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

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ABSTRACT

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

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

Key findings: EEMz showed mainly phenolic compounds and pentacyclic triterpenes from Δ^{12} -oleane/ Δ^{12} -ursane series. Chlorogenic acid, myricetin-3-O- β -D-glucopyranoside, mearnsitrin, germanicol, and germanicol acetate were reported to *M. zapota* leaves for the first time in this work. EEMz, HPFMz, LPFMz showed significative activity against *C. albicans* (16 $\mu\text{g/mL}$), while isolated flavonoids were active against *S. aureus* (<32 $\mu\text{g/mL}$). EEMz, phenolic-rich compounds (F_2), and chlorogenic acid were potentially active against *S. venezuelensis* at 24 h.

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

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

Highlights

- Phenolic and pentacyclic triterpenes from Δ^{12} -oleane/ Δ^{12} -ursane were detected
- Chlorogenic acid, myricetin-3-O- β -D-glucopyranoside, mearnsitrin were identified
- The pentacyclic triterpenes presented promising antimicrobial activity
- Ethanolic extract and phenolic fractions showed high antifungal activity
- Ethanolic and phenolic fractions, and chlorogenic acid exhibited anthelmintic action

Abbreviation List

- CAD: Charged Aerosol Detector
- Compound 1: chlorogenic acid
- Compound 2: myricetin-3-O- β -D-glucopyranoside
- Compound 3: myricitrin
- Compound 4: mearnsitrin
- Compound 5: germanicol
- Compound 6: β - amyrin
- Compound 7: α -amyrin
- Compound 8: germanicol acetate
- COSY: Correlation Spectroscopy
- EEMz: dry ethanolic extract
- ESI: Electrospray interface
- F: fraction obtained from High polarity insoluble fraction
- HMBC: Heteronuclear Multiple Bond Correlation
- HPFMz: High polarity insoluble fraction
- HRMS: High Resolution Mass Spectroscopy
- HSQC: Heteronuclear Single Quantum Coherence
- LPFMz: lower polarity fraction
- Mz: *Manilkara zapota*
- PVP: polyvinylpyrrolidone
- TOF: Time-of-flight

1. INTRODUCTION

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

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

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

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

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

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

2. MATERIALS AND METHODS

2.1 Materials

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

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

2.2. Bioactive compounds of the *M. zapota*

2.2.1. Ethanolic extract of the *M. zapota*

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

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

2.2.2. Fractions of the *M. zapota*

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

2.2.3. Isolation of the *M. zapota* compounds

1.5 g of the HPFMz was purified by flash chromatography using a Combi Flash[®] Rf⁺ Teledyne ISCO system (Lincoln, NE, EUA) equipped with a UV detector and C18 column (86 g). A flow rate of 60 mL/min and H₂O/CH₃CN gradient of: 80/20 for 6 min (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 (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 min (F₅), was used to obtain five diferents fractions. Subsequently, the fractions F₂ and F₃ were combined and purified by preparative HPLC system (Gilson, Middleton, WI, USA) equipped with a model 322 pump, a GX-271 fraction collector, a DAD 171 diode array detector, and a prepELSII electrospray accessory. A Phenomenex Luna preparative C18 column (21.2 × 250 mm, 5 µm) (Torrance, CA, USA) was employed at a flow rate of 17 mL/min. The gradient apllied was H₂O/CH₃CN (98/2 to 77/23 in 5 min, 77/23 to 55/45 in 15 min, 55/45 to 0/100 in 5 min). Four compounds **1** (3.5 mg, Rt 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) were isolated and chracterized.

2.3 Identification of the components by chromatography analyses

2.3.1 Thin layer chromatography

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

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

2.3.2 Gas chromatography-mass spectrometry (GC-MS)

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

2.3.3 HPLC analyses

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

2.4 Structural elucidation of the HPFMz

2.4.1 Optical rotation

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

2.4.2. ^1H and ^{13}C NMR and 2D-NMR spectra

NMR analyses were performed in a Bruker (Billerica, MA, USA) 500 MHz spectrometer equipped with a 1 mm inverse detection probe. The samples were dissolved in deuterated methanol (CD_3OD) in 5 mm tubes. The chemical shifts (δ , in ppm) were reported based on the signal for tetramethylsilane (TMS).

2.4.3 High resolution mass analyses

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

Compound 1: Yellow powder; $R_f \sim 0.45$ with blue fluorescence at 366 nm. λ_{max} : 327 nm; ^1H NMR (CD_3OD) 7.56 (1H, d, $J = 15.8$ Hz, H-7), 7.05 (1H, d, $J = 1.8$ Hz, H-2'), 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, $J = 15.8$ Hz, H-8'), 4.20 (1H, m), 3.85 (1H, m), 2.05 (1H, br.s), 1.93 (br.s); HRESITOFMS $[\text{M} - \text{H}]^-$ calculated for $\text{C}_{16}\text{H}_{17}\text{O}_9$, m/z 353.0872.

Compound 2: Yellow powder; $R_f \sim 0.65$ with orange fluorescence at 366 nm. $[\alpha]_D^{25}$: -12° (c 0.1 g/100 mL, CH_2OH); λ_{max} : 261, 356 nm; ^1H NMR (CD_3OD): δ 3.23 (1H, ddd, $J = 9.5, 5.2, 2.4$ Hz, H-5''), 3.38 (1H, dd, $J = 9.5, 8.8$ Hz, H-3''), 3.42 (1H, t, $J = 8.8$ Hz, H-4''), 3.51 (1H, dd, $J = 8.8, 7.9$ Hz, H-2''), 3.62 (1H, d, $J = 11.9, 5.2$ Hz, H-6 β ''), 3.73 (1H, dd, $J = 11.9, 2.4$ Hz, H-6 α ''), 5.26 (1H, d, $J = 7.9$ Hz, H-1''), 6.20 (1H, d, $J = 2.1$ Hz, H-6), 6.38 (1H, d, $J = 2.1$ Hz), 7.30 (2H, s); HRESITOFMS $[\text{M} - \text{H}]^-$, m/z 479.0820 (calculated for $\text{C}_{21}\text{H}_{19}\text{O}_{13}$, m/z 479.0826) and $[\text{M} + \text{H}]^+$, m/z 481.0986 (calculated for $\text{C}_{21}\text{H}_{21}\text{O}_{13}$, m/z 481.0982).

Compound 3: Yellow powder; $R_f \sim 0.8$ with orange fluorescence at 366 nm. $[\alpha]_D^{25}$: -61° (c 0.1 g / 100 mL, CH_2OH), λ_{max} : 261, 350 nm; ^1H NMR (CD_3OD): δ 0.96 (3H, d, $J = 6.1$, 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, 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, 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'); ^{13}C NMR (CD_3OD): 179.58 (C-4), 166.55 (C-7), 163.15 (C-5), 159.34 (C-2), 158.55 (C-9), 146.84 (C-3', C-5'), 137.91 (C-4'), 136.25 (C-3), 121.91 (C-1'), 109.55 (C-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-

3''), 72.00 (C-5''), 71.87 (C-2''), 17.64 (C-6''). HRESITOFMS $[M - H]^-$, m/z 463.0877 (calculated for $C_{21}H_{19}O_{12}$, m/z 463.0877) and $[M + H]^+$, m/z 465.1032 (calculated for $C_{21}H_{21}O_{12}$, m/z 465.1033).

Compound 4: Yellow powder; R_f ~0.85 with yellow fluorescence at 366 nm $[\alpha]_D^{25}$: -58° (c 0.1 g/100 mL, CH_2OH), λ_{max} : 263, 337 nm. 1H NMR (CD_3OD): δ 0.95 (3H, d, J = 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, 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 (1H, d, J = 2.2 Hz, H-6), 6.27 (1H, d, J = 2.2 Hz), 6.87 (2H, s, H-2', H-6'); ^{13}C NMR (CD_3OD): 168.27 (C-7), 158.77 (C-2), 152.28 (C-3', C-5'), 139.28 (C-4'), 127.42 (C-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 (C-4''), 72.15 (C-3''), 72.05 (C-2''), 61.04 (4'-OCH₃), 17.86 (C-6''). HRESITOFMS $[M - H]^-$, m/z 477.1039 (calculated for $C_{22}H_{21}O_{12}$, m/z 477.1033) and $[M + H]^+$, m/z 479.1180 (calculated for $C_{22}H_{23}O_{12}$, m/z 479.1190).

2.5 Antimicrobial activity

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

2.6 Anthelmintic activity

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

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

2.7 Statistical analyses

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

3. RESULTS AND DISCUSSION

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

3.1 Identification of compounds in the LPFMz fraction

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

Please, insert here the Figure 1 and Table 1

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

Please, insert here the Figure 2

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

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

3.2 Identification of compounds isolated from HPFMz

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

Please, insert here the Figure 3

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

Please, insert here the Figure 4

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

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

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

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

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

3.3 *In vitro* antimicrobial activity

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

Please, insert here the Table 2

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

3.4 *In vitro* anthelmintic activity assays

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

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

Please, insert here the Figure 5

Ivermectin (pharmacological control) reduced the motility of the organisms, but there was survival of all the individuals after 72 h. Similar behavior to infective larvae (L3) of *S. stercoralis* using 250 µg/mL of ivermectin has been observed with reduction of the parasite motility and survive during 6 days ^[44]. The lower polarity fractions were also tested (data not shown) but showed unsatisfactory and imprecise results at all concentrations evaluated due the formation of insoluble clusters in the culture medium. At 100 µg/mL, chlorogenic acid was lethal in the first 24 h, while EEMz and F₂ caused total mortality at the end of the incubation time (Fig. 6). At 200 µg/mL, only chlorogenic acid was lethal after 24 h. However, there was no survival for any of the samples tested after 72 h. Images of the parasites after 72 h of incubation are shown in Fig. 7. Parasites exposed to myricitrin (Fig. 7B) exhibited distortions and cuticular wrinkling. Those treated with F₂ (Fig. 7C) showed distortions, depressions, and alterations in cuticular integrity, suggestive of direct cytotoxic effects on the morphology of the worms. The parasites exposed to ivermectin (Fig. 7D) exhibited reduced motility, but the integrity of the cuticular surface remained similar to that of the positive control (Fig. 7A).

Please, insert here the Figure 6 and 7

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

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

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

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

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

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

CONCLUSIONS

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

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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FIGURE LEGENDS

Fig. 1- Expanded chromatogram profile of the LPFMz

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

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

Fig. 4- Compounds isolated from HPFMz.

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

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

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

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.

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

Microorganism	MIC (µg/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

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

Figure 1 Expanded chromatogram profile of the LPFMz.

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

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

Figure 4 Compounds isolated from HPFMz.

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

Figure 6 Comparison of the anthelmintic activities of the extract, fractions, and compounds obtained from the leaves of *M. zapota* at 100 $\mu\text{g/mL}$ (A) and 200 $\mu\text{g/mL}$ (B). Control (●), EEMz (■), HPFMz (▲), F2 (▼), F3 (◆), chlorogenic acid (compound 1) (○) and myricitrin (compound 3) (□).

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