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Biological activity an *in silico* toxicity of phenolic compounds found in cupuaçu seed (*Theobroma grandiflorum*)

Atividade biológica e toxicidade in silico de compostos fenólicos encontrados na semente do cupuaçu (*Theobroma grandiflorum*)

Actividade biológica y toxicidad *in silico* de compuestos fenólicos encontrados en la Semilla de cupuaçu (*Theobroma grandiflorum*)

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Abstract
Cupuaçu (*Theobroma grandiflorum*) from the Malvaceae family, has seeds rich in flavonoids. The biological activities of seeds are diverse, however, there are scarce data on these metabolites. The study aimed to evaluate, through in silico methods, the biological, toxicological, and pharmacokinetic potential of metabolites present in the cupuaçu seed. The metabolites hypolaetina 3'-methylether 8-O-β-D glucuronide, theograndin II, hypolaetina 3'-methylether 8-O-β-D 3"-sulfated glucuronide, isoscutellarein 8-0-β-D glucuronide and theograndin I were analysed through Pubchem to search for structures, and PreADMET (pharmacokinetics and toxicity) and PASS online for biological activities (PA >98%). The results demonstrated low human intestinal absorption, in addition to weak to moderate distribution across the blood-brain barrier. All phenolic compounds showed weak to moderate binding to plasma proteins and low permeability in $CaCo_2$ cells, except for isoscutelarein 8-0-β-D glucuronide and hypolaetina 3'-methylether 8-O-β-D glucuronide with moderate permeability, being non-mutagenic. Furthermore, they had the potential to inhibit CYPs, such as CYP-2C9 and CYP-3A4. Also, the structures showed toxicity in *Medaka* fish, but they did not present toxicity in algae and *Daphnia* sp. Finally, the molecules presented the following main properties: hemostatic, cardioprotective, and antioxidant action, among others. The compounds presented varied biological activity profiles, but their pharmacokinetic and toxicity were not promising, limiting the potential for developing new drugs. The use of technologies is suggested to transform them into prodrugs, carriers, and the use of molecular modeling.
Keywords: Biological potencial; Flavonoids; Seeds; Toxicity.

Resumo
O cupuaçu (*Theobroma grandiflorum*) da família Malvaceae, possui sementes ricas em flavonoides. As atividades biológicas das sementes são diversas, entretanto, há escassez de estudos desses metabólitos. O objetivo do estudo foi avaliar, por métodos *in silico*, o potencial biológico, toxicológico e farmacocinética de metabólitos presentes na semente do cupuaçu. A análise ocorreu nos metabólitos: hipolaetina 3'-metiléter 8-O-β-D glicuronídeo, theograndina II, hipolaetina 3'-metiléter 8-O-β-D glicuronídeo 3"-sulfatado, isoscutelareína 8-0-β-D glicuronídeo e theograndina I, através do Pubchem para busca das estruturas, PreADMET (farmacocinética e toxicidade) e o PASS online para atividades biológicas (PA >98%). Os resultados das moléculas demonstraram baixa absorção intestinal humana, além

de fraca a moderada distribuição na barreira hematoencefálica. Todos os compostos fenólicos apresentaram fraca a moderada ligação a proteína plasmática e baixa permeabilidade em células CaCo2, com exceção da isoscutelareína 8-0-β-D glicuronídeo e da hipolaetina 3'-metiléter 8-O-β-D glicuronídeo com moderada permeabilidade, sendo não mutagênica. Ademais, apresentaram potencial para inibição de CYPs, como a CYP-2C9 e a CYP-3A4. Também, as estruturas apresentaram toxicidade em peixes *Medaka*, no entanto, não apresentaram toxicidade em algas e *Daphnia* sp. Enfim, em relação às atividades biológicas, as moléculas apresentaram como principais propriedades: ação hemostática, cardioprotetora, antioxidante, entre outras. Os compostos apresentaram variados perfis de atividades biológicas, mas seus perfis farmacocinéticos e de toxicidade não foram promissores, limitando o potencial para desenvolvimento de novos fármacos, sugere-se a utilização de tecnologias, como transformar em pró-farmacos, carreadores e utilização da modelagem molecular.
Palavras-chave: Flavonoides; Potencial biológico; Sementes; Toxicidade.

Resumen
El cupuaçu (Theobroma grandiflorum) de la familia Malvaceae, tiene semillas ricas en flavonoides. Las actividades biológicas de las semillas son diversas, aunque hay escasez de estudios sobre estos metabolitos. El objetivo del estudio fue evaluar, mediante métodos in silico, el potencial biológico, toxicológico y farmacocinético de los metabolitos presentes en las semillas de cupuaçu. Se analizaron los metabolitos: hipolaetina 3'-metiléter 8-O-β-D glucurónido, theograndina II, hipolaetina 3'-metiléter 8-O-β-D glucurónido 3''-sulfatado, isoscutelareína 8-0-β-D glucurónido y theograndina I, utilizando PubChem para buscar las estructuras, PreADMET (farmacocinética y toxicidad) y PASS online para actividades biológicas (PA > 98%). Los resultados de las moléculas mostraron baja absorción intestinal humana, así como distribución débil a moderada en la barrera hematoencefálica. Todos los compuestos fenólicos presentaron una unión débil a moderada a proteínas plasmáticas y baja permeabilidad en células CaCo2, excepto isoscutelareína 8-0-β-D glucurónido y hipolaetina 3'-metiléter 8-O-β-D glucurónido, que presentaron permeabilidad moderada y no fueron mutagénicos. Además, mostraron potencial para la inhibición de CYPs, como CYP-2C9 y CYP-3A4. Las estructuras también presentaron toxicidad en peces *Medaka*, sin embargo, no mostraron toxicidad en algas y *Daphnia* sp. En cuanto a las actividades biológicas, las moléculas presentaron propiedades principales como acción hemostática, cardioprotectora, antioxidante, entre otras. Aunque los compuestos mostraron diversos perfiles de actividades biológicas, sus perfiles farmacocinéticos y de toxicidad no fueron prometedores, lo que limita el potencial para el desarrollo de nuevos fármacos. Se sugiere explorar tecnologías como la transformación en profármacos, transportadores y el uso de modelado molecular.
Palabras clave: Flavonoides; Potencial biológico; Semillas; Toxicidade.

1. Introduction

From the Malvaceae family, the species *Theobroma grandiflorum* is popularly known as cupuaçu in the Amazon region (Niu et al., 2019) but its name may vary depending on the region (Coradin et al., 2022). The fruit is an oblong, ellipsoid or oboval drupaceous berry, with obtuse or rounded contours. Its peel is composed of epicarp with a woody characteristic and mesocarp with a whitish color, and the endocarp is the pulp of the fruit (Souza et al., 2017). The endocarp has a yellowish color, an acidic and characteristic aroma, and is widely used in food (Instituto de Desenvolvimento Agropecuário e Florestal Sustentável do Estado do Amazonas [IDAM], 2021). The fruit contains around 20 to 60 ovoid or ellipsoid-ovoid grains, reasonably flat, ranging from 20 to 30 mm in length and 20 to 25 mm in width (Serejo et al., 2021). This grain is composed of the almond (two cotyledons and an embryo) and the shell (thin layer that surrounds it), with the germ (seed) corresponding to 43% to 57% of the total fruit (Jean-Marie et al., 2022).

The endocarp is widely used, especially in northern Brazil (Souza et al., 2017), but its other residues, such as seeds, are not reused and mostly discarded during the process of food derivatives production, which can cause pollution to the environment (Coradin et al., 2022). However, seeds are sources of proteins, fats, and carbohydrates (Rebouças et al., 2020), in addition, they are made up of promising secondary metabolites for biological actions and bioactive substances, such as methylxanthines and flavonoids (Sakiroh et al., 2019).

Nine flavonoids present in the seed of Theobroma grandiflorum were found and identified, including: (+)-catechin, (-)-epicatechin, isoscutelarein 8-O-β-D-glucuronide, hypolaetin 8-O-β-glucuronide, quercetin 3 -O-β-D-glucuronide, quercetin 3-O-β-D glucuronide 6″-methyl ester, quercetin, kaempferol and isoscutellarein 8-O-β-D-glucuronide 6″-methyl ester. In addition to two new sulfated flavononic glycosides, theograndins I and II (Pugliese et al., 2013).

It is noteworthy that flavonoids are polyphenols present in several plants, where more than 8,000 metabolites have already been identified. They have in their composition a common flavilum nucleus of phenylchromanoma, containing 15 carbons distributed in two aromatic rings (rings A and B), the benzenes, interconnected to a central heterocyclic structure, the pyran (ring C), where the first benzene is condensed with the sixth carbon of the pyran, which in position 2 carries a phenyl group (Almeida & Santos, 2018), with substitution in one or more hydroxyls, including those linked to sugars (glycosides).

Flavonoids presented several health benefits. In vivo and in vitro studyes showed activities such as antimicrobial, antioxidant (Torres et al., 2018), anti-inflammatory, antitumor, immune response modulation, enzyme inhibition function (Mercês & Santos, 2022), cardiovascular protective properties (Calvário & Oliveira, 2021), hypoglycemic (Baltazar et al., 2021) and antiviral actions (Valente et al., 2022). However, there is a lack of scientific studies regarding the analysis of the relationship between structure and biological activity of flavonoids present in cupuaçu seeds. In this way, the use of in silico methodologies can optimize the discovery of new drug candidates, as they are theoretical methods used as first alternative to analyze physicochemical properties of substances (Carvalho et al., 2018).

In this sense, in silico studies allows to evaluate the possibility of a compound having a potential biological effect, as well as to evaluate its pharmacokinetics and toxicity. It is of great relevance to the analysis of isolated secondary metabolites and discards unpromising metabolites (Chagas et al., 2022). The aim of carrying out the present study was to identify the main biological activities, pharmacokinetics, and toxicity, in silico, of the isolated flavonoids present in the cupuaçu seed.

2. Methodology

A This work used the scientific model of comparison for diversification in the predictions of pharmacokinetic, toxicological, physical-chemical, and biological activity, in which the designed molecules were compared with others in the databases of the programs used (Pereira, et al., 2018).

To select the molecules, present in the seed of *Theobroma grandiflorum* (cupuaçu), a literature review was initially carried out. Studies evaluating existing biological activities were selected. This search was developed using data from PubMed from the National Library of Medicine, Google Scholar, and Scientific Electronic Library online (SciELO).

After reviewing the literature, certain flavonoids found in the cupuaçu seeds were selected: hypolaetina 3'-methylether 8-O-β-D glucuronide, hypolaetina 8-0-β- D glucuronide 3”-sulfate, hypolaetina 3'-methylether 8-O-β-D glucuronide 3”-sulfated, isoscutellarein 8-0-β-D glucuronide, isoscutelarein 8-0-β-D glucuronide 3”-sulfate (Pugliese et al., 2013).

From this, PubChem® (available at: https://pubchem.ncbi.nlm.nih.gov/) was used to obtain the two-dimensional structures of the secondary metabolites, and the Marvinsketch software (version 2.0; available at: https://l1nq.com/rdgUm) for the design of the structures. The PreADMET application (version 2.0, Preadmet 2020; available at: https://encr.pw/RDYuC) for pharmacokinetic and toxicological predictions, and the online PASS software (predicting activity spectra for substances - Way2Drug.com © 2011 - 2023 • Version 2.0; available at: http://www.way2drug.com/passonline/) for prediction of biological activities.

The pharmacokinetic prediction was carried out in the freely available online PreADMET, where the following parameters were analyzed: HIA (human intestinal absorption), being: 0-20% (low absorption), 20-70% (moderate absorption), 70-100 % (high absorption); BBB (ability to cross blood-brain barrier), being: <1 crosses to a reduced extent or not at all, and >2 crosses moderately; Permeability in Caco2 cells: <4 low permeability, ≥4 and ≤70 moderate permeability and >70 high permeability; Permeability in MDCK cells: <25 low permeability, 25-500 moderate permeability, >500 high permeability; and binding to the plasma protein albumin (PPA): >90% strong binding to albumin and <90% weak to moderate binding (PreADMET, 2020).

For evaluating the action on metabolism, the CYP inhibition by molecules was assessed. The substances that do not

inhibit and/or induce any CYP were considered ideal because they probably do not interfere with the metabolism of other substances including the endogenous ones (Sousa, 2012).

In toxicity analyzes on marine organisms, parameters were used for algae, crustaceans *Daphnia* sp. and *Medaka* fish, with algae indicating acute toxicity, the crustaceans *Daphnia* sp. acute and subchronic toxicity, and *Medaka* fish indicating chronic toxicity (Dolabela et al.,2018). According to Costa et al. (2008), to be considered toxic for algae the result must be greater than 1mg/mL, and non-toxic the result must be less than 1mg/mL. Toxicity in *Daphnia* is evaluated as: toxic = greater than 22 μg/mL, and non-toxic = less than 22 μg/mL (Guilhermino et al., 2000). For toxicity in *Medaka* fish, the values are: <1mg/L = very toxic; toxic between 1-10 mg/L, harmful between 10-100 mg/L and non-toxic > 100 mg/L (Zucker & Jonhson, 1985). In PreADMET, for mutagenicity analyses, the Ames test with *Salmonella typhimurium* was used. This test has the potential to identify substances that can cause gene mutations (Ames & Yamasaki, 1975). The strains analyzed were TA98, TA100 and TA1535, being considered positive when a mutation is seen in the bacteria reversion and negative when there is no mutation reversion (Sousa, 2012).

Carcinogenicity prediction was carried out by PreADMET, which uses data from the national toxicology program (NTP) and the food and drug administration (FDA). The result concerns the carcinogenic potential in rats and mice, being (+) for carcinogens and (-) for non-carcinogens (PreADMET, 2020).

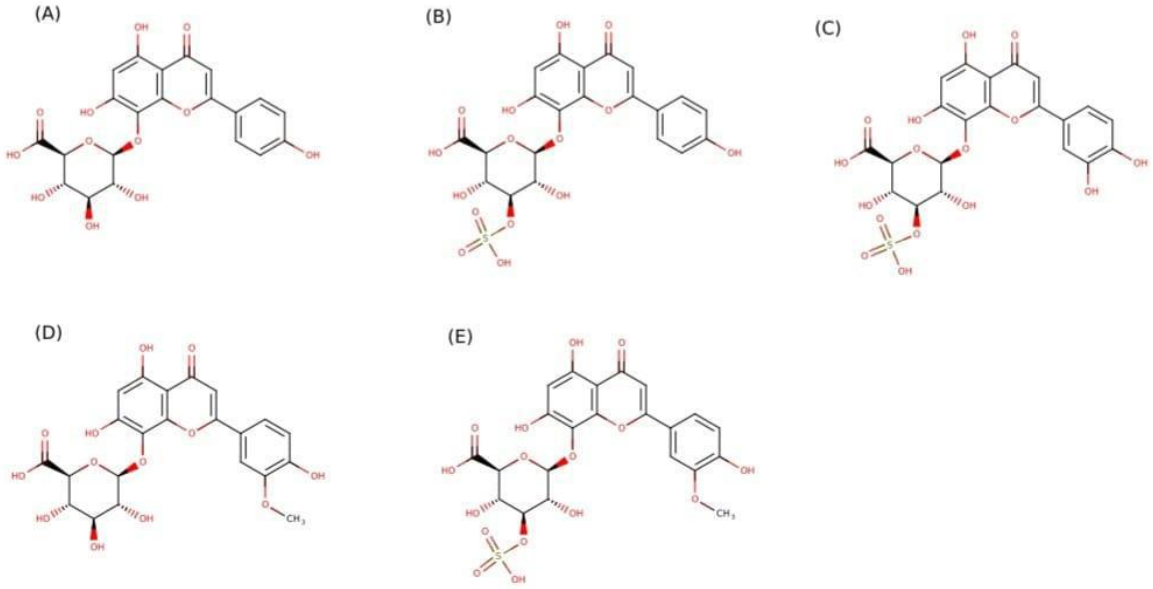
Finally, the biological activity of the metabolites was evaluated after crossing more than 2,500 biologically active substances present in the online PASS software database, with a value equal to or above 0.98 being standardized, that is, 98% for the activity probability (PA) (Filimonov et al., 2014).

3. Results and Discussion

3.1 Prediction of biological activities

In the cupuaçu seed, five secondary metabolites that have few studies in the scientific literature were identified: isoscutellarein 8-O-beta-D glucuronide, isoscutellarein 8-O-beta-D glucuronide 3``-sulfate (theograndin I), hypolaetina 8-O-beta-D glucuronide 3``-sulfate (theograndin II), hypolaetina 3`-methyl ether 8-O-beta-D glucuronide and hypolaetina 3`-methyl ether 8-O-beta-D glucuronide 3 ``-sulfate named, respectively, isoscutelarein A, theograndin I, theograndin II, hypolaetin A and hypolaetin B. These secondary metabolites, structurally represented in Figure 1, were subjected to in silico analyzes to identify their biological activities, pharmacokinetic profile, and toxicity.

Figure 1 - Structure of secondary metabolites from cupuaçu seed (*Theobroma grandiflorum*).



(A): isoscutellarein 8-O-beta-D glucuronide; (B): isoscutellarein 8-O-beta-D glucuronide 3``-sulfate (theograndin I); (C): hypolaetin 8-O-beta-D glucuronide 3``-sulfate (theograndin II); (D): hypolaetin 3`-methyl ether 8-O-beta-D glucuronide; (E): hypolaetin 3`-methyl ether 8-O-beta-D glucuronide 3``-sulfate. Source: Authors (2023).

After analyzing the results of biological activities (Table 1), the molecules appear to be promising as hemostatic (isoscutelarein A, theograndin I, theograndin II, hypolaetin A and hypolaetin B). These results demonstrate great biological importance, since hemostatic action can control and stop bleeding, being clinically relevant in situations of surgery, trauma, and people with hemolytic disorders (Prado et al., 2014).

The cardioprotective action of the molecules isoscutelarein A and hypolaetin A was suggested (Table 1). The cardioprotective action can help reduce damage to the heart muscle, improving cardiovascular health, potentially preventing serious complications as heart attacks (Faria et al., 2020). In addition to the activities mentioned, the substances isoscutelarein A and hypolaetin A were suggested to have antioxidant activity by eliminating free radicals (Table 1). Antioxidant activity helps to neutralize free radicals, eventually reducing the risk of certain conditions, generating improvements in general health (Filhos et al., 2023).

Theograndin I and theograndin II were shown to act as sulfotransferase substrates (Table 1). Sulfotransferase substrates are specific molecules modified by the sulfotransferase enzyme, and thus regulate the activities of hundreds of small molecules through the transfer of the sulfuryl moiety (-SO3; Wang, Cook & Leyh, 2016). In this sense, it is clinically important in the biotransformation of drugs since the activation or inactivation of certain drugs occurs through these sulfation reactions. Also, according to the results in Table 1, the action as a membrane integrity antagonist for isoscutelarein A was suggested, it probably can preserve and strengthen cell membranes, fundamental structures for the proper functioning of the body's cells. (Montenegro et al., 2017).

Finally, Table 1 shows that isoscutelarein A acts as a UDP-glucuronosyltransferase substrate and UGT1A substrate. According to Mackenzie at al. (2003), a substrate of UDP-glucuronosyltransferase refers to a molecule that is modified by the UGTs enzyme, being of paramount importance in the drug conjugation process. It is a significant action, determining the response to chemicals that are eliminated mainly by conjugation with glucuronic acid. Therefore, the clinical importance lies in the metabolization of many medications and toxins by the liver, making them more soluble in water for excretion.

The UGT1A substrate, uridine diphosphate glucuronosyltransferase (UGT) 1A is involved in the formation of inactive metabolites. Genetic variations in these enzymes can impact the way the body metabolizes medications (Man et al., 2018),

affecting their concentrations in the body.

Table 1 - Prediction of biological activities of substances isolated from *T. grandiflorum.*

Secondary metabolites	Main properties predicted by PASS online	AP%
Isoscutellarein 8-O-β-D glycorunide	Hemostatic	98,7%
	Membrane Integrity Agonist	98,3%
	Cardioprotective	98,1%
	Free radical scavenger	98,0%
	UDP-glucuronosyltransferase substrate	98,1%
	UGT1A substrate	98,0%
Isoscutellarein 8-O-β-D glycorunide 3``-sulfate	Hemostatic	99,4%
	Sulfotransferase substrate	98,5%
Hypolaetin 8-O-β-D glycorunide 3``-sulfate	Hemostatic	99,3%
	Sulfotransferase substrate	98,3%
Hypolaetin 3`-methyl ether 8-O-β-D Glycorunide	Hemostatic	98,7%
	Free radical scavenger	98,3%
	Cardioprotective	98,1%
Hypolaetin 3`-methyl ether 8-O-β-D Glycorunide 3``-sulfate	Hemostatic	99,2%

PA%: Probability of Activity in percentage, PA ≥ 0.980: probable activity equal to or greater than 98%. The results of the online PASS prediction were interpreted as follows: only activities with PA equal to or above 98% were considered possible for a given compound, the closer to 100% indicated a high chance of experimentally finding the predicted activity. Source: Pass online (2023).

3.2 Pharmacokinetic profile prediction

In the pharmacokinetic data estimated by the PreADMET Program the substances presented the following results:

3.2.1 Absorption

Permeability in MDCK cells

All structures showed low permeability in MDCK cells, with the following results: isoscutelarein A: 0.99703, theograndin I: 0.0448005, Theograndin II: 0.045115, hypolaetin A: 0.542677 and hypolaetin B: 0.0446855 (Table 2). MDCK (Madin-Darby canine kidney) cells come from the kidneys of Madin-Darby dogs which, when grown in the laboratory, form structures like ducts, differentiating into columnar epithelial cells with semipermeable membranes with junctions similar to Caco-2 cells, exhibiting features that resemble the epithelial cells found in kidney tubules (Ferreira, Brandão & Dolabela, 2020). MDCK cells are widely used to study renal distal tubular physiology through the transport of substances and ions (Sousa, Freitas & Storpirtis, 2007). Furthermore, they are used to check the permeability and absorption of substances, providing information on the oral absorption of compounds, being very important in the initial phase of drug development, helping to predict how a substance can be absorbed by the body when administered orally (Bokulic et al., 2022). In this sense, the results demonstrated that all molecules presented low permeability for MDCK, demonstrating they are not well absorbed by the body.

Permeability in CaCo2 cells

The substances theograndin I, theograndin II and hypolaetin B presented the respective results: 3.30465, 3.02004 and 2.46378, being interpreted as structures that have low permeability in CaCo2 cells. On the other hand, Isoscutelarein A and hypolaetin A showed moderate permeability in Caco2 cells with the following results, Isoscutelarein A: 14,609 and hypolaetin A: 13,6522 (Table 2).

CaCo2 is a cell line obtained from human colon adenocarcinoma that has characteristics similar to intestinal epithelial cells. They form a polarized monolayer when grown in the laboratory, developing tight junctions that resemble those found in

the the small intestine. This polarization is crucial to replicate the natural absorption conditions in the human body, making studies with CaCo2 cells essential to understand how substances are absorbed or metabolized in the digestive system, helping to predict drugs bioavailability (Menezes, 2009).

Except for isoscutelarein A and hypolaetin A, which showed moderate permeability, the other substances demonstrated low permeability (Table 2), indicating that isoscutelarein A and hypolaetin A, even if absorption is limited, have a greater chance of being absorbed, more considerably in the large intestine (Caco2 cells) when compared to absorption in the kidneys (MDCK cells).

Human intestinal absorption (HIA)

Compared to the Human Intestinal Absorption (HIA) parameter, all molecules showed low absorption (Table 2): isoscutelarein A: 15.784278, theograndin I: 6.923619, theograndin II: 3.150528, hypolaetin A: 13.089938 and hypolaetin B: 5,759287.

Human Intestinal Absorption (HIA) is an important parameter in the development of medicines, as it directly affects the effectiveness and bioavailability of a substance (Corrêa & Oliveira, 2023). It is used to evaluate the absorption capacity of a specific compound by the human gastrointestinal tract, to predict how a substance will be absorbed by the body after oral administration (Pinheiro et al., 2022). Considering that ideal HIA of a drug is directly linked to the drug intestinal absorption via oral administration, it can be suggested that these molecules do not have characteristics to be candidates for oral drugs, as they have low bioavailability.

Most of the structures do not appear to be promising in terms of absorption parameter, except for isoscutelarein A and hypolaetin A, which indicated moderate absorption in CaCo2 cells. It is inferred that the low absorption may be related to the association of phenolic compounds with sugar molecules, as the presence of the glycoside group may hinder their absorption by the gastrointestinal tract and, consequently, their bioavailability (Hollman, 2004). However, glycosylation can confer other properties to flavonoids, such as increasing their water solubility and stability, which can have a positive impact in other areas, such as antioxidant activity or interaction with specific enzymes in the human body (Amaretti et al., 2015).

Table 2 - Results of pharmacokinetic predictions of molecules isolated from *T. grandiflorum*.

Secondary metabolites	Absorption			Distribution		Metabolism	
	HIA	Caco2	MDCK	BBB	PPB	Fase I	Inibição
Isoscutellarein 8-O-B-D glycorunide	15.784278	14.609	0.99703	0.033499	73.846049	Weakly	CYP-2C9 CYP-3A4
Isoscutellarein 8-O-b-D glucuronide 3``-sulfate	6.923619	3.30465	0.0448005	0.0294342*	74.804253	Weakly	CYP-2C9 CYP-3A4
Hypolaetin 8-O-b-D glucuronide 3``-sulfate	3.150528	3.02004	0.045115	0.0373023*	70.169998	Weakly	CYP-2C9 CYP-3A4
Hypolaetin 3`-methyl ether 8-O-b-D glucuronide	13.089938	13.6522	0.542677	0.0349036	61.580035	Weakly	CYP-2C9 CYP-3A4
Hypolaetin 3`-methyl ether 8-O-b-D glucuronide 3``-sulfate	5.759287	2.46378	0.0446855	0.0371595*	65.041128	Weakly	CYP-2C9 CYP-3A4

HIA: (0-20%) low absorption, (20-70%) moderate absorption, (70-100%) high absorption; Caco2: (<1nm/s) low permeability, (≥4 to ≤70nm/s) moderate permeability, (>70nm/s) high permeability; MDCK: (<25nm/s) low permeability, (25-500nm/s) moderate permeability, (>500nm/s) high permeability; BBB: (<1) crosses in a reduced way or not at all, (>2) crosses moderately; PPB: (>90%) strong binding to albumin, (>90) weak to moderate binding. Source: PreADMET (2023).

3.2.2 Distribution

Ability to cross the blood-brain barrier (BBB)

According to the parameters in Table 2, all metabolites cross in a reduced way or cannot cross the BBB, as the substances presented values lower than 1, namely: isoscutelarein A: 0.033499, theograndin I: 0.0294342, theograndin II: 0.0373023, hypolaetin A: 0.0349036 and hypolaetin B: 0.0371595.

The BBB is formed by endothelial cells that are aligned with the capillaries, preventing or hindering the passage of substances from the blood to the nervous tissue. The BBB membrane contains P-glycoprotein, which is an ATP transporter and participates in drug efflux. Due to its high level of expression, its location in the luminal membrane, multispecificity and high transport potential make this protein a selective element (Vieira & Sousa, 2013).

The blood-brain barrier is very relevant to the central nervous system, as it acts as a selective barrier, controlling the entry of medications and toxic substances (Carreño, 2015). The phenolic compounds studied do not cross the BBB due to their hydrophilic characteristics, as well as the molecules size, which become large due to the glycosides presence, which makes it difficult for them to pass through the blood-brain barrier.

In this sense, BBB allows the passage of compounds necessary for the proper functioning of the brain, such as nutrients and hormones, preventing toxins, antibiotics and other medications from reaching it. Therefore, if a drug crosses the BBB, it can result in significant consequences in the central nervous system (CNS), causing neurological disorders through neurotoxicity, which results in part from an increase in BBB permeability (Pimentel, Sivalingam, Doke & Samikkannu, 2020). The studied metabolites have adequate parameters, given the fact they do not have the ability to cross the BBB.

Plasma protein binding (PPB)

The binding of the five substances to the albumin protein was weak to moderate given that the metabolites presented the following results: isoscutelarein A: 73.846049, theograndin I: 74.804253, theograndin II: 70.169998, hypolaetin A: 61.580035, hypolaetin B PPB: 65.041128 (Table 2).

The function of plasma proteins as drug carriers can facilitate access to the site of action and reduce side effects. In particular, the application of plasma proteins in drug release may be important in antineoplastic therapy (Lambrinidis et al., 2015). It is possible to infer that of all drug interactions, those related to pharmacokinetics are of great importance, such as in distribution, where the basic interaction is the drugs competition for albumin, when both have high affinity. When the interaction occurs, the free fraction of one of the drugs may increase along with its toxicity (Vieira & Sousa, 2013).

With values lower than 90%, the parameter of binding to plasma proteins is considered good, as it would not generate drugs competition for binding to plasma proteins, which reduces the chances of a drug interaction (Pimentel et al., 2020).

3.2.3 Metabolism

For metabolism, interaction with CYPs was verified. The metabolites have been shown not to inhibit CYP2C19 and CYP2D6 and were not considered a CYP2D6 substrate. However, they had the ability to inhibit CYP2C9 and CYP3A4, and to be weakly a CYP3A4 substrate (Table 2).

CYP450 (cytochrome P450) is a family of enzymes responsible for the metabolism of a wide variety of compounds, including drugs, toxins and endogenous substances. It is divided into several subfamilies such as the CYP1A2, CYP2B6, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 isoenzymes, with CYP3A4 being responsible for metabolizing most medications (Zhao et al., 2021).

According to Monteiro et al. (2007), CYP3A4 represents around 30% of the hepatic cytochrome and is in the gastrointestinal tract, kidneys and liver. Its anatomical and functional association with Gp-P and the modulation of its catalytic

capacity (inhibition/induction) by several compounds, make this isoform a preferential target for drug interactions. Furthermore, CYP2C9 represents 20% of the hepatic cytochrome total and this isoform is widely cited for drug metabolism and possible interactions. In addition to being subject to genetic polymorphism and having its functional activity affected by inhibition and induction processes, several of its substrates are drugs with reduced therapeutic margins.

Drugs that act on the same subfamily of CYP450 enzymes, when administered concomitantly, can interact with each other, and affect the systemic clearance of one of them and result in reduced effectiveness, synergism or toxicity (Braz, Figueiredo, Barroso & Reis, 2018). The nature, extent and clinical relevance of these drug interactions depend on whether the drug exerts substrate activity, where the structure competes for metabolization in the same CYP, which can cause an inductive effect and an increase in the enzymatic process speed, resulting in a accelerated metabolism of the xenobiotic and/or CYP450 enzyme inhibitor, where there is a reduction in metabolism rate with prolonged effects and toxicity (Hakkola et al., 2020).

Drug interactions resulting from the inhibition of CYP450 enzymes are more frequent than those caused by enzyme induction, and generally occur when the inhibitor is a substrate and compete directly for the enzyme's binding site (Braz et al., 2018).

Regarding metabolism by CYP, drugs that are metabolized by CYP3A4 have a more lipophilic nature and tend to remain in the body. In this way, these substances go through the biotransformation process and transform into metabolites with more hydrophilic characteristics, but drugs with a polar nature are also metabolized by CYP3A4, becoming even more hydrophilic (Dolabela et al., 2018).

In this sense, non-glycosylated molecules can inhibit the CYP2C9 and CYP3A4 enzymes, since the potency of an inhibitor is determined by its lipophilic character and binding strength (Guido et al., 2010). Therefore, when evaluating the lipophilic nature of the molecules, the metabolites isoscutelarein A, theograndin I, theograndin II, hypolaetin A and hypolaetin B are possibly less potent inhibitors of these enzymes when compared to non-glycosylated ones.

An example from drugs already available on the market, ketoconazole, from the imidazole family, is a potent CYP inhibitor (high lipid solubility). On the other hand, cimetidine is a relatively weaker reversible inhibitor (low lipid solubility and lower affinity for CYP; Lin & Lu, 1998). Thus, as glycosylated metabolites have lower lipid solubility, it is suggested that the inhibition of CYP3A4 and CYP2C9 is weak. Finally, it is suggested that even by weakly inhibiting CYP3A4 and CYP2C9, the substances can reduce the effectiveness of a drug and, possibly, toxicity. Thereby, the metabolites were not considered promising in terms of metabolism parameter.

3.3 Toxicological profile prediction

In the context of toxicity testing of substances with pharmacological potential in marine organisms, regulatory authorities recommend the use of more than one species, covering different levels in the food chain. It is common to use algae (as a food base), crustaceans (which feed on the algae) and fish (as subsequent consumers) for these assessments (Rebouças et al., 2013). In this sense, the five substances were evaluated against algae, *Daphnia* sp and *Medaka* fish.

Algae

The results for algae in Table 3, suggest that no structure was considered toxic: Isoscutelareina A: 0.0164792, theograndina I: 0.00828153, theograndina II: 0.00608718, hypolaetina A: 0.0118827 and hypolaetina B: 0.00600983.

Algae as a biological indicator have important uses because, as primary producers, they are the base of food chain and any change in the dynamics of their communities can affect the upper trophic levels. One of the advantages of using algae in toxicity tests is their high sensitivity to changes in the environment, combined with their short life cycle, which makes it possible to detect toxic effects over multiple generations. Algae are used to assess acute toxicity, focusing on death counts (Vidotti &

Rollemberg, 2004).

Acute toxicity tests aim to evaluate the impacts of toxic agents on aquatic species over a short period of time, compared to the life cycle of the test organism. Generally, in these studies, the effect measured is lethality or some previous manifestation, such as the state of immobility, which occurs in exposed aquatic organisms (Costa et al., 2008).

***Daphnia* sp.**

For Daphnia sp., no structure was considered toxic, as demonstrated by the following results: isoscutelarein A: 0.44536, theograndin I: 0.297374, theograndin II: 0.345388, hypolaetin A: 0.436065 and hypolaetin B: 0.291685 (Table 3).

The *Daphnia* sp are freshwater crustaceans, from the order Cladocera and the genus Daphnia, which are commonly known as water fleas. They are frequently used in substance toxicity studies, as they play a fundamental role in evaluating the harmful effects of different substances in the aquatic environment, being very sensitive to toxic agents. Furthermore, they are important in many food chains, being a significant source of food for fish, in addition to having a relatively short life cycle, a significant parameter for evaluating acute and subchronic toxicity (Ferreira et al., 2020).

***Medaka* Fish**

On the other hand, all structures were considered very toxic to Medaka fish, as shown in Table 3: isoscutelareina A: 0.377886, theograndina I: 0.208158, theograndina II: 0.283511, hypolaetina A: 0.371075 and hypolaetina B: 0.20506.

Medaka fish, scientifically known as Oryzias latipes, are crucial in toxicity studies of substances. Their importance lies in the sensitivity of these animals to a wide range of chemical compounds, which makes them a valuable model organism for evaluating the impacts of these substances on the environment and human health. *Medaka* fish are used to demonstrate both acute and chronic poisoning. Furthermore, as they are larger animals, they allow the observation of toxic changes in specific organs (Arenzon et al., 2013).

In accordance with Costa et al. (2008), chronic toxicity tests on aquatic species aim to evaluate the impacts of chemical substances over a period that encompasses part or even the entire life cycle, but the absence of toxic effects of a substance in acute toxicity tests does not imply its non-toxicity in chronic tests. Furthermore, chronic tests evaluate possible toxic impacts in prolonged exposure to sublethal concentrations, that is, at levels that allow the survival of organisms, but affect biological functions.

The metabolites did not present acute toxicity, but showed chronic toxicity, indicating an accumulation of these substances over time and consequently toxicity, requiring more detailed studies to enable the improvement of this parameter.

Table 3 - Predictions of toxic events of molecules isolated from *Theobroma grandiflorum*.

Secondary metabolites	Toxicity			Mutagenicity	Carcinogenicity	
	Algae	*Daphnia sp*	*Medaka* Fish	Ames Test	Mice	Rats
Isoscutellarein 8-O-b-D glucuronide	0.0164792	0.44536	0.377886	-	+	+
Isoscutellarein 8-O-b-D glucuronide 3``- sulfate	0.00828153	0.297374	0.208158	+	+	+
Hypolaetin 8-O-b-D glucuronide 3``- sulfate	0.00608718	0.345388	0.283511	+	+	+
Hypolaetin 3`-methyl ether 8-O-b-D glucuronide	0.0118827	0.436065	0.371075	-	+	+
Hypolaetin 3`-methyl ether 8-O-b-D glucuronide 3``- sulfate	0.00600983	0.291685	0.20506	+	+	+

Algae: (>1mg/mL) toxic, (<1mg/mL) non-toxic; Daphnia: (>22µg/mL) toxic, <22µg/mL non-toxic; Medaka: (<1mg/L) very toxic, (1-10mg/L) toxic, (10-100mg/L) harmful, (>100mg/L) non-toxic; Mutagenic: (+) positive, (-) negative; Carcinogenic: (+) positive, (-) negative. Source: PreADMET (2023).

3.3.1 Mutagenicity

The mutagenicity is important in the initial assessment of the safety of chemical substances. For this, the Ames test was used, which consists of evaluating the ability of chemical substances to induce reverse mutations in specific genes of specific bacterial strains, such as *Salmonella typhimurium* (TA98, TA100 and TA1535). This analysis is sensitive to certain compounds that can cause DNA damage and lead to mutations (Ames et al., 1975).

The Ames test was considered positive when there was a reversion of the mutation in one or more bacteria, and negative when no reversion was observed in them. According to the results (Table 3), theograndin I, theograndin II and hypolaetin B were considered mutagenic, causing reversion in the bacteria. However, isoscutelarein A and hypolaetin A were non-mutagenic, which are considered more viable for future studies. These results may be related to the presence of sulfate in the molecules, since hypolaetin A and isoscutelarein A do not present this group.

Mutogenic agents can alter the base sequence of DNA, potentially triggering carcinogenicity. In this sense, in Table 3, as well as compounds with mutagenic potential, compounds with non-mutagenic potential presented carcinogenic potential.

3.3.2 Carcinogenicity

All molecules showed carcinogenic potential for both genders in rats and mice (Table 3). It is inferred that in silico models seek to estimate the acute and chronic toxicity of substances, as well as their ability to cause long-term adverse effects in mice and rats, through the prediction of the carcinogenic potential of compounds in rodents (Rodent Carcingogenicity) carried out based on data from the National Toxicology Program (NTP) and FDA (Food and Drug Administration) (PreADMET, 2020). These results may be related to the accumulation of the substances in these larger organisms, which, consequently, can cause carcinogenicity through genotoxicity (Kishino et al., 2019). It is understood that the metabolites of the cupuaçu seed are not promising in terms of toxicity, however, more specific scientific studies are necessary to understand the mechanisms involved in these flavonoids on mutagenicity and carcinogenicity.

After analyzing the results, all molecules were not favorable for human intestinal absorption parameters, MDCK cells and Caco2 cells, although isoscutelarein A and hypolaetin A indicate moderate permeability and toxicity. Regarding distribution, they were not promising on the ability to cross the blood-brain barrier, however, they presented a good profile in terms of binding to plasma proteins, as the metabolites bind weakly, consequently there would be no competition and drug interactions. The metabolization parameter was ineffective, having the potential to inhibit CYPs, therefore it could cause interaction with other

drugs or endogenous substances, with the possibility of toxicity. The flavonoids had unfavorable results on the toxicity parameters, they showed toxicity in *Medaka* fish, however, they did not show toxicity in algae and Daphnia sp. Furthermore, they were carcinogenic, with only hypolaetin A and isoscutelarein A considered non-mutagenic. Finally, in relation to the prediction of biological activities, the molecules showed promise.

4. Conclusion

In short, in relation to pharmacokinetic prediction, no metabolite obtained satisfactory results in the analyzed parameters, except for isoscutelarein A and hypolaetin A, which showed moderate permeability in Caco2 cells, but with non-significant relevance. Furthermore, based on toxicity, no metabolites were promising as they presented positive results for at least one toxicity parameter. On the other hand, the biological activities found were promising, with results that demonstrate great clinical importance, requiring in vitro studies, followed by in vivo evaluation to better understand their mechanisms of action. In our study, flavonoids did not present promising pharmacokinetic and toxicological profiles, limiting the potential for the development of new drugs based on their structures. However, the applicability of technologies could improve the molecules, preserving their biological activities, such as molecular modeling which allows the adjustment of molecules, improving their stability, as well as the use of nanotechnology, use of transporters and formulation of prodrugs to improve the solubility, bioavailability, and stability, allowing more efficient absorption.

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