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Antileishmanial Activity of Coumarin from *Amburana cearensis* Seeds

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Abstract

Visceral leishmaniasis is caused by parasites of the genus Leishmania. Prevalent in developing countries, the disease is on the list of the world's most neglected diseases. Most cases (90%) in Latin America occur in Brazil, especially in the Northeast. The condition is treated mainly with pentavalent antimonials, an expensive and relatively inefficient drug known to induce resistance. In search of new antileishmanial drugs, much attention has been given to the medicinal properties of coumarin. In this study, coumarin was isolated from seeds of *Amburana cearensis*, purified and evaluated with regard to its ability to inhibit *Leishmania chagasi* promastigotes using the MTT test. The cell viability of macrophages treated with coumarin was also evaluated. The findings were submitted to one-way ANOVA for paired data, followed by the Bonferroni correction. The level of statistical significance was set at 5% ($p < 0.05$). Coumarin displayed low toxicity to macrophages in the MTT test ($p > 0.05$), but was toxic to *Leishmania chagasi* promastigotes ($p < 0.05$). Our results represent a contribution to the development of new drugs for the control or prophylaxis of visceral leishmaniasis.

Subject Areas

Parasitology

Keywords

Leishmaniasis, Coumarin, *Amburana cearensis*, Cell Viability

1. Introduction

Leishmaniasis is a zoonotic disease caused by protozoa of the genus Leishmania. The parasite undergoes two development stages, one in the cells of the human mononuclear phagocyte system (amastigotes) and one in invertebrates (promastigotes) [1]. Classified as a "neglected disease", the condition is prevalent in socioeconomically challenged populations, especially in developing countries. About 12 million people are affected worldwide, with approximately 2 million new cases of the clinical form and 60,000 deaths each year [2].

Visceral leishmaniasis (VL) is distributed worldwide. A Latin American form of the disease named American visceral leishmaniasis (AVL), or neotropical kala-azar, has been registered in at least 12 countries, with 90% of the reports coming from Brazil, especially from the Northeast [3]. Between 2006 and 2010, 18,168 new cases of VL were reported in Brazil, 47.1% of which from the Northeast [3]. In this country, *Leishmania (L) infantum chagasi* is the etiologic agent of visceral leishmaniasis [4]. Transmission occurs between humans and dogs bitten by infected sandflies of the genus Lutzomyia [5].

Leishmaniasis is treated mainly with pentavalent antimonials, with a success rate of 26% - 100%. However, the use of this medication is limited by serious side effects and parasite resistance [6]. Pentamidine or Amphotericin B is the choice of treatment in such cases [7]. Lately, much attention has been given to the search for phytotherapeutic compounds with potential antileishmanial activity [8]. In Brazil, about 80,000 species of plants with medicinal properties have been described. Coumarin is found in several of these [9].

Amburana cearensis (Allemão) AC Smith is native to Northeastern Brazil but is currently widely distributed in South America, including regions of Argentina and Bolivia. Its bark and seeds have long been used to prepare homemade remedies for respiratory illnesses [10].

The tree produces coumarin, a biologically active compound with antileishmanial properties [11]. In the present study, we evaluated the antileismananial activity of coumarin isolated from seeds of *Amburana cearensis* by testing for cell viability. The study is intended as a contribution to the development of new drugs for the control or prophylaxis of visceral leishmaniasis.

2. Materials and Methods

2.1. Plant Material

Seeds of *Amburana cearensis* were harvested in the month of January 2009, 2010 and 2011 from plants growing in Quixeramobim (a hinterland municipality in Ceará, Northeastern Brazil). **Figure 1** shows *Amburana cearensis* seeds. A voucher specimen was deposited at Prisco Bezerra herbarium (Department of Biology, Federal University of Ceará) under no. 51673.

2.2. Extraction and Chromatographic and Spectroscopic Identification

The collected seeds were fragmented and defatted with hexane. The dried seed

Figure 1. *Amburanacearensis* seeds.

powder was extracted overnight with 0.15 M NaCl under refrigeration at 8˚C. The extract was centrifuged at 10,000 ×g for 30 min and filtered in membrane with pore 0.45 μm.

Coumarin was extracted by partition from the total extract using chloroform. An 80-mL aliquot was placed in a separatory funnel and homogenized with 90 mL of chloroform. The coumarin present in the organic phase was collected in a beaker with anhydrous sodium sulfate and then transferred to a tared becker. The sample was covered with aluminum foil and left at room temperature overnight to dry the sample. The mass of coumarin was established by calculating the difference between the weight of the empty becker and the dry sample. Coumarin was submitted to thin-layer chromatography (TLC) for identification. To do so, a cellulose acetate plate with a sample of the extracted coumarin and a control sample of >99% pure standard coumarin (Sigma®) were analyzed in a chamber under UV light at 365 nm. The mobile phase consisted of chloroform and ethyl acetate (1:1) [12].

The structure of the extracted compound was determined by analysis of nuclear magnetic resonance (NMR). The proton nuclear magnetic resonance (^{1}H NMR) and carbon-13 magnetic resonance (^{13}C NMR) spectra were obtained on a Bruker Avance (DPX-300) spectrometer belonging to the Northeastern Center for the Application and Use of Nuclear Magnetic Resonance of the Federal University of Ceará (CENAUREMN/UFC).

For the determination of ^{1}H and ^{13}C, the sample (20 μg) was diluted in 0.6 mL aliquots of deuterated solvent: chloroform (CDCl3) ACROS®; water (D2O) Cambridge Isotope Laboratories®. The Bruker Avance DRX-300 spectrometer with a 5 mm reverse detection probr and 7046 T magneto was operated on the frequencies of 300.13 and 75.47 MHz respectively.

Chemical shifts (δ) were expressed were expressed in parts per million (ppm) according to spectra. The proton spectra were referenced by peaks of the hydrogens belonging to the non-deuterated residual molecules of the solvent borne

(chloroform-δ 7.27). While for the carbon-13 spectra, the shifts were referenced by chemical shifts of the central chloroform carbon-13 peaks (δ 77.23).

In the ^{1}H and ^{13}C experiments, the values for the acquisition parameters, respectively: spectral widths of 24 and 260 ppm; relaxation time of 1s and 90°pulse width of 9.60 us (0 dB) and 10.90 us (−3 dB). To obtain the data, the programs ^{1}H (zg), ^{13}C-BB (zppg), 13C-DEPT135 (dept135) were used. The DEPT (Distortionless Enhancement by Polarization Transfer) method was used to determine the

^{13}C NMR hydrogenation pattern as follows: C (non-hydrogenated carbon), CH (methylene carbon), CH_2 (methylene or methylidene carbon) and CH_3 (methyl carbon).The characterization of the non hydrogenated carbons was done by subtracting the DEPT 135 spectrum from the BB spectrum [13].

2.3. Cytotoxicity Assay

Macrophages (AMJ2C11) were obtained from the cell bank (BCRJ) of the Federal University of Rio de Janeiro (UFRJ). The cells were cultured in 75 cm^2 plates with DMEM medium supplemented with 10% foetal calf serum, L-glutamine and penicillin and incubated at 37˚C in an atmosphere of 5% CO_2 and 100% humidity. For the in vitro tests, the confluent cell monolayer was trypsinized, washed with culture medium, distributed in sterile flat-bottomed 96-well plates and incubated at 37˚C for 24 h to ensure cell adherence.

Leishmania chagasi promastigotes (MHOM/2002/LPC-RPV BR) were obtained from the Leishmania collection of the Oswaldo Cruz Institute (CLIOC). The cells were cultured in culture bottles filled with Schneider medium supplemented with 10% foetal calf serum and incubated at 22˚C in an atmosphere of 5% CO_2 and 100% humidity.

Cytotoxicity was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method. Macrophages (4 × 107 cells/well) and promastigotes (10^6 cells/well) were incubated in 100 µL coumarin solution at five different concentrations (25, 50, 100, 250 and 500 mg/mL). The plates were incubated for 24 hours in an atmosphere of 5% CO_2 at 37˚C (macrophages) or 22˚C (promastigotes). Suspensions of macrophages and non-treated Leishmania were used as negative controls. For the MTT assay, the plates were centrifuged, the supernatant was carefully removed from the wells, 200 µL MTT solution (10%) was added and the plates were incubated for another 3 hours. Subsequently, the plates were centrifuged, the supernatant was removed and 150 µL DMSO was added to the wells to lyse the cells and solubilize the formazan, with thorough mixing. The optical density at 540 nm was determined using an ELISA reader (Biotek®).

The MTT test evaluates mitochondrial viability by quantifying mitochondrial reduction of MTT to formazan. Reduced optical density indicates decreased cell viability. The absorbance obtained from non-treated cells was considered as 100% cell viability. Cell viability was expressed as the percentage of absorbance

obtained from untreated control cells and treated cells after subtracting the background absorbance (DMSO). All tests were conducted sixfold on three different days [13].

2.4. Statistical Analysis

The results of the in vitro tests were analyzed with the software Graph Pad Prism® 6.0. Mean values ± standard error of the mean (SEM) for each group were entered in a bar chart for comparison. Differences between treatments were analyzed with one-way ANOVA for paired data, followed by the Bonferroni correction. The level of statistical significance was set at 5% ($p < 0.05$).

3. Results

In this study, we evaluated the antileishmanial activity of coumarin isolated from seeds of *Amburana cearensis*. The crude extract yielded 0.0145 g of coumarin. Characterized by a pattern of intense fluorescence under UV light at 365 nm, the chromatographic profile of the sample was similar to that of pure standard coumarin.

The isolated coumarin was identified as 1,2 benzopyrone by comparing the results of1H and 13C NMR. The **Figure 2** shows the 1H NMR profile of the coumarin spectrum (300 MHz, $CDCl_3$) featured two multiplets at δ 7.51 and 7.29 (2H, m) linked to aromatic hydrogens.

The **Figure 3** shows the two doublets observed at δ 7.71 and 6.42 (1H, d, J = 9.5 Hz) were related to cis-olefinic protons. On 13C NMR (75 MHz, $CDCl_3$) nine spectral lines were visible. The line at δ 160.9 (C-2) was associated with α, β-un-

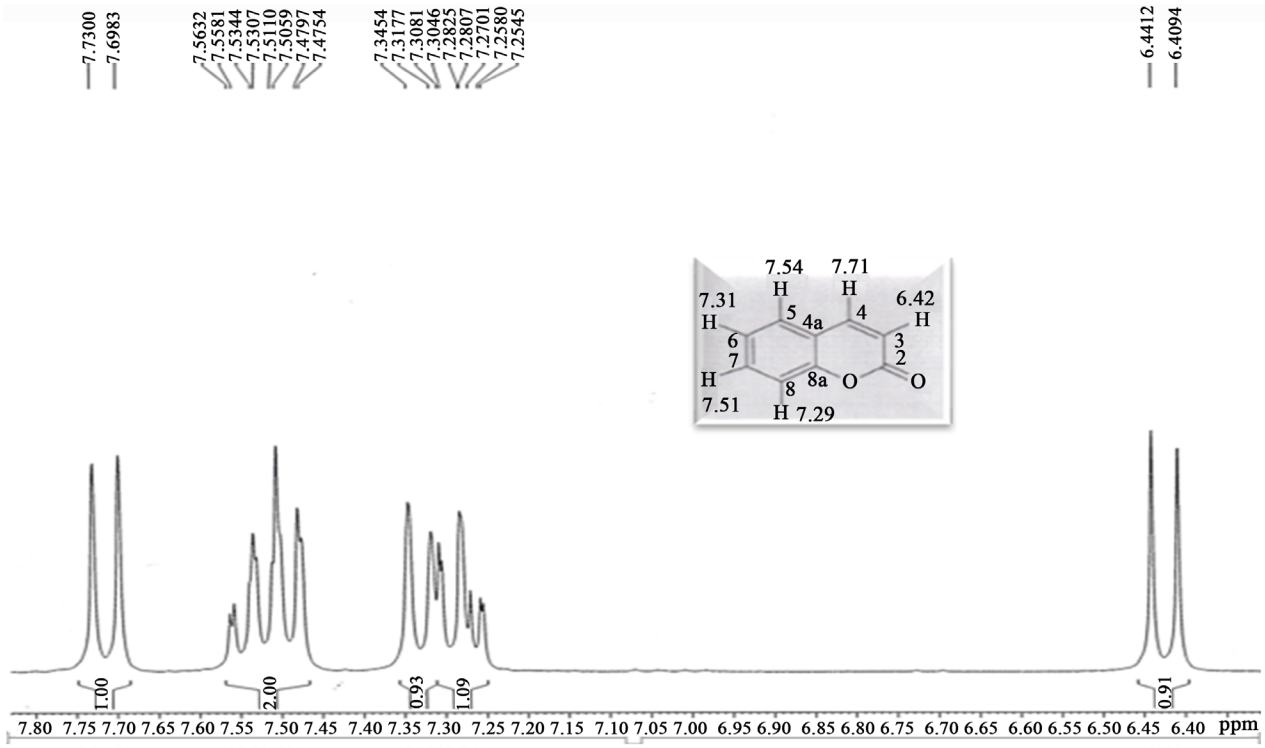

Figure 2. 1H NMR spectrum (CDCl3, 300 MHz) of coumarin isolated from an aqueous extract of seeds of *Amburana cearensis.*

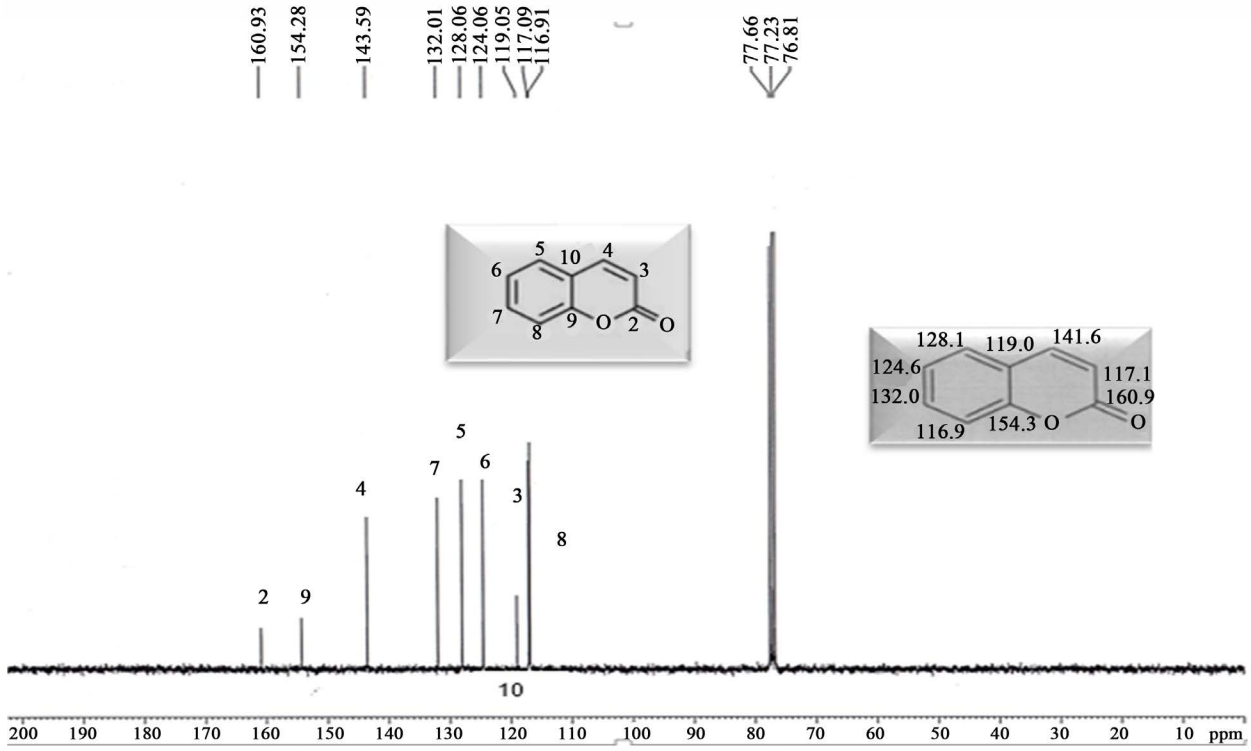

Figure 3. Composite pulse-decoupled 13C NMR spectrum (CDCl3, 75 MHz) of coumarin isolated from an aqueous extract of seeds of *Amburana cearensis.*

saturated lactone carbonyl, while the others were attached to sp2 carbons (δ 154.3 - 116.9).

The Table 1 demonstrates the results of the NMR scan allowed to deduce the molecular formula of coumarin ($C_9H_6O_2$) as being composed of a benzene ring and an α, β-unsaturated lactone ring (Table 1).

In the cytotoxicity test, *L. chagasi* promastigotes were challenged in vitro with coumarin solutions at different concentrations (25, 50, 100, 200 and 500 μg/mL). Figure 4 shows that none of the coumarin concentrations displayed toxicity against macrophages ($p > 0.05$).

All coumarin concentrations (25, 50, 100, 250 and 500 μg/mL) presented anti-leishmanial activity when compared to untreated control Leishmania ($p < 0.05$). As shown in Figure 5, the antileishmanial activity of the coumarin solutions used in this study was not dose-dependent: the effect was statistically similar for all the concentrations tested (25, 50, 100, 250 and 500 μg/mL).

As shown in Figure 5, the antileishmanial activity of the coumarin solutions used in this study was not dose-dependent: the effect was statistically similar for all the concentrations tested (25, 50, 100, 250 and 500 μg/mL).

4. Discussion

Known for its medicinal properties, coumarin (1,2 benzopyrone) has been isolated from many different plant species, including *Amburana cearensis* [14]. Extracts from the bark and seeds of this tree are used to treat disorders of the res-

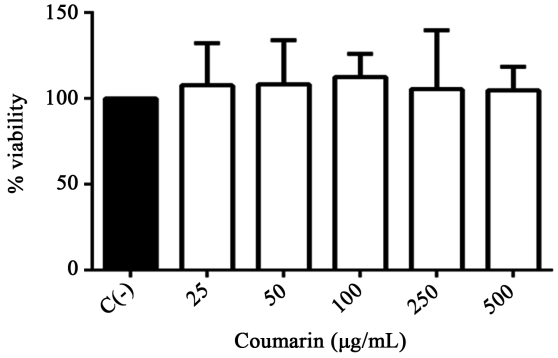

Figure 4. Macrophages challenged with coumarin isolated from seeds of *Amburana cearensis*. No statistically significant difference was observed between the groups ($p > 0.05$). The bars represent mean values ± standard error of the mean (SEM) in each group. After subtracting the background absorbance (DMSO), the data were analyzed with ANOVA followed by the Bonferroni correction. C(-) = negative control.

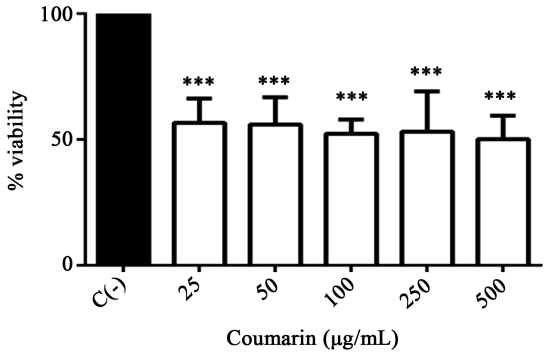

Figure 5. *Leishmania chagasi* promastigotes challenged with coumarin isolated from seeds of *Amburana cearensis* at different concentrations. The bars represent mean values ± standard error of the mean (SEM) in each group. The experiment was conducted six-fold on three different days. After subtracting the background absorbance (DMSO), the data were analyzed with ANOVA followed by the Bonferroni correction. * = $p < 0.001$ in relation to the negative control C(-).

Table 1. ^{13}C NMR profile of coumarin isolated from an aqueous extract of seeds of *Amburana cearensis* compared to 13C NMR profiles from two other studies.

#C	Coumarin (Pouchert) δ_c (ppm) $CDCl_3$	ACS-1 (Canuto) δ_c (ppm) $CDCl_3$	Current study δ_c (ppm) $CDCl_3$
2	160.6	160.9	160.9
3	116.4	117.1	117.0
4	143.3	141.6	143.5
5	127.7	128.1	128.0
6	124.3	124.6	124.6
7	131.6	132.0	132.0
8	116.6	116.9	116.9
9	118.8	119.9	119.5
10	153.8	154.3	154.2

piratory tract and to relieve spasms and rheumatic pain [15]. Most phytochemical studies of *A. cearensis* have focused on compounds isolated from the bark, especially coumarin [15].

Spectral data for coumarin isolated from *A. cearensis* have been published before [16]. Based on ethanol extracts of seeds, [17] used NMR data to deduce the molecular formula of coumarin ($C_9H_6O_2$). This formula was confirmed in the present study by ^{1}H and ^{13}C NMR imaging.

The previously reported high level of activity of coumarim from seeds of *A. cearensis* against *L. chagasi* was confirmed in our experiments. [18] and [19] found coumarin extracted from the bark of *A. cearensis* to be efficient against *Leishmania amazonensis, L. braziliensis* and *L. donovani* at a concentration of 50 μg/mL. In addition, coumarin displayed antileishamanial action against *L. chagasi* at a concentration of 73 mg/mL [19]. To evaluate the antileishmanial activity of coumarin (-) mammea A/BB isolated from extracts of the leaves of *Calophyllum brasiliensis, L. amazonensis* promastigotes were challenged with 3.0 mg/mL (-) mammea A/BB for 72 hours. On electron microscopy, ultrastructural changes were observed, including binucleated cells, multiple vacuolation, intense exocytic activity in the flagellar pocket region and swollen mitochondria with concentric membranes in the mitochondrial matrix [20].

The coumarin solutions tested in this study displayed no toxicity against macrophages in vitro. Macrophages are the target cells for Leishmania. They provide nutrients for amastigote development and favor the establishment of infection [21]. Phagocytosis occurs by adhesion of Leishmania promastigotes to macrophages by way of lectin-like ligands in the membrane. The induction of immune response involves the processing of antigenic material and the presentation to lymphocytes of this material on macrophage surfaces following phagocytosis and digestion [22]. Essentially, the parasite restricts the action of the immune system and inhibits the microbicidal action of the macrophages [23]. The presence of the parasite leads to defects in macrophage-lymphocyte cooperation, making leishmaniasis a difficult-to-treat condition [24].

In conclusion, leishmaniasis is a neglected disease, the treatment of which has remained unaltered for over 60 years despite serious side effects and the worldwide emergence of drug resistance. New strategies and therapies are needed to prevent and treat leishmaniasis. *In vitro* tests of leishmanicidal compounds extracted from plants like *Amburana cearensis* is a promising line of study towards the development of new antileishmanial drugs. The present study confirmed the antileishmanial activity of coumarin against *L. chagasi* promastigotes *in vitro*.

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