Vol.67: e24231132, 2024
https://doi.org/10.1590/1678-4324-2024231132
ISSN 1678-4324 Online Edition

BABT
Brazilian Archives of Biology and Technology

Article – Biological and Applied Sciences

Cytotoxicity Evaluation and Dereplication of Flavonoids-Guided by Antioxidant Activity and Total Phenolics Content from *Ephedrantus amazonicus* Leaves

Alisson Martins Albino[1,3]
https://orcid.org/0000-0002-1104-4119

Débora Luciano Gomes[1]
https://orcid.org/0009-0007-5756-0921

Arthur Henrique dos Santos[1]
https://orcid.org/0009-0003-5542-8039

Giovanna de Souza Lima Malta[1]
https://orcid.org/0009-0003-8519-7323

David Henrique Lima de Oliveira[1]
https://orcid.org/0009-0004-9973-9311

Luís Fernando Lira Souto[1]
https://orcid.org/0000-0001-9760-8642

Carolina Bioni Garcia Teles[2]
https://orcid.org/0000-0002-4529-2137

Saara Neri Fialho[2]
https://orcid.org/0000-0002-8667-2953

Minelly Azevedo da Silva[1]
https://orcid.org/0000-0002-6052-6341

Nilton Fagner de Oliveira Araújo[1]
https://orcid.org/0000-0002-1211-1838

Márcia Bay[1*]
https://orcid.org/0000-0001-5109-0497

[1]Instituto Federal de Educação, Ciência e Tecnologia de Rondônia - IFRO, Câmpus Porto Velho Calama, Porto Velho, Rondônia, Brasil; [2]Fundação Oswaldo Cruz - FIOCRUZ/RO, Porto Velho, Rondônia, Brasil. [3]Universidade Federal de Rondônia - UNIR, Programa de Ciências Ambientais, Porto Velho, Rondônia, Brasil

Editor-in-Chief: Paulo Vitor Farago
Associate Editor: Jane Manfron Budel

Received: 18-Nov-2023; Accepted: 06-May-2024

*Correspondence: marcia.bay@ifro.edu.br; Tel.:(+55) 69-98493-8110 (M.B)

HIGHLIGHTS

- The phenolic content shows a positive correlation with antioxidant activity;
- The leaf ethanolic extract from *Ephedranthus amazonicus* has antioxidant activity;
- The leaf ethanolic extract from *Ephedranthus amazonicus* didn't show cytotoxic activity against L929 (fibroblast) cell lines.

Abstract: Plants synthesize secondary metabolites as a means of survival and competition. These metabolites are a potential source of natural antioxidants that may promote activity, favoring the control of cellular damage caused by oxidative stress. The Annonaceae family occurs in tropical and subtropical regions worldwide. The medicinal benefits of plants from this family are caused by secondary metabolites present in these species. Thus, this study aimed to determine the total phenolic content, evaluate the antioxidant potential, and *in vitro* cytotoxicity of the leaf ethanolic extract from *Ephedrantus amazonicus* R.

E. Fries. The phenolic content and antioxidant activity were evaluated by the Folin-Ciocalteu and DPPH methods, respectively. The assays were adapted for 96-well microplates, with absorbance reading at λ = 630 and 517 nm, respectively, using a UV-Vis spectrophotometer. The *in vitro* cytotoxicity assay was performed using fibroblasts (L929 cell line). The MS/MS analyses were performed through direct infusion using a LCQ Fleet mass spectrometer equipped with APCI font and ion trap analyzer, in the positive and negative mode. A molecular network was created using the online workflow on the GNPS website (http://gnps.ucsd.edu). The phenolic content at 400µg was 25.24 ± 0.98µg.GAE/g, while the antioxidant activity showed an EC_{50} of 13.37±2.63 µg.mL^{-1}. Dereplication led to the annotation of three flavonoids that may contribute to the observed antioxidant activity. The extract showed low cytotoxicity at the highest tested concentration (> 500µM). The results demonstrate that the extract from *Ephedrantus amazonicus* R.E. Fries leaves is a promising source of natural antioxidants.

Keywords: Antioxidant; natural; cytotoxic activity; *Ephedranthus amazonicus*.

GRAPHICAL ABSTRACT

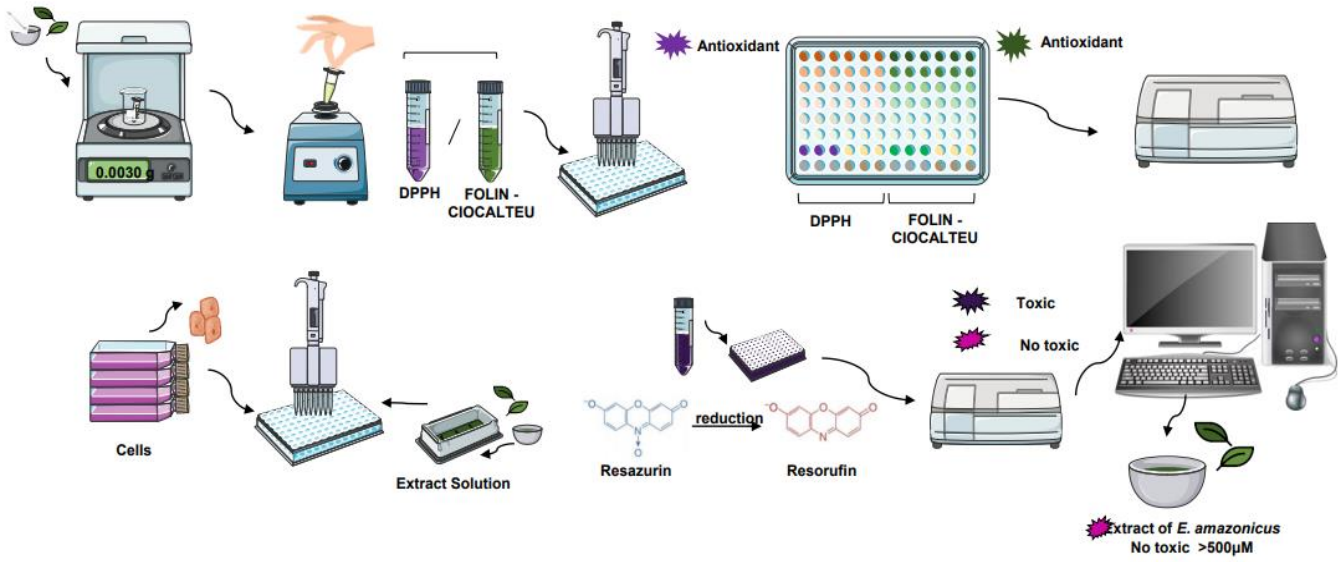

Figure: http;//smart.service.com

INTRODUCTION

Annonaceae is a family of flowering plants of the order Magnoliales belonging to the Magnoliidae class. It occurs in tropical and subtropical regions throughout the globe and currently contains 110 genera and over 2,500 species of trees, shrubs, and lianas. Most species belonging to the Annonaceae family have been traditionally used in the treatment of several diseases [1].

Ephedranthus S. Moore is a Neotropical genus of Annonaceae distributed from Colombia and the Guianas to Bolivia and the southeastern region of Brazil, composed of seven species: *E. amazonicus* RE Fr., *E. boliviensis* Chatrou & Pirie, *E. colombianus* Maas & Setten, *E. dimerus* JC Lopes, Chatrou & Mello-Silva, *E. guianensis* RE Fr., *E. parviflorus* S. Moore and *E. pisocarpus* RE Pe., distributed in the Amazon region of Brazil, Colombia, and Peru, with a single known collection in Venezuela. It occurs in non-flooded forests on lateritic soil, at altitudes ranging from 50 to 200 m. It flowers from April to June, rarely in October, and fruits throughout the year, mainly from April to August [2].

The medicinal benefits of the family are associated with the bioactivity of secondary metabolites present in the species. Plants synthesize these metabolites as a means of protection against animals, bacterial and viral infections, and competition with other plants [3].

Plant secondary metabolites have emerged as a potential source of natural antioxidants. These compounds perform a synergistic action that promotes activity, favoring the control of cellular damage caused by oxidative stress [4], and have also been widely used as food additives to prevent or delay oxidative degradation of foods [5].

Antioxidant activity can be measured through colorimetric techniques such as DPPH and Folin-Ciocalteu [6,7] assays. Several methods for evaluating antioxidant activity are proposed in the literature; however, some are more appropriate than others. Therefore, the method of choice will depend on the

nature of the compounds present in the species. To be considered a good antioxidant, the sample must, at low concentrations, delay or prevent the oxidation of the substrate. For this, some aspects are necessary, such as the presence of electron-donating substituents or a hydroxyl group [8, 9, 10].

Microplates are a standard tool in various biological activity investigation laboratories [11]. Therefore, to make the model more applicable to different concentration ranges, adaptations were made to the protocols for total phenolic content and antioxidant activity (DPPH method), for the 96-well microplate model, following the authors' guidelines [6,7]. The microplate assay reduces the amount of reagents and samples required, increases the number of simultaneous analyses and concentrations, and allows for the automation of absorbance readings, enabling the analysis of more robust results that allow for the discovery of antioxidant agents that can be used by Society [12].

Thus, considering the biological potential of species belonging to the Annonaceae family and the limited amount of scientific records involving the species *Ephedrantus amazonicus* RE Fries, popularly known as "envira-dura" [12] the present study aimed to determine the total phenolic content, antioxidant potential, and *in vitro* cytotoxicity of the ethanolic extract from leaves.

MATERIAL AND METHODS

Botanical material

The plant material of *E. amazonicus* (leaves) was collected in a forest fragment in the municipality of Porto Velho-RO, Brazil with the following coordinates: 9º 16' 3" S, 64º 24' 20" W. An exsicata of the plant was selected and identified by Mendes-Silva, I, and the specimen of the species was deposited in the Herbário Rondoniense João Geraldo Kuhlmann (RON) under the number CEN00093531. Authorization requests for the activities were forwarded to the Environmental Agencies: Genetic Heritage and Associated Traditional Knowledge Management System (SISGEN) under the registration A0EC19D.

Preparation of the ethanolic extract

The leaves (100g), collected in September 2021, were washed with running water to remove possible dirt residues before being exposed to dehydration. They were also crushed to reduce their size, thus increasing the surface area of contact with the solvent. Subsequently, the samples were placed in autoclaved glass jars, and were macerated with 500 mL of ethanol for a period of 72h. On the third day, the suspension was filtered and dried in a rotary evaporator (Fisatom-802) under reduced pressure. The prepared extract was stored in an amber bottle and kept in kept in freezing temperatures.

Evaluation of phenolic content and antioxidant potential

Initially, the assays for determining the total phenolic content by the Folin-Ciocalteau method (wavelength (λ) of 630 nm); and scavenging of the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) (wavelength (λ) of 517 nm) were standardized [6,7].

Folin-Ciocalteau method

The 10% sodium carbonate solution (Na_2CO_3) (Êxodo) was prepared using a volumetric flask. For this, 10.06 g of the reagent were weighed in the final volume of 100 mL of distilled water. The solution was stirred until complete sample dilution and subsequently stored in an amber glass bottle. The 3% Folin-Ciocalteau solution was prepared using a volumetric flask. For this, 3 mL of Folin-Ciocalteau Phenol 2M reagent (Êxodo) were diluted to the final volume of 100 mL. The solution was homogenized and stored in an amber glass bottle. A stock solution of gallic acid (Neon) at 0.2 mg/mL was prepared, as well as the standard solution at 3 mg/mL, both diluted with ethanol. After homogenization, they were transferred to amber glass bottles and stored in the refrigerator for up to 5 days [6].

DPPH method

The solution was prepared using the DPPH reagent (2,2-diphenyl-1-picrylhydrazyl) (Sigma Aldrich) at a concentration of 32 µg/mL (80 µM; MM = 394.32 $g.mol^{-1}$). For this, 1.6 mg of the reagent were dissolved in 50 mL of Ethyl Alcohol P.A. (Êxodo). The solution was homogenized in an ultrasonic bath (GlassLab) for 25 min [7].

Sample testing

Folin-Ciocalteu assay

In a 96-well plate, the extract was added at concentrations of 400, 200, 100, 50, 25, and 12.5 µg/mL. Distilled water, Folin-Ciocalteu solution, and saturated sodium carbonate solution were sequentially added. The plate also included Folin Ciocalteu control, blank (water), sample blank (extract), and positive control (gallic acid). The plate was protected from light and incubated for 20 minutes. After this period, absorbance was measured at λ=630 nm using the UV-vis spectrophotometer (model MB-580-Heales). From the equation (linear regression) generated through calibration with gallic acid, as shown in Figure 1, it was possible to quantify the content of phenolic compounds in the samples based on the reference (gallic acid). The assay was performed in triplicate.

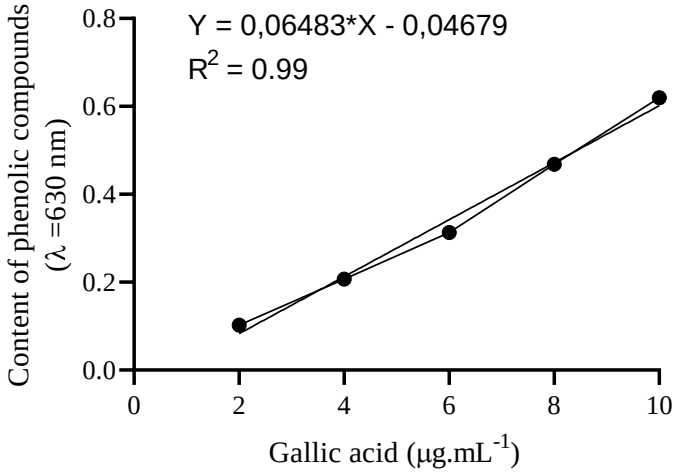

Figure 1. Standard curve of gallic acid at concentrations ranging from 2 to 10 µg/mL versus the respective absorbances read at λ=630 nm.

DPPH assay

In a 96-well plate, the extract was added at concentrations of 400, 200, 100, 50, 25, and 12.5 µg, and then the DPPH solution was added. The plate also included controls for DPPH, blank (ethanol reagent), blank of the analyzed sample (extract), and positive control (gallic acid). The plate was protected from light and incubated for 30 minutes. After this period, the absorbance was read at λ=517 nm using a UV-Vis spectrophotometer (model MB-580-Heales). From the equation (linear regression) generated through the calibration with gallic acid, demonstrated in Figure 2, it was possible to quantify the antioxidant activity of the samples from the reference (gallic acid). The experiment was performed in triplicate.

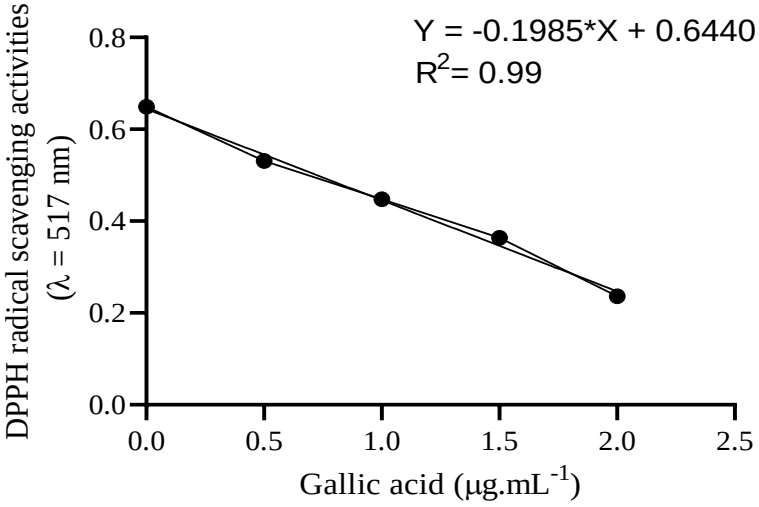

Figure 2. Standard curve of gallic acid at concentrations ranging from 0 to 2 µg/mL versus the respective absorbances read at λ=517 nm.

Cytotoxicity test

For the cytotoxicity test, a fibroblast cell line derived from mouse connective tissue, L929, was cultured in RPMI medium, supplemented with 50 µg/mL gentamicin, and 10% fetal bovine serum (FBS). The cultures were incubated in a CO_2 incubator at 37ºC. For the cytotoxicity assays, in 96-well plates, after fibroblasts adhered to the plate for a period of 24 hours, they were treated with 20 µL of the extracts at serial concentrations (500 – 7.81 µg). Positive control consisted of cells without treatment, negative control consisted of cells treated with Lysis buffer – 100 µL, and the blank consisted of RPMI-1640 medium supplemented with FBS. After 72 hours of treatment, 20 µL of a resazurin solution was added to each well at an initial concentration of 2 mM. After 5 hours of incubation at 37°C, fluorescence was determined using the Synergy HT spectrophotometer (BioTek) with excitation at λ=530/25 and emission at λ=590/35 [13]. The tests were carried out at Fiocruz/RO.

Statistical analysis of the data

Statistical analyses were performed using GraphPad Prism version 8.0. In order to determine the total phenolic content (TPC), the absorbance values obtained for each sample in the test were correlated with the standard curve of gallic acid, and the TPC was expressed in micrograms of gallic acid equivalent (GAE) per gram of extract sample. For the calculation of DPPH reduction, non-linear regressions of the means and values for each concentration were performed, with values observed based on at least two independent experiments and three replicates.

MS/MS data acquisition

The MS/MS analyses were performed using LCQ Fleet (Thermo Fisher Scientific, Waltham, MA, USA) through direct infusion (3 µL/min using integrated syringe pump). The mass spectrometer, equipped with APCI font and ion trap analyzer, was operated in the positive and negative mode. Sheath gas (35 arbitrary units) and auxiliary gas (15 arbitrary units). Mass spectrometry parameters used were a source voltage of 5.0 kV, a capillary voltage of 35 V, a tube lens voltage of 110 V, a capillary temperature of 200 °C, and an APCI vaporizer temperature of 350 °C. Full-scan data acquisition (mass range: m/z 100–1000). The normalized collision energy of the collision-induced dissociation (CID) cell was set at 35 eV. The spectra were processed using the Xcalibur software, version 2.2 SP1.

Molecular Networking Workflow

Data were converted to mzML using ProteoWizard MSConvert [14]. After processing, a molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu) [15]. The data was filtered by removing all MS/MS fragment ions within +/- 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/- 50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da. A network was then created where edges were filtered to have a cosine score above 0.70 and more than 6 matched peaks. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks. The networks were plotted using Cytoscape [16,17].

RESULTS

Determination of Total Phenols

As shown in Figure 3, the leaf extract from *E. amazonicus* R.E.FR exhibited a phenolic compound content of 25.24 ± 0.98 µg.GAE/g at the highest concentration tested (400 µg/mL).

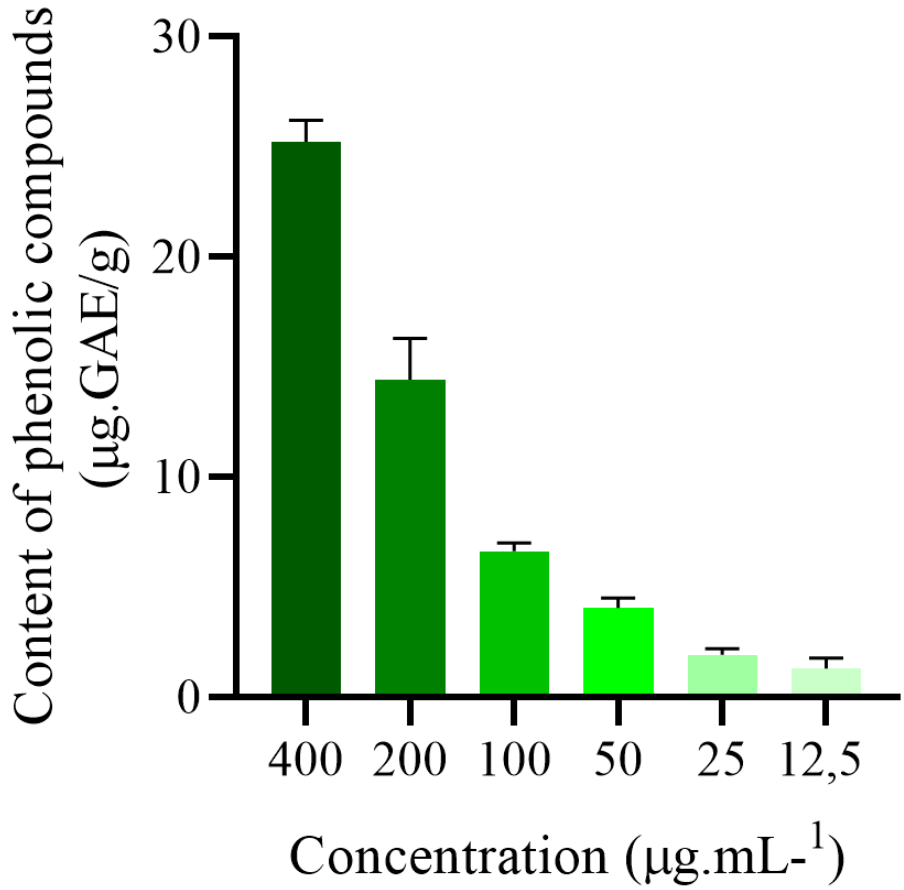

Figure 3. Total phenolic compound content (TPC), expressed in gallic acid equivalents (GAE), for crude extract from leaves of *Ephedranthus amazonicus* R.E.FR. The graph was plotted as a function of the extract concentration (µg.mL^{-1}) and the respective absorbances read at 630nm. Data refer to the mean and standard deviation (±) of two independent experiments.

DPPH radical method

When analyzing the results by the DPPH method, we observe that the extract from *Ephedranthus amazonicus* R.E.FR leaves presented an EC_{50} (half maximal Effective Concentration) - which is the amount of antioxidants necessary to decrease the initial concentration of DPPH - by 50%, of 13.37±2.63 µg. mL^{-1}. Additionally, gallic acid, used as a positive control, was tested at concentrations ranging from 2 µg/mL to 0.5 µg/mL, resulting in an EC_{50} of 0.7327 µg/mL (Figure 4).

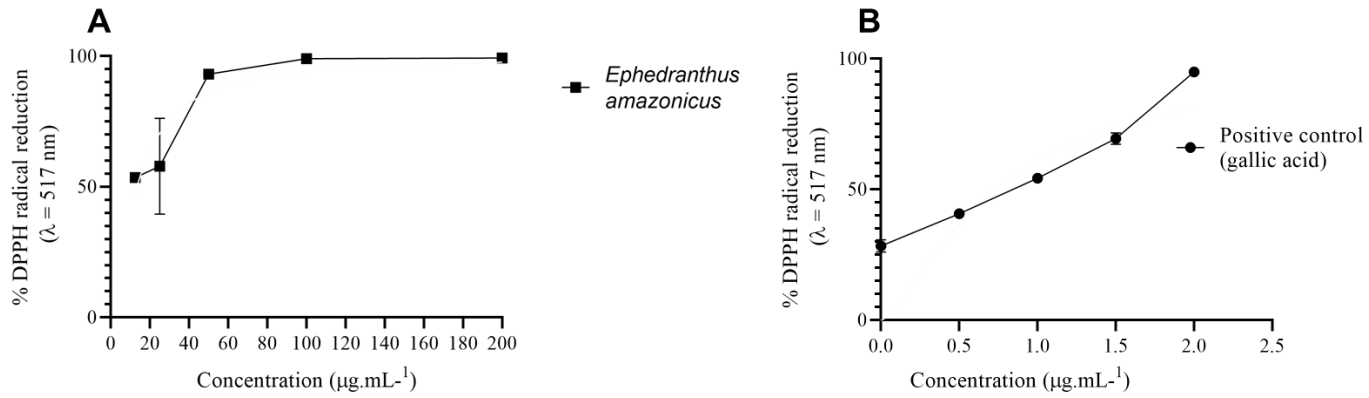

Figure 4. Antioxidant activity of the crude extract from *Ephedranthus amazonicus* R.E.FR. (A) leaves and (B) Gallic acid, evaluated by the DPPH radical scavenging capacity method. The graphs were plotted as a function of extract concentration (µg.mL^{-1}) and respective absorbances read at 517 nm. Data refer to the mean and standard deviation (±) of two independent experiments.

Cytotoxic activity of the extract

In this study, the plant extract showed cytotoxic concentration (CC_{50}) >500 µg.mL^{-1} against the L929 cell line (fibroblastic cell line derived from mouse connective tissue) suggesting low cytotoxicity of the crude extract, as shown in Figure 5. A lysis buffer was used as a positive control for cytotoxicity, resulting in 100% cell mortality.

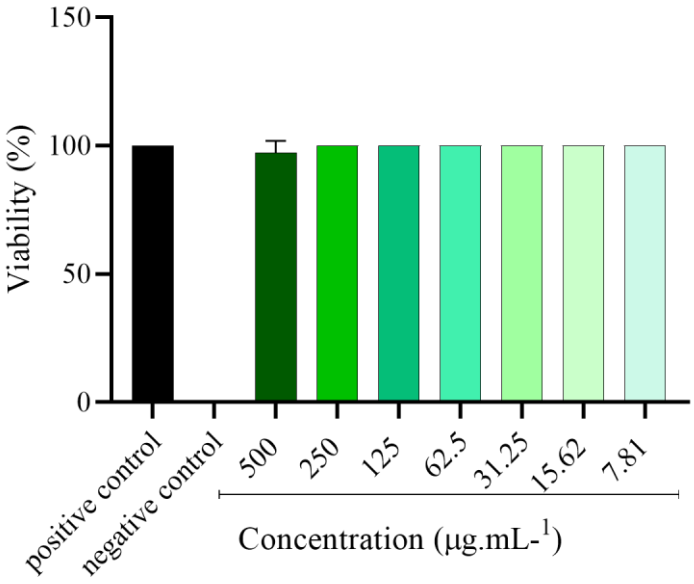

Figure 5. Determination of the cytotoxic effect of *Ephedranthus amazonicus* extract, serial dilution (500 – 7.81 µg), against the L929 murine fibroblasts lineage, after 72 hours of incubation through the resazurin assay. **Notes:** Positive control= cells without treatment; negative control= cells treated with Lysis buffer

Flavonoids annotation by mass spectrometry

The acquired and pre-processed MS/MS datasets were used for flavonoid annotation through MN generated by GNPS. The MN processed data can be accessed here: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=de5ad0f4fbb8484c95c617b1e0e8895b. In the network arbitrarily identified as MN1 (Figure 6), the 3 nodes were annotated with high confident spectral matches (cosine > 0.9) to the flavonoids isovitexin (**1**), luteolin-7-glucoside (**2**), and luteolin 4'-O-glucoside (**3**) (Table 1).

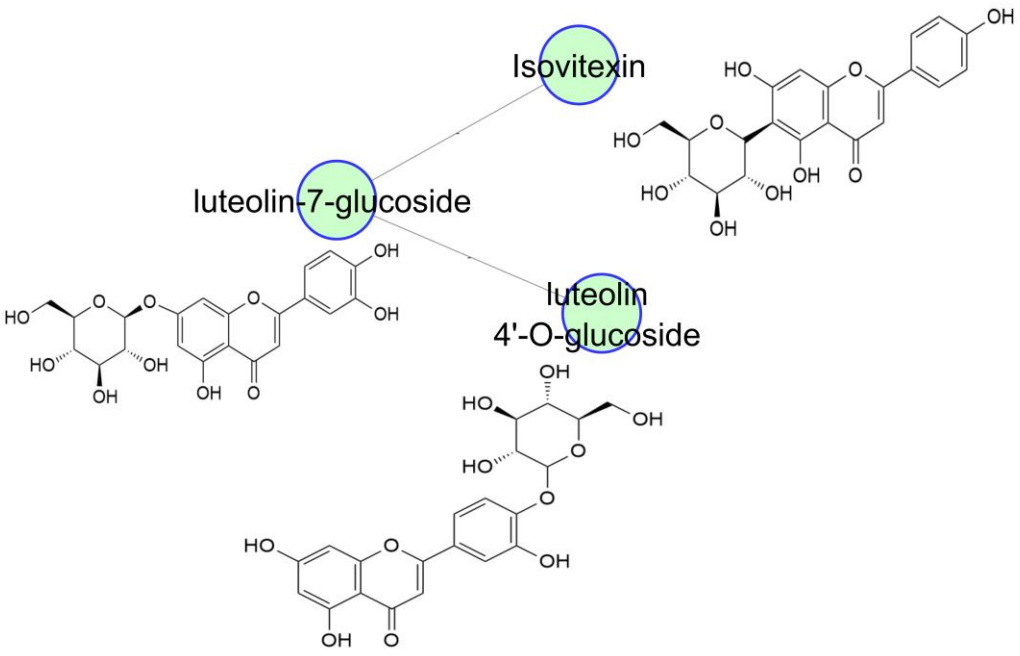

Figure 6. Flavonoids annotated in the GPNS data base.

Table 1. APC^+ MS/MS data from raw extract from *E. amazonicus*.

Compound	RT (min)	Composition	$[M+H]^+$ (*m/z*)	MS/MS - (*m/z*)
1	3.75	$C_{21}H_{20}O_{10}$	433.69388	**433** $[M+H]^+$; **415** $[M+H-H_2O]^+$; **397** $[M+H-2H_2O]^+$; **379** $[M+H-3H_2O]^+$; **313** $[M+H-C_4H_8O_4]^+$; **283**$[M+H-C_4H_8O_4-CH_2O]^+$; **271** $[M+H-C_6H_{10}O_5]^+$
2	4.83	$C_{21}H_{20}O_{11}$	449,69583	**449** $[M+H]^+$; **431** $[M+H-H_2O]^+$; **413** $[M+H-2H_2O]^+$; **395** $[M+H-3H_2O]^+$; **329** $[M+H-C_4H_8O_4]^+$; **299** $[M+H-C_4H_8O_4-CH_2O]^+$; **287** $[M+H-C_6H_{10}O_5]^+$
3	4.11	$C_{21}H_{20}O_{11}$	449,51152	**449** $[M+H]^+$; **431** $[M+H-H_2O]^+$; **413** $[M+H-2H_2O]^+$; **299** $[M+H-C_4H_8O_4-CH_2O]^+$; **287** $[M+H-C_6H_{10}O_5]^+$

DISCUSSION

Polyphenols present in natural extracts sometimes have high antioxidant activity [18]. In the study carried out by [19], the crude extract from the leaves of *Ephedranthus parviflorus* presented 102.67 ± 1.89 mg.EAG/g of total phenols. The difference between the results of total phenols in this study (25.24 µg.EAG/g or 0.02524 mg.EAG/g) and those found by [19] can be explained by the higher concentration of phenols in the extracts. Thus, it is inferred that samples with greater amounts of phenols may have greater antioxidant capacity.

In the study by [20] the ability to eliminate DPPH free radicals increases according to the concentration of phenols. Thus, phenols are considered important constituents, due to the presence of hydroxyl groups and their ability to scavenge free radicals. Therefore, the phenol content in plant extracts directly contributes to their antioxidant action [20, 21, 22].

Considering the content of phenols obtained as satisfactory, a correlation with the presence of phenolic compounds in the sample is suggested, taking into account that compounds that have a hydroxyl group are among the main agents responsible for the antioxidant effects of plants. There are other factors involved in the antioxidant action, such as the chemical structure, composition of active phytochemicals, position, the number of hydroxyls present in the molecules, among others [23].

The study by [12] highlighted the antioxidant potential of methanolic extracts from *Ephedrantus amazonicus* by the $ORAC_{FL}$ method. The activity was associated with the presence of a high concentration of compounds from the alkaloid class. In this sense the alkaloid fraction of the branches of the investigated species showed greater antioxidant capacity. As for isolated compounds, the isomoschatoline alkaloid extracted from leaves and branches of the *Ephedrantus amazonicus* species showed significant antioxidant activity by the $ORAC_{FL}$ method when compared to positive controls [12].

L929 fibroblasts are recommended by ISO 10993-5 for cytotoxicity tests [24]. The study by Alcântara (2015) presents the cytotoxicity of the essential from *Ephedranthus amazonicus* leaves in three human cell lines: ACP02 (gastric adenocarcinoma), MCF-7 (breast adenocarcinoma) and SK-MELL3 (melanoma). The results showed that cell viability decreased with increasing oil concentration, and at lower concentrations, the oil showed moderate cytotoxicity for ACP02 and SK-MELL3, and mild cytotoxicity for the MCF-7 strain. The oil also showed cytotoxic activity at the highest concentration tested (50 $\mu g.mL^{-1}$) against cell lines HCT-116 (colorectal carcinoma) and MRC-5 (human lung fibroblast) [25].

To complement the GNPS platform annotation, the three flavonoids were putatively identified at level 2 [26] by comparison with data from literature [27, 28, 29]. Mass spectra obtained under APCI for putative compounds **1**, **2**, and **3** are in agreement with literature data.

Flavonoids are very common and widespread secondary plant metabolites. The predominant form of naturally occurring flavonoids in plants is that of a flavonoid glycoside. Flavonoids were isolated and identified from several species of Annonaceae native from Brazil [30, 31, 32]. Our findingss represent the first report on the presence of flavonoids isovitexin, luteolin-7-glucoside, and luteolin 4'-O-glucoside in *E. amazonicus*. These results, therefore, provide evidence that these flavonoids may contribute to the antioxidant activity shown to the extract from leaves of *E. amazonicus*.

CONCLUSION

Based on the DPPH reduction mechanism, we infer that the antioxidant capacity of the alcoholic extract from *Ephedrantus amazonicus* R.E. Fries leaves is correlated with the concentration of phenol content. Our findings suggest that flavonoids contribute to the antioxidant activity shown to the extract from

leaves of *E. amazonicus*. The extract was not shown to be cytotoxic at the highest concentration tested, thus, the *in vitro* results suggest that the species can be considered as a potential source of natural antioxidant compounds. As perspectives for this group, isolation and identification of the chemical constituents of the extract will be carried out, as well as studies with other antioxidant models and *in vivo* assays that are essential to further confirm it as a biological antioxidant, in order to explore the bioavailability of phytomolecules for domestic use in the food industries, cosmetics and pharmaceuticals.

Acknowledgments: The authors would like to thank the instituto Federal de Educaçao, Ciência e Tecnologia de Rondônia (IFRO), Centro de Bionegócios da Amazônia (CBA) and the Oswaldo Cruz Foundation-RO for their support in conducting the cytotoxicity assay.
Financial support: Financial support: Instituto Federal de Educação, Ciência e Tecnologia de Rondônia (IFRO) Porto Velho campus – Calama, public notice nº 14/2023 (Support for scientific and literary communication) and public notice nº 09/2022 (Technological Initiation Modality - Cycle 2022 - PROPESP); public note nº 13/2023/REIT - PROPESP/IFRO - Edital de Inovação; public note nº 15/2023/REIT - PROPESP/IFRO-RENOVAÇÃO PROJETOS IC.
Conflict of interest: The authors declare that they have no financial interests or personal relationships that could have influenced the work reported in this article.

REFERENCES

1. Erkens RHJ, Blanpain LMP, Carrascosa JI, Runge K, Verspagen N, Cosiaux A, et al. [Spatial distribution of Annonaceae across biomes and anthromes: *Knowledge gaps* in spatial and ecological data]. Plants People Planet. 2022 Sep;5(4):520-35.
2. Lopes JDC, Mello-Silva R. [Revision and morphological phylogeny of *Ephedranthus*, a Neotropical genus of Annonaceae]. Ann Mo Bot Gard. 2019 Jun;104(2):244-61.
3. Wink M. [Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective]. Phytochemistry. 2003 Sep;64(1):3-19.
4. Díaz-Rivas JO, González-Laredo RF, Chávez-Simental JA, Montoya-Ayón JB, Moreno-Jiménez MR, Gallegos-Infante JA, et al. [Comprehensive characterization of extractable phenolic compounds by UPLC-PDA-ESI-QqQ of *Buddleja scordioides* plants elicited with salicylic acid]. J Chem. 2018 Nov;2018:1-10.
5. Benamar-Aissa B, Gourine N, Ouinten M, Harrat M, Benarfa A, Yousfi M. Synergistic effects of essential oils and phenolic extracts on antioxidant activities responses using two *Artemisia species* (A. campestris and A. herba alba) combined with *Citrus aurantium.* Biocatal Agric Biotechnol. 2023 Jan;47:102570.
6. Pires JS, Torres PB, Santos DYAC, Chow F. [Microplate assay of reducing substances using the Folin-Ciocalteu method for algae extracts]. São Paulo: Instituto de Biociências, Universidade de São Paulo; 2017 Sep [cited 2023 May 12]. 5 p. Available from: https://www.ib.usp.br/comunicacao-m/materiais-didaticos-em-elaboracao/file/9-ensaio-antioxidante-folin.html
7. Pires JS, Torres PB, Santos DYAC, Chow F. [Microplate assay of antioxidant potential using the DPPH free radical scavenging method for algae extracts]. São Paulo: Instituto de Biociências, Universidade de São Paulo; 2017 Sep [cited 2023 May 12]. 6 p. Available from: https://www.ib.usp.br/comunicacao-m/materiais-didaticos-em-elaboracao/file/10-ensaio-antioxidante-dpph.html
8. Sucupira NR, Silva AB, Pereira G, Costa JN. [Methods for determining antioxidant activity of fruits]. J Health Sci. 2012 Jul;14(4):263-9.
9. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic. 1965 Jan;16(3):144-58.
10. Waterman PG, Mole S. Extraction and chemical quantification. In: Waterman PG, Mole S, editors. Analysis of phenolic plant metabolites. Boston: Blackwell Scientific; 1994. p. 66-103.
11. Jacob JAM. [Development of paper microtiter plates] [dissertation]. Lisbon: Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa; 2013. 125 p.
12. Alencar DC. [Alkaloid profile of Annonaceae from the UFAM campus and phytochemical and biological study of *Ephedranthus amazonicus* RE Fries (Annonaceae)] [dissertation]. Manaus: Universidade Federal do Amazonas; 2010. 139 p.
13. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. [Cell viability assays]. In: Markossian S, Grossman A, Arkin M, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004 [updated 2016 Jul; cited 2023 May 12].1-40.
14. Kessner D, Chambers M, Burke R, Agus D, Mallick P. ProteoWizard: open-source software for rapid proteomics tools development. Bioinformatics. 2008 Nov;24(21):2534-6.
15. Wang M, Carver JJ, Phelan VV, Sanchez LM, Garg N, Peng Y, et al. Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. Nat Biotechnol. 2016 Aug;34(8):828-37.
16. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. [Cytoscape: a software environment for integrated models of biomolecular interaction networks]. Genome Res. 2003 Nov;13(11):2498-504.
17. Smoot ME, Ono K, Ruscheinski J, Wang PL, Ideker T. Cytoscape 2.8: new features for data integration and network visualization. Bioinformatics. 2011 Feb;27(3):431-2.

18. Viteri R, Giordano A, Montenegro G, Zacconi F. Eucryphia cordifolia extracts: phytochemical screening, antibacterial and antioxidant activities. Nat Prod Res. 2022 Aug;36(16):4177-81.
19. Silva AACA, Sousa EA, Chaves MH. [Antioxidant activity and total phenols of the ethanolic extract fractions of Ephedranthus parviflorus]. In: Proceedings of the 62ª Reunião Anual da SBPC; 2010 Jul 25-30; Natal, RN. São Paulo: SBPC; 2010.
20. Sultana B, Anwar F. Flavonols (kaempeferol, quercetin, myricetin) contents of selected fruits, vegetables and medicinal plants. Food Chem. 2008;108(3):879-84.
21. Tosun M, Ercisli S, Sengul M, Ozer H, Polat T, Ozturk E. Antioxidant properties and total phenolic content of eight salvia species from turkey. Biol Res. 2009;42(2):175-81.
22. Geremu M, Tola YB, Sualeh A. Extraction and determination of total polyphenols and antioxidant capacity of red coffee (*Coffea arabica* L.) pulp of wet processing plants. Chem Biol Technol Agric. 2016 Sep;3(25):1-6.
23. Lemos AR, Rêgo Júnior NO, São José AR, Pereira MLA, Silva MV. [Antioxidant activity and correlation with total phenolics in annatto (*Bixa orellana* L.) genotypes]. Rev Inst Adolfo Lutz. 2011 Jan;70(1):62-8.
24. International Standards Organization. ISO 10993-5: Biological Evaluation of Medical Devices—Part 5. Tests for Cytotoxicity: In Vitro methods. Geneva (Switzerland): ISO; 1999.
25. Alcântara JM. [Chemical composition and biological potential of Annonaceae essential oils from the INPA and UFAM campuses] [doctoral thesis]. Manaus (AM): Universidade Federal do Amazonas, Instituto de Ciências Exatas; 2015. 132 p.
26. Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, et al. [Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI)]. Metabolomics. 2007 Sep;3(3):211-21. doi: 10.1007/s11306-007-0082-2. PMID: 24039616; PMCID: PMC3772505.
27. Cuyckens F, Claeys M. Mass spectrometry in the structural analysis of flavonoids. J Mass Spectrom. 2004 Jan;39(1):1-15.
28. Ouedraogo M, Hema A, Bazié BSR, Zida SW, Batieno BJT, Kabré E, Nacro M. Identification of major flavonoids using HPLC-MS/MS and determination of antioxidant potential of Vigna radiata seed extracts. J Soc Ouest-Afr Chim. 2021 Jan;50:57-67.
29. March RE, Lewars EG, Stadey CJ, Miao XS, Zhao X, Metcalfe CD. A comparison of flavonoid glycosides by electrospray tandem mass spectrometry. Int J Mass Spectrom. 2006 Jan;248(1-2):61-85.
30. Santos DYACD, Salatino MLF. Foliar flavonoids of Annonaceae from Brazil: taxonomic significance. Phytochemistry. 2000 Nov;55(6):567-73.
31. Brito HO, Noronha EP, França LM, Brito LMO, Prado MSA. [Analysis of the phytochemical composition of the ethanolic extract from the leaves of *Annona squamosa* (ATA)]. Rev Bras Farm. 2008;89(3):180-4.
32. Chokchaisiri R, Chaichompoo W, Chalermglin R, Suksamrarn A. Potent antiplasmodial alkaloids and flavonoids from *Dasymaschalon acuminatum*. Rec Nat Prod. 2015 Jan;9(2):243-6.