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Traditional medicine, an overview of natural products with medicinal interest.
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The background of the slide is a photograph of a green medicinal plant with small white flowers. Overlaid on the lower right is a white chemical structure of a complex molecule. In the bottom right corner, there is a circular gold seal with a lion and a book, surrounded by Latin text.

Antiparasitic metabolites from medicinal plants used in the Tacana Bolivian native tribe

Traditional medicine, an overview of natural products with medicinal interest.

IVAN LIMACHI VALDEZ

CENTRE FOR ANALYSIS AND SYNTHESIS | LUND UNIVERSITY

And I say luckily because there are a lot of methods to write a script but the true is that all are useless: every story brings with it its own technique. The important thing for the screenwriter is to discover it.

Gabriel Garcia Márquez

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Centre for Analysis and Synthesis
Department of Chemistry
Faculty of Science
Lund University



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native tribe

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Traditional medicine, an overview of natural products
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Ivan Limachi Valdez



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DOCTORAL DISSERTATION

By due permission of the Faculty of Engineering, Lund University, Sweden.
To be defended at the Centre of chemistry on 5th December at 9:15, lecture hall
K:B

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Title and subtitle Antiparasitic metabolites from medicinal plants used in the Tacana Bolivian native tribe. Traditional medicine, an overview of natural products with medicinal interest		
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Key words Tacana Traditional Medicine, Antiparasitic, cytotoxicity, <i>Hyptis brevipes</i> , <i>hyptis mutabilis</i> , <i>Tessaria integrifolia</i> , <i>Trichilia adolfi</i>		
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Traditional medicine, an overview of natural products
with medicinal interest

Ivan Limachi Valdez



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To my family

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Abstract

The thesis aims to contribute to the preservation of traditional medicinal knowledge of the Tacana Bolivian native tribe, as well as the isolation of antiparasitic metabolites from plants used to the treatment of endemic diseases. A selection of medicinal plants, carried out with the cooperation of Tacana communities who decided which plants have a specific medicinal propose, plants collected were identified in the national herbarium of Bolivia, and confirmed by healers from the most representative communities. From the screened plants the most active species were selected, using as selection criteria the *in vitro* antiparasitic activity in *Leishmania* strains. These species were subjected to chemical analysis that includes the chromatographic isolation of their metabolites and structural elucidation by spectroscopic techniques. The main metabolites identified were evaluated in a broad battery of parasite assays, besides the cytotoxicity, to determine the potency as well as selectivity to obtain a better understanding of the medicinal properties of the medicinal plants.

In the first chapter, a general description about Tacana native tribe is given in order to understand the intimate connection that this people have with their environment. A cooperation project between the Tacana people, La Paz university and international organizations, facilitated the collection and taxonomical identification of 38 plants with medicinal uses to be added to the ethnobotany list of Tacana traditional medicine.

In the second chapter, laboratory work was carried out to prepare ethanolic extracts of plants collected, and the antiparasitic activity against leishmania promastigotes of each extract have been evaluated *in vitro*, besides the cytotoxicity in HeLa cells. This gave the selectivity index (SI). Thus, three vegetable species were selected as the most active antiparasitic plants, identified as *Hyptis mutabilis*, *Hyptis brevipes* and *Tessaria integrifolia*. Additionally, two species were selected due the extended use among Tacana people: *Renealmia breviscapa* and *Trichilia adolfi*.

The last chapter is concentrated on the chemical exploration of selected plants, the ethanolic extracts of *H. brevipes* and *H. mutabilis*, both belong the same family, afforded the isolation of nine metabolites with diverse antiparasitic activity, some of them part of the brevipolide chemical family. The most active compound was found in *H. mutabilis* and identified as olguine. Super critical fluid extraction technique was applied to extract the chemical content of *T. integrifolia*, and eleven metabolites were isolated. Seven of them were identified as eremophilane-type sesquiterpenoids, the remaining were flavonoids and terpenoids. The relative chemical contents of the crude extract and fractions were compared using LC-MS techniques. The ethanolic extract of *T. adolfi* afforded nine new tetranortriterpenoids, and an extensive spectroscopic analysis was necessary to elucidate their complex structures. The trivial name trichilianone-type was proposed for compounds that possess a bicyclo-cyclopropane-hexane system as part of the terpenoic skeleton. The antiparasitic activity and cytotoxicity was reported together with a short analysis of the hypothetical biosynthetic pathway.

Popular Science

Traditional medicine (TM) is an ancestral practice conserved by several cultures. A well-known example of TM is the Chinese traditional medicine, sociably accepted around the world, well documented, and in constant development also including scientific support. As well as in Chinese TM, every culture around the world keep these practices, but in most of the cases it is not well documented. This is the case of the Tacana TM. The state of Bolivia recognizes 35 native ethnic groups and Tacana is one of them, and Tacana also includes some minor tribes of the Amazon basin. The Tacana live in the tropical humid forest located in borderline between Andes mountains and the Amazon basin in the department of La Paz – Bolivia. Even with the pressure from modern society, Tacana people still conserve traditions and customs, and efforts to document their ethnobotany started twenty years ago. In a first approach were collected and identified around of 450 plants with a traditional use, among them near to 150 had a medicinal use, since this effort not updates were done until now. In our study were collected, identified and added to the ethno-botany list of Tacana medicinal plants, 38 vegetable species with a medicinal propose. The results presented contribute to the preservation of valuable knowledge about the traditional medicine. Moreover, the plants collected were extracted in laboratory to obtain the crude extract, which contain all the main active components, In our interest to have a better understanding of the TM, a systematic investigation of the crude extracts, using *in vitro* antiparasitic activity as selection criteria, allowed us to select the three most promising species (*Hyptis brevipes*, *Hyptis mutabilis* and *Tessaria integrifolia*). In addition, two more plants were selected due to the chemical interest and the broad use among Tacana people (*Renalmia breviscapa*, *Trichilia adolfi*). The extracts of the selected plants were subjected to chromatographic fractionation to isolate the main metabolites, and these components were further analyzed by spectroscopic techniques that determined the structures of a variety of chemical compounds. Among them were found brevipolide-, curcumin-, flavonoid-, sesquiterpenoid- and limonoid-type compounds. Antiparasitic activity of every purified compound was evaluated in our *in vitro* model against leishmania parasites, and the cytotoxicity was evaluated on HeLa cell cultures. With our result we want to contribute with the preservation of TM knowledge and the exploration of the chemical diversity of natural products.

List of Papers

This doctoral thesis was written on the base of six scientific papers. They are referred as follow:

Paper 1: D. Arévalo-Lopéz, N. Nina, J. Ticona, **I. Limachi**, E. Salamanca, E. Udaeta, C. Paredes, B. Espinoza, A. Serato, D. Garnica, A. Limachi, D. Coaquira, S. Salazar, N. Flores, O. Sterner, A. Giménez. Leishmanicidal and cytotoxic activity from plants used in Tacana traditional medicine (Bolivia)., *Journal of Ethnopharmacology* **216**, 120-133 (2018)

Paper 2: **I. Limachi**, C. Condo, C. Palma, N. Nina, E. Salamanca, J.C. Ticona, E. Udaeta, N. Flores, A. Serato, N. Marupa, B. Chao, G. Ibaguari, C. Nay, S. Manner, O. Sterner and A. Giménez. Antiparasitic metabolites from *Hyptis brevipes*, a Tacana medicinal plant. *Natural Product Communications* **14**, 55-58 (2019).

Paper 3: **I. Limachi**, C. Condo, C Palma, N. Nina, E. Salamanca, J. C. Ticona, E. Udaeta, N. Serato, N. Marupa, B. Chao, G. Ibaguari, C. Naye, S. Manner, O. Sterner and A. Giménez Antiparasitic Metabolites of plants from Tacana's traditional medicine. in manuscript.

Paper 4: **I. Limachi**, C. Palma, J.C. Ticona, N. Nina, E. Salamaca, S. Manner, A. Gimenez and O. Sterner. Supercritical Fluid Extraction (SFE) as “green” technique to obtain antiparasitic metabolites from *Tessaria integrifolia*. in manuscript.

Paper 5: **I. Limachi**, M.A. Gonzales, J.C. Ticona, E. Salamanca, S. Manner, A. Gimenez and O. Sterner. Trichilianone A-D, novel cyclopropane-type limonoids from *Trichilia adolfi*. in manuscript

Paper 6: **I. Limachi**, M.A. Gonzales, J.C. Ticona, E. Salamanca, S. Manner, A. Gimenez and O. Sterner. Additional limonoids from *Trichilia adolfi*. in manuscript.

My contribution to the papers

- Paper 1:** I have participated actively planning the project, as well as in collection, extraction and preparation of vegetable samples. I took active part in the revision of the manuscript.
- Paper 2:** I planned and performed all the experiments that include the plant collection, extraction, chemical isolation and structural identification of active metabolites, E. Salamanca performed biological evaluations. I wrote the manuscript and designed the paper outline and participate in the revision.
- Paper 3:** I planned, performed all the chemical analysis and wrote the manuscript, chemical analysis includes plant collection, extraction, purification and structural elucidation of active compounds, and E. Salamanca performed Biological evaluation.
- Paper 4:** I conceived the project and wrote the draft manuscript, I performed all the chemical work that includes plant collection, supercritical fluid extraction, chromatographic purification and structural elucidation of active compounds, E. Salamanca performed antiparasitic evaluations and cytotoxic activity.
- Paper 5:** I planned and performed the chemical analysis that includes plant collection, extraction, chromatographic purification and structural elucidation of chemical compounds. Antiparasitic activity and cytotoxicity were performed by E. Salamanca. I wrote the draft manuscript and participate in the revision.
- Paper 6:** I planned, performed and wrote the draft manuscript. Laboratory work includes plant collection, extraction, purification and chemical elucidation of chemical compounds. Antiparasitic activity and cytotoxicity were performed by E. Salamanca.

Introduction

Traditional medicine (TM) is an ancestral practice that has been preserved by several cultures through generations, and certified by international organizations such as the World Health Organization (WHO). WHO defines TM as “the total sum of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in prevention, diagnosis, improvement or treatment of physical and mental illnesses”¹. In some countries the use of TM is well documented and supported by clinical data, constantly subjected to development and regulation. Countries such as China, with the well known Chinese Traditional Medicine², or the Ayurveda in India³, are on top of the list with long histories of traditions and practices that are recognized together with other Complementary Medicines (CM) as part of the dominant healthcare system.

Nowadays the incorporation of TM and others CM into the healthcare system has become a priority in developing countries, mainly due to the fact that they are more accessible in regions where modern drugs are less available. Moreover, WHO have been promoting such initiatives and made efforts to develop projects that integrates TM/CM with the healthcare system. The project “WHO traditional medicine strategy 2014 -2024”⁴ incentives central governments to create policies to facilitate such integration. In the 2019 report, of 61 member states that were asked whether they had an existing national plan for integrating TM/CM into their national health service 13 confirmed that they do, and Bolivia is one of these⁵.

TM is important in Bolivia, a country where TM is legally recognized since 1985⁶. Bolivia has a strong culture that keep ancient traditions alive, and the Bolivians to a large extent prefer the use of TM over the modern medicine⁷. Indigenous people in Bolivia represent more than 60% of the total population and it has been estimated that 60-79% of them use TM, preferentially herbal medicines. Bolivia is since 2014 one of five countries in Americas region, together with Brazil, Cuba, Mexico and Peru, that allow herbal medicines to be part of the National Essential Medicines List⁵.

However, while the intentions in Bolivia are the best, the lack of organization and documentation necessary to create regulations and control procedures for herbal medicines is a tough challenge for the country. In addition, Bolivia has 36 ethnic groups that are recognized by the state ⁸, and several of these were not recognized 50 years ago. Some of them reclaim their identity in the state through indigenous organizations. A prominent example of the many ethnic groups is the Tacana, a culture that can be traced since the XVIII century that survived the colonization, keeping its own traditions and customs through the centuries, and still resisting the modern society ⁹.

Tacana's people have an intimate connection with their environment, and TM always was part of their culture. The knowledge that they keep is preserved through generations but it is partially documented as great efforts to document their ethnobotanical knowledge were made some 20 years ago ¹⁰. Thereby, approximately 450 vegetable species with traditional cultural uses were documented, and of them at least 150 species were associated with medicinal uses. Thus, in 1999 a pharmacopeia was created, with a good description of practices and herbal preparations ¹¹. Such herbal preparations provide alternatives to the treatment of parasitic diseases (leishmania, malaria, etc.) that are endemic in the region, diseases that always have been present in South America, and nowadays are as unattended or neglected. This is due to the difficulty to control them, and because they mainly affect people in poor regions that do not constitute profitable markets for the pharmacy companies. Such regions may not have access to primary medical assistance, and modern drugs may simply not be available. In such case TM becomes the primary choice of medicine and the vast majority of traditional treatments in such regions are sociable accepted, although not well understood by the scientific community. The pharmacological potential of plant medicines was only described superficially by the documented TM and the chemical diversity that can be anticipated to relay the pharmacological effects is still waiting to be explored.

The aim of the studies described in this thesis is to contribute to the documentation of Tacana's TM and explore its potential, through a systematic search for secondary metabolites in medicinal plants. The work is carried out with the cooperation of Tacana people and focus on effects on parasitic diseases. Various parasites are used in biological assays used to evaluate biological activity of selected plants in an *in vitro* model. By performing phytochemical analyses of extracts of active plants, or bioactive fractionation, the chemical diversity of the plants may generate lead compounds that can be useful for the fight against the parasites.

Scope of the thesis

The thesis has been divided in six articles: two published and four manuscripts

Paper 1 and **paper 3** describes procedures for the selection, collection and taxonomical identification of plants used in Tacana's TM, as well as the preparation of crude extracts and biological data obtained with them from an *in vitro* model analysis against kinetoplast parasites of: *Leishmania amazonensis*, *L. braziliensis* (promastigotes) and *Trypanosoma cruzi* (epimastigotes); *Plasmodium falciparum* (asynchronous cultures) and *Giardia lamblia* (trophozoites). From this first screening several interesting plants were identified as species suitable for a phytochemical analysis, focusing primarily on the isolation of natural products with the potential to treat the *Leishmania* parasite.

Paper 2 describes our first approach to isolate active metabolites, through a bioassay-guided fractionation that led us to identify, among others, brevipolide-type compounds from *Hyptis brevipes*. The pure compounds were evaluated in an *in vitro* model against *L. amazonensis* and *L. braziliensis*, and compared with the cytotoxicity in mammalian cells. The selective index (SI) obtained from these data indicates the potential usefulness of each active compound.

In **Paper 4**, a green technology for extraction was applied to obtain a crude extract of *Tessaria integrifolia*, followed by a bioassay-guided fractionation to isolated the main active terpenoids. Such metabolites were subjected to LC-MS analysis, and the data were used to measure the terpenoic contents in the crude extracts traditionally used in Tacana's medicine to heal leishmania sores.

Finally, **Paper 5** and **Paper 6** describes the isolation of novel limonoids by semi-preparative HPLC from extracts of *Trichilia adolfi*. The structural elucidations were challenging, and the structures were determined through the extensive analysis of spectroscopic data. This is described in detail together with the biological evaluation of the pure metabolites.

Chapter 1: General aspects about the Tacana Culture

1.1 Geographical location

Tacana is located in the heart of South America in Bolivia (Figure 1), an Andean country with a hugely diverse geography divided in nine departments that have their own characteristic topography. 60% of its territory is part of the lowlands area of the Amazon basin and the Chaco ecosystem, and most of the Tacana's communities are located in the northwest part of the department of La Paz. The limit with the Beni department together with up to 35 ethnic groups that live in the Amazon area and represent some 3% of the total population of the country, it is remarkable that these ethnic groups possess an intimate knowledge of their natural environment.

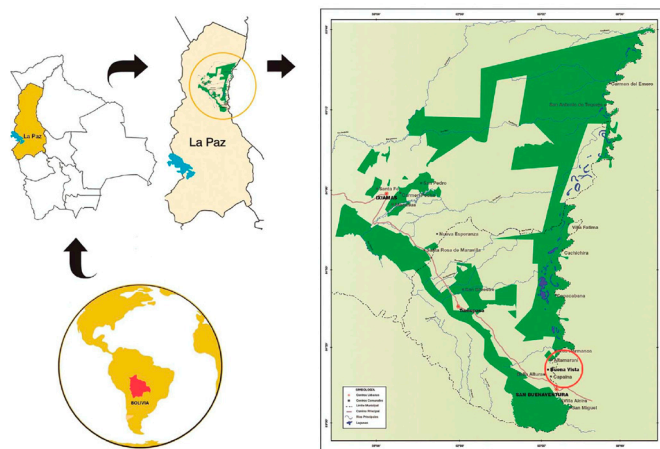


Fig. 1. Map of location of Bolivia, La Paz department and Tacana territory (green area) with the main communities, highlighting Buena Vista community, collection's place of Tacana plants. (The image file was taken from CIPTA/CIMTA, 2014 with some modifications).

The Tacana culture is spread in the transition zone between the humid tropical and dry subtropical climates, including lowlands at less than 300 m above sea level (masl) as well as high mountains reaching an altitude of almost 7000 masl. In 1992 the Tacana Indigenous Council (CIPTA, Consejo Indígena de Pueblos Tacana) was created, and in 1997 a land claim of their Indigenous Territory (called TCO-Tacana 1) was submitted to INRA (Bolivian Land Reform Institute) on behalf of 20 communities (621 families and 2,849 inhabitants). The location is on the southeast border of the Madidi protected area, achieving to date an area covering 389 303 ha (of which 39 430 overlap with Madidi) ¹².

This region is composed of all the Amazonian forest types, and the plants described are the same as those found in the moist humid forest where a permanent moisture is supplied by cloud drizzle and rainfall brought from the Amazon basin by the eastern winds ¹³. The Amazon forests are the regions richest in biodiversity, it is estimated that 20 000 plant species are growing in Bolivia ¹⁴, and that 8 500 species are present only in these specific lowlands forests. However, this is poorly documented and to carry out a better study of the ecosystem is easier said than done. Besides the plant biodiversity, the chemical diversity representing the secondary metabolites was only explored by scratching the surface, most of the plants present in the region are still waiting for phytochemical studies to disclose their contents of metabolites with potential as new drugs.

1.2 The organization of the Tacana communities

The Tacana people are part of the Tacana linguistic family, that collects other Amazonian groups such as the Reyesano, Cavineño, Araona and Ese Ejja (Figure 2). They are organized in the “Consejo Indígena de los Pueblos Tacana” (CIPTA, Indigenous Council of the Tacana People) and the “Consejo Indígena de Mujeres Tacana” (CIMTA, Indigenous Council of the Tacana Women). Both organizations represent 20 Tacana communities of the Abel Iturralde province, departments of La Paz and Beni. CIPTA was founded in 1992, with the objectives to defend, consolidate and take action for the conservation of the natural resource found in their territory, while CIMTA was founded in 1996, responsible for overseeing cultural affairs of the Tacana people. The role of CIMTA in the

community was also to encourage women to participate actively in roles outside the home, holding positions of authority which increases the community equality

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Fig 2: Map of Bolivian ethnic groups. Image file was taken from Internet with some modifications (<https://achacachi.tripod.com>)

The Tacana community is the most representative human source in the region. In 2015 they were recipients of the Equator Prize at the U.N. Paris climate conference, for their commitment to forest protection. Their intimate knowledge of their natural environment led them to prioritize strategies to create sustainable ways of life, and promote the conservation of the biodiversity. These efforts are reflected in a deforestation rates four times lower than the surrounding areas.¹⁶ Before starting our fieldwork we signed a letter of agreements with both organizations (CIPTA/CIMTA) and coordinated fieldwork at Buena Vista Community.

1.3 The Ethnobotany of Tacana’s Culture

Tacana’s people have conserved a traditional land usage and natural sources management practices, which is reflected in a well-conserved knowledge of their ethnobotany (although only partially documented). Several approaches to document the botany of Tacana’s culture have been made over time. In 1997

Dewalt et al.¹⁷ collected and documented 185 species of two permanent plots near to a Tacana community, 115 of them had traditional uses, for example for construction, fishing, hunting and for food, while 40 plants were recognized as medicinal plants. In the next years, approximately 450 different plant species were added to this collection also associated to cultural uses. Consequently, some 150 species were described as medicinal to treat several diseases such as gastrointestinal disorders, skin afflictions, respiratory problems, gynecological disorders, febrifuges, rheumatic disorders, leishmaniasis, and for snakebites¹⁰. These plants officially represented 33% of the total number of documented plants, although we believe that the true number is much greater than this report is indicating, and that the potential of the additional plants is not explored at all.

Nowadays, the Tacana people consider themselves to be farmers, fishermen and hunters, and the plants that they use for medicinal purposes are in most cases grown in their own small greenhouses. This is a practical way to conserve traditions while making use of new techniques, but they experience a constant pressure from the outside world and are afraid that their traditions and knowledge will be lost with time. Since the Dewalt report almost 20 years ago no updates have been recorded, and some information should be more complete. In the following study we want to contribute to the documentation of medicinal plants, saving traditions of Tacana's culture, and explore the chemistry of traditional medicine.

1.4 Selection of vegetable species (Paper 1 and 3)

Our journey started in San Buena Ventura (La Paz-Bolivia), in the period 2013 to 2017 with the cooperation of CIPTA-CIMTA and the financial support of the international agency SIDA. We performed several workshops with the intention to document plants and their uses, to update the traditional pharmacopeia and consolidate plants with traditional uses.

Consequently, a letter of agreement was signed with both organizations CIPTA and CIMTA, and fieldwork to collect vegetable species was planned. The criteria for the selection include all those plants used for leishmaniasis, skin sores, ulcers and wound healing, and in addition those used for anti-inflammatory, analgesic and febrifuge effects.

Fieldwork was made in the region of Abel Iturralde province, in the department of La Paz, Bolivia (S 14°21'969" and W 67°33'764"; elevation 205 masl). Plants were collected from November 2013 to July 2017, with the collaboration of well prepared Tacana guides that were certified by Tacana councils. They identified the species *in situ* and documented it, and once a specie was collected it was divided in its components, including leaves, root, bark, stem, flowers and fruits, for further analysis. The Tacana names, the way the plants were prepared and their medicinal uses were verified in a local workshop with healers from eight Tacana communities, before the plant material was sent to the National Herbarium of Bolivia (LPB) for taxonomical identification. Voucher specimens of the identified plants were deposited and the scientific names were confirmed using International Plant Name Index database (IPNI) (Table 1).

During our different fieldtrips we collected 46 plants with traditional medicinal uses, they are reported in **papers 1 and 3** and added to the arsenal of medicinal plants of Tacana's culture. With the identified plants, bibliographic searches were carried out looking for some kind of antiparasitic background also for the traditional uses. In these records we found that 24 plants have a documented antiparasitic use (52.1%), and that most of the records focus on the evaluation of biological activity of a crude extract while fewer were directed towards isolated compounds.

The next step in our journey will be focused on the biological evaluation of plants collected, in an *in vitro* model for leishmanicidal activity.

Table 1

Scientific name, plant family, voucher herbarium specimen number, Tacana name, traditional uses, plant part and preparation from the selected plant species

Entry	Scientific name	Plant family	Voucher specimen	Tacana name	Traditional use	plant part	plant mode preparation	Literature
1	<i>Abuta grandifolia</i> (Mart.) Sandwith	Menispermaceae	AS-087	Nurri jaja	Skin and intestinal infection Fruits are edible	I, B	Young mashed leaves applied on affected area for skin infection. Decoction of handful of B in 2 L Water, until 1L, drinks 1 cup/day/8days, before breakfast for No intestinal infection.	No
2	<i>Adiantum latifolium</i> Lam. cf. <i>Adiantaceae</i>		NM-054	Atarisi Huachidhi	Skin infections and wounds	WP	Fresh plant sap applied topically on affected area	No
3	<i>Alocasia macrorrhizos</i> (L.) G. Don	Araceae	AS-096	Bes'a bes'a	Skin infection, anti-inflammatory. The plant is poisonous	R, L	Roots mashed applied as poultice on affected area for skin infection. Young L applied as poultice on affected area for inflammation.	No
4	<i>Amaranthus spinosus</i> L.	Amaranthaceae	AS-076	Naru naru Id'rene	Skin infection, anti-inflammatory	S, L	Sap from mashed L applied topically only, twice/day/3 days, for skin infection take to boiling a handful of L and S in 1 L water, then take a hot bath, and after drink 1 hot cup, for inflammation.	¹⁸
5	<i>Andropogon bicornis</i> L.	Poaceae	AS-035	Batsumo tidja	Anti-inflammatory, hernia	R	Decoction of a handful of R in 2 L water until 1 L, drink some 250 mL/day, for two weeks, for inflammation and hernia	No
6	<i>Aspidosperma</i> spp.	Apocynaceae	AS-048	Kipabi tid'i	Skin infection, malaria, contusions, and bleeding or menstrual pain. Timber is used in construction, for fire and utensils	B	Dried powdered B applied on affected area, for skin infection. Decoction of a handful of B in 2 L water until 1 L, and used in bath, against malaria. Poultice of dry B powdered and applied on affected area, for contusion take to boiling 1 spoon of powdered B in 1 L of water and drink 1/4cup/day/3 days, for bleeding or menstrual pain.	^{19, 20-22}
7	<i>Bocageopsis</i> spp.	annonaceae	AS-086	Djipicu	Skin infection, stem is used in building	L	Fresh mashed L applied on the affected area, twice/day during 5 days.	²³
8	<i>Calyophyllum apricaeanum</i> (Benth.) K.Schum	Rubiaceae	AS-114	Buwe aqui	Diarthrea, acne, skin infection	B	Decoction of a handful of B in 2L water until 1 L, drink 1cup/3times/day until improvement, for diarrhea. Same decoction is used fro washing the face, or affected areas, for acne and skin infections.	²⁴
9	<i>Cecropia Concolor</i> Willd.	Cecropiaceae	AS-004	Tahua midha	Skin infection	B	Fresh M mashed in small amount of water and apply the liquid on affected area, 3 times/day until improvement	No
10	<i>Cedrela</i> spp.	Meliaceae	AS-019	Kuabadhu	Antiparasitary, skin infection, to stop bleeding and blood cleansing after childbirth. Stem is used in building	B	Decoction of a handful of B in 2 L water until 1 L, drink until parasite removal, for antiparasitary, same decoction is used in a bath or applied on affected areas, for skin infection. The same decoction, drink 1-3 cups/days until stop bleeding, or for blood cleansing after childbirth.	^{19, 25, 26}

(Continued in the next page)

Table 1 (continued)

Entry	Scientific name	Plant family	Voucher specimen	Tacana name	Traditional use	Plant part	Mode of preparation	Literature
11	<i>Celtis iguanaea</i> (Jacq.) Sarg.	Ulmaceae	AS-026	D'ije badju quid'a	Anti-inflammatory, skin infection, to strength after childbirth and hemorrhage. Fruits are edible	L, F, R, B	Decoction of handful of L in 2 L water until LL, used in a hot bath and drink 1 hot cup, for anti-inflammatory. The crushed fruits are rubbed on affected area to treat skin infection. F are eaten after childbirth, for strengthener. Decoction of handful of B in 2 L water until 1 L, drink 1 cup/3times/day, until stop bleeding.	No
12	<i>Cyathea spp.</i>	Cyatheaceae	AS-001	Atarisi tse ai	Wounds, bruises and diabetes	M	Poultice from tender fresh and grated M applied on wounds or bruises. Decoction of a handful of fresh smashed M in 2 L water until 1 L, drink 1 cup/day until 1 L, No drink 1 cup/day until improvement, for diabetes (often with sap of <i>Uncaria tomentosa</i> "uña de gato").	No
<i>Erechtites</i>								
13	<i>Hieracifolius</i> (L.) Raf. Ex DC. var. <i>cacalioides</i> Griseb.	Asteraceae	AS-071	Maransera	Cicatrizantion and pimples	L	Fresh L mashed in oil, warm the mix and apply the warm L on wound, change when L dries, until cicatrization or healing of pimples.	No
14	<i>Erythrochiton jallax</i> Kallunki	Rutaceae	AS-057	Huabu quere ina	Dog skin infections. For luck in hunting	L	Fresh L mashed applied on affected area, for dogs skin infection	No
15	<i>Dendropanax arboreus</i> (L.) Decne & Planch	Decne Araliaceae	AS-127A	Yanama huana dheja	Intestinal parasites	B	It is used in combination with <i>G. longiflora</i> . Decoction of a handful of both plants in 2 L water until 1 L, drink 1 cup, for diarrhea and vomits.	No
16	<i>Hippeastrum puniceum</i> Kunzke	(Lam.) e Amaryllidaceae	NM-061	Bacua rudhu	Skin infection and pregnancy	Bu	Sap from fresh mashed Bu applied on affected areas. The red FI should be kept by women and men for conceiving a child	No
17	<i>Hirrelta bullata</i> Benth.	Chrysobalanaceae	AS-023	Chadhii aquí Shaka	Skin infection and rash	L	About 1 kg of fresh L are mashed in water in a bathtub and take 3 baths/days/3 days (for elder or child), for skin infection or rash.	No
18	<i>Hypis brevipes</i> Poit.	Lamiaceae	AS-055	Id'ene ejdhue	Intestinal parasites	L	Handful of fresh L squeezed in a small glass of fresh water, drink on empty stomach 3times/day/8 days	27
19	<i>Hypis mutabilis</i> Briq.	Lamiaceae	AS-088	Tapacha ina	Leishmaniasis, skin infection, urinary infection, diarrhea, fright vomits, diarrhea and fever.	L, R	Dried and powdered L applied on the clean ulcers or infected area until improvement, for leishmaniasis and/or skin infection. Handful of fresh L squeezed in a small glass of fresh water, drink on empty stomach 3times/day/8 days, for urinary infection Decoction of a handful of R in 2 L water until 1 L, drink 1cup, for diarrhea and vomits. With the same R decoction take a bath, for fever.	No
20	<i>Iresine diffusa</i> & Bonpl. ex Willd.	Amaranthaceae	AS-065	Quidi' juno	Skin infection	R	Fresh R mashed applied on affected area, until improvement	28

(Continued in the next page)

Table 1 (continued)

Entry	Scientific name	Plant family	Voucher specimen	Tacana name	Traditional use	Plant part	Mode of preparation	Literature
21	<i>Jacaranda glabra</i> Bureau & K.Schum.	Sapindaceae	AS-120	Chepererqui	Antiparasitary, stomachache, skin infection, scabies, leishmaniasis, and repellent	B, L, F	Decoction of a handful of fresh smashed B in 2 L water until 1 L, drink 1 cup before eating, for stomachache and antiparasitary. Fresh L crushed and applied on affected area, until improvement. Better results when bathing with a decoction of a handful of fresh B and L in 2 L water until 1 L, for skin infection. Dried L crushed and applied on affected area, until improvement, for scabies. Fruit sap applied on ulcer, for leishmaniasis. Burned L used as repellent.	No
22	<i>Jatropha curcas</i> L.	Euphorbiaceae	AS-036	Tara	Antifungal and scabies	S, L	Boiled fresh S in water until soft and squeeze it hot on affected area, for fungal infection. Fresh L mashed applied on affected area, for scabies	29
23	<i>Lunania parviflora</i> Spruce ex Benth.	Flacourtiaceae	AS-115	Hueruru	Skin infection	L	Fresh leaves mashed applied on the affected area, until improvement	No
24	<i>Nicotiana glauca</i> L. cf. <i>Nicotiana glauca</i> L.	Solanaceae	AS-099	Uma'sa ina	Pimples	WP	Buds and leaves in oil as poultice in affected areas, change 3-4 times/day for maturing the pimples.	No
25	<i>Otoba cf. parvifolia</i> (Markgr.) A.H. Gentry	Myristicaceae	AS-049	Naiqui, Naiki	Cicatrization	Re	The fresh resin is applied on the clean wound.	30 18
26	<i>Ourotea cf. flexuosa</i> Rusby	Ochnaceae	AS-080	Aqui tseru	Intestinal parasites	L	Handful of fresh L squeezed in a small glass of fresh water, drink on empty stomach 3 times/day/8 days	31
27	<i>Phlebotomum</i> <i>decumanum</i> J. Sm.	Polypodiaceae	AS-040	Dhutida	Cicatrization, gallbladder	S, L, IS	Fresh IS applied on affected area until improvement, for cicatrization. L and S decoction, drink 1cup/3 times/day until improvement, for gallbladder.	No
28	<i>Pharus latifolius</i> L.	Gramineae	AS-011	Bacua ina	Stomach pain, dysentery, snakebites	L	Dried and powdered L applied on the wound area until improvement. Handful of fresh L squeezed in a small glass of fresh water, drink on empty stomach 3 times/day/8 days, for dysentery.	32
29	<i>Physalis angulata</i> L.	Solanaceae	AS-105	Teimi tumati	Malaria, febrifuge, skin infection	WP, F	WP squeezed in 1L of water, drink 1cup/day until improvement, for Malaria and febrifuge. 2F squeezed in cold water and take a bath, for skin infection	33 19 34
30	<i>Porophyllum ruderale</i> (Jacq.) Cass. <i>Renealmia</i>	Asteraceae	AS-043	Ebus'a ina, Chadh'i ina	Leishmaniasis, infection, pimples	skin L	Dry powdered L applied on ulcer, for leishmaniasis. Tender L mashed applied on affected area and should be applied on new moon, for pimples and skin infection	35 36
31	<i>Renealmia</i> & Endlicher	Poeppig Zingiberaceae	SD-467	Mashahui	Food dye	R	Smashed R is used to dye rice similar to <i>C. longa</i>	37 28
32	<i>Salacia impressifolia</i> (Miers) A. C. Sm.	Celastraceae	AS-031	Panu	Rheumatism, cramps, cold and male impotence	R	Decoction of a handful of R in 2 L water until 1 L, drink 1 cup /3times/day, until feel improvement	No
33	<i>Scoparia dulcis</i> L.	Scrophulariaceae	AS-068	Bacua etse	Tuberculosis, headache, anti-inflammatory	WP	Prepare a poultice with WP mashed in boiled water, put on the back, and a handful of WP mashed in 1L of water, drink before breakfast until improvement generally 1 month, for treating tuberculosis. Wash the head with the "serenado" of the WP mashed in water, for headache. Poultice from WP mashed in boiled water, applied on the affected area as anti-inflammatory.	38 32

(Continued in the next page)

Table 1 (continued)

Entry	Scientific name	Plant family	Voucher specimen	Tacana name	Traditional use	Plant part	Mode of preparation	Literature
34	<i>Scleria macrophylla</i> J.Presl & C.Presl	Cyperaceae	NM-064	Kawasha baba	Fever	R	Decoction of a handful of R in 2 L water until 1 L, drink 1 cup, for fever.	39
35	<i>Senna hirsuta</i> (L.) H.S. Irwin&Barneby	Caesalpiniaceae	AS-044	Ina Paque Inama aqwi	Skin infections, Rush cutaneous produced by warm Urinary infection	L, R	Tender L mashed, applied on affected area, for skin infection and Rush cutaneous	No
36	<i>Senna pendula</i> (Willd.) H.S. Irwin&Barneby	Caesalpiniaceae	AS-058	Inama ina	Skin infection R are used as soap	L	Fresh L mashed applied on affected area, for skin infection	No
37	<i>Solanum</i> <i>Caricaefolium</i> Rusby	Solanaceae	AS-022	Adjadja ay	Skin pimples	F	Sap from mashed F applied on affected area and wash after 15min	No
38	<i>Spondias</i> spp.	Anacardiaceae	AS-018	Dhiji	Skin infection, antiparasitary, urinary infection	B, F	Mashed F applied on affected areas, until improvement, for skin infection. Sap from mashed F boiled in 1L water and drunk instead water, for antiparasitary. Syrup of 1 handful B, in 2L of water until thick syrup, drink instead of water until improvement, for urinary infection.	40 41 42
39	<i>Stachytarpheta</i> <i>cayennensis</i> (Rich.) Vahl	Verbenaceae	AS-112	Huira huira	Skin infection, diarrhea and vomits	R, F L	Fresh R and/or F mashed applied on affected areas, 3times/day/3days, for skin infection. Decoction of a handful of R, F, L in 2 L water until 1 L, drink 1 L same decoction, drink 1 spoon for babies and ½ cup cup/3times/day, during 3 days, for diarrhea.	43 44 28 45
40	<i>Talinum paniculatum</i> (Jacq.) Gaertn.	Portulacaceae	AS-110	Bechu ina	Febrifuge, skin infection	L	Fresh L soaked in "tumi" oil (Attalea phalerata) are applied on temple, for fever. 24 fresh mashed L applied on affected area until improvement, for skin infection	24
41	<i>Texaria integrifolia</i> Ruiz et Pav.	Asteraceae	AS-014	Cawuara	Lishmaniasis Anti-inflammatory, ray sting	L	L Fresh mashed Leaves on ulcer to clean and after apply powdered dry L as poultice on the ulcer, until poultice falls of, for leishmaniasis.	46
42	<i>Thalia geniculata</i> L.	Marantaceae	AS-083	Japauro, jatima baba	Diarrhea, cicatrization F are used for handicrafts, F are ornamentals.	Bd, L, R	Decoction of a handful of R in 2 L water until 1 L, drink 1cup/day/3 days, for diarrhea. Tender Bd wet with water, applied as poultice on the wound, for cicatrization.	47
43	<i>Tilisia baccata</i> (L.) Pruski	Asteraceae	AS-039	Djeru ina	Stye and skin pimple Fruits are edible.	L, F	Crushed F sap applied on stye at night before sleep. Crush fresh L, either alone or mixed with Attalea phalerata oil, applied on affected area, for skin pimples.	24
44	<i>Trichilia adolfi</i> Harms	Meliaceae	AS-092	Sapuraqui ina	Lung, liver and kidney disorders	B	Decoction of a handful of R in 2 L water until 1 L, drink 1cup a day until feel improvement	48
45	<i>Vitex</i> spp.	Lamiaceae	AS-049	Siringuero ina	Skin infection	L	Fresh mashed L applied on affected areas, once/day/5 days	49 50
46	Genus and specie not defined	Euphorbiaceae	AS-003	Ejije bidua	Cicatrization, skin infection, burns and muscle pain	L, S	Fresh smashed L applied directly on wound, and/or affected areas, for cicatrization, skin infection and burns. Hot bath with Infusion of L and S, before getting to bed, repeat until improvement, for muscle pain.	

Plant part: AP aerial parts; B: bark; Bu: bulb; Bd: bud; F: fruit; Fl: flower; IS: inner stem; L: leaves; M: meristem; R: roots; Re: resin; S: stem; T: twigs; WP: whole plant

1.5 Summary and conclusion

In summary, we have seen that Tacana traditional medicine has long history and is rich in traditions. Our contribution to the ethnobotany of medicinal plants includes 46 vegetable species used to treat skin affections, among them are leishmania ulcers, skin infections, as anti-inflammatory and wound healing. The plants collected were taxonomically identified and the traditional uses were defined by healers from eight Tacana communities in a local workshop. From the 46 vegetable species 22 species were documented for first time as medicinal plants in Tacana TM, and only four were specifically cited for leishmaniasis treatment, *Porophyllum rudale* (Asteraceae), *Hyptis mutabilis* (Lamiaceae), *Jacaranda glabra* (Sapindaceae) and *Tessaria integrifolia* (Asteraceae). The extensive literature research shown that 24 species has previous been studied as antiparasitic. This part of the study contribute to the preservation of Tacana TM, and show the importance of including traditional knowledge as criteria for selecting plants for possible alternative treatments.

Chapter 2: Leishmaniasis, a neglected tropical disease

In chapter 1 were collected and identified medicinal species to be added to the list of plants of Tacana TM, and contribute with the preservation of such a valuable culture. But our wish to understand how these vegetable species can be used as medicine for specific diseases, led us to subject such plants in an *in vitro* model and evaluate their antiparasitic properties against parasites of endemic diseases.

2.1 General aspects of Neglected Tropical Diseases

The concept of "Neglected Diseases" is understood as infectious diseases that occur mainly in developing countries and affect people that live in poverty, besides that they have difficulty to access health services and are in close contact with the infectious vector. In a broad definition that may include 49 diseases⁵¹.

In 1975, the Special Programme for Research and Training in Tropical Diseases (TDR)⁵² was created on the initiative of WHO, with the objective of developing improved tools for control of tropical diseases and strengthening the research capability of the affected countries themselves. In the list of action were included malaria, schistosomiasis, filariasis, trypanosomiasis (both African sleeping sickness and the American form called Chagas disease), leishmaniasis and leprosy⁵³. However, later they noted that the problem was bigger than they believed and with time new tropical diseases were included. In January 2012 the WHO published a list of targets that includes 17 neglected tropical diseases, that are considered as high priority in the international agenda. The list includes: Buruli ulcer, chagas disease, taeniasis/cysticercosis, dengue, dracunculiasis, echinococcosis, endemic treponematoses, foodborne trematodiasis, human African trypanosomiasis, leishmaniasis, leprosy, lymphatic filariasis,

onchocerciasis, rabies, schistosomiasis, trachoma and soil-transmitted helminthiasis⁴.

The efforts are concentrated to develop five strategies to overcome the NTDs that include: preventive chemotherapy; intensified case-management; vector control; the provision of safe water, sanitation and hygiene; and veterinary public health⁵⁴. Furthermore, through the London declaration in 2015, nations commit to take part to sustain, expand and extend drug access programs to ensure the necessary supply of drugs and other interventions to facilitate the control of schistosomiasis, soil-transmitted helminthes, Chagas disease, visceral leishmaniasis and river blindness (onchocerciasis) by 2020⁴.

The leishmaniasis surveillance and control program is based on case detection and treatment, and it is evident that there is a persistent insufficiency in drug and vaccine development for neglected diseases. This is reflected in a study of 850 new therapeutic products registered, from 2000 to 2011, only 4% were indicated for ND, and from them only four new chemicals were approved for the treatment (three for malaria, one for diarrheal disease). Since the development of miltefosine in 2014, no more new therapeutic products were approved or recommended for leishmaniasis disease⁵¹. This is an urgent signal to develop new alternatives for the treatment of leishmaniasis, considering that it leads the second most studied disease, after geohelminthic diseases⁵⁵.

2.2 Leishmaniasis

Leishmaniasis is a group of diseases caused by protozoan parasites, and it is considered endemic to 88 countries around the world with over 12 million people suffering the disease. *Leishmania* parasites belong to the Trypanosomatidae family and are taxonomically divided in two subgenera (*Leishmania-Leishmania* and *Leishmania-Viannia*) that include over 20 human pathogenic parasite species. The vector is the female phlebotomine (sandflies). There are three main clinical manifestations of the disease: visceral leishmaniasis (VL, also known as kala-azar); cutaneous leishmaniasis (CL); and mucocutaneous leishmaniasis (MCL)⁵⁶. While CL is the most common form of the disease in the Americas generating open sores; MCL is also widespread and can cause severe and persistent lesions with disfigurement (nose, throat and esophagus) and often require prolonged treatment regimens, with low degree of success. The VL infection, mainly occurs

in Africa, Brazil and India, is the most serious clinical form, and can be fatal if untreated⁵⁷.

According to PAHO 2019⁵⁸ Americas has a report of 940 396 new cases in 2017, with an annual average of 55 317 cases, incidence that is maintained since 2001, being Brazil the most affected country (72.6%, 17 526 cases), Also is reported that 89.9% were CL and 10.1% were MCL, with a predominance of national epidemiological indices of *L. amazonensis* (LC), *L. braziliensis* (CL and MCL) and *L. lainsoni* (CL and MCL). Tropical regions among Brazil, Peru and Bolivia are reported as the most affected endemic zones (Figure 2.1)⁵⁹

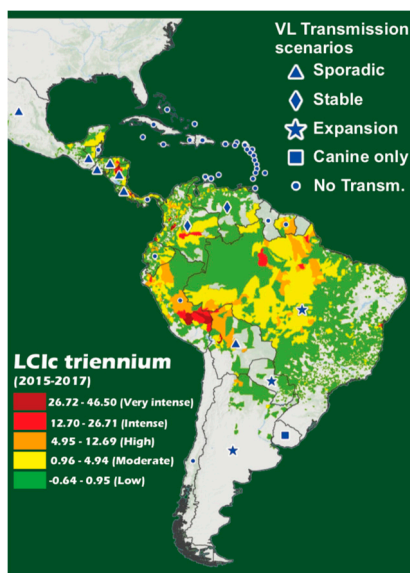


Figure 2.1: CL and MCL reported in Americas at 2017. Image file was taken from WHO 2019 report⁵⁸.

As mentioned above, even when Leishmaniasis is the second most studied neglected disease, no new drugs were approved since the release of Miltefosine (2.1). The first election drug for the treatment, Meglumine antimoniate (2.2), came into medical use in 1946⁶⁰, years later, amphotericin B (2.3) from *Streptomyces nodosus* was discovered in 1955 and launched to market in 1957 as antifungal agent, but the FDA approved it for VL in 1997⁶¹. Miltefosine was approved in India in 2002 for VL, then approved by the FDA in 2008 and has been added to the WHO Model List of Essential Medicines for treating VL and CL in 2010⁶². All of these treatments have been reported as highly toxic and shown severe side effects. Therefore, it is important to develop new alternative treatments. More so

when natural products from plants and other organisms continue to play a crucial role in the discovery of new drugs ⁶³.

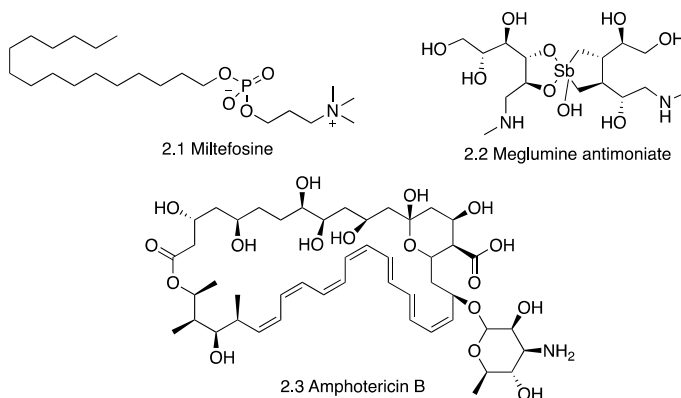


Figure 2.2 Antiparasitic drugs approved for the treatment of leishmania disease.

2.3 Antiparasitic activity of plants from Tacana TM

Our journey to explore chemical diversity of natural products, afforded us to obtain several ethanolic extract from the collected plants to be evaluated as antiparasitic in our *in vitro* models (figure 2.3). In the first phase of the project we selected 46 species, some were divided in leafs, roots, bark, flowers, etc. and as a result we obtained 84 crude extracts that were evaluated against two strains of *Leishmania* promastigotes (*L. amazonensis* (*L.a*), *L. braziliensis* (*L.b*)) (paper 1 and 3). The most active extracts are presented in table 2.1. From 84 crude extracts, 37 shown some activity (45.2%), and they were included in 3 groups according to their biological behavior. In group 1 were included 13 extracts (16.6%) that showed the highest potencies ($IC_{50} < 50$ mg/dL), in the group 2 were included those extracts with a moderate activity ($IC_{50} > 50$ but < 100 mg/dL), some 14 extracts, and finally in the group 3 those extracts that showed activity only in 1 parasite were included.

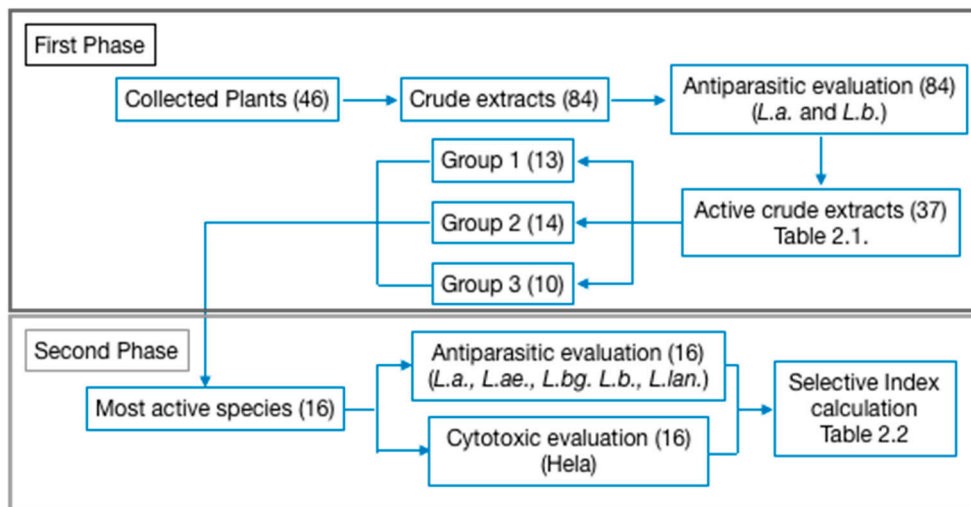


Figure 2.3: Bioguided assay to select antiparasitic plants

Group 1 was selected for further studies including seven additional species that showed activity against *L. braziliensis*: leaves of *Adiantum* cf. *latifolium*, *Erythrochiton fallax*, *T. integrifolia*, *P. angulate*, *Vitex* spp. and *Talinum paniculatum*; roots from *S. dulcis* and *amaranthus spinosus*, *T. adolfi*. In total, some 16 species were selected for bioguided studies in the second phase of investigation.

In the second phase, selected plants were subjected to chromatographic column separations to obtain the main fractions [less polar, medium polar (F3) and polar fractions (F4)]. Each fraction was tested again in a broader battery of parasites, including *L. Aethiopica*, *L. braziliensis* native and *L. Lainsoni* (Table 2.2). For some experiments, amount collected from the less polar fraction was insufficient, and for most of the cases the medium polar F3 fraction was the most active fraction (table 3, paper 1). In order to calculate the selectivity index (SI) value, additionally cytotoxic activity was evaluated in HeLa cell cultures. The following discussion is focused on the results presented in table 2.2, a bibliography research that has been made of the active plants. For this study we considered as potentially interesting extracts those with selectivity index up to $SI > 2$, as criteria of selection was taken as average of the SI for natural products suggested by other authors^{64 65} that refer as active natural extract an SI value between 1.0 and 3.0. The extract of *G. longiflora* (SI:2.0) (CAT in table 3), a plant widely studied due its antiparasitic properties, was used as internal control.

Table 2.1

Antiprotozoal activity (IC50 in µg/mL±SD) on promastigotes of *Leishmania amazonensis* (Lma) and *L. braziliensis* (M2904) of crude extracts and extraction yields from the Tacana plants.

No	Scientific name	Plant part	Collection coordinates	% w/w yield	Parasite strains	
					<i>L.a</i>	<i>L.b</i>
GROUP ONE						
1	<i>Abuta grandifolia</i>	L	S 14°21'434" W 67°34'744"	9.4	38.3±4.2	31.1±13.1
2	<i>Bocageopsis spp.</i>	L	S 14°21'428" W 67°34'626"	5.4	37.9±6.2	19.1±2.3
3	<i>Cedrela spp.</i>	B	S 14°21'917" W 67°33'781"	10.1	36.8±2.8	18.2±1.5
4	<i>Hyptis mutabilis</i>	L	S 14°22'007" W 67°34'004"	5.0	29.7±1.1	9.8±0.8
5	<i>Hyptis brevipes</i>	L	S 14°21'630" W 67°33'496"	8.3	13.1±1.0	5.3±0.4
6	<i>Iresine diffusa</i>	Fl	S 14°22'035" W 67°34'010"	15.3	30.5±7.1	11.1±2.5
7	<i>Iresine diffusa</i>	L	S 14°22'035" W 67°34'010"	10.8	37.3±1.4	29.4±2.5
8	<i>Jacaranda glabra</i>	B	S 14°21'558" W 67°34'477"	1.4	29.8±3.6	17.4±2.7
9	<i>Physalis angulata</i>	F	S 14°21'967" W 67°33'764"	3.1	17.6±1.8	43.5±0.3
10	<i>Renealmia breviscapa</i>	R	S 14°21'960" W 67°33'769"	12.1	21.0±2.5	37.0±3.7
11	<i>Salacia impressifolia</i>	R	S 14°22'410" W 67°34'203"	9.8	9.3±0.2	21.6±1.6
12	<i>Scoparia dulcis</i>	AP	S 14°21'583" W 67°33'388"	10.4	23.9±4.1	25.1±1.2
13	<i>Thalia geniculata</i>	R	S 14°22'688" W 67°33'957"	1.4	29.5±5.1	17.6±0.8
GROUP TWO						
15	<i>Adiantum cf. latifolium</i>	WP	S 14°22'538" W 67°33'880"	6.5	78.4±3.3	25.0±2.2
16	<i>Cecropia cf. concolor</i>	L	S 14°22'678" W 67°34'398"	5.4	97.4±3.2	88.4±11.0
17	<i>Celtis iguanaea</i>	L	S 14°22'048" W 67°33'764"	2.1	59.3±3.6	76.8±10.0
18	<i>Celtis iguanaea</i>	L, S	S 14°22'048" W 67°33'764"	3.6	83.6±16.4	71.9±22.2
19	<i>Erechtites hieraciifolius var. cacalioides</i>	L	S 14°21'820" W 67°33'692"	3.3	75.9±2.2	84.0±5.2
20	<i>Physalis angulata</i>	L	S 14°21'967" W 67°33'764"	7.6	72.4±10.1	41.3±0.4
21	<i>Stachytarpheta cayennensis</i>	R	S 14°21'820" W 67°33'692"	1.3	28.2±3.0	65.6±19.0
22	<i>Talinum paniculatum</i>	L	S 14°21'967" W 67°33'764"	2.2	66.2±2.9	41.5±7.1
23	<i>Talinum paniculatum</i>	S	S 14°21'967" W 67°33'764"	1.9	93.5±4.6	93.0±1.0
24	<i>Talinum paniculatum</i>	F, L	S 14°21'967" W 67°33'764"	4.2	75.5±0.8	89.5±7.2
25	<i>Thalia geniculata</i>	S	S 14°22'688" W 67°33'957"	1.9	78.2±21.9	60.6±6.5
26	<i>Tessaria integrifolia</i>	L	S 14°22'048" W 67°34'064"	4.3	54.2±9.6	31.6±14.8
27	<i>Tessaria integrifolia</i>	S	S 14°22'048" W 67°34'064"	1.4	78.5±4.0	58.6±5.4
28	<i>Vitex spp.</i>	L	S 14°21'441" W 67°34'711"	4.0	76.0±0.3	37.7±3.3
GROUP THREE						
29	<i>Amaranthus spinosus</i>	R	S 14°22'035" W 67°34'010"	7.6	>100	31.7±12.6
30	<i>Erythrochiton fallax</i>	L	S 14°21'434" W 67°34'744"	6.4	>100	55.5±16.2
31	<i>Hippeastrum puniceum</i>	Bu	S 14°22'154" W 67°34'218"	1.4	79.1±10.6	>100
32	<i>Hyptis mutabilis</i>	S	S 14°22'007" W 67°34'004"	1.2	>100	92.2±7.9
33	<i>Hyptis mutabilis</i>	R	S 14°22'007" W 67°34'004"	2.0	97.8±2.3	>100
34	<i>Scoparia dulcis</i>	R	S 14°21'583" W 67°33'388"	3.3	>100	53.2±3.4
35	<i>Senna pendula</i>	L	S 14°21'820" W 67°33'692"	8.0	>100	87.6±6.2
36	<i>Tilesia baccata</i>	L	S 14°21'716" W 67°33'632"	2.7	>100	92.7±1.3
37	<i>Trichilia adolfi</i>	B	S 14°21'438" W 67°33'728"	2.2	>100	39.2±0.2
CONTROL DRUGS						
CAT					20.4±1.9	20.1±7.0
Miltefosine					10.6±1.4	3.1±1.0
Amphotericin B					0.2±0.06	0.3±0.1

Plant part: AP aerial parts; B: bark, Bu: bulb; F: fruit; Fl: flower; L: leaves; R: roots; S: stem; WP: whole plant.

Table 2.2

Leishmanicidal activity (IC50 in µg/mL±SD) against a panel of protozoa and cytotoxicity with HeLa cells (LD50 in µg/mL±SD) of the most active crude extracts and fractions.

Nº	Scientific name	Plant part	Sample	L.a.	SI	L.a.e	SI	L.b.	SI	L.b.G	SI	L.lan	SI	HeLa
1	<i>Abutia grandifolia</i>	L	Crude	38.3±4.2	0.4	21.9±0.4	0.8	31.1±13.1	0.5	21.4±0.2	0.8	19.5±1.0	0.9	16.7±2.7
		F3		14.9±3.4	0.8	7.9±0.1	1.5	10.9±0.9	1.1	6.3±0.05	1.9	5.6±0.7	2.1	11.9±1.6
2	<i>Adiantum cf. latifolium</i>	WP	Crude	78.4±3.3	0.9	73.9±21.6	1.0	25.0±2.2	3.0	34.7±5.1	2.1	28.5±9.3	2.6	74.5±2.5
		F3		37.3±3.8	0.9	49.0±15.3	0.7	14.6±7.9	2.4	31.6±10.6	1.1	18.9±1.1	1.9	35.5±14.8
3	<i>Bocageopsis spp.</i>	L	Crude	37.9±6.2	0.7	31.4±2.8	0.8	19.1±2.3	1.4	21.9±0.1	1.2	16.6±7.2	1.6	26.1±4.0
		F3		18.8±2.5	1.1	16.9±1.7	1.2	9.4±1.2	2.1	10.8±0.2	1.8	7.3±2.9	2.7	19.8±0.5
4	<i>Cedrela spp.</i>	B	Crude	36.8±2.8	1.8	54.5±2.5	1.2	18.2±1.5	3.7	22.7±1.2	2.9	59.7±18.2	1.1	66.5±7.5
		F3		8.3±0.2	1.2	7.7±0.9	1.3	5.2±0.9	1.9	6.2±0.2	1.6	7.2±1.0	1.4	9.8±2.6
5	<i>Erythronchiton fallax</i>	L	Crude	>100	1.2	77.7±2.2	1.5	55.5±16.2	2.2	74.8±6.2	1.6	58.0±16.2	2.1	121.1±8.8
		F3		47.9±1.7	1.1	34.2±14.9	1.6	19.0±3.7	2.9	31.6±10.8	1.7	28.9±17.5	1.9	54.6±8.5
6	<i>Hypsis brevispes</i>	L	Crude	5.3±0.4	3.2	12.3±1.5	1.4	13.1±1.0	1.3	10.9±0.5	1.6	15.2±7.3	1.1	17.3±2.3
		F3		29.7±1.2	2.1	15.4±2.4	4.2	9.8±0.8	6.6	21.7±0.9	3.0	27.4±7.1	2.4	64.4±7.4
8	<i>Iresine diffusa</i>	L	Crude	37.3±1.4	1.3	39.4±0.5	1.3	29.4±2.5	1.7	40.6±1.5	1.2	26.0±3.8	1.9	50.7±15.4
		F3		17.9±0.7	1.9	16.9±0.9	2.0	12.8±2.1	2.6	14.7±0.9	2.3	15.6±0.3	2.2	33.7±3.6
9	<i>Jacaranda glabra</i>	B	Crude	29.8±3.6	6.4	45.4±4.6	4.2	17.4±2.7	11.0	22.4±0.4	8.6	27.5±16.0	7.0	192.0±8.0
		F3		22.1±3.0	1.5	12.5±0.9	2.6	5.6±0.6	5.7	6.2±0.2	5.2	6.9±2.3	4.7	32.1±7.1
10	<i>Physalis angulata</i>	F	Crude	17.6±1.8	1.1	41.8±1.2	0.5	43.5±0.3	0.4	41.4±2.1	0.5	30.4±0.1	0.6	19.5±2.1
		L		72.4±10.1	2.1	97.5±2.6	1.6	41.3±0.4	3.7	85.6±4.6	1.8	87.0±13.0	1.8	154.5±37.5
	<i>Renealmia breviscapa</i>	F3		16.9±1.0	2.5	15.2±0.9	2.8	9.3±1.5	4.6	13.5±2.7	3.2	10.5±5.6	4.1	43.0±15.0
		R		21.0±2.5	0.7	NE	0.4	37.0±3.7	NE	NE	NE	NE	NE	15.4±3.0
12	<i>Scoparia dhicis</i>	AP	Crude	23.9±4.1	3.0	19.1±0.4	3.8	25.1±1.2	2.9	13.8±3.3	5.3	27.9±8.4	2.6	72.7±22.4
		F3		20.4±4.0	2.2	15.8±2.8	2.9	25.4±4.5	1.8	8.2±2.0	5.5	19.0±0.7	2.4	45.2±1.0
	R		Crude	>100	-	>100	-	53.2±3.4	2.0	47.6±0.6	2.3	28.7±16.6	3.8	108.3±8.3
		F3		19.0±1.3	1.8	25.9±3.2	1.3	6.5±3.0	5.2	10.4±0.2	3.3	10.3±0.9	3.3	33.8±8.9
13	<i>Tessaria integrifolia</i>	L	Crude	54.2±9.6	2.2	48.0±3.4	2.5	31.6±14.8	3.8	39.0±0.5	3.0	34.8±0.7	3.4	119.3±24.9
		F3		22.5±1.4	3.0	23.4±0.9	2.9	9.7±1.3	7.0	19.7±0.1	3.5	24.1±5.0	2.8	68.0±5.10
	S		Crude	78.5±4.0	1.3	88.5±11.6	1.2	58.6±5.4	1.8	81.2±7.4	1.3	43.9±5.9	2.4	105.5±10.5
		F3		43.4±4.9	1.1	24.6±1.4	1.9	33.3±0.6	1.4	36.9±2.0	1.3	21.3±2.9	2.2	47.0±18.5
14	<i>Thalia geniculata</i>	R	Crude	29.5±5.1	1.7	20.9±1.6	2.4	17.6±0.8	2.9	24.7±1.4	2.1	26.6±0.1	1.9	51.0±2.0
		F3		12.7±1.7	3.1	10.5±1.4	3.8	10.1±0.3	3.9	14.6±3.3	2.7	11.9±1.0	3.3	39.5±6.5
15	<i>Trichilia adolfi</i>	B	Crude	>50		>50		39.2±0.2	5.1	>50		>50		>200
		L		76.0±0.3	1.0	94.6±5.5	0.8	37.7±3.3	2.0	44.7±0.4	1.7	52.3±14.2	1.4	75.0±12.6
16	<i>Vitex spp.</i>	F3		32.7±2.0	0.5	21.3±2.2	0.8	20.1±7.0	0.9	14.5±3.7	1.2	14.7±3.0	1.2	17.6±5.6
		L		20.4±1.9	2.0	17.3±1.0	2.3	19.8±1.9	2.0	18.0±2.1	2.2	17.3±0.9	2.3	40.0±25.7
CAT			23.7±5.9	3.5	25.4±10.7	3.3	26.6±8.8	3.1	21.9±10.1	3.8	25.4±7.7	3.3	83.7±20.1	
2FQ			10.6±1.4	1.7	2.5±0.08	7.2	8.5±1.2	2.1	11.3±1.7	1.6	3.98±0.6	4.5	18.1±7.0	
Miltefosine			0.2±0.06	176.0	0.1±0.06	353.0	0.1±0.06	353.0	0.1±0.01	353.0	0.1±0.06	353.0	0.1±0.06	353.0
Amphotericin B														

Protozoa Panel: L.a.: Leishmania amazonensis; L.a.e: L. acchiopica; L.b.: L. braziliensis; L.b.G: L. braziliensis native; L.lan: L. lainsoni; SI: selectivity index.

2.3.1 *Abuta grandifolia* (Mart.) Sandwith (Menispermaceae)

A. grandifolia (Table 3, entry 1) was used in folk tradition for the treatment of malaria, diabetes, stomachache, as anti-inflammatory and antimicrobial, among other uses⁶⁶, chemically it is composed of bisbenzylisoquinoline alkaloids (**2.4**) with anticholinesterase properties⁶⁷, that also have antimalarial activity⁶⁸. Similar compounds isolated from Ranunculaceae (**2.5**), a sister taxon to Menispermaceae, has also been reported to possess leishmanicidal properties⁶⁹ suggesting that they are responsible for the activity of this plant. Together with other components among which can be noted bidesmosidic and triterpenoid saponins (**2.6**), aphorpine alkaloids (**2.8**) can be also found in leaves and twigs⁷⁰. In our study it shown the SI <1.0, being the most cytotoxic extract. The fraction F3 showed slight improvement activity than the crude.

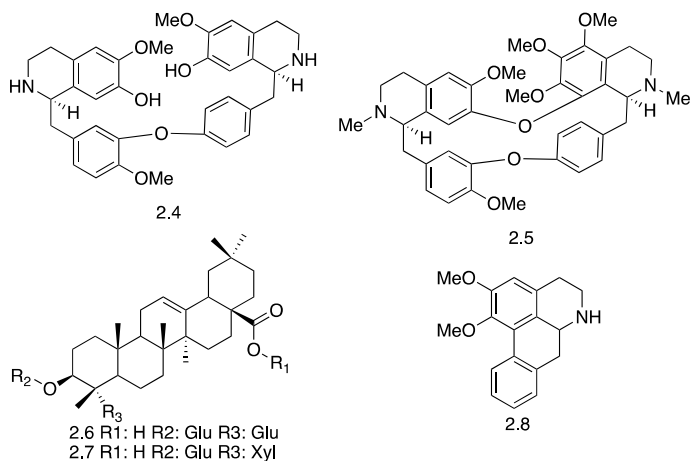


Figure 2.4: Main metabolites reported from *Abuta grandifolia*

2.3.2 *Adiantum cf. latifolium* Lam. (Adiantaceae)

Leafs infusion of *A. latifolium* is employed in Latin American as an anxiolytic, anti-inflammatory and analgesic⁷¹, while not much work was done on its chemical contents. Some steroids were the main compounds found in the leafs (**2.9-2.12**)⁷². In this study, F3 showed higher antiparasitic activity than the crude extract (Table 3, entry 2), even though it also showed high cytotoxicity as the SI value does not change, a similar behavior was reported by Lopez, 2001⁷³.

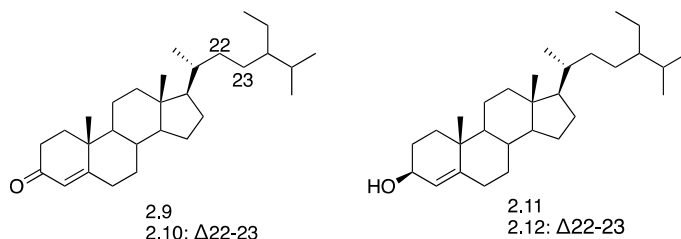


Figure 2.5: Triterpenoids isolated from *Adiantum latifolium*

2.3.3 *Bocageopsis* spp. (Annonaceae)

Studies focused in the essential oil, and its antimicrobial and leishmanicidal activity have been reported. They describe the chemical content to be mainly composed of sesquiterpenoids and alkaloids (**2.13** – **2.20**)^{74 75}, with a moderate leishmanicidal activity (IC_{50} :14.6 μ g/mL). In this study the fraction F3 reported a similar activity (Table 3, entry 3). Additionally, combined with the cytotoxicity, a low SI value is reported ($SI < 1.1$) which is less than the average to be considered as an antiparasitic.

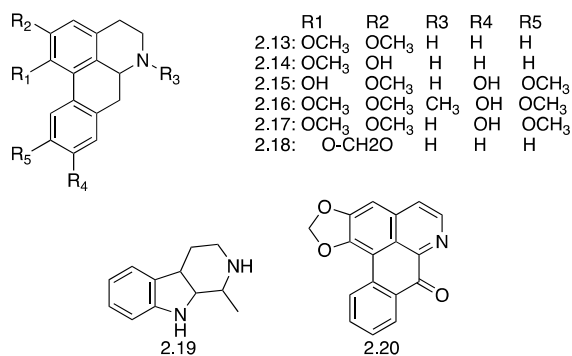


Figure 2.6 Isolation of aporphine-type alkaloids from *Bocageopsis* spp

2.3.4 *Cedrela* spp. (Meliaceae)

Cedrela species are grown in subtropical climates across Latin America and they are appreciated for their commercial value. The chemical content found in these species were described by several authors, highlighting the content of triterpenoids (**2.21**)⁷⁶, and several limonoids of cedrelone-type (**2.22**)⁷⁷, gedunin-type (**2.23**)⁷⁸, nomilin-type (**2.24**)^{79 80}, and cedrenolide-type (**2.26**)⁸¹. Regarding to biological activity, limonoids are known for their insecticidal properties, and similar compounds found in this genus exhibit such characteristic⁸². Also

antiprotozoal activity was reported against *L. infantum*, *L. major* and *Trypanosoma cruzi*^{26 25}, and in this study fraction F3 showed better antiparasitic values (SI>2.0) than the crude extracts against *L. braziliensis* and *L. lainsoni*.

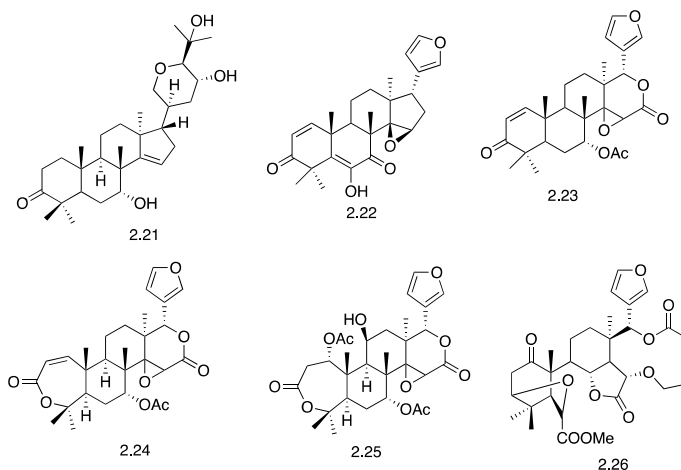


Figure 2.7: Some Tetranortriterpenoids from *Cedrela* genus

2.3.5 *Erythrochiton fallax* kallunki (Rutaceae)

We have not found references regarding chemical or biological studies, according to tropicos.org, in 2019 this species was reported only in the South Americas region⁸³. Tacana people use the leaves to treat dogs skin infections. This is the first report of biological activity that shown slight sensitivity of *L. braziliensis* than other strains to the extract, with SI values SI>2.0.

2.3.6 *Hyptis brevipes* Poit. (Lamiaceae)

Hyptis species are disseminated along the America and Asia continents, they were studied in Mexico and Indonesia and brevipolide-type compounds were isolated (2.27 – 2.30) among other compounds (2.31 – 2.33)^{84 85}. The biological activities of this family of compounds show cytotoxic, anti-oxidative and insecticidal properties^{84 86}, and the crude extract show an interesting SI value (SI>3.0). For this reason it was selected for further analysis (Paper 2) and will be described in the next chapter.

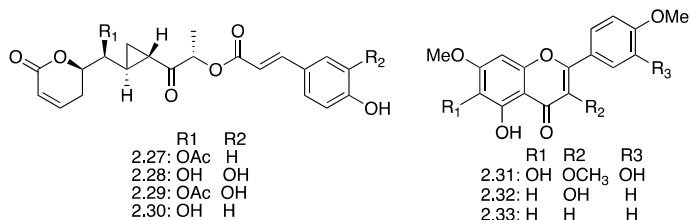


Figure 2.8: Active brevipolide-type and flavonoids from *Hyptis brevipes*

2.3.7 *Hyptis mutabilis* Briq. (Lamiaceae)

From the same genus of *H. brevipes*, *H. mutabilis* shown the best SI value in our study (SI>12.0). Previous studies of this species were directed to investigate the essential oils and its sedative and anesthetic properties⁸⁷, the components present in it are widely described in species from the region⁸⁸. There is a report of a high content of sesquiterpenes with antimicrobial properties⁸⁹. Major components are germacrene D (**2.34**), curzerene (**2.35**), and globulol (**2.36**), and this plant was also selected for further analysis and will be described in the next chapter (paper 3).

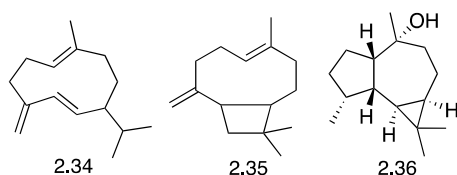


Figure 2.9: Main components present in the essential oil of *Hyptis mutabilis*.

2.3.8 *Iresine diffusa* Humb. & Bonpl. exWilld (Amaranthaceae)

Previous studies on axenic amastigotes of *L. braziliensis* showed some activity of the crude extract (63.3 ug/mL)²⁸, in contrast our study shown a similar behavior in promastigotes with SI values up to SI>2.0. A chemical investigation revealed the presence of sesquiterpene lactones described as iresine-type (**2.37**)⁹⁰ and drimene-type (**2.38** – **2.39**)⁹¹ as the main metabolites, and studies of the *Iresine* genus reports its use for wound healing and as an antioxidant agent⁹².

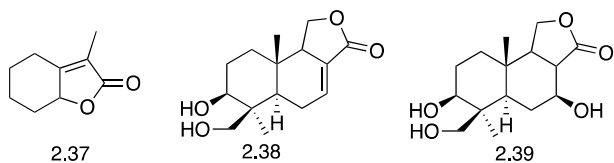


Figure 2.10: Sesquiterpenes from *Iresine diffusa*

2.3.9 *Jacaranda glabra* Bureau & K. Schum (Sapindaceae)

Several reports shown that *Jacaranda* genus was used in folk medicine, and there are reports of leishmanicidal activity but with high cutaneous toxicity of crude extract. However, not much studies of pure compounds were described⁹³. The representative diterpenoid jacaranone (**2.40**) and glycosidic derivatives (**2.41**) exhibit promising anti-protozoal activity⁹⁴. In our study, it showed good SI values ($4.2 < SI < 11.0$) as crude extracts, that tend to suffer a decrease with fractionation. Unfortunately, not enough material was available to continue with the study, but it is a remarkable specie to consider for a future study.

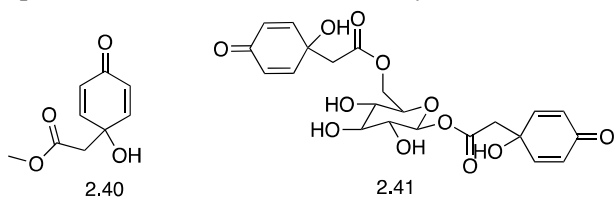


Figure 2.11: Jacaranone-type and jacaglabrosides-type metabolites from *Jacaranda glabra*

2.3.10 *Physalis angulate* I. (Solanaceae)

Physalis genus was widely studied and was reviewed for the folk uses, the chemistry and pharmacology beside the chemical constituents and their biological activities⁹⁵⁻⁹⁶. Chemically it is composed with a great diversity of versatile components, mainly those derived from withanolide-type compounds with an unmodified skeleton (**2.43**) or triterpenoids as physalin-type (**2.44**) with a modified skeleton⁹⁷, among others labdane-type triterpenoid glycosides (**2.42**)⁹⁸ and phenolic compounds⁹⁹. Additionally, evaluation reported attribute anti-inflammatory, anti-proliferative¹⁰⁰, anti-protozoal¹⁰¹, and leishmanicidal properties.¹⁰² In this study, fruits and leaves were studied, showing that the fruits gave better results, but it was not further studied due the low amount available. The fraction F3 of the leaves showed an increment of the SI values for all strains.

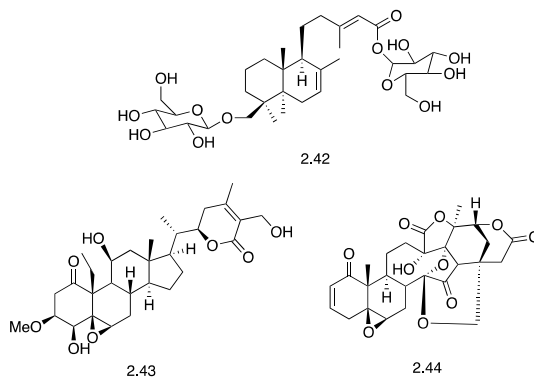


Figure 2.12: Withanolide-type metabolites from *Physalis angulata*

2.3.11 *Renalmia breviscapa* Poeppig & Endlicher (Zingeraaceae)

R. breviscapa is used as a food dye by Tacana people and showed moderate antiparasitic activity (*L.a.* 21.0±2.5, *L.b.* 37.0±3.7). It belongs to the same family as *Curcuma longa*, a widely studied plant with several biological properties, including antiparasitic¹⁰³. Studies in rhizomes showed that curcumin-type compounds can act as immune-modulators (2.45 – 2.47)³⁷. While the leaves were reported to contain constituents that have leishmanicidal properties, described mainly as sesquiterpenes and benzofuranone-type compounds (2.48 – 2.50), this plant got our attention due to the daily use in the Tacana culture, and the natural products found will be described in chapter 3.

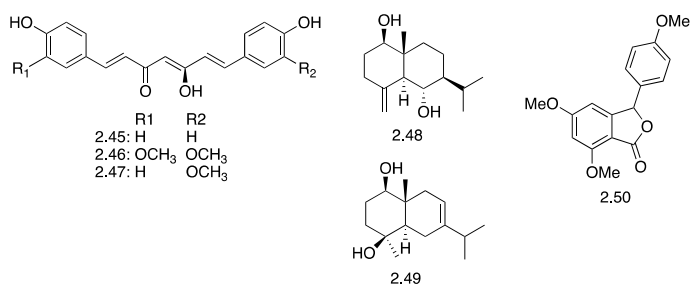


Figure 2.13: Main metabolites isolated from *Renalmia* genus

2.3.12 *Scoparia dulcis* L. (Scrophulariaceae)

Traditional uses and chemical studies report that the metabolites found in this species mainly are diterpenes of the scoparic acid-type (2.51), labdane-derived diterpenes (2.52) and flavonoids, and that the extract has healing properties^{104 105}

¹⁰⁶. Antiparasitic studies report that the crude extract has leishmanicidal activity, but with no references to pure metabolites ¹⁰⁷. During our studies we investigated aerial parts and roots, and both extracts shown acceptable SI values but they were excluded from further studies due the small amount of crude material available.

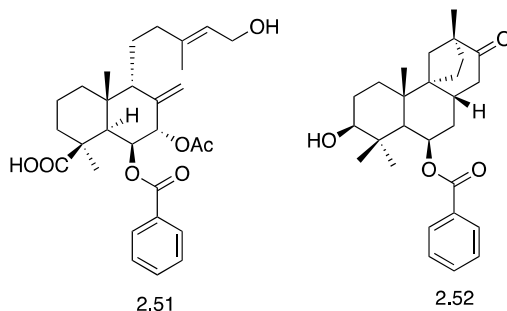


Figure 2.14: Main metabolites from *Scoparia dulcis*

2.3.13 *Tessaria integrifolia* Ruiz et Pav. (Asteraceae)

Previous studies on *Tessaria* species showed that essential oil consist of about 32% of oxygenated monoterpenes, 36% of oxygenated sesquiterpenes and 17% of sesquiterpene hydrocarbons ¹⁰⁸. In this high concentration of oxygenated metabolites were found sesquiterpenes as tessaric acid (**2.53**), several types of eremophilanes- (**2.54**), eudesmona-type (**2.55**) compounds ^{109-110 111 112} and flavonoids ¹¹³⁻¹¹⁴. Regarding biological activity studies on crude extracts showed that it was specific against leishmania, more active on intracellular forms and active with an $IC_{50} < 1 \mu\text{g/mL}$ (intracellular form). However, the chemical composition is still unclear ⁴⁶. In our study, stem extracts are less active and selective than leave extracts. Calculated SI values for the leaves extract indicate that this is one of the most selective extracts with $SI > 2$. For this reason it was selected for a chemical analysis and its compounds will be discussed in the next chapter.

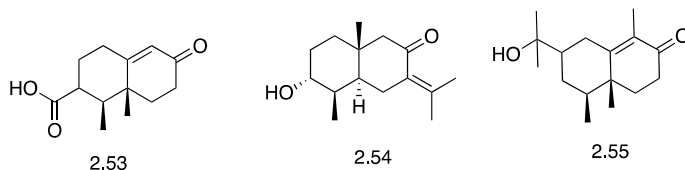


Figure 2.15: Sesquiterpene compounds isolated from *Tessaria* species

2.3.14 *Thalia geniculata* L. (Marantaceae)

Important phenolic metabolites like rosmarinic acid (**2.56**) and chlorogenic acid (**2.57**) were detected in the leaves of this plant ¹¹⁵, together with several phytosterols that have been reported to possess activity against *P. falciparum* and *L. donovani* ¹¹⁶. In our study, the crude extract showed good SI values, and a general improvement in the fractions is observed ($2.5 < SI < 3.9$).

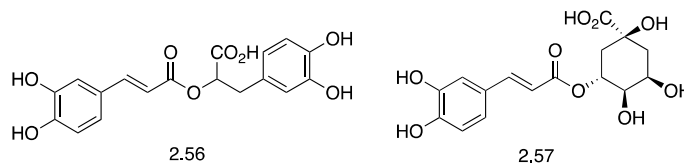


Figure 2.16: Main metabolites isolated from *Thalia geniculata*

2.3.15 *Trichilia adolfi* Harms (Meliaceae)

This genus was widely studied for its chemical contents, the most representative examples reported are trichilin-type limonoids (**2.58**) ¹¹⁷ that conserve the intact four-terpenoic ring system, mexicanolide-type (**2.59**) ¹¹⁸ that is characterized by A, B-*seco* rings and contain a δ -lactone D ring; the phragmalin-type limonoids (**2.60**) ¹¹⁹ that are highly oxygenated A-*seco* rings as well as the prieurianin-type compounds (**2.61**) ¹²⁰ that have modifications in the A, B-*seco* rings with the characteristic of a 5 membered-D ring. Regarding their biological activities, the earliest reports suggest the use of limonoids as bio-pesticides but recent studies are more oriented towards antibiotic¹²¹, cytotoxic¹²² and anti-cancer ¹²³ properties. Only *T. emetica* has been reported as leishmanicidal ¹²⁴. In our study extracts of the bark of *T. adolfi* has shown some activity towards *L. braziliensis*, but the chemical interest of this plant led us to a phytochemical investigation of its components that will be described in the next chapter.

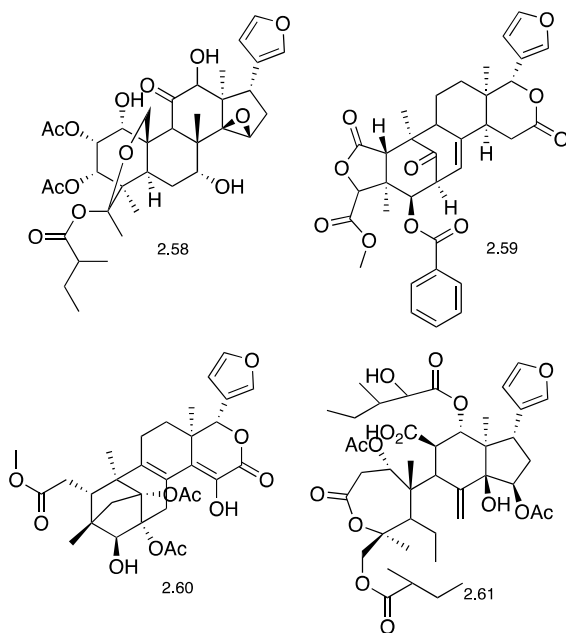


Figure 2.17: limonoids isolated from *Trichilia* species

2.3.16 *Vitex* spp. (Lamiaceae)

Chemical studies of this genus describe iridoid-type terpenes (**2.62**) as the main metabolites, possessing a broad spectrum of biological activities that include anti-tumor, anti-inflammatory and anti-bacterial activities¹²⁵. Also flavonoids (**2.63**) that were reported to possess leishmanicidal properties¹²⁶, besides lignans (**2.64**)¹²⁷ and caffeoylquinic acid derivatives (**2.65**) that show anti-oxidative properties¹²⁸. The IC₅₀ for fraction F3 shows a general improvement, but a deterioration of the calculated SI values. For this reason we did not continue with a chemical study of this extract.

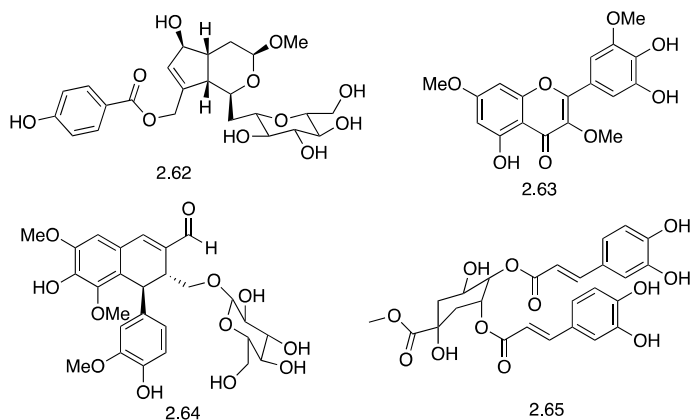


Figure 2.18: Metabolites isolated from *Vitex* species

2.4 Summary and conclusions

In summary, from 46 vegetable species collected were obtained 84 crude extracts that were evaluated in a first instance against two leishmania strains that gave us an overview of their potential. To classify these plants they were included into three groups according their biological activities: group 1 with the most active in two strains, group 2 with moderate active in two strains and group 3 with activity in only one strain. From this screening, the selected 16 vegetable species were subjected to chromatographic fractionation to obtain the main fractions. Further analysis of the crude extracts and the fractions in a broad battery of leishmania strains and the cytotoxic evaluation on HeLa cell cultures, enabled us to calculate the selectivity index (SI) value.

The SI values pointed us towards the most promising extracts to continue with the phytochemical analysis. Among the most active extracts, five species were selected for further studies, leaves from both species of the *Hyptis* genera: *H. brevipes* (entry **6**) and *H. mutabilis* (entry **7**), and leaves from *T. integrifolia* (entry **13**) as being the most active species. In addition, Rhizomes extract of *R. breviscapa* (entry **11**) and bark extract of *T. adolfi* (entry **15**), got our attention due to their extended use among Tacana people.

J. glabra, *S. dulcis*, *I. diffusa* and *T. geniculata*, even though they were very active species, were excluded from the study due to the lack of crude material.

As a result of the literature search, we had an overview of the chemical diversity and the medicinal potential of the active species studied here, as extensive studies in several species have given an impression of the chemical contents. However, phytochemical studies and biological evaluations of pure compounds are still insufficient, the reported compounds are in most of the cases not related with antiparasitic activities, which shows that this is a wide research field waiting to be explored.

Chapter 3: Natural Products with medicinal interest

In the chapters 1 and 2 medicinal plants were selected through a bioassay-guided study, and as a result three vegetable species that showed high selectivity for leishma strains (*H. brevipipes*, *H. mutabilis*, *T. integrifolia*) were selected. Two additional species were selected due to the extensive use among the Tacana people (*R. breviscapa*) and due its chemical interest (*T. adolfi*). From the crude extracts were isolated as many metabolites as possible through phytochemical techniques, and the structural elucidation was achieved by spectroscopic analysis. Finally, the pure metabolites were evaluate against parasites in our *in vitro* models.

3.1 Antiparasitic metabolites from *Hyptis brevipipes* and *Hyptis mutabilis* (Paper 2 and 3)

Hyptis genera (lamiaceae) are native and widely disseminated in America's continent, and with a pantropical distribution it is listed in some traditional pharmacopeias. Investigations of specimens collected in Mexico and Malasia reported a family of natural products with versatile biological activities, named the brevipolides¹²⁹⁻¹³⁰. These were originally isolated due to their cytotoxicity activity, in particular those listed as brevipolides (A-J)⁸⁴⁻⁸⁵. These compounds share a *cis*- α - β -unsaturated carbonyl system as part of a δ -lactone ring attached to a hydroxylated and esterified hexyl chain bearing a cyclopropane moiety. The brevipolides have several stereogenic centers in their skeleton, and the absolute stereochemistry of these chiral carbons was assigned as 6R, 1'S, 2'S, 4'S and 6'S by spectroscopic, chemical and synthetic methods. No other brevipolides with different stereochemistry have so far been reported¹³¹. On the other hand *H. mutabilis* belong to the same family (lamiaceae) and genus as *H. brevipipes*, showed a different chemical profile^{132 133}. Previous studies were focused on the

characterization of essential oils due their anesthetic properties ¹³⁴, while little work was done with pure compounds.

Our study (Figure 3.1) is the first report applying antiparasitic activity criteria to isolate active metabolites from *H. brevipes*, using the *L. amazonensis* strain for the bioguided isolation. The ethanolic extract yielded seven isolated compounds, among them four brevipolides H (**3.1**, 0.58%), G (**3.4**, 0.13%), C (**3.6**, 1.17%) and J (**3.5**, 0.11%); a catechol derivative (**3.2**, 0.66%); the three flavonoids chrysoptanol C (**3.3**, 0.08%), tomentin (**3.7**, 0.16%) and rutin (**3.8**, 0.07%) (Figure 3.1). These were characterized chemically by spectroscopic analysis, and their different antiparasitic profiles were determined by *in vitro* assays.

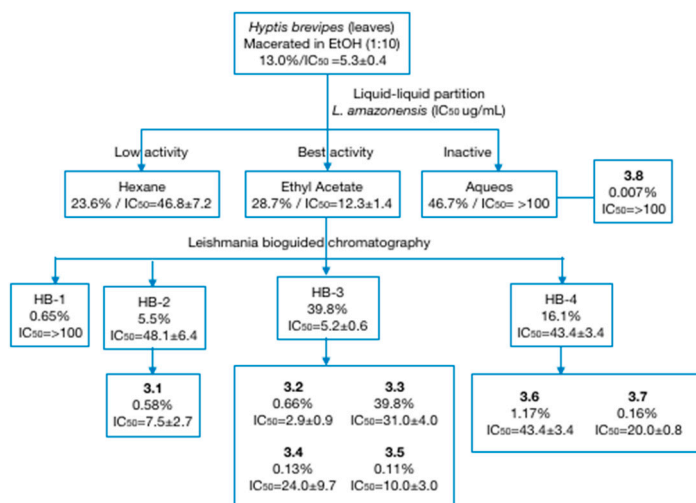


Figure 3.1. Bioguided fractionation of *Hyptis brevipes* against *L. amazonensis*, fractions and pure compounds yields are related to raw extract weight

From *H. mutabilis*, the most abundant metabolite that was obtained was identified as olguine (**3.9**), isolated as amorphous white solid (98.0 mg, 0.8% of the raw extract), and it was characterized fully by analysis of the NMR spectra and the MS data (Figure 3.2) ¹³⁵.

Biological evaluation of the pure, isolated compounds show that all brevipolides possess anti-parasitic activity and a moderate cytotoxicity. However, the most active metabolite isolated from *H. brevipes* was not a brevipolide, instead it is compound **3.2** which can be described as a catechol-type of metabolite. It was previously isolated from *Pletranthus sylvestris* (Lamiaceae family) for its antioxidant activity, but it turns out to be the most cytotoxic metabolite with good active against all parasites of our battery. The brevipolides showed a variable activity, as some strains are more sensitive than others. The flavonoids isolated are

potent against leishmania, but also highly cytotoxic. Olguine (**3.9**) isolated from *H. mutabilis*, was the most active compound in this investigation, it is strongly active against all parasite strains while being only modestly cytotoxic (44 ± 1.1 $\mu\text{g/ml}$).

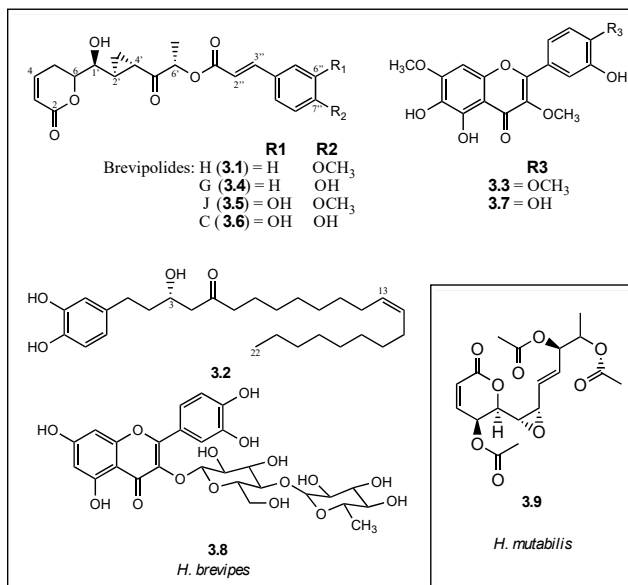


Figure 3.2: Natural products from *H. brevipes* and *H. mutabilis*

The calculation of the SI of the pure metabolites (Table 3.1) shown that compounds **3.1**, **3.2**, **3.5**, and **3.9** are the most selective for the leishmania strains. Good results were obtained for **3.1** in all leishmania strain ($2.9 < \text{SI} < 7.6$), while **3.5** was active only in *L.a.* ($\text{SI} 3.0$). The catechol-type metabolite **3.2** showed variable activity ($0.8 < \text{SI} < 5.8$), while olguine **3.9** by far was the most selective as well as potent compound with SI values almost comparable to Amphotericin B (with impressive SI values between $20.9 < \text{SI} < 88.0$ against leishmania strains). Olguine **3.9** also showed a SI of 44.0 against *T. cruzi*. This is the first report of antiparasitic activities of this compound.

Table 3.1: Selectivity index of metabolites from *H. brevipes* and *H. mutabilis*

Sample/Compounds	Leishmania strains					Parasites		
	<i>L.a</i>	<i>L.ae</i>	<i>L.b</i>	LbG	<i>L.lan</i>	<i>T.c</i>	<i>P.f</i>	<i>G.l</i>
Crude extract EtOH	3.3	1.4	1.3	1.6	1.1	1.2	1.0	0.3
Brevipolide H (3.1)	5.1	5.4	7.6	2.9	4.0	1.9	2.8	1.2
Catechol-type (3.2)	3.2	5.8	1.3	0.8	2.9	2.4	0.9	0.4
Chrysosplenol C (3.3)	0.8	1.2	0.9	1.1	0.9	-	-	0.9
Brevipolide G (3.4)	1.3	0.7	0.5	-	0.5	-	1.5	0.7
Brevipolide J (3.5)	3.0	1.7	0.6	0.5	1.1	0.5	2.0	1.6
Brevipolide C (3.6)	0.2	-	0.3	0.3	-	-	2.2	-
Tometin (3.7)	0.8	0.5	0.4	0.5	0.7	-	1.1	0.2
Olguine (3.9)	33.8	55.0	73.3	88.0	20.9	44.0	-	-
Miltefosine	2.7	5.6	1.9	1.1	3.5			
Amphotericin B	117.7	117.7	176.5	117.7	88.3			
Benznidazol						13.4		
Nifurtimox						14.2		
Quinine							10.0	
Tinidazol								4000.0

L.a.: *Leishmania amazonensis*, *L.ae.*: *L. aethiopica*, *L.b.*: *L. braziliensis*, LbG: *L. braziliensis native*, *L.lan.*: *L. lainsoni, native*, *T.c.*: *Trypanosoma cruzi*, *P.f.*: *Plasmodium falciparum*, *G.l.*: *Giardia lamblia*, EtOH: Ethanol.

3.2 Antiparasitic metabolites from *Tessaria integrifolia* (Paper 4)

In our approach to analyze the chemical composition of *T. integrifolia* we selected the Supercritical Fluid Extraction (SFE) as a “green” technique that allows the extraction of herbal chemical components with high quality, and that differs greatly from traditional organic solvent extraction¹³⁶. The main quality of the SFE process is that it avoids the extraction of chlorophylls, that is a problem for the fractionation procedures, and that it not leave any solvent residues. Furthermore, SFE uses carbon dioxide (CO₂) in super critical state as solvent, and CO₂ is considered as non-toxic, chemically stable, and is used in food and pharmaceutical industries¹³⁷.

3.2.1 Global yields of Supercritical fluid extraction (SFE) and ethanolic maceration (Et)

In the present study, we have compared two different types of extractions, SFE and Et. We have compared the yields and the anti-parasitic activity against *in vitro* leishmania strains (**Table 3.2**). Milled dried leaves (500 g) were extracted under several different SFE conditions (pressure, temperature) to produce SFE extract 1 - 4 (see **Table 3.3**). The best extraction yield was obtained at 2100 psi and 34 °C, with 15.5 g, 3.1%, **SFE1**. This is still lower compared to the **Et 1** extraction of the same material (21.5 g, 4.3 %). Notably, **SFE1** has a yellow oily consistence, different to **Et 1** that is a green pasta due the presence of chlorophylls. An important remark is the advantage of the use of SFE that do not extract chlorophylls that are undesired components for further fractionations. HPLC analyses gave us the relative amounts of the main metabolites present in the different extracts.

In addition, the biological activities of the different extracts were tested on promastigotes of *Leishmania amazonensis* (*L.a.*) and *Leishmania braziliensis* (*L.b.*), while their cytotoxicity was evaluated in HeLa cell cultures. The results show that all SFE extracts have similar antiparasitic potency in, strain *L.b.*, which was shown to be more sensitive than *L.a.* Significant difference was observed in the cytotoxic effect. In general, the **SFE** extracts show lower cytotoxic activity than the ethanol extract **Et 1**.

Table 3.2: Yields of extraction at different conditions, and biological activity of crude extracts from *T. Integrifolia* leaves

Type of extraction	Conditions	Yield (%)	IC ₅₀ ug/mL				DL ₅₀ ug/mL
			Cutaneous Infection		Muco-cutaneous Infection		Cytotoxicity
			L.a.	SI	L.b.	SI	HeLa
SFE1	Pressure: 2100 psi T°: 34°C, 6H	3.1	36.5±1.2	5.5	21.6±5.6	9.2	>200
SFE2	Pressure: 1300 psi T°: 34°C, 6H	2.7	39.1±1.7	5.1	12.6±2.0	15.9	>200
SFE3	Pressure: 2100 psi T°: 55°C, 6H	2.8	45.5±3.7	1.9	33.6±4.5	2.6	87.5±7.0
SFE4	Pressure: 1300 psi T°: 55°C, 6H	2.2	36.8±0.1	5.4	25.3±0.3	7.9	>200
Et1	Maceration: Ethanol, r.t., 3 days	4.3	54.2±9.6	2.2	31.6±14.8	3.8	119.3±24.9
CAT			21.9±2.2	1.3	19.4±4.2	1.5	29.5±5.8
Miltefosine			10.3±3.3	1.6	9.6±2.8	1.7	16.6±5.3
Amphotericin B			0.3±0.1	33.3	0.2±0.04	50	>10

After chromatographic separations of **SFE1** yielded four main fractions, named (according to polarity from least to most polar) **F1 - F4**. Different amounts were obtained in the four fractions and they have different biological activities (**Table 3.3**). The most abundant fraction **F1** (6.3 g, 41% of the crude extract) showed the lowest antiparasitic activity and moderate cytotoxicity ($DL_{50} > 50 \mu\text{g/mL}$). The most active fraction was **F2** that showed good antiparasitic activity ($IC_{50} < 50 \mu\text{g/mL}$) and a low cytotoxicity ($DL_{50} > 100 \mu\text{g/mL}$). SI calculations gave a value of 8.7 and 8.5 for *L.a* and *L.b.*, respectively, for **F2**, which can be considered as promising for a medicinal herbal extract. **SFE1** and its fractions were selected for further HPLC-MS and phytochemical analysis.

Table 3.3. Yields of main fractions of SFE 1 and Et 1, and comparison of biological activity

		Yield	L.a	SI	L.b	SI	HeLa	
6	SFE1	Crude	36.5±1.2	5.5	21.6±5.6	9.2	>200	
		F1	41	>100	0.5	79.8±2.6	0.7	57.7±3.4
		F2	12.5	19.6±1.3	8.7	20.1±3.1	8.5	171.9±11.1
		F3	4.6	16.9±2.1	1.5	9.1±2.5	2.9	26.8±1.6
		F4	11	12.5±2.4	5.6	7.9±1.9	8.9	70.8±0.1
7	Et1	Crude	4.3	54.2±9.6	2.2	31.6±14.8	3.8	119.3±24.9
		F3	43	22.5±1.4	3	9.7±1.3	7	68.0±5.1
CAT			21.9±2.2	1.3	19.4±4.2	1.5	29.5±5.8	
Miltefosine			10.3±3.3	1.6	9.6±2.8	1.7	16.6±5.3	
Amphotericin B			0.3±0.1	33.3	0.2±0.04	50	>10	

Biological activity values in $\mu\text{g/mL}$, *L.a*: *Leishmania amazoniensis*, *L.b*: *leishmania braziliensis*, yields in percentage

3.2.2 Chemical identification of metabolites

The chemical composition of *T. integrifolia* vary from region to region, and studies in different countries report several related compounds (**Table 3.4**). Most of the studies agree to a high presence of oxygenated sesquiterpenes such as the eudesmane-type and cuauthemone-type, besides flavonoids. On the other hand, the presence of more polar compounds like caffeoyl quinic acid derivatives is of particular interest due their anti-oxidative properties.

From **SFE1**, 11 pure compounds were isolated by chromatographic techniques, and subsequently identified by NMR and HMRS techniques (**Figure 3.3**). Seven were identified as eremophilane-type derivatives (1 new and 6 previously reported), two were reported flavonoids and two reported triterpenes. Previous

GC-MS studies on *Tessaria* genus showed that the crude extract consist of about 32% of oxygenated monoterpenes, 36% of oxygenated sesquiterpenes and 17% of sesquiterpene hydrocarbons.¹⁰⁸ In this study, the following oxygenated metabolites were isolated: **3.10** 2,11-dihydroxyeremophil-9-en-8-one¹³⁸, **3.11** 11-hydroxy-valenc-1(10)-en-2-one¹³⁹, **3.12** 2-oxo-hinesol¹⁴⁰, **3.13** described in table 3.6, **3.14** artemetin¹⁴¹, **3.15** (-)-eremophila-9-en-8 β ,11-diol¹⁴², **3.16** ligudicin C¹⁴³, **3.17** quercetogetin¹⁴⁴, **3.18** dehydrofukinone¹⁴⁵, **3.19** α -amyrin¹⁴⁶, and **3.20** α -amyrone¹⁴⁷.

Table 3.4: Chemical compounds found in *Tessaria integrifolia*

Specie	Author	Country	Compounds	N° of compounds
	Ono (2000) ^{112a}	Peru	Eudesmane –type	5
	Jakupovic (1985) ¹¹¹	Costa Rica	Cuauthemone-type	9
	Guerreiro (1990) ¹⁴⁸	Argentina	Flavonoids	2
	Silva-Correa (2018) ¹⁴⁹	Peru	Eudesmane-type	1
			Eudesmane-type	5
<i>Tessaria integrifolia</i>			Phenolic compounds	11
<i>Ruiz et Pav.</i>	Ono (2000) ^{150b}	Peru	Flavonoids	4
			Caffeoyl quinic acids derivatives	5
	De Feo, (1990) ¹⁵¹	Peru	Caffeoyl quinic acid derivatives	2
			Flavonoids	3
	Eto, (2008) ¹⁵²	Peru	Flavonoids	
<i>Tessaria Absinthioides</i>	Bholmann (1977) ¹⁰⁹	Chile	Eremophilane-type	7
			Triterpene	1
	Kurina-Sanz, (1997) ¹⁵³	Argentina	Eudesmane-type	

Fractions of **SFE1** have been subjected to purifications on SiO₂ chromatographic column (CC) using mixtures of Hex: EtOAc (1:0 to 8:2) as eluent solvents. The four main fractions obtained were subjected to CC for further purifications. From F1 were obtained compounds **3.18** (186.0 mg, 1.21% from the crude extract) and **3.20** (98.0 mg, 0.64%). In fraction F2 only one compound was present (according to NMR spectra) and this was identified as **3.16** (1.9 g, 12.5% of the crude extract). Further purifications of F3 on CC gave compound **3.19** (30.0 mg, 0.19%). CC of F4 eluted with Hep: EtOAc gradient (8:2 to 2:8) gave compounds **3.15** (4.9 mg, 0.03%) and **3.16** (0.9 g, 5.88%) as the least polar compounds. In a Hep: EtOAc (6:4) gradient system we obtained fractions containing compounds **3.13**, **3.10**, **3.11** and **3.12**, and further purification of these by preparative-TLC plate (toluene: EtOAc, 6:4, elution system) afforded **3.13** (68.0 mg, 0.44%), **3.10** (35.1 mg, 0.22%), **3.11** (25.2 mg, 0.16%) and **3.12** (35.5 mg, 0.22%). Finally, in the

system Hep: EtOAc (2:8) were obtained fractions containing compounds **3.14** and **3.17**, that after preparative-TLC plate purification (toluene: EtOAc, 4:6, elution system) afforded **3.14** (87.9 mg, 0.56%) and **3.17** (91.0 mg, 0.59%).

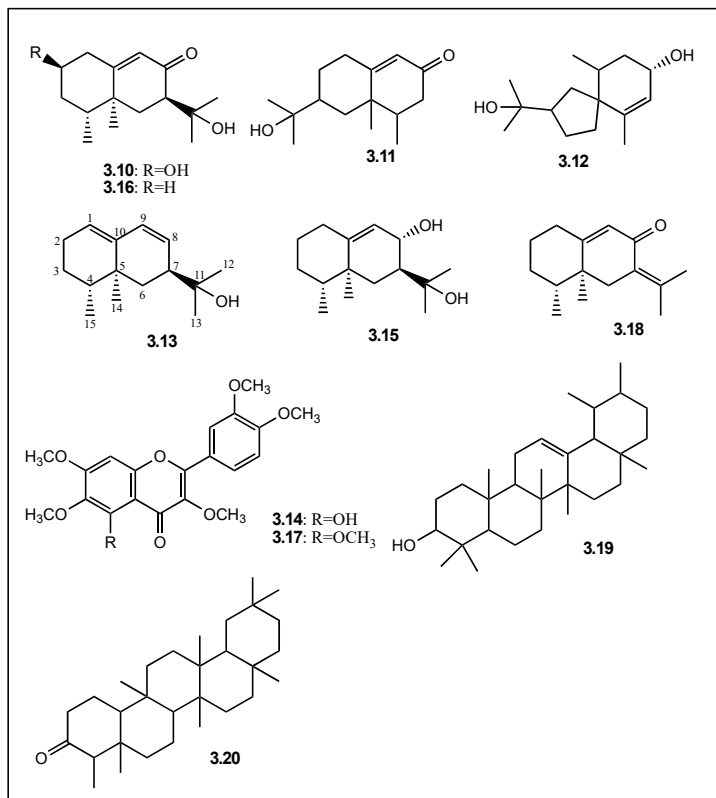


Figure 3.3: Metabolites isolated from *T. integrifolia*

3.2.3 Metabolite quantification in extracts SFE1 and Et1

The pure compounds were analyzed by HPLC-MS (Figure 3.4), and the retention times (t_R) were compared with the content in **SFE 1**, **Et1** and fractions to construct a chemical profile of metabolite content on each sample **Table 3.5**. In the HPLC-MS analyses were recognized signals at t_R 2.63 min (**3.11**, **3.12**), 12.80 min (**3.16**) and 21.67 min (**3.18**), which were the most abundant compounds. **3.16** was recognized as ligucidin C and is the most abundant metabolite Its presence in the SFE1 is 62.7% while in Et1 it only reaches 43.2%. The contents of compounds **3.11**, **3.12** and **3.18** vary in both samples, and suggests that maceration extraction

is more efficiently for those three compounds. Fractions obtained from **SFE1** were used to construct the polarity profile, with F1 as the least polar fraction shown to be reach in triterpenoids. F1 also has a high content of compound **3.16**. The medium polar fractions F2 and F3 have essentially the same metabolites present in the fraction F3 of Et 1, which is the medium polar fraction of Et 1. While F4, that should contain the most polar components, also contain significant amounts of less polar metabolites.

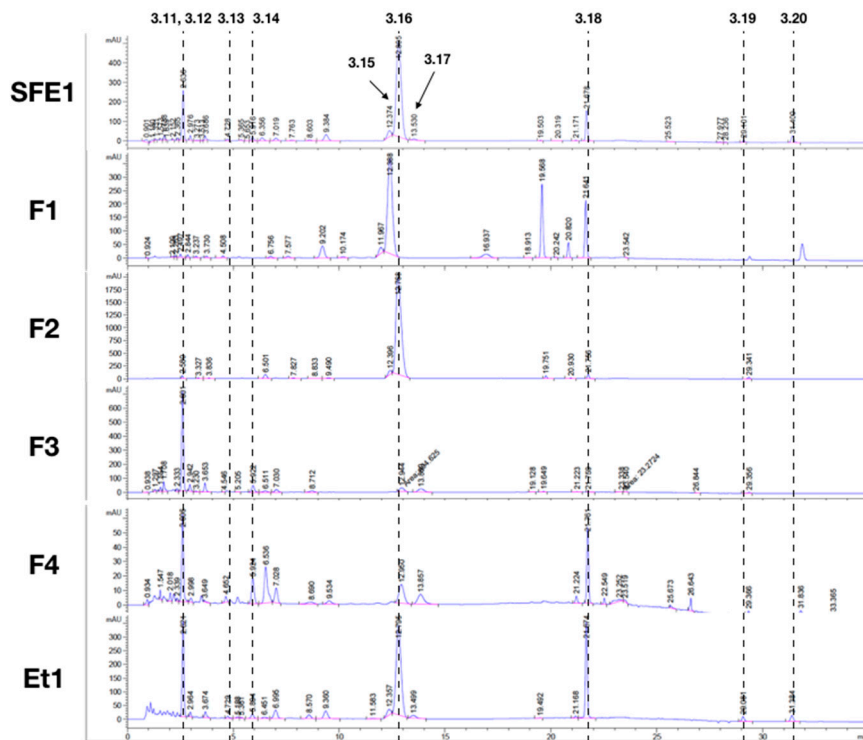


Figure3.4: Comparative LC-MS spectra of crude and fraction

Table 3.5: HPLC-MS analysis of metabolite content in SFE1 and Et1 extracts

Compound	Molecular Formula	RT (min)	Yield (%)						Et 1	Et-F3
			SFE1	F1	F2	F3	F4			
3.10	C ₁₅ H ₂₄ O ₃	1.73	0.68	-	-	4.50	-	0.45	0.35	
3.11, 3.12	C ₁₅ H ₂₄ O ₂	2.63	11.70	-	-	54.33	17.84	15.75	14.18	
3.13	C ₁₅ H ₂₄ O	4.72	1.18	-	-	5.87	8.03	1.19	1.06	
3.14	C ₂₁ H ₂₂ O ₈	5.91	1.26	-	2.18	1.45	14.41	0.56	0.23	
3.15	C ₁₅ H ₂₆ O ₂	12.37	2.87	48.52	2.18	-	-	1.96	2.22	
3.16	C ₁₅ H ₂₄ O ₂	12.80	62.71	-	90.73	6.69	11.30	43.25	60.64	
3.17	C ₂₀ H ₂₀ O ₈	13.53	0.69	-	-	6.13	7.25	1.34	1.71	
3.18	C ₁₅ H ₂₂ O	21.67	7.26	11.02	1.27	0.28	15.97	17.33	0.03	
3.19	C ₃₀ H ₄₈ O	29.10	0.56	0.72	0.55	0.78	0.88	1.19	1.27	
3.20	C ₃₀ H ₅₀ O	31.40	2.35	4.98	-	-	1.83	1.60	2.19	
TOTAL			91.26	65.24	96.91	80.03	77.51	84.62	83.88	

3.2.4 MS fragmentation analysis of SFE 1

An analysis of fragmentation pattern of pure compounds (**Table 3.6**) showed that common fragments generated follow a pattern that is illustrated in **Figure 3.5**. The main ion observed at 253 m/z [M+1] (**3.10**) may lose an oxygen in one of the two positions and generate two fragments of 237 m/z [M+1] (**3.16**) and 235 m/z [M+1], followed by a loss of 28 m/z and generate a remaining fragment at 207 m/z [M+1]. While the molecular ion at 237 m/z [M+1] (**3.16**), may follow the loss of -44, -32 or -46 m/z . but principally the loss of water (-18 m/z), giving a fragment of 219 m/z [M+1] (**3.18**). From **3.18** is observed a fragment at 203 m/z [M+1] that also is observed in **3.13** and **3.15**.

Table 3.6: LC-MS analysis of SFE 1

Retention Time	N°	Molecular formula	MS [M+1]	Fragmentation			Identification
1.716	3.10	C ₁₅ H ₂₄ O ₃	253.1600	235.2	207.2		ID NMR, 2D NMR, HRMS, LC-MS
2.152		C ₁₉ H ₃₂ O ₂	293.2	253.1	235.2	219.2 195.1	LC-MS
2.353		C ₁₅ H ₂₄ O ₃	253.1		235.2	219.0	LC-MS
2.652	3.11	C ₁₅ H ₂₄ O ₂	237.1848	219.2	201.2	177.2	ID NMR, 2D NMR, HRMS, LC-MS
	3.12	C ₁₅ H ₂₄ O ₂	237.1849	219.2	201.2		ID NMR, 2D NMR, HRMS, LC-MS
2.976		C ₁₈ H ₂₆ O ₂	275.2		235.2	219.2 207.2	LC-MS
3.701		C ₁₅ H ₂₄ O ₂	237.2			219.2 203.2	LC-MS
4.728	3.13	C ₁₅ H ₂₄ O	221.1900	203.2	185.0	177.1	ID NMR, 2D NMR, HRMS, LC-MS
5.444		C ₁₅ H ₂₄ O ₂	237.1	205.2	193.2	149.2	LC-MS
5.916	3.14	C ₂₁ H ₂₂ O ₈	403.1392	359.1	343.1	291.2	¹ H NMR, 2D NMR, HRMS, LC-MS
6.356		C ₁₅ H ₂₆ O ₃	255.2		231.2	219.1 214.1	LC-MS
7.019		Unknown	375.1	257.2	235.2	217.1	LC-MS
9.426		Unknown	217.2	179.2	109.1		LC-MS
12.284	3.15	C ₁₅ H ₂₆ O ₂	239.2008	221.2	203.2	179.1	ID NMR, 2D NMR, HRMS, LC-MS
12.805	3.16	C ₁₅ H ₂₄ O ₂	237.1850	219.2	191.2	179.1 149.1	ID NMR, 2D NMR, HRMS, LC-MS
13.53	3.17	C ₂₀ H ₂₀ O ₈	389.1227	329.0	266.1		ID NMR, 2D NMR, HRMS, LC-MS
21.678	3.18	C ₁₅ H ₂₂ O	219.1744	203.2	195.1		ID NMR, 2D NMR, HRMS, LC-MS
22.256		C ₁₅ H ₂₆ O ₂	237.2	219.2	205.2		LC-MS
22.522		C ₁₇ H ₃₀ O ₃	282.2	237.2	219.2	205.2 149.1	LC-MS

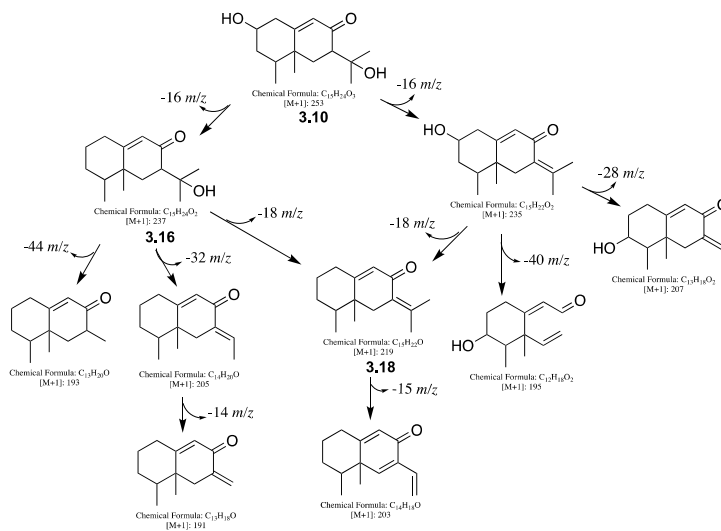


Figure 3.5 Fragmentation patterns of the terpenoids

3.2.5 Biological evaluation of compounds 3.10-3.20

In addition to *L. amazoniensis* and *L. braziliensis*, pure compounds were also evaluated against promastigotes of *L. aethiopica*, *L. lainsoni* and *T. cruzi*, as well as against trophozoites of *G. Lamblia*. The cytotoxicity was evaluated in *Raw* cell culture. Results shown that sesquiterpenoids (**3.10 – 3.13, 3.15, 3.16, 3.18**) show variable behavior in our *in vitro* models, the compound **3.16** being the most active and selective against leishmania parasites and *T. cruzi* with selective index between $2.2 < SI < 5.1$. Compounds **3.15** and **3.18** showed activity only in *L.Lan* strain and *T.cruzi*. Flavonoid **3.17** shown high selectivity in all strains ($2.6 < SI < 4.1$), while the triterpenoids were inactive in our *in vitro* models. *G. lamblia* was insensitive to all compounds tested here (**Table 3.7**)

Table 3.7. Biological Activity of metabolites isolated from *Tessaria integrifolia*.

Comp.	Cutaneous Infection				Muco-cutaneous Infection				DL ₅₀				
	IC ₅₀ ug/mL Lma	SI	L.ae	SI	IC ₅₀ ug/mL M2904	SI	L.lan	SI	T. cruzi	SI	G. Lambliia	SI	Raw
3.10	35.6±0.9	1.0	17.3±1.0	2.1	22.1±3.4	1.6	35.6±6.8	1.0	16.6±0.1	2.1	60.0±27	0.6	36.0±2.6
3.11	>50.0	-	>50.0	-	>50.0	-	>50.0	-	>50.0	-	80.0±27	0.8	62.4±16.7
3.12	>50.0	-	>50.0	-	>50.0	-	>50.0	-	>50.0	-	73.0±10.2	1.1	83.8±0.03
3.13	28.2±3.1	0.6	18.4±4.1	0.9	14.9±3.4	1.2	12.0±0.3	1.5	19.7±0.9	0.9	49.0±4.1	0.4	18.0±2.6
3.14	28.7±4.1	1.9	28.4±8.3	1.9	20.8±1.4	2.6	14.9±5.7	3.7	21.2±1.4	2.6	65.0±26	0.8	55.5±6.6
3.15	18.7±0.9	1.4	14.0±3.5	1.9	10.0±0.7	2.7	6.9±0.4	4.0	5.8±0.9	4.7	30.0±1.1	0.9	27.8±6.7
3.16	22.3±0.3	3.4	34.6±3.1	2.2	22.0±1.1	3.4	15.0±5.1	5.1	17.1±0.9	4.4	62.1±8.2	1.2	76.2±4.6
3.17	11.3±2.9	3.2	8.9±2.3	4.1	14.2±4.4	2.6	11.7±0.9	3.1	9.7±1.7	3.8	68.0±29	0.5	27.5±7.7
3.18	24.8±2.0	1.4	10.5±0.7	3.5	19.1±0.2	1.9	10.3±0.3	3.5	5.8±0.7	6.2	74.1±30	0.4	36.4±4.7
3.19	>50.0	-	>50.0	-	>50.0	-	>50.0	-	>50.0	-	>100.0	-	31.7±2
3.20	>50.0	-	>50.0	-	>50.0	-	>50.0	-	>50.0	-	>100.0	-	22.3±2.5
CAT	21.9±2.2	1.8	18.5±2.7	2.1	19.4±4.2	2.1	22.1±2.8	1.8	12.6±2.5	3.2	-	-	40.5±1.9
Miltefosine	10.3±3.3	2.8	<3.1	9.6	9.6±2.8	3.1	4.8±0.9	6.4	-	-	-	-	29.8±9.1
Amphotericin B	0.3±0.1	46.0	0.3±0.1	46.0	0.2±0.04	69.0	>0.6	23.0	-	-	-	-	13.8±2.7
Benznidazole	-	-	-	-	-	-	-	-	18.0±6.6	4.1	-	-	74.7±9.1
Nifurtimox	-	-	-	-	-	-	-	-	1.4±0.6	6.6	-	-	9.2±0.7
Timidazole	-	-	-	-	-	-	-	-	-	-	0.3±0.1	-	-

3.3 Limonoid compounds from *Trichilia adolfi* (Paper 5 and 6)

Bolivian biodiversity includes plants from the *Trichilia* genus (Meliaceae), a tropical genus widely disseminated around the world, which has reported more than 472 names of species only in this genera⁸³. In Bolivia have been reported three species used in TM: *T. emetic*, *T. pleeana* and *T. adolfi*¹¹. Among the traditional uses in the region, this genus is used to treat liver, lung and kidney pain¹⁰. The Brazil Meliaceae family has been used in folk medicine as anthelmintic, anti-inflammatory, antiparasitic and immunomodulatory, among others¹²⁴. As well as in several African tribes, trees from this family have medicinal and non-medicinal purposes, such as ornamental, for furniture and for household implements, while the decoction of leaves, roots or bark can be used to treat several conditions or diseases, such as digestive infections, eye infections, urinary infections, wound healing, malaria, teniasis and pneumonia¹⁵⁴.

The biological and pharmacological properties have been reviewed by Longhini et al.¹²⁴, Garg¹⁵⁵, Curcino-Vieira, et al.¹⁵⁶, Komane et al.¹⁵⁴. More recently this genus has been studied for their inflammatory^{157 158} and cytotoxic properties^{159 119 160}. Some report strong activity in mouse lymphoma cell¹⁶¹ as well as good selective for proteins involved in oncogenesis and chemotherapy resistance¹⁶², giving possibilities as new anti-cancer agents¹²³.

Studies of the chemical contents has received great attention, mainly because of the advance of analytical techniques (NMR, MS, X-ray diffraction) that allow elucidating such complex structures found in this family. The secondary metabolites consist mainly of limonoids^{124 155 163}, which are a huge family of highly-oxygenated terpenoids. They are named tetranortriterpenoids and have versatile structures. Examples of limonoid-type compounds were described above (section 2.3.15).

3.3.1 Isolation of compounds 3.21 – 3.29

In the present study were found 9 novel limonoid compounds from the bark of *Trichilia adolfi*, the structural elucidation was made applying an extensive analysis of 1D and 2D NMR data (figure 3.6).

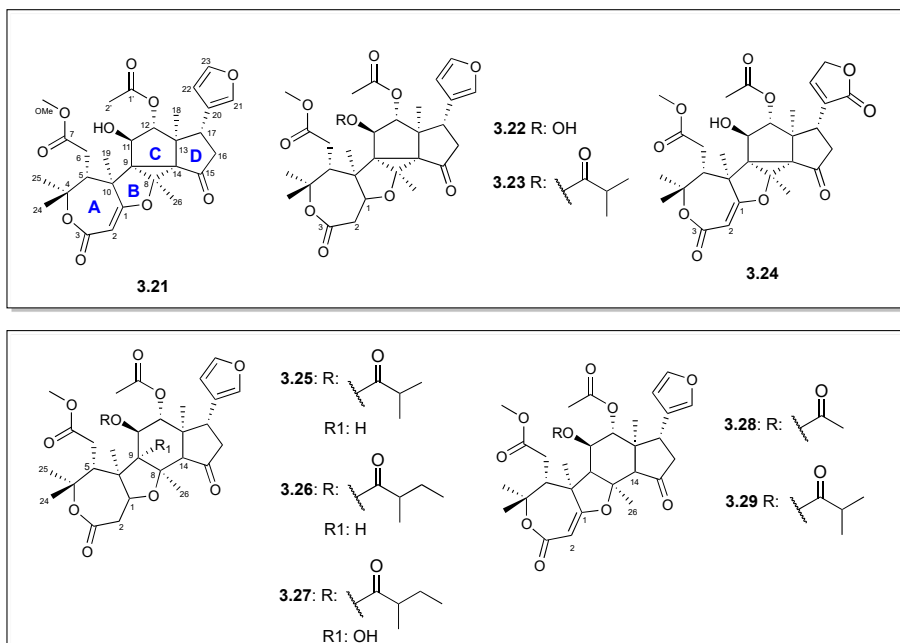


Figure 3.6: Limonoids isolated from *Trichilia adolfi*

From the milled dry bark of *T. adolfi* (1000 g), was obtained a crude ethanolic extract (22.0 g, 2.2 % of bark weight), such extract was subjected to liquid/liquid extraction with water: *n*-heptane, yielding 3.1 g (14 % of the crude extract), followed by chloroform extraction 6.4 g (29 % of the crude extract), the remained aqueous phase was freeze-dried to yield 12.5 g (57 % of the crude extract).

The CHCl_3 fraction was subjected to SiO_2 gel column chromatography (1:30, w/w) eluted with mixtures of *n*-heptane: EtOAc (from 8:1 to 2:8 v/v). TLC analysis provided four main pools according to polarity from least to most polar: F1 (2.5g, 39.1% of CHCl_3 extract), F2 (0.27g, 4.2%), F3 (0.77g, 12.3%) and F4 (0.68g, 10.6%). Further fractionations were performed with fractions F1, F2 and F3 using a semi-preparative HPLC (Vikram, A. et al 2007)¹⁶⁴ with the binary solvent system that includes solvent A, composed of 3mM of formic acid and solvent B, acetonitrile. The elution was performed in gradient mode, starting with 85% of solvent A, followed by a linear decreasing to 77% in 5 min, a second linear decrease to 74% at 25 min, further reduced to 60% at 30 min, finishing at 54% at 45 min. The flow rate was kept at 4.7 ml/min. All samples were filtered through 0.25 μm membrane and detected at 210 nm at 25°C. From F1 was purified **3.23** (30.0 mg, t_R = 15.6 min, 0.14% of raw extract). F2 was subjected to CC column eluting with Hep: EtOAc (8:2, v/v, isocratic) afforded crude **3.21** (50.5

mg, 0.22% of raw extract, re-crystallized in methanol: water 1:2) further purified by HPLC (16.1 mg, t_R = 10.7 min), from the same fraction were purified **3.26** (5.1 mg, t_R = 15.35 min, 0.023% of raw extract) and **3.24** (4.9 mg, t_R = 6.63 min, 0.022%). Fraction F3 was chromatographed on SiO₂ open column, using a mixture of Hep: EtOAc (6:4, v/v, isocratic) to obtain two sub-fractions both purified by HPLC to afford from F3.1 compound **3.22** (5.0 mg, t_R = 9.85 min, 0.022% of raw extract) and **3.25** (3.0 mg, t_R =12.3 min, 0.013% of raw extract) and from F3.2 compound **3.27** (6.3 mg, t_R =18.3 min, 0.028% of raw extract). Finally, F4 was directly applied to HPLC and allow the isolation of **3.28** (12.0 mg, t_R = 15.8 min, 0.054% of raw extract) and **3.29** (14.6 mg, t_R = 18.0 min, 0.066% of raw extract).

3.3.2 Structural elucidation of compounds 3.21 – 3.29

Complete elucidation of compounds **3.21** to **3.29** required the application of NMR and MS techniques, trivial names also were proposed by us as Trichilianone A – D (**3.21** – **3.24**) for those compounds that beared a cyclopropane ring and trichilone A – E (**3.25** – **3.29**) for those that did not have such modification in their structure. Detailed analysis of their spectroscopic data is in manuscripts **5** and **6**.

3.3.3 Hypothetical biosynthesis

A study of biogenesis of these limonoids was conduct, based on the evidence found in the literature of chemical reactions related to the isolated compounds.

In a possible biosynthetic pathway, we propose that the compound **3.21** (**trichilianone A**) has as precursor an azadirone-type derivative (Intermediate 1, **Figure 3.7**)^{165 166}. If **Int. 1** is subjected to an opening reaction of the B-ring through a Baeyer-Villiger-type oxidation^{167 168 169}, followed by rotation about C-9/C-10 bond is possible the formation of the tetrahydro furan ring via a Michael addition and a hydroxyl group formed to the unsaturated (**Int. 2**)¹⁷⁰. It is reasonable to assume that the biosynthetic pathways leading to the trichilianones share critical steps with those producing the hortolides (Hortiolide A, **Figure 3.7**). The major difference between the two systems is that the cyclopentanone ring of the former has been subjected to a Baeyer-Villiger-type oxidation in the latter, while the same type of oxidation has introduced the ϵ -lactone functionality in the trichilianones between C-3/C-4 (**Int. 3**)¹⁶⁹. Some reports include mono- and di-oxygenases as the case of styrene epoxidase that describe two pathways, the first involve epoxydation in the first step, while the second pathway starts with the

dihydroxylation of a double bond, in both cases the following steps may lead the reduction of the α,β unsaturated ϵ -lactone ring and the esterification of C-11 to produce compounds **3.25** – **3.29**¹⁷¹. While the mechanism for the formation of the cyclopropane ring, that trichilianones and hortiolides share, is not understood in the limonoids, it has been studied in diterpenoids from the thujane group.¹⁷² Especially in the trichilianones this biosynthetic step introduces a considerable amount of ring tension, and a deeper insight of how this is achieved would require studies involving labeled precursors. Nevertheless, it is important to make a hypothesis about the cyclopropane ring formation, as such reactions require an stable tertiary cation that in our case may be the enol form of C-15 that attacks C-9 and forms the cyclopropane ring (**Int. 4**) forming compound **3.21**¹⁷³.

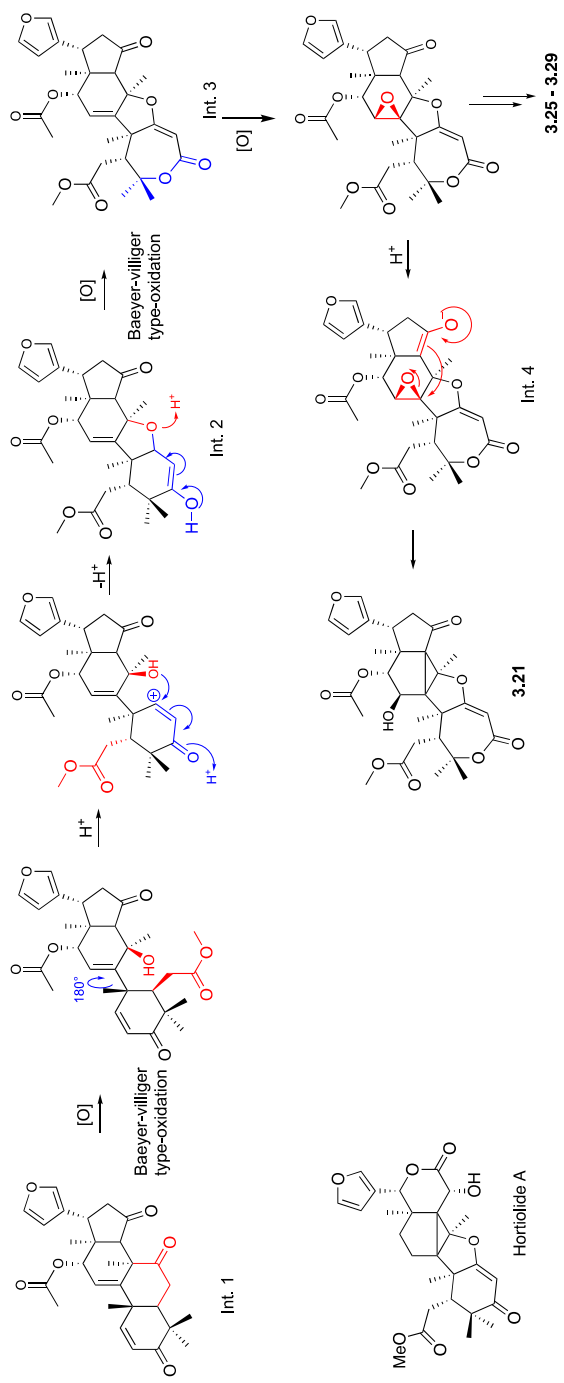


Figure 3.7: hypothetical biosynthetic pathway for trichiliane-type compound

3.4 Natural products with antiparasitic activity from *Renealmia breviscapa* (Paper 3)

R. breviscapa is widely used in the Tacana culture, mainly because the rhizomes can be used as food dye. It belongs to the same family of *Curcuma longa*, a widely studied plant with several biological properties, including antiparasitic.¹⁰³

In our study, the air-dried and powdered rhizomes (382 g), were extracted with ethanol (2.5 L) the crude extract was concentrated to dryness (46.2 g, 12.1% of dried raw material). The extract was then dissolved in water (0.25 L) and subjected to L/L extraction with different solvents with increasing polarity, petroleum ether (1.7 g, 3.6% of crude extract), chloroform (27.80 g, 60.1% of crude extract), ethyl acetate (12.6 g, 27.3% of crude extract), then the organic solvents were evaporated under reduced pressure. The chloroform extract (27.80 g) was repeatedly subjected to Sephadex LH-20 column chromatography using as elution system chloroform: methanol (1:1, v/v), then each fraction obtained was subjected to chromatographic column on silica gel, eluted with mixtures of (CHCl₃: MeOH, 98:2 to 95:5) to isolate compounds **3.30** (6.9 g, 25.1%), **3.31** (4.3 g, 15.6%) and **3.32** (2.8 g, 10.1%) as yellow powders, while compound **3.33** (0.05 g, 0.17%), **3.34** (0.035 g, 0.12%) and **3.35** (0.01 g, 0.035%) were obtained as white powders. NMR experiments allow the identification of three 5C-curcumin derivatives **3.30**, **3.31** and **3.32**, together with its precursor **3.33**, **3.34** and a bisabolane terpene **3.35**¹⁷⁴ as main metabolites. Their biological activities are reported in **table 3.8**, *Lb* was found to be more sensitive to 5C-curcuminoids, while **7** was more active against *La* than *Lb*. **3.33** and **3.34** were not active in our study.

Table 3.8: Cytotoxicity, antiparasitic activity and selective index (SI) of isolated compounds

Compound	DL ₅₀		IC ₅₀		SI	Gi	SI
	HeLa	La	SI	Lb			
1	38.0±1.0	1.4±0.2	27.1	0.6±0.02	63.3	NE	-
2	11.0±1.5	10.0±1.4	1.1	3.6±1.0	3.1	40.0±3	0.3
3	21.0±0.9	23.0±1.3	0.9	4.3±1.0	4.9	40.0±0.1	0.5
4	12.5±1.1	11.0±1.5	1.1	2.0±0.7	6.3	13.3±5.0	0.9
7	152.0±68	14.8±4.1	10.2	38.8±4.1	3.9	181.0±16.0	0.8
Amphotericin B	35.3±18.7	0.3±0.1		0.3±0.1			
Tinidazole	>100					0.3±0.1	

La: *L. amazonensis*, Lb: *L. braziliensis*, Gi: *Giardia lamblia*, DL₅₀ and IC₅₀ values in ug/mL

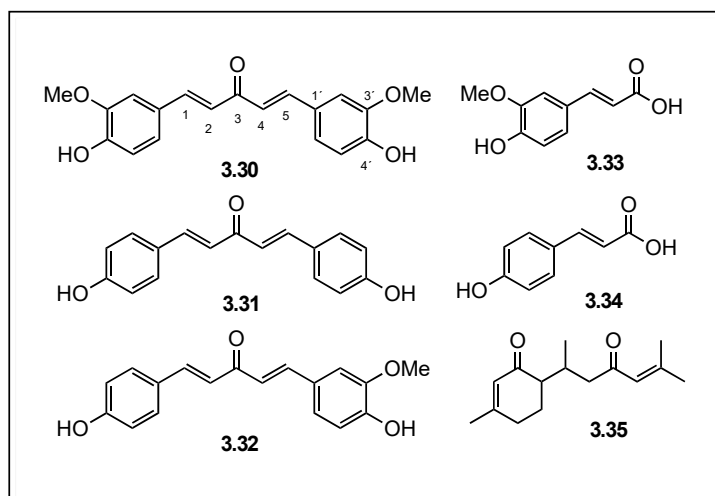


Figure 3.8: Isolated metabolites from *Renealmia breviscapa*

3.4 Summary and conclusions

For the selected plants, phytochemical analyses yielded pure metabolites and the biological evaluation of these pure compounds helped us to understand the complexity of the traditional medicine. The systematic research with the cooperation of the Tacana people, and using anti-parasitic activity as criteria to select plants, afforded several compounds with diverse chemical structures. Further studies of these compounds are necessary to consolidate the use of medicinal herbs to treat endemic tropical diseases.

From *H. mutabilis* and *H. brevipes*, the main compounds show interesting biological activity *in vitro*, and gave scientific support for their use to treat leishmania diseases. The implementation of green techniques to extract the main active compounds from *T. integrifolia*, enabled us to increase the selectivity compared to traditional solvent extraction. The chemical contents were measured by HPLC-MS, aided by an analysis of the fragmentation pattern. The purified compounds show good selective index values, and their potential usefulness will be further analysed.

Our interest to explore chemical diversity lead us to investigate in the bark of *T. adolfi*, which yielded nine new limonoid-type compounds. Four contain an unusual cyclopropane moiety, and even though they were not responsible for the anti-parasitic activity of the crude extract, the medicinal potential is still unexplored.

Concluding remarks

Traditional medicine is a vast source of knowledge, and the chemistry involved behind the use of medicinal plants has only been investigated to a small extent. As we saw in Paper 1 and 4, the cooperation of native organizations with the scientific community was key to contributing with the knowledge of medicinal plants and their uses. With this work we want to encourage to put attention and develop programs to keep such knowledge. The plants included in this work have a great pharmacological value that was not explored yet.

From the 38 species collected, and after our biological evaluation, we observed that the most active species had previous Tacana uses as an antiparasitic agent, these plants were identified as *Hyptis brevipes*, *Hyptis mutabilis* and *Tessaria integrifolia*.

H. brevipes and *H. mutabilis* are good antiparasitic medicinal plants, in our study the main metabolites from both species were identified and characterized. This also gave us the insight that they at least partly are responsible for the biological activity. However, more detailed studies are needed to support the biological activity data. In *H. mutabilis* was found the chemical compound with the highest biological activity in this study (olguine), which combined the high activity with low cytotoxicity. These preliminary data, especially those for olguine, has to be supported by more extensive biological experiments.

Tessaria integrifolia was also selected due the high antiparasitic activity and broad use among the Tacana people to treat leishmania sores. It proved to be a good antiparasitic medicinal plant, and the biological activities showed that this is attributed to the high content of sesquiterpenes. Our study identified the majority of the metabolites present in decent amounts, but some minor compounds are still present without being detected. Further studies of the minor components in the mid-polar fractions as well as in the most polar fraction are required in order to have a better overview of the chemical contents of the plant, and how that may vary with growth location and other growth related variations.

Trichilia adolfi, even if it was not selected due to a high antiparasitic activity, was subjected to a study due the chemical interest. nine new limonoid-type compounds were isolated and characterized, which actually was a challenge due to the complexities of their structures. The high chemical diversity demonstrated by limonoids isolated from related species, make this class of compounds extremely interesting for including in other biological activity screening programs. Not to much is know about the limonoids, a hypothetical biosynthetic pathway was proposed based on the reports in literature, but this preliminary analysis require of much more data that may include the use of labeled precursors. Limonoids are associated with several applications and exploring these compounds further might provide new applications.

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