Research Paper

ANTIPROTOZOAL ACTIVITIES OF COMPOUNDS ISOLATED FROM *CROTON LOBATUS L.*

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Abstract

In a preliminary evaluation of ethnobotanically selected Beninese medicinal plants for their *in vitro* antiplasmodial activity, the methanolic extract of the aerial parts of *C. lobatus* was found to have significant activity against *P. falciparum,* antileishmainal and antiprotoazoal assays carried out on some of the isolated compounds. Phytochemical investigation of this extract resulted in the isolation of five compounds: tiliroside (kaempferol-3-*O*-β-D-(6-*E-p*-coumaroyl) glycopyranoside) (**1**), isovitexin (apigenin-6-*C*-β-D-glucopyranoside) (**2**), vitexin (apigenin-8-*C*- β-D-glucopyranoside) (**3**), chlorogenic acid (acid-5-*O*-cafféoylquinic) (**4**) and 4,5-*O*-dicaffeoylquinic acid (**5**). Vitexin and tiliroside showed the best *in vitro* antiplasmodial activity against *P. falciparum*, with IC_{50} values of 4.4 and 7.1 µM, respectively. Vitexin also exhibited strong *in vitro* leishmanicidal and antitrypanosomal activities against *Leishmania donovani* amastigotes and *Trypanosoma brucei rhodensiense* trypomastigotes, with IC_{50} values of 0.6 and 0.1 µM, respectively.

Key words: *Croton lobatus*, tiliroside, vitexin, antiprotozoal activity

Introduction

Protozoal diseases, such as malaria, trypanosomiasis, and leishmaniasis, are a major threat for public health. Every year, malaria causes 300 to 500 million clinical cases and more than one million deaths, and there is increasing prevalence of malaria exhibiting resistance of *Plasmodium falciparum* to inexpensive, cheap standard treatments (Wellems and Plowe, 2001). In the same order of idea, leishmaniasis and trypanosomiasis are major causes of mortality and cause much economic hardship, particularly in the developing world. Unfortunately, there are few drugs available to treat these two diseases, and most of these drugs suffer from poor clinical efficacy and unwanted effects. There is an urgent need to discover new therapeutic agents for these parasitic diseases, and traditional medicine knowledge can be useful to open new ways in the field of antiprotozoal therapy. In a previous work, we reported *in vitro* antiprotozoal activity of *Croton lobatus* L. (Euphorbiaceae), a traditional plant used in Benin and in neighbouring country Togo, against malaria in folk medicine, towards both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* (Adjanohoun et al., 1989; Weniger et al., 2004).

In this study, we report the isolation and identification of five compounds from *C. lobatus* aerial parts and their evaluation for anti-trypanosomal *(Trypanosoma brucei rhodesiense* STIB 900 strain), leishmanicidal (*Leishmania donovani* MHOMET- 67/L82 strain), and antiplasmodial *(P. falciparum* Kl multidrug-resistant strain) activities.

Materials and Methods
Plant material

Aerial parts of *Croton lobatus* L. was collected in January 2005 in the area of Weto, in the Atlantic region (Southern Benin). Botanical determination was performed by taxonomists from the Herbier National of Abomey-Calavi University in Benin, and voucher specimens (Hp 565a) were deposited at the same Herbarium. The aerial parts were air-dried, powdered (0.2 mm sieve), and subjected to extraction with methanol.

Extraction and isolation

Dry powdered aerial parts of *C. lobatus* (515g) were extracted with methanol using a Soxhlet extractor (Soxtec Avanti 2055 apparatus, Foss Tecator AB, Box 70, Se-263 21, Höganäs, Sweden). The dry methanolic extract (29.27g) dissolved in water and then partitioned with cyclohexane, methylene chloride and ethyl acetate. Each fraction was taken to dryness under vacuum and the residues were stored at room temperature. The ethyl acetate fraction (2.73g) was first chromatophied using Sephadex LH-20 exclusion chromatography (Sephadex LH20, 25 – 100 µm, Merck), according to the method described by Houghton and Raman (1998). Two fractions (A, 855 mg and B, 962 mg) were obtained and purified using a combination of chromatographic methods such as high pressure liquid chromatography (HPLC), atmospheric column chromatography (CC) and preparative TLC chromatography.

Fraction A was purified by HPLC (Gilson VP 250/21, Nucléodur 100-10 C_{18}ec, Macherey-Nagel, UV detection 254 nm) by gradient elution (flow rate 10 ml/min) using acetonitrile-water (20:80 to 80:20 v/v) with 0.7% formic acid as mobile phase, to obtain compounds **1** (20.7 mg) and **2** (7,2 mg). Fraction B was purified using C_{18} Flash column chromatography (BIOTAGE System, Sweden, Flash 40 + M, KP-Sil, 40 x 150 mm) using water-methanol as mobile phase at a flow rate of 40ml/min, to obtain three sub-fractions (B_1, B_2 and B_3). Fractionation of B_1 using preparative HPLC gave two subfractions named Sfb1 and Sfb2. The sub-fraction Sfb1, subjected to a chromatography on sephadex LH-20 led to the isolation of compound **2** (3.5mg). Fraction Sfb2 was subjected to a preparative TLC to give compound **3** (7.8 mg). Successive preparative TLC chromatographies (1.25 mm thick, 20x20 cm Si gel 60 F_{254} plates) of sub-fraction B2 in a mixture of ethyle acetate-methanol-water (100:17:13, v/v/v), gave compounds **4** (4.5mg) and **5** (5.6mg).

Structural determination of the isolated compounds was carried out by spectrophotometric methods (1D and 2D NMR, mass and UV spectrometry). 1D and 2D NMR spectrum were recorded at room temperature with a Bruker NMR spectrometer (200MHz and 300 MHz).

Biological assays
Antiplasmodial activity

Quantitative assessment of *in vitro* antimalarial activity against the Kl resistant strain was determined by means of the microculture radioisotope technique based on the method previously described by Desjardins et al. (1979) and modified by Ridley et al. (1996). The assay uses the uptake of [^{3}H]hypoxanthine by parasites as an indicator of viability. Continuous *in vitro* cultures of asexual erythrocytic stages of *Plasmodium falciparum* were maintained following the methods of Trager and Jensen (1976). Compounds were tested against Kl strain (multi-drug pyrimethamine/chloroquine resistant strain) (Thaithong and Beale, 1981). Initial concentration of each compound was 30 μg/ml diluted with two-fold dilutions to make seven concentrations, the lowest being 0.47 μg/ml. After 48 h incubation of the parasites with the compound at 37∘C, [^{3}H]hypoxanthine (Amersham 115 Int., Buckinghamshire, UK) was added to each well and the incubation was continued for another 24 h at the same temperature. IC50 was calculated by linear interpolation between the two drug concentrations above and below 50% (Huber and Koella, 1993). Chloroquine and artemisinin were used as positive references. The values are means of two independent assays. Each assay was run in duplicate.

Antitrypanosomal and leishmanicidal assays
Trypanosoma brucei rhodesiense

The assays were performed according to the procedures described by Freiburghaus et al. (1996). The compounds were dissolved in 10% DMSO, and working stock solutions of 1 mg/mL in serum containing culture medium were prepared. Diluted compounds (100 μL) were pipetted in duplicate into the first row of a 96-well microtiter plate (Costar, Corning, NY, USA). With complete culture medium, three-fold serial dilutions were prepared. After the addition of *Trypanosoma brucei rhodesiense* bloodstream-form trypanosomes from axenic culture, the concentrations of the compounds ranged from 500 to 0.07μg/mL. The total number of trypanosomes in each well was $2 \times 10^2/100$ μL. The plate was then incubated for 72 h at 37∘C in 5% CO_2. Two hours before the end of the incubation 10 μL of Alamar blue solution was added. Fluorescence was measured after 2 h of incubation with the dye Alamar blue in a fluorescence plate reader at 530 nm excitation and 590-nm emission

wavelength (Cytofluor 2300, Millipore, Bedford, MA, USA) (Räz et al., 1997). IC50 values were calculated from the sigmoidal inhibition curve. The values are means of two independent assays. Each assay was run in duplicate.
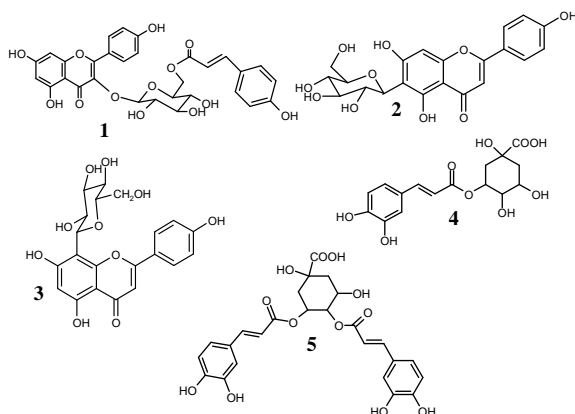
Leishmanicidal activity

Fifty microliters of culture medium, a 1:1 mixture of SM medium (Cunningham, 1977) and SDM-79 medium (Brun and Schönenberger, 1979) at pH 5.4 supplemented with 10% heat-inactivated FBS, was added to each well of a 96-well microtiter plate (Costar). Serial drug dilutions in duplicates were prepared covering a range from 30 to 0.041 μg/mL. Then, 105 axenically grown *L. donovani* amastigotes (strain MHOM/ET/67/L82) in 50 μL medium were added to each well and the plate incubated at 37°C under a 5% CO_2 atmosphere for 72 hrs. Ten microliters of resazurin solution (12.5 mg resazurin dissolved in 100mL distilled water) were then added to each well and incubation continued for a further 2- 4 hrs. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm (Räz et al., 1997). Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic programe Softmax Pro (Molecular Devices), which calculated IC_{50} values. The values are means of two independent assays. Each assay was run in duplicate.

Results and Discussion

In a previous work, the methanolic extract of aerial parts of *C. lobatus* was found to have significant activity against *P. falciparum in vitro* (Weniger et al., 2004). The phytochemical analysis of the active extract of aerial parts of *C.lobatus* led to the isolation and characterization of five compounds, identified as tiliroside (kaempferol-3-*O*-β-D-(6-*E*-*p*-coumaroyl) glycopyranoside) (**1**), isovitexin (apigenin-6-*C*-β-D-glucopyranoside) (**2**), vitexin (apigenin-8-*C*- β-D-glucopyranoside) (**3**), chlorogenic acid (acid-5-*O*-cafféoylquinic) (**4**) and 4,5-*O*-dicaffeoylquinic acid (**5**) (Figure 1). All the data were consistent with the literature (Agrawal P.K., 1989; Phan et al., 2004; Ahmad et al., 2003; Lendl et al., 2005; Beker et al., 2005).

Tiliroside was isolated previously from *Waltheria indica* (Rao et al., 2005)*, Helichrysum italicum* (Sala et al., 2003) and *Platanus orientalis* L. (Dimas et al., 2000). It showed a good antioxidizing property revealing using DPPH assay (IC_{50} = 6μM) (Sala et al., 2003). It also showed a significant inhibition without cytotoxicity of the production of inflammatory intermediaries (NO, TNF alpha, IL-12, LPS, IFN) to murins macrophages.
Isovitexin was isolated previously from *Lythrum salicaria* (Becker et al., 2005), *Patrinia villosa* (Peng et al., 2005), *Thlaspi arvense* (Pedras et al., 2003). It showed an antioxidative property (Pedras et al., 2003). Vitexin was isolated previously from the flowers and leaves of *Ficaria verna* (Tomczyk et al., 2002) and *Terminalia catappa* (Yun-Lian L. et al., 2000).

Figure 1: Structures of compounds isolated from aerial parts of *C. lobatus*

Table 1: *In vitro* antiprotozoal activity of isolated compounds

Compounds	Antiprotozoal activity IC$_{50}$ (µM)		
	antiplasmodial activity[a]	leishmanicidal activity[b]	antitrypanosomal activity[c]
Tiliroside	7.1	>30	>30
Isovitexin	>10	>30	>30
Vitexin	4.4	0.6	0.1
Chlorogenic acid	>10	>50	>30
4,5-*O*-dicafféoylquinic acid	9.7	>50	>30
Standards			
Chloroquine	0.6	-	-
Artemisinin	0.007	-	-
Miltefosin	-	0.47	-
Melarsoprol	-	-	0.005

Data shown are values from replicate experiments
[a]*P. falciparum* K1 resistant strain
[b]*L. donovani* MHOM/ET/67/L82 strain axenic amastigotes
[c]*T. brucei rhodensiense* STIB 900 strain trypomastigotes

Chlorogenic acid showed anti-MMP-9 activity (Un-Ho Jin et al., 2005). It also possessed antiradicalaire properties towards the superoxide anion (Kima et al., 2005). Dicaffeoylquinic acid activity concerns the inhibition of the growth of the tumoral HL-60 cells by induction of apoptose (Satoshi et al., 2005). All these compounds were evaluated for *in vitro* antiplasmodial activity to check whether or not they contribute to activity. As *C. lobatus* is also used to treat parasitic diseases in Togo folk medicine, the isolated compounds were also assayed for leishmanicidal and antitrypanosomal activities. IC$_{50}$ values are presented in Table 1. Three of the five compounds tested, vitexin (**3**), tiliroside (**1**) and 4,5-*O*-dicaffeoylquinic acid (**5**) showed mild antiplasmodial activity, with IC$_{50}$ values of 4.4, 7.1 and 9.7 µM, respectively. These results provide scientific evidence supporting the use of *C. lobatus* as an antimalarial remedy in folk medicine in Benin. Moreover, vitexin exhibited a prominent leishmanicidal effect with an IC$_{50}$ value comparable to that of the reference compound miltefosin, as well as significant antitrypanosomal activity against *T. b. rhodensiense*. These results provide scientific evidence supporting the use of *C. lobatus* to treat parasitic disease in traditional medicine in Togo.

Conclusion

The results obtained in this study showed the interest of the ethnopharmacological approach in the search for active substances against the parasitic pathology. The active tested compounds may, either to be models for the synthesis of more active or less toxic analogues, or to be used in association with antimalarial commercial drugs, as it was shown in recent works (Ramanitrahasimbola et al., 2006), as they may reverse the resistance of the parasite to antimalarial drugs.

Acknowledgement

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