Trypanocidal Constituents in Plants 1. Evaluation of Some Mexican Plants for Their Trypanocidal Activity and Active Constituents in Guaco, Roots of *Aristolochia taliscana*

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Crude extracts of Mexican medicinal plants were screened for trypanocidal activity against *Trypanosoma cruzi*, which is the etiological agent for Chagas' disease, one of the most serious protozoan diseases in Latin America. There were 43 kinds of methanolic and other organic extracts from 39 plants which were examined by the preliminary screening test to see immobilization of epimastigotes of *T. cruzi in vitro*. Eighteen of them showed activity at the concentration of 2 mg/ml after incubation for 2 h, while 13 showed activity at the concentration of 1 mg/ml after incubation for 48 h. Among them, the MeOH extract of roots of *Aristolochia taliscana* (Aristolochiaceae), locally known as "Guaco," immobilized all the epimastigotes even at lower concentration of 0.5 mg/ml (48 h). In order to identify principal compounds for this activity, the MeOH extract of Guaco was subjected to bioassay-guided fractionation. From the active fractions, four neolignans, eupomatenoid-7 (1), licarin A (2), eupomatenoid-1 (5) and licarin B (6), and two lignans, austrobailignan-7 (3) and fragransin E_1 (4) were isolated. Compounds 1—4 immobilized all the epimastigotes at the minimum concentration of 25—75 μg/ml after incubation for 48 h, while compounds 5 and 6 were inactive. Corresponding concentration of gossypol, berberine chloride and harmine was 280 μg/ml, 300 μg/ml and $>$500 μg/ml, respectively.

Key words trypanocidal activity; *Trypanosoma cruzi*; *Aristolochia taliscana*; Chagas' disease; neolignan; lignan

Protozoan *Trypanosoma cruzi* is the etiological agent of Chagas' disease (American trypanosomiasis), which affects 16—18 million people in Latin America and is responsible for the death of more than 45000 patients per year.[1] It is transmitted to humans by triatomine bugs or through blood transfusion. During its life cycle, *T. cruzi* differentiates into three stages, namely epimastigote in the insect gut, trypomastigote, an infectious form in the blood stream, and amastigote, an intracellular form.

Medication for Chagas' disease is usually effective when given during the acute stage of infection. Once the disease has progressed to later stages, no medication has been proven to be effective. Moreover, synthetic drugs, such as nifurtimox and benznidazole, have severe side effects.[2,3] There rises an urgent need to develop new drugs. In order to seek new chemotherapeutic agents from natural resources, we started a survey of trypanocidal constituents in Mexican plants. Investigation on a trypanocidal constituent in *Piqueria trinervia*, one of Mexican plants, was already performed by Castro *et al.*[4] As a preliminary screening test, we examined crude extracts of Mexican medicinal plants for trypanocidal activity against epimastigotes of *T. cruzi in vitro* as previously reported in the case of plants in Guatemala.[5] Among them the MeOH extract of roots of *Aristolochia taliscana* (Aristolochiaceae) showed trypanocidal activity. *A. taliscana* and some other species of *Aristolochia* are locally called "Guaco" and used as remedies for diarrhea, snake bites, and dermatological affections.[6] Chemical investigation of *A. taliscana* has been previously accomplished by Enriquez *et al.* and four neolignans have been isolated.[7]

In this paper we describe the results of preliminary screening tests for trypanocidal activity in some Mexican plants, and identification of the active constituents in Guaco, *A. taliscana*, one of the plant materials which showed activity.

MATERIALS AND METHODS

Plant Materials and Preparation of Their Extracts Plant materials including *A. taliscana* were mainly purchased at Sonora medicinal plant market in Mexico City and collected in the fields. Identification of the plants was done by M. E. López-Villafranco of National University of Mexico (Iztacala) and A. Aguilar, one of the co-authors. The voucher specimens were deposited in the Herbariums IZTA and IMSSM of National University of Mexico (Campus Iztacala) and Mexican Institute for Social Security, respectively. The plants examined are listed in Table 1. Dried and powdered materials were extracted with MeOH, MeOH–CH_2Cl_2 or acetone at room temperature overnight. In the case of fresh materials, chopped materials were soaked in MeOH and filtered. The residue was extracted again with MeOH. The solvent was concentrated *in vacuo* to give each extract.

Cultivation of *T. cruzi* The strain of *T. cruzi* used in this study was H6 (international code: MHOM/GT/95/SMI-06), which was originally collected from a patient of Chagas' disease in Guatemala by Dr. T. Yanagi of Nagasaki University, Dr. C. Monroy and Dr. V. Matta of San Carlos University in Guatemala, and H. Higo, one of the co-authors. The epimastigotes of *T. cruzi* have been cultured in liver infusion-tryptose (LIT) medium as described by Baum.[8] Hemin was replaced by hemoglobin.

Reagents Tryptose and liver infusion broth were ob-

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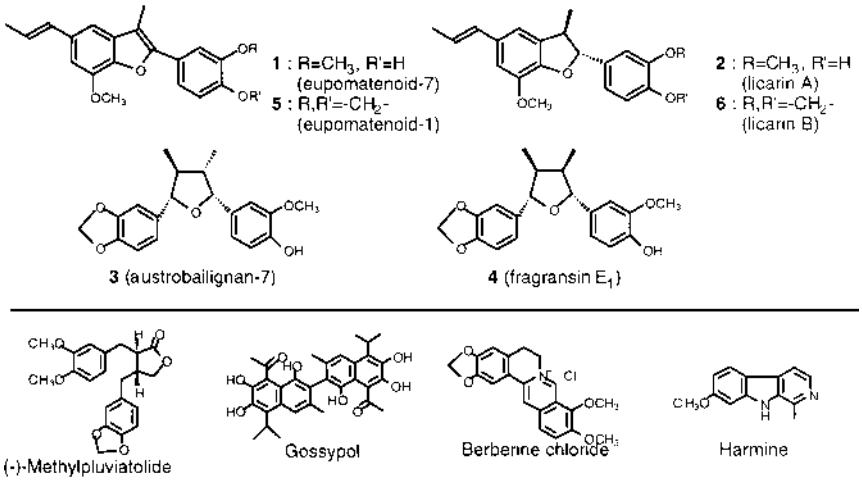

Fig. 1. Isolated Compounds from Roots of *A. taliscana*

tained from Difco, fetal bovine serum from GIBCO and hemoglobin from Japan Biotest Institute. Gossypol and harmine were purchased from Sigma Chemical Company, and berberine chloride (*n*-hydrate) from Tokyo Chemical Industries Co. Ltd.

Trypanocidal Assay Preliminary Screening: Each extract was dissolved in dimethyl sulfoxide (DMSO) first and then diluted with LIT medium to get certain concentration. The final DMSO concentration was less than 1%. 1% DMSO solution itself caused no affection on motion of epimastigotes. Under condition 1, the final concentration of each extract was 2 mg/ml and incubation time was 2 h. Under condition 2, the final concentration was 1 mg/ml and incubation time was 48 h. Each 50 μl of sample solution and cell suspension (*ca.* 2×10^6 epimastigotes/ml) was placed in a 96-well micro plate in duplicate and incubated at 26 °C. The control was free from samples. The motion of epimastigotes both in the sample well and in the control well was observed under inverted light microscope ($\times$100). Each test was run twice. The results are shown in Table 1. The mark (+) means that all the epimastigotes became immobilized, while the mark ($\pm$) means 80—90% of the whole epimastigotes became immobilized. The mark (−) means that more than 50% of the epimastigotes kept mobility.

Estimation of Trypanocidal Activity: Sample solutions in different concentration were treated as mentioned above. The activity is shown by MC_{100} value, which was defined as the minimum concentration at which all the epimastigotes become immobilized after 48 h-incubation at 26 °C.

Extraction and Isolation of the Active Constituents from the Roots of *Aristolochia taliscana* Dried and powdered roots (142 g) of *A. taliscana* were extracted with MeOH (500 ml) under reflux for 1 h and filtered. Further extraction for 30 min was done twice. The filtrates were combined, concentrated and dried *in vacuo* to give a dark brown residue (19.4 g). The MC_{100} value of the MeOH extract was 0.5 mg/ml. The residue was suspended in 60% MeOH and centrifuged. The precipitates were extracted with MeOH, and then with AcOEt. The supernatant of 60% MeOH 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. The 60% MeOH eluate was concentrated *in vacuo* to remove MeOH and the aqueous solution was passed through a new Diaion HP-20 column. The column was eluted with H_2O and then 60% MeOH. Each eluate was concentrated and dried *in vacuo* to obtain brown resin: H_2O eluate (fr. 1, 10.1 g); 60% MeOH eluate (fr. 2, 1.4 g); MeOH eluate (fr. 3, 5.2 g); AcOEt eluate (fr. 4, 2.3 g). MC_{100} values of frs. 1—4 are $>$1000 μg/ml, $>$1000 μg/ml, 60 μg/ml, and 400 μg/ml, respectively. Fractions 3 and 4 were chromatographed with silica gel (hexane–AcOEt), Sephadex LH-20 ($CHCl_3$), and ODS (YMC gel) (70% MeOH) column, successively, to afford eupomatenoid-7[9] (**1**, 826 mg), licarin-A[10] (**2**, 324 mg), mixture of **3** and **4** (230 mg), eupomatenoid-1[11] (**5**, 109 mg), and licarin B[12] (**6**, 368 mg). Austrobailignan-7[13] (**3**, 139 mg) and fragransin E_1[14] (**4**, 35 mg) were separated by preparative HPLC (ODS, KC-Pack, 60% MeOH). Identification of compounds **1**—**6** was performed by analyses of MS and NMR spectra as well as the comparison of physical data with those reported. MC_{100} values of compounds **1**—**6** were estimated as described above and shown in Table 2.

RESULTS AND DISCUSSION

Many trypanocidal constituents from natural resources have been isolated and were first reviewed by Sepúlveda-Boza and Cassels in 1996.[15] In this review, various kinds of natural compounds were reported such as hydroquinones, naphthoquinones, diterpenes and many types of alkaloids. It is difficult to compare activity of each compound due to the diversity of strains, stages of its life cycle, and experimental conditions applied. Since Schlemper *et al.* mentioned the positive correlation between activity against epimastigotes *in vitro* and activity against trypomastigote *in vivo*,[16] we used epimastigotes to estimate trypanocidal activity. The method of assay by Hocquemillar *et al.* was modified for estimation of trypanocidal activity *in vitro*.[17]

For preliminary screening tests, we chose plants and herbal medicines traditionally used mainly for antiparasitic purposes in Mexico[18] and Guatemala.[19] The list of crude extracts is shown in Table 1 with scientific names, local names and parts examined. It is comprised of 20 families and 37

Table 1. List of Plants Examined and Their Trypanocidal Activity[a] (Epimastigotes, *in Vitro*)

Family	Scientific name	Local name	Part (Solvent)[b]	(1)[c]	(2)[d]
Annonaceae	*Annona reticulata*	Anona	LT (M)	+	+
	Annona muricata	Guanábana	S (M)	+	+
Aristolochiaceae	*Aristolochia taliscana*	Guaco	R (M)	+	+
Burseraceae	*Bursera simaruba*	Palo mulato	LT (M)	−	−
			F (M)	−	−
Cecropiaceae	*Cecropia obtusifolia*	Guarumbo	L (M)	+	−
Chenopodiaceae	*Chenopodium graveolens*	Epazote de zorrillo	G (M)	+	±
	Chenopodium ambrosioides	Epazote morado	G (M)	−	−
Compositae	*Artemisia ludoviciana* var. *mexicana*	Estafiate	L (M)	±	−
	Bidens odorata	Mosote blanco	G (M)	+	±
Cucurbitaceae	*Maximowitzia sonorae*	Guareque	R (M)	−	−
Elaeocarpaceae	*Muntingia calabura*	Capulín rojo	L (M)	+	−
			F (M)	−	−
Euphorbiaceae	*Croton draco*	Sangre de grado	L (M)	−	−
	Hura polyandra	Haba o Habilla	S (M)	−	−
Guttiferae	*Calophyllum brasiliense*[e]	Bari	L (A)	+	+
	Calophyllum brasiliense[f]	Bari	L (M)	−	−
	Clusia salvinii	Lobo de tigre	L (M+C)	−	−
	Clusia guatemalensis	Lobo de tigre	L (M)	−	−
	Garcinia intermedia	Limoncillo	L (M+C)	+	+
	Mammea americana	Zapote Domingo	P (A)	+	+
	Vismia baccifera	Vismia	L (M)	−	−
Lauraceae	*Persea americana*	Aguacate	S (M)	+	±
Leguminosae	*Brongniartia podalyrioides*	Hierba de la Víbora	R (M)	−	−
	Eysenhartia polystachia	Palo Dulce	S (M)	−	−
	Gliricidia sepium	Cocuite	L (M)	+	−
	Haematoxylum brasileto	Palo de Brasil	S (M)	+	±
	Lonchocarpus unifoliolatum		L (M)	−	−
	Lonchocarpus oaxacensis		R (M)	−	−
	Senna hirsuta	Yecapahtzin	LT (M)	+	±
	Zornia thymifolia	Hierba de la vibora	L (M)	+	±
Marvaceae	*Malvaviscus arboreus*	Azocopacle	L (M)	−	−
Myrtaceae	*Psidium guajava*	Guayaba	L (M)	−	−
			S (M)	−	−
Piperaceae	*Piper* sp.		L (M)	+	+
	Piper auritum	Acuyo	L (M)	−	−
Polypodiaceae	*Phlebodium aureum*	Lengua de ciervo	G (M)	−	−
			R (M)	−	−
Rubiaceae	*Hamelia patens*	Balletilla	LT (M)	−	−
Sapotaceae	*Pouteria sapota*	Mamey	S (M)	±	−
Urticaceae	*Urtica dioica*	Ortiga	LT (M)	−	−
Verbenaceae	*Aloysia triphylla*	Té cedrón	L (M)	−	−
	Lippia dulcis	Hierba dulce	L (M)	−	−

a) The mark (+) means all the epimastigotes were immobilized. The mark (±) means 80—90% of the whole epimastigotes were immobilized. *b*) LT: leaves and twigs, R: roots, F: fruits, L: leaves, G: ground parts, S: stems, P: peels of fruits, M: MeOH, A: acetone, C: CH_2Cl_2. *c*) Activity at 2 mg/ml (2 h). *d*) Activity at 1 mg/ml (48 h). *e*) Collected at Santa Martha, Veracruz. *f*) Collected at Los Tuxtlas, Veracruz.

species. Eighteen out of 43 extracts showed trypanocidal activity so far at 2 mg/ml, while 13 showed activity even at 1 mg/ml. We started the chemical investigation of these active plant materials.

The MeOH extracts of roots of *A. taliscana* immobilized all the epimastigotes at 0.5 mg/ml. When the MeOH extract was separated into 4 fractions (frs. 1—4) by a Diaion HP-20 column, activity was observed in less polar fraction 3 eluted with MeOH and fraction 4 eluted with AcOEt. Fractions 3 and 4 were combined and subjected to various kinds of chromatographies to afford four neolignans, eupomatenoid-7 (**1**), licarin A (**2**), eupomatenoid-1 (**5**) and licarin B (**6**), and two lignans, austrobailignan-7 (**3**) and fragransin E_1 (**4**). Although these neolignans were already isolated from Guaco by Enriquez *et al.*,[7] this is the first isolation of lignans **3** and **4** from Guaco. MC_{100} values of these compounds are listed in Table 2. Trypanocidal natural compounds, gossypol,[20]

Table 2. MC_{100} Values of Compounds **1**—**6**, Gossypol, Berberine Chloride, and Harmine against Epimastigotes of *T. cruzi in Vitro*

Compounds	MC_{100}	
	μg/ml	μM
1	25	77
2	40	123
3	75	219
4	50	146
5	>1000	
6	>1000	
Gossypol	280	540
Berberine Cl	300	807
Harmine	>500	

berberine chloride[21] and harmine[21] were also estimated and MC_{100} values of them are listed in Table 2. Among six compounds isolated from Guaco, **1**—**4** exhibited higher activity than the above mentioned three compounds. Comparisons of **1** with **5**, and **2** with **6** suggest that the loss of hydroxyl group reduces activity. The differences of activity between **1** and **2**, and **3** and **4** are not negligible. It suggests that steric structures might influence on activity. Judging from the yield and MC_{100} value, trypanocidal activity of Guaco is mainly due to **1** (eupomatenoid-7).

Recently Bastos *et al.* have isolated seven lignans from *Zanthoxylum naranjillo* (Rutaceae) and revealed that (−)-methylpulviatolide is highly active against trypomastigotes of the two strains of *T. cruzi*, the Bolivia and Y.[22] As for trypanocidal lignans, lignans from Guaco are the second instance.

Chemical investigation of the other active plant extracts and bioassay against trypomastigotes *in vitro* are now under progress.

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