UNIVERSITY OF YAOUNDE I
UNIVERSITÉ DE YAOUNDÉ I
****
POST-GRADUATE AND TRAINING SCHOOL OF LIFE SCIENCE-HEALTH AND ENVIRONMENT
CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCE DE LA VIE-SANTE ET ENVIRONNEMENT

FACULTY OF SCIENCES
FACULTE DES SCIENCES
****
POST-GRADUATE AND TRAINING UNIT OF LIFE SCIENCE-HEALTH
UNITE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCE DE LA VIE

DEPARTMENT OF BIOCHEMISTRY
DEPARTEMENT DE BIOCHIMIE

LABORATORY FOR PHYTOBIOCHEMISTRY AND MEDICINAL PLANTS STUDIES
LABORATOIRE DE PHYTOBIOCHIMIE ET D'ETUDE DES PLANTES MEDICINALES

ANTIMICROBIAL AND BIOCONTROL AGENTS UNIT
UNITE DES AGENTS ANTIMICROBIENS ET DE BIOCONTROLE

Bio-guided Isolation of the *in vitro* anti-leishmanial active natural products from *Diospyros grascilesens* L. (Ebenaceae) and *Rothmania hispida* K. Schum (Rubiaceae).

Thesis submitted in partial fulfillment of the requirements for the award of a Doctorate/PhD

in Biochemistry

By:

NJANPA NGANSOP Cyrille Armel
Master of Science (*MSc*) in Biochemistry
Registration N° 10R0594

Under the direction of:

FEKAM BOYOM Fabrice
Professor
University of Yaoundé I

2022/2023

UNIVERSITE DE YAOUNDE I

CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCE DE LA VIE, SANTE ET ENVIRONEMENT

UNITE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCE DE LA VIE

DEPARTEMENT DE BIOCHIMIE

THE UNIVERSITY OF YAOUNDE I

CENTRE FOR RESEARCH AND TRAINING IN GRADUATE STUDIES IN LIFE, HEALTH AND ENVIRONMENTAL SCIENCES

RESEARCH DOCTORATE TRAINING UNIT IN LIFE SCIENCES

DEPARTMENT OF BIOCHEMISTRY

CERTIFICATE OF CORRECTION OF THE DOCTORAL THESIS
ATTESTATION DE CORRECTION DE LA THESE DE DOCTORAT

We, the undersigned Thesis Director, and Board of Assessors of the Doctorate/PhD thesis in Biochemistry entitled: «**Bio-guided Isolation of the *in vitro* anti-leishmanial active natural products from *Diospyros grascilesens* L. (Ebenaceae) and *Rothmania Hispida* K. Schum (Rubiaceae)** defended on **July 28, 2022**, by **Mr NJANPA NGANSOP Cyrille Armel**- registration number **10R0594**, hereby certify that the candidate has completed the corrections of the above-mentioned thesis as requested by the Examiners.

We hereby certify that the Board of Assessors is satisfied with the corrections and recommend that the Doctorate/PhD degree be awarded to the candidate.

Yaounde, the 11 SEPT 2023

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ACADEMIC YEAR 2022/2023

(*by Department and by Grade*)

LAST UPDATED: May 31, 2023

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Head of Academic Affairs division, Keeping of Terms and Research: AJEAGAH Gideon AGHAINDUM, Professor

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20	MOFOR née TEUGWA Clotilde	Associate Professor	**Dean FS/Uds**
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25	NOAH EWOTI Olive Vivien	Associate Professor	On duty
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38	KOGA MANG DOBARA	Senior Lecturer	On duty
39	LEME BANOCK Lucie	Senior Lecturer	On duty
40	MAPON NSANGOU Indou	Senior Lecturer	On duty
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42	MVEYO NDANKEU Yves Patrick	Senior Lecturer	On duty
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45	NWANE Philippe Bienvenu	Senior Lecturer	On duty
46	TADU Zephyrin	Senior Lecturer	On duty
47	YEDE	Senior Lecturer	On duty
48	YOUNOUSSA LAME	Senior Lecturer	On duty
49	AMBADA NDZENGUE GEORGIA ELNA	Assist. Lecturer	On duty
50	KODJOM WANCHE Jacguy Joyce	Assist. Lecturer	On duty
51	NDENGUE Jean De Matha	Assist. Lecturer	On duty
52	ZEMO GAMO Franklin	Assist. Lecturer	On duty
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2	DJOCGOUE Pierre François	Professor	On duty
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4	MOSSEBO Dominique Claude	Professor	On duty
5	YOUMBI Emmanuel	Professor	On duty
6	ZAPFACK Louis	Professor	On duty
7	ANGONI Hyacinthe	Associate Professor	On duty

8	BIYE Elvire Hortense	Associate Professor	On duty
9	MAHBOU SOMO TOUKAM. Gabriel	Associate Professor	On duty
10	MALA Armand William	Associate Professor	On duty
11	MBARGA BINDZI Marie Alain	Associate Professor	***DAAC /UDla***
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19	DJEUANI Astride Carole	Senior Lecturer	On duty
20	GONMADGE CHRISTELLE	Senior Lecturer	On duty
21	MAFFO MAFFO Nicole Liliane	Senior Lecturer	On duty
22	NNANGA MEBENGA Ruth Laure	Senior Lecturer	On duty
23	NOUKEU KOUAKAM Armelle	Senior Lecturer	On duty
24	NSOM ZAMBO EPSE Pial Annie Claude	Senior Lecturer	**UNESCO MALI**
25	GODSWILL NTSOMBOH NTSEFONG	Senior Lecturer	On duty
26	KABELONG BANAHO Louis-Paul-Roger	Senior Lecturer	**UNESCO MALI**
27	KONO Léon Dieudonné	Senior Lecturer	On duty
28	LIBALAH Moses BAKONCK	Senior Lecturer	On duty
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30	TAEDOUNG Evariste Hermann	Senior Lecturer	On duty
31	TEMEGNE NONO Carine	Senior Lecturer	On duty
32	MANGA NDJAGA JUDE	Assist. Lecturer	On duty
33	DIDA LONTSI Sylvere Landry	Assist. Lecturer	On duty
34	METSEBING Blondo-Pascal	Assist. Lecturer	On duty
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4	NENWA Justin	Professor	On duty
5	NGAMENI Emmanuel	Professor	**Dean F.S. U. Ndere**
6	NGOMO Horace MANGA	Professor	**Vice Chancellor/U.B**.
7	NJOYA Dayirou	Professor	On duty
8	ACAYANKA Elie	Associate Professor	On duty
9	EMADAK Alphonse	Associate Professor	On duty
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11	KEMMEGNE MBOUGUEM Jean C.	Associate Professor	On duty
12	KENNE DEDZO GUSTAVE	Associate Professor	On duty
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28	BOYOM TATCHEMO Franck W	Assist. Lecturer	On duty
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4	PEGNYEMB Dieudonné Emmanuel	Professor	**Director MINESUP/Head of**

			Department
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6	MBAZOA née DJAMA Céline	Professor	On duty
7	AMBASSA Pantaléon	Associate Professor	On duty
8	EYONG Kenneth OBEN	Associate Professor	On duty
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10	KAMTO Eutrophe Le Doux	Associate Professor	On duty
11	KENMOGNE Marguerite	Associate Professor	On duty
12	KEUMEDJIO Félix	Associate Professor	On duty
13	KOUAM Jacques	Associate Professor	On duty
14	MKOUNGA Pierre	Associate Professor	On duty
15	MVOT AKAK CARINE	Associate Professor	On duty
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17	NGONO BIKOBO Dominique Serge	Associate Professor	**CEA/MINESUP**
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31	SIELINOU TEDJON Valérie	Senior Lecturer	On duty
32	TCHAMGOUE Joseph	Senior Lecturer	On duty
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34	TSAMO TONTSA Armelle	Senior Lecturer	On duty
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6	AMINOU Halidou	Senior Lecturer	**Head of Department**
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9	EBELE Serge Alain	Senior Lecturer	On duty
10	HAMZA Adamou	Senior Lecturer	On duty
11	JIOMEKONG AZANZI Fidel	Senior Lecturer	On duty
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13	DJIADEU NGAHA Michel	Senior Lecturer	On duty
14	DOUANLA YONTA Herman	Senior Lecturer	On duty
15	KIKI Maxime Armand	Senior Lecturer	On duty
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17	MBAKOP Guy Merlin	Senior Lecturer	On duty
18	MBATAKOU Salomon Joseph	Senior Lecturer	On duty
19	MENGUE MENGUE David Joël	Senior Lecturer	**Head of dep./ENS Univ.of Eblwa**
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21	NGUEFACK Bernard	Senior Lecturer	On duty
22	NIMPA PEFOUKEU Romain	Senior Lecturer	On duty
23	OGADOA AMASSAYOGA	Senior Lecturer	On duty
24	POLA DOUNDOU Emmanuel	Senior Lecturer	On duty
25	TCHEUTIA Daniel Duviol	Senior Lecturer	On duty
26	TETSADJIO TCHILEPECK M. Eric.	Senior Lecturer	On duty
27	BITYE MVONDO Esther Claudine	Assist. Lecturer	On duty
28	FOKAM Jean Marcel	Assist. Lecturer	On duty
29	GUIDZAVAI KOUCHERE Albert	Assist. Lecturer	On duty
30	MANN MANYOMBE Martin Luther	Assist. Lecturer	On duty
31	MEFENZA NOUNTU Thiery	Assist. Lecturer	On duty
32	NYOUMBI DLEUNA Christelle	Assist. Lecturer	On duty
33	TENKEU JEUFACK Yannick Léa	Assist. Lecturer	On duty
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5	BOYOMO ONANA	Associate Professor	On duty

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7	RIWOM Sara Honorine	Associate Professor	On duty
8	NJIKI BIKOÏ Jacky	Associate Professor	On duty
9	SADO KAMDEM Sylvain Leroy	Associate Professor	On duty
10	ESSONO OBOUGOU Germain G.	Senior Lecturer	On duty
11	LAMYE Glory MOH	Senior Lecturer	On duty
12	MEYIN A EBONG Solange	Senior Lecturer	On duty
13	MONI NDEDI Esther Del Florence	Senior Lecturer	On duty
14	NKOUDOU ZE Nardis	Senior Lecturer	On duty
15	TAMATCHO KWEYANG Blandine Pulchérie	Senior Lecturer	On duty
16	TCHIKOUA Roger	Senior Lecturer	Head of Service/FS
17	TOBOLBAÏ Richard	Senior Lecturer	On duty
18	NKOUE TONG Abraham	Assist. Lecturer	On duty
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21	EHETH Jean Samuel	Assist. Lecturer	On duty
22	MAYI Marie Paule Audrey	Assist. Lecturer	On duty
23	NGOUENAM Romial Joël	Assist. Lecturer	On duty
24	NJAPNDOUNKE Bilkissou	Assist. Lecturer	On duty
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4	ESSIMBI ZOBO Bernard	Professor	On duty
5	HONA Jacques	Professor	On duty
6	NANA ENGO Serge Guy	Professor	On duty
7	NANA NBENDJO Blaise	Professor	On duty
8	NDJAKA Jean Marie Bienvenu	Professor	**Head of Department**
9	NJANDJOCK NOUCK Philippe	Professor	**Vice Director/MINRESI**
10	NOUAYOU Robert	Professor	On duty

11	SAIDOU	Professor	**Head of Center/IRGM/MINRESI**
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13	TCHAWOUA Clément	Professor	On duty
14	WOAFO Paul	Professor	On duty
15	ZEKENG Serge Sylvain	Professor	On duty
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17	BODO Bertrand	Associate Professor	**G. D./HYDRO Mekin**
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21	MBINACK Clément	Associate Professor	On duty
22	MBONO SAMBA Yves Christian U.	Associate Professor	On duty
23	MELI'I Joelle Larissa	Associate Lecturer	On duty
24	MVOGO ALAIN	Associate Lecturer	On duty
25	NDOP Joseph	Associate Professor	On duty
26	SIEWE SIEWE Martin	Associate Professor	On duty
27	SIMO Elie	Associate Professor	On duty
28	VONDOU Derbetini Appolinaire	Associate Professor	On duty
29	WAKATA née BEYA Annie	Associate Professor	**Director/ENS/UY1**
30	WOULACHE Rosalie Laure	Associate Professor	**In stage from February 2023**
31	ABDOURAHIMI	Assist. Lecturer	On duty
32	AYISSI EYEBE Guy François Valérie	Senior Lecturer	On duty
33	CHAMANI Roméo	Senior Lecturer	On duty
34	DJIOTANG TCHOTCHOU Lucie Angennes	Senior Lecturer	On duty
35	EDONGUE HERVAIS	Senior Lecturer	On duty
36	FOUEJIO David	Senior Lecturer	**Chief Cell /MINADER**
37	KAMENI NEMATCHOUA Modeste	Senior Lecturer	On duty
38	LAMARA Maurice	Senior Lecturer	On duty
39	OTTOU ABE Martin Thierry	Senior Lecturer	Director/IMPM
40	TEYOU NGOUPOU Ariel	Senior Lecturer	On duty
41	WANDJI NYAMSI William	Senior Lecturer	On duty
42	NGA ONGODO Dieudonné	Assist. Lecturer	On duty

43	SOUFFO TAGUEU Merimé	Assist. Lecturer	On duty
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37	TCHAPTCHET TCHATO De P.	Senior Lecturer	On duty
38	TEHNA Nathanaël	Senior Lecturer	On duty
39	FEUMBA Roger	Senior Lecturer	On duty
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41	KOAH NA LEBOGO Serge Parfait	Assist. Lecturer	On duty
42	NGO'O ZE ARNAUD	Assist. Lecturer	On duty
43	TENE DJOUKAM Joëlle Flore, épouse KOUANKAP NONO	Assist. Lecturer	On duty

Classification of teaching staff at the faculty of Science of the University of Yaoundé 1

NUMBER OF LECTURERS					
Department	**Professor**	**Associate Professor**	**Senior Lecturer**	**Assist. Lecturer**	**Total**
BCH	8 (01)	15 (11)	13 (03)	07 (05)	**43 (20)**
A. B. P.	14 (01)	16 (09)	18 (04)	04 (02)	**52 (16)**
P. B. P.	06 (01)	12 (02)	13 (07)	03 (00)	**34 (10)**
I. C.	07 (01)	15 (04)	05 (01)	01 (00)	**28 (06)**
O. C.	06 (01)	18 (04)	11 (04)	02 (00)	**37 (09)**
C. S.	02 (00)	02 (00)	14 (01)	04 (00)	**22 (01)**
MAT	01 (00)	08 (00)	17 (01)	07 (02)	**33 (03)**
MIB	02 (01)	07 (03)	08 (04)	07 (02)	**24 (10)**
PHY	15 (01)	15 (04)	11 (01)	02 (00)	**43 (06)**
E. S.	08 (00)	17 (03)	15 (04)	03 (01)	**43 (08)**
Total	**69 (07)**	**125 (40)**	**125 (30)**	**40 (12)**	**359 (89)**

A total of **359 (89)** including:

Professors	**69 (07)**
Associate Professors	**125 (40)**
Senior Lecturers	**125 (30)**
Assist. Lecturers	**40 (12)**
() = Number of women	**89**

The Dean of the Faculty of Science

Prof. TCHOUANKEU Jean-Claude

DEDICATION

I dedicate this work to:

All the family ***NGANSOP.***

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LIST OF ABBREVIATIONS

CC_{50}: Cytotoxic Concentration 50

CL: Cutaneous Leishmaniasis

DAD: Diode Array Detector

DAT: Direct Agglutination Test

DMEM: *Dulbecco's Modified Eagle's Medium*

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic Acid

DPPH: 1,1-diphényl-2-picrylhydrazyl

EDTA: Ethylene Diamine Tetracetic Acid

ESI: Electrospray Ionization

FAST: Fast Agglutination Screening Test

FBS: Fetal Bovin Serum

FIC_{50}: Fractional Inhibitory Concentration 50

FRAP: Ferric Ion Reducing Antioxidant Power

HIFBS: Heat-Inactivated Fetal Bovine Serum

HIV: Human Immunodeficiency Virus

HPLC: High-Performance Liquid Chromatography

HR: High Resolution

HRESI: High Resolution Electrospray Ionization

IC_{50}: Inhibitory Concentration 50

ICT: Immune-Chromatographic Test

IFAT: Immunofluorescence Assay Test

IFN-γ: Interferon-γ

iNOS: inducible Nitric Oxide Synthase

MCL: Mucocutaneous leishmaniasis

MS: Mass Spectrometry

MS/MS: Mass Spectrometry/Mass Spectrometry

NO: Nitrite Oxide

NTDs: Neglected Tropical Diseases

OA: Oleanolic Acid

OD: Optical Density

OECD: Organization for Economic Coperation and Development

ONP: Ortho-Nitro-Phenantroline

PBS: Phosphate Buffer Solution

PCR: Polymerase Chain Reaction

PKDL: PostKala-Azar Dermal Leishmaniasis

rRNA: ribosomal Ribonucleic Acid

RNA: Rinonucleic Acid

RPM: Rotation Per Minute

SD: Standard Deviation

SI: Selectivity Index

SPI: Specificity Index

TLC: Thin Layer Chromatography

TNF-α: Tumor Necrosis Factor alpha

UA: Ursolic Acid

UPLC: Ultraperformance Liquid Chromatography

UV: Ultraviolet

VL: Visceral Leishmaniasis

WHO: World Health Organization

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ABSTRACT

Leishmaniasis is a disabling neglected tropical disease (NTD) caused by *Leishmania spp.* transmitted through the bite of infected female sandflies. The disease leads to disfigurement and social stigma, particularly for women and children. This disease remains a serious public health problem around the world. The treatment of the disease is hampered by the limited availability of drugs, their toxicity, severe side effects and the emergence of drug resistance. In addition, in the absence of a suitable vaccine, there is a need to search new molecules for the treatment of leishmaniasis. In an attempt to discover novel chemical scaffolds as starting points for new drug development against leishmaniasis, this work described the bio-guided investigation of the *in vitro* antileishmanial active extracts from *Diospyros gracilescens* (Ebenaceae) and *Rothmania hispida* stem (Rubiaceae), targeting the visceral leishmaniasis extracellular (promastigotes) and intracellular (amastigotes) forms of *Leishmania donovani*.

Plant extracts were prepared by maceration using Water: Ethanol (H_2O: EtOH) (30:70, v/v) and Methylen Chloride: Methanol (CH_2Cl_2-MeOH) (1:1, v/v) and further fractionated using liquid–liquid partition and silica gel column chromatography approaches. Different concentrations of *D. gracilescens* and *R. hispida* stem extracts and fractions (100, 20, 4, 0.8 and 0.16 µg/mL) and isolated compounds (50, 10, 2, 0.4 and 0.08 µg/mL) were tested against *L. donovani* (1S (MHOM/SD/62/1S) promastigotes and intracellular amastigotes forms *in vitro*. The antileishmanial potency was determined using the rezazurin colorimetric assay. The cytotoxicity was assayed in RAW 264.7 cells using the rezazurin colorimetric assay. The immunomodulatory and invasion assays, the time kill kinetic profile, the antioxidant properties; the interaction studies of the two (02) most active compounds with the positive control (amphotericin B) and the acute toxicity of the most active crude extract were also investigated.

The hydroethanolic crude extract of *D. gracilescens* trunk and CH_2Cl_2-MeOH crude extract of *R. hispida* stem showed the most potent antileishmanial activity against promastigotes of *L. donovani* (Inhibitrice Concentration 50 (IC_{50}) = 5.84 and 5.74 µg/mL, respectively). The bio-guided fractionation of *D. gracilescens* trunk extract led to four (04) fractions, of which the hexane fraction showed the most potent activity (IC_{50} = 0.79 µg/mL). The purification of the most active fractions led to the isolation of six (06) compounds that exhibited decreased potency (IC_{50} = 5.50-38.75 µg/mL) compared to the parent fraction. The lupeol, mixture of β-sitosterol and stigmasterol were isolated

from the *n*-hexane fraction, the betulin and betulinic acid from the dichloromethane fraction while the *β*-sitosterol glucoside and 1-deoxyinositol were isolated from the n-butanol fraction.

The bio-guided fractionation of *R. hispida* stem extract led to three (03) fractions, of which the hexane fraction showed the most potent activity (IC_{50} = 4.38 µg/mL). The purification of the most active fractions led to the isolation of nine (09) compounds, of which ursolic acid (UA) showed the most potent activity (IC_{50} = 0.75 µg/mL) compared to the parent fraction. The mixture of stigmasterol and β-sitosterol, lichexanthone, the mixture uvaol and erythrodiol, docosanoic acid, UA and OA were isolated from the hexane fraction while, sitosterol 3-*O*-*β*-D- glycoside was isolated from the EtOAc fraction.

The promising trunk extract, derived hexane fraction from *D. grascilesens*, UA and OA compounds from *R. hispida* showed acceptable selectivity (Selectivity Index (SI)>18). However, most of the derived compounds isolated from *D. grascilesens* were weakly active and non-selective (SI<1), while the compounds isolated from *R. hispida* were active and selective. The immunomodulatory assay showed that high levels of nitrite oxide (NO) were produced by macrophages treated with UA and OA, suggesting that it could be the mechanism involved in the *in vitro* intracellular amastigotes assay. The invasion assay showed that the IC_{50} value was decreased when UA was added before the infection of macrophages, suggesting that this compound might act as a preventive agent, by preventing the inhibition of macrophages by metacyclic promastigotes forms of *L donovani*. The promising hexane fraction, UA and OA showed significant inhibition of parasite growth at different concentrations but with no evidence of a cidal effect over a screening period of 120 hours. The interaction studies of the two (02) most active compounds, UA and OA, with amphotericin B showed a synergistic effect against both promastigotes and intracellular amastigotes of *L. donovani*. The two (02) most active compounds exhibited good antioxidant properties. The acute toxicity assay of the most active extract showed that the lethal dose 50 (LD_{50}) of *R. hispida* stems was higher than 2000 mg/kg.

The results obtained indicated that the derived hexane fraction from *D. gracilescens*, UA and OA from *R. hispida* have very potent inhibitory effects on promastigotes and intracellular amastigotes of *L. donovani* parasites in culture. The isolated compounds from *D. gracilescens* showed a lesser extent of potency and selectivity, while compounds isolated from *R. hispida* were active and highly selective. The combination of UA and OA with amphotericin B showed a synergistic effect on both extracellular promastigotes and intracellular amastigotes forms of *L. donovani*. However, further structure-activity relationship studies could lead to more potent and selective hit derivatives of interest for detailed drug discovery programs against visceral leishmaniasis (VL).

KEYWORDS: Antileishmanial Activity, Bio-guided isolation, *Diospyros gracilescens*, *Rothmania hispida,* ursolic acid and oleanolic acids.

RESUME

La leishmaniose est une maladie parasitaire tropicale négligée causée par les espèces appartenant au genre *leishmania* et transmise par la piqure du phlébotome femelle infecté. Cette maladie constitue un véritable problème de santé publique avec d'énormes conséquences sur les plans social et économique. Le traitement de la leishmaniose est limité par de nombreux facteurs tels que: la non-disponibilité des médicaments, leur cout élevé, de nombreux effets secondaires enregistrés, leur toxicité et l'émergence des phénomènes de résistance de plus en plus rencontrés. De plus, en l'absence d'un vaccin éfficace, il devient important de rechercher de nouveaux principes actifs comme point de départ de nouveaux médicaments pour lutter contre cette maladie, l'objectif de ce travail était focalisé sur l'activité antileishmaniale *in vitro* bio-guidée des extraits bruts de *D. gracilescens* et *R. hispida*, ciblant les formes extracellulaire (promastigotes) et intracellulaire (amastigotes) de *L. donovani*.

Les différents extraits de plantes ont été préparés par macération utilisant un mélange eau-éthanol (H_2O: EtOH) (30:70, v/v) et Chlorure de methylène-methanol (CH_2Cl_2-MeOH) (1:1, v/v). Par la suite, les extraits bruts les plus actifs ont été fractionnés en utilisant la partition liquide-liquide et la chromatographie sur colonne comme approches. Différentes concentrations des extraits bruts et fractions (100 ; 20 ; 4 ; 0.8 and 0.16 µg/mL), et des composés isolés (50, 10 ; 2 ; 0.4 and 0.08 µg/mL) de *D. gracilescens* et *R. hispida* ont été testés *in vitro* sur les formes promastigotes et intracellulaires amastigotes de *L. donovani* (1S (MHOM/SD/62/1S). L'activité antileishmaniale a été effectuée par la méthode colorimétrique utilisant la résazurine. Le test de cytotoxicité a été effectué sur la lignée cellulaire des macrophages RAW 264.7 par la méthode colorimétrique utilisant la résazurine. Les tests d'invasion et d'immunomodulation, la cinétique de mortalité, l'étude des interactions des deux (02) composés les plus actifs entre eux et avec le médicament de référence, l'activité anti-oxydante des composés les plus actifs de même que la toxicité aigüe de l'extrait brut le plus actif ont aussi été investigués.

Les résultats obtenus ont montré que l'extrait brut hydroethanolique du tronc de *D. gracilescens* et l'extrait brut au mélange CH_2Cl_2-MeOH des tiges de *R. hispida* ont été les les plus actifs sur les formes promastigotes de *L. donovani* avec des valeurs de Concentrations Inhibitrices 50 (CI_{50}) respectives de 5.84 et 5.74 µg/mL. Le fractionnement bio-guidé de l'extrait brut du tronc de *D. gracilescens* a permis l'obtention de quatre (04) fractions parmi lesquelles, la fraction à l'hexane qui s'est révélée comme étant la plus active (CI_{50} = 0.789 µg/ml). La purification des fractions les plus

actives a conduit à l'isolement de six (06) composés qui ont présenté une activité modérée comparée à celle de la fraction à l'hexane (CI_{50} = 5.50-38.75 µg/mL). Le lupeol, le mélange β-sitosterol/stigmasterol ont été isolés de la fraction hexanique, la betuline et l'acide bétulinique de la fraction au dichloromethane tandis que le *β*-sitosterol glucoside et le 1-deoxyinositol ont été isolés de la fraction au n-butanol.

Le fractionnement bioguidé de l'extrait des tiges de *R. hispida* a donné trois (03) fractions dont, la fraction à l'hexane s'est revélée la plus active avec une CI_{50} de 4.38 µg/ml**.** La purification des fractions actives conduit à l'isolement de neuf (09) composés parmi lesquels, l'acide ursolique a présenté la meilleure activité avec une CI_{50} de 0.75 µg/ml. Le mélange stigmasterol-β-sitosterol, lichexanthone, le mélange uvaol et erythrodiol, les acides docosanoique, ursolique et oleanolique ont été isolés de la fraction à l'hexane tandis que le 3-*O*-*β*-D-sitosterolglycoside de la fraction à l'acétate d'éthyle.

La fraction à l'hexane provenant de *D. gracilescens*, les acides ursolique et oléanolique isolés des *R. hispida* ont tous montré des indices de sélectivité (IS) supérieures à 18 (IS>18). Le test d'immunomodulation a montré un taux ce production élévé d'oxyde nitrique dans les macrophages traités par les acides ursolique et olénolique suggérant ainsi que ces composés pourraient etre impliqués dans le mécanisme de mort des amastigotes intracellaulaires du parasite *Leishmania*. Le test d'invasion a montré que la CI_{50} a été diminuée lors que l'acide ursolique a été ajouté avant infection des macrophages suggérant ainsi que ce composé pourrait agir comme un agent préventif, ceci à travers l'inhibition de l'infection des macrophages par les promastigotes métacycliques du parasite *Leishmania*. La plupart des composés isolés de *D. gracilescens* étaient faiblement actifs et non-sélectifs (IS<1) comparés aux composés isolés de *R. hispida* qui ont été tous actifs et sélectifs. La fraction à l'hexane de *D. gracilescens*, les acides ursolique et oléanolique ont montré un potentiel significativement inhibiteur sur la croissance des promastigotes de *L. donovani*, mais aucun effet sur l'inhibition totale n'a été observé sur une période de 120 heures. Le test d'interaction des 2 composés les plus actifs entre eux et avec le médicament de référence l'amphotéricine B, a montré un effet synergique sur les formes promastigotes et intracellulaires amastigotes du parasite *leishmania*. Les acides ursolique et oléanique ont montré une bonne capacité de piégeage du radical DPPH et un fort potentiel dans l'activité chélatrice du fer. Le test de toxicité aigüe de l'extrait brut des tiges de *R. hispida* a montré que la Dose Létale 50 (DL_{50}) est supérieure à 2000 mg/kg.

Les résultats obtenus dans cette étude ont montré que la fraction à l'hexane du tronc de *D. gracilescens*, les acide ursolique et oléanolique isolés des tiges de *R. hispida* ont montré un grand potentiel inhibiteur sur les formes promastigotes et intracellulaires amastigotes du parasite *L. donovani*. La plupart des composés isolés de *D. gracilescens* étaient faiblement actifs et non-sélectifs

(IS<1) comparés aux composés isolés de *R. hispida* qui ont été tous actifs et sélectifs. Toutefois, des études futures portant sur la relation structure-activité pourrait conduire à l'obtention de dérivés d'intérêts plus actifs qui pourront ainsi servir comme point de départ pour la découverte de nouveaux médicaments contre la leishmaniose viscérale.

Mots clés: Activité Antileishmaniale, Isolement Bioguidé, *Diospyros gracilescens*, *Rothmania hispida*, Acide urosolique, Acide oléanolique.

CHAPTER I

INTRODUCTION

INTRODUCTION

Leishmaniasis is a Neglected Tropical Disease (NTD) caused by an intracellular flagellate protozoan parasite belonging to the kinetoplastidae family and the genus *Leishmania*. The disease is generally transmitted between man and animals during a blood meal by the female sandfly. Approximately 20 different *Leishmania* species, including *L. donovani* are pathogenic to humans **(Akhoundi *et al*., 2016)**.

Leishmaniasis is endemic in more than 98 countries worldwide, with around 370 million people considered to be at risk; furthermore, 12 million people are infected with 1 to 2 million new cases reported annually **(Abdolmajid *et al*., 2015)**. Among the different species that cause leishmaniasis, *L. donovani* is one of the most dangerous causing Visceral Leishmaniasis (VL). In fact, VL is the most dangerous and fatal form of the disease which can be lethal in humans without treatment. The reported global annual number of deaths caused by VL infection is approximately 20,000 **(Steverding, 2017)**.

In 2017, 20792 out of 22145 (94%) new cases reported by WHO occurred in seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan. Concerning the cutaneous leishmaniasis (CL), they majority of cases occur in Afghanistan, Algeria, Brazil, Colombia, the Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia and the Syrian Arab Republic. Almost 90% of mucocutaneous leishmaniasis (MCL) cases occur in the Plurinational State of Bolivia, Brazil and Peru **(WHO, 2017)**.

For the case of Cameroon, many studies have been investigated concerning the disease. Between October 1987 and January 1988, a study performed in Kousseri in the North region of the country showed that among 120 persons selected, 46 presented symptoms of the disease, and 9 were confirmed to be positive for VL after diagnosis **(Dondji *et al*., 2001)**. In addition, the Hospital of Roua of the north region of the Cameroon reported the majority of cases, with 9 cases out of 16 cases (56%) reported in 2019 **(Rapport Biennal, 2019)**. The disease is considered as a serious public health problem worldwide, especially in Africa, where a high percentage of deaths every year, then requiring effective chemotherapy since not much progress have been made in the development of a potent vaccine.

The Current chemotherapy treatment includes first-line treatment drugs such as pentavalent antimonials, meglumine antimoniate (glucantime) and sodium stibogluconate (pentostam) **(Arevalo *et al*., 2007)** and second-line drugs such as amphotericin B, pentamidine, paromomycin and miltefosine **(Goto et Lindoso, 2010)**. However, the use of these drugs is limited by factors such as the emergence of drug resistance, especially pentavalent antimonials and challenges of

toxicity, short half-life and high cost of drugs, as well as failure of patients to comply with treatment (**Berman *et al*., 1998; Croft et Coombs, 2003)**. In addition, there is no vaccin against the disease.

Due to the limitations of current chemotherapeutic treatment and in the absence of a suitable vaccin, there is an urgence to search for new bioactive molecules for the treatment of leishmaniasis. In this respect, natural products from medicinal plants represent an alternative for the new drug discovery, because they contain many secondary metabolites classes responsible of multiple biological activities **(WHO, 2000).** In fact, plants from Rubiaceae and Ebenacea are sources of new bioactives substances that could be used in the treatment of the disease.

More specifically, *D. gracilescens,* a plant of the Ebenaceae family, is a forest tree widely distributed in the western and central regions of Cameroon. To the current extent of our knowledge, no previous biological work has been reported on this plant. There is no mention of the use of *D. gracilescens* in traditional medicine in Cameroon. However, related species such as *D. bipindensis, D. conocarpa* and *D. malabarica* are widely used by the Baka Pygmies for the treatment of malaria, sleeping sickness and respiratory disorders **(Kaushik *et al*., 2013)**. Globally, *Diospyros* spp. are known, above all, as fishing poisons, especially in Southeast Asia and in the Philippines. They are also widely used as medications in traditional African medicine, mainly against leprosy. The roots are used as purgative in the Central African Republic, against pneumonia in Zimbabwe and schistosomiasis in Malawi **(Kerharo and Bouquet, 1949; Gafner *et al*., 1987)**. The first chemical study of *D. gracilescens* was reported by **Mbi and Waterman** in **1978**. The results of their studies led to the isolation of a few compounds, such as lupeol, betulin, betulinic acid, isodiospyrin (II) and sitosterol. To the best of our knowledge, no other previous study on *D. gracilescens* has been published in the literature. In this thesis, we therefore describe for the first time, the isolation of the chemical constituents of *D. gracilescens* guided by their antileishmanial activity.

Another plant is *R. hispida,* a plant of the Rubiaceae family. It is widely distributed around the world and in Cameroon. Its usage in traditional medicine is very widely known in Cameroon, and the plant is used traditionally for the treatment of many various diseases, such as malaria, filariasis, diarrhea, itching skin diseases, ulcers, and diabetes (**Cardon, 2005)**. To the best of our knowledge, very few chemical and biological studies have investigated this plant.

With the aim of the valorization of Cameroonian medicinal flora and specifically plants from the Rubiaceae and Ebenaceae families, the objective of this study was to investigate the bio-guided isolation of the *in vitro* antileishmanial active natural products from *D. grascilisens* and *R.*

hispida stem extract, targeting extracellular (promastigotes) and intracellular (amastigotes) forms of *L. donovani*, the causative agent of VL. More specifically, we have:

1. Determine the *in vitro* antileishmanial activity of extracts, fractions and isolated compounds from *D. gracilescens* and *R. hispida* stem extracts against promastigotes and intracellular amastigotes of *L. donovani* parasites using bioactivity-guided fractionation.

2. Determine the potential modes of action of the most potent fractions/compounds using invasion and immunomodulatory assays and the acute toxicity of the most active extract.

CHAPTER I

LITERATURE REVIEW

I- LITERATURE REVIEW

I-1- History and biology of leishmaniasis

I-1-1-History of Leishmaniasis

The earliest Old World records describing lesions with Cutaneous Leishmaniasis (CL) character dates to the seventh century BCE. The detailed reports from Arab physicians in the 10th century describe CL in various regions of what is today called the Middle East **(Manson-Bahr, 1996)**. The Old World VL, or kala azar, characterized by an enlarged spleen, was first recognized in India in 1824. However, the symptoms were confused with those of malaria **(Ross, 1899)**. The clear recognition of VL as a distinct disease was achieved in 1900 after William Leishman and Charles Donovan independently identified *L. donovani* parasites in the spleen of kala azar patients. At the same time, *Leishmania* parasites were also observed in samples obtained from CL lesions. In 1908, Nicolle isolated the parasite from a cutaneous lesion and established the similarity between cutaneous and visceral forms of the disease with regard to the causative agent **(Nicolle, 1908)**. The majority of CL cases in the Old World are caused by two *Leishmania* species: *L. major* and *L. tropica*. In the New World, CL and MCL cause disfiguring conditions, and these have been depicted on sculptures dating back to the fifth century. The references to leishmaniasis are also found in the writings of Spanish missionaries from the 16th century **(Lainson, 1996)**. In 1911, Gaspar Vianna discovered that leishmaniasis in South America was caused by a different *Leishmania* species from that in the Old World and gave a new name, *L. brazilienses*, for this species **(Vianna, 1911)**. The species name was later corrected to *L. braziliensis*. In the 1960s, additional *Leishmania* species causing CL in Latin America were recognized, such as *L. mexicana* **(Lainson et Strangways-Dixon, 1964)**. In 1937, the causative agent of VL in the New World was designated a distinct species, named *L. chagasi* **(Cunha et Chagas, 1937)**. However, this specie is indistinguishable from *L. infantum*, the specie that causes VL in southern Europe **(Kuhls *et al.*, 2011)**.

I-1-2-Definition and classification

Leishmaniasis is caused by *Leishmania spp*. belonging to the Kinetoplastida order, Trypanosomatidae family and *Leishmania* genus. Based on the clinical manifestations, we have four different forms of the disease: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral leishmaniasis/kala azar (VL), and post kala azar dermal leishmaniasis (PKDL). Promastigotes are the extracellular forms generally found in the insect vector, while amastigotes are the intracellular forms generally found in the human host **(Burton *et al.*, 2012)**.

I-1-3- Geographical Distribution

Leishmaniasis is a NTD mainly distributed in many tropical and subtropical regions around the world. The repartition of the disease depends mainly on the distribution of the different species of the insect vector. Ther is two main geographical situations: the Old world (The South of Europe, Africa, Near-Orient, Middle-Orient and Asia) and the new world (South and Center of America) **(Table I)**. More than 20 *Leishmania spp*. distributed in approximately 88 countries on 5 continents have been found to cause human leishmaniasis **(figures 1 and 2) (WHO, 2018)**.

For the case of VL, 31% were reported by the African Region, 30% by the Asia South-East Region, 22% by the Eastern Mediterranean Region and 15% by the Americas Region; 2% and 1% of cases worldwide were reported by the European and Western Pacific Regions respectively. The three ecoepidemiological foci of VL are East Africa (Ethiopia, Kenya, Uganda, Somalia, Sudan and South Sudan), the Indian subcontinent (Bangladesh, India, and Nepal) and Brazil **(WHO, 2018).**

For the case of CL, more than 90% of new cases were reported in the Mediterranean Eastern (69%) and the Americas (24%) regions. The Oriental Mediterranean region and Algeria are part of the same ecoepidemiological focis because they notify two three-quarters of all CL cases. Brazil, Colombia and Peru in the Americas are also considered scale homes worldwide (15%). Six countries (Afghanistan, Algeria, Brazil, Colombia, Pakistan and Syria) represent more than 70% of the cases reported worldwide. These 6 countries, including Morocco, Nicaragua, Peru, Sudan, Tunisia and Yemen, account for 90% of the burden of CL around the world **(WHO, 2018)**.

In Cameroon, CL is mainly found in the North Region of the country (Mokolo the most affected) and the frontier area with Chad. This form has been found in Cameroon since 1930 **(Hervé, 1937)**. The visceral form is frequently present in the Kousseri region of the country **(Dondji, 2001; Ngouateu *et al*., 2012)**.

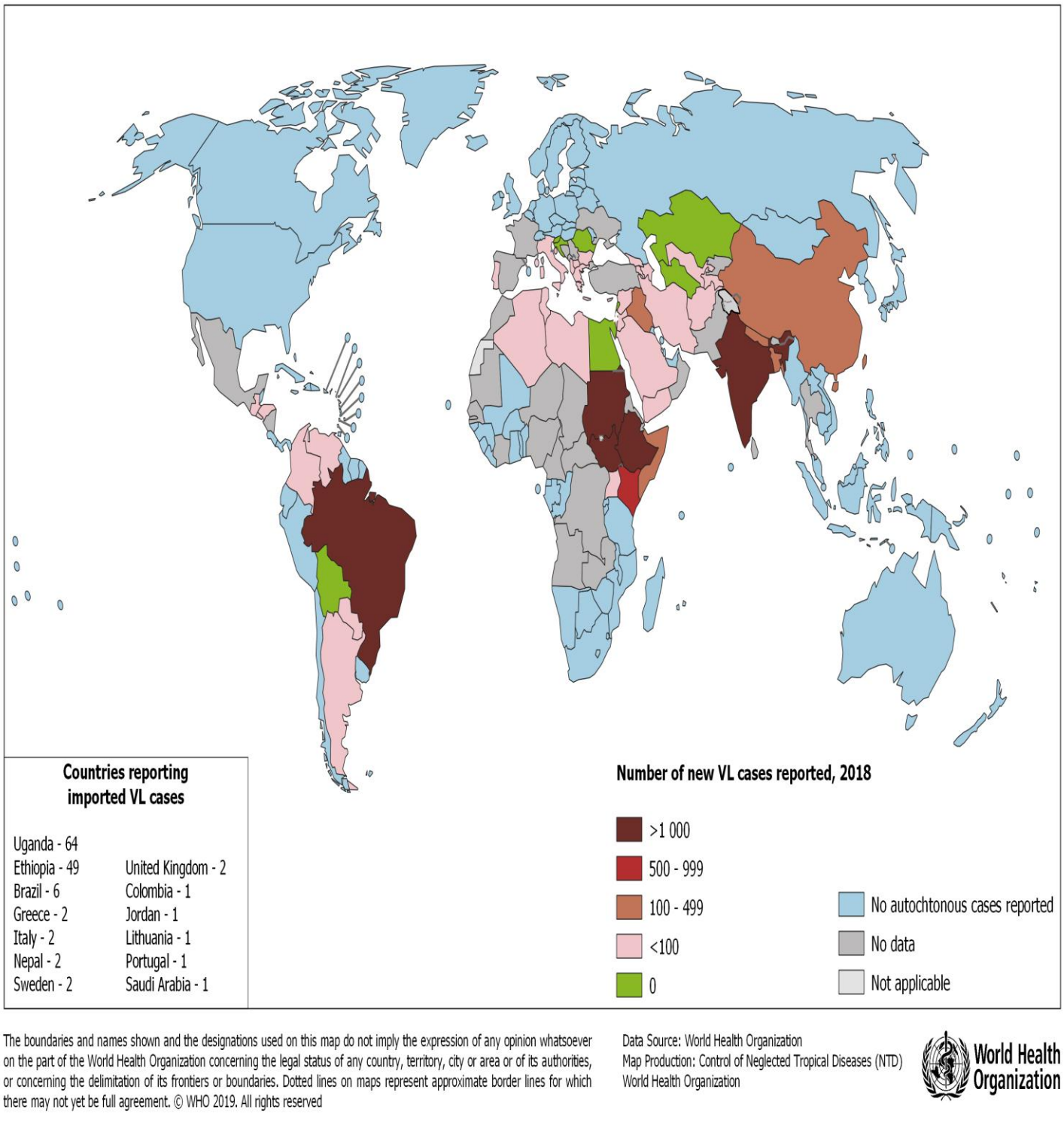


Figure 1: Distribution and endemicity of visceral leishmaniasis (VL) according to 2018 annual country reports. Countries in gray have no reliable epidemiological data or do not report disease incidence to the WHO NTD section. Countries in green had no autochthonous cases of VL reported in 2018. (Source: WHO Global Health Observatory, 2018).

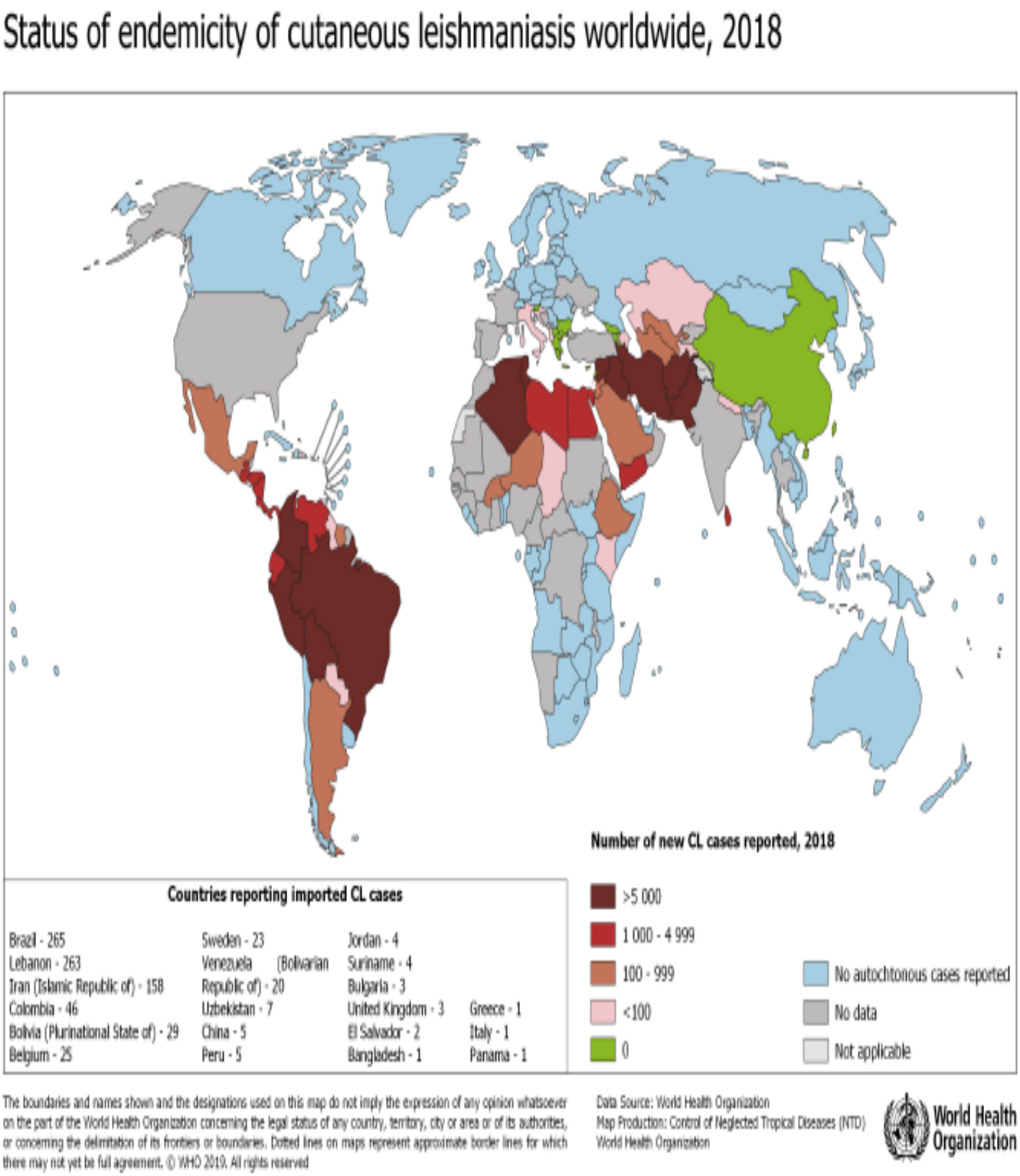


Figure 2: Distribution and endemicity of CL according to 2018 annual country reports. Countries in gray have no reliable epidemiological data or do not report disease incidence to the WHO NTD section. Countries in green had no autochthonous cases of VL reported in 2018. (Source: WHO Global Health Observatory, 2018).

The geographical repartition of the different agents of leishmaniasis depends mainly on the species from one country to another **(Table I)**.

Table I: *Leishmania* Species Reported to Cause Human Infections and Associated Leishmaniasis Syndromes **(Nagle *et al*., 2014)**.

Species	Clinical presentation	Epidemiology	Symptoms
***L.donovani* (anthroponotic)**	Visceral leishmaniasis/kala azar (VL)	• India, Bangladesh, Nepal, East Africa • 300,000 new cases per year, with India having the highest incidence • 40,000 death annually	• Prolonged fever, splenomegaly, pancytopenia, progressive anemia and weigh loss • Darkening of the skin
***L.infantum* (Zoonotic)**	Visceral leishmaniasis	• Mediterranean Region, Central and South America • Primary animal reservoir, dog	• Same as above
***L.siamensis* (Zoonotic)**	Visceral leishmaniasis	• Thailand	• Same as above
***L.donovani* (anthroponotic)**	Post kala-azar dermal leishmaniasis (PKDL)	• East Africa (Soudan), India, Bangladesh and Nepal • Occurs in 50-60% of Sudanese and 10-20% in India VL patients with 0.5-7 years of infection	• Severe dermatitis entailing parasites containing facial skin lesions and plaques on body • Potential to lead to nerve damage or blindness
L. aethipica* *L. killicki* *L. major* *L. tropica* *L. turanica	Cutaneous leishmaniasis (CL. Old World)	•Southern Europe, Middle East and Southwest Asia and Africa • 0.7 to 1.2 million case per year, with greatest number of infected individuals residing in Algeria Afghanistan, Syria and Ethiopia	• Erythematous papulae at the site of san-fly bite with eventual scaring • Potential for becoming more severe and diffuse
L. amazonensis* *L. brazilensis* *L.colombiensis* *L. garnhami* *L. guyanensis* *L. lainsoni* *L. Mexicana* *L. panamensis* *L. peruviana* *L. shawi* *L. venezeluensis	Cutaneous leishmaniasis (CL. New World)	• Central and South America • Greatest number of infected individuals residing in Brazil, Colombia, Costa Rica and Peru • Infected patients included military workers, international travelers and endemic area migrants	• Erythematous papulae at the site of san-fly bite (for *L. Mexicana*) • Metastasizing lesions that can lead to MCL (for *L. Brazilensis*) and diffuse cutaneous leishmaniasis (DCL for (for *L. amazonensis*) • DCL and MCL are complications that occur (90% of cases in Brazil, Bolivia and Peru)
L. aethiopica* *L. bazilensis* *L. guyanensis* *L. panamensis	Muco-cutaneous leishmaniasis (MCL)	• Ethiopia • Central and South America	• Disfiguring and Obstructive lesions of the mucosal membrane occurring in 1-10% of CL patients • Destruction of the oronasopharyngeal mucosa

I-1-4- Epidemiology

I-1-4-1- Visceral Leishmaniasis and Post Kala Azar Leishmaniasis

VL is the most severe form of leishmaniasis. There are two types of VL, defined by the causative *Leishmania* species and the parasite reservoir. The zoonotic form, caused by *L. infantum*, occurs in the Mediterranean basin, Central and South America, with dogs being the main parasite reservoir **(Van Griensven et Diro, 2012; Sundar et Chakravarty, 2013; WHO, 2010)**. The more common anthroponotic form is caused by *L. donovani* and is predominant in India, Bangladesh, Nepal, and East Africa. VL is endemic in rural areas of developing countries and has been reported in approximately 98 countries worldwide; 90% of all cases occur in tropical/subtropical regions of six countries: India, Bangladesh, Sudan, South Sudan, Brazil, and Ethiopia **(Chappuis *et al*., 2007; Van Griensven et Diro, 2012)**. Approximately 300 000 new cases of VL occur each year, leading to an estimated 40 000 deaths. Approximately 90% of all VL cases occur in 3 endemic foci: 1. India, Bangladesh, and Nepal; 2. East Africa; and 3. Brazil. India has the highest incidence of the disease, with approximately 60% of all new cases occurring in Bihar state **(Chappuis *et al*., 2007; Alvar *et al*., 2008; Van Griensven et Diro, 2012; Ejazi et Ali, 2013)**. Outbreaks are common during migration or entry of naive hosts into endemic areas, and an increase in the immunosuppressed patient population (such as HIV) has contributed to the escalation in VL incidence in East Africa **(Sundar et Chakravarty, 2013)**. Additionally, an absence of implementation of cost-effective control strategies makes VL a major public health concern **(Chappuis *et al*., 2007)**.

Four (04) countries (Brazil, India, Sudan and South Sudan) have each reported more than 3000 cases of VL, which accounts for 78% of cases worldwide. Together with Ethiopia, Kenya and Somalia, these seven (07) countries account for 90% of the VL reported globally. Countries such as South Sudan, Bangladesh and Nepal are actively fighting to eliminate VL **(WHO, 2018)**.

PKDL is prevalent in areas where *L. donovani* is endemic (India and East Africa) and occurs in 50−60% of Sudanese and 10−20% of Indian VL patients within 6 months to 2−7 years after initial infection **(Ramesh et Mukherjee, 1995; Zijlstra *et al*., 1994; Singh *et al*., 2011)**. Of these cases, approximately 15− 20% (India) and 8% (Sudan) of patients do not have a history of VL, indicating the existence of an asymptomatic infection **(Ramesh et Mukherjee, 1995; Zijlstra *et al*., 1994)**. Few cases of PKDL caused by *L. infantum* or *L. tropica* have been reported **(Sacks *et al*., 1995)**. It has been previously shown that the presence of a small population of infected individuals (0.5%) may lead to a widespread epidemic of VL infection in India and other regions

of Asia; therefore, PDKL patients play a major role in the spread of the disease, and parasite eradication should be a high priority **(Dye et Wolpert., 1988; Kedzierski, 2011)**.

I-1-4-2- Cutaneous leishmaniasis

Approximately 0.7 to 1.2 million cases of CL occur each year in the Americas, Mediterranean Basin, the Middle East, and Central Asia. A large fraction (75%) of CL patients resides in the following ten countries: Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica, and Peru **(Alvar *et al*., 2008)**. The disease is caused by *L. tropica*, *L. major,* and *L. aethiopica* in the Old World (Southern Europe, Middle East, Southwest Asia, and Africa) or by *L. mexicana, L. braziliensis* and additional *Leishmania* species in the New World (Central and South America) **(Table 1) (Berman, 2005, WHO, 2010)**. CL cases caused by *L. major* and *L. tropica papulae* typically heal within a few months without medical intervention, whereas CL caused by *L. braziliensis* is distinguished by lesions that frequently metastasize to mucosal tissues (MCL) and are treated with anti-leishmanial therapeutics **(Markle et Makhoul, 2004; Weina *et al*., 2004; Sundar et Chakravarty, 2013; WHO, 2010)**. DCL (*L. amazonensis*) and MCL are complications of CL that occur primarily in the New World (90% of cases found in Brazil, Bolivia, and Peru), respectively **(Markle et Makhoul, 2004)**.

An increasing number of CL cases have been reported in international travelers, and endemic area migrants **(Weina *et al*., 2004; Ul Bari, 2006)**. Travels to Central and South America account for approximately 40% of CL cases in tourists and workers in the USA **(Herwaldt et Berman; 1992)**, while some cases of leishmaniasis introduced into industrialized nations involve VL; greater than 80% of these cases are caused by CL. In fact, CL is one of the most frequent skin disorders in the New World and accounts for approximately 60% of all cases in nonendemic areas. The disease specifically affects persons living in sub-Saharan countries **(WHO, 2010)**.

An increasing number of cases of leishmaniasis have been noticed in some regions of the world. For the example of CL cases in Brazil, 21800 persons were affected in 1998, and this number increased to 40000 in 2002. For VL in northern Brazil, there were 1840 cases in 1998, and this number increased to 6000 in 2002 **(Desjeux, 2004)**.

✓ *Leishmaniasis in Cameroon*

For the case of Cameroon, many studies were done concerning the disease. Between October 1987 and January 1988, a study performed in Kousseri in the North region of the country showed that among 120 persons selected, 46 presented symptoms of the disease, and 9 were positively confirmed for VL after diagnosis **(Dondji *et al*., 2001)**. Studies in the past showed the presence of some coinfection cases of many *Leishmania* species (mainly visceral form) with other

diseases, such as malaria, pneumonia, tuberculosis, and HIV-AIDS, increasing the percentage of mortality of the disease. With the increase of travel, immigration, and military work in endemic areas of this disease, the risk levels and incidence are predicted to increase, hence making implementation of precautionary measures crucial in this selected group. In addition, according to the work done by the WHO, 2000 cases of coinfections were reported in the Mediterranean Basin, with 90% present in Spain, Italy, France and Portugal **(Cruz *et al.*, 2006; Desjeux, 2001)**. We may, for example, highlight a study showing VL-HIV coinfection in Cameroun **(Ngouateu *et al.*, 2015)**. However, it is also important to note that a recent study performed at Kumbo in the northwest region of Cameroon showed the presence of a new CL-HIV coinfection in one death Cameroonian woman with 28 years old **(Tangie *et al.*, 2017)**.

In addition, the Hospital of Roua of the north region of the Cameroon reported the majority of cases, with 9 cases on 16 cases (56%) reported in 2019 **(Rapport Biennal, 2019)**.

I-2- The pathogen and vector

I-2-1- The pathogen: Leishmania

I-2-1-1- Classification

The pathogen agent of the leishmaniasis belongs to the Trypanosomatidae family, Kinetoplastidae order and the *Leishmania* genus. The following classification was established by **Mendoza-Leon *et al.* (2002)**. **(Table II)**.

Table II: Systematic classification of the *Leishmania* genus **(Mendoza-Leon *et al.*, 2002)**.

Classification of *Leishmania* genus	
Domain	Eucaryotes
Reign	Protista
Sub-reign	Protozoa
Branch	Sarcomastigophora
Class	Zoomastigophorea
Rank	Kinetoplastida
Sub-Rank	Trypanosomatina
Family	Trypanosomatidae
Genus	*Leishmania*
Subgenus	*Leishmania (L.)* et *Viannia (V.)*

The development of the parasite in the digestive tract of the insect vector at the level of the pylore is the criteria that allow the division of the *Leishmania* genus into two subgenuses: the

Suprapylaria section, characterized by multiplication at the level of tthe anterior intestine, called the *Leishmania* subgenus and the Peripylaria section, characterized by multiplication at the level of the posterior part followed by an anterior migration called the *Viannia* subgenus **(Botero, 2006)**.

The *Leishmania* genus is composed of many morphologically similar species and causes many clinical manifestations, starting from cutaneous affections that are resorbed themselves to fatal visceral infections passing through inflammation causing serious disfigurement. We actually group *Leishmania* species in the «complex» according the biochemical similarities **(Table I)**.

I-2-1-2- Reproductive Cycle

The life cycle of the *Leishmania spp*. is characterized by two morphologically distinct forms **(Figure 4)**: the elongated, flagellated and very high motile promastigote form, found in the alimentary tract of the female sandfly vector, and the round non-motile amastigote form, present in the bloodstream and tissues of the mammalian host **(Vannier-Santos *et al*., 2002; Ouellette *et al*., 2003)**. There are two main stages during the parasite life cycle:

- ***Extracellular stage: development in the insect vector***

The parasites at this stage are mobile, extracellular and fusiform, 5 to 20 μm in length and 1 to 4 μm in wide, prolonged by flagella reaching up to 20 μm in length and emerging in the anterior pole **(Dedet, 1999; Vannier-Santos *et al*., 2002)**. According to the species, the period of the life cycle on the insect vector varies between 4 and 18 days.

As an infected sandfly takes a blood meal from a naive host, it regurgitates infective promastigotes at the bite site. The parasites are subsequently taken up by host dendritic cells and macrophages in the dermal layer of the skin. Then, they differentiate into amastigotes and multiply within phagolysosomes (via binary fission) and resists to the degradation by lysosomal enzymes. Upon lysis of infected macrophages and dendritic cells, the parasites disseminate via the lymph and circulatory systems and infect other macrophages of the reticulo-endothelial system. The parasites persist in macrophages, spleen, bone marrow, liver, and lymphnodes and induce extensive inflammation and increased hematopoiesis (**Van Griensven et Diro, 2012)**.

At this stage, two differentiation conditions may be considered: some nectomonades become oval haptomonades, giving paramastigotes whose exact role is not yet known. Other nectomonades are transformed into metacyclic promastigotes (between the 5^{th} and 7^{th} day), more infectious, elongated form, flagellated and more mobile, not able to divide again. These last forms migrate by the esophagus, pharynx and proboscis. They will be ingested by insect vectors during

the next blood meal **(Figure 3)** (**Dedet, 1999; Ouellette *et al*., 2003; Sacks et Kamhawi, 2001; Sacks, 1989)**.

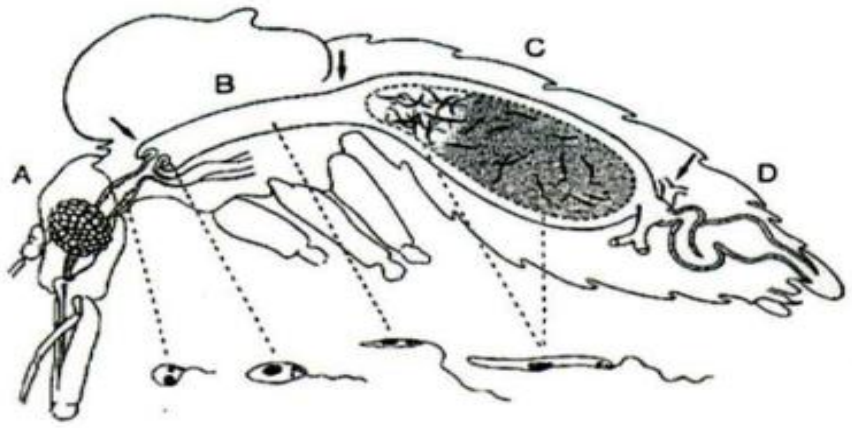


Figure 3: Differentiation process at the level of the digestive gut on the vector agent of leishmaniasis **(Schlein, 1993)**. Processing by a non-infectious form (procyclic) of the promastigote stage of *Leishmania* is differentiated into a highly infectious form (metacyclic). This differentiation occurs between five and seven days. The metacyclic forms migrate by the pharynx of the insect. Head and pharynx (A), median thoracic intestine (B), median abdominal intestine (C), posterior intestine (D). Blood (area gray in C) is enclosed by a peritrophic membrane.

- ***Intracellular stage: development in the mammalian host***

This form of parasite, generally oval with a diameter of 2 to 5 μm, is the intracellular and non-mobile form found in the mammalian host.

Infected patients serve as parasite reservoirs and can infect naive sandflies when infected macrophages are ingested as part of the sandfly blood meal. After the parasite-infected macrophage is ingested by the sandfly, the amastigotes transform into promastigotes in the insect midgut, multiply, and migrate to the proximal end of the gut, where they remain until the next cycle of vector host infection and transmission **(Chappuis *et al*., 2007; Van Griensven et Diro, 2012)**.

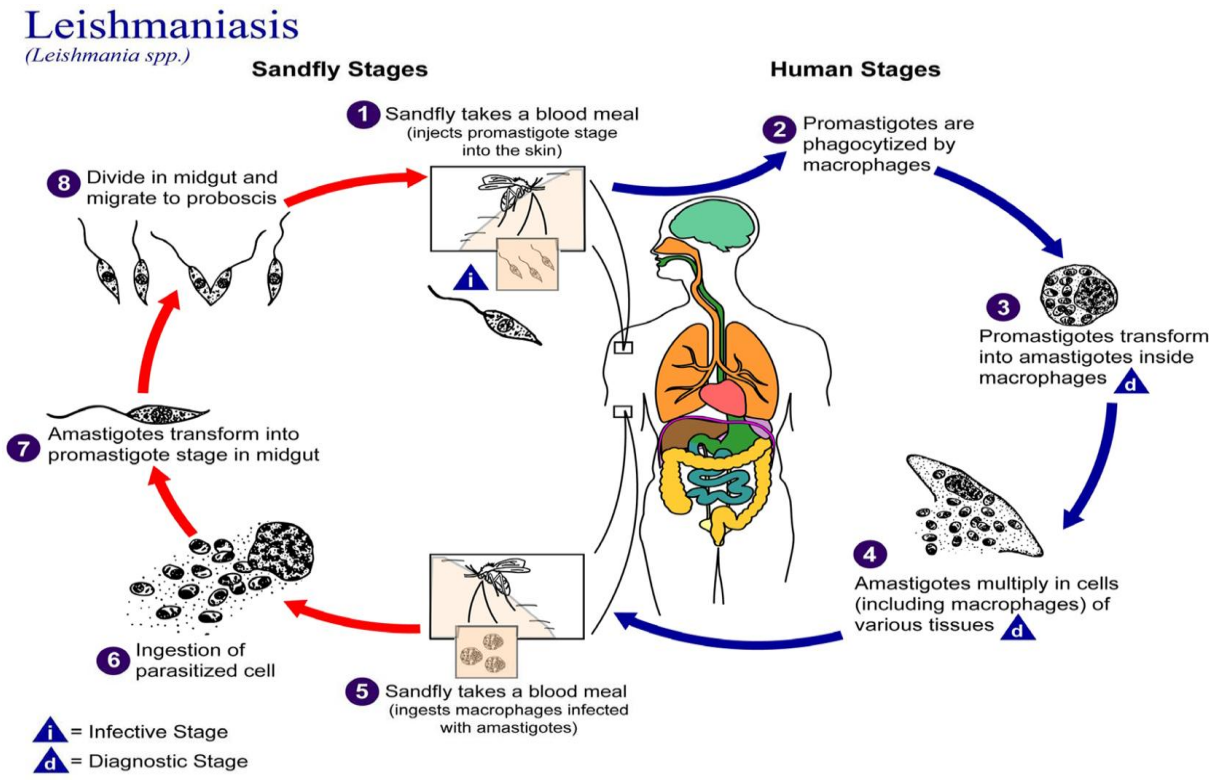


Figure 4: Life cycle of *Leishmania* parasites (source: Public Health Image Library provided by CDC- DPDx/Alexander J. da Silva, PhD, Blaine Mathison).

The cycle is completed when a phlebotome, taking infected blood from humans, ingests amastigotes that will be differentiated to promastigotes and then will be multiplied inside the insect to finally migrate though the proboscis **(Esch et Petersen, 2013)**.

I-2-2- The vector and transmission

The vector agents of transmission of leishmaniasis are insects belonging to the Diptera order, Psychodidae family and Phlebotominae subfamily. There are approximately 700 phlebotome species among approximately thirty are possible vectors. These species are grouped into three main genera: *Phlebotomus*, *Lutzomyia* and *Sergentomyia*. Only the *Lutzomyia* and *Phlebotomus* genera are involved in the epidemiology of leishmaniasis and are responsible of the transmission of the disease. The first is divided into 16 subgenuses, and the second is divided into 8 subgenuses **(Dedet, 1999)**. The insects of the *Phlebotomus* genus are responsible for the transmission of leishmaniasis in the Old World and are located in the subtropical areas of arid and semiarid Asia, Africa and southern Europe. Those of the *Lutzomyia* genus are responsible for the transmission of the disease in the new world and are mainly located in tropical and subtropical areas of America **(Killick-Kendrick, 1990).**

Phlebotomes are small insects (2 to 5 mm in length) with a slender and elongated body, and the maximal activity is crepuscular. The colors vary from white to black. Only females

(Figure 5) are hematophagous (necessary to give eggs) and responsible for the transmission of the disease **(Pinto *et al.*, 2001)**. The distribution of the vectors also depends on the ecology where they are located (altitudes, climates, temperature and pluviometry). Their lifetime depends mainly of temperature and humidity. They generally live between two weeks and two months. In addition, it has been reported that phlebotomes are insects that live in burrow, chink, rivers, trees and rodent houses in the old world, while in the new world, they live in vault and stretcher forests. These insects generally have nocturne activity **(Dedet, 1999)**.

Figure 5: The vector of leishmaniasis during a blood meal **(WHO, 2009b)**.

The propagation of leishmaniasis cases is based on two main ecoepidemiological forms. In the first or zoonotic form, mammalian or domestic savages (mainly the dog) serve as natural reservoirs and do not generally show the clinical signs of the disease, where the human plays the role of facultative host when it is exposed accidentally to the transmission cycle. The second form or anthroponotic form (human is definitive host) illustrated by the following figure is characterized by the presence of one reservoir that is also an infection way: human **(Figure 6) (Dedet, 1999; Desjeux, 2001, 2004; Ouellette *et al.*, 2003)**.

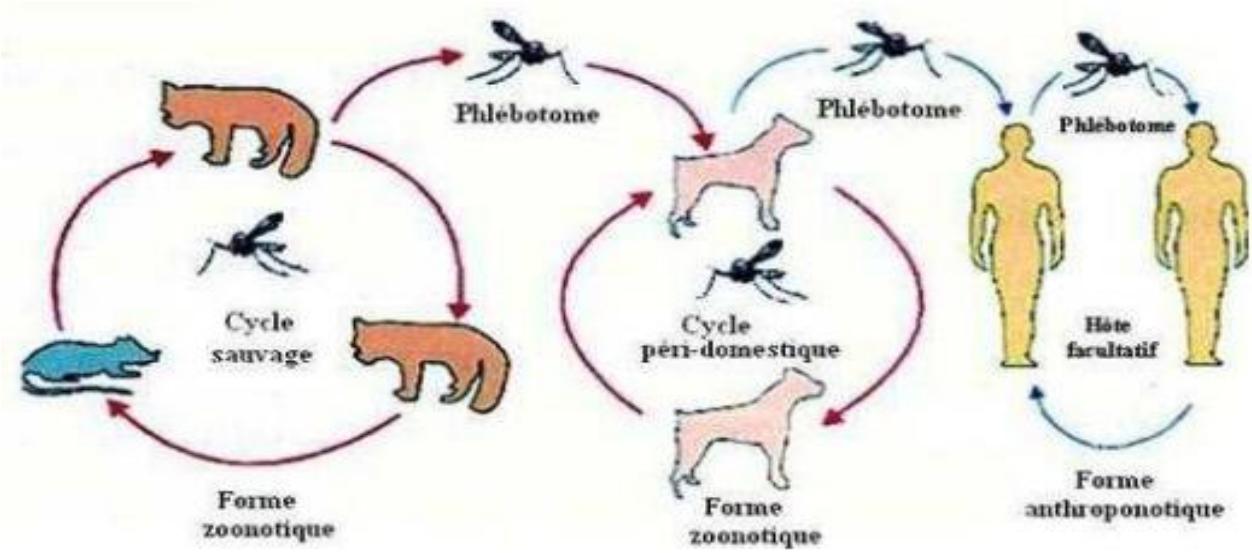


Figure 6: Epidemiological cycle of leishmaniasis **(unanleon)**

I-3- Clinical manifestations of leishmaniasis in humans

The parasites of the *Leishmania* genus are responsible for different pathologies. The symptomatic clinical manifestations generally involve the skin, mucous membranes and viscera with severe consequences that were diagnosed in the infected persons. They can be classified into four groups **(Desjeux, 2004)**:

- ***Cutaneous Leishmaniasis (CL)***

The most common form of the disease, CL, exhibits various clinical presentations depending on the *Leishmania* species **(Table I)** and the mode of transmission. CL starts with an erythematous preulcer papule at the site of the sandfly bite. This may self-cure within months or undergo slow healing with severe scarring **(Markle et Makhoul, 2004)**. Rarer manifestations of CL include DCL and MCL, which are life-threatening conditions **(Markle et Makhoul, 2004; Khandelwal *et al*., 2011)**.

The clinical symptoms include papule skin lesions on the face, which gradually increase in size to form nodules on the body and which can further transform into large plaques (Indian) or ulcers (Sudanese). These nodules have been shown to contain *Leishmania* parasites **(van Griensven et Diro, 2012; Zijlstra *et al*., 1994; Singh *et al*., 2011)**. All is generally resorbed after some months or one year, leaving many deep scars non-pigmented. In the case of *L. mexicana*, the lesions heal spontaneously except for an infection at the level of the auricle ear, which may remain for many years (some date of 40 years) and is known as «ulcéra del chiclero» **(figure 7) (Roberts et Janovy, 2000)**.

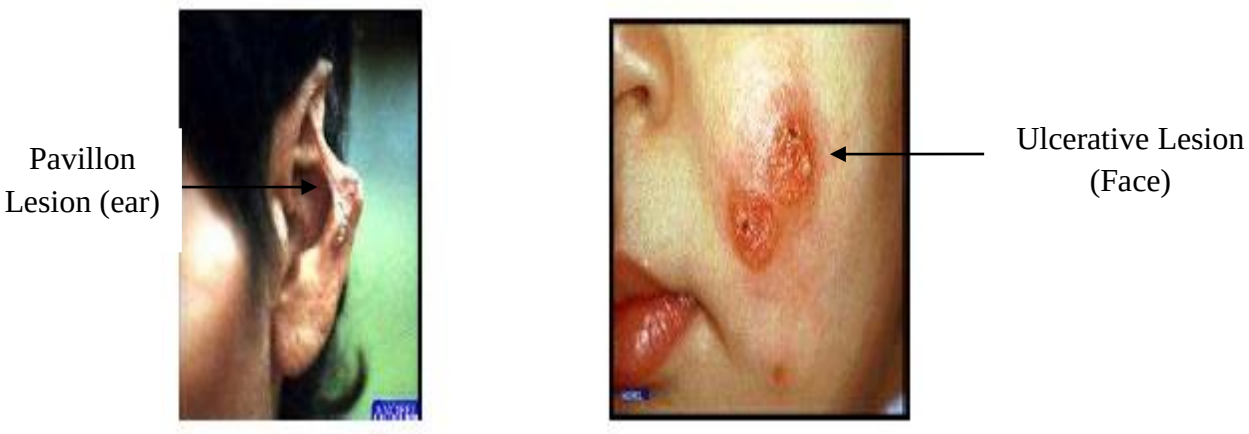


Figure 7: Ulcerative locations of CL **(Acebey, 2007)**

- ***Muco-Cutanaeous leishmaniasis (MCL)***

MCL is characterized by disfiguring and destructive lesions of the mucosal membranes and is usually observed months or even years after CL lesions in approximately 1−10% of CL patients **(Markle et Makhou, 2004; Kedziersk, 2011)**. In addition to ulcerative lesions and erythema

around the nose and lips, MCL patients initially present with nasal congestion and nasal septal granulomas (both anterior and posterior), lymphadenopathy, fever, hepatomegaly, and scars from previous CL incidence. Later-stage MCL patients may exhibit additional complications within the nasal cavity (edema, septum perforation) and periodontitis, with eventual destruction of the oronasopharyngeal mucosa and airway obstruction **(Figure 8) (Reithinger *et al.*, 2007)**.

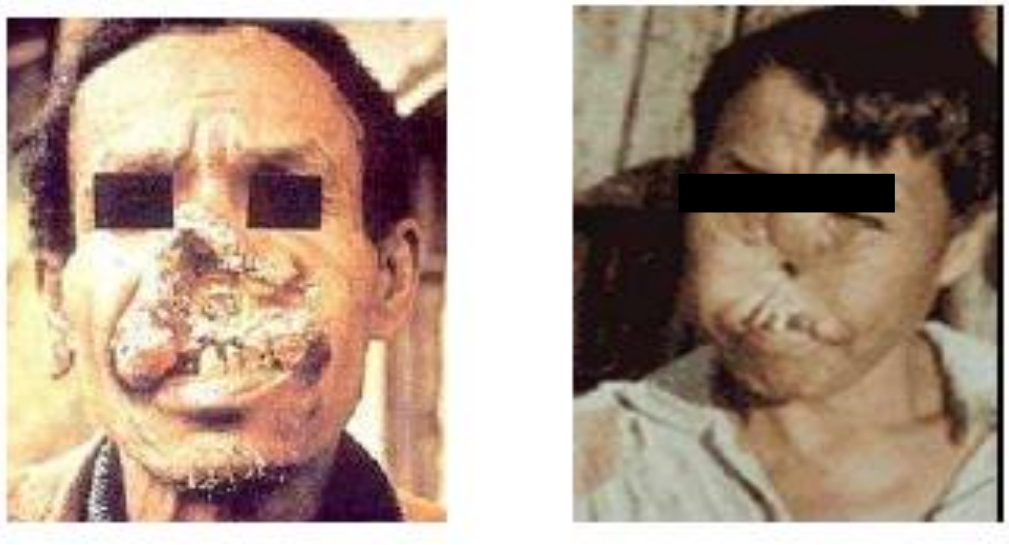


Figure 8: Mucocutaneous lesions of leishmaniasis **(Acebey, 2007)**.

- ***Diffuse-cutaneous leishmaniasis (DCL)***

This form is characterized by the dissemination of nodular lesions or in patches such as the strongly leproïdes form, often present on the face. These lesions do not heal spontaneously. This form is rare even in countries where leishmaniasis is endemic. It is present as a red papule in the inoculation zone and then dispersed as an erythaematous macula or non-violaceous ulcer. It is more frequent in persons with a defective immune system. The face, superior and inferior extremities and buttock are generally more affected **(Figure 9) (Alidadi et Oryan, 2014)**.

Figure 9: Diffuse-cutaneous leishmaniasis **(Acebey, 2007)**.

- ***Post kala azar dermal leishmaniasis, or PKDL***

Post-kala azar dermal leishmaniasis, or PKDL, is a form of dermal leishmaniasis that may appear months to years after effective treatment of VL and exhibits distinct features based on geography (Indian and Sudanese PKDL) **(Zijlstra *et al*., 1994; van Griensven et Diro., 2012; Sundar et Chakravarty, 2013)**. While most cases of PKDL present as severe dermatitis, the spread of infection can lead to blindness (via the mucosal membranes) and nerve damage (primarily in Indian PKDL) **(Ramesh et Mukherjee, 1995)**.

- ***Visceral leishmaniasis (VL)***

VL is the most severe form of the disease and typically results in death if left untreated **(Ejazi et Ali, 2013)**. The clinical features generally manifest 2−6 months after infection, and these include prolonged fever, splenomegaly, hepatomegaly, pancytopenia, progressive anemia, and weight loss **(Chappuis *et al*., 2007; van Griensven et Diro., 2012; Berman, 2005)**. Latent cases may remain undiagnosed until the patient becomes immune-compromised, with symptoms then appearing only several years after infection **(Chappuis *et al*., 2007; van Griensven et Diro., 2012; Ejazi et Ali, 2013; Sundar et Chakravarty, 2013)**. The darkening of the skin occurs in patients (particularly in South Asia) and defines the origin of the disease synonym kala azar (black fever in the Hindi language) **(van Griensven et Diro, 2012; Dorlo *et al*., 2012).**

VL patients are at high risk for bacterial coinfections, including pneumonia, tuberculosis, and gastrointestinal (GI) infection **(Chappuis *et al*., 2007).** Both *Leishmania* and HIV target the immune system, and coinfections are found in overlapping HIV/VL endemic areas, specifically Ethiopia, Brazil, and India **(Jarvis and Lockwood, 2013)**. Furthermore, the risk of developing VL is approximately 100 to 2000 times greater in patients infected with HIV than in non-HIV individuals. HIV/VL Coinfected patients have a reduced $CD4^+$ T-cell count (below 200 cells/μL) and generally present symptoms similar to those observed in HIV-negative patients, including fever, splenomegaly, pancytopenia, lymphadenopathy, lethargy, and gastrointestinal issues. Coinfections are also more refractory to treatment and often require VL rescue therapy with an alternative drug **(Alexandrino-de-Oliveira *et al*., 2010)**. Early detection and treatment are crucial determinants of the prognosis for infected patients and for the prevention of transmission **(Figure 10) (Roberts et Janovy, 2000)**.

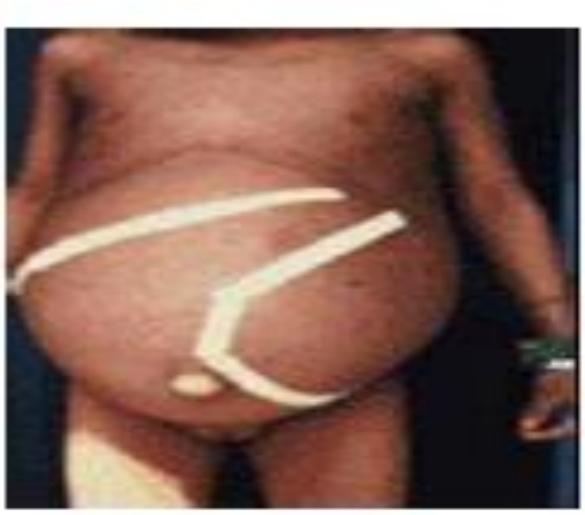


Figure 10: Children with visceral leishmaniasis **(Acebey, 2007)**.

- ***Leishmaniasis and Oxidative Stress***

During its life cycle, *Leishmania* encounters and readily adapts to various hostile conditions such as oxidative stress due to heme digestion in the blood meal, proteases in the sandfly midgut, complement-mediated lysis in the blood upon transmission, and reactive oxygen and nitrogen species (ROS and RNS) generated during phagocytosis by host macrophages (**Almeida *et al.* (2012) ; Miao *et al.* (2009)**). The two important molecules that are critical in controlling *Leishmania* infection are superoxide anion (O_2^-) and nitric oxide (NO). During the initial phase of infection by *Leishmania*, superoxide is produced as part of the oxidative burst of macrophages in response to phagocytosis (**Almeida *et al.* (2012); Miao *et al.* (2009)**). The second oxidant produced by macrophages is nitric oxide, which, in contrast to superoxide, is generated after activation of macrophages by IFN-γ and TNF-α **(Gantt *et al.*, 2001)**. Nitric oxide is derived from the oxidation of the terminal guanidine nitrogen of L-arginine by an NADPHdependent enzyme, NO synthase. In murine systems, IFN-γ has been shown to synergize with TNF, to activate inducible nitric oxide synthase (iNOS or NOS2) to produce nitric oxide (NO) resulting in eradication of intracellular parasites **(Bogdan *et al.*, 2000)**.

- ***Physiopathology of Leishmaniasis***

The sand fly vector of genus *Phlebotomus* (old world) or *Lutzomyia* (new world) becomes infected when feeding on the blood of an infected individual or an animal reservoir. The *Leishmania* parasites live in the macrophages as round, non-motile amastigotes (3-7 μm in diameter). The sandfly ingests the macrophages during the blood meal and the amastigotes are released into the stomach of insect **(Killick-Kendrick, 1990)**. Almost immediately the amastigotes transform into

the motile, elongated (10-20 μm), flagellate promastigote form. The promastigotes then migrate to the alimentary tract of the sandfly, where they live extracellularly and multiply by binary fission **(Guevara *et al*., 2001)**. Sandfly saliva selectively inhibits parasite killing by macrophages and nitric oxide production **(Hall et Titus, 1995)**. The major surface glycoconjugate lipophosphoglycan (LPG) constitutes a dense glycocalyx that covers the entire surface of the parasite including the flagellum. Immature organisms, termed procylics, express shorter LPG molecules but mature metacyclics bear the capping at the terminal β-galactose residues with α-arabinose and elongation by increasing the numbers of repeating disachharides unit by two to three folds. This mature metacylic form of the organism is released from the midgut and migrates to the proboscis. Whereas procyclic organisms from log phase cultures are extremely sensitive to complement–mediated lysis through the alternative pathway, metacylic organism activates the classical pathway but is not lysed. When the sandfly next feeds on a mammalian host, it transfers the metacyclic *Leishmania* promastigotes to the host along with the saliva **(Sacks et Kamhawi, 2001)**.Once in the host, the promastigotes are taken up by the macrophages where they rapidly revert to the amastigote form, survive and multiply inside the macrophages, eventually leading to the lysis of the macrophages. The released amastigotes are taken up by additional macrophages and so the cycle continues. Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow **(Pulvertaft et Hoyle, 1960)**.

. *I-4- Diagnosis of Leishmaniasis*

Diagnostic tests include direct parasite detection (by microscopic visualization), use of polymerase chain reaction (PCR) for quantification and determination of the infecting species, serological tests, and antigen-detection tests **(Chappuis *et al*., 2007; Mondal et Bhattarcharya, 2010).** The presence of amastigotes can be microscopically observed in patient lymphnodes, bone marrow, or splenic aspirates and has been used for both diagnosis and evaluation of successful therapy. Quantitative assessment of parasite burden has been improved with the use of PCR to amplify *Leishmania* gene targets such as 18S ribosomal ribonucleic acid (rRNA), kinetoplast (mitochondrial) deoxyribonucleic acid (DNA), ß-tubulin, and cytochrome b **(Foulet *et al*., 2007; Mondal et Bhattarcharya, 2010)**. While direct parasite detection is the most dependable method for disease confirmation, complications from hemorrhage during splenic aspiration (0.1% of individuals) do arise, and examination requires high fidelity, skilled expertise, and established laboratories for sample collection and evaluation. Serological tests monitor specific anti-leishmanial antibodies and include the direct agglutination test (DAT) or fast agglutination screening test (FAST), indirect immunofluorescence assay test (IFAT), and the rK39-based

immune-chromatographic test (ICT) **(Chappuis *et al*., 2007; Mondal et Bhattarcharya, 2010)**. The antigen detection tests represent an alternative to antibody detection. KAtex, a latex agglutination test that detects a low molecular weight glyco-conjugate antigen in the urine of patients, shows high selectivity for parasites but has low sensitivity **(Attar *et al*., 2001; Chappuis *et al*., 2007; Mondal et Bhattarcharya, 2010)**. For HIV/VL coinfected patients, diagnosis by direct visualization and quantification are highly reliable and sensitive, as the parasite burden has been shown to be more than 10-fold higher in HIV-positive (versus HIV-negative) patients **(Jarvis and Lockwood, 2013; ter Horst *et al*., 2009)**.

The diagnosis of PKDL is based on a previous history of VL and results from various clinical and serological tests. As sample collection (via tissue biopsy) is quite invasive and parasite loads tend to be low in papulae, detection of infection is not always straightforward, and misdiagnosis of leprosy is common. The splenic aspirate collection method is less invasive and currently shows the greatest promise for the diagnosis of PKDL in a reliable and noninvasive manner **(Verma *et al*., 2013)**. Overall, the diagnostic tests need to be improved for greater sensitivity and specificity, low cost and convenience, greater throughput, and ease of sample collection and test administration. **(Chappuis *et al*., 2007; Mondal et Bhattarcharya, 2010; Berman, 2003).**

Diagnostic tests for the various forms of CL are similar to those used to identify VL and include parasite collection (cutaneous skin scraping of the center/margin of ulcer) and subsequent microscopic visualization via Giemsa staining, punch biopsy, needle aspirate and parasite cultuvation, serological antibody detection, and PCR quantification **(Markle and Makhoul, 2004; Paniz Mondolfi *et al.*, 2013).**

I-5- The antileishmanial drugs and their therapeutic targets

I-5-1- Current Treatments

The different drugs used for the treatment of VL include pentavalent antimonials, pentamidine, various formulations of amphotericin B, paromomycin, and miltefosine **(Table III)**. As some of these drugs are used for the treatment of CL and MCL, the corresponding regimens for these syndromes (including PKDL) are also briefly described when applicable. Treatment of VL varies from one endemic region to another; the WHO recommended regimens for major VL endemic foci are summarized in **table 3**. In general, as discussed earlier, summarized in **table 3** and described in more detail in the next sections, the current treatment options are inadequate, and new chemical entities are urgently needed (target product profile in **annexe**).

Table 3: Overview of Existing VL Drugs and Recommendation Regimens for the treatment of VL and PKDL in Different Endemic Regions.

Drug	Efficacy	Advantage	Limitations	Cost
Amphotericine B (Fungizone)	> 95%	• Effective against Sb^v resistance	• Deoxycholate form requires hospitalization and can cause myocarditis, hypokalemia, renal toxicity, and reactions in the infusion site	~$100
Liposomal Amphotericine B (AmBisome)	~ 100%	• No documented cases of drug resistance • Effective with low toxicity profile	• High cost • Fever and rigor during infusion • Renal toxicity	$280
Miltefosine	94-97%	• Highly potent, first effective oral treatment for VL and CL	• Highly toxic (liver and kidneys) • Gastrointestinal complications • No safe for pregnant patients (teratogenicity)	~$70
Paramomycine Sulfate	95% (India) 46-85% (Africa)	• Low cost	• Reversible ototoxicity (2%) • Pain in injection site (55%) • Highly hepatotoxicity (6%)	$10
Pentamidine	70-80%	• Potential use in combination therapy at low dosage	• Renal toxicity • Myocarditis • Insulin-dependant diabetes mellitus as irreversible side effect (4-12% patients) • Hypoglycemia and hypotention • Fever	~$100
Pentavalent Antimonials : Sodium Stilbogluconate Meglumine antimoniate	35-95%	• Low cost • Can be used in combination with Amphotericine B in pregnant or elderly patients	• Drug resistance in Bihar, India (>60%) • Heart ventricle complications (prolonged QTc interval, premature beats, tachycardia, fibrillationa and torsades de pointes) and fatal cardiac amythmias • Arthralgia, myalgia, pancreatitis, elevated hepatic enzymes • Highest toxicity in HIV patients	~$50-70

I-5-1-1- Pentavalent Antimonials

Antimony has been used as a therapeutic for several centuries. The first use of antimony in the modern era dates to 1905, when trivalent sodium antimonial tartrate was used to treat trypanosomiasis **(Plimmer et Thomson, 1908).** The use of trivalent antimonials for the treatment of CL was first reported by Vianna and for VL by Di Cristina and Caronia in Sicily and Rogers in India in 1915 **(Vianna, 1912; Di Cristina et Caronia, 1915; Cook, 2006)**. Later, this drug was found to be highly toxic and exhibited side effects such as cough, chest pain, and depression. The key breakthrough in the use of antimony for the treatment of leishmaniasis was achieved in 1925 by Brahmachari, who synthesized the pentavalent antimony compound urea stibamine and discovered it was an effective chemotherapeutic agent against VL **(Brahmachari, 1928)**. This discovery saved millions of lives in India, especially in Assam state, where many villages were depopulated by VL epidemics. Further progress in antimony therapy of VL was achieved through the synthesis of antimony gluconate (Solustibosan) in 1937 and sodium stibogluconate (Pentostam) in 1945 **(Kikuth et Schmidt, 1947; Goodwin, 1995)**.

Currently, there are two formulations of pentavalent antimonials in use: sodium stibogluconate (100 mg antimony (SbV+)/100 mL) and meglumine antimoniate (85 mg antimony/100 mL). Both formulations have poor oral absorption and are given via intramuscular injections or intravenous infusions **(Chulay *et al*., 1988).** Common side effects of pentavalent antimonials include prolonged QTc interval, ventricular premature beats, ventricular tachycardia, ventricular fibrillation, and torsades de pointes **(Lawn *et al*., 2006; Ortega-Carnicer *et al*., 1977)**. Prolongation of the interval (>0.5 s) is often associated with serious or even fatal cardiac arrhythmias **(Chulay *et al*., 1985).** Arthralgia and myalgia, elevated hepatic enzymes and pancreatitis are other common adverse events (**Delgado *et al*., 1999).** Antimonial use causes more toxicity and mortality in HIV-positive patients than in HIV-positive patients treated with miltefosine or AmBisome or HIV-negative patients treated with antimonials **(Ritmeijer *et al*., 2006)**.

In India, sodium stibogluconate was initially administered at low doses of 10 mg/kg/day for 6−10 days **(Wilcocks et Manson, 1972)**. These regimens were successful in curing most of the patients until the late seventies, when several unconfirmed reports of unresponsiveness appeared. In the 1980s, clinical studies were performed to determine the most effective regimen, and these concluded in the recommendation in 1992 to treat VL in India with 20 mg SbV+/kg for 28−30 days **(Herwaldt et Berman, 1992; Thakur *et al*., 1984)**. During the 1990s and 2000s, the clinical efficacy of antimonials in Bihar state (where ∼90% of VL cases in India occur) gradually

declined, and more than 60% of VL cases in this state are now refractory to this treatment, although the drug continues to be effective in surrounding areas (e.g., Uttar Pradesh state) **(Sundar *et al.*, 2000)**. It is not established with certainty what factors drove the emergence of antimony-resistant *L. donovani* in Bihar. According to one hypothesis, resistance to antimonials emerged as the result of large-scale misuse of the drug in Bihar, where in one survey, only 26% of patients were treated according to the WHO guidelines **(Sundar *et al.*, 1994)**. The alternative hypothesis is based on the observation that exposure of *L. don*ovani to low concentrations of arsenic leads to the emergence of parasite resistance to pentavalent antimonials. Starting in the 1970s, there was a large-scale tapping of aquifers in Bihar to provide clean drinking water. The Bihari population was at risk from arsenic exposure due to contamination from naturally occurring trivalent arsenic in the groundwater. Thus, chronic exposure of the Bihar population to arsenic in drinking water could have driven the emergence of antimony-resistant *L. donovani* strains **(Perry *et al.*, 2013)**. Even though pentavalent antimonials continue to be efficacious in other parts of Southeast Asia, the WHO currently recommends alternative drugs (AmBisome infusion) as the first-line therapy options in this region **(WHO, 2010).** As in India, VL in Africa is caused by *L. dono*vani with major disease foci in Sudan, South Sudan, and Ethiopia and a lower number of cases found in Kenya and Uganda. Recommended treatment consists of 20 mg/kg sodium stibogluconate for 30 days **(Anabwani *et al.*, 1983)**. This regimen typically yields >90% cure rates in HIV-negative patients across the East Africa region **(Musa *et al.*, 2012).** However, monotherapy with pentavalent antimony is not considered the first-line treatment in East Africa according to the WHO, which recommends combination treatment with pentavalent antimony and paromomycin **(WHO, 2010)**.

Unlike in India and Africa, VL in South America is caused by *L. infantum* (formerly referred to as *L. chagasi*). There is no evidence of significant resistance to pentavalent antimonials in Brazil, and meglumine antimoniate is the first choice for the treatment of mild and moderate cases of VL **(Brustoloni *et al.*, 2010)**. For severe cases (age less than six months or over 65 years with signs of malnourishment, renal or hepatic insufficiency) and pregnant women, the Brazilian Health Ministry recommends treatment with liposomal amphotericin B (AmBisome) **(Ministerio Da Saude *et al.*, 2011)**. A recent retrospective study focusing on a cohort of children treated with 20 mg/kg meglumine antimoniate per day for 20−40 days reported an efficacy of 96.9% in mild-to-moderate cases and over 60% in severe cases.

VL in Mediterranean countries is also caused by *L. infantum*. During the 1990s, antimonials were the first-line treatment in most countries of this region (France, Greece, Italy, Malta, Spain, Portugal, Albania, Israel, Turkey, Morocco, Algeria, and Tunisia), with cure rates

>95% in immunocompetent patients using regimens of 20 mg SbV+/kg for 20−30 days **(Gradoni *et al*., 2008)**. More recently, pentavalent antimonials have been replaced by AmBisome as the first line of treatment in European countries **(Rosenthal *et al*., 2009)**.

Most countries endemic for VL also have HIV-infected populations, with the highest coinfection rates found in East Africa (up to 25−40% in parts of Ethiopia), followed by Brazil (~5%) and India (2−5%). The use of pentavalent antimonials in HIV-infected patients is no longer recommended by most experts in the field due to their unacceptable toxicity in this patient group and high rates of treatment failure. **(Ritmeijer *et al*., 2006; Ritmeijer *et al*., 2001)**. However, because of their low cost, antimonials at a dose of 20 mg/kg for 28−30 days are still used when alternative treatments are prohibitively expensive. HIV infection has consistently been a predictor of poor outcome of VL treatment (e.g., only a 44% cure rate in HIV-positive versus 92% in HIV-negative patients in one trial in Ethiopia) and associated with high rates of relapse (15−57%) **(Ritmeijer *et al*., 2001)**.

Antimonials have also been used extensively as the primary treatment option for CL and ML, particularly in the New World, where there is a greater risk of mucosal involvement **(Minodier et Parola, 2007)**. Administration is either by intralesion injections (limited to Old World CL infections - up to 5 individual doses separated by 3−7 days) or systemically (20 mg/kg for 20 days for CL and 28−30 days for MCL). Several studies of this drug therapy indicate differences in effectiveness, with 85−90% cure rates in Old World CL and 26%−100% in South America, depending on country and parasite species **(Llanos-Cuentas *et al*., 2008).**

I-5-1-2- Pentamidine

Pentamidine has been in use since the 1940s for the treatment of sleeping sickness **(Lourie et Yorke, 1938)**. The first use for VL treatment was reported in India in 1949 and in Spain in 1950 **(Garcia *et al*., 1950)**. Most regimens are based on intramuscular injection or intravenous infusion of 4 mg/kg pentamidine (isethionate or methanosulfonate) per day for a variable number (up to 30) of days. Safety is a major concern, with insulin-dependent diabetes mellitus being the most feared and irreversible adverse event **(Belehu et Naafs, 1982)**. This complication, while not uniformly reported, occurs in 4−12% of cases. Additional side effects include hypoglycemia, hypotension, fever, myocarditis and renal toxicity **(Sands et al., 1985)**. Pentamidine was used as the second-line therapy for the treatment of antimony-refractory cases of VL in India. However, due to its toxicity and rapidly emerging resistance (frequently to both pentamidine and antimonials), pentamidine use in India was abandoned in the 1990s and replaced with amphotericin B deoxycholate as the recommended treatment **(Sundar, 2001)**. During the early

years of increased pentamidine use in India (1978), 10 injections were sufficient to affect cure in all treated patients. By the early 1990s, 15 or more injections were required to produce cure in only 67−77% of patients **(Jha *et al*., 1991)**. More recently, pentamidine was successfully used in several cases of HIV-positive patients to prevent VL relapse following the initial treatment with an alternative drug **(Patel et Lockwood, 2009)**. Pentamidine is the first option for the treatment of CL caused by *L. guyanensis* and is recommended as the first-line treatment in French Guiana and Suriname, where it is the only available antileishmanial. The typical treatment consists of a single intramuscular injection of 7 mg/kg pentamidine isethionate and can be repeated 48 h later in complicated cases. In one study, these regimens yielded 78.8 and 83.6% cure rates, respectively **(Roussel *et al*., 2006).**

I-5-1-3- Amphotericin B

Amphotericin B is a polyene antibiotic isolated from Streptomyces nodosus in 1955, which was identified because of its antifungal activity **(Gold *et al*., 1956)**. The *in vitro* activity of amphotericin B on *Leishmania* was reported for the first time in 1960, and the first successful treatment of patients with VL was reported in 1963 in Brazil **(Furtado *et al*., 1960; Prata, 1963)**. The drug increases membrane permeability by binding to ergosterol present in the *Leishmania* plasma membrane **(Matsumori *et al*., 2009)**. Amphotericin B is used in complex with deoxycholate or various lipids, and all formulations are administered by intravenous infusion. The deoxycholate form of the drug has many adverse effects, including infusion reactions, nephrotoxicity, hypokalemia, and myocarditis, and requires close monitoring and hospitalization for 4−5 weeks.

Lipid formulations of amphotericin B are efficacious at lower doses and have reduced toxicity, but the high cost complicates the treatment of patients in low-income settings **(Messori *et al*., 2013)**. In India, amphotericin B was traditionally a second-line treatment for VL, but the decreased efficacy of antimonials and pentamidine led to recommendations for use as a first-line treatment starting in the 1990s in Bihar. Amphotericin B deoxycholate has been used with different dosing regimens, with a total dose ranging from 7 to 20 mg/kg, and treatment administered on alternate days or daily for up to 43 days with either constant or incremental dosing. Amphotericin B regimens typically produce high cure rates (close to 100%) for both antimony-sensitive and refractory infections **(Giri et Singh, 1994).** Several lipid formulations of amphotericin B (liposomal-AmBisome, lipid complex-Abelcet, colloidal dispersion-Amphocil, lipid emulsion - Amphomul) have also been tested, all enabling regimens with ∼100% cure rates **(Olson *et al*., 2005)**. Lipid formulations lead to the rapid concentration of the drug in organs such

as the liver and spleen **(Olson *et al.*, 2005).** This greatly reduces adverse effects, including nephrotoxicity, and allows the delivery of large doses of the drug over short periods of time. In an open label study in Bihar in 2010, a single dose of 10 mg/kg of AmBisome produced a 96.3% cure rate **(Sundar *et al.*, 2010)**. The outcome prompted the WHO to recommend this regimen as the first-line treatment for VL in South Asia **(WHO, 2010)**. The efficacy of amphotericin B deoxycholate in East Africa (Uganda) was extensively evaluated from 2003−2004 during an interruption in the supply of antimonial drugs. The regimen consisted of slow infusion of 1 mg/kg amphotericin B on alternate days for 30 days (total dose 15 mg/kg) and produced a 92.4% cure rate **(Mueller *et al.*, 2008)**. Experience with AmBisome treatment in East Africa suggests that higher total doses than in India are required to achieve >90% cure rates. Treatment with 30 mg/kg AmBisome in 6 doses on alternate days in Sudan produced a 92.6% initial cure rate in HIV-negative patients but only 59.5% in the HIV-positive group. AmBisome was even less effective in HIV-positive VL relapses (38.0% initial cure, 55.7% parasitological failure). Of additional interest, a study to determine the optimal single dose of AmBisome (tested doses include 7.5, 10, 12.5, and 15 mg/kg) in HIV-negative patients in East Africa was concluded, and the results are expected to be published soon. In Latin America, there are few data on AmBisome's efficacy. In Brazil, a total dose of 20 mg/kg has been proven to be efficacious **(Freire *et al.*, 1997)**. The Pan American Health Organization guidelines for the treatment of leishmaniasis in the Americas have established liposomal amphotericin B (3−5 mg/kg per day IV for 3−6 days, with a total dose of 20 mg/kg) as one of the firstline therapeutic options. In southern Europe, doses of 3−5 mg/kg per day, up to a total of 20 mg/kg in different regimens, have been demonstrated to be effective in up to 99−100% of patients. Total doses of 15, 18, and 24 mg/kg were tested in Italy, with response rates of 91, 98 and 100%, respectively. In Greece, one study administered a total dose of 20 mg/kg in a short regimen of 2 days, with a cure rate of 98%, versus 90%, when it was administered over 5 days. Because of the large number of published case series, there is an important accumulation of evidence regarding the use of liposomal amphotericin B in pediatric populations in Europe, with high response rates (97% with total doses of 18−24 mg/kg in different regimens). It has been shown that liposomal amphotericin B reduces the average duration of hospitalization when compared with antimonials and that it was effective in cases that did not respond to treatment with antimonials **(Davidson *et al.*, 1994)**. For all of these reasons and despite the absence of randomized clinical trials, liposomal amphotericin B is considered a reference treatment for VL in the Mediterranean countries in both adults and children. Amphotericin B deoxycholate (0.7 mg/kg per day, by infusion, for 25−30 doses) and AmBisome (2−3 mg/kg per day, by infusion, up to 20−40 mg/kg total dose) are also used for the treatment of CL and MCL infections caused by *L.*

braziliensis and other species, including *L. guyanensis*, *L. infantum*, and *L. aethiopica* **(Rapp *et al*., 2003)**. In a study completed by Solomon and colleagues, a dosage of 18 mg/kg total given to patients afflicted with *L. braziliensis* CL resulted in an approximately 85% complete cure in patients within two months **(Solomon *et al*., 2011)**.

I-5-1-4- Paromomycin

Paromomycin is an aminoglycoside broad-spectrum antibiotic that was first isolated in the 1950s from Streptomyces krestomuceticus. Paromomycin inhibits proteosynthesis by binding to 16S rRNA **(Vicens et Westhof, 2001)**. It was shown to be efficacious for the treatment of CL in 1966 and for VL in 1990 in Kenya **(Moskalenko et Pershin, 1966)**. The most common adverse event with paromomycin is injection site pain (55%); however, this typically does not lead to the discontinuation of therapy. A small fraction of patients experience reversible ototoxicity (2%) and a rise in hepatic transaminases (6%) **(Sundar *et al*., 2007)**.

In a phase III study in Bihar in 2003−2004, a paromomycin regimen of 11 mg/kg (15 mg/kg as the sulfate) i.m. for 21 days was shown to be noninferior to amphotericin B (1 mg/kg i.v. alternate days for 30 days), with final cure rates of 94.6 versus 98.8%, respectively **(Sundar *et al*., 2007)**. The cure rate among those previously treated with SbV+ or miltefosine was 98%. The cure rate was 96% in pediatric patients and 95% in females. The main advantage of paromomycin is its affordability: the cost of the treatment is only ∼$10 per patient. A study conducted in 5 centers in Sudan, Kenya, and Ethiopia compared the efficacy of paromomycin as monotherapy at a dose of 15 mg/kg per day for 21 days, antimonials (20 mg/kg per day) as monotherapy for 30 days or the combination of both drugs for 17 days. At 6 months after the end of treatment, paromomycin monotherapy provided only a 63.8% average cure rate, with very low cure rates observed in 2 Sudanese centers (14.3% and 46.7%) **(Hailu *et al*., 2010)**. A follow-up study in East Africa evaluated paromomycin regimens of 15 mg/kg per day for 28 days and 20 mg/kg per day for 21 days with final cure rates of 81% and 80%; however, both regimens were still inferior to the standard treatment (20 mg/kg of sodium stibogluconate for 30 days yielded a 94.1% cure rate) **(Musa *et al*., 2010).** There are no reports on paromomycin use in VL treatment in Latin America and Mediterranean countries. Paromomycin in the form of ointment (15% paromomycin/12% methylbenzethonium chloride) is also used for the local treatment of noncomplicated Old World CL by application to the lesion twice daily for 20 days. Experience with paromomycin ointment for the treatment of New World CL is limited. In one trial, a 20-day treatment twice daily produced 70−90% cure rates for CL caused by *L. mexicana*, *L. panamensis*, and *L. braziliensis* in Ecuador and Guatemala **(Arana *et al*., 2001)**.

More recently, a novel paromomycin ointment was described (15% paromomycin, 0.5% gentamycin) and found to be efficacious for CL treatment caused by *L. major* **(Ben Salah *et al.*, 2013)**.

I-5-1-5- Miltefosine

Miltefosine or hexadecylphosphocholine was originally developed as an anticancer drug. In the 1990s, several laboratories discovered that miltefosine has antileishmanial activity **(Croft *et al.*, 1996),** and in 2002, it was approved in India as the first oral treatment of VL. The most common adverse events include gastrointestinal side effects and occasional hepato- and nephrotoxicity. Another miltefosine limitation is teratogenicity, and women of child-bearing age must take contraceptives for the duration of treatment and for an additional 3 months afterward due to the long half-life of miltefosine (∼1 week). In 2002, a phase III trial in India with a regimen of 50−100 mg/day for 28 days resulted in a 94% cure rate, and miltefosine was selected for the VL elimination program in India, Nepal, and Bangladesh. However, a recent study suggests that miltefosine efficacy is starting to decline, and a study in 2012 yielded a reduced cure rate of 90.3% **(Sundar *et al.*, 2012)**. Miltefosine is also efficacious for the treatment of PKDL cases, and the recommended regimen includes treatment with 50−100 mg/day for 12 weeks **(Sundar *et al.*, 2013)**. The efficacy of miltefosine in East Africa was determined during a trial in Ethiopia in 2006. A regimen of 100 mg/kg per day of miltefosine for 28 days was found to be equivalent to sodium stibogluconate treatment (20 mg/kg per day for 40−60 days) in HIV-negative patients (final cure rate of approximately 94% for patients who could be traced during follow-up) **(Ritmeijer *et al.*, 2006)**. A phase II trial to evaluate the efficacy of miltefosine in Sudan and Kenya is ongoing. Miltefosine is considered to be the first effective oral treatment regimen for CL, with greater accessibility and lower toxicity than antimonials **(Machado *et al.*, 2010)**. Miltefosine at a dose of 2 mg/kg per day for 28 days is effective against CL in Colombia caused by *L. panamensis* (70−90% cure rate) but has only a limited effect against the disease caused by *L. braziliensis* and *L. mexicana* (<60% cure rate). Treatment extension to six months for CL in Brazil originating from *L. braziliensis* infection resulted in a 75% cure rate compared to the 53% cure rate following treatment with antimony, with efficacy shown to be greater in adults than in children **(Machado *et al.*, 2010).** In **Table 3,** the WHO regimens for the treatment of VL and PKDL in various endemic regions are described.

I-5-1-6- Ketoconazole

Azoles are oral antifungal drugs that inhibit fungal ergosterol biosynthesis at the lanosterol demethylase step, resulting in the accumulation of 14 α-methyl sterols. As ***Leishmania*** parasites

rely on ergosterol for their sterol needs and share this biosynthetic pathway with fungi, azoles have been explored for their therapeutic potential against *Leishmania* infections. For CL, the efficacy of compounds varies depending on species **(Rangel *et al.*, 1996)**. Ketoconazole was tested for a month in both adults and children on CL caused by *L. braziliensis* (either 600 mg or 100 mg daily, respectively, for 28 days) and resulted in a 76% cure with mild side effects **(Saenz *et al.*, 1990)**. Similar testing in patients afflicted with CL caused by *L. mexicana* resulted in an 89% cure in another study completed by Navin and colleagues **(Navin *et al.*, 1992)**. Another ergosterol biosynthesis inhibitor, fluconazole (200 mg daily for 6 weeks), was also previously tested in patients with CL originating from *L. major* and resulted in a 59% cure and shorter healing time for patients residing in Saudi Arabia **(Alrajhi *et al.*, 2002)**. In the case of itraconazole, minimal response rates were observed in cases of CL resulting from *L. major* and in MCL originating from *L. braziliensis* **(Momeni *et al.*, 2002)**. Among the several azole drugs tested (fluconazole, itraconazole, ketoconazole), only ketoconazole was found to be consistently efficacious and is now used for the treatment of CL infections caused by *L. mexicana* (600 mg per day for 28 days).

I-5-2- Treatments with Drug Combinations

There are only a limited number of new chemical entities in the drug development pipeline to address the limitations of the current VL treatments. Instead, treatments with combinations of existing drugs have become the main short- to medium-term strategy to combat emerging drug resistance, reduce adverse events, and shorten therapy duration. The earliest attempts to explore this approach occurred in the early 1990s, with a combination of sodium stibogluconate and paromomycin tested in Kenya, Bihar state, and Sudan. A study in Bihar evaluating combinations of various paromomycin and sodium stibogluconate doses found that a combination of 12 mg/kg of paromomycin and 20 mg/kg of sodium stibogluconate (both administered daily) for 20 days yielded an 88% cure rate. Seventeen-day treatment with the combination of sodium stibogluconate (20 mg/kg) and paromomycin (15 mg/kg) in Sudan affected a 97% initial cure rate and was found to be superior to sodium stibogluconate alone (20 mg/kg for 30 days) **(Seaman *et al.*, 1993)**. Similar results were also observed in a subsequent East Africa multicenter trial, and this combination regimen is now the preferred treatment in this region **(WHO, 2010; Musa *et al.*, 2012)**. Another approach to combination treatment relies on the sequential use of 2 different drugs. During recent trials in India, it was established that a single infusion of 5 mg/kg AmBisome followed by either 7 days of 50 mg/kg per day miltefosine or 10 days of 11 mg/kg per day paromomycin both yielded 97.5% cure rates 6 months after the end of treatment. As a part of this

trial, a treatment arm with daily coadministration of miltefosine and paromomycin (50 mg/kg and 11 mg/kg per day, respectively) for 10 days was also evaluated and yielded a 98.7% final cure rate **(Sundar *et al.*, 2012)**. In summary, combination therapies have been established as safe and effective treatment options, and their implementation into primary treatment centers in India and East Africa is ongoing. Combination therapy with antimonials has been used to enhance efficacy for CL **(Minodier et Parola, 2007)**. Allopurinol supplementation led to a 2-fold reduction in the required antimony dosage and resulted in a cure rate of 75−80% in Iranian patients infected with *L. major* and improved treatment outcomes for patients treated with a single agent while infected with *L. tropica* **(Momeni *et al.*, 2002).** To treat *L. braziliensis*, pentavalent antimony (15−20 mg/kg daily) has been used in conjunction with pentoxifylline (400 mg, three times a day) for a month to cure 90% of patients with MCL and lesions resistant to single-agent therapy **(Lessa *et al.*, 2001)**.

I-5-3- Prophylaxis and anti-vector struggle

There is no vaccine or prophylactic drug against leishmaniasis. The only prophylactic method currently used consists to the utilization of mosquitos for a long period of action impregnated with persistent pyréthrinoïdes, which does not leave pass the phlebotomes **(WHO, 2012)**.

I-6- Traditional Medicine: An alternative

The development of resistance mechanisms to antileishmanial available drugs, the toxicity and severe side effects observed, highlights the urgency to look for new natural antileishmanial drugs with new mechanisms of action without secondary side effects. As an alternative, natural products are a potential alternative because they are sources of many secondary metabolites classes with multiples biological activities. According to the WHO in 2000, approximately 80% of the world's population uses traditional medicine for their health problems **(WHO, 2000)**.

In addition, plants constituted the base of all traditional knowledge in the domain of medicine. Today, more than 60% of commercialized drugs are of natural origin. Only 10 to 20% of world flora has been studied to a phytochemical point, and the potential of this natural resource remains very important **(Beniddir, 2012)**.

Plants of the Ebenaceae family are widely distributed around the world. The *Diospyros* genus is one of the most important members of this family. One of the plants belonging to this genus is *D. gracilescens*. It is a forest tree widely distributed in the western and central regions of Cameroon. Furthermore, there is no mention concerning the use of *D. gracilescens* in traditional

medicine in Cameroon. However, related species such as *D. bipindensis*, *D. conocarpa* and *D. malabarica* are widely used by Baka Pygmies for the treatment of malaria, sleeping sickness and respiratory disorders **(Kaushik *et al.*, 2013)**. Globally, *Diospyros* spp. are known, above all, as fishing poisons, especially in Southeast Asia and in the Philippines. They are also widely used as medications in traditional African medicine, mainly against leprosy. The roots are used as purgative in the Central African Republic, against pneumonia in Zimbabwe and schistosomiasis in Malawi **(Gafner *et al.*, 1987; Kerharo and Bouquet, 1949)**. The first chemical study of *D. gracilescens* was reported by **Mbi and Waterman** in **1978**. The results of their studies led to the isolation of few compounds, such as lupeol, betulin, betulinic acid, isodiospyrin (II) and sitosterol. To the best of our knowledge, no other previous study on *D. gracilescens* has been published in the literature. In this thesis, we therefore describe for the first time the isolation of the chemical constituents of *D. gracilescens* guided by their antileishmanial activity.

Another family is Rubiaceae, which is widely distributed in Cameroon. Plants of this family in many previous studies have been investigated and shown to possess many antiparasitic activities. One medicinal plant of this family widely used is *Rothmania hispida*. It is a forest tree widely distributed in the center region of Cameroon. His usage in traditional medicine is very widely known in Cameroon, and the plant is traditionally used for the treatment of many various diseases, such as malaria, filariasis, diarrhea, itching skin diseases, ulcers, and diabetes **(Cardon, 2005)**. To the best of our knowledge, very few chemical and biological studies have been investigated this plant.

In this study, the bio-guided isolation of *in vitro* anti-leishmanial active extracts from *D. gracilescens* (Ebenaceae) and *R. hispida* (Rubiaceae), targeting the extracellular (promastigotes) and intracellular (amastigotes) forms of *Leishmania donovani*, was investigated.

I-7- Generalities on Ebenaceae

Ebenaceae is a medium-sized perennial woody plant family, and it mainly occurs in the tropics and subtropics with a smaller representation in the temperate zone. The family is pantropical in distribution and encompasses seven genera, namely, *Diospyros, Euclea, Maba, Oncothea, Rhaphidanthe, Royena* and *Tetraclis.* Thus, most of the botanists consider that it is composed of the three genera *Diospyros, Euclea and Lassiocarpa*. However, at present, *Maba, Rhaphidanthe, Royena* and *Tetraclis* are included under *Diospyros,* and *Oncotheca* is included as a monotypic family **(Hegnauer, 2012; Duangjai *et al.*, 2006)**. Plants of the Ebenaceae family are widespread in the tropics and subtropics, occasionally in temperate areas. They are trees or erect shrubs, occasionally with spine-tipped branchless. Leaves are alternated, rarely opposite, entire.

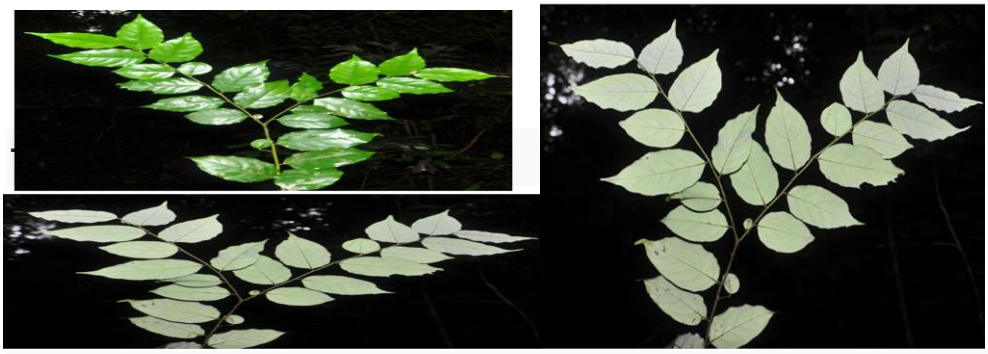
Stipules are absent. Flowers are actinomorphic, usually unisexual, dioeciously, or polygamous and rarely bisexual. Staminate flowers are often in cymes, sometimes in clusters or solitary; pistil rudimentary or absent. Pistillate flowers are often solitary, axillary, imperfect or without stamens. Calyx is 3–7-lobed, persistent and often becoming enlarged in pistillate or bisexual flowers; lobes valvate or overlap in bud. The corolla is 3–7-lobed; lobes convolute, rarely overlapping or valvate. Stamens are hypogynous or at base of corolla, 2–4 × as many as corolla lobes, rarely as many as corolla lobes and alternate with them. Filaments are free or united in pairs. The ovary is superior, with 2–16-locular, 1 or 2 ovules per locule. There are 2–8 Styles, free or basally united. Stigmas are entire or 2-lobed. Fruits are generally fleshy, few- to several-seeded berries. Seeds are usually oblong; endosperm sometimes ruminate; hilum small (**Hegnauer, 2012)**.

i- Diospyros genus

Diospyros is one of the most important genera of the *Ebenaceae* family and is widespread in the tropics and subtropics, occasionally in temperate areas. According to **Hegnaeur (2012)**, the family consists of seven genera, namely, *Diospyros, Euclea, Maba, Oncothea, Rhaphidanthe, Royena* and *Tetraclis.* However, at present, *Maba, Rhaphidanthe, Royena* and *Tetraclis* are included under *Diospyros,* and *Oncotheca* is included as a monotypic family **(Willis, 1973)**. Thus, most of the botanists consider that it is composed of the three genera *Diospyros, Euclea and Lassiocarpa*. The genus *Diospyros* is by far the largest, with more than 500 species, of which 59 species are more distributed in India **(Pearson et Brown, 1932),** mostly in the evergreen forests of Deccan, Assam and Bengal, China, Thailand, Japan, Nigeria, South Africa, Indonesia, Malay Peninsula and the Philippines **(Jain et Yadava 1994, Willis, 1966;**. A few of them also occur in N. India **(The Wealth of India, 1952)**. Terminal buds are absent. Branchlet tips sometimes form a spine. Leaves are alternate, occasionally minutely translucent dotted or with gland pits. Flowers are dioeciously or polygamous. They are staminate flowers in axillary cymes, rarely solitary, usually on the basal part of the current year's branchlets, deciduous soon after anthesis; stamens 4 to numerous, often paired and forming 2 whorls; ovary rudimentary. Pistillate flowers are usually solitary, axillary; staminodes 0–16; stigma often 2-cleft. The calyx is usually 3–5(–7)-lobed, sometimes truncate; corolla urceolate, campanulate, or tubular, 3–5(–7)-lobed, deciduous. Berry fleshy to somewhat leathery, usually with an enlarged persistent calyx. Seeds 1–10 (or more), often laterally compressed. It is a large genus of trees or shrubs, deciduous or evergreen, well known for their medicinal uses since ancient times in many traditional medicinal systems, such as Ayurveda, traditional Chinese medicine and the African folklore **(Mallavadhani *et al*., 1998)**. A

few of them also occur in northern India. Its bark is bitter, astringent and febrifuge. The unripe fruit is a more powerful astringent **(Willis, 1973)**.

i-1- Diospyros grascilisens. (Photograph 1)



- **Synonyms:** Maba.

i-1-1- Botany

D. grascilisens is a persistent tree with subcoriaceous leaves, with lanceolate ellipticals (measuring 110 mm in length and 50 mm in width). It has an obtuse base to cuneiform, an acuminate apex, entire margins, and a glabrescent petiole measuring approximately 5 mm in length. Its flowers are unisexual (dioecia). Its male flowers are grouped by 1-7 axillary cymes and have a calyx with 3 obtuse lobes, a butoliform and yellow corolla that measures 7-8 mm in length, 3 lobes and 6-9 stamens. The female flowers are solitary and axillary, similar to males, with 3-6 staminodes. The fruits are globose berries and measure 26 mm in length and have a diameter of approximately 30 mm (**Hegnauer, 2012).**

i-1-2- Ecology

D. grascilisens is a plant native to Africa (Nigeria, Cameroon, and Gabon). It is also found much more frequently in evergreen forests and rainforests **(Mallavadhani *et al*., 1998)**.

i-1-3- Ethnobotanical Usage

The traditional use of *D. grascilis* is not well known in Cameroon and around the world. However, many plants of the *Diospyros* genus are used in traditional medicine for the treatment of many various diseases. For example, *D. montana*, *D. bipindensis*, *D. conocarpa* and *D. malabarica* are widely used by Baka Pygmies for the treatment of asthma, abdominal pains, dysentry, leprosy, whopping cough, menstrual troubles and as antibiotics, several parts of the plant have been used for a long time **(Watt and Breyer-Brandwijk, 1962; Kokwaro, 1976).**

i-1-4- Previous studies

- The only chemical study of *D. gracilescens* was reported by **Mbi and Waterman** in **1978**. The results of their studies led to the isolation of a few compounds, such as lupeol, betulin, betulinic acid, isodiospyrin (II) and sitosterol.

➢ To the best of our knowledge, no previous biological activities on this plant have been investigated.

I-8- Generalities on Rubiaceae

The Rubiaceae family is considered to be the fourth largest angiosperm and is composed of approximately 600 genera and 13 000 species distributed worldwide **(Robbrecht, 1988).** ArBased on molecular phylogenetic studies, this family is partitioned into three subfamilies: Cinchonoideae, Ixoroideae and Rubioideae **(Bremer *et al.*, 1999; Rova *et al.*, 2002)**. The family is easily recognized but has a problematic and much-discussed intrafamilial phylogeny and classification **(e.g., Verdcourt, 1958; Bremekamp, 1966; Robbrecht and Puff, 1986; Robbrecht, 1986, 1988, 1993; Bremer and Jansen, 1991; Andersson and Persson, 1991; Andersson, 1993; Manen, 1996)**. It is widely distributed, mainly in tropical and subtropical regions but also in cold and temperate regions in Europe and northern Canada **(Judd *et al.*, 2007)**. Most botanists who encounter unidentified Rubiaceae specimens observed that they are difficult to identify or classify. The family is biologically diverse, and one problem with identification is that unique morphological characters are uncommon, whereas most features concerning life forms, flowers, and fruits are homoplastic. The early classification was simple but artificial, with two recognized subfamilies based on ovule number per carpel (e.g., in **Schumann, 1891**). Much progress and deeper biological insight into the family were incorporated into the two classification systems from the middle of this century by **Verdcourt (1958)** and **Bremekamp (1966)**.

i- Rothmania genus

Rothmannia is a genus of flowering plants in the family Rubiaceae. Around 11 species of *Rothmania* are present in Cameroon. It was described in 1776 and is named for Göran Rothman (1739–1778) by Thunberg – both were pupils of Linnaeus. Although Rubiaceae flowers are generally organized in many-flowered inflorescences, solitary flowers are also found in this genus. The reduction in the number of flowers per inflorescence is often inversely proportionate to the size of the flowers, which explains the large solitary flowers of some *Rothmannia.* They are shrubs or small trees, with stipules triangular and acuminate. They leaves are opposite, occasionally in threes, sometimes with domatia. *Flowers* are large and *terminal*. The calyx tube is gradually funnel-shaped or cylindrical. The corolla funnel is shaped or campanulate with 5 lobes. They are ovaries with 1 locula and 2 parietal placentas. Fruits are spherical or ellipsoid. Seeds are very numerous, fused into a pulpy solid mass ***(Robbrecht, 1986).***

i-1- Rothmania hispida (photograph 2)

- Scientific Name: ***Rothmannia hispida* (K Schum.) Fagerlind**
- Common names:

➢ **Ivory Coast**: *ABE* tivi (Banco; Aub.) *AKYE* bakapri (Aub.) *ANYI* bakablé (Aub.)

➢ **Ghana**: *AKAN-ASANTE* tukubo (CV; FRI) *ANYI* bakable (FRI) bakalile (FRI)

➢ **Nigeria**: *BOKYI*étông (KO&S) *EDO* asun (auctt.) asun-leghere (JMD) asun-nékhūí (JMD; Lowe) *EFIK*, "okukin", "obong", "asun", "asogbodu", "uriohia" and "owuruokumuo"

i-1-1- Botany

Rothmannia hispida (K. Schum) Fagerl (Syn. *Randia hispida*) is a shrub or small tree of approximately 10 meters tall. The fruits are 6-11 cm long and are cylindrical. The wood, when cut and exposed to air, takes on a blue color. The leaves are silky and more hairy in appearance, with purple marking on its white corolla tube than the leaves of other species **(Lewis and Elvis-Lewis, 2003)**. In Nigeria, a dye and an ink-like extract called "katambiri" is made from finely crushed seeds.

i-1-2- Ecology

Rothmannia comprises approximately 40 species distributed in tropical Africa, tropical Asia and Madagascar. In tropical Africa, approximately 18 species are present. It is of the forest understory or in secondary jungle occurring in Guinea, DR Congo, West Cameroon, and in Zaïre **(Delprete, 1999).**

i-1-3- Ethnobotanical usage

R. hispida is used in West African traditional medicine for the treatment of various elements, such as fever, dysentery, skin infections, abdominal pain and diabetes mellitus (leaf-sap, fruit-juice). Their medicines are used for cutaneous, subcutaneous parasitic infection (fruit). Their products are used as dyes, stains, inks, tattoos and mordants. The plant has been used to treat

many ailments, including diabetes mellitus **(Antai *et al*, 1995; 2005)** and skin infections **(Etukudo, 2003)**.

In tropical Africa and Nigeria, leaf sap and fruit juice are commonly used to make black designs on the body and to blacken tattoos; mixed with palm oil, they are applied on the skin against fungal infections. They are also used in coloring fibers of vegetable origin, e.g., local mats. The fruits have traditionally been used for years for dyeing mats and for drawing tribal marks on the body of fattened women **(Aubréville, Delprete, 1999)**.

The leaves of *R. hispida* are used as enema against kidney pain and diarrhea and for the treatment of diabetes mellitus. Drinking of leaf juice is used to relieve pain during labor and child birth. The stems of these plants are used in making shafts of long-handled chisels in Sierra Leone, which are used in harvesting a bunch of oil palm. The stems are also used in making spear handles and as chewing sticks in Ghana **(Cardon, 2005)**. It is also used for the treatment of fever, filariasis, dysentery, itching, skin diseases, ulcers, and as an emetic **(Cardon, 2005; Lewis et Elvin, 1977)**.

i-1-4- Previous studies

Some previous studies have been investigated *R. hispida*.

- **Antai *et al.* (1995)** studied the acute effects of *R. hispida* on alloxan-induced diabetes in rats.
- **Antai *et al.* (2010)** showed that 100 mg/kg body weight of the *R. hispida* leaves extract is equally as potent as 1 unit of protamine-zinc insuslin and act as an antidiabetic agent.
- **Udia *et al*. (2013)** evaluated the phytochemistry and elemental compositions of extracts from the leaves of *R. longiflora* and *R. hispida* and showed that they contain protein, fat, ash, crude fiber carbohydrate and contain Elemental composition such as sodium (Na), calcium (Ca), magnesium (Mg), manganese (Mn). Their results obtained unraveled the pharmacological basis of the therapeutic applications of *Rothmannia hispida* in traditional medical practice and as potential sources of useful drugs.
- **Pius *et al*. (2016)** studied the insulin and alpha amylase levels in alloxan-induced diabetic rats and the effect of *Rothmannia hispida* (K. Schum) Fagerl leaf extract.
- **Womkam *et al*. (2020)** demonstrated the *in vitro* antilesihmanial activity of the leaf extract of *Rothmannia hispida* against promastigotes of *L. donovani*.

CHAPTER II

MATERIALS AND METHODS

II- MATERIALS AND METHODS

II-1- Material

II-1-1- Plant Material

The different parts of *D. grascilisens* and *R. hispida* were respectively collected on March 29th, 2017, at Eloumdem Mountain (**GPS coordinates**: Latitude 3°49'00″N, Longitude 11°25′60″E), and on June 20th, 2017, at Nkol-Afamba (**GPS coordinates**: Latitude 3°51′32″N, Longitude 11°39′53″ E) two localities in the Centre Region of Cameroon. The identity of the two plants was confirmed by Mr. Victor Nana, a botanist at the National Herbarium of Cameroon, by comparison with a voucher specimen under the reference numbers, N°. 2016/SRFK for *D. grascilisens* and 46515 HNC for *R. hispida*. The different organs were the roots, trunk, stem bark and leaves for *D. grascilisens* and the stem for *R. hispida*.

II-1-2- Strain

The *in vitro* antileishmanial activities were performed on both promastigotes and intracellular amastigotes of the *L. donovani* 1S (MHOM/SD/62/1S) strain. The strain was graciously offered by BEI Resources and was maintained in continuous culture in the Laboratory for Phytobiochemistry and Medicinal Plant Studies, Antimicrobial and Biocontrol Agents Unit, at the Department of Biochemistry of the Faculty of Science of the University of Yaoundé I in Cameroon. The evaluation of the inhibitory potential of extracts, fractions and isolated compounds on parasite growth was performed in the same laboratory.

II-1-3- Cell lines

The RAW 264.7 murine macrophage Cells were used for cytotoxicity and *ex vivo* antileishmanial assays against intracellular amastigotes of the *L. donovani* parasite. The cells were kindly provided by **Dr. TCHOKOUAHA YAMTHE Lauve**, under the approval of Professor **APPIAH-OPONG** of the Clinical Pathology Department from the Noguchi Memorial Institute for Medical Research at the University of Ghana. They were maintained in continuous culture at the Laboratory for Phytobiochemistry and Medicinal Plant Studies, Antimicrobial and Biocontrol Agents Unit, at the Department of Biochemistry of the faculty of sciences of the University of Yaoundé I in Cameroon.

II-1-4- Animals

The evaluation of the acute toxicity was performed on female mice, having six to eight weeks, weighing 100-130 g. The mices were maintained at ambient temperature (25±1°C) in the animal house near to the laboratory of pharmacology and toxicology of the University of Yaoundé

I. They received complete food and water daily, and they were acclimatised for at least 14 days before the experiment.

II-2- Methods

II-2-1- Collection of plant material and preparation of the crude extracts

The collected plant materials were harvested, dried under shelter at room temperature for 1 or 2 weeks and then ground to obtain the powders. The tinctures were prepared by maceration of 4000 g of each powder in 70% aqueous ethanol (15L, 48h x 3) for *D. grascilisens* and 200.6 g of the powder with CH_2Cl_2-MeOH (1/1; v/v, 48 h x 3) for *R. hispida*. The mixture was agitated two times per day. The resulting macerates were filtered using Whatman filter paper No. 2, and the filtrates concentrated on a Büchi rotary evaporator under reduced pressure at 45–55°C and further lyophilized to obtain the crude extracts. The yield of extraction of each extract was calculated according to the following formula:

$$\text{Yield of extraction (\%)} = \frac{mass\ of\ extract\ obtained\ (g)}{mass\ of\ plant\ material\ (g)} \times 100$$

Each crude extract was dried and stored in the bottles at 4°C until it was used for the biological assays.

II-2-2- Phytochemical investigation

II-2-2-1- Phytochemical study of D. grascilisens

i) UPLC–DAD–ESI-MS analysis of extracts

i-1) Sample preparation

Each extract was dissolved in High Performance Liquid Chromatography (HPLC) grade methanol at a concentration of 0.5 mg/mL and then filtered through a syringe-filter membrane. Each aliquot obtained (5 µL) was injected into the UPLC–DAD-HRESI/MS Dionex Ultimate 3000 HPLC (Germany) apparatus used to perform the analyses.

i-2) HPLC–MS conditions

High-resolution mass spectra were obtained with an OTOF Spectrometer (Bruker, Germany) equipped with an HRESI source and a UV–vis absorbance detector. The spectrometer was operated in positive mode (mass range: 100-1500, with a scan rate of 1.00 Hz) with automatic gain control to provide high-accuracy mass measurements with 2 ppm deviation using Na Formate as calibrant. Mass spectra were simultaneously acquired using electrospray ionization (ESI) in positive ionization mode. The following parameters were used for experiments: spray voltage of 4.5 kV and capillary temperature of 200°C. Nitrogen was used as the sheath gas (10 l/min). The

spectrometer was attached to an Ultimate 3000 (Thermo Fisher, USA) HPLC system consisting of an LC pump. UV traces were measured at 215, 218, 254, 280 and 330 nm, and UV spectra—diode array detector— (DAD) was recorded between 190 and 600 nm, auto sampler (injection volume 5 µl) and column oven (35°C). The separations were performed using a Synergi MAX-RP 100A (50x2 mm, 2.5 µ particle size) with a H_2O (+0.1% HCOOH) (A)/acetonitrile (+0.1% HCOOH) (B) gradient (flow rate 500 µL/min). Samples were analyzed using a gradient program as follows: 95% A isocratic for 1.5 min, linear gradient to 100% B over 6 min, after 100% B isocratic for 2 min, the system returned to its initial condition (90% A) within 1 min and was equilibrated for 1 min.

i-3) Identification of peaks

Identification of all constituents was performed by UPLC–DAD-HRESI/MS analysis and by comparing the UV, MS spectra and MS/MS fragmentation of the selected peaks in the sample chromatogram with those of data reported in the SciFinder database.

ii) Liquid–Liquid partition of the most active crude extract

The crude extract of the trunk that showed the best leishmanicidal activity was fractionated by liquid–liquid partition according to the procedure described by **Xie *et al*. (2011)**. Briefly, 4000 g of each powder were macerate in aqueous ethanol 70% to obtain 140 g of the extract. Then each extract was suspended in water and then successively extracted with n-hexane, dichloromethane, ethyl acetate and n-butanol. Each fraction was evaporated under reduced pressure at 45–55°C, and then the aqueous fraction was lyophilized. Five residues were obtained and named fraction A from n-hexane [6.5 g, 4.6% yield], fraction B from dichloromethane [20.0 g, 14.3% yield], fraction C from ethyl acetate [23.4 g, 16.7% yield], fraction D from n-butanol [31.3 g, 22.35% yield] and fraction E for the remaining aqueous residue (45.5 g, 32.50% yield). Each of the afforded fractions was submitted to antileishmanial screening, and promising fractions (fractions A, B and D) with IC_{50} values below 2 µg/mL were selected and submitted for chromatographic separation.

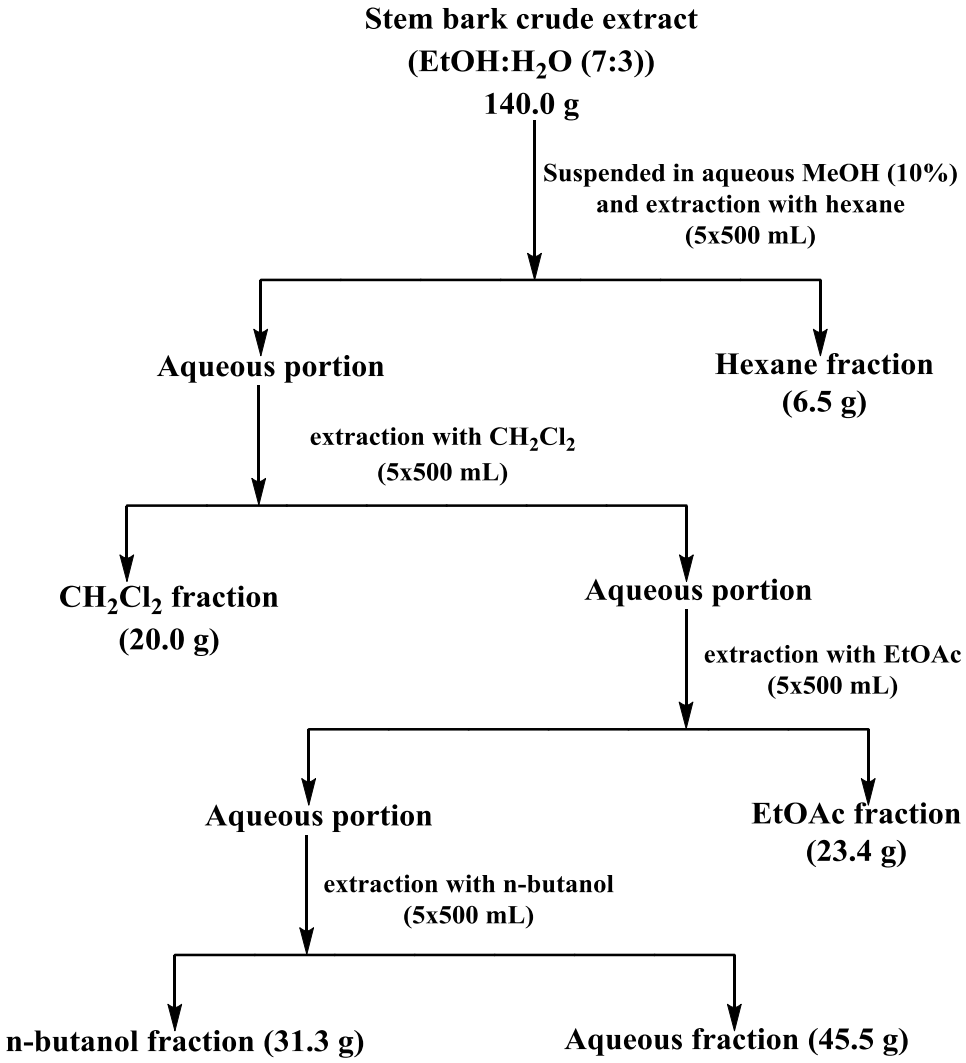


Figure 11: Fractionation procedure of the crude extract from the trunk of *D. gracilescens*.

iii) Chromatography of the active fractions

iii-1) Chromatography of fraction A

A portion of the *n*-hexane fraction that showed the most potent activity was subjected to silica gel column chromatography eluting with an isocratic system of n-hexane/ethyl acetate (95/5). Three hundred subfractions of ca. 100 mL each were collected and combined into 10 subfractions A1-A10 based on their TLC profiles. Subfractions A1 to A6 consisted of a mixture of fatty acids and were not investigated further. Lupeol (**1**) (11 mg; **Adzu *et al.*, 2015**) was filtered from subfractions A7-8 (n-hexane/EtOAc (95/5), 1.5 g) and purified by recrystallization in acetone/methanol/water (6/3/1). In subfractions A9 (n-hexane/EtOAc (90/10), 2.2 g) and A10 (n-hexane/EtOAc (80/20), 22 mg), a mixture of β-sitosterol **(3/4)** (**4**) and stigmasterol **(1/4)** (**5**) (14.8 mg) was filtered **(Akak *et al.*, 2010)**.

iii-2) Chromatography of fraction B

The dichloromethane fraction, which showed good antileishmanial activity, was subjected to silica gel column chromatography eluting with a gradient of ethyl acetate in n-hexane. Ninety subfractions of approximately 250 mL each were collected and combined into 4 major subfractions B1—B4 according to their TLC profiles. Subfraction B1 (n-hexane/EtOAc (95/5), 8.1 g) was found to contain mainly fatty substances and traces of hexane soluble compounds. Subfraction B2 (n-hexane/EtOAc (90/10), 2.1 g) was subjected to repeated silica gel column chromatography using a mixture of n-hexane:ethyl acetate in gradient mode as the eluent to afford a mixture of β-sitosterol **(3/4)** (**4**) and stigmasterol **(1/4)** (**5**) (17.6 mg; **Akak *et al*., 2010**). Subfraction B4 (n-hexane/EtOAc (65/35), 4.5 g) was fractionated on silica gel column chromatography with an isocratic solvent system of n-hexane/ethyl acetate (30/70) to afford a mixture of compounds **2** and **3,** which were further purified using Sephadex LH-20 column chromatography with a mixture of CH_2Cl_2/MeOH (20/80) as eluent to afford pure betulin (**2**) (2.0 mg; **Zhong *et al*., 1984**) and betulinic acid (**3**) (27 mg; **Kim *et al*., 2016**). Subfraction B3 was not further studied due to its limited quantity.

iii-3) Chromatography of fraction D

The n-butanol fraction, which showed good antileishmanial activity, was subjected to silica-gel column chromatography eluting with a mixture of n-hexane/acetone/methanol in gradient mode. Twenty subfractions of approximately 400 mL each were collected and combined into 5 major subfractions D1—D5 based on their TLC profiles. The β-sitosterol glucoside (**6**) (185 mg; **Kaushik *et al*., 2012**) precipitated in subfraction D1 (n-hexane/acetone 30/70). Compound 1-deoxyinositol (7) (30 mg; **Merchant *et al*., 2007**) precipitated in subfraction D5 (n-hexane/acetone/methanol 3/6/1) and was filtered before being purified by recrystallization in acetone/water (90/10). Data on subfractions D2-3 are not presented in this thesis because they are under further scrutiny.

II-2-2-2- Phytochemical study of R. hipsida stem

i) Qualitative identification of compounds using UPLC–DAD–(HR) ESI-MS

UPLC-DAD-MS analysis was carried out to identify the compounds present in the crude extract of *R. hispida* stem. This identification was performed according to the protocol described by **Wouamba *et al*. (2020b)**. Briefly, the crude extract was dissolved in HPLC grade methanol at a concentration of 5 mg/ml and then filtrated through a syringe-filter membrane. Aliquots of 5.00 µl were injected into the UPLC–DAD/MS Dionex Ultimate 3000 HPLC (Germany), used to

performe the analyses. High-resolution mass spectra were obtained with a spectrometer (QTOF Bruker, Germany) equipped with an HRESI source. The spectrometer operates in positive mode (mass range: 100-1500, with a scan rate of 1.00 Hz) with automatic gain control to provide high-accuracy mass measurements within 0.40 ppm deviation using Na Formate as calibrant. The following parameters were used for experiments: spray voltage of 4.5 kV and capillary temperature of 200°C. Nitrogen was used as the sheath gas (10 L/min). The spectrometer was attached to an Ultimate 3000 (Thermo Fisher, Germany) UHPLC system consisting of an LC pump, diode array detector (DAD) (λ: 190-600 nm), autosampler (injection volume 10 µl) and column oven (40°C). The separations were performed using a Synergi MAX-RP 100A C-18 (50x2 mm, 2.5 µm particle size) with a H_2O (+0.1% HCOOH) (A)/acetonitrile (+0.1% HCOOH) (B) gradient (flow rate 500 µL/min, injection volume 5 µl). Samples were analyzed using a gradient program as follows: 95% A isocratic for 1.5 min, linear gradient to 100% B over 6 min, after 100% B isocratic for 2 min, the system returned to its initial condition (90% A) within 1 min and was equilibrated for 1 min.

ii) Fractionation by silica gel column chromatography of the stem crude extract and compound isolation

Several compounds of interest and isomers were identified by UPLC-DAD-MS analysis. It was therefore important to isolate them according to a bioguided procedure with the aim of characterizing the active ingredients responsible for the leishmanicidal activity of the crude extract. Therefore, the stem crude extract was subjected to fractionation using silica gel column chromatography. Briefly, 200.6 g of the powder was macerated in CH_2Cl_2-MeOH (1/1; v/v) to yield 50.5 g of the extract. Then, each extract was fractionated using n-hexane, ethyl acetate and *n*-butanol (each 300 mL×5) to afford three fractions, namely, F_1 (9.6 g), F_2 (4.1 g) and F_3 (13.9 g), respectively.

A portion of the hexane fraction (F1, 8.5 g) that showed the most potent activity was subjected to silica gel column chromatography and eluted with gradients of n-hexane, ethyl acetate and ethyl acetate-methanol to afford 3 subfractions (F_1A-F_1C). The mixture of stigmasterol **(1/4)** (**5**) (8.2 mg; **Habib *et al*., 2007**) and β-sitosterol **(3/4)** (**4**) (12.1 mg; **Moreira *et al*., 2017**) precipitated as white powders in subfraction F_1A (2.6 g; n-hexane-EtOAc, 100:0, 85:15, v/v). Repeated column chromatography of F_1B (4.4 g; *n*-hexane/EtOAc, 80:20—40:60, v/v) on silica gel with *n*-hexane-EtOAc (98:2 to 0:100) gave docosanoic acid (**12**) (7.1 mg; **Martins *et al*., 2013**), lichexanthone (**13**) (6.3 mg; **El-Seedi *et al*., 1994**), the mixture uvaol (**10**) and erythrodiol (**11**) (27.3 mg; **Mahato *et* Kundu, 1994**), ursolic acid (**8**) (48.6 mg; **Dumaro *et al*., 2017**) and

oleanolic acid (**9**) (7.1 mg; **Martins *et al.*, 2013**). Fraction F_2 (3.2 g) was subjected to silica gel column chromatography with *n*-hexane-EtOAc (85:15 to 0:100) and EtOAc-MeOH (100:0 to 80:20) to afford oleanolic acid (**9**) (3.0 mg; **Martins *et al.*, 2013**), ursolic acid (**8**) (8.3 mg; **Dumaro *et al.*, 2017**) and sitosterol 3-*O*-*β*-D- glycoside (**6**) (6.7 mg, **Koagne *et al.*, 2017**).

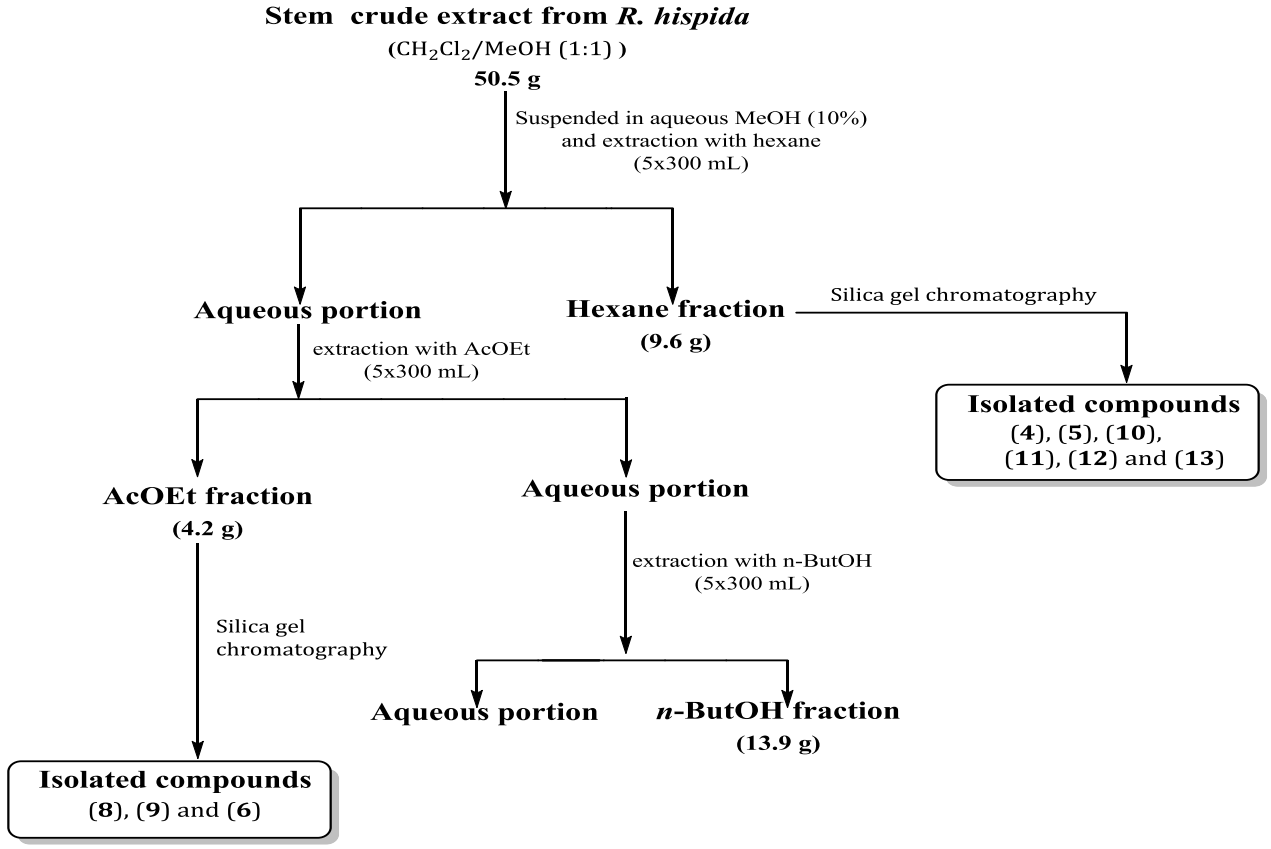


Figure 12: Protocol of extraction, isolation and purification of stems of *R. hispida*.

II-2-3- Screening of extracts for biological activity

II-2-3-1- Preparation of Completed Culture Medium (M-199 10%)

The M-199 10% was prepared following the manufacturer's instructions (annex section).

II-2-3-2- Preparation of stock solutions

Stock solutions were prepared at initial concentration of 100 mg/ml for extracts and fractions and 10 mg/ml for compounds in 100% DMSO. The solutions were sterilized by filtration using a 0.22 µm filter membrane and conserved at 4°C for the biological assays.

II-2-3-3- Preparation of intermediate solutions

With the aim to have a final concentration of DMSO less than 1%, the concentration of DMSO non-toxic for parasitic and cell growth, intermediate plates were prepared at initial concentrations of 1000 µg/ml for the extracts and fractions and 500 µg/ml for the compounds. A 5

serial-fold dilution was performed to obtain the other concentrations and the plates were conserved at -80°C for the *in vitro* assays.

II-2-3-4- Evaluation of the "in vitro" anti-leishmanial activities

The evaluation of *in vitro* anti-leishmanial activities was carried out at the Laboratory for Phytobiochemistry and Medicinal Plant Studies, Antimicrobial and Biocontrol Agents Unit of the Department of Biochemistry at the University of Yaoundé I in Cameroon. The study was investigated on both promastigotes and intracellular amastigotes forms of *L. donovani* by the rezazurin colorimetric method. All the assays were investigated under the Biohazard cabinet Level II in the strict conditions of sterility.

II-2-3-4-1- Inhibitory assay against L. donovani promastigotes:

➢ *In vitro parasite culture*

The cryopreserved promastigotes form of *Leishmania donovani* (1S (MHOM/SD/62/1S)) was obtained from Bei Resources (https://www.beiresources.org/). The *L. donovani* promastigotes were routinely cultured at the Antimicrobial and Biocontrol Agents Unit of the University of Yaoundé I, in Medium 199 supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) (Sigma), 100 UI/mL penicillin and 100 µg/mL streptomycin. The culture was maintained in 75 and 25 Cm^2 cell culture flasks at 26°C and subcultured every 72 hours. When the culture reached or was near the peak density, approximately 0.1 to 0.2 mL of promastigotes from the first culture were transferred into a new flask containing 5 to 10 mL fresh M199 10%. The mixture was homogenized, incubated at 26°C and renewed every 72 hours. To verify the growth of parasites and any contamination, parasites were observed daily under an inverted microscope **(Khanjani *et al.*, 2015).**

The assays were performed on promastigotes in the exponential growth phase. The parasitic load was determined by counting on the Neubauer cells for the anti-leishmanial activity. The final parasitic load for the assays was 4×10^5 cells/ml.

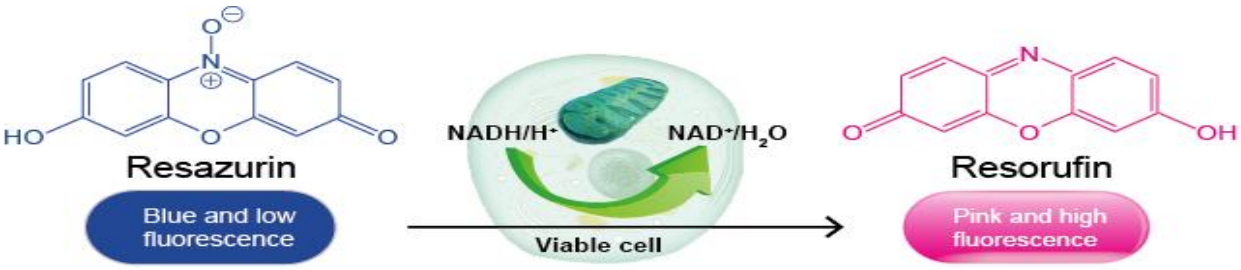
i) Antileishmanial activity against promastigotes

The antileishmanial assays for crude extracts, derived fractions and compounds against *L. donovani* promastigotes was evaluated using the resazurin colorimetric assay as previously described by **Siqueira-Neto *et al.* (2010)**.

✓ *Principle of the method*

The method used is based on the reduction of rezazurin (blue color) to rezorufin (pink color) by mitochondrial dehydrogenases present in *Leishmania* parasites. The complex formed absorbing

at 570 nm by direct measurement of metabolic activity in viable parasites. The quantity of rezorufin produced is directly proportional to the quantity of viable parasites in the medium.



The promastigote forms of *L. donovani* were taken in the exponential growth phase by centrifugation at 2500 rotations per minute (RPM) for 10 minutes. The number of parasites was determined by counting using a Neubauer Cell.

The antileishmanial assay was performed in 96-well plates. Briefly, promastigotes from a logarithmic phase culture (4×10^5 cells/mL; 90 µL) were seeded in 96-well microtiter plates and were treated with 10 µL of inhibitors at different triplicate concentrations ranging from 100, 20, 4, 0.8 and 0.16 µg/mL for extracts and fractions, and 50, 10, 2, 0.4 and 0.08 µg/mL for compounds for the final volume per well of 100 µL. The plates were incubated for 28 hours at 26°C, followed by the addition of 10 µL of resazurin concentrated at 1 mg/mL. The sterility and negative control were respectively 10% M-199 without parasites and complete M-199 medium with parasites without extract. A positive control was amphotericin B and prepared at concentrations of 10, 2, 0.4, 0.08 and 0.016 µg/mL. After an additional incubation for 44 hours, plates were then read on a Magelan Infinite M200 fluorescence multiwell plate reader (Tecan) at excitation and emission wavelengths of 530 and 590 nm respectively.

✓ ***Expression of Results***

For each sample, the percentage of inhibition was calculated on Microsoft Excel Software using the formula:

I (%) = 100 x (Absorbance of untreated cells - Absorbance of treated cells)/Absorbance of untreated cells.

Finally, dose–response curves were constructed to determine the 50% inhibitory concentration (IC_{50}) using GraphPad Prism version 5.0 software.

II-2-3-4-2-Inhibitory assay against the intracellular form of L. donovani

i) In vitro RAW 264.7 Cells Culture

RAW 264.7 murine macrophage Cells were cultured in 25 cm^2 cell culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 13.5 g/L DMEM, 0.2% sodium

bicarbonate (w/v), 2 g/L $NaHCO_3$ and 50 µg/mL gentamycin supplemented with 10% FBS, 100 UI/mL penicillin and 100 µg/mL streptomycin and incubated at 37°C for 72 hours under a 5% CO_2 atmosphere. They were routinely maintained in continuous culture, and the medium was renewed every 72 hours. When confluent, the medium was changed, and the cells were subcultured at $1x10^5$ cells/ml. The cytotoxicity and antileishmanial assays against intracellular amastigotes were performed on macrophages in the exponential growth phase.

The adherent RAW 264.7 cells were detached by using Trypsin/EDTA 0.4%. The monolayer of macrophages was recovered with 1 or 2 ml of trypsin/EDTA 0.4%, and the T-flasks were lightly agitated and incubated for 5 minutes at 37°C under a 5% CO_2 atmosphere. After incubation, 10 or 20 ml of DMEM 10% was introduced into the flask to stop the action of trypsin. The cells were then centrifuged at 1800 RPM for 5 minutes. After the elimination of the supernatant, the pellet was resuspended in fresh medium.

Cell counting was performed using an inverse microscope with Neubauer cells, and the viability was measured using trypan blue solution.

ii) Transformation of procyclic promastigotes to metacyclic promastigotes (Metacyclogenesis)

To allow the infection of macrophages by metacyclic promastigotes, matacyclogenesis was induced following the recommendations of **Jain *et al*. (2012)**. For this, 3 ml of fresh solution containing previously procyclic promastigotes of *L. donovani* was introduced into a new T-flask containing 12 ml of fresh 10% M-199 for a final volume of 15 ml. Then, the T-flasks were incubated at 26°C for 5 to 6 days. After incubation, the metacyclic forms obtained were used for the infection of macrophages.

iii) Infection of macrophages

For the infection, macrophages were seeded in plates at 4×10^4 cells/ml and incubated at 37°C, 95% oxygen and 5% CO_2 for 6 hours to allow the adhesion of macrophages on the plates. After incubation, the medium was discarded, and the wells were washed four times with sterile PBS to remove non-adherent cells. Then, 100 µl of metacyclic promastigotes (at $4x10^5$ parasites/ml) prepared in freshly M-199 10% were added to each well to obtain a macrophage-metacyclic promastigote ratio of 1:10. Finally, the plates were incubated for 24 hours at 37°C under 5% CO_2 to allow the infection of macrophages.

iv) Inhibitory assay against intracellular amastigotes:

The effect of the most active extract/fraction/compound against the intracellular amastigotes form of *L. donovani* was evaluated by the method described by **Jain *et al*. (2012)** with

some modifications. Briefly, the Raw 264.7 Cells ($4x10^4$ cells/ml) were seeded in 96-well plates and incubated for 6 hours at 37°C under 5% CO_2 for adhesion of the cells. Afterwards, the non-adherent cells were washed with sterile PBS, and the adherent Raw 264.7 cells were infected with metacyclic promastigotes (4×10^5 cells) at an infection ratio of 1:10 macrophage:parasite and incubated for 24 hours at 37°C under 5% CO_2 to allow infection of macrophages by metacyclic promastigotes. Thereafter, the overlying medium was removed, and the monolayer cells with internalized amastigotes were carefully washed four times with PBS to remove free parasites. Freshly prepared M199 medium containing 10% FBS and the extracts/fractions/compounds were added in triplicate to the infected cells at serially diluted concentrations and incubated for 48 hours at 37°C under 5% CO_2. After incubation, sodium dodecyl sulfate (SDS) at 0.05% was added to each well for 30 seconds for the lysis followed by the addition of the M199 10% FBS as a macrophage lysis stopper. Finally, Resazurin reagent (250 µg/mL) was thereafter added to each well, and the plates were incubated for 24 hours followed by fluorescence recording at λexcitation = 530 nm and λemission = 590 nm using a Tecan Infinite M200 microplate reader (Tecan).

- ✓ ***Expression of Results***

The inhibition percentages were calculated using Microsoft Excel Software, and the median inhibitory concentration (IC_{50}) was obtained from dose–response curves using GraphPad Prism 5.0. Software.

II-2-3-4-3- Invasion assay of the most active compounds

To determine whether our most active compounds are able to prevent the infection of macrophages by metacyclic promastigotes of *L. donovani*, we performed an invasion test. Briefly, the Raw 264.7 cells (4×10^4 cells/ml) were seeded in 96-well plates and incubated for 6 hours at 37°C under 5% CO_2 for adhesion of the cells. Afterwards, the non-adherent cells were washed with sterile PBS, and the active compounds were added. Thereafter, the adherent Raw 264.7 cells were infected with metacyclic promastigotes (4×10^5 cells) at an infection ratio of 1:10 macrophage:parasites and incubated for 24 hours at 37°C under 5% CO_2 to determine whether our extracts can prevent the infection of macrophages by metacyclic promastigotes. Thereafter, the overlying medium was removed, and the monolayer cells with internalized amastigotes were carefully washed four times with PBS to remove free parasites. After incubation, 0.05% sodium dodecyl sulfate (SDS) was added to each well for 30 seconds for the lysis followed by the addition of M199 with 10% FBS as a macrophage lysis stopper. Then, Resazurin reagent (250 µg/mL) was thereafter added to each well, and the plates were incubated for 24 hours followed by fluorescence

recording at λexcitation = 530 nm and λemission = 590 nm using a Tecan Infinite M200 microplate reader (Tecan).

- ✓ ***Expression of Results***

The Inhibition percentages were calculated using Microsoft Excel Software, and the median inhibitory concentration (IC_{50}) was obtained from dose–response curves using GraphPad Prism 5.0. Software.

II-2-3-5- Cytotoxicity assay

The cytotoxicity profile of extracts, fractions and isolated compounds was assessed using the Alamar blue assay against Raw 264.7 cells duly cultivated in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 13.5 g/L DMEM, 10% fetal bovine serum, 0.2% sodium bicarbonate (w/v), 100 IU/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL gentamycin as described by **Bowling et *al.* (2012)**. Globally, macrophages were seeded into 96-well cell-culture flat-bottomed plates at a density of 1 x 10^4 cells in a final volume of 100 µL of complete medium/well and incubated for 18 hours at 37°C and 5% CO_2 (ICO 105 memmert incubator; 70% RH) to allow cell adhesion. Then, 10 µl of each serially diluted test sample solution was added, and assay plates were then incubated for 48 hours under the same experimental conditions. Growth control (0.1% DMSO-100% growth) and positive control wells (podophyllotoxin at 20 µM) were included in the experimental plates. Cell proliferation was checked by adding 10 µL of a stock solution of resazurin (0.15 mg/mL in sterile PBS) to each well followed by incubation of the plates for 4 hours. Fluorescence was then read on a Tecan Infinite M200 fluorescence multiwell plate reader (Tecan) at an excitation/emission of 530/590 nm.

- ✓ ***Expression of Results***

The percentage of inhibition for each sample was calculated using Microsoft Excel Software. The 50% cytotoxic concentrations (CC_{50}) were determined using GraphPad Prism 5.0 version. Finally, SI, defined as CC_{50} (on RAW 264.7)/IC_{50} (on parasite), defines the balance between cytotoxicity and antileishmanial activity was calculated for each test substance. The experiments were performed in triplicate.

II-2-3-6-Nitrite oxide (NO) production assay of the most active compounds

Because of the good activity shown by our promising compounds against intracellular amastigotes forms of *L. donovani*, it was interesting to investigate whether this activity could involve some modulator mechanisms of the cellular response. The ability of our most active compounds to induce the production of NO by the infected macrophages was assessed using

Griess reagent in the culture supernatants of the RAW cells following the procedure described by **Kalliopi Karampetsou *et al*. (2019)**.

i) Principle of the method

The Griess colorimetric nitrite assay relies on the conversion of nitric oxide (incolore) in a sample to a stable azo-chromophore compound (pink) that absorbs at approximately 540 nm **(Sun *et al.*, 2003)**.

ii) Preparation of Solutions

ii-1) Preparation of Griess reagent

The Griess reagent consists of two separate solutions (A and B) that are mixed together (ratio 1:1) at the moment of the experimental operation and was prepared according to the manufacturer's instructions.

(annexe section)

- ✓ ***Expression of Results***

The percentage of NO production of each sample at each concentration was calculated using Microsoft Excel Software, and the histograms were plotted using GraphPad Prism 5.0. Software.

II-2-3-7- Antioxidant properties of the most active compounds

II-2-3-7-1- DPPH radical scavenging assay

The DPPH radical scavenging assay was performed following the procedure described by **Bassene (2012)** with some modifications.

- ✓ ***Principle of the method***

The principle of the method is based on the capacity of the compounds to supply protons to 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. In fact, the DPPH radical is unstable, and when it reacts with an antioxidant compound, it can be reduced and becomes stable by taking hydrogen ions. This reducing power of the compound is revealed by a change in the color from purple to yellow. A decrease in absorbance at 517 nm is proportional to the antioxidant potential of the compounds.

- ✓ ***Preparation of DPPH stock solution***

The DPPH stock solution was prepared in methanol at an initial concentration of 0.02%. For this, 20 mg of DPPH was completely dissolved in 100 mL of 100% methanol. The solution was conserved in a closed bottle away from light and against any source of heat before usage.

The assay was performed in 96-well microplates. A 2 serial-fold dilution was performed to obtain compounds at final concentrations ranging from 2000 to 31.25 µg/ml. Then, 25 µl from each dilution was introduced into a new microplate followed by the addition of 75 µl of DPPH prepared at final concentrations from 500 to 7.8125 µg/ml. The plates were incubated in dark at room temperature for 30 min. Then, after incubation, the OD values were recorded at 517 nm in an Infinite M200 TECAN microplate reader. The negative control was DPPH diluted in methanol. The positive control was ascorbic acid prepared at final concentrations of 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/ml. The assays were performed in duplicate.

✓ *Expression of Results*

The radical scavenging activity (RSA) of the compounds was calculated using the following formula:

$$\mathbf{RSA\ (\%) = \frac{Ao - As}{Ao}\ x\ 100}$$

Where:

RSA = Radical scavenging activity;

A_o = Absorbance of the negative control (DPPH radical + methanol);

As = Absorbance of the test sample (DPPH radical + compound or positive control).

From the RSA, the following parameters were determined: RSA_{50}, EC_{50}, and ARP.

- **RSA_{50}** = Concentration of compound at which 50% of the free radicals are scavenged. It is obtained from a graph of the percentage of RSA *versus* the concentration of the compounds.
- **EC_{50}** = **Efficient concentration 50,** defined as the concentration of compound required to scavenge ½ mole of DPPH and calculated using the following formula:

$$\mathbf{EC_{50} = \frac{RSA_{50}}{[DPPH]}}$$

- **ARP=** Antiradical power, the inverse of the EC_{50}. It measures the efficiency of the antiradical compound. If the is value lager, more the antiradical is efficient.

$$\mathbf{ARP = \frac{1}{EC_{50}}}$$

✓ *Statistical analysis*

The results obtained are expressed as the mean ± standard deviation. The IC_{50} values were determined using SPSS 17.0 software. Microsoft Excel 2016 software for Windows was used to calculate the means, EC_{50}, and ARP and to plot the graph.

II-2-3-7-2- Ferric Ion Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed according to the method described by **Path Canada (1994)** with some modifications.

✓ *Principle of the method*

The principle is based on the reduction of Fe^{3+} to Fe^{2+} by the antiooxidant compounds, which in the presence of ortho-phenanthroline forms a brown or orange–red colored complex. This complex absorbs at 505 nm, and the intensity of the coloration is proportional to the amount of Fe^{3+} converted into Fe^{2+} by the compound.

✓ *Preparation of Fe^{3+} solution:*

The Fe^{3+} solution was prepared at 1.2 mg/ml. For this, 1.2 mg of $FeCl_3$ was dissolved into 1 ml distilled water.

✓ *Preparation of Ortho-Phenantroline (ONP) solution:*

The ortho-phenanthroline solution used for the assay was prepared at 0.2%. For this, 200 mg of the powder was dissolved in 100 µl of methanol.

The assay was performed in 96-well microplates. First, the compounds were diluted to final concentrations ranging from 2000 to 31.25 µg/ml. Then, 25 µl from each dilution was introduced into a new microplate, followed by the addition of 25 µl of Fe^{3+} solution. The plates were pre-incubated for 15 minutes at ambient temperature. After incubation, 50 µl of ortho-phenanthroline was added to each well, and the plates were further incubated for 15 min at ambient temperature. The OD values were recorded at 505 nm using an Infinite M200 TECAN microplate reader. The negative control was methanol with Fe^{3+} and ortho-phenanthroline. The positive control was ascorbic acid prepared at final concentrations of 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/ml. The assay was performed in duplicate.

✓ *Expression of Results*

The chelating activity percentage of the compounds and vitamin C was calculated using the following formula:

$$\textbf{Chelating activity (\%)} = \frac{\mathbf{Ao - As}}{\mathbf{Ao}} \; \boldsymbol{x}\; \mathbf{100}$$

where:

- Ao = absorbance of the blank (methanol);
- As = absorbance of Fe^{3+} + ortho-phenanthrolin + extract or positive control.

The different values of the percentage of chelating were obtained after plotting the graph percentage of chelation *versus* different concentrations of the compounds.

II-2-4- Concentration/time inhibition kinetics of the most active fraction/compounds

The growth inhibitory effect of the most active fraction/compounds against *L. donovani* promastigotes was examined by culturing parasites (4×10^5 cells/ml) in freshly prepared complete M199 medium in the presence and absence of varying concentrations of the fraction/compounds (1/2 IC_{50}, IC_{50}, 2x IC_{50}, 4x IC_{50}), using amphotericin B as a positive control (1/2 IC_{50}, IC_{50}, 2x IC_{50}, 4x IC_{50}) for 120 hours. The number of viable parasites was determined every 24 hours for 120 hours by staining with trypan blue. Quantification of viable parasites was achieved by counting the parasites with clear cytoplasm (nonstained) using a Neubauer hemocytometer with cover slips. Three independent experiments were performed for each sample.

II-2-5- Interaction studies between the most active compounds and the positive control amphotericin B

Drug resistance related to monotherapy regimens was found in approximately 10 to 15% and 50 to 60% of CL and VL patients, respectively **(Salehabadi *et al.*, 2014)**. Combination therapy with antileishmanial drugs is currently considered one of the most rational and promising approaches and has advantages such as reduced toxicity, synergic effects, limited drug resistance development, low treatment failure rate, and shorter treatment regimens **(Gazanion *et al.*, 2011; Monzote, 2011)**. For example, combined therapy of the botulin derivative BT06 and the betulinic acid derivative AB13 with miltefosine via synergistic interactions has been considered a promising compound in the field of new alternative therapy for leishmaniasis **(Sousa *et al.*, 2014)**. In addition, **Jabini *et al.* (2015)** suggested that silymarin in conjunction with glucantime, but not alone, can have promising effects on CL caused by *L. major*. The combination of treatment with cryosurgery and an antileishmanial drug such as glucantime can be much more effective than each of the two modalities when used alone **(Alavi-Naini *et al.*, 2012)**.

In the same way, to see if our most active compounds could be more active in combination with the positive control amphotericin B, we therefore describe here in this study the interaction studies between our most active compounds amphotericin B against both promastigote and amastigotes forms of *L. donovani*.

✓ *Preparation of stock solutions of compounds and amphotericin B*

The most active compounds and the positive control were prepared at initial concentrations of 10 and 1 mg/ml, respectively. For this, 2 mg of each compound was dissolved in 200 µl of 100% DMSO, and 1 mg of amphotericin B was dissolved in 1 ml of 100% DMSO. Then, a solution of each compound and amphotericin B was prepared at 5 x IC_{50}.

✓ *Preparation of drug combination*

Potent antileishmanial compounds were examined for *in vitro* antileishmanial potency in combination with amphotericin B. The IC_{50} values of compounds were obtained from the dose response experiment as described earlier. For the combination studies, 5 x IC_{50} stock solutions of individual compounds were prepared. Stock solutions of the compounds were then combined with stock solutions of amphotericin B in volumetric ratios of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5. The stock mixtures of each of the compounds were placed into the wells of sterile 96-well plates and serially fivefold diluted. Then, the plates were incubated at -80°C for the assay.

i) Inhibitory assay of the drug combination against L. donovani promastigotes

The interaction studies were carried out following the methodology described by **Sharma *et al.* (2018)**. For the assay, 10 µl of the above mentioned stocks and their respective dilutions were transferred into new 96-well flat-bottomed cell culture plates, followed by the addition of 90 µl of $4x10^5$ promastigotes/mL/well for a final volume of 100 µl. The plates were incubated for 28 hours at 26°C. After incubation, 10 µl of resazurin (at 1 mg/mL) was added to each well. After an additional incubation for 44 hours, plates were then read on a Magelan Infinite M200 fluorescence multiwell plate reader (Tecan) at excitation and emission wavelengths of 530 and 590 nm, respectively. The sterility and negative control were 10% M-199 with no parasites and complete M-199 medium with parasites without extract, respectively. A positive control was amphotericin B (10-0.016 µg/mL).

ii) Inhibitory assay of the drug combination against L. donovani amastigotes

For the *in vitro* antileishmanial activity of the drug combination against amastigotes, macrophage Raw 264.7 cells (4 x 10^4 cells/mll) were seeded in 96-well plates and incubated for 6 hours at 37°C under 5% CO_2 for adhesion of the cells. Afterwards, the nonadherent cells were washed with sterile PBS. The adherent Raw 264.7 cells were infected with metacyclic promastigotes (4 x 10^5 cells) at an infection ratio of 1:10 macrophage:parasite and incubated for 24 hours at 37°C under 5% CO_2 to allow infection of macrophages by metacyclic promastigotes. Thereafter, the overlying medium was removed, and the monolayer cells with internalized

amastigotes were carefully washed four times with PBS to remove free parasites. Freshly prepared M199 medium containing 10% FBS and the compounds from the intermediate plate combination were added in triplicate to the infected cells at serially diluted concentrations and incubated for 48 hours at 37°C under 5% CO_2. After incubation, 0.05% sodium dodecyl sulfate (SDS) was added to each well for 30 seconds for controlled lysis followed by M199 with 10% FBS as a macrophage lysis stopper. Resazurin reagent (250 µg/mL) was thereafter added to each well, and the plates were incubated for 24 hours followed by fluorescence recording at λexcitation = 530 nm and λemission = 590 nm using a Tecan Infinite M200 microplate reader (Tecan).

✓ *Expression of Results*

For each sample, growth percent inhibition was calculated on Microsoft Excel Software using the formula:

I (%) = 100 x (Absorbance of untreated cells - Absorbance of treated cells)/Absorbance of untreated cells.

Finally, inhibition percentages were calculated using Microsoft Excel software, and the median inhibitory concentration (IC_{50}) was obtained from dose–response curves using GraphPad Prism 5.0. Software.

To study the effect of each combination, two IC_{50} values of each drug were derived from two differents dose response curves, each containing a drug alone curve and drugs in fixed concentration ratios. These IC_{50} values were used to derive a series of 50% fractional inhibitory concentration (FIC_{50}) values of each drug by using the following equation:

$$FIC_{50} = \frac{IC_{50}\ for\ drug\ in\ combination}{IC_{50}\ for\ drug\ alone}$$

The FIC_{50} values of the different drugs in each combination were used to plot an isobologram, a graphical representation of drug–drug interactions.

In the isobologram, the FIC_{50} of the different drugs for each ratio is plotted on the X and Y axes, respectively, using a linear scale. A diagonal connecting FIC=1 on each axis then allows one to see the significance of each point: those that lie on the diagonal indicate additive action, those that are below the diagonal indicate synergy and those that are above the diagonal indicate antagonism. The isobologram numerically represents the sum of individual FIC_{50} from each of the two axes as $\sum FIC_{50}$. Thus, the $\sum FIC_{50}$ of each combination ratio was calculated to determine the combined effects of the two drugs against *leishmania* parasites taken in combination using the following formula:

$$\sum FIC_{50} = FIC_{50} \text{ (Drug A)} + FIC_{50} \text{ (Drug B)}$$

$\sum FIC_{50}$ values of <1, 1 and >1 were ascribed to synergy, additivity and antagonism, respectively.

II-2-6- Acute toxicity of the most active extract

Due to the lack of quantity of our most active compounds, we decided to investigate the acute toxicity of the most active crude extract. The test was performed according to the guidelines described by **OECD 423 (2001)** with the ethic consent rules.

The crude extract was dissolved in distilled water. For the assay, six (06) female animals were divided into two (02) groups, each group containing 3 animals: one normal group (receiving only the vehicle, distilled water) and the other the test group (receiving the extract). After dissolution, the volume of extract administered to each animal was calculated according to the following formula:

$$V\,(m) = \frac{D \times W}{C}$$

Where:

V = Volume administered

D = Dose in mg/kg

C = concentration of extract

W = Weight of the animal

The extract was orally administered only to the animals of the test group at a single dose of 2000 mg/kg, and distilled water was administered to the animals of the normal group. Directly after administration, animals were observed for a short period of 30 minutes, then 1 and 4 hours, every day for a long period of 14 days. During the period of observation, the following parameters were evaluated: body weight, horripilation, sensibility to sound and touch, mobility, aggressiveness, and the aspect of feces. For a long period of 14 days, the animals received daily food and water, and the weights of both groups during the experiments were recorded to determine the influence of the extract on the weight of the animals.

CHAPTER III
RESULTS AND DISCUSSION

III- RESULTS AND DISCUSSION

III-1- Results

III-1-1-Phytochemical analysis

III-1-1-1-Phytochemical analysis data of *D. gracilescens*

The crude extract, the hexane and dichloromethane soluble fractions of the trunk of *D. gracilescens* were analyzed by UPLC coupled to both diode array and mass spectrometry detectors. The latter was used with an electrospray ionization (ESI) source in positive ion mode. A representative base peak chromatogram and all-ion MS (shown in **Figure 13**) indicated that the UPLC conditions allowed good separation of a large percentage of compounds. The UPLC-DAD-HRESI/MS identification of all constituent was performed by analysis of total ions chromatogram (TIC) traces of several compounds, the MS fragmentation and databases (Scifinder, NIST/EPA/NIH Mass Spectral Library (NIST 14) and MassBank of North America (NoNA)) and subsequent confirmation by comparison with literature data. The chromatographic and spectroscopic data are shown in **Figure 13 and Table IV** below.

Table 4: FT-MS product ions of detected compounds in the trunk extract of *D. gracilescens*.

N°	Tr (min)	$[M+H]^+$		UV, λ_{max} (nm)	molecular formular	Name of compound
		Exp.	Calcd.			
1	7.19	663.4740	663.4772	222	$C_{46}H_{62}O_3$	chlorobiumquinone
2	6.99	431.3689	431.3672	222	$C_{32}H_{46}$	NI
3	6.82	483.3621	483.3621	450	$C_{30}H_{46}O$	NI
4	6.77	427.3946	427.3946	222	$C_{30}H_{50}O$	Lupeol
5	6.65	469.3465	469.3421	222	$C_{32}H_{46}O$	NI
6	6.57	479.3887	479.3884	222	$C_{33}H_{50}O_2$	NI
7	6.21	391.3003	391.2995	222	$C_{28}H_{38}O$	NI
8	5.13	579.3116	579.3105	218	$C_{38}H_{42}O_5$	NI

NI: Not Identified

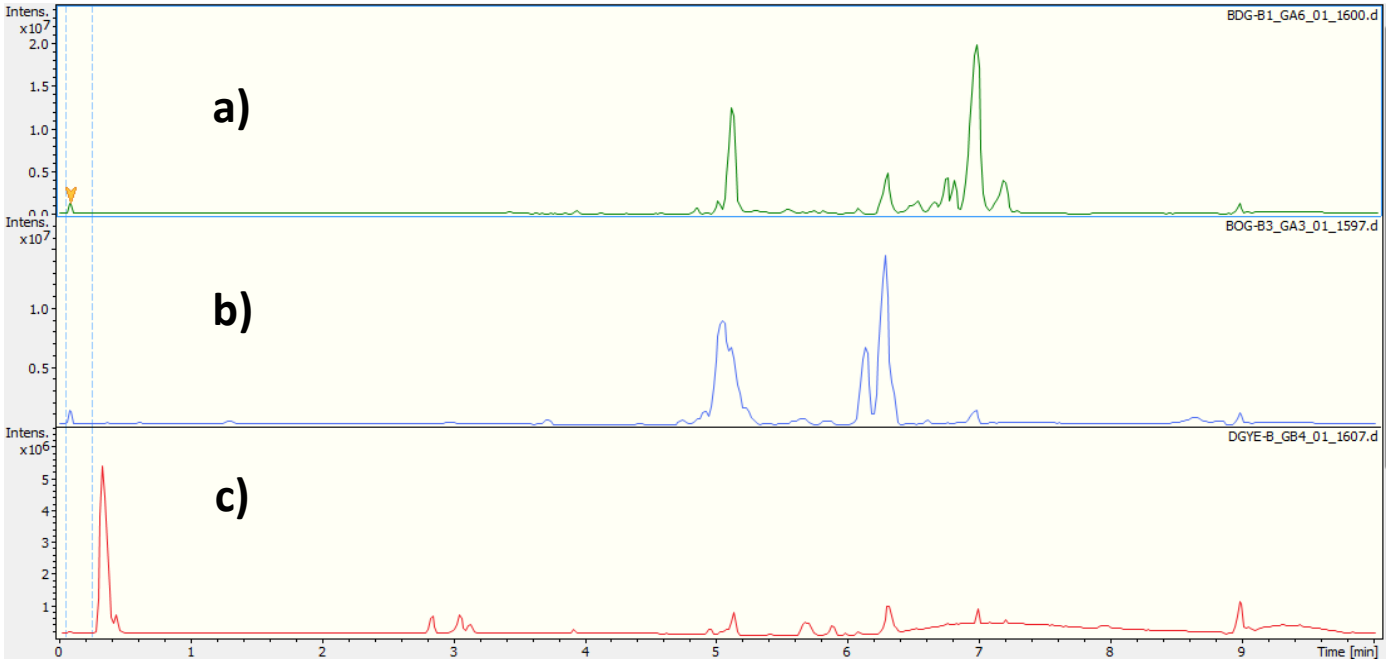


Figure 13: HPLC chromatograms (TIC: m/z 150-1000) of a) crude extract, b) dichloromethane and c) hexane fractions from the trunk of *D. gracilescens*.

III-1-1-2- Identification of peaks from UPLC–DAD–(HR)-ESI-MS from *R. hispida* crude extract

The identification of all constituents was performed by UPLC–DAD–MS analysis and by comparing the MS spectra and fragmentation of the peaks in the samples with those of compounds from the *Rothmania* genus reported in the literature using the Scifinder database. The compounds were putatively identified.

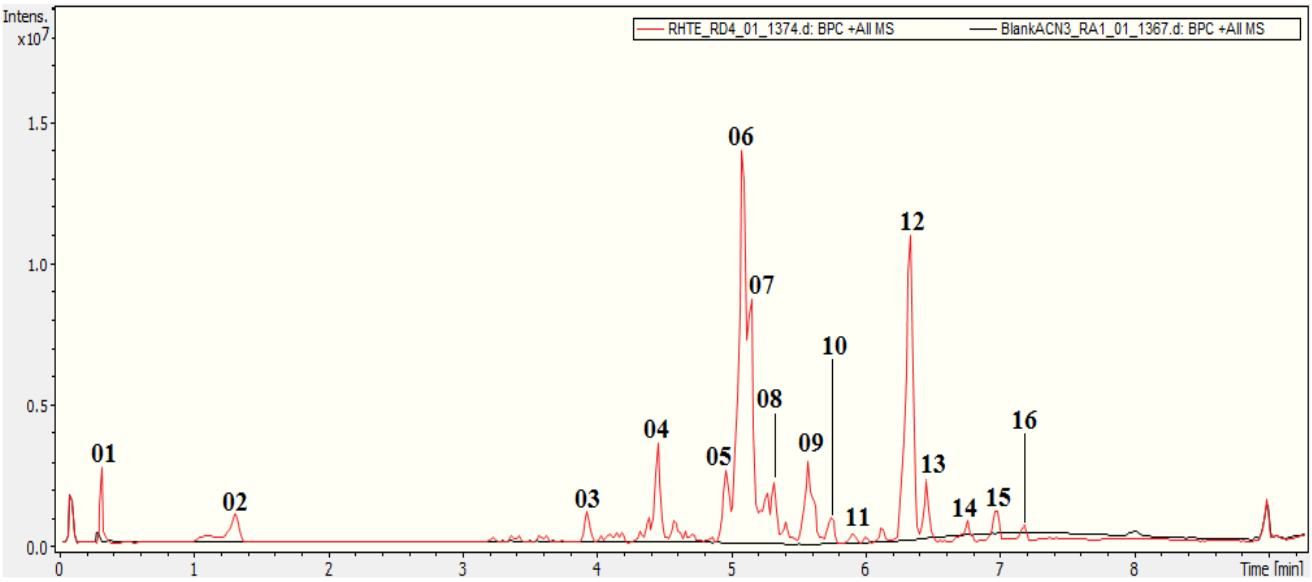


Figure 14: Chromatographic profiles of *R. hispida* crude extract

Table 5: Main signals exhibited in the UPLC-DAD-MS spectra of compounds detected in *R. hispida* crude extract and proposed attribution.

N°	Tr (min)	$[M+H]^+$ or $[M+Na]^+$		Ions peaks	Molecular Formular	Name of Compound
		Exp.	Calcd.			
01	0.31	205.0678	205.0688	$[M+Na]^+$	$C_9H_{12}O_3$	D-mannitol
02	1.31	169.0857	169.0859	$[M+H]^+$	$C_9H_{12}O_3$	NI
03	3.92	353.2294	353.2298	$[M+Na]^+$	$C_{18}H_{34}O_5$	NI
04	4.46	467.1946	467.1982	$[M+Na]^+$	$C_{32}H_{28}O_2$	NI
05	4.97	219.2247	219.2244	$[M+Na]^+$	$C_{18}H_{32}O_3$	NI
06	5.07	295.2267	295.2268	$[M+H]^+$	$C_{18}H_{30}O_3$	NI
07	5.16	295.2270				NI
08	5.33	287.0922	287.0915	$[M+H]^+$	$C_{16}H_{14}O_5$	Lichexanthone
09	5.58	331.2251	331.2245	$[M+Na]^+$	$C_{19}H_{32}O_3$	NI
10	5.63	331.2252				NI
11	5.77	333.2412	333.2400	$[M+Na]^+$	$C_{19}H_{34}O_3$	NI
12	6.35	393.2977	393.2975	$[M+Na]^+$	$C_{22}H_{42}O_4$	NI
13	6.46	429.2986	429.2975	$[M+Na]^+$	$C_{25}H_{42}O_4$	NI
14	6.77	449.3616	449.3601	$[M+Na]^+$	$C_{26}H_{50}O_4$	NI
15	6.99	551.4666	551.4670	$[M+H]^+$	$C_{34}H_{62}O_5$	NI
16	7 .19	685.4288	685.4280	$[M+Na]^+$	$C_{49}H_{58}O$	NI

NI: Not Identified

III-1-2- Biological activities data

III-1-2-1- Antileishmanial activity of plant samples

The antileishmanial assays were performed on roots, stem, bark, trunk and leaf hydroethanolic extracts of *D. grascilesens* and stem CH_2Cl_2-MeOH extract of *R. hispida*. All the different crude extracts were first tested against promastigotes forms of the *L. donovani* parasite. Only the extracts that showed very good activity against this extracellular form of the parasite were then selected for the fractionation, and the antileishmanial activity of the different fractions and isolated compounds

against both promastigotes and intracellular amastigotes forms and their cytotoxicity profiles were also evaluated.

The antileishmanial activity was classified according to the IC_{50} value obtained, following the criteria previously established by **Camacho *et al*. (2003)** indicating that:

- ✓ With an IC_{50} < 10 µg/ml, the extract is **highly active**;
- ✓ 10 < IC_{50} < 50 µg/ml, extract is **good active**;
- ✓ 50 < IC_{50} <100 µg/ml, extract is **moderately active**;
- ✓ IC_{50} >100 µg/ml, the extract was **inactive**.

The following table shows the *in vitro* antileishmanial potential of all the crude extracts against the promastigotes form of *L. donovani*:

Table 6: Anti-leishmanial activity of crude extracts against promastigotes of *L. donovani*.

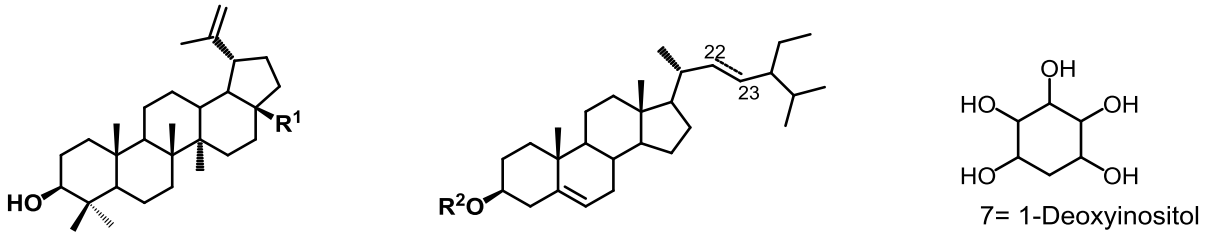
Name of the Plant	Part	Solvent	*Promastigotes of L. donovani* 1S (MHOM/SD/62/1S)
			IC_{50} ± SD (µg/ml)
D. grascilisens	Root	H_20 : EtOH	**> 100**
	Bark	H_20 : EtOH	**> 100**
	Leaf	H_20 : EtOH	**> 100**
	stem	H_20 : EtOH	**> 100**
	Trunk	H_20 : EtOH	**5.84 ± 0.20**
R. hispida	Stem	CH_2Cl_2-MeOH	**5.74 ± 0.12**
Positive Control	Amphotericin B		**0.33 ± 0.21**

Legend: IC_{50}: 50% inhibitory concentration; SD: standard deviation

The results shown in table 6 showed that crude extracts from the trunk and stem of *D. grascilisens* of *R. hispida* showed the promising antileishmanial activity with IC_{50} values of 5.84 and 5.74 µg/mL respectively. The other extracts were non-active up to 100 µg/mL. According to the criteria described by **Camacho *et al*. (2003)** in the table above, these two crude extracts are classified as highly active because they showed IC_{50} values lesser than 10 µg/ml (**IC_{50} < 10 µg/ml**).

Based on their antileishmanial activity, these two crude extracts were therefore processed for the bio-guided investigation using liquid–liquid partition and silica gel column chromatography approaches. The different active fractions obtained were subjected to the purification to isolate the compounds.

Fractionation of the trunk crude extract of *D. grascilesens* was performed by liquid–liquid partition to afford five (05) main fractions. The purification of the most active fractions led to isolation of six (06) compounds that are shown below:

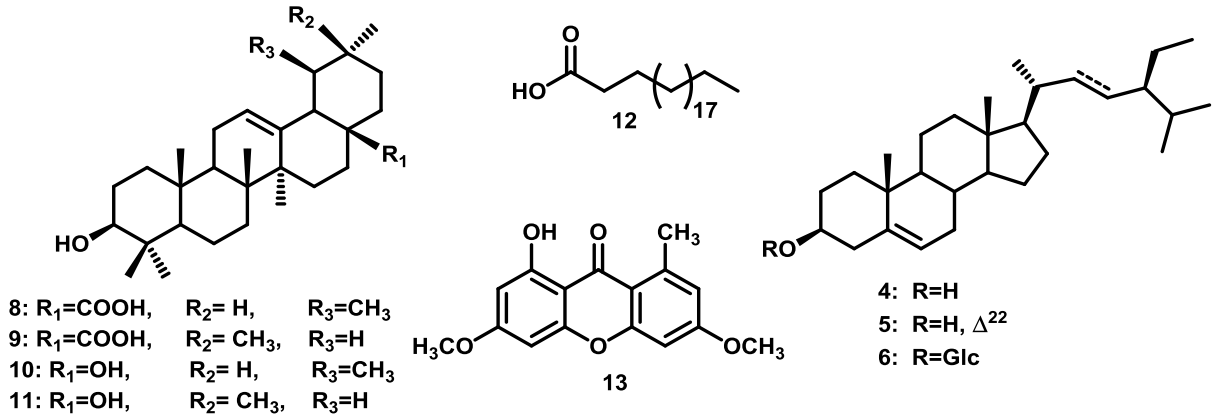


1 R^1= Me lupeol
2 R^1= -CH_2OH betulin
3 R^1 = COOH betulinic acid

4 R^2= H ß-sitosterol
5 R^2= H, Δ^{22} stigmastérol
6 R^2= Glc ß-sitosterol glucoside

Figure 15: Isolated compounds from *D. gracilescens*.

On the other part, the fractionation of the stem crude extract of *R. hispida* was performed by silica gel column chromatography to afford three (03) main fractions. The purification of the most active fractions led to the isolation of nine (09) compounds. The names and their structures are shown below:



8: R_1=COOH, R_2= H, R_3=CH_3
9: R_1=COOH, R_2= CH_3, R_3=H
10: R_1=OH, R_2= H, R_3=CH_3
11: R_1=OH, R_2= CH_3, R_3=H

4: β-sitosterol; **5:** stigmasterol; **6:** sitosterol 3-*O*-*β*-D- glycoside; **8:** ursolic acid; **9:** oleanolic acid; **10:** uvaol; **11:** erythrodiol; **12:** docosanoic acid; **13:** lichexanthone.

Figure 16: Isolated compounds from *R. hispida*.

Concerning the isolated compounds, three (03) among the nine (09) isolated from *R. hispida* and five (05) among the six (06) isolated from *D. grascilesens* were tested for their antileishmanial potential. All the fractions and isolated compounds were tested against both promastigotes and intracellular amastigotes forms of *L. donovani* parasite, and their cytotoxicity profile against RAW 264.7 cells was also evaluated.

The selectivity index (SI) and specificity index (SPI) of the samples were also determined for each sample.

The criteria of selectivity of each sample were adopted following the criteria established by **De Lima *et al*. (2012)** stating that:

- ✓ With a selectivity index higher than 1 **(SI > 1)**, the extract is selective and is more active on the parasite than on the host cell.
- ✓ With a selectivity index lesser than 1 **(SI < 1)**, the extract is not selective and is more active on the host cell than the parasite.

The specificity index (SPI) is the ratio between the IC_{50} values on promastigotes and amastigotes forms of the parasite. These values allow us to identify on which form of the parasite the sample is more active. This index has been classified according to the criteria defined by **De Muylder *et al*. (2011)**:

- ✓ With SPI higher than 2 **(SPI > 2)**, the sample is more active in amastigote form,
- ✓ With SPI less than 0.4 **(SPI <0.4)**, the sample is more active on promastigote form,
- ✓ With SPI between 0.4 and 2 **(0.4< SPI < 2)**, the sample is considered as equally active on both promastigotes and amastigotes forms of the parasite.

The results of the antileishmanial activity of each tested sample are summarized in **table 7** below:

Table 7: Anti-leishmanial activity against both promastigotes and intracellular amastigotes forms of *L. donovani*, Specificity and Selectivity Indexes.

Plants	Organs	Nature	Codes	CC_{50} (µg/ml)	Activity on *L. donovani* 1S (MHOM/SD/62/1S)				
					Promastigotes		Amastigotes		SPI
					IC_{50} ± SD (µg/ml)	IS	IC_{50} ± SD (µg/ml)	SI	
R. hispida	**Stem**	Ext CH_2Cl_2/MeOH	RHE	> **100**	5.74 ± 0.12	> **17.39**	15.70 ± 0.16	> **6.36**	0.36
		Fraction	RTF1	> **100**	4.38 ± 0.10	> **22.78**	6.72 ± 0.21	> **14.88**	0.65
		Fraction	RTF2	> **100**	12.74 ± 0.07	> **7.84**	8.41 ± 0.17	> **14.48**	1.84
		Fraction	RTF3	> **100**	86.98 ± 0.13	> **1.14**	6.90 ± 0.17	> **11.88**	10.33
		Sub-fraction	RTF1S1	> **100**	6.04 ± 0.14	> **16.55**	10.49 ± 0.16	> **9.53**	0.57
		Sub-fraction	RTF1S2	**78.74 ± 0.31**	29.22 ± 0.12	**2.69**	11.90 ± 0.13	6.61	2.45
		Sub-fraction	RTF3S1	> **100**	> **100**	**ND**	24.36 ± 0.08	> **4.10**	ND
		Sub-fraction	RTF3S2	> **100**	0.77 ± 0.15	> **129.87**	8.19 ± 0.12	> **12.20**	0.09
		Compound	RHT2	> **100**	3.12 ± 0.23	> **32**	11.94 ± 0.04	> **8.37**	0.26
		Compound	RHT3	**58.74 ± 0.14**	1.78 ± 0.16	**32.96**	9.36 ± 0.03	6.27	0.19
		Compound	RHT4	**72.74 ± 0.46**	0.75 ± 0.12	**154.76**	2.34 ± 0.24	30.97	0.20
		Compound	RHT5	> **100**	2.05 ± 0.14	> **48.63**	12.83 ± 0.21	> **7.79**	0.16
D.grascilesens	**Trunk**	Extr EtOH: H_20	$DGYES_2E$	> **100**	5.38 ± 0.16	> **18.56**	35.69 ± 0.25	> **2.80**	0.15
		Fraction	DGF1	> **100**	0.78 ± 0.09	> **126.74**	8.06 ± 0.39	> **1.28**	0.09
		Fraction	DGF2	> **100**	1.63 ± 0.11	> **61.16**	10.97 ± 0.10	> **9.11**	0.14
		Fraction	DGF3	> **100**	2.36 ± 0.06	> **42.30**	16.05 ± 0.12	> **6.23**	0.14
		Fraction	DGF4	> **100**	1.11 ± 0.10	> **89.60**	22.08 ± 0.09	> 4.52	0.05
		Compound	DGC1	**37.64 ± 0.3**	6.21± 0.07	**6.05**	8.43 ± 0.01	4.46	0.73
		Compound	DGC2	**8.28 ± 0.11**	9.91 ± 0.08	**0.83**	19.91 ± 0.65	0.41	0.49
		Compound	DGC3	**15.4 ± 0.09**	38.75 ± 0.19	**0.397**	14.75 ± 0.16	1.04	2.62
		Compound	DGC4	**16.65 ± 0.07**	28.46 ± 0.11	**0.58**	18.46 ± 0.21	0.90	1.54
		Compound	DGC5	**21.42 ± 0.07**	39.68 ± 0.16	**0.53**	19.68 ± 0.18	1.08	2.01
		Compound	DGC6	**NT**	**NT**	**ND**	**NT**	**ND**	**ND**
Amphotericin B				**0.33 ± 0.21**		**ND**	**0.12 ± 0.11**		**ND**

CC_{50}: 50% Cytotoxic Concentration; **IC_{50}:** 50% Inhibitory Concentration; **SD:** Standard deviation;; **SPI:** Specificity Index; **SI:** Selectivity Index; **ND:** Not-Determined; NT: Non-Tested; **Ext CH_2Cl_2/MeOH:** *R. hispida* CH_2Cl_2-MeOH Stem crude extract; **RTF1:** Hexanic fraction ;

RTF2: EtOAc Fraction ; **RTF3:** n-butanol fraction; **RTF1S1:** Hexanic subfraction 1; **RTF1S2:** Hexanic subfraction 2 ; **RTF3S1:** n-butanol subfraction 1; **RTF3S2:** n-butanol subfraction 2; **RHT2:** Ergosterol ; **RHT3:** Oleanolic acid; **RHT4:** Ursolic acid; **RHT5:** Erythrodiol/Uvaol mixture ; **DGYES$_2$E:** hydroethanolic stem-bark of *D. grascilesens*; **DGF1:** Hexanic fraction; **DGF2:** DCM fraction; **DGF3:** Ethylacetate Fraction; **DGF4:** n-butanol fraction ; **DGC1:** Betulinic acid; **DGC2:** Lupéol ; **DGC3:** Phytostérol ; **DGC4:** β-sitostérol glucoside ; **DGC5:** 1-Deoxyinositol ; **DGC6:** betulin.

The **Table VII** above shows the results of the antileishmanial assays of extracts, fractions and isolated compounds on both promastigotes and intracellular amastigotes forms of *L. donovani*.The results shows that the crude extracts, fractions and isolated compounds showed antileishmanial activity varying from 0.75 to 86.98 µg/ml. The activity was depending of the plant, organ, solvent of extraction and the form of the pathogen.

For the case of *R. hispida,* the CH_2Cl_2-MeOH stem crude extract was highly active (IC_{50}= 5.74 µg/ml) against promastigotes forms, but showed good activity against intracellular amastigotes forms ($IC_{50=}$ 15.70 µg/ml) of *L. donovani*. With SI greater than 1 (SI>1), this crude extract is selective against both forms of the parasite.

After the fractionation of this crude extract, the different fractions obtained (Hexanic, EtOAc and n-butanol fractions) exhibited good activity on the promastigotes forms of the parasite. Among the tested fractions, the hexanic fraction showed the best anti-leishmanial activity (IC_{50}= 4.38 µg/ml) and was found to be the most active compared to the crude extract on promastigote form. The EtOAc fraction exhibited good anti-leishmanial activity (IC_{50}= 12.74 µg/ml), while the n-butanol fraction showed moderate activity (IC_{50} = 86.98 µg/ml) against promastigotes forms. All three fractions were selective on promastigote form (SI>1).

All the three fractions exhibited very high antileishmanial activity (IC_{50}<10 µg/ml) against the intracellular amastigotes forms of the parasite. The most active fraction was the hexanic fraction, with an IC_{50} value of 6.72 µg/ml, and was the most selective (SI >14) against the amastigotes forms**.** The EtOAc and n-butanol fractions were also selective against intracellular amastigotes forms of the parasite.

Globally, most of the four (04) subfractions tested were active against both promastigotes and amastigotes of the parasite. Hexanic subfraction 1 shows high activity against promastigotes and good activity against amastigotes. Hexanic subfraction 2 shows good activity against both forms of the parasite. The n-butanol subfraction 1 was not active against promastigotes (IC_{50} > 100 µg/ml) and showed good activity against intracellular amastigotes. The n-butanol subfraction 2 exhibited very good antileishmanial activity against promastigotes and intracellular amastigotes,

with IC_{50} values of 0.77 and 8.19 µg/ml, respectively. Among the entire tested sample, the n-butanol subfraction 2 was the most active against promastigotes forms. All the subfractions were selective.

All three (03) compounds tested (oleanolic acid, ursolic acid and erythrodiol/uvaol mixture) showed high activity (IC_{50} < 10 µg/ml) against promastigotes forms of *L. donovani*. The oleanolic and ursolic acids showed a very strong potential (IC_{50} < 10 µg/ml), while ergosterol and erythrodiol/uvol exhibited good activity (10 < IC_{50} < 50 µg/ml) against the intracellular amastigotes forms. The best inhibition growth against promastigotes and intracellular amastigotes was observed with ursolic acid, with IC_{50} values of 0.75 and 2.34 µg/ml, respectively. Generally, all the compounds were selective (SI>1) against both forms of the parasite. Ursolic acid was found to be the most selective against promastigotes and amastigotes (SI>30).

For the case of *D. grascilesens*, the hydroethanolic trunk crude extract showed very good antileishmanial activity against promastigotes forms (IC_{50}= 5.38 µg/ml) and moderate activity against intracellular amastigotes (IC_{50}=35.69 µg/ml). With SI greater than 1 (SI>1), this crude extract is selective against both forms of the parasite.

Contrary to the case of *R. hispida*, we noticed for *D. grascilesens*, the amelioration of the anti-leishmanial activity on promastigotes forms of the parasite after the liquid–liquid partition of the crude extract. The increase in this activity is followed by an increase in the SI values. All the fractions obtained exhibited very high activity on the promastigotes form of the parasite. Among the tested fractions, the hexanic fraction was the most active on this form of the parasite. All the fractions were mostly active in promastigotes forms compared to the crude extract and were the most selective on this form of the parasite (SI>1).

All the fractions exhibited very good antileishmanial activity (IC_{50}<10 µg/ml) on the intracellular amastigotes forms of the parasite. The most active fraction was the hexanic fraction with an IC_{50} value of 8.06 µg/ml, with a SI >1.28. All the fractions were also selective on intracellular amastigotes forms of the parasite.

Among the six (06) compounds isolated, betulinic acid and lupeol showed very good activity (IC_{50} <10 µg/ml), while stigmastérol, β-sitostérol glucoside and 1-deoxyinositol showed good activity (10 < IC_{50}< 50 µg/ml) against promastigotes forms of the parasite. Only betulinic acid showed a very good activity, while the other compounds exhibited good activity against the intracellular amastigotes forms. Betulinic acid was selective against both forms of the parasite (SI>1). Most of the compounds were non-selective (SI<1).

Among the two (02) plants investigated in this study, *D. grascilesens* is less active and less selective. Five (05) organs of this plant were tested: leaves, roots, stem, bark and trunk. Apart

from the hydroethanolic trunk extract, none of the other parts of the plant showed any activity against promastigotes forms of *L. donovani* (IC_{50} >100 µg/ml). We noticed that after the liquid–liquid partition of the trunk extract, the amelioration of the activity on promastigotes forms of the parasite.

The reference drug, amphotericin B, was the most active among the entire sample tested, showing a high activity against both promastigotes and intracellular amastigotes, with IC_{50} values of 0.33 and 0.12 µg/ml, respectively.

Compared to the data found in the literature, with IC_{50} < 10 µg/ml and SI > 1:

- ✓ For the *R. hispida*: the hexanic fraction, erythrodiol/uvol mixture, oleanic and ursolic acids could be an interest against both the promastigotes and intracellular amastigotes forms of *L. donovani*, with ursolic acid being the most active (IC_{50} = 0.75 and 2.34 µg/ml) and the most selective (SI= 154.76 and 30.97) against both promastigotes and amastigotes, respectively.
- ✓ For *D. grascilesens*, the hexanic, DCM and Ethyl-acetate fractions could be an interest against both promastigotes forms of *L. donovani*, with the hexanic fraction being the most active (IC_{50} = 0.78 and 8.06 µg/ml against promastigotes and amastigotes respectively) and the most selective on promastigotes form (SI=126.74).

Concerning the SPI, we observed that most of the samples were active on the promastigotes forms of *L. donovani* (SPI<0.4). Some of them were equally active on promastigotes than on amastigotes forms (0.4<SPI<2). Only a few samples were more active on amastigotes than promastigote forms (SPI>2). We observed that promastigotes are more sensitive than amastigotes. The difference in the sensitivity of intracellular amastigotes could be explained by the difference in adaptation that facilitates the intracellular survival of the *L. donovani* parasite (**Croft et Coombs, 2003)**.

III-1-2-2-Invasion assay of the most active compounds

The ability of our most active compounds (ursolic and oleanolic acids) to prevent the infection of macrophages by metacyclic promastigotes of the parasite was evaluated using the rezazurin colorimetric spectrophotometric method, and **table XIII** below shows the results obtained:

Table 8: Invasion assay of the most active compounds.

Name of Compound	Metacyclics promastigotes of *L. donovani* 1S (MHOM/SD/62/1S)
	IC_{50} ± SD (µg/ml)
Ursolic acid	1.34 ± 0.08
Oleanolic acid	11.47 ± 0.04
Amphotericin B	0.12 ± 0.11

Globally, the results obtained from the table above indicated that ursolic and oleanolic acids showed high and good antileishmanial activity, respectively, when they were added before the infection of macrophages by metacyclics promastigotes. Additionally, when we compare the IC_{50} values obtained during the *ex vivo* antileishmanial assay against intracellular amastigotes (IC_{50} values of 2.34 and 9.36 µg/ml for ursolic and oleanolic acids, respectively), the important information that we can notice here is the decreasing in the IC_{50} value when ursolic acid is introduced before infection of macrophages compared to when it is added for the treatment of the infected macrophages. Otherwise, we noticed an increase in the IC_{50} value with oleanolic acid when it was added before the infection of macrophages compared to the treatment. This result might allow us to conclude that ursolic acid might act as a preventive agent by inhibiting the infection of macrophages by metacyclic promastigotes of *L. donovani*.

III-1-3- Nitrite oxide (NO) production assay of the most active compounds

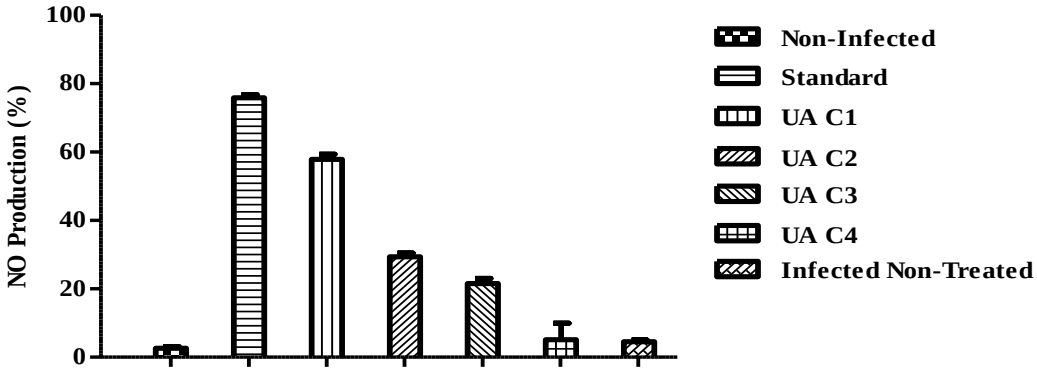
The interest in immunomodulatory drugs is currently increasing as possible anti-*Leishmania* adjuvant treatments. Whereas immunostimulation could be a promising strategy to enhance efficacy and/or reduce drug intake, it should be adapted to the clinical setting, as the expected benefit may vary according to the cutaneous or visceral setting of the disease **(Murray *et al.*, 2000)**. The fate of *Leishmania spp*. parasites is determined by the activation status of macrophages **(Tomiotto-Pellissier *et al.*, 2018)**. It is well known that NO is one of the crucial molecules in the control of parasite load during the development of CL **(Khouri *et al.*, 2009)**.

In fact, as nitric oxide (NO) is an effector molecule critical for the elimination of intracellular *Leishmania* parasites, disease progression is ensured via enhancement of Th2 responses that cause deactivation of macrophages and decreased production of NO **(Naderer et McConville, 2008)**. Therefore, parasite removal should entail activation of infected macrophages by increased expression of inducible nitric oxide synthase (iNOS) to form NO **(Holzmuller *et al.*, 2002)**. During *Leishmania* infection, decreased expression or inactivation of iNOS may also be associated with increased activation of arginase, as deprivation of L-arginine impairs *L. major*-specific T-cell responses **(Munder *et al.*, 2009)**.

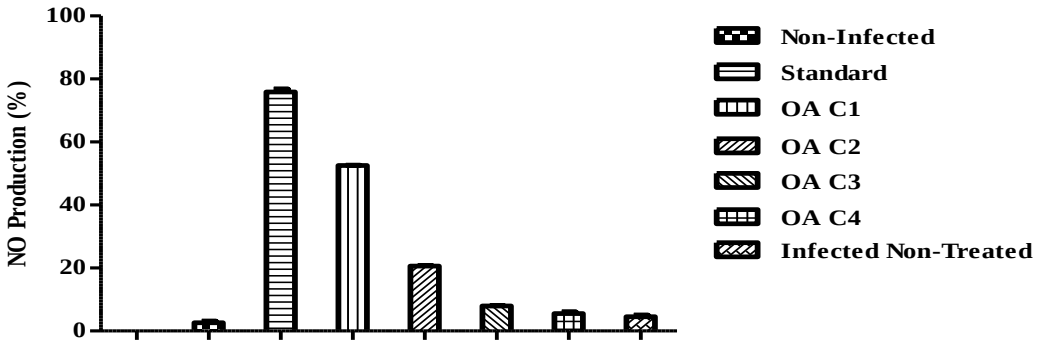
The obligatory intracellular parasites of the genus *Leishmania* have deployed several sophisticated mechanisms to escape, evade and modulate the host's immune system for their benefit, and consequently, they are able to survive and persist within macrophages **(Evans *et al.*, 1993)**. For survival, *Leishmania* can further evade cytotoxic mechanisms by reducing NO production through the increased expression of arginase, which cleaves L-arginine, a precursor of NO synthesis **(Bogdan and Röllinghoff, 1999; Calegari-Silva *et al*; 2009)**. To evade host

immunity, an important one is that parasites of the genus *Leishmania* modulate the response in macrophages by decreasing or suppressing the expression of inducible nitric oxide synthase (iNOS) activity in macrophage activity, which consequently results in depletion of NO, a key mediator of leishmanicidal activity, able to impair the replication of *L. donovani*, as well as increasing the production of essential polyamines that are needed for the growth and differentiation of the parasites **(Wanasen and Soong, 2008)**.

The following graphs show the percentage of NO produced by ursolic and oleanolic acids at different concentrations during the amastigote assay:



Ursolic Acid NO Production



Oleanolic Acid NO Production

UA= Ursolic Acid; OA= Oleanolic acid; C_1= 50 µg/ml; C_2= 10 µg/ml; C_3= 2 µg/ml; C_4= 0.4 µg/ml

Figure 17: NO generation by ursolic and oleanolic acids in RAW macrophages infected by *L. donovani* parasites.

By observing the graphs above, we noticed that low basal levels of NO were detected in the cell-free culture supernatants of infected macrophages, correlating with the progression of the infection. The NO release in the culture medium from infected macrophages compared to untreated infected cells upon subsequent incubation after 48 h of treatment with UA and OA was

dose dependent, and the levels of NO were higher than those produced by normal macrophages **(Figure 17)**.

III-1-4- Antioxidant properties of the most active compounds

Oxidative stress is known to be involved in the pathogenesis of various chronic diseases; hence, antioxidant therapy is a promising strategy for the management and treatment of these diseases **(Bajpai *et al*., 2016)**.

III-1-4-1- DPPH radical scavenging activity

The DPPH assay measures the ability of the sample to act as a proton donor and eventually help stop the damaging effect of reactive free radicals. Despite some limitations, the method of DPPH scavenging has been widely used in trials to assess the potential of natural products in reducing free radicals **(Masoko and Eloff 2007, Shah *et al*. 2010)**. According to this strategy, products with IC_{50} values lower than 50 µg/mL indicate very high antioxidant properties, while values ranging from 50-100 µg/mL, 100-200 µg/mL and above 200 µg/mL indicate good, moderate and lack of antioxidant activity, respectively **(Reynertson *et al*. 2005)**.

The **Table IX** below shows the results of the DPPH radical scavenging activity of the ursolic and oleanolic acids and the positive control, vitamin C.

Code of compound	IC_{50} ± SD (µg/ml)	EC_{50}	ARP
UA	75.85 ± 0.001	0.37×10^{4}	26.36×10^{-5}
OA	94.91 ± 1.59	0.47×10^{4}	21.05×10^{-5}
Vitamin C	8.92 ±1.06	0.04×10^{4}	224.21×10^{-5}

Table 9: DPPH radical scavenging parameters of compounds and vitamin C.

IC_{50}: inhibitory concentration 50**; EC_{50}:** efficient concentration 50**; ARP:** antiradical power; UA= ursolic acid; OA= oleanolic acid

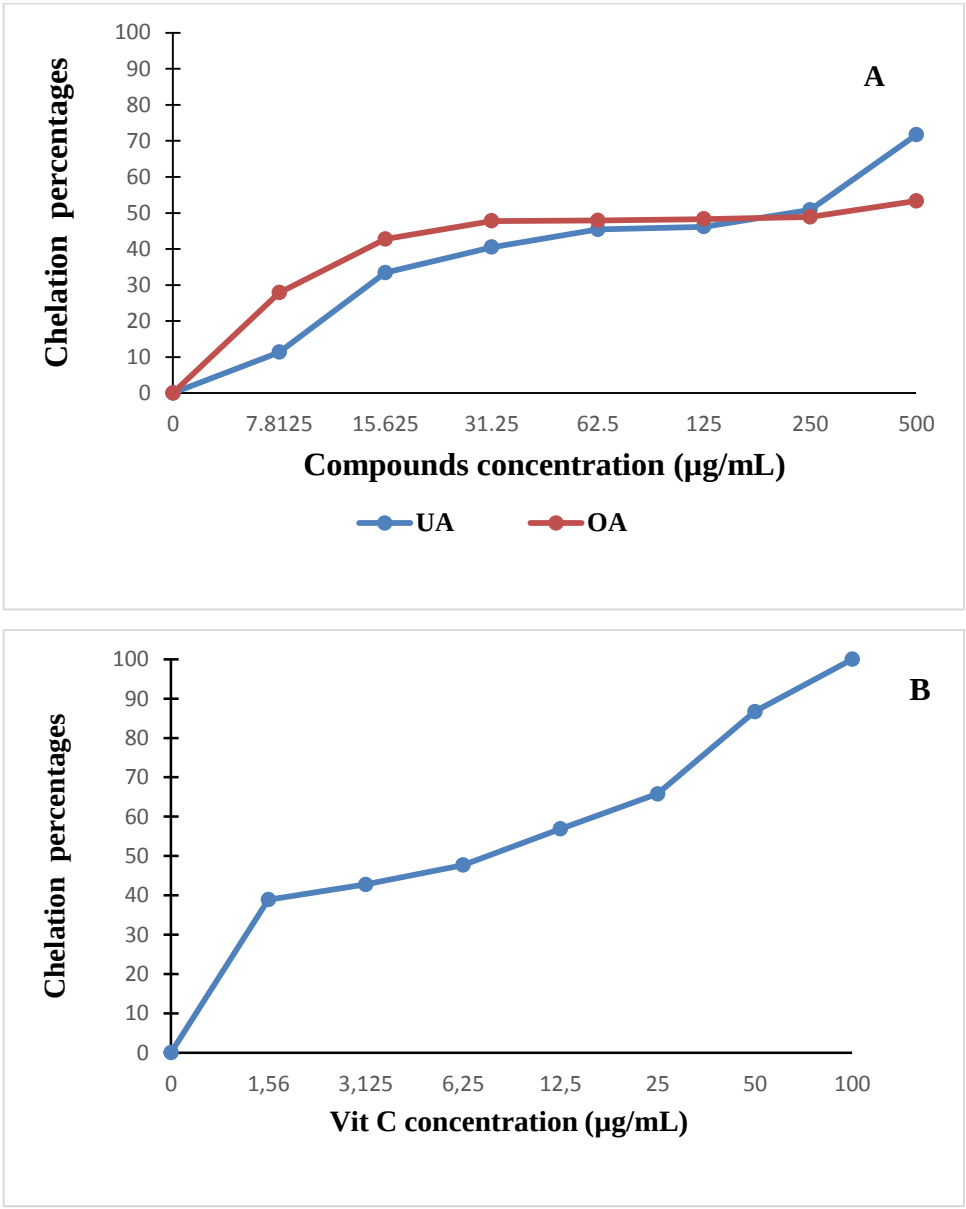
Therefore, following the criteria previously elucidated, UA and OA show good antioxidant potential with IC_{50} values of 75.85 and 94.91µg/ml, respectively. Vitamine C, the positive control, showed the highest antioxidant activity, with an IC_{50} value of 8.92 µg/ml and showed the highest ARP, followed by UA and then OA.

The results obtained in this study are similar to those obtained by **Patrícia *et al*. (2014),** which showed the antioxidant activity of UA to scavenge the DPPH radical with an IC_{50} value of 59.7 µg/mL. In the same way, **Joquebede *et al*. (2017)** showed the higher potential of UA and OA, with an IC_{50} value of 49.4 µg/mL**.** On the other hand**, Santiago *et al*. (2014)** showed the highest scavenging activity of UA and OA acids, with an IC_{50} value of 333.33 mM.

III-1-4-2- Ferric Ion Reducing Antioxidant Power (FRAP) Assay

On the other hand, the reducing power of these compounds was evaluated by their ability to reduce Fe^{3+} ions to $Fe^{2+,}$ as shown by the formation of a Prussian blue solution.

Figure 18 below shows the results of the percentages of Fe^{3+} chelation of our most active compounds and vitamin C:



Vit C: Vitamin C

Figure 18: Percentages of Fe^{3+} chelation as a function of the different concentrations; A) compounds; B) vitamin C (positive control).

By observing the figures above, we can observe that the percentages of Fe^{3+} chelation of UA, OA and vitamin C depended mainly on the concentration of the compound. At the lowest concentration of 7.8125 µg/ml, UA and OA showed percentages of Fe^{3+} chelation of 10 and 27%, respectively, while at a higher concentration of 500 µg/mL, they showed percentages of 70 and

50%, respectively. Vitamin C remained the most active among the three compounds tested, with 100% Fe^{3+} chelation at 100 µg/mL.

III-1-5- *Kinetics of parasite killing as a relation to time and inhibitor concentration*

The following graphs A, B, C and D (figure 19) below show the time kill kinetics of the most active fraction (Hexanic fraction) and the most active compounds (UA and OA) compared to the positive control, amphotericin B.

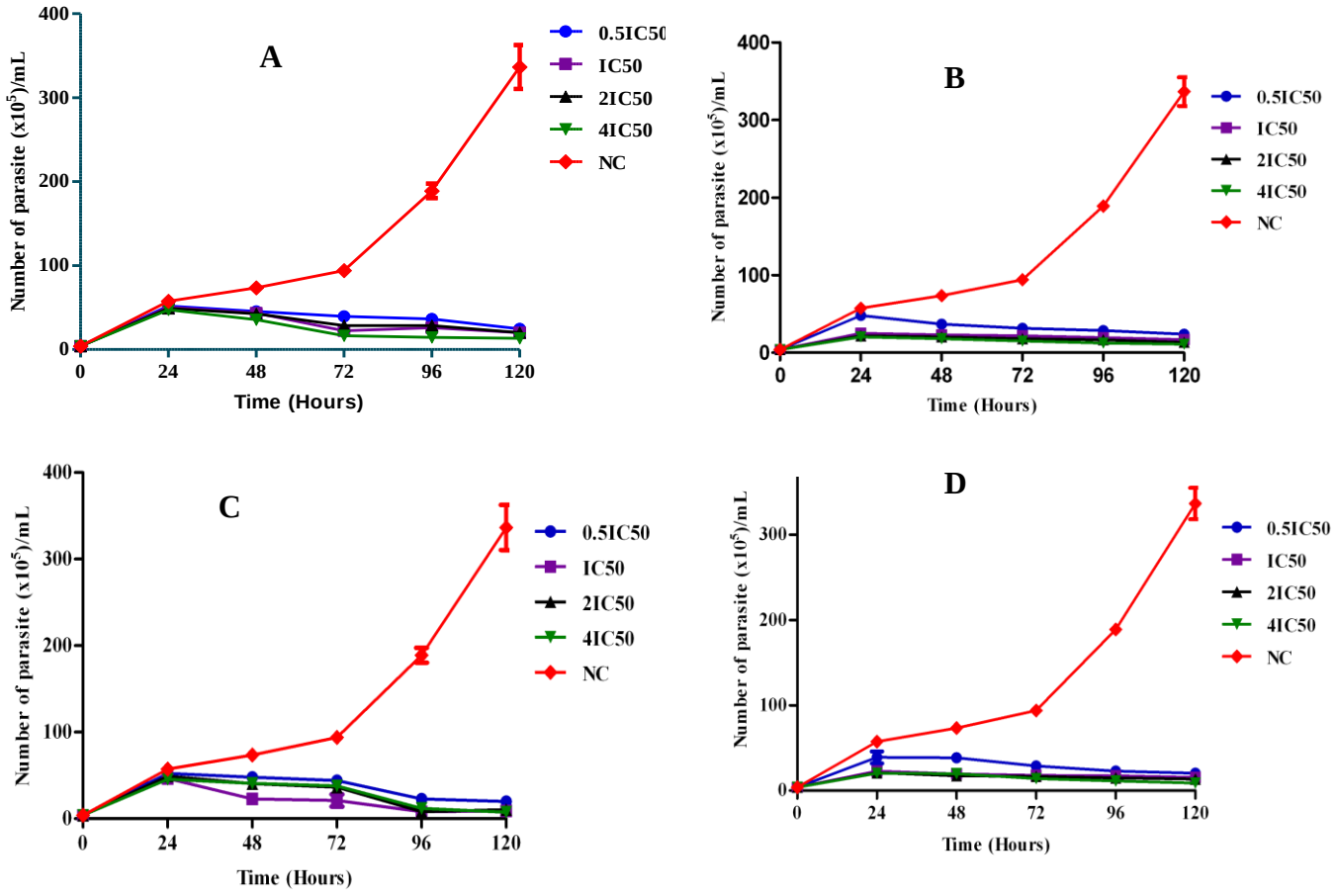


Figure 19: Kill kinetics of *L. donovani* promastigotes in relation to drug concentration and time**.** Cells were treated with different concentrations of (A) Hexanic fraction, (B) UA, (C) OA and (D) amphotericin B. The growth kinetic curves were plotted as the *number of viable parasites vs time* with data collected every 24 hours over a period of 120 hours; NC= Negative control. Each data point represents the mean ± SD from three experiments.

The ability of the hexane fraction, UA and OA to fast-kill the promastigotes of *L. donovani* was assessed at different concentration points (0.5x IC_{50}, IC_{50}, 2x IC_{50} and 4x IC_{50}) relative to untreated parasite culture over a period of 120 hours. The results showed that treatment with

increasing concentrations of the hexane fraction, compounds and amphotericin B resulted in a significant reduction in promastigote replication after 24 hours (**figure 19**). In the meantime, exponential growth was observed in untreated parasite culture.

At all tested concentrations of hexane fraction, compounds and amphotericin B, a regular reduction in the viability of treated parasite cultures was observed up to 120 hours, with no evidence of a cidal effect.

III-1-6- Interaction studies of the most active compounds with the positive control amphotericin B

The following isobolograms represent the results of the interaction studies of the most active compounds with the positive control, amphotericin B:

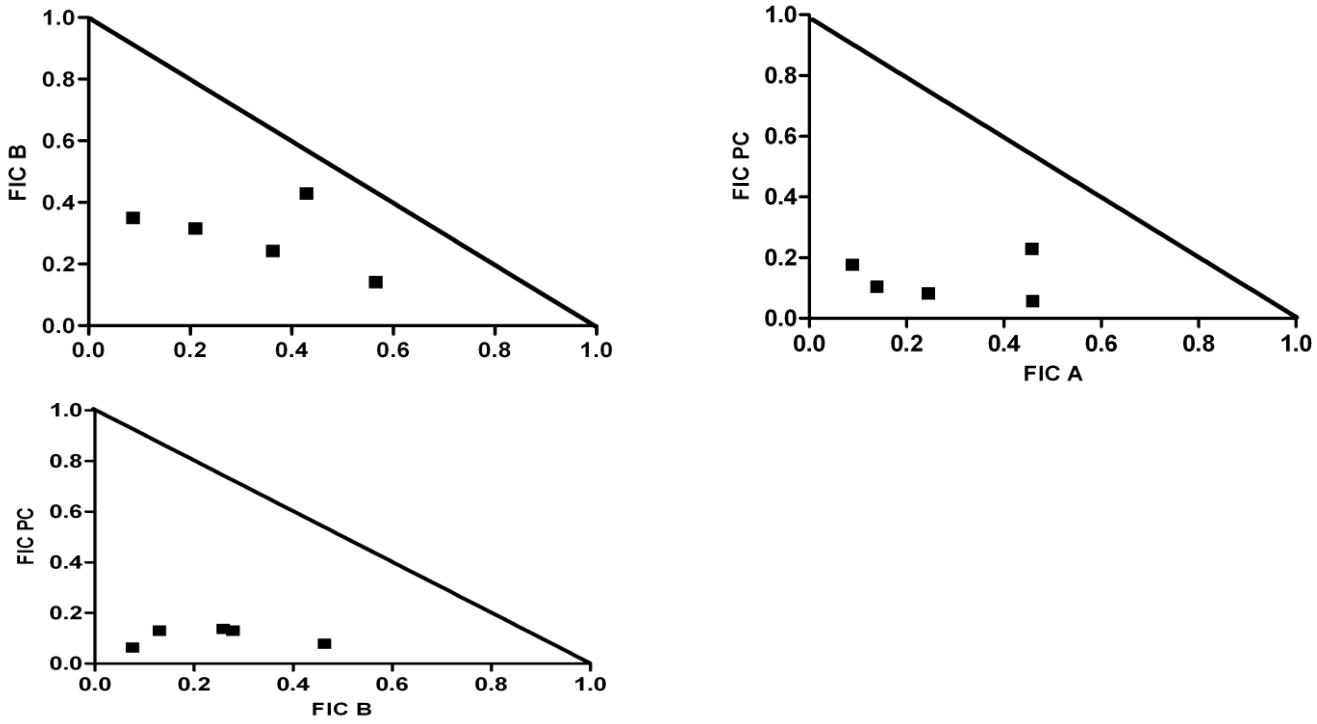
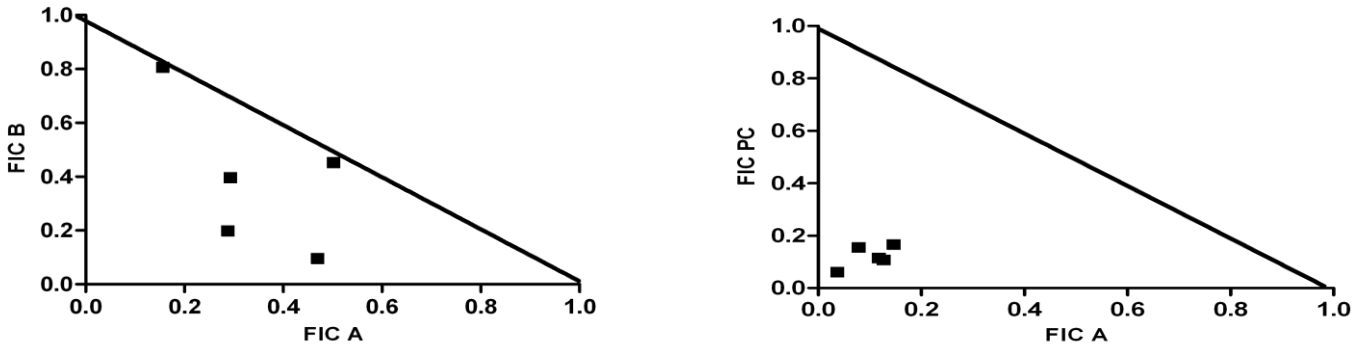


Figure 20: Antileishmanial activities of combinations of UA/Amphotericin B and Ursolic Acid/Oleanolic acid against promastigotes form of *L. donovani*.



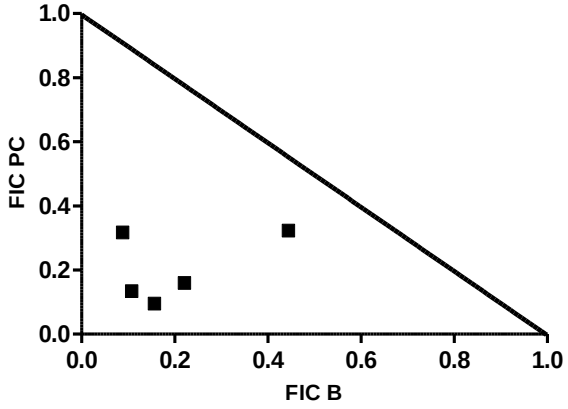


Figure 21: Antileishmanial activities of combinations of OA/amphotericin B and oleanolic acid/ursolic acid against the amastigote form of *L. donovani***.**

By observing all the above graphs, we note that all the points are below the diagonal, indicating a synergistic mechanism. The synergistic effect obtained after combination confirms some results previously obtained in the literature. In fact, a similar result was obtained by **Juliana *et al*. (2011)**, showing that a mixture of UA and OA displayed increased antileishmanial activity, indicating a possible synergistic effect. In the same way, similar results had been previously observed when a mixture of UA and OA showed a higher anti-inflammatory effect compared with the isolated compounds in the paw edema assay **(Vasconcelos *et al*., 2006)**.

III-1-7-Acute toxicity of R. hispida stem extract

The acute toxicity of the *R. hispida* stem extract was determined at a single dose of 2000 mg/kg. After 14 days of experimentation:

- ✓ **No signs of toxicity** were observed 30 min, 1 h or 4 h after administration.
- ✓ **No** deaths were recorded during the 14 days of experimentation.

According to OECD 423 guidelines, we may conclude that the LD_{50} of the extract is higher than 2000 mg/kg **(LD50> 2000 mg/kg).**

III-2- Discussion

The UPLC–MS analysis allows us to detect some compounds that have not been isolated in the crude extract and active fractions. This result has double importance. Firstly, *D. grascilesens* is a rich source of secondary metabolites, and further exploration of the phytochemical studies remains to be encouraged due to the presence of some new uncharacterized compounds detected in this study. Secondly, the antileishmanial activity of the crude extract being more significant than of the isolated compounds could be due to the presence of those non-identified new

secondary metabolites classes or due to the synergy mechanism between the crude extracts and isolated compounds.

Investigating medicinal plants remains one of the credible strategies to find natural products with improved biological activity and safety. The extracts and chemical constituents of *D. grascilesens* were screened for their *in vitro* antileishmanial activity, and the results showed that the hydroethanolic trunk extract exhibited promising antileishmanial potential against *L. donovani* promastigotes. To the best of our knowledge, extracts from *D. grascilesens* have not yet been investigated for their antileishmanial activity. However, extracts from other *Diospyros* spp. have been previously investigated and showed interesting *in vitro* biological properties. In this regard, **Lenta *et al*. (2015)** demonstrated the capacity of the dichloromethane-methanol (1:1) extract of *D. canaliculata* to inhibit the growth of axenic amastigotes of *L. donovani* with an IC_{50} value of 2.99 µg/ml. On the other hand, **Dhar *et al*. (1968)** reported that ethanolic extracts of *D. montana* and *D. pereqrina* possess antiprotozoal activity against *Entamoeba histolytica*, antiviral activity against Ranikhet disease virus and hypoglycemic activities in albino rats. **Rocío *et al*. (2013)** also demonstrated the *in vitro* antimycobacterial potency of the stem bark extract from *D. anisandra* against a resistant strain of *M. tuberculosis*. In other studies, **Hazra *et al*. (1984)** demonstrated the antitumour activity of bark extract from *D. ferrea*. **Asolkar *et al*. (1992)** showed the antibacterial activity of leaf and seed extracts from *D. montana*. **Satish et Sunil (2016)** demonstrated the antidiabetic and antioxidant potential of the ethanolic bark extract of *D. malabarica*.

According to the antileishmanial activity criteria defined by **Camacho *et al*. (2003)**, the hydroethanolic trunk extract of *D. grascilesens* was therefore selected and further processed for fractionation, yielding 5 fractions, among which the hexanic fraction exhibited the best activity against the extracellular and intracellular forms of the parasite and the best selectivity. This result is not surprising because many nonpolar components exhibit a synergistic effect to produce leishmanicidal activity against *L. donovani* promastigotes **(Elham *et al*., 2017)**.

The purification of the most active fractions led to six (6) compounds identified as lupeol, betulin, betulinic acid, a mixture of sterols, β-sitosterol glucoside and 1-deoxyinositol. Among these compounds, betulinic acid showed the most potent activity and selectivity against both promastigotes and intracellular amastigotes forms of *L. donovani*. There are few studies in the literature reporting the activity of betulin and betulinic acid and their derivatives against *Leishmania* parasites. Indeed, **Sousa *et al*. (2014)** reported the antileishmanial activity of semisynthetic lupane triterpenoids, betulin and betulinic acid and described their synergistic effects with miltefosine, an alkylphosphocholine drug with demonstrated activity against various

parasite species (including *Leishmania* parasites and amoeba) and cancer cells as well as some pathogenic bacteria and fungi (**Dorlo *et al.*, 2012)**. **Alakurtti *et al.* (2010)** also determined the activity of heterocyclic betulin derivatives on *L. donovani* amastigotes and showed the *in vitro* antileishmanial activity of betulin and betulinic acid derivatives against *L. donovani* promastigotes and amastigotes of *L. amazonensis,* respectively. **Dominguez *et al.* (2010)** found activity against promastigotes of *L. amazonensis* of betulinic acid acetate and of betulinic acid methyl ester.

Betulinic acid has already been reported in the literature to possess a wide range of biological and medicinal properties, including anti-human immunodeficiency virus (HIV), antibacterial, antimalarial, anti-inflammatory, anthelmintic, antinociceptive, anti-herpes simplex viruses-1 (HSV-1), immune-modulatory, antiangiogenic, and anticancer activities **(Yogeeswari et Sriram, 2005; Gheorgheosu *et al.*, 2014)**. The activity of betulinic acid and its derivatives against the erythrocytic stage of the chloroquine-sensitive 3D7 *Plasmodium falciparum* strain and the antileishmanial activity on different *Leishmania* spp. have also been reported **(Alakurtti *et al.*, 2010; Chen *et al.*, 2010; Innocente *et al.*, 2012)**. **Cassio *et al.* (2016)** also showed that the semisynthetic derivatives of betulinic acid were able to prevent parasite development and invasion into host cells, which are crucial events for *T. cruzi* infection establishment, with potency similar to benznidazole.

In this study, lupeol was also found to be active against promastigotes and moderately active against amastigotes. The antileishmanial potential of this compound has been reported in the literature. Indeed, **Das *et al.* (2017)** evaluated the antileishmanial activity of lupeol against both promastigotes and intracellular amastigotes of *L. donovani* and found that the IC_{50} values were 65 and 15 µg/mL, respectively. Other previous studies indicated that lupeol isolated from aerial parts of *Vernonia scorpioides* displayed weak antileishmanial activity with an IC_{50} value of 100 µg/mL **(Fournet *et al.* 1992, Araujo *et al.* 2016)**.

Some authors in the literature have also evaluated the mechanism of action of lupeol. In this regard, **Ramos *et al.* (1996)** observed that this compound mediates increased cytoplasmic membrane depolarization, which may promote enhanced cell membrane damage. They also suggested that the leishmanicidal activity is explained through disruption of the cytoplasmic membrane of *L. donovani* promastigotes, as evidenced by DISC3-mediated fluorometric analysis. However, the lupeol-mediated reduction in intracellular parasitic load was found to be executed through the induction of a proinflammatory cytokine response and the generation of nitrite oxide (NO) in *L. donovani*-infected macrophages. The heightened Th1 response thereby suppresses the intracellular survival of *L. donovani* amastigotes. They concluded that lupeol might act by inducing cytoplasmic membrane damage in *L. donovani* promastigotes, enhancing the host

protective immune response by regulating NO generation and Th1/Th2 cytokine production **(Das *et al*., 2017)**. Many studies have also highlighted the therapeutic effects of lupeol, where lupeol from latex of *E. Resinifera* and *E. officinarum* showed an anti-promastigote effect on *L. infantum* **(Mazoir *et al*., 2011)**.

Among the isolated compounds, 1-deoxyinositol exerted promising activity against both promastigotes and intracellular amastigotes forms of *L. donovani*. This compound was previously detected by GC–MS with a moderately active (with IC_{50} value of 126.4 µg/mL) methanolic extract from the aerial part of *Scutellaria havanensis* against *L. amazonensis* promastigotes **(Fernandez-Calienes *et al*., 2016)**. The antileishmanial activity of 1-deoxyinositol adds to the novelty of this work given that, to the extent of our knowledge, no previous report has been published on the antileishmanial activity of this compound. More interestingly, 1-deoxyinositol showed the highest selectivity against *L. donovani* promastigotes (SI> 17.75) as well as an acceptable preference for intrazcellular amastigotes (SI> 1.09).

In the case of *R. hipida*, the stem CH_2Cl_2-MeOH extract exhibited very good antileishmanial activity against promastigotes and intracellular amastigotes of *L. donovani*. To the best of our knowledge, this study is the first report of the *in vitro* antileishmanial activity of stems from *R. hipida*. Additionally, **Womkam *et al*. (2020)** demonstrated the good antileishmanial activity of the CH_2Cl_2-MeOH leaf extract from the same plant against promastigotes forms of *L. donovani* with an IC_{50} value of 15.50 µg/mL. However, some extracts from other plants from the Rubiaceae family were studied and showed interesting results. Previous studies reported very good antileishmanial activity of the root extract of *Ixora brachiata* against *L. major* promastigotes, with IC_{100} and IC_{50} values of 2500 µg/mL and 78 µg/mL, respectively **(Sadeghi-Nejad B et Saki, 2014)**. Recently, **Eskandari *et al*. (2019)** demonstrated a good antileishmanial potency of ethanolic extracts from the roots of *Ixora brachiata* against promastigotes and intracellular amastigotes of *L. major* and *L. infantum* with an IC_{50} value of 41.90 µg/mL. Additionally, *Ixora coccinea* leaves showed antileishmanial activity against *L. donovani* promastigotes **(Naskar *et al*., 2013)**.

The stem crude extract of *R. hipida* was further processed for fractionation, yielding 3 fractions, of which the hexane fraction exhibited the best activity against the extracellular and intracellular forms of the parasite and the best selectivity. The most active fractions were selected for purification, and nine (09) compounds were obtained and identified as stigmasterol, mixture stigmasterol and *β*-sitosterol, mixture uvaol and erythrodiol, oleanolic acid, ursolic acid, docosanic acid, *β*-sitosterol glucoside, and lichexanthone. Among the 09 compounds obtained, only three

(03) were tested against both promastigotes and intracellular amastigotes of the *leishmania donovani* parasite, and its cytotoxicity profile against the RAW 264.7-cell line was evaluated.

These compounds are commonly found in many plants in the Rubiaceae family. Recently, it was demonstrated that UA displayed activity against promastigotes and amastigotes of *L. (L.) amazonensis, L. (V.) braziliensis, L. (L.) chagasi* and *L. (L.) major*, suggesting that UA presents multispectral action **(Gnoatto *et al*., 2008; da Silva *et al*. (2009); Bero *et al*., (2011); Inocêncio *et al*., 2011; Passero *et al*., 2011; Johann *et al*., 2012; Begum *et al*., 2014) (Torres-Santos *et al*., 2004)**. In this regard, **Oliveira *et al*. (2012)** isolated Uvaol/erythrodiol, ursolic and oleanolic acids, a mixture of sitosterol and stigmasterol steroids in the ethanolic extract from leaves of *Lecythis pisonis*. They submitted the compounds to cytotoxicity assays, and their results showed that UA and OA acid mixtures showed toxicity against human leukemia (HL-60), human colon (HCT-8), glioblastoma (SF-295), and human melanoma (MDA-MB-435) with IC_{50} values of 3.4, 7.1, 2.4 and 10.6 µg/mL, respectively. In the same way**, Sifaoui *et al*. (2017)** reported that OA exhibited a relatively higher activity against *L. infantum* amastigotes with an IC_{50} value of 0.999 µg/ml and selectivity index of 8.11 against peritoneal macrophages of mice, while UA displayed the best antileishmanial activity and selectivity in this study. Similarly, **Da silva Filho *et al*. (2004, 2009)** isolated UA from *Baccharis dracunculifolia* and reported an IC_{50} value of 3.7 µg/ml. **Peixoto *et al.* (2011)** demonstrated the antileishmanial activity of UA and OA with IC_{50} values of 164.54 µg/ml and 200.7 µg/ml, respectively. **Eduardo *et al*. (2015)** showed the *in vitro* antileishmanial potency of UA against *L. (L.) amazonensis* promastigotes with an IC_{50} value of 6.2 µg/ml. The CC_{50} of this compound was 56.1 µg/ml. However, the antileishmanial and cytotoxicity activities values of OA were greater than 100 µg/ml. Similarly, **Melo *et al*. (2016)** demonstrated that OA exhibited anti-leishmanial activity against *L. amazonensis, L. braziliensis* and *L. infantum*. Moreover, **Jessica *et al*. (2017)** demonstrated that UA isolated from *Baccharis uncinella* showed a therapeutic effect at a dose of 20 mg/kg on hamsters infected with *L. donovani* promastigotes. In the same work, the authors mentioned that the compound did not show significant changes in the spleen, liver, lung, or heart in hamsters treated with 1.0 mg/kg and 2.0 mg/kg ursolic acid or 5.0 mg/kg amphotericin B. The bio-guided fractionation of acetone extract of the roots of *Salvia cilicica* afforded the purification of ursolic acid (UA) and oleanolic acid (OA), with UA being the most active compound against *L. major* promastigotes (**Tan *et al*., 2002**). *Pourouma guianensis*-isolated UA showed significant activity against *L. (L.) amazonensis* promastigotes with an EC_{50} of 5.0 µg/mL **(Torres-Santos *et al*., 2004)**. Moreover, UA isolated from *Baccharis dracunculifolia* was effective against *L. donovani* promastigotes with an EC_{50} of 3.7 µg/mL. **Yamamoto *et al*. (2014)** showed that UA eliminated *L. amazonensis* promastigotes with an EC_{50} of 6.2 µg/mL,

comparable with miltefosine, while its isomer, OA, presented only an effect on promastigote forms at 100 μg/mL. Regarding macrophage toxicity, UA was not toxic to peritoneal macrophages from BALB/c mice, with a CC_{50} value of 56.1 μg/mL, and it was able to eliminate intracellular amastigotes associated with nitric oxide (NO) production. The divergent results may be related to the diversity of parasite strains, parasitic load, and stages of the parasite life cycle. Differences in the applied experimental conditions can also lead to different IC_{50} values of amphotericin B, which was used as a positive control in both studies, also reinforcing the divergent results obtained for the compounds (**Juliana *et al*., 2011)**.

The very high activity of lupeol, UA, OA and betulinic acid in this study, which showed the most potent activity, may also be attributed to their constitution. All these compounds belong to the class of triterpenoids. Triterpenoids are the most representative group of phytochemicals, comprising more than 20,000 known compounds that can be classified into groups based on their structural skeletons, such as cycloartanes, dammaranes, euphanes, friedelanes, lanostanes, lupanes, oleananes, tirucallanes, and ursanes **(Hill and Connolly, 2012)**. The diversity of triterpenes is highly associated with their broad range of pharmacological effects, and different studies have already shown that these compounds present multispecies action against *Leishmania sp.* Different authors have suggested that the antileishmanial activity of these compounds could be related to the inhibition of protein and nucleic acid synthesis or of a membrane-associated calcium-dependent ATPase pump **(Goijman *et al*., 1984; Mishina *et al*., 2007)**. Indeed, previous studies have suggested that lipophilic compounds, such as triterpenes, act by a peculiar mechanism. These compounds can pass easily through the cytoplasmic membranes, affecting the structures of their different layers of polysaccharides, fatty acids, and phospholipids, thus making them permeable **(DiPasqua *et al*. 2007)**. Once they cross the membrane, coagulation of the cytoplasm can occur. These events are able to promote the interruption of specific metabolic pathways of lipids and proteins **(Ultee *et al*., 2000)**, interfere with cell division **(Fortes *et al*., 2013; De Almeida *et al*. 2003)**, or stimulate the depolarization of the mitochondrial membranes, which can lead the cell to trigger necrosis or apoptosis mechanisms **(Armstrong *et al*., 2006)**. The OA and its isomer, ursolic acid (UA), are triterpenoid compounds that widely occur in nature in the free acid form or as an aglycone precursor for triterpenoid saponins. These triterpenoid acids frequently occur simultaneously because they share similar structural features. The leishmanicidal effects of ursolic and oleanic acids have already been evaluated against several *leishmania sp*. suggesting that UA can be considered a prototype drug. The UA and OA acids are capable of activating the immune system to induce the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) **(Mukherjee *et al*., 1999)**, cytokines that act in synergy in the activation of macrophages

to produce nitric oxide and control the infection by *Leishmania* **(Bogdan *et al.*, 1999)**. An ultrastructural study revealed that UA caused parasite morphological alterations, which can be associated with apoptosis or even autophagy **(Saraste et Pulkki, 2000)**. Studies dealing with the antitumoral effect of UA-treated R-HepG2 and SW480 tumoral cells presented ultrastructural alterations similar to those found in UA-treated *L. amazonensis*, suggesting that at least one of the possible mechanisms of action triggered by ursolic acid is programmed cell death **(Yang *et al.*, 2010; Nam et Kim, 2013)**.

In addition, the leishmanicidal effect observed on intracellular amastigotes could be attributed to the activation of NO production by macrophages. The immunomodulatory properties of marine compounds have been described along with their inhibitory activity on NO production **(Mayer *et al.*, 2011)**. Among the main factors of the immunity that are recruited to confront *Leishmania* infection are the macrophages that produce effector molecules such as nitric oxide (NO) and reactive oxygen species (ROS). In fact, nitric oxide (NO) has been demonstrated to be the principal effector molecule mediating the intracellular killing of *Leishmania*, and it is a key molecule in the macrophage defense system against intracellular parasites. Activated macrophages are capable of killing parasites effectively via the production of large amounts of nitric oxide (NO) upon activation of inducible nitric oxide synthase (iNOS) and other leishmanicidal molecules, such as reactive oxygen species (ROS), which have many biological functions, including defense against intracellular pathogens and particularly *Leishmania* **(reviewed in Nathan and Shiloh, 2000; Brunet, 2001; Colasanti *et al.*, 2002). Horta *et al.*, 2012; Roy *et al.*, 2017)**. The most efficient mechanism of parasite death involves the production of IFN-γ and tumor necrosis factor alpha (TNF-α) by $CD4^+$ Th1 cells. These stimulate the synthesis of inducible iNOS, generating NO, a potent cytotoxin involved in *Leishmania* parasite clearance or inhibition **(Horta *et al.*, 2012)**.

Some authors in the literature have shown that lipophilicity is an important parameter in the development of biological agents, so molecules with carbon chains above C-10 are fairly lipophilic and good candidates for pharmacological evaluation **(Koch *et al.*, 2005; Irungu *et al.*, 2007; Terrazzino *et al.*, 2013; Mallavadhani and coworkers; 2004)**.

In the time killing assay, the action of the hexanic fraction, UA and OA acids depended on the time and concentration. After a period of 24 h, the inhibition effect was evident at doses equal to or above its IC_{50} even if complete clearance was not observed until 120 h. The rapid growth inhibition comparable to the effect of the positive control suggests that UA has a leishmania-static effect.

CONCLUSION AND PERSPECTIVES

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This study aimed to investigate the *in vitro* antileishmanial bioguided isolation of active natural products from *D. gracilescens* (Ebenaceae) and *R. hispida* stems (Rubiaceae). At the end of this study, the following conclusions were made:

- ✓ The hydroethanolic crude extract of *D. gracilescens* trunk showed the most potent antileishmanial activity against promastigotes of *L. donovani* (**IC_{50} = 5.84 µg/mL**). The bio-guided fractionation of *D. gracilescens* trunk extract led to four (04) fractions, of which the hexane fraction showed the most potent activity (**IC_{50} = 0.79 µg/mL**). The purification of the active fractions led to the isolation of six (06) compounds that exhibited decreased potency (**IC_{50} = 5.50-38.75 µg/mL**) compared to the parent fraction. The promising trunk extract and the derived hexane fraction from *D. grascilesens* showed acceptable selectivity (SI>18). However, most of the derived compounds isolated from *D. grascilesens* were not selective (SI<1).The promising hexanic fraction showed significant inhibition of parasite growth at different concentrations, but with no evidence of a cidal effect over a screening period of 120 hours.
- ✓ The CH_2Cl_2-MeOH crude extract of *R. hispida* stem showed the most potent antileishmanial activity against promastigotes of *L. donovani* (**IC_{50} = 5.74 µg/mL**). The bio-guided fractionation of this extract led to three (03) fractions, of which the hexane fraction showed the most potent activity (**IC_{50} = 4.38 µg/mL**). The purification of the active fractions led to the isolation of nine (09) compounds, of UA showed the most potent activity (**IC_{50} = 0.75 µg/mL**) compared to the parent fraction. The ursolic and oleanolic acids compounds showed acceptable selectivity (SI>18), and significant inhibition of parasite growth at different concentrations, but with no evidence of a cidal effect over a screening period of 120 hours. The interaction studies of the two (02) most active compounds, UA and OA, with the positive control amphotericin B showed a synergetic effect against promastigotes and the intracellular form amastigotes forms of *L. donovani*. UA and OA showed good scavenging DPPH and Fe^{3+} chelation activities. The acute toxicity assay showed that the lethal dose 50 (LD_{50}) of *R. hispida* stem extract was higher than 2000 mg/kg, and the compound was classified as non-toxic.

Globally, the results obtained in this study indicate that the derived hexane fraction from *D. gracilescens,* the UA and OA from *R. hispida* have very potent inhibitory effects on cultivated promastigotes and intracellular amastigotes forms of the *L. donovani* parasite.

For future studies, we plan to:

- ✓ Carry out some chemical transformations on UA and OA and evaluate the antileishmanial activity of the derivatives against promastigotes and intracellular amastigotes forms of *L. donovani*.
- ✓ The *in vivo* toxicological profile and the *in vivo* antileishmanial activity of the potent fraction/compounds and their combinations will be determined.
- ✓ Evaluate the activity of our active compounds/combinations on some enzymes (phosphatase acid, phosphatase alkaline) and other components of the immune system.
- ✓ Formulate a phytodrug that could be used alone or in combination that could serve as treatment against visceral leishmaniasis that exacts a very heavy toll to poor patients in remote endemic settings in Africa and elsewhere.

BIBLIOGRAPHIC REFERENCES

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Abdolmajid, F., Ghodratollah, S. S., Hushang, R., Mojtaba, M. B., Ali, M. M., Abdolghayoum, M. (2015). Identification of *Leishmania* species by kinetoplast DNA-polymerase chain reaction for the first time in Khaf district, Khorasan-e-Razavi province, Iran. *Tropical Parasitology*, ***5***(1), 50.

Acebey Castellon, I. L. (2007). *Caractérisation de terpènes antileishmaniens isolés par bioguidage d'une plante bolivienne Hedyosmum angustifolium (Ruiz et Pavon) Solms Chloranthaceae* (Doctoral dissertation).

Adzu, B., Chindo, B. A., Tarfa, F. D., Salawu, O. A., Igoli, O. J. (2015). Isolation and analgesic property of lupeol from *Diospyros mespiliformis* stem bark. *Journal of Medicinal Plants Research*, ***9***(30), 813-819.

Akak, C. M., Djama, C. M., Nkengfack, A. E., Tu, P. F., Lei, L. D. (2010). New coumarin glycosides from the leaves of *Diospyros crassiflora* (Hiern). *Fitoterapia*, ***81***(7), 873-877.

Akhoundi, M., Kuhls, K., Cannet, A., Votýpka, J., Marty, P., Delaunay, P., Sereno, D. (2016). A historical overview of the classification, evolution, and dispersion of *Leishmania* parasites and sandflies. *PLoS Neglected Tropical Diseases*, ***10***(3), e0004349.

Alavi-Naini, R., Fazaeli, A., O'dempsey, T. (2012). Topical treatment modalities for old world cutaneous leishmaniasis: a review. *Prague Medical Report*, ***113***(2), 105-118.

Alidadi, S., Oryan, A. (2014). Cutaneous leishmaniasis and the strategies for its prevention and control. *Tropical Medicine and Surgery*.

Alexandrino-de-Oliveira, P., Santos-Oliveira, J. R., Dorval, M. E. C., Da-Costa, F. D. C. B., Pereira, G. R. O. L., Cunha, R. V. D., Paniago, A. M., Da-Cruz, A. M. (2010). HIV/AIDS-associated visceral leishmaniasis in patients from an endemic area in Central-west Brazil. *Memórias do Instituto Oswaldo Cruz*, ***105***(5), 692-697.

Alakurtti, S., Heiska, T., Kiriazis, A., Sacerdoti-Sierra, N., Jaffe, C. L., Yli-Kauhaluoma, J. (2010). Synthesis and anti-leishmanial activity of heterocyclic betulin derivatives. *Bioorganic and Medicinal Chemistry*, ***18***(4), 1573-1582.

Almeida, T. F., Palma, L. C., Mendez, L. C., Noronha-Dutra, A. A., Veras, P. S. (2012). *Leishmania amazonensis* fails to induce the release of reactive oxygen intermediates by CBA macrophages. *Parasite Immunology*, **34**(10):492–498.

Alrajhi, A. A., Ibrahim, E. A., De Vol, E. B., Khairat, M., Faris, R. M., Maguire, J. H. (2002). Fluconazole for the treatment of cutaneous leishmaniasis caused by *Leishmania major*. *New England Journal of Medicine*, ***346***(12), 891-895.

Alvar, J., Aparicio, P., Aseffa, A., Den Boer, M., Canavate, C., Dedet, J. P., Gradoni, L.; Ter Horst, R.; Lopez-Velez, R., Moreno, J. (2008). The relationship between leishmaniasis and AIDS: the second 10 years. *Clinical Microbiology Reviews*, ***21***(2), 334-359.

Armstrong, J. S. (2006). Mitochondrial membrane permeabilization: the sine qua non for cell death. *Bioessays*, 28(3), 253-260.

Anabwani, G. M., Dimiti, G., Ngira, J. A., Bryceson, A. D. M. (1983). Comparison of two dosage schedules of sodium stibogluconate in the treatment of visceral leishmaniasis in Kenya. *The Lancet*, ***321***(8318), 210-212.

Andersson, L. (1993). Pollen characteristics of the tribes Calycophylleae, Cinchoneae, and Hillieae (Rubiaceae). *Nordic Journal of Botany*, ***13***(4), 405-417.

Andersson, L., Persson, C. (1991). Circumscription of the tribeCinchoneae (Rubiaceae)-a cladistic approach. *Plant Systematics and Evolution*, ***178***(1), 65-94.

Antai, A. B., Anaele, B. A., Etta, K. M. (2005). Hypoglycemic actions of a medicinal herb, *Rothmannia hispida* in diabetic rats. *Mary Slessor Journal of Medicine*, **5**(2), 21-24.

Antai, A. B., Ndep, A. O., Etta, K. M. (1995). Acute effects of a medicinal herb, *Rothmannia hispida* on alloxan-induced diabetes in the rat. In *Proceeding of the Annual Scientific Conference of the Nigerian Physiological Association*, **11**(1-2), p 68.

Antai, A. B., Ofem, O. E., Nwosu, O. J., Ukafia, S. O., Iyadi, K. C., Nia, R., Osim, E. E. (2010). Comparative effects of *Rothmannia hispida* leaves extract and protamine-zinc insulin on alloxan-induced diabetic rats. *African Journal of Biomedical Research*, **13(**1), 47-54.

Arana, B. A., Mendoza, C. E., Rizzo, N. R., Kroeger, A. (2001). Randomized, controlled, double-blind trial of topical treatment of cutaneous leishmaniasis with paromomycin plus methylbenzethonium chloride ointment in Guatemala. *The American journal of Tropical Medicine and Hygiene*, ***65***(5), 466-470.

Araujo, C., Silva, R., Silva, M., Takahashi, A., Sales-Junior, A., Dessimoni-Pinto, V., Souza-Fagundes, M., Romanha, J., Murta, F., Alcantara, A. (2016). Constituents from stem barks of *Luehea ochrophylla* Mart and evaluation of their antiparasitic, antimicrobial, and antioxidant activities. Natural Product Research, **31**(16), 1948–53.

Arevalo, J., Ramirez, L., Adaui, V., Zimic, M., Tulliano, G., Miranda-Verástegui, C., Lazo, M., Loayza-Muro, R., De Doncker, S., Maurer, A., Chappuis, F., Dujardin, J. C., Llanos-Cuentas, A. (2007). Influence of *Leishmania* (Viannia) species on the response to antimonial treatment in patients with American tegumentary leishmaniasis. *The Journal of Infectious Diseases*, ***195***(12), 1846-1851.

Asolkar, L. V., Kakkar, K. K., Chakre, O. J. (1992). *Second supplement to glossary of Indian medicinal plants with active principles: part-1 (A–K), (1965-1981).*

Attar, Z. J., Chance, M. L., el-Safi, S., Carney, J., Azazy, A., El-Hadi, M., Dourado, C., Hommel, M. (2001). Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. *Acta Tropica*, ***78***(1), 11-16

Aubréville, 1959: **3**: 278. 2.

Bajpai, V. K., Rather, I. A., Shukla, S. (2016). Oxidative stress: role of natural antioxidant compounds. *In Microbes in Food and Health*, Springer, Cham, 65-76.

Bassene, E. (2012). *Initiation à la recherche sur les substances naturelles: Extraction-Analyze-Essais Biologiques*. Presses Universitaires de Dakar.

Begum, S., Ayub, A., Qamar, Z. S., Shaheen, S. B, Iqbal, C. M., Samreen. (2014). Leishmanicidal Triterpenes from *Lantana camara*. *Chemistry Biodiversity*; **11**(5), 709–718.

Belehu, A., Naafs, B. (1982). Diabetes mellitus associated with pentamidine mesyiate. *Diabetes mellitus associated with pentamidine mesyiate, **1***, 1463-1464.

Beniddir, A. M. (2012). *Recherche de composés à activité antiplasmodiale à partir de la biodiversité malgache* (Doctoral dissertation, Université Paris Sud-Paris XI).

Ben Salah, A., Ben Messaoud, N., Guedri, E., Zaatour, A., Ben Alaya, N., Bettaieb, J., Gharbi, A., Belhadj Hamida, N., Boukthir, A., Chlif, S., Abdelhamid, K., El Ahmadi, Z., Louzir, H., Mokni, M., Morizot, G., Buffet, P., Smith, P. L., Kopydlowski, K. M., Kreishman-Deitrick, M., Smith, K. S., Nielsen, C. J., Ullman, D. R., Norwood, J. A., Thorne, G. D., McCarthy, W. F., Adams, R. C., Rice, R. M., Tang, D., Berman, J., Ransom, J., Magill, A. J., Grogl, M. (2013). Topical paromomycin with or without gentamicin for cutaneous leishmaniasis. *New England Journal of Medicine*, ***368***(6), 524-532.

Berman, J. (2003). Current treatment approaches to leishmaniasis. *Current Opinion in Infectious Diseases*, ***16***(5), 397-401.

Berman, J. (2005). Recent developments in leishmaniasis: epidemiology, diagnosis, and treatment. *Current Infectious Disease Reports*, ***7***(1), 33-38.

Berman, J. D., Badaro, R., Thakur, C. P., Wasunna, K. M., Behbehani, K., Davidson, R., Kuzoe, F., Pang, L., Weerasuriya, K., Bryceson, A. D. (1998). Efficacy and safety of liposomal amphotericin B (AmBisome) for visceral leishmaniasis in endemic developing countries. *Bulletin of the World Health Organization*, ***76***(1), 25.

Bero, J., Hannaert, V., Chataigné, G., Hérent, M. F., Quetin-Leclercq, J. (2011). *In vitro* antitrypanosomal and antileishmanial activity of plants used in Benin in traditional medicine

and bioguided fractionation of the most active extract. *Journal of Ethnopharmacology*, ***137***(2), 998-1002.

Bogdan, C., Röllinghoff, M. (1999). How do protozoan parasites survive inside macrophages? *Parasitology Today*, ***15***(1), 22-28.

Bogdan C, Rollinghoff M, Diefenbach A. (2000). The role of nitric oxide in innate immunity. ***Immunology Review***, 173:17–26.

Botero, D. (2006). Marcos Restrepo. *Parasitosis humana. 4a ed. Medellín, Colombia. Corporación para Investigaciones Biológicas (CIB).*

Brahmachari, U. (1928). A Treatise on Kala Azar. *A Treatise on Kala Azar.*

Bremer, B., Jansen, R. K. (1991). Comparative restriction site mapping of chloroplast DNA implies new phylogenetic relationships within Rubiaceae. *American Journal of Botany*, ***78***(2), 198-213.

Bremer, B., Jansen, R. K., Oxelman, B., Backlund, M., Lantz, H., Kim, K. J. (1999). More characters or more taxa for a robust phylogeny—case study from the coffee family (Rubiaceae). *Systematic Biology*, ***48***(3), 413-435.

Bremekamp, C. E. B. (1966). Remarks on the position, the delimitation and the subdivision of the Rubiaceae. *Acta Botanica Neerlandica*, ***15***(1), 1-33.

Brunet, L. R. (2001). Nitric oxide in parasitic infections. *International Immunopharmacology*, ***1***(8), 1457-1467.

Brustoloni, Y. M., Cunha, R. V., Consolo, L. Z., Oliveira, A. L. L., Dorval, M. E. C., Oshiro, E. T. (2010). Treatment of visceral leishmaniasis in children in the Central-West Region of Brazil. *Infection*, ***38***(4), 261-267.

Burton, J. B., Clint, E. C., Thomas, N. O. (2012). *Human parasitology*. Fourth edition. *Elsevier*, 430p.

Calegari-Silva, T. C., Pereira, R. M., De-Melo, L. D. B., Saraiva, E. M., Soares, D. C., Bellio, M., Lopes, U. G. (2009). NF-κB-mediated repression of iNOS expression in *Leishmania amazonensis* macrophage infection. *Immunology Letters*, ***127***(1), 19-26.

Camacho, M. D. R., Phillipson, J. D., Croft, S. L., Solis, P. N., Marshall, S. J., Ghazanfar, S. A. (2003). Screening of plant extracts for antiprotozoal and cytotoxic activities. *Journal of Ethnopharmacology*, ***89***(2-3), 185-191.

Cardon, D. (2005). *Dyes and Tannins* (Vol. **3**). PROTA.

Cassio, S. M., Jose, M. B., Lanfredi-Rangel, A., Elisalva, T. G., Diogo, M., Milena, S. (2016). Antiparasitic evaluation of betulinic acid derivatives reveals effective and selective anti-*Trypanosoma cruzi* inhibitors. *Experimental Parasitology*, **66**:108–15.

Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R. W., Alvar, J., Boelaert, M. (2007). Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nature Reviews Microbiology*, ***5***(11), 873-882.

Chen, Y., Li, S., Sun, F., Han, H., Zhang, X., Fan, Y., Tai, G., Zhou, Y. (2010). *In vivo* antimalarial activities of glycoalkaloids isolated from Solanaceae plants. *Pharmaceutical Biology*, ***48***(9), 1018-1024.

Chulay, J. D., Spencer, H. C., Mugambi, M. (1985). Electrocardiographic changes during treatment of leishmaniasis with pentavalent antimony (sodium stibogluconate). *The American Journal of Tropical Medicine and ygiene*, ***34***(4), 702-709.

Chulay, J. D., Fleckenstein, L., Smith, D. H. (1988). Pharmacokinetics of antimony during treatment of visceral leishmaniasis with sodium stibogluconate or meglumine antimoniate. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **82(**1), 69-72. PMID: 2845611.

Colasanti, M., Gradoni, L., Mattu, M., Persichini, T., Salvati, L., Venturini, G., Ascenzi, P. (2002). Molecular bases for the anti-parasitic effect of NO. *International Journal of Molecular Medicine*, ***9***(2), 131-134.

Collins, S. W. N., Happi, G. M., Pouofo, M. N., Tchamgoue, J., Longo, F., Lenta N.B., Sewald, N., Kouam, S. F. (2020). Antibacterial flavonoids and other compounds from the aerial parts of Vernonia guineensis Benth.(Asteraceae). *Chemistry and Biodiversity*, ***17***(9), e2000296.

Cook, G. C. (2006). Leonard Rogers KCSI FRCP FRS (1868–1962) and the founding of the Calcutta School of Tropical Medicine. *Notes and Records of the Royal Society*, ***60***(2), 171-181.

Croft, S. L., Coombs, G. H. (2003). Leishmaniasis–current chemotherapy and recent advances in the search for novel drugs. *Trends in Parasitology*, ***19***(11), 502-508.

Croft, S. L., Snowdon, D., Yardley, V. (1996). The activities of four anticancer alkyllysophospholipids against *Leishmania donovani*, *Trypanosoma cruzi* and *Trypanosoma brucei*. *Journal of Antimicrobial Chemotherapy*, ***38***(6), 1041-1047.

Cruz, I., Nieto, J., Moreno, J., Cañavate, C., Desjeux, P., Alvar, J. (2006). Leishmania/HIV coinfections in the second decade.

Cunha, A. M., Chagas, E. (1937). New species of protozoa of the genus *Leishmania* pathogenic to man *Leishmania* chagasi n. sp previous note. *O Hospital*, ***11***, 3-9.

De Matta, A. A. (1916). Sur les leishmanioses tegumentaires. Classification generale des leishmainoses. *Bulletin de la Societe de Pathologie Exotique et de Ses Filiales*, ***9***, 494-503.

da Silva Filho, A. A., Pires, B. P. C., Gregório, L. E., Andrade, S. M. L., Albuquerque, S., Bastos, J. K. (2004). *In vitro* trypanocidal activity evaluation of crude extract and isolated compounds from *Baccharis dracunculifolia* D.C. (Asteraceae). *Journal of Pharmacy and Pharmacology*, **56**(9), 1195-1199.

da Silva Filho, A. A., Resende, D. O., Fukui, M. J., Santos, F. F., Pauletti, P. M., Cunha, W. R., Nanayakkara, N. P. D. (2009). *In vitro* antileishmanial, antiplasmodial and cytotoxic activities of phenolics and triterpenoids from *Baccharis dracunculifolia* DC (Asteraceae). *Fitoterapia*, ***80***(8), 478-482.

Das, A., Jawed, J. J., Das, M. C., Sandhu, P., De, U. C., Dinda, B., Bhattacharjee, S. (2017). Antileishmanial and immunomodulatory activities of lupeol, a triterpene compound isolated from *Sterculia villosa*. *International Journal of Antimicrobial Agents*, ***50***(4), 512-522.

Davidson, R. N., Martino, L. D., Gradoni, L., Giacchino, R., Russo, R., Gaeta, G. B., Pempinello, R., Scott, S., Raimondi, F., Cascio, A. T., Prestileo, T., Caldeira, L., Wilkinson, R. J., Bryceson, A. D. M. (1994). Liposomal amphotericin B (AmBisome) in Mediterranean visceral leishmaniasis: a multicenterentre trial. *An International Journal of Medicine*, ***87***(2), 75-81.

De Almeida, M. C., Vilhena, V., Barral, A., Barral-Netto, M. (2003). Leishmanial infection: analysis of its first steps. A review. *Memorias do Instituto Oswaldo Cruz*, ***98***(7), 861-87.

Dedet, J. P. (1999). *Les Leishmanioses*. Paris: Ellipses.

Delgado, J., MacÃas, J., Pineda, J. A., Corzo, J. E., de la Rosa, R., Leal, M., Lissen, E. (1999). High frequency of serious side effects from meglumine antimoniate given without an upper limit dose for the treatment of visceral leishmaniasis in human immunodeficiency virus type-1-infected patients. *The American Journal of Tropical Medicine and Hygiene*, ***61***(5), 766-769.

De Lima, J. P., Pinheiro, M. L., Santos, A. M. G., Pereira, J. L. D. S., Santos, D. M., Barison, A., Silva Jardim, I., Costa, E. V. (2012). *In vitro* antileishmanial and cytotoxic activities of *Annona mucosa* (Annonaceae). *Rev Virtual Quim*, ***4***(6), 692-702.

Delprete, P. G. (1999). Rondeletieae (Rubiaceae): Part I (Rustia, Tresanthera, Condaminea, Picardaea, Pogonopus, Chimarrhis, Dioicodendron, Molopanthera, Dolichodelphys, and Parachimarrhis). *Flora Neotropica*, 1-225.

De Muylder, G., Ang, K. K., Chen, S., Arkin, M. R., Engel, J. C., McKerrow, J. H. (2011). A screen against Leishmania intracellular amastigotes: comparison to a promastigote screen and identification of a host cell-specific hit. *PLoS Neglected Tropical Diseases*, ***5***(7), e1253.

Dhar, M. L., Dhar, M. M., Dhawan, B. N., Mehrotra, B. N., Ray, C. (1968). Screening of Indian plants for biological activity: *Indian Journal of Experimental Biology* : Part I.

Desjeux, P. (2001). The increase in risk factors for leishmaniasis worldwide. *Transactions of the royal society of tropical medicine and hygiene*, **95(**3), 239-243.

Desjeux, P. (2004). Leishmaniasis: current situation and new perspectives. *Comparative immunology, microbiology and infectious diseases*, ***27***(5), 305-318.

Di Cristina, G., Caronia, G. (1915). Sulla terapia della leishmaniosi interna. *Pathologica*, ***7***, 82-83.

Di Pasqua, R., Betts, G., Hoskins, N., Edwards, M., Ercolini, D., Mauriello, G. (2007). Membrane toxicity of antimicrobial compounds from essential oils. *Journal of Agricultural and Food Chemistry*, ***55***(12), 4863-4870.

Domínguez-Carmona, D. B., Escalante-Erosa, F., García-Sosa, K., Ruiz-Pinell, G., Gutierrez-Yapu, D., Chan-Bacab, M. J., Giménez-Turba, A., Peña-Rodríguez, L. M. (2010). Antiprotozoal activity of betulinic acid derivatives. *Phytomedicine*, ***17***(5), 379-382.

Dondji, B. (2001). Leishmanioses et phlébotomes du Cameroun: le point sur les données actuelles. *Bulletin de la Société de pathologie exotique*, ***94***(3), 277-279.

Dorlo, T. P., Balasegaram, M., Beijnen, J. H., de Vries, P. J. (2012). Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *Journal of Antimicrobial Chemotherapy*, ***67***(11), 2576-2597.

Duangjai, S., Wallnöfer, B., Samuel, R., Munzinger, J., Chase, M. W. (2006). Generic delimitation and relationships in Ebenaceae sensu lato: evidence from six plastid DNA regions. *American Journal of Botany*, ***93***(12), 1808-1827.

Dumaro, C. A., Etim, E., Ahmadu, A. (2017). Anti-Inflammatory constituents of *Randia hispida* K. Schum (Rubiaceae). *Journal of Chemical and Pharmaceutical Research*, 9, 160-164.

Dye, C., Wolpert, D. M. (1988). Earthquakes, influenza and cycles of Indian kala-azar. *Transactions of the royal society of tropical medicine and hygiene*, ***82***(6), 843-850.

Ejazi, S. A., Ali, N. (2013). Developments in diagnosis and treatment of visceral leishmaniasis during the last decade and future prospects. *Expert review of anti-infective therapy*, ***11***(1), 79-98.

Elham, A., Abeer, Moawad., Hossam, H. (2017). Biologically guided isolation of leishmanicidal secondary metabolites from *Euphorbia peplus* L. *Saudi Pharmaceutical Journal*, **25**, 236–240.

El-Seedi, H. R., Hazell, A. C., Torssell, K. B. (1994). Triterpenes, lichexanthone and an acetylenic acid from *Minquartia guianensis*. *Phytochemistry*, ***35***(5), 1297-1299.

Eskandari, K., Khademvatan, S., Nejad, B. S., Naanaie, S. Y. (2019). Evaluation of the Antileishmanial Properties of *Ixora brachiata* Roxb on *Leishmania major* and *Leishmania infantum* by Colorimetric MTT Assay. *International Journal of Enteric Pathogens*, ***7(***4), 126-129.

Etukudo, I. (2003). Ethnobotany conversational and traditional uses of plants, Calabar, Nigeria. p. 53.

Esch, K. J., Petersen, C. A. (2013). Transmission and epidemiology of zoonotic protozoal diseases of companion animals. *Clinical microbiology reviews*, ***26***(1), 58-85.

Evans, T. G., Thai, L., Granger, D. L., Hibbs, J. B. (1993). Effect of *in vivo* inhibition of nitric oxide production in murine leishmaniasis. *The Journal of Immunology*, ***151***(2), 907-915.

Fernández-Calienes Valdés, A., Monzote, F. L., Sariego, R. I., Marrero, D. D., Morales, R. C. L., Mendiola, M. J., Cuéllar, A. C. (2016). Antiprotozoal screening of the Cuban native plant Scutellaria havanensis. *Pharmaceutical Biology*, ***54***(12), 3197-3202.

Fortes, A., Almeida, A., Mendonc¸a-Junior, F., Freitas, R., Soares-Sobrinho, L., De La Roca Soares, F. (2013). "Anxiolytic properties of new chemical entity, 5TIO1," *Neurochemical Research*, **38**(4), 726–731.

Foulet, F., Botterel, F., Buffet, P., Morizot, G., Rivollet, D., Deniau, M., Pratlong, F., Costa, J. M., Bretagne, S. (2007). Detection and identification of *Leishmania* species from clinical specimens by using a real-time PCR assay and sequencing of the cytochrome B gene. *Journal of Clinical Microbiology*, *45*(7), 2110-2115.

Fournet, A., Angelo, A., Muñoz, V., Roblot, F., Hocquemiller, R., Cavé, A. (1992). Biological and chemical studies of *Pera benensis*, a Bolivian plant used in folk medicine as a treatment of cutaneous leishmaniasis. *Journal of Ethnopharmacology*, ***37***(2), 159-164.

Freire, M., Badaró, F., Avelar, M. E., Luz, K., Nakatani, M. S., Teixeira, R., Martins Netto, E., Badaró, R. (1997). Efficacy and tolerability of liposomal amphotericin B (Ambisome) in the treatment of visceral leishmaniasis in Brazil. *Brazilian Journal of Infectious Diseases*, ***1***(5), 230-40.

Furtado, T. A., Cisalpino, E. O., Santos, U. M. (1960). *In vitro* Studies of the Effect of Amphotericin B in *Leishmania* brasiliensis. *Antibiotics & Chemotherapy*, ***10***(11), 692-693.

Gafner, F., Chapuis, J. C., Msonthi, J. D., Hostettmann, K. (1987). Cytotoxic naphthoquinones, molluskicidal saponins and flavonols from *Diospyros zombensis*. *Phytochemistry*, ***26***(9), 2501-2503.

Gantt K, R., Goldman, T. L., McCormick, M. L., Miller, M. A., Jeronimo, S. M., Nascimento, E. T., et al. Oxidative responses of human and murine macrophages during

phagocytosis of Leishmania chagasi. J Immunol. 2001; 167(2):893–901. Epub 2001/07/07. PMID: 11441096.

Garcia, P. M., Guasch, J., Llaurado, F. (1950). First cases of infantile kala-azar treated in Spain with diamidines (stilbamidine and pentamidine). *Revista espanola de pediatria*, ***6***(3), 319-346.

Gazanion, E., Vergnes, B., Seveno, M., Garcia, D., Oury, B., Ait-Oudhia, K., Sereno, D. (2011). *In vitro* activity of nicotinamide/antileishmanial drug combinations. *Parasitology International*, ***60***(1), 19-24.

Gheorgheosu, D., Duicu, O., Dehelean, C., Soica, C., Muntean, D. (2014). Betulinic acid as a potent and complex antitumor phytochemical: a minireview. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, ***14***(7), 936-945.

Giri, O. P., Singh, A. N. (1994). Experience with amphotericin B in sodium stibogluconate-unresponsive cases of visceral Leishmaniasis in north Bihar. *The Journal of the Association of Physicians of India*, ***42***(9), 690-691.

Gnoatto, S. C., Vechia, L. D., Lencina, C. L., Dassonville-Klimpt, A., Da Nascimento, S., Mossalayi, D., Sonnet, P. (2008). Synthesis and preliminary evaluation of new ursolic and oleanolic acids derivatives as antileishmanial agents. *Journal of Enzyme inhibition and Medicinal chemistry*, ***23***(5), 604-610.

Goijman, S. G., Turrens, J. F., Marini-Bettolo, G. B., Stoppani, A. O. (1984). Inhibition of growth and macromolecular biosynthesis in *Trypanosoma cruzi* by natural products. Effects of miconidine and tingenone. *Medicina*, ***44***(4), 361-370.

Gold, W., Stout, H. A.; Pagano, J. F.; Donovick, R. (1956). Antibiotic, **579**, 955−956.

Goodwin, L. G. (1995). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **89**, 339.

Goto, H., Lindoso, J. L., (2010). Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. *Expert Review of Anti Infective Therapy*, **8**, 419–433.

Gradoni, L., Soteriadou, K., Louzir, H., Dakkak, A., Toz, S. O., Jaffe, C., Dedet, J. P., Campino, L., Canavate, C., Dujardin, J. C. (2008). Drug regimens for visceral leishmaniasis in Mediterranean countries. *Tropical Medicine & International Health*, ***13***(10), 1272-1276.

Guevara, P., Pinto-Santini, D., Rojas, A., Crisante, G., Añez, N., Ramirez, J. L. (2001). Green fluorescent protein-tagged Leishmania in phlebotomine sand flies. *Journal of medical entomology*, ***38***(1), 39-43.

Habib, M. R., Nikkon, F., Rahman, M., Haque, M. E., Karim, M. R. (2007). Isolation of stigmasterol and beta-sitosterol from methanolic extract of root bark of *Calotropis gigantea* (Linn). *Pakistan Journal of Biological Sciences*, ***10***(22), 4174-4176.

Hailu, A., Musa, A., Wasunna, M., Balasegaram, M., Yifru, S., Mengistu, G., Hurissa, Z., Hailu, W. Weldegebreal, T., Tesfaye, S., Makonnen, E., Khalil, E., Ahmed, O., Fadlalla, A., El-Hassan, A., Raheem, M., Mueller, M., Koummuki, Y., Rashid, J., Mbui, J., Mucee, G., Njoroge, S., Manduku, V., Musibi, A., Mutuma, G., Kirui, F., Lodenyo, H., Mutea, D., Kirigi, G., Edwards, T., Smith, P., Muthami, L., Royce, C., Ellis, S., Alobo, M., Omollo, R., Kesusu, J., Owiti, R., Kinuthia, J. (2010). Leishmaniasis East Africa Platform (LEAP) group. Geographical variation in the response of visceral leishmaniasis to paromomycin in East Africa: a multicenter, open-label, randomized trial. *PLoS Neglected Tropical Diseases*, ***4***(10), e709.

Hall, L. R., Titus, R. G (1995). Sandfly vector saliva selectively modulates macrophage functions that inhibit killing of *Leishmania major* and nitric oxide production. *Journal of Immunoogyl*, 155: 3501-3506.

Hazra, B., Sur, P., Roy, D. K., Sur, B., Banerjee, A. (1984). Biological activity of diospyrin toward Ehrlich ascites carcinoma in Swiss A mice. *Planta Medica*, ***50***(04), 295-297.

Hegnauer, R. (2012). Plants as taxonomic markers. In *The Plant Diversity of Malesia: Proceedings of the Flora Malesiana Symposium commemorating Professor Dr. CGGJ van Steenis Leiden, August 1989* (p. 93). Springer Science & Business Media.

Hervé, M. (1937). Note sur la leishmaniose cutanée au Cameroun. *Ann Med Phar Colon*, ***35***, 928-934.

Herwaldt, B. L., Berman, J. D. (1992). Recommendations for treating leishmaniasis with sodium stibogluconate (Pentostam) and review of pertinent clinical studies. *The American Journal of Tropical Medicine and Hygiene*, ***46***(3), 296-306.

Hill, R. A., Connolly, J. D. (2012). Triterpenoids. *Natural Product Reports*, **29**(7), 780-818.

Holzmuller, P., Sereno, D., Cavaleyra, M., Mangot, I., Daulouede, S., Vincendeau, P., Lemesre, J. L. (2002). Nitric oxide-mediated proteasome-dependent oligonucleosomal DNA fragmentation in *Leishmania amazonensis* amastigotes. *Infection and Immunity*, ***70***(7), 3727-3735.

Horta, M. F., Mendes, B. P., Roma, E. H., Noronha, F. S. M., Macêdo, J. P., Oliveira, L. S., Duarte, M. M., Vieira, L. Q. (2012). Reactive oxygen species and nitric oxide in cutaneous leishmaniasis. *Journal of Parasitology Research*, *2012*.

Innocente, A. M., Silva, G. N., Cruz, L. N., Moraes, M. S., Nakabashi, M., Sonnet, P., Gosmann, G., Garcia, R., Gnoatto, S. C. (2012). Synthesis and antiplasmodial activity of betulinic acid and ursolic acid analogs. *Molecules*, ***17***(10), 12003-12014.

Inocêncio da Luz, R. A., Vermeersch, M., Deschacht, M., Hendrickx, S., Van Assche, T., Cos, P., Maes, L. (2011). *In vitro* and *in vivo* prophylactic and curative activity of the triterpene saponin PX-6518 against cutaneous Leishmania species. *Journal of Antimicrobial Chemotherapy*, ***66***(2), 350-353.

Irungu, B. N., Rukunga, G. M., Mungai, G. M., Muthaura, C. N. (2007). *Invitro* antiplasmodial and cytotoxicity activities of 14 medicinal plants from Kenya. *South African Journal of Botany*, ***73***(2), 204-207.

Jabini, R., Jaafari, M. R., Hasani, F. V., Ghazizadeh, F., Khamesipour, A., Karimi, G. (2015). Effects of combined therapy with silymarin and glucantime on leishmaniasis induced by *Leishmania major* in BALB/c mice. *Drug Research*, ***65***(03), 119-124.

Jain, N., Yadava, R. (1994). Peregrinol, a lupane type triterpene from the fruits of *Diospyros peregrina*. *Phytochemistry*, ***35***(4), 1070-1072.

Jain, S. K., Sahu, R., Walker, L. A., & Tekwani, B. L. (2012). A parasite rescue and transformation assay for antileishmanial screening against intracellular Leishmania donovani amastigotes in THP1 human acute monocytic leukemia cell line. *Journal of Visualized Experiments)*, (70), 4054.

Jarvis, J. N., Lockwood, D. N. (2013). Clinical aspects of visceral leishmaniasis in HIV infection. *Current opinion in infectious diseases*, ***26***(1), 1-9.

Jessica, A., Jesus., Thais, F., Eduardo, S., Yamamoto, Marcia, L., Marcelo, S., Aurea, F., Jo~ao, G., Lago, Gabriela, S., Gomes, Luiz, D., Passero. (2017). Therapeutic effect of ursolic acid in experimental visceral Leishmaniasis. *International Journal for Parasitology*: *Drugs and Drug Resistance*, (7)1-11.

Jha, S. N., Singh, N. K., Jha, T. K. (1991). Changing response to diamidine compounds in cases of kala-azar unresponsive to antimonial. *The Journal of the Association of Physicians of India*, ***39***(4), 314-316.

Johann, S., Oliveira, F. B., Siqueira, E. P., Cisalpino, P. S., Rosa, C. A., Alves, T. M., Carlos, L. Z., Betania, B. C. (2012). Activity of compounds isolated from *Baccharis dracunculifolia* D.C. (Asteraceae) against *Paracoccidioides brasiliensis*. *Medical Mycology*, **50**(8), 843–851.

Joquebede, R. P., Raphael, F., Queiroz, E. A. D. S, Ana, C. B. V., Antônio, E. G., Sant'ana, D. M. S., Paulo, R. A. D. M A. (2017). Evaluation of cytogenotoxicity, antioxidant and

hypoglycemiant activities of isolate compounds from *Mansoa hirsuta* D.C. (Bignoniaceae). Anais da Academia Brasileira de Ciências **89**(1): 317-331.

Judd, W. S., Campbell, C. S., Kellogg, E. A. and Stevens, P. F. (2007). Plant Systematics: A Phylogenetic Approach. 3rd Edition, Sinauer Associates, Inc., Sunderland.

Juliana, A., Peixoto, Márcio, L. A. S., Antônio, E. M. Crotti, Rodrigo, C. S. V., Valéria, M. M. G., Ana, H., Januário, M. G., Lizandra, G., Magalhães, F. F., dos Santos, S. A., Ademar, A., da Silva, F., Wilson, R. C. (2011). *Molecules **16***, 1825-1833.

Kaptue, L., Zekeng, L., Fomekong, E., Nsangou, A., Tagu, J. P., Tchuela, J. (1992). La leishmaniose viscérale au Cameroun: à propos de quelques observations et d'une prospection clinique dans la région de Kousseri, extrême-nord camerounais. *Bulletin de la Société de Pathologie Exotique*, ***85***(2), 156-158.

Karampetsou, K., Koutsoni, O. S., Dotsika, E. (2019). Quantification of Nitric Oxide and Reactive Oxygen Species in Leishmania-infected J774A. 1 Macrophages as a Response to the *in vitr*o treatment with a Natural Product Compound. *Bioprotocol*, ***9***(23).

Kavatagimath, S. A., Jalalpure, S. S. (2016). Screening of ethanolic extract of *Diospyros malabarica* Desr. Bark for anti-diabetic and antioxidant potential. *Indian Journal of Pharmaceutical Education and Research*, ***50***, 179-189.

Kaushik, V., Saini, V., Pandurangan, A., Khosa, R. L., Parcha, V. (2012). A review of phytochemical and biological studies of *Diospyros malabarica*. *International Journal of Pharmacy and Pharmaceutical Sciences*. ***2***(6), 167-9.

Kedzierski, L. (2011). Human.Vaccines.

Kerharo, J., Bouquet, A. (1949). La chasse en Côte d'Ivoire et en Haute-Volta. *Acta Tropica*, ***6***(3), 193-220.

Khandelwal, K., Bumb, R. A., Mehta, R. D., Kaushal, H., Lezama-Davila, C., Salotra, P., Satoskar, A. R. (2011). Case report: A patient presenting with diffuse cutaneous leishmaniasis (DCL) as a first indicator of HIV infection in India. *The American Journal of Tropical Medicine and Hygiene*, ***85***(1), 64.

Khanjani Jafroodi, S., Farazmand, A., Amin, M., Doroodgar, A., Shirzadi, M. R., Razavi, M. R. (2015). Methanolic extract's activity of Artemisia absinthium, Vitex agnus-castus and Phytolaca americana against *Leishmania major in vitro* and in vivo. *International Archives of Health Sciences*, ***2***(2), 69-74.

Khouri, R., Bafica, A., Silva, M. D. P. P., Noronha, A., Kolb, J. P., Wietzerbin, J., Aldina, B., Manoel, B. N., Van Weyenbergh, J. (2009). IFN-β impairs superoxide-dependent

parasite killing in human macrophages: evidence for a deleterious role of SOD1 in cutaneous leishmaniasis. *The Journal of Immunology*, ***182***(4), 2525-2531.

Killick-Kendrick, R. (1990). The life-cycle of Leishmania in the sandfly with special reference to the form infective to the vertebrate host. *Annales de Parasitologie humaine et comparée*, *65*, 37-42.

Kikuth, W., Schmidt, H. (1937). Chinese Medical Journal. **52**, 425.

Killick-Kendrick, R. (1990). Phlebotomine vectors of the leishmaniases: a review. *Medical and Veterinary Entomology*, ***4***(1), 1-24.

Kim, S., Lee, S., Kim, C., Yoon, S., Ko, O., Kim, C. (2016). Anti-inflammatory effects and mechanisms of action of coussaric and betulinic acids isolated from *Diospyros kaki* in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Molecules*, **21**(9):e1206.

Koagne, R., Bitchagno, T., Fobofou, A., Konga, S., Tamokou, D., Wessjohann, A., Tane, P. (2017). Rothtalazepane, a new azepane from the wood of *Rothmannia talbotii* (Rubiaceae). *Natural Product Communications*, **12**, 1435-1436.

Koch, A., Tamez, P., Pezzuto, J., Soejarto, D. (2005). Evaluation of plants used for antimalarial treatment by the Maasai of Kenya. *Journal of Ethnopharmacology*, ***101***(1-3), 95-99.

Kokwaro, J. O. (1976). *Medicinal plants of east Africa*. East African Literature Bureau.

Kuhls, K., Alam, M. Z., Cupolillo, E., Ferreira, G. E. M., Mauricio, I. L., Oddone, R., Feliciangeli, M. D., Wirth, T., Miles, M. A., Schönian, G. (2011). Comparative microsatellite typing of new world Leishmania infantum reveals low heterogeneity among populations and its recent old world origin. *PLoS Neglected Tropical Diseases*, ***5***(6), e1155.

Lainson, R., Strangways-Dixon. (1964).*Transactions of the Royal Society of Tropical Medicine and Hygiene*. **58**, 470.

Lainson, R. (1996). New world leishmaniasis. *The Wellcome Trust illustrated history of tropical diseases.*, 218-229.

Lawn, S. D., Armstrong, M., Chilton, D., Whitty, C. J. (2006). Electrocardiographic and biochemical adverse effects of sodium stibogluconate during treatment of cutaneous and mucosal leishmaniasis among returned travelers. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, ***100***(3), 264-269.

Lenta, B. N., Ngamgwe, R. F., Kamdem, L. M., Ngatchou, J., Tantangmo, F., Antheaume, C., Kaiser, M., Ngouela, S., Tsamo, E., Sewald, N. (2015). Compounds from *Diospyros canaliculata* (Ebenaceae) and their antiparasitic activities. *International Research Journal of Pure and Applied Chemistry*, ***6***(2), 56.

Lessa, H. A., Machado, P., Lima, F., Cruz, A. A., Bacellar, O., Guerreiro, J., Carvalho, E. M. (2001). Successful treatment of refractory mucosal leishmaniasis with pentoxifylline plus antimony. *The American Journal of Tropical Medicine and Hygiene*, ***65***(2), 87-89.

Lewis, W. H., and Elvin-Lewis, M. P. (2003). *Medical botany: plants affecting human health*. John Wiley & Sons.

Llanos-Cuentas, A., Tulliano, G., Araujo-Castillo, R., Miranda-Verastegui, C., Santamaria-Castrellon, G., Ramirez, L., Lazo, M., De Doncker, S., Boelaert, M., Robays, J., Dujardin, J. C., Arevalo., Chappuis, F. (2008). Clinical and parasite species risk factors for pentavalent antimonial treatment failure in cutaneous leishmaniasis in Peru. *Clinical Infectious Diseases*, ***46***(2), 223-231.

Lourie, E. M., Yorke, W. A. (1938). *Tropical Medicine and parasitology*, **33**, 289.

Machado, P. R., Ampuero, J., Guimarães, L. H., Villasboas, L., Rocha, A. T., Schriefer, A., Sousa, R. S., Talhari, A., Penna, G., Carvalho, E. M. (2010). Miltefosine in the treatment of cutaneous leishmaniasis caused by *Leishmania braziliensis* in Brazil: a randomized and controlled trial. *PLOS Neglected Tropical Diseases*, ***4***(12), e912.

Mahato, S. B., Kundu, A. P. (1994). ^{13}C NMR spectra of pentacyclic triterpenoids-a compilation and some salient features. *Phytochemistry*, ***37***(6), 1517-1575.

Mallavadhani, U. V., Panda, A. K., Rao, Y. R. (1998). Review article number 134 pharmacology and chemotaxonomy of *diospyros*. *Phytochemistry*, ***49***(4), 901-951.

Mallavadhani, U. V., Mahapatra, A., Jamil, K., Reddy, P. S. (2004). Antimicrobial activity of some pentacyclic triterpenes and their synthesized 3-O-lipophilic chains. *Biological and Pharmaceutical Bulletin*, ***27***(10), 1576-1579.

Manen, J. F. (1996). The chloroplast atpB-rbcL spacer in Rubiaceae. In *Second International Rubiaceae Conference, Proceedigs*, **7**, 51-57.

Manson-Bahr, P. E. C. (1996). Old World leishmaniasis. *The Wellcome Trust illustrated history of tropical diseases.*, 206-217.

Markle, W. H., Makhoul, K. (2004). Cutaneous leishmaniasis recognition and treatment. *American Family Physician*, ***69***(6), 1455-1460.

Masoko, P., Eloff, J. N. (2007). Screening of twenty-four South African Combretum and six Terminalia species (Combretaceae) for antioxidant activities. *African Journal of Traditional, Complementary and Alternative Medicines*, ***4***(2), 231-239.

Matsumori, N., Tahara, K., Yamamoto, H., Morooka, A., Doi, M., Oishi, T., Murata, M. (2009). Direct interaction between amphotericin B and ergosterol in lipid bilayers as

revealed by 2H NMR spectroscopy. *Journal of the American Chemical Society*, ***131***(33), 11855-11860.

Martins, D., Carrion, L. L., Ramos, D. F., Salomé, K. S., da Silva, P. E. A., Barison, A., Nunez, C. V. (2013). Triterpenes and the antimycobacterial activity of *Duroia macrophylla* Huber (Rubiaceae). *BioMedical Research International*.

Mayer, A. M., Rodríguez, A. D., Berlinck, R. G., Fusetani, N. (2011). Marine pharmacology in 2007–8: Marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous system, and other miscellaneous mechanisms of action. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, ***153***(2), 191-222.

Mazoir, N., Benharref, A., Bailén, M., Reina, M., González-Coloma, A., Martínez-Díaz, R. A. (2011). Antileishmanial and antitrypanosomal activity of triterpene derivatives from latex of two Euphorbia species. *Zeitschrift für Naturforschung C*, ***66***(7-8), 360-366.

Mbi, C. N., Waterman, P. G. (1978). Sterols and naphthoquinones from *Diospyros gracilescens*. *Journal of Pharmacy and Pharmacology*, ***30***, 86p.

Melo, T. S., Gattass, C. R., Soares, D. C., Cunha, M. R., Ferreira, C., Tavares, M. T., Saraiva, E., Parise-Filho, R., Braden, H., Delorenzi, J. C. (2016). Oleanolic acid (OA) as an antileishmanial agent: Biological evaluation and in silico mechanistic insights. *Parasitology International*, ***65***(3), 227-237.

Mendoza-León, A., Luis, L., Fernandes, O., Cupolillo, E., García, L. (2002). Molecular markers for species identification in the *Leishmania* subgenus Viannia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, ***96***, 65-70.

Merchant, M. M., Welsh, A. K., McLean, R. J. (2007). Rheinheimera texasensis sp. nov., a halointolerant freshwater oligotroph. *International Journal of Systematic and Evolutionary Microbiology*, ***57***(10), 2376-2380.

Messori, A., Fadda, V., Maratea, D., Trippoli, S., Marinai, C. (2013). Nephrotoxicity of different formulations of amphotericin B: summarizing evidence by network meta-analysis. *Clinical Infectious Diseases*, ***57***(12), 1783-1784.

Miao, L., Clair, D. K. S (2009)**.** Regulation of superoxide dismutase genes: implications in disease. Free Radical Biology and Medicine.; 47(4):344–56.

Ministério Da Saúde. (2011). Secretaria De Vigilância Em Saúde, Leishmaniose Visceral: RecomenDacọ ẽ s Cliń icas Para Reducą̃o Da letalidade, Departamento De Vigilância Epidemiológica; Departamento De Vigilañ cia Epidemioloǵ ica: Brasiĺia, Brazil

Minodier, P., Parola, P. (2007). *Travel Medicine and Infectious Disease*, **5**, 150.

Mishina, Y. V., Krishna, S., Haynes, R. K., Meade, J. C. (2007). Artemisinins inhibit *Trypanosoma cruzi* and *Trypanosoma brucei rhodesiense* in vitro growth. *Antimicrobial agents and Chemotherapy*, ***51***(5), 1852-1854.

Momeni, A. Z., Reiszadae, M. R., Aminjavaheri, M. (2002). Treatment of cutaneous leishmaniasis with a combination of allopurinol and low-dose meglumine antimoniate. *International Journal of Dermatology*, ***41***(7), 441-443.

Mondal, S., Bhattacharya, P., Ali, N. (2010). Current diagnosis and treatment of visceral leishmaniasis. *Expert review of anti-infective therapy*, ***8***(8), 919-944.

Monzote, L. (2011). Antileishmanial patents antileishmanial current drugs and relevant patents. *Recent Patents on Anti-infective Drug Discovery*, ***6***(1), 1-26.

Moreira, V. F., Vieira, I. J., Braz-Filho, R. (2017). Angelocunhol: New Erythroxylane Diterpene and Other Compounds from Simira sampaioana (Rubiaceae). *Journal of the Brazilian Chemical Society*, ***28***, 152-157.

Moskalenko, N. I., Pershin, G. N. (1966). Comparative study of the chemotherapeutic effect of paromomycin and monomycin in experimental cutaneous leischmaniasis in albino mice. *Farmakologiia i toksikologiia*, ***29***(1), 90-94.

Mosmann T. (1983). Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. *Journal of Immunological Methods*, **65**:55−63.

Mueller, Y., Nguimfack, A., Cavailler, P., Couffignal, S., Rwakimari, J. B., Loutan, L., Chappuis, F. Ann. (2008). *Tropical Medicine and Parasitology*. **102**,11.

Mukherjee, S., Garg, S., Talwar, G. P. (1999). Early post implantation contraceptive effects of a purified fraction of neem (*Azadirachta indica*) seeds, given orally in rats: possible mechanisms involved. *Journal of Ethnopharmacology*, ***67***(3), 287-296.

Munder, M., Choi, B. S., Rogers, M., Kropf, P. (2009). L-arginine deprivation impairs *Leishmania major*-specific T-cell responses. *European Journal of Immunology*, ***39***(8), 2161-2172.

Murray, H. W., Montelibano, C., Peterson, R., Sypek, J. P. (2000). Interleukin-12 regulates the response to chemotherapy in experimental visceral leishmaniasis. *The Journal of Infectious Diseases*, ***182***(5), 1497-1502.

Musa, A., Khalil, E., Hailu, A., Olobo, J., Balasegaram, M., Omollo, R., Edwards, T., Rashid, J., Mbui, J., Musa, B., Abuzaid, A. A., Ahmed, O., Fadlalla, A., El-Hassan, A., Mueller, M., Mucee, G., Njoroge, S., Manduku, V., Mutuma, G., Apadet, L., Lodenyo, H., Mutea, D., Kirigi, G., Yifru, S., Mengistu, G., Hurissa, Z., Hailu, W., Weldegebreal, T., Tafes,

H., Mekonnen, Y., Makonnen, E., Ndegwa, S., Sagaki, P., Kimutai, R., Kesusu, J., Owiti, R., Ellis, S., Wasunna, M. (2012). *PLoS Neglected Tropicol Diseases*, **6**, 1674.

Musa, A. M., Younis, B., Fadlalla, A., Royce, C., Balasegaram, M., Wasunna, M., Hailu, A., Edwards, T., Omollo, R., Mudawi, M., Kokwaro, G., El-Hassan, K. E. (2010). Paromomycin for the treatment of visceral leishmaniasis in Sudan: a randomized, open-label, dose-finding study. *PLoS Neglected Tropical Diseases*, ***4***(10), e855.

Naderer, T., McConville, M. J. (2008). The Leishmania–macrophage interaction: a metabolic perspective. *Cellular Microbiology*, ***10***(2), 301-308.

Nagle, A. S., Khare, S., Kumar, A. B., Supek, F., Buchynskyy, A., Mathison, C. J., Naveen, K. C., Nagendar, P., Frederick, S. B., Michael. H. Gelb., Molteni, V. (2014). Recent developments in drug discovery for leishmaniasis and human African trypanosomiasis. *Chemical Reviews*, ***114***(22), 11305-11347.

Nam, H., Kim, M. M. (2013). Ursolic acid induces apoptosis of SW480 cells via p53 activation. *Food and chemical toxicology*, ***62***, 579-583.

Navin, T. R., Arana, B. A., Arana, F. E., Berman, J. D., Chajón, J. F. (1992). Placebo-controlled clinical trial of sodium stibogluconate (Pentostam) versus ketoconazole for treating cutaneous leishmaniasis in Guatemala. *Journal of Infectious Diseases*, ***165***(3), 528-534.

Naskar, M., Bhattacharya, S., Biswas, M. (2013). Antileishmanial effect of *Ixora coccinea* leaf extracts on the *in vitro* growth of *Leishmania donovani* promastigotes. *Journal of Advanced Pharmacy Education & Research Oct-Dec*, ***3***(4).

Ngouateu, O. B., Kollo, P., Ravel, C., Dereure, J., Kamtchouing, P., Same-Ekobo, A., von, S. E., Maurer M., Dondji, B. (2012). Clinical features and epidemiology of cutaneous leishmaniasis and Leishmania major/HIV coinfection in Cameroon: results of a large cross-sectional study. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, ***106***(3), 137-142

Nicolle, C. C. R. (1908).Hebd. Seances Academy of Sciences, **146**, 842.

Oliveira, J. P., Ferreira, E. L., Chaves, M. H., Militão, G. C. V., Júnior, G. M., Costa, A. D. M., Costa-Lotufo, L. V. (2012). Chemical constituents of *Lecythis pisonis* and cytotoxic activity. *Revista Brasileira de Farmacognosia*, ***22***(5), 1140-1144.

Olson, J. A., Adler-Moore, J. P., Smith, P. J., Proffitt, R. T. (2005). Treatment of *Candida glabrata* infection in immunosuppressed mice by using a combination of liposomal amphotericin B with caspofungin or micafungin. *Antimicrobial Agents and Chemotherapy*, ***49***(12), 4895-4902.

Ouellette, M., Olivier, M., Sato, S., Papadopoulou, B. (2003). Le parasite *Leishmania* à l'ère de la postgénomique. *M/S: médecine sciences*, 19: 900-909.

Ortega-Carnicer, J., Alcazar, R., De la Torre, M., AND Benezet, J. (1997). Pentavalent antimonial-induced torsade de pointes. *Journal of electrocardiology*, *30*(2), 143-145.

Paniz M. A. E., Duffey, G. B., Horton, L. E., Tirado, M., Reyes Jaimes, O., Perez-Alvarez, A., Zerpa, O. (2013). Intermediate/borderline disseminated cutaneous leishmaniasis. *International Journal of Dermatology*, ***52***(4), 446-455.

Passero, L. F. D., Bonfim-Melo, A., Corbett, C. E. P., Laurenti, M. D., Toyama, M. H., De Toyama, D. O., Lago, J. H. G. (2011). Anti-leishmanial effects of purified compounds from aerial parts of *Baccharis uncinella* C. DC. (Asteraceae). *Parasitology Research*, ***108***(3), 529-536.

Patel, T. A., Lockwood, D. N. (2009). Pentamidine as secondary prophylaxis for visceral leishmaniasis in the immunocompromised host: report of four cases. *Tropical medicine and International Health*, ***14***(9), 1064-1070.

Path Canada. (1994). Field test methods for the determination of iron in fortified foods.

Patrícia G. G., do Nascimento, T. L. G., Lemos, A. M. C., Bizerra, Â. M. C., Arriaga, D. A., Ferreira, G. M. P., Santiago, R. B., José Galberto, M. (2014). Antibacterial and Antioxidant Activities of Ursolic Acid and Derivatives. *Molecules*, ***19***, 1317-1327.

Pearson, R. S., A and Brown, H. P. (1932). Commercial timbers of India: their distribution, supplies, anatomical structure, physical and mechanical properties and uses. Volumes I & II. *Commercial timbers of India: their distribution, supplies, anatomical structure, physical and mechanical properties and uses. Volumes I & II.*

Peixoto, J. A., Andrade S. M. L., Crotti, A. E., Cassio S. V. R., Gimenez, V. M., Januário, A. H., Milton, G., Lizandra, G. M., Fransérgio, F., dos Santos, Sérgio, A., Ademar, A., da Silva Filho C. W. R. (2011). Antileishmanial activity of the hydroalcoholic extract of Miconia langsdorffii, isolated compounds, and semisynthetic derivatives. *Molecules*, ***16***(2), 1825-1833.

Perry, M. R., Wyllie, S., Raab, A., Feldmann, J., Fairlamb, A. H. (2013). Chronic exposure to arsenic in drinking water can lead to resistance to antimonial drugs in a mouse model of visceral leishmaniasis. *Proceedings of the National Academy of Sciences*, ***110***(49), 19932-19937.

Pinto, M. C., Campbell-Lendrum, D. H., Lozovei, A. L., Teodoro, U., Davies, C. R. (2001). Phlebotomine sandfly responses to carbon dioxide and human odor in the field. *Medical and Veterinary Entomology*, ***15***(2), 132-139.

Plimmer, H. G., Thomson, J. D. (1908). Further results of the experimental treatment of trypanosomiasis in rats; being a progress report of a committee of the Royal Society. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character*, ***80***(536), 1-10.

Prata, A. (1963). Treatment of kala-azar with amphotericin B. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, ***57***(4), 266-268.

Pulvertaft, R. J. V., & Hoyle, G. F. (1960). Stages in the life-cycle of Leishmania donovani. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *54*(2), 191-196.

Ramesh, V., Mukherjee, A. (1995). Post-kala-azar dermal leishmaniasis. *International Journal of Dermatology*, ***34***(2), 85-91.

Ramos, H., Valdivieso, E., Gamargo, M., Dagger, F., Cohen, B. E. (1996). Amphotericin B kills unicellular leishmanias by forming aqueous pores permeable to small cations and anions. *The Journal of Membrane Biology*, ***152***(1), 65-75.

Rangel, H., Dagger, F., Hernandez, A., Liendo, A., Urbina, J. A. (1996). *Antimicrobial Agents and Chemotherapy*, **40**, 2785.

Rapp, C., Imbert, P., Darie, H., Simon, F., Gros, P., Debord, T., Roué, R. (2003). Traitement par amphotéricine B liposomale d'une leishmaniose cutanée contractée à Djibouti et résistante à l'antimoniate de méglumine. Bulletin de la Société de Pathologie Exotique ***96***(3), 209-211.

Reithinger, R., Dujardin, J. C., Louzir, H., Pirmez, C., Alexander, B., Brooker, S. (2007). Cutaneous leishmaniasis. *The Lancet infectious diseases*, ***7***(9), 581-596.

Ritmeijer, K., Dejenie, A., Assefa, Y., Hundie, T. B., Mesure, J., Boots, G., den Boer, M., Davidson, R. N. (2006). A comparison of miltefosine and sodium stibogluconate for treatment of visceral leishmaniasis in an Ethiopian population with high prevalence of HIV infection. *Clinical Infectious Diseases*, **43**(3), 357-364.

Ritmeijer, K., Veeken, H., Melaku, Y., Leal, G., Amsalu, R., Seaman, J., Davidson, R. N. (2001). Ethiopian kala-azar: generic sodium stibogluconate and Pentostam are equivalent; HIV coinfected patients have a poor outcome. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, **95**(6), 668-672.

Reynertson, K. A., Basile, M. J., Kennelly, E. J. (2005). Antioxidant potential of seven myrtaceous fruits. *Ethnobotany Research and Applications*, ***3***, 025-036.

Roberts, L. S., and Janovy Jr, J. (2000). Gerald D. Schmidt e Larry S. Roberts' foundations of parasitology. In *Gerald D. Schmidt e Larry S. Roberts' foundations of parasitology* (pp. xviii-670).

Robbrecht, E., Puff, C. (1986). Survey of the Gardenieae and related tribes (Rubiaceae). *Botanische Jahrbucher fur Systematik, Pflanzengeschichte und Pflanzengeographie*.

Robbrecht, E. (1988). Tropical woody Rubiaceae. Opera Bot. Belg. **1**:1–271.

Robbrecht, E. (1993). Supplement to the 1988 outline of the classification of the Rubiaceae. Index to genera. *Advances in Rubiaceae Macrosystematics.*, ***6***, 173-196.

Rocío, B. A., Andrés, U. C., Carlos, Q. N., Gonzalo, C; E., Mirbella, C. (2013). A selective chemical method for the separation of quinones from the stem bark of *Diospyros Anisandra*. *International Journal of Current Pharmaceutical Research*. **5**(4), 13-17.

Rosenthal, E., Delaunay, P., Jeandel, P. Y., Haas, H., Pomares-Estran, C., Marty, P. (2009). Liposomal amphotericin B as treatment for visceral leishmaniasis in Europe, 2009. *Medecine et Maladies Infectieuses*, ***39***(10), 741-744.

Ross, S. R. (1899). *Report on the nature of Kala-Azar*. Calcutta: Office of the Superintendent of Government Printing.

Roussel, M., Nacher, M., Fremont, G., Rotureau, B., Clyti, E., Sainte-Marie, D., Carme, B., Pradinaud, R., Couppie, P. ('2006). *Tropical Medicine and parasitology*, **100**, 307.

Rova, J. H., Delprete, P. G., Andersson, L., Albert, V. A. (2002). A trnL-F cpDNA sequence study of the Condamineeae-Rondeletieae-Sipaneeae complex with implications on the phylogeny of the Rubiaceae. *American Journal of Botany*, ***89***(1), 145-159.

Roy, S., Dutta, D., Satyavarapu, E. M., Yadav, P. K., Mandal, C., Kar, S., Mandal, C. (2017). Mahanine exerts *in vitro* and *in vivo* antileishmanial activity by modulation of redox homeostasis. *Scientific Reports*, ***7***(1), 1-16.

Sacks, D. L. (1989). Metacyclogenesis in *Leishmania* promastigotes. *Experimental Parasitology*, ***69***(1), 100-103.

Sacks, D., Kamhawi, S. (2001). Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. *Annual Reviews in Microbiology*, ***55***(1), 453-483.

Sacks, D. L., Kenney, R. T., Neva, F. A., Kreutzer, R. D., Jaffe, C. L., Gupta, A. K., Sharma, M. C., Sinha, S. P., Neva, F. A., Saran, R. (1995). Indian kala-azar caused by *Leishmania tropica*. *The Lancet*, ***345***(8955), 959-961.

Sadeghi-Nejad, B., Saki, J. (2014). Effect of aqueous *Allium cepa* and *Ixora brachiata* root extract on *Leishmania major* promastigotes. *Jundishapur journal of natural pharmaceutical products*, ***9***(2).

Saenz, R. E., Paz, H., Berman, J. D. (1990). Efficacy of ketoconazole against *Leishmania braziliensis panamensis* cutaneous leishmaniasis. *The American Journal of Medicine*, ***89***(2), 147-155.

Sands, M., Kron, M. A., Brown, R. B. (1985). Pentamidine: a review. *Reviews of Infectious Diseases*, ***7***(5), 625-6344.

Salehabadi, A., Karamian, M., Farzad, M. H., Namaei, M. H. (2014). Effect of root bark extract of *Berberis vulgaris* L. on *Leishmania major* on BALB/c mice. *Parasitology Research*, ***113***(3), 953-957.

Santiago, L. A., Dayrit, K. C., Correa, P., Mayor, A. B. R. (2014). Comparison of antioxidant and free radical scavenging activity of triterpenes αamyrin, oleanolic acid and ursolic acid. *Journal of Natural Prod*ucts, ***7***, 29-36.

Saraste, A., Pulkki, K. (2000). Morphologic and biochemical hallmarks of apoptosis. *Cardiovascular Research*, ***45***(3), 528-537.

Satish, A. K., Sunil, S. J. (2016). Screening of ethanolic extract of *Diospyros malabarica* Desr. bark for anti-diabetic and antioxidant potential. *International Journal of pharmacy and Education Research*, **50**(1):179-89.

Schlein, Y. (1993). *Leishmania* and sandflies: interactions in the life cycle and transmission. *Parasitology Today*, ***9***(7), 255-258.

Seaman, J., Pryce, D., Sondorp, H. E., Moody, A., Bryceson, A. D. M., Davidson, R. N. (1993). Epidemic visceral leishmaniasis in Sudan: a randomized trial of aminosidine plus sodium stibogluconate versus sodium stibogluconate alone. *Journal of Infectious Diseases*, ***168***(3), 715-720.

Shah, R., Kathad, H., Sheth, R., Sheth, N. (2010). *In vitro* antioxidant activity of roots of *Tephrosia purpurea* Linn. *International Journal of Pharmacy and Pharmaceutical Sciences*, ***2***(3), 30-33.

Sharma, U. K., Mohanakrishnan, D., Sharma, N., Equbal, D., Sahal, D., Sinha, A. K. (2018). Facile synthesis of vanillin-based novel bischalcones identifies one that induces apoptosis and displays synergy with Artemisinin in killing chloroquine resistant *Plasmodium falciparum*. *European Journal of Medicinal Chemistry*, ***155***, 623-638.

Sifaoui, I., López-Arencibia, A., Martín-Navarro, C. M., Reyes-Batlle, M., Mejri, M., Valladares, B., Lorenzo-Morales, J., Abderabba, M., Piñero, J. E. (2017). Selective activity of oleanolic and maslinic acids on the amastigote form of *Leishmania spp*. *Iranian Journal of Pharmaceutical Research*, ***16***(3), 1190.

Singh, S., Sharma, U., Mishra, J. (2011). Post-kala-azar dermal leishmaniasis: recent developments. *International Journal of Dermatology*, ***50***(9), 1099-1108.

Siqueira-Neto, J. L., Song, O. R., Oh, H., Sohn, J. H., Yang, G., Nam, J., Jiyeon, J., Jonathan, C., Chang, B. L., Seunghyun, M., Auguste, G., Eric, C., Thierry, C., Freitas-

Junior, L. H. (2010). Antileishmanial high-throughput drug screening reveals drug candidates with new scaffolds. *PLoS Neglected Tropical Diseases*, ***4***(5), e675.

Schumann, K. (1891). Rubiaceae. Die nat ¨ urlichen P • anzenfamilien 4 (4).WilhelmEngelmann, Leipzig, Germany.

Solomon, M., Pavlotsky, F., Leshem, E., Ephros, M., Trau, H., Schwartz, E. (2011). Liposomal amphotericin B treatment of cutaneous leishmaniasis due to *Leishmania tropica*. *Journal of the European Academy of Dermatology and Venereology*, ***25***(8), 973-977.

Sonke B and Simo K. A. Révision systématique du genre *Rothmannia* (Rubiaceae-Gardenieae) au Cameroun. *Bulletin du Jardin botanique National de Belgique*. 65: 219-247 (31-05-1996)

Sousa, M. C., Varandas, R., Santos, R.C., Santos-Rosa, M., Alves, V., Salvador, J. A. (2014). Antileishmanial activity of semisynthetic lupane triterpenoids betulin and betulinic acid derivatives: synergistic effects with miltefosine. *PLoS ONE*, **9**(3): 89939.

Steverding, D. (2017). The history of leishmaniasis. *Parasites and vectors*, ***10***(1), 1-10.

Sun, J., Zhang, X., Broderick, M., Fein, H. (2003). Measurement of nitric oxide production in biological systems by using Griess reaction assay. *Sensors*, ***3***(8), 276-284.

Sundar, S., Sinha, P., Jha, T. K., Chakravarty, J., Rai, M., Kumar, N. and Arana, B. (2013). Oral miltefosine for Indian post-kala-azar dermal leishmaniasis: a randomised trial. *Tropical Medicine & International Health*, *18*(1), 96-100.

Sundar, S.; Thakur, B. B.; Tandon, A. K.; Agrawal, N. R., Mishra, C. P., Mahapatra, T. M., Singh, V. P. (1994). *British Medical Journal*, **308**, 307.

Sundar, S. (2001). Treatment of visceral leishmaniasis. *Medical microbiology and Immunology*, ***190***(1), 89-92.

Sundar, S., More, D. K., Singh, M. K., Singh, V. P., Sharma, S., Makharia, A., Kumar, P. C., Murray, H. W. (2000). Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clinical Infectious Diseases*, ***31***(4), 1104-1107.

Sundar, S., Chakravarty, J., Agarwal, D., Rai, M., Murray, H. W. (2010). Single-dose liposomal amphotericin B for visceral leishmaniasis in India. *New England Journal of Medicine*, ***362***(6), 504-512.

Sundar, S., Chakravarty, J. (2013). Leishmaniasis: an update of current pharmacotherapy. *Expert Opinion on Pharmacotherapy*, *14*(1), 53-63.

Sundar, S., Jha, T. K., Thakur, C. P., Sinha, P. K., Bhattacharya, S. K. (2007). Injectable paromomycin for visceral leishmaniasis in India. *New England Journal of Medicine*, ***356***(25), 2571-2581.

Sundar, S., Singh, A., Rai, M., Prajapati, V. K., Singh, A. K., Ostyn, B., Boelaert, M., Dujardin, J. C., Chakravarty, J. (2012). Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clinical Infectious Diseases*, ***55***(4), 543-550.

Tan, N., Kaloga, M., Radtke, O. A., Kiderlen, A. F., Oksüz, S., Ulubelen, A. (2002). Abietane diterpenoids and triterpenoic acids from *Salvia cilicica* and their antileishmanial activities. *Phytochemistry*, **61** (8):881–884. PMID: 12453510.

Tangie, L. N., Desmond, A., Aminde, L. N., Ako, A. M., Halle, P. M. (2017). Cutaneous leishmaniasis in a severely immunocompromised HIV patient in Kumbo, Northwest region of Cameroon: case report. *BMC Research Notes*, ***10***(1), 1-4.

Terrazzino, S., Cargnin, S., Del Re, M., Danesi, R., Canonico, P. L., Genazzani, A. A. (2013). DPYD IVS14+ 1G> A and 2846A> T genotyping for the prediction of severe fluoropyrimidine-related toxicity: a meta-analysis. *Pharmacogenomics*, ***14***(11), 1255-1272.

ter Horst, R., Tefera, T., Assefa, G., Ebrahim, A. Z., Davidson, R. N., Ritmeijer, K. (2009). Field evaluation of rK39 test and direct agglutination test for diagnosis of visceral leishmaniasis in a population with high prevalence of human immunodeficiency virus in Ethiopia. *The American Journal of Tropical Medicine and Hygiene*, ***80***(6), 929-934.

Thakur, C. P., Kumar, M., Singh, S. K., Sharma, D., Prasad, U. S., Singh, R. S., Dhawan, P. S., Achari, V. (1984). Comparison of regimens of treatment with sodium stibogluconate in kala-azar. ***British Medical Journal*** *(Clin Res Ed)*, ***288***(6421), 895-897.

The Wealth of India. (1952). *A Dictionary of Indian Raw Materials and Industrial Products*. Vol. **III,** CSIR, New Delhi, 76.

Tomiotto-Pellissier, F., Bortoleti, B. T. D. S., Assolini, J. P., Gonçalves, M. D., Carloto, A. C. M., Miranda-Sapla, M. M., Pavanelli, W. R. (2018). Macrophage polarization in leishmaniasis: broadening horizons. *Frontiers in immunology*, 2529.

Torres-Santos, E. C., Lopes, D., Oliveira, R. R., Carauta, J. P. P., Falcao, C. B., Kaplan, M. A. C., Rossi-Bergmann, B. (2004). Antileishmanial activity of isolated triterpenoids from *Pourouma guianensis*. *Phytomedicine*, ***11***(2-3), 114-120.

Toxicity–Up, A. O. (2001). OECD guideline for testing of chemicals. *Organisation for Economic Co-Operation and Development: Paris, France*.

Udia, P. M., Takem, L. P., Ufot, U. F., Antai, A. B., Owu, D. U. (2016). Insulin and alpha amylase levels in alloxan-induced diabetic rats and the effect of *Rothmannia hispida* (K. Schum) Fagerl leaf extract. *The Journal of Phytopharmacology*, ***5***(1), 1-5.

Ul Bari, A. (2006). Epidemiology of cutaneous leishmaniasis. *Journal of Pakistan Association of Dermatologists*, ***16***, 156-162.

Ultee, A., Kets, E. P., Alberda, M., Hoekstra, F. A., Smid, E. J. (2000). Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. *Archives of microbiology*, ***174***(4), 233-238.

van Griensven, J., Diro, E. (2012). Visceral leishmaniasis. *Infectious Disease Clinics*, ***26***(2), 309-322.

Vannier-Santos, M. A., Martiny, A., Souza, W. D. (2002). Cell biology of *Leishmania spp.*: invading and evading. *Current pharmaceutical design*, ***8***(4), 297-318.

Vasconcelos, M. A. L., Royo, V. A., Ferreira, D. S., Crotti, A. E. M., Andrade, M. L., Carvalho, J. C. T., Bastos, J.K., Cunha, W. R. (2006). *In vivo* analgesic and anti-inflammatory activities of ursolic acid and oleanoic acid from *Miconia albicans* (Melastomataceae). *Zeitschrift für Naturforschung C*, ***61***(7-8), 477-482.

Verdcourt, B. (1958). Remarks on the classification of the Rubiaceae. *Bulletin du Jardin botanique de l'Etat, Bruxelles/Bulletin van den Rijksplantentuin, Brussel*, 209-290.

Verma, S., Bhandari, V., Avishek, K., Ramesh, V., Salotra, P. (2013). Reliable diagnosis of post-kala-azar dermal leishmaniasis (PKDL) using slit aspirate specimen to avoid invasive sampling procedures. *Tropical Medicine & International Health*, ***18***(3), 268-275.

Vianna, G. (1911). Sobre uma nova espécie de Leishmania (nota preliminar). *Brasil-Médico*, ***25***, 411.

Vianna, G. (1912). Tratamento da leishmaniose pelo tártaro emético. Comunicação ao 7 o Congresso Brasileiro de Medicina e Cirurgia. Belo Horizonte, Minas Gerais.

Vicens, Q., & Westhof, E. (2001). Crystal structure of paromomycin docked into the eubacterial ribosomal decoding A site. *Structure*, *9*(8), 647-658.

Wanasen, N., Soong, L. (2008). L-arginine metabolism and its impact on host immunity against *Leishmania* infection. *Immunologic research*, ***41***(1), 15-25.

Watt, J. M., Breyer-Brandwijk, M. G. (1962). The Medicinal and Poisonous Plants of Southern and Eastern Africa being an Account of their Medicinal and other Uses, Chemical Composition, Pharmacological Effects and Toxicology in Man and Animal. *The Medicinal and Poisonous Plants of Southern and Eastern Africa being an Account of their Medicinal and other Uses, Chemical Composition, Pharmacological Effects and Toxicology in Man and Animal.*, (Edition 2).

Weina, P. J., Neafie, R. C., Wortmann, G., Polhemus, M., Aronson, N. E., Strausbaugh, L. J. (2004). Old world leishmaniasis: an emerging infection among deployed US military and civilian workers. *Clinical Infectious Diseases*, ***39***(11), 1674-1680.

WHO. (2009b). *Leishmaniasis Control*. Dossier complet réalisé par l'organization mondiale de la santé, division de surveillance des maladies tropicales (2009).

WHO. (2010). Working to Overcome the Global Impact of Neglected Tropical Diseases: First WHO Report on Neglected Tropical Diseases. WHO: Geneva, Switzerland, No.1.

WHO. (2012). Research priorities for Chagas disease, Human African Trypanosomiasis a WHO Expert Committee on the Control of the Leishmaniases. Meeting, & World Health Organization. (2010). *Control of the Leishmaniases: Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010* (No. 949). World Health Organization.nd Leishmaniasis. WHO Technical Report.Serie No 975.

WHO. (2017). Leishmaniasis report. http://www.who.int/leishmaniasis/en/. Serie N°375.

WHO. (2018). Leishmaniasis report. http://www.who.int/leishmaniasis/en/.

Wilcocks, C., Manson-Bahr, P. E. C. (1972). Manson's tropical diseases. *Manson's tropical diseases.* (Edn 17).

Willis, J. C. (1966). A dictionary of the flowering plants and ferns. *A dictionary of the flowering plants and ferns.* (**7**).

Willis, J. C. (1973). *A Dictionary of the Flowering Plants and Ferns* (revised by H. K. Airy Shaw), Cambridge University Press, London, 8th edn, 397.

Wonkam, A. K. N., Ngansop, C. A. N., Wouamba, S. C. N., Jouda, J. B., Happi, G. M., Boyom, F. F., Sewald, N. Lenta, B. N. (2020). Rothmanniamide and other constituents from the leaves of *Rothmannia hispida* (K. Schum.) fagerl.(Rubiaceae) and their chemophenetic significance. *Biochemical Systematics and Ecology*, ***93***, 104-137.

World Health Organization. (2000). *General guidelines for Methodologies on Research and evaluation of traditional medicine* (No. WHO/EDM/TRM/2000.1). World Health Organization.

Xie, K., Huang, K., Yang, L., Yu, P., Liu, H. (2011). Three-liquid-phase extraction: A new approach for simultaneous enrichment and separation of Cr (III) and Cr (VI). *Industrial & engineering chemistry research*, ***50***(22), 12767-12773.

Yang, L., Liu, X., Lu, Z., Chan, J. Y. W., Zhou, L., Fung, K. P., Ping W., Wu, S. (2010). Ursolic acid induces doxorubicin-resistant HepG2 cell death via the release of apoptosis-inducing factor. *Cancer Letters*, ***298***(1), 128-138.

Yamamoto, E. S., Campos, B. L. S., Laurenti, M. D., Lago, J. H., Grecco, S. D. S., Corbett, C. E., Passero, L. F. D. (2014). Treatment with triterpenic fraction purified from *Baccharis uncinella* leaves inhibits *Leishmania* (*Leishmania*) *amazonensis* spreading and improves Th1 immune response in infected mice. *Parasitology Research*, ***113***(1), 333-339.

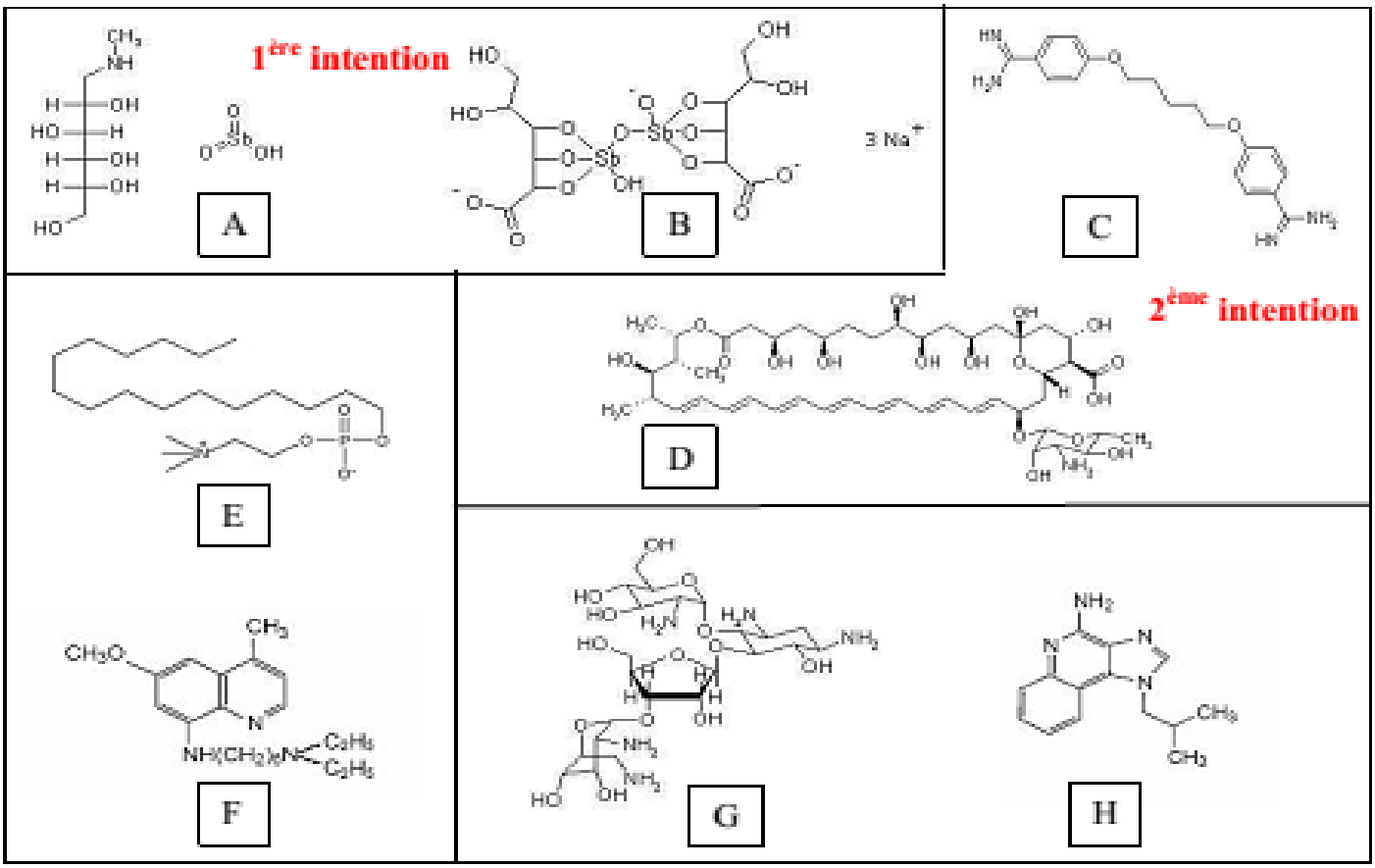
Yogeeswari, P., Sriram, D. (2005). Betulinic acid and its derivatives: a review on their biological properties. *Current Medicinal Chemistry*, ***12***(6), 657-666.

Zijlstra, E. E., El-Hassan, A. M., Ismael, A., Ghalib, H. W. (1994). Endemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical infection and postkala-azar dermal leishmaniasis. *The American Journal of Tropical Medicine and Hygiene*, ***51***(6), 826-836.

APPENDICE

APPENDIX

Appendice 1: Structure of some antileishmanial drugs



Légend: A: Glucantime®; B: Stibogluconate de Sodium; C: Pentamidine iséthionate; D: Amphotéricine B; E: Miltéfosine; F: Sitamaquine; G: Paromomycine; H: Imiquimod.

Appendice 2: Preparation of M-199 Medium (1L)

- ✓ 1 L of distilled water was sterilized by autoclaving at 200°C; the water was left for a few minutes to cool.
- ✓ M199 in powdered form was added to the water, and the solution was mixed.
- ✓ 100 ml of FBS was added to the solution followed by the addition of 10 ml of the antibiotic streptomycin/penicillin

  -The solution was sterilized by filtering.

  -The filtered solution was distributed in 50 ml Falcon tubes and was stored at 4°C until further use.

Appendice 3: Target Product Profile for VL (Adapted from DNDi)

Tim	Optimal target profile	Minimal target profile
***Leishmania* species**	All species	*L. donovani*
Distribution	All areas	Either India or Africa
Target population	Immunocompetent and Immunosuppressed	Immunocompetent
Clinical efficacy	>95%	>90%
Resistance	Active against resistant strains	Active against resistance strains
Safety and tolerability	No adverse events requiring monitoring	1 monitoring visit in mid/end –point
Contraindications	None	Pregnancy/lactation
Interactions	None-compatible for combination therapy	None for malaria, TB and HIV therapies
Drug formulation	Oral or intramuscular depot	Oral or intramuscular depot
Drug stability	3 years in hot and humid countries (zone 4)	Stable under conditions that can be reasonably achieved in the target region (> 2 year)
Treatment regimen	Q.d for 10 days p.o or 3 shots given over 10 days	B.i.d for < 10 days p.o. or < 3 shots over 10 days
Cost	<$10 per drug course	<$80 per drug course

Appendice 4: Spectroscopic data of compounds 1–7

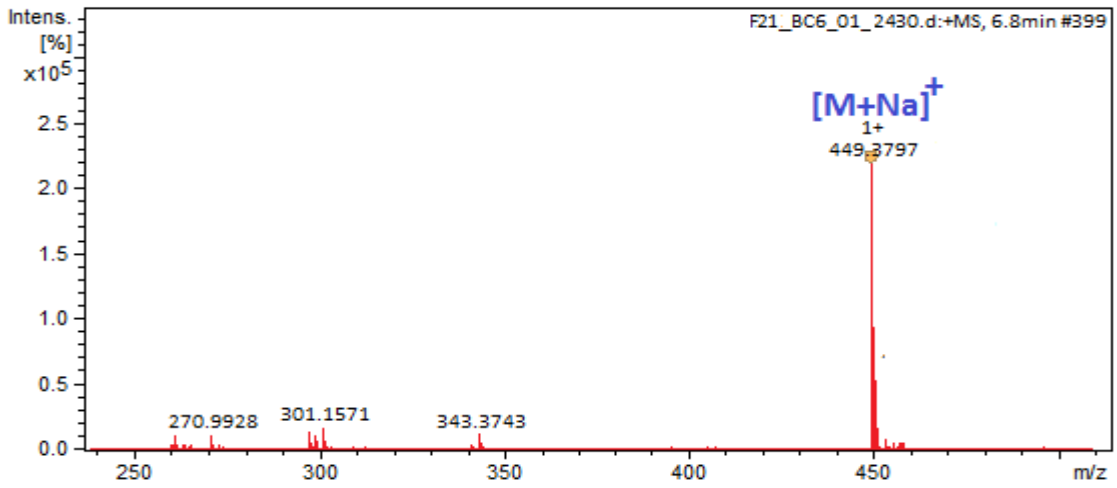


Figure S1: HRESI mass spectrum of lupeol (**1**)

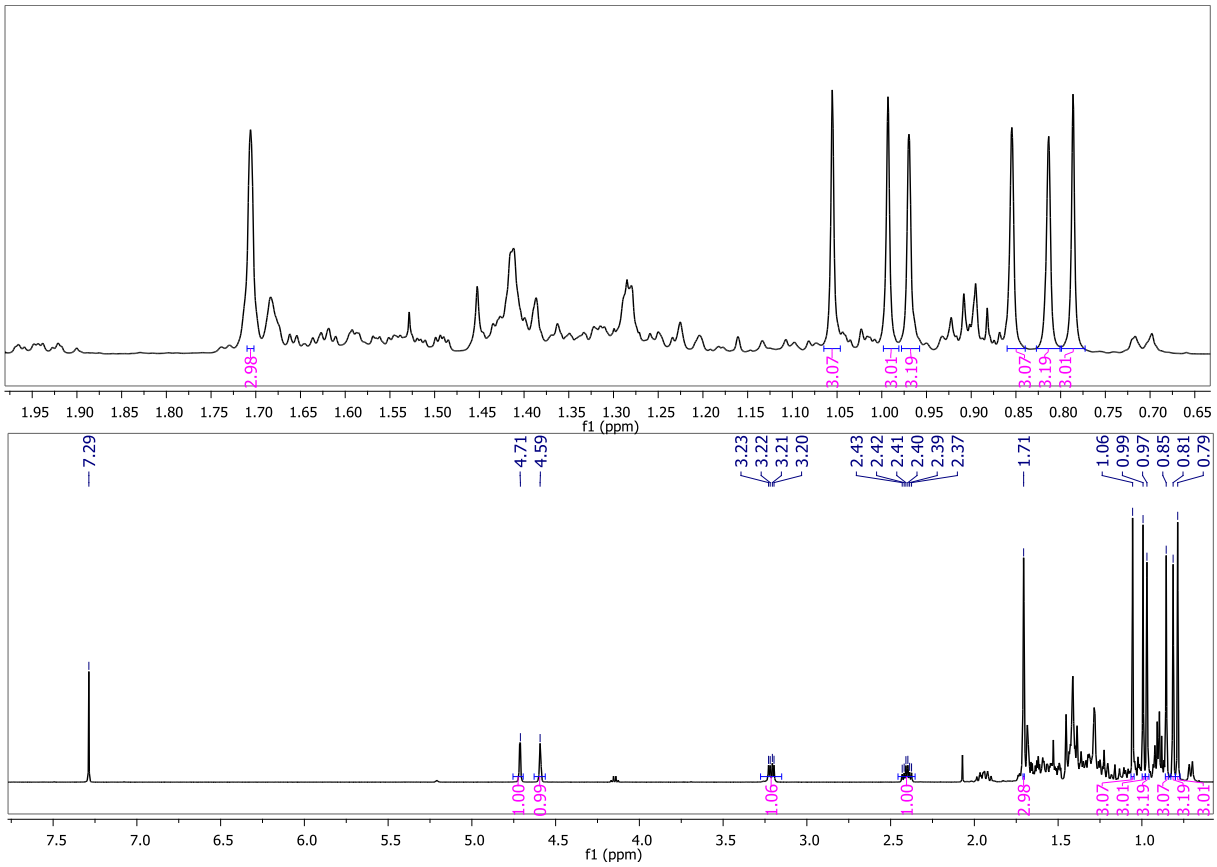


Figure S2: ^{1}H NMR spectrum of lupeol (**1**) ($CDCl_3$, 500 MHz)

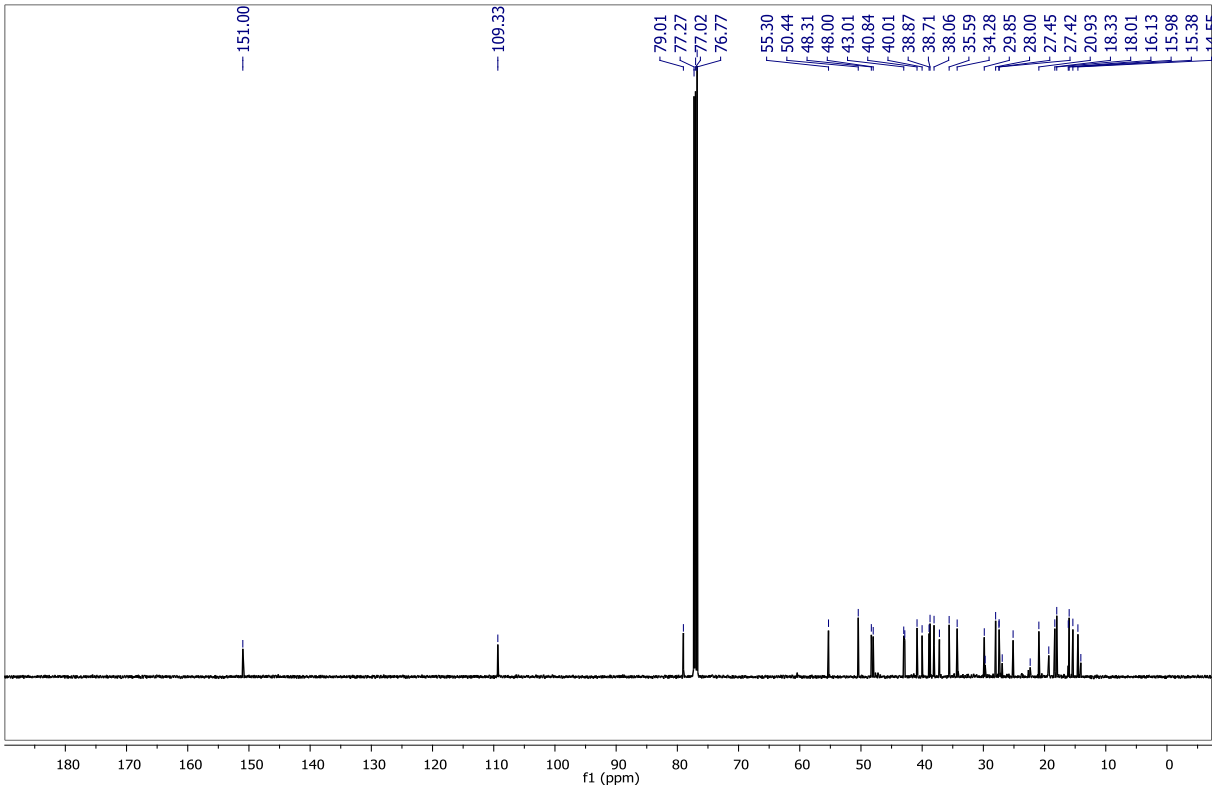


Figure S3: ^{13}C NMR spectrum of lupeol (**1**) ($CDCl_3$, 125 MHz)

d

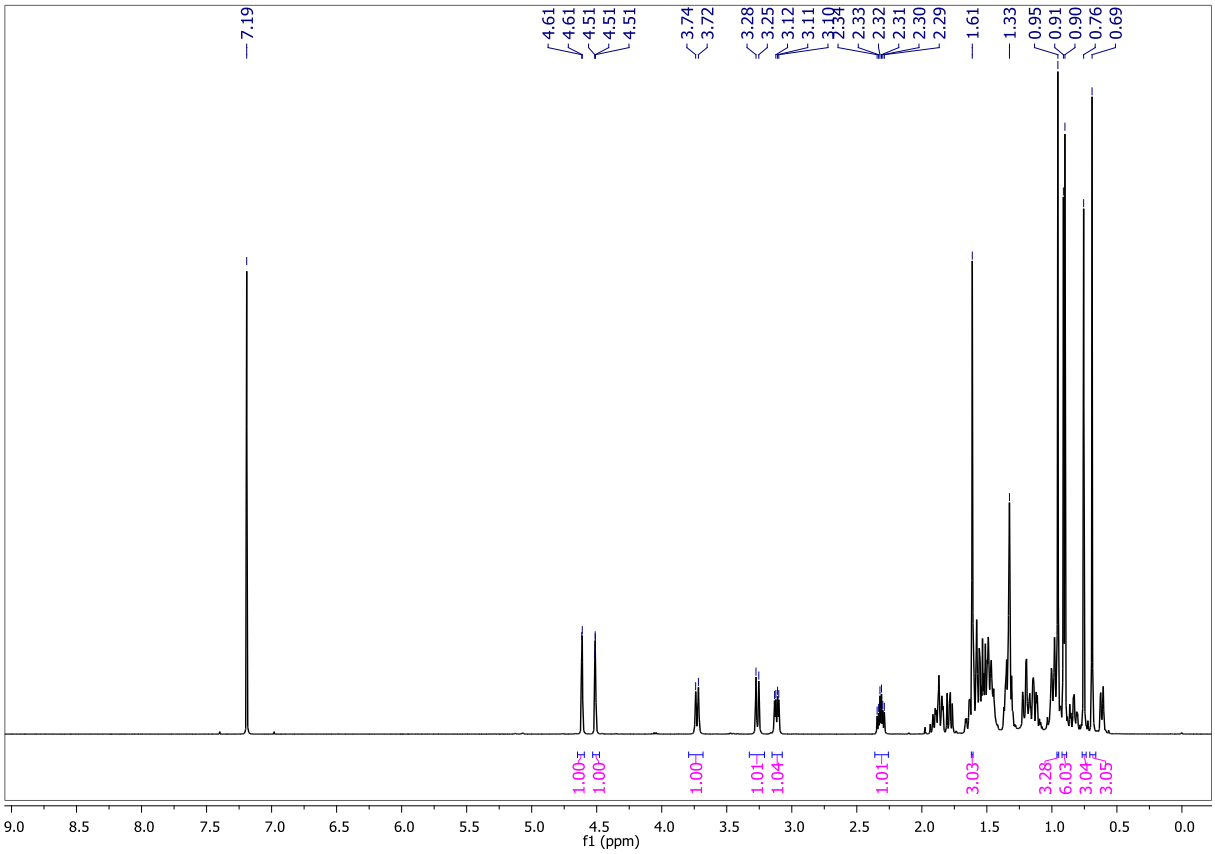


Figure S4: ^{1}H NMR spectrum of betulin (**2**) ($CDCl_3$, 500 MHz)

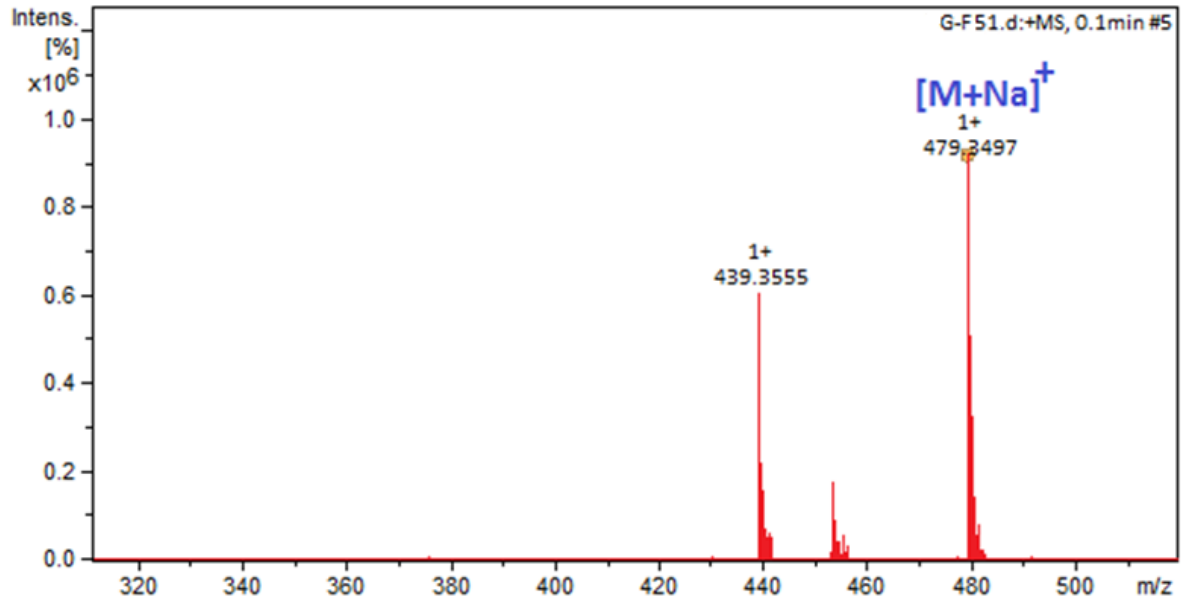


Figure S5: HRESI mass spectrum of betulinic acid (**3**)

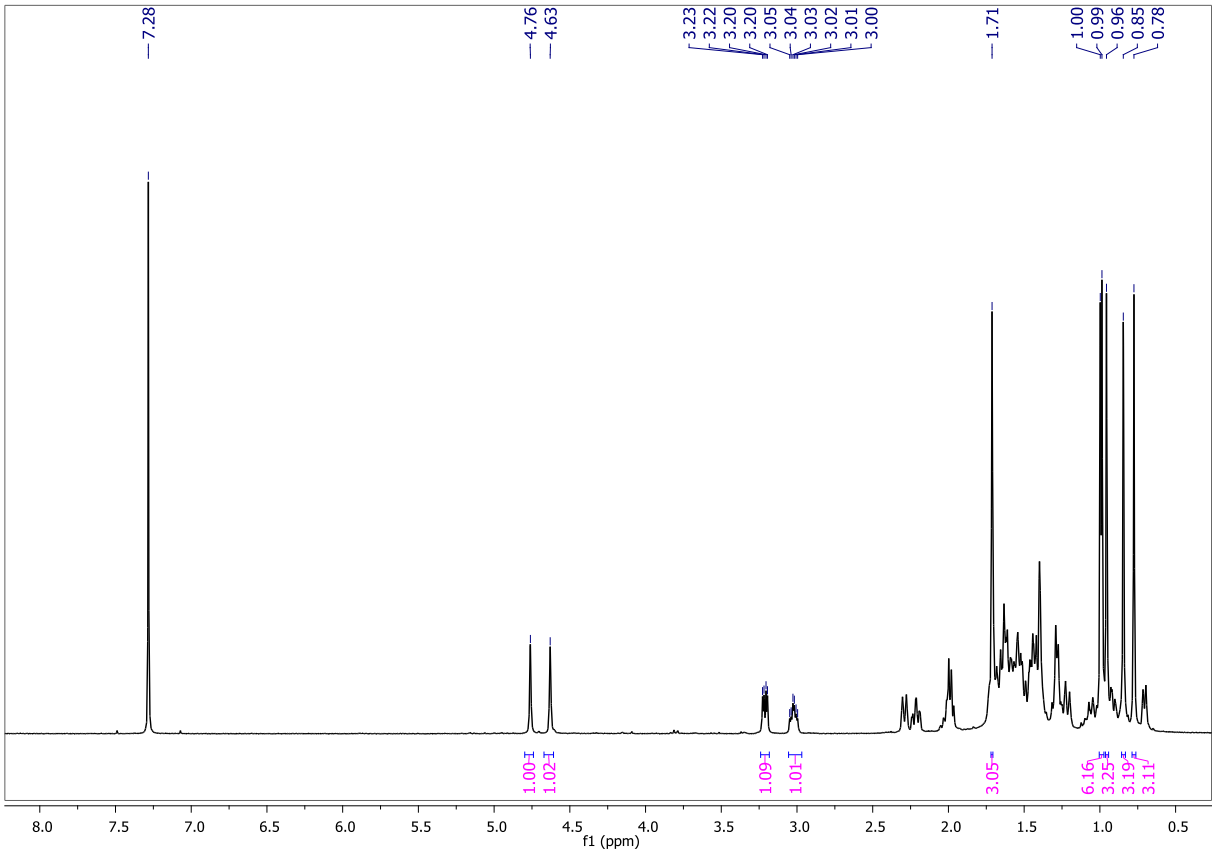


Figure S6: ^{1}H NMR spectrum of betulinic acid (**3**) ($CDCl_3$, 500 MHz)

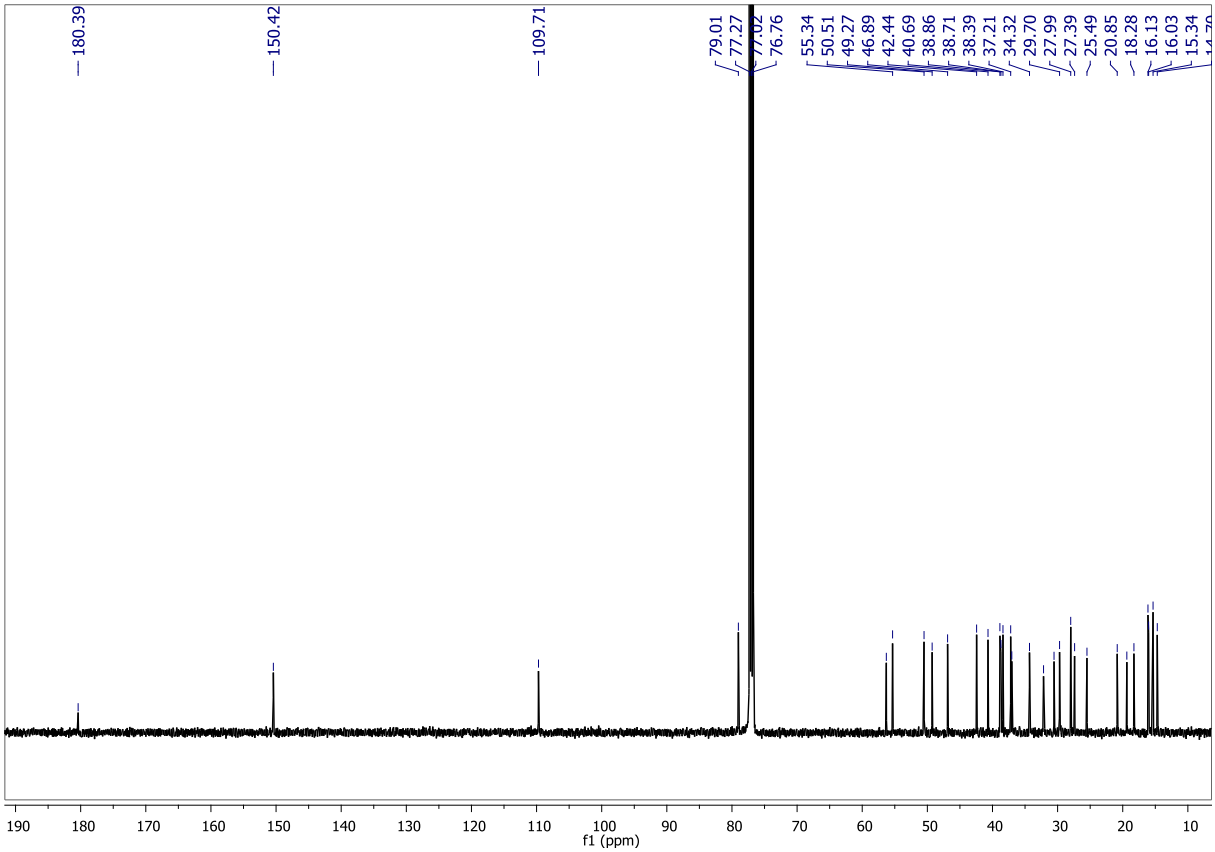


Figure S7: ^{13}C NMR spectrum of betulinic acid (**3**) ($CDCl_3$, 125 MHz)

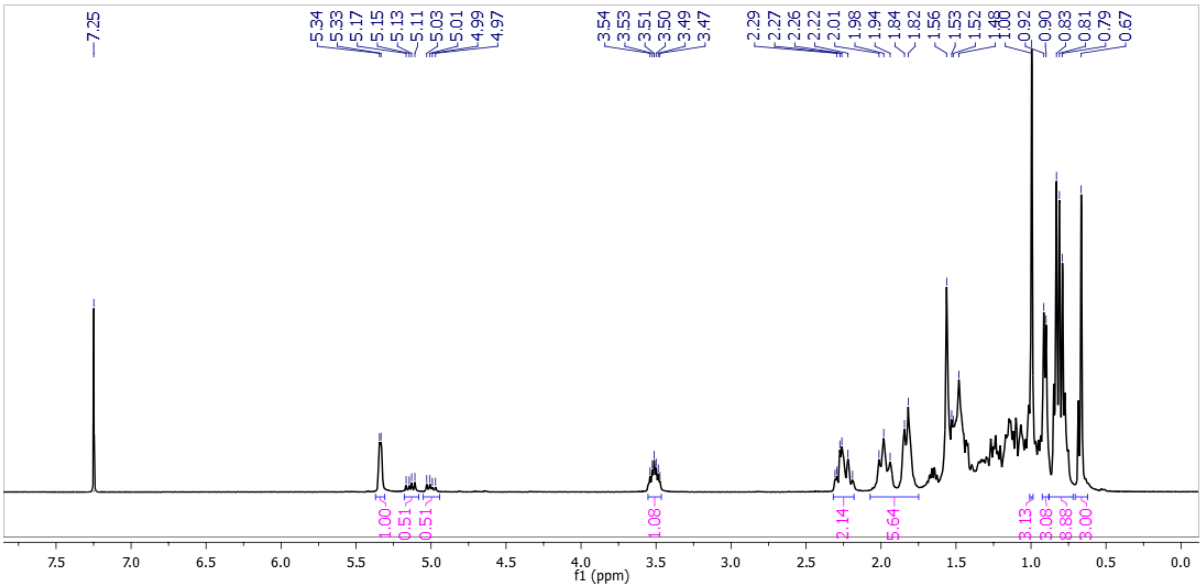


Figure S8: ^{1}H NMR spectrum of a mixture of compounds **4** and **5** ($CDCl_3$, 500 MHz)

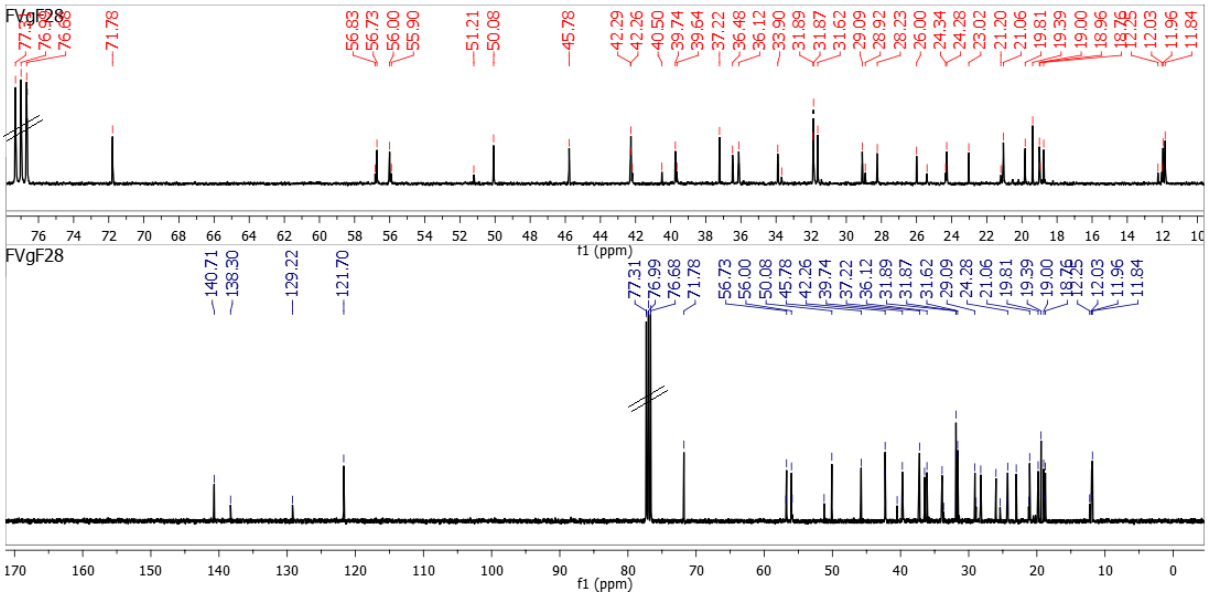


Figure S9: ^{13}C NMR spectrum of a mixture of compounds **4** and **5** ($CDCl_3$, 125 MHz)

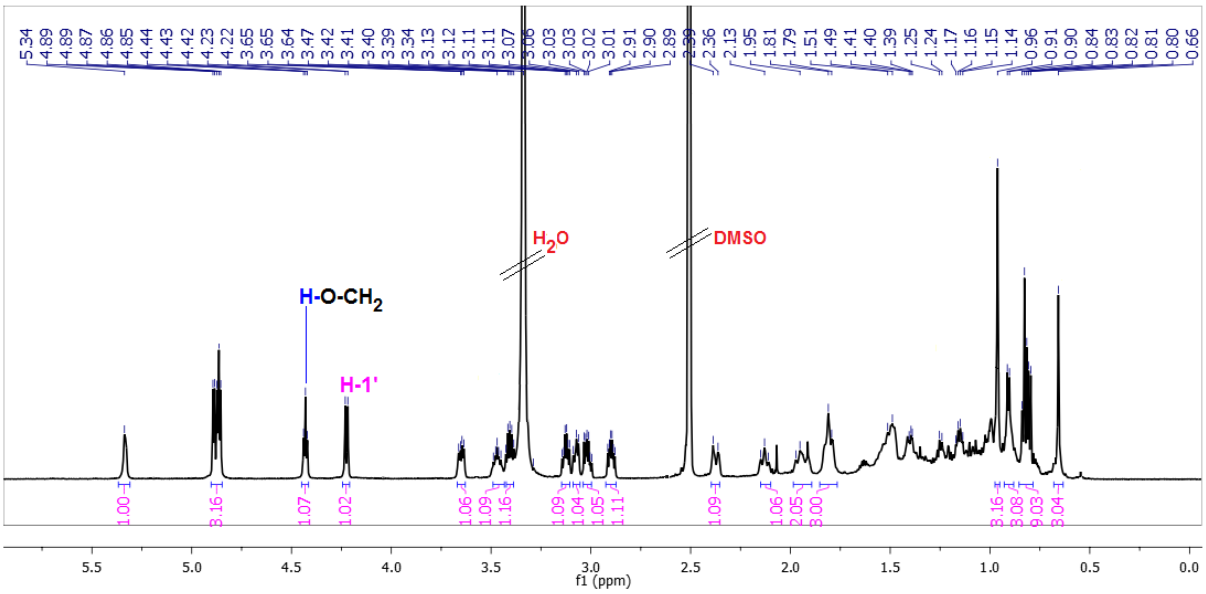


Figure S10: ^{1}H NMR spectrum of β-sitosterol 3-O-D-glucopyranoside (6) (DMSO-d_6, 500 MHz)

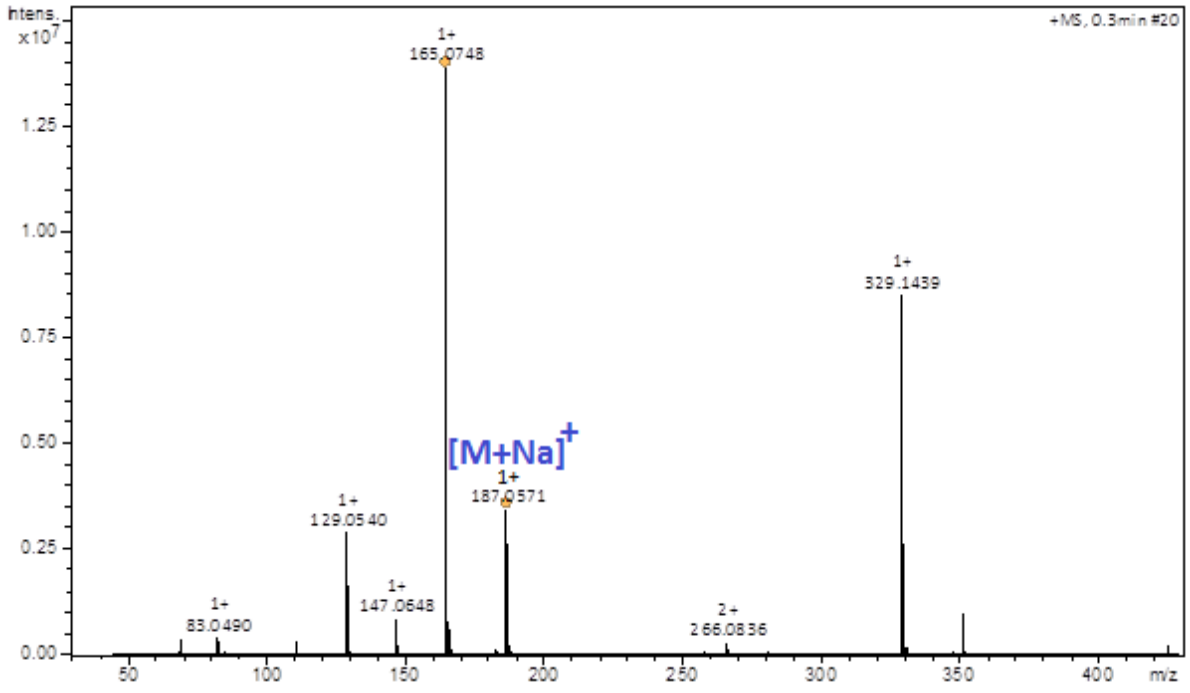


Figure S11: HRESI mass spectrum of 1-deoxyinositol (**7**)

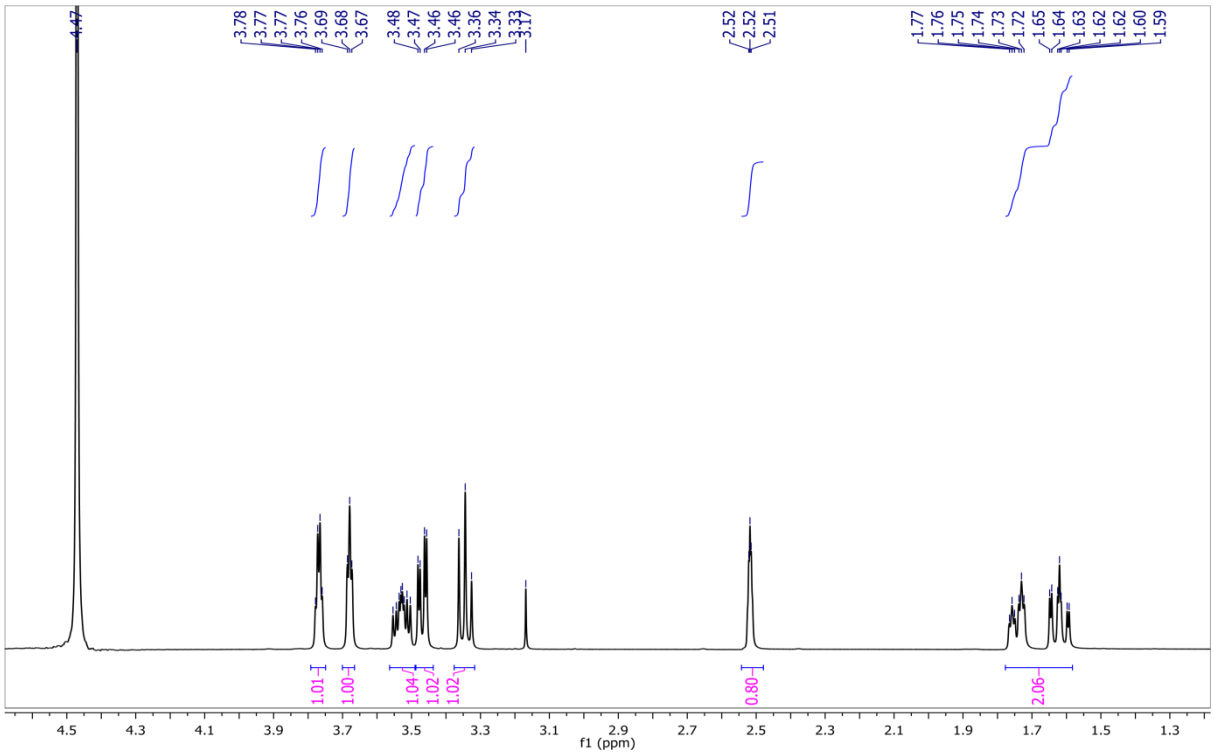


Figure S11: ^{1}H NMR spectrum of 1-deoxyinositol (**7**) (DMSO-d_6, 500 MHz)

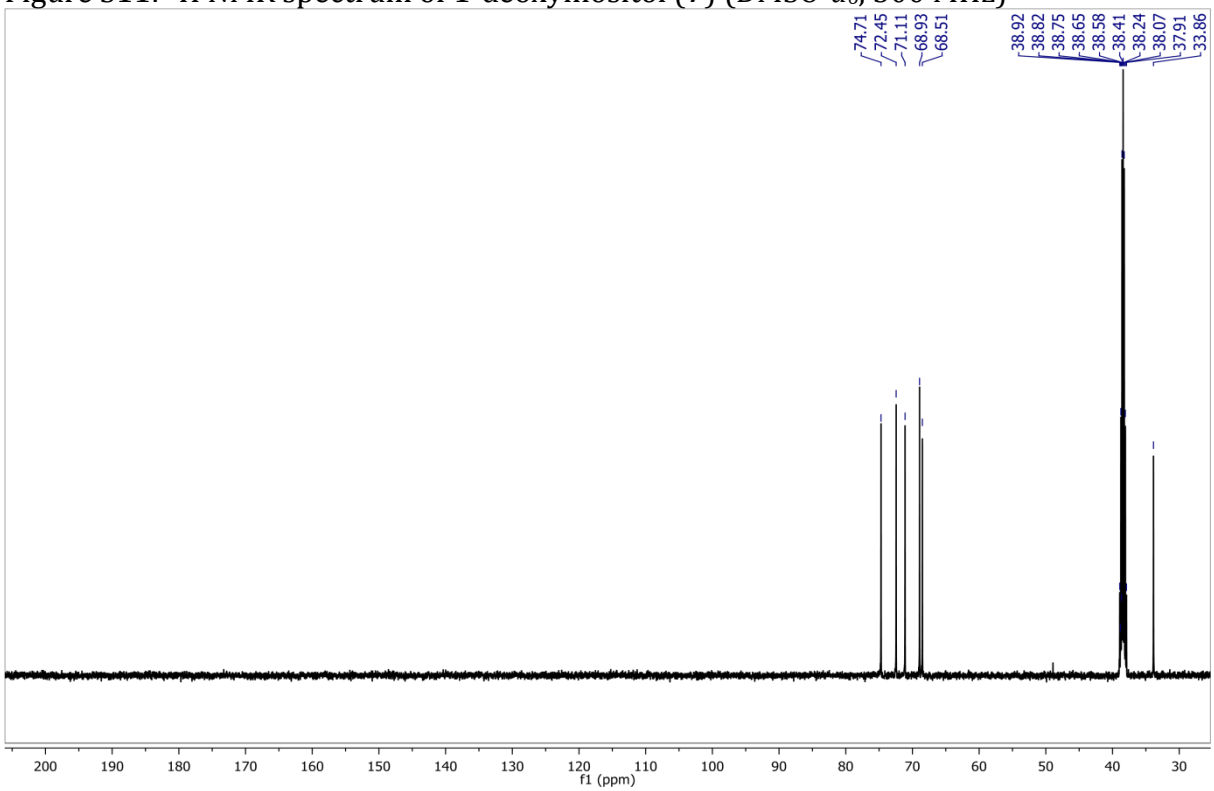


Figure S12: ^{13}C NMR spectrum of 1-deoxyinositol (**7**) (DMSO-d_6, 125 MHz)

BMC Complementary Medicine and Therapies

RESEARCH ARTICLE Open Access

Bio-guided isolation of anti-leishmanial natural products from *Diospyros gracilescens* L. (Ebenaceae)

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Abstract

Background: Plants represent an intricate and innovative source for the discovery of novel therapeutic remedies for the management of infectious diseases. The current study aimed at discovering new inhibitors of *Leishmania* spp., using anti-leishmanial activity-guided investigation approach of extracts from *Diospyros gracilescens* Gürke (1911) (Ebenaceae), targeting the extracellular (promastigotes) and intracellular (amastigotes) forms of *Leishmania donovani*.

Methods: The plant extracts were prepared by maceration using H_20: EtOH (30:70, v/v) and further fractionated using a bio-guided approach. Different concentrations of *D. gracilescens* extracts, fractions and isolated compounds were tested in triplicate against *L. donovani* promastigotes and amastigotes in vitro. The antileishmanial potency and cytotoxicity on RAW 264.7 cells were determined using the resazurin colorimetric assay. The time kill kinetic profile of the most active sample was also investigated. The structures of all compounds were elucidated on the basis of extensive spectroscopic analyses, including 1D and 2D NMR, and HR-ESI-MS and by comparison of their data with those reported in the literature.

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(Continued from previous page)

Results: The hydroethanolic crude extract of *D. gracilescens* trunk showed the most potent antileishmanial activity (IC_{50} = 5.84 μg/mL). Further fractionation of this extract led to four (4) fractions of which, the hexane fraction showed the most potent activity (IC_{50} = 0.79 μg/mL), and seven (07) compounds that exhibited moderate potency (IC_{50} = 13.69–241.71 μM) against *L. donovani*. Compound 1-deoxyinositol (7) inhibited the promastigote and amastigote forms of *L. donovani* with IC_{50} values of 241.71 μM and 120 μM respectively and also showed the highest selectivity against *L. donovani* promastigotes (SI > 5.04). To the best of our knowledge, the antileishmanial activity of this compound is being reported here for the first time. The promising hexane fraction showed significant inhibition of parasites growth at different concentrations, but with no evidence of cidal effect over an exposure period of 120 h.

Conclusions: The results obtained indicated that the hydroethanolic extract from the *D. gracilescens* trunk and the derived hexane fraction have very potent inhibitory effect on cultivated promastigotes and amastigotes of *L. donovani* parasite. The isolated compounds showed a lesser extent of potency and selectivity. However, further structure-activity-relationship studies of 1-deoxyinositol could lead to more potent and selective hit derivatives of interest for detailed drug discovery program against visceral leishmaniasis.

Keywords: *Diospyros gracilescens*, Ebenaceae, Hexane fraction, Isolated compounds, Antileishmanial, Cytotoxicity, 1-deoxyinositol

Background

Leishmaniasis is a severe, widespread zoonotic and parasitic disease caused by an intracellular flagellate protozoan of the genus *Leishmania*. The disease is generally transmitted between man and animals during a blood meal by the phlebotome female sandfly. About 20 different *Leishmania* species including *L. donovani* have been discovered to be pathogenic to human. The clinical features of the disease include a wide range of manifestations, including skin ulcers at the site of the infection or dissemination in visceral organs followed by anemia, leucopenia, fever and weakness [1, 2]. The World Health Organization estimates that 1.3 million new cases of leishmaniasis occur every year with 20,000 to 30,000 deaths annually [3]. Therefore, a great concern has been expressed by the WHO, as leishmaniases are considered as neglected tropical diseases [4]. Visceral leishmaniasis (VL), caused by *L. donovani* is the most dangerous form of the disease that can be lethal in human when untreated. It is considered as a serious public health problem worldwide, and especially in Africa where its significant morbidity and mortality require more effective chemotherapy [5]. Current available chemotherapy includes the first line treatment drugs such as pentavalent antimonials, meglumine antimoniate (glucantime) and sodium stibogluconate (pentostam) and second line drugs such as amphotericin B, pentamidine, paromomycin and miltefosine [6, 7]. However, these drugs are limited by factors such as emergence of drug resistance, especially with the pentavalent antimonials and challenges of toxicity, short half-life and high cost of drugs, as well as failure of patient to comply with treatment [8, 9]. Due to the limitations of current chemotherapeutic regimes and in the absence of effective and sustainable vaccines, there is a persistent need for alternative and readily available sources for treatment of leishmaniasis. In this respect, natural products offer good sources for new drug discovery [10].

This paper describes the in vitro antileishmanial activity of natural products from *D. gracilescens*, a plant of Ebenaceae family. It is a forest tree widely distributed in West and Centre regions of Cameroon. Furthermore, there is no mention of use of *D. gracilescens* in traditional medicine in Cameroon. However, related species such as *D. bipindensis* (Gürke), *D. conocarpa* (Gürke & K. Schum.) and *D. malabarica* ((Descr.) Kostel.) are widely used by Baka Pygmies for the treatment of malaria, sleeping sickness and respiratory disorders [11]. Globally, *Diospyros* spp. are known above all, as fishing poisons, especially in South East Asia and in the Philippines. They are also widely-used medications in traditional African medicine, mainly against leprosy. The roots are used as purgative in the Central African Republic, against pneumonia in Zimbabwe and schistosomiasis in Malawi [12, 13]. The first chemical study of *D. gracilescens* led to the isolation of few compounds such as: lupeol, betulin, betulinic acid, isodiospyrin (II) and sitosterol [14]. Of note, this is the first report of antileishmanial guided isolation of the chemical constituents of *D. gracilescens*.

Methods

Phytochemical investigation of D. gracilescens

Collection of plant material and preparation of the crude extracts

The plant materials (root, trunk, stem bark and leaf) of *D. gracilescens*, were collected in March 2017 at Eloumdem mountain (GPS coordinates: Latitude 3°49′00″N,

Longitude 11°25′60″E), in the Centre Region of Cameroon and were identified by comparison with a voucher specimen (No. 2016 / SRFK) from the National Herbarium of Cameroon by Mr. Victor Nana, a botanist.

The collected plant materials were dried under shelter at room temperature and further ground to obtain the powders. The tinctures were prepared by maceration of 4000 g of each powder in aqueous ethanol 70% (15 L, 48 h × 3). The resulting macerates were filtered using Whatman filter paper No. 2 and the filtrates concentrated on a Büchi rotary evaporator (Büchi Labortechnik AG - Flawil, Switzerland) under reduced pressure at 45–55 °C and further lyophilized using a freeze-dryer Alpha 2–4 LD plus (Christ, Germany) to yield the crude extracts. Each extract was kept dried in tightly stoppered bottles at 4 °C until it was used for the biological screenings.

Liquid-liquid partition of the trunk crude extract

The crude extract of the trunk (150 g) which showed the best leishmanicidal activity was fractionated by liquid-liquid partition as shown in Fig. 1, according to the procedure described by Xie et al. [15]. Briefly, 140 g of the crude extract was suspended in water and then extracted with n-hexane, dichloromethane, ethyl acetate and *n*-butanol successively (Fig. 1). Each fraction was evaporated under reduced pressure at 45–55 °C and then the aqueous fraction was lyophilized. Five residues were obtained and respectively named fraction A from *n*-hexane [6.5 g, 4.6% yield], fraction B from dichloromethane [20.0 g, 14.3% yield], fraction C from ethyl acetate [23.4 g, 16.7% yield], fraction D from *n*-butanol [31.3 g, 22.35% yield] and fraction E for the remaining aqueous residue (45.5 g, 32.50% yield). Each of the afforded fractions was submitted to antileishmanial screening and the promising ones (fractions A, B and D) having IC_{50} values below 2 μg/mL were selected and submitted to chromatographic separation.

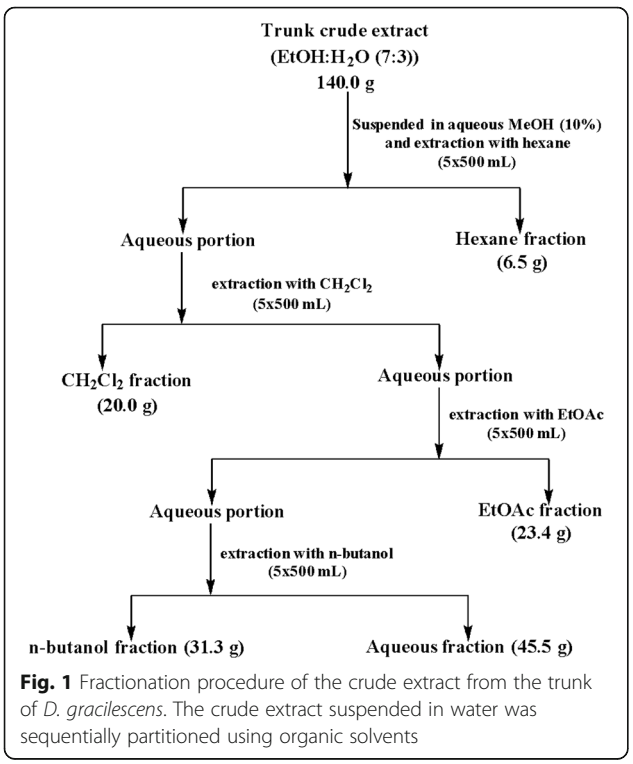

Fig. 1 Fractionation procedure of the crude extract from the trunk of *D. gracilescens*. The crude extract suspended in water was sequentially partitioned using organic solvents

Chromatography of the active fractions

Chromatography of fraction A A portion of *n*-hexane fraction (m = 6 g; IC_{50} = 0.78 μg/mL) was subjected to silica gel column chromatography eluting with an isocratic system of *n*-hexane/ethyl acetate (95/5). Three hundred sub-fractions of ca 100 mL each were collected and combined into 10 sub-fractions A1—A10 based on their TLC profiles. Sub-fractions A1 to A6 consisted of a mixture of fatty acids and were not investigated further. Lupeol (1) (1.11 mg) and a mixture of *β*-sitosterol (4) and stigmasterol (5) (14.8 mg) were obtained from sub-fractions A7–8 (n-hexane/EtOAc (95/5), 1.5 g) and sub-fractions A9&10 (*n*-hexane/EtOAc 90/10, 2.2 g and 80/20, 22 mg) respectively [16, 17].

Chromatography of fraction B The dichloromethane fraction (m = 19 g; IC_{50} = 1.63 μg/mL) was subjected to column chromatography on 230–400 mesh silica gel (Merck, Darmstadt, Germany) eluting with a gradient of ethyl acetate in *n*-hexane. Ninety sub-fractions of ca 250 mL each were collected and combined into 4 major sub-fractions B1—B4 according to their TLC profiles. Sub-fraction B1 (*n*-hexane/EtOAc (95/5), 8.1 g) was found to contain mainly fatty substances and traces of hexane soluble compounds. Sub-fraction B2 (n-hexane/EtOAc (90/10), 2.1 g) was subjected to repeated silica gel column chromatography using a mixture of *n*-hexane:ethyl acetate in a gradient mode as eluent to afford the mixture of *β*-sitosterol (4) and stigmasterol (5) (17.6 mg). Sub-fraction B4 (*n*-hexane/EtOAc (65/35), 4.5 g) was fractionated on silica gel column chromatography with an isocratic solvent system of n-hexane/ethyl acetate (30/70) to afford a mixture of compounds 2 and 3 which were further purified using a sephadex LH-20 (Sigma-Aldrich, Munich, Germany) column chromatography with a mixture of CH_2Cl_2/MeOH (20/80) as eluent to afford pure betulin (2) (2.0 mg) and betulinic acid (3) (27 mg) [17–19]. Sub-fraction B3 was not further studied due to limited quantity.

Chromatography of fraction D The n-butanol fraction (m = 30 g; IC_{50} = 1.11 μg/mL) was subjected to silica-gel column chromatography eluting with a mixture of *n*-

hexane/acetone/methanol in a gradient mode. Twenty sub-fractions of ca 400 mL each were collected and combined into 5 major sub-fractions D1—D5 based on their TLC profiles. Beta-Sitosterol glucoside (6) (185 mg) precipitated in sub-fraction D1 (*n*-hexane/Acetone 30/70). Compound 1-deoxyinositol (7) (30 mg) precipitated in sub-fraction D5 (*n*-hexane/Acetone/Methanol (3/6/1)) and was filtered before being purified by recrystallization in acetone/water (90/10) [11, 20]. Data on sub-fractions D2–3 are not presented in this paper because they are under further scrutiny.

HPLC–DAD–ESI-MS analysis of extracts from D. gracilescens

Sample preparation Each extract was dissolved in HPLC grade methanol at a concentration of 0.5 mg/mL, then filtrated through a syringe-filter-membrane. Each aliquot obtained (5 µL) was injected into the UPLC–DAD-HRESI/MS Dionex Ultimate 3000 HPLC (Germany) apparatus used to perform the analyses.

HPLC-MS conditions High resolution mass spectra were obtained with an OTOF Spectrometer (Bruker, Germany) equipped with a HRESI source and a UV–vis absorbance detector. The spectrometer was operated in positive mode (mass range: 100–1500, with a scan rate of 1.00 Hz) with automatic gain control to provide high-accuracy mass measurements with 2 ppm deviation using Na Formate as calibrant. Mass spectra were simultaneously acquired using electrospray ionization in the positive ionization mode. The following parameters were used for experiments: spray voltage of 4.5 kV, capillary temperature of 200 °C. Nitrogen was used as sheath gas (10 l/min). The spectrometer was attached to an Ultimate 3000 (Thermo Fisher, USA) HPLC system consisting of LC-pump, UV traces were measured at 215, 218, 254, 280 and 330 nm and UV spectra—Diode Array Detector— (DAD) was recorded between 190 and 600 nm, auto sampler (injection volume 5 µl) and column oven (35 °C). The separations were performed using a Synergi MAX-RP 100A (50 × 2 mm, 2.5 µ particle size) with a H_2O (+ 0.1% HCOOH) (A)/acetonitrile (+ 0.1% HCOOH) (B) gradient (flow rate 500 µL/min). Samples were analyzed using a gradient program as follows: 95% A isocratic for 1.5 min, linear gradient to 100% B over 6 min, after 100% B isocratic for 2 min, the system returned to its initial condition (90% A) within 1 min, and was equilibrated for 1 min.

Identification of peaks Identification of all constituents was performed by UPLC–DAD-HRESI/MS analysis and by comparing the UV, MS spectra and MS/MS fragmentation of the selected peaks in the sample chromatogram with those of data reported the literature of SciFinder database.

Screening of extracts for biological activity

Parasite culture and maintenance

The cryopreserved promastigote form of *L. donovani* (1S (MHOM/SD/62/1S) was obtained from Bei Resources (https://www.beiresources.org/) and is routinely cultured at the Antimicrobial and Biocontrol Agents Unit, University of Yaoundé I, in Medium 199 (Sigma, Darmstadt, Germany) supplemented with 10% Heat-Inactivated fetal Bovine Serum (HIFBS) (Sigma, Darmstadt, Germany) and 100 IU/mL penicillin and 100 µg/mL streptomycin. The culture was maintained in 75 Cm^2 cell culture flask at 28 °C and checked for growth daily and sub-cultured everyday 72 h [21].

Determination of the antileishmanial activity of plant extracts and fractions

Inhibitory assay against *L. donovani* promastigotes The antileishmanial activity of *D. gracilescens* crude extracts, derived fractions and compounds against cultured *L. donovani* promastigotes was evaluated using the resazurin colorimetric assay as described by Siqueira-Neto et al. [22]. The stock solutions were prepared by dissolving each sample in 100% dimethyl sulfoxide (DMSO) and subsequently diluted serially in non-supplemented culture medium. To assess the antileishmanial activity, 4×10^5 promastigotes/mL/well were seeded in a 96 well microtiter plate and treated with 5-fold diluted concentrations of *D. gracilescens* extracts (0.16, 0.8, 4, 20 and 100 µg/mL) for 72 h at 28 °C. The viability rate of promastigotes positively correlated with the amount of pink resorufin that was produced through the reduction of blue resazurin by the dehydrogenase enzymes in the inner mitochondrial membrane of the living parasites. Briefly, promastigotes from a logarithmic phase culture (4×10^5 cells/mL; 90 µL) were seeded in 96-well microtiter plates and were treated with 10 µl of inhibitors at different triplicate concentrations ranging 100 µg/mL-0.16 µg/mL for extracts and fractions and 50 µg/mL-0.08 µg/mL for compounds. The final concentration of DMSO in each well was not higher than 1%. Plates were incubated for 28 h at 28 °C, followed by the addition of 1 mg/mL resazurin (Sigma, Darmstadt, Germany). The negative and positive controls were 0.1% DMSO and amphotericin B (Sigma, Darmstadt, Germany) (10–0.016 µg/mL) respectively. After an additional incubation for 44 h, plates were then read on a Magelan Infinite M200 fluorescence multi-well plate reader (Tecan, Männedorf, Switzerland) at an excitation and emission wave lengths of 530 and 590 nm respectively. For each sample, growth percentages were calculated and dose–response

curves were constructed to determine the 50% inhibitory concentration (IC_{50}) using the GraphPad Prism-version 5.0 software (San Diego, California, USA).

Inhibitory assay against *L. donovani* amastigotes The effect of plant isolates against the intracellular amastigote form of *L. donovani* was evaluated essentially as described by Jain et al. [23] with some modifications. Briefly, macrophage Raw 264.7 cells (4×10^3 cells/well) were seeded in 96 well plates and incubated for 6 h at 37 °C under 5% CO_2 for adhesion of the cells. Afterwards, the non-adherent cells were washed-out with sterile PBS. The adherent Raw 264.7 cells were infected with metacyclic promastigotes (4×10^5 cells) at an infection ratio of 1:10 macrophage: parasites and incubated for 24 h at 37 °C under 5% CO_2 in order to allow infection of macrophages by metacyclic promastigotes. Thereafter, the overlying medium was removed, and the monolayer cells with internalized amastigotes were carefully washed four times with PBS to remove free parasites. Freshly prepared M199 medium containing 10% FBS and the test extracts were added in triplicate to the infected cells at serially diluted concentrations and incubated for 48 h at 37 °C under 5% CO_2. After incubation, 0.05% Sodium dodecyl sulfate (SDS) was added in each well for 30 s for controlled lysis followed by M199 with 10% FBS as macrophage lysis stopper. Resazurin reagent (250 μg/mL) was thereafter added in each well and the plates were incubated for 24 h followed by fluorescence recording at λexcitation = 530 nm and λemission = 590 nm using a Tecan Infinite M200 microplate reader (Tecan). Inhibition percentages were calculated using Microsoft Excel Software and median inhibitory concentration (IC_{50}) obtained from dose-response curves using GraphPad Prism 5.0. Software.

Cytotoxicity assay

The cytotoxicity profile of extracts, fractions and compounds was assessed using the Alamar blue assay [24] against Raw 264.7 cells duly cultivated in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 13.5 g/L DMEM (Sigma Aldrich), 10% fetal Bovine Serum (Sigma Aldrich), 0.2% sodium bicarbonate (w/v) (Sigma, Darmstadt, Germany) and 50 μg/mL gentamycin (Sigma Aldrich). Globally, macrophages were seeded into 96-wells cell-culture flat-bottomed plates at a density of 10^4 cells in final volume of 100 μL of complete medium/well and incubated for 24 h at 37 °C, 5% CO_2, 70% RH in a ICO 105 memmert incubator (memmert, Schwabach, Germany) to allow cell adhesion. Ten μL of each serially diluted test sample were added in triplicate wells and assay plates were then incubated for 48 h in the same experimental conditions. Growth control consisted of 0.1%DMSO (100% growth) and positive control of podophyllotoxin (Sigma, Darmstadt, Germany) at 20 μM. Cell proliferation was checked by adding 10 μL of a stock solution of resazurin (0.15 mg/mL in sterile PBS) to each well followed by plates incubation during 4 h. Fluorescence was then read on a Tecan Infinite M200 fluorescence multi-well plate reader at an excitation/emission of 530/590 nm. Results were expressed as 50% cytotoxic concentrations (CC_{50}). Selectivity indices (CC_{50}/IC_{50}, defining the balance between cytotoxicity and antileishmanial activity) were calculated for each test substance.

Concentration/time inhibition kinetics of the most active fraction

The growth inhibitory effect of the most active fraction against *L. donovani* promastigotes was examined by culturing parasites (4×10^5 cells/ml) in freshly prepared complete M199 medium in the presence and absence of varying concentrations of the hexane fraction (1/2 IC_{50}, IC_{50}, 2x IC_{50}, 4x IC_{50}), using amphotericin B as positive control (1/2 IC_{50}, IC_{50}, 2x IC_{50}, 4x IC_{50}) for 120 h. The number of viable parasites was determined after every 24 h over 120 h by staining with trypan blue. Quantification of viable parasites was achieved by counting the parasites with clear cytoplasm (non-stained) using a Neubauer hemocytometer with cover slips. Three independent experiments were performed for each sample.

Data analysis

All the activity data represent mean ± standard deviation (SD) from three independent experiments. Microsoft Excel Software was used to calculate the percentage of inhibition. The IC_{50} and CC_{50} values were determined using GraphPad Prism 5.0 Software with data fitted by non-linear regression.

Statistical analysis

Data were expressed as mean ± SEM (standard error of mean). Statistical analysis was performed by one-way ANOVA (analysis of variance) followed by the Bonferroni post-test using GraphPad 7 software. Difference was considered as significant at $p < 0.05$.

Results

Phytochemical analysis data

The crude extract, the hexane and dichloromethane soluble fractions of the trunk of *D. gracilescens* were analyzed by UPLC coupled to both diode array and mass spectrometry detectors. The latter was used with an electrospray ionization (ESI) source in positive ion mode. A representative base peak chromatogram and all ions MS (Fig. 1) indicating that the used UPLC conditions allowed a good separation of a large percentage of compounds. The compounds were recognizable from

their characteristic UV spectra, which were identified based on the UPLC–DAD–HRESI-MS data and subsequent confirmation by comparison with literature data. The chromatographic profile and spectroscopic data are presented in Table 1 and Fig. 2 below.

Biological activities data

Antileishmanial activity of plant samples

The inhibitory potential of *D. gracilescens* extracts against *L. donovani* promastigotes was measured by direct counting of live promastigotes after parasite exposure to various concentrations of extract. The IC_{50} values of the different crude extracts are shown in Table 2 below.

The results shown in Table 2 indicate that only the extract from the trunk of *D. grascilisens* exerted antileishmanial activity with an IC_{50} value of 5.84 μg/mL. The other extracts were non-active up to 100 μg/mL. The trunk extract was therefore progressed for bio-guided investigation.

Bio-guided fractionation of the trunk extract of D. grascilisens

Fractionation of the trunk extract was performed by liquid-liquid partition to afford four (04) main fractions that were tested for activity against the promastigotes and amastigotes forms of *L. donovani* (Table 3).

Results from Table 3 indicate that bio-guided fractionation of the crude trunk extract (IC_{50} = 5.84 μg/mL) has resulted in more potent fractions, with increase in activity in the range of 7.4–2-fold against *L. donovani* promastigotes (IC_{50} = 0.79–2.36 μg/mL). The hexane fraction showed the highest potency (IC_{50} = 0.79 μg/mL), followed respectively by the n-butanol (IC_{50} = 1.11 μg/mL), dichloromethane (IC_{50} = 1.63 μg/mL) and ethyl acetate (IC_{50} = 2.36 μg/mL) fractions. The water residue showed no activity up to 100 μg/mL. Activity data against *L. donovani* amastigotes indicated decreased potency of the crude trunk extract (IC_{50} = 35.69 μg/mL) and the hexane fraction (IC_{50} = 8.06 μg/mL). Overall, the extract and fractions showed greater selectivity against *L. donovani* promastigotes (18 < SI < 127) than against the amastigotes (2.8 < SI < 12.4). The hexane fraction exhibited the highest selectivity against *L. donovani* promastigotes (SI > 126.7) and amastigotes (SI > 12.4).

Further fractionation of fraction A led to the isolation of lupeol (1) and a mixture of sterols (4). Fraction B led to betulin (2) and betulinic acid (3) and fraction D led to *β*-sitosterol glucoside (6) and 1-deoxyinositol (7) as shown in Fig. 3 below. The structures of these compounds were elucidated on the basis of spectroscopic analyses, including 1D and 2D NMR, and HR-ESI-MS and by comparison of their data with those reported in the literature (see supplementary information). These compounds were also tested for activity against the promastigote and amastigote forms of *L. donovani*. The results achieved are presented in Table 4.

Globally, the isolated compounds exhibited moderate inhibition of *L. donovani* promastigotes (IC_{50}: 13.69–241.71 μM) and amastigotes (17.83–120 μM). Of note, the activity of 1-deoxyinositol (IC_{50} of 241.71 μM and 120 μM against promastigotes and amastigotes respectively) is being reported here for the first time.

Kinetics of parasite killing as a relation to time and inhibitor concentration

The following graphs below (Fig. 4) show the time kill kinetic of the most active (Hexane) fraction (A), compared to the positive control, amphotericin B (B).

The ability of the hexane fraction to fast-kill *L. donovani* promastigotes was assessed at different concentrations points (0.5x IC_{50}, IC_{50}, 2x IC_{50} and 4x IC_{50}) relative to untreated parasites culture over a period of 120 h. The results showed that treatment with increasing concentrations of the hexane fraction and amphotericin B resulted in a significant reduction in promastigote replication after 24 h (Fig. 4). In the meantime, an exponential growth was observed in untreated parasite culture.

Table 1 FT-MS product ions of detected compounds in the trunk extract of *D. gracilescens*

N°	RT (min)	$[M+H]^+$		UV, λ_{max} (nm)	molecular formula	Name of compound
		Exp.	Calcd.			
1	7.19	663.4740	663.4772	222	$C_{46}H_{62}O_3$	Chlorobiumquinone
2	6.99	431.3689	431.3672	222	$C_{32}H_{46}$	NI
3	6.82	483.3621	483.3621	450	$C_{30}H_{46}O$	NI
4	6.77	427.3946	427.3946	222	$C_{30}H_{50}O$	Lupeol
5	6.65	469.3465	469.3421	222	$C_{32}H_{46}O$	NI
6	6.57	479.3887	479.3884	222	$C_{33}H_{50}O_2$	NI
7	6.21	391.3003	391.2995	222	$C_{28}H_{38}O$	NI
8	5.13	579.3116	579.3105	218	$C_{38}H_{42}O_5$	NI

NI Not Identified

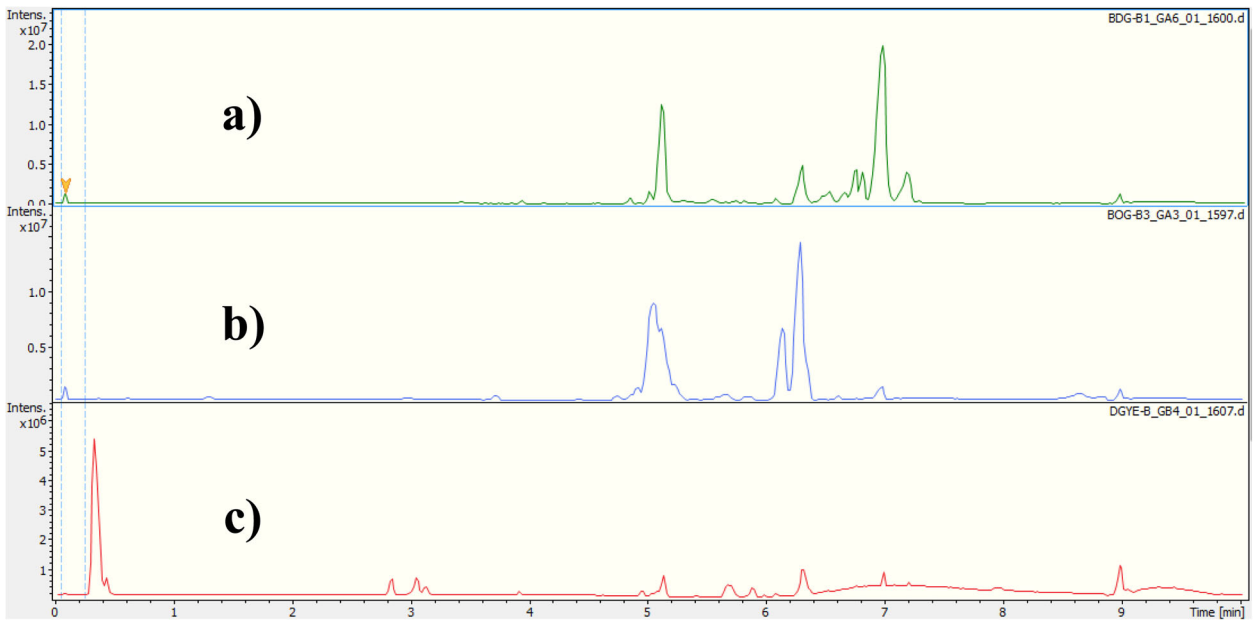

Fig. 2 HPLC profiles of the crude extract and fractions from the trunk of *D. gracilescens* (TIC: m/z 150–1000). a crude extract; b Dichloromethane fraction; c Hexane fraction

At all tested concentrations of hexane fraction and amphotericin B, a regular reduction of the viability of treated parasite cultures was observed up to 120 h, with however no evidence of cidal effect.

Discussion

The investigation of extracts from *D. grascilesens* for antileishmanial activity identified the hydroethanolic trunk extract as a promising starting point for bio-guided study. This is the first report describing the antileishmanial activity of extracts from *D. grascilesens*. However, extracts from other *Diospyros* spp. have been previously investigated in this direction. Of note, Lenta et al. [25] demonstrated the capacity of the dichloromethane-methanol (1:1) extract of *D. canaliculata* (De Wild.) to inhibit the growth of axenic amastigotes of *L. donovani*. On another hand, Dhar et al. [26] reported that ethanolic extracts of *D. montana* (Roxb) and *D. peregrina* ((Gaertn.) Gürke) possess antiprotozoal activity against *Entamoeba histolytica*, antiviral activity against Ranikhet disease virus and hypoglycemic activities in albino rats. Rocío et al. [27] also demonstrated the in vitro antimycobacterial potency of the stem bark extract from *D. anisandra* (S.F. Blake) against a resistant strain of *M. tuberculosis*. In other studies, Hazra et al. [28] demonstrated the anti-tumour activity of bark extract from *D. ferrea* ((Willd.) Bakh). Asolkar et al. [29] showed the antibacterial activity of leaf and seed extracts from *D. montana* and Satish and Sunil [30] demonstrated the anti-diabetic and antioxidant potential of the ethanolic bark extract of *D. malabarica*.

Based on the criteria set for antileishmanial activity of plant extracts by Camacho et al. [31], the promising hydroethanolic trunk extract of *D. grascilesens* was further fractionated yielding the hexanic fraction as the more active and selective against the extracellular and the intracellular forms of *L. donovani* parasite. Further fractionation of this fraction led to six (6) compounds

Table 2 Anti-leishmanial activity of *D. gracilescens* crude extracts against promastigotes of *L. donovani*

Plant	Part	Solvent	Promastigote of *L. donovani* (MHOM/SD/62/1S) IC_{50} ± SD (μg/ml)
D. grascilisens	Root	H_20: EtOH	> 100
	Stem bark	H_20: EtOH	> 100
	Leaf	H_20: EtOH	> 100
	Trunk	H_20: EtOH	5.84 ± 0.20
Positive control	Amphotericin B		0.34 ± 0.22

Activity data are expressed as mean ± Standard deviation (SD) from triplicate experiments; IC_{50}: 50% Inhibitory Concentration

Table 3 Anti-leishmanial activity and selectivity Indexes of fractions from the trunk crude extract against promastigotes and amastigotes of *L. donovani*

Extract/ fraction	IC_{50} (Promastigotes) (μg/ml ± SD)	IC_{50} (Amastigotes) (μg/ml) ± SD	Raw267.4 CC_{50} (μg/ml ± SD)	SI (Promastigotes)	SI (Amastigotes)
Trunk crude extract	5.84 ± 0.20^{f}	35.69 ± 0.26^{f}	> 100	> 18.56	> 2.80
Hexane fraction (A)	0.79 ± 0.09^{b}	8.06 ± 0.39^{b}	> 100	> 126.74	> 12.40
Dichloromethane fraction (B)	1.63 ± 0.11^{d}	10.97 ± 0.11^{c}	> 100	> 61.16	> 9.11
Ethyl-acetate fraction (C)	2.36 ± 0.06^{e}	16.05 ± 0.12^{d}	> 100	> 42.30	> 6.23
n-butanol fraction (D)	1.11 ± 0.10^{c}	22.08 ± 0.09^{e}	> 100	> 89.60	> 4.53
Water residue (E)	> 100	–	–	–	–
Positive control	0.34 ± 0.22^{a}	0.12 ± 0.11^{a}	/	/	/

Activity data are mean ± Standard deviation (SD) from triplicate experiments; IC_{50}: 50% Inhibitory Concentration; CC_{50}: 50% Cytotoxic Concentration; SI: Selectivity Index. Along each column, IC_{50} values with the same letter superscripts are not significantly different, Bonferroni test ($p > 0.05$)

identified as lupeol, betulin, betulinic acid, mixture of sterols, β-sitosterol glucoside and 1-deoxyinositol. Among these compounds, betulinic acid showed the most potent activity and selectivity against both promastigote and amastigote forms of *L. donovani*. There are few studies in the literature reporting the activity of betulin and betulinic acid and derivatives against *Leishmania* parasites. Indeed, similarly to our findings, Sousa et al. [32] have reported moderate antileishmanial activity (23-55 μM) of semisynthetic lupane triterpenoids, betulin and betulinic acid when tested alone, and their synergistic effects with miltefosine, an alkylphosphocholine drug with demonstrated activity against various parasite species (including *Leishmania infantum* parasites and amoeba) and cancer cells as well as some pathogenic bacteria and fungi [33]. Alakurtti et al. [34] also determined the activity of heterocyclic betulin derivatives on *L. donovani* amastigotes and the in vitro activity of betulin and betulinic acid derivatives against *L. donovani* amastigotes and promastigotes of *L. amazonensis*. Dominguez et al. [35] also reported the activity of betulinic acid acetate and betulinic acid methyl ester against promastigotes of *L. amazonensis*.

Betulinic acid has been already reported in the literature to possess a wide range of biological and medicinal properties, including anti-human immunodeficiency virus (HIV), antibacterial, antimalarial, anti-inflammatory, anthelmintic, antinociceptive, anti-herpes simplex viruses-1 (HSV-1), immune-modulatory, antiangiogenic, and anticancer activities [36, 37]. The activity of betulinic acid and its derivatives against the erythrocytic stage of the chloroquine-sensitive 3D7 *Plasmodium falciparum* strain was previously reported, as well as moderate antileishmanial activity on different *Leishmania* spp. [34, 38, 39]. Cassio et al. [40] also showed that the semi-synthetic derivatives of betulinic acid were able to prevent the parasite development and invasion into host cells, that are crucial events for *Trypanosoma cruzi* infection establishment, with potency similar to benznidazole.

In this study, lupeol was found to be active against promastigotes and moderately active against amastigotes

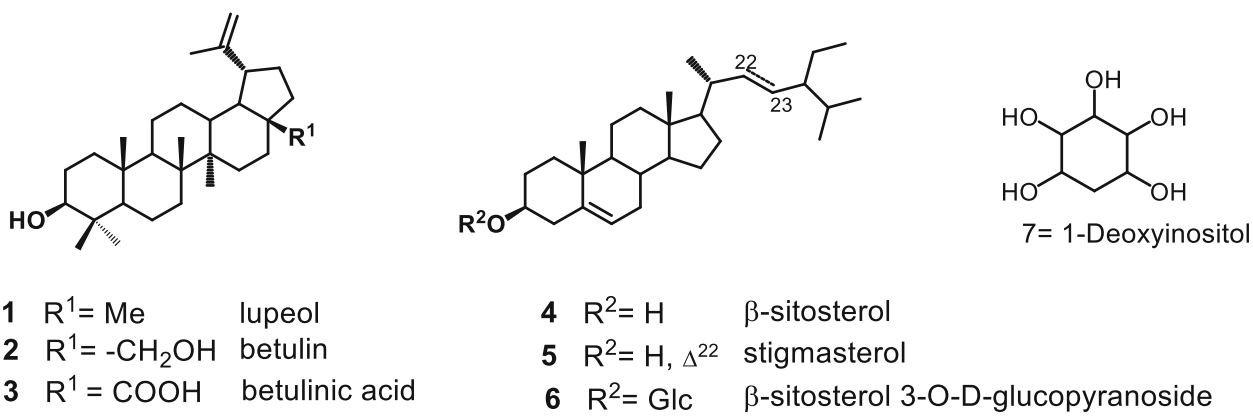

Fig. 3 Isolated compounds from *D. gracilescens*. Structures of the isolated compounds were elucidated by means of physical and spectroscopic methods

Table 4 Anti-leishmanial activity and selectivity indexes of isolated compounds against *L. donovani* promastigotes and amastigotes

Compound	IC_{50} (Promastigotes) (μM ± SD)	IC_{50} (Amastigotes) (μM ± SD)	Raw267.4 CC_{50} (μM ± SD)	SI (Promastigotes)	SI (Amastigotes)
Lupeol (1) from fraction A	23.22 ± 0.08	46.66 ± 0.65	19.28 ± 0.3	0.82	0.41
Betulin (2) from fraction B	NT	NT	ND	ND	ND
Betulinic acid (3) from fraction B	13.69 ± 0.07	18.46 ± 0.01	82.39 ± 0.09	6.02	4.46
Mixture of sterols (4) from fraction A	46.83 ± 0.20	17.83 ± 0.17	18.26 ± 0.18	0.39	1.02
β-sitosterol glucoside (6) from fraction D	49.34 ± 0.12	32 ± 0.22	28.60 ± 0.07	0.58	0.89
1-deoxyinositol (7) from fraction D	241.71 ± 0.16	120 ± 0.18	> 1218 ± 0.13	> 5.04	> 10.15
Amphotericin B	0.37 ± 0.22	0.13 ± 0.11		/	/

Activity data are expressed as mean ± Standard deviation (SD) from triplicate experiments; IC_{50}: 50% Inhibitory Concentration, ND: Non-Determined; NT: Non-Tested (Betulin not tested due to insufficient quantity)

of *L. donovani*, corroborating the previous findings. Indeed, the antileishmanial activity of lupeol against both promastigotes and amastigotes of *L. donovani* has been demonstrated in the literature [41]. Other previous studies indicated that lupeol isolated from aerial parts of *Vernonia scorpioides* displayed a weak antileishmanial activity [42, 43]. Also, studies have highlighted the activity of lupeol from the latex of *E. resinifera* and *E. officinarum* against promastigote of *L. infantum* [44]. Other studies attempting to establish the mechanism of action of lupeol were conducted by Ramos et al. [45] and showed that this compound mediates an increased cytoplasmic membrane depolarization which may promote enhanced cell membrane damage. They also suggested that the leishmanicidal activity could lead to disruption of the cytoplasmic membrane of *L. donovani* promastigotes as evidenced by DISC3 mediated fluorometric analysis. Whereas lupeol might mediate reduction in intracellular parasitic load was found to be executed through the induction of pro-inflammatory cytokine response and generation of Nitrite Oxide (NO) in *L. donovani* infected macrophages [41].

Betulinic acid and lupeol that showed the most potent activity in this study belong to the class of terpenoids. In fact, a number of terpenes are reputed to possess antileishmanial activity. Different authors suggested that the antileishmanial activity of these compounds could be related to the inhibition of proteins and nucleic acids synthesis or of a membrane-associated calcium-dependent ATPase pump [43, 46]. Indeed, previous studies have suggested that lipophilic compounds, such as triterpenes, act by a peculiar mechanism. These compounds can pass easily through the cytoplasmic membranes, affecting structures of their different layers of polysaccharides, fatty acids, and phospholipids, thus making them permeable [47]. Once they cross the membrane, the coagulation of cytoplasm can occur. These events are able to promote the interruption of specific metabolic pathways of lipids and proteins [48], interference in cell division [49, 50], or stimulate the depolarization of the mitochondrial membranes, which can lead the cell to trigger necrosis or apoptosis mechanisms [51].

Among the isolated compounds, 1-deoxyinositol exerted a promising activity against both promastigote and amastigote forms of *L. donovani*. This compound was previously detected by GC-MS in a moderately active (with IC_{50} value of 126.4 μg/mL) methanolic extract from the aerial part of *Scutellaria havanensis* against *L.*

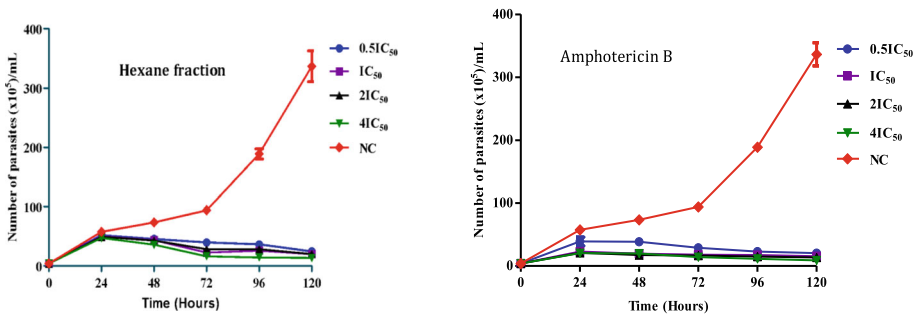

Fig. 4 Kill kinetics of *L. donovani promastigotes* in relation to drug concentration and time. Cells were treated with different concentrations of (a) hexane fraction and (b) amphotericin B. The growth kinetic curves were plotted as *number of viable parasites* vs *time* with data collected every 24 h over a period of 120 h; NC = Negative control; Each data point represents mean ± SD from three experiments

amazonensis promastigotes [52]. The antileishmanial activity of 1-deoxyinositol adds to the novelty of this work given that, to the extent of our knowledge, no previous report has been published on the antileishmanial activity of this compound. More interestingly, 1-deoxyinositol showed the highest selectivity against *L. donovani* promastigotes (SI > 5.04) as well as acceptable preference for amastigotes (SI > 10.15).

Overall, this study has indicated that the hexane fraction was 12 to 49-fold and 1.8 to 2.4-fold more active than the derived lupeol and mixture of sterols against the promastigote and amastigote forms of *L. donovani* respectively. Also, selectivity indexes greater than 152 and 11 for promastigotes and amastigotes respectively were obtained compared to the derived components. The activity profile of the hexane fraction portents a very probable synergistic interaction between its non-polar components to increase activity and selectivity (safety profile). The implication of these findings is of high significance in the use of *D. grascilesens* plant in traditional medicine to treat neglected tropical diseases (NTDs).

Conclusion

This study reports the first detailed investigation aiming at determining the antileishmanial activity of natural products from *D. gracilescens* using a bio-guided approach. The hydroethanolic extract of the trunk showed promising profile, (IC_{50} = 5.84 μg/mL) and its bio-guided fractionation led to the most potent hexane fraction (IC_{50} = 0.79 μg/mL). Further fractionation of this fraction led to six compounds that also exhibited antileishmanial potency. The promising hexane fraction and derived active compounds represent potential raw materials for detail-oriented drug discovery against visceral leishmaniasis that exacts a very heavy toll to poor patients in remote endemic settings in Africa and elsewhere. Among the isolated compounds, 1-deoxyinositol has shown acceptable profile for further structure-activity-relationship studies in drug discovery program to unveil hits or leads adhering to the criteria defined earlier against visceral leishmaniasis. Of particular note is the activity profile of the hexane fraction that exerted the greatest potency and selectivity. It is a promising candidate for the development of a phytodrug against leishmaniasis.

Abbreviations

CC_{50}: 50% cytotoxic concentration; *D.*: *Diospyros*; DMEM: Dulbecco's Modified Eagle's Medium; DMSO: Dimethylsulfoxide; ESI: Electrospray Ionization; FBS: Fetal Bovine Serum; FT-MS: Fourier Transform Mass Spectrometry; HIFBS: Heat-Inactivated fetal Bovine Serum; HIV: Human Immunodeficiency Virus; HPLC: High Performance Liquid Chromatography; HRESI: High Resolution Electrospray Ionization; HSV-1: Herpes simplex viruses-1; IC_{50}: 50% inhibitory concentration; L.: *Leishmania*; M199: Medium 199; M. tuberculosis: *Mycobacterium tuberculosis*; MS: Mass Spectrometry; NC: Negative control; NI: Not Identified; NO: Nitrite Oxide; NT: Non-tested; NTD: Neglected Tropical Disease; PBS: Phosphate Buffer Solution; SD: Standard deviation; SDS: Sodium Dodecyl sulfate; SI: Selectivity Index; *T. cruzi*: *Trypanosoma cruzi*; TIC: Total Ion Chromatogram; UPLC: Ultra Performance Liquid Chromatography; VL: Visceral Leishmaniasis; WHO: World Health Organization

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12906-021-03279-1.

Additional file 1.

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Authors' contributions

FFB and SFK designed the study. CANN, CNW, DD and BMTT carried out studies and drafted the manuscript. LRTY, PVTF supervised the biological experiments. MNP collected the plant material. JBJ performed the acquisition of LC-MS data. FFB, SKF, BLN and NS revised the manuscript. All the authors approved the final version of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Akhoundi M, Kuhls K, Cannet A, Votypka J, Marty P, Delaunay P, et al. Historical overview of the classification, evolution, and dispersion of *Leishmania* parasites and sandflies. PLoS Negl Tropl Dis. 2016;10(3): e0004349. https://doi.org/10.1371/journal.pntd.0004349.
2. Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Esmenjaud J, Arenas R. Leishmaniasis: a review. F1000Res. 2017;6:750.
3. World Health Organization. Leishmaniasis. Fact sheet N°375. World Health Organization,Thailand.http://www.searo.who.int/thailand/factsheets/fs0013/n/. 2014. (Accessed 16 June 2018).
4. WHO. Working to Overcome the Global Impact of Neglected Tropical Diseases: First WHO Report on Neglected Tropical Diseases. Geneva No.1: WHO; 2010.
5. Steverding D. The history of leishmaniasis. Parasit Vectors. 2017;10(1):82. https://doi.org/10.1186/s13071-017-2028-5.
6. Arevalo J, Ramirez L, Adaui V, Zimic M, Tulliano G, Miranda-Verástegui C, et al. Influence of *Leishmania* (Viannia) species on the response to antimonial treatment in patients with American tegumentary leishmaniasis. J Infect Dis. 2007;195(12):1846–51. https://doi.org/10.1086/518041.
7. Goto H, Lindoso JL. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. Expert Rev Anti-Infect Ther. 2010;8(4):419–33. https://doi.org/10.1586/eri.10.19.
8. Croft SL, Coombs GH. Leishmaniasis; current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol. 2003;19(11):502–8. https://doi.org/10.1016/j.pt.2003.09.008.
9. Berman JD, Badaro R, Thakur CP, Wasunna KM, Behbehani K, Davidson R, et al. Efficacy and safety of liposomal amphotericin B (AmBisome) for visceral leishmaniasis in endemic developing countries. B WHO. 1998;76(1): 25–32.
10. WHO. General guideline for methodologies on research and evaluation of traditional medicine. W.H.O /E.D.M. /T.R.M; 2000. p. 1. Pp 27
11. Kaushik V, Saini V, Pandurangan A, Khosa RL, Parcha V. A review of phytochemical and biological studies of *Diospyros malabarica*. Inter J Pharm Sci Lett. 2012;2:167–9.
12. Gafner F, Chapuis JC, Msonthi JD, Hosttemann K. Cytotoxic naphtoquinones, molluscidal saponins and flavonoids from *Diospyros zombensis*. Phytochem. 1987;26(9):2501–3. https://doi.org/10.1016/S0031-9422(00)83864-9.
13. Kerharo J, Bouquet A. La chasse en Côte d'Ivoire et en Haute-Volta. Acta Trop. 1949;6(3):193–220.
14. Mbi CN, Waterman PG. Sterols and naphthoquinones from *Diospyros gracilescens*. J Pharm Pharmacol. 1978;30(S1):86P. https://doi.org/10.1111/j.2042-7158.1978.tb10793.x.
15. Xie K, Huang K, Yang R, Yu H, Liu Z. Three liquid-phase extraction: a new approach for simultaneous enrichment and separation of Cr(III) and Cr(VI). Ind Eng Chem Res. 2011;50(22):12767–73. https://doi.org/10.1021/ie201816j.
16. Abdullahi SM, Musa AM, Abdullahi MI, Sule MI, Sani YM. Isolation of lupeol from the stem-bark of *Lonchocarpus sericeus* (Papilionaceae). J Biosci. 2013; 1(1):18–9.
17. Wouamba SCN, Happi GM, Lenta BN, Sewald N, Kouam SF. Vernoguinamide: A new ceramide and other compounds from the root of Vernonia guineensis Benth and their chemophenetic significance. Biochem Syst Ecol. 2020;88:103988.
18. Tijjani A, Ndukwe IG, Ayo RG. Isolation and characterization of lup-20 (29)-ene-3, 28-diol (Betulin) from the stem-bark of Adenium obesum (Apocynaceae). Trop J Pharm Res. 2012;11(2):259–62.
19. SCN W, Happi GM, Nguiam Pouofo M, Tchamgoue J, Jouda JB, Longo F, et al. Antibacterial flavonoids and other compounds from the aerial parts of Vernonia guineensis Benth.(Asteraceae). Chem. Biodivers. 2020;17(9):e2000296.
20. Venkateswara RG, Sahoo MR, Madhavi MSL, Mukhopadhyay T. Phytoconstituents from the leaves and seeds of Manilkara zapota Linn. Der Pharm Lett. 2014;6:69–73.
21. Khanjani JS, Farazmand A, Amin M, Doroodgar A, Shirzadi M, Razavi M. Methanolic extract's activity of *Artemisia absinthium*, *Vitex agnus-castus* and *Phytolaca americana* against *Leishmania major in vitro* and *in vivo*. IAHS. 2015;2(2):69–74.
22. Siqueira-Neto JL, Song OR, Oh H, Sohn JH, Yang G, Nam J, et al. Antileishmanial high-throughput drug screening reveals drug candidates with new scaffolds. PLoS Negl Trop Dis. 2010;4(5):675.
23. Jain K, Sahu R, Walker A, Tekwani L. A parasite rescue and transformation assay for anti-leishmanial screening against intracellular *Leishmania donovani* amastigotes in THP1 human acute monocytic leukemia cell line. J Vis Exp. 2012;70:e4054.
24. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth. 1983; 65(1-2):55–63. https://doi.org/10.1016/0022-1759(83)90303-4.
25. Lenta BN, Ngamgwe RF, Kamdem LM, Ngatchou J, Tantangmo F, Antheaume C, et al. Compounds from *Diospyros canaliculata* (Ebenaceae) and their Antiparasitic activities. Res J Pure Ap Chem. 2014;6(2):56–65.
26. Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN, Ray C. Screening of Indian plants for biological activity. In J Exp Biol. 1968;6(4):232–47.
27. Rocío BA, Andrés UC, Carlos QN, Gonzalo CE, Mirbella C. A selective chemical method for the separation of quinones from the stem bark of *Diospyros Anisandra*. Int J Curr Pharm Res. 2013;5(4):13–7.
28. Hazra B, Sur P, Roy DK, Sur B, Banerjee A. Biological activity of diospyrin towards Ehrlich ascites carcinoma in Swiss a mice. Planta Med. 1984;50(4): 295–7. https://doi.org/10.1055/s-2007-969713.
29. Asolkar LV, Kakkar KK, Chakre OJ. Second supplement to glossary of Indian medicinal plants with active principles', CSIR, New Delhi; 1965. p. 271–9.
30. Satish AK, Sunil SJ. Screening of ethanolic extract of *Diospyros malabarica* Desr. Bark for anti-diabetic and antioxidant potential. J Pharm Educ Res. 2016;50(1):179–89.
31. Camacho MDR, Phillipson JD, Croft SL, Solis PN, Marshall SJ, Ghazanfar SA. Screening of plant extracts for antiprotozoal and cytotoxic activities. J Ethnopharmacol. 2003;89(2-3):185–91. https://doi.org/10.1016/S0378-8741(03)00269-1.
32. Sousa MC, Varandas R, Santos RC, Santos-Rosa M, Alves V, Salvador JA. Antileishmanial activity of semisynthetic Lupane triterpenoids betulin and betulinic acid derivatives: synergistic effects with miltefosine. PLoS One. 2014;9(3):e89939. https://doi.org/10.1371/journal.pone.0089939.
33. Dorlo TPC, Balasegaram M, Beijnen JH, de Vries PJ. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. J Antimicrob Chemother. 2012;67(11):2576–97. https://doi.org/10.1093/jac/dks275.
34. Alakurtti S, Heiska T, Kiriaziz A, Sacerdoti-Sierra N, Jaffe L, Yli-Kauhaluoma J. Synthesis and anti-leishmanial activity of heterocyclic betulin derivatives. Bioorg Med Chem. 2010;18(4):1573–82. https://doi.org/10.1016/j.bmc.2010.01.003.
35. Domínguez-Carmona DB, Escalante-Erosa F, García-Sosa K, Ruiz-Pinell G, Gutierrez-Yapu D, Chan-Bacab MJ, et al. Antiprotozoal activity of betulinic acid derivatives. Phytomedecine. 2010;17(5):379–82. https://doi.org/10.1016/j.phymed.2009.08.002.
36. Yogeeswari P, Sriram D. Betulinic acid and its derivatives: a review on their biological properties. Curr Med Chem. 2005;12(6):657–66. https://doi.org/10.2174/0929867053202214.
37. Gheorgheosu D, Duicu O, Dehelean C, Soica C, Muntean D. Betulinic acid as a potent and complex antitumor phytochemical: a minireview. Anti Cancer Agents Med Chem. 2014;14(7):936–45. https://doi.org/10.2174/1871520614666140223192148.
38. Chen Y, Li S, Sun F, Han H, Zhang X, Fan Y, et al. In vivo antimalarial activities of glycoalkaloids isolated from Solanaceae plants. Pharm Biol. 2010; 48(9):1018–24. https://doi.org/10.3109/13880200903440211.
39. Innocente M, Silva N, Cruz N, Moraes S, Nakabashi M, Sonnet P, et al. Synthesis and antiplasmodial activity of betulinic acid and ursolic acid analogues. Molecules. 2012;17(10):12003–14. https://doi.org/10.3390/molecules171012003.
40. Cassio SM, Jose MB, Lanfredi-Rangel A, Elisalva TG, Diogo M, Milena S. Antiparasitic evaluation of betulinic acid derivatives reveals effective and selective anti-*Trypanosoma cruzi* inhibitors. Exp Parasitol. 2016;166:108–15.
41. Das A, Junaid J, Manash D, Padmani S, Utpal C, Biswanath D, et al. Antileishmanial and immunomodulatory activity of lupeol a triterpene compound isolated from *Sterculia villosa*. Inter J Antimicrob Agents. 2017; 50(4):512–22. https://doi.org/10.1016/j.ijantimicag.2017.04.022.
42. Fournet A, Angelo A, Munoz V, Roblot F, Hocquemiller R, Cave A. Biological and chemical studies of *Pera benensis*, a Bolivian plant used in folk medicine as a treatment of cutaneous leishmaniasis. J Ethnopharmacol. 1992;37(2): 159–64. https://doi.org/10.1016/0378-8741(92)90074-2.
43. Goijman G, Turrens F, Marini-Bettolo B, Stoppani O. Inhibition of growth and macromolecular biosynthesis in *Trypanosoma cruzi* by natural products. Effects Miconidine Tingenone. Medicina (Buenos Aires). 1984;44:361–70.

44. Araujo C, Silva R, Silva M, Takahashi A, Sales-Junior A, Dessimoni-Pinto V, et al. Constituents from stem barks of *Luehea ochrophylla* Mart and evaluation of their antiparasitic, antimicrobial, and antioxidant activities. Nat Prod Res. 2016;31(16):1948–53. https://doi.org/10.1080/14786419.2016.1266346.
45. Ramos H, Valdivieso E, Gamargo M, Dagger F, Cohen E. Amphotericin B kills unicellular leishmanias by forming aqueous pores permeable to small cations and anions. J Membr Biol. 1996;152(1):65–75. https://doi.org/10.1007/s002329900086.
46. Mishina V, Krishna S, Haynes K, Meade C. Artemisinins inhibit *Trypanosoma cruzi* and *Trypanosoma brucei rhodesiense in vitro* growth. Antimicrob Agents Chemother. 2007;51(5):1852–4. https://doi.org/10.1128/AAC.01544-06.
47. DiPasqua GRBR, Hoskins MN, Edwards ED, Mauriello G. Membrane toxicity of antimicrobial compounds from essential oils. J Agric Food Chem. 2007; 55(12):4863–70.
48. Ultee KA, Alberda M, Hoekstra FA, Smid EJ. Adaptation of the food-borne pathogen Bacillus cereus to carvacrol. Arch Microbiol. 2000;174(4):233–8. https://doi.org/10.1007/s002030000199.
49. Fortes A, Almeida A, Mendonc A-Junior F, Freitas R, Soares-Sobrinho L, De La Roca Soares F. Anxiolytic properties of new chemical entity, 5TlO1. Neurochem Res. 2013;38(4):726–31.
50. De Almeida M, Vilhena V, Barral A, Barral-Netto M. Leishmanial infection: analysis of its first steps. A review. Memorias do Instituto Oswaldo Cruz. 2003;98(7):861–70. https://doi.org/10.1590/S0074-02762003000700001.
51. Armstrong JS. Mitochondrial membrane permeabilization: the sine qua non for cell death. BioEssays. 2006;28(3):253–60. https://doi.org/10.1002/bies.20370.
52. Fernandez-Calienes VA, Monzote FL, Sariego RI, Marrero DD, Morales RCL, Mendiola MJ, et al. Antiprotozoal screening of the Cuban native plant *Scutellaria havanensis*. Pharm Biol. 2016;54(12):3197–202. https://doi.org/10.1080/13880209.2016.1216130.

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