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Deciphering anti-infectious compounds from Peruvian medicinal Cordoncillos extract library through multiplexed assays and chemical profiling

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Author contribution statement

Conceptualization: PGVO, AM, GM, and LRM; Methodology: PGVO, AM, GM, and LRM; Chemical data acquisition/curation: PGVO, KL, GM, AG, and AM; Statistical analysis: PGVO and GM; Biological data curation: SC, VR, SB, and SP; Investigation: PGVO, SC, VR, GM, SP, AG, KL, ID, LRV, HRC, WRM, KL, SB, LRM, and AM. PGVO wrote the original draft and all the authors contributed to the final manuscript.

Keywords

Anti-infectious diseases, Metabolomics, Peruvian amazonia, Piper, Cordoncillos

Abstract

Word count: 274

High prevalence of parasitic or bacterial infectious diseases in some world areas is due to multiple reasons, including a lack of an appropriate health policy, challenging logistics and poverty. The support to research and development of new medicines to fight infectious diseases is one of the sustainable development goals promoted by World Health Organization (WHO). In this sense, the traditional medicinal knowledge substantiated by ethnopharmacology is a valuable starting point for drug discovery. This work aims at the scientific validation of the traditional use of Piper species ("Cordoncillos") as firsthand anti-infectious medicines. For this purpose, we adapted a computational statistical model to correlate the LCMS chemical profiles of 54 extracts from 19 Piper species to their corresponding anti-infectious assay results based on 37 microbial or parasites strains. We mainly identified two groups of bioactive compounds (called features as they are considered at the analytical level and are not formally isolated). Group 1 is composed of 11 features being highly correlated to an inhibiting activity on 21 bacteria (principally Gram-positive strains), one fungus (C. albicans), and one parasite (Trypanosoma brucei gambiense). The group 2 is composed of 9 features having a clear selectivity on Leishmania (all strains, both axenic and intramacrophagic). Bioactive features in group 1 were identified principally in the extracts of Piper strigosum and P. xanthostachyum. In group 2, bioactive features were distributed in the extracts of 14 Piper species. This multiplexed approach provided a broad picture of the metabolome as well as a map of compounds putatively associated to bioactivity. To our knowledge, the implementation of this type of metabolomics tools aimed at identifying bioactive compounds has not been used so far.

Contribution to the field

It describes an original approach using computational statistical data treatment to identify putative bioactive compounds in natural extracts. In a nutshell, by crossing the results of the testing of 54 extracts on 37 anti-infectious assays (bacteria, fungus and parasites), with the HPLC-HR MSMS profiling of these extracts, we identified 20 compounds most likely responsible for the activity. Among these compounds, 17 were annotated and given a putative structure. The efficacy of such an approach to point out compounds of biological relevance in complex extracts is of great interest and is a significant improvement compared to more traditional approaches used for natural product-based drug discovery. Therefore, we believe this work deserves to be published in Frontiers in Pharmacology.

Ethics statements

Studies involving animal subjects Generated Statement: No animal studies are presented in this manuscript.

Studies involving human subjects

Generated Statement: No human studies are presented in this manuscript.

Inclusion of identifiable human data

Generated Statement: No potentially identifiable human images or data is presented in this study.

Data availability statement

Generated Statement: The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Inteview



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- 34 Abstract
- 35 High prevalence of parasitic or bacterial infectious diseases in some world areas is due to multiple
- 36 reasons, including a lack of an appropriate health policy, challenging logistics and poverty. The support
- 37 to research and development of new medicines to fight infectious diseases is one of the sustainable
- 38 development goals promoted by World Health Organization (WHO). In this sense, the traditional
- medicinal knowledge substantiated by ethnopharmacology is a valuable starting point for drug 39
- 40 discovery. This work aims at the scientific validation of the traditional use of Piper species
- 41 ("Cordoncillos") as firsthand anti-infectious medicines. For this purpose, we adapted a computational

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- 42 statistical model to correlate the LCMS chemical profiles of 54 extracts from 19 *Piper* species to their
- 43 corresponding anti-infectious assay results based on 37 microbial or parasites strains. We mainly
- 44 identified two groups of bioactive compounds (called features as they are considered at the analytical
- 45 level and are not formally isolated). Group 1 is composed of 11 features being highly correlated to an
- 46 inhibiting activity on 21 bacteria (principally Gram-positive strains), one fungus (C. albicans), and one
- 47 parasite (Trypanosoma brucei gambiense). The group 2 is composed of 9 features having a clear
- 48 selectivity on *Leishmania* (all strains, both axenic and intramacrophagic). Bioactive features in group
- 49 1 were identified principally in the extracts of *Piper strigosum* and *P. xanthostachyum*. In group 2,
- 50 bioactive features were distributed in the extracts of 14 Piper species. This multiplexed approach
- 51 provided a broad picture of the metabolome as well as a map of compounds putatively associated to
- 52 bioactivity. To our knowledge, the implementation of this type of metabolomics tools aimed at
- 53 identifying bioactive compounds has not been used so far.
- 54 Keywords: Anti-infectious diseases, *Cordoncillos*, metabolomics, Peruvian Amazonia, *Piper*



55 INTRODUCTION

56 In Amazonia, tropical diseases have a high prevalence due to multiple reasons, including a lack of an 57 appropriate health policy, a climate conducive to diseases, challenging logistics and poverty. Neglected 58 tropical diseases (NTDs) are a group of 20 conditions (caused by viruses, protozoa, helminths and 59 bacteria) prioritized by the World Health Organization (WHO). The NTDs are responsible for 60 approximately 200,000 deaths and the loss of 19 million disability-adjusted life years (DALYs) 61 annually. In 2020, new infections by *Plasmodium* spp. and *Leishmania* spp. worldwide were estimated 62 at 241 and 1 million, respectively. Even if *Plasmodium* is not strictly classified as a NTD, it caused around 627,000 deaths in 2020, with two-thirds of these deaths (470,000) being due to treatment 63 64 disruptions during the COVID-19 pandemic (WHO, 2021). Leishmania spp. and Trypanosoma spp. are protozoan parasites responsible forof diseases labeled as NTDs stricto sensu. The ambitious 10-65 year WHO plan to defeat NTDs is based on three pillars: control, elimination and eradication (WHO, 66 2022a). The support of research and development of new medicines to fight infectious diseases is one 67 68 of the sustainable development goals promoted by WHO (WHO, 2022b). Drug resistance is a 69 widespread concern in medical care, and the increase of drug-resistant infections is faster than the pace 70 of the development of new drugs approved for use in humans. Therefore, every input into the search 71 for new antimicrobial agents is welcome (Yan et al., 2021). Ethnopharmacology is the interdisciplinary 72 study of the knowledge or practices of traditional cultures related to plants, animals, or mineral used 73 for therapeutic purposes (SFE, 2022). Such knowledge can be valued as the starting point for drug 74 discovery, especially in the case of infectious diseases which are prevalent among such populations.

75 Piperaceae is a pantropical family composed of eight genera. Two genera are the most representative 76 in this family: Piper and Peperomia (Vásquez-Ocmín et al., 2017; Salehi et al., 2019). Among these, 77 *Piper* is the most diverse and representative genus, encompassing *ca.* 2600 species (Trujillo et al., 78 2022). Besides pungent compounds, many *Piper* species produce essential oils and are hence highly 79 aromatic, explaining their use for cooking and medicinal purposes (Ruiz-Vásquez et al., 2022). In Peru, 80 *Piper* spp. (called "Cordoncillos") have been used for a very long time in traditional medicine as a "first-hand treatment", especially in the villages far away from major cities and medical care. The 81 82 necessary scientific validation of traditional uses implies the isolation of the main bioactive compounds 83 by successive fractionations using chromatographic techniques and biological activity testing (bio-84 guided isolation). Such an approach has evidenced interesting activities for Piper spp. as anti-85 inflammatory, antiparasitic, antibacterial, etc. (Mgbeahuruike et al., 2017; Durant-Archibold et al., 86 2018). Even if bio-guided fractionation is still used with some success in the natural product chemistry 87 field, current trends involve streamlining the cost, effort, and time (Vásquez-Ocmín et al., 2022a). 88 Metabolomics is a holistic approach allowing rapid detection and putative identification (*i.e.* 89 annotation) of numerous metabolites, along with data mining on multiple datasets. Metabolomics aims 90 to comprehensively map all biochemical reactions in a given system and has become a key to 91 deciphering their biological roles, hence becoming mainstream in natural product chemistry and drug 92 discovery. This approach can be divided into targeted and untargeted analyses (Alarcon-Barrera et al., 93 2022). Two spectral techniques are regularly employed for metabolomics analysis: mass spectrometry 94 (MS) and nuclear magnetic resonance (NMR) spectroscopy, both being assisted by bioinformatics and 95 statistical analysis. Our team has solid expertise in implementing straightforward and effective 96 workflows to decipher anti-infectious compounds using untargeted metabolomics (Vásquez-Ocmín et 97 al., 2021a). 98 This work aims at the scientific validation of the use of *Piper* species as firsthand anti-infectious

99 medicines. We adapted a statistical model to correlate the LCMS chemical profiles of 54 extracts from

- 100 19 *Piper* species to their corresponding anti-infectious assay results based on 37 microbial or parasites
- 101 strains. This multiplexed approach led to the annotation of compounds bearing these activities.

102 MATERIALS AND METHODS

103 Ethnopharmacology and Plant Material

104 Based on the encouraging previous results of our research group on the anti-infectious activities of 105 Piper species (Vásquez-Ocmín et al., 2021a), ethnopharmacological surveys were realized as part of 106 the project "Compuestos bioactivos in vitro a partir de especies vegetales Amazónicas". The surveys 107 were undertaken between July and December 2020 in communities of three Amazonian regions of 108 Peru: Cusco, Loreto and San Martin. People of these communities were interrogated about the main 109 use of medicinal Piper species ("Cordoncillos"), including their use against malaria, "uta" (local name 110 for leishmaniasis) and bacteria. According to ethnopharmacological studies, we collected 8 species in 111 Cusco, 10 species in Loreto, and 1 species in San Martin.

- 112 Thus, different parts of these nineteen plants were collected, then identified and deposited in the
- 113 Herbarium Amazonense (AMAZ), Iquitos, Peru. This project was realized in accordance with the
- 114 guidelines pertaining to ethnopharmacological studies and edited by the Laboratorio de Investigacion
- 115 de Productos Naturales Antiparasitarios de la Amazonia (LIPNAA) of the Universidad Nacional de la
- 116 Amazonia Peruana (UNAP) (Resolucion Rectoral Nº 1312-2020-UNAP).

117 **Preparation of plant extracts**

118 One hundred grams of each air-dried and ground plant (leaves, leaves and stems, aerial parts) were

soaked in 1 L of each of the following solvents successively: hexane, methylene chloride, methanol,

ethanol/water (7:3, v/v), and aqueous. <u>These macerations</u> were <u>made performed during</u>for

- 21 days <u>each</u> for hexane, methylene chloride and ethanol/water, and for 15 days in methanol and
- aqueous extracts, with solvents being changed every 3 days. The extracts were then filtered through a paper filter and evaporated under reduced pressure below 40°C. Dry extracts were stored at -4 °C until
- use. Extracts were solubilized in DMSO at a concentration of 10 mg/mL for *in vitro* bioassays and
- 125 HPLC-MS analysis.

5

126 Cell lines and microorganism culture

127 HUVEC cells: Human umbilical vein endothelial cells (HUVECs) were maintained in culture in RPMI

128 1640 medium (Invitrogen, Life Technologies) supplemented with 10 % heat-inactivated fetal bovine

129 serum (Invitrogen Life Technologies) and 1 mM glutamine (Invitrogen Life Technologies) (Vásquez-

- 130 Ocmín et al., 2018).
- 131 RAW264.7: The mouse monocyte/macrophage cell line RAW264.7 was maintained in culture in
- 132 DMEM (Invitrogen, Life Technologies) supplemented with 10 % heat-inactivated fetal bovine serum
- 133 (Vásquez-Ocmín et al., 2018).

Plasmodium: The *P. falciparum* chloroquine-sensitive strain 3D7 was obtained from the Malaria
 French National Reference Center (CNR Paludisme, Hôpital Bichat Claude Bernard, Paris) and was
 maintained in O⁺ human erythrocytes in RPMI 1640 medium (Invitrogen, Life Technologies)
 supplemented with 25 mM HEPES (Sigma), 25 mM NaHCO₃ (Sigma) and 0.5 % Albumax II

- 137 supplemented with 25 mM HEPES (Sigma), 25 mM NaHCO₃ (Sigma) and 0.5 % Albumax II 138 (Invitrogen, Life Technologies) at 37°C in a candle-jar method following the Trager and Jensen
- 139 conditions (Trager and Jensen, 1976; Lambros and Vanderberg, 1979; Vásquez-Ocmín et al., 2018).

140 Leishmania: The L. donovani MHOM/ET/67/HU3, L. amazonensis MHOM/BR/73/M2269, and L.

- 141 *braziliensis* MHOM/BR/75/M2903b strains were maintained routinely in *in vitro* culture. Passages in
- 142 macrophages RAW 264.7 were carried out regularly to preserve the virulence of the strain, then they
- 143 were recovered for culture maintenance in the promastigote form. For assays, parasites were
- maintained as promastigote forms in M-199 medium (Sigma) supplemented with 40 mM HEPES, 100
- 145 mM adenosine, 0.5 mg/L hemin and 10 % fetal bovine serum (FBS) at 25° C in a dark environment
- 146 (Balaraman et al., 2015; Vásquez-Ocmín et al., 2018).

147 *Trypanosomes*: Trypomastigotes of *T. b. gambiense* (FéoITMAP/1893 strain) were grown in HMI9 148 medium constituted of prepacked Iscove's modified Dulbecco's medium (Thermo-Fisher, Les Ulis,

France) supplemented with 36 mM NaHCO₃, 1 mM hypoxanthine, 0.05 mM bathocuproine, 0.16 mM

150 thymidine, 0.2 mM 2-mercapthoethanol, 1.5 mM L-cysteine, 10 % heat-inactivated foetal bovine

- serum, 100 IU penicillin and 100 μ g.mL⁻¹ streptomycin. Parasites were incubated in a Series 8000
- 152 direct-heat CO₂ incubator (Thermo-Fisher, Les Ulis, France) at 37°C in a water-saturated atmosphere
- 153 containing 5 % CO₂ (Pomel et al., 2015; Vásquez-Ocmín et al., 2018).
- 154 Bacteria and yeast:
- 155 Most microbial strains were diluted in BH medium (Brain Heart), MH medium (Mueller Hinton) for
- 156 Candida sp. and Mycobacterium sp., or WW medium (Wilkins-West) for Streptococcus sp. and stored
- 157 at -20°C. Then strains were subcultured at 20°C on RC (Ringer Cysteine) medium for 24 hours before
- 158 tests (Bocquet et al., 2019).

159 In vitro antiprotozoal activity

- 160 In vitro antiplasmodial activity on P. falciparum
- Assays were realized with a suspension of erythrocytes at 1 % parasitemia containing more than 85 % ring stage obtained by repeated sorbitol treatment and incubated with the compounds at concentrations ranging between 0.49 and 100 μ M or μ g/mL, obtained by serial dilution, in duplicates. Two controls
- 164 were used, parasites without drug and parasites with chloroquine at concentrations ranging between
- 165 0.49 and 1000 nM. Plates were incubated for 44 h at 37 °C in a candle jar (Vásquez-Ocmín et al., 2018,
- 166 2021a).
- 167 In vitro antileishmanial activity on L. donovani, L. amazonensis, and L. braziliensis axenic amastigotes
- 168 A suspension of promastigotes in growth plateau-phase was incubated at 37° C in 5 % CO₂ for 3 days
- to obtain the amastigote form in promastigote medium supplemented with 2 mM CaCl2, 2 mM MgCl₂,
- and a pH adjusted to 5.5. The axenic amastigote suspension containing 1.106 parasites/mL was
- incubated for 72h with the compounds at 37° C in 5 % CO₂ in the dark. Tested compounds or extracts
- were obtained by serial dilution and ranged between 0.49 and 100 μ M or μ g/mL. There were two
- 173 controls: parasites without drug and parasites treated with miltefosine at the same concentrations as the 174
- 174 compounds tested (Vásquez-Ocmín et al., 2018, 2021a).

175 In vitro antileishmanial activity on L. donovani, L. amazonenesis and L. braziliensis intramacrophage 176 amastigote form

- 177 Macrophages were seeded into a 96 well microtitration plate at a density of 100,000 cells/well in 100
- 178 μ L and incubated in a 5 % CO₂ at 37°C for 24h. After removing the medium, cells were incubated with
- 179 $100 \,\mu\text{L}$ of fresh DMEM containing a suspension of promastigotes in the growth plateau phase at a rate
- 180 of 1 cell per 10 parasites. After incubation under a 5 % CO₂ atmosphere at 37° C for 24h (the time
- 181 needed by the parasite to infect the macrophage), the culture medium was replaced with 100 μ L of

182 fresh DMEM with different concentrations of compounds as previously for a new incubation period of

183 48h. Controls were parasites alone in DMEM medium, axenic amastigotes, macrophages alone,

184 infected macrophages and infected macrophages with different concentrations of miltefosine

185 (Balaraman et al., 2015; Vásquez-Ocmín et al., 2018, 2021a).

186 Determination of IC50, CC50 and Selectivity Index for Plasmodium, Leishmania, and Trypanosma

187 After incubation, the plates were subjected to 3 freeze/thaw cycles to achieve complete cell lysis. The 188 cell lysis suspension was diluted 1:1 except for Plasmodium plates that have been diluted 1:10 in lysis 189 buffer (10 mM NaCl, 1 mM Tris HCl pH 8, 2.5 mM EDTA pH 8, 0.05 % SDS, 0.01 mg/mL proteinase 190 K and 1X SYBR Green). SYBR Green incorporation in cell DNA amplification was determined using the Master epRealplex cycler[®] (Eppendorf, France) and the following program to increase SYBR 191 Green incorporation: 90°C for 1 min, decrease in temperature from 90°C to 10°C for 5 min with 192 193 fluorescence reading at 10°C for 1 min and a new reading at 10°C for 2 min. Molecules were tested in 194 duplicate. Compounds with different SYBR Green fluorescence values from duplicates were retested. 195 The cytotoxicity of the compounds was expressed as IC₅₀ (concentration of drug inhibiting the parasite 196 growth by 50 %, comparatively to the controls), CC₅₀ (Cytotoxic Concentration 50 %: concentration 197 inhibiting macrophages growth by 50 %). IC₅₀ and CC₅₀ were calculated by nonlinear regression using 198 ICEstimator 1.2 version (http://www.antimalarial-icestimator.net/MethodIntro.htm). Selectivity index 199 (SI) for antiplasmodial and anti-Trypanosoma activities were calculated as the HUVEC's CC50 divided 200 to IC₅₀ of *Plasmodium* 3D7 and *Trypanosoma*, and for *Leishmania* assays as the ratio of RAW 364.7

201 CC₅₀ to intramacrophage amastigote IC₅₀ value (Vásquez-Ocmín et al., 2018, 2021a).

202 In vitro evaluation on bloodstream form of Trypanosoma brucei gambiense

203 For each extract, twelve two-fold serial dilutions from 100 µg/mL to 0.049 µg/mL were performed in 204 96-well microplates in 100 µL HMI9 medium. Parasites were then added to each well to reach a final 205 density of 4.104 cells/mL in 200 µL. Following 3 days of incubation at 37°C with 5 % CO₂ in a water-206 saturated atmosphere, 20 µL of resazurin at 450 µM in water was added to each well for a final 207 concentration of 40.9 nM to evaluate cell viability. Plates were then incubated for 6h at 37°C with 5 % 208 CO₂ in water-saturated atmosphere and the conversion of resazurin to resorufin was quantified by 209 measuring the absorbance at 570 nm (resofurin) and 600 nm (resazurin) using the microplate reader 210 Spark[®] (Lyon, France) Pentamidine di-isethionate was used as the reference compound (Pomel et al.,

211 2015; Vásquez-Ocmín et al., 2018, 2021a).

212 Antimicrobial Assay

Minimal Inhibitory Concentration (MIC) determinations of crude extracts were carried out using the agar dilution method stipulated by the Clinical and Laboratory Standards Institute (CLSI, 2006). Antimicrobial activity was evaluated for the first time against a panel of 36 pathogenic and multi-drugresistant bacteria, which, in most cases, have been recently isolated from human infections. For comparison, reference strains from the American Type Culture Collection (ATCC) were included.

The inhibitory concentrations ranged between 0.075 and 1.2 mg/mL in five dilutions (1.2, 0.6, 0.3,

219 0.15 and 0.075 mg/mL); 0.075 mg/mL was considered a low enough concentration for a preliminary

screening. Petri dishes were inoculated with strains (104 CFU, obtained by dilution in brain heart

221 medium, BH) using a Steer's replicator and were incubated at 37°C for 24 h. MIC was defined as the

222 lowest concentration of extract without bacterial growth after incubation. The extracts with MIC ≤ 1.2

- 223 mg/mL were tested in triplicate at lower concentrations (mean absolute deviation is done for values:
- 224 1.2 ± 0.4 ; 0.6 ± 0.2 ; 0.3 ± 0.1 ; 0.15 ± 0.05 ; 0.075 ± 0.03). The standards (gentamicin, vancomycin,

225 amoxicillin, amphotericin B, fluconazole, and sertaconazole) were tested in triplicate in 12 226 concentrations ranging from 0.03 to 64 mg/mL.

227 Cytotoxic assay

Cytotoxicity was evaluated on RAW 264.7 macrophages for *Leishmania* assays and HUVECs for *Plasmodium* and *Trypanosoma*. The cells were seeded at a density of 50,000 cells per well in 100 μ l of DMEM in a 96-well microtiter plate. After incubation in a 5 % CO₂ at 37°C for 24h, the culture medium was replaced with 100 μ l of fresh DMEM containing serial dilutions of the compounds tested. The concentrations of the compounds are the same as for intramacrophage *Leishmania* or *Plasmodium* assays. The plates were incubated for 48h at 37°C with 5 % CO₂. Antiprotozoal assays have been performed only with compounds not demonstrating cytotoxicity (Vásquez-Ocmín et al., 2021a).

235 Liquid chromatography and mass spectrometry data mining

236 HPLC-MS analyses

237 Extracts were analyzed by liquid chromatography performed on an Agilent 1260 series HPLC coupled 238 to a 6530 QToF (Agilent Technologies). Chromatography separations were performed on a XSelect 239 CSH C18 column-C18, 130Å, 2.5 µm, 2.1 x 75 mm - 2.5 µm (Waters). The mobile phase comprised 240 water (0.1 % formic acid) (A) and acetonitrile (ACN) (B). A stepwise gradient method at a constant 241 flow rate of 0.35 mL/min was applied as follows: 5–100% B (0–9.5 min), 100% B (4.5 min) and 4 min 242 equilibration at 5% B. The mass spectrometer settings were: positive ESI mode, 50-3200 mass range 243 calibration, and 2 GHz acquisition rate. Ionization source conditions were drying gas temperature 325 244 °C, drying gas flow rate 10 L/min, nebulizer 35 psig, fragmentor 150 V, and skimmer 65 V. Range of 245 m/z was 200-1700. Purine C₅H₄N₄ [M+H]⁺ ion (m/z 121.050873) and the hexakis-(1H,1H,3H-246 tetrafluoropropoxy)-phosphazene C₁₈H₁₈F₂₄N₃O₆P₃ $[M+H]^+$ ion (m/z 922.009798) were used as 247 internal lock masses. Full scans were acquired at a resolution of 11 000 (at m/z 922). MS-MS 248 acquisitions were performed using three collision energies: 10, 20, and 40 eV. Three of the most intense 249 ions (top 3) per cycle were selected. MS-MS acquisition parameters were defined as follows: m/z range 250 100-1200, default charge of 1, minimum intensity of 5000 counts, rate/time = 3 spectra/s, isolation 251 width: Narrow (1.3 u).

252 Data processing

253 For untargeted metabolomics, the LC-MS data were processed according to the MSCleanR workflow 254 (Fraisier-Vannier et al., 2020; Vásquez-Ocmín et al., 2021b). Briefly, a batch in positive ionization 255 (PI) was processed with MS-DIAL version 4.90 (Tsugawa et al., 2015). MS1 and MS2 tolerances were 256 set to 0.01 and 0.05 Da, respectively, in centroid mode for each dataset. Peaks were aligned on a OC 257 reference with an RT tolerance of 0.2 min, a mass tolerance of 0.015 Da, and a minimum peak height 258 detection at 1×10^5 . MS-DIAL data was deconvoluted together with MS-CleanR by selecting all filters 259 with a minimum blank ratio set to 0.8 and a maximum relative standard deviation (RSD) set to 40. The 260 maximum mass difference for feature relationship detection was set to 0.005 Da, and the maximum 261 RT difference was set to 0.025 min. Pearson correlation links were used with a correlation > 0.8 and a 262 p-value significance threshold of 0.05. Two peaks were kept in each cluster for further database 263 requests and the kept features were annotated with MS-FINDER version 3.52 (Tsugawa et al., 2016). 264 The MS1 and MS2 tolerances were set to 5 and 15 ppm, respectively. The formula finder was 265 exclusively based on C, H, O, and N atoms. Three levels of compounds annotation from several sets 266 of data were carried out. Metabolite annotation at level 1 using MS-DIAL: i) the experimental LC-267 MS/MS data of 500 compounds (retention time, exact mass and fragmentation) were used as

- 268 references; ii) the Mass spectral records from MS-DIAL, MONA (MassBank of North America), and
- 269 GNPS (Global Natural Product Social Molecular Networking) databases were used for spectral match
- applying a dot product score cut-off of 800. Metabolite annotation at level 2 was prioritized according
- to: i) a search would be made using MSFinder for a match with compounds identified in the literature
- 272 for the *Piper* genus (genus level) and Piperaceae family (family level) (Dictionary of Natural Products
- version 28.2, CRC Press) based on exact mass and *in silico* fragmentation. For metabolite annotation
- level 3, a search was made using MS-FINDER for a match with natural compounds included in their
 databases embedded (PlantCyc, ChEBI, NANPDB, COCONUT, and KNApSAcK) (generic level)
- 276 (Vásquez-Ocmín et al., 2022b).
- 277 Mass spectra similarity networking was carried out from PI mode using MetGem (Olivon et al., 2018)
- 278 on the final annotated .msp file and metadata file for PI obtained with MSCleanR. Values used were
- MS2 m/z tolerance = 0.02 Da, minimum matched peaks = 4 and minimal cosine score value = 0.7. The
- visualization of the molecular network (MN) was performed on Cytoscape version 3.9.1 (Shannon et
- al., 2003). The list of compound mass, retention time, row ID and peak height was exported in CSV
- 282 format. Then, this format was used for the statistical data analysis.

283 Statistical analyses

284 The final annotated untargeted metabolome feature matrix and biological assay results datasets were 285 analyzed using the R package MixOmics (http://mixomics.org/), which is dedicated to the integrative 286 analysis of 'omics' data (Le Cao et al., 2016). Biological assays results expressed in IC50 were transformed in pIC₅₀ (-log10 [IC50]) for further statistical correlation analysis. MIC values were log 287 288 transformed. The LC-MS dataset ($m/z \times RT \times peak$ area) was normalized to total ion chromatogram 289 and scaled to unit variance. A vertical integration approach has been afforded to leverage on 290 multiplexed measurements for the same extract (González et al., 2008). To highlight the overall 291 correlation between chemical fingerprints and biological assays., <u>Aa</u> regularized Canonical Correlation 292 Analysis (rCCA) was done using a cross validation approach for tuning regularization parameters. The 293 correlation structure of the two-block data matrices was displayed on clustered image map (CIM) and 294 relevance network using 0.3 as cutoff correlation value.

295 **RESULTS**

296 Ethnopharmacological survey

Our set of 19 samples was collected in three Amazonian regions of Peru, where the use of these species of "*Cordoncillos*" in traditional medicine is widespread. All information about the collected samples are presented in **supp info 1**. Among the plants studied in this work, 10 (52.6 %) had not been subject to chemical analysis so far. In our previous work (Vásquez-Ocmín et al., 2021a), *Piper casapiense*, *P. strigosum* and *P. pseudoarboreum* showed promising antiprotozoal activity. Thus, these plants were re-collected in other areas of the Loreto region.

303 Anti-infectious activity

304 Antiprotozoal activity

The IC₅₀ values for antiplasmodial, antileishmanial and antitrypanosomal activities of each extract are shown in **Table 1**. We considered an extract active and worthy to be further studied when its IC₅₀ value was below 10 μ g/mL. Four extracts were active in this case regarding the 3D7 *P. falciparum* chloroquine-sensitive strain assay: the hexane and methylene chloride extracts of *P. crassinervium*, the methanol extract of *P. stellipilum*, and the methylene chloride extract of *P. xanthostachyum*. Only the 310 methylene chloride extracts of P. crassinervium, and P. oblongum were active on three strains of 311 Leishmania (both axenic and intra-macrophagic amastigotes). For the other active extracts, six were 312 not active on intra-macrophagic forms: hexane and methylene chloride of P. heterophyllum, hexane, 313 methylene chloride and water extracts of *P. sancti-felicis* (*L. amazonensis*) and a hexane extract of *P.* 314 crassinervium (L. donovani). Hexane extract of P. "cordatomentosa" was active on all the strains, both 315 axenic and intramacrophagic amastigotes, except on axenic amastigote forms of L. donovani. Five extracts were active only on L. amazonenesis, and L. braziliensis (both axenic and intramacrophagic 316 amastigotes): methylene chloride extracts of P. calvescentinerve, Piper crassinervium, and P. 317 318 oblongum, and the methylene chloride and hexane extracts of Piper "cordatomentosa", and Piper 319 crassinervium. Five extracts were active only on L. donovani, and L. braziliensis (both axenic and 320 intramacrophagic amastigotes), the methylene chloride extract of P. crassinervium, and the hexane and 321 methylene chloride extracts of *P. heterophyllum*, and *P. oblongum*. Hexane extract of *P. glabribaccum* 322 was not active on axenic amastigote forms of L. donovani. Two extracts were active on L. donovani, 323 and L. amazonenesis, both forms, methylene chloride extracts of Piper crassinervium, and P. 324 oblongum. Selective activity on L. braziliensis were observed for twelve extracts, the hexane and 325 methylene chloride extracts Piper sancti-felicis, Piper stellipilum, and P. xanthostachyum; hexane and methylene chloride extracts of P. stellipilum, and hexane and methylene chloride extracts of P. 326 calvescentinerve and of P. divaricatum. None of the extracts showed selective activity on L. donovani 327 328 or L. amazonensis.



Table 1. *In vitro* antiprotozoal and cytotoxicity activities of *Piper* extracts. M = methanol; H = hexane; D = methylene chloride (D stands for dichloromethane); HA = hydroalcoholic; Aq = aqueous; NT = not tested. *Species showing no reference in phytochemical literature on Web of Science and PubMed. **Extracts previously tested (Vásquez-Ocmín et al., 2021a) (the plants in this work come from different collection site). L = leaves; L&S = Leaves and stems; AP= aerial parts; "" = species with temporary botanical name (these species need more taxonomical studies).

Species, Region (Part tested)	Extract (code for metabolomic analyses)	<i>P. falciparum</i> 3D7 strain, IC ₅₀ ± SD, μg/mL	L. donovani LV9 strain (axenic amastigotes), IC50 ± SD, µg/mL	L. donovani LV9 strain (intra- macrophagic amastigotes), IC ₅₀ ± SD, µg/mL	L. amazonensis (axenic amastigotes), IC ₅₀ ± SD, μg/mL	L. amazonensis (intra- macrophagic amastigotes), $IC_{50} \pm SD$, $\mu g/mL$	L. braziliensis (axenic amastigotes), IC ₅₀ ± SD, μg/mL	L. braziliensis (intra- macrophagic amastigotes), $IC_{50} \pm SD$, $\mu g/mL$	Trypanosoma brucei gambiense IC50 ± SD, μg/mL	HUVEC CC ₅₀ ± SD, µg/mL	RAW 264.7 macrophages CC ₅₀ ± SD, μg/mL
Piper	H (P4H)	>100	>100	NT	>100	NT	>100	NT	0.68 ± 0.14	>100	>100
<i>casapiense</i> (Mig.) C. DC	D (P4D)**	>100	>100	NT	>100	NT	>100	NT	4.06 ± 1.11	45.31 ± 1.97	61.12 ± 4.41
Loreto (L&S)	M (P4M)	>100	>100	NT	60.64 ± 6.07	44.22 ± 3.3	>100	NT	18.59 ± 11.62	30.48 ± 2.55	64.88 ± 6.98
Piper strigosum	H (P12H)**	>100	>100	NT	74.24 ± 6.33	22.41 ± 3.12	17.41 ± 0.71	9.07 ± 0.22	0.10 ± 0.02	>100	>100
Trel.; Loreto	D (P12D)**	>100	>100	NT	54.43 ± 4.22	31.12 ± 2.66	12.33 ± 0.43	8.12 ± 0.87	0.028 ± 0.004	41.66 ± 4.01	66.01 ± 5.13
(L&S)	M (P12M)**	>100	>100	NT	54.21 ± 4.32	20.97 ± 2.08	20.22 ± 0.20	17.64 ± 1.64	4.22 ± 1.59	51.22 ± 4.20	>100
Piper	H (P14H)**	>100	>100	NT	>100	NT	>100	NT	12.06 ± 3.80	61.94 ± 4.64	>100
pseudoarboreum	D (P14D)**	>100	>100	NT	>100	NT	>100	NT	5.07 ± 2.17	$40.21{\pm}4.12$	>100
Y unck; Loreto (L&S)	M (P14M)**	>100	>100	NT	>100	NT	>100	NT	28.56 ± 15.97	25.49 ± 1.87	49.09 ± 3.88
*Piper armatum	H (P18H)	>100	>100	NT	>100	NT	>100	NT	25.04 ± 5.33	>100	>100
Trel. & Yunck;	D (P18D)	>100	>100	NT	>100	NT	>100	NT	1.50 ± 0.16	55.31 ± 4.12	>100
Loreto (L&S)	M (P18M)	>100	>100	NT	>100	NT	>100	NT	11.26 ± 1.90	35.86 ± 2.88	33.03 ± 2.99
*Piper	H (P19H)	>100	>100	NT	23.45 ± 2.74	20.12 ± 1.66	14.20 ± 1.33	12.66 ± 1.64	29.47 ± 12.31	>100	>100
DC; Loreto (L&S)	D (P19D)	20.19 ± 1.44	>100	NT	12.66 ± 1.64	10.22 ± 1.99	18.41 ± 1.84	11.22 ± 1.88	1.11 ± 0.13	20.19 ± 1.44	21.99 ± 2.37
* "Piper	H (P20H)	>100	>100	NT	>100	NT	>100	NT	5.05 ± 1.51	>100	77.64 ± 6.88
bullatum Vahl";	D (P20D)	>100	>100	NT	>100	NT	>100	NT	4.40 ± 0.54	43.79 ± 4.41	41.66 ± 3.64
Cusco (L&S)	M (P20M)	>100	>100	NT	>100	NT	>100	NT	21.47 ± 12.60	25.09 ± 2.40	>100
*Piper calvescentinerve Trel; Cusco (L&S)	H (P21H) D (P21D) M (P21M)	>100 >100 >100	29.29 ± 2.12 >100 >100	22.64 ± 2.01 NT NT	$\begin{array}{c} 17.44 \pm 1.55 \\ 2.55 \pm 1.08 \\ > 100 \end{array}$	15.84 ± 1.11 1.94 ± 0.84 NT	$\begin{array}{c} 6.33 \pm 0.63 \\ 3.54 \pm 0.16 \\ 43.61 \pm 3.87 \end{array}$	$\begin{array}{c} 1.92 \pm 0.07 \\ 1.64 \pm 1.02 \\ 20.64 \pm 1.55 \end{array}$	$\begin{array}{c} 3.97 \pm 0.25 \\ 6.08 \pm 0.49 \\ 17.75 \pm 1.32 \end{array}$	>100 38.83 ± 5.41 38.55 ± 1.13	>100 44.88 ± 4.34 72.22 ± 6.66

Piper "cordatomentos a"; Cuzco (L&S)	H (P22H) D (P22D) M (P22M)	>100 >100 >100	$\begin{array}{c} 12.41 \pm 0.88 \\ 17.99 \pm 0.77 \\ >100 \end{array}$	$\begin{array}{c} 9.78 \pm 1.55 \\ 18.34 \pm 0.66 \\ \text{NT} \end{array}$	$\begin{array}{c} 8.77 \pm 1.12 \\ 10.44 \pm 1.45 \\ > 100 \end{array}$	5.66 ± 1.88 10.44 ± 1.84 NT	$\begin{array}{c} 6.92 \pm 0.71 \\ 4.59 \pm 0.45 \\ > 100 \end{array}$	3.22 ± 0.48 1.08 ± 0.33 NT	$\begin{array}{c} 4.50 \pm 1.27 \\ 2.62 \pm 1.11 \\ 34.58 \pm 13.10 \end{array}$	>100 58.32 ± 5.68 61.50 ± 2.62	$>100 \\ 61.44 \pm 4.11 \\ >100$
Piper crassinervium Kunth.; Cusco (L&S)	H (P23H) D (P23D) M (P23M)	$5.66 \pm 0.87 \\ 7.41 \pm 0.99 \\ >100$	$\begin{array}{c} 8.05 \pm 0.74 \\ 2.95 \pm 0.32 \\ > 100 \end{array}$	>100 0.99 ± 1.33 NT	8.55 ± 1.61 3.18 ± 1.08 >100	4.55 ± 1.09 2.66 ± 1.88 NT	$\begin{array}{c} 6.48 \pm 1.22 \\ 5.77 \pm 1.12 \\ 2.70 \pm 0.19 \end{array}$	3.18 ± 1.08 1.25 ± 1.46 1.12 ± 1.46	$\begin{array}{c} 7.08 \pm 0.85 \\ 3.25 \pm 0.62 \\ 14.44 \pm 9.07 \end{array}$	$\begin{array}{c} 3.51 \pm 0.71 \\ > 100 \\ 15.28 \pm 3.07 \end{array}$	$\begin{array}{c} 10.44 \pm 0.79 \\ 37.38 \pm 4.52 \\ 37.38 \pm 4.52 \end{array}$
<i>Piper</i> <i>divaricatum</i> G. Mey; San Martin (L)	H (P24H) D (P24D)	>100 >100	>100 >100	NT NT	>100 >100	NT NT	>100 2.61 ± 0.27	NT 5.44 ± 0.88	$\begin{array}{c} 4.57 \pm 0.94 \\ 4.02 \pm 0.39 \end{array}$	30.04 ± 4.95 >100	55.95 ± 4.12 >100
*Piper	H (P25H)	>100	12.05 ± 3.25	8.33 ± 1.48	12.44 ± 0.77	11.41 ± 1.61	3.58 ± 0.45	3.88 ± 0.44	4.83 ± 1.36	>100	>100
<i>glabribaccum</i> Trel: Cusco	D (P25D)	>100	5.04 ± 1.2	10.33 ± 2.10	13.41 ± 0.45	11.22 ± 0.43	3.57 ± 0.45	6.66 ± 0.64	3.85 ± 1.26	35.62 ± 2.54	77.20 ± 6.61
(L&S)	M (P25M)	>100	>100	NT	>100	NT	>100	NT	14.98 ± 2.75	84.72 ± 1.25	>100
<i>Piper</i> <i>heterophyllum</i> Ruiz & Pav.; Loreto (L)	H (P26H) D (P26D)	>100 >100	$\begin{array}{c} 4.32 \pm 0.76 \\ 6.56 \pm 0.61 \end{array}$	3.11 ± 0.99 7.0 ± 0.61	$\begin{array}{c} 15.66 \pm 1.46 \\ 10.41 \pm 0.64 \end{array}$	$\begin{array}{c} 11.51 \pm 0.98 \\ 11.32 \pm 1.61 \end{array}$	$\begin{array}{c} 5.55 \pm 0.95 \\ 2.07 \pm 0.20 \end{array}$	$\begin{array}{c} 3.15 \pm 0.99 \\ 3.44 \pm 0.88 \end{array}$	$\begin{array}{c} 17.27 \pm 0.59 \\ 11.59 \pm 3.93 \end{array}$	$\begin{array}{c} 81.67 \pm 2.17 \\ 17.14 \pm 2.19 \end{array}$	89.66 ± 5.78 50.53 ± 5.11
*Piper	H (P27H)	>100	8.02 ± 1.16	7.66 ± 1.11	13.45 ± 1.99	15.64 ± 1.66	5.03 ± 1.39	4.13 ± 1.22	4.91 ± 0.75	48.32 ± 1.18	51.64 ± 4.30
oblongum Kuntha Carren	D (P27D)	>100	3.70 ± 0.71	1.36 ± 0.88	8.77 ± 1.65	7.44 ± 0.78	3.58 ± 0.43	4.13 ± 0.99	5.94 ± 1.88	26.23 ± 1.01	>100
(L&S)	M (P27M)	>100	>100	NT	>100	NT	5.03 ± 1.39	5.03 ± 1.40	10.65 ± 2.21	21.43 ± 1.99	77.56 ± 4.81
Piper	H (P28H)	>100	>100	NT	>100	NT	>100	NT	11.84 ± 1.29	>100	>100
reticulatum L;	D (P28D)	>100	>100	NT	>100	NT	>100	NT	6.65 ± 1.31	>100	84.46 ± 5.88
Loreto (L)	M (P28M)	>100	>100	NI 19.41 + 1.22	>100	NI 10.22 × 1.62	>100	N1	24.92 ± 0.00	>100	//.54 ± /.12
*Piper sancti- felicis Trel:	H (P29 H) D (P29 D)	>100	$1/.88 \pm 1.11$ 13 11 + 0.88	18.41 ± 1.33 10.45 ± 0.46	21.55 ± 1.46 18 41 + 1 34	18.22 ± 1.62 17.51 ± 1.99	4.10 ± 0.57 4.21 ± 0.52	3.44 ± 0.99 6.44 ± 0.99	6.60 ± 1.24 9.23 + 2.58	>100 49 40 + 4 04	>100
Loreto (L&S)	Aq (P29 Aq)	>100	4.63 ± 0.63	1.12 ± 0.77	8.64 ± 0.44	11.51 ± 1.41	6.07 ± 0.16	10.31 ± 0.11	48.13 ± 8.38	72.22 ± 4.98	>100
*Piper stellipilum (Miq.) C. DC; Loreto (L&S)	H (P30H) D (P30D) M (P30M)	>100 13.44 ± 0.21 8.22 ± 0.77	>100 >100 >100	NT NT NT	>100 >100 >100	NT NT NT	$\begin{array}{c} 3.74 \pm 0.65 \\ 4.45 \pm 0.45 \\ 12.33 \pm 1.08 \end{array}$	$\begin{array}{c} 8.41 \pm 1.31 \\ 9.99 \pm 1.34 \\ 15.64 \pm 1.88 \end{array}$	$\begin{array}{c} 6.78 \pm 3.78 \\ 9.64 \pm 0.46 \\ 13.15 \pm 2.01 \end{array}$	>100 18.05 ± 1.04 25.03 ± 3.09	>100 66.54 ± 5.12 51.44 ± 2.99
*Piper trigonum	H (P31H)	>100	>100	NT	>100	NT	>100	NT	8.84 ± 0.29	>100	>100
C. DC.; Cusco	D (P31D)	>100	>100	NT	>100	NT	>100	NT	4.84 ± 1.40	30.51 ± 3.05	77.45 ± 4.44
(L&S)	M (P31M)	>100	>100	NT	>100	NT	>100	NT	10.93 ± 2.53	>100	70.67 ± 6.12
*Piper verruculosum C. DC.; Cusco (L&S)	H (P32H) D (P32D) M (P32M)	>100 >100 >100	>100 >100 >100	NT NT NT	>100 >100 >100	NT NT NT	>100 >100 >100	NT NT NT	$\begin{array}{c} 8.54 \pm 2.90 \\ 5.64 \pm 0.41 \\ 27.38 \pm 6.81 \end{array}$	>100 >100 >100	>100 >100 >100
Piper xanthostachyum	H (P33H) D (P33D)	>100 6.33 ± 0.74	$17.54 \pm 0.99 \\ 14.79 \pm 0.77$	$\frac{11.12 \pm 1.01}{15.32 \pm 0.99}$	21.46 ± 1.89 29.44 ± 1.46	18.44 ± 1.46 15.63 ± 1.77	6.51 ± 0.64 2.98 ± 0.29	9.55 ± 0.55 5.45 ± 0.66	$\frac{14.00 \pm 1.26}{2.25 \pm 0.83}$	>100 6.18 ± 1.74	>100 52.12 ± 4.03

Running Title

C.DC.; Loreto (L)	HA (P33HA)	>100	23.11 ± 1.08	18.47 ± 1.12	30.48 ± 2.66	15.66 ± 1.63	23.88 ± 0.89	21.41 ± 1.48	4.11 ± 0.69	49.84 ± 4.31	45.89 ± 4.08
Chloroquine		$\begin{array}{c} 0.007 \ (21.40 \pm \\ 1.56 \ nM \end{array}$	NT	NT	NT	NT	NT	NT	NT	NT	NT
Miltefosine		NT	$\begin{array}{c} 1.41 \pm 0.50 \; (3.46 \\ \pm 1.22 \\ \mu M) \end{array}$	$\begin{array}{c} 2.49 \pm 0.45 \\ (6.10 \pm \\ 1.10 \ \mu M) \end{array}$	$\begin{array}{c} 1.88 \pm 1.39 \\ (4.61 \pm 3.41 \\ \mu M) \end{array}$	$\begin{array}{c} 2.15 \pm 1.78 \\ (5.28 \pm 4.37 \\ \mu M) \end{array}$	$\begin{array}{c} 3.49 \pm 1.85 \\ (8.56 \pm 4.54 \\ \mu M) \end{array}$	$\begin{array}{c} 2.22 \pm 1.64 \\ (5.44 \pm 4.02 \\ \mu M) \end{array}$	NT	NT	NT
Pentamidine		NT	NT	NT	NT	NT	NT	NT	$\begin{array}{c} 0.006 \pm 0.002 \\ (17.6 \pm 5.8 \\ nM) \end{array}$	NT	NT
4											



335 Selectivity index (SI) values for antiprotozoal activities (**Table 2**) were ranked as low >10<50, high 336 >51<99, and very high >100. For antimalarial activity, the only active extract displaying a low SI 337 (13.50) was the methylene chloride extract of P. crassinervium. For the antileishmanial activity, 338 methylene chloride extract of P. crassinervium and hexane extracts of Piper glabribaccum and P. 339 heterophyllum showed a low SI on L. donovani with 37.76, 12.0, and 28.82 respectively; methylene 340 chloride and aqueous extracts of P. oblongum and P. sancti-felicis showed high SI with 73.53 and 341 89.29 respectively. For *L. amazonenesis*, three extracts presented a low SI, methylene chloride extracts 342 of P. "cordatomentosa", P. crassinervium, and P. oblongum with 13.39, 14.05, and 13.44 respectively. 343 For L. braziliensis, 17 extracts displayed low SI, the hexane extracts of P. strigosum, P. 344 "cordatomentosa", P. glabribaccum, P. heterophyllum, P. oblongum, P. sancti-felicis, P. stellipilum, and P. xanthostachyum (11.02, 31.06, 25.77, 28.46, 12.50, 29.07, 11.89, 10.47), methylene chloride 345 346 extracts of Piper calvescentinerve, P. crassinervium, P. divaricatum, P. glabribaccum, P. 347 heterophyllum, P. oblongum, and P. sancti-felicis (27.37, 29.90, 18.38, 11.59, 14.69, 24.21, 15.53, 348 respectively); methanol extracts of *Piper crassinervium*, and *P. oblongum* (33.36 and 15.42). Only the 349 hexane and methylene chloride extracts of P. calvescentinerve and P. "cordatomentosa" presented a 350 high SI with 52.08 and 56.89, respectively.

351 At least one extract of all nineteen plants, except P. heterophyllum, was active on T. b. gambiense at 352 $IC_{50} \le 10 \,\mu g/mL$ (**Table 1**). The only two plants that displayed activity in all extracts were *P. strigosum* 353 and *P. divaricatum*. Among all active extracts, five had very good activity with a IC₅₀ \leq 1 µg/mL, 354 hexane and methylene chloride extracts of P. strigosum, hexane extract of P. casapiense, and 355 methylene chloride extracts of P. armatum, and P. brasiliense. Especially, the methylene chloride 356 extract from *Piper strigosum* was very active with an IC₅₀ at 0.028 µg/mL. Three extracts presented a 357 high SI, hexane extract of *P. casapiense* with 147.06 and the hexane and methylene chloride extracts 358 of *P. strigosum* with 1000 and 1487.86, respectively. Twenty extracts displayed a lower yet interesting 359 SI between 10.58 and 36.87.

Table 2. Selectivity index of *Piper* extracts calculated with CC_{50} on HUVEC cells for *P. falciparum* and *T. b. gambiense*, and CC_{50} on RAW 264.7 cells for *Leishmania* strains. M = methanol; H = hexane; D = methylene chloride (D stands for dichloromethane); Aq = aqueous; ND = not determined. *Species with no phytochemical reference in Web of Science and PubMed. **Extracts previously tested (Vásquez-Ocmín et al., 2021a) (the plants come from different collection site). L = leaves; L&S = Leaves and stems. "" = species with a temporary botanical name (these species need further taxonomic study).

Species	Extract (code for metabolomic analyses)	SI on P. falciparum 3D7 strain, CC ₅₀ /IC ₅₀	SI on <i>L.</i> donovani (intra- macrophagic amastigotes) , CC ₅₀ /IC ₅₀	SI on L. amazonenesis (intra-macrophagic amastigotes), CC50/IC50	SI on <i>L.</i> braziliensis (intra- macrophagic amastigotes), CC50/IC50	SI on <i>T. b.</i> gambiense, CC ₅₀ /IC ₅₀
	H (P4H)	≤ 1	ND	ND	ND	147.06
Piper casapiense	D (P4D)**	0.45	ND	ND	ND	11.16
	M (P4M)	0.30	ND	1.46	ND	1.64
	H (P12H)**	≤ 1	ND	4.46	11.02	1000
Piper strigosum	D (P12D)**	0.42	ND	2.12	8.13	1487.86
	M (P12M)**	0.51	ND	4.77	5.67	12.14
Piper	H (P14H)**	0.62	ND	ND	ND	5.14
pseudoarboreum	D (P14D)**	0.40	ND	ND	ND	7.93

	M (P14M)**	0.25	ND	ND	ND	0.89
	H (P18H)	< 1	ND	ND	ND	3.99
*Piper armatum	D (P18D)	0.55	ND	ND	ND	36.87
<u>.</u>	M (P18M)	0.36	ND	ND	ND	11.26
	H (P19H)	≤ 1	ND	4.97	7.90	3.39
*Piper brasiliense	D (P19D)	1.00	ND	2.15	1.96	18.19
	H (P20H)	≤1	ND	ND	ND	19.80
*"Piper bullatum"	D (P20D)	0.44	ND	ND	ND	9.95
	M (P20M)	0.25	ND	ND	ND	1.17
	H (P21H)	≤1	4.42	6.31	52.08	25.19
Piper	D (P21D)	0.39	ND	2.31	27.37	6.39
calvescentinerve	M (P21M)	0.39	ND	ND	ND	2.17
(D)	H (P22H)	≤1	10.22	1.77	31.06	22.22
"Piper	D (P22D)	0.58	3.35	13.39	56.89	22.26
coraatomentosa	M (P22M)	0.62	ND	ND	ND	1.79
Piper	H (P23H)	0.62	0.10	2.99	3.28	0.50
crassinervium	D (P23D)	13.50	37.76	14.05	29.90	30.77
Kunth	M (P23M)	0.15	ND	ND	33.36	10.58
Dia an dia mia ntana	H (P24H)	0.30	ND	ND	ND	6.57
Piper alvaricatum	D (P24D)	≤ 1	ND	ND	18.38	24.88
*D:	H (P25H)	≤ 1	12.00	8.76	25.77	20.70
*Piper alabribaccum	D (P25D)	0.36	7.47	0.15	11.59	9.25
glabribaccum	M (P25M)	0.85	ND	ND	ND	5.66
Piper	H (P26H)	0.82	28.82	7.79	28.46	4.73
heterophyllum	D (P26D)	0.17	7.22	4.46	14.69	1.48
	H (P27H)	0.48	6.74	3.30	12.50	9.84
*Piper oblongum	D (P27D)	0.26	73.53	13.44	24.21	4.42
	M (P27M)	0.21	ND	ND	15.42	2.01
	H (P28H)	≤ 1	ND	ND	ND	8.45
Piper reticulatum	D (P28D)	≤ 1	ND	ND	ND	15.04
	M (P28M)	≤ 1	ND	ND	ND	4.01
*Piper sancti-	H (P29 H)	≤ 1	5.13	5.49	29.07	15.15
felicis	D (P29 D)	0.49	9.57	5.71	15.53	5.35
	Aq (P29 Aq)	0.72	89.29	8.69	9.70	1.50
	H (P30H)	≤ 1	ND	ND	11.89	14.75
*Piper stellipilum	D (P30D)	1.34	ND	ND	6.66	1.87
	M (P30M)	3.04	ND	ND	3.29	1.90
	H (P31H)	≤ 1	ND	ND	ND	11.31
*Piper trigonum	D (P31D)	0.31	ND	ND	ND	6.30
	M (P31M)	≤ 1	ND	ND	ND	9.15
Piner	H (P32H)	≤ 1	ND	ND	ND	11.70
verruculosum	D (P32D)	≤ 1	ND	ND	ND	17.73
	M (P32M)	≤ 1	ND	ND	ND	3.65
Piner	H (P33H)	≤ 1	8.99	5.42	10.47	7.14
xanthostachvum	D (P33D)	0.98	3.40	3.33	9.56	2.75
Mannostachymh	HA (P33HA)	0.50	2.48	2.93	2.14	12.13

367 Antimicrobial activity

Antimicrobial assay results are presented in **Table 3** (17 Gram-positive bacteria, 13 Gram-negative bacteria, 2 yeasts). Extracts were more active on Gram-positive bacteria and yeast. The IC₅₀ cut-off values were set at ≤ 0.5 mg/mL for good activity and ≤ 0.09 mg/mL for very good activity. Among the very active extracts, methylene chloride extract of *P. strigosum* is the most representative, being very active on all Gram-positive strains [(excepted: *Staphylococcus warneri* (T26A1)], only on two Gram-

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negative strains [(*Stenotrophomonas maltophilia* (21170) and *Burkholderia cepacia* (13003)], and on
the two *Candida albicans* strains. This extract was also quite active on *Streptococcus pyogenes*(16135). Other extracts with good activity on Gram-positive bacteria can be pointed out: methylene
chloride extracts of *P. xantochyma* and *P. brasiliense*, and hydroalcoholic extract of *P. xantochyma*.
Extracts with very good activity on Gram-negative bacteria are: methylene chloride extracts of *P. xantochyma*.

378 xantochyma and P. divaricatum on Burkholderia cepacia (13003) and Pseudomonas aeruginosa

- 379 (ATCC 27583), respectively. Extracts with very good activity on *Candida* strains were methylene
- 380 chloride extracts of *P. pseudoarboreum*, *P. divaricatum*, and *P. heterophyllum* and hexane extract of
- 381 *P. sancti-felicis.* Extracts with good activity on Gram-positive bacteria and *Candida* were principally
- methylene chloride of *P. brasiliense* and a hydroalcoholic extract of *P. xantochyma*.



Table 3. *In vitro* antimicrobial activity of *Piper* extracts (minimal inhibitory concentration MIC), in mg/mL). ND = not determinate, NA = not active.

			_	_					_								_	_							_								_
	Extracts	Candida albicans (10286)	Candida albicans (ATCC 1023)	Mycobacterium smegmatis (500	Enterococcus faecalis (C159-6	Enterococcus sp (8153)	Staphylococcus aureus (8146)	Staphylococcus aureus (8241)	Staphylococcus aureus (ATCC 65	Staphylococcus aureus (T28-1	Staphylococcus aureus (T17-4)	Staphylococcus epidermidis (T19.	Staphylococcus epidermidis (T21	Staphylococcus warneri (T12A1	Staphylococcus warneri (T26A)	Staphylococcus pettenkoferi (T47	Streptococcus agalactia e (T38.)	Streptococcus agalactiae (T53C	Streptococcus pyogenes (16135	Corynebacterium striatum (T40A	Citrobacter freundii (11041)	Acinetobacter baumannii (901)	Stenotrophomonas maltophilia (2)	Burkholderia cepacia (13003)	Escherichia coli (ATCC 25922	Escherichia coli (T20AI)	Escherichia coli (8138)	Proteus mirabilis (11060)	Proteus mirabilis (T28-3)	Pseudomonas aeruginosa (813)	Pseudomonas aeruginosa (ATCC 2	Pseudomonas aeruginosa (812)	Salmonella sp. (11033)
Species / Type of extract	:		e	3)					(88)			41)	45)	2)	0	A6)	2)	9)		3))		Ŋ	170)							0	7583)	9	
Piper casapiense (#4)	D U	1.2	0.6	0.3	- 1.2	•	1.2	-	1.2	0.6	1.2	0.6	0.6	0.6	0.6	0.6	0.6	0.3	0.6	0.6	•	0.15	0.6	- 0.6	•	•	•	0.6	1.2	- 1.2	0.6	0.3	•
Piper strigosum (#12)	M D H	0.0375	1.2 0.0375 0.15	0.6 0.0375 0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	1.2 0.0375 0.15	0.0375	1.2	1.2 0.0375 1.2	1.2 0.0375 0.3	1.2 0.6 1.2	0.0 1.2 0.0375 0.3	1.2 0.0375 0.15	0.0375	0.15 0.3	1.2 0.0375 0.3	•	0.6	0.0375	0.0375	- 0.6 -	0.6	0.6	•	-	•	1.2 0.6	0.6	-
Piper pseudoarboreum (#14)	M D H	0.075 0.3	1.2 1.2 -	1.2 0.3 0.6	- 1.2 -	•	1.2 - -	1.2 - -	0.6 -	0.6 - -	0.6 - -	0.6 -	0.6 - -	0.6 - -	0.6 -	0.6 1.2	0.6 - -	1.2 1.2	0.6 - 1.2	0.6	•	-	0.3 0.6 1.2	-	-	-	•	0.6 -	0.6 - -	0.6 - -	0.6 0.15	0.6 - -	-
Piper armatum (#18)	M D H	0.6 0.3	0.3 0.3	0.6 0.075 1.2	- 0.6 -	- 1.2 -	1.2 0.6	1.2 0.6	1.2 0.6	1.2 0.6	1.2 0.6	0.6 0.6 -	1.2 0.6	1.2 0.6	1.2 -	1.2 0.3	1.2 0.6	- 0.6 -	1.2 0.3 0.15	1.2 0.6 -	• • •	- 0.6 -	0.3 0.3	1.2 0.3	•		•	1.2 -	-	1.2	0.3 0.6 1.2	0.3 1.2	•
Piper brasiliense (#19)	M D H	0.15 0.3	0.15	0.15 0.3	0.3		0.3	0.3	0.3	0.3	0.3 -	0.15	0.15 0.6	0.3	- 1.2	- 0.6 -	0.3 -	0.3	- 0.3 0.075	0.3	0.6	0.3	0.15	0.3	- 1.2 -	- 0.6 -	- 1.2 -	0.3	0.3	- 0.3 -	- 0.3 0.6	0.3	-
Piper bullatum (#20)	M D H	- 0.15	0.3 - 0.6	0.3 0.3 0.075	- 0.6 0.3	•	1.2 - 0.6	0.6 - 0.6	1.2 - 0.6	0.6 - 0.6	0.6 - 0.6	0.3 - 1.2	0.3 - 0.6	0.6 - 0.6	0.6 - 0.6	0.3 - 0.3	0.3 - 0.6	0.6 0.15 0.15	0.6 0.15 0.075	0.3 0.6 0.15	•	1.2 - -	0.6 0.6 0.3	1.2 0.15 0.15	•	•	•	0.6 - -	0.6 - -	1.2 -	0.3 -	0.3 - -	
Piper calvescentinerve (#21)	M D H	0.3 0.15	0.6 - -	- 0.6 0.6	•	-	1.2 - -	1.2 -	1.2	1.2 -	1.2	-	0.6 -	0.6 - -	0.6 -	0.6 - -	0.6 -	1.2 1.2	1.2 0.6 0.3	0.6 - 1.2		1.2 - -	0.6	1.2 -	-	-	-	-	1.2 -	1.2 -	0.6 - -	0.6 -	-
Piper « cordotamentosa » (#22)	M D H	- 0.6 0.3	0.6 - -	0.6 0.3	- 0.6 0.6	•	1.2 -	1.2	1.2	1.2	1.2 -	0.6 - -	0.6	1.2 -	0.6 -	0.6 1.2 0.6	0.6 - -	- 0.6 0.6	- 0.3	1.2 1.2 0.6	•	•	0.6 0.6 -	- 1.2	•	•	•	•	-	•	0.6 1.2 -	0.6 - -	-
Piper crassinervium (#23)	M D H	0.3 0.15	0.6 0.15 -	1.2 0.6 0.3	- 0.15 1.2	•	0.3	1.2 0.3	0.3 -	1.2 0.15 -	1.2 0.3	1.2 0.3	1.2 0.3	1.2 0.6 1.2	1.2 1.2	0.6 0.3 1.2	1.2 0.3 1.2	1.2 0.15 0.6	1.2 0.15 0.6	1.2 0.15 1.2	•	-	0.3 0.15 0.6	1.2 0.15	-	-	•	1.2 -	1.2 - -	0.6 1.2 -	0.3 1.2 1.2	0.6 1.2 -	-
Piper divaricatum (#24)	M D H	- 0.075 1.2	- 1.2 -	0.3 0.3	- 0.6 1.2		-	÷		-	•	- 1.2 -	- 1.2 0.6	- 1.2 1.2	• • •	- 0.6 1.2	- 1.2	- 0.3 0.6	- - 0.3	- 0.6 1.2	•	- 1.2	1.2	- 1.2 -	•			•			- 0.075 -		-
Piper glabribaccum (#25)	M D H	- 0.15	0.6	0.6	•	•	•	•	-	•	•	•	•	•	•	- 1.2	- - 1.2	- - 1.2	- 0.6 0.3	- - 1.2	•	- 1.2	- 1.2 -	•	•	•	•	•	•	•	0.6 - -	0.6 - -	•
Piper heterophyllum (#26)	M D H	0.075	- 0.6 -	- 0.3 0.6	- 0.6 1.2	•	•	•	-	•	•	•	•	-	•	-	- - 1.2	- - 0.6	0.075	1.2	•	•	- - 1.2	•	•	•	•	•	•	•	- 0.6 -	•	
Piper oblongum (#27)	M D H	1.2 1.2 0.15	0.3 0.6 0.6	0.6 0.3 0.15	- 0.6 0.3	- 1.2	1.2 0.6 0.6	1.2 0.6 0.6	0.6 0.6 0.6	0.6 0.3 0.3	0.6 0.6 0.6	0.6 0.6 0.6	0.6 0.6 0.3	0.6 0.6 0.3	0.6 1.2 0.3	0.3 0.6 0.15	0.3 0.6 0.3	1.2 0.15 0.15	0.6 0.6 0.3	0.6 0.3 0.3	•	-	0.3 0.6 0.3	0.3 0.3	•	•	•	1.2 -	1.2 -	1.2 -	0.3	0.3	
Piper reticulatum (#28)	M D H	0.3 0.3	0.6 1.2	- 0.6 0.6	- 0.6 -	•	•	•	-	1.2 -	•	1.2 -	1.2 -	-	1.2 -	1.2 0.6	1.2 -	- 1.2 1.2	- 1.2 0.075	- 1.2 -	•	- 0.6 -	0.6 0.6 0.6	•	•	•	•	- 1.2	•	- 1.2	0.6 0.6	0.3 -	
Piper sancti-felicis (#29)	Aq D H	0.3 0.075	- 1.2 1.2	0.3 0.3	- 1.2 1.2	•	•	•	-	-	•	•	0.3 1.2 0.6	0.6 1.2 0.6	- 1.2	0.6 - 0.6	- 1.2 0.6	- - 0.6	0.075 0.3 0.075	- 1.2 -	•	- 1.2 1.2	- 0.6 0.6	•	•	-	•	•	-	•	- 1.2 1.2		•
Piper stellipilum (#30)	M D H	- 1.2 0.3	0.3 - 1.2	- 0.6 1.2	- - 0.6	1.2 -	0.6 - 0.6	- 1.2	0.6 - 0.6	0.6 - 0.6	0.6 - 0.6	0.3 - 1.2	0.3 0.6	0.3 - 0.6	0.3 - 0.6	0.3 - 0.6	0.3 - 0.6	1.2 - 1.2	0.6 0.3 0.6	0.3 - 0.6	•	1.2 - 1.2	0.3 - 1.2	- - 0.6	•	-	•	0.6 - -	0.6 - -	0.6 - -	0.3 -	0.3 -	-
Piper trigonum (#31)	M D H	- 1.2	0.6	1.2 0.3 1.2	- 1.2	•	- 1.2 1.2	•	- 1.2 1.2	1.2 1.2 1.2	- 1.2 1.2	1.2	1.2 0.3	1.2 0.6 0.3	1.2	1.2 0.6 1.2	1.2 1.2	- 0.6 -	0.6 - -	- 0.6 0.6	•	•	1.2 0.6	- 0.3 -	•	•		•	-	•	1.2 -	0.6 - -	• •
Piper verruculosum (#32)	M D H	0.3 0.6	0.3	0.3 0.6 1.2	1.2	•	1.2 -	0.6 - -	0.6	0.6 - -	0.6	0.3	0.3 - 0.3	0.6	0.6 - -	0.3 1.2 0.6	0.3	0.6	0.3	0.3	1.2 -	1.2 0.6	0.3	1.2 -	•	-	•	0.6 - -	1.2 -	0.6 -	0.15	0.3	-
Piper xanthostachyum (#33)	HA D H	0.3	0.15	0.15 0.075 1.2	0.3 0.075 1.2	0.6	0.15 0.15 1.2	0.3 0.15 1.2	0.15 0.15 1.2	0.3 0.15 1.2	0.15 0.15 1.2	0.3 0.15 1.2	0.15 0.15 1.2	0.3 0.15 1.2	0.15 1.2	0.15 0.15 1.2	0.15 0.15 1.2	0.3	0.15 0.15 1.2	0.15 0.075 1.2	•	1.2 0.6	0.3 0.15 1.2	0.15 0.075 1.2	•	•	•	1.2 0.6	1.2 0.6	1.2	0.6	0.6 0.6	-
Contemucin					4		0.5	0.5	0.25	0.5	0.5	22	0.06	0.06	0.06	0.04	2		0.125	0.02	0.25	0.25	4		0.5	0.25	0.5	0.5	0.5		,	0.02	0.25
Gentamycin		ND	ND	0.03	4	2	0.5	0.5	0.25	0.5	0.5	32	0.06	0.06	0.06	0.06	2	1	0.125	0.03	0.25	0.25	4	8	0.5	0.25	0.5	0.5	0.5	1	4	0.03	0.25
Vancomycin		ND	ND	0.5	0.5	4	2	2	2	2	2	2	2	2	2	2	0.5	0.5	0.25	1	NA	16	16	16	NA	NA	NA	NA	NA	NA	NA	NA	NA
Amoxicilin		ND	ND	2	64	2	4	16	0.125	16	1	8	16	1	0.25	0.25	0.06	0.03	0.25	0.25	2	0.5	2	16	16	NA	NA	2	1	NA	NA	NA	2
Amphotericin B		4	0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fluconazole		32	8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sertaconazole		NA	64	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Set meetimeone		1.17	1 04	1 10	100	110	<u>ערו</u>	ιψ.	10	411	100	110	110	100	110	100		110	100	110	110	100	110	110	100	110	TW I	110	110	ιw.	nu -	110	100

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386 LCMS data mining and statistical correlation analysis

After the application MS-CleanR workflow to the LCMS data, we obtained 123 unique metabolite features ($m/z \times RT \times Peak$ area). Among these, 56 compounds were annotated at the genus level (45.53

features ($m/z \times RT \times Peak$ area). Among these, 56 compounds were annotated at the genus level (45.53 %), 6 compounds at the family level (4.88 %), and 55 compounds at the generic level (44.72 %),

leaving 6 compounds unannotated (4.88 %). The MSMS fragmentation pathway of these compounds

391 was used to establish a molecular network (**Figure 1**). Using NPClassifire and ClassyFire (Djoumbou

Feunang et al., 2016; Kim et al., 2021), major phytochemical classes and subclasses of compounds

were identified. The main classes of compounds identified were alkaloids, amino acids, fatty acids,

394 polyketides, shikimates, phenylpropanoids, and terpenoids (metadata in **supp info 2**).



395

Figure 1. Molecular networking of putative compounds. A feature ID is allocated in each node. The colors in the network and pie chart relate to the phytochemical classes. The number of features for each class is also shown in the pie chart.

A heatmap of correlation analysis (Clustered Image Map, CIM) is shown in **Figure 2-A**. For this analysis, we compiled all *in vitro* assay results (30 bacterial strains, 2 fungal strains, 4 parasitic strains and 2 cell lines, **Tables 1** and **3**) *versus* the LCMS features. This figure results from a setting of the threshold of correlation at 0.3 (the heatmap is shown *in extenso* in **supp info 3**). Two groups of features responsible for activities can be distinguished. **Group 1** is composed of 11 features (12, 21, 23, 27, 31, 35, 41, 45, 87, 95, and 119) having activity on 21 bacteria, one fungus (*C. albicans* ATCC 10231), and one parasite (*T. b. gambiense*). Activity on bacteria was mostly concentrated on Gram-positive strains

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406 [(except for Burkholderia cepacia (13003), Stenotrophomonas maltophilia (21170), and the three 407 strains of *Escherichia coli*)]. Group 2 is composed of 9 features (4, 6, 7, 16, 53, 63, 65, 77, and 117). 408 This group was selective on Leishmania (all strains, both axenic and intramacrophagic). We also 409 performed a relevance network (RN) analysis on the same datasets to verify and complete the CIM 410 results (Figure 2-B). RN showed two main clusters of features linked to the activity, the same as in the 411 CIM. The LC-MSMS molecular network (Figure 1) allowed to determine that six features identified 412 in the **Group 1** belong to the alkaloids class, while others are mostly self-loops (*i. e.* compounds not having structurally-related analogs in the extract) belonging to varied phytochemical classes. In the 413 414 case of Leishmania-specific compounds (Group 2), they mostly belong to the polyketides or 415 shikimates and phenylpropanoids classes, and are found only as self-loops or only related to one compound and not correlated to any activity (*i.e.* features 16 and 4). 416



Figure 2. A) Clustered Image Map (CIM) performed on the two-blocks of data sets. The plot depicts the correlation between X = LCMS features (p = peak) data and Y = bioassays. Variables were selected across two dimensions and clustered with a complete Euclidean distance method. Blue squares represent the two main groups of features identified by activities B) The two main clusters of interaction represented by the relevance network (RN). The two analyses were tuned to a threshold of interaction of 0.3.

417

424 **Table 4.** Features identified as responsible for bioactivity. $^{\dagger} = [M+H-H_2O]^+$; $^{\ddagger} = [M+Na]^+$; $^{\ddagger} =$ based on 425 molecular networking.

Feature ID	<i>m/z</i> [M+H] ⁺	RT	Formula	Annotation	Annotation level	Phytochemical class
				Group 1		
12	344.335	9.745	C ₂₄ H ₄₁ N	(1-{12,16-dimethylpentacyclo octadecan-15- yl}ethyl)dimethylamine	generic	terpenoid

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21	362.342	9.745	C ₂₄ H ₄₃ NO	2,4,14-eicosatrienoic acid, 2- methylpropylamide	rienoic acid, 2- opylamide genus				
23	396.363	10.225	C ₂₃ H ₄₅ N ₃ O ₂	5-(4-aminobutyl)-1,5- diazacyclohenicosane-6,14- dione	generic	alkaloids and derivatives			
27	337.573 [†]	10.391	C ₂₆ H ₄₇ NO	filfiline derivative	genus	alkaloids and derivatives [‡]			
31	400.394 [†]	11.035		unknown		alkaloids and derivatives [‡]			
35	428.426	11.628		unknown		alkaloids and derivatives [‡]			
41	456.457	12.118		unknown		alkaloids and derivatives [‡]			
45	489.155	10.287	$C_{28}H_{24}O_8$	ferrudiol	genus	shikimates and phenylpropanoids			
87	271.060	9.28	$C_{15}H_{10}O_5$	apigenin	generic	shikimates and phenylpropanoids			
95	279.232	10.707	$C_{18}H_{30}O_2$	gamma-linolenate	generic	fatty acids and conjugates			
119	316.301	9.077	C ₂₂ H37N	daphnane	generic	alkaloids and derivatives			
				Group 2					
4	151.0755	6.5	$C_{9}H_{10}O_{2}$	acetanisole derivative	genus	shikimates and phenylpropanoids			
6	$221.081^{\text{¥}}$	8.087	$C_{10}H_{14}O_4$	decarestrictin H	generic	polyketides			
7	340.116 [¥]	7.486	$C_{17}H_{19}NO_5$	aduncamide	genus	shikimates and phenylpropanoids			
16	355.118	9.49	$C_{20}H_{18}O_{6}$	sesamin	genus	shikimates and phenylpropanoids			
53	593.277 [¥]	13.09	$C_{32}H_{42}O_9$	swietenin E	generic	terpenoids			
63	627.246	12.539	$C_{33}H_{38}O_{12}$	thielavin L	generic	polyketides			
65	197.117	6.022	$C_{11}H_{16}O_3$	isololiolide	family	terpenoids			
77	221.081	7.516	$C_{10}H_{14}O_4$	modiolide A	generic	polyketides			
117	312.159	8.705	$C_{19}H_{21}NO_3$	piperettine	genus	alkaloids and derivatives			

426

427 Figure 3 shows a heatmap depicting how the features are distributed among the extracts. Features of Group 1 are mainly distributed in all extracts of *P. strigosum* (features 12, 21, 23, 31, 41, 27 and 35) 428 429 and P. xanthostachyum (features 45 and 87). Features of Group 2 are found in the hexane and 430 methylene chloride extracts of P. sancti-felicis and hexane extracts of P. calvescentinerve, P. 431 cordatomentosa and P. crassinervium (features 6, 7, 16 and 77), methylene chloride extracts of P. 432 pseudoarboreum, P. calvescentinerve, P. divaricatum, P. glabribaccum, P. bullatum, P. 433 heterophyllum, P. reticulatum, P. oblongum, P. trigonum, P. crassinervium and P. cordatomentosa, 434 and the aqueous extract of P. sancti-felicis (features 4, 53, 63, 65 and 117). Feature 95 is found in the 435 methanol and hexane extracts of P. reticulatum and the hexane extracts of P. pseudoarboreum, P 436 armatum, P. glabribaccum, P. oblongum and P. verruculosum.





- **Figure 3.** Heatmap of correlation between plant extracts and presumed bioactive peak features. Y =
- *Piper* extracts, and X = features of LC-MSMS analysis as shown in Figure 2A-B. Green and yellow
- 441 squares represent the distribution of the bioactive features identified for the **Groups 1** and **2** in the 442 *Piper* extracts
- *Piper* extracts.



443 **DISCUSSION**

444 Plants from the Piper genus are currently used by the Amerindian communities in Peru in different 445 preparations (infusion of different parts, juice directly ingested, cataplasms made of crushed leaves or stems. etc.) to treat different diseases (Odonne et al., 2009, 2011; Vásquez-Ocmín et al., 2021a). For 446 447 the present study, we conducted ethnopharmacological surveys in Loreto and Cusco, Peruvian 448 Amazonian regions departments heavily impacted by protozoal diseases. Indeed, Loreto has always 449 been the department with the greatest number of malaria cases (principally *P. vivax*), mostly affecting 450 children from birth to age 11 (84.5 % in 2020) (Ministerio de Salud del Perú, 2021). Leishmaniasis 451 occurrence in Peru is principally due to cutaneous (L. amazonesis, 17 892 cases over the last five years) 452 and mucosal forms (L. braziliensis, L. peruviana, L. lainsoni, L. guyanensis, 1 642 cases in the last five 453 years). To date, in 2022 70% of the cases of both forms were concentrated in 7 departments: Madre de 454 Dios, Cusco (Echarate and Kosñipata districts), Piura, Junín, Loreto, Cajamarca and San Martín 455 (Ministerio de Salud del Perú, 2022). In Peru, plant-based treatments are very common and even 456 recommended by the health personnel. It is estimated that approximately 5000 species are used in the 457 traditional medicine (Vásquez-Ocmín et al., 2018) among which the Piper species studied in this work 458 represent 0.34 %. We targeted the ethnopharmacological survey on "Cordoncillos" (little cords), a 459 word used to describe pepper vines in Amazonia because the presence of nodes on the stems reminds 460 us of a nudo de soga (node of cord). While this terminology would initially direct the sample collection, 461 we took great care of properly identifying the species upon collection. In two cases, we could not fully 462 ascertain the species and further taxonomic examination is underway.

463 Chemical profiling of natural extracts can be readily obtained, but for metabolomic purposes, data need 464 to be 'preprocessed' (peak picking, alignment, clustering, integration and normalization) to obtain a clean dataset able to provide meaningful information upon statistical analysis. The large abundance 465 and complexity of variables are also challenging and require to apply an appropriate statistical method 466 to answer a specific biological question (Hervé et al., 2018). Few metabolomic studies on Piper species 467 using NMR (Yamaguchi et al., 2011; Uckele et al., 2021) or LCMS have been reported so far (Vásquez-468 469 Ocmín et al., 2021a). The Piper genus is a good model to apply our framework for two reasons: i) 470 *Piper* species are particularly abundant in Peru (~260 according to www.tropicos.org), many of them 471 used in traditional medicine; ii) many studies, including numerous phytochemical ones, were carried 472 out on this genus (~9000 results found in www.webofscience.com for "Piper", consulted on July 1st, 473 2022), allowing a good metabolome coverage.

474 We propose in this work a computational statistical analysis to integrate two heterodimensional 475 datasets: the features (compounds) obtained by LC-MSMS analysis of *Piper* extracts (supp info 2), 476 and the *in vitro* biological activity of these extracts on parasites, bacteria and mammalian cells (**Tables** 477 1 and 3), with the aim to outline most active compounds. To leverage on this multiplexed approach, 478 we applied a regularized Canonical Correlation Analysis (rCCA). This method aims to extract 479 correlated information by maximizing their correlation via canonical variates between two datasets 480 acquired on the same samples (vertical integration). The results of rCCA model were displayed using 481 Clustered Image Maps (CIM) to highlight the correlation level of variables from all datasets, ordered 482 through unsupervised clustering on both samples and compounds simultaneously (González et al., 483 2012). Another approach for displaying net-like correlation structures between two data sets is to use 484 Relevance Networks (RN). This method generates a graph where nodes represent variables, and edges 485 represent variable associations (Butte et al., 2000). Given an appropriate threshold, the RN highlights 486 main correlation between both datasets (Figure 2-B). To our knowledge, the implementation of this 487 type of metabolomics framework aimed at identifying bioactive compounds has not been used so far.

488 However, a study has been recently published using rCCA to combine multiparametric analysis of

489 adjuvanticity in vivo with immunological profiles in vitro (cytokines, chemokines, and growth factor

490 secretion analyzed by flow cytometry) of a library of compounds derived from hot-water extracts of

491 herbal medicines (Hioki et al., 2022).

492 Figure 2 strikingly shows that the antibacterial and antileishmanial activities are correlated with two 493 distinct groups of compounds. This is by no means surprising as these organisms have significantly 494 different physiological organization, leading to different drugs currently used against these infections. 495 The species showing the most significant antibacterial activities are *P. strigosum* and *P.* 496 xanthostachyum, and the species showing the most significant antileishmanial activities are P. 497 pseudoarboreum, P. calvescentinerve, P. glabribaccum, P. reticulatum and P. sancti-felicis. The 498 compounds correlated with these activities within these extracts are rather specific to these species 499 (Figure 3). An overall weak activity of these *Piper* extracts on *P. falciparum* (Table 1) is an 500 unexpected result, as *Piper* species are usually reputed for their antimalarial potential.

501 Features from Group 1 were active not only on bacteria but also on one fungus and one parasite. 502 Although the high cross-activity observed for this group of compounds (Figure 2 and supp info 3) 503 could foretell of a lack of selectivity, these compounds did not cause cytotoxicity in mammalian cells. 504 Six of the features of **Group 1** annotated as alkaloid derivatives were grouped in a same cluster in the 505 molecular networking (MN), suggesting a similar biosynthesis pathway. Only three of them were 506 annotated in our workflow, suggesting that the three unannotated ones may have an original structure. 507 The reliability of the annotation or classification (NPClassifire and ClassyFire) part of the workflow 508 may nevertheless be tempered, as shown by the case of feature 27, classified by the workflow as an 509 alkaloid, but annotated as filfiline, a fatty amide previously isolated from the roots of P. retrofractum 510 (Banerji et al., 2002). The broad bioactivity spectrum of alkaloids is well known, *i.e.*, antibacterial, 511 antiparasitic, anticancer, but also their cytotoxicity (Newman and Cragg, 2007, 2020; Daley and 512 Cordell, 2021; Yan et al., 2021). The mechanism of action of antibacterial alkaloids is mainly described 513 as disrupting the bacterial membrane, affecting DNA function and inhibiting protein synthesis 514 (Ananthan et al., 2009; Pan et al., 2014; Kelley et al., 2013; Li et al., 2014; Larghi et al.). Antibacterial 515 alkaloids (MIC $<10 \mu$ M/mL) are as diverse as isoquinolines, aporphines, phenanthrenes, quinolines 516 and indoles and their activity is mostly oriented against Gram-positive bacteria (Porras et al., 2021). 517 Quinoleines like 8-hydroxyquinoline and its derivatives and evocarpine are active on bacteria 518 associated with respiratory system infections: M. tuberculosis, S. aureus, and MRSA (Methicillin-519 resistant *Staphylococcus aureus*) (MIC $\leq 10 \mu$ M/mL). Aporphine alkaloid derivatives exhibit high 520 broad-spectrum activity against Gram-positive bacteria: S. agalactiae, S. aureus, S. epidermidis, E. faecalis, and as in our study E. coli (Hamoud et al., 2015; Tan et al., 2015). Even if the chemical array 521 522 within *Piper* spp. is impressively diverse, nitrogen-containing compounds are limited, *i.e.*, alkaloids 523 like piperines or amides like piplartine, phenethyls, and diaminodiamides. Amides are known to have 524 fungicidal, cytotoxic, or antiprotozoal activities. With regard to fungicidal activity. 525 dehydropipernonaline and nigramide R previously isolated from P. retrofractum displayed potent 526 growth inhibition of *Cladosporium cladosporioides* and cytotoxicity against the L5178Y mouse 527 lymphoma cell line (IC₅₀ values of 8.9 µM and 9.3 µM, respectively) (Muharini et al., 2015). Amide 528 piplartine isolated from a methanol extract of P. retrofractum showed good activity on L. donovani 529 with an IC₅₀ value at 7.5 µM (Bodiwala et al., 2007). In Group 1, features correlated with a fungicidal 530 activity would only target C. albicans. Other active compounds identified in Group 1 were one 531 terpenoid, two fatty acids, one shikimate and phenylpropanoid derivative and one polyketide.

532 Compounds from the **Group 2**, characterized as being rather specific to *Leishmania*, are labeled as 533 polyketides (3), shikimates and phenylpropanoids (3), terpenoids (2) and alkaloids (1). Their lack of 534 activity on *Trypanosoma* is not surprising (Figure 2), as even though both parasites belong to the same 535 Trypanosomatidae order, the current treatments are not similar. These two parasites cause different 536 pathologies, in distinct geographical areas. They evolved differently and show evolutionary 537 discrepancies in their mechanisms that may explain variations in sensitivity to treatments (Fernandes 538 et al., 2020; Van den Broeck et al., 2020). A cross-reading of Figures 2 and 3, suggests that extracts 539 of *P. calvescentinerve* and *P. glabribaccum* and features from Group 2 have not only a selectivity for 540 *Leishmania* but also a good selectivity index. Selectivity is a key parameter for antileishmanial drugs: 541 antimonials, amphotericin B, paromomycin sulfate and miltefosine have variable efficacy against the 542 20+ Leishmania species but have significant adverse effects (Rao et al., 2019). Tremendous efforts 543 have been put into the understanding of the Leishmania biology, leading to the identification of 544 numerous putative targets: ergosterol and its biosynthetic pathway [*i.e.* amphotericine B and enzymes 545 like squalene synthase (SQS) or sterol methyltransferase (SMT)], the glycolytic pathway necessary to 546 provide glucose as an energy source, DNA topoisomerases, enzymes of the polyamine biosynthetic 547 pathway (*i.e.* arginase, ornithine decarboxylase, s-adenosylmethionine decarboxylase, and spermidine 548 synthase), redox metabolism pathway (Nagle et al., 2014; Raj et al., 2020).

549 The compounds labeled as shikimates or phenylpropanoids (acetanisole, aduncamide, and sesamin) or 550 alkaloid (piperettine) were annotated at the genus level because they had previously been isolated from 551 P. tuberculatum, P. nigrum, P. longum, P. aduncum, P. puberulum, P. austrosinense, P. 552 brachystachyum, P. mullesua, P. retrofractum, P. sarmentosum and P. sylvaticum (Spring and Stark, 553 1950; Orjala et al., 1993; De Araujo-Junior et al., 1997; Puri et al., 1998; Umezawa, 2003; 554 Tuntiwachwuttikul et al., 2006). Among these compounds, only the lignan sesamin was reported to 555 show activity on L. amazonensis with an IC₅₀ of 44.6 µM/mL and was not cytotoxic for mouse 556 macrophage cells ($CC_{50} > 100 \mu g/mL$, SI > 6) (Pulivarthi et al., 2015). A study based on computational 557 methods suggests that sesamin could be a promising inhibitor of the L. donovani CRK12 receptor (binding affinity of -8.5 kcal/mol) (Broni et al., 2021). Nevertheless, sesamin, isolated from a hexane 558 559 extract of *P. retrofractum* (IC₅₀ of the extract = $5 \mu g/mL$), was inactive on *L. donovani* (Bodiwala et 560 al., 2007) (Bodiwala et al., 2007). This compound was also inactive on *Plasmodium falciparum* K1 561 multidrug resistant strain, Mycobacterium tuberculosis H37Ra, and Candida albicans (EC₅₀ > 20 562 μ g/mL, MIC > 200 μ g/mL and IC₅₀ > 50 μ g/mL, respectively). Cubein, another lignan isolated from 563 *P. cubeba*, was shown to be active on *L. donovani* (IC₅₀ = $28.0 \,\mu$ M). Interestingly, sesamin is connected 564 in the MN with an unknown compound (RT = 6.577, $[M+H]^+ = 360.145$). Piperettine has been 565 previously isolated from P. nigrum and P. aurantiacum and was shown to be active on epimastigotes 566 and amastigotes of *Trypanosoma cruzi* (IC₅₀ = 10.67 and 7.40 μ M, respectively) (Ribeiro et al., 2004). 567 In our work, the compound annotated as piperettine was only detected in *P. calvescentinerve* extracts 568 (Figure 3) but was not labeled as being correlated with any activity on T. b. gambiense (supp info 3). 569 The use of this *Piper* species as a medicinal plant with various indications can partly be validated by the fact that it is an inhibitor of 5-lipoxygenase (76.02 µM) (Muthuraman et al., 2019), a key enzyme 570 571 involved in the biosynthesis of pro-inflammatory leukotrienes, provided its concentration is sufficient 572 in the traditional preparations. Aduncamide was shown to present a moderate antineuroinflammatory 573 activity (IC₅₀ at $26 \pm 8.3 \,\mu$ M) by the Griess method on LPS-stimulated BV-2 cells (Zheng et al., 2021), 574 to be cytotoxic for KB nasopharyngeal carcinoma cells (ED₅₀ = $5.7 \mu g/mL$) and to inhibit growth 575 Gram-positive B. subtilis and M. luteus, while being less active towards Gram-negative E. coli (Orjala 576 et al., 1993). In our work, the compound annotated as aduncamide was identified in extracts of P. 577 glabribaccum, P. calvescentinerve and P. cordatomentosa, whose extracts are among the most active 578 on Leishmania. Two compounds were labeled as 10-membered lactone macrolides and annotated as 579 decarestrictin H and modiolide A, previously isolated from the fungi Penicillium simplicissimum and Stagonospora cirsii, respectively (Grabley et al., 1992; Evidente et al., 2008). These two last 580 581 annotations, although resulting from similar fragmentation patterns, may be subject to caution. Indeed,

582 these compounds were annotated at the generic level and no macrolide has been identified in *Piper* 583 spp. so far (only a macrolactam, laevicarpin, previously isolated from *P. laevicarpus*) (da Silva A. 584 Maciel et al., 2016). Furthermore, macrolides are well-known antibacterial compounds and thus should logically rather be clustered in Group 1, if ever present in the extracts. A compound annotated as 585 586 apigenin was detected only in P. xanthostachyum and was identified as being correlated to the 587 antibacterial activity (Figure 2A). This may appear surprising given that apigenin is a common and ubiquitous flavonoid with no antibacterial activity. The number of possible flavonoid isomers being 588 589 quite large, this annotation may also be subject to caution, notwithstanding the fact that the actual 590 compound (p87) being present in the extract remains of interest from the antibacterial perspective. 591 Other compounds were annotated as flavonoids in the extracts (most of the compounds in Group 3, 592 see **supp info 3**), characterized by a moderate activity of the extracts containing them, a moderate 593 correlation with all the activities without any clear selectivity. These compounds were annotated at the 594 genus level, as they were previously isolated from Piper species (Matsui and Munakata, 1976; Lago et al., 2004; González et al., 2022). Antiprotozoal or antimicrobial activity of flavonoids is well 595 596 documented (Graf et al., 2005; Ortiz et al., 2017, 2020). Two compounds were annotated as 597 pinocembrine and pinostrobin, both belonging to the flavanones subclass. Pinocembrine displays 598 antifungal (C. cladosporioides and C. sphaerospermum) or antibacterial (Enterococcus faecalis, 599 Mycobacterium tuberculosis) potential (Lago et al., 2004; Jeong et al., 2009; Gröblacher et al., 2012), 600 while showing either no cytotoxicity on healthy and cancerous cell lines (RAW 264.7, epidermoid 601 carcinoma of the oral cavity (KB), human small cell lung cancer (NCI-H187), metastatic murine colon 26-L5 carcinoma, PANC-1 human pancreatic cancer, metastatic human HT-1080 fibrosarcoma), or 602 603 high cytotoxicity (Awale et al., 2009; Yenjai and Wanich, 2010; Lee et al., 2013). Pinostrobin showed 604 weak antimalaria activity on P. falciparum (Kaur et al., 2009) but significant potential as a cancer 605 chemopreventive agent (Gu et al., 2002).

606 Some of these *Piper* compounds are therefore of interest in an isolation and factual testing perspective. 607 Compounds annotated as aduncamide, sesamin, and apigenin, along with the unknown compounds 608 correlated to the activity, could be subjected to mass-targeted isolation (Vásquez-Ocmín et al., 2022a), 609 in order to confirm their annotation or identify their structure, as well as confirm their biological 610 potential.

611 CONCLUSION

612 Hyphenated analytical techniques are very useful to provide highly informative chemical profiling of complex metabolomes. Nevertheless, deciphering such profiles to determine the compounds 613 614 responsible for the biological activity remains a challenge. By correlating these chemical profiles with 615 biological assay results, we propose a workflow of integrated metabolomics statistical tools to provide 616 a broad picture of the metabolome as well as a map of compounds putatively associated to the 617 bioactivity. The structure of these compounds can either be annotated from previous works or remain 618 unknown at this stage, but in any case, they require a formal isolation step to confirm their structure 619 and activity. Nevertheless, an initial data mining on analytical-scale data proves very effective for prioritizing the compounds to target. The relevance of our approach has been validated on a set of 620 621 Piper species tested for their anti-infectious diseases at the extract level. Such an approach can be 622 extended to any type of natural extract, particularly when prior phytochemical data is available in the 623 literature.

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625 DATA AVAILABILITY STATEMENT

626 The raw data from the LCMS were uploaded to zenodo (DOI:10.5281/zenodo.7317966).

627 AUTHOR CONTRIBUTIONS

Conceptualization: PGVO, AM, GM, and LRM; Methodology: PGVO, AM, GM, and LRM; Chemical
data acquisition/curation: PGVO, KL, GM, AG, and AM; Statistical analysis: PGVO and GM;
Biological data curation: SC, VR, SB, and SP; Investigation: PGVO, SC, VR, GM, SP, AG, KL, ID,
LRV, HRC, WRM, KL, SB, LRM, and AM. PGVO wrote the original draft and all the authors
contributed to the final manuscript.

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Terpenoids







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