Trypanocidal Activity of Meliaceae and Rutaceae Plant Extracts

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The in vitro trypanocidal activity of 22 extracts and 43 fractions of plants belonging to the families Meliaceae and Rutaceae was evaluated. The extracts from leaves of Conchocarphus heterophyllus *and branches of* Trichilia ramalhoi *were the most active. The trypanocidal activity seems to be increased by fractionation of the extracts. Fractions from* C. heterophyllus *and* Galipea carinata *were the most active and a 100% lysis of the parasites was observed for five fractions. From one of them were isolated two flavonoids: flavone and 7-methoxyflavone, which showed weak trypanocidal activity. The results obtained from the extracts and fractions revealed that the order Rutales is a promising source for the search of new drugs for Chagas disease. Phytochemical studies with the other active fractions are underway in order to isolate compounds, which could be associated with observed activities.*

Key words: Meliaceae - Rutaceae - Chagas disease - trypanocidal activity

Chagas disease (American trypanosomiasis) is caused by the flagellate protozoan *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatina), and affects more than 18 million people in Latin America, leading to approximately 400,000 deaths per year (WHO 1997). Its treatment is still a challenge, since the only drug commercially available (benznidazole) possesses severe side effects and its activity is limited to the acute phase of the disease (De Castro 1993, Fairlamb 1999). Coura and De Castro (2002) mention that an effective chemotherapy is needed for the people who are already infected. The demonstration of parasites in chronic patients reinforces the need of finding more efficient and less toxic drugs.

In the context of efforts to improve the therapy of Chagas disease, higher plants are a potential source of new drugs, with high activity and low toxicity (Phillipson & Wright 1991). Sepúlveda-Boza and Cassels (1996) mentioned a broad spectrum of chemical classes of substances showing activity against the parasite. Other promising compounds are the 2-aryl and 2-alkylquinoline alkaloids isolated from the extracts of the stem bark, root bark and leaves of *Galipea longiflora* (Rutaceae) (Fournet et al. 1994) and lignans from *Zanthoxylum naranjillo* (Rutaceae) (Bastos et al. 1999). In addition, we have been studying species of the order Rutales (Rutaceae, Meliaceae, Simaroubaceae, Burseraceae, and Cneoraceae) and several active substances have been isolated, mainly trypanocidal compounds (Mafezoli et al. 2000, Tomazela et al. 2000, Vieira et al. 2001).

Financial support: Fundação de Amparo à Pesquisa do Estado de São Paulo
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Received 5 September 2003
Accepted 12 January 2004

In this paper, we present the results of the trypanocidal activity of some extracts and fractions of *Almeidea coerulea*, *Almeidea rubra*, *Conchocarpus heterophyllus*, and *Galipea carinata* (from Rutaceae family), as well as *Trichilia ramalhoi* (from the family Meliaceae). Also, the results of the in vitro trypomastigote bioassay with flavone (**1**) and 7-methoxyflavone (**2**), the major compounds of one active fraction, are described.

MATERIALS AND METHODS

Plant material - All screened plants were collected in Southeastern Brazil, and identified by Dr José R Pirani from the Department of Botany, University of São Paulo, Brazil. The voucher herbarium specimens were deposited at the Herbarium of that Department (Table I).

Preparation of crude extracts - Selected parts of the plants (leaves, stems, and/or branches) were dried carefully by forced air at 40°C and reduced to powder, followed by extraction three times with hexane by maceration at room temperature for 72 h. After the evaporation of the solvent under reduced pressure, crude hexane extracts were obtained. This process was repeated with methanol. The hexane and methanol extracts so obtained were assayed against *T. cruzi*.

Fractionation of crude extracts - Methanol extracts of *T. ramalhoi* were fractionated through liquid-liquid partition with hexane-methanol-water, dichloromethane-methanol-water, ethyl acetate-methanol-water, and butanol-methanol-water. The crude extracts of *A. coerulea*, *A. rubra*, *C. heterophyllus*, *G. carinata*, and the hexane extract of the leaves of *T. ramalhoi* were submitted to vacuum liquid chromatography over silica gel using a gradient hexane, dichloromethane, ethyl acetate, and methanol, to yield the corresponding fractions, which were subsequently tested for their tryponocidal activity.

Isolation of flavone and 7-methoxyflavone - The ethyl acetate fraction of the hexane extract from the leaves of *C. heterophyllus* (AHFHA) (7.3 g) was chromatographed on a Florisil column (8 x 5.9 cm) and eluted with solvents of

TABLE I
Botanical identification of plants assayed

Plant family	Botanical name	Collected in	Voucher number
Rutaceae	*Almeidea coerulea* A. St.-Hil.	06/02/93	Pirani & Kallunki 2747
	Almeidea rubra A. St.-Hil.	19/05/00	Pirani et al. 4746
	Conchocarpus heterophyllus (A. St.-Hil.) Kallunki & Pirani	28/01/93	Pirani & Kallunki 2693
	Galipea carinata Pirani (sp. nov.)	18/01/93	Kallunki & Pirani 336
	Galipea carinata Pirani (sp. nov.)	18/05/00	Pirani et al. 4722
Meliaceae	*Trichilia ramalhoi* Rizzini	15/01/85	Pirani & Kallunki 2632

increasing polarity (hexane → methanol) to afford 6 fractions. The third fraction (1.6 g) was rechromatographed on a Silica gel column (230-400 mesh, 3.9 x 25.2 cm) using hexane-EtOAc mixtures. Eight fractions were obtained. The seventh one (1.27 g) was identificated as flavone (**1**) by comparison of the ^{13}C NMR data with the literature (Kingsburry & Looker 1975). Further purification of fraction 4 (3.12 g) performed on a Silica gel column (230-400 mesh, 3.9 x 25.2 cm; CH_2Cl_2 → MeOH) followed by gel filtration on Sephadex LH-20 (3.2 x 49.2 cm; MeOH) led to the isolation of 7-methoxyflavone (**2**) (22.7 mg) (Kingsburry & Looker 1975). These compounds were assayed against *T. cruzi*.

Trypanocidal activity in vitro - The bioassays were carried out using blood of infected Swiss albino mice, which was collected by cardiac puncture at the peak of parasitemic infection (7th day of infection for Y strain of *T. cruzi*). The infected blood was diluted with healthy mice blood to achieve a concentration of 2.10^6 forms/ml. Stock solutions of the extracts/fractions/compounds were prepared by dissolving in dimethylsulfoxide (DMSO). The activity of crude extracts was evaluated at 4 mg/ml, fractions at 2 mg/ml, pure substances at 500, 250, and 100 µg/ml. The bioassays were performed in triplicate on titration microplates (96 wells) which contained 400 µl of mixture/well. The plates were incubated at 4°C, and the number of parasites counted after 24 h, following the method described by Brener (1962). Infected blood with the same volume of DMSO was used as control, and gentian violet to a concentration of 250 µg/ml was used as positive control. The activity is expressed as percent reduction of the parasite number (lysis) and IC_{50} (mg/ml) for flavone and 7-methoxyflavone were calculated using the program GraphPad Prims v.3.0.

TABLE II
Trypanocidal activity of the crude extracts Meliaceae and Rutaceae species

Species	Plant part	Extraction solvent	Crude extract	Lysis %
Almeidea coerulea	Branch	Hexane	AGH	29.91
		Methanol	AGM	55.11
Almeidea rubra	Leaf	Hexane	ALFH	35.43
		Methanol	ALFM	54.33
	Stem	Hexane	ALCH	40.94
		Methanol	ALCM	55.90
Conchocarpus heterophyllus	Leaf	Hexane	AHFH	99.22
		Methanol	AHFM	59.44
	Stem	Hexane	AHCH	71.65
		Methanol	AHCM	68.89
Galipea carinata [a]	Leaf	Hexane	GFH	61.42
		Methanol	GFM	64.56
	Stem	Hexane	GCH	51.97
		Methanol	GCM	44.09
Galipea carinata	Leaf	Hexane	GCFH	54.33
		Methanol	GCFM	20.47
	Stem	Hexane	GCCH	50.00
		Methanol	GCCM	62.99
Trichilia ramalhoi	Leaf	Hexane	TRFH	59.44
		Methanol	TRFM	47.63
	Branch	Hexane	TRGH	84.25
		Methanol	TRGM	81.89

a: *G. carinata* specimen collected in 18/01/93

RESULTS AND DISCUSSION

In the present study, the trypanocidal activity of 22 extracts and 43 fractions of plants of Meliaceae and Rutaceae family was evaluated. Table II summarizes the results obtained from the crude extracts. Sixteen extracts showed significant activity (lysis % ≥ 50). The extracts from the leaves of *C. heterophyllus* (AHFH) and from the branches of *T. ramalhoi* (TRGH, TRGM) were the most active ones. Also the results obtained from the extracts (Table II) revealed that these species are rich sources of trypanocidal compounds, therefore the order Rutales is a promising source of new drugs for Chagas disease. The species *A. coerulea* and *C. heterophyllus* had already been tested by Mafezoli et al. (2000), however different parts of the plants were investigated.

Table III shows the results obtained from the in vitro assay of fractions against the trypomastigote form of *T. cruzi*. Trypanocidal activity seems to be enriched by fractionation of the extracts. Only fractions of *T. ramalhoi* showed a lower percentage of lysis than those of the ex-

TABLE III

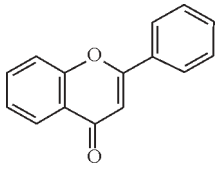
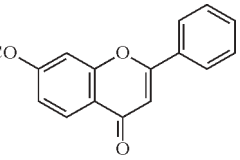
Trypanocidal activity of fractions of Meliaceae and Rutaceae species

Species	Crude extract	Vacuum liquid chromatography	Fraction	Lysis %
Almeidea coerulea	AGM	Dichloromethane	AGMD	100
		Ethyl acetate	AGMA	68.6
		Methanol	AGMM	12.9
Almeidea rubra	ALFM	Dichloromethane	ALFMD	41.4
		Ethyl acetate	ALFMA	62.9
		Methanol	ALFMM	40.0
	ALCM	Dichloromethane	ALCMD	65.7
		Ethyl acetate	ALCMA	80.0
		Methanol	ALCMM	17.1
Conchocarpus heterophyllus	AHFH	Hexane	AHFHH	25.7
		Dichloromethane	AHFHD	45.0
		Ethyl acetate	AHFHA	100
		Methanol	AHFHM	7.1
	AHFM	Hexane	AHFMH	97.1
		Dichloromethane	AHFMD	25.6
		Ethyl acetate	AHFMA	56.4
		Methanol	AHFMM	98.6
	AHCM	Ethyl acetate	AHCMA	100
		Methanol	AHCMM	37.0
Galipea carinata [a]	GFM	Dichloromethane	GFMD	82.6
		Ethyl acetate	GFMA	96.4
		Methanol	GFMM	50.0
Galipea carinata	GCFH	Hexane	GCFHH	100
		Dichloromethane	GCFHD	9.3
		Ethyl acetate	GCFHA	11.3
		Methanol	GCFHM	65.6
	GCCM	Dichloromethane	GCCMD	100
		Ethyl acetate	GCCMA	44.4
		Methanol	GCCMM	31.1
Trichilia ramalhoi	TRFH	Hexane	TRFHH	49.7
		Dichloromethane	TRFHD	8.6
		Ethyl acetate	TRFHA	28.5
		Methanol	TRFHM	15.2
	TRFM	Liquid-liquid partition		
		Hexane	TRFMH	12.6
		Dichloromethane	TRFMD	25.8
		Ethyl acetate	TRFMA	21.9
		Methanol	TRFMM	31.1
		Butanol	TRFMB	29.8
	TRGM	Liquid-liquid partition		
		Hexane	TRGMH	68.2
		Dichloromethane	TRGMD	76.2
		Ethyl acetate	TRGMA	13.9
		Methanol	TRGMM	23.2
		Butanol	TRGMB	12.6

a: *G. carinata* specimen collected in 18/01/93

TABLE IV

Trypanocidal activity of flavone (**1**) and 7-methoxyflavone (**2**) isolated from *Conchocarpus heterophyllus*

Compound	Concentration (μg/ml) x Lysis % (± S.D.)			IC_{50} (μg/ml)
	100	250	500	
(**1**) 	34.5 ± 10.9	38.6 ± 8.1	48.6 ± 6.4	2116.0
(**2**) 	12.1 ± 7.4	31.8 ± 3.1	38.6 ± 8.1	787.1

S.D.: standard deviation

tracts. Ten fractions exhibited lysis above 80%, among them: the dichloromethane fraction from the methanolic extract of *A. coerulea*, AGMD (100%); the ethyl acetate fraction from the methanolic extract of the stem of *A. rubra*, ALCMA (80%); the ethyl acetate fraction from the hexane extract and the hexane and methanol fractions from the methanolic extract of the leaves and the ethyl acetate fraction from the methanolic stem extract of *C. heterophyllus* - AHFHA (100%), AHFMH (97.1%), AHFMM (98.6%), and AHCMA (100%); the dichloromethane and ethyl acetate fractions from the methanolic extract and the hexane fraction from the hexane extract of leaves of *G. carinata* GFMD (82.6%), GFMA (96.4%), GCFHH (100%) as well as the dichloromethane fraction from the methanolic extract of the stem of the same plant - GCCMD (100%). It will be noted that the best results were obtained from *Conchocarpus* and *Galipea* fractions. Phytochemical studies of all active fractions are underway in order to isolate the compounds which could be associated with observed activities.

One active fraction from *C. heterophyllus* (AHFHA) was investigated leading to the isolation of flavone (**1**) and 7-methoxyflavone (**2**), which were assayed against *T. cruzi* (Table IV). The results showed that these compounds have weak trypanocidal activity, particularly when compared to other flavonoids isolated from *Trixis vauthieri* (Ribeiro et al. 1997). The trypanocidal effect of the fraction AHFHA may be due to a combination effect between flavone and 7-methoxyflavone, which are the major compounds in this fraction. This possibility will be assessed by new bioassays with mixtures of these substances, which exist in the same proportion in the plant.

It is possible that the activity of the Rutaceae may be associated with coumarins and alkaloids (derived from anthranilic acid), which are characteristic of this family (Gray 1983, Mester 1983). C-glucosyl flavones (Jay et al. 1979, Wirasutisna et al. 1986), 2-quinolone and other alkaloids (Moulis et al. 1983), cycloartane triterpenoids and further alkaloids (Santos et al. 1998) have already been isolated from *Almeidea*. *Galipea* has afforded a hydroxychalcone (López et al. 1998), a chromone (López et al. 1997), a coumarin (Wirasutisna et al. 1987), O- and C-glycosylflavones (Bakhtiar et al. 1990, 1994), and several 2-quinoline alkaloids (Fournet et al. 1989, 1993, Vieira & Kubo 1990, Rakotoson et al. 1998, Jacquemond-Collet et al. 1999). The structural diversity of metabolites from Rutaceae and the trypanocidal activity of 2-quinoline alkaloids and the lignan methylpluviatolide isolated from *G. longiflora* and *Z. naranjillo* (Fournet et al. 1994, Bastos et al. 1999) stimulated us to choose these plants. The trypanocidal activities observed confirm the previously noted potential of plants of the Rutaceae.

T. ramalhoi was the only Meliaceae species tested and no previous work on this plant was found. Meliaceae are a rich source of limonoids, however it seems that this class of compounds has never been evaluated for trypanocidal activity before (Champagne et al. 1992).

Finally, the results obtained in this study confirm the order Rutales as a fruitful source of new antichagasic compounds, since in the present work 16 active extracts and several fractions have been obtained, 5 of them showing 100% reduction of the parasite number. The more promising species seem to be *C. heterophyllus* and *G. carinata* although the weakly trypanocidal flavone (**1**) and 7-methoxyflavone (**2**) in their isolated form do not reproduce the activity of the crude extract. The full explanation of the observed activity of these fractions must await the results of the ongoing phytochemical studies.

ACKNOWLEDGEMENTS

To Dr José Rubens Pirani for collecting and identificating all the mentioned plants.

REFERENCES

Bakhtiar A, Gleye J, Moulis C, Fouraste I, Stanislas E 1990. C-Glycosylflavones from *Galipea trifoliata*. *Phytochemistry 29*: 1339-1340.

Bakhtiar A, Gleye J, Moulis C, Fouraste I 1994. O-Glycosyl-C-glycosylflavones from *Galipea trifoliata*. *Phytochemistry 35*: 1593-1594.

Bastos JK, Albuquerque S, Silva MLA 1999. Evaluation of the

trypanocidal activity of lignans isolated from the leaves of *Zanthoxylum naranjillo*. *Planta Med 65*: 541-544.

Brener Z 1962. Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi*. *Rev Inst Med Trop São Paulo 4*: 389-396.

Champagne DE, Koul O, Isman MB, Scudder GGE, Towers GHN 1992. Biological activity of limonoids from the Rutales. *Phytochemistry 31*: 377-394.

Coura JR, De Castro SL 2002. A critical review on Chagas disease chemotherapy. *Mem Inst Oswaldo Cruz 97*: 3-24.

De Castro SL 1993. The challenge of Chagas' disease chemotherapy: an update of drugs assayed against *Trypanosoma cruzi*. *Acta Trop 53*: 83-98.

Fairlamb AH 1999. Future prospects for the chemotherapy of Chagas disease. *Medicina (B Aires) 59*: 179-187.

Fournet A, Barrios AA, Munoz, V, Hocquemiller R, Roblot F, Cavé A, Richomme P, Bruneton J 1994. Antiprotozoal activity of quinoline alkaloids isolated from *Galipea longiflora*, a Bolivian plant used as a treatment for cutaneous leishmaniasis. *Phytother Res 8*: 174-178.

Fournet A, Hocquemiller R, Roblot F, Cavé A, Richomme P, Bruneton J 1993. Les chimanines, nouvelles quinoleines substituées en 2, isolées d'une plante bolivienne antiparasitaire: *Galipea longiflora*. *J Nat Prod 56*: 1547-1552.

Fournet A, Vagneur B, Richomme P, Bruneton J 1989. Aryl-2 et alkyl-2 quinoléines nouvelles isolées d'une Rutacée bolivienne: *Galipea longiflora*. *Can J Chem 67*: 2116-2118.

Gray AI 1983. Structural diversity and distribution of coumarins and chromones in the Rutales. In PG Waterman, Grundon MF (eds), *Chemistry and Chemical Taxonomy of the Rutales,* Academic Press, London, p. 97-146.

Jacquemond-Collet I, Hannedouche S, Fabre N, Fourasté I, Moulis C 1999. Two tetrahydroquinoline alkaloids from *Galipea officinalis*. *Phytochemistry 51*: 1167-1169.

Jay M, Gleye J, Bouillant ML, Stanislas E, Moretti C 1979. Nouvelles C-arabinosyl flavones extraites de *Almeidea guianensis* (Rutaceae). *Phytochemistry 18*: 184-185.

Kingsburry CA, Looker JH 1975. Carbon-13 spectra of methoxyflavones. *J Org Chem 40*: 1120-1124.

López JA, Barillas W, Gomes-Laurito J, Martin GE, Al-Rehaily AJ, Zematis MA, SchiffJr PL 1997. Granulosin: a new chromone from *Galipea granulosa*. *J Nat Prod 60*: 24-26.

López JA, Barillas W, Gomes-Laurito J, Martin GE, Lin F, Al-Rehaily AJ, Zematis MA, Schiff Jr PL 1998. Galiposin: a new β-hydroxychalcone from *Galipea granulosa*. *Planta Med 64*: 76-77.

Mafezoli J, Vieira PC, Fernandes JB, Da Silva MFGF, Albuquerque S 2000. *In vitro* activity of Rutaceae species against the trypomastigote form of *Trypanosoma cruzi*. *J Ethnopharmacol 73*: 335-340.

Mester I 1983. Structural diversity and distribution of alkaloids in the Rutales. In PG Waterman, MF Grundon (eds), *Chemistry and Chemical Taxonomy of the Rutales*, Academic Press, London, p. 31-96.

Moulis C, Wirasutisna KR, Gleye J, Loiseau P, Stanislas E, Moretti C 1983. A 2-quinolone alkaloid from *Almeidea guianensis*. *Phytochemistry 22*: 2095-2096.

Phillipson JD, Wright CW 1991. Medicinal plants against protozoal diseases. *Trans R Soc Trop Med Hyg 85*: 155-165.

Rakotoson JH, Fabre N, Jacquemond-Collet I, Hannedouche S, Fourasté I, Moulis C 1998. Alkaloids from *Galipea officinalis*. *Planta Med 64*: 762-763.

Ribeiro A, Piló-Veloso D, Romanha AJ, Zani CL 1997. Trypanocidal flavonoids from *Trixis vauthieri*. *J Nat Prod 60*: 836-838.

Santos CS, Januário AH, Vieira PC, Fernandes JB, Da Silva MFGF, Pirani JR 1998. Cycloartane triterpenoid and alkaloids from *Almeidea* spp. *J Braz Chem Soc 9*: 39-42.

Sepúlveda-Boza S, Cassels BK 1996. Plant metabolites active against *Trypanosoma cruzi*. *Planta Med 62*: 98-105.

Tomazela DM, Pupo MT, Passador EAP, DaSilva MFGF, Vieira PC, Fernandes JB, Rodrigues-Filho E, Oliva G, Pirani JR 2000. Pyrano chalcones and a flavone from *Neoraputia magnifica* and their *Trypanosoma cruzi* glycosomal glyceraldehyde-3-phosphate dehydrogenase-inhibitory activities. *Phytochemistry 55*: 643-651.

Vieira PC, Kubo I 1990. Molluscicidal quinoline alkaloids from *Galipea bracteata*. *Phytochemistry 29*: 813-815.

Vieira PC, Mafezoli J, Pupo MT, Fernandes JB, Da Silva MFGF, Albuquerque S, Oliva G, Pavão F 2001. Strategies for the isolation and identification of trypanocidal compounds from the Rutales. *Pure Appl Chem 73*: 617-622.

WHO-World Health Organization 1997. Chagas disease. Thirteenth Programme Report UNDP/TDR, Geneve.

Wirasutisna KR, Gleye J, Moulis C, Stanislas E, Moretti C 1986. Flavone C-Glycosides of *Almeidea guyanensis*. *Phytochemistry 25*: 558-559.

Wirasutisna KR, Gleye J, Moulis C, Stanislas E, Moretti C 1987. Galipein, a coumarin from *Galipea trifoliata*. *Phytochemistry 26*: 3372.