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SHORT COMMUNICATION

Antileishmanial activity of leaf extract from *Calophyllum rivulare* against *Leishmania amazonensis*

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Abstract

Calophyllum rivulare is an endemic tree from Cuba that belongs to Clusiaceae family. This plant had revealed it to be a rich source of pyranochromanone acids including compounds with a variety of biological potentialities such as antioxidant, antiulcer, anticancer and anti-*Helicobacter pylori* activities. Herein, antileishmanial and cytotoxic activities of extracts obtained from *C. rivulare* leaves (rich in pyranochromanone acids) and amentoflavone were compared. Extracts were prepared by maceration employing ethyl acetate (CR-1) and methanol (CR-2) as solvents and amentoflavone was isolated from both extracts. The CR-2 extract showed the better activity against the parasite, with an IC_{50} value of 95.1 ± 5.4 µg/mL and 20.6 ± 1.4 µg/mL against promastigote and amastigote forms, respectively. This extract showed lower cytotoxicity (IC_{50}=182.4 ± 2.3 µg/mL) against peritoneal macrophage from BALB/c mice and a selectivity index of 9. Amentoflavone showed an IC50 of 5.9 ± 0.3 µg/mL against amastigote form and a selectivity index of 9. Results suggest that CR-2 extract and amentoflavone could be explored as new antileishmanial alternatives.

Key words: Amentoflavone, *Calophyllum rivulare*, *Leishmania*

Introduction

Leishmaniasis is a tropical affliction that has been considered one of the most important tropical infection diseases by World Health Organization. An estimated of 350 million population lives in risk and 12 million of peoples are affected for this worldwide disease. Chemotherapy remains the mainstay for the control of leishmaniasis, as effective vaccines have not been developed (den Boer et al., 2011; Kedzierski, 2011). Available drugs are limited in number and present several disadvantages (Croft and Olliaro, 2011). In recent years, growing interest has been observed in alternative therapies and natural products, particularly the use of plants as sources of new chemotherapeutic compounds with better activity and fewer side effects (Rates, 2001). In view of the present unsatisfactory scenario, the study of new molecules obtained from medicinal plants for leishmaniasis treatment is highly desirable.

The genus *Calophyllum* belongs to Clusiaceae family and comprises about 200 species with pantropical distribution. Many species of this genus have shown medicinal properties, including the treatment of gastric ulcers, infections and inflammatory processes (Dharmaratne et al., 2009). Diverse compounds have been isolated from this genus, including xanthones, steroids, triterpenes, biflavonoids, coumarins and piranochromanone acids, which exhibited analgesic (Isaias et al., 2004), anticarcinogenic (Guilet et al., 2001), antimicrobial (Yasunaka et al., 2005), antiprotozoan (Abe et al., 2004) and antiviral (Ito et al., 2003) activities. Species of this genus have received considerable attention from pharmacological point of view because some of them produce potent inhibitors of reverse transcriptase of Human Immunodeficiency Virus (HIV) type 1 (Dharmaratne et al., 2001). In Cuba, five species have been reported: *C. antillanum* Britt., *C. inophyllum* L., *C. pinetorium* Bisse, *C. rivulare* Bisse and *C. utile* Bisse. *C. rivulare* is known as "ocuje blanco", an endemic tree from

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Cuba, which could be found near of rivers or in the calcareous soil of mountains (Roig, 1988). A previous study suggested that pyranochromanone acids including apetalic acid, isoapetalic acid, calolongic acid, pinetoric acid I and pinetoric acid II are secondary metabolites common to some Cuban *Calophyllum*. In this work, both *in vitro* antileishmanial activity and cytotoxicity effect of extracts from *C. rivulare* leaves were evaluated.

Materials and Methods

Parasite culture

Strain MHOM/77BR/LTB0016 of *Leishmania amazonensis* was kindly provided by the Department of Immunology, Oswaldo Cruz Foundation (FIOCRUZ), Brazil. Parasites were routinely isolated from mouse lesions and maintained as promastigotes at 26ºC in Schneider's medium (SIGMA, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (HFBS) (SIGMA, St. Louis, MO, USA), 100 μg of streptomycin/mL, and 100 U penicillin/mL. The parasites were not used after the tenth passage.

Extraction

The dried and powered leaves of *C. rivulare* (151 g) were extracted successively with ethyl acetate (1.5 L) and methanol (1.5 L) for 7 days each. After partial concentration under reduced pressure, a pale yellow precipitated was observed and obtained by filtration from both extracts. Mother liquors were finally concentrated to obtain 18.9 g of ethyl acetate extract (CR-1) and 21.2 g of methanol extract (CR-2), respectively.

Detection of pyranochromanone acids by HPLC-PDA-ESI-MS analysis

Apetalic acid, isoapetalic acid, calolongic acid, pinetoric acid I and pinetoric acid II (Figure 1) were detected in both extracts by HPLC analysis as previously reported [(Piccinelli et al., 2013)]. In brief, MeOH/H_2O 8:2 (v/v) solution of each extract (3 mg mL^{-1}) was prepared. HPLC separations were performed on a Luna C18 column (150 mm × 2.0 mm, 5 μm, Phenomenex) with a gradient of water (solvent A) and MeOH (solvent B), both containing 0.1% (v/v) formic acid. The following gradient was adopted: 0–10 min, isocratic on 75% B; 10–15 min, linear gradient from 75 to 80% B; 15–42 min, isocratic on 80% B. Elution was performed at flow rate of 250 μL min−1 and the volume of the injection was 10 μL. Detection by diode array was performed simultaneously at two different wavelengths: 280 and 320 nm. The mass analyses were performed with an ESI interface in the positive modes.

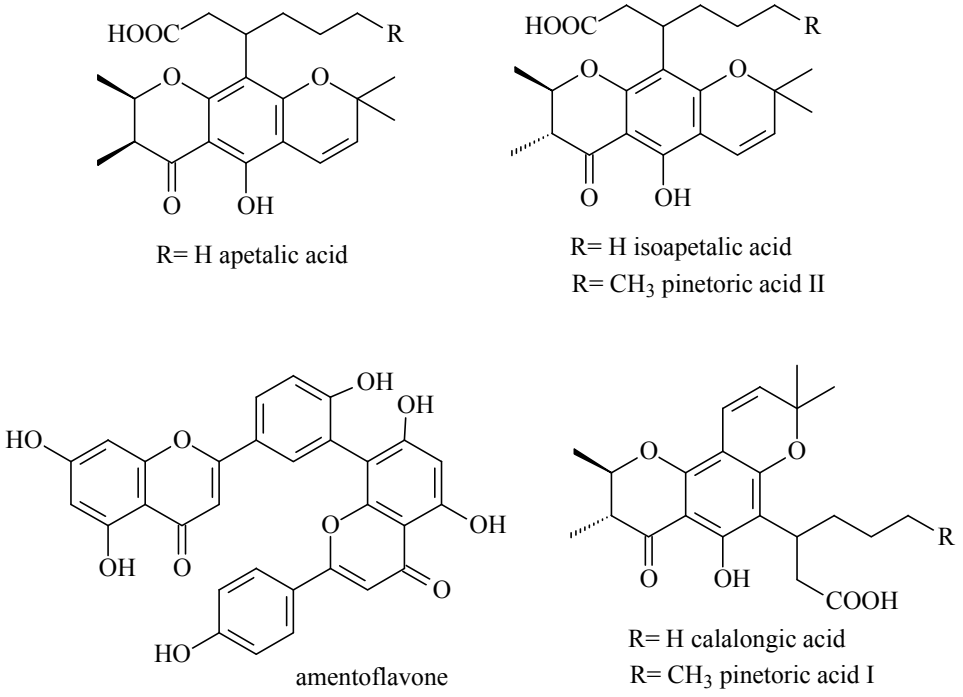

Figure 1. Amentoflavone and main compounds isolated from the leaves of *Calophyllum rivulare*.

Identification of amentoflavone

The pale yellow compound obtained from both extracts was identified as amentoflavone. The structural elucidation was performed on the basis of NMR analysis. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^{1}H and 150.858 MHz for ^{13}C with UXNMR software package was used. ^{1}H-^{1}H DQF-COSY, ^{1}H-^{13}C HSQC, and HMBC experiments were obtained using conventional pulse sequences. Amentoflavone (Figure 1) was identified by comparison of their spectroscopic data with literature values (Agrawal et al., 1989).

Laboratory animals

Female BALB/c mice, with a body weight of approximately 20 to 22 g, were obtained from The National Center for Laboratory Animals Production (CENPALAB). The maintenance and care of mice was followed according Institutional Ethical Committee from Institute of Tropical Medicine Pedro Kouri (Reference number: CEI-IPK 14-12).

Reference drug

Pentamidine (Richet, Buenos Aires, Argentina) prepared at a concentration of 10 mg/mL was used as control.

Antipromastigote activity

Exponentially growing promastigotes (10^5 promastigotes/mL, 198 µL) were plated in 96-well plates. Two microliters of products were added to the wells at a final concentration between 6.25 and 100 µg/mL. Plates were incubated at 26ºC for 72 h. After 3 days of exposure, parasites were incubated for 3h with p-nitrophenyl phosphate (20 mg/mL) dissolved in buffer of sodium acetate 1M (BDH, Poole, England), pH 5.5, with 1% Triton X-100 (BDH, Poole, England) at 37ºC. The absorbance was determined in an EMS Reader MF Version 2.4-0, at a wavelength of 405 nm. The 50% inhibitory concentration (IC_{50}) was obtained from dose-response curves fit to data by means of the equation for the sigmoidal E_{max} model (Bodley et al., 1995).

Cytotoxicity assay

Resident macrophages were collected from peritoneal cavity of healthy BALB/c mice in RPMI 1640 medium (Sigma, St. Louis, Mo, USA) supplemented with antibiotics (penicillin 200 UI, streptomycin 200 µg/mL), plated at 10^6/ml in 96-Well Lab-Tek (Costar®, USA) and left to adhere for 2 h at 37°C in 5% CO_2. Non-adherent cells were removed by washing with PBS after 2 h of incubation at 37ºC in 5% CO_2. Then, 198 µL of medium with 10% of HFBS and antibiotics (penicillin 200 UI, streptomycin 200 µg/mL) was added in each well, later 2 µL of products-dilutions, previously prepared in medium, was added. Macrophages were treated with the products from 12.5 to 200 µg/mL for 72 h. Cultures with DMSO were included as control treated. The cytotoxicity was determined using the colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) solution (15 µL at 5 mg/mL in PBS) (Sladowski et al., 1993). The median cytotoxic concentration (CC_{50}) was obtained from lineal dose-response curves. Three replicates were performed and the results were expressed as their average and standard deviation.

Antiamastigote activity

Peritoneal macrophages from BALB/c mice were collected, plated at 10^6/mL in 24-Well Lab-Tek (Costar®, USA) and incubated 2 h at 37°C in 5% CO_2. Non-adherent cells were removed and stationary-phase *L. amazonensis* promastigotes were added at a 4:1 parasite/macrophage ratio for 4 h and cell monolayers were washed to remove free parasites. Then, 1990 µL of the RPMI complete medium and 10 µL of the different products were added, following serial dilutions 1:2 (at concentrations from 12.5 to 100 µg/mL) for a further 48 h (Caio et al., 1999). Cultures were then fixed with absolute methanol, stained with Giemsa, and examined under light microscopy. The number of intracellular amastigotes was determined by counting the amastigotes resident in 100 macrophages per each sample. Results were expressed as percent of reduction of the infection rate (% IR) in comparison with those obtained with positive controls. The infection rates were obtained by multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophages (Delorenzi et al., 2001). The IC_{50} value was calculated by linear regression analysis. Each experiment was performed twice. The results were expressed as their average and standard deviation.

Selectivity indices (SI) were then calculated by dividing the CC_{50} for peritoneal macrophage of BALB/c mice by the IC_{50} for *Leishmania* amastigotes.

Statistical analyses

Statistical differences, classified as $p < 0.05$, between IC_{50} of extracts was identified using a Mann-Whitney test and the STATISTICA for Windows Program (Release 4.5, StatSoft, Inc. 1993).

Table 1. Antileishmanial activity and cytotoxicity of two extracts and amentoflavone from *Calophyllum rivulare* leaves.

Products	IC_{50}[a] ± SD[b] (µg/mL)		CC_{50}[c] ± SD[b] (µg/mL)	SI[d]
	Promastigote	Amastigote		
CR-1	104.3 ± 0.5	37.5 ± 1.9	97.5 ± 1.7	3
CR-2	95.1 ± 5.4	20.6 ± 1.4	182.4 ± 2.3	9
Amentoflavone	ND[e]	5.9 ± 0.3	50.5 ± 0.03	9
Pentamidine	0.37 ± 0.01	1.3 ± 0.1	11.7 ± 1.7	9

[a]: IC_{50}: Concentration of drug that caused 50 % of inhibition growth of promastigotes of *L. amazonensis*.
[b]: SD: Standard deviation.
[c]: CC_{50}: Concentration of drug that caused 50 % of mortality of peritoneal macrophage from BALB/c.
[d]: SI: Selectivity index. SI = IC_{50} macrophage / IC_{50} *Leishmania* amastigote.
[e]: ND: Not done.

Results and Discussion

In the present study, we report for the first time the evaluation of leaves from *C. rivulare* against *L. amazonensis*. Table 1 shows the antileishmanial activity and cytotoxicity of evaluated products. The CR-2 extract caused a significant activity against both forms of the parasite; while CR-1 caused a significant lower ($p < 0.05$) activity and selectivity. This extract showed a SI of 9, which suggest a specific activity for *Leishmania*. In the literature, no reports about biological evaluation of this plant species were found. However, some extracts from other species of the genus *Calophyllun* have been evaluated against *Leishmania* and other micro-organisms. The dichloromethane crude extract from leaves of *C. brasiliense* showed a significant *in vitro* (Honda et al., 2010; Brenzan et al., 2007) and *in vivo* activity against *L. amazonensis* (Honda et al., 2010). A coumarin was isolated from this extract and its derivatives showed significant activity against *L. amazonensis,* with IC_{50} between 0.6 and 34.0 µg/mL (Brenzan et al., 2007; Brenzan et al., 2008). On other hand, the extract from roots of *C. brasiliense* was active against *L. chagasi* and *Plasmodium falciparum,* with an IC_{50} of 6.7 and 27.6 µg/mL, respectively (Albernaz et al., 2010). Other study reported the purification of three xanthones from heartwood, which exhibited activity against epimastigote forms of *Trypanosoma cruzi* (Abe et al., 2004).

It is known that plant extracts are constituted by a complex mixture of substances, for that reason, both extracts were evaluated in order to confirm the presence of pyranochromanone acids as previously reported. The existence of apetalic acid, isoapetalic acid, calolongic acid, pinetoric acid I and pinetoric acid II in CR-1 and CR-2 extracts was confirmed. Additionally, amentoflavone was identified as a constituent of CR-1 and CR-2 extracts and its potentialities were also evaluated (Table 1). Thus, five pyranochromanone acids and a biflavonoid were recognized as chemical constituents of analysed extracts. All the samples under investigation were active against *Leishmania* but pure amentoflavone exhibited a more potent activity that those extract containing a mixture of pyranochromanone acids mainly. Amentoflavone showed leishmanicidal activity with an IC_{50} of 5.9 µg/mL, which was superior effect ($p < 0.05$) compared with both extracts and same selectivity (SI=9) that extract CR-2.

This biflavonoid has been found in some plant species and was tested against *Leishmania* and other protozoan parasites. Camacho and collaborators reported also the activity of amentoflavone isolated from leaves of *Celanodendron mexicanum* against *L. donovani* (Camacho et al., 2000), causal agent to visceral leishmaniasis. In addition, in a study of plants-derived agents, the isolation of twelve biflavonoids including amentoflavone from the Indian medicinal herb *Selaginella bryopteris* was reported, where eleven compounds were active *in vitro* against *L. donovani, P. falciparum*, *T. brucei rhodesiense* and *T. cruzi* (Batista et al., 2009). All these results suggest that biflavonoids are active against different protozoan parasites; while it seems that pyranochromanone acids are less active than amentoflavone against *Leishmania*.

Conclusions

In conclusion, antileishmanial potentialities of *C. rivulare* have been demonstrated, probably by presence of amentoflavone, which could be explored as leading compounds against *Leishmania* parasite.

Conflict of interest

The authors declare that they have no conflict of interest.

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