UNIVERSITY OF SÃO PAULO
SCHOOL OF PHARMACEUTICAL SCIENCES OF RIBEIRÃO PRETO

In vitro and *in vivo* activities of guajiru fruit (*Chrysobalanus icaco* L.) in oxidative stress, DNA damage, and inflammation biomarkers

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Toxicologia para obtenção do título de Doutor em Ciências

Área de Concentração: Toxicologia

Doctoral thesis submitted to the Toxicology Graduate Program in fulfillment of the requirements for the degree of Doctor of Science

Field of Study: Toxicology

Doctoral Candidate: Vinicius de Paula Venancio

Advisor: Prof. Dr. Lusânia Maria Greggi Antunes

Ribeirão Preto

2016

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Publication Cataloguing
School of Pharmaceutical Sciences of Ribeirão Preto

Venancio, Vinicius de Paula

In vitro and *in vivo* activities of guajiru fruit (*Chrysobalanus icaco* L.) in oxidative stress, DNA damage, and inflammation biomarkers. Ribeirão Preto, 2016.
100 p. : il. ; 30cm.

Thesis (Doctorate) submitted to the School of Pharmaceutical Sciences of Ribeirão Preto/USP – Field of Study: Toxicology.
Advisor: Antunes, Lusânia Maria Greggi

1. Amazon fruit. 2. Comet assay. 3. Micronucleus test.
4. Nutrigenomics

RESUMO

VENANCIO, V. P. **Atividades *in vitro* e *in vivo* do fruto do guajiruzeiro (*Chrysobalanus icaco* L.) em biomarcadores de estresse oxidativo, danos ao DNA e inflamação.** 2016. 100 f. Tese (doutorado) – Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, Ribeirão Preto, 2016.

O guajiru (*Chrysobalanus icaco* L.) é um fruto rico em antocianinas, as quais exercem vários efeitos benéficos à saúde. Embora as folhas do guajiru sejam utilizadas na medicina popular como hipoglicemiante e antioxidante, os efeitos do fruto na saúde permanecem inexplorados. O objetivo deste estudo foi avaliar os efeitos do fruto do guajiruzeiro sobre danos ao DNA e estresse oxidativo *in vivo* e inflamação *in vitro* e *in vivo*. Ratos machos Wistar (4-5 semanas, 110 g) foram divididos em oito grupos e tratados por 14 dias com água ou fruto do guajiruzeiro liofilizado (100, 200 ou 400 mg/kg p.c.) por gavagem. No 14º dia, os animais receberam solução fisiológica ou DXR (15 mg/kg p.c. i.p.) e foram eutanasiados após 24 horas. A genotoxicidade e antigenotoxicidade foram avaliadas pelo ensaio do cometa em sangue periférico, fígado, rins e coração. A mutagenicidade e antimutagenicidade foram investigadas pelo teste do micronúcleo em medula óssea e sangue periférico. O *burst* oxidativo foi avaliado em neutrófilos do sangue periférico. Parâmetros de estresse oxidativo envolveram: concentração de substâncias reativas ao ácido tiobarbitúrico, razão glutationa reduzida e oxidada e atividade da catalase em fígado, rins e coração. As expressões de genes de dano/reparo de DNA *Gadd45a* (*growth arrest and DNA damage-inducible alpha*), *Parp1* (*Poly(ADP-ribose) polymerase 1*) e *Xrcc2* (*X-Ray Repair complementing defective repair in Chinese hamster cells 2*) e dos marcadores pró-inflamatórios *Il-1β* (*interleukin 1 beta*), *Il-6* (*interleukin 6*), *Nf-κb* (*nuclear factor kappa B*) e *Tnf-α* (tumor *necrosis factor alpha*) foram realizadas por PCR quantitativo em tempo real. Células de cólon humano CCD-18Co (fibroblastos) e HT-29 (adenocarcinoma) foram tratadas com antocianinas do guajiru (1,0 a 20,0 mg/L equivalentes de ácido gálico - GAE) e as expressões de IL-1β, IL-6, NF-κB e TNF-α analizadas a nível de RNA mensageiro e proteína. TNF-α foi utilizado para induzir inflamação em células CCD-18Co. Os polifenois do fruto do guajiruzeiro foram quantificados/caracterizados por métodos cromatográficos e espectrométricos. As concentrações de 19 elementos químicos foram determinadas por plasma indutivamente acoplado a espectrometria de massas. Delfinidina, cianidina, petunidina e peonidina foram as antocianinas majoritárias encontradas no fruto. Concentrações significantes de polifenois, magnésio e selênio foram encontradas nesse fruto. O fruto do guajiruzeiro exibiu atividade antioxidante *in vivo* em neutrófilos, antigenotoxicidade em sangue periférico e antimutagenicidade em sangue periférico e medula óssea. O guajiru diminuiu os danos ao DNA no fígado, rins e coração. O fruto também diminuiu as expressões de *Gadd45a*, *Il-1β,* e *Tnf-α* nos tecidos. A proliferação celular foi suprimida em células HT-29, acompanhado por aumento na produção de ROS e diminuição nas expressões de *TNF-α*, *IL-1β*, *IL-6* e *NF-κB*. Não foi observado efeito citotóxico das antocianinas em células CCD-18Co. As expressões das proteínas IL-1β, IL-6 e TNF-α foram reduzidas em células CCD-18Co tratadas com TNF-α e com as antocianinas. Os resultados deste trabalho demonstram que os fitoquímicos e elementos químicos no fruto do guajiruzeiro possuem efeitos antigenotóxico,

antimutagênico, antioxidante e anti-inflamatório e encorajam a realização de outros ensaios *in vivo* e estudos clínicos com esse fruto subutilizado.

Palavras-chave: Ensaio do cometa, ensaio do micronúcleo, fruto da Amazônia, nutrigenômica.

ABSTRACT

VENANCIO V. P. ***In vitro* and *in vivo* activities of guajiru fruit (*Chrysobalanus icaco* L.) in oxidative stress, DNA damage, and inflammation biomarkers.** 2016. 100 p. Thesis (Doctorate) – Faculdade de Ciências Farmacêuticas de Ribeirão Preto - Universidade de São Paulo, Ribeirão Preto, 2016.

Guajiru (*Chrysobalanus icaco* L.) is a fruit rich in anthocyanins, which exert several beneficial effects on health. Although guajiru leaves are used in folk medicine as hypoglycemic and antioxidant, the fruit effects on health remain unknown. The aim of this study was to evaluate the effects of guajiru fruit against *in vivo* DNA damage and oxidative stress and *in vivo/in vitro* inflammation. Male Wistar rats (4-5 weeks old, 110 g) were divided into eight groups and treated for 14 days with water or lyophilized guajiru fruit (100, 200 or 400 mg/kg b.w.) by gavage. On the 14th day, animals received physiologic solution or DXR (15 mg/kg b.w. i.p.) and were euthanized after 24 hours. Genotoxicity and antigenotoxicity were evaluated by comet assay in peripheral blood, liver, kidney, and heart. Mutagenicity and antimutagenicity of guajiru fruit were investigated by micronucleus test in peripheral blood and bone marrow. The oxidative burst was measured in peripheral blood neutrophils. Oxidative stress parameters involved the concentration of thiobarbituric acid reactive substances, reduced/oxidized glutathione ratio, and catalase activity in liver, kidney and heart. The expressions of DNA damage/repair genes *Gadd45a* (growth arrest and DNA damage-inducible alpha), *Parp1* (Poly(ADP-ribose) polymerase 1), and *Xrcc2* (X-Ray Repair complementing defective repair in Chinese hamster cells 2) and pro-inflammatory markers *Il-1β* (interleukin 1 beta), *Il-6* (interleukin 6), *Nf-κb* (nuclear factor kappa B), and *Tnf-α* (tumor necrosis factor alpha) were evaluated by real-time quantitative PCR. Human colon cell lines CCD-18Co (fibroblasts), and HT-29 (adenocarcinoma) were treated with guajiru anthocyanins (1.0 – 20.0 mg/L gallic acid equivalents - GAE) and the expressions of IL-1β, IL-6, NF-κB and TNF-α were analyzed at mRNA and protein levels. TNF-α was used to induce inflammation in CCD-18Co cells. Guajiru fruit phytochemicals were quantified and characterized by chromatographic and spectrometric methods. The concentrations of 19 chemical elements were determined by inductively coupled plasma mass spectrometry (ICP-MS). Delphinidin, cyanidin, petunidin and peonidin were the major anthocyanins in this fruit. Significant amounts of phytochemicals, magnesium, and selenium were found in this fruit. Guajiru fruit displayed *in vivo* antioxidant activity in neutrophils, antigenotoxicity in peripheral blood and antimutagenicity in bone marrow and peripheral blood. Guajiru fruit decreased DNA damage in liver, kidney, and heart. This fruit decreased the expression of *Gadd45a*, *Il-1β,* and *Tnf-α* in tissues. Cell proliferation was suppressed in HT-29 cells, and this was accompanied by increased intracellular ROS production as well as decreased *TNF-α*, *IL-1β*, *IL-6*, and *NF-κB* expressions. There was no cytotoxic effect of guajiru fruit anthocyanins in CCD-18Co cells. IL-1β, IL-6, and TNF-α protein expressions were reduced in TNF-α-treated CCD-18Co cells by guajiru fruit anthocyanins. The findings from this investigation demonstrated that phytochemicals and chemical elements in guajiru fruit possess antigenotoxic, antimutagenic, antioxidant and anti-inflammatory effects and encourage other *in vivo* and clinical studies with this underutilized fruit.

Keywords: Amazon fruit, comet assay, micronucleus test, nutrigenomics.

Chapter 1

1. Introduction

1.1 Fruit and vegetable intake and guajiru (*Chrysobalanus icaco* L.)

Several studies have demonstrated the relationship between the intake of natural products and the reduction of mortality by cardiac and degenerative diseases and cancer (MUSCARITOLI; AMABILE; MOLFINO, 2016; RAUTIAINEN et al., 2015). A few years ago, the "World Cancer Research Fund" performed an extensive literature review, describing evidence of the effect of the diet in colon, lung, stomach, esophagus and pharynx cancer, and probable evidence for larynx, pancreas, breast and bladder cancer (DE KOK et al., 2010).

The protective effects of fruits and vegetables are attributed to the chemical composition of food due to the presence of antioxidant molecules (such as vitamins, beta-carotene, and polyphenols such as anthocyanins). It is estimated that more than 4.000 phytochemical compounds can be found in fruits and vegetables, with the ability to mitigate damage induced by reactive oxygen species (ROS) to proteins, lipids, carbohydrates and the DNA. Therefore, the scientific interest in fruits, vegetables and isolated compounds from these sources have encouraged research in this area (MAGALHAES et al., 2009).

There are several fruits with functional properties already described in the literature. Mango (*Mangifera indica* L.) and pomegranate (*Punica Granatum* L.) decreased intestinal inflammation in a murine model of colitis (KIM et al., 2016). Java plum (*Syzygium cumini*) restored the body weight, glucose, urea and creatinine levels of diabetic rats to normal levels. Amazon fruits, such as açaí and pequiá (*Caryocar villosum*) exerted *in vivo* antigenotoxic and antimutagenic effects (ALMEIDA et al., 2012; RIBEIRO et al., 2010).

The Amazon Biome is the biggest tropical forest area in the world, and its flora comprises several fruit species that remain underexplored. In the last years, there is a general concern from scientists to improve the quality of life, aiming at the decrease of degenerative diseases. In this context, the interest in exploring native fruits has been growing (SCHRECKINGER et al., 2010). Thus, the promising species also represent an excellent opportunity for those local producers who reach this marketing niche (ALVES et al., 2008). However, several edible fruits still don't

possess economic importance since they are not sufficiently studied and by consequence, their cultivation and commercialization are not promoted (RODRIGUES; MARX, 2006).

Guajiru (*Chrysobalanus icaco* L.) belongs to the Chrysobalanaceae family, that comprises around 20 genera and 500 plant species (PRANCE, 1979). It is native from coastal areas around the globe, such as South Florida, Bahamas, and the Caribbean. In Brazil, this plant is found in the Northern region, in the Amazon Biome (LITTLE; WOODBURY; WADSWORT, 1974). Guajiru trees have shrubby form, with 3 meters maximum height and evergreen life cycle. Germination normally occurs within 20 to 30 days (MATTOS, 1999).

The guajiru leaf extract is used in the folk medicine to control glucose levels in diabetic individuals, and this effect was already described in the literature (BARBOSA et al., 2013; WHITE et al., 2016). Other effects of the leaf extract from guajiru trees are described, such as diuretic (PRESTA; PEREIRA, 1987), antiangiogenic (PAULO et al., 2000), cytotoxic against K562 – chronic myeloid leukemia – cells (FERNANDES et al., 2003), and antioxidant (FERREIRA-MACHADO et al., 2004). These effects are associated with the presence of terpenoids (diterpenoids and triterpenoids), flavonoids, steroids, and tannins, with functional properties described in the literature (LI et al., 2015; SIENIAWSKA, 2015).

Guajiru fruits are characterized by their elliptical or almost round shape, pink or purple-black peel (Figure 1.1). They are succulent, edible and have 20-29 mm diameter, containing a single, whitish seed inside. The flesh is white, sweet when ripe and astringent when unripe. Guajiru fruits are usually used fresh, but also as processed preserves. Vargas et al. (2000) highlight the fruit as a delicacy highly appreciated in Mexico. Fruiting and flowering occur mostly between January and April (PRANCE, 1979).

While guajiru leaves are widely explored, their fruits lack studies that prove their functional activity. A previous investigation with this fruit (DE BRITO et al., 2007) reported the presence of anthocyanins in the concentration of 104 mg/100 g in the fresh fruit. Anthocyanins are colored compounds responsible for the red, purple and blue pigmentation of fruits and vegetables (DE BRITO et al., 2007). There is evidence, reported by many studies, demonstrating the importance of this class of compounds to human health, since they are powerful antioxidants. Among the

beneficial effects of anthocyanins, are included the modulation of cardiac disease progression by decreasing inflammation (AMIN et al., 2015), protection against neurodegenerative disorders (BADSHAH; KIM; KIM, 2015) and antimutagenic effect (AZEVEDO et al., 2007).

Considering that many natural products remain unexplored, it becomes necessary to evaluate native fruits and vegetables, to know their effects after their consumption from the diet. Genetic toxicology tests are widely known and used to determine the influence of chemical compounds in the occurrance of mutations and chromosomal damage that could lead to cancer, developmental abnormalities and genetic diseases (CIMINO, 2006; LYNCH et al., 2011). Genotoxicity and mutagenicity assays are often part of the guidelines adopted by national and international regulatory agencies (ANVISA, 2010; FDA, 2012; OECD, 2014; OECD, 2014).

Figure 1.1 – Guajiru (*Chrysobalanus icaco* L.) fruits. Photo: Marcella Camargo Marques.

1.2 Genetic toxicology and DNA repair

The micronucleus (MN) test is one of the most used mutagenicity tests (BOLT; STEWART; HENGSTLER, 2011), being employed for detecting clastogenic (chromosomal breakage) and aneugenic agents (abnormal chromosome segregation) (HAYASHI et al., 2007; HAYASHI; SOFUNI; MORITA, 1991). Several researchers have described the relationship between micronuclei frequency and carcinogenesis. Cancer is associated with accumulated genetic damage (BONASSI et al., 2011) and therefore, genomic instability plays a role as a predisposition factor in cancer initiation (STRATTON; CAMPBELL; FUTREAL, 2009). Currently, high MN frequency has been associated with high risk of cancer, as described by many researchers (BONASSI et al., 2011; BONASSI et al., 2007; HOLLAND; CLEVELAND, 2012).

The micronucleus can be observed in dividing cells, as a result of chromosomal breaks, acentric fragments or as the result of whole chromosomes that are not attached to the spindle fibers. In telophase, these fragments or whole chromosomes are encapsulated in a small nucleus and are found in the cytoplasm, separated from the main nucleus. During maturation of erythroid cells in the bone marrow, the main nucleus is expelled from the nucleated erythrocytes, while the MNi are retained. These small nuclei are analyzed in polychromatic erythrocytes (PCEs) (RIBEIRO; SALVADORI; MARQUES, 2003).

The first protocol for MN test in mice was developed by Schmid (1975). MNi are typically rounded, with a diameter of 1/20 to 1/5 of erythrocyte diameter and correspond to what is called, in hematology, Howell-Jolly bodies (RABELLO-GAY; RODRIGUES; MONTELEONE-NETO, 1991). In the bone marrow, the cytotoxicity of treatment can also be evaluated by the PCE/NCE ratio (NCE – normochromatic erythrocytes). The decrease of this index reflects the occurrence of cytotoxicity or cell depletion (ZAIZUHANA et al., 2006).

To improve the efficiency of the *in vivo* toxicity tests, it is often discussed the association of MN test with the comet assay in the same animals, to allow reducing sample size and the required amount of the test compound (ROTHFUSS et al., 2011).

The alkaline comet assay (single cell gel electrophoresis), described by Singh et al. (1988) and modified by Speit and Hartman (1999) is a technique used to evaluate the genotoxicity of compounds. Considered to be of simple and quick execution, comet assay presents other advantages, such as high sensitivity and specificity, and versatility (can be performed in different tissues). Also, this assay does not require large amounts of sample test substance compared to other genotoxicity and mutagenicity tests (COLLINS, 2004; SINGH et al., 1988). Comet assay allows the detection of DNA breaks that, different from the mutations detected in the MN test, are likely to be repaired (ROJAS; LOPEZ; VALVERDE, 1997). Due to its versatility and reliability for detecting DNA damage, Glei, Schneider and Schlormann (2016) consider comet assay an essential tool in toxicological research.

Compared to other genotoxicity assays, the advantages of comet assay are: (1) high sensitivity to detect low levels of DNA damage; (2) ability to detect single- and double-strand breaks, alkali-labile sites, and DNA-DNA and DNA-protein cross-linking; (3) ability to detect DNA breaks in non-dividing cells (4) requires low number of cells per sample; (5) low cost; (6) easy application; (7) relatively fast (TICE et al., 2000). Furthermore, comet assay can be performed in several types of tissues and cell lines, being liver and kidney the most recommended (GLEI; SCHNEIDER; SCHLORMANN, 2016; HARTMANN et al., 2003; TICE et al., 2000).

Regulatory agencies such as the United States Food and Drug Administration – US FDA (2012) and the European Food Safety Authority – EFSA (2011) currently recommend comet assay as part of their genotoxicity testing strategies. In 2014, Organization for Economic Cooperation and Development (OECD) published the Test Guideline 489 for the *in vivo* mammalian alkaline comet assay, which summarizes the principles and limitations, and presents detailed descriptions of this method (OECD, 2014).

In antimutagenicity tests, using known agents recognized as DNA damage inducers is crucial and recommended by many protocols to investigate the protective effect of substances (MACGREGOR et al., 1987). Among the chemicals used as positive control in antigenotoxicity and antimutagenicity investigations, doxorubicin (DXR) is an anthracycline antitumor antibiotic that has been consistently used in several studies, including some of our research group (ANTUNES; TAKAHASHI, 1998; CHEQUER et al., 2012; RIBEIRO et al., 2010). DXR is efficient in the

generation of DNA damage in both *in vivo* (WANG et al., 2014) and *in vitro* (CHEQUER et al., 2012) experiments. Therefore, this drug was chosen and used in this doctoral thesis as the positive control in all *in vivo* assays to evaluate the protective effect of guajiru fruit in the animals.

The main causes of DNA damage with implications for mutations are environmental agents (ultraviolet light, chemicals, ionizing radiation), products of cell metabolism (e.g. ROS), and the tendency to spontaneously disintegration of some chemical bonds in the DNA (GLEI; SCHNEIDER; SCHLORMANN, 2016). Therefore, a cellular machinery towards the counteraction of the genetic degeneration is vital for cell survival. DNA repair mechanisms involve base excision repair (BER), nucleotide excision repair (NER), recombinational repair and mismatch repair (HOEIJMAKERS, 2001). Cells respond to DNA damage by the activation of signaling pathways that determine cell fate, promoting cell death or DNA repair and cell survival (ROOS; THOMAS; KAINA, 2016). The DNA repair capacity is considered a marker of susceptibility to cancer and mutations, and it is often determined by the transcription levels of genes involved with DNA damage and repair by DNA microarray or real-time quantitative PCR (RT-qPCR) (GLEI; SCHNEIDER; SCHLORMANN, 2016; LIU et al., 2016).

Growth arrest and DNA-damage-inducible, alpha (*Gadd45a*) is a gene rapidly induced by genotoxic stress (GUPTA et al., 2005). *Gadd45a* expression is often upregulated in response to environmental stressors and DNA-damaging agents, including ultraviolet and ionizing radiations and chemical compounds such as methyl methanesulfonate (MOSKALEV et al., 2012). This gene induces cell cycle arrest at G2/M stages, allowing DNA repair to occur (WANG et al., 1999; WINGERT; RIEGER, 2016). Several chemicals modulate the expression of *Gadd45a*, including 5-azacytidine, cisplatin, and DXR (KRUSHKAL et al., 2016).

Poly(ADP-Ribose) Polymerase 1 (*Parp1*) gene is also upregulated by different types of damage, such as single-strand breaks, DNA crosslinks, stalled replication forks and double-strand breaks (KRISHNAKUMAR; KRAUS, 2010). For almost two decades, this gene was considered a central component of base excision repair and single-strand break repair processes. Recently, accumulated evidence shows that PARP1 also plays a role in double-strand break repair (BECK et al., 2014). This

protein can also bind to nucleosomes and chromatin-associated proteins, especially in regions affected by DNA damage (KRISHNAKUMAR; KRAUS, 2010).

X-ray repair complementing defective repair in Chinese hamster cells 2 (*Xrcc2*) is another gene associated with the repair of double strand breaks. However, this gene acts through homologous repair. Severe forms of DNA damage must be repaired efficiently for cells to survive and homologous recombination is essential in the repair of such damage in mammals (TAMBINI et al., 2010). *Xrcc2* along with other genes (e.g., *Rad51* and *Xrcc3*) play a major role in homologous recombination, ensuring the proper repair of the damaged DNA strand using homologous segments of the undamaged strand (TAMBINI et al., 2010).

In summary, the association between the genotoxicity/mutagenicity tests and DNA damage/repair biomarkers may provide useful information about the mechanism of antigenotoxicity and antimutagenicity of compounds, including those obtained from the diet, in both *in vivo* and *in vitro* systems.

1.3 Oxidative stress and oxidative burst of neutrophils

Several mechanisms are involved in the damage induced to the DNA structure, including the effects related to ROS (KIRSCH-VOLDERS et al., 2003; WINCZURA; ZDZALIK; TUDEK, 2012). The investigation of oxidative stress biomarkers is critical in the evaluation of compounds named antioxidants, since this class of molecules is described by protecting the cells and the genome against damage (LAUVER; KAISSARIAN; LUCCHESI, 2013; OTERO-LOSADA et al., 2013). Therefore, the evaluation of processes such as ROS generation and lipid peroxidation and the assessment of the antioxidant system components (e.g., glutathione and catalase) have been used in chemopreventive studies involving extracts and molecules from fruit and other dietary compounds (SAHREEN; KHAN; KHAN, 2014; SALEEM; CHETTY; KAVIMANI, 2013).

The main byproduct of lipid peroxidation is malondialdehyde (MDA), produced by the reaction between a polyunsaturated fatty acid and molecular oxygen, with the production of peroxyl radicals. The reduction of these radicals leads to the formation of MDA (VOULGARIDOU et al., 2011). Both mutagenicity and carcinogenicity of MDA are already known since this molecule diffuses throughout the cell and interacts with DNA and proteins (KANNER, 2007; KEW, 2009).

Glutathione is an important tripeptide of the antioxidant system, and its intracellular concentration is used as oxidative stress indicator. Two forms of glutathione co-exist in the intracellular environment: the reduced (GSH) and the oxidized (GSSG) glutathione. The oxidative stress leads to the imbalance of thiols and change (decrease) the GSH/GSSG ratio in tissues. ROS, particularly superoxide anions, hydroxyl radicals and hydrogen peroxide and hydroperoxide, are scavenged by glutathione through detoxification reactions involving the enzymes glutathione peroxidase, glutathione-S-transferase, and glutathione reductase. Additionally, glutathione acts in processes related to signal transcription, gene expression, and apoptosis. Thus, the GSH/GSSG ratio is frequently investigated in physiological and pathological situations (RAHMAN; KODE; BISWAS, 2006).

Catalase is a ubiquitous antioxidant enzyme found in the cells and catalyzes the reduction of hydrogen peroxide (H_2O_2) to water and can neutralize some organic hydroperoxides and oxidize xenobiotics such as phenols, formic acid, and alcohols

(NAZIROGLU, 2012). In experimental systems, oxidative stress is characterized by the decrease of the activity of this enzyme, affecting the efficiency of the antioxidant system (BALAJI; MUTHUKUMARAN; NALINI, 2014; HU et al., 2014).

Oxidative burst is the functional response of neutrophils and other phagocytes, characterized by the rapid release of high concentrations of ROS. These cells play a fundamental role in the defense against pathogens and the modulation of the inflammatory response. Although ROS levels released by neutrophils are useful for immune defense, the overproduction of these molecules can lead to cellular and tissue damage (CIZ et al., 2012).

The production of ROS by neutrophils is characterized by the release of superoxide radicals by the NADPH oxidase enzyme complex (LOJEK et al., 2002; PEKAROVA et al., 2011). It has been demonstrated that the intracellular redox status can be pharmacologically modulated by using chemical compounds with antioxidant characteristics, that act donating electrons to ROS, converting these molecules into their non-radical forms or inhibiting the NADPH oxidase complex. Thus, phytochemicals obtained from the diet have been regarded as substances of interest due to their capacity to modulate the oxidative burst of neutrophils and by consequence, decrease the production of ROS and tissue damage in the inflammation sites (ČÍŽ et al., 2010; CIZ et al., 2012; DENEV et al., 2010).

1.4 Inflammation, colon cancer, and intestinal bowel disease

Inflammation is a ubiquitous process that happens in response to tissue injury and involves the activation and migration of leucocytes to the site of damage and the activity of mast cells in the injured tissue. A family of chemotactic cytokines, named chemokines, recruit effector cells and are the responsible for the natural evolution of the inflammatory response. However, dysregulation in the inflammatory process can lead to abnormalities and ultimately, pathogenesis, including cancer and intestinal bowel disease. In carcinogenesis, neoplastic promotion is associated with the exposure of initiated cells to the factors released at the site of wounding, that could lead to induced cell proliferation, increased production of ROS, DNA damage, and reduced DNA repair. Due to the lack of cell death and DNA repair, cells with abnormal growth control start proliferating (COUSSENS; WERB, 2002).

Intestinal bowel diseases (IBDs) are chronic gastrointestinal disorders characterized by intestinal inflammation and epithelial injury (BAUMGART; SANDBORN, 2012; DANESE; FIOCCHI, 2011). Cytokines have been associated with the pathogenesis of IBD and may play a major role in controlling intestinal inflammation and the clinical symptoms of the disease (NEURATH, 2014; STROBER; FUSS; BLUMBERG, 2002). IBD pathogenesis involves critical alterations in the epithelial barrier function, allowing the translocation of bacterial antigens into the bowel wall. The excessive cytokine responses triggered by the inflammatory stimuli cause subclinical or acute inflammation in genetically susceptible individuals (STROBER; FUSS; BLUMBERG, 2002). The inability to resolve acute intestinal inflammation leads to chronic inflammation in the intestinal tissue, induced by the overstimulation of the mucosal immune system (STROBER; FUSS; BLUMBERG, 2002). Therefore, the high levels of cytokines are the main responsible for the intestinal inflammation and associated symptoms (e.g., diarrhea), but also for the extra-intestinal manifestations of this disease (arthralgia or arthritis), and complications such as intestinal stenosis, abscess and fistula formation, and the development of colitis-associated neoplasias (PEYRIN-BIROULET et al., 2011).

Evaluating the expression of pro-inflamatory cytokines is a useful tool for investigating the severity of inflammation in biological systems. The generation of ROS at the site of wounding can activate NF-κB through the phosphorylation of IκBα,

initiating an inflammatory response (MORGAN; LIU, 2011). In colorectal cancer, NF-κB increases angiogenesis and cell proliferation, inhibits cell death, and promotes cell invasion and metastasis (NAUGLER; KARIN, 2008). Elevated activity of NF-κB is also involved in cellular resistance to chemotherapy and ionizing radiation in human cells (WANG; MAYO; BALDWIN, 1996), complicating cancer prognosis and treatment. NF-κB overexpression in myeloid and epithelial colonic cells is also associated with IBD (CHUNG, 2000). Many drugs used to treat IBD aim to inhibit NF-κB-dependent mechanisms (MAJUMDAR; AGGARWAL, 2001; WAHL et al., 1998).

TNF-α, IL-1β, and IL-6 are cytokines associated with both colorectal and colitis-associated tumorigenesis (POPIVANOVA et al., 2008; WANG et al., 2009). TNF-α initiates an inflammatory response, and is followed by the production of cytokines, chemokines, and adhesion molecules in the colonic endothelium (TERZIC et al., 2010). TNF-α is often upregulated in colon tumorigenesis and in intestinal tissue of patients with Crohn's disease or other forms of IBD (KOLLIAS, 2004; POPIVANOVA et al., 2008). IL-1β is an acute pro-inflammatory cytokine that is increased in colitis-associated and other forms of gastrointestinal cancer (POPIVANOVA et al., 2008). IL-6 induces colon cancer cell growth, stimulating tumor growth and the proliferation of premalignant enterocytes (BECKER et al., 2005). While this cytokine plays a significant role in colitis and the pathogenic immune response, tissue regeneration process could also be modulated by IL-6, as described in a murine infection model by Dann et al. (2008).

DNA damage and inflammation are critical pathways in health promotion since these processes are highly interrelated (PALMAI-PALLAG; BACHRATI, 2014) and both have shown to be modulated by dietary compounds (FENECH, 2014; LYONS; KENNEDY; ROCHE, 2016). Therefore, investigating the effects of food, such as fruits and vegetables, at cellular and molecular levels becomes a valuable tool to elucidate their mechanism of action.

Considering the existing data regarding the effects of anthocyanins in disease prevention (SODAGARI et al., 2015; WALLACE; SLAVIN; FRANKENFELD, 2016), it is possible that guajiru fruit could be used for health promotion purposes.

1.5. Objectives

To evaluate the protective effects of guajiru fruit against *in vivo* DXR-induced DNA damage, oxidative stress, and inflammation, and *in vitro* TNF-α-induced inflammation.

1.5.1 Specific objectives

✓ To assess the *in vivo* antigenotoxicity, antimutagenicity and antioxidant activity of guajiru fruit against DXR-induced damage in peripheral blood and bone marrow cells, and to stablish the relationship between genomic instability and oxidative stress in this fruit chemoprevention mechanism;

✓ To investigate the *in vivo* antigenotoxicity and anti-inflammatory effects of guajiru fruit against DXR-induced DNA damage and inflammation in liver, kidney and heart tissues;

✓ To assess the antiproliferative, antioxidant and anti-inflammatory activities of guajiru anthocyanins in *in vitro* models of intestinal bowel disease and colon cancer.

Chapter 5

5. Conclusions

✓ Data presented in Chapter 2 suggest that guajiru fruit can act as dietary antioxidant and, by consequence, protect the DNA against DXR-induced damage *in vivo*;

✓ From Chapter 3, guajiru fruit reduced DXR-induced DNA damage (by decreasing comet assay parameters and the levels of *Gadd45a*) and inflammation (by reducing expressions of *Tnf-α* and *Il-1β*) in tissues of rats;

✓ The in vitro experiment described in Chapter 4 indicated that guajiru anthocyanins exerted selective cytotoxicity in HT-29 colon cancer cells and modulated the ROS generation and inflammation in colon cancer and inflamed normal colon cells. The results indicate the protective effects of this fruit in intestinal cells, shown by the decrease in inflammation markers;

✓ The results may be explained by this fruit chemical (polyphenol and inorganic elements) composition;

✓ Since there was no information in the literature regarding guajiru fruit effects on health, this investigation provides new and innovative information about this polyphenol-rich fruit, which can help future research as well as the optimization of the use of this underutilized fruit on human health;

✓ Future mechanistic and *in vivo* studies should clarify the mechanisms of action and the potential of this fruit as a prospective nutraceutical in the prevention of intestinal inflammation and inflammatory diseases. Additionally, pharmacokinetic studies need to be performed to determine effective dose levels.

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