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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.

In review

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Abstract

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

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*

In review

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
63 around 627,000 deaths in 2020, with two-thirds of these deaths (470,000) being due to treatment
64 disruptions during the COVID-19 pandemic (WHO, 2021). *Leishmania* spp. and *Trypanosoma* spp.
65 are protozoan parasites responsible ~~for~~ diseases labeled as NTDs *stricto sensu*. The ambitious 10-
66 year WHO plan to defeat NTDs is based on three pillars: control, elimination and eradication (WHO,
67 2022a). The support of research and development of new medicines to fight infectious diseases is one
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
81 “first-hand treatment”, especially in the villages far away from major cities and medical care. The
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 N° 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
119 soaked in 1 L of each of the following solvents successively: hexane, methylene chloride, methanol,
120 ethanol/water (7:3, v/v), and aqueous. ~~These macerations~~ ~~Extractions~~ were ~~made-performed during~~ ~~for~~
121 21 days ~~each~~ for hexane, methylene chloride and ethanol/water, and for 15 days in methanol and
122 aqueous extracts, with solvents being changed every 3 days. The extracts were then filtered through a
123 paper filter and evaporated under reduced pressure below 40°C. Dry extracts were stored at -4 °C until
124 use. Extracts were solubilized in DMSO at a concentration of 10 mg/mL for *in vitro* bioassays and
125 HPLC-MS analysis.

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).

134 *Plasmodium*: The *P. falciparum* chloroquine-sensitive strain 3D7 was obtained from the Malaria
135 French National Reference Center (CNR Paludisme, Hôpital Bichat Claude Bernard, Paris) and was
136 maintained in O⁺ human erythrocytes in RPMI 1640 medium (Invitrogen, Life Technologies)
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
144 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,
149 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
151 serum, 100 IU penicillin and 100 µg.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*

161 Assays were realized with a suspension of erythrocytes at 1 % parasitemia containing more than 85 %
162 ring stage obtained by repeated sorbitol treatment and incubated with the compounds at concentrations
163 ranging between 0.49 and 100 µM or µg/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
169 to obtain the amastigote form in promastigote medium supplemented with 2 mM CaCl₂, 2 mM MgCl₂,
170 and a pH adjusted to 5.5. The axenic amastigote suspension containing 1.106 parasites/mL was
171 incubated for 72h with the compounds at 37°C in 5 % CO₂ in the dark. Tested compounds or extracts
172 were obtained by serial dilution and ranged between 0.49 and 100 µM or µg/mL. There were two
173 controls: parasites without drug and parasites treated with miltefosine at the same concentrations as the
174 compounds tested (Vásquez-Ocmín et al., 2018, 2021a).

175 *In vitro* antileishmanial activity on *L. donovani*, *L. amazonensis* 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 µL and incubated in a 5 % CO₂ at 37°C for 24h. After removing the medium, cells were incubated with
179 100 µL 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 µL 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 Trypanosoma*

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
191 the Master epRealplex cyclor[®] (Eppendorf, France) and the following program to increase SYBR
192 Green incorporation: 90°C for 1 min, decrease in temperature from 90°C to 10°C for 5 min with
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**

213 Minimal Inhibitory Concentration (MIC) determinations of crude extracts were carried out using the
214 agar dilution method stipulated by the Clinical and Laboratory Standards Institute (CLSI, 2006).
215 Antimicrobial activity was evaluated for the first time against a panel of 36 pathogenic and multi-drug-
216 resistant bacteria, which, in most cases, have been recently isolated from human infections. For
217 comparison, reference strains from the American Type Culture Collection (ATCC) were included.
218 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
220 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

228 Cytotoxicity was evaluated on RAW 264.7 macrophages for *Leishmania* assays and HUVECs for
229 *Plasmodium* and *Trypanosoma*. The cells were seeded at a density of 50,000 cells per well in 100 μ l
230 of DMEM in a 96-well microtiter plate. After incubation in a 5 % CO₂ at 37°C for 24h, the culture
231 medium was replaced with 100 μ l of fresh DMEM containing serial dilutions of the compounds tested.
232 The concentrations of the compounds are the same as for intramacrophage *Leishmania* or *Plasmodium*
233 assays. The plates were incubated for 48h at 37°C with 5 % CO₂. Antiprotozoal assays have been
234 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 \$\mu\$ m](#), 2.1 x 75 mm ~~2.5 μ m~~ (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 QC
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
270 applying a dot product score cut-off of 800. Metabolite annotation at level 2 was prioritized according
271 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
273 version 28.2, CRC Press) based on exact mass and *in silico* fragmentation. For metabolite annotation
274 level 3, a search was made using MS-FINDER for a match with natural compounds included in their
275 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
279 MS2 m/z tolerance = 0.02 Da, minimum matched peaks = 4 and minimal cosine score value = 0.7. The
280 visualization of the molecular network (MN) was performed on Cytoscape version 3.9.1 (Shannon et
281 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
287 transformed in pIC₅₀ (-log₁₀ [IC₅₀]) for further statistical correlation analysis. MIC values were log
288 transformed. The LC-MS dataset (*m/z* × RT × 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, a 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**

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

303 **Anti-infectious activity**

304 **Antiprotozoal activity**

305 The IC₅₀ values for antiplasmodial, antileishmanial and antitrypanosomal activities of each extract are
306 shown in **Table 1**. We considered an extract active and worthy to be further studied when its IC₅₀ value
307 was below 10 µg/mL. Four extracts were active in this case regarding the 3D7 *P. falciparum*
308 chloroquine-sensitive strain assay: the hexane and methylene chloride extracts of *P. crassinervium*, the
309 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
316 extracts were active only on *L. amazonensis*, and *L. braziliensis* (both axenic and intramacrophagic
317 amastigotes): methylene chloride extracts of *P. calvescentinerve*, *Piper crassinervium*, and *P.*
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. amazonensis*, 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
326 methylene chloride extracts of *P. stellipilum*, and hexane and methylene chloride extracts of *P.*
327 *calvescentinerve* and of *P. divaricatum*. None of the extracts showed selective activity on *L. donovani*
328 or *L. amazonensis*.

In review

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

Species, Region (Part tested)	Extract (code for metabolomic analyses)	<i>P. falciparum</i> 3D7 strain, IC ₅₀ ± SD, µg/mL	<i>L. donovani</i> LV9 strain (axenic amastigotes), IC ₅₀ ± SD, µg/mL	<i>L. donovani</i> LV9 strain (intra- macrophagic amastigotes), IC ₅₀ ± SD, µg/mL	<i>L.</i> <i>amazonensis</i> (axenic amastigotes), IC ₅₀ ± SD, µg/mL	<i>L. amazonensis</i> (intra- macrophagic amastigotes), IC ₅₀ ± SD, µg/mL	<i>L. braziliensis</i> (axenic amastigotes), IC ₅₀ ± SD, µg/mL	<i>L. braziliensis</i> (intra- macrophagic amastigotes), IC ₅₀ ± SD, µg/mL	<i>Trypanosoma</i> <i>brucei</i> <i>gambiense</i> IC ₅₀ ± SD, µg/mL	HUVEC CC ₅₀ ± SD, µg/mL	RAW 264.7 macrophages CC ₅₀ ± SD, µg/mL
<i>Piper</i> <i>casapiense</i> (Miq.) C. DC., Loreto (L&S)	H (P4H)	>100	>100	NT	>100	NT	>100	NT	0.68 ± 0.14	>100	>100
	D (P4D)**	>100	>100	NT	>100	NT	>100	NT	4.06 ± 1.11	45.31 ± 1.97	61.12 ± 4.41
	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
<i>Piper</i> <i>strigosum</i> Trel.; Loreto (L&S)	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
	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
	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
<i>Piper</i> <i>pseudoarboresum</i> Yunck; Loreto (L&S)	H (P14H)**	>100	>100	NT	>100	NT	>100	NT	12.06 ± 3.80	61.94 ± 4.64	>100
	D (P14D)**	>100	>100	NT	>100	NT	>100	NT	5.07 ± 2.17	40.21 ± 4.12	>100
	M (P14M)**	>100	>100	NT	>100	NT	>100	NT	28.56 ± 15.97	25.49 ± 1.87	49.09 ± 3.88
* <i>Piper</i> <i>armatum</i> Trel. & Yunck; Loreto (L&S)	H (P18H)	>100	>100	NT	>100	NT	>100	NT	25.04 ± 5.33	>100	>100
	D (P18D)	>100	>100	NT	>100	NT	>100	NT	1.50 ± 0.16	55.31 ± 4.12	>100
	M (P18M)	>100	>100	NT	>100	NT	>100	NT	11.26 ± 1.90	35.86 ± 2.88	33.03 ± 2.99
* <i>Piper</i> <i>brasiliense</i> C. DC; Loreto (L&S)	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
	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
* “ <i>Piper</i> <i>bullatum</i> Vahl”; CUSCO (L&S)	H (P20H)	>100	>100	NT	>100	NT	>100	NT	5.05 ± 1.51	>100	77.64 ± 6.88
	D (P20D)	>100	>100	NT	>100	NT	>100	NT	4.40 ± 0.54	43.79 ± 4.41	41.66 ± 3.64
	M (P20M)	>100	>100	NT	>100	NT	>100	NT	21.47 ± 12.60	25.09 ± 2.40	>100
* <i>Piper</i> <i>calvescentinerve</i> Trel; Cusco (L&S)	H (P21H)	>100	29.29 ± 2.12	22.64 ± 2.01	17.44 ± 1.55	15.84 ± 1.11	6.33 ± 0.63	1.92 ± 0.07	3.97 ± 0.25	>100	>100
	D (P21D)	>100	>100	NT	2.55 ± 1.08	1.94 ± 0.84	3.54 ± 0.16	1.64 ± 1.02	6.08 ± 0.49	38.83 ± 5.41	44.88 ± 4.34
	M (P21M)	>100	>100	NT	>100	NT	43.61 ± 3.87	20.64 ± 1.55	17.75 ± 1.32	38.55 ± 1.13	72.22 ± 6.66

<i>Piper "cordatomentos a"</i> ; Cuzco (L&S)	H (P22H)	>100	12.41 ± 0.88	9.78 ± 1.55	8.77 ± 1.12	5.66 ± 1.88	6.92 ± 0.71	3.22 ± 0.48	4.50 ± 1.27	>100	>100
	D (P22D)	>100	17.99 ± 0.77	18.34 ± 0.66	10.44 ± 1.45	10.44 ± 1.84	4.59 ± 0.45	1.08 ± 0.33	2.62 ± 1.11	58.32 ± 5.68	61.44 ± 4.11
	M (P22M)	>100	>100	NT	>100	NT	>100	NT	34.58 ± 13.10	61.50 ± 2.62	>100
<i>Piper crassinervium</i> Kunth.; Cusco (L&S)	H (P23H)	5.66 ± 0.87	8.05 ± 0.74	>100	8.55 ± 1.61	4.55 ± 1.09	6.48 ± 1.22	3.18 ± 1.08	7.08 ± 0.85	3.51 ± 0.71	10.44 ± 0.79
	D (P23D)	7.41 ± 0.99	2.95 ± 0.32	0.99 ± 1.33	3.18 ± 1.08	2.66 ± 1.88	5.77 ± 1.12	1.25 ± 1.46	3.25 ± 0.62	>100	37.38 ± 4.52
	M (P23M)	>100	>100	NT	>100	NT	2.70 ± 0.19	1.12 ± 1.46	14.44 ± 9.07	15.28 ± 3.07	37.38 ± 4.52
<i>Piper divaricatum</i> G. Mey; San Martin (L)	H (P24H)	>100	>100	NT	>100	NT	>100	NT	4.57 ± 0.94	30.04 ± 4.95	55.95 ± 4.12
	D (P24D)	>100	>100	NT	>100	NT	2.61 ± 0.27	5.44 ± 0.88	4.02 ± 0.39	>100	>100
<i>*Piper glabribaccum</i> Trel; Cusco (L&S)	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
	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
	M (P25M)	>100	>100	NT	>100	NT	>100	NT	14.98 ± 2.75	84.72 ± 1.25	>100
<i>Piper heterophyllum</i> Ruiz & Pav.; Loreto (L)	H (P26H)	>100	4.32 ± 0.76	3.11 ± 0.99	15.66 ± 1.46	11.51 ± 0.98	5.55 ± 0.95	3.15 ± 0.99		81.67 ± 2.17	
	D (P26D)	>100	6.56 ± 0.61	7.0 ± 0.61	10.41 ± 0.64	11.32 ± 1.61	2.07 ± 0.20	3.44 ± 0.88	17.27 ± 0.59	17.14 ± 2.19	89.66 ± 5.78
<i>*Piper oblongum</i> Kunth; Cusco (L&S)	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
	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
	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
<i>Piper reticulatum</i> L.; Loreto (L)	H (P28H)	>100	>100	NT	>100	NT	>100	NT	11.84 ± 1.29	>100	>100
	D (P28D)	>100	>100	NT	>100	NT	>100	NT	6.65 ± 1.31	>100	84.46 ± 5.88
	M (P28M)	>100	>100	NT	>100	NT	>100	NT	24.92 ± 6.60	>100	77.54 ± 7.12
<i>*Piper sanctifelicis</i> Trel; Loreto (L&S)	H (P29 H)	>100	17.88 ± 1.11	18.41 ± 1.33	21.55 ± 1.46	18.22 ± 1.62	4.10 ± 0.57	3.44 ± 0.99	6.60 ± 1.24	>100	>100
	D (P29 D)	>100	13.11 ± 0.88	10.45 ± 0.46	18.41 ± 1.34	17.51 ± 1.99	4.21 ± 0.52	6.44 ± 0.99	9.23 ± 2.58	49.40 ± 4.04	>100
	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
<i>*Piper stellipilum</i> (Miq.) C. DC; Loreto (L&S)	H (P30H)	>100	>100	NT	>100	NT	3.74 ± 0.65	8.41 ± 1.31	6.78 ± 3.78	>100	>100
	D (P30D)	13.44 ± 0.21	>100	NT	>100	NT	4.45 ± 0.45	9.99 ± 1.34	9.64 ± 0.46	18.05 ± 1.04	66.54 ± 5.12
	M (P30M)	8.22 ± 0.77	>100	NT	>100	NT	12.33 ± 1.08	15.64 ± 1.88	13.15 ± 2.01	25.03 ± 3.09	51.44 ± 2.99
<i>*Piper trigonum</i> C. DC.; Cusco (L&S)	H (P31H)	>100	>100	NT	>100	NT	>100	NT	8.84 ± 0.29	>100	>100
	D (P31D)	>100	>100	NT	>100	NT	>100	NT	4.84 ± 1.40	30.51 ± 3.05	77.45 ± 4.44
	M (P31M)	>100	>100	NT	>100	NT	>100	NT	10.93 ± 2.53	>100	70.67 ± 6.12
<i>*Piper verruculosum</i> C. DC.; Cusco (L&S)	H (P32H)	>100	>100	NT	>100	NT	>100	NT	8.54 ± 2.90	>100	>100
	D (P32D)	>100	>100	NT	>100	NT	>100	NT	5.64 ± 0.41	>100	>100
	M (P32M)	>100	>100	NT	>100	NT	>100	NT	27.38 ± 6.81	>100	>100
<i>Piper xanthostachyum</i>	H (P33H)	>100	17.54 ± 0.99	11.12 ± 1.01	21.46 ± 1.89	18.44 ± 1.46	6.51 ± 0.64	9.55 ± 0.55	14.00 ± 1.26	>100	>100
	D (P33D)	6.33 ± 0.74	14.79 ± 0.77	15.32 ± 0.99	29.44 ± 1.46	15.63 ± 1.77	2.98 ± 0.29	5.45 ± 0.66	2.25 ± 0.83	6.18 ± 1.74	52.12 ± 4.03

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		0.007 (21.40 ± 1.56 nM)	NT	NT	NT	NT	NT	NT	NT	NT	NT
Miltefosine		NT	1.41 ± 0.50 (3.46 ± 1.22 μM)	2.49 ± 0.45 (6.10 ± 1.10 μM)	1.88 ± 1.39 (4.61 ± 3.41 μM)	2.15 ± 1.78 (5.28 ± 4.37 μM)	3.49 ± 1.85 (8.56 ± 4.54 μM)	2.22 ± 1.64 (5.44 ± 4.02 μM)	NT	NT	NT
Pentamidine		NT	NT	NT	NT	NT	NT	NT	0.006 ± 0.002 (17.6 ± 5.8 nM)	NT	NT

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. amazonensis*, 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*,
 345 and *P. xanthostachyum* (11.02, 31.06, 25.77, 28.46, 12.50, 29.07, 11.89, 10.47), methylene chloride
 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 $IC_{50} \leq 10 \mu\text{g/mL}$ (**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 $IC_{50} \leq 1 \mu\text{g/mL}$,
 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 IC_{50} at $0.028 \mu\text{g/mL}$. 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.

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

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

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

367 Antimicrobial activity

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

373 negative strains [(*Stenotrophomonas maltophilia* (21170) and *Burkholderia cepacia* (13003)], and on
374 the two *Candida albicans* strains. This extract was also quite active on *Streptococcus pyogenes*
375 (16135). Other extracts with good activity on Gram-positive bacteria can be pointed out: methylene
376 chloride extracts of *P. xantochyma* and *P. brasiliense*, and hydroalcoholic extract of *P. xantochyma*.
377 Extracts with very good activity on Gram-negative bacteria are: methylene chloride extracts of *P.*
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. pseudoarboresum*, *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
382 methylene chloride of *P. brasiliense* and a hydroalcoholic extract of *P. xantochyma*.

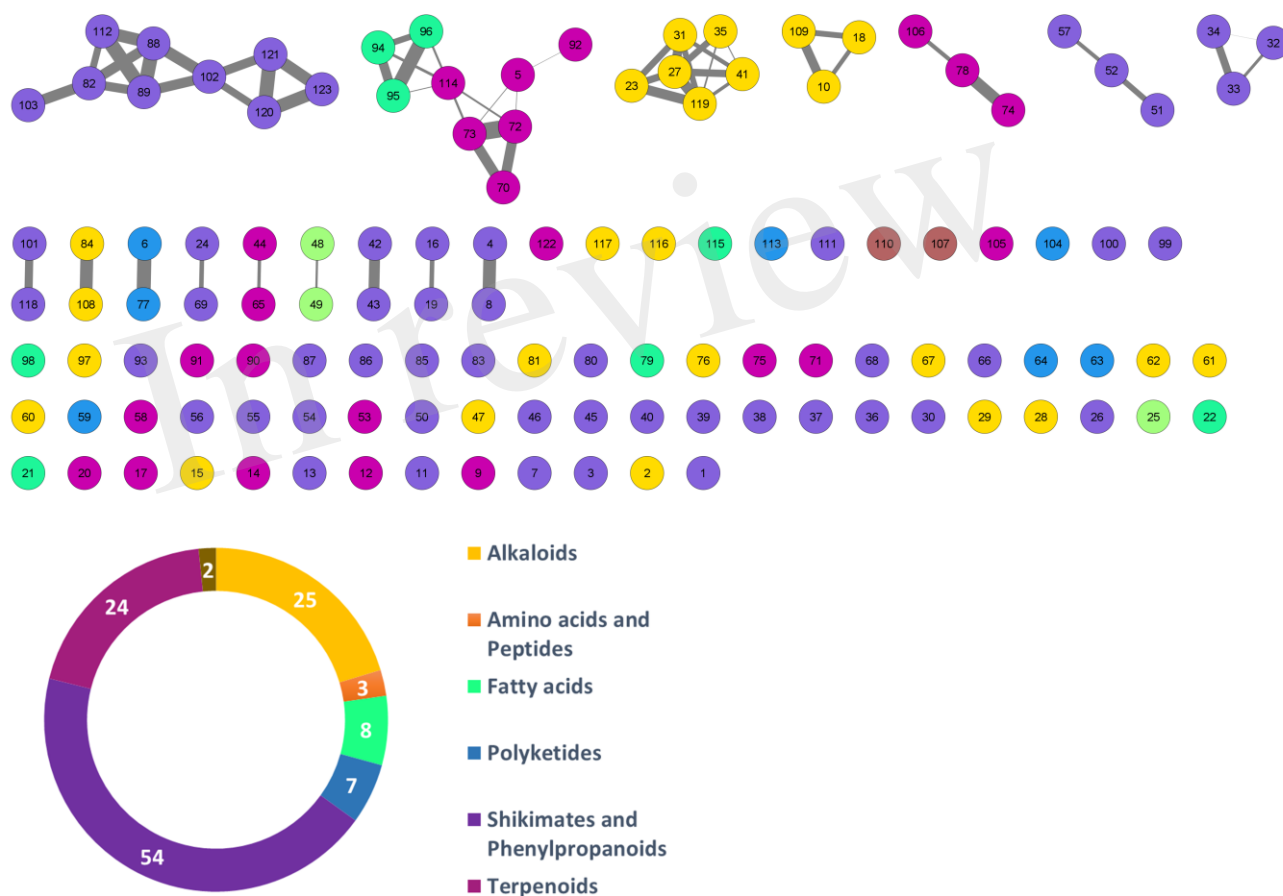
In review

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

Species / Type of extract	Extracts																				
	<i>Candida albicans</i> (ATCC 10231)	<i>Candida glabrata</i> (ATCC 22019)	<i>Enterococcus faecalis</i> (ATCC 29218)	<i>Enterococcus faecium</i> (ATCC 29533)	<i>Enterococcus sp.</i> (ATCC 49619)	<i>Staphylococcus aureus</i> (ATCC 6538)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	<i>Staphylococcus aureus</i> (ATCC 6539)	
<i>Piper casapiense</i> (#4)	M	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	1.2	0.3	0.3	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper strigosum</i> (#12)	M	-	1.2	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375	0.0375
	H	0.6	0.15	0.0375	0.15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper pseudoarborescens</i> (#14)	M	-	1.2	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.075	1.2	0.3	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	0.3	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper armatum</i> (#18)	M	-	0.3	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.6	0.3	0.075	0.6	1.2	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
	H	0.3	-	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper brasiliense</i> (#19)	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.15	0.15	0.15	0.3	-	0.3	0.3	0.3	0.3	0.3	0.15	0.15	0.3	1.2	0.6	0.3	0.3	0.3	0.3	0.3
	H	0.3	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper bullatum</i> (#20)	M	-	0.3	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	-	0.3	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	0.15	0.6	0.075	0.3	-	0.6	0.6	0.6	0.6	0.6	0.6	0.3	0.6	0.6	0.3	0.6	0.15	0.15	-	-
<i>Piper calvescens</i> (#21)	M	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.3	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	0.15	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper cordatamentosa</i> (#22)	M	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.6	-	0.6	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	0.3	-	0.3	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper crassinervium</i> (#23)	M	-	0.6	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.3	0.15	0.6	0.15	-	0.3	0.3	0.3	0.15	0.3	0.3	0.3	0.6	1.2	0.3	0.3	0.15	0.15	-	-
	H	0.15	-	0.3	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper divaricatum</i> (#24)	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.075	1.2	0.3	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	1.2	-	0.3	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper glabribaccum</i> (#25)	M	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	0.15	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper heterophyllum</i> (#26)	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.075	0.6	0.3	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	-	-	0.6	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper oblongum</i> (#27)	M	1.2	0.3	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	1.2	0.6	0.3	0.6	1.2	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	1.2	0.6	0.6	0.15	0.6	0.3	-
	H	0.15	0.6	0.15	0.3	-	0.6	0.6	0.6	0.3	0.6	0.6	0.3	0.3	0.3	0.15	0.3	0.15	0.3	0.3	-
<i>Piper reticulatum</i> (#28)	M	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.3	1.2	0.6	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	0.3	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper sancti-felices</i> (#29)	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.3	1.2	0.3	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	0.075	1.2	0.3	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper stellipilum</i> (#30)	M	-	0.3	-	-	1.2	0.6	-	0.6	0.6	0.6	0.3	0.3	0.3	1.2	0.6	0.3	-	-	-	-
	D	1.2	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	0.3	1.2	1.2	0.6	-	0.6	1.2	0.6	0.6	0.6	1.2	0.6	0.6	0.6	1.2	0.6	0.6	-	-	-
<i>Piper trigonum</i> (#31)	M	-	0.6	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	1.2	1.2	0.3	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	-	-	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper verruculosum</i> (#32)	M	-	0.3	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	0.3	0.6	0.6	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	H	0.6	-	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Piper xanthostachyum</i> (#33)	HA	0.3	0.15	0.15	0.3	0.6	0.15	0.3	0.15	0.3	0.15	0.3	0.15	0.3	0.15	0.15	0.15	0.15	0.15	0.15	0.15
	D	0.15	0.075	0.075	0.075	0.3	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
	H	-	-	1.2	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentamycin	ND	ND	0.03	4	-	0.5	0.5	0.25	0.5	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25
Vancomycin	ND	ND	0.5	0.5	4	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Amoxicillin	ND	ND	2	64	2	4	16	0.125	16	1	8	16	1	0.25	0.25	0.06	0.06	0.06	0.06	0.06	0.06
Amphotericin B	4	8.5	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
Sertaconazole	NA	64	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

386 LCMS data mining and statistical correlation analysis

387 After the application MS-CleanR workflow to the LCMS data, we obtained 123 unique metabolite
 388 features ($m/z \times RT \times Peak\ area$). Among these, 56 compounds were annotated at the genus level (45.53
 389 %), 6 compounds at the family level (4.88 %), and 55 compounds at the generic level (44.72 %),
 390 leaving 6 compounds unannotated (4.88 %). The MSMS fragmentation pathway of these compounds
 391 was used to establish a molecular network (**Figure 1**). Using NPClassifier and ClassyFire (Djoumbou
 392 Feunang et al., 2016; Kim et al., 2021), major phytochemical classes and subclasses of compounds
 393 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**).

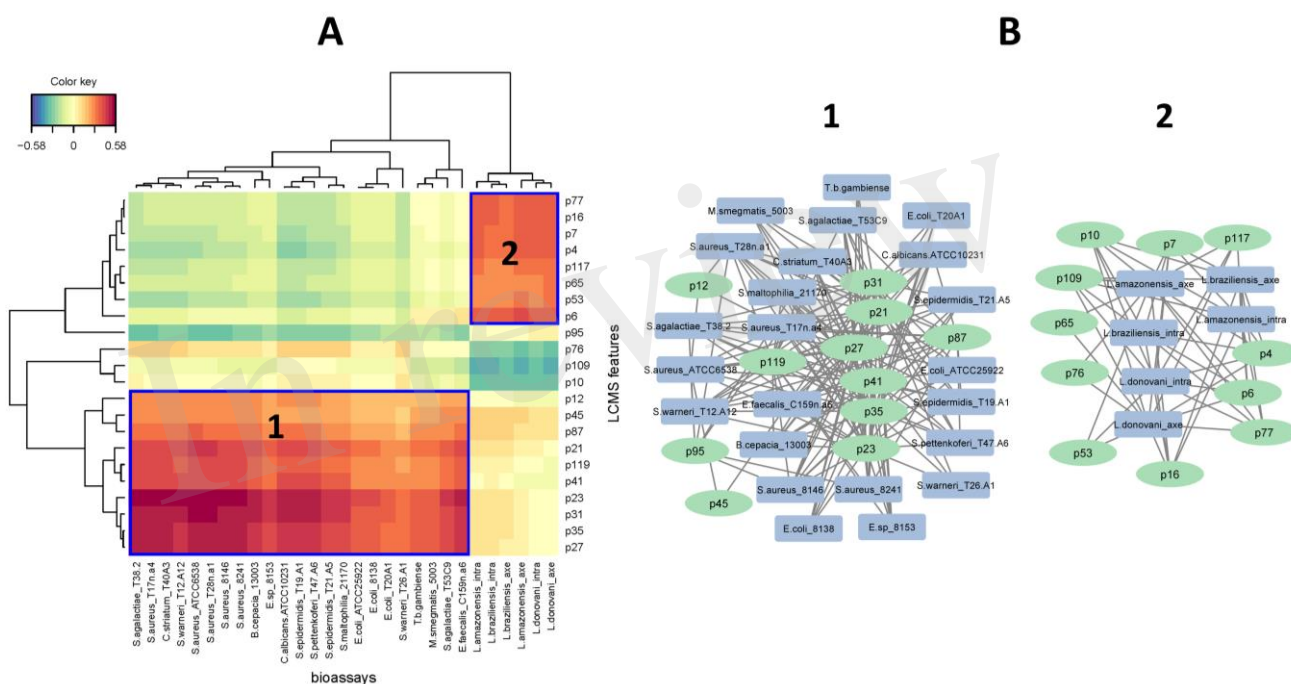


395

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

399 A heatmap of correlation analysis (Clustered Image Map, CIM) is shown in **Figure 2-A**. For this
 400 analysis, we compiled all *in vitro* assay results (30 bacterial strains, 2 fungal strains, 4 parasitic strains
 401 and 2 cell lines, **Tables 1 and 3**) versus the LCMS features. This figure results from a setting of the
 402 threshold of correlation at 0.3 (the heatmap is shown *in extenso* in **supp info 3**). Two groups of features
 403 responsible for activities can be distinguished. **Group 1** is composed of 11 features (12, 21, 23, 27, 31,
 404 35, 41, 45, 87, 95, and 119) having activity on 21 bacteria, one fungus (*C. albicans* ATCC 10231), and
 405 one parasite (*T. b. gambiense*). Activity on bacteria was mostly concentrated on Gram-positive strains

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
 413 having structurally-related analogs in the extract) belonging to varied phytochemical classes. In the
 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
 416 compound and not correlated to any activity (*i.e.* features 16 and 4).



417

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

424 **Table 4.** Features identified as responsible for bioactivity. [†] = [M+H-H₂O]⁺; [‡] = [M+Na]⁺; [‡] = based on
 425 molecular networking.

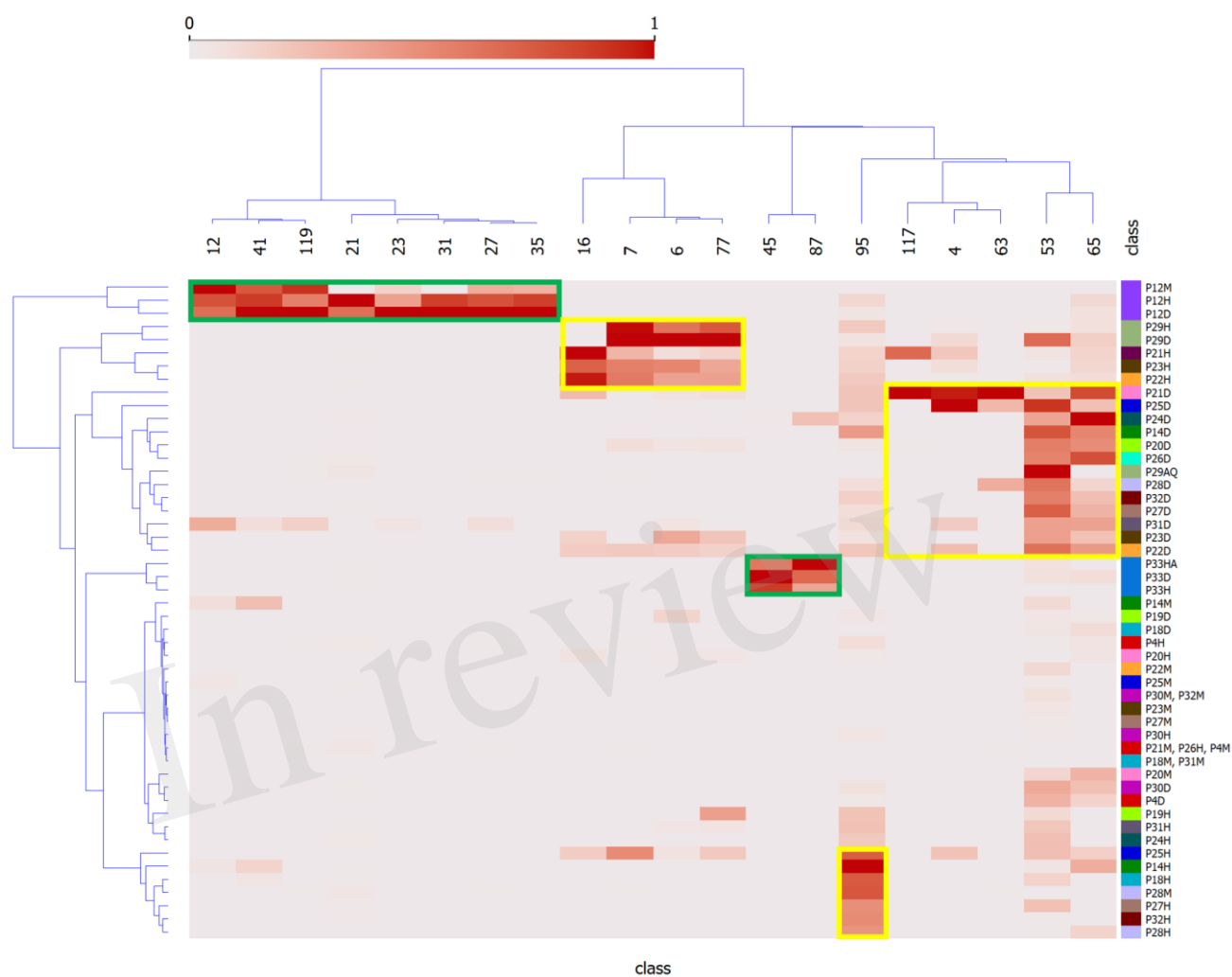
Feature ID	m/z [M+H] ⁺	RT	Formula	Annotation	Annotation level	Phytochemical class
Group 1						
12	344.335	9.745	C ₂₄ H ₄₁ N	(1-{12,16-dimethylpentacyclo octadecan-15- yl}ethyl)dimethylamine	generic	terpenoid

21	362.342	9.745	C ₂₄ H ₄₃ NO	2,4,14-eicosatrienoic acid, 2-methylpropylamide	genus	fatty acids and conjugates
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	filifiline 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 H ₃₇ N	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 [¥]	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
 428 **Group 1** are mainly distributed in all extracts of *P. strigosum* (features 12, 21, 23, 31, 41, 27 and 35)
 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*.

437



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
446 stems, etc.) to treat different diseases (Odonne et al., 2009, 2011; Vásquez-Ocmín et al., 2021a). For
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. amazonensis*, 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 sogá* (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
465 clean dataset able to provide meaningful information upon statistical analysis. The large abundance
466 and complexity of variables are also challenging and require to apply an appropriate statistical method
467 to answer a specific biological question (Hervé et al., 2018). Few metabolomic studies on *Piper* species
468 using NMR (Yamaguchi et al., 2011; Uckele et al., 2021) or LCMS have been reported so far (Vásquez-
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 *pseudoarboresum*, *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 (NPClassifier 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 filifiline, 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 µM/mL) are as diverse as isoquinolines, aporphines, phenanthrenes, quinolines
516 and indoles and their activity is mostly oriented against Gram-positive bacteria (Porrás 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 ≤ 10 µM/mL). Aporphine alkaloid derivatives exhibit high
520 broad-spectrum activity against Gram-positive bacteria: *S. agalactiae*, *S. aureus*, *S. epidermidis*, *E.*
521 *faecalis*, and as in our study *E. coli* (Hamoud et al., 2015; Tan et al., 2015). Even if the chemical array
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 dehydropiperonaline 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.* amphotericin 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 IC_{50} of 44.6 $\mu\text{M}/\text{mL}$ and was not cytotoxic for mouse
556 macrophage cells ($CC_{50} > 100 \mu\text{g}/\text{mL}$, $SI > 6$) (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
558 (binding affinity of $-8.5 \text{ kcal}/\text{mol}$) (Broni et al., 2021). Nevertheless, sesamin, isolated from a hexane
559 extract of *P. retrofractum* (IC_{50} of the extract = 5 $\mu\text{g}/\text{mL}$), 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* ($EC_{50} > 20$
562 $\mu\text{g}/\text{mL}$, $MIC > 200 \mu\text{g}/\text{mL}$ and $IC_{50} > 50 \mu\text{g}/\text{mL}$, respectively). Cubein, another lignan isolated from
563 *P. cubeba*, was shown to be active on *L. donovani* ($IC_{50} = 28.0 \mu\text{M}$). 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* ($IC_{50} = 10.67$ and $7.40 \mu\text{M}$, 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
570 the fact that it is an inhibitor of 5-lipoxygenase (76.02 μM) (Muthuraman et al., 2019), a key enzyme
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 (IC_{50} at $26 \pm 8.3 \mu\text{M}$) by the Griess method on LPS-stimulated BV-2 cells (Zheng et al., 2021),
574 to be cytotoxic for KB nasopharyngeal carcinoma cells ($ED_{50} = 5.7 \mu\text{g}/\text{mL}$) 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
580 *Stagonospora cirsii*, respectively (Gabley et al., 1992; Evidente et al., 2008). These two last
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. laevicaropus*) (da Silva A.
584 Maciel et al., 2016). Furthermore, macrolides are well-known antibacterial compounds and thus should
585 logically rather be clustered in **Group 1**, if ever present in the extracts. A compound annotated as
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
588 ubiquitous flavonoid with no antibacterial activity. The number of possible flavonoid isomers being
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
595 al., 2004; González et al., 2022). Antiprotozoal or antimicrobial activity of flavonoids is well
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
602 26-L5 carcinoma, PANC-1 human pancreatic cancer, metastatic human HT-1080 fibrosarcoma), or
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
613 complex metabolomes. Nevertheless, deciphering such profiles to determine the compounds
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
620 prioritizing the compounds to target. The relevance of our approach has been validated on a set of
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.

624

625 DATA AVAILABILITY STATEMENT

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

627 AUTHOR CONTRIBUTIONS

628 Conceptualization: PGVO, AM, GM, and LRM; Methodology: PGVO, AM, GM, and LRM; Chemical
629 data acquisition/curation: PGVO, KL, GM, AG, and AM; Statistical analysis: PGVO and GM;
630 Biological data curation: SC, VR, SB, and SP; Investigation: PGVO, SC, VR, GM, SP, AG, KL, ID,
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Figure 1.TIF

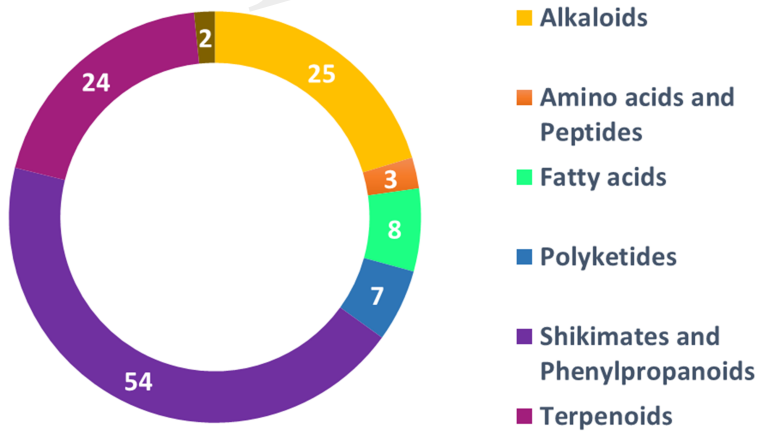
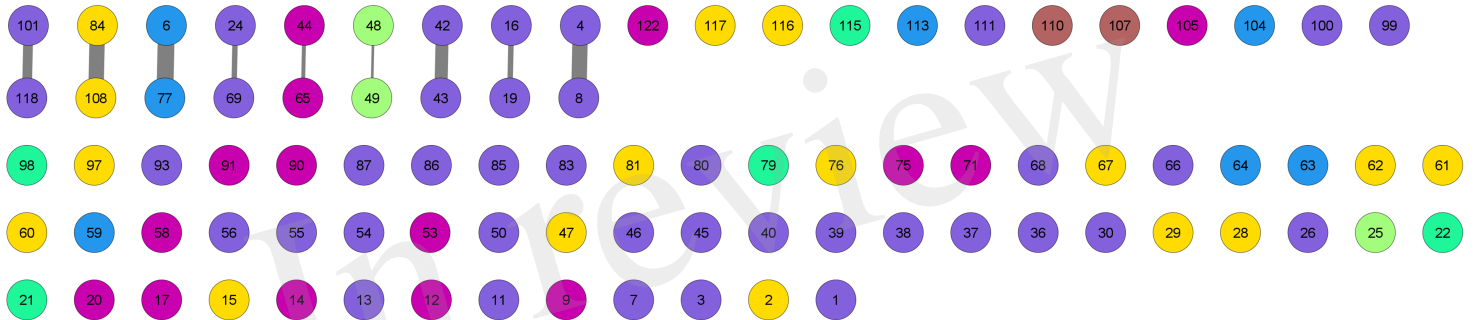
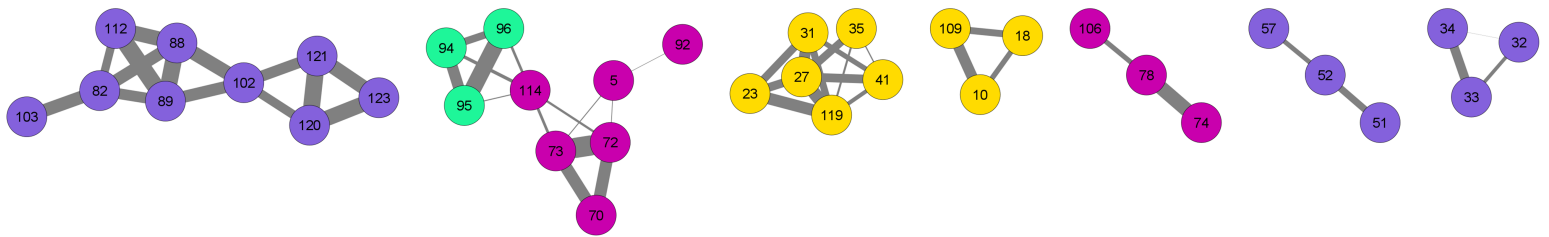
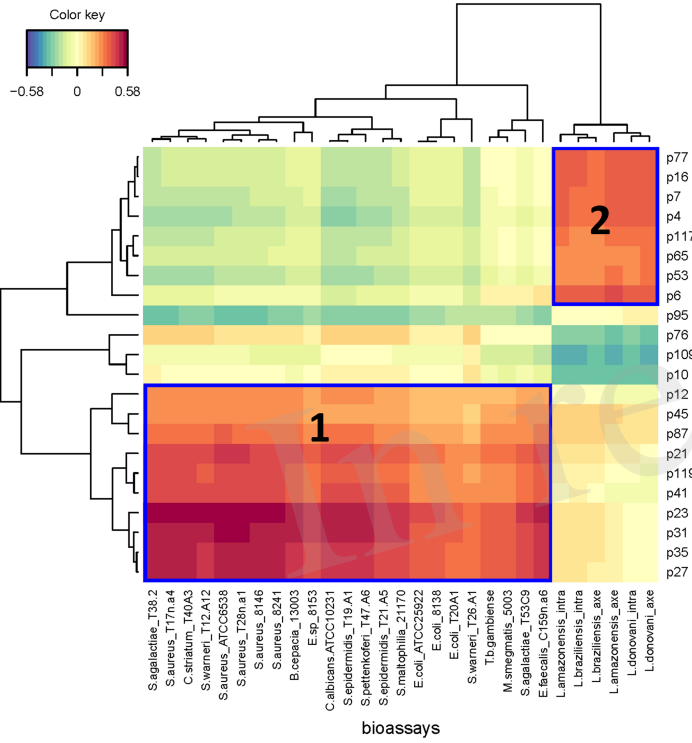


Figure 2.TIF

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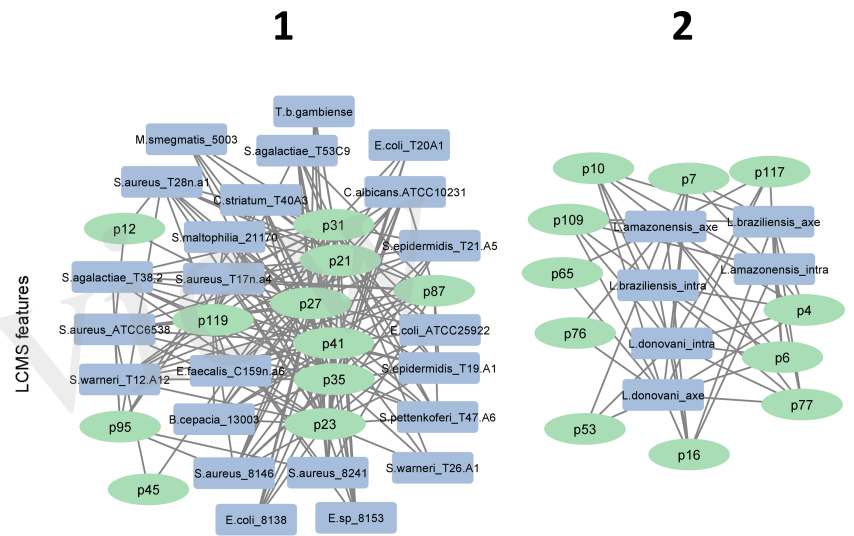


Figure 3.TIF

