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EFEITO TERAPÊUTICO DO EXTRATO DE BACCHARIS ANOMALA EM CÉLULAS ESTRELADAS HEPÁTICAS ATIVADAS

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EFEITO TERAPÊUTICO DO EXTRATO DE *BACCHARIS ANOMALA* EM CÉLULAS ESTRELADAS HEPÁTICAS ATIVADAS

Dissertação apresentada como requisito para a obtenção do grau de Mestre pelo Programa de Pós-graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientador: Prof. Dr. Jarbas Rodrigues de Oliveira

Co-orientador: Profa. Dra. Eliane Romanato Santarém

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RESUMO

O gênero Baccharis pertence à família Asteraceae e tem várias espécies amplamente utilizadas na medicina popular, possuindo muitos compostos de interesse farmacêutico. O objetivo deste trabalho foi avaliar o efeito citotóxico, antiproliferativo e a reversão fenotípica do extrato de *Baccharis anomala* em células estreladas hepáticas ativadas (HSCs) e identificar os compostos presentes no extrato. Para avaliar o efeito citotóxico e antiproliferativo do extrato fracionado, foram utilizadas a liberação de lactato desidrogenase (LDH) e o método de exclusão de azul de Tripan. A reversão do fenótipo foi avaliada por coloração com Oil Red e pela expressão de PPAR-γ por RT-PCR. As frações obtidas a partir do extrato metanólico foram caracterizadas pela sua composição fenólica por HPLC. Os resultados mostraram que duas frações do extrato metanólico diminuíram a proliferação celular sem aumentar os níveis de liberação de LDH. A parada do ciclo celular foi avaliada usando 7-AAD e coloração por DAPI foi utilizada para avaliar a apoptose celular. As frações de B.anomala induziram a parada do ciclo celular em fase G1, a coloração por DAPI não revelou morte celular por apoptose. Coloração por Oil-RedO (ORO) mostrou a capacidade de indução da reversão fenotípica, a avaliação da expressão mRNA de PPAR-y por RT-PCR não foi alterada, sugerindo que exista uma via independente de PPAR-y envolvida. Os principais componentes identificados nas frações que apresentaram atividade biológica foram os ácidos hidroxibenzóico, clorogênico e cumárico. Nosso estudo foi capaz de mostrar o efeito antiproliferativo das frações de extrato metanólico de *B.anomala*, seu potencial para reverter o fenótipo ativado das células estreladas hepáticas e mostrou seu potencial para o tratamento da fibrose hepática.

Palavras-chave: Fibrose hepática; células estreladas hepáticas; GRX; HPLC; ciclo celular; compostos fenólicos.

Abstract

The genus Baccharis belongs to the family Asteraceae and has several species widely used in folk medicine and own many compounds of pharmaceutical interest. The aim of this work was to evaluate the cytotoxic, antiproliferative effect and phenotypic reversion of *Baccharis anomala* extract on activated hepatic stellate cells (HSCs) and identify the compounds present in the extract. In order to evaluate the cytotoxic and antiproliferative effect of the fractionated extract, lactate dehydrogenase (LDH) release and the trypan blue exclusion method were used. The phenotype reversion was evaluated by staining with Oil Red and PPAR-γ expression by RT-PCR. The fractions obtained from the methanolic extract were characterized for their phenolic composition by HPLC. The results showed that two fractions of methanolic extract decreased cell proliferation without increasing LDH release levels. Cell cycle arrest was evaluated using 7-AAD and DAPI staining was used to evaluate indications of apoptosis. Fractions of *B.anomala* induced cell cycle arrest in G1 phase, while DAPI staining did not reveal cell death by apoptosis. Oil-Red (ORO) staining showed the ability of fractions to induce phenotypic reversion and the evaluation of PPAR-γ mRNA expression was not altered, suggesting that there is an independent PPAR-γ pathway involved. The main components identified in the fractions that presented biological activity were hydroxybenzoic, chlorogenic and coumaric acid. Our study was able to show the antiproliferative effect of methanolic extract fractions of *B.anomala*, its potential to reverse the activated phenotype of hepatic stellate cells and showed its potential for the treatment of liver fibrosis.

Key words: Hepatic fibrosis; hepatic stellate cells; GRX; HPLC; cell cycle; phenolic compounds.

LISTA DE ABREVIATURAS

α-SMA: Actina de músculo liso alfa

Col1: Colágeno tipo 1

CLAE: Cromatografia líquida de alta eficiência

DAPI: 4',6-diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagle Medium

ECM: Matriz extracelular

HGF: Fator de crescimento hepático

HPLC: High performance liquid chromatography

HSC: Célula hepática estrelada

LDH: Desidrogenasse láctica

NAC: N-acetilcisteína

ORO: OilRed-O

PPAR: Receptor ativado por proliferador de peroxissoma

ROS: Espécies reativas de oxigênio

SFB: Soro fetal bovino

TGF-β: Fator de transformação do crescimento beta

TIMPs: Inibidores de metaloprotease tecidual

SUMÁRIO

1. INTRODUÇÂO

1.1 Fibrose Hepática

A fibrose hepática é o resultado de lesões crônicas no fígado, que podem ser causadas por infecções virais, como as hepatites B e C, abuso alcoólico, toxinas, acúmulo de metais e doenças hereditárias (1). A fibrose se caracteriza por diversas modificações no tecido hepático, como o aumento da deposição de componentes da matriz extracelular (ECM), tais como colágeno e fibronectina. Devido à ativação destes processos celulares, ocorrem alterações na arquitetura do tecido hepático localizado ao redor da camada sinusoidal no espaço de Disse (1).

O dano crônico ao fígado, somado à deposição de ECM, pode resultar em um estágio mais avançado de fibrose, conhecido como cirrose, que pode levar ao risco de insuficiência hepática devido à distorção do parênquima hepático com formação de nódulos e septos, fluxo sanguíneo alterado e modificação da arquitetura hepática (2-4).

As células hepáticas estreladas (HSC) são consideradas as estruturas celulares chaves no processo fibrótico hepático e são encontradas normalmente no estágio quiescente. Em condições normais, o fenótipo quiescente é mantido, por fatores de transcrição, como os receptores ativados por proliferador de peroxissoma; PPAR-α, PPAR-β / δ e PPAR-γ. O fator de transcrição PPAR-γ tem sido considerado como um dos fatores chave para a regulação da adipogênese em HSCs (5, 6) e sua ativação é necessária para a manutenção do fenótipo quiescente (7). Estudos também revelaram que a modulação do metabolismo lipídico pode ocorrer via PPAR-α (8). As HSCs em estado quiescente acumulam retinol (vitamina A) em seu citoplasma, por essa característica, também são conhecidas como lipócitos, exercendo a função tanto de manutenção no tecido hepático quanto de síntese de proteínas responsáveis pela formação e degradação de componentes da ECM (5).

Quando ativadas, estas células perdem a capacidade de armazenar retinol em seu citoplasma e também o controle do equilíbrio da síntese de componentes da ECM. Com isso, tornam-se responsáveis pela deposição desordenada de elementos da ECM, como fibronectina e colágeno do tipo I. Este

processo leva à fibrogênese hepática, que por consequência resulta na desestruturação do tecido e perda de sua funcionalidade. Na Figura 1 podemos ver as células estreladas em seu fenótipo quiescente (normal liver) e quando apresentam-se em seu estado ativado, ou seja, em um fenótipo miofibroblástico (liver injury).

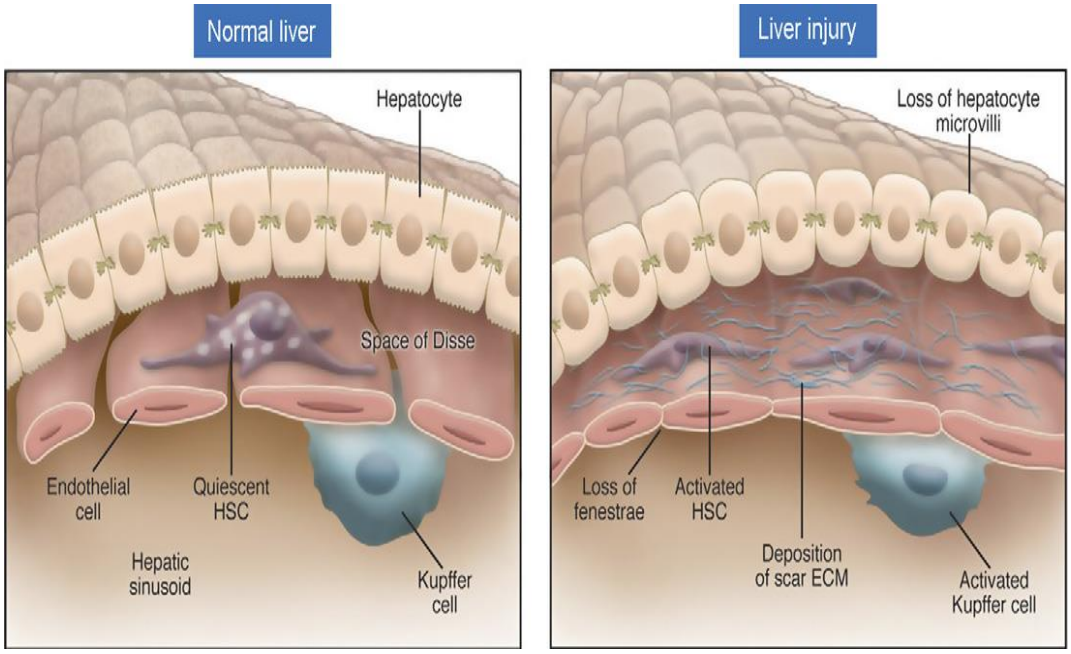

Figura 1 - Representação esquemática da ativação de células hepáticas estreladas (adaptada de Iredale, 2008) (1).

Diversas vias celulares participam do processo de ativação das HSCs e na modificação do microambiente hepático e dentre elas podemos citar o aumento da síntese de colágeno tipo I (Col-1) e de actina de músculo liso alfa (α-SMA). Além disso, também existe um aumento da expressão e secreção do fator de fator de transformação do crescimento beta1 (TGF-β1) e de inibidores de metaloprotease tecidual (TIMPs) 1 e 2 (2).

1.2 Linhagem celular GRX

A linhagem celular GRX é uma linhagem imortalizada de células hepáticas obtidas a partir de granulomas hepáticos de camundongos infectados com *Schistosoma mansoni.* É considerada como um dos melhores modelos para a representação de fibrose hepática *in vitro* por possuir semelhança nos aspectos morfológicos e bioquímicos com células de origem humana (9).

Sob cultivo, a linhagem GRX apresenta normalmente o fenótipo transicional de miofibroblasto (10), pois se encontra entre o estágio quiescente e ativado. Para a ativação dos miofibroblastos é necessária a presença de citocinas profibrogênicas e do estresse oxidativo que levam à reorganização do citoesqueleto e aumento na produção de colágeno. Já a reversão ao fenótipo quiescente pode ser induzida quando o miofibroblasto transicional é exposto a retinóides, indometacina ou β-caroteno (11) (Figura 2).

Atualmente, a diminuição da proliferação e a reversão fenotípica das células estreladas ativadas é um alvo terapêutico para o tratamento da fibrose hepática.

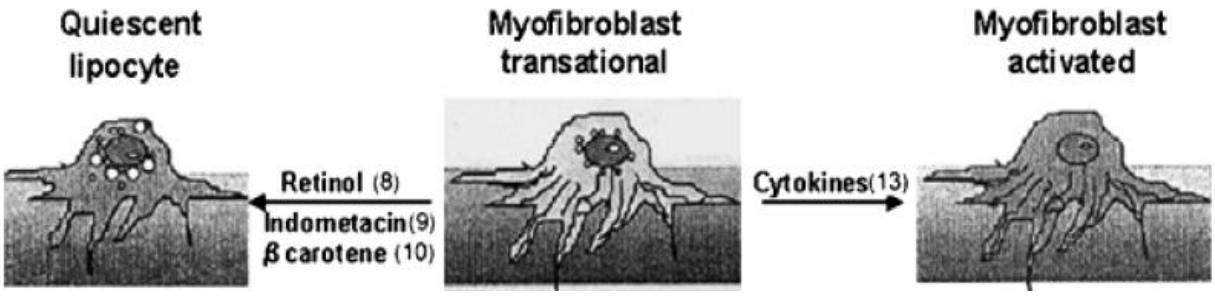

Figura 2 - Representação do processo de ativação e reversão fenotípica de miofibroblasto transicional (adaptada de Guimarães, 2006) (11).

1.3 Tratamento da fibrose hepática

Muitas moléculas têm sido usadas com sucesso como agentes antifibróticos. Entre elas se encontram inibidores do TGF-β1 (fator de transformação do crescimento beta) e o fator de crescimento hepático (HGF), uma citocina produzida pelas células estreladas hepáticas e implicadas da regeneração celular (2). Entre os fármacos mais usados pode-se destacar a N-acetilcisteína (NAC), um fármaco conhecido principalmente por seu efeito antioxidante, sendo normalmente utilizado em pacientes em casos de intoxicação por paracetamol (12). Também foi demonstrado seu efeito preventivo *in vitro* sobre modelo experimental de cirrose, onde foram observados dois mecanismos de ação da NAC, diminuindo o estresse oxidativo e a concentração de TGF-β (13). Além disso, a NAC também apresentou efeito sobre o ciclo celular de células estreladas hepáticas ativadas, levando à parada do ciclo em fase G1(14).

1.4 Plantas medicinais

A ampla ocorrência de compostos com propriedades farmacológicas em diversos gêneros de plantas revela a riqueza que se pode encontrar na natureza. Plantas que possuem esses compostos são frequentemente utilizadas na medicina popular de diversas culturas. Dentre os efeitos de interesse, podemos destacar a atividade antioxidante e anti-inflamatória (15, 16). Esses efeitos se devem a compostos produzidos pelos vegetais, conhecidos como metabólitos secundários.

A produção e o acúmulo de metabólicos secundários pelo vegetal estão diretamente ligados à interação da planta com o ambiente, pois fatores como temperatura, predação e luminosidade podem influenciar na composição química da planta (17). Os compostos fenólicos estão muito presentes na alimentação humana e compreendem muitas moléculas com atividades biológicas, representando uma das principais e mais estudadas classes de metabólitos secundários (18, 19). Dentre os efeitos destas moléculas, podemos destacar sua alta atividade antioxidante, o que leva a busca de suas aplicabilidades para o desenvolvimento de novas terapias (18, 20).

1.4.1 O gênero *Baccharis*

O gênero *Baccharis* pertence à família *Asteraceae* e possui aproximadamente 500 espécies conhecidas (21, 22). Muitas espécies do gênero são utilizadas na medicina popular para o tratamento de diversas patologias, como doenças gastrointestinais, hepáticas, inflamação, diarreia, febre, infecções e diabetes (23, 24). Estudos realizados com diferentes extratos das partes aéreas de espécies do gênero *Baccharis* mostraram a presença de compostos antioxidantes, anti-inflamatórios, analgésicos e antimicrobianos (17, 21). Dentre os metabólitos secundários encontrados no gênero, os que possuem maior destaque por seus efeitos farmacológicos são os compostos fenólicos (25). Estas plantas são utilizadas normalmente na forma de chás, bebidas alcoólicas ou macerados.

Algumas espécies do gênero *Baccharis* são alvo de interesse devido suas atividades biológicas e há muitos estudos demonstrando suas propriedades antivirais, antibióticas, antidiabéticas, antimicrobianas, hepatoprotetoras e antiproliferativa em modelos *in vitro* e *in vivo*, utilizando extratos brutos ou fracionados (26-29). Os compostos mais descritos para este gênero são ácido cafeico, ácido cinâmico, drupanina, bacarina, artepilina C e ácido *p*-cumárico (26, 28).

Os extratos aquosos e etanólicos de *B. trimera* apresentam atividade antiulcerogênica, antiinflamatória e anti-helmíntica, devido à presença de compostos fenólicos, como ácido clorogênico, rutina, ácido elágico, ácido rosmarínico, luteolina e quercetina (30, 31). A *B. trimera*, também conhecida popularmente como “carqueja” ou “carqueja-amarga”, é amplamente distribuída pela América do Sul e utilizada na medicina popular por seus efeitos digestivos e hepatoprotetores (26).

B. uncinella é bastante utilizada como anti-inflamatório na medicina popular (16). Estudos sobre a composição química da espécie revelaram a presença de hispidulina, ácido cafeico, ácido clorogênico e pectolinarigenina (26). Outras espécies do gênero que também possuem efeito antinflamatório são *B. illinita*, *B. coridifolia* e *B. grisebachii*.

Da mesma forma, existem espécies que possuem efeitos diuréticos, como o caso da *B. articulata*, também conhecida como “caquejinha-doce”, que é muito

confundida com a *B. trimera*, sendo muito utilizada por povos do Sul do Brasil, Uruguai e Argentina, devido aos seus efeitos diuréticos e digestivos. Suas parte aéreas são consumidas principalmente na forma de infusão em água (21, 26).

Muitos desses metabólicos secundários podem ser obtidos através de extratos hidroalcoólicos, utilizando diferentes solventes e técnicas. Solventes como etanol, metanol e água ou a combinação deles têm demonstrado resultados positivos para a extração destes compostos (32). Estudos revelam que a utilização de solventes hidroalcóolicos, como metanol 80% ou etanol 80% resultam em maior obtenção de compostos fenólicos, devido à maior polaridade e, por conseguinte, melhor afinidade destes compostos pelo solvente (33-35).

Dentre as espécies conhecidas do gênero *Baccharis*, cerca de 90% são encontradas na América do Sul, sendo mais comum no Brasil, Argentina, Uruguai, Chile, Colômbia e México. A espécie *Baccharis anomala*, também conhecida como cambará-de-cipó ou parreirinha, possui sua distribuição geográfica nas regiões sudeste do Brasil (Minas Gerais, São Paulo) e sul (Paraná, Santa Catarina, Rio Grande do Sul) (36), porém, é também encontrada na Argentina, Uruguai e Paraguai (37).

B. anomala (Figura 3) caracteriza-se por ser um arbusto escandente, com caule folhoso, cilíndrico e castanho, com folhas verdes ovado-lanceoladas com pecíolo, e possui inflorescência paniculada de cor creme-claro (38). Sua floração/frutificação ocorre na primavera e no verão, tendo sua dispersão de forma anemocórica (36). As partes aéreas de *B. anomala* são utilizadas na medicina popular como diurético (39).

Figura 3 – *Baccharis anomala.* Schwirkowski, *B. anomala* DC. 2011. Flora SBS (40).

1.5 Compostos Fenólicos

Compostos fenólicos são compostos hidroxilados comumente encontrados em muitas fontes alimentares e com isso, muito presentes em nossa dieta. Alguns destes compostos são substâncias com alta atividade biológica e

podem ser consideradas como potentes agentes terapêuticos (18, 20). Os compostos fenólicos naturais acumulam-se como produtos finais das vias shikimato e acetato e podem variar desde moléculas relativamente simples, como ácidos fenólicos, fenilpropanóides, flavonoides, até compostos altamente polimerizados como por exemplos as ligninas, melaninas e taninos (19).

Os ácidos fenólicos consistem em dois subgrupos, os hidroxibenzóicos e os hidroxicinâmicos. Os ácidos hidroxibenzóicos incluem ácido gálico, p-hidroxibenzóico, ácido protocatecúico, ácido vanílico e ácido siringico, que possuem em comum a estrutura C6-C1. Por outro lado os ácidos hidroxicinâmicos, são compostos aromáticos que possuem uma cadeia lateral composta por três carbonos (C6-C3), sendo os ácidos cafeico, ferúlico, p-cumárico e sinápico os representantes mais comuns deste grupo (20)

Entre os mecanismos mais importantes encontrados para a aplicação terapêutica dos compostos fenólicos, está o seu efeito direto sobre o ciclo celular. Com isso, a investigação acerca desses compostos tem ganhado visibilidade, devido ao seu potencial para o tratamento de muitas doenças, entre elas o câncer (41). Estudos recentes indicam que os compostos fenólicos, de vários subgrupos, inibem significativamente a proliferação de diferentes células cancerosas (18, 41-43). A diversidade estrutural destes compostos influencia vários componentes envolvidos na regulação do ciclo celular.

A formação de metabólitos biologicamente ativos, através de efeitos sinérgicos do uso concomitante de compostos fenólicos e a sensibilização de células cancerosas para medicamentos quimioterapêuticos, têm despertado interesse da indústria farmacêutica, por revelar-se uma terapia alternativa e eficaz ao tratamento tradicional do câncer. E com isso impulsionando a busca por espécies de plantas que se destacam pela produção e acumulo destes compostos (44).

2. JUSTIFICATIVA

Os fármacos com fortes efeitos antioxidantes são alvo de interesse para o tratamento de diversas doenças, entre elas a fibrose hepática, uma doença resultante do dano crônico ao tecido hepático, levando à modificação na homeostase tecidual até complicações mais sérias. Na natureza, é possível observar a produção de compostos antioxidantes como metabólitos secundários de diferentes famílias e espécies vegetais. Muitas das plantas com propriedades antioxidantes são utilizadas na medicina popular para o tratamento de doenças gastrointestinais, hepáticas e infecções. O gênero *Baccharis* é conhecido por possuir diversas espécies utilizadas na medicina popular de povos da América do Sul. Com isso, a investigação das propriedades farmacológicas de plantas que apresentam compostos com atividade biológica de interesse e são consumidas na medicina popular do estado do Rio Grande do Sul, se torna necessária, devido à sua disponibilidade na natureza e seus efeitos benéficos à saúde, podendo tornar-se uma alternativa para o tratamento de fibrose hepática, além de contribuir para o estudo do gênero *Baccharis*, descrevendo suas características fitoquímicas e seu potencial para o desenvolvimento de novas terapias.

3. OBJETIVOS

3.1 Geral:

Avaliar os efeitos do extrato metanólico de *Baccharis anomala* e de suas frações sobre linhagem celular GRX.

3.2. Específicos:

Avaliar o efeito do extrato metanólico e suas frações sobre a proliferação de células hepáticas estreladas da linhagem celular GRX;

Fracionar o extrato metanólico bruto;

Determinar o potencial antioxidante das frações;

Avaliar a citotoxicidade das frações;

Avaliar o efeito das frações na indução de morte celular por apoptose;

Avaliar o efeito das frações sobre o ciclo celular;

Avaliar o efeito das frações sobre a reversão fenotípica de células GRX;

Identificar os componentes majoritários das frações.

4. ARTIGO CIENTÍFICO

Os resultados do presente trabalho foram submetidos ao *periódico Phytomedicine.*

Fator de impacto: 3.526

Therapeutic effect of *Baccharis anomala* extracts on activated hepatic stellate cells

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ABSTRACT

Background

The genus *Baccharis* belongs to the family *Asteraceae* and has several species widely used in folk medicine and own many compounds of pharmaceutical interest.

Objective

The aim of this work was to evaluate the cytotoxic, antiproliferative effect and phenotypic reversion of *Baccharis anomala* extract on activated hepatic stellate cells (HSCs) and identify the compounds present in the extract.

Methods

In order to evaluate the cytotoxic and antiproliferative effect of the fractionated extract, lactate dehydrogenase (LDH) release and the trypan blue exclusion method were used. The phenotype reversion was evaluated by staining with Oil Red and PPAR-γ expression by RT-PCR. The fractions obtained from the methanolic extract were characterized for their phenolic composition by HPLC. The results showed that two fractions of methanolic extract decreased cell proliferation without increasing LDH release levels. Cell cycle arrest was evaluated using 7-AAD and DAPI staining was used to evaluate indications of apoptosis.

Results

Fractions of *B.anomala* induced cell cycle arrest in G1 phase, while DAPI staining did not reveal cell death by apoptosis. Oil-Red (ORO) staining showed the ability of fractions to induce phenotypic reversion and the evaluation of PPAR-γ mRNA expression was not altered, suggesting that there is an independent PPAR-γ pathway involved. The main components identified in the fractions that presented biological activity were hydroxybenzoic, chlorogenic and coumaric acid.

Conclusion

Our study was able to show the antiproliferative effect of methanolic extract fractions of *B.anomala*, its potential to reverse the activated phenotype of hepatic stellate cells and showed its potential for the treatment of liver fibrosis.

Key words: Hepatic fibrosis; hepatic stellate cells; GRX; HPLC; cell cycle; phenolic compounds.

Abbreviations:

α-SMA: Alpha smooth muscle actin

Col1: Type 1 collagen

DAPI: 4',6-diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagle Medium

DPPH: 2,2-diphenyl-1-picrylhydrazyl

ECM: Extracellular Matrix

FBS: Fetal bovine serum

HGF: Hepatic growth factor

HPLC: High performance liquid chromatography

HSC: Hepatic stellate cell

LDH: Lactate dehydrogenase

NAC: N-acetylcysteine

ORO: OilRed-O

PPAR: Peroxisome proliferator-activated receptor

ROS: Reactive oxygen species

TGF-β: Transforming growth factor beta

TIMPs: Tissue inhibitors of metalloproteinases

1. Introduction

Hepatic fibrosis is associated with chronic liver damage, caused by different factors such as hepatitis B and C, alcohol abuse, toxins, metal accumulation and hereditary diseases (Iredale, 2008), resulting in the activation of fibrotic processes in cells known as hepatic stellate cells (HSCs), which have been identified as the trigger of fibrosis. Activation of HSCs leads to phenotypic changes in cells, losing their lipocytic character and promoting the accumulation of extracellular matrix, which can lead to the destruction of hepatic tissue architecture and in more severe cases, to the development of cirrhosis followed by hepatic insufficiency (Friedman, 2008; Gressner, 1998; Wynn and Ramalingam, 2012). HSCs are considered the key cellular structures in hepatic fibrotic process and are usually found in quiescent stage (Friedman, 2008; Iredale, 2008; Wynn and Ramalingam, 2012). Under normal conditions, the quiescent phenotype is maintained by transcription factors such as peroxisome proliferator-activated receptors PPAR-α, PPAR-β/δ and PPAR-γ. The PPAR-γ transcription factor has been considered as one of the key factors for the regulation of adipogenesis in HSCs (Guimaraes et al., 2007; Tsukamoto et al., 2006) and its activation is necessary to maintain the quiescent phenotype (Braganca de Moraes et al., 2014; de Mesquita et al., 2013). Studies also revealed that modulation of lipid metabolism could occur via PPAR-α (Chen et al., 2015). When activated, these cells lose the ability to store retinol in their cytoplasm and control the balance of ECM component synthesis. Therefore, the inhibition of HSCs' activation seems to be an effective strategy for therapy of liver fibrosis.

GRX cell line is an immortalized lineage of liver cells obtained from hepatic granulomas of mice infected with *Schistosoma mansoni* and is considered an excellent model for

representation of liver fibrosis *in vitro* (Borojevic et al., 1985). GRX lineage normally presents the myofibroblast transitional phenotype (Herrmann et al., 2007), since it is between the quiescent and activated stages. In order to activate myofibroblasts is necessary the presence of profibrogenic cytokines and oxidative stress that cause cytoskeleton reorganization and increase in collagen production. The quiescent phenotype expression can be induced when the transitional myofibroblast is exposed to retinol, indomethacin or β-carotene (Guimaraes et al., 2006).

Several molecules have been successfully used as antifibrotic agents, including inhibitors of TGF-β1 and hepatic growth factor (HGF) (Friedman, 2008). N-acetylcysteine, a drug mainly known for its antioxidant effect, can be highlighted amongst the most used drugs for controlling fibrosis, and it is often used in cases of paracetamol intoxication (Vargha et al., 2014). Plants have been pointed as natural resources for traditional medicine and for the modern pharmaceutical industry. The wide occurrence of compounds with pharmacological properties found in various plant species reveals the richness that can be found in nature. Plants that possess these compounds are often used in folk medicine of different cultures. These effects are due to compounds produced by the plant, known as secondary metabolites. The production and accumulation of these metabolites by the plant are related to the interaction of the plant with the environment, since factors such as temperature, predation and luminosity can influence the phytochemical profile (Sartor T, 2013). Phenolic compounds represent one of the main and most studied classes of secondary metabolites, mainly due to their antioxidant activity (Nagendran Balasundram KS, 2006; Neuhouser, 2004). The genus *Baccharis* has approximately 500 known species (Abad, 2007; J.M. Budel, 2005) and many species are used in folk medicine in form of teas, alcoholic beverages or macerated, for the treatment of various diseases, such as gastrointestinal diseases, liver diseases, inflammation, diarrhea, fever, infections and diabetes (Hocayen Pde et al., 2016; Ramos Campos et al., 2016). Among the known species of the genus, about 90% are found in South America, being more common in Brazil, Argentina, Uruguay, Chile, Colombia and Mexico. The main constituents found in the *Baccharis* genus currently described in literature are phenolic compounds and terpenoids (Ramos Campos et al., 2016). The species *B. megapotamica, B. incarum, B. trimera, B. trinervis, B. salicifolia, B.uncinella, B. coridifolia, B. dracunculifolia, B. grisebachii* and *B. tricuneata* have been extensively studied for their chemical composition (Luiz Gonzaga Verdi, 2005).

The species *Baccharis anomala*, also known as "cambará-de-cipó" or "parreirinha", is geographically distributed in the southeastern (Minas Gerais, São Paulo) and south (Paraná, Santa Catarina, Rio Grande do Sul) regions of Brazil (Heiden, 2010), although it is also found in Argentina, Uruguay, and Paraguay. The aerial parts of *B. anomala* are mainly used for its diuretic effect in folk medicine (Jane M. Budel, 2008). Since few studies on the phytochemical composition and medicinal properties of the species are currently available, the aim of this study was to evaluate the effect of the extract of *B. anomala* on activated hepatic stellate cell line GRX.

2. Material and Methods

2.1 Plant material and extraction

Leaves and twigs of plants were collected in São Francisco de Paula in the state of Rio Grande do Sul (Southern Brazil; latitude, 29◦29S; longitude, 50◦11 W; altitude, 950 m). The specimen was deposited in the Herbarium of the Science and Technology Museum of the Pontifícia Universidade Católica do Rio Grande do Sul (Herbarium MPUC) under the voucher specimen number Herbarium 3354. The plant material was dried, powdered and then stored at -20ºC until preparation of extracts. Extracts were prepared by combining 3 g of dried plant material with either 100 mL of distilled water (aqueous extract), 100 mL of ethanol 96º (ethanolic extract) or 100 mL 80% aqueous methanolic solution (80:20; methanolic extract). Aqueous and ethanolic extracts were agitated for 5 and 72 h at room temperature, respectively. Methanolic extract was kept in ultrasonic bath for 15 min at room temperature. Extracts were then centrifuged for 10 min at 1000 rpm, the supernatant was collected and dried in a rotary evaporator.

2.2. Fractionation by column chromatography

The fractionation of the methanolic extract was performed in chromatography column using silica gel 60 (Merk) as stationary phase and the mobile phase consisted in eluents of increasing polarity (v/v): dichloromethane (100:0), dichloromethane: methanol (95:5), dichloromethane: methanol (90:10), methanol (100:0) and methanol: water (80:20). Four different fractions were obtained (FI, FII, FIII and FIV) at the end of the chromatography. Obtained fractions were filtered, dried and weighed for their use in the experiments.

2.3. Evaluation of antioxidant activity of the methanolic extract

Fractions from the methanolic extract were analyzed for its antioxidant activity. The reduction of DPPH (2,2-diphenyl-1-picrylhydrazyl) through electron transfer by the action of an antioxidant was measured by spectrophotometry in an ELISA reader at the wavelength of 515 nm. All samples analyzed were dissolved in methanol 100%. Ascorbic acid (550 μg/mL) was used as a positive control for antioxidant activity.

2.4. Effect of B. anomala *extracts on GRX cells*

2.4.1 GRX cell culture

Hepatic stellate cell line GRX was obtained from the Rio de Janeiro Cell Bank. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (GIBCO), 1% penicillin and streptomycin (Invitrogen) and pH 7.4. Cells

were seeded in tissue culture plates and after 24 h they were treated with crude extracts of *B. anomala* (25, 50, 500 μg/mL) or fractions FIII and FIV (1.25, 2.5, 5.0, 50.0 and 100.0 μg/mL)and incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were performed in triplicate.

2.4.2. Proliferative and cytotoxicity evaluation

In order to evaluate cell proliferation and cytotoxity of GRX cells, cells were seeded in a 24 wells tissue culture plate ($3x10^3$ cells/well) and incubated with *B. anomala* crude extracts at concentrations of 25, 50, 500 μg/mL. NAC (400 μg/mL) was used as a comparative of a well-known medicine used in the treatment of hepatic fibrosis. Proliferation was assessed by cell count with Trypan blue exclude method. The evaluation of cytotoxicity was performed using a lactate dehydrogenase (LDH) kit (LabTest, Brazil) in the culture supernatants. As LDH is a known enzyme related to membrane cell damage, its activity was measured by a colorimetric assay at 420 nm and compared with the control group. For the control of total cell lysis, 5% Tween was used. Analyses were performed after 72 h of treatment.

2.4.3. Oil Red assay

Phenotypic reversion in hepatic stellate cells was observed using Oil Red assay. Cells were plated in a 24 wells tissue culture plate ($3x10^3$ cells/well), and 72 h after treatment with fractions III and IV, cells were fixed with 10% formaldehyde for 1 h and stained with Oil Red-O (ORO; Sigma Chemical). Intracellular lipid accumulation was observed after 30 min, using an inverted light microscope at magnification of 400x. For estimation of lipid accumulation, the ORO within the lipid droplets was extracted using isopropanol and the absorbance was read at 492 nm using ELISA plate reader. Specific lipid content was calculated as the ratio of absorbance value obtained for ORO and number of cell count.

2.4.4. RNA extraction and RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). RNA was reversely transcribed into cDNA, using Superscript III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's instructions. Table 1 shows the primer sets used. Polymerase chain reaction products were electrophoresed using 1.5% agarose gel containing 5 μg/mL ethidium bromide. The gel was visualized using UV light and photographed. The band intensities were measured using the public domain National Institutes of Health Image program (Image J) and the signals were expressed relatively to the intensity of the β-actin amplicon in each co-amplified sample.

2.4.5. DAPI nuclei staining

The nuclear morphology of cells was evaluated using the cell-permeable DNA dye 6-diamidino-2-phenylindole (DAPI). Cells were seeded at a cell density of 3 x 10^3 cells per well in 24-well plates and incubated for 24 h at 37 ºC in a 5% CO_2 incubator. Cells were then treated with FIII and FIV fractions, NAC (400 µg/mL) and Cisplatin as positive control at concentration of 2.5 µM. After 72 h incubation, cells were fixed with 4% paraformaldehyde for 2 h. Fixed cells were permeabilized with 0.3% 4-(1,1,3,3 Tetramethylbutyl)phenyl-polyethyleneglycol,t-Octylphenoxypolyethoxyethanol, Polyethylene glycol tert-octylphenyl ether (Triton X-100) in phosphate buffered saline (PBS) for 30 min and subsequently marked with a solution containing 300 nM of DAPI for 2 min. Incubation was performed at room temperature and cells were photographed using a fluorescence microscope (IX71, Olympus).

2.4.6. Cell cycle evaluation

Cell cycle arrest was evaluated using 7-AAD staining. GRX cells were seeded into 24-well plates at 3x10^3 cells/well and treated either with FIII at concentration of 50 µg/mL, FIV at 5 µg/mL or NAC at 400 µg/mL. Cells were collected by trypsinization, washed twice with PBS and then, while vortexing, 2.5 mL of ethanol 70% was added per sample, drop by drop. Cells were incubated at -20 ºC for 2 h and then washed twice to remove ethanol. Cells were centrifuged, the supernatant was aspirated and cells were resuspended in 100 µL of Stain Buffer and 4µL of 7-AAD, and incubated for 15min at room temperature. Samples were analyzed by flow cytometry to identify the cell cycle phases. Data were analyzed using FlowJo 7.6.5 software (Tree Star). Analysis allows discrimination among Sub-G1, Go/G1, S, G2/M.

2.4.7. Assessment of GRX cell contraction by collagen gel analysis

Collagen from rat tail tendon was extracted and prepared as described elsewhere (Preparation of ready-to-use, storable and reconstituted type I collagen from rat tail tendon for tissue engineering applications). Collagen gels (125µl of 4x DMEM and 125 µl of 4 mg/mL Rat Tail Tendon Collagen) were impregnated with 1x10^5 cells resuspended in 250 µl of PBS. The gels were added to a 24-well plate, left to polymerize at 37 °C for 30 min, detached and suspended in 600 µL of DMEM with 5% FBS alone or with FIII, FIV and NAC as required. Images were made after 24 h and the surface of the area of each gel was determined as percentage of well area using ImageJ (http://rsb.info.nih.gov/ij).

2.5 Determination of phenolic compounds

Phenolic compounds (chlorogenic acid, cumaric acid and hidroxidbenzoic acid) were analyzed in fractions III and IV by HPLC. Chromatographic analysis was performed using a

Sykam System S600 with nm equipped with a MetaSil ODS reverse phase column (5 μm, 150 x 4.6 mm) and DAD UV-VIS detector set at 280 nm). The column was maintained at 40 °C, and the injected sample volume was 20 μL. Chromatographic data were obtained and processed by the Clarity Chromatography Software® system. The following eluents were used: 2% phosphoric acid in water (eluent A) and methanol (eluent B). The gradient elution program was 10% eluent B from 0 to 10 min, 20% to 80% B from 10 to 25 min, 80% to 100% B from 25 min to 32 min and 100% B from 32 to 35 min. The flow rate was maintained at 1 mL/min. Compounds were identified based on the retention time of pure standard and quantified by reference to peak areas of the standard curves.

2.6. Statistical analysis

Data are reported as mean ± SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test at a significance level of $p < 0.05$. The statistical program used was GraphPad Prism Version 5.00.

3. Results

3.1. Effect of aqueous, ethanolic and methanolic extracts of B. anomala on cell proliferation

Extracts of *B. anomala* affected GRX cell proliferation. Results showed that all extracts reduced cell proliferation in 72 h of treatment at concentrations of 50 and 500 μg/mL. However,only the methanolic extract decreased the cell proliferation at concentration of 25 μg/mL when compared with control group (Fig. 1). Therefore, the methanolic extract was selected for the next experiments.

3.2 Antioxidant activity of methanolic fractions

The antioxidant potential of each fraction obtained in chromatography column was determined with the DPPH assay. At concentration of 100 μg/mL, fractions FIII and FIV had a significant antioxidant activity when compared to control group, while FI and FII showed no significant activity. NAC exhibit a great antioxidant activity reaching the same level of activity as the positive control with ascorbic acid (fig. 2).

3.3 Effect of methanolic fractions FIII and FIV on cell proliferation

Fractions FIII and FIV were selected for evaluating the effect on GRX cell proliferation because their high antioxidant activity. FIII significantly decreased cell number compared to control group at concentration of 50 and 100 μg/mL, while FIV decreased at 5, 50 and 100 μg/mL (fig. 3). The results evidenced that FIV had a stronger effect on GRX cell line, decreasing the cell number at a lower concentration (5 μg/mL) than FIII (50 μg/mL). For the next experiments the

mentioned doses from each fraction were used, based on the minimum concentration for a significant reduction of cell number.

3.4 Evaluation of cytotoxicity of the fractions

To evaluate the cytotoxicity of the fractions FIII and FIV obtained from the methanolic extract LDH assay was performed on supernatant of cell culture 72 h after treatment with fractions. The results showed that the fractions are capable to reduce cell number without cell damage, indicating that both fractions do not exhibit cytotoxicity activity in GRX cells at tested concentrations (fig.4).

3.5 Visualization of apoptotic cells by DAPI

To investigate whether the decrease in proliferation caused by fractions III and IV was due to apoptosis, the cells nuclei were stained with DAPI. The morphological profile indicated nuclear condensation and fragmentation identified a possible pro-apoptotic effect of Cisplatin on GRX cells (fig. 5 B). However, cells treated with NAC as well as the fractions III, IV showed no alteration on the morphological profile (fig. 5 A, C, D and E).

3.6 Effects of fractions on cell cycle

In an attempt to understand the decrease in cell number with the treatment of methanolic fractions III and IV, evaluation of the cell cycle was carried out. Both fractions and NAC significantly induced cell cycle arrest in G1-phase in GRX cells when compared to the control group. FIII promote cell cycle arrest and significantly differed from FIV, which presented the lower induction of cell cycle arrest when compared to the other treatment groups (fig. 6).

3.7 Effect of fractions on phenotype reversion

We investigate the ability of fractions to reverse GRX cell phenotype by inducing the accumulation of lipids in the cytoplasm. When observed under inverted microscope, GRX cells showed increased ability of fat storing in cytoplasmic content when treated with both fractions when compared to the control (fig. 7A-C). The NAC treatment also induced the accumulation of lipids in the cytoplasm (fig. 7D). Lipid accumulation was quantified by absorbance at 492nm and confirmed the positive result of the fractions on storage of lipids (fig. 7E).

3.8 Fractions deactivate GRX cells by a PPAR-γ independent mechanism

As ORO staining revealed the formation of fat droplets, the expression of PPAR-γ was assessed, in order to elucidate the pathway of the phenotypic reversion mechanism. No changes were observed in PPAR-γ mRNA expression in the treated groups when compared to control

group (fig. 8). The result suggests that another pathway might be involved in accumulation of lipids droplets on cytoplasm of GRX cells.

3.9 Collagen gel contraction assay

After 24 hours of treatment, it was possible to observe that the area occupied by the collagen gel that received treatment with the fractions was larger in relation to the area of the control group. Therefore, it indicates that both fractions (FIII and FIV) were able to decrease cell contraction, while control group presented a greater contraction due to its activated phenotype (fig. 9).

3.10 Determination of phenolic compounds

For the identification of compounds in fractions III and IV HPLC analysis was performed. In FIII, chlorogenic acid (26.66%), coumaric acid (1.39%) and hydroxybenzoic acid (0.78%) were the identified phenolic compounds. In FIV, only chlorogenic acid (1.9%) and coumaric acid (0.53%) were detected. The retention times and percentages of each compound identified in fractions III and IV are shown in Table 2.

4. Discussion

Studies have shown that the consumption of foods rich in phenolic compounds can prevent disease development, and among them, the cancer (Neuhouser, 2004). Since the results of such studies have demonstrated the health benefits and the applicability of these compounds to the development of new therapies, the search for natural sources of phenolic compounds seems to be promising. Some groups of plants have gained visibility due to the high production and accumulation of phenolics, mainly the genus *Baccharis* (J.M. Budel, 2005; Ramos Campos et al., 2016). This genus has many species used in the popular medicine of several cultures of South America. Out of the 500 known species approximately 30% has the phytochemical composition determined (Abad, 2007). Thereby, the search for species with potential for the development of new applicability and therapies is necessary.

The present study investigated the cytotoxicity of *Baccharis anomala* extract, as well as antiproliferative effect and de-activation of hepatic stellate cells (GRX). We also try identifies the main compounds present in the extract.

Amongst the *Baccharis anomala* extracts (aqueous, ethanolic and methanolic) tested, the methanolic showed a significant reduction of cell proliferation in the lowest concentration tested (25 µg/mL). These results made us look for the main compounds present in the extract. For this reason, the methanolic extract was fractionated by column chromatography and four fractions

were obtained. Methanolic fractions (FIII and FIV) showed high antioxidant activity, a chemical characteristic that seems to be promising for treatment of hepatic fibrosis (Di Francia et al., 2016).

The effect on cell proliferation of fractions III and IV showed that FIV was able to significantly decrease proliferation at the dose of 5 µg/mL, while FIII was efficient at 50 µg/mL. These doses were selected for cytotoxicity assessment by LDH test, because the activity of LDH in cell culture supernatant is a marker of cellular injury. Cytotoxicity was not observed in fractions III, IV and NAC, showing that the decrease in proliferation was not due to necrosis.

A study with *Baccharis articulata* revealed the ability of aqueous extract to induce apoptosis in human peripheral blood mononuclear cells (Cariddi et al., 2012) and another study conducted with the polyphenol resveratrol on activated hepatic stellate cells demonstrated the ability to induce apoptosis through the evaluation performed with DAPI staining (Souza et al., 2008). To verify a possible pro-apoptotic action of the fractions that could lead to a decrease in proliferation of GRX cells, nuclear morphology was visualized through the DAPI staining. As positive control of apoptosis cisplatin was used, which is a well-known chemotherapeutic drug used for treatment of numerous human cancers (Dasari and Tchounwou, 2014). Cisplatin form adducts on DNA, causing damage and subsequently, inducing apoptosis (Siddik, 2003). The results showed that the cells treated with the fractions III, IV and NAC showed no alteration in their nuclear morphology in relation to the control group. On the other hand, in addition to significantly reducing cell proliferation, cisplatin has shown nuclei with a condensed morphology, indicating the formation of apoptotic bodies. This led us to believe that the decrease in cell proliferation was not due to death by apoptosis, driven our investigation to the action of FIII and FIV methanolic fractions on the cell cycle.

Several studies have reported the action of phenolic compounds on the cell cycle (Jafari et al., 2014). Cell cycle is modulated and controlled by complexes of cyclins and cyclin-dependent kinase enzymes (CDKs). The expression of these enzymes can be regulated by simple phenols or complex polymeric structures, such as, tannins (Jafari et al., 2014). Treatment of HL-60 cells with gallic acid resulted in G0/G1 phase arrest, which was associated with up-regulation of p21 and p27 (Yeh et al., 2011). So, the investigation of the cell cycle seems to be necessary to understand the activity of these compounds on cell proliferation. Our results showed that both fractions and NAC induces cell cycle arrest in G1-phase in GRX cells. Cell cycle arrest in G1-phase observed in NAC treatment is in agreement with the literature, since NAC mediates cell cycle arrest through a Sp1- dependent mechanism (Kim et al., 2001),(Grinstein et al., 2002).

In quiescent state HSCs accumulate retinol (vitamin A) in their cytoplasm, therefore they are also known as lipocytes, exerting the function of both maintenance in the hepatic tissue and synthesis of proteins responsible for the formation and degradation of ECM components

(Borojevic et al., 1985; Iredale, 2008). This study showed lipid deposits in intracellular content of GRX cells as well as changes in cell morphology observed in phase contrast microscopy. The GRX cells treated with fractions III, IV and NAC, lost their elongated and parallel strand appearance and acquired a larger and polygonal shape, while control cells preserved their myofibroblast-like morphology, devoid of large lipid droplets. Quantification of the total lipid content was possible through the absorbance corrected by number of $50x10^3$ cells, which confirmed the significant increase in lipid production in cells treated with the fractions and NAC during 72 h. Our results show that the fractions III and IV obtained from the methanolic extract showed antiproliferative effect and the ability to deactivate HSCs, transforming the fibroblastic phenotype into quiescent cells.

There are three peroxisome proliferator-activated receptors (PPARs) members of the nuclear hormone receptor family of ligand-activated transcription factors and consisting of three different isoforms; PPAR-α, PPAR-β/δ and PPAR-γ, involved in the regulation of lipid synthesis. PPAR-γ is a transcription factor that is related to induction of quiescent phenotype in HSCs (Guimaraes et al., 2007; Tsukamoto et al., 2006). Currently, there are studies of molecules capable of inducing the expression of PPAR-α, leading to deactivation of HSCs through pathways independent of PPAR-γ expression (Chen et al., 2015). To investigate the mechanism involved in restoring the quiescent phenotype, the PPPAR-γ mRNA expression was evaluated by RT-PCR. The results showed that PPAR-γ mRNA expression was not altered by treatment with the fractions III, IV and NAC. The non-alteration of PPAR-γ mRNA expression in NAC treatment appears to be in agreement with the literature, since its effect is mainly related to the expression of PPAR-α (Calzadilla et al., 2011; Paintlia et al., 2008). So, it is possible that the restoration of the quiescent phenotype of the HSCs treated with the fractions III and IV could be by an independent pathway to PPAR-γ.

Analysis by HPLC performed in fraction III allowed the identification of the major phenolic compounds, using internal phenolic standards. It was possible to identify in FIII the presence of chlorogenic, hydroxybezoic and coumaric acids. The chlorogenic acid was the most abundant phenolic in FIII. We cannot ignore the possibility of presence of others compounds, however they were not identified by the used method. Some of the phenolic compounds identified by HPLC analysis are in agreement with the phytochemical composition of species found in the genus (Ramos Campos et al., 2016).

Species that have several studies regarding their chemical composition, such as, *B. dracucunlifolia*, are known to produce a large amount of phenolic compounds, such as caffeic acid, cinnamic acid, drupanin, baccharin, artepillin C and *p*-coumaric acid (Hocayen Pde et al., 2016; Ramos Campos et al., 2016). Studies have demonstrated the antiviral, antibiotic, antidiabetic, antimicrobial, hepatoprotective properties and antiproliferative activity, in *in vitro*

and *in vivo* models, using crude or fractionated extracts of this species (Hocayen Pde et al., 2016; Pereira et al., 2016; Ramos Campos et al., 2016; Rezende et al., 2014). Similarly, aqueous and ethanolic extracts of *B. trimera* showed antiulcerogenic activity, anti-inflammatory and anthelmintic activity (de Oliveira et al., 2014; Dos Reis Livero et al., 2016; Menezes et al., 2016; Nogueira et al., 2011) due the presence of phenolic compounds such as chlorogenic acid, rutin, ellagic acid, rosmarinic acid, luteolin and quercetin (Menezes et al., 2016). *B. uncinella* is fairly used as anti-inflammatory in folk medicine (Zalewski et al., 2011). Studies on the chemical composition of this species have revealed the presence of hispidulin, caffeic acid, chlorogenic acid and pectolinarigenin (Ramos Campos et al., 2016). Moreover, benzoic acid and it derivatives, such as, *p*-hydroxybenzoic acid, found in both fractions, are phenolic compounds that exhibit an well known antioxidant activity (Yamaguchi et al., 2006) and are widely used as a food preservatives (Ding et al., 2015). Chlorogenic acid belongs to the group of hydroxycinnamates, being the most abundant element in human diet from this group. Chlorogenic acid is the main phenolic compounds in coffee and it consists of the conjugate ester of caffeic, ferulic or *p*-coumaric acids with quinic acid. The molecule shows a strong antioxidant activity (Zhaohui Zhao, 2010) and it is the third most abundant compound in FIII. Thus, we believe that at least in part, the antioxidant, antiproliferative and the reversion of phenotype effects observed in the treatments with the methanolic fractions are due the presence of hydrobenzoic acid and chlorogenic acid. In the FIV, chlorogenic and coumaric acids were detected in smaller proportions than in the FIII fraction. For this reason, we believe that there is a molecule or synergism of molecules in greater proportion which could be responsible for the strong antiproliferative effect observed by the FIV fraction. Further investigations are necessary to identify these molecules.

During the development of liver fibrosis occurs the process of transformation HSCs quiescent (lipocytes) into myofibroblasts and this process can result in alterations in contraction capacity of cells and, therefore, lead to a physiological effect in the liver, an effect that can result in a more serious problem, such as portal hypertension (Iredale, 2008). The deactivation of the HSCs cells is an important therapeutic target. With the deactivation of the HSCs, the production of components of extracellular matrix decreases, resulting in reduction of contraction. In our work, this reduction was observed in collagen gel with cells treated with fractions FIII and FIV of methanolic extracts of *B.anomala.*

Even fractions III and IV possessing some components in common, they vary greatly in quantity. FIV was able to decrease proliferation and reverse cell phenotype with a concentration ten times lower than FIII. However, FIII presented better results in cell cycle arrest and antioxidant activity, showing that both fractions have potential for the treatment of liver fibrosis, even though they own distinctive features. FIV appears to be more purified than FIII, since it was the last fraction to be collected in column chromatography. Thereby, we believe that the

predominance of a certain compound in this fraction may be responsible for the effect observed in the HSCs in a lower concentration.

As perspective, it would be necessary to compare the effect of the fractions with the isolated molecules, in order to better understand the effect that each one exerts on HSCs and how they could act concomitantly and thus search for mechanisms of action. Phenolic acids are natural compounds that can act synergistically with other molecules present in the plant extract. Therefore, their effects can complement each other acting more effective on a biological system and likely do not exhibit as significant biological activity when used isolated as they do in sinergism (Vaz et al., 2012).

5. Conclusion

The ability of methanolic fractions III and IV to deactivate hepatic stellate cells was evidenced by decreased in proliferative activity without cytotoxicity. In addition, the fractions restored the lipocytic quiescent cell phenotype, observed by increased accumulation of lipid droplets. The results also indicate that the mechanism by which there was increased lipid synthesis is independent of the expression of PPAR-γ. Finally, our results reveal for the first time the main phenolic constituents of *Baccharis anomala* methanolic extract. The study was able to evaluate the cytotoxicity, phenotypic reversion and antiproliferative effect of the fractions obtained from methanolic extract of *Baccharis anomala* and showed the potential for the treatment of liver fibrosis.

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Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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FIGURE AND TABLE LEGENDS

Fig. 1. Effect of crude extracts of *Baccharis anomala* on cellular proliferation of GRX cells during 72 h of treatment. Cellular proliferation assessed by Typan blue exclude method. Data represent the mean ± SD (n = 3). Results were expressed as cell number. * $P < 0.05$ compared with control.

Fig. 2. Antioxidant activity of the fractions obtained from the methanolic extract at concentration of 100 µg/mL. The antioxidant activity of NAC was evaluated at concentration of 400 µg/mL. Ascorbic acid was used as the control for antioxidant activity at concentration of 550 µg/mL. The results are presented as percentage of DPPH reduction in relation to the control group. Data represent the mean ± SD (n = 3). * $P < 0.05$ compared with control.

Fig. 3. Effect of fractions on GRX cells proliferation during 72 h of treatment. **(A)** FIII;**(B)** FIV at concentrations of 1.25, 2.5, 5, 50, and 100 µg/mL. Cellular proliferation assessed by Trypan blue exclude method. Data represent the mean ± SD (n = 3). Results were expressed as cell number. * $P < 0.05$ compared with control.

Fig. 4. Citotoxicity of fractions III and IV was evaluated by measuring LDH release levels in the supernatant after 72 h of treatment. FIII (50 µg/mL), FIV (5 µg/mL) and NAC (400 µg/mL). Data represent the mean ± SD (n = 3). The results were presented as percentage of LDH release in the supernatant in relation to the total content of LDH culture obtained by cell lysis.

Fig. 5. DAPI nuclei staining. Effect of fractions IIIand IV, and the controls NAC and Cisplatin on apoptosis of GRX cells analyzed by nuclear morphology. **(A)** Control group, **(B)** Cisplatin at 2.5 µM, **(C)** NAC at 400 µg/mL, **(D)** FIII 50 µg/mL and **(E)** FIV at 5 µg/mL. Cells were treated for 72h. Cisplatin were used as positive control of apoptotic inducer; apoptotic cells demonstrated nuclear condensation (arrows).

Fig. 6. Cell cycle arrest in GRX cells were evaluated by 7-AAD. FIII at 50 µg/mL, FIV at 5 µg/mL and NAC at 400 µg/mL. Samples were analyzed by flow cytometry to identify cell cycle phases. Results were presented as percentage of cells at each phase of the cell cycle. Data were analyzed using FlowJo 7.6.5 software (Tree Star Inc., Ashland OR) (n=3). ***P < 0.0001, **P < 0.001 and * P < 0.05 compared with control.

Fig. 7. Oil Red-O (ORO) staining and lipid quantification of GRX cells at 72 h. **(A)** Control, **(B)** FIII at 50 µg/mL, **(C)** FIV at 5 µg/mL and **(D)** NAC at 400 µg/mL, 400x magnification. Lipid droplets indicated by arrows. **(E)** Lipid quantification. Results are shown as the absorbance value obtained for ORO adjusted for number of $50x10^3$ cells. Results are expressed as mean ± SD (n=3). * P < 0.05 compared with control.

Fig. 8. Effect of FIII (5 µg/mL), FIV (50 µg/mL) and NAC (400 µg/mL) on PPARγ mRNA expression of GRX cells treated for 72 h. β-actin was an internal control for equal loading. Results are presented as relative optical density PPARγ/β-actin. Data are expressed as mean ± SD (*n*=3).

Fig. 9. Cell contraction assessed by collagen gel assay in GRX cells. Mean ± SD are shown. n= 4 per group. ***P < 0.001 vs. Control, *P < 0.05 vs. Control, by one-way ANOVA and Tukey test.

Table 1 - Sequence of primers used for RT-PCR.

Table 2 - HPLC analysis of phenolic compounds in *B. anomala* obtained fractions. Results expressed as mean ± standard deviations (SD) of three samples.

FIGURES AND TABLES

FIGURE 1:

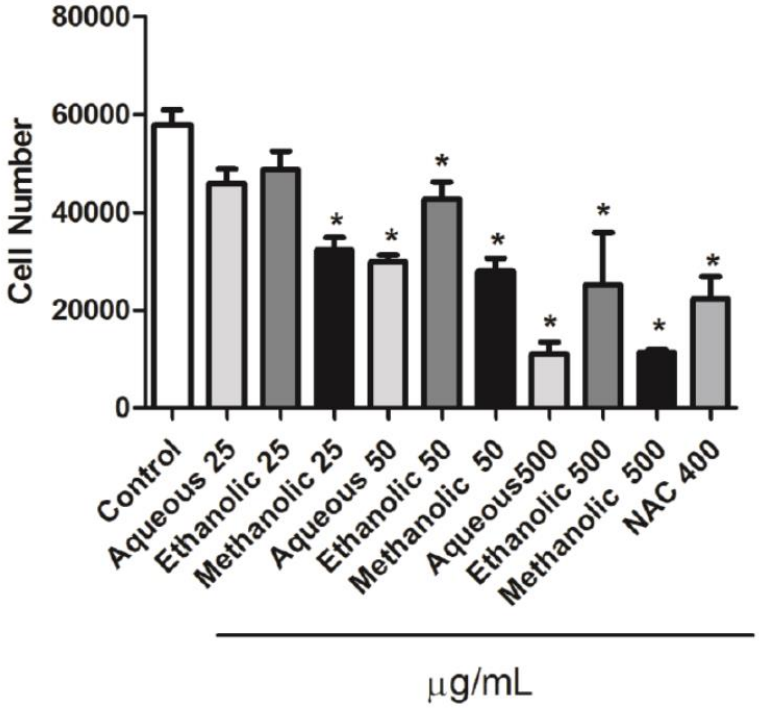
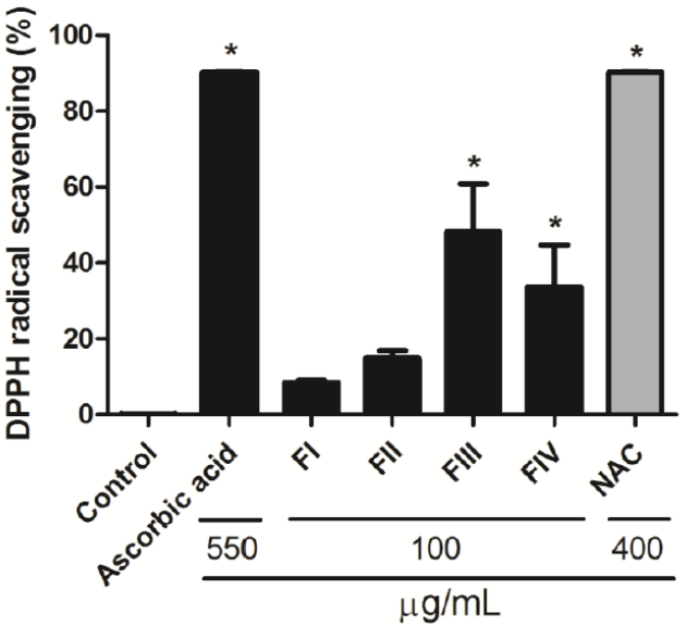


FIGURE 2:



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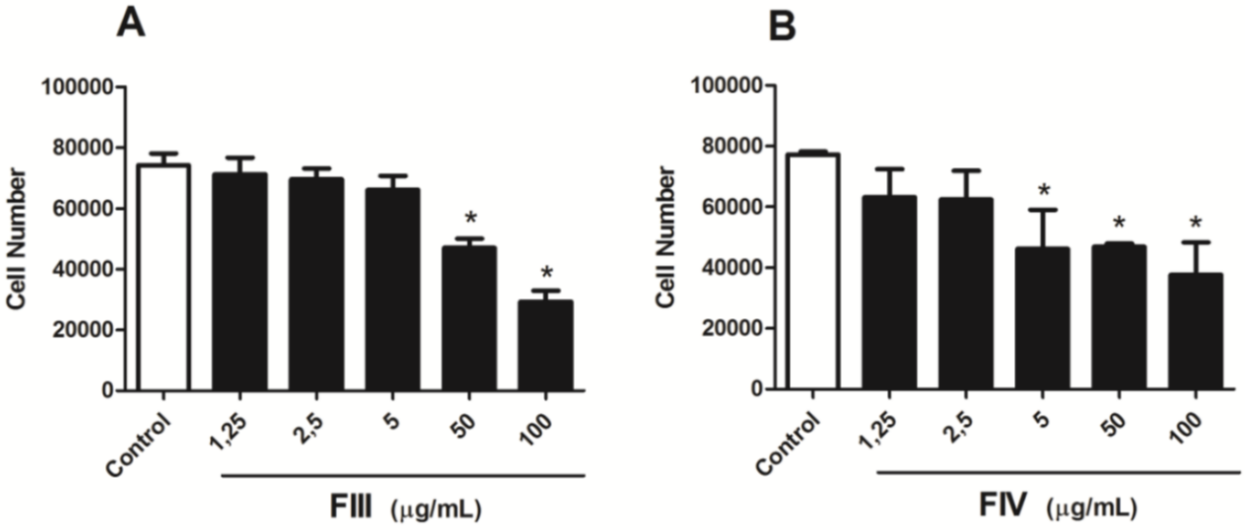

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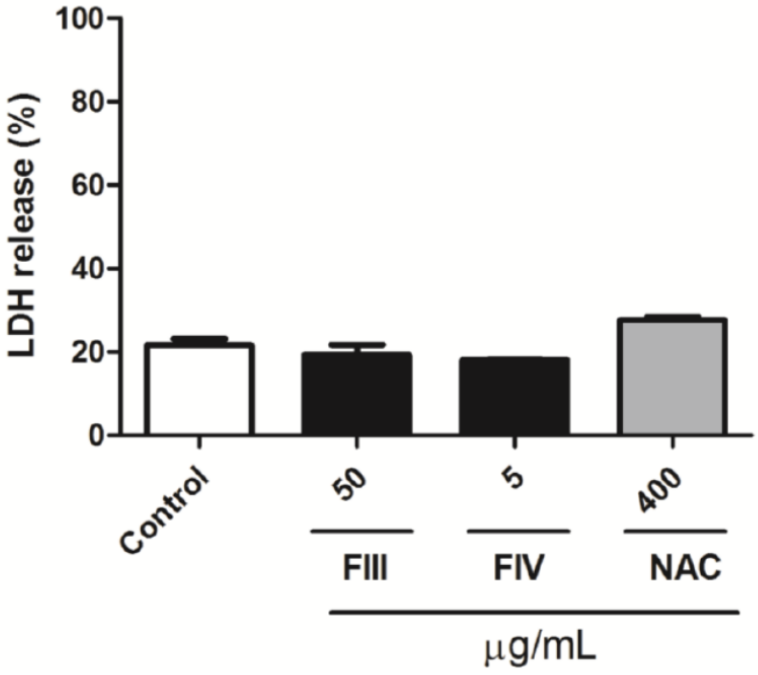

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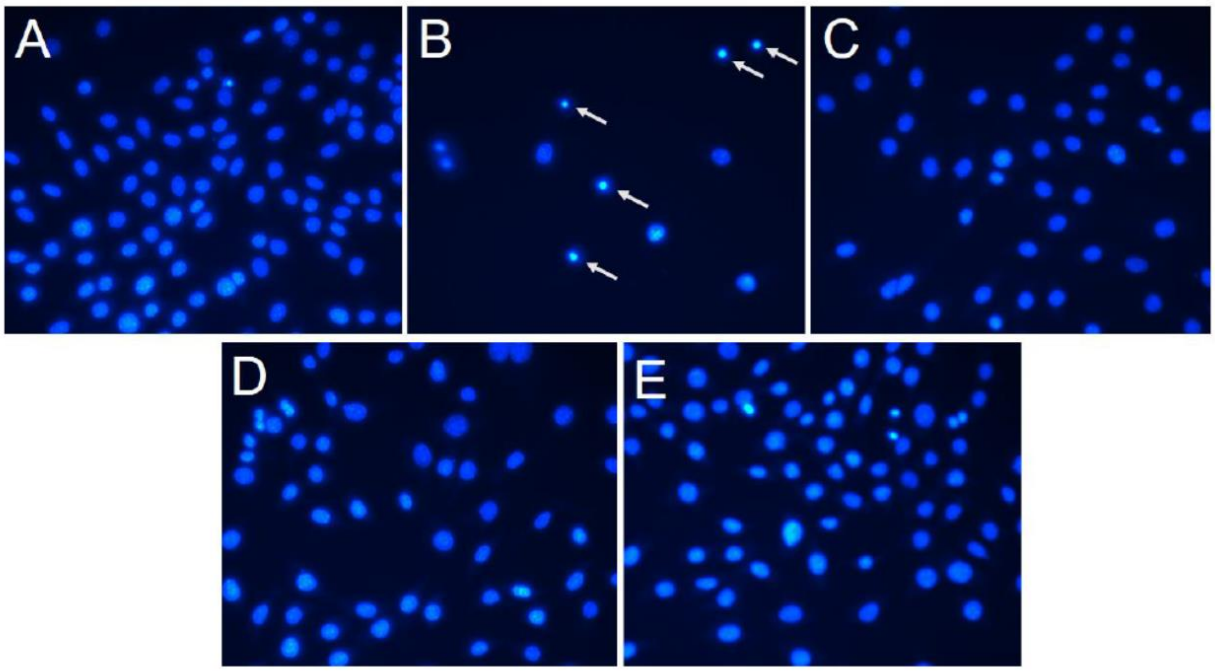

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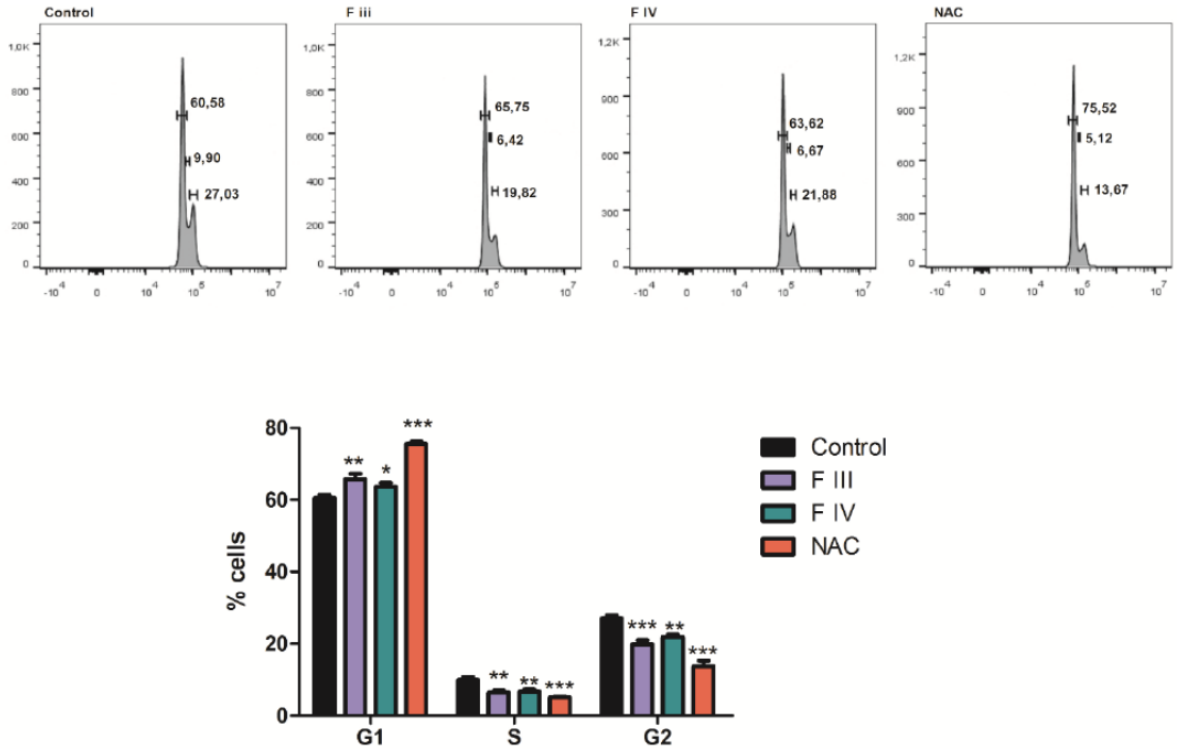

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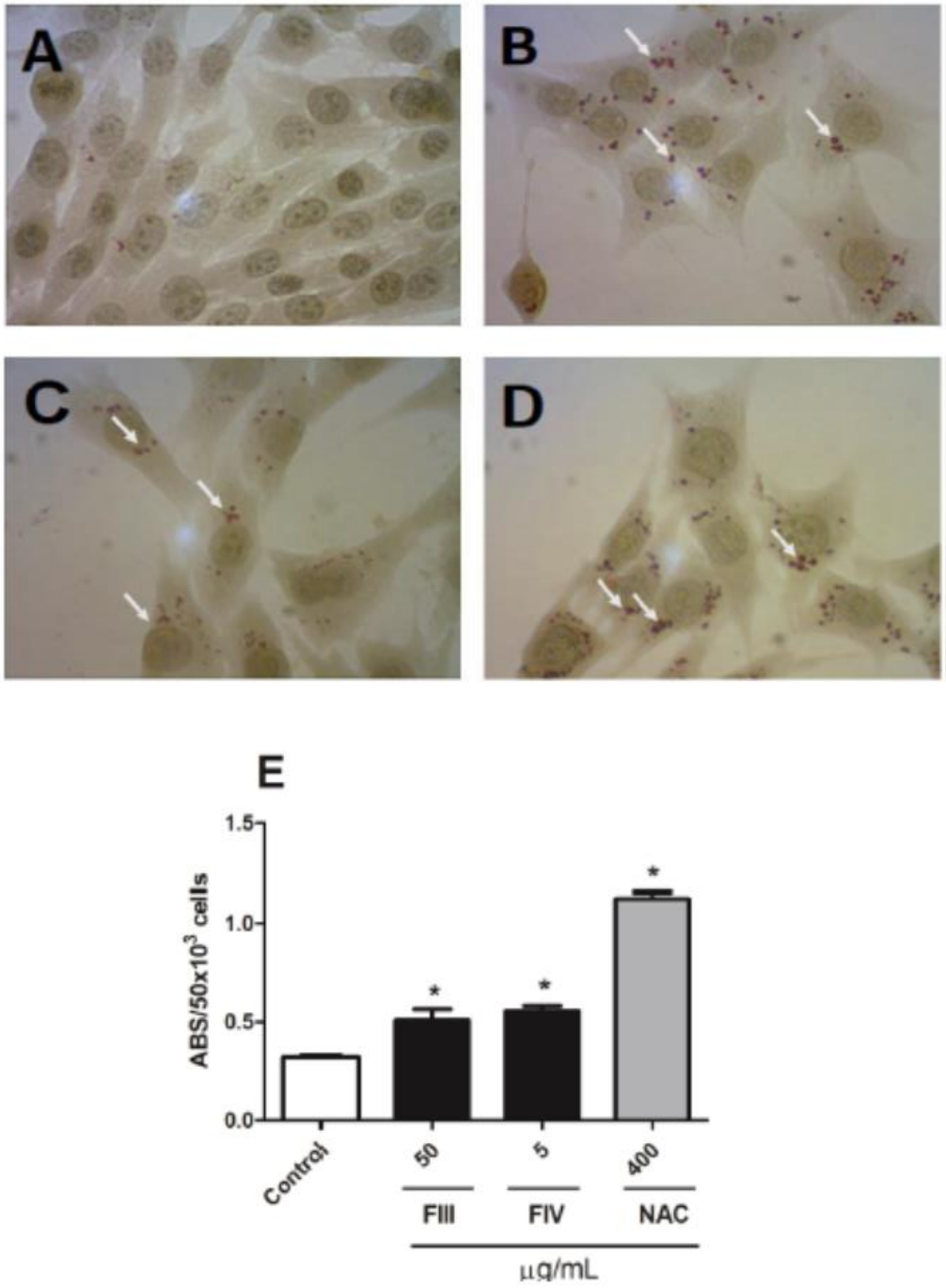
A
B
C
D
E
ABS/50x10^3 cells
1.5
1.0
0.5
0.0
*
*
*
Control
50
5
400
FIII
FIV
NAC
µg/mL

FIGURE 8:

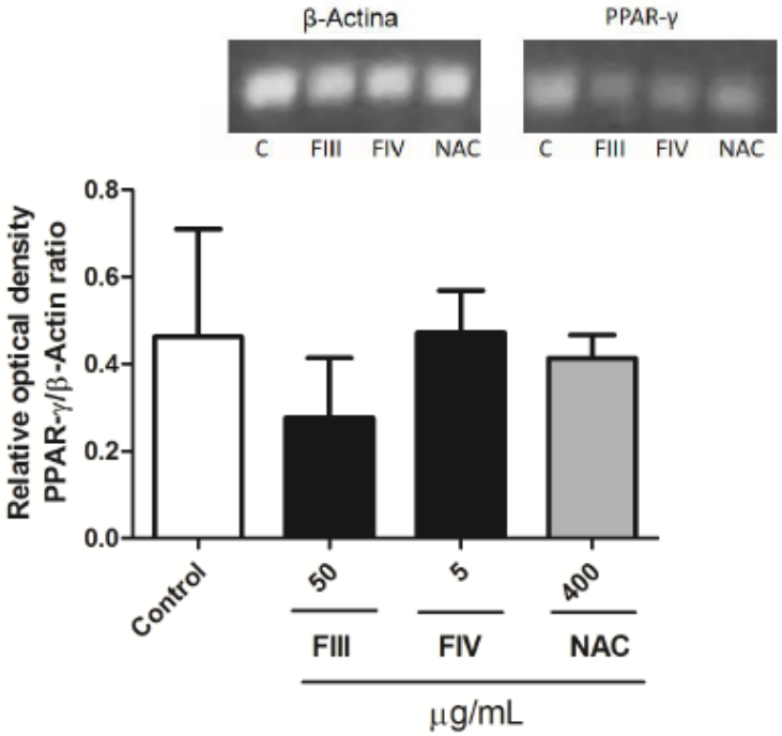

FIGURE 9:

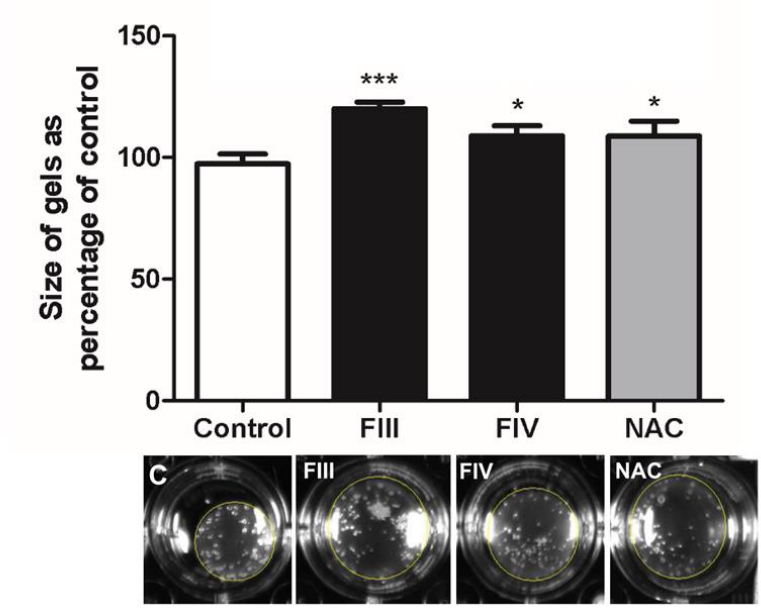

TABLE 1:

Primers	Forward primer (5'– 3')	Reverse primer (5'– 3')	Reference
PPAR-γ	TGGAATTAGATGACAGTGACTTGG	CTCTGTGACGATCTGCCTGAG	(Guimaraes et al. 2007)
β-actin	TATGCCAACACAGTGCTGTCTGG	TACTCCTGCTTGCTGATCCACAT	(Guimaraes et al. 2007)

Table 1 - Sequence of primers used for RT-PCR.

TABLE 2:

		Fraction III	Fraction IV
Compound	Retention time (min)	% mg of compound / mg dry weight	% mg of compound / mg dry weight
Hydroxybenzoic acid	8,7	0,78	-
Chlorogenic acid	12,3	26,66	1,90
Coumaric acid	21,1	1,39	0,53

Table 2 - HPLC analysis of phenolic compounds in *B. anomala* obtained fractions. Results expressed as mean ± standard deviations (SD) of three samples.

5. CONSIDERAÇÕES FINAIS

A carência de estudos sobre a composição fitoquímica da espécie *B. anomala* e o aumento de interesse pelo gênero, nos impulsionaram para a realização deste trabalho. Seu potencial para o desenvolvimento de novas terapias tem despertado interesse pela indústria farmacêutica, visto que, o gênero *Baccahris* possui muitas espécies utilizadas na medicina popular para o tratamento de doenças gastrointestinais, renais, hepáticas e inflamações. A *B. anomala* é encontrada principalmente na região Sul do Brasil, presente na cultura de diversos povos desta região, tendo suas partes aéreas comumente utilizadas em forma de chá devido ao seu efeito diurético.

Compostos fenólicos encontrados em formas naturais, presente em plantas, possuem alta aplicabilidade para o desenvolvimento de terapias alternativas a tratamentos convencionais de diversas doenças. Moléculas pertencentes à classe de composto fenólicos são conhecidas por suas atividades antioxidante, anti-inflamatória, antibiótica, antiviral e anti-helmíntica. Como a fibrose hepática é uma doença associada ao dano crônico no fígado, que em muitos casos, quando é diagnosticada, já apresenta um estágio avançado de fibrose, podendo levar ao desenvolvimento de cirrose e ao risco de insuficiência hepática. No entanto, a doença em estágios iniciais pode ser reversível. A atividade antioxidante tem sido uma característica química almejada para o tratamento da fibrose hepática, devido aos ótimos resultados observados, visto que a NAC, um fármaco reconhecido por possuir alta atividade antioxidante, é atualmente utilizado para o tratamento da doença.

O presente estudo demonstrou a habilidade das frações do extrato metanólico em reverter o perfil fibrótico para quiescente e diminuir a proliferação de células hepáticas estreladas ativadas. Através da análise em CLAE foi possível identificar alguns dos compostos fenólicos presentes nas frações. Os resultados revelaram o potencial dos compostos encontrados nas frações avaliadas para o tratamento da fibrose hepática.

Para melhor compreender os mecanismos de ação envolvidos na atividade observada pelos compostos identificados, trabalhos futuros deverão investigar o efeito da associação concomitante destas moléculas e também sua ação de

forma isolada. Uma vez que já se tem conhecimento de que estes compostos podem apresentar efeitos sinérgicos e assim apresentar uma atividade biológica mais significativa do que as mesmas quando isoladas. Com isso, desejamos ter atingindo aos objetivos propostos pelo trabalho e também ter acrescentado de forma positiva ao estudo do gênero *Baccharis.*

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