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In vitro antioxidant, antitumor and leishmanicidal activity of riparin A, an analog of the Amazon alkamides from *Aniba riparia* (Lauraceae)

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ABSTRACT

Aniba riparia (Lauraceae) is an important medicinal plant found in the Amazon region and presents alkaloids of the type alkamide known as riparins. Riparin A is structurally represented as the fundamental core of all Amazon riparins. This work aimed to assess the *in vitro* antioxidant, antitumor and antileishmanial effects of riparin A. Riparin A presented weak antioxidant capacity by tecniques of DPPH• (EC50 of 296.2 μg mL^{-1}) and ABTS•+ (EC50 of 450.1 μg mL^{-1}), showed moderate activity against colon carcinoma (HCT-116: IC50 of 21.7 μg mL^{-1}) and leishmanicidal activity on promastigotes of *L. amazonensis* (IC_{50} of 307.0 ± 79.6, 193.7 ± 44.3 and 81.8 ± 11.2 μg mL^{-1}, respectively, after 24, 48 and 72 h of incubation). Then, in addition to its structural simplicity, riparin A revealed promising biological activities and remarkable *in vitro* leishmanicidal action, an important result in epidemiological point of view to control leishmaniasis in Brazil, including in the Amazon region.

KEYWORDS: Bioprospecting, Chemoprevention, Cytotoxicity, Antiparasitic drug.

Atividade antioxidante, antitumoral e leishmanicida *in vitro* da riparina A, um análogo das alcamidas amazônicas de *Aniba riparia* (Lauraceae)

RESUMO

Aniba riparia (Lauraceae) é uma importante planta medicinal encontrada na região amazônica que apresenta alcaloides do tipo alcamida e conhecidos como riparinas. Este trabalho teve como objetivo avaliar os efeitos antioxidantes, antitumorais e leishmanicidas *in vitro* da riparina A. Riparina A apresentou fraca capacidade antioxidante pelas técnicas do DPPH• (CE50 de 296,2 μg mL^{-1}) e ABTS•+ (CE50 de 450,1 μg mL^{-1}), mostrou moderada atividade contra carcinoma de cólon (HCT-116: CI50 de 21,7 μg mL^{-1}) e atividade leishmanicida sobre formas promastigotas de *Leishmania amazonensis* (CI_{50} de 307,0 ± 79,6; 193,7 ± 44,3 e 81,8 ± 11,2 μg mL^{-1}, respectivamente, após 24, 48 e 72 h de incubação). Assim, além de sua simplicidade estrutural, a riparina A revelou atividades biológicas promissoras e significativa ação leishmanicida *in vitro*, resultado importante diante da relevância epidemiológica para controle da leishmaniose no Brasil, inclusive na região amazônica.

PALAVRAS-CHAVE: Bioprospecção, Quimioprevenção, Citotoxicidade, Droga antiparasitária.

INTRODUCTION

Aniba riparia (Nees) Mez is an important medicinal plant belonging to the Lauraceae family. The Lauraceae family stands out both ecologically and economically. Many of its species are used in folk medicine to treat skin lesions, gastric disorders and circulatory problems, and some of them have anti-inflammatory and hypoglycemic properties and central effects. The various activities are attributed to alkamide alkaloids, which are known as riparins (Santos *et al.* 2011). Riparins possess anxiolytic, antidepressant, anticonvulsant, antimicrobial and myorelaxing action. Natural riparins n-benzoyl tyramine (riparin I), n-(-2-hydroxybenzoyl) tyramine (riparin II) and n-(2,6-dihydroxybenzoyl) tyramine (riparin III) have been isolated from an unripe fruit of *Aniba riparia*, a plant of typical occurrence in the Amazon region, popularly known as "*louro*" (Catão *et al.* 2005; Teixeira *et al.* 2013).

In addition to the naturally occurring molecules in *A. riparia*, using the Schotten-Bauman reaction, Gutierrez *et al.* (2005) obtained synthetic analogues called riparins A, B, C, D, E and F (Figure 1). Thus, the amount of molecules that can be tested regarding their pharmacological and toxicological properties have increased, since these substances share the same fundamental structure of natural riparins, without requiring the exploitation of the Brazilian flora.

Therefore, riparin A, represented as the core structure of all Amazonian riparins, emerges as a promising molecule. Tests regarding its antioxidant and myorelaxing effects in animal models indicated that riparin A has neuroprotective capacity, without reducing the muscle tone of rodents (Nunes *et al.* 2014; 2015). Riparin A has also recently demonstrated anti-inflammatory potential in acute inflammation models, in which it reduced inflammatory response through inhibition of cellular events, neutrophils migration modulation and inhibition of proinflammatory cytokines (TNF-α and IL-1β) production.

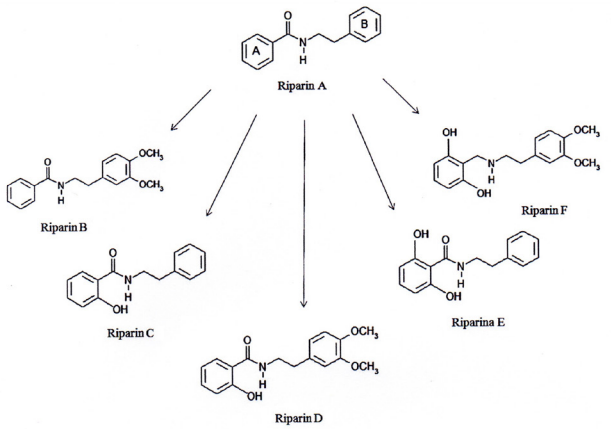

Figure 1. Molecular structure of the synthetic riparinas (B, C, D, E and F) based on the fundamental structure of the natural riparin A.

These events are often triggered by parasites, microorganisms and local circulatory changes (Silva *et al.* 2015). Then, this study aimed to assess the *in vitro* the antioxidant, antitumor and antileishmanial effects of riparin A.

MATERIALS AND METHODS

Sample collection

Riparin A was obtained following the Schoten-Bauman reaction, through the mixture of 0.41 mL acyl chloride and 0.89 mL 2-phenylethylamine with triethylamine, followed by magnetic stirring and purification by column chromatography, as described by Gutierrez *et al.* (2005) and Nunes *et al.* (2015).

Antioxidant Activity Evaluation by DPPH• and ABTS•+ Methods

For *in vitro* antioxidant evaluation, stock solutions of riparin A, DPPH$^{\bullet}$ (9.8 mM), ABTS$^{\bullet+}$ (7 mM) and vitamin C standard (20 mM) were prepared in 4% DMSO (dimethylsulfoxide). All solutions had a final concentration of 4% DMSO. In order to determine the antioxidant activity by ABTS assay, the methodology described by Re *et al.* (1999) was used. The DPPH$^{\bullet}$ method was based in Blois (1958) and adapted by Brand-Williams *et al.* (1995). Following dilution, concentrations of 24, 120, 240, 480 and 1200 μg mL^{-1} of riparin were obtained. Vitamin C (Vit. C) was used as positive control (176 μg mL^{-1}).

The 50% effective concentration (EC_{50}) of riparin A was determined spectrophotometrically (T80+ UV/VIS Spectrometer, PG Instruments Ltd®, Leicestershire, UK) at 517 nm for DPPH$^{\bullet}$ and in 734 nm for ABTS$^{\bullet+}$, 30 minutes after the reaction started. Antioxidant evaluation was performed in triplicate and absorbance values were converted to the inhibition percentage (I) of radicals using the equation of Reanmongkol *et al.* (1994): I (%) = [(Abs.control - Abs.sample) x 100]/Abs.control, where Abs.control is the DPPH$^{\bullet}$ or ABTS$^{\bullet+}$ solution initial absorbance and Abs.sample is the reaction mixture absorbance (DPPH$^{\bullet}$ or ABTS$^{\bullet+}$ and sample).

Cytotoxic evaluation

Cytotoxicity evaluation was conducted using the MTT assay (Mosmann 1983) in three human tumor lines: HCT-116 (colon cancer), OVCAR-8 (ovarian) and SF-295 (glioblastoma). Cell lines were grown in plastic flasks using the RPMI 1640 culture medium supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin). Cells were incubated at 37 °C with an atmosphere of 5% CO_2 and 95% humidity (CO_2 Incubator, Shel Lab, Cornelius, USA). Afterwards, cells were observed every 24h in relation to cell growth and contamination control in an inverted microscope (Nikon Inverted Microscope, City Labs, Tokyo, Japan) and, when necessary, cells were subcultured on fresh culture medium.

Cells were plated in 96-well plates (0.3-0.7 x 10^5 cells/well) and incubated in order to allow cell adhesion. After 24h, riparin A was added to each well in increasing concentrations (0.004 - 50 µg mL^{-1}). Cells of the negative control, positive control and those treated with riparin were exposed to the same DMSO percentage (0.1%) (Ferreira *et al.* 2014). After 69h, plates were centrifuged at 1,000 g per 15 min, the supernatant was aspirated and 200 µL of 10% MTT solution was added in RPMI 1640. The plate was placed in an incubator at 5% CO_2 for additional 3h. Then, plates were centrifuged again at 1,000 g per 15 min, the supernatant was aspirated and its precipitate was resuspended in pure DMSO. Afterwards, it was stirred for about 10 min, until formazan crystals were completely dissolute. The chemotherapeutic doxorubicin (Sigma Aldrich, USA) was used as positive control (0.005 - 5.0 µg mL^{-1}). Plates were read in a plate spectrophotometer at the wavelength of 595 nm (DTX 880 Multimode Detector, Beckman Coulter, Harbor Boulevard, Fullerton, USA).

Leishmanicidal activity avaluation

For the leishmanicidal test, *L. amazonensis* strains grown in Schneider's medium (Sigma Aldrich, USA), supplemented with 10% fetal bovine serum (Sigma Aldrich, USA), penicillin and streptomycin were used. Promastigote forms in their logarithmic growth phase were distributed into 96-well plates for cell culture, in the amount of 1 x 10^6 leishmanias/well. Starting from a riparin A stock solution, serial dilutions in the range of 1:2 (25 - 800 µg mL^{-1}) were carried out in a Schneider's medium. Plates were incubated at 26 °C temperature in biochemical oxygen demand (BOD) incubator and observed at 24, 48 and 72h of exposure to the substance, analyzing leishmania growth and viability in a Neubauer chamber. Amphotericin B was used as positive control (40 µg mL^{-1} in 4% DMSO).

Statistical analysis

EC_{50} and IC_{50} values and their confidence intervals of 95% were obtained by linear regression. Results shown as mean [± standard error of the mean (S.E.M.)], from two independent experiments, were assessed using ANOVA followed by Newman-Keuls test (GraphPad Prism 5.0, Intuitive Software for Science, San Diego, CA, USA), considering p <0.05.

RESULTS

Antioxidant activity

Riparin A decreased DPPH• (2,2-diphenyl-1-picryl-hydrazyl) levels, regardless of its concentration, in 27.7 ± 1.3; 26.5 ± 0.2; 27.3 ± 0.2; 30.4 ± 1.9 and 34.0 ± 0.1%, in the concentrations of 24, 120, 240, 480 and 1200 µg mL^{-1}, respectively, with CE_{50} of 296.2 µg mL-1. Meanwhile, vitamin C showed inhibition of 61.3 ± 0.9% (p <0.05) (Figure 2A).

Similarly, riparin A caused $ABTS^{\bullet+}$ radical inhibition of 33.2 ± 1.3; 33.3 ± 1.0; 34.5 ± 0.2; 35.0 ± 0.2 and 35.3 ± 1.2% in concentrations of 24, 120, 240, 480 and 1200 µg mL^{-1}, respectively, and EC_{50} value of 450.1 µg mL-1. Vitamin C reduced the radical 93.5 ± 1.6% (p <0.05) (Figure 2B).

Cytotoxic activity

In this study, riparin A showed moderate antiproliferative activity against colon carcinoma (HCT-116) tumor cells, with an IC_{50} of 21.7 (19.8 to 23.8) µg mL^{-1} (Table 1).

In relation to the leishmanicidal potential, riparin A acted on *L. amazonensis* promastigotes with IC_{50} values of 307.0 ± 79.6; 193.7 ± 44.3; and 81.8 ± 11.2 µg mL^{-1} after 24, 48 and 72h of incubation, respectively (p <0.05) (Figure 3).

DISCUSSION

Innovative molecules endowed with biological activity, that can be obtained at an affordable cost, with low toxicity and in a sustainable way, have been the focus of the pharmaceutical industry and academic institutions in recent years, in order to show alternatives to processes based on predatory extraction of active substances of natural species (Nunes *et al.* 2013; Cardoso *et al.* 2015; Ferreira *et al.* 2015). These bioactive substances have been highlighted, especially in research for the treatment or prevention of chronic diseases associated with oxidative stress, such as cancer or infectious diseases. The latter, although neglected, are responsible for high morbidity

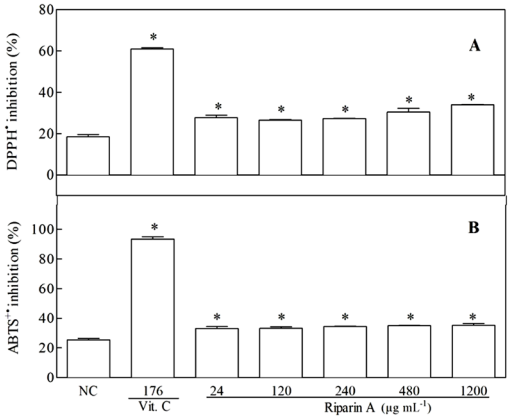

Figure 2. Effects of riparin A on the DPPH• (A) and ABTS•+ (B) reduction. The results are expressed as average of the percentage of inhibition ± standard error of the mean (S.E.M.) of independent experiments (n= 2). Negative control (NC) was treated with the solution used for diluting the tested substance. Vitamin C (Vit. C) was used as positive control (176 µg mL^{-1}). *p <0.05 when compared to the negative control by ANOVA followed by Student-Neuman-Keuls test.

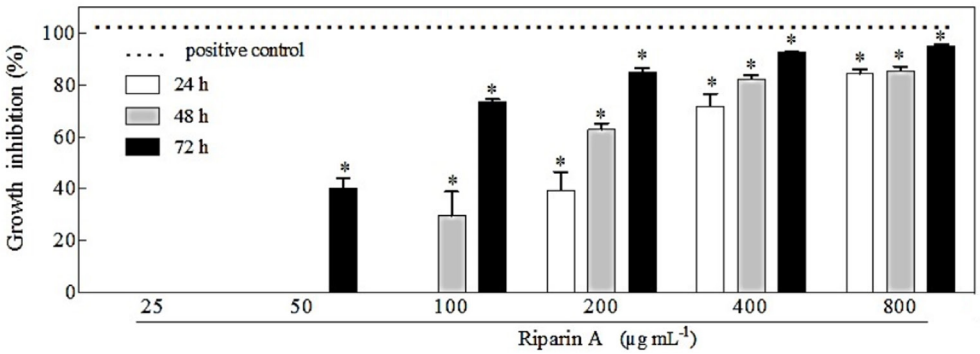

Figure 3. Cytotoxicity of riparin A on *Leishmania amazonensis* promastigotes after 24, 48 and 72 h exposure. The results are expressed as average of the percentage of inhibition ± standard error of the mean (S.E.M.) of independent experiments (n= 2). Amphotericin B was used as positive control (40 μg mL^{-1}). *p <0.05 when compared to the negative control by ANOVA followed by Student-Neuman-Keuls test.

Table 1. *In vitro* cytotoxic activity of riparin A on cancer lines determined by MTT assay after 72 h of incubation.

Sample	IC_{50} (μg mL^{-1})*		
	HCT-116	OVCAR-8	SF-295
Riparin A	21.7 (19.8 – 23.8)	> 50	> 50
Doxorubicin	0.1 (0.1 – 0.2)	1.3 (1.0 – 1.9)	0.2 (0.2 – 0.3)

* Data are presented as IC_{50} values and 95% confidence intervals for colon (HCT-116), ovarian (OVCAR-8) and glioblastoma (SF-295) tumor lines. Doxorubicin was used as positive control. Experiments were performed in duplicate.

and mortality rates in developing countries (Alves *et al*. 2010; Oliveira *et al*. 2012; Farias *et al*. 2013).

The oxidation phenomenon naturally occurs in cellular processes as part of the biochemical reaction mechanism, in cellular energy production, in intercellular signaling and phagocytosis. However, excessive oxidation may cause damage to cells, resulting in the evolution or aggravation of diseases (Alam *et al*. 2012). The DPPH$^{\bullet}$ method has been used in many substance antioxidant evaluation studies based on the capacity of these substances to sequester the radical (Sharma and Bhat 2009; Nascimento *et al.* 2011; Farias *et al*., 2013). In this study, riparin A reduced DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ levels, proving to be a molecule with chemopreventive potential.

The ABTS$^{\bullet+}$ radical, which is a chromophore, soluble and stable compound produced from the 2,2-azino-bis(3-ethylbenzothiazoline)-6-sulfonic precursor, when captured by potentially antioxidant molecules, causes absorbance decrease and subsequent reduction in the concentration of the tested sample (Villaño *et al*. 2004; Sucupira *et al*. 2012). The riparin A molecule, due to having potential electron donors only in the nitrogen and oxygen and few potential acceptor in the hydrogen atoms, proved to be a substance endowed with limited antioxidant capacity.

There are records showing that essential oils of *Piper divaricatum* species from the Brazilian Amazon are rich in mono and sesquiterpenes, or phenylpropanoids. A study conducted by Silva *et al.* (2010) showed that the oil extracted from the species was effective in inhibiting the DPPH$^{\bullet}$ radical formation in up to 74%, with a CE_{50} value of 16.2 ± 1.9 µg mL^{-1}. On the other hand, Santana *et al.* (2014) reported that the *Mikania glomerata* leaf ethanol extract was able to reduce DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ levels (CE_{50} values of 138.91 µg mL^{-1} and 175.68 µg mL^{-1}, respectively).

The group of this study previously showed that synthetic riparins (A-F) have antiproliferative potential (Nunes *et al.* 2014). In this study, cytotoxic activity was confirmed in other cancer cell lines, with selective effect on colon carcinoma. In addition, it was found that riparins C, D, E and F showed cytotoxic activity in laryngeal (HEP-2) and lung (NCIH-292) carcinoma lines and in leukemia cells (HL-60), with IC_{50} values ranging from 1.9 to 11.4 µg mL^{-1}. Moreover, cell proliferation inhibition capacity was higher than 90% on colon adenocarcinoma cells (HT-29), confirming the most recent studies by the group of this study, in which the riparin A showed cytotoxic activity against colon carcinoma. It is believed that synthetic riparins cytotoxic activity is related to the substituents of their aromatic rings (Shayne *et al.* 2007). It is possible that hydroxyl presence and methoxy groups absence increases the antiproliferative activity of riparins C, D and E. Similarly, the fact that the fundamental structure (represented by riparin A), which is devoid of substituents, showed no relevant activity, endorses the hypothesis that hydroxyl insertion in lateral rings is associated with antitumor activity.

Like cancer, protozoa are responsible for millions of disease cases with varied features worldwide, although such diseases mainly affect countries with medium to low economic and social development levels, besides areas with sanitation, environmental education and health program deficits (Rodrigues *et al.* 2013). In Central and South American countries, including the perimeter represented by the Amazon forest, as well as in Africa, South Asia and Middle East, leishmaniasis has large impact on individuals and communities. Thus, researches for novel molecules with anti-parasitic efficacy are stimulated, against, for example, *Leishmania braziliensis* and *Leishmania amazonensis*, which are the most prevalent species in the maintenance of the leishmaniasis epidemiological chain in Brazil (Dorval *et al*. 2006; Teles *et al*. 2014). Therefore, riparin A showed leishmanicidal activity against *L. amazonensis* promastigotes within 72h of incubation. In a similar study, Silva *et al.* (2014) showed that the methanol extract and a hexane fraction of the *Lacistema pubescens* Mart. Amazonian species had IC_{50} values of 3.9 and 3.5 μg mL^{-1} against *L. amazonensis*. The results obtained in this study are considerable, since riparin A showed lower IC_{50} values against *L. amazonensis* than the N-methyl-glucamine compound (120.3 to 400.3 μg mL^{-1}) (Costa-Filho *et al.* 2008), which is used as standard antimony drug for cutaneous leishmaniasis treatment. N-methyl-glucamine causes several side effects, such as arthralgia, myalgia, appetite loss, nausea, vomiting, epigastric pain, heartburn, abdominal pain, rash, fever, weakness, headache, dizziness, palpitations, insomnia, nervousness, edema and acute renal failure, in addition to being inappropriate for the treatment of pregnant women and patients with pulmonary tuberculosis, malaria, heart diseases, kidney diseases, liver diseases and Chagas disease, requiring rigorous and constant assessment and monitoring in clinical use. Associated with prominent side effects, antimonial drugs are also capable of inducing parasitic resistance (Rath *et al*. 2003; Rodrigues *et al*. 2006; Pelissari *et al*. 2011).

CONCLUSION

The N-phenyl benzamide compound, or riparin A, due to its structural simplicity, is easy to be obtained, it has shown promising biological activities and has significant *in vitro* leishmanicidal action. These findings are important because of the epidemiological importance of leishmaniasis in Brazil, including in the Amazon region. Given this bioactive potential, studies aiming at elucidating the pharmacological mechanism and pharmacophore group(s) of riparins in nanocarrier systems are being conducted, in order to enhance their bioavailability and their therapeutic activities.

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