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ORIGINAL PAPER

Antiparasitic activity of biochanin A, an isolated isoflavone from fruits of *Cassia fistula* (Leguminosae)

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Abstract The fractionation through bioguided antileishmanial activity of the dichloromethane extract of *Cassia fistula* fruits (Leguminosae) led to the isolation of the active isoflavone biochanin A, identified by spectroscopic methods. This compound showed 50% effective concentration (EC_{50}) value of 18.96 μg/mL against promastigotes of *Leishmania* (L.) *chagasi*. The cytotoxicity of this substance against peritoneal macrophages resulted in an EC_{50} value of 42.58 μg/mL. Additionally, biochanin A presented an anti-*Trypanosoma-cruzi* activity, resulting in an EC_{50} value of 18.32 μg/mL and a 2.4-fold more effectiveness than benznidazole. These results contribute with novel antiprotozoal compounds for future drug design studies.

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Introduction

Cassia fistula is an ornamental tree commonly known as the golden shower, and it is one of the 400 different species that comprises the genus *Cassia* (Leguminosae). This species is native in India, Amazon, and Sri Lanka and is widely used in traditional medicine for children and pregnant women (Bahorun et al. 2005) as mild laxative and also as purgative (Iyengar et al. 1966). Other important therapeutic applications include its use as cicatrizing, antipyretic, analgesic, and hypoglicemiant and also for the treatment of hypercholesterolemia (Bahorun et al. 2005). In ayurvedic medicine, it is used against various disorders such as hematemesis, pruritus leukoderma, and diabetics (Luximon-Ramma et al. 2002). It has also been described as a cathartic action as a result of the anthraquinone derivatives isolated from the pulp of the fruits of *C. fistula* (Iyengar et al. 1966). Antioxidant activity of phenolic compounds like proanthocyanidins and flavonoids, as well as antitumor activity from extracts of seeds have been reported (Luximon-Ramma et al. 2002). In general species of genus, *Cassia* have been used as laxative, purgative, antimicrobial, antipyretic, antiviral, and anti-inflammatory agents (Viegas et al. 2006).

Phytochemical analysis of this specie led to the isolation of flavonoids, catechins, and proanthocyanidins, also known as condensed tannins (Kashiwada et al. 1990, Morimoto et al. 1988, Yadava and Verma 2003). Other compounds include sennosides, a mixture of anthrones (Asseleih et al. 1990) and fistulic acid (Agrawal et al. 1972). Parasitic diseases constitute the major cause of mortality in developing countries. Leishmaniasis is a neglected disease found in 16 developed and 72 developing countries, with 12 million cases (Croft et al. 2006; Trouiller et al. 2001). The dramatic hepatosplenomegaly of the

visceral form (VL) of the disease kills 100% of untreated patients (Singh and Sivakumar 2004). The therapeutic arsenal in VL is very limited due to highly toxic drugs. The first-line drug still remains an antimonial therapy for over a hundred years, with severe nephrotoxicity as severe side effects (Balaña-Fouce et al. 1998). Chagas' disease or American trypanosomiasis is another parasitic neglected disease which afflicts 18 million of people in Latin American. The current chemotherapy is based on the nitroaromatic compounds as benznidazole and nifurtimox, which are low-efficacy drugs especially against the chronic phase of infection. Elevated toxicity during treatment has also been the main barrier (Tempone et al. 2007). Consequently, there is an urgent need for new drugs to treat these neglected diseases. In this work, the bioguided fractionation of the fruits of *C. fistula*, led to the isolation of a novel isoflavone in this species, with a potent antileishmanial activity. In addition, we have evaluated the in vitro activity against *Trypanosoma cruzi* of the isolated compound and its cytotoxicity against mammalian cells.

Materials and methods

Plant material Fruits from *C. fistula* were collected at São Paulo City, São Paulo State, Brazil (GPS S 23° 30 564' W 46° 36 501') in September 2004. A voucher specimen was deposited at the Herbarium of Instituto Florestal de São Paulo, SP, Brazil (SPSF 30711).

Instruments ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on Bruker AC-200 and Bruker DPX-300 operating at 200 and 300 MHz, respectively. Spectra were performed in CD_3OD and CD_3OCD_3 with tetramethylsilane as internal standard. The chemical shifts (δ) are given in parts per million and coupling constants (J) are given in Hertz.

Extraction and isolation Dried fruits of *C. fistula* were cut into pieces and milled yielding 1.223 g of plant material. The material was extracted three times with methanol (MeOH) at room temperature. The solutions were dried under reduced pressure, suspended in water, and sequentially partitioned with hexane, dichloromethane, ethyl acetate, and butanol. The extracts were concentrated under reduced pressure and each extract was assayed for antileishmanial activity against promastigotes of *Leishmania* (L.) *chagasi*. The dichloromethane extract (1,632.3 mg) presented the antileishmanial activity and was further chromatographed on a silica gel column. Elution was carried out through the addition of an increasing solvent polarity using hexane and ethyl acetate. This procedure yielded 19 fractions based in the chromatographic profile using thin-layer chromatography. The most active fractions, DF-19, were submitted to high-performance liquid chromatography analysis (Shimadzu LC-10) using a C18 column (250×10 mm, 5 μm i.d., Phenomenex) with ACN in H_2O (25:75). Fractions were manually collected based on its absorbance at 254 nm. The antileishmanial fraction of this study resulted in the isolated substance identified as biochanin A.

Antileishmanial activity The antileishmanial activity of extracts, fractions, and the isolated substance were determined against *L. chagasi* (MHOM/BR/1972/LD) promastigotes. Parasites were maintained in M-199 medium supplemented with 10% calf serum and 0.25% hemin at 24°C. The antileishmanial activity against promastigotes was determined using 96-well microplates. Briefly, 1×10^6 promastigotes per well were seeded with different sample concentrations, previously dissolved in MeOH and diluted in M-199 medium. The viability of parasites was colorimetrically determined by the mitochondrial oxidation of 3-[4,5-dimethylthiazol-2-y1]-2,5diphenyl-tetrazolium bromide (MTT; Tada et al. 1986). Pentamidine was used as standard drug and controls with MeOH and without samples were performed. The isolated biochanin A was also tested against intracellular amastigotes of *L. chagasi.* Briefly, macrophages were isolated from the peritoneal cavity of BALB/c mice using Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% calf serum, and were dispensed into 24-well microplates containing 13-mm glass coverslips at 4×10^5 cells per well for 24 h prior to infection. Amastigotes were purified through differential centrifugation (Tempone et al. 2004) from a hamster spleen and dispensed to the macrophage wells at 10:1 rate (amastigotes–macrophage). The isolated biochanin A and standard drug Glucantime® were diluted in RPMI 1640 medium and incubated for 120 h at 37°C in a 5% CO_2-humidified incubator. Infected macrophages were fixed with MeOH and stained with Giemsa prior to light microscopy studies. The infection rate was determined by the number of infected macrophages out of 500 cells in duplicate. The EC_{50} was calculated with a sigmoid dose–response model using the Graph Pad Prism Software 3.0.

Antitrypanosomal activity Trypomastigotes of *T. cruzi* were maintained in LLC-MK2 cells (ATCC CCL 7). Trypomastigotes were counted in a Neubauer hemocytometer and seeded at 1×10^6 cells per well in 96-well microplates. Biochanin A was incubated to the highest concentration of 150 μg/mL for 24 h at 37°C in a 5% CO_2-humidified incubator, with benznidazole as standard drug. The trypomastigote viability was based on the cellular conversion of the soluble tetrazolium salt MTT into the insoluble formazan by mitochondrial enzymes. The formazan extraction was carried out with 10% (*v*/*v*) sodium dodecyl sulfate for 18 h (100 μL per well) at 24°C (Lane et al. 1996).

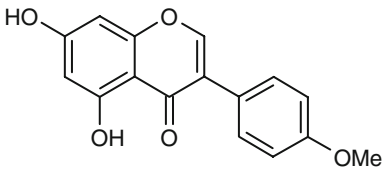

Fig. 1 Structure of biochanin A

Cytotoxicity assay LLC-MK2 cells were seeded (4×10^4 per well) in 96-well microplates and incubated with different drug concentrations for 48 h at 37°C in a 5% CO_2-humidified incubator. The viability of cells was determined with the MTT assay as described above. The selectivity index was determined considering the following equation: EC_{50} against LLC-MK2 cells/EC_{50} against parasites.

Hemolytic activity The hemolytic activity of biochanin A was evaluated in BALB/c erythrocytes to the highest concentration of 100 μg/mL (Moreira et al. 2007). A 3% suspension of mice erythrocytes was incubated for 2 h with isolated compound (diluted in MeOH) in a 96-well U-shaped microplates at 25°C and the supernatant was read at 550 nm in a Multiskan reader (Conceição et al. 2006).

Results and discussion

The subfraction DF19-1, obtained from dichloromethane extract of *C. fistula*, was submitted to the spectroscopic analysis of ^{1}H NMR. The spectra showed the presence of two aromatic moieties, methoxyl group and carbinolic hydrogen of double bond which singlet at δ 7.55 was attributed to H-2 linked to unsaturated carbon, a characteristic signal of isoflavonoid. Signals at 7.86 $\delta(d, J = 8.8\text{Hz})$ referring to H-2'/H-6' and at 6.82 δ (*d*, *J*=8.8 Hz) referring to hydrogens H-3'/H-5' with orto coupling H-2'/H-3' e H-6'/H-5', respectively, was also observed. The signal at 3.88 δ (*s*) corresponding to hydrogens of methoxy group linked to aromatic ring. The position of methoxy group at C-4' was confirmed by the ^{1}H-NOE difference spectra performed by irradiation at 3.88 δ (*s*, OMe-4') resulting in enhancement of the signal at 6.82 δ (*d*, J=8.8 Hz). A singlet at 9.75 δ referring to hydrogen of hydroxyl chelated to carbonyl was also observed. From the spectroscopic data and comparison with described data, we could identify the isoflavone biochanin A (Fig. 1), which was previously isolated from *Virola caducifolia* (Santos et al. 1995). Isoflavonoids show a restricted distribution in the vegetable kingdom, including Leguminosae family. In the vegetable, it plays a role as the phytoalexins, i.e., substances produced by plant in response to an infection caused by pathogenic agents (Torssell 1997). Biochanin A, along with others isoflavonoids, were isolated from the roots of *Gynerium sagittatum*, a species known as a rich source of isoflavonoids (Benavides et al. 2007). Biochanin A, isolated from roots of *Virola surinamensis*, has been presenting antifungal activities (Lopes et al. 1999).

Our data clearly demonstrated an antileishmanial activity of biochanin A against *L. chagasi* promastigotes (Table 1), with an EC_{50} value of 18.96 μg/mL (95% CI 18.01–19.96 μg/mL). Pentamidine was used as internal control and resulted in an EC_{50} value of 0.09 μg/mL (95% CI 0.05–0.15 μg/mL). The intracellular amastigote assay using biochanin A indicated lack of activity, since higher concentrations needed to eliminate the parasites were too much toxic for host cells. Despite a low antileishmanial activity of biochanin A when compared to standard drug pentamidine, it was about tenfold less toxic than pentamidine against peritoneal macrophages, suggesting that this substance could be used as prototype to QSAR studies in the development of new drugs against *Leishmania* sp. parasites.

Antileishmanial activity of flavonoids has been described in a variety of plant species. Previous studies have shown a strong antileishmanial activity of 5,7,4'-trihydroxyflavan against amastigotes forms of *Leishmania amazonensis*, while the biflavonoids amentoavone, podocarpusavone A and B, isolated from the leaves of *Celanodendron mexicanum*, showed only a weak activity against promastigotes of *Leishmania donovani* (Chan-Bacab and Peña-Rodriguez

Table 1 Antiparasitic activity and cytotoxicity of biochanin A

Compound	EC_{50} μg/mL (95% CI)				(%) μg/mL
	T. cruzi trypomastigotes	*L. chagasi* promastigotes	*L. chagasi* amastigotes	Cytotoxicity	Hemolytic activity
Biochanin A	18.32 (9.7 a 34.5)	18.96 (18.0 a 19.9)	ne	42.58 (29.0 a 62.5)	>100
Pentamidine	–	0.096 (0.059 a 0.156)	nd	4.22 (3.67 a 4.86)	nd
Glucantime®	–	–	29.55 (28.09 a 31.09)	>100	nd
Benznidazole	44.86 (25.9 a 77.5)	nd	nd	>500	nd

The parasites were incubated with biochanin A and its viability was determined by incubation with MTT (promastigotes of *L. chagasi*–trypomastigotes of *T. cruzi*) or by optical microscopic (amastigotes of *L. chagasi*).
nd not determined, *ne* not effective, *EC_{50}* effective concentration 50%, *95% CI* 95% confidence interval

2001). Synthetic biochanin A has been described with activity against axenic *L. donovani* amastigotes, the causative agent of visceral leishmaniasis in India, showing an EC_{50} of 3.2 μg/mL (Tadesmir et al. 2006). Through QSAR studies, the authors demonstrated the activity of other isoflavone and flavone analogs by considering the substitution pattern of aromatic ring (hydroxylation and/or methylation).

In order to evaluate the antiparasitic activity against other protozoans, we studied the in vitro activity of the isolated biochanin A against the trypomastigotes forms of *T. cruzi* (Y strain), the etiologic agent of Chagas' disease. Thus, biochanin A showed an EC_{50} value of 18.32 μg/mL (95% CI 9.7 to 34.5), being 2.5-fold more effective than the standard drug benznidazole (EC_{50} of 44.86 μg/mL). The cytotoxicity was also evaluated against rhesus monkey kidney cells (LLC-MK2- ATCC), showing an EC_{50} value of 42.58 μg/mL (95% CI 29.0 to 62.5), after 48 h of incubation. Furthermore, no hemolytic activity was found to the highest concentration of 100 μg/mL using mice erythrocytes. These results indicate that biochanin A can be used as toll for drug design studies in the development of new therapeutics especially against Chagas' disease.

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