Antiprotozoal Activity Against *Entamoeba histolytica* of Flavonoids Isolated from *Lippia graveolens* Kunth

Ramiro Quintanilla-Licea [1],* , **Javier Vargas-Villarreal [2]**, **María Julia Verde-Star [1]** , **Verónica Mayela Rivas-Galindo [3]** and **Ángel David Torres-Hernández [1]**

[1] Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León (UANL), Av. Universidad S/N, Cd. Universitaria, San Nicolás de los Garza, C.P. 66455 Nuevo León, Mexico; maria.verdest@uanl.edu.mx (M.J.V.-S.); angel.torreshr@uanl.edu.mx (Á.D.T.-H.)

[2] Laboratorio de Bioquímica y Biología Celular, Centro de Investigaciones Biomédicas del Noreste (CIBIN), Dos de abril esquina con San Luis Potosí, C.P. 64720 Monterrey, Mexico; jvargas147@yahoo.com.mx

[3] Facultad de Medicina, Universidad Autónoma de Nuevo León (UANL), Madero y Aguirre Pequeño, Mitras Centro, Monterrey, C.P. 64460 Nuevo León, Mexico; veronica.rivasgl@uanl.edu.mx

* Correspondence: ramiro.quintanillalc@uanl.edu.mx; Tel.: +52-81-83763668

check for updates

Abstract: Amebiasis caused by *Entamoeba histolytica* is nowadays a serious public health problem worldwide, especially in developing countries. Annually, up to 100,000 deaths occur across the world. Due to the resistance that pathogenic protozoa exhibit against commercial antiprotozoal drugs, a growing emphasis has been placed on plants used in traditional medicine to discover new antiparasitics. Previously, we reported the in vitro antiamoebic activity of a methanolic extract of *Lippia graveolens* Kunth (Mexican oregano). In this study, we outline the isolation and structure elucidation of antiamoebic compounds occurring in this plant. The subsequent work-up of this methanol extract by bioguided isolation using several chromatographic techniques yielded the flavonoids pinocembrin (**1**), sakuranetin (**2**), cirsimaritin (**3**), and naringenin (**4**). Structural elucidation of the isolated compounds was achieved by spectroscopic/spectrometric analyses and comparing literature data. These compounds revealed significant antiprotozoal activity against *E. histolytica* trophozoites using in vitro tests, showing a 50% inhibitory concentration (IC_{50}) ranging from 28 to 154 μg/mL. Amebicide activity of sakuranetin and cirsimaritin is reported for the first time in this study. These research data may help to corroborate the use of this plant in traditional Mexican medicine for the treatment of dyspepsia.

Keywords: infectious diseases; amoebiasis; Mexican oregano; bioguided isolation; flavonoids; antiprotozoal agents

1. Introduction

Amoebiasis is caused by *Entamoeba histolytica*, which is a protozoan of the family Endomoebidae [1]. It is related to elevated morbidity and mortality worldwide, and has become a serious public health problem in developing countries [2]. Traveling to endemic countries is a risk factor for acquiring an *E. histolytica* infection [3]. After malaria, amoebiasis is the second cause of death due to parasitic diseases [4,5]. The symptoms vary from mild diarrhea to dysentery, but, occasionally, *E. histolytica* can invade the intestinal mucosal barrier and trigger liver abscesses [6]. Asymptomatic infections occur in 90% of individuals, whereas the remaining 10% contract symptomatic infections [7]. Around 50 million people suffer from severe amoebiasis, and 40,000–100,000 deaths occur annually due to this parasitosis [8,9].

Currently, metronidazole is the most used commercial drug for the treatment of amoebiasis, however, since drug resistance by *E. histolytica* is increasing, the use of higher doses to overcome the infection is needed, thus causing unpleasant side effects [10,11]. Considering these undesired side effects as well as the development of resistant strains of *E. histolytica* against metronidazole, more efficient and safer antiamoebic agents are required [12–14].

Natural products occurring in medicinal plants have proved to be an important source of leading compounds for the design of new drugs [15]. Mexican oregano (*Lippia graveolens* Kunth) has been used in traditional Mexican medicine for curing inflammation-related diseases, such as respiratory and digestive disorders, headaches, and rheumatism, among others [16,17]. Oregano's essential oil, regardless of the species, shows a broad range of effects on bacteria, with some of them being resistant to antibiotics of clinical use, as well as on fungi and parasites [18–22].

Recently, we reported the in vitro antiamoebic activity of a methanolic extract of *Lippia graveolens* Kunth and the bioguided isolation of carvacrol, as one of the bioactive compounds with antiprotozoal activity [23]. This work aims to isolate and achieve the structure elucidation of additional antiamoebic compounds present in this plant.

2. Results

2.1. Bioguided Isolation of Flavonoids from Lippia graveolens Kunth

As previously reported, the partition of a methanolic extract of *Lippia graveolens* by extraction with *n*-hexane and fractionation of the hexane phase led to the isolation of carvacrol with excellent antiamoebic activity [23]. The subsequent handling of the remaining methanol (MeOH) by partition between methanol-water and ethyl acetate (EtOAc), carried out in this research, yielded an EtOAc residue with 93.3% growth inhibition of *E. histolytica*. After column chromatography (silica gel, Sephadex), this residue afforded the known flavonoids pinocembrin (**1**), sakuranetin (**2**), cirsimaritin (**3**), and naringenin (**4**), with significant antiamoebic activity (Figure 1).
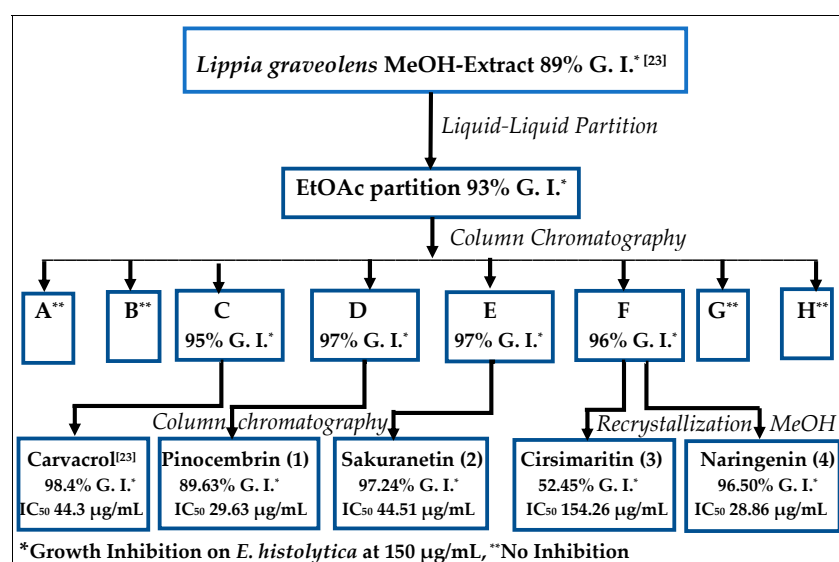
Figure 1. General scheme for the bioguided isolation of compounds with antiamoebic activity from *Lippia graveolens Kunth* (Mexican oregano).

The isolated flavonoids were identified by comparing their physical and spectral data with those reported in the literature.

The electron ionization (EI) mass spectrum showed a molecular ion with $m/z = 256$ for pinocembrin **1** (calcd. for $C_{15}H_{12}O_4$, 256.253), $m/z = 286$ for sakuranetin **2** (calcd. for $C_{16}H_{14}O_5$, 286.283), $m/z = 314$

for cirsimaritin **3** (calcd. for $C_{17}H_{14}O_6$, 314.28), and *m/z* = 272 for naringenin **4** (calcd. for $C_{15}H_{12}O_5$, 272.252).

The infrared (IR) spectrum of all isolated flavonoids (see Supplementary material) contained absorption bands at 1600–1650 cm^{-1} (medium) and 1100–1250 cm^{-1} (strong), consistent with a C=O bond in the molecules [24–27].

One- and two-dimensional nuclear magnetic resonance (NMR) spectra were recorded for the isolated compounds using deuterated dimethyl sulfoxide (DMSO-d_6). ^{1}H- and ^{13}C-NMR chemical shifts (see Supplementary Material) were in accordance with those reported for pinocembrin **1** [28–30], sakuranetin **2** [31–34], cirsimaritin **3** [26,35–37], and naringenin **4** [27,28,38–41]. An unambiguous assignment of the ^{13}C-NMR spectrum of these compounds was deduced from ^{1}H-^{1}H COSY, NOESY, HSQC, and HMBC spectra (see Supplementary Material).

The four isolated flavonoids have the common characteristic that the rotameric hydroxy group at C-5 forms an intramolecular H-bond with the carbonyl group [42]. That explains the shift of this proton absorption to the range of about δ 13.0–12.0 as a sharp singlet in DMSO-d_6 [43].

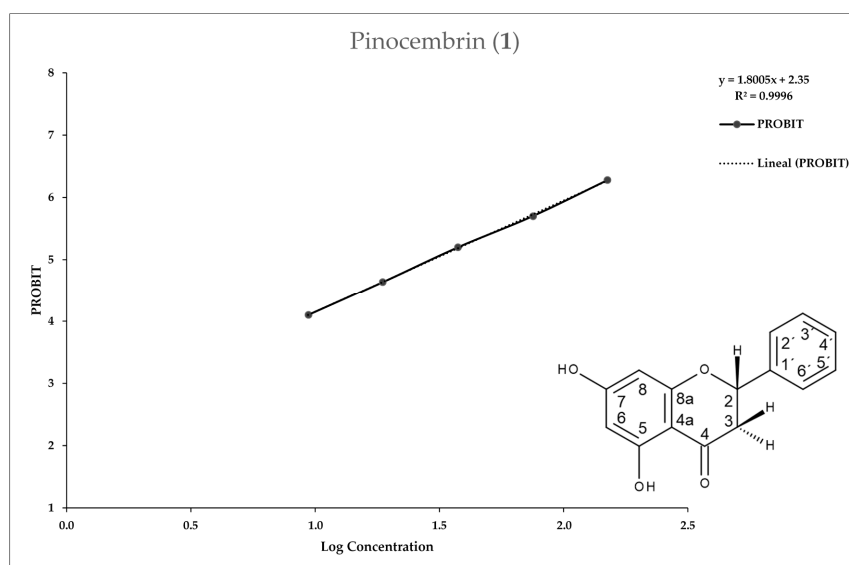## 2.2. Entamoeba Histolytica Growth Parameters

After collecting data from *E. histolytica* growth kinetics experiments, it was estimated that the generation time is 14.76 h and the duplication time is 21.30 h.

The ideal growth time of *E. histolytica* for evaluating the amebicide activity of the compounds was set at 72 h because, at this point, protozoa are still in the exponential and sustained growth stage, thus decreasing the number of false positives.

2.3. In Vitro Assay for Entamoeba histolytica

The pure compounds were dissolved in DMSO to a concentration of 150 μg/mL in a suspension of *E. histolytica* trophozoites in a logarithmic phase in peptone, pancreas, and liver extract plus 10% bovine serum (PEHPS medium). They showed significant growth inhibition of *E. histolytica* at this concentration. The 50% inhibitory concentration (IC$_{50}$) values of these compounds ranged from 28.86 to 154.26 μg/mL (metronidazole IC$_{50}$ 0.205 μg/mL).

Figures 2–5 show the 50% inhibitory concentration of each compound calculated by using a Probit analysis, considering a 95% confidence level.
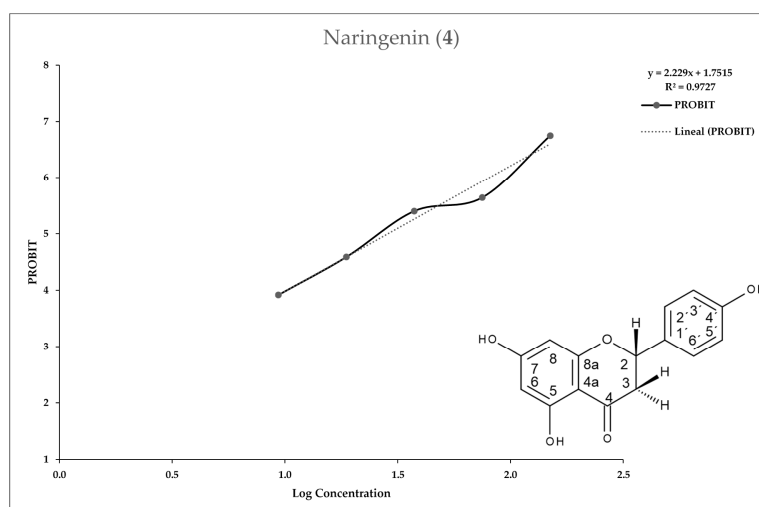

Figure 2. Antiprotozoal activity of Pinocembrin **1** against *Entamoeba histolytica*. Growth inhibition of 89.63% at 150 μg/mL, 50% inhibitory concentration value (IC$_{50}$) = 29.63 μg/mL.
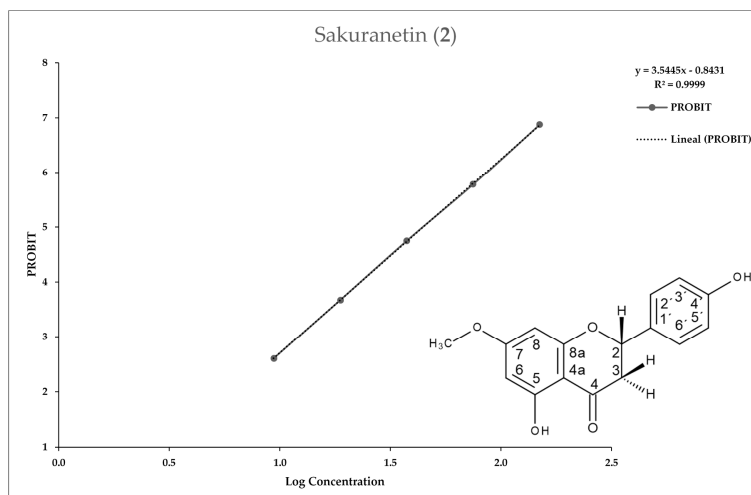
Figure 3. Antiprotozoal activity of Sakuranetin **2** against *Entamoeba histolytica.* Growth inhibition of 97.24% at 150 μg/mL, IC_{50} = 44.51 μg/mL.
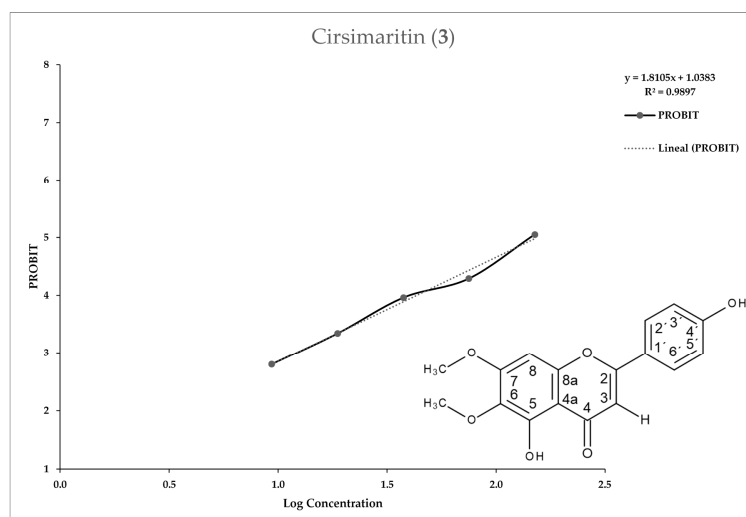
Figure 4. Antiprotozoal activity of Cirsimaritin **3** against *Entamoeba histolytica.* Growth inhibition of 52.45% at 150 μg/mL, IC_{50} = 154.26 μg/mL.

Figure 5. Antiprotozoal activity of Naringenin **4** against *Entamoeba histolytica.* Growth inhibition of 96.50% at 150 μg/mL, IC_{50} = 28.86 μg/mL.

3. Discussion

It is estimated that about 6000 flavonoids are present in different plants worldwide [44,45], and many of them are common ingredients of our daily food. Flavonoids exhibit a variety of biological properties, such as antioxidant, anticancer, antibacterial, antifungal, antiparasitic [46–53], as well as those for treating other kinds of illness [54–57].

More than 20 flavonoids have been identified in the leaves of *Lippia graveolens* by high pressure liquid chromatography (HPLC), according to previous reports [58]. Pinocembrin, sakuranetin, naringenin, and cirsimaritin have already been isolated from this plant [58–62], but, in the respective studies, no antiamoebic activity is reported. Nevertheless, some research groups have reported antiprotozoal activity of these flavonoids isolated from sources other than *Lippia graveolens.*

A weak antiparasitic activity of pinocembrin has been shown against trypomastigotes of *Trypanosoma cruzi*, with inhibition values in the range of 40.13% (at 250 µg/mL) to 43.68% (at 500 µg/mL), according to Grael et al. [63]. Weak inhibition was also observed against *Giardia lamblia* trophozoites, with an IC_{50} of 174.4 µg/mL reported by Alday-Provencio et al. [64] and an IC_{50} of 57.39 µg/mL reported by Calzada et al. [65], respectively.

Sakuranetin presented activity against *Leishmania amazonensis*, *Leishmania brazilians*, *Leishmania major*, and *Leishmania chagasi*, with a range of 43–52 µg/mL, as well as against *T. cruzi* trypomastigotes, with an IC_{50} of 20.17 µg/mL, according to Grecco et al. [39].

Regarding parasitic diseases, cirsimaritin showed potent inhibition against *Plasmodium falciparum* resistant to chloroquine, with an IC_{50} of 16.9 µM [66], and similar activity against *Leishmania donovani* (IC_{50} = 3.9 µg/mL), *Trypanosoma brucei rhodesiense* (IC_{50} = 3.3 µg/mL), and *T. cruzi* (IC_{50} = 19.7 µg/mL), according to Tasdemir et al. [49].

Antiparasitic research has found giardicidal activity in naringenin (**4**), with an IC_{50} of 125.7 µg/mL [64] and 47.84 µg/mL [65], according to Alday-Provencio et al. and Calzada et al., respectively, but no damage against *Trypanosoma cruzi* and *Leishmania* spp. was observed, according to Grecco et al. [39].

Some results of pinocembrin and naringenin tested against the strain *E. histolytica* HM1:IMSS have been published; pinocembrin isolated from *Teloxys graveolens* and naringenin from a commercial source exhibited an IC_{50} of 80.76 and 98.24 µg/mL, respectively [65,67]. This indicates a lower effectiveness compared with our results (IC_{50} of 29.51 µg/mL for pinocembrin and 28.85 µg/mL for naringenin, respectively), which could be explained by the difference between the methodology reported by Calzada [65] and that used by our group. The principal difference was the time that the trophozoites were exposed to the chemical compound. In our methodology, we incubated the trophozoites with the flavonoids for 72 h, while Calzada [65] incubated them for 48 h, which could represent a factor in the certainty of the biological activity.

In our view, this is the first report on the amebicide activity of sakuranetin and cirsimaritin.

The antiprotozoal activity of pinocembrin and naringenin (IC_{50} of 29.63 and 28.86 µg/mL, respectively) was higher compared with sakuranetin (44.51 µg/mL), and the most remarkable comparison was with cirsimaritin (154.26 µg/mL), revealing that a 5,7-dihydroxylated A ring is essential for antiprotozoal activity, as remarked by Calzada [65]. The structure–effect correlations also showed that a 2,3-double bond in ring B (as in cirsimaritin) reduces the antiprotozoal activity.

Currently, there are no available studies on the mechanism of action against *E. histolytica* of the flavonoids isolated from *L. graveolens*, although there are reports on some structurally related flavonoids [68,69]. The ultrastructural changes in the morphology of *Entamoeba histolytica* when it was assayed with (−)-epicatechin, a flavan-3-ol flavonoid, have previously been demonstrated, showing an IC_{50} of 1.9 µg/mL. The results indicated programmed cell death activation with nuclear alterations (small clumps around the nuclear membrane), in addition to cytoplasmatic modifications, such as an increase of glycogen deposits and a reduction of the size and number of vacuoles [70]. Recently, Bolaños et al. [68] have also demonstrated that the flavonoids (−)-epicatechin and kaempferol affect cytoskeleton proteins and functions in *E. histolytica* [68,71], leading to changes in essential cellular

mechanisms, such as adhesion, migration, phagocytosis, and cytolysis. These findings lead us to have an idea of the possible targets and mechanisms of action of the flavonoids isolated from *L. graveolens*. None of the flavonoids isolated from *L. graveolens* present a hydroxyl group at position 3, as in the case of epicatechin and kaempferol, so there must be subtle differences in the mechanism of action of pinocembrin, sakuranetin, naringenin, and cirsimaritin, and part of the future work of our group will be devoted to this issue.

The significant inhibitory effect against *E. histolytica* observed for the methanolic extract of *Lippia graveolens* (IC_{50} = 59.15 µg/mL) can be attributed to the presence of the flavonoids isolated from the ethyl acetate partition as well as the carvacrol mainly obtained from the hexane partition [23]. Compounds with a higher polarity occurring in *L. graveolens*, soluble in methanol or water, are not involved in the antiprotozoal activity of this plant against *E. histolytica*.

4. Materials and Methods

4.1. General

An Electrothermal 9100 apparatus (Electrothermal Engineering Ltd., Southend-on-Sea, UK) was used for melting point acquisition. IR spectra were measured on a Frontier Fourier transform infrared (FT-IR) spectrometer (PerkinElmer, Waltham, MA, USA) with an ATR accessory. NMR spectra were recorded on an Avance DPX 400 spectrometer (Bruker, Billerica, MA, USA) running at 400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C. EI-MS were obtained on a MAT 95 spectrometer (70 eV, Finnigan, San Jose, CA, USA). Thin layer chromatography (TLC) was realized on precoated silica gel glass plates (5 × 10 cm, Merck silica gel 60 F_{254}, Darmstadt, Germany). Column chromatography was carried out on silica gel (60–200 mesh) purchased from J. T. Baker (Phillipsburg, NJ, USA). Size-exclusion chromatography was performed on Sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd., purchased from Sigma-Aldrich Chemie, Steinheim, Germany).

4.2. Plant Material

Aerial parts of *Lippia graveolens* were collected near the town General Cepeda (Mexican State Coahuila) in March 2011 and identified by Maria del Consuelo González. A voucher specimen (No. 025554) was deposited at the Herbario de la Facultad de Ciencias Biológicas (UANL), Nuevo León, México. The plant name has been checked with http://www.theplantlist.org. The vegetal material was dried and ground to powder.

4.3. Plant Extraction and Bioguided Isolation of Antiamoebic Compounds from Lippia graveolens Kunth

In total, 600 g of dried and ground *Lippia graveolens* leaves were extracted in a Soxhlet apparatus for 40 h with MeOH. After filtration, the solvent was removed in a rotatory evaporator to yield 260 g of crude extract. This extract was analyzed for its amebicide activity on trophozoites of *E. histolytica* (HM1:IMSS strain), showing a significant inhibition percentage (89%; IC_{50} 59.15 µg/mL) in terms of the standard concentration of 150 µg/mL. Afterward, the extract was redissolved in 2 L methanol and divided into four portions of 500 mL each for conducting liquid-liquid partition with *n*-hexane. After solvent evaporation, 19.9 g of a residue with high amebicidal activity (90.9% growth inhibition) was obtained. Bioguided fractionation of this hexane partition, using column chromatography on silica gel, provided 2.2 g of carvacrol (98.4% growth inhibition; IC_{50} 44.30 µg/mL), as previously reported [23].

The methanolic phase was concentrated under reduced pressure up to a volume of ca. 500 mL, and 1.5 L distilled water was then added, gradually and under constant stirring. Afterward, the methanol/water mixture was divided into four portions of 500 mL each and submitted to liquid–liquid partition with ethyl acetate to yield, after solvent evaporation, 67.3 g of a combined residue with 93.3% growth inhibition against *E. histolytica*.

The EtOAc partition was suspended in 400 mL of chloroform ($CHCl_3$), and, after stirring, filtration, and solvent evaporation, 25.8 g of $CHCl_3$-soluble residue was obtained. The material recovered

from the filter was then suspended in 400 mL of EtOAc, and, after stirring, filtration, and solvent evaporation, 8.8 g of EtOAc-soluble residue was obtained. This second material recovered from the filter was then suspended in 100 mL MeOH, and the same procedure was applied to yield 8.8 g of MeOH-soluble residue and ca. 24 g of an insoluble powder from the final filtration. Each residue was analyzed for its anti-*Entamoeba histolytica* activity, and remarkable results were obtained, mainly for the $CHCl_3$-residue, with 90.9% growth inhibition, followed by the EtOAc residue, with 61.6% growth inhibition. The MeOH residue and insoluble powder did not show any activity.

The $CHCl_3$ fraction was divided into five portions of ca. 5 g, and each of them was chromatographed on a silica gel (100 g) column (60×2.6 cm) and eluted with stepwise gradients of chloroform-ethyl acetate, and finally with methanol. For each column, a total of 110 subfractions (50 mL) were obtained and collected, and considering their TLC ($CHCl_3$–EtOAc, 9:1) profiles, split into eight main fractions (A-H). These main fractions, containing the nonpolar to the more polar compounds, were used for amebicide assays. Out of the eight fractions, only fractions C, D, E, and F showed amebicide activity, with 95.34%, 96.89%, 97.24%, and 95.85% growth inhibition, respectively.

Fraction C (showing the main compound, according to TLC, with $R_f = 0.77$; $CHCl_3$-Ethyl acetate, 9:1) was divided into five portions of approx. 1 g, and each of them was chromatographed on a silica gel (20 g) column (39×2 cm) and eluted with a stepwise gradient solvent system of chloroform and ethyl acetate, and finally with methanol. Sixty subfractions (10 mL) were collected for each column and combined, based on their TLC ($CHCl_3$-Ethyl acetate, 9:1) profiles, into five main fractions (CA-CE). Fraction CB presented the main compound, with $R_f = 0.77$, so it was subjected to subsequent purification with three columns packed with silica gel as stationary phase (data not shown). Afterward, 315 mg of a viscous liquid with a high amebicide activity (96.76% growth inhibition; IC_{50} 44.1 µg/mL) was recovered. The spectroscopic results indicated that this compound is carvacrol, previously isolated from hexane partition [23].

Fraction D (showing the main compound, according to TLC, with $R_f = 0.51$; $CHCl_3$-Ethyl acetate, 9:1) was divided into four portions of ca. 1 g and each of them was submitted to chromatography on a silica gel (20 g) column (39×2 cm) and eluted with a stepwise gradient solvent system of chloroform and ethyl acetate, and finally with methanol. Sixty subfractions (10 mL) were collected for each column and combined, based on their TLC ($CHCl_3$-Ethyl acetate, 9:1) profiles, into five main fractions (DA-DE). Fraction DB presented the main compound, with $R_f = 0.51$, so it was subjected to subsequent purification with several columns packed with Sephadex as stationary phase (data not shown). Afterward, 32 mg of a solid with a high amebicide activity (89.63% growth inhibition; IC_{50} 29.63 µg/mL) was recovered. The spectroscopic results indicated that this compound is pinocembrin **1** ($C_{15}H_{12}O_4$; M.p. 210 °C: Lit. 223–236 °C [72], 191–193 °C [24]).

Fraction E (showing the main compound, according to TLC, with $R_f = 0.33$; $CHCl_3$-Ethyl acetate, 9:1) was divided into three portions of approx. 1 g and each of them was chromatographed on a silica gel (20 g) column (39×2 cm) and eluted with a stepwise gradient solvent system of chloroform and ethyl acetate, and finally with methanol. Sixty subfractions (10 mL) were collected for each column and combined, based on their TLC ($CHCl_3$-Ethyl acetate, 9:1) profiles, into five main fractions (EA-EE). Fractions EB and EC presented the main compound, with $R_f = 0.33$, so they were subjected to subsequent purification with several columns packed with Sephadex as stationary phase (data not shown). Afterward, 102 mg of a solid with a high amebicide activity (97.24% growth inhibition; IC_{50} 44.51 µg/mL) was recovered. The spectroscopic results indicated that this compound is sakuranetin **2** ($C_{16}H_{14}O_5$; M.p. 160 °C: Lit. 151–153 °C [73], 143–144 °C [74]).

Fraction F showed only a main compound, according to TLC, with $R_f = 0.10$ ($CHCl_3$-Ethyl acetate, 9:1). Using an eluent of a higher polarity ($CHCl_3$-Ethyl acetate, 1:1), this compound was revealed to be a mixture of two compounds, with an R_f of 0.70 and 0.53, respectively. This mixture was no longer soluble in chloroform and it was therefore not possible to use column chromatography with silica gel of a normal phase to try to separate the two compounds. Then, this fraction was suspended in 30 mL of chloroform and heated to reflux for 15 min, noting that a large quantity of the material remained

insoluble. After cooling and filtering, 645 mg of an insoluble solid consisting of the two compounds of the mixture (TLC) was recovered, while in the chloroform solution, a mixture of the same compounds was also observed, but with additional impurities, mainly of a lower polarity. The insoluble solid (645 mg) recovered from the filter was suspended in 30 mL methanol and heated to reflux until complete solubility was observed. After cooling, the MeOH solution was kept refrigerated for 72 h with hermetic closure, and during that time, 50.6 mg of a greenish powder containing only the compound with R_f = 0.53 (CHCl$_3$-Ethyl acetate, 1:1) was separated. This solid presented no significant amebicide activity (52.45% growth inhibition; IC$_{50}$ 154.26 µg/mL). The spectroscopic results indicated that this compound is cirsimaritin **3** (C$_{17}$H$_{14}$O$_6$; M.p. 268 °C: Lit. 256–258 °C [26], 267–268 °C [75]).

The filtered MeOH solution was concentrated and chromatographed on a Sephadex (50 g) column (160 × 1.5 cm) and eluted with methanol (300 mL). A total of 60 subfractions (5 mL) were collected and combined, based on their TLC (CHCl$_3$-Ethyl acetate, 1:1) profiles, into seven main fractions (FA-FG). From fraction FC, additional cirsimaritin with R_f = 0.53 was recovered. Fraction FF showed only the pure compound, with R_f = 0.70. This solid also presented high amebicide activity (96.50% growth inhibition; IC$_{50}$ 28.86 µg/mL). The spectroscopic results indicated that this compound is naringenin **4** (C$_{15}$H$_{12}$O$_5$; M.p. 254 °C: Lit. 250–252 °C [27], 248–250 °C [73]).

4.4. Antiprotozoal Assay

4.4.1. Test Microorganisms

Strain HM-1:IMSS of *Entamoeba histolytica* was obtained from the microorganism culture collection of the Centro de Investigación Biomédica del Noreste (CIBIN) in Monterrey, Mexico. The trophozoites were grown axenically and maintained in peptone, pancreas, and liver extract plus bovine serum and employed at the log phase of growth (2×10^4 cells/mL) by all of the bioassays performed [76,77].

The procedure for determining the growth curve for *E. histolytica* was performed in 13 × 100 mm screw cap test tubes, by inoculating 20,000 trophozoites of *E. histolytica* in 5 mL of PEHPS medium, to which 10% bovine serum was added. Subsequently, they were incubated at 36.5 °C for 120 h, and every 24 h, the number of trophozoites was determined and the growth parameters in the medium were evaluated. The process was conducted in three separate experiments per triplicate.

4.4.2. In Vitro Assay for Entamoeba histolytica

Each compound was dissolved in DMSO and adjusted to a concentration of 150 µg/mL in a suspension of *E. histolytica* trophozoites at a logarithmic phase in PEHPS medium with 10% bovine serum. Vials were incubated for 72 h, and then chilled in iced water for 20 min, and, by using a hemocytometer, the number of dead trophozoites per milliliter was calculated. Each extract assay was carried out in triplicate. Metronidazole was used as a positive control, and as a negative control, an *E. histolytica* suspension in PEHPS medium with no extract added was used. The percentage of inhibition was estimated as the number of dead trophozoites compared to the negative controls.

4.4.3. In Vitro IC$_{50}$ Determination

Each compound was dissolved in dimethyl sulfoxide and adjusted to 150, 75, 37.5, 18.75, and 9.375 µg/mL by adding a suspension of *E. histolytica* trophozoites at a logarithmic phase in PEHPS medium with 10% bovine serum. Vials were incubated for 72 h, and then chilled in cold water for 20 min, and the number of dead trophozoites per milliliter was evaluated using a hemocytometer. All assays were performed in triplicate. Metronidazole was used as a positive control, and as a negative control, an *E. histolytica* suspension in PEHPS medium with no extract added was used. The percentage of inhibition was estimated as the number of dead trophozoites compared to the negative controls. The 50% inhibitory concentration of each compound was calculated by using a Probit analysis, considering a 95% confidence level.

5. Conclusions

The isolated and pure flavonoids from *L. graveolens* showed significant growth inhibition against *E. histolytica* (52% to 97% at a concentration of 150 µg/mL). The IC$_{50}$ values of these compounds ranged from 28.86 to 154.26 µg/mL, so they were not as effective as metronidazole (IC$_{50}$ 0.205 µg/mL), but these IC$_{50}$ values can be used as a guideline for further research on this plant as a source of potential antiamoebic agents.

The main contribution of this research work lies in the fact that it has shown that the presence of the flavonoids described herein in *Lippia graveolens* has a direct relationship with the antiprotozoal activity of extracts of this plant against *Entamoeba histolytica*. These flavonoids could be used as biomarkers [78] for the quality control of phytotherapeutics developed based on this work.

The results of our research may also form the basis for directly incorporating the use of *Lippia graveolens* extracts into conventional and complementary medicine for the treatment of amebiasis, as well as other infectious diseases.

References

1.   Ávila-García, R.; Valdés, J.; Jáuregui-Wade, J.M.; Ayala-Sumuano, J.T.; Cerbón-Solórzano, J. The metabolic pathway of sphingolipids biosynthesis and signaling in Entamoeba histolytica. *Biochem. Biophys. Res. Commun.* **2020**, *522*, 574–579. [CrossRef] [PubMed]

2.   Carrero, J.C.; Reyes-López, M.; Serrano-Luna, J.; Shibayama, M.; Unzueta, J.; León-Sicairos, N.; De La Garza, M. Intestinal amoebiasis: 160 years of its first detection and still remains as a health problem in developing countries. *Int. J. Med. Microbiol.* **2020**, *310*, 151358. [CrossRef] [PubMed]

3.   Broucke, S.V.D.; Verschueren, J.; Van Esbroeck, M.; Bottieau, E.; Ende, J.V.D. Clinical and microscopic predictors of Entamoeba histolytica intestinal infection in travelers and migrants diagnosed with Entamoeba histolytica/dispar infection. *PLoS Negl. Trop. Dis.* **2018**, *12*, e0006892. [CrossRef]

4.   Aguilar-Rojas, A.; Olivo-Marin, J.-C.; Guillen, N. The motility of Entamoeba histolytica: Finding ways to understand intestinal amoebiasis. *Curr. Opin. Microbiol.* **2016**, *34*, 24–30. [CrossRef] [PubMed]

5.   Muñoz, P.L.A.; Minchaca, A.Z.; Mares, R.E.; Ramos-Ibarra, M.A. Activity, stability and folding analysis of the chitinase from Entamoeba histolytica. *Parasitol. Int.* **2016**, *65*, 70–77. [CrossRef]

6.   Guo, F.; Forde, M.; Werre, S.R.; Krecek, R.C.; Zhu, G. Seroprevalence of five parasitic pathogens in pregnant women in ten Caribbean countries. *Parasitol. Res.* **2016**, *116*, 347–358. [CrossRef]

7.   Singh, R.S.; Walia, A.K.; Kanwar, J.R.; Kennedy, J.F. Amoebiasis vaccine development: A snapshot on E. histolytica with emphasis on perspectives of Gal/GalNAc lectin. *Int. J. Boil. Macromol.* **2016**, *91*, 258–268. [CrossRef]

8.   Iyer, L.R.; Verma, A.K.; Paul, J.; Bhattacharya, A. Phagocytosis of Gut Bacteria by Entamoeba histolytica. *Front. Microbiol.* **2019**, *9*, 34. [CrossRef]

9.   Institut-Pasteur Amoebiasis. Available online: https://www.pasteur.fr/en/medical-center/disease-sheets/amoebiasis-0 (accessed on 19 April 2020).

10. Gómez-García, C.; Marchat, L.A.; López-Cánovas, L.; Pérez-Ishiwara, D.G.; Rodriguez, M.A.; Orozco, E. Drug Resistance Mechanisms in Entamoeba histolytica, Giardia lamblia, Trichomonas vaginalis, and Opportunistic Anaerobic Protozoa. In *Antimicrobial Drug Resistance. Mechanisms of Drug Resistance*; Mayers, D.L., Sobel, J.D., Oullette, M., Kaye, K.S., Marchaim, D., Eds.; Springer International Publishing AG: Cham, Switzerland, 2017; pp. 613–628. ISBN 978-3-319-47264-5.

11. Luevano, J.H.E.; Castro-Ríos, R.; Sánchez-García, E.; Hernández-García, M.E.; Villarreal, J.V.; Rodríguez-Luis, O.E.; Chávez-Montes, A. In Vitro Study of Antiamoebic Activity of Methanol Extracts of Argemone mexicana on Trophozoites of Entamoeba histolytica HM1-IMSS. *Can. J. Infect. Dis. Med. Microbiol.* **2018**, *2018*, 1–8. [CrossRef]

12. Singh, B.; Singh, J.P.; Kaur, A.; Singh, N. Bioactive compounds in banana and their associated health benefits—A review. *Food Chem.* **2016**, *206*, 1–11. [CrossRef]

13. Hayat, F.; Azam, A.; Shin, D. Recent progress on the discovery of antiamoebic agents. *Bioorganic Med. Chem. Lett.* **2016**, *26*, 5149–5159. [CrossRef] [PubMed]

14. Varghese, S.S.; Ghosh, S.K. Stress-responsive Entamoeba topoisomerase II: A potential antiamoebic target. *FEBS Lett.* **2020**, *594*, 1005–1020. [CrossRef] [PubMed]

15. Newman, D.J.; Cragg, G.M. Natural Products as Sources of New Drugs from 1981 to 2014. *J. Nat. Prod.* **2016**, *79*, 629–661. [CrossRef]

16. Leyva-López, N.; Nair, V.; Bang, W.Y.; Cisneros-Zevallos, L.; Heredia, J.B. Protective role of terpenes and polyphenols from three species of Oregano (Lippia graveolens, Lippia palmeri and Hedeoma patens) on the suppression of lipopolysaccharide-induced inflammation in RAW 264.7 macrophage cells. *J. Ethnopharmacol.* **2016**, *187*, 302–312. [CrossRef]

17. Ramirez-Moreno, E.; Soto-Sanchez, J.; Rivera, G.; Marchat, L.A. Mexican Medicinal Plants as an Alternative for the Development of New Compounds Against Protozoan Parasites. In *Natural Remedies in the Fight Against Parasites*; Hanem, K., Govindarajan, M., Benelli, G., Eds.; INTECH: London, UK, 2017; pp. 61–91. ISBN 978-953-51-3290-5.

18. Martínez-Rocha, A.; Puga, R.; Hernández-Sandoval, L.; Loarca-Piña, G.; Mendoza, S. Antioxidant and Antimutagenic Activities of Mexican Oregano (Lippia graveolens Kunth). *Plant Foods Hum. Nutr.* **2007**, *63*, 1–5. [CrossRef] [PubMed]

19. Arana-Sánchez, A.; Estarrón-Espinosa, M.; Obledo-Vázquez, E.; Padilla-Camberos, E.; Silva-Vázquez, R.; Lugo-Cervantes, E. Antimicrobial and antioxidant activities of Mexican oregano essential oils (Lippia graveolens H. B. K.) with different composition when microencapsulated inβ-cyclodextrin. *Lett. Appl. Microbiol.* **2010**, *50*, 585–590. [CrossRef] [PubMed]

20. Rivero-Cruz, I.; Duarte, G.; Navarrete, A.; Bye, R.; Linares, E.; Mata, R. Chemical composition and antimicrobial and spasmolytic properties of Poliomintha longiflora and Lippia graveolens essential oils. *J. Food Sci.* **2011**, *76*, C309–C317. [CrossRef]

21. Adame-Gallegos, J.; Ochoa, S.A.; Nevárez-Moorillón, G.V. Potential Use of Mexican Oregano Essential Oil against Parasite, Fungal and Bacterial Pathogens. *J. Essent. Oil Bear. Plants* **2016**, *19*, 553–567. [CrossRef]

22. García-Heredia, A.; Garcia, S.; Merino-Mascorro, J.Á.; Feng, P.; Heredia, N. Natural plant products inhibits growth and alters the swarming motility, biofilm formation, and expression of virulence genes in enteroaggregative and enterohemorrhagic Escherichia coli. *Food Microbiol.* **2016**, *59*, 124–132. [CrossRef]

23. Quintanilla-Licea, R.; Mata-Cárdenas, B.D.; Villarreal, J.V.; Bazaldúa-Rodríguez, A.F.; Kavimngeles-Hernández, I.; Garza-González, J.N.; Hernández-García, M.E.; Ángeles-Hernández, I.K. Antiprotozoal Activity against Entamoeba histolytica of Plants Used in Northeast Mexican Traditional Medicine. Bioactive Compounds from Lippia graveolens and Ruta chalepensis. *Molecules* **2014**, *19*, 21044–21065. [CrossRef]

24. Ching, A.Y.L.; Wah, T.S.; Sukari, M.A.; Lian, G.E.C.; Rahmani, M.; Khalid, K. Characterization of Flavonoid Derivatives From Boesenbergia Rotunda (L.). *Malays. J. Anal. Sci.* **2007**, *11*, 154–159.

25. Jerz, G.; Waibel, R.; Achenbach, H. Cyclohexanoid protoflavanones from the stem-bark and roots of Ongokea gore. *Phytochemistry* **2005**, *66*, 1698–1706. [CrossRef] [PubMed]

26. Suleimen, Y.; Raldugin, V.A.; Adekenov, S.M. Cirsimaritin from Stizolophus balsamita. *Chem. Nat. Compd.* **2008**, *44*, 398. [CrossRef]

27. Jain, R.; Mittal, M. Naringenin, a flavonone from the stem of Nyctanthes arbortristis Linn. *Int. J. Biol. Pharm. Allied Sci.* **2012**, *1*, 964–972.

28. Bertelli, D.; Papotti, G.; Bortolotti, L.; Marcazzan, G.L.; Plessi, M. 1H-NMR Simultaneous Identification of Health-Relevant Compounds in Propolis Extracts. *Phytochem. Anal.* **2011**, *23*, 260–266. [CrossRef]

29. Katerere, D.R.; Gray, A.I.; Nash, R.J.; Waigh, R.D. Phytochemical and antimicrobial investigations of stilbenoids and flavonoids isolated from three species of Combretaceae. *Fitoterapia* **2012**, *83*, 932–940. [CrossRef]

30. Ramírez, J.; Cartuche, L.; Morocho, V.; Aguilar, S.; Malagón, O. Antifungal activity of raw extract and flavanons isolated from Piper ecuadorense from Ecuador. *Rev. Bras. Farm.* **2013**, *23*, 370–373. [CrossRef]

31. Guzmán-Avendaño, A.J.; Torrenegra-Guerrero, R.D.; Rodríguez-Aguirre, O.E. Flavonoids from Chromolaena subscandens (Hieron.) R.M. King & H. Rob. *Rev. Prod. Nat.* **2008**, *2*, 1–5.

32. McNulty, J.; Nair, J.J.; Bollareddy, E.; Keskar, K.; Thorat, A.; Crankshaw, D.; Holloway, A.C.; Khan, G.; Wright, G.D.; Ejim, L. Isolation of flavonoids from the heartwood and resin of Prunus avium and some preliminary biological investigations. *Phytochemistry* **2009**, *70*, 2040–2046. [CrossRef]

33. Grecco, S.D.S.; Dorigueto, A.C.; Landre, I.M.; Soares, M.G.; Martho, K.; Lima, R.; Pascon, R.C.; Vallim, M.A.; Capello, T.M.; Romoff, P.; et al. Structural Crystalline Characterization of Sakuranetin—An Antimicrobial Flavanone from Twigs of Baccharis retusa (Asteraceae). *Molecules* **2014**, *19*, 7528–7542. [CrossRef]

34. Yamashita, Y.; Hanaya, K.; Shoji, M.; Sugai, T. Simple Synthesis of Sakuranetin and Selinone via a Common Intermediate, Utilizing Complementary Regioselectivity in the Deacetylation of Naringenin Triacetate. *Chem. Pharm. Bull.* **2016**, *64*, 961–965. [CrossRef]

35. Alwahsh, M.A.A.; Khairuddean, M.; Chong, W.K. Chemical constituents and antioxidant activity of Teucrium barbeyanum Aschers. *Rec. Nat. Prod.* **2015**, *9*, 159–163.

36. Exarchou, V.; Kanetis, L.; Charalambous, Z.; Apers, S.; Pieters, L.; Gekas, V.; Goulas, V. HPLC-SPE-NMR Characterization of Major Metabolites in Salvia fruticosa Mill. Extract with Antifungal Potential: Relevance of Carnosic Acid, Carnosol, and Hispidulin. *J. Agric. Food Chem.* **2015**, *63*, 457–463. [CrossRef] [PubMed]

37. Lee, S.-J.; Jang, H.-J.; Kim, Y.; Oh, H.-M.; Lee, S.; Jung, K.; Kim, Y.-H.; Lee, W.-S.; Lee, S.W.; Rho, M.-C. Inhibitory effects of IL-6-induced STAT3 activation of bio-active compounds derived from Salvia plebeia R.Br. *Process. Biochem.* **2016**, *51*, 2222–2229. [CrossRef]

38. Gaggeri, R.; Rossi, D.; Christodoulou, M.S.; Passarella, D.; Leoni, F.; Azzolina, O.; Collina, S. Chiral Flavanones from Amygdalus lycioides Spach: Structural Elucidation and Identification of TNFalpha Inhibitors by Bioactivity-guided Fractionation. *Molecules* **2012**, *17*, 1665–1674. [CrossRef]

39. Grecco, S.D.S.; Reimão, J.Q.; Tempone, A.G.; Sartorelli, P.; Cunha, R.L.O.R.; Romoff, P.; Ferreira, M.J.P.; Fávero, O.A.; Lago, J. In vitro antileishmanial and antitrypanosomal activities of flavanones from Baccharis retusa DC. (Asteraceae). *Exp. Parasitol.* **2012**, *130*, 141–145. [CrossRef]

40. Kyriakou, E.; Primikyri, A.; Charisiadis, P.; Katsoura, M.; Stamatis, H.; Gerothanassis, I.P.; Tzakos, A. Unexpected enzyme-catalyzed regioselective acylation of flavonoid aglycones and rapid product screening. *Org. Biomol. Chem.* **2012**, *10*, 1739. [CrossRef]

41. Ashour, M.A.-G. New diacyl flavonoid derivatives from the Egyptian plant Blepharis edulis (Forssk.) Pers. *Bull. Fac. Pharm. Cairo Univ.* **2015**, *53*, 11–17. [CrossRef]

42. Joseph-Nathan, P.; Gordillo-Román, B. Vibrational Circular Dichroism Absolute Configuration Determination of Natural Products. In *Progress in the Chemistry of Organic Natural Products*; Kinghorn, A.D., Falk, H., Kobayashi, J., Eds.; Springer International Publishing Switzerland: Cham, Switzerland, 2015; Volume 100, pp. 311–452.

43. Silverstein, R.M.; Bassler, G.C. Spectrometric identification of organic compounds. *J. Chem. Educ.* **1962**, *39*, 546. [CrossRef]

44. Grotewold, E. *The Science of Flavonoids*; Springer Science+Business Media, Inc.: New York, NY, USA, 2006; ISBN 9780387288222.

45. Pinheiro, P.F.; Justino, G.C. Structural analysis of flavonoids and related compounds—A review of spectroscopic applications. In *Phytochemicals–A Global Perspective of Their Role in Nutrition and Health*; Rao, V., Ed.; InTech Europe: Rijeka, Croatia, 2012; pp. 33–56. ISBN 978-953-51-0296-0.

46. Ren, W.; Qiao, Z.; Wang, H.; Zhu, L.; Zhang, L. Flavonoids: Promising anticancer agents. *Med. Res. Rev.* **2003**, *23*, 519–534. [CrossRef]

47. Cai, Y.; Luo, Q.; Sun, M.; Corke, H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci.* **2004**, *74*, 2157–2184. [CrossRef] [PubMed]

48. Cushnie, T.T.; Lamb, A.J. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents* **2005**, *26*, 343–356. [CrossRef] [PubMed]

49. Tasdemir, D.; Kaiser, M.; Brun, R.; Yardley, V.; Schmidt, T.J.; Tosun, F.; Ruedi, P. Antitrypanosomal and Antileishmanial Activities of Flavonoids and Their Analogues: In Vitro, In Vivo, Structure-Activity Relationship, and Quantitative Structure-Activity Relationship Studies. *Antimicrob. Agents Chemother.* **2006**, *50*, 1352–1364. [CrossRef] [PubMed]

50. Plochmann, K.; Korte, G.; Koutsilieri, E.; Richling, E.; Riederer, P.; Rethwilm, A.; Schreier, P.; Scheller, C. Structure–activity relationships of flavonoid-induced cytotoxicity on human leukemia cells. *Arch. Biochem. Biophys.* **2007**, *460*, 1–9. [CrossRef] [PubMed]

51. Delgado, L.; Fernandes, I.; González-Manzano, S.; De Freitas, V.; Mateus, N.; Santos-Buelga, C. Anti-proliferative effects of quercetin and catechin metabolites. *Food Funct.* **2014**, *5*, 797. [CrossRef] [PubMed]

52. Resende, F.A.; Almeida, C.P.D.S.; Vilegas, W.; Varanda, E.A. Differences in the hydroxylation pattern of flavonoids alter their chemoprotective effect against direct- and indirect-acting mutagens. *Food Chem.* **2014**, *155*, 251–255. [CrossRef]

53. Martínez-Castillo, M.; Pacheco-Yépez, J.; Flores, N.; Guzman, P.G.; Jarillo-Luna, R.A.; Cárdenas-Jaramillo, L.M.; Campos-Rodríguez, R.; Shibayama, M. Flavonoids as a Natural Treatment Against Entamoeba histolytica. *Front. Microbiol.* **2018**, *8*, 1–14. [CrossRef]

54. Vaya, J.; Tamir, S. The relation between the chemical structure of flavonoids and their estrogen-like activities. *Curr. Med. Chem.* **2004**, *11*, 1333–1343. [CrossRef]

55. Agrawal, A.D. Pharmacological activities of flavonoids: A review. *Int. J. Pharm. Sci. Nanotechnol.* **2011**, *4*, 1394–1398.

56. Beekmann, K.; Actis-Goretta, L.; Van Bladeren, P.J.; Dionisi, F.; Destaillats, F.; Rietjens, I.M.C.M. A state-of-the-art overview of the effect of metabolic conjugation on the biological activity of flavonoids. *Food Funct.* **2012**, *3*, 1008. [CrossRef]

57. Kumar, S.; Pandey, A.K. Chemistry and Biological Activities of Flavonoids: An Overview. *Sci. World J.* **2013**, *2013*, 1–16. [CrossRef] [PubMed]

58. Lin, L.-Z.; Mukhopadhyay, S.; Robbins, R.J.; Harnly, J.M. Identification and quantification of flavonoids of Mexican oregano (Lippia graveolens) by LC-DAD-ESI/MS analysis. *J. Food Compos. Anal.* **2007**, *20*, 361–369. [CrossRef] [PubMed]

59. Dominguez, S.; Sánchez, H.; Suárez, M.; Baldas, J.; González, M.D.R. Chemical Constituents of Lippia graveolens. *Planta Med.* **1989**, *55*, 208–209. [CrossRef]

60. González-Güereca, M.C.; Soto-Hernández, M.; Kite, G.; Martínez-VÁzquez, M. Antioxidant activity of flavonoids from the stem of the Mexican oregano (Lippia graveolens HBK var. berlandieri Schauer). *Rev. Fitotec. Mex.* **2007**, *30*, 43–49.

61. Rufino-González, Y.; Ponce-Macotela, M.; González-Maciel, A.; Reynoso-Robles, R.; Jiménez-Estrada, M.; Sanchez-Contreras, A.; Martínez-Gordillo, M.N. In vitro activity of the F-6 fraction of oregano against Giardia intestinalis. *Parasitology* **2012**, *139*, 434–440. [CrossRef] [PubMed]

62. Moreno-Rodríguez, A.; Vázquez-Medrano, J.; Hernández-Portilla, L.B.; Peñalosa-Castro, I.; Canales-Martínez, M.; Orozco-Segovia, A.; Jiménez-Estrada, M.; Colville, L.; Pritchard, H.W.; Flores-Ortiz, C.M. The effect of light and soil moisture on the accumulation of three flavonoids in the leaves of Mexican oregano (Lippia graveolens Kunth). *J. Food, Agric. Environ.* **2014**, *12*, 1272–1279.

63. Grael, C.; De Albuquerque, S.; Lopes, J.L. Chemical constituents of Lychnophora pohlii and trypanocidal activity of crude plant extracts and of isolated compounds. *Fitoterapia* **2005**, *76*, 73–82. [CrossRef]

64. Alday-Provencio, S.; Diaz, G.; Rascon, L.; Quintero, J.; Alday, E.; Robles-Zepeda, R.; Garibay-Escobar, A.; Astiazaran, H.; Hernández, J.; Velázquez, C. Sonoran Propolis and Some of its Chemical Constituents Inhibit in vitro Growth of Giardia lamblia Trophozoites. *Planta Med.* **2015**, *81*, 742–747. [CrossRef]

65. Calzada, F.; Meckes, M.; Cedillo-Rivera, R. Antiamoebic and Antigiardial Activity of Plant Flavonoids. *Planta Med.* **1999**, *65*, 78–80. [CrossRef]

66. Tasdemir, D.; Lack, G.; Brun, R.; Rüedi, P.; Scapozza, L.; Perozzo, R. Inhibition of Plasmodium falciparum fatty acid biosynthesis: Evaluation of FabG, FabZ, and FabI as drug targets for flavonoids. *J. Med. Chem.* **2006**, *49*, 3345–3353. [CrossRef]

67. Calzada, F.; Velazquez, C.; Cedillo-Rivera, R.; Esquivel, B. Antiprotozoal activity of the constituents of Teloxys graveolens. *Phytother. Res.* **2003**, *17*, 731–732. [CrossRef] [PubMed]

68. Bolaños, V.; Díaz-Martínez, A.; Soto, J.; Marchat, L.A.; Sánchez-Monroy, V.; Ramírez-Moreno, E. Kaempferol inhibits Entamoeba histolytica growth by altering cytoskeletal functions. *Mol. Biochem. Parasitol.* **2015**, *204*, 16–25. [CrossRef] [PubMed]

69. Fatima, S.; Gupta, P.; Agarwal, S.M. Insight into structural requirements of antiamoebic flavonoids: 3D-QSAR and G-QSAR studies. *Chem. Boil. Drug Des.* **2018**, *92*, 1743–1749. [CrossRef] [PubMed]

70. Soto, J.; Gómez, C.; Calzada, F.; Ramírez, M.E. Ultrastructural Changes on Entamoeba histolytica HM1-IMSS Caused by the Flavan-3-Ol, (-)-Epicatechin. *Planta Med.* **2009**, *76*, 611–612. [CrossRef] [PubMed]

71. Bolaños, V.; Díaz-Martínez, A.; Soto, J.; Rodríguez, M.A.; López-Camarillo, C.; Marchat, L.A.; Ramírez-Moreno, E. The flavonoid (-)-epicatechin affects cytoskeleton proteins and functions in Entamoeba histolytica. *J. Proteom.* **2014**, *111*, 74–85. [CrossRef]

72. Yi, W.; Wei, Q.; Di, G.; Jing-Yu, L.; Luo, Y.-L. Phenols and flavonoids from the aerial part of Euphorbia hirta. *Chin. J. Nat. Med.* **2012**, *10*, 40–42.

73. Vasconcelos, J.M.; Silva, A.M.S.; Cavaleiro, J.A.S. Chromones and flavanones from artemisia campestris subsp. maritima. *Phytochemistry* **1998**, *49*, 1421–1424. [CrossRef]

74. Oyama, K.-I.; Kondo, T. Total Synthesis of Flavocommelin, a Component of the Blue Supramolecular Pigment from Commelinacommunis, on the Basis of Direct 6-C-Glycosylation of Flavan. *J. Org. Chem.* **2004**, *69*, 5240–5246. [CrossRef]

75. Isobe, T.; Doe, M.; Morimoto, Y.; Nagata, K.; Ohsaki, A. The anti-Helicobacter pylori flavones in a Brazilian plant, Hyptis fasciculata, and the activity of methoxyflavones. *Boil. Pharm. Bull.* **2006**, *29*, 1039–1041. [CrossRef]

76. Mata-Cárdenas, B.D.; Vargas-Villarreal, J.; González-Salazar, F.; Palacios-Corona, R.; Salvador, S.-F. A new vial microassay to screen antiprotozoal drugs. *Pharmacologyonline* **2008**, *1*, 529–537.

77. González-Salazar, F.; Mata-Cárdenas, B.D.; Vargas-Villarreal, J. Sensibilidad de trofozoitos de Entamoeba histolytica a Ivermectina. *Medicina (Buenos Aires)* **2009**, *69*, 318–320.

78. Hirudkar, J.R.; Parmar, K.M.; Prasad, R.S.; Sinha, S.K.; Jogi, M.S.; Itankar, P.R.; Prasad, S.K. Quercetin a major biomarker of Psidium guajava L. inhibits SepA protease activity of Shigella flexneri in treatment of infectious diarrhoea. *Microb. Pathog.* **2019**, *138*, 103807. [CrossRef] [PubMed]

Sample Availability: Not available.