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Article

Chemical study of *Adenocalymma axillarum* crude leaf extract and isolated compounds

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Adenocalymma axillarum (K.Schum.) L.G. Lohmann is a liana belonging to the family Bignoniaceae. In traditional medicine, the genus *Adenocalymma* is used to treat fever, skin ailments, and body, joint, and facial muscle pains, and it is also applied as cosmetic. Biological assays conducted with the *A. axillarum* crude leaf ethanol extract have indicated leishmanicidal activity and absence of cytotoxicity. This study aimed to analyze the *A. axillarum* leaf ethanol crude extract by high-performance liquid chromatography–high-resolution mass spectrometry–diode array detector (HPLC–HRMS–DAD) and to evaluate the leishmanicidal and cytotoxic activities of this crude extract, its fractions, and isolated compounds. HPLC–HRMS–DAD analysis of this extract revealed that it consisted mainly of flavonoids, with nine major compounds. Extract purification yielded 4-hydroxy-*N*-methylproline, 6-β-hydroxyipolamiide, quercetin-3-*O*-robinobioside, hyperin, isorhamnetin-3-*O*-robinobioside, and 3'-*O*-methylhyperin, which were identified by Nuclear Magnetic Resonance. The isolated compounds were inactive against *Leishmania amazonensis* promastigotes and human lung fibroblast cells.

Keywords: Cytotoxicity. Flavonol. HPLC-HRMS-DAD. Iridoid. Leishmanicidal.

INTRODUCTION

Adenocalymma axillarum (K.Schum.) L.G.Lohmann, synonym *Memora axillaris*, popularly known as "ciganinha" and "caroba-amarela", is an Angiosperm belonging to the family Bignoniaceae and to the tribe Bignonieae, which predominates in the Neotropics and comprises important neotropical forest components. This family consists of approximately 82 genera and 827 species that occur as shrubs, trees, and climbing plants (Lohmann, Taylor, 2014; Lorenzi, Souza, 2001; Olmstead *et al*., 2009). From a chemical viewpoint, the genus *Adenocalymma* is characterized by the presence of allantoin, terpenoids, diallyl di-, tri-, and tetrasulfides, and flavonoids (de Oliveira *et al*., 2017; Misra *et al*., 1995; Apparao *et al*., 1978; Florentino *et al*., 2016; Grassi *et al*., 2005). Additionally, in folk medicine, this genus is used to treat fever, skin ailments, and body, joint, and facial muscle pains, and it is also applied as cosmetic, to keep the skin soft and moist (Gentry, 1999). Although *A. axillarum* has not been studied yet, its crude extract displays antileishmanial activity.

Leishmaniasis is a tropical disease that is caused by more than twenty *Leishmania* species, including

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Leishmania (*Viannia*) *braziliensis*, *Leishmania* (*Viannia*) *guyanensis*, and *Leishmania* (*Leishmania*) *amazonensis*. The parasite is transmitted through the bite of an insect belonging to the genus *Lutzomyia* (Ghorbani, Farhoudi, 2018). In Brazil, the drugs that are usually employed to treat cutaneous leishmaniasis include meglumine antimoniate, amphotericin B, pentamidine, and pentoxifylline (Ministério da Saúde, 2017). Side effects, high cost, parasite resistance, and the impossibility of administering these drugs to pregnant women justify research into new drugs and alternative treatment to fight leishmaniasis (Hendrickx, Caljon, Maes, 2019).

This study uses a high-performance liquid chromatography–high-resolution mass spectrometry–diode array detector (HPLC–HRMS–DAD) method to analyze the *A. axillarum* bioactive crude leaf extract aiming at improving phytomedicine characterization. In addition, the leishmanicidal action and cytotoxicity of the crude leaf extract, its fractions, and isolated compounds have been investigated.

MATERIAL AND METHODS

General

For TLC analysis, Sigma–Aldrich silica gel plates in aluminum foil with fluorescent indicator were used. For HPLC analysis in the preparative and analytical modes, a Shimadzu LC-6AD chromatograph coupled to a UV-Vis detector model SPD-20A was employed. In addition, a Shimadzu LC-20AD chromatograph linked to an automatic injector model SIL-20A-HT, an oven model CTO-20A, and a DAD detector model SPD-M20A was used in the analytical mode. Shimadzu Shim-pack ODS columns (5 µm, 250 x 4.60 mm, and 250 x 20 mm) and Phenomenex Luna ODS columns (5 µm, 250 x 4.60 mm, and 250 x 10 mm) were employed in the HPLC studies. The crude leaf extract was analyzed by HPLC–HRMS–DAD on a micrOTOF-QII-ESI-TOF Mass Spectrometer (Bruker Daltonics) Shimadzu HPLC LC-20AD; an ODS column (Phenomenex Luna) was used. The data were obtained according to previous conditions (Bertanha *et al*., 2020). Silica ODS from Sigma–Aldrich and Sephadex LH-20 from GE Healthcare were applied as chromatographic support. ^{1}H and ^{13}C Nuclear Magnetic Resonance spectra and 2D and DEPT experiments of the isolated compounds were recorded on the Bruker Advance DRX 400 and 500 spectrometers; the samples were dissolved in D_2O, DMSO-d_6, or CD_3OD from Sigma–Aldrich. The standard verbascoside had been isolated in a previous study (Alvarenga *et al*., 2015).

Plant Material

The *A. axillarum* (K. Schum.) L.G. Lohmann leaves were harvested at Estação Ecológica Jataí, Luiz Antônio, Brazil (21º 35′ 49.5″ S, 47º 47′ 20.2″ W), in October 2015, and they were identified by V.M.M. Gimenez. A voucher specimen (SPFR 16314) was deposited in the Herbarium of the Department of Biology, Laboratory of Plant Systematics, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, University of São Paulo, Brazil (Herbarium, SPFR).

Extraction and isolation

The *A. axillarum* leaves were dried in an oven at 40 ºC, which resulted in 305.4 g of dried material. After drying and milling, the materials were extracted with ethanol by maceration. After the extraction, the material was filtered, and the solvent was removed in a rotary evaporator, which gave 44 g of crude extract. Then, the crude extract (30 g) was dissolved in 500 mL of methanol/water (8:2, v/v) and extracted with hexane and ethyl acetate. The solvents were removed in a rotary evaporator, to afford the following fractions: hexane (3.3 g), ethyl acetate (5.3 g), and hydromethanol (19.7 g). The ethyl acetate fraction (5.3 g) was subjected to solid phase extraction on silica ODS; methanol/water 3:7 (v/v), 5:5 (v/v), and 7:3 (v/v), methanol, and ethyl acetate were used as the mobile phases. This procedure furnished five sub-fractions. Sub-fraction 1 (1.71 g) was submitted to elution with methanol on a Sephadex LH-20 column, to provide 45 sub-fractions. Sub-fraction 10 (51 mg) yielded a mixture of compounds **2** and **3**. A precipitate was separated from sub-fractions 31 and 32, to afford compound **8** (12 mg). Sub-fractions 11–14 (486 mg) were purified on a silica ODS column; the

mobile phase consisted of a methanol/water gradient, which provided 29 fractions. Fractions 2–4 (198 mg) were injected in a semi-preparative-HPLC column and eluted with acetonitrile/water (6:94, v/v) at 4 mL/min. Compounds **2** and **3** were obtained from this experiment at retention times (t_R) 3.4 min (20 mg) and 4.3 min (11 mg), respectively. Sub-fractions 24–25 (100 mg) were purified by preparative-HPLC; the mobile phase was composed by methanol/water (4:6, v/v) at 5 mL/min, which gave compounds **7** (12 mg, t_R 25.3min) and **9** (9 mg, t_R 31.4 min). Fractions 26–28 were subjected to preparative-HPLC in the same condition (methanol/water (4:6, v/v)), which yielded a new fraction (13.4 mg) with t_R 29.9 min, which was again purified by semi-preparative-HPLC in methanol/water (35:65, v/v) at 4 mL/min, to afford compound **10** (1 mg, t_R 55.0 min).

4-hydroxy-*N*-methylproline (**2**). ^{1}H NMR (500 MHz, CD_3OD) δ: 4.49 (m, 1H, H-4), 4.06 (dd, *J*= 7.5 and 11.0 Hz, 1H, H-2), 3.82 (dd, *J*= 4.5 and 12.5 Hz, 1H, H-5β), 3.08 (brd, *J*= 12.5 Hz, 1H, H-5α), 3.00 (s, 3H, N-CH_3), 2.44 (dd, *J*= 7.5 and 14.0 Hz, 1H, H-3α), 2.17 (ddd, *J*= 4.5, 11.0 and 14.0 Hz, 1H, H-3β). ^{13}C NMR (125 MHz, CD_3OD) δ: 171.4 (COOH), 70.5 (C-2), 69.5 (C-4), 62.8 (C-5), 42.7 (N-CH_3), 38.9 (C-3).

6β-hydroxyipolamiide (**3**). ^{1}H NMR (500 MHz, CD_3OD) δ: 7.49 (s, 1H, H-3), 5.84 (brs, 1H, H-1), 4.59 (d, 1H, H-1′), 4.04 (m, 1H, H-6), 3.90 (m, 1H, H-6′), 3.73 (s, 3H, H-12), 3.65 (m, 1H, H-6′), 3.36 (m, 2H, H-5′and H-3′), 3.27 (m, 1H, H-4′), 3.18 (m, 1H, H-2′), 2,56 (s, 1H, H-9), 2,00 (dd, *J*= 6.1 and 13.0 Hz, 1H, H-7), 1.89 (dd, *J*= 8.0 and 13.0 Hz, 1H, H-7), 1.13 (s, 3H, H-10). ^{13}C NMR (100 MHz, D_2O) δ: 168.2 (C-11), 153.7 (C-3), 111.9 (C-4), 98.5 (C-1′), 93.3 (C-1), 76.3 (C-5′), 75.2 (C-3′), 73.9 (C-8), 73.6 (C-6), 72.4 (C-2′), 70.2 (C-5), 69.6 (C-4′), 60.6 (C-6′), 58.2 (C-9), 51.9 (12-$COOCH_3$), 46.1 (C-7), 22.6 (C-10).

Quercetin-3-*O*-robinobioside (**7**). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.58 (brs, 1H, 5-OH), 7.65 (dd, *J*= 2.0 and 8.5 Hz, 1H, H-6′), 7.52 (d, *J*= 2.0 Hz, 1H, H-2′), 6.82 (d, *J*= 8.5 Hz, 1H, H-5′), 6.38 (brs, 1H, H-8), 6.18 (brs, 1H, H-6), 5.31, (d, *J*= 7.5 Hz, 1H, H-1″), 4.42 (brs, 1H, H-1‴), 3.60-3.55 (m, 4H, H-2″, H-4″, H-5″ and H-6″), 3.40-3.30 (m, 4H, H-3″, H-2‴, H-3‴ and H-5‴), 3.24 (dd, *J*= 6.2 and 9.5, 1H, H-6″), 3.09 (t, *J*= 9.3, 1H, H-4‴), 1.06 (d, *J*= 6.2 Hz, 3H, H-6‴).^{13}C NMR (100 MHz, DMSO-d_6) δ: 177.3 (C-4), 164.4 (C-7), 161.1 (C-5), 156.3 (C-2 and C-9), 148.5 (C-4′), 144.8 (C-3′), 133.4 (C-3), 121.8 (C-6′), 120.9 (C-1′), 115.9 (C-2′), 115.1 (C-5′), 103.7 (C-10), 102.0 (C-1″), 99.9 (C-1‴), 98.7 (C-6), 93.5 (C-8), 73.5 (C-5″), 73.0 (C-3″), 71.8 (C-4‴), 71.0 (C-2″), 70.5 (C-3‴), 70.3 (C-2‴), 68.2 (C-5‴), 68.0 (C-4″), 65.0 (C-6″), 17.8 (C-6‴).

Hyperin (**8**). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.61 (brs, 1H, 5-OH), 7.66 (dd, *J*= 2.2 and 8.5 Hz, 1H, H-6′), 7.53 (d, *J*= 2.2 Hz, 1H, H-2′), 6.80 (d, *J*= 8.5 Hz, 1H, H-5′), 6.36 (d, *J*= 2.0 Hz, 1H, H-8), 6.16 (d, *J*= 2.0 Hz, 1H, H-6), 5.36, (d, *J*= 7.6 Hz, 1H, H-1″), 3.64 (d, *J*= 3.2 Hz, 1H, H-4″), 3.57 (dd, *J*= 7.6 and 9.2 Hz, 1H, H-2″), 3.46 (dd, *J*= 5.0 and 9.8 Hz, 1H, H-6″), 3.37 (dd, *J*= 3.2 and 9.2 Hz, 1H, H-3″), 3.32 (dd, *J*= 5.0 and 9.8 Hz, 1H, H-5″), 3.28 (t, *J*= 5.0 Hz, 1H, H-6″).^{13}C NMR (100 MHz, DMSO-d_6) δ: 177.2 (C-4), 161.1 (C-5 and C-7), 156.3 (C-2), 155.9 (C-9), 148.5 (C-4′), 144.8 (C-3′), 133.3 (C-3), 121.9 (C-6′), 120.9 (C-1′), 115.8 (C-2′), 115.1 (C-5′), 103.4 (C-10), 101.9 (C-1″), 98.9 (C-6), 93.6 (C-8), 75.7 (C-5″), 73.1 (C-3″), 71.1 (C-2″), 67.8 (C-4″), 60.0 (C-6″).

Isorhamnetin-3-*O*-robinobioside (**9**). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.55 (brs, 1H, 5-OH), 7.99 (brs, 1H, H-2′), 7.49 (dd, *J*= 1.5 and 8.5 Hz, 1H, H-6′), 6.89 (d, *J*= 8.5 Hz, 1H, H-5′), 6.35 (brs, 1H, H-8), 6.12 (brs, 1H, H-6), 5.43, (d, *J*= 8.0 Hz, 1H, H-1″), 4.42 (brs, 1H, H-1‴), 3.85 (s, 3H, 3′-OCH_3), 3.64-3.56 (m, 4H, H-2″, H-4″, H-5″ and H-6″), 3.44-3.40 (m, 3H, H-3″, H-2‴and H-5‴), 3.30 (m, 2H, H-6″and H-3‴), 3.09 (t, *J*= 9.3, 1H, H-4‴), 1.05 (d, *J*= 6.2 Hz, 3H, H-6‴).^{13}C NMR (100 MHz, DMSO-d_6) δ: 176.9 (C-4), 164.6 (C-7), 161.0 (C-5), 156.5 (C-2 and C-9), 149.4 (C-3′), 146.9 (C-4′), 132.9 (C-3), 121.8 (C-6′), 121.0 (C-1′), 115.1 (C-5′), 113.3 (C-2′), 103.2 (C-10), 101.9 (C-1″), 100.0 (C-1‴), 99.2 (C-6), 93.9 (C-8), 73.4 (C-5″), 72.9 (C-3″), 71.8 (C-4‴), 71.1 (C-2″), 70.5 (C-3‴), 70.3 (C-2‴), 68.2 (C-5‴), 67.9 (C-4″), 65.1 (C-6″), 55.8 (3′-OCH_3), 17.8 (C-6‴).

3’-*O*-methylhyperin (**10**). ^{1}H NMR (500 MHz, CD_3OD) δ: 8.03 (d, *J*= 2.0 Hz, 1H, H-2′), 7.58 (dd, *J*= 2.0 and 8.5 Hz,

1H, H-6′), 6.90 (d, *J*= 8.5 Hz, 1H, H-5′), 6.39 (d, *J*= 2.0 Hz, 1H, H-8), 6.19 (d, *J*= 2.0 Hz, 1H, H-6), 5.33, (d, *J*= 8.0 Hz, 1H, H-1″), 3.96 (s, 3H, 3′-OCH_3), 3.84 (m, 1H, H-4″), 3.81 (m, 1H, H-2″), 3.66 (dd, *J*= 6.0 and 11.0 Hz, 1H, H-6″), 3.58 (m, 1H, H-6″), 3.55 (m, 1H, H-3″), 3.47 (t, *J*= 6.0 Hz, 1H, H-5″).^{13}C NMR (125 MHz, CD_3OD) δ: 165.0 (C-7), 157.0 (C-2 and C-9), 149.0 (C-3′), 147.0 (C-4′), 134.0 (C-3), 122.1 (C-6′), 122.0 (C-1′), 114.5 (C-5′), 113.0 (C-2′), 104.0 (C-10), 101.0 (C-1″), 98.5 (C-6), 93.1 (C-8), 75.8 (C-5″), 73.8 (C-3″), 72.0 (C-2″), 68.5 (C-4″), 60.8 (C-6″), 55.6 (3′-OCH_3).

Leishmanicidal assay

The leishmanicidal activity was determined by using *Leishmania amazonensis* promastigotes (IFLA/BR/67/PH8) according to an earlier reference (Andrade *et al*., 2018). Amphotericin B was employed as positive control.

Cytotoxicity assay

The cytotoxic activity was evaluated by measuring the effects of the samples on the normal human lung fibroblast cell line (GM07492A); XTT was used according to a previously described method (Alvarenga *et al*., 2015).

RESULTS AND DISCUSSION

The *Adenocalymma axillarum* crude leaf ethanol extract (CE) at 50 µg/mL presented leishmanicidal activity with 48.77 ± 10.99 % of flagellar motility inhibition against *Leishmania amazonensis* promastigotes, as shown in Table I. CE was further tested against the human lung fibroblasts cell line (GM07492A), which revealed that and was not cytotoxic (CC_{50} > 2500 µg/mL).

Table I - Antileishmanial activity toward *Leishmania amazonensis* promastigotes and cytotoxicity against the human lung fibroblast cell line (GM07492A) of *Adenocalymma axillarum* leaf crude ethanol extract, fractions, and isolated compounds.

	***Leishmania amazonensis* promastigote % flagellar motility inhibition ± S.D.**		**Cell line GM07492A CC_{50}**	
samples	**[50 µg/mL]**	**[50 µM]**	**[µg/mL]**	**[µM]**
CE	48.77 ± 10.99	-	> 2500	-
F-H	n.a.	-	1550 ± 90	-
F-AC	n.a.	-	466.6 ± 23.3	-
F-HM	n.a.	-	> 2500	-
2	-	n.a.	> 2500	> 17241
3	-	n.a.	> 2500	> 5924
7	-	n.a.	> 1000	> 1639
8	-	n.a.	> 2000	> 4303
9	-	n.a.	> 500	> 801
10	-	n.a.	> 1000	> 2092
Amphotericin B	-	100±0.00[a]		

Means ± S.D. (standard deviation), GM07492A (human lung fibroblasts), CC_{50} 50% cytotoxic concentration values after treatment for 24 h, n.a. not active, [a]Amphotericin B was tested at 1.56 µM, CE *Adenocalymma axillarum* leaf crude ethanol extract, F-H hexane fraction, F-AC ethyl acetate fraction, F-HM hydromethanol fraction, **2** 4-hydroxy-*N*-methylproline, **3** 6-β-hydroxyipolamiide, **7** quercetin-3-*O*-robinobioside, **8** hyperin, **9** isorhamnetin-3-*O*-robinobioside, and **10** 3'-*O*-methylhyperin.

Next, we analyzed CE by HPLC–HRMS–DAD between 200 and 800 nm, in the positive and negative modes. The chromatogram was acquired by using a methanol/water (+ 0.1% acetic acid) gradient from 5 to 100% methanol for 35 min, which was followed by elution with 100% methanol for 10 min. We aimed to characterize the CE components better, to obtain additional information about the metabolic profile. The DAD detector provided a chromatogram (Figure 1a) that displayed nine main peaks. The UV spectra of the peaks at t_R 10.6, 11.2, 12.2, and 13.0 min (Supplementary information) closely resembled the spectra of flavonols, with λ_{max} around 255 and 353 nm (de Villiers, Venter, Pasch, 2016). Figure 1b contains the data that we obtained for the base peak chromatogram (BPC) in the positive mode; the chromatogram revealed six peaks. In the negative mode, nine peaks emerged in the chromatogram (Figure 1c).

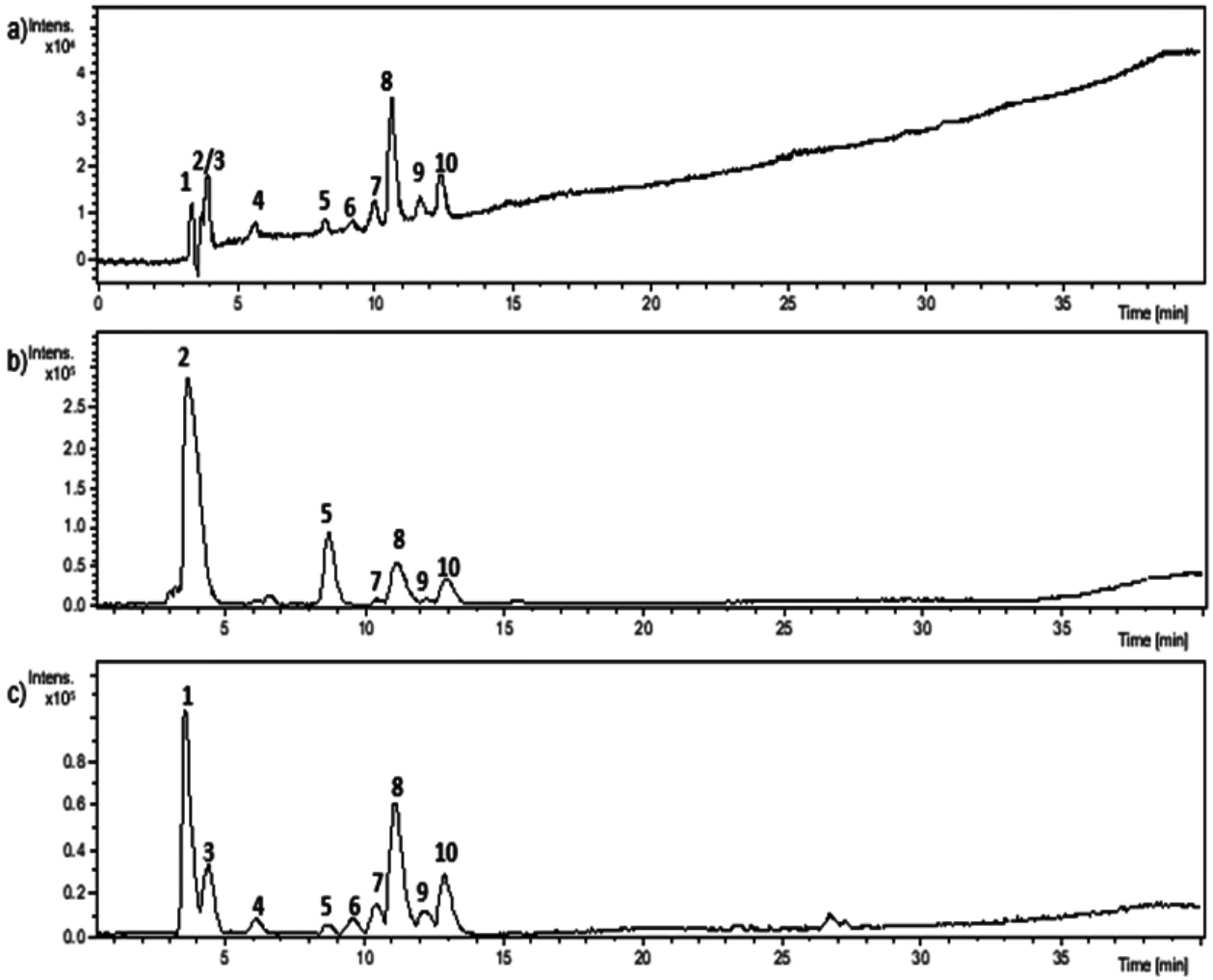

Figure 1 - a) Chromatogram and spectrum in the Ultraviolet/Visible region (200–800 nm); b) Base peak chromatogram in the positive mode and c) Base peak chromatogram in the negative mode of the *Adenocalymma axillarum* crude leaf ethanol extract. Chromatographic conditions: methanol/water (+ 0.1% acetic acid) linear gradient from 5 to 100% methanol for 35 min, and 100% methanol for 10 min. The flow-rate was 1.0 mL/min. An ODS column (Phenomenex Luna) was employed.

The mass spectra helped to identify the ions that corresponded to each peak. We verified one or two protonated and/or deprotonated ions ($[M + H]^+$ and/or $[M + H]^-$) in the analysis. We used the exact mass that we had achieved to determine the possible molecular formulae. We also calculated the errors in ppm. We searched the obtained formulae in the Natural Products Dictionary and SciFinder database; we used *Adenocalymma* and *Memora* to restrict the search. The mass spectra suggested the presence of the compounds quinic acid, 4-hydroxy-*N*-methylproline, 6β-hydroxyipolamiide, *O*-caffeoyl quinic acid, verbascoside, hyperin, and 3'-*O*-methylhyperin (Table II). Isolation of the compounds later confirmed their presence. Quercetin-3-*O*-robinobioside and isorhamnetin-3-*O*-robinobioside had not been reported in the genus yet, and isolation and re-analysis of the HRMS data confirmed their presence in CE. Additionally, we identified verbascoside by comparison with the standard compound. CE consisted mainly of flavonols. According to Blatt, dos Santos, Salatino (1998), flavones are rarely found in species belonging to the tribe Bignonieae, so our results agree with previous data.

Table II - Compounds identified by high-performance liquid chromatography–high-resolution mass spectrometry (HPLC–HRMS) data in *Adenocalymma axillarum* leaf crude ethanol extract.

peak	*m/z*	t_R (min)	ion	Molecular formula	Identification	Error (ppm)	Ref.
1	191.0557	3.7	$[M-H]^-$	$C_7H_{11}O_6$	Quinic acid	0.52	de Oliveira *et al*., 2017
2	146.0808	3.9	$[M+H]^+$	$C_6H_{12}NO_3$	4-hydroxy-*N*-methylproline	-2.74	Grassi *et al*., 2005
3	421.1325	4.5	$[M-H]^-$	$C_{17}H_{25}O_{12}$	6β-hydroxylpolamiide	-4.98	Grassi *et al*., 2005
4	353.0840	6.2	$[M-H]^-$	$C_{16}H_{17}O_9$	*O*-caffeoyl quinic acid	-9.35	de Oliveira *et al*., 2017
5	307.1761	8.8	$[M+H]^+$	$C_{14}H_{27}O_7$	n.i.	1.30	A
5	305.1577	8.8	$[M-H]^-$	$C_{14}H_{25}O_7$		-7.54	
6	623.1952	9.7	$[M-H]^-$	$C_{29}H_{35}O_{15}$	verbascoside	-3.85	B
7	609.1435	10.6	$[M-H]^-$	$C_{27}H_{29}O_{16}$	Quercetin-3-*O*-robinobioside	-3.44	C
7	611.1588	10.6	$[M+H]^+$	$C_{27}H_{31}O_{16}$		-3.93	
8	465.1018	11.2	$[M+H]^+$	$C_{21}H_{21}O_{12}$	Hyperin	1.07	Grassi *et al*., 2005, c
8	463.0851	11.2	$[M-H]^-$	$C_{21}H_{19}O_{12}$		-5.61	
9	623.1606	12.2	$[M-H]^-$	$C_{28}H_{31}O_{16}$	Isorhamnetin-3-*O*-robinobioside	-0.96	C
9	625.1752	12.2	$[M+H]^+$	$C_{28}H_{33}O_{16}$		-2.72	
10	479.1177	13.0	$[M+H]^+$	$C_{22}H_{23}O_{12}$	3'-*O*-methylhyperin	-2.71	Grassi *et al*., 2005, c
10	477.1011	13.0	$[M-H]^-$	$C_{22}H_{21}O_{12}$		-4.61	

m/z mass/charge, t_R retention time, ppm parts-per-million, n.i. not identified, Ref. references, a three possible compounds by using Dictionary of Natural Products database, b identified by comparison with standard compound, and c identified by isolation and NMR data.

Thus, we selected CE for fractionation to isolate its bioactive compounds. We suspended the crude extract in methanol/water (8:2, v/v) and submitted it to liquid-liquid extraction with hexane and ethyl acetate, to obtain three main fractions. We evaluated the resulting fractions against *L. amazonensis* promastigotes and human lung fibroblasts (GM07492A) (Table I). For the leishmanicidal activity, the results listed in Table I indicated that this activity was lost during fractionation when CE was evaluated at 50 µg/mL. As for cytotoxicity, the obtained fractions; that is, the hexane (F-H, CC_{50}= 1550 ± 90 µg/mL) and the ethyl acetate (F-AC, CC_{50}= 466.6 ± 23.3 µg/mL) fractions, presented increased cytotoxic activity as compared to CE, especially F-AC. The hydromethanol fraction (F-HM) was inactive and displayed CC_{50} > 2500 µg/mL. Among the obtained fractions, F-AC presented increased cytotoxic activity and provided the best CC_{50} values among the samples evaluated in this study. Therefore, we decided to use F-AC, the most promising fraction, in the isolation steps.

We subjected F-AC to solid phase extraction by using silica ODS and methanol/water. We purified sub-fraction 1 on a Sephadex LH-20 column eluted with methanol, which furnished compound **8** and a mixture of compounds **2**, **3**. We further purified compounds **2**, **3**, **7**, **9**, and **10** by preparative-RP-HPLC. We identified the major isolated compounds (Figure 2) as 4-hydroxy-*N*-methylproline (**2**), 6-β-hydroxyipolamiide (**3**), quercetin-3-*O*-robinobioside (**7**), hyperin (**8**), isorhamnetin-3-*O*-robinobioside (**9**), and 3'-*O*-methylhyperin (**10**) on the basis of the 1D and 2D NMR data and by comparison with previously reported data (Grassi *et al*., 2005; Sciuto *et al*., 1983; Ajaghaku *et al*., 2018; Buschi, Pomilio, 1982; Agrawal, 1989; Mendez, Bilia, Morelli, 1995). Moreover, this is the first report on the presence of quercetin-3-*O*-robinobioside and isorhamnetin-3-*O*-robinobioside in this genus.

We assayed the isolated compounds (**2**, **3**, **7**–**10**) against human lung fibroblast (GM07492A) cells and *Leishmania amazonensis* promastigotes (Table I). Compounds **2** and **3** and **7**–**10** were cytotoxic to the normal cells, with CC_{50} > 17241 µg/mL, CC_{50} > 5924 µg/mL, CC_{50} > 1639 µg/mL, CC_{50} > 4303 µg/mL, CC_{50} > 801 µg/mL, and CC_{50} > 2092 µg/mL, respectively. The *Leishmania amazonensis* promastigotes were not susceptible to the isolated compounds (**2**, **3**, **7**–**10**) at 50 µM.

The *in vitro* leishmanicidal activity of several plant crude extracts have been screened, and active compounds, such as alkaloids, furanocoumarins, flavonoids, terpenoids, and phenylpropanoids, have been well identified for some species (Ullah *et al*., 2016; Tiwari *et al*., 2018). Nevertheless, the obtained compounds were inactive even though the crude extract was active. Furthermore, verbascoside, which was detected in the crude extract, has antileishmanial activity. Verbascoside presented an IC_{50} of 19 µM (11.9 µg/mL) against *Leishmania amazonensis* promastigotes (Maquiaveli *et al*., 2016). On the other hand, the compounds of various herbal extracts are known to act synergistically (Caesar, Cech, 2019).

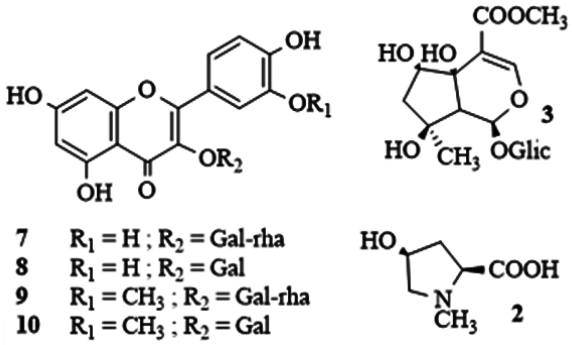

Figure 2 - Compounds isolated from *Adenocalymma axillarum* crude leaf ethanol extract.

CONCLUSION

We have described the leishmanicidal and cytotoxic activities of *Adenocalymma axillarum* crude leaf ethanol extract and the isolation of compounds **7** and **10** for the first time in the genus. The isolated compounds were inactive against *Leishmania amazonensis* promastigotes and human lung fibroblast cells. The isolated compounds confirmed that the genus *Adenocalymma* accumulates flavonols, which is important in chemotaxonomy studies, since the tribe Bignoniae has recently been reclassified.

SUPPLEMENTARY INFORMATION

NMR data of compounds **2** and **3** and **7**–**10**. HPLC-HRMS-DAD data.

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