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

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

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Doctoral Candidate: Vinicius de Paula Venancio

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DEDICATION

To my parents, Lourdes de Fátima Paula Venâncio and José Antônio Venâncio. What a divine blessing is being your son. Thank you for all knowledge, your support and for being examples of integrity, honesty and hard work. This achievement is also yours.

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“It had long since come to my attention that people of accomplishment rarely sat back and let things happen to them. They went out and happened to things”

Leonardo da Vinci

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.

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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 in 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 2

2. Guajiru (*Chrysobalanus icaco* L.) fruits inhibit NADPH oxidase complex and protect DNA against doxorubicin-induced damage *in vivo*

2.1 Abstract

Guajiru (*Chrysobalanus icaco* L.) is an underexplored plant found in tropical areas around the globe. Currently, there is no apparent information regarding the effects guajiru fruits may exert *in vivo* or their potential role in health promotion. This study aimed at providing evidence regarding the *in vivo* influence of this fruit on antigenotoxicity, antimutagenicity, and oxidative stress in rats. Male Wistar rats were treated with 100, 200 or 400 mg/kg b.w./day guajiru fruit for 14 days. Doxorubicin (DXR, 15 mg/kg b.w, i.p.) was used for DNA damaging and as an oxidant to generate reactive oxygen species (ROS). Genomic instability was assessed by comet assay and micronucleus (MN) test while antioxidant activity was determined by oxidative burst of neutrophils. Guajiru fruit phytochemicals were quantified and characterized by high-performance liquid chromatography coupled to a diode array detector and mass spectrometer (HPLC-DAD-MS/MS). The concentrations of 19 chemical elements were determined by inductively coupled plasma-mass sprectroscopy (ICP-MS). Significant amounts of phytochemicals, magnesium, and selenium were found in guajiru fruit. This fruit displayed *in vivo* antioxidant activity against DXR-induced damage in rat peripheral blood neutrophils, antigenotoxicity in peripheral blood cells, and antimutagenicity in bone marrow cells and peripheral blood cells. Correlation analyses between endpoints examined indicated that the mechanism underlying chemopreventive actions of guajiru fruit was attributed to inhibition of NADPH oxidase complex manifested as low levels of DNA damage in animals exposed to DXR. Data indicate that phytochemicals and minerals in guajiru fruit protect DNA against damage *in vivo* associated with their antioxidant properties.

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2.2 Introduction

Chrysobalanus icaco L. (guajiru, cocoplum or abajeru) belongs to the Chrysobalanaceae family, which consists of approximately 20 genera and 500 different species (PRANCE, 1979). *Chrysobalanus icaco* L. is native to coastal regions such as southern Florida, Brazil, the Bahamas, and the Caribbean. No apparent information regarding the effects of guajiru fruits was found in the literature; however, leaf extracts were noted to exert biological activities such as decreasing blood sugar levels (BARBOSA et al., 2013), insulin sensitivity (WHITE et al., 2016) and angiogenesis (ALVES DE PAULO et al., 2000). These fruits are rich in anthocyanins (DE BRITO et al., 2007), natural pigments that possess antioxidant capacity and are responsible for many beneficial effects as evidenced by protection of endothelial progenitor cells against angiotensin II-mediated dysfunction (PARZONKO et al., 2015) and attenuation of manganese (Mn)-induced oxidative stress in rat astrocytes (DA SILVA SANTOS et al., 2014).

Cells in the organism generate reactive oxygen species (ROS), and overproduction of these molecules leads to deleterious interactions with DNA, RNA, proteins, and lipids (GHIO; CARRAWAY; MADDEN, 2012; MARALDI, 2013). Production and elimination of ROS are usually well balanced due to finely regulated systems (GHIO; CARRAWAY; MADDEN, 2012; LUSHCHAK, 2014). Antioxidant compounds obtained from the diet might reduce or prevent excessive production of ROS by promoting endogenous antioxidant activity and rapidly neutralizing these molecules (ARGENTIN; DIVIZIA; CICCHETTI, 2015; HARASYM; OLEDZKI, 2014). Therefore, this investigation aimed at providing novel information regarding *in vivo* antigenotoxic, antimutagenic and antioxidant actions induced by guajiru fruit in rats previously administered doxorubicin (DXR), with the view to potentially use guajiru fruit in humans suffering from adverse oxidant exposure.

2.3 Material and Methods

2.3.1 Guajiru fruit and chemicals

Ripe guajiru fruits were harvested at Praia do Farol (1° 7' 59.98" S, 48° 27' 33.98" W), Belém, Pará, Brazil. The moisture content of the fresh fruit (pulp + peel) was 81.95 ± 1.48 g/100g (*n* = 3). Seeds were removed, and pulp and peel immediately frozen in liquid nitrogen before lyophilization (-60 °C, 50 μmHg, 7 days, Liotop L101, Liobras, São Paulo, Brazil). The lyophilized fruit was homogenized in a food processor (Walita, Barueri, Brazil), vacuum-packed and stored at -36°C until use.

All-*trans*-β-carotene (99.9%, CAS 7235-40-7), quercetin (95%, CAS 6151-25-3), gallic acid (GA, 98%, CAS 149-91-7), acridine orange (AO, CAS 10127-02-3), and trypan blue (CAS 72-57-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ellagic acid (EA, 95%, CAS 476-66-4), cyanidin 3-glucoside (97%, CAS 7084-24-4), delphinidin 3-glucoside (95%, CAS 6906-38-3), peonidin 3-glucoside (95%, CAS 68795-37-9), and petunidin 3-glucoside (95%, CAS 6988-81-4) were acquired from Extrasynthèse (Genay, France). All-*trans*-lutein (98.8%, CAS 38327-39-8) was donated by DSM Nutritional Products (Basel, Switzerland), and all-*trans*-violaxanthin (95%, CAS 126-29-4) and 9'-*cis*-neoxanthin (97%, CAS 14660-91-4) were purchased from CaroteNature (Lupsingen, Switzerland). Doxorubicin hydrochloride (DXR, CAS 25316-40-9) was obtained from Laboratório Químico Farmacêutico Bérgamo (São Paulo, Brazil). Low (CAS 39346-81-1) and normal melting point agaroses (CAS 9012-36-6) were purchased from Invitrogen (Carlsbad, CA, USA). Dimethylsulfoxide (DMSO, CAS 67-68-5) was purchased from Merck Chemicals (Rio de Janeiro, Brazil). GelRed™ Nucleic Acid Gel Stain 10,000× was purchased from Biotium (Hayward, CA, USA). All other reagents used were of the highest possible purity.

2.3.2 Phytochemical characterization

Anthocyanins were extracted from 0.05 ± 0.006 g lyophilized sample with 1% (v/v) HCl and identified and quantified by high-performance liquid chromatography

coupled to diode array detector and tandem mass spectrometer (HPLC-DAD-MS/MS) (FARIA; MARQUES; MERCADANTE, 2011).

Carotenoids were extracted from 2 ± 0.4 g lyophilized sample by maceration with 5 mL ethyl acetate and 5 mL ethyl ether, filtered and partitioned in a mixture of petroleum ether: ethyl ether (1:1, v/v). The extract was dried in a rotary evaporator (Büchi, Flawil, Switzerland) and dissolved in 10 mL petroleum ether. After saponification, carotenoids were identified and quantified by HPLC-DAD-MS/MS (ROSSO; MERCADANTE, 2007). Phenolic compounds were extracted from 0.1 ± 0.01 g of lyophilized sample with methanol: water (8:2, v/v). The identification and quantification of phenolic compounds were carried out by HPLC-DAD-MS/MS (CHISTE; MERCADANTE, 2012).

All extracts for identification and quantification of bioactive compounds were prepared in triplicate. The chromatographic analyses for identification and quantification of bioactive compounds were carried out in a Shimadzu HPLC (Kyoto, Japan) coupled in series to a diode array (DAD, model, SPD-M20A, Shimadzu) and mass spectrometer (Amazon Speed, Bruker Daltonics, Bremen, Germany) detectors.

2.3.3 Mineral characterization

Freeze-dried guajiru fruit was digested in triplicate using a microwave oven decomposition system (Milestone Ethos D, Italy) in closed vessels, according to NARDI et al. (2009). A total of 19 elements (sodium, potassium, magnesium, calcium, rubidium, iron, manganese, aluminum, zinc, copper, barium, nickel, chromium, selenium, arsenic, lead, cobalt, vanadium, and thallium) were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) (Elan DRC II, Perkin Elmer, Norwalk, CT).

2.3.4 Animals, experimental design, and dose selection

The local Ethics Committee for Animal Use approved the experimental protocol used in this investigation (approval number 11.1.1517.53.0). Male Wistar rats (*Rattus norvegicus*), 4-5 weeks old, weighting 110 ± 10 g, were provided by the animal facility of “Prefeitura do Campus USP de Ribeirão Preto” and kept in polycarbonate

cages at 22 ± 2 °C, 12-hour light/dark cycle with *ad libitum* access to food (Nuvilab, Colombo, PR, Brazil) and fresh water.

Since there are no apparent data regarding guajiru fruit toxicity, 400 mg/kg b.w./day was selected as the maximal dose because it mimics consumption of this fruit as a 200-mL daily juice preparation. Animals were divided into 8 groups (*n* = 6 animals/group) and treated as follows: Controls received water for 14 days. DXR alone group was intraperitoneally injected (ip) with 15 mg/kg b.w. 24 hours before euthanasia. Groups of animals were administered daily by gavage 100, 200 or 400 mg/kg b.w/day guajiru fruit (G) alone for 14 days. In addition, groups of rats were also administered daily by gavage 100, 200 or 400 mg/kg b.w/day guajiru fruit (G) for 14 days followed by DXR (15 mg/kg b.w., i.p.) 24 hours before euthanasia. The freeze-dried guajiru fruit was dispersed in fresh water and administered daily by gavage to the experimental groups. Controls received water for 14 days. Twenty-four hours after last injection, all animals were anesthetized (ketamine/xylazine 100/10 mg/kg b.w.) and euthanized by cardiac puncture. Blood and bone marrow cells were collected.

2.3.5 Peripheral blood alkaline comet assay

In vivo genotoxicity and antigenotoxicity of guajiru fruit were assessed as previously described by Singh et al. (1988) and Tice et al. (2000). Blood samples were collected with K_2 EDTA preservative (Labtest, Lagoa Santa, Brazil), mixed in 0.5% (w/v) low melting point agarose and spread on 1.5% (w/v) agarose pre-treated microscope slides. After agarose solidification (20 min, 4 ºC), the slides were subjected to lysis (overnight, 4 °C), DNA unwinding (20 min, 4 °C), electrophoresis (20 min, 0.85 V/cm, 300 mA, 4 ºC), neutralization (5 min, pH 7.5, 4 ºC), and fixation (ethanol, 2 min). Immediately before analysis, slides were stained with GelRed™ (1:10,000 v/v). Tail Intensity (% DNA in tail) and Tail Moment were analyzed in 100 nucleoids per animal using Comet Assay IV software (Perceptive Instruments, Suffolk, UK) and a fluorescence microscope (Axiostarplus, Zeiss, Germany) equipped with 515-560 nm excitation and 590 nm barrier filters. Cell viability was determined in cell suspensions by trypan blue exclusion method, and all results were above 90% (data not shown).

2.3.6 Micronucleus test

The *in vivo* mutagenicity and antimutagenicity of guajiru fruit were investigated by the bone marrow and peripheral blood micronucleus tests. The bone marrow cells were collected in fetal bovine serum as described by Schmid (1975). Cells were smeared on clean microscope slides, dried, fixed in methanol for 2 min and stained with Giemsa (in pH 5.5 Sorenson's buffer). The frequency of micronuclei (MN) in polychromatic erythrocytes (PCEs) among 2,000 PCEs and the ratio between PCEs and normochromatic erythrocytes (NCEs) among 500 bone marrow erythrocytes were assessed using light microscopy (Carl Zeiss Axiostar Plus) at 1,000× magnification.

Peripheral blood micronucleus test was performed as described by Holden, Majeska and Studwell (1997). A drop of peripheral blood from tail veins was collected and smeared on clean microscope slides. The slides were dried, fixed in methanol and stained with 16 µg/mL acridine orange. The frequency of MN in 1,000 reticulocytes (RETs) was scored using a fluorescence microscope (Axiostarplus, Zeiss) equipped with a 488 nm excitation filter.

2.3.7 Oxidative burst of neutrophils

Neutrophils were isolated from peripheral blood according to Russo-Carbolante et al. (2002). Cell viability was performed by trypan blue exclusion method and the purity of neutrophil population assessed by Giemsa staining. The oxidative burst was measured by chemiluminescence using 1×10^5 neutrophils per reaction, 10^{-7} M phorbol 12-myristate 13-acetate (PMA) as stimulus and 10^{-4} M luminol as the luminescent label. The luminescence intensity was followed for 20 minutes at 37 °C by a luminometer (Autolumat Plus LB 953, EG&G Berthold, Bad Wildbad, Germany) and the area under curve calculated for each measurement.

2.3.8 Statistical analysis

All results are expressed as mean ± standard deviation (SD). The normality of variable distributions was analyzed by Kolmogorov-Smirnov test. Data were analyzed

by ANOVA-Tukey using the software GraphPad Prism 6.0. p-values < 0.05 were considered significant. For the micronucleus test, the reduction percentage was calculated according to Waters et al. (1990).

2.4 Results

2.4.1 Phytochemical and mineral composition

Phytochemical characterization is shown in Figure 2.1 and described in details in Table 2.1. Four anthocyanins, namely delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside and peonidin 3-glucoside, were identified based on the comparison of elution order on C_{18} column and mass spectra features compared to authentic standards analyzed under the same conditions. Cyanidin 3-glucoside and peonidin 3-glucoside were identified for the first time in guajiru fruit. The MS data show the coelution of petunidin 3-glucoside and an acylated anthocyanin tentatively identified as delphinidin 3-(6″-acetoyl)galactoside or delphinidin 3-(6″-oxaloyl)arabinoside. These two coeluted anthocyanins corresponded to more than 47% of the anthocyanins in this fruit. The assignment of sugar and acid moieties identity in all acylated compounds was based on the loss of 204 u in the MS/MS spectra of the molecular ion, which can correspond to the loss of one hexose and one acetyl group $[M-162-42]^+$ or to the loss of one arabinose and one oxaloyl group $[M-132-72]^+$ (WU; PRIOR, 2005); thus, it is not possible to differentiate these compounds by mass spectrometry. The other three anthocyanins found in guajiru were tentatively identified as petunidin 3-(6″-acetoyl)galactoside or petunidin 3-(6″-oxaloyl)arabinoside, peonidin 3-(6″-acetoyl)galactoside or peonidin 3-(6″-oxaloyl)arabinoside and petunidin 3-(6″-acetoyl)glucoside or petunidin 3-(6″-oxaloyl)arabinoside.

All-*trans*-lutein was the major carotenoid found in guajiru fruit, representing more than 22% of total carotenoids. Three phenolic compounds were tentatively identified as an ellagic acid derivative, a myricetin pentoside, and a quercetin derivative.

The concentrations of 19 chemical elements found in the guajiru fruit are also shown in Table 2.1.

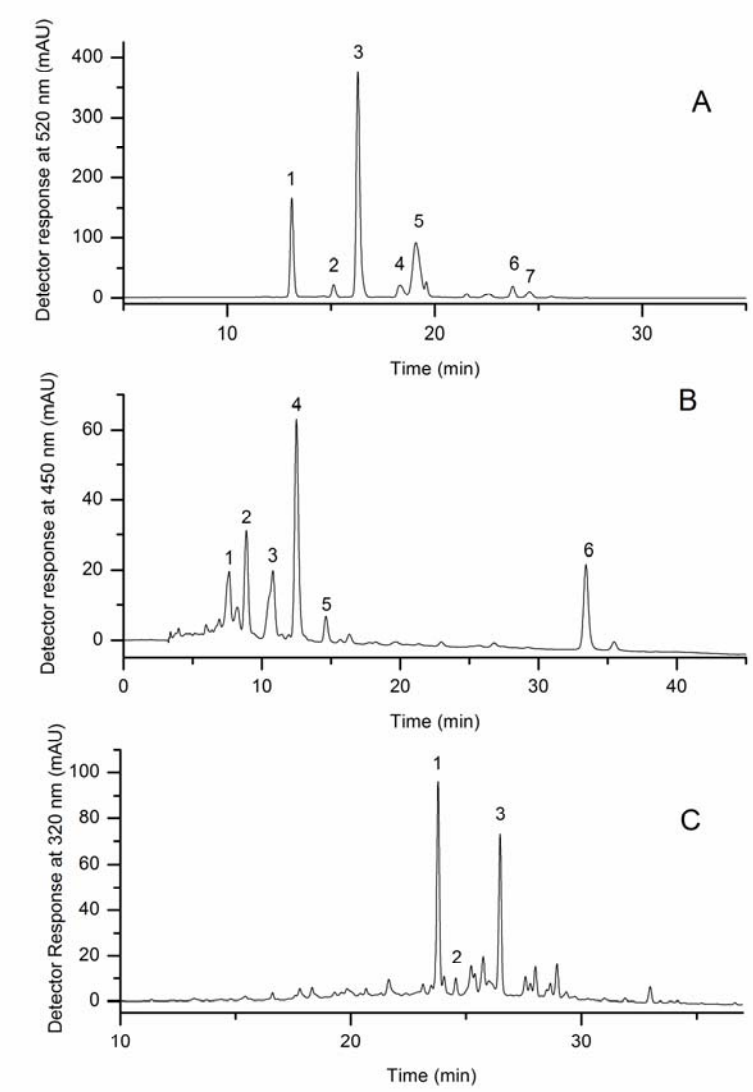

Figure 2.1 - Chromatograms obtained by HPLC–DAD from guajiru fruit. (A) **Anthocyanins** (processed at 520 nm): 1: delphinidin 3-glucoside, 2: cyanidin 3-glucoside, 3: petunidin 3-glucoside + delphinidin 3-(6″-acetoyl)galactoside or delphinidin 3-(6″-oxaloyl)arabinoside, 4: peonidin 3-glucoside, 5: petunidin 3-(6″-acetoyl)galactoside or petunidin 3-(6″-oxaloyl)arabinoside, 6: peonidin 3-(6″-acetoyl)glucoside or peonidin 3-(6″-oxaloyl)arabinoside, 7: petunidin 3-(6″-acetoyl)glucoside or petunidin 3-(6″-oxaloyl)arabinoside. (B) **Carotenoids** (processed at 450 nm): 1: all-*trans*-violaxanthin, 2: 9′-*cis*-neoxanthin, 3: 9-*cis*-violaxanthin, 4: all-*trans*-lutein, 5: all-*trans*-zeaxanthin, 6: all-*trans*-β-carotene. (C) **Non-anthocyanic** (processed at 320 nm): 1: ellagic acid derivative, 2: myricetin pentoside, 3: quercetin derivative.

Table 2.1 - Phytochemical and mineral composition of guajiru fruit

Compound classes		Major compounds
Non-anthocyanic phenolic compounds	213.33 ± 0.37[a]	ellagic acid derivative (133.43 ± 1.04)[a], myricetin pentoside (18.68 ± 0.70)[b], quercetin derivative (61.22 ± 2.59)[b]
Total anthocyanins	547 ± 44[c]	petunidin 3-glucoside + delphinidin 3-(6″-acetoyl)galactoside or delphinidin 3-(6″-oxaloyl)arabinoside (257 ± 20)[c]
Total carotenoids	850 ± 60[d]	all-*trans*-lutein (188.6 ± 9.4)[e], 9′-*cis*-neoxanthin (99.8 ± 10.9)[f], all-*trans*-β-carotene (79.2 ± 12.4)[d], 9-*cis*-violaxanthin (76.7 ± 13.4)[g], all-*trans*-violaxanthin (75.7 ± 8.6)[g]
Chemical elements		sodium (1642 ± 44)[h], potassium (1469 ± 35)[h], magnesium (282 ± 6.8)[h], calcium (216 ± 6.0)[h], rubidium (3.8 ± 0.07)[h], iron (2.7 ± 0.4)[h], manganese (1.3 ± 0.03)[h], aluminum (1.2 ± 0.2)[h], zinc (1.2 ± 0.04)[h], copper (0.9 ± 0.02)[h], barium (0.3 ± 0.01)[h], nickel (0.3 ± 0.01)[h], chromium (0.2 ± 0.02)[h], selenium (0.09 ± 0.04)[h], arsenic (20.6 ± 1.9)[i], lead (9.8 ± 3.6)[i], cobalt (6.7 ± 0.7)[i], vanadium (2.5 ± 0.9)[i], thallium (0.0 ± 0.1)[i]

All results are expressed in dry weight (DW) or fresh weight (FW), as the mean ± SD of triplicate analyses

[a] mg ellagic acid equivalent/100 g DW. [b] mg quercetin equivalent/100 g DW. [c] mg cyanidin 3-glucoside equivalent/100 g DW. [d] µg all-*trans*-β-carotene/100 g DW. [e]µg all-*trans*-lutein/100 g DW. [f] µg 9′-*cis*- neoxanthin equivalent/100 g DW. [g] µg all-*trans*-violaxanthin/100 g DW. [h] µg/g FW. [i] ng/g FW

2.4.2 Antigenotoxicity of guajiru fruit in peripheral blood by comet assay

The comet assay was performed to evaluate whether guajiru fruit could induce DNA damage or reduce DXR-induced DNA damage in peripheral blood cells. Guajiru fruit did not cause genotoxicity but decreased DXR-induced Tail Moment and Tail Intensity in these cells (Figure 2.2). Both DNA damage parameters observed in the

association (G+DXR) groups were statistically different from DXR treatment group, demonstrating the protective effects of guajiru in those cells. These data indicate that guajiru fruit was antigenotoxic against DXR-induced DNA damage in peripheral blood cells ($p < 0.05$).

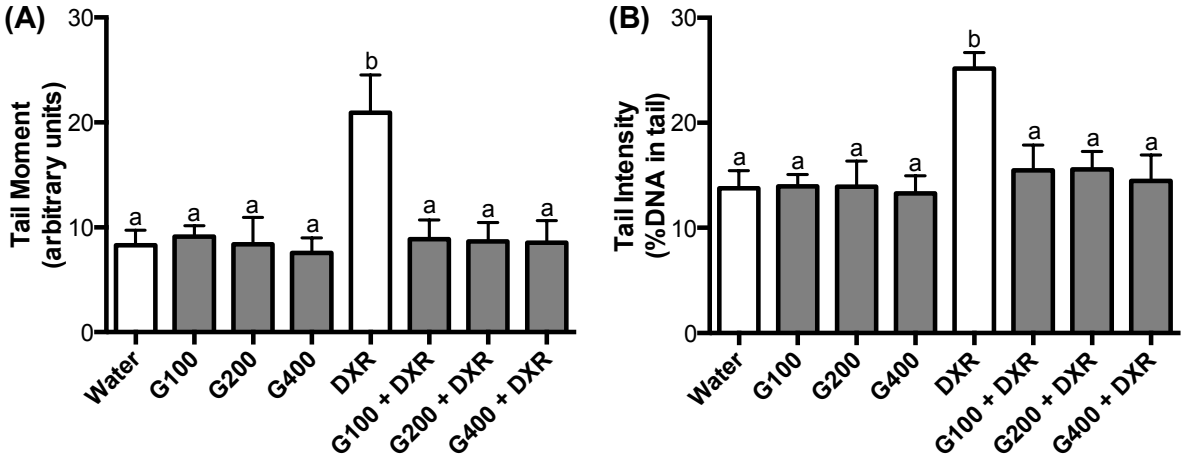

Figure 2.2 - Antigenotoxicity of guajiru fruit (G) on (A) Tail Moment and (B) Tail Intensity of peripheral blood cells by alkaline comet assay. Data are mean ± SD. DXR: doxorubicin (15 mg/kg b.w. – i.p.); G100, G200, G400: guajiru fruit at 100, 200 or 400 mg/kg b.w./day. Different letters indicate significant differences between groups ($p < 0.05$, ANOVA-Tukey). One hundred nucleoids were analyzed per animal. $n = 6$.

2.4.3 Guajiru fruit antimutagenicity by micronucleus test

Besides genotoxicity, the mutagenicity was also evaluated by micronucleus assay in peripheral blood and bone marrow cells. The results are shown in Table 2.2.

Table 2.2 - Antimutagenicity of guajiru fruit (G) in peripheral blood and bone marrow cells by micronucleus test

Groups	Peripheral blood		Bone marrow		
	MN/ 1,000 RET	Reduction (%)	PCE/NCE ratio	MN/ 1,000 PCE	Reduction (%)
Water	1.7 ± 1.6[a]		0.4 ± 0.1[a]	1.5 ± 1.1[a,d]	
G100	1.1 ± 1.1[a]		0.4 ± 0.1[a]	0.8 ± 0.7[a]	
G200	1.3 ± 0.8[a]		0.4 ± 0.1[a]	1.2 ± 0.9[a,e]	
G400	1.3 ± 0.5[a]		0.4 ± 0.1[a]	0.9 ± 0.8[a]	
DXR	13.3 ± 2.3[b]		0.4 ± 0.1[a]	16.0 ± 3.2[b]	
G100 + DXR	7.2 ± 2.1[c]	52.8	0.5 ± 0.1[a]	7.9 ± 1.5[c]	55.9
G200 + DXR	2.2 ± 1.2[a]	95.7	0.4 ± 0.1[a]	4.4 ± 0.5[d]	80.0
G400 + DXR	2.0 ± 1.4[a]	97.2	0.4 ± 0.2[a]	4.2 ± 2.2[d,e]	81.6

MN: micronuclei; RET: reticulocytes; PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes; G100, G200, G400: guajiru fruit at 100, 200 or 400 mg/kg b.w./day; DXR: doxorubicin (15 mg/kg b.w. – i.p.). Data are mean ± SD. Different letters indicate significant differences between groups ($p < 0.05$, ANOVA-Tukey). $n = 6$.

No statistical difference was observed between control and experimental groups for the PCE/NCE ratio. The sensitivity of this *in vivo* experiment was demonstrated by the increased MN frequency found in DXR treatment group compared to the control group in both peripheral blood and bone marrow cells. All three guajiru fruit tested doses exerted protective effects regarding the mutagenicity of DXR, with comparable reduction percentages in both cells. In peripheral blood cells, the micronuclei frequencies observed for 200 and 400 mg/kg b.w./day guajiru fruit were statistically the same as the control group, establishing substantial antimutagenic effect of this fruit.

2.4.4 Oxidative burst of neutrophils

The oxidative burst of neutrophils from peripheral blood was performed to assess the antioxidant activity of guajiru fruit in these cells. The results are shown in Figure 2.3.

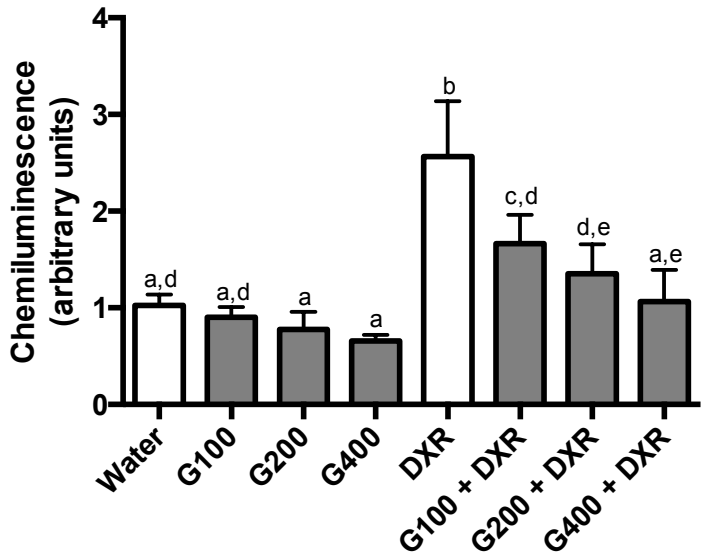

Figure 2.3 – Chemiluminescence response of guajiru fruit in peripheral blood neutrophils. Data are mean ± SD. G100, G200, G400: guajiru fruit at 100, 200 or 400 mg/kg b.w./day; DXR: doxorubicin (15 mg/kg b.w. – i.p.). Different letters indicate significant differences between groups ($p < 0.05$, ANOVA-Tukey). n=6.

DXR increased the chemiluminescence response of neutrophils compared to the control group. Guajiru fruit did not induce oxidative burst in neutrophils but instead reduced the DXR-induced response from this cell at all three tested doses (100, 200 and 400 mg/kg b.w./day).

2.4.5 Correlation between the oxidative burst of neutrophils and DNA damage biomarkers

Taken together, the DNA damage biomarkers evaluated by comet assay and the antimutagenicity activity assessed by micronucleus test are related to the oxidative burst decrease. Correlation analysis showed a high correlation between the oxidative burst of neutrophils and comet assay Tail Moment ($r^2 = 0.750$), comet assay Tail Intensity ($r^2 = 0.805$), bone marrow micronucleus test ($r^2 = 0.922$) and peripheral blood micronucleus test ($r^2 = 0.891$).

2.5 Discussion

This is the first apparent report on the composition of carotenoids and phenolic compounds present in guajiru fruits. The most abundant carotenoid in guajiru fruit, all-*trans*-lutein, was previously shown to modulate the expression of antioxidant genes and decrease DNA damage in mice (SERPELONI et al., 2014). Diets high in β-carotene, another carotenoid found in guajiru fruit, are associated with reduced risk of type 2 diabetes in healthy men and women (SLUIJS et al., 2015). The content of anthocyanins (104 mg cyanidin 3-glucoside equivalents /100 g fresh weight) detected in this study is similar to that previously reported for the same fruit (DE BRITO et al., 2007). These compounds form a complex with the DNA strand, which protects the DNA structure from damage (SARMA; SHARMA, 1999). Ellagic acid was the major phenolic compound found in guajiru fruit. Previous studies have shown that this molecule improved the antioxidant defense system in T cells against lymphoma (MISHRA; VINAYAK, 2014) and protected cardiomyocytes against DXR-induced toxicity (LIN; YIN, 2013).

In addition to assessing the phytochemical composition of guajiru fruits, determination of the concentration of certain inorganic chemical elements in these fruits is also important (DA SILVA SANTOS; DE ALMEIDA TEIXEIRA; BARBOSA, 2014). There are no apparent data in the literature concerning the inorganic composition of this fruit. Several “essential trace elements” have an important role in immunological, endocrinological, and antioxidant reactions in the body and their deficiency may lead to diseases (HOUSTON, 1994).

The chemical element composition indicates guajiru fruit have as much magnesium as avocados (290 μg/g fresh weight) or bananas (270 μg/g fresh weight), as well as three- to sevenfold more selenium than other selenium-rich fruits, such as kiwi fruit (0.031 μg/g fresh weight), grapefruit (0.014 μg/g fresh weight), and tamarinds (0.013 μg/g fresh fruit) (USDA, 2016). The roles of these elements on health were previously studied: magnesium is a micronutrient involved in more than 600 enzymatic reactions, and its supplementation has been recommended for treating migraines, depression, and epilepsy (BAAIJ; HOENDEROP; BINDELS, 2014). Selenium plays important roles in oxidative stress and antioxidant capacity in humans (GAĆ et al., 2015).

The phytochemical composition of fruits and vegetables is often responsible for their antigenotoxic and antimutagenic effects (NUNES et al., 2013). Besides guajiru fruit, other delphinidin- and petunidin-rich fruits or extracts possess *in vivo* and *in vitro* antigenotoxic activities (HABERMEYER et al., 2005). Both major phenolic compound and carotenoid found in guajiru fruit exert antigenotoxic and/or antimutagenic actions, as described for ellagic acid by Rehman et al. (2012) and for lutein by Serpeloni et al. (2010).

In this investigation, anthocyanins, carotenoids, phenolic compounds, and minerals from guajiru fruit are probably responsible for decreasing both Tail Moment and Tail Intensity comet assay parameters. Since the DNA structure of the guajiru-treated animals was more intact due to the protective effect of this fruit's compounds on the genome, this resulted in a decrease in both bone marrow and peripheral blood micronucleus frequencies.

The oxidative burst of neutrophils showed that guajiru fruit decreased the ROS production induced by DXR in blood neutrophils after PMA stimulation. It is possible that the phenolic compounds (including anthocyanins) and carotenoids present in this fruit may act either by deactivating the NADPH oxidase complex leading to a decrease in superoxide ion generation by PMA or by increasing the scavenging of the ROS generated by neutrophils (CIZ et al., 2012). Other anthocyanin-rich fruits were also shown to act as oxidative stress inhibitors, such as blueberries (BRAGA et al., 2013) and açaí (DA SILVA SANTOS et al., 2014).

Correlation analyses provide stronger evidence regarding the chemoprevention mechanism underlying guajiru fruit-mediated effects. The high correlation coefficients obtained allow us to predict that inhibition of the NADPH oxidase complex or enhanced ROS scavenging rate by guajiru led to a significant fall in both comet assay parameters and reduced frequencies of chromosal damage assessed in bone marrow and peripheral blood erythrocytes.

Chapter 3

3. Antigenotoxic and anti-inflammatory effects of guajiru fruit (*Chrysobalanus icaco* L.) against doxorubicin-induced damage *in vivo*

3.1 Abstract

DNA damage and inflammation are promising targets to be evaluated in disease prevention and health promotion studies. Since these pathways have shown to be modulated by dietary compounds, investigating the molecular effects of food provides useful information regarding disease prevention and protection against chemical agents. This study aimed at investigating the tissue-specific activities and the protective effects of guajiru fruit (*Chrysobalanus icaco* L.) against doxorubicin (DXR)-induced damage. Wistar rats were treated with guajiru fruit (100, 200 or 400 mg/kg/day) for 14 days, associated or not with a single intraperitoneal injection of DXR (15 mg/kg b.w., 24 hours before euthanasia). Tissue-targeted comet assay and the oxidative stress parameters thiobarbituric acid reactive substances (TBARS), oxidized/reduced glutathione ratio (GSH/GSSG) and catalase (CAT) activity were investigated in liver, kidney, and heart tissues. The gene expressions of DNA damage/repair targets (*Gadd45a*, *Parp1*, *Xrcc2*) and pro-inflammatory markers (*Il-1β*, *Il-6*, *Nf-κb*, *Tnf-α*) were performed by real-time quantitative PCR. Guajiru fruit decreased DNA damage in liver, kidney and heart by comet assay. This fruit also decreased the DXR-induced expression of *Gadd45a*, *Il-1β,* and *Tnf-α* in tissues. The findings from this investigation demonstrated that phytochemicals and chemical elements in guajiru fruit possess antigenotoxic and anti-inflammatory effects against DXR-induced damage and encourage other *in vivo* and clinical studies with this underutilized fruit.

3.2 Introduction

Nutrigenomics focuses on the effects of dietary components on genomic stability, RNA and protein alterations, and metabolic changes (BERNA et al., 2014). Several studies have been performed aiming at investigating the effects of compounds obtained from the diet and their roles on several molecular mechanisms in the organism: recently, vitamin B6 deficiency was described by Almeida et al.(2016) for affecting the expression of genes related to GABA, glutamate, and serotonin metabolisms in a multi-generational study; phytochemicals in mango and pomegranate fruits exerted anti-inflammatory activity by downregulating the IGF-1R-AKT/mTOR axis and the mTOR downstream pathway, respectively (KIM et al., 2016).

DNA damage and inflammation are critical processes in health promotion since they 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.

Although the chemopreventive effect of guajiru fruit (*Chrysobalanus icaco* L.) have already been reported (VENANCIO et al., 2016), tissue-specific activities remain unknown. Our hypothesis is that, by possessing significant concentrations of phytochemicals (anthocyanins, carotenoids, and phenolic compounds) and chemical elements (such as magnesium and selenium), guajiru fruit could modulate DNA damage and inflammation pathways. This study evaluated the protective effect of this fruit in rats against doxorubicin (DXR)-induced damage by tissue-targeted comet assay, oxidative stress biomarkers and gene expression by real-time quantitative PCR (RT-qPCR).

3.3. Material and Methods

3.3.1 Chemicals

Doxorubicin hydrochloride (DXR, CAS 25316-40-9) was obtained from Laboratório Bérgamo (Taboão da Serra, São Paulo, Brasil). Normal and low melting point agarose (CAS 9012-36-6) were purchased from Invitrogen (Carlsbad, CA, USA). Triton X-100 (CAS 9002-93-1), Tris (CAS 77-86-1), glutathione reductase (GR, CAS 9001-48-3), nicotinamide adenine dinucleotide phosphate (NADPH, CAS 100929-71-3), 5,5-dithiobis-2-nitrobenzoic acid (DTNB, CAS 69-78-3), and 2-vinylpiridine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethylsulfoxide (DMSO, CAS 67-68-5) and hydrogen peroxide (CAS 7722-84-1) were purchased from Merck Chemicals (Rio de Janeiro, RJ, Brazil). GelRed™ was obtained from Biotium (Hayward, CA, USA). All other reagents had the highest possible purity.

3.3.2 Guajiru fruit and dose selection

Ripe guajiru fruits were harvested at Praia do Farol (1° 7' 59.98" S, 48° 27' 33.98" W), Belém, Pará, Brazil. In the laboratory, the seeds were removed, and the fruits (peel + pulp) were immediately frozen in liquid nitrogen. Fruits were then lyophilized for seven days at -60 ºC and 50 µmHg (Liotop L101, Liobras, São Paulo, SP, Brazil). The lyophilized fruit was homogenized in a food processor (Walita, Barueri, SP, Brazil), vacuum-sealed and kept at -36 ºC until use. The phytochemical profile (anthocyanin, carotenoid and phenolic compound identification and quantification) of this batch of guajiru fruits was described previously (VENANCIO et al., 2016).

For administration to the animals, the lyophilized fruit powder was rehydrated in water. The fruit suspension was prepared daily and immediately before use. The same batch of lyophilized guajiru powder was used throughout the study. The maximum dose employed in this study, 400 mg/kg b.w./day, mimics this fruit consumption as a 200-mL juice preparation.

3.3.3 In vivo experimental design

The *in vivo* experimental design used in this investigation was approved by the local Ethics Committee for Animal Use (approval number 11.1.1517.53.0). Four- to five-week-old Male Wistar rats (*Rattus norvegicus*) weighting 110 ± 10 g from the animal facility of the "Prefeitura do Campus USP de Ribeirão Preto" were divided into eight groups of six animals per group. Animals were kept in proper conditions (22 ± 2 °C, 12-hour light/dark cycle), and had *ad libitum* access to food (Nuvilab, Colombo, PR, Brazil) and fresh water.

Animals were randomly assigned to one of the eight experimental groups and treated with guajiru fruit (G) or water daily, by gavage, for 14 days, with 24-hour intervals between treatments. Immediately after the last dose, the animals were intraperitoneally injected with saline (0.9% NaCl) or doxorubicin (DXR, 15 mg/kg b.w.). Twenty-four hours after the intraperitoneal injection, the animals were euthanized. The experimental groups are described as follows: Water group: animals received water by gavage and i.p. saline injection (control group); Guajiru (G) groups: animals received guajiru fruit at one of the three doses (100, 200 or 400 mg/kg b.w.) by gavage and i.p. saline injection; DXR group: animals received water by gavage and i.p. DXR injection; G + DXR groups: animals received guajiru fruit at one of the three doses cited above by gavage and i.p. DXR injection.

DXR was used as DNA damage, oxidative stress and inflammation inductor, obtained as its commercial formulation doxorubicin hydrochloride (Rubidox®). DXR solutions were prepared immediately before use, protected from light.

Following the treatment schedule, the animals were intraperitoneally anesthetized with ketamine and xylazine (100 mg/kg b.w. and 10 mg/kg b.w., respectively). Animals were euthanized by cardiac puncture and liver, kidney, and heart samples were immediately weighed (for organ weight/body weight measurements) and processed for comet assay. Samples from these organs were immediately frozen in liquid nitrogen or immersed in RNAlater® (QIAGEN, Hilden, Germany), and stored at -80 ºC until use.

3.3.4 Alkaline comet assay

The alkaline comet assay was performed according to Singh et al. (1988) and Tice et al. (2000) for *in vivo* studies. Samples of liver, kidney and heart tissues (0.2 g) were manually fragmented with scissors in Hanks balanced saline solution and filtered through a gauze. The cell suspensions were mixed with low melting point agarose (0.5% w/v), added on top of normal melting point (1.5% w/v) pre-coated slides and covered with a coverslip. Slides were kept at 4 ºC for 20 minutes to allow agarose solidification, the coverslips removed and the slides immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10% v/v DMSO, 1% v/v Triton X-100 and 10 mM Tris, pH 10) overnight. The slides were then immersed in electrophoresis buffer (300 mM NaOH and 1mM EDTA, pH>13) for 20 minutes to allow DNA unwinding. Then, the slides were transferred to a horizontal unit and submitted to electrophoresis (0.85 V/cm, 300 mA) for 20 minutes. Finally, the slides were neutralized using an appropriate buffer (0.4 M Tris-HCl, pH 7.5) for 5 minutes and air-dried. The slides were fixed in 100% ethanol and stored at room temperature until analysis.

Immediately before analysis, the slides were stained with GelRed™ (1:10,000 v/v) and scored using a fluorescence microscope (Axiostar, Zeiss, Germany) equipped with a 515–560 nm excitation filter, a 590 nm barrier filter and an integrated digital camera. The Tail Moment (product of the proportion of the tail's intensity and the displacement of the tail's center of mass relative to the center of the head) and Tail Intensity (% DNA in the tail) were evaluated using Comet Assay IV software (Perceptive Instruments, Suffolk, UK) at 200× magnification. One hundred randomly chosen nucleoids were analyzed per tissue, per animal. Cell viability was determined in cell suspensions by trypan blue exclusion method, and all results were above 90% (data not shown).

3.3.5 Determination of thiobarbituric acid reactive substances (TBARS)

The concentrations of TBARS were determined in liver, kidney and heart samples by the method previously described by Draper and Hadley (1990). Samples were homogenized with cold 1.15% (w/v) potassium chloride (KCl). The homogenate

was added to a tube containing thiobarbituric acid (0.8% w/v), acetic acid buffer (pH 3.4) and sodium dodecyl sulfate (8.1% w/v). The tubes were incubated at 95 ºC for 2 hours, and the absorbance of the supernatant was determined at 532 nm. The TBARS concentrations were calculated using a malondialdehyde standard curve (1.5 – 9.0 nmol/mL) obtained from the same method. All determinations were performed in duplicate, and the results were expressed as nmol TBARS/mg protein.

3.3.6 Determination of total protein content

The protein content of the samples was carried out by the protocol previously described by Hartree (1972). In this method, samples are mixed with sodium tartrate, copper sulfate, and Folin-Ciocalteau reagent. The absorbance of the supernatant was determined by spectrophotometry at 650 nm. For the protein content calculations, a curve of bovine serum albumin (0.5 – 0.125 mg/mL) was analyzed by the same method. All experiments were performed in duplicate.

3.3.7 Reduced (GSH) and oxidized (GSSG) glutathione

The concentrations of GSH and GSSG were performed by the method described by Rahman, Kode and Biswas (2006). The level of total glutathione (GSH + GSSG) was assessed at 412 nm after mixing samples with GR, NADPH, and DTNB. The GSSG content was determined by the same method, after GSH derivatization by 2-vinylpyridine. GSH and GSSG standard curves (26.4 – 0.4125 nM) were performed by the same procedure, and all determinations were assessed in duplicate. The GSH concentration was determined by the difference of total GSH and GSSG. The GSH/GSSG ratio was calculated and compared among groups.

3.3.8 Catalase activity

Catalase activity was spectrophotometrically measured by the procedure described by Beers and Sizer (1952). Liver, kidney, and heart samples were mixed with 50 mM phosphate buffer (pH 7.4) and centrifuged at 15,300 ×g for 12 minutes at 4 ºC. Triton X-100 was added, and the tubes were homogenized. After proper

dilution, catalase activity was measured by decomposition of hydrogen peroxide, followed at 240 nm for 1 minute. The result was expressed by U catalase/mg protein, considering molar extinction coefficient of hydrogen peroxide ($\varepsilon = 4 \times 10^{-2}$ mL/µmol cm). All experiments were performed in duplicate.

3.3.9 Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from 25 mg liver, kidney and heart samples from each animal using QIAzol (QIAGEN, Venlo, Netherlands) and purified by miRNeasy Mini Kit (QIAGEN, Venlo, Netherlands), following the manufacturer's protocol. Both quality and quantity of the obtained RNA samples were assessed using NanoDrop ND-2000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 230, 260 and 280 nm.

Complementary DNA (cDNA) was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol. The expressions of genes involved in DNA damage/repair (growth arrest and DNA-damage-inducible, alpha - *Gadd45α*, poly (ADP-ribose) polymerase 1 - *Parp1*, and X-ray repair complementing defective repair in Chinese hamster cells 2 - *Xrcc2*) were analyzed by real-time quantitative PCR, using specific TaqMan probes for these targets (Rn01425130_g1, Rn00565018_m1, Rn01765703_m1 and Rn00667869_m1, respectively) and TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA). Actin beta (*Actb*) was used as the reference gene. The expressions of the inflammatory markers interleukin 1 beta (*Il-1β*), interleukin 6 (*Il-6*), nuclear factor kappa B (*Nf-κb*) and tumor necrosis factor alpha (*Tnf-α*) were performed by the same methodology, but using primers (Sigma-Aldrich, St. Louis, MO, USA) and Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). *Actb* was again used as the reference gene. The sequences of primers used are described in Table 3.1. The levels of transcripts were calculated relatively to the control group by $2^{-\Delta\Delta Ct}$ method (SCHMITTGEN; LIVAK, 2008).

Table 3.1 – Sequence of the primers used in the expression of inflammatory markers. *Actb* was used as reference gene

Gene	Forward (5' – 3')	Reverse (5' – 3')
Il-1β	TAAGCCAACAAGTGGTATTC	AGGTATAGATTCTTCCCCTTG
Il-6	CAGAGTCATTCAGAGCAATAC	CTTTCAAGATGAGTTGGATGG
Nf-κb	AAAAACGAGCCTAGAGATTG	ACATCCTCTTCCTTGTCTTC
Tnf-α	CTCACACTCAGATCATCTTC	GAGAACCTGGGAGTAGATAAG
Actb	AAGACCTCTATGCCAACAC	TGATCTTCATGGTGCTAGG

3.3.10 Statistical analyses

All results are described as the mean ± standard deviation and were analyzed regarding their normality by Kolmogorov-Smirnov's test. Data were submitted to analysis of variance (ANOVA), and then the means were compared by Tukey's test using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). The p-values < 0.05 were considered statistically different for all analyzed parameters.

3.4 Results

3.4.1 Body weight gain and organ weight/body weight measurements

There were no differences among groups on body weight gain and organ weight/body weight percentages (Table 3.2). In this investigation, liver, kidney and heart relative weights were evaluated.

Table 3.2 – Body weight variation (difference between body weight on the last day of treatment and the body weight on the first experimental day) and percentage of liver weight/body weight, kidney weight/body weight and heart weight/body weight of Wistar rats treated with guajiru fruit for 14 days, associated or not to a single administration of doxorubicin (DXR, 15 mg/kg b.w., i.p.)

Group	Body weight gain (g)	Relative liver weight (%)	Relative kidney weight (%)	Relative heart weight (%)
Water	122.1 ± 25.2	5.0 ± 0.7	1.2 ± 0.2	0.5 ± 0.1
G100	135.5 ± 4.7	4.7 ± 0.5	1.1 ± 0.1	0.4 ± 0.1
G200	131.1 ± 10.9	4.5 ± 0.2	1.1 ± 0.1	0.4 ± 0.1
G400	144.5 ± 10.0	4.8 ± 0.5	1.1 ± 0.2	0.4 ± 0.1
DXR	132.0 ± 9.5	4.6 ± 0.3	1.1 ± 0.1	0.4 ± 0.1
G100 + DXR	135.0 ± 12.9	4.9 ± 0.2	1.2 ± 0.2	0.5 ± 0.1
G200 + DXR	144.3 ± 11.1	5.0 ± 0.5	1.1 ± 0.1	0.5 ± 0.1
G400 + DXR	123.3 ± 16.9	4.8 ± 0.3	1.1 ± 0.1	0.5 ± 0.1

Data are mean ± SD. No statistical difference was observed between groups ($p > 0.05$, ANOVA-Tukey). $n = 6$.

3.4.2 Antigenotoxicity of guajiru fruit in liver, kidney, and heart tissues

The data obtained from liver, kidney and heart comet assays are shown in Table 3.3. A single i.p. administration of 15 mg/kg b.w. DXR induced DNA damage in all analyzed tissues (liver, kidney, and heart) by increasing both %DNA in tail and Tail Moment parameters. Guajiru fruit did not cause DNA damage but decreased the DXR-induced injury in the experimental animals in guajiru + DXR groups.

Table 3.3 – Percentage of DNA damage in tail (%DNA in tail) and Tail Moment of nucleoids from liver, kidney, and heart of Wistar rats treated with guajiru fruit for 14 days, associated or not to a single administration of doxorubicin (DXR, 15 mg/kg b.w., i.p.)

	Liver		Kidney		Heart	
Group	% DNA in tail	*Tail Moment*	% DNA in tail	*Tail Moment*	% DNA in tail	*Tail Moment*
Water	14.6 ± 2.8[a]	9.4 ± 1.3[a]	11.7 ± 1.2[a]	8.3 ± 1.7[a,c]	10.7 ± 2.4[a]	3.8 ± 0.7[a]
G100	17.1 ± 3.5[a]	8.7 ± 0.9[a]	12.3 ± 2.1[a]	5.8 ± 2.2[a]	9.2 ± 1.7[a]	3.1 ± 0.8[a]
G200	13.2 ± 3.3[a]	8.2 ± 2.0[a]	11.7 ± 1.2[a]	5.7 ± 1.9[a]	9.5 ± 1.7[a]	2.6 ± 0.6[a]
G400	12.9 ± 1.0[a]	9.1 ± 0.9[a]	13.5 ± 1.8[a]	9.0 ± 1.4[a,c]	9.7 ± 1.4[a]	3.0 ± 0.5[a]
DXR	27.4 ± 2.2[b]	17.2 ± 2.7[b]	25.0 ± 2.6[b]	27.0 ± 1.8[b]	17.6 ± 2.5[b]	6.2 ± 0.8[b]
G100 + DXR	17.3 ± 2.3[a]	12.3 ± 2.1[a]	15.3 ± 3.2[a]	5.7 ± 2.1[a]	10.5 ± 1.4[a]	3.5 ± 0.7[a]
G200 + DXR	17.0 ± 1.6[a]	10.4 ± 1.8[a]	15.4 ± 1.7[a]	5.9 ± 0.4[a]	11.2 ± 1.6[a]	3.3 ± 0.6[a]
G400 + DXR	16.8 ± 2.1[a]	10.8 ± 2.0[a]	19.0 ± 1.7[c]	10.9 ± 3.0[c]	10.2 ± 1.9[a]	3.5 ± 0.9[a]

Data are mean ± SD. G100, G200 and G400: guajiru fruit at 100, 200 and 400 mg/kg b.w., respectively. Different letters indicate significant differences between groups ($p < 0.05$, ANOVA-Tukey). One hundred nucleoids were analyzed per animal. $n = 6$.

3.4.3 Oxidative stress biomarkers in liver, kidney, and heart tissues

The levels of TBARS, the GSH/GSSG ratio and the catalase activity were measured in liver, kidney and heart samples. The results are shown in Table 3.4. Neither DXR nor guajiru fruit was able to modulate the analyzed oxidative stress biomarkers in this experimental protocol since no statistical differences were observed among groups.

Table 3.4 – Oxidative stress markers: concentration of thiobarbituric acid reactive substances (TBARS), reduced and oxidized glutathione ratio (GSH/GSSG) and catalase activity (CAT) in Wistar rats treated with guajiru fruit for 14 days, associated or not to a single administration of doxorubicin (DXR, 15 mg/kg b.w., i.p.)

Groups	TBARS (nmol/mg)			GSH/GSSG			CAT (U/mg)		
	Liver	Kidney	Heart	Liver	Kidney	Heart	Liver	Kidney	Heart
Water	0.33 ± 0.07	0.59 ± 0.20	0.58 ± 0.15	5.72 ± 1.79	4.67 ± 1.23	6.57 ± 1.28	189.2 ± 53.5	195.1 ± 28.7	49.5 ± 18.2
G100	0.33 ± 0.07	0.49 ± 0.25	0.73 ± 0.48	6.19 ± 2.09	4.35 ± 1.17	6.39 ± 1.43	154.8 ± 25.7	186.9 ± 42.5	47.1 ± 31.2
G200	0.33 ± 0.06	0.58 ± 0.26	0.70 ± 0.23	7.28 ± 1.78	4.92 ± 1.45	6.17 ± 1.69	126.0 ± 50.5	201.3 ± 20.2	42.9 ± 29.3
G400	0.35 ± 0.05	0.39 ± 0.10	0.56 ± 0.15	5.92 ± 1.97	5.21 ± 1.73	5.92 ± 1.62	134.0 ± 49.4	201.7 ± 9.6	37.4 ± 8.1
DXR	0.38 ± 0.09	0.57 ± 0.34	0.77 ± 0.34	6.09 ± 1.85	4.42 ± 1.61	5.69 ± 1.28	111.9 ± 35.1	224.1 ± 70.8	55.8 ± 40.7
G100+DXR	0.40 ± 0.14	0.47 ± 0.42	0.91 ± 0.53	5.48 ± 2.31	5.14 ± 2.09	6.21 ± 1.89	154.1 ± 22.0	198.6 ± 26.6	44.5 ± 13.9
G200+DXR	0.43 ± 0.16	0.34 ± 0.24	0.62 ± 0.29	6.23 ± 0.35	4.31 ± 1.84	6.57 ± 1.92	148.4 ± 33.8	205.2 ± 32.2	43.8 ± 33.0
G400+DXR	0.37 ± 0.15	0.97 ± 0.51	0.55 ± 0.20	5.62 ± 1.21	4.77 ± 1.59	5.91 ± 1.36	119.0 ± 30.6	226.2 ± 30.8	39.8 ± 12.7

Data are mean ± SD. G100, G200 and G400: guajiru fruit at 100, 200 and 400 mg/kg b.w., respectively. No statistical difference was observed between groups ($p > 0.05$, ANOVA-Tukey). $n = 6$.

3.4.4 Expression of genes related to DNA damage and repair

The expression of the genes *Gadd45a*, *Parp1* and *Xrcc2* were performed in liver, kidney and heart samples. The results are shown in Figure 3.1. DXR induced the expression of *Gadd45a* in liver and heart tissues and *Parp1* in liver. Guajiru fruit did not induce any of these biomarkers, but decreased the DXR-induced expression of *Gadd45a* in liver and heart. *Xrcc2* was not modulated by DXR or guajiru fruit.

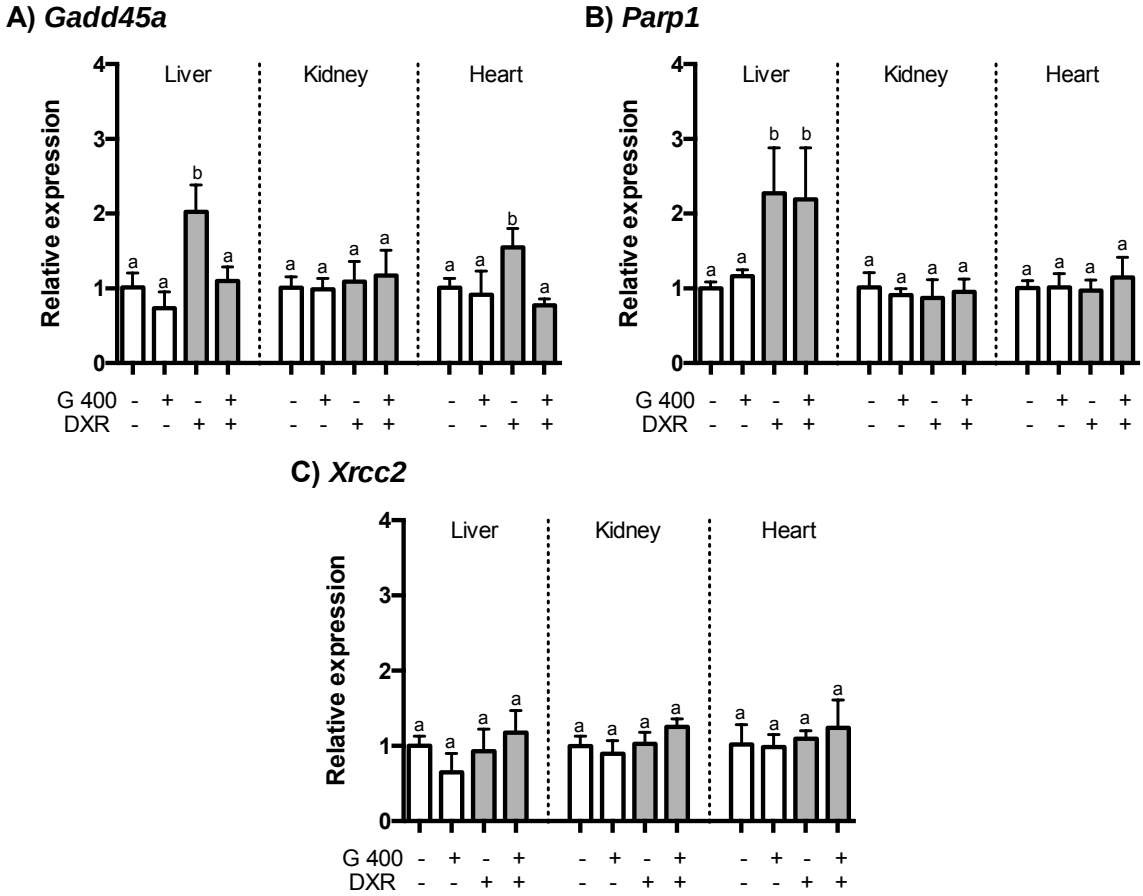

Figure 3.1 – Gene expression of (A) *Gadd45a*, (B) *Parp1* and (C) *Xrcc2* in liver, kidney, and heart of rats treated with doxorubicin (DXR, 15 mg/kg b.w., i.p.), guajiru fruit at 400 mg/kg b.w./day (G 400) or their association. Data are mean ± SD. Different letters within the same tissue indicate significant differences ($p < 0.05$, ANOVA-Tukey). $n = 5$.

3.4.5 Effect of guajiru fruit against DXR-induced inflammation

The protective effect of guajiru fruit against DXR-induced inflammation is shown in Figure 3.2. DXR increased the expression of *Tnf-α* and *Il-1β* in all analyzed tissues

(liver, kidney, and heart). Guajiru fruit at 400 mg/kg b.w./day for 14 days was able to decrease the expressions of these biomarkers compared to the DXR group.

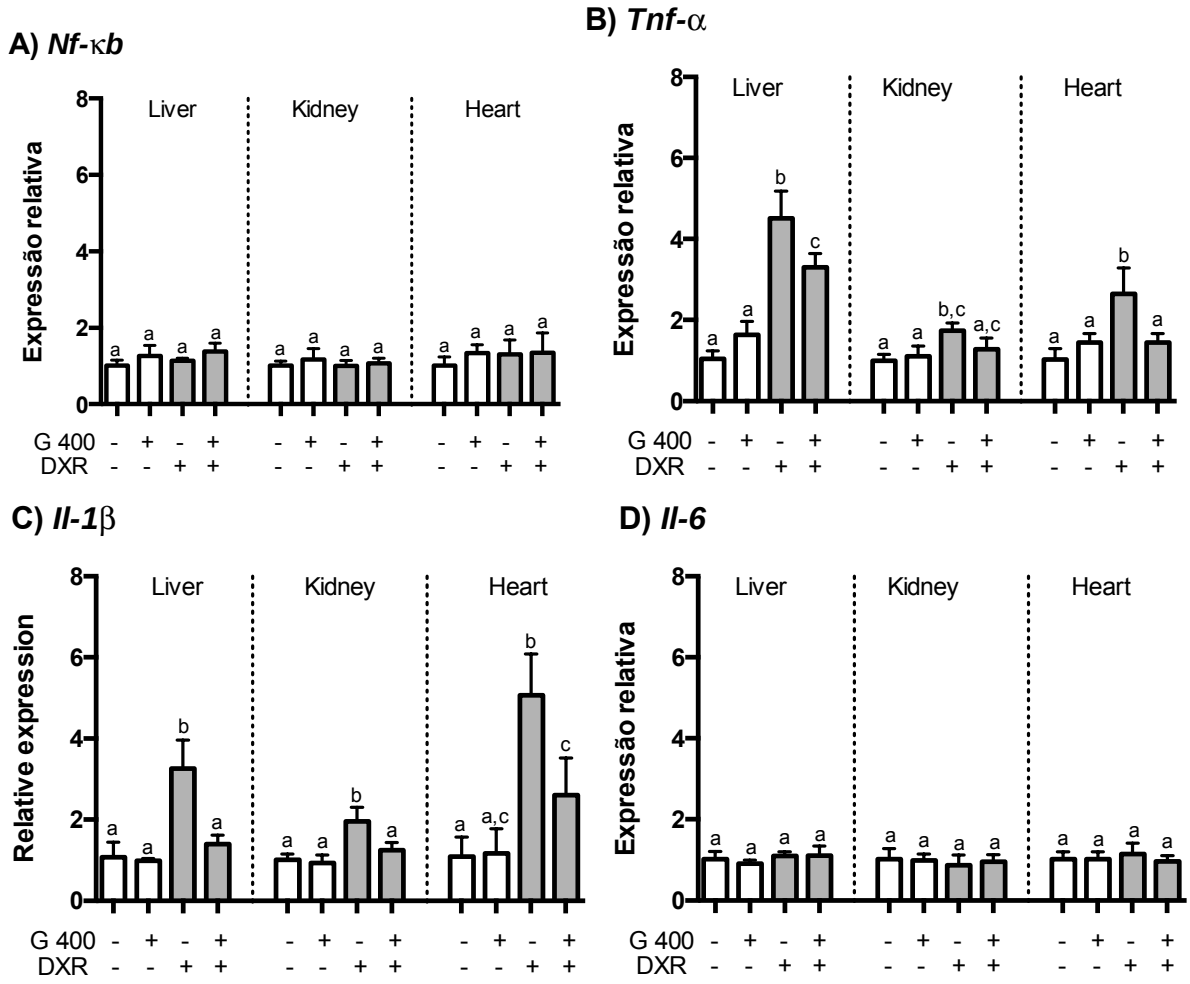

Figure 3.2 – Effect of guajiru fruit at 400 mg/kg b.w./day (G 400) for 14 days on the gene expression of (A) *Nf-κB*, (B) *Tnf-α*, (C) *Il-1β* and (D) *Il-6* in liver, kidney, and heart. Data are mean ± SD. DXR: doxorubicin, 15 mg/kg b.w., i.p. Different letters within the same tissue indicate significant differences ($p < 0.05$, ANOVA-Tukey). *n* = 5.

3.5 Discussion

This investigation provides more information regarding the *in vivo* tissue-specific activities of guajiru fruit. The fruit proved to exert protective effects against DXR-induced genomic instability and inflammation.

The highest dose of guajiru fruit used in this investigation (400 mg/kg b.w./day) is equivalent to 15 ripe fruits (20-29 mm diameter each), achievable by humans by the daily intake of 200 mL of a juice preparation. This was the dose in which the dispersion of the freeze-dried fruit in water was possible, allowing its administration by gavage, using appropriate apparatus. Doses higher than 400 mg/kg/day resulted in high viscosity solutions that were not suitable for administration. Based on this dose, 200 and 100 mg/kg/day were proposed to evaluate dose-dependent effects.

Body weight gain and relative organ weight data suggest absence of toxicity, as described by Wolfsegger et al. (2009). According to this author, the organ weight/body weight ratio can predict toxic effects due to the treatment, as well as help identifying target tissues related to the exposition to that compound. Changes in organ weight can be associated with hyperplasia, e.g., in renal tissue, or to hypertrophy, e.g., in hepatic and cardiac tissues (SELLERS et al., 2007). In this investigation, neither guajiru fruit nor DXR induced changes (macroscopic hypertrophy, for example) in liver, kidney or heart of the experimental animals.

Comet assay is a versatile genotoxicity test and can be performed in multiple tissues (COLLINS, 2004). Its use proved to be suitable for mechanistic investigations and to assess tissue-specific and “side-of-contact” genotoxic activities (HARTMANN et al., 2003). Liver comet assay, for example, can complement bone marrow and peripheral blood micronucleus assay in genotoxicity studies (ROTHFUSS et al., 2011). Accordingly, the present study demonstrates that reduction in the DNA damage induced by DXR in liver measured by the comet assay corroborate our previously findings where guajiru fruit also decreased the mutagenicity of DXR evaluated by the micronucleus assay in peripheral blood and bone marrow cells (VENANCIO et al., 2016).

The phytochemicals in guajiru fruit may be the responsible for the antigenotoxicity (decrease in DXR-induced DNA damage) observed in liver, kidney and heart comet assay. According to the chemical composition of guajiru fruit

(VENANCIO et al., 2016), the major carotenoid and non-anthocyanic phenolic identified in this fruit are all-trans-lutein and ellagic acid derivative, respectively. Notable concentrations of acylated petunidin and delphinidin were also present in this fruit, as well as significant levels of magnesium and selenium. These compounds and chemical elements have been described as possessing *in vivo* antigenotoxicity against different DNA damage inducers: ellagic acid (84 mg/kg b.w.) decreased benzo[a]pyrene-induced Tail Moment of nucleoids from rat peripheral blood cells (GRADECKA-MEESTERS et al., 2011). The anthocyanin delphinidin decreased the frequency of micronuclei in the bone marrow of cyclophosphamide-treated mice (AZEVEDO et al., 2007) and the carotenoid lutein reduced cisplatin-induced crosslink formation in peripheral blood cells of mice (SERPELONI et al., 2010). A study performed by Petrovic et al. (2016) showed magnesium supplementation decreased basal levels and exogenous hydrogen peroxide-induced DNA damage in human peripheral blood lymphocytes. The antigenotoxicity of manganese was previously described by Grotto et al. (2009).

In a recent study, our research group proposed that guajiru fruit chemical compounds reduced the oxidative burst of peripheral blood neutrophils and this ROS scavenging effect led to the decreased DNA damage (in peripheral blood comet assay) and frequency of mutations (in bone marrow and peripheral blood) (VENANCIO et al., 2016).

Although DXR mechanism of action involves ROS generation, this chemical did not modulate the oxidative stress biomarkers in any of the analyzed tissues 24 hours after its intraperitoneal administration. Recent studies indicate that oxidative stress may not be the primary mechanism of toxicity of anthracyclines. Some researchers report that DXR also influences processes such as topoisomerase II inhibition (DAMIANI et al., 2016; NITISS, 2009), mitochondrial biogenesis (JIRKOVSKY et al., 2012) and ceramide generation and accumulation (ANDRIEU-ABADIE et al., 1999).

On genotoxicity (TICE et al., 2000) and mutagenicity assay protocols (HAYASHI et al., 2000), it is indicated that the samples should be obtained 24 hours after the last treatment. Authors show that the clinical manifestations of DXR administration can occur minutes after a single dose of this antitumor drug (HORENSTEIN; VANDER HEIDE; L'ECUYER, 2000). Au and Hsu (1980) demonstrated that DXR treatment requires 5-24 hours to achieve the peak induction of chromosomal

aberrations in bone marrow cells and 3-5 days in testicular tissue. Therefore, the 24-hour treatment used in this study may not be enough for DXR to induce lipid peroxidation, GSH/GSSG imbalance or to decrease catalase activity in the analyzed tissues.

To better understand the molecular mechanism of antigenotoxicity in the analyzed tissues, we selected genes involved in the DNA damage signaling and DNA repair pathways (*Gadd45a*, *Parp1*, and *Xrcc2*) and others related to inflammation (*Nf-κB*, *Tnf-α*, *Il-1β*, and *Il-6*). Speit et al. (2015) mentioned that assessing inflammation biomarkers such as Tnf-α and Il-6 may be useful in combination with tissue comet assay since perturbation in the tissue homeostasis can be detected at low doses (below histopathological examination, for example). Moreover, minimal to moderate inflammation has been correlated to increased *in vivo* DNA migration in comet assay (DOWNS et al., 2012; VASQUEZ, 2012).

While *Gadd45a* has been related to genotoxic activity and in the response to physiological and environmental stress (GUPTA et al., 2005), *Parp1* and *Xrcc2* have roles in single- and double-strand break DNA repairs, respectively (GODON et al., 2008; THACKER; ZDZIENICKA, 2004). Although the expressions of *Parp1* and *Xrcc2* were not different among treatments, the expression of DXR-induced *Gadd45a* was decreased by guajiru fruit in liver and heart. These findings corroborate the antigenotoxicity activity (decrease in primary DNA breaks) obtained by comet assay. However, DXR genotoxicity in kidney tissue was not accompanied by increased *Gadd45a* expression. Johansen (1981) described that over the course of 24 hours after a 12 mg/kg (i.p.) DXR injection, the concentration of this drug in kidney decreases up to three times, suggesting its elimination and metabolization. Doxorubicinol, the DXR metabolite responsible for the antitumor activity of this drug, was described as being 20 times less cytotoxic and genotoxic than its precursor in mouse fibrosarcoma cells (FERRAZZI et al., 1991). Therefore, since comet assay is a very sensitive technique and that damage detected by this method can be accordingly repaired, it is possible that the low DXR concentration in the kidney due to the treatment and its metabolism (24 hours after i.p. administration) led to DNA damage but did not change the expression of DNA signaling genes in this tissue.

This study also proves that guajiru fruit compounds may act in different pathways, such as reducing inflammation. Experimental animals treated with guajiru

+ DXR showed decreased expressions of *Tnf-α* and *Il-1β* compared to the DXR treatment. After intraperitoneal administration, DXR induces apoptosis-associated inflammation and apoptotic cells can lead to Tnf-α production (KACZMAREK et al., 2013; KRYSKO et al., 2011). According to Sauter et al. (2011), Il-1β may play a role in the symptoms associated with antracyclin treatment (fatigue, lethargy, sleep disturbance, and pain) and the mechanism occurs due to the activation of NLRP3 inflammasome. Zhu et al. (2010) demonstrated that Il-1β levels were increased in DXR-treated mice. As for *Gadd45a* expression, the induction of *Tnf-α* and *Il-1β* by DXR were significantly lower in kidney than in liver and heart, probably due to the metabolism of this drug.

Chapter 4

4. Guajiru (*Chrysobalanus icaco* L.) anthocyanins exert anti-inflammatory activity in human colon cancer and non-malignant colon cells

4.1 Abstract

Guajiru (*Chrysobalanus icaco* L.) is an anthocyanin-rich fruit found in tropical areas around the globe. Guajiru fruit phytochemicals are associated with beneficial effects on health, including reduction of inflammation and oxidative stress. Due to its functional properties, the consumption of this fruit may be beneficial in the promotion of human health and reducing the risk for chronic disease. The objective of this study was to assess the anti-inflammatory and anti-proliferative activities of anthocyanins extracted from guajiru fruit at 1.0 to 20.0 mg/L gallic acid equivalents (GAE) in CCD-18Co non-malignant colonic fibroblasts and HT-29 colorectal adenocarcinoma cells. Tumor necrosis factor alpha (TNF-α, 10 ng/mL) was used to induce inflammation in CCD-18Co cells. Guajiru fruit anthocyanins were identified and quantified using high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS^n). The chemical analysis of guajiru fruit extract identified delphinidin, cyanidin, petunidin and peonidin derivatives as major components. Cell proliferation was suppressed in HT-29 cells starting at 10.0 mg/L GAE and this was accompanied by increased intracellular ROS production as well as decreased *TNF-α*, *IL-1β*, *IL-6*, and *NF-κB* expressions at 20.0 mg/L GAE. Within the same concentration range, there was no cytotoxic effect of guajiru fruit anthocyanins in CCD-18Co cells, and TNF-α-induced intracellular ROS production was decreased by 17.3%. IL-1β, IL-6 and TNF-α protein expressions were also reduced in TNF-α-treated CCD-18Co cells by guajiru fruit anthocyanins at 20.0 mg/L GAE. These results suggest that guajiru fruit anthocyanins possess cancer-cytotoxic and anti-inflammatory activities in both inflamed colon and colon cancer cells.

4.2 Introduction

Intestinal bowel disease (IBD) presents a major risk factor for colon cancer (BARRAL et al., 2016). Studies indicate that IBD affects 1.5 million individuals in the US, 2.2 million people in Europe, and many more in other countries (COSNES et al., 2011). The American Cancer Society estimates 134,490 new cases of colorectal cancer, responsible for 49,190 deaths in the U.S. in 2016 (SIEGEL; MILLER; JEMAL, 2016) and in Brazil, Instituto Nacional do Cancer estimates 34.280 new cases of this disease (INCA, 2016) and registered 15.415 deaths in 2013 (INCA, 2013). Although IBD and cancer have different pathogenesis, both inflammation and carcinogenesis are partially attributed to the generation of reactive oxygen species (ROS) (WARIS; AHSAN, 2006; WINROW et al., 1993). ROS-induced DNA damage and its consequences in cancer initiation and progression has been widely discussed (COLIN et al., 2014; COOKE et al., 2003). Additionally, the upregulation of NF-κB and other inflammation biomarkers by ROS (such as hydrogen peroxide) has been identified as an initiating contributor in carcinogenesis (WINROW et al., 1993).

Several classes of non-nutrient phytochemicals, including phenolic acids, and flavonoids such as anthocyanins, have been described as anti-inflammatory agents that can modulate the expression of cytokines and modulate oxidative stress (HUR et al., 2012; MILEO; MICCADEI, 2016). Therefore, there is a need to identify anti-inflammatory compounds that could be beneficial in IBD and cancer prevention, alone or in combination with current pharmacological treatments (MILEO; MICCADEI, 2016; SOMANI et al., 2015).

Anthocyanins are natural pigments responsible for the blue, red or purple color of fruits and vegetables. Anthocyanins from different sources proved to exert anti-inflammatory activity by decreasing several biomarkers (IL-6, TNF-α and IL-1β) (LEE et al., 2014; PAIXAO; DINIS; ALMEIDA, 2012; POULOSE et al., 2012). These compounds were also described regarding their selective cytotoxicity to cancer cell lines (MALIK et al., 2003; ZHAO et al., 2004).

Guajiru (*Chrysobalanus icaco* L.), an anthocyanin-rich fruit (DE BRITO et al., 2007), is native to coastal regions around the globe, including the Brazilian Amazon forest. Bioactive compounds in this plant's leaf extracts were previously described as hypoglycemic (BARBOSA-FILHO et al., 2005), antiangiogenic (ALVES DE PAULO et

al., 2000), and cytotoxic against K562 chronic myeloid leukemia cells (FERNANDES et al., 2003). Guajiru trees are grown around the world, but the fruits are still underutilized. Any beneficial effects of these fruits still lack investigation, and there is no information about the anti-inflammatory activities of anthocyanins from this fruit in cancer or inflamed cells.

Considering the previously reported activities of anthocyanins on inflammation and carcinogenesis (SODAGARI et al., 2015; WALLACE; SLAVIN; FRANKENFELD, 2016), it is possible that polyphenolic compounds in this understudied fruit could be beneficial in disease prevention. This study was designed to investigate an anthocyanin-rich extract from guajiru fruit on cell viability, ROS generation, and pro-inflammatory biomarkers in inflamed CCD-18Co non-malignant and HT-29 colon cancer cells.

4.3 Material and Methods

4.3.1 Chemicals and reagents

Folin-Ciocalteau reagent and 2'7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Fischer Scientific (Pittsburgh, PA, USA). Tumor necrosis factor alpha (TNF-α), resazurin solution, and cyanidin-3-glucoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bradford reagent, RIPA buffer, and proteinase inhibitor cocktail were purchased from Bio-Rad (Hercules, CA, USA). Primers for real-time quantitative PCR (RT-qPCR) were purchased from Integrated DNA Technologies (San Diego, CA, USA) and the beads for protein expression analyses were purchased from EMD Millipore (Billerica, MA, USA). All other reagents had the highest possible purity.

4.3.2 Anthocyanin extraction

Anthocyanins were extracted from freeze-dried guajiru fruit using methanol:water (80:20) and C_{18} Sep-Pack columns (Waters Corporation, Milford, MA, USA). The non-anthocyanin phenolic compounds were eluted with 100% ethyl acetate; a predominantly anthocyanin fraction was collected after elution with 100% methanol containing 0.01% (v/v) HCl (DE AGUIAR CIPRIANO et al., 2015; RODRIGUEZ-SAONA; WROLSTAD, 2001). Methanol was evaporated under vacuum conditions at 35 ºC and re-dissolved in 0.5 M citric acid buffer at pH 3. Total anthocyanins were quantified by differential pH spectrophotometric assay (LEE; DURST; WROLSTAD, 2005) and expressed as mg/L equivalents of cyanidin-3-glucoside. Before use in cell culture, the extract was diluted accordingly based on the total soluble polyphenol content, determined by the Folin-Ciocalteau assay (SINGLETON; ROSSI, 1965) and expressed as mg/L gallic acid equivalents (GAE). The extracts were stored at -20 ºC until use in cell culture.

4.3.3 Chemical analyses

Guajiru fruit anthocyanins were tentatively characterized by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS^n) using a Thermo Finnigan LCQ Deca XP Max ion trap mass spectrometer equipped with an ESI ion source run in positive ionization mode (ThermoFischer, San Jose, CA, USA) (PACHECO-PALENCIA; HAWKEN; TALCOTT, 2007). Compounds were separated on a Phenomenex (Torrace, CA, USA) Synergi 4µ Hydro-RP 80A (2 x 150 mm) in a linear gradient of methanol with 0.1% formic acid into water with 0.1% formic acid over 30 min. Electrospray ionization was conducted with sheath gas (N_2) at 60 units/min and auxiliary gas (N_2) at 5 units/min with spray voltage at 3.3 kV, capillary temperature at 250 °C, capillary voltage at 1.5 V, and tube lens offset at 0 V. Anthocyanin extracts were also manually infused and target ions subjected to MS^n fragmentation to help elucidate compound identity. Quantification was then carried out on a Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA) equipped with a Waters 996 photodiode array detector with separations conducted on a 250 × 4.6 mm Acclaim 120 C_{18} column (Dionex, Sunnyvale, CA) with a C_{18} guard column, as previously described (DE AGUIAR CIPRIANO et al., 2015). Individual anthocyanins were monitored at 520 nm and the concentrations of the compounds were calculated according to peak area against a standard curve of cyanidin-3-glucoside.

4.3.4 Cell cultures

Human non-cancer colon fibroblast CCD-18Co and colorectal adenocarcinoma HT-29 cells were obtained from ATCC (Manassas, VA, USA) and cultured using Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% (v/v) fetal bovine serum, and 1% (v/v) penicillin/streptomycin mix. Cells were maintained in an incubator at 37 ºC and 5% CO_2 atmosphere and used between third and ninth passages. All treatments with guajiru fruit anthocyanins were diluted in complete culture media immediately before use. TNF-α (10 ng/mL) was used to induce ROS generation and inflammation in CCD-18Co cultures.

4.3.5 Cell proliferation assay

Cell proliferation was evaluated according to the methodology described (WANG; MAZZA, 2002) with minor modifications. CCD-18Co and HT-29 cells (1×10^4 cells/well) were seeded onto 96-well plates and incubated for 24 hours to allow cell attachment. The cultures were exposed to guajiru fruit anthocyanins (1.0 – 20.0 mg/L GAE) for 48 hours, and untreated wells were also included. Resazurin solution (20 μL) was added to each well, and the plates were incubated for 3 hours. The fluorescence intensity was analyzed using a microplate reader (BMG Labtech Inc. Durhan, NC, USA) at 560 nm excitation and 590 nm emission. Relative cell viability was quantified with the fluorescence intensity in the control group considered as 100%. Three independent experiments were performed.

4.3.6 Reactive oxygen species generation (ROS) assay

CCD-18Co and HT-29 cells (5×10^3 and 1×10^4, respectively) were seeded in 96-well plates and incubated for 24 hours. The CCD-18Co cells were treated with TNF-α (10 ng/mL) for ROS induction, and all cultures were treated with guajiru fruit anthocyanins (1.0 – 20.0 mg/L GAE) for 24 hours. Cells were then washed twice with PBS, incubated with 10 μM DCFH-DA for 30 minutes at 37 ºC and the fluorescence intensity of each well was measured using a microplate reader (BMG Labtech Inc. Durhan, NC, USA) at 480 nm excitation and 520 nm emission. Relative ROS generation was quantified with the fluorescence intensity in the control group considered as 100% (MERTENS-TALCOTT et al., 2005). Three independent experiments were performed.

4.3.7 Quantitative RT-qPCR

CCD-18Co and HT-29 cells (1×10^5 and 1.5×10^5, respectively) were seeded in 12-well plates and incubated for 24 hours. Cells were treated with guajiru fruit anthocyanins (5.0 – 20.0 mg/L GAE) for 4 hours. In CCD-18Co cells, 10 ng/mL TNF-α was used to induce inflammation. Negative (culture media only) and positive (TNF-α) controls were also performed. Total RNA was isolated and purified using an

RNeasy mini kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's protocol. RNA quality and quantification were assessed with NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was performed with a Reverse Transcription Kit (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. RT-qPCR reactions were performed using SYBR Green PCR MasterMix (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). mRNA expression of nuclear factor kappa B (*NF-κB*), tumor necrosis factor alpha (*TNF-α*), interleukin-1 beta (*IL-1β*) and interleukin 6 (*IL-6*) were analyzed using beta actin (*ACTB*) as a reference gene. Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) (Table 4.1). Each primer was selected based on its homology using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi); their specificity was assessed by dissociation curve analysis. The levels of transcripts were calculated relatively to the control group by $2^{-\Delta\Delta Ct}$ method (SCHMITTGEN; LIVAK, 2008).

Table 4.1 - Human primer sequences used in mRNA analyses

Gene	Forward	Reverse
ACTB	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
NF-κB	GCGAGAGGAGCACAGATACC	CTGATAGCCTGCTCCAGGTC
TNF-α	TCCTTCAGACACCCTCAACC	AGGCCCCAGTTTGAATTCTT
IL-1β	GGGCCTCAAGGAAAAGAATC	TTCTGCTTGAGAGGTGCTGA
IL-6	TACCCCCAGGAGAAGATTCC	TTTTCTGCCAGTGCCTCTTT

4.3.8 Multiplex Bead Assay

CCD-18Co and HT-29 cells (3×10^5 and 2×10^5, respectively) were seeded in 6-well plates and incubated for 24 hours. Cells were treated with guajiru fruit anthocyanins (5.0 – 20.0 mg/L GAE) for 24 hours. In CCD-18Co cells, 10 ng/mL TNF-α was used to induce inflammation (positive control). Cells were lysed with RIPA buffer and proteinase inhibitor cocktail (Bio-Rad, Hercules, CA, USA) for 30 minutes at 4 ºC. Solid debris was removed by centrifugation (10,500 ×g at 4 ºC for 10 minutes), and total protein content was determined using Bradford reagent (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. Protein expression assays were performed using xMAP Multiplex Assay and a human cytokine/chemokine

magnetic bead panel (EMD Millipore, Billerica, MA, USA) in 96-well plates. Protein lysate (25 μg protein/well) was mixed with beads for TNF-α, IL-1β and IL-6 overnight at 4 ºC. Detection antibody was added to each well, and the plates were incubated for 1 hour at room temperature. Streptavidin-Phycoerythrin was added, and the plate was incubated again for 30 minutes at room temperature. After successive plate washing steps, the beads were suspended in sheath fluid and analyzed with a Luminex 200 flow cytometer using Luminex xPONENT software (Luminex Corporation, Austin, TX, USA).

4.3.9 Statistical analyses

All data were analyzed by one-way analysis of variance (ANOVA) with Tukey's posttest using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Data were considered significantly different when $p < 0.05$.

4.4 Results

4.4.1 Anthocyanin quantification and identification

The chemical composition provides information about the molecules in the study treatment and helps discussing its biological effects in the cell lines. The guajiru fruit extract contained 4,586 ± 33 mg/L of total anthocyanins. A chromatogram of the anthocyanins in guajiru shows six predominant anthocyanin peaks that were tentatively identified and semi-quantified based on HPLC-MS fragmentation patterns (Figure 4.1). However, exact confirmation of esterified glycoside moieties or acylated organic acids could not be fully elucidated under these analytical conditions. Compounds identified included delphinidin-3-glucoside (1,162 mg/L), cyanidin 3-glucoside (382 mg/L), petunidin 3-glucoside that coeluted with either delphinidin 3-(6″-acetoyl) galactoside or delphinidin 3-(6″-oxaloyl) arabinoside (1,396 mg/L), peonidin 3-glucoside (345 mg/L), petunidin 3-(6″-acetoyl) galactoside or petunidin 3-(6″-oxaloyl) arabinoside (611 mg/L) and peonidin 3-(6″-acetoyl) glucoside or peonidin 3-(6″-oxaloyl) arabinoside (689 mg/L).

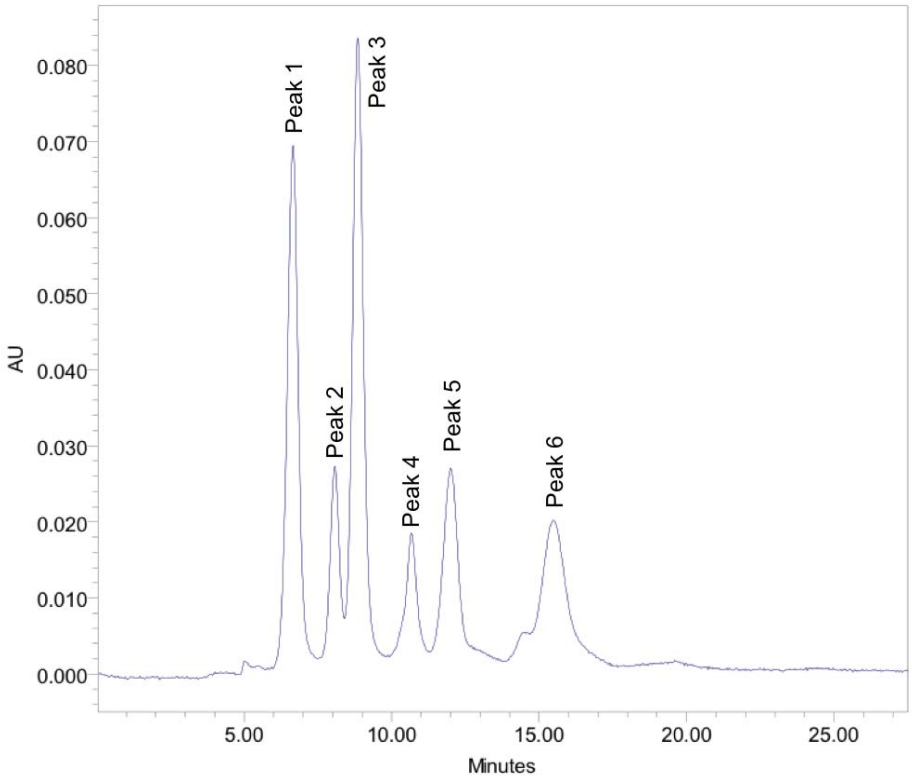

Figure 4.1 – Anthocyanin chromatographic profile of guajiru fruit extract. HPLC-ESI-MS^n analysis allowed the characterization of each peak as follows: delphinidin-3-glucoside (peak 1), cyanidin 3-glucoside (peak 2), petunidin 3-glucoside + delphinidin 3-(6″-acetoyl)galactoside or delphinidin 3-(6″-oxaloyl)arabinoside (peak 3), peonidin 3-glucoside (peak 4), petunidin 3-(6″-acetoyl)galactoside or petunidin 3-(6″-oxaloyl)arabinoside (peak 5) and peonidin 3-(6″-acetoyl)glucoside or peonidin 3-(6″-oxaloyl)arabinoside (peak 6).

4.4.2 Cell proliferation and ROS generation assays

CCD-18Co cell growth (Fig. 4.2A) did not change after 48 hours of treatment with guajiru anthocyanin-rich extract. However, in HT-29 cells, the extract significantly decreased the cell viability at 10.0 and 20.0 mg/L GAE by up to 50% (Fig. 4.2B; $p < 0.05$).

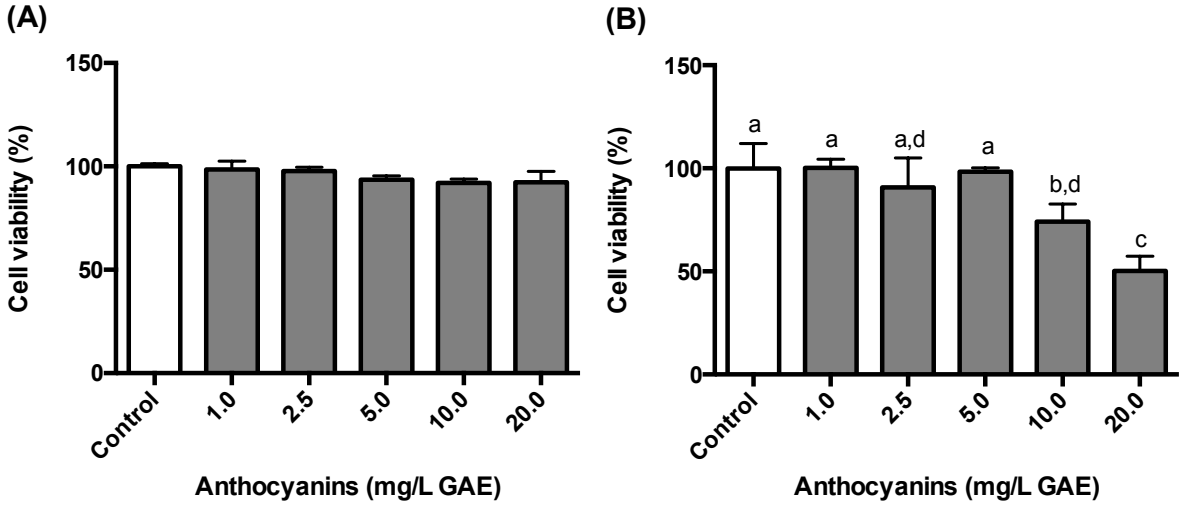

Figure 4.2 - Cell viability of (A) CCD-18Co and (B) HT-29 cells treated with guajiru extract for 48 hours. Data are mean ± SD. Different letters indicate significant differences between groups ($p < 0.05$, ANOVA-Tukey). *n* = 3.

In CCD-18Co cells, TNF-α-induced ROS were decreased when treated with 20.0 mg/L GAE guajiru extract treatment for 24 hours (Fig. 4.3A). In HT-29 cells, all tested guajiru extract concentrations (1.0 – 20.0 mg/L GAE) induced ROS generation compared to the control group (Fig. 4.3B).

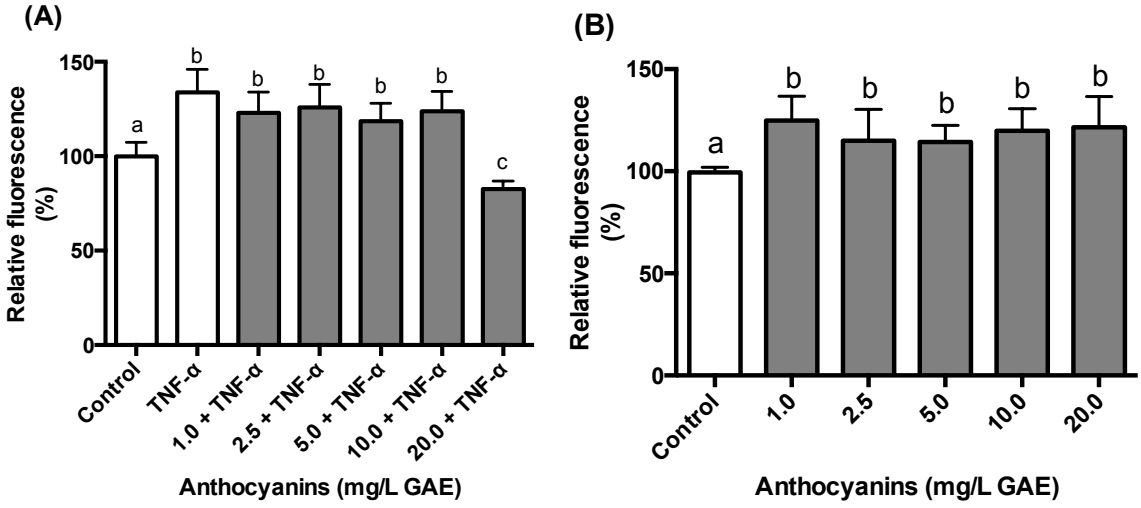

Figure 4.3 - ROS generation in (A) TNF-α-challenged CCD-18Co and (B) HT-29 cells treated with guajiru extract for 24 hours. Data are mean ± SD. Different letters indicate significant differences between groups ($p < 0.05$, ANOVA-Tukey). *n* = 3.

4.4.3 mRNA expression

Pro-inflammatory cytokines are biomarkers used to quantify the severity of inflammation in cells. Anti-inflammatory activity is characterized by decreased expression of these molecules. The mRNA expression of *NF-κB*, *TNF-α*, *IL-1β* and *IL-6* in inflamed CCD-18Co colon cells and HT-29 colon cancer cells were investigated (Fig. 4.4). In CCD-18Co cells, treatments of 10.0 and 20.0 mg/L GAE of guajiru extract decreased the expression of NF-κB compared to TNF-α group. All four target genes were decreased when treated with 20.0 mg/L GAE guajiru extract in HT-29 cells.

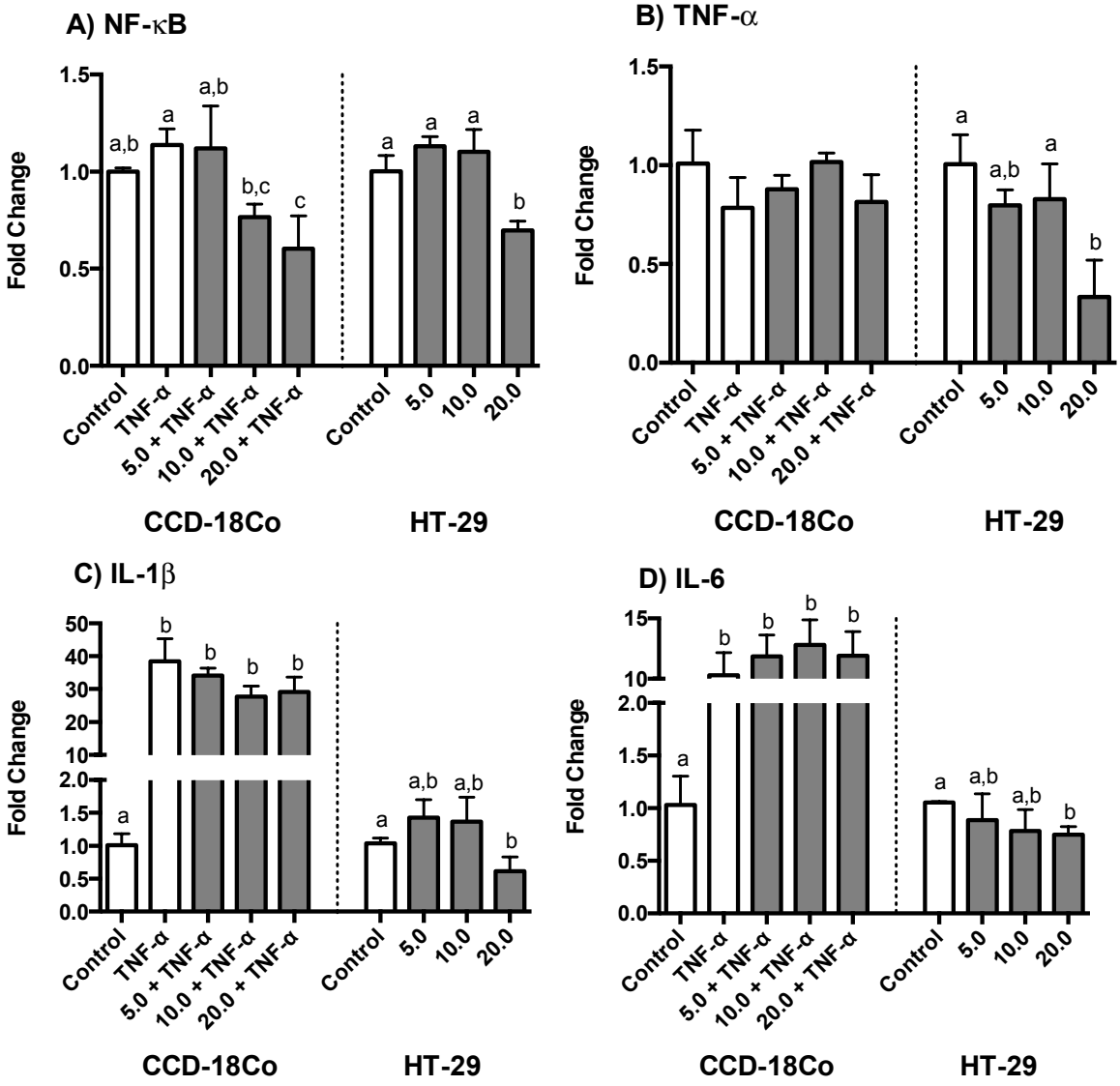

Figure 4.4 - Differential mRNA expression of genes (A) *NF-κB*, (B) *TNF-α*, (C) *IL-1β* and (D) *IL-6* in TNF-α-challenged CCD-18Co and HT-29 cells. Data are mean ± SD. Different letters within the same cell line indicate significant differences between groups ($p < 0.05$, ANOVA-Tukey). $n = 3$.

4.4.4 Protein expression

The protein expressions of TNF-α, IL-1β and IL-6 in both cell lines are shown in Fig. 4.5. TNF-α induced the expression of all three inflammatory biomarkers analyzed in CCD-18Co cells. In this cell line, guajiru extract decreased the expression of TNF-α (5.0 – 20.0 mg/L GAE), IL-1β (10.0 – 20.0 mg/L GAE) and IL-6 (5.0 – 20.0 mg/L GAE) by up to 2.4-fold, 2.5-fold and 2.3-fold respectively. TNF-α protein was also downregulated in HT-29 cells after 20.0 mg/L GAE guajiru anthocyanin treatment.

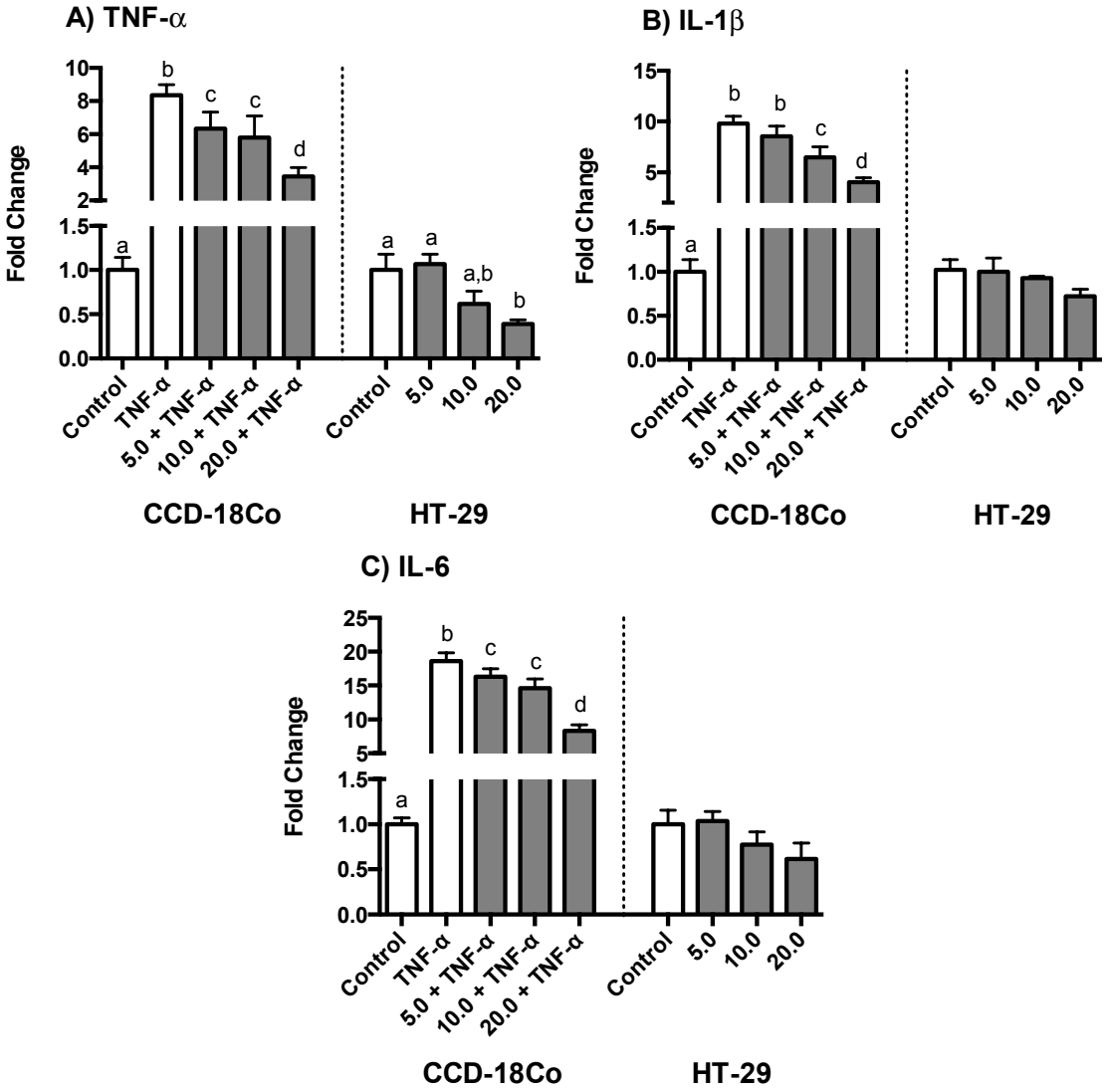

Figure 4.5 - Expression of pro-inflammatory biomarkers (A) TNF-α, (B) IL-1β and (C) IL-6 in TNF-α-challenged CCD-18Co and HT-29 cells. Data are mean ± SD. Different letters within the same cell line indicate significant differences between groups ($p < 0.05$, ANOVA-Tukey). *n* = 3.

4.5 Discussion

Overall, in this *in vitro* investigation, guajiru anthocyanins exhibited selective cytotoxicity to the cancer cell line (HT-29). These compounds also affected the generation of ROS and inflammation in human intestinal cells and therefore have the potential to be used in disease prevention.

In our investigation, guajiru extract showed substantial amounts of acylated anthocyanin glycosides with delphinidin, cyanidin, petunidin, or peonidin base aglycones. This extract was not cytotoxic to the CCD-18Co normal cell line, but did decrease cell viability of HT-29 colon cancer cells after 10 mg/L and 20 mg/L GAE treatment for 48 hours by up to 49.9%. Previously, anthocyanins from *Aronia meloncarpa* primarily composed of cyanidin-3-galactoside exerted cytotoxicity and cell cycle arrest in HT-29 colon cancer, but not in NCM460 normal colon cells (MALIK et al., 2003). Peach and plum varieties have been shown to exert selective cytotoxicity – affecting cell viability of breast cancer cells (MDA-MB-435 and MCF-7), but not MCF-10A normal breast cell lines (VIZZOTTO et al., 2014) and this has previously been demonstrated for many polyphenols (SAK, 2014).

Phytochemicals from açaí (*Euterpene oleracea* M.), another Amazon-native, anthocyanin-rich fruit, are described as ROS scavenging agents in lipopolysaccharide (LPS)-treated CCD-18Co cells at 1.0 – 5.0 mg GAE/L (DIAS et al., 2015). Anthocyanins from red cabbage that contains acylated cyanidin derivatives also decrease LPS- and thrombin-induced ROS in human platelets (SALUK et al., 2015). In this investigation, the decrease in ROS generation due to guajiru anthocyanins may improve the antioxidant status of colon cells, since ROS are highly reactive molecules that can damage lipids, proteins and DNA (BAYNES, 1991), that may lead to carcinogenesis (LINHART; BARTSCH; SEITZ, 2014). In HT-29 cells, guajiru anthocyanin-rich extract induced ROS at all tested concentrations (1.0 – 20.0 mg/L GAE). Increased ROS levels may cause apoptosis and cell death (KUETE et al., 2015; ZUGIC et al., 2016). Anthocyanins induce apoptosis in cancer cell lines due to increased ROS generation in chronic lymphocytic leukemia and teratocarcinoma cancer stem-like cells (ALHOSIN et al., 2015; SHARIF et al., 2013). Therefore, it is possible that the increased ROS generation in HT-29 cells is at least in part responsible for the cytotoxicity observed in this cell line.

The generation of ROS can activate NF-κB through the phosphorylation of IκBα, initiating an inflammatory response (MORGAN; LIU, 2011). In this investigation, TNF-α was used to induce inflammation in CCD-18Co cells. Inflammation was not induced in HT-29 cells since this cell line already expresses high ROS levels and inflammation. The investigated pro-inflammatory biomarkers include genes and proteins associated with colorectal cancer promotion, colitis-associated tumorigenesis, and IBD (BECKER et al., 2005; CHUNG, 2000; NEURATH, 2014; WANG et al., 2009).

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, (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-involved mechanisms (MAJUMDAR; AGGARWAL, 2001; WAHL et al., 1998). In this study, NF-κB mRNA was downregulated at 10.0 – 20.0 mg/L GAE guajiru anthocyanins by up to 39%.

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 proliferation, stimulating tumor growth and the proliferation of premalignant enterocytes (BECKER et al., 2005). While this cytokine plays an important role in colitis and the pathogenic immune response, tissue regeneration process could also be modulated by IL-6 (DANN et al., 2008). All TNF-α, IL-1β and IL-6 mRNA were downregulated after treatment with 20.0 mg/L GAE guajiru extract, and also TNF-α protein levels were decreased by this treatment.

As observed with guajiru in this investigation, other anthocyanin-rich fruits demonstrated to be anti-inflammatory by decreasing the expression of these biomarkers: Açaí downregulated the expression of LPS-induced NF-κB in CCD-18Co cells (DIAS et al., 2015). Anthocyanins from purple carrot (cyanidin derivatives) decrease the mRNA expression of IL-1β and IL-6 in LPS-induced inflammation in a co-culture of intestinal Caco-2 cells and RAW264.7 macrophages (OLEJNIK et al., 2016).

In this study, guajiru anthocyanins decreased pro-inflammatory biomarker expressions in both human colon cancer and non-malignant colon cells. Therefore, these compounds may have potential as disease prevention agents in colon cancer cells, as well as in inflammatory intestinal diseases, such as IBD.

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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