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Original Article

Molecular docking studies and evaluation of the antiretroviral activity and cytotoxicity of the species *Lafoensia pacari* Saint-Hilaire

Estudos de docking molecular e avaliação da atividade antirretroviral e citotoxicidade da espécie *Lafoensia pacari* Saint-Hilaire

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Abstract

Interest in antiviral plant species has grown exponentially and some have been reported to have anti-HIV properties. This research aims to perform the bio-guided phytochemical fractionation by antiretroviral activity of *Lafoensia pacari* stem barks. This in vitro experimental study involved the preparation of plant material, obtention of ethanolic extract, fractionation, purification, identification and quantification of fractions, acid-base extraction, nuclear magnetic resonance, HIV-1 RT inhibition test and molecular docking studies. From the bio-guided fractionation by the antiretroviral activity there was a higher activity in the acetanolic subfractions, highlighting the acetate subfraction – neutrals with 60.98% of RT inhibition and ellagic acid with 88.61% of RT inhibition and absence of cytotoxicity. The macrophage lineage cytotoxicity assay showed that the chloroform fraction was more toxic than the acetate fraction. The analysis of the J-resolved spectrum in the aromatic region showed a singlet at 7.48 and 6.93 ppm which was identified as ellagic acid and gallic acid, respectively. The 5TIQ enzyme obtained better affinity parameter with the ellagic acid ligand, which was confirmed by the HSQC-^{1}H-^{13}C spectra. Gallic acid was also favorable to form interaction with the 5TIQ enzyme, being confirmed through the HSQC-^{1}H-^{13}C spectrum. From the PreADMET evaluation it was found that ellagic acid is a promising molecule for its RT inhibition activity and pharmacokinetic and toxicity parameters.

Keywords: antiviral, HIV, molecular docking, macrophages.

Resumo

O interesse por espécies vegetais com ação antiviral tem crescido exponencialmente e algumas tem sido relatadas como possuídoras de propriedades anti-HIV. Essa pesquisa tem como objetivo realizar o fracionamento fitoquímico biodirecionado pela atividade antirretroviral das cascas do caule da espécie *Lafoensia pacari*. Trata-se de um estudo experimental *in vitro* e a metodologia envolve preparo do material vegetal, obtenção do extrato etanólico, fracionamento, purificação, identificação e quantificação das frações, extração ácido-base, ressonância magnética nuclear, teste de inibição da TR do HIV-1 e estudos de docking molecular. A partir do fracionamento biodirecionado pela atividade antirretroviral verificou-se uma maior atividade nas subfrações acetanólica. Com destaque para a subfração acetanólica neutros com 60,98% de inibição de TR e o ácido elágico com 88,61% de inibição de TR e ausência de citotoxicidade. Verificou-se com o teste de citotoxicidade em linhagem de macrófagos que a fração clorofórmica foi mais tóxica que a fração acetanólica. A análise do espectro J-resolvido na região aromática apresentou um simpleto em 7.48 e 6.93 ppm que foram identificados como ácido elágico e ácido gálico respectivamente. A enzima

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5TIQ obteve melhor parâmetro de afinidade com o ligante ácido elágico que foi confirmado pelos espectros HSQC-^{1}H-^{13}C. O ácido gálico mostrou-se também favorável a formar interação com a enzima 5TIQ, sendo confirmado através do espectro HSQC-^{1}H-^{13}C. Através da avaliação do PreADMET verificou-se que o ácido elágico é uma molécula promissora pela sua atividade de inibição da TR e parâmetros farmacocinéticos e de toxicidade.

Palavras-chave: antiviral, HIV, docking molecular, macrófagos.

1. Introduction

The Human Immunodeficiency Virus (HIV) belongs to the Retroviridae family and to the *Lentivirus* genus. This virus is responsible for the pathological basis of the Acquired Immunodeficiency Syndrome (AIDS) (Ferreira et al., 2010), which epidemy is one of the major challenges in public health and was accounted for devasting consequences for families, communities, and countries over the past 30 years (Martins et al., 2014).

Numbers provided show that in 2020, 27.4 million of the 37.6 million people living with HIV were on treatment, with only 7.8 million in 2010. The implementation of affordable, quality treatment is estimated to have prevented 16.2 million aids-related deaths since 2001. Deaths have declined largely due to antiretroviral therapy. AIDS-related deaths have declined by 43% since 2010, reaching 690,000 in 2020 (Unaids, 2018).

Brazil was the first developing country to initiate a global distribution of pharmaceuticals via public health policies and, since 1996, offers a law-guaranteed access to antiretroviral drugs for all HIV-infected individuals (Silva, 2015).

However, despite of this support, therapeutic failure still occurs mainly due to persistence and mutagenicity of HIV. The high rate of reverse transcriptase (RT) mutation promotes the emergence of new inhibitors-resistant stirpes of HIV, including efavirenz, a non-nucleoside reverse transcriptase inhibitor (NNRTI) (Yang et al., 2001; Wang et al., 2007; Azevedo, 2013).

The use of efavirenz is the initial regimen component for patients with satisfactory results and good tolerance to this drug, but its use is related to neuropsychiatric reactions. It is also preferably indicated as an initial regimen for women of childbearing potential. Efavirenz has been gradually replaced by dolutegravir, the most recent antiretroviral incorporated into the SUS. This switch is recommended, because of the greater viral suppression, less likelihood of resistance, greater immune system recovery, once-daily dosing, and few adverse reactions attributed to dolutegravir (Gerage et al., 2020).

Such aspects have been promoting and justifying the arousing interest by several research groups that aim to identify and develop new substances to replace or to combine with antivirals, from researches and isolated plant molecules.

The investigation regarding anti-HIV activity of plant extracts and isolated plant molecules has been performed using compounds that interfere in several parts of the viral replication cycle, which, in this context, the reverse RT inhibitors stand out. Phytochemical studies were performed on plant species with proved anti-RT property aiming to identify substances involved on this action and to shed some light on the inhibitory mechanism (Ferreira, 2010).

Lafoensia pacari A. St.-Hil. is a species of the Lythraceae family, which comprises 22 widely distributed genus on tropical and subtropical regions, though are also present at moderate climate zones. It usually lives in humid areas and fluvial coasts and the *Lafoensia* sp. is restricted to the South America continent. This family is represented by a mean of 500 species, among which the *L. pacari* stands out with distribution on Brazil and Paraguay (Cabral and Pasa, 2009).

On folk medicine, this species is used as antipyretic, healing agent, tonic, analgesic, antiulcerogenic, antidiarrheal, and on cancer treatment (Solon et al., 2000; Mundo and Duarte, 2007).

Studies carried out with extracts obtained from the leaves and bark of *L. pacari* demonstrated antimicrobial, antinociceptive, antidepressant, antidyspeptic, antifungal, free radical scavenger and anti-inflammatory activities (Lima et al., 2006; Galdino et al., 2009; Guimarães et al., 2010; Solon et al., 2000; Galdino, 2015).

In the last years, the interest in antiviral plant species has grown exponentially and some have been reported to have anti-HIV properties. Thus, the aim of the present study was to assess the antiretroviral activity of *L. pacari* species as well as to identify its chemical components and to perform molecular docking studies.

2. Materials and Methods

2.1. Collection of botanic material and obtention of crude ethanolic extract

The collection of stem barks of *Lafoensia pacari* was performed in 2007 in the state of Goiás, Brazil, and was identified with the registration number 3535 in the herbarium of the University of Brasília (UnB).

The ethanolic extract of stem barks of *L. pacari* was prepared by percolation. This procedure was repeated until exhaustive extraction of plant material. The ethanolic extract from the stem bark of *L. pacari* was previously prepared by another researcher and stored in a glass container. The obtained liquid sample was concentrated in a rotatory evaporator under the temperature between 35 to 45°C, with reduced pressure until a crude ethanolic extract (CEE) was obtained (Sonaglio et al., 2004).

2.2. Methods of fractionation and purification

The liquid-liquid extraction was performed to separate compounds. The CEE of stem barks of *L. pacari* was solubilized in water and methanol at 99.9% (MeOH:H_2O 8:2) and fractioned using the liquid-liquid extraction method (LLE), using the following solvents in ascending order of

polarity: chloroform ($CHCl_3$), ethyl acetate (AcOET), and buthanol (BuTOH).

Initially, a solution of MeOH:H_2O was added to the CEE in the proportion of 8:2 and then $CHCl_3$. After the separation of the chloroform fraction, AcOET was added to the hydromethanolic fraction from the first separation. After the separation of the acetanolic fraction, BuTOH was added to the resulting hydromethanolic fraction. All fractions were concentrated in a rotary evaporator and subjected to reverse transcriptase assay.

2.3. Reverse transcriptase inhibition assay (RT)

In this study, a quantitative immunoenzymatic colorimetric method was used to determine RT inhibition activity according to the manufacturer's protocol (Reverse Transcriptase Assay, Roche, Germany), as previously described by Ferreira et al. (2010). In the inhibition test of RT of HIV-1, RT is required for proviral DNA synthesis and uses viral genomic RNA as a template for this synthesis. Recombinant RT of HIV-1 was used diluted in a 2 ng/well lysis buffer and with an incubation period of 2 hours. The following were added in a 96-well plate: 40µL of nucleotide solution and the template/primer; 40µL of lysis buffer with 4ng of recombinant RT of HIV-1; and 40µL of lysis buffer with the tested extracts.

This plaque was incubated for 2 hours at 37 °C, in order to the RT using the template/primer could produce proviral DNA, incorporating digoxigenin and biotin-labeled nucleotides. After incubation, 60 µL of this mixture was transferred to each well of a streptavidin-treated plate, incubating for 1h at 37 °C so that biotin could bind the synthesized proviral DNA to the surface of the streptavidin-coated microplate. Then, 128 well was washed fivefold with 250 µL of washing buffer and 200 µL/well of the peroxidase-conjugated antidigoxigenin antibody solution (anti-DIG-POD) were added at a 200 mU/mL concentration. The plaque was incubated for 1 hour at 37 °C in order to the anti-DIG-POD could bind to digoxigenin-labeled DNA.

Then, the solution was removed from wells and these were washed fivefold with 250 µL of washing buffer. In the final step, 200 µL/well of the peroxidase substrate ABTS were added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colorimetric reaction product. The intensity of staining of the samples was determined using a 490nm wavelength microplate reader. The intensity of the staining, and consequently the absorbance, is directly correlated to the RT activity level in the sample.

2.4. Acid-base extraction

The bioactive fractions were separately subjected to the acid-base extraction (ABE) process using solutions of 0.1 M of hydrochloric acid (HCl) and 0.1 M of sodium hydroxide (NaOH) (Conegero et al., 2003).

The $CHCl_3$ fraction was solubilized in $CHCl_3$ and added to the aqueous solution of 0.1 M of NaOH, which allowed to obtain the acid salts fraction (aqueous fraction) and the basic and neutral compounds fraction (chloroform fraction). To the acid salt fraction was added 0.1 M of HCl to pH 1.0, thus restoring the acid character of the compounds. These compounds were extracted with AcOET and dried over anhydrous sodium sulfate (Na_2SO_4), resulting in the acid compound and acid emulsion fractions.

The $CHCl_3$ fractions neutrals and acids were treated with 0.1M of HCl, which allowed to extract their basic salts (aqueous fraction). To these, NaOH to pH 10 and a solution of a solution of $CHCl_3$ were added. In the organic fraction, the basic compounds were obtained after being washed with water and dried with Na_2SO_4. The fraction water – neutrals was washed with water and dried with Na_2SO_4 to originate the neutral compounds. All subfractions were concentrated in a rotatory evaporator.

The AcOET fraction was solubilized with MeOH and 0.1 M of NaOH was added, thus forming acid salts (aqueous fractions) that were treated posteriorly with 0.1 M of HCl, restoring its acid character, and AcOET, obtaining its acidic compounds (aqueous fraction), acidic compounds insoluble in AcOET and H_2O, and AcOET fraction, which was washed in water and dried with Na_2SO_4, thus originating the acidic compounds (AcOET fraction) and washing water – acidic compounds (aqueous fraction).

The AcOET fractions – basics and neutrals were treated with 0.1M of HCl, obtaining the neutral compounds (AcOET fraction) and basic salts (aqueous fraction), which were treated with 0.1 M of NaOH, thus restoring the basic character of its compounds, and AcOET, originating its basic compounds (AcOET fraction) and washing water – basic compounds (aqueous fraction). All obtained subfractions were concentrated in a rotatory evaporator.

2.5. Nuclear magnetic resonance analysis

Nuclear magnetic resonance (NMR) spectra were analyzed at 20°C on a Bruker AVANCE spectrometer operating at 400 MHz at the hydrogen frequency. MeOD-d4 and DMSO-d6 were used for magnetic field switching (lock). The signal of the solvent was used as a reference for calibration of the spectra.

2.6. Determination of cell viability

Macrophages of the lineage J774.A1 were grown in triplicates in 96-well plates at a concentration of 1 x 10^5 cells/well and incubated in a 37ºC greenhouse with a humid atmosphere containing 7% CO_2 overnight, for macrophage adhesion to the plaque. After this period, the supernatant medium was removed to remove non-adherent cells, and different concentrations of the substances (1, 10, 100 µg) were added to the wells and kept in a CO_2 greenhouse for 48 hours (Mosmann, 1983).

Then, 100 µL of MTT (3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide), 5 mg/10mL, was added to the wells and kept for 1 hour in a CO_2 greenhouse. After this period, the supernatant was discarded and 100 µL of DMSO was added to the wells and then read on a 550 nm ELISA reader. Cells of control wells were grown with culture medium, only, or in the presence of sample diluent (DMSO, Sigma). Cell viability of the substance-treated cultures was compared to the death standard obtained in the control cultures (Mosmann, 1983).

2.7. Molecular docking

The 3D structures of the Major Capsid protein of PBCV-1 was obtained from the Protein Data Bank (PDB) with the code (5TIQ). The 3D structures of gallic acid (GA), ellagic acid (EA), and punicalagin (PU) were obtained from PUBCHEM with the codes 370, 5281855, and 16129869, respectively.

The Autodock Tools (ADT) version 4.2 package was used for all Molecular Docking Simulations (Goodsell et al., 1996; Goodsell, 2005; Morris et al., 2008). Proteins and binders were prepared for docking simulations using AutoDock Tools (ADT) version 1.5.6 (Sanner, 1999). The receiver was considered rigid while each binder was considered flexible. Gasteiger partial loads (Gasteiger and Marsili, 1980) were calculated after the addition of all hydrogens. The nonpolar hydrogen atoms of proteins and ligands were subsequently merged. A 60 x 60 x 60 points cubic box was generated with 0,35 Å of spacing between grid points for all protein target. The Lamarckian Genetic Algorithm (LGA) (Morris et al., 1998) and the local search (LS) pseudo-Solis and Wets (Solis and Wets, 1981) were applied on the docking research. Each ligand was subjected to 100 independent runs of docking simulations (Ramos et al., 2012). The coordinates of the selected complexes for a more detailed analysis were chosen using the least energy cluster fit conformation criterion combined with visual inspection.

The obtaining of the physicochemical and pharmacokinetic properties of the gallic GA, EA and PU was determined by querying online databases from the initial molecular structures (2D, 3D or SMILES) submitted to digital platforms: FAF-Drugs4 (Lagorce et al., 2017), SwissADME (Daiana et al., 2017), PreADMET (Lee et al., 2003; Molinspiration Cheminformatics, 2020; Nisha et al., 2016; Krishnan et al., 2017; Voet and Voet, 2013) and PASS Oline (Filimonov et al., 2014). Some of the parameters analysed were: Human Intestinal Absorption (HIA), Penetration in the Blood–brain Barrier (BBB), Lipinski's rule, permeability in Caco2 and MDCK cells (Madin-Darby canine kidney), aqueous solubility, the mutagenicity of species Ames Salmonella (TA100 and TA1535), the carcinogenicity, mutagenicity and bioactivity assays (Kramer et al., 2018)

PreADMET was performed, which corresponds to a web-based application to predict ADMET data and create a drug-like library using the *in silico* method. Through ADMET it is possible to predict analyzes such as absorption, distribution, metabolism, excretion, and toxicity, which constitute the pharmacokinetics of a drug candidate molecule. There are currently a considerable number of online and offline computational tools that help predict and analyze ligand ADMET profiles based on their structure and the interaction between ligand and receptor (Atkins and Paula, 2006). Prediction data and drug descriptors such as mutagenicity, toxicological dosage in different tissues and pharmacologically relevant properties of the tested compound can be predicted using the PreADMET server (Nisha et al., 2016; Krishnan et al., 2017; Voet and Voet, 2013).

2.8. Statistical analysis

Multivariate analysis was performed in which the optimized ^{1}H-NMR spectra were reduced (δ 0.3-10.0 ppm) and divided into regions of width (bucket = 0.0005 ppm), standardized and aligned using the software MATLAB version 2015a. The region of δ 4.7-5.0 ppm was excluded from analysis because of the residual methanol signal. The principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using the SIMCA-P software (version 14.0, Umetrics, Umeå, Sweden) with single-variance scaling (SV).

Data obtained from the determination of cytotoxicity of plant samples of the species *L. pacari* in macrophages were reported as mean ± standard error of mean (M ± S.D.M.) after statistical analysis using the One-Way ANOVA test followed by the Dunnett post-hoc test, where differences between means were considered significant when $p^* < 0.05$, $p^{**} < 0.01$ and $p^{***} < 0.001$ compared to the control group.

3. Results and Discussion

3.1. Bio-guided fractionation by RT inhibition activity

All fractions obtained after LLE/ABE underwent RT assay. Possibly, the high polarity and complexity of chemical compounds of this species made atypical subfractions emerge in this acid-base extraction process. These subfractions were coded as water/acid fraction, water – acidic compounds, washing water – acidic compounds, and washing water – basic compounds.

In a study by Firmo et al. (2015) on genus *Lafoensia*, the acid compounds were identified in a higher quantity, followed by triterpenes, saponins, flavonoids and acetophenones, ensuring that acid compounds represent the biological activity factor of the species (Firmo et al., 2015).

A higher activity of acetanolic subfractions was verified using the bio-guided fractionation by antiretroviral activity. The acetanolic subfraction washing water - acids obtained 88.82% of RT inhibition. The acetanolic subfraction water - acids presented 87.63% of RT inhibition. The acetanolic subfraction neutrals showed 60.98% of RT inhibition, followed by chloroform subfraction - neutrals with 41.46% and chloroform subfraction - acids with 37.6% of RT inhibition. Comparing to Efavirenz, a RT inhibition of 88.61% and 0% were verified for ellagic acid and for gallic acid, respectively (Table 1).

Similar to our results, effects of ellagic acid on HIV-1 IN activity have previously been observed (Sanna et al., 2021; Siwe-Noundou et al., 2019). Ellagic acid was also found to be able to suppress the replication of X4-tropic HIV-1 in the target cells without cytotoxicity (Promsong et al., 2018). This compound was also reported to have the ability to inhibit HIV-1 protease expression (Modi et al., 2013). Differently from our results, Modi et al. (2013) reported the inability of ellagic acid to inhibit HIV RT. However, they assayed the ability of ellagic acid to inhibit RT DNA polymerase activity, not RNase H activity. This is a possible reason for the discrepancy with respect to our data on HIV RT inhibitory activity.

Experiments of nuclear magnetic resonance were conducted on samples that underwent the RT% test. The profile of ^{1}H-NMR spectra of subfractions obtained from

Table 1. Antiretroviral activities of extract, fractions, and subfractions of *L. pacari.*

Type of extraction	Fractions	Inhibition activity (RT)%
CEE	----------	7.15
Fraction of LLE	Fraction $CHCl_3$	53.05
Fractions of ABE	$CHCl_3$ SubF. (neutrals)	41.46
	$CHCl_3$ SubF. (water/acids)	30
	$CHCl_3$ SubF. (acids)	37.6
	$CHCl_3$ SubF. (emulsion acids)	29.22
	$CHCl_3$ SubF. (bases)	22.55
Fraction of LLE	AcOEt fraction insoluble	69.14
Fraction of LLE	AcOEt fraction	74.82
Fractions of ABE	AcOET SubF. (neutrals)	60.98
	AcOET SubF. (H2O acids)	87.65
	AcOET SubF. (washing water - bases)	25.69
	AcOET SubF. (washing water - acids)	88.82
	AcOET SubF. (acids)	87.65
Fraction of LLE	Aqueous fraction	72.55
Patterns		
Ellagic acid	----------	88.61
Gallic acid	----------	0
Efavirenz (control)	----------	100

CEE: crude ethanolic extract; LLE: liquid-liquid extraction; ABE: acid-base extraction; $CHCl_3$: chloroform; AcOEt: acetate; SubF: subfraction.

the ethanolic extract of *L. pacari* classified by the activity (RT%) of each subfraction showed a considerable complexity in its compositions, since it regards a mix of compounds with different polarities. For most subfractions is possible to observe characteristic signals of aromatic compounds (6 to 9 ppm), sugars (3 to 5.5 ppm), and aliphatic compounds (0.5 to 2 ppm) (Figure 1).

The principal component analysis was performed and showed a total variance of principal components 1 and of 83.11% of hydrogen NMR data of each subfraction, aiming to identify differences between subfractions and thus identify the compounds through signals indicated on PCA, where each point refers to a spectrum of hydrogen resonance (Figure 2). The PCA showed that it was not possible to separate the more active subfractions and that they are randomly organized due to the variance of metabolite profiles detected on similar subfractions. The more effective separation of more active subfractions did not occur. Only subfractions 41.46%, 25.69%, and 29.22% were separated by principal component 1 (PC1).

Aiming to separate the more and the less active fractions, a PCA was performed containing 6 subfractions, with 3 of them with a higher percentage of activity (88.82, 87.65 and 87.65%) and 3 of them with percentage of activity (37.6, 22.55 and 60.98%). The PCA explained 92.8% of total variance of data through components 1 and 2, and, as presented on Figure 3A, the more active fractions were separated from the less active fractions through PC1 (71.5%). The analysis of loads of PC1 was performed to identify the compounds that characterize the class of more active fractions that were separated on PCA (Figure 3B). Figure 3B indicates the peaks of discriminants for each class; the more active fractions were characterized by the high concentration of aromatic compounds (6 to 9 ppm) and sugars (3 to 5 ppm, positive peak amplitudes), whereas the less active fractions feature a high concentration of aliphatic compounds (0.8 to 2.5 ppm), probably triterpenes (negative peak amplitudes). Signals with positive amplitude refer to subfractions 87.65%, 88.82% and 87.65%, and signals with negative amplitude refer to subfractions 22.55%, 37.60% and 60.98%.

With the aim to identify the descriminating compounds of the more active subfractions presented on PC1 loadings, the J-resolved experiments ^{1}H-^{1}H, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC of the acetanolic subfraction (washing water – acids) 88.82%, the more active subfraction, were performed. The analysis of the ^{1}H-NMR spectrum of the acetanolic subfraction (washing water – acids – 88.82%) presented characteristic signals of aliphatic compunds, sugars, and aromatic compounds.

Since PCA demonstrated a high concentration of aromatic compounds and sugars, we decided to focus on the identification of methabolits on the aromatic region, since the region of sugars features a high complexity for presenting overlapping signals. The analysis of the J-resolved spectrum in the aromatic region between 6 and 8 ppm showed a large number of singlets (Figure 4); singlets at 7.48 and 6.93 ppm were identidied as ellagic acid and gallic acid, respectively (Figure 4).

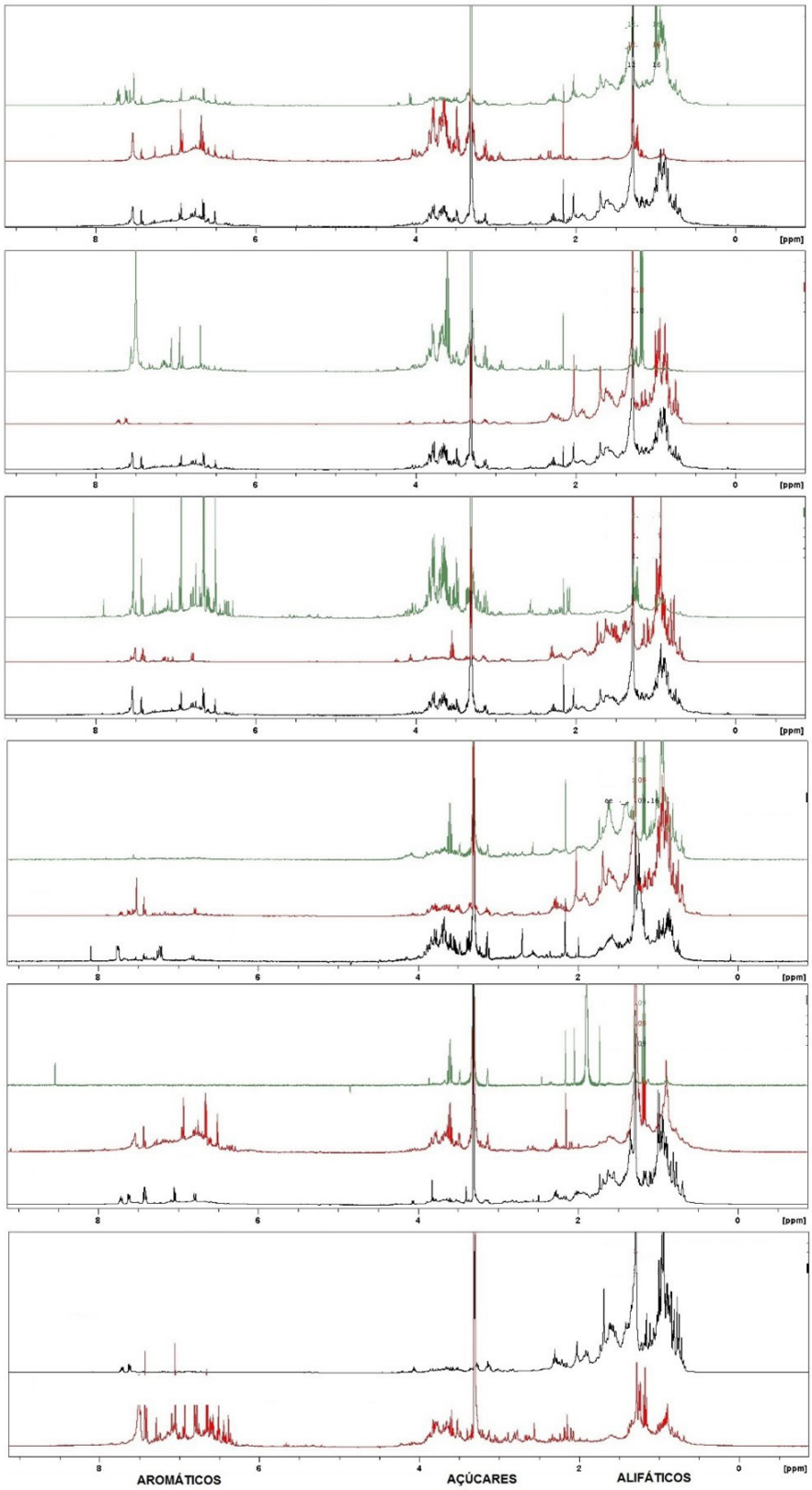

Figure 1. Profile of 1H-NMR spectra of subfractions obtained from the ethanolic extract of L. pacari classified by (RT%) activity of each subfraction.

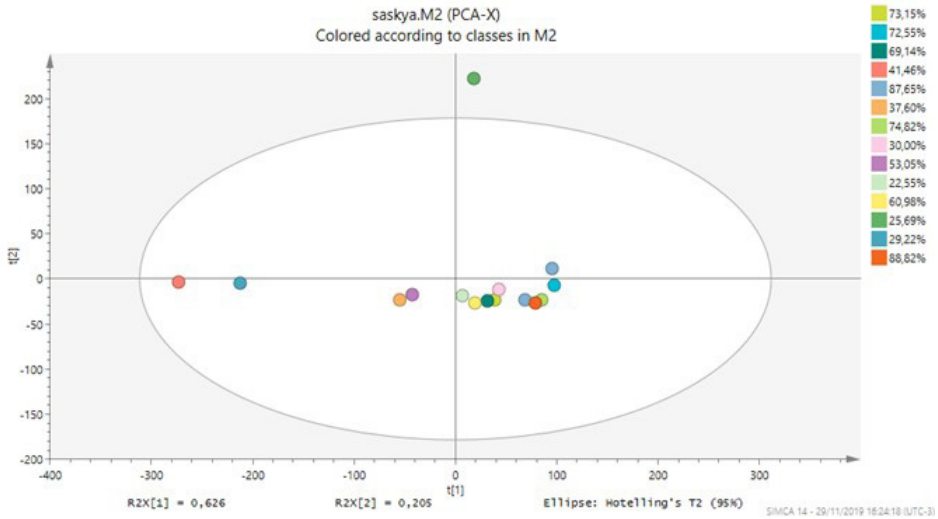

Figure 2. Scores of PCA of obtained subfractions from the ethanolic extract of L. pacari classified by the (RT%) activity of each subfraction.

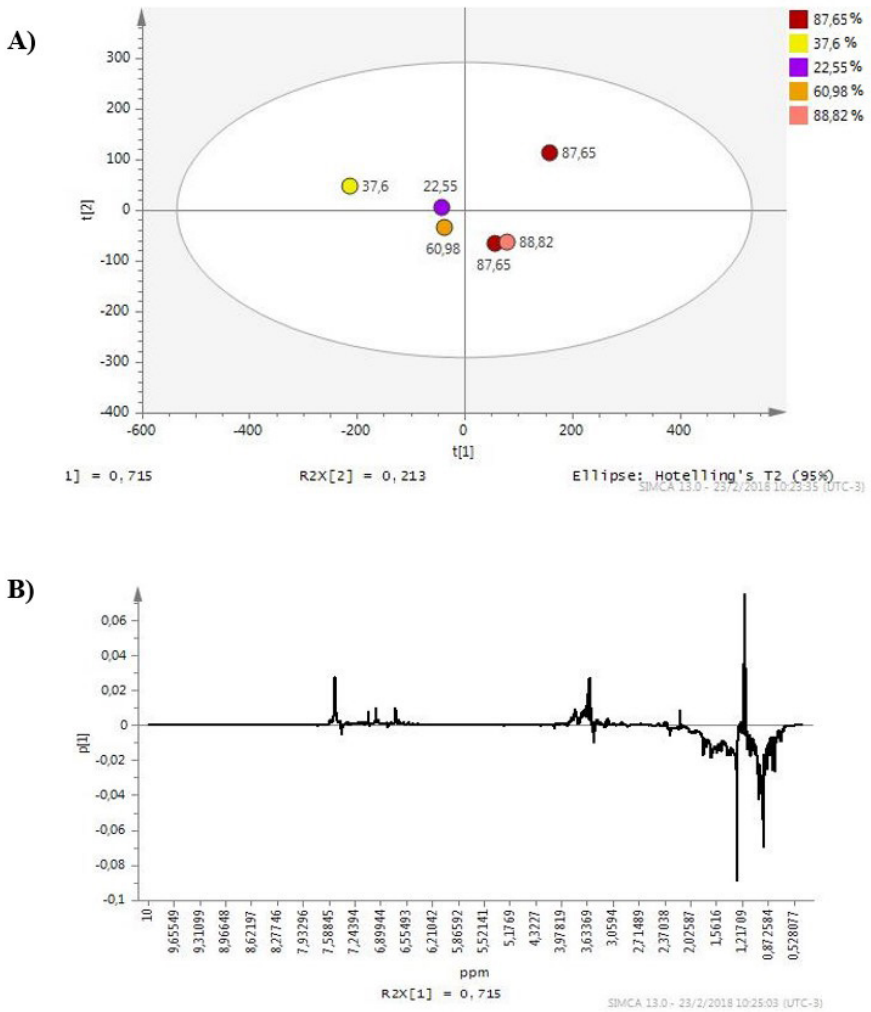

Figure 3. (A) Scores of PCA (71.5% PC1 and 21.3% PC2) of 6 subfractions obtained from the ethanolic extract of L. pacari classified by (RT%) activity of each subfraction. (B) Loadings from PC1 (71.5%) with NMR signals discriminating between subfractions.

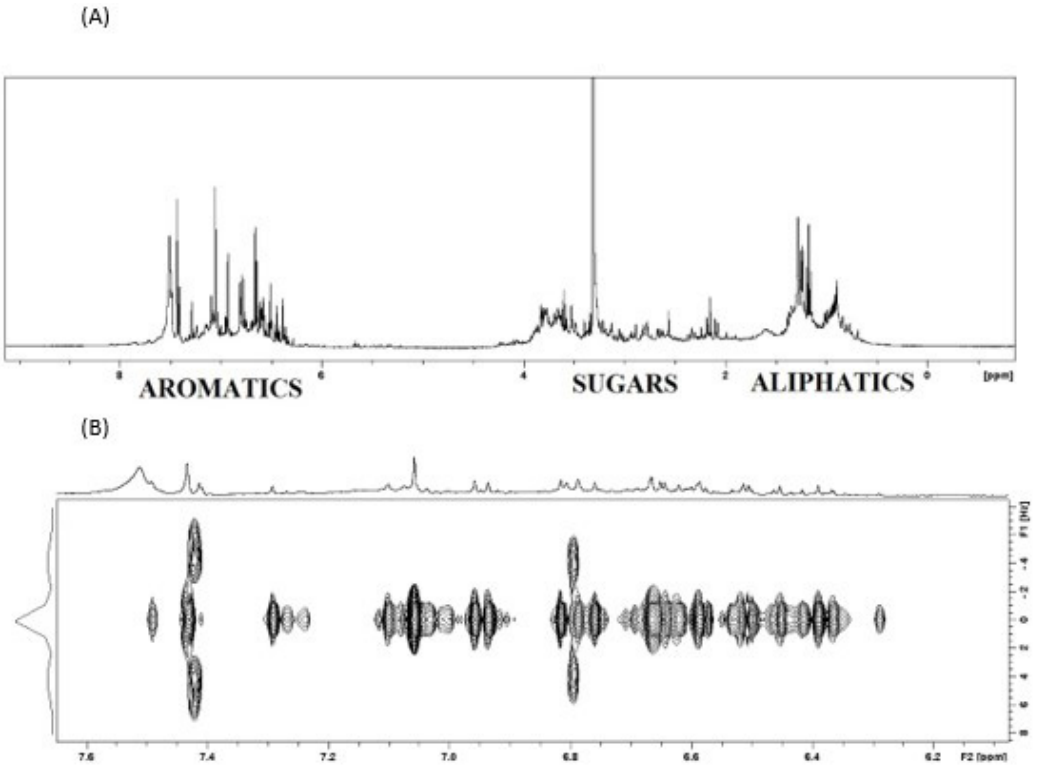

Figure 4. Spectra of 1H-NMR (A) and J-resolved 1H-1H (B) expanded on the region of aromatic signals of the acetanolic subfraction - washing water - acids (88.82%) of L. pacari obtained in methanol-d4.

Ellagic acid was confirmed through the spectra ^{1}H-^{13}C HSQC, which resulted on the coupling of H-5 (7.48 ppm) with C-5 at 110.63 ppm, and ^{1}H-^{13}C HMBC which allowed to detect the long-range correlations of H-5 with carbons C-1 at 112.4 ppm, C-2 at 136.8 ppm, C-3 at 139.2 ppm, C-4 at 148.2 ppm, C-6 at 107.6 ppm, and C-7 at 159.4 ppm (Table 1).

Gallic acid was confirmed through the spectrum ^{1}H-^{13}C HSQC which showed a direct correlation between H-2 and H-6 at 6.93 ppm and the C-2 and C-6 at 109.1 ppm (Figure 5); on the spectrum ^{1}H-^{13}C HMBC, was possible to observe the correlations of H-2 with the carbons C-1 at 121.01 ppm, C-7 at 167.8 ppm, C-3 at 145.2 ppm, and C-4 at 138.3 ppm.

When the aromatic region from 6.40 to 6.85 was analyzed, a considerable number of singlets were observed on spectrum ^{1}H-^{13}C HMBC of the acetate subfraction washing water 0000- acids (88.83%) with a coupling pattern suggesting elagitanine structure. Hydrogen couplings were identified at 6.40 ppm, 6.52 ppm, 6.58 ppm, 6.66 ppm, and 6.77 ppm with the respected carbons at 168.5 ppm, 144.1 ppm, 135.0 ppm, and 114.2 ppm, which allowed to especulate the presence of α and β punicalagin (Figure 6) (Doig et al., 1990). However, the hydrogen couplings of the sugar unit with the punicalagin carbonyls were not observed on the spectrum HMBC; nevertheless, one must highlight that these molecules were already identified on *L. pacari* (Carneiro, 2016).

Researches regarding the chemical compounds of *L. pacari* demonstrated the presence of phenolic compounds. Among the phenolic compounds found that belong to the group of tannins are the polyphenols, which can be divided into two classes: elagitanines and galotanines (Sampaio, 2010; Costa, 2008).

Phenolic compounds found in this species include benzoic acid derivatives such as gallic acid and its dimerization product (hexahydroxy diphenic acid) and derivatives, especially ellagic acid, mainly in the form of hydrolysable tannins, as well as polyphenols derived from the acetate-polymalate pathway, mainly flavonoids and their heterosides as well as condensed tannins (Sampaio, 2010).

Punicalagin is the main elagitanine constituent identified in the *L. pacari* species (Carneiro, 2016). However, the active principles responsible for most of the biological effects of the species have not yet been fully elucidated.

Samples in sufficient amounts underwent cytotoxicity assays of plant samples of *L. pacari* (1, 10, 100 µg/mL) in macrophages J774.A1 (MTT assay) (Table 2).

The MTT method assesses the metabolic activity of cells, quantifying the metabolic reduction of MTT (3-[4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide) performed by mitochondrial enzyme succinate dehydrogenase associated with NADPH and NADH, which results in the production of formazan crystals (purplish and water-insoluble staining), thus allowing to evaluate the

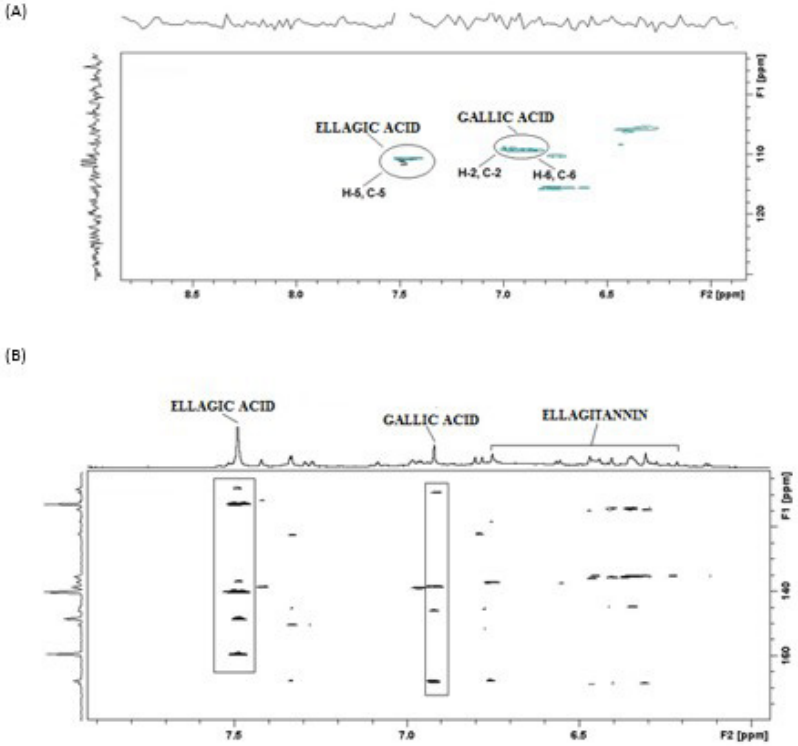

Figure 5. Spectrum region HSQC 1H-13C (A) and HMBC 1H-13C (B) with region of aromatics compounds of the acid wash water acetate subfraction (88.83%) of L. pacari obtained in methanol-d4.

Table 2. Long-range correlations ^{1}H and ^{13}C (HMBC) regarding ellagic acid, gallic acid, and elagitanine obtained in methanol-d4.

Compound	^{1}H	^{13}C
Ellagic Acid	7.48 (H-5-5')	112.4 (C-1-1'), 13.8 (C-2-2'), 139.2 (C-3-3'), 148.2 (C-4-4'), 107.6 (C-6-6'), 159.4 (C-7-7')
Gallic Acid	6.93 (H-2)	121.0 (C-1), 167.8 (C-7), 145.2 (C-3), 138.3 (C-4)
Elagitanine	6.40	114.2, 135.0, 144.1, 168.5
	6.52	114.2 (C-14), 135.0 (C-16), 144.1 (C-15), 168.5 (C-20)
	6.58	114.2 (C-14), 135.0 (C-16), 144.1 (C-15), 168.5 (C-20)
	6.66	114.2 (C-14), 135.0 (C-16), 144.1 (C-15), 168.5 (C-20)
	6.77	114.2 (C-14), 135.0 (C-16), 144.1 (C-15), 168.5 (C-20)

activity of mitochondrial dehydrogenases, being quantified by spectrophotometry (Mosmann, 1983).

Despite its high activity (73.15% of RT inhibition), the crude ethanolic extract promoted maximum cytotoxicity on the concentration of 100 ± 0 µg/mL. Its bio-guided fractionation resulted in more active fractions: acetanolic subfraction - washing water - acids that promoted 88.82% of RT inhibition and cytotoxicity of 38.2 ± 7.6 up to 100 µg/L. One must highlight the acetanolic subfraction - neutrals with 60.98% of RT inhibition and the ellagic acid with 88.61% of RT inhibition and absence of cytotoxicity.

Samples that showed no statistically significant toxicity for J774.A1 macrophages up to the maximum concentration tested (100 µg / L) were the following: acetate subfraction – neutral compounds, acetanolic subfraction - acids, aqueous subfraction – acidic compounds, aqueous fraction, Efavirenz, and ellagic acid.

From this experiment it was possible to observe that the following samples presented toxicity up to the maximum concentration tested (100 µg/L): crude ethanolic extract (100 ± 0), acetanolic fraction (69.6 ± 2.3), acetanolic subfraction – washing water – basic compounds (90.8 ± 2.2), acetanolic subfraction – washing water - acids (38.2 ± 7.6), chloroform fraction (97.7 ± 2.1), chloroform subfraction - acids (94.6 ± 7.6), chloroform subfraction - neutrals (74.0 ± 2.0), chloroform subfraction – basic compounds (63.0 ± 3.8), chloroform subfraction – water/acids (27.0 ± 7.4), chloroform subfraction – emulsion acids (43.3 ± 4.5), Atazanavir (86.0 ± 4.3), Raltegravir (100 ± 0) and gallic acid (100 ± 0) (Table 2).

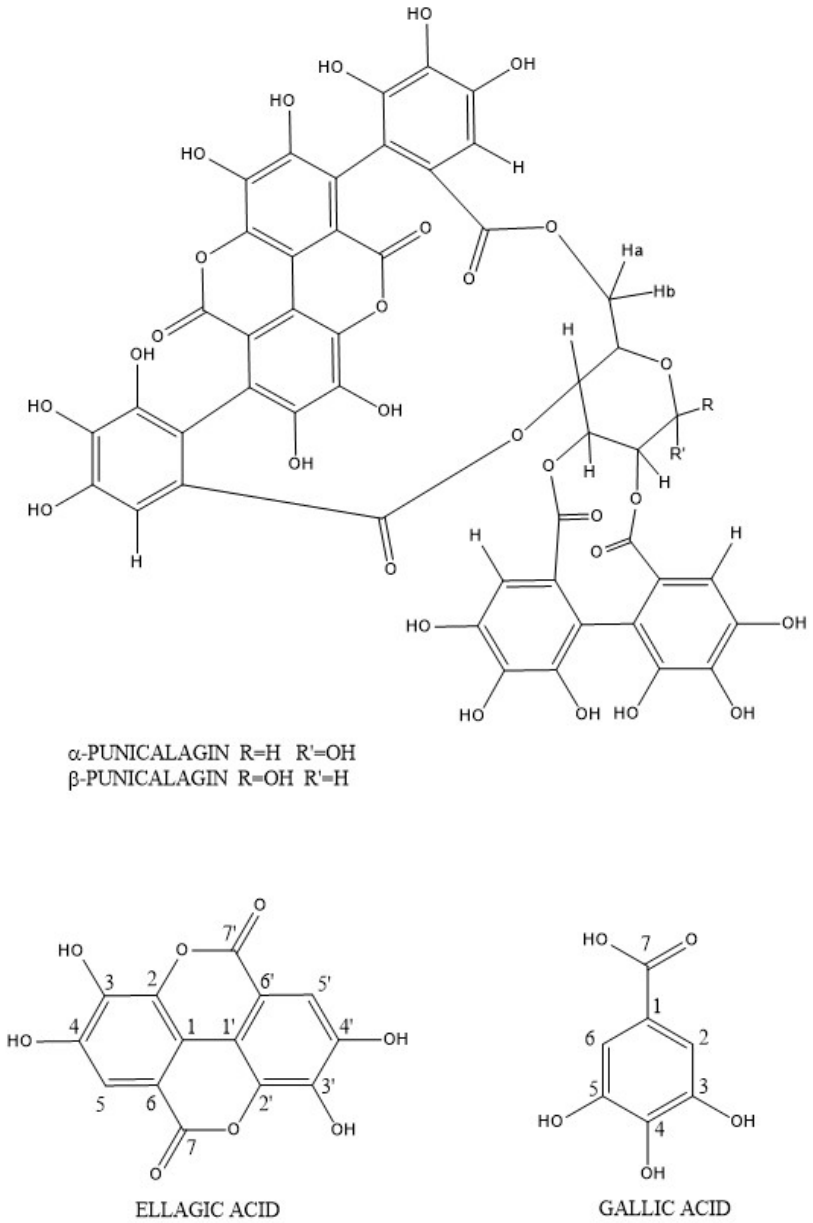

Figure 6. Structures of Ellagic Acid, Gallic Acid, α and β Punicalagins.

Of these, the more toxic ones were: crude ethanolic extract, which was lethal for 100% of macrophages at 55 ± 0 µg/L; Raltegravir was lethal for 100% of macrophages at 32.4 ± 1.8 µg/L; gallic acid was lethal for 100% of cells at 19.8 ± 0.2 µg/L; Atazanavir was lethal for 86.0 ± 4.3% of macrophages at 61.7 ± 1.5 µg/L; and Chloroform fraction, which was lethal for 97.7 ± 2.1% of macrophage cells at 56 ± 0.6 µg/L.

The following samples were of low toxicity (maximum toxicity of less than 50%) and therefore had no IC_{50} calculated: acetanolic subfraction – washing water – acidic compounds; chloroform subfraction – water/acids; and chloroform subfraction – emulsion acids.

One must highlight that the vehicle used to solubilize all substances (DMSO 0.1%) did not present a significant toxic effect for the cell (data not shown), when compared to the negative control (cells cultivated in growth medium, only). Thus, the toxicity of substances could not be attributed to the presence of DMSO in the culture.

The results show that the toxicity of a substance is a complex event that can cause cell damage and promote physiological and systemic effects. The cytotoxicity of a substance refers to its ability to induce cell death. It can also be used to define optimal concentrations of substances, reflecting on the investigation of their potential *in vitro* activities (Costa, 2008).

Atazanavir and Raltegravir were the most toxic drugs used on the cytotoxicity assay. Adverse events associated with therapy have been described in people with HIV undergoing antiretroviral therapy (ART), bringing other demands to the services responsible for comprehensive healthcare for these patients. One must highlight the lipodystrophy syndrome of HIV, which features dyslipidemia, glycemic alterations, and morphological alterations with lipoatrophy and lipohypertrophy. Observational studies have shown that the incidence of cardiovascular events in HIV-infected patients undergoing ART is higher than in the general population (Almeida et al., 2009).

Cytotoxicity assays of the species *L. pacari* are reported on scientific literature using other methodologies such as micronucleus test and human cancer cell lines. Methanolic extract prepared from the peels induces cells to apoptosis on the behavior of human cancer cell lines U-937, Daudi, Jurkat, HeLa, HRT-18 and SY5Y and mononuclear cells of peripheral blood and of mice *in vitro* (Marcondes, 2013). Gemin D and punicalagin isolated from *L. pacari* have been shown to have relevant antigenotoxic and cytotoxic effects, indicating that they may be likely candidates for chemoprevention or development of new cancer therapies (Carneiro, 2016).

The cytotoxic effect of the ethanolic extract of *L. pacari* leaves moderated in bacterial strains of *Salmonella typhimurium* TA98 and TA100 was also verified (Lima, 2012).

Molecular docking affinity parameters of gallic acid (GA), ellagic acid (EA), and punicalagin (PU) with the enzyme 5TIQ were analyzed using the software Autodock® 4.2 (Table 3).

The 5TIQ enzyme obtained a better affinity parameter with the ellagic acid ligand. The Gibbs energy obtained in this interaction was -8.01 Kcal/mol. Ellagic acid makes hydrogen bonds with the enzyme active site residues PRO236, LYS102, as well as hydrophobic interactions with the residues PHE227, LEU234, HIS235, TYR318, LEU100, LYS108.

Gallic acid was also favorable to form interaction with the enzyme 5TIQ, with energy of -5.48 Kcal/mol. The residues HIS96, VAL381, LYS101 form hydrogen bonds with GA, while residues ILE382, GLY99, LEU100 form Van der Waals bonds with this acid.

The punicalagin ligand was not favorable to interact with the enzyme, since the Gibbs energy obtained is highly positive of 249 Kcal/mol. In this complex, hydrogen bonds of PU at the active site of the enzyme with amino acid residues ILE180, LYS103, LYS101, as well as hydrophobic

Table 3. Determination of cytotoxicity of vegetable samples of *L. pacari* (1, 10, 100 µg/mL) in macrophages J774.A1 (MTT assay).

Type of extraction	Fractions	IC_{50} (µg/mL)[a]	Maximum toxicity (%)[b]
CEE		55 ± 0	100 ± 0***
Fraction of CEE	Chloroform fraction	56 ± 0.6	97.7 ± 2.1***
Fractions of ABE	Chloroform subfraction – acids	48.7 ± 2.9	94.6 ± 7.6***
	Chloroform subfraction – neutrals	70.7 ± 1.2	74.0 ± 2.0***
	Chloroform subfraction – bases	81.3 ± 2.2	63.0 ± 3.8**
	Chloroform subfraction – water acids	>100	27.0 ± 7.4*
	Chloroform subfraction – emulsion acids	>100	43.3 ± 4.5*
Fraction of LLE	Acetanolic fraction	74.1 ± 1.2	69.6 ± 2.3***
Fractions of ABE	Acetanolic subfraction – neutral compounds	>100	NA
	Aqueous subfraction –acidic compounds	>100	NA
	Acetanolic subfraction – washing water basic compounds	59.3 ± 0.7	90.8 ± 2.2***
	Acetanolic subfraction – washing water acidic compounds	>100	38.2 ± 7.6*
	Acetanolic subfraction – acids	>100	NA
Fraction of LLE	Aqueous fraction	>100	NA
Patterns		**IC50** (µg/mL)	**Maximum toxicity (%)**
Efavirenz		>100	NA
Atazanavir		61.7 ± 1.5	86.0 ± 4.3***
Raltegravir		32.4 ± 1.8	100 ± 0***
Gallic acid		19.8 ± 0.2	100 ± 0***
Ellagic acid		>100	NA

[a]Inhibitory concentration 50 (IC_{50}) calculated from the dose-response curve. [b]Mean ± standard error of the mean maximum cytotoxicity in triplicate. Values of maximum effect were considered significant when *p <0.05, **p <0.01, and ***p <0.001 compared to group DMSO 0.1%. NA: did not show maximum cytotoxicity up to the concentration of 100 µg/mL compared to group DMSO.

interactions with the residues ILE178, VAL179, TYR181, TYR193, PHE227, VAL106, PRO226 are observed.

Molecular docking results show that GA, EA, and PU ligands interact within the same active site as 5TIQ, and the LYS101 residue forms hydrogen bond with all ligands, thus suggesting that this residue is important for ligand complexation in the ligand in the active site of the enzyme. The molecular size of EA allows better interaction within the active site; however, the large molecular volume of PU makes this ligand unsuitable for complexation, thus justifying the positive value of interaction energy. All interactions occurring within the 5TIQ enzyme active site with ligands GA, EA and PU were detected, as well as the isosurface of the formed complexes (Figure 7 and 8).

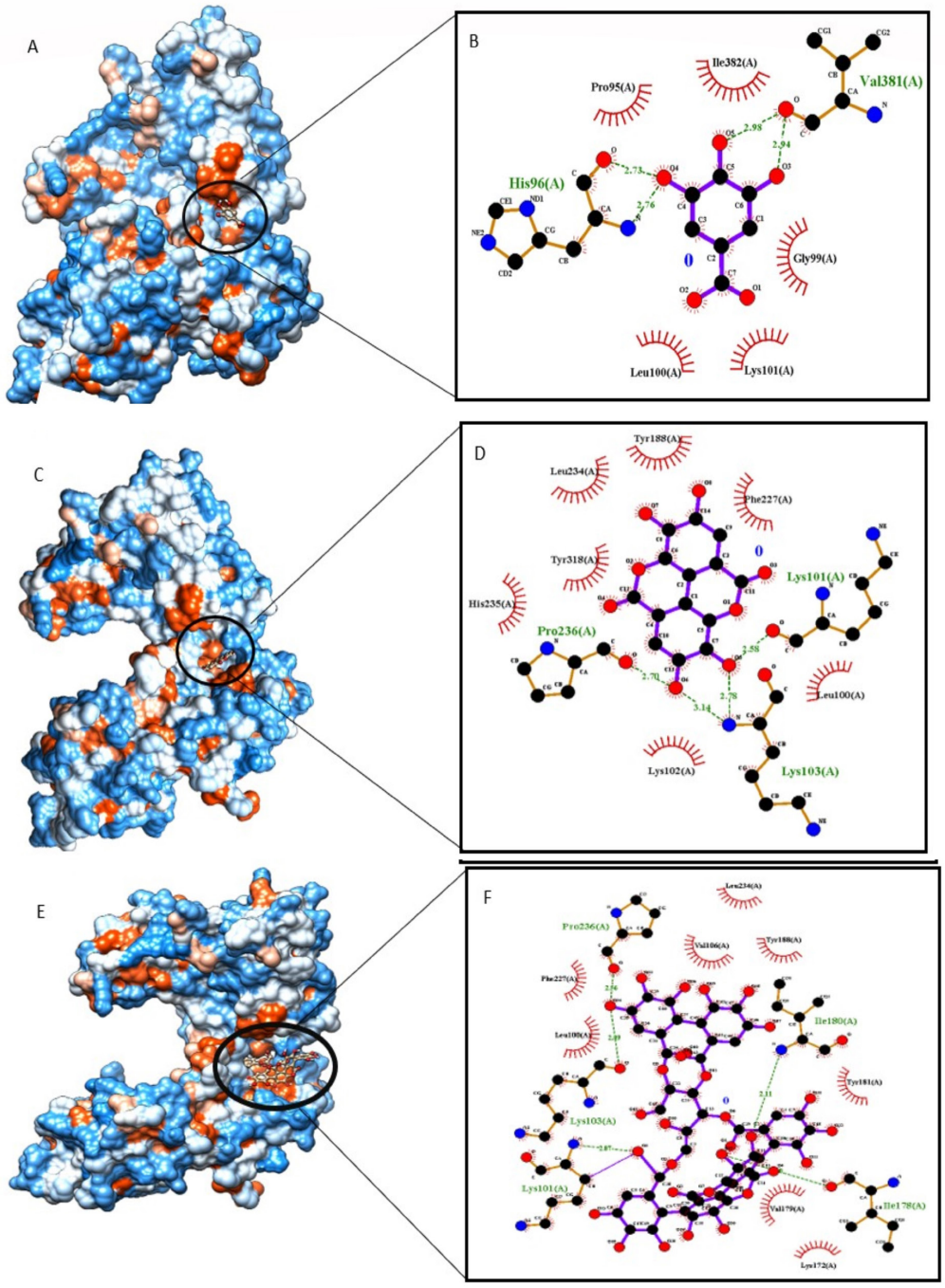

Figure 7. Representation of complex ribbons (A, C, and E) and 2D interactions within the enzyme active site (B, D, and F): AG/5TIQ (A, B); AE/5TIQ (C, D); PU/5TIQ (E, F).

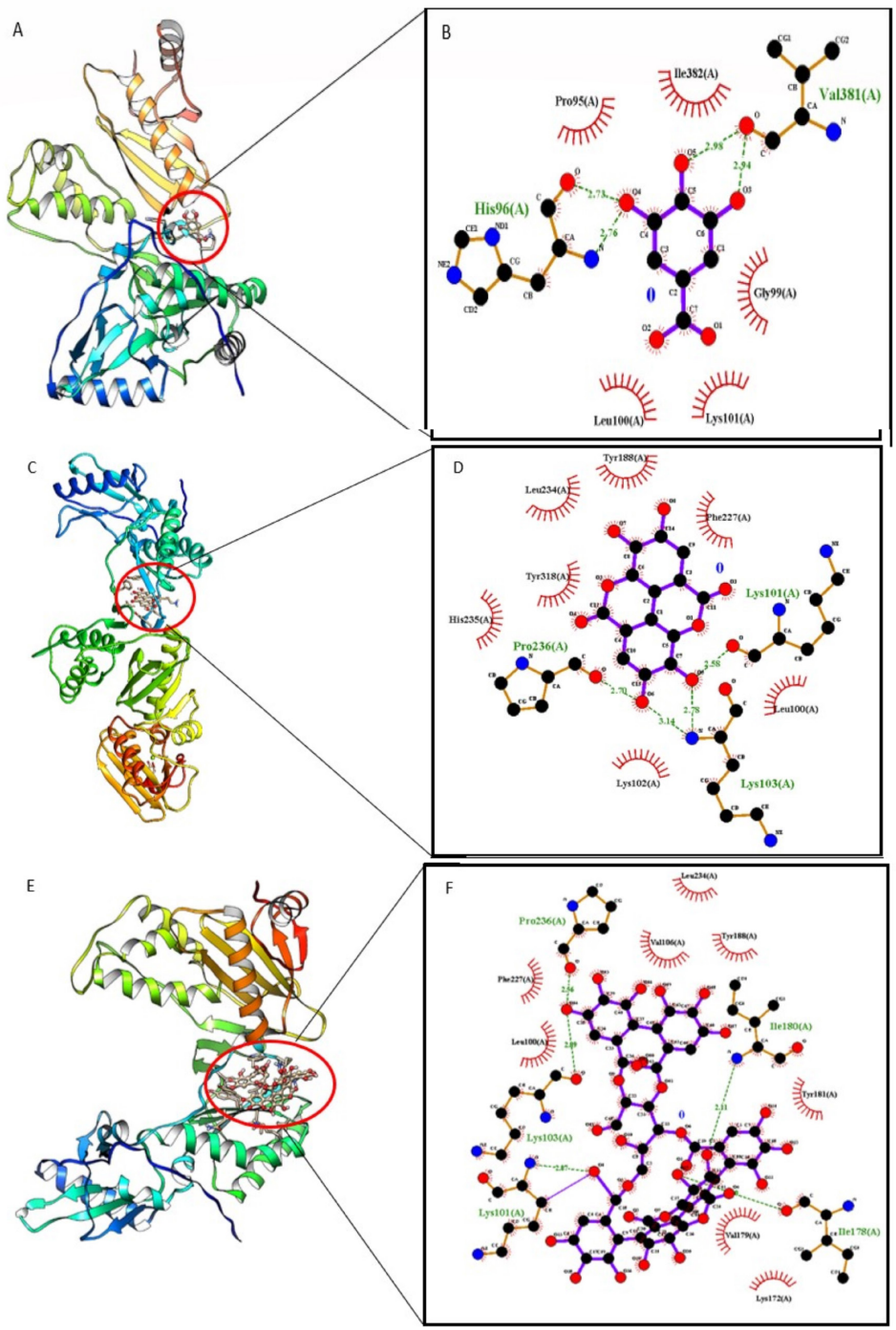

Figure 8. Representation of complex isosurface (A, C, and E) and 2D interactions within the enzyme active site (B, D, and F): AG/5TIQ (A, B); AE/5TIQ (C, D); PU/5TIQ (E, F).

The results obtained in silico allow us to use the scope of the computer simulation and related areas to indicate something that occurred through a computer simulation (Nisha et al., 2016; Krishnan et al., 2017).

Pharmacokinetic and toxicity parameters of molecules of ellagic acid, gallic acid, and punicalagin were evaluated by PreADMET (Table 4).

Data analysis was initiated by BBB (Brain barrier penetration), which corresponds to penetration used to assess whether compounds pass the blood-brain barrier, which acts as a barrier between blood and cerebrospinal fluid. In ADMET terms, penetration tends to be high when higher than >2, moderately when it is between 0.1 and 2 and reduced <0.1 (Kramer et al., 2018). The blood-brain

Table 4. Molecular docking affinity parameters of gallic acid (GA), ellagic acid (EA), and punicalagin (PU) with the enzyme 5TIQ using the software Autodock® 4.2.

Protein-ligand complex	[a]ΔG_{bind} (kcal/mol)	[b]Ki (μM)	[c]Amino acids that interact by hydrogen bonds	[c]Amino acids with hydrophobic interactions
5TIQ/AG	-5.48	96μM	HIS96, VAL381, LYS101	ILE382, GLY99, LEU100
5TIQ/AE	-8.01	1.34μM	PRO236, LYS101	PHE227, LEU234, HIS235, TYR318, LEU100, LYS108
5TIQ/PU	249.41	-	ILE180, LYS103, LYS101	ILE178, VAL179, TYR181, TYR193, PHE227, VAL106, PRO226

[a]Bond energy of the best conformation. [b]Inhibitory constant of the best conformation. [c]Amino acids interations. HIS: histidine; GLY:glycine; ILE: isoleucine; LEU: leucine; LYS: lysine; PRO: proline; PHE: phenylalanine; TYR: tyrosine; VAL: valine.

Table 5. Pharmacokinetic and toxicity parameters evaluated by PreADMET.

Parameters	Ellagic acid	Gallic acid	Punicalagin
BBB	0.321339	0.348084	0.0269945
Caco-2	20.4888	13.8492	15.6233
HIA	61.39513	53.69685	0.000000
MDCK	17.2974	9.53976	0.0434156
Solubility in pure water (mg/L)	922.464	72.3334	1.076e+007
Ames test	Mutagenic	Mutagenic	Non mutagenic
Carcino Mouse	Negative	Negative	Negative
Carcino Rat	Positive	Positive	Negative
Risk Herg inhibition	Low risk	Low risk	Low risk

BBB: brain barrier penetration; Caco-2: intestinal epithelium; HIA: human intestinal absorption; MKCK: Madin-Darby canine kidney.

barrier functions as a physiological barrier that restricts the passage of most blood compounds to the brain, thus having a protective property for this organ (Nisha et al., 2016). In this simulation, this parameter showed low absorption of these compounds when passing through the blood-brain barrier. In Table 5 the Ellagic acid and Gallic acid demonstrate intermediate penetration values of 0.32 and 0.34, respectively, and Punicalagin show low penetration. Values below 0.1 indicate that the compounds have low potential to penetrate the BBB, showing they are less likely to cause side effects (neurotoxicity) in the central nervous system (Mafud et al., 2018).

Absorption of a drug molecule, proposed for oral administration, depends on the extent of transport through the walls of the gastrointestinal tract (GIT). Human Intestinal Absorption (or HIA%) is another crucial factor that helps predict the viability of absorption of a drug through the small intestine (Souza et al., 2007). One may consider the following parameters: between 70% and 100% (high), between 20% and 70% (average), and lower than 20% (low). Ellagic and gallic acids were found to have a medium intestinal absorption and punicalagin had a very low absorption.

Other data of utmost importance are the permeabilities, such as Caco-2 (intestinal epithelium) and MDCK (Madin-Darby Canine Kidney). These systems aloow to assess the dissolution and permeation of water-soluble drugs, predicting their absorption rate after oral administration to humans (Kobayashi et al., 2001). The rate and extent to which a drug is absorbed may vary due to its physicochemical characteristics and factors related to disintegration and dissolution (Souza et al., 2007). Regarding Caco-2 permeability, the simulation showed an average result for both, and in terms of MDCK, a low value was found.

For the permeability assay in Caco-2 and MDCK cells, the values are high when greater than 70 nm/s, average between 4 and 70 nm/s and low when less than 4 nm/s (Yazdanian et al., 1998); therefore, the Ellagic and Gallic acid molecules in Table 5 have an average permeability, and low for Punicalagin. As for the permeability tests in MDCK cells, they tend to be high when greater than 500 nm/s, average between 25 and 500 nm/s and low when less than 25 nm/s (Velasco-Chong et al., 2020). With this, we can classify that the molecules with average values.

According to the Biopharmaceutics Classification System (BCS), dissolution and intestinal permeation of the drug may limit absorption and therefore therapeutic action (Gonçalves and Storpirtis, 2011). The aqueous solubility of a drug is considered high according to the BCS as to dose ratio, if it is equal to or less than 250 mg/L. Good solubility was observed in both analyzed molecules.

The mutagenicity of a drug is extremely important in its characterization, as mutations alter the normal functioning and cell division (Willey et al., 2010). The results denote gallic acid and ellagic acids as mutagenic, however, tests on Carcino Mouse showed negative results. In addition, punicalagin was not mutagenic.

Other topic of extreme importance is the risk of cardiac diseases that the drug may present, which is a concerning factor according to the Pan American Health Organization (PAHO). In Brazil, 300.000 people suffer from heart attacks every year, which is fatal in 30% of cases (Brasil, 2007). The obtained results showed a low risk of both ellagic acid and gallic acid; punicalagin, however, showed an unclear risk.

The ames test is a simple method to test the mutagenicity of the compounds, which uses mutant strains of the bacterium Salmonella typhimurim. Carcinogenicity is a toxicity that causes cancer in the body, the tests in rats and mice were positive in Table 5 only for acid molecules and showed negative for Punicalagin. The risk of heart disease (hER risk) is an important factor, the molecules analyzed here have shown low risk.

Ellagic acid is a dimeric derivative of gallic acid. It features very high thermodynamic stability, a lipophilic character conferred by the two aromatic rings, and a hydrophilic region represented by four phenolic groups, which act as hydrogen donors, and by two lactones, receptors of hydrogen bonds. It is a promising molecule for its RT inhibiting activity and pharmacokinetic and toxicity parameters.

4. Conclusion

From the bio-guided fractionation by antiretroviral activity, a higher activity of acetanolic subfractions was shown. Despite its high activity (73.15% of RT inhibition), the crude ethanolic extract promoted maximum cytotoxicity of 100 ± 0 µg/mL. Its bio-guided fractionation resulted in the more active fraction acetanolic subfraction – washing water – acids, which promoted 88.82% of RT inhibition and cytotoxicity of 38.2 ± 7.6 up to 100 µg/L. One must highlight the acetanolic subfraction – neutrals with 60,98% of RT inhibition and the ellagic acid with 88.61% of RT inhibition and absence of cytotoxicity in the tested concentrations. The cytotoxicity assay in macrophages lineages showed that the chloroform fraction was more toxic than the acetanolic fraction.

The analysis of the J-resolved spectrum of the aromatic region between 6 and 8 ppm presented a considerable number of singlets. One singlet at 7.48 and 6.93 ppm was identified as ellagic acid and gallic acid, respectively.

Results of molecular docking showed that the ligands gallic acid, ellagic acid and punicalagin interact within the same active site of 5TIQ. The 5TIQ enzyme obtained a better affinity parameter with the ellagic acid ligand, which was confirmed by spectra HSQC-^{1}H-^{13}C. Gallic acid was also favorable to form interaction with the 5TIQ enzyme. Through the PreADMET evaluation, ellagic acid has been found to be a promising molecule for its RT inhibiting activity and pharmacokinetic and toxicity parameters.

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