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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

EFEITOS DE COMPOSTOS FENÓLICOS NATURAIS SOBRE A PROLIFERAÇÃO E VIABILIDADE DE CÉLULAS ESTRELADAS HEPÁTICAS ATIVADAS

Tese de doutorado

CRISTIANE CASAGRANDE DENARDIN

Porto Alegre, RS
2013

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de Doutor em Ciências Biológicas-Bioquímica.

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APRESENTAÇÃO

A presente Tese de Doutorado encontra-se organizada em três partes principais:

A **Parte I** representa a **Introdução**, a qual contém o referencial teórico utilizado para a construção das hipóteses investigadas nesta tese. Em seguida, os **Objetivos** (Geral e Específicos) trazem o principal questionamento do trabalho realizado e as questões de pesquisa específicas que nortearam a realização desta Tese.

A **Parte II** está subdividida em três **Capítulos**. O **Capítulo I** traz um manuscrito submetido para publicação; o **Capítulo II** contém um artigo já aceito para publicação; o **Capítulo III** contém um manuscrito em preparação para submissão à publicação; As seções Materiais e Métodos, Discussão e Referências Bibliográficas encontram-se nos próprios manuscritos e artigos e representam na íntegra este estudo.

A **Parte III** abrange **Discussão, Conclusões, Perspectivas e Referências Bibliográficas**. A **Discussão** representa uma interpretação geral dos dados obtidos em todos os trabalhos. As **Conclusões** contêm um resumo dos principais resultados da tese. Em seguida, está apresentada a seção **Perspectivas**, a qual sugere possíveis estudos futuros a partir dos resultados obtidos nesta investigação. A seção **Referências Bibliográficas** apresenta a bibliografia citada nas seções Introdução e Discussão desta tese.

PARTE I

RESUMO

A presença de compostos fenólicos nas dietas ricas em frutas e vegetais tem atraído a atenção de diversos pesquisadores devido aos seus efeitos antioxidantes, os quais atuam na prevenção e combate de diversos tipos de enfermidades. O Brasil é um país com rica diversidade de flora e fauna, o qual apresenta um grande potencial para a descoberta de substâncias que já são utilizadas há muito tempo na medicina popular, mas ainda não possuem comprovação científica. Portanto, o presente trabalho teve como objetivo verificar o potencial antioxidante de extratos de frutas nativas do Brasil através da determinação dos compostos bioativos e capacidade antioxidante *in vitro*, a fim de selecionar uma fruta que apresentasse efeitos biológicos promissores sobre a proliferação, viabilidade celular e mecanismos de resolução do estado de ativação de células estreladas hepáticas ativadas. Os extratos de amora-preta (*Rubus sp.*) Xavante e pitanga roxa (*Eugenia uniflora* L.) apresentaram o maior conteúdo de compostos fenólicos totais, sendo que diversos compostos foram identificados nestas frutas, como derivados de quercetina, quercitrina, isoquercitrina e cianidina-3-glicosídio, entre outros, os quais podem estar contribuindo para a elevada capacidade antioxidante destas frutas. A maior atividade antioxidante no ensaio DPPH foi observada no extrato de pitanga roxa, que também apresentou maior atividade nos ensaios de FRAP e TRAP, sendo, portanto a fruta de escolha para os testes de atividade biológica em cultura de células. O extrato de pitanga roxa reduziu significativamente, e de modo dose-dependente, a viabilidade e proliferação celular das células estreladas hepáticas ativadas (linhagem celular GRX), além de promover alterações no ciclo celular. Também observamos uma redução significativa na massa e potencial de membrana mitocondrial nas células tratadas com 5, 50 e 100 µg/mL do extrato, o que pode estar relacionado com o aumento na morte celular por apoptose e necrose observada neste estudo. Além disso, observamos um aumento significativo na granulosidade citoplasmática das células tratadas com 50 e 100 µg/mL do extrato de pitanga roxa, o que foi provocado pelo aumento no número de autofagossomos e autolisossomos observados por microscopia eletrônica de transmissão. A indução da autofagia e mitofagia foram comprovadas pela presença de organelas vacuolares ácidas coradas por acridine orange, pelo aumento da expressão da proteína relacionada com a autofagia, Atg7, e pelo aumento da colocalização de mitocôndrias e lisossomas nas células tratadas. Este é o primeiro estudo demonstrando o efeito do extrato de pitanga roxa sobre a resolução do estado de ativação das células estreladas hepáticas. Porém, mais estudos devem ser realizados para verificar o efeito desta fruta sobre as outras células hepáticas envolvidas na fibrose hepática, além de avaliar o efeito *in vivo* deste extrato em modelos experimentais de fibrose em animais.

ABSTRACT

The presence of phenolic compounds in the diets rich in fruits and vegetables has attracted the attention of many researchers due to its antioxidant effects, which act to prevent various types of diseases. Brazil is a country with rich diversity of flora and fauna, which has great potential for the discovery of substances that are already used for a long time in folk medicine, but do not yet have scientific proof. Therefore, this study aimed to determine the antioxidant potential of fruit extracts native to Brazil through the determination of bioactive compounds and antioxidant capacity *in vitro*, in order to select a fruit that could promising biological effects on proliferation, cell viability and resolution mechanisms of activation of hepatic stellate cells. The extracts of blackberry (*Rubus sp.*) Xavante and purple pitanga (*Eugenia uniflora* L.) had the highest total phenolic content, and several compounds were identified in these fruits, as quercetin derivatives, quercitrin, isoquercitrin and cyanidin-3-glycoside, among others, which may be contributing to the high antioxidant capacity of these fruits. The highest antioxidant activity in DPPH assay was observed in purple pitanga extract, which also showed the highest antioxidant activity in FRAP and TRAP assays and therefore was the fruit of choice for testing your biological activity in cell culture. The purple pitanga extract reduced significantly and dose-dependent manner, the viability and proliferation of activated hepatic stellate cells (GRX cell line), as well as promoting changes in the cell cycle. We also observed a significant reduction in the mass and mitochondrial membrane potential in cells treated with 5, 50 e 100 μg/mL of the extract, which may be related to the increase in cell death by apoptosis and necrosis in this study. Moreover, we observed a significant increase in cytoplasmic granularity of cells treated with 50 e 100 μg/mL of purple pitanga extract, which was caused by the increase in the number of autophagosomos and autolysosomes observed by transmission electron microscopy. Induction of autophagy and mitophagy were confirmed by the presence of acidic vacuolar organelles stained by acridine orange, increased expression of autophagy-related protein, Atg7, and increased colocalization of mitochondria and lysosomes in cells treated. This is the first study demonstrating the effect of purple pitanga extract on the resolution of activation state of hepatic stellate cells. However, more studies should be conducted to verify the effect of this fruit on other liver cells involved in liver fibrosis, and to evaluate the effect of this extract in experimental models of fibrosis *in vivo*.

LISTA DE ABREVIATURAS

AO – acridine Orange

AVOs – organelas vacuolares ácidas

ATG 7 – Proteína relacionada com a autofagia 7

DPPH – 2,2-difenil-1-picrilidrazil

ECM – matriz extracelular

ET-1 – endotelina 1

FRAP – poder antioxidante de redução do ferro

HCC – carcinoma hepatocelular

HSCs – células estreladas hepáticas

MCP-1 – proteína quimiotática de monócitos tipo 1

MMP-2 – metaloproteinase de matriz 2

MTG – MitoTracker Green

NAFLD – doença hepática gordurosa não-alcóolica

NK – “natural killers”

NO – óxido nítrico

PDGF – fator de crescimento derivado de plaquetas

PPARγ – receptor ativado por proliferadores de peroxissoma

ROS – espécies reativas de oxigênio

SREBP-1 – proteína 1 ligadora do elemento regulado por esteróis

TAR – reatividade antioxidante total

TEM – microscopia eletrônica de transmissão

TGF-β – fator de transformação do crescimento β

TIMP-1 – inibidor de tecido de metaloproteinase 1

TNF-α – fator de necrose tumoral α

TRAP – potencial antioxidante reativo total

α-SMA – α-actina do músculo liso

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Figura 1: Células estreladas hepáticas e sinusóides hepáticos no fígado normal e com dano.

Figura 2: Ativação da célula estrelada hepática.

INTRODUÇÃO

1. Fibrose Hepática

A fibrose hepática é um importante problema de saúde pública em todo o mundo, e possui uma mortalidade de cerca de 1,5 milhões de mortes por ano, geralmente atribuída à cirrose e ao câncer de fígado (Poynard et al., 2010). Segundo a Organização Mundial da Saúde (OMS), o consumo de álcool é considerado como o responsável por cerca de 20 a 50% dos casos de cirrose hepática. Já no Brasil, estudos epidemiológicos sobre fibrose e cirrose hepática são muito escassos.

A fibrose hepática é o acumulo de matriz extracelular (ECM), ou cicatriz, em resposta à lesão hepática aguda ou crônica. A fibrogênese é uma resposta de cicatrização de feridas após uma lesão, e, geralmente conduz à cirrose. A cirrose é o estágio final da fibrose no parênquima hepático, resultando na formação de nódulos que podem levar a alterações na função hepática e fluxo sanguíneo. Tanto a fibrose como a cirrose são consequências de uma resposta de cicatrização provocada por múltiplas formas de lesão, incluindo hepatites, doença metabólica (ou seja, síndrome metabólica), doenças no trato biliar, presença de toxinas (incluindo o álcool), e presença de metais pesados (Boyer et al., 2012; Rockey, 2013).

Fisiologicamente, a deposição de ECM no espaço de Disse leva ao rompimento da microanatomia fenestrada normal dos sinusóides hepáticos em um processo denominado de capilarização destes vasos (Figura 1). A capilarização dos sinusóides prejudica as trocas bidirecionais normais entre o sangue venoso portal e os hepatócitos, prejudicando a chegada de substâncias que seriam degradadas ou metabolizadas nos hepatócitos, e a saída de substâncias produzidas no fígado. Este processo pode gerar complicações principalmente causadas pela hipertensão portal e redução na função sintética hepatocelular, tais como hiperbilirrubinemia, encefalopatia hepática, hipoalbuminemia e deficiência de fatores de coagulação (Boyer et al., 2012; Moreira, 2007).

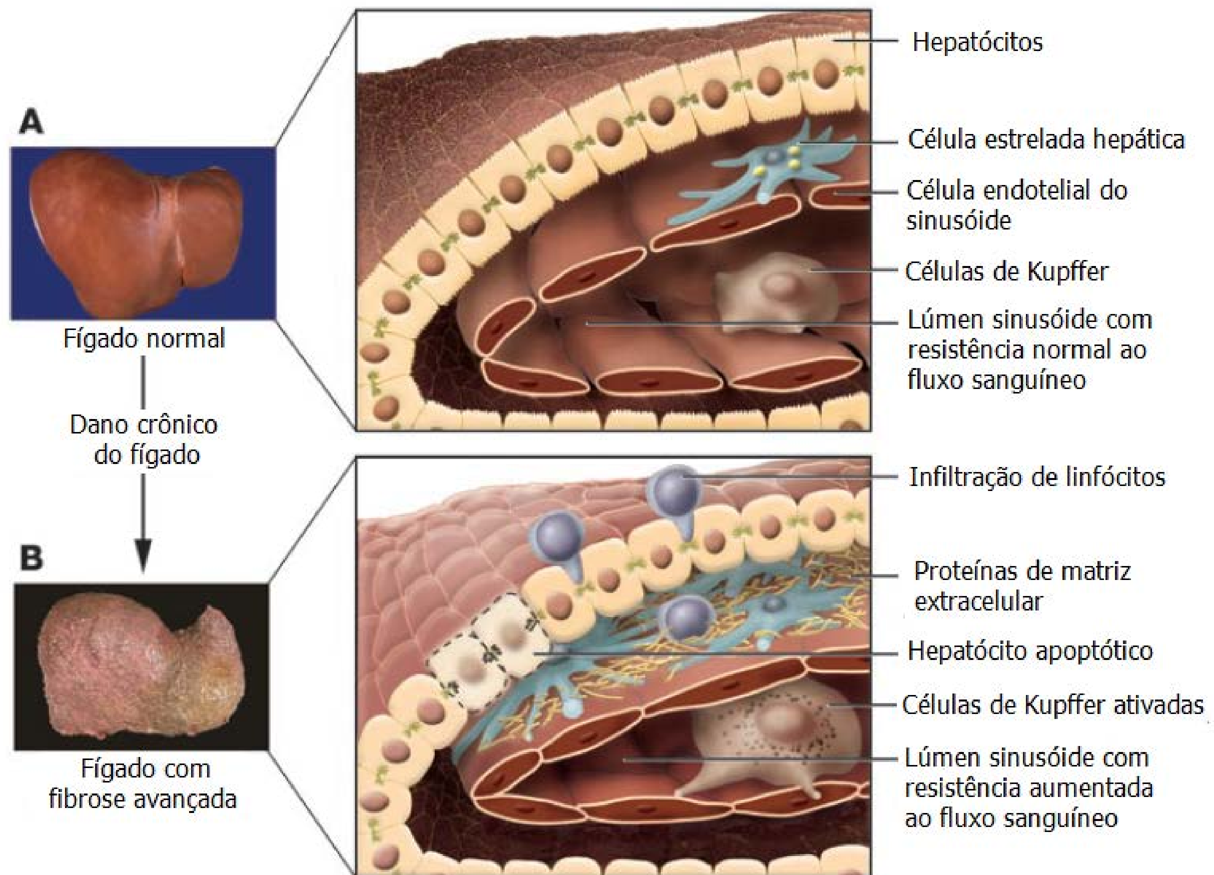

Figura 1. Células estreladas hepáticas e sinusóide hepático no fígado normal e com dano. No painel superior (A) observamos os elementos celulares específicos do fígado normal, incluindo hepatócitos, células endoteliais, células de Kupffer e células estreladas. As células estreladas estão localizadas dentro do espaço subendotelial de Disse (ou seja, entre o endotélio sinusóide e os hepatócitos). Após o dano hepático (B – painel inferior), ocorrem mudanças em vários tipos celulares; por exemplo, ativação das células estreladas e de Kupffer; perda dos microvilos de hepatócitos e perda da característica fenestrada das células endoteliais. Todas estas características contribuem para a perpetuação da ativação celular e dano, assim como para a disfunção do órgão como um todo. Adaptado de Boyer et al. (2012).

As manifestações clínicas da cirrose variam amplamente, desde a ausência de sintomas até a presença de sintomas de insuficiência hepática, e são determinadas tanto pela natureza e gravidade da doença hepática de origem, quanto pelo grau de fibrose instalada. Até 40% dos pacientes com cirrose são assintomáticos e podem continuar assim por longos períodos, mas a deterioração progressiva do órgão conduz à morte ou ao transplante de fígado devido a diversas complicações

que surgem com o passar do tempo, como ascite, hemorragia por varizes ou encefalopatia (Boyer et al., 2012).

O processo fibrogênico é caracterizado pelo aumento de vários componentes da matriz, incluindo colágenos intersticiais, colágenos da membrana basal, proteoglicanos e glicoproteínas de matriz, tais como laminina e fibronectina; e as mudanças específicas na composição da matriz são muito semelhantes em todas as formas de lesão hepática e fibrogênese. Entre as mais importantes proteínas da ECM estão os colágenos (tipo I > III > IV), mas o aumento de outras proteínas também é relevante. É importante enfatizar que o processo de dano é um processo dinâmico que inclui aspectos da síntese e deposição de matriz, bem como degradação. Existem diversos dados na literatura atual indicando que a fibrose tanto experimental quanto clínica, e até mesmo a cirrose, são, em alguns casos, reversíveis (Bataller and Brenner, 2005; Lim and Kim, 2008; Rockey, 2013).

Diversos trabalhos tem demonstrado o papel fundamental das células estreladas hepáticas (HSCs) no desenvolvimento e perpetuação da fibrose hepática (Atzori et al., 2009; Friedman, 2008; Rockey, 2013; Sato et al., 2003). Com isso, a compreensão dos complexos mecanismos fisiopatológicos envolvendo estas células é essencial para o desenvolvimento de terapias antifibróticas. Atualmente, existem evidencias substanciais mostrando que as HSCs são as principais células produtoras de matriz extracelular (ECM) no processo de fibrose hepática. O reconhecimento das HSCs como elemento chave na fibrose levou a um crescente interesse nestas células (principalmente na sua forma ativada) como um indicador da progressão da fibrose hepática, sendo assim um alvo em potencial para intervenções terapêuticas que visam prevenir o desenvolvimento da cirrose (Moreira, 2007).

2. Célula Estrelada Hepática (HSC)

As células estreladas hepáticas (também conhecidas como células Ito, células de armazenamento de vitamina A, lipócitos ou células perisinusoidais) têm recebido grande atenção

como efetores da resposta fibrogênica. No fígado normal, as HSCs compreendem aproximadamente 1,4% do volume total do fígado e estão presentes em uma proporção de aproximadamente 3,6 - 6 células por 100 hepatócitos (ou 1:20). As células estreladas hepáticas são tipicamente localizadas no espaço perisinusoidal de Disse, um espaço localizado entre células endoteliais dos sinusóides e hepatócitos (Figura 1). No seu estado quiescente (fígado normal), as HSCs são o principal sítio de armazenamento de retinóides (metabólitos da vitamina A) o que corresponde a cerca de 40-70% dos retinóides do corpo. A maioria destes retinóides está na forma de retinil ésteres que são armazenados em gotas lipídicas no citoplasma (Boyer et al., 2012; Friedman, 2008; Sato et al., 2003). Com isso, as células estreladas apresentam um papel importante na regulação da homeostase de ácido retinóico. As células estreladas também parecem ter um papel chave na manutenção dos níveis normais de componentes da ECM (principalmente colágenos do tipo IV e VI) nos sinusóides hepáticos e na regulação do fluxo sanguíneo hepático e pressão venosa portal (Boyer et al., 2012; Moreira, 2007).

Em resposta ao dano, as HSCs sofrem "ativação" ou transdiferenciação, passando de uma célula quiescente que armazena vitamina A para uma célula do tipo miofibroblasto, a qual apresenta várias novas características fenotípicas, tais como: aumento na migração e adesão celular, expressão de α-actina do músculo liso (α-SMA), aumento na proliferação, produção de substâncias quimiotáxicas capazes de recrutar células inflamatórias ou outras HSCs, contratibilidade, perda da capacidade normal de armazenar retinóides, aumento no retículo endoplasmático rugoso, mudanças na organização do citoesqueleto e morfologia celular e, mais importante, aquisição de capacidade fibrogênica (Friedman, 2008; Moreira, 2007; Rockey, 2013; Sato et al., 2003).

Este processo de ativação consiste em duas fases distintas: iniciação e perpetuação, seguido pela resolução da fibrose, caso o dano seja atenuado ou removido (Friedman, 2008; Moreira, 2007) (Figura 2). Na fase de iniciação (ou estágio pré-inflamatório), as HSCs sofrem as primeiras

mudanças na expressão gênica e no fenótipo que promovem a diferenciação para uma célula com características de miofibroblasto, o qual se torna responsivo a proliferação e citocinas fibrogênicas.

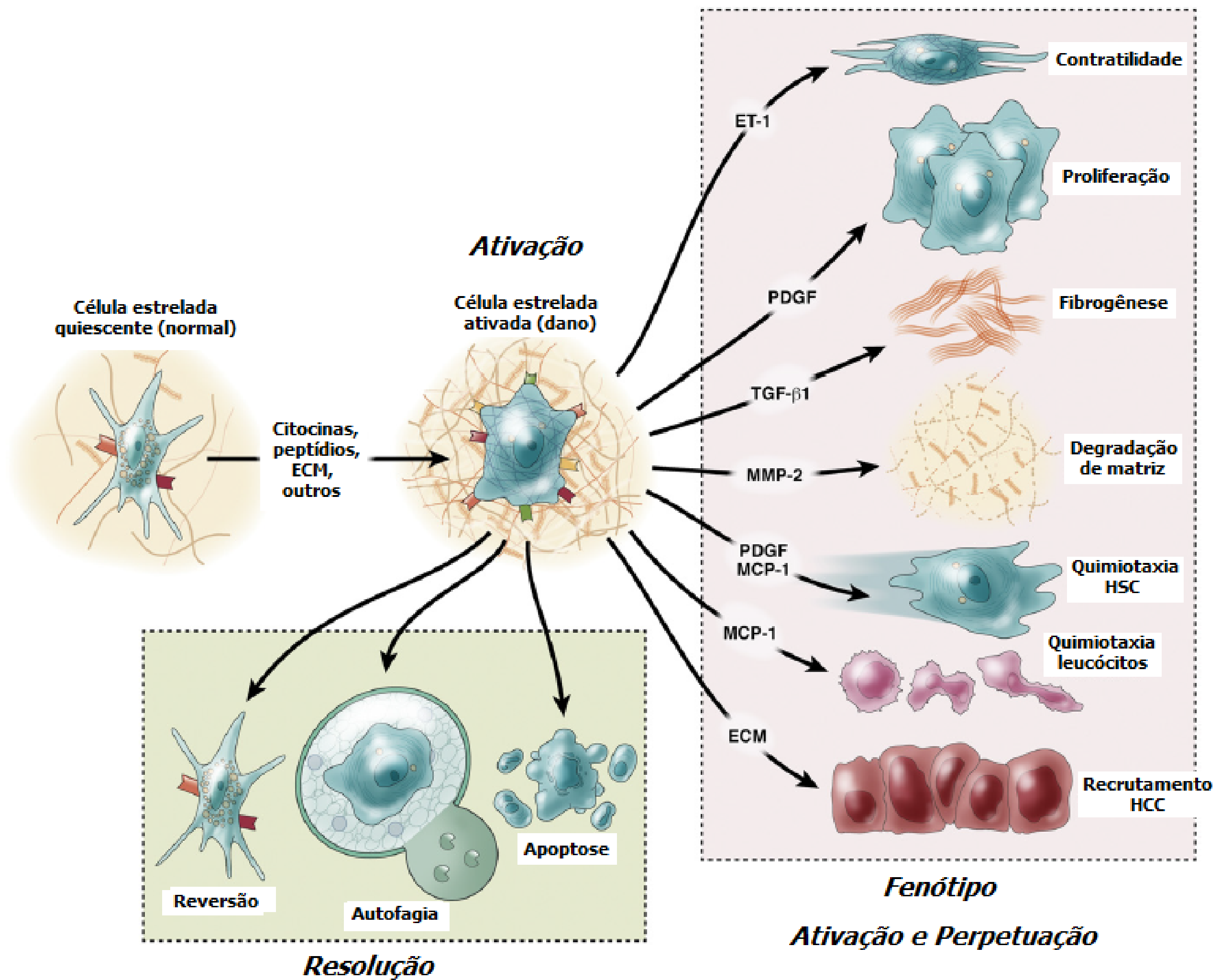

Figura 2. Ativação da célula estrelada. O processo de ativação é complexo, tanto no que diz respeito aos eventos que induzem a ativação quanto aos efeitos da ativação. ET-1 = endotelina 1; PDGF = fator de crescimento derivado de plaquetas; TGF-β1 = fator de transformação do crescimento β1; MMP-2 = metaloproteinase de matriz 2; MCP-1 = proteína quimiotática de monócitos tipo 1; ECM = matriz extracelular; HCC = carcinoma hepatocelular. Adaptado de Rockey (2013).

Embora as principais características da ativação incluam a produção da ECM e a expressão de α-SMA, a ativação também está associada a outros importantes fenótipos celulares, incluindo aumento na proliferação, liberação de citocinas pró-inflamatórias, liberação de enzimas de degradação da matriz e seus inibidores; e recrutamento e ativação de outros tipos celulares, tais como outras HSCs e células inflamatórias (Boyer et al., 2012; Rockey, 2013) (Figura 2).

Uma vez que as HSCs tenham sido induzidas para superexpressar receptores de citocinas, um aumento na fibrogênese, proliferação e outras características do fenótipo ativado irão perpetuar pela continuada liberação de mediadores do tecido cronicamente inflamado e danificado. Este segundo estágio do processo de ativação tem sido denominado de fase de perpetuação. Vários tipos celulares presentes no fígado normal tais como hepatócitos, células de Kupffer, células do endotélio sinusóide, plaquetas e HSCs ativadas, têm sido implicados na produção de citocinas e outros mediadores e podem apresentar um papel importante nesta parte do processo (Friedman, 2008; Moreira, 2007; Rockey, 2013).

O fator de crescimento derivado de plaquetas (PDGF) e o fator de transformação do crescimento beta (TGF-β) são as duas citocinas mais bem caracterizadas responsáveis pela ativação das células estreladas. Vários estudos caracterizaram o PDGF como o principal mediador para o aumento na proliferação e o TGF-β como a citocina mais importante na estimulação da fibrogênese (produção de ECM) nas células estreladas (Boyer et al., 2012; Friedman, 2008; Rockey, 2013). Uma variedade de outros mediadores, contudo, tem sido relacionados com a ativação das HSCs e a fibrogênese, incluindo a proteína quimiotática de monócitos tipo 1 (MCP-1), endotelina 1, angiotensina II e algumas adipocinas como a leptina, entre outros. Além disso, várias moléculas, incluindo o fator de necrose tumoral α (TNF-α), TGF-β, inibidor de tecido de metaloproteinases 1 (TIMP-1), colágeno 1 e integrinas apresentam atividade fibrogênica por causar inibição na apoptose das HSCs, portanto, contribuindo para o aumento no número destas células no local do dano hepático. Espécies reativas de oxigênio produzidas pelas células de Kupffer e hepatócitos

danificados também tem mostrado ter um papel na ativação das HSCs, assim como no recrutamento de células inflamatórias (Friedman, 2008; Moreira, 2007; Rockey, 2013).

A resolução da fibrose refere-se ao processo em que as células estreladas morrem por apoptose, senescência ou autofagia; ou sofrem uma transformação ou reversão para o fenótipo mais quiescente (Figura 2). Embora a reversão para o fenótipo mais quiescente seja observada em células estreladas em cultura, ainda não foi validada *in vivo* (Friedman, 2008; Kisseleva and Brenner, 2011). Este fenótipo quiescente das HSCs está associado com a expressão de genes lipogênicos e com o armazenamento de vitamina A em gotas lipídicas. A depleção do receptor ativado por proliferadores de peroxissoma (PPAR-γ) constitui o evento molecular chave para a ativação das HSCs, e a expressão deste receptor nuclear resulta na reversão fenotípica das HSCs ativadas para quiescentes em cultura de células. Desta forma, o tratamento das HSCs ativadas com um coquetel de diferenciação adipogênica ou super-expressão de SREBP-1c resulta na super-regulação de fatores de transcrição adipogênicos que promovem a reversão morfológica e bioquímica das HSCs ativadas para células quiescentes (Kisseleva and Brenner, 2011). Além disso, diversos estudos utilizando a linhagem celular GRX, que apresenta o fenótipo ativado quando em condições normais de cultura, observaram que o tratamento com o agente adipogênico indometacina (Borojevic et al., 1990), com retinol (Margis and Borojevic, 1989), com capsaicina (Bitencourt et al., 2012) ou com outros carotenóides, como o β-Caroteno (Martucci et al., 2004) ou licopeno (Teodoro et al., 2009) levam a célula GRX a um fenótipo lipocítico, com gotas lipídicas perinucleares características do fenótipo quiescente das HSC.

3. Reversão e tratamento da fibrose hepática

O fígado tem uma capacidade extraordinária de regenerar e restaurar o tecido após danos químicos ou mecânicos. Devido a sua relevância como uma das principais causas de morbidade e mortalidade em todo o mundo, a remoção da causa subjacente da injúria hepática crônica e o

transplante de fígado são as únicas intervenções terapêuticas disponíveis capazes de modificar a história natural da fibrose hepática. A eliminação dos agentes causadores nem sempre é possível, e o transplante de fígado tem várias desvantagens, incluindo a escassez de doadores, altos custos e riscos associados ao procedimento, além de complicações de imunossupressão. Até o momento, nenhum medicamento antifibrótico foi aprovado para uso clínico.

A fibrose hepática tem sido considerada tradicionalmente como um processo irreversível. Porém, diversas evidencias indicam que até a fibrose avançada, de fato, pode ser uma condição reversível. Fisiopatologicamente, a fibrose hepática tem sido considerada um processo dinâmico em que as vias fibrogênicas e fibrolíticas coexistem e interagem. Após a remoção dos agentes causadores da injúria hepática, as HSCs entram em apoptose e liberam mediadores com atividade fibrolítica que iniciam um processo denominado remodelamento (Fallowfield, 2011; Kisseleva and Brenner, 2011; Moreira, 2007).

Atualmente existem vários passos chave no processo de ativação das células estreladas e da fibrogênese que podem ser utilizados como alvos terapêuticos potenciais para uso clínico na prevenção e tratamento da fibrose hepática. Entre os mediadores envolvidos na fibrogênese, o TGF-β e o PDGF apresentam papel central. Consequentemente, vários estudos têm sido publicados mostrando o efeito de inibidores destas substâncias em modelos experimentais de fibrose hepática, sendo que compostos com atividade inibitória direta ou indireta sobre o TGF-β tem reduzido significativamente a fibrose hepática em modelos animais (Fallowfield, 2011; Moreira, 2007; Rockey, 2013; Wu and Zern, 2000). A indução da apoptose nas HSCs também têm sido explorada como uma possível estratégia antifibrótica. E muitos agentes com atividade antioxidante, tais como N-acetilcisteína, resveratrol, quercetina, glutationa e α-tocoferol têm sido avaliados e apresentam atividade antifibrogênica *in vitro*, uma vez que o estresse oxidativo também apresenta um papel na fibrose hepática por atuar na ativação das células estreladas (Moreira, 2007; Rockey, 2013; Wu and Zern, 2000).

Além disso, estudos experimentais têm demonstrado que muitas outras intervenções diferentes são capazes de inibir (geralmente prevenir) a fibrogênese, sendo que o alvo de tais terapias tem sido geralmente a inibição da síntese de colágeno e deposição de matriz; a modulação da ativação de células estreladas; a estimulação da degradação da matriz; ou a estimulação da morte de células estreladas. Algumas destas abordagens pré-clínicas têm sido transferida para os ensaios clínicos em seres humanos (Fallowfield, 2011; Rockey, 2013; Wu and Zern, 2000).

4. HSCs e apoptose

A apoptose ou morte celular programada é um processo homeostático onipresente envolvido em numerosos sistemas biológicos. Sob condições fisiológicas, a apoptose é crítica não somente no "turnover" das células nos tecidos, mas também durante o desenvolvimento normal e senescência. Além disso, sua desregulação tem sido observada tanto como uma causa quanto consequência de diversas patologias, incluindo o câncer, doenças autoimunes e neurodegenerativas. A apoptose é um processo dependente de ATP e altamente organizado induzido por diversos estímulos, sendo caracterizado pela progressiva ativação de vias que conduzem a alterações bioquímicas e morfológicas específicas nas células, sem envolver uma resposta inflamatória. Os estágios iniciais da apoptose são caracterizados pela ativação de caspases iniciadoras (caspases 3 e 7, entre outras), retração das células, perda da assimetria lipídica da membrana plasmática, e condensação da cromatina. A fase seguinte envolve a ativação de outras caspases (por exemplo, caspase 9) e endonucleases, formação de corpos apoptóticos e fragmentação celular. No fígado, a apoptose é predominantemente mediada pela ativação de receptores Fas, que são super-expressados nos hepatócitos danificados (Guicciardi and Gores, 2010; Witek et al., 2009).

A morte celular por apoptose é proeminente em células estreladas, e parece ser um mecanismo importante para a regressão da fibrose. Diversos trabalhos sugerem que o equilíbrio entre a proliferação celular e a apoptose é importante, pois determina a dinâmica da população total

de células estreladas do fígado. Com base nestes dados, a ativação da apoptose de células estreladas poderia ser uma abordagem terapêutica atraente (Friedman, 2008; Kisseleva and Brenner, 2011; Rockey, 2013). Por outro lado, a apoptose de hepatócitos tem sido considerada um mecanismo que contribuiria para a fibrogênese e cirrose. Os corpos apoptóticos são fagocitados pelas células adjacentes, e a fagocitose destes corpos pelas HSCs quiescentes é um dos mecanismos que promovem a sua ativação (Guicciardi and Gores, 2010; Witek et al., 2009).

Vários mecanismos estão implicados com a apoptose das HSCs ativadas: ativação de vias de morte celular mediadas por receptores (receptores Fas ou TNFR-1) e caspases 8 e 3; up-regulação de proteínas pró-apoptóticas (por exemplo, p53, Bax, caspase 9); e redução de genes pró-sobrevivência (por exemplo, Bcl-2). Uma população de células do fígado associadas a “natural killers” (NK) e células γδ T (NKT) também estimulam a apoptose nas HSCs ativadas. Além disso, drogas que induzem a apoptose, como gliotoxina, sulfasalazina, inibidores IKK e anticorpos anti-TIMP, causam regressão da fibrose hepática (Friedman, 2008; Kisseleva and Brenner, 2011).

Atualmente, diversos estudos tem demonstrado o efeito de compostos naturais e ervas sobre a inibição da ativação, redução na proliferação e indução da apoptose das HSCs ativadas. Ding e colaboradores (2011) observaram que a neferina, principal alcalóide das sementes de *Nelumbo nucifera*, uma erva tradicional da medicina chinesa, reduziu significativamente os níveis de TGF-β1 e a produção de colágeno do tipo 1 em cultura de células estreladas hepáticas (HSC-T6). Além disso, foi demonstrado que a neferina induz a apoptose destas células de uma maneira dose dependente, através do aumento da expressão de Bax e caspase 3, e redução na expressão de Bcl-2 e α-SMA. De forma semelhante, estudos avaliando o efeito do ácido rosmarínico sobre a proliferação e apoptose de HSCs ativadas, mostraram que este composto fenólico inibe a proliferação celular e induz a apoptose, tanto *in vitro* como *in vivo* (Li et al., 2010; Zhang et al., 2011).

A fitoterapia ou uso de ervas medicinais tem sido utilizada há séculos na China para tratar doenças hepáticas, porém muito pouco deste conhecimento popular é comprovado cientificamente.

Um estudo avaliando o efeito de 14 ervas medicinais comumente utilizadas para tratar a fibrose hepática verificou que 5 destas ervas possuíam efeito anti-proliferativo e pró-apoptótico em cultura de células (HSC-T6). Além disso, observou-se que as vias apoptóticas ativadas envolviam o receptor Fas e a família Bcl-2 (Chor et al., 2005). Além disso, um extrato de sete tipos de ervas japonesas denominado Sho-saiko-to (TJ-9) também demonstrou uma inibição da ativação das HSCs através de parada no ciclo celular e reduziu a expressão de procolágenos do tipo I e II (Kayano et al., 1998).

Portanto, a administração de compostos que possuem atividade anti-proliferativa e pró-apoptótica poderia ser uma estratégia promissora no tratamento da fibrose hepática. Porém, devemos ter em mente que as HSCs possuem uma localização anatômica muito específica, e que estes efeitos devem possuir uma grande seletividade, ou seja, devem atingir somente as HSCs ativadas sem provocar danos nas células vizinhas. Além disso, como citado anteriormente, a indução da apoptose em hepatócitos promove um aumento no dano hepático e deve ser evitada no quadro da fibrose. Portanto, a seletividade de ação destes compostos deve ser seriamente observada para evitar a indução da fibrose e garantir sua atuação antifibrótica.

5. HSCs e autofagia

A autofagia é um processo de degradação intracelular por via lisossomal, e é considerado o principal mecanismo de degradação de proteínas de vida longa, e a única via reconhecida de degradação de organelas celulares. Durante autofagia, ocorre a formação de uma membrana de isolamento, provavelmente resultante de um compartimento vesicular conhecido como estrutura preautofagossomal; que invagina e sequestra constituintes citoplasmáticos, incluindo retículo endoplasmático, mitocôndrias e ribossomas. As bordas desta membrana se fundem para formar uma estrutura de dupla membrana ou multimembranosa, conhecido como autofagossomo ou vacúolo autofágico. A membrana externa do autofagossoma se funde com o lisossomo iniciando o processo

de digestão no lúmen do compartimento agora denominado autolisossoma. A degradação do material sequestrado gera nucleotídeos, aminoácidos e ácidos graxos livres que são reciclados para a síntese de macromoléculas e geração de ATP (Levine and Yuan, 2005; Rautou et al., 2010).

A maioria dos tecidos tem um nível basal de autofagia que contribui para a homeostase celular através da regulação do turnover de componentes citoplasmáticos. No entanto, a autofagia também pode ser induzida por várias condições e estresses, tais como: fome, agregados de proteínas, infecção por vírus, estresse oxidativo e estresse no retículo endoplasmático. Alem disso, evidências atuais indicam que a autofagia está envolvida em várias condições fisiopatológicas, incluindo o desenvolvimento, diferenciação e remodelamento de tecidos, lesão tecidual, envelhecimento e câncer (Levine and Yuan, 2005; Ni et al., 2012). O fígado é um dos órgãos mais dinâmicos em mamíferos e humanos, e a autofagia apresenta importantes papéis na fisiologia e patologia deste órgão.

No fígado, a autofagia parece exercer predominantemente funções protetoras, como a promoção da função hepática no envelhecimento; proteção contra o carcinoma hepatocelular; proteção em doenças hepáticas devido a deficiência de α1-antitripsina; e proteção contra a formação de corpos de Mallory-Denk no dano hepático induzido por álcool (Ni et al., 2012; Rautou et al., 2010). No entanto, sob algumas condições a autofagia pode também promover o dano hepático; por exemplo, em pacientes com hepatite viral onde os vírus da hepatite B e C utilizam a maquinaria da autofagia em benefício próprio (Rautou et al., 2010).

Recentemente, alguns estudos tem demonstrado que a autofagia parece desempenhar um papel importante na ativação das células estreladas. Camundongos com deleção específica de proteínas relacionadas com a autofagia (Atg 7) nas células estreladas, apresentam uma redução na ativação das HSCs após a lesão hepática, levando à redução da fibrose *in vivo* (Rockey, 2013). Além disso, fígados fibróticos de camundongos tratados com tetracloreto de carbono apresentaram uma expressão aumentada de LC3-II, uma das proteínas envolvidas na elongação de

autofagossomas, e uma medida indireta útil de autofagossomas. Já a utilização de inibidores da autofagia, como bafilomicina A1, 3-metiladenina e hidroxicloroquina reduziram significativamente a ativação de HSCs de células humanas e de ratos *in vitro* (Thoen et al., 2011).

Outro papel controverso da autofagia no fígado é a sua atuação sobre o metabolismo de lipídios hepáticos. Alguns estudos sugerem que a autofagia pode atuar degradando seletivamente as gotas lipídicas num processo denominado lipofagia. Com isso a ativação da autofagia seria benéfica na doença hepática gordurosa não-alcoólica (NAFLD) devido ao aumento da lipofagia e redução no conteúdo de triglicerídeos hepáticos. Por outro lado, a lipofagia poderia promover uma redução das gotas lipídicas das HSCs quiescentes promovendo a ativação destas células (Ni et al., 2012).

Portanto, ainda existem muitas dúvidas e questionamentos no que diz respeito ao papel da autofagia na fibrose hepática, sendo este um campo promissor para novas pesquisas. Além disso, deve-se ressaltar que são praticamente inexistentes as pesquisas avaliando o efeito da autofagia na resolução da fibrose, ou seja, utilizando HSCs já ativadas.

6. HSCs e antioxidantes

Os hepatócitos e as células de Kupffer são uma fonte importante de espécies reativas de oxigênio (ROS) e nitrogênio (RNS) que exercem uma atividade parácrina na estimulação e ativação das HSCs. Além disso, sua atividade é amplificada *in vivo* pela depleção das defesas antioxidantes como tipicamente ocorre nas doenças hepáticas (Urtasun and Nieto, 2007). Durante o processo de inflamação hepática, ROS, incluindo metabólitos intermediários (acetaldeído) e radicais livres; como H_2O_2, $O_2^{\cdot-}$ e óxido nítrico (NO), podem ser derivados da infiltração de neutrófilos, células de Kupffer ativadas e hepatócitos danificados. Diversas evidências demonstram que estes ROS atuam na ativação das HSCs *in vitro* e podem mostrar um importante papel *in vivo*. Sabe-se que a administração de quaisquer agentes que possuem efeitos antioxidantes, como vitamina E, adenosil-

L-metionina (SAMe) e polienilfosfatidilcolina (PPC), protegem o fígado do dano induzido por hepatotoxinas bloqueando assim a progressão da fibrogênese (Wu and Zern, 2000).

Sabe-se que a fibrose hepática é invariavelmente precedida pela inflamação e estresse oxidativo. Portanto, diversos compostos que apresentam capacidade antioxidante como, silimarina, curcumina, resveratrol, catequina, entre outros, são geralmente seguros e apresentam resultados promissores na regressão da fibrose hepática, embora ainda sejam escassos os estudos utilizando seres humanos (Fallowfield, 2011). Estudos utilizando a linhagem celular GRX mostraram que a catequina reduz a proliferação celular por mecanismos que não incluem a apoptose e autofagia, mas sim, devido a parada no ciclo celular e propriedades antinflamatórias (Braganca de Moraes et al., 2012). Além disso, as catequinas do chá verde também apresentaram efeito sobre a fibrose hepática induzida em ratos, uma vez que reduziram o estresse oxidativo e controlaram a expressão de fatores de transcrição envolvidos na ativação das HSCs (Kobayashi et al., 2010).

O resveratrol é um composto fenólico com elevada capacidade antioxidante que apresenta diversos efeitos benéficos em várias patologias, incluindo a fibrose hepática. Estudo realizado por Souza et al. (2008) demonstraram que o resveratrol promove redução na proliferação celular (GRX) através da indução de parada no ciclo celular e apoptose. De forma semelhante, foi observado que os compostos resveratrol, quercetina e N-acetilcisteína são potentes inibidores da proliferação das HSCs através de parada no ciclo celular e inibem as mudanças estruturais na ativação das células estreladas, além de apresentarem efeitos antinflamatórios (Kawada et al., 1998; Stefano et al., 2011).

Portanto, atualmente encontramos diversos trabalhos comprovando os efeitos antifibróticos de alguns compostos fenólicos naturais e flavonóides, que atuam principalmente na atenuação do estresse oxidativo, inibição da ativação das HSCs e como antinflamatórios, tanto *in vitro* quanto *in vivo*. Dentre eles destacam-se a cianidina-3-O-β-glicopiranosídeo, a baicaleina, a luteolina, o resveratrol, a quercetina e catequinas (Bendia et al., 2005; Domitrovic et al., 2009; Inoue and

Jackson, 1999; Kawada et al., 1998). Porém, ainda são muito escassos os trabalhos avaliando o efeito de frutas ou vegetais que apresentam elevada capacidade antioxidante e possuem diversos compostos fenólicos naturais atuando em sinergismo sobre a fibrose hepática.

7. Pitanga roxa (*Eugenia uniflora* L.)

A pitanga, fruto da pitangueira (*Eugenia uniflora* L.) pertence a família Myrtaceae e é uma planta frutífera nativa do Sul e Sudeste do Brasil, Argentina, Uruguai e Paraguai. Ela adapta-se favoravelmente em diferentes condições climáticas e, portanto, pode ser encontrada em muitos lugares, como América Central, Flórida, Califórnia, Havaí, Holanda, França, China, Tunísia, Argélia e Sri Lanka (Gomes, 2007). Devido ao seu sabor altamente desejável e ao elevado conteúdo de carotenoides (Rodriguez-Amaya et al., 2008), a pitangueira é uma das árvores frutíferas mais promissoras para programas de exploração sustentável na Mata Atlântica Brasileira. No Brasil, as pitangueiras são cultivadas principalmente em hortas, pequenas propriedades agrícolas, ou de forma nativa. Atualmente, a Embrapa Clima Temperado localizada no município de Pelotas – RS possui um programa de melhoramento genético e expansão de produção desta árvore frutífera, o qual atua no fornecimento de mudas e programas informativos junto à comunidade.

O fruto da pitangueira pode atingir cerca de 30 mm de diâmetro e apresenta de 8 a 10 ranhuras longitudinais na casca. Aproximadamente 66% dos frutos é constituído de polpa que possui um sabor doce e ácido únicos, com aroma intenso e característico (Gomes, 2007). Durante a maturação, o epicarpo da fruta evolui de verde para laranja e vermelho, nas variedades laranja e vermelha e, do verde ao roxo profundo ou quase preto, na variedade roxa. Além de serem altamente desejáveis para o consumo in natura, as pitangas são também utilizados para a produção de sucos e sorvetes, assim como na indústria de cosméticos. O crescente interesse por estas frutas está relacionado com as grandes quantidades de catequinas, flavonóides, proantocianidinas e compostos fenólicos, conhecidos por sua atividade antioxidante, que elas podem apresentar (Bagetti et al.,

2011; Celli et al., 2011). Além dos diversos efeitos na saúde humana, os compostos antioxidantes são também importantes na inibição e/ou prevenção da oxidação de produtos alimentares.

As folhas da pitangueira têm sido utilizados a muito tempo na medicina popular, devido a suas diversas atividades biológicas, sendo geralmente preparadas como infusão para o tratamento da febre, reumatismo, bronquite, doenças do estômago, e distúrbios digestivos, bem como hipertensão, febre amarela e gota (Alice et al., 1991; Bagetti et al., 2011; Velazquez et al., 2003). Também podem reduzir o peso corporal e a pressão arterial, servir como um diurético, além de sua comprovada atividade calmante e antinflamatória (Schapoval et al., 1994; Schmeda-Hirschmann et al., 1987). Recentemente, o extrato das folhas da pitangueira apresentou atividade citotóxica e anti-*Trypanosoma cruzi* com baixa toxicidade em estudos *in vitro* (Santos et al., 2012).

A pitanga, ou seja, os frutos da pitangueira, também apresentam atividade antioxidante e atuam inibindo a peroxidação lipídica e na remoção de radicais livres (Bagetti et al., 2011; Celli et al., 2011; Velazquez et al., 2003). Porém, ainda são muito escassos ou praticamente inexistentes os trabalhos avaliando o efeito dos extratos ou compostos isolados da pitanga, que apresenta comprovadamente diversos compostos com elevada capacidade antioxidante, sobre condições fisiológicas e patológicas, tanto *in vitro* como *in vivo*. Portanto, este é um campo de pesquisa promissor que pode alavancar o consumo e a produção desta fruta nativa brasileira.

Sabe-se que o consumo de frutas e hortaliças pode trazer diversos benefícios a saúde humana inclusive atuando na prevenção de várias doenças crônicas. Porém, embora o Brasil seja um grande produtor mundial de frutas e hortaliças, com grande abundância de variedades nas diferentes regiões do país, o brasileiro ainda é um péssimo consumidor destes alimentos. De acordo com a Organização Mundial da Saúde (OMS), o consumo diário mínimo para um adulto deve ser de cinco porções, ou 400 gramas de frutas e legumes. No Brasil, a ingestão não chega a um terço destes valores. Segundo a mais recente pesquisa feita pelo Instituto Brasileiro de Geografia e Estatística (IBGE/2008-2009), frutas, verduras e legumes correspondem a apenas 2,3% das calorias

totais ingeridas pela população. Portanto, o incentivo ao consumo e produção de frutas pode ser melhorado com pesquisas que promovam um maior conhecimento sobre os compostos com propriedades benéficas presentes nestes vegetais que podem atuar na prevenção ou tratamento de enfermidades.

OBJETIVOS

1. Objetivo geral

O presente trabalho tem como objetivo geral investigar o efeito do extrato de pitanga (*Eugenia uniflora* L.) roxa, uma fruta nativa do Brasil, sobre a proliferação, viabilidade celular e resolução do estado de ativação de células estreladas hepáticas ativadas, bem como verificar o potencial antioxidante deste extrato através da determinação dos compostos bioativos e capacidade antioxidante *in vitro*.

2. Objetivos específicos

- Determinar o conteúdo de fenólicos totais, perfil de compostos fenólicos e carotenóides e a atividade antioxidante *in vitro* do extrato de pitanga roxa.

- Avaliar o possível efeito deste extrato sobre a proliferação e viabilidade celular em cultura de células estreladas hepáticas ativadas utilizando a linhagem celular GRX.

- Avaliar o efeito do extrato de pitanga roxa sobre os mecanismos de apoptose e autofagia das células estreladas hepáticas ativadas (GRX).

PARTE II

CAPÍTULO I

Antioxidant capacity and bioactive compounds of some Brazilian native fruits

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1 **Abstract**

2 The purpose of this study was to evaluate the antioxidant activity and bioactive compounds of
3 extracts from araçá (*Psidium cattleianum*), butiá (*Butia eriospatha*) and pitanga (*Eugenia*
4 *uniflora*) fruits with different flesh colors (purple, red and orange), and blackberries (*Rubus*
5 *sp.*) (cv. 'Xavante' and 'Cherokee') collected in the Southern region of Brazil. The ascorbic
6 acid content, total carotenoids and phenolics contents were determined. The profile of
7 phenolic compounds was assessed by HPLC-DAD. The antioxidant activity was determined
8 using the FRAP, DPPH, TRAP and TAR assays. The 'Xavante' blackberry and purple-
9 fleshed pitanga showed the highest total phenolic contents (816.50 and 799.80 mg
10 GAE/100g). The araçá and red-fleshed pitanga showed the highest carotenoid content (6.27
11 and 5.86 ug β-carotene/g). Several phenolic compounds were identified in fruits, such as
12 quercetin derivatives, quercitrin, isoquercitrin and cyanidin derivatives, among others, which
13 may contribute differentially to the antioxidant capacity. The highest scavenging activity in
14 the DPPH assay was found for purple-fleshed pitanga (IC_{50} 36.78 mg/L), blackberries (IC_{50}
15 44.70 and 78.25 mg/L) and araçá (IC_{50} 48.05 mg/L), which also showed the highest ferric-
16 reducing antioxidant power (FRAP) followed by orange and red-fleshed pitanga. Our results
17 revealed that some fruits grown in Southern Brazil, such as purple-fleshed pitanga,
18 blackberries and araçá are rich sources of phenolic compounds and have great antioxidant
19 activity.

20 *Keywords:* Antioxidant activity; *Eugenia uniflora*; *Psidium cattleianum*; *Rubus sp.*; *Butia*
21 *eriospatha*.

22

23 1. Introduction

24 The relationship between nutrition and health has become a topic of great interest and
25 there is substantial evidence of the beneficial effects of diets that are rich in fruits and

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1 vegetables. Brazil has a great biological diversity that can be explored to yield extracts for
2 therapeutic application to control and/or prevent chronic diseases. Polyphenols from fruits and
3 vegetables [1] can be divided into several classes (i.e., hydroxybenzoic acids,
4 hydroxycinnamic acids, anthocyanins, proanthocyanidins, flavonols, flavones, flavanols,
5 flavanones, isoflavones, stilbenes and lignans). They contribute substantially for the
6 antioxidant effect of many small-fruited species, having potential healthy effects.
7 *Psidium cattleianum* Sabine (araçá; Myrtaceae), which is also known as wild guava or
8 Brazilian guava, is found from the state of Minas Gerais to the state of Rio Grande do Sul [2].
9 According to folk medicine, araçá is indicated to treat diarrhea, hemorrhage and cramp. *Butia*
10 *eriospatha* (Mart. ex Drude) Becc. (butiá; Arecaceae) is a palm tree that is native from South
11 America. In Brazil, it occurs in the states of Parana, Santa Catarina and Rio Grande do Sul.
12 The ripe fruit can be eaten raw or used for preparation of juices, wine and liqueurs. We found
13 no study on the literature evaluating the content of phenolic compounds and antioxidant
14 activity of araçá and butiá fruits.
15 *Eugenia uniflora* L. (Myrtaceae) is a widely distributed tree species in South America,
16 mainly in Brazil, Argentina, Uruguay and Paraguay. The leaves are used in popular medicine
17 as infusion for the treatment of fever, rheumatism, stomach diseases, and digestive disorders,
18 as well as hypertension, yellow fever and gout. It may also reduce weight, blood pressure, and
19 serve as a diuretic [3, 4]. Its fruit, which is known as pitanga, brazilian cherry or suriname
20 cherry, also shows antioxidant activity inhibiting lipid peroxidation and removing free
21 radicals [5]. Pitanga fruits contain various volatile compounds that are also found in the
22 essential oil from pitanga leaves [6]. Like the leaves, pitanga fruits could also have healthy
23 benefits. In the Brazilian food industry, the pitanga fruit has mostly been used to produce
24 juice, which shows good economic potential due to the consumer appeal arising from its high
25 concentrations of antioxidant compounds, such as anthocyanins, flavonols and carotenoids.

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1 Despite being native from Asia, Europe, North and South America, *Rubus sp.*
2 (blackberry; Rosaceae) grows only in specific regions, because most blackberry species are
3 not adapted to regions with mild winter [7]. In Brazil, blackberry was introduced in the 70s by
4 the Brazilian Agricultural Research Corporation (Embrapa Temperate Agriculture).
5 Thereafter, Embrapa has conducted a genetic improvement program that developed various
6 blackberry cultivars adapted to the Southern Region of Brazil, such as Guarani, Caingangue,
7 Xavante and Tupy. Due to its subtropical climate, Rio Grande do Sul was the first state in
8 Brazil to produce blackberries and it is still the main one [8].
9 Blackberry fruits are good sources of natural antioxidants. Extracts from blackberry
10 fruits may have some health benefits such as antioxidant [9, 10], anticonvulsant and muscle
11 relaxant [11] and anti-inflammatory properties [12]. Their extracts are rich in secondary
12 metabolites such as anthocyanins and phenolic acids [9], but little is known about the
13 presence and antioxidant activity of these compounds in genotypes growing in the Rio Grande
14 do Sul state (Brazil).
15 The presence of phenolic compounds in fruit and vegetable-rich diets have attracted
16 researchers' attention due to their health-promoting effects, which include lowering the risk of
17 cardiovascular diseases, cancer, or other conditions associated with aging. The biological
18 mechanisms behind these effects include protection against free radicals, free radical-
19 mediated cellular signaling, inflammation, allergies, platelet aggregation, ulcers, viruses,
20 tumors and hepatotoxicity [13]. However there are few studies on the identification of
21 phytochemical compounds and antioxidant activity of extracts from Brazilian native fruits.
22 Moreover, the knowledge concerning the antioxidant activity and content of bioactive
23 compounds in different fruit genotypes may be useful for genetic improvement programs to
24 select those varieties with higher nutritional values. Thus, the objective of this study was to
25 evaluate the antioxidant activity and bioactive compounds of extracts from araçá, butiá and

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pitanga fruits with different flesh colors (purple, red and orange), and blackberry fruits (cv. Xavante and Cherokee) collected in different regions of Rio Grande do Sul (Brazil).

2. Materials and methods

2.1. Chemicals

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) 97%, 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) ≥98%, luminol (3-aminophthal-hydrazide) 97%, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) 97%, Folin-Ciocalteu reagent 2N, p-hydroxybenzoic ≥99%, chlorogenic ≥95%, p-coumaric ≥98%, caffeic ≥98%, ferulic 99%, syringic ≥95%, gallic 97.5-102.5%, ellagic ≥95% and ascorbic ≥99% acids, beta-carotene ≥95%, rutin ≥94%, kaempferol ≥90%, kaempferol-3-glucoside ≥97%, malvidin ≥95%, delphinidin ≥95%, pelargonidin ≥95%, cyanidin ≥95%, cyanidin-3-glucoside ≥95%, quercetin-3-O-galactoside (hyperoside) ≥97%, quercetin-3-beta-D-glucoside (isoquercitrin) ≥90% and quercetin-3-rhamnoside (quercitrin) ≥78% were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Quercetin ≥98% was from Jassem Chemical (Beerse, Belgium). Vanillic acid ≥97% was from Fluka Chemical (Bochus, Switzerland).

2.2. Preparation of fruit extracts

Samples of orange, red and purple-fleshed breeding lines of pitanga fruits (*Eugenia uniflora*) as well as blackberry (*Rubus sp.*) cultivars "Xavante" and "Cherokee" were obtained from harvest 2009/2010 at Embrapa Temperate Agriculture (Pelotas, Rio Grande do Sul, Brazil, 31°40'47''S, 52°26'24''W, 60 m) and immediately frozen. Samples of araçá (*Psidium cattleianum*) and butiá (*Butia eriospatha*) fruits were collected at Tuparendi (Rio Grande do Sul, Brazil; 27°77'24''S, 54°49'76''W, 60 m) and Santa Maria (Rio Grande do Sul, Brazil;

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1 29°42'27''S, 53°40'29''W, 318 m), respectively, in January 2008. Each species was sampled
2 searching for a mixture of completely ripe fruits from various plants of the same genotype.
3 Three independent samples were collected, frozen at -18 °C and transported to the Federal
4 University of Santa Maria.
5 Fruit extracts were prepared from the edible portions of fruits. Briefly, fresh fruit
6 samples were homogenized with an ultra-turrax homogenizer for 5 min in 95% ethanol (1:3
7 w/v). The homogenates were blended for 30 minutes, at room temperature, and centrifuged at
8 1500 x g for 5 min. The supernatant was collected and the extraction procedure was repeated.
9 The pooled supernatants were concentrated in rotary evaporator at 40ºC. The samples were
10 reconstituted in water and stored at -80ºC. The extracts obtained from each fruit were always
11 adjusted to the same final volume, so that the yield of extraction was 0.15 mL of extract g^{-1}
12 fruit.
13
14 *2.3. Ascorbic acid, total carotenoids and phenolics contents*
15 The ascorbic acid content of sampled extracts was assessed as described by Sánchez-
16 Mata *et al.* [14] with some modifications. Ethanolic extracts were filtered through a 0.22 mm
17 Millipore filter (Bedford, Md., USA) and 10 μL were analyzed using an Intralab HPLC
18 system (5100 model) coupled with an UV-Visible detector (Intralab 5100) and reverse phase
19 Microsorb - MW C18 column (4.6 x 250 mm, particle size 5 μm) (Varian). The flow rate was
20 0.9 mL/min (isocratic gradient) and the mobile phase was a solution of sulfuric acid 0.01% in
21 Milli-Q water (final pH 2.8). Total run time was 8 min and the wavelength of detection was
22 set at 245 nm. Quantification of ascorbic acid was achieved using calibration curves with 7
23 concentrations of ascorbic acid (R^2=0,9992).
24 The carotenoids were exhaustively extracted from the fruits with ethyl acetate, by
25 vortexing during 1 min. The organic phases were transferred to a separatory funnel, washed

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with water until obtaining neutral pH, and then dried under a N_2 stream and dissolved in petroleum ether [15]. The total carotenoid content was subsequently measured spectrophotometrically at 450 nm using an extinction coefficient of 2590 and the results were expressed as β-carotene equivalents (μg g^{-1} of fresh weight).

Total phenolic content were measured according to the Folin-Ciocalteu method adapted from Swain and Hillis [16]. The extract (100 μL) was mixed with 1600 μL of Milli-Q water, and 100 μL of 0.25 N Folin-Ciocalteu reagent. The mixture was allowed to react for 3 min then 200 μL of 1 N Na_2CO_3 was added and the solution was incubated at room temperature (23±1°C) in the dark for 2 h. The absorbance was measured at 725 nm and the results were expressed as gallic acid equivalents (GAE; mg 100 g^{-1} fresh weight) using a gallic acid (0–0.4 mg/mL) standard curve.

2.4. High-performance liquid chromatography combined with diode array detection (HPLC-DAD) analysis

Samples were filtered through a 0.45 μm filter (Millipore). The HPLC analysis was carried out on a Waters 2695 HPLC system equipped with a Luna C18 reversed-phase silica 100 Å (250 x 4.6 mm, particle size 3 μm) column (Phenomonex, Torrance, CA, USA), a Waters 996 DAD detector (Waters Corp., Milford, MA) and Empower Software (Waters). The solvents were 2.5% aqueous formic acid (pH 2.4; solvent A) and acetonitrile (solvent B). Anthocyanins were analyzed in the ethanolic extracts (10 μL injection volume) (at 520 nm) using the following gradient: from 12 to 50% B in 20 min, from 50 to 12% B in 22 min and isocratically with 12% B up to 30 min, at a flow rate of 0.5 mL/min. For other phenolic compounds, gradient conditions were as follows: from 0 to 30% B in 80 min, from 30 to 50% B in 90 min, from 50 to 100% B in 95 min, and then isocratically with 100% B up to 98 min, at a flow rate of 0.5 mL/min, after which the column was washed during 5 min and

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1 equilibrated for 8 min. The injection volume was 10 μL, and the detection was carried out
2 between 200 and 600 nm. Peak identification and quantification was based on comparison
3 with the retention times and UV- vis spectra of authentic standards.
4
5 *2.5. Antioxidant activity*

6 **FRAP assay.** The ferric-reducing antioxidant power of each ethanolic extract (three different
7 dilution of the sample) was estimated according to the procedure described by Pulido *et al.*
8 [17]. Briefly, 2.7 mL of FRAP reagent, prepared freshly and warmed at 37 °C, was mixed
9 with 270 μL of Milli-Q water and 90 μL of test sample, water, or methanol as appropriate for
10 the reagent blank. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ solution in 40 mM
11 HCl plus 2.5 mL of 20 mM $FeCl_3.6H_2O$ and 25 mL of 0.3 M acetate buffer, pH 3.6. The
12 reaction mixture was incubated at 37ºC for 30 min and the absorption maximum was assessed
13 at 595 nm. An intense blue colour is formed when the ferric-tripyridyltriazine (Fe^{3+}-TPTZ)
14 complex is reduced to the ferrous (Fe^{2+}) form. Aqueous solutions of known Fe^{2+}
15 concentrations in the range of 500-1500 μM ($FeSO_4$. $7H_2O$) were used for calibration.

16 The total antioxidant activity was defined as the concentration of antioxidant having a
17 ferric-TPTZ reducing ability equivalent to that of 1 mM $FeSO_4.7H_2O$/g of fruit. Total
18 antioxidant activity was calculated as the concentration of antioxidant giving an absorbance
19 increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mM
20 $FeSO_4.7H_2O$ solution determined using the corresponding regression equation.

21 **DPPH assay.** The capacity to scavenge the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical
22 was assessed according to Brand-Williams *et al.* [18] with some modifications. Fruit extracts
23 (100 μL) were allowed to react with 3.9 mL of the DPPH solution for 90 min in the dark. The
24 blank sample consisted of 0.1 mL of methanol added to 3.9 mL of DPPH. Then the
25 absorbance was taken at 515 nm. The radical scavenging activity was calculated as follows:

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1 $I\% = [(Abs_0 - Abs_1)/Abs_0] \times 100$, were Abs_0 was the absorbance of the blank and Abs_1 was
2 the absorbance in the presence of the test compound at different concentrations. The IC_{50}
3 (concentration providing 50% inhibition of DPPH absorbance) was calculated graphically
4 using a calibration curve in the linear range by plotting the extract concentration vs. the
5 corresponding scavenging effect.

6 **Total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR).**
7 TRAP assay has been described by Lissi *et al* [19]. We used this test as an index of the non-
8 enzymatic antioxidant capacity of each ethanolic extract, based on the peroxyl radical
9 scavenge by sample compounds. The peroxyl radical was generated by mixing 2,20-azobis[2-
10 amidinopropane] (AAPH) solution with luminol (system). The first reading of
11 chemiluminescence emission was done 2h after system preparation to allow the stabilization.
12 After the sample addition the readings were taken for nearly 30 min. Results were
13 transformed as percent of the first reading and plotted against time. The area under curve
14 (AUC) was calculated using GraphPad 5.0 Software Inc. (San Diego, CA, USA). The total
15 antioxidant reactivity (TAR) was also analyzed of each ethanolic extracts and it is based on
16 the same technical principles of TRAP, but TAR is more related to the quality of samples
17 antioxidants. The TAR results were calculated as the ratio of light in the absence of sample
18 (I_0)/ light intensity right after sample addition (I) [19].
19

20 *2.6. Statistical analysis*

21 Data were reported as means ± standard deviations (SD) of three replicates of each sampled
22 species. Results were analyzed by one-way analysis of variance (ANOVA) followed by
23 Tukey's test ($p<0.05$). The relationship between antioxidant compounds (phenolics,
24 carotenoids or ascorbic acid) and antioxidant activity was evaluated by Pearson's correlation.
25 All analyses were performed using the statistical software SPSS (SPSS Inc., Chicago, USA).

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2 **3. Results and discussion**

3 *3.1. Total phenolics, carotenoids and ascorbic acid*

4 Significant differences ($p < 0.05$) were found among fruit extracts for total phenolics,
5 carotenoids and ascorbic acid contents (Table 1). The total phenolic content ranged from
6 359.5 to 816.5 mg GAE/100g fw. The 'Xavante' blackberry and the purple-fleshed pitanga
7 showed the highest total phenolic contents, followed by 'Cherokee' blackberry, araçá, orange
8 and red-fleshed pitanga and butiá (Table 1). The high content of phenolic compounds found
9 in blackberry in the present study was also reported by several authors for different cultivars
10 found in the United States, Brazil and Italy, with values ranging from 192.8 to 499.0 mg
11 GAE/100g [9-10, 20]. Jacques *et al.* [21] also observed a higher content of phenolic
12 compounds in purple pitanga (420.8 mg GAE/100g) compared to red (239.2 mg GAE/100g)
13 and orange pitanga (201.8 mg GAE/100g), and similar content in butiá (328.6 mg
14 GAE/100g). The total phenolic content observed for the araçá was higher than reported by
15 Biegelmeyer *et al.* [22] for the same yellow araçá species (292.03 mg /100 g) probably due to
16 the use of other methods for the determination of phenolic compounds. Phenolic compounds
17 are secondary products of plant metabolism that constitute a large and complex group. These
18 molecules are essential for growth and reproduction of plants, and its synthesis is induced
19 under conditions of biotic and abiotic stresses, such as infections, injury, UV radiation, ozone,
20 salinity, water stress, heat, among others. In foods, they are partially responsible for the color,
21 astringency, aroma and oxidative stability [1].

22 The total carotenoids ranged from 6.27 to 0.87 µg β-caroteno/g fw. The araçá and red-
23 fleshed pitanga showed the highest carotenoid content, followed by orange-fleshed pitanga,
24 butiá, purple-fleshed pitanga and 'Xavante' and 'Cherokee' blackberries (Table 1). Sixteen
25 carotenoids had been isolated from Guava (*Psidium guajava* L.) and identified as phytofluene,

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1 β-carotenes, γ-carotene, lycopenes, β-cryptoxanthin, rubixanthin, cryptoflavin, lutein and
2 neochrome [23]. We have not found studies evaluating the carotenoid content in araçá.
3 According to Coimbra and Jorge [24], the Brazilian palm species guariroba (*Syagrus*
4 *oleraces*), jerivá (*Syagrus romanzoffiana*) and macaúba (*Acrocomia aculeata*) contains high
5 levels of carotenoids (158.44; 1219 and 300.01 μg β-carotene/g, respectively), however, the
6 concentration of carotenoids observed in this study to butiá was only 3.8 μg β-carotene/g.
7 Among the three selections of pitanga, the red-fleshed one showed higher total carotenoids
8 (5.9 μg β-carotene/g) than orange (4.0 μg β-carotene/g) and purple-fleshed pitanga (3.0 μg β-
9 carotene/g), which is in agreement with results obtained in the literature [21]. During the
10 ripening process, the pitanga fruits change from green to yellow, to orange, to red and then to
11 dark red, becoming almost black in some cases when lycopene is the major carotenoid found.
12 The following carotenoids have been found in pitanga fruits in decreasing quantitative order:
13 lycopene, rubixanthin, cis-rubixanthin, β-cryptoxanthin, cis-lycopene, β-carotene, γ-carotene,
14 zeaxanthin, lutein, violaxanthin and β-carotene-5,6-epoxide [25]. Blackberries showed the
15 lowest levels of carotenoids when compared to the other fruits. This is because the color of
16 these fruits in the mature stage is mainly due to the presence of anthocyanins, while the
17 carotenoid content decreases with maturation [21]. The composition of carotenoids in the
18 plant is affected by several factors such as variety, part of the plant, degree of maturity,
19 climate, soil type, growing conditions and geographical area of production, as well as harvest
20 conditions, processing and storage. This may explain the lower total carotenoid content of all
21 fruits examined when compared to results found in the literature for the same species and fruit
22 varieties.
23 The content of ascorbic acid of the butiá was about 73 times greater than all other
24 fruits studied (Table 1). Ascorbic acid, besides being the biologically active form of vitamin
25 C, is also the most commonly found and widely distributed in products of plant origin, mainly

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1 in citrus fruits and leafy vegetables. The extracts of pitanga and araçá showed low levels of
2 ascorbic acid, which are characterized as poor sources of vitamin C. The vitamin content of
3 fruit can vary depending on the species, maturity stage at harvest time, genetic variants, post-
4 harvest handling, storage conditions and processing. The content of these nutrients in the
5 fresh food and its stability can influence its nutritional quality [26]. The extracts of
6 blackberries had very low ascorbic acid levels. Accordingly, Hassimotto *et al.* [20] found only
7 the oxidized form of vitamin C (L-dehydroascorbic acid) in Tupy and Guarani cultivars
8 grown in a tropical climate region of Brazil. This finding was attributed to the fast oxidation
9 of vitamin C and absence of de novo synthesis of ascorbic acid during development or
10 ripening.

11 We found a negative correlation between total phenolic content and ascorbic acid
12 content (r^2 = -0.674; $p<0.05$). It suggests that the lower the ascorbic acid content, the higher
13 the total phenolic content in these fruits studied.

14

15 *3.2. Profile of phenolic compounds*

16 Phenolic compounds or polyphenols are a complex group of phytochemicals
17 possessing several hydroxyl groups on aromatic rings. They are widely distributed throughout
18 the plant kingdom and thus form an integral part of the human diet.

19 The HPLC-DAD chromatograns (at 280 nm and 360 nm) for the extracts of pitanga
20 fruits are shown in Figure 1, and the main phenolic compounds identified are listed in Table
21 2. The chromatograms of three varieties of pitanga have many similarities in the profile of
22 phenolic compounds. Gallic acid derivatives, quercetin derivatives, quercitrin, isoquercitrin,
23 kaempferol derivatives, and cyanidin-3-glucoside were found in the three varieties of pitanga,
24 while cyanidin derivatives and quercetin were found only in red and purple-fleshed pitanga.
25 Protocatechuic acid derivatives were found in red-fleshed pitanga and malvidin derivatives in

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1 purple-fleshed pitanga (Table 2). There are a range of phytochemicals already identified in
2 pitanga leaves such as flavonoids (myricetin, quercetin and its quercetrin 3-l-ramnoside),
3 steroids and triterpenoids, tanins, antraquinons and phenols, sineol and essential oils [3-4], but
4 there are few studies evaluating these compounds in pitanga fruits. Celli *et al*. [27] evaluated
5 the flavonoids profile in red and purple pitanga fruits and identified several flavonoid
6 derivatives of cyanidin, myricetin and quercetin. Some anthocyanins such as cyanidin-3-
7 glucoside and delfinidin-3-glucoside were also identified.

8 As observed for pitanga fruits, the two cultivars of blackberries also showed
9 chromatograms with very similar profiles (Figure 2). The phenolic compounds identified in
10 the two cultivars studied were: ellagic acid derivatives, quercetin derivatives, isoquercitrin,
11 cyanidin-3-glucoside and delphinidin derivatives. Protocatechuic acid derivatives, cyanidin
12 derivatives, quercetin and quercitrin were found in the Xavante cultivar, whereas kaempferol
13 derivatives were found in the Cherokee cultivar (Table 2). There are many studies on the
14 phenolic compounds of blackberries, but few studies were performed on blackberries grown
15 and adapted to the southern region of Brazil. Mertz *et al.* [28] analyzed the phenolic
16 compounds in two blackberry species and identified gallic acid and galloyl esters, caffeic
17 acid, p-coumaric acid, ferulic acid, epicatechin, ellagic acid, quercetin derivatives, kaempferol
18 derivatives, cyanidin-3-glucoside, among others. Hassimotto *et al.* [20] found cyanidin,
19 flavan-3-ol epicatechin, quercetin and kaempferol in blackberry cultivars from the southern
20 region of Brazil. Some recent studies suggest that blackberries have among the highest
21 antioxidant capacity of any fruits and vegetables, mainly due to its high content of cyanidin-3-
22 glucoside [29].

23 The chromatograns (at 280 nm and 360 nm) of araçá and butiá extracts are shown in
24 Figure 3. Although these two fruits have yellow flesh color, the profile of these phenolic
25 compounds showed significant differences, due to variations in the botanical family as the

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araçá belongs to the Myrtaceae and butiá belongs to the Arecaceae family. The major phenolic compounds identified in araçá were: gallic acid derivatives, quercetin derivatives, apigenin derivatives and isoquercitrin, although most chromatographic peaks were not identified (Table 2). In addition, the following compounds were identified in butiá: gallic acid derivatives, protocatechuic acid derivatives, caffeic acid derivatives, chlorogenic acid derivatives, isoquercitrin, quercetin derivatives, hyperoside and rutin (Table 2). Few reports in the literature do assess the profile of phenolic compounds in these fruits. However, hyperoside was the main phenolic compound found in araçá, followed by cyanidin [22].

The phenolic composition of fruits is determined by genetic and environmental factors, but may be modified by oxidative reactions during processing and storage [30]. The phenolic compounds are metabolized as a defense response against intense solar radiation and other adverse factors and variations in the fruits are due to the route of formation of these compounds, since their presence differs in each fruit. Thus, the different contributions of individual phenolics in the extracts are expected to yield different antioxidant effects by the extracts.

3.3. Antioxidant capacity

Polyphenols have powerful antioxidant activity *in vitro* being capable of scavenging a wide range of reactive oxygen, nitrogen, and chlorine species, such as superoxide anion, hydroxyl radical, peroxyl radicals, hypochlorous acid and peroxynitrous acid. They also chelate metal ions, thus decreasing their pro-oxidant activity. Since considerable evidence indicates that increased oxidative damage is associated with the development of most major age-related degenerative diseases, it has been speculated that polyphenols may have protective effects against such conditions [31].

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Because of multiple reaction characteristics and mechanisms, a single antioxidant assay will not accurately reflect all antioxidant in a mixed or complex system. Thus, the use of different antioxidant assays help to identify variations in the response of the compounds extracted from fruits [31-32]. For this reason, three different antioxidant assays were conducted to clarify different aspects of the antioxidant capacity of extracts from fruits produced in the southern region of Brazil. DPPH and FRAP assays were chosen because they are simple and rapid methods for assessing the antioxidant capacity of fruits and vegetables [32]. These two assays are based on different principles, and thus may be used to screen compounds that have different antioxidant mechanisms. While the FRAP assay measures the ferric reducing capacity of antioxidants [33], the DPPH assay measures the ability of antioxidants to scavenge the DPPH radical [18].

The evaluation of antioxidant activity by the DPPH method showed a large variation among the different fruit extracts (Table 3), which may be related to differences in the profile of phenolic compounds among these fruits. The lowest value IC_{50} value, which corresponds to the highest scavenging activity of DPPH radicals, was obtained for the extracts of purple-fleshed pitanga, 'Xavante' blackberry, araçá and 'Cherokee' blackberry, whereas orange and red-fleshed pitanga had intermediate and butiá had the lowest scavenging capacity (Table 3). Purple-fleshed pitanga, araçá and 'Xavante' and 'Cherokee' blackberries also showed the highest ferric-reducing antioxidant power (FRAP) followed by orange and red-fleshed pitanga, whereas butiá had the lowest FRAP value (Table 3).

The total phenolic content of fruit extracts was negatively correlated with the IC_{50} value for the DPPH antioxidant assay (r^2 = -0.758; $p<0.05$) and positively correlated with the FRAP value (r^2 =0.773; $p<0.05$). In addition, a significant positive correlation was found between the ascorbic acid content of fruits and the IC_{50} value for the DPPH antioxidant assay (r^2 =0.918; $p<0.05$) and a negative correlation was found between ascorbic acid content and

1 the FRAP value ($r^2 = -0.718$; $p<0.05$). These correlations found between bioactive compounds
2 and the antioxidant activity of extracts suggests that phenolic compounds are the major
3 responsible for the antioxidant activity in the DPPH and FRAP assays and the ascorbic acid
4 might not be important here.
5 Several authors demonstrated a strong positive correlation between total phenolic
6 content and the antioxidant capacity of fruits [9, 34] including pitanga [35] and blackberries
7 [29]. According to Bagetti *et al.* [35] both the ferric-reducing power and the DPPH radical
8 scavenging capacity were higher for the extracts from purple-fleshed pitanga than for the red
9 and orange-fleshed fruits. Moreover, recently it was found that araçá had high antioxidant
10 activity, possibly attributed to the synergism of their phenolic compounds and the antioxidant
11 activity varied in a concentration-dependent manner in red and yellow fruits of same species
12 [22].
13 The ability of species to scavenge reactive oxygen depends on the type of antioxidant.
14 Fruits contain many different antioxidant components [36] and their relative quantities may
15 also vary, affecting the total antioxidant capacity of fruits. The antioxidant activity of
16 polyphenols is carried out by different mechanisms, the most important being the
17 sequestration of free radicals, which depends on the structure of the compound involved. The
18 intensity of the antioxidant activity exhibited by these phytochemicals is different, mainly
19 because of the number and position of hydroxyl groups present in the molecule [37]. These
20 facts may explain the higher antioxidant activity observed in fruits with red and purple flesh
21 color, which had higher phenolic content and predominance of flavonoids and anthocyanins
22 as cyanidin and quercetin derivatives. However, it should be noted that araçá, a yellow-
23 fleshed fruit, also showed strong antioxidant activity, which can be explained by the presence
24 of several phenolic compounds that could not be identified in the HPLC chromatogram, and
25 the presence of flavonoid derivatives as quercetin and isoquercetrin, among others.

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1 The TRAP was determined using a method based on the quenching of luminol-
2 enhanced chemiluminescence derived from the thermolysis of a water-soluble azo compound,
3 AAPH, used as a reliable and quantifiable source of alkyl peroxyl radicals [19]. This widely
4 used assay has proved to be a simple, sensitive, and reproducible method that can be used to
5 determine the antioxidant capacity in complex mixtures such as plant extracts [38]. TRAP
6 measurement is an index of the non-antioxidant capacity and indicates the quantity of
7 antioxidants present in the plant extract, whereas TAR indicates the quality (given by the
8 reactivity) in those extracts with antioxidant activity. In this study we observed the highest
9 non-enzymatic antioxidant potential, seen by decreased AUC in the TRAP assay, for the
10 purple-fleshed pitanga followed by the 'Cherokee' blackberry and butiá, whereas the other
11 fruits had the lowest antioxidant potential (Table 3). However, the TAR was higher for the red
12 and orange-fleshed pitanga, followed by 'Xavante' blackberry, butiá, araçá, 'Cherokee'
13 blackberry and purple-fleshed pitanga (Table 3). No significant correlation was found
14 between the total phenolic content and the antioxidant activity assessed by the TRAP or TAR
15 assays.
16 We found that the purple-fleshed pitanga, despite its higher content of phenolic
17 compounds, higher antioxidant activity in the DPPH and FRAP assays, and greater amount of
18 antioxidants observed by TRAP method, had a lower quality (reactivity) of these antioxidants
19 observed in the TAR assay as compared with the other fruits studied. Moreover, red and
20 orange-fleshed pitanga, which showed intermediate phenolic content and antioxidant activity
21 in the DPPH and FRAP assays, showed a higher TAR value, indicating greater reactivity of
22 phenolic compounds in these fruits as compared to the others, regardless of their quantity.
23 These differences can be the result of efficient and inefficient antioxidants in the
24 extracts [37]. All the extracts studied were active in reducing the luminol-enhanced
25 chemiluminescence, indicating the presence of compounds with peroxyl scavenging

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1 properties. However, the amount of total phenolic compounds was not directly associated
2 with the peroxyl scavenging property and possible beneficial effects of these fruits, which was
3 probably because different bioactive compounds can act in synergism or antagonism.

4 The antioxidant activity of a specific phenolic compound is related with the number of
5 available hydroxyl groups present in the chemical structure and will depend on their donor-
6 proton capacity [37]. Therefore the manner these compounds neutralize free radicals will
7 depend on their relative concentrations in the sample matrix. In addition, phenolic compounds
8 can act synergistically, additively, or antagonistically to inhibit reactive species.

9 For flavonoids (flavonols, isoflavones, and so on), which have a diphenylpropane
10 skeleton, their efficiency as free-radical scavengers seems to depend mainly on the number of
11 hydroxyl groups and their position on the molecule. The antioxidant potency is related to
12 structure in terms of electron delocalization of the aromatic nucleus. Additionally, the
13 presence of glycosylations on the molecule may decrease its antioxidant activity (rutin for
14 example). In general, flavonoids structural arrangements are considered to impart greatest
15 antioxidant activity [37]. Thus, quercetin satisfies all the above mentioned determinants and is
16 a more effective antioxidant.

17 For phenolic acids (hydroxybenzoic, hydroxyphenylacetic and hydroxycinnamic
18 acids) and their ester derivates it is known that antioxidant activity depends on the number of
19 hydroxyl groups in the molecule that are affected by steric hindrance from their carboxylate
20 group [37]. The closeness of the carboxylate group and the hydroxyl groups on the phenolic
21 ring in hydroxybenzoic acids negatively affects their donor–proton ability. As a result, higher
22 antioxidant activities are usually observed on hydroxycinnamic acids (that is, coumaric,
23 caffeic, and ferulic acid) compared to their hydroxybenzoic counterparts [37].

24 Thus, the fruits with the highest content of phenolics (purple-fleshed pitanga,
25 blackberries, and araçá) and that had show quercetin derivatives and cyanidin derivatives,

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which possess a high antioxidant activity as mentioned above, also had the highest antioxidant activity in the DPPH and FRAP assays, since these phenolic compounds may differentially contribute to the antioxidant capacity in these fruits.

4. Conclusions

Results revealed that the fruits with the highest content of phenolics (purple-fleshed pitanga, blackberries, and araçá) also had the highest antioxidant activity in the DPPH and FRAP assays. Moreover, we observed that although the butiá had the highest content of ascorbic acid its antioxidant activity in the DPPH and FRAP assays was the lowest one. In the TRAP assay, purple-fleshed pitanga, 'Cherokee' blackberry and butiá showed the highest capacity to scavenge the peroxyl radical. However, the amount of total phenolic compounds was not directly associated with the peroxyl scavenging property, probably because different bioactive compounds can provide increased efficiency or inefficiency in the antioxidant response as observed in TRAP and TAR assays. Several phenolic compounds were identified in fruits, such as gallic acid derivatives, quercetin derivatives, quercitrin, isoquercitrin, cyanidin derivatives, among others, which may differentially contribute to the antioxidant capacity. These data reinforce the importance of a regular fruit intake to provide antioxidant polyphenols for the human diet and indicate that purple-fleshed pitanga, blackberries and araçá had a great antioxidant potential. However, more studies are necessary to identify and quantify all the phenolic compounds present in these fruits and determine the contribution of the major compounds to the antioxidant activity.

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4

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Table 1. Total phenolics content, total carotenoids content and ascorbic acid of some Brazilian native fruits

Fruit	Total phenolics content (mg GAE 100 g^{-1} fw)	Total carotenoids (ug β-carotene g^{-1})	Ascorbic Acid (mg 100 g^{-1})
Butia	359.50±45.2^{d}	3.85±0.74^{b}	9.351±0.06^{a}
Araça	660.19±47.6^{b}	6.27±0.06^{a}	0.095±0.01^{bc}
Orange Pitanga	457.43±15.2^{c}	4.02±0.05^{b}	0.128±0.03^{b}
Red Pitanga	433.84±60.5^{cd}	5.86±0.03^{a}	0.086±0.00^{bc}
Purple Pitanga	799.80±54.7^{a}	3.04±0.06^{b}	0.101±0.01^{bc}
Blackberry “Xavante”	816.50±63.6^{a}	1.04±0.04^{c}	0.010±0.00^{c}
Blackberry “Cheroquee”	718.65±59.0^{b}	0.87±0.05^{c}	0.004±0.00^{c}

Results as mean ± standard deviations (n=3). $^{a\text{-}e}$ Values with the same letters in the same column do not present a significant difference (Tukey’s test, $p<0.05$). Butia: *Butia eriospatha;* Araça: *Psidium cattleianum;* Pitanga: *Eugenia uniflora*; Blackberry: *Rubus sp.*

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Table 2. Tentative identification of phenolic compounds in ethanolic extract of some Brazilian native fruits

peak no.[c]	t_R (min)	DAD characteristics absorption maxima (nm)	Fruit	tentative characterization[a]
Benzoic acid derivatives, Hydroxycinnamic acid derivatives and Ellagic acid derivatives (280 nm)				
1; 3; 15; 16; 24; 69; 70; 71	18.3; 18.3; 18.5; 18.5; 18.7; 25.6; 25.9; 25.7; 25.6; 28.5; 24.1; 19.1; 20.7; 24.9	225, 272	Orange, red and purple pitanga; Xav.; Cher.; butiá	gallic acid derivative
2; 58	20.6; 20.8; 20.9; 20.9; 20.3	225, 277	Orange, red and purple pitanga; araçá	gallic acid derivative
57	18.9	216, 272	Araçá	gallic acid derivative
72	25.8	272	Butiá	gallic acid derivative
20; 80	68.9; 63.9	263, 325	Red pitanga; Butiá	protocatechuic acid derivative
42	69.5	268, 325, 357	Xav.	protocatechuic acid derivative
74	56. 8	225, 287, 325	Butiá	protocatechuic acid derivative
79	52.5	216, 239, 325	Butiá	caffeic acid derivative
77	46.9	249, 325	Butiá	chlorogenic acid derivative
37	71.6	216, 249, 362	Xav.	ellagic acid derivative
38	62.6; 63.3	253, 362	Xav. and Cher.	ellagic acid derivative
44	71.6	216, 249, 362	Xav.	ellagic acid derivative
48	61.2	216, 253, 362	Cher.	ellagic acid derivative
Flavonols (360 nm)				
4	66.1; 66. 2; 64.2	225, 258, 348	Orange, red and purple pitanga	quercetin derivative
6; 60; 75	67.7; 67.7; 66.9; 66.7	220, 253, 354	Orange and red pitanga; Araçá; Butiá	quercetin derivative
7; 25; 51; 61; 67; 68	71.9; 71.9; 69.9; 65.1; 68.4; 68.7; 66.9; 68.7	225, 253, 354	Orange, red and purple pitanga; Cher.; Araçá	quercetin derivative
8; 10; 27; 50	73.3; 73.3; 71.3; 68.9; 59.2; 67.2	225, 258, 354	Orange, red and purple pitanga; Cher.	quercetin derivative
21; 34; 35; 49; 81	70. 6; 63.2; 66.6; 63.9; 69.9	220, 258, 354	Red pitanga, Xav.; Cher.; Butiá	quercetin derivative
29; 30;	68.6; 69.4; 62.1	225, 263, 354	Purple pitanga; Araçá	quercetin derivative

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65				
36; 41	67.8; 67.9	225, 354	Xav.	quercetin derivative
39	63.2	263, 354	Xav.	quercetin derivative
40; 73; 78	65.9; 49.9; 49.9	253, 354	Xav.; Butiá	quercetin derivative
43	71.1	225, 272, 354	Xav.	quercetin derivative
53	71.9	216, 253, 354	Cher.	quercetin derivative
66	66.4	216, 354	Araçá	quercetin derivative
83	79.9	216, 253, 349, 357	Butiá	quercitrin derivative
82	70.6	253, 354	Butiá	quercetin-3-rutinoside
84	93.9	253, 354	Butiá	quercetin-3-O-galactoside
32; 22	91.5; 92.2; 92.7	225, 253, 371	Red and Purple pitanga; Xav.	Quercetin
62; 63	73.9; 76.6	225, 282, 335	Araçá	apigenin derivative
5	67.0; 67.1; 65.7; 66.6; 66.6; 67.5; 64.6	225, 253, 354	Orange, red and purple pitanga; Xav.; Cher.; Araçá; Butiá	quercetin-3-b-D-glucoside
9	74.0; 74.0; 71.9; 72.4	225, 253, 349	Orange, red and purple pitanga; Xav.	quercetin-3-rhamnoside
11; 13	71. 4; 71.4; 87.5	220, 263, 344	Orange and red pitanga	kaempferol derivative
19	87.5	263, 349	Red pitanga	kaempferol derivative
12; 17; 26; 52; 54	79.9; 79.9; 77.6; 79.9; 85.0; 69.2; 72.7	225, 263, 349	Orange, red and purple pitanga; Cher.	kaempferol derivative
		Anthocyanins (520 nm)[b]		
14	43.8; 43.9; 42.6; 43.0; 43.6	230, 277, 517	Orange, red and purple pitanga; Xav.; Cher.	cyanidin-3-glucoside
23; 46	40.7; 39.6; 52.1	225, 517, 527	Red and purple pitanga; Xav.	cyanidin derivative
33	47.6	225, 287, 537	Purple pitanga	malvidin derivative
47	56.8; 57.5	225, 277, 532	Xav.; Cher.	delphinidin derivative

Xav. = blackberry “Xavante”; Cher. = Blackberry “Cheroquee”.

[a] Characterization based on standard retention time and standard UV – vis spectra.

[b] Chromatograms not showed.

[c] Peak numbers correspond to Figures 1, 2 and 3. The peaks were numbered according to their t_R and DAD characteristcs absorption maxima (nm), being the same peaks received the same number in all samples, but different peaks with the same DAD characteristcs absorption maxima (nm) received different number.

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Table 3. Antioxidant capacity and non-enzymatic potential of some Brazilian native fruits extracts

	DPPH (IC_{50} mg L^{-1})*	FRAP (µmol $FeSO_4.7H_2O$ g^{-1} fw)	TRAP (AUC)**	TAR (I_0/I)**
Butia	253.80±25.4^{a}	9.32±0.9^{d}	25.73±0.23^{ab}	72.05±0.01^{c}
Araça	48.05±12.1^{de}	89.09±13.0^{a}	26.14±0.34^{a}	71.62±10.63^{c}
Orange Pitanga	110.91±18.9^{bc}	33.17±2.8^{cd}	26.19±0.17^{a}	81.80±9.27^{ab}
Red Pitanga	121.87±8.3^{b}	23.43±4.4^{d}	25.90±0.40^{a}	88.94±4.89^{a}
Purple Pitanga	36.78±5.8^{e}	81.62±10.1^{a}	25.27±0.31^{b}	68.30±8.97^{c}
Blackberry "Xavante"	44.70±2.1^{de}	52.51±3.3^{bc}	26.21±0.15^{a}	72.85±7.01^{ab}
Blackberry "Cheroquee"	78.25±8.1^{cd}	66.60±4.3^{ab}	25.59±0.17^{ab}	68.78±4.77^{c}

Results as mean ± standard deviations (n=3). $^{a-e}$ Values with the same letters in the same column do not present a significant difference (Tukey's test, $p<0.05$). TRAP - total reactive antioxidant potential.TAR - total antioxidant reactivity. Butia: *Butia eriospatha;* Araça: *Psidium cattleianum;* Pitanga: *Eugenia uniflora*; Blackberry: *Rubus sp.* * The results are expressed as mg of fresh fruit per liter of extract. ** For all fruits TRAP and TAR values were obtained using an extract amount equivalent to 66 µg fruit.

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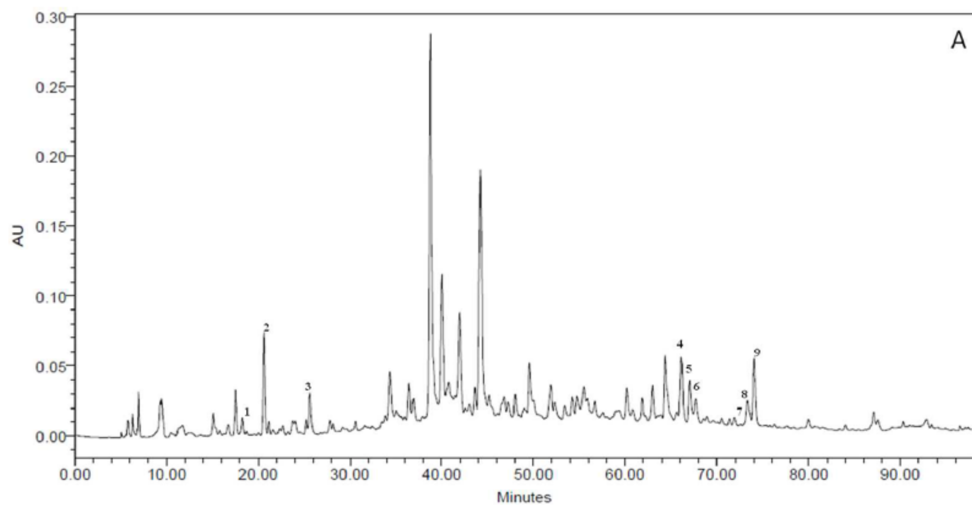

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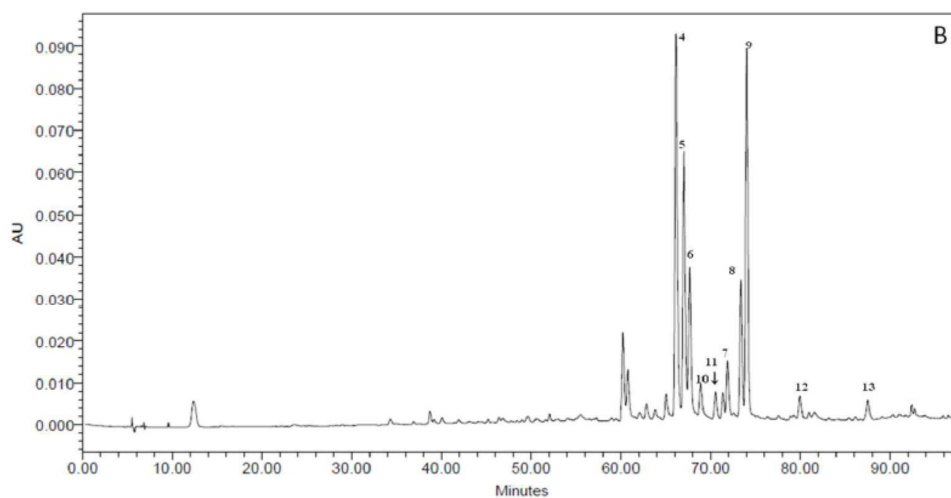

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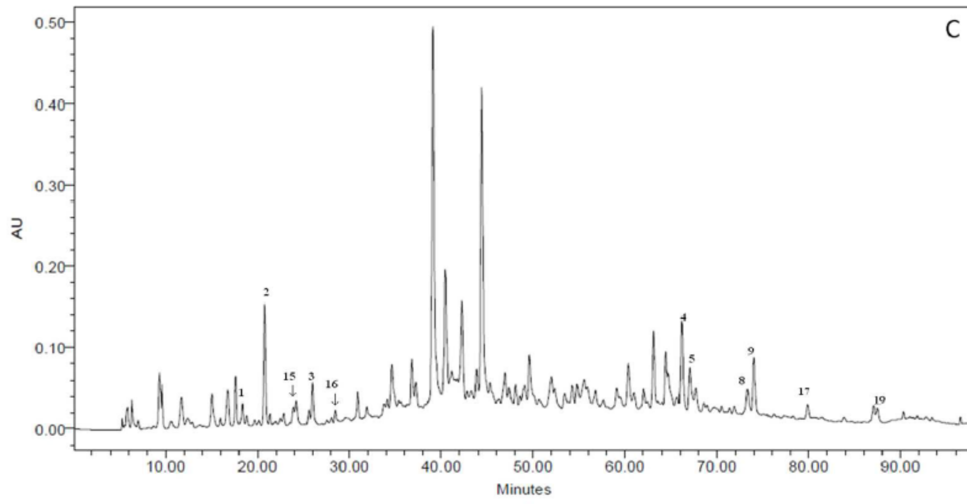

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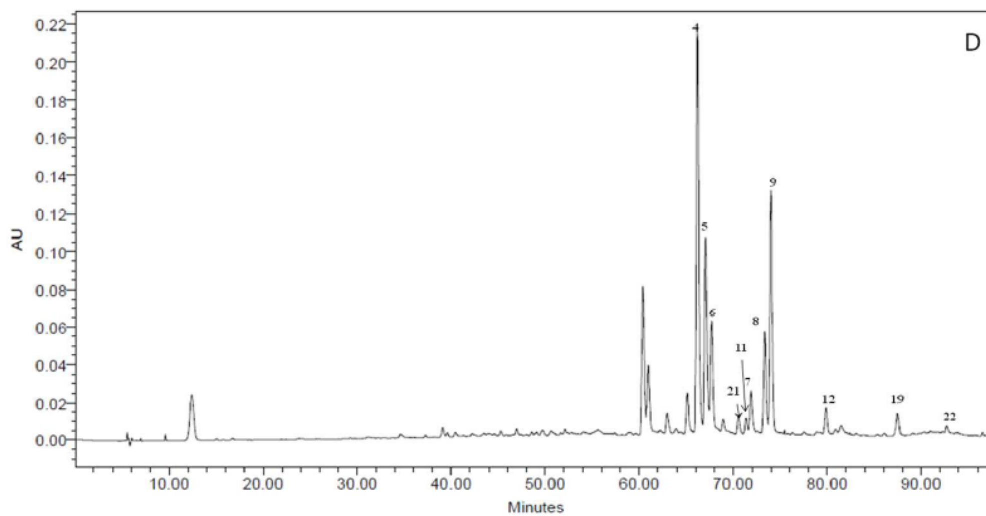

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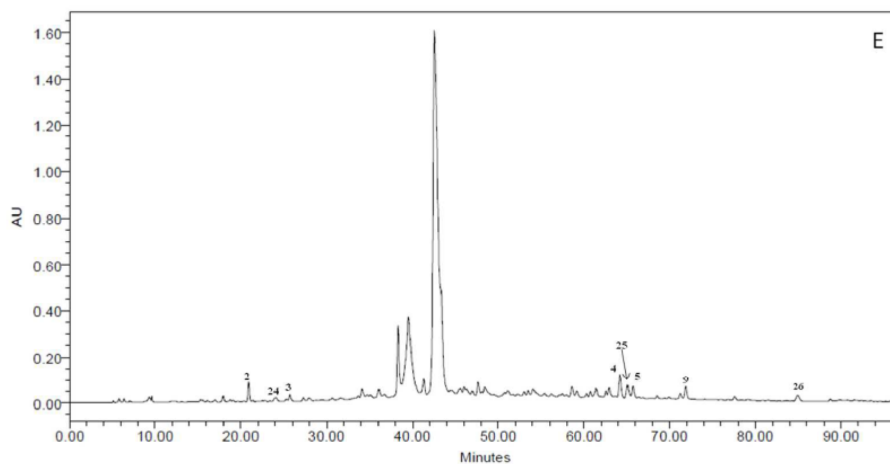

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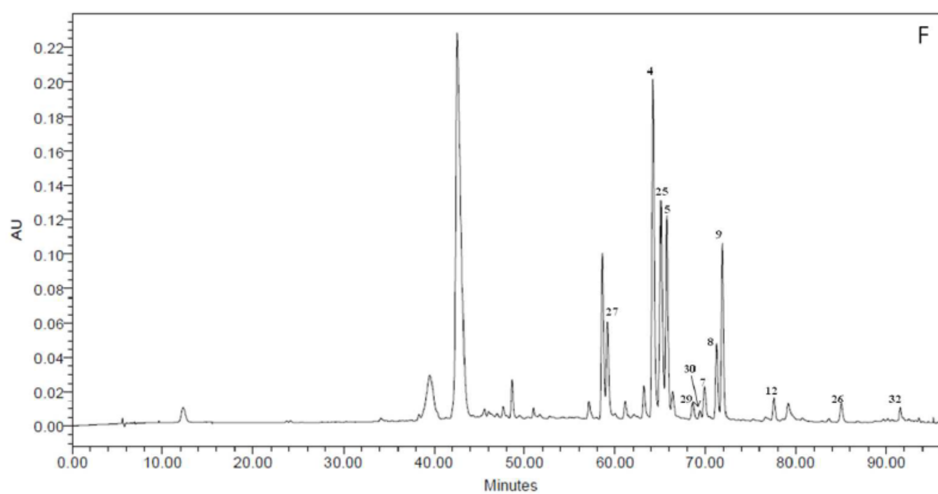

Figure 1. HPLC chromatograms of pitanga extracts of three different varieties: orange 280 nm (A) and 360 nm (B); red 280 nm (C) and 360 nm (D); purple 280 nm (E) and 360 nm (F). For peak numbers, see Table 2.
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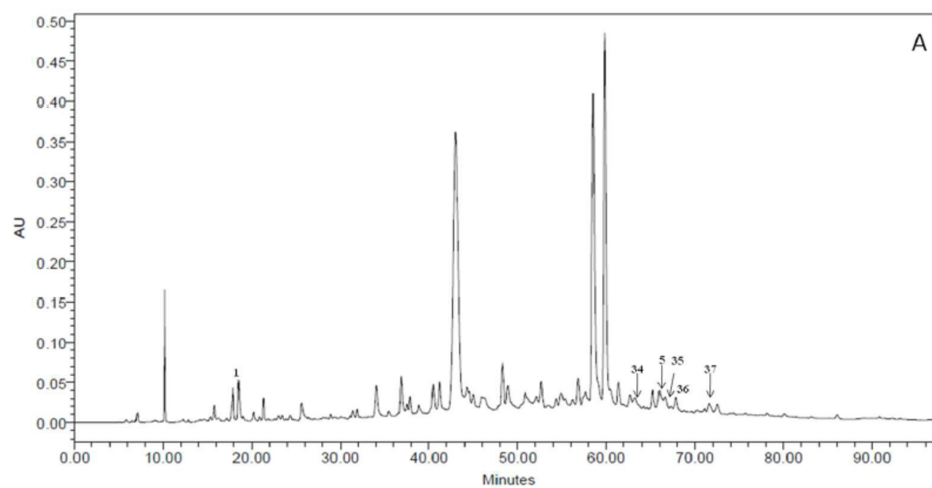

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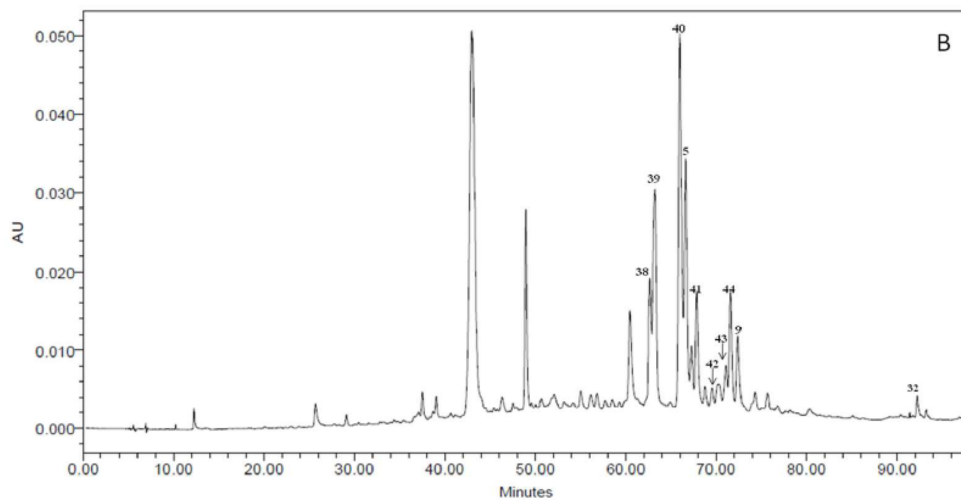

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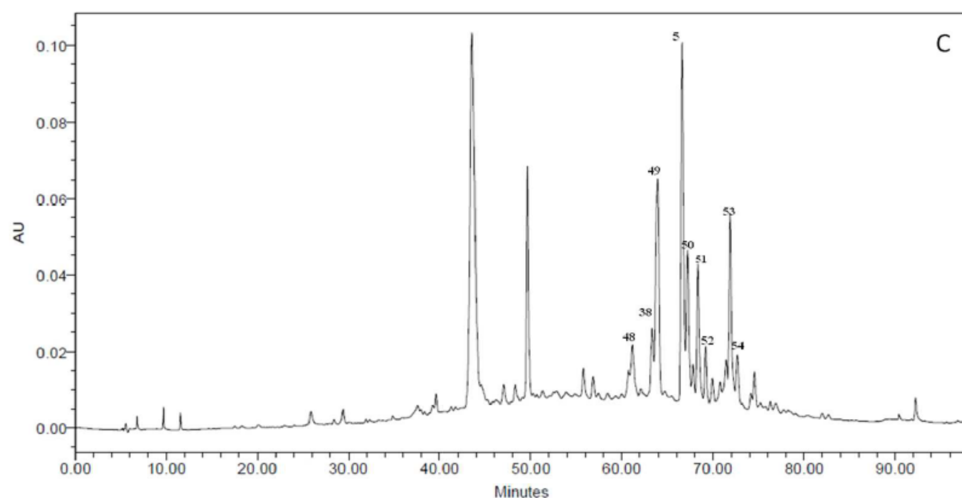

Figure 2. HPLC chromatograms of blackberry extracts of two different varieties: Xavante 280 nm (A) and 360 nm (B); Cheroquee 360 nm (C). For peak numbers, see Table 2.
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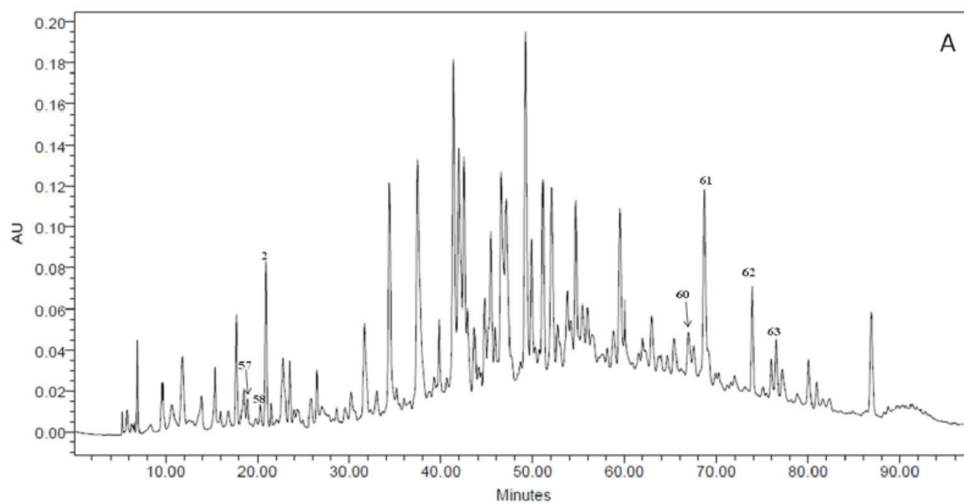

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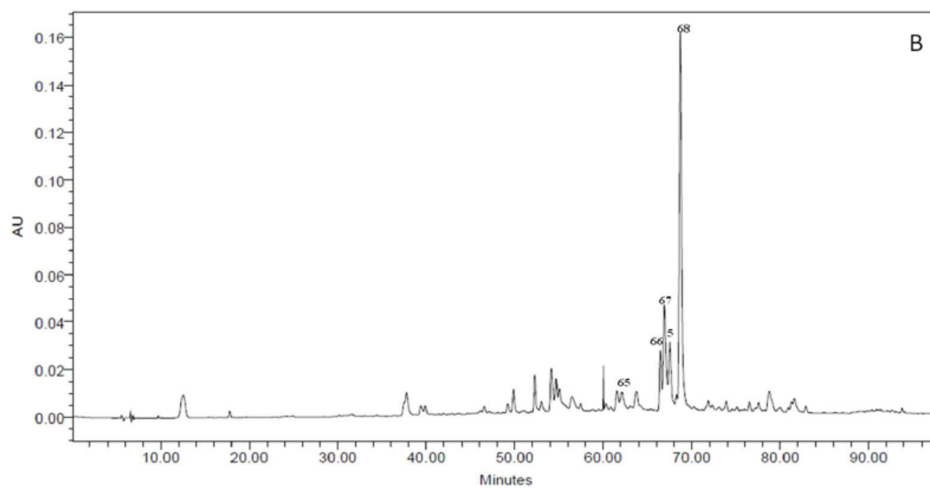

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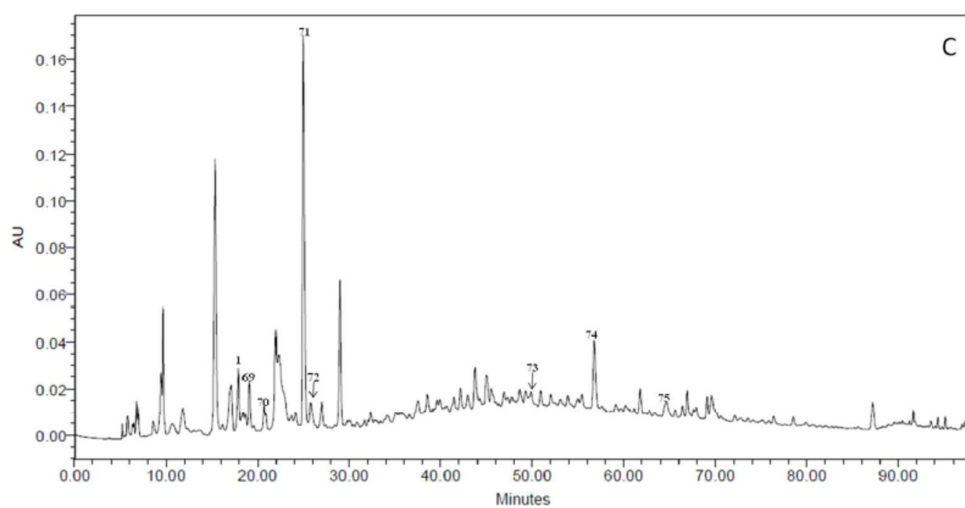

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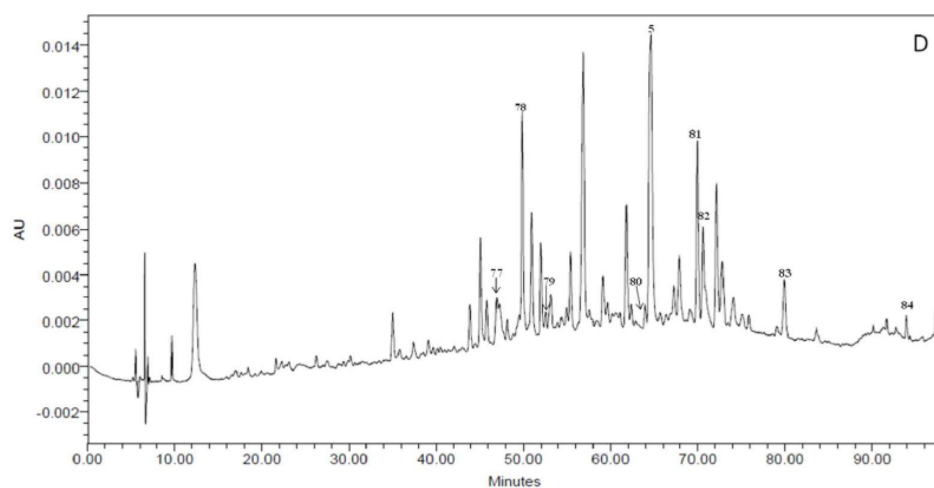

Figure 3. HPLC chromatograms of araçá and butiá extracts: araçá 280 nm (A) and 360 nm (B); butiá 280 nm (C) and 360 nm (D). For peak numbers, see Table 2.
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For Peer Review

CAPÍTULO II

Antiproliferative and cytotoxic effects of purple pitanga (Eugenia uniflora L.) extract on activated hepatic stellate cells

Cristiane C. Denardin, Mariana M. Parisi, Leo A. M. Martins, Silvia R. Terra, Radovan Borojevic, Márcia Vizzotto, Marcos L. S. Perry, Tatiana Emanuelli, Fátima T. C. R. Guma

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Antiproliferative and cytotoxic effects of purple pitanga (*Eugenia uniflora* L.) extract on activated hepatic stellate cells

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The presence of phenolic compounds in fruit- and vegetable-rich diets has attracted researchers' attention due to their health-promoting effects. The objective of this study was to evaluate the effects of purple pitanga (*Eugenia uniflora* L.) extract on cell proliferation, viability, mitochondrial membrane potential, cell death and cell cycle in murine activated hepatic stellate cells (GRX). Cell viability by 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was significantly decreased on cells treated with 50 and 100 $\mu g\ ml^{-1}$ of purple pitanga extract for 48 and 72 h, and the percentage of dead cell stained with 7-amino-actinomycin D was significantly higher in treated cells. The reduction of cell proliferation was dose dependent, and we also observed alterations on cell cycle progression. At all times studied, GRX cells treated with 50 and 100 $\mu g\ ml^{-1}$ of purple pitanga showed a significant reduction in cellular mitochondrial content as well as a decrease in mitochondrial membrane potential. Furthermore, our results indicated that purple pitanga extract induces early and late apoptosis/necrosis and necrotic death in GRX cells. This is the first report describing the antiproliferative, cytotoxic and apoptotic activity for *E. uniflora* fruits in hepatic stellate cells. The present study provides a foundation for the prevention and treatment of liver fibrosis, and more studies will be carried to elucidate this effect. Copyright © 2013 John Wiley & Sons, Ltd.

KEY WORDS—liver fibrosis; *Eugenia uniflora*; cytotoxicity; cell cycle arrest; death cell

INTRODUCTION

Hepatic fibrosis is a very common disease and results from many forms of chronic liver injuries, for example, persistent viral infections, autoimmune liver diseases, toxins, alcohol and hereditary metal overload. Regardless of causes, liver fibrosis is characterized by increased and altered deposition of newly formed extracellular matrix (ECM) components such as collagen, proteoglycans, fibronectin and hyaluronic acid, leading to the complications of portal hypertension, esophageal varices and hepatic failure. This is a severe disease with high morbidity and mortality, representing a serious worldwide healthcare problem and effective antifibrotic treatments are urgently needed.[1]

Hepatic stellate cells (HSCs), also called Ito cells, vitamin A–storing cells, lipocytes or fat-storing cells, have now been clearly identified as the primary cellular source involved in the pathogenesis of liver fibrosis. During the development of liver fibrosis, stellate cells undergo activation, a process characterized by increased cell proliferation, morphological transformation into myofibroblast-like cells and synthesis of excessive ECM components.[2] It has been reported that HSCs are involved in the development and regeneration of liver tissue, reorganization of hepatic ECM, development of hepatic fibrosis or cancer cell invasiveness.[3] The activation and the proliferation of HSC play a key role in fibrogenesis, whereas the apoptosis of HSC is associated with resolution of fibrosis. Inhibiting the activation of stellate cells seems to be an attractive strategy for therapy of liver fibrosis.

Eugenia uniflora L. (Myrtaceae) is a widely distributed tree species in South America, mainly in Brazil, Argentina, Uruguay and Paraguay. The leaves are used in popular medicine as infusion for the treatment of fever, rheumatism, stomach diseases and digestive disorders, as well as hypertension, yellow fever and gout. It may also reduce weight, blood pressure and serve as a diuretic.[4,5] Its fruit, which is known as pitanga, Brazilian cherry or Suriname cherry, also shows antioxidant activity inhibiting lipid peroxidation and removing free radicals.[6–8] Recently, pitanga

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leaves were shown to have anti-*Trypanosoma* activity with low toxicity [9]. Like the leaves, pitanga fruits could also have healthy benefits. In the Brazilian food industry, the pitanga fruit has mostly been used to produce juice, which shows good economic potential due to the consumer appeal arising from its high concentrations of antioxidant compounds, such as anthocyanins, flavonols and carotenoids [7,8].

The presence of phenolic compounds in fruit- and vegetable-rich diets have attracted researchers' attention due to their health-promoting effects, which include lowering the risk of cardiovascular diseases, cancer, or other conditions associated with aging. The biological mechanisms behind these effects include protection against free radicals, free radical–mediated cellular signaling, inflammation, allergies, platelet aggregation, ulcers, viruses, tumors and hepatotoxicity.[10] Thus, the objective of this study was to evaluate the effects of purple pitanga extract on cell proliferation, viability, mitochondrial membrane potential, cell death and cell cycle in murine HSCs.

MATERIALS AND METHODS

Preparation of fruit extracts

Samples of purple-fleshed breeding line of pitanga fruits (*E. uniflora*) were obtained from harvest 2009/2010 at Embrapa Temperate Climate (Pelotas, Rio Grande do Sul, Brazil, 31°40′47″S, 52°26′24″W, 60 m) and immediately frozen. The fruits were sampled searching for a mixture of completely ripe fruits from various plant selections with purple flesh. Three independent samples were collected, frozen at −18 °C and transported to the Federal University of Santa Maria.

Fruit extract was prepared from the edible portions of fruits. Briefly, fresh fruit samples were homogenized with an ultra-turrax homogenizer for 5 min in 95% ethanol (1:3, w/v). The homogenates were blended for 30 min, at room temperature, and centrifuged at 1500*g* for 5 min. The supernatant was collected, and the extraction procedure was repeated. The pooled supernatants were concentrated in rotary evaporator at 40 °C. The samples were reconstituted in water and stored at −80 °C.

Total phenolic content was measured according to the Folin–Ciocalteu method adapted from Swain and Hillis.[11] The absorbance was measured at 725 nm, and the results were expressed as chlorogenic acid equivalents (CAE; μg ml^{-1}) using a chlorogenic acid (0–0.4 mg ml^{-1}) standard curve. The stock concentration of purple pitanga extract was 20.725 mg CAE ml^{-1}.

Cell culture and treatments

The immortal HSC line was obtained from livers of C3H/HeN mice that were infected by transcutaneous penetration of cercariae from the *Schistosoma mansoni* BH strain.[12]

The murine HSC cell line, GRX, was established by Borojevic *et al.* [12] and kindly provided by the Cell Bank of Rio de Janeiro (HUCFF, UFRJ, RJ). Cells were routinely maintained in Dulbecco's modified minimum essential medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (GIBCO, Carlsbad, CA, USA) and 2 g L^{-1} HEPES buffer, pH 7.4, lower than 37 °C and 5% CO_2 conditions. The cells were plated (5×10^4 ml^{-1}) in 12- or 24-well plates and cultured for 24 h to reach 60%–70% of confluence before treatment with purple pitanga. Purple pitanga extract was diluted in culture medium to final concentrations of 5, 50 and 100 μg CAE ml^{-1} just before use. The GRX cells were treated with the above-mentioned extract concentrations for 24, 48 and 72 h. Each concentration group included three or four wells. The routinely cultured cells were used as normal controls.

Colorimetric MTT assay and cell number count

MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma Inc., Saint Louis, MO, USA) is a yellow tetrazolium salt that is reduced to purple formazan crystals. The MTT assay is widely used for the assessment of cytotoxicity, cell viability and proliferation studies in cell biology.[13] Preconfluent GRX cells were incubated with different concentrations of purple pitanga extract for 24, 48 and 72 h. Cells were then incubated with 1 mg ml^{-1} MTT for 2 h at 37 °C. Purple crystals were dissolved in dimethylsulfoxide (Sigma Inc.). The absorbance was measured using a spectrophotometric microtitre plate reader (Spectra Max M5; Molecular Devices, Sunnyvale, CA, USA) at 570 nm and 630 nm.

For counting of cell number, preconfluent GRX cells were incubated with different concentrations of purple pitanga extract for 24, 48 and 72 h. Cells were dislodged with 0.25% trypsin-EDTA and counted using a counting chamber.

Tritiated thymidine incorporation assay

GRX cells treated, or not, with purple pitanga extract were incubated (24 h, 1 μCi ml^{-1}) with [6-^{3}H] thymidine ([^{3}H]dT) (specific activity 23.0 Ci $nmol^{-1}$; Amersham Biosciences, Hillerod, Denmark). Subsequently, the medium was removed, cells were washed with phosphate-buffered saline (PBS) and 10% of trichloroacetic acid was added to each well. The cell pellet was then dissolved in 200 μL of 0.1 N NaOH, and the incorporated DNA radioactivity was determined by scintillation counting.[14] The protein content was measured according to Peterson.[15]

MitoTracker Green

MitoTracker® Green FM (MTG; Invitrogen) is a probe that becomes fluorescent once it accumulates in the lipid environment of mitochondria, being an important indicator of cellular mitochondrial content. Briefly, after treatment with purple pitanga extracts, GRX cells were washed with PBS before incubation with 100 nM of MTG diluted in free serum DMEM for 30 min under growth conditions. Then cells were washed with PBS, and the MTG fluorescence was measured in a plate spectrofluorometer (Spectra Max M5; Molecular Devices) after exciting at 490 nm and collecting

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the emission at 516 nm. The results were expressed as relative fluorescence units per microgram of protein.

For confocal images, GRX cells were seeded under coverslips placed on 12-well plates. After treatment, cells were washed with PBS then incubated for 30 min in the dark with 100 nM of MTG (Invitrogen) diluted in serum free DMEM. Cells were fixed with 2% of paraformaldehyde for 20 min at 4 °C and rewashed with PBS before blades mounting with ProLong Gold antifade reagent (Invitrogen). Images were obtained on FV1000 Olympus Confocal Microscope and deconvolved using the free Image J analysis software.

Flow cytometry assays

Cell viability, mitochondrial membrane potential, changes of cell cycle and apoptosis were analyzed by flow cytometry. For all analysis, GRX cells treated or not with the aforementioned extracts were harvested by trypsinization and counted.

The cell viability was evaluated using 7-amino-actinomycin D (7-AAD; BD Bioscience, San Jose, CA, USA). The cells (1×10^6 cells ml^{-1}) were incubated with 5 μL (0.25 μg) of 7-AAD at room temperature for 10 min, washed in PBS and resuspended for FL3-H analysis.

Tetrachloro-1,1,3,3-tetraethylbenzimidazol-carbocynine-iodine (JC1; BD Bioscience) is a fluorescent dye that exists as green-emitting monomers at low mitochondrial membrane potential, or as red-emitting aggregates when mitochondrial membrane potential increases.[16] The cells (1×10^6 cells ml^{-1}) were incubated with 500 μL of JC1 solution for 15 min, washed two times with buffer according to the manufacturer's manual and analyzed for FL1-H and FL2-H.

For cell cycle analysis, the cells were washed in PBS and resuspended in 400 μL (1×10^6 cells ml^{-1}) of cell cycle solution (3.5 mM trisodium citrate, 0.5 mM Tris, 0.1% Nonidet, 100 μg ml^{-1} RNAse A, 50 μg ml^{-1} propidium iodide) and incubated in the dark at room temperature for 15 min. The DNA content was then analyzed.

Apoptosis was analyzed with annexin-V FITC conjugate kit (Invitrogen). The cells were washed with PBS and resuspended at 1×10^6 cells ml^{-1} in annexin-V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$); 10^5 cells ml^{-1} (100 μL/tube) were incubated with 5 μL of Annexin-V FITC and 5 μg ml^{-1} of propidium iodine, mixed and incubated for 15 min at room temperature in the dark. Four hundred microlitres of binding buffer was added to each tube and cells were analyzed for FL1-H and FL3-H.

The detection of caspase activation was performed using the "CaspACE, FITC-VAD-fmk In Situ Marker" (Promega, Madison, USA). Briefly, 1×10^6 cells were washed in PBS, suspended in 100 μL staining solution containing 50 μM of the fluorescein isothiocyanate conjugate of z-VAD-fmk (FITC-VAD-fmk) and incubated for 20 min at 30 °C in the dark. Then cells were washed once and suspended in PBS, and cells were analyzed for FL1-H.

All analyses were performed using a FACScan Calibur flow cytometer (BD Bioscience) equipped with a blue laser emitting 488 nm and a red laser emitting 633 nm.

Statistical analysis

Data were reported as mean ± SD. Results were analyzed by one-way ANOVA followed by Tukey's test ($P < 0.05$). All analyses and graphical were performed using the statistical software GraphPad Prism 5 for Windows (GraphPad Software Inc., version 5.01, San Diego, USA).

RESULTS

Purple pitanga extract inhibits cell growth and interferes with cell viability in GRX cells

The effects of purple pitanga extract on cell viability were assessed by MTT assay and flow cytometry 7-AAD stain (Figures 1A and 1B). The results show that the cell viability by MTT assay was significantly decreased on cells treated with 50 μg ml^{-1} of purple pitanga extract for 72 h and on cells treated with 100 μg ml^{-1} for 48 and 72 h (Figure 1A). Furthermore, the percentage of dead cell stained with 7-AAD was significantly higher in cells treated with 50 μg ml^{-1} of purple pitanga extract for 48 and 72 h and 100 μg ml^{-1} of purple pitanga extract at all times studied (Figure 1B). 7-AAD is used as a cell viability stain: cells with compromised membranes will stain with 7-AAD, whereas live cells with intact cell membranes will remain dark.

The antiproliferative effects of purple pitanga extract were assessed by cell counting and thymidine incorporation assay (Figures 1C and 1D). The reduction of cell proliferation was dose dependent at the cell counting assay and the cells treated with 100 μg ml^{-1} of purple pitanga extract visually not increased in three days of treatment (Figure 1C). This effect was confirmed by the thymidine incorporation assay, where the decrease in GRX cell proliferation was dose and time dependent (Figure 1D). In three days of treatment, all concentrations of purple pitanga extract significantly decreased GRX cell proliferation.

Impaired mitochondrial membrane potential and decreased mitochondrial content induced by purple pitanga extract in GRX cells

Mitochondrial content was assessed by MTG probe (Figures 2A and 2C), which is now commonly used for measurement of mitochondrial shape changes, mass or swelling.[16,17] Mitochondrial content was significantly reduced in GRX cells treated with 50 and 100 μg ml^{-1} of purple pitanga at all times studied (Figures 2A and 2C).

The mitochondrial membrane potential was measured by JC1 (Figures 2B and 2D), which is a dye that changes its fluorescence emission from red to green when mitochondria loss its membrane polarization. The results show that JC-1 exists as aggregates within the mitochondria (red) in control cells. In cells treated with 50 (for 72 h) and 100 μg ml^{-1} of purple pitanga extract (for 48 and 72 h), JC-1 exists primarily as the monomeric form (green) within the cytoplasm, indicating less membrane potential related to the reduced mitochondrial content as shown by MTG (Figures 2B and 2D).

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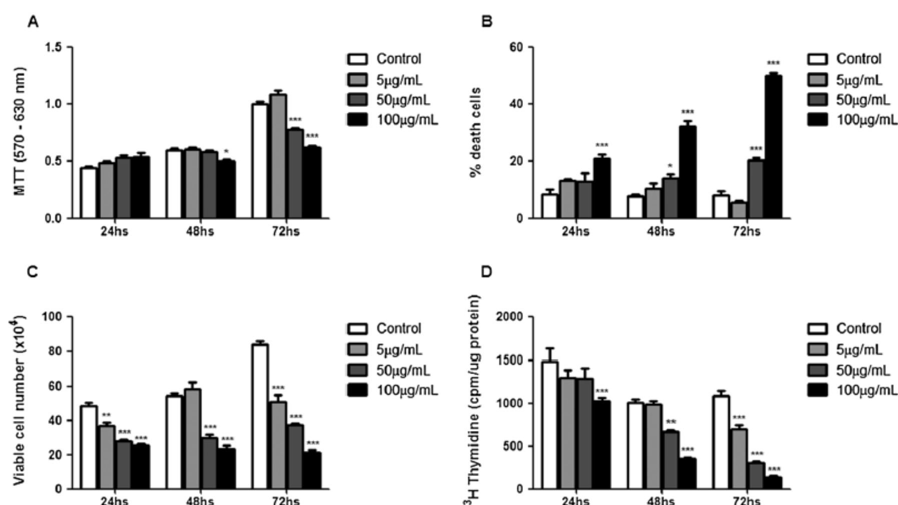

Figure 1. Effect of purple pitanga (*Eugenia uniflora* L.) extract on cell viability and proliferation in GRX cells. The cells were treated with 5, 50 and 100 µg CAE ml^{-1} concentrations of purple pitanga extract for 24, 48 and 72 h. Cell viability measured by (A) MTT assay and (B) 7-AAD analyzed by flow cytometry. Cell proliferation measured by (C) cell counting and (D) [3H]dT incorporation assay. Values are shown as mean ± SD of three independent experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus untreated control cells; 278 × 168 mm (96 × 96 DPI)

Purple pitanga extract affect the cell cycle progression in GRX cells

The effects of purple pitanga extract on cell proliferation could be due to its actions on cell cycle (Figure 3). Cell cycle progression in GRX cells treated with purple pitanga extract was analyzed by flow cytometry. The cells treated with 50 and 100 µg ml^{-1} of purple pitanga extract for 24 h showed a 13% increase in the number of GRX cells in G_0G_1 phase and reduction in the S phase (Figure 3). Although the increase in the G_0G_1 phase persists with time, only treatment with 50 µg ml^{-1} of purple pitanga extract was significant at 48 and 72 h. Furthermore at 72 h, the treatment with 50 and 100 µg ml^{-1} of purple pitanga leads to an increase of cells in the G_0G_1 phase with a concomitant decrease in S phase and the disappearance of G_2M phase. The 100-µg ml^{-1} treatment also results in an increase in apoptotic sub-G_1 population (Figure 3). The sub-G_1 population represents cells with significant DNA damage that was confirmed by the annexin V/PI assay.

Apoptotic effect of purple pitanga extract in GRX cells

Purple pitanga extract-induced apoptosis in GRX cells was examined and confirmed by annexin V and PI staining, using FACS analysis. We did not observe an increase in apoptosis in cells treated for 24 and 48 h. However, the percentage of necrotic cells increased significantly in cells treated with 50 and 100 µg ml^{-1} for 48 h (Figure 4A). As shown in Figure 4B, the percentage of viable cells was reduced from 91.07% to 51.58% by the higher treatment with purple pitanga extract for 72 h. The largest apoptotic and necrotic effects were observed on cells treated for 72 h, where we observed a significant increase in early apoptosis in cells treated with 5 µg ml^{-1}, an increase in late apoptosis and necrosis in cells treated with 50 µg ml^{-1} and an increase in early and late apoptosis in cells treated with 100 µg ml^{-1} of purple pitanga extract. These results indicate that purple pitanga extract induces the apoptotic process in early and late stages and induces necrosis in GRX cells.

DISCUSSION

Brazil features the largest biodiversity in the world; however, only 8% have been studied in search for bioactive compounds [9]. *E. uniflora* L. is often used as a food and in folk medicine due to many biological activities. Their leaves are used in infusions or decoctions in popular medicine to treat hypercholesterolemia, gout, hypertension, digestive disease, rheumatism, inflammations, fever and hepatic disease and as a diuretic, antimicrobial and antioxidant [4,6,18–22]. However, we found no study evaluating the effects of the fruits of this plant that also have a high antioxidant activity. Several phytoconstituents of *E. uniflora* leaves have been isolated, such as flavonoids myricitrin, quercetin and quercetin 3-ramnoside, and steroids, mono- and triterpenoid compounds, tannins, anthraquinones, phenols, cineol and essential oils [23,24] and several phenolic compounds were identified in fruits, such as myricetin and quercetin derivatives, quercitrin, isoquercitrin and cyanidin derivatives, among others, which may contribute differentially to the antioxidant capacity (our unpublished observations)[7].

The liver has an extraordinary capacity to regenerate and restore from damage tissue after chemical or mechanical injury. As previously mentioned, in response to liver injury, HSC lose vitamin A droplets and undergo significant morphological and functional changes, a complex process defined as “activation,” leading to the acquisition of a

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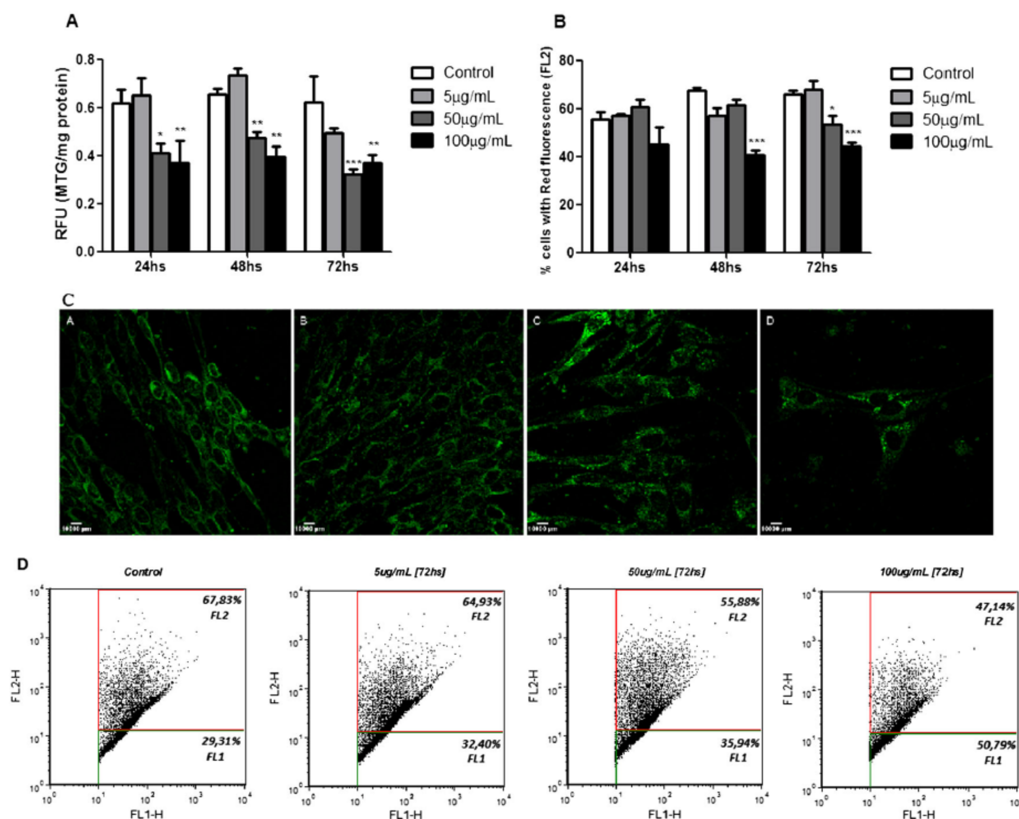

Figure 2. Purple pitanga (*Eugenia uniflora* L.) extract alter mitochondrial membrane potential and mitochondrial mass in GRX cells. The cells were treated with 5, 50 and 100 µg CAE ml^{-1} concentrations of purple pitanga extract for 24, 48 and 72 h. (A and C) Mitochondrial mass assessed by MTG fluorescent probe. (C) A, control untreated cells; B, 5 3 µg ml^{-1}; C, 50 3 µg ml^{-1}; D, 100 3 µg ml^{-1}. (B and D) Mitochondrial membrane potential measured by JC1 fluorescent probe FL2 channel shows the red-emitting aggregates formed when mitochondrial membrane potential increases. Values are shown as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus untreated control cells; 282 × 235 mm (96 × 96 DPI)

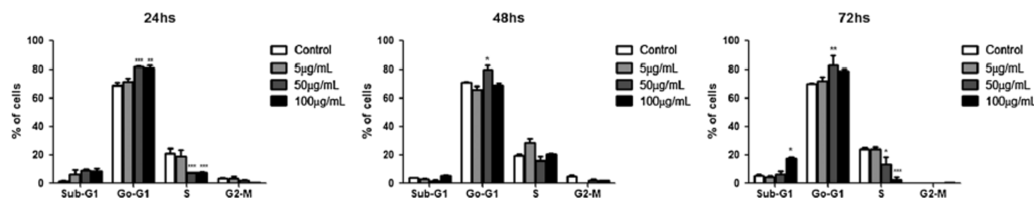

Figure 3. The effect of purple pitanga (*Eugenia uniflora* L.) extract on cell cycle progression in GRX cells. The cells were treated with 5, 50 and 100 µg CAE ml^{-1} concentrations of purple pitanga extract for 24, 48 and 72 h. Values are shown as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus untreated control cells; 406 × 81 mm (96 × 96 DPI)

myofibroblast-like cell phenotype and to the excessive production of collagen. Fibrosis resolution refers to pathways that either drive the stellate cell to apoptosis or contribute to their reversion to a more quiescent phenotype.[2] Thus, the inhibition of HSC activation and proliferation and the induction of the apoptosis of activated HSC have been proposed as potential antifibrotic strategies.

Our results show that the treatment with purple pitanga extract reduced the proliferation and viability of GRX cells. Kawada *et al.* [14] demonstrated that resveratrol, quercetin and *N*-acetylcysteine inhibited the proliferation of rat stellate cells and their expression of smooth muscle α-actin. Similarly, cyanidin 3-glucoside inhibited the cell proliferation of HSC isolated from rat liver induced by a pro-oxidant agent. This effect appeared to be directly related to the inhibition of type I collagen synthesis rather than to the antioxidant activity.[25] Previous studies from our research group evaluating the profile of phenolic compounds in purple pitanga extract observed the presence of various flavonoids and anthocyanins, and cyanidin 3-glucoside and quercetin derivatives were

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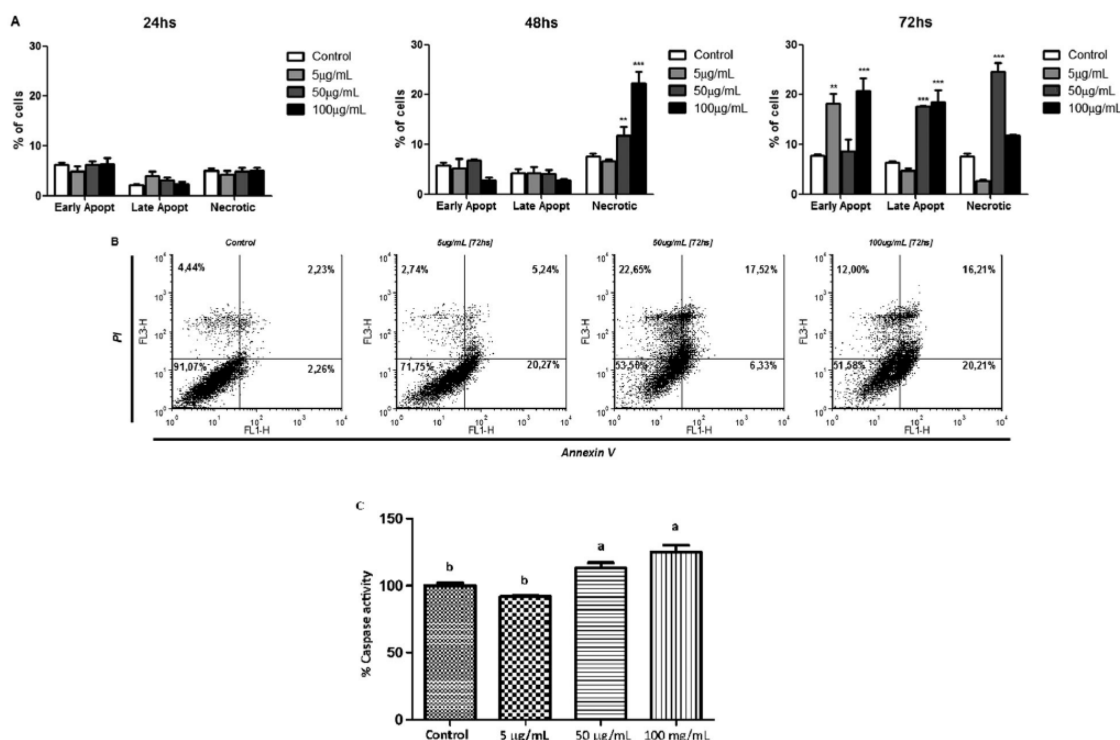

Figure 4. Apoptotic effects of purple pitanga (*Eugenia uniflora* L.) extract in GRX cells. The cells were treated with 5, 50 and 100 µg CAE ml^{-1} concentrations of purple pitanga extract for 24, 48 and 72 h. (A) Apoptosis analyzed with annexin-V FITC Conjugate Kit. (B) Nonapoptotic cells: annexin-V negative and PI negative; early apoptotic cells: annexin-V positive and PI negative; late apoptotic cells: annexin-V positive and PI positive; necrotic cells: annexin-V negative and PI positive. (C) Detection of caspase activation using the "CaspACE, FITC-VAD-fmk In Situ Marker" for 72 h; different letters indicate statistical difference. Values are shown as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus untreated control cells; 400 × 274 mm (96 × 96 DPI)

found at significant amounts. In addition, preliminary results evaluating the type I collagen expression by RT-PCR has observed a significant reduction in GRX cells treated with 5 and 50 µg ml^{-1} of purple pitanga extract (data not shown). Thus, the antiproliferative effect and the reduced viability observed in the present study may be related to the effect of these phenolic compounds in the resolution of fibrosis and may be related to alterations on cell cycle progression and/or activation of cell death via apoptosis and/or necrosis, as we observed.

Because DNA content increases due to the cell proliferation, the potential of stellate cells to proliferate can be assessed as the increased number of the cells in the S and G_2/M phases as well as the decreased number in the G_0G_1 phase. Our data suggest that purple pitanga extract has an inhibitory effect on GRX cell proliferation, which may be associated with G_0G_1 cell cycle arrest. Studies using several kinds of popular herbs of Japanese medicine also found antiproliferative effects by inducing arrest at the G_0G_1 phase in the cell cycle of HSCs.[26,27] Furthermore, studies using isolated phenolic compounds as quercetin and baicalein also found similar results. Quercetin arrested HSCs at G_1 phase with a selective decrease in the cellular levels of cyclin D1 a cell cycle-related protein of G_1 phase.[14,28] Many flavonoids alter the expression and activities of numerous enzymes involved in the regulation of cell cycle in cancer cell lines.[29–31] Thus, the cell cycle arrest observed in this study may be attributed to the presence of phenolic compounds in the purple pitanga extract, like quercetin and other flavonoids that may be acting individually or synergistically.

Mitochondria are membrane-enclosed organelles found in most eukaryotic cells. In addition to supplying cellular energy, mitochondria are involved in a range of other processes, such as signaling, cellular differentiation, cell death, and the control of the cell cycle and cell growth.[32] The respiratory chain pumps protons to the intermembrane space, generating an electrochemical gradient across the mitochondrial inner membrane consisting of mitochondrial membrane potential and a minor pH gradient. Mitochondrial membrane potential is a widely used bioenergetic parameter affecting multiple mitochondrial functions including ATP synthesis, Ca^{++} sequestration, protein import, mitochondrial fusion, mitochondrial autophagy and the generation of reactive oxygen species.[33] Our results demonstrate a reduction of mitochondrial membrane potential measured by JC1 fluorescent probe accompanied by decrease of mitochondrial mass observed by MTG. These results can be related with the antiproliferative effect of purple pitanga extract, the increase of

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cytotoxicity observed by 7-AAD and mainly the increase in early and late apoptosis and necrosis observed in this study.

Mitochondrial depolarization is frequently attributed to mitochondrial respiratory dysfunction.[16] Furthermore, the opening of mitochondrial membrane permeability transition pores results in a collapse of mitochondrial membrane potential and cessation of adenosine triphosphate synthesis. In addition, the release of intermembrane proteins, such as apoptosis-inducing factor and endonuclease G and their translocation to the nucleus, leads to nuclear DNA fragmentation. Together, these events trigger cell death. Alternatively, the release of cytochrome c and other proapoptotic factors from mitochondria can promote caspase activation and apoptotic cell death.[34] Thus, the marked reduction in mitochondrial mass and mitochondrial membrane potential observed in cells treated with purple pitanga extract could be caused by activation of apoptosis and necrosis pathways as observed as an increase in sub-G1 cell populations and annexin V and PI positive cells.

As previously mentioned, the inhibition of HSCs activation and proliferation and the induction of apoptosis have become potentially important for the prevention and treatment of hepatic fibrosis. Apoptosis is a normal physiological process during development and cellular differentiation. "Programmed cell death" can be triggered experimentally by a variety of physical or chemical stressors. It can be induced by a range of stimuli such as ultraviolet irradiation, hyperthermia and cytotoxic chemotherapy. In contrast to necrosis, apoptosis is a well-regulated physiological process, and any disturbance of the balance between cell proliferation and cell death maintained by apoptosis can result in serious diseases. The property of many flavonoids to alter the expression and activities of numerous enzymes involved in the regulation of apoptosis may be the reason for the observed cytostatic properties and the induction of apoptosis in many cell types. Myricetin, quercetin, isorhamnetin and kaempferol have been shown to induce apoptosis in human acute myeloid leukemic cells (HL-60).[31] Furthermore, neferine and rosmarinic acid reduced cell proliferation and induced apoptosis in rat HSC line HSC-T6.[35,36] According to Ding *et al.* [35], neferine induces the apoptosis of HSC-T6 cells by increasing the activation of caspase 3, that is, by mitochondrial pathway. Therefore, the marked increase in apoptosis and necrosis observed in cells treated with purple pitanga extract for 72 h may be related to the mitochondrial apoptotic route because it also observed a reduction in mitochondrial mass and membrane potential. Moreover, we observed by flow cytometry an intense activation of caspases, which reinforces this theory. Further studies are being conducted to elucidate the exact route that is being activated.

In the present study, we demonstrated that purple pitanga extract leads to an inhibition of proliferation, alterations on cell cycle progression and GRX cells apoptosis. This is the first report on the antiproliferative, cytotoxic and apoptotic activity for *E. uniflora* fruits in HSCs. Further studies are being conducted to determine the molecular mechanisms underlying the cell cycle arrest and the apoptosis induced by purple pitanga extract.

In conclusion, our results suggest that the inhibitory effect of purple pitanga extract on HSCs was promoted by the reduction on proliferation probably via G_0G_1 cell cycle arrest and cytotoxicity observed with a consequent increase in apoptosis and necrosis. Our results also suggest that apoptosis is triggered via mitochondria mainly due to the activation of caspases and the reduction in mitochondrial membrane potential and cellular mitochondria content. The present study provides a foundation for the prevention and treatment of liver fibrosis, and more studies *in vivo* will be carried to elucidate this effect.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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CAPÍTULO III

Purple pitanga (*Eugenia uniflora* L.) extract induces autophagy on activated hepatic stellate cells

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1 **Purple pitanga (*Eugenia uniflora* L.) extract induces autophagy on activated hepatic**

2 **stellate cells**

3

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18 RUNNING HEAD: PURPLE PITANGA INDUCES AUTOPHAGY IN HSCs

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Conflicts of interest: None.

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1 **ABSTRACT**

2 Liver fibrosis has traditionally been regarded as an irreversible process. However,
3 accumulating evidence indicates that even advanced fibrosis may, in fact, be a reversible
4 condition, but no drug has yet been approved for the treatment of liver fibrosis in humans.
5 Therefore, the aim of this work was to verify the presence of autophagy in activated hepatic
6 stellate cells (GRX) treated with purple pitanga (*Eugenia uniflora* L.) extract that has a high
7 antioxidant capacity. The cytoplasmatic granular intensity analyzed by flow cytometry was
8 significantly increased in treatments with 50 and 100 μg/mL of purple pitanga extract for 72h.
9 The presence of acidic vacuolar organelles stained by acridine orange indicate that the
10 presence of autophagosomes were significantly higher in GRX cells treated with 50 and 100
11 μg/mL of purple pitanga extract. Autophagy was molecularly confirmed by increase in the
12 expression of autophagy-related protein Atg7 in the cells treated with 5 and 50 μg/mL of
13 purple pitanga extract. We observed that the purple pitanga extract increase the number of
14 autophagosomes and autolysosomes when ultrastructural analysis of the cells was performed
15 by transmission electron microscopy. Furthermore our results indicate that purple pitanga
16 extract induces the mitophagy in a dose-dependent manner. This is the first report indicating
17 the increase of autophagic activity in GRX cells treated with *E. uniflora* fruits. The present
18 study provides a foundation for the prevention and treatment of liver fibrosis and more studies
19 will be carried to elucidate this effect.

20

21 **KEY WORDS:** LIVER FIBROSIS; EUGENIA UNIFLORA; AUTOPHAGY; DEATH
22 CELL

23

24

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1 Fibrosis is the essential pathophysiologic consequence of chronic liver injury, and it
2 represents the common underlying mechanism for hepatic insufficiency and for most clinical
3 complications of end-stage liver disease. This fibrotic process results from the accumulation
4 of extracellular matrix (ECM) proteins including collagens, proteoglycans, and glycoproteins.
5 Hepatic stellate cells (HSCs) (previously known as Ito cells, lipocytes, or fat storing cells) are
6 liver-specific pericytes which were identified as a major source of collagen in pathologic
7 fibrosis following their activation to miofibroblast-like cells [Friedman, 2006; Friedman,
8 2008]. HSCs are involved in development and regeneration of liver tissue, reorganization of
9 hepatic ECM, development of hepatic fibrosis, or cancer cell invasiveness [Atzori et al., 2009;
10 Friedman, 2008; Sato et al., 2003].

11 Stellate cell activation refers to conversion of a resting vitamin A-rich cell to one that
12 is proliferating, fibrogenic, and contractile. While it is increasingly clear that other
13 mesenchymal cell populations also contribute to extracellular matrix accumulation, stellate
14 cell activation remains the most dominant pathway leading to hepatic fibrosis [Atzori et al.,
15 2009; Friedman, 2008]. Activation consists of two major phases: initiation (early changes in
16 gene expression and phenotype) and perpetuation (activated phenotype and generating
17 fibrosis), followed by resolution (clearance through apoptosis or reversion to a more quiescent
18 phenotype) if injury subsides. Putative anti-fibrogenic drugs mainly includes agents able to
19 reduce inflammation, agents able to reduce HSC activation, agents with a pro-apoptotic
20 potential for HSC, agents with antioxidant effects, and agents able to increase fibrillar ECM
21 degradation [Atzori et al., 2009; Friedman, 2008; Moreira, 2007; Rockey, 2013].

22 Autophagy is a genetically programmed, evolutionarily conserved process coordinated
23 by a family of genes, called Atg, that lead to the degradation of organelles and proteins. It
24 involves the formation of double-membrane vesicles, containing cellular components that
25 merge to lysosomes, forming the autophagolysosome, where the components are degraded

1 and the products generated are reused by the cell [Levine and Yuan, 2005; Mizushima et al.,
2 2010]. Most tissues have a basal level autophagy which contributes to cellular homeostasis by
3 regulating the routine turnover of cytoplasmic components. However, autophagy can also be
4 induced by various conditions and stresses such as starvation, protein aggregates, virus
5 infection, and oxidative and endoplasmic reticulum (ER) stresses. Increasing evidence now
6 indicates that autophagy is involved in many pathophysiological conditions in various
7 organisms including development, differentiation, tissue remodeling, tissue injury, aging and
8 cancer [Ni et al., 2012]. Liver is one of the most dynamic organs in mammals and humans,
9 and autophagy plays important roles in liver physiology and pathology [Ni et al., 2012;
10 Rautou et al., 2010].

11 Pitangueira (*Eugenia uniflora* L.) is a widely distributed tree species in South
12 America, mainly in Brazil, Argentina, Uruguay and Paraguay, and its fruits are called pitanga
13 or Brazilian cherry with have different flesh colors (purple, red and orange). The leaves are
14 used in popular medicine as infusion for the treatment of fever, rheumatism, stomach
15 diseases, and digestive disorders, as well as hypertension, yellow fever and gout. It may also
16 reduce weight, blood pressure, and be used as a diuretic [Alice et al., 1991; Schmeda-
17 Hirschmann et al., 1987]. Its fruit also shows antioxidant activity inhibiting lipid peroxidation
18 and removing free radicals [Bagetti et al., 2011; Celli et al., 2011; Velazquez et al., 2003]. As
19 the leaves, pitanga fruits could also have healthy benefits due to high concentrations of
20 antioxidant compounds, such as anthocyanins, flavonols and carotenoids [Bagetti et al., 2011;
21 Celli et al., 2011].

22 Liver fibrosis has traditionally been regarded as an irreversible process. However,
23 accumulating evidence indicates that even advanced fibrosis may, in fact, be a reversible
24 condition [Rockey, 2013]. Unfortunately, no drug has yet been approved for the treatment of
25 liver fibrosis in humans. Moreover, due the great Brazilian flora and fauna, several plants that

1 may have therapeutic potential have not been studied as purple pitanga. Therefore, the aim of
2 this work was to verify the presence of autophagy in activated HSCs treated with purple
3 pitanga extract that has a high antioxidant capacity.
4
5 **MATERIALS AND METHODS**
6 REAGENTS
7 Fluorescent dry Acridine Orange (AO) was purchased from Sigma-Aldrich Chemical
8 Co. (St. Louis, MO, USA). MitoTracker® Green FM (MTG), Lysotracker Red DND-99
9 (LYSR) and all culture reagents and medium were purchased from Invitrogen (Carlsbad, CA,
10 USA). All reagents were analytical grade.
11
12 PREPARATION OF FRUIT EXTRACTS
13 Samples of purple-fleshed breeding line of pitanga fruits (*Eugenia uniflora*) were
14 obtained from harvest 2009/2010 at Embrapa Temperate Climate (Pelotas, Rio Grande do Sul,
15 Brazil, 31°40'47''S, 52°26'24''W, 60 m) and immediately frozen. The fruits were sampled
16 searching for a mixture of completely ripe fruits from various plant selections with purple
17 flesh. Three independent samples were collected, frozen at -18 °C and transported to the
18 Federal University of Santa Maria.
19 Fruit extract was prepared from the edible portions of fruits. Briefly, fresh fruit
20 samples were homogenized with an ultra-turrax homogenizer for 5 min in 95% ethanol (1:3,
21 w/v). The homogenates were blended for 30 minutes, at room temperature, and centrifuged at
22 1500 x g for 5 min. The supernatant was collected and the extraction procedure was repeated.
23 The pooled supernatants were concentrated in rotary evaporator at 40°C. The samples were
24 reconstituted in water and stored at -80°C.

1 Total phenolic content was measured according to the Folin-Ciocalteu method adapted
2 from Swain & Hillis [1959]. The absorbance was measured at 725 nm and the results were
3 expressed as chlorogenic acid equivalents (CAE; μg/mL) using a chlorogenic acid (0–0.4
4 mg/mL) standard curve. The stock concentration of purple pitanga extract was 20.725 mg
5 CAE/mL.
6
7 CELL CULTURE AND TREATMENTS
8 The immortal HSC line was obtained from livers of C3H/HeN mice that were infected
9 by transcutaneous penetration of cercarias from the *Schistosoma mansoni* BH strain
10 [Borojevic et al., 1985]. The murine HSC cell line, GRX, was established by Borojevic
11 [1985] and kindly provided by the Cell Bank of Rio de Janeiro (HUCFF, UFRJ, RJ). Cells
12 were routinely maintained in Dulbecco's Modified Minimum Essential Medium (DMEM,
13 Invitrogen) supplemented with 5% fetal bovine serum (FBS – GIBCO) and 2g/L HEPES
14 buffer, pH 7.4, under 37ºC and 5% CO_2 conditions. The cells were plated (5×10^4/mL) in 12
15 or 24-well plates and cultured for 24 h to reach 60-70% of confluence before treatment with
16 purple pitanga. Purple pitanga extract was diluted in culture medium to final concentrations of
17 5, 50 and 100 μg CAE/mL just before use. The GRX cells were treated with the above
18 mentioned extract concentrations for 72 h. Each concentration group included three or four
19 wells. The routinely cultured cells were used as normal controls.
20
21 ANALYSIS OF CYTOPLASMATIC GRANULAR INTENSITY
22 For granularity analysis, GRX cells treated or not with the aforementioned extracts
23 were harvested by trypsinization and counted. The percentage of cells with different levels of
24 granularity was analyzed by flow cytometry using a FACScan Calibur flow cytometer (BD
25 Bioscience).

1

2 QUANTIFICATION OF ACIDIC VACUOLAR ORGANELLES (AVOs) BY AO
3 STAINING

4 AO is a marker of AVOs that fluoresces green in the whole cell except in acidic
5 compartments (mainly late autophagosomes), where it fluoresces red. Development of AVOs
6 is a typical feature of autophagy, and its formation indicates the maturation of
7 autophagosomes and an efficient autophagic process, since only mature/late autophagosomes
8 are acidic [Klionsky et al., 2008]. GRX cells were treated or not with the aforementioned
9 extracts and incubated with AO (2.7 μM) for 15 min at room temperature. To quantify the
10 percentage of cells with AVOs (red marked cells), treated cells were analyzed by flow
11 cytometry as describe previously [Filippi-Chiela et al., 2011] using a FACScan Calibur flow
12 cytometer (BD Bioscience).

13

14 q-RT-PCR

15 Total RNA of GRX cells was extracted using TRIzol reagent (Invitrogen; Biogen,
16 Brazil) according to the manufacturer's protocol. After extraction, total RNA was quantified
17 by UV absorbance in the apparatus BioPhotometer Plus (Eppendorf) and its integrity analyzed
18 on 1% agarose gel. Reverse transcription of the mRNA into cDNA was performed using the
19 kit Superscript First-Strand Synthesis System (Invitrogen), from 3 μg of RNA for all samples,
20 using oligodT primers, according to manufacturer's instructions. The synthesized cDNA was
21 used for polymerase chain reaction in real time (q-RT-PCR). The q-RT-PCR was performed
22 with the qPCR kit GoTaq ® Master Mix (Promega, Brazil) StepOnePlus ™ thermocycler
23 (Applied Biosystems, Brazil) in a final volume of 25 μL containing 0.2 μM of each specific
24 primer and 1μL of cDNA at a dilution of 1:10. The sequences of primers were designed with
25 software Design Software IDT (Integrated DNA Technologies Inc., USA) using gene

sequences available at GenBank (www.ncbi.nlm.nih.gov) and the site of genomes Ensembl.org (www.ensembl.org / Homo_sapiens). The sequences of the primers atg7 (NCBI accession number: NM_001253717 e 3925) were designed between exon1 and exon 2: 5' AGC CTG TTC ACC CAA AGT TC 3' Reverse 5' CGT CAC TCA TGT CCC AGA TC 3'. The constitutive gene used for normalization of reactions was the TBP (NCBI accession number: NM_003194): TBP forward (ex 6) 5'-GGGTTTTCCAGCTAAGTTCTTG-3'; TBP reverse (ex 6/7) 5'-CAGGAAATAACTCTGGCTCATAAC-3'.

The cycle conditions were: 95 ºC for 120s, followed by 40 cycles at 95 °C for 15s and 60 °C for 30s. At the end of the PCR reaction was performed melting curve to confirm the presence of the particular amplicon expected for each primer pair and used to check the possible formation of dimers between the primers. The reactions were performed in triplicate for each sample of cDNA synthesized. As a negative control we used an aliquot of water (reaction without cDNA). The reactions containing cDNA amplified only expected *amplicoms* and negative control samples did not amplify any *amplicom*, demonstrating the quality of reactions. The Ct mean values (*cycle threshold*) of the samples in triplicates were used to calculate the expression of the target gene, normalizing the results by gene constitutive and using the formula 2 ΔCt [Schmittgen and Livak, 2008].

ULTRASTRUCTURAL ANALYSIS BY TRANSMISSION ELECTRON MICROSCOPY

Semi-confluent GRX cells were collected by trypsinization. The cells were harvested by centrifugation and washed twice and 0,1M phosphate buffered (pH 7.3), After, the cells were fixed in a mixture of 4% paraformaldehyde and 2,5% glutaraldehyde buffered with 0.1M phosphate (pH 7.3) at room temperature, and then postfixade in osmium tetroxido in the same buffer for 45 minutes before dehydration. Dehydration was done in a graded acetone series (30-100%) and embedding in araldite (Durcupan ACM, Fluka) for 72h at 60C°.Thin

sections (70nm) were stained with 2% uranyl acetate followed with lead citrate. Ultrastructural analysis was performed on randomly selected fifty fields of each group in a JEM 1200EX II transmission electron microscopy.

CONFOCAL ANALYSIS

For laser-scanning confocal analysis, experiments were performed at least three times for each sample. For mitophagy evaluating, cells were incubated with MitoTracker® Green FM (MTG), a mitochondria fluorescent probe, and Lysotracker Red DND-99 (LYSR), a lysossome fluorescent probe. Briefly, LYSR was diluted in prewarmed (37°C) DMEM at 75 nM, and then incubated with MTG for 20 minutes in the dark. Several cells of each sample were observed under Olympus F1000 confocal microscopy; it was chosen six fields for image acquisition by Olympus FluoView FV1000 software (Olympus, Tokyo, Japan). Each sample was scanned in 10 layers with UPLSAPO 60 oil-immersion lenses. The MTG fluorescence was measured after laser exciting at 473 nm and collecting the emission at 520 nM. The MTO and LYSR fluorescences were measured after laser exciting at 559 nm and collecting the emission at 520 nM, respectively. The colocalization analysis and images blind deconvolution were performed with imageJ software. The product of difference from the mean (PDM) was used for show the positive correlations between the green/red probes in each image. The Pearson's and Mander's coefficients were used to quantify this correlation (REF).

STATISTICAL ANALYSIS

Data were reported as mean ± standard deviations (SD). Results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test ($p<0.05$). All analyses and graphical were performed using the statistical software GraphPad Prism 5 for Windows (GraphPad Software Inc., version 5.01).

RESULTS

INCREASE OF INTRACELLULAR GRANULARITY IN GRX CELLS TREATED WITH PURPLE PITANGA EXTRACT

Increases in intracellular granularity are associated with cellular endpoints, such as terminal growth arrest and cell death [Haynes et al., 2009]. The cytoplasmatic granular intensity was performed by flow cytometry (Fig. 1A and B) where FSC (Forward Scatter) is related to cell size, and SSC (Side Scatter) is related to the internal granularity or complexity of a particle. The results show that the number of cells in Gates 2 and 3 increases in treatments with 50 and 100 μg/mL of purple pitanga extract, indicating an increase in cytoplasmic granularity in these cells compared with the control (Fig. 1A). There were no changes in cell size in the cells treated with purple pitanga extract. These results were surprising and we tried to find the reason for this increase of intracellular granularity evaluating autophagy, cell death and cell cycle. We had already seen an increase in cell death by apoptosis and cell cycle arrest in GRX cells treated with purple pitanga extract (Fig. 4) [Denardin et al., 2013].

PURPLE PITANGA EXTRACT INDUCES AUTOPHAGOSOME FORMATION IN GRX CELLS

The presence of acidic vacuolar organelles (AVOs) was evaluated by AO staining (Fig 1C and D). Autophagosomes were observed through AO staining, which significantly increased after purple pitanga extract treatment (Fig. 1C). The results show that the proportion of GRX cells with red staining with AO increased in the cells treated with 50 and 100 μg/mL of purple pitanga extract (Fig 1D). To molecularly confirm the induction of autophagy, we measured the expression of autophagy-related protein Atg7. Purple pitanga extract induced a

1 significant increase in Atg7 at 72 h in the cells treated with 5 and 50 μg/mL of purple pitanga
2 extract, which enhances the evidences for the induction of autophagy in cells treated with
3 purple pitanga extract (Fig. 1E).

4 The presence of autophagosomes and autolysosomes was confirmed by ultrastructural
5 analysis by transmission electron microscopy (TEM) (Fig 2). Analyzing the images observed
6 that all cells have a normal architecture without major changes in the structure of the cells
7 treated or untreated with the extract. However, the increase in the number of autophagosomes
8 and autolysosomes is evident in the cells treated with 5 and 50 μg/mL of purple pitanga
9 extract (Fig 2). Together, these results demonstrate the effect of the purple pitanga extract in
10 increased autophagy in GRX cells.

11

12 MITOPHAGY IS INDUCED IN GRX CELLS BY PURPLE PITANGA EXTRACT

13 The presence of mitochondria in lysosomes and autophagosomes is a type of
14 autophagy called mitophagy and has a important role in the removal and degradation of these
15 important organelles [Goldman et al., 2010]. We evaluated the presence of mitophagy by
16 visualizing co-load cells with MTG and LYSR using confocal analysis (Fig 3). The images
17 show the mitochondrial translocation into acid organelles during the treatment with 50 μg/mL
18 of purple pitanga extract (Fig 3A). The colocalization analyses coefficients show that
19 mitophagy increased to a dose-dependent manner, i.e., cells treated with 50 and 100 μg/mL
20 were those with highest values of Mander's coefficient, with 0.914 and 0.841, respectively
21 (Fig 3B).

22

23 **DISCUSSION**

24 The central component to the liver fibrosis process is transforming quiescent stellate
25 cells (normal) to an activated (injury) state. The activation process is remarkably complex,

1 with multiple and dynamic features. Phenotypically, it consists of many important cellular
2 changes; characteristic features include loss of vitamin A, acquisition of stress bundles, and
3 development of prominent rough endoplasmic reticulum. Perhaps the most prominent feature
4 of activation is the striking increase in production and secretion of extracellular matrix
5 proteins, including types I, III, and IV collagens, fibronectin, laminin, proteoglycans, and
6 others. Activation also is associated with enhanced expression of smooth muscle α actin,
7 proliferation, and release of proinflammatory cytokines [Friedman, 2008; Rockey, 2013].

8 Experimental studies have shown that many different interventions are capable of
9 inhibiting or reverse fibrogenesis. For example, apoptosis (ie, programmed cell death) is
10 prominent in stellate cells and appears to be an important mechanism for fibrosis regression.
11 The data suggest that a balance between cell proliferation and apoptosis is important in
12 determining the dynamics of the total overall stellate cell population in the liver. In addition,
13 stellate cells may undergo senescence or revert to a normal phenotype. Recently, autophagy, a
14 catabolic mechanism involving cell degradation of unnecessary or dysfunctional cellular
15 components through the lysosomal pathway, appears to play a role in stellate activation. Most
16 current studies have demonstrated that inhibition of autophagy (through deletion of Atg genes
17 or the use of inhibitors) has reduced the activation of these cells after liver injury, leading to
18 reduced fibrosis [Ni et al., 2012; Rautou et al., 2010; Rockey, 2013], but the role of autophagy
19 in the resolution of fibrosis, i.e., after activation, remains unknown.

20 Autophagy is the cell's major regulated mechanism for degrading long-live proteins
21 and the only known pathway for degrading organelles. Upon activation, a small vesicular sac
22 called the isolation membrane or phagophore elongates and subsequently encloses a portion
23 of cytoplasm, which results in the formation of a double-membrane structure, the
24 autophagosome. Then, the outer membrane of the autophagosome fuses with lysosomes (to
25 form an autolysosome), leading to the degradation of the enclosed materials together with the

1 inner autophagosomal membrane. Amino acids and other small molecules that are generated
2 by autophagic degradation are delivered back to the cytoplasm for recycling or energy
3 production [Levine and Yuan, 2005; Mizushima et al., 2010; Rautou et al., 2010].

4 The nature of cellular changes leading to increases in intracellular granularity is
5 dependent on the agent and cell type under investigation. The increase of cytoplasmatic
6 granularity observed in this work was due to activation of autophagy and production of
7 autophagic vacuoles. Haynes and colleagues [2009] observed increases in intracellular
8 granularity in prostate cancer cells exposed to chemotherapeutics and conclude that this
9 phenotypic changes that increase the cell granularity are useful markers for identify small
10 molecules that induce cellular growth arrest or death. The presence of autophagy was first
11 visualized by the presence of AVOs stained by AO in cells treated with purple pitanga extract,
12 indicating the maturation of autophagosomes and efficient autophagic process. These results
13 were corroborated at the ultrastructural level by TEM where we visualize the increase of
14 autophagosomes and autolysosomes in GRX cells treated with purple pitanga extract.

15 Although there are no studies in the literature evaluating the effect of purple pitanga
16 extract on the induction of autophagy, there are studies showing the beneficial effects of
17 natural compounds, such as resveratrol and tocotrienols, in the induction of autophagy in
18 cancer and pancreatic stellate cells [Filippi-Chiela et al., 2011; Rickmann et al., 2007]. A
19 study by Filippi-Chiela et al. [2011] has shown that there a crosstalk among autophagy, cell
20 cycle and apoptosis in glioma cells treated by resveratrol, and conclude that autophagy may
21 be a promising therapeutic intervention for cancer treatment. The antiproliferative and
22 cytotoxic effects of some phenolic compounds in activated hepatic stellate cells has
23 demonstrate the antifibrotic effect of various natural compounds such as resveratrol, catechin,
24 quercetin, and others [Braganca de Moraes et al., 2012; Kawada et al., 1998; Souza et al.,

1 2008], but were not found published papers demonstrating the effect of autophagy on fibrosis
2 reversion.
3 To molecularly confirm the induction of autophagy, we measured the expression of
4 autophagy-related protein Atg7. The execution of autophagy involves a set of evolutionarily
5 conserved gene products known as the Atg proteins that are required for the formation of the
6 isolation membrane and the autophagosome. Two ubiquitin-like conjugation systems that
7 participate in a coordinate manner are necessary in early stages of autophagosome formation.
8 Both protein conjugation systems are dependent on the activity of Atg 7 and others Atg
9 proteins [Fader and Colombo, 2009]. In this study, we observed an increase in the expression
10 of Atg7 in GRX cells treated with the purple pitanga extract, indicating an activation of
11 autophagy, which was confirmed by the presence of AVOs and visualization of
12 autophagosomes by TEM.
13 The presence of mitophagy also was investigated in this work and we observed that
14 the purple pitanga extract increase the co-load cells with mitochondria and lysosomes.
15 Originally it was believed by many that autophagy was a strictly non-seletive process,
16 randomly engulfing cytosolic components and subject only to universal up- or down-
17 regulation. Later, the concept of "targeted" or specific autophagy began to evolve in which
18 autophagosomes were observed to preferentially degrade particular macromolecular
19 constituents within the cytosol, such as mitochondria [Goldman et al., 2010; Klionsky et al.,
20 2008]. The mitophagic activity can be activated in response to damaged mitochondria,
21 resulting in the targeted removal and degradation of those mitochondria; and under
22 physiological conditions when mitochondria plays a role in the process of cellular
23 differentiation or function [Goldman et al., 2010]. In a recent study, we observed a reduction
24 of mitochondrial membrane potential measured by JC1 fluorescent probe in GRX cells treated
25 with the same doses of purple pitanga extract [Denardin et al., 2013]. Thus, this increase of

1 mitophagy observed in this study may be due to this reduction of membrane potential which
2 signals the removal of these damaged mitochondria.

3 Liver is one of the most dynamic organs in mammals and humans, and autophagy
4 plays important roles in liver physiology and pathology. In the liver, autophagy appears to
5 predominantly exert protective functions including the promotion of hepatic function during
6 aging, protection from hepatocellular carcinoma, protection from liver diseases due to α1-
7 antitrypsin deficiency, and protection from Mallory-Denk body formation and liver injury in
8 alcoholic liver disease [Harada et al., 2008; Hidvegi et al., 2010; Qu et al., 2003; Rautou et
9 al., 2010; Zhang and Cuervo, 2008]. However, some studies explore the relationship between
10 autophagy and HSC activation, and introduce the idea of targeting autophagy for the
11 prevention of HSC activation [Ni et al., 2012; Thoen et al., 2011]. Fibrotic livers from CCl_4-
12 treated mice displayed increased expression of LC3-II, one of the proteins involved in
13 elongation of autophagosomes, and a useful indirect measure of autophagosomes. Cells
14 treated with bafilomycin (autophagy inhibitor) had increased large lipid droplets, which are
15 more characteristics of quiescent rather than activated HSCs, and treatment of HSCs with
16 PDGF induced co-localization of lipid droplets and LC3-B fluorescence suggesting that
17 autophagy is responsible for the lipid droplet metabolism [Thoen et al., 2011]. Moreover,
18 there is much contradictory evidence on the role of autophagy in liver fibrosis, and no work
19 currently available evaluated the effect of autophagy in activated HSCs with the purpose to
20 verify the fibrosis resolution via cell death.

21 In this study, we used the GRX cell line, established from hepatic fibrogranulomatous
22 reactions, which mobilizes adjacent stellate cells. Under standard conditions, these cells
23 express a transitional myofibroblast phenotype and present morphological and biochemical
24 aspects of hepatic connective tissue. Therefore, we observe an induction of autophagy in
25 activated HSCs, which may be beneficial when viewed as a resolution of fibrosis via

programmed cell death. Moreover, our recent study demonstrated the effect of the purple pitanga extract on the induction of apoptosis and cell cycle arrest in GRX cells [Denardin et al., 2013]. Together, these results may indicate that phenolic compounds present in purple pitanga extract may be activating the programmed cell death of GRX cells by apoptosis and autophagy, and which could be used as a therapeutic target in the resolution of hepatic fibrosis (see figure 4). However, more studies should be conducted to prove this theory and assess the effect of purple pitanga on HSCs activation *in vivo* and its effects on other liver cells involved in liver fibrosis.

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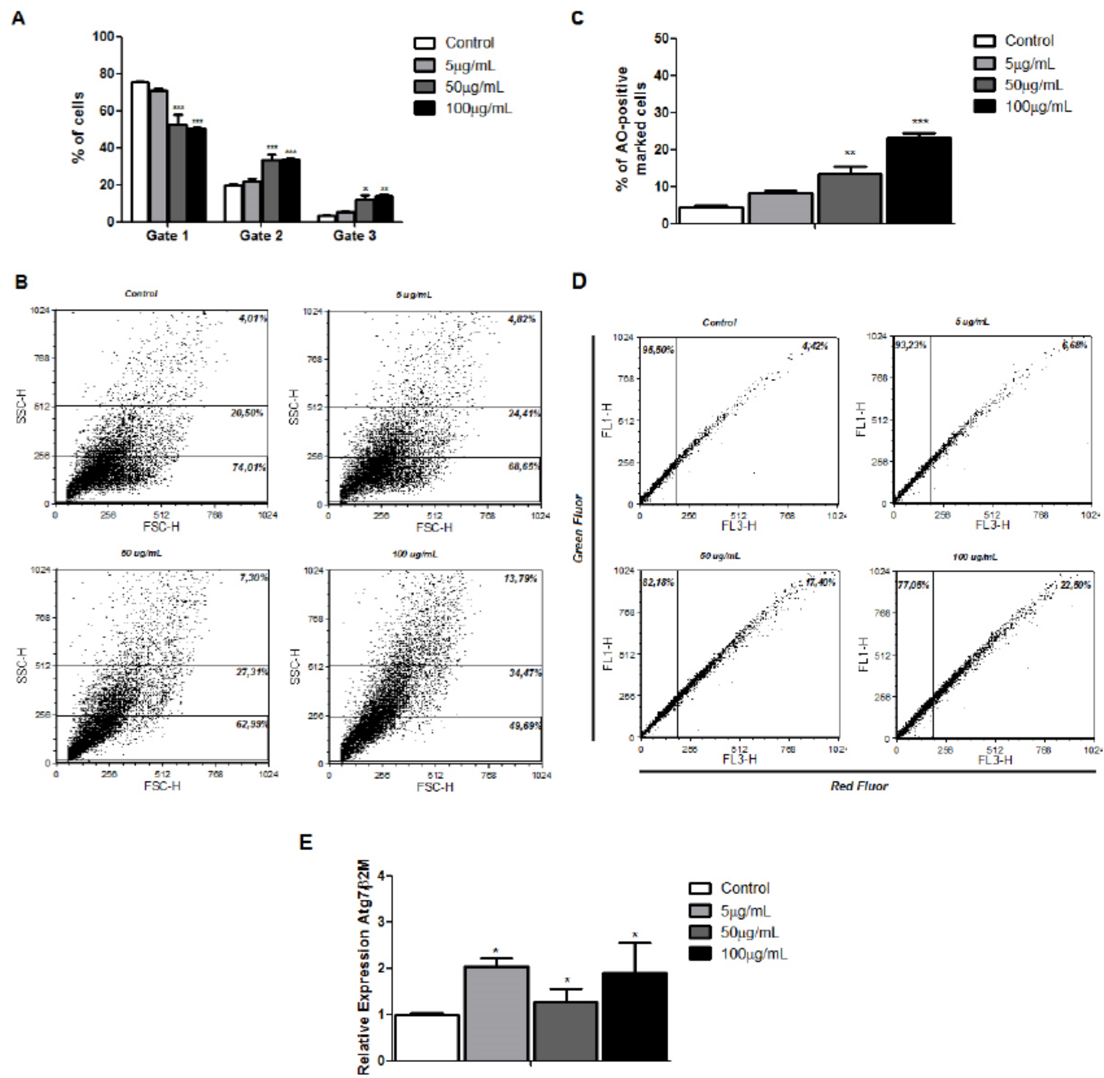

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2 **Figure 1. Purple pitanga (*Eugenia* uniflora L.) extract increase cytoplasmatic granular**
3 **intensity and induces autophagy in GRX cells.** The cells were treated with 5, 50 and 100 μg
4 CAE/mL concentrations of purple pitanga extract for 72 h. (A) Percentage of cells with
5 different levels of granularity analyzed by flow cytometry. (B) The *x* axis, FSC, represents the
6 cell size, whereas the *y* axis, SSC, represents cytoplasmic granular intensity. (C) Percentage
7 of cells with positive red fluorescence as analyzed by flow cytometry. (D) The *x* axis, FL3-H,
8 represents red fluorescence, whereas the *y* axis, FL1-H, represents green fluorescence. (E)

Expression of autophagy-related protein Atg7 by q-RT-PCR. Values are show as mean±SD of three independent experiments. *$p<0.05$; **$p<0.01$ and ***$p<0.001$ vs. untreated control cells.

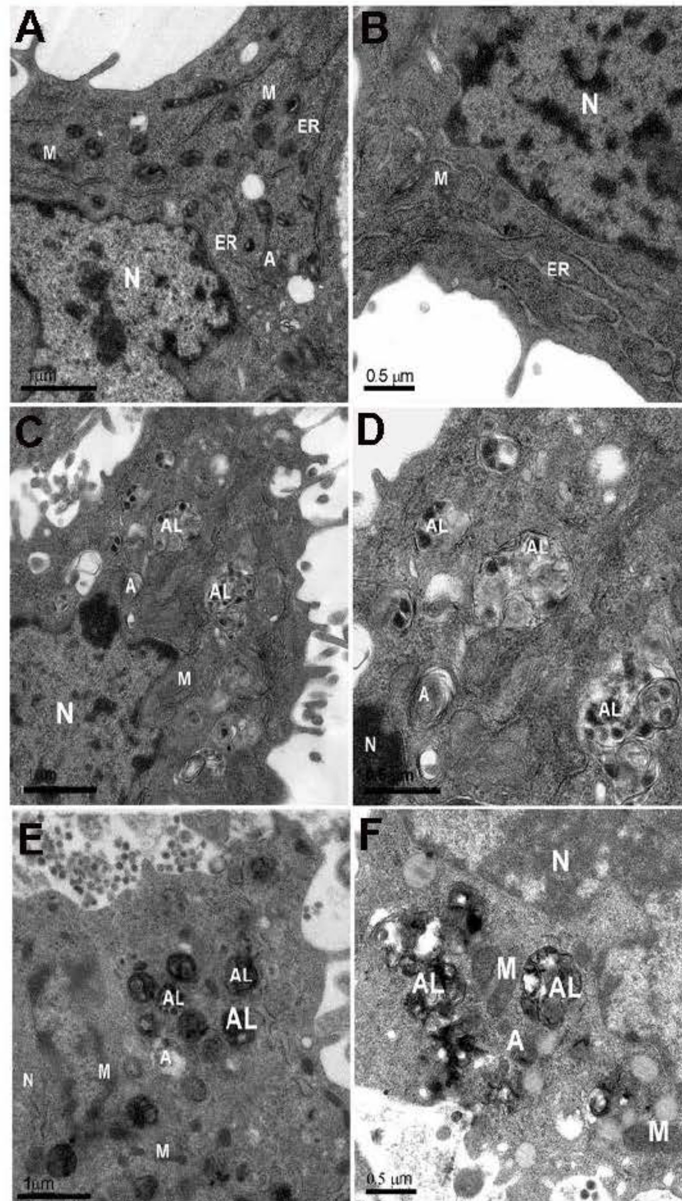
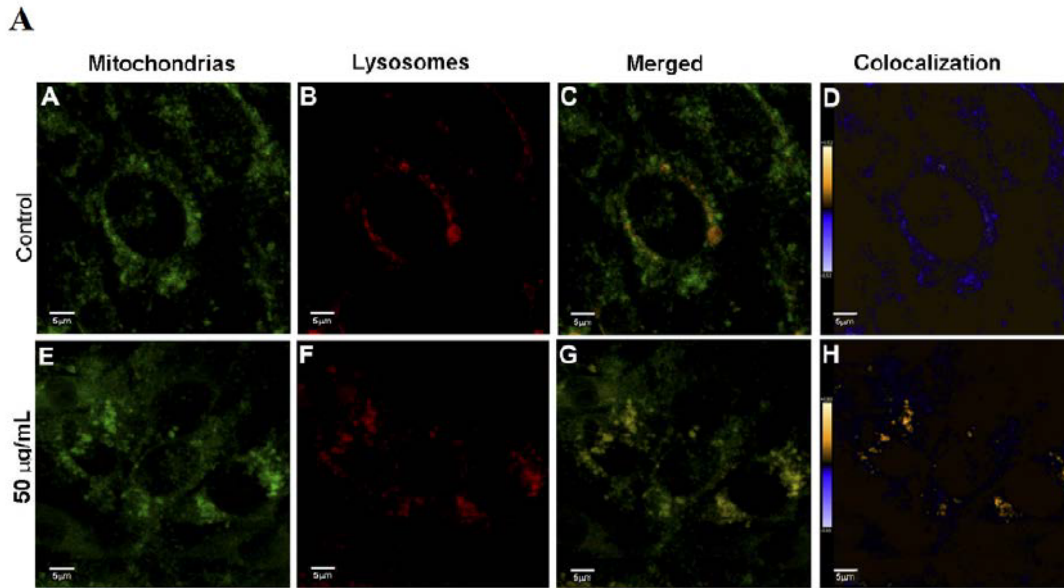


Figure 2: **Accumulation of autophagosomes and autolysosomes in GRX cells treated with purple pitanga (*Eugenia* uniflora L.) extract.** Representative electron microscopic (TEM) images of cytoplasmic regions of cells are shown. GRX cells were cultured in DMEN containing 5% FBS (control cells, **A** and **B**), or incubated with DMEN 5 % FBS plus purple pitanga extract, 5 µg/mL (**C** and **D**) or 100 µg/mL (**E** and **F**). **A**, indicates autophagosomes; **AL**, autolysosomes; **ER**, endoplasmic reticulum; **M**, mitochondria; **N**, nucleus. **A**, **C** and **E** = 30 000x, **B** and **F** = 50 000x and **D** = 60 000x.



B

Colocalization of lysosomes and mitochondrias

	Control	5 μg/mL	50 μg/mL	100 μg/mL
Pearson's coefficient	0.113	0.582	0.783	0.733
Mander's coefficient	0.172	0.562	0.914	0.841

Figure 3. Mitochondrial translocation into acidic organelles during the treatment with purple pitanga (*Eugenia* uniflora L.) extract. GRX cells were cultured in DMEM containing 5% FBS, or incubated with DMEM 5 % FBS plus purple pitanga extract (5 to 100 μg/mL). Then, cells were co-loaded with MTG and LTR and imaged, as described in Material and Methods. (A) Representative images of co-load cells with MTG and LTR. A and E: green fluorescing mitochondrias; B and F, red fluorescing lysosomes; C and G, autolysosomes, superimposition of green and red fluorescence (orange-yellow in color overlay); D and H, colocalization analysis. (B) Colocalization analysis coeffients.

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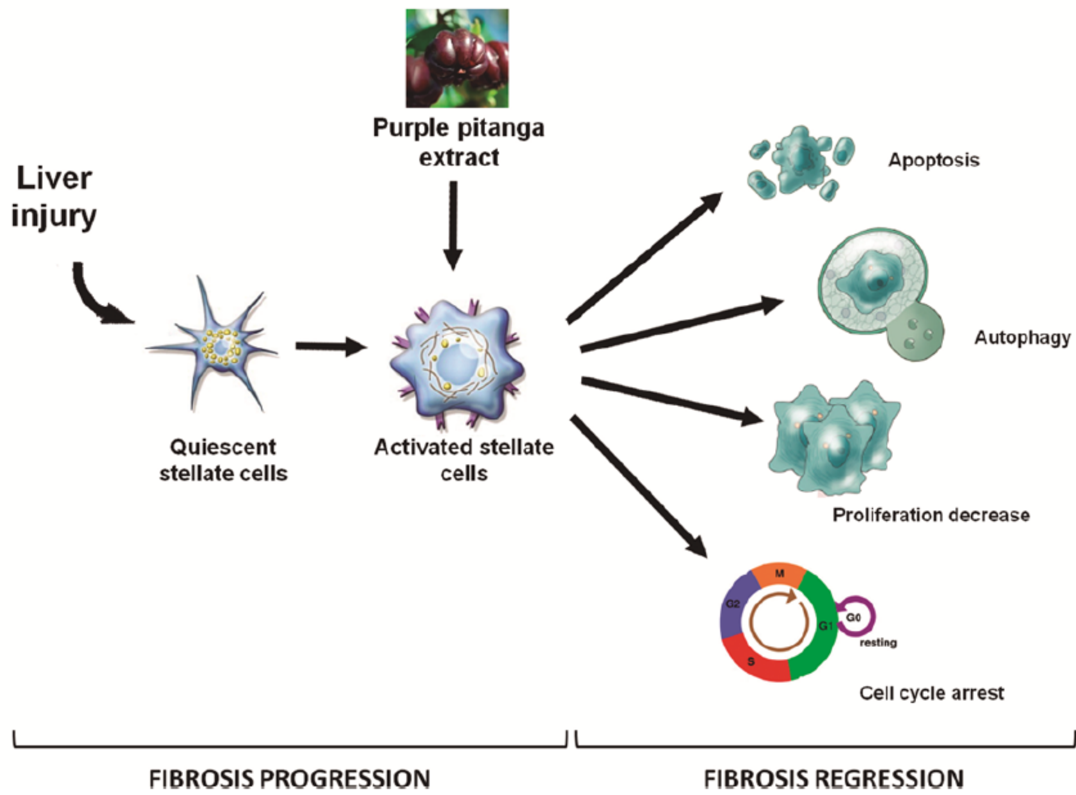

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3 **Figure 4. Main effects of purple pitanga (*Eugenia* uniflora L.) extract on activated**
4 **hepatic stellate cells (GRX cells).** The purple pitanga extract promotes reduction in cell
5 proliferation and cell cycle arrest; and acts in the resolution of fibrosis through the activation
6 of cell death by apoptosis and autophagy.

PARTE III

DISCUSSÃO

A relação entre nutrição e saúde tornou-se um tema de grande interesse nos dias atuais, sendo que existem evidências substanciais dos efeitos benéficos de dietas ricas em frutas e vegetais. O Brasil possui uma grande biodiversidade, tanto de flora quanto de fauna, que ainda pode ser explorada para produzir extratos e compostos isolados destinados à aplicação terapêutica no controle e/ou prevenção de diversas doenças crônicas. A presença de compostos fenólicos nas dietas ricas em frutas e vegetais tem atraído a atenção dos pesquisadores devido aos seus efeitos na promoção da saúde, que incluem a redução do risco de doenças cardiovasculares, câncer e outras condições associadas ao envelhecimento. Os mecanismos biológicos por trás desses efeitos incluem a proteção contra os radicais livres, a sinalização mediada por radicais livres, inflamação, alergias, agregação plaquetária, úlceras, tumores, vírus e hepatotoxicidade (Dillard and German, 2000). No entanto, existem poucos estudos sobre a identificação de quais compostos fitoquímicos exercem esta atividade antioxidante em diversas frutas nativas brasileiras, que já são a muito tempo utilizadas na medicina popular.

Os compostos fenólicos são produtos secundários do metabolismo das plantas e são constituídos por um grupo grande e complexo de substâncias. Estas moléculas são essenciais para o crescimento e reprodução das plantas, e sua síntese geralmente é induzida por condições de estresse biótico e abiótico, como: infecções, ferimentos, radiações UV, ozônio, salinidade, estresse hídrico, calor, entre outros. Nos alimentos, eles são parcialmente responsáveis pela cor, adstringência, aroma e estabilidade oxidativa (Manach et al., 2004). O perfil de compostos fenólicos nas frutas é determinado por fatores genéticos e ambientais, mas também pode ser modificado por reações de oxidação durante o processamento e armazenamento (Robards et al., 1999). Assim, como estes compostos são metabolizados como uma resposta de defesa contra a radiação solar intensa e outros fatores adversos, as diferenças na composição dos compostos fenólicos nos frutos podem promover variações na capacidade antioxidante de diferentes frutos, ou até mesmo de frutos de uma mesma

variedade, mas produzidos em diferentes locais e safras. Os polifenóis presentes em frutas e vegetais podem ser divididos em várias classes (ácidos hidroxibenzóicos, ácidos hidroxicinâmicos, antocianinas, proantocianidinas, flavonóis, flavonas, flavonóides, flavanonas, isoflavonas, lignanas e estilbenos) (Manach et al., 2004). Eles contribuem substancialmente para o efeito antioxidante de muitas espécies de frutos pequenos, apresentando diversos efeitos saudáveis.

Os polifenóis têm uma potente atividade antioxidante *in vitro* sendo capazes de remover uma vasta gama espécies reativas de oxigênio, nitrogênio e cloro, como por exemplo: ânion superóxido, radicais hidroxil, radicais peroxil, ácido hipocloroso e ácido peroxinitroso. Eles também quelam íons metais diminuindo assim a sua atividade pró-oxidante. Portanto, uma vez que diversas evidências indicam que o aumento do dano oxidativo está associado com o desenvolvimento da maioria das principais doenças degenerativas relacionadas com a idade, tem-se especulado que os polifenóis podem ter efeitos protetores contra tais condições (Tabart et al., 2009).

No presente estudo, nosso objetivo inicial foi escolher uma fruta nativa do Brasil que apresentasse uma elevada capacidade antioxidante e possuísse características promissoras para ser testada biologicamente. Portanto, nós avaliamos a capacidade antioxidante e perfil de compostos fenólicos de quatro frutas nativas: araçá amarelo, butiá, pitanga (variedades laranja, vermelha e roxa) e amora-preta (cultivares Xavante e Cheroque). Nós observamos que as frutas que apresentaram o maior conteúdo de compostos fenólicos (pitanga roxa, amora-preta e araçá), também tiveram a maior capacidade antioxidante nos ensaios de DPPH, FRAP e TRAP. Além disso, observamos que embora o butiá tenha apresentado o maior conteúdo de ácido ascórbico, sua capacidade antioxidante foi muito baixa em todos os ensaios realizados. Portanto concluímos que os extratos de pitanga roxa, amora-preta e araçá, seriam os mais promissores para serem testados na cultura de células estreladas hepáticas ativadas (GRX). Assim, o extrato de pitanga roxa foi escolhido para este trabalho de pesquisa devido a sua elevada capacidade antioxidante, melhor caracterização de compostos fenólicos e por ser uma fruta nativa do Brasil.

Analisando os cromatogramas do perfil de compostos fenólicos determinado por HPLC-DAD, observamos que os extratos de pitanga das três variedades (laranja, vermelha e roxa) foram muito semelhantes. Derivados do ácido gálico, derivados da quercetina, quercitrina, isoquercitrina, derivados do kampferol e cianidina-3-glicosídio foram encontrados nas três variedades de pitanga, enquanto derivados de cianidina e quercetina foram encontrados somente nas pitangas vermelha e roxa. Além disso, derivados do ácido protocatecuico foram encontrados na pitanga vermelha e derivados da malvidina na pitanga roxa. Vários fitoquímicos já foram identificados nas folhas da pitangueira, tais como: flavonoides (miricetina, quercetina e quercitrina), esteróides e triterpenóides, taninos, antraquinonas e fenóis, sineol e óleos essenciais (Alice et al., 1991; Schmeda-Hirschmann et al., 1987), mas existem poucos estudos avaliando a presença destes compostos nas frutas. Celli et. al. (2011) avaliaram o perfil de flavonoides nas pitangas vermelha e roxa e identificaram vários derivados de flavonoides como cianidina, miricetina e quercetina. Algumas antocianinas como cianidina-3-glicosídio e delfinidina-3-glicosídio também foram identificadas, corroborando com os resultados encontrados no presente estudo.

Como já relatado neste trabalho, a fibrose hepática é uma doença muito comum, caracterizada pela deposição de componentes da ECM, como colágeno, proteoglicanos, fibronectina e ácido hialurônico, que leva a complicações de hipertenção portal e falência hepática. É uma doença severa com alta morbidade e mortalidade, representando um sério problema de saúde pública, e tratamentos antifibróticos efetivos são muito necessários (Wynn, 2008). Sabe-se que as HSCs são as fontes celulares primárias envolvidas na patogênese da fibrose hepática através da sua ativação, ou seja, aumento na proliferação, modificação do fenótipo e síntese excessiva de componentes da EMC. Portanto, a ativação e proliferação das HSCs é um papel chave na fibrogênese, enquanto que a apoptose ou morte celular está associada com a resolução da fibrose (Friedman, 2008; Sato et al., 2003).

No presente estudo, nós observamos que o extrato de pitanga roxa inibe o crescimento e interfere na viabilidade celular nas HSCs ativadas (GRX). Kawada et al. (1998) demonstrou que resveratrol, quercetina e N-acetilcisteína também inibiram a proliferação e a expressão de α-SMA em células estreladas de ratos. De forma semelhante, a cianidina-3-glicosídio inibiu a proliferação celular em HSC isolada de fígado de ratos, e este efeito parece estar diretamente relacionado com a inibição na síntese de colágeno tipo I mais do que devido a atividade antioxidante deste composto (Bendia et al., 2005). Resultados preliminares do nosso grupo de pesquisa avaliando a expressão de colágeno do tipo I por RT-PCR, demonstraram uma significativa redução na expressão nas células tratadas com 5 e 50 μg/mL de extrato de pitanga roxa (dados não mostrados). Além disso, nós observamos que o perfil de compostos fenólicos do extrato de pitanga roxa apresenta vários flavonoides e antocianinas em quantidades significativas, principalmente cianidina-3-glicosídio e derivados da quercetina. Assim, os efeitos anti-proliferativo e de redução na viabilidade celular observados neste trabalho podem estar relacionados com a presença destes compostos fenólicos nos extratos das frutas que teriam papel na resolução da fibrose.

O ciclo celular é um processo complexo e ubíquo envolvido no crescimento e proliferação celular, regulação da reparação de danos ao DNA, hiperplasia tecidual como resposta a injúrias, e doenças como câncer. Sendo assim, o ciclo celular pode ser usado para explorar o mecanismo de ação várias drogas e toxinas. A determinação da ploidia celular é uma das abordagens experimentais mais usadas para análise da distribuição das células nas diferentes fases do ciclo celular e é facilmente medida por citometria de fluxo. Portanto, nossos resultados sugerem que esta inibição na proliferação das células GRX tratadas com o extrato de pitanga roxa pode estar associada com uma parada no ciclo celular na fase G0G1. De forma semelhante ao observado neste trabalho, estudos utilizando vários tipos de ervas da medicina japonesa popular também observaram efeitos anti-proliferativos e indução na parada do ciclo celular em G0G1 nas células estreladas hepáticas (Chor et al., 2005; Kayano et al., 1998). Além disso, estudos usando compostos fenólicos

isolados, como quercetina e baicaleina, também encontraram resultados semelhantes. A quercetina promoveu parada no ciclo celular na fase G1 em HSCs devido a seletiva redução nos níveis de ciclina D1, uma proteína relacionada com a fase G1 do ciclo celular (Inoue and Jackson, 1999; Kawada et al., 1998). Além disso, muitos flavonoides podem alterar a expressão e atividade de várias enzimas envolvidas na regulação do ciclo celular em linhagens celulares cancerígenas (Casagrande and Darbon, 2001; Rusak et al., 2005). Assim, a parada no ciclo celular observada neste estudo pode ser atribuída a presença de alguns compostos fenólicos no extrato de pitanga roxa, como quercetina e outros flavonoides, que podem atuar individualmente ou sinergicamente.

Neste estudo foi observada uma redução no potencial de membrana mitocondrial avaliado pelo marcador fluorescente JC1, que foi acompanhada por uma redução na massa mitocondrial observada pelo MTG. Estes resultados podem estar relacionados com o efeito anti-proliferativo, aumento da citotoxicidade e, principalmente, o aumento na morte celular (apoptose, necrose e autofagia) promovida pelo extrato de pitanga roxa. As mitocôndrias são organelas responsáveis pelo fornecimento de energia celular, além de estarem envolvidas em diversos outros processos, como sinalização, diferenciação celular, morte celular, controle do ciclo celular e crescimento celular (McBride et al., 2006). A cadeia respiratória mitocondrial bombeia prótons para o espaço intermembrana gerando um gradiente eletroquímico que é conhecido como potencial de membrana mitocondrial. Este potencial de membrana é um parâmetro bioenergético que pode afetar diversas funções mitocondriais, incluindo síntese de ATP, sequestro de Ca^{++}, fusão mitocondrial, autofagia mitocondrial e a geração de espécies reativas de oxigênio (Nicholls and Ward, 2000). Portanto, a despolarização mitocondrial é frequentemente atribuída a disfunções na respiração mitocondrial (Wikstrom et al., 2009), uma vez que ocorre a abertura dos poros de transição de permeabilidade mitocondrial. Além disso, este processo pode promover a liberação de proteínas intermembranas, como fatores indutores de apoptose e autofagia, levando a morte celular. Assim, esta marcante redução na massa e potencial de membrana mitocondrial observados nas células tratadas com

extrato de pitanga roxa podem ser causados pela ativação da apoptose, necrose e autofagia como observados no aumento da população de células em sub-G1 no ciclo celular, aumento nas células anexina e PI positivas e aumento na presença de autofagossomos e autolisossomos.

Como mencionado anteriormente, a inibição da ativação das HSCs e a indução da apoptose são estratégias importantes na prevenção e tratamento da fibrose hepática. A apoptose é um processo fisiológico normal durante o desenvolvimento e diferenciação celular, e ao contrário da necrose, a apoptose é um processo fisiológico bem regulado. Muitos flavonoides tem a propriedade de alterar a expressão e atividade de diversas enzimas envolvidas na regulação da apoptose, possuindo propriedades citostáticas e de indução da apoptose em diversos tipos celulares. Miricetina, quercetina e kaempferol tem apresentado atividade de induzir a apoptose em células de leucemia mieloide humana (HL-60) (Rusak et al., 2005). Além disso, neferina e ácido rosmarínico reduzem a proliferação e induzem a apoptose na linhagem de célula estrelada de ratos HSC-T6 (Ding et al., 2011; Zhang et al., 2011). De acordo com Ding et al. (2011), a neferina induz a apoptose nas células HSC-T6 pelo aumento na ativação de caspase-3, ou seja, por via mitocondrial. Portanto, o aumento marcante na apoptose e necrose observado nas células tratadas com extrato de pitanga roxa por 72hs podem estar relacionados com esta rota mitocondrial, uma vez que observamos o aumento na ativação das caspases e a redução no potencial de membrana e massa mitocondrial.

A natureza das mudanças celulares que leva ao aumento na granulosidade intracelular é dependente do agente indutor e do tipo de célula avaliada. O aumento na granulosidade citoplasmática observado neste trabalho foi devido a ativação da autofagia e produção de vacúolos autofágicos. Haynes and colleagues (2009) observaram um aumento na granulosidade intracellular em células de câncer de próstata expostas a quimioterapêuticos e concluíram que esta mudança no fenótipo celular pode ser um marcador útil para identificar moléculas que inibem o crescimento celular e induzem a morte celular. Neste trabalho, nós comprovamos a presença da autofagia através

da presença de AVOs corados por AO nas células GRX tratadas com extrato de pitanga roxa, o que indica uma maturação dos autofagossomos e comprova a eficiência do processo autofágico. Além disso, estes resultados foram reforçados pela visualização ultraestrutural das células através da TEM mostrando a presença de autofagossomos e autolisossomos, e pelo aumento na expressão da Atg 7, uma proteína relacionada com a autofagia, nas células tratadas com extrato de pitanga roxa.

Embora não tenham sido encontrados na literatura trabalhos avaliando o efeito do extrato de pitanga roxa sobre a indução da autofagia em HSCs, existem alguns estudos mostrando os efeitos benéficos de compostos naturais, como resveratrol e tocotrienóis, na indução da autofagia em células estreladas pancreáticas e células cancerígenas (Filippi-Chiela et al., 2011; Rickmann et al., 2007). Um estudo recente de Filippi-Chiela et al. (2011) mostrou que existe uma interação entre autofagia, ciclo celular e apoptose em células de glioma tratadas com resveratrol, e concluíram que a autofagia pode ser uma intervenção terapêutica promissora no tratamento do câncer. Além dos efeitos anti-proliferativos e citotóxicos de alguns compostos fenólicos nas HSCs ativadas, tem sido demonstrado o efeito antifibrótico de vários compostos naturais, como resveratrol, quercetina entre outros (Braganca de Moraes et al., 2012; Kawada et al., 1998; Souza et al., 2008), entretanto, não foram encontrados trabalhos na literatura avaliando o papel da autofagia na reversão da fibrose.

A presença de mitofagia também foi avaliada neste trabalho e observamos que o extrato de pitanga roxa aumentou a colocalização de mitocôndrias e lisossomas quando observados por microscopia confocal. Originalmente acreditava-se que a autofagia não era um processo seletivo, ou seja, atuava captando componentes citosólicos aleatoriamente e possuía uma regulação universal e pouco seletiva. Mais tarde, o conceito de autofagia específica foi introduzido após a observação de que autofagossomos poderiam degradar alguns tipos de macromoléculas e organelas em particular, como por exemplo, as mitocôndrias (Goldman et al., 2010; Klionsky et al., 2008). A atividade mitofágica pode ser ativada em resposta ao dano mitocondrial, o que promove a degradação e remoção destas mitocôndrias; e sob condições fisiológicas quando esta organela atua nos processos

de diferenciação e função celular (Goldman et al., 2010). Portanto, a redução no potencial de membrana mitocondrial observados nas células tratadas com o extrato de pitanga roxa pode estar ativando a sinalização para a remoção destas mitocôndrias danificadas através da mitofagia, o que pode ser comprovado pela redução na massa mitocondrial também observado neste trabalho.

Em resumo, este trabalho sugere que o extrato de pitanga roxa poderia ser utilizado como um possível alvo terapêutico na fibrose hepática, uma vez que apresentou efeitos antiproliferativos e citotóxicos, além de induzir a morte celular por apoptose e necrose em células estreladas hepáticas ativadas. Além disso, observamos uma indução da autofagia e mitofagia nas células tratadas com o extrato de pitanga roxa, o que poderia estar relacionado com um mecanismo adicional de resolução do estado de ativação destas células. Portanto, torna-se interessante e necessário mais estudos avaliando o efeito desta fruta no cenário da resolução da fibrose hepática, tanto *in vitro* como *in vivo*.

CONCLUSÕES

Os resultados obtidos na presente Tese permitem concluir que:

1. A pitanga roxa foi uma das frutas que apresentou o maior conteúdo de compostos fenólicos (pitanga roxa, amora-preta e araçá) e maior capacidade antioxidante nos ensaios de DPPH, FRAP e TRAP, o que pode ser atribuído a presença dos seguintes compostos fenólicos: derivados do ácido gálico, derivados da quercetina, quercitrina, isoquercitrina, derivados da cianidina e cianidina-3-glicosidio; sendo portanto a fruta de escolha para análise do potencial biológico nos ensaios com cultura de células.

2. O extrato de pitanta roxa promoveu redução na proliferação celular e citotoxicidade, além de aumentar a morte celular por apoptose e necrose sobre as HSCs ativadas. Nossos resultados sugerem que a redução na proliferação celular pode estar associada a parada no ciclo celular em GoG1, e que a apoptose ocorre por via mitocondrial devido a ativação das caspases, e redução no potencial de membrana e massa mitocondrial.

3. O extrato de pitanga roxa induziu autofagia e mitofagia nas células estreladas hepáticas ativadas, o qual foi comprovado pelo aumento no número de autofagossomos e autolisossomos nas células tratadas, além da maior expressão da proteína Atg7. Estes resultados indicam que a indução da autofagia e mitofagia pode ser um possível alvo terapêutico na resolução da fibrose hepática.

PERSPECTIVAS

- Avaliar o perfil de compostos fenólicos por HPLC-MS-MS, a fim de melhor caracterizar e quantificar os compostos fenólicos e carotenoides das frutas estudadas nesta tese.
- Avaliar a expressão de genes e proteínas envolvidos no ciclo celular e morte celular por apoptose e necrose em HSCs tratadas com extrato de pitanga roxa.
- Elucidar o mecanismo molecular de indução da autofagia nas HSCs ativadas.
- Verificar o efeito do extrato de pitanga roxa sobre outras células hepáticas, como células quiescentes presentes no fígado saudável, hepatócitos e células de Kupffer.
- Realizar estudos avaliando os efeitos do extrato de pitanga roxa sobre marcadores do estado de ativação das células estreladas hepáticas quiescentes e ativadas.
- Verificar o efeito dos extratos de frutas nativas sobre a adipogênese em células mesenquimais derivadas de tecido adiposo e pré-adipócitos (3T3-L1), uma vez que alguns estudos preliminares mostraram que o extrato de pitanga roxa inibe significativamente o acúmulo de gotas lipídicas quando a adipogênese é induzida.

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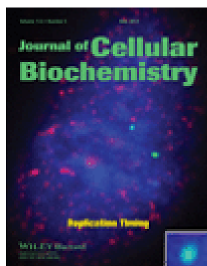

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