Acta Brasiliensis 5(1): 44-47, 2021 *Note*
http://revistas.ufcg.edu.br/ActaBra http://dx.doi.org/ 10.22571/2526-4338429

In vitro antileishmanial activity of *Anacardium othonianum* and isolated compounds against *Leishmania amazonensis*

Tavane Aparecida Alvarenga[a], Osvaine Junior Alvarenga Alves[a], Mariana Cintra Pagotti[a], Wilson Roberto Cunha[b], Márcio Luís Andrade e Silva[b], Juliana de Fátima Sales[c], Fabiano Guimarães Silva[c], Ana Helena Januario[b], Lizandra Guidi Magalhães[b], Patrícia Mendonça Paulettia[b*]

[a] *Programa de Pós-graduação em Ciências, Universidade de Franca, Franca, 14404-600, São Paulo, Brasil.*
** patricia.pauletti@unifran.edu.br*
[b] *Universidade de Franca, Franca, 14404-600, São Paulo, Brasil.*
** patricia.pauletti@unifran.edu.br*
[c] *Instituto Federal de Educação, Ciência e Tecnologia Goiano, Rio Verde, Rio Verde, 75901-970, Goiás, Brasil.*

Received: July 20, 2020 / Accepted: October 27, 2020/ Published online: January 27, 2021

Abstract

This study analyzes the antileishmanial activity of the crude ethanol extract, fractions, and isolated compounds of *A. othonianum* nuts. Antileishmanial activity was evaluated against *Leishmania amazonensis* promastigotes *in vitro*. The phytochemical study was performed by high-performance liquid chromatography-high-resolution mass spectrometry-diode array detector (HPLC-HRMS-DAD) and by preparative HPLC. HPLC-HRMS-DAD analysis of the bioactive extract confirmed the presence of ten alkyl phenol derivatives that had previously been isolated from *A. occidentale*. Bioassay-guided isolation afforded cardanol triene, cardanol diene, cardanol monoene, cardol triene, anacardic acid triene, anacardic acid diene, and anacardic acid monoene. Cardol triene gave an IC_{50} of 80.66 µM. The obtained data suggest that the evaluated extract, fractions, and cardol triene had moderate activity against *L. amazonensis* promastigotes. This is the first description of alkyl phenols in *A. othonianum*.

Keywords: Alkyl phenols, Anacardiaceae, anacardic acid, cardol.

Atividade antileishmania in vitro de *Anacardium othonianum* e substâncias isoladas em *Leishmania amazonensis*

Resumo

Este estudo visou avaliar a atividade antileishmanial do extrato bruto etanólico, frações e substâncias isoladas obtidos das castanhas de *A. othonianum*. A atividade antileishmania foi avaliada por ensaio *in vitro* nas formas promastigotas de *Leishmania amazonensis*. O estudo fitoquímico foi realizado por cromatografia líquida de alta eficiência - espectrometria de massa de alta resolução - detector de arranjo de diodos (CLAE-EMAR-DAD) e por CLAE-preparativa. A análise por CLAE-EMAR-DAD do extrato bioativo confirmou a presença de dez derivados de alquil-fenóis previamente isolados de *A. occidentale*. A investigação bioguiada resultou no isolamento do cardanol trieno, cardanol dieno, cardanol monoeno, cardol trieno, ácido anacárdico trieno, ácido anacárico dieno e ácido anacárico monoeno. O cardol trieno apresentou uma CI_{50} de 80,66 µM. Os dados obtidos sugeriram que o extrato, frações e o cardol trieno possuem atividade moderada no ensaio nas formas promastigotas de *L. amazonensis*. Este é o primeiro relato de alquil-fenóis em *A. othonianum*.

Palavras-chave: alquil-fenóis, Anacardiaceae, ácido anacárdico, cardol.

Anacardium othonianum (Rizz.) (Anacardiaceae) is a native species of the Brazilian Cerrado. This species produces cashew fruit, which is popularly known as "caju-do-cerrado", "cajuzinho", and "cajuí". *A. othonianum* has regional importance: its pseudofruit is consumed fresh or in juices, liqueurs, and sweets, and its fruit (cashew nuts) is consumed after being roasted with salt (Souza & Silva, 2015). In traditional medicine, *A. othonianum* is used to treat infections, inflammations, respiratory pathologies (cough and flu), diabetes, and pain (Bessa *et al*., 2013).

The crude ethanol extract of *A. othonianum* leaves presents antifungal activity. In addition, the isolated compounds amentoflavone, gallic acid, protocatechuic acid, and ethyl 3,4,5-trimethoxybenzoate have been isolated and

also shown to display antifungal activity (Curado *et al*., 2016). Nevertheless, the crude extract obtained from *A. othonianum* nuts has not yet been investigated.

A. occidentale L. nuts are known to be a source of alkyl phenols, which exhibit several biological activities. These phenols inhibit *Trypanosoma cruzi* sirtuin and exert anti-*T. cruzi* (Matutino Bastos *et al*., 2019), antiplasmodial (Gimenez *et al*., 2019), schistosomicidal (Alvarenga *et al*., 2016), and molluscicidal activity (Kubo, Komatsu & Ochi, 1986), among other activities.

Protozoan parasites of the genus *Leishmania* are the etiological agent of the disease leishmaniasis, which affects the skin, mucous membranes, and internal organs. Between 700,000 and 1 million new cases of leishmaniasis are estimated to emerge annually (WHO, 2020). This disease affects poor regions in Africa, Asia, and Latin America (WHO, 2020). The treatment of leishmaniasis consists of the use of antimonials, amphotericin B, pentamidine, paromomycin, and miltefosine. However, these drugs are costly, difficult to administer, and highly toxic, not to mention the emerging resistance to them (Ghorbani & Farhoudi, 2018; Hendrickx, Caljon & Maes, 2019; Charlton, Rossi-Bergmann, Denny & Steel, 2018).

As part of our ongoing research into *Anacardium* (Alvarenga *et al*., 2016; Curado *et al*., 2016; Gazzola *et al*., 2017; Gimenez *et al*., 2019), and because the crude extract of *Anacardium othonianum* (Rizz.) (Anacardiaceae) nuts has been demonstrated to display antileishmanial activity *in vitro* (Alvarenga, 2020), this study analyzes the antileishmanial activity of the crude ethanol extract, fractions, and isolated compounds of *A. othonianum* nuts.

Analytical HPLC data were obtained on a Shimadzu LC-20AD chromatograph (Kyoto, Japan) equipped with a DAD detector (SPD-M20A), an automatic injector (SIL-20A-HT), and an oven (CTO-20A). Preparative HPLC purification steps were run on a Shimadzu LC-6AD chromatograph (Kyoto, Japan) equipped with a UV-Vis detector (SPD-20A). HPLC columns were as follows: Shimadzu Shim-pack ODS columns (5 μm, 250 x 4.60 mm, and 250 x 20 mm) and Phenomenex Luna ODS column (5 μm, 250 x 4.60 mm). TLC analyses were performed on Sigma–Aldrich silica gel plates on aluminum foil with fluorescence indicator. Octadecyl silica (ODS) and silica gel 60 (Sigma–Aldrich) were used as chromatographic support.

The Nuclear Magnetic Resonance data of the compounds dissolved in $CDCl_3$ (Sigma-Aldrich) were acquired on Bruker Avance DRX 400 and 500 spectrometers (Billerica, MA, USA). The HPLC-HRMS-DAD data of the crude ethanol extract of cashew nuts were obtained on an LC-20AD HPLC (Shimadzu, Kyoto, Japan) and micrOTOF-QII ESI mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The same parameters described by Bertanha *et al*. (2020) were applied. An ODS column (Phenomenex) was employed. The mobile phase consisted of 70:30 acetonitrile/water (+ 0.2% formic acid) for 60 min, followed by a linear gradient from 70 to 100% acetonitrile for 10 min, and 100% acetonitrile for 10 min. The flow rate was 1.0 mL/min.

The fruits of species *Anacardium othonianum* Riss. (Anacardiaceae) were provided by Prof. Dr. Fabiano Silva. A voucher specimen (HJ3793) was deposited in the Herbarium Jataiense Germano Guarim Neto of the Federal University of Goiás, Brazil (Herbarium HJ). The license to access genetic heritage was SisGen #AC7C911.

The air-dried powdered nuts (62.3 g) were extracted with ethanol (Alvarenga *et al*., 2016). After filtration, the solvent was removed under reduced pressure to yield 14.3 g of the extract. The crude ethanol extract (CEE, 7.0 g) was submitted to solid-phase extraction on octadecyl silica (ODS); methanol/water (80:20, 90:10, and 100:0 v/v) was used as mobile phase. This procedure yielded three fractions (FR1, methanol/water 80:20, 4.24 g; FR2, methanol/water 90:10, 2.26 g; and FR3, methanol, 0.40 g). Fraction 1, which was eluted with methanol/water 80:20 (v/v, 4.2 g), was fractionated by chromatography on silica; hexane/EtOAc (ethyl acetate) was the mobile phase, yielding two hundred and thirty-two subfractions of 10 mL, which were combined in twenty-six subfractions by using TLC data. Subfractions 12–20 (75 mg) were submitted to preparative RP-HPLC purification with acetonitrile/water (98:2, v/v), with UV detection at 220 nm and flow rate of 5 mL/min, which afforded eight subfractions. Subfractions 3, 5, and 7 gave compounds **1** (3.5 mg, t_R 22.9 min), **2** (1.7 mg, t_R 28.2 min), and **3** (3.4 mg, t_R 37.5 min), respectively. Subfractions 30−85 (1.3 g) were chromatographed on octadecyl silica (ODS), and an acetonitrile/water gradient was employed as mobile phase. Subfraction 2 gave compound **4** (193.9 mg).

Subfractions 172–220 (318 mg) were also chromatographed by preparative RP-HPLC with acetonitrile/water (99:1, v/v), with UV detection at 220 nm and flow rate of 5 mL/min, yielding four fractions. These procedures led to the isolation of compounds **5** (71.0 mg, t_R 22.0 min), **6** (18.1 mg, t_R 27.7 min), and **7** (12.0 mg, t_R 37.2 min).

The extract, fractions, and compounds were evaluated against *Leishmania amazonensis* (MHOM/BR/PH8) promastigotes (1 x 10^6 parasites per well) according to a previous reference (Andrade *et al*., 2018). The extract and fractions were tested at 50 μg/mL; compounds were tested at 100, 80, 50, 25, and 12.5 μM. Amphotericin B was used as positive control; the negative control was RPMI 1640 medium containing 0.1% DMSO. The IC_{50} (50% *L. amazonensis* promastigote flagellar motility inhibition) was obtained by using GraphPad Prism 6.

The crude ethanol extract (CEE) inhibited 96.00% ± 1.47% of the *Leishmania amazonensis* promastigote flagellar motility. The CEE was purified by solid-phase extraction, which yielded three fractions. Fractions 1 (FR1) and 3 (FR3) inhibited flagellar motility the most effectively, by 86.48 ± 0.95 and 83.54 ± 1.15%, respectively, in comparison with Fraction 2 (FR2 = 50.47 ± 2.52%). Thus, activity was maintained after CEE fractionation.

The CEE chromatogram was obtained between 200 and 800 nm, and in the negative mode. High-resolution mass spectra provided the exact mass for the obtained peaks and was employed to calculate the molecular formulae and errors (Table 1). The molecular formulae already described in the literature (Correia, David & David, 2006) for compounds from the genus *Anacardium* were compared with the molecular formulae obtained in this study. Thus, CEE analysis by HPLC-HRMS-DAD revealed the presence of ten

known alkyl phenols, namely cardol triene, cardol diene, anacardic acid triene, cardanol triene, anacardic acid diene, cardanol diene, anacardic acid monoene, cardanol monoene, anacardic acid, and cardanol. This indicates that *A. othonianum* is also a rich source of these compounds.

Table 1. HPLC–HRMS data for the compounds identified in the crude ethanol extract (CEE) obtained from *A. othonianum*.

t_R (min)	*m/z*	Ion	Molecular formula	Tentative Identification	Error (ppm)
19.8	313.2173	$[M-H]^-$	$C_{21}H_{29}O_2$	Cardol triene	-0.0
	627.4416	$[2M-H]^-$	$C_{42}H_{59}O_4$	Cardol triene	-0.28
28.7	315.2316	$[M-H]^-$	$C_{21}H_{31}O_2$	Cardol diene	-1.35
	631.4713	$[2M-H]^-$	$C_{42}H_{63}O_4$	Cardol triene	-1.88
45.5	341.2137	$[M-H]^-$	$C_{22}H_{29}O_3$	Anacardic acid triene	1.48
	683.4310	$[2M-H]^-$	$C_{44}H_{59}O_6$	Anacardic acid triene	-0.71
	297.2220	$[M-H]^-$	$C_{21}H_{29}O$	Cardanol triene	-0.39
66.4	343.2280	$[M-H]^-$	$C_{22}H_{31}O_3$	Anacardic acid diene	0.13
	687.4600	$[2M-H]^-$	$C_{44}H_{63}O_6$	Anacardic acid triene	-3.01
	299.2374	$[M-H]^-$	$C_{21}H_{31}O$	Cardanol diene	-0.64
68.5	343.2267	$[M-H]^-$	$C_{22}H_{31}O_3$	n.i.	-1.17
	299.2372	$[M-H]^-$	$C_{21}H_{31}O$	n.i.	-0.84
69.3	331.2265	$[M-H]^-$	$C_{21}H_{31}O_3$	n.i.	-1.37
70.6	369.2425	$[M-H]^-$	$C_{24}H_{33}O_3$	n.i.	-1.02
	325.2517	$[M-H]^-$	$C_{23}H_{33}O$	n.i.	-1.99
71.3	369.2422	$[M-H]^-$	$C_{24}H_{33}O_3$	n.i.	-1.32
71.7	387.2523	$[M-H]^-$	$C_{24}H_{35}O_4$	n.i.	-1.78
72.2	319.2265	$[M-H]^-$	$C_{20}H_{31}O_3$	n.i.	-1.37
	275.2371	$[M-H]^-$	$C_{19}H_{31}O$	n.i.	-0.94
73.8	345.2419	$[M-H]^-$	$C_{22}H_{33}O_3$	Anacardic acid monoene	-1.62
	301.2527	$[M-H]^-$	$C_{21}H_{33}O$	Cardanol monoene	-0.99
79.0	347.2580	$[M-H]^-$	$C_{22}H_{35}O_3$	Anacardic acid	-1.17
	373.2726	$[M-H]^-$	$C_{24}H_{37}O_3$	n.i.	-2.22
	303.2689	$[M-H]^-$	$C_{21}H_{35}O$	Cardanol	-0.44

n.i. not identified

FR1 had higher yield and was purified by various chromatographic purification steps to give the main compounds (Figure 1): cardanol triene (1), cardanol diene (2), cardanol monoene (3), cardol triene (4), anacardic acid triene (5), anacardic acid diene (6), and anacardic acid monoene (7). These compounds were identified by NMR and by comparison with previously published data (Alvarenga *et al.*, 2016; Lomonaco *et al.*, 2012).

Compound 4 displayed leishmanicidal activity. Its IC_{50} was 80.66 µM (95% confidence interval [CI], 80.44 to 80.87 µM). Compounds 1–3 and 5–7 provided IC_{50} > 100 µM. These data suggest that the presence of an extra hydroxyl group at the aromatic ring as well as the absence of the acid group improved the activity of compound 4 in relation to compounds 1 and 5, which also presented three double bonds at the C_{15} carbon chain.

According to previously published data (Alvarenga *et al.*, 2016), cardol diene and 2-methyl cardol diene present schistosomicidal activity against *Schistosoma mansoni* adult worms, with LC_{50} of 32.2 and 14.5 µM, respectively. Additionally, these compounds are active against *Plasmodium falciparum* D6 strain, with IC_{50} of 5.69 and 5.39 µM, respectively (Gimenez *et al.*, 2019). Cardol triene has higher activity against trypomastigote and amastigote forms of *T. cruzi*, with EC_{50} of 23.36 ± 0.12 and 11.75 ± 0.40 µM, respectively, in comparison to cardanol triene and anacardic acid triene (Matutino Basto *et al.*, 2019). Mixtures of anacardic acid, cardol, and cardanol display good larvicidal action against *Aedes aegypti*, with LC_{50} of 12.40 ± 0.10 ppm, 5.55 ± 0.07 ppm, and 8.20 ± 0.15 ppm, respectively (Oliveira *et al.*, 2011). For *Biomphalaria glabrata*, the snail vector of schistosomiasis, evaluations showed that anacardic acid triene is the most active molluscicidal agent (LD_{50} = 0.3 ppm), followed by cardol triene (LD_{50} = 15 ppm), with cardanol triene being the least active agent (LD_{50} = 80 ppm) (Kubo *et al.*, 1986).

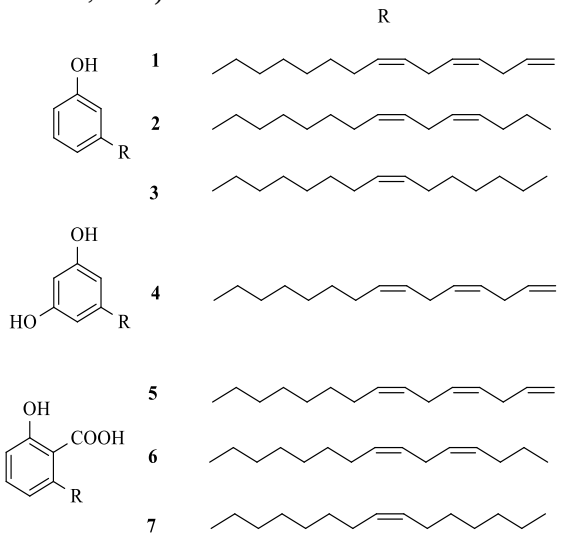

Figure 1. Chemical structure of the isolated compounds.

Anacardium othonianum extract, fractions, and cardol triene can moderately inhibit *L. amazonensis* promastigote flagellar motility. Additionally, the occurrence of alkyl phenols in *A. othonianum* has been confirmed for the first time. Nevertheless, further *in vivo* studies are important to evaluate the leishmanicidal potential of the *Anacardium othonianum* extract, fractions, and compounds.

Acknowledgements

The National Council for Scientific and Technological Development (CNPq), the Coordination for the Improvement of Higher Education Personnel (CAPES, Finance Code 001), and the São Paulo Research Foundation - FAPESP (grant number 2017/24860-0) are acknowledged for fellowships. This research was supported by the São Paulo Research Foundation (grant number 2016/10313-4).

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