ESCOLA DE CIÊNCIAS DA SAÚDE E DA VIDA
PROGRAMA DE PÓS-GRADUAÇÃO EM
BIOLOGIA CELULAR E MOLECULAR

BRUNO DE SOUZA BASSO

AVALIAÇÃO DO METOXIEUGENOL E DO EXTRATO DE *Baccharis anomala DC.* NO TRATAMENTO DE FIBROSE HEPÁTICA

Porto Alegre

2021

BRUNO DE SOUZA BASSO

AVALIAÇÃO DO METOXIEUGENOL E DO EXTRATO DE *Baccharis anomala DC.* NO TRATAMENTO DE FIBROSE HEPÁTICA

Tese apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Escola de Ciências da Saúde e da Vida da Pontifícia Universidade Católica do Rio Grande do Sul

Orientador: Prof. Dr. Jarbas Rodrigues de Oliveira

Co-orietadora: Prof. Dr. Eliane Romanato Santarém

Porto Alegre

2021

Ficha Catalográfica

B322a Basso, Bruno de Souza

Avaliação do metoxieugenol e do extrato de Baccharis anomala DC. no tratamento de fibrose hepática / Bruno de Souza Basso. – 2021.

80.

Tese (Doutorado) – Programa de Pós-Graduação em Biologia Celular e Molecular, PUCRS.

Orientador: Prof. Dr. Jarbas Rodrigues de Oliveira.
Co-orientadora: Profa. Dra. Eliane Romanato Santarém.

1. Compostos fenólicos. 2. Fibrose. 3. Fitoquímicos. 4. Doenças hepáticas. I. Oliveira, Jarbas Rodrigues de. II. Santarém, Eliane Romanato. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da PUCRS
com os dados fornecidos pelo(a) autor(a).
Bibliotecária responsável: Clarissa Jesinska Selbach CRB-10/2051

RESUMO

A fibrose hepática é uma doença que tem o seu desenvolvimento ligado ao dano crônico no fígado, sendo muitas vezes consequência de infecções virais, abuso alcoólico, toxinas, ou acúmulo de metais. O dano crônico no fígado leva a um processo inflamatório que pode resultar na perda do equilibro da síntese e degradação de elementos de matriz extracelular, sendo a ativação das células hepáticas estreladas um dos principais fatores responsáveis por iniciar o processo fibrótico. Geralmente, a progressão da fibrose hepática, é detectada somente em estágios avançados, podendo resultar no último estágio de doenças crônicas hepáticas, conhecido como cirrose, com isso levando ao risco de insuficiência hepática. Atualmente, não existe um tratamento bem estabelecido, porém, se busca primeiramente identificar e remover o agente causador do dano crônico e utilizar juntamente terapias capazes de reduzir a ativação, proliferação ou até mesmo induzir apoptose em células estreladas hepáticas. No entanto, moléculas com efeito antioxidante têm mostrado efeitos significativos na diminuição do dano por estresse oxidativo. Sendo assim, a busca por novos candidatos ao tratamento da fibrose hepática é necessária, e para isso, a investigação de moléculas de fontes naturais e renováveis parece ser promissora, pois sabe-se que os metabólitos secundários de muitas espécies de plantas possuem potencial para o desenvolvimento de novos fármacos. Dessa forma, o presente estudo investigou os efeitos de dois tratamentos que possuem em comum, sua fonte de origem natural e sua composição fitoquímica, composta principalmente por compostos fenólicos. No primeiro estudo *in vitro* e *in vivo*, a molécula do metoxieugenol,

demonstrou efeito modulador em células hepáticas estreladas ativadas, reduzindo seus marcadores de ativação (Col-1, α-SMA e TGF-β e) e revertendo ao seu fenótipo quiescente. Da mesma forma, o tratamento foi capaz de ativar o fator de transcrição PPAR-γ, e quando na presença do seu inibidor, GW9662, o efeito do metoxieugenol foi suprimido, mostrando a interação da ativação de PPAR-γ nos efeitos observados. O efeito hepatoprotetor *in vivo* em camundongos foi observado, atenuando o desenvolvimento da fibrose e diminuindo a ativação de vias inflamatórias mediadas por NF-kB. No segundo estudo *in vivo*, o tratamento com o extrato de *Baccharis anomala DC.* diminui o dano por estresse oxidativo (TBARS e DCFH-DA), reduziu a ativação de genes pro inflamatórios (TNF-α, NF-kB, IL-6 e iNOS) e protegeu o fígado dos animais do desenvolvimento da fibrose. Os principais componentes do extrato identificados por HPLC foram os ácidos fenólicos caféico, gálico e cumárico.

Palavras chave: Compostos fenólicos, fibrose, fitoquímicos, doenças hepáticas.

ABSTRACT

Liver fibrosis is a disease that has its development linked to chronic liver damage, often due to viral infections, alcohol and drugs abuse toxins or metal accumulation. Chronic liver damage leads to a continuous inflammatory process that can result in activation of hepatic stellate cells. The activated phenotype of hepatic stellate cells disrupts the balance between synthesis and degradation of extracellular matrix elements, and this process is responsible for initiating the fibrotic process. The progression of liver fibrosis is often detected in late stages of disease and may result in the last stage of chronic liver disease, known as cirrhosis, leading to the risk of liver failure. Currently, liver fibrosis has no well-established treatment, however, the first goal for therapy is to identify and remove the cause of chronic damage and to use therapies capable of reducing activation, proliferation or even inducing apoptosis in hepatic stellate cells. In addition, molecules with an antioxidant effect have shown good effects in decreasing oxidative stress damage. Thus, the search for new candidates for the treatment of liver fibrosis is necessary, and for this, the investigation of molecules from natural and renewable sources seems to be promising, since it is currently known that the secondary metabolites of many plant species have high applicability for the development of new drugs. Thus, the present study investigated the effects of two treatments that have in common, their natural source and their phytochemical composition, mainly composed of phenolic compounds. In the first *in vitro* and *in vivo* study, the methoxyeugenol molecule demonstrated a modulatory effect on activated hepatic stellate cells, reducing its activation markers (Col-1, α-SMA and

TGF-β e) and inducing to its quiescent phenotype. Also, the treatment was able to activate the PPAR-γ transcription factor, and when in the presence of its inhibitor, GW9662, the effect of methoxyeugenol was suppressed, showing the interaction of PPAR-γ activation in the observed effects. Furthermore, it showed hepatoprotective effect *in vivo* in mice, attenuating the development of fibrosis and decreasing the activation of NF-kB-mediated inflammatory pathways. In the second *in vivo* study, treatment with *B.anomala* extract decreases oxidative stress damage (TBARS and DCFH-DA), reduced activation of pro-inflammatory genes (TNF-α, NF-kB, IL-6 and iNOS) and protected the animals' livers from the development of fibrosis. The main components of the extract identified by HPLC were caffeic, gallic and coumaric phenolic acids.

Key words: Fibrosis, liver diseases, phenolic compounds, phytochemicals.

AGRADECIMENTOS

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Agradeço, primeiramente, à minha família por todo apoio e suporte durante minha formação.

Agradeço ao meu orientador, Jarbas Rodrigues de Oliveira, por todas as oportunidade e pelo aprendizado durante toda minha formação acadêmica, desde a iniciação cientifica.

Agradeço à minha co-orientadora, Eliane Romanato Santarém, pelos ensinamentos na minha formação como biólogo e em todo suporte durante a pós-graduação.

Agradeço ao meu orientador no exterior, Jordi Gracia-Sancho, por me receber em seu laboratório no exterior e pela oportunidade de trabalhar com a sua equipe.

Agradeço a todos meus colegas de laboratório pela ajuda e companheirismo durante todo o período de doutorado.

Agradeço a todos, de que alguma forma tenha contribuído de forma positiva para esta etapa da minha vida.

Obrigado.

LISTA DE SIGLAS

α-SMA: Actina de músculo liso alfa

ALT- Alanina transaminase

AST- Aspartato transaminase

CCl_4: Tetracloreto de carbono

CLD: Doenças crônicas do fígado

CAT: Catalase

DMEM: Dulbecco's Modified Eagle Medium

ECM: Elementos de matriz extracelular

EROS: Espécies reativas de oxigênio

HGF: Fator de crescimento hepático

HSC: Célula hepática estrelada

IP: Intraperitoneal

LDH: Desidrogenasse láctica

NAC: N-acetilcisteína

NF-κB: Fator nuclear kappa B

ORO: Oil Red O

PDGF- βr: Receptor do fator de crescimento derivado de plaquetas subunidade B

PPAR: Receptor ativado por proliferador de peroxissoma

PUCRS: Pontifícia Universidade Católica do Rio Grande do Sul

SFB: Soro fetal bovino

SLM: Silimarina

TAA: Tioacetamida

TBA: Ácido tiobarbitúrico

TBARS: Thiobarbituric acid reactive substances

TGF-β: Fator de transformação do crescimento beta

TNF- α: Fator de necrose tumoral alfa

TIMPs: Inibidores de metaloprotease tecidual

SUMÁRIO

1. INTRODUÇÃO

1.1 Fibrose Hepática

O desenvolvimento de doenças crônicas hepáticas (CLD) se deve ao dano persistente as células do fígado, levando a um processo inflamatório crônico no órgão, somado a um processo de ativação de células responsáveis pelo equilibro de síntese e degradação de elementos da matriz extracelular (ECM), instalando assim um quadro de fibrose hepática (Wynn e Ramalingam, 2012).

O dano crônico no tecido hepático pode ser resultado de infecções virais (hepatites B e C), assim como, o abuso de drogas e álcool, e acumulo de toxinas ou metais, e em alguns casos doenças hereditárias (Friedman, 2008; Iredale, 2008). Quando o dano crônico persiste, uma resposta cicatricial continua se estabelece no fígado, e essa deposição desordenada de ECM somada ao processo inflamatório crônico, pode levar ao risco de insuficiência hepática. Esse último estágio das CLD, também conhecido como cirrose, se caracteriza pela distorção do parênquima hepático devido a formação de nódulos e septos, alterações no fluxo sanguíneo e modificação da arquitetura celular hepática. Estima-se que, anualmente, ocorram aproximadamente 2 milhões de mortes por CLD no mundo (Asrani et al., 2019).

Primeiramente, o processo de deposição de ECM leva a alterações na região do tecido hepático localizado ao redor da camada sinusoidal no espaço de Disse, onde se encontram as células hepáticas estreladas (HSC), que são consideradas uma das principais estruturas celulares no desenvolvimento da

fibrose hepática e as responsáveis pelo desequilíbrio da síntese e degradação de ECM (Friedman, 2008). Os hepatócitos compreendem aproximadamente 80% das células hepáticas e são as células mais afetadas pelo dano de substâncias no órgão, pois são responsáveis pelo metabolismo de diversas substâncias no organismo. O dano aos hepatócitos leva à produção de espécies reativas de oxigênio e citocinas inflamatórias e prófibrogênicas, que ativam as células estreladas hepáticas HSC, direta e indiretamente, através das células de Kupffer e das células endoteliais sinusoidais (Roehlen et al., 2020). As células de Kupffer podem estimular a ativação das HSCs através de fatores como fator de crescimento transformante-β (TGF-β) e o Fator de necrose tumoral alfa (TNF-α), estimulando a síntese de ECM, a proliferação celular e o decréscimo dos depósitos de lipídeos intracelular das HSCs (Kolios et al., 2006; Marrone et al., 2016). Na Figura 1, observa-se os principais mecanismos envolvidos na ativação das HSC, onde diferentes tipos celulares promovem um estado inflamatório crônico, sendo a sinalização pro inflamatória capaz de ativar tanto células HSC quanto células do sistema imune, com isso perpetuando o fenótipo ativado das HSC.

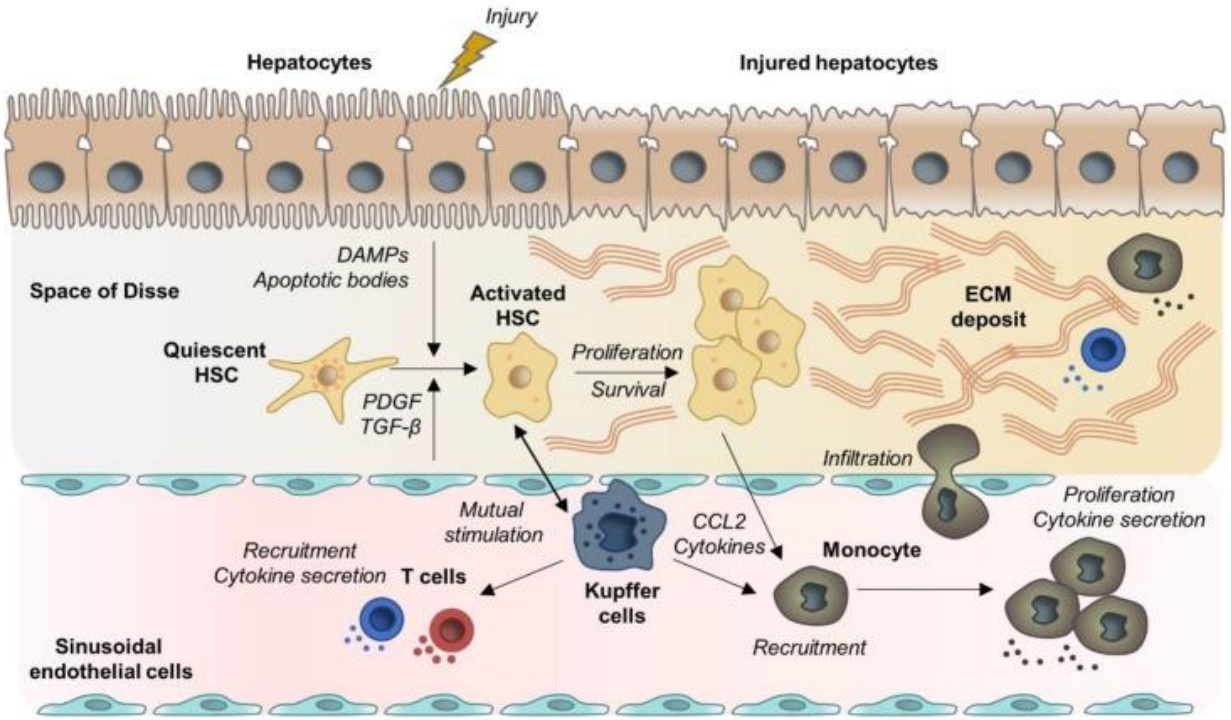


Figura 1: Principais mecanismos envolvidos na fibrose hepática. (Roehlen N, Crouchet E, Baumert TF, 2020).

1.2 Células hepáticas estreladas

As HSC correspondem a aproximadamente 8% da população celular total no tecido hepático, sendo os hepatócitos (células parenquimatosas) presente em maior quantidade seguido de células endoteliais (sinusoidais e vasculares) e por último, em menor quantidade, as células de Kupffer (macrófagos hepáticos).

Em condições normais, as HSC apresentam o seu fenótipo quiescente, e possuem como característica o acúmulo de gotículas de lipídeos em seu citoplasma (principalmente de vitamina A), outra característica é a baixa taxa proliferativa e a regulação no balanço entre a síntese e degradação de componentes da ECM (Guimaraes et al., 2007). O fenótipo quiescente das HSC

pode ser regulado por fatores de transcrição como o grupo de proteínas receptoras nucleares, os receptores ativados por proliferador de peroxissoma (PPAR), que participam na transcrição de diversos genes capazes de regular seu fenótipo lipocitico, as isomorfas mais conhecidas dos PPAR são o PPAR-α, PPAR-β / δ e PPAR-γ. Importante destacar o fator de transcrição PPAR-γ, pois essa isoforma de PPAR tem sido considerado como um dos principais reguladores da lipogênese em HSCs (Guimaraes et al., 2007; Tsukamoto et al., 2006), já que sua ativação é necessária para a manutenção do fenótipo quiescente em HSC ativadas (de Mesquita et al., 2013). Além disso, estudos tem demostrado a relação entre a ativação de PPAR- γ e a supressão de vias inflamatórias mediadas por NF-kB (He et al., 2019; Zong et al., 2013), com isso, a regulação desse fator de transcrição pode ser considerado um alvo terapêutico interessante para o desenvolvimento de tratamentos para a fibrose hepática, já que possui relação com a manutenção do fenótipo quiescente e a sinalização inflamatória (Mirza et al., 2019).

Quando o dano crônico persistente no tecido hepático leva a sinalização de vias inflamatórias ocorre a ativação das HSC, e o resultado da ativação dessas células é a perda na capacidade de armazenar lipídeos em seu citoplasma, aumento em sua taxa proliferativa, e também, na perda do seu controle sobre o equilíbrio entre a síntese e degradação de componentes da ECM, levando a uma deposição desordenada de ECM (Mederacke et al., 2013; Testerink et al., 2012). A deposição de ECM é composta principalmente por fibronectina e fibras de colágeno do tipo I, e assim caracterizando o início da fibrogênese no fígado, que

por consequência, pode resultar na desestruturação do tecido e perda de sua funcionalidade (Gracia-Sancho et al., 2018).

Quando ocorre a ativação das HSCs, seu fenótipo passa de lipocítico para miofibroblástico que é caracterizado pela perda das gotículas de gordura em seu citoplasma, aumento da proliferação celular e também por alterações em outras células sinusoidais (por ex. células endoteliais) envolvidas no processo de fibrogênese hepática (Gracia-Sancho et al., 2018). A α-SMA é considerada um marcador fundamental para a formação de miofibroblastos em diferentes tecidos, pois sua expressão se encontra em níveis basais nos tecidos normais, enquanto que é altamente expressa em tecidos que possuem o desenvolvimento de um processo fibrótico. Além disso, também ocorre um aumento nos níveis de expressão e secreção do fator de crescimento TGF-β1 e de inibidores de metaloprotease tecidual (TIMPs) 1 e 2 (Friedman, 2008).

1.3 Tratamento da fibrose hepática

Atualmente não existe uma terapia padrão para o tratamento da fibrose hepática, porém sabe-se que a identificação e remoção do agente causador do dano crônico ao fígado devem ser feitas, e são fundamentais para o início do tratamento (Roehlen et al., 2020). Os fármacos testados para o tratamento da fibrose hepática podem variar segundo o seu alvo terapêutico. Muitos buscam formas de controlar o estresse oxidativo formado no ambiente hepático inflamado, sendo capazes de diminuir a formação de radicais livres ou restaurar os

antioxidantes endógenos, estratégia esta, que apresenta bons resultados para diminuição do dano causado no tecido hepático (Odagiri et al., 2021).

Cada vez mais se sabe que fatores de crescimento são capazes de ativar tanto as HSC como as células de Kupffer, instalando um estado inflamatório crônico no fígado e favorecendo a proliferação das HSCs (Kolios et al., 2006), dessa forma, inibidores da ativação de TGF-β são possíveis candidatos para o tratamento, sendo que esse efeito pode ser obtido através da utilização de RNAs de interferência (Pragyan et al., 2021; Tighe et al., 2020). Muitos fármacos têm como alvo a desativação das HSC, por serem consideradas peças fundamentais no processo fibrótico. Dentre eles pode-se citar, os agonistas de PPAR-γ (pioglitazone), o agonista de FXR (ácidos obeticólicos) e os inibidores de fatores de crescimento (pirfenidona) (Odagiri et al., 2021).

Mais recentemente, a importância das células endoteliais sinusoidais para a manutenção do parênquima hepático tem sido estudada (Lafoz et al., 2020). Em condições normais, as células endoteliais sinusoidais representam um endotélio com fenestras (em condições normais não apresentam membrana basal), que comunica o sangue sinusoidal com as células hepáticas através de seus pequenos poros (fenestras). São responsáveis pela homeostasia do metabolismo, regulação da sinalização inflamatória, angiogênese e regulação do tônus vascular (Lafoz et al., 2020). Quando ativadas devido ao dano hepático, as células endoteliais sinusoidais desenvolvem uma camada basal e passam a formar um endotélio continuo, sendo essa uma das características do estágio inicial da fibrose, conhecida como capilarização (Sørensen et al., 2015). No fenótipo

ativado, passam a favorecer processos vasoconstritores, fibróticos, inflamatórios e angiogênico, além de contribuírem para ativação das HSCs e das células de Kuppfer (Maretti-Mira et al., 2019; DeLeve et al., 2008). Existem poucos estudos a respeitos de terapias específicas visando as células sinusoidais endoteliais, porém, trabalhos recentes têm explorado seus numerosos receptores e formas de entregar fármacos por nanopartículas, nanocápsulas e nanogels, por exemplo, devido sua capacidade de endocitar macromoléculas solúveis (Chen et al., 2019; Lafoz et al., 2020; Szafraniec et al., 2017).

Compostos de origem natural também têm recebido atenção para o desenvolvimento de novos tratamentos, como por exemplo, o resveratrol, curcumina e silimarina (Pragyan et al., 2021; Popov, 2009; Tighe et al., 2020; Liedtke et al., 2013). Esses compostos alternativos com potencial antifibrótico possuem em comum o seu alto poder antioxidante, sendo essa uma característica importante para diminuição do estresse oxidativo no tecido hepático. Além disso, estudos demonstraram que essas moléculas naturais são capazes de regular fatores de crescimento, como TGF-β e PDGF- βr, diminuir a ativação de HSCs por reduzir a sinalização de citocinas pro inflamatórias (TNF-α e NF-kB) ou induzir morte celular por apoptose (Popov, 2009; Tighe et al, 2020). Alguns compostos de origem natural já foram testados em ensaios clínicos, para validar seu efeito protetivo em casos de fibrose hepática, como a espirulina, resveratrol e a sylibina (http://clinicaltrials.gov/). A Figura 2, mostra a diversidade de estratégias para o tratamento da fibrose hepática.

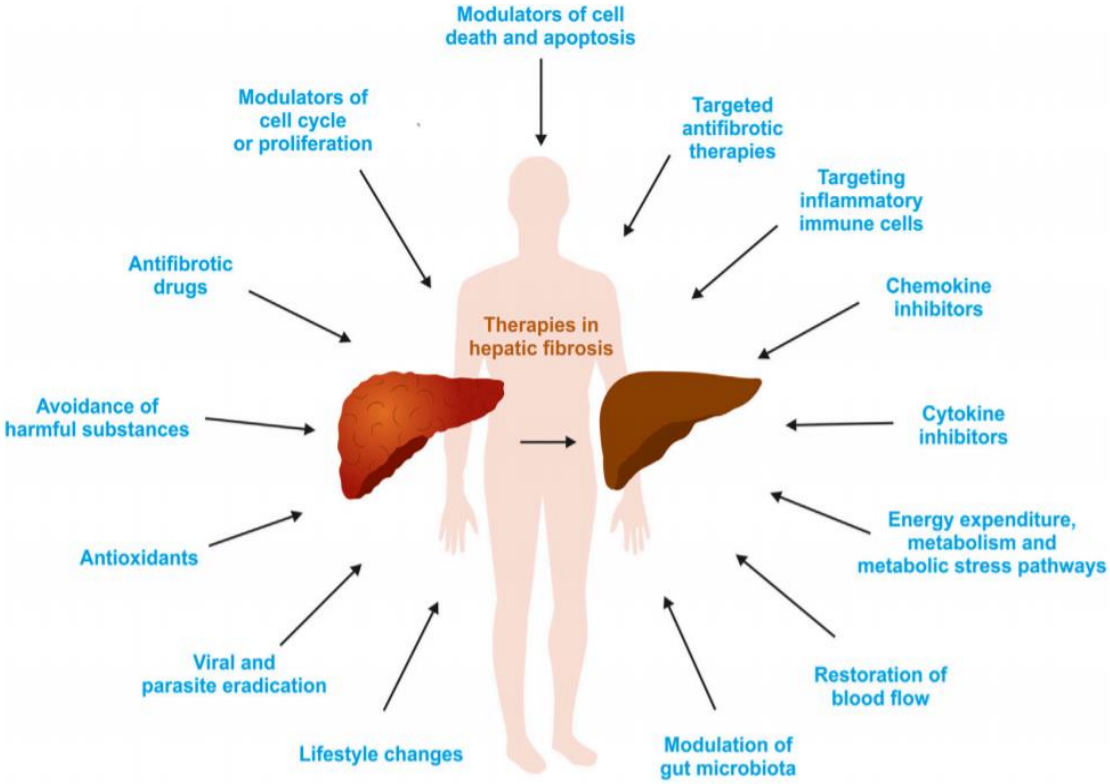


Figura 2: Estratégias propostas para o tratamento da fibrose hepática. (Acharya Pragyan, 2021)

Considerando que atualmente não existem tratamentos estabelecidos para a fibrose hepática, a busca por novas alternativas se faz necessária, incluindo a investigação de compostos naturais, a partir de fontes renováveis e sustentáveis, uma vez que existe uma ampla ocorrência de compostos com propriedades farmacológicas em diversos gêneros de plantas (Kuete et al., 2016).

1.4 Compostos fenólicos

Fármacos capazes de reverter o fenótipo ativado, diminuir a proliferação das HSCs, reduzir o estresse oxidativo ou os fatores inflamatórios no fígado, são testados para o desenvolvimento de tratamentos para a fibrose hepática. Muitas

plantas que possuem compostos com propriedades terapêuticas estão presentes na medicina popular de diversas culturas, e muitas vezes, essas plantas são utilizadas em forma de infusão, macerados ou extratos mais complexos. Seus efeitos farmacológicos são frequentemente atribuídos a sua atividade antioxidante e anti-inflamatória (Denardin et al., 2014; Zalewski et al., 2011)

Esses compostos, muitas vezes descritos como terapêuticos, são conhecidos como metabólitos secundários. Os metabólitos secundários são produzidos pelo vegetal como resposta direta das interações da planta com o ambiente. Com isso, a produção e o acúmulo desses metabólicos pelo vegetal, está relacionada com a temperatura, atração de polinizadores, luminosidade e ataque de predadores, por exemplo. Sendo esses fatores ambientais responsáveis na composição química dos metabólitos secundários que se podem encontrar em uma planta (Sartor, 2013). Dentre os metabólitos secundários, os compostos fenólicos, são uma das classes mais conhecidas e estudas e incluem muitas moléculas com atividade biológica comprovada e estão muito presentes nossa alimentação humana. Por isso, é uma das principais e mais estudadas classes de metabólitos secundários (Bravo, 1998; Neuhouser, 2004).

Os compostos fenólicos possuem uma enorme diversidade de estruturas químicas e representam um grupo heterogêneo de fitoquímicos. Em comum, essas moléculas apresentam anéis de fenol (Figura 3), e são classificadas em vários grupos, dentre os quais destacam-se ácidos fenólicos, flavonoides e ligninas (Saha et al., 2019). Por possuírem uma característica naturalmente antioxidante, eles combatem os danos oxidativos através da doação de hidrogênio

ou elétron para radicais livres e assim, neste processo, auxiliam na estabilização da membrana celular e inibindo a ativação e expressão de citocinas inflamatórias como TNFα, TGF-β e diversas outras interleucinas (IL-6, IL-2, IL-8) (Saha et al., 2019; Zhang e Tsao, 2016; Zhen et al., 2016).

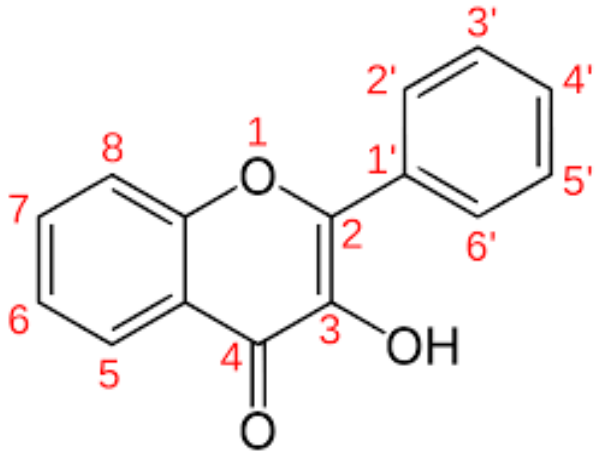


Figura 3: Estrutura básica de um composto fenólico.

1.5 Metoxieugenol

O Metoxieugenol (4-Alil-2,6-dimetoxifenol) é encontrado em ervas e especiarias, como em óleo de sassafrás (*Sassafras albidum*), noz-moscada (*Myristica fragrans*) e *Cinnamomum glanduiferum* (Agnihotri et al., 2012; Guridip Singh, 2005; Kamdem e Gage, 1995). Por apresentar um aroma descrito muitas vezes como defumado é uma molécula utilizada na indústria de alimentos como aditivo, e, por esta razão, já existem estudos a respeito de seus efeitos tóxicos e doses seguras para humanos (European Food Safety Authority , 2011). Estudos com o extrato de noz-moscada (*Myristica fragrans*) revelaram atividade anti-

helmíntica, antifúngica e antioxidante (López, 2015; Maeda et al., 2008). Também foi demonstrado seu efeito anti-inflamatório em doenças hepáticas, onde o extrato etanólico de noz-moscada reduziu a ativação das citocinas inflamatórias IL-6 e TNF-α (Zhao et al., 2020).

O metoxieugenol também foi identificado no própolis vermelho brasileiro, que é produzido por abelhas da espécie *Apis melifera* a partir de espécies de plantas da família *Leguminosae* encontradas em Maceió, no estado de Alagoas, no nordeste do Brasil. O extrato metanólico do própolis vermelho apresentou atividades antioxidante e antimicrobiana (Boeing et al., 2020; Righi et al., 2011) e, em estudos *in vivo*, demonstrou efeito hepatoprotetor em ratos submetidos a administração tóxica de tioacetamida (Silva et al., 2019). Além disso, em camundongos, foi demonstrada a proteção contra o dano causado por álcool no trato gastrointestinal, através da redução da produção de radicais livres e aumento na atividade dos antioxidantes endógenos catalase (CAT) e glutationa (GSH) (Boeing et al., 2020). O metoxieugenol pertence à classe de compostos orgânicos conhecidos como fenilpropanóides, caracterizados por possuir uma cauda de três carbonos ligados ao anel benzeno de um fenol. Na Figura 4 está representada a molécula do metoxieugenol, junto de duas fontes dessa molécula (própolis vermelho e noz-moscada).

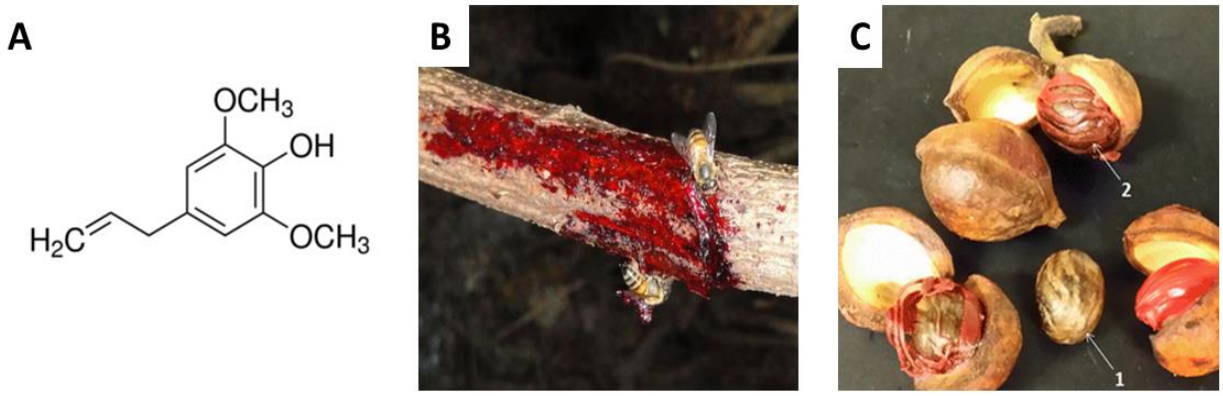


Figura 4: Molécula do methoxieugenol (4-Alil-2,6-dimetoxifenol) **(A).** Esudato, conhecido como *própolis-vermelho-brasileiro* **(B)**. Semente de *Myristica fragrans* (noz-moscada) **(C)**.

1.6 *Baccharis anomala*

As espécies do gênero Baccharis estão distribuídas pela América do Sul (Budel, 2008) e são muito presentes na medicina popular dos povos dessa região, sendo utilizadas para o tratamento de diversas patologias como doenças gastrointestinais, hepáticas, inflamação, diarreia, febre, infecções e diabetes (Pereira et al., 2016; Ramos Campos et al., 2016; Rezende et al., 2014). Estas plantas são utilizadas normalmente na forma de chás, bebidas alcoólicas ou macerados. Pelas reconhecidas propriedades medicinais encontradas no gênero *Baccharis*, a busca por espécies deste gênero com potencial para o desenvolvimento de tratamentos alternativos, é promissora, visto que a maior parte dos estudos realizados foram *com B. trimera, B. dracunculifolia e B. uncinella*, que compreendem apenas uma pequena parte de todo potencial fitoquímico que as espécies do gênero possuem (Nogueira et al., 2011; Ramos Campos et al., 2016; Zalewski et al., 2011).

Baccharis anomala (Figura 5), também conhecida como cambará-de-cipó ou parreirinha, possui sua distribuição geográfica nas regiões Sudeste (Minas Gerais, São Paulo) e Sul (Paraná, Santa Catarina, Rio Grande do Sul) do Brasil e suas partes aéreas são utilizadas na medicina popular como diurético (Budel, 2008). A espécie tem sido estudada quanto suas propriedades medicinais, o extrato de *B. anomala* apresentou atividades antiviral e anti-inflamatórias (Venturi et al., 2018; Basso et al., 2019). Em estudo prévio publicado do nosso grupo de pesquisa, o extrato metanólico fracionado de *B. anomala* demonstrou efeito antiproliferativo em células GRX (HSC de camundongos ativadas), além de reverter seu fenótipo ativado para quiescente (Basso et al., 2019).

As principais propriedades encontradas nos extratos das partes aéreas de espécies de *Baccharis* parecem estar relacionadas com a presença de compostos antioxidantes, anti-inflamatórios, analgésicos e antimicrobianos (Cariddi et al., 2012; Hocayen et al., 2015). Dentre os metabólitos secundários encontrados nas espécies do gênero, destacam-se a presença de compostos fenólicos, por seus efeitos farmacológicos conhecidos (Abad, 2007).

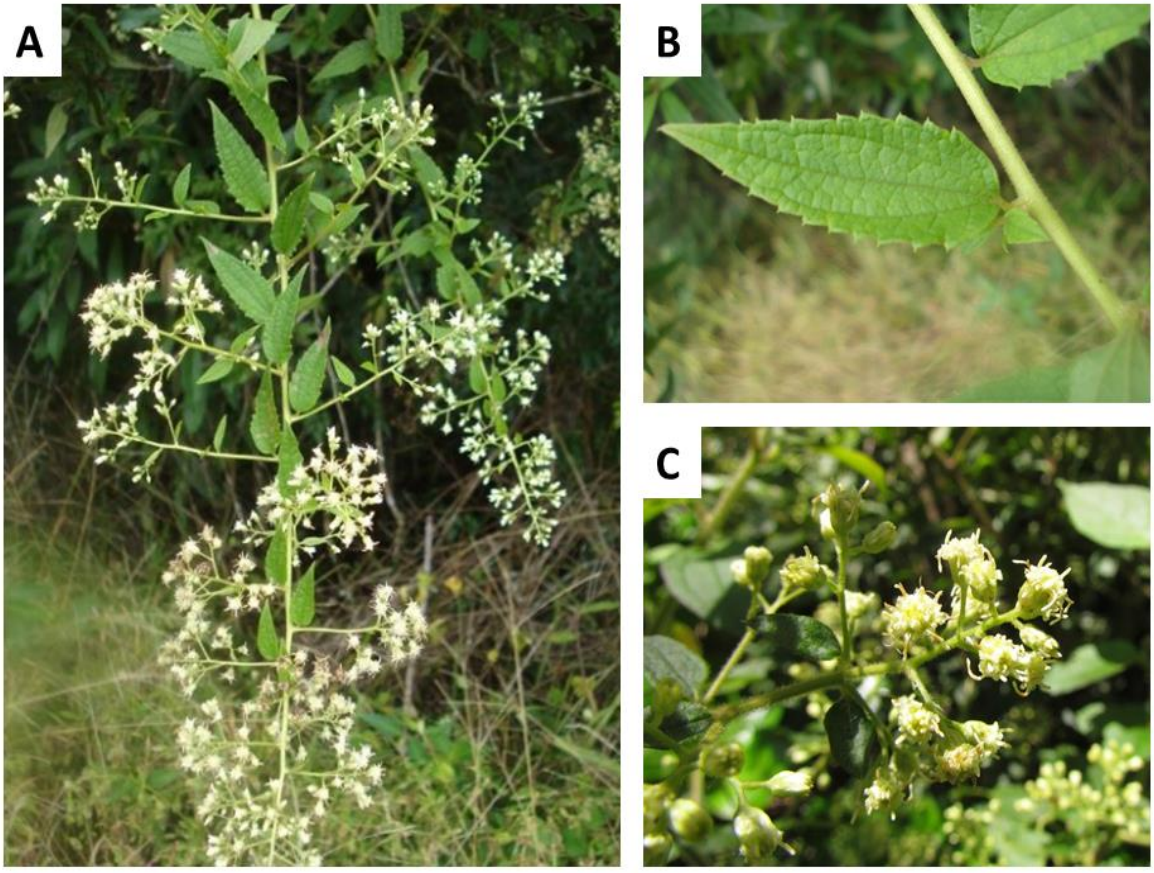


Figura 5: Espécime de *B.anomala* **(A)**. Partes aéreas vegetativas de *B.anomala* **(B)**. Partes reprodutivas de *B.anomala* **(C)**.

Compostos fenólicos encontrados em formas naturais, presente em plantas, possuem alta aplicabilidade para o desenvolvimento de terapias alternativas a tratamentos convencionais de diversas doenças (Jafari et al., 2014). A utilização de extratos de planta para o desenvolvimento de novas terapias para o tratamento de muitas doenças ainda é investigada, uma vez que já se tem conhecimento de que estes compostos podem apresentar efeitos sinérgicos, fazendo com que a composição química de um extrato obtido da planta possa apresentar um efeito terapêutico superior ao de moléculas isoladas (Vaz et al., 2012).

2. JUSTIFICATIVA

A busca por moléculas ou compostos fitoquímicos de plantas disponíveis na natureza e de fontes renováveis tem se mostrado uma área promissora na farmacologia e essas moléculas ou compostos com alta atividade biológica podem ampliar o leque de opções terapêuticas para o tratamento de várias doenças. Os compostos fenólicos pertencem a este grupo de moléculas que já tiveram seu potencial para o desenvolvimento de fármacos revelado, por apresentaram atividade antiproliferativa celular e pelo seu conhecido efeito antioxidante. Porém, ainda há muitas moléculas a serem descobertas e estudadas. Atualmente, não existe um tratamento específico para a fibrose hepática e, desta forma, o presente trabalho buscou avaliar o potencial de tratamentos provenientes de fontes naturais e renováveis para o desenvolvimento de novas terapias. Para isso, investigamos o potencial terapêutico do metoxieugenol, um fenilpropanóide, bem como do extrato de *Baccharis anomala*, uma mistura fitoquímica de diversos metabólitos secundários.

3. OBJETIVOS

(1) Avaliar o efeito do tratamento do metoxieugenol em modelos de fibrose hepática *in vitro* e *in vivo* e elucidar o seu possível mecanismo de ação.

(2) Avaliar o efeito do tratamento do extrato de *B.anomala* em modelo de fibrose hepática *in vivo* e identificar os principais compostos fenólicos presente no extrato.

4. MATERIAIS E MÉTODOS

Os materiais e métodos utilizados no presente trabalho estão descritos nos artigos científicos produzidos.

5. ARTIGO 1.

Artigo publicado no periódico Journal of Ethnopharmacology (IF: 4.36)

Objetivo 1

Avaliar o efeito do tratamento do metoxieugenol em modelos de fibrose hepática *in vitro* e *in vivo* e elucidar o seu possível mecanismo de ação.

Journal of Ethnopharmacology 280 (2021) 114433

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Methoxyeugenol deactivates hepatic stellate cells and attenuates liver fibrosis and inflammation through a PPAR-ɣ and NF-kB mechanism

Bruno de Souza Basso [a,*], Gabriela Viegas Haute [a], Martí Ortega-Ribera [b], Carolina Luft [a], Géssica Luana Antunes [a], Matheus Scherer Bastos [a], Leonardo Pfeiff Carlessi [a], Vitor Giancarlo Levorse [a], Eduardo Cassel [c], Márcio Vinícius Fagundes Donadio [a], Eliane Romanato Santarém [a,1], Jordi Gracia-Sancho [b,1], Jarbas Rodrigues de Oliveira [a,1]

[a] School of Health and Life Sciences, Pontifical Catholic University of Rio Grande do Sul PUCRS, Porto Alegre, Rio Grande do Sul, Brazil
[b] Liver Vascular Biology Research Group, Barcelona Hepatic Hemodynamic Lab, IDIBAPS Biomedical Research Institute - CIBEREHD, Barcelona, Spain
[c] School of Technology, Pontifical Catholic University of Rio Grande do Sul – PUCRS, Porto Alegre, Rio Grande do Sul, Brazil

ARTICLE INFO

Keywords:
Liver disease
Antifibrotic drugs
Nutraceutical
HSCs
Liver inflammation
Chronic liver disease
Cirrhosis

ABSTRACT

Ethnopharmacological relevance: Studies have shown interest in nutraceuticals for the prevention of liver diseases. Methoxyeugenol, is a molecule found in foods, such as nutmeg (*Myristica fragrans Houtt.*) and Brazilian red propolis. These two sources of methoxyeugenol, propolis and nutmeg, are used in folk medicine for the treatment of hepatic and gastrointestinal disorders, although little is known about their effects on the prevention of liver fibrosis. Natural PPAR (Peroxisome proliferator-activated receptor) agonists would represent unique molecules for therapy, considering the lack of therapeutics to treat liver fibrosis in chronic liver disease. Thus, investigation on new alternatives are necessary, including the search for natural compounds from renewable and sustainable sources. Liver fibrosis is a pathological process characterized by an exacerbated cicatricial response in the hepatic tissue, which compromises liver function. Therefore, inhibition of HSC (hepatic stellate cell) activation and hepatocyte damage are considered major strategies for the development of new anti-fibrotic treatments.

Aim of the study: This study aimed to investigate the effects of methoxyeugenol treatment on HSC phenotype modulation in human and murine cells, hepatocyte damage prevention, and protective effects *in vivo*, in order to evaluate its therapeutic potential for liver fibrosis prevention.

Methods: We investigated the effects of methoxyeugenol in (i) *in vitro* models using human and murine HSC and hepatocytes, and (ii) *in vivo* models of CCl_4 (carbon tetrachloride) -induced liver fibrosis in mice.

Results: We herein report that methoxyeugenol decreases HSC activation through the activation of PPAR-ɣ, ultimately inducing a quiescent phenotype highlighted by an increase in lipid droplets, loss of contraction ability, and a decrease in the proliferative rate and mRNA expression of fibroblast markers. In addition, methoxyeugenol prevented hepatocytes from oxidative stress damage. Moreover, in mice submitted to chronic liver disease through CCl_4 administration, methoxyeugenol decreased the inflammatory profile, liver fibrosis, mRNA expression of fibrotic genes, and the inflammatory pathway signaled by NF-kB (Nuclear factor kappa B).

Conclusion: We propose methoxyeugenol as a novel and potential therapeutic approach to treat chronic liver disease and fibrosis.

1. Introduction

Natural compounds, crude extracts, or isolated molecules obtained from plants have progressively attracted attention for devising antifibrotic therapies (Bravo, 2009; Singh et al., 2005). Methoxyeugenol (4-Allyl-2,6-dimethoxyphenol) is a molecule present in the human diet, as it is widely used as an additive in the food industry, and it is also present in the composition of several plant that showed beneficial effects for treatment of innumerous diseases (Agnihotri et al., 2012; Righi et al.,

* Corresponding author. Laboratório de Pesquisa em Biofísica Celular e Inflamação, Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga, 6681, Sala 221, bloco C, prédio 12, Porto Alegre, RS, Brazil.
E-mail address: bruno.basso.001@acad.pucrs.br (B. de Souza Basso).
[1] **Co-senior authors**: E. Romanato Santarém, J. Gracia-Sancho, J. Rodrigues de Oliveira are co-senior authors.

https://doi.org/10.1016/j.jep.2021.114433
Received 12 May 2021; Received in revised form 2 July 2021; Accepted 14 July 2021
Available online 16 July 2021

Abbreviations list

HSC	Hepatic stellate cell
TGF-β:	Transforming growth factor beta
PPAR	Peroxisome proliferator-activated receptor
NF-kB	Nuclear factor kappa B
DMEM	Dulbecco's modified eagle medium
CCl_4	Carbon tetrachloride
FBS	Fetal bovine serum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
DMSO	Dimethyl sulfoxide
NAC	N-acetylcysteine
DPPH	2,2-Diphenyl-1-picrylhydrazyl
TBARS	Thiobarbituric acid reactive substances
LDH	Lactate dehydrogenase
SLM	Silymarin
ALT	Alanine transaminase
AST	Aspartate transaminase
NAS	Nonalcoholic Fatty Liver Disease Activity Score
PBS	Phosphate Buffered Saline
DAB	3,3′-Diaminobenzidine
α-SMA	Alpha-smooth muscle actin
p-NF-kB	Phosphorylated nuclear factor kappa B
Col1a1	Type I collagen
PDGFrβ	Platelet-derived growth factor receptor beta
IL:	Interleukin
TNF-α:	Tumor necrosis factor alpha
CD163	Cluster of differentiation 163
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
TTBS	Tween-tris-buffered saline
BSA	Bovine serum albumin
EDTA	Ethylenediamine tetraacetic acid
iNOS	Inducible NO synthase
LPS	Lipopolysaccharide

2011). It is found in herbs, spices, sassafras oil (*Sassafras albidum*), nutmeg (*Myristica fragrans Houtt.*), *Cinnamomum glanduiferum*, and Brazilian red propolis (Agnihotri et al., 2012; Guridip Singh et al., 2005; Kamdem and Gage, 1995; Righi et al., 2011).

As mentioned, Methoxyeugenol has been identified in Brazilian red propolis, which is produced by bees of the species *Apis mellifera,* collected from plants exudate of the *Leguminosae* family (*Dalbergia ecastaphyllum (L.) Taub)* found in Maceió, in the northeast of Brazil (Boeing et al., 2020; Righi et al., 2011). Propolis therapeutic effects are known for centuries in folk medicine and used to treat infections, gastric disorders and also to improve wound healing (Sforcin, 2016; Ghisalberti, 1979). Moreover, the therapeutic effect of red propolis has been investigated for treatment of hepatic and gastric disorder and showed hepatoprotective and gastroprotective properties in mice (Boeing et al., 2020; Silva et al., 2019).

Nutmeg (*Myristica fragrans Houtt.*), another important source of methoxyeugenol, is a seed used as spice, originally from Indonesians islands, that was widely spread to the world by the English exploration in the 17th century (Abourashed and El-Alfy, 2016). Its use in folk medicine is reported mainly in the treatment of gastrointestinal problems and has long been used as a therapy for this kind of illness (Asgarpanah and Kazemivash, 2012). Moreover, studies showed that nutmeg could be useful in the treatment of nonalcoholic fatty liver disease (Zhao et al., 2020), promoting lipid metabolism regulation and anti-inflammatory effects. More importantly, extract containing nutmeg could effectively protect liver against hepatic toxic-induced damage (Yimam et al., 2016). Nevertheless, the effects of methoxyeugenol on liver fibrogenesis and the associated molecular mechanisms of its possible beneficial effects are still unknown.

Liver fibrosis has its development linked to chronic liver damage, often due to viral infections, alcoholism, and toxins (Friedman, 2008). Chronic injury leads to an inflammatory process that ultimately results in HSCs activation, with the acquisition of a myofibroblastic phenotype, and the initiation of the fibrotic process, evidenced by the loss of synthesis/degradation balance of extracellular matrix elements (Iredale, 2008). The maintenance of HSCs phenotype is primarily performed by transcription factors, which tightly modulate the function of these cells. In a quiescent state, HSCs act on glucose regulation, lipid synthesis and degradation and synthesis of extracellular matrix components (Guimarães et al., 2007; Marrone et al., 2016). Nevertheless, upon activation, HSCs become pro-contractile, pro-inflammatory, and pro-fibrogenic (Gracia-Sancho et al., 2018), actively contributing to chronic liver disease progression.

Among the transcription factors regulating HSCs, the PPAR family (mainly PPAR-ɣ and PPAR-α) has demonstrated to play an important role in promoting their quiescent phenotype (Ivanova et al., 2015; Tyagi et al., 2011; Chen et al., 2015) and inhibiting the activation of inflammatory pathways, manly those activated by NF-kB signaling (Mirza et al., 2019; Qu et al., 2017). Therefore, modulators of these transcription factors are promising candidates to initiate the process of HSCs deactivation.

It is nowadays clear that hepatic fibrosis treatment requires the identification and removal of the chronic damage agent and/or the administration of drugs with beneficial activities on proliferation and activation of the hepatic stellate cells, oxidative stress, and inflammation. Currently, treatments for liver fibrosis includes curcumin, quercetin, silymarin and probiotics that reduce oxidative stress and inflammation in the liver. Furthermore, more recently, the potential for treatment with small interfering RNAs to decrease TGF-β signaling has been demonstrated to be a promising strategy. (Pragyan et al., 2021; Popov and Schuppan, 2009; Tighe et al., 2020; Liedtke et al., 2013). On the other hand, natural PPAR agonists would represent unique molecules for therapy, as they exert beneficial effects with fewer side effects than specific agonists of these receptors (Wang et al., 2014).

In a previous work by our research group, methoxyeugenol showed an effect in decreasing cell proliferation in human endometrial cancer (Costa et al., 2021). HSCs are responsible for extracellular matrix deposition and synthesis, and their activation and proliferation rate are closely linked to the development of fibrosis. Considering the lack of therapeutics to treat liver fibrosis in chronic liver disease, investigation on new alternatives are necessary, including the search for natural compounds from renewable and sustainable sources (Bravo, 2009). In the present study, we investigated the effects of methoxyeugenol on HSCs phenotype in human and murine cells, hepatocyte damage prevention, and protective effects *in vivo*, in order to evaluate its therapeutic potential for liver fibrosis prevention.

2. Materials and methods

2.1. In vitro experiments

2.1.1. Cell culture

The murine HSC cell line GRX was obtained from the Rio de Janeiro Cell Bank (HUCFF, UFRJ, Rio de Janeiro, Brazil) and the LX-2 was kindly provided by Dr. Bataller. Cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium, Sigma, 5030) supplemented with 10% FBS (fetal bovine serum, Gibco, 26140079), 1% penicillin and streptomycin (Gibco, 15140122) and pH 7.4. Cells were seeded in tissue culture plates and, after 24 h, treated and incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. HepG2 (obtained from the Rio de Janeiro Cell

Bank - HUCFF, UFRJ, Rio de Janeiro, Brazil) and VERO cells (kindly provided by Dr. Pablo Machado) were maintained in DMEM supplemented with 10% FBS and subjected to the same conditions and temperature mentioned above. All *in vitro* experiments were performed four independent times, in triplicate.

Methoxyeugenol molecule (4-Allyl-2,6-dimethoxyphenol) was purchased from Sigma-Aldrich (Cat.#W365505/Lot#STBD5682V) and stored in room temperature. For experiments, methoxyeugenol was solubilized in DMSO (dimethyl sulfoxide).

2.1.2. Cellular viability

Cellular viability of GRX, VERO and HepG2 cells were determined by colorimetric MTT (2,2-Diphenyl-1-picrylhydrazyl, Sigma, 298-93-1) assay (De Mesquita et al., 2013). Briefly, 3×10^3 cells per well were seeded in 24-well plate, grown for 24 h and treated with methoxyeugenol at 15, 30, 60, 125 and 250 μM diluted in 0.5% DMSO (Synth, 01D1011) for 72 h. After that, MTT solution was added and cells were incubated for 4 h. NAC (N-acetylcysteine) was used as positive control at 2.5 mM. Formazam crystals were dissolved with DMSO and quantified in an ELISA microplate reader at absorbance of 492 nm. For cell number determination, cells were counted in Neubauer chamber with Trypan blue Solution 0.4% (Gibco, 15250061) and live cells expressed as control percentage. Considering that 30 μM was the lowest concentration that induced a decrease in cell proliferation and did not show cellular toxicity, this dose was chosen for further experiments.

2.1.3. Antioxidant activity

Methoxyeugenol was evaluated for its antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma, 1898-66-4) reduction (Dias et al., 2017). The antioxidant activity 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 positive control due to its well-known antioxidant activity.

2.1.4. Oxidative stress in HepG2 cells

For oxidative stress evaluation, HepG2 cells were seeded in 6-well plate at concentration of 1×10^5 cells per well. After 6 h, cells were adhered to the well bottom and received the pre-treatment of methoxyeugenol at 30 μM for 12 h. Later, cells were challenged with CCl_4 (4 mM) for 6 h. At the end of the experiment, both supernatant and cell lysate were collected. The concentration of TBARS (thiobarbituric acid reactive substances) and LDH (lactate dehydrogenase, Labtest, 86) released were analyzed (Krause et al., 2017).

2.1.5. Detection of lipid droplets in HSC

Lipid droplets accumulation in HSC was observed using Oil Red staining (De Mesquita et al., 2013). Cells were plated in a 24-well tissue culture plate (3×10^3 cells/well), and 72 h after treatment with methoxyeugenol, cells were fixed with 10% formaldehyde and stained with Oil Red-O (Sigma, 1320-06-5). Intracellular lipid accumulation was observed after 30 min, using an inverted light microscope at magnification of 400×. The dye from stained cells was extracted using isopropanol and specific lipid content was calculated as the ratio of absorbance value and number of cells. Oil Red-O quantification was accessed by optic density at 492 nm.

2.1.6. Assessment of cell contraction by collagen gel assay

Collagen gels consist of a solution with collagen 4 mg/mL and DMEM 4× concentrated. Each gel was impregnated with 1×10^5 cells and added to a 24-well plate, left to polymerize for 30 min at 37 °C, detached and suspended in 600 μL of DMEM with 5% FBS solely or with either methoxyeugenol or NAC. Images were obtained 24 h after and surface area of each gel was determined as a percentage of the total well area occupied (De Oliveira et al., 2020).

2.1.7. PPAR- ɣ antagonist pre-treatment

Human activated HSC LX-2 were seeded in a 6-well plate at concentration of 4×10^4 cells per well and pre-treated with specific PPAR-ɣ antagonist GW9662 (Sigma, M6191-5 MG), at concentration of 10 μM for 24 h (Gionfriddo et al., 2020). Later, cellular medium was changed to a new medium contain DMSO, for the control and GW992 groups, and DMSO + methoxyeugenol 30 μM for the treated group. Cells were maintained in incubation for an additional of 72 h and mRNA were collected to evaluate the activation HSC markers.

2.2. In vivo experiments

2.2.1. Animals and experimental design

Male BALB/c mice, 8 weeks old and weighing 25–30 g, were bred and maintained at the university animal facilities (CeMBE, PUCRS), under specific pathogen free conditions, 12/12 h light–dark cycle, temperature of 22 °C with free access to water and food on individually ventilated cages.

Liver fibrosis was induced using the chronic CCl_4 administration model. Forty BALB/c mice were randomly allocated to one of the following 4 groups (ten mice per group): Control, CCl_4, CCl_4 + methoxyeugenol 0.25 mg/kg and CCl_4 + methoxyeugenol 1.0 mg/kg. Mice were i.p. (intraperitoneally) injected with CCl_4 (10% in olive oil, 1 mL/kg body weight) three times per week, for a total of 10 weeks. We have decided to administrate via i.p. to assure that all animals received the correct dosage, considering that oral administration by gavage for long periods of treatment are stressful to the animals and oral administration by capsules or food may not deliver the same amount of the molecule to all animals. In order to validate the liver fibrosis model through chronic CCl_4 administration used in the present study, we have used Silymarin (SLM) as a positive treatment control, as it is a substance known for its treatment effects in *in vivo* experiments. A SLM dose of 200 mg/kg was administered (Zhang et al., 2018).

Methoxyeugenol treatment was given twice a week, on alternating days with CCl_4 induction, intraperitoneally at 0.25 and 1.0 mg/kg. Doses were selected according to European Food Safety Authority that reports a safety dose for methoxyeugenol ranging from 1 to 30 mg/kg (European Food Safety Authority (EFSA) and Parma, 2011). Therefore, a dose of 1.0 mg/kg was chosen in addition to a lower dose of 0.25 mg/kg, which would not cause any side effect to animals. Animals were euthanized at 24 h after the last administration. Trunk blood was collected by decapitation and the liver was removed and stored. Serum was separated by centrifugation at 5×10^3 rpm. The study was approved by the University (PUCRS) Animal Ethics Committee (CEUA 8318).

For *in vivo* experiments, methoxyeugenol molecule (4-Allyl-2,6-dimethoxyphenol) was administered from the same batch (Sigma-Aldrich - Cat.#W365505/Lot#STBD5682V) used in the *in vitro* experiments, also solubilized in DMSO.

2.2.2. Serum analysis

Blood samples were collected at the end of the experiment and centrifuged at 4 °C. Serum ALT (alanine transaminase), AST (aspartate transaminase), alkaline phosphatase activity and albumin concentration were analyzed by using Labtest Kits (Lagoa Santa, Minas Gerais, Brazil), following the manufacturer's recommendations.

2.2.3. Liver histopathology

After euthanasia, liver samples were fixed with 10% buffered formalin, and embedded in paraffin blocks. Tissues were cut into 4.5 μm sections and stained with hematoxylin and eosin stain (H&E – Cytological Products Soldan, RBP-1700-02A). A semi-quantitative assessment was performed: steatosis (0–3), inflammation (0–2), and ballooning (0–2); according to the Nonalcoholic Fatty Liver Disease Activity Score (NAS) (Kleiner et al., 2005). In order to demonstrate fibrosis, liver sections were stained with Picro Sirius Red. Liver fibrosis also was semi-quantitatively determined: fibrosis (0–4). Images of the

sections were captured through a BMX 43 microscope equipped with a digital DP73 camera (Olympus, Tokyo, Japan). For immunohistochemistry, tissue samples were cut in 4.5 μm thick sections of paraffin-embedded blocks and mounted in slides. Slides were dehydrated and the antigen retrieval was performed in microwave (3 cycles of 5 min each), in citrate buffer pH = 6.0. Samples were washed in PBS (phosphate buffered saline). Peroxidase step (3% in PBS) was performed in slides for 10 min and afterwards washed in PBS. For the blocking step, 5% goat serum in PBS solution with 0.1% of TritonX and incubation at room temperature for 1 h were used. Primary antibodies for αSMA (alpha-smooth muscle actin, #19245, Cell Signaling), NF-kB (nuclear factor kappa B, p-65, #8242, Cell Signaling), p-NF-kB (phosphorylated nuclear factor kappa B, p-p65, #3036, Cell Signaling) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase, #97166, Cell Signaling) in a concentration of 1:200 diluted in blocking solution were incubated at 4 °C overnight. At the following day, samples were washed with PBS and incubated with a secondary anti-rabbit antibody (A16160, Invitrogen), diluted 1:200 in PBS with TritonX 0.1%, for 1 h. After secondary incubation, samples were washed with PBS. DAB (3,3′-diaminobenzidine) was used as chromogen and counterstained with hematoxylin, followed by a rehydration step. Samples were mounted with DPX mountain medium and let to completely dry at room temperature. Images were obtained in a BMX 43 microscope, equipped with DP73 (Olympus, Tokyo, Japan) digital camera. Analysis of stained area were performed by calculation of % area stained with DAB with ImageJ software.

2.3. General techniques

2.3.1. mRNA extraction and real-time qPCR

Gene expression was determined by Real-time qPCR (Applied Biosystems StepOne) in GRX (α-SMA, TGF-β, PPAR-γ and PPAR-α) and LX-2 (α-SMA, Col-1(type 1 collagen) and PDGFrβ (platelet-derived growth factor receptor beta) cells treated with methoxyeugenol at 30 μM during 72 h, and also in hepatic tissues from animals submitted to the CCl_4 fibrosis protocol (α-SMA, Col-1, IL-6 (interleukin-6), TNF-α (tumor necrosis factor) and CD 163 (cluster of differentiation 163), iNOS (inducible NO synthase)). mRNA was extracted using TRIzol reagent (Invitrogen, 15596026) and reversely transcribed into cDNA, using the Superscript III SuperMix (Invitrogen, 18080400). All samples had the total mRNA concentration normalized at 5 μg. Relative expression levels of interest genes were performed using the GAPDH as a reference gene. The reactions were catalyzed by using the SYBR Green (Applied Biosystems). Results, expressed as $\Delta\Delta CT^2$, represent the x-fold increase of gene expression compared with the corresponding control group.

2.3.2. Western blot

For protein extraction, liver tissues from mice were homogenized in a solution containing CHAPS 0.5%, β-mercaptoethanol and proteases (Amresco, M221). HSCs LX-2 cells were lysated with a solution containing Triton (Sigma, 9002-93-1), TBS5x, EDTA (ethylenediamine tetraacetic acid) and protease inhibitors. Samples were normalized to 30 μg of protein, separated with electrophoresis (polyacrylamide gel 10% w/v) and transferred to a nitrocellulose membrane (Biorad, 1620112). The blot was washed with Tris-HCl, NaCl, and 0.05% Tween (Sigma, P9416), followed by 30 min incubation in blocking solution TTBS (tween-tris-buffered saline) containing 5% BSA (bovine serum albumin). After, the blot was washed again with TTBS and incubated overnight at 4 °C in blocking solution containing the following primary antibodies: anti-GAPDH, anti-αSMA, anti-NF-kβ (p65) or anti-phosphorylated-NF-kβ (p-p65). After an overnight incubation, the blot was washed and incubated again for 2 h with horseradish peroxidase-conjugated anti-IgG secondary antibody. The band was detected by a gel documentation system (Fujifilm, LAS-3000). Band intensities were quantified through the ImageJ software.

2.3.3. Statistical analysis

Data are reported as mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test at a significance level of $p < 0.05$. Otherwise, comparisons were assessed with the non-parametric Mann-Whitney U test. Differences were considered significant at a p-value < 0.05. The statistical program used was the GraphPad Prism Version 5.00.

3. Results

3.1. Methoxyeugenol decreases HSC proliferation without evidence of cytotoxicity

Murine HSC GRX cells treated for 72 h with methoxyeugenol (30, 60, 125 and 250 μM) showed decreased cellular number using the MTT assay (Fig. 1A) and cell counting (Fig. 1B). The investigation of LDH release was performed to determine whether the decrease in the number of GRX cells was due to necrotic cell death. The results showed that all tested concentrations presented released LDH levels similar to the control group (Fig. 1C), indicating that the decrease in the number of cells was not caused by necrosis. In addition, possible effects on cell viability on two other well-known cell lines used for cytotoxicity investigations, VERO - derived from kidney epithelial cells (Fig. 1D), and HepG2 - derived from human hepatocellular carcinoma (Fig. 1E), were investigated. Neither cell lines presented changes in their cellular viability, at the same condition. Thus, based on the minimum concentration for a significant reduction on GRX cell proliferation, 30 μM of methoxyeugenol was selected for the following *in vitro* experiments.

3.2. Methoxyeugenol promotes a quiescent phenotype in HSC

Methoxyeugenol treatment resulted in significant lipid droplets accumulation into the cellular cytoplasm when compared to the vehicle group (Fig. 2A–D). Additionally, methoxyeugenol reduced HSC contractility, as evidenced by significant inhibition of the reduction in collagen gel area (Fig. 2E). Analysis of gene expression in GRX cells treated with methoxyeugenol evidenced significant decrease in profibrotic genes, α-SMA (Fig. 2F) and TGF-β (Fig. 2G), an overexpression of PPAR-γ (Fig. 2H), and no changes in PPAR-α (Fig. 2I) compared to vehicle-treated cells. Importantly, deactivation properties of methoxyeugenol in murine HSC cell line were validated in primary HSC isolated from CCl_4-induced cirrhotic rats (Fig. S1).

3.3. Methoxyeugenol protects HepG2 hepatocytes from oxidative stress damage

Methoxyeugenol exhibited strong basal antioxidant capacity, as demonstrated by the DPPH assay (Fig. 3A). Its antioxidant capacity was maintained under high oxidative stress conditions, as those produced by CCl_4 *in vitro* (Fig. 3B). In addition, cell damage was also verified in HepG2 cells that received CCl_4 by measuring LDH release in the supernatant, demonstrating that methoxyeugenol prevented LDH release (Fig. 3C).

3.4. Methoxyeugenol decreases liver injury, inflammation and fibrosis in vivo

Animals treated with CCl_4 showed an increase in lobular inflammation score compared to the vehicle group in an effect prevented in the animals receiving methoxyeugenol (Fig. 4). The effects of methoxyeugenol preventing liver injury was further confirmed by significant reduction in ALT serum levels (Fig. 4E), with no changes in albumin, alkaline phosphatase and AST levels (Fig. S2). Expression of pro-inflammatory genes (IL-6, TNF-α, iNOS and IL-8) was significantly increased in the CCl_4 group compared to both control and methoxyeugenol treated groups (Fig. 4G-M). The NF-kB protein expression

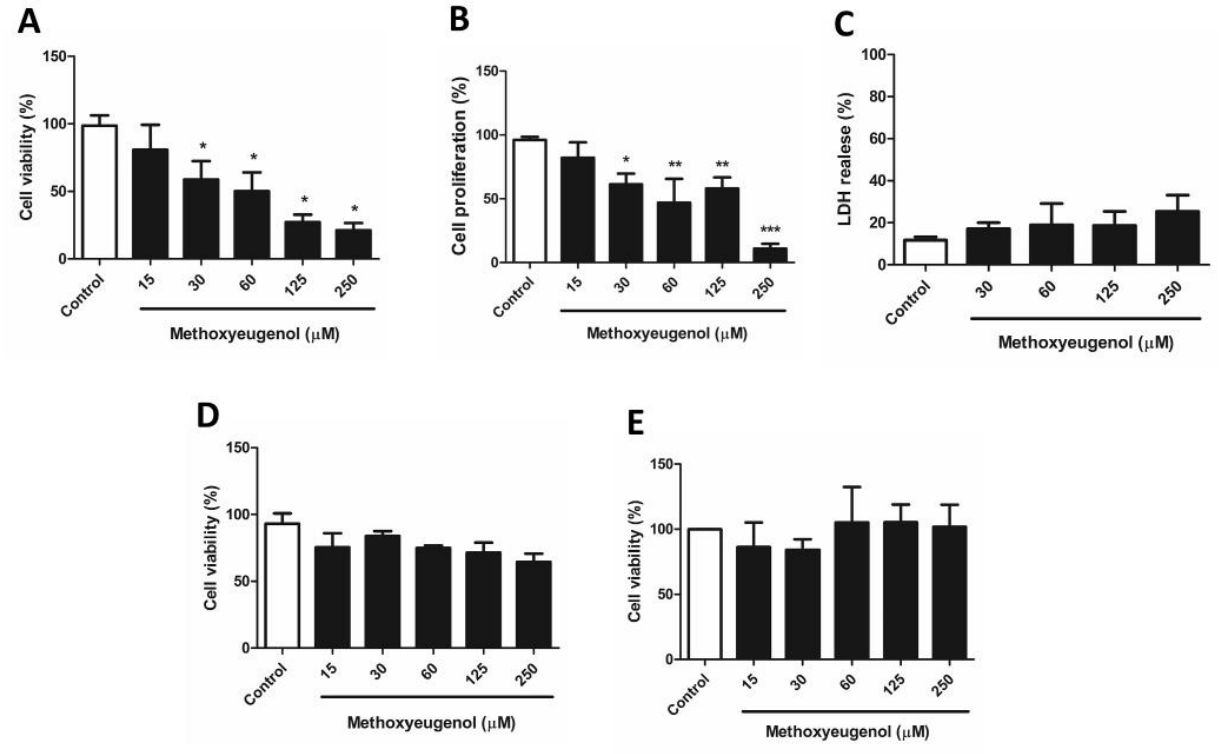


Fig. 1. Effects of methoxyeugenol on cell viability. Effects of methoxyeugenol were determined at concentrations of 15, 30, 60, 125 and 250 μM, during 72 h of treatment, in GRX cells through MTT (A); cell counting (B); and LDH release in the supernatant (C). Cellular viability was also evaluated by MTT assay in endothelial cells from VERO strain (D) and hepatocytes HepG2 cells (E) at concentrations of 30, 60, 120 and 250 μM. Data represent mean ± standard deviation (SD) (n = 4). $^{*}p < 0.05$ compared with control. $^{**}p < 0.01$ compared with control. $^{***}p < 0.001$ compared with control.

showed a similar result, as animals treated with CCl_4 presented a greater expression ratio than animals from both control and methoxyugenol treated groups (Fig. 4I). The protein CD163, a cluster of differentiation of M2 macrophages, was significantly induced in animals treated with CCl_4 and this effect was attenuated by methoxyeugenol (Fig. 4J).

Liver sections were stained with H&E and Picro Sirius Red and scores for ballooning, steatosis and fibrotic areas were analyzed. The CCl_4 treated group exhibited increased steatosis, ballooning, Sirius red-stain and α-SMA area in comparison to the vehicle group (Fig. 5A–F), confirming that animals developed a fibrotic process. Treatment with methoxyeugenol was able to attenuate all the analyzed parameters, both at 0.25 and 1.0 mg/kg (Fig. 5D–G). Levels of mRNA expression of α-SMA and Col-1 genes were evaluated in the hepatic tissue and animals treated with CCl_4 showed a high expression ratio of Col-1 and α-SMA genes, while methoxyeugenol treated animals maintained the expression rate similar to the control group (Fig. 5H–I). In addition, treatment with SLM also decreased the liver fibrosis development in the same experimental model (Fig. S3).

3.5. *Methoxyeugenol improves human HSCs phenotype via PPAR-ɣ activation*

Similarly to murine HSC, methoxyeugenol reduced the expression of α-SMA in human HSC LX-2 cells (Fig. 6A). In order to assess the role of PPAR-ɣ in this beneficial effect, cells were pre-treated with a specific PPAR-ɣ antagonist (GW9662) for 24 h, and then treated with methoxyeugenol or vehicle for 72 h. Results demonstrated that the effect of methoxyeugenol in LX-2 cells was suppressed when PPAR-ɣ was antagonized, exhibiting no improvement in the expression of Col1a1 (Fig. 6B) and α-SMA (Fig. 6C) in comparison to the control group.

4. Discussion

Chronic damage in liver tissue triggers a regenerative process, which leads to hepatic fibrosis development, characterized as a continuous healing response with hepatocytes and HSCs playing a central role. Hepatocytes injury may trigger HSCs to an activated phenotype by an inflammatory signaling pathway, guided either indirectly by Kupffer cells or directly by the release of growth factors and pro-inflammatory cytokines. A previous study reported the beneficial effects of nutraceutical supplementation in liver diseases, such as steatosis, metabolic syndrome and liver fibrosis (Bravo, 2009). Molecules as caffeine, resveratrol, curcumin, vitamin E and quercetin have shown positive effects against oxidative stress, inflammation and HSCs activation (Li et al., 2017). Nevertheless, liver fibrosis and its clinical complications still represent an important clinical problem and therefore novel therapeutic approaches are needed. Our objective was to evaluate the effects of methoxyeugenol in the regulation of activated HSCs phenotype and in the protection of hepatocytes from oxidative stress, as well as its protective effects on hepatic fibrosis.

Our *in vitro* results demonstrated that methoxyeugenol promotes HSCs deactivation, defined as reduced proliferation and lower expression of phenotypic activation markers. Indeed, treatment with methoxyeugenol inhibited the increment in cultured GRX cell number, without evidence of cell death, which suggests a reduction in the proliferative rate. Possible toxicity of the compound was discarded by analyzing the expression of cell death markers and these effects were further corroborated by results from two other cell lines used as internal controls. Vero cells of epithelial origin are commonly used in assays to evaluate cytotoxicity, as well as the HepG2 cell line, which has a carcinogenic origin, but maintain many similarities to human hepatocytes.

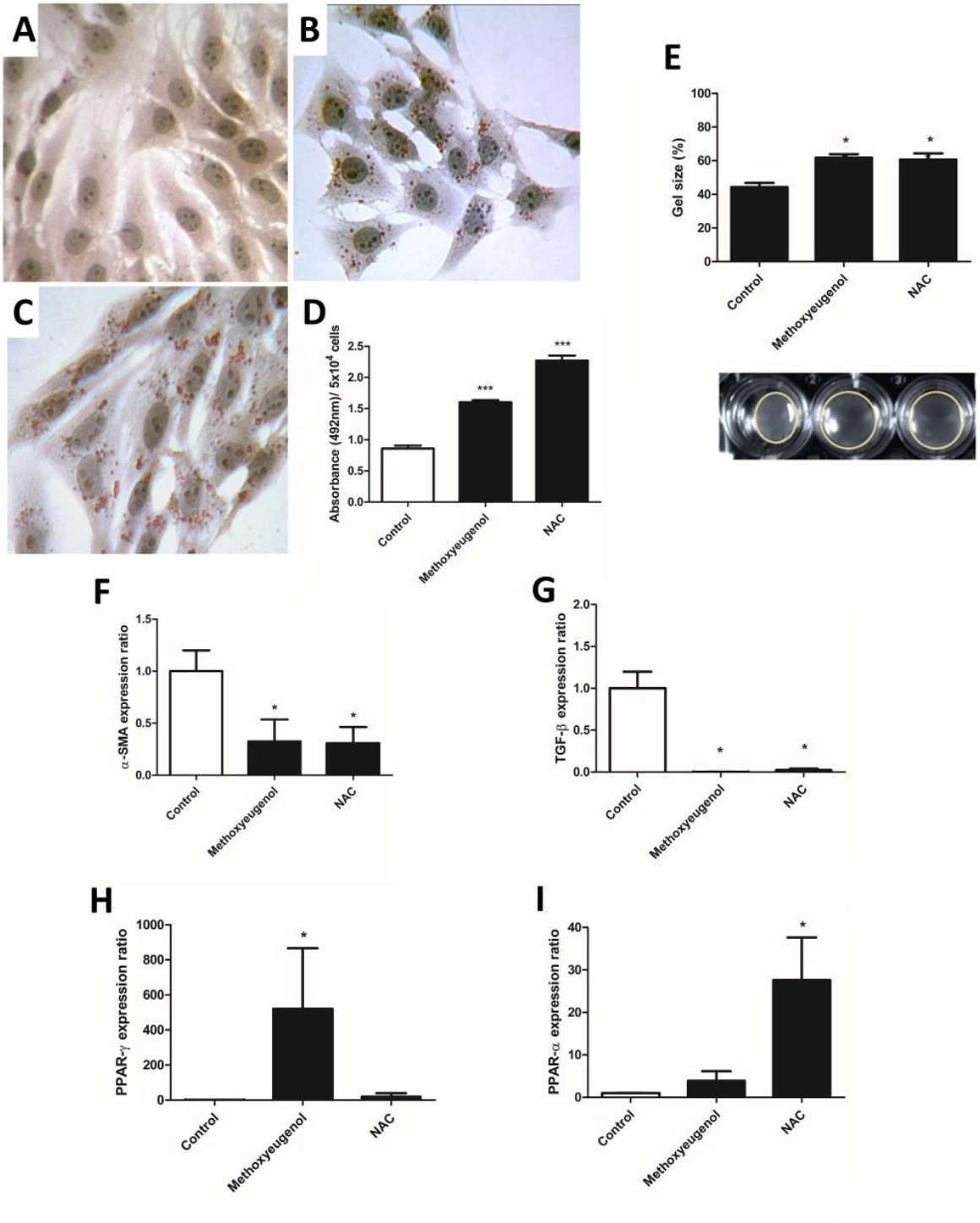


Fig. 2. Evaluation of HSCs activation markers. Lipid droplets staining and cellular contraction. Oil Red-O lipid staining of GRX cells (A–D). Control (A), methoxyeugenol at 30 μM (B), NAC at 2.5 mM (C) after 72 h; 400× magnification. Lipid quantification (D) are shown as the absorbance (492 nm) value obtained for Oil Red adjusted for the number of cells (5×10^4). Cellular contraction of GRX cells was evaluated by measuring the gel area after treatment with methoxyeugenol. Values represent the percentage occupied by the gel relative to the total area of the well (E). Data represent mean ± standard deviation (SD) (n = 4). $^*p < 0.05$ compared with control. $^{***}p < 0.001$ compared with control. Expression of mRNA ratio of α-SMA (F), TGF-β (G), PPAR-γ (H) and PPAR-α (I) in GRX cells treated with methoxyeugenol at 30 μM during 72 h. NAC was used as a positive control at 2.5 mM. Data represent mean ± standard deviation (SD) (n = 4). $^*p < 0.05$ compared with control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

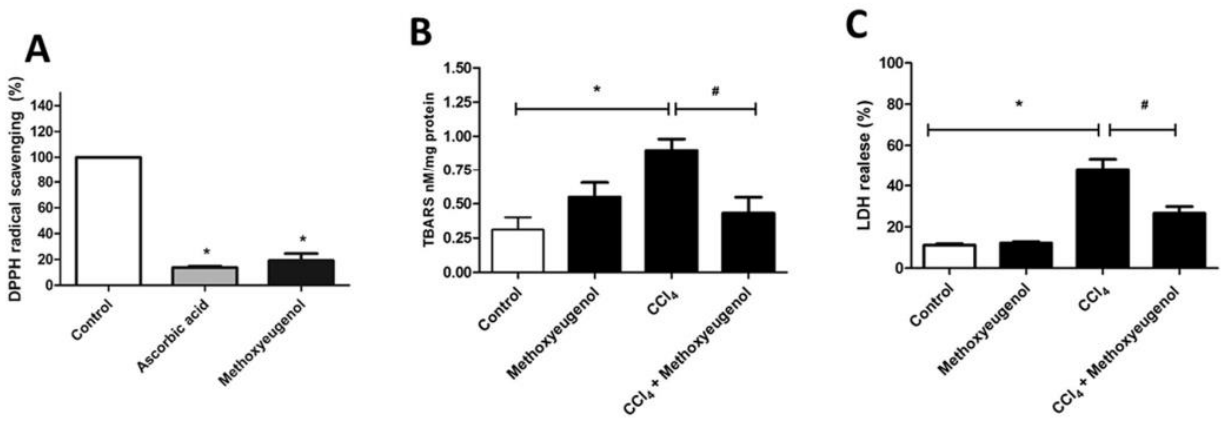


Fig. 3. Antioxidant effects of methoxyeugenol. Methoxyeugenol (30 μM) antioxidant activity was evaluated by DDPH scavenging assay and the ascorbic acid (550 μg/mL) was used as a positive control (A). Oxidative stress damage on hepatocytes challenged with CCl_4 for 6 h were determined by the measurement of TBARS (B) and by the quantification of percentage of LDH release in cell culture supernatant (C). Data represent mean ± standard deviation (SD) (n = 4). $^*p < 0.05$ compared with control. $\#p < 0.05$ compared with the CCl_4 group.

In order to determine the possible effects of the compound in the HSCs phenotype, expression levels of profibrotic genes TGF-β and α-SMA were investigated. Results have shown that the mRNA expression of these genes was suppressed by treatment with methoxyeugenol. The cytokine TGF-β is a growth factor that plays an important role in the development of liver fibrosis, particularly by activating quiescent HSCs, which loses the characteristic of accumulating vitamin A in its cytoplasm and additionally enhances its contractibility (Hernandez-Gea and Friedman, 2011). The contraction capacity of HSC is related to increased expression of α-SMA, since this actin isoform is highly expressed in myofibroblasts and their activation play a key role in the development of the fibrotic response. Collagen gel test confirmed the decrease of contraction capacity of HSC cells treated with methoxyeugenol. In addition, in human activated HSCs (LX-2), the treatment reduced mRNA expression levels of α-SMA and Collagen1, as well as protein levels of α-SMA, indicating a modulating effect of methoxyeugenol on activated HSCs phenotype.

HSCs are the main storage source of vitamin A in the human body, which is located in quiescent HSCs cytoplasm, into lipid droplets. The loss of cytoplasmatic lipid accumulation is a morphological characteristic of activated HSCs. The maintenance of the lipid metabolism occurs by nuclear receptors of the PPAR's family, mainly by PPAR-α/ɣ (Chen et al., 2015). The treatment with methoxyeugenol increased PPAR-ɣ mRNA expression, which associated with the results obtained with Oil Red-O staining, indicates a more quiescent phenotype by lipid droplets formation in GRX cells. In addition, treatment with NAC also induced lipid droplets development in the cytoplasm, although this seems to be through a PPAR-α activation pathway (Kim et al., 2001). More importantly, the pre-treatment of LX-2 cells with specific PPAR-ɣ antagonist (GW9662) suppressed the effects of methoxyeugenol treatment on activated HSCs phenotype markers collagen1 and α-SMA. Taken together, these results suggest that the beneficial effects of methoxyeugenol in murine and human HSCs is, at least in part, through the activation of PPAR-ɣ. Our results are in agreement with previous studies using synthetic activators of PPARs, although the present study demonstrates such effects using a natural compound that may present much lower undesired side-effects then full agonists of PPARs (Chen et al., 2015; Nan et al., 2013). Several studies have shown that the PPAR-γ activation can inhibit macrophage activation and inflammatory pathways mediated by NF-kβ (Mirza et al., 2019; Qu et al., 2017). The PPAR-γ activation may improve the phenotype modulation of activated HSC by restoring the lipid metabolism and by inhibiting the inflammatory signaling. These combined effects show that natural activators of PPARs are great alternatives for the development of new therapies for chronic liver diseases.

Moreover, we have investigated the potential of methoxyeugenol to prevent hepatocyte injury *in vitro*. One important pathway for HSCs activation is through hepatocyte signaling, since these cells represent approximately 80% of the hepatic cell population and are responsible for metabolizing many substances. The damage to the liver tissue is often due to oxidative stress derived from metabolism or inflammatory processes. Therefore, the ability of methoxyeugenol to scavenge free radicals was evaluated. Results revealed that methoxyeugenol has high antioxidant activity, both under basal *in vitro* conditions and upon oxidative stress challenge achieved through CCl_4 treatment.

In summary, the *in vitro* results demonstrated that methoxyeugenol were able to deactivate human and murine HSC, and to protect hepatocytes. Therefore, *in vivo* activity of methoxyeugenol in CCl_4-induced liver fibrosis model was investigated. The periodic administration of CCl_4 leads to lipid accumulation (steatosis), increased inflammatory infiltrate, loss of normal hepatocytes, collagen deposition, and fiber segmentation formation. These hepatic tissue alterations can be classified by the degree of hepatic fibrosis according to NAFLD Activity Score in HE stained sections. Interestingly, treatment with methoxyeugenol attenuated the inflammatory process and fibrosis observed in mice chronically treated with CCl_4. In addition, treatment with methoxyeugenol was able to decrease ALT serum levels when compared to the vehicle group. Exacerbated deposition of extracellular matrix components during liver fibrosis is a result of HSC activation and related to the expression of type 1 collagen and α-SMA genes. Both genes and α-SMA protein expression were suppressed with methoxyeugenol treatment. Moreover, in the same experimental model, we showed that the use of SLM as a positive control decreases the liver fibrosis development.

Inflammation is a beneficial process for the regeneration of damaged tissues and the elimination of pathogens, however chronic inflammation may result in a permanent healing state in the liver, initiating the fibrotic process. NF-kB is a nuclear transcription factor involved in inflammatory regulation and cell death in several cell types. NF-kB is a heterodimer consisting of two subunits, p65 and p50. When not stimulated, the NF-kB factor is found in the cytoplasm, linked to the Ik-B inhibitory protein. This complex prevents the translocation of the NF-kB to the nucleus. The phosphorylation of Ik-B, a family of inhibitory proteins, by Ik-B kinases in critical serine residues (Ser32 and Ser36) determines the release of NF-kB to the cell nucleus, where it will play its role as a factor of transcription for inflammatory interleukins. Thus, phosphorylation and degradation of Ik-B are necessary for translocation to occur. Different stimuli can activate NF-kB, including pathogen-related molecules, such as LPS (lipopolysaccharide), or inflammatory

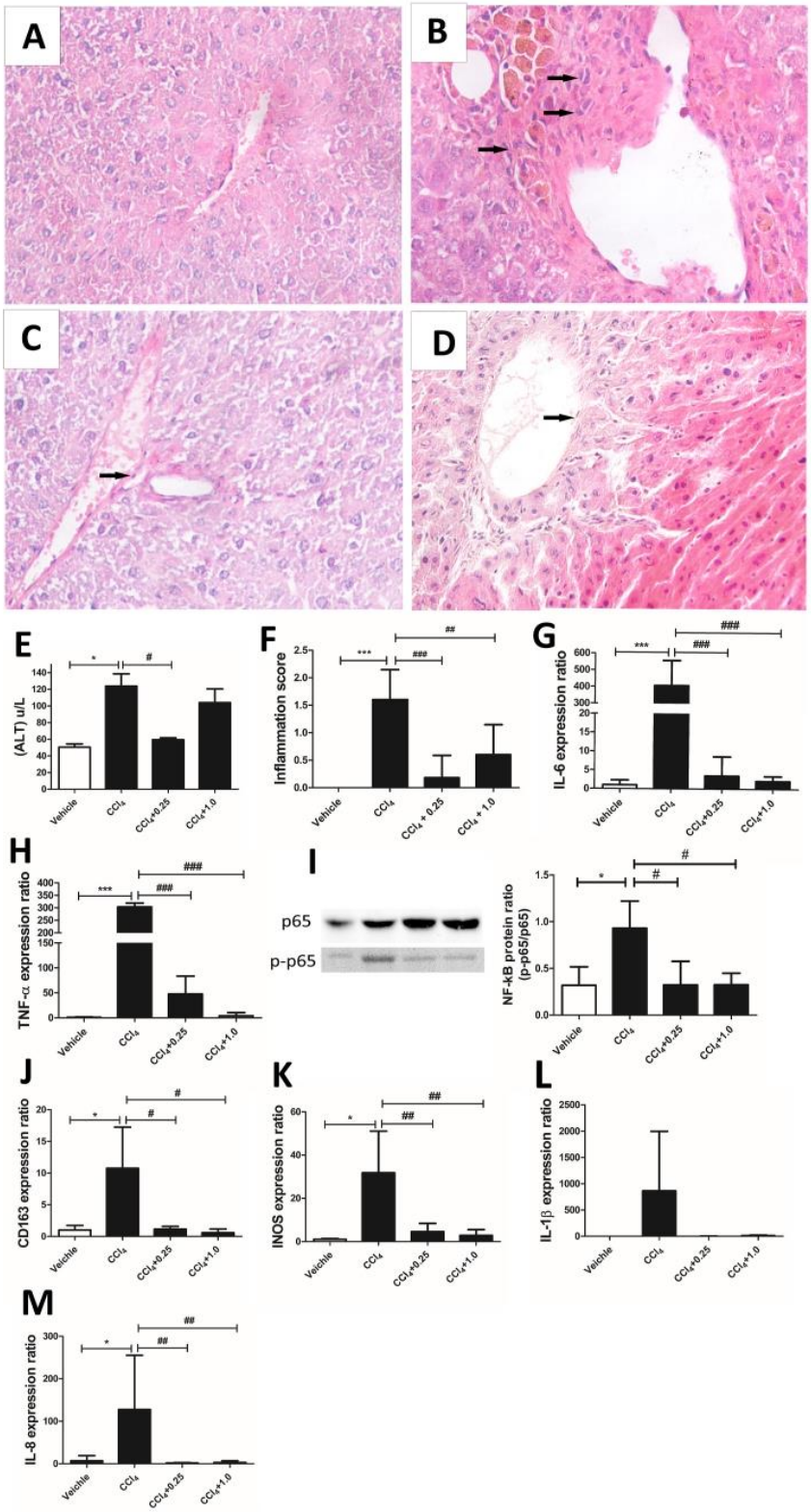


Fig. 4. *In vivo* effects of methoxyeugenol on liver inflammation. Lobular inflammation was evaluated on H&E staining of mice treated with vehicle (A), CCl_4 (B), methoxyeugenol 0.25 mg/kg + CCl_4 (C) or methoxyeugenol 1.0 mg/kg + CCl_4 (D). Black arrows indicate hepatic infiltrates. Magnification of 400×. ALT serum levels (E), quantification of lobular inflammation score (F), IL-6 (G), TNF-α (H) and protein expression ratio of NF kβ (p-p65/p65) (I). Expression of mRNA ratio of CD163 (J), INOS (K) IL-1b (L), IL-8 (M). Data represent mean ± standard deviation (SD) (n = 3–8). $^{*}p < 0.05$ compared with the vehicle group. $^{**}p < 0.01$ compared with the vehicle group. $^{***}p < 0.001$ compared with the vehicle group. $\#p < 0.05$ compared with the CCl_4 group. $\#\#p < 0.01$ compared with the CCl_4 group. $\#\#\#p < 0.001$ compared with the CCl_4 group.

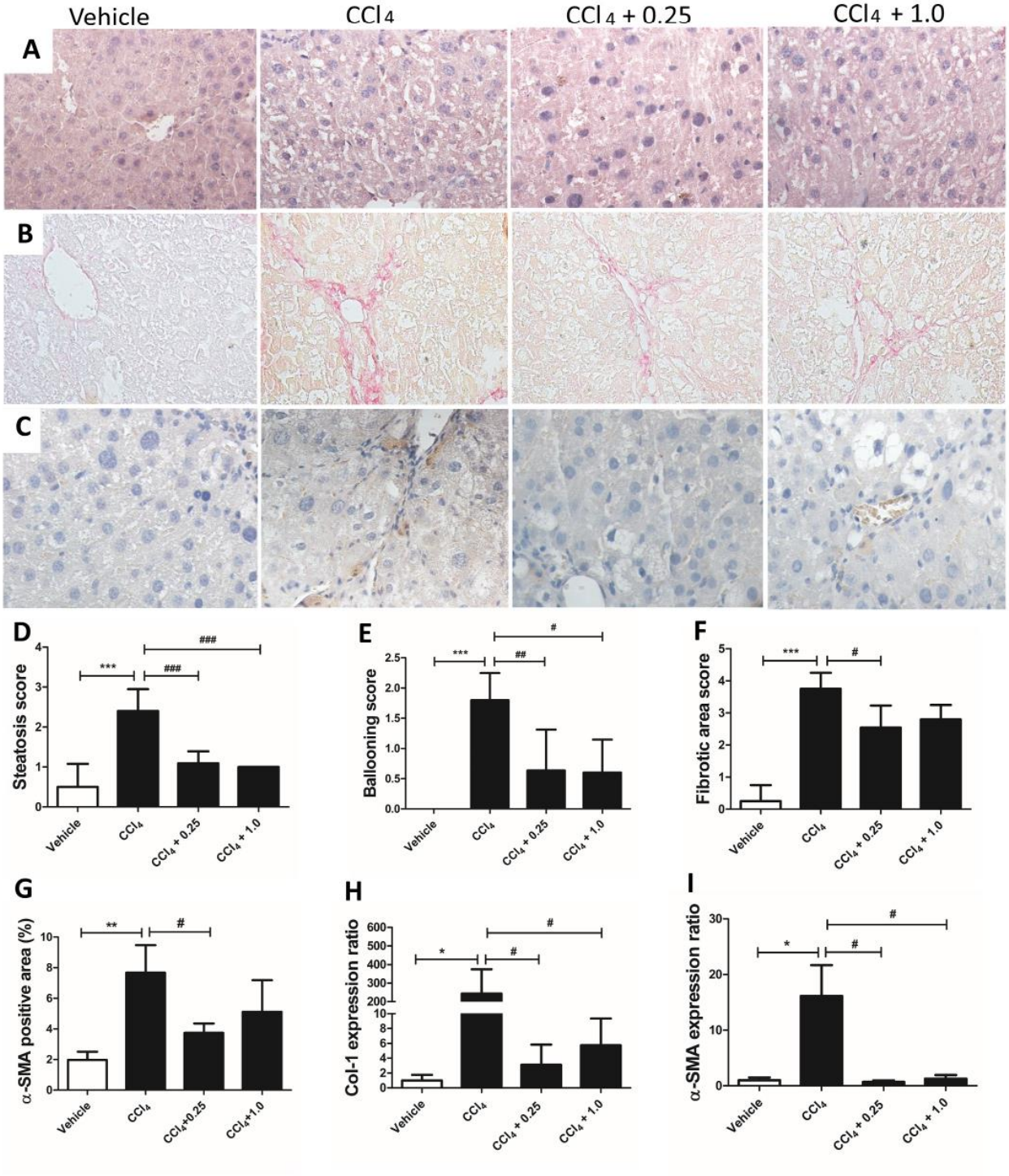


Fig. 5. ***In vivo*** **effects of methoxyeugenol on liver fibrosis.** H&E (A), Picro Sirius (B), α-SMA immunohistochemistry (C) staining of representative mice treated with vehicle, vehicle + CCl_4, methoxyeugenol 0.25 mg/kg + CCl_4 or methoxyeugenol 1.0 mg/kg + CCl_4. Magnification of 400×. Quantification of Steatosis score (D), Ballooning score (E), Fibrotic area score (F), % of positive α-SMA area (G), expression of mRNA ratio of Col-1 (H) and α-SMA (I). Data represent mean ± standard deviation (SD) (n = 8). $^{*}p < 0.05$ compared with the vehicle group. $^{***}p < 0.001$ compared with the vehicle group. $\#p < 0.05$ compared with the CCl_4 group. $\#\#p < 0.01$ compared with the CCl_4 group. $\#\#\#p < 0.001$ compared with the CCl_4 group.

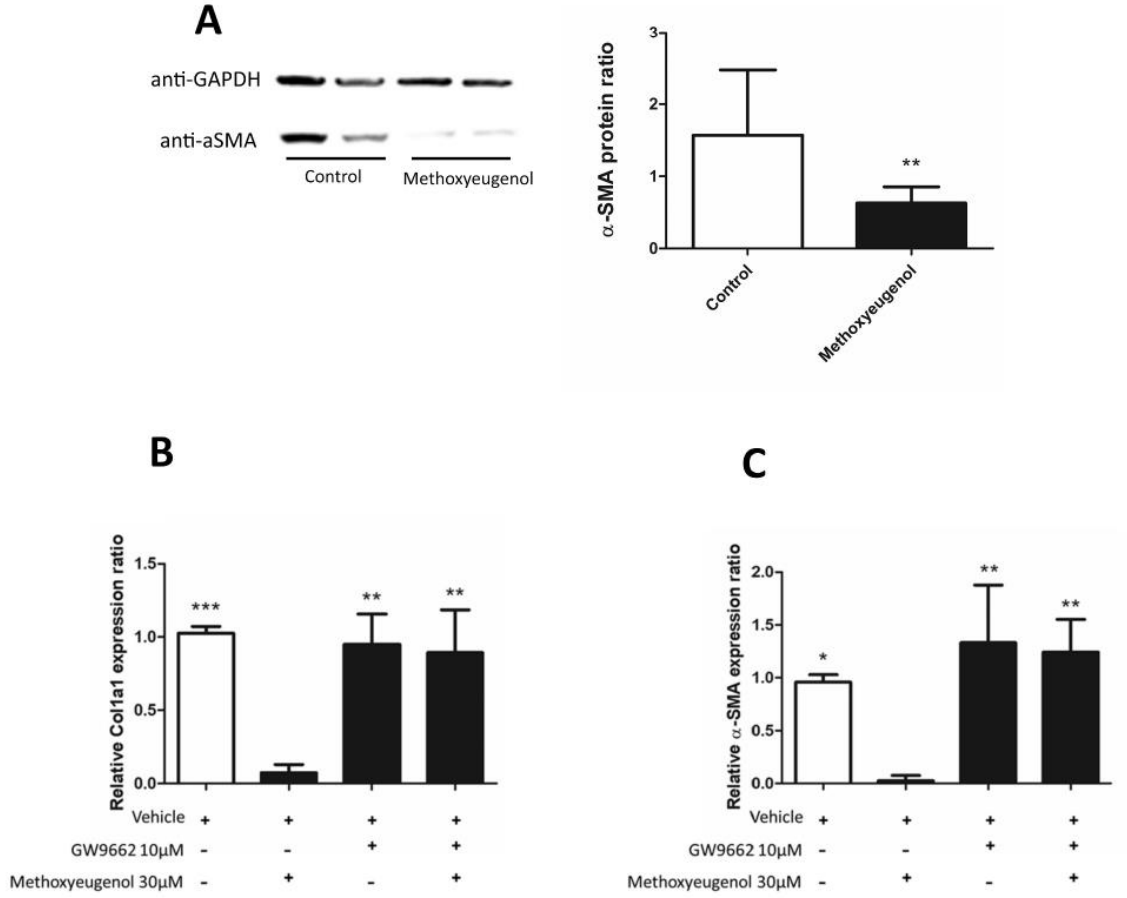


Fig. 6. Effect of PPAR-γ antagonist pre-treatment on human activated HSC. Protein expression ratio of α-SMA (A) in LX-2 cells treated with methoxyeugenol for 72 h. Evaluation of activation markers of HSC on LX-2 cells pre-treatment with GW9662 10 μM for 24 h, followed by treatment with methoxyeugenol 30 μM or vehicle for 72 h, Col1a1 (B) and α-SMA (C). Data represent mean standard deviation (SD) (n = 3). *$p < 0.05$ compared with the methoxyeugenol group. **$p < 0.01$ compared with the methoxyeugenol group. ***$p < 0.001$ compared with the methoxyeugenol group.

cytokines, such as tumor necrosis factor-α (TNF-α) (Luedde and Schwabe, 2011). NF-kB activation (phosphorylation) leads to transcription of many genes related to inflammatory cytokines, such as iNOS and interleukins (IL-1b, IL-6, IL-8). NF-kB activation also leads to the recruitment of inflammatory infiltrates that are characterized by intralobular inflammation, which consists of a variety of inflammatory cells (lymphocytes, neutrophils, eosinophils, and Kupffer cells). Even though the inflammatory role of NF-kB is not completely elucidated, it is known that its basal levels are beneficial for maintaining liver tissue homeostasis.

Moreover, studies have shown that NF-kB deletion may lead to spontaneous development of liver fibrosis (Luedde and Schwabe, 2011). However, pronounced activation of NF-kB may also result in the development of fibrosis, considering that its activation leads to HSC activation. Even with this dual role of NF-kB, drugs that influence the regulation of inflammation by one of its pathways are considered as research candidates for the development of new therapies (Papa et al., 2009). Treatment with methoxyeugenol was able to reduce intralobular inflammation, decrease TNF-α, iNOS, IL-6, and IL-8 gene expression, as well as NF-kB protein expression. Methoxyeugenol increases the expression of PPAR-γ, which is considered an important factor in the metabolism of lipids and glucose, also participating in several biological responses, such as anti-inflammatory and antiproliferative actions. Its anti-inflammatory effects cause inhibition of NF-kB and consequent suppression of the expression of pro-inflammatory proteins, including IL-6, IL-8, TNF-α, and MCP-1 (Jung-Hoon et al., 2015). The hemoglobin scavenger receptor, CD163, is a M2 macrophage-specific protein and higher CD163 expression in macrophages is characteristic of tissues responding to inflammation (Etzerodt and Moestrup, 2012). The treatment with methoxyugenol decreased the inflammation *in vivo* and the CD163 results have also shown a decrease, indicating that there is less scavenger process.

In order to validate the effects on human HSC and to investigate the PPAR-γ role in the use of methoxyeugenol, we have used the irreversible and selective PPAR-γ inhibitor, GW9662. The use of GW9662 on cell lines, aiming to test PPAR-γ potential ligands are well established on previous studies, normally using the concentration of 10 μm (Ma et al., 2017). The pre-treatment of LX-2 cells with the specific PPAR-γ antagonist (GW9662) suppressed the effects of methoxyeugenol treatment on activated HSC phenotype markers collagen1 and α-SMA. Regarding HSC activation, the PPAR-γ expression is dramatically reduced, whereas the opposite effect is observed on HSC quiescent phenotype, demonstrating that PPAR-γ is an important modulator of HSC activation (He et al., 2019). In addition, studies have shown that an increase in PPAR-γ expression leads to a NF-κB suppression (Zong et al., 2013). Thus, the regulation of this nuclear receptor can bring different benefits for liver fibrosis treatment, modulating HSC phenotype and reducing inflammatory signaling.

5. Conclusions

In conclusion, the present study has shown the use of a nutraceutical molecule, which is currently mainly used as food additive, as a new therapeutic agent for the treatment of hepatic fibrosis. Results showed that methoxyeugenol was able to modulate the activated phenotype of HSC by activating PPAR-ɣ and demonstrated a protective effect against oxidative stress damage in hepatocytes. *In vivo* experiments further indicated that methoxyeugenol promotes a protective effect, improving inflammatory parameters and fibrosis, probably due to an increase of PPAR-ɣ activation that inhibits the NF-kB inflammatory signaling.

Declaration of competing interest

All the authors have no conflict of interest and all of them have read the manuscript and approved the submitted manuscript.

Financial support

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, and the Instituto de Salud Carlos III – Ministerio de Ciencia e Innovación (FIS PI17/00012).

Ethics approval

All procedures performed in this study involving animals were approved by PUCRS Animal Ethics Committee, code 8318.

Author contributions

Conceptualization, Bruno de Souza Basso; Formal analysis, Bruno de Souza Basso; Funding acquisition, Jordi Gracia-Sancho and Jarbas Rodrigues de Oliveira; Investigation, Bruno de Souza Basso, Gabriela Viegas Haute, Martí Ortega Ribera, Carolina Luft, Géssica Luana Antunes, Matheus Scherer Bastos, Eduardo Cassel, Eliane Romanato Santarém, Jordi Gracia-Sancho and Jarbas Rodrigues de Oliveira; Methodology, Bruno de Souza Basso, Gabriela Viegas Haute, Martí Ortega Ribera, Carolina Luft, Géssica Luana Antunes, Matheus Scherer Bastos, Leonardo Pfeiff Carlessi, Vitor Giancarlo Levorse, Eliane Romanato Santarém, Jordi Gracia-Sancho and Jarbas Rodrigues de Oliveira; Resources, Eliane Romanato Santarém; Writing – original draft, Bruno de Souza Basso; Writing – review & editing, Bruno de Souza Basso, Márcio Vinícius Fagundes Donadio, Eliane Romanato San-tarém, Jordi Gracia-Sancho and Jarbas Rodrigues de Oliveira.

CRediT authorship contribution statement

Bruno de Souza Basso: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Gabriela Viegas Haute:** Investigation, Methodology. **Martí Ortega-Ribera:** Investigation, Methodology. **Carolina Luft:** Investigation, Methodology. **Géssica Luana Antunes:** Investigation, Methodology. **Matheus Scherer Bastos:** Investigation, Methodology. **Leonardo Pfeiff Carlessi:** Methodology. **Vitor Giancarlo Levorse:** Methodology. **Eduardo Cassel:** Investigation. **Márcio Vinícius Fagundes Donadio:** Writing – review & editing. **Eliane Romanato Santarém:** Investigation, Methodology, Resources, Writing – review & editing. **Jordi Gracia-Sancho:** Funding acquisition, Investigation, Methodology, Writing – review & editing. **Jarbas Rodrigues de Oliveira:** Funding acquisition, Investigation, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, 142335/2015-0). GVH received a fellowship from CNPq and CL, GLA and BSB received fellowships from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, financial code 001).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.114433.

References

Abourashed, E., El-Alfy, A., 2016. Chemical diversity and pharmacological significance of the secondary metabolites of nutmeg (Myristica fragrans Houtt.). Phytochemistry Rev. 15 (6), 1035–1056. https://doi.org/10.1007/s11101-016-9469-x.

Agnihotri, S., Wakode, S., Ali, M., 2012. Essential oil of Myrica esculenta Buch. Ham.: Composition, antimicrobial and topical anti-inflammatory activities. Nat. Prod. Res. 26 (23), 2266–2269.

Asgarpanah, J., Kazemivash, N., 2012. Phytochemistry and pharmacologic properties of Myristica fragrans Hoyutt.: a review. Afr. J. Biotechnol. 11 (65), 12787–12793.

Boeing, T., Mejía, J.A.A., Ccana-Ccapatinta, G.V., Mariott, M., Silva, R., de Souza, P., Mariano, L.N.B., Oliveira, G.R., da Rocha, I.M., da Costa, G.A., de Andrade, S.F., da Silva, L.M., Bastos, J.K., 2020. The gastroprotective effect of red própolis extract from Northeastern Brazil and the role of its isolated compounds. J. Ethnopharmacol. https://doi.org/10.1016/j.jep.2020.113623.

Bravo, L., 2009. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr. Rev. 56 (11), 317–333.

Chen, L., Li, L., Chen, J., Li, L., Zheng, Z., Ren, J., Qiu, Y., 2015. Oleoylethanolamide, an endogenous PPAR-α ligand, attenuates liver fibrosis targeting hepatic stellate cells. Oncotarget 6 (40), 42530–42540.

Costa, B.P., Nassr, M.T., Diz, F.M., Fernandes, K.H., Antunes, G.L., Grun, L.K., Barbé-Tuana, F.M., Nunes, F.B., Branchini, G., Oliveira, J.R., 2021. Methoxyeugenol regulates the p53/p21 pathway and suppresses human endometrial cancer cell proliferation. J. Ethnopharmacol. 267, 113645.

De Mesquita, F.C., Bitencourt, S., Caberlon, E., Da Silva, G.V., Basso, B.S., Schmid, J., De Oliveira, J.R., 2013. Fructose-1,6-bisphosphate induces phenotypic reversion of activated hepatic stellate cell. Eur. J. Pharmacol. 720 (1–3).

De Oliveira, C.M., Martins, L.A.M., de Sousa, A.C., Moraes, K.D.S., Costa, B.P., Vieira, M. Q., Coelho, B.P., Borojevic, R., de Oliveira, J.R., Guma, F.C.R., 2020. Resveratrol increases the activation markers and changes the release of inflammatory cytokines of hepatic stellate cells. Mol Cell Biochem. 476 (2), 649–661. https://doi.org/10.1007/s11010-020-03933-1. Feb.

Dias, H.B., Krause, G.C., Squizani, E.D., et al., 2017. Fructose-1,6-bisphosphate reverts iron-induced phenotype of hepatic stellate cells by chelating ferrous ions. Biometals 30, 549–558. https://doi.org/10.1007/s10534-017-0025-y.

Etzerodt, A., Moestrup, S., 2012. CD163 and inflammation: biological, diagnostic, and therapeutic aspects. Antioxidants Redox Signal. 18 (17), 2352–2363. https://doi.org/10.1089/ars.2012.4834. Jun 10.

European Food Safety Authority EFSA, Parma, Italy, 2011. Scientific Opinion on the safety and efficacy of allylhy-droxybenzenes (chemical group 18) when used as flavourings for all animal species. EFSA J. 9 (12), 2440.

Friedman, S.L., 2008. Mechanisms of hepatic fibrogenesis. Gastroenterology 134 (6), 1655–1669.

Ghisalberti, E., 1979. Propolis: a review. Bee World 60 (2), 59–84. https://doi.org/10.1080/0005772X.1979.11097738.

Gionfriddo, G., Plastina, P., Augimeri, G., Catalano, S., Giordano, C., Barone, I., Morelli, C., Giordano, F., Gelsomino, L., Sisci, D., Witkamp, R., Andò, S., van Norren, K., Bonofiglio, D., 2020. Modulating tumor-associated macrophage polarization by synthetic and natural PPARγ ligands as a potential target in breast cancer. Cells 9 (1), 174. https://doi.org/10.3390/cells9010174. Jan 10.

Gracia-Sancho, J., Marrone, G., Fernández-Iglesias, A., 2018. Hepatic microcirculation and mechanisms of portal hyper-tension. Nat. Rev. 16, 221–234.

Guimarães, E.L.M., Franceschi, M.F.S., Andrade, C.M.B., Guaragna, R.M., Borojevic, R., Margis, R., Guma, F.C.R., 2007. Hepatic stellate cell line modulates lipogenic transcription factors. Liver Int. 27 (9), 1255–1264.

He, J., Hong, B., Bian, M., Jin, H., Chen, J., Shao, J., Zhang, F., Zheng, S., 2019. Docosahexaenoic acid inhibits hepatic stellate cell activation to attenuate liver fibrosis in a PPARγ-dependent manner. Int. Immunopharm. 75, 105816.

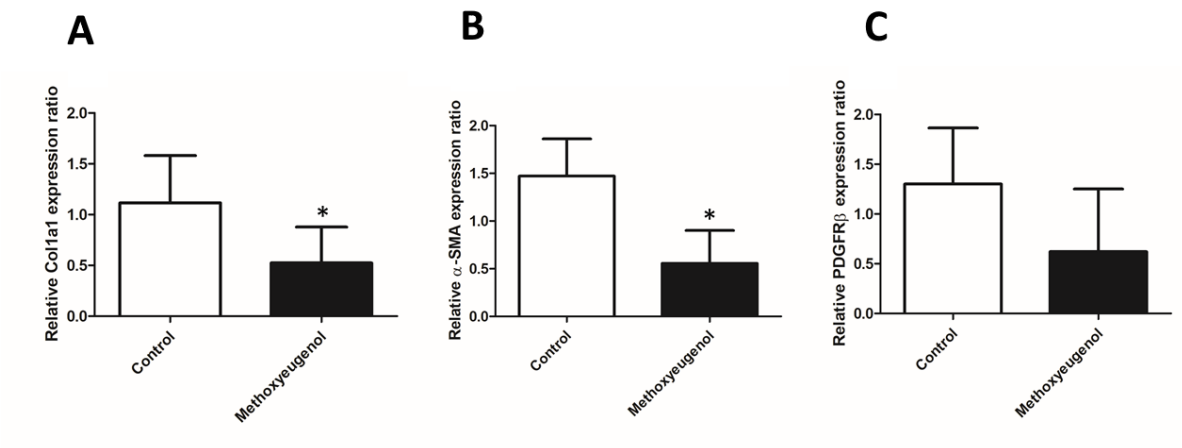
Hernandez-Gea, V., Friedman, S.L., 2011. Pathogenesis of liver fibrosis. Annu. Rev. Pathol. 6, 425–456.

Iredale, J., 2008. Defining therapeutic targets for liver fibrosis: exploiting the biology of inflammation and repair. Pharmacol. Res. Commun. 58, 129–136.

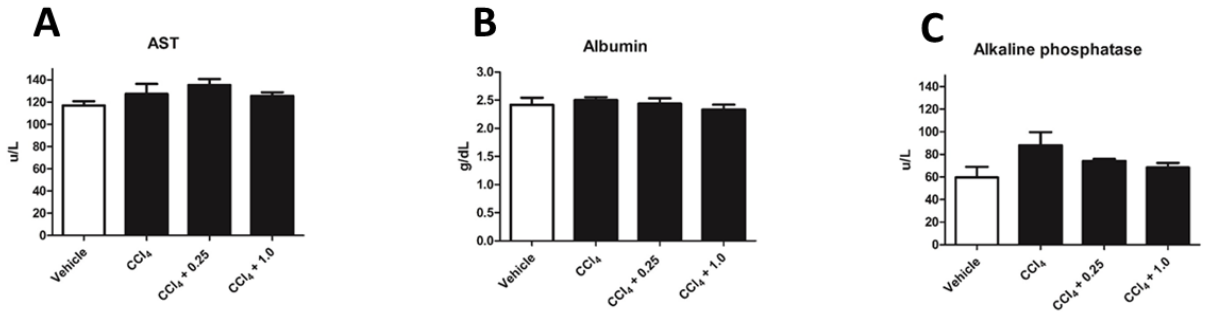
Ivanova, E.A., Parolari, A., Myasoedova, V., Melnichenko, A.A., Bobryshev, Y.V., Orekhov, A.N., 2015. Peroxisome proliferator-activated receptor (PPAR) gamma in cardiovascular disorders and cardiovascular surgery. J. Cardiol. 66, 271–278.

Jung-Hoon, K., Jaewhan, S., Kye Won, P., 2015. The multifaceted factor peroxisome proliferator-activated receptor c (PPARc) in metabolism, immunity, and cancer. Pharm. Res. (N. Y.) 38, 302–312.
Kamdem, D.P., Gage, D.A., 1995. Chemical composition of essential oil from the root bark of Sassafras albidum. Planta Med. 61 (6), 574–575. https://doi.org/10.1055/s-2006-959379. Dec.
Kim, K.Y., Rhim, T.Y., Choi, I., Kim, S.S., 2001. N-acetylcysteine induces cell cycle arrest in hepatic stellate cells through its reducing activity. JBC 276 (44), 40591–40598.
Kleiner, D.E., Brunt, E.M., Van Natta, M., Behling, C., Contos, M.J., Cummings, O.W., Sanyal, A.J., 2005. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 41 (6), 1313–1321.
Krause, G.C., Lima, K.G., Haute, G.V., Schuster, A.D., Dias, H.B., Mesquita, F.C., Pedrazza, L., Marczak, E.S., Basso, B.S., Velasque, A.C., Martha, B.A., Nunes, F.B., Donadio, M.V., de Oliveira, J.R., 2017. Fructose-1,6-bisphosphate decreases IL-8 levels and increases the activity of pro-apoptotic proteins in HepG2 cells. Biomed. Pharmacother. 89, 358–365. https://doi.org/10.1016/j.biopha.2017.01.178. May.
Li, F., Yang, Y., Yang, L., Wang, K., Zhang, X., Zong, Y., Ji, G., 2017. Resveratrol alleviates FFA and CCl4 induced apoptosis in HepG2 cells via restoring endoplasmic reticulum stress. Oncotarget 8 (27), 43799–43809.
Liedtke, C., Luedde, T., Sauerbruch, T., Scholten, D., Streetz, K., Tacke, F., Weiskirchen, R., 2013. Experimental liver fibrosis research: update on animal models, legal issues and translational aspects. Fibrogenesis Tissue Repair 6 (1), 19. https://doi.org/10.1186/1755-1536-6-19. Oct 1.
Luedde, T., Schwabe, R.F., 2011. NF-κB in the liver-linking injury, fibrosis and hepatocellular carcinoma. Nat. Rev. Gastroenterol. Hepatol. 8, 108–118.
Ma, Z.G., Yuan, Y.P., Zhang, X., Xu, S.C., Wang, S.S., Tang, Q.Z., 2017. Piperine attenuates pathological cardiac fibrosis via PPAR-γ/AKT pathways. EBioMedicine 18, 179–187. https://doi.org/10.1016/j.ebiom.2017.03.021, 2017.
Marrone, G., Shah, V.H., Gracia-Sancho, J., 2016. Sinusoidal communication in liver fibrosis and regeneration. J. Hepatol. 65 (3), 608–617. Sep.
Mirza, A., Althagafi, I., Shamshad, H., 2019. Role of PPAR receptor in different diseases and their ligands: physiological importance and clinical implications. Eur. J. Med. Chem. 166 (15 March), 502–513.
Nan, Y.M., Kong, L.B., Ren, W.G., Wang, R.Q., Du, J.H., Li, W.C., Yu, J., 2013. Activation of peroxisome proliferator activated receptor alpha ameliorates ethanol mediated liver fibrosis in mice. Lipids Health Dis. https://doi.org/10.1186/1476-511X-12-11. Feb 6;12:11.
Pragyan, A., Komal, C., Sabine, W., Ralf, W., 2021. Cellular mechanisms of liver fibrosis. Front. Pharmacol. 12, 1072.
Papa, S., Bubici, C., Zazzeroni, F., Franzoso, G., 2009. Mechanisms of liver disease: cross-talk between the NF-κB and JNK pathways. Biol. Chem. 390, 965–976.
Popov, Y., Schuppan, D., 2009. Targeting liver fibrosis: strategies for development and validation of antifibrotic therapies. Hepatology 50, 1294–1306. https://doi.org/10.1002/hep.23123.
Qu, Y., Zhou, L., Wang, C., 2017. Mangiferin inhibits IL-1β-induced inflammatory response by activating PPAR-γ in human osteoarthritis chondrocytes. Inflammation 40 (1) (February).
Righi, A.A., Alves, T.R., Negri, G., Marques, L.M., Breyer, H., Salatino, A., 2011. Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. J. Sci. Food Agric. 91 (13), 2363–2370.
Silva, J.B., Paiva, A.R., Costa, M.F.M., Viana, A., Araújo, N., Bezerra, S., Freitas, I.A., Batista, S., 2019. Hepatoprotective and antineoplastic potential of red propolis produced by the bees Apis mellifera in the semiarid of Rio Grande do Norte, Brazil. Pesqui. Vet. Bras. 39 (9), 744–756. https://doi.org/10.1590/1678-5150-pvb-6214.
Singh, G., Marimuthu, P., De Heluani, C.S., Catalan, C., 2005. Antimicrobial and antioxidant potentials of essential oil and acetone extract of Myristica fragrans Houtt. (aril part). J. Food Sci. 70 (2), M141–M148. https://doi.org/10.1111/j.1365-2621.2005.tb07105.x.
Sforcin, J.M., 2016. Biological properties and therapeutic applications of propolis. Phytother Res. 30 (6), 894–905. https://doi.org/10.1002/ptr.5605. Jun.
Tighe, S., Akhtar, D., Iqbal, U., Ahmed, A., 2020. Chronic liver disease and silymarin: a biochemical and clinical review. J. Clin. Transl. Hepatol. 8 (4), 454–458. https://doi.org/10.14218/JCTH.2020.00012. Dec 28.
Tyagi, S., Gupta, P., Saini, A., Kaushal, C., Sharma, S., 2011. The peroxisome proliferator-activated receptor: a family of nuclear receptors role in various diseases. "J. Adv. Pharm. Technol. Research"" (JAPTR)" 2, 236–240.
Wang, L., Waltenberger, B., Pferschy-Wenzig, E., Blunder, M., Liu, X., Malainer, C., Blazevic, T., Schwaiger, S., Rollinger, J., Heiss, E., Kopp, D., Bauer, R., Stuppner, H., Dirsch, V., Atanasova, A., 2014. Natural product agonists of peroxisome proliferator-activated receptor gamma (PPARγ): a review. Biochem. Pharmacol. 92 (1), 73–89.
Yimam, M., Jiao, P., Hong, M., Jia, Q., 2016. Hepatoprotective activity of an herbal composition, MAP, a standardized blend comprising myristica fragrans, Astragalus membranaceus, and poria cocos. J. Med. Food. 19 (10), 952–960. https://doi.org/10.1089/jmf.2016.0048. Oct.
Zhang, Y., Miao, H., Yan, H., Sheng, Y., Ji, L., 2018. Hepatoprotective effect of Forsythiae Fructus water extract against carbon tetrachloride-induced liver fibrosis in mice. J. Ethnopharmacol. 218, 27–34. https://doi.org/10.1016/j.jep.2018.02.033. May 23.
Zhao, W., Song, F., Hu, D., Chen, H., Zhai, Q., Lu, W., Zhao, J., Zhang, H., Chen, W., Gu, Z., Wang, G., 2020. The protective effect of myristica fragrans Houtt. Extracts against obesity and inflammation by regulating free fatty acids metabolism in nonalcoholic fatty liver disease. Nutrients 12, 2507. https://doi.org/10.3390/nu12092507.
Zong, L., Qu, Y., Xu, M.Y., Dong, Y.W., Lu, L.G., 2013. Effect of 18α-GA on HSCs. J. Digest. Dis. 14, 328–336. https://doi.org/10.1111/1751 2980.12041.

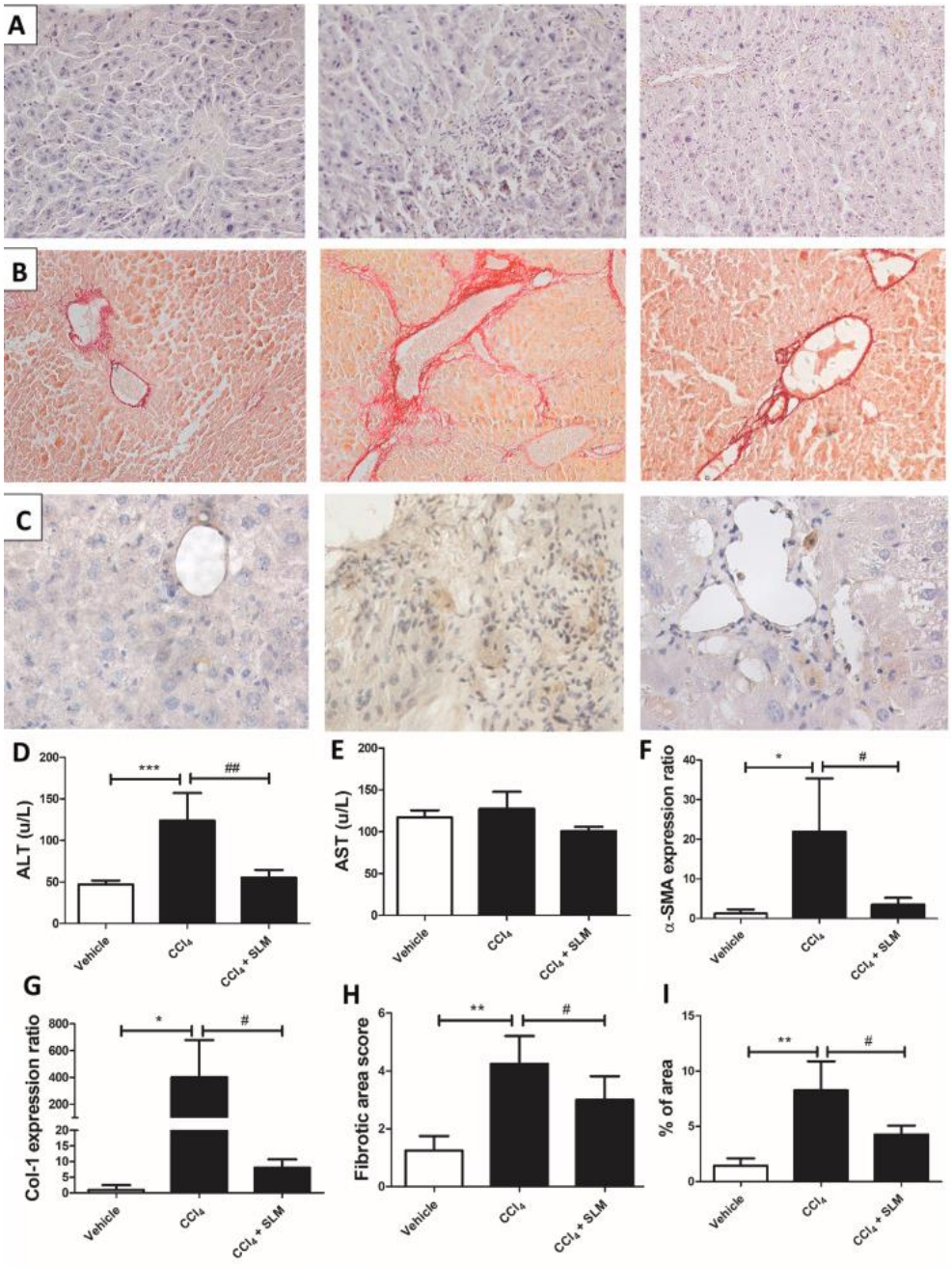
Supplementary data



Supplementary Figure 1. Effect of methoxyeugenol on primary HSC isolated from cirrhotic rats induced with CCl_4. mRNA expressions levels of αSMA (A), Collagen1 (B) and PDGFRβ (C) were evaluated. Data represent mean ± standard error of the mean (n=3). *P<0.05 compared with vehicle group.



Supplementary Figure 2. Effect of methoxyeugenol on the serum markers of fibrotic mice induced with CCl_4. Concentration AST (A), albumin (B) and alkaline phosphatase (C). Animals received CCl_4, methoxyeugenol at 0.25 and 1.0 mg/Kg twice a week for 10 weeks. Data represent mean ± standard error of the mean (n = 8).



Supplementary Figure 3. Effect of Sylimarin on liver fibrosis development. H&E (A), Picro Sirius (B), α-SMA immunohistochemistry (C) staining of representative mice treated with vehicle, vehicle+CCl4, SLM 200mg/Kg +CCl4. Magnification of 400x. ALT (D) and AST (E) serum levels. Expression of mRNA ratio of α-SMA (F) and Col-1 (G). Quantification of Fibrotic area score (H) and % of positive α-SMA area (I). Data represent mean ± standard deviation (SD) (n = 4). *p<0.05 compared with the vehicle group. ***p<0.001 compared with the vehicle group. #p<0.05 compared with the CCl_4 group. ##p<0.01 compared with the CCl_4 group.

6. ARTIGO 2. *Artigo submetido ao periódico Planta Medica (IF: 3.35)*

Objetivo 2

Avaliar o efeito do tratamento do extrato de *B. anomala* em modelo de fibrose hepática *in vivo* e identificar os principais compostos fenólicos presente no extrato.

Leaf extract of Baccharis anomala reduces NF-kB activation and attenuates liver fibrosis in mice

Bruno de Souza Basso[1,], Géssica Luana Antunes[1], Lucas Strassburger Matzenbacher[1], Matheus Scherer Bastos[1], Fernando Mendonça Diz[2], Leonardo Pfeiff Carlessi[1], Vitor Giancarlo Levorse[1], Marcella Nassr Tornquist[1], Camille Kirinus Reghelin[1], Arieli Cruz de Sousa[3], Carolina Luft[1], Eliane Romanato Santarém[1], Jarbas Rodrigues de Oliveira[1]*

[1] School of Health and Life Sciences, Pontifical Catholic University of Rio Grande do Sul - PUCRS, Porto Alegre, Rio Grande do Sul, Brazil.

[2] School of Technology, Pontifical Catholic University of Rio Grande do Sul - PUCRS, Porto Alegre, Rio Grande do Sul, Brazil.

[3]Department of Biochemistry, Federal University of Rio Grande Do Sul - UFRGS, Porto Alegre, Rio Grande do Sul,3 Brazil.

** Corresponding authors:*

Bruno de Souza Basso, Laboratório de Pesquisa em Biofísica Celular e Inflamação, Pontifícia Universidade Católica do Rio Grande do Sul, Av. Ipiranga, 6681, Sala 221, bloco C, prédio 12, Porto Alegre, RS, Brasil. Tel.: 00+55+51 3320.4147; Fax: 00+55+51 3320.3500, E-mail: bruno.basso.001@acad.pucrs.br.

ABSTRACT

The beneficial effect of *Baccharis anomala* extract treatment on liver fibrosis was evaluated through an *in vivo* model of liver fibrosis induced by CCl_4 in mice. Previously reported, *B. anomala* extract was able to modulate hepatic stellate cells (HSC) activated phenotype, which play a central role on extracellular matrix (ECM) deposition on liver fibrosis development. *B.anomala* is a plant found in South America and is used as diuretic and for gastrointestinal disorder, and studies have recently reported its antiviral and anti-inflammatory activity. Treatment with extract of *B.anomala* was able to reduce fibrosis hallmarkers expression (αSMA and col-1), to decrease the reactive oxygen species formation and lipid peroxidation. The extract showed an anti-inflammatory effect on liver, suppressing NF-kβ activation, and reducing downstream targets signaling (IL-6 and iNOS). The data obtained showed that *B. anomala* extract has antifibrotic and anti-inflammatory effect.

KEY WORDS: *Baccharis anomala, nutraceutical, oxidative stress, liver disease.*

1. INTRODUCTION

Baccharis anomala is a species used on folk medicine as an antiseptic agent on wounds, as diuretic and for gastroenteritis (Campos et al., 2016; Xavier et al., 2013). It is mainly found in south of South America (Argentina, Uruguay, and Paraguay) and in southeastern and south regions of Brazil (Heiden and Schneider, 2010).

As previously reported by our research group, extracts of *B. anomala* deactivates the activated phenotype of hepatic stellate cells (HSC) (Basso et al., 2019). The treatment with two fractions (fractions III and IV) obtained from the crude extract of *B. anomala* was able to reduce the cellular proliferation and pro-fibrotic markers, as well to induce the quiescent phenotype of activated HSC (GRX cells). The main phenolic constituents in the fractions from the methanolic extract were the chlorogenic, hydroxybenzoic and coumaric acids. Studies have also reported that *B. anomala* extract presents antiviral activity and beneficial effects on inflammatory background diseases (Venturi et al., 2018; Basso et al., 2019). HSCs are the main source of extracellular matrix (ECM) elements deposition on liver during the fibrosis process. HSC on activated phenotype also leads to an increase in the proliferation profile and pro-inflammatory signaling. The modulation of these cells is target of therapeutics for reducing the fibrosis development on hepatic tissue (Higashi et al., 2017).

Liver fibrosis consists of a constant wound healing process on the liver, due to a chronic damage on hepatic cells. Most chronic liver diseases are due to viral infections and alcohol abuse or induced by the use of drugs. Liver cell population is composed mostly by hepatocytes, followed by endothelial cells, HSC and Kupffer cells (Friedman, 2008). Hepatocytes are responsible for the metabolism of many substances and are therefore the first target of toxins. Damaged hepatocytes can trigger the fibrotic development by signaling HSC and contribute to activation of the specialized macrophages from liver, Kuppfer cells, which in turn when activated, enhance proinflammatory signaling pathways. As consequence, a continuous harmful process is established in the hepatic tissue (Marrone et al., 2016).

There is no standard established treatment for liver fibrosis, although current therapeutic strategies consist mostly of decreasing the chronic damage and improving liver function (Odagiri et al., 2021). Drugs, such as N-acetylcysteine (NAC), which can reduce oxidative stress and increasing the synthesis of endogenous antioxidants are considered treatments for fibrosis, (Kim et al., 2001, Iredale, 2008). Natural compounds, crude extracts or isolated molecules obtained from plants have progressively attracted attention for devising antifibrotic therapies. (Bravo, 2009). Being obtained from renewable sources, natural compounds can be considered excellent candidates for new molecules, biocompounds or extracts with a biotechnological application.

In the present study, we evaluated the therapeutic effect of the methanolic extract of *B. anomala* leaves in mice submitted to the liver fibrosis protocol induction by CCl_4. The effects of the extract on fibrotic and inflammatory markers and on damage from oxidative stress were evaluated.

2. METHODS

2.1 *Plant material and extract preparation*

The extract was prepared with leaves of *Baccharis anomala DC* collected in São Francisco de Paula (latitude, 29º29S; longitude, 50º 11 W; 950 m) southern Brazil (Rio Grande do Sul state). The specimen was identified and deposited (voucher specimen number 3354) in the Pontifícia Universidade Católica do Rio Grande do Sul Herbarium. For extract preparation the plant material was completely dried at room temperature, then the dried leaves were powdered and stored at -20 ºC. The methanol extract was prepared at concentration of 3 µg/mL, in a methanolic solution (80% methanol + 20% distilled water). In a volumetric flask, containing the powdered vegetal material and the methanol solution, the mixture was ultrasonified in an ultrasonic bath for 15 min at room temperature. Then, the mixture was transferred to a falcon and centrifugated for 10 min at $4x10^3$ g. After centrifugation, the supernatant was collected and dried in a rotary

evaporator (Basso et al.,2019). For animal treatment, the extract was solubilized in saline solution and administered via i.p. 50 or 100 mg/Kg.

2.2 *Identifications of major components of crude extract*

The major phenolic compounds from crude extract were determined by High Performance Liquid Chromatography (HPLC), in a Sikam Chromatography™ S 600, and UV/VIS detector Mod. 3345DAD. Separation of phenolic compounds was done in a MetaSil ODS column (5 µm; 250 x 4.6 mm), with a C_{18} guard column. Temperature of the column oven was set at 25 °C. Chromatographic data were processed by Clarity Chromatography Software. Molecules present in the extract were compared by the retention times to the commercial standards caffeic acid, coumaric acid, gallic acid, hydroxybenzoic acid and chlorogenic acid. The mobile phase was composed of methanol (elution solvent A) and acidic water with formic acid (2.5%; v/v) (elution solvent B). The linear gradient started with 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 0.5 mL/min, and detection was carried out at 280 nm.

2.3 *Animals*

For the *in vivo* experiments, 8 weeks old male BALB/c mice were bread, till they weighing 25-30 g at the university animal facility (CeMBE, PUCRS). CeMBE facilities provides ventilated cages with specific pathogen free conditions, controlled temperature and humidity, the light cycle for experiments using mice is 12h of light and 12h dark. The animals have free access to water and ration.

For the liver fibrosis model, were used the chronic carbon tetrachloride (CCl_4) protocol by periodic CCl_4 injection. In the experiment *in vivo*, 24 BALB/c mice were randomly allocated to four groups (six mice per group): Control group; CCl_4 group; CCl_4 + 50mg/Kg of extract; CCl_4+ 100 mg/Kg of extract. The administration of CCl_4 was done via i.p. (20% of CCl_4 in olive oil, 1ml/Kg) three times a week for a total of ten weeks. The treatments with 50 or 100 mg/Kg (in saline) were administered via i.p. in alternate days to the CCl_4 injection on groups

that received the treatment with extract, and only saline was used in CCl_4 group. After ten weeks of protocol, animals were sacrificed by decapitation, and for the following experiments the blood and liver were collected. The work is in agreement with all Animals procedure terms of CeMBE (project number 8318).

2.4 *Serum analysis*

From the collected blood, the serum was separate by centrifuged at 4 °C, 3000 rpm for 10 min. Using commercial kits from Labtest Kits (Lagoa Santa, Minas Gerais, Brazil) was analyzed the enzymatic activities of alanine transaminase (ALT) and aspartate transaminase (AST). The experiments were carried out following the manufacturer's recommendations.

2.5 *Liver histopathology*

From the collected liver, samples were fixed with 10% buffered formalin and were subjected to tissue processing for the next experiments. Paraffin blocks contain liver tissues slices were cut into 5 μm sections. From the mounted slides, stanning with hematoxylin and eosin stain (H&E) and Picro Sirius Red were performed. For immunohistochemistry, the antigen retrieval in the mounted slides was performed using microwave heat (3 cycles of 5 min), in a glass recipient with citrate buffer (pH= 6). After antigen retrieval step, slides were submitted to a peroxidase step (3% in PBS) for 10 min. After peroxidase step, slides were washed in PBS then a blocking step was performed (5% of goat serum in PBS solution with 0.1% of Triton X) at room temperature for 1h. After blocking step, the primary antibody (αSMA) was added to the tissue on the slides. For secondary antibody incubation, was used anti-rabbit antibody, diluted in PBS with Triton X 0.1% (1:300) for 1 h. For visualization of positive areas for the primary antibody, DAB (3, 3′ diaminobenzidine tetrahydrochloride) was used. Samples were counterstained with Hematoxylin and mounted using DPX mounting medium. Images were obtained using microscope BMX 43 equipped with DP73 digital camera (Olympus, Tokyo, Japan).

2.6 *Oxidative stress on liver*

For oxidative stress and enzymatic assays, liver was macerated in PBS, centrifuged at $4x10^3$ g for 10 min and supernatant was collected. The total protein in each sample was determined using the Nanodrop Spectrophotometer (Thermo Scientific).

For the thiobarbituric acid reactive substance (TBARS) assay, 10 µl of the collected supernatant was added to a mixture of 10 µl of sodium dodecyl sulfate + 400 µl of thiobarbituric acid. Then, the mixture was heated for 30 min following a centrifugation step ($4x10^3$ g for 10 min). After, the collected supernatant was measured at 532 nm. The results were represented as mM TBARS/mg protein.

For catalase (CAT) activity assay measuring, 102 µl of H_2O_2 30% and 100 µl of Triton X were combined to 10 µl of the supernatant from the liver macerate and read at 240 nm. One CAT unit is defined as 1 µmol of H_2O_2 consumed per minute. The results were presented as CAT units/mg protein.

For reduced glutathione (GSH) analysis, 250 µl of metaphosphoric acid was added to 50 µl of liver supernatant. The mixture was heated and 650 µl of Na_2HPO_4 + 100 µl of the color reagent were added. The resultant compound was measured at 412 nm. The results were represented as OD 412 nm/g protein.

The measurement of reactive species of oxygen (ROS) was determined with the DCFH-DA assay. The liver supernatant was incubated in 100 µM of 2′ 7′-dichlorofluorescein diacetate (H2DCF-DA) solution. After the incubation, samples were measured at λem = 488 nm and λex = 525 nm. The results were represented as nmol DCF/mg protein.

2.7 *mRNA extraction and real-time qPCR*

Real-time qPCR was performed in a StepOne equipment (Applied Biosystems). The RNA from liver samples, were extracted using TRIzol reagent (Invitrogen). Using Superscript III SuperMix kit (Invitrogen), the RNA was reversely transcribed into cDNA. The total RNA concentration of all samples was normalized

at 5 µg. For determination of the relative expression levels of target genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression were used as reference gene. The qPCR reaction was catalyzed by SYBR Green (Applied Biosystems) and results were expressed as $\Delta\Delta CT$[2].

2.8 *Western Blot*

For Western Blot assay, the protein from liver tissue was extracted by homogenization the sample in solution containing CHAPS 0.5%, β-mercaptoethanol and proteases. All protein samples concentration were normalized to 40 µg of protein. For protein separation, electrophoresis in a polyacrylamide gel 10% was performed. After electrophoresis, the bands were transferred to a nitrocellulose membrane (Biorad, 1620112). The blot was incubated in blocking solution TTBS containing 5% BSA for 30 min. After the blocking step, the blot was washed in TTBS and incubated overnight at 4°C in blocking solution containing the following primary antibodies: anti-GAPDH, anti-NF-kβ p65, anti-P- NF-kβ p65, anti-iNOS or anti-αSMA. The concentration of each antibody was performed following the manufacturer's instructions. After the overnight incubation, the blot was washed and incubated again for 2 h with horseradish peroxidase-conjugated anti-IgG secondary antibody. The band was detected by a gel documentation system (Fujifilm, LAS-3000). Band intensities were quantified through the ImageJ software.

2.9 *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 $P < 0.05$. Differences were considered significant at a P value < 0.05. The statistical program used was GraphPad Prism Version 5.00.

3. RESULTS

3.1 *B. anomala extract decreases serum markers of liver damage.*

To determine the development of fibrosis through liver damage, the enzymatic activities of ALT and AST was evaluated, since their release into the serum is related to permeabilization and rupture of the hepatocyte membrane, respectively. Both enzymes showed an increase in the group that received only CCl_4, whereas groups treated with the extract at 50 or 100 mg/Kg showed a decrease in serum ALT levels (Figures 1a and 1b). Due to the continuous deposition of extracellular matrix components in the liver during the fibrotic process, we evaluated the relationship between the animal's body weight and liver weight. Animals that received only CCl_4 showed a higher liver index than the control animals and those treated with the extract at 100 mg/Kg (Figure 1c).

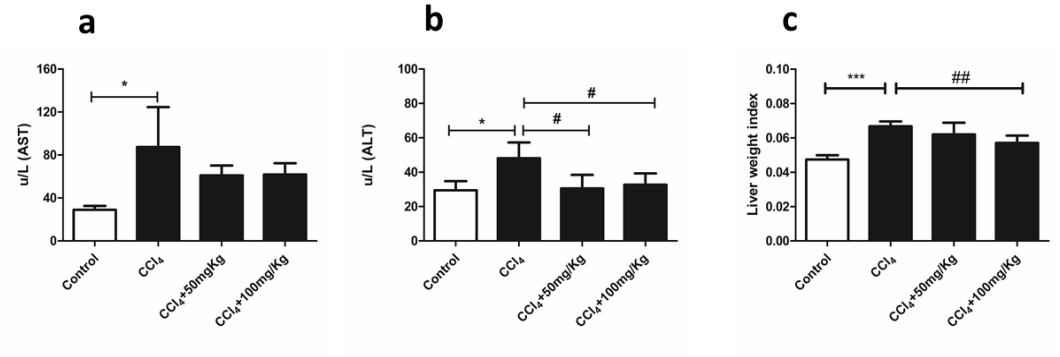


Figure 1: *B. anomala* extract decreases serum markers of liver damage. **(a)** AST levels in the serum of mice. **(b)** ALT levels in the serum of mice. **(c)** Liver index (liver weight / body weight) of mice. Data represent mean ± standard error of the mean (n=6). *P < 0.05 compared with vehicle group. ***P<0.001 compared with vehicle group. #P< 0.05 compared with CCl_4 group. ##P<0.01 compared with CCl_4 group.

3.2 *B. anomala extract attenuates fibrosis development on liver*

During the development of liver fibrosis, fiber formation and deposition of ECM in the hepatic tissue is observed. The evaluation of those ECM markers is crucial to determine the fibrosis in the liver, since exacerbated ECM deposition contributes to the stiffness of the liver and can result in loss of liver function. To evaluate the formation of collagen fibers, liver samples were stained with Picro

Sirius, and immunohistochemistry was used to visualize the αSMA distribution on the samples. Animals that received CCl_4 injections showed a greater area in red, due collagen stained by Picro Sirius, and a higher positive area for αSMA (brown spots) than control. Treatment with extract was able to reduce both stains, especially the dose of 100 mg/Kg (Figure 2a and 2b). Moreover, we evaluated the expression of these markers (Col-1 and αSMA). Animals that only underwent CCl_4 induction showed a higher expression of collagen type1 and αSMA. The treatment with 100 mg/Kg of the extract decreased both levels of expression, whereas the treatment with 50 mg/Kg reduced only the levels of Col-1 (Figure 2c and 2d). As αSMA is a specific marker to assess fibrosis, we decided to quantify its protein expression by western blot. The results showed a decrease protein level induced by extract treatment with 50 and 100 mg/Kg and these results suggest that the treatment with extract of *B. anomala* was able to reduce the development of fibrosis on the liver (Figure 2e).

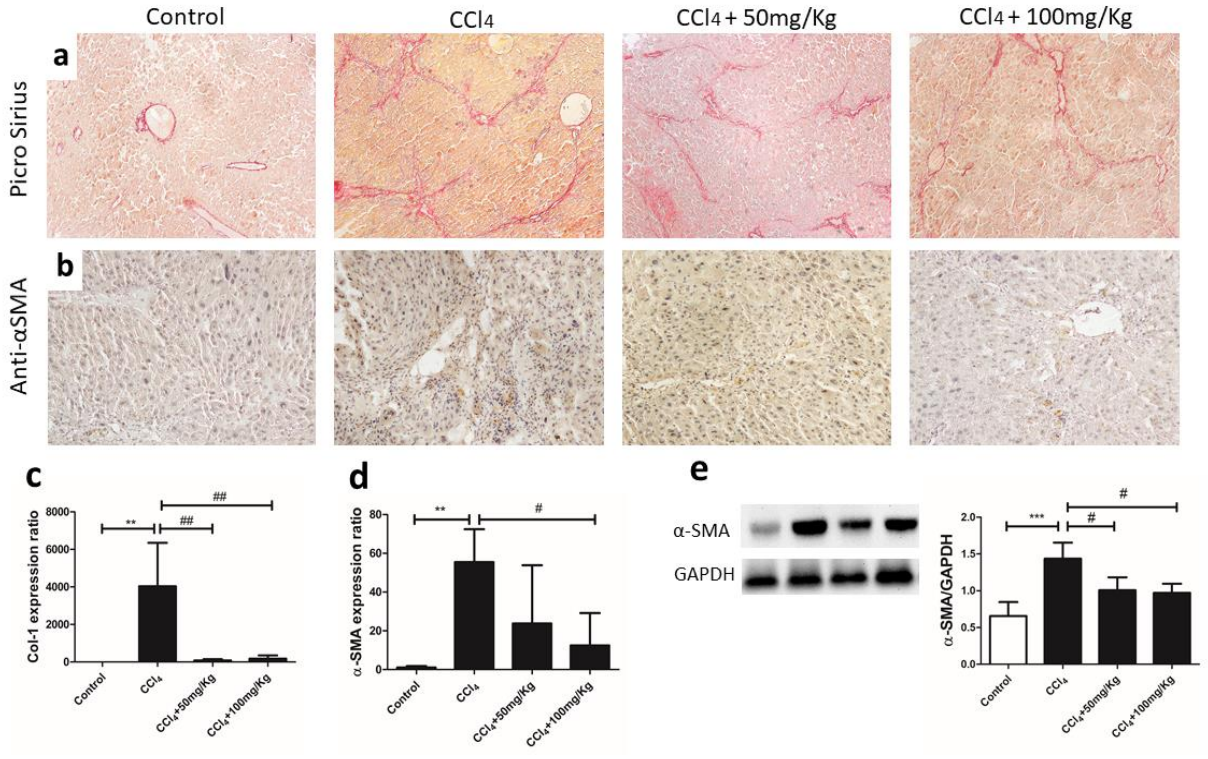


Figure 2*: B. anomala* extract attenuates liver fibrosis. **(a)** Picro Sirius staining of liver. **(b)** αSMA immunohistochemistry on liver. mRNA expression levels of Collagen1 **(c)** αSMA **(d)** and

protein levels of αSMA **(e)**. **P<0.01 compared with vehicle group. ***P<0.001 compared with vehicle group. #P< 0.05 compared with CCl_4 group. ##P<0.01 compared with CCl_4 group.

3.3 *B. anomala extract attenuates the oxidative stress*

Oxidative stress leads to lipid peroxidation, and the product resulting from this process (malondialdehyde) can be determined by TBARS assay. The treatment with the extract from *B. anomala* prevented lipid peroxidation (TBARS) on liver tissue of mice subjected to CCl_4 administration (Figure 3a). ROS formation can be determined by achieving activity of hydroxyl, peroxyl and other reactive oxygen species by the fluorometric assay DCFH-DA. Results showed that mice treated with 50 and 100 mg/Kg showed a substantial reduction in ROS formation (Figure 3b). However, the treatment with the extract failed to prevent the drench of antioxidant enzymes CAT and GSH (Figure 3c and 3b).

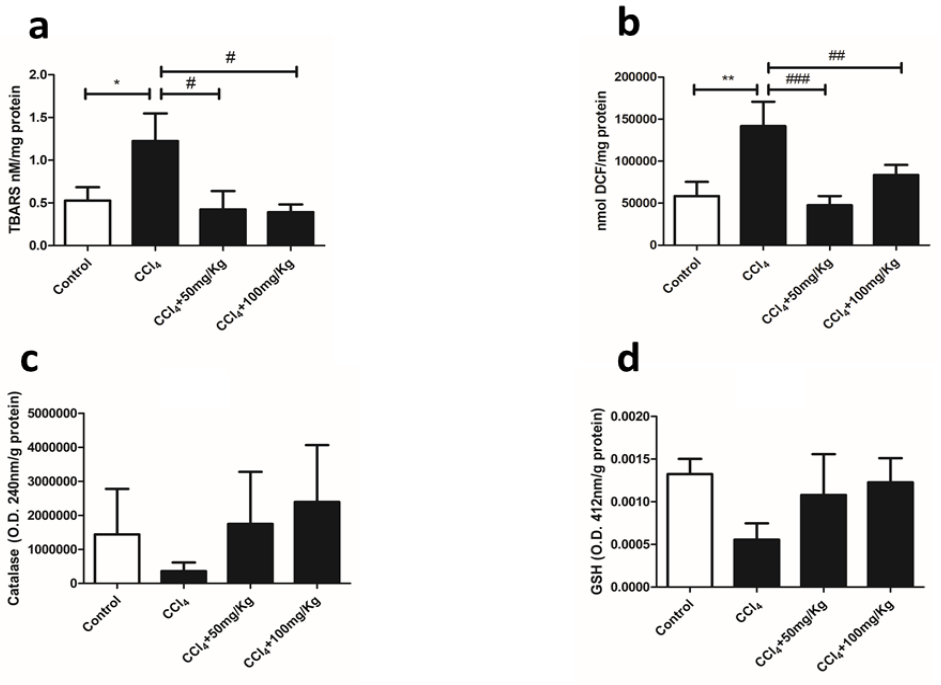


Figure 3: *B. anomala extract attenuates the oxidative stress*. **(a)** TBARS levels on mice liver. **(b)** DCFH-DA levels on mice liver. **(c)** Catalase activity on mice liver. **(d)** GSH levels on mice liver. *P < 0.05 compared with vehicle group. **P<0.01 compared with vehicle group. #P< 0.05 compared with CCl_4 group. ##P<0.01 compared with CCl_4 group. ###P< 0.001 compared with CCl_4 group.

3.4 *B. anomala extract decreased liver inflammation*

During liver fibrosis, the constant inflammation leads to an increase in activation of pro-fibrotic signaling and the reduction of inflammation on liver represents an important goal to enhance liver function. Animals that received the extract at 50 and 100 mg/Kg showed a decrease in TNF-α gene expression (Figure 4a), and NF-kB activation (Figure 4b). The NF-kB activation mediates the transcription of several proinflammatory cytokines and molecules, such as iNOS and IL-6. Our results show that iNOS protein expression (Figure 4c) and both gene expressions (Figures 4d and 4e) were suppressed by the treatment with the *B. anomala* extract. Chronic damage on the liver leads to a continuous inflammatory state, inducing the migration of inflammatory cells and activation of liver macrophages resident (Kupffer cells). These inflammatory infiltrates, mainly macrophages, can be visualized on H&E staining and, also, through of expression of CD163, phenotypic marker of M2 macrophages. Treatment with extract was able to reduce the inflammatory infiltrates showed by H&E staining (Figure 5a-d) and decreased the gene expression of CD163 (Figure 5e) compared with CCl_4 group.

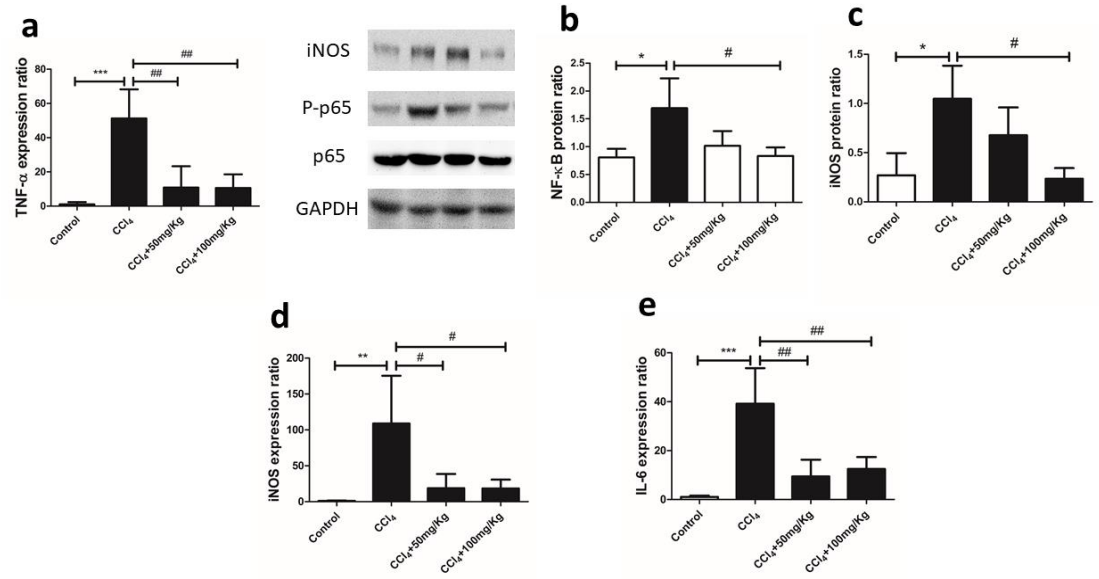


Figure 4: *B. anomala extract decreases liver inflammation*. mRNA expressions levels of TNF-α **(a)**, protein levels of NF-kB (total/phosphorylated) **(b)** and iNOS **(c),** and mRNA expressions levels of iNOS **(d)** and IL-6 **(e).** *P < 0.05 compared with vehicle group. **P<0.01 compared with vehicle group. ***P<0.001 compared with vehicle group. #P< 0.05 compared with CCl_4 group. ##P<0.01 compared with CCl_4 group.

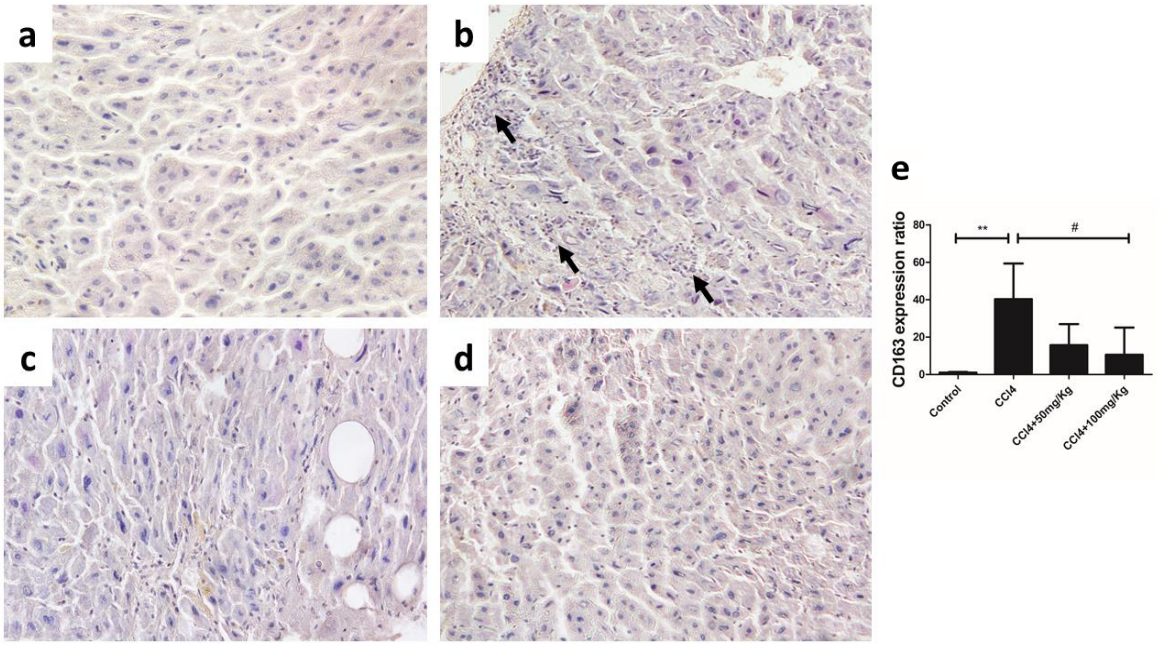


Figure 5: *B.anomala extract reduce inflammatory infiltrates on liver.* Representative H&E staining of liver fibrosis control **(a)**, CCl_4 **(b),** CCl_4 + 50mg/Kg **(c)** and CCl_4 + 100mg/Kg **(d)**. Arrows indicate inflammatory infiltrates. mRNA expressions levels of CD163 **(e).** **P<0.01 compared with vehicle group. #P< 0.05 compared with CCl_4 group.

3.5 *Compounds identification by HPLC analysis*

The methanolic extract of *B. anomala* had its major phenolics compounds identified and quantified HPLC analysis. The phenolic in greater concentration in the extract was caffeic acid (0.191 mg/g), followed by gallic (0,032 mg/g) and Coumaric (0,031 mg/g) acids and in lower concentrations chlorogenic (0,020 mg/ g) and hydroxybenzoic acid (0,016 mg/ g). The concentration of each compound identified in the extract are shown in Table 1.

Compound	mg/g of dry weight
Caffeic acid	0,191
Gallic acid	0,032
Coumaric acid	0, 031
Chlorogenic acid	0,020
Hidroxybenzoic acid	0,016

Table 1: HPLC analysis concentration is represented as *mg* of compound in *g* of vegetal dry weight.

4. DISCUSSION

The present study aimed to evaluate the effect of *B. anomala* extract treatment on mice that underwent liver fibrosis model. Our results showed that treatment with 50 and 100 mg/Kg of extract decrease the liver fibrosis development, As previously reported, *B. anomala* extracts could de-activate HSC *in vitro*, cells responsible for ECM deposition on liver tissue (Basso et al., 2019). Our *in vivo* results demonstrated that the treatment with extract leads to a decrease in liver index and ALT release on serum, showing a decrease in liver damage caused by CCl_4. During fibrosis development on the liver, the continuous fiber formation, mainly collagen type1, re-structures and stiffens the organ, events that can compromise the hepatic function and leads to liver failure (Friedman et al., 2008). Another ECM element that is highly expressed in fibrosis development is the α-SMA, an actin isoform that is a hallmark of fibrosis and is present in organs that have a contracting capacity (bladder and lungs, for example) (Rockey et al., 2019). The treatment with *B. anomala* extract could prevent the exaggerated deposition of Col-1 and αSMA on hepatic tissue.

Hepatocytes represent the major cell population on the liver and are responsible for metabolization of many compounds, being the primary target of

toxic agents. When damaged by harmful substances, hepatocytes secreted molecules that activate signaling pathways which can activate HSC and Kupffer cells, initiating a chronic inflammatory state in the liver (Roehlen et al., 2020). Therefore, the increase in the production of free radicals, combined with the action of toxic substances formed by metabolized by-products in the liver, result in lipid peroxidation and consequently a substantial increase in ROS levels, causing oxidative stress damage (Abrams et al., 1995).

Drugs, such as NAC (N-acetylcysteine), are used due to its antioxidant activity and for improving CAT storage. These two effects lead to a reduction of oxidative stress on liver and for this reason NAC is used for treatment of liver injury, such as acetaminophen intoxication (Kim et al., 2001; Ghanem, 2016). Therefore, the effect of *B. anomala* extract on the activity of endogenous antioxidant enzymes (CAT and GSH), on the production of ROS and on oxidative stress damage was analyzed. No increase in GSH and CAT activities was observed with the extract treatment compared to CCl_4 group, but there was an important decrease in the production of ROS and damage caused by oxidative stress. We believe that the mechanism by which the extract decreases oxidative stress is through the inflammation reduction and, due the positive interference of the phenolic composition of the extract. Indeed, we have previously reported the antioxidant effect of the extract from *B. anomala*, suggesting the role of phenolics as free radical scavengers on the damage reduction caused by the oxidative stress (Basso et al., 2019).

The activation of the proinflammatory nuclear factor NF-kB by different stimuli including pathogen-related molecules such as lipopolysaccharide (LPS) or also inflammatory cytokines such as tumor necrosis factor-α (TNF-α), leads to a signaling pathway that increases the expression of cytokines, such as IL-6, IL-8 and IL-1β. NF- kB plays a central role in several diseases with an inflammatory background and its suppression is essential to reduce the inflammatory signal cascade (Papa et al., 2009). In liver tissue, NF- kB activation can bind to nitric oxide synthase (iNOS) promoter and induce iNOS expression, and being its

expression highly related to several types of liver diseases (Iwakiri et al., 2015). In normal conditions, nitric oxide (NO) is found in small amounts in the liver, while in pathological conditions, higher NO levels act as an antimicrobial agent and plays an important role in immune regulation (Tache et al., 2014). Chronic increased NO levels may cause the hyperdynamic circulation, as seen in cirrhosis and portal hypertension (Abrams et al., 1995). Higher levels of NO also can activate HSC and Kupffer cells, and initiate the fibrotic and inflammatory process on the liver via expression of several cytokines (IL-6, for example) and growth factors, such as TGF-β. The regulation of iNOS expression plays a central role in liver disease and is a target of therapeutics.

Activated Kupffer cells leads to a recruitment of new inflammatory cells to the hepatic lesion, including macrophages, neutrophils and monocytes (Tanwar et al., 2020). Neutrophils and monocytes have a peak in their cell population on the early hours of the hepatic damage, while activated macrophages represent the principal inflammatory cells on liver (Tanwar et al., 2020). Kupffer cells release several inflammatory mediators including cytokines, superoxide, nitric oxide and grow factor such as TGF-β. Kuppfer cells represent the main source of TGF- β on liver, and this cytokine is highly related with HSC activation and the initiation of the fibrogenesis on liver injury (Kolios et al., 2006).

The specific marker of activated macrophages, CD163, seen to be an important biomarker for cirrhosis prognostic (Grønbaek et al., 2012), and these positive CD163 cells are responsible for the inflammatory signaling on hepatic liver injury. Our results showed that the extract was able to reduce the inflammatory infiltrates (decrease of positive CD163 cells) and the expression of TNF-α, NF-kB, and, also, downstream targets of NF- kβ, IL-6 and iNOS.

Previous studies reported the beneficial effects of nutraceutical supplementation in liver diseases, such as steatosis, metabolic syndrome and liver fibrosis (Bravo, 2009). Molecules as caffeine, resveratrol, curcumin, vitamin E and quercetin showed activity against oxidative stress, inflammation, and HSCs activation (Li et al., 2017). The results of identifying the extract components by

HPLC are similar to those obtained in the previous study, varying the concentration of each compound, but with a similar composition. The major compound identified in the extract, caffeic acid, had its protective effects against liver fibrosis development reported on previously studies, playing a role on restoring endogenous antioxidants, SOD and CAT, improving gut microbiota and decreasing inflammation (Mu et al., 2021; Li et al., 2015). Chlorogenic acid, the constituent reported in *B. anomala* extract attenuated the liver fibrosis through the TGF-β/Smad7 signaling pathway regulation (Shi et al., 2016; Yang et al., 2017, Basso et al., 2019). Moreover, in a CCl_4 liver fibrosis-induced model, the treatment with chlorogenic acid decreased expression of toll-like receptor 4 signaling pathway, leading to NF-kB suppression and consequently an anti-inflammatory response (Shi et al., 2013). Another molecule present on *B. anomala* extract, the coumaric acid, had its effect evaluated on liver injury on cisplatin induced-hepatotoxicity *in vivo* study (Akdemir et al., 2017). The study reported the beneficial effects of coumaric treatment on prevention of oxidative stress damage, by decreasing lipid peroxidation and improving the levels of antioxidants enzymes (SOD and GSH).

Our results suggest that *B. anomala* has an anti-inflammatory effect on liver, through NF- kB signaling suppression. The anti-inflammatory effect also could decrease the activation of HSC, and result in a less fibrotic development. The two major compounds found in the extract could be responsible, at least in part, for the results observed. Therefore, we suggest the hepatoprotective effect of the extract of *B. anomala in vivo* and its potential for development of new drugs to the treatment of liver fibrosis. The beneficial effect of *B. anomala* extract could be due to the rich phenolic composition and these molecules could exert its effect synergistically on the liver, decreasing the lipid peroxidation and improving the inflammatory state

5. CONCLUSION

In this work we demonstrated the *in vivo* effects of the administration of *B. anomala* extract in fibrotic animals induced with CCl_4. Our findings indicate that periodic administration of *B. anomala* extract leads to decrease in the development of liver fibrosis, as well as presented an anti-inflammatory in the liver, by decreasing the activation of NF-kB.

6. FUNDING

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

7. ETHICS APPROVAL

All procedures performed in this study involving animals were approved by PUCRS Animal Ethics Committee, code 8318.

8. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

9. REFERENCES

Abrams GA, Trauner M, Nathanson MH. Nitric oxide and liver disease. Gastroenterologist. 1995; Sep;3(3):220-33. PMID: 8535773.

Basso BS, Mesquita FC, Dias HB, Krause GC , Scherer M , Santarém ER , Oliveira JR, Therapeutic effect of Baccharis anomala DC. extracts on activated hepatic stellate cells. EXCLI Journal 2019; 18:91-105.

Bravo L, Polyphenols: Chemistry, Dietary Sources, Metabolism, and Nutritional Significance. Nutr. Rev. 2009; 56(11), 317–333,.

Campos FR, Bressan J, Godoy Jasinski VC, Zuccolotto T, da Silva LE, Bonancio Cerqueira L. Baccharis (Asteraceae): Chemical constituents and biological activities. Chem Biodivers. 2016; 13:1-17.

Akdemir E, Nur F. “The Protective Effects of p-Coumaric Acid on Acute Liver and Kidney Damages Induced by Cisplatin.” Biomedicines. 2017; vol. 5,2 18. 28.

Friedman, S. L., Mechanisms of Hepatic Fibrogenesis. Gastroenterology, 2008; 134(6), 1655–1669.

Li M, Wang XF, Shi JJ, et al. Caffeic acid phenethyl ester inhibits liver fibrosis in rats. World J Gastroenterol. 2015;21(13):3893-3903. doi:10.3748/wjg.v21.i13.3893

Mu HN, Zhou Q, Yang RY, Tang WQ, Li HX, Wang SM, Li J, Chen WX, Dong J. Caffeic acid prevents non-alcoholic fatty liver disease induced by a high-fat diet through gut microbiota modulation in mice. Food Res Int. 2021 May;143:110240. doi: 10.1016/j.foodres.2021.110240.

Ghanem CI, Pérez MJ, Manautou JE, Mottino AD. Acetaminophen from liver to brain: New insights into drug pharmacological action and toxicity. Pharmacol Res. 2016 ; Jul;109:119-31.

Grønbaek H, Sandahl TD, Mortensen C, Vilstrup H, Møller HJ, Møller S. Soluble CD163, a marker of Kupffer cell activation, is related to portal hypertension in patients with liver cirrhosis. Aliment Pharmacol Ther. 2012; Jul, 36(2):173-80.

Shi H, Dong L, Jiang J, Zhao J, Zhao G, Dang X, Lu X, Jia M, Chlorogenic acid reduces liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway, Toxicology. 2013; Volume 303, Pages 107-114,

Heiden G, Schneider A. Baccharis em lista de espécies da flora do Brasil. Jardim Botânico do Rio de Janeiro. 2010. http://dipeq.jbrj.gov.br/conservacao/lista-daflora-do-brasil-reflora/.

Higashi T, Friedman SL, Hoshida Y. Hepatic stellate cells as key target in liver fibrosis. Adv Drug Deliv Rev. 2017; Nov 1;121:27-42.

Iredale, J. Defining therapeutic targets for liver fibrosis: Exploiting the biology of inflammation and repair. Pharmacological Research. 2008; Vol. 58, pp. 129–136.

Iwakiri Y. Nitric oxide in liver fibrosis: The role of inducible nitric oxide synthase. Clin Mol Hepatol. 2015; 21(4):319-325.

Kim KY, Rhim TY, Choi I, Kim SS. N-Acetylcysteine Induces Cell Cycle Arrest in Hepatic Stellate Cells through Its Reducing Activity. JBC. 2001; 276(44), 40591–40598.

Kolios G, Valatas V, Kouroumalis E. Role of Kupffer cells in the pathogenesis of liver disease. World J Gastroenterol. 2006; 12(46):7413-7420.

Li F, Yang Y, Yang L, Wang K, Zhang X, Zong Y, Ji G. Resveratrol alleviates FFA and CCl4 induced apoptosis in HepG2 cells via restoring endoplasmic reticulum stress. Oncotarget. 2017; 8(27), 43799–43809.

Marrone G, Shah VH, Gracia-Sancho J. Sinusoidal communication in liver fibrosis and regeneration. J Hepatol. 2016; Sep, 65(3):608-17.

Odagiri N, Matsubara T, Sato-Matsubara M, Fujii H, Enomoto M, Kawada N. Anti-fibrotic treatments for chronic liver diseases: The present and the future. Clin Mol Hepatol. 2021 Jul;27(3):413-424.

Papa S, Bubici C, Zazzeroni F, Franzoso G. Mechanisms of liver disease: Cross-talk between the NF-κB and JNK pathways. Biol. Chem. 2009; Vol. 390, pp. 965–976.

Rockey DC, Du Q, Weymouth ND, Shi Z. Smooth Muscle α-Actin Deficiency Leads to Decreased Liver Fibrosis via Impaired Cytoskeletal Signaling in Hepatic Stellate Cells. Am J Pathol. 2019 Nov;189(11):2209-2220

Roehlen N, Crouchet E, Baumert TF. Liver Fibrosis: Mechanistic Concepts and Therapeutic Perspectives. Cells. 2020; 9(4):875.

Shi H, Shi A, Dong L, Lu X, Wang Y, Zhao J, Dai F, Guo X. Chlorogenic acid protects against liver fibrosis in vivo and in vitro through inhibition of oxidative stress. Clin Nutr. 2016; Dec, 35(6):1366-1373.

Tache DE, Stănciulescu CE, Baniţă IM, Purcaru ŞO, Andrei AM, Comănescu V, Pisoschi CG. Inducible nitric oxide synthase expression (iNOS) in chronic viral hepatitis and its correlation with liver fibrosis. Rom J Morphol Embryol. 2014; 55(2 Suppl):539-43.

Tanwar S, Rhodes F, Srivastava A, Trembling PM, Rosenberg WM. Inflammation and fibrosis in chronic liver diseases including non-alcoholic fatty liver disease and hepatitis C. World J Gastroenterol. 2020; 26(2):109-133.

Venturi CR, Bordignon SAL, Roehe PM, Montanha JA, Cibulski SP, Gosmann G, Chemical analysis and antiviral activity evaluation of Baccharis anomala. Natural Product Research. 2018; 32:16, 1960-1962.

Xavier VB, Vargas RMF, Minteguiaga NU, Dellacassa E, Cassel E, Evaluation of the key odorants of Baccharis anomala DC essential oil: New applications for known products. Ind Crops Prod. 2013; 49:492–496.

Yang F, Luo L, Zhu ZD, Zhou X, Wang Y, Xue J, Zhang J, Cai X, Chen ZL, Ma Q, Chen YF, Wang YJ, Luo YY, Liu P, Zhao L. Chlorogenic Acid Inhibits Liver Fibrosis by Blocking the miR-21-Regulated TGF-β1/Smad7 Signaling Pathway in Vitro and in Vivo. Front Pharmacol. 2017; Dec 19;8:929.

7. APÊNDICES

SIPESQ

Sistema de Pesquisas da PUCRS

Código SIPESQ: 8318 Porto Alegre,27 de março de 2018

Prezado(a) Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou o Projeto de Pesquisa "AVALIAÇÃO DE DIFERENTES COMPOSTOS FENÓLICOS COMO CANDIDATOS PARA O TRATAMENTO DE FIBROSE HEPÁTICA" coordenado por JARBAS RODRIGUES DE OLIVEIRA.

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está autorizada a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Duração do Projeto: 27/03/2018 - 27/03/2021

Nº de Animais	Espécie
540	Mus musculus
Total de Animais: 540	

Atenciosamente,

Comissão de Ética no Uso de Animais(CEUA)

Identificação do Documento: 8B7V-7FZV-USEY-2JS Página 2 de 35

8. CONSIDERAÇÕES FINAIS

A motivação por trás do presente trabalho se deve principalmente ao intuito de selecionar possíveis moléculas para o desenvolvimento de novos tratamentos para a fibrose hepática. É importante salientar que outros fatores além da parte experimental em si, também foram essenciais para a realização deste trabalho, como a integração entre os laboratórios de pesquisa, Laboratório de Biofísica Celular e da Inflamação (PUCRS-Brasil), Laboratório de Biotecnologia Vegetal (PUCRS-Brasil) e o Laboratório de Hemodinâmica Hepática (IDIBAPS-Espanha), levando a uma troca de conhecimentos e fornecendo novas ferramentas para o desenvolvimento deste projeto, e possivelmente, de novos estudos sobre a fibrose hepática e seu tratamento.

O início do projeto se deu com a avaliação *in vitro* do efeito do metoxieugenol e extrato metanólico da planta *Baccharis anomala*, as quais foram selecionadas baseando-se em publicações de estudos prévios, buscando efeitos benéficos que poderiam ter ação na diminuição do processo fibrótico no fígado. Nossos primeiros resultados mostraram a capacidade do metoxieugenol em modular a ativação das HSC, através da diminuição da proliferação, diminuição da síntese de colágeno e α-SMA. Após estes resultados promissores, decidimos avaliar o efeito do metoxieugenol *in vivo*. Nosso laboratório não possuía uma técnica padronizada para este estudo, porém decidimos implementar a técnica e validar este efeito em modelo *in vivo* de fibrose hepática. Utilizando as instalações do CEMBE, conseguimos padronizar e aplicar a metodologia de indução de fibrose hepática em murinos, utilizando a administração crônica de CCl_4. Os

resultados *in vivo* demostraram um efeito protetivo do metoxieugenol no fígado dos animais.

Afim de elucidar o mecanismo de ação por trás desse efeito, durante o período de doutorado de sanduiche realizado no Hospital Clínico de Barcelona, foi possível avaliar o efeito do metoxieugenol em células HSC de origem humana. Os resultados obtidos com a linhagem celular humana mostraram que o efeito antifibrótico do metoxieugenol ocorria também nestas células, e usando um inibidor específico do PPAR-γ, foi possível verificar que agia como um ativador deste receptor intracelular. Com esses resultados concluímos o artigo 1 do presente trabalho, onde acreditamos que pelo menos, em parte, a ativação do PPAR-γ está relacionada com os efeitos observados. A investigação por ligantes naturais que ativam o PPAR-γ é de interesse da indústria farmacêutica, visto que essas moléculas, além de possuírem propriedades terapêuticas, possuem menos efeitos colaterais que agonistas específicos. Portanto, a regulação do PPAR-γ representa uma estratégia promissora, já que sua ativação está intimamente ligada a inibição da transcrição de diversos fatores inflamatórios, como o NF-kB, que se soma a sua propriedade de reverter o fenótipo ativado das HSC

A molécula do metoxieugenol, investigada no artigo 1, está presente na alimentação humana de diversas formas, tanto em alimentos *in natura* como industrializados. Sua biossegurança para consumo é conhecida e aprovada, com isso acreditamos que essa molécula possui um grande potencial para o desenvolvimento de novos tratamentos, seja pela elaboração de fórmulas farmacêuticas, complexação com íons metálicos para fornecer maior estabilidade

e diferentes formas de distribuição no organismo, ou simplesmente pela suplementação alimentar, por exemplo. Trabalhos futuros a respeito de outras vias que podem estar envolvidas no mecanismo de ação do metoxieugenol, tendem a aumentar a visibilidade dessa molécula como uma futura terapia para a fibrose hepática e outras doenças. Com isso, esperamos ter acrescentado de forma positiva para a investigação dessa molécula e revelado uma parte de seu potencial.

O trabalho com o extrato de *B.anomala* teve seu início durante o mestrado, onde os resultados obtidos com o extrato fracionado metanólico de *B.anomala* em células estreladas hepáticas revelaram um efeito de diminuição da proliferação e ativação dessas células. Com isso, para dar continuidade a investigação do potencial terapêutico do extrato, durante o período de doutorado foram realizados experimentos *in vivo.* Os resultados demonstraram que o extrato possui a capacidade de atenuar o desenvolvimento da fibrose, e também reduzir o dano causado pelo estresse oxidativo. Contudo, a atividade de antioxidantes endógenos não foi restaurada pelo tratamento. Acreditamos que o tratamento com o extrato, por se tratar em uma mistura rica em compostos fenólicos, tenha uma alta atividade antioxidante e com isso exerceu o efeito hepatoprotetor observado.

Os resultados obtidos, mesmo sendo preliminares, demonstram um potencial terapêutico do extrato. Por se tratar de uma planta presente na medicina popular do estado do Rio Grande do Sul, dados a respeito de seus efeitos e mecanismos de ação são importantes para o estudo da composição fitoquímica e

também para o enriquecimento do conhecimento acerca do efeito terapêutico deste gênero botânico.

9. REFERÊNCIAS

- Asrani SK, Devarbhavi H, Eaton J, Kamath PS Burden of liver diseases in the world. J. Hepatol. 2019. 70:151–171.
- Bravo L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev. 1998. 56, 317-333.
- Budel J, Pharmacobotanical study of aerial vegetative parts of Baccharis anomala DC. Asteraceae. Brazilian Journal of Pharmacognosy. 2008.18, 761-768.
- Chen L, Gu T, Li B, Li F, Ma Z, Zhang Q, Cai X, Lu. Delta-like Ligand 4/DLL4 Regulates the Capillarization of Liver Sinusoidal Endothelial Cell and Liver Fibrogenesis. Biochim. Biophys. Acta Mol. Cell Res. 2019.
- Chen J, Li L, Zheng Z, Ren J, Qiu Y. Oleoylethanolamide, an endogenous PPAR-α ligand, attenuates liver fibrosis targeting hepatic stellate cells. Oncotarget. 2015. 6(40), 42530–42540.
- Costa BP, Nassr MT, Diz FM, Fernandes KH, Antunes GL, Grun LK, Barbé-Tuana FM, Nunes FB, Branchini G, Oliveira JR. Methoxyeugenol regulates the p53/p21 pathway and suppresses human endometrial cancer cell proliferation, Journal of Ethnopharmacology. 2021. Vol. 267, 113645.
- DeLeve LD, Wang X, Guo Y. Sinusoidal Endothelial Cells Prevent Rat Stellate Cell Activation and Promote Reversion to Quiescence. Hepatology. 2008. 48, 920–930.

- De Mesquita FC, Bitencourt S, Caberlon E, Da Silva GV, Basso BS, Schmid J, De Oliveira JR. Fructose-1,6-bisphosphate induces phenotypic reversion of activated hepatic stellate cell. Eur. J. Pharmacol. 2013. 720(1–3).
- Gracia-Sancho J, Marrone G, Fernández-Iglesias A. Hepatic microcirculation and mechanisms of portal hyper-tension. Nat. Rev. 2018. 16, pages 221–234(2019).
- Guimarães ELM, Franceschi MFS, Andrade CMB, Guaragna RM, Borojevic, R, Margis R, Guma FCR. Hepatic stellate cell line modulates lipogenic transcription factors. LIVER INT. 2007. 27(9), 1255–1264.
- He J, Hong B, Bian M, Jin H, Chen J, Shao J, Zhang F, Zheng S. Docosahexaenoic acid inhibits hepatic stellate cell activation to attenuate liver fibrosis in a PPARγ-dependent manner, International Immunopharmacology. 2019. Volume 75, 2019, 105816.
- Hocayen PA, Grassiolli S, Leite NC, Pochapski MT, Pereira RA, da Silva LA, Snack AL, Michel RG, Kagimura FY, da Cunha MA, Malfatti CR. Baccharis dracunculifolia methanol extract enhances glucose-stimulated insulin secretion in pancreatic islets of monosodium glutamate induced-obesity model rats. Pharm Biol. 2015. 1-9.
- Kamdem DP, Gage DA. Chemical composition of essential oil from the root bark of Sassafras albidum. Planta Med. 2015. 61(6).

- Kuete V, Mbaveng A, Nono E, Simo CC, Zeino M, Nkengfack AE, Efferth T. Cytotoxicity of seven naturally occurring phenolic compounds towards multi-factorial drug-resistant cancer cells. Phytomedicine. 2016. 23, 856-863.
- Kolios G, Valatas V, Kouroumalis E. Role of Kupffer cells in the pathogenesis of liver disease. World J Gastroenterol. 2006. 12(46):7413-7420.
- Lafoz E, Ruart M, Anton A, Oncins A, Hernández-Gea V. The Endothelium as a Driver of Liver Fibrosis and Regeneration. Cells. 2020;9(4):929.
- Liedtke C, Luedde T, Sauerbruch T, Scholten D, Streetz K, Tacke F, Weiskirchen R. Experimental liver fibrosis research: Update on animal models, legal issues and translational aspects. Fibrogenesis and Tissue Repair. 2013. Vol. 6.
- López JG, Langa E, Berzosa C, Valero MS, Gómez-Rincón C. Antihelmintic effects of nutmeg (Myristica fragans) on Anisakis simplex L3 larvae obtained from Micromesistius potassou Research in Veterinary Science. 2015. 148–152.
- Iredale J. Defining therapeutic targets for liver fibrosis: Exploiting the biology of inflammation and repair. Pharmacol. Res. Commun. 2008. Vol. 58, pp. 129–136.
- Roehlen N, Crouchet E, Baumert TF. Liver Fibrosis: Mechanistic Concepts and Therapeutic Perspectives. Cells. 2020. 9(4):875.

- Maeda A, Tanimoto S, Abe T, Kazama S, Tanizawa H, Nomura M. Chemical constituents of Myristica fragrans Houttuyn seed and their physiological activities]. Yakugaku zasshi: Journal of the Pharmaceutical Society of Japan. 2008. 128, 129-133.

- Maretti-Mira AC, Wang X, Wang L, DeLeve LD. Incomplete Differentiation of Engrafted Bone Marrow Endothelial Progenitor Cells Initiates Hepatic Fibrosis in the Rat. Hepatology. 2019. Mar;69(3):1259-1272.

- Marrone G, Shah VH, Gracia-Sancho J. Sinusoidal communication in liver fibrosis and regeneration. J Hepatol. 2016. Sep;65(3):608-17.

- Mederacke I, Hsu CC, Troeger JS, Huebener P, Mu X, Dapito DH, Pradere JP, Schwabe RF. Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. Nat. Commun. 2013. 4:2823.

- Mirza A, Althagafi I, Shamshad H. Role of PPAR receptor in different diseases and their ligands: Physiological importance and clinical implications. Eur. J. Med. Chem. 2019. Volume 166, 15 March 2019, 502-513.

- Nogueira NP, Reis PA, Laranja GA, Pinto AC, Aiub CA, Felzenszwalb I, Paes MC, Bastos FF, Bastos VL, Sabino KC, Coelho MG. In vitro and in vivo toxicological evaluation of extract and fractions from Baccharis trimera with anti-inflammatory activity. J Ethnopharmacol. 2011. 138, 513-522.

- Ramos Campos F, Bressan J, Godoy Jasinski VC, Zuccolotto T, da Silva LE, Bonancio Cerqueira L. Baccharis (Asteraceae): Chemical Constituents and Biological Activities. Chem Biodivers. 2016. 13, 1-17.
- Rezende TP, Aarestrup BJ, Aarestrup FM, de Sousa OV, da Silva Filho AA. Protective effects of Baccharis dracunculifolia leaves extract against carbon tetrachloride- and acetaminophen-induced hepatotoxicity in experimental animals. Molecules. 2014. 19, 9257-9272.
- Righi AA, Alves TR, Negri G, Marques LM, Breyer H, Salatino A. Brazilian red propolis: unreported substances, antioxidant and antimicrobial activities. Journal of the science of food and agriculture. 2011. 91, 2363-2370.
- Silva Jardel B, Paiva K, Costa M, Viana G, Araújo N, Bezerra S, Freitas A, Batista. Hepatoprotective and antineoplastic potencial of red propolis produced by the bees Apis mellifera in the semiarid of Rio Grande do Norte, Brazil. Pesquisa Veterinária Brasileira. 2019. 39(9), 744-756.
- Pereira CA, Costa AC, Liporoni PC, Rego MA, Jorge AO. Antibacterial activity of Baccharis dracunculifolia in planktonic cultures and biofilms of Streptococcus mutans. J Infect Public Health. 2016. 9, 324-330.
- Odagiri N, Matsubara T, Sato-Matsubara M, Fujii H, Enomoto M, Kawada N. Anti-fibrotic treatments for chronic liver diseases: The present and the future. Clin Mol Hepatol. 2021. Jul;27(3):413-424.

- Papa S, Bubici C, Zazzeroni F, Franzoso G. Mechanisms of liver disease: Cross-talk between the NF-κB and JNK pathways. Biol. Chem. 2009. 390, pp. 965–976.
- Pragyan A, Komal C, Sabine W, Ralf W. Cellular Mechanisms of Liver Fibrosis. rontiers in Pharmacology. 2021. 12, 2021, 1072.
- Popov Y and Schuppan D. Targeting liver fibrosis: Strategies for development and validation of antifibrotic therapies. Hepatology. 2009. 50: 1294-1306.
- Sartor T, Falcão MA, Mondin CA, dos Santos MA, Cassel E, Astarita LV, Santarém ER. Seasonal changes in phenolic compounds and in the biological activities of Baccharis dentata (Vell.) Industrial Crops and Products. 2013. 51, 355– 359.
- Szafraniec J, Błazejczyk A, Kus E, Janik M, Zajac G, Wietrzyk J, Chlopicki S, Zapotoczny S. Robust Oil-Core Nanocapsules with Hyaluronate-Based Shells as Promising Nanovehicles for Lipophilic Compounds. Nanoscale 2017. 9, 18867–18880.
- Sørensen KK, Simon-Santamaria J, McCuskey R, Smedsrød B. Liver Sinusoidal Endothelial Cells. In Comprehensive Physiology; John Wiley & Sons, Inc.: Hoboken, NJ, USA. 2015; Volume 5, pp. 1751–1774.
- Testerink N, Ajat M, Houweling M, Brouwers JF, Pully VV, van Manen HJ, Otto C, Helms JB, Vaandrager AB. Replacement of Retinyl Esters by Polyunsaturated Triacylglycerol Species in Lipid Droplets of Hepatic Stellate

Cells during Activation. PLoS ONE. 2012. 7 doi: 10.1371/journal.pone.0034945.

- Tighe S, Akhtar D, Iqbal U, Ahmed A. Chronic Liver Disease and Silymarin: A Biochemical and Clinical Review. J Clin Transl Hepatol. 2020. Dec 28;8(4):454-458.
- Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med. 2012. 18, 1028-1040.
- Zalewski CA, Passero LF, Melo AS, Corbett CE, Laurenti MD, Toyama MH, Toyama DO, Romoff P, Favero OA, Lago J. Evaluation of anti-inflammatory activity of derivatives from aerial parts of Baccharis uncinella. Pharm Biol. 2011. 49, 602-607.
- Zong L, Qu Y, Xu Y, Dong W, Lu LG. Effect of 18α-GA on HSCs. Journal of Digestive Diseases. 2013. 14: 328-336.

Pontifícia Universidade Católica do Rio Grande do Sul
Pró-Reitoria de Graduação
Av. Ipiranga, 6681 - Prédio 1 - 3º. andar
Porto Alegre - RS - Brasil
Fone: (51) 3320-3500 - Fax: (51) 3339-1564
E-mail: prograd@pucrs.br
Site: www.pucrs.br