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Trypanocidal activity of (−)-cubebin derivatives against free amastigote forms of *Trypanosoma cruzi*

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Abstract—Five (−)-cubebin derivative compounds, (−)-*O*-acetyl cubebin (**3**), (−)-*O*-benzyl cubebin (**4**), (−)-*O*-(*N*,*N*-dimethylaminoethyl)-cubebin (**5**), (−)-hinokinin (**6**) and (−)-6,6′-dinitrohinokinin (**7**), previously synthesised by our research group, were evaluated on in vitro assay against free amastigote forms of *Trypanosoma cruzi*, the asogic agent of Chagas' disease. It was observed that **6** was the most active compound (IC_{50} = 0.7 μM), and that **4** and **5** displayed moderate activity against the parasite, giving IC_{50} values of 5.7 and 4.7 μM, respectively. In contrast, it was observed that compound **3** was inactive and that **7** displayed low activity with IC_{50} values of $\cong 1.5 \times 10^4$ and 95.3 μM, respectively.

Since it was discovered in 1909,[1] Chagas' disease infection has been a difficult disease to control, due to its multiple characteristics. Almost the entire Latin American population is at risk of infection, where more than 18 million individuals have been already infected, causing the death of approximately 400,000 people per year.[2]

Trypanosoma cruzi, the disease aetiologic agent, shows a pathogenicity, which is influenced by characteristics of both the human body and the lineage of *T. cruzi* strain. Thus, the infection course in the susceptible vertebrates is influenced by factors such as: the environmental temperature, age, sex, genetic constitution of human body, genetic and morphologic characteristics of the infectant strain, as well as therapeutic agents.[3]

One of the main causes for the difficulties in finding an efficient compound to combat *T. cruzi* is directly linked to the morphologic characteristics of its strains, mainly due to the occurrence of various sub-populations of the parasite, leading to a different host tissue's tropism.[1,2,4,5] As reported by Andrade[2,4] strains that present predominance of slander forms, display differentiated tropism for cells of the phagocitary mononuclear system, infecting a larger number of splenocites, cells of the liver and cells of the marrow bone. Furthermore, these parasite strains characteristics usually lead to a high and precocious parasitaemic pick. Moreover, in despite of the high susceptibility of these slander form strains to the action of antibodies, the infection determines a high mortality rate for the great majority of the infected experimental animals in its acute phase. In addition, there are strains bearing broad forms, which have an intense tropism for muscle cells, such as skeletal and cardiac, as well as for granular tissues.[6,7] This type of morphologic conformation provides to this strain a larger resistance to the antibodies. As a consequence, they stay longer in the blood stream, determining late parasitaemic picks and lingering infection duration.

Keywords: Lignans dibenzylbutyrolactones; Trypanocidal activity; Amastigote forms; Cubebin derivatives.
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Clinical treatment of infected patients has been made by using beznidazole, which causes serious side effects and is not effective for the treatment of the chronic phase of the disease.[8] Therefore, there is an urgent demand for the discovery of new therapeutic compounds to treat Chagas' disease. In this regard, natural products have drawn special attention to researchers, due to the conspicuous chemical structures found in nature, bearing important biological activities, in this regard, the Brazilian flora stands out as a potential source of new lead compounds.[9,10] For instance, natural products, like podophyllotoxin and stagnancies, which belong to lignan class, are used for the treatment of cancer and other diseases.[11–14]

The compounds investigated in this work belong to the dibenzylbutyrolactone lignan class of compounds, which is among the natural products of interest, since many of its compounds display a broad range of biological activities with therapeutic potential.[15,16] Recently, Bastos et al.[17] reported the highly significant trypanocidal activity of seven dibenzylbutyrolactone lignans isolated from a Brazilian plant species, particularly for (−)-methylpluviatolide (**1**). Therefore, the aim of this work was to evaluate the trypanocidal activities of compounds obtained by partial synthesis, using (−)-cubebin (**2**), in the search for new potential trypanocidal drugs, as well as to investigate the correlation between the chemical structures and the biological activities for this group of compounds (Fig. 1).

The compounds obtained by partial synthesis from (−)-cubebin (**2**): (−)-*O*-acetyl cubebin (**3**), (−)-*O*-benzyl cubebin (**4**), (−)-*O*-(*N*,*N*-dimethylaminoethyl)-cubebin (**5**), (−)-hinokinin (**6**) and (−)-6,6′-dinitrohinokinin (**7**) are outlined in Scheme 1.

(−)-Cubebin (**2**) was isolated from the seeds of *Piper cubeba* L., which was acquired from the market. For this purpose, the powdered seeds were exhaustively extracted by maceration with 96% aqueous ethanol. The extract was filtered and concentrated under vacuum to furnish the crude extract, which was fractionated by partition between the phases of hexane and methanol/water 9:1. 430 g of the crude methanol/water fraction were submitted to repeated column chromatography over 1.0 kg of silica gel (12 × 120 cm). Elution with increasing proportions of hexane, dichloromethane and ethyl acetate yielded six fractions. Then, cubebin-rich fractions (1:1 hexane/dichloromethane and 100% dichloromethane) were submitted to repeated crystallisation in hexane/acetone to furnish crystalline cubebin (37 g). Its chemical structure was confirmed by analysis of its ^{1}H NMR and IR data, in comparison to the data published in the literature.[18] Its purity was estimated at 99% by both HPLC and ^{13}C NMR. (−)-*O*-Acetyl cubebin (**3**), (−)-*O*-benzyl cubebin (**4**) and (−)-*O*-(*N*,*N*-dimethylaminoethyl)-cubebin (**5**) were obtained by the reaction of (−)-cubebin (**2**) with Ac_2O/Py[19] (room temperature, 24 h, 85%), NaH/BrBn[20] (THF, room temperature, overnight, 74%) and $EtONa/(CH_3)_2N(CH_2)_2Cl$[19] (EtOH, reflux, 6 h, 71%), respectively. Hinokinin (**6**) was prepared by oxidation of (−)-cubebin (**2**) with 2 equiv of PCC (CH_2Cl_2, room temperature, 24 h, 98%).[21] After oxidation, **6** was dissolved in chloroform and submitted to nitration by dropping nitric acid into the reaction mixture (6 equiv HNO_3, $CHCl_3$, −10 °C, 4 h, 90%) of the dinitro derivative **7**, functionalised at positions 6 and 6′ of the aromatic rings.[22] The obtained spectroscopic data for compound 7 ~ fits with its established chemical structure.[23]

The trypanocidal assay was undertaken by using amastigote forms of *T. cruzi*,[24] which were obtained by culture of $LLMCK_2$ cell lineage. The cells were cultured in DEMEM medium (GIBCO), supplemented with 2 mM of the L-glutamine, 10 mM of $NaHCO_3$, 100 U/mL of penicillin, 100 μg/mL of streptomycin and 5% of inactivated fetal calf serum. The culture in a 96 wells microplate was kept at 37 °C in an atmosphere of 5% of CO_2 and 95% of humidity. Each well was filled with 5×10^5 cells, which were counted by using a Neubauer chamber. The trypomastigote forms were added into the culture medium at a ratio of 10:1, and cultured for five days. After cultivation, the supernatant was removed and centrifuged, furnishing free amastigote forms of the parasite

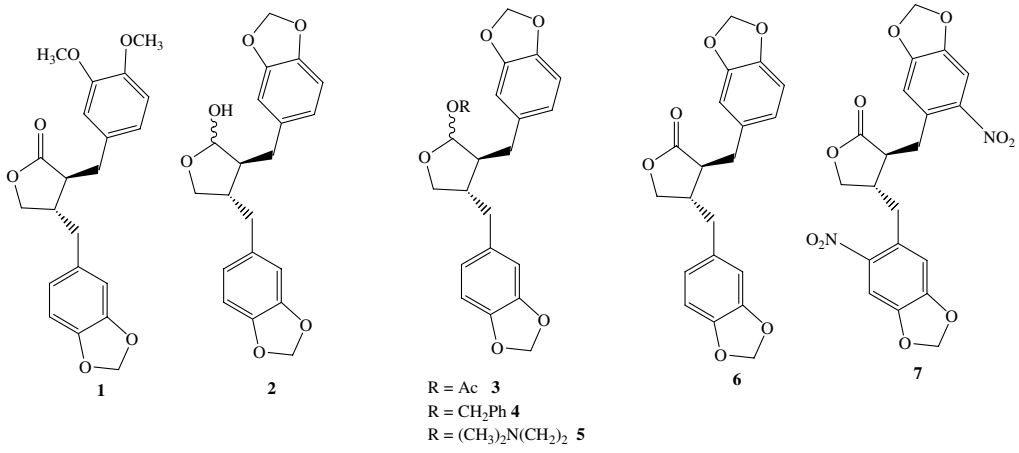
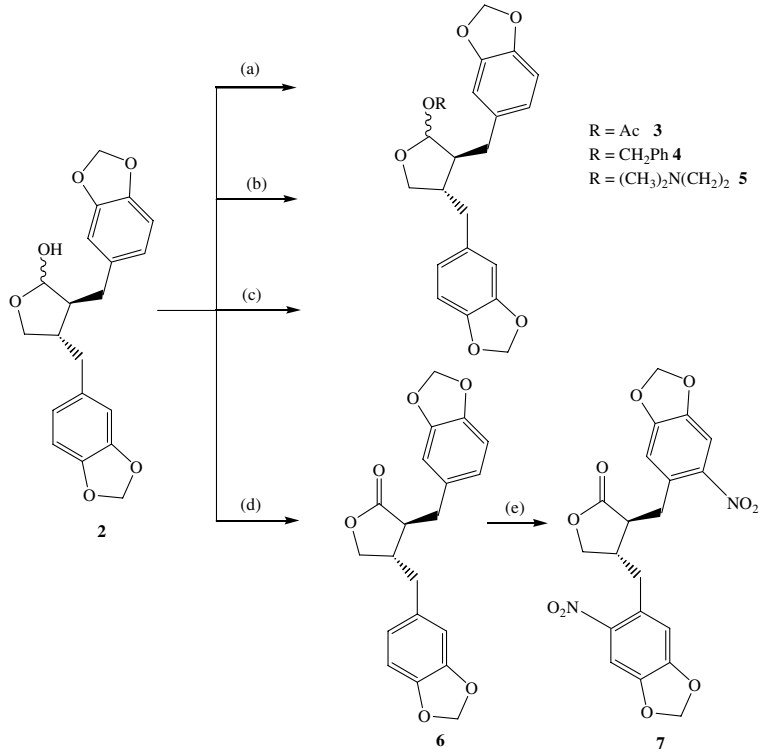


Figure 1. Chemical structures of (−)-methylpluviatolide (**1**), (−)-cubebin (**2**), (−)-*O*-acetyl cubebin (**3**), (−)-*O*-benzyl cubebin (**4**), (−)-*O*-(*N*,*N*-dimethylaminoethyl)-cubebin (**5**), (−)-hinokinin (**6**): and (−)-6,6′-dinitrohinokinin (**7**).



Scheme 1. Reagents and conditions: (a) Ac_2O, Py rt, 24 h; (b) NaH, BnBr, THF, rt, 24 h; (c) EtONa, $(CH_3)_2CH_2Cl$, EtOH, reflux, 6 h; (d) PCC, CH_2Cl_2, rt, N_2, 12 h; (e) 6 equiv HNO_3, 2 h, −10 °C.

for the bioassay. The supernatant was transferred to another microtitre plate, and each test compound was added into the biological system, which was kept for 24 h at the same above described conditions, and 1×10^6 amastigotes/mL were used for each assay.

Stock solutions were prepared by dissolving the compounds in pure dimethylsulfoxide (DMSO), to obtain a final concentration of 20 mM for each compound. Aliquots of the stock solution were added into the parasite suspension to obtain final concentrations of 0.5, 2.0, 8.0 and 32.0 μM concentrations of each compound.

Biological activity was evaluated by using the MTT colorimetric method [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], in a microplate reader at 570 nm, as described by Muelas-Serrano et al.[25] The negative control group containing solvent and medium, the negative control group containing medium, the positive control group containing benznidazole, and the lignan compounds tested groups were all run in parallel. All the assays were undertaken in triplicate.

For the statistical analysis the One-Way ANOVA test was used, which was complemented by the Tukey analysis, aiming to establish the significant levels of the activity of the compounds in the in vitro assay. For the IC_{50} determination, the sigmoidal dose-response curve was used as statistical method.

The natural (−)-cubebin (**2**), used as the starting compound to obtain the evaluated dibenzylbutyrolactone derivatives, did not display activity against trypomastigote forms of *T. cruzi*[17] Nevertheless, it was selected as starting compound because of its availability, being easily isolated in large amounts from the seeds of *P. cubeba*.

The trypanocidal activities of compounds **1** and **2** against both trypomastigote and amastigote forms of *T. cruzi* were previously reported, along with (−)-methylpluviatolide, which displayed 100% of trypomastigote lysis in the in vitro assay. However, it did not improve a significant reduction in the number of the intracellular amastigote forms of the parasite in an in vivo assay, probably due to the low permeability of the compound to both host and amastigote cell membranes.[17] Hence, the biological evaluation against amastigote forms of *T. cruzi* was undertaken only for compounds **3, 4, 5, 6** and **7**, as reported here. Compounds **3, 4** and **5** differ from compound **2** by the presence of acetyl, benzyl and dimethylaminoethyl groups replacing the lactol hydrogen, respectively. Compounds **6** and **7** are lignan-lactones, like (−)-methylpluviatolide (**1**), and differ from compound **2** by the presence of a carbonyl group at C-9.

The production of compound **3** by substitution of the lactol hydrogen of cubebin by an acetyl group led to a strong reduction of its trypanocidal activity, in comparison with all other tested compounds belonging to the same group ($IC_{50} \cong 1.5 \times 10^4$ μM). Furthermore, the comparison of compounds **3** and **4** indicate that the biological activity against the amastigote forms of *T.* cruzi were significantly affected by the nature of the substituting group at position C-9, which played an important

Table 1. Results of the trypanocidal activity evaluation of compounds **3**, **4**, **5**, **6**, **7** and benznidazole, against amastigote forms of Y strain of *Trypanosoma cruzi*

Compounds	Concentration (μM) × % de lyse (±SD)				IC_{50} (μM)
	0.5	2.0	8.0	32.0	
3	14.5 ± 1.9	26.0 ± 2.5	29.0 ± 5.2	26.4 ± 1.2	$\cong 1.5 \times 10^4$
4	37.0 ± 1.4	38.0 ± 7.0	46.8 ± 6.4	68.8 ± 2.9	5.7
5	32.6 ± 2.6	55.4 ± 4.3	50.8 ± 1.0	57.8 ± 8.0	4.7
6	47.6 ± 9.5	57.0 ± 1.1	57.6 ± 8.9	63.6 ± 5.2	0.7
7	34.6 ± 7.9	48.7 ± 1.4	38.9 ± 2.0	48.5 ± 6.1	95.3
Benznidazole	38.4 ± 3.0	67.0 ± 7.2	69.0 ± 4.0	68.6 ± 1.7	0.8

Negative control—RPMI-1640 medium plus 1% DMSO displayed 0% lysis.

role in the reduction of the calculated IC_{50} value for compound **4** (IC_{50} = 5.7 μM). Likewise, cubebin derivative **5**, bearing an amino group at the lactol ring, displayed an activity quite similar to compound **4**.

Analysis of the obtained results, displayed in Table 1, indicate that compound **6** was the most active, with an IC_{50} value of 0.7 μM similar to that displayed by benznidazole (0.8 μM), a standard drug used as the positive control. On the other hand, most of the other evaluated compounds displayed much lower activity, with the exception of compounds **4** (IC_{50} = 5.7 μM) and **5** (IC_{50} = 4.7 μM), which showed significant activity. Compounds bearing nitro groups on the aromatic ring, like benznidazole[8] and **7**, displayed much lower biological activity than compound **6**.

It should be pointed out that hinokinin **6** is a promising compound to continue examining, since at 0.5 μM it displayed higher activity than benznidazole and at the other assayed concentrations (2.0, 8.0 and 32.0 μM) it showed similar activity. Moreover, the commercialised therapeutic drug, benznidazole, causes serious side effects,[8] making the dibenzylbutyrolactone derivatives a very important potential source for obtaining new compounds for the treatment of Chagas' disease, as observed for compound **6**, which was the most active one in this study.

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23. (−)-*O*-Acetyl cubebin (**3**): $[\alpha]_D^{26}$ −123 (*c* 0.0057, $CHCl_3$); ^1H NMR δ ($CDCl_3$): 6.8–6.5 m (6H aromatic), 5.9 (m, 2H), 5.25 (broad s, H2 of epimer in major proportion), 5.15 (s, H2 of the epimer in minor proportion), 4.1 (m, H5a of both epimers), 4.0 (m, H5b of both epimers), 3.8 (m, 2H), 3.6 (m, 2H), 2.8–2.0 (m, 2H), 2.0 (s, 1H).
(−)-*O*-Benzyl cubebin (**4**): $[\alpha]_D^{26}$ −2.1 (*c* 0.008, $CHCl_3$) ^1H NMR δ ($CDCl_3$): 6.8–6.3 (m, 6H aromatic), 2.8–2.4 (m, 4H), 2.3–2.4 (m, 1H), 2.3–1.9 (m, 1H), 2.9–2.5 (m, 1H), 5.20 (s, 1H), 4.05 (dd, *J* = 12 and 6 Hz, 1H), 3.72 (dd, *J* = 12 and 8 Hz, 1H), 4.70 (d, 1H) and 4.75 (d, 1H); ^{13}C NMR δ ($CDCl_3$): 134.5, 108.2, 146.4, 146.3, 109.3, 121.8, 33.9, 52.5, 103.9, 133.9, 108.3, 146.4, 146.4, 109.3, 121.7, 38.8, 46.2, 72.5, 100.8, 69.0.
(−)-*O*-(*N*,*N*-Dimethylaminoethyl)-cubebin (**5**): $[\alpha]_D^{26}$ −4.4 (*c* 0.067, $CHCl_3$); ^1H NMR δ ($CDCl_3$): 6.70–6.40 (m, 6H aromatic), 5.90 (s, 4H, –O–CH_2–O–), 4.75 (s, 1H), 4.05 (m,

1H), 3.80 (m, 1H), 3.66–3.52 (m, 2H, O-CH_2), 2.80–2.30 (m, 4H), 2.30–1.90 (m, 1H), 2.80–2.40 (m, 1H), 1.12 (t, 1H, J = 7.07 Hz) and 1.09 (t, 1H, J = 7.07 Hz), 1.09 (s, 6H); ^{13}C NMR δ ($CDCl_3$): 134.5, 108.3, 147.8, 146.2, 109.6, 122.0, 39.5, 52.5, 104.5, 72.3, 101.1, 63.2, 43.7, 46.2.

(−)-Hinokinin (**6**): $[\alpha]_D^{26}$ −30 (*c* 0.99, $CHCl_3$); ^{1}H NMR δ ($CDCl_3$): 6.8–6.4 (m, 1H), 5.9 (broad s, 2H), 4.15 (dd, 1H, J = 7.1 and 9.3 Hz), 3.85 (dd, 1H, J = 7.1 and 9.1 Hz), 3.0 (dd, 1H, J = 5.1 and 14.2 Hz), 2.85 (dd, 1H, J = 7.3 and 14.2 Hz), 2.6 (d, 1H, J = 7.1 Hz), 2.55 (m, 1H), 2.45 (d, 1H, J = 8.6 Hz), 2.4 (m, 1H); ^{13}C NMR δ ($CDCl_3$): 178.4, 147.9, 147.8, 146.5, 146.4, 131.6, 131.3, 122.2, 121.55, 109.4, 108.8, 108.4, 108.3, 101.0, 71.2, 46.4, 41.3, 38.4, 34.8.

(−)-6,6′-Dinitrohinokinin (**7**): mp 191–193 °C; $[\alpha]_D^{26}$ −29 (*c* 0.008, $CHCl_3$); ^{1}H NMR δ ($CDCl_3$) 7.5 (s, 1H), 7.48 (s, 1H), 6.8 (s, 1H), 6.6 (s, 1H), 6.1 (m, 2H), 4.35 (dd, 1H, J = 7.1 and 9.1 Hz), 4.0 (dd, 1H, J = 7.3 and 9.1 Hz), 3.26 (d, 2H, J = 7.1 Hz), 3.2 (dd, 1H, J = 6.3 Hz and J = 13.6 Hz), 3.0 (dd, 1H,J = 7.8 and 13.6 Hz), 2.8 (m, 2H); ^{13}C NMR δ ($CDCl_3$): 178.0, 152.3, 152.2, 147.6, 143.1, 142.9, 130.9, 130.7, 112.5, 111.2, 106.6, 106.2, 103.6, 103.5, 71.4, 45.7, 41.7, 37.2, 34.2.

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