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1 **Antimicrobial and anthelmintic activities of the ethanolic extract, fractions, and**
2 **isolated compounds from *Manilkara zapota* L. P. Royen (Sapotaceae)**

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20 **ABSTRACT**

21

22 **Objectives:** To evaluate the antibacterial, antifungal, and anthelmintic activities of the
23 ethanolic extract (EEMz), fractions (LPFMz and HPFMz), and compounds isolated
24 from the leaves of *Manilkara zapota* L. P. Royen.

25 **Methods:** Extract and fractions were produced by turbolization. LPFMz fraction was
26 analyzed by GC-MS. The isolated compounds from HPFMz were purified by flash and
27 preparative chromatographic methods, and chemically characterized by UPLC-
28 ESITOFMS, optical rotation, and one- and two-dimensional ¹H and ¹³C NMR
29 techniques. Anthelmintic against *Strongyloides venezuelensis* and antimicrobial
30 activities against *Candida albicans*, *Trichophyton rubrum* and *Staphylococcus aureus*
31 were evaluated.

32 **Key findings:** EEMz showed mainly phenolic compounds and pentacyclic triterpenes
33 from Δ¹²-oleane/Δ¹²-ursane series. Chlorogenic acid, myricetin-3-O-β-D-
34 glucopyranoside, mearnsitrin, germanicol, and germanicol acetate were reported to *M.*
35 *zapota* leaves for the first time in this work. EEMz, HPFMz, LPFMz showed
36 significant activity against *C. albicans* (16 μg/mL), while isolated flavonoids were
37 active against *S. aureus* (<32 μg/mL). EEMz, phenolic-rich compounds (F₂), and
38 chlorogenic acid were potentially active against *S. venezuelensis* at 24 h.

39 **Conclusions:** *M. zapota* and its bioactive compound can be eligible such as a potential
40 phytomedicine for the treatment of microbial and strongyloidiasis drug-resistant
41 infections.

42 **KEY-WORDS:** Traditional medicine Meso- and Southern America; phytochemistry;
43 anthelmintic; antimicrobial; chromatography.

44

45 **Highlights**

- 46 • Phenolic and pentacyclic triterpenes from Δ 12-oleane/ Δ 12-ursane were detected
- 47 • Chlorogenic acid, myricetin-3-O- β -D-glucopyranoside, mearnsitrin were
- 48 identified
- 49 • The pentacyclic triterpenes presented promising antimicrobial activity
- 50 • Ethanolic extract and phenolic fractions showed high antifungal activity
- 51 • Ethanolic and phenolic fractions, and chlorogenic acid exhibited anthelmintic
- 52 action

53 **Abbreviation List**

- 54 CAD: Charged Aerosol Detector
- 55 Compound 1: chlorogenic acid
- 56 Compound 2: myricetin-3-O- β -D-glucopyranoside
- 57 Compound 3: myricitrin
- 58 Compound 4: mearnsitrin
- 59 Compound 5: germanicol
- 60 Compound 6: β - amyrin
- 61 Compound 7: α -amyrin
- 62 Compound 8: germanicol acetate
- 63 COSY: Correlation Spectroscopy
- 64 EEMz: dry ethanolic extract
- 65 ESI: Electrospray interface
- 66 F: fraction obtained from High polarity insoluble fraction
- 67 HMBC: Heteronuclear Multiple Bond Correlation
- 68 HPFMz: High polarity insoluble fraction
- 69 HRMS: High Resolution Mass Spectroscopy
- 70 HSQC: Heteronuclear Single Quantum Coherence
- 71 LPFMz: lower polarity fraction
- 72 Mz: *Manilkara zapota*
- 73 PVP: polyvinylpyrrolidone
- 74 TOF: Time-of-flight
- 75

76 1. INTRODUCTION

77 Infectious diseases caused by bacteria, viruses, fungi, and parasites are the greatest
78 cause of death, disability, and social and economic difficulties for millions of people
79 worldwide ^[1]. This problem is especially high in developing countries, due to poor
80 availability of drugs and the emergence of widespread drug resistance ^[2].

81 Infections caused by microorganisms such as *Staphylococcus aureus* can promote
82 disorders in the skin and mucosa, toxic shock syndrome, endocarditis, osteomyelitis,
83 and infections of the urinary and intestinal tracts.^[3] Additionally, dermatophyte fungi
84 are also able to invade the keratinized tissues of animals and humans ^[4]. Among them,
85 *Trichophyton rubrum* is most widespread, representing 80% of clinical isolates ^[5].
86 *Candida albicans* is another important opportunistic pathogenic fungus that causes
87 candidiasis responsible for a variety of conditions in humans, ranging from superficial
88 cutaneous lesions to generalized infection ^[6].

89 In the same way, helminthiasis caused by intestinal nematodes and transmitted via the
90 soil are the most common and persistent neglected parasitic infections, especially in
91 developing countries. *Strongyloides stercoralis* is the main neglected geohelminths
92 responsible to infect 30-100 million people in the world ^[7, 8]. The autoinfection ability
93 of *S. stercoralis* leads to syndromes of persistent infection that can result in chronic
94 illness with various clinical manifestations. In immunocompromised patients, the
95 uncontrolled multiplication of the parasite (hyperinfection) and the potentially fatal
96 distribution of larvae in various organs represents a major concern in terms of public
97 health ^[8; 9].

98 Therefore, the discovery of new compounds with chemical structures and mechanisms
99 of action different from those current antimicrobial and anthelmintic drugs are urgently
100 need. This fact is due to the increased incidence of new and reemerging infectious
101 diseases, the ineffectiveness of treatments using broad-spectrum anthelmintics, and
102 especially the increasing inefficacy of the drugs employed in the current therapy against
103 pathogenic microorganisms and intestinal parasites ^[10; 11].

104 *Manilkara zapota* (L.) (Synonyms: *M. zapotilla*, *M. achras*, *Mimusops manilkara*,
105 *Achras zapota*), commonly known as sapoti, is a member of the Sapotaceae family and
106 it represents a potential natural resource to treat infectious process ^[12]. In folk medicine,
107 the leaves are used to treat coughs, colds, and diarrhea, the crushed seeds are used as a

108 diuretic and febrifuge, and the husks have astringent and febrifuge activities ^[13]. In the
109 literature, *M. zapota* has been reported to exhibit antioxidant properties ^[14; 15], as well as
110 analgesic ^[16], antimicrobial ^[17; 18], antihyperglycemic, and hypocholesterolemic
111 activities ^[19; 20], due the presence of phenolic acids, flavonoids, tannins (mainly in the
112 immature fruit), triterpenes, and saponins (mainly in seeds) compounds ^[19; 21-23]. Despite
113 these important properties, the bioguided phytochemical study of the compounds from
114 the *M. zapota* leaves has not yet been developed. In this sense, the goal of this work was
115 to characterize the phytochemistry of the ethanolic extract, fractions, and isolated
116 compounds from the *Manilkara zapota* L. P. Royen leaves and to evaluate their
117 antimicrobial action and, for the first time, their anthelmintic activity.

118

119 **2. MATERIALS AND METHODS**

120 **2.1 Materials**

121 Leaves of *Manilkara zapota* (Mz) were collected from the orchard of the
122 Multidisciplinary Center for Chemical, Biological, and Agricultural Research (CPQBA)
123 in Campinas, São Paulo State, Brazil (22° 47' 52" S, 47° 6' 49" W). A sample was
124 deposited in the Herbarium of the Institute of Biology of the State University of
125 Campinas, under registration no. UEC 186541.

126 Ivermectin and chlorogenic acid (95%) were purchased from Sigma-Aldrich (St. Louis,
127 MO, USA) and used as a control drug and an analytical standard, respectively, in the
128 anthelmintic activity assays. Polyvinylpyrrolidone (PVP-K30) was obtained from Synth
129 (São Paulo, Brazil). Oxacillin, itraconazole, and fluconazole from Molekula (Dorset,
130 UK) were used as control drugs in the antimicrobial activity assays. All solvents
131 employed in the study were HPLC or UPLC grade.

132

133 **2.2. Bioactive compounds of the *M. zapota***

134 **2.2.1. Ethanolic extract of the *M. zapota***

135 The ethanolic extract was produced by turbolization. 900 g of the triturated and dried
136 leaves of *M. zapota* were dispersed in 3 L of ethanol under constant stirring at 16,000
137 rpm for 10 min using a mechanical disperser (Ultra Turrax T50, IKA Works Inc.,
138 Wilmington, NC, USA) at 25 °C. The suspension was filtrated in nylon filter to remove

139 non-soluble particles. Afterthat, the residue was re-extracted with 3 L of ethanol and
140 filtrated. The extracts were combined and the solvent was evaporated under vacuum
141 until complete dryness, resulting in the dry ethanolic extract (EEMz, 96.47 g).

142

143 **2.2.2. Fractions of the *M. zapota***

144 30 g of the EEMz was suspended in 300 mL of hexane/ethyl acetate mixture (1:1) and
145 extracted in the Ultra Turrax system for 5 min at 16,000 rpm. Lower polarity fraction
146 (LPFMz) and the high polarity insoluble fraction (HPFMz) were obtained. These
147 fractions were gently separated by centrifugation at 5,000 g for 15 min and posteriorly
148 dried under vacuum in order to produce 16.25 g and 11.44 g of LPFMz and HPFMz,
149 respectively.

150

151 **2.2.3. Isolation of the *M. zapota* compounds**

152 1.5 g of the HPFMz was purified by flash chromatography using a Combi Flash[®] Rf⁺
153 Teledyne ISCO system (Lincoln, NE, EUA) equipped with a UV detector and C18
154 column (86 g). A flow rate of 60 mL/min and H₂O/CH₃CN gradient of: 80/20 for 6 min
155 (F₁), 80/20 to 70/30 in 1 s, 70/30 for 6 min (F₂), 70/30 to 60/40 in 1 s, 60/40 for 6 min
156 (F₃), 60/40 to 50/50 in 1 s, 50/50 for 6 min (F₄), 50/50 to 0/100 in 1 s, and 0/100 for 6
157 min (F₅), was used to obtain five diferents fractions. Subsequently, the fractions F₂ and
158 F₃ were combined and purified by preparative HPLC system (Gilson, Middleton, WI,
159 USA) equipped with a model 322 pump, a GX-271 fraction collector, a DAD 171 diode
160 array detector, and a prepELSII electrospray accessory. A Phenomenex Luna
161 preparative C18 column (21.2 × 250 mm, 5 μm) (Torrance, CA, USA) was employed at
162 a flow rate of 17 mL/min. The gradient appllied was H₂O/CH₃CN (98/2 to 77/23 in 5
163 min, 77/23 to 55/45 in 15 min, 55/45 to 0/100 in 5 min). Four compounds **1** (3.5 mg, Rt
164 2.14 min); **2** (0.7 mg, Rt 3.51 min); **3** (13.8 mg, Rt 4.14 min); **4** (4.6 mg, Rt 5.26 min)
165 were isolated and chracterized.

166

167 **2.3 Identification of the components by chromatography analyses**

168 **2.3.1 Thin layer chromatography**

169 Thin layer chromatography analyses were performed using aluminum plates coated with
170 silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany). The lower polarity compounds were

171 eluted with hexane: ethyl acetate (70:30) and ethanolic anisaldehyde was used as stain.
172 For the high polarity compounds, the mobile phase was ethyl acetate: formic acid:
173 glacial acetic acid: water (52:11:11:26) and a solution of diphenylboryloxyethylamine
174 and polyethyleneglycol (NP/PEG) was used as the developer. UV detection was
175 performed at 254 and 366 nm.

176

177 **2.3.2 Gas chromatography-mass spectrometry (GC-MS)**

178 A gas chromatograph (Agilent, model 6890, Santa Clara, CA, USA) coupled to an
179 Agilent 5975 mass spectrometer was used to identify compounds present in LPFMz.
180 The analyses were performed using an HP-5MS capillary column (30 m × 0.25 mm,
181 0.25 μm) (J&W Scientific, Santa Clara, CA, USA). The column temperature was
182 increased from 110 °C to 280 °C during 26 min, using a heating rate of 5 °C/min. The
183 injector and detector were maintained at 250 °C and 300 °C, respectively. The carrier
184 gas was helium at a flow rate of 1 mL/min. The mass spectra were obtained using an
185 ionization energy of 70 eV and *m/z* from 40 to 600 a.m.u.. Injection of 1 μL of sample
186 was made in split mode (1:30). The compounds were identified by comparison of the
187 fragmentation patterns with data in the NIST 11 spectral library and data reported in the
188 literature.

189

190 **2.3.3 HPLC analyses**

191 The analytical profiles were obtained with a Dionex UltiMate 3000 system equipped
192 with dual PDA and Corona CAD detectors (Thermo Fisher Scientific, Waltham, MA,
193 USA). A Cortecs C18 column was used (4.6 × 150 mm, 2.7 μm) (Waters, Milford, MA,
194 USA). Elution was performed using a linear gradient of H₂O mixed with an increasing
195 proportion of CH₃CN. The flow rate was of 1.6 mL/min. The data were obtained with
196 Chromeleon™ Chromatography Data System (CDS) Software.

197

198 **2.4 Structural elucidation of the HPFMz**

199 **2.4.1 Optical rotation**

200 The optical rotation was measured in methanol (MeOH) using an Anton Paar MCP 300
201 polarimeter (Graz, Austria) equipped with a 350 μL cell with a length of 100 mm.

202

203 **2.4.2. ¹H and ¹³C NMR and 2D-NMR spectra**

204 NMR analyses were performed in a Bruker (Billerica, MA, USA) 500 MHz
205 spectrometer equipped with a 1 mm inverse detection probe. The samples were
206 dissolved in deuterated methanol (CD₃OD) in 5 mm tubes. The chemical shifts (δ, in
207 ppm) were reported based on the signal for tetramethylsilane (TMS).

208

209 **2.4.3 High resolution mass analyses**

210 High resolution mass measurements (ESITOFMS) were performed in Acquity UPLC
211 system (Waters, Milford, MA, USA) coupled to a Waters Micromass LCT Premier
212 mass spectrometer equipped with an electrospray interface (ESI) and a time-of-flight
213 (TOF) detector.

214 **Compound 1:** Yellow powder; R_f ~0.45 with blue fluorescence at 366 nm. λ_{max}: 327
215 nm; ¹H NMR (CD₃OD) 7.56 (1H, d, *J* = 15.8 Hz, H-7), 7.05 (1H, d, *J* = 1.8 Hz, H-2'),
216 6.96 (1H, dd, *J* = 7.9, 1.8 Hz, H-6'), 6.77 (1H, d, *J* = 7.9 Hz, H-5'), 6.37 (1H, d, *J* =
217 15.8 Hz, H-8'), 4.20 (1H, m), 3.85 (1H, m), 2.05 (1H, br.s), 1.93 (br.s); HRESITOFMS
218 [M - H]⁻ calculated for C₁₆H₁₇O₉, *m/z* 353.0872.

219 **Compound 2:** Yellow powder; R_f ~0.65 with orange fluorescence at 366 nm. [α]²⁵_D: -
220 12° (*c* 0.1 g/100 mL, CH₂OH); λ_{max}: 261, 356 nm; ¹H NMR (CD₃OD): δ 3.23 (1H, ddd,
221 *J* = 9.5, 5.2, 2.4 Hz, H-5''), 3.38 (1H, dd, *J* = 9.5, 8.8 Hz, H-3''), 3.42 (1H, t, *J* = 8.8 Hz, H-
222 4''), 3.51 (1H, dd, *J* = 8.8, 7.9 Hz, H-2''), 3.62 (1H, d, *J* = 11.9, 5.2 Hz, H-6β''), 3.73
223 (1H, dd, *J* = 11.9, 2.4 Hz, H-6α''), 5.26 (1H, d, *J* = 7.9 Hz, H-1''), 6.20 (1H, d, *J* = 2.1
224 Hz, H-6), 6.38 (1H, d, *J* = 2.1 Hz), 7.30 (2H, s); HRESITOFMS [M - H]⁻, *m/z*
225 479.0820 (calculated for C₂₁H₁₉O₁₃, *m/z* 479.0826) and [M + H]⁺, *m/z* 481.0986
226 (calculated for C₂₁H₂₁O₁₃, *m/z* 481.0982).

227 **Compound 3:** Yellow powder; R_f ~0.8 with orange fluorescence at 366 nm. [α]²⁵_D: -
228 61° (*c* 0.1 g / 100 mL, CH₂OH), λ_{max}: 261, 350 nm; ¹H NMR (CD₃OD): δ 0.96 (3H, d, *J*
229 = 6.1, H-6''), 3.52 (1H, dd, *J* = 9.2, 6.1, H-5''), 3.34 (1H, t, *J* = 9.2 Hz, H-4''), 3.79 (1H,
230 dd, *J* = 9.2, 3.0 Hz, H-3''), 4.22 (1H, dd, *J* = 3.0, 1.2 Hz, H-2''), 5.32 (1H, d, *J* = 1.2 Hz,
231 H-1''), 6.20 (1H, d, *J* = 1.8 Hz, H-6), 6.36 (1H, d, *J* = 1.8 Hz), 6.95 (2H, s, H-2', H-6');
232 ¹³C NMR (CD₃OD): 179.58 (C-4), 166.55 (C-7), 163.15 (C-5), 159.34 (C-2), 158.55
233 (C-9), 146.84 (C-3', C-5'), 137.91 (C-4'), 136.25 (C-3), 121.91 (C-1'), 109.55 (C-
234 2',6'), 105.67 (C-10), 103.60 (C-1''), 100.00 (C-6), 94.83 (C-8), 73.34 (C-4''), 72.11 (C-

235 3''), 72.00 (C-5''), 71.87 (C-2''), 17.64 (C-6''). HRESITOFMS [M - H]⁻, *m/z* 463.0877
236 (calculated for C₂₁H₁₉O₁₂, *m/z* 463.0877) and [M + H]⁺, *m/z* 465.1032 (calculated for
237 C₂₁H₂₁O₁₂, *m/z* 465.1033).

238 **Compound 4:** Yellow powder; R_f ~0.85 with yellow fluorescence at 366 nm [α]²⁵_D: -
239 58° (c 0.1 g/100 mL, CH₂OH), λ_{max}: 263, 337 nm. ¹H NMR (CD₃OD): δ 0.95 (3H, d, *J*
240 = 5.7, H-6''), 3.32 (m, H5''), 3.33 (m, H4''), 3.75 (1H, dd, *J* = 9.2, 3.5 H-3''), 3.88 (3H, s,
241 4'-OCH₃), 4.22 (1H, dd, *J* = 3.5, 1.9 Hz, H-2''), 5.30 (1H, d, *J* = 1.9 Hz, H-1''), 6.13
242 (1H, d, *J* = 2.2 Hz, H-6), 6.27 (1H, d, *J* = 2.2 Hz), 6.87 (2H, s, H-2', H-6'); ¹³C NMR
243 (CD₃OD): 168.27 (C-7), 158.77 (C-2), 152.28 (C-3', C-5'), 139.28 (C-4'), 127.42 (C-
244 1'), 109.91 (C-2', C-6'), 103.63 (C-1''), 100.65 (C-6), 95.85 (C-8), 73.40 (C-5''), 72.27
245 (C-4''), 72.15 (C-3''), 72.05 (C-2''), 61.04 (4'-OCH₃), 17.86 (C-6''). HRESITOFMS [M
246 - H]⁻, *m/z* 477.1039 (calculated for C₂₂H₂₁O₁₂, *m/z* 477.1033) and [M + H]⁺, *m/z*
247 479.1180 (calculated for C₂₂H₂₃O₁₂, *m/z* 479.1190).

248

249 **2.5 Antimicrobial activity**

250 The antimicrobial activities of the EEMz, HPFMz, LPFMz, and isolated flavonoids **2**, **3**,
251 and **4** were analyzed against *S. aureus* (ATCC 29213), *C. albicans* (ATCC 10231), and
252 *T. rubrum* (SNB-TR1). The broth microdilution technique was used to determine the
253 minimal inhibitory concentration (MIC) according to the guidelines of the Clinical and
254 Laboratory Standards Institute (CLSI) [24; 25]. Previously, the samples were solubilized
255 with dimethyl-sulfoxide (DMSO). Then, the solutions were placed in 96-well
256 microplates, diluted in a growth medium. Lastly, the pathogen suspension was added,
257 resulting in concentrations ranging from 512 to 1 µg/mL. The microplates were for
258 incubated at 37 °C for 24 h (bacteria), 48 h (yeast), and 5 days (dermatophyte).
259 Oxacillin (16-0.03 µg/mL) was used as a reference antibiotic, while itraconazole (16-
260 0.03 µg/mL) and fluconazole (64-0.125 µg/mL) were employed as positive controls for
261 antifungal activity. The concentrations of DMSO were less than 0.5 %, thus not toxic
262 for the micro-organisms. MIC was determined as the lowest concentration that
263 prevented microbial growth.

264

265 **2.6 Anthelmintic activity**

266 All the experiments employing animals were approved by the Ethics Committee for the
267 Use of Animals (CEUA/UNICAMP, protocol no. 4089-1/2015) and complied with the
268 ethical principles for animal experimentation adopted by CEUA, State University of
269 Campinas. Adults (triploid parthenogenetic females) of *Strongyloides venezuelensis*
270 were isolated from the rodent *Bolomys lasiuris* and maintained in *Rattus norvegicus*.
271 Infective larvae (L3) of *S. venezuelensis* were obtained by coproculture. The cultures
272 were collected, concentrated and maintained at 28 °C for 48 h. For the recovery of adult
273 female parasites, male SPF Wistar rats were deliberately infected with *S. venezuelensis*
274 (2,000 larvae) during 15 days. Subsequently, rats were euthanased (by concussion) and
275 15 cm of the intestines were removed, longitudinally sectioned, and placed in Petri
276 dishes containing RPMI 1640 medium. The parasites leave the intestinal mucosa after 2
277 h of incubation at 37 °C.

278 EEMz, HPFMz, LPFMz, F₂, F₃, and compounds **1** (chlorogenic acid) and **3** (myricitrin)
279 were analysed. In order to increase the solubility in the culture medium, samples were
280 dispersed in polyvinylpyrrolidone (PVP-K30) at 1:4 ratio. The assays were performed
281 in 24-well plates containing RPMI 1640 medium (2 mL/well) supplemented with
282 gentamicin (50 mg/L) and amphotericin B (2.5 mg/L). Two parasites were transferred to
283 each well and the plates were incubated under 5% CO₂ at 37 °C for 72 h. 50, 100, 200
284 and 400 µg/mL were analysed. Positive control was treated under the same
285 concentrations of ivermectin, while negative control only fresh medium containing PVP
286 was used. At 6, 24, 48, and 72 h, the motility and survival of the worms were evaluated
287 and morphological changes was observed by DM500 microscope (Leica, Wetzlar,
288 Germany). All the tests were performed in triplicate and survival curves were plotted
289 using Prism 5.0 software (GraphPad, San Diego, CA, USA).

290

291 **2.7 Statistical analyses**

292 All analyses were performed in triplicate. Statistical significance was determined by the
293 Mantel-Cox test, using GraphPad Prism 5.0 software. The differences between the
294 survival curves were considered statistically significant when the p-value was smaller
295 than 0.001.

296 **3. RESULTS AND DISCUSSION**

297

298 Bioguided studies of the antibacterial, antifungal, and anthelmintic (against *S.*
299 *venezuelensis*) activities of the extract, fractions, and isolated compounds obtained from
300 the leaves of *M. zapota* was firstly developed in this work. EEMz was obtained with a
301 yield of 10.71% while LPFMz and HPFMz showed yields of 54.16% and 38.13%,
302 respectively.

303

304 **3.1 Identification of compounds in the LPFMz fraction**

305 GC-MS analyses revealed a predominance of pentacyclic triterpene compounds
306 showing typical fragmentation patterns of ursane and olean skeletons, with retention
307 times between 40 and 47 minutes corresponding at 75% of the all LPFMz fraction (Fig.
308 1). Table 1 shows the compounds identified from their fragmentation patterns,
309 compared to data obtained from the NIST 11 library. The presence of long chain
310 hydrocarbons and sterols in the aerial parts of *M. zapota* has been reported previously
311 [21; 26] and was confirmed here by the presence of compounds **A**, **B**, and **C**.

312 **Please, insert here the Figure 1 and Table 1**

313 Compound **5** presented a base peak at m/z 204, accompanied by the ions with m/z 189
314 and 177, which were characteristic of the compound germanicol (olean-18-en-3 β -ol) [27]
315 (Fig. 2). Peaks at m/z 218, 203, and 189 were associated with retro-Diels-Alder type
316 fragmentation of ring C of olean-12-enes and ursan-12-enes. These characteristic peaks
317 were observed for compounds **6** and **7** identified as β - and α -amyrin, respectively.
318 Triterpene analogues of β -amyrin show greater abundance of the peak at m/z 203,
319 compared to the peak at m/z 189, while the α -amyrin analogues show similar
320 abundances of these peaks [27; 28]. The mass spectra of triterpene esters reflect the
321 fragmentation pattern of the main skeleton, with smaller ions derived from the acid
322 portion [29]. Hence, the fragmentation pattern of compound **8** corresponded to that
323 expected for germanicol acetate (m/z 468), with the fragments characteristic of
324 germanicol (at m/z 204 and 169), together with fragments at m/z 453 and 408 reflecting
325 the loss of methyl and acetyl groups, respectively, and a fragment at m/z 43
326 corresponding to COCH_3 [27].

327 **Please, insert here the Figure 2**

328 Compounds **9** and **10** corresponded to α - and β -amyrin acetates, respectively, with
329 fragments peaks at m/z 218, 203, and 189 characteristics of amyryns and fragments with

330 loss of the acetyl group ^[27]. Compound **11** was identified as lupeol acetate, with peaks at
331 *m/z* 453, 408, 365, 218, 189, and 43 ^[30]. Amyrins, amyrin acetates, and lupeol acetate
332 have been reported in leaves of *M. zapota* ^[14; 16]. However, germanicol and its acetate
333 were firstly reported in the leaves of *M. zapota* in the current study. However, the
334 current study is the first time that germanicol and its acetate have been reported in *M.*
335 *zapota* leaves.

336

337 **3.2 Identification of compounds isolated from HPFMz**

338 Four compounds from HPFMz can be isolated and purified by preparative HPLC.
339 Compounds **1** (3.5 mg), **2** (0.7 mg), **3** (13.8 mg), and **4** (4.6 mg) showed retention times
340 of 2.01, 3.51, 4.14, and 5.26 min, respectively, detected in the HPLC analysis (Fig. 3).
341 The structures of the compounds were determined based on the one- and two-
342 dimensional NMR (¹H, ¹³C, COSY, HSQC, and HMBC), high-resolution mass
343 spectrometry, and optical rotation analyses.

344 **Please, insert here the Figure 3**

345 Compound **1** presented the molecular formula C₁₆H₁₈O₉, with a mass of 354. Its
346 structure was identified as that of chlorogenic acid (Fig. 4). The high-resolution
347 negative mode mass spectrometry analysis resulted in extraction of the [M-H]⁻ ion with
348 *m/z* 353.0872. The structural elucidation data corroborates Fayek et al. (2013) ^[19], who
349 isolated this compound from the fruit of *M. zapota*. Other earlier studies have also been
350 identified chlorogenic acid in the fruit and seeds of *M. zapota* ^[31; 32]. It could be inferred
351 from the ¹H NMR spectra of substances **2**, **3**, and **4** that they possessed similar base
352 nuclei and belonged to the flavonoid class of compounds (Fig. 4). The pair of doublets
353 at δ 6.18 (± 0.8) (J = 2.0 δ ± 0.5 1H) and δ 6.35 (± 0.8) (J = 2.1 ± 0.4 1H) could be
354 attributed to the 5,7,9,10-tetrasubstituted ring A, while the presence of two singlets at δ
355 6.95 (± 0.8) was indicative of a flavonol with a 1',3',4',5'-tetrasubstituted ring B,
356 corresponding to myricetin ^[33].

357 **Please, insert here the Figure 4**

358 Considering the sugars linked to C-3 of the aglycones, compound **2** showed the
359 presence of β-D-glucopyranoside unit, due to the high coupling constants (*J*_{HH})
360 involving each consecutive pair of H-1, H-2, H-3, H-4, and H-5 protons of the sugar,
361 varying between 8.8 and 11.9 Hz, which is characteristic of protons in axial positions.

362 For compounds **3** and **4**, the same saccharide unit was attached to C-3. The signal at δ_C
363 17.6 ± 0.2 , assigned to the C-6 methyl carbon of the sugar, together with the set of
364 signals at δ_C 71.8 (± 0.2), 72.1 (± 0.4), 73.3 (± 0.1), and 72.0 (± 0.2), enabled its
365 classification as a rhamnose.

366 HRESITOFMS spectrum of compound **2** presented pseudomolecular ion peaks $[M +$
367 $H]^+$ with m/z 481.0986 (calculated for $C_{21}H_{21}O_{13}$, with m/z 481.0982) and $[M - H]^-$ with
368 m/z 479.0820 (calculated for $C_{21}H_{19}O_{13}$, with m/z 479.0826), giving the molecular
369 formula $C_{21}H_{19}O_{13}$ with mass of 480. The structural elucidation of this compound was
370 reported in other plants and identified as myricetin-3-O- β -D-glucopyranoside^[34; 35]. The
371 absence of a smaller value of J_{HH} between the H-3'' and H-4'' protons confirmed that it
372 was not a 3-O-galactoside^[34]. The literature confirms the presence of myricetin-3-O- β -
373 D-galactopyranoside in species of the Sapotaceae family^[36].

374 Compound **3** presented the molecular formula $C_{21}H_{19}O_{13}$, with mass of 464, derived of
375 the base formula $C_{21}H_{19}O_{12}$ resulting from the negative mode adduct $[M - H]^-$ with m/z
376 463.0877 (calculated for $C_{21}H_{19}O_{12}$, with m/z 463.0877) and the positive mode ion $[M +$
377 $H]^+$ with m/z 465.1032 (calculated for $C_{21}H_{21}O_{12}$, with m/z 465.1033). This compound
378 was identified as myricetin-3-O- α -L-rhamnopyranoside, also known as myricitrin. This
379 compound has also been identified in previous studies using the leaves of *M. zapota*^{[21;}
380 33; 37].

381 1H NMR data for compound **4** showed a signal characteristic of a methoxyl (δ 3.88).
382 The location of this group was determined from the HMBC data, which showed its
383 correlation with C-4' (δ 139.2) and with the two protons H-2' and H-6' responsible for
384 the singlet at 6.87 ppm of the aglycone. Using the ions $[M - H]^-$ with m/z 477.1039
385 (calculated for $C_{22}H_{21}O_{12}$, with m/z 477.1033) and $[M + H]^+$ with m/z 479.1180
386 (calculated for $C_{22}H_{23}O_{12}$, with m/z 479.1190), it was possible to obtain the molecular
387 formula of compound **4** as $C_{22}H_{22}O_{12}$, with mass of 478, identified as myricetin-3-O-4'-
388 methyl-ether-3-O- α -L-rhamnopyranoside, also known as mearnsetin-3-O- α -L-
389 rhamnopyranoside (mearnsitrin). Take all results together, it is important to highlight
390 that chlorogenic acid, myricetin-3-O- β -D-glucopyranoside, and mearnsitrin compounds
391 in leaves of *M. zapota* were firstly detected in this work.

392

393 **3.3 In vitro antimicrobial activity**

394 MIC of the extracts, fractions, and isolated flavonoids of *M. zapota* are shown in Table
395 2. These results showed that EEMz, HPFMz, LPFMz were potentially active against *C.*
396 *albicans* (MIC = 16 µg/mL). In the case of *T. rubrum*, EEMz and HPFMz presented
397 moderate activity, while LPFMz was ineffective. HPFMz showed high activity against
398 *S. aureus* and moderate activity against the other microorganisms. About the flavonoid,
399 compound **2** showed significant activity against *S. aureus* (MIC = 8 µg/mL) and
400 promising activity against *T. rubrum* (MIC = 16 µg/mL). Myricitrin (compound **3**)
401 presented promising activity against the gram-positive bacterium and the dermatophyte
402 (MIC values of 32 and 64 µg/mL, respectively). Mearnsitrin (compound **4**) presented
403 activity against *S. aureus* (MIC = 32 µg/mL) and was ineffective against the other
404 microorganisms.

405 **Please, insert here the Table 2**

406 It has been found that the presence of terpenoids and glycosidic flavonoids in crude
407 extracts plays an important role in determining their antimicrobial activity ^[38]. The
408 presence of such compounds in the extract and the higher polarity fraction could
409 provide an explanation for the observed antimicrobial action against the pathogens
410 tested. The results obtained to *C. albicans* corroborate previous studies using aqueous
411 extract of leaves collected in India ^[39] and various *Candida* species ^[40]. The
412 antimicrobial properties of *M. zapota* extracts against *S. aureus* have been observed
413 previously ^[39; 41]. These activities may be related to the isomeric mixture of α - and β -
414 amyryn detected in the LPFMz that possesses known antimicrobial activity ^[42]. The
415 presence of one or more sugar units at different positions of the aglycone can increase
416 solubility and enhance the inherent medicinal properties of flavonoid compounds ^[43].
417 Taken together, all the samples originated from the leaves of *M. zapota* presented
418 antimicrobial activities (of different magnitudes). These findings demonstrate the high
419 antimicrobial potential of the leaves of *M. zapota* in combating three important human
420 pathogenic microorganisms.

421

422 **3.4 In vitro anthelmintic activity assays**

423 The anthelmintic effect of EEMz, HPFMz, LPFMz, F₂, F₃, and isolated flavonoids
424 (compounds 1 and 3) were evaluated by the survival percentage and cuticular integrity
425 of female (L4) *S. venezuelensis* parasites. The positive control (with addition of PVP)

426 presented 100% survival of the parasites. All samples tested at 400 µg/mL were lethal
427 after 24 h to the parasites (Fig. 5). For EEMz, no survival of parasites at 72 h was
428 observed to 100 and 200 µg/mL (Fig. 5A). HPFMz was lethal at 200 µg/mL after 48 h,
429 although survival at 72h have been occurred for 100 and 50 µg/mL corresponding at
430 5.5% and 72%, respectively (Fig. 5B). F₂ was lethal at all concentrations tested after 72
431 h of incubation (Fig. 5C), while F₃ was only lethal at the two highest concentrations
432 (Fig. 5D). The parasites exposed to chlorogenic acid at 200 µg/mL died within the first
433 24 h, while after 72 h there was no survival at any concentration (Fig. 5E). At 72 h,
434 there was no survival of the parasites exposed to myricitrin (compound **3**) at 400 and
435 200 µg/mL. However, the survive of the 11.1 and 61.1% were determined to 100 and 50
436 µg/mL, respectively (Fig 5F). It was possible to observe that all treatment had
437 statistically significant difference of the control ($p < 0.0001$).

438 **Please, insert here the Figure 5**

439 Ivermectin (pharmacological control) reduced the motility of the organisms, but there
440 was survival of all the individuals after 72 h. Similar behavior to infective larvae (L3) of
441 *S. stercoralis* using 250 µg/mL of ivermectin has been observed with reduction of the
442 parasite motility and survive during 6 days ^[44]. The lower polarity fractions were also
443 tested (data not shown) but showed unsatisfactory and imprecise results at all
444 concentrations evaluated due the formation of insoluble clusters in the culture medium.

445 At 100 µg/mL, chlorogenic acid was lethal in the first 24 h, while EEMz and F₂ caused
446 total mortality at the end of the incubation time (Fig. 6). At 200 µg/mL, only
447 chlorogenic acid was lethal after 24 h. However, there was no survival for any of the
448 samples tested after 72 h. Images of the parasites after 72 h of incubation are shown in
449 Fig. 7. Parasites exposed to myricitrin (Fig. 7B) exhibited distortions and cuticular
450 wrinkling. Those treated with F₂ (Fig. 7C) showed distortions, depressions, and
451 alterations in cuticular integrity, suggestive of direct cytotoxic effects on the
452 morphology of the worms. The parasites exposed to ivermectin (Fig. 7D) exhibited
453 reduced motility, but the integrity of the cuticular surface remained similar to that of the
454 positive control (Fig. 7A).

455 **Please, insert here the Figure 6 and 7**

456 Thus, the first study describing the direct action of extracts, fractions, and isolated
457 compounds obtained from *M. zapota* leaves on adult females of *S. venezuelensis* have

458 been presented in this work, highlighting the potential anthelmintic activities, especially
459 of the ethanolic extract, F₂, and chlorogenic acid. The chemical analyses showed that
460 both the ethanolic extract and the fractions contained glycosidic flavonoids as well as
461 phenolic acids and their derivatives, suggesting that the effective anthelmintic action
462 against the nematode was due to the presence of these compounds in the leaves of *M.*
463 *zapota*.

464 Various studies of plant species have been undertaken in the search for new treatments
465 for veterinary and human parasitosis. Free-living females of *Strongyloides* ssp. exposed
466 to a methanolic extract of *Lawsonia inermis* have been analyzed taking into account the
467 presence of distortions ,and longitudinal and transverse cracks ^[45]. Sarojini et al.
468 (2012)^[46] reported strong anthelmintic action of *L. inermis* extract due to the presence
469 of glycosides, terpenoids, and flavonoids. Carvalho et al. (2012)^[47] described the action
470 of extracts of *Piper tuberculatum*, *Lippia sidoides*, and *Mentha piperita* against
471 *Haemonchus contortus*

472 Ndhlala et al. (2016) ^[48] described the chemical composition and anthelmintic activity
473 of extracts of *Achyranthes aspera* against the free-living nematode *Caenorhabditis*
474 *elegans*. The aqueous and acetone extracts of *A. aspera* exhibited strong activity, with
475 UHPLC-MS/MS analyses revealing the presence of rutin, chlorogenic acid, and
476 genistein, which were associated with the activity.

477 Comparison of the analogous flavonoids revealed a close relationship between their
478 structures and antiparasitic activity. The presence of substituents (hydroxyl, methyl,
479 methoxy, and glycosides) is fundamental to determine its effectiveness. In the case of
480 hydroxyls, the number and pattern of hydroxylations in ring B has been found to have a
481 major influence on the antiparasitic activity of flavonoids ^[49]. However, the mechanisms
482 of action of flavonoids in helminths remain to be fully elucidated, even if several
483 possibilities have been proposed: an increase in the supply of nitric oxide acting as a
484 neurotransmitter in neuromuscular junctions and causing myoinhibition; nonspecific
485 inhibition of esterases and cholinesterases leading to neuromuscular disorganization of
486 the parasites; increased efflux of calcium causing depolymerization of the
487 microtrabecular network, with consequent disorganization and disaggregation of the
488 myofilaments; and decreased levels of free amino acids and increased levels of glutamic

489 acid, glutamine, citrulline, and gamma-aminobutyric acid (GABA), leading to the
490 release of nitric acid and resulting in neurological alterations ^[49].

491

492 **CONCLUSIONS**

493 Antimicrobial, antifungal, and anthelmintic activities of the extract, fractions, and
494 isolated compounds obtained from the leaves of *Manilkara zapota* were firstly
495 evaluated. Another innovative find of this work was the identification of the compounds
496 **1** (chlorogenic acid), **2** (myricetin-3-O- β -D-glucopyranoside), **4** (mearnsitrin), **5**
497 (germanicol), and **8** (germanicol acetate) in *M. zapota* leaves. Ethanolic extract and
498 fractions showed high antifungal activity against *C. albicans*. Compounds **3** (myricetin-
499 3-O- α -L-rhamnopyranoside) and **4** presented promising activity against *S. aureus* while
500 the high polarity fraction and compound **2** exhibited high activity against *S. aureus* and
501 *T. rubrum*. The ethanolic extract and F₂ containing phenolic compounds, and
502 chlorogenic acid showed effective antiparasitic action against female (L4) *S.*
503 *venezuelensis* parasites. This study contributes to the scientific basis for the possibility
504 of using plants and compounds of natural origin to combat strongyloidiasis and
505 microbial infections. The development of new therapeutic agents from *M. zapota* leaves
506 will require further in-depth studies, but nonetheless this plant material appears to offer
507 an effective alternative for the treatment of these diseases that are widespread and
508 difficult to control.

509

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518

519 **CONFLICTS OF INTEREST**

520 The authors declare that they have no conflicts of interest.

521

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646

647 **FIGURE LEGENDS**

648 **Fig. 1-** Expanded chromatogram profile of the LPFMz

649 **Fig. 2-** Compounds detected by GC-MS in the LPFMz

650 **Fig. 3-** HPLC elution of the compounds 1, 2, 3, and 4 in the HPFMz.

651 **Fig. 4-** Compounds isolated from HPFMz.

652 **Fig. 5-** Anthelmintic activity of *M. zapota* against *S. venezuelensis* after 72 h of
653 incubation. Percentage survival of parasites exposed to: EEMz (A), HPFMz (B), F₂ (C),
654 F₃ (D), chlorogenic acid (compound 1) (E), and myricitrin (compound 3) (F). Control
655 (●), 50 µg/mL (■), 100 µg/mL (▲), 200 µg/mL (▼), and 400 µg/mL (◆).

656 **Fig. 6-** Comparison of the anthelmintic activities of the extract, fractions, and
657 compounds obtained from the leaves of *M. zapota* at 100 µg/mL (A) and 200 µg/mL
658 (B). Control (●), EEMz (■), HPFMz (▲), F₂ (▼), F₃ (◆), chlorogenic acid (compound
659 1) (○), and myricitrin (compound 3) (□).

660 **Fig. 7-** Parthenogenetic females of *S. venezuelensis* after exposure to 400 µg/mL for 72
661 h of incubation. Parasite of the control group with preserved cuticle (A); Parasite
662 exposed to myricitrin, with cuticular wrinkling (arrow) (B); Parasite exposed to F₂, with
663 cuticular alterations (arrows) (C); Parasite treated with ivermectin, with preserved
664 cuticular integrity (D).

665

666 **Table 1:** GC-MS analysis of the chemical composition of the LPFMz

Type	RT ^a (mints)	Compound ^b	m/z	Molecular formula	Peak area (%)
A	32.76	Squalene	410	C ₃₀ H ₅₀	5.94
B	36.61	n-Hentriacontane	436	C ₃₁ H ₆₄	2.83
C	37.35	α -Tocopherol	430	C ₂₉ H ₅₀ O ₂	0.86
5	41.69	Germanicol	426	C ₃₀ H ₅₀ O	14.79
6	42.13	β -Amyrin	426	C ₃₀ H ₅₀ O	3.89
7	43.46	α -Amyrin	426	C ₃₀ H ₅₀ O	6.54
8	44.54	Germanicol acetate	468	C ₃₂ H ₅₂ O	4.84
9	45.15	β -Amyrin acetate	468	C ₃₂ H ₅₂ O ₂	13.90
10	45.59	α -Amyrin acetate	468	C ₃₂ H ₅₂ O ₂	31.04
11	46.60	Lupeol acetate	468	C ₃₂ H ₅₂ O ₂	

667 ^a Retention time (mints).668 ^b Compounds listed in order of elution from an HP-5MS capillary column.

669 **Table 2:** Minimum inhibitory concentrations (MICs) of the EEMz, HPFMz, LPFMz,
 670 and isolated flavonoids (compounds 2, 3, and 4) obtained from the leaves of *M. zapota*.

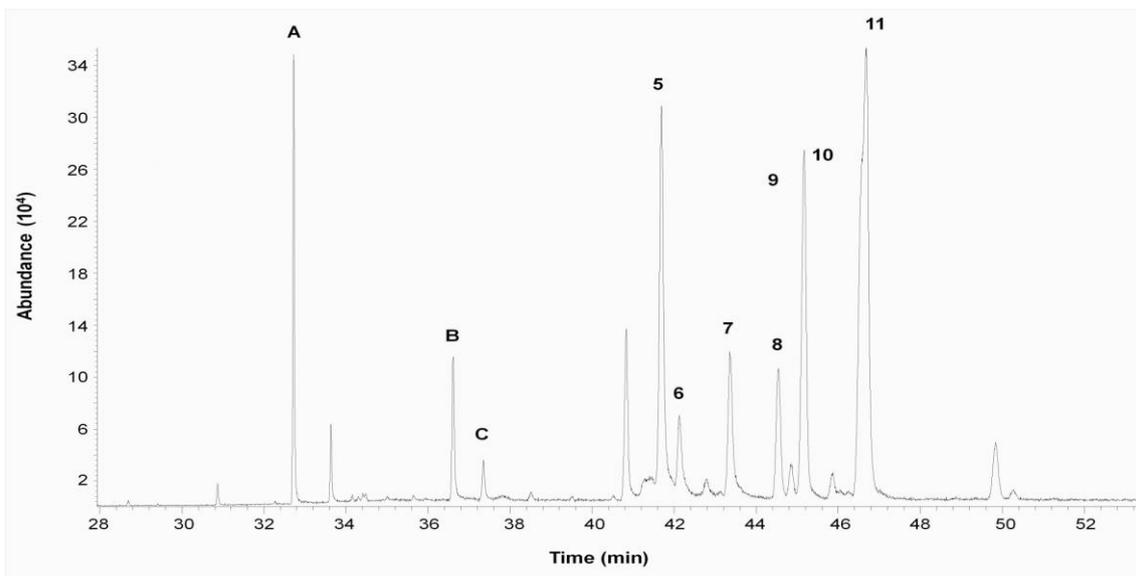
Microorganism	MIC ($\mu\text{g/mL}$)						Control [*]
	EEMz	HPFMz	LPFMz	2	3	4	
<i>C. albicans</i> (ATCC 10231)	16	16	16	>128	>128	>128	8 ^a
<i>T. rubrum</i> (SNB-TR1)	256	128	>512	16	64	128	3.125x10 ⁻³ ^a 2 ^b
<i>S. aureus</i> (ATCC 29213)	128	32	128	8	32	32	2 ^c

671 ^{*} Positive control; ^a fluconazole; ^b itraconazole; ^c oxacillin.

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674 **Figure 1** Expanded chromatogram profile of the LPFMz.

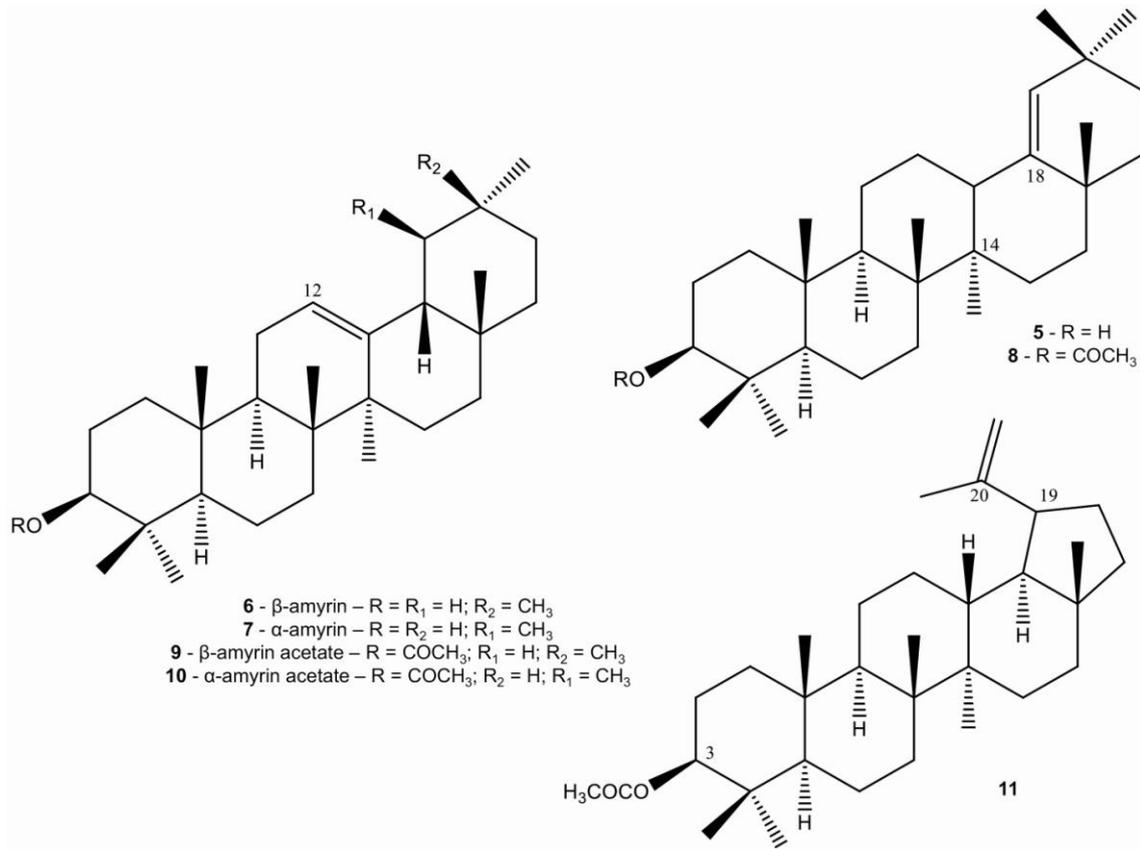


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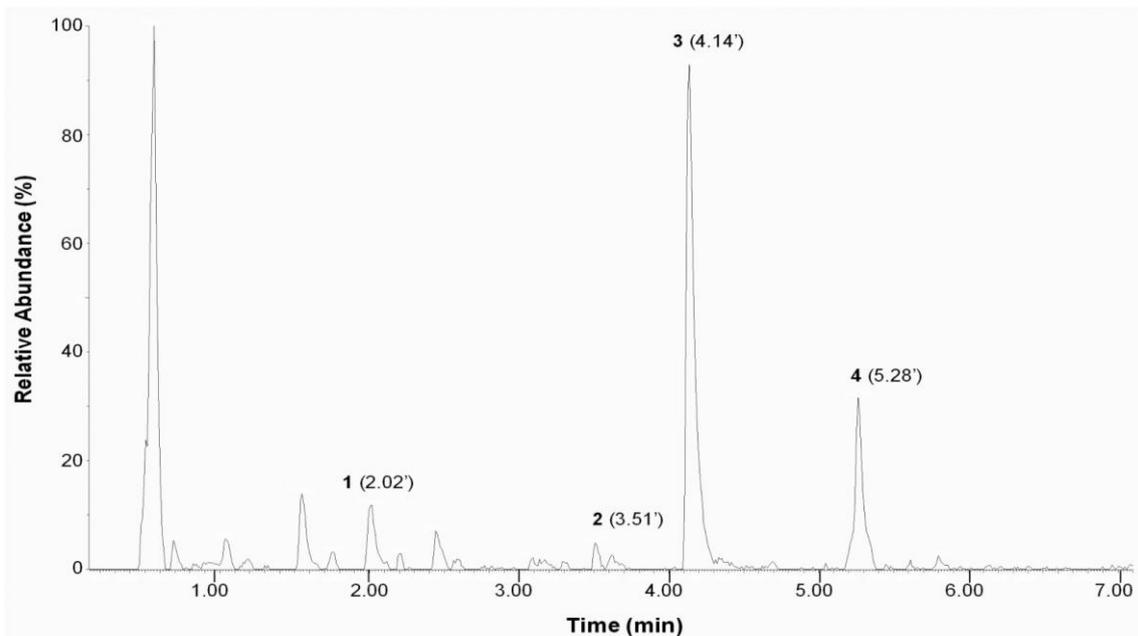
678 **Figure 2** Compounds detected by GC-MS in the LPFMz.



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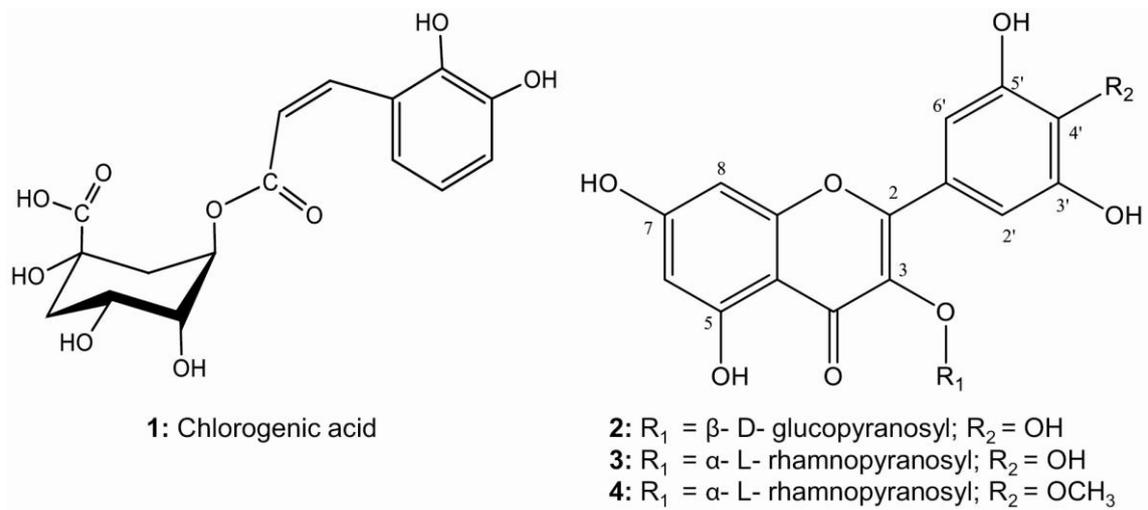
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681 **Figure 3** HPLC elution of the compounds 1, 2, 3 and 4 in the HPFMz.



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683 **Figure 4** Compounds isolated from HPFMz.

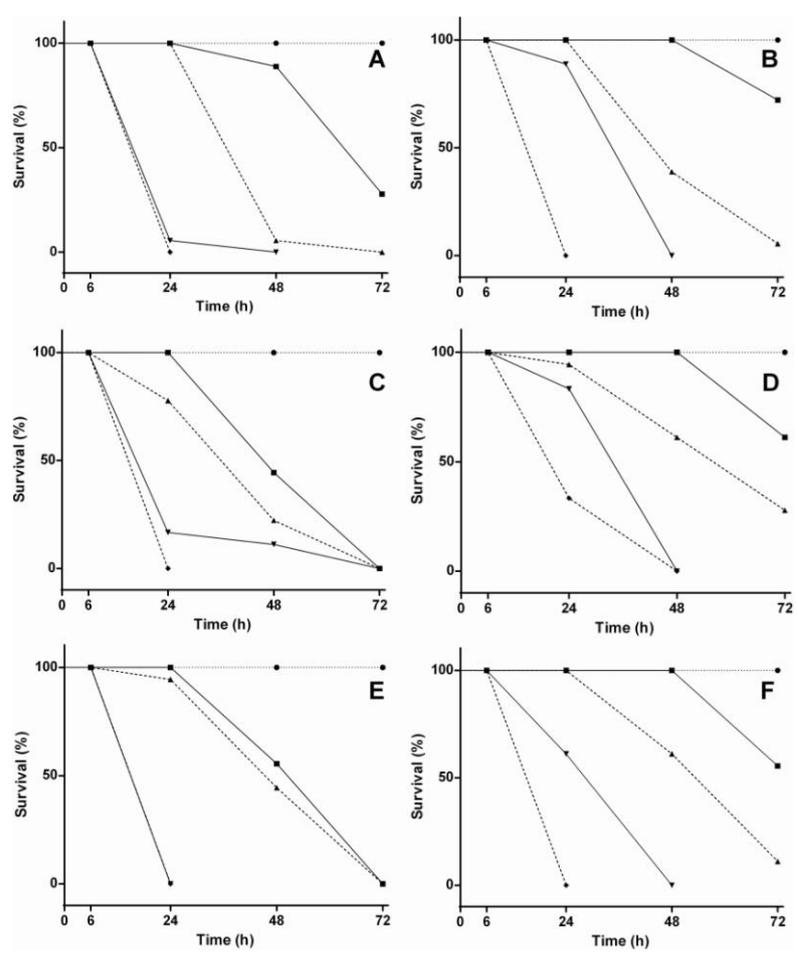


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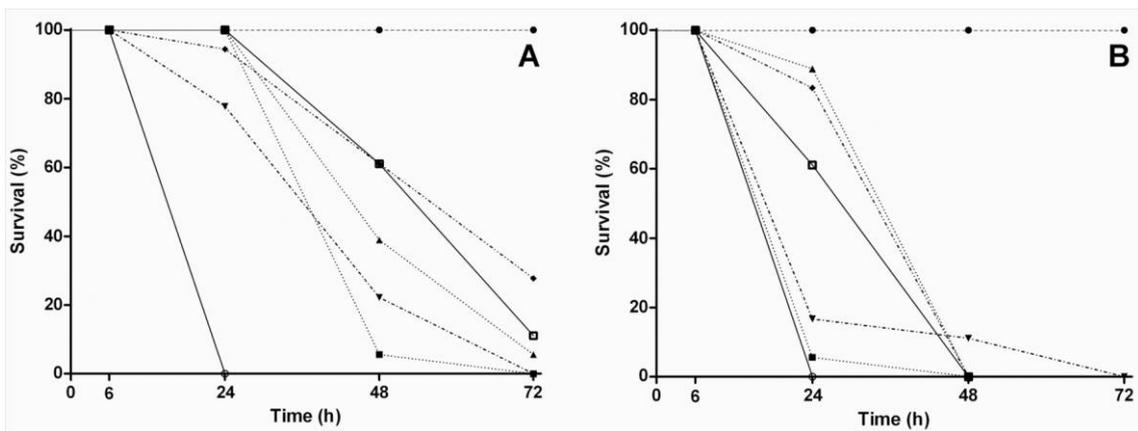
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687 **Figure 5** Anthelmintic activity of *M. zapota* against *S. venezuelensis* after 72 h of
 688 incubation. Percentage survival of parasites exposed to: EEMz (A), HPFMz (B), F₂ (C),
 689 F₃ (D), chlorogenic acid (compound 1) (E) and myricitrin (compound 3) (F). Control
 690 (●), 50 μg/mL (■), 100 μg/mL (▲), 200 μg/mL (▼) and 400 μg/mL (◆).



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694 **Figure 6** Comparison of the anthelmintic activities of the extract, fractions, and
695 compounds obtained from the leaves of *M. zapota* at 100 µg/mL (A) and 200 µg/mL
696 (B). Control (●), EEMz (■), HPFMz (▲), F2 (▼), F3 (◆), chlorogenic acid (compound
697 1) (○) and myricitrin (compound 3) (□).

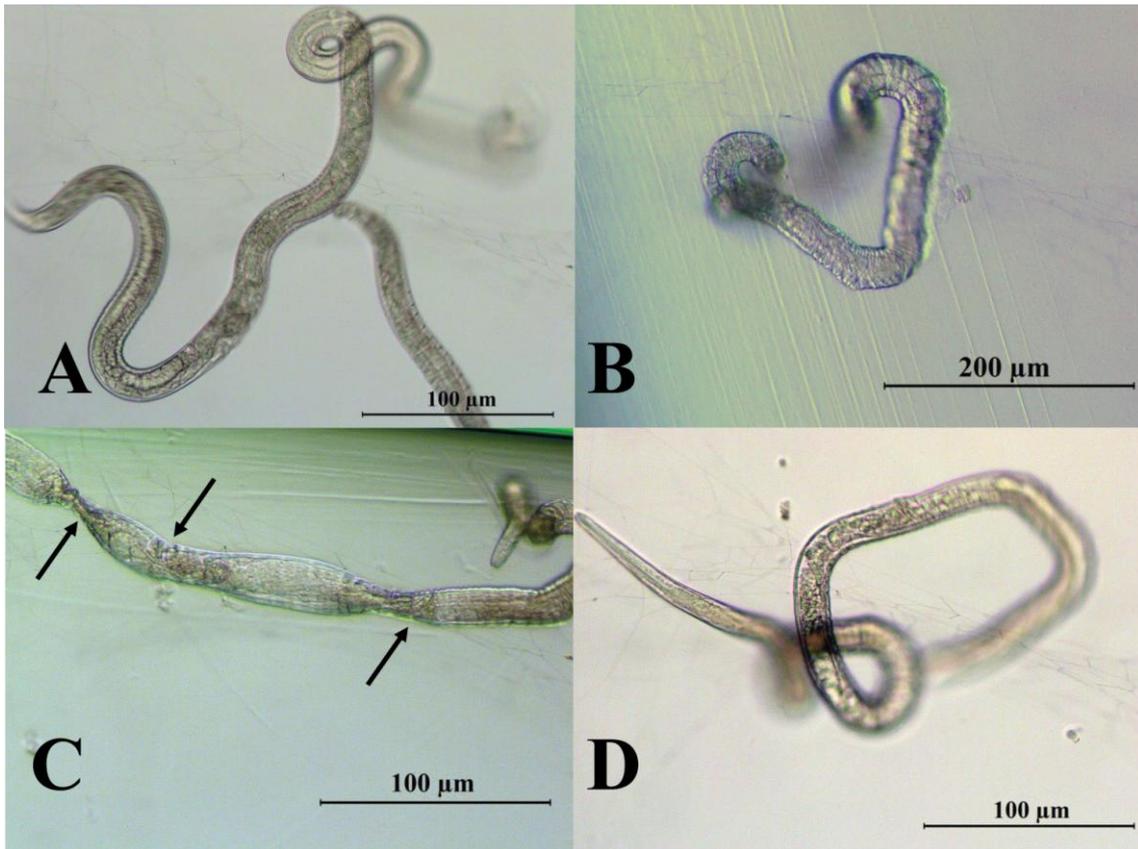


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701 **Figure 7** Parthenogenetic females of *S. venezuelensis* after exposure to 400 µg/mL for
702 72 h of incubation. Parasite of the control group with preserved cuticle (A); Parasite
703 exposed to myricitrin, with cuticular wrinkling (arrow) (B); Parasite exposed to F2, with
704 cuticular alterations (arrows) (C); Parasite treated with ivermectin, with preserved
705 cuticular integrity (D).



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