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Microwave-Assisted Semisynthesis and Leishmanicidal Activity of Some Phenolic Constituents from Lichens

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Abstract: Leishmaniasis is considered one of the most untreated tropical diseases in the world. In this study, we investigated the in vitro leishmanicidal activity and cytotoxicity of various isolated lichen substances, including atranorin (**1**), usnic acid (**2**), gyrophoric acid (**3**), salazinic acid (**4**), galbinic acid (**5**), and parietin (**6**), and some semi-synthetic imine derivatives of usnic acid (**7**, **8**, **9**) and atranorin (**10**, **11**, **12**, **13**). Imine condensation reactions with hydrazine and several amines were assisted by microwave heating, an efficient and eco-friendly energy source. The most interesting result was obtained for compound **2**, which has high leishmanicidal activity but also high cytotoxicity. This cytotoxicity was mitigated in its derivative, **9**, with better selectivity and high antileishmanic activity. This result may indicate that the usnic acid derivative (**9**) obtained using condensation with two cyclohexylamine groups is a promising lead compound for the discovery of new semisynthetic antiparasitic drugs.

Keywords: atranorin derivatives; cytotoxicity; green chemistry; imine; leishmania; pyrazoline; usnic acid derivatives

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1. Introduction

Leishmaniasis is a group of diseases caused by protozoan parasites. More than 12 million people suffer from this disease, which is considered endemic in 88 countries around the world. Leishmaniasis is generally zoonotic in origin, including over 20 human pathogenic parasite species. Leishmaniasis is an intracellular infection, with different parasite species associated with various clinical forms of this disease. Many species cause skin ulcers and nodules leading to cutaneous leishmaniasis, which may be local or diffuse. Some species of these organisms can also affect the mucous membranes and cause lesions that disfigure the nose and cause mucocutaneous leishmaniasis. Other species damage internal organs and cause visceral leishmaniasis, which is fatal if not treated in time [1]. Leishmaniasis is considered by the WHO to be both a highly relevant endemic infectious

disease and a public health threat. The pentavalent antimony preparations commonly used to treat this disease are limited. Therefore, new natural products could serve as alternatives to existing treatments [2] or could pave the way for the discovery of new leishmanicidal agents [3].

On the other hand, microwave-assisted organic synthesis facilitates more rapid reaction times and screening for chemical substances in order to discover potential new drugs with desirable properties [4]. Additionally, this technique is regarded as an eco-friendly source of energy in chemical synthesis, with better yields and higher purities when compared with traditional processes [5]. This approach has been employed to obtain new antileishmanial compounds such as natural-product-inspired functionalized spiro[indoline-3,2′-pyrrolidin]-2-one/spiro[indoline-3,3′-pyrrolizin]-2-one derivatives [6], γ-butyrolactones derivatives [7], methylene isoindolinone compounds [8], and 5-substituted benzylidene amino-2-butyl benzofuran-3-yl-4-methoxyphenyl methanones [9]. Keeping this in mind, we focused our interest on the condensation of amines with ketones to form imines, a reaction that has proven to be feasible under microwave conditions with promising results [10].

Taking this into account, our research has focused on natural products isolated from lichens. The classical definition states that lichens are organisms that arise from symbiosis between a fungus (mycobiont) and microscopic algae or cyanobacteria (photobiont). This symbiosis leads to a stable taxonomic unit with a long evolutionary history [11,12] that biosynthesizes some characteristic compounds called "lichen substances", such as depsides, depsidones, dibenzofurans, anthraquinones, and pulvinic acid derivatives [13,14]. These compounds have a wide range of biological activities, such as antioxidant, anti-inflammatory, antiviral, analgesic, cytotoxic, antibiotic, antitumor, anti-HIV, antifungal, and antiproliferative properties [12,15]. This study aims to isolate lichen compounds, obtain new semisynthetic imine derivatives, and evaluate their leishmanicidal activity and cytotoxicity in vitro.

2. Materials and Methods

2.1. General

The reagents used for the derivatizations were aniline, 99.5%; phenylhydrazine, 97.0%; cyclohexylamine 95%; and naphthylamine, 99.0% (Sigma-Aldrich, St. Louis, MA, USA); absolute ethanol, 99.7% (Winkler, Lampa, Santiago, Chile); and chloroform, 99.99% (JT Baker, Puebla, Mexico). The deuterated solvents were purchased from Sigma-Aldrich (St. Louis, MA, USA). All NMR spectra were recorded using a spectrometer, the Bruker DRX 300 (Sao Paulo, Brazil, 300 MHz (^{1}H) and 75 MHz (^{13}C)), using TMS as the internal standard. MestReNova (Mestrelab Research, Santiago de Compostela, Coruña, Spain) was used to process the NMR spectra. Chemical shift values were referenced to the residual solvent signals for CDCl3 (δH/δC, 7.26/77.16) and DMSO (δH/δC, 2.50/39.51). Reactions requiring heating were performed using a microwave device (Monowave 450, Anton Paar, Buchs, Switzerland) with adjustable operating parameters. Finally, the melting point of the derivatives was determined with a fusiometer (Fisatom 430D, Sao Paulo, Brazil).

2.1.1. HPLC Parameters

A Thermo Scientific Dionex Ultimate 3000 UHPLC system equipped with a quaternary Series RS pump, and a PDA detector controlled by the Chromeleon 7.2 software (Thermo Fisher Scientific, Bremen, Germany), hyphenated, was used for analysis. A UHPLC C18 column (Acclaim, 150 mm × 4.6 mm ID, 5 µm, Thermo Fisher Scientific, Bremen, Germany) operated at 25 °C was employed. The PDA value was recorded from 200 to 800 nm, and the mobile phases were 0.1% formic aqueous solution (A) and acetonitrile (B). The gradient program (time (min), % B) was (0.00, 5); (5.00, 5); (10.00, 30); (15.00, 30); (20.00, 70); (25.00, 70); (35.00, 5). The flow rate was 1.00 mL min^{-1}, and the injection volume was 10 µL. Samples (2 mg) were dissolved in methanol (10 mL) for the evaluation of purity.

2.1.2. MS Parameters

A Thermo Q Exactive focus orbitrap mass spectrometer with an electrospray ionization (ESI) source (Thermo, Bremen, Germany) was used for analysis. Sheath gas flow rate (N_2), 75 units; aux. gas unit flow rate (N_2), 20; capillary temperature, 400 °C; aux gas heater temperature, 500 °C; spray voltage, 2500 V; and S-lens RF level, 30. Full positive scan data were acquired at a resolving power of 70,000 FWHM (full width at half maximum) at m/z 200. For the compounds of interest, a scan range of m/z 100–1000 was chosen; the automatic gain control (AGC) was set at 3×10^6 and the injection time was set to 200 ms. The scan rate was set at 2 scans/s^{-1}. External calibration was performed using a calibration solution in positive and negative modes. For confirmation purposes, a targeted MS/MS analysis was performed using the mass inclusion list, with a 30 s time window, with an Orbitrap spectrometer operating both in positive and negative mode at 17,500 FWHM (m/z 200). The AGC target was set to 2×10^5, with a maximum injection time of 20 ms. The precursor ions were filtered by a quadrupole, which operated at an isolation window of m/z 2. The fore vacuum, high vacuum, and ultrahigh vacuum were maintained at approximately 2 mbar, from 10^5 and below 10^{10} mbar. Collision energy (HCD cell) was operated at 30 kV. The mass tolerance window was set to 5 ppm for the two modes. Total analysis runtime was 1.0 min via direct injection.

2.2. Lichen Material

Lichen species were collected from different locations in Bolivia (May 2018) and Chile (September 2018). These places were chosen since there is a large population of these lichens. Sampling locations are listed in Table 1.

Table 1. Lichen species from Bolivia and Chile.

Lichen	Providence (Site)	Jurisdiction
Usnea patagonica *	Sud Yungas (Pongo)	La Paz
Stereocaulon ramulosum *	Sud Yungas (Unduavi)	La Paz
Teloschistes chrysophthalmus *	Murillo (Cota-Cota)	La Paz
Xanthoparmelia lineola *	Omasuyos (Apuvillque)	La Paz
Usnea Cornuta ‡	Chillan	VIII region
Parmotrema paramoreliensis ‡	Longavi	VII region

* The lichens were identified at the Herbario Nacional de Bolivia at Universidad Mayor de San Andrés (UMSA), La Paz, Bolivia. ‡ The lichens were identified at the Biology Department of the Universidad Metropolitána de Ciéncias de la Educación (UMCE), Santiago, Chile.

2.3. Isolation of Lichen Substances

Atranorin (**1**): This compound was isolated from *S. ramulosum*, as described in the literature [16]. In total, 100 g of dry lichen was extracted with EtOH (1.0 L) via maceration (48 h). The ethanolic extract (11.0 g) was eluted with a DCM:MeOH mixture (1:0; 9.5:0.5; 9:1; 8:2; 7:3; 6:4; 1:1; 0:1) to yield compound **1** (2.81 g): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (ppm); 2.71 (3H, *s*, CH_3-9), 2.11(3H, *s*, CH_3-8′), 2.56 (3H, *s*, CH_3-9′), 4.00 (3H, *s*, CH_3-O), 6.39 (1H, *s*, H-5), 10.37 (1H, *s*, H-8), 6.54 (1H, *s*, H-6′), 12.52 (1H, *s*, OH-2), 12.57 (1H, *s*, OH-4), 11.95 (1H, *s*, OH-3′). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ (ppm); 102.2 (C-1), 169.1 (C-2), 108.5 (C-3), 167.5 (C-4), 112.8 (C-5), 152.4 (C-6), 169.7 (C-7), 193.0 (C-8), 25.6 (C-9), 152.4 (C-1′), 116.8 (C-2′), 162.9 (C-3′), 110.2 (C-4′), 139.9 (C-5′), 116.0 (C-6′), 172.2 (C-7′), 9.4 (C-8′), 24.0 (C-9′), 52.3 (CH_3O-). This was confirmed via comparison with data from the literature [16,17].

Usnic acid (**2**): This natural product was isolated from *X. lineola*. Dry lichenic material (133.5 g) was extracted with EtOAc (0.9 L) for 48 h at room temperature; then, 80% of the solvent was removed under vacuum. The remaining 20% was left at room temperature to obtain a brown precipitate, which was filtered and washed with acetone followed by EtOH to yield compound **2** (3.01 g).

This natural product was also isolated from *U. patagónica*. Dry lichenic material (61.0 g) was extracted with acetone (1.0 L) for 72 h at room temperature; the extract was filtered;

and the solvent was removed under vacuum. The obtained dry extract was then extracted with chloroform to collect yellow crystals corresponding to compound **2** (4.80 g): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (ppm); 1.78 (3H, *s*, CH_3-15), 2.12 (3H, *s*, CH_3-10), 2.68(3H, *s*, CH_3-12), 2.69(3H, *s*, CH_3-14), 6.00(1H, *s*, H-4), 11.04 (1H, *s*, OH-9),13.33 (1H, *s*, OH-7). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ (ppm); 198.05 (C-1), 105.23 (C-2), 191.71 (C-3), 98.32 (C-4), 179.36 (C-4a), 155.20 (C-5a), 101.52 (C-6), 163.88 (C-7), 109.32 (C-8), 157.50 (C-9), 103.95 (C-9a), 59.07 (C-9b), 7.52 (C-10), 201.76 (C-11), 27.87 (C-12), 200.31 (C-13), 31.25 (C-14), 32.11 (C-15). This was confirmed via comparison with data from the literature [18].

Gyrophoric acid (**3**): This natural product was isolated from *U. patagónica*. Dry lichenic material (61.0 g) was extracted with acetone (1.0 L) for 72 h at room temperature; the extract was filtered; and the solvent was removed under vacuum. The dry extract obtained was then extracted with chloroform, and the remaining white amorphous solid was recrystallized in MeOH to yield compound **3** (3.50 g): ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm); 2.50 (3H, *s*, CH_3-8), 2.51 (3H, *s*, CH_3-8′), 2.52 (3H, *s*, CH_3-8″), 6.36 (1H, *d*, *J* = 2.1 Hz, H-3), 6.38 (1H, *d*, *J* = 2.1 Hz, H-5), 6.72 (1H, *d*, *J* = 1.7 Hz, H-3′), 6.74 (1H, *d*, *J* = 1.7 Hz, H-5′), 6.76 (1H, *d*, *J* = 2.0 Hz, H-3″), 6.78 (1H, *d*, *J* = 2.0 Hz, H-5″). ^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm); 109.3 (C-1), 160.9 (C-2), 101.4 (C-3), 162.0 (C-4), 110.7 (C-5), 141.2 (C-6), 168.0 (C-7), 21.2 (C-8), 118.9 (C-1′), 157.2 (C-2′), 118.0 (C-3′), 153.0 (C-4′), 115.3 (C-5′), 138.9 (C-6′), 166.6 (C-7′), 21.2 (C-8′), 118.2 (C-1″), 157.2 (C-2″), 108.0 (C-3″), 153.0 (C-4″), 115.1 (C-5″), 140.4 (C-6″), 171.4 (C-7″), 21.7 (C-8″). This was confirmed via comparison with data from the literature [17,19].

Salazinic acid (**4**): This natural product was isolated from *P. paramoreliensis*. Dry lichenic material (100.1 g) was extracted with EtOH (1.2 L) for 48 h at room temperature; the extract was filtered; and 85% of the solvent was removed under vacuum. The remaining 15% was left at room temperature to obtain a precipitate, which was filtered and washed with warm EtOH to yield compound **4** (2.60 g): ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm); 2.46 (3H, *s*, CH_3-8), 4.66 (2H, *s*, CH_2-9′), 10.46 (1H, s, H-9), 6.89 (1H, *s*, H-5), 6.81 (1H, *s*, H-8′), 8.31 (1H, *s*, OH-8′), 12.06 (1H, *s*, OH-2′). ^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm); 112.43 (C-1), 164.03 (C-2), 111.14 (C-3), 164.47 (C-4), 117.89 (C-5), 152.74 (C-6), 160.76 (C-7), 21.88 (C-8), 193.20 (C-9), 110.12 (C-1′), 153.27 (C-2′), 123.92 (C-3′), 148.62 (C-4′), 138.57 (C-5′), 137.78 (C-6′), 166.35 (C-7′), 95.33 (C-8′), 53.16 (C-9′). This was confirmed via comparison with data from the literature [20,21].

Galbinic acid (**5**): This compound was isolated from *U. cornuta* [22]. From 50 g of dry lichen was macerated with methanol (1.2 L) for 24 h. The crude extract was submitted to flash chromatography and then CC using DCM/MeOH mixtures to yield compound **5** (400 mg): ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm); 2.47 (3H, *s*, CH_3-8), 5.18 (2H, *s*, CH_2-9′), 10.45(1H, *s*, H-9), 6.89 (1H, *s*, H-5), 6.82 (1H, *s*, H-8′), 8.34 (1H, *s*, OH-8′), 12.06 (1H, *s*, OH-2′), 1.99 (3H, *s*, CH3-O) [9]. ^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm); 112.09 (C-1), 164.21 (C-2), 111.71 (C-3), 164.54 (C-4), 117.98 (C-5), 152.77 (C-6), 160.52 (C-7), 21.76 (C-8), 193.10 (C-9), 110.19 (C-1′), 156.73 (C-2′), 119.02 (C-3′), 149.34 (C-4′), 138.10 (C-5′), 136.55 (C-6′), 166.09 (C-7′), 95.68 (C-8′), 55.90 (C-9′), 170.04, 21.04 (Acetyl). This was confirmed via comparison with data from the literature [22].

Parietin (**6**): This natural product was isolated from *T. chrysophthalmus*. Dry lichenic material (39.9 g) was extracted with acetone (0.7 L) for 30 h at room temperature; then, 80% of the solvent was removed under vacuum. The remaining 20% was cooled to 0 °C in an ice bath, yielding a precipitate that was filtered and washed with warm EtOAc to obtain compound **6** (1.05 g): ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm); 2.44 (3H, *s*, CH_3-15), 3.93 (3H, *s*, CH_3-16), 6.69 (1H, *d*, *J* = 2.48, H-10), 7.07 (1H, *s*, H-2), 7.34 (1H, *d*, *J* = 2.52, H-8) 7.61 (1H, *s*, H-5), 12.11 (1H, *s*, OH-1), 12.30 (1H, *s*, H-10). ^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm); 162.48 (C-1), 124.49 (C-2), 148.45, (C-3),121.27, (C-4), 133.19 (C-5), 182.01 (C-6), 135.23 (C-7), 108.23 (C-8), 166.54 (C-9), 106.73 (C-10), 165.18 (C-11), 110.22 (C-12), 190.78, (C-13), 133.65 (C-14), 22.15 (C-15), 56.08 (C-16). This was confirmed via comparison with data from the literature [23,24].

2.4. Semi-Synthesis of Derivatives

(*S*)-*5-hydroxy-1,4,5b,7-tetramethyl-3,9-diphenyl-5b,9-dihydrofuro* [*2,3-e*:*4,5-f*′]*bis*(*indazole*)-*6*(*3H*)-*one* (**7**): Compound **2** (502 mg, 1.46 mmol) was dissolved in an Erlenmeyer flask with EtOH (96 °GL, 20 mL) and then phenylhydrazine (0.5 mL, 5.08 mmol) was added. The mixture was irradiated with microwaves (170 W) for 15 min at intervals of 20 s; during the rest periods, the Erlenmeyer flask was cooled at 0 °C in an ice bath. After the completion of the reaction, the mixture was left in the ice bath at 0 °C for 1 h to precipitate the crude product. The solid was filtered and washed with EtOH, yielding the following: 511 mg (1.05 mmol, 71.9%), mp 285 °C, ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (ppm); 1.82 (3H, *s*, CH_3-15), 2.21 (3H, *s*, CH_3-10), 2.61 (3H, *s*, CH_3-12), 2.46 (3H, *s*, CH_3-14), 6.18 (1H, *s*, H-4), 11.61 (1H, *s*, OH-9), 7.59–6.94 (10H, H- [a, b, c, d, a′, b′, c′ d′]). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ (ppm); 196.43 (C-1), 108.29 (C-2), 148.59 (C-3), 88.15 (C-4), 173.75 (C-4a), 143.98 (C-5a), 100.15 (C-6), 1652.38 (C-7), 108.29 (C-8), 158.07 (C-9), 103.65 (C-9a), 60.52 (C-9b), 8.20 (C-10), 151.40 (C-11), 13.34 (C-12), 145.65 (C-13), 15.16 (C-14), 30.58 (C-15), 138.09 (C-a), 123.96 (C-b), 129.43 (C-c), 128.58 (C-d), 153.23 (C-a′), 113.02 (C-b′), 129.65 (C-b′), 120.94 (C-d′). ESI-MS: 489.19 ([M + H]$^+$, *m*/*z*); (calcd. for $C_{30}H_{25}N_4O_3^+$, 489.19).

(*S*,*E*)-*6-acetyl-3,7,9-trihydroxy-8,9b-dimethyl-2-*(*1-*(*phenylimino*)*ethyl*)*dibenzo*[*b,d*] *furan-1*(*9bH*)-*one* (**8**): A mixture of compound **2** (877 mg, 2.55 mmol), aniline (0.5 mL, 5.48 mmol), and EtOH (10 mL) was irradiated with microwaves (170 W) at 30 s intervals for 12 min in an Erlenmeyer flask, and the mixture was cooled at 0 °C in an ice bath between each heating interval. At the end of the reaction time, the mixture was cooled in an ice bath at 0 °C for 60 min. After this time, a yellow amorphous solid formed, which was recovered via filtration and washed with EtOH:H_2O 1:1, yielding: 863 mg of product **8** (2.06 mmol, 80.8%), mp 226 °C, ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (ppm); 1.77 (3H, *s*, CH_3-15), 2.12 (3H, *s*, CH_3-10), 2.59 (3H, *s*, CH_3-12), 2.71 (3H, *s*, CH_3-14), 5.89 (1H, *s*, H-4), 11.88 (1H, *s*, OH-9), 13.39 (1H, *s*,OH-7), 15.08 (1H, *s*,OH-3), 7.73–7.21 (5H, H-[a, b, c, d]). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ (ppm); 198.84 (C-1), 102.65 (C-2), 191.03 (C-3), 102.26 (C-4), 174.78 (C-4a), 155.80 (C-5a), 101.39 (C-6), 163.56 (C-7), 108.18 (C-8), 158.22 (C-9), 104.96 (C-9a), 57.55 (C-9b), 7.52 (C-10), 173.98 (C-11), 20.65 (C-12), 200.70 (C-13), 31.35 (C-14), 31.92 (C-15), 136.06 (C-a), 125.75 (C-b), 129.70 (C-c), 128.28 (C-d). ESI-MS: 420.13 ([M + H]$^+$, *m*/*z*); (calcd. for $C_{24}H_{22}NO_6^+$, 420.14).

(*S*)-*2-*((*E*)-*1-*(*cyclohexylimino*)*ethyl*)-*6-*(*1-*(*cyclohexylimino*)*ethyl*)-*3,7,9-trihydroxy-8,9b-dimethyldibenzo*[*b,d*]*furan-1*(*9bH*)-*one* (**9**): A mixture of compound **2** (802 mg, 2.33 mmol), cyclohexylamine (0.6 mL, 5.23 mmol), and EtOH (25 mL) was irradiated with microwaves (170 W) in an Erlenmeyer flask for 15 min at intervals of 20 s, and the mixture was cooled to 0 °C in an ice bath between each heating interval. At the end of the reaction time, the mixture was cooled for 2 h at 0 °C in an ice bath. After this time, a yellow amorphous solid formed, which was recovered via filtration and washed with EtOH, yielding: 1042 mg of product **9** (2.06 mmol, 88.4%), mp 235 °C, ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (ppm); 1.69 (3H, *s*, CH_3-15), 2.13 (3H, *s*, CH_3-10), 2.57 (3H, *s*, CH_3-12), 2.64 (3H, *s*, CH_3-14), 5.72 (1H, *s*, H-4), 11.50 (1H, *s*, OH-9), 13.57 (1H, *s*, OH-7),3.75, 3.72 (2H, CH=N), 1.95-1.25 (20H, H- [a, b, c, a′, b′, c′]). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ (ppm); 198.81 (C-1), 102.02 (C-2), 190.83 (C-3), 101.32 (C-4), 168.66 (C-4a), 154.69 (C-5a), 98.97 (C-6), 163.71 (C-7), 108.91 (C-8), 155.45 (C-9), 103.50 (C-9a), 54.30 (C-9b), 8.12 (C-10), 171.50 (C-11), 17.59 (C-12), 167.06 (C-13), 18.33 (C-14), 32.72 (C-15), 54.30 (C-a), 33.29 (C-b), 25.35 (C-c), 24.47 (C-d), 52.41 (C-a′), 32.72 (C-b′), 25.08 (C-b′), 24.03 (C-d′). ESI-MS *m*/*z*: 507.28 ([M + H]$^+$, *m*/*z*); (calcd. for $C_{30}H_{39}N_2O_5^+$, 507.29).

3-hydroxy-4-(*methoxycarbonyl*)*-2,5-dimethylphenyl 4-hydroxy-6-methyl-1-phenyl-1H-indazole-7-carboxylate* (**10**): Compound **1** (1080 mg, 2.88 mmol) was dissolved in an Erlenmeyer flask with EtOH (96 °GL, 20 mL) and then phenylhydrazine (0.5 mL, 5.1 mmol) was added. The mixture was irradiated with microwaves (170 W) for 3 min at 12 s intervals. During the rest periods, the Erlenmeyer flask was cooled to 0 °C in an ice bath. The reaction was monitored using TLC. When the reaction was complete, the mixture was cooled at 4 °C for 30 min to precipitate the crude product. The solid was filtered, yielding: 1101 mg of compound **10** (2.5 mmol, 86.2%), mp 187 °C, ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (ppm); 2.69 (3H, *s*, CH_3-9), 2.12 (3H, *s*, CH_3-8′), 2.56 (3H, *s*, CH_3-9′), 4.00 (3H, *s*, CH_3-O), 6.50 (1H, *s*, H-5),

8.38 (1H, *s*, H-8), 6.55 (1H, *s*, H-6′), 12.11 (1H, *s*, OH-4), 11.97 (1H, *s*, OH-3′), 7.55–6.97 (6H, *m*, 1″-4″ aromatic). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ (ppm); 102.79 (C-1), 163.72 (C-2), 105.39 (C-3), 162.88 (C-4), 112.77 (C-5), 144.35 (C-6), 170.23 (C-7), 136.83 (C-8), 25.03 (C-9), 152.35 (C-1′), 116.91 (C-2′), 162.84 (C-3′), 110.05 (C-4′), 139.77 (C-5′), 116.25 (C-6′), 172.29 (C-7′), 9.39 (C-8′), 24.06 (C-9′), 52.32 (CH_3O-), 143.41 (C-1″), 112.54 (C-2″), 129.57 (C-3″), 120.84 (C-4″). ESI-MS *m*/*z*: 447.15 ([M + H]$^+$, *m*/*z*); (calcd. for $C_{25}H_{23}N2O_6^+$, 447.16).

3-hydroxy-4-(*methoxycarbonyl*)*-2,5-dimethylphenyl* (*E*)*-2,4-dihydroxy-6-methyl-3-*((*phenylimino*)*methyl*)*benzoate* (**11**): A mixture of compound **1** (325 mg, 0.87 mmol), aniline (0.15 mL, 1.58 mmol), and EtOH (10 mL) was irradiated with microwaves (170 W) in an Erlenmeyer flask for 3 min at 15 s intervals. Between each heating interval, the mixture was cooled to 0 °C in an ice bath. The reaction was monitored using TLC. Once complete, the reaction mixture was cooled for 60 min at 0 °C in an ice bath for 60 min. After this time, a yellow amorphous solid formed, which was recovered via filtration, corresponding to 345 mg of product **11**: (0.77 mmol, 88.5%), mp 167 °C, ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (ppm); 2.67 (3H, *s*, CH_3-9), 2.13 (3H, *s*, CH_3-8′), 2.57 (3H, *s*, CH_3-9′), 4.00 (3H, *s*, CH_3-O), 6.41 (1H, *s*, H-5), 9.17 (1H, *s*, H-8), 6.55 (1H, *s*, H-6′), 15.65 (1H, *s*, OH-2), 12.67 (1H, *s*, OH-4), 11.97 (1H, *s*, OH-3′), 7.49-7.28 (6H, *m*, a-d aromatic). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ (ppm); 100.22 (C-1), 168.04 (C-2), 105.99 (C-3), 170.24 (C-4), 115.27 (C-5), 148.38 (C-6), 171.94 (C-7), 156.54 (C-8), 25.41 (C-9), 152.35 (C-1′), 116.93 (C-2′), 162.87 (C-3′), 110.04 (C-4′), 139.74 (C-5′), 116.27 (C-6′), 172.28 (C-7′), 9.37 (C-8′), 24.05 (C-9′), 52.30 (CH_3O-), 144.79 (C-a), 120.64 (C-b), 129.65 (C-c), 127.24 (C-d). ESI-MS *m*/*z*: 450.15 ([M + H]$^+$, *m*/*z*); (calcd. for $C_{25}H_{24}NO_7^+$, 450.16).

3-hydroxy-4-(*methoxycarbonyl*)*-2,5-dimethylphenyl* (*E*)*-2,4-dihydroxy-6-methyl-3-*((*naphthalen-2-ylimino*)*methyl*)*benzoate* (**12**): A mixture of compound **1** (505 mg, 1.35 mmol), 1-naphthylamine 300 mg, 2.10 mmol), and EtOH (15 mL) was irradiated with microwaves (170 W) at 15 s intervals for 5 min in an Erlenmeyer flask. Between each heating interval, the mixture was cooled to 0 °C in an ice bath. The reaction was monitored using TLC. Once complete, the reaction mixture was cooled for 60 min at 0 °C in an ice bath. After this time, a yellow amorphous solid formed, which was recovered via filtration, yielding: 592 mg of product 13 (1.19 mmol, 88.1%), mp 177 °C, ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (ppm); 2.71 (3H, *s*, CH_3-9), 2.13 (3H, *s*, CH_3-8′), 2.56 (3H, *s*, CH_3-9′), 3.99 (3H, *s*, CH_3-O), 6.50 (1H, *s*, H-5), 9.27 (1H, *s*, H-8), 6.56 (1H, *s*, H-6′), 15.80 (1H, *s*, OH-2), 12.57 (1H, *s*, OH-4), 11.97 (1H, *s*, OH-3′), 7.92-7.22 (10H, *m*, a-j aromatic) ^{13}C-NMR ($CDCl_3$, 75 MHz) δ (ppm); 101.07 (C-1), 170.18 (C-2), 106.58 (C-3), 167.53 (C-4), 114.61 (C-5), 148.49 (C-6), 170.30 (C-7), 158.23 (C-8), 25.42 (C-9), 152.32 (C-1′), 116.90 (C-2′), 162.85 (C-3′), 110.07 (C-4′), 139.76 (C-5′), 116.25 (C-6′), 172.27 (C-7′), 9.37 (C-8′), 24.02 (C-9′), 52.29 (CH_3O), 143.49 (C-a), 114.48 (C-b), 126.75 (C-c), 127.28 (C-d), 134.04 (C-e), 128.10 (C-f), 125.92 (C-g), 126.80 (C-h), 127.78 (C-i), 122.69 (C-j). ESI-MS *m*/*z*: 500.16 ([M + H]$^+$, *m*/*z*); (calcd. for $C_{29}H_{26}NO_7^+$, 500.17).

Ethyl (*E*)*-2,4-dihydroxy-6-methyl-3-*((*phenylimino*)*methyl*)*benzoate* (**13**): Compound **1** (500 mg, 1.34 mmol) was dissolved in a mixture of ethanol:chloroform (2:1) (15 mL), and aniline (0.2 mL, 2.19 mmol) was added; then, the reaction was sonicated for 30 min at room temperature in an ultrasonic bath. The mixture was then left at room temperature overnight, which enabled the crystallization of **13** into yellow needles, yielding: 329 mg (1.10 mmol, 82.1%). mp 117 °C, ^{1}H-NMR ($CDCl_3$, 300 MHz) δ (ppm); 1.45 (3H, *t*, J = 7.08, CH_3-11), 2.54 (3H, *s*, CH_3-9), 4.42 (2H, *q*, J = 7.04, CH_2-10), 6.32 (1H, *s*, H-5), 9.15 (1H, *s*, H-8), 13.03 (1H, *s*, OH-2), 15.36 (1H, *s*, OH-4), 7.28-7.47 (5H, m, 1′-4′aromatic). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ (ppm); 101.96 (C-1), 169.63 (C-2), 106.03(C-3), 166.71 (C-4), 113.87 (C-5), 146.00 (C-6), 172.7 (C-7), 157.18 (C-8), 25.13 (C-9), 61.44 (C-10), 14.25 (C-11), 148.28 (C-1′), 120.82 (C-2′), 129.52 (C-3′), 126.91 (C-4′). ESI-MS *m*/*z*: 300.10 ([M + H]$^+$, *m*/*z*); (calcd. for $C_{17}H_{18}NO_4^+$, 300.12).

All synthesized compounds were identified via qualitative analysis using a combination of both TLC and HPLC, and we observed the purity of the compounds. In relation to the compounds containing an imine group (**8**, **9**, and **11**–**13**), one E isomer was detected. After TLC and HPLC analysis, only one compound was observed and confirmed with NMR analysis. Rotation is common in imine groups, with the E isomer being favored owing to

steric effects. It was not possible for it to show up clearly on X-rays since not all compounds were crystalline material. Compounds **7**–**9** were eluted at retention times (Rt) of 26.9, 27.0, and 26.5 min on HPLC, respectively, while compounds **10**–**13** were eluted at Rts of 27.0, 26.7, 27.3, and 28.8 min. TLC analysis (SiO_2) was conducted using dichloromethane, and a spot was observed under UV light (254–366 nm). The analysis was determined via the retention factor (Rf). Compounds **7**–**13** showed Rf values of 0.45, 0.57, 0.42, 0.18, 0.33, 0.12, and 0.10 on TLC, respectively.

2.5. Evaluation against Leishmania Parasites

Promastigotes of *Leishmania-Leishmania*: *L. amazonensis*, clone 1, NHOM-BR-76-LTB-012 (Lma), *Leishmania-Viannia*: *L. braziliensis*, M2904 C192 RJA (M2904). Strains were cultured in Schneider's insect medium (pH 6.2) supplemented with 10% fetal bovine serum (FBS) and incubated in 96-microwell plates at 26 °C. The biological assay was performed as described elsewhere [25]. Briefly, promastigotes in the logarithmic growth phase at a concentration of 3×10^6 parasites/mL were treated with samples dissolved in DMSO (1%) at different concentrations (3.1–100 µg/mL). Miltefosine (3.1–100 µg/mL, Profounda Company, Orlando, FL, USA) and total alkaloid extracts from *Galipea longiflora* (CAT, 3.1–100 µg/mL) [26] were used as control drugs. The total alkaloid extract, referred to as CAT y/o AEE, was used as a positive natural extract control for *Leishmania* and other protozoa parasites as quoted from different authors [27]. The main component of the *G. longiflora* of the active fraction was 2-phenyl-quinoline, but the bulk total alkaloids are cited as an active natural reference since the values of other synthetic drugs are active at very low concentrations when compared with raw extracts. The leishmanicidal model used was based on promastigotes, and activity values were quoted against cytotoxicity against RAW cells, indicating whether these substances present some selective activity. The microwell plates were incubated at 26 °C for 72 h, the optical density of each well was determined, and the IC_{50} values were calculated. The assays were performed in triplicate.

2.6. Cytotoxicity

The RAW 264.7 cell line (Murine Macrophages, Carlsbad, CA, USA) was used and grown in DMEM-HG medium (Dulbeccos Modified Eagle's Medium-High Glucose, Grand Island, NY, USA), supplemented with 1% streptomycin–penicillin, sodium bicarbonate (2.2 g/L), and 10% fetal bovine serum in sterile 25 cm^2 culture boxes incubated under standard conditions (5% CO_2 in air, 37 °C, and 100% relative humidity). The culture medium was changed every 72 h. The biological assay was performed with cells at a concentration of 5×10^4 cells/mL incubated for 24 h and then treated with samples dissolved in DMSO (1%) at different concentrations. The microwell plates were incubated for 72 h; then, resazurine (2 mM, 10 µL/well) prepared in PBS (2.86 g/L Na_2HPO_4, 0.31g/L NaH_2PO_4, pH = 7.4) was added and incubated for another 3 h. The fluorescence of each well was measured (excitation 540 nm and emission 590 nm), and IC_{50} values were calculated. Assays were performed in triplicate.

2.7. 96-Well Plate Preparation

After 72 h of incubation and cell confluence of approximately 95%, the cells were mechanically detached (scraped), the cell suspension was counted in a Neubauer chamber, and the population was adjusted to 5×10^4 cells/mL and distributed into 96-well plates (100 µL). They were incubated for 24 h. Then, the concentrations of the compounds (100 µL) (200-100-50-25-12.5-6.2 ug/mL) were added to the indicated wells. DMEM-HG medium blank (100 µL) plus study drugs (100 µL) were the cell growth controls. Plates were incubated at 37 °C for 72 h.

2.8. IC_{50} by Fluorometric Method (Rezasurin)

After 72 h of incubation at 37 °C, resazurin (2 mM) was added to each well (10 µL) and incubated for another 3 h under the same conditions. Plates were read in a SYNERGY

spectrofluorometer at 540 nm excitation and 590 nm emission (Santa Clara, CA, USA). The Gen5 program (Bio tek) and linear trend calculations using Microsoft Excel (Version 2110) were used to determine the mean inhibitory concentration (IC_{50}).

2.9. Selective Index

Selective index values were calculated as the ratio between cell LD_{50} values and parasite IC_{50} values ($SI = LD_{50}/IC_{50}$).

3. Results and Discussion

3.1. Lichen Compounds

Depside atranorin (**1**) was isolated from *Stereocaulon ramulosum*. This known dibenzofuran usnic acid (**2**) was isolated from various species, that is, *Xanthoparmelia lineola* and *Usnea patagonica*; tridepside gyrophoric acid (**3**) was isolated from *U. patagonica*; depsidones salazinic acid (**4**) and galbinic acid (**5**) were isolated from *Parmotrema paramoreliensis* and *Usnea cornuta*, respectively; and finally, the anthraquinone called parietin (**6**) was isolated from *Teloschistes chrysophthalmus*. All these structures (Figure 1) were elucidated via NMR spectroscopy (Supplementary Material).

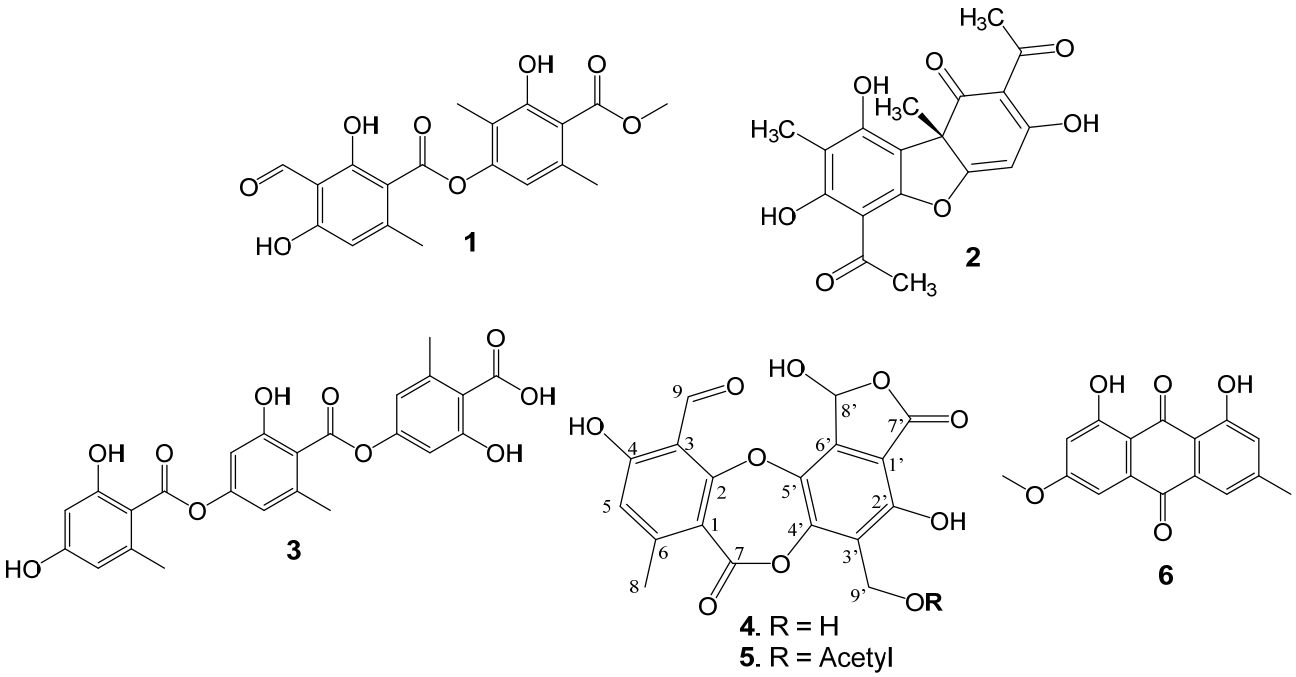

Figure 1. Chemical structure of the isolated lichen compounds.

3.2. Microwave Semisynthesis

Microwaves (MWs) have been used in lichen chemistry, especially for the extraction of lichen substances, with analytical proposals [28–30]. On the other hand, MW-assisted reactions have been widely and successfully used for the derivatization of natural products in pharmaceutical research, with the advantage of being an environmentally friendly tool that offers improved reaction times and yields [31]. Moreover, MW heating improves the results and experimental procedures for the formation of imine and Schiff bases [10], which are interesting functional groups for lichen compounds given their potential biological activities [32].

Two lichen substances were selected as starting materials given their fast derivatization from the lichens under study. They are common compounds in lichens: usnic acid (**2**) was used to obtain compounds **7**, **8**, and **9**, and atranorin (**1**) was used to obtain compounds **10**, **11**, **12**, and **13** (Figure 2A). The condensation of usnic acid (**2**) with various nitrogen-containing compounds has been described previously under conventional heating with

aromatic and aliphatic amines and phenylhydrazines [11,33–37] and atranorin (**1**), which were condensed using hydrazides under conventional heating [38]. The nitrogen-based reagents that were used in this study were pheynylhydrazine, aniline, 2-naphtilamine, and cyclohexylamine (Figure 2B). Preliminary experiments with aliphatic amines have shown complex reaction mixtures, possibly related to degradation; therefore, we focused our study on cyclic and aromatic compounds. An additional advantage was that the adducts with cyclic amines were much easier to isolate and purify via crystallization.

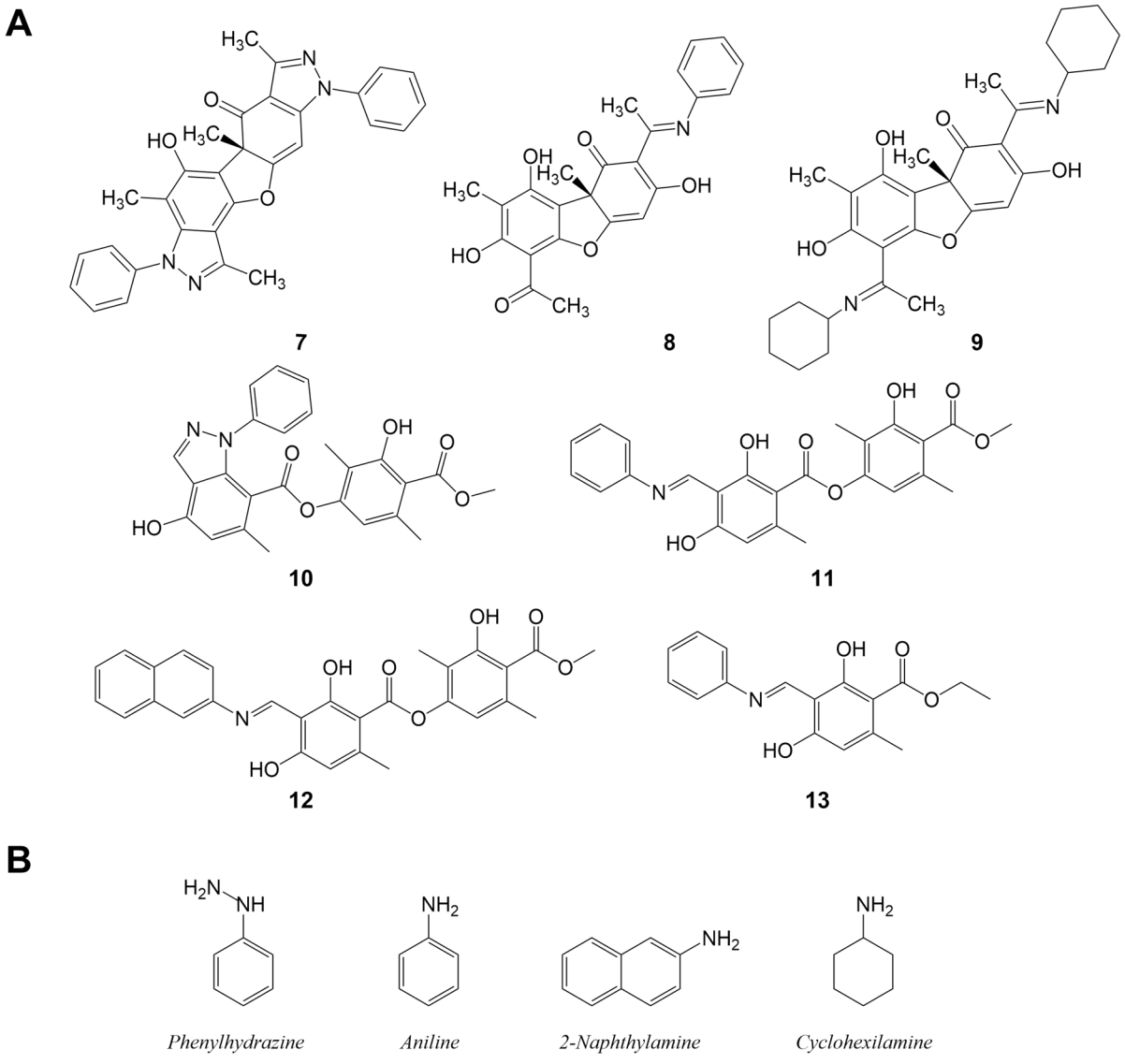

Figure 2. (**A**) Chemical structure of semisynthetic compounds. (**B**) Nitrogen-based reagents used for the derivatization.

The condensation reaction between **2** and phenylhydrazine provided compound **7** with a good yield (71.9%). This product contains two heterocyclic pyrazoline moieties, which have not been reported for conventional heating. This last product had a pyrazoline ring and an imine intermediate that could not cyclize even in the presence of a large excess of phenylhydrazine [11]. Although the reaction products were different, the reaction times could be compared, showing that MW heating is much faster than conventional heating. Under MWs, the reaction was completed in only 15 min, but under conventional heating, it took 2 h without completing product **7** [11]. Compound **8** was obtained via the condensation of **2** with aniline. The formation of imine adducts has been studied and compared under MW conditions and conventional heating. The authors reported a

reaction between **2** and several aromatic amines. The reported reaction conditions were an irradiation power of 60 W for 20 min, a usnic acid (**2**):aromatic amine ratio of 1:1, and ethanol as solvent. On the other hand, our conditions were an irradiation power of 170 W for 6 min (intervals of 30 s), a usnic acid (**2**):aromatic amine ratio of 1:1.8, and ethanol as solvent. In some cases where aniline is used as an aromatic amine, the reported yield was 64.8%, and our procedure provided a yield of 80.8%. As expected, more irradiation power and a higher usnic acid (**2**):aniline ratio, as employed in our procedure, can improve yields [39]. Finally, compound **9** was the condensation product of **2** with cyclohexylamine. This product has not been reported previously, but reports of coupling with aliphatic amines under conventional heating have been published [33–37,39,40]. Under conventional heating, the yields are nearly 80%, but under MW irradiation, the yield is nearly 90%. However, the most important difference is the reaction time, which was significantly improved from an average of 1.5 h under conventional heating to 12 min under MWs.

The condensation of **1** with phenylhydrazine yielded compound **10** (86.2%). The reaction provided similar results to **7**, forming a pyrazoline ring, but with regioselectivity in this case. To explain this regioselectivity, a reaction mechanism is proposed in Figure 3 in which an aromatic nucleophilic substitution of the hydroxyl group from the phenol by the nitrogen of the coupled hydrazine is facilitated by a 1,4 addition to a vicinal α,β-unsaturated carbonyl group. It has been reported that the addition of hydrazines to **1** under conventional heating provides intermediate imines [38]. Therefore, the formation of the pyrazoline ring in product **10** may indicate that only MW irradiation can provide the necessary energy to complete the reaction. Compound **11** was obtained via the condensation of **1** with aniline. Unlike the other products, there is no information about this reaction. The result shows that MW heating provides a high yield (88.5%) in a short reaction time (3 min). The same result was observed when the reaction was repeated with 2-naphtilamine to obtain compound **12**, for which the yield and reaction time were also higher (87.9%, 5 min).

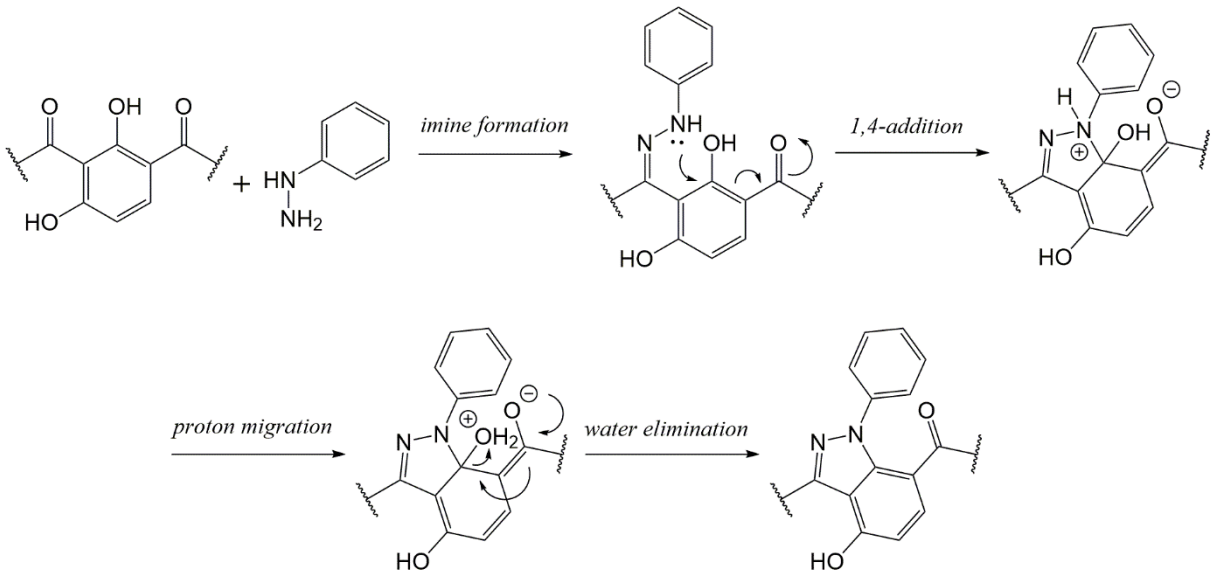

Figure 3. Proposed reaction mechanism for the formation of a pyrazoline ring for compounds **7** and **10**.

Finally, compound **13** was an artifact produced by the chemical reaction of compound **11**. To obtain a homogeneous mixture in ethanol:chloroform, compound **1** and aniline were sonicated for 30 min. After this time, yellow needle crystals, identified as **13**, were observed. Even considering that the reaction was not planned, the yield was high (82.1%). The formation of **13** can be explained as follows: ultrasound methods have been shown to be a fast and efficient method of producing Schiff bases [41]. The additional transesterification of orsellinic acid with an ethoxy group in 3-formyl orsellinic acid can be facilitated via

ultrasound in the same way as the transesterification of fatty acids under ultrasound for the production of biodiesel [42].

3.3. Leishmania Activity and Cytotoxicity

Previous studies on lichen compounds with antileishmanial activity have been poorly explored. Similar studies have shown that depside isodivaricatic acid, depsidonas 1′-chloropannarine, and pannarine have good activity against *Leishmania amazonensis*, *L. brasiliensis*, and *L. infantum* [43]. Usnic acid, one of the most representative and accessible lichen compounds, is a candidate for the treatment of infections caused by *L. major*, *L. infantum*, *L. tropica* [44,45], *L. donovani* [44], *L. brasiliensis*, and *L. infantum chagasi* [2], but its use as a treatment is not recommended because it has hepatoxic effects [46].

Gyroporic acid (**3**), salazinic acid (**4**), galbinic acid (**5**), parietin (**6**), and derivatives **8** and **10** showed no activity against *Leishmania* parasites, suggesting that they are more cytotoxic than the antiparasitic ones (Table 2). Atranorin (**1**) showed moderate activity, whereas usnic acid (**2**) showed strong activity against both *Leishmania* strains. The SI for **1** was 1.8 for both strains. The SI for **2** was 1.2 for *L. amazonensis* and 1.7 for *L. braziliensis*. These results may indicate that these active natural products could be promising lead compounds for the synthesis of leishmanicidal compounds. The semisynthetic derivatives **10**, **11**, **12**, and **13** obtained from **1** showed a significant increase in cytotoxicity or loss of leishmanicidal activity (Figure 4). Interestingly, the semisynthetic derivative **9** from **2** provided a better SI value (about four) than its precursor, suggesting that **2** could be an interesting drug leader providing selective and highly active leishmanicidal compounds. In Table 2, only compounds 1 and 9 could be compared with Miltefosine and CAT since all others are mainly cytotoxic and cannot be classified as leishmanicidal.

Table 2. In vitro leishmanicidal activity and cytotoxicity in RAW line cell.

Compound	*Leishmania amazonensis* IC_{50} (μg/mL)	SI	*Leishmania braziliensis* IC_{50} (μg/mL)	SI	RAW Cells LD_{50} (μg/mL)
1	28.0 ± 11.1	1.7	26.2 ± 11	1.8	48.0 ± 11.4
2	4.3 ± 0.5	1.2	3.0 ± 0.6	1.7	5.0 ± 1.2
3	>100	-	>100	-	58.0 ± 3.0
4	>100	-	>100	-	70.0 ± 2.2
5	>100	-	>100	-	46.5 ± 1.0
6	>100	-	>100	-	20.0 ± 6.3
7	33.1 ± 6.5	0.2	29.0 ± 8.0	0.2	5.4 ± 1.0
8	>100	-	>100	-	83.1 ± 2.0
9	10.5 ± 0.3	4.2	12.2 ± 2.2	3.6	44.3 ± 7.0
10	>100	-	>100	-	10.3 ± 5.0
11	21.0 ± 2.0	0.9	20.0 ± 1.0	1.0	19.1 ± 1.0
12	85.1 ± 13	0.2	78.0 ± 16	0.2	17.0 ± 1.0
13	25 ± 2.0	0.3	11.0 ± 0.2	0.7	8.0 ± 2.0
Miltefosine	3.5 ± 0.6	4.4	3.4 ± 1.0	4.5	15.3 ± 0.3
CAT	19.5 ± 1.4	1.2	19.0 ± 2.4	1.3	24.0 ± 0.3

SI: Selective index.

The reason why these reported compounds exert activity remains unknown. There are many natural products that show activity, but no studies for understanding the mechanisms of action have been performed. Some antifungal agents (such as Amphotericine-B) show anti-leishmanial activity because fungi and leishmania parasites share some cell wall characteristics [47], but other compounds, such as Glucantime® [48], have a very different mechanism of action. Therefore, detailed studies on the mechanisms of action of these lichen compounds should be carried out.

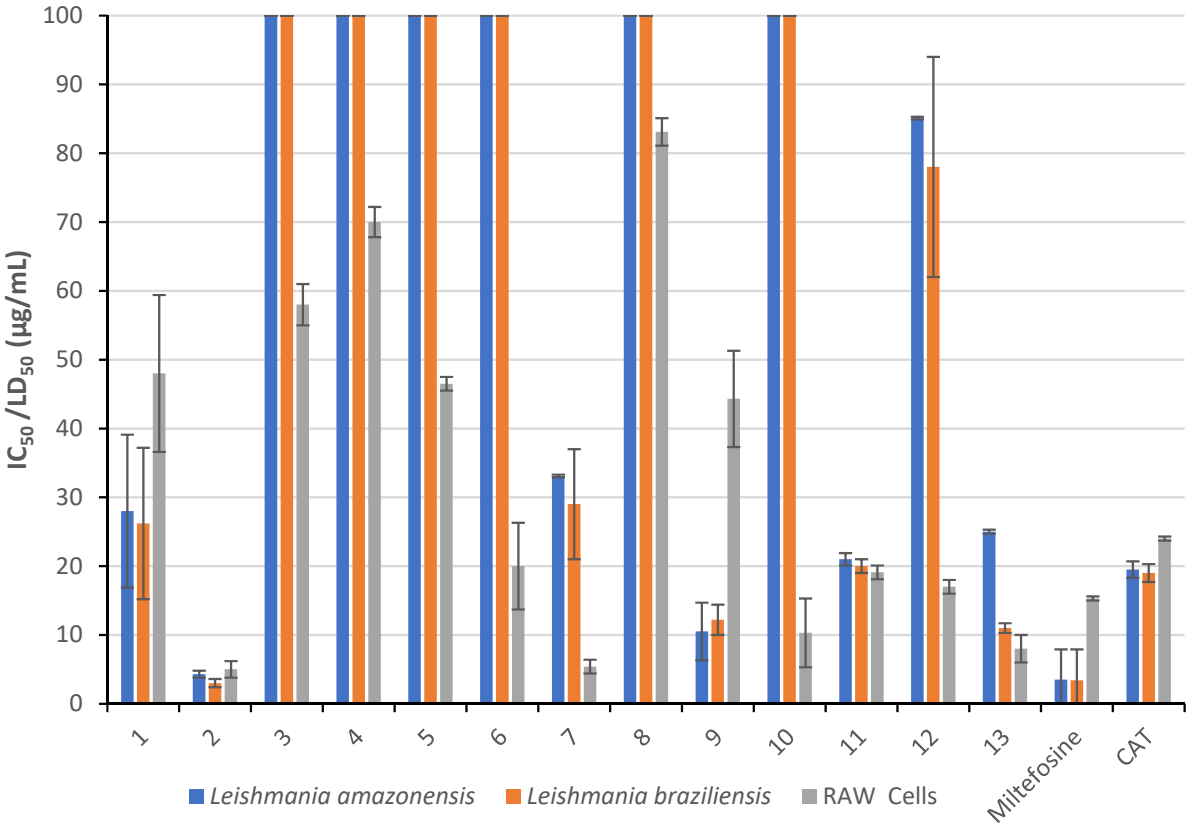

Figure 4. In vitro leishmanicidal activity (IC_{50}) and cytotoxicity in RAW line cell (LD_{50}) against *Leishmania amazonensis* and *Leishmania braziliensis*.

4. Conclusions

Our results show that novel derivatives were semi-synthesized via the condensation of lichen compounds with amines. The reactions were carried out under MW conditions following green chemistry procedures. They proved to be favorable for the formation of the products in high yields, especially in short reaction times. Although the results for leishmanicidal activity were not encouraging given the cytotoxicity of the compounds, we believe that usnic acid is still a promising lead compound for the development of new antiparasitic drugs if selectivity is improved. Additionally, semisynthetic derivative **9** provided a better SI than **2**, suggesting that aliphatic imines can have better selectivity and activity. We suggest carrying out further research on this issue.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10100524/s1, Figure S1: ^{13}C-NMR (DMSO-d_6, 75 MHz) spectrum of galbinic acid (**5**); Figure S2: ^{1}H-NMR ($CDCl_3$, 300 MHz) spectrum of compound (**7**); Figure S3: ^{13}C-NMR ($CDCl_3$, 75 MHz) spectrum of compound (**7**); Figure S4: ^{1}H-NMR ($CDCl_3$, 300 MHz) spectrum of compound (**8**); Figure S5: ^{13}C-NMR ($CDCl_3$, 75 MHz) spectrum of compound (**8**); Figure S6: ^{1}H-NMR ($CDCl_3$, 300 MHz) spectrum of compound (**9**); Figure S7: ^{13}C-NMR ($CDCl_3$, 75 MHz) spectrum of compound (**9**); Figure S8: ^{1}H-NMR ($CDCl_3$, 300 MHz) spectrum of compound (**10**); Figure S9: ^{13}C-NMR ($CDCl_3$, 75 MHz) spectrum of compound (**10**); Figure S10: ^{1}H-NMR ($CDCl_3$, 300 MHz) spectrum of compound (**11**); Figure S11: ^{13}C-NMR ($CDCl_3$, 75 MHz) spectrum of compound (**11**); Figure S12: ^{1}H-NMR ($CDCl_3$, 300 MHz) spectrum of compound (**12**); Figure S13: ^{13}C-NMR ($CDCl_3$, 75 MHz) spectrum of compound (**12**); Figure S14: ^{1}H-NMR ($CDCl_3$, 300 MHz) spectrum of compound (**13**); Figure S15: ^{13}C-NMR ($CDCl_3$, 75 MHz) spectrum of compound (**13**).

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