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Artigo

Screening of *Trypanosoma cruzi* glycosomal glyceraldehyde-3-phosphate dehydrogenase enzyme inhibitors

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RESUMO: "Busca de inibidores da enzima glicossomal gliceraldeído 3-fosfato desidrogenase de *Trypanosoma cruzi*". Nesse trabalho foi avaliada a atividade inibitória sobre a enzima glicossomal gliceraldeído-3-fosfato desidrogenase de *T. cruzi* (gGAPDH) de extratos vegetais oriundos de plantas das famílias Meliaceae e Rutaceae, na concentração de 100 µg/mL. Foram testados 46 extratos, dos quais 15 apresentaram atividade inibitória significativa (% AI ≥ 50). A maioria dos extratos de plantas da família Meliaceae (*Cedrela fissilis*, *Cipadessa fruticosa* e *Trichilia ramalhoi*) apresentou grande potencial em inibir a atividade enzimática. O fracionamento do extrato hexânico dos galhos de *C. fruticosa* permitiu o isolamento de três flavonóides: flavona, 7-metoxiflavona e 3',4',5',5,7-pentametoxiflavona. Os dois últimos foram ativos na inibição da atividade de gGAPDH. Desta forma, as três espécies de Meliaceae testadas podem ser consideradas promissoras na busca de compostos protótipos para o controle da doença de Chagas.

Unitermo: Rutaceae, Meliaceae, flavonóides, gGAPDH, *Trypanosoma cruzi*, inibidores.

ABSTRACT: The inhibitory activity of crude extracts of Meliaceae and Rutaceae plants on glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) enzyme from *Trypanosoma cruzi* was evaluated at 100 µg/mL. Forty-six extracts were tested and fifteen of them showed significant inhibitory activity (IA % ≥ 50). The majority of the assayed extracts of Meliaceae plants (*Cedrela fissilis*, *Cipadessa fruticosa* and *Trichilia ramalhoi*) showed high ability to inhibit the enzymatic activity. The fractionation of the hexane extract from branches of *C. fruticosa* led to the isolation of three flavonoids: flavone, 7-methoxyflavone and 3',4',5',5,7-pentamethoxyflavone. The two last compounds showed high ability to inhibit the gGAPDH activity. Therefore, the assayed Meliaceae species could be considered as a promising source of lead compounds against Chagas' disease.

Keywords: Rutaceae, Meliaceae, flavonoids, gGAPDH, *Trypanosoma cruzi*, inhibitors.

INTRODUCTION

Chagas' disease, caused by the protozoan *Trypanosoma cruzi*, affects some 16-18 million people, mostly from South and Central America, where 25 % of the total population are at risk of contamination (WHO, 2007). Control of the insect vector (*Triatoma infestans*) in endemic areas has led to the virtual elimination of transmission by insect bites, and as consequence, blood transfusion and congenital transmission are the major causes for the spread of the disease (Dias, 1993). Its treatment is still a challenge, since the only drug commercially available (benznidazole) has strong side effects (De Castro, 1993).

The bloodstream form of parasites of the family Trypanosomatidae possesses a microbody-like

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organelle, where glycolysis takes place, the glycosome (Opperdoes & Borst, 1977). The bloodstream form of the parasite *T. cruzi* has no functional tricarboxylic acid cycle, and it is highly dependent on glycolysis for ATP production. This great dependence on glycolysis as a source of energy makes the glycolytic enzymes attractive targets for trypanocidal drug design (Souza et al., 1998). Glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) is a glycolytic enzyme which catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate (GAP) to 1,3-bisphosphoglycerate (BGP). The three dimensional structure of *T. cruzi* gGAPDH previously determined shows differences, when compared to the homologous human enzyme that could be exploited for selective inhibition (Souza et al., 1998).

The need for the development of more efficient drugs against Chagas' disease has stimulated us to initiate a project aiming at the discovery of lead compounds from several plants of Rutales order. In this way, we have proposed the utilization of the glycolytic enzyme gGAPDH as a target for the search of new natural trypanocides (Tomazela et al., 2000; Vieira et al., 2001; Pavão et al., 2002; Moraes et al., 2003). In this paper, we report the gGAPDH inhibition activity of 46 crude extracts of Meliaceae and Rutaceae plants, along with three flavones isolated from an active fraction.

MATERIAL AND METHODS

Plant material

The fruits, branches, stems, roots, and leaves of *Cedrela fissilis* Vell. were collected in 01/06/01 in São Carlos (São Paulo, Brazil) and identified by Dr. Maria Inês Salgueiro Lima from the Department of Botany, Universidade Federal de São Carlos, where a voucher specimen (6701) was deposited. The fruits, branches and leaves of *Cipadessa fruticosa* Bl. were collected in 01/20/01 in Viçosa (Minas Gerais, Brazil) and identified by Dr. José R. Pirani from the Department of Botany, Universidade de São Paulo and deposited at the herbarium from this department with a voucher number 110.664. The branches of *Almeidea coerulea* A. St.-Hil., stems, and leaves of *A. rubra* A. St.-Hil., *Conchocarpus heterophyllus* (A. St.-Hil.) Kallunki & Pirani, *Galipea carinata* Pirani (sp. nov.) and *Trichilia ramalhoi* Rizzini were collected in Southeastern Brazil, and identified by Dr. José R. Pirani from the Department of Botany, Universidade de São Paulo, Brazil. The specimens were deposited at the Herbarium of that Department. The voucher numbers and the dates of collection were described in Ambrozin et al. (2004).

Preparation of crude extracts

The powdered air-dried plant material (branches, fruits, leaves, roots, and/or stems) was extracted by maceration three times with hexane, at room temperature for 72 h. This process was repeated with dichloromethane and methanol for *C. fissilis* and *C. fruticosa*, and only with methanol for the other species. The solvent was removed under reduced pressure by rotary evaporation and the extracts obtained were assayed on *T. cruzi* gGAPDH enzyme.

Isolation of compounds

The hexane extract from branches of *C. fruticosa* (12.2 g) was submitted to vacuum liquid chromatography over silica gel using a hexane-dichloromethane-ethyl acetate-methanol gradient. The ethyl acetate fraction (6.8 g) was chromatographed on silica gel, eluting with a hexane-dichloromethane-acetone gradient to give 9 fractions. Fraction 3 was fractionated as above, using hexane-dichloromethane-acetone (6:3:1), affording 9 fractions. Fraction 5 was three times chromatographed through column chromatography on silica gel, eluting with hexane-dichloromethane-methanol (7:2.5:0.5) to afford compounds **1** (16.7 mg) and **2** (8.2 mg). Fraction 6 was twice submitted to column chromatography with hexane-dichloromethane-methanol (6:3:1) giving compound **3** (30.6 mg). Compounds **1**-**3** were characterized by comparison of ^{13}C NMR data with the literature (Kingsburry & Looker, 1975; Passador et al., 1997) and submitted to the enzymatic assay.

Preparation and purification of recombinant *T. cruzi* gGAPDH

TcGAPDH was overexpressed and purified as reported by Souza et al. (1998). It is maintained in the Crystallography Laboratory of the Universidade de São Paulo, São Carlos, SP, Brazil.

T. cruzi gGAPDH inhibitory activity

gGAPDH activity was determined according to a previously reported procedure (Barbosa & Nakano, 1987; Vieira et al., 2001). Reduced NADH was spectrophotometrically measured at 340 nm during 30 s. The reaction medium contains 50 mmol L^{-1} Tris-HCl pH 8.6 buffer, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} β-mercapto-ethanol, 30 mmol L^{-1} Na_2HAsO_4, 2.5 mmol L^{-1} NAD^+, 0.3 mmol L^{-1} glyceraldehyde-3-phosphate and 0.15 mg protein, in a total volume of 500 μL. The reaction was initiated by the addition of substrate. The extracts were tested at a final concentration of 100 μg/mL.

RESULTS

In this work, we evaluated the inhibitory activity of 46 extracts of Meliaceae and Rutaceae plants on *T. cruzi* gGAPDH enzyme (Table 1). Fifteen of them showed high ability in inhibit the enzyme (IA % ≥ 50) at 100 μg/mL.

The hexane extract from branches of *C. fruticosa* (CFGH) and the methanol extracts from Meliaceae plants were the most active ones, whereas the extracts from Rutaceae did not present significant results.

The investigation of the ethyl acetate fraction (inhibitory activity = 81.9 % at 100 μg/mL) from the hexane extract from branches of *C. fruticosa* (CFGH) led to the isolation of three flavonoids: flavone (**1**), 7-methoxyflavone (**2**) and 3',4',5',5,7-pentamethoxyflavone (**3**). The flavones **1** and **2** were assayed (Table 2) and the results showed that only **2** presented high inhibitory activity against gGAPDH enzyme.

DISCUSSION

The results indicated that, among the assayed species, *C. fissilis*, *C. fruticosa* and *Trichilia ramalhoi* are promising sources of lead compounds for the rational design of new trypanocidal drugs. The inhibitory activity of the flavones **1** and **2** against gGAPDH has been reported for the first time. Recently, Ambrozin et al. (2004) published their activity against trypomastigote forms of *T. cruzi* and Tadesmir et al. (2006) described the trypanocidal activity of some flavones. A previous study showed that compound **3** completely inhibited the gGAPDH activity at 268.8 μmol/L (Tomazela et al., 2000). Thus, the activity of the studied extract (CFGH) (Table 1) is probably related to the presence of the active flavones **2** and **3**. However, their enzymatic activity were considered weak, particularly in comparison with chalepin, a coumarin isolated from *Pilocarpus spicatus* (Rutaceae), which reduced the gGAPDH activity by 75 % at 93 μmol/L (IC_{50} = 64 μmol/L) (Vieira et al., 2001; Pavão et al., 2002; Menezes et al., 2003; Moraes et al., 2003; Leitão et al., 2004).

Similar studies involving the enzyme gGAPDH have been reported by our group. Vieira et al. (2001) showed the enzymatic activity of several plants of Rutaceae and Meliaceae families and thirteen coumarins isolated from different species, which are considered as one of the most promising class of substances with gGAPDH inhibitory activity. Moreover, the gGAPDH activity of several flavonoids isolated from Meliaceae, Rutaceae and Leguminosae plants, including pyrano chalcones, flavones, flavanones, flavonols and isoflavones, have also been reported (Tomazela et al., 2000; Moraes et al., 2003). Recently, Januário et al. (2005) described the activity of 7-hydroxy-4',6-dimethoxyisoflavone on gGAPDH. All these data support the hypothesis that highly oxygenated flavones possess the structural requirements to inhibit gGAPDH.

The family Meliaceae is characterized by the frequent occurrence of limonoids (da Silva et al., 1984). This class of compounds has been attracted considerable interest because of their biological properties and diversity of structures (Champagne et al., 1992).

Previous studies of *C. fissilis* showed the isolation of limonoids and triterpenes (Taylor, 1984; Zelnik, 1966, 1970; Leite et al., 2005a; Ambrozin et al., 2006) and *C. fruticosa* has been reported to contain *ent*-clerodanes and labdanes diterpenoids (Rojatkar and Nagasampagi, 1994; Rojatkar et al., 1994), limonoids, steroids, sesquiterpenoids, heneicosene derivatives and one coumarin (Luo et al., 2000, 2001; Leite et al., 2005a,b). There are no phytochemical studies about *T. ramalhoi*, but several compounds have been isolated from the *Trichilia* genus, such as, limonoids (Cortez et al., 1992; Garcez et al., 1997; Garcez, et al., 2000; Cortez et al., 2000), lignan glycosides (Cortez et al., 1998), ω-phenyl alkanoic and alkenoic acids (Pupo et al., 1996), terpenoids, steroids (Pupo et al., 2002; Pupo et al., 1996) and γ-lactones (Pupo et al., 1998). Phytochemical studies with other active extracts from these plants are in progress and shall reveal molecules that can be used as potential lead compounds in the search for potent and selective *T. cruzi* gGAPDH inhibitors.

In conclusion, most of the active extracts belong to the family Meliaceae. New investigations with these extracts are undertaken in order to find inhibitors that could act as potential lead molecules on the described assay. Moreover, the isolated flavonoids bring about the enzymatic inhibition, showing that these compounds are potential lead molecules for the development of new drugs against Chagas' disease.

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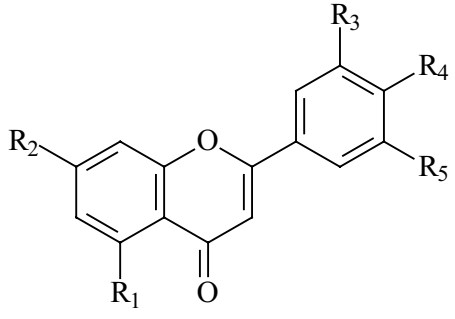

1: $R_1 = R_2 = R_3 = R_4 = R_5 = H$

2: $R_2 = OCH_3$, $R_1 = R_3 = R_4 = R_5 = H$

3: $R_1 = R_2 = R_3 = R_4 = R_5 = OCH_3$

Figure 1. Compounds isolated from *Cipadessa fruticosa*.

Table 1. gGAPDH inhibitory activity of Meliaceae and Rutaceae extracts at 100 µg/mL.

Family/species	Plant part	Extraction solvent	Crude extract	IA %
MELIACEAE				
Cedrela fissilis	fruits	hexane	CFRH	50.8
		dichloromethane	CFRD	70.7
		methanol	CFRM	94.0
	branches	hexane	CGH	32.4
		dichloromethane	CGD	85.4
		methanol	CGM	93.2
	stems	hexane	CCH	25.4
		dichloromethane	CCD	9.8
		methanol	CCM	94.2
	roots	hexane	CRH	3.1
		dichloromethane	CRD	5.0
		methanol	CRM	94.4
	leaves	hexane	CFH	36.2
		dichloromethane	CFD	65.5
		methanol	CFM	91.6
Cipadessa fruticosa	fruits	hexane	CFFRH	12.2
		dichloromethane	CFFRD	9.6
		methanol	CFFRM	84.8
	branches	hexane	CFGH	98.3
		dichloromethane	CFGD	11.7
		methanol	CFGM	90.2
	leaves	hexane	CFFH	0
		dichloromethane	CFFD	0
		methanol	CFFM	66.7
Trichilia ramalhoi	branches	hexane	TRGH	9.7
		methanol	TRGM	95.3
	leaves	hexane	TRFH	73.5
		methanol	TRFM	92.4
RUTACEAE				
Almeidea coerulea	branches	hexane	AGH	0
		methanol	AGM	0
Almeidea rubra	stems	hexane	ALCH	0
		methanol	ALCM	0
	leaves	hexane	ALFH	0
		methanol	ALFM	0
Conchocarpus heterophyllus	stems	hexane	AHCH	0
		methanol	AHCM	8.2
	leaves	hexane	AHFH	0
		methanol	AHFM	8.2
Galipea carinata[a]	stems	hexane	GCH	0
		methanol	GCM	10.9
	leaves	hexane	GFH	0
		methanol	GFM	0
Galipea carinata[b]	stems	hexane	GCCH	0
		methanol	GCCM	0
	leaves	hexane	GCFH	0
		methanol	GCFM	0

[a]*Galipea carinata* specimen collected in 01/18/93, [b]*Galipea carinata* specimen collected in 05/18/00.

Table 2. gGAPDH inhibitory activity of flavone (**1**), 7-methoxyflavone (**2**) and 3',4',5',5,7-pentamethoxyflavone (**3**).

Compound	Concentration (μmol/L)	% IA
1	450	9.7
2	397	78.3
3[a]	268	100.0

[a]reported by Tomazela et al. (2000).

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