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Emeline Houël[a,*], Marine Ginouves[b,c], Nadine Azas[d], Eliane Bourreau[e], Véronique Eparvier[f], Sébastien Hutter[d], Adeline Knittel-Obrecht[g], Arnaud Jahn-Oyac[a], Ghislaine Prévot[b,c], Pascal Villa[g], Catherine Vonthron-Sénécheau[h], Guillaume Odonne[i]

[a] CNRS, UMR EcoFoG, AgroParisTech, Cirad, INRAE, Université des Antilles, Université de Guyane, 97300 Cayenne, France

[b] TBIP, Université de Guyane, 97300 Cayenne, French Guiana

[c] Université de Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019-UMR9017-CIIL Center for Infection and Immunity of Lille, 59000 Lille, France

[d] Aix Marseille Univ, IHU Méditerranée Infection, UMR VITROME - Tropical Eukaryotic Pathogens, 19-21 Boulevard Jean Moulin, 13005 Marseille, France

[e] Institut Pasteur de la Guyane, 23 Avenue Pasteur, BP6010, 97306 Cayenne Cedex, French Guiana

[f] CNRS – Institut de Chimie des Substances Naturelles, Université Paris-Saclay, 1 Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France

[g] Plate-forme de Chimie Biologique Intégrative de Strasbourg UAR 3286 CNRS-Université de Strasbourg, Institut du Médicament de Strasbourg, ESBS Pôle API, Bld Sébastien Brant, 67412 Illkirch Cedex, France

[h] Laboratoire d'Innovation Thérapeutique UMR 7200 CNRS - Université de Strasbourg, Institut du Médicament de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch cedex, France

[i] Laboratoire Ecologie, évolution, interactions des systèmes amazoniens (LEEISA), CNRS, Université de Guyane, IFREMER, 97300 Cayenne, French Guiana

*Corresponding author: emeline.houel@ecofog.gf; +594 594 293134

Abstract

Ethnopharmacological relevance: Leishmaniasis are widely distributed among tropical and subtropical countries, and remains a crucial health issue in Amazonia. Indigenous groups across Amazonia have developed abundant knowledge about medicinal plants related to this pathology.

Aim of the study: We intent to explore the weight of different pharmacological activities driving taxa selection for medicinal use in Amazonian communities. Our hypothesis is that specific activity against *Leishmania* parasites is only one factor along other (anti-inflammatory, wound healing, immunomodulating, antimicrobial) activities.

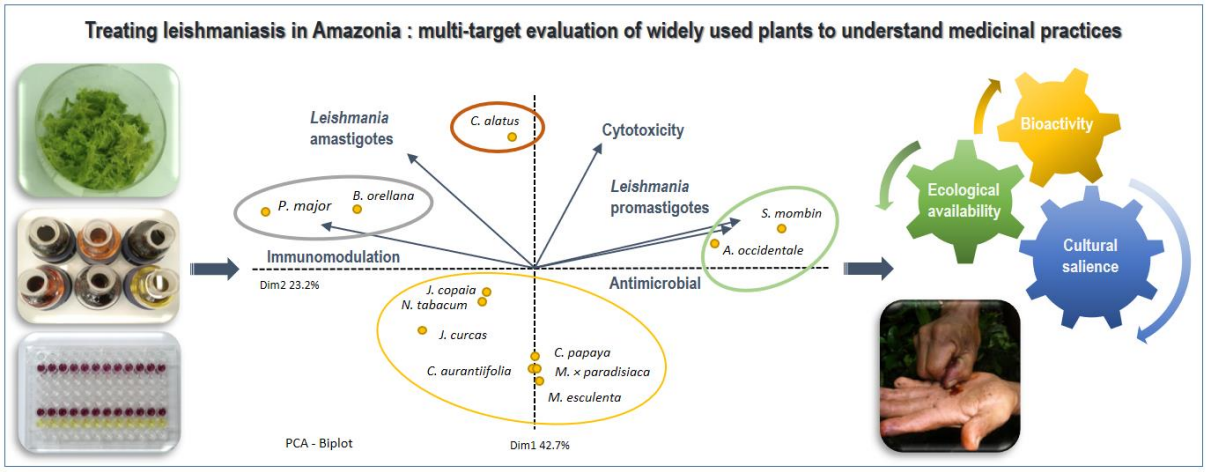
Materials and methods: The twelve most widespread plant species used against leishmaniasis in Amazonia, according to their cultural and biogeographical importance determined through a wide bibliographical survey (475 use reports), were selected for this study. Plant extracts were prepared to mimic their traditional preparations. Antiparasitic activity was evaluated against promastigotes of reference and clinical New-World strains of *Leishmania* (*L. guyanensis, L. braziliensis* and *L. amazonensis*) and *L. amazonensis* intracellular amastigotes. We concurrently assessed the extracts immunomodulatory properties on PHA-stimulated human PBMCs and RAW264.7 cells, and on *L. guyanensis* antigens-stimulated PBMCs obtained from *Leishmania*-infected patients, as well as antifungal activity and wound healing properties (human keratinocyte migration assay) of the selected extracts. The cytotoxicity of the extracts against various cell lines (HFF1, THP-1, HepG2, PBMCs, RAW264.7 and HaCaT cells) was also considered. The biological activity pattern of the extracts was represented through PCA analysis, and a correlation matrix was calculated.

Results: Spondias mombin L. bark and *Anacardium occidentale L*. stem and leaves extracts displayed high anti-promatigotes activity, with IC_{50} ≤ 32 µg/mL against *L. guyanensis*

promastigotes for *S. mombin* and IC_{50} of 67 and 47 µg/mL against *L. braziliensis* and *L. guyanensis* promastigotes, respectively, for *A. occidentale*. In addition to the antiparasitic effect, antifungal activity measured against *C. albicans* and *T. rubrum* (MIC in the 16 - 64 µg/mL range) was observed. However, in the case of *Leishmania* amastigotes, the most active species were *Bixa orellana* L. (seeds), *Chelonantus alatus* (Aubl.) Pulle (leaves), *Jacaranda copaia* (Aubl.) D. Don. (leaves) and *Plantago major* L. (leaves) with IC_{50} < 20 µg/mL and infection rates of 14 - 25% compared to the control. Concerning immunomodulatory activity, *P. major* and *B. orellana* were highlighted as the most potent species for the wider range of cytokines in all tested conditions despite overall contrasting results depending on the model. Most of the species led to moderate to low cytotoxic extracts except for *C. alatus*, which exhibited strong cytotoxic activity in almost all models. None of the tested extracts displayed wound healing properties.

Conclusions: We highlighted pharmacologically active extracts either on the parasite or on associated pathophysiological aspects, thus supporting the hypothesis that antiparasitic activities are not the only biological factor useful for antileishmanial evaluation. This result should however be supplemented by *in vivo* studies, and attracts once again the attention on the importance of the choice of biological models for an ethnophamacologically consistent study. Moreover, plant cultural importance, ecological status and availability were discussed in relation with biological results, thus contributing to link ethnobotany, medical anthropology and biology.

Graphical abstract



Keywords

Leishmania; Infected macrophages; Immunomodulation; Plant use pattern; *Bixa orellana* L.; *Plantago major* L.

1. Introduction

Among well-known parasitic diseases in the Neotropics stands leishmaniasis, a neglected tropical disease caused by several parasitic species of *Leishmania*. Notably, the three most common species of *Leishmania* encountered in Amazonia, i.e. *L. (V.) guyanensis*, *L. (V.) braziliensis* and *L. (L.) amazonensis*, are responsible of cutaneous leishmaniasis (CL) and its variants (diffuse and disseminated CL, and *Leishmania recidivans*) and of the muco-cutaneous (MCL) form of the disease (Burza et al., 2018; Espir et al., 2014; Simon et al., 2017). As suggested by historical, anatomical, and histological evidence, this disease has likely been present in South America since pre-Columbian times (Altamirano-Enciso et al., 2003; Tuon et al., 2008), and still remains a crucial health issue in tropical and neotropical regions (Burza et al., 2018; Pigott et al., 2014). In Amazonia, leishmaniasis symptoms are well described and recognized by the concerned populations, with a high overlapping of local and biomedical definitions. Moreover, Amazonian populations have also developed abundant knowledge

about medicinal plants related to this pathology, these phytoremedies being numerous and of frequent use (Odonne et al., 2009, 2011, 2013, 2017).

Quantitative methods have been developed in ethnobiology, ethnobotany or ethnopharmacology, leading to a more reflexive, integrative and interdisciplinary science (Gaoue et al., 2017; Leonti et al., 2020; Tareau et al., 2020). These methods are in particular useful to understand the use of biodiversity in ethnomedicinal systems, and show that the selection of useful taxa can be explained by objective factors more than driven by a random selection. The fact that cultural factors (religion, historical context, organoleptic perception and meaning effect in general), ecological parameters (phylogeny, ecological distribution, plant habitat) and pharmacochemical aspects (chemical composition, pharmacological activity) impact at once medicinal plants use patterns is indeed widely discussed (Albuquerque et al., 2018, 2020; Coe and Gaoue, 2020; da Silva et al., 2020; Geck et al., 2017; Leonti et al., 2013; Menendez-Baceta et al., 2015; Moerman, 1979; Reinaldo et al., 2020; Santos et al., 2018; Savo et al., 2015; Shepard, 2004). However, the “evidence-based medicines paradox” shows that despite the large number of medicinal plants described, few biologically active compounds have been identified from these traditional medicines (Bourdy et al., 2008; Gertsch, 2009, 2011). Among possible explanations stands the fact that a single *in vitro* bioassay, which can neither represent and describe the complexity of a living organism, nor the intricate interactions between the pathogen and its host, often fails to explain traditional remedies’ good reputation and actual biological activity (Butterweck and Nahrstedt, 2012; Houghton et al., 2007). *Leishmania* infection outcomes are notably linked with host immunity, parasite species and co-infection with other pathogens (de Freitas e Silva and von Stebut, 2021; Soong et al., 2012). Cutaneous lesions provoked by leishmaniasis are susceptible to host bacteria and fungi, leading to numerous cases of secondary infections, and creating a favorable immune environment promoting both infections concurrent development and maintenance (Alamilla-Fonseca et al., 2018; Antonio et al., 2017; Hartley et al., 2013; Salgado et al., 2016). As a consequence, WHO currently recommends treating patients presenting infected ulcers both

with antibiotic and antileishmanial drugs (World Health Organization, 2010, 2013). On their behalf, immunomodulating properties are of crucial importance when it comes to leishmaniasis pathogenesis. In particular, the survival of the parasite in the mammalian host is dependent on the outcome of multiple host–parasite interactions throughout the duration of the infection (Kaye and Scott, 2011; Reithinger et al., 2007). These interactions modulate more particularly the immunological response, through cytokines and inflammatory reactions, and can therefore have an impact on associated symptoms, like fever and pain, or chronicity and severity of the lesion (Alexander and Bryson, 2005; Scott and Novais, 2016; Soong et al., 2012).

Therefore, not only considering the antiparasitic activity of selected extracts to evaluate their antileishmanial properties, but also taking into account leishmaniasis pathophysiology is crucial when trying to understand the use of antileishmanial remedies and select pertinent biological models to evaluate their pharmacological activity. In this perspective, and concurrently to antileishmanial assays performed on Amazonian strains, four pathogenic microorganisms were selected to evaluate the potential antimicrobial activity of the extracts. *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Trichophyton rubrum* are frequent and opportunistic pathogens, associated to numerous infections, including *Leishmania* cutaneous lesions (Antonio et al., 2017; Pinto e Silva et al., 2009; Santos and Hamdan, 2007; Shirazi et al., 2007; Ziaie and Sadeghian, 2008). A set of cytokines was also selected according to their relevance in the context of the study. TNF-α and INF-γ are important macrophage activators, leading to parasite killing by inducing NO production (Oliveira et al., 2012). However, these two cytokines may also present a detrimental role in leishmaniasis pathogenesis by increasing lesion development, severity and chronicity (Oliveira et al., 2014; Scott and Novais, 2016). IL-6 is a pro-inflammatory cytokine which promote the acute phase of inflammation by stimulating Th2 response and inhibiting Th1 response (Castellucci et al., 2006; Espir et al., 2014; Gomes et al., 2014). Alike TNF-α and INF-γ, IL-10 is a dual anti-inflammatory cytokine, concurrently helping to manage the severity of the lesion and exhibiting an inhibitory effect on leishmanicidal activity of phagocytes and INF-γ, and thus on NO

151 production (Oliveira et al., 2014; Salhi et al., 2008; Gomes et al., 2014). IL-13 is a down-
152 regulating factor of macrophage function and known to inhibit the production of pro-
153 inflammatory cytokines such as IL-6 or TNF-α and of nitrite oxide (Bourreau et al., 2001). IL-
154 17 has a detrimental effect by favouring tissue damage in the case of excessive production,
155 notably in the case of *L. braziliensis* infection (Soong et al., 2012; Hartley et al., 2016). Overall,
156 this information emphasizes the complexity of the immunological response and dysregulation
157 mechanisms due to *Leishmania* infection, implying both the host immune status and the
158 parasite species implicated (Espir et al., 2014; Reithinger et al., 2007; Silveira et al., 2009).
159 Eventually, wound healing effect was evaluated using scratch (or cell migration) assay, a
160 reference method to evaluate the formation of skin tissues during scarring process (Liang et
161 al., 2007; Zubair et al., 2016).
162 Our objective was therefore, in the context of an intercultural study of medicinal plant use
163 against leishmaniasis throughout Amazonia (Odonne et al., 2017), to question the
164 interrelations between different pharmacological assays and the perceptions of the
165 phytoremedies across different cultures and geographical areas.
166

167 ## 2. Materials and methods

168 ### 2.1. Taxa selection and collection

169 Twelve antileishmanial remedies reputed across Amazonia among various cultural groups
170 were selected according to our previous work (Odonne et al., 2017). Plants having the highest
171 geographical range of antileishmanial use were selected for pharmacological evaluation.
172 Selected taxa and studied plant parts were the following: *Spondias mombin* L. (bark), *Nicotiana*
173 *tabacum* L. (leaves), *Citrus aurantiifolia* (Christm.) Swingle (fruits), *Jatropha curcas* L. (leaves),
174 *Musa* × *paradisiaca* L. (stem and sap), *Carica papaya* L. (stem and leaves), *Anacardium*
175 *occidentale* L. (stem and leaves), *Chelonanthus alatus* (Aubl.) Pulle (stem and leaves),
176 *Manihot esculenta* Crantz (roots), *Plantago major* L. (leaves), *Jacaranda copaia* (Aubl.) D. Don
177 (leaves), and *Bixa orellana* L. (seeds) (Table 1). Above cited plants are not protected and could

be collected without restriction around Cayenne (Elysée, Macouria, N4°57'25", W52°25'14"), French Guiana, in July 2013. Only *Nicotiana tabacum* L. leaves were bought as commercial handrolling golden tobacco (Orlando™), corresponding to the type of botanical material used by Amazonian societies for this particular species. Collect authorizations were unnecessary at the time of the collect. Herbarium vouchers were deposited in French Guiana Herbarium (CAY), and plant names were checked with http://www.theplantlist.org (01/10/2021) before the study's publication.

Table 1: Plant species, botanical families, and plant part collected. Cayenne herbarium voucher number (CAY) is indicated between brackets when available. In all cases, plant species identifications were however validated in Cayenne herbarium.

Plant species	Botanical families	Plant part
Anacardium occidentale L.	Anacardiaceae	Stems and leaves
Bixa orellana L.	Bixaceae	Seeds
Carica papaya L.	Caricaceae	Stem and leaves
Chelonantus alatus (Aubl.) Pulle (CAY Odonne 810)	Gentianaceae	Leaves
Citrus aurantiifolia (Christm.) Swingle	Rutaceae	Fruits
Jacaranda copaia (Aubl.) D. Don (CAY Odonne 813)	Bignoniaceae	Leaves
Jatropha curcas L.	Euphorbiaceae	Leaves
Manihot esculenta Crantz (CAY Odonne 812)	Euphorbiaceae	Roots
Musa × *paradisiaca* L.	Musaceae	Stem and sap
Nicotiana tabacum L.	Solanaceae	Leaves
Plantago major L.	Plantaginaceae	Leaves
Spondias mombin L.	Anacardiaceae	Bark

2.2. Extraction

Chemicals were purchased from Carlo Erba Reagents, Dasit Group, Cornaredo, MI, Italy. Fresh material was extracted after being roughly crushed using a manual corn grinder to mimic the matter of a cataplasm. As remedies are applied as poultices of fresh crude plants, both hydrophilic and lipophilic compounds contained in the crude vegetal material are thus directly in contact with the wound. Three successive extractions with acidic water (pH = 5 adjusted

with acetic acid 96%), ethanol (RPE grade) and ethyl acetate (RPE grade) were therefore performed, and the extracts pooled together in a single complex mixture for biological assays, to represent the chemical diversity available on the skin through the galenic form used by Amazonian societies. For each plant, 50 g of crushed vegetal material were extracted with successively 200 mL of each solvent during 24 hours for each extraction. Solvents were evaporated under reduced pressure using rotatory evaporator and speedvac concentrator (Savant SPD121P, Thermo Scientific, ThermoFisher Scientific, Waltham, MA, USA) or freeze-dried (Labconco FreeZone 2.5, Labconco, Kansas City, MO, USA) in the case of water extracts. All extracts were pooled afterwards in a single complex extract kept at -18°C until submitted to bioassays and chemical analysis.

2.3. *In vitro* evaluation of antileishmanial activity

2.3.1. Parasites and cultures

For the evaluation of the extracts anti-promastigotes activity, tests were carried out on four New-World strains of *Leishmania*, including two reference strains [*Leishmania (Viannia) guyanensis* MHOM/GF/97/LBC6 (LG-R) and *Leishmania* (*Leishmania*) *amazonensis* MPRO/BR/72/M1845 (LA-R)] and two isolates of patients from French Guiana [*L.* (*V.*) *guyanensis* (LG) and *L*. (*V.*) *braziliensis* (LB)]. The reference strains were obtained from the national reference center for leishmaniasis in Montpellier, France, and the other strains were kindly supplied by the parasitology and mycology laboratory of Cayenne Hospital (Centre Hospitalier Andrée Rosemon), French Guiana. Parasite culture was performed according to previously published protocols (Ginouves et al., 2014, 2017). Briefly, parasites were cultured in RPMI 1640 medium (Gibco, ThermoFisher Scientific, Waltham, MA, USA) containing L-glutamine, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), without phenol red, supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA), 50 IU/mL penicillin (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA), 0.05 mg/mL streptomycin (Invitrogen ThermoFisher Scientific,

223 Waltham, MA, USA), nonessential amino acids (Gibco, ThermoFisher Scientific, Waltham, MA,
224 USA), until reaching exponential phase. This medium is referred to as RPMIØRP medium.
225
226 For the *in vitro* evaluation of the extracts antileishmanial activity on intracellular amastigotes
227 forms, tests were carried out on one New-World strain of *Leishmania* (*Leishmania*)
228 *amazonensis* MHOM/GF/99/LBC19 obtained from the national reference center for
229 leishmaniasis in Montpellier. Parasites were cultured in Schneider's Drosophila Medium
230 (Dutscher, Brumath, France) without phenol red, containing 2 mM L-glutamine (Sigma Aldrich,
231 Saint Louis, MO, USA), supplemented with 10% heat-inactivated FCS (Gibco, ThermoFisher
232 Scientific, Waltham, MA, USA), 100 U/mL penicillin / 0.1 mg/mL streptomycin (Sigma Aldrich,
233 Saint Louis, MO, USA).
234
235 ### 2.3.2. Promastigotes sensitivity test
236 Plant extracts stock solutions were prepared at 20 mg/mL in DMSO. Serial 2-fold dilutions in
237 culture medium were performed to obtain the final testing concentrations, which were 200 -
238 6.25 µg/mL for plant extracts and 0.0125 - 0.00039 µg/mL for pentamidine, used as a positive
239 control. The optimal concentration ranges for extracts and drugs were chosen to allow for the
240 calculation of IC_{50} values, through the obtaining mortalities from 0 to 100% for the tested
241 parasites, and consistently with previously published protocols including the evaluation of
242 reference drugs and botanical extracts (Ginouves et al., 2014). Promastigotes sensitivity
243 assays were performed in a 96 wells plate, using 90 µL of 10^6 parasites in the exponential
244 growth phase / well placed in contact with 10 µL of the different extracts and reference drug
245 solutions. We added 10 µl of RPMIØRP to the control wells. The background noise, due to the
246 color of the extracts, was subtracted from the assay OD values (90 µL of culture medium with
247 10 µL of each concentration of extract, treated under the same conditions as the assays).
248 Following a 48 h incubation at 26°C, 10% of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-

nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, Dojindo Laboratories, Japan) was added and parasites were incubated for a further 24 h at 26°C.

Absorbance (*A*) was measured at 450 nm using a Multiskan (Thermo Scientific, ThermoFisher Scientific, Waltham, MA, USA). The percentage of inhibition was obtained as follows: % inhibition = [(*A*control - *A*test)/*A*control] x 100. The 50% inhibitory concentration (IC_{50}) was then calculated by non-linear regression analysis processed on dose-response inhibition curves using the GraphPad Prism6 software program (GraphPad Software, San Diego, CA, USA).

2.3.3. *In vitro* evaluation of the antileishmanial activity on intracellular amastigotes forms

The *in vitro* evaluation of the antileishmanial activity on intracellular amastigotes forms of the tested extracts was assessed according to the da Luz et al. protocol (2009) with minor modifications. Firstly, 400 µL of acute monocytic leukemia cells (THP-1, ATCC TIB-202) with PMA (Phorbol 12-myristate 13-acetate, Sigma Aldrich, Saint-Louis, MO, USA, final concentration 50 ng/ml) were seeded in sterile chamber-slides at an average density of 1×10^5 cells/ml and incubated for 48 h at 37°C, 5% CO_2. Leishmanial promastigotes forms were centrifuged at 3000 rpm for 10 min and the supernatant replaced by the same volume of Schneider 20% FCS acidified at pH 5.4 by HCl 10N and incubated for 24 hours at 27°C. Differentiated THP-1 cells were then infected by acidified promastigotes with an infection ratio of ten parasites for one macrophage and incubated for 24 hours at 37°C, 5% CO_2. After washing to remove extracellular leishmanial promastigotes forms, 400 µL of medium containing 20 µg/mL of tested-extracts was added in duplicate (final DMSO concentration being inferior to 0.5% v/v). This subtoxic concentration was chosen to obtain a classification of the extracts consistent with the criteria proposed by Deharo et al. (2001) and Gertsch (2009) for antiparasitic activity of natural bioactive compounds selected from a traditional use. Amphotericin B (Sigma-Aldrich, Saint-Louis, MO, USA), an antileishmanial reference drug,was added in duplicate as positive control at final concentration 0.924, 0.462, 0.0924 and 0.0462 µg/mL (i.e. 1, 0.5, 0.1, 0.05 µM) to each set of experiments to allow for the calculation of an

IC_{50} value and consistently with previously published protocols (Fersing et al., 2019) . Untreated cells (DMSO 0.5%) are considered as a negative control After 120 hours incubation at 37°C, 5% CO_2, well supernatant was removed. Cells were then fixed with analytical grade methanol and stained with 10% Giemsa (Sigma-Aldrich, Saint-Louis, MO, USA). The percentage of infected macrophages in each assay was determined microscopically by counting at least 200 cells in each sample. IC_{50} was defined as the concentration of drug necessary to produce 50% decrease of infected macrophages compared to the control. IC_{50} were calculated by non-linear regression analysis processed on dose-response curves, using Table-Curve 2D V5.0 software (Systat Software Inc., Chicago, IL, USA). IC_{50} values represent the mean value calculated from at least three independent experiments.

2.3.4. Cytotoxicity assays

Cytotoxicity was concurrently assessed for each antileishmanial assay. Concerning the anti-promastigote assay, Human Fibroblast Foreskin (HFF1, ATCC SCRC-1041) cells were cultivated in the same medium used for promastigote growth as described above. Confluent HFF1 cells were washed with 5 ml of Phosphate Buffered Saline (PBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA) and cells were decollated from the flask with 5 mL of trypsin 0.25% EDTA and a 1 to 3 minutes incubation at 37 °C. Then, 10 mL of RPMI medium (10% FCS) was added to stop the digestion by trypsin and cells were centrifuged 5 minutes at 1 500 rpm. Medium was removed and 5 mL of culture medium was added. Cells were counted and diluted in order to seed 90 µL of 5.10^3 cells per wells, in a 96-wells plate. Cells were incubated 1 hour at 37°C with 5% CO_2 to allow adhesion on the wells. The same extracts and pentamidine concentrations as for promastigotes were used, with pentamidine used as a positive control. After adding 10 µL of extract or pentamidine, plate was incubated 72 h at 37°C; 5% CO_2. We added 10 µl of culture medium to the control wells. Then, 10% of WST-8 was added and HFF1 cells were incubated for 4 hours at 37°C; 5% CO_2. Absorbance was measured at 450 nm using a Multiskan (Thermo Scientific, ThermoFisher Scientific, Waltham, MA, USA), and percentage

of inhibition was calculated as for promastigotes. The background noise, due to the color of the extracts, was subtracted from the assay OD values (90 µL of culture medium with 10 µL of each concentration of extract, treated under the same conditions as the assays).

The evaluation of the tested extracts cytotoxicity on the THP-1 cell line (acute monocytic leukemia cell line ATCC TIB-202), used for the *in vitro* evaluation of the antileishmanial activity on intracellular amastigotes forms, was performed according to the method of Mosmann (Mosmann, 1983) with slight modifications. Cells were incubated at an average density of 1×10^5 cells/well in 100 µL of complete RPMI medium supplemented with 50 ng/ml PMA (Phorbol 12-myristate 13-acetate, Sigma Aldrich, Saint-Louis, MO, USA) for 48 h at 37°C, 5% CO_2. After 48h incubation, 100 µL of medium with 1 µL of various concentrations of extracts, optimized to allow for the calculation of IC_{50} values as previously published (Delmas et al., 2000), and dissolved in DMSO (final concentration less than 0.5% v/v) were added in duplicate. Untreated cells (DMSO 0.5%) are considered as a negative control. The plates were incubated for 72 h at 37°C, 5% CO_2. Each well was then microscope-examined for detecting possible precipitate formation before the medium was aspirated from the wells. 100 µL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, Sigma Aldrich, Saint-Louis, MO, USA) solution (0.5 mg/mL in medium without FCS) were then added to each well. Cells were incubated for 2 h at 37°C. After this time, the MTT solution was removed and DMSO (100 µL) was added to dissolve the resulting blue formazan crystals. Plates were shaken vigorously (300 rpm) for 10 min. The absorbance was measured at 570 nm with 630 nm as reference wavelength spectrophotometer using a BIO-TEK ELx808 Absorbance Microplate Reader (BioTek Instruments, Winooski, VT, USA). DMSO was used as blank and doxorubicin and amphotericin B (Sigma Aldrich, Saint-Louis, MO, USA) as positive controls. Cell viability was calculated as percentage of control (cells incubated without compound). The 50% cytotoxic concentration (CC_{50}) was determined from the dose–response curve by using the TableCurve

2D v.5.0 software (Systat Software Inc., Chicago, IL, USA). CC_{50} values represent the mean value calculated from three independent experiments.

Eventually, HepG2 hepatocarcinoma cell line (HepG2, ATCC HB-8065) was used as a cytotoxicity control concurrently to the *in vitro* evaluation of the antileishmanial activity on intracellular amastigotes forms. Cell line was maintained at 37°C, 5% CO_2 with 90% humidity in MEM (Minimum Essential Media, Thermofisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 1% L-glutamine (200 mM), penicillin (100 U/mL) and streptomycin (0.1 mg/mL) (complete MEM medium). The evaluation of the tested molecules cytotoxicity on the HepG2 cell line was performed according to the method of Mosmann (Mosmann, 1983) with slight modifications. Firstly, 5.10^3 cells in 100 µL of complete medium were inoculated into each well of 96-well plates and incubated at 37°C in a humidified 5% CO_2. After 24 h incubation, 100 µL of medium with 1 µL of various concentrations of extracts, optimized to allow for the calculation of IC_{50} values as previously published (Fersing et al., 2019), and dissolved in DMSO (final concentration less than 0.5% v/v) were added and the plates were incubated for 72 h at 37°C. Untreated cells (DMSO 0.5%) are considered as a negative control. Triplicate assays were performed for each sample. Each plate-well was then microscope-examined for detecting possible precipitate formation before the medium was aspirated from the wells. 100 µL of MTT solution (0.5 mg/mL in medium without FCS) were then added to each well. Cells were incubated for 2 h at 37°C. After this time, the MTT solution was removed and DMSO (100 µL) was added to dissolve the resulting blue formazan crystals. Plates were shaken vigorously (700 rpm) for 10 min. The absorbance was measured at 570 nm with 630 nm as reference wavelength using a TECAN Infinite F-200 Microplate Reader (Männedorf, Switzerland). DMSO was used as blank and doxorubicin (Sigma Aldrich, Saint-Louis, MO, USA) as positive control. Cell viability was calculated as percentage of control (cells incubated without compound). The 50% cytotoxic concentration (CC_{50}) was determined from the dose–response curve by using the TableCurve software 2D v.5.0 (Systat Software Inc.,

Chicago, IL, USA). CC_{50} values represent the mean value calculated from three independent experiments.

2.3.5. Statistical analysis

The 95% confidence interval (95% CI) was determined using GraphPad Prism6 software for each test (GraphPad Software, San Diego, CA, USA).

2.4. *In vitro* antimicrobial activity

Four pathogenic microorganisms were selected to evaluate the antimicrobial activity of the extracts: two bacteria (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213), a yeast (*Candida albicans* ATCC 10231) and a filamentous dermatophytic fungus (*Trichophyton rubrum* SNB-TR1). *T. rubrum* clinical isolate was kindly provided to Dr V. Eparvier by Prof. P. Loiseau, Université Paris-Saclay. This strain was identified by P. Loiseau and C. Bories, with molecular analysis conducted by BACTUP and ITS sequence deposited in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under the registry numbers KC692746. Extracts were tested according to the reference protocol of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org) adapted as previously published (Nirma et al., 2015). Notably, microbial suspensions were adjusted to 0.5 McFarland standard, and fungal suspensions then diluted 1:1000 (v/v) and bacterial suspensions then diluted 1:100 (v/v) using RPMI 1640 medium in all cases. The standard microdilution test as described by the Clinical and Laboratory Standards Institute guidelines (M27-A2, M7-A8 and M38-A) was used to determine minimal inhibition concentrations (MIC) against dermatophyte fungi, bacteria and yeasts (CLSI, 2008a; 2008b; 2009). Gentamicin, oxacilin, fluconazole and itrconazole were used as positive controls depending on the microbial strain. The MIC values refer to the lowest concentration preventing visible fungal or bacterial growth. Crude extracts were tested at concentrations ranging from 256 to 0.5 µg/mL, in accordance with previously published protocols aiming at evaluating the antimicrobial activity of natural products, notably

selected on an ethnobotanical basis (Houël et al., 2015b; Roumy et al., 2020) Untreated wells (culture medium inoculated with microbial suspension and without extract solution or reference drug) are considered as a negative control. The microplates were incubated at 32°C, and MIC values were calculated after 24h for bacteria, 48h for yeast and 5 days for *T. rubrum*.

2.5. *In vitro* anti-inflammatory activity through non-selective stimulation

2.5.1. Inhibitory activity on NO production from macrophage-like cell line RAW 264.7

The bioassays were performed as reported previously in the context of ethnopharmacological studies (Houël et al., 2015a, 2016). Briefly, $2x10^5$ RAW 264.7 cells (ATCC TIB-71) in 100 µL into each well of 96-well plates were stimulated with lipopolysaccharide (LPS, Sigma Aldrich L-6636, Saint-Louis, MO, USA) at 5 µg/mL and treated with 2 concentrations of each extract (50 µg/mL or 10 µg/mL) diluted in DMSO. Quercetin (Sigma Aldrich Q4951, Saint-Louis, MO, USA) at 50 µg/mL and 10 µg/mL (final DMSO concentration 0.1% v/v) was used as a positive control. Unstimulated cells (DMSO 0.1%, without LPS) are considered as negative control. Plates were then incubated for 24 hours at 37°C in a humidified 5% CO_2 and nitrite (NO_2^-) accumulation was determined as an indicator of NO production in culture media as previously described (Kumar-Roiné et al., 2009, Houël et al., 2015a). Each measurement was performed in duplicate and results were expressed as means for three independent experiments. Cytotoxicity was measured using the tretrazolium salt WST-1 assay (Ozyme, Saint-Cyr-l'École, France) according to supplier protocol. All experiments were performed on a fully automated platform (Beckman Coulter, Brea, CA, USA).

2.5.2. Peripheral blood mononuclear cells (PBMCs) isolation and culture

PBMCs were prepared from the peripheral blood of healthy donors (Etablissement Français du Sang, Strasbourg) following legal rules specified in a contract established between CNRS and EFS with specification that informed consent was obtained. All was done in the respect of guidelines of the Declaration of Helsinki and Tokyo for humans, and was approved by the

409 French legal authorities as reported previously (Houël et al., 2015a, 2016). $8x10^4$ Isolated
410 PBMCs in 80 µL into each well of 96-well plates were stimulated with 5 µg/mL lectin from
411 *Phaseolus vulgaris* (PHA, Sigma Aldrich, Saint-Louis, MO, USA). All extracts were dissolved
412 in DMSO so that final DMSO concentration is 0.1% and tested at 10 and 50 µg/mL in duplicate.
413 Positive control dexamethasone (Sigma Aldrich D4902, Saint-Louis, MO, USA) (dissolved in
414 ethanol so that final EtOH concentration is 0.1%) was simultaneously tested at 10 and 50
415 µg/mL. Cells without PHA stimulation (DMSO 0.1%) were considered as negative control
416 (basal level of cytokine). Cells were incubated at 37°C (5% CO_2).
417
418 2.5.3. Characterization of cytokine secretion.
419 After 72 h, PBMCs supernatants were transferred into a 384-well plate. Cytokine detection was
420 performed using HTRF technology (Homogeneous Time Resolved Fluorescence, Cisbio
421 bioassays, Codolet, France) for TNFα (62TNFPEC), IL-6 (62IL6PEB), IL-10 (6FH10PEB) and
422 INF-γ (62IFNPEB) according to supplier recommendations. Reading was performed after 3 h
423 (TNFα, IL-6) or 20 h (IL-10, INF-γ) of incubation using Envision multi-labelled reader (Perkin
424 Elmer, Waltham, MA, USA) with supplier recommended parameters. A standard curve was
425 performed for each cytokine to determine the concentration of released cytokines (in pg/mL)
426 by PBMCs in the supernatant. The cytokine secretion is expressed as a percentage of the
427 cytokine concentration measured in the negative control experiment conducted on the same
428 plate.
429
430 2.5.4. Cell viability assay
431 Cell viability was measured using the WST-1 (Ozyme, Saint-Cyr-l'École, France) assay
432 according to the manufacturers' protocol. Briefly, after supernatant transfer for cytokine
433 determination, WST-1-containing medium was added to cells and cell viability was determined
434 by measuring absorbance at 450 nm using Envision multi-labelled reader (Perkin Elmer,

435 Waltham, MA, USA) after 45 min incubation at 37°C. Each measurement was performed in
436 triplicate and results were expressed as means of three independent experiments.
437
438 ## 2.6. *In vitro* anti-inflammatory activity in experimental leishmaniasis
439 ### 2.6.1 Patients
440 Patients were received at the Cayenne Hospital (Centre Hospitalier Andrée Rosemon), French
441 Guiana. Blood samples were obtained from 2 patients with active cutaneous leishmaniasis
442 caused by *L. guyanensis*. Both were human immunodeficiency virus seronegative. Blood
443 samples were collected by venipuncture into sterile tubes (Veinoject; Terumo Medical
444 Corporation, Somerset, NJ, USA). Samples from patient 1 were collected at 5-6 weeks of
445 evolution and from patient 2 at 3-4 weeks of evolution; none were receiving leishmaniasis
446 therapy. This study was approved by CPP Ile-de-France (N°IDRCB 2014-A01009-38) and
447 CNIL (DR2014364 (914625)), prior informed consent was obtained from patients.
448
449 ### 2.6.2. Reagents
450 MILLIPLEX® MAP kit (Millipore, Burlington, MA, USA) for Luminex were used for cytokine
451 detection (TNF-α, IL-6, IL-10, INF-γ, IL-13 and IL-17).
452
453 ### 2.6.3. Preparation of *L. guyanensis* antigens.
454 *L. guyanensi*s promastigotes (M4147) were cultured in Schneider medium complemented with
455 10% FCS (Sigma Aldrich, Saint-Louis, MO, USA). Extracellular proteins were removed from
456 the pellets by 2 washes with PBS. Antigen lysate was prepared by freezing (-70°C) and
457 thawing (37°C) the *Leishmania* preparation 10 times. Then lysate was centrifuged and was
458 maintained at a final concentration of an equivalent of 10^8 parasites/mL.
459

2.6.4. PBMCs culture and detection of cytokines

PBMCs were isolated after blood puncture over a ficoll-hypaque gradient (dp 1.077) and were resuspended in RPMI medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin (all from Sigma Aldrich, Saint-Louis, MO, USA), and 5% human heat-inactivated AB serum. Cultures for cytokine production (10^6 cells in 1 mL of culture medium) were plated on flat-bottom 24-well plates (Costar, Corning, Thermo Scientific, ThermoFisher Scientific, Waltham, MA, USA) with or without *L. guyanensis* antigens (equivalent to 10^6 parasites) and extracts at 10 and 50 µg/mL in DMSO (final concentration in DMSO: 1%). The concentrations were chosen to correspond to those of the non-selective assay and allow for comparison between the two protocols and unstimulated cells (DMSO 1%, without antigens) are considered as negative control. Culture supernatants were harvested after 7 days for TNF-α, IL-6, IL-10, INF-γ, IL-13 and IL-17 production and were stored at -20°C. Cytokine production was analyzed by using a Luminex (Austin, TX, USA) system. Extracts were tested in duplicate, and cytokine secretion inhibition is expressed as a percentage of the cytokine concentration measured in the negative control experiment.

2.7. Cell migration assay

The protocol used is based on Abboud et al. (Abboud et al., 2015) and modified as described in Demay et al. (Demay et al., 2021) works. Briefly, the immortalized human keratinocytes cell line HaCaT (DKFZ, Heidelberg) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L D-glucose (Sigma Aldrich, Saint-Louis, MO, USA, reference D5796). Culture media was supplemented with 10% FBS (fetal bovine serum, Invitrogen, ThermoFisher Scientific, Waltham, MA, USA, reference 10270-106) and 100 U/mL penicillin / 100 µg/mL streptomycin (Sigma Aldrich, Saint-Louis, MO, USA, reference P0781). HaCaT cells were seeded at a density of $3x10^4$ cells/well in a 96-well ImageLock™ tissue culture plate (Essen Bioscience, Ann Arbor, MI, USA, reference 4379) and incubated in a humidified incubator (5% CO_2, 37°C) to form a cell monolayer. At 90% confluency, the medium was changed to DMEM

without FBS and cells were incubated for 48 hours. A scratch wound along the cell monolayer was created with the 96-well WoundMaker™ (Essen Bioscience, Ann Arbor, MI, USA). Cells were washed once with DMEM without FBS to remove detached cells, before treatment with the extract at 10 µg/mL or 50 µg/mL (final DMSO concentration 0.1% v/v). Mitomycin C at 0.5 µg/mL (Sigma Aldrich, Saint-Louis, MO, USA, reference M0503) was added at the treatment to prevent cell proliferation. EGF (Epidermal Growth Factor) at 20 ng/mL and FBS at 0.5% or 10% were used as positive controls. Untreated cells (DMSO 0.1%, no serum) are considered as a negative control. Images of the wounds were automatically acquired in CO_2 incubator using the IncuCyteTM FLR (Essen Bioscience, Ann Arbor, MI, USA) for 72 hours. Each measurement was performed in triplicate with four independent experiments. The Relative Wound Density metric relies on measuring the spatial cell density in the wound area relative to the spatial cell density outside of the wound area at every time point. It is designed to be zero at t=0, and 100% when the cell density inside the wound is the same as the cell density outside the initial wound. In this respect, the metric is self-normalizing for changes in cell density which may occur outside the wound as a result of cell proliferation and/or pharmacological effects. Importantly, the RWD metric is robust across multiple cell types as it does not rely on finding cell boundaries. The result analysis was performed using the IncuCyteTM FLR software (version 2011a rev2, Essen Bioscience, Ann Arbor, MI, USA). Cell viability was evaluated 72 h after treatment using CellTiter-Glo Luminescent Assay (Promega, Madison, WI, USA, reference G7571) following manufacturer's recommendations.

2.8. Analysis of the extracts biological activity pattern

To allow a better understanding of the biological activities pattern of the analyzed extracts, scores representing the various biological activities were attributed to each extract, similarly to a previously used procedure (Rodrigues et al., 2019) and using a logarithmic scale for a better discrimination of the extracts. As the objective is to not to provide a global holistic antileishmanial score, but to display an efficient visualization of each concurrent biological

activity in an independent manner, without considering if this activity is beneficial or not, the same scoring system was used for all activities, including cytotoxicity.

A summary of scores calculation methods is presented in Table 2. Viability of RAW 264.7 cells, PBMCs and HaCaT cells measured at the highest tested dose (50 µg/ml) was used to calculate the cell viability score. Eventually, the total score was calculated as the sum of all bioassays scores for each extract and assay category (antileishmanial activity / promastigotes, antileishmanial activity / amastigotes, antimicrobial activity, immunomodulation / non selective assay, immunomodulation / selective assay, cytotoxicity / cell viability). Concerning immunomodulation assays, a large amount of non-dose-dependent data was available (Supplementary Material Tables S1-S5). Therefore, each cytokine secretion and NO production inhibition rate (in absolute value, inhibition % / DMSO 0.1 % - PHA or LPS 5 µg/L, Tables S1, S3 and S5) was attributed a score as presented in Table 2, and the mean value of these scores was calculated for each extract to obtain the final immunomodulating score for non-selective and selective assay, respectively. PCA analysis was computed in R software version 4.0.2 (https://www.r-project.org/) using the factoMineR, factoextra and ggplot2 packages. Correlation matrix was obtained using *corrplot* and *ade4* packages, and the function *rcorr* in *Hmisc* package was used to compute the significance levels for Pearson correlations and obtain *p* values for all possible pairs. The *ropls* package (https://www.bioconductor.org/packages/release/bioc/html/ropls.html) was used for PCA validation and OPLS analysis.

Table 2 : Summary of score calculation method. Concerning immunomodulation assays, inhibition rates are calculated as cytokine secretion inhibition mean absolute values for all cytokines and NO at the two tested doses (10 and 50 µg/mL).

Score	Anti-promastigotes, antimicrobial and cytotoxicity (HHF1, THP-1, HepG2)	Anti-amastigotes activity	Immunomodulation (inhibition rate)	Cell viability at 50 µg/mL (RAW264.7, PBMCs, HaCaT)

1	IC_{50} and MIC > 200 µg/ml CC_{50} > 100 µg/ml	Infection rate 100-75%		0-24%	≥ 95%
10	200 µg/mL ≥ IC_{50} ; MIC > 100 µg/ml 100 µg/mL ≥ CC_{50}> 50 µg/ml	Infection rate 74-50%	IC50 > 20 µg/ml	25-49%	95-75%
100	100 µg/mL ≥ IC_{50} ; MIC > 50 µg/ml 50 µg/mL ≥ CC_{50} > 25 µg/ml	Infection rate 49-25%	IC50 = 20 µg/ml	50-99%	≤ 75%
1000	IC_{50} and MIC ≤ 50 µg/mL CC_{50} ≤ 25 µg/ml	Infection rate 24-0%	IC50 < 20 µg/ml	≥ 100%	≤ 50%
10000				≥ 1000%	

2.9. Analysis of the medicinal practices

For each species, the use was analyzed through putting in perspective biological activity scores (BAS) and activity patterns with notably the distribution indices as defined by Odonne et al. (2017). As a reminder, the geographical distribution index (I_g) was designed to measure the extend of the spatial distribution of the antimeishmanial use of a given species. The cultural distribution index (I_c) aimed at evaluating the ratio of cultural groups mentioning the use of a given species in case of leishmaniasis outcomes. Eventually, the general distribution index (I_d) was calculated as the mean value of the two former indices.

3. Results

3.1. *In vitro* sensitivity of cutaneous *Leishmania* promastigotes from reference and clinical strains and activity against *L. amazonensis* amastigotes

The obtained results are displayed in Table 3. Only *S. mombin* and *A. occidentale* extracts displayed significant anti-promastigote activity according to the endpoint criteria (IC_{50} < 100 µg/ml for crude extracts) proposed by Cos et al. (Cos et al., 2006). *S. mombin* extract exhibited an effect against *L. guyanensis* (LG) promastigotes, with no activity against the other strains, and IC_{50} values of 32 µg/mL (CI 26-38) for the LG reference strain and <31 µg/mL for the clinical isolate. *A. occidentale* extract led to IC_{50} values of 67 µg/mL (CI 54-83) and 47 µg/mL (CI 42-53) against *L. amazonensis* (LA-R) and *L. guyanensis* (LG-R) reference strains promastigotes, respectively. This latter extract had no effect on *Leishmania* clinical isolates.

In the amastigote assay, *B. orellana*, *J. copaia*, *P. major* and in a lesser extend *N. tabacum* stood forth, with IC_{50} values < 20 µg/mL and infection rates in the 14 - 45% range compared to the control, and were therefore considered active as defined in the chosen assay protocol. *C. alatus* also exhibited an interesting activity against *L. amazonensis* amastigotes.

Table 3 *In vitro* sensitivity of cutaneous *Leishmania* promastigotes from reference (R) and clinical (C) strains (IC_{50}, µg/mL) and of *Leishmania amazonensis* amastigotes. CI: confidence interval. Pentamidine was used as control in the promastigote model and amphotericin B in the amastigote model. For the amastigotes assay, all extracts were tested at 20 µg/mL and the control infection rate was 20%.

Plant species and positive controls	***Leishmania* promastigotes**				***Leishmania* amastigotes (LA)**	
	LA-R	**LB-C**	**LG-R**	**LG-C**	**% infection / control**	**IC_{50} (µg/mL)**
A. occidentale	**67 (54-83)**	> 200	**47 (42-53)**	> 200	75%	> 20
B. orellana	> 200	> 200	> 200	> 200	**14%**	**< 20**
C. papaya	> 200	> 200	> 200	> 200	50%	20
C. alatus	> 200	> 200	> 200	> 200	**20%**	**< 20**
C. aurantiifolia	> 200	> 200	> 200	> 200	50%	20
J. copaia	> 200	> 200	> 200	> 200	**25%**	**< 20**
J. curcas	> 200	> 200	> 200	> 200	55%	> 20
M. esculenta	> 200	> 200	> 200	> 200	n.t.	n.t.
M. × paradisiaca	> 200	> 200	> 200	> 200	50%	20
N. tabacum	> 200	> 200	> 200	> 200	**45%**	**< 20**
P. major	> 200	> 200	> 200	> 200	**25%**	**< 20**
S. mombin	>200	> 200	**32 (26-38)**	**<31**	55%	> 20
Pentamidine	0.005 (very wide)	> 0.01	0.003 (very wide)	> 0.01	n.t.	n.t.
Amphotericin B	n.t.	n.t.	n.t.	n.t.	-	0.46

LA : *L. amazonensis*; LB : *L. braziliensis*, LG : *L. guyanensis* ; R: reference strain ; C: clinical strain; n.t. : not tested
In bold: species for which the noticeable biological activity could account for the maintenance of their medicinal use

571

572 ## 3.2. Antimicrobial activities

573 Two taxa exhibited antimicrobial activity: *S. mombin* extract was strongly active against *C.*
574 *albicans* only (MIC value of 16 µg/mL), whereas *A. occidentale* extract exhibited significant
575 antifungal activity against both *C. albicans* and *T. rubrum* (MIC values of 32 and 64 µg/mL
576 respectively) according to endpoint criteria proposed in the ethnopharmacological literature
577 (Cos et al., 2006; Gertsch et al., 2009) (Table 4).

578

579 **Table 4:** Antimicrobial activities (MIC, µg/mL) of the plant extracts against bacteria (*E. coli* and
580 *S. aureus*), yeasts (*C. albicans*) and filamentous dermatophyte (*T. rubrum*) (MIC, µg/mL).
581 Gentamicin, oxacilin, fluconazole and itraconazole were used as controls.

Plant species and positive controls	***E.coli* ATCC 25922**	***S. aureus* ATCC 29213**	***C. albicans* ATCC 10231**	***T. rubrum* SNB-TR1**
A. occidentale	>256	>256	**32**	**64**
B. orellana	>256	>256	>256	>256
C. papaya	>256	>256	>256	>256
C. alatus	>256	>256	>256	>256
C. aurantiifolia	>256	>256	>256	>256
J. copaia	>256	>256	>256	>256
J. curcas	>256	>256	>256	>256
M. esculenta	>256	>256	>256	>256
M. × paradisiaca	>256	>256	>256	>256
N. tabacum	>256	>256	>256	>256
P. major	>256	>256	>256	>256
S. mombin	>256	>256	**16**	>256
Gentamicin	8	n.t	n.t	n.t.
Oxacilin	n.t.	0.5	n.t.	n.t.
Fluconazole	n.t	n.t.	4	n.t.
Itraconazole	n.t.	n.t.	n.t.	0.03

In bold: species for which the noticeable biological activity could account for the maintenance of their medicinal use
n.t. : not tested

582

3.3. Immunomodulatory properties of the extracts

For these assays, we selected TNF-α, IL-6, IL-10 and INF-γ for the non-selective assay, adding IL-13 and IL-17 in the case of non-naïve cells. The global influence of the tested extracts on cytokine production is presented in Table 5. The full dataset is available as Supplementary Material (Tables S1-S5).

Table 5: Immunomodulatory effects of the plant extracts in the case of non-selective (PHA and LPS-stimulated human PBMCs and RAW cells respectively, for IL-10, IL-6, INF-γ and TNF-α) and selective (*L. guyanensis* antigens-stimulated PBMCs obtained from *Leishmania*-infected patients for IL-10, IL-6, INFγ-, TNF-α, IL-13, IL-17) assays. The number of arrows was assigned based on the biological activity score (BAS) for each independent cytokine as defined in Table 2, and plant extracts obtaining two arrows for more than one cytokine are highlighted in bold.

Plant species	Non-selective assay	Selective assay
A. occidentale	↘ INF-γ	↗↗ IL-13
B. orellana	**↗↗ (TNF-α, IL-6, IL-10)**	↗ IL-6 ↘ (IL-13, TNF-α)
C. papaya	-	↗ (IL-6, INF- γ)
C. alatus	↘ (TNF-α, INF-γ, IL-10, IL-6, NO)	↗ (TNF-α, IL-6, INF-γ, IL-13)
C. aurantiifolia	-	↗ (TNF-α, INF-γ, IL-10, IL-6, IL-13, IL-17)
J. copaia	↗↗ IL-6	↗↗ INF-γ ↗ (TNF-α, IL-6, IL-13)
J. curcas	↘↘ IL-10 ↘ (IL-6, INF-γ) ↗ TNF-α	↗ (INF-γ, IL-10, IL-6, IL-13, IL-17)
M. esculenta	↗ TNF-α	↗ (IL-6, INF-γ)
M. × paradisiaca	-	↗ (IL-6, INF-γ)
N. tabacum	↗ IL-6 ↘ INF-γ	↗↗ INF-γ ↗ (TNF-α, IL-10, IL-6, IL-13, IL-17)
P. major	**↗↗ (TNF-α, IL-6, IL-10) ↘ INF-γ**	**↗↗ (TNF-α, IL-6) ↘ INF-γ**
S. mombin	↘ INF-γ	↘ (TNF-α, INF-γ)

In bold: species for which the noticeable biological activity could account for the maintenance of their medicinal use

Non-selective stimulation highlighted clear and contrasted activities of 8 over 12 extracts. *P. major* and *B. orellana* extracts demonstrated marked to strong immunoenhancing properties

along a large spectrum of cytokines, with an increase of TNF-α, IL-6 and IL-10 concentrations of 100-170% for TNF-α, 42-48% for IL-6 and 168-328% for IL-10 for both extracts. The immunostimulating effect was not dose-dependent and was observed even without PHA stimulation. Interestingly, *P. major* extract also notably inhibited INF-γ secretion (66%) at the concentration of 50 µg/mL. *J. curcas* and C*. alatus* extracts were highlighted as the two most active extracts leading to the inhibition of a wide range of cytokines secretion. *J. curcas* extract inhibited the secretion of INF-γ (89% at 50 µg/mL) and IL-10 (57% at 10 µg/mL and 76% at 50 µg/mL), alongside with IL-6 (83% at 50 µg/mL) and in a lesser way TNF-α (58% at 50 µg/mL). Concerning C*. alatus* extract, the secretion of all tested cytokines was strongly inhibited as well as NO secretion. Lastly, three extracts exhibited a more selective activity. Interestingly, *N. tabacum* extract significantly upregulated IL-6 (6 times the basal signal at 50 µg/mL) without PHA induction, while inhibiting the secretion of INF-γ (61% at 50 µg/mL) in the case of stimulated cells. *A. occidentale* extract only strongly inhibiting INF-γ secretion (70% at 50 µg/mL). *S. mombin* extract for its part also strongly inhibited the secretion of INF-γ (80%) at 50 µg/mL, concurrently with a less marked effect on TNF-α (48%), and IL-10 (38%) at the same concentration. Eventually, *J. copaia* extract only led to enhancing IL-6 secretion in a non-dose dependent manner. *C. aurantia*, *M.* × *paradisiaca*, *C. papaya* and *M. esculenta* did not exhibit any or clear immunomodulatory activity, even if a slight increase of TNF-α (51%) was observed at 50 µg/mL for *M. esculenta* extract.

Concerning specific stimulation (*L. guyanensis* (LG) antigen on non-naïve PBMCs), *P. major* extract also highly upregulated the secretion of TNF-α (more than 400% concentration increase at 10 and 50 µg/mL) and IL-6 (~ 4000% concentration increase at 10 and 50 µg/mL). As highlighted in the non-selective stimulation assay, *P. major* extract also inhibited INF-γ secretion in a 70% range at 10 and 50 µg/mL. All responses were non dose-dependent. On the contrary, *B. orellana* extract significantly induced TNF-α, IL-6 and IL-10 expression in the non-selective assay, but only markedly increased the secretion of IL-6 at 50 µg/mL (up to 600% at 50 µg/ml) in the selective assay, with also a slight inhibiting effect measured for IL-13

(around 60%) and TNF-α (56% at 10 µg/mL). Concurently, *S. mombin* was the only other extract found to exert such an inhibiting effect in the case of a selective stimulation of non-naïve cells. An inhibiting effect was indeed measured for INF-γ, with 86% of cytokine secretion inhibition at 10 µg/mL, and TNF-α, with 69% of cytokine secretion inhibition at 10 µg/mL. At 50 µg/mL inhibitions were less marked than at 10 mg/mL indicating a non-dose dependent effect. Concerning *A. occidentale*, a less marked inhibiting profile was observed in the non-selective assay, similarly to *S. mombin*, with only ~ 50% inhibition for INF-γ at both concentrations in this case, and alongside with a strong increase of IL-13 expression (700% at 10 µg/mL). Interestingly, whereas *P. major* and *B. orellana* were the only two extracts to notably increase the secretion of a wide range of cytokines in the first non-selective assay, several clearly immunoenhancing extracts were identified in this selective assay. *N. tabacum* extract increased TNF-α (up to 176% at 50 µg/mL), IL-6 (778 - 723% concentration increase at 10 and 50 µg/mL), IL-10 (192% at 10 µg/mL, but with no effect at 50 µg/mL), INF-γ (~ 200% with a non-dose-dependent effect), IL-17 (115% at 10 µg/mL, but with no effect at 50 µg/mL), and IL-13 (99 - 82%). Almost the same profile was observed for *C. aurantiifolia* extract, only with a less marked effect on IL-6 and TNF-α secretion, and a slightly stronger effect on INF-γ and IL-17 secretion at 10 µg/mL. *J. curcas* extract also strongly increased IL-6, IL-10 and INF-γ secretion, alongside with significant effects on TNF-α at 50 mg/mL and IL-13 and IL-17 at 10 mg/mL. Concerning *J. copaia*, the most significant enhancing effect concerned INF- γ secretion (113-137% in a non-dose dependent manner), alongside with the promotion of the secretion of TNF-α and IL-6 at both concentrations, and a slight effect on IL-13 production (59% at 10 µg/mL). The profile observed for *C. alatus* extract was similar. Generally speaking, the results observed for these extracts are completely different in terms of cytokine range and immunostimulating or inhibiting action compared to the non-selective assay. The remaining extracts (*C. papaya*, *M. esculenta* and *M.* × *paradisiaca*), exerted more limited effects, with equivalent immunoenhancing activity profiles for the three species.

3.4. Wound-healing properties of the extracts

Wound-healing properties of the selected extracts were searched using a cellular migration assay of the keratinocyte cell line HaCaT for 48 h after having injured the monolayer. In our experimental conditions, we added mitomycin C in the medium to block cell division, in order to measure only cell growth and migration leading to the wound recovery. We could see a fast recovery with controls (EGF or 10% serum). The treatment with extracts behaved like the negative control (without serum), with a low recovery of the wound (Supplementary material Tables S6 and S7). We can conclude that 9 out of 12 extracts did not increase the recovery of wound, but did not show any adverse effect compared to negative controls. The 3 other extracts (*A. occidentale*, *C. alatus*, *S. mombin*) showed a cell viability lower than 50 % and/or precipitated at 50 µg/mL.

3.5 Cytotoxicity and cell viability

Concurrently to the above presented bioassays, plant extracts cytotoxicity and evaluation of cell viability were assessed towards the human cell lines HFF1 (Human Fibroblast Foreskin), HepG2 (hepatocarcinoma cell line), THP-1 (acute monocytic leukemia cell line), HaCaT (skin immortalized keratinocytes) and PBMCs (peripheral blood mononuclear cells) on the one hand, and the murine RAW 264.7 cell line on the other hand. The obtained results are presented in Table 6, and full cell viability datasets are available in Supplementary Material (Tables S8-S13). The species exerting the highest cytotoxicity / lowest cell viability rate for each of the tested cell lines are indicated in bold. The cytotoxic effects are evaluated according to endpoint criteria proposed in the ethnopharmacological literature (Cos et al., 2006; Gertsch et al., 2009).

Table 6: Cytotoxicity towards HFF1 (Human Fibroblast Foreskin), THP-1 cell line (acute monocytic leukemia), HepG2 cell line (hepatocarcinoma), and cell viability of RAW 264.7 cells, PBMCs (prepared from the peripheral blood of healthy donors) and human keratinocytes cell

680 line HaCaT. CC_{50} and IC_{50} are expressed in µg/mL. For PBMCs, RAW 264.7 and HaCaT cells,
681 cell viability corresponds to the value measured at the highest tested dose (50 µg/mL).

Plant species and positive controls	Cytotoxicity (µg/mL)			Cell viability (%)		
	IC_{50} HFF1	**CC_{50} THP-1**	**CC_{50} HepG2**	**PBMCs**	**RAW 264.7**	**HaCaT**
A. occidentale	31 (23-41)	> 100	79	108	95	23
B. orellana	113 (very wide)	> 100	> 100	105	107	94
C. papaya	> 200	> 50	> 50	96	96	87
C. alatus	**20 (17-25)**	**13**	**28**	**56**	108	48
C. aurantiifolia	> 200	> 100	> 100	95	100	89
J. copaia	83 (49-141)	> 100	> 100	106	104	91
J. curcas	> 200	> 50	> 50	72	**91**	79
M. esculenta	> 200	n.t.	n.t.	99	97	84
M. × paradisiaca	> 200	> 100	> 100	94	97	92
N. tabacum	> 200	> 50	> 50	93	98	79
P. major	102 (very wide)	> 25	> 25	96	94	99
S. mombin	93 (76-114)	> 50	> 50	117	98	**10**
Pentamidine	0.005 (very wide)	n.t.	n.t.	-	-	-
Doxorubicin	n.t.	1.4	0.11	-	-	-
Amphotericin B	n.t.	3.3	n.t.	-	-	-
Miltefosin	n.t.	> 16	n.t.	-	-	-
Dexamethasone	-	-	-	125	-	-
Quercetol	-	-	-	-	106	-
EGF	-	-	-	-	-	124
FCS 0.5%	-	-	-	-	-	109
FCS 10%	-	-	-	-	-	179

n.t. : not tested ; - : not relevant for the considered assay; EGF: Epidermal growth factor; FCS: fetal calf serum
In bold: species with the highest cytotoxicity / lowest cell viability levels in the considered bioassay

682

683 Overall, the obtained results indicated the highest cytotoxicity for *C. alatus*. Notably, *C. alatus*
684 exhibited an IC_{50} value of 20 µg/mL (CI 17-25) against HFF1 cells, and CC_{50} values of 13 and
685 28 µg/mL against THP-1 and HepG2 cell lines respectively. In terms of cell viability, a viability
686 of only 56% was observed for PBMCs exposed to this extract at the highest concentration
687 used (50 µg/mL). No deleterious effect was however observed in the case of the more sensitive
688 murine cell line RAW 264.7, which usually show less survival than other cells at the same
689 extracts concentrations. Alongside to *C. alatus* extract, *A. occidentale* extract had a noticeable

effect on fibroblasts (IC_{50} = 31 µg/mL, CI 23-41), and a slight cytotoxic effect against HepG2 cells (CC_{50} value of 79 µg/mL). Both extracts displayed noticeable toxicity at the highest tested concentration (50 µg/mL) in the context of the migration assay performed on HaCaT cells (Table S12), and the mean values obtained for the two doses were of 48% and 23% respectively. For this same assay, *S. mombin* extract showed the highest toxicity of all plant extracts, with only 10% of viable cells at 50 µg/ml. HFF1 cells were slightly sensitive to *S. mombin* extract, with an IC_{50} value around 100 µg/mL, as also observed for *P. major*, *J. copaia* and *B. orellana* extracts. As well, THP-1 and HepG2 cells were sensitive to *S. mombin* and *P. major* extracts, with CC_{50} values > 50 and > 25 µg/mL respectively. Eventually, *J. curcas* extract displayed the lowest cell viability value against RAW264.7 cells, but with noneless a high survival of the cells (91%). A CC_{50} value > 50 µg/mL was however observed towards THP-1 and HepG2 cells for this extract.

3.6 Biological activity pattern of the extracts

The biological activity scores (BAS), calculated for each independent performed bioassay as indicated in the materials and methods section are presented in Table 7. Cell migration assay was not included in this calculation, as no wound-healing activity was measured in this case for any of the tested extracts.

Table 7: Biological activity scores (BAS) obtained for antileishmanial, antimicrobial, immunomodulation and cytotoxicity bioassays for the tested plant extracts

Species	Antileishmanial activity		Antimicrobial activity	Immunomodulation assays		Cytotoxicity & Cell viability
	Promastigotes	Amastigotes		Non selective	Selective	
A. occidentale	1002	11	1102	1	1000	1014
B. orellana	3	2000	4	1000	1000	24
C. papaya	3	110	4	1	1000	33
C. alatus	3	2000	4	100	1000	3201
C. aurantiifolia	3	110	4	1	1000	15
J. copaia	3	1100	4	1	1000	114
J. curcas	3	20	4	1000	1000	141

M. esculenta	3	2	4	1	1000	15
M. × paradisiaca	3	110	4	1	1000	24
N. tabacum	3	1100	4	1	1000	42
P. major	3	1100	4	1000	10000	222
S. mombin	2001	20	1003	1	1	1122

A principal component analysis performed using R software allow obtaining the biplot presented in Figure 1, and the correlation matrix obtained for BAS is presented in Figure 2. Complementary information about PCA (square cosines, contribution of variables, description of dimensions 1 and 2 and PCA summary) are available in Supplementary Material Tables S14-S16 and Figure S1. The cumulative R^2X of 0.659 suggest a good reliability of the model, and dimensions 1 and 2 account for 42.7% and 23.2% of the variance respectively.

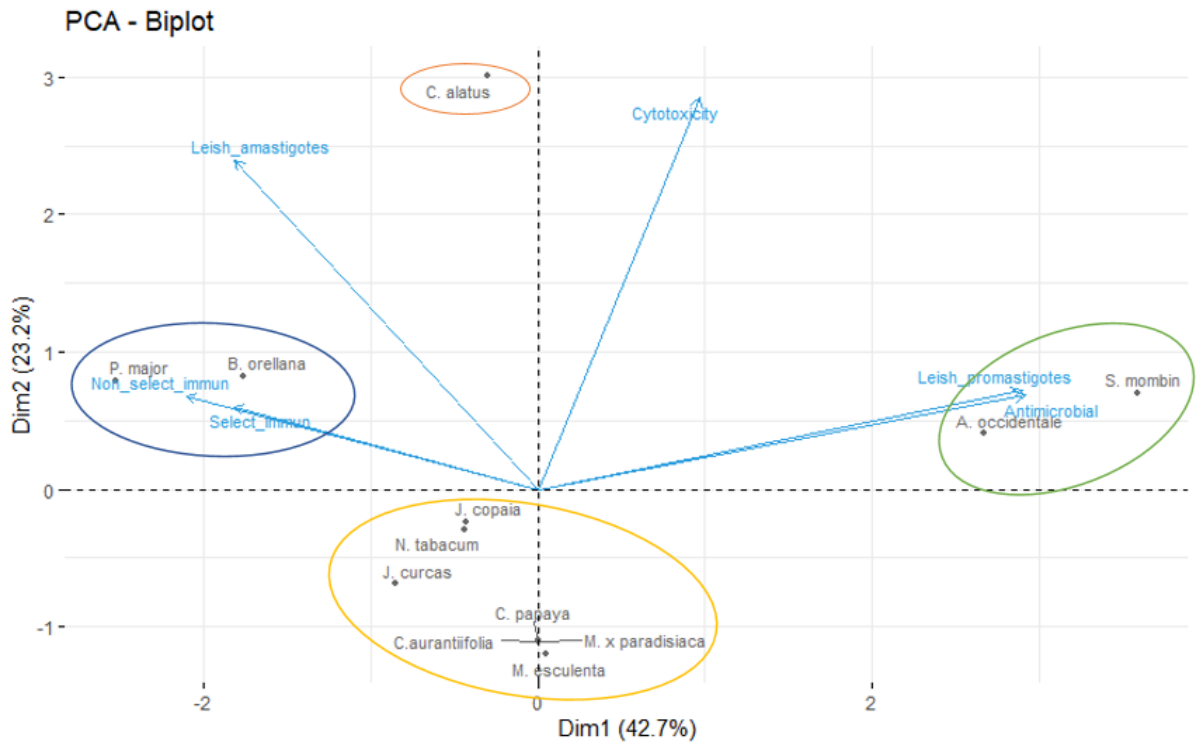


Figure 1 : Biplot obtained for PCA analysis of the scores attributed to plant extracts biological activities scores (BAS). Ellipses were not calculated due to an insufficient number of samples for each group, but added manually to improve results visualization.

Variables observation in the biplot enables us to highlight four plant extracts or groups of plant extracts. These groups were consistent with the total BAS, with BAS > 4000 for the blue, green and red groups, and in the 1000-2000 range for the yellow group. First, *S. mombin* and *A. occidentale* are both characterized by strong anti-promastigotes and antimicrobial activities. On the other hand, *B. orellana* and *P. major* are both representative of plant extracts exhibiting remarkable anti-amastigotes and immunomodulatory activities. Eventually, *C. alatus* stands out for its elevated cytotoxicity, possibly explaining its good activity against *Leishmania* amastigotes. To confirm these groups discrimination from PCA, we performed an OPLS analysis. The following parameters were obtained: cumulatives R^2X of 0.637, R^2Y of 0.912, and a Q^2 value of 0.689, assessing the statistical significance of the groups segregation. OPLS summary is available in Figure S2. The biplot observation indicating parallel results for anti-promastigotes and antimicrobial activities was confirmed by the positive coefficient and *p* value < 0.0001 in the correlation matrix (Figure 2). Other correlations exhibited *p* values in the 0.02 – 0.05 range. Notably, *Leishmania* anti-amastigotes and promastigotes assays results were demonstrated to be negatively correlated (*p* value of 0.0271). Logically, anti-amastigote activity was therefore also negatively correlated to antimicrobial activity (*p* value of 0.0244). The type of immunomodulation assay overall lead to comparable results, non-selective and selective assays results being slightly positively correlated (*p* value of 0.0468). Also, non-selective immunomodulating effect was negatively correlated both with anti-promastigote activity (*p* value of 0.0394) and with antimicrobial activity (*p* value of 0.0382). Cytotoxicity exhibited no statistically significant correlation with any of the other bioassay.

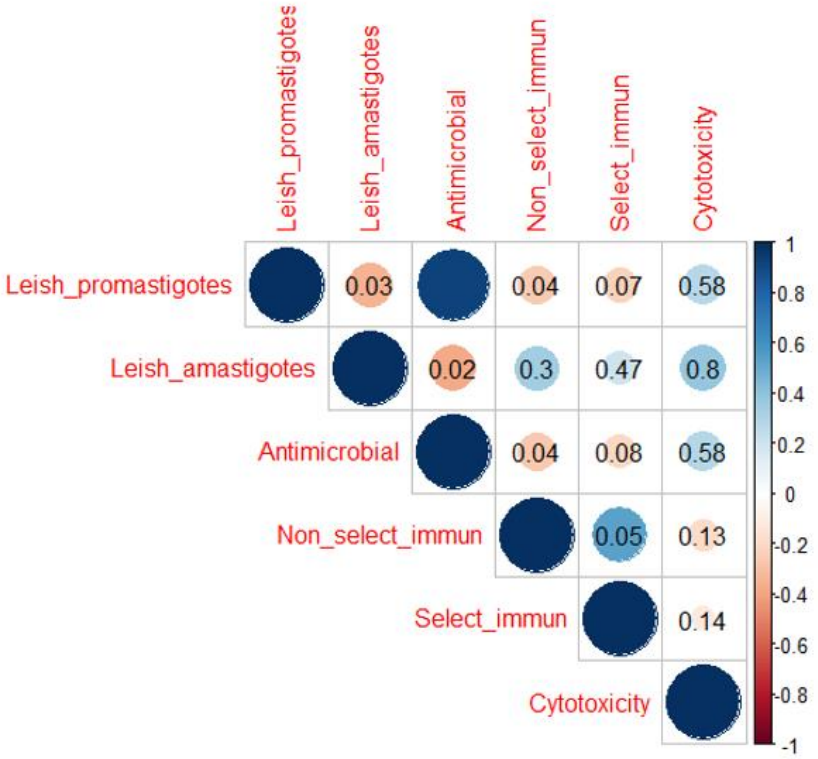


Figure 2: Correlation matrix obtained for biological activity scores of the tested plant extracts. Pearson correlation coefficients are graphically represented as dots in the matrix, depending on their strengh and direction. *P* values ≥ 0.0001 are indicated in the matrix. Without any indications, *p* values are < 0.0001.

4. Discussion

4.1 "Visualizing an elephant": towards a complementary and optimized choice of biological models

Evaluating a range of complementary biological activities can be an asset for natural products valorization in drugs or therapies. It however also help to obtain a more complete picture of the effects of plant extracts in the context of a given pathology, and contribute to take into account the perception of the population concerning the remedies' efficacy in the context of an integrative biological evaluation (Demay et al., 2021; Leduc et al., 2006). This latter approach

761 can thus contribute to “rebuild” the whole elephant, as earlier advocated by Houghton et al.
762 (Houghton et al., 2005, 2007), and to decipher the biological criteria on which a plant use could
763 rely. For antileishmanial assays, both promastigotes and amastigotes were used in our study.
764 In the promastigotes assay, *Leishmania* sensitivity varied widely from one species to another,
765 but also from one strain to another within the same species. Notably, *L. braziliensis* (LB) clinical
766 isolate showed no sensitivity to any extracts compared to the other strains. Laboratory
767 reference strains are good models allowing comparisons between experiments as well as
768 stable and reproducible averages on reference drugs, but sometimes have a pathogenicity far
769 from that of recently isolated parasites. A cultured strain, maintained over several cycles, can
770 indeed have its sensitivity modified, hence the interest to also use patient isolates, which reflect
771 more precisely the sensitivity of circulating strains. The two models are thus complementary,
772 as previously highlighted (Ginouves et al., 2017). Interestingly, plant extracts identified as
773 active against one or several strains of *Leishmania* promastigotes displayed no significant
774 effect in the amastigote model, which was confirmed by the significant negative correlation
775 obtained through statistical analysis of the biological activity scores (*p* value of 0.0271). This
776 result could be related to the species/strains tested in both models, as plant extracts activity
777 on both promastigotes and amastigotes forms have already been observed, for example on *L.*
778 *mexicana* or *L. tropica* (Alamilla-Fonseca et al., 2018; Ilaghi et al., 2021). However, literature
779 defends the intracellular amastigote model as the gold standard, this form better reflecting the
780 observed sensitivity in patients. This model also includes host cell-mediated effects, notably
781 host cell membranes crossing and potential metabolization of the products by the macrophage
782 (De Muylder et al., 2011; Vermeersch et al., 2009). Contrasting results between promastigotes
783 and amastigotes assays could therefore arise from these distinct modes of action.
784 Interestingly, it was previously described that cutaneous leishmaniasis is known in Peru by the
785 Quechua name *uta*, differentiated in *uta de agua* and *uta seca* by the Yanesha community. If
786 *uta de agua* seems to clearly correlate to CL symptoms, *uta seca* could more probably describe
787 fungal infections, in particular due to *Trichophyton* sp. (Valadeau et al., 2009). Investigating

788 plant extracts activity against *T. rubrum* is therefore consistent with some local perceptions of
789 leishmaniasis. We observed in our experimental results that antimicrobial activity parallel anti-
790 promastigotes activity, which was confirmed by the *p* value < 0.0001 calculated in the
791 correlation matrix. This observation is consistent with previous studies concerning natural
792 products such as essential oils (Houël et al., 2015b). It can be put in perspective with the
793 results obtained for several commercial drugs such as miltefosine, amphotericin B and azoles,
794 exhibiting bioactivity against both filamentous dermatophytic fungi and *Leishmania* sp.
795 amastigotes (Moskowitz and Kurban, 1999; Shakya et al., 2011; Tong et al., 2007), due to
796 involvement of these compounds with the sterols of fungal or parasitic membrane.
797 In the case of *in vitro* immunomodulation bioassays, we embarked upon comparing reference
798 assays (naïve cells with non-selective stimulation) and biological assays more closely linked
799 to the medical context of leishmaniasis in Amazonia, by using specific stimulation (*Leishmania*
800 antigen) on non-naïve cells (PBMCs isolated from patients suffering from cutaneous
801 leishmaniasis due to *L. guyanensis* infection). Using PBMCs from patients, stimulated with
802 *Leishmania* antigens or parasites, is a common protocol when trying to decipher the immune
803 response associated with *Leishmania* infection (Bourreau et al., 2001; Gomes et al., 2014;
804 Oliveira et al., 2014). However, to our knowledge, immunomodulatory properties of natural
805 products on human PBMCs in experimental leishmaniasis are still poorly documented
806 (Amarante et al., 2012; dos Santos Thomazelli et al., 2017). Some studies are however
807 performed on human cells infected with *Leishmania* parasite (Dayakar et al., 2015; López et
808 al., 2009). In our case, the sometimes-divergent results obtained between the two models
809 demonstrate the impact of host and parasite interaction, even if non-selective and selective
810 assays results were slightly positively correlated (*p* value of 0.0468).
811 Cell migration results were not included in the biological activity score analysis, as none of the
812 tested extracts displayed any biological activity in this assay. However, as for the immune
813 response, wound healing is a complex process. It first involves inflammatory phenomena, with
814 a preponderant role of IL-6, IL-8, VGEF (vascular endothelial growth factor) and IL-10 (Wedler

et al., 2014). Wound healing then relies on keratinocytes and fibroblasts proliferation, before collagen lattice formation and tissue remodelling. Interestingly, it also depends on oxidative conditions and microbial context (Houghton et al., 2005; Kandimalla et al., 2016). Therefore, anti-inflammatory, antioxidant or antimicrobial activities of the plant extracts may also contribute to wound healing activity. The results obtained in our study can thus be discussed as such, even if the absence of measurable effect in the scratch assay deprives us from discussing correlations between the above-cited effects through our BAS analysis. As well, if no concluding results were obtained concerning HaCaT keratinocytes migration, toxicity results on HFF1 fibroblasts can however lead to some informative data on the extracts effect towards human skin cells, and wound healing process.

4.2. Biological activity scores analysis: plants and patterns

4.2.1. *S. mombin* and *A. occidentale* (with *J. curcas* joining the team): pharmacological, ecological or cultural importance?

S. mombin was highlighted in our previous study as the species having the most elevated I_d (general distribution index), and being in particular the species used by the widest number of cultural groups (highest I_c (cultural distribution index) of all listed species) (Odonne et al., 2017). In this case, we hypothesized that compromises between ecological availability, cultural relevance and pharmacological activity were potentially at stake, notably according to (Menendez-Baceta et al., 2015) and (Saslis-Lagoudakis et al., 2012). Eventually, our biological results may rather plead in favor of availability (*S. mombin* is both found wild and cultivated among Amazonian societies, and its fruits are appreciated) or cultural factors, despite the elevated I_c. In our hands, *S. mombin* was indeed highlighted as a taxa leading to strongly pharmacologically active extract, adding novel and complementary information to literature, already widely reviewed in Odonne et al. (2017), with some new additions (Roumy et al., 2020). The strong biological effects observed in our study were measured against a very limited range of biological targets (*L. guyanensis* reference and clinical strains, alongside with *C. albicans*).

842 The parasite targets furthermore consisted in the promastigote form of *Leishmania* parasites,
843 i.e. the most distant parasite model in terms of biological complexity of the disease (Passero
844 et al., 2021). In addition, according to the immunomodulatory and cytotoxicity results, as well
845 as the absence of effect of *S. mombin* extract in the scratch assay, only the antimicrobial
846 activity alone could account for an interesting activity in a wound healing perspective. When it
847 comes to phytochemistry, few data are available in the literature about this species bark
848 chemical composition. A high content in polyphenols (35%), alongside with the presence of
849 flavonoids, was recently highlighted in *S. mombin* bark ethanolic extract (Clementino et al.,
850 2018). Flavonoids, ellagitanins and phenolic acids are also the main compounds found in
851 leaves extracts, as reviewed in our previous work (Odonne et al., 2017). However, these widely
852 ubiquitous compounds cannot easily be related to a specific biological activity, but could
853 reasonably account for the antimicrobial and antipromastigote activity observed in this study.
854 The question that remains is to understand if anti-promastigotes and antimicrobial activity
855 alone are sufficient biological effects to drive *S. mombin* selection as renowned antileishmanial
856 species. Several previous studies could however shed interesting light on the importance of
857 cultural factors when it comes to *S. mombin* medicinal use in case of *Leishmaniasis*
858 occurrence. This species is indeed included in the numerous plants used for disinfect or heal
859 injuries and selected by Peruvian populations to treat leishmaniasis, there considered as a
860 wound outbreak and not a parasitic infection (Kvist et al., 2006). Alongside with some
861 antimicrobial activity and high availability, this could be responsible of the widespread use of
862 *S. mombin* across Amazonia. Eventually, the use of this species could also be related to the
863 important suberization of its bark, which may symbolically be associated with the regeneration
864 of the skin (Odonne et al., 2011, 2017). Confronting physiological or anatomical properties of
865 the plant and the disease perceived characteristics is indeed consistent with a widely found
866 way of memorizing and disseminating information among traditional cultures (Bennett, 2007).
867 Complementary, the perceived toxicity of this species could also be investigated, as its extracts
868 exhibited noticeable effect on one of the tested cell lines.

869 *A. occidentale* was characterized by a rather high I_d (7th rank on 31 listed species) and high I_c,
870 but a medium I_g in our previous analysis (Odonne et al., 2017). These medium values, and the
871 absence of information about cultural significance of this species in the literature, make
872 hypothesis and interpretations of the weight of the different factors (ecological, cultural and
873 pharmacological) quite difficult. The biological activity profile obtained in our hands for *A.*
874 *occidentale* extract strictly mirror the one observed for *S. mombin*, with an antileishmanial
875 effect observed against *L. amazonensis* and *L. guyanensis* reference strains promastigotes
876 and an antimicrobial effect against *C. albicans* and *T. rubrum*, without any other remarkable
877 activity among the selected assays. Interestingly, the chemical composition of *A. occidentale*
878 leaves and bark ethanolic extracts described in the literature is also close to the one of *S.*
879 *mombin* bark extracts in terms of major phytochemical classes, with the presence of phenolic
880 acids, flavonoids and tannins (Costa et al., 2020). Gallic acid, catechin and quercetin were
881 notably identified from a methanol bark extract (Duangjan et al., 2021). Thus, the observed
882 biological activity could be related to such compounds, which is for example highlighted in a
883 recent study about *A. occidentale* bark anti-candidal activity (Costa et al., 2021), but also to
884 the presence of essential oil in leaves (Kossouch et al., 2008; Montari et al., 2012). Indeed,
885 mono- and sesquiterpenes are known for their antimicrobial, but also antiparasitic activities
886 (Houël et al., 2015b). Other factors than pharmacological activity should however explain this
887 species' reputation. Notably, the presence of corrosive substances in *A. occidentale* nut
888 (anacardic acid, cardanol, cardol) places this species in the category of irritating and burning
889 plants (Carvalho et al., 2019). In relation with the words of Ramdas "*cruel disease, cruel*
890 *medicine"*, this species physiological particularity could therefore account for its stem bark or
891 leaves use against leihmaniasis (França et al., 1996; Ramdas, 2012). Moreover, *A. occidentale*
892 is a widely distributed cultivated species, of alimentary importance and easily recognizable,
893 and these parameters may also explain its use and good reputation (Santos et al., 2018).
894 *J. curcas* was also part of the species exhibiting the highest I_ds, and its use could also be linked
895 to the caustic properties of the plant latex, through the convergent use of irritating plants for

ulcerating diseases and despite the rather low biological activity pattern observed in our study. Painful treatment using plant latex against leishmaniasis were also described in Peru, for example with *Acalypha macrostachya* Jacq. (Euphorbiaceae) latex by the Yanesha community (Valadeau et al., 2009), or *Hura crepitans* L. and *Sapium marmieri* Huber (Euphorbiaceae) sap by the Chayahuita (Odonne et al., 2009). However, numerous uses and pharmacological effects against *Leishmania* spp. were already highlighted, as reviewed in our previous paper. This species could therefore deserve further investigation to disentangle such contradictory results. (Dzoyem and Eloff, 2015; Othman et al., 2015; Prasad et al., 2012; Ribeiro Neto et al., 2020; Villegas et al., 1997).

4.2.2. *P. major* and *B. orellana*: Old and New world taxa against leishmaniasis

P. major is a ubiquitous species in many parts of the world, and can even be cited as one of the most commonly used medicinal species. It is indeed a major component of numerous pharmacopeias, especially for wound healing, skin diseases, infections or disorders (burns, bruises, cuts…) among other uses (Adom et al., 2017; Gonçalves and Romano, 2016; Mazzei et al., 2020; Moerman, 2007; Samuelsen, 2000). *B. orellana* on its part is native from South America, and more specifically from the Amazon region, where its seeds are notably renowned in cases of bruises and wounds, and more generally for wound healing objectives (Odonne et al., 2011; Vilar et al., 2014). The inclusion of *P. major* as an exotic species for wound healing purposes concurrently to *B. orellana* can be interestingly noted as discussed in the literature (De Medeiros et al., 2017; Gama et al., 2018).

In our previous study, we identified *P. major* as one of the species characterized by a medium to low I_c and a high I_g, and therefore a commonly used plant throughout Amazonia. Our hypothesis was then that a convergence of practices due to effective pharmacological activities drove these taxa selection rather than cultural factors (Odonne et al., 2017). Although obtaining slightly lesser I_g and I_c scores, *B. orellana* could also be included in the same category. With marked antileishmanial activities in the intracellular amastigotes model, and remarkable pro-

inflammatory effects notably in the case of *P. major* extract, these two species indeed exhibited a similar and original profile in our assay, alongside with low cytotoxicity, pleading in favor of a safe use of the extract. These data are globally consistent with previously published information (Adom et al., 2017; Braga et al., 2007; Chariandy et al., 1999; Gomez-Flores et al., 2000; Hussan et al., 2015; Monzote et al., 2014; Samuelsen, 2000; Ulbricht et al., 2012; Vilar et al., 2014), even if some disparities could be observed concerning antimicrobial and wound healing properties, possibly due to differences in chemical composition of the extracts (Grozdanova et al., 2020; Kartini et al., 2021; Mazzei et al., 2020). To our knowledge, this is notably the first report of an antileishmanial activity for a *P. major* leaves extract, even if the use of this species was specified for the treatment of *L. brasiliensis* ulcers in Brazil (França et al., 1996). In terms of immunomodulatory or anti-inflammatory properties, few studies appear available concerning *B. orellana* crude plant extracts, and concern leaves (Lima Viana et al., 2018; Yong et al., 2018). Bixin however, the major natural carotenoid extracted from *B. orellana* seeds, is highlighted for its anti-inflammatory properties, notably in various *in vivo* models (Pacheco et al., 2019; Somacal et al., 2015; Xu and Kong, 2017). Many other carotenoids were also isolated from the seeds and could also account for the observed biological effect (Vilar et al., 2014). On their side, *P. major* leaves extracts are well known for the presence of numerous classes of anti-inflammatory or immunostimulatory compounds (polysaccharides, triterpenic acids such as oleanolic and ursolic acids, caffeic acid derivatives such as plantamajoside, flavonoids (scutellarein, baicalein, hispidulin) and iridoid glycosides, notably aucubin), as previously reviewed (Samuelsen, 2000, Odonne et al., 2017). Overall, these biological profiles, supported by the extracts chemical composition, could very reasonably account for the good reputation of these species across Amazonia in case of *Leishmania* infections.

However, it would be presumptuous to conclude at this stage of the study about the precise mode of action of this given plant extracts in patients. Indeed, in the case of leishmaniasis infection, cytokines play complex and dual role, also depending on infection stage in the patient

and parasite species, having either protective or detrimental role, inside a network of regulatory, counter-regulatory and inter-regulatory interactions (Alexander and Bryson, 2005; Espir et al., 2014; Oliveira et al., 2014; Scott and Novais, 2016; Soong et al., 2012). This could for example explain the absence of effect of *P. major* extract on IL-10 in the selective assay compared to the non-selective one. The extract was indeed tested on a patient affected by a lesion older than 1 month thus implicating a Th1 response driven by INF- γ and not IL-10, whereas this cytokine plays a central role in leishmaniasis pathophysiology (Th2 response) in the case of more recent lesions (Bourreau et al., 2003). Also, our immunomodulatory results highlighted strong pro-inflammatory effects of the two extracts, promoting TNF-α and/or IL-6 secretion in both non-selective and selective assays for *P. major* and *B. orellana*, respectively. Yet, if both TNF-α and IL-6 are necessary to initiate and promote wound healing process in its inflammatory phase, high concentrations are detrimental and regulation of these cytokines is necessary (Wedler et al., 2014). Thus, a balance has to be found between pro- and anti-inflammatory response, and plant extracts upregulating the secretion of pro-inflammatory cytokines may also present interest in the search for active remedies against leishmaniasis (Chouhan et al., 2014; Rodrigues et al., 2015).

4.2.3. Use and activity pattern for other plant extracts and the particular case of *C. alatus*: what's the story?

In our previous work, which led to this study's taxa selection, several species (*N. tabacum*, *M. x paradisiaca*, *C. papaya*, *C. alatus* and *J. copaia*) were grouped as characterized by a medium to low I_c and a high I_g alongside with *P. major*. To this group could also reasonably be included *C. aurantiifolia* and *B. orellana*, also tested in this study. As explained earlier, we postulated that these species should lead to biologically strongly active extracts, cultural factors being less predominant in this case (Odonne et al., 2017). This was also previously assumed by (Kvist et al., 2006) or (Saslis-Lagoudakis et al., 2011), based on the hypothesis of a gradual geographical diffusion of positive experiences with remedies. However, when evaluating a

network of pharmacological activities linked to leishmaniasis, only *P. major*, *B. orellana* and *C. alatus* standed out for this group of species. In our hands, *C. alatus* was shown to exhibit an interesting activity against *L. amazonensis* amastigotes, consistently with previously published data (Valadeau et al., 2009). This may be due to the presence of irlbacholine, which is an original dimer of the synthetic molecule miltefosine used as antifungal but also antileishmanial compound (Lu et al., 1999; Passero et al., 2018). However, this result has to be related to the elevated cytotoxicity observed against most of the tested cell lines used in our assay. *C. alatus* was indeed highlighted as the most cytotoxic extract against HFF1, THP-1, HepG2 cells and PBMCs, and exerted marked toxicity against HaCaT cells. These cytotoxic activities are indeed a strong biological effect as postulated for this taxa category, but cannot explain the wide use of this plant across Amazonia.

According to biological activity scores (BAS) and PCA analysis, and contrary to our initial hypothesis, *N. tabacum*, *M.* × *paradisiaca*, *C. papaya*, *C. aurantiifolia* and *J. copaia* were not characterized by any particularly strong biological activity, or specific biological activity pattern. Only noticeable effect in the intracellular *L. amazonensis* amastigotes model was observed for *N. tabacum* and *J. copaia* extracts, supporting previous data in this field (Ilaghi et al., 2021; Sauvain et al., 1993; Valadeau et al., 2009), and alongside with some immunomodulating activity. If the chemical composition of *N. tabacum* leaves is extensively described, as previously reviewed (Odonne et al., 2017) with notably the well-known alcaloid nicotine, but also isocoumarins, flavononoids, lignans and lignan derivatives, phenylpropanoids and sesquiterpenes, to our knowledge no link between antileishmanial activity and specific metabolites was explored up to date. Concerning *J. copaia*, few data are on the contrary currently available concerning leaves extracts. Two compounds, ursolic acid and jacaranone, a quinoid derivative, were however isolated from a dichloromethane leaves extract (Gachet and Schühly, 2009; Sauvain et al., 1993). Interestingly, the pure compound ursolic acid was shown to be active against *L. amazonensis* amastigotes (ED_{50} of 20 µM) in vitro, without toxicity

towards BALB/c mice macrophages, and also diplayed in vivo results of interest (Sauvain et al., 1993). This compounds could therefore account for the activity observed in our assay. On the contrary, jacaranone was previously found active against *L. amazonensis* promastigotes, but with a noticeable toxicity against macrophages, this toxic effect being confirmed by in vivo studies (Sauvain et al., 1993). In terms of ethnobotanical considerations, *N. tabacum* is notably renowned as one of the major hallucinogenic plant and a central cultural species among Amazonian indigenous groups of Amazonia (Bennett, 1992; Odonne et al., 2013; Schultes and Raffauf, 1990; Valadeau et al., 2010). This strong cultural significance could therefore counterbalance a limited pharmacological efficacy. However, this species is also favored in other traditional medicinal systems for skin infections, and could thus deserve further pharmacological investigation (Mazzei et al., 2020). *Jacaranda* species for their part are pioneer trees common in Amazonia and known for numerous ethnopharmalogical uses, notably *J. copaia* (Gachet and Schühly, 2009; Kffuri et al., 2016; Scotti-Saintagne et al., 2012). In our case, this species' reputation could therefore be based on the good knowledge of this plant. Also, the "wildness" of a plant was described as a possible guarantee of effectiveness among the Chayahuita community in Peru (Odonne et al., 2013). Being both wild and accessible could therefore account for *J. copaia* good reputation, even if complementary biological evaluation, for example including other extract preparation modes, could be of interest.

The three last species of this group, *M. x paradiasiaca*, *C. papaya* and *C. aurantiifolia*, were highlighted by PCA as the least globally active species, alongside with *M. esculenta*. Various biological evaluations of extracts and compounds isolated from these species or related species were previously reviewed (Chariandy et al., 1999; Kvist et al., 2006; Mans et al., 2016; Mazzei et al., 2020; Odonne et al., 2017; Roumy et al., 2020; Valadeau et al., 2009). Interestingly, *M.* × *paradisiaca*, *C. papaya* and the above discussed species *N. tabacum* were highlighted in a recent review a promising candidates for the discovery of bioactive

antileishmanial products, based on their frequency of citation in a literature review (Passero et al., 2021). However, our current results clearly argue for much complex scheme to explain a marked medicinal reputation of a given species. *M. esculenta* was for its part included in the taxa having the highest general distribution index (I_d), suggesting the existence of various factors explaining their use and good reputation, like *S. mombin*, *A. occidentale* and *J. curcas*. Interestingly, *M. x paradiasiaca*, *C. papaya*, *C. aurantiifolia* and *M. esculenta* are all cultivated for edible purpose. Therefore, and as highlighted in our previous survey (Odonne et al., 2017), the low activity/high availability compromise could be the major pattern explaining the antileishmanial use of these species, independently of I_c and I_g values. This possibility was also proposed by Kvist *et al*. in the case of antileishmanial and antimalarial plants in Peru, assuming that the use of commonly and easily accessible species could be an alternative for the populations while waiting to identify highly efficient and easily accessible species (Kvist et al., 2006). Another example of cultivated plants of dietary interest widely used in local pharmacopeia was given in a maroon community in Brazil, yards and homegarden hosting the majority of the cited medicinal species (De Santana et al., 2016). Depending on the cultural and geopolitical context, the importance of cultivated plants of edible interest may either reflect land-use changes and the increase of disturbed ecological habitats around villages alongside a loss of medicinal knowledge associated to wild / forest species, or a learning process of old growth forest species medicinal properties still in progress (Hoffman, 2013; Odonne et al., 2011).

Conclusion

As a first result, we highlighted pharmacologically active extracts according to different biological patterns, with effects exerted either on the parasite or on associated pathophysiological aspects. Notably, *P. major* and *B. orellana* appeared as the most promising species, acting on *Leishmania* amastigote forms and immunomodulatory processes, alongside with low cytotoxicity, even if their potent immunomodulatory properties should be more finely

explored due to the subtlety of the implied mechanisms. *S. mombin* and *A. occidentale* displayed strong antiparasitic and antimicrobial activities, but the lower consistency of the biological target (*Leishmania* promastigotes) alongside with a more marked cytotoxicity appeal for careful investigations. These data overall support the fact that the most comprehensive and integrated biological approaches possible are necessary to obtain a better picture of phytotherapeutic remedies activity, notably in the case of complex diseases such as leishmaniasis. Moreover, besides gaining insight into the pharmacological bases that could account for the maintenance of these plants use, these results, which should be supplemented by clinical studies, could also provide new data for the search of new natural products in the case of *Leishmania* infections. They could also be an opportunity to deepen the phytochemical knowledge of these renowned antileishmanial species, notably through network pharmacology approaches, taking into account chemical complexity, synergies and network targets interacting with multi-compounds extracts. Eventually, this information may also allow increasing the global comprehension of the factors explaining a similarity of medicinal use for plants across different cultures and geographical areas. Indeed, plant symbolism, major cultural importance as magical or dietary species, use shifts linked to leishmaniasis etiology perception, and ecological status and availability were highlighted as potentially strong factors. They accompanied pharmacologically significant extracts, but also counterbalanced biological activities limited in terms of intensity or range highlighted in our assays, thus illustrating that considering a wide geographical use alone is not a sufficient factor to predict relevant pharmacological effects. These results appeal for the development of methods to quantify more precisely the weight of the different parameters. Eventually, the obtained results allowed us to bring to light and discuss the importance of evolution, transformation and hybridization when it comes to medicinal plant use. Changing the paradigm and re-integrating traditional remedies in what they really are, that is a complex mix of more or less pharmacologically active products used in a given geographical and cultural context by living people could thus help to

progress towards a better understanding of the dynamic patterns underlying the maintenance of phytotherapeutic preparations through cultures and spaces.

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CRediT author statement

Emeline Houël: Project administration, Funding acquisition, Conceptualization, Formal analysis, Writing - Original draft preparation. **Marine Ginouves**; **Sébastien Hutter; Adeline Knittel-Obrecht; Eliane Bourreau**: Methodology, Investigation, Formal analysis. **Pascal Villa:** Methodology, Supervision, Resources. **Nadine Azas; Ghislaine Prévot;** Methodology, Supervision. **Véronique Eparvier:** Funding acquisition, Investigation, Formal analysis. **Catherine Vonthron-Sénécheau:** Funding acquisition, Methodology. **Arnaud Jahn-Oyac:** Resources, Investigation. **Guillaume Odonne:** Project administration, Funding acquisition, Conceptualization, Resources, Writing - Original draft preparation. **All authors participated in:** Writing- Review and Editing.

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