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Leishmanicidal activity of alkaloids from *Hamelia* patens

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Abstract

The crude ethanolic extract from the leaves of *Hamelia patens* Jacq. was active *in vitro* against *Leishmania mexicana*. Partition of the extract, showed that the activity was confined to the dichloromethane extract. The four alkaloids isolated from this extract: isopteropodine **(1)**, palmirine **(2)**, rumberine **(3)** and mitrajavine **(4)** were evaluated *in vitro* for antileishmanial activity. Palmirine **(2)**, one of the main alkaloids, showed the highest leishmanicidal activity with IC_{50}= 56 μM, the alkaloid mitrajavine **(4)** and the two flavonoids isolated from the ethyl acetate extract: kaempferol-3-*O*-rutinoside **(5)**, and (-) epicatechine **(6)** are reported for the first time in this plant.

Key words: *Rubiaceae*, *Hamelia patens*, leishmanicidal, alkaloids, flavonoids.

Actividad leishmanicida de alcaloides de *Hamelia patens*

Resumen

El extracto etanólico obtenido de las hojas de *Hamelia patens* Jacq. fue activo en pruebas *in vitro* contra *Leishmania mexicana.* La partición del extracto mostró que la actividad estaba confinada al extracto de diclorometano. Cuatro alcaloides aislados de ese extracto, isopteropodina (**1**), palmirina (**2**), rumberina (**3**) y mitrajavina (**4**), fueron evaluados in vitro en actividad leishmanicida. Palmirina (**2**) mostró la más alta actividad con un IC_{50}= 56 μM. El alcaloide mitrajavine (**4**) y los dos flavonoides aislados del extracto de acetato de etilo, kaempferol-3-O-rutinosido (**5**) y (-) epicatequina (**6**), son reportados por primera vez en esta planta.

Palabras claves: *Rubiaceae*, *Hamelia patens*, leishmanicida, alcaloides, flavonoides.

Introduction

*Hamelia patens***,** a plant belonging to the *Rubiaceae*, is a species distributed throughout tropical America. The plant is known commonly as "coralito" and, is widely used in the Venezuelan ethnomedicine to treat different diseases and health conditions such as: malaria, rheumatism, gastritis, dysentery, ulcers, and as an antihemorragic (1). As part of our search for bioactive compounds from plants, a screening of a number of plants used in Venezuela to treat malaria and leishmania showed that a dichloromethane extract of the leaves from *Hamelia patens* exhibited strong *in vitro* activity

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against *Leishmania mexicana*. For that reason, it was decided to evaluate the compounds isolated from the mentioned extract. The aerial parts of *Hamelia patens* were investigated previously by Aquino *et ál.* (2) who reported the presence of 5,7,2,5´-tetrahidroxyflavone-7-rutinoside; Prabir *et ál.* (3) isolated ephedrine and rosmarinic acid, Borges *et ál.* (4) reported three of the alkaloids here mentioned: isopteropodine (**1**), palmirine (**2**), and rumberine (**3**), and more recently Rios & Aguilar-Guadarrama (5) isolated cycloartanols and other triterpenes. This paper deals with the isolation, structural determination, and the leishmanicidal activity of the alkaloids found in our research (figure 1).

Materials and methods

General procedure

Melting points were determined using a Kofler hot-stage instrument and were uncorrected, IR spectra were measured on a Perkin-Elmer 1320 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or in MeOD using TMS as internal reference, employing a JEOL 270 MHz. High resolution mass measurements were obtained from UCR Mass Spectrometry Facility at Riverside, California. Isolation procedures were monitored by employing thin-layer chromatography on pre-coated silica gel plates (Merck, Kieselgel 60 F-254).

Plant material

Hamelia patens Jacq. was collected in San Diego de los Altos, Miranda State, Venezuela, and identified by Dr. Stephen Tillett. A voucher specimen was deposited in the Herbarium "Victor Manuel Ovalles" of the Facultad de Farmacia, Universidad Central de Venezuela, with the accession number MYF 11810.

Extraction and isolation

Air-dried and powdered leaves (136 g) were exhaustively extracted with MeOH in a Soxhlet apparatus. The residue from MeOH

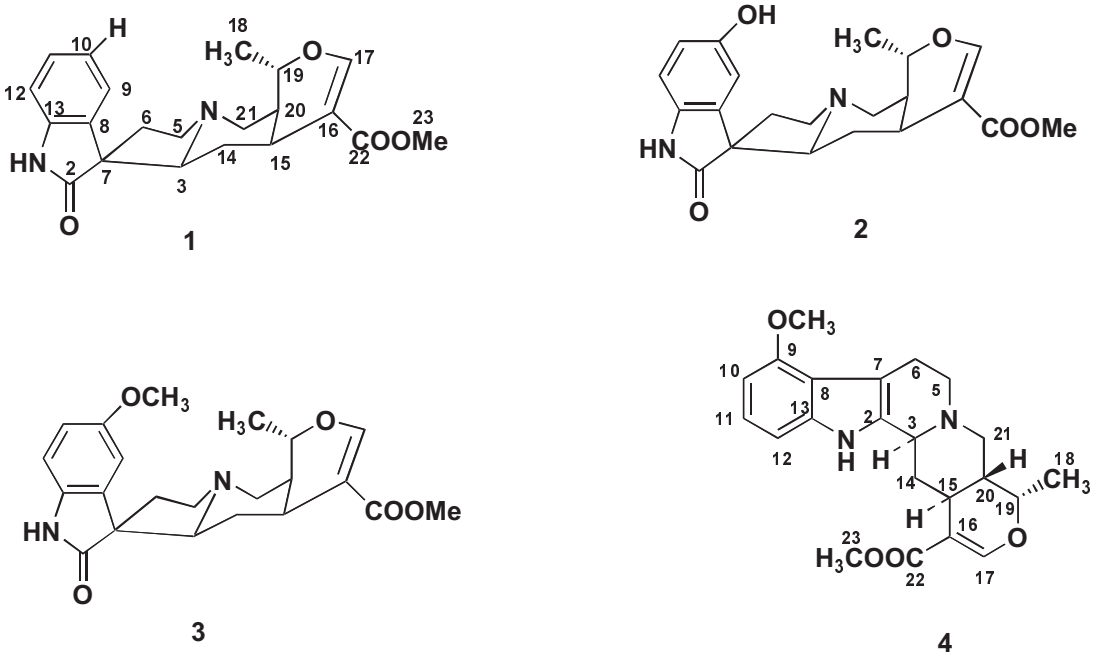
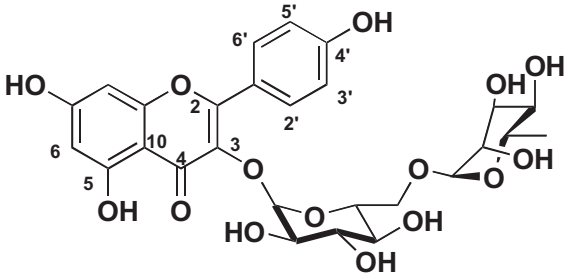


Figure 1. Structures of alkaloids tested for leishmanicidal activity: isopteropodine (1), palmirine (2), rumberine (3) and mitrajavine (4).

(28.5 g) was dissolved in MeOH-H_2O 1:1, and partitioned between hexane, CH_2Cl_2, EtOAc, and H_2O. The antileishmanial activity was tested, and as the CH_2Cl_2 and EtOAc extracts showed the highest values, these were subjected to column chromatography over silica gel and eluted with a gradient of $CHCl_3$-MeOH (from 100:0 to 80:20). From the CH_2Cl_2, six fractions were collected to yield compounds **1** (85 mg), **2** (43 mg), **3** (32 mg), and **4** (72 mg). From the EtOAc extract, compounds **5** (75 mg), and **6** (162 mg) were obtained by silica gel column chromatography eluted with EtOAc-MeOH (5-40%). All the isolated were further purified by crystallization in appropriate solvents. Structural elucidation of these pure compounds was obtained using spectroscopic techniques such as nuclear magnetic resonance, infrared, mass spectroscopy. All compounds were identified by comparison of their physical and spectroscopic data with those of the literature (figure 2).



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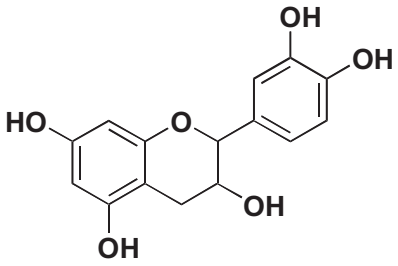

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Figure 2. Structures of flavonoids from *Hamelia patens*.

Isolates

Isopteropodine (**1**). Yellow needles (MeOH), mp 205-208°C, $[\alpha]_D^{20}$ -53 (c, 0.50 $CHCl_3$). MS, 368.43, IR (KBr) γ_{max} cm^{-1}: 3300 (NH), 1730 (COOR), 1690 (CONH). ^1H ($CDCl_3$, 270 MHz), δ: 0.86 (1H, dd, J =12.1, 12.3 Hz, H-14a), 1.37 (3H, d, J = 6.2 Hz. H-18), 1.84 (1H, m, H-20), 1.97 (1H, m, H-15), 2.36-2.43 (8H, m, H-6, H-14, H-21), 2.56 (1H, m, H-5a), 3.23 (1H, m, H-15), 3.38 (1H, m, H-5b), 3.58 (3H, s, OCH_3), 4.34 (1H, m, H-3), 4.40 (1H, m, H-19), 6.85 (1H, d, J = 8.2 Hz, H-12), 6.99 (1H,ddd, J = 8.2, 8,1, 1.1 Hz, H-10), 7.16 (1H,ddd, J = 8.2, 8,1, 1.1 Hz, H-11), 7.26 (1H, d, J= 8.1 Hz, H-9), 7.39 (1H, s, H-17), 8.04 (1H, brs, NH). ^{13}C NMR ($CDCl_3$, 67 MHz) ä: 181.5 (C-2), 72.1 (C-3), 53.9 (C-5), 30.2 (C-6), 56.7 (C-7), 134.1 (C-8), 124.8 (C-9), 122.7 (C-10), 127.8 (C-11), 109.6 (C-12), 140.4 (C-13), 34.6 (C-14), 29.9 (C-15), 109.6 (C-16), 155.4 (C-17), 18.3 (C-18), 71.3 (C-19), 37.6 (C-20), 53.3 (C-21), 168.2 (C-22), 50.8 (C-23).

Palmirine (**2**). Colorless amorphous solid, m.p. 103-104 °C; $[A]_D^{20}$ -48 (c, 0.40 $CHCl_3$). MS, 398.26, IR (KBr) γ_{max} cm^{-1} 3320 cm^{-1} (NH), 1740 (COOR), 1695 (CONH). ^1H ($CDCl_3$, 270 MHz), δ: 1.25 (1H, m, H-20), 1.45 (3H, d, J = 6.5 MHz, H-18), 3.58 (3H, s, OCH_3), 3.75 (3H, s, OCH_3), 4.35 (1H, m, H-3), 6.87 (1H, d, J = 7.6 Hz, H-12), 7.15 (1 H,dd, J= 7.5, 0.8 Hz, H-11), 7.30 (1H, d, J= 1.3 Hz, H-9), 9.30 (1H, brs, NH). ^{13}C NMR ($CDCl_3$, 67 MHz) δ: 181.0 (C-2), 71.3 (C-3), 54.1 (C-5), 30.3 (C-6), 57.6 (C-7), 133.8 (C-8), 111.9 (C-9), 156.0 (C-10), 111.2 (C-11), 109.9 (C-12), 135.3 (C-13), 34.9 (C-14), 30.7 (C-15), 110.0 (C-16), 155.1 (C-17), 18.6 (C-18), 72.3 (C-19), 38.2 (C-20), 53.6 (C-21), 167.6 (C-22), 51.0 (C-23), 56.7 (CH_3OAr).

Rumberine (**3**). Amorphous yellow solid, m.p. 185-188 °C; $[\alpha]_D^{20}$ -52 (c, 023 $CHCl_3$), MS , 384.32 m/z , IR (KBr) $\gamma_{max}$$cm^{-1}$: 3300 cm^{-1} (NH, OH), 1730 (COOR), 1700 (CONH). ^1H ($CDCl_3$, 270 MHz), δ: 1.25 (1H, m, H-20), 1.43 (3H, d, J = 6.5 MHz, H-18), 3.56 (3H, s, OCH_3), 4.42 (1H, m, H-3), 6.80 (1H, d, J = 7.6 Hz, H-12), 7.18 (1H,dd, J = 7.5, 0.8 Hz,

H-11), 7.37 (1H, d, *J* = 1.3 Hz, H-9), 8.50 (1H, brs, NH).[13]C NMR ($CDCl_3$, 67 MHz) *δ*: 182.0 (C-2), 71.2 (C-3), 53.9 (C-5), 30.2 (C-6), 56.8 (C-7), 134.0 (C-8), 124.8 (C-9), 140.5 (C-10), 127.7 (C-11), 133.9 (C-12), 134.0 (C-13), 34.5 (C-14), 30.2 (C-15), 109.9 (C-16), 155.4 (C-17), 18.6 (C-18), 72.5 (C-19), 37.6 (C-20), 53.3 (C-21), 168.0 (C-22), 50.8 (C-23).

Mitrajavine (**4**). White solid, mp 113-115°C $[\alpha]^{20}$ -45 (c, 0.30 $CHCl_3$), IR (KBr) γ_{max} cm^{-1}: 3400 (NH), 1710 (COOR), 1615 (aromatic ring). FAB-MS: 382.1860. C ^{1}H NMR ($CDCl_3$, 270 MHz) *δ*: 1.37 (3H, d, *J* = 6.5 Hz, H-18), 1.49 -1.68 (2H, m, H-14), 2.04 (2H, m, H-20, H-21), 3.26 (1H, d, *J* = 11.6 Hz, H-3), 2.60 (1H, m, H-5), 3.06 (2H, d, *J* = 12.12 Hz, H-6), 3.72 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 4.71 (1H, m, H-19), 6.76 (1H, dd, *J* = 2.48, 8.64 Hz), 6.87 (1H, d; *J* = 2.21 Hz,), 7.14 (1H, d, *J* = 8.64 Hz), 7.54 (1H, s, H-17), 7.86 (1H, brs, NH). 13C NMR ($CDCl_3$, 67 MHz) *δ*: 131.2 (C-2), 59.9 (C-3), 53.6 (C-5), 21.7 (C-6), 127.6 (C-7), 109.5 (C-8), 154.1 (C-9), 111.5 (C-10), 100.5 (C-11), 111.2 (C-12), 131.2 (C-13), 34.5 (C-14), 31.3 (C-15), 107.9 (C-16), 155.8 (C-17), 18.6 (C-18), 72.5 (C-19), 38.5 (C-20), 53.6 (C-21), 168.1 (C-22), 51.2 (C-23).

Kaempferol-3-O-rutinoside (**5**). ^{1}H (methanol-d_4, 270 MHz) *δ*: 0.98 (1H, d, *J* = 5.9 Hz, H-6'"), 3.00 (2H, m, H-5'", H-2'"), 3.10 (2H, m, H-4'", H-3'"), 3.16 (1H, m, H-4"), 3.26 (1H, m, H-5"), 3.69 (1H, m, H-3"), 4.51 (1H, brs, H-1'"), 5.09 (1H, d, *J* = 7.1 Hz, H-1"), 5.32 (1H, d, *J* = 7.2 Hz, H-2"), 6.88 (2H, d, *J* = 8.6, H-3',H-5'), 6.19 (1H, brs, H-6), 6.41 (1H, brs, H-8), 7.99 (2H, d, *J* = 8.6 Hz, H-2', H-6'), 12.56 (1H, s, OH-5).[13]C NMR (methanol-d_4, 67 MHz) ä: 157.2 (C-2), 134.5 (C-3), 178.0 (C-4), 161.5 (C-5), 98.6 (C-6), 164.6 (C-7), 93.6 (C-8), 158.9 (C-9), 104.0 (C-10), 121.5 (C-1'), 103.4 (C-1"), 114.8 (C-2'), 131.5 (C-3'), 160.1 (C-4'), 131.1 (C-5'), 114.8 (C-6'), 75.8 (C-2"), 76.8 (C-3"), 70.7 (C-4"), 75.8 (C-5"), 67.0 (C-6"), 107.1 (C-1'"), 71.0 (C-2'"), 70.8 (C-3'"), 72.6 (C-4'"), 68.4 (C-5'"), 16.7 (C-6'").

(-)Epicatechine (**6**) ^{1} H NMR (methanol-d_4, 270 MHz) *δ*; 2.80 (2H, dd, *J* = 4.2, 16.3 Hz, H-4), 4.18 (1H, brs, H-3), 4.85 (1H, brs, H-2), 5.90 (1H, d, *J* = 2.3 Hz, H-6), 6.01(1H, d, *J* = 2.3 Hz, H-8), 6.76 (1H, d, *J* = 8.2 Hz, H-5'), 6.83 (1H, dd, *J* = 1.8, 8.2 Hz, H-6'), 7.02 (1H, d, *J* = 1.8 Hz, H-2'). [13]C NMR (methanol-d_4, 67 MHz) *δ*: 78.5 (C-2), 66.2 (C-3), 27.9 (C-4), 156.3 (C-5), 94.6 (C-6), 156.6 (C-7), 95.0 (C-8), 156.0 (C-9), 98.8 (C-10), 130.9 (C-1'), 114.6 (C-2'), 143.4 (C-3'), 144.8 (C-4'), 114.8 (C-5'), 118.1 (C-6').

Assay for leishmanicidal activity

Leishmania mexicana, strain NR, characterized by Ramirez and Guevara (6), was cultured in LIT medium supplemented with 10% inactivated fetal bovine serum, at 26 °C. The bioassays were carried out in duplicate with the promastigote form of the parasite, and monitored during 76 hours by direct counting in a Neubauer chamber. The drug was dissolved in DMSO and added to cultures of *L. mexicana* in the log phase at a density of 2 X 10^6 cells/mL (figures 3, 4, 5 y 6).

Results and discussion

Upon repeated column chromatography the CH_2Cl_2 extract of the leaves afforded isopteropodine (**1**), palmirine (**2**), rumberine (**3**) and the other alkaloid characterized as mitrajavine (**4**).

Compound **4,** was obtained as yellow syrup and, crystallized in EtOH/H_2O. Its IR displayed an intense absorption band at 1710 cm^{-1}, associated to –COOR group and broad band at 3400 cm^{-1} –NH. The high resolution FAB-MS analysis gave m/z 382.1860 and established the molecular formula as $C_{22}H_{26}N_2O_4$. The ^{1}H NMR spectrum allowed the identification of an ABX aromatic system for three aromatic protons which resonated at *δ* 7.15 (d, *J*= 8.6 Hz), 6.86 (d, *J*= 2.2 Hz), and 6.75 (dd, *J* = 8.6, 2.2 Hz). The resonance of the indolic NH proton was found at *δ* 7.87 ppm as well the olefinic proton 17 which resonated at *δ* 7.54 ppm. Two signals singlet of

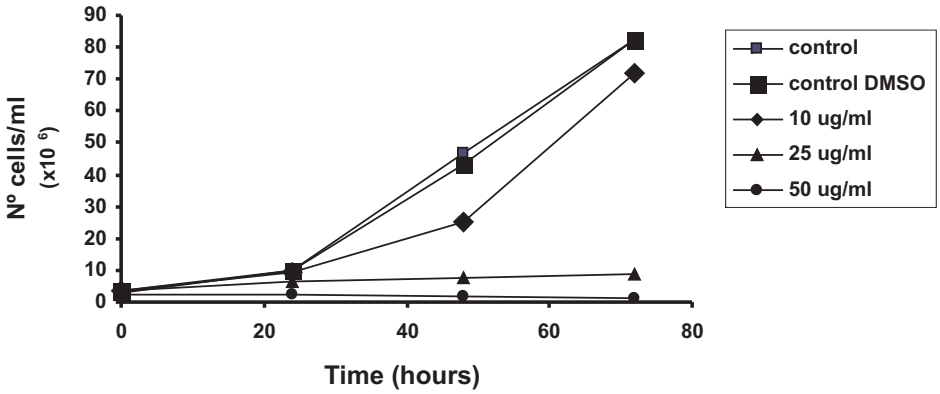

Figure 3. Effect of palmirine (2) on growth and proliferation of *Leishmania mexicana* promastigotes.

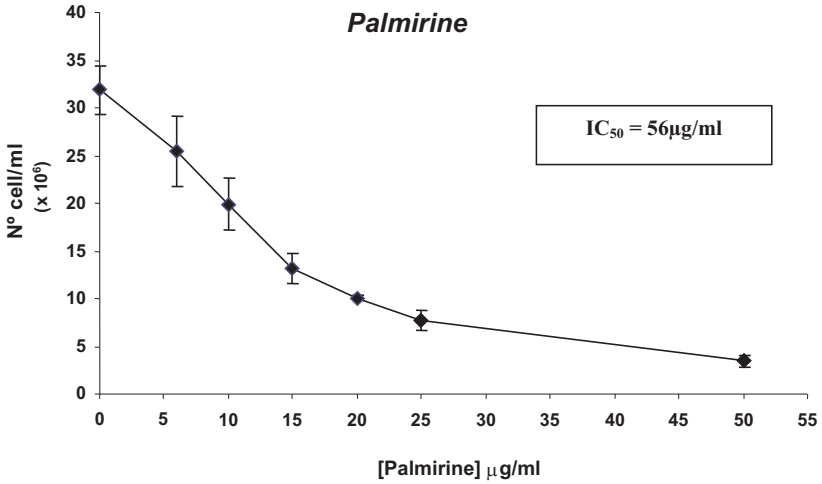

Figure 4. Dosis-answer curve for palmirine (2) against *L. mexicana* promastigotes.

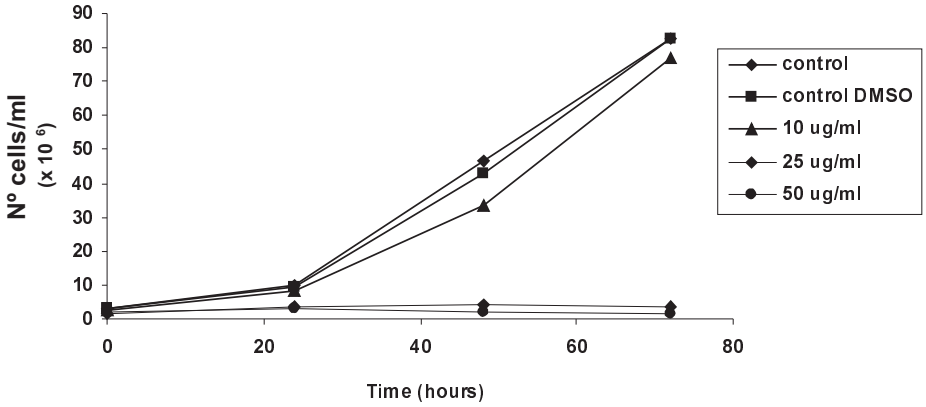

Figure 5. Effect of rumberine (3) on the growth and proliferation of *Leishmania mexicana* promastigotes.

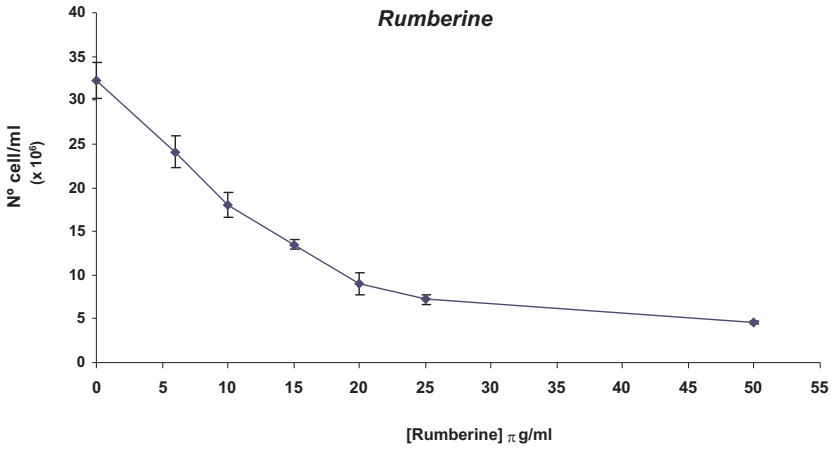

Figure 6. Dosis-answer curve for rumberine (3) against *L. mexicana* promastigotes.

methoxy groups showed resonance at δ 3.78 and 3.74 ppm. Protons clearly belonging to pyrane and quinolizidine rings presents in indole alkaloids, which are frequently isolated in the Rubiaceae, were also observed as multiplets between 2.5 and 4.5 ppm. Finally one doublet integrating for three protons was found at 1.37 ppm, this signal suggested one methyl group coupled to one proton. All this analyzed data were consistent with a yohimbine type structure. The ^{13}C spectrum confirmed by HMQC was consistent with the proposed structure. 21 signals resonances indicated the presence of eleven sp^2 carbons including one ester carbonyl (168.1 ppm); the signal at 131.2 ppm was unambiguously assigned by HMBC to the carbons C-2 and C-13, the presence of the methoxy groups were confirmed by the resonances at δ 51.2 and 56.0 ppm; carbons belonging to three substituted olefin were identified at 107.9 (C-16) and 155.8 (C-17). One oxygenated methine carbon was found at δ 72.5 ppm and the methyl group at δ 18.6 ppm. Comparing our data with yohimbane alkaloids in the literature, we found a good correlation with the reported data for mitrajavine **(4)** (7, 8), this alkaloid was previously found in *Mitragina* species of Asia.

Isopteropodine ($C_{21}H_{24}N_2O_4$) **1,** rumberine ($C_{21}H_{24}N_2O_5$) **2**, and palmirine ($C_{22}H_{26}N_2O_5$) **3**, had been fully characterized (4). Both the physical and spectral data of the compounds we isolated were in good agreement with those had been reported. However, we report here the unequivocal assignment of the ^{13}C NMR spectrum using 2D NMR techniques, providing additional information with respect to previously published data (table 1).

Compound **5** was shown by FAB-MS to have the molecular formula $C_{27}H_{30}O_{15}$. The UV spectra showed absorption maxima at λ_{max} 207, 268, and 365 nm. These are typical signals of the flavonol skeleton. The ^{1}H NMR (CD_3OD) spectrum of compound **5** suggested a 3-glycosylated flavonoid which displayed the characteristic signals of the kaempferol nucleus: two doublets at δ 6.19 and 6.39 ppm (J = 1.8 Hz), assigned to the H-6 and H-8 protons, respectively, of the A-ring; and a pair of doublets integrating for two protons each one indicated a typical A_2B_2 aromatic systems at δ 6.87 and 8.06 ppm (J = 8.8 Hz) in the C ring. The glycosidic nature was confirmed by the two anomeric protons at δ 4.51 (brs, 1H) and 5.09 (d, J = 7.1 Hz, 1H). The glucose β-linked to 3-OH, was evident from the large constant coupling of H-1 while L-rhamnose is á-linked to glucose δ 4.51 (brs, 1H). The ^{13}C spectrum was compared with literature spectra and all the signals indicated a flavone substituted at the 3 position. Finally we found all our data matched with the known kaempferol-rutinoside (9).

Table 1
^{13}C NMR data ($CDCl_3$) for *H. patens* alkaloids

δC	1 (exp)	1 (lit)[4]	2(exp)	2(lit)	3(exp)	3(lit)[4]	4(exp)
2	181.5	180.7	181.0	181.0	181.0	181.0	131.2
3	72.1	70.9	71.3	71.3	71.3	71.3	59.9
5	53.9	53.8	54.1	54.1	54.1	54.1	53.6
6	30.2	30.0	30.3	30.3	30.3	30.3	21.7
7	56.7	56.7	57.6	57.6	57.6	57.6	127.6
8	134.1	133.2	133.8	133.80	133.8	133.8	109.5
9	124.8	124.0	111.9	111.9	111.9	111.9	154.1
10	122.7	121.0	156.0	156.0	156.0	156.0	111.5
11	127.8	127.2	111.2	112.2	111.2	112.2	100.5
12	109.6	109.3	109.9	109.3	109.9	109.3	111.2
13	140.4	139.5	135.3	135.3	135.3	135.3	131.2
14	34.6	34.7	34.9	34.9	34.9	34.9	34.5
15	29.9	30.7	30.7	30.7	30.7	30.7	31.3
16	109.6	109.5	110.0	110.0	110.0	110.0	107.9
17	155.4	154.4	155.1	155.1	155.1	155.1	155.8
18	18.3	18.5	18.6	18.6	18.6	18.6	18.6
19	71.3	71.8	72.3	72.3	72.3	72.3	72.5
20	37.6	37.8	38.2	38.2	38.2	38.2	38.5
21	53.3	53.3	53.6	53.6	53.6	53.6	53.6
22	168.2	166.9	167.60	167.6	167.6	167.6	168.1
23	50.8	50.7	51.0	51.0	51.0	51.0	51.2
MeO-Ar			56.7	55.7	56.7	55.7	56.0

Compound **6**, was isolated from the EtOAc extract as yellow amorphous solid. The FAB MS exhibited a molecular ion peak at 290, and the composition of the molecular formula was determined as $C_{15}H_{14}O_6$. The ^1H NMR spectra showed an ABX system represented by the resonances at 7.02 ppm (d, *J*= 1.8 Hz); 6.86 ppm (d, *J*= 1.8, 8.6 Hz) and, 6.76 ppm (d, *J*= 8.6 Hz). At ä 5.90 (d, *J*=2.3 Hz) and 6.01 (d, *J*= 2.3 Hz) were found two protons meta-coupled. Two broad singlet signals integrating for one proton were observed at 4.18 and 4.85 ppm , assigned by HMQC to H-3 and H-4. Finally a doublet of doublet signal at 2.82 (dd, *J*= 4.2, 16.3 Hz) belonging to the protons H-4 confirmed the presence of one flavane skeleton.

The 15 carbon signals in the ^{13}C-NMR spectrum were characterized by a DEPT experiment, which shows that **6** was a flavonoid which have seven quaternary carbons, seven methines, and one methylene. The absence of carbonyl suggested an isoflavan structure. Comparison of the ^{13}C NMR data of **6** with those of reported isoflavan (10, 11) was used to make the assignment of all

peaks in the spectra. The HETCOR experiment and a literature survey established the structure of compound **6** as (-) epicatechine.

Pure indole alkaloids **1-4** were tested *in vitro* for their ability to inhibit the growth of *Leishmania mexicana*. Comparison of the leishmanicidal activity showed that compounds **2** and **3** have the highest values, with IC_{50} of 56 μM and 61 M respectively. (figures 3-6). These values are slightly higher than those reported for two alternative drugs used for the treatment of leishmaniasis: the allylamine terbinafine, IC_{50} 8.5 μM, (12), which blockage at sterol biosynthesis at the level of squalene epoxidase (13), and cationic peptide dermaseptine, IC_{50} 3 μM, which binds to the surface membrane, inducing alteration in the lipid bilayer (14) . We can not give any structure-activity relationships with our results, but it is interesting to point out that mitrajavine **4**, did not show any activity in our assays; however, compounds with the same indole skeleton were reported with high leishmanicidal activity against *Leishmania major* promastigotes (15). Nor did compound **1** show any meaningful difference, in comparison with the results of the control groups.

Although all compounds **1**-**4** have been reported (4, 8), to our knowledge, this is the first report of antileishmanial activities for these alkaloids. The results obtained with the oxindole alkaloids **1**-**3** indicate that the activity could be related to the presence of a substituent with oxygen in the 5 position of the indole ring. Further studies with derivatives of these compounds will be necessary to determine the structure-activity relationship, but they could be considered templates for future antileishmanial drugs.

References

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