotonated in the negative ion mode) is therefore obtained easily. In order to facilitate positive ionization, volatile acids such as an acetic or formic acid are often added in the mobile phase. On the other hand, volatile bases such as ammonia are added to help the deprotonation of the studied molecules in the negative mode. However, in a case of unknown compounds, very little structural information is obtained. In such cases (especially when dealing with natural products of unknown composition), a tandem mass spectrometry (MS/MS) is used to fragment the molecular ion formed and deduce more information (El-Aneed *et al*., 2009).

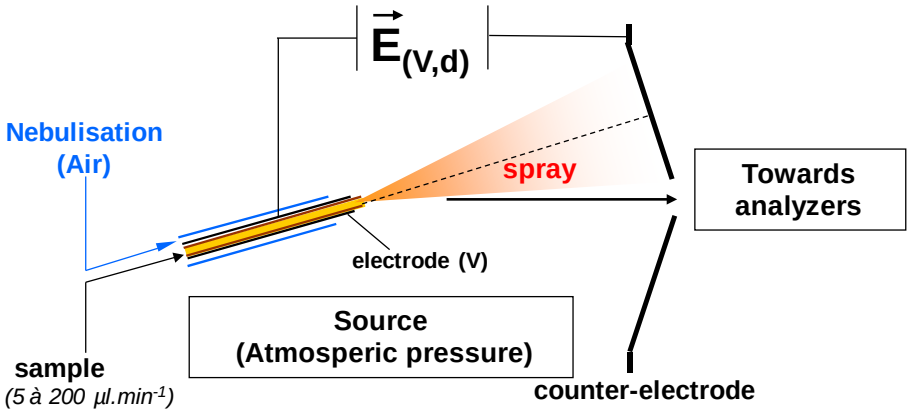


Figure 3.4: Schematic view of an electrospray ionization (ESI) interface.

In our study, ESI was used during the LC-MS metabolic profiling experiments on the most polar fractions of our refined plant extracts (Retrieved and adapted from Chaimbault, 2000).

4.3.2.4. Laser desorption/ionization and Matrix Assisted Laser Desorption/ Ionization (MALDI)

In our study, Matrix Assisted Laser Desorption/Ionisation was used only on purified compounds during the step of the exact mass determination. Obviously, Laser desorption/ionization without a matrix or assisted by a matrix can be applied on complex mixtures and even in imaging mass spectrometry. It was the case for example in the MALDI Mass Spectrometry Imaging for the simultaneous location of resveratrol, pterostilbene and viniferins in grapevine leaves (Becker *et al*., 2014). During laser ionization, a laser beam (at very short pulses, in the order of a nanosecond or even picosecond) is sent onto the sample deposited on a metal support or a sample holder. During this step, complex phenomenon yielding to charge transfers occurs, *i.e*. ionization, while the molecules are desorbed from the surface of the support (Figure 3.5). This desorption ionization can be assisted by a matrix. In MALDI-MS, there are 3 main sample preparation methods: the dried droplet, the thin layer and the sandwich methods. In our case, we have used the thin layer methods which will be described in section 6.5.3. An excessive amount of an appropriate matrix; among which 2,5 dihydroxybenzoic acid (DHB), α-cyano-hydroxy-cinnamic acid (HCCA) and Sinnapinic acid (SA) are probably the most commonly used; is cocrystallized with the sample on the target plate. Finally, the components in the mixture are brought into the gaseous phase via a laser beam (usually a nitrogen laser at a wavelength of 337 nm) that hits the sample-matrix crystal, leading to absorption of the laser energy by the matrix and subsequent desorption and ionization of the analytes in the sample (Figure 3.5). MALDI was initially operated under vacuum but nowadays it could also be run under atmospheric pressure (AP) MALDI (El-Aneed *et al*., 2009). In addition to its dependency on the nature of the analytes, the choice of the matrix, which is yet rather empiric, can also be influenced by the ionization mode, whether positive or negative. Basic matrices, such as 9-aminoacridine (Vermillion-salsbury *et al*., 2002) are favored in the case of the negative mode, whereas acidic ones (DHB, HCCA or SA) are more efficient in the case of the positive mode (El-Aneed A *et al*., 2009). In positive ion mode, one would expect to reveal the protonated molecular ion $[M+H]^+$ but very often, when the sample is also containing salt, sodium $[M+Na]^+$ or potassium $[M+K]^+$adduct can be observed. In negative ion mode, one tries to favor the presence of the deprotonated molecular ion $[M-H]^-$.

Laser ionization is applied to inorganic volatiles; thermolabile and high molecular mass organic molecules (1200 to 300 u.a.m) needing a soft ionization (Hamon *et al*., 1990, Figure

3.5). This ionization method is the one used for the identification of some of the compounds isolated from the roots of *P. erinaceus*.

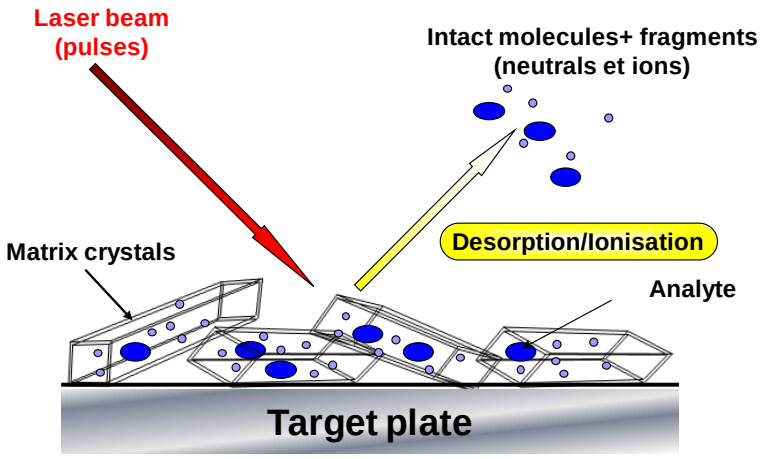


Figure 3.5: Schematic view of MALDI ionization

On the target plate, could be seen the cocrystallized matrix and sample. This method was used during our LC-MS experiments.

4.3.3. Analyzer

The role of the analyzer is to separate the ions based on their mass-to-charge (*m/z*) ratio. This separation is done using various principles. They are summarized in Table 3.1.

Table 3.1: Types of analyzers used in mass spectrometry

Retrieved from de Hoffman and Stroobant, 2012.

Type of analyzer	Symbol	Principle of separation
Quadrupole	Q	*m/z* (trajectory stability)
Ion trap	IT	*m/z*(resonance frequency)
Time-of-flight	TOF	Velocity (flight time)
Fourier transform ion cyclotron resonnance	FTICR	*m/z* (resonance frequency)
Fourier transform orbitrap	FT-OT	*m/z* (resonance frequency)

IT and Q analyzers are low-resolution analyzers whereas TOF is a high-resolution analyzer and FTICR and Orbitrap are very high-resolution analyzers. The mass accuracy measurement of the very high-resolution analyzers allows the determination of molecular formulae.

4.3.3.1. Quadrupole analyzers

As their name suggests, they are made up of four rods of circular or ideally hyperbolic section which are perfectly parallel to each other (Figure 3.6). In this analyzer, ions are separated based on the stability of their trajectories in the oscillating electric fields that are applied to the rods. Indeed, a radio frequency (RF) voltage with a DC offset voltage is applied between one pair of rods and the other. In such oscillating field, some ions will have unstable trajectories and will collide with the rods (Figure 3.6). Only the ones with stable trajectories will make it to the detector (Groos, 2011; de Hoffman and Stroobant, 2012). The quadrupole is the analyzer used for the GC-MS analysis of samples in this study. Although the mass accuracy measurement is low, the scan speed of quadrupoles is very interesting when used during the coupling of mass spectrometry with separative techniques.

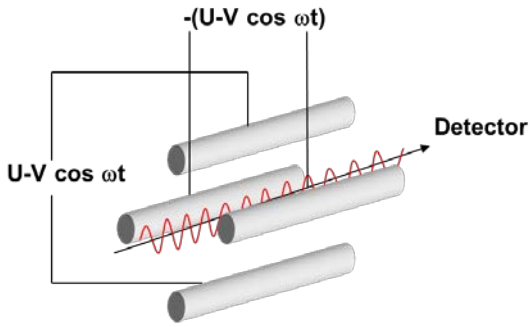


Figure 3.6: Schematic representation of a quadrupole analyzer

A radio frequency voltage is applied between one pair of rods and the other: U-V cost wt and –(U-V cost wt) (Retrieved from Chaimbault, 2000).

4.3.3.2. Ion trap analyzers

Ion trap analyzers first aim is to store ions (Figure 3.7). The trap is achieved by using a radiofrequency quadrupolar field that traps ions in two or three dimensions: 3D ion traps (Quadrupole ion trap or Paul ion traps) and 2D ion traps (Linear ions trap).

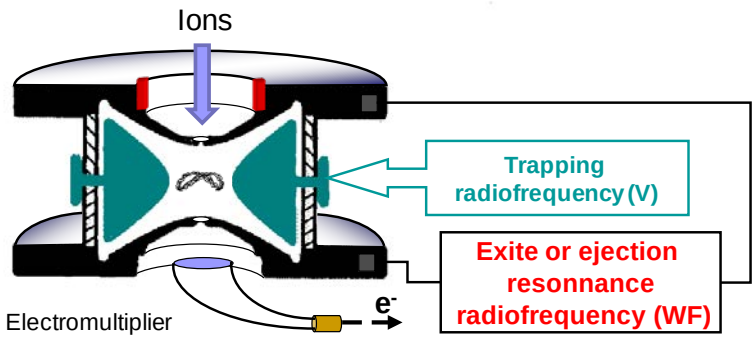


Figure 3.7: Schematic representation of an Ion Trap (IT) analyzer

The trapping radiofrequency (V) corresponds in fact to the static DC electric fields. The exit or ejection resonance radiofrequency WF corresponds to the oscillating AC radiofrequency.

Ion trap analyzers (classic)

It consists of two hyperbolic metal electrodes with their foci facing each other and a hyperbolic ring electrode halfway between the other two electrodes. The ions are trapped in the space between these three electrodes by oscillating AC and static DC electric fields. The AC radio frequency voltage oscillates between the two hyperbolic metal and caps electrodes. If ion excitation is desired, the driving AC voltage is applied to the ring electrode. Firstly, the ions are pulled up and down axially while being pushed in radially. Secondly, the ions are pulled out radially and pushed in axially (from the top and bottom). In this way, the ions move in a complex motion that generally involves the cloud of ions being long and narrow and short and wide, back and forth, oscillating between the two states. Ions of a given mass could be expelled out of the trap by adjusting the potentials (Groos, 2011; de Hoffman and Stroobant, 2012).

2D ion trap analyzers

In this analyzer, ions are trapped radially by a set of quadrupole rods and axially by a static electrical potential on the end electrodes. The oscillating fields are therefore no longer used. The trapping potential is achieved by placing electrodes of slightly higher potential adjacent to the front and rear ends of the quadrupole. The advantages of linear ion traps in comparison to 3D ion traps is a higher ion trapping capacity (more than 10-fold) and a higher ion trapping or injection efficiency (more than 50% is achieved when ions are injected into the 2D ion trap from an external source in comparison to only 5% for the 3D ion trap). These advantages increase the sensitivity and the dynamic range (Groos, 2011; de Hoffman and Stroobant, 2012).

LC-MS experiments have been carried out using an ion trap as an analyzer. Although the mass accuracy measurement is rather low, an ion trap allows MS^2 to MS^{10} study by fragmentation (in practice no more than MS^6 because of the weak number of ion remaining in the trap after each fragmentation series). This device is thus particularly interesting when using to study the fragment ion filiations (genealogical tree of ions) and thus helping to rebuild the initial structure from the fragment to get the molecule identification.

4.3.3.3. Time-of-flight analyzers

Those analyzers are based on the following principle: ions of different *m/z* are dispersed in time during their flight along a field-free drift path of known length. Provided all the ions start their journey at the same time or at least within a sufficiently short time interval, the lighter ones will arrive earlier at the detector than the heavier ones. This demands that they emerge from a pulsed ion source which can be realized either by pulsing ion packages out of a continuous beam or more conveniently by employing a true pulsed ionization method (Groos, 2011).

Linear time of flight analyzers

Used in combination with a laser desorption/ionization or a matrix-assisted laser desorption/ionization (MALDI). The sample is supplied as a thin layer on a sample holder upon which a pulsed laser is focused. An acceleration voltage U is applied between this target and a grounded counter electrode. Ions formed and desorbed during the laser pulse are continuously extracted and accelerated as they emerge from the target into the gas phase. When leaving the acceleration region, the ions should possess equal kinetic energies. They drift down a field-free flight path and finally hit the detector (Groos, 2011).

Reflector time of flight analyzers or reflections

The energy of the ions in a normal TOF is not only due to the electric field (which is not 100% homogenous) but also to the thermal energy coming from the ions brought from the ionization process in the ionization chamber. In a reflector TOF, this kinetic energy is corrected to have it stable. It uses a constant electrostatic field, a retarding field, acting as an ion mirror that sends the ions back through the flight tube. Consequently, the more energetic ions penetrate deeper into the reflector and take a slightly longer path to the detector. The less energetic ions of the same m/z ratio penetrate a shorter distance into the reflector and

correspondingly take a shorter path to the detector (Groos, 2011; de Hoffman and Stroobant, 2012). This analyzer, coupled to MALDI was used for the exact mass measurement (chemical formulae determination) of isolated compounds.

4.3.3.4. Ion cyclotron resonance and Fourier transform mass spectrometry

In a magnetic field, the ions trajectories are curved. When an intense field is applied to an ion that has a low velocity, the radius of the trajectory becomes small and the ion can be trapped on a circular trajectory in the magnetic field: that is the principle of the ion cyclotron or the penning trap. When an electromagnetic field having the same frequency as the trapped ion is applied to the cyclotron, the wave could be absorbed by the ion, leading to its excitation. This leads to an increase of the radius of the trajectory of the ions. The electromagnetic field is then removed after the excitation allowing the ions to come back to their initial state by emitting energy, just like in the case of an NMR experiment; it is the resonance. This energy could be set or fixed upon a set of electrodes near the ions at that moment. The 'image current' of such movement could be measured and by applying the Fourier Transform to the data obtained, the *m/z* are deduced (Groos, 2011; de Hoffman and Stroobant, 2012). This method has been used to identify a saponin isolated from the trunk barks of *P. erinaceus*.

4.3.4. Tandem mass spectrometry

The idea of a tandem mass spectrometry is to give information as much as possible on a molecule, the different radicals, and functional groups present. It could either be realized in space by coupling two distinct instruments (obtaining a hybrid instrument) or in time by performing an appropriate sequence of events in an ion storage device. The common space tandem mass spectrometry is realized using the Hybrid Quadrupole Orthogonal Time-of-Flight instrument (Q-ToF). The time one could be realized using analyzers such as ions traps and FTICR and program them so that the different MS^n steps are performed in the same instruments (El-Aneed *et al*., 2009, de Hoffman and Stroobant, 2012). In the present work, an MS^n experiment was carried out using an Ion Trap on one polar fraction of the trunk barks of *P. erinaceus*.

4.4. GC-MS on the apolar fractions obtained from the parts of *P. erinaceus*

The apolar fractions were the petroleum ether (PE), the dichloromethane (DCM) and the ethyl acetate (EA) fractions.

4.4.1. Roots of *P. erinaceus*

4.4.1.1. Chromatographic and mass spectrometry conditions

Samples were prepared by dissolving fractions in ethyl acetate for analytical use to obtain solutions at 1µg/mL.

Gas chromatography electro-ionisation mass spectrometry (GC–EIMS) analysis was performed on a fused silica column (HP-5-MS, 5% phenyl methyl polysiloxane, 25 m, 0.32 mm i.d., 0.32 µm film thickness; Phenomenex) in a GC 2010 chromatography (Thermo). Helium was used as a carrier gas at the flow rate of 1 mL/min. The program temperature was set as follow: incubator temperature was raised from 70 °C to 260 °C using a ramp of 10 °C/min. One µL of the sample was injected onto the column with a split ratio of 1/10. The mass spectrometer GCMS-DSQII SE was equipped with a quadrupole analyzer. The MS detector was set as follows: electron impact mode with ion source temperature set at 200 °C, analyzed mass interval m/z 40–700 and mass spectra were generated at 70 eV. Spectrum acquisition was performed during 21 min. The confirmation of the chemical structures was done by comparison with a library of mass spectra (NIST MS Search 2.0).

4.4.1.2. Results

In general, for all the apolar extracts investigated, a chromatogram was obtained that could roughly be subdivided into three parts (Figure 3.8): phenolic compounds, fatty acids and phytosterols or pentacyclic triterpenes. Tens of peaks were observed most of the time, corresponding to tens of compounds.

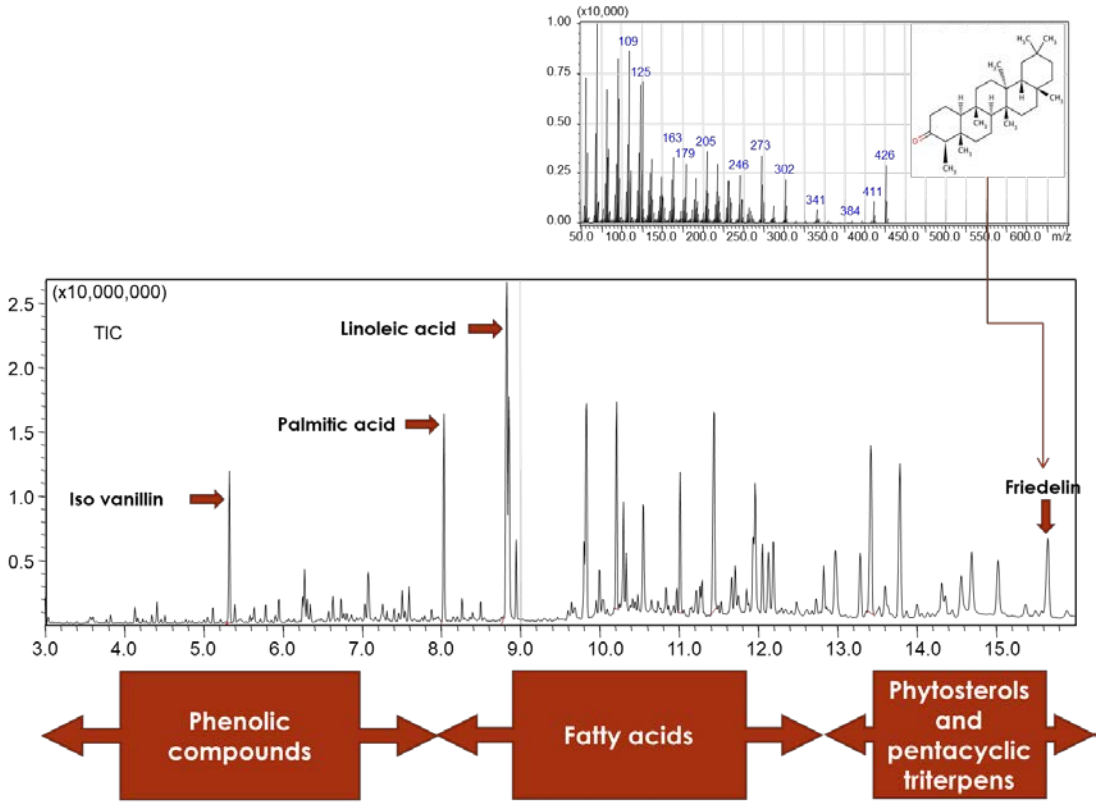


Figure 3.8: General presentation of a GC-MS chromatogram obtained on apolar fractions of the two plants.

Tens of peaks are observed corresponding to tens of compounds. Not all the peaks did hit a compound present in the library.

Compounds identified with a similitude more than 80% through the GC-MS are more likely to correspond to the real compounds. The ones with a similitude of around 70% could imply to just have some similar structures with the compounds.

The following saturated fatty acids were identified in the three fractions of the roots with around 70% structure equivalence according to NIST: hexadecanoic acid (palmitic acid); stearic acid (octadecanoic acid) and eicosanoic acid (arachidic acid). Some unsaturated fatty acids were also detected with a structure equivalence higher than 80%: hexadecenoic acid (palmitoleic acid), linolenic acid and stearolic acid (Tables 3.2 and 3.3). The following sesquiterpenes, cadina-1(10),4-diene (hydrocarbon sesquiterpenes) and nerolidol (alcohol sesquiterpenes), and the aromatic compound asarone were also been noticed in the PE fraction (Table 3.2).

Table 3.2: GC-MS results of petroleum ether fraction of *P. erinaceus* root

Relative Selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in minutes, INH: compound did not hit a compound present in the library.

n°	RT	RSI %	Area %	Name
1	10.16	74.8	0.83	Cadina-1(10),4-diene
2	10.63	80.0	0.79	Nerolidol
3	11.72	80.0	0.02	Asarone
4	13.94	-	-	INH
5	14.37	75.4	0.04	Cinnamic acid, 3,4-dimethoxy, methyl ester
6	14.98	85.4	13.92	Hexadecenoic acid
7	15.92	73.1	1.65	Arachadic acid
8	16.64	89.1	31.31	Stearolic acid
9	16.70	83.9	31.31	Linolenic acid (coeluted with compound 8)
10	16.87	78.5	3.65	Stearic acid

Table 3.3: GC-MS results of dichloromethane and ethyl acetate fractions of *P. erinaceus* root

Relative selectivity index (RSI: the similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library and EAc: compound identified in the ethyl acetate fraction.

n°	RT	RSI %	Area %	Name
1	14.96	75.7	5.14	Hexadecanoic acid
2	16.62	79	7.1	Linolenic acid
3	16.68	68.3	4.01	Stearic acid
1	14.94	71.1	1.46	Arachidic acid (EAc)
2	18.53	78	5.19	Cholestan-5,7,9, (11) trien-3-ol acetate (EAc)

4.4.2. Trunk barks of *P. erinaceus*

The same parameters as the ones described in 4.4.1 were applied.

The following sesquiterpenes alcohol were identified in the PE fraction: cubenol (70% structurally equivalent); *t*-muurolol and or *α*-cadinol (more than 80% equivalence) (Table 3.4).

Table 3.4: GC-MS results of petroleum ether fraction of *P. erinaceus* trunk bark

Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library.

n°	RT	RSI %	Area %	Name
1	10.64	70.5	1.32	Dodecanoic acid
2	11.60	79.2	0.84	*T*-muurolol
3	11.75	83	1.27	*α* -cadinol/ *T*-muurolol
4	12.64	83	2.41	3-methylbut-2-enoic, 4-nitrophenyl ester
5	13.25	83.6	1.32	Cinnamic acid, 3,4-dimethoxy, methyl ester
6	13.97	90.5	3.33	Cyclobutanecarboxylic acid, trydec-2-ynyl ester, 2-methylen cholestan-2-ol (3 *α*, 5 *α*)
7	14.34	74.7	7.98	Cinnamic acid, 3,4-dimethoxy, methyl ester
8	14.96	74	4.87	Hexadecanoic acid
9	15.66	77.9	0.7	3,12 octadecadienoic acid methyl ester
10	16.64	70	17.7	Stearolic acid
11	16.71	87.2	8.1	*Cis*, *cis*, *cis* 7,10,13 hexadecatrienal/ Linoleic acid
12	19.39	77.9	2.64	Cubenol

Besides, the hexadecenoic or palmitic acid (saturated fatty acid, C_{16}) was found in all the three (03) fractions with a molecule equivalence higher than 70% in PE and DCM fractions and higher than 80% in EA fraction. The dodecanoic acid (lauric acid) (saturated fatty acid, C_{12}) found in the PE and DCM fractions, respectively with a NIST equivalence of more than 70% and 80% (Tables 3.4 and 3.5). The following unsaturated fatty acids were also identified in the barks: octadecadienoic acid (linoleic acid) in the PE fraction (more than 70% structure equivalence), octadecatrienoic acid (linolenic acid) in the PE and DCM fractions (more than 80% equivalence) and in the EA fraction (more than 70% structure equivalence); stearolic acid or 9-octadecynoic acid in the PE fraction (70%) and in the DCM and EA fractions (more

than 80%). The following aromatic compounds have also been found: vanillin in the DCM fraction (structure equivalence higher than 70%) and cinnamic acid derivatives in the PE fraction (more than 80%) (Tables 3.4 and 3.5).

Table 3.5: GC-MS results of dichloromethane and ethyl acetate fractions of *P. erinaceus* trunk bark

Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library. EAc: compound identified in the ethyl acetate fraction.

n°	RT	RSI %	Area %	Name
1	9.38	83.9	3.2	Vanilin
2	10.64	88.8	0.92	Dodecanoic acid
3	10.91 12.98 13.18 13.98 14.34	-	-	INH
8	14.95	78.8	5.18	Hexadecanoic acid
9	16.63	80.5	14.47	Steraolic acid
10	16.69	84.9	6.85	Linolenic acid
11	16.86 18.53 19.37 20.14	-	-	INH
1	14.94	82	5.75	Hexadecanoic acid (EAc)
2	16.62	86.7	15.12	Stearolic acid (EAc)
3	16.68	77.5	4.82	Linolenic acid (EAc)
4	18.53	69.3	3.97	Armid ow (EAc)

4.4.3. Leaves of *P. erinaceus*

The results were obtained by applying the same parameters as described in 4.4.3. Compounds were identified in the apolar fractions of the leaves using GC-MS. Eicosatrienoic acid (C_{20}), an unsaturated fatty acid, was identified in the PE fraction under an ester form (eicosatrienoic methyl ester acid) (Table 3.6).

Table 3.6: GC-MS results of *P. erinaceus* leave's petroleum ether fraction

Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library.

n°	RT	RSI %	Area %	Name
1	13.68	85.7	2.72	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
2	13.94	73.8	0.77	2-chloroethyl linoleate
3	14.13	80.5	1	1,2-diexyl cyclopropen
4	14.95	80.6	5.71	Hexadecanoic acid
5	16.70	88.8	11.48	Eicosatrienoic acid methyl ester
6	16.85	68.8	2.04	Aqua cera

The fatty saturated acids, hexadecanoic acid or palmitic acid (C_{16}), have been identified in the PE, under its unsaturated forms in DCM (under alcene form: hexadecenoic acid, its aldehyde form: *cis*, *cis*, *cis* 7-10-13 hexadecatrienal) and EA (under its aldehyde form: hexadecadienal and its diglyceride form 1,2-di palmitin) fractions with a similitude greater than 80% (Table 3.7). The unsaturated acid, linoleic acid (C_{18}) has also been identified in the PE and EA fractions under respectively its ester (2-chloroethyl linoleate) and amide (9-octadecenamide) forms but with a similitude just around 70% (Tables 3.6 and 3.7). Interestingly, heptatriacontanol (alcoholic fatty acid) and cinnamic acid were also found in the EA fraction but with a respective similitude of more than 80% and just 70%. The EA fraction was the one containing many terpenes: menthol and with a similitude higher than 90% and 2-caranone with a similitude just around 70%. The terpene, hexadecenol (terpene alcohol), has also been identified in the PE and DCM fractions with a similitude more than 80% (Tables 3.6 and 3.7).

Table 3.7: GC-MS results of dichloromethane and ethyl acetate fractions of *P. erinaceus* leaves

Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library. EAc: compound identified in the ethyl acetate fraction.

n°	RT	RSI %	Area %	Name
1	3.58	74.8	2.75	Hexenoic acid
2	9.41	85.3	0.68	Anti-2-acetoxyacetaldoxime
3	12.51	-	-	INH
4	13.68	82.9%	2.65	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
5	14.28	76.8%	8.73	1-hydroxy-7a-methyloctahydro-5h-inden-5-one
6	14.95	82.4%	3.55	Hexadecenoic acid
7	16.68	82.1%	13.15	*Cis*, *cis*, *cis* 7-10-13 hexadecatrienal
8	18.55	-	-	INH
1	5.51	90.3	6.45	Menthol (EAc)
2	6.67	76.6	1.69	2-caranone (EAc)
3	14.95	71.5	1.6	1,2-di palmitin (EAc)
4	16.09	74.2	1.99	Hydrocinnamic acid (EAc)
5	16.68	83.9	4.36	*Cis*, *cis*-7,10-hexadecadienal (EAc)
6	18.55	71	4.27	9-Octadecenamide (Armid ow) (EAc)
7	19.71	81.7	0.52	Heptatriacontanol (EAc)

4.4.4. GC-MS analysis of the apolar fractions obtained from the parts of *D. oliveri*

Considering the amount of work required, analytical work was focused only on the most active fractions, especially the apolar ones where GC-MS analysis was performed: petroleum ether fractions (leaves, trunk barks); dichloromethane fractions (trunk barks, roots) and ethyl acetate fractions (trunk barks, roots). The GC-MS analysis was done using two different approaches because with the first method (first parameters precedently described), a temperature higher than 300 °C could not be attained although compounds such as sterols need such temperature to evaporate. Contrary to the first plant (*P. erinaceus*), it was not possible to perform the isolation of compounds on *D. oliveri* active extracts and fractions.

Performing the two types of GC-MS helped to get as much information as possible that could be obtained using GC-MS on this plant.

4.4.5. Roots of *D. oliveri*

4.4.5.1. First parameters of the GC-MS

- Same parameters as described in 4.4.1.1.
- Results

Different types of terpenes were recognized. Among them, a furano-diterpene lactone, Columbin (73.7% structure equivalence) in EA fraction; a monoterpene alcohol, 1-tridecanol (83.2%) in DCM fraction; an alcohol sesquiterpene, *t*-muurolol (74.5%) in EA fraction; hydrocarbon sesquiterpenes, cedrene (81.5%) and cadina-1(10),4-diene (83.3%) in DCM fraction, germacrene D (more than 85%) in DCM and EA fractions; oxygenated sesquiterpenes, aromadrene oxide (81.9%) and iso-aromadendrene epoxide (81.1%) in EA fractions (Table 3.8). Saturated fatty acids have also been unbedded: hexadecadienoic acid under its aldehyde form, *cis*, *cis* 7-10 hexadecadienal (more than 88%) in DCM and EA fractions and, pentadecanoic acid methyl ester (79.2%) in DCM. And for unsaturated fatty acids, there was hexadecenoic acid (more than 80%) in DCM and EA fractions; octadecadienoic acid methyl ester (82.8%) in DCM; octadecenoic acid methyl ester (81.2%) in DCM fraction and 10-12 pentacosadienyoic acid (81%) in EA fraction. The phenolic compounds described were naphthalene carboxylic acid (more than 90%) in DCM and EA fractions, and naphthalene (83.2%) in EA fraction (Table 3.8).

Table 3.8: GC-MS results of dichloromethane fraction (1) of *D. oliveri* root

Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library. EAc: compound identified in the ethyl acetate fraction.

n°	RT	RSI %	Area %	Name
1	7.92	83.5	0.68	Germacrene D
2	8.42	83.2	0.6	1-tridecanol
3	8.47	87.2	0.6	Germacrene D
4	9.58	81.5	0.28	Cedrene
5	10.15	83.3	1.35	Cadina-1(10),4-diene
6	14.58	79.2	0.25	Pentadecanoic acid methyl ester
7	14.95	81	0.65	Hexadecanoic acid
8	16.23	82.8	0.41	Octadecadienoic acid methyl ester
9	16.28	81.2	0.36	Octadecenoic acid methyl ester
10	16.62	88.7	1.51	*Cis*, *cis* 7-10 headecadienal
11	18.91	81.9	2.04	Aromadrene oxide
12	19.48	91	78.87	1-naphtalencarboxylic ac
1	7.92	89.3	0.58	Germacrene D (EAc)
2	10.15	83.2	1.33	Naphtalene (EAc)
3	11.60	74.5	0.28	*T*-murolol (EAc)
4	11.75	73.7	0.22	Columbin (EAc)
5	14.95	83.4	0.71	Hexadecanoic acid (EAc)
6	16.23	91	0.11	*Cis*, *cis* 7-10 hexadecadienal (EAc)
7	16.62	88.9	1.77	*Cis*, *cis* 7-10 hexadecadienal (EAc)
8	18.12	81	0.36	10-12 pentacosadienyoic acid (EAc)
9	18.91	81.1	1.47	Iso-aromadendren epoxid (EAc)
10	19.49	91.3	81.29	1-naphtalencarboxylic acid (EAc)

4.4.5.2. Second parameters

- Chromatographic and spectrometric conditions

Samples were prepared by dissolving fractions in dichloromethane (for analytics) for petroleum ether and dichloromethane fractions and in ethyl acetate (for analytics) for ethyl acetate fractions to obtain solutions at 1 mg/ml.

GC–EIMS analysis was then performed on a fused silica column (ZB-5-MS, 5% phenyl methyl polysiloxane, 30 m, 0.25 mm i.d., 0.25 μm film thickness; Phenomenex) in a GC 2010 chromatograph (Shimadzu). Helium was used as a carrier gas at the flow rate of 1 mL/min. The program temperature was set as follow: incubator temperature was raised from 100 °C to 325 °C using a ramp of 20 °C/min. The final temperature was maintained for 5 min (end of the analysis). One μL of the derivatized sample was injected onto the column with a split ratio of 1/10. The mass spectrometer GCMS-QP2010 SE was equipped with a quadrupole analyzer. The MS detector was set as follows: electron impact mode with ion source temperature set at 200 °C, analyzed mass interval m/z 40–700 and mass spectra were generated at 70 eV. Spectrum acquisition was performed during 16.25 min. The confirmation of the chemical structures was achieved by comparison with a library of mass spectra (NIST MS Search 2.0).

- Results

Applying these parameters has helped to identify many terpenes and one sterol.

The diterpene polyalthic acid or daniellic acid (96%, DCM fraction) and its derivative, 1-naphthalenecarboxylic acid, 5-[2-(3-furanyl)ethyl]decahydro-1,4a-dimethyl-6-methylene (95%) have also been recognized in the EA fraction. An alcohol sesquiterpene, *α*-cadinol (85%); an oxygenated sesquiterpene 2(3H)-benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl) or dehydrosasurealactone (72%) and a phytosterol, *β*-stigmast-8(14)-en-3-ol (66%) were identified in the EA fraction (Table 3.9).

Table 3.9: GC-MS results of ethyl acetate fraction (2) of *D. oliveri* root

Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), N°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library.

N°	RT	RSI %	Area %	Name
1	4.20	-	-	INH
2	4.48	86	0.27	D-Germacrene
3	5.03	-	-	INH
4	5.18	-	-	INH
5	5.28	85	3.39	Cadina-1(10),4-diene
6	6.05	82	0.41	*T*-Muurolol
7	6.11	72	0.32	α-Cadinol
8	6.12	68	0.32	Cadala-1(10),3,8-triene
9	9.35	76	0.57	2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)
10	9.75	80	1.38	Bicyclo [5.2.0] nonane, 4-methylene-2,8,8-trimethyl-2-vinyl-
11	9.94	95	32.05	1-Naphthalenecarboxylic acid, 5-[2-(3-furanyl)ethyl]decahydro-1,4a-dimethyl-6-methylene
12	13.23	65	0.38	Benzene-1-isopropyl-4-methyl-
13	13.63	66	0.25	β-Stigmast-8(14)-en-3-ol

Besides, some hydrocarbon sesquiterpenes have also been unveiled: cadina-1(10),4-diene (more than 90% structure equivalence) and bicyclo[5.2.0]nonane, 4-methylene-2,8,8-trimethyl-2-vinyl (more than 70%) in DCM and EA fractions; germacrene D (86%) and cadala-1(10),3,8-triene (68%) in EA fraction (Tables 3.9 and 3.10).

Table 3.10: GC-MS results of dichloromethane fraction (2) of *D. oliveri* root

Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library.

n°	RT	RSI %	Area %	Name
1	4.20	-	-	INH
2	5.04	-	-	INH
3	5.27	94	1.89	Cadina-1(10),4-diene or delta.-Cadinene, (+)-
4	5.60	-	-	INH
6	9.75	72	0.7	Bicyclo[5.2.0]nonane, 4-methylene-2,8,8-trimethyl-2-vinyl
7	9.93	96	8.77	Enantio-Polyalthic acid or Daniellic acid

4.4.6. Trunk barks of *D. oliveri*

4.4.6.1. First parameter results

Many fatty acids have been recognized in the sample using the first parameters of GC-MS. Some terpenes, an amine, and a phenolic compound have also been identified. Among fatty acids, the following unsaturated fatty acids were found: linoleic acid (9,12 - octadecadienoic acid) found in all the three fractions but as its aldehyde form (octadienal) in the DCM and EA fractions with a structure equivalence higher than 80%. The same acid is also present as its 3-saturated form, octadecatrienoic acid (linolenic acid) in the PE fractions and as its saturated form, octadecanoic acid (stearic acid), in the DCM fraction. The polyunsaturated fatty acids, eicosapentaenoic acid, and docosatetraenoic acids methyl esters have also been identified respectively in the PE and DCM fractions (more than 70% structure correspondence). The following saturated fatty acids (more than 80% structure correspondence in most cases) have also been deciphered: hexadecanoic acid in the three fractions; myristic and nonanoic acids in the PE fraction; pentadecanoic acid in the PE and DCM fractions and dodecanoic acid in the DCM fraction under a chlorine form (chloromethyl chloro-dodecanoate) (Tables 3.11 and 3.12). Concerning terpenes, a diterpene (λ-8(20),13(16),14-trien-18-oic acid); a hydrocarbon sesquiterpene (α-caryophyllene) and two oxygenated sesquiterpenes (12 oxabicyclic [9,10] dodeca-3,7-diene and caryophyllene oxide, more than 80% equivalence) have been identified

in the PE fraction. In the same fraction, an amine (hydroxylamine) and phenolic compounds (phenol 2,4 bis (1,1, -dimethyl ethyl), benzene propanoic acid, isocoumarins and naphthalene) were found (Table 3.11).

Table 3.11: GC-MS results of petroleum ether fraction (1) of *D. oliveri* trunk bark
Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library.

n°	RT	RSI %	Area %	Name
1	4.49	80.7	0.46	Hydroxyl amine
2	6.95	82.35	1.28	Nonanoic acid
3	9.30	77.4	0.29	α-caryophyllen
4	10.15	87.9	0.74	Naphtalene
5	10.64	88.7	2.44	Isocoumarin
6	10.93	83.4	1	Caryophyllen oxide
7	11.24	82.6	1.08	12 oxabicyclic [9,10]dodeca-3,7-diene
8	12.88	83.7	0.9	Myristic acid
9	13.95	83.1	1.94	Pentadecanoic acid
10	14.77	70.1	2.05	Benzenpropanoic acid
11	15	88	17.34	Hexadecanoic acid
12	15.68	85.3	3.57	10-dodecyn-1-ol
13	15.70	73.2	3.57	Octadecadienoic acid (partially coeluted with the precedent peak)
14	16.57	85.9	20.02	Octadecadienoic acid Cis, cis 7-10-hexadecadienal
15	17.92	89.8	1	Eicosapentaneoic acid methyl ester
16	18.28	70.8	1.02	Linolenic acid
17	19.39	72.7	2.08	Labda-8(20),13(16),14-trien-18-oic acid
18	19.96	-	-	INH

Table 3.12: GC-MS results of dichloromethane and ethyl acetate fractions (1) of *D. oliveri* trunk bark

Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library. EAc: compound identified in the ethyl acetate fraction.

n°	RT	RSI %	Area %	Name
1	12.88	-	-	INH
2	13.94	78.2	1.47	Pentadecanoïc acid
3	14.98	88.1	14.16	Hexadecanoic acid
4	15.71	84.4	1.8	Chloromethyl chlorododecanoate
5	15.92	74.4	0.71	Hexadecanoic acid 1,1-dimethyl ester
6	16.28	74.8	0.81	Docosatetranoic acid methyl ester
7	16.68	87.2	22.33	Octadecadienal
8	16.86	75.7	2.08	Octadecanoic acid
9	18.54	-	-	INH
1	14.95	73.1	4.9	Hexadecanoic acid (EAc)
2	16.64	85.3	6.3	3,12-Octadecadienal (EAc)
3	18.53	83.5	8.46	8-methyl 6 nonenamide (EAc) 5,9-dimethyl cyclodecanol (EAc)

4.4.6.2. Second parameter results

This GC-MS setting has helped identify phytosterols and glycerides, in addition to the type of compounds already described with the first parameters.

The saturated fatty acids identified are: hexadecanoic acid (PE and DCM fractions, more than 90%); nonanoic acid (86%) in the PE fraction; succinic acid (92%), 2-methyl butanoic acid (more than 60%), myristic acid, pentadecanoic acid; heptadecanoic or margaric acid; octadecanoic or stearic acid; arachidic acid; behenic or docosanoic acid; tetracosanoic or lignoceric acid; pentacosanoic or hyenic acid and hexacosanoic or serotic acid (more than 90% in most cases) in the DCM fraction (Tables 3.13 and 3.14). Only three unsaturated fatty acids were identified: linoleic acid (9,12 octadecadienoic acid) in PE and DCM fractions (more than 90% respectively); oleic acid (octadecenoic acid); *cis*-10-heptadecenoic acid in DCM fraction (more than 90%). For the terpenes, in addition to the already described terpenes

in the precedent lines *ie* oxygenated sesquiterpene (caryophyllene oxide and humulene-1,2-epoxide in PE fraction, more than 85%) and alcohol terpene (hexadecane-1,2-diol and 3-tetradecyn-1-ol in PE fraction, more than 85%), one pentacyclic triterpene was unveiled, *β*-amyrinin in the DCM fraction (more than 90%) (Table 3.14). Other interesting discovered compounds were phytosterols: campesterol, sitosterol (*β* in PE fraction 78%, *γ* in DCM fraction 90%), stigmasterol (60% in PE, in DCM more than 90%). A monoglyceride, 1-monopalmitoylglycerol (more than 90%) in DCM fraction has also appeared as a new structure in comparison to the precedently described ones (Table 3.14). Finally, phenolic already described compounds were also unveiled: mellein (isocoumarin) 91% and benzenpropanoic acid (more than 80%) in the PE fraction; vanillin, benzoic acid and its derivatives (70 to more than 90%) in the DCM fraction (Tables 3.13 and 3.14). The ethyl acetate fraction showed interesting Gaussian peaks that did not hit already described compounds in the library.

Table 3.13: GC-MS results of petroleum ether fraction (2) of *D. oliveri* trunk bark
Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library.

n°	RT	RSI %	Area %	Name
1	3.53	86	0.6	Nonanoic acid (or pelargonic acid)
2	5.49	91	2.2	Mellein (3,4-Dihydro-8-hydroxy-3-methylisocoumarin)
3	5.74	88	0.59	Caryophyllene epoxide/
4	5.9	85	0.85	Humulene-1,2-epoxide
6	7.5	81	0.76	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester.
7	7.59	95	1.67	*n*-Hexadecanoic acid (Palmitic acid)
8	8.45	92	3.31	Linoleic acid (9,12-Octadeca dienoic acid)
9	8.54	86	0.49	3-Tetradecyn-1-ol
11	13.37	60	1.17	Stigmasta-5,22-dien-3-ol, (3.*β*.)- / Stigmasterin
12	13.73	78	3.49	*γ*-sitosterol

Table 3.14$_1$: GC-MS results of dichloromethane fraction (2) of *D. oliveri* trunk bark Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library.

n°	RT	RSI %	Area %	Name
1	3.04	62	0.44	2-Methylbutanoic acid
	3.16 3.17 3.23 3.28 3.31 3.36	-	-	INH
8	3.84	95	0.66	Succinic acid
9	5.31	91	0.35	Vanillin
10	5.44	87	0.42	Benzoic acid
11	5.81	-	-	INH
12	6.26	-	-	INH
13	6.34	92	0.17	Benzoic acid
14	6.57	-	-	INH
15	6.66	71	0.18	Benzoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-
16	6.73	-	-	INH
17	6.86	93	1.02	Benzoic acid
18	7.03	88	0.5	Tetradecanoic acid/ Myristic acid
19	7.27	-	-	INH
20	7.45	94	0.48	Hexadecanoic acid methyl ester (palmitic acid methyl ester).
21	7.53	93	1.42	*n*-Pentadecanoic acid ester
22	8.02	94	10.02	Hexadecanoic acid / Palmitic acid
23	8.3	92	0.35	Linoleic acid, methyl ester.

Table 3.14$_2$: GC-MS results of dichloromethane fraction (2) of *D. oliveri* trunk bark

Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library.

n°	RT	RSI %	Area %	Name
24	8.32	91	0.41	6-Octadecenoic acid, methyl ester, (*Z*)-/ Methyl cis-6-octadecenoate.
25	8.36	76	0.89	9,12-Octadecadienoic acid
26	8.38	91	1.14	cis-10-Heptadecenoic acid
27	8.49	93	0.85	Heptadecanoic acid (Margaric acid)
28	8.71	87	0.46	Hexadecane-1,2-diol
29	8.81	95	9.44	12-Octadecadienoic acid (*Z*, *Z*)-/ Linoleic acid
30	8.83	94	10.23	trans-9-Octadecenoic acid/ (9*E*)-9-octadecenoate
31	8.94	96	2.97	Octadecanoic acid/ Stearic acid
32	9.78	91	0.89	Arachidic acid
33	10.32	94	0.69	Hexadecanoic acid/ 1-Monopalmitin / 1-Monopalmitoylglycerol
34	10.55	92	0.7	Behenic acid / Docosanoic acid
35	10.84	81	0.88	9-Octadecenoic acid
36	10.97	89	1.06	Oleic acid
37	11.29	91	2.16	Tetracosanoic acid/ tetracosanoate
38	11.65	85	0.99	Pentacosanoic acid
39	12.04	92	1.37	Hexacosanoic acid
40	12.21	-	-	INH
41	13.26	91	1.47	Campesterol
42	13.40	92	1.81	Stigmasterol
43	13.77	93	5.42	*β*-sitosterol
44	14.56	91	10.79	*β*-Amyrin

4.4.7. Leaves of *D. oliveri*

4.4.7.1. First measurements

Same parameters as described in 4.5.1.1.

The following compounds have been identified in the PE fraction of the leaves applying those parameters of GC-MS. Terpene alcohols (6-linalool; *t*-muurolol; 3,7,11,15- tetramethyl-2-hexadecen-1-ol); sesquiterpene hydrocarbons (aromadendrene; α-caryophyllen or humulene, germacrene D, 6-chamigrene); oxygenated sesquiterpenes (tricyclo [5.2.2.0(1,6)] undecane-3-ol; 1,5,5,5,8-tetramethyl-12-oxabicyclo [9,10] dodeca-3,7-diene or humulene-1,2-epoxide or humulene epoxide II); saturated fatty acids (hexadecanoic acid); unsaturated fatty acids (*cis*, *cis*, *cis*, 7-10-13-hexadecatrienal) and aromatic compounds (naphthalene and aromadendrin oxide) (Table 3.15).

Table 3.15: GC-MS results of petroleum ether fraction (1) of *D. oliveri* leaves

Relative selectivity index (RSI: similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in min, INH: compound did not hit a compound present in the library.

n°	RT	RSI %	Area %	Name
1	4.48	77.5	0.38	6-linalool
2	8.86	85.3	0.66	Aromadendrene
3	9.3	87.3	1.33	α-caryophyllen
4	9.58	89.9	0.29	Germacrene D
5	9.87	87.1	0.56	Naphtalene
7	10.93	82.3	1.89	Caryophyllene oxide
8	11.24	79.4	2.72	1,5,5,5,8-tetramethyl-12-oxabicyclo [9,10] dodeca-3,7-diene
9	11.60	84.2	0.98	*T*-murolol
10	12.13	83.6	1.32	Tricyclo [5.2.2.0(1,6)] undecane-3-ol.
11	14.96	84.1	3.35	Hexadecanoic acid
12	16.40	78.8	9.18	3,7,11,15- tetramethyl-2-hexadecen-1-ol
13	16.68	85	3	*Cis*, *cis*, *cis*, 7-10-13-hexadecatrienal
14	18.01	85.7	0.11	6-chamigrene
15	18.09	88.3	9.95	(7a-isopropenyl-4,5-dimethyloctahydroinden-4-y) methanol
16	18.96	80.7	37.66	Aromadendrin oxide

4.4.7.2. Second measurements

The following compounds have been identified: sesquiterpene hydrocarbons (humulene, cadina-1(10),4-diene); oxygenated sesquiterpenes (caryophyllene oxide, humulene-1,2-epoxide); terpenes alcohol (10-epi-α-muurolol) and unsaturated fatty acids (9,12-octadecadienoic acid) (Table 3.16).

Table 3.16: GC-MS results of petroleum ether fraction (2) of *D. oliveri* leaves

Relative selectivity index (RSI): similarity of the spectrum with the structure according to the library), n°: Compound number, RT: Retention time in minutes, INH: compound did not hit a compound present in the library.

n°	RT	RSI %	Area %	Name
1	4.95	88	0.6	Humulene/ α-Caryophyllene/ α-Humulene
2	5.27	86	0.54	Cadina-1(10),4-diene/ δ-Cadinene
3	5.74	88	0.33	Caryophylene oxide/ Caryophyllene epoxide.
4	5.9	87	0.63	Humulene-1,2-epoxide/ Humulene epoxide 2
5	6.05	77	0.53	*T*-Muurolol / 10-epi-α-Muurolol
6	14.45	65	0.68	9,12-Octadecadienoic acid

4.5. LC-MS on the polar fractions obtained from the trunk barks of *P. erinaceus*

The LC-MS was carried out on the polar fractions of the trunk barks, namely the butanol and water fractions, considering the results obtained with their anti-bacterial activities: a MIC at 64 µg/mL and an inhibition of 50% of bacterial growth at 1 µg/mL (see section 4.1.1. of the second chapter).

4.5.1. Chromatographic and spectrometric conditions

For LC-MS analysis, the high performance liquid chromatography system (Dionex Ultimate 3000, Dionex, France) was connected to a dual-pressure linear ion trap mass spectrometer (LTQ Velos Pro, Thermo Fisher Scientific, San José, CA, USA). The separation was realized on a C_{18} reverse phase column (Symmetry Shield, 4.6 × 50 mm, 3.5 µm, Waters). 20 µL of sample was injected. The flow rate was kept to 500 mL/min and a constant elution gradient was applied from 0 (5% acetonitrile / 95% water) to 55 min (100% acetonitrile) during the liquid chromatography (LC) run. HESI (*Heated Electrospray Ionization Source*) interface was plugged into the ion source of the LTQ mass spectrometer with a capillary temperature set at

300 °C, a source heater at 250 °C, a sheath gas flow at 10.00, an auxiliary gas flow at 5 with the injection waveforms on.

MS system was running from 110 to 2000 *m/z* at MS scan rate of 9 Hz. To confirm chromatographic peak assignment, MS/MS by CID was systematically conducted on the five most intense mass peaks of each mass spectrum.

Besides, the spectrometry was also run in two modes: positive and negative. During the positive mode, the source voltage was set at +5 kV and the source current at 100 µA. During the negative mode, the source voltage was set at -4 kV, the source current at 100 µA, the S-Lens RF Level at 60%, the multipole RF Amplifier at 800.00 (Vp-p), the multipole 00 Offset at 2 V, the lens voltage at 3 V, the multipole offset at 9 V, the lens voltage at 15 V, the gate lens offset at 90 V and the multipole 1 offset at 20 V.

4.5.2. Identification of compounds

A list of m/z ratio was obtained from the LC-MS report. An MS^2 (MS/MS) experiment was run on the highest peaks (as defined in the methodology) simultaneously by applying the following strategies:

- the observed MS^2 spectrum was compared to the literature via search engines (general search level).
- the neutral loss observed between the molecular ion and the fragments were calculated. Indeed, in the plants' kingdom, some well-known neutral loss is particularly informative such as -162 u for glucoside derivatives, -132 for a xylose or arabinose loss and the loss of 44 u in negative ion mode indicates the presence of a carboxylic acid function.
- in negative ion mode particularly, we checked the presence of fragments corresponding to the hydroxycinnamic acids (coumaric, caffeic and ferulic acids) but also gallic acids or catechins, etc. Indeed, those phenolic compounds are commonly present in the plants' kingdom.
- some compounds were identified using the literature review on species belonging to the genus *Pterocarpus* and on other plants (with the notable fact that plants from the genus *Pterocarpus* are rather poorly documented in term of secondary metabolites).

Besides, some isolated compounds, *a posteriori* identified in NMR, have helped us to reconsider some abandoned spectra leading to the successful identification of some molecules.

4.5.3. Results

The data were acquired on the two fractions: butanol and water fractions of the trunk barks. Since, the butanol fraction presented more compounds than the water fraction, we have focussed on the butanol fraction for the identification of compounds.

4.5.3.1. Positive ion mode

In the positive mode, at least 500 compounds were detected excluding isomers and isobars. The chromatogram (Figure 3.9) obtained could roughly be subdivided into 4 parts:

- a first large peak containing very polar compounds like free sugars radicals and amino acids, eluted in the void volume (corresponding to a retention time of 1.50 min).
- a second large peak from 5 to 15 minutes. Many compounds were unidentified in this chromatogram area but as it will be seen in the following lines, some compounds were identified as isoflavone glycosides.
- the next area is a plate were some detected compounds exhibit interesting MS fragmentation spectrum. The compounds present in this area are for sure a little bit more "apolar" because of their higher retention time but it was impossible to connect these spectra with putative structures except for two of them (a pterocarpine or calycosin glucoside and maackiain).
- the last area is corresponding to the rinsing step. Some compounds were still eluted from the column but it was impossible to get their MS fragmentation, even at a collision energy higher than 35 eV.

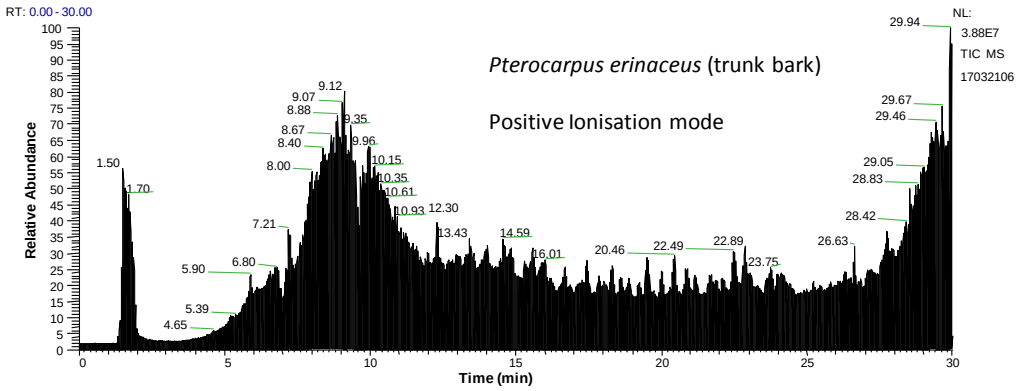
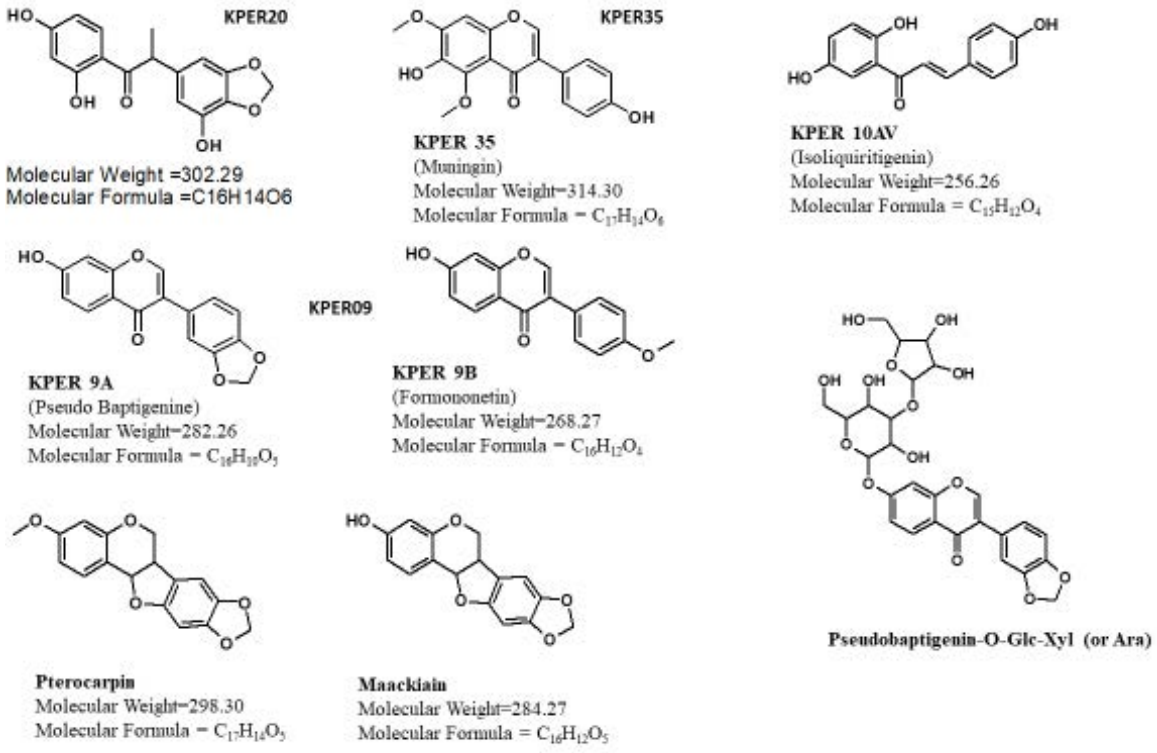


Figure 3.9: LC-MS-MS chromatogram of the butanol fraction of the trunk barks of *P. erinaceus* in the positive mode

The retention time is the number writing on top of some peaks. The intensity of the peaks is judged on the peak length. The spectrum could roughly be subdivided into 4 parts.

From those 500 detected compounds, 10 putative structures of compounds have been established using the strategy described in Section 4.2.2.2.

Some of the compounds isolated and formally identified in the roots of *P. erinaceus*, have also been identified in the butanol extract, either linked with sugar moieties or as aglycone: formononetin; pseudobaptigenin; pseudobaptigenin-glucose-xylose or pseudobaptigenin-glucose-arabinose; formononetin-glucose-xylose or formononetin-glucose-arabinose and muningin-glucose-xylose or muningin-glucose-arabinose (Figure 3.10).



<u>Figure 3.10:</u> Different types of flavonoids found in *P. erinaceus* roots and trunk barks during this study.

KPER 09, KPER 10AV, KPER 20 and KPER 35 were the compounds isolated from the roots of *P. erinaceus* and identified by NMR. They were, *a posteriori*, used to identify some of the molecules in the positive mode. The other compounds were identified using data from the bibliographic review on the genus *Pterocarpus*.

Other isoflavonoids have also been detected, alone or with sugar moieties: genistein-glucose-xylose or genistein-glucose-arabinose and isomer; santal-rhamnose-glucose; pterocarpine-

glucose-xylose or pterocarpine-glucose-arabinose or calycosin-glucose-xylose or calycosin-glucose-arabinose and maackiain (Table 3.17, Figures 3.10 and 3.11).

Table 3.17: List of compounds identified by LC-MS and LC-MS/MS positive ion mode in the butanol fraction of the trunk barks of *P. erinaceus*.

RT: retention time in min; n°: compound number; Glc: glucose, Xyl: xylose, Ara: arabinose, Rha: rhamnose. $[M+H]^+$: protonated (+) molecular ion.

n°	$[M+H]^+$ m/z	RT	Fragments	Putative structure
1	577	12.50	445-283	Pseudobaptigenin-Glc-Xyl (or Ara)
2	563	12.69	431-269	Formononetin-Glc-Xyl (or Ara)
3 and 3'	565	11.42 12.68	433-271	Genistein-Glc-Xyl (or Ara) and isomer
4	267	16.87	252	Formononetin
5	281	16.70	253	Pseudobaptigenin
6	607	12.59	477-315	Muningin-Glc-Xyl (or Ara)
7	607	13.36	461-299	Santal-Rha-Glc
9	579	15.66	447-285	Pterocarpine-Glc-Xyl (or Ara) or Calycosin-Glc-Xyl (or Ara)
10	299	17.80	284	Maackiain

The fragmentation of the glycosylated flavonoids occured step by step. The proces helped to establish that the two sugar moieties were connected to the flavonoid not at two different positions but as a disaccharide moiety connected to the flavonoid through *O*-links. Indeed, as shown in Figure 3.12, the loss of the two sugar moieties did not occur at the same time but in a consecutive way. Taking the example of Pseudobaptigenin-Glc-Xyl ($[M+H]^+$: 577 u), it would have directly led to a molecular peak at 283 u with 2 others at 162 u (glucose) and 132 u (xylose or arabinose) without an intermediate molecular peak at 445 u if the loss of the two sugars moieties had occured at the same time. The molecular peak at 445 u, in fact, corresponds to the fragment obtained after a loss of 132 u. The presence of the *O*-link was established based on the fragment obtained and the fact that *C*-*C* bonds are less easily breakable. In addition, when *C*-*C* bonds are broken, entire sugar moieties are not obtained but fragments.

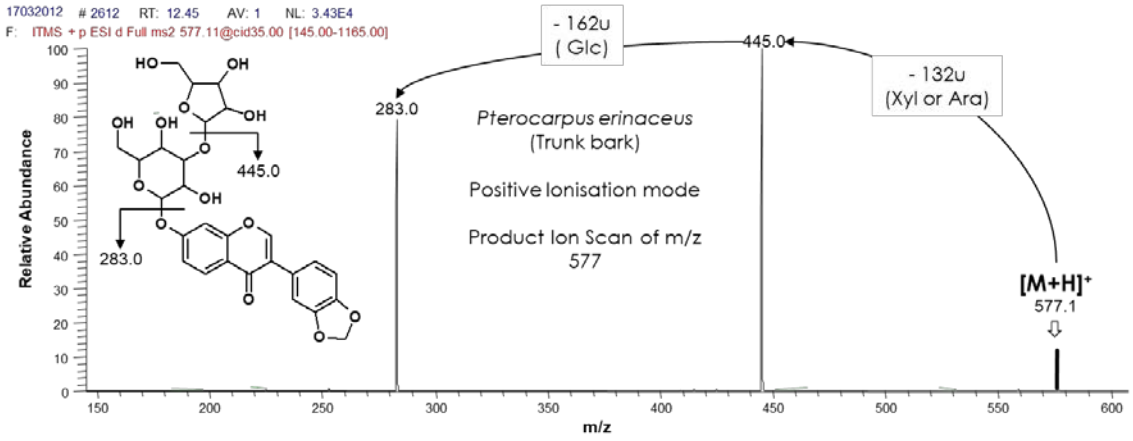


Figure 3.11: Example of a glycoside flavonoid fragmentation, Pseudobaptigenin-*O*-Glc-Xyl (or Ara).

The position of substitution indicated in the figure is a hypothesis. This structure is only given to show where the fragmentation occurs. [M+H]+: protonated (+) molecular ion. Glc: Glucose; Xyl; xylose and Ara: Arabinose.

4.5.3.2. Negative ion mode

In the negative ion mode, around 300 compounds were detected excluding the isomers and isobars. The chromatogram obtained (Figure 3.12) using that mode was like the one obtained in the positive mode, and could roughly be subdivided into the same 4 parts. However, the large peak from 5 to 15 min exhibits large amounts of proanthocyanidin oligomers and the following plate shows many hydroxycinnamic acid derivatives.

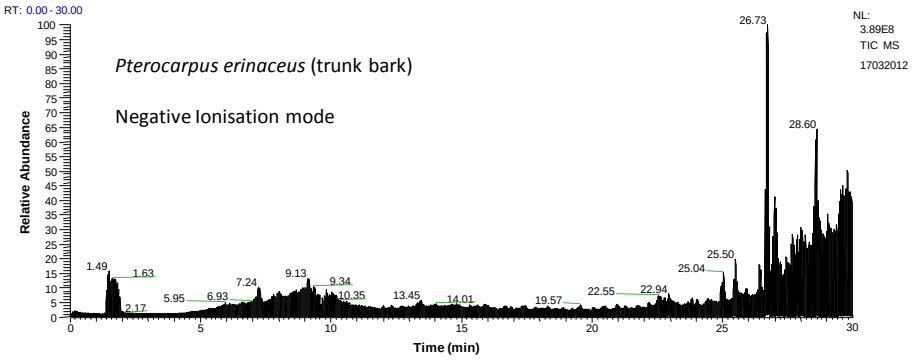


Figure 3.12: Mass spectrum of the butanol fraction of the trunk barks of *P. erinaceus* in the negative mode

The retention time is the number writing on top of some peaks. The intensity of the peaks is judged on the peak heigth. The spectrum could be roughly subdivided into 4 parts.

The following types of compounds have been identified: huge quantities of pro-anthocyanidins derivatives (dimers, trimers, tetramers, etc…), cinnamic acid derivatives (feruloyl-caffeoyl, tartaric acid, di-coumaryl ester derivative, di-feruloyl ester derivative, feruloyl-coumaroyl ester derivative, etc..), some triterpene pentacyclic glycosylated saponins, flavonoids-C-glycosylated and one acid (Table 3.18).

Table 3.18$_1$: List of compounds identified by LC-MS (negative mode) in the butanol fraction of the trunk barks of *P. erinaceus*

RT: retention time in minutes and n°: compound number.

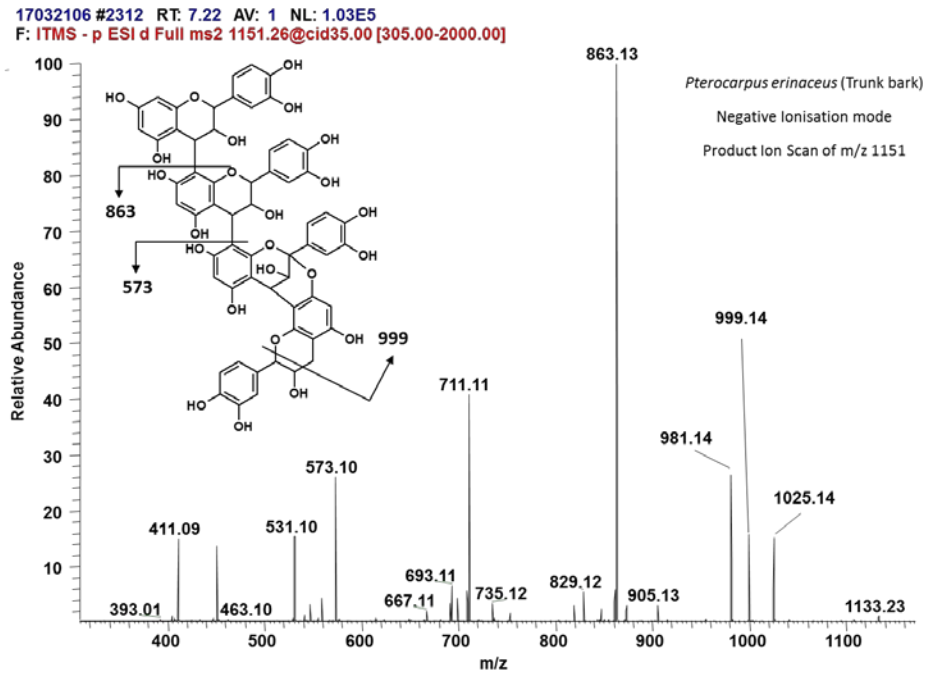
n°	[M-H]- m/z	RT	Fragments	Putative structure
1	341	1.50	179, 161, 143	Disaccharide
2	1153	7.22 9.31	1027, 1001, 983, 865, 863, 693, 577, 575, 425, 407, 289	Proanthocyanidin type B tetramers
3	1151	7.24 8.91 9.32	1025, 999, 981, 863, 861, 711, 693, 691, 573, 411	Single linked Proanthocyanidin type A tetramers
4	1439	7.98 10.80	1313, 1287, 1151, 1025, 863, 711, 573, 531, 451	Single linked Proanthocyanidin type A pentamers
5	863	8.66 8.73 10.06	711, 693, 573, 559, 449, 451, 423, 411, 407, 289	Single linked Proanthocyanidin type A trimers
6	577	8.66 10.98	559, 451, 425, 407, 289	Proanthocyanidin type B dimers
7	865	9.38 9.46	739, 713, 695, 577, 575, 449, 451, 425, 407, 289, 287	Proanthocyanidin type B trimers

Table 18_2: List of compounds identified by LC-MS (negative mode) in the butanol fraction of the trunk barks of *P. erinaceus*

RT: retention time in minutes; n°: compound number; *: only compound on which an MS^3 was realized.

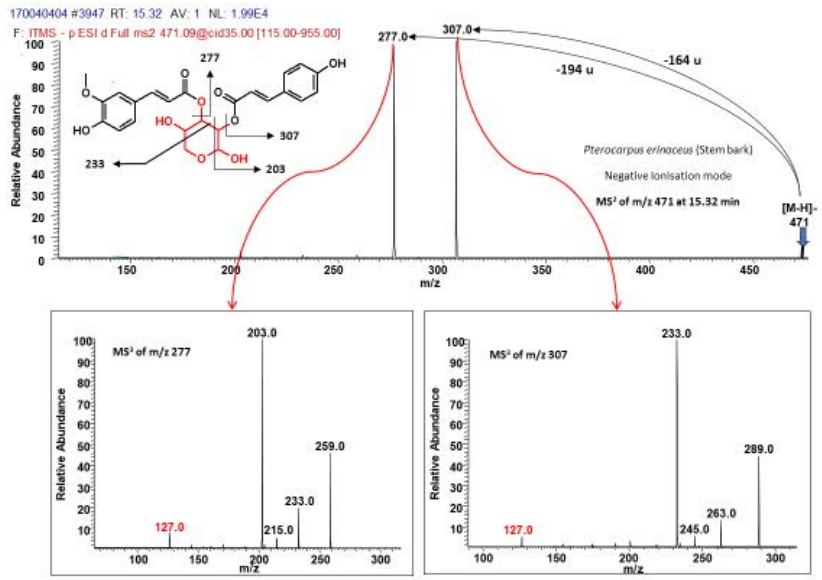
n°	[M-H]- m/z	RT	Fragments	Putative structure
8	295	9.52	163	Coutaric acid
9	467	13.57	325, 307, 293	Feruloyl-cafeoyl tartaric acid
10	441	14.48	277	Dicoumaryl ester derivative (xylose, arabinose or tartaric acid)
11	501	14.71	307	Diferuloyl ester derivative (xylan, arabinoxylan or tartaric acid)
12	471	15.32	307, 277	Feruloyl-coumaroyl ester derivative (xylan, arabino, xylan or tartaric acid (see MS^3 figures 14 and 15)*
13	329	17.11	311, 293, 229, 211, 171	Tri-hydroxyoctadecenoic acid (Oxylipin)
14	957	17,43 18.05 19.55	939, 895, 811, 749, 631, 541, 503, 473 (aglycone)	Pentacyclic triterpen saponins with a sugarchain composed of rhamnose-glucose-glucuronic acid

Instead of the flavonoids-O-glycosylated identified in the positive mode, flavonoids-C-glycosylated have been identified in the negative mode. This identification was established based on the observation of the loss of 60 u, 90 u and 120 u by many compounds. This loss is characteristic of internal fragmentation of sugar moieties. The structure of pro-anthocyanidins was established through observation of the fragments (as described in Figure 3. 13 for the pro-anthocyadinin type B tetramer identified at a RT of 7.22 and 9.33) and comparison to the literature (Zhang S and Zhu MJ, 2015; Lanming Gou *et al*., 2016).



<u>Figure 3.13:</u> Spectrum of fragmentation of a pro-anthocyadinin Type B tetramer.
The different breaks leading to various molecules are shown on the molecule. The Epicatechine loss and the fragment at 999 are characteristic of the molecule because they are the highest peaks obtained.

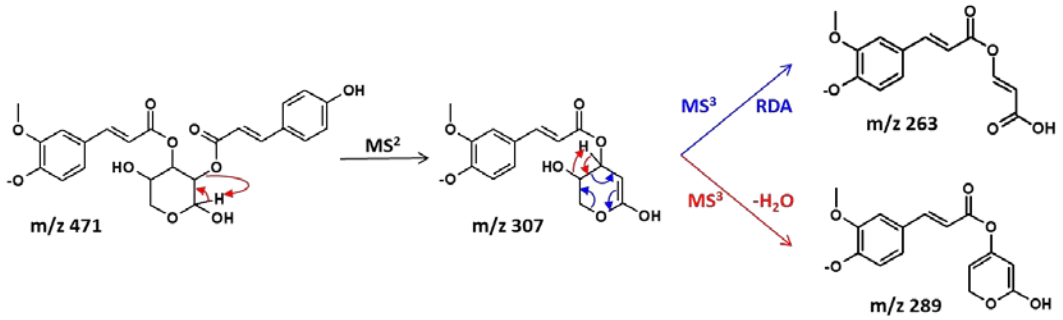
The structure of the hydroxycinnamic acid derivatives was also deduced through observation of the fragments and comparison with literature (Akin *et al*., 1993; Flamini, 2013). One example is the feruloyl-coumaroyl ester derivative (xylan, arabinoxylan or tartaric acid). Its fragmentation patterns are shown in Figure 3.14.



<u>Figure 3.14</u>: Spectrum of fragmentation of a Feruloyl-coumaroyl ester derivative (a cinnamic acid compound)

$[M+H]^-$: molecular ion. After an MS^2, two fragments were obtained at 277 and 307. An MS^3 was then carried out on the fragments 277 and 307. The different patterns obtained were corresponding to the ones reported in the literature (Schnitzler Miriam *et al*., 2007).

It is the only compound on which an MS^3 has been performed with the different patterns obtained presented in Figures 3.14 and 3.15. The structure of the feruloyl-coumaroyl ester derivative was also established through observation of the patterns obtained and comparison with bibliography (Schnitzler Miriam *et al*., 2007).



<u>Figure 3.15</u>: Possible fragmentation patterns of the compound Feruloyl-coumaroyl ester derivative

The formation of the fragments obtained at m/z 263 and 289 were due to a loss of water and a Retro-Diels-Alter-Reaction (RDA), an intern electronic rearrangement of the compound.

The description of glycosylated pentacyclic triterpenes was achieved based on the observation of the patterns as in Figure 3.16 and previous work (Llorent-Martínez *et al*., 2015) where the aglycone was identified as soyasapogenol. In the present study, the same m/z was obtained at 473. Unfortunately, the aglycone could not be confirmed to exactly correspond to this compound. There is a possibility that it could be an isomer. An acid, the tri-hydroxy-octadecenoic acid (oxylipin family) was also detected. The spectrum obtained was the same as the one reported of an oxypilin present in bamboo (Table 3.18) (Van Hoyweghen *et al*., 2014).

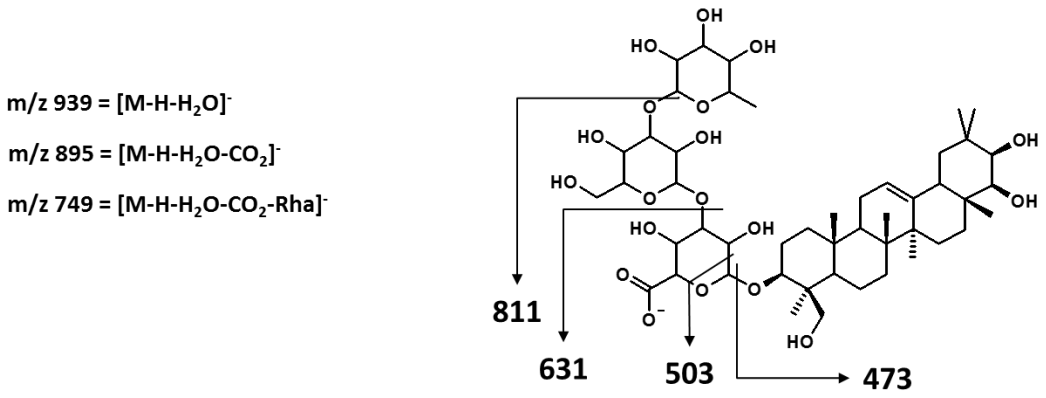


Figure 3.16: Fragmentation of a saponin triterpene

The following fragments 811, 631, 503 and 403 have been obtained after different breaks. The 473 fragment corresponds to the aglycone Soyasapogenol. The same patterns have been obtained on the saponin triterpene identified in our work with an aglycone mass at 473 u. But it is not sufficient to certify that the aglycone obtained in our study is also the Soyasapogenol (Retrieved from Llorent-Martínez *et al*., 2015).

5. Purification

5.1. Definition and methods

Purification was aimed at isolating individual, pure compounds from the extracts and/or active fractions. It was carried out using liquid and planar chromatography. The liquid chromatography used were Flash chromatography (FC) and Low-Pressure Liquid Chromatography (LPLC) while Thin Layer Chromatography (TLC) was used as the planar chromatography.

- Low-pressure liquid chromatography (LPLC)

Column chromatographic methods which allow the flow of the mobile phase at atmospheric pressure without additional forces either by vacuum or pressure are still a major tool in the fractionation protocols for natural products isolation. They are also named Open column chromatography. All the different types of chromatographic mechanisms and stationary phases already described, could be used under this type (Bucar *et al*., 2013).

- Flash chromatography (FC)

Flash chromatography is mainly used for rapid fractionation of crude extracts or coarsely purified fractions. By applying nitrogen or compressed air, the mobile phase is flushed through the stationary phase in a tightly closed glass column or prepacked cartridges. In comparison to open-column chromatography, smaller particle size (ca. 40 μm in the case of silica) can be used, hence increasing peak resolution. Online peak detection is also possible, usually by coupling to a UV detector (Bucar *et al*., 2013).

- Thin layer chromatography (TLC)

Planar chromatography refers when the stationary phase is a large flat surface of small thickness. The flat surface could either be a paper (Paper chromatography) or a sheet of glass, plastic or aluminum foil (Thin layer chromatography). Paper chromatography is no longer used, the TLC is the common one nowadays.

In TLC, the sample to be analyzed is spotted near the bottom line of the sheet, dried and put in an airtight chamber containing the mobile phase (MP). The MP will separate the different components of the sample by capillarity. The sheet of glass, plastic or aluminum foil is coated with an adsorbent material of different thicknesses (silica gel, alumina or cellulose) containing an organic binder and a fluorescent indicator. The most used plates are 60F plates containing silica gel with a 60 Å (6 nm) pore diameter and phosphor element fluorescing at 254 nm (to observe analytes under UV). The use of bonded C-18 (also designated RP-18 by some manufacturers) and a diol, CN, and NH_2 (Normal Phase or Reverse Phase depending on the MP and analyte) layers, is also increasing. The advantage of the TLC resides in the fact that the desired substance could be obtained, identified or recognized by zone detection/ identification and or separation (removal of the compound by scratching its zone on the TLC and recovering with dissolution in an appropriate solvent). Zone detection and identification could be realized using natural color, fluorescence or UV absorption, chemical, biological and other detection methods directly applied onto the plate or associated to it. Some of them are versatile, allowing the removal of a compound. The separation is monitored using the Retention factor (R_f). R_f is equal to the ratio of the distance traveled by the center of a spot to

the distance traveled by the solvent front ($Rf = \frac{distance\ of\ migration}{total\ distance}$). And the R_f is unique to every molecule. By using standards on the TLC plate and comparing the different R_fs, one could also be able to identify and or recognize compounds of particular interest (Sherma, 2010; Bucar *et al*., 2013).

Concerning the chemical detection in our work, we have used di-iode and molybdate to recognize organic compounds in general and particularly identify compounds with saturated links with the diode and steroids with the molybdate.

5.2. Purification performed on the roots of *Pterocarpus erinaceus*

The raw extract (MeOH-DCM; 1:1) of the roots has been separated further using normal phase open column chromatography. The stationary phase was composed of normal silica gel (Standard silica 60, 0.063-0.200 mm, Merck) and the mobile phase was made of solvents and mixtures of solvent of different polarities: petroleum ether (100%), petroleum ether-ethyl acetate (95:5, 90:10, 85:15, 75:25, 65:35, 50:50, 30:70, 10:90 in volume); ethyl acetate (100% in volume), ethyl acetate-methanol (98.5:2.5, 95:5, 90:10, 80:20 in volume) and methanol (100% in volume) to obtain 170 fraction- vials drops. Vials drop 1-11 obtained under petroleum ether-ethyl acetate (95:5) developed white crystals after removal of the solvent. A TLC revealed them to be alike and they were named **KPER1**.

Vials drop 151-155 obtained under ethyl acetate-methanol (90:10) were observed to develop reddish/caramelize crystals after removal of the solvent. A comparative TLC revealed the crystals to be alike, they were put together and named **KPER 7**.

- Sub-fraction 1

After a comparative TLC, vials 74-76 were pulled together to obtain sub-fraction 1. An open column chromatography was realized as precedent on this sub-fraction using cyclohexane (100% in volume), cyclohexane-dichloromethane (6:4; 2:8 in volume), dichloromethane (100% in volume); dichloromethane-methanol (99:1, 98:2, 97:3 in volume) and methanol (100% in volume) to obtain 24 vials drops. The vials drop 15-16 developed an off-white powder after removal of the solvent. A TLC revealed the compounds to be the same, they were put together and named **KPER 9**.

Vials 12-14 obtained under dichloromethane-methanol (99:1 and 98:2 in volume) were pulled together after TLC and subject to another normal phase open column chromatography with cyclohexane-dichloromethane (9:1; 7:3; 6:4; 1:1; 2:3 in volume), dichloromethane (100% in volume), dichloromethane-methanol (9.5:0.5; 9:1; 8:2; 1:1 in volume) and methanol (100% in

volume). Vials 26-30 obtained under dichloromethane-methanol (9.5:0.5) after a TLC were pulled together and subject to a preparative TLC followed by a normal phase flash chromatography with dichloromethane-methanol (9.5:0.5) and yield a purple powder named **KPER 20**. Vial 17 obtained under dichloromethane 100% was also subject to a preparative TLC followed by a normal phase flash chromatography with dichloromethane-methanol (99:1 in volume) and yield a yellow bright powder named **KPER 10AV**.

- Sub-fraction 3

Vials drop 19-21 obtained under cyclohexane-ethyl acetate (60:40 in volume) were observed to develop translucent white crystals after removal of the solvent. The crystals were put together after TLC and named **KPER 35**.

- Sub-fraction 4

Vials drops 20-44 obtained under ethyl acetate - methanol (62.5: 27.5; 67.5: 22.5; 75:25; 0: 100; 98: 2 in volume) were put together after a comparative TLC, subject to repetitive preparative TLC followed by a flash chromatography with dichloromethane-methanol (8:2) to yield a dark yellow powder (in comparison to **KPER 10AV** a bright yellow compound) named **KPER 37B** (more polar than **KPER 10AV**).

- Sub-fraction 5

Vials drops 92 to 153, obtained under petroleum ether-ethyl acetate (90:10 in volume); ethyl acetate (100% in volume), ethyl acetate-methanol (98.5:2.5, 95:5, 90:10 in volume) were put together after a comparative TLC and subject to a normal phase open column chromatography using cyclohexane - ethyl acetate (2:8 in volume); ethyl acetate (100% in volume), ethyl acetate-methanol (98:2; 95:5; 90: 10; 85:15; 80:20; 70:30; 60:40 in volume) and methanol (100%). A white crystalline compound precipitated in the vials drops 14 to 33. After a TLC which proved the compounds obtained in the different vials to be alike, they were put together and named **KPER 39**.

5.3. Purification performed on the trunk barks of *P. erinaceus*

The ethyl acetate fraction (12.9 g) has been separated further using normal phase open column chromatography (Standard silica gel 60, 0.063-0.200 mm, Merck) with cyclohexane-dichloromethane (1:1, 2:3, 3:7, 2:8, 1:9; v:v); dichloromethane (100%); dichloromethane-methanol (98:2, 95:5, 80:20; v:v); methanol (100%) to obtain 747 fraction- vials drops. Vials drop 70-100 obtained under cyclohexane-dichloromethane (2:3; v:v) afforded white crystals. A TLC (TLC silica gel 60 F254, Merck Millipore, 1.05554.0001) on these crystals revealed

them to be alike. They were put together and named **Compound C 1**. Crystalline powder precipitated in vials drops 641-651 obtained under dichloromethane-methanol (80:20; v:v). After TLC analysis, these crystals were combined and named **Compound C 2**. Vials drop 272-310 obtained under cyclohexane-dichloromethane (2:3, v:v) for 272-291 and (3:7; v:v) for 292-310, were observed to develop crystals after removal of the solvent. After TLC analysis, crystals were combined and named **Compound C 3**. Vial drops 695-704 afforded a white crystalline powder corresponding to **Compound C 4**.

6. Qualitative analysis of the isolated compounds

The first analysis performed on pure compounds when they are obtained is the Nuclear Magnetic Resonance (NMR) to give the complete structure of the compound. Then the mass spectrum is carried out to confirm the molecular mass of the compound obtained. The ultraviolet (UV) and infrared (IR) are then performed to give the wavelengths of compounds which absorb in those fields. The melting point is realized to give the temperature at which the compound melts. All those parameters are characteristic of a compound and give its identity card.

6.1. Nuclear Magnetic Resonance

6.1.1. Definition

Electrons and some specific atoms nuclei have the capacity to rotate around themselves. This capacity has been named spin and in the case of the electron, the Quantic number related to this property, which is called spin number "s" could only take 2 values: +1/2 and -1/2 depending on the direction of the rotation. In the case of the nucleus of an atom, their spins are depending on their constitution. Thus, when the nucleus has an even number of neutrons and protons, the resulting spin (nuclear spin) from the addition is equal to zero (eg: ^{12}C, ^{16}O, ^{32}S). in contrast, when one of its constituents is odd, the resulting is a multiple of ½ and the atom has consequently a nuclear spin (^{1}H, ^{13}C, ^{19}F, ^{31}P, ^{15}N, etc..). It could also happen that both constituents of the nucleus are odd, in that case, the nuclear spin is equal to 1 and can take 3 values, -1, 0 and +1. Spinning nuclei naturally exhibit a magnetic moment (analogous to the magnétic field of the Earth) whose orientation depends on the sign of the spin number. The intensity of this magnetic moment is also proportional to the sign of the spin number. When nuclei possessing spin are placed in a magnetic field, they do no longer turn around

themselves but around the direction of the magnetic field applied. This movement is called Larmor movement. By having such a movement, the nucleus consequently acquires an energy that leads it from a fundamental state to an excited state. Thus, when a sample is introduced in an NMR apparatus, its nuclei are in this state. When a radiofrequency pulse (10-800 MHz, corresponding to a wavelength of 30-40 cm) is applied, in addition to the magnetic field already existing, more energy is giving to the nuclei, absorbed by them, leading almost all of them to the excited state consequently leading to the saturation at the excited state (no more absorption could be done), obliging the nuclei to come back to the fundamental state. When coming back to the fundamental state, they emit an electromagnetic wave: it is the relaxation, corresponding to the magnetic resonance. This electromagnetic wave is characteristic or particular to every nucleus or particle (it does depend not only on the passage of the nucleus from an excited to a fundamental state but also to its neighboring molecular environment and the energetic interactions between them). By recording the different sound when a sample is put in an NMR apparatus, one could deduce information on the chemical structure of the molecules.

There are two types of NMR experiments: the ^{1}D and the ^{2}D NMR experiments.

In the following lines, we will use the example of one compound we have isolated from the roots of *P. erinaceus*, KPER 35 (which has been identified as Muningin, after running all the types of NMR experiments), to explain the different types of NMR experiments run on a pure compound in natural products chemistry.

6.1.2. First order NMR (NMR ^{1}D)

This NMR 1D is the widely used in chemical experiments especially the ^{1}H and ^{13}C experiments (because almost all the elements existing in nature do contain those two types of the element). In the spectrum of such an experiment, the chemical shift is reported on the horizontal axis and the intensity on the vertical axis. To explain a 1D NMR spectrum, the following parameters are to be considered: the number of signals; the chemical shift δ; the signal multiplicity; the coupling constant J; and the signal integrals (Darbeau, 2006).

As said before, illustrations will be realized using the NMR results of the compound KPER35 which has been identified as an isoflavone (Figure 3.17). After the decrypting of all the NMR data, this isoflavone was identified as the Muningin.

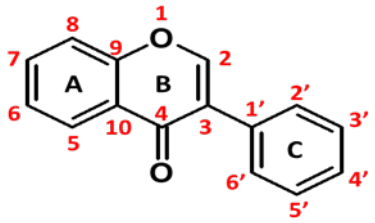


Figure 3.17: Structure and numbering of an isoflavone skeleton

A, B and C represent the different cycles of an isoflavone

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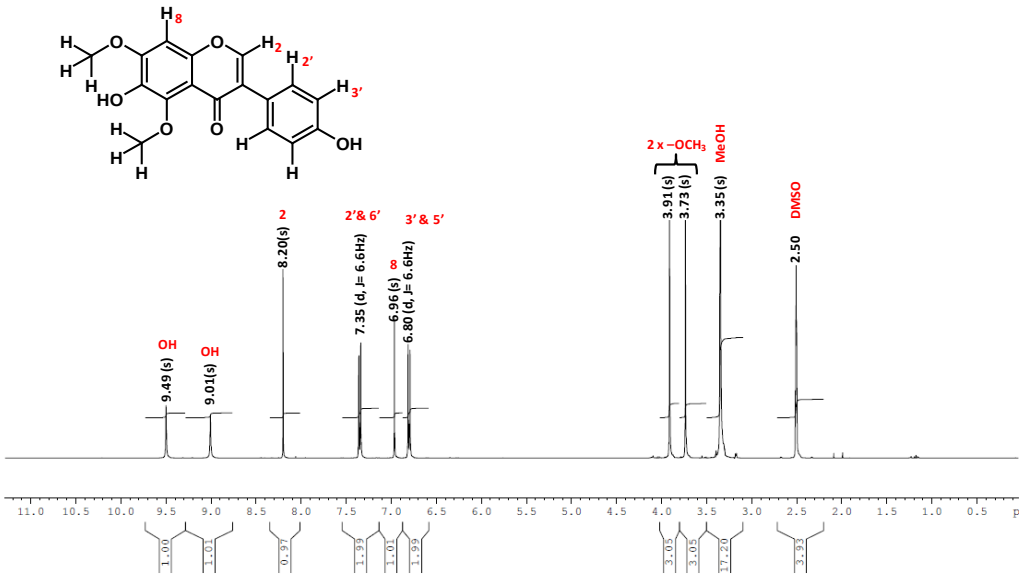
6.1.2.1. ^{1}H NMR

The ^{1}H NMR spectrum provides several types of information on the studied molecule necessary to get its structure.

The first set of information is obtained from the chemical shifts. Isochrone protons have the same chemical shift (δ). Depending on the distance betwenn the chemical shift and the reference signal (TetraMethylSilane or TMS), the chemical shift is said to be ”shielded” when close to the TMS signal and ”unshielded” when far from the TMS signal.

How shielded the proton is, depends on the type of atom bearing it (related to its electronegativity), the chemical substituents carried by this atom and the nature of the neighboring atoms and the chemical substituents carried by them. This shield could be explained by the fact that the electrons around the proton create a magnetic field that opposes the applied field. This reduces the field experienced by the nucleus and therefore decreases the frequency required for the absorption. Consequently, the chemical shift (delta /ppm) will change depending on the electron density around the proton. Since electronegative groups decrease the electron density, there will be less shielding (deshielding) and the chemical shift will increase. For example, protons in the neighborhood of an oxygen atom exhibit a higher chemical shift than in a carbonaceous environment because oxygen is more electronegative and thus more attractive than carbon toward the electron shell. This is the case for example of the protons of the 2 methoxy groups of KPER 35 (Figure 3.18) which appears at around 3.5 ppm whereas protons of methyl groups linked to hydrocarbon chains are expected at around 1ppm and even less. Attractive effects (mesomeric, inductive effects) on the protons will result in it to have signals at down fields (unshielded) and additive effects on it will lead it to have signals at high fields (shielded signal). It is important to note that the chemical shift of protons is strongly dependent on the solvent used to perform the NMR experiment.

The integration of the area of each signal represented on the spectrum of ^{1}H is proportional to the number of corresponding protons. This integration represents somehow a way (even if it is sometimes more or less easy because of symmetry plan in the molecule) of counting the hydrogen atoms present in a functional group. Thus the ^{1}H NMR spectrum of the KPER 35 (Figure 3.18) shows an integration of 3 for the methoxy groups, 2 for the protons 2' and 6' (they are equivalents because of the symmetry in the substitution the aromatic ring) and 1 for phenol groups.



<u>Figure 3.18:</u> The ^{1}H-NMR spectrum of KPER 35 in d6-DMSO.

The ^{1}H-NMR is the first spectrum acquired from the series. Even if some information can be obtained from this simple experiment, it is very often not enough informative to get the whole structure elucidated. At this stage, the molecule appears to contain 14 atoms of hydrogen in its structure.

The spin-spin coupling of studied protons with its neighbors are traduced on the spectrum by the multiplicity of its signal, i.e. the number of the peak (N_{peaks}) in which the signal is split and the value(s) of the associated coupling constant(s) J (expressed in Hz). When a proton has **n** equivalent neighbor protons, The multiplicity is expressed by the Equation 3.1:

$$N_{peaks} = n + 1 \qquad \text{(Equation 3.1)}$$

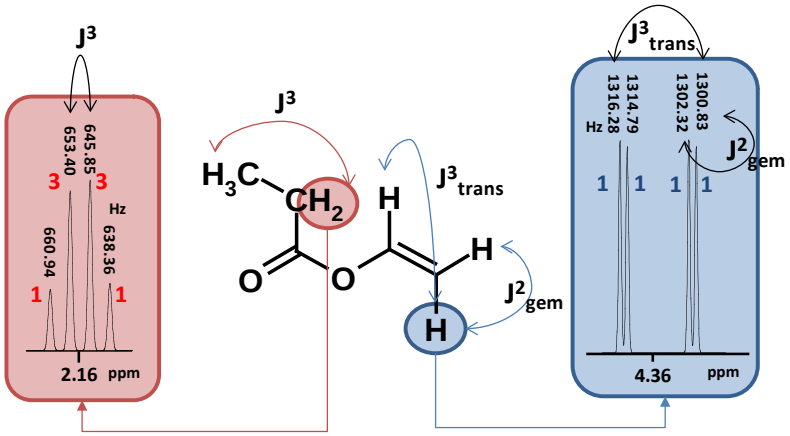
This equation remains under a single peak called singlet (s) if the proton is isolated (no neighbour) as for most of the protons of the Muningin. The only coupled protons appear for the C-ring of this isoflavone. Protons 2' and 6' (equivalents because of the symmetry in the substitution the aromatic ring) are coupled to protons 3' and 5' (they are also equivalent) because the distance between 2' and 3' is less than 4 bonds. Indeed, they are far away from

only 3 bounds (called J^3) and the expected value of such a coupling constant is between 6 and 8 Hz (here 6.6 Hz). This signal is observed as a doublet (n=1) with the same intensity for each peak (relative intensity of 1/1) When the number of equivalent neighbours is two, a triplet is observed (with relatives intensities of 1/2/1), a quadruplet (with relative intensities of 1/3/3/1) for 3 (Figure 3.19, signal in the red box), etc.

Some protons can also be coupled with other protons with different coupling constants. In the case of neighboring groups possessing $\mathbf{n_1}$ and $\mathbf{n_2}$ protons, the multiplicity of its signal is given by the Equation 3.2.

$$N_{peaks} = (n_1 + 1) \times (n_2 + 1) \qquad \text{(Equation 3.2)}$$

The relative intensity of these multiplets helps distinguish those systems from systems containing only equivalent protons. For example, if one proton is coupled with two other protons having two different J values (J_1 and J_2), 4 peaks are expected. With two equivalent protons, a quadruplet is expected with relative intensities of 1/3/3/1 for the four peaks.



<u>Figure 3.19:</u> Illustration of the difference between a quadruplet in red (J^3 = 7.5 Hz) and a double doublet in blue (J^2_{gem} = 1.6 Hz and J^3_{trans} = 14 Hz).

The distance between each peak corresponds to the coupling constant. For two different protons, 4 peaks are also expected but their relative abundance is 1/1/1/1 and the two coupling constant can be measured from their distances. The highest distance corresponds to the highest coupling constant and the smallest one to the smallest coupling constant. This type of multiplet is not a quadruplet but a double doublet. Many other combinations exist such as double triplets, triple triplets but these kinds of signals are often observed in ethylenic or aromatic systems and are not present on the KPER 35 spectrum (Figure 3.18).

<u>Example</u>: ^{1}H of the compound KPER 35 (Figure 3.18).

Knowing nothing about the molecule, the ^{1}H NMR of KPER35 (isolated from the roots of *P.erinaceus*), two signals corresponding to 2 different methoxy groups (3H) appears clearly at 3.75 and 3.91 ppm. Then the area corresponding to 6.5 to 8.5 ppm is very often attributable to

aromatic protons. 6 protons appear in this area among which a system of 2 x 2 protons coupled together with a 6.6 Hz constant. This is typical of an aromatic ring substituted in the para position. The two last signals are corresponding of the two de-shielded signal of H of the two OH (singlet, the spin-spin coupling through heteroatoms is very rare). This in addition to the presence of a high number of peaks in area 6 to 10 ppm is characteristic of an isoflavone (Figure 17) which are moreover found in many *Pterocarpus* species (Krishnaveni KS and Rao J, 2000; Mitra J and Joshi T, 1982). We could also notice two additional signals which could be attributed to the solvents: residual methanol traces and DMSO the solvent of dissolution of the compound.

6.1.2.2. ^{13}C NMR

The ^{13}C NMR is less sensitive than the ^{1}H NMR because of the low natural abundance of the ^{13}C and also its weakest gyromagnetic ratio compared to proton. Consequently, in practice, the ^{13}C NMR requires more quantity than the ^{1}H one. To gain in sensitivity, the ^{13}C-NMR spectrum is decoupled and consequently, every signal appears as a singlet (Figure 3.20, blue spectrum). The rules governing its spectrum are the same as the ones of the ^{1}H NMR. The difference between carbons lies in the chemical shift which varies from 0 to about 250 ppm depending on their chemical environment. In contrast to the ^{1}H NMR, the integration of the signal is not relevant but the spectrum helps getting the number of carbons in the molecule and the chemical shift is not influenced by the solvent. The highest chemical shifts, over 170 ppm, usually correspond to carbonyl function. The signal at 173.9 ppm reflects the presence of a unique carbonyl as expected in an isoflavone (C10).

Something very special with the ^{13}C NMR is the Distortionless Enhancement by Polarization Transfer (DEPT) experiment. This experiment allows to observe and differentiate the types of carbons present in the spectrum depending on their substitution with protons: quaternary (C), tertiary (CH), secondary (CH_2) or primary (CH_3) carbons. In such experiment, the pulse has a flip angle of 45° (DEPT 45); 90° (DEPT 90) and 135° (DEPT 135). The sign of the signals will reveal the substitution of the carbons. In a DEPT 45, all the signals will be positive. In a DEPT 90, only signals of CH groups will show and in a DEPT 135, signals of CH_2 will be negative while the ones of CH and CH_3 will be positive. Most of the time, the DEPT 135 and 90 are run together with the ^{13}C NMR to be able to differentiate all the types of carbons present in the spectrum.

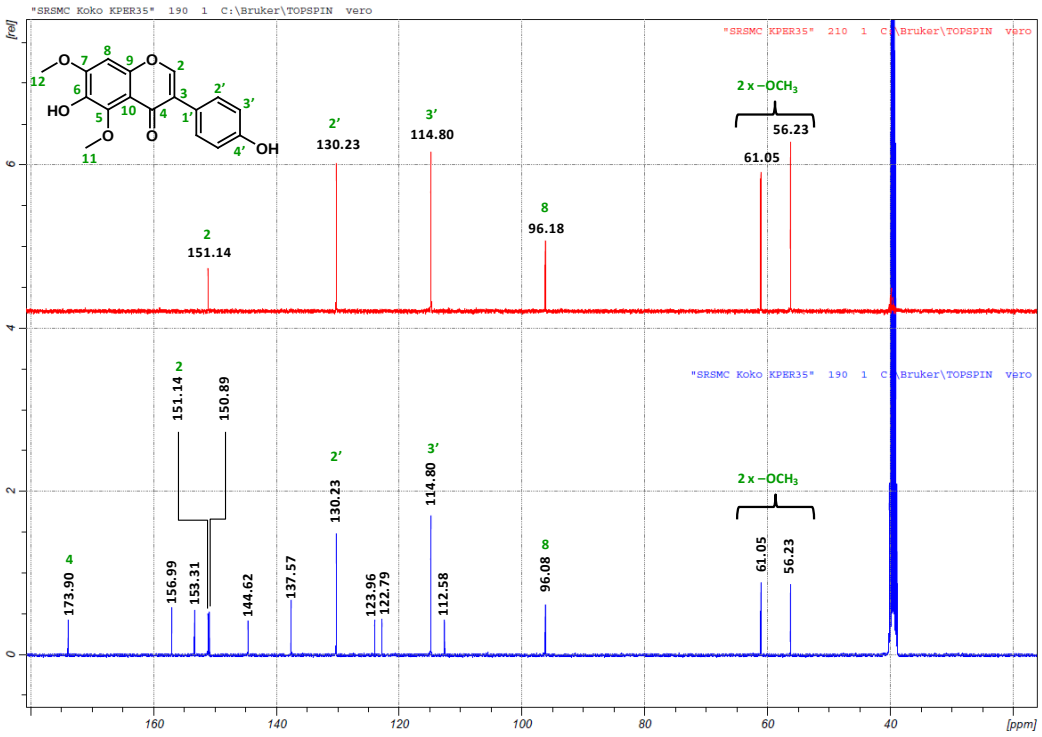


Figure 3.20: The ^{13}C NMR (in blue) spectrum in d6-DMSO of KPER 35 run together with a DEPT 135 (in red).

The ^{13}C-NMR (including DEPT 135) is the second spectrum acquired from the series. Even if some additional information has been obtained from this new experiment, it is very often still not enough informative to get the whole structure elucidated. At this stage, the molecule appears to contain 15 atoms of carbon (we also have to remember from the isoflavone structure that there are 2 x 2 equivalent carbons in the C ring) in its structure.

Example: ^{13}C of the compound KPER 35.

In the ^{13}C spectrum of Muningin (Figure 20), a total number of 14 C is revealed in the molecule and bearing in mind the isoflavone skeleton, one could deduce the presence of one ethyl radical or two methyl radicals. In the DEPT 135 (Figure 11, red spectrum), we have positive signals only, confirming the absence of CH_2 in this molecule, just CH, and CH_3.

6.1.3. ^{2}D NMR

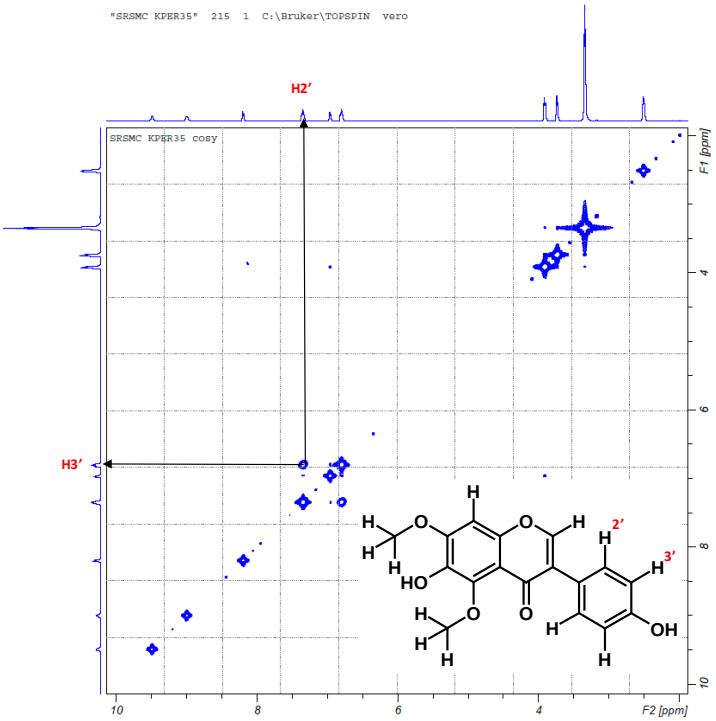
In a ^{2}D NMR spectrum, a point has as coordinates one intensity and 2 frequencies. The implications of the coordinates depend on the types of experiments to be performed. The ones most used in natural products chemistry are going to be described: the COSY (Correlation Spectroscopy) COSY, the Nuclear Overhauser Effect Stereoscopy (NOESY), the Rotating

frame Nuclear Overhauser Effect Spectroscopy (ROESY), the Heteronuclear Single Quantum Correlation (HSQC) and the Heteronuclear Multiple Bond Correlation (HMBC).

The COSY experiment is run for proton spectra. It is an homonuclear ^{1}H-^{1}H spin scalar coupling (coupling through bonds) experiment. It helps attributing the various corresponding chemical shifts to coupling protons of a ^{1}H NMR spectrum. The NOESY ^{1}H-^{1}H and the ROESY ^{1}H-^{1}H are also homonuclear experiment, not scalar coupling but coupling trough space. The space coupling arises in ^{1}H when protons are within 5-6 Å from each other: is the Overhauser effect. They help determine which signals arise from protons close to each other in space even if they are not bonded through spin-lattice relaxation for the first one and spin-spin relaxation for the second one. The main difference between both is that the first one is used for small molecules and the second one for big molecules (1000-2000 g/mol).

<u>Example</u>: The COSY and ROESY spectra of KPER 35

By drawing orthogonal axes, one could easily correlate the H2' and H3'in the COSY spectrum, suggesting those two protons to be separated by a direct link (Figure 3.21) confirming our hypothesis formulated from the ^{1}H-NMR spectrum. This is the only correlation that could be expected to be observed if the studied molecule is the Muningin.



<u>Figure 3.21</u>: COSY spectrum of KPER 35 showing the correlation between H2' and H3'. Two axes were drawn (in black on the figure) to establish the link between the two protons.

In the ROESY spectrum (Figure 3.22), knowing already the positions of the protons as described in the ^{1}H spectrum of KPER 35 (Figure 3.18), perpendicular axes are drawn to correlate protons on the two axes, it helps realize that the proton H8 is closed to one of the two methoxy groups. Knowing the position of H8, we could conclude that this proton could only be on the methoxy group in position 7, accordingly the position of the second methoxy is position 5. It also shows us that the proton H3' is close to one proton belonging to one of the two phenol groups, consequently this phenol is in position 4 (Figure 3.22).

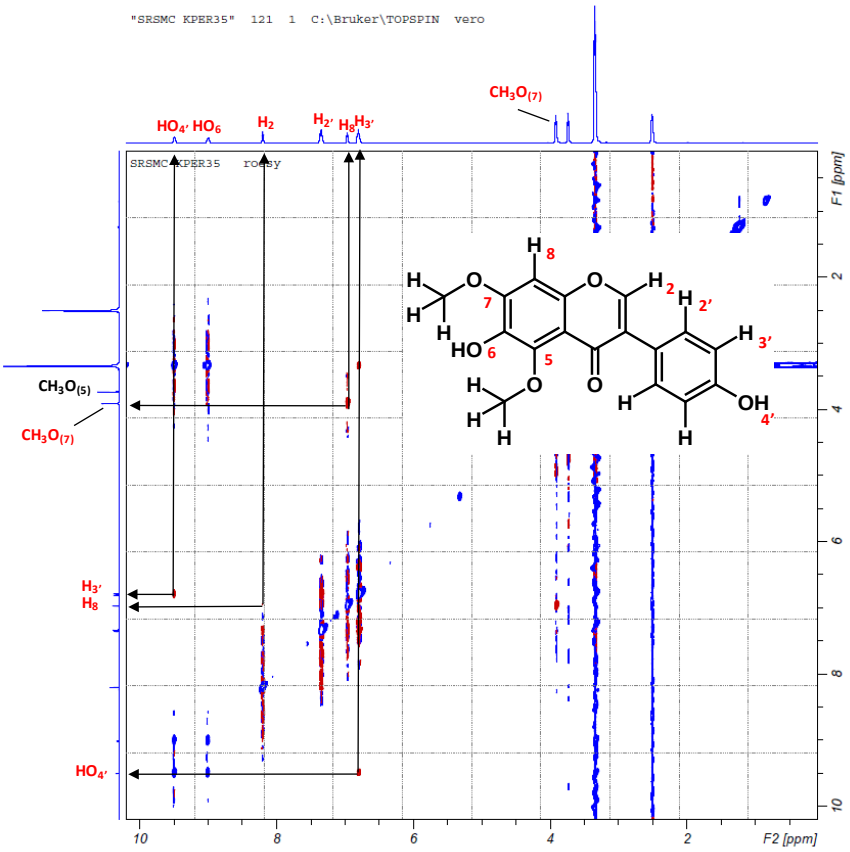


Figure 3.22: ROESY spectrum of KPER35 and some of the connectivities helping the spectrum indexation.

Axes are drawn, coming from the abscissa and coordinate, departing from a blue spot on the figure. It helps attribute the different protons that are close to each other and deduce the positions of some of them.

The ^{2}D NMR could also be realized to correlate the chemical shifts of two different nuclei such as the ^{13}C with the ^{1}H (or the ^{15}N if the molecule contains nitrogen). Such experiments are called heteronuclear ^{2}D NMR. The most commonly used are the HMBC and the HSQC experiments which correlate the chemical shifts of the ^{13}C with the ^{1}H. the HSQC gives the

correlations between directly bonded ^{13}C and ^{1}H with the observation being centered onto protons ($^{1}J_{CH}$). The HMBC gives the correlations between ^{13}C and ^{1}H separated by two, three and sometimes 4 bonds, the direct correlations are suppressed.

Example: The HSQC and HMBC spectra of KPER 35

In the HSQC and HMBC spectra of KPER 35 (Figure 3.23), the ^{1}H is represented on the absciss axis and the ^{13}C on the ordinate abscissa. Having already identified the proton positions, by drawing perpendicular axes we could immediately identify the carbons directly bonded to those different protons.

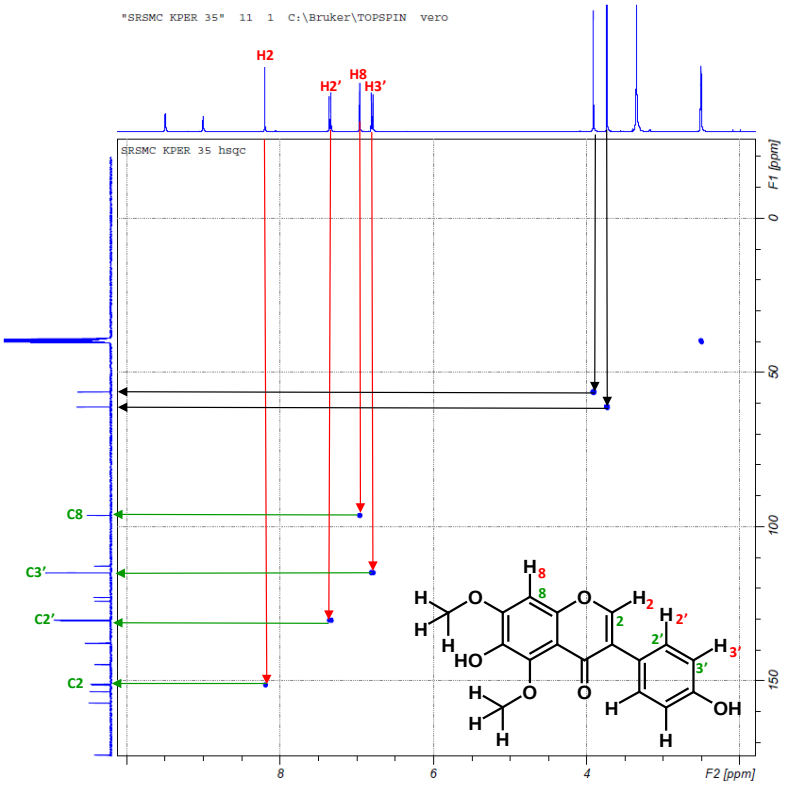
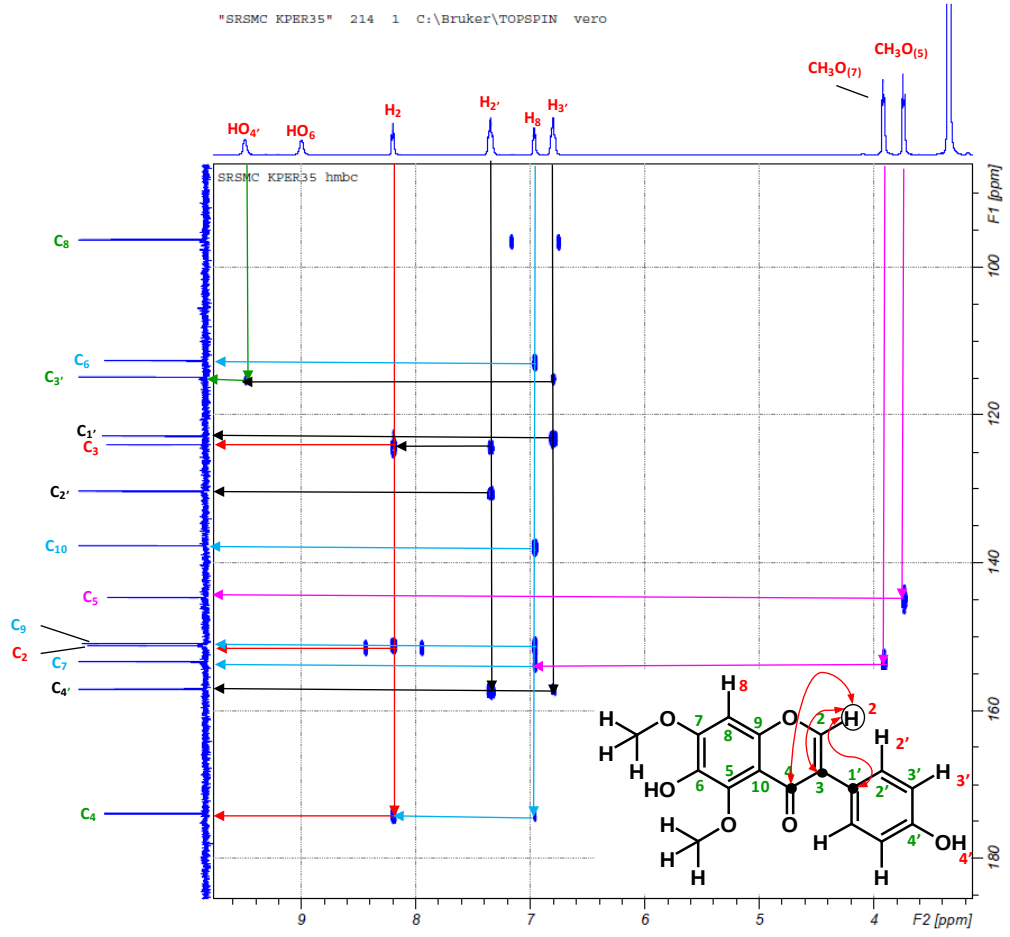


Figure 3.23: HSQC spectrum of KPER 35 and the connectivities helping the spectrum indexation

The ^{1}H spectrum is represented on the abscissa axis and the ^{13}C spectrum on the ordinate axis. Departing from the blue spots on the figure, red straight lines are drawn from the ^{1}H spectrum and green lines from the ^{13}C spectrum to link protons that are directly connected to carbons. Carbons bearing the previously identified protons are immediately recognized. Black axes were also drawn to connect the protons not already identified to the carbons that bear them.

In the HMBC spectrum (Figure 3.24), we could distinguish the different correlations of the protons with the carbons. For example, the proton H2 with the carbons in position 3 and 4,

therefore, confirming their respective positions. The two methyls are also correlating with carbons in positions 5 and 7, confirming their respective positions.



<u>Figure 3.24</u>: HMBC spectrum of KPER 35 and some of the connectivities helping the spectrum indexation.

The ^{1}H spectrum is represented on the abscissa axis and the ^{13}C spectrum on the ordinate axis. Departing from the blue spots on the figure, red, black, pink, green and blue straight lines are drawn from the ^{1}H spectrum and to connect with their counterparts from the ^{13}C spectrum. It helps establish the distant correlation that every carbon has with all its neighboring protons as shown on the figure with the carbon in position 4.

Based on all those experiments, a complete structure of the compound KPER 35 could be established (Figure 3.25). Every chemical shift on the ^{1}H spectrum and ^{13}C could be attributed. It is the Muningin.

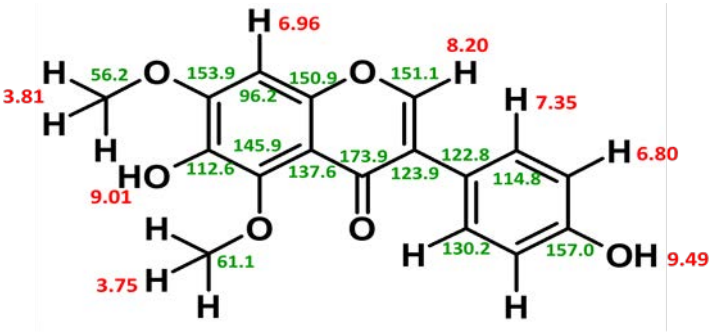


Figure 3.25: Complete structure of KPER 35 = Muningin

With an overview of the chemical shifts for ^{1}H (in red) and ^{13}C (in green) obtained from all the experiments carried out in NMR on KPER35 which has been identified as Munigin.

6.2. Mass spectrometry

The analysis carried out is identical to the ones described in section 4.3 of this chapter.

6.3. Ultraviolet and infrared absorbance

Ultraviolet and infrared absorbance are carried out to obtain wavelengths at which a compound may absorb in the ultraviolet and infrared. It gives information on the types of organic functions that the compound may bear and are therefore important characteristics.

6.4. Melting point

It gives information at the temperature at which the compound changed its form from solid state to liquid state at standard atmospheric pressure.

6.5. Results of the purification process run on the roots of *P. erinaceus*

6.5.1. NMR spectra recording

The NMR spectra were generated on a Bruker DRX 400, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The 2.50 ppm and 40.0 ppm resonances of residual CD_3SOCD_3 were used as internal references for ^{1}H and ^{13}C NMR spectra, respectively. The structures of the compounds were confirmed by comparison with reference data from the literature.

6.5.2. Melting point measurement

Melting points were recorded on a melting point apparatus and are uncorrected.

6.5.3. Mass spectra data generation

Compounds were analyzed by MALDI-TOF mass spectrometry (Table 3.21). The analysis was run using an Ultraflex III (Bruker Daltonic) apparatus equipped with a Smart beam® Nd-YAG wavelength of $\lambda = 355$ nm.

The deposit on the MALDI plate is achieved using the dry drop method: (1µL of extract analyte obtained by dissolving the analyte in methanol) is deposited on the plate and then 1 µL of the matrix is deposited on top of it and mixed up with the analyte by several cycles of suction-delivery to the micropipette. In positive mode, the matrix used is the 2,5-DHB (2,5-dihydroxy benzoic acid) at 1M in 50:50 ACN / H_2O solution (acetonitrile/water). In negative mode, the 9-AA (9-AminoAcridine) 7 mg / mL in methanol was used but did not yield results. To calibrate the device, we used the monoisotopic peaks of a solution of PEG600 at 10-4M for a first external calibration. The spectra were then recalibrated internally using the matrix peaks, *i.e* $[(DHB+H-H_2O]^+ = 173.0233$, $[DHB+H]^+ = 155.0339$, $[2DHB+Na]^+ = 273.0394$. Calibration is considered acceptable when the average error is less than 15 ppm.

6.5.4. Compounds identified

The structure of the compounds was established mainly by NMR aided by mass spectrometry for molecular mass confirmation.

- **KPER1** (white crystals) corresponding to **Friedeline** and **KPER 39** (white crystalline powder) corresponding to ***β*-sitosteryl-*β*-D-glucoside** as described in the section 6.6.4. of this chapter.
- **KPER 7** (reddish/caramelize crystals) was identified as ***β*-D-glucopyranose** (m.p: 150°C; $C_{16}H_{12}O_{6;}$ m/z 180.156). The ^1H and ^{13}C NMR spectral data are in close agreement with the values reported by Roslund *et al*., 2008.
- **KPER 9** (off-white powder, m.p: 257-261°C) was identified as a mixture of two isoflavones, **formononetin** (m/z 269.0821) and **pseudobaptigenin** (m/z 283.0631), in the respective proportion 5:1. The ^1H and ^{13}C spectra were corresponding to the values described in Matin *et al*., 2009. Formononetin was previously reported in the Roots of *Pueraria lobata* by Rong *et al*., 1998 and pseudobaptigenin in the roots of *Millettia speciosa* by Ping *et al*., 2014 (Figure 3.26, the mass spectrum of the compound KPER 9).

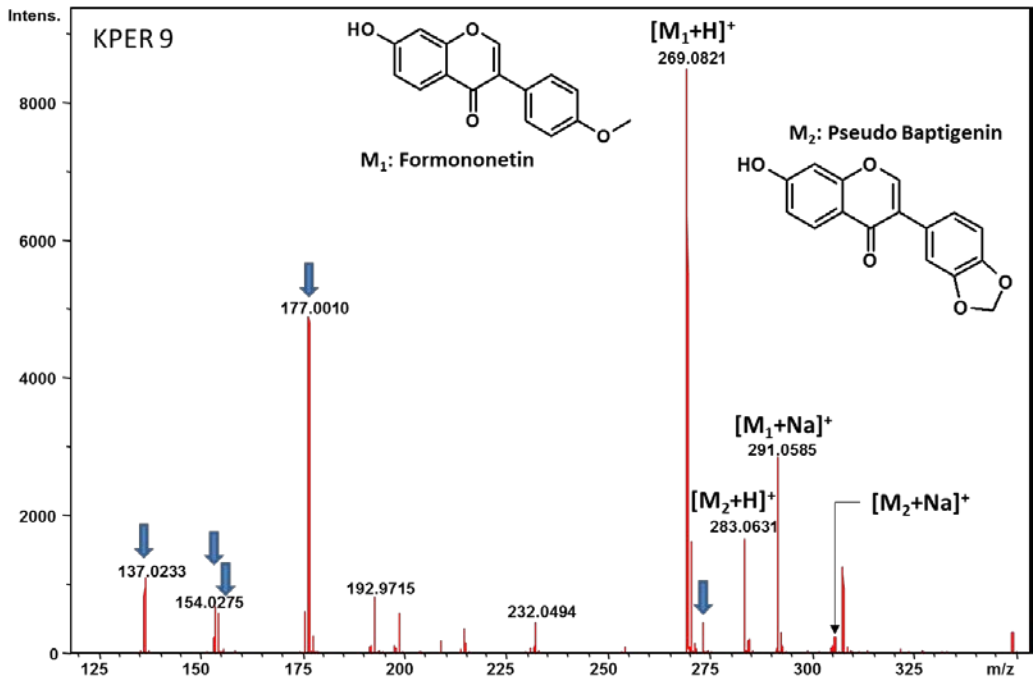


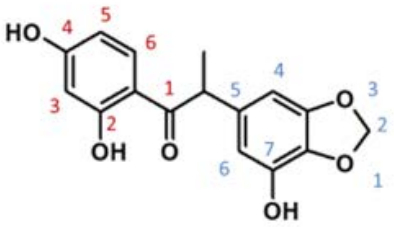
Figure 3.26: Mass spectrum of KPER 9

The spectrum confirms the presence of two molecular peaks $[M+H]^+$ at 269 for Formononetin and at 283 for Pseudobaptigenin. The blue arrows show the different adducts formed during the experiment.

- **KPER 10AV** (yellow bright powder) corresponding to **isoliquiritigenin** (a chalcone; m.p: 206-210; m/z 257.0821; $C_{15}H_{12}O_4$). The ^1H and ^{13}C NMR spectral data are in close agreement with the values reported by Liu *et al*., 2016 in the seeds of *Whitfordiodendron filipes*.
- **KPER 35** (translucent white crystals), corresponding to **muningin** (an isoflavone, m.p: 285°C, m/z 315.0883), previously reported in the heartwood of *Pterocarpus angolensis* by King *et al*., 1952. ^1H NMR (DMSO-d6): δ = 3.73 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 6.8 (d, J = 6.6 Hz, 2H, 2CH), 6.96 (s, H, CH), 7.35 (d, J = 6.6 Hz, 2H, 2CH), 8.2 (s, H, CH), 9.01 (s, H, OH), 9.49 (s, H, OH).
- **KPER 37B** (dark yellow powder in comparison to KPER 10AV which is a bright yellow compound) a new isoflavone and **KPER 20** (purple powder) a new arylpropanoid are the two never described compounds in nature which were isolated from the roots of *P. erinaceus* (Figure 3.27; Tables 3.19 and 3.20). They are undergoing further chemical analysis to finalize their chemical identity card.

Novel Arylpropanoid

KPER 20



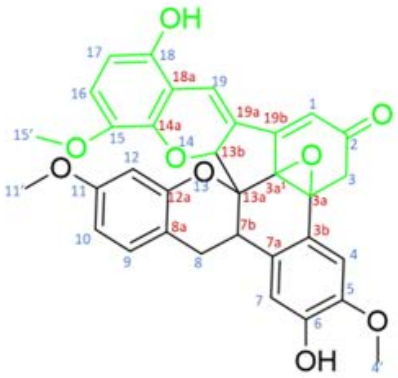
1-(2,4-dihydroxyphenyl)-2-(7-hydroxybenzo[d][1,3]dioxol-5-yl)propan-1-one

Molecular weight: 302.29

Molecular formula: $C_{16}H_{17}O_6$

Novel Dimeric Isoflavonoid

KPER 37B



6,18-dihydroxy-5,11,15-trimethoxy-3,7b,8,13b-tetrahydro-2*H*-3a,3a^1 epoxybenzo[*a*]chromeno[2',3':2,3]indeno [7,1-*cd*]xanthen-2-one

Molecular weight: 589.14

Molecular formula: $C_{33}H_{26}O_9$

Figure 3.27: Structures of KPER 20 and KPER 37B

Structures of the two new compounds identified in the roots of *P. erinaceus*. A further chemical investigation is underway to finalize their chemical information and identity card.

Table 3.19: Structure description KPER 20

The data provided are based on the different NMR experiments carried out in $CDCl_3$. δ: chemical shifs, Multiplicity (s : singulet ; d : doublet ; dd : doublet of doublet; q: quintuplet)

	RMN ^{13}C	RMN ^{1}H			RMN ^{2}D	
	δ (ppm)	δ (ppm)	Multiplicity	J (Hz)	HMBC	COSY
1'	135					
2	111	6.77	s		1', 2	
3'	145.7				2, 1'	
4	119.1	6.76	d	2.0	1'	
5	134.5				1', 2'	
6	114	6.88	d	2.0		2'
7	146	3.84 (OH)	s			
1	O ; 204.8					
2	166	12.85 (OH)	s			

3	103.7	6.33	d	2.5	2	
4	164.3	6	s			
5	107.7	6.27	dd	8.9 ; 2.		
6	132.7	7.68	d	8.9	3, 2’	
7	113.3					
1’	46.5	4.53	q	6.9		
2’	19.2	1.45	d	6.9	1’, 2’	

Table 3.19: Structure description KPER 37B

The data provided are based on the different NMR experiments carried out in DMSO. δ: chemical shifs, Multiplicity (s : singulet ; d : doublet ; dd : doublet of doublet)

	RMN ^{13}C	RMN ^{1}H			RMN ^{2}D
	δ (ppm)	δ (ppm)	Multiplicity	J (Hz)	HMBC
1	119.6	5.68	s		
2	199.9				
3	2.67	2.62	s		
4	114.2	6.89	s		
5	146.5				
6	144.6				
7	113.4	6.78	s		
8	37.8	2.71 / 3.20	dd/dd	12.8, 2.2 13.2, 3.5	9 ; 13b; 8 ; 4’
9	131.8	7.03	d	8	
10	107.6	6.32	dd	8, 2.3	
11	159.4				
12	99.9	6.36	d	2.3	
13					
14					
15	150.5				

16	107.6	6.49	d	8.7	
17	119.6	6.18	d	8.7	
18	155.4				
19	122	6.44	s		
3a	52.2				7b
3b	130.6				
3a[1]	78.9				1 ; 8
7a	129.6				
7b	56.5	3.42	dd	8.3, 0.8	
13a	84.5				1 ; 13b ; 7b ; 8 ; 12
8a	120.1				
12a	159.3				
14a	140.7				
18a	119.2				
13b	82.4	5	s		
19a	122.7				
19b	157.7				
5’	60.6	3.71	s		
11’	55.5	3.76	s		
15’	56.5	3.85	s		7 ; 13b ; 8 ; 4’

Table 3.21: Molecular masses compounds identified in the roots of *P. erinaceus*.

The mass of those compounds was determined using MALDI-TOF. $[M+H]^+$: molecular protonated ion. δ: Chemical incertitude on the molecular mass obtained.

Compound	Metabolites	$[M+H]^+$	m/z observed	m/z theoretical	δ (ppm)
KPER 9	Formononetin	$C_{16}H_{13}O_4$	269.0821	269.0813	+ 3.0
	Pseudobaptigenin	$C_{16}H_{11}O_5$	283.0631	283.0606	+ 8.8
KPER 10AV	Isoliquiritigenin	$C_{15}H_{13}O_4$	257.0821	257.0808	+ 5.1
KPER 20	KPER 20	$C_{16}H_{15}O_6$	303.0900	303.0863	+ 12
KPER 35	Muningin	$C_{17}H_{15}O_6$	315.0883	315.0863	+ 6.3

With $$\delta_{(ppm)} = \frac{m/z_{observed} - m/z_{theoretical}}{m/z_{theoretical}} \cdot 10^6$$

6.5.5. Biological activities of compounds isolated

The compounds isolated have been tested against *Aspergillus fumigatus* using the methodology described in section 3.1. of the second chapter.

Compounds were active against *A. fumigatus* with MIC from 8 µg/mL to 256 µg/mL. The most active is KPER 9 that happens to be a mixture of Formononetin and *ψ*-baptigenin**,** in the respective proportion 5:1. The second most active is the KPER 37B at 256 µg/ml. KPER 10 AV (isoliquiritigenin) and KPER 35 are slightly active with a respective inhibition of 35.03 and 25.6 of bacterial growth at the highest concentration tested (256 µg/mL). The other compounds did not show any activity at 256 µg/mL (Table 3.22).

Table 3.22: Antifungal activities of the pure compounds from the roots of *P. erinaceus* against *A. fumigatus*

MICs are marked in 'bold'. *X%*: Percentage of inhibition (PI) at the highest concentration of extract tested (256 µg/mL). n. o: no activity observed at the highest concentration tested 256 µg/mL.

Tests were repeated 3 times at least. *: Test made once; **: test made 2 times.

X: KPER

X 1	X 7	X 9	X 10 AV	X 20	X 33	X 35	X 37B	X 39
n. o	n. o	**8** **	*35.03**	n. o*	n. o	*25.6*	**256***	n. o

6.6. Results of the purification process run on the trunk barks of *P. erinaceus*

6.6.1. NMR spectra recording and melting point measurements

They were realized as precedently described in the sections 6.5.1 and 6.5.2.

6.6.2. Mass spectrometry data generation

The **Compounds C 1** and **C 3** were analyzed using GC-EIMS as follow. The compound was first evaporated to dryness and subjected to silylation with a mixture of *N, O*-bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich, St Quentin-Fallavier, France) and acetonitrile (1:1; v:v) at 100 °C for 10 min. GC–EIMS analysis was then performed on a fused silica column (ZB-5-MS, 5% phenyl methyl polysiloxane, 30 m, 0.25 mm i.d., 0.25 µm film thickness; Phenomenex) in a GC 2010 chromatograph (Shimadzu). Helium was used as a carrier gas at the flow rate of 1 mL/min. The program temperature was set as follow: incubator temperature was raised from 100 °C to 340 °C using a ramp of 20 °C/min. The final temperature was maintained for 5 min (end of the analysis). One µL of the derivatized sample was injected onto the column with a split ratio of 1/10. The mass spectrometer GCMS-QP2010 SE was equipped with a quadrupole analyzer. The MS detector was set as follows: electron impact mode with ion source temperature set at 200 °C, analyzed mass interval m/z 40–700 and mass spectra were generated at 70 eV. Spectrum acquisition was performed during 16.25 min. The confirmation of the chemical structures was achieved by comparison with a library of mass spectra (NIST MS Search 2.0).

It was not possible to obtain significant MS results with GC-EIMS for **compounds C 2** and **C 4** because their structures after the NMR analysis are corresponding to a monoglyceride and a saponin respectively (see next paragraph). Consequently, ESI-FTICRMS (Electrospray-Fourier Transform Ion Cyclotron Resonance Mass Spectrometry) was used to confirm their molecular formula (exact mass measurement). ESI-FTICRMS measurements (High-Resolution MS) were carried out on an Explorer HiRes FTICR mass spectrometer (Varian Corporation, Palo Alto, CA, USA) fitted with a 9.4 T shielded superconducting magnet and a Micromass Z-spray electrospray ionization (ESI) source in the negative ionization mode. The ESI source was operated using the following parameters: needle potential, +3.8 kV; source temperature, 85 °C; probe temperature, 100 °C. The sampling and extractor cone voltages were set to 45 V and 10 V, respectively. The flow rate was fixed at 2 µL min^{-1}. Ions were accumulated for 3 s in a storage hexapole. Then, ions were transferred to the ICR cell through a hexapole ion guide. Mass spectra were acquired with Omega software in manual mode

(v.9.1.18, Varian Corporation, CA). For each sample, mass spectra were recorded during 30 s in triplicate. Between each acquisition, the ESI source was cleaned during 1 min with ethyl acetate. The external calibration was carried out using a commercial calibration solution (Pierce™ LTQ Velos ESI Positive Ion Calibration Solution, Thermo Scientific). The mass calibration was considered as successful if the root-mean-square (rms) error of the ion assignment was in the range of 0–1.5 ppm.

6.6.3. Compounds identified

Compound C1 has been identified as friedeline (mp: 262-265 °C). The NMR spectra were the same as described in the literature by Queiroga *et al*., 2000 and Ouedraogo *et al*., 2012. And the experimental mass spectrum of this compound fit the NIST MS library with a confidence higher than 95% (Figure 3.28).

Compound C2 was identified as 2,3 dihydroxypropyloctacosanoate (mp: 58-59°C): ^{1}H NMR (DMSO-d6): δ = 0.85 (t, J = 6.6 Hz, 3H, CH_3), 1.20-1.35 (m, 46H, CH_2), 1.63 (quint, J = 7.5 Hz, 2H, CH_2), 2.08 (br, 2H, OH), 2.41 (t, J = 7.5 Hz, 2H, CH_2), 4.11 (m, 2H, CH_2), 4.43 (m, 1H, CHOH), 4.66 (m, 2H, CH_2); ^{13}C NMR: δ = 14.0 (CH_3), 23.4 (CH_2), 24.1 (CH_2), 25.7 (CH_2), 27.3 (CH_2), 29.8 (CH_2), 30.0 (CH_2), 30.1 (CH_2), 30.2 (CH_2), 30.3 (CH_2), 30.5 (CH_2), 30.5 (CH_2), 32.5 (CH_2), 32.6 (CH_2), 64.7 (CH_2), 67.2 (CH_2), 71.4 (CHOH), 174.5 (C=O). The molecular formula was confirmed to be $C_{31}H_{62}O_4$ from its ESI-FTICR mass spectrum, showing a molecular ion $[M+Na]^+$ at m/z 521.4536 ($C_{31}H_{62}O_4Na_1$, calc. m/z 521.4540) (Figure 3.28).

Compound C3: The ^{1}H and ^{13}C spectra revealed a mixture of *β*-sitosterol and Stigmasterol as already described by Luhata Lokadi *et al*., in *Odontonema strictum* (2015). However, the GC-EI-MS analysis of this fraction led to the conclusion that Compound 3 (m. p: 160-164°C) is actually a mixture of 3 phytosterols: 39.6 % of Stigmasterol, 40.4 % of *β*-sitosterol and 20% of Campesterol. All the experimental mass spectra fit the NIST MS library with a confidence higher than 95% (Figure 3.28).

Compound C4 was identified as *β*-sitosteryl-*β*-D-glucopyranoside (m. p: 280°). ^{1}H NMR and ^{13}C NMR spectral data are in close agreement with the values reported by Arora and Kalia (2013) in DMSO-d6. The molecular formula was confirmed to be $C_{35}H_{60}O_6$ from its HR mass spectrum, showing a molecular ion [M+Na]+ at m/z 599.4282 4285 ($C_{35}H_{60}O_6Na_1$, calc. m/z 599.4282) (Figure 3.28).

The structures of the compounds are drawn in Figure 3.28.

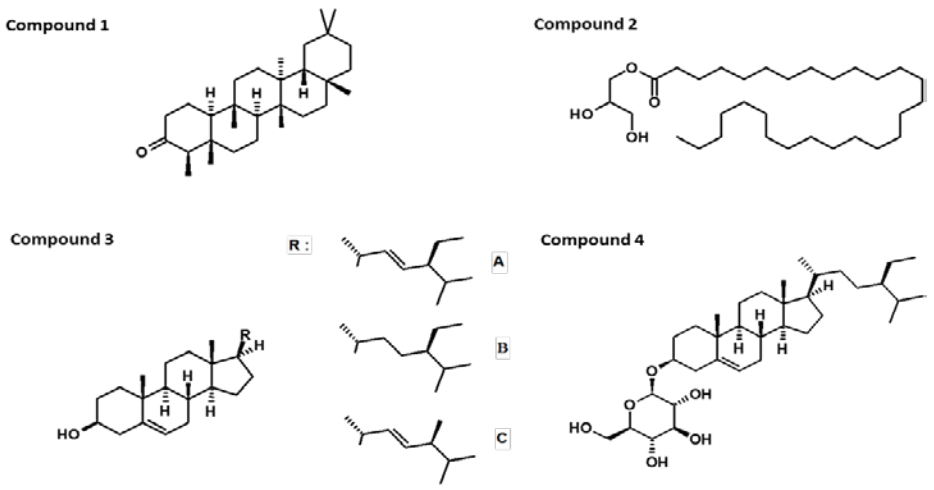


Figure 3.28: Chemical structure of the compounds isolated from the trunk barks.

Compound 1: Friedeline. Compound 2: 2,3 dihydroxy-propyl-octacosanoate. Compound 3: a mixture of phytosterols: 39.6 % of Stigmasterol (A), 40.4 % of *β*-sitosterol (B) and 20% of Campesterol (C). Compound 4: *β*-sitosteryl-*β*-D-glucopyranoside.

6.6.1. Biological activities of the compounds identified

The best activities were obtained with Friedeline (Compound 1) and the mixture of phytosterols (Compound 3) with a MIC at 4 µg/mL on *Staphylococcus aureus* and *Staphylococcus aureus* Resistant to Methicillin (SARM). The 2,3 dihydroxy-propyl-octacosanoate (compound 2) and *β*-sitosteryl-*β*-D-glucopyranoside (compound 4) show an anti-bacterial activity at 256 µg/mL (the highest concentration tested) (Table 3.23).

Table 3.23: Antibacterial activities of the pure compounds from the trunk barks of *P. erinaceus* on some bacteria belonging to the genus *Staphylococcus*.
MICs are marked in 'bold'.
X%: Percentage of inhibition (PI) at the highest concentration of extract tested (256 µg/mL).
SARM: *Staphylococcus aureus* Resistant to Methicillin.
Compound 1: Friedeline. Compound 2: 2,3 dihydroxypropyloctacosanoate. Compound 3: mixture of phytosterols (39.6 % of Stigmasterol + 40.4 % of *β*-sitosterol + 20% of Campesterol). Compound 4: *β*-sitosteryl-*β*-*D*-glucopyranoside.
Tests were carried out once (n=1).

	Compound 1	Compound 2	Compound 3	Compound 4
S. aureus	**4**	*90%*	**4**	*85%*
SARM	**4**	*90%*	**4**	*85%*

7. Discussion

7.1. *Pterocarpus erinaceus*

7.1.1. Roots

By performing the GC-MS, it was possible to name some of the somehow volatile compounds contained in the apolar fractions of the roots: saturated and unsaturated fatty acids, terpenes and aromatic compounds. Confidence could be given to such identification except for the compounds with just 70% of equivalence where some doubt could persist. Nonetheless, the structure identified is closed to the one presented to be corresponding by the library. Such compounds have also already been described in other plants. For example, asarone has been reported in the roots of *Acorus calamus* (Acoraceae) (Bisht *et al*., 2011); friedeline, stearic and palmitic acids have been isolated from the roots barks of *Tripterygium hypoglaucum* (Celastraceae) (Liu *et al*., 2011); stearic, palmitic, palmitoleic, linoleic and arachidic acids in the roots of *Nyctanthes arbortristis* (Oleaceae) (Mizanur *et al*., 2011); nerolidol in the roots of *Aristolochia elegans* (Aristolochiaceae) (Vila *et al*., 1997) and cadina-1(10),4-diene in the roots of *Kadsura oblongifolia* (Schisandraceae).

Concerning the isolated compounds, except friedeline (Ouédraogo *et al*., 2012), none of the isolated compounds have been reported in this species before to the best of our knowledge. But, muningin has been reported in another species belonging to the genus *Pterocarpus*, namely *P. angolensis* (King *et al*., 1952). Besides, the following non-specific compounds are documented to be present in *P. santalinus*: isoflavones, glycosylated isoflavonoids, triterpenes, sesquiterpenes, phenolic compounds, lignans, pterostilbenes, nonflavonoid polyphenols containing different sub-group compounds such as simple phenols, benzoic acids, hydrolysable tannins, cinnamic acid, acetophenones, phenylacetic acid, lignans, coumarins, benzophenones, xanthones, and stilbenes (Saradamma Bulle *et al*., 2015). The types of compounds could also easily be found in any other species belonging to the genus *Pterocarpus*. Indeed, isoliquiritigenin was reported in the heartwood of *P. marsupium* (Maurya R. *et al*., 1984); formononetin was first reported in the heartwood of *P. vidalianus* (Orth and Forschner, 1965) and pseudobaptigenin has been previously isolated from the roots of *P. marsupium* (Adinarayana Dama *et al*., 1982). Compounds such as *β*-sitosteryl-*β*-*D*-Glucoside and *β*-*D*-Glucopyranose are compounds commonly found in plants. Interestingly, 2 compounds never reported in nature have also been discovered: an arylpropanoid KPER 20 and a dimer of isoflavone KPER 37B. The dimer is a very complex molecule and with interesting anti-fungal activity at 256 µg/ml corresponding to around $0.43x10^{-3}$ mol/mL.

7.1.2. Trunk barks

7.1.2.1. GC-MS analysis of the apolar fractions of the trunk barks

The GC-MS helps us to obtain information on the compounds contain therein: fatty acids, terpenes, and aromatic compounds have been identified in the non-polar compounds. Such compounds are not surprising in the plant kingdom. For example: palmitic acid, linoleic acid, and stearic acid have been isolated from the stems barks of *Millettia richardiana* (Fabaceae) (Rajemiarimiraho *et al*., 2014); lauric acid was determined in the stems barks of *Prunus Africana* (Rosaceae) (Nyaimai, 2015); octadecadienoic acid (linoleic acid) and octadecatrienoic acid (linolenic acid) in the stems barks of *Berberis tinctorial* (Berberidaceae) (Deepak and Gopal 2014); stearolic acid in the stems barks of *Alvaradoa amorphoides* (Simaroubaceae) (Quintal-Novelo *et al*., 2015); α-cadinol in the stems barks of *Zanthoxylum nitidum* (Rutaceae) (Yang *et al*., 2009); cubenol, linoleic acid and linolenic acid in the stems barks of *Guarea macrophylla* Vahl. ssp. tuberculata Vellozo (Meliaceae) (Ghilardi *et al*., 2002) and *t*-muurolol was identified in the stems barks of *Kadsura coccinea* (Schisandraceae) (Dai *et al*., 2015). The compounds 9-oleamide (or armid ow) and cyclobutanecarboxylic acid, trydec-2-ynyl ester, 2-methylen cholestan-2-ol (3a, 5a), are contaminants from the solvent. Other compounds have presented very good peaks but did not correspond to compounds present in the NIST library. They could be corresponding to compounds never described in nature.

7.1.2.2. Analytical work on the ethyl acetate fraction of the trunk barks

Except for Friedeline (Ouédraogo *et al*., 2012), none of the six compounds characterized from the ethyl acetate fraction to the best of our knowledge, has been reported previously in *P. erinaceus*. This is rather surprising as extracts of *P. erinaceus* are exceptionally active against different bacteria (see above), and all the compounds in question have been identified in other plants before, and hence may provide a focus for antimicrobial activity already. In order to investigate the antimicrobial activity associated with such substances, the relevant antimicrobial assays have been performed, and, indeed, interesting anti-bacterial activities could be seen for these four compounds, with MIC values of 4 µg/mL and a PI ranging from 85% to 90% at 256 µg/mL (the highest concentration under investigation). Expressed in molar concentrations, this activity resides in the lower micromolar range. These findings agree with existing literature on these compounds. Indeed, Viswanathan *et al*., 2012 have reported the

antibacterial activities associated with friedeline and stigmasterol, which they isolated from the leaves of *Jatropha tanjorensis*, a plant found in tropical areas including Africa and Asia and use for a wide range of pharmacological effects (e.g. antidiabetic, antimicrobial, anti-inflammatory activities). In that study, the activity was observed at concentrations from 2 to 10 mg/mL using the disc and agar-well diffusion methods.

Besides during our preliminary work (Tittikpina *et al*., 2013), a basic phytochemical screening revealed the presence of catechuic tannins in these barks. Indeed, Ouédraogo *et al*., 2012 have isolated epicatechin from the trunk bark, and Hage *et al*., 2015 have studied the kind of the trunk bark of this plant and have noted the presence of dimers, trimers, and tetramers of epicatechin. The presence of these tannins, i.e. oligomers and polymers of epicatechin, could also be responsible for the superior activities associated with the water fractions (in comparison to the ethyl acetate fraction). Indeed, tannins are documented to possess antibacterial activity by their capacity to form bonds with glycoproteins present on the external membranes of bacteria, consequently disorganizing the exchanges between the intracellular and extracellular environments in the bacteria, leading to their death. Karou *et al*., 2005, for instance, have demonstrated the antibacterial activities of such polyphenolic compounds derived from the barks of *P. erinaceus*. Moving on from such studies with extracts and fractions, the present study is, for the first time, reporting such antibacterial activities for pure compounds isolated from this plant.

7.1.2.3. LC-MS investigation of the polar fractions of the trunk barks

During the LC-MS experiment on the butanol and water extract, some time has been spent to reach the best chromatographic conditions: the elution was too high with a high proportion of acetonitrile, it was not enough with a high proportion of methanol and it was better only with a mixture of acetonitrile and methanol.

From the compounds identified using the positive mode, some were part of the compounds we have isolated in the roots of *P. erinaceus* and described in other species belonging to this genus. The other compounds were also reported in other species by other studies. Indeed, maackiain and santal were first reported in the heartwood wood of *P. santalinus* and pterocarpine and calycosine, in the heartwood of *P. dalbergioides* (Seshradi, 1972). Genistein was also reported (linked to glucose: genistein-7-*O*-*ß*-*D*-glucopyranoside) in the trunk bark of *P. dalbergioides* (Camilia *et al*., 2013). The mass spectrometric parameters applied have helped identify the different compounds, establish their glycosylated forms but unfortunately,

we were unable to differentiate between pterocarpine and calycosine and, between xylose and arabinose because they have the same molecular weights. To achieve such distinction, one solution would be to run a high-resolution mass spectrometry or to isolate the compounds and perform a full identification by NMR. The problem with the last solution is the probability of breaking the anomeric link during the purification process. Nevertheless, we were able to propose structures that have never been reported in nature to the best of our knowledge: pseudobaptigenin-glucose-xylose or pseudobaptigenin-glucose-arabinose; formononetin-glucose-xylose or formononetin-glucose-arabinose; muningin-glucose-xylose; muningin-glucose-arabinose; genistein-glucose-xylose or genistein-glucose-arabinose; santal-rhamnose-glucose; pterocarpine-glucose-xylose or pterocarpine-glucose-arabinose or calycosin-glucose-xylose or calycosin-glucose-arabinose.

The compounds identified using the negative mode were also reported in other plants species. Cinnamic acid, coumarins, xanthones, flavonoids, phenolic compounds, etc, have also been reported in other species belonging to the genus *Pterocarpus* (Saradamma Bulle *et al*., 2015). But those compounds have never been reported in *P. erinaceus*, to the best of our knowledge. The documentation of those structures was not without some ambiguity or uncertainty on some fragments. For example, the presence of the *C*-glycosylated flavonoids was deduced from the observation of fragments but the complete structural identification was not achieved. To achieve such identification, the analysis needs to be run using an LC-MS/MS high resolution on an Orbitrap, for example. Besides, as in the positive mode, a discrimination was not possible between the xylose, arabinose or tartaric acid. To achive such discrimination, a high-resolution mass spectrometry would be used. However, the probabilities for the fragment to be either a xylose and its derivative or an arabinose and its derivative are higher than to be a tartaric acid, considering the spectra already published on plants. Interestingly, one compound never identified in nature has also been discovered in the negative mode: the feruloyl-coumaroyl ester derivative (xylan, arabinoxylan or tartaric acid), the structure with the highest probability is the feruloyl-coumaroyl-xylan or feruloyl-coumaroyl-arabinoxylan.

In general, the trunk barks of *P. erinaceus* contain a huge variety of compounds: terpenes, fatty acids, sterols, flavonoids, pro-anthocyadines, cinnamic acid derivatives, etc. All the compounds including the formally isolated (except friedeline), the identified ones and the ones with predicted structures have never been reported in this plant species.

7.1.3. Leaves

Compounds identified with a similitude more than 80% through the GC-MS are more likely to correspond to the real compounds. The ones with a similitude of around 70% could imply to just have some similar structures with the compounds. The following compounds could be impurities from the solvent: 1,2-diexyl cyclopropen; aqua cera; anti-2-acetoxyacetaldoxime and 1-hydroxy-7α-methyloctahydro-5h-inden-5-one. The Fatty acids identified, saturated (palmitic acid) and unsaturated (linolenic acid), are common fatty acid in plants. They are not present in high quantity in the leaves where they are stored in oily inclusions. It is also the same for terpenes (Jean Bruneton, 2016). For example, hexadecanoic acid and tetramethylhexadecenol have been reported in the leaves *Leucaena leucocephala* L. (Fabaceae family) (Zayed and Sammling, 2016); heptatriacontanol, hexadecanoic acid and hexadecenoic acid in the leaves of *Sesamum radiatum* (Pedaliaceae family) (Ogunlesi *et al*., 2010). 9-octadecenamide was reported in the leaves of *Astragalus gombiformis* Pomel (Fabaceae); 4-caranone in the leaves of *Ledum groenlandicum* (Ericaceae); menthol in the leaves of *Gliricidia sepium* (Fabaceae) (Teyeb *et al*., 2011, Collin, 2015; Chaverri and Cicció, 2015). Cinnamic acid is aromatic compounds, they occur naturally in plants and have a key role in the various physiological phenomenon of the plant (growth, fight against the disease, etc…) (Guzman, 2014).

Some compounds which exhibited interesting peaks did not match any compound of the NIST library. It may imply the existence of new compounds that have never been reported in the literature.

7.2. *Daniellia oliveri*

7.2.1. Roots

Apart from the fatty acids and terpenes already identified in this plant parts and in *P. erinceaus* and also in other plants, some new compounds have been unveiled. Those compounds were also identified by other researchers. It is the case for example of Al-Rahmah *et al*., 2013 who have identified cedrene in the roots of *Zingiber officinale* (Zingiberaceae). Qirong *et al*., 2007 have isolated columbin from *Radix tinosporae* (Menispermaceae). Abhishek Mukherjee and Anandamay Barik have identified 1-tridecanol in the leaves of *Momordica cochinchinensis* (Menispermaceae). Kai-yue Cao *et al*., 2014 have isolated and identified pentacosadienoic acid from the leaves of *Oplopanax horridus* (Arialaceae). Atolania and Olatunji have isolated polyalthic or daniellic acid from the oleoresin of *D. oliveri* (Fabaceae). Dehydrosasurealactone has been identified in the volatile oil of the wood

of *Laurus nobilis* (Lauraceae). Yagi *et al*., 2016 have identified bicyclo[5.2.0]nonane, 4-methylene-2,8,8-trimethyl-2-vinyl- in the essential oil extracted from the leafy stems of *Cymbopogon schoenanthus* (Poaceae). Finally, Suleimen *et al*., 2014 have identified cadala-1(10),3,8-triene in the essential oil from the aerial parts of *Angelica ursina* (Apiaceae).

7.2.2. Trunk barks

It could be surprising to have identified polyunsaturated fatty acids in the trunk barks. But, various studies such as Abedi and Sahari, 2014 have reported such acids in different plant parts material. Pentadecanoic acid, a fatty acid not reported in *P. erinaceus* for example, has been unveiled in *D. oliveri* stems barks. Lograda *et al*., 2010 have also identified it in the aerial parts of *Genista vepres* (Fabaceae). Isocoumarins such as bergenin, norbergenin have been isolated from the roots and stems of *Cissus pteroclada* (Vitaceae) by Lin *et al*., 2012; Mellein has been isolated from the leaves and stems of *Stevia lucida* Lagasca (Asteraceae) by Chacón-Morales *et al*., 2013. Dodecanoic, pentadecanoic, heptadecanoic, octadecanoic, octadecenoic, octadecadienoic, eicosanoic, docosanoic, tetracosanoic and heptadecenoic acids were identified in the tuber of *Lepidium meyenii* (Brasicacceae) by Dini *et al*., 1994. Hexacosanoic or serotic acid has been characterized in the peanut seed oil by Dean and Sanders, 2009. Caryophyllene oxide, humulene epoxide II, *β*-sitosterol, stigmasterol, and campesterol have been isolated from the stems barks of *Annona vepretorum* (Annonaceae) (Dutra *et al*., 2014). Labda-8(20),13(16),14-trien-18-oic acid is a precursor in the formation of plants diterpenes (Moreira *et al*., 2009). The pentacyclic triterpene *β* and *α*-amyrin are present in plants leaves, barks and roots (Vázquez *et al*., 2012). Also without no surprise, 1-monopalmitoylglycerol, a monoglyceride resulting from the esterification of palmitic acid and glycerol was unveiled in the stems barks. Indeed, glycerid compounds are some of the main components of plants bark.

Other compounds such as a succinic acid may be a laboratory contaminant or coming from the stationary phase of the GC system, hydroxylamine and naphthalene could be laboratory contaminants. Compounds such as dodecynol and 8-methyl-6-nonenamide 5,9-dimethylcyclodecanol and benzenpropanoic acid may be provided by the solvent.

7.2.3. Leaves

Apart from the compounds that were previously reported in the leaves of *P. erinaceus*, some new compounds are identified in the leaves of *D. oliveri* and already described in other plants parts. For example, among the alcohol terpenes, 6-linalool has been identified in the leaves of

Cinnamomum camphora (Lauraceae) (Fan Yang *et al*., 2014); *t*-muurolol in the leaves of *Microbiota decussata* (Cupressaceae) (Krauze-Baranowska *et al*., 2002). The following hydrocarbons sesquiterpenes have also been reported in other plants leaves: humulene and germacrene D in the leaves of *Zornia brasiliensis* (Fabaceae) (Costa *et al*., 2015); aromadendrene in the leaves of *Eucalyptus globulus* (Myrtaceae) (Mulyaningsih *et al*., 2011) and 6-chamigrene in the leaves of *Chamaecyparis taiwanensis* (Cupressaceae) (Shô Itô *et al*., 1967). Concerning the oxygenated sesquiterpenes, tricyclo [5.2.2.0(1,6)] undecane-3-ol has been identified in the leaves and stems of *Anacyclus Clavatus* (Asteraceae) (Aliboudhar *et al*., 2013); caryophyllene oxide in the leaves of *Lantana camara* (Verbenaceae) (Shô Itô *et al*., 1967) and humulene-1,2-epoxide in the leaves of *Allophylus africanus* (Sapindaceae) (Balogun and Zhiqiang, 2014). Interestingly, a flavonoid has also been identified, never reported in this species: aromadendrin. Aromadendrin is an aglycone moiety of a glycoside flavonoids (aromadendrin-7-*O*-glucoside). It has been previously isolated from the barks of *Populus alba* (Salicaceae) (Chuan-Ling Si *et al*., 2009). Aromadendrin oxide is the aglycone which has the oxygen after a break of the glycosylic link during the massic event of the GC-MS procedure. Besides, Schwob *et al*., 2008 have studied the chemical composition of the essential oil coming from the leaves of *D. oliveri* and have identified among other compounds: germacrene D, allo-aromadendrene, humulene, caryophyllene oxide, *δ*-cadinene and muurolene.

The aromatic compound naphthalene could be a contamination during extraction or handling of the extracts and fractions in the laboratory. The fatty acids identified are as described in the precedent lines commonly happening in the plants.

We were unable to study the polar fractions of *D.oliveri*. But, some other studies have revealed interesting activities and or compounds with more polar extracts and or fractions. For example, when investigating the antimicrobial activity of the leaves of *D. oliveri* against bacteria and fungi, researchers found the *n*-butanol to be the most effective one. The *n*-butanol fraction has consequently been studied analytically to identify the responsible compounds, 4 glycoside flavonoids have been identified: rutin, quercitin-3$^{/}$-*O*-methyl-3-*O*-*α*-rhamnopyranosyl-(→)-*β*-*D*-glucopyranoside (narcissism), quercitrin and quercimeritrin (Ahmadu *et al*., 2004 and Ahmadu *et al*., 2007). The anti-bacterial activity associated with the apolar fractions of this plant is not surprising because lots of studies have documented compounds such as terpenes, fatty acids, sterols, etc…..to possess antibacterial activity (Gachkar *et al*., 2007 and Okoh *et al*., 2010).

8. Conclusions

Using analytical chemistry, from separation to detection methods, we could identify compounds, isolate some of them and test their biological activities. The GC-MS analysis realized on the nonpolar fractions of the leaves, trunk barks and roots of *P. erinaceus* revealed its trunk barks and roots to be rich in fatty acids with terpenes, sterols and phenolic compounds as second and third compounds. In contrast, its leaves were richer in terpenes than in fatty acids. When comparing those results with the ones obtained from the different parts of *Daniellia oliveri*, it was noticed that the leaves of the latter were richer in terpernes, compared to the leaves of *P. erinaceus*. The trunk barks of *D. oliveri* were mainly composed of fatty acids and then sterols, terpenes, and phenolic compounds. The roots, in contrast, were primarily made of terpenes and then fatty acids and phytosterols as most abundant compounds. The antibacterial and antifungal activities observed with those apolar fractions are not surprising as terpenes, fatty acids, monoglycerides and phenolic compounds are widely documented to possess such biological activities.

Using LC-MS, an *in-situ* chemical profiling was realized on the butanol fraction of the trunk barks of *P. erinaceus*, leading to identity a lot of compounds never reported in this plant species and never reported in nature. Those structures could be finalized by running a high-resolution spectrometry to clearly identify the type of sugar (xylose or arabinose) or isolate those compounds to fully establish the structures by NMR spectroscopy. Another procedure will also be the combination of mass spectrometry to NMR for the *in-situ* identification of those structures. The NMR has also allowed categorising a certain number of molecules in the roots and trunk barks of this plant. Most of those compounds have never been reported in the plant species and some of them never reported in nature at all, to the best of our knowledge.

Unfortunately, in this study, we were unable to carry out an isolation of compounds on the different parts of *D. oliveri*. Nevertheless, the GC-MS profiling of its apolar fractions has given information on the composition of its leaves, trunk barks, and roots. And a preliminary check-up of its polar fractions by LC-MS has also conducted to notice a huge number of anthocyanidines. More work is consequently needed to fully document the chemical composition of this plant. Besides, even with *P. erinaceus* (with for example more than 500 compounds present in the butanol extract of its trunk barks in the positive mode of the LC-MS), more is to be discovered.

Chapter 4: Nanoparticles of plant powders

Turning waste into value: Nanosized material of *Pterocarpus erinaceus* Poir trunk barks with promising anti-bacterial activities

This chapter is based on results already published in the following paper: Griffin, S., Tittikpina, N. K., Al-marby, A., Alkhayer, R., Denezhkin, P., Witek, K., Gbogbo, K. A., Batawila, K., Duval, R. E., Jawad Nasim, M. J., Awadh-Ali, N. A., Kirsch, G., Chaimbault, P., Schafer, K.-H., Keck, C. M., Handzlik, J. Jacob, C., Turning Waste into Value: Nanosized Natural Plant Materials of *Solanum incanum* L. and *Pterocarpus erinaceus* Poir with Promising Antimicrobial Activities, Pharmaceutics, 8(2): 11, doi:10.3390/pharmaceutics8020011.

1. Introduction

Extraction is the commonly used method to unlock the active compounds contained in the plants. This implies the use of organic solvents that most of the time are not safer not only for the person running the extraction but also not for the environment. From extraction to the pure active compound, several steps need to be followed: separation and isolation using chromatographic methods, structure identification using advanced techniques such as Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) as described in the previous lines of this work. The pure compounds are then processed into forms suitable for medical delivery such as creams, sprays, pills, etc. And here again, there is a need to use advanced equipment that most of the time is not available in developing countries such as Togo. In such countries, the plants material is usually applied after maceration, decoction or infusion of fresh or dried parts of the plants in water, in alcohol or a mixture of both. It will, therefore, be interesting to find cheap, readily available ways to unlock those bioactive compounds from plants in a safe and environment friendly way.

To solve this issue, we have turned our attention to nanosizing little-processed, crude plant materials (please note that the expression "nanosizing" is employed here to describe the arsenal of milling and homogenization methods employed and does not imply that the materials obtained by these methods necessarily also consist of particles with diameters in the nanometer range). We would like to test such techniques and see whether they are able to unlock the active molecules in a way to obtain the same effect as the one that may be obtained with the organic extract from the same plant part.

To achieve this goal, we should be able to go from a simple milling using, for example, a coffee or corn miller (as used in many African countries), to obtain particles of various diameters (millimeters roughly) to particles of controlled sized with diameters around nanometres. To obtain the latter, wet milling presents itself as a method of choice as it allows to obtain very fine particle sizes (Fahr, 2015). Among the different processes for wet milling available today, *i.e.* milling with colloid mills, bead milling and High-Pressure Homogenization (HPH), HPH is the method of choice for the samples discussed here: the high energy input during the homogenization process enables an efficient diminution of the material within a short period of time (Jahnke, 2001). HPH is also a well-known and hence well-established, straightforward and safe technique which is frequently applied in practice, not only in the pharmaceutical industry but also in the field of cosmetics and food production.

In addition, large-scale production is possible (Keck, 2006). It should also be noted that the basic HPH equipment is accepted by regulatory authorities, is of relatively low cost (around 10 000 euros) and available worldwide.

Pterocarpus erinaceus trunk barks, whose extracts have proven themselves as very active against different bacteria, were therefore selected as the plant material on which such kind of experiments has been carried out.

2. Material and methods

2.1. Plant material

The plant part used to obtain nanomaterial is the trunk barks of *P. erinaceus* described in the precedent lines. 2 types of material have been produced and their biological activities compared: methanolic raw extract (as previously described) and the nanosized material from the powder of the trunk barks.

Fresh stems barks *P. erinaceus*

Dried stems barks *P. erinaceus*

Grinded (powder) stems barks *P. erinaceus*, original sample for the nanomaterial

Figure 4.1: **Trunk barks of *P. erinaceus* (fresh, dried after cutting and ground)**

The nanosizing of the powder was realized in cooperation with Dipl. Pharm. Sharoon Griffin, PhD student at the Institute of Bioorganic Chemistry, University of Saarland.

The nanosizing of the powder was performed by a combination of rotor-stator high-speed stirring (HSS) and subsequent High-Pressure Homogenization (HPH) in the presence of the natural surfactant Plantacare. The latter is a plant derived, food-grade uncharged tenside commonly used to stabilize particles destined for medical or agricultural applications. Particle size analysis was performed using Photon Correlation Spectroscopy (PCS), Laser Diffraction (LD) and light microscopy.

The nanosizing of the powdered trunk barks was realized in 3 consecutive steps: firstly a dry milling using a FastPrep 24 Instrument (MP Biomedicals, Solon, OH, USA). Precellys Kits

(Bertin Technologies, Montigny-le-Bretonneux, France); secondly a stabilization by suspension in 1% Plantacare® 2000 UP (alkyl-polyglycoside, BASF, Ludwigshafen, Germany) in distilled water to yield 1% macro-suspensions of finely milled plant materials and thirdly a pre-homogenization using a MICCRA D-9 Homogenizer–Disperser (MICCRA GmbH, Müllheim, Germany) at different cycles, followed by successive homogenization employing an APV Gaulin LAB 40 (APV GmbH, Mainz, Germany) High Pressure Homogenizer.

Throughout those 3 different steps, the quality and the properties of the different material obtained was assessed employing: LD, PCS and light microscopy. LD measurements were performed on a Mastersizer 2000, PCS measurements on a Zetasizer Nano ZS (both from Malvern Instruments, England, UK). The light microscopy, employing a Leica DM 1000 LED microscope (Leica Microsystems, Wetzlar, Germany) to provide basic information regarding the homogeneity of the samples and the size and shape of the particles (Griffin *et al*., 2015).

2.2. Biological activity against nematode (*Steinernema feltiae*), bacterium (*Escherichia coli*) and yeast (*Saccharomyces cerevisiae*).

The biological assay was performed by myself.

The 1% macro-suspensions of finely milled plant materials in water were used to realize the bio-assays and compare it to the results previously obtained with the methanolic extracts. The tests against the nematodes, the bacterium, and the yeast were performed like described in Chapter 2 in sections 3.1. and 3.3.

Statistical calculations carried out in this part were performed in collaboration with Dipl. Biol. Polina Denezhkin, PhD student at the Institute of Bioorganic Chemistry, University of Saarland.

3. Results

3.1. Homogenized particles of *P. erinaceus* barks

The dry milling, stabilization with Plantacare and pre-homogenization at different cycles led to obtaining particles of around 100 µm (size confirmed by LD). Subsequent High-Pressure Homogenization (HPH) led to obtaining particles of 4 µm of diameter (size given by PCS analysis) which were kept in a cool environment to reduce the possibility of long-term agglomeration till further use. The different sizes of the particles are presented in Figure 4.2 (Griffin *et al*., 2015).

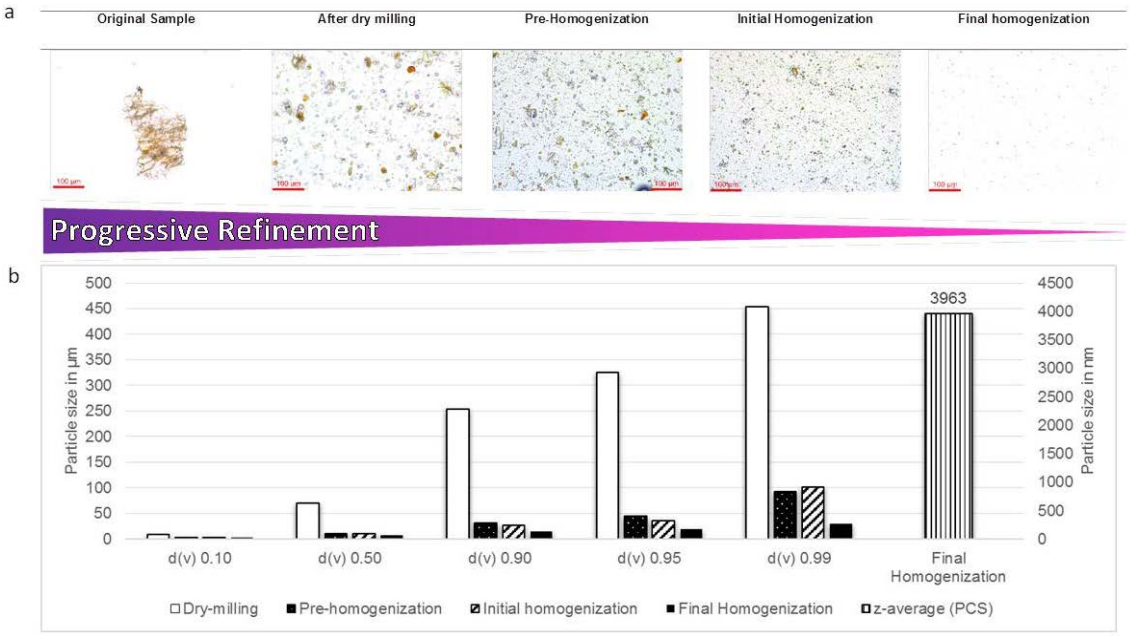


Figure 4.2: (**a**) Optical microscopy of the sequentially processed bark of *P. erinaceus*, from the crude plant material ground down with a simple coffee grinder at the University of Lomé in Togo on the left to initially and finally homogenized materials on the right (200-fold magnification). (**b**) LD and PCS analysis of the different samples confirming a progressive refinement of the particles to a final average particle size of just below 4 µm achieved by HSS and subsequent HPH.

3.2. Biological Activity of *P. erinaceus* barks nanomaterial and respective extracts against *S. feltiae*

The nanosized particles from the bark of *P. erinaceus* exhibited a concentration-dependent activity against *S. feltiae*, reducing the viability of the nematode significantly, to less than 80%, when employed at a concentration of 1%. In contrast, the bark extract of *P. erinacues* did not affect the viability of *S. feltiae* (as reported in the precedent lines: Chapter 2, Section 4.4).

3.3. Biological Activity of *P. erinaceus* barks nanomaterial and respective extracts against *E. coli*

The particles, as well as the extracts of the *P. erinaceus*, reduce the survival of *E. coli* in a concentration-dependent and statistically significant manner. At a particle concentration of 1%, for instance, the bacterial count is reduced to zero when compared to the negative control (water), whilst the stabilizer Plantacare at this concentration exhibited no significant impact on bacterial growth. This finding is in agreement with the pronounced activity of the

methanolic extract of the bark of *P. erinaceus* which also reduced bacterial growth from an OD_{593} of 0.8 in the control to just an OD_{593} of 0.2 (*i.e.*, to around 25% of the original growth), when employed at a concentration of 256 µg/mL (Figure 4.3). It then appears that processing the bark of *P. erinaceus* to small particles provides a viable method to "unlock" the biological activity of this material and hence to render it suitable to reduce the viability of certain bacteria, such as *E. coli*.

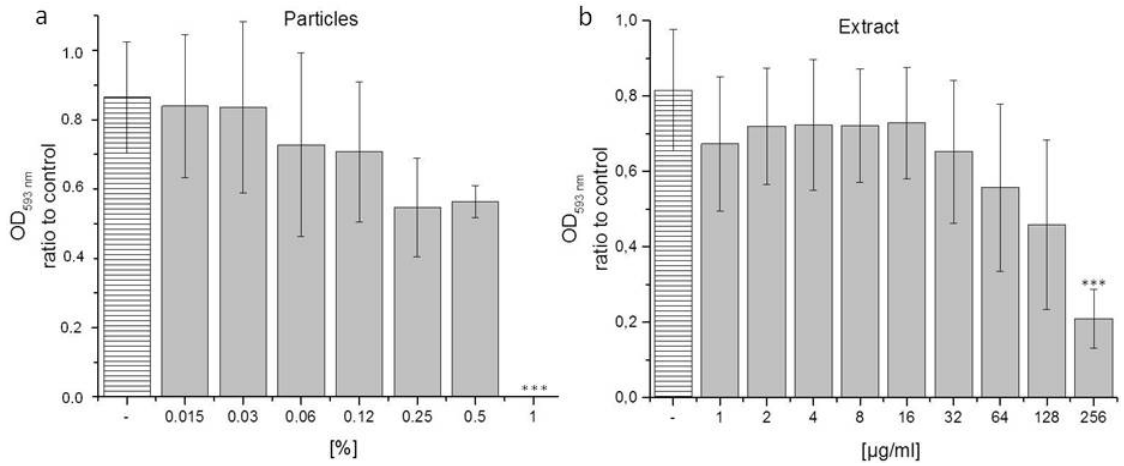


Figure 4.3: Activity of homogenized particles (a) and methanolic extracts (b) of *P. erinaceus* bark against the Gram-negative bacterium *E. coli*.

LB broth (−) and Plantacare (not active, not shown) were used negative controls. All experiments were performed in triplicate and on at least two different occasions. Statistical significances were calculated using one-way ANOVA (Origin Plus). *** $p < 0.005$.

4. Discussion

The results obtained with the processed, homogenized particles of *P. erinaceus* support the use of such crude materials against pathogenic organisms, such as bacteria and agriculturally relevant nematodes related to *S. feltiae* (*S. feltiae* itself is a model but not a target). On the other hand, the results also point towards possible limitations of the method and scope for further improvement, particularly in the context of particle quality and stability, aspects of release and level of activity.

The nanosizing process deserves further attention. Whilst a combination of HSS and subsequent HPH seems to provide a simple, straight-forward and comparably rapid method to produce samples for initial biological testing, it is also apparent that there is still considerable room for further improvement. Whilst the shapes and sizes of the particles obtained by these procedures, no doubt are adequate for biological tests, it would be desirable to achieve

particles of more uniform shapes and smaller sizes, and particularly of higher stability for practical applications in the future. Here, more refined methods, such as more appropriate stabilizers or more effective nanonization methods, e.g., ART Crystal Technology, may be considered, bearing in mind that one of the prime aims of this study has been the investigation of *simple* methods which explicitly may be applied locally in developing countries (Scholz *et al*., 2014). Hence future studies may have to consider a balance between economical, straight-forward methods on the one side and good quality and stable particles on the other.

Similarly, our study should also be seen as preliminary when considering issues of why such particles are active. Such considerations almost certainly will raise questions related to nanosafety and/or the release of active substances from those particles. Here, we still lack information regarding the release of biologically active compounds from them. Still, one may speculate that the particles employed serve as natural delivery systems for toxic agents such as alkaloids, whilst other effects on cells and organisms, such as interactions with membranes or the entire blockage of pores (in nematodes) would also have to be to be considered.

In any case, to unlock the biological activity of active ingredients contained within plants and plant cells, it should be possible to nanosize a wide variety of such different plants and parts thereof. As long as they can be dried, do not contain excessive fats or oils, such natural plant materials should be suitable for simple milling and homogenization procedures. In fact, the activity of the particle preparations derived from the bark of *P. erinaceus*, compares rather well with the ones observed for the respective extract. This, in turn, may point to wider practical applications, not only of the nanosizing method but also of the plant involved in this study. *P. erinaceus* may possess antibacterial properties useful in the context of simple infections, for instance affecting the gastrointestinal tract or the skin. In both cases, toxic effects on human cells obviously need to be investigated in earnest. Yet as far as we can judge, neither agricultural nor topical applications seem to bear any excessive risks.

Besides the African barwood, which has been used here simply to showcase the potential of the homogenization method, one may also envisage a wide range of additional plants commonly grown and highly abundant often even as weed or waste in developing countries with a rich flora, in particular in Africa, Asia and South America. Promising examples include, for instance, *Nauclea latifolia Sm.* or *Ocimum gratissimum* L., which already have some reputation as being effective against malaria and intestinal parasites (Tchacondo *et al*., 2012). Other sources of particular interest may well include various parasitic plants, which often also behave as weeds, and on one side are rich in biologically active ingredients and on

the other side tend to lack chlorophyll and other readily degrading substances. Indeed, there is plenty of choices as far as suitable plants are concerned.

Eventually, comprehensive studies on the nanosizing techniques, the particles obtained and their respective biological activities, physico–chemical and release properties will decide if such methods indeed provide a viable alternative to the extraction, isolation, formulation and delivery methods traditionally employed to move from a crude plant material to a practical application. As nanosizing "in one go" covers all these conventional methods from extraction to the formulation, and since our initial results are certainly not negative, it is worthwhile to give such methods and the resulting particles further consideration.

5. Conclusions

In essence, our study has lent support to the idea that nanosizing of plant materials may enable us to move from a crude, dried plant material to an applicable, "complete particle"-based delivery and release system in just one or a few simple steps. Whilst there are plenty of questions which remain to be addressed and answered in the future, none of these issues are insurmountable. Depending on the funds available, the methods for nanosizing can be varied and refined, ultimately resulting in more defined and stable particles. Release properties can also be controlled by such processes, and so can be the (physical) properties of the particle itself and the biological activity caused by the substances released from it.

Future studies will therefore not only focus on the preparation of a wide range of particles from an equally wide range of local plants, and of different, application-specific quality. They will also consider a much wider spectrum of possible applications in the fields of nutrition and cosmetics, in disease prevention and therapy. Here, cardiovascular, anti-inflammatory, anti-cancer and anti-infective agents may be at the forefront of the investigation. Particles of *P. erinaceus*, for instance, have already shown some activity in this context and, after a more in-depth examination, could herald a new era for locally grown and produced particles against dysentery and simple skin infections.

Ultimately, a prime focus will also be on agricultural applications, as the amounts required in agriculture are considerably higher than in medicine, whilst the potential risk for humans is lower.

General conclusions

Togo is a country with a diverse climate from south to north which has consequently given birth to a diverse flora. People all over the country take advantage of this diverse flora and use it for various purposes: food, housing, healthcare, etc.

By carrying out the ethnobotanical survey in Tchamba prefecture, we could record the knowledge of 53 traditional healers. The treatment of the recorded knowledge has led to identifying 43 plants belonging to 43 genera and 27 families of which the *Fabaceae* were the most represented. To identify the most promising plants out from the 43 species, a computer-aided method CAPITURE, like an algorithm, has been established with statistical numerical values calculated using Excel tables. The general values such as the Reported Use (RU), the Frequency of Citation (FC), the Use Value (UV), the Informant Consensus Factor (ICF), the Fidelity level (Fl) were applied to the data to identify the first group of interesting plants. The more specific values such as the Plant Part Value (PPV), the Specific Use (SU), the Intraspecific Use Value (IUV) were then applied to the first group to identify the most promising plants parts and the diseases they are supposed to be efficient against. This way, we could identify *Pterocarpus erinaceus* sap against ringworm, its roots against candidiasis and *Daniellia oliveri* sap against intertrigo and its trunk barks against candidiasis as the most interesting targets. Candidiasis is usually caused by *Candida albicans*, the ringworm is due to a dermatophyte *Tricophyton rubrum* and the Intertrigo is caused by either *Candida albicans* or *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Considering the number of fungi and bacteria involved in those different diseases, it could be implied that *P. erinaceus* will be more active against fungi and *D. oliveri* will be more efficient against bacteria but with also some activity on fungi. Consequently, the two plants have been evaluated against a wide range of bacteria and fungi (including the ones mentioned in the precedent lines). The results obtained during the biological studies have confirmed the predictions of the CAPITURE method *ie D. oliveri* exhibited more activity on bacteria than *P. erinaceus*. Indeed, only the stems barks of *P. erinaceus* showed interesting activity against seven bacteria commonly encountered in infectious diseases and used in the biological tests: *Enterococcus faecalis*; *Staphylococcus aureus*; *Escherichia coli*; *Klebsiella pneumoniae*; *Enterobacter cloacae*; *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. All the three parts of *D. oliveri* (leaves, stems barks and roots) showed interesting activity against the bacteria, the opposite results have been obtained during the anti-fungal tests. Indeed, our previous study has shown

that *D. oliveri* was less active against *C. albicans* in comparison to *P. erinaceus*. Another study has also proven that *P. erinaceus* was active against *Tricophyton rubrum*. The present study has demonstrated the activity of *P. erinaceus* against *Aspergillus fumigatus*. In contrast, *D. oliveri* was not active against this germ. From those different results, we could conclude that *P. erinaceus* is more active against fungi than *D. oliveri*. Finally, the CAPITURE method finds its confirmation here: *P. erinaceus* is more active against fungi than *D. oliveri* which is more active on bacteria than *P. erinaceus*.

The extracts and fractions that have shown the best activities during the biological tests were in a third step chemically analyzed to identify the substances that may be responsible for the observed activities. To achieve this aim, analytical chemistry methods have been used: from separation methods (extraction and chromatography) to detection methods (mass spectrometry and nuclear magnetic resonance). During this third step, a focus was placed on *P. erinaceus*. The following compounds have been isolated in the stems barks of this plant, identified mainly by NMR, with MIC values around 4 µg/mL against bacteria for some of them: Friedeline; 2,3 dihydroxypropyloctacosanoate; *β*-sitosterol; stigmasterol; campesterol and *β*-sitosteryl-*β*-*D*-glucopyranoside. In the roots, some compounds have also been isolated, identified mainly by NMR, with MIC values of 8 µg/mL to 256 µg/mL against fungi: Friedeline; *β*-sitosteryl-*β*-*D*-glucopyranoside; formononetin; *ψ*-baptigenin; isoliquiritigenin; nuningin, a new arylpropanoid and a new isoflavone. All those compounds except friedeline have never been reported in this plant species and the arylpropanoid and the isoflavone have never been reported in nature at all. Using liquid chromatography coupled to mass spectrometry (LC-MS), an *in-situ* chemical identification has led to propose structures of compounds never reported in nature at all such as formononetin-glc-xyl (or ara); pseudobaptigenin-glc-xyl (or ara) and genistein-glc-xyl (or ara) and isomer (trunk barks). Using also only gas chromatography coupled to mass spectrometry (GC-MS), compounds have been identified in apolar fractions of the different parts of *P. erinaceus* and *D. oliveri*: many fatty acids (saturated, unsaturated with sometimes poly-saturated fatty acids), many terpenes (sesquiterpenes), some sterols and phenolic compounds. Many compounds did also not hit a known compound in the library, suggesting such compounds to be compounds never reported in nature at all.

In countries such as Togo, the plant parts are used after infusion or decoction with water or ethanol. Such countries do not have a pharmaceutical industry where new compounds such as the ones discovered in this study could be turned into pills, sprays or creams. By nanonisizing

the powder from the trunk barks of *P. erinaceus*, it was demonstrated that without having to use an organic solvent for the extraction and huge quantity of plant material, the biological activity of the plant was kept. The nanosized plant powder could be if further investigated, a new formulation to be used to cure diseases.

From traditional medicine to modern investigations, from plants to a medicine, this study has once more underlined the efficacy of plants, in this case, used in traditional medicine in the Tchamba district of Togo, to treat different infectious diseases. The study has helped address the most apparent question always raised after an ethnobotanical survey namely are the plants active? As part of this investigation, a computational approach has been proposed to predict and subsequently select the most interesting plants. Even though such a method needs improvements, it yields interesting results as confirmed by the biological activities of the plants selected by this method. Concerning the biological part, it will be worth investigating further the activity of the two plants against other types of Gram-positive bacteria, especially the ones belonging to the genus *Staphylococcus* as there is a preferential activity against this kind of germ. The analytical chemistry part has provided considerable structural information on the two plants. Further investigations are, however, required, especially with *D. oliveri* where no isolation has been carried out but also with the mass spectrometric studies on *P. erinaceus* extracts and fractions. The new structures reported should also be studied further to evaluate their whole range of biological activities and to see whether new pharmaceutical products could be derived from them. This way, the transfer from traditional to modern medicine will be complete. In general, more in-depth studies will provide a complete map of those plants: biological activities, pharmacological properties, toxicology, bio-active compounds, etc. Subsequent studies will be extensive and comprehensive and will by far exceed the scope of one PhD thesis.

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APPENDIX

1. Chapter one

Table 1: General data on the plants used to treat fungal diseases (Obtained from the ethnobotanical survey).

Local names. Tch: Tchamba, Te: Tem, La: Lamba, K (Kounssountou), Lo: Logba, Ka: Kabyè. **Mode of preparation**. Inf: infusion; Sol: solution; Pa: paste; Cr: cream; Pow: powder; Dec: decoction. **Mode of administration**. T: topical; O: oral. **Type of fungal and related diseases**. R: Ringworm; I: Intertrigo; OC: Oral Candidiasis; SC: Sexual Candidiasis; F: Felon; Ony: Onychomycosis. **Plants parts**. Bu: bulb; B: barks; Fr: fruits; FrS: fruits shell; R: roots; Rhi: rhizome; S: sap; Wp: whole plant; L: leaves; Se: seeds; Tr: Trunk. **Phytogeographic type**. Pan: Pantropical; I: Introduced; AT: Afro-Tropical; GC: Guineo-Congolese; SZ: soudano-zambezian; Pal: Paleotropical; S: Soudanian; G: Guinean; Pra: Pluri-regional African; AA: Afro-American, AM: Afro-Malgach. **Biological type**. LmP: Liana mesoPhanerophyt; Lge: Liana geophyt; He: Hemicryptophyte; Ch: Chamaephyte; MPh: MegaPhanerophyt; Th: Therophyte; Lmp: Liana microphanerophyte; Ge: geophyte; mPh: mesoPhanerophyt; nph: nanophanerophyte; mph: microphanerophyte. **Other uses**. N: none. **Voucher number**. NP: not possible.

Family	Scientific name	Biologic type	Phyto geographic type	Local name	Voucher specimen	Used part	Mode of preparation/ administration	Disease	Other uses	∑ (RU)	UV
Alliaceae	*Allium sativum* L. [cult.]	He	I	Ayo (Tch)	NP	Bu	Mac / T, O	R	High blood pressure, snake bite.	2	0.04
Anacardiaceae	*Mangifera indica* L. [cult.]	mph	Pan	Mango (Tch)	TOGO 01797	B	Inf / O, T (bath)	OC and SC	N	1	0.02
	Anacardium occidentale L. [cult.]	mph	Pan	Atchan (Tch)	TOGO 01768	B, Fr	Inf, Sol/ T	OC and SC, I	High blood pressure, malaria, abscess	2	0.04
Annonaceae	*Uvaria chamae* P. Beauv.	Lmph	PRA	Djablow (Tch)	TOGO 01950	R	Inf/	SC	Abscess	1	0.02
Araceae	*Anchomanes difformis* (Blume) Engl.	Ge	G	Agnantr (La)	TOGO 09515	Rhi	Pa/ T	F	Haemorrhoids	1	0.02
Arecaceae	*Cocos nucifera* L. [cult.]	mPh	Pan	Koubéboutchi (Tch)	NP	FrS	Inf	F	N	1	0.02
Asclepiadaceae	*Calotropis procera* (Aiton) R.Br.	nph	Pal	Tchovô (Te), Koulalapem (Tch)	TOGO 02209	S	Sol /T	R	Epilepsy, sickle cell disease	2	0.04
Asteraceae	*Tridax procumbens* L.	Th	Pan	Dwatsè (Tch)	TOGO 01151	Wp	Cr / T, Inf / O	OC and SC	Earache	1	0.02

Combretaceae	*Cochlospermum planchonii* Hook.f.	nph	SZ	Sissinô (Te), Tchèlira (La)	TOGO 00466	B, R	Pow / T, O (maceration in food)	SC, I	Stomachache	2	0.04
	Quisqualis indica L.	Lmp	Pan	Ofantignô (Tch)	TOGO 15085	R	Pow/ O (maceration in food) Inf/ O, T (bath)	OC and SC	N	2	0.04
Connaraceae	*Brysocarpus coccineus* Thonn. ex Schumach.	Lmp	GC	Sanbâlâ (Tch)	TOGO 15081	R	Pow/ T, O (maceration in food)	OC and SC	Haemorrhoids, rheumatism, stomachache	1	0.02
Dioscoreaceae	*Dioscorea alata* L. [cult.]	G	I	Sakata (Tch)	TOGO 10414	Rhi	Pow	OC and SC	N	1	0.02
	Dioscorea cayenensis Lam. [cult.]	Lge	At	Dnô (Tch)	TOGO 10424	Rhi	Pow / O (maceration in food)	OC and SC	N	1	0.02
Euphorbiaceae	*Bridelia ferruginea* Benth.	mph	PRA	Kalâ (Lo)	TOGO 03089	L	Cr / T	R	N	1	0.02
	Jatropha curcas L. [cult.]	nph	I	Koutchatchamoi (Tch)	TOGO 15092	Fr	Pow / O (maceration in food)	OC	Irregular periods	1	0.02
	Ricinus communis L.	nph	Pan	Ounwolou (Tch)	TOGO 03732	Fr, Se	Cr, Pow / T	OC, SC, R	Stomach ache	2	0.04

	Flueggea virosa (Roxb. ex Willd.) Voigt	Ph	Pal	Sakassaka (K), Tchâmalidô (Te), Tchatchata (La)	TOGO 03760	R, B	Cr, Sol / T Pow, Inf / O (maceration in food), T	SC, Ony	Stomachache, anaemia.	4	0.08
Fabaceae	*Desmodium gangeticum* (L.) DC	Ch	Pal	Kougnindoudoum (Ka)	TOGO 05946	R	Cr / T	SC, R	Stomachache	2	0.04
	Pterocarpus erinaceus Poir.	mPh	SZ	Boutô (Tch)	TOGO 15077	S, B, R	Sol, Cr/ T Inf/ O	R, OC and SC	Headache, tomachache, tonic, release from spells, infertility, irregular periods, anaemia, haemorrhoids, lipstick,	15	0.28
	Cassia alata L.	nph	GC	Tchakô (Lo)	TOGO 15094	L	Pa / T	R		1	0.02
	Cassia occidentalis L.	np	pan	N'tchamtcham (Tch)	TOGO 15088	Wp	Pow / T	Ony	Vomiting	1	0.02
	Detarium microcarpum Guill. & Perr.	mph	S	Kpôr (La)	TOGO 00176	B	Dec / O (maceration in food), T (bath)	OC and SC		1	0.02
	Daniellia oliveri (Rolfe) Hutch. & Dalziel	mPh	SZ	Boussâ (Tch), Tchâlo (La)	TOGO 15076	S, B	Sol /T Inf / O, T	I, OC and SC	Galactogenic, haemorrhoids	6	0.11
Loganiaceae	*Strychnos spinosa* Lam.	mph	AM	Kokosinatchi (Tch)	TOGO 12659	R, B	Cr/ T	R	Stomachache, painful periods (amenorrhoea)	1	0.02

Meliaceae	*Khaya senegalensis* (Desr.) A.Juss.	MPh	S	Koutcha (K)	TOGO 01797	B	Inf/ O	OC	Hernia, abscess	1	0.02
	Pseudocedrela kotschyi (Schweinf.) Harms	mph	S	Azimtélmr (La)	TOGO 12736	B	Inf/ O, T (bath)	OC and SC	Dizziness	1	0.02
	Xeroderris stuhlmannii (Taub.) Mendonça & E.C. Sousa	mph	SZ	Bougbé (Tch), Tchéou (La)	TOGO 06769	Wp, B	Cr/ T	R	Anaemia	2	0.04
Mimosaceae	*Parkia biglobosa* (Jacq.) R.Br. ex G.Don	MPh	Pal	Boudô (Tch)	TOGO 15084	Tr	Pow/T	F	N	1	0.02
Moraceae	*Ficus thonningii* Blume	mPh	AT	Koussôssô (Tch)	TOGO 05199	L	Sol/ T	R	N	1	0.02
	Milicia excelsa (Welw.) C.C.Berg	MPh	G	Frimou (Te)	TOGO 12751	Sa, B	Pow/T, O (maceration in food)	F, SC	Conjunctivitis	2	0.04
Musaceae	*Musa sapientum* L.	Ge	I	Ayaba (Tem)	TOGO 15091	Fr	Pow / T, O (maceration in food)	OC and SC	Impotence	3	0.06
Piperaceae	*Piper guineense* (Schum and Thonn.)	LmP	GC	Djèyam (Tch)	TOGO 06862	Fr	Cr / T Inf/ O, T (bath)	F, OC and SC	N	2	0.04
Poaceae	*Zea mays* L. [cult.]	Th	I	Barafotia (K), Wamladêni (Te)	TOGO 11532	Fr	Pow / T, O (maceration in food)	SC and OC	N	2	0.04
Rubiaceae	*Gardenia aqualla* Stapf & Hutch	nph	SZ	D'nabô (Tch)	TOGO 07309	Tr	Pow/ O (topical, mouth wash)	OC	N	1	0.02

	Morinda lucida Benth	mp	Pan	Katchinligan (Te)	TOGO 07498	R	Pow, Cr / O (maceration in food or water), T	OC and SC	Stomachache, headache	1	0.02
Rutaceae	*Citrus limon* (L.) Burm.f. [cult.]	mph	AT	Kankayo (Te), Anga (La), Sonssontiya (K), Sonssonboutchi (Tch)	TOGO 15089	Fr, R, B	Sol, Cr /T Pow/ T or O (maceration in food)	F, Ony, I, OC and SC	Breathing stop, angina, stomachache	1	0.02
	Citrus sinensis (L.) Osbeck	mph	AT	Akotsouboutchi (Tch)	TOGO 15093	Fr	Pow, Cr/ T	SC	N	1	0.02
Sapindaceae	*Paullinia pinnata* L.	Lmph	AA	Kindjidjifèyi (Tch)	TOGO 15082	R	Dec / T	Ony, F	Incurable wounds, haemorrhage	4	0.08
Solanaceea	*Nicotiana tabacum* L. [cult.]	Th	I	Agboké (K)	TOGO O8500	L	Pa/ T	R	Epilepsy, fever, vomiting	2	0.04
Sterculiaceae	*Cola gigantea* A. Chev	mP	GC	Dikponkpori (Tch)	TOGO 08587	B	Pow/O (maceration in food)	SC	N	1	0.02
	Heliotropium indicum L.	Th	Pan	Dinalantobiri (Tch)	TOGO 02508	L	Inf / O	OC	N	1	0.02
Verbenaceae	*Vitex doniana* Sweet	mPh	AT	Bognan (Tch), Sangourô (K)	TOGO 09273	L, B	Sol, Inf/ T	R	N	2	0.04
Zingiberaceae	*Aframomum melegueta* [Roscoe] K. Schum	Ge	GC	Yibalèdessa (K), Abalôtchangai (Te)		Fr	Pow	OC	N	1	0.02

Table 2: Bibliographic review on the species with RU ≥ 2 or UV ≥ 0.04

Scientific name	Other surveys related to antifungal activities	Antifungal activities (MIC) in vitro or in vivo	Chemical compounds responsible of the observed antifungal activities	Other chemical compounds	Toxicity
Allium sativum L. [cult.]	NRAS	MIC not determined in most cases, just zones of inhibition on the agar (Singh *et al*., 1990; Khan *et al*., 2000; Sumbul *et al*., 2004; Shams-Ghahfarokhi *et al*., 2006; Okere *et al*., 2014; Bodhankar *et al*., 2011; Akinmusire *et al*., 2004).	Phytochemical screening: alkaloids, flavonoids, glycosides and tannins; Allicin (Bodhankar *et al*., 2011; Akinmusire *et al*., 2004).	Cycloalliin, γ-Glutamylpeptides, Ajoene and homologues, vinyldithiines (Sendl *et al*., 1995).	LD50 3034 mg/ kg in experimental rabbits (Mikail *et al*., 2010).
Anacardium occidentale L. [cult.]	NRAS	**Leaves**: MIC on *Candida albicans* and *Aspergillus niger*: 15.62-31.25 µg/ml. **Nut shell oil:** MIC on *Saccharomyces, cerevisiae*and, *Penicillium chrysogenum* > 1600µg/ml (Rajesh *et al*., 2009; Himejima *et al*., 1991).	Anacardic acids, cardols, 2-methylcardols, cardanols and phenolics (Himejima *et al*., 1991; Assunçao *et al*., 2003).	**Fruit:** β-carotene, β-cryptoxanthin, α-carotene and 9-cis- + 13-cis-β-carotene, ascorbic acid, provitamin A (Assunçao *et al*., 2003). **Flavonols:** 3-O-galactoside, 3-O-glucoside, 3-O-rhamnoside, 3-O-xylopyranoside, 3-O-arabinopyranoside, 3-O-arabinofuranoside of quercetin and myricetin, kaempferol 3-O-glucoside. **Anthocyan:** 3-O-hexoside of methyl-cyanidin (de Brito *et al*., 2007).	**Leaves:** oral acute toxicity in **wistar rats:** LD50 higher than 2000 mg/ Kg (Konan *et al*., 2007)
Calotropis procera (Aiton) R.Br.	NRAS	**Leaf:** MIC: 250µg/ml on *Trichophyton mentagrophytes*, *T. rubrum*, *Microsporum canis*, *M.gypreum*, *Epidermophyton floccousm*. **Latex crude extract:** MIC: 300 µL on *C. albicans*, *T. rubrum* and *Aspergillus terreus* (Goyal	A protein CpOsm from the latex: IC 50 of 67 mg/ml against *Fusarium solani*; IC 50 of 57.5 mg/ml against *Neurospora* sp. IC 50 of 32.1 mg/ml against *Colletotrichum Gloeosporioides* (de Freitas *et al*., 2011).	**Vegetative stems**: 5-hydroxy-3,7-dimethoxyflavone-4-*O*-*β*-glucopyranoside; 2*β*,19-epoxy- 3b,14β-dihydroxy-19-methoxy-5*α*-card-20(22)-enolide; β-anhydroepidigitoxigenin-3*β*-Oglucopyranoside, uzarigenine and β-anhydroepidigitoxigenin (Shaker *et al*., 2010) **Latex:** urs-19(29)-en-3-yl acetate, *β*-sitosterol and stigmasterol, multiflorenol, urs-19(29)-en-3-β-ol and 3β,27-dihydroxy-urs-18-en-	**Latex and leaves:** hepatotoxic and cardiotoxic respectively in rats and sheeps at doses of 1ml of fresh latex /kg and 60g/Kg of leaves (de

		et al., 2013)		13,28-olide[18]; Procerain (cysteine protease) (Dubey *et al*., 2003).	Lima *et al*., 2011)
Cochlospermum planchonii Hook.f.	NRAS	**Stem leaf:** MIC on *Tricophyton* spp: 20 mg/ml (Isah *et al*., 2013).	Phytochemistry screening: Carbohydrate, Glycoside, Antraquinone, Saponins, Steroidal triterpenes, Flavonoids, Tannins (Isah *et al*., 2013).	**Rhizomes essential oil:** β-elemene, β and α-selinene, tridecan-2-one, undecyl acetate, 7-diepi-α-selinene, tetradecen-3-one (Ouattara *et al*., 2007).	**Rhizome:** 50mg/kg orally alterate the biochemical parameter in albino rats (Nafiu *et al*., 2011).
Milicia excelsa (Welw.) C.C.Berg	NRAS	**Heartwood:** MIC on *Trametes versicolor*: 50µg/ml [24]. **Stems barks:** On *C. albicans*, IC 50: 8.705-6.563 mg/ml and MFC: 25-100 mg/ml (Kra Adou *et al*., 2014).	Heartwood: phenolic compound (Huang *et al*., 2009).	**Roots:** 2-(2,4-dihydroxyphenyl)-5-hydroxy-8,8-dimethyl-4H,8H-pyrano[2,3-f]chromen-4-one (2'-hydroxyatalantoflavone); atalantoflavone, neocyclomorusin, 6-geranylnorartocarpetin, cudraxanthone and betulinic acid. **Stem barks:** melicilamide (ceramide). **Leaves**: 3,4-dimethoxybenzyl β-D-xylopyranosyl (1 --> 2)-beta-D-glucopyranoside, lupeol acetate, ursolic acid, triacontyl (E)-ferulate and 2-(3,5dihydroxyphenyl)benzofuran-5,6-diol (Hussain *et al*., 2013; Ouete *et al*., 2014).	NRAS
Quisqualis indica L.	NRAS	Diameters of fungi and dermatophytes inhibition (Jahan *et al*., 2008; Sangur *et al*., 2012; Sarika *et al*., 2013).	NRAS	**Fruits and leaves:** quisqualin A; quisqualin B; 2,3-(S)-HHDP-D-glucose; 2,3-(S)-HHDP-4-0-galloyl-D-glucose; 2,3-(S)HHDP- 6-0-galloyl-D-glucose; 2,3-(S)-HHDP-4,6-di-O-galloyl-D-glucose; pedunculagin; punicalagin; 6-0-galloyl-D-glucose;1,6-d.i-O-ganoyl-~-D-glucose (Lin *et al*., 1977).	NRAS
Ricinus communis L.	Leaves: yeast (Manpreet Rana *et al*., 2012).	Inhibition of *A. fumigatus* and *A; flavus* by the leaves (Rabia *et al*., 2012).	Casbene (Manpreet Rana *et al*., 2012).	**Leaves:** Disaccharide glycoside rutin, gentistic acid, quercetin, gallic acid, kaempferol-3-O-β-drutinoside, kaempferol-3-O-β-d-xylo pyranoid. **Seeds:** Ricin A, B and C and one ricinus agglutinin.	**Leaves:** 10g/Kg no toxic effect in Swiss mice (Pingale Shirish S., 2011]).

				Roots: Indole-3-acetic acid (Manpreet Rana *et al*., 2012). **Aerial parts essential oil:** α-thujone, 1,8-cineole, α-pinene, camphor, camphene (Kadri *et al*., 1996).	
Nicotiana tabacum L. [cult.]	NRAS	NRAS	Enzymes : chitinases and 1,3-glucanases (Sela-Buurlage *et al*., 1993) ; Chitin binding protein 20 kd (Ponstein *et al*., 1994).	**Leaf:** Alcaloids: nicotine, nicotein, nicotimine. **Roots:** Anatabine and (+) nornicotine. **Flowers:** Quercetin-3,3´- dimethyl ether and quercetin-3-methyl ether (Aarti *et al*., 2013).	NRAS
Vitex doniana Sweet	NRAS	**Leaves:** Inhibition of *Aspergillus* spp sporulation and mycelia development (Lagnika *et al*., 2012).	NRAS	**Stem bark extract:** 11β-hydroxy-20-deoxyshidasterone; 21-hydroxyshidasterone; 2,3-acetonide-24-hydroxyecdysone [Ishola *et al*., 2014]. **Leaves essential oil:** β-phellandrene, phytol, β-caryophyllene, α-caryophyllene, caryophyllene oxide, α-pinene and bicyclogermacrene (Sonibare *et al*., 2009).	**Root bark:** LD50 in rats 980 mg/Kg [Akan *et al*., 2012]. **Leaf:** LD 50 greater than 5000 mg/ kg (Dawang *et al*., 2015).
Xeroderris stuhlmannii (Taub.) Mendonça & E.C. Sousa	NRAS	**Leaves:** slight inhibition activity on *C. albicans* and no activity on *C. crusei* (Mangoyi *et al*., 2011).	NRAS	NRAS	NRAS
Zea mays L. [cult.]	NRAS	**Seeds:** MIC of 5µg/ml on *Alternaria* spp (Delavalle *et al*., 2011). Anthocyans from Corn: MIC 0.625-2.5 mg/ml on different species of Candida (Suket *et al*., 2014).	NRAS	**Style:** chrysoeriol 6-C-α-boivinopyranosyl-7-O-α-glucopyranoside (Suzuki *et al*., 2003). Lumichrome, Chrysoeriol, genistein, adenosine, guanosine, uracil, acetovanillone, vanillin, vanillic acid, 6-methoxy-benzoxazolinone (Suzuki *et al*., 2007). **Corn:** cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, p-coumaric, vanillic acid, protocatechuic acid (Pedreschi *et al*., 2007).	NRAS

Desmodium gangeticum (L.) DC	NRAS	NRAS	NRAS	**Stems :** 4,5,7-Trihydroxy-8-Prenylflavone 4-O-α-Lrhamnopyranosyl-(1→6)-β-d-glucopyranoside; Whole plant: 8-C-prenyl-5,7,5'- trimethoxy-3',4'-α Methylenedioxyflavone, Kaempferol, 7-O-β-d-glucopyranoside, Rutin Desmodium gangeticum, Quercetin (Ma *et al*., 2011).	**Aerial parts:** no toxicity (Ma *et al*., 2011).
Piper guineense	NRAS	**Seeds:** MIC 125-250 µg/ml on *Microsporum gypseum*, *T. mentagrophytes*, *T. rubrum*, *Cryptococcus neoformans*. **Fruits:** no activity on *C. albicans*. **Leaves:** MIC on *C. albicans* and *S. cerevisiae*: 10-20 mg/ml (Ngane *et al*., 2003; Okigbo *et al*., 2007; Anyanwu *et al*., 2014).	Phytochemical screening. Leaves: flavonnoids, tannins, glycosides, saponins and alkaloids (Anyanwu et Nwosu); Seeds: alkaloids, coumarins and polyoses (Ngane *et al*., 2003).	**Roots:** N-Isobutyl-Trans-2-Trans-4-Eicosadienamide, αβ-dihydropiperine, wisanine and wisanidine. **Fresh fruits essential oil:** β-sesquiphellandrene, linalool, limonene, Z-β-bisabolene and α-pinene (Addae-Mensah *et al*., 1977 a; Addae-Mensah *et al*., 1977 b Oyemitan *et al*., 2015).	**Seeds:** no toxicity has been observed in mice (Ngane *et al*., 2003).
Musa sapientum L.	NRAS	**Leaf:** MIC of 0.0625 mg/ml – 0.5 mg/ ml on *Microsporum canis* and *Tricophyton tonsurans.* No activity on *T. rubrum* (Ige *et al*., 2015).	NRAS	**Pulp and fruit pulp:** a review on the main components with examples such as carbohydrates, flavonoids, tannins (Zafar *et al*., 2011).	NRAS
Flueggea virosa (Roxb. ex Willd.) Voigt	NRAS	**Roots barks:** MIC on *Candida* spp: 3.125-50 mg/ml (Kouangbé *et al*., 2015)	Phytochemical screening: alkaloids, tannins, sterols, polyphenols, flavonoids, quinone substances (Kouangbé *et al*., 2015).	**Twigs and leaves:** Alkaloids; Flueggedine fluevirines A, B, C and D; fluevirosinines B, C, D, E, F, G, H, I and J; flueggethers B and C (1 and 2) as dimers and flueggether D (3) as a trimer. **Roots :** Trinorditerpenes: flueggrenes A and B (Li *et al*., 2014; Zhang *et al*., 2015; Zhang *et al*., 2016 ; Chao *et al*., 2014).	NRAS
Paullinia pinnata L.	NRAS	**Stem bark:** no MIC on C. albicans but MIC from	Stem barks: (3β)-3-O-(2′-Acetamido-2′-deoxy-β-D-	**Leaves**: diosmetin-7-O-(2"-O-β-D-apiofuranosyl-6"-acetyl-β-D-	**Leaves:** toxic at doses higher

		3125-12500µg/ml on other yeasts and at 12500µg/ml on different dermatophytes, except *Tricophyton rubrum*. **Leaves:** MIC on a range of yeasts including *Candida albicans* and dermatophytes except for *T. rubrum*, 1562-12500 µg/ml (Lunga *et al*., 2014).	glucopyranosyl) oleanolic acid and (3β)-3-O-[β-D-glucopyranosyl-(1″-3′)-2′-acetamido-2′-deoxy-β-D-galactopyranosyl] oleanolic acid with MIC ranging from 3.125-100µg/ml on a range of yeasts and dermatophytes (Lunga *et al*., 2014).	glucopyranoside) and tricetin-4'-O-methyl-7-O-(2"-O-β-D-apiofuranosyl-6"-acetyl-β-D-glucopyranoside). **Roots:** paullinomide A, β-amyrin, 2-(4-hydroxy-3,5-dimethoxyphenyl)-3-hydroxymethyl-2,3-dihydro-1,4,5-trioxaphenanthren-6-one, 5α-poriferastane-3ß,6α-diol, *β*-sitosterol, *l*-quebrachitol, and *β*-sitosterol glucopyranoside and 3-O-isovanilloyl-3R,5R,8R,9R,10R,13R,14S,17S,18R,19R-lup-20(29)-en (Paullinoyl) (Abourashed *et al*., 1991; Lasisi *et al*., 2015; Adeyemo-Salami *et al*., 2013).	than 200 mg/ kg in male Wistar albino rats. **Roots:** oral consumption is safe for doses up to 850 mg/Kg in Wistar albino rats (Adeyemo-Salami *et al*., 2013; Adinortey *et al*., 2012).
Daniellia oliveri (Rolfe) Hutch. & Dalziel	NRAS	**Leaves:** MIC on *C alicans* :7.5 mg/ml; **stem barks:** 1.875-30 mg/ml; **roots:** 1.875-30 mg/ml (Tittikpina *et al*., 2013)	Phytochemical screening: flavonoids, tannins, saponins (Tittikpina *et al*., 2013).	Leaves: Rutin, narcissin, quercitrin, quercimeritrin; Oleoresin : polyalthic acid (Ahmadu *et al*., 2004 ; Atolania *et al*., 2014).	**Stem bark:** LD50 in mice > 3500 mg/Kg (Kabore *et al*., 2010).
Pterocarpus erinaceus Poir.	**Stem bark:** Ringworm (Etuk *et al*., 2008).	**Leaves:** MIC on *C. albicans*: 1.875mg/ml. **Stem barks:** 7.5 mg/ml. **Roots:** 1.875-3.75 mg/ml. **Stem bark on C. albicans:** 0.5 mg/ml (Tittikpina *et al*., 2013; Gabriel *et al*., 2010).	Phytochemical screening: saponines, tanins, alcaloids and flavonoids (Tittikpina *et al*., 2013; Gabriel *et al*., 2010).	**Stems barks:** Friedelin, lupeol and epicatechin (Ouédraogo *et al*., 2012).	**Stem barks:** intraperitoneal LD_{50} 447.21 mg/kg, oral LD_{50} was > 5000 mg/kg in albino wistar rats (Slawu *et al*., 2008).

Table 3: Questionnaire used during the survey

Name	
Age	
Sex	
Level of education	
Ethnicity	
Living place	
Specialty	
Knowledge origin	
Local name of the plant	
Fungal disease	
Name of the disease in local name	
Symptoms to recognize the disease	
Plant part used	
Mode of preparation administration	
Other uses of the plant	

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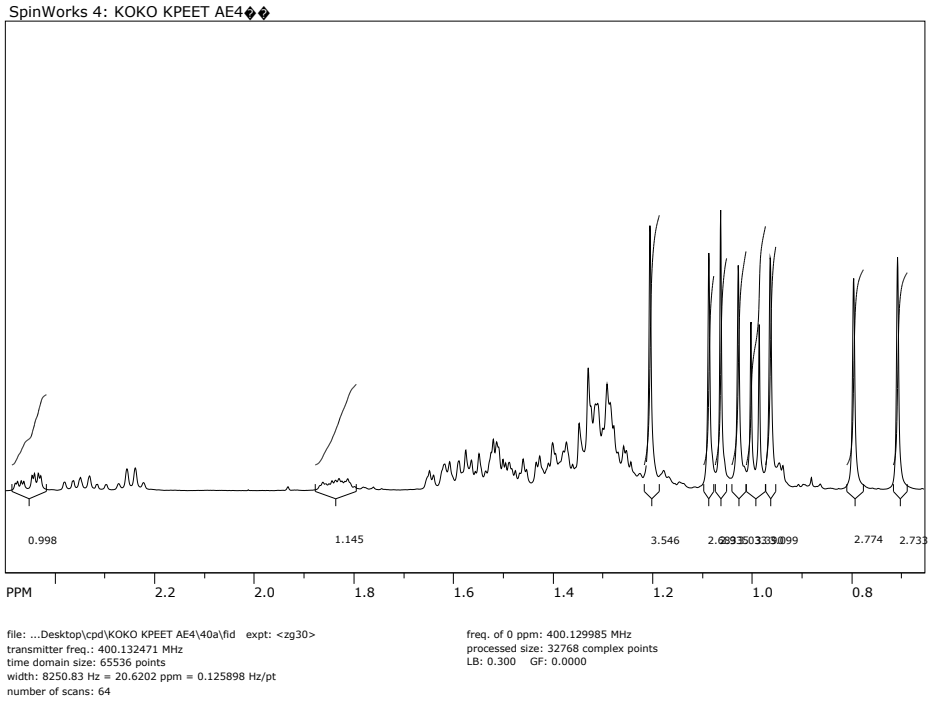
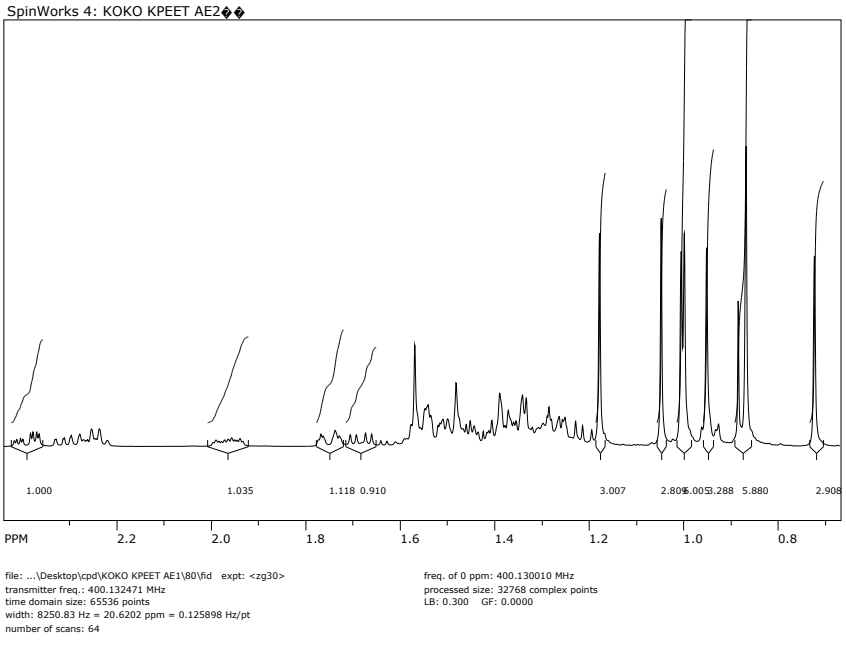
2. Chapter 3

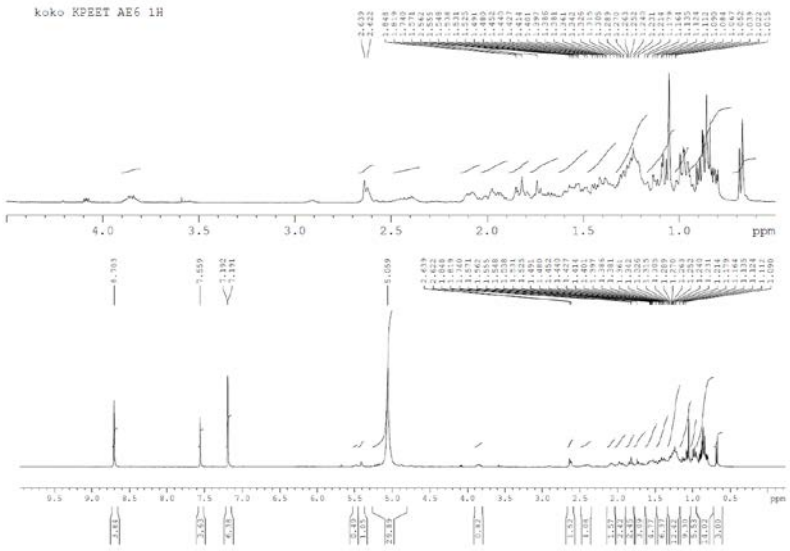
Compound 1= KPEETAE 4 (Friedeline, 1 H)



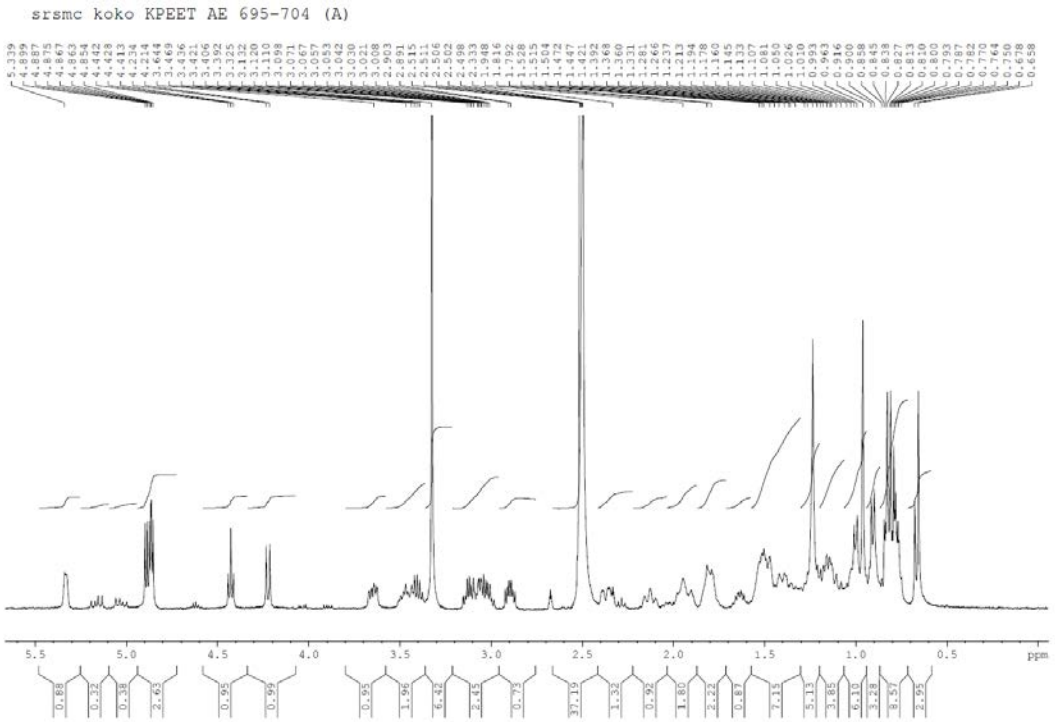
Compound 2= KPEETAE 2 (2,3 dihydroxypropyloctacosanoate, ^{1}H)



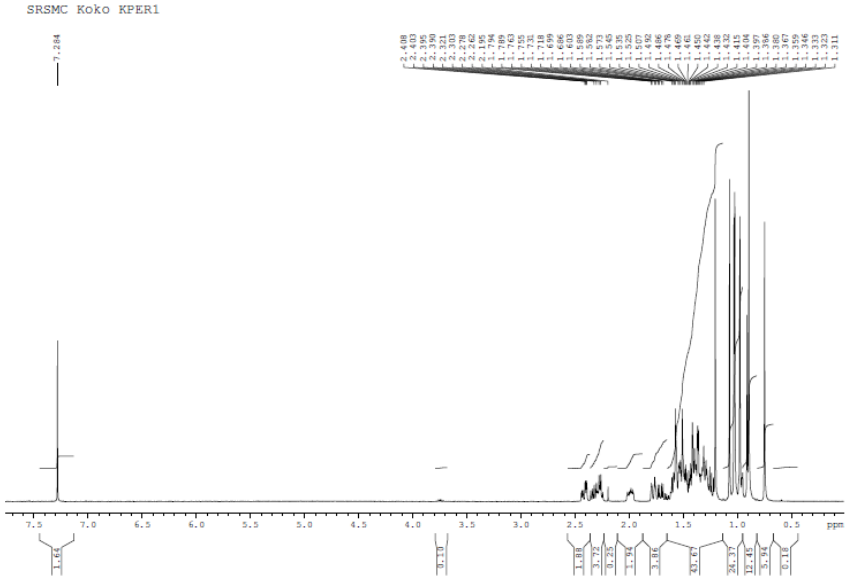
Compound 3 = KPEETAE 6 (*β*-sitosterol, campesterol and stigmasterol)



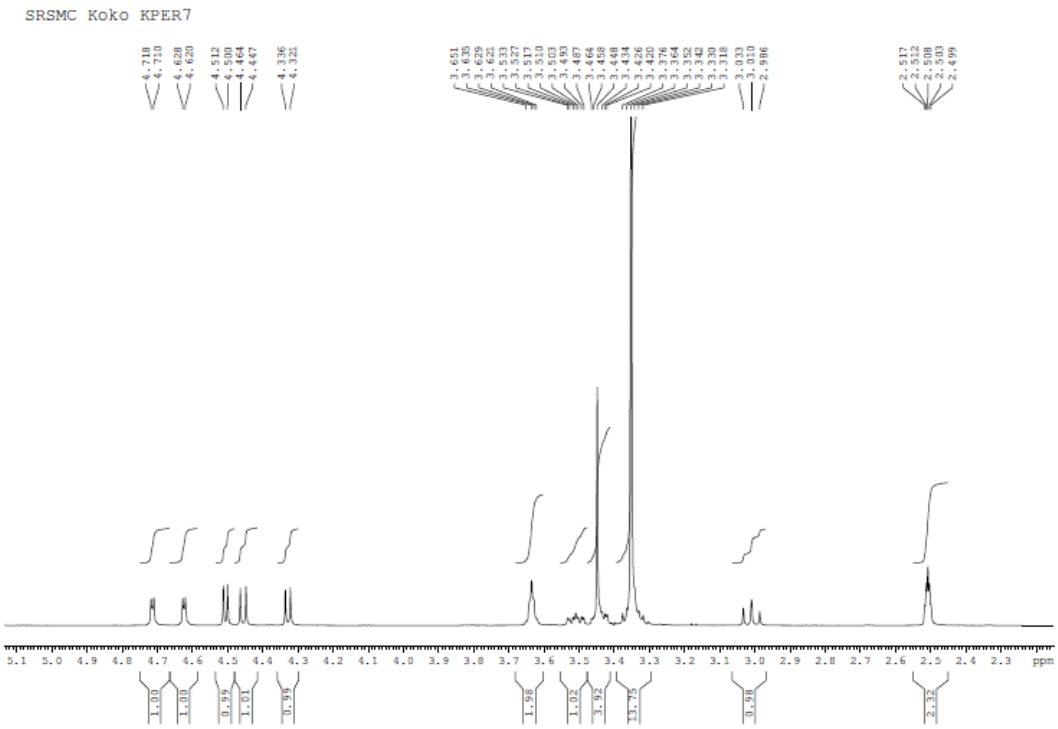
Compound 4= KPEETAE 695-704 (A) (*β*-sitosteryl-*β*-*D*-glucopyranoside, 1H)



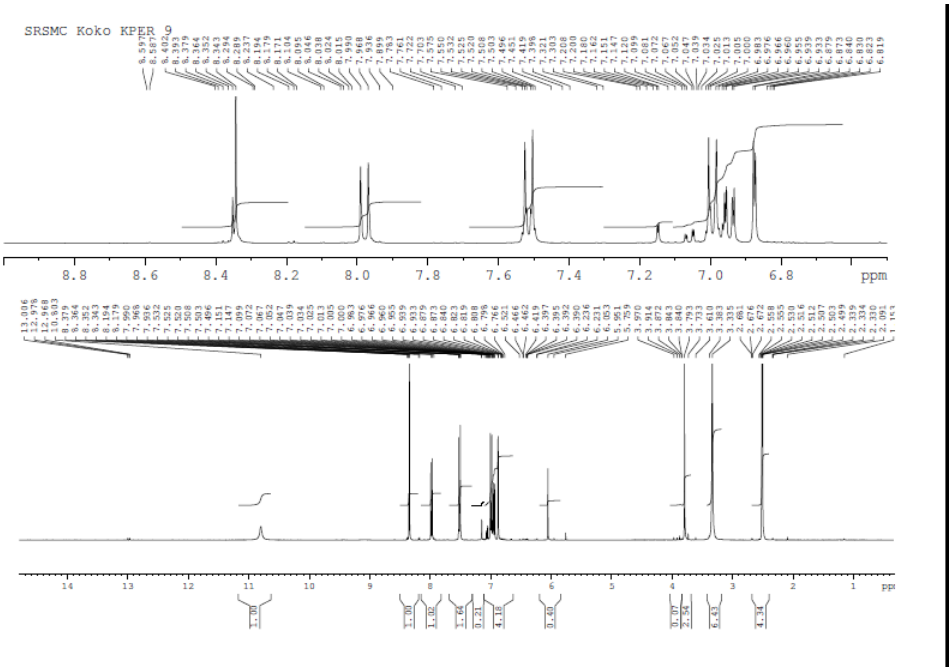
KPER 1 (Friedeline, 1H)



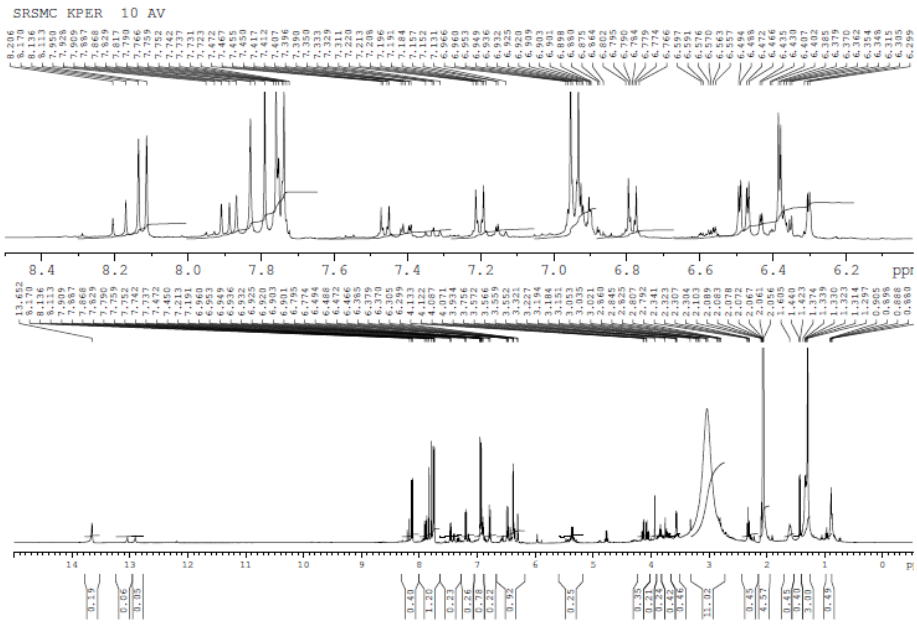
KPER 7 (Glucopyranose, ^{1}H)



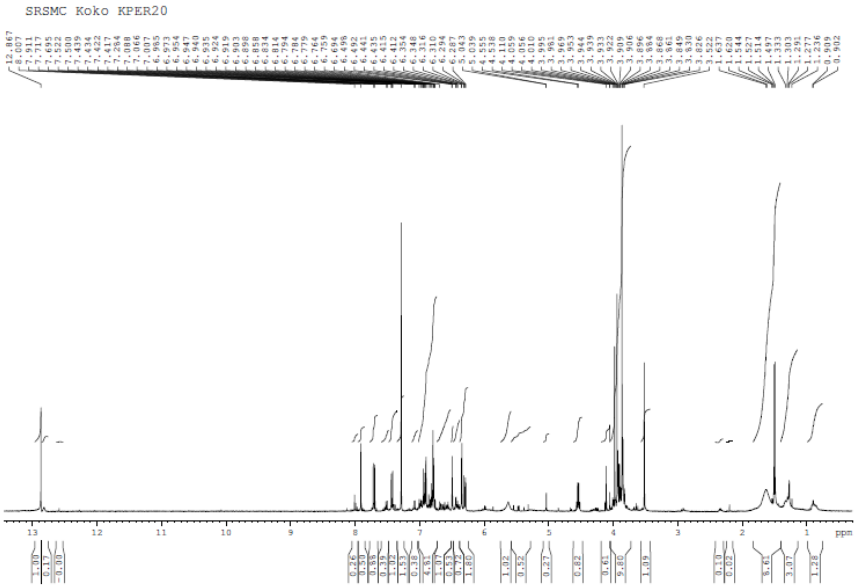
KPER 9 (Formononetin + pseudobaptigenin, 5:1, ^{1}H)



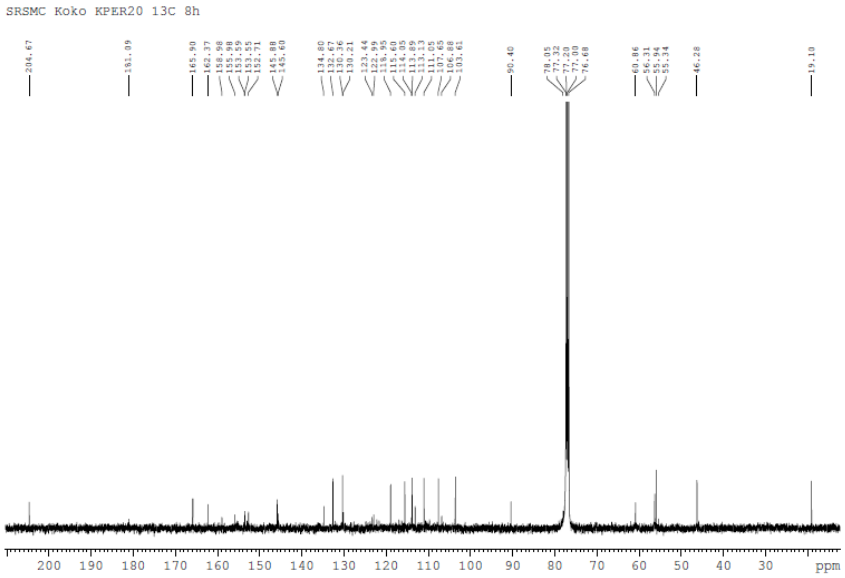
KPER 10AV (Iosliquiritigenin, ^{1}H)



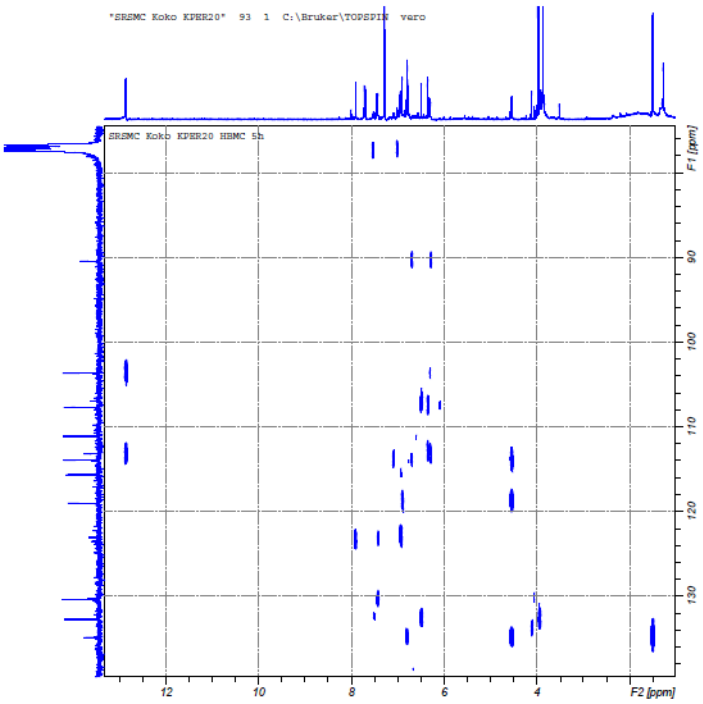
KPER 20 (1-(2,4-dihydroxyphenyl)-2-(7-hydroxybenzo[d][1,3]dioxol-5-yl)propan-1-one; ^{1}H)



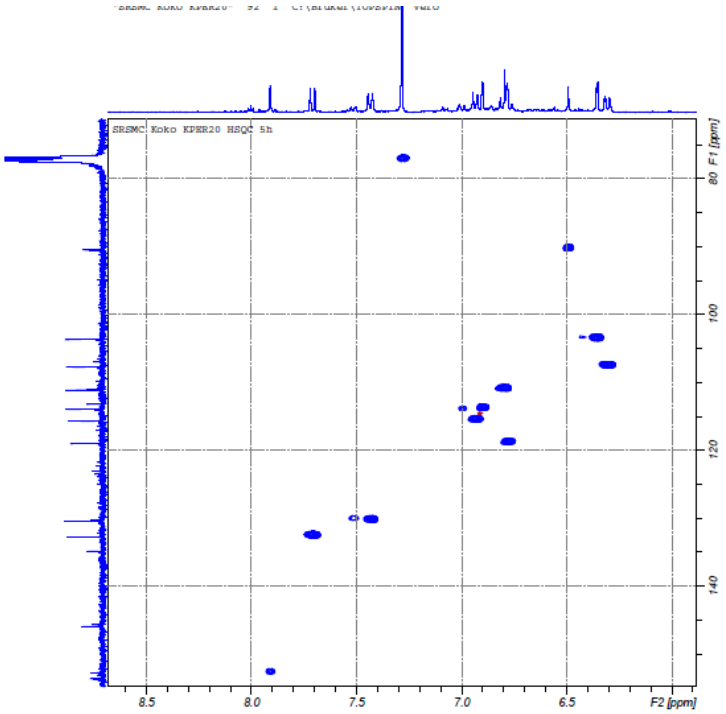
KPER 20 (1-(2,4-dihydroxyphenyl)-2-(7-hydroxybenzo[d][1,3]dioxol-5-yl)propan-1-one; ^{13}C)



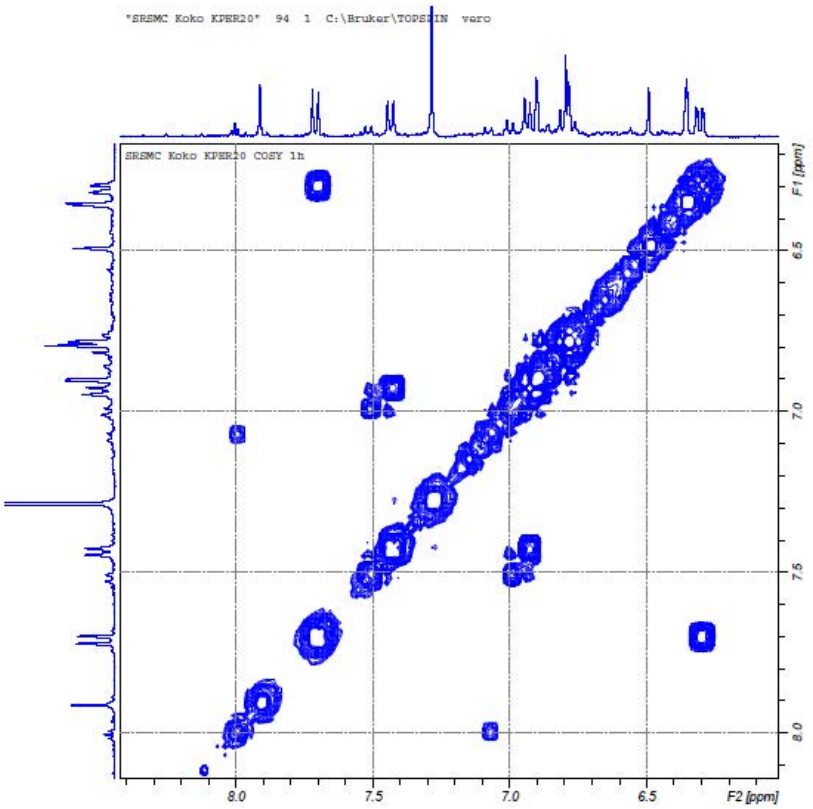
KPER 20 (1-(2,4-dihydroxyphenyl)-2-(7-hydroxybenzo[d][1,3]dioxol-5-yl)propan-1-one; HMBC)



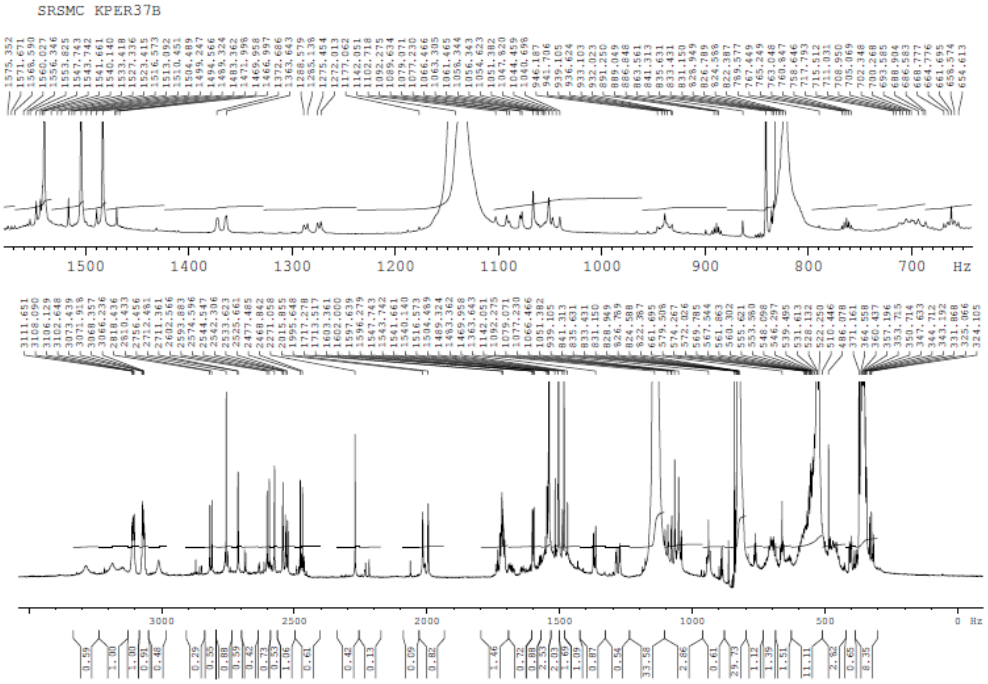
KPER 20 (1-(2,4-dihydroxyphenyl)-2-(7-hydroxybenzo[d][1,3]dioxol-5-yl)propan-1-one; HSQC)



KPER 20 (1-(2,4-dihydroxyphenyl)-2-(7-hydroxybenzo[d][1,3]dioxol-5-yl)propan-1-one; COSY)



KPER 37 B (6,18-dihydroxy-5,11,15-trimethoxy-3,7b,8,13b-tetrahydro-2*H*-3a,3a[1]epoxybenzo[*a*]chromeno[2',3':2,3]indeno[7,1-*cd*]xanthen-2-one; [1]H)



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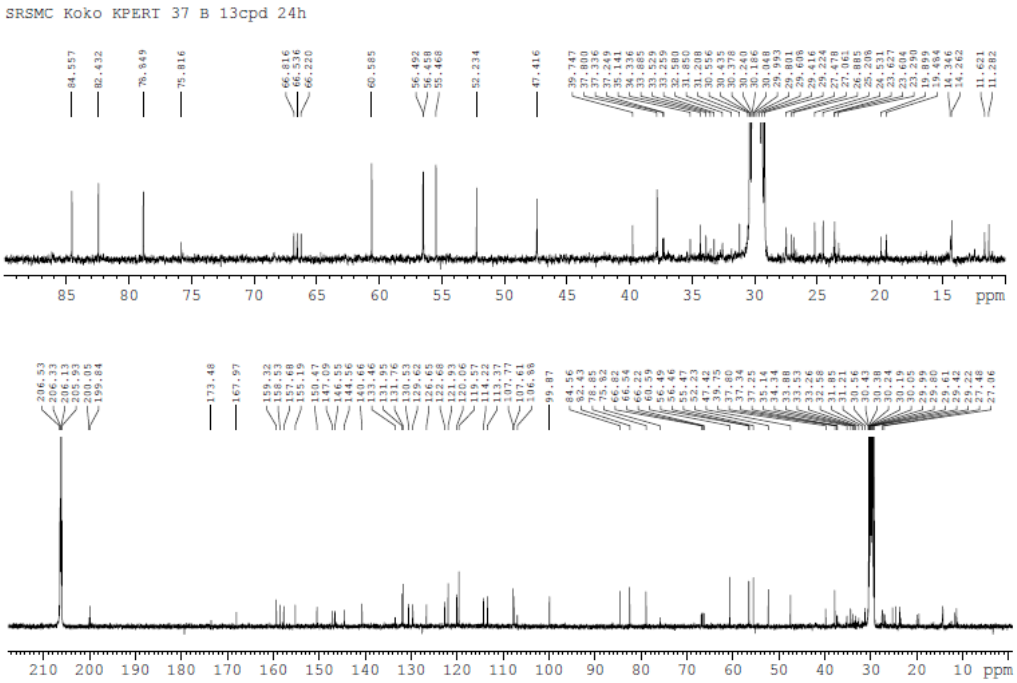
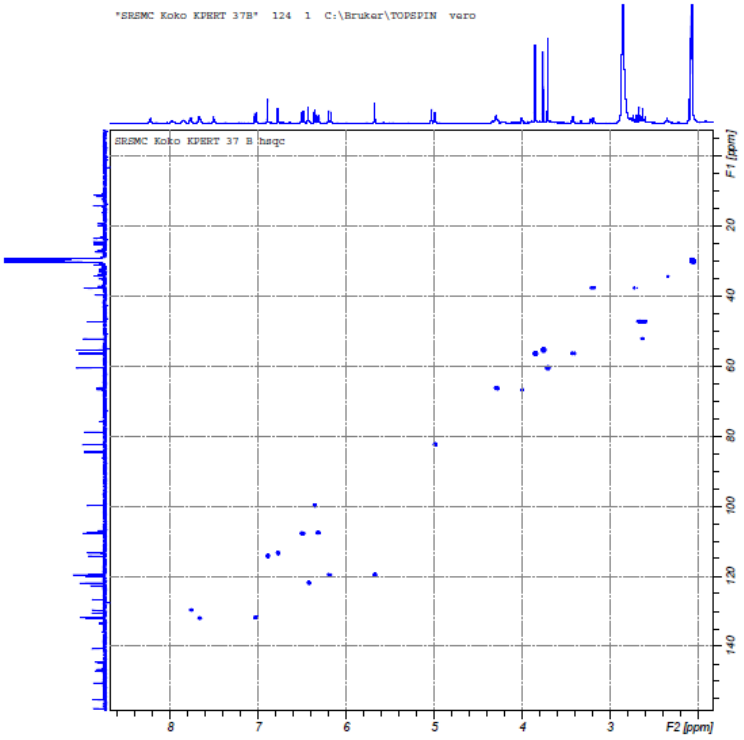
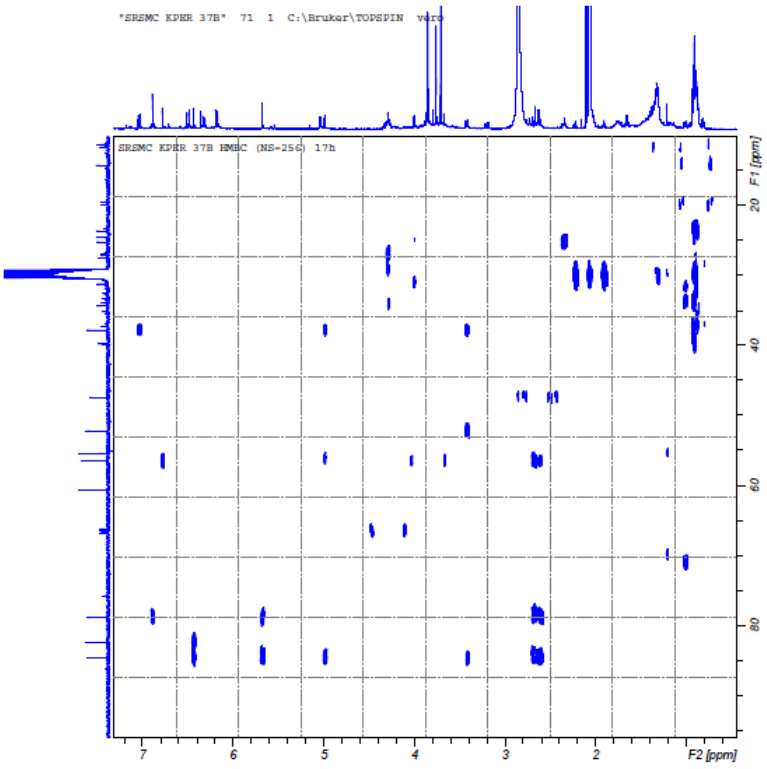


KPER 37 B (6,18-dihydroxy-5,11,15-trimethoxy-3,7b,8,13b-tetrahydro-2*H*-3a,3a[1]epoxybenzo[*a*]chromeno[2',3':2,3]indeno[7,1-*cd*]xanthen-2-one;HSQC)



KPER 37 B (6,18-dihydroxy-5,11,15-trimethoxy-3,7b,8,13b-tetrahydro-2*H*-3a,3a[1]epoxybenzo[*a*]chromeno[2',3':2,3]indeno[7,1-*cd*]xanthen-2-one;HMBC)



LIST OF PUBLICATIONS

(1) Tittikpina, N. K., Ejike, ECC C., Estevam, E. C., Nasim, M. J., Griffin, S., Chaimbault, P., Kirsch, G., Atakpama, W., Batawila, K. and Jacob, C., Togo to go: Products and compounds derived from local plants for the treatment of diseases endemic in sub-saharan Africa, AJTCAM, 13 (1), 85-94. http://dx.doi.org/10.4314/ajtcam.v13i1.3664.

(2) Griffin, S., Tittikpina, N. K., Al-marby, A., Alkhayer, R., Denezhkin, P., Witek, K., Gbogbo, K. A., Batawila, K., Duval, R. E., Jawad Nasim, M. J., Awadh-Ali, N. A., Kirsch, G., Chaimbault, P., Schafer, K.-H., Keck, C. M., Handzlik, J. Jacob, C., Turning Waste into Value: Nanosized Natural Plant Materials of *Solanum incanum* L. and *Pterocarpus erinaceus* Poir with Promising Antimicrobial Activities, Pharmaceutics, 8(2): 11, doi:10.3390/pharmaceutics8020011.

(3) Tittikpina, N. K., Nana, F., Fontanay, S., Batawila, K., Akpagana, K., Kirsch, G., Duval, R. E., Chaimbault, P., Jacob, C. Study of *Pterocarpus erinaceus*, a promising plant from Togo to treat infectious diseases, Planta Med, 2016, 82(S 01): S1-S381, DOI: 10.1055/s-0036-1596502

(4) Tittikpina, N. K., Atakpama, W., Pereki, H., Nasim, M. J., Ali, W., Fontanay, S., Nana, F., Ejike, ECC C., Kirsch, G., Duval, R. E., Chaimbault, P., Karou, S.D., Batawila, K., Akpagana, K. and Jacob, C. 'Capiture' plants with interesting biological activities: a case to go. Open chemistry, 2017; 15:1-11.

(5) Tittikpina, N. K., Nana, F., Fontanay, S., Philippot, S., Batawila, K., Akpagana, K., Kirsch, G., Duval, R. E., Chaimbault, P., Jacob, Antibacterial activity and cytotoxicity of *Pterocarpus erinaceus* Poir ex-tracts, fractions and isolated compounds. *Submitted to* Journal of Ethnopharmacology.

LIST OF COMMUNICATIONS

(1) The International Union for Forests Research Organizations (IUFRO) XXIV World Congress, Salt Lake City (USA); 5th - 11th 10. 2014.

Tittikpina N. K., Agban A., Gbogbo K. A., Hoekou Y. P., Pereki H., Batawila K. & Akpagana K. Plants used to treat infectious diseases in Togo: *Pterocarpus erinaceus* Poir (Faboïdeae) and *Daniellia oliveri* (Rolfe) Hutch. and Dalz (Caesalpinoïdeae). Poster

(2) NUTRIOX meeting, Luxembourg (Luxembourg); 19th - 20th 11. 2015.

Tittikpina, N. K., Batawila K., Akpagana K., Duval R. E., Kirsch G., Chaimbault P. and Jacob C. *Pterocarpus erinaceus* Poir (Fabaceae) and *Daniellia oliveri* (Rolfe) Hutch. and Dalz (Caesalpinaceae): promising plants of Togo to treat infection and cancer diseases. Oral communication.

(3) Phytoday 2016 in Nancy (France), 24th June 2016

Tittikpina N.K., Nana F., Fontanay S., Batawila K., Akpagana K., Kirsch G., Duval R.E., Chaimbault P. and Jacob C. Promising antibacterial activities of *P. erinaceus* Poir, a plant from Togo. Oral communication.

(4) 9th Joint Natural Products Conference in Copenhagen (Danemark); 24th - 27th 07. 2016.

Tittikpina N. K., Nana F., Fontanay S., Batawila K., Akpagana K., Kirsch G., Duval R. E., Chaimbault P. and Jacob C. Study of *Pterocarpus erinaceus*, a promising plant from Togo to treat infectious diseases. Poster

(5) Schlumberger Foundation, Faculty For the Future Fellows and Alumnae Forum at the University of Cambridge (Cambridge, United Kingdom); 30th 10. 2016 - 2nd 11. 2016.

Tittikpina, N. K. Biological and chemical investigations of extracts coming from two plants used in traditional medicine in Togo. Poster.

(6) 65th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Products Research in Basel (Switzerland); 3rd -7th 09. 2017.

Tittikpina N. K., Atakpama W., Pereki H., Nasim M. J., Ali W., Fontanay S., Nana F., ECC Ejike C., Kirsch G., Duval R. E., Chaimbault P., Karou S. D., Batawila K., Akpagana K. and Jacob C. From ethnobotany to the laboratory: a computer aided ethnobotanical method for the identification of plants with interesting biological activities. Poster.