Trypanocidal Constituents in Plants 3.[1] Leaves of *Garcinia intermedia* and Heartwood of *Calophyllum brasiliense*

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The constituents of the leaves of *Garcinia intermedia* and heartwood of *Calophyllum brasiliense* were investigated based on their trypanocidal activity against epimastigotes of *Trypanosoma cruzi*, the etiologic agent of Chagas' disease. As the active components, the polyisoprenylated benzophenone derivative guttiferone A and the xanthone 8-desoxygartanin were isolated along with the biflavonoids podocarpusflavone A and amentoflavone, and friedelin from the former. Three xanthones, jacareubin, 6-deoxyjacareubin, and 1,3,5,6-tetrahydroxy-2-(3-methyl-2-butenyl)xanthone from the latter showed activity. The trypanocidal activity of these compounds against trypomastigotes, an infectious form of *T. cruzi*, was examined as well as gossypol, berberine chloride, and harmine for comparison.

Key words *Trypanosoma cruzi*; *Garcinia intermedia*; *Calophyllum brasiliense*; trypanocidal activity; Chagas' disease; Guttiferae

In the previous paper in this series,[2] we described the results of preliminary screening tests for trypanocidal activity of some Mexican plants against epimastigotes of *Trypanosoma cruzi* and identification of the active constituents in Guaco (*Aristolochia taliscana*). The protozoan *Trypanosoma cruzi* is the etiologic agent of Chagas' disease (American trypanosomiasis), one of the most serious diseases in Latin America.

In the course of our search for trypanocidal constituents in plants, we have investigated the leaves of *Garcinia intermedia* (Pittier) Hammel and heartwood of *Calophyllum brasiliense* Cambess., which showed activity in preliminary screening tests.[2] Both *G. intermedia* and *C. brasiliense* belong to Guttiferae.

G. intermedia is a tree that grows up to 20 m high and is distributed from Mexico to Panama. It is known as *limoncillo* in the local language, meaning "small lemon," and its fruit is edible. No chemical study on it has been reported. *C. brasiliense*, on the other hand, grows up to 50 m high and is distributed throughout Latin America. Two of the coauthors reported the isolation of antifungal xanthones from the heartwood.[3] Ito *et al.* reported the isolation of xanthones from the stem bark and their cancer chemopreventive activity.[4]

T. cruzi exhibits three developmental stages: epimastigote in the insect gut; trypomastigote, an infectious form in the mammalian bloodstream; and amastigote, a proliferative form in mammalian cells. In the course of the isolation procedure, trypanocidal activity was monitored against epimastigotes, which are uninfectious and easy to cultivate. However, it is necessary to examine trypanocidal activity against the infectious bloodstream form of trypomastigotes or intracelluar amastigotes for evaluation of the chemotherapeutic potential. In the case of the isolates, activity against trypomastigotes was examined *in vitro*. The trypanocidal activity of gossypol, berberine chloride, and harmine was also assayed for comparison.

MATERIALS AND METHODS

Plant Materials Leaves of *G. intermedia* were collected at Ejido Benigno Mendoza, State of Veracruz, Mexico, in 1998. The identification was done by Dr. Mario Vasquez Torres. A voucher specimen is deposited in the Herbarium of Instituto de Investigaciones Biológicas, Universidad Veracruzana (CIB), Xalapa, Mexico. The heartwood of *C. brasiliense* was bought at a lumber store in Mexico City.

Reagents Newborn calf serum (NCS) was purchased from Nacalai Tesque, and Dulbecco's modified Eagle's medium (DMEM) from Sigma. Other reagents were the same as used in the previous study.[2]

Cultivation of *T. cruzi* The strain of *T. cruzi* used in this study and the method of cultivation of epimastigotes were the same as those described in the previous paper.[2] The trypomastigotes of *T. cruzi* were harvested from the culture supernatant of mouse kidney carcinoma (LLC-MK2) cells infected with *T. cruzi*.[5] LLC-MK2 cells were maintained in DMEM containing 10% fetal bovine serum (FBS). After inoculation of trypomastigotes into LLC-MK2 cells, the medium was changed to DMEM containing 10% NCS.

Assay of Trypanocidal Activity The detailed assay procedure against epimastigotes was described in the previous paper.[2] The activity was expressed as the MC_{100} value (μg/ml or μM), which was defined as the minimum concentration at which all the epimastigotes were terminated after 48-h incubation at 26 °C.

To determine the trypanocidal activity against trypomastigotes, each compound was first dissolved in dimethyl sulfoxide (DMSO) and then diluted with DMEM to obtain the desired concentration. Fifty microliters of each cell suspension (*ca.* 2×10^6 trypomastigotes/ml) and sample solution prepared by the serial two-fold dilution method were placed in 96-well microplates in duplicate and incubated at 37 °C for 48 h under 5% CO_2. The activity was expressed as the MC_{100} value as in the case of epimastigotes.

Extraction and Isolation of the Active Constituents from Leaves of *G. intermedia* The dried and powdered

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leaves of *G. intermedia* (121 g) were extracted with CH_2Cl_2–MeOH (1 : 1) at room temperature and filtered. The filtrate was concentrated and dried *in vacuo* to give a dark brown residue (11.7 g). The MC_{100} value of the MeOH extract against epimastigotes was 0.5 mg/ml. The MeOH extract was suspended in 60% MeOH and centrifuged. The precipitates were extracted with MeOH and then with AcOEt. The supernatant of 60% MeOH suspension was passed through a column of styrene polymer, Diaion HP-20, and the column was washed with 60% MeOH. The MeOH solution was passed through the same column, and the column was washed with MeOH. The AcOEt solution was treated in the same way. Each eluate was concentrated and dried *in vacuo* to give brown resin: 60% MeOH eluate (fr. 1, 1.7 g); MeOH eluate (fr. 2, 5.4 g); and AcOEt eluate (fr. 3, 2.6 g). MC_{100} values (μg/ml) of frs. 1—3 were >1000, 500, and >1000, respectively. Fraction 2 was chromatographed successively on silica gel ($CHCl_3$–MeOH), Sephadex LH-20 ($CHCl_3$), and ODS (YMC gel) (CH_3CN–H_2O, MeOH–H_2O) columns to afford guttiferone A[6] (**1**, 720 mg), 8-deoxygartanin[7] (**2**, 12 mg), podocarpusflavone A[8] (**3**, 88 mg), and amentoflavone[9] (**4**, 47 mg). Silica gel column chromatography of fraction 3 yielded friedelin[10] (**5**, 209 mg). Compounds **1**—**5** were identified by comparison of MS and NMR spectral data with those reported. MC_{100} values of compounds **1**—**5** against epimastigotes and trypomastigotes were assayed and are shown in Table 1.

Extraction and Isolation of the Active Constituents from Heartwood of *C. brasiliense* The dried and powdered heartwood of *C. brasiliense* (50 g) was extracted with MeOH–acetone (2 : 1) at room temperature and filtered. The filtrate was concentrated and dried *in vacuo* to give a dark brown residue (2.0 g). The MC_{100} value of the extract against epimastigotes was 0.5 mg/ml. The MeOH–acetone (2 : 1) extract was treated in the same way as in the case of *G. intermedia*. Fractions 1 (0.2 g, MC_{100} >1000 μg/ml), 2 (1.5 g, 250 μg/ml), and 3 (0.3 g, >1000 μg/ml) were obtained. Fraction 2 was chromatographed successively on Sephadex LH-20 (MeOH) and silica gel (hexane–AcOEt) columns to afford jacareubin[11] (**6**, 175 mg), 6-deoxyjacareubin[12] (**7**, 30 mg), 1,3,5,6-tetrahydroxy-2-(3-methyl-2-butenyl)xanthone[13] (**8**, 319 mg), and 1,3,5,6-tetrahydroxy-2-(3-hydroxy-3-methylbutyl)xanthone[14] (**9**, 162 mg). Compounds **6**—**9** were identified by comparison of their MS and NMR spectral data with those reported. MC_{100} values of **6**—**9** against epimastigotes and trypomastigotes are shown in Table 1.

The trypanocidal activity of gossypol,[15] berberine chloride,[16] and harmine,[16] which were examined against epimastigotes in the previous paper,[2] was also estimated against trypomastigotes.

Table 1. Trypanocidal Activity (MC_{100}) of **1**—**10**, Gossypol, Berberine Chloride, and Harmine against Epimastigotes and Trypomastigotes of *T. cruzi in Vitro*

Compound	MC_{100}			
	(Epimastigotes)		(Trypomastigotes)	
	μg/ml	μM	μg/ml	μM
1	60	100	50	83
2	45	118	50	131
3	>500		>200	
4	>500		>200	
5	>500		>200	
6	50	153	15	46
7	50	161	200	645
8	70	213	40	122
9	>300		>200	
10	25	66	3	8
Gossypol	280	540	50	96
Berberine chloride	300	807	7	19
Harmine	>500		>200	

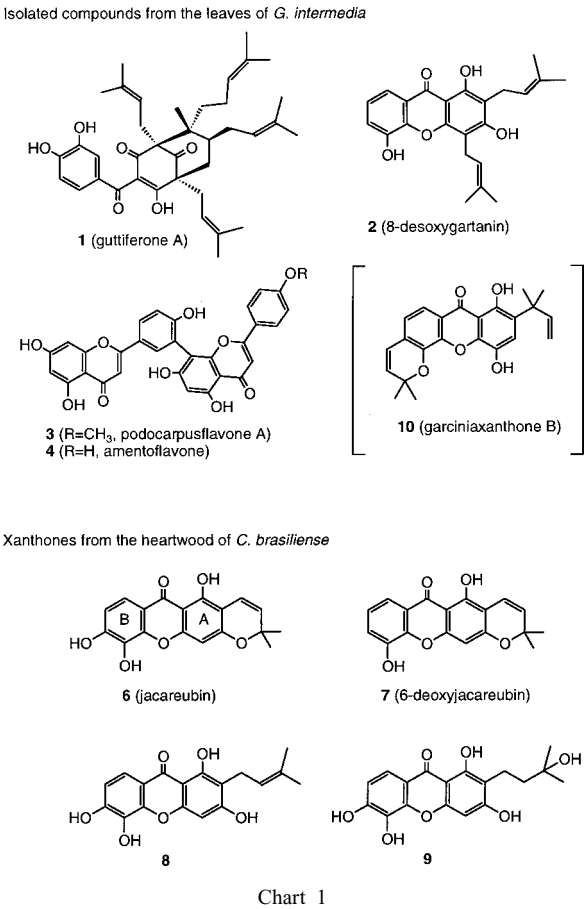

Chart 1

RESULTS AND DISCUSSION

The CH_2Cl_2–MeOH extract of leaves of *G. intermedia* were separated guided by the trypanocidal activity against epimastigotes *in vitro*. As active components, guttiferone A (**1**) and 8-desoxygartanin (**2**) were obtained. Compound **1** is the principle of the activity of the extract due to the high yield (0.6%) and MC_{100} values (epimastigotes 100 μM; trypomastigotes 83 μM). Guttiferone A (**1**) was first isolated as the active anti-HIV constituent from *Symphonia globulifera* (Guttiferae).[6] To the best of our knowledge, this is the first time that the trypanocidal activity of guttiferones has been described. Biflavonoids **3** and **4** showed no activity, like fukugetin in *Garcinia subelliptica*.[1] From the MeOH–acetone extract of the heartwood of *C. brasiliense*, four xanthones (**6**—**9**) were obtained in the same way. Three (**6**—**8**) of these four showed activity.

In the preceding paper, we described the trypanocidal activity of xanthones from *G. subelliptica*.[1] They have 1 to 3

isoprenyl units in their structures, and garciniaxanthone B (**10**) with one prenyl unit in both rings A and B showed the highest activity (epimastigotes 66 μM; trypomastigotes 8 μM). It is not easy to establish a structure-activity relationship from the limited number of samples, although the following are the effects of substitution patterns on the activity against trypomastigotes. Comparing **7** with **6**, the formation of a pyrocatechol function increased the activity greatly. Compounds **6**, **8**, and **9** have the same substitution pattern in ring B. A decrease in the degree of unsaturation in the ring A side chain was accompanied by a decrease in activity. Compound **9**, with a saturated side chain, loses activity completely. Compounds **2** and **10**, with two prenyl units in their structures, showed different activity. This might be due to a higher degree of unsaturation in **10**.

It is noteworthy that the trypanocidal activity of berberine chloride was markedly different in two life stages: weak against epimastigotes and much stronger against trypomastigotes. The respective ratios of MC_{100} values of other compounds examined in this study were in the range 0.25—8.25, although it was 42.5 in the case of berberine chloride. This may be due to the difference in the mode of action between berberine chloride and these xanthones. Xanthones appear to influence the redox balance of *T. cruzi* like quinones[17] and flavonoids.[18] It is known that the specific neuraminidase activity of *T. cruzi* varies greatly among developmental stages, with the highest in the trypomastigote stage.[19] Further investigation is needed to clarify whether high activity of berberine chloride against trypomastigotes correlates with this enzyme activity. Since the trypomastigote stage is an infectious form in the mammalian bloodstream, the strong trypanocidal activity of berberine chloride is promising for therapeutic purposes.

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