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Trypanocide activity of *Castela coccinea* Griseb. extracts

[Actividad tripanocida de extractos de *Castela coccinea* Griseb.]

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Abstract

Castela coccinea Griseb is a small tree that belongs to Simaroubaceae family. This work shows that this plant extracts present *in vitro* a strong activity against *Trypanosoma cruzi* epimastigotes. Particularly, wood dichloromethane extract displayed the most intensive trypanocide effect. A fraction of this extract, purified by column chromatography over Silica-gel, was enriched in coumarins, phenolic acids and alkaloids. This fraction was even more effective with an IC_{50} of 15 µg/mL. Our results suggest that alkaloids could be the responsible compounds for this activity. The 8-amino-6-methoxyquinoline group was detected by GC-MS, even though the whole molecule could not be identified yet. On the other hand, all extracts have exhibited cytotoxicity against the human cell line K-562, at high concentrations. However, the selectivity index of wood dichloromethane extract has been 7.5.

Keywords: *Castela;* Simaroubaceae; Trypanocide activity; Cytotoxicity.

Resumen

Castela coccinea Griseb es un árbol de pequeño porte que pertenece a la familia Simaroubaceae. En el presente trabajo los extractos de esta planta muestran una fuerte actividad *in vitro* contra epimastigotes de *Trypanosoma cruzi*. El extracto diclorometánico de madera es el que presenta mayor efecto tripanocida. Una fracción de este extracto, purificada por cromatografía en columna, está enriquecida en cumarinas, ácidos fenólicos y alcaloides. Esta fracción es aún más activa con una CI_{50} =15 µg/mL. Nuestros resultados sugieren que los alcaloides son los responsables de esta actividad. Más aún, se ha detectado la presencia del grupo 8-amino-6-metoxiquinolina por cromatografía gaseosa acoplada a un espectrómetro de masa, pero todavía la molécula entera no ha sido identificada. Por otro lado, todos los extractos mostraron actividad citotóxica contra la línea celular humana K-562, a altas concentraciones. Sin embargo, el índice de selectividad para el extracto diclorometánico de la madera es de 7.5.

Palabras Clave: *Castela;* Simaroubaceae; Actividad tripanocida; Citotoxicidad.

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INTRODUCTION

Castela coccinea Griseb. mainly grows in South America and belongs to the Simaroubaceae family. This family has played a prominent role in folk medicine because of its anthelmintic and antiamoebic properties (Polonsky, 1973). Chemical examination of these plants has been carried out by several groups resulting in the isolation of quassinoids (Ogura et al.,1977; Grieco et al., 1999; Jiwajinda et al., 2001; Jacobs et al., 2007), alkaloids (Sanchez and Comin, 1971; Ogura et al., 1978; Ouyang et al., 1995; Readel et al., 2003) and terpenoids (Sherman et al., 1980). In recent years, attention has been focused on quassinoids, bitter principles of the family, as several of them have shown promising antitumor, antiviral, antimalarial, antileukemic and antifeedant properties (Polonsky, 1985).

Bourdy et al. has demonstrated that leaf extracts of *Castela coccinea* Griseb display strong activity against *Plasmodium falciparum*, which is the etiological agent of the most severe form of malaria (Bourdy et al., 2004). Malaria has low incidence in South America. On the other hand, South America is the endemic zone for Chagas, another parasitic disease caused by *Trypanosoma cruzi*. Although the incidence of Chagas has declined over the past 20 years mainly due to the efforts made towards the control and elimination of the vector, American trypanosomiasis remains a sickness difficult to eradicate (Moncayo, 2003). Current therapy is based on two drugs, nifurtimox and benznidazole, which have been proved effective against the acute phase of the disease. Otherwise, their effectiveness in the chronic stage is controversial. Additionally, serious side effects have been reported in about 50 % of treated patients (Castro et al., 2006). Therefore, it still remains a compelling need to develop safer and more efficient drugs, as well as new approaches to overcome the drawbacks of this chemotherapy strategy.

The aim of this work is to examine anti-trypanosomal activity of *Castela coccinea* Griseb extracts. Cell viability was also evaluated after treating cells with different extracts of *Castela coccinea* Griseb to investigate the behaviour in biological systems, because it is well-know that many Simaroubaceae plants show cytotoxic activity (Among others, Cuendet and Pezzutto, 2004; Guo et al., 2005; Lopez Saez et al., 2008).

MATERIALS AND METHODS

Plant Material

Castela coccinea Griseb. (Simaroubaceae) was collected from Argentina: Santa Fe Province, General Department Obligado, Berna Locality in December 2004 and October 2007 and was authenticated by Luis Oakley, Departamento de Biología y Recursos Naturales, Universidad Nacional de Rosario (UNR). A voucher specimen was deposited in the Herbarium of SF referring Pensiero 6943 to material collected in 2004 and in the Herbarium of UNR referring Oakley et al. 053 to material collected in 2007.

Chemicals

1,1-diphenyl-2-picryldydrazyl (DPPH), dimethyl sulfoxide (DMSO), 2-aminoethyl diphenylborinate and anisaldehide were purchased from Sigma Chemicals, St. Louis, MO, USA. The culture media Eagle's minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), trypsin, penicillin G, streptomycin were purchased from Gibco BRL (Gaithersburg, MD, USA). All other chemicals and reagents used were of the highest commercially available purity.

Preparation of extracts and partial purification

The bark (100 g) and the wood (100 g) of *Castela coccinea* Griseb were extracted three times with ethanol (**EtOH**) or dichloromethane (**DCM**) at room temperature and then concentrated under reduce pressure yielding the residues: $\mathbf{Bark_{EtOH}}$ (2.0 g), $\mathbf{Wood_{EtOH}}$ (2.0 g), $\mathbf{Bark_{DCM}}$ (1.24 g) and $\mathbf{Wood_{DCM}}$ (0.93 g). Each of them was resuspended in the corresponding solvent or in DMSO.

$Wood_{DCM}$ extract was subjected to chromatography on a Silica gel-60 column (40 cm length, 2 cm i.d.) equilibrated with hexane and eluted with a stepwise gradient of hexane and ethyl acetate. 90 fractions of 5 mL each were collected and analyzed by TLC aluminium sheets. On the basis of the TLC profile the similar fractions were pooled into 5 fractions: **I, II, III, IV** and **V.**

Trypanocide assay

Epimastigotes from CL Brener and Y strains were cultured in modified liver infusion tryptose (LIT) medium supplemented with 10% fetal bovine serum (FBS) at 28 ± 0.5 °C in 24 wells culture flasks (Camargo, 1964). Cultures (3 - 4 x 10^6 parasites/mL)

where incubated with increasing amounts of each extract. The parasite's growth was monitored by counting them in a Neubauer chamber. Percentage of inhibition was calculated as the *ratio* between parasite's growth in the presence or absence of each extract, after 72 h of culture. IC_{50} was obtained by plotting the percentage of inhibition against extract concentration. Each experiment was performed in triplicate and trypanocide drug benznidazole (BZL) was used as positive control.

Cytotoxicity assay

Human myelogenous leukaemia K-562 cell line was cultured in DMEM supplemented with penicillin G (100 units/mL), streptomycin (100 µg/mL) and 10-15% fetal bovine serum in a 5% CO_2 humidified incubator at 37 °C.

The extracts were first dissolved in DMSO and then in MEM. Confluent cultures were treated with medium containing the extracts at concentrations ranging from 1 up to 450 µg/mL or the solvent alone (control) for 24, 48 and 72 h. Each drug concentration was tested in quadruplicate. Final concentration of DMSO in the test medium and controls was 1%. After the indicated treatments, cells were removed from culture flasks by treatment with trypsin.

The effect of the DMSO 1% was analyzed in all experimental procedures and there was no significant difference between the solvent and the control on all the measured parameters.

Cell death was determined by incubating with 0.4% Trypan Blue, and then the stained cells were counted in a Neubauer haemocytometer. The results were expressed as the percentage of cell death relative to solvent-treated control incubations and the IC_{50} values were calculated using non-linear regression analysis (percentage of cell death vs. concentration). *In vitro* cytotoxicity effect was calculated as the *ratio* between cell's growth in the presence or absence of each extract, after 72 h of culture.

Morphological features of the cells (variation in size or nuclear/cytoplasmic ratio, presence of granules) were monitored by light microscopy.

General experimental procedures

The extracts were subjected to thin layer chromatography (TLC) examination on aluminium sheets pre-coated with silica gel 60 F 254 (Merck). Mobile phase **I** Toluene: ethylacetate: formic acid (9:3:0.7), **II**: ethylacetate: Acetic Acid: Formic acid: water (10:1.1:1.1:2.6), **III** Toluene: ether (1:1 saturated with 10% acetic acid). The chromatograms were observed first without chemical treatment, under UV 254 and UV 365 nm light, and then using the spray reagents: NP/PEG, Dragendorff, KOH 5%, Liebermann-Burchard reagent or $FeCl_3$ 3% (Wagner et al., 2001). TLC sheet was also immersed in a 0.4 mM DPPH methanolic solution for 10 s.

GC-MS analysis was carried out on PERKIN ELMER Gas Chromatograph attached to a TURBOMASS Mass Spectrometer. SE-30, 25 m x 0.22 mm de ID column was used for fraction IV separation. Helium was used as the carrier gas and kept at 1 mL/min. Oven temperature was programmed at 100 ºC (5 min) to 310 ºC at a rate of 10 ºC/min (10 min). The mass-scanning was done by electron impact (EI) source with electron energy set to 70 eV and mass range *m/z* 50-450.

RESULTS

Cytotoxicity

The *in vitro* cytotoxic effect was determined using the haemocytometer to estimate the total number of viable and non-viable cells and was also evaluated by Trypan-blue exclusion method (Fig. 1A). All extracts have shown significant cell death at the highest dosage of 0.45 mg/mL. IC_{50} was estimated from graphical interpolation. Among the tested fractions, $Bark_{EtOH}$ extract has shown the strongest inhibition with an IC_{50} of 0.20 mg/mL. IC_{50} for wood extracts was 0.45 mg/mL. Microscopically detectable alterations were observed in the cell morphology. When cells were incubated with bark extract granulation and vacuolization in the cytoplasm were observed, as well as irregular borders and a lower number of cells were detected (Fig. 1B).

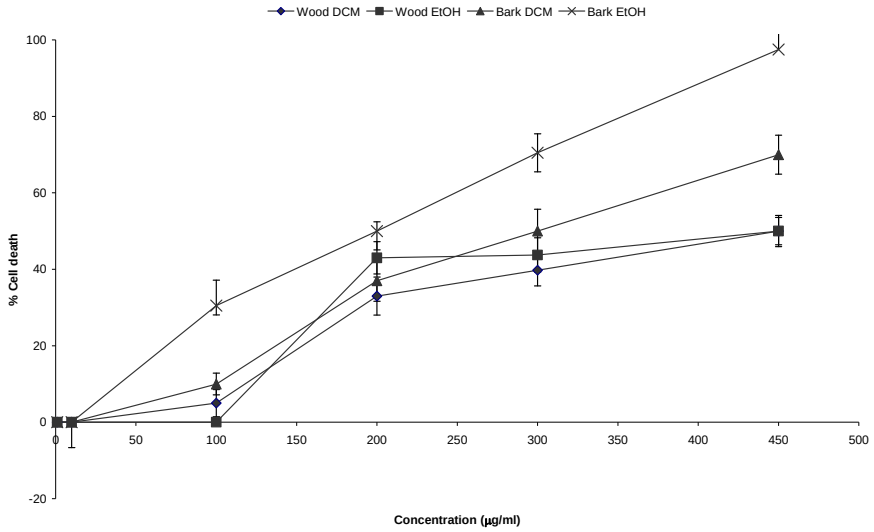
Antitrypanosomal activity of the extracts

Ethanolic and DCM extracts of *Castela coccinea* Griseb bark and wood were screened for antiprotozoal activity against *Trypanosoma cruzi* epimastigotes (Fig. 2). $Wood_{DCM}$ extract showed the strongest activity with an IC_{50} = 60 µg/mL. BZL was used as positive control and showed an IC_{50} = 10 µg/mL. Cytotoxicity did not correlate with antiprotozoal activity. Wood extracts were more selective against the parasites than against mammalian cells. This was demonstrated by their Selectivity Index (SI = IC_{50} for K-562 cells/ IC_{50} antiprotozoal activity) values > 1. $Wood_{DCM}$ SI has been 7.5.

Figure 1. Effect of *Castela coccinea* Griseb extracts on inhibition of the leukemic cell line K-562.

A. *In vitro* cytotoxicity effect calculated as the *ratio* between cell's growth in the presence or absence of each extract, after 72 h of culture. **B.** Photomicrographs **1)** Cells incubated with solvent alone (Control) **2)** $Bark_{EtOH}$ (100 µg/mL) treated cells and staining with Trypan blue. Death staining cells are shown with an arrow. It was observed a lower number of cells, irregular borders and cytoplasmic granules. **3)** $Bark_{EtOH}$ (200 µg/mL) treated cells without staining. A very low number of cells and abundant granules were observed.

A



B

Fractionation and phytochemical screening of $Wood_{DCM}$ extract

The $Wood_{DCM}$ extract was purified by column chromatography. When tested against the parasite, Fraction **I,** the high non-polar fraction, showed an IC_{50} value of 185 µg/mL, while fraction **III** exhibited an IC_{50} value of 60 µg/mL. The highest activity was concentrated at fraction **IV** (Fig. 3), and this fraction also had activity against DPPH.

Phytochemical analysis by TLC has demonstrated the presence of alkaloids and terpenoids in fraction III and alkaloids, phenolic acids and coumarin in fraction IV. When this fraction (IV) was subjected to GC-MS, scopoletin and an 8-amino-6-methoxyquinoline group were detected, however the identity of the whole alkaloid molecule could not be determined. Despite scopoletin being the main component of fraction IV (Fig. 4), it did not show any trypanocide activity up to 6 µg/mL. On the other hand, the phenolic acids detected in fraction IV were also detected in fraction V (the most polar fraction). It is a very interesting fact that this last fraction had not trypanocide activity at all. Therefore, trypanocide activity would not be caused by effect of these phenolic acids. On the contrary, they were responsible for DPPH scavenging activity.

Figure 2. *In vitro* trypanocide activity of *Castela coccinea* Griseb extracts expressed as percentage of parasite's growth inhibition..

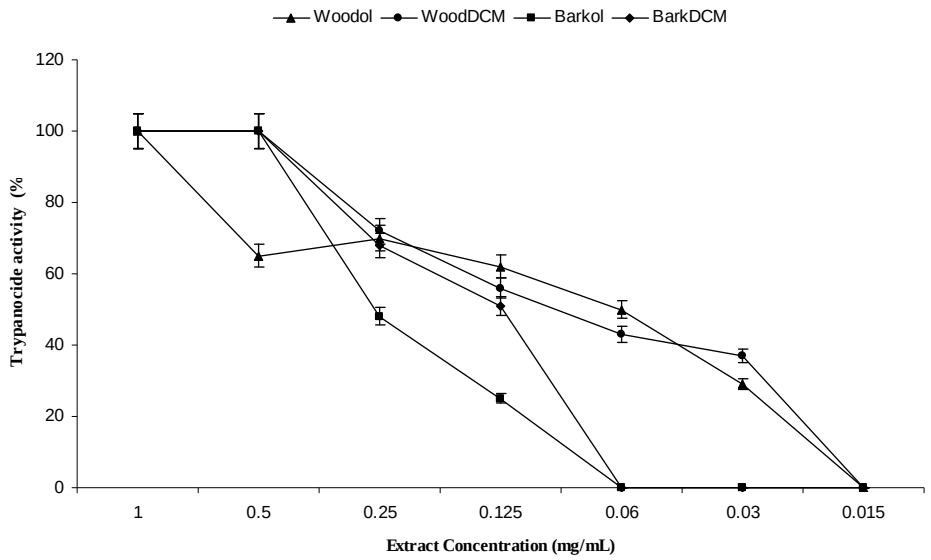


Figure 3. *In vitro* trypanocide activity of different wood $_{DCM}$ fractions expressed as percentage of parasite's growth inhibition.

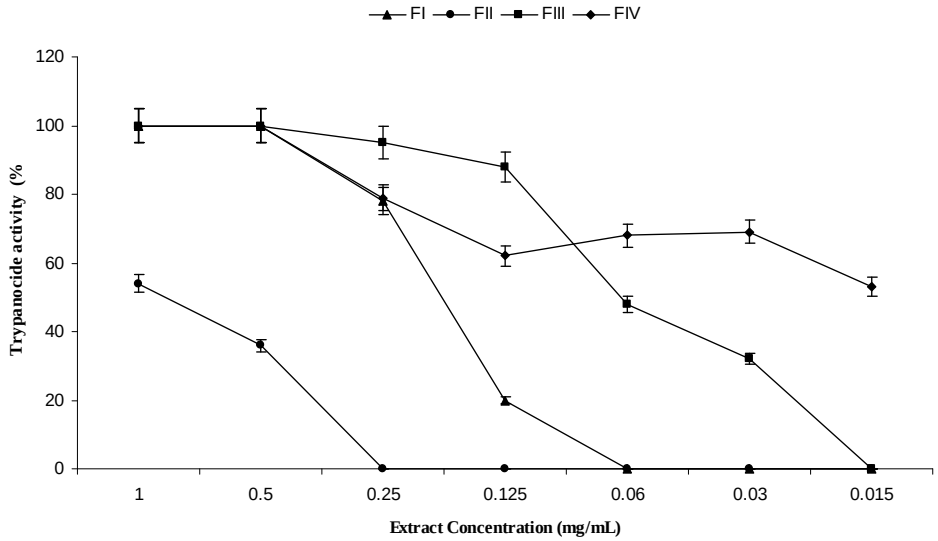
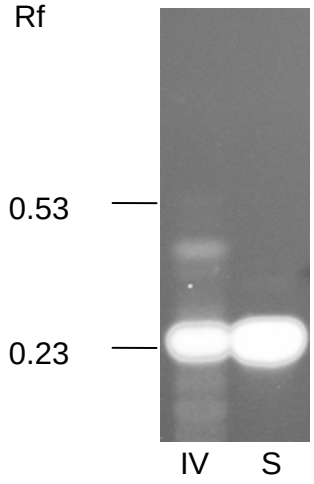


Table 1. *In vitro* trypanocide activity and cytotoxicity of *Castela coccinea* Griseb extracts.

	Cytotoxicity IC_{50} (µg /mL)		Selectivity index[a]
	T. cruzi	**K-562**	
$Wood_{ol}$	81	450	5.5
FI	185	-	-
FII	-	-	-
FIII	60	-	-
FIV	15	-	-
FV	-	-	-
$Wood_{DCM}$	60	450	7.5
$Bark_{ol}$	220	200	0.9
$Bark_{DCM}$	168	300	1.78

(a) Selectivity index was determined by the ratio of the IC_{50} value on K-562 cells to the IC_{50} value on *T. cruzi*.

Figure 4. Analysis of fraction IV by TLC. TLC plate was stained with NP-PEG. Fraction IV exhibited eight to ten blue fluorescent zones from the start up to Rf 0.53. Solvent system III.
S: commercial scopoletin 0.1% in ethanolic solution (5 µL).



DISCUSSION

This work showed that *Castela coccinea* Griseb presents a strong trypanocide activity and particularly wood extracts displayed the most intensive effect. On the other hand, all extracts have exhibited cytotoxicity at high concentrations. However, $Wood_{DCM}$ was more selective against parasites than against mammalian cells with a selectivity index of 7.5. A fraction of this extract enriched in coumarins, phenolic acids and alkaloids was more effective against *Trypanosoma cruzi* with an IC_{50} of 15 µg/mL. Several coumarins and indol or quinoline alkaloids exhibit remarkable parasitaemia suppression against different strains of *Plasmodium* or *Trypanosoma* (among others Phillipson, 1999; Camacho, 2004; Muhammad et al,. 2003 and 2004; Argotte-Ramos et al., 2006; Huang et al., 2006; Fournet et al., 2007; Bringmann, 2007; Frederich, 2008). Our results suggest that alkaloids could be responsible for this activity. The 8-amino-6-methoxyquinoline group was detected but the whole molecule could not be identified yet. Interestingly, compounds containing this group, as for example, primaquine (CAS Nº 63-45-6) are already being used to produce radical cure and prevent relapse of vivax and oval malarias following treatment with blood schizontocide (Dongre et al., 2005; Musonda et al., 2007; Kaur et al., 2007).

The fraction with the highest trypanocide activity also showed antioxidant capacity. It has been proven that *Trypanosoma* is sensitive to oxidative stress, in particular by reactive oxidant species produced during the immune response of the host. The fact that this fraction both show radical scavenging and kill parasites suggests that these activities should be associated with two different molecules present in the extract. The detected antioxidant compounds were phenolic acids, also present in the most polar fraction without show trypanocide activity. However, the possibility that the same molecule works as a radical scavenger in mammalian cells and as a radical inducer in parasites could not be completely miss-considered.

The screening of natural products provides the chance to discover new molecules of unique structure with high activity and selectivity, which can be further optimized by semi- or fully synthetic procedures. Alkaloids are one of the most fascinating natural products, providing many drugs for human use (Phillipson et al., 1999; Kayser et al., 2003). The results of this work show that *Castela coccinea* Griseb extracts are potentially a rich source of bioactive compounds and the search of these entities is carried out. A preliminary phytochemical study is done but further studies are needed as this plant is unknown from the phytochemical point of view.

CONCLUSION

Castela coccinea Griseb has not been previously studied for their trypanocidal effect. Wood $_{DCM}$ showed strong activity against *Trypanosoma cruzi* with an IC_{50} of 15 µg/mL. Our results suggest that alkaloids could be responsible for this activity. Interestingly, although the whole molecule identity could not be assessed, an 8-amino-6-methoxyquinoline group was detected. A bioguided fractionation is in progress to identify the active compounds.

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