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CAMU-CAMU (*MYRCIARIA DUBIA)* SEED EXTRACTS AS A NOVEL SOURCE OF BIOACTIVE COMPOUNDS WITH PROMISING ANTIOXIDANT, ANTIMUTAGENIC, CYTOTOXIC, ANTIMALARIAL AND ANTISCHISTOSOMICIDAL PROPERTIES

Alfenas/MG
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DEDICATION

To God for being my source of faith and peace.

To my parents, Luiz e Luciene for being my inspirations of goodness, honesty, integrity and work hard. Thank you for the warm lunch, clean house and washed clothes over these years. This achievement is our!

To my sister Luíza and my boyfriend Leandro for the moments of outburst, affection, love and understanding.You rejoice my days!

To my advisor Luciana for opening so many ways and believing in my work.

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LIST OF ILUSTRATIONS

SUMMARY

1 INTRODUCTION

Currently, there has been an increase in the scientific interest regarding natural products in relation to their potential application, chemical composition and positive biological effects to human health. There is a large number of underexploited native and exotic fruit species in Brazilian biomes, representing a potential interest for the industries and for the local population (ALMEIDA et al., 2011). *Myrciaria dubia* is a native Amazonian bush from the Myrtaceae family commonly known as camu–camu, "caçari" or "araçá d'água", with natural occurrence during periods of flooding. This species grows near courses of rivers and lakes in the Amazon Forest and Peru (AZEVEDO et al., 2019; DA SILVA et al., 2012; FIDELIS et al., 2018; FUJITA et al., 2017).

The camu-camu seeds, which comprehend about 20% of the fruit weight, is discarded without taking benefit of their chemical components and potential application by the food and pharmaceutical industries (CARMO et al., 2019). Camu-camu byproducts, such as seeds, have been associated with *in vitro* and *in vivo* functional properties, such as anti-inflammatory (YAZAWA et al., 2011), antimicrobial (MYODA et al., 2010) antihypertensive and neuroprotective (FIDELIS et al., 2018).

Cancer has attracted worldwide attention, although traditional therapies like surgery, chemotherapy, and radiotherapy have some effects to treat cancer, besides the drug resistance and toxicities (YI et al., 2019). Moreover, parasitic diseases such as malaria, schistosomiasis and leishmaniasis, are one of the world's most devastating and prevalent infections, causing millions of morbidities and mortalities annually. (MOMCILOVIC et al., 2018). Taking into account the interst of biopharmaceutical application of natural products, researches have shown that some polyphenols may act as antiproliferative agents against several types of cancer (LEÓN-GONZÁLEZ; AUGER; SCHINI-KERTH, 2015; TAKASHINA et al., 2017), besides the close relationship between flavonoids and antiparasitic properties (KRETTLI, 2009; MWANGI et al., 2017).

The present dissertation aimed to review the role of phenolic compounds on oxidative stress and development of functional products. Futhermore, as we believe in camu-camu seeds potential, we also described their chemical profile and evaluate their *in vitro* cytotoxic, antioxidant, anti-mutagenic, anti-hemolytic and antiparasitic properties. For this, the study was conducted into three chapters, (1) polyphenols as potential antiproliferative agents: scientific trends; (2) hydroalcoholic *Myrciaria dubia* (camu-camu) seed extracts prevent chromosome

damage and act as antioxidant and cytotoxic agents; (3) camu-camu (*Myrciaria dubia*) seeds as a novel source of bioactive compounds with promising antimalarial and antischistosomicidal properties.

2 OBJECTIVES

2.1 GENERAL OBJECTIVES

The present work aims to review the relationship among polyphenols, oxidative stress and antiproliferative activity, besides to evaluate the biological properties of camu-camu (*Myrciaria dubia*) seed extracts.

2.2 SPECIFIC OBJECTIVES

This study was carried into three main specific goals:

a) To review the effects of polyphenols from food matrix under their activities on oxidative stress and antiproliferative activity, besides their technological applications by the industries. This goal is presented in the Chapter 1.

b) To study the properties of camu-camu seed extract compounds, such their phenolic profile, antioxidant capacity, *in vitro* cytotoxicity against cancer and normal cells, protective effects on cisplatin-induced chromosome damage and finally, to investigate the correlation between the phenolic composition and biological activities. This aim is showed in the Chapter 2.

c) To evaluate the effects of camu-camu seed extracts against *in vitro* cultures of *Plasmodium falciparum, Schistosoma mansoni, Leishmania amazonensis,* besides to correlate individual phenolic constituints with the antiparasitic and anti-hemolytic activities. This goal is pointed out in the Chapter 3.

3 LITERATURE REVIEW

3.1 CAMU-CAMU (*Myrciaria dubia*)

The Amazon region is home to a vast diversity of fruit very rich in vitamins, minerals and bioactive compounds; however, there is very little knowledge about chemical, biological and toxicological properties of many of these species (DA SILVA et al., 2012). *Myrciaria dubia* is a native Amazonian bush from the Myrtaceae family commonly known as camu–camu, "caçari" or "araçá d'água", with natural occurrence during periods of flooding, near courses of rivers and lakes in the Amazon Forest and Peru (AZEVEDO et al., 2019; FIDELIS et al., 2018; FUJITA et al., 2017). The shrub grows to a height of 1 to 3 m, with globular fruits with a diameter of 1.0–3.2 cm (Figure 1). It has a thin, shiny skin with a juicy, pink pulp surrounding one to four seeds. In general, the fruit is not consumed *in natura*, except for the indigenous people who inhabit the fruit's natural territories, because of its very high acidity; rather, it is normally consumed in the form of juices, purees, and pulp, the last to support beverage production and powder as a food additive (LANGLEY et al., 2015).

Camu-camu pulp has great nutritional value, mainly due to the higher amounts of bioactive compounds and ascorbic acid besides the considerable antioxidant capacity (AZEVEDO et al., 2019; FIDELIS et al., 2018; MYODA et al., 2010). Phenolic compounds are secondary metabolites, widely found in fruits, vegetables and grains (CARMO et al., 2019). Some of these phenolic compounds from camu-camu presented antioxidant, antimicrobial (MYODA et al., 2010), antyhipertensive (FIDELIS et al., 2018), antimutagenic and anti-inflammatory (YAZAWA et al., 2011) properties and may contribute to counter oxidative stress-induced chronic diseases when consumed as part of the diet, due to high vitamin C, and rich phenolic profiles, such as flavonoids and ellagitannins. The phenolic compounds found in camu-camu pulp are quercetin, cyanidin-3-glucoside, ellagic acid and ellagitannins (FRACASSETTI et al., 2013; NASCIMENTO et al., 2017). The seeds and peels of camu-camu may present higher antioxidant potential and important levels of phenolic compounds (MYODA et al., 2010) when compared to pulp, once most bioactive compounds are retained on these fruit parts. However, these by-products are usually discarded without taking benefit of their chemical components. Recently, the extraction and analyzes of bioactive compounds from by-products of fruits has been increasingly studied in order to avoid important losses and wastes, besides representing potential benefits for applications in the industry (FIDELIS et al., 2018; MYODA et al., 2010). With such a high phenolic bioactive profile and antioxidative

potential, camu camu can be incorporated with other functional ingredients and foods for diet based management of oxidative stress linked non-communicable chronic diseases (NCDs) and parasitic diseases, such as cancer and malaria, respectively.

Figure 1- Camu-camu fruit.

Source: https://www.sitiodamata.com.br/camu-camu-myrciaria-dubia

3.2 OXIDATIVE STRESS AND CANCER

Oxygen-free radicals, more generally known as reactive oxygen species (ROS) are well recognised for playing a dual role as both deleterious and beneficial species ROS act as secondary messengers in cell signaling and are required for various biological processes in normal cells. Under physiological conditions, ROS are continuously generated by ROS producers and eliminated through ROS scavenging systems in order to maintain redox homeostasis (VALKO et al., 2006). Cells aim to maintain a redox balance that is ideal to support cellular processes like differentiation and proliferation and allow for the adaptation to metabolic and immune stress. Changes in redox balance, which can have endogenous or exogenous causes, can either lead to an increase in ROS levels or rate of production, resulting in cell damaging oxidative stress and aberrant cell signaling, or a decrease in ROS, leading to a disruption of cell signaling and therefore disruption of cellular homeostasis. Redox imbalance, oxidative stress, which are often a result of changes in cancer cell metabolism, and aberrant antioxidant levels to balance this stress, are hallmarks of many cancers. The role of ROS in cancer is two-sided. One the one hand, ROS can contribute to cancer initiation, progression and spreading through the activation and maintenance of signaling pathways that regulate cellular proliferation, survival, angiogenesis and metastasis. Through this role in promoting tumorigenic cell signaling events, ROS are considered oncogenic. However, on the other hand, the excessive

levels of ROS in cancer cells can also induce cell death signaling, senescence and cell cycle arrest. In the context of this imbalanced redox status, oncogene-induced cancer cells adapt and increase antioxidant pathways and regulators leading to increased ROS scavenging, in order to maintain ROS levels that allow pro-tumorigenic signaling pathways to be activated without inducing cell death. Various studies have shown that further ROS elevation, either through ROS producers or antioxidant inhibitors, can selectively kill cancer cells and suppress tumor growth and progression in various cancer cell lines (DO CARMO et al., 2018; GLASAUER; CHANDEL, 2014) .

3.3 PARASITIC DISEASES

Neglected diseases (NDs) are a great public health problem and induce an important socioeconomic impact worldwide. Their treatments are still precarious, and, in general, NDs affect poor and marginalized people. Therefore, there is an urgent need for new drugs to treat them (SANTOS et al., 2020). Parasitic diseases are one of the world's most devastating and prevalent infections, causing millions of morbidities and mortalities annually. In the past, many of these infections have been linked predominantly to tropical or subtropical areas. Nowadays, however, climatic and vector ecology changes, a significant increase in international travel, armed conflicts, and migration of humans and animals have influenced the transmission of some parasitic diseases from ‘book pages’ to reality in developed countries (MOMCILOVIC et al., 2018).

Leishmaniasis is considered an extremely ND, caused by flagellate protozoan of the genus *Leishmania*, prevalent in tropical and subtropical areas in Africa, Asia and Latin America. This disease affects approximately 2 million people per year, and the infection is mainly related to environmental changes, poor socioeconomic conditions and immunosuppression. Transmission occurs through the sandflies bite, as showed in Figure 2. *Leishmania spp*. causes a set of diseases with different severity, thus it can manifest in the visceral, mucosal, cutaneous or mucocutaneous forms, depending on the strains and host immune response (SANTOS et al., 2020).

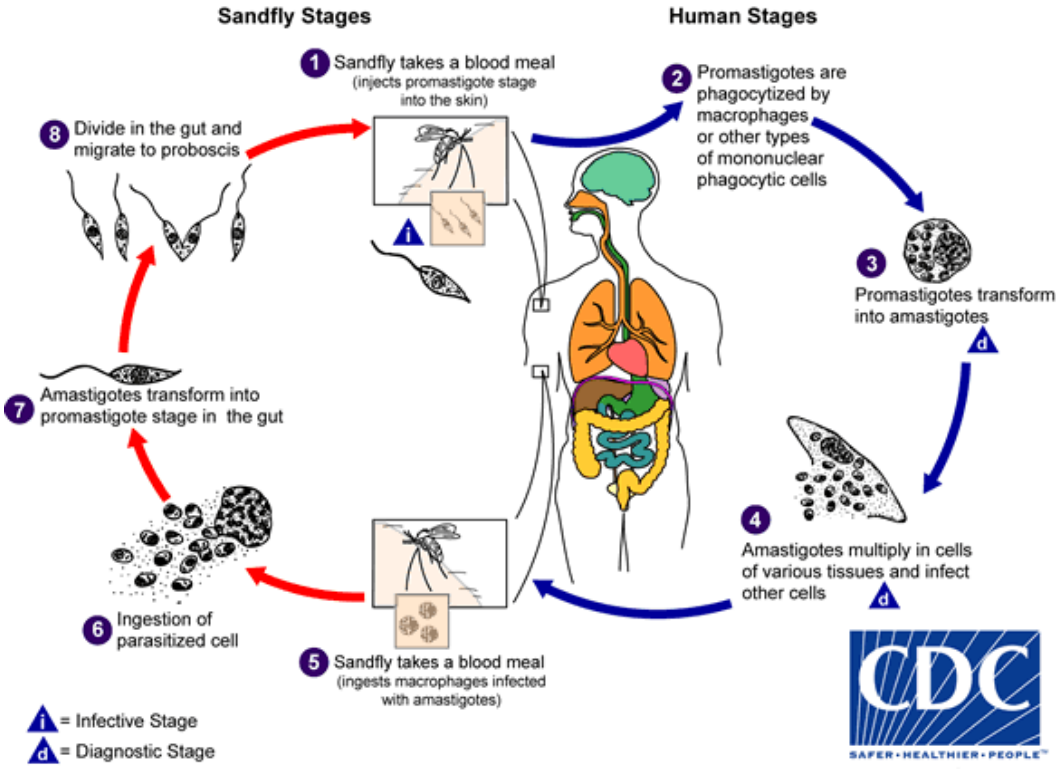
Malaria in humans is caused by five species of parasites belonging to the genus *Plasmodium*. Four of these- *P. falciparum, P. vivax, P. malariae* and *P.ovale* infect humans and spread from one person to another by the bite of female mosquitoes of the genus *Anopheles.* Malaria is endemic throughout most of the tropics. Around 3.2 billion people live in areas affected by malaria, 1.2 billion are at high risk; the World Health Organization (WHO) reported

that there were 214 million cases of symptomatic malaria in 2015 (SAHEB, 2018). Common complications like circulatory sequestration, kidney failure, respiratory dysfunction, and anemia are well-known characteristics of the *P. falciparum* severe malaria. The latest countrywide epidemiological status by the National Vector Borne Disease Control Programme (NVBDCP) reveals that *P. falciparum* infections are accountable for 49.99% of the total malaria cases. Its life cycle is pointed out in Figure 3. Antimalarial treatment plays a critical role in avoiding complications of malaria infection, often these do not protect from the malaria related complications, thus leading to life-threatening situations (BHARDWAJ et al., 2020).

Schistosomiasis is a helminthiasis caused by digeneic trematodes, belonging to the genus *Schistosoma* and it is considered the most important helminthiases in terms of mortality and morbidity. Major species causing for human schistosomiasis are *Schistosoma mansoni, S. japonicum, S. mekongi, S. guineensis* and *S. intercalatum*, responsible for intestinal schistosomiasis, whereas *S. haematobium* provokes urogenital schistosomiasis. The infection happens through contact with water contaminated with cercariae, infective larval phase for the definitive host (Figure 4). Schistosomiasis is endemic in 78 countries and just in 52 the preventive chemotherapy has been applied, affecting more than 250 million people, though this number likely is underestimated. In world, around 206.4 million people need to preventive treatment, being that 118.5 million are in school-aged children. In 2016, more than 89 million people received the preventive chemotherapy for schistosomiasis and the mortality is estimated of 200,000 deaths every year (SANTOS et al., 2020).

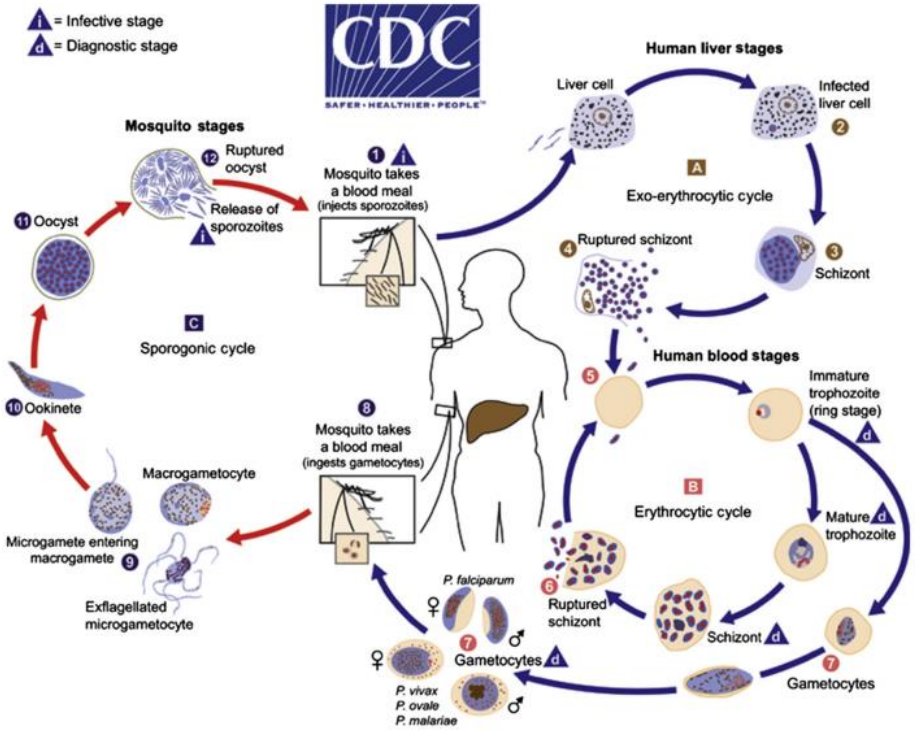
In light of these above concerns, several scientific studies on safety and efficacy have been conducted with medicinal herbs that are used for management of malaria, leishmaniasis, and schistosomiasis (KRETTLI, 2009; MWANGI et al., 2017). Thus, camu-camu seed extracts, due their bioactive compounds content, may be considered a promising source of novel drug structures for malaria, schistosomiasis and leishmaniasis treatments.

Figure 2- Life cycle of human Leishmaniasis



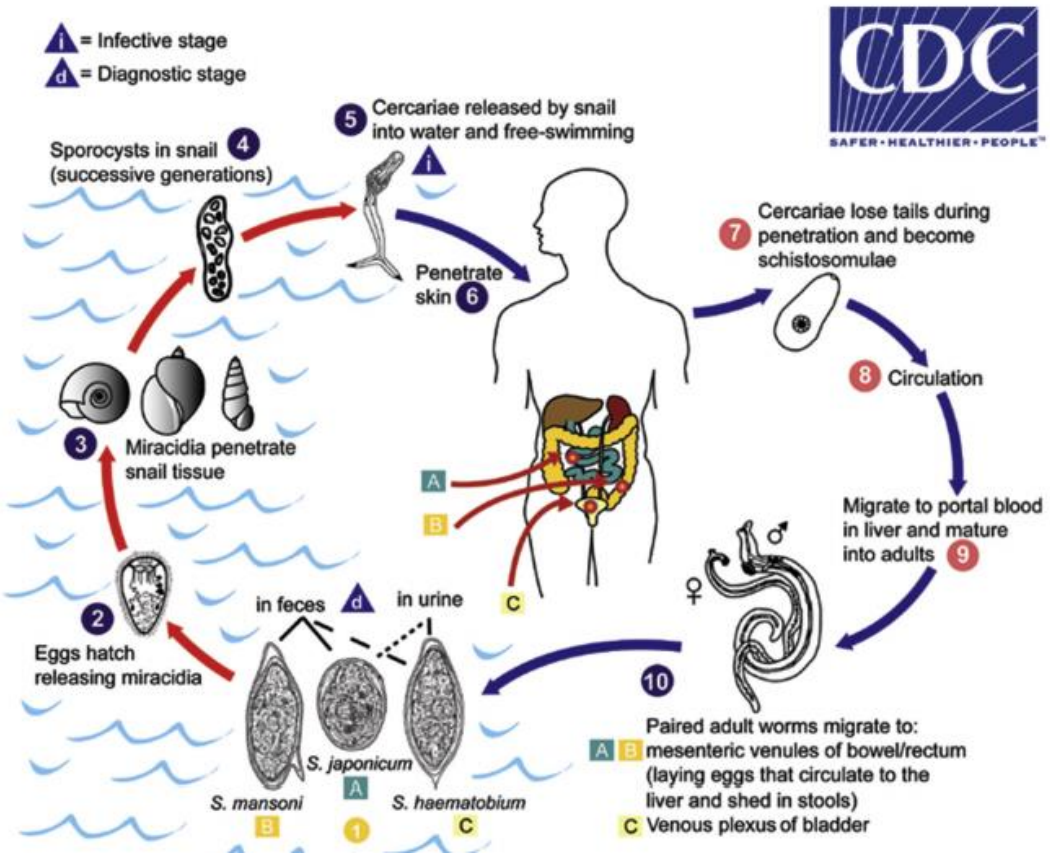
Source: CDC Division of Parasitic Diseases

Figure 3- Life cycle of *Plasmodium falciparum*



Source: CDC Division of Parasitic Diseases.

Figure 4- Life cycle of human schistosomes



Source: CDC Division of Parasitic Diseases

Chapter 1

Polyphenols as potential antiproliferative agents: scientific trends

ABSTRACT

Bioactive compounds are associated with decreased in oxidative stress, inflammation and consequently in risk of non-communicable diseases. Polyphenols have demonstrated potential biological activity in many diseases, such as cancer, diabetes, inflammation, obesity-related diseases, neurodegenerative disorders, bacterial and viral infections or cardiovascular diseases due to antioxidant and prooxidant capacities. Indeed, these compounds may still have many applications in the pharmaceutical and food industries, with potential functional properties in vivo. This review article discusses the relationship between polyphenols, their antiproliferative effects and the mechanisms involved, oxidative stress, technological applications and future perspectives in this research field.

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Current Opinion in Food Science

Polyphenols as potential antiproliferative agents: scientific trends

Mariana Araújo Vieira do Carmo[1], Carolina Girotto Pressete[1], Marcos José Marques[3], Daniel Granato[2] and Luciana Azevedo[1]

Bioactive compounds are associated with decreased in oxidative stress, inflammation and consequently in risk of non-communicable diseases. Polyphenols have demonstrated potential biological activity in many diseases, such as cancer, diabetes, inflammation, obesity-related diseases, neurodegenerative disorders, bacterial and viral infections or cardiovascular diseases due to antioxidant and prooxidant capacities. Indeed, these compounds may still have many applications in the pharmaceutical and food industries, with potential functional properties *in vivo*. This review article discusses the relationship between polyphenols, their antiproliferative effects and the mechanisms involved, oxidative stress, technological applications and future perspectives in this research field.

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Polyphenols effect on oxidative stress and cell proliferation

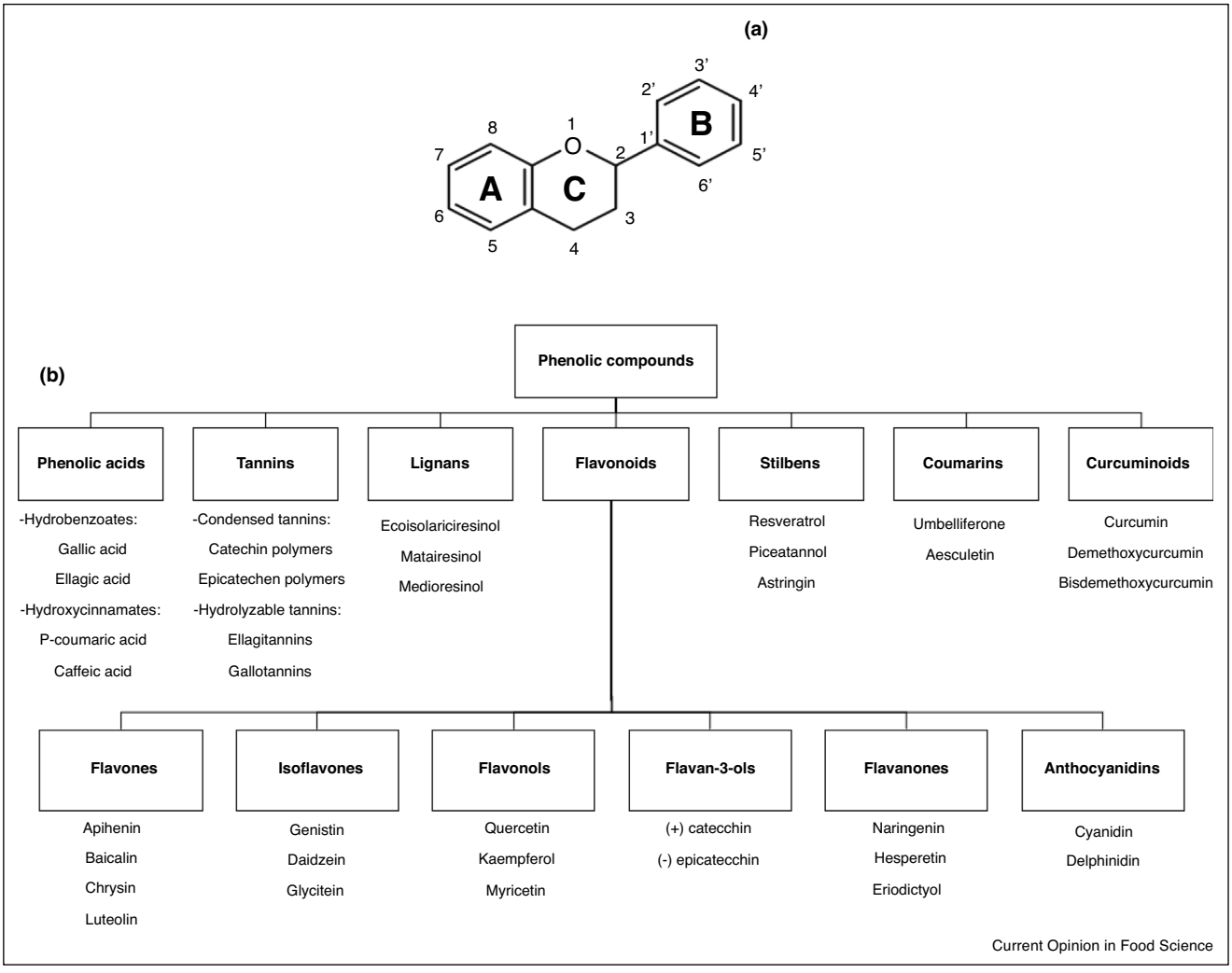
Polyphenols are secondary metabolites in plants with a common aromatic ring bearing one or more hydroxyl groups [1–3]. More than 8000 natural phenolic compounds have been identified so far [2–4]. These compounds can be classified into different subclasses according to the number of aromatic rings in their structure, the elements that bind the rings, and the substituents linked to the rings [2]. Polyphenols have heterogeneous structures, which range from low molecular weight single aromatic ring structure to high molecular weight polymeric compounds, thus being broadly classified into simple and complex phenolics [3]. The typical structural characteristic shared by most polyphenols is the three-membered flavan skeleton [5] (Figure 1a).

Polyphenols as potential antiproliferative agents

These compounds are basically divided into several subclasses, such as flavonoids, coumarins, stilbenoids, lignans, tannins, and phenolic acids (Figure 1b), which have been characterized and studied extensively for their health-promoting properties [1,4]. Their antioxidant capacity can be attributed by radical scavenging mechanism, in which the polyphenols sacrificially reduce ROS/RNS, such as $^{\bullet}OH$, $O_2^{\bullet}$-, $NO^{\bullet}$, or OONO-, preventing more ROS generation, and biomolecules damage [5]. Furthermore, their hydroxyl functions are potent H^+ donors for free radical acceptors due to electron delocalization across the molecule, and, as consequence, they may lead to DNA damage and genetic mutations [6]. Interestingly, some polyphenols are effective metal chelators [7]. In this sense, when H_2O_2 is present, as a result of oxidative stress, redox active metal ions such as Fe^{+2} or Cu^{+2} that are localized or bound to the DNA react with H_2O_2 to form highly reactive $^{\bullet}OH$ by Fenton and Haber-Weiss reactions. Preventing this event, polyphenols are easily deprotonated at physiological pH in the presence of iron and form very stable complexes. Therefore, iron binding has been also proposed as another mechanism for polyphenols antioxidant activity [8]. Thus, polyphenols molecules could be potent scavengers of reactive oxygen species (ROS), reactive nitrogen species (RNS), and metal chelating agents. Accordingly, these compounds perform a preventative strategy against mutation-related diseases [9], acting dynamically in balance between the ROS generation and the antioxidant capacity [10,11•,12].

The homeostasis of reactive oxygen and nitrogen species is essential for cell survival and normal cell signaling, which is achieved by non-enzymatic molecules include glutathione, polyphenols, vitamin A, C and E, and enzymatic antioxidants include superoxide dismutase (SOD), superoxide reductase, catalase, glutathione peroxidase (GPX), glutathione reductase, peroxiredoxin (PRX), and thioredoxin (TRX) [13••,14••]. Protein products of tumor suppressor genes can also behave as antioxidants. For example, p53 has been shown to decrease ROS accumulation by regulating the

Figure 1



(a) Flavan general structure, showing the ring labeling and numbering system (Adapted from Perron e Brumaghim, 2009). **(b)** Classification of phenolic compounds (Adapted from Liu, 2003).

expression of various endogenous antioxidant enzymes, such as catalase. However, it is well established that p53 is lost or mutated in more than 50% of cancers, allowing for ROS accumulation and pro-tumourigenic signaling to occur [15]. For instance, mutagenesis is a determinative event in the initiation stage of carcinogenesis and polyphenols decrease DNA damage induced by various carcinogens, due in part to their antioxidant properties, acting as ROS scavengers, transition metal chelators, or by modulating the activity and/or expression level of oxidative stress-related enzymes [16]. This occurs by different signaling pathway and proteins, involving markers of cell proliferation, such as increase of p53 [17–19], p21 [19], Bax and ROS [20,21] and decrease of Bcl-2 [22,23], viable strategy to inhibit tumor growth [3].

Healthy cells have developed specific adaptations to overcome the damaging effects of ROS, through the balanced generation of these species, sufficient antioxidant activity and cellular repair, which result in low concentrations of ROS, toward to limited cell survival and proliferation. Metabolic activity of tumour cells yields high ROS concentrations enhancing their cell survival and proliferation, through many pathways including PI3K/AKT, MAPK/ERK1/2 and PKD and inactivation of their downstream targets, such as Bad, Bax, Bim, Foxo, and PTEN. These event leads to DNA damage, decreased cellular repair by faithful DNA damage repair pathways and genetic instability. Elevated ROS levels can cause cellular damage. However, tumour cells readjust with adequate adaptations to conditions including hypoxia and increased antioxidant activity to remove excessive ROS/RNS while maintaining pro-tumourigenic signaling. If ROS/RNS levels increase dramatically to toxic concentrations, the JNK pathway can be activated resulting in apoptosis initiated by intrinsic

apoptotic signaling in the mitochondria or extrinsic apoptotic signaling by death receptor pathways [13••]. In this respect, there is enough evidence that exogenous antioxidants (i.e. phenolics) produce positive effects in several cancers, but whether an antioxidant supplement would be helpful, harmful or neutral depends on the specific antioxidant, its dose, the chemotherapy drugs being used, and the type and stage of cancer [24]. Indeed, the antioxidant action of phenolic compounds may effectively exhibit cytoprotective effect on normal cells by inhibiting neoplastic transformation. However, when the tumor is already installed, these compounds could accelerate tumor growth and favor metastases [25] or they could exert cytotoxic effect on neoplastic cells by acting as prooxidant [26]. Polyphenols also could produce antimetastatic efficacy by significantly downregulate the expression of matrix metalloproteinases, such as MMP-2 and MMP-9, which promote cellular invasion [27].

Despite polyphenols are widely known for high antioxidant capacity, they are able to exert multiple health benefits by acting as prooxidant [28]. It has been observed that epigallocatechin-3-gallate (EGCG), *trans*-resveratrol, quercetin, and curcumin, which produce hydrogen peroxide, can efficiently kill tumor cells, without dramatically affecting normal cells [16]. The curcumin induces ROS production by activation of mitochondrial enzymes driving to apoptotic effects and cell death [29,30]. The induction of ROS by curcumin could occur through its interaction with thioredoxin reductase and thus changing its activity to NADPH oxidase [29]. Curcumin was shown to be responsible for the inhibition of AK-5 tumor (a rat histiocytoma) growth by inducing apoptosis in AK-5 tumor cells via caspase activation, due to the hyperproduction of reactive oxygen species, which leaded a loss of mitochondrial membrane potential and cytochrome C release to the cytosol [30]. These observations point out that polyphenols may interfere in the "two-faced" character of ROS, which acts as secondary messengers in intracellular signaling cascades, with induction and maintenance of cancer cell oncogenic phenotype, however, ROS can also induce cellular senescence and apoptosis and can therefore function as anti-tumourigenic species [31].

The cumulative production of ROS/RNS through either endogenous or exogenous insults induce a cellular redox imbalance and plays a main role in the cytotoxic activity on cell proliferation, wherein oxidative damage to membrane lipids and other cellular constituents is a major reason in its toxicity [31,32].

Thus, bearing these considerations, the cytostatic and cytotoxic effects are important to attenuate uncontrolled cell proliferation. Cytostatic drugs have the pharmacological function of inhibiting or preventing cell multiplication, so this group of drugs is capable of slowing the evolution of the tumor. On the other hand, cytotoxic drugs are able to cause cancer cells death [33]. Ramirez-Mares, Kobayashi & de Mejia [34] described some important parameters to assess the tumour cell viability when an *in vitro* method is used: IC_{50}, GI_{50} and LC_{50}. The IC_{50} is the concentration of the agent that inhibits growth by 50% at moment of treatment, where $(T/C) \times 100 = 50$, T = number of cells at treatment time; C = control cells at time t of treatment. The GI_{50} is the concentration of the agent that inhibits growth by 50%, taking into account the untreated cells and number of cells from start of treatment, where $([T - T0]/[C - T0]) \times 100 = 50$, T0 is the number of cells at time zero, T and C are the number of treated and control cells, respectively, at time t of treatment and $T > T0$. LC_{50} is the concentration of the agent that results in a net loss of 50% cells, relative to the number at the start of treatment, where $([T - T0]/T0) \times 100 = -50$; $T < T0$.

Regarding the cytostatic and cytotoxic effects, it is necessary to highlight that the transformation in the human gastrointestinal tract by the enzymes and microbiota can change the mechanisms of absorption, transportation, bioavailability, and bioactivity of polyphenols and consequently in their IC_{50} values [18,35]. In addition, the absorption of these compounds can be also influenced by solubility, interaction with some dietary constituents, molecular changes, protein transporters, human organisms metabolism and, lastly, effects of gut microbiota [36•]. Although most studies on polyphenol bioavailability use mainly pure compounds, the interactions between diet and extract compounds give substantial difference in bioavailability assays, which happens with using of single molecules (isolated from food or chemically synthesized) and whole foods. When dealing with plant extracts, one must be careful because the biological activities of individual phenolics may not be able to accurately implicate the observed activities for the whole extract [37]. In this sense, only polyphenols released from the food matrix during the digestive process (named *bioaccessible polyphenols*) are potentially bioavailable or susceptible to absorption through the gut barrier [38].

Bearing these considerations, biochemical interactions (between phytochemicals and foods/drugs) could eliminate, reduce and even improve their bioactivity, making these compounds harmful or beneficial to the organisms. It is very important not forget that through the entire human metabolism numerous compounds are converted on their active forms, while others are inactivated or even linked to several biomolecules that can change the original effect and none of those aspects could be achieved by using *in vitro* studies. Despite these considerations the *in vivo* experiments remain poorly investigated, while the *in vitro* studies have increased exponentially [36•].

In this respect and taking into account the difference between the polyphenol content in the food matrix and its absorption, studies have shown that the bioavailability of bioaccessible polyphenols in the small intestine is very low,

Table 1

Summary of literature data regarding the anticancer activity of the phenolic compounds against various cell lines with their IC_{50} values

Extracts/product	Major phenolic compounds	Experimental models	Pathway/proteins involved	IC_{50}	Reference
Ajuga bracteosa extract	Pyrocatechol, gallic acid, resorcinol, catechin, chlorogenic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, vanillic acid, coumarin, sinapinic acid, *trans*-cinnamic acid, rutin, quercetin and kaempferol	THP-1 leukemia cells and human hepatocarcinoma cells (HepG2)	–	THP-1cells: methanol-acetone extract (4.70 +/− 0.43 μg/mL); HepG2 cells: n-hexane, ethyl acetate and methanol-distilled water extracts (8.65–8.95 μg/mL)	Zahra *et al.* [40]
Quercus dilatata extract	Chlorogenic acid, coumarin, *p*-coumarin, gallic acid, quercetin, catechin	THP-1 leukemia cells, human hepatocellular carcinoma cells (HepG2)	–	HepG2 cells > 20 μg/mL (all extracts); THP-1 cells: ethyl acetate + acetone (3.88 ± 0.53 μg/mL); methanol + ethyl acetate (5.59 ± 0.25 μg/mL); ethanol (4.95 ± 0.53 μg/mL); distilled water (9.24 ± 0.53 μg/mL) and > 20 μg/mL for all other extracts	Ahmed *et al.* [41]
Seventeen trihydroxyflavone derivatives	Seventeen trihydroxyflavone derivatives, including apigenin and baicalein	Human lung carcinoma cell (A549), human breast adenocarcinoma cell (MCF-7), and human glioblastoma cell (U87)	–	A549 cell: apigenin (77.5 ± 9.2 μM), baicalein (68.2 ± 3.6 μM); MCF-7 cell: apigenin (71.5 ± 15.6 μM), baicalein (26.1 ± 7.1 μM); U87 cell: apigenin (>100.0 μM), baicalein (>100.0 μM)	Grigalius e Petrikaite, [42]
Morus alba L. extract	Caffeic acid, chlorogenic acid, cyanidin-3-O-glucoside, cyanidin-3-O-galactose, quercetin, procyanidins B2 and myricetrin	Human hepatocellular carcinoma cells (HepG2)	–	Acetone/ acetic acid/water (28.2 mg/mL)	Li et al. [43]
Resveratrol	Resveratrol analogs	Acute lymphoblastic leucemia cells	Compounds caused G1 ↑ phase arrest p53	Resveratrol (10.5 μM), resveratryl triacetate (3.4 μM), resveratryl triisobutyrate (5.1 μM), resveratryl triisovalerate (4.9 μM) and >10 μM for all other analogs	Urbaniak *et al.* [17]
Red wine pomace seasonings extract	Hydroxybenzoic acid, hydroxycinnamic acid	Human colon adenocarcinoma cell line (HT-29)	↑ p53	Seedless wine pomace (845 μg/mL), isolated seeds prior digestion (1085 μg/mL) and digested fractions (814 μg/mL)	Del Pino-Garcia *et al.* [18]
Red and White Wine extract	Resveratrol, quercetin, gallic acid and tyrosol	Prostate Cancer Cells (PC-3)	↑ ROS, H2O2, NO GSH ↓		Tenta *et al.* [21]
Analogs of gut microbe-derived metabolites	*trans*-Cinnamic acid	Human colon carcinoma cells (HT29), human lung carcinoma cells (H460), human lung adenocarcinoma cells (A549) and human pancreatic carcinoma cells (MIA PaCa-2)	↑ BAX, activation of cleavage of poly ADP ribose polymerase ↓ PARP, Bcl-2, histone deacetylases markers	HT29 (1.07 ± 0.38 mM) H460 (2.10 ± 0.43 mM); A549 (3.54 ± 0.34 mM); MIA PaCa-2 (1.33 ± 0.07 mM)	Zhu *et al.* [22]
Ferulic acid	Ferulic acid	Human pancreatic carcinoma cells (MIA PaCa-2)	↓ CCND1, CDK 4/6, Bcl2, caspase 8, caspase 10 ↑ p53, Bax, PTEN caspase 3, caspase 9	500 μM	Fahrioglu *et al.* [23]

Table 1 (*Continued*)

Extracts/product	Major phenolic compounds	Experimental models	Pathway/proteins involved	IC_{50}	Reference
p-Coumaric acid product	*p*-Coumaric acid	Neuroblastoma cells (N2a)	↑ ROS, p53, caspase 8, LC3-II protein and acridine orange-stained autophagosomes (autophagy markers) ↓ GSH	104 μM	Shailasree *et al.* [20]
Ferulic acid product	Ferulic acid	Prostate cancer cell (PC -3 and LNCAP)	↑ gene expressions of ATR, ATM, CDKN1 A, CDKN1B, E2F4, RB1, and TP53 (PC -3 cells) ↓ gene expressions of CCND1, CCND2, CCND3, CDK2, CDK4, and CDK6 (PC -3 cells) ↑ gene expressions of CASP1, CASP2, CASP8, CYCS, FAS, FASLG, and TRADD (LNCAP cells) ↓ gene expressions of BCL2 and XIAP (LNCAP cells)	PC -3 (300 μM); LNCAP (500 μM)	Eroglu *et al.* [44]
Propolis extract	Caffeic acid phenyl ester	Breast cancer cell (MDA-MB-23 and Hs578 T)	↓ NF-κB, BCL2 ↑ Caspase 3, Bax	MDA-MB-23 cell: ethanol extract of propolis (48.35 μg/mL) and caffeic acid phenyl ester (14.08 μM); Hs578 T cell: ethanol extract of própolis (33.68 μg/mL) and caffeic acid phenyl ester (8.01 μM)	Rzepecka-Stojko *et al.* [45]
Rosmarinus officinalis L. extract	Rosmarinic acid, carnosol, carnosoic acid, and methyl carnosate	Human adenocarcinoma cell (CACO-2), human immortalized macrophage (U937)	↑ cell cycle arrest in S phase (U937) ↓ G1 and G2/M phases (U937)	CACO-2 cell (14.95 ± 2.32 μg/mL); U937 cell (14.85 ± 0.20 μg/mL)	Amar *et al.* [4]
Nannochloropsis gaditana extract	Fucoxanthin, violaxanthin, lycopene, neoxanthin, lutein, and cantaxanthin.	Human lung adenocarcinoma cells (A549)		Acetonic extract (0.412 mg/mL), methanolic extract (0.512 mg/mL), dichloromethanic extract (0.521 mg/mL), hexanic extract (1.16 mg/mL), aqueous extract (2.308 mg/mL)	Al, (2018) [46]

Table 2

Patent overview of polyphenol for several purposes

Phenolic compounds	Title	Publication number	Publication date	Technology domain	Applicant/Assignee
Cocoa polyphenols (procyanidins)	Cacao polyphenols and their use in the treatment or prevention of eosinophilic esophagitis	BR 11 2016 005727 9	2017/08/01	Pharmaceuticals	Nestec S.A. (CH)
Red grapes polyphenols	Grape-based functional beverage as the main agent containing polyphenols of an intermediate size in a liquid grape "pais" juice	BR 11 2016 013649 7 A2	2017/08/08	Food chemistry	Universidad de Concepción (CL)
Euterpe oleracea polyphenols	Process for the preparation of ointments containing antioxidants obtained from plants rich in polyphenols for use in the treatment of wounds of various etiologies in which there is an increase in pro-oxidant factors and/or high formation of reactive species derived from oxygen and/or minor formation of nitric oxide	PI 1003215-0 A2	2012/03/20	Pharmaceuticals	University of the State of Rio de Janeiro - UERJ (BR/RJ)
Plant polyphenols	Process for obtaining toothpaste and/or mouthwashes (oral antiseptic agents) containing natural or synthetic antioxidants and antioxidants obtained from plants rich in polyphenols used in the prevention and treatment of oral diseases in which there is an increase in pro -oxidants and/or large formation of reactive species derived from oxygen	PI 0705003-8 A2	2009/08/25	Pharmaceuticals	University of the State of Rio de Janeiro - UERJ (BR/RJ)
Polyphenols	A method for significantly reducing levels of astringency and bitterness in polyphenol compositions, and composition comprising polyphenols	PI 0704096-2	2008/08/19	Food chemistry	Kraft Foods Group Brands LLC (US)
	Topical application of nucleic acid inhibitor polyphenols, inducible nitric oxide synthase and nuclear transcription factor alpha in the treatment of inflammatory skin diseases	PI 0401980-6 A2	2005/12/06	Pharmaceuticals	Henry Okigami (BR/GO) / Paulo Takao Okigami (BR/GO)
Red wine polyphenols	Process for obtaining lyophilized and/or similar, polyphenol rich from red wine, pharmaceutical compositions containing the lyophilisate and therapeutic use of the compositions in the prevention and treatment of arterial hypertension and other cardiovascular diseases	PI 0605018-1 A2	2008/03/04	Pharmaceuticals	University of the State of Rio de Janeiro - UERJ (BR/RJ)
Green tea (*Camellia sinensis*) polyphenols	Use of a nutrient composition comprising green tea polyphenols for osteosarcoma treatment	PI 0520221-3 A2	2009/04/22	Pharmaceuticals	Matthias Rath (NL)
Chamomilla recutita polyphenols (apigenin)	Standardized extract of chamomile polyphenols (*Chamomilla recutita*) and similar varieties applied in the gynoid lipodystrophy treatment	PI 0402674-8 A2	2006/02/14	Pharmaceuticals	Henry Okigami (BR/GO) / Paulo Takao Okigami (BR/GO)
Cynara scolymus polyphenols (apigenin)	Standardized extract of artichoke polyphenols (*Cynara scolymus*) applied in the gynoid lipodystrophy treatment	PI 0402673-0 A2	2006/02/14	Pharmaceuticals	Henry Okigami (BR/GO)/ Paulo Takao Okigami (BR/GO)
Epigallocatechin gallate, epicatechin gallate, epigallocatechin, epicatechin, catechin	A pharmaceutical nutrient formulation comprising polyphenols and their uses in the treatment of cancer	PI 0302672-8 A2	2004/02/25	Pharmaceuticals	Matthias Rath (NL)
Carnosic acid	Synergistic composition comprising propolis and carnosic acid for use thereof in treating and preventing infections caused by species of the cryptococcus neoformans fungus	3278798	2018/02/07	Pharmaceuticals/ Food chemistry	Vitalgaia España S L
Flavonoids, anthocyanins, tannins, curcumin	Anti-age composition comprising a combination of antioxidant agents in association with bifidobacteria and cell walls isolated from probiotics	20180050071	2018/02/22	Pharmaceuticals	Bioimmunizer Sagl

Table 2 (*Continued*)

Phenolic compounds	Title	Publication number	Publication date	Technology domain	Applicant/Assignee
Rosmarinic acid, chicoric acid, or caftaric acid	Probiotic and polyphenol against neurodegeneration	US2018028582 (A1)	2018/02/01	Pharmaceuticals	Nestec S.A. [CH]
Tea polyphenols	Water dispersible sterol/stanol enriched polyphenol rich herbal teas in aqueous or powdered forms to reduce total and low-density lipoprotein cholesterol levels	KR20170129176 (A)	2017/11/24	Pharmaceuticals	Tubitak [TR]
	Oral disinfectant and spray bottle for preparation of oral disinfectant	CN107296870 (A)	2017/10/27	Pharmaceuticals	Suzhou kanglijie disinfection tech co LTD
Olive polyphenols	Treatment of early stage Parkinson's disease with a hydroxytyrosol-containing polyphenol formulation	WO2017222598 (A1)	2017/12/28	Pharmaceuticals	Allevium therapeutics inc [US]
Black tea polyphenols	Formula and preparation method of fermented plant beverage with high nutrient content	CN107296122 (A)	2017/10/27	Food chemistry	Henan xiaoyi biological tech co LTD
Gallic acid	A beverage containing wood components	TW201716566 (A)	2017/05/16	Food chemistry	Suntory holdings LTD [JP]
Anthocyanin	Blueberry anthocyanin composite tablet capable of effectively relieving teenager asthenopia	CN107296205 (A)	2017/10/27	Pharmaceuticals	Harbin institute of tech
Tannin	Compositions comprising *Lactobacillus plantarum* strains in combination with tannin and new *Lactobacillus plantarum* strains	USRE46718 (E)	2018/02/20	Food chemistry	Molin Goran [SE]; Ahrne Siv [SE]; Jeppsson Bengt [SE]; Probi Ab [SE]
Epigallocatechin gallate, myricetin, and luteolin	Pharmaceutical composition for preventing or treating Zika virus	KR20170125484 (A)	2017/11/15	Pharmaceuticals	Seoul national univ r&db foundation [KR]
Luteolin, quercetin, kaempferol, isoramnetin, rutin hyperoside, astragaline, ferulic acid, coffee acid, caffeolic acid, rosmaric acid, tannins, catechin	Drugs for toxic hepatitis prevention and treatment	RU2633590 (C1)	2017/10/13	Pharmaceuticals	Fed gosudarstvennoe byudzhetnoe nauchnoe uchrezhdenie vserossijskij nauchno-issledovatelskij inst le [RU]
Carnosic acid, quercetin, resveratrol and gallic acid	Synergistic combinations of carotenoids and polyphenols	WO2010082205	2015/09/27	Pharmaceuticals	ЗЕЛКХА Моррис (IL) ЛЕВИ Рахель (IL) ПАРАН Эстер (IL) ШАРОНИ Йоав (IL) ЛЕВИ Иосеф (IL) ZELKKhA Morris (IL) LEVI Rakhel' (IL) PARAN Ehster (IL) ShARONI Joav (IL) LEVI Iosef (IL)
Proanthocyanidin	Method for aging control of salted fish guts, pickle of fish and shellfish, salted processed food or the like	JP2001252009 (A)	2001/09/18	Food chemistry	Nakajima suisan co LTD
Proanthocyanidin monomers and oligomers (OPC), hydroxystilbenes, flavonoid monomers and oligomers	Composition for combating the signs of ageing of the skin and hair and nails	EP3278798 (A1)	2018/02/07	Pharmaceuticals	Caudalie Ip [GB]; ThomaS Mathilde [FR]; Thomas Bertrand [FR]

eaching values between 5% and 10% [38]. Different results can be observed with a pure compound such as the gallic acid that showed to be less bioaccessible after digestion (from 28.43 to 32.58%) [39••], however with higher percentage than bioaccessible polyphenol from food matrix. In spite of the digestion be able to decrease the bioavailability, the digestive process seems to increase the overall capacity to inhibit cancer cell viability. Del Pino-Garcia *et al.* [18], working with HT-29 cells (human colorectal adenocarcinoma), observed that *in vitro* digestion decreased the IC_{50} values of seedless wine pomace, whole wine pomace, and isolated seeds, which levels ranging from 846–1085 μg/mL before to 718–800 μg/mL after digestion process. Overall, recent studies have demonstrated that phenolic compounds undergo transformation in gut microbiota thereby acquire additional properties that promote their biological activities [3].

The activity of some phenolic compounds found in many plants against a variety of cell lines is summarized in Table 1.

Polyphenols: innovation and applications

Polyphenols - crude extracts or isolated compounds - have been widely used in development of new products (nutraceuticals or foods) and technologies (Table 2). In terms of patents granted, in a search from the Espacenet Patent Search using the internet: <URL: https://worldwide.espacenet.com> it was possible to find roughly 2656 patent claims (as of July 2018) for "Polyphenol" (title/abstract) in all kinds of classifications to date. Espacenet Patent Search is a database covering patent registrations from more than 90 countries while INPE (www.inpe.br) covers records made in Brazil. Between 2001 and 2010, there was an evolution of the global production of articles in the field of technologies of polyphenols and antioxidants extracted from plants. However, the overall production of patent deposits in this same field did not show the same steady growth. In this same period, comparing the number of articles found, it was verified that the production of articles was much more intense than the production of patents, with a ratio of 14.1 articles per patent [47]. On the other hand, comparing 2010 with 2017, there was an increase of approximately 50% of new patent registrations in the fields of pharmaceuticals and the food industry (Table 3). This suggests that there has been an increase in the applicability of scientific research with polyphenols in the development of new products that may offer therapeutic and pharmaceutical properties, ranging from cosmetics to functional drinks, aiming to reduce the oxidative stress and inflammation *in vivo*, such as cancer and cardiovascular diseases. However, the focus is on scientific production, which can lead to the assumption that technology is emerging, at the moment and therefore, the patenting process is still limited, as there are several factors that may be involved, such as bureaucratic and financial issues. More than 8000 phenolic compounds have already been identified and many of them already have known antiproliferative, anticarcinogenic and antimutagenic properties. Nevertheless, the technological application and *in vivo* evaluation (pre-clinical and clinical trials) of these compounds for the development of potentially functional foods is needed [48].

Table 3

Number of polyphenol patent records in Human Necessities domain

Publication year	Health, Amusement	Foodstuffs; Tobacco
	Medical or veterinary science; hygiene (A61)	Foods or foodstuffs; their treatment, not covered by other classes (A23)
2010	72	47
2011	72	51
2012	75	47
2013	84	47
2014	102	64
2015	107	64
2016	92	58
2017	124	96
2018*	6	9

Note: Search conducted in July 2018.

Finals remarks and conclusions

In this review, we highlight the role of polyphenols as potential antiproliferative agents due to antioxidant and prooxidant properties. The fact that these compounds affect numerous essential pathways and targets associated with antiproliferative effects is recognized, although, to date, there is still few clinical trials as such testing the role of plant phenolic compounds for inhibiting tumor growth in humans. The antiproliferative activities, and consequently in the IC_{50} values, can be influenced by digestive process and depending if their compounds are isolated or in a complex food matrix this event can improve or decrease their bioaccessibility, bioavailability, and biological activities.

As a final recommendation on this topic, scientists and companies should bear in mind that although the chemistry and bioactivity of phenolic compounds are already well established, a methodological approach could be improved even further to test the efficacy of these compounds on the antiproliferative activity and the subsequent development of functional foods and/or nutraceuticals. For this purpose, the effects of both *in vitro* and *in vivo* methods, including compound interactions, digestion, absorption and metabolism pathways should also be more studied to understand the bioaccessibility and functional properties of nutraceuticals or foods added with extracts containing phenolic compounds. Finally, the technological application should be assessed, such as the effects of thermal and nonthermal processing on the levels and bioactivity of phenolic compounds. Obviously, sensory characteristics and regulatory aspects should be considered in the complex and time-consuming

for the development of innovative food structures and functionalities to satisfy consumer needs and expectations and offer multitude health benefits.

Conflict of interest statement

Nothing declared.

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Chapter 2

Hydroalcoholic *Myrciaria dubia* (camu-camu) seed extracts prevent chromosome damage and act as antioxidant and cytotoxic agents

ABSTRACT

The camu-camu seeds, which comprehend about 20% of the fruit weight, is discarded without taking benefit of their chemical components and potential application by the industry. In the current study, we characterized the phenolic composition, the *in vitro* chemical antioxidant effects, cytotoxic activity, and the inhibition of inducedcisplatin chromosomal aberrations of five camu-camu seed extracts obtained with different proportions of water (H_2O) and ethyl alcohol (EtOH). The 50% H_2O+50% EtOH was the most promising extract because it presented higher total phenolic content (4802 mg GAE/100 g), antioxidant capacity (DPPH = 3694 mg AAE/100 g; FRAP=6604 mg AAE/100 g; FCRC=4918 mg GAE/100 g) and inhibited the cell growth of four cancer cell lines (GI_{50}=7.49 μg GAE/mL A549; 13.3 μg GAE/mL Caco-2; 15.57 μg GAE/mL HepG2 and 14.89 μg GAE/mL HCT8) without cytotoxic effects against normal cells (GI_{50} IMR90 > 43.2 μg GAE/mL). The cytotoxic effects presented high correlation with the (−)-epicatechin and methylvescalagin contents, while gallic and 2,5-dihydroxybenzoic acids were associated with cytoprotective effects of HCT8 cancer cell line. The 50% H_2O+50% EtOH extract also presented protective effect by decreasing 37% of the induced-cisplatin chromosomal breaks index, suggesting its antimutagenic potential, which may be associated to its antioxidant and cytotoxic activities.

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Hydroalcoholic *Myrciaria dubia* (camu-camu) seed extracts prevent chromosome damage and act as antioxidant and cytotoxic agents

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ABSTRACT

The camu-camu seeds, which comprehend about 20% of the fruit weight, is discarded without taking benefit of their chemical components and potential application by the industry. In the current study, we characterized the phenolic composition, the *in vitro* chemical antioxidant effects, cytotoxic activity, and the inhibition of induced-cisplatin chromosomal aberrations of five camu-camu seed extracts obtained with different proportions of water (H_2O) and ethyl alcohol (EtOH). The 50% H_2O + 50% EtOH was the most promising extract because it presented higher total phenolic content (4802 mg GAE/100 g), antioxidant capacity (DPPH = 3694 mg AAE/100 g; FRAP = 6604 mg AAE/100 g; FCRC = 4918 mg GAE/100 g) and inhibited the cell growth of four cancer cell lines (GI_{50} = 7.49 μg GAE/mL A549; 13.3 μg GAE/mL Caco-2; 15.57 μg GAE/mL HepG2 and 14.89 μg GAE/mL HCT8) without cytotoxic effects against normal cells (GI_{50} IMR90 > 43.2 μg GAE/mL). The cytotoxic effects presented high correlation with the (−)-epicatechin and methylvescalagin contents, while gallic and 2,5-dihydroxybenzoic acids were associated with cytoprotective effects of HCT8 cancer cell line. The 50% H_2O + 50% EtOH extract also presented protective effect by decreasing 37% of the induced-cisplatin chromosomal breaks index, suggesting its antimutagenic potential, which may be associated to its antioxidant and cytotoxic activities.

1. Introduction

Currently, there has been an increase in the scientific interest regarding natural products in relation to their potential application, chemical composition and positive biological effects to human health. There is a large number of underexploited native and exotic fruit species in Brazilian biomes, representing a potential interest for the agroindustry and for the local population (Almeida et al., 2011). *Myrciaria dubia* is a native Amazonian bush from the Myrtaceae family commonly known as camu–camu, “caçari” or “araçá d'água”, with natural occurrence during periods of flooding. This species grows near courses of rivers and lakes in the Amazon Forest (Azevedo et al., 2018; da Silva et al., 2012; Fidelis et al., 2018; Fujita et al., 2017).

Camu-camu pulp has a well-known nutritional value, mainly due to its amounts of bioactive compounds and ascorbic acid (864 mg/100 g pulp), besides the considerable antioxidant capacity (Azevedo et al., 2018; Fidelis et al., 2018; Myoda et al., 2010; Nascimento, Boleti, Yuyama, & Lima, 2013). Camu-camu seed and peel present higher levels of polyphenols, 369.4 ± 9.6 mg/g and 203.8 ± 7.7 mg/g respectively (Myoda et al., 2010) when compared to the pulp power (4.85 mg/g) (Fracassetti, Costa, Moulay, & Tomás-Barberán, 2013) and other fruit juice residues, such as acerola (94.6 mg/g), pineapple (9.1 mg/ g) and passion fruit (41.2 mg/g) (de Oliveira et al., 2009). Most research articles relatethe use of the fruit pulp (Fujita et al., 2017; Nascimento et al., 2013; Neves, Tosin, Benedette, & Cisneros-Zevallos, 2015) and peel (Neves et al., 2015). Camu-camu by-products, such as seeds are usually discarded without taking benefit of their chemical components (Fidelis et al., 2018; Myoda et al., 2010). The extraction and analyzes of bioactive compounds from by-products of fruits has been increasingly studied in order to avoid important losses and wastes,

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besides representing potential benefits for applications in pharmaceutical and food industries (Fidelis et al., 2018; Myoda et al., 2010).

Regarding the bioactive effects, studies have already shown the role of camu-camu in inflammatory condition (Yazawa, Suga, Honma, Shirosaki, & Koyama, 2011), besides antimicrobial activity (Myoda et al., 2010). Moreover, camu-camu pulp extracts decreased the *in vivo* oxidative stress and exerted protection against the mutagenic effects of the drugs on bone marrow and gut micronuclei, apoptosis, and comet assay (Azevedo et al., 2018). Cancer has attracted worldwide attention, although traditional therapies such as surgery, chemotherapy, and radiotherapy have some effects to treat cancer, besides the drug resistance and toxicities. Hence, it is necessary to screen efficient cytotoxic compounds from natural product resources (Yi et al., 2019). In this sense, research has shown that some polyphenols may act as antiproliferative agents against several types of cancer (León-gonzález, Auger, & Schini-kerth, 2015; Takashina et al., 2017).

In this context, we aimed to evaluate different hydroalcoholic extracts of camu-camu seeds in relation to their phenolic composition, antioxidant, cytotoxic activities, and to assess the capacity of the extracts to inhibit chromosome aberrations. For this purpose, we chose the chromosomal aberration assay, which detects of both aneugen, and clastogen damages (Sendão, Behling, dos Santos, Antunes, & Bianchi, 2006), and cytotoxicity evaluation. Additionally, the possible mechanisms involved in the reactive oxygen species generation, cytotoxic and antioxidant activities were proposed.

2. Material and methods

2.1. Camu-camu seeds and extraction

Camu-camu fruit was cultivated in Iguapé in the state of São Paulo/Brazil, geographical coordinates 24° 41′51″ south, 47° 34′16″ west at 6 m altitude, and harvested in March 2017. The fruits were sanitized (NaClO at 200 mg/L for 15 min) and the seeds removed manually. The seeds were dried in an oven with air circulation at 35 °C for 31 h (~12% moisture) and then they were ground using a knife mill (Kika Werke, model M 20) and sieved with a 42 Tyler mesh (0.354 mm) sieve and the prepared material was kept in a high-density polyethylene 8 °C until the beginning of the extractions. The extractions were performed in the ratio 1:20 (sample: solvent, m/v), *i.e.*, 10 g of flour obtained from the camu-camu seeds were mixed with 200 mL of solvent mixture. In all, 5 different extractions were carried out with ultrapure water and ethyl alcohol: 100% ultrapure water, 100% ethyl alcohol, 50% ultrapure water +50% ethyl alcohol, 25% ultrapure water +75% ethyl alcohol, and 75% ultrapure water (H_2O) + 25% ethyl alcohol (EtOH). The technique used for this purpose was the extraction by continuous agitation, with temperature control and the solution was kept in a bath at 45 °C for 45 min. The filtered extract was transferred to a rotary evaporator and, finally, lyophilized and analyzed for their phenolic composition, functional and biological properties.

2.2. Determination of phenolic composition

The total phenolic content (TPC) was quantified using the Prussian Blue method described by Margraf, Karnopp, Rosso, and Granato (2015). The results were expressed as mg of gallic acid equivalent (GAE) per 100 g of seed (mg GAE/100 g). Total condensed tannins were estimated using the vanillin-H_2SO_4 method according to Horszwald and Andlauer (2011) and the values were expressed as mg of (+)-catechin equivalent per 100 g (mg CTE/100 g).

Phenolic compounds of different classes (phenolic acids, flavonoids, ellagic acid, and stilbene) were determined by high-performance liquid chromatography (HPLC), Shimadzu LC-20 T, equipped with DAD (diode detector array) and fluorescence detectors, degasser system, auto sampler, and oven column, according to the method validated by Fidelis et al. (2018). The ellagitannin (methylvescalagin) identification and quantification was performed by means of a HPLC (JASCO LC-2000 Plus HPLC system) equipped with a MD-2010 Plus photodiode array detector and an Atlantis T3 column (3 µm, 4.6 mm i.d. × 150 mm, Waters, Milford, MA, USA). The mobile phase for gradient elution was as follows: solvent A was 5% acetonitrile containing 0.2% formic acid, and B was 100% acetonitrile. The gradient condition was as follows: 0 min, 0% B; 5 min, 10% B; 25 min, 15% B; 40 min, 50% B; 45–50 min, 100 B; 51 min, 0% B. Results were expressed as mg/100 g of seed.

2.3. Antioxidant activity

In the current research, the camu-camu seed extracts were assessed in relation to the antioxidant activity using the following assays: DPPH radical scavenging method proposed by Brand-Williams, Cuvelier, and Berset (1995), and the values were expressed as mg of ascorbic acid equivalent per 100 g (mg AAE/100 g). The ferric-reducing antioxidant power (FRAP) of the extracts was quantified according to Benzie and Strain (1996) and data were expressed in mg AAE/100 g. The Folin-Ciocalteu reducing capacity was assessed according to Singleton (1985) and the results were expressed in mg GAE/100 g.

2.4. In vitro assays of cytotoxicity and proliferation

The *in vitro* cytotoxic effect of the camu-camu seed extracts were analyzed in relation to the following cell lines: A549 (lung adenocarcinoma epithelial cells); Caco-2 (colorectal adenocarcinoma epithelial cells); HepG2 (human hepatoma carcinoma cells) HCT8 (human colon carcinoma) and IMR90 (human lung fibroblast), which were cultured as described by Santos et al. (2018) Briefly the cells were plated into 96-well plates at a density of 1×10^4 cells/well (HepG2 and Caco), 5×10^3 cells/well (A549 and HCT8) and 2×10^3 (IMR90), 100 µL/well. After adhesion, the cells were treated for 48 h with serial concentrations of 100–900 µg/mL of camu-camu seed extracts; which represents 1.35–43.2 µg GAE/mL. The cell viability was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Maciel et al., 2018) In accordance with the method described by do Carmo, Pressete, Marques, Granato, and Azevedo (2018) the $IC_{50,}$ GI_{50} and LC_{50} parameters were performed. The camu-camu selectivity index (SI) was calculated by the ratio IC_{50} (IMR90)/IC_{50} (cancer cell lines) (Boechat et al., 2014). SI value indicates selectivity of the sample to the cell lines tested. Any sample which has SI value higher than 3 will be considered to have high selectivity (Prayong, Barusrux, & Weerapreeyakul, 2008).

2.5. Intracellular reactive oxygen species (ROS) activity

All cell lines (6×10^4 per well) were treated for 1 h at 37 °C with camu-camu seed extracts, which were diluted in DCFH-DA solution (25 mmol/L) at different concentrations (10, 50 and 100 µg/mL). For the positive control, the cells were treated with 15 µmol/L H_2O_2 and for the negative control; the cells were only treated with culture medium. Following the treatment, it was added pos-treatment with H_2O_2 at 15 µmol/L for all the wells (Escher et al., 2018). The fluorescence intensity was measured at an excitation wavelength of 485 nm and at an emission wavelength of 538 nm. The data were expressed as percentage of fluorescence intensity relative to the untreated group (negative control).

2.6. Chromosomal aberration assay in A549 cells

The A549 cell line was plated into 75 cm^3 flasks at a density of 5×10^5 cells/flask and cultures were divided into four groups. Group 1 was the negative control *i.e.* cells received only culture medium. In group 2, cells were added of 4 µM cisplatin (positive control). In group 3, cells were treated with 50% H_2O + 50% EtOH camu-camu seed extract (GI_{50} value for A549) and group 4 with a combination of 4 µM

cisplatin and 50% H_2O + 50% EtOH (GI_{50}) camu-camu seed extract, all for 48 h. The procedures were adapted and performed as described El-Mahdy Sayed Othman (2000) and Surendran, Geetha, and Mohanan (2012). For the chromosomal aberrations test was used the chromosomal breakage criteria described by Auerbach, Rogatko, and Schroeder-Kurth (1989), with minor modifications. This criterion converts the aberrations into break events. Thus, gaps were not counted as chromosome breaks; both chromatidic and chromosomal breaks were counted as single break events; and finally, ring chromosomes, dicentric chromosomes, tri- and tetra-radial figures, and complex rearrangements were counted as two break events each (Auerbach et al., 1989). The chromosomal breaks index (CBI) was determined by the average number of chromosomal breaks observed by metaphase.

2.7. Statistical analysis

The experimental values were expressed as mean ± standard deviation ($n = 3$). Equality of variances was analyzed by the Brown–Forsythe test ($n \geq 3$ extracts) or F-test for two extracts. Differences between the extracts were evaluated by the analysis of unifatorial variances, the means being compared by the Duncan test ($n \geq 3$ extracts) or Student's *t*-test for independent samples ($n = 2$ extracts). Linear correlations between the independent variables were calculated by the Pearson correlation coefficient, considering the replicates ($n = 15$ data). Probability values below 0.05 were used to reject the null hypothesis. Multiple linear regression was employed to evaluate the effect of the solvent system, that is, different proportions of water and ethyl alcohol, in the phenolic composition and antioxidant activity (Alberti et al., 2016). For this purpose, $n = 15$ results were used in the modeling step and the regression coefficients were calculated using the cubic model, as shown in Eq. (1):

$$y_i = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1 * x_2 + \beta_4 x_1 * x_2 (x_1 - x_2) \quad (1)$$

where x1 = water, x2 = ethyl alcohol. The magnitude of the coefficient β allows to compare the relative contribution of water (x_1) and ethyl alcohol (x_2) in the prediction of the dependent variable (chemical composition and antioxidant activity). As evaluation measures of the models generated, the coefficient of determination (R^2) and the adjusted R^2 were calculated. For the interpretation of the generated models, $p < .10$ was used.

In the attempt to associate all the variables and design the extracts of camu-camu in the bidimensional plane-factor, the principal component analysis (PCA) was used. For this, the data set was composed of $n = 390$ data points ($n = 5$ extracts with $n = 3$ replicates and $n = 26$ variables), autoscaled, and decomposed according to Eq. (2):

$$X = TP^t + E \quad (2)$$

where $T = \{t_{ia}\}$ represents the matrix of scores (camu-camu extracts), $P = \{p_{ja}\}$ represents the loadings matrix and $E = \{e_{ij}\}$ is the residue matrix. The PCA was based on linear correlations and the variances were computed as the sum of the squares divided by *n*-(Nunes, Alvarenga, de Souza Sant'Ana, Santos, & Granato, 2015). The statistical software TIBCO Statistica v. 13.3 (TIBCO Statistica Ltd., USA) was used in all statistical analysis.

3. Results and discussion

3.1. Phenolic composition by HPLC-DAD

The results of total phenolic content and condensed tannins of camu-camu seed extracts shown a significant difference among the extracts ($p < .001$) (Table 1), suggesting that the solvent system highly affects the extraction of phenolic compounds and these data are in-line with the results obtained with camu-camu seed coat extracted with propanone, water, and ethyl alcohol (Fidelis et al., 2018). The mean total phenolic content of all samples ranged from 128 (100% EtOH) to 675 (50% H_2O + 50% EtOH) mg GAE/100 g. The sample extracted with 50% H_2O + 50% EtOH had higher levels of total phenolic content, including rosmarinic acid, 2,4-dihydroxybenzoic acid, ellagic acid, cyanidin-3-glucoside, methylvescalagin, *trans*-resveratrol and quercetin. Similarly, Fidelis et al. (2018) evaluated the bioactive compounds of camu-camu seed coat (*Myrciaria dubia* Mc Vaugh) and detected the presence of the same compounds. Herein, the content of total phenolics obtained with the five different proportions of solvents are much higher than those reported for other fruit sources, such as *Myrciaria dubia* seed coat (108–185 mg/100 g) (Fidelis et al., 2018) and *Myrciaria dubia* pulp, peel, pulp powder, seed and flour (8.66–336 mg/100 g) (Fracassetti et al., 2013). Herein, the capacity of this proportion of water and ethyl alcohol to extract a greater content of phenolic compounds could be related to the affinity between the phenolic compounds present in the camu-camu seed and the polarity of the extracting medium.

Regarding the condensed tannins content, it was observed a similar behavior once the camu-camu seed extracted with 50% H_2O + 50% EtOH presented the highest amount, whereas the aqueous extract presented the opposite behavior. Interestingly, the aqueous extract of camu-camu seed coat presented similar results (Fidelis et al., 2018), indicating that these compounds do not have greater affinity with water.

Taking into account the individual phenolic composition (Table 1), the 75% H_2O + 25% EtOH extract presented the highest levels of gallic acid (37.09 ± 0.59 mg/100 g). Similarly, Fidelis et al. (2018) observed high levels of the same compound in the aqueous extract of camu-camu seed coat. This compound is highly soluble in aqueous medium, which justifies the highest levels in the samples extract with more water proportion compared with the ethyl alcoholic extract. Interestingly, caffeic acid was better extracted in the 25% H_2O + 75% EtOH (3.73 ± 0.04 mg/100 g) extract and was not detected in 100% H_2O, 100% EtOH and 50% H_2O + 50% EtOH. 2,5-Dihydroxybenzoic acid was only found in the 75% H_2O + 25% EtOH extract (2.21 ± 0.04 mg/100 g). In the current research, among the phenolic compounds identified in the 50% H_2O + 50% EtOH extract, methylvescalagin presented the highest content (559.07 ± 9.47 mg/100 g).

3.2. Antioxidant activity

Similar to what was observed for the phenolic composition, the antioxidant activity of the different camu-camu seed extracts presented statistically significant differences ($p < .001$) (Table 1). The 50% H_2O + 50% EtOH extract had the highest antioxidant activity using the FRAP (8076 ± 511 mg AAE/100 g), DPPH (4340 ± 117 mg AAE/100 g), and the Folin-Ciocalteau reducing capacity (4918 ± 85 mg GAE/100 g) assays, while the 100% EtOH extract exhibited the lowest antioxidant capacity (DPPH: 1111 ± 48 mg AAE/100 g and FRAP: 2248 ± 39 mg AAE/100 g). Fidelis et al. (2018) also observed greater antioxidant activity values in the aqueous extract of the camu-camu seed coat – 7425 mg AAE/100 g, 2838 mg AAE/100 g and 8522 ± 318 mg GAE/100 g in FRAP, DPPH and Folin-Ciocalteau reducing capacity assays, respectively. In another study on phenolic-antioxidant capacity of mango seed kernel methanol extract, Abdel-Aty, Salama, Hamed, Fahmy, and Mohamed (2018) also observed substantial antioxidant activity measured by the DPPH (IC_{50} = 47.3 ± 0.85 μg GAE/mL) and ABTS assays (IC_{50} = 7.9 ± 0.14 μg GAE/mL).

3.3. Cell viability

The results indicated that all extracts presented cytotoxic effects against the cancer cell lines tested (A549, HCT8, HepG2 and Caco-2), at higher or lower concentrations depending on the type of cell (Table 2). Among the cancer cell lines, HCT8 cells have been shown to be more resistant to extracts by exhibiting high values of GI_{50} (294 to > 900 μg/mL), indicating that very high concentrations are required to inhibit the

Table 1
Chemical composition and antioxidant activity of camu-camu seed extracted with different proportions of water and ethyl alcohol.

Fenolic compounds	100% H_2O	75% H_2O + 25% EtOH	50% H_2O + 50% EtOH	25% H_2O + 75% EtOH	100% EtOH	*p*-Value[1]	*p*-Value[2]
Phenolic composition (HPLC)							
Gallic acid (mg/100 g)	18.48 ± 0.25^{b}	**37.09 ± 0.59^{a}**	2.56 ± 0.09^{d}	6.90 ± 0.60^{c}	4.36 ± 0.82^{e}	.654	< .001
3,4-Dihydroxybenzoic acid (mg/100 g)	1.26 ± 0.02	ND	ND	ND	ND	NA	NA
2,4-Dihydroxybenzoic acid (mg/100 g)	5.11 ± 0.01^{b}	5.09 ± 0.02^{b}	**5.25 ± 0.03^{a}**	4.13 ± 0.01^{c}	4.10 ± 0.02^{c}	.716	< .001
2,5-Dihydroxybenzoic acid (mg/100 g)	ND	2.21 ± 0.04	ND	ND	ND	NA	NA
2-Hydroxycinnamic acid (mg/100 g)	1.34 ± 0.03^{e}	1.99 ± 0.02^{c}	2.28 ± 0.04^{b}	**2.34 ± 0.04^{a}**	1.92 ± 0.04^{d}	.958	< .001
Rosmarinic acid (mg/100 g)	0.84 ± 0.00^{e}	0.93 ± 0.01^{c}	**1.03 ± 0.01^{a}**	1.00 ± 0.01^{b}	0.85 ± 0.00^{d}	.736	< .001
Syringic acid (mg/100 g)	ND	**9.94 ± 0.06^{a}**	9.77 ± 0.06^{b}	3.71 ± 0.09^{c}	2.29 ± 0.12^{d}	.432	< .001
Caffeic acid (mg/100 g)	ND	1.03 ± 0.00^{b}	ND	**3.73 ± 0.04^{a}**	ND	.246	< .001
p-Coumaric acid (mg/100 g)	ND	0.22 ± 0.01^{b}	ND	**0.53 ± 0.01^{a}**	ND	.186	< .001
Ellagic acid (mg/100 g)	4.31 ± 0.02^{e}	11.95 ± 0.09^{d}	**20.10 ± 0.01^{a}**	19.85 ± 0.16^{b}	14.53 ± 0.04^{c}	.430	< .001
trans-Resveratrol (mg/100 g)	0.63 ± 0.00^{c}	0.92 ± 0.01^{d}	**0.93 ± 0.02^{a}**	0.88 ± 0.01^{b}	0.61 ± 0.01^{d}	.679	< .001
Quercetin-3-rutinoside (mg/100 g)	3.23 ± 0.01^{d}	8.07 ± 0.19^{c}	10.04 ± 0.03^{b}	**10.48 ± 0.09^{a}**	8.20 ± 0.19^{c}	.347	< .001
Quercetin (mg/100 g)	0.73 ± 0.00^{e}	1.03 ± 0.01^{c}	**1.17 ± 0.01^{a}**	1.15 ± 0.01^{b}	0.91 ± 0.00^{d}	.910	< .001
Proanthocyanidin A2 (mg/100 g)	14.81 ± 0.16^{e}	22.63 ± 0.46^{d}	55.68 ± 2.51^{b}	**60.63 ± 1.32^{a}**	46.19 ± 0.32^{c}	.469	< .001
Cyanidin-3-glucoside (mg/100 g)	4.03 ± 0.02^{c}	**4.56 ± 0.01^{a}**	**4.55 ± 0.03^{a}**	4.45 ± 0.07^{b}	4.35 ± 0.03^{c}	.722	< .001
(+)-Catechin (mg/100 g)	0.52 ± 0.01^{b}	0.65 ± 0.01^{a}	0.55 ± 0.01^{b}	0.65 ± 0.01^{a}	0.29 ± 0.01^{c}	.230	< .001
(−)-Epicatechin (mg/100 g)	3.07 ± 0.04^{a}	2.52 ± 0.05^{b}	2.38 ± 0.03^{bc}	3.04 ± 0.08^{a}	2.26 ± 0.13^{c}	.867	< .001
Methylvescalagin (mg/100 g)	327.32 ± 12.77^{d}	457.74 ± 3.84^{b}	**559.07 ± 9.47^{a}**	436.10 ± 7.94^{c}	38.15 ± 0.86^{e}	.407	< .001
Total phenolic content – HPLC (mg/100 g)	385.63 ± 12.73^{c}	568.57 ± 3.63^{b}	**675.36 ± 10.11^{a}**	559.59 ± 8.13^{b}	128.73 ± 0.61^{d}	.397	< .001
Total phenolic content – UV/VIS spectrophotometry (mg GAE/100 g)	2502 ± 46^{d}	3974 ± 142^{b}	**4802 ± 139^{a}**	3403 ± 170^{c}	1353 ± 19^{e}	.736	< .001
Condensed tannins (mg CTE/100 g)	350 ± 44^{d}	**1219 ± 36^{a}**	**1164 ± 19^{a}**	863 ± 19^{b}	510 ± 53^{c}	.849	< .001
Non-tannin phenolics (mg/100 g)	2152 ± 32^{c}	2756 ± 176^{b}	**3639 ± 121^{a}**	2540 ± 179^{b}	844 ± 66^{d}	.728	< .001
Antioxidant activity							
DPPH (mg AAE/100 g)	2045 ± 22^{c}	3694 ± 81^{b}	**4340 ± 117^{a}**	3527 ± 276^{b}	1111 ± 48^{d}	.545	< .001
FRAP (mg AAE/100 g)	4667 ± 37^{d}	6604 ± 221^{b}	**8076 ± 511^{a}**	6084 ± 106^{c}	2248 ± 39^{e}	.174	< .001
Folin-Ciocalteu reducing capacity (mg GAE/100 g)	2114 ± 99^{d}	4165 ± 85^{c}	**4918 ± 85^{a}**	4354 ± 115^{b}	1502 ± 64^{e}	.990	< .001

Notes: ND = not detected; NA = not applicable. Different letters in the same row represent statistically different results according to the Duncan test ($n \geq 3$ extracts) or Student's *t*-test for independent samples when $n = 2$ extracts ($p < .05$).
The bold numbers in Table 1 highlight the highest amount of phenolic compound among the camu-camu seed extracts.
[1] Probability values for the homoscedasticity by the Brown–Forsythe test ($n \geq 3$ extracts) or F test for $n = 2$ extracts.
[2] Values of probability according to the one-factor analysis of variances.

Table 2
Cytotoxicity, cell growth inhibition and selectivity index of Caco-2, HepG2, A549, HCT8 and IMR90 cells after 48 h exposure to camu-camu seed extracts expressed in μg/mL and μg GAE/mL (in parentheses).

Cell lines		Extracts									
		100% H_2O	SI	100% EtOH	SI	50% H_2O + 50% EtOH	SI	25% H_2O + 75% EtOH	SI	75% H_2O + 25% EtOH	SI
Caco-2	IC_{50}	204.0 (5.6)	> 4.4	794.2 (10.5)	> 1.13	337.2 (16.1)	> 2.66	366.1 (10.8)	> 2.46	976.1 (39.5)	> 0.92
	GI_{50}	104.4 (2.7)		694.5 (9.3)		218.2 (13.3)		215.7 (5.6)		762.6 (29.4)	
	LC_{50}	559 (14.0)		> 900 (12.1)		776.0 (37.2)		736.7 (25.0)		> 900 (35.7)	
HepG2	IC_{50}	428.2 (10.7)	> 2.1	1014.0 (13.7)	> 0.88	476.1 (12.9)	> 1.89	532.6 (12.7)	> 1.71	853.6 (25.7)	> 1.05
	GI_{50}	317.0 (7.6)		832.3 (11.2)		324.4 (7.5)		454.4 (10.5)		740.1 (21.2)	
	LC_{50}	745.2 (18.6)		> 900(12.1)		1309 (21.2)		> 900 (19.9)		> 900 31.35	
A549	IC_{50}	343.1 (8.1)	> 2.62	785.1 (10.2)	> 0.87	278.0 (13.0)	> 3.2	371.3 (12.7)	> 2.42	650.0 (25.8)	> 1.38
	GI_{50}	282.8 (7.3)		712.8 (9.4)		251.0 (7.5)		304.4(10.5)		602.4 (21.2)	
	LC_{50}	784.719.6		> 900 (12.1)		441.4 (21.2)		585.8(20.0)		784.7(31.3)	
HCT8	IC_{50}	521.4 (13.0)	> 1.72	692.4 (9.3)	> 1.3	610.8 (29.3)	> 1.47	513.7 (17.5)	> 1.75	818.9 (32.5)	> 1.09
	GI_{50}	294.0 (7.3)		493.4 (6.7)		310.1 (14.9)		289.0 (9.8)		> 900 (22.0)	
	LC_{50}	1308(32.7)		1051 (14.2)		> 900 (> 43.2)		1035(35.1)		> 900 (35.4)	
IMR90	IC_{50}	> 900 (> 22.5)	–	> 900 (> 12.1)	–	> 900 (> 43.2)	–	> 900 (> 30.6)	–	> 900 (> 35.7)	–
	GI_{50}	> 900 (> 22.5)		> 900 (> 12.1)		> 900 (> 43.2)		> 900 (> 30.6)		> 900 (> 35.7)	
	LC_{50}	> 900 (> 22.5)		> 900(> 12.1)		> 900(> 43.2)		> 900(> 30.6)		> 900 (> 35.7)	

Notes: NC = Not converged. IC_{50}: the concentration of the agent that inhibits growth by 50%, is the concentration at which (T/C) × 100 = 50, where T = number of cells, at time t of treatment; C = control cells at time t of treatment. GI_{50}: the concentration of the agent that inhibits growth by 50%, relative to untreated cells, is the concentration at which ([T − T0]/[C − T0]) × 100 = 50, where T and C are the number of treated and control cells, respectively, at time t of treatment and T > T0; T0 is the number of cells at time zero. LC_{50}: the concentration of the agent that results in a net loss of 50% cells, relative to the number at the start of treatment, is the concentration at which ([T − T0]/T0) × 100 = − 50; T < T0. Selectivity index (SI) = IMR90 IC_{50}/cancer cells IC_{50}.

cell proliferation. On the other hand, Caco-2 cells exhibited most sensitivity toward the extracts, since they presented low values of GI_{50} (104.4–762.6 μg/mL), meaning substantial cytotoxicity for this cancer cell. Other cancer cells, HepG2 and A549, were susceptible to the camu-camu seed extracts (Table 2). In contrast, Correia et al. (2016) revealed that dichloromethanolic extract from *Myrciaria dubia* fruit, showed to be not cytotoxicity for HepG2 cells (IC_{50} > 500 μg/mL). Considering this result, the main factors that affect the phenolic content in extraction are the solvent, contact area, temperature, time, and molecular structure of the matrix (Escher et al., 2018; Setford, Jeffery, Grbin, & Muhlack, 2017), besides the part of the plant used for extraction. Herein, the solvents used for the extraction were water and ethyl alcohol, substances that have no toxicity and are efficient in extracting phenolic compounds (Dias et al., 2014; Liao et al., 2016).

Interestingly, growth of non-cancer IMR90 lung fibroblast cells was not inhibited by the extracts, which exhibited high IC_{50} and GI_{50} values (> 900 μg/mL), suggesting that it is necessary to use higher concentrations to inhibit the proliferation of half of the cells, meaning low cytotoxicity and low cell inhibition. These results confirmed the chemopreventive potential of camu-camu polyphenols that inhibited cancer cell growth without toxicity to the non-cancer cells. A similar behavior was observed in a studies with açai polyphenolic (Dias et al., 2014) and aqueous açai seed (Barros et al., 2015) extracts where the cytotoxicity effects of the extracts were more evidenced in cancer cell lines, when compared with normal cells. Herein, the malignant cells seemed to be more susceptible to our treatments than normal cells indicating a possible therapeutic window as an anti-cancer agent.

Overall and considering all the cell lines results together, we also could point out that among the extracts, the 75% H_2O + 25% EtOH seems to exert less activity among the others, since it presented high values of GI_{50} (602.4 to > 900 μg/mL). In contrast, the 100% H_2O extract, appears to exert better capacity in inhibit the cell growth with lower GI_{50} values (104.4–317.6 μg/mL). High cytotoxic and selectivity (SI ≥ 3) were observed for 100% H_2O (Caco-2) and for 50% H_2O + 50% EtOH (A549), indicating their potential for biopharmaceutical application among the sample tested.

3.4. Measurement of intracellular ROS

In the present study, the ROS produced in cell lines exposed to various concentrations of camu-camu seed extracts was accessed using DCFH-DA. The levels of ROS induced by H_2O_2 was higher than the control and similar to some treatment groups (Fig. 1). We highlight that all the extracts exhibited a suitable decrease in ROS generation, suggesting a protective effect in Caco-2, A549, HCT8, HepG2 and IMR90 cells. Similarly, Dias et al. (2014) showed that açai polyphenolic extract also reduced significantly ROS production for cancer and non-cancer cells. Indeed, our the extracts exhibited a suitable decrease in ROS induction in cancer cells, highlighting that cell death observed in the cell viability test may involve other mechanisms not related to the ROS generation, which is one of the cell death pathways. Different kinds of physical-chemical stress stimuli can initiate necrosis or apoptosis, including cell membrane damage, mitochondrial dysfunction and destabilization (Peixoto, de Oliveira Galvão, & Batistuzzo de Medeiros, 2017), tumor necrosis factor α (TNFα) production, ischemia–reperfusion injury, glutamate, and calcium overload (Fulda, 2013).

Bayele, Debnam, and Srai (2016) observed that some phenolic compounds, such as quercetin, caffeic acid, quercetin-3-rutinoside, *t*-resveratrol and ferulic acid may enhance cellular defense genes against oxidative stress by attenuating Nrf2 (transcription factor which regulates cytoprotective genes) inhibition *in vivo* and in HepG2 cells. This ability can underlie the cytoprotection conferred by polyphenols in the camu-camu seed extracts against oxidative stress damage. Herein, differently for HCT8 cells, the extract 100% EtOH induced ROS for all tested concentrations, while 25% H_20 + 75% EtOH and 50% H_20 + 50% EtOH exhibited protective activity against oxidative stress. Oxidative damage caused by ROS seems to be a contributing event in the initiation of cytotoxicity in cancer cells (Azevedo et al., 2016). Indeed, if ROS levels increase dramatically to toxic concentrations, the JNK pathway can be activated resulting in apoptosis and cell death (Moloney & Cotter, 2018), which was observed in cell viability test. Thus, as our results suggested that phenolic compounds present in the 100% EtOH extract can induce ROS generation in HCT8 cells, they could be considered a chemotherapeutic effect in suppressing cancer growth. A similar mechanism was observed in a study with pistachio kernel extracts (Reboredo-Rodríguez et al., 2018), where the acute production of ROS was induced causing alterations of intracellular redox status and consequently cell death. In this respect and taking into account the difference among the extracts content, specific compounds can present pro-oxidant effects, which lead to cell death by the ROS generation (Santos et al., 2018).

All the extracts, except for the 100% EtOH extract for HCT8 cells, have shown a decrease in ROS levels, indicating cytoprotective activity in all concentrations. Herein, our results suggest that *Myrciaria dubia* seed extracts can increase or decrease the ROS generation levels. This adjustment can be attributed to the antioxidant components according to each extract chemical profile and cellular adaptations, conferring cytoprotective or cytotoxicity activities.

3.5. Chromosomal aberrations

In this study, the possible protective effects of camu-camu seed extract (50% H_2O + 50% H_2O) on cisplatin-induced chromosome damage in A549 cells were evaluated. In the current study, chromosome aberrations, whatever the type, were significantly increased in cells treated with 4 μmol/L cisplatin (positive control) (1.62 ± 2.2) compared with the negative control group (0.22 ± 0.55) (Fig. 2A). Furthermore, camu-camu treatment alone did not induce significant aberrations (0.06 ± 0.24) indicating its non-clastogenicity/aneugenicity. Besides the non-toxic effect, the extract presented protective action by decreasing 37% the chromosomal breaks index (1.02 ± 1.39) compared to positive control group, which suggest that camu-camu seed extract may attenuate cisplatin-induced mutagenic damage (Fig. 2B). Similarly, other studies observed that lycopene (Sendão et al., 2006) and ascorbic acid (Nefic, 2001) attenuated the cisplatin-induced chromosome aberrations in 33–66% and 57% in rats' bone marrow and in human lymphocyte cultures, respectively.

Cisplatin is an antineoplastic drug with high mutagenic effect, which causes sister-chromatid exchange and enhancement of chromosome aberrations (Rjiba-Touati et al., 2012). The camu-camu anticlastogenic effect observed on A549 cells can be explained by mechanisms based on decreasing the cisplatin action. Considering that the components of cisplatin hydrolysis can interfere in DNA replication and transcription leading to its disrupt structure, camu-camu seed extracts could may interfere in the cisplatin interaction with N7-N3 position of nuclear DNA (Surendran et al., 2012), which induces activation of proteins involved in apoptosis (Gómez-sierra, Eugenio-pérez, Sánchez-chinchillas, & Pedraza-chaverri, 2018). Moreover, according to the following authors, the extracts from camu-camu seeds also could have acted by different ways: 1) changing the cell cycle or selective cell killing (Sendão et al., 2006); 2) acting as an antioxidant by scavenging mitochondrial ROS released by the cisplatin, thereby preventing cell damage (Kilic et al., 2019); 3) decreasing the cisplatin effect on mitochondrial bioenergetics, thereby reducing the blockage of glutamate oxaloacetate transaminase enzymatic activity, which catalyzes the conversion of aspartate and α-ketoglutarate into glutamate and oxaloacetate (Gómez-sierra et al., 2018).

Since mutations induced at the cytogenetic levels represent the first step of cancer development (Siddiqui, Sanna, Ahmad, Sechi, & Mukhtar, 2015), the inhibition of chromosomal aberrations by camu-camu seed extract suggest its antimutagenic potential, which could be related to its antioxidant and cytotoxic activities.

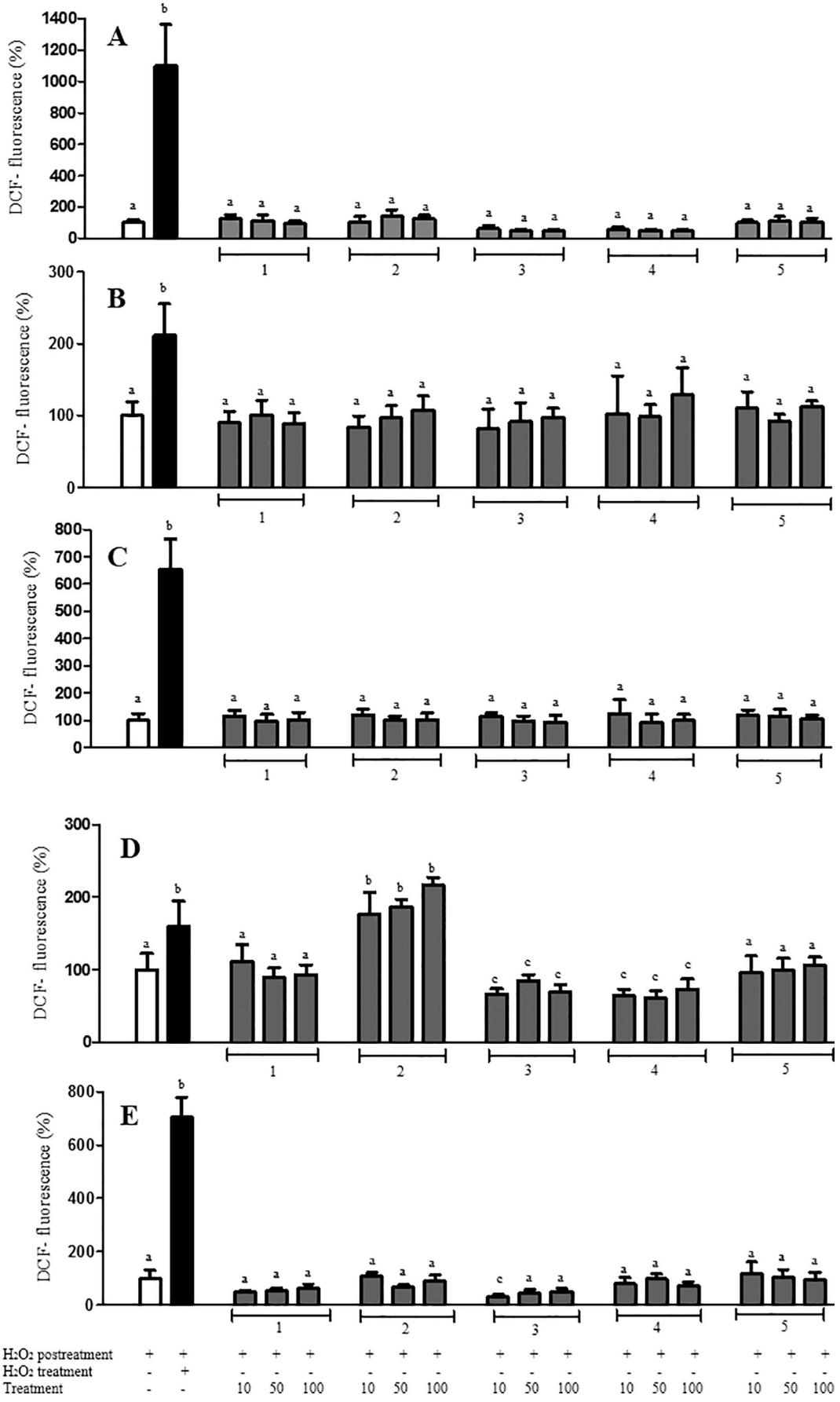

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Fig. 1. Results of intracellular ROS measurement in Caco-2 (A), HepG2 (B), A549 (C), HCT8 (D) and IMR90 (E) cells by spectrofluoremetry. 1, 2, 3, 4 and 5 = 100% H_2O, 100% EtOH, 50% H_2O + 50% EtOH, 25% H_2O + 75% EtOH and 75% H_2O + 25% EtOH, respectively, at 10–100 μg/mL. Quantitative data are the mean ± standard deviation. ANOVA one-way was made within the same extract and your related controls ($p \leq .05$).

3.6. Multiple linear regression analysis

Multiple linear regression analysis (supplementary material) was employed to evaluate the effect of the solvent system, that is, different proportions of water and ethyl alcohol, on the phenolic composition (total phenolics and isolated compounds, non-tannin phenolics, condensed tannins), antioxidant (DPPH, FRAP and FCRC) and cytotoxicactivities (A549, HCT8, Caco-2 and HepG2 cells).

All the proposed multiple regression models were considered significant ($p < .001$) to explain the effects of water and ethyl alcohol on the chemical composition, antioxidant and cytotoxic activities of camu-camu hydroalcoholic extracts. These results demonstrate that the generated models presented good statistical quality, once > 71% ($R^2 > 0.71$) of the variability were explained, except for gallic, caffeic, and *p*-coumaric acids (adjusted $R^2 = 0.5815$, 0.5037 and 0.2468 respectively). These results confirm that the models can be used for prediction purposes, that is, the phenolic composition, the antioxidant and cytotoxic activities may be determined if the concentration of water and EtOH are available. From the industrial/technological standpoint, one may use the generated models to obtain a camu-camu hydroalcoholic extract with higher *in vitro* antioxidant and/or cytotoxic activities in relation to certain cell lines.

Water significantly and positively affected the extraction of gallic acid 2,4-dihydroxybenzoic acid, total phenolic content, non-tannin phenolics and, as a consequence, enhanced the antioxidant capacity of the extracts (DPPH, FRAP, FCRC). In contrast, ethyl alcohol significantly increased the content of condensed tannins, while the interaction AB between the solvents, affected the extraction of *p*-coumaric acid, proanthocyanidin A2, *trans*-resveratrol, rutin, ellagic acid, methylvescalagin and quercetin. On the other hand, the quadratic interaction [AB(A-B)] between water and ethyl alcohol showed synergistic effect on the cell growth inhibition of all cell lines, and a negative effect on the methylvescalagin content. The linear interaction between ethyl alcohol and water had a negative impact on the cytotoxic activity in relation to HepG2 and A549 cell lines. We emphasize that this is the first report on the literature that used a mathematical/statistical approach to assess the effects of two atoxic solvents (water and ethyl alcohol), alone and in binary mixtures, on the phenolic composition, *in vitro* antioxidant and cytotoxic activities of camu-camu seed hydroalcoholic extracts.

3.7. Correlation analysis

It is known that biochemical interactions between extract matrix's phytochemicals could eliminate, reduce and even improve their bioactivity, making these compounds harmful or beneficial to the organisms, which turn a challenge to obtain conclusions from these complex matrix (do Carmo et al., 2018). Herein, the Pearson's correlation (Fig. 3) established the relationship between individual phenolic constituents from camu-camu seed extracts and their *in vitro*

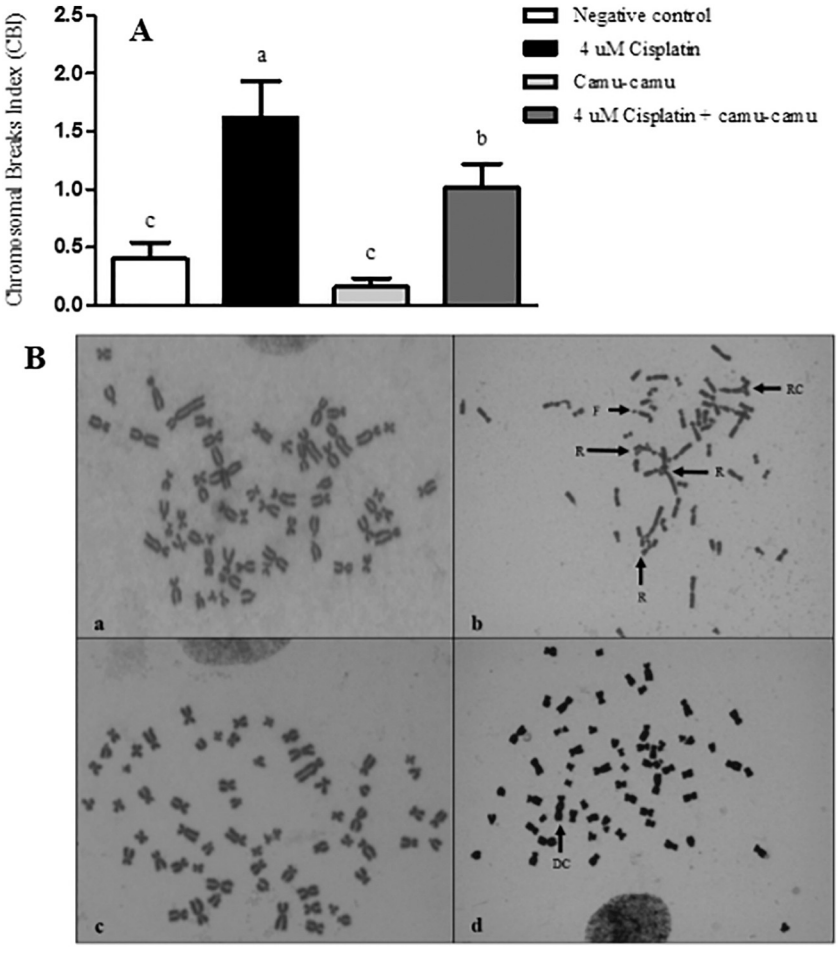

Fig. 2. (A) Chromosomal breaks index in A549 cells after cisplatin and camu-camu seed extract treatments. CBI is defined by the average number of chromosomal breaks observed by metaphase. For each type of chromosomal abnormality, a specific value is assigned for the calculation of CBI. Thus, a point is assigned for fragments, and both chromatidic and chromosomal breaks; and two points for ring chromosomes, dicentric chromosomes, tri- and tetra-radial figures, and complex rearrangements Quantitative data are the mean ± standard deviation. One-way ANOVA was made within all group with Fisher's post test. Different letters represent statistically different results ($p \leq .05$). (B) Photomicrographs of metaphase plate of A549 cells: Negative control (a), 4 μM cisplatin (b), 50% H_2O + 50% EtOH camu-camu seed extract (c) and 50% H_2O + 50% EtOH camu-camu seed extract + 4 μM cisplatin (d). Different kinds of aberrations were observed, like radial configuration (RC), rearrangement (R), fragment (F), and dicentric chromosome (DC). 1000×.

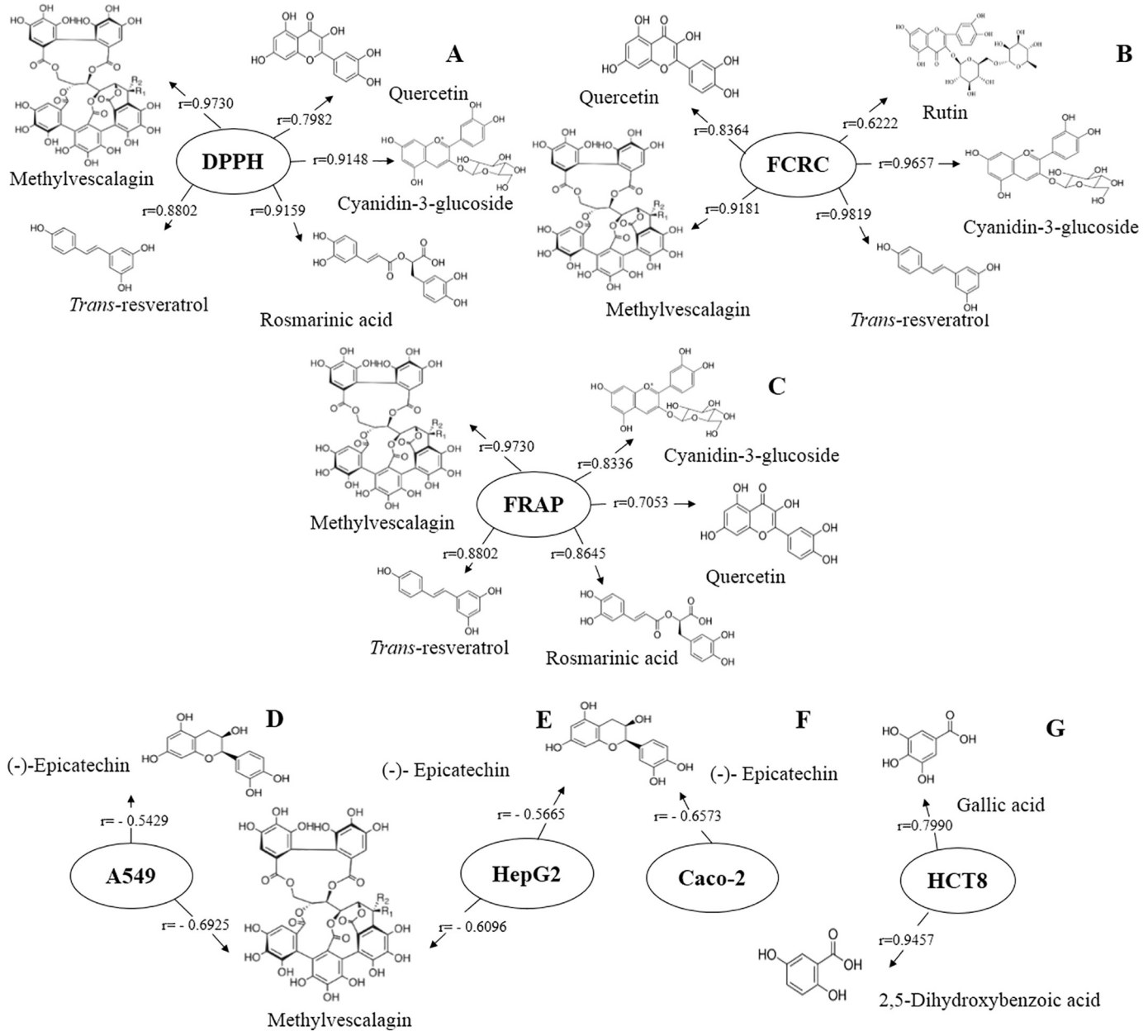

Fig. 3. Correlation between phenolic composition and DDPH (A), Folin-Ciocalteu reducing capacity – FCRC (B), FRAP (C), *in vitro* cytotoxic or cytoprotective activity in A549 (D), HepG2 (E), Caco-2 (F) and HCT8 cells (G).

antioxidant and cytotoxicactivities, excluding the interactions among the total compounds of the matrix.

Herein, we noted that there is a significant difference in the phenolic composition of camu-camu seed extracts obtained with different water/ethyl alcohol ratios. In summary, the lower the content of phenolic compounds, the lower the antioxidant capacity of the extracts. In fact, the chemical antioxidant activity, we observed a positive and significant correlation ($p \leq .05$) between total phenolic content and DPPH ($r = 0.9724$), FRAP ($r = 0.9839$) and FCRC ($r = 0.9457$) was observed. Moreover, it is well-known that the chemical antioxidant capacity of polyphenols (*i.e.*, DPPH, FCRC, and FRAP) is closely related to its chemical structure, once the presence of hydroxyl groups in their structures are capable of donating electrons to free radicals (Fidelis et al., 2018). For instance, the presence of the adjacency of the two hydroxyl groups in the *ortho*-diphenolic arrangement in the B ring can enhance the antioxidant activity (Rice-Evans, Miller, & Paganga, 1996), especially by scavenging free radicals *in vitro* and *in vivo*. This event can be observed in structures such as quercetin, which showed the greater and significant correlation with the chemical antioxidant assays (FRAP, DPPH and FCRC) (Fig. 3). Interestingly, an *in vivo* study showed that quercetin was effective for the prevention of *t*-BHP-induced hepatic damage in mice; and this protective effect may have been linked to its higher antioxidant capacity, free radical scavenging effect and inhibition of lipid peroxidation (Kalantari et al., 2018). Methylvescalagin presented a significant and positive correlation with DPPH ($r = 0.9504$), FRAP ($r = 0.9730$) and FCRC ($r = 0.9181$) (Fig. 3A, C and B respectively). Therefore, considering the antioxidant capacity and its amount present in 50% H_2O + 50% EtOH extract, methylvescalagin is highly associated with the chemical antioxidant activity of the camu-camu seed extracts. Kaneshima, Myoda, Toeda, Fujimori, and Nishizawa (2013) observed that the extract of camu-camu seeds and peel exhibited potent DPPH radical scavenging activity (IC_{50} = 32.2 μg/mL), and some C-glycosidic ellagitannins, namely vescalagin and castalagin, were shown to be responsible for the

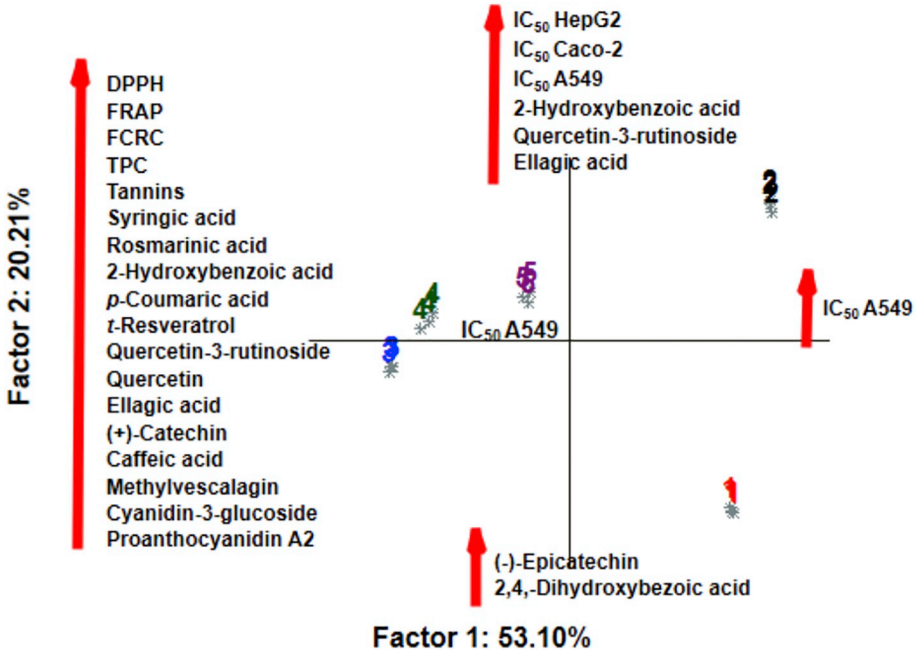

Fig. 4. Principal components analysis (PC1 *versus* PC2) of the camu-camu seed extracted with different concentrations of water and ethyl alcohol based on the chemical composition and antioxidant activity. Note: **1** = 100% H_2O; **2** = 100% EtOH; **3** = 50% H_2O + 50% EtOH; **4** = 25% H_2O + 75% EtOH and **5** = 75% H_2O + 25% EtOH. FCRC = Folin-Ciocalteu reducing capacity; TPC = total phenolic content.

observed antioxidant activity.

Considering the capacity on cell growth inhibition, in the current study, methylvescalagin showed a significant and negative correlation with A549 ($r = -0.6925$) and HepG2 ($r = -0.6096$) cells, suggesting that the higher amount of such a compound the lower is the concentration of extract necessary to kill half of the cells (IC_{50} value). This was also observed by Yang et al. (2018) with two new ellagitannins, isolated from the *Trigonostemon lutescens*, which showed potent cytotoxic activities against HeLa, HCT116 and HepG2 cells.

In the same way, our correlation analysis also showed a negative and significant correlation. ($p \leq .05$) between the GI_{50} values and epicatechin ($r = -0.5429$ for A549; $r = -0.6573$ for Caco-2 and $r = -0.5665$ for HepG2), indicating its important relationship with the cytotoxicity. Similarly, Santos et al. (2018) found that an optimized *Camellia sinensis* var. *sinensis*, *Ilex paraguariensis*, and *Aspalathus linearis* blend presented high total phenolic content, especially (−)-epicatechin, and highcytotoxic activities against cancer cell lines (HepG2 and Caco-2). Thus, epicatechin together with methylvescalagin, may be responsible, in parts, for inhibiting the proliferation capacity of cancerous cell lines.

Considering the high values of GI_{50} for HCT8 cell line, the gallic and 2,5-dihydroxybenzoic acids may have acted as cytoprotector agents against cytotoxic effects from extract component, as these compounds presented a high and positive correlation ($p < .001$) with HCT8 cells ($r = 0.7990$ and 0.9457, respectively) (Fig. 3G). Therefore, the higher the amount of these phenolic compounds, the higher the amount of extract needed to kill half of the cells. Another observation should be made: HCT8 cell line was not significantly and negatively correlated to any of the phenolic compounds evaluated herein. In this case, the cell death observed in cell viability test may have occurred by the combinations among the phenolic compounds or by an unidentified compound in the extracts. This association turn evident that the although individual phenolic constituents exist in different proportions in whole natural matrices, the final observed potential is not always the sum of each one of the individual phenolic compounds present (Martins, Barros, & Ferreira, 2016). Harish Nayaka, Sathisha, and Dharmesh (2010) observed that *Decalepis hamiltonii* phenolic acid extracts showed cytoprotectivity in NIH 3 T3 fibroblast cells, reducing power, radical scavenging ability and protection to DNA damage induced by hydroxyl radical and among the phenolic acids identified, gallic acid was one of the major contributors for these effects. Therefore, gallic acid may act as cytoprotector agent in different cell lines.

Another important observation based on the linear correlation analysis should be made: non-tannin phenolics was significantly ($p < .05$) correlated to the cytotoxic effect in relation to A549 ($r = -0.7663$) and HepG2 ($r = -0.6816$), while total tannins content was not significantly correlated to the cytotoxicity all four cancer cell lines ($r < 0.39$, $p > .15$). The antioxidant activity measured by FRAP ($r = -0.7126$, $p = .003$ for A549 and $r = -0.6114$, $p = .015$) and DPPH ($r = -0.5819$, $p = .023$ for A549) were also significantly correlated to the cytotoxic effect of camu-camu seed extracts.

In order to reduce the dimension of the data set, and to distinguish the camu-camu seed extracts, Principal component analysis (PCA) was applied. The first principal component (PC1) explained nearly 53% of the data variability and the second PC (PC2) explained roughly 20%, retaining about 73% of all variability in the experimental data (Fig. 4). In summary, the 50% H_2O + 50% EtOH and the 25% H_2O + 75% EtOH extracts had the highest antioxidant capacity (DPPH, FRAP), total phenolics, tannins, non-tannin phenolics, methylvescalagin, quercetin, 2-hydroxybenzoic, *p*-coumaric, caffeic rosmarinic and ellagic acids. In contrast, the aqueous extract presented a higher mean level of (−)-epicatechin which is similar to the data obtained by Fidelis et al. (2018), who observed the same compound in aqueous camu-camu seed coat extract.

It is known that the lower the IC_{50}, the greater the cell viability inhibition (Santos et al., 2018). Regarding the cell viability of the camu-camu hydroalcoholic extracts, higher IC_{50} values of HepG2, Caco-2 and A549 cells are associated with quercetin-3-rutinoside, 2-hydroxycinnamic and ellagic acids, which corroborates the Pearson's correlation coefficients. In contrast, Yanez et al. (2004) observed correlation between some phenolic compounds, such as quercetin and gallic acid, and their substantial antiproliferative effects against three melanoma cell lines. Herein, extract 2 (100% EtOH) presented the highest mean IC_{50} values, indicating low cell viability inhibition for all cell lines, while the other extracts, especially extracts 3 (50% H_2O + 50% EtOH) and 1 (100% H_2O) presented the lowest IC_{50} values, indicating higher cytotoxicity. In this sense, Tauchen et al. (2016) also showed that pericarp and leaves of *Myrciaria dubia* exhibited great cytotoxic activity (IC_{50} = 124 and 149.5 μg/mL, respectively) against HepG2 cells, however low correlation between this effect and phenolic compounds content.

PCA alone was not able to associate the cytotoxiceffect of the camu-camu seed hydroalcoholic extracts with the individual phenolic composition. Nonetheless, if all results (antioxidant and cytotoxic activities and the phenolic composition) are considered simultaneously, camu-camu seed extracted with either 50% H_2O + 50% EtOH is the most promising antioxidant and cytotoxic extract with the highest concentrations of phenolic compounds.

4. Conclusions

The current study provides novel information on the *in vitro* inhibition chromosomal damage and antioxidant and cytotoxicactivities of camu-camu seed extracts. None of the tested extracts exerted toxicity toward normal IMR90 cells, pointing their relative safety. In contrast, camu-camu hydroalcoholic seed extracts presented great cytotoxic effect against all cancer cell lines (HepG2, A549, Caco-2 and HCT8), especially 50% H_2O + 50% EtOH once it exhibited promising antioxidant and cell growth inhibition, besides its antimutagenic potential by preventing chromosomal aberration induced-cisplatin. (−)-Epicatechin and methylvescalagin were the major phenolic compounds associated with cytotoxicity, while gallic and 2,5-dihydroxybenzoic acids showed close relationship with cytoprotective effects in HCT8 cancer cell line. Detailed analysis of their chemical composition and *in vivo* antioxidant,cytotoxic activities should be performed in order to verify their potential practical application.

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Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.108551.

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Chapter 3

Camu-camu (*Myrciaria dubia*) seeds as a novel source of bioactive compounds with promising antimalarial and antischistosomicidal properties

ABSTRACT

Parasitic diseases have attracted worldwide attention due to mortality and morbidity, and several plants have been screened for antiparasitic activity. Although camu-camu (*Myrciaria dubia*) seeds are a rich source of phenolic compounds, with antioxidant, antimutagenic, cytotoxic, anti-inflammatory, antimicrobial, antihypertensive and neuroprotective properties, there is no information on their antiparasitic effects. In the present study we aimed to evaluate five hydroalcoholic extracts of camu-camu seeds in relation to their *in vitro* antimalarial, antischistosomicidal, leishmanicidal and anti-hemolytic effects. The extracts exhibited antischistosomicidal (ED_{50} values from 418.4 to >1000.0 µg/mL) and antimalarial activities (IC_{50} values from 24.2 to 240.8 µg/mL) for both W2 and 37 strains in all intra-erythrocytic stages. According to the correlation analysis, this toxic effects may mainly be attributed to methylvescalagin ($r = -0.548$ to -0.951, $p<0.05$) and 2,4-dihydroxybenzoic acid ($r = -0.612$ to -0.917, $p<0.05$) contents. Moreover, the anti-hemolytic effect was associated to methylvescalagin ($r = -0.597$, $p<0.05$). No toxic effects were observed for leishmaniasis and IMR90 normal cells. Herein, methylvescalagin was the bioactive compound of greatest interest once it presented simultaneous relation with antiparasitic and anti-hemolytic activities.

1 INTRODUCTION

Parasitic diseases are one of the world's most devastating and prevalent infections, causing millions of morbidities and mortalities annually. (MOMCILOVIC et al., 2018). Among them, *Plasmodium falciparum* is the most virulent of the malaria parasites that infect humans and is responsible for most of the malaria-related deaths. Despite the global epidemic of malaria decreased, according to the latest WHO estimates released, there were still 212 million cases of malaria in 2015 and 429,000 deaths (BOUCHUT et al., 2019). Schistosomiasis is another severe debilitating neglected tropical disease that affects almost 240 million people worldwide, and more than 700 million people live in endemic areas (WHO, 2016), while Leishmaniasis is reported that the disease currently threatens about 350 million people in 88 countries around the world, with about 2 million affected annually (MUGANZA et al., 2012).

In this sense, a large number of Brazilian, indigenous and African plants have been screened for antiplasmodial activity *(Artemisia annua, Combretum micranthum, Securidaca longepedunculata),* antischistosomicidal (*Curcuma longa, Plectranthus neochilus* and *Artemisia annua)* and leishmanicidal (*Warbugia ugandensis, Acacia nilotica,* and *Ambrosia miratima*) and flavonoids have been closely associated with this property (KRETTLI, 2009; MWANGI et al., 2017). Considering these concerns, the close association between medicine and natural products has been established through the application of traditional therapies and natural drugs many years ago. Moreover, the remarkable activity of quinine and the success of related drugs, such as artemisinin to treat malaria, have stimulated the search for new plant derived with biopharmaceutical properties (TEINKELA et al., 2018).These findings were a result of a study, which investigated more than 2,000 Chinese herb preparations and identified 640 hits that had possible antimalarial activities (TU, 2011). Therefore, camu-camu seed extracts have a rich and varied chemical profile that may be a promising source of novel drug structures for malaria, schistosomiasis and leishmaniasis treatments, which has been a challenge for treatment and control strategies.

Camu-camu byproducts, such as seeds, have been associated with *in vitro* and *in vivo* functional properties, such as antioxidant, antimutagenic, cytotoxic (CARMO et al., 2019), anti-inflammatory (YAZAWA et al., 2011), antimicrobial (MYODA et al., 2010) antihypertensive and neuroprotective (FIDELIS et al., 2018) properties. Thus, in this context, we used a series of statistical methods to assess the bivariate and multivariate association between the phenolic composition of different hydroalcoholic extracts of camu-camu seeds and their *in vitro* antimalarial, antischistosomicidal, antileishmanial and anti-hemolytic activities.

2 MATERIALS AND METHODS

2.1 CAMU-CAMU SEEDS EXTRACTION AND PHENOLIC COMPOSITION

The procedures of camu-camu seed extracts were previously describe by Carmo et al. (2019). Briefly, the camu-camu seeds were removed from fruit, dried in an oven with air circulation at 35° C for 31 h (~12% moisture) and then they were ground using a knife mill. The extractions were performed in the ratio 1:20 (sample: solvent, m/v), i.e., 10 g of flour obtained from the camu-camu seeds were mixed with 200 mL of solvent mixture. In all, 5 different extractions were obtained with ultrapure water and ethyl alcohol: 100% ultrapure water (H_2O), 100% ethyl alcohol (EtOH), 50% ultrapure water + 50% ethyl alcohol, 25% ultrapure water + 75% ethyl alcohol, and 75% ultrapure water + 25% ethyl alcohol. The filtered extract was transferred to a rotary evaporator and, finally, lyophilized for biological analyses. The phenolic profiling of the camu-camu seed extracts was determined by high-performance liquid chromatography (HPLC) in our previous work, in which a total of 18 phenolic compounds were detected and tentatively identified, including phenolic acids, flavonols, flavan-3-ols, tannins and anthocyanins (CARMO et al., 2019).

2.2 *IN VITRO* ANTIPLASMODIAL TEST

The *in vitro* antiplasmodial effect of the camu-camu seed extracts were analyzed in relation to W2 (chloroquine resistant) and 3D7 (chloroquine sensitive) strains. Briefly, parasites were cultured in Petri dishes containing RPMI culture medium supplemented with 10% albumax II (Gibco, USA) with 4% hematocrit. Plates were incubated at 37° C using the candle jar method. The culture medium was replaced daily and parasitaemia checked in Giemsa-stained smears. The parasites were synchronized with sorbitol solution as described by Lambros & Vanderberg (1979) to get mostly ring forms, diluted and incubated in 96 well plates containing the extracts, chloroquine, or culture medium (positive control). All cultures were started using a single synchronized culture from which aliquots were taken at 12 h (young rings - Figure 1 IIA), 24 h (trophozoites - Figure 1 IIB) and 36 h (schizonts - Figure 1 IIC) and treated with different concentrations (10 – 500 µL/mL) of camu-camu seed extracts at 37º C with the parasite suspensions (0.5% parasitaemia and 2% hematocrit) into 96-well microplates. After 48 h, the culture supernatant was removed and replaced by 100 µL of lysis buffer solution [Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%; wt/vol), and Triton X-100 (0.08%; v/v)]

followed by addition of 0.2 µL/mL Sybr Safe (Sigma-Aldrich, Carlsbad, CA, USA). The microplates were incubated in the dark for 30 min and the reading was made using a microplate reader (Synergy ™ H1, Biotek) with excitation at 485 nm and emission at 535 nm (ADEBAYO; ADEWOLE; KRETTLI, 2017).

Intra-erythrocytic images of *P. falciparum* stages were obtained from AFM for the qualitative examination of parasites inside erythrocytes. The 256 × 256 pixel resolution AFM images were obtained with a Park NX10 microscope using the True Non-Contac™ Mode. The initial scan size was 50 × 50 µm made with a NSC15 cantilever (from MikroMasch) at 0.15 Hz scan rate, 3.5 Z servo gain and 6 nm set point.

2.3 ASSAY FOR HAEM POLYMERIZATION

The hemozoin (β-haematin) formation inhibition assay was performed as described by Silva et al. (2015). Briefly, solutions with different concentrations (10 to 80 mg/mL) of 50% H2O + 50% EtOH extract was added (20 µL) in quadruplicate to a 96-well plate. Next, bovine hematin (SigmaAldrich, Germany) solution (101 µL; 1.68 mM in 0.1 M sodium hydroxide) was added to each well followed by addition of pH 5 sodium acetate buffer (12 M; 58 µL) with constant stirring at 60° C. After incubation at 60° C for 1 h, the plate was centrifuged at 500 g for 8 min. The supernatant was discarded and the crystals of hemozoin were dissolved in 200 µL of 0.1 M sodium hydroxide. The reaction was monitores using a spectrophotometer at 405 nm, and the results were expressed as the percent inhibition of hemozoin formation. The optical density of the untreated controls corresponded to 100% hemozoin formation and the known inhibitor chloroquine was used as a positive control.

2.4 *IN VITRO* ANTI-HEMOLYTIC ACTIVITY

The anti-hemolytic activity of the camu-camu seed extracts was performed according to the procedures described by Escher et al. (2018). The O+ blood sample was obtained from the Clinical Laboratory of Federal University of Alfenas, Brazil. The erythrocytes were concentrated with phosphate buffered saline (PBS pH 7.3) and centrifuged at 700 *g* for 10 min. The extract was diluted in PBS from 10 to 50 µg/mL and incubated with 0.8% hematocrit at 25 °C for 15 min. The 96-well microplates were centrifuged at 700 g for 10 min and the absorbance was recorded at λ=576 nm. Total hemolysis (positive control) consisted of erythrocytes in ultrapure water. The hemolytic effect was obtained according to the following equation:

% Hemolysis = (Aa/ATH) × 100

where Aa corresponds to the absorbance of the extract and ATH corresponds to the absorbance of the total hemolysis.

2.5 *IN VITRO* EVALUATION OF THE EFFECT OF CAMU-CAMU SEED AGAINST ADULT WORMS OF *SCHISTOSOMA MANSONI* AND *IN VITRO* ANTILEISHMANIAL ACTIVITY AGAINST PROMASTIGOTES

The *S. mansoni* LE (Luiz Evangelista) strain was maintained by serial passages in Biomphalaria glabrata models and mice of Swiss lineage in the Research Center René Rachou/Oswaldo Cruz Foundation. All the experiments and procedures were approved by the Ethical Committee for Animal Research (Protocol #408/2012) 20th, 2014 in accordance with the ethical principles required for animal. Thus, mice infected with *S. mansoni* cercariae (LE strain) were sacrificed 45 days after infection by "overdose" with 10.0% ketamine chloride (Ketamina Agener) and 2.0% xylazine hydrochloride (Rompun) dissolved in saline, administered intraperitoneally (±0.2 mL per animal). Subsequently, the retrograde liver perfusion was completed for obtaining the parasites and the recovered parasites were cultivated in twelve-well culture plates (four couples/well) in RPMI-1640 culture medium supplemented with 5.0% fetal bovine serum heat-inactivated; besides 1.0% penicillin (10,000 IU/mL) and streptomycin (10.0 mg/mL) (Sigma, USA). Then, the samples of each camu-camu seed extract, (100% H_2O, 75% H_2O + 25% EtOH, 50% H_2O + 50% EtOH, 25% H_2O + 75% EtOH and 100% EtOH) were added to the cultures at different concentrations (250, 300, 400, 500, 600, 700 and 1000 μg/mL). As described by Viegas et al. (2017) the plates were then kept in an incubator at 37 °C and 5.0% of CO_2; and analyzed within 2 and 24 h after contact with the samples. The test groups were compared to the controls of supplemented RPMI 1640 medium, DMSO 0.25% (used as solvent for all samples) and praziquantel (2 μg/mL). After 24 h, the wells were washed five times to remove the samples from contact with the parasites by removing the culture medium from the wells and adding the same amount of sterile medium. The cultures were analyzed daily for eight days using inverted microscopy (Nikon Eclipse TS100 microscope, increase of 4×, 10×, 20× and 40×), and records of the adult worms were documented.

In respect of antileishmanial assay, promastigotes of *Leishmania (L.) amazonensis* (strain MHOM/BR/71973/M2269) were grown on 24-well plates in LIT medium, supplemented with 10.0% (v/v) heat-inactivated foetal bovine serum and 1.0% penicillin (10000UI/mL)/streptomycin (10.0 mg/mL) (Sigma, USA). Cells were harvested in the log phase, suspended in fresh medium, counted in Neubauer chambers and adjusted to a

concentration of 1 x 10^6 cells/mL, using 24-wells plates. The camu-camu seed extracts were added to promastigote cultures (1 x 10^6 cells/mL) in the range of 0.10–40.00 µg/mL, solubilized in dimethyl sulfoxide (DMSO) (0.6%, v/v in all wells) and incubated at 25 ºC. After 72 h of incubation, the surviving parasites were counted in a Neubauer's chamber and compared with controls, with just DMSO in concentration of 0.6% v/v, for the determination of 50.0% inhibitory growth concentration (IC_{50}). All tests were performed in triplicate at three different times and amphotericin B (Sigma) was used as the reference drug (ESPURI et al., 2019).

2.6 CYTOTOXICITY TEST

The in vitro cytotoxic effect of the camu-camu seed extracts against normal human lung fibroblast cells (IMR90) and its 50% cytotoxic concentration (IC_{50}) were previously described by Carmo et al. (2019). Briefly, cells were seeded at 5 x 10^3/well and after 48 h of treatment with the camu-camu seed extracts (100 – 900 µg/mL) the cytotoxicity was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method. The camu-camu selectivity index (SI) was evaluated by the ratio IC_{50} (IMR90)/IC_{50} (*P. falciparum* or *S. mansoni)* (BOECHAT et al., 2014).

2.7 STATISTICAL ANALYSIS

In order to check for quantitative association between chemical composition and antimalarial and antischistosomicidal activities, Pearson's correlation coefficients were calculated the probability values below 5% were regarded as significant. For the multivariate projection of samples on the two-dimensional plane, principal component analysis (PCA) was carried out according to the methodology recommended by Granato, Santos, Escher, Ferreira, & Maggio (2018). For that purpose, the data matrix (five extracts analyzed in triplicate for a total of 25 responses) was autoscaled to unit variance, totaling 375 data points. Factor loadings ≥0.60 were considered to project the responses on the factor-plane. The software TIBCO Statistica v. 13 (TIBCO Statistica Ltd, Palo Alto, USA) was used in the analyses.

3 RESULTS AND DISCUSSION

3.1 ANTIMALARIAL ACTIVITY OF CAMU-CAMU SEED EXTRACTS

Bioactive compounds of camu-camu seeds have not previously been explored as an antimalarial treatment and as observed in Figure 1, the strains W2 and 3D7 exhibited high sensitivity toward all extracts, moreover the antimalarial test revealed that the different extracts presented differential pattern effect in both strains. For instance, in respect of chloroquine resistant strain (W2), the ring was the most sensible stage, once it exhibited lower IC_{50} value (24.2 µg/mL) for 100% H_2O extract. In contrast, regarding chloroquine sensitive strain (3D7), the trophozoite was the most vulnerable form (IC_{50} = 26.8 µg/mL) for 75% H_2O + 25% EtOH extract. In fact, camu-camu seed extracts, which are rich in phenolic compounds, may interfere on *P. falciparum* fatty acid metabolism, once Tasdemir et al. (2006) pointed out that phenolic compounds, such as flavonoids (i.e. quercetin), presented antimalarial effects by inhibiting enzymes (β-ketoacyl-ACP-reductase, β-hydroxacyl-ACP-dehydratase and enoyl-ACP-reductase) involved on type-II fatty acid biosynthesis pathway of *P. falciparum.* In general, herein, the schizont form from both strains seemed to be more resistant to the treatments (IC_{50} = 81.5 to 240.8 µg/mL).

Considering the different response of camu-camu seed extracts among intra-erythrocytic *P. falciparum* stages, Bozdech et al. (2003) showed that trophozoite stage presented several genes related to general cellular growth functions such as transcription, translation and hemoglobin degradation, while schizont form exhibited more genes associated with merozoites maturation. Moreover, Weißbach, Golzmann, Bennink, Pradel, & Julius, (2017) identified nine protease genes (*pfαβh, falcipain 1 (pffp1), pffp3, pfdpap1, pfm1-ap, pfsub3, pfpm9, pfpm10 and pffln*) which showed transcript expression in ring, trophozoite and schizont stages, however, the transcripts of three genes (*pfdpap3, pfsub1 and pfsera5*) were exclusively present in schizonts. Most notably, Ferreira et al. (2019) pointed out that the nonanthocyanin phenolics fraction from açaí (*Euterpe oleracea*) pulp presented antimalarial properties by targeting the CCT complex, an essential protein folding machinery, which plays a key role in *P. falciparum* proteostasis and cell survival. Herein, taken together the chemical composition of extracts and the different gene-expression profiles among the intra-erythrocytic stages, it is expected that *P. falciparum* ring, trophozoite and schizont can exhibit different behavior towards the same camu-camu extract/compound. This situation opens the field for the development of new treatments with increased efficacy (three *P. falciparum* stages as targets) and decreased risk of developing resistance. According to the correlation analyses (Figure 5), we proposed that the antimalarial

effects observed against both *P. falciparum* strains, may mainly be correlated to methylvescalagin and 2,4-dihydroxybenzoic acid contents. These compounds could gain access to the red blood cell (RBC) cytosol via pores, which appear in the host membrane of infected RBC several hours after merozoites invasion (KUTNER et al., 1987). Moreover, the toxic effect of hydrolysable tannins, such as methylvescalagin, may be attributed to their ability of binding to proteins and other molecules interfering on their biological pathways (ADRAR; MADANI; ADRAR, 2019; MONTEIRO et al., 2005). In light of these concerns, we hypothesize that methylvescalagin go into the infected RBC by membrane pores and it complexes with specific binding sites of protein molecules, thus inhibiting the *P. falciparum* intra-erytrocytic development. In fact, these findings may explain why our extracts were toxic to parasite and simultaneously did not exert harmful effects toward non-infected RBC. Careful consideration of interaction between *P. falciparum* and phenolic compounds will be necessary in future studies in order to clarify the pathways involved in antimalarial mechanism of action.

Together, IC_{50} and selective index (SI) values correspond a efficacy parameters for *in vitro* antimalarial activity and for crude extracts, IC_{50} values should certainly be below 100 mg/mL (TEINKELA et al., 2018) while the most promising antimalarial extracts are those with IC_{50} values below 15 µg/mL (JANSEN et al., 2012). In this study, the extracts revealed moderate (15 µg/mL < IC_{50} ≤50 µg/mL) and weak (IC_{50} >50 µg/mL) activities depending on the parasite's stages. The weak activity observed for some extracts did not indicate the absence of antimalarial activity only that it was required higher concentrations to achieve the desired therapeutic effect. Interestingly, Correia et al. (2016) reported that dichloromethanolic extract from *Myrciaria dubia* pulp, impacted on W2 strain activity (IC_{50} = 2.35 µg/mL). This disagreement may be explained by the affinity between the phenolic compounds present and the polarity of the extracting solvent, besides the part of the plant used for extraction (SETFORD et al., 2017).

Regarding the SI, any sample which its value higher than 3 will be considered to have high selectivity (PRAYONG; BARUSRUX; WEERAPREEYAKUL, 2008). Fortunately, growth of non-cancer IMR90 cells was not inhibited by the extracts, which exhibited high IC_{50} values (> 900 µg/mL), suggesting that it is necessary to use higher concentrations to inhibit the proliferation of half of the cells, meaning low cytotoxicity against this normal cell line. Herein, the SI (Table 1) obtained for all the extracts and both strains were much higher than 3 (SI from 3.7 to 37.2), indicating that camu-camu seed extracts were more toxic to *Plasmodium falciparum* than IMR90 normal cells and suggesting safety, selectivity and their potential for biopharmaceutical applications against malaria.

Figure 5- (I) Antiplasmodial activity and cytotoxicity of camu-camu seed extracts, against chloroquine resistant strain (W2) and chloroquine sensitive strain (3D7). Antiparasitic activities of extracts were classified into four classes according to their IC_{50} values: high activity ($IC_{50} \leq 5$ µg/mL); promising activity (5 µg/mL < $IC_{50} \leq 15$ µg/mL); moderate activity (15 µg/mL < $IC_{50} \leq 50$ µg/mL); weak activity ($IC_{50} > 50$ µg/mL). (II) Different synchronized plasmodial stages at beginning of treatment with camu-camu seed extracts. Representative light-microscopy fields of cultures subjected to the treatment are shown after panoptic staining at the times 12h (A), 24h (B) and 36h (C). Images (D, E, F) obtained from 792 Atomic Force Microscope (AFM, Park NX10), exhibiting infected red blood cells by *Plasmodium falciparum*

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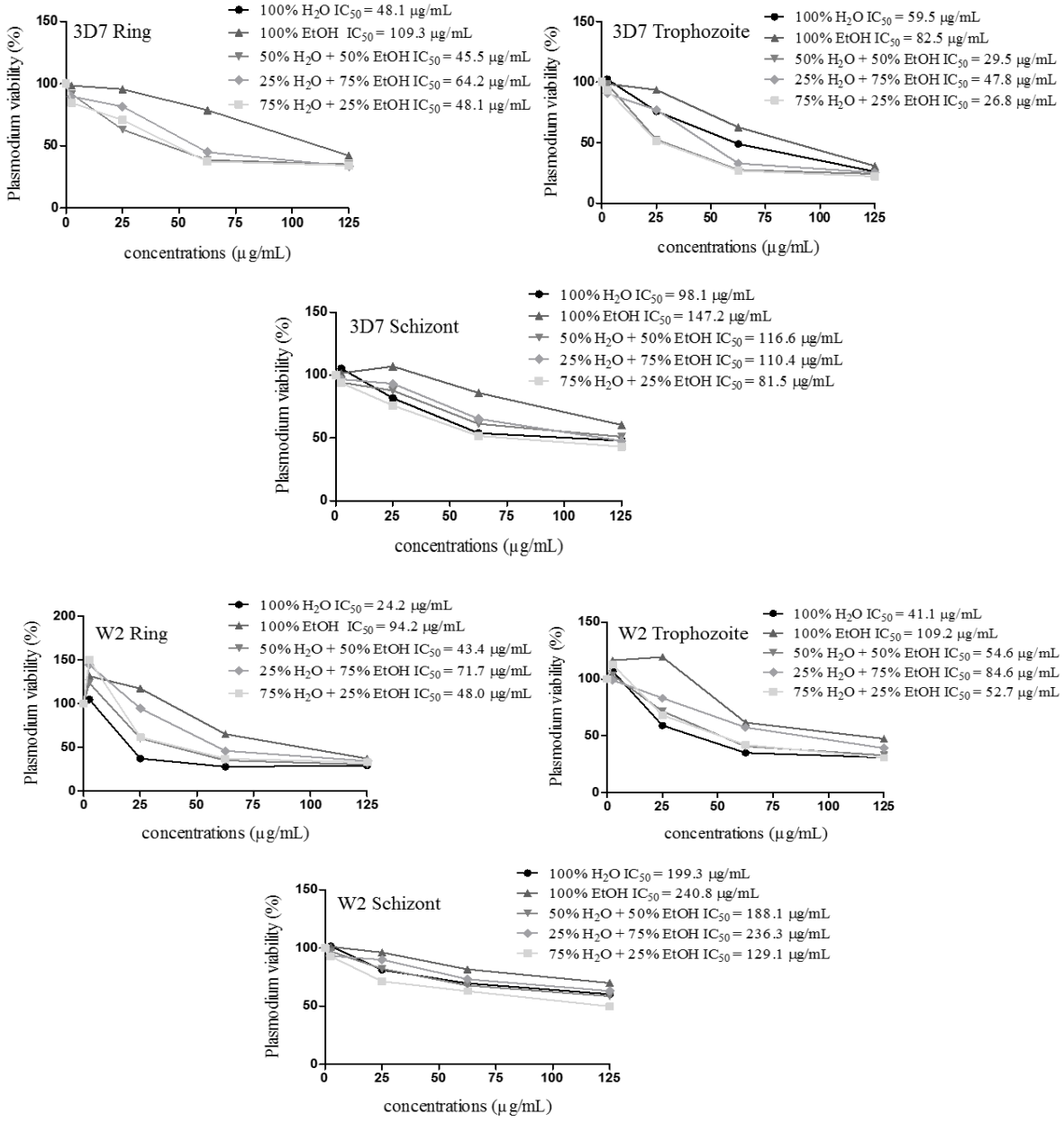

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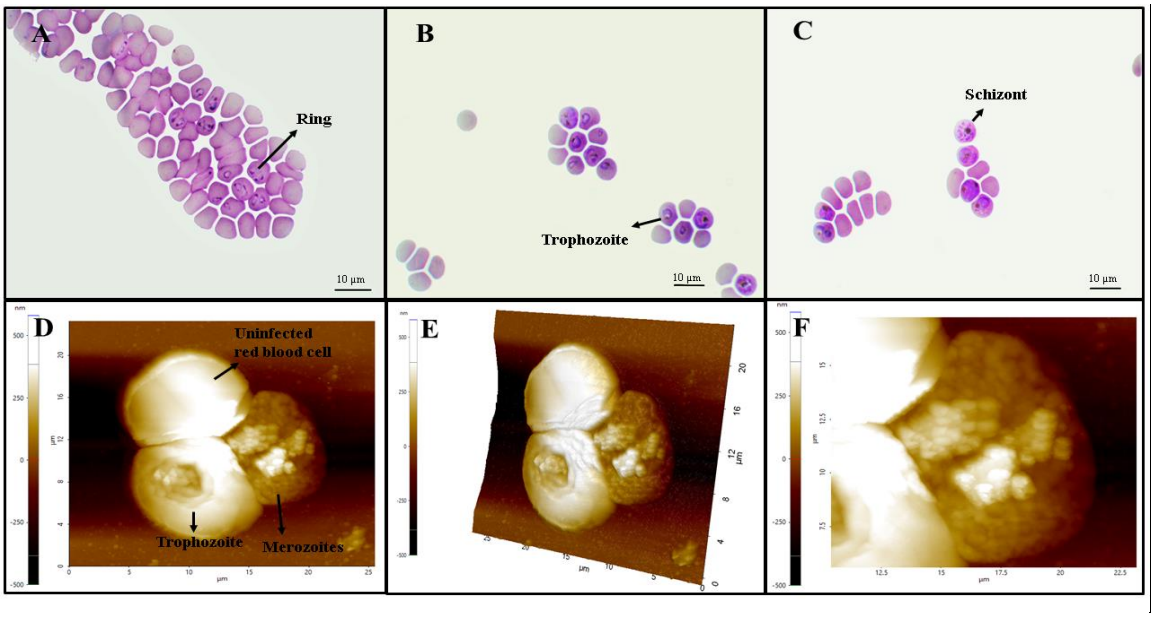


Source: Author, 2020.

Table 1- Selectivity index (SI) in *S. mansoni* and ring (R), trophozoite (T) and schizont (S) stages of *P. falciparum*

Extracts	Selectivity Index (SI) IC_{50} IMR90 cell/IC_{50} *P. falciparum* (W2-3D7) *or S. mansoni*			
	R (W2-3D7)	T(W2-3D7)	S(W2-3D7)	*S.mansoni*
100% H_2O	>37.2 - >18.7	>21.9 - > 15.1	> 4.5 - >9.2	>1.86
100% EtOH	>9.5 - >8.2	>8.3 - >10.9	>3.7 - >6.1	-
50% H_2O + 50% EtOH	>20.7 - >19.8	>16.5 - >30.5	>4.8 - >7.7	>2.5
25% H_2O + 75% EtOH	>12.6 - >14.0	>10.6 - >18.8	>3.8 - >8.1	>1.35
75% H_2O + 25% EtOH	>18.8 - >18.7	>17.0 - >33.6	>7.0 - >11.0	>1.77

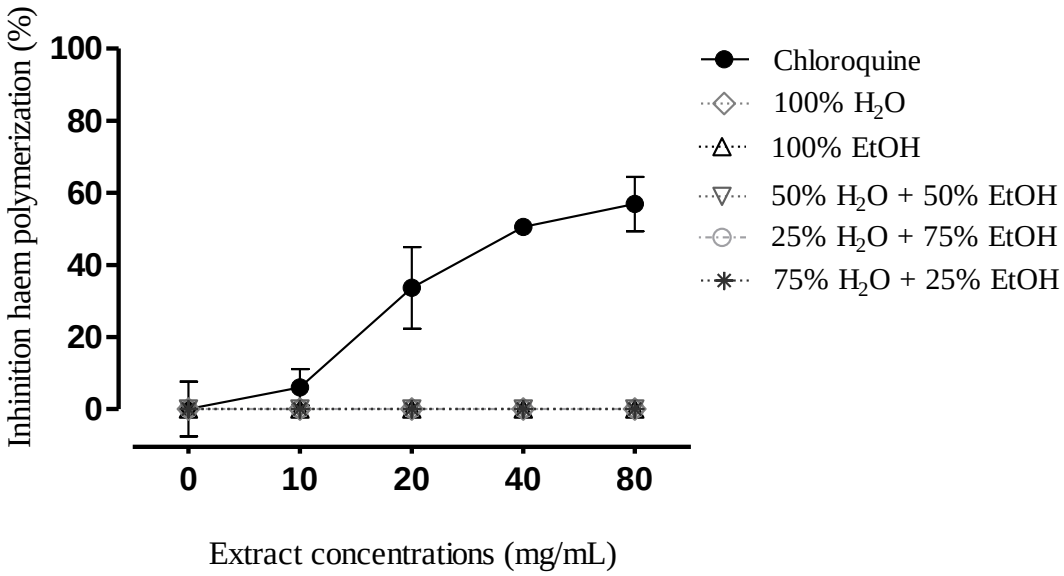
Source: Author, 2020.

3.2 ASSAY OF HAEM POLYMERIZATION

According to the above results, all the camu-camu seed extracts revealed antiplasmodial activity. *Plasmodium* is hematophagous organism, and the hemozoin formation is crucial for the survival of this parasite (WANG et al., 2017). Heme detoxification into hemozoin was believed to be one of the most attractive drug development targets against malaria (HU et al., 2017). In order to verify the hypothesis that our extracts could interfere on hemozoin polymerization, the inhibition assay of β -haematin (synthetic hemozoin) formation was performed. Our results revealed that the antimalarial activity of chloroquine involves the inhibition of formation of hemozoin as observed by Moneriz et al. (2011), Silva et al. (2015) and Wang et al. (2017). Herein, chloroquine 80 mg/mL inhibited in 60% the β-haematin formation (Figure 2). In contrast, all the tested extracts did not interacted with the hemozoin

process. Pathways such as DNA intercalation, alteration of digestive food vacuole pH and formation of a toxic ferriprotoporphyrin IX complex can be related with the extracts toxicity (TEWARI et al., 2017). Following a similar approach, Moneriz et al. (2011) presented that maslinic acid, a natural triterpene obtained from olive pomace, did also not interfere in haem polimerization. So, our data confirmed the antimalarial activity by camu-camu seed extracts, but investigations are needed, once inhibiting hemozoin formation was discarded as antiplasmodial mechanisms of action.

Figure 6-. Assay of β-haematin polymerization. Haem was incubated in the presence of increasing amounts of camu-camu extracts and chloroquine for 1 h, and the formation of β-haematin was determined spectrophotometrically at 405 nm. Results are the mean ± SD.



Source: Author, 2020.

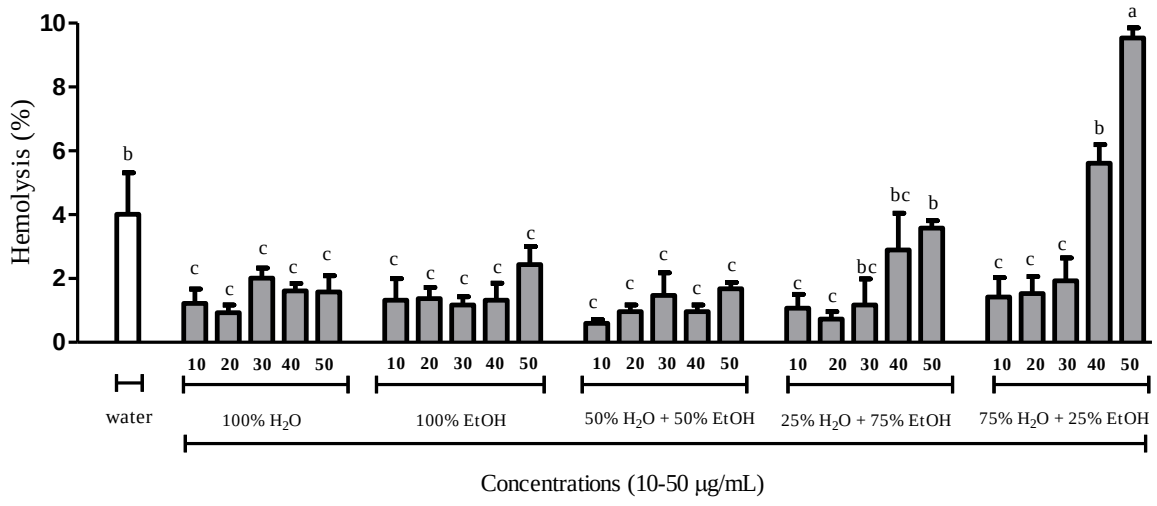
3.3 *IN VITRO* ANTI-HEMOLYTIC ACTIVITY

Hemolytic activity is considered as an indicator of general cytotoxicity of drug towards normal healthy cells, and herbal drugs contain phytoconstituents, which might disrupt red cell membrane inducing hemolytic anemia (SUGANTHY; MUNIASAMY; ARCHUNAN, 2018). The *in vitro* anti-hemolytic activity of camu-camu seed extracts (0 – 50 µg/mL) was evaluated in relation to type O+ human blood erythrocytes. Figure 3 shows that the different extracts exhibited differential hemolytic effect towards human erythrocytes. Results indicated that the extracts 100% H_2O, 100% EtOH and 50% H_2O + 50% EtOH exhibited a beneficial interaction with the erythrocytes, as well as a protective effect against hemolysis when compared to mechanically hemolysed erythrocytes (0 µg/mL). Similar findings were observed by Escher et

al. (2018) with *C. cyanus* petals extract, which demonstrated dose-dependent anti-hemolytic capacity. Herein, on the other hand, the extract 75% H_2O + 25% EtOH, showed protective effects with the lower concentrations, but elevated hemolytic activity with the highest tested concentrations. In this case, lysis of erythrocytes was found to be increased with an increase of extract concentration. Shabbir, Khan, & Saeed (2013) showed that fractions of *M. royleanus* leaves, despite the anti-hemolytic activity, presented a dose dependent increased cell lysis. Ralph, Guest, & Green (1998) rated the degree of *in vitro* toxicity in hemolytic assay, as 0–9% non-toxic, 10–49% slightly toxic, 50–89% toxic and 90–100% as highly toxic. All extracts exhibited a non-toxic profile, moreover anti-hemolytic activity, except the sample 75% H_2O + 25% EtOH in the highest concentration.

The hypotonic hemolysis is a situation of erythrocyte osmotic instability as the low osmotic pressure in the extracellular medium makes the erythrocyte to absorb water to achieve the osmotic balance. The excess of extracellular water leads to extreme distension and consequently the cell membrane disrupts, releasing the hemoglobin content (ZHANG et al., 2019). Within this context, extracts rich in phenolic compounds, like camu-camu seed extracts, may reduce the erythrocyte hemolysis under hypotonic conditions by decreasing the fluidity of the membrane cell (PHAN et al., 2014), and by increasing the osmotic pressure caused by the presence of ions (SATO; YAMAKOSE; SUZUKI, 1993). Thinking about the phenolic composition of camu-camu seed extracts, the correlation analysis (Figure 5) showed that this protector effect observed against hemolysis could be attributed by rosmarinic acid and metylvescalagin contents.

Figure 7- Anti-hemolytic activity of camu-camu seed extracts. Different letters represent statistically significant differences within the same group and control ($p < 0.05$). 1, 2, 3, 4 and 5 = 100% H_2O, 100% EtOH. 50% H_2O + 50% EtOH, 25% H_2O + 70% EtOH and 75% H_2O + 25% EtOH, respectively, at 0 – 50 µg/mL.



Source: Author, 2020.

3.4 *IN VITRO* EFFECT OF CAMU-CAMU SEED EXTRACTS ON ADULT WORMS OF *S. MANSONI* AND *L. (L.) AMAZONENSIS* PROMASTIGOTES

Our results show that all camu-camu seed extracts exhibited no toxicity against *L. (L.) amazonensis promastigotes* at tested concentrations, indicating ED_{50} values > 1000 µg/mL. In relation to *S. mansoni* worms, except 100% EtOH, all extracts revealed a promising bioactive source of active metabolites, which killed 100% of the worms at 1000 µg/mL (Figure 4I). Among the samples, the 50% H_2O + 50% EtOH extract presented better activity (ED_{50} = 418.4 µg/mL), while 100% EtOH extract exhibit no antischistosomicidal effect. Through the microscopic images, we observed that our extracts caused flaccid paralysis, reduction on motor activity, as well as tegument damage in parasites of both sexes (Figure 4II B, C, D and F). In contrast, when adult worms were maintained in the RPMI medium containing 0.25% DMSO, their appearance were similar to those maintained in the same medium without DMSO. During the incubation period, all parasites revealed normal motor activity with natural peristalsis of the worm body and peristalsis of the gut. PZQ (2 µg/mL), used as positive control, caused the death of all the parasites after incubation period. Dias et al. (2017) also observed that hydroalcoholic extract of *Arctium lappa L.*caused decrease of motor activity and tegumental alteration, besides 100% of *S. mansoni* death at 200 µg/mL. These findings could be related to some natural compounds, which may act as agonists or antagonists of neuroreceptors/ion channels leading *S. manasoni* to death by neurotoxic effects. *S. mansoni* parasites have a nervous system that utilizes neurotransmitters such as acetylcholine (ACh) and AChreceptors, which are responsible for controlling worm muscle activity (WINK, 2012). Therefore, inhibition of the activity of these neuroreceptors may result in behavioral changes, such as muscle paralysis and worms death. So, according to correlations analyses, we hypothesized that *S. mansoni* mortality may be correlated to *trans*-resveratrol, methylvescalagin and 2,4-dihydroxybenzoic acid contents found in the extracts. The *S. mansoni* SI obtained for all the extracts varied between >1.35 and >2.5, which were much lower than to *P. falciparum* (SI = 3.7 to 37.2), indicating that camu-camu seed extracts were more toxic to *Plasmodium falciparum* followed *S. mansoni* and IMR90 cells.

Figure 8- Adult *S. mansoni* worms exposed to the camu-camu seed extracts. (A) Control, DMSO; (B) 75% H_2O + 25% EtOH; (C) 50% H_2O + 50% EtOH; (D) 25% H_2O + 75% EtOH; (E) 100% EtOH; (F) 100% H_2O. WE = worms eggs; MW = male worm and FW = female worm. (Scale bar = 200 µm).

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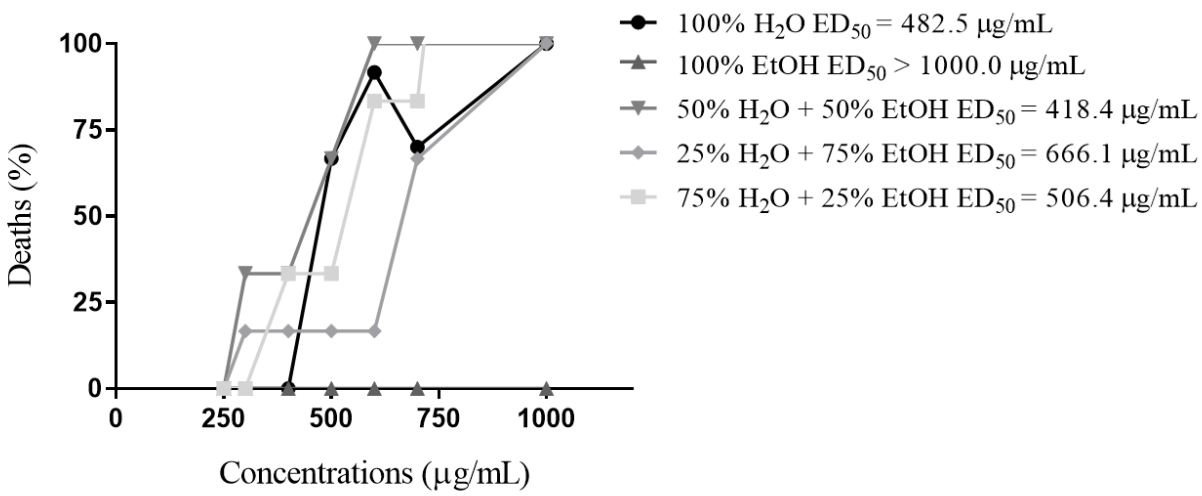

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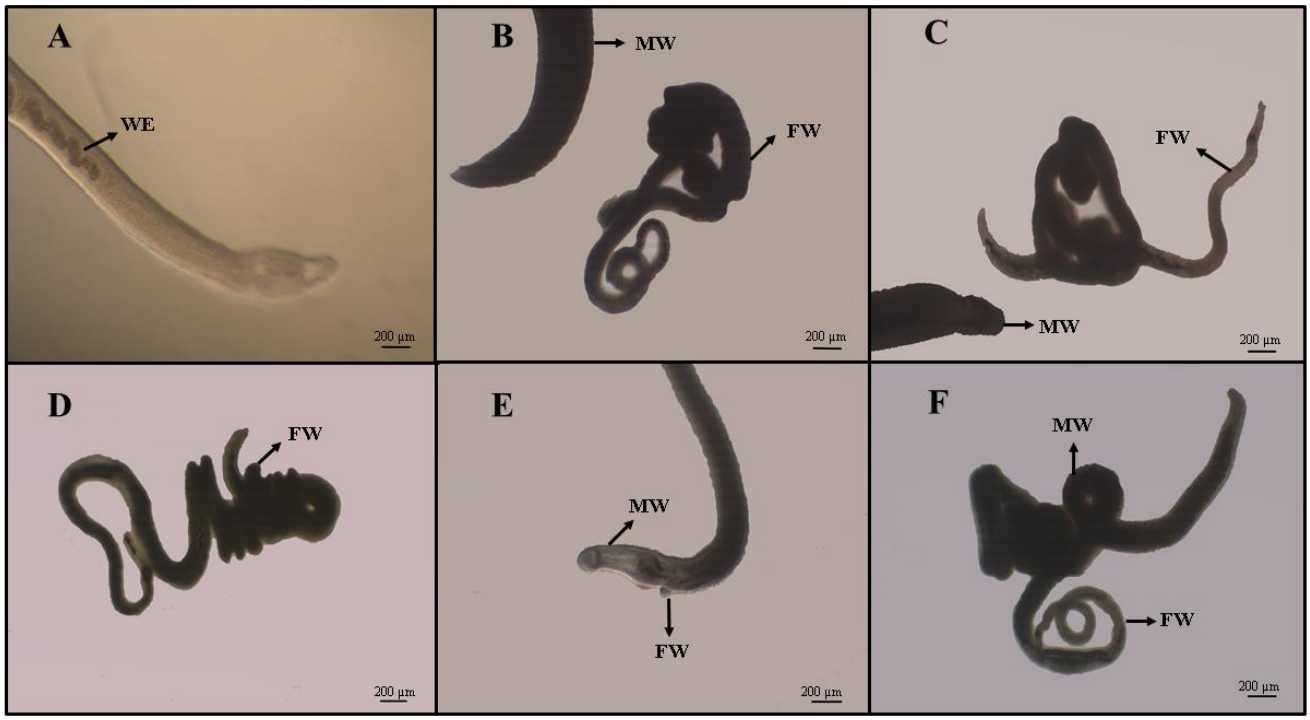


Source: Author, 2020.

3.5 CORRELATION ANALYSES

To the best of our knowledge, this is the first study that establishes the relationship between individual phenolic constituents from camu-camu seed extracts and their *in vitro* antimalarial, antischistosomicidal and anti-hemolytic activities, in order to identify the main compounds responsible to the observed toxicity. In light of these considerations, methylvescalagin and 2,4-dihydroxybenzoic acid showed strong and negative correlation

(Figure 5) with S. *mansoni* (r = - 0.883; r = - 0.872) and all stages and strains of *P. falciparum* (r values between - 0.548 and - 0.904) cells, suggesting that the higher amount of these compounds the lower is the concentration of extract necessary to kill half of the cells (IC_{50} value). Similarly, Carmo et al. (2019) observed that the same extracts presented cytotoxic effects against cancer cells associated to methylvescalagin content.

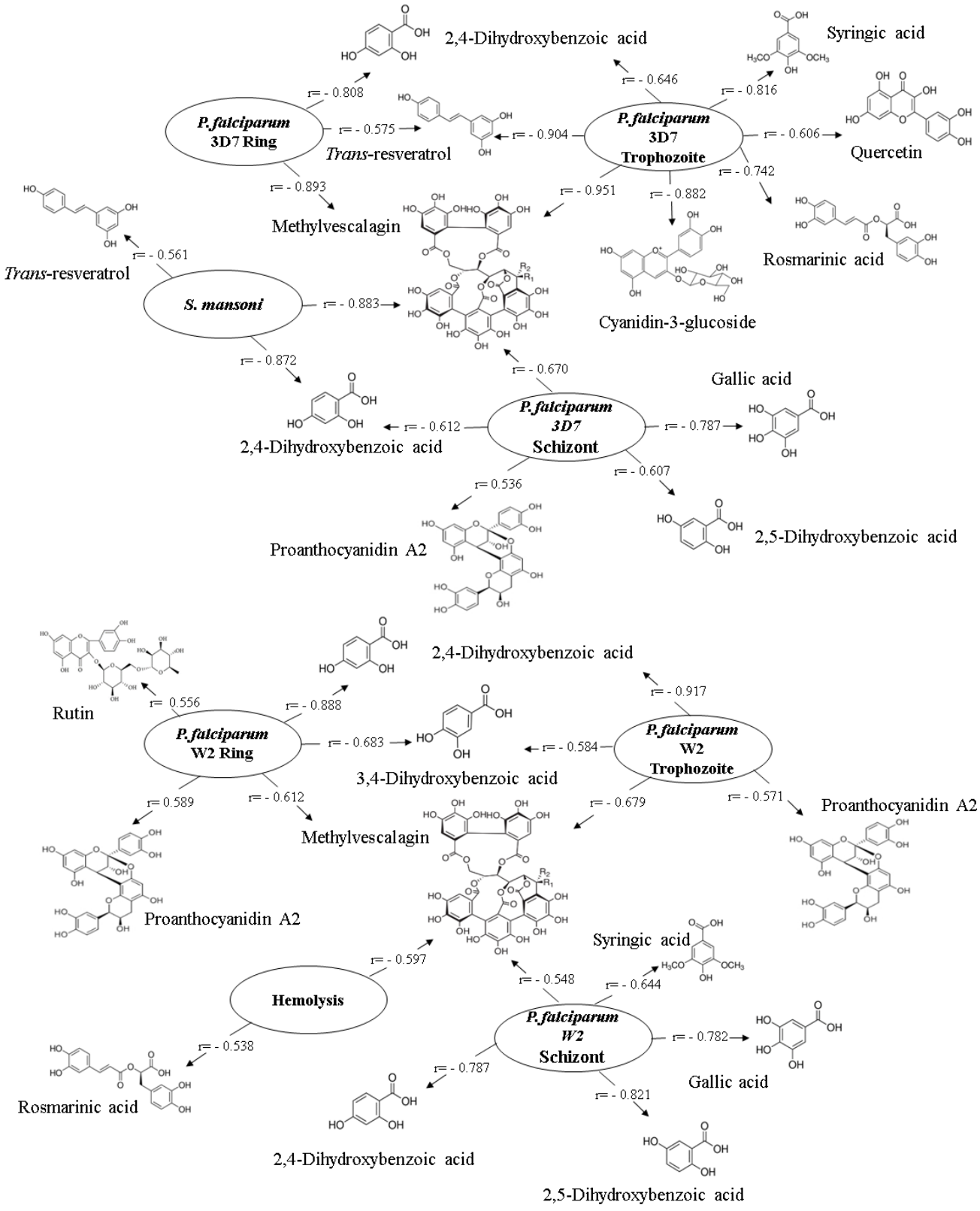
In the same way, the correlation analysis also showed a negative and significant correlation ($p \leq 0.05$) between *trans*-resveratrol and *S. mansoni* (r = - 0.561) and 3D7 *P. falciparum* strain on both ring (r = - 0.576) and trophozoite (r = -0.904) stages. Other compounds correlated with the toxic parasite potential of extracts were: rosmarinic acid (r = - 0.742 for 3D7 trophozoite), syring acid (r = -0.816 for 3D7 trophozoite and r = -0.644 for W2 schizont), gallic acid (r = -0.787 for 3D7 schizont and r = -0.782 for W2 schizont), quercetin (r = - .606 for 3D7 trophozite), 2,5 dihydroxybenzoic acid (r = -0.607 for 3D7 schizont), 3,4 dihydroxybenzoic acid (r = -0.683 for W2 ring and r = -0.584 for W2 trophozoite) and cyanidin-3-glucoside (r = -0.882 for 3D7 trophozoite). In contrast, proanthocyanidin A2 may have acted as protector agent against toxic effects from extract component, once this compound presented a high and positive correlation ($p < 0.001$) with 3D7 on schizont stage (r = 0.563), W2 on ring and trophozoite stages (r = 0.589; r = 0.571 respectively). Therefore, the higher the amount of these phenolic compounds, the higher the concentration of extract needed to kill half of the parasites. In this sense, it also can highlighted that the extract 25% H_2O + 75% EtOH presented the higher content of proanthocyanidin A2 (60.63 ± 1.32 mg/100 g) in comparison to the others. Consequently this extract exhibited one of the highest values of IC_{50} for 3D7 on schizont stage (IC_{50} = 110.4 µg/mL), W2 on ring stage (IC_{50} = 71.7 µg/mL) and W2 trophozoite stage (IC_{50} = 84.6 µg/mL). On the other hand, the extract 100% H_2O had the lower content of proanthocyanidin A2 (14.81 ± 0.16 mg/100 g), thus lower values of IC_{50} for the same stages of *P. falciparum* (IC_{50} values from 24.2 to 98.1 µg/mL), which reinforce our hypothesis of proanthocyanidin A2 protection against the toxic compounds (i.e. methylvescalagin) presented in the extracts.

In respect of anti-hemolytic effect, the content of methylvescalgin and rosmarinic acid were negatively correlated with hemolysis (r= -0.597 and 0.538, respectively), meaning that the higher amount of these compounds the lower hemolysis (e.g., higher cytoprotection). Therefore, methylvescalgin and rosmarinic acid acted as protector agents. Interestingly, the methylvescalagin exhibited dual-face action, once it was toxic against all parasites, however, on the hand, it protected erythrocytes against hemolysis, showing selectivity. In light of these findings, methylvescalagin is the promising bioactive compound presented in our extracts.

Principal component analysis (PCA) was applied in order to reduce the dimension of the data set, and to distinguish the camu-camu seed extracts. The first principal component (PC1) explained nearly 43% of the data variability and the second PC (PC2) explained roughly 36%, retaining roughly 79% of all variability in the experimental data (Figure 6). Regarding the potential antimalarial and antischistosomicidal activities observed in toxicity assays, the 100% H_2O extract had the highest content of 2,4-dihydroxybenzoic and 3,4-dihydroxybenzoic acids, and this same extract was correlated with low values of IC_{50} for all erythrocytic stages of W2 *P. falciparum* strain. Moreover, the 50% H_2O + 50% EtOH and 75% H_2O + 25% EtOH extracts were associated with low values of IC_{50} for *S. mansoni*, all stages of 3D7 *P. falciparum* strain and schizont stage of W2 strain, highlighting their toxic potential for these parasites, which may be related to high amounts of rosmarinic acid, *trans*-resveratrol, quercetin, siringic acid, methylvescalagin and cyanidin-3-glucoside.

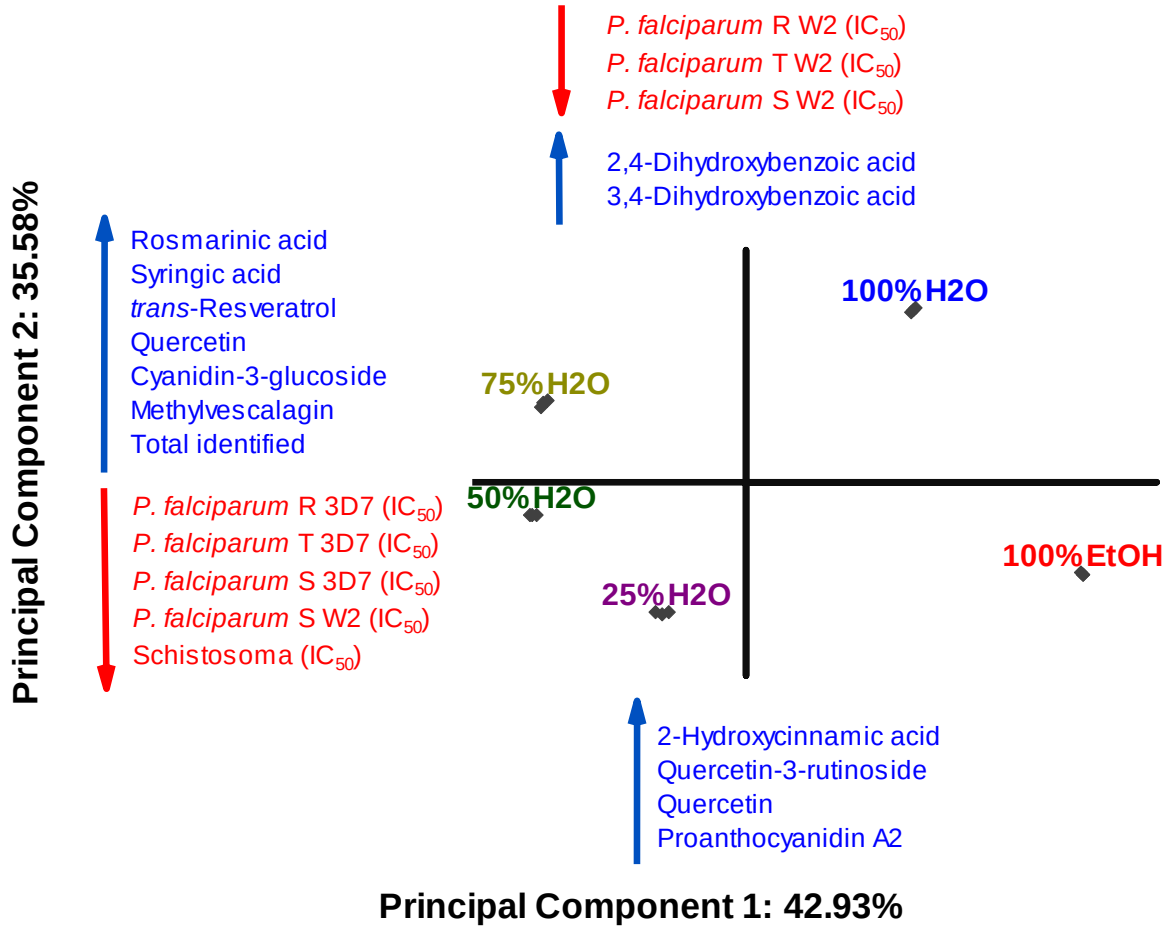
Overall, takin into account all results (*in vitro* antiparasitic activities and the phenolic composition), camu-camu seed extracted with either 50% H_2O + 50% EtOH, 75% H_2O + 25% EtOH and 100% H_2O are the most promising antimalarial and antischistosomicidal extracts with the highest concentrations of phenolic compounds and lower IC_{50} values.

Figure 9- Correlation between phenolic composition and hemolysis, *in vitro* toxicity against *S. mansoni* and *P. falciparum.*



Source: Author, 2020.

Figure 10- Principal component analysis based on the phenolic composition and on the antimalarial and antischistosomicidal activities of camu-camu seed extracts. Note: R = ring, T = trophozoite, S = schizont.



Source: Author, 2020.

4 CONCLUSIONS

Results of this study provide new and promising findings of hydroalcoholic camu-camu seed extracts on antimalarial and antischistosomicidal activities. These toxic effects may mainly be attributed by 50% H_2O + 50% EtOH, 75% H_2O + 25% EtOH and 100% H_2O extracts, in special their methylvescalagin and 2,4 dihydroxybenzoic acid contents. No toxicity was observed against *L. (L.) amazonensis* and IMR90 normal cells. Moreover, the extracts exhibited protective effect against hemolysis, pointing their relative safety. The present investigation demonstrated that camu-camu seed extracts is a great candidate for the discovery of potential antiparasitic compounds, taking into account principally the methylvescalagin bioactivity. Further study needs to be performed to verify antimalarial and antischistosomicidal activities in *in vivo* models.

Chapter 4

FINAL CONCLUSIONS

Data presented in Chapter 1 recognized that phenolic compounds affect numerous essential pathways and targets associated with antiproliferative effects and suggested the development of innovative food structures and functionalities to satisfy consumer needs and expectations and offer multitude health benefits.

The *in vitro* experiment described in Chapter 2 indicated that camu-camu hydroalcoholic seed extracts presented great cytotoxic effect against all cancer cell lines (HepG2, A549, Caco-2 and HCT8) and they exhibited no cytotoxicity against normal cells (IMR90). 50% H2O + 50% EtOH was considered the most promising extract, due to antioxidant and cell growth inhibition, besides its antimutagenic potential by preventing chromosomal aberration induced-cisplatin. (−)-Epicatechin and methylvescalagin were the major phenolic compounds associated with cytotoxicity, while gallic and 2,5-dihydroxybenzoic acids showed close relationship with cytoprotective effects in HCT8 cancer cell line.

From Chapter 3, camu-camu seed extracts exhibited anti-hemolytic, antimalarial and antischistosomicidal properties. These toxic effects may specially be attributed by methylvescalagin and 2,4 dihydroxybenzoic acid contents.

In fact, these above results providing a novel source of bioactive compounds, which open avenues to apply camu-camu seed phenolic compounds as potential new antiparasitic and anticancer candidates.

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The addition of inulin and *Lactobacillus casei* 01 in sheep milk ice cream

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ABSTRACT

The effect of the *Lactobacillus casei* 01 and inulin addition on sheep milk ice cream during storage (−18 °C, 150 days) was investigated. Control, probiotic and synbiotic ice cream (10% w/w sheep milk cream; 10% w/w sheep milk cream, *L. casei* 01, 6 log CFU/mL; 10% w/w inulin, *L. casei* 01, 6 log CFU/mL, respectively) were manufactured. Microbiological counts (probiotic count, survival after *in vitro* gastrointestinal resistance, Caco-2 cell adhesion), bioactivity and microstructure were analysed. Physical and textural characteristics, colour parameters, thermal analysis and organic acids/volatile compounds were also evaluated. All formulations supported *L. casei* 01 viability and maintained above the minimum therapeutic level (> 6 log CFU/mL) during storage. Inulin did not affect *L. casei* 01 survival after the passage through simulated gastrointestinal tract and adhesion to Caco-2 cells while improved the ACE-inhibitory and antioxidant activity. *L. casei* 01 addition produced several volatile compounds, such as carboxylic acids, alcohols, aldehydes and ketones. Also, scanning electron microscopy showed an interaction between probiotic bacteria and inulin fibre on synbiotic ice cream and the adhesion of *L. casei* to Caco-2 cells was observed.

1. Introduction

Probiotics are defined as live microorganisms which when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). The probiotic effects are strain-specific (Espitia, Batista, Azeredo, & Otoni, 2016) and they should possess resistant to the gastric and bile acids, adhere to mucus or human enteric epithelial cells, antimicrobial activity against pathogenic bacteria, bile salt hydrolase activity, and ability to reduce pathogen adhesion to surfaces of gastrointestinal tract (Kolaček et al., 2017).

Prebiotics are "substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). The prebiotic fibres consist of inulin and other oligosaccharides. Thus, the term *synbiotic* refers to the synergistic effect between prebiotic foods and selective probiotic microorganisms (Cencic & Chingwaru 2010). Many animal and human studies have demonstrated that the consumption of products containing both probiotics and prebiotics can provide health benefits, improving the survivability and deployment of probiotics in the food supplements into the gastrointestinal tract by promoting the selective growth and/or activating bacteria metabolism (Miremadi, Sherkat, & Stojanovska, 2016).

The wide use of inulin in the food industry is based on its technological attributes and the great interest to develop healthy products aiming at the consumers' requirement. These products include fibre-enriched, prebiotic, low fat and low sugar foods (Ahmed & Rashid, 2017). As supplement in skim dairy products, inulin considerably increases the growth and sustainability of *Lactobacillus* spp. and *Bifidobacterium* spp. in non-fat fermented milk (Closa-Monasterolo et al., 2013), including *L. casei*-01 (Paseephol & Sherkat, 2009). Various prebiotic dairy desserts with low fat content have been prepared using

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Clitoria ternatea L. petal bioactive compounds display antioxidant, antihemolytic and antihypertensive effects, inhibit α-amylase and α-glucosidase activities and reduce human LDL cholesterol and DNA induced oxidation

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Chemical compounds:
Caffeic acid (PubChem CID: 689043)
Delphinidin-3-O-glucoside (PubChem CID: 443650)
2,4-Dihydroxybenzoic acid (PubChem CID: 1491)
Gallic acid (PubChem CID: 370)
p-Coumaric acid (PubChem CID: 637542)
Procyanidin A2 (PubChem CID: 124025)
Protocatechuic acid (PubChem CID: 72)
Quercetin-3-rutinoside (PubChem CID: 5280805)
Syringic acid (PubChem CID: 10742)

ABSTRACT

The purpose of this study was to use a statistical approach to optimise the experimental conditions regarding the extraction of bioactive compounds, and to analyse the *in vitro* functional properties of crude lyophilized extracts (CLE) and partially purified (PPE) extracts of *Clitoria ternatea* petals. The results showed that the factors of temperature and time influenced the extraction of phenolic compounds, antioxidant activity and the physicochemical parameters. Simultaneous optimisation showed that the same levels of bioactive compounds were extracted when using temperatures from 11.7 to 68.3 °C and times from 8.47 to 51.12 min. Principal component analysis revealed the experimental conditions that provided the extraction producing the highest level of phenolic content (40 °C/30 min). The CLE showed antimicrobial activity; protective effect against hemolysis of erythrocytes; inhibition of α-amylase, α-glucosidase and angiotensin-I-converting (ACE-I) enzymes; and inhibition of lipid peroxidation. The CLE and PPE demonstrated oxygen radical absorption capacity; inhibition of DNA strand scission; inhibition of LDL cholesterol oxidation; intracellular antioxidant activity against reactive oxygen species (> 100 μg/mL); and no cytotoxicity (IC_{50}, GI_{50} and LC_{50} > 900 μg/mL) against A549, HCT8 and IMR90 cell lines.

Abbreviations: ACE-I, angiotensin-I-converting enzyme; CCA, Cu^{2+} chelating ability; RCRC, reducing capacity of the Folin-Ciocalteu reagent; CLE, crude lyophilized extract; H_2O_2, hydrogen peroxide; HPLC-PAD-UV, high-performance liquid chromatography - photodiode array detector - fluorescence detector; LDL, low-density lipoprotein cholesterol; NAF, non-anthocyanic flavonoid; NFP, non-flavonoid phenolic; ORAC, oxygen radical absorption capacity; PCA, principal components analysis; PPE, partially purified extract; ROS, reactive oxygen species; RSM, response surface methodology; TPC, total phenolic content

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From byproduct to a functional ingredient: Camu-camu (*Myrciaria dubia*) seed extract as an antioxidant agent in a yogurt model

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ABSTRACT

This work aimed to characterize the phenolic composition and in vitro antioxidant and antiproliferative properties of lyophilized camu-camu (*Myrciaria dubia*) seed extract (LCE), and to assess the effects of LCE on the antioxidant and sensory traits of yogurt. The LCE contained 46.3% (wt/wt) total phenolic content; the main compounds quantified were vescalagin, castalagin, gallic acid, procyanidin A2, and (−)-epicatechin. The LCE had antioxidant activity, as measured by different chemical assays (2,2-diphenyl-1-picrylhydrazyl, Folin–Ciocalteu reducing capacity, total reducing capacity, ferric reducing antioxidant power, and Cu^{2+} chelating capacity), and inhibited the cell proliferation of HepG2 cells (human hepatoma carcinoma; IC_{50} = 1,116 μg/mL) and Caco-2 cells (human colorectal adenocarcinoma epithelial cells; IC_{50} = 608.5 μg/mL). In addition, LCE inhibited the in vitro activity of α-amylase, α-glucosidase, and angiotensin-converting enzyme, and protected DNA from peroxyl radical–induced scission. When added to yogurts, different concentrations of LCE (0, 0.25, 0.5, 0.75, and 1.0 g/100 g) increased the chemical antioxidant and reducing capacities. The camu-camu yogurt containing LCE at 0.25 g/100 g had an acceptance index of 84%, showing that camu-camu seed extract may be a potential ingredient for addition to yogurts.

Key words: food innovation, phenolic compound, ellagitannin, antioxidants, antiproliferative activity

INTRODUCTION

Camu-camu [*Myrciaria dubia* (HBK) McVaugh] is a fruit from South America that is mainly consumed on its own or as frozen pulp. The frozen pulp is exported to many European and Asian countries because of its taste and versatility in household cooking. However, its peel and seeds are discarded by frozen pulp companies, generating a large quantity of byproduct. The consumption of camu-camu pulp is related to its unique taste and high ascorbic acid content, but recently chemical and functional analyses of camu-camu byproducts have indicated in vitro and in vivo functional properties, such as antioxidant, antihyperglycemic, antihyperlipidemic, antihypertensive, antimicrobial, and neuroprotective effects (de Souza Schmidt Gonçalves et al., 2014; Azevêdo et al., 2015; Fujita et al., 2015; Miyashita et al., 2017; Fidelis et al., 2018). Camu-camu byproducts have the potential to be used in developing new bioactive-rich foods and possibly eliminating the use of synthetic chemical preservatives in processed foods. No patents are registered with the Brazilian Industrial Property Institute (INPI; search on June 3, 2019, using the key words "camu-camu yogurt" and "camu-camu dairy food"), indicating that the dairy sector should explore the use of camu-camu pulp, seeds, and peel to develop new potentially functional dairy foods. To date, yogurts manufactured with pomegranate (*Punica granatum*) and jacaranda (*Jacaranda mimosifolia*) seed flours (0.5 g of flour/100 g of yogurt) have been shown to increase antioxidant activity, as measured by 2,2-diphenyl-1-picrylhydrazyl (**DPPH**) assay and the degree of liking of taste (Van Nieuwenhove et al., 2019).

According to Sloan (2019), 38% of US consumers are willing to pay a premium for foods that offer health benefits beyond basic nutrition, and roughly 54% of those consumers actively seek out functional foods. In

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antioxidants

Article

From the Field to the Pot: Phytochemical and Functional Analyses of *Calendula officinalis* L. Flower for Incorporation in an Organic Yogurt

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Abstract: Edible flowers have been used as ingredients because of their biological activities, taste, and overall appearance. This research was aimed to characterize the chemical composition and in vitro antioxidant activity of the marigold flower (*Calendula officinalis* L.) extracted with different proportions of water and ethyl alcohol, and the lyophilized extract with higher content of antioxidant compounds was incorporated into an organic yogurt. Results showed that the hydroalcoholic extract (50:50 v/v) presented the highest total phenolic content (TPC), flavonoids, and antioxidant activity (ferric reducing antioxidant power (FRAP), total reducing capacity (TRC), and Cu^{2+}/Fe^{2+} chelating ability). Phenolic acids and flavonoids were quantified in the extract by LC-DAD, while 19 compounds were tentatively identified by ESI-MS/MS. The lyophilized marigold extract (LME) also inhibited 12% of Wistar rat's brain lipid oxidation *in vitro*, inhibited α-amylase, and α-glucosidase activities, but showed no cytotoxicity towards cancerous cells (HCT8 and A549). However, marigold flower extract protected human erythrocytes against mechanical stress. When added into an organic yogurt model (0 to 1.5%), LME increased TPC and antioxidant activity (2,2-diphenyl-1-picrylhydrazyl (DPPH) and TRC), and the sensory analysis showed that the organic yogurt had an acceptance of 80.4%. Our results show that the use of LME may be a technological strategy to increase the content of bioactive compounds in yogurts.

Keywords: antihemolytic effect; free radicals; antiproliferative activity; natural products; functional foods; edible flowers

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Antioxidants-rich ice cream containing herbal extracts and fructooligossaccharides: manufacture, functional and sensory properties

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ABSTRACT

This work aimed to optimize an aqueous extract rich in phenolic compounds and potential functional properties made of *Ilex paraguariensis*, *Melissa officinalis*, and *Cymbopogon citratus*. The lyophilized extract was used for the development of an ice cream. Total phenolics, FRAP, DPPH, Folin-Ciocalteu's reducing capacity, and total reducing capacity of different combinations of herbal extracts were tested and modeled using response surface methodology. Simultaneous optimisation was employed to maximize the bioactive compounds in the extract and the lyophilized optimum combination was added to ice cream. The lyophilized extract contained quercetin-3-rutinoside, hesperidin, isoquercetin, caffeic acid, and 5,7-dihydroxyflavone. The optimised extract, which showed antihypertensive, antidiabetic, and antioxidant activity using *in vitro* protocols, increased total phenolics and antioxidant activity in comparison to the control ice cream. The ice cream presented a sensory acceptance index of 83%. After 72 days of storage (−18 °C), total phenolics and antioxidant activity significantly decreased.

1. Introduction

In addition to being accepted by all age groups, ice cream is the most consumed product within the category of dairy desserts (Goff & Hartel, 2013). According to Brazilian regulation, ice cream is a food product obtained from a fat and protein emulsion, either with or without the addition of other ingredients and substances, or a mixture of water, sugars, and other ingredients and substances that have been subjected to freezing (Brasil, 2005).

Increasing knowledge and research regarding the relationship between food and health, together with the technological need for innovations, have generated new products, some of which have the functional potential to benefit human health (Granato et al., 2018). For instance, Gabbi, Bajwa, and Goraya (2018) tested the use of ginger (*Zingiber officinale*) powder (0.5–2 g/100 g) on the manufacture of ice creams and observed that the ginger powder decreased the total lipids and increased the antioxidant activity, titratable acidity, and the total phenolic content. Kavaz, Yüksel, and Dağdemir (2016) produced ice creams with dried Besni grape (*Vitis vinifera* L.) at 5–15 g/100 g and observed that the grape pomace increased the viscosity, redness, ashes, total phenolics, and flavonoids but no statistical difference was observed for the overall sensory acceptability, flavor, and texture. Similarly, Borrin, Georges, Brito-Oliveira, Moraes, and Pinho (2017) manufactured ice creams added with curcumin-loaded nanoemulsion aiming to replace synthetic yellow dyes in the ice cream formulation as the curcumin-based nanoemulsion did not provoke significant effects in the physicochemical, rheological, and sensory properties of the test samples. With these examples, it is obvious that consumers want to

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Hibiscus sabdariffa anthocyanins-rich extract: Chemical stability, *in vitro* antioxidant and antiproliferative activities

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ABSTRACT

Hibiscus sabdariffa calyx is a rich source of anthocyanins and other bioactive compounds but no study reported the effects of experimental conditions on the extraction of these chemical compounds. Therefore, the effects of time and extraction temperature on the bioactive compounds and antioxidant activity of *Hibiscus sabdariffa* calyx were evaluated. In addition, the effects of copigmentation and pH on the stability of anthocyanins were assessed and the cytotoxic effects (LC_{50}, IC_{50}, and GC_{50}) of the extracts were determined in relation to tumor cell lines - Caco-2, HepG-2, HCT8, and A549. The temperature significantly influenced the total anthocyanins and flavonoids contents. The interaction between time/temperature influenced the total phenolic content and ascorbic acid. The $t_{1/2}$ and the percentage of colour retention decreased markedly at temperatures above 80 °C. Variations in pH conserved the antioxidant activity of the anthocyanins, and the protonation-deprotonation process of the extract was reversible. The treatment of cells with purified anthocyanin extract or crude extracts at 5–800 μg mL^{-1} did not show significant cytotoxic effects on the cell lines, corroborating the chemical antioxidant effect of the extracts (DPPH assay). Cyanidin-3-glucoside, delphinidin-3-sambubioside, delphinidin-3-glucoside, and cyanidin-3-sambubioside were identified in the extracts by LC-ESI-MS.

1. Introduction

Teas are used in different industrial sectors and of those that have been studied *Hibiscus sabdariffa* L. (Roselle) deserves special attention because of its chemical composition and functional properties (Higginbotham et al., 2014). There are more than 300 cultivated species of *Hibiscus* sp. and *Hibiscus sabdariffa* is widely consumed worldwide because of its considerable content of bioactive compounds, specifically flavonoids (Sindi et al., 2014).

Roselle calyxes have been used as a basis for infusions, jams, jellies, sauces and fermented products (Gradinaru et al., 2003). *In vitro* studies have already shown the ferric reducing antioxidant activity and free-radical scavenging activity in relation to 2,2-diphenyl-1-picrylhydrazyl, DPPH, and 2,2′-azinobis 3-ethylbenzthiazoline-6-sulfonic acid radicals, and the oxygen radical absorbance capacity, ORAC, of the extracts from *H. sabdariffa* (Fernández-Arroyo et al., 2011; Mohd-Esa et al., 2010; Owoade et al., 2015). Furthermore, delfinidin-3-sambubioside has been shown to induce the apoptosis of HL-60 cells through a pathway of mitochondrial dysfunction that is mediated by reactive oxygen species. Beltran-Debon et al. (2010) observed that extract of *H. sabdariffa* protected mononuclear cells from H_2O_2-induced death. Studies have shown that extracts of *H. sabdariffa* have presented therapeutic effects regarding the prevention of atherosclerosis and oxidative stress, antibacterial, antioxidant, and hepato-protective effects, regulation of the lipid metabolism, as well as anti-diabetic and anti-hypertensive effects (Da-Costa-Rocha et al., 2014; Lin et al., 2015).

In terms of extraction, the yield of the target compound must be maximized, with the minimum of degradation. Anthocyanins are soluble in polar solvents and in the presence of HCl or organic acids (Naczk and Shahidi, 2004). The lower the particle size the greater the content of anthocyanins in the extract (Cissé et al., 2012). Factors such as temperature and time, as well as the type and volume of solvent, have an influence on the yield of anthocyanins. Mathematical models can accurately describe isolated effects as well as combinations of factors; they can also be used to maximize a functional extract (Zhang et al., 2006; Pedro et al., 2016). Heat treatment is a method that is used

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Sclerotinia Sclerotiorum (White Mold): Cytotoxic, Mutagenic, and Antimalarial Effects *In Vivo* and *In Vitro*

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Abstract: This work aimed includes performing the sclerotia chemical profile and evaluates their biological effects on mutagenesis, oxidative stress, cancer, and malaria. A chemical profile was determined by ultraperformance liquid chromatography mass spectrometry (UHPLC–HRMS) analysis dereplicating norditerpenoid dilactone, sclerolide, and other compounds. The GI_{50} values to cancer cells (19.8 to 277.6 µg/mL) were higher than normal (16.05 µg/mL), meaning high cytotoxicity. Regarding the oxidative stress, the results showed that the all AcOET fraction concentrations tested on IMR90 noncancer cell increased reactive oxygen species (ROS) production in more intense way (by fivefold) than in tested cancer cells. The *in vivo* study showed an increase of the following biomarkers (by 296.00%): % DNA in comet tail in peripheral blood and liver cells; micronucleated erythrocytes and colon cells and lipid serum peroxidation. These results indicate the sclerotia as genotoxic and mutagenic agent and its contamination may lead to fungal toxic effects with a risk to human health.

Keywords: apoptosis, cytotoxicity, fungus, malaria, mutagenicity, oxidative stress

Introduction

It is known that natural compounds may behave as beneficial or toxic to health and we confronted with a variety of those ones within our everyday life (Wang, Ouyang, & Lin, 2018). Toxins are hazardous substances, causing illness or damage to an exposed organism if inhaled, swallowed, or absorbed through the skin (Schilter, Constable, & Perrin, 2014). Fungi (yeasts and molds) and bacteria are capable of producing toxic secondary metabolites that can contaminate food and cause adverse effects on human health (Aichinger et al., 2018), which effects include acute or chronic toxicity, genotoxicity, mutagenicity, carcinogenicity, neurotoxicity (Tournas, 2005), pulmonary infection, allergies, osteomyelitis, endocarditis, keratitis (Frąc, Jezierska-Tys, & Yaguchi, 2015), hepatotoxicity, and teratogenicity upon consumption (Kong et al., 2014; Shin, Bae, Choi, & Woo, 2014). Conversely, many of them cause a broad variety of beneficial biological activities including anticancer, anti-inflammatory, antimicrobial (Chow & Ting, 2015; Liu, Zhao, Sun, Li, & Liu, 2018; Shin et al., 2014), antibacterial, antiviral, and antiprotozoal therapies (Bhadury, Mohammad, & Wright, 2006). Thus, a large number of secondary metabolites from fungi occupy a significant position in the pharmaceutical industry and have been integrated into drug development (Bhadury et al., 2006). Compounds that are ultimately selected for development of new drugs must meet the requirements of safe drugs and that do not show any overt toxicity to human health (Fidock, Rosenthal, Croft, Brun, & Nwaka, 2004), however, this requirement is not always met. Sadorn et al. (2018) showed that crude extracts from fungus *Cytospora eugeniae* had cytotoxic activity against human breast adenocarcinoma MCF-7, papilloma carcinoma KB, and lung NCI-H187 cancer cells, but also demonstrate toxic effect in noncancerous cells (Vero, African green monkey kidney fibroblasts).

The fungus *Sclerotinia sclerotiorum* is remarkable for its extremely broad host range and for its aggressive host tissue colonization (Liang & Rollins, 2018). This nonspecific plant pathogen may infect economically important crops and vegetables such as sunflower, bean, soybean, canola, cotton, potatoes, peas, and tomatoes (Duan et al., 2018; Heard, Brown, & Hammond-Kosack, 2015; Xu, Liang, Hou, & Zhou, 2015). Its infection causes stem rot or white mold and represents one of the major challenges for agricultural production (Bolton, Thomma, & Nelson, 2006; Malenčić et al., 2010). The food contamination with *S. sclerotiorum* may be derived from their sclerotia. These structures are characterized by hard melanized rind enclosing compact dark bodies and they are not always removed during harvest and postharvest procedures, which may lead to their human consumption (Azevedo et al., 2016). Unfortunately, little is still known about the effects of human consumption of contaminated food with *S. sclerotiorum* since their compounds can be a potential health hazard to individuals. Azevedo et al. (2016) pointed out that sclerotia aqueous extract presented mutagenic and cytotoxic effects against colon adenocarcinoma (HT29). Herein, we aimed at performing the chemical profile of sclerotia and to assay their *in vivo* and *in vitro* toxicological activities, furthermore determined their possible antimalarial and anticancer potential.

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Optimized *Camellia sinensis* var. *sinensis*, *Ilex paraguariensis*, and *Aspalathus linearis* blend presents high antioxidant and antiproliferative activities in a beverage model

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ABSTRACT

A statistical optimization study was conducted to obtain a tea containing fermented rooibos (*Aspalathus linearis*), white tea (*Camellia sinensis* var. *sinensis*), and roasted mate (*Ilex paraguariensis*). An optimal combination of these species was proposed. This optimized tea inhibited 64% the lipoperoxidation *in vitro* and presented a high phenolic content, especially kaempferol, (+)-catechin, (−)-epicatechin, rutin, (−)-epigallocatechin, and (−)-epicatechin-2-*O*-gallate. Indeed, the antioxidant effect was confirmed by decreasing 30% the reactive oxygen species generation in human hepatoma carcinoma cells (HepG2, 100 and 240 μg/mL). In the cell viability assay, the GI_{50} for human colorectal adenocarcinoma epithelial cells (Caco-2) was about 547 μg/mL and 481 μg/mL for HepG2. The pasteurization process (65 °C/30 min) did not affect the total phenolic content and antioxidant activity of the optimized tea formulation. The sensory test indicated an acceptability index of 78%, showing that the analytical approach adopted was feasible to develop a phenolic-rich beverage.

1. Introduction

Teas are among the most popular and widely consumed beverages in the world. The increase in tea consumption worldwide has been related to *in vitro* and *in vivo* functionalities that have showed a direct relationship between the continuous consumption of teas and the reduced risk of non-communicable degenerative diseases (Rossi, Bassett, & Sammán, 2016; Santos, Brizola, & Granato, 2017). Some of the great variety of teas that are produced are noteworthy because of their varied phenolic composition. This is the case of white, mate and rooibos teas, which are of different botanical and endemic origins and are from distinct global regions. These factors give them specific chemical and sensory characteristics.

White tea is mainly produced in Asia from the shoots and young leaves of *Camellia sinensis*. This plant is covered in small silvery hairs and is harvested before the flowers bloom (Rusak, Komes, Likić, Horžić, & Kovač, 2008). The latter authors state that for this reason, white tea maintains the highest levels of antioxidant compounds and the lowest caffeine levels compared with any other *C. sinensis* tea. Flavan-3-ols are the main phenolic compounds in *C. sinensis* but their chemical composition also shows significant levels of flavonols, flavones, and phenolic acids (Rusak et al., 2008).

Mate tea is produced from the leaves of *Ilex paraguariensis* St. Hilaire, a plant from the Aquifoliaceae family that is typical of subtropical and native regions of South America. It is a drink that is rich in phenolic compounds, such as isomers of chlorogenic acid, i.e. 3-, 4- and 5-mono caffeoylquinic acids and 3,5- and 4,5-dicaffeoylquinic acids (Peres, Tonin, Tavares, & Rodriguez-Amaya, 2013). There are literature reports regarding the presence of rutin, gallic, caffeic, syringic, ferulic, and *p*-coumaric acids in aqueous extracts of mate (Da Silveira, Meinhart, De Souza, Teixeira Filho, & Godoy, 2016).

Fermented rooibos tea is made from the leaves and stems of an African shrub, *Aspalathus linearis*. Its aqueous extract is caffeine-free and it contains low levels of tannins, which gives it a mild taste (Koch,

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Flaxleaf Fleabane Leaves (*Conyza bonariensis*), A New Functional Nonconventional Edible Plant?

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Abstract: This work aimed to investigate the phytochemical composition, nutritional value, antioxidant, antihemolytic, antihyperglycemic, and antiproliferative activities of flaxleaf fleabane (*Conyza bonariensis*) leaves. Different concentrations of water and ethanol (0:100, 25:75, 50:50, 75:25, and 100:0 v/v) were used in the extraction process and results showed that the hydroalcoholic extract (50:50 v/v) presented the highest total phenolics, *ortho*-diphenolics, Folin-Ciocalteu reducing capacity, FRAP, and Fe^{2+} chelating ability values. Flaxleaf fleabane leaves (FFL) contained 19.6 g/100 g of fibers and 26 g/100 g of proteins. Ellagic acid, procyanidin A2, caffeic, rosmarinic, gallic, and 2,5-dihydroxybenzoic acids were the main phenolics. This phenolic-rich extract inhibited the lipid oxidation of Wistar rat brain (IC_{50} = 863.0 mg GAE/L), inhibited α-glucosidase activity (IC_{50} = 435.4 µg/mL), protected human erythrocytes against mechanical hemolysis at different osmolarity conditions, and showed cytotoxic/antiproliferative effects against human ileocecal adenocarcinoma cells (HCT8; IC_{50} = 552.6 µg/mL) but no cytotoxicity toward noncancerous human lung fibroblast (IMR90). Overall, FFL showed potential to be explored by food companies to be a source of proteins, natural color substances, and phenolic compounds.

Keywords: antihemolytic effects, antihyperglycemic effect, cytotoxicity, free radicals, natural colorants, phenolic compounds

Practical Application: Flaxleaf fleabane leaves (FFL) are usually burnt or partially given to cattle, without a proper utilization as a source of nutrients for human nutrition. Here, we studied the nutritional composition, phenolic composition, and toxicological aspects of FFL using different biological protocols. FFL was proven to be a rich source of proteins and dietary fibers and showed antioxidant activity measured by chemical and *in vitro* biological assays. Additionally, as it did protected human red cells and did not show cytotoxicity, we assume FFL has relative safety to be consumed as a nonconventional edible plant.

Introduction

Flaxleaf fleabane (*Conyza bonariensis*, Asteraceae) is considered a major cropping weed in many tropical and subtropical countries, such as Brazil and Australia. Its uncontrolled growth caused by its resistance to glyphosate (a largely used herbicide) may reduce the production yield of grains produced in a large scale, such as soy, wheat, maize, and sorghum. According to Walker et al. (2012), flaxleaf fleabane grows up to 1 m in height and has erect multiple-branching stems covered with stiff hairs. Leaves are green, deeply indented, coarsely toothed and covered in fine gray hairs.

Flaxleaf fleabane leaf (FFL) has not being explored commercially as a conventional edible plant but is considered a medicinal plant in Saudi Arabia (Daur, 2015) and Brazil (de Albuquerque, Monteiro, Ramos, & de Amorim, 2007). However, some people use this plant as salad and salad dressing preparations, which makes the study of the chemical composition and possible health effects a demanding task. In fact, some researches have been conducted to assess the total phenolic content (TPC), essential minerals, and chemical antioxidant activity, and to evaluate the antimicrobial effects of FFL extracts (Daur, 2015; Thabit et al., 2015). Moreover, FFL extracts have been tested for the treatment of smallpox, chickenpox, sore throat, and skin-related diseases (Shinwari & Khan, 2000). Additionally, this plant has shown to be gut modulator *in vivo* by displaying spasmolytic effects (Bukhari et al., 2013) and anti-inflammatory activity (Ribeiro, Arruda, Abd El-Salam, & Bastos, 2018). In a toxicological study, de Paula et al. (2018) showed that leaves and root extracts of *Conyza bonariensis* did not demonstrate acute oral toxicity in mice at 500, 1,000, 2,000, and 5,000 mg/kg.

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Multivariate effects of Chinese keemun black tea grades (*Camellia sinensis* var. *sinensis*) on the phenolic composition, antioxidant, antihemolytic and cytotoxic/cytoprotection activities

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ABSTRACT

The main objectives of the study were to compare the phenolic composition, chemical and biological antioxidant activities, and cytotoxicity towards IMR90, HCT8, and A549 cell lines of eight grades of Chinese keemun black tea (*Camellia sinensis* var. *sinensis*) using a statistical approach. No cytotoxic effects were observed on IMR90 normal cells. Our results all together show that the chemical antioxidant capacity of high-grade black teas measured by DPPH, FRAP, and total reducing capacity assays was correspondingly higher than the mean values of low-grade teas and these antioxidant assays were not associated with cytotoxicity towards cancerous cell lines (HCT8 and A549). High grades of Chinese keemun black teas contained higher contents of total phenolics, flavonoids and *ortho*-diphenols than lower grades and theaflavin-3,3′-di-gallate could only be detected in high black tea grades (T1 and T2). Intermediate-high keemun black tea grades - C1, C3, T1, and T2 - which also had the highest mean values of TPC, flavonoids, *o*-diphenols, theaflavin-3-gallate, theaflavin-3′-gallate, Fe^{2+} chelating ability, and chemical antioxidant activity, presented the highest inhibition of Wistar rat's brain oxidation. No clear differentiation and trend were observed between erythrocyte protection and Chinese black tea grades as results clearly showed that intermediate black tea grades (C3 and C4) protected more the human erythrocytes against mechanical stress. Our study shows that although higher Chinese keemun black tea grades (T1 and T2) presented the highest TPC, flavonoids, and chemical antioxidant activity, these *in vitro* chemical assays were not translated into higher biological activity.

1. Introduction

Keemun black tea is one of the most famous teas in China and has been widely consumed worldwide. Keemun black tea may be produced using *Camellia sinensis* leaves and its quality is related to the raw materials' tenderness, tea infusion taste and flavor, also the tea appearance. With so many factors influencing its quality traits, keemun black tea may be classified into eight quality grades according to dry tea/brewed tea leaf appearance, infusion color, taste and flavor, namely T1, T2, C1 C2, C3, C4, C5 and C6, which are ordered from the highest to the lowest grade. Traditionally, the grade of produced keemun black tea is classified by experienced tea specialists, mainly based on the sensory evaluation. As this analysis is rather subjective even when experienced assessors are used, there is insufficient objective analytical methods and quantitative indexes to classify keemun black tea into different grades. For example, the main phenolic compounds of different grades are similar, but significant differences are observed in the contents of some compounds by non-targeted metabolomics, such as theasinensins, flavonoid glucosides (Guo, Long, Meng, Ho, & Zhang, 2018). It suggested the varied levels of these bioactive compounds in different grades may also affect the biological activities. There have been many reports about the biological activities of black tea or their major compounds, but

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Red Chicory (*Cichorium intybus*) Extract Rich in Anthocyanins: Chemical Stability, Antioxidant Activity, and Antiproliferative Activity *In Vitro*

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Abstract: Red chicory leaves are appreciated sensorially and their constituents contain bioactive properties. The objectives of this study were as follows: to use an experimental design to extract anthocyanins from red chicory in aqueous solution at pH 2.5; to determine the stability of the extracts in relation to temperature and pH; and to evaluate the antioxidant activity and *in vitro* cytotoxic effect of the lyophilized and purified extracts. The best extraction conditions for the bioactive compounds from red chicory were a temperature of 64.2 °C for 25 min; the anthocyanin content was 73.53 ± 0.13 mg per 100 g fresh weight basis sample. The EC_{50} (Half maximal effective concentration) value for the antioxidant activity assay in relation to DPPH (2,2-diphenyl-1-picrylhydrazyl) with optimized extract was 0.363, which corresponds to a concentration of 39.171 µmol/L of anthocyanins. The activation energy for the degradation reaction of the anthocyanins from the red chicory extract was 84.88 kJ/mol. The optimized extract, which was rich in anthocyanins, showed chemical and biological antioxidant activity (protection against erythrocyte hemolysis) and inhibited lipid peroxidation *in vitro*. The *Cichorium intybus* L. extracts interfered on the levels of reactive oxygen species generation and the crude extract did not present procarcinogenic effect.

Keywords: *Cichorium intybus* L., free radicals, functional foods, thermal stability

Practical Application: Red chicory is basically consumed as a part of traditional dishes worldwide. Here, we developed a process to extract and purify the anthocyanins from *Cichorium intybus* leaves and test the extracts in terms of the chemical composition, thermal stability, antioxidant activity, and antiproliferative effects. The anthocyanin-rich extract presented antioxidant activity in chemical and biological assays and low cytotoxicity and cytoprotective effects in relation to HepG2, HCT8, and Caco-2 cell lines. Additionally, the red chicory extract protected human erythrocytes against hemolysis. This extract may be used as a natural colorant/antioxidant in foods.

Food Chemistry

Introduction

Agricultural by-products are abundant, renewable sources of natural compounds. Raw materials from plants are sources of dietary fiber, carotenoids, tocopherols, and polyphenols. Among vegetables, red chicory (*Cichorium intybus* L.) has been the focus of research regarding its content of phenolic compounds and anthocyanins. Red chicory leaves are appreciated sensorially and are used in various culinary preparations (Innocenti et al., 2005). Sixty-four chemical compounds were detected in the leaves of *C. intybus* (var. "Treviso," "Treviso Belgium," and "Verona"), including derivatives of hydroxycinnamic acid, mono and dicaffeoylquinic acids, and three derivatives of tartaric acid. Thirty-one flavonols, two flavone glucosides, and 10 anthocyanins were also identified (Carazzone, Mascherpa, Gazzani, & Papetti, 2013). Reif, Arrigoni, Schärer, Nyström, and Hurrell (2013) detected considerable concentrations of lutein (1.76 to 6.98 mg/100 g) and β-carotene (1.05 to 4.16 mg/100 g) in *C. intybus* var. *foliosum*. The hydroalcoholic extraction of leaves of *C. intybus* L. detected the presence of flavonoids, phenolic acids, tannins, saponins, and relevant amounts of Mg and Zn (Abbas et al., 2015).

Varieties of *C. intybus* have been used in folk medicine to treat liver disorders. The major anthocyanin identified in Intybus Balou, Indigo, Manchini, Leonardo, and Erfano varieties of chicory was cyanidin-3-O-(6-malonyl-glucopyranoside); the level was over 95% and the highest content was detected in the Indigo variety. The aqueous extract of Indigo, Balou, Leonardo, Manchini, and Erfano varieties inhibited lipid peroxidation *in vitro*, and the anthocyanins isolated from this species presented the best inhibition of lipid peroxidation and cyclooxygenase (Mulabagal, Wang, Ngouajio, & Nair, 2009).

Red chicory extracts presented antioxidant, cytoprotective, and antiproliferative activities in Caco-2 intestinal cell models. Red chicory extract had a modulating effect on the oxidative stress induced by 4-*tert*-OP (4-*tert*-octylphenol) and hepatotoxicity.

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FULL ARTICLE

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Effect of *Pereskia aculeata* Mill. in vitro and in overweight humans: A randomized controlled trial

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Abstract

Objectives: The objective of this study was to investigate the influence of ora-pro-nobis (*Pereskia aculeata* Mill.) flour on the adhesion of probiotics to intestinal epithelial cells and to evaluate the effect of a product based on this flour on gastrointestinal symptoms, weight, body fat, glycemia, and lipid profile in overweight men. *Methods*: Microbiological counts (probiotic count, survival after in vitro gastrointestinal resistance, Caco-2 cell adhesion) were analyzed. A randomized, cross-over intervention was performed. Intestinal microbiota was indirectly assessed on the basis of consistency, color of feces, and gastrointestinal symptoms. *Results*: *P. aculeata* did not affect *Lactobacillus casei* adhesion to Caco-2 cells. Ora-pro-nobis flour improved gastrointestinal symptoms and increased satiety. *Conclusion*: The consumption of ora-pro-nobis flour improved intestinal health. In addition, it maintained the high adherence of *L. casei* to intestinal cells as well as patient anthropometric and biochemical parameters.

Practical applications

Pereskia aculeata Mill. is well known in folk medicine and has several nutrients; however, there are few studies on this plant. This is the first study to analyze the influence of *P. aculeata* on bacterial adherence and the first cross-over clinical trial to evaluate the beneficial potential of ora-pro-nobis flour in overweight men. Thus, this study will contribute to the promotion of ora-pro-nobis as a functional ingredient and will arouse the interest of industries to develop related healthy foods. In addition, it is an effective dietary strategy to improve the gastrointestinal health of men.

KEYWORDS
dietary fiber, functional ingredient, gastrointestinal symptoms, ora-pro-nobis

1 | INTRODUCTION

Pereskia aculeata Mill., commonly known as ora-pro-nobis, is an underutilized plant that belongs to the Cactaceae family (Calixto et al., 2012; Pinto, Duque, et al., 2015). Food and pharmaceutical industries have been interested in this vegetable because of its high nutrient content. The leaves of ora-pro-nobis have remarkable levels of fibers, proteins, calcium, magnesium, manganese, zinc, vitamin A, vitamin C, folic acid, and bioactive compounds, such as carotenoids (Pinto et al., 2016).

Takeiti, Antonio, Motta, Collares-Queiroz, and Park (2009) reported a high concentration of dietary fiber in *P. aculeata* (39.1%), which is responsible for the functionality of this plant. The association of fibers with probiotics for the improvement of intestinal health has

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In vitro antioxidant and antihypertensive compounds from camu-camu (*Myrciaria dubia* McVaugh, Myrtaceae) seed coat: A multivariate structure-activity study

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ABSTRACT

Camu-camu (*Myrciaria dubia*) pulp, seeds, and skin are widely known because of their nutritional properties. However, the seed coat has never been studied as a source of bioactive compounds. Herein, we characterized the phenolic composition, the antioxidant activity, and inhibition of angiotensin-converting enzyme (ACE) of three different extracts (water, propanone, and ethanol) from this residue and assessed the structure-activity using bivariate and multivariate statistical approaches. Phenolic acids and flavonoids were quantified by high-performance liquid chromatography while the ferric reducing antioxidant power (FRAP), inhibition of lipid peroxidation using egg yolk and Wistar rat brain, scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) radical, Folin-Ciocalteu reducing capacity (FCRC), and the inhibition of angiotensin-converting enzyme (ACE) by the extracts were also analyzed. *t*-Resveratrol was found in camu-camu seed coat for the first time. The aqueous extract had the highest total phenolic content, FRAP, DPPH˙, FCRC, and inhibition of lipid oxidation using both chemical and biological assays, while the propanone extract showed the opposite behavior but it presented higher *in vitro* antihypertensive activity. The ethanolic extract exhibited intermediate values for the responses. The association between chemical composition and the functional properties of the camu-camu seed coat extracts were revealed using correlation analysis and principal component analysis.

1. Introduction

Tropical fruits are attractive to the food industry mainly because of their unique appearance, flavor, and nutritional value (Kaneshima et al., 2013). Among the fruits classified as berries, camu-camu has received attention due to its high content of bioactive compounds, such as phenolic compounds (flavonoids and ellagitannins), ascorbic acid, and β-carotene (Inoue et al., 2008; Gonçalves et al. al., 2010; Akter et al., 2011).

Camu-camu (*Myrciaria dubia* [H.B.K] McVaugh), also known as "caçarí" or "araçá d'água", was discovered in 1958. It belongs to the Myrtaceae family and is spontaneously present on riverbanks and lakes of the Amazon basin, between Peru and Brazil (Zapata and Dufour, 1993; Silva and Andrade, 1997). Due to its high content of ascorbic acid and phenolic compounds, camu-camu reveals a bitter taste, which minimizes its consumption *in natura* (Myoda et al., 2010). As it is not widespread in the entire Brazilian territory, the commercialization is achieved on a small scale, basically in the form of frozen pulp. In Europe, Japan, Canada, and in the United States, there is great interest in the fruit, which is imported and its pulp transformed into sparkling drinks, vinegar, ice cream, and sweets (Yuyama, 2011).

The seeds and peels of camu-camu represent about 40% of the fruit (Rodrigues et al., 2001). However, these by-products are generally discarded without taking advantage of their chemical constituents. In this aspect, these residues may present higher antioxidant potential when compared to pulp because most bioactive compounds are retained on the fruit parts, which are treated as by-products by the food industry (Guo et al., 2003; Myoda et al., 2010; Azevêdo et al., 2014). Therefore, the processing of these by-products has the potential to become a segment of the agribusiness contributing to a better utilization,

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