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FRANCISLAINE APARECIDA DOS REIS LÍVERO

AVALIAÇÃO FARMACOLÓGICA DOS EFEITOS HEPÁTICOS E GÁSTRICOS DA *Baccharis trimera* EM LESÕES INDUZIDAS POR ETANOL

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Tese apresentada ao Programa de Pós-Graduação em Farmacologia, da Universidade Federal do Paraná (UFPR), como requisito parcial à obtenção do título de Doutor em Farmacologia.

Orientadora: Profª. Dra. Alexandra Acco

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Doutorado de **FRANCISLAINE APARECIDA DOS REIS LIVERO**, intitulada: "**AVALIAÇÃO FARMACOLÓGICA DOS EFEITOS GÁSTRICOS E HEPÁTICOS DA Baccharis trimera EM LESÕES INDUZIDAS POR ETANOL**", após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua aprovação, completando-se assim todos os requisitos previstos nas normas desta Instituição para a obtenção do Grau de **Doutor em FARMACOLOGIA**.

Curitiba, 12 de Fevereiro de 2016.

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Prof ADRIANA DE OLIVEIRA CHRISTOFF (UNIBRASIL)

Prof JORGETE CONSTANTIN (UEM)

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Esta tese é apresentada em formato alternativo – artigos publicados e submetidos para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná, constando de uma revisão de literatura, objetivos do trabalho e dois artigos científicos abordando os experimentos realizados, com resultados e discussão, além das considerações finais.

Dedicatória

Aos meus pais, José Claudionor e Cleonice, por me incentivarem a dar os primeiros passos e por incansavelmente me impulsionarem a sempre dar um passo a mais na vida. Por todos os sonhos que vocês abriram mão de realizar para que eu pudesse realizar os meus, todo meu amor, respeito, gratidão e admiração.

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"(...) diariamente eu chego à simples conclusão de que a vida é tão maravilhosa porque também é feita de colos, de feridas que cicatrizam, de amigos que celebram ou choram junto, de café coado com coador de pano, de gente que pega ônibus ou faz caminhada pela manhã, de quem planta o que se pode comer. Que a vida é feita de algumas pessoas que direcionam todo o seu potencial criativo para melhorar a qualidade de vida de gente que eles nem conhecem. Que é feita de e-mails que chegam recheados de saudade e de cartas extraviadas solitárias numa gaveta de um correio qualquer. De muros e pontes e cais. De aviões que suprimem distâncias e de barcos que chegam. De bicicletas que atravessam cidades. De redes que balançam gente. De rostos que recebem beijos. De bocas que beijam. De mãos que se dão. Que existem pessoas altamente gostáveis, altamente rabugentas, altamente generosas, pessoas distraídas que perdem as coisas, mal-educadas que buzinam sem necessidade, pessoas conectadas que se preocupam com o lixo, pessoas sedutoras e seduzíveis, possíveis e impossíveis, pessoas que se entregam, pessoas que se privam, pessoas que machucam, pessoas que chegam pra curar, desencadeadores de poemas, de sorrisos, de lições de vida que ficarão guardadas para sempre … A vida é tão maravilhosa porque ela nos compensa com ela mesma."

(Marla de Queiroz)

“... Se não puderes ser um pinheiro, no topo de uma colina,

sê um arbusto no vale, mas sê

o melhor arbusto à margem do regato.

Sê um ramo, se não puderes ser uma árvore.

Se não puderes ser um ramo, sê um pouco de relva

e dá alegria a algum caminho.

Se não puderes ser uma estrada, sê apenas uma senda,

Se não puderes ser o Sol, sê uma estrela.

Não é pelo tamanho que terás êxito ou fracasso...

Mas sê o melhor no que quer que sejas...”

(Pablo Neruda)

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ARTIGO CIENTÍFICO 1 - "Molecular basis of alcoholic fatty liver disease: from incidence to treatment."

AC: alcoholic cirrhosis; *ADH*: alcohol dehydrogenase; *AFLD*: alcoholic fatty liver disease; *AH*: alcoholic hepatitis; *ALD*: alcoholic liver disease; *ALDH*: aldehyde dehydrogenase; *AMPK*: 5′-adenosine monophosphate activated protein kinase; *ALT:* alanine aminotransferase; *AST*: aspartate aminotransferase; *Cat*: catalase; *CoA*: coenzyme A; *CYP2E1*: cytochrome P450 2E1; *FXR*: farnesoid X receptor; *GSH*: reduced glutathione; H_2O_2: peroxide hydrogen; *HIF*: hypoxia-inducible factors; *IL*: interleukin; *LPO*: lipoperoxidation; *LXR*: liver X receptor; *NAC*: N-acetylcysteine; NAD^+: nicotinamide adenine dinucleotide; *NADH*: reduced nicotinamide adenine dinucleotide; *NAFLD*: nonalcoholic fatty liver disease; *NR*: nuclear receptors; *PPAR*: peroxisome proliferator-activated receptors; *ROS*: reactive oxygen species; *SAM*: S-adenosylmethionine; *SOD*: superoxide dismutase; *SREBP1*: Sterol regulatory element-binding protein-1; *STAT3*: activator of transcription 3; *TNF-α*: tumor necrosis factor-α; *γ-GT*: γ-glutamyltransferase; *6-ECDCA*: 6α-ethylchenodeoxycholic acid.

ARTIGO CIENTÍFICO 2 – "Hydroethanolic extract of *Baccharis trimera* ameliorates alcoholic fatty liver disease in mice."

AC: cirrhosis; *ADH*: alcohol dehydrogenase; *AFLD*: alcoholic fatty liver disease; *ALD*: alcoholic liver disease; *ALDH*: aldehyde dehydrogenase; *AMPK*: adenosine monophosphate activate protein kinase; *ANOVA*: one-way analysis of variance; *ASH*: steatohepatitis; *ALT*: alanine aminotransferase; *AST*: aspartate aminotransferase; *Cat*: catalase; *CHO*: cholesterol; *CYP2E1*: cytochrome P450 2E1; *DCFA*: 2',7'-dichlorofluorescein-diacetate; *DTNB*: 5,5'-dithiobis; Fe^{2+}, iron; *GPx*: glutathione peroxidase; *GSH*: reduced glutathione; *GST*: glutathione S-transferase; H_2O_2: peroxide hydrogen; *HDL:* high density lipoprotein; *HE*:

hematoxylin/eosin; *HEBT*: hydroethanolic extract of *Baccharis trimera*; *LDL*: low density lipoprotein; *LOOH*: lipid hydroperoxide; NAD^+, nicotinamide adenine dinucleotide; *NADH*, reduced nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; *NADPH*, reduced nicotinamide adenine dinucleotide phosphate; *Nrf2*: nuclear factor erythroid 2-related factor 2; $O_2^{\cdot-}$: superoxide anion; OH^-: hydroxyl; *ROS*: reactive oxygen species; *scd1*: stearoyl-CoA reductase; *SEM*: standard error of the mean; *SOD*: superoxide dismutase; *TG*: triglycerides; *UGT*: UDP-glucuronosyltransferase.

ARTIGO CIENTÍFICO 3 – "*Baccharis trimera* hydroethanolic extract promotes gastroprotection and healing of gastric lesions induced by acute and chronic ethanol consumption."

DPPH: 2,2-Diphenyl-1-picrylhydrazyl; *EV*: ethanol + vehicle; *EHEBT*: ethanol + HEBT; *HEBT*: hydroethanolic extract of *B. trimera*; *HPLC*: high performance liquid chromatography; *GSH*: reduced glutathione; *GST*: glutathione *S*-transferase; LD_{50}: median lethal dose; *LOOH*: lipid hydroperoxide; *NMR*: nuclear magnetic resonance; *NSAIDs*: antiinflammatory drugs; *ROS*: reactive oxygen species; *SEM*: standard error of the mean; *SOD*: superoxide dismutase; *WV*: water + vehicle.

RESUMO

Estima-se que 2 bilhões de pessoas consumam o etanol em todo o mundo, e destas 76,3 milhões apresentam doenças relacionadas ao seu consumo. Dentre elas, a esteatose hepática alcoólica (EHA), estágio inicial das doenças hepáticas alcoólicas, destaca-se como uma enfermidade diretamente relacionada ao estresse oxidativo e a desarmonia da homeostase lipídica. Já a úlcera gástrica é uma doença multifatorial que decorre do desequilíbrio entre fatores agressivos e protetores. Tais lesões merecem atenção especial devido à ausência de tratamento preconizado, ou ainda, à vasta quantidade de efeitos colaterais observados. Buscando uma nova alternativa de tratamento, investigamos o potencial farmacológico da *Baccharis trimera* (“carqueja”), planta popularmente utilizada para tratar distúrbios gastrointestinais, em modelos de lesão gástrica e EHA. O extrato hidroetanólico (HEBT) foi obtido das partes aéreas da planta e caracterizado por cromatografia líquida de alta eficiência. Para investigar a atividade farmacológica do HEBT frente à EHA, submetemos camundongos à ingestão de etanol a 10% e dieta hipoprotéica por 6 semanas. Nas duas últimas semanas os animais foram tratados diariamente com o HEBT (30 $mg.kg^{-1}$, via oral). O estresse oxidativo induzido pelo etanol foi revertido pelo HEBT, que normalizou os níveis de LPO, ROS total e GSH, bem como a atividade das enzimas SOD, Cat, GPx e GST. Além disso, o HEBT corrigiu os níveis de colesterol (CHO) e triglicerídeos (TG), HDL e LDL plasmáticos, normalizou os níveis de TG, HDL e LDL hepáticos e aumentou a excreção fecal de TG. O HEBT também reverteu alterações histológicas e ultraestruturais induzidas pelo etanol e normalizou a expressão dos genes *Cyp2e1*, *Nrf2* e *Scd1*. Adicionalmente, úlceras induzidas por uso agudo ou crônico de etanol e por ácido acético, ligadura do piloro e motilidade gastrointestinal foram avaliados em ratos e camundongos a fim de examinar a atividade gastroprotetora do HEBT. O extrato preveniu a ulceração aguda e crônica, diminuindo significativamente a área da lesão induzida por etanol e ácido acético, mas não protegeu contra a depleção de muco. Além disso, o HEBT não alterou o volume e acidez gástricos. Histologicamente, o tratamento acelerou a cicatrização, refletida pela contração da base da úlcera. A atividade antiulcerogênica do HEBT pode ser atribuída, em partes, à inibição da geração de radicais livres e consequente prevenção da lipoperoxidação, promovida pelos ácidos cafeilquínicos, componentes principais do extrato. Nenhum sinal de toxicidade foi observado. Nossos resultados indicam que o HEBT possui efeitos hepato- e gastroprotetores e que pode ser uma terapia promissora para o tratamento de doenças hepáticas e gástricas decorrentes do consumo do etanol.

ABSTRACT

An estimated 2 billion people consume ethanol worldwide, and 76.3 million have ethanol-related disorders. Among them, alcoholic fatty liver disease (AFLD), early stages of alcoholic liver diseases, is directly related to oxidative stress and lipogenesis disruption. Regarding gastric ulcer, it is a multifactorial process that occurs through an imbalance between aggressive and protective factors. These injuries deserve special attention due the absence of preconized treatment, or even the vast amount of side effects observed. Searching for a new alternative treatment, we investigated the pharmacologic activity of *Baccharis trimera* ("carqueja"), a plant popularly used for gastrointestinal disorders, in gastric lesions models and AFLD. The hydroethanolic extract (HEBT) was obtained from the plant aerial parts and characterized by high-performance liquid chromatography. To investigate the pharmacologic HEBT activity against AFLD, we submitted mice to 10% ethanol ingestion and low-protein diet, during 6 weeks. In the last 2 weeks, mice were treated with HEBT (30 $mg.kg^{-1}$, p.o.). The oxidative stress induced by ethanol was reversed by HEBT, which normalized LPO, total ROS and GSH levels, as well as SOD, Cat, GPx and GST activity. Beside this, HEBT corrected plasmatic cholesterol (CHO), triglycerides (TG), HDL and LDL levels, normalized hepatic TG, HDL and LDL levels and increased fecal TG excretion. HEBT also reverted histologic and ultrastructural alterations induced by ethanol and normalized *Cyp2e1*, *Nrf2* e *Scd1* gene expression. Additionally, gastric ulcers induced by acute or chronic ethanol or acetic acid consumption, pylorus ligature and gastrointestinal motility were evaluated in mice and rats to examine HEBT gastrointestinal protective effects. HEBT prevented acute and chronic gastric ulceration, decreasing significantly the lesion area induced by ethanol and acetic acid but not protect against mucus depletion. Besides this, HEBT did not altered gastric volume and acidity. Histologically, HEBT accelerated the healing, reflected by contractions of the ulcer base. HEBT antiulcerogenic activity may be partially attributable to the inhibition of free radical generation and subsequent prevention of lipid peroxidation, promoted by caffeoylquinic acids, the major components of extract. No signs of toxicity were observed. Our results indicate that HEBT have hepatic and gastroprotective effects and may be a promising therapy for hepatic and gastric disorders, due to ethanol consumption.

1. INTRODUÇÃO

1.1 Etanol: do consumo ao desenvolvimento de doenças gástricas e hepáticas

Consumir etanol é um hábito em muitas culturas e seu abuso é comum em todo o mundo, sendo considerado um problema de saúde pública mundial (U.S. Department of Health and Human Services, 2010). Os efeitos nocivos do consumo de etanol, em especial o consumo pesado e crônico, estão bem estabelecidos e são causa e/ou fator de risco para mais de 60 tipos de doença, tonando o alcoolismo o terceiro principal fator de risco mundial para morbidades “evitáveis”, invalidez e mortalidade. Estima-se que o uso prejudicial da substância resulta em aproximadamente 2,5 milhões de mortes a cada ano, grande parte em decorrência de doenças hepáticas alcoólicas (Massey et al., 2015; Who, 2011).

Dentre os muitos sistemas que medeiam os efeitos do etanol sobre o organismo e sua homeostase, o trato gastrointestinal (TGI) desempenha um papel particularmente importante. Vários processos estão envolvidos nesta resposta: *1)* é no TGI que ocorre a absorção e a conversão do etanol para compostos mais tóxicos e deletérios, como o acetaldeído; *2)* O contato direto do etanol com a mucosa do TGI superior pode induzir diversas alterações metabólicas e funcionais que levam a acentuado dano de mucosa, resultando em diversas doenças agudas e/ou crônicas, como hemorragia e diarreia, por exemplo; *3)* Alterações funcionais e danos na mucosa intestinal alteram a digestão de nutrientes, bem como sua utilização pelo organismo, contribuindo assim para a desnutrição e perda de peso observada em indivíduos alcoolistas; *4)* As lesões induzidas pelo etanol na mucosa do intestino delgado permitem o translocamento de grandes moléculas, como endotoxinas e outras toxinas bacterianas, que desta maneira atingem mais rapidamente a circulação sanguínea e linfática. Tais moléculas têm efeitos bastante deletérios, especialmente sobre o fígado (Bode & Bode, 1997).

Ao longo das últimas décadas, até os dias de hoje, as pesquisas estão voltadas à elucidação dos mecanismos através dos quais o consumo agudo e

crônico do etanol pode afetar a estrutura e a função do TGI, com atenção especial para as ações gástricas e hepáticas (Bode & Bode, 1997).

1.2 Etanol e úlcera gástrica

Apesar da associação entre a ingestão excessiva de etanol e o risco de sangramento gástrico datar de 170 anos, os efeitos nocivos do etanol sobre o TGI superior têm sido sistematicamente estudados apenas nos últimos 15 anos (Rocco et al., 2014). Tanto o consumo agudo quanto o consumo crônico da substância podem interferir com a funcionalidade do estômago através de múltiplos e complexos mecanismos, dependendo tanto do contato direto do etanol e seu metabólito acetaldeído com a mucosa, quanto dos componentes não alcoólicos das bebidas, como produtos da fermentação, por exemplo (Bode & Bode, 1997; Rocco et al., 2014). Estes mecanismos resultam em *1)* inflamação da mucosa esofagiana e gástrica; *2)* modificação da pressão do esfíncter e falha na motilidade; e *3)* alteração da produção ácida gástrica. Entretanto, todos estes mecanismos são dose-dependentes e reversíveis com abstinência (Rocco et al., 2014; Teyssen & Singer, 2003).

Úlceras gástricas são enfermidades comuns do TGI que afetam milhares de pessoas mundialmente (O'Malley, 2003). A ocorrência dessas lesões desencadeada pelo consumo de etanol é relatada há mais de 40 anos e o alcoolismo é considerado um fator de risco independente para a instalação e complicações de úlceras gástricas (Birdane et al., 2007; Robert, 1972). Entretanto, os mecanismos através dos quais o etanol danifica a mucosa gástrica ainda não estão totalmente elucidados. A ingestão de etanol pode causar edema, erosão, hemorragia e necrose por afetar diretamente a mucosa gástrica e assim afetar a capacidade de defesa da mucosa contra o ácido gástrico, a bile e as enzimas digestivas (Robert, 1972; Rocco et al., 2014). Estudos recentes demonstram que a alteração da microcirculação gástrica acompanhada pelo aumento dos níveis plasmáticos de endotelina (ET-1) e diminuição dos níveis de óxido nítrico (NO) e prostaglandina E2 (PGE2) podem contribuir criticamente para o dano da mucosa gástrica (Ning et al., 2012). O NO e a PGE2 intensificam a microcirculação gástrica, promovem a secreção de

bicarbonato, medeiam a resposta imune, aumentam a síntese de proteínas e a renovação celular e, desta forma, aprimoram a capacidade de reparação tecidual (Ning et al., 2012). Em contrapartida, a ET-1 exerce uma atividade vasoconstritora gástrica bastante acentuada. Estudos prévios relatam elevados níveis plasmáticos de ET-1 e diminuição dos níveis de NO e PGE2 em ratos expostos a um consumo alto de etanol, quando comparados com animais basais (Lazaratos et al., 2001), sugerindo que os danos de mucosa e a diminuição da capacidade de reparação são consequência da estimulação de ET-1 e da inibição e síntese de NO e PGE2 (Lazaratos et al., 2001).

Em relação às alterações que o etanol provoca sobre o esvaziamento gástrico, as pesquisas são contraditórias, e dependem da dose e do tipo de bebida ingerida. De fato, o esvaziamento gástrico parece ser acelerado após a ingestão de baixas doses de etanol, enquanto doses mais elevadas atrasam o esvaziamento e reduzem a motilidade (Bujanda, 2000). Entretanto, neste cenário, o excesso de produção de NO é apontado por diversas pesquisas como a principal causa dos distúrbios de motilidade gastrintestinal relacionadas ao consumo de etanol (Bagyánszki et al., 2011; Mashimo et al., 1996). Adicionalmente, o etanol pode afetar a secreção ácida gástrica. Baixas doses de etanol estimulam a secreção gástrica enquanto que elevadas doses podem ou não exercer efeito inibitório sobre a secreção (Teyssen & Singer, 2003). Efeitos contraditórios similares também são relatados quanto aos efeitos do etanol sobre a regulação celular endócrina de secreção ácida, promovida pelas células G. Enquanto alguns pesquisadores apontam diminuição do número de células G e aumento dos níveis plasmáticos de gastrina após o consumo crônico de etanol, outros grupos relatam que o consumo agudo ou crônico de etanol não afeta número de células G ou os níveis plasmáticos de gastrina (Koko et al., 1998; Todorović et al., 2008; Yamada et al., 1998).

Os sinais clínicos da úlcera gástrica causam bastante desconforto ao paciente e incluem dor e queimação epigástrica que ocorrem à noite ou quando o estômago está vazio e são aliviados com alimentação ou uso de antiácidos (Fendrick et al., 2005). O diagnóstico da doença é feito com base no histórico do paciente e através de endoscopia do TGI alto (Fendrick et al., 2005).

Dentre os fármacos utilizados para o tratamento de úlceras gástricas, destacam-se os antibióticos para a erradicação de *Helicobacter* pylori, retirada de antinflamatórios não esteroidais e as drogas antiácidas, como os antagonistas do receptor tipo 2 da histamina e os inibidores da bomba de prótons (Kangwan et al., 2014). Entretanto, esta abordagem terapêutica é insuficiente para a completa cicatrização da úlcera e está diretamente associada à recorrência da lesão (Kangwan et al., 2014). Além disso, diversos efeitos colaterais como osteoporose, hipergastrinemia e desenvolvimento de tumores carcinoides são relatados (DeVault & Talley, 2009; Eom et al., 2011; Penston & Wormsley, 1987; Poynter et al., 1985; Sheen & Triadafilopoulos, 2011). Neste sentido, é cada vez maior o interesse por novos agentes farmacológicos que tenham ação antiulcerogênica e apresentem menos efeitos adversos.

1.3 Etanol e esteatose hepática alcoólica

O fígado é o principal órgão biotransformador do etanol e consequentemente um dos principais alvos de lesões induzidas por tal substância. As doenças hepáticas alcoólicas (DHA) estão listadas entre as 20 maiores causas de morte mundiais e também são responsáveis por elevada morbidade (Rehm & Shield, 2013). Nos Estados Unidos estima-se que 1 a cada 3 transplantes hepáticos ocorram em consequência da DHA (Singal et al., 2013). O risco de desenvolvimento da enfermidade aumenta de maneira dose e tempo-dependente e a susceptibilidade hepática para os efeitos tóxicos mediados pelo etanol é resultado das elevadas concentrações sanguíneas portais (*versus* sistêmica) da substância e das alterações metabólicas provenientes de seu metabolismo (Massey et al., 2015).

As DHA induzidas pelo consumo crônico de etanol são caracterizadas por um amplo espectro de lesões (Liu, 2014). A alteração mais precoce é a esteatose hepática alcoólica (EHA), popularmente conhecida como “fígado gorduroso”, que ocorre em cerca de 80% de alcoolistas que consomem acima de 80 g de etanol por dia (Levene & Goldin, 2012). Destes indivíduos, aproximadamente 20 a 40% progridem para o estágio seguinte, a

esteatohepatite, que é caracterizada por inflamação e morte celular (Day, 2002; Levene & Goldin, 2012). Aproximadamente 40% dos indivíduos com esteatohepatite desenvolvem necroinflamação e fibrose (Levene & Goldin, 2012) e cerca de 10% desenvolvem cirrose, o estágio final da DHA (Bellentani et al., 1997; Friedman, 2000; Levene & Goldin, 2012). Já o hepatocarcinoma celular ocorre em aproximadamente 1 a 2% dos indivíduos cirróticos (Seitz & Stickel, 2010).

A EHA é uma condição predominantemente assintomática (exceto pela hepatomegalia) e reversível com abstinência, porém, é um fator de risco à progressão para estágios mais avançados da doença, como fibrose e cirrose, especialmente em pacientes não abstêmios (Beckingham, 2001). Histologicamente, a doença é caracterizada pelo acúmulo de lipídeos, em especial triglicerídeos e colesterol, no parênquima hepático, sendo a zona 3 (região perivenular) a mais afetada devido à atividade metabólica mais elevada (Liu, 2014).

Dentre os diversos fatores que favorecem a instalação e/ou progressão da EHA, destacam-se: a *1)* alteração no estado redox provocada pela metabolização do etanol, *2)* o estresse oxidativo, apontado por muitos pesquisadores como a força motriz para a instalação e progressão da DHA, e *3)* o desbalanço da homeostase lipídica (Sozio & Crabb, 2008; Szabo & Mandrekar, 2010). A hepatotoxicidade provocada pelo etanol pode ser direta ou indireta, através dos metabólitos provenientes de sua oxidação. Inicialmente, a enzima álcool desidrogenase (ALD) biotransforma o etanol à acetaldeído, que então é convertido a acetato pela enzima aldeído desidrogenase (ALDH; Ceni et al., 2014; Wilfred de Alwis, 2007). Durante o metabolismo, a nicotinamida adenina dinucleotídeo (NAD^+) é utilizada como cofator, ocasionando assim um acúmulo de nicotinamida adenina dinucleotídeo reduzida (NADH) e consequente redução da razão NAD^+/NADH (Ceni et al., 2014). Como resultado, diversas vias metabólicas, como o ciclo do ácido cítrico e a oxidação de ácidos graxos, são impactadas e favorecem a instalação da EHA (Ceni et al., 2014). Além disso, o acetaldeído altera a homeostase lipídica, através da indução da síntese e da diminuição da oxidação de lipídeos; e altera a permeabilidade da mucosa intestinal, permitindo a translocação de

endotoxinas, com consequente indução das células de Kupffer e produção de espécies reativas de oxigênio (ERO) (Ceni et al., 2014). Ademais, há envolvimento do citocromo P450 2E1 (CYP2E1) na biotransformação do etanol, promovendo a conversão do etanol a acetaldeído e em seguida a acetato, em uma reação bastante nociva que gera diversas ERO, como superóxido, peróxido de hidrogênio e radicais hidroxietil (Cederbaum, 2006; Terelius et al., 1991; Wu et al., 1998).

Outro mecanismo clássico através do qual o etanol promove hepatotoxicidade é a indução de estresse oxidativo, que é definido, em um conceito mais contemporâneo, como uma interrupção do controle e da sinalização do estado redox celular (Jones, 2006; Nagata et al., 2007). A oxidação do etanol através de CYP2E1, distúrbio na cadeia respiratória mitocondrial, ativação das células de Kupffer, desequilíbrio na lipogênese e produção de citocinas são mecanismos através dos quais o etanol induz um ambiente celular pró-oxidante (Albano et al., 1996; Bailey et al., 2009; Nagata et al., 2007). Entretanto, para combater as diferentes ERO geradas e manter a homeostase celular, diversos sistemas antioxidantes enzimáticos e não enzimáticos são estimulados. Dentre as principais enzimas antioxidantes destacam-se a superóxido dismutase (SOD), a catalase (Cat) e o sistema da glutationa, que inclui a glutationa reduzida (GSH), peroxidase (GPx) e S-transferase (GST; Ha et al., 2010). Quando o sistema antioxidante falha em combater ou converter as ERO em espécies reativas menos nocivas pode ocorrer lesão celular, especialmente em nível de membrana celular, refletida por aumento da lipoperoxidação (LPO; de Groot, 1994). A LPO é provavelmente a reação mais relevante associada à hepatotoxicidade induzida pelo etanol, uma vez que as membranas e as organelas subcelulares são os maiores alvos dos radicais lipídicos, produtos da LPO (Albano, 2002; Nagata et al., 2007).

Finalmente, a esteatose hepática é resultado do desequilíbrio do metabolismo lipídico, refletido pelo aumento da síntese e pela diminuição da oxidação de lipídeos, especialmente triglicerídeos. Dentre os mecanismos envolvidos na quebra da homeostase lipídica, está o aumento da razão NAD^+/NADH (Ceni et al., 2014), aumento da atividade da proteína de ligação

ao elemento regulador de esterol-1 (SREBP-1), diminuição da atividade de receptores ativados pelo proliferador de peroxissoma-*α* (PPAR-α) e diminuição da atividade da proteína quinase dependente do AMP cíclico (AMPK; Gao & Bataller, 2011; Violet et al., 2009; You et al., 2002).

O diagnóstico da doença é baseado no relato do paciente de consumo de etanol por períodos prolongados, aumento dos níveis plasmáticos das enzimas γ-glutamiltransferase (γ-GT), aspartato e alanina aminotransferase (AST e ALT, respectivamente), além de exames de imagem, como ultrassonografia e tomografia computadorizada, que indicam hepatomegalia e presença de esteatose (European Association for the Study of the liver, 2012; Menon et al., 2005).

Em relação ao tratamento, não há uma terapia singular capaz de atuar em todas as vias envolvidas na patogênese da EHA. Dentre as estratégias utilizadas para o tratamento, destacam-se a abstinência, mudanças de estilo de vida e uso de antioxidantes (Brown, 2011; Dixit et al., 2007; Frazier et al., 2009; Ratziu et al., 2015; Samuhasaneeto et al., 2007; Sodem et al., 2007; Tsukamoto et al., 2009). Entretanto, tais estratégias não são totalmente eficazes. Assim, pesquisas por novos agentes farmacológicos capazes de atuar em todas as vias envolvidas na instalação e/ou progressão desta enfermidade são extremamente necessárias, a fim de reverter a lesão tecidual e evitar a progressão para estágios mais severos da doença hepática alcoólica.

1.4 *Baccharis trimera* e suas implicações terapêuticas

O potencial das plantas como fonte de novas drogas ainda oferece grande campo para investigação científica, pois das cerca de 250 a 500 mil espécies conhecidas, somente uma pequena porcentagem foi investigada fitoquimicamente e apenas uma fração destas já foi avaliada quanto ao potencial farmacológico (Rates, 2001). Além disso, um grande número de espécies com uso medicinal tradicional ainda continua sem comprovação da eficácia e da segurança de seu uso (Ruiz et al., 2008).

Uma planta com intenso uso na medicina popular no Brasil e América do Sul e com reputação atrelada à ação antiácida e antiulcerogênica é a *Baccharis trimera* (Less.) DC, da família Asteraceae (Biondo et al., 2011). Conhecida popularmente como “carqueja”, a *Baccharis trimera* é uma das 120 espécies do gênero *Baccharis* encontradas no Brasil (Verdi et al., 2005). As partes aéreas da planta são utilizadas na medicina tradicional sob a forma de infusão, decocção ou tinturas para os mais diversos fins, como problemas hepáticos, digestivos, malária, diabetes, anemia, diarreia, inflamações urinárias, verminoses, (Verdi et al., 2005), hipercolesterolemia, disfunção erétil e reumatismo (Alonso, 1998). Algumas de suas atividades biológicas, como antihepatotóxica, antidiabética, antioxidante, antinociceptiva, antinflamatória e antiulcerogênica já foram relatadas e são atribuídas aos flavonoides, diterpenos, triterpenos, saponinas, óleos essenciais e ácidos cafeilquínicos presentes na planta (Biondo et al., 2011; Gené et al., 1996; Lorenzi & Matos, 2002; Oliveira et al., 2005; Paiva et al., 2015; Simões-Pires et al., 2005; Soicke & Leng-Peschlow, 1987).

Entretanto, os resultados encontrados na literatura são insuficientes para elucidar os mecanismos gastro- e hepatoprotetores atribuídos a essa planta, em especial os relacionados aos sistemas redox e antioxidante, que estão fortemente envolvidos na patogênese das doenças hepáticas alcoólicas e da úlcera gástrica induzida pelo consumo prolongado de etanol. Além disso, não há pesquisas especificamente apontando o potencial da *B. trimera* como agente farmacológico para o tratamento da esteatose hepática alcoólica, o que motivou este estudo.

2. OBJETIVOS

2.1. Objetivo geral

Investigar a atividade farmacológica hepato- e gastroprotetora do extrato hidroetanólico da *Baccharis trimera* (HEBT) frente a diversos modelos de lesão hepática e gástrica.

2.2. Objetivos específicos

1. No modelo de estudo de esteatose hepática alcoólica (EHA):

- ✓ Investigar a ação hepatoprotetora do HEBT;
- ✓ Avaliar alterações histológicas e o possível efeito benéfico do HEBT, através das técnicas de coloração por Hematoxilina/Eosina e Azul do Nilo;
- ✓ Examinar alterações ultraestruturais, através de microscopia eletrônica de transmissão;
- ✓ Dosar colesterol, triglicerídeos, lipoproteína de alta densidade e lipoproteína de baixa densidade plasmáticos, hepáticos e fecais, nos diferentes grupos experimentais;
- ✓ Dosar os níveis plasmáticos de aspartato aminotransferase, alanina aminotransferase, glicose, amilase, albumina, proteínas totais, creatinina e ureia;
- ✓ Explorar o sistema antioxidante e o envolvimento do estresse oxidativo na EHA, através da avaliação da atividade das enzimas superóxido dismutase (SOD), catalase (Cat), glutationa peroxidase e S-transferase (GPx e GST, respectivamente), bem como dos níveis de glutationa reduzida (GSH), espécies reativas de oxigênio totais e lipoperoxidação (LOOH);
- ✓ Pesquisar o efeito do HEBT sobre a expressão de genes envolvidos no metabolismo hepático, no sistema antioxidante e na lipogênese, especificamente *Cyp2e1*, *Nrf2* e *Scd1*.

2. Nos modelos de estudo de lesão gástrica:

- ✓ Investigar os efeitos farmacológicos do HEBT frente ao modelo de úlcera gástrica aguda, induzida por etanol;
- ✓ Analisar a ação farmacológica do HEBT em um modelo de úlcera gástrica crônica, induzida por ácido acético;
- ✓ Explorar a atividade farmacológica gástrica do HEBT em um modelo de consumo prolongado de etanol;
- ✓ Induzir hipersecreção gástrica através da ligadura do piloro e investigar a ação do HEBT sobre a secreção e o pH gástrico;
- ✓ Determinar quais são os efeitos do HEBT sobre a motilidade gastrointestinal;
- ✓ Investigar a capacidade antioxidante *in vitro* do HEBT, através da avaliação do sequestro do radical livre estável DPPH; e *in vivo* através da atividade das enzimas SOD e GST, bem como dos níveis de GSH e LOOH;
- ✓ Examinar a ação do HEBT sobre a produção de muco gástrico;
- ✓ Analisar modificações histológicas induzidas pela administração de etanol e ácido acético, através da coloração com Hematoxilina/Eosina;
- ✓ Verificar a toxicidade do HEBT através da determinação da dose letal 50 (DL_{50}).

3. ARTIGO CIENTÍFICO 1 – “Molecular basis of alcoholic fatty liver disease: from incidence to treatment.”

Artigo publicado na revista ***Hepatology Research*****, 46(1): 111-123, 2016.**

Molecular basis of alcoholic fatty liver disease: from incidence to treatment

Francislaine AR Lívero[a], Alexandra Acco[a]

[a] Department of Pharmacology, Federal University of Paraná, Curitiba, PR, Brazil

Abstract

Alcoholic liver diseases have complex and multiple pathogenic mechanisms but still no effective treatment. Steatosis or alcoholic fatty liver disease (AFLD) has a widespread incidence and is the first step in the progression to more severe stages of alcoholic liver disease, with concomitant increases in morbidity and mortality rates. The ways in which this progression occurs and why some individuals are susceptible are still unanswered scientific questions. Research with animal models and clinical evidence have shown that it is a multifactorial disease that involves interactions between lipid metabolism, inflammation, the immune response, and oxidative stress. Each of these pathways provides a better understanding of the pathogenesis of AFLD and contributes to the development of therapeutic strategies. This review emphasizes the importance of research on alcoholic steatosis based on incidence data, key pathogenic mechanisms, and therapeutic interventions and discusses perspectives on the progression of this disease.

Key words: alcohol, alcoholic fatty liver disease, ethanol, pathogenesis, steatosis, treatment.

1. General Background

Alcohol is a psychoactive substance whose consumption and health problems associated with it are widely prevalent around the world. The burden of alcohol-related disease and mortality remains alarming in most countries.[1] Harmful alcohol use ranks among the world's five largest risk factors for disease development, disability, and death worldwide.[2] In fact, the World Health Organization estimated that alcohol consumption was responsible for 3.3 million deaths in 2013.[3] This corresponds to 5.9% of all deaths or one of every 20 deaths worldwide (7.6% for men, 4.0% for women). Alcohol use can also have social and economic consequences for individuals other than the drinker and society as a whole.[4,5] Several social, environmental, and individual factors, such as culture, the availability of alcohol, public policy, age, gender, family risk factors, socioeconomic status, and culture, are relevant when explaining differences in vulnerability to alcohol-related problems between societies and individuals.[6-8]

Alcohol consumption can be directly responsible for the development of a disease state *per se* or indirectly contribute to the onset and progression of other disorders. Such harmful effects of alcohol are determined in three spheres that are related to drinking: (1) initially, the volume of alcohol consumed (e.g., for all alcohol-attributable cancers, there is a dose-dependent relationship), (2) consumption pattern (e.g., chronic consumption or heavy episodic drinking), and (3) the quality of the beverage ingested (e.g., homemade or illegally produced alcoholic beverages contaminated with very toxic substances).[9-12] More than 200 health conditions have been associated with alcohol consumption. Among these are neuropsychiatric conditions, gastrointestinal diseases, cancers, intentional injuries, unintentional injuries, cardiovascular disease, fetal alcohol syndrome, diabetes mellitus, infectious disease, and liver diseases, which have a very strong relationship with alcohol consumption.[12-14]

Alcoholic liver disease (ALD) is one of the major chronic liver diseases. It is highly prevalent and listed among the top 20 causes of death worldwide.[15,16] The number of patients with ALD around the world is unclear and probably underestimated. In the United States, the incidence may exceed 2 million cases.[17] Alcoholic liver disease comprises a broad clinical and histological spectrum. One associated condition is alcoholic fatty liver disease (AFLD),

namely steatosis, which is reversible with abstinence and/or improvements in lifestyle. Severe alcoholic hepatitis (AH) and fibrosis can also occur, which may or may not improve with abstinence. Another related disorder is alcoholic cirrhosis (AC). This is the end stage of ALD, an irreversible disease with an unfavorable prognosis.[18] Patients with AH and AC present mortality of 65% in a period of 4 years and can die within the first months, which makes the prognosis for this disease more threatening than many frequent cancers, including colon cancer, breast cancer, and prostate cancer.[19] Moreover, continued alcohol use combined with “second hits” may further increase the risk of hepatocellular carcinoma.[20] Figure 1 shows the steps of ALD progression.

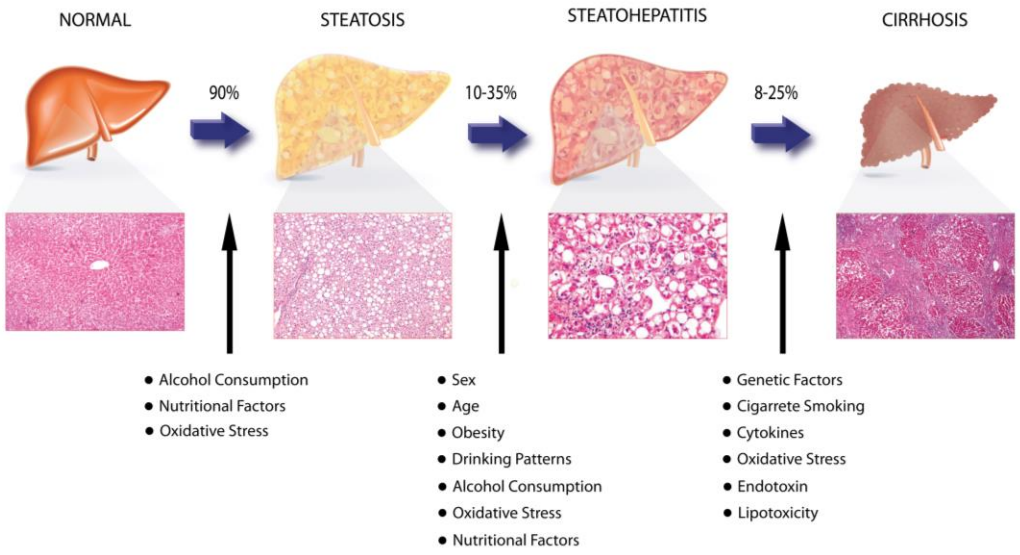

Figure 1. Steps of macro and microscopy alcoholic liver disease progression. ALD comprises a broad clinical and histological spectrum. Most of drinkers develop AFLD, but only some of them develop advanced ASH and progresses to AC. Factors like alcohol consumption, drinking patterns, nutrition, cigarette smoking, obesity, sex, age, genetic factors, oxidative stress, cytokines, endotoxin and lipotoxicity are trigger points for disease progression. Histologically, steatosis is defined as accumulation of fat molecules in droplets within hepatocytes, steatohepatitis is steatosis associated with an intense inflammatory process and cirrhosis is the end stage, with intense fibrosis and diffuse nodular formation. Abbreviations: ALD, alcoholic liver disease; AFLD, alcoholic fatty liver disease; ASH, alcoholic steatohepatitis; AC, alcoholic cirrhosis.

Approximately 90% of heavy drinkers develop steatosis, but only some of them (35%) develop advanced ALD. This indicates two important points: (1) other factors, like gender, age, obesity, drinking patterns, dietary factors, genetic factors, and cigarette smoking, are involved, and (2) there are possibilities to intervene in the initial/reversible stages of the disease to prevent

its progression to more severe stages.[20-22] Considering the high incidence of steatosis, which is the initial stage of ALD, and lack of approved treatments, AFLD is an interesting research field that may lead to the development of therapeutic strategies that can lessen the profound health and economic impact of this disease. The present review discusses the pathogenesis of AFLD and possible therapeutic interventions.

2. AFLD Definition

One of the best-known biological effects of significant alcohol intake is the induction of fatty liver disease.[23] AFLD is the first response of the liver to alcohol abuse. It is usually asymptomatic, except for hepatomegaly. It is defined histologically by the accumulation of fat molecules in both small (microvesicular) and large (macrovesicular) droplets within hepatocytes as a result of increased intracytoplasmic triglyceride formation.[18,24]

3. Pathogenesis

Studies in humans and rodents began to reveal the biological effects of alcohol on liver in the 1960s.[25] The original hypothesis to explain this effect introduced redox shifts that are generated by the oxidation of alcohol by alcohol and aldehyde dehydrogenases, oxidative stress, and the mobilization of peripheral triglycerides from adipose tissue to the liver.[26] Subsequent research showed that these mechanisms are insufficient to explain the initial theory. With regard to lipid accumulation in hepatocytes, various mechanisms have been described. Alcohol may cause steatosis by the induction of tumor necrosis factor-α (TNF-α), a decrease in fatty acid oxidation, and an increase in lipogenesis in hepatocytes. Cytokines can impair the transport and secretion of triglycerides. Sterol regulatory element-binding protein-1 (SREBP-1) and adenosine monophosphate activated protein kinase (AMPK) are two main lipogenic signaling pathways in the liver that are affected by alcohol.[18] Several factors have been proposed to explain the pathogenesis of AFLD (Figure 2), which are discussed below.

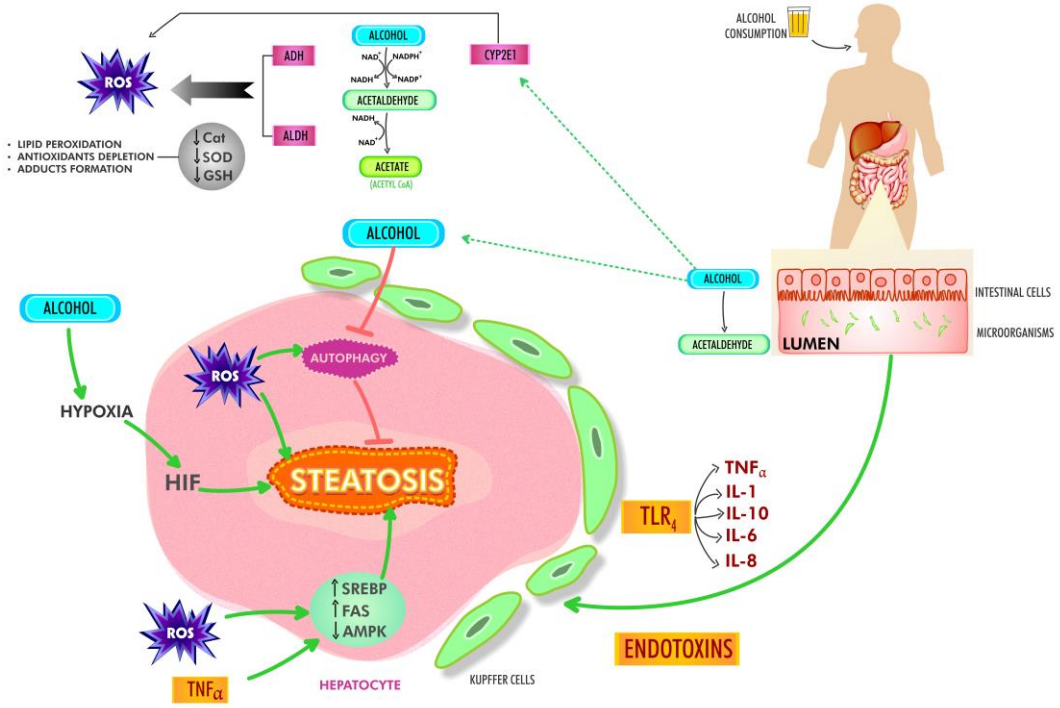

Figure 2. Pathogenesis of AFLD. Alcohol can exert hepatotoxicity directly or indirectly through its metabolites. After consumption, ADH and CYP2E1 oxidize alcohol to acetaldehyde that is converted to acetate by ALDH and introduced into the citric acid cycle as acetyl-CoA. ROS are generated in several steps of alcohol metabolism and is highly deleterious since inhibit the antioxidant capacity of the hepatocyte, decreasing Cat, SOD and GSH or increasing lipid peroxidation and adducts formation. Alcohol increases the permeability of intestinal mucosa and sensitizes Kupffer cells to activation by endotoxins via TLR4. As consequence, increased production of TNF-□, ROS, IL-1, IL-6, IL-8 and IL-10 occurs, which contribute to the onset of AFLD because cytokines can impair the transport and secretion of triglycerides. Alcohol also may cause steatosis decreasing fatty acid oxidation and increasing lipogenesis in hepatocytes. SREBP-1, FAS and AMPK are two main lipogenic signaling pathways affected by alcohol. Beside this, acute alcohol intake generates ROS that can activate autophagy that prevents lipid accumulation in early stages of ALD. However, chronic alcohol intake inhibits autophagy resulting in steatosis. Ultimately, HIFs activation occurs during periods of cellular hypoxia induced by chronic alcohol consumption, resulting in steatosis. Abbreviations: AFLD, alcoholic fatty liver disease; ADH, alcohol dehydrogenase; CYP2E1, cytochrome P450 2E1; ALDH, aldehyde dehydrogenase; ROS, reactive oxygen species; Cat, catalase; SOD, superoxide dismutase; GSH, reduced glutathione; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha; IL, interleukin; SREBP-1, sterol regulatory element-binding protein-1; FAS, fatty acid synthetase; AMPK, adenosine monophosphate activated protein kinase; ALD, alcoholic liver disease; HIFs, hypoxia inducible factors.

3.1. *Oxidative stress*

A classic mechanism of alcohol hepatotoxicity is its ability to induce free radical formation and consequent oxidative stress.[27] Free radicals are molecules or molecular fragments that contain one or more unpaired electrons in atomic or molecular orbitals that are able to induce oxidative stress.[28] In a more contemporary definition, oxidative stress is the “disruption of redox

signaling and control".[29] Hepatocytes have various potential sources of reactive oxygen species (ROS), which are induced or altered by chronic alcohol consumption, leading to an increase in the production of oxidants.[29] Among these are oxidation that is induced by CYP2E1, the mitochondrial respiratory chain, the cytosolic enzyme aldehyde oxidase, Kupffer cell activation, lipogenesis disruption, and cytokine production.[27,31]

Given that ROS production is a natural and persistent process, several enzymatic and non-enzymatic antioxidant systems are linked in the cellular protection. These include superoxide dismutase (SOD; which detoxifies the superoxide anion), catalase (Cat), and glutathione peroxidase (GPx), whose function is to detoxify cellular peroxides. Moreover, reduced glutathione (GSH), ubiquinone, and vitamins A, C, and E, which are low-molecular-weight non-enzymatic antioxidants, also comprise this system.[32] Although most ROS are converted to less reactive species or water by antioxidant systems before they can cause cellular damage, some of them can induce cellular injury, including lipid peroxidation (LPO), enzyme inactivation, and DNA mutations.[33] Lipid peroxidation is probably the most relevant reaction that is associated with alcohol-induced hepatotoxicity because biomembranes and subcellular organelles are the major sites of lipid peroxide damage.[27,34] Lívero et al. reported increasing levels of LPO in mice that developed steatosis after 6-week feeding with 10% alcohol and a low-protein diet. The same was observed with Cat and SOD activity, including increased levels of total ROS, indicating that oxidative stress contributed to the establishment of steatosis in that model.[35] In a binge model, acute alcohol drinking also increased LPO and induced hepatic steatosis in mice. Treatment with cannabidiol, which has been reported to function as an antioxidant, protected the liver from alcohol-generated oxidative stress-induced steatosis.[36] Tsedensodnom et al. found that ascorbic acid and *N*-acetylcysteine (NAC) blocked steatosis, and low doses of H_2O_2 and alcohol synergistically interact to cause hepatocyte dysfunction.[37]

3.2. *Alcohol metabolism*

Alcohol can exert hepatotoxicity directly or indirectly through its metabolites. In recent decades, significant progress has been made in understanding the molecular mechanisms by which alcohol-related hepatotoxic mechanisms

contribute to the onset and progression of disease.[38] The oxidation of alcohol is a two-step process that involves the enzyme alcohol dehydrogenase (ADH), which converts alcohol to acetaldehyde, which is then oxidized to acetate by aldehyde dehydrogenase (ALDH), with nicotinamide adenine dinucleotide (NAD^+) as a cofactor.[39] During alcohol metabolism, reduced nicotinamide adenine dinucleotide (NADH) accumulation occurs, with a consequent reduction of the NAD^+/NADH ratio. This reduction has an important impact on several biochemical pathways, such as glycolysis, the citric acid cycle, fatty acid oxidation, and glucogenesis.[39] In parallel, acetaldehyde has several hepatotoxic effects and alters hepatic lipid homeostasis, decreasing the transcriptional activity of peroxisome proliferator-activated receptors (PPARs) and increasing sterol regulatory element binding protein (SREBP) activity by an AMPK-dependent mechanism.[39] Furthermore, acetaldehyde alters the intestinal barrier and promotes endotoxin translocation and the consequent induction of Kupffer cells to release ROS, cytokines, and chemokines.[39]

Cytochrome P450 (CYP450) is another metabolic system that connected with alcohol metabolism. CYP2E1 catalyzes the oxidation of alcohol to acetaldehyde and can also catalyze the oxidation of acetaldehyde to acetate in a harmful reaction that generates several ROS, such as superoxide, hydrogen peroxide, hydroxyl radicals, and hydroxyethyl radicals.[40-42] Passeri et al. found that 32 h of continuous exposure to 2% alcohol in zebrafish caused steatosis and hepatomegaly through alcohol metabolism and oxidative stress.[43] Homologous ADH and CYP2E1 are expressed in the zebrafish liver and metabolize alcohol, leading to hepatic damage, reflected by changes in hepatic gene expression and steatosis intensity.[37]

Catalase is an additional metabolic pathway attached with the oxidation of alcohol. In the liver, Cat plays no significant function, but in the brain, it is closely related to the metabolism of alcohol to acetaldehyde, which appears to have a function in alcohol tolerance and addiction.[44,45]

3.3. *Nuclear receptors and lipid homeostasis*

Nuclear receptors (NRs) belong to a family with 48 members of ligand-activated transcriptional factors and have an important regulatory function in several physiological, developmental, and toxicological processes.[46,47] Much

evidence suggests that some members of this family contribute to the control of drug disposition over the synchronized regulation of genes that are linked in hepatic uptake, phase I and phase II metabolism, the excretion of lipids, and bile acid homeostasis.[48] NRs have been identified as lipid sensors. Their activation induces a metabolic cascade that maintains lipid homeostasis at the level of the transcription of genes that are involved in lipid metabolism, storage, transport, and elimination.[47]

Nuclear receptor activity is controlled by the intracellular concentration of their specific ligands. Of these, bile acids are critical regulators of lipid metabolism and essential for lipid absorption and cholesterol homeostasis.[49] Several studies have suggested that the farnesoid X receptor (FXR; a bile acid receptor) plays a central function in hepatic lipid metabolism through the regulation of its related target genes.[35,50] The activation of FXRs by small heterodimer partner (SHP) downregulates the liver X receptor (LXR) and its target genes, SREBP-1C and fatty acid synthetase (FAS), which inhibit the synthesis of triglycerides and promote the degradation of triglycerides, respectively. Liver X receptors inhibit fatty acid oxidation by activating PPAR-α. Thus, FXRs play an essential role in triglyceride metabolism.[50]

Several studies have indicated that alcohol intake may directly or indirectly regulate lipid metabolism athwart the upregulation of SREBP-1c and downregulation of PPAR-α.[21,51] Alcohol ingestion downregulates AMPK, which in turn inactivates acetyl CoA carboxylase, leading to a reduction of fatty acid synthesis and an increase in fatty acid oxidation over its effects on malonyl-CoA and carnitine palmitoyltransferase, promoting steatosis.[52] Therefore, AMPK is a key element in the regulation of cellular energy homeostasis by limiting anabolic pathways and facilitating catabolic pathways.[39] Additionally, the contribution of PPAR-α to fatty acid homeostasis has been clearly demonstrated in PPAR-α knockout mice that lack the ability to increase rates of fatty acid oxidation.[53] Clearly, PPAR-α is emerging as a pivotal player in fatty acid metabolism.[54]

Acting strictly in parallel to PPARs, SREBPs comprise a family of transcriptional factors that bind sterol regulatory element and control several enzymes that are involved in the synthesis of fatty acids.[39,55,56] SREBP-2 regulates gene-encoding proteins that are linked with cholesterol metabolism.[55]

SREBP-1 (including SREBP-1a, SREBP-1c, and SREBP-2), the dominant form in cell lines, regulates gene-encoding proteins that are attached in both cholesterol genesis and lipogenesis.[55,56] Lívero et al. reported an increase in the expression of SREBP-1 mRNA in mice that were fed 10% alcohol and a low-protein diet, and these mice presented intense hepatic steatosis.[35] Other researchers found that SREBP-1a overexpression in the liver is associated with high rates of fatty acid biosynthesis and the development of fatty liver. Ablation of the SREBP-1 gene results in the low expression of lipogenic genes.[55-57]

3.4. *Cytokine modulation and innate immunity*

Cytokines are soluble molecules that are produced by a wide range of cells in the body, including the major types of hepatic cells. They are attached in intercellular communication processes and mediate diverse fundamental biological activities, such as body growth, hematopoiesis, adiposity, lactation, inflammation, and immunity.[58] In most tissue, including the liver, the constitutive production of cytokines is minimal or absent, but pathologic and/or physiologic stimuli can activate cells, leading to an increase in the production of these effector molecules and consequently tissue responses.[59] Hepatocytes are targets of cytokine toxicity and also an increasingly recognized source of cytokine production.[19] Although cytokines are essential for liver regeneration that is caused by injury, they may also play an important channel in the development and progression of ALD by the stimulation of inflammation, necrosis, apoptosis, and fibrosis.[59, 60]

Alcohol consumption increases the permeability of intestinal mucosa and sensitizes Kupffer cells to activation by endotoxins via toll-like receptor 4. Deleterious paracrine effects of Kupffer cells activation include ROS or TNF-α, interleukin-6 (IL-6), and IL-10-mediated damage to endothelial cells, which contribute to the onset and/or progression of ALD.[61-64] Clinical and experimental approaches were used by Li et al. to evaluate whether steatosis has inflammatory biomarkers. These researchers found that serum TNF-α levels were significantly higher in the steatosis group, coinciding with an increase in the severity of histological liver lesions.[65] Furthermore, alcohol metabolism by ADH and CYP2E1 leads to acetaldehyde production, which interacts with proteins, forms protein adducts, and stimulates cytokine production.[66]

Additionally, ROS formation as a consequence of CYP2E1 metabolism and antioxidant depletion, especially GSH, significantly contributes to the production of cytokines that precipitate cellular apoptosis mechanisms.[66]

The initiation of ALD is associated with an increase in the levels of proinflammatory cytokines, including IL-1, IL-6, IL-8, and TNF-α, and decreases in the production of protective antiinflammatory cytokines, such as IL-10, by monocytes and Kupffer cells. In addition to its anti-fibrotic effects, IL-10 plays an important role in modulating the effects of TNF-α.[19] IL-6, IL-10, and IL-22 appear to play a protective function in ameliorating AFLD over the activation of *signal transducer and activator of transcription 3 (*STAT-3).[63,67] STAT-3 is a cell survival signal and appears to be linked in protection against hepatocellular damage in many models of liver injury.[68] IL-6 has been reported to be an important factor that induces an acute phase response, liver regeneration, and hepatoprotection.[68] Elevations of IL-6 associated with ALD may play a compensatory role in preventing hepatocellular damage in AFLD.[63] Increasing evidence suggests that IL-22 plays an important function in preventing T-cell hepatitis, improving fatty liver, and stimulating liver recovery.[69-71]

Chronic alcohol consumption inhibits autophagy, an essential process that attenuates lipid accumulation in hepatocytes.[72,73] However, as mentioned above, acute alcohol intake generates ROS that can activate autophagy, thus indicating that this compensatory function can prevent lipid droplet congestion in early stages of ALD.[74]

3.5. *Methionine-folate cycle*

Several studies have indicated that alterations of the methionine-folate cycle can contribute to the development of ALD.[75-77] Methionine, a sulphur-containing essential amino acid, exerts its metabolic effects athwart its conversion to *S*-adenosylmethionine (SAM) in a methionine adenosyltransferase-dependent process.[78] *S*-adenosylmethionine is a methionine metabolite that plays an important role in many vital functions and cell survival processes. For example, SAM is a precursor of polyamines and glutathione.[79] Hepatic SAM depletion is associated with early and more advanced stages of ALD and a reduction of the formation of glutathione and polyamines. It also affects the methylation of RNA, DNA, and proteins.[79-82] Alcohol consumption suppresses methionine

adenosyltransferase, decreases the hepatic concentrations of betaine and folate (i.e., an endogenous precursor of methionine), and increases circulating levels of homocysteine.[83,84]

3.6. *Centrilobular hypoxia*

Chronic alcohol consumption leads to cell death in hepatic oxygen-poor pericentral regions, both in humans and animal models.[85] During periods of cellular hypoxia, hepatocytes adapt to consume less oxygen and activate gene transcription to regulate glucose uptake and metabolism, erythropoiesis, angiogenesis, cell death, and cell proliferation.[86] When oxygen concentrations are low, hypoxia inducible factors (HIFs) are activated. HIF-1 is a heterodimeric protein complex that has three subunits (HIF-1α, HIF-2α, and HIF-3α). The redox-sensitive HIF-1α subunit is considered the major regulator of O_2 tension-sensitive genes in cells.[87] HIF-2α has been shown to play a prominent route in regulating hepatic lipid metabolism.[88] Increases in hepatic HIF-1α and HIF-2α expression occur with both acute and chronic alcohol ingestion in mice, and this may explain why steatosis occurs early in hepatocytes of zone 3 (perivenular). It can also affect zone 2 and even zone 1 (periportal) when liver injury is more severe.[21]

Some authors have reported that hepatocyte-specific HIF-1α knockout mice had more severe steatosis than wildtype mice after 6% alcohol exposure for 4 weeks.[89] In contrast, Nath et al. reported that hepatocyte-specific HIF-1α knockout mice were protected against steatosis after 5% alcohol exposure over the same period of time.[90] To resolve these disparate findings, Ni et al. recently demonstrated that hepatocyte-specific HIF-1β knockout mice were resistant to alcohol-induced steatosis, thus providing evidence to support the detrimental involvement of alcohol-induced HIF-1 activation in ALD.[86] These findings may also help clarify previously conflicting findings in hepatocyte-specific HIF-1α knockout mice. Besides these alterations at hepatocytes after exposure to toxins and development of coagulative necrosis and neutrophilic inflammation, sinusoidal cells also participate of pathogenesis.[64] The most important changes like cellular swelling, blood cell aggregation and microcirculation disturbance occurs initially at sinusoidal endothelium.[64]

4. Clinical signs and diagnosis

The clinical distinction between AFLD and nonalcoholic fatty liver disease (NAFLD) is a great challenge. The misclassification of disease due to difficulties in gathering patient information and molecular process are analyzed individually rather than together. Thus, the liver disorders are rarely treated as complex diseases that really are.[91] AFLD and NAFLD have histological and clinical similarity and in some situations the differential diagnosis of them may be difficult because some patients do not report alcoholism or under-report their use.[92] Knowledge of whether patients ingest alcohol in an abusive manner is essential to determine the presence of ALD.

4.1. *Histological features*

Histological diagnosis of ALD requires liver biopsy, which can be done percutaneously.[93] Four types of histopathological lesions determine ALD: a) steatosis; b) ballooning, c) inflammatory infiltrate and d) variable degree of fibrosis and change in parenchymal structure.[94] Because liver biopsy is an invasive procedure with significant morbidity it is recommend only for patients with suspect of more severe forms of ALD providing a better prediction of the patients outcome.[93]

4.2. *Clinical diagnosis*

AFLD is predominantly an asymptomatic disease, but some clinical aspects of steatohepatitis (e.g., fever, anorexia, cachexia, neutrophilia, and hypoalbuminemia) have been linked to abnormal serum TNF-α levels.[62] The diagnosis of ALD is suspected upon relate of excess alcohol consumption and the presence of clinical abnormalities suggestive of liver injury.[93] Because of the difficulty to obtaining an accurate historic of alcohol consumption, several biochemical blood markers are used to detect alcohol use and liver damage. Increased aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (γ-GT), and mean corpuscular volume (MCV) can indicate early stages of ALD whereas decreased albumin, prolonged prothrombin time and increased bilirubin level or thrombocytopenia show more aggressive stages.[93] A parameter that can be used to differential diagnosis between NAFLD and AFLD is the AST/ALT ratio, since values below 1 strongly suggest

NAFLD and values above 1 indicate AFLD.[92] In AFLD, transaminase levels are elevated more than 5 to 10 times the normal value and AST values is generally higher than ALT level.[95]

4.3. *Hepatic imaging tests*

In addition to hepatic biomarkers, the presence of lipid infiltrates on radiological images (e.g., ultrasonography and computed tomography) and hepatomegaly may indicate the presence of steatosis.[95] Liver biopsy is generally unnecessary for diagnosis, however can be useful to determine the degree of ALD and to exclude the presence of AC.[95] This technique reveals polymorphonuclear infiltrates, centrilobular hepatocyte swelling and degeneration, macro and microvesicular steatosis, Mallory bodies and pericentral-perisinusoidal fibrosis.[95]

5. Treatment

An ideal pharmacological treatment for AFLD would reduce inflammatory parameters, oxidative stress, and lipid accumulation and prevent fibrotic events. However, developing such a drug that is able to acting on so many different pathways is extremely difficult. For this reason, no single drug therapy has been developed, but combination therapies have been devised in an attempt to reverse hepatocyte injury. Among the strategies for the treatment of hepatic steatosis are lifestyle changes that seek to decrease alcohol consumption, smoking, and obesity and promote adequate nutrition. Because of the lack of clinical trials that have evaluated the efficacy of pharmacological agents for the treatment of AFLD, we discuss possible therapies based on animal researches and some clinical trials.

5.1. *Lifestyle changes and diet*

The first step in AFLD treatment is the cessation of alcohol use because persistent alcohol intake is the most causal risk factor for the progression of ALD.[96] Abstinence at any stage of ALD dramatically changes the prognosis. The early identification of alcoholics and early classification of their liver disease may aid in ceasing or reducing alcohol consumption.[97]

Because of the vast number of mechanisms that are connected in the pathogenesis of AFLD, the diagnosis and treatment of comorbid diseases and symptoms, such as malnutrition, can be difficult. This is extremely important because of the high incidence of malnutrition among these patients.[98] The nutritional status of alcoholics is hindered by primary malnutrition (e.g., anorexia, resulting in lower food intake) and secondary malnutrition (e.g., alterations in intestinal mucosa that result in the poor absorption and digestion of nutrients).[78,99] Regular and chronic alcohol consumers are usually overweight because of the added calories from alcohol. These individuals substitute nutrients with calories from alcohol, that are considered “empty calories” that are devoid of biological value.[78] Moreover, alcohol consumption profoundly affects the metabolism of macro- and micronutrients, decreases the uptake of amino acids, decreases the synthesis and secretion of proteins (lipoproteins, albumin, and fibrinogen) by the liver, and increases protein catabolism by the intestines through intense cellular regeneration.[100] Correcting nutritional intake positively affects the production of proinflammatory cytokines because it helps maintain the integrity of the intestinal mucosa, which prevents the translocation of endotoxins.[19] Furthermore, increases in the levels of antioxidants from an adequate diet potentially decrease cytokine production over the blockade of oxidative stress.[19] Deficiencies in micronutrients, such as thiamine, folic acid, methionine, and pyridoxine, are found in almost all alcoholics. This imbalance should be corrected because nutritional support can improve liver function and prevent progression of the disease.

Other lifestyle changes can significantly contribute to reversing alcohol-induced liver damage. Obesity, which itself can cause non-alcoholic fatty liver disease, acts as an independent risk factor that negatively affects the intensity of damage. Furthermore, smoking has been associated with an increase in the risk of developing ALD and progression of the disease to more severe forms.[20]

5.2. *Antioxidants*

Owing to the great importance of oxidative stress in the pathogenesis of AFLD, several studies have focused on the use of antioxidants to prevent oxidative damage and improve liver function. Investigators have tested the effects of the antioxidant vitamin E on hepatic lipid accumulation. Vitamin E is

the best-researched fat-soluble compound with protective effects on lipid membranes and unsaturated fatty acids. It also provides protection against oxidative damage that is induced by free radicals.[101,102] In addition to its antioxidant properties, vitamin E protects the liver by blocking intrinsic apoptotic pathways or mitochondrial toxicity or downregulating inflammatory mediators that depend of nuclear factor-κB.[102-104] Kaur et al. evaluated the effects of vitamin E on molecular mechanisms associated with alcohol-induced oxidative stress in mice. Vitamin E supplementation restored redox status, reduced apoptosis, and prevented oxidative stress, the major cause of alcohol hepatotoxicity in this model.[105] Although many studies have indicated beneficial effects of vitamin E in animal models, human trials have not been encouraging. Mezei et al. related that 1000 I.U. vitamin E per day, for 3 months, in patients with mild to moderate alcoholic hepatitis, improves serum hyaluronic acid but has no beneficial effects on tests of liver function. [106] However, studies evaluating non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) indicates that vitamin E supplementation had a positive effect in the ballooning degeneration, improvement of steatosis, lobular inflammation and fibrosis. [107]

Another compound with well-documented antioxidant effects is silymarin, which is obtained from *Silybum marianum* (milk thistle), an edible plant and one of the most popular forms of alternative medicinal therapies for liver injury.[108,109] The beneficial effects of silymarin in ALD can be explained by its antioxidant, antiinflammatory, immunomodulatory, and anti-fibrotic properties.[110] As discussed above, alcohol consumption decreases the hepatic levels of GSH and increases AST, ALT, and γ-GT levels. Many studies have found that silymarin decreases LPO, reduces liver alterations, and retards the development of fibrosis in both acute and chronic models of alcohol consumption.[110-112] However, other researchers failed to show promising results in patients with more severe forms of ALD.[113-115] The effect of silymarin on liver function in patients with drug-induced elevation of ALT, AST and γ-GT was evaluated in a non-interventional study. Treatment with silymarin for 2 or 3 months was considered safe, efficacious and promoted benefit in terms of liver-related symptoms and quality of life.[116] Regardless the actions of silymarin on

ALD, the results are controversial. It was reported no changes in the evolution and mortality of patients that received treatment for 15 months[117]. However, increase of GSH and decrease of LPO levels in patients with alcoholic liver cirrhosis treated with silymarin for 6 months were also found.[114]

A potent antioxidant that is used for steatosis treatment is *N*-acetylcysteine (NAC), a precursor of GSH.[118] The rationale for using NAC only in early stages of ALD is based on the key involvement of oxidative stress in this disorder. NAC stimulates GSH synthesis, increases GST activity and detoxification, and interacts with ROS to scavenge free radicals.[119,120] Evidence from animal models suggests that NAC is a potent hepatic antioxidant that abolishes LPO, depletes GSH, and stimulates the formation of protein adducts after chronic alcohol exposure.[121,122] However, these benefits have not been observed in more severe stages of ALD, when used alone or in combination with corticosteroids.[123,124]

5.3. Emerging therapies

5.3.1. Folate, betaine, and metadoxine

Deficiencies in folate, which plays an important channel in homocysteine metabolism, are also involved in the pathogenesis of ALD.[125] Folate administration increases the conversion of homocysteine to methionine in the hepatic methionine cycle, thus stimulating the synthesis of SAM to restore glutathione levels.[126] In mice, SAM treatment was connected with the recovery of mitochondrial glutathione concentrations, reduction of LPO, and significantly reduction of steatosis and ALT levels.[127]

Betaine (trimethylglycine) is an indispensable nutrient from foods or dietary supplements. As a methyl donor, betaine provides a methyl group to homocysteine to form methionine.[79] In a Lieber-DeCarli alcohol-containing diet model, betaine administration for 2-4 weeks attenuated fatty liver, reduced homocysteine concentrations, and elevated SAM concentrations.[128]

Metadoxine, also known as pyridoxine-pyrrolidone carboxylatethe, is a synthetic drug, the plasma concentrations of which can be four- to five-times higher than conventional pyridoxine.[126] Metadoxine helps restore NAD, ATP,

glutathione, and adenosine concentrations in the liver and brain and acts in ALD by decreasing alcohol levels and acetaldehyde accumulation.[126] In a double-blind randomized study, 136 patients who were diagnosed with alcoholic liver steatosis received 150 mg metadoxine for 3 months. After 1 month of treatment, considerable decreases in AST, ALT, and γ-GT were observed, with a concomitant reduction of steatosis signals on ultrasonography.[129]

5.3.2. Nuclear receptor modulators

Given their importance in various metabolic pathways, NRs have been the subject of research and an attractive target for drug discovery.[130] FXRs and PPARs are ligand-regulated transcriptional factors that are responsible for several regulatory effects on glucose, bile acids, and lipid homeostasis. A large number of synthetic FXR agonists are being tested for the treatment of lipid-related diseases.[131,132] Manley et al. reported that FXRs are essential for protection against acute alcohol-related hepatotoxicity. FXR knockout mice had higher ALT and hepatic triglyceride levels and presented impairments in autophagy compared with wildtype animals.[133] After 6 weeks of alcohol exposure and a low-protein diet, the FXR agonist 6-ECDCA reversed alcohol-induced increases in ALT, AST, triglycerides, and cholesterol in mice. 6-ECDCA also acted against oxidative stress and hepatic triglyceride and cholesterol accumulation, significantly reducing AFLD.[35]

Zhang et al. described the protective effects of berberine, an AMPK regulator, against alcohol-induce oxidative stress and steatosis in mice. Blunted hepatic lipid accumulation, a decrease in oxidative stress by a reduction of LPO, GSH depletion, and mitochondrial oxidative damage were found and attributed to the restoration of PPAR-α by berberine.[134]

5.3.3. Cytokine modulation

Increasing evidence indicates that IL-22 plays a role in homeostasis, the control of bacterial infection, tissue repair, and fatty liver improvements, and it has been proposed to be a possible therapeutic target.[70,135,136] Ki et al. (2010) reported that IL-22 treatment ameliorated alcohol-induced liver injury in a murine model, decreased AST, ALT, and hepatic triglyceride levels, increased

the hepatic expression of antimicrobial genes, prevented LPO, and restored GSH levels, thus suggesting its therapeutic use in ALD.[67]

6. Concluding Remarks

In summary, alcohol consumption can lead to alcoholic hepatic steatosis. This condition occurs worldwide, has a high incidence, and is associated with socioeconomic costs that can be compounded by morbidity and progression to more severe stages of ALD. Among the pathophysiological mechanisms are alcohol metabolism, oxidative stress, the modulation of lipogenic genes, the modulation of cytokines, and centrilobular hypoxia. Despite the well-known pathophysiology of the disease and advances in the search for new treatments, no approved pharmacological treatments are available. Therefore, prevention, abstinence, and lifestyle changes remain the pillars of treatment to reverse hepatic lipid accumulation. Further investigations in the field are encouraged to elucidate the overall pathogenesis of AFLD, pharmacological targets, and new therapies.

References

1. World Health Organization. The global status report on alcohol and health 2011. Geneva. 2011.
2. Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; **380**: 2224−2260.
3. World Health Organization. Global Health Estimates 2013: Deaths by Cause, Age and Sex, by Country, 2000-2012. Geneva. 2014.
4. Anderson P, Baumberg B. Alcohol in Europe – A public health perspective. A report for the European Commission. England: Institute of Alcohol Studies. 2006.
5. Sacks JJ, Roeber J, Bouchery EE, Gonzales K, Chaloupka FJ, Brewer R. State costs of excessive alcohol consumption, 2006. *Am J Preventive Med* 2013; **45**: 474–85.
6. World Health Organization. WHO Expert Committee on Problems Related to Alcohol Consumption. Second report, Geneva. 2007.
7. Babor T, Caetano R, Casswell S, Edwards G, Giesbrecht N, Graham K, et al. Alcohol: No Ordinary Commodity—Research and Public Policy. Oxford, UK: Oxford University Press. 2010.

8. Nelson DE, Jarman DW, Rehm J, Greenfield TK, Rey G, Kerr WC. Alcohol-attributable cancer deaths and years of potential life lost in the United States. *Am J Public Health* 2003; **103**: 641–648.
9. Rehm J, Room R, Graham K, Monteiro M, Gmel G, Sempos CT. The relationship of average volume of alcohol consumption and patterns of drinking to burden of disease − An overview. *Addiction* 2003; **98**: 1209−1228.
10. Rehm J, Kanteres F, Lachenmeier DW. Unrecorded consumption, quality of alcohol and health consequences. *Drug Alcohol Rev* 2010; **29**: 426−36.
11. World Health Organization. Global Strategy to reduce the harmful use of alcohol 2010. Geneva. 2010.
12. Shield KD, Parry C, Rehm J. Chronic diseases and conditions related to alcohol use. *Alcohol Research Current Reviews* 2013; **35**: 155–171.
13. World Health Organization. WHO Statistical Classification of Diseases and Related Health Problems (ICD) 10th revision. Geneva. 1992.
14. Rehm J, Baliunas D, Borges GL, Graham K, Irving H, Kehoe T, et al. The relation between different dimensions of alcohol consumption and burden of disease − an overview. *Addiction* 2010; **105**: 817−43.
15. Xie Y, Feng B, Gao Y, Wei L. Characteristics of alcoholic fatty liver disease and predictive factors for mortality of patients with alcoholic cirrhosis. *Hepatobiliary Pacreat Dis Int* 2013; **12**: 594-601.
16. Rehm J, Shield, KD. Alcohol and mortality: global alcohol-attributable deaths from cancer, liver cirrhosis and injury in 2010. *Alcohol Research: Current Reviews* 2013; **35**, 174−183.
17. Arteel G, Marsano L, Mendez C, Bentley F, McClain CJ. Advances in alcoholic liver disease. *Best Pract Res Clin Gastroenterol* 2003; **17**: 625–647.
18. Szabo G, Mandrekar P. Focus on: alcohol and liver. *Alcohol Res Health* 2010; **33**: 87-96.
19. McClain CJ, Zhenyuan S, Shirish SB, Hill DB, Deaciuc I. Recent Advances in Alcoholic Liver Disease IV. Dysregulated cytokine metabolism in alcoholic liver disease. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: 497–502.
20. Tsukamoto H, Machida K, Dynnyk A, Mkrtchyan H. “Second hit” models of alcoholic liver disease. *Semin Liver Dis* 2009; **29**: 178–187.
21. Gao B, Bataller R. Alcoholic fatty liver disease: Pathogenesis and new therapeutic targets. *Gastroenterology* 2011; **141**: 1572-1585.
22. O’Shea RS, Dasarathy S, McCullough AJ. Alcoholic liver disease. *Hepatology* 2010; **51**: 307–328.
23. Sozio M, Crabb DW. Alcohol and lipid metabolism. *Am J Physiol Endocrinol Metab* 2008; **295**: 10-16.
24. Orman ES, Odena G, Bataller R. Alcoholic liver disease: Pathogenesis, management, and novel targets for therapy. *J Gastroenterol Hepatol* 2013; **28**: 77-84.
25. Lieber CS, Jones DP, Decarli LM. Effects of prolonged ethanol intake: production of fatty liver despite adequate diets. *J Clin Invest* 1965; **44**: 1009–1021.
26. Baraona E, Lieber CS. Effects of ethanol on lipid metabolism. *J Lipid Res* 1979; **20**: 289–315.

27. Nagata K, Suzuki H, Sakaguchi S. Common pathogenic mechanism in development progression of liver injury caused by non-alcoholic or alcoholic steatohepatitis. *J Toxicol Sci* 2007; **32**: 453-468.
28. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd edn. Oxford University Press, 1999.
29. Jones, DP. Redefining oxidative stress. *Antioxid Redox Signal* 2006; **8**: 1865-1879.
30. Bailey SM, Mantena SK, Millender-Swain T, Cakir Y, Jhala NC, Chhieng D, et al. Ethanol and tobacco smoke increase hepatic steatosis and hypoxia in the hypercholesterolemic ApoE-/- mouse: Implications for a "multihit" hypothesis of fatty liver disease. *Free Rad Biol Med* 2009; **46**: 928-938.
31. Albano E, Clot P, Morimoto M, Tomasi A, Ingelman-Sundberg M, French SW. Role of cytochrome P4502E1-dependent formation of hydroxyethyl free radicals in the development of liver damage in rats intragastrically fed with ethanol. *Hepatology* 1996; **23**: 155–163.
32. Ha HL, Shin HJ, Feitelson MA, Yu DY. Oxidative stress and antioxidants in hepatic pathogenesis. *World J Gastroenterol* 2010; **16**: 6035-6043.
33. de Groot H. Reactive oxygen species in tissue injury. *Hepatogastroenterology* 1994; **41**: 328-332.
34. Albano E. Free radical mechanisms in immune reactions associated with alcoholic liver disease. *Free Radic Biol Med* 2002; **32**: 110-114.
35. Lívero FAR, Stolf AM, Dreifuss AA, Bastos-Pereira AL, Chicorski R, Oliveira LG, et al. The FXR agonist 6ECDCA reduces hepatic steatosis and oxidative stress induced by ethanol and low protein diet in mice. Chem Biol Interact 2014; **217**: 19-27.
36. Yang L, Rozenfeld R, Wu D, Devi LA, Zhang Z, Cederbaum A. Cannabidiol protects liver from binge alcohol-induced steatosis by mechanisms including inhibition of oxidative stress and increase in autophagy. *Free Radic Biol Med* 2014; **68**: 260–267.
37. Tsedensodnom O, Vacaru AM, Howarth DL, Yin C, Sadler KC. Ethanol metabolism and oxidative stress are required for unfolded protein response activation and steatosis in zebrafish with alcoholic liver disease. *Dis Model Mech* 2013; **6**: 1213-1226.
38. Wilfred de Alwis NM, Day CP. Genetics of alcoholic liver disease and nonalcoholic fatty liver disease. *Semin Liver Dis* 2007; **27**: 44-54.
39. Ceni E, Mello T, Galli A. Pathogenesis of alcoholic liver disease: Role of oxidative metabolism. *World J Gastroenterol* 2014; **20**: 17756-17772.
40. Terelius Y, Norsten-Höög C, Cronholm T, Ingelman-Sundberg M. Acetaldehyde as a substrate for ethanol-inducible cytochrome P450 (CYP2E1). *Biochem Biophys Res Commun* 1991; **179**: 689-694.
41. Wu YS, Salmela KS, Lieber CS. Microsomal acetaldehyde oxidation is negligible in the presence of ethanol. *Alcohol Clin Exp Res* 1998; **22**: 1165-1169.
42. Cederbaum AI. CYP2E1--biochemical and toxicological aspects and role in alcohol-induced liver injury. *Mt Sinai J Med* 2006; **73**: 657-672.
43. Passeri MJ, Cinaroglu A, Gao C, Sadler KC. Hepatic Steatosis in Response to Acute Alcohol Exposure in Zebrafish Requires Sterol

Regulatory Element Binding Protein Activation. *Hepatology* 2009; **49**: 443-452.
44. Zimatkin SM, Liopo AV, Deitrich RA. Distribution and kinetics of ethanol metabolism in rat brain. *Alcohol Clin Exp Res* 1998; **22**: 1623-1627.
45. Deng XS, Deitrich RA. Putative role of brain acetaldehyde in ethanol addiction. *Curr Drug Abuse Rev* 2008; **1**: 3-8.
46. Laudet, V. Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *J Mol Endocrinol* 1997; **19**: 207–226.
47. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. Nuclear receptors and lipid physiology: opening the X-files. *Science* 2001; **294**: 1866-1870.
48. Zollner G, Wagner M, Trauner M. Nuclear receptors as drug targets in cholestasis and drug-induced hepatotoxicity. *Pharmacol Ther* 2010; **126**: 228-243.
49. Jiang C, Xie C, Li F, Zhang L, Nichols RG, Krausz KW, et al. Intestinal farnesoid X receptor signaling promotes nonalcoholic fatty liver disease. *J Clin Invest* 2015; **125**: 386–402.
50. Yang Z-X, Shen W, Sun H. Effects of nuclear receptor FXR on the regulation of liver lipid metabolism in patients with non-alcoholic fatty liver disease. *Hepatol Int* 2010; **4**: 741–748.
51. You M, Fischer M, Deeg MA, Crabb DW. Ethanol induces fatty acid synthesis pathways by activation of sterol regulatory element-binding protein (SREBP). *J Biol Chem* 2002; **277**: 29342–29347.
52. Viollet B, Guigas B, Leclerc J, Hébrard S, Lantier L, Mounier R, et al. AMP-activated protein kinase in the regulation of hepatic energy metabolism: from physiology to therapeutic perspectives. *Acta Physiol (Oxf)* 2009; **196**: 81–98.
53. Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desbvergne B, Wahli W. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* 1999; **103**: 1489–1498.
54. Clarke SD. Nonalcoholic Steatosis and Steatohepatitis. I. Molecular mechanism for polyunsaturated fatty acid regulation of gene transcription. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: 865–869.
55. Brown MS, Goldstein JL. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc Natl Acad Sci* 1999; **96**: 1041–1048.
56. Osborne TF. Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. *J Biol Chem* 2000; **275**: 32379–32282.
57. Duplus E, Glorian M, Forest C. Fatty acid regulation of gene transcription. *J Biol Chem* 2000; **275**: 30749–30752.
58. Tracey KJ, Cerami A. Tumor necrosis factor, other cytokines and disease. *Annu Rev Cell Biol* 1993; **9**: 317-343.
59. Tilg H, Diehl AM. Cytokines in alcoholic and nonalcoholic steatohepatitis. *N Engl J Med* 2000; **343**: 1467-1476.
60. Braunersreuther V, Viviani GL, Mach F, Montecucco F. Role of cytokines and chemokines in non-alcoholic fatty liver disease. *World J Gastroenterol* 2012; **18**: 727-735.

61. McClain JC, Shedlofsky S, Barve S, Hill DB. Cytokines and alcoholic liver disease. *Alcohol Health Res World* 1997; **21**: 317-320.
62. Tilg H, Moschen AR, Kaneider NC. Pathways of liver injury in alcoholic liver disease. *J Hepatol* 2011; **55**: 1159-1161.
63. Miller AM, Horiguchi N, Jeong W-IL, Radaeva S, Gao B. Molecular Mechanisms of Alcoholic Liver Disease: Innate Immunity and Cytokines. *Alcohol Clin Exp Res* 2011; **35**: 787–793.
64. Tsukamoto H, Lu SC. Current concepts in the pathogenesis of alcoholic liver injury. *The FASEB Journal* 2001; **15**: 1335-1349.
65. Li Y, Liu L, Wang B, Wang J, Chen D. Simple steatosis is a more relevant source of serum inflammatory markers than omental adipose tissue. *Clin Res Hepatol Gastroenterol* 2014; **38**: 46-54.
66. Neuman MG. Cytokines - central factors in alcoholic liver disease. *Alcohol Res Health* 2003; **27**: 307-316.
67. Ki SH, Park O, Zheng M, Morales-Ibanez O, Kolls JK, Bataller R, et al. Interleukin-22 treatment ameliorates alcoholic liver injury in a murine model of chronic-binge ethanol feeding: Role of STAT3. *Hepatology* 2010; **52**: 1291–1300.
68. Gao B. Cytokines, STATs and liver disease. *Cell Mol Immunol* 2005; **2**: 92–100.
69. Radaeva S, Sun R, Pan HN, Hong F, Gao B. Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation. *Hepatology* 2004; **39**: 1332–1342.
70. Ren XHB, Colletti LM. IL-22 is involved in liver regeneration after hepatectomy. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**: 74–80.
71. Yang L, Zhang Y, Wang L, Fan F, Zhu L, Li Z, et al. Amelioration of high fat diet induced lipogenesis and hepatic steatosis by interleukin-22. *J Hepatol* 2010; **53**: 339-347.
72. Czaja MJ. Functions of autophagy in hepatic and pancreatic physiology and disease. *Gastroenterology* 2011; **140**: 1895–1908.
73. Donohue TM Jr. Autophagy and ethanol-induced liver injury. *World J Gastroenterol* 2009; **15**: 1178–1185.
74. Ding WX, Li M, Chen X, Ni HM, Lin CW, Gao W, et al. Autophagy reduces acute ethanol-induced hepatotoxicity and steatosis in mice. *Gastroenterology* 2010; **139**: 1740–1752.
75. Halsted CH, Villanueva JA, Devlin AM. Folate deficiency, methionine metabolism, and alcoholic liver disease. *Alcohol* 2002; **27**: 169–172.
76. Lu SC, Tsukamoto H, Mato JM. Role of abnormal methionine metabolism in alcoholic liver injury. *Alcohol* 2002; **27**: 155–62.
77. Mato JM, Cámara J, Fernández de Paz J, Caballeria L, Coll S, Caballero A, et al. S-adenosylmethionine in alcoholic liver cirrhosis: a randomized, placebo-controlled, double-bind, multicenter clinical trial. *J Hepatol* 1999; **30**: 1081-1089.
78. Stickel F, Hoehn B, Schuppan D, Seitz HK. Review article: nutritional therapy in alcoholic liver disease. *Aliment Pharmacol Ther* 2003; **18**: 357–373.
79. Purohit V, Abdelmalek MF, Barve S, Benevenga NJ, Hasteld CH, Kaplowitz N, et al. Role of S-adenosylmethionine, folate, and betaine in

the treatment of alcoholic liver disease: summary of a symposium. *Am J Clin Nutr* 2007; **86**: 14-24.
80. Song Z, Zhou Z, Uriarte S, Wang L, Kang YJ, Chen T, et al. S-Adenosyl homocysteine sensitizes to TNF-alpha hepatotoxicity in mice and liver cells: a possible etiological factor in alcoholic liver disease. *Hepatology* 2004; **40**: 989–97.
81. Lee TD, Sadda MR, Mendler MH, Bottiglieri T, Kanel G, Mato JM, et al. Abnormal hepatic methionine and glutathione metabolism in patients with alcoholic hepatitis. *Alcohol Clin Exp Res* 2004; **28**: 173–81.
82. Martinez-Chantar ML, Garcia-Trevijano ER, Latasa MU, Pérez-Mato I, Sánchez del Pino MM, Corrales FJ, et al. Importance of a deficiency in S-adenosyl-L-methionine synthesis in the pathogenesis of liver injury. *Am J Clin Nutr* 2002; **76**: 1177–1182.
83. Halsted CH, Villanueva J, Chandler CJ, Stabler SP, Allen RH, Muskhelishvili L, et al. Ethanol feeding of micropigs alters methionine metabolism and increases hepatocellular apoptosis and proliferation. *Hepatology* 1996; **23**: 497–505.
84. Villanueva JA, Halsted CH. Hepatic transmethylation reactions in micropigs with alcoholic liver disease. *Hepatology* 2004; **39**: 1303–1310.
85. Arteel GE, Iimuro Y, Yin M, Raleigh JA, Thurman RG. Chronic enteral ethanol treatment causes hypoxia in rat liver tissue in vivo. *Hepatology* 1997; **25**: 920-926.
86. Ni H-M, Bhakta A, Wang S, Li Z, Manley S, Huang H, et al. Role of Hypoxia Inducing Factor1b in Alcohol-Induced Autophagy, Steatosis and Liver Injury in Mice. *Plos One* 2014; **9**: 115849 1-25.
87. Semenza, GL. Hypoxia-inducible factor 1: master regulator of O_2 homeostasis. *Curr Opin Genet Dev* 1998; **8**: 588-594.
88. Rankin EB, Rha J, Selak MA, Unger TL, Keith B, Liu Q, Haase VH. Hypoxia-inducible factor 2 regulates hepatic lipid metabolism. *Mol Cell Biol* 2009; **29**: 4527–4538.
89. Nishiyama Y, Goda N, Kanai M, Niwa D, Osanai K, Yamamoto Y, et al. HIF-1alpha induction suppresses excessive lipid accumulation in alcoholic fatty liver in mice. *J Hepatol* 2012; **56**: 441–447.
90. Nath B, Levin I, Csak T, Petrasek J, Mueller C, Kodys K, et al. Hepatocyte-specific hypoxia-inducible factor-1alpha is a determinant of lipid accumulation and liver injury in alcohol-induced steatosis in mice. *Hepatology* 2011; **53**: 1526–1537.
91. Sookoian S, Pirola CJ. Systems biology elucidates elucidates common pathogenic mechanisms between nonalcoholic and alcoholic-fatty liver disease. *Plos One* 2013; **8**: e58895.
92. Zanin Jr I, Mattos AA, Perin C, Ramos GZ. A importância do índice AST/ALT no diagnóstico da esteatohepatite não alcoólica. *Arq Gastroenterol* 2002; **39**: 22-26.
93. European Association for the Study of the liver. EASL Clinical practical guidelines: management of alcoholic liver disease. *Journal of hepatology* 2012; **57**: 399-420.
94. MacSween RN, Burt AD. Histologic spectrum of alcoholic liver disease. *Semin Liver Dis* 1986; **6**: 221–232.

95. Menon KVN, Gores GJ, Shah VH. Pathogenesis, Diagnosis, and Treatment of Alcoholic Liver Disease. *Mayo Clin Proc* 2001; **76**: 1021-1029.
96. Brown RS Jr. Transplantation for alcoholic hepatitis: time to rethink the 6-month “rule”. *N Engl J Med* 2011; **365**: 1836–8.
97. Bergheim I, McClain CJ, Arteel GE. Treatment for alcoholic liver disease. *Dig Dis* 2005; **23**: 275-284.
98. Carvalho L, Parise ER. Evaluation of nutritional status of nonhospitalized patients with liver cirrhosis. *Arq Gastroenterol* 2006; **43**: 269-274.
99. Seitz HK, Suter PM. Ethanol toxicity and nutritional status. In: Kotsonis, FN, Mackey, MA, eds. Nutritional Toxicology, 2nd edn. New York: Taylor & Francis. 2002, 122–154.
100. Lieber CS. Alcohol: its metabolism and interaction with nutrients. *Ann Rev Nutr* 2000; **20**: 395–430.
101. Ratziu V, Goodman Z, Sanyal A. Current efforts and trends in the treatment of NASH. *Journal of Hepatology* 2015, **62**: 65-75.
102. Soden JS, Devereaux MW, Haas JE, Gumpricht E, Dahl R, Gralla J, et al. Subcutaneous vitamin E ameliorates liver injury in an in vivo model of steatocholestasis. *Hepatology* 2007, **46**: 485-495.
103. Sokol RJ, McKim Jr JM, Goff MC, Ruyle SZ, Devereaux MW, Han D, et al. Vitamin E reduces oxidant injury to mitochondria and the hepatotoxicity of taurochenodeoxycholic acid in the rat. *Gastroenterology* 1998, **114**: 164-174.
104. Morante M, Sandoval J, Gomez-Cabrera MC, Rodriguez JL, Pallardo FV, Vina JR et al. Vitamin E deficiency induces liver nuclear fator-KappaB DNA-binding activity and changes in related genes. *Free Radic Res* 2005; **39**: 1127-1138.
105. Kaur J, Shalini S, Bansal MP. Influence of vitamin E on alcohol-induced changes in antioxidant defenses in mice liver. *Toxicol Mech Methods* 2010; **20**: 82-89.
106. Mezey E, Potter JJ, Rennie-Tankersley L, Caballeria J, Pares A. A randomized placebo controlled trial of vitamin E for alcoholic hepatitis. *J Hepatol* 2004; **41**: 40-46.
107. Xu R, Tao A, Zhang S, Deng Y, Chen G. Association beteween vitamin E and non-alcoholic steatohepatitis: a meta-analysis. *Int J Clin Exp Med* 2015; **8**: 3924:3934.
108. Dixit N, Baboota S, Kohli K, Ahmad S, Ali J. Silymarin: A review of pharmacological aspects and bioavailability enhanced approaches. *Indian J Pharmacol* 2007; **39**: 172-179.
109. Frazier TH, Stocker AM, Kershner NA, Marsano LS, McClain CJ. Treatment of alcoholic liver disease. *Therap Adv Gastroenterol* 2011; **4**: 63-81.
110. Lieber C, Leo MA, Cao Q, Ren C, DeCarli LM. Silymarin retards the progression of alcohol-induced hepatic fibrosis in baboons. *J Clin Gastroenterol* 2003; **37**: 336–339.
111. Wang M, Grange LL, Tao J. Hepatoprotective properties of Silybum marianum herbal formulation on ethanol induced liver damage. *Fitoterapia* 1996; **67**: 167-171.

112. Velenzuela A, Garrido A. Biochemical bases of the pharmacological action of flavanoid silymarin and its structural isomer silibinin. *Biol Res* 1994; **27**: 105-112.
113. Ferenci P, Dragosics B, Dittrich H, Frank H, Benda L, Lochs H, et al. Randomized controlled trial of silymarin treatment in patients with cirrhosis of the liver. *J Hepatol* 1989; **9**: 105–113.
114. Lucena MI, Andrade RJ, de la Cruz JP, Rodriguez-Mendizabal M, Blanco E, Sanchez de la Cuesta F. Effects of silymarin MZ-80 on oxidative stress in patients with alcoholic cirrhosis. Results of a randomized, double-blind, placebo-controlled clinical study. *Int J Clin Pharmacol Ther* 2002; **40**: 2–8.
115. Pares A, Planas R, Torres M, Caballeria J, Viver JM, Acero D, et al. Effects of silymarin in alcoholic patients with cirrhosis of the liver: results of a controlled, double-blind, randomized and multicenter trial. *J Hepatol* 1998; **28**: 615–621.
116. Gillessen A, Herrmann HA, Kemper M, Morath H, Mann K. Effect of silymarin on liver health and quality of life. Results of a non-interventional study. *MMW Fortschr Med* 2014; **156**: 120-126.
117. Bunout D, Hirsch S, Petermann M, de la Maza MP, Silva G, Kelly M, Ugarte G, Iturriaga H. Controlled study of the effect of silymarin on alcoholic liver disease. *Rev Med Chil* 1992; **120**: 1370-1375.
118. Samuhasaneeto S, Thong-Ngam D, Kulaputana O, Patumraj S, Klaikeaw N. Effects of N-acetylcysteine on oxidative stress in rats with non-alcoholic steatohepatitis. *J Med Assoc Thai* 2007; **90**: 788-797.
119. De Vries N, De Flora S. N-acetyl-l-cysteine. *J Cell Biochem Suppl* 1993; **17**: 270–277.
120. Videla LA, Rodrigo R, Orellana M, Fernandez V, Tapia G, Quinones L, et al. Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. *Clin Sci (Lond)* 2004; **106**: 261–268.
121. Ronis MJJ, Butura A, Sampey BP, Shankar K, Prior RL, Korourian S, et al. Effects of N-acetylcysteine on ethanol-induced hepatotoxicity in rats fed via total enteral nutrition. *Free Radic Biol Med* 2005; **39**: 619–630.
122. Ozaras R, Tahan V, Aydin S, Uzun H, Kaya S, Senturk H. N-acetylcysteine attenuates alcohol-induced oxidative stress in the rat. *World J Gastroenterol* 2003; **9**:125-128.
123. Nguyen-Khac E, Thevenot T, Piquet M-A, Benferhat S, Chatelain D, Tramier B, et al. Glucocorticoids plus N-Acetylcysteine in Severe Alcoholic Hepatitis*. N Engl J Med* 2011; **365**: 1781-1789.
124. Stewart S, Prince M, Bassendine M, Hudson M, James O, Jones D, Record C, Day CP. A randomized trial of antioxidant therapy alone or with corticosteroids in acute alcoholic hepatitis. J Hepatol 2007; **47**: 277-83.
125. Ji C, Kaplowitz N. Hyperhomocysteinemia, endoplasmic reticulum stress, and alcoholic liver injury. *World J Gastroenterol* 2004; **10**: 1699–708.
126. Váli L, Blázovics A, Fehér J. The therapeutic effect of metadoxine on alcoholic and non-alcoholic steatohepatitis. *Ovr Hetil* 2005; **146**: 2409-2414.

127. Song Z, Zhou Z, Chen T, Hill D, Kang J, Barve S, et al. S-Adenosylmethionine (SAMe) protects against acute alcohol induced hepatotoxicity in mice. *J Nutr Biochem* 2003; **14**: 591–597.
128. Barak AJ, Beckenhauer HC, Badakhsh S, Tuma DJ. The effect of betaine in reversing alcoholic steatosis. *Alcohol Clin Exp Res* 1997; **21**: 1100–1102.
129. Caballeria J, Pares A, Brú C, Mercader J, García Plaza A, Caballeria L, et al. Metadoxine accelerates fatty liver recovery in alcoholic patients: results of a randomized double-blind, placebo-control trial. *J Hepatol* 1998; **28**: 54–60.
130. Wang Y-D, Chen W-D, Moore DD, Huang W. FXR: a metabolic regulator and cell protector. *Cell Research* 2008; **18**: 1087-1095.
131. Fiorucci S, Rizzo G, Antonelli E, Renga B, Mencarelli A, Riccardi L, et al. Cross-Talk between Farnesoid-X-receptor (FXR) and Peroxisome Proliferator-Activated Receptor γ Contributes to the Antifibrotic Activity of FXR Ligands in Rodent Models of Liver Cirrhosis. *J Pharmacol Exp Ther* 2005; **315**: 58-68.
132. Musso G, Gambino R, Cassader M. Emerging Molecular Targets for the Treatment of Nonalcoholic Fatty Liver Disease. *Annu Rev Med* 2010; **61**: 375–392.
133. Manley S, Ni H-M, Williams JA, Kong B, DiTacchio L, Guo G, Ding W-X. Farnesoid X receptor regulates forkhead Box O3a activation in ethanol-induced autophagy and hepatotoxicity. *Redox Biol* 2014; **2**: 991–1002.
134. Zhang P, Ma D, Wang Y, Zhang M, Qiang X, Liao M, Liu X, Wu H, Zhang Y. Berberine protects liver from ethanol-induced oxidative stress and steatosis in mice. *Food Chem Toxicol* 2014; **74**: 225-232.
135. Wolk KWE, Witte K, Warszawska K, Sabat R. Biology of interleukin-22. *Semin Immunopathol* 2010; **32**: 17-31.
136. Aujla SJ, Kolls JK. IL-22: a critical mediator in mucosal host defense. *J Mol Med* 2009; **87**: 451– 454.

4. ARTIGO CIENTÍFICO 2 - “Hydroethanolic extract of *Baccharis trimera* ameliorates alcoholic fatty liver disease in mice.

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Hydroethanolic extract of *Baccharis trimera* ameliorates alcoholic fatty liver disease in mice

Francislaine A. dos Reis Lívero[a], Gracianny Gomes Martins[a], José Ederaldo Queiroz Telles[b], Olair Beltrame[c], Stellee Marcela Petris Biscaia[d], Célia Regina Cavichiolo Franco[d], Ronald P.J. Oude Elferink[e], Alexandra Acco[a]

[a] Department of Pharmacology, Federal University of Paraná, Curitiba, PR, Brazil

[b] Department of Medical Pathology, Federal University of Paraná, Curitiba, PR, Brazil

[c] Department of Veterinary Medicine, Federal University of Paraná, Curitiba, PR, Brazil

[d] Department of Cell Biology, Federal University of Paraná, Curitiba, PR, Brazil

[e]Tytgat Institute for Liver and Intestinal Research, University of Amsterdam, Amsterdam, The Netherlands

Corresponding author:

Alexandra Acco, Federal University of Paraná (UFPR), Biological Science Sector, Department of Pharmacology, Centro Politécnico, Cx. P. 19031, Curitiba – Paraná – Brazil, Zip Code 81531-980

Phone: +55 (41) 3361-1742; Fax: +55 (41) 3266-2042

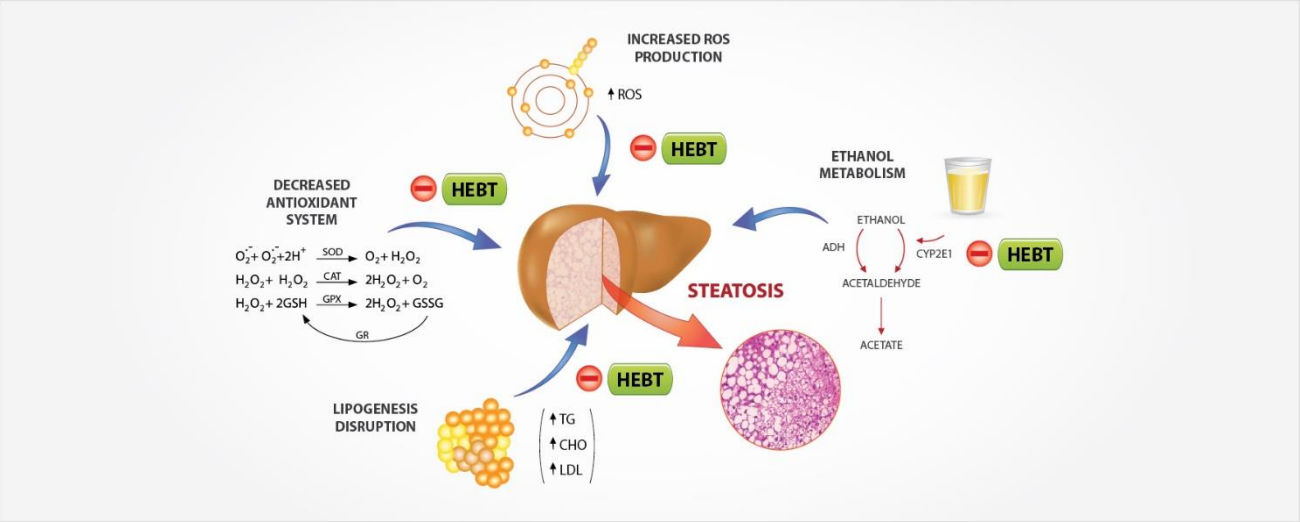
E-mail: aleacco@ufpr.br

Abstract

Ethanol abuse is a serious public health problem and is associated with several alcoholic liver diseases (ALD), with high incidence of morbidity and mortality. Among them, alcoholic fatty liver disease (AFLD), the earliest stage of ALD, is a multifactorial disease that involves especially oxidative stress and lipid metabolism disruption. Although benign and reversible, there is no pharmacological treatment registered for this disease. Thus, we proposed to treat mice bearing-AFLD, induced by 10% ethanol and low protein diet, with oral hydroethanolic extract of *Baccharis trimera* (HEBT; 30 $mg \cdot kg^{-1}$). HEBT was able to reverse the oxidative status induced by ethanol in the liver, reducing the lipoperoxidation, and normalizing GSH level, GPx, GST, SOD and Cat activity, and total ROS levels. The reverser effect of HEBT was observed upon ethanol increased levels of plasmatic and hepatic triglycerides, plasmatic cholesterol and HDL; and plasmatic and hepatic LDL. Moreover, HEBT increased fecal triglycerides. HEBT also reduced the histological lesions in liver provoked by ethanol consumption. Finally, HEBT was able to alter the expression of genes involved in ethanol metabolism, antioxidant system and lipogenesis, such as CYP2E1, Nrf2 and Scd1, respectively. No signs of toxicity were observed in HEBT treated mice. For these reasons, we propose the hydroethanolic extract of *Baccharis trimera* as a promising pharmacological agent for the treatment of alcoholic fatty liver disease.

Key words: liver, hepatology, alcoholic fatty liver, *Baccharis trimera*, carqueja, ethanol.

Graphical abstract



1. *Introduction*

The harmful use of ethanol is one of the risk factors of greatest impact on morbidity, mortality and disability worldwide, being directly or indirectly responsible for 3.3 million deaths per year (World Health Organization, 2014). Among the consequences of excessive use of ethanol is alcoholic liver disease (ALD), one of the major chronic liver disease, that appears in the top 20 causes of death worldwide (Rehm & Shield, 2013; Xie et al., 2013). The spectrum of ALD comprises alcoholic fatty liver disease (AFLD, also namely steatosis), the earliest response to exceeding ethanol consumption, which is followed by more severe lesions, such as steatohepatitis (ASH) and cirrhosis (AC) stages (Gao & Bataller, 2011).

AFLD, an asymptomatic condition, is characterized by triglyceride accumulation in hepatocytes (Orman et al., 2013). The ALFD triggering pathophysiological mechanism includes lipogenesis imbalance and generation of reactive oxygen species (ROS), and consequent oxidative stress generated by Kupffer cell activation, lipogenesis disruption, cytokines production, decrease in hepatic antioxidant defense, and ethanol metabolism mediated by alcohol dehydrogenase and cytochrome P450 2E1 (CYP2E1; Nagata et al., 2007;

Polavarapu et al., 1998). Ethanol metabolism by CYP2E1 produces superoxide anion ($O_2^{\cdot-}$), peroxide hydrogen (H_2O_2) and hydroxyethyl radicals that are responsible for oxidative stress and lipid peroxidation (Sakaguchi et al., 2011). Lipid peroxidation is probably the most significant event associated with ethanol-induced hepatotoxicity (Albano, 2002; Nagata et al., 2007). The review of Lívero & Acco (2016) presents more details about AFLD pathogenesis.

Regarding pharmacological treatment, there is no single drug therapy capable of acting in so many pathways to reverse hepatocyte injuries present in AFLD. An ideal agent would be able to reduce oxidative stress, lipid accumulation, inflammatory mediators, and prevent fibrotic events. Currently, the most effective in AFLD treatment is ethanol abstinence. However, therapies are urgently needed for those patients who are unable to stop drinking and to prevent the progression of AFLD, since mortality in patients with ASH and AC is around 65% in a period of four years (Altamiro & Bataller, 2011; McClain et al., 2004).

A vast field of research for new pharmacological agents is medicinal plant extracts, that have been used for health-related purposes since more than 5000 years (Stickel & Shouval, 2015). The popularity and use of natural products grow exponentially over the past decades due to various reasons, including their use in curative and preventive medicine or just as healthy tonics; however, scientific evidence providing the beneficial effects of many medicinal plants is mostly lacking (Stickel & Shouval, 2015). One of the natural products used in folk medicine is *Baccharis trimera* (Less.) DC (*B. trimera*), a widespread South America plant (Paul et al., 2009). In Brazil it is popularly known as ‘carqueja’ and its aerial parts are used, in the form of tea, for the treatment of diabetes, inflammatory processes, and gastrointestinal and liver disease (Garcia et al., 2014; Lermen et al., 2009). Some biological effects of *B. trimera* compounds include relaxant effect on vascular smooth muscle, blockade of the voltage-dependent calcium channels, hepatoprotective effects, hypoglycemic, antioxidant and anti-inflammatory (Biondo et al., 2011; Brandão Torres et al., 2000; Garcia et al., 2014; Oliveira et al., 2005; Paul et al., 2009; Rodrigues et al., 2009; Soicke & Leng-Peschlow, 1987). However, there is no research with this plant extract in AFLD pathogenesis.

In view of all problems exposed and searching for a possible AFLD pharmacological treatment, we evaluated if a hydroethanolic extract of *Baccharis trimera* (HEBT) can prevent the ethanol related-hepatotoxicity, reverting steatosis and oxidative stress in liver of mice under an ethanol and low-protein diet condition.

2. Material and methods

2.1. Chemicals

Bovine serum albumin, DTNB (5,5'-dithiobis), DCFA (2',7'-dichlorofluorescein-diacetate), reduced glutathione, glutathione reductase, NADPH, xylenol orange, K_2HPO_4, KH_2PO_4, Tris 1M, EDTA 5mM, TRIS HCl (all from Sigma®, St. Louis, USA); CDNB (1-chloro-2,4-dinitrobenzene), pyrogallol, absolute ethanol and methanol, ferrous ammonium sulfate, hydrogen peroxide, trichloroacetic acid, formaldehyde, sodium azide (Vetec®, Rio de Janeiro, Brazil); Bradford (BioRad® Protein Assay), and ultra-pure water from a Milli-Q system were used for the eluent preparation.

2.2. Botanical material, preparation and chemical analysis of HEBT

Aerial parts of *B. trimera* [Less.] DC was harvested in the Garden of Medicinal Plants of Paranaense University (UNIPAR), Umuarama, Paraná, Brazil, which is located at 430 m of altitude above sea level (coordinates S23°47′55–W53°18′48). The herbarium of UNIPAR received a voucher specimen (no.220). The hydroethanolic extract (ethanol: water, 9:1) was prepared according to described by Lívero et al. (2016, "submitted"), stored in a tightly sealed glass bottle and kept in a freezer until its utilization. The freeze-dried extract was dissolved in distilled water and 20 µl of 2% Tween immediately before the experiments. Chemical analysis of HEBT was previously described by Lívero et al. (2016, "submitted").

The main classes of compounds in HEBT were investigated by detailed analysis of selected regions of the NMR 1D and 2D spectra and HPLC-UV/PAD. Free sucrose, clerodane diterpenes, flavones, caffeoylquinic, dicaffeoylquinic and tricaffeoylquinic acid was found. Previous research of our group had described the HEBT analysis with more details (Lívero et al., 2016, “submitted”).

2.3. Animals and diet

Swiss male mice, 8-10 weeks old weighing 25-35 g were housed into individual cages, at 22 ± 2ºC under a 12/12 h light dark cycle with *ad libitum* access to food, water or ethanol, with consume weekly controlled. Ethical commit for animal use of Federal University of Paraná approved all of the procedures (no. 619) and experiments were performed in accordance with international standards and ethical guidelines on animal welfare. Chow, namely AIN-93G 6% Protein and AIN-93G 23% Protein Diet Pelleted (low-protein and normal-protein diet, respectively), were acquired from Rhoster® Industry and Commerce Ltda, São Paulo – SP - Brazil.

2.4. Induction of hepatic steatosis and experimental design

Hepatic steatosis was induced according to Lívero et al. (2014). During 6 experimental weeks, the mice received a low-protein diet and fluid that contained either 10% ethanol (n = 16) or water (n = 6). In the last 2 weeks, the animals were redistributed into three groups for the initiation of treatment with HEBT (30 $mg \cdot kg^{-1}$, p.o.) or vehicle (water plus 2% Tween, 1 $ml \cdot kg^{-1}$) once a day for 14 days. At the same time, a naïve group feed with a normal-protein diet and water was evaluated. Thus, the final groups were the following: Naïve (water + normal-protein diet + vehicle), WV (water + low-protein diet + vehicle), EV (steatotic group; ethanol + low-protein diet + vehicle), and EHEBT (ethanol + low-protein diet + 30 $mg \cdot kg^{-1}$ dose of HEBT).

A naïve and a WV treated with 30 mg.kg^{-1} dose of HEBT were investigated in the same conditions aforementioned in relation to effects triggered only by the extract. No differences were found and the results are not show.

The dose of HEBT was selected based on a curve dose-response performed with several oral doses (30 mg·kg^{-1}, 90 mg·kg^{-1}, 270 mg·kg^{-1}, 810 mg·kg^{-1}, once a day for 14 days, and 270 mg·kg^{-1} once a day during 21 days) in the same conditions above-mentioned. The criteria for the dose choice was the reversal of increase levels of hepatic transaminases and triglyceride accumulation induced by 10% ethanol and low-protein diet (data not show).

2.5. Sample collection

At the end of 6 experimental weeks, mice were fasted for 12 h and anesthetized with 80 mg·kg^{-1} dose of ketamine and 10 mg·kg^{-1} dose of xylazine, intraperitoneally. After laparotomy, blood was collect with heparinized syringes from the abdominal cava vein. Plasma was separated through centrifugation (4000 rpm for 10 minutes) and stored at -80ºC for biochemical analyses. Abdominal fat, kidneys and liver were harvested and weighed. Liver samples were rapidly separated and frozen in liquid nitrogen for oxidative stress, biochemical and molecular evaluations. Hepatic major lobe was stored for histological analyses. The euthanasia of mice was performed by puncture of the diaphragm, under anesthesia.

Additionally, feces were collected directly from the animal cages in the last day of experiment and stored at -20ºC until processing. This material was representative of 3 days' feces accumulation.

2.6. Liver histology

Two samples of hepatic major lobe were quickly harvested, fixed in buffered 10% formalin solution (distillated water, 35-40% formaldehyde, monobasic and dibasic sodium phosphate) and stored for posterior staining. One of them was dehydrated with alcohol and xylene, embedded in paraffin

wax, and sectioned at 6 µm for histological evaluation after hematoxylin/eosin (HE) staining. Other one was transferred to 10%, 20%, 30% sucrose solution, for 24 h at each concentration. After saturation, samples were stored in Tissue Tek® (O.C.T. Sakura®) and rapidly frozen in liquid nitrogen and sectioned at 6 µm for Nile Blue staining. The hepatic sections were observed and photographed with a slide scanner from MetaSystems (MetaViewer® version 2.0.100) at 20 and 40 × magnification. Scores of steatosis and other lesions (inflammation, ballooning and presence of Mallory hyaline bodies) were evaluated according to proposed by Kleiner et al. (2005), with few modifications, which evaluate the liver zones: zone 1 encircles the portal veins, zone 3 located around central veins, and zone 2 located in between. The evaluation was performed in degrees: 0 means lesions within 5% of tissue; 2 between 6 to 33%; 3 between 34 to 66% and degree 4 represents lesions between 67 to 100% of tissue.

2.7. Transmission electron microscopy

Samples were prepared according to described by Guimaraes et al., 2009. Briefly, 1 mm^3 of hepatic major lobe were fixed during 20 minutes with Karnovsky's fixative (2% glutaraldehyde, 4% paraformaldehyde, 5 mM $CaCl_2$, in 0.1 M cacodylate buffer, pH 7.2). Then, samples were washed with cacodylate buffer and fixed with 1 mM $CaCl_2$, 0.8% potassium ferricyanide, 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) during 15 minutes and rinsed twice for 1 minute with the same buffer. Hepatic pieces were dehydrated with 50, 70, 90 and 100% acetone (twice, for 3 minutes). After, samples were moved to bean capsules with 90% acetone and infiltrated in epoxy resin (Epon)/acetone solution (1:1) during 2 hours, following by 4 hours of pure Epon and a new Epon solution overnight. Polymerization was carried out for 48 hours at 60°C. Ultra-thin sections were stained with aqueous uranyl acetate (15 min) and with lead citrate (2 min). Samples were visualized with a Jeol JEM 1011 transmission electron microscope. Images were obtained using a GATAN digital micrograph.

2.8. Measurement of hepatic triglycerides (TG), cholesterol (CHOL), high density lipoprotein (HDL), low density lipoprotein (LDL) and fecal TG and CHOL

Liver and fecal samples were submitted to lipid extraction by gravimetric technique proposed by Folch et al. (1957) with few modifications. Liver and fecal lyophilized samples (200 mg) were mixed with 1.8 mL of hexane (98.50% pure) as solvent and heated at 80ºC. After resting overnight, the supernatant was transferred to a second flask and naturally evaporated. This procedure was repeated 3 times. Then the lipid content was weigh and suspended in 1 mL of chloroform (99.50% pure) and 2 mL of isopropanol (99.50% pure) for determination of hepatic levels of TG, CHOL, HDL, LDL and fecal levels of TG and CHOL using commercial kits (Kovalent®, São Gonçalo, Brazil) in a Mindray BS-200® automated device. The results are expressed as $mg \cdot dL^{-1}$ of hepatic or fecal homogenates.

2.9. Plasma biochemistry analysis

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), CHOL, TG, HDL, LDL, glucose, amylase, albumin, globulin, total protein, creatinine, and urea were evaluated in the plasma of mice using commercial kits (Labtest Diagnostica®, Lagoa Santa, Brazil and Kovalent®, São Gonçalo, Brazil) in a Mindray BS-200® automated device, which results are expressed as $mg \cdot dL^{-1}$.

2.10. Preparation of hepatic homogenates for antioxidant system analyses

Liver samples were homogenized with potassium phosphate buffer (0.1 M, pH 6.5) in a 1:10 dilution. Then, 100 µL were separated, suspended in 80 µL of trichloroacetic acid (12.50%), vortexed and centrifuged at 6000 rpm, during 15 min; at 4ºC for glutathione reduced (GSH) analyze. The remainder homogenate was centrifuged at 9700 rpm, during 20 min, at 4ºC for glutathione S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD),

catalase (Cat), lipoperoxidation (LOOH), total content of ROS and amount of protein.

2.11. Evaluation of antioxidant system

Determination of reduced glutathione (GSH) levels

Reduced glutathione levels were determined in hepatic homogenates according to propose by Sedlak and Lindsay (1968), with few modifications. For the assay, 20 µL of supernatant was added to 280 µL of TRIS buffer (0.4 M, pH 8.9) and 5 µL of DTNB (0.01 M). Absorbance was read at 415 nm. The individual values were interpolated into a standard curve of GSH (0.375 – 3 µg) and are expressed as µg·g of tissue^{-1}.

2.11.1. *Determination of peroxidase glutathione (GPx) activity*

GPx was measured according to the method described for Paglia et al. (1967). Supernatant was diluted 1:30 in potassium phosphate buffer (0.1 M, pH 6.5). Briefly, 10 µL of diluted supernatant and 130 µL of reaction solution [sodium phosphate buffer (0.1 M, pH 7); sodium azide (3.078 mM); NADPH (0.307 mM); GSH (3.07 mM) and 1.795 U·mL^{-1} of glutathione reductase) was mixed and incubated for 2 min. Then, 60 µL of hydrogen peroxide (1.5 mM) were added. The reaction was read at 340 nm and expressed as nmol·min^{-1}·mg of protein^{-1}.

2.11.2. *Determination of glutathione S-transferase (GST) activity*

GST activity was measured using the method of Habig et al. (1974). Supernatant was diluted 1:80 in potassium phosphate buffer (0.1 M, pH 6.5). Reactions were performed in the presence of 100 µL of diluted supernatant and 200 µL of reagent solution [CDNB (3 mM), GSH (3 mM), and potassium phosphate buffer (0.1 M, pH 6.5)] at room temperature. The conjugation of CDNB with GSH was monitored at 340 nm for 180 s. Specific activity was calculated using an extinction coefficient of 9.6·mM^{-1}·cm^{-1} for GSH, and the results are expressed as mmol·min^{-1}·mg of protein^{-1}.

2.11.3. *Determination of superoxide dismutase (SOD) activity*

SOD activity was measured through the ability of SOD to inhibit pyrogallol autoxidation, according to Gao et al. (1998). Supernatant was diluted 1:10 in potassium phosphate buffer (0.1 M, pH 6.5). Then, 60 µL of dilution was added to 1327.5 µL of Tris EDTA buffer solution (0.4 M, pH 8), vortexed and mixture with 75 µL of pyrogallol solution (15 mM). The reaction was incubated for 30 min at room temperature and stopped with the addition of 37.5 µL of 1N HCl. The absorbance of the resulting supernatant was measured at 405 nm. The amount of SOD that inhibited the oxidation of pyrogallol by 50% (relative to the control) was defined as one unit of SOD and the enzymatic activity of SOD was expressed as U SOD·mg of protein^{-1}.

2.11.4. Determination of catalase (Cat) activity

The activity of Cat was evaluated according to proposed by Aebi (1984). Briefly, 5 µL of 1:10 supernatant dilution (in 0.1 M potassium phosphate buffer, pH 6.5) was mixed with a hydrogen peroxide solution (Tris EDTA buffer, pH 8.0; ultrapure water; and 30% hydrogen peroxide) and read at 240 nm. Results are expressed as mmol.min^{-1}.mg of protein^{-1}.

2.11.5. Determination of lipid hydroperoxide (LOOH) content

The levels of hepatic LOOH were determined using the ferrous oxidation-xylenol orange (FOX2) method as described by Jiang et al. (1992). Briefly, 100 µL of methanol P.A. was added to 100 µL of supernatant, vortexed, and centrifuged at 5000 rpm for 5 min at 4°C. 100 µL of this supernatant was mixed with 900 µL of FOX2 reagent [BHT (4 mM), $FeSO_4$ (250 µM), H_2SO_4 (250 mM), and xylenol orange (100 mM)], vortexed and incubated in the dark for 30 min at room temperature. Absorbance was read at 560 nm and the concentration of LOOH is expressed as mmol hydroperoxide·mg of protein^{-1}.

2.11.6. *Total ROS*

The total ROS content was quantified through the 20-70-dichlorofluorescein-diacetate (DCFH-DA) assay, previously proposed by Driver et al (2000). Briefly, 200 µL of 1:10 supernatant dilution (in 0.1 M potassium phosphate buffer, pH 6.5) was mixed with a DCFA solution (DCFA, ethanol and

dimethyl sulfoxide), incubate during 40 min, at room temperature, in the dark. The formation of DCF was measured with a spectrofluorimeter in which the excitation and emission wavelengths were set at 485 and 506 nm, respectively, and results are expressed as fluorescence.

2.11.7. *Protein assay*

The protein content was measured to express the results of the oxidative stress parameters. Protein concentrations of the supernatants were determined by the Bradford method (1976). Supernatant was diluted 1:10 in potassium phosphate buffer (0.1 M, pH 6.5). Then, 10 µL of diluted supernatant was mixed with 250 µL of Bradford solution (Bio-Rad®, Hercules, CA, USA). Reading was performed at 595 nm. The individual values were interpolated into a standard curve of bovine serum albumin (125 – 1000 µg) and are expressed as mg of protein.

2.12. Gene expression

Measurements of genes involved in ethanol metabolism, oxidative stress, and lipogenesis were performed in liver tissue. The mRNA levels were determined for the following genes: *Cyp2e1* (cytochrome P450 2E1), *Nrf2* (nuclear factor E2-related factor 2) and *Scd1* (Stearoyl-CoA reductase). The complementary DNA (cDNA) was synthesized from 2 µg of RNA, while following all of the reaction steps in a PCR-thermo-cycler. The expression of the mentioned genes was obtained using LightCycler 480 System (Roche®), and described subsequently as mRNA relative expression, using *18S* as the housekeeping gene. For this purpose, specific primers for murine genes were used, which sequences (5’ → 3’) were prepared by Invitrogen® (The Netherlands).

2.13. Statistical analysis

The data were analyzed for homogeneity of variance and normal distribution. Differences between means were determined by one-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test. The level of significance was

set at 95% ($p < 0.05$). The data are expressed as mean ± standard error of the mean (SEM).

3. *Results*

4.

4.1. Chow intake and body weight of mice

No differences in chow intake, fluid consumption or body weight gain were observed between experimental groups (Supplementary table). The animals receiving HEBT did not shown signals of toxicity during the 15 days of treatment.

Supplementary Table – Body weight and chow consumption of mice submitted to AFLD model and treated with vehicle or HEBT.

	At 1 week		At 3 weeks		At 6 weeks	
Groups	Body weight (g)	Chow consumption (g)	Body weight (g)	Chow consumption (g)	Body weight (g)	Chow consumption (g)
Naïve	37.83±2.12	64.33±3.84	38.85±2.29	65.33±9.83	38.89±2.30	68.00±8.32
WV	36.41±1.01	65.67±4.33	38.30±0.90	59.33±2.72	39.97±1.41	61.33±11.67
EV	36.57±1.39	68.25±7.92	39.49±2.08	54.00±9.33	42.62±3.14	51.25±4.32
EHEBT	33.76±1.73	79.00±1.78	38.29±1.17	70.00±2.04	40.78±1.99	63.50±4.62

Values are expressed as means ± S.E.M. (*n* = 6-8). Statistical comparison was performed using one-way ANOVA.

4.2. Histological characterization of EHA and HEBT effects

Histopathological analyses confirmed the presence of steatosis already suggested by the macroscopic appearance of the liver (enlarged and pale), confirming the effectiveness of the disease model used. No alterations occurred in livers of naïve group. Ethanol induced significant cellular changes in liver of mice. Ballooning code 0 and 1, micro- and macro-vesicular steatosis grade 2 and 3, and Mallory's hyaline bodies, more prominent in zone 3 (central vein

surrounds), were observed. HEBT was able to revert most of these alterations and only macro-vesicular steatosis grade 1 was observed. Nile blue staining, in which triglycerides stained in pink, confirmed the accumulation of lipids reveled by HE technique (vacuoles in with). Low-protein diet alone (group WV – water + low-protein diet + vehicle) caused few cellular modifications represented by hepatocyte ballooning code 1 and absence of steatosis (grade 0), localized mainly in zone 3. Figure 1 shows representative slides of these histological findings.

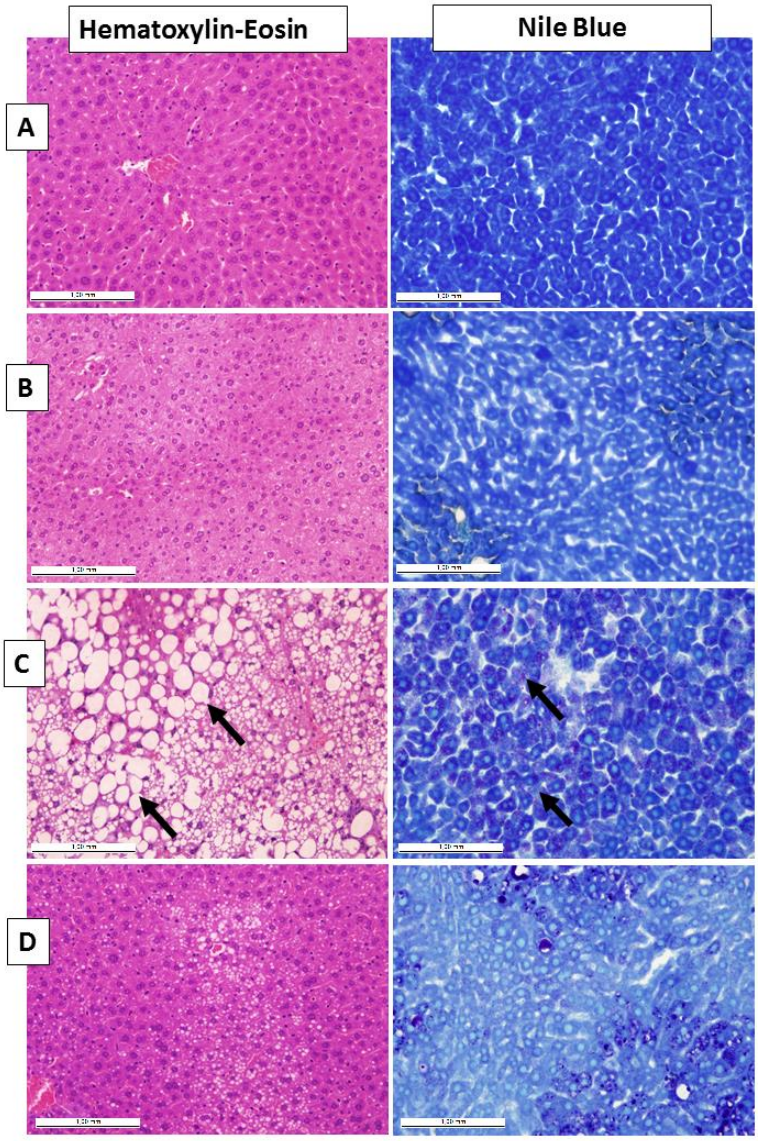


Figure 1: Hepatic histology of mice staining with Hematoxylin-Eosin and Nile Blue, with 20x magnification**.** (A) Naïve group; (B) water + vehicle, (C) ethanol + vehicle, (D) ethanol + HEBT. Arrows show fatty vacuoles.

4.3. Transmission electron microscopy

An ultrastructural overview by transmission electron microscopy showed preservation of hepatic architecture, absence of macrolipids and organelles similar to lysosomes in naïve group. Several alterations were observed in ethanol fed mice: intense reduction of glycogen, presence of macrolipids and microvesicular bodies, many merging. Furthermore, we observed evidence of lysosomes and Kupffer cells (data not show), loss of membrane integrity, mitochondria surrounded by rough endoplasmic reticulum and a electrondense material surrounding macrolipids, compatible with peroxisomes. HEBT ameliorates lesions induced by ethanol even at ultrastructural level, represented by lower incidence of macrolipids and microvesicular bodies, and more evidence of glycogen content. Additionally, the cytoplasmic extend showed a region of vesicular aggregates similar with tubular and vesicular smooth endoplasmic reticulum. Figure 2 shows the main alterations found at transmission electron microscopy.

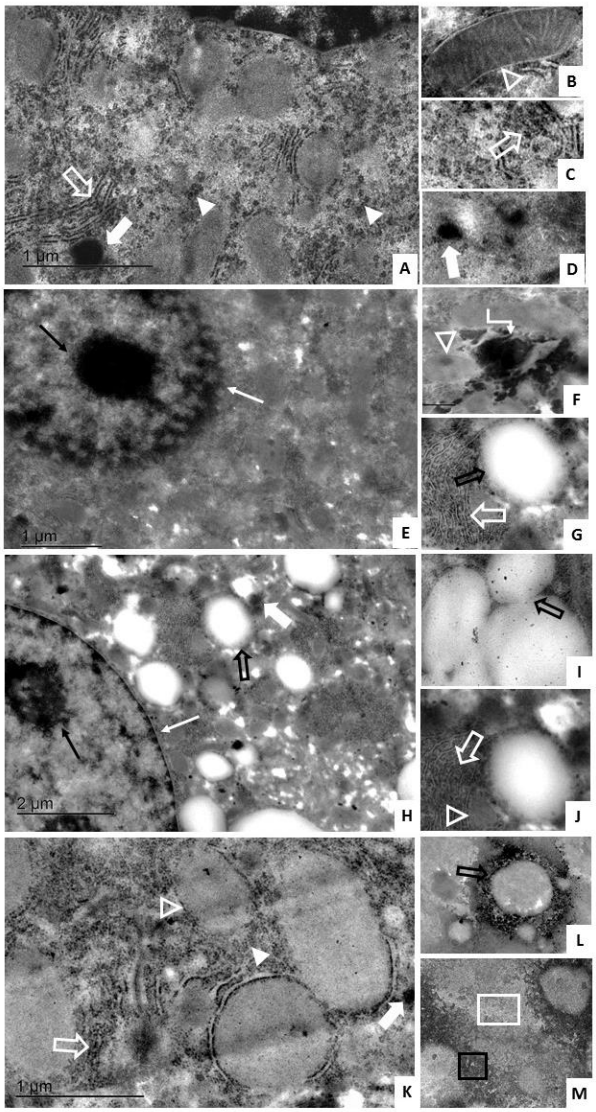


Figure 2: Ultrastructural overview by transmission electron microscopy of mice submitted to AFLD model. (A, B, C, D) Naïve group; (E, F, G) water + vehicle, (H, I, J) ethanol + vehicle, (K, L, M) ethanol + HEBT. Symbols: large white arrow: lysosomes; large arrow delineated in white: rough endoplasmic reticulum; head white arrow: glycogen; head arrow delineated in white: mitochondria; thin white arrow: nuclear pores; thin black arrow: nucleolus; angled arrow: electrondense material similar to peroxisomes; large arrow delineated in black: macrolipids; square delineated in with: tubular smooth endoplasmic reticulum; square delineated in black: vesicular smooth endoplasmic reticulum.

4.4. Measurement of plasmatic and hepatic TG, CHOL, HDL and LDL; and fecal TG and CHOL

Administration of 10% ethanol increased plasmatic TG and CHOL levels by 130.23% and 64.75%, respectively, compared with non-lesioned group (Naïve: 69.06 ± 6.83 $mg \cdot dL^{-1}$ and 83.76 ± 3.94 $mg \cdot dL^{-1}$, Fig. 3A and 3B, respectively). Treatment with HEBT revert totally the increase in TG and CHOL

(Fig. 3A and 3B, respectively). Regardless plasmatic HDL and LDL levels, an increase of 41.78% and 378.16% happened in ethanol group, compared with the naïve group (Naïve: 57.96 ± 2.42 mg·dL^{-1} and 57.38 ± 2.99 mg·dL^{-1}, respectively). Treatment with HEBT normalized HDL and LDL levels (Fig. 3C and 3D, respectively).

HEBT was able to reverse the increase in hepatic TG triggered by ethanol compared with basal values (331.60 ± 15.88 mg·dL^{-1}, Figure 3A). Ethanol also increased hepatic LDL levels by 21.77% compared with naïve group (213.50 ± 4.33 mg·dL^{-1}, Fig. 3D). No significant differences occurred in hepatic CHOL and HDL levels (Fig. 3B and 3C, respectively).

Fecal lipids dosages point out that HEBT was able to induce (≅ 35.73%) the excretion of TG compared with naïve group (104.1 ± 1.88 mg·dL^{-1}, Fig. 3A). No significant differences among the groups were observed in fecal levels of CHOL (Fig. 3B).

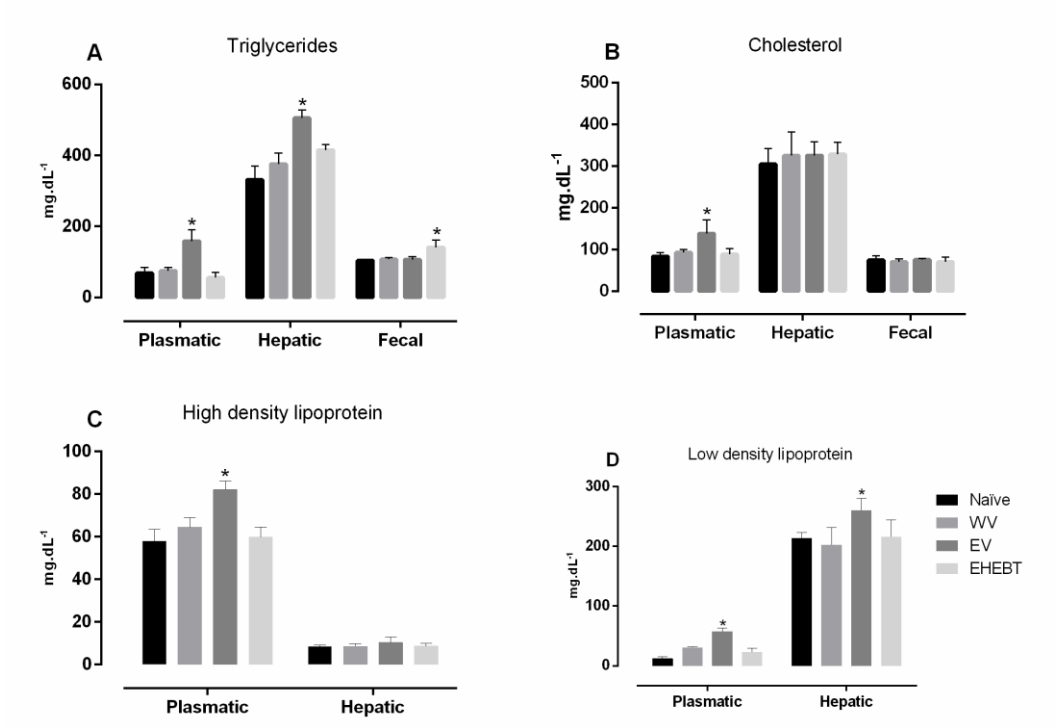


Figure 3: Plasmatic, hepatic and fecal levels of (A) triglycerides and (B) cholesterol; or plasmatic and hepatic levels of (C) high density lipoprotein and (D) low density lipoprotein of mice submitted to AFLD model. Values are expressed as mean ± SEM (*n* = 6-8). Statistical comparison was performed using one-way ANOVA followed by Bonferroni's test. * p<0.05 when compared with naïve.

4.5. Plasma biochemistry

Administration of 10% ethanol increased ALT and AST levels by 182.77% and 51.20%, respectively, compared with the naïve group (21.94 ± 2.64 and 51.34 ± 4.70 mg·dL^{-1}). Treatment with HEBT revert totally the increase in AST levels and decrease ALT levels by 92.88%, compared with steatotic group (EV). HEBT also restored glucose levels affected by ethanol (naïve: 195.70 ± 17.64 mg·dL^{-1}). Furthermore, 10% ethanol increased globulin and total protein levels by 106.03% and 28.97%, compared with naïve group (1.32 ± 0.13 mg·dL^{-1} and 3.52 ± 0.14 mg·dL^{-1}, respectively). The effect of low-protein diet was evident in urea levels, pointed by a decreased level in all groups that received the diet, compared with naïve group. HEBT did not revert this alteration. No significant differences between groups occurs in amylase, albumin and creatinine levels. Plasma biochemistry results are present in Table 1.

TABLE 1 – Plasma biochemistry of mice fed to 10% ethanol and treated with HEBT or vehicle.

	Naïve	**Water**	**Ethanol**	**Ethanol + HEBT**
ALT (mg.dL^{-1})	21.94 ± 2.64	29.05 ± 3.66	62.04 ± 1.91*	41.66 ± 4.27*
AST (mg.dL^{-1})	51.34 ± 4.70	41.60 ± 3.30	77.63 ± 2.47*	47.73 ± 1.99
Creatinine (mg.dL^{-1})	0.23 ± 0.02	0.20 ± 0.28	0.17 ± 0.25	0.35 ± 0.49
Urea (mg.dL^{-1})	47.05 ± 2.82	23.40 ± 7.70*	22.40 ± 4.87*	28.13 ± 1.29*
Amylase (mg.dL^{-1})	908.10 ± 52.48	924.1 ± 65.49	1011.00 ± 56.85	872.6 ± 39.09
Glucose (mg.dL^{-1})	195.7 ± 17.64	239.4 ± 18.67	118.1 ± 11.78*	215.9 ± 20.25
Globulin (mg.dL^{-1})	1.32 ± 0.13	2.43 ± 0.18*	2.73 ± 0.29*	1.76 ± 0.18
Albumin (mg.dL^{-1})	2.17 ± 0.10	1.76 ± 0.17	1.80 ± 0.26	1,76 ± 0.17
Total protein (mg.dL^{-1})	3.52 ± 0.14	4.71 ± 0.19*	4.54 ± 0.18*	3.38 ± 0.18

Values are expressed as means ± S.E.M. (*n* = 6-8). Statistical comparison was performed using one-way ANOVA followed by Bonferroni's test. * p<0.05 when compared with naive.

4.6. Effect of HEBT on markers of oxidative stress in AFLD

Administration of 10% ethanol increased total ROS and LPO levels by 63.81% and 23.19%, respectively, compared with non-lesioned group (naïve: 2739.00 ± 197.30 nmol·mg of protein^{-1} and 23.45 ± 28.89 mmol hydroperoxides·mg of protein^{-1}. A decrease occurs in Cat and SOD levels by 24.90% and 19.78%, respectively, compared with the naïve group (301.20 ± 22.66 mmol·min^{-}1·mg of protein^{-1} and 46.15 ± 0.73 U SOD·mg of protein^{-1}). Treatment with HEBT revert the increase in total ROS, LPO and the decrease in Cat and SOD levels. Regardless GSH system, increased levels of GST, GSH and GPx was observed in steatotic group, compared with naïve group (26.70 ± 1.71 µmol·min^{-1}·mg of protein^{-1}; 161.60 ± 33.54 µg GSH·g of tissue^{-1} and 2.38 ± 0.04 µmol·min^{-1}·mg of protein^{-1}, respectively). HEBT revert the elevation in GST, GSH and GPx levels. Low-protein diet alone was able to increased GSH levels by 239.78%, compared with naïve values. Results of oxidative stress parameters are present in Table 2.

TABLE 2 – Hepatic markers of oxidative stress in mice bearing AFLD and treated with HEBT or water.

	Naïve	**Water**	**Ethanol**	**Ethanol + HEBT**
LOOH	23.45 ± 0.91	19.60 ± 1.79	28.89 ± 1.42*	2171 ± 0.83*
Total ROS	2739 ± 197.30	2943 ± 154.10	4487 ± 112.80*	3497 ± 127.30*
GSH	161.60 ± 33.54	549.10 ± 17.62*	586.20 ± 36.82*	265.10 ± 39.93
GPx	2.38 ± 0.04	2.99 ± 0.05*	3.61 ± 0.09*	2.43 ± 0.08
GST	26.70 ± 1.71	23.83 ± 2.63	41.07 ± 2.68*	25.36 ± 1.82
SOD	46.15 ± 0.73	54.78 ± 1.60*	37.02 ± 1.21*	50.02 ± 0.96
Cat	301.20 ± 22.66	355.20 ± 15.25	226.20 ± 8.69*	366.50 ± 13.28*

Values are expressed as means ± S.E.M. (*n* = 6-8). Statistical comparison was performed using one-way ANOVA followed by Bonferroni's test. LOOH: mmol hydroperoxides·mg of protein^{-1}; Total ROS: nmol·mg of protein^{-1}; GSH: µg GSH·g of tissue^{-1}; GPx: µmol·min^{-1}·mg of protein^{-1}; GST: µmol·min^{-1}·mg of protein^{-1}; SOD: U SOD·mg of protein^{-1}; Cat: mmol·min^{-1}·mg of protein^{-1}. * $p<0.05$ when compared with naïve.

4.7. Gene expression

Ethanol consumption increased the gene expression of *Cyp2e1* and *Scd1* by 132.00% and 615.00%, respectively (Fig. 4A and 4B, respectively). Daily treatment with HEBT reversed these alterations and elevated *Nrf2* expression by 66.20% (Fig. 4C).

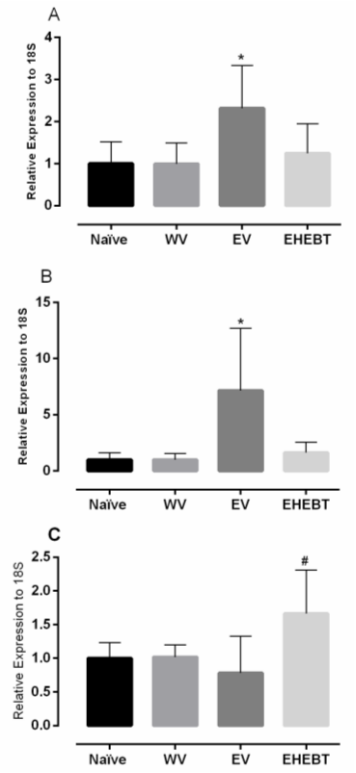


Figure 4: Gene expression of A) *Cyp2e1*; (B) *Scd1* and (C) *Nrf2* of mice submitted to AFLD model. Values are expressed as relative expression, using *18S* as the housekeeping gene (*n* = 6-8) Statistical comparison was performed using one-way ANOVA followed by Bonferroni's test. * $p<0.05$ when compared with naïve; $^{\#}p<0.05$ when compared with ethanol.

5. Discussion

In this research we evaluated the effects of a hydroethanolic extract of *Baccharis trimera* in a mice model of AFLD, combining 10% ethanol and low-protein diet. The modified protein diet was should to be associated with onset and progression of ALD induced by nutritional deficiencies (Gramenzi et al., 2006). Using this model previously proposed by our group (Lívero et al., 2014), we observed hepatic histopathological damage, disruption in lipid profile, increases in plasma transaminase levels, induction of oxidative stress and altered expression of some genes in liver. Daily administration of 30 $mg.kg^{-1}$ of

HEBT reverted these AFLD characteristics, as summarized at Figure 5. In consequence, we investigated HEBT as a possible pharmacological agent for this disease.

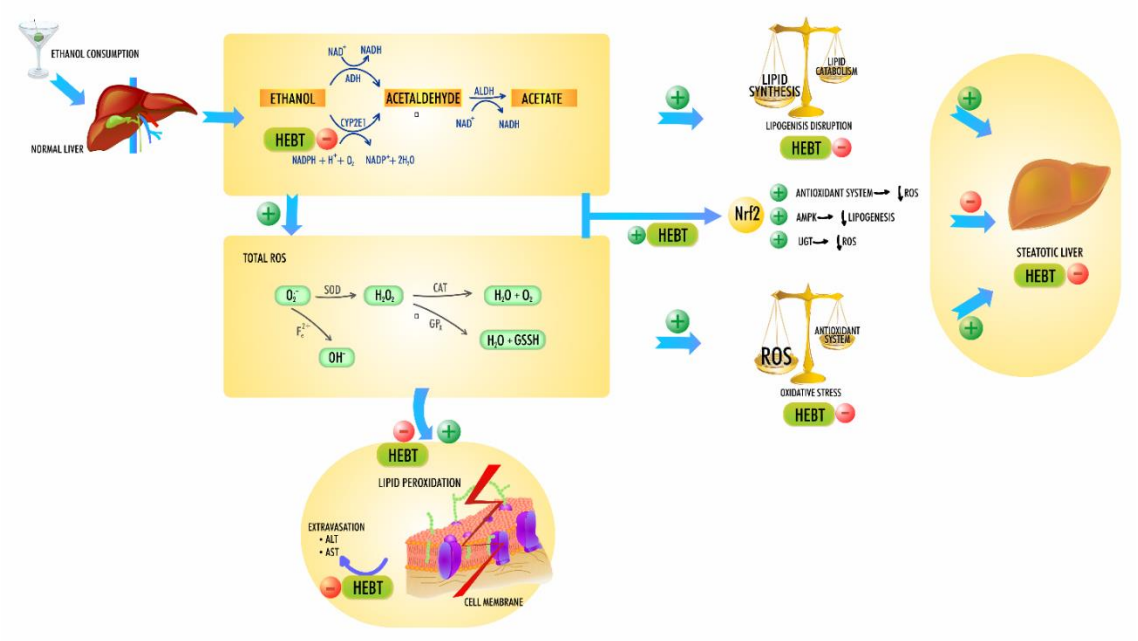


Figure 5: Pathological pathways of AFLD induction and the interference of HEBT in this process. After consumption, ADH and CYP2E1 oxidize ethanol to acetaldehyde that is converted to acetate by ALDH. Ethanol can induce steatosis decreasing fatty acid oxidation and increasing lipogenesis in hepatocytes. Besides this, during ethanol metabolism, several ROS like $O_2^{\cdot-}$, H_2O_2 and OH^- are generated and inhibit the antioxidant capacity of the hepatocyte, decreasing Cat, SOD and GSH; and increasing lipid peroxidation, resulting in hepatic oxidative stress. Lipid peroxidation allow ALT and AST extravasation. Finally, ethanol interferes with Nrf2, a transcriptional factor of antioxidant, lipogenesis and detoxification genes. Taken together, these alterations induced by ethanol favors lipid accumulation and fatty liver disease initiation. HEBT played an important role in all steps, decreasing lipid peroxidation and oxidative stress, normalizing lipogenesis and inducing Nrf2. As consequence, ameliorating fatty liver disease. Abbreviations: ADH, alcohol dehydrogenase; AFLD, alcoholic fatty liver disease; ALDH, aldehyde dehydrogenase; AMPK, adenosine monophosphate activated protein kinase; ALT, alanine aminotransferase; AMPK, adenosine monophosphate activated protein kinase; AST, aspartate aminotransferase; Cat, catalase; CYP2E1, cytochrome P450 2E1; Fe^{2+}, iron; GPx, peroxidase glutathione; GSH, reduced glutathione; GSSG, oxidizing glutathione; H_2O, water; H_2O_2, hydrogen peroxide; HEBT, hydroethanolic extract of Baccharis trimera; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Nrf2, nuclear factor erythroid 2-related factor 2; O_2, oxygen; $O_2^{\cdot-}$, superoxide anion; OH^- hydroxyl; ROS, reactive oxygen species; SOD, superoxide dismutase; UGT, UDP-glucuronosyltransferase.

Fatty liver is well characterized by large accumulation of lipids (mainly TG) spread into zone 1 and 2 hepatocytes (Liu, 2014). Previous reports show that lipid accumulation *per se* is not a key event of liver injury, since *1)* lipid droplets are specialized organelles that enable cells to regulate store and drop lipids,

and *2)* this compartmentation in lipid droplets per se does not induce damage pathways (Schwabe & Maher, 2012). Instead, lipid flow in and out of these vesicles, being converted into potentially toxic mediators, is emerging as an important role of lipid-mediated hepatic damage (Schwabe & Maher, 2012). Thus, eliminating these droplets and consequent lipid efflux is a crucial way to preserve tissue integrity. In our study, besides TG accumulation indicated by histology, biochemical measurement of hepatic and plasmatic concentrations also reflected fatty liver and hyperlipidemia, with an accentuated elevation in TG and CHOL levels. Fernando et al., (2011) evaluated lipidomic changes in rats after long term-exposure to ethanol, and described vacuolization, fatty deposition and higher plasmatic levels of TG and CHOL after 2 and 3 experimental months. Lu & Cederbaum (2015) also described increment in TG levels and hepatic fat accumulation produced by 4 weeks of ethanol. In our study, treatment with HEBT was able to revert hepatic TG accumulation, increase TG fecal excretion, and reduce hepatic and plasmatic levels of CHOL and TG induced by ethanol. Thus, HEBT protects hepatocytes against lipid-mediated damage.

Plasma biochemistry also revealed marked elevation of ALT and AST levels induced by ethanol, which was reversed when mice received treatment with HEBT. Our results are in accordance with previous reports that described increased transaminase levels of rodents submitted to ethanol consumption (Fernando et al., 2011; Lívero et al., 2014; Lu & Cederbaum, 2015; Segawa et al., 2008). The same occurs in humans. In a clinical trial, Kirpich et al., (2008) reported elevate levels of AST, ALT and GGT in alcoholics that were diminished when individuals were treated with probiotics.

Prolonged ethanol consumption is also linked to enzymatic induction of CYP2E1, an important pathway of ethanol metabolism that has been recognized as a major contributor to ethanol-induced oxidative stress and liver injury (Gramenzi et al., 2006). Lu, Zhang and Cederbaum (2012) reported a 2.4-fold increase in *Cyp2e1* in mice treated with ethanol. Induction of *CYP2E1* expression also occurs in humans, with 4- to 10-fold rise in mRNA levels in liver biopsy from subjects who drunk ethanol (Sakaguchi et al., 2011). Our results are in agreement, since we found that mice fed ethanol have an induction of

Cyp2e1 expression by 2.3-fold. However, simultaneous treatment with HEBT maintained levels within the naïve range. The regulation of CYP2E1 is crucial for liver homeostasis since activation of this CYP may contribute indirectly to ALD development by interaction with cytochrome reductase, leading to electron leaks in respiratory chain and consequent overproduction of superoxide anion (Lieber, 1999; Malaguarnera et al., 2009; Polavarapu et al., 1998; Wu & Cederbaum, 2005), inducing oxidative stress. The ability of ethanol to induce oxidative stress is well recognized (Beier & McClain, 2010; Cederbaum et al., 2009; Lívero et al., 2014). Besides ethanol-induced ROS formation and antioxidants depletion, malnutrition per se also promotes the depletion of endogenous antioxidants like glutathione and vitamin A, E and C (Gramenzi et al., 2006). However, in our study, despite a low-protein diet, we found an interesting augment in GSH levels induced by diet. Surprisingly, HEBT treatment reverted this elevation. Apart from these observation, and in line with augmented ROS formation and a decrease in the antioxidant system, we found increased levels of oxidative stress biomarker. Hydroperoxides, and lipid peroxidation biomarkers, were significantly elevated in mice that received ethanol. Several studies indicate that tissue damage caused by ethanol is mediated by lipid peroxidation (Nagata et al., 2007; Sakaguchi et al., 2011). Through reversion of lipid peroxidation, HEBT prevented hepatic damage and consequent leakage of liver transaminases, such as ALT, for example. Furthermore, the importance of maintaining redox state balance to reverse AFLD was evident in our study. Ethanol induced hepatic oxidant environment reflected by elevate levels of lipoperoxidation, total ROS, GSH, GPx and GST and diminished levels of Cat and SOD. These capacity of ethanol to induce oxidative stress rationalizes the use of antioxidants to protect liver against oxidative damage. The antioxidant activity of *B. trimera* previously described in others models (Lívero et al., 2016 “submitted”; Pádua et al., 2010; Pádua et al., 2014) was confirmed also in AFLD model and revert all oxidative parameters altered by ethanol in the liver.

The antioxidant effect and the reduced lipogenesis diminished in HEBT-treated mice of our study can be correlated, at least in part, with induction of the nuclear factor erythroid-related factor 2 (Nrf2). Nrf2 has emerged as an

essential transcriptional factor of antioxidant, detoxification, biotransformation and lipogenesis genes (Bataille & Manautou, 2013; Tanaka et al., 2008; Tanaka et al., 2012; Xing et al., 2015). Preliminary studies established that ethanol-induced lipid peroxidation, oxidative stress and liver-associated mortality are increased in Nrf2-knocked down cells or Nrf2-null mice (Gong & Cederbaum, 2006; Lamlé et al., 2008). Beside its strong involvement with antioxidant system, Nrf2 also upregulates the expression of phase II enzymes, like UGT (UDP-glucuronosyltransferase), that has significant ethanol-metabolizing activity with less ROS production, that is advantageous for liver homeostasis (Buckley & Klaassen, 2009). Finally, Nrf2 downregulates the expression of Scd1, a lipogenic enzyme, probably through induction of adenosine monophosphate activate protein kinase (AMPK) activity (Dobrzyn et al., 2004; Dobrzyn et al., 2005; Kim et al., 2011). AMPK is a key element in lipogenesis by limiting anabolic pathways and facilitating catabolic pathways (Ceni et al., 2014). Thus, the elevation in *Nrf2* levels induced by HEBT promotes beneficial effects upon several parameters of AFLD, like oxidative stress and lipogenesis control, for example.

Finally, no signs of toxicity were observed with HEBT treatment. Previous studies of acute toxicity test of HEBT showed no evidence of toxicity, such as no alterations in water or food intake, behavioral changes, or body and organ weight, suggesting the absence of toxicity of different doses. HEBT were administered orally (50-5000 $mg \cdot kg^{-1}$) or intraperitoneally (1000 $mg \cdot kg^{-1}$), indicating the safety of HEBT, even in higher doses (Lívero et al., 2015; “submitted”) than used in the present experiment (30 $mg \cdot kg^{-1}$).

Conclusions

Considering the absence of approved drugs to revert, to control or prevent ALD progression, and taking in account our results exposed in an AFLD model, the hydroethanolic extract of *Baccharis trimera* is a promising pharmacological agent for the treatment of alcoholic fatty liver disease.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

FARL was responsible for every step of the experiments, data analysis, manuscript preparation and data discussion. GGM contributed with the *in vivo* experiments. JEQT, CRCF and SMPB contributed with histological analyses. OB contributed with biochemical analyses. RPJOE helped in data discussion. AA were responsible for data discussion and manuscript correction. AA was the senior researcher responsible for the project.

References

Adachi M, Ishii H. Role of mitochondria in alcoholic liver injury. Free Radical Biology & Medicine, Vol. 32, No. 6, pp. 487–491, 2002.

Aebi H. Catalase in vitro. Methods Enzymol.1984, 105: 121–126.

Albano, E. Alcohol, oxidative stress and free radical damage. Proceedings of the Nutrition Society (2006), 65, 278–290.

Altamirano J, Bataller R. Alcoholic liver disease: pathogenesis and new targets for therapy. Nature reviews Gastroenterology and Hepatology. 2011, 8: 491-501.

Bataille AM, Manautou JE. Nrf2: A Potential Target for New Therapeutics in Liver Disease. Clin Pharmacol Ther. 2012, 92: 340-348.

Beier JI, McClain CJ. Mechanisms and cell signaling in alcoholic liver disease. Biol Chem. 2010; 391:1249–64.

Biondo TMA, Tanae MM, Coletta ED, Lima-Landman MTR, Lapa AJ, Souccar C. Antisecretory actions of *Baccharis trimera* (Less.) DC aqueous extract and isolated compounds: Analysis of underlying mechanisms. Journal of Ethnopharmacology, 2011, 136: 368-373.

Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry 1976; 72:248-254.

Brandão Torres, L. M., M. T. Gamberini, N. F. Roque, M. T. LimaLandman, C. Souccar, and A. J. Lapa. 2000. Diterpene from Baccharis trimera with a relaxant effect on rat vascular smooth muscle. Phytochemistry. 55:617–619.

Buckley DB, Klaassen CD (2009) Induction of mouse UDP-glucuronosyltransferase mRNA expression in liver and intestine by activators of aryl-hydrocarbon receptor, constitutive androstane receptor, pregnane X receptor, peroxisome proliferator-activated receptor alpha, and nuclear factor erythroid 2-related factor 2. Drug Metab Dispos 37: 847–856.

Cederbaum AI, Lu Y, Wu D. Role of oxidative stress in alcohol-induced liver injury. Arch Toxicol. 2009; 83:519–48.

Dobrzyn P, Dobrzyn A, Miyazaki M, Cohen P, Asilmaz E, Hardie DG, Friedman JM, Ntambi JM. Stearoyl-CoA desaturase 1 deficiency increases fatty acid oxidation by activating AMP-activated protein kinase in liver. Proc. Natl. Acad. Sci. 2004,101: 6409–6414.

Dobrzyn A, Dobrzyn P, Lee SH, Miyazaki M, Cohen P, Asilmaz E, Hardie DG, Friedman JM, Ntambi JM. Stearoyl-CoA desaturase-1 deficiency reduces ceramide synthesis by downregulating serine palmitoyl-transferase and increasing beta-oxidation in skeletal muscle. Am. J.Physiol. Endocrinol. Metab. 2005, 288: E599–607.

Driver AS, Kodavanti PRSK, Mundy WR. Age-related changes in reactive oxygen species production in rat brain homogenates. Neurotoxicol. Teratol. 2000, 22: 175–181.

Fernando H, Bhopale KK, Kondraganti S, Kaphalia BS, Shakeel Ansari GA. Lipidomic changes in rat liver after long-term exposure to ethanol. Toxicol Appl Pharmacol. 2011 Sep 1;255(2):127-37.

Folk J, Lees M, Stanley GA. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957; 226: 497-509.

Gao R, Yuan Z, Zhao Z, Gao X. Mechanism of pyrogallol autoxidation and determination of superoxide dismutase enzyme activity. Bioelectrochemistry and Bioenergetics 1998; 45(1):41-45.

Gao B, Bataller R. Alcoholic Liver Disease: Pathogenesis and New Therapeutic Targets. Gastroenterology 2011, 141: 1572-1585.

Garcia FAO, Tanae MM, Torres LMB, Lapa AJ, Lima-Landman MTR, Souccar C. A comparative study of two clerodane diterpenes from *Baccharis trimera* (Less.) DC. on the influx and mobilization of intracellular calcium in rat cardiomyocytes. Phytomedicine. 2014. 21: 1021-1025.

Gong P, Cederbaum AI. Nrf2 is increased by CYP2E1 in rodent liver and HepG2 cells and protects against oxidative stress caused by CYP2E1. Hepatology. 2006; 43:144–153.

Gramenzi A, Caputo F, Biselli M, Kuria F, Loggi E, Andreone P, Bernardi M. Review article: alcoholic liver disease – pathophysiological aspects and risk factors. Aliment Pharmacol Ther 2006. 24: 1151-1161.

Guimaraes FSF, Oliveira SM, Oliveira CC, Donatti L, Buchi DF: A shorter fixation protocol for transmission electron microscopy: An alternative to spend less time. Ultrastr Pathol 2009, 33(4):169-74.

Habig WH, Papst MJ, Jakoby WB. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 1974; 249:7130-7139.

Jiang, Z.Y., Hunt, J.V., Wolff, S.P., 1992. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. Anal Biochem 202, 384–389.

Kim E, Lee JH, Ntambi JM, Hyun CK. Inhibition of stearoyl-CoA desaturase 1 activates AMPK and exhibits beneficial lipid metabolic effects in vitro. Eur. J. Pharmacol. 2011, 672: 38 –44.

Kirpich IA, Solovieva NV, Leikhter SN, Shidakova NA, et al. Probiotics restore bowel flora and improve liver enzymes in human alcohol-induced liver injury: a pilot study. Alcohol. 2008, 42: 675-682.

Kleiner DE, Brunt EM, Van NM et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005, 41: 1313-1321.

Lamlé J, et al. Nuclear factor-eythroid 2-related factor 2 prevents alcohol-induced fulminant liver injury. Gastroenterology. 2008; 134:1159–1168.

Lermen C, Cagnini CZ, Pelisson DC, Girotto D, Rodirgues KPLC, Nicareta L, Poyer M, Castelli S, Blanco RF, Benedetti VP. Ação do óleo essencial obtido da carqueja – *Baccharis trimera* D.C. (Asteracae) sobre *Cândida albicans*. Biology and Healthy Journal, 2009, 3: 30-36.

Lieber CS. Microsomal ethanol-oxidizing system (MEOS): the first 30 years (1968– 1998) - a review. Alcohol Clin Exp Res 1999; 23: 991–1007.

Liu J. Ethanol and liver: recent insights into the mechanisms of ethanol-induced fatty liver. World J Gastroenterol, 2014, 28: 14672-14685.

Lívero FAR, Stolf AM, Dreifuss AA, et al., 2014. The FXR agonista 6ECDCA reduces hepatic steatosis and oxidative stress induced by ethanol and low protein diet in mice. Chem Biol Interact 217, 19-27.

Lívero FAR, Silva LM, Ferreira DM, et al., 2016. *Baccharis trimera* hydroethanolic extract promotes gastroprotection and healing of gastric lesions induced by acute and chronic ethanol consumption. “Submitted”.

Lu Y, Cederbaum AI. Autophagy protects against CYP2E1/chronic ethanol-induced hepatotoxicity. Biomolecules, 2015, 5: 2659-2674.

Lu Y, Zhang XH, Cederbaum AI. Ethanol induction of CYP2A5: Role of CYP2E1-ROS-Nrf2 pathway. Toxicological Sciences. 2012; 128: 427-438.

Malaguarnera M, Di Rosa M, Nicoletti F, Malaguarnera L. Molecular mechanisms involved in NAFLD progression. J Mol Med. 2009. 87: 679-695.

McClain CJ, Zhenyuan S, Shirish SB, Hill DB, Deaciuc I. Recent Advances in Alcoholic Liver Disease IV. Dysregulated cytokine metabolism in alcoholic liver disease. Am J Physiol Gastrointest Liver Physiol. 2004; 287: 497–502.

Miller AM, Horiguchi N, Jeong WI, Radaeva S, Gao B. Molecular mechanisms of alcoholic liver disease: innate immunity and cytokines. Alcohol Clin Exp Res 2011; 35: 787-793.

Nagata K, Suzuki H, Sakaguchi S. Common pathogenic mechanism in development progression of liver injury caused by non-alcoholic or alcoholic steatohepatitis. The Journal of Toxicological Sciences. 2007; 32: 453-468.

Orman ES, Odena G, Bataller R. Alcoholic liver disease: Pathogenesis, management, and novel targets for therapy. J Gastroenterol Hepatol. 2013 August ; 28(0 1): 77–84.

Oliveira ACP, Endringer DC, Amorim LAS, Brandão MGL, Coelho MM. Effect of the extracts and fractions of *Baccharis trimera* and *Syzygium cumini* on glycaemia of diabetic and non-diabetic mice. Journal of Ethnopharmacology. 2011, 102: 465-469.

Pádua BC, Silva LD, Júnior JVR, Humberto JL, et al. Antioxidant properties of *Baccharis trimera* in the neutrophils of Fisher rats. Journal of Ethnopharmacology. 2010, 129: 381–386.

Pádua BC, Júnior JVR, Magalhães CLB, Chaves MM, et al. Protective Effect of *Baccharis trimera* Extract on Acute Hepatic Injury in a Model of Inflammation Induced by Acetaminophen. Mediators of Inflammation. 2014, 2014: 196598.

Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70, 158–169.

Paul EL, Lunardelli A, Caberlon E, Oliveira CB, Santos RCV, Biolchi V, Bastos CMA, Moreira KB, Nunes FB, Grace G, de Oliveira JR. Anti-inflammatory and

immunomodulatory effects of *Baccharis trimera* aqueous extract on induced pleurisy in rats and lymphoproliferation *in vitro*. Inflammation 2009, 32: 419-425.

Polavarapu P, Spitz DR, Sim JE, Follansbee MH, Oberley LW, Rahemtulla A, Nanji AA. Increased lipid peroxidation and impaired antioxidant enzyme function is associated with pathological liver injury in experimental alcoholic liver disease in rats fed diets high in corn oil and fish oil. Hepatology 1998, 27: 1317-1323.

Prista, L.N., Correia, A.A., Morgado, R. Técnica Farmacêutica e Farmácia Galênica. Lisboa: Fundação Calouste Gulbenkian, 2.ed., 1975. v. 1, p. 1220.

Rehm J, Baliunas D, Borges GL, Graham K, Irving H, Kehoe T, et al. The relation between different dimensions of alcohol consumption and burden of disease − an overview. Addiction. 2010; 105: 817−43.

Rodrigues CRF, Dias JH, Mello RN, Richter MF, Picada JN, Ferraz ABF. Genotoxic and antigenotoxic properties of *Baccharis trimera* in mice. Journal of Ethnopharmacology. 2009, 125: 97-101.

Ronis MJ, Korourian S, Blackburn ML, Badeaux J, Badger TM. The role of ethanol metabolism in development of alcoholic steatohepatitis in the rat. Alcohol 2010, 44: 157-169.

Sakaguchi S, Takahashi S, Sasaki T, Kumagai T, Nagata K. Progression of alcoholic and non-alcoholic steatohepatitis: common metabolic aspects of innate immune system and oxidative stress. Drug Metab Pharmacokinetic 2011, 26: 30-46.

Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Analytical Biochemistry 1968; 25:192-205.

Schwabe RF, Maher JJ. Lipids in liver disease: looking beyond steatosis. Gastroenterology 2012, 142: 8-11.

Segawa S, Wakita Y, Hirata H, Watari J. Oral administration of heat-killed *Lactobacillus brevis* SBC8803 ameliorates alcoholic liver disease in ethanol-

containing diet-fed C57BL/6N mice. International Journal of Food Microbiology. 2008, 128: 371-377.

Soicke H, Leng-Peschlow E. Characterisation of flavonoids from *Baccharis trimera* and their antihepatotoxic properties. Planta Medica 53, 37-39.

Stickel, F., Shouval, D. Hepatotoxicity of herbal and dietary supplements: an update. Arch Toxicol 2015, 89: 851-865.

Tanaka Y, Ikeda T, Yamamoto K, Ogawa H, Kamisako T. Dysregulated expression of fatty acid oxidation enzymes and iron-regulatory genes in livers of Nrf2-null mice. J Gastroenterol Hepatol 2012; 27: 1711–1717.

Tanaka Y, Aleksunes LM, Yeager RL, et al. NF-E2-related factor 2 inhibits lipid accumulation and oxidative stress in mice fed a high-fat diet. J Pharmacol Exp Ther 2008; 325: 655–664.

Thompson KJ, McKillop IH, Schrum LW. Targeting collagen expression in alcoholic liver disease. World J Gastroenterol 2011 May 28; 17(20): 2473-2481.

Tsukamoto H, Machida K, Dynnyk A, et al. “Second hit” models of alcoholic liver disease. Semin Liver Dis. 2009; 29:178–187.

Wu D, Cederbaum AI. Oxidative stress mediated toxicity exerted by ethanol-inducible CYP2E1. Toxicol Appl Pharmacol. 2005, 207: 70-76

Xie Y, Feng B, Gao Y, Wei L. Characteristics of alcoholic fatty liver disease and predictive factors for mortality of patients with alcoholic cirrhosis. Hepatobiliary Pacreat Dis Int. 2013; 12: 594-601.

Xing H, Jia K, He J, Shi C, et al. Establishment of the tree shrew as an alcohol-induced fatty liver model for the study of alcoholic liver diseases. Plos One. 2015, 10: e0128253.

5. ARTIGO 3 – "*Baccharis trimera* hydroethanolic extract promotes gastroprotection and healing of gastric lesions induced by acute and chronic ethanol consumption."

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Baccharis trimera hydroethanolic extract promotes gastroprotection and healing of gastric lesions induced by acute and chronic ethanol consumption

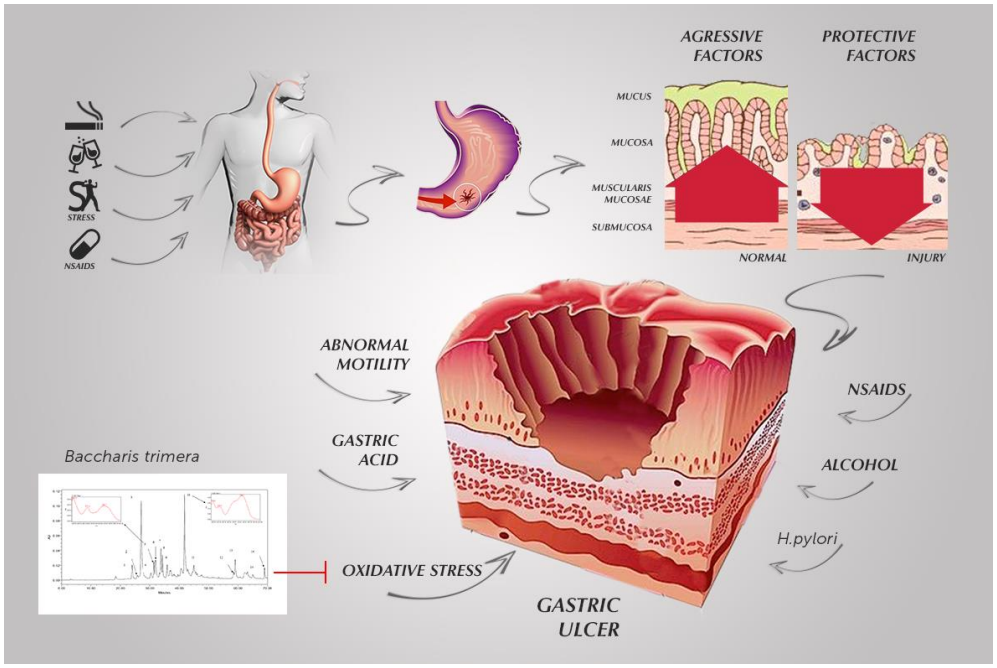
Francislaine Aparecida dos Reis Lívero[a], Luisa Mota da Silva[a], Daniele Maria Ferreira[a], Larissa Favaretto Galuppo[a], Debora Gasparin Borato[a], Thiago Bruno Lima Prando[b], Emerson Luiz Botelho Lourenço[b], Regiane Lauriano Batista Strapasson[c], Maria Élida Alves Stefanello[c], Maria Fernanda de Paula Werner[a], Alexandra Acco[a]

[a] Department of Pharmacology, Federal University of Paraná, Curitiba, PR, Brazil

[b] Laboratory of Toxicology and Pharmacology, Paranaense University, Umuarama, PR, Brazil

[c] Department of Chemistry, Federal University of Paraná, Curitiba, PR, Brazil

Graphical Abstract



Abstract

Purpose Ethanol is a psychoactive substance highly consumed around the world whose health problems include gastric lesions. Baccharis trimera is used in folk medicine for the treatment of gastrointestinal disorders. However, few studies have evaluated its biological and toxic effects. To validate the popular use of B. trimera and elucidate its possible anti-ulcerogenic and cytotoxic mechanisms, an hydroethanolic extract of B. trimera (HEBT) was evaluated in models of gastric lesions. **Methods** HEBT was characterized using HPLC. Rats and mice were used to evaluate the protective and anti-ulcerogenic effects of HEBT on gastric lesions induced by ethanol, acetic acid, and chronic ethanol consumption. The effects of HEBT were also evaluated in a pylorus ligature model and on gastrointestinal motility. The LD50 of HEBT in mice was additionally estimated. **Results** HEBT presented caffeoylquinic acids, which contributed to the HEBT therapeutic efficacy, preventing or reverting ethanol- and acetic acid-induced ulcers, respectively. Oral HEBT administration significantly reduced the lesion area and the oxidative stress induced by acute and chronic ethanol consumption. However, HEBT did not protect against gastric wall mucus depletion and did not alter gastric secretory volume, pH, or total acidity in the pylorus ligature model. Histologically, HEBT accelerated the healing of chronic gastric ulcers in rats, reflected by contractions of the ulcer base. HEBT antiulcerogenic activity may be partially attributable to the inhibition of free radical generation and subsequent prevention of lipid peroxidation. **Conclusion** Our results indicate that HEBT has both gastroprotective and curative activity in animal models, with no toxicity.

Key words: gastric ulcer, ethanol, oxidative stress, anti-ulcerogenic, Baccharis trimera

Abbreviations: NSAIDs: antiinflammatory drugs; ROS: reactive oxygen species; B. trimera: Baccharis trimera; HEBT: hydroethanolic extract of B. trimera; NMR: nuclear magnetic resonance; HPLC: high performance liquid chromatography; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; WV: water+vehicle; EV: ethanol+vehicle; EHEBT: ethanol+HEBT; GSH: reduced glutathione; LOOH: lipid hydroperoxide; SOD: superoxide dismutase; GST: glutathione S-

transferase; LD50: median lethal dose; ROS: reactive oxygen species; SEM: standard error of the mean.

1. Introduction and background

Alcoholic beverages have been consumed worldwide for centuries in cultural, social, religious, and medical contexts (Chai 2011). An estimated 2 billion people consume ethanol worldwide, and 76.3 million have ethanol-related disorders (WHO 2008). Among the systems of the body that are affected by prolonged ethanol exposure, the gastrointestinal tract deserves special attention because gastric lesions are a frequent problem in ethanol abusers. Direct contact between ethanol and mucosa induces many functional and metabolic modifications (Chai 2011). Damage to the stomach in alcoholics differs from damage to other organs, such as the liver, because ethanol consumption affects the upper gastrointestinal tract through multiple and complex mechanisms. These mechanisms depend on contact with ethanol that can cause direct mucosal damage (Franke et al. 2005) or nonalcoholic components (e.g. fermentation products) (Rocco et al. 2014). Thus, alcoholism is considered an independent risk factor for the initiation and complications associated with ulcerative disease, similar to smoking, stress, Helicobacter pylori infection, and the chronic use of nonsteroidal antiinflammatory drugs (NSAIDs) (Birdane et al. 2007; Galuska et al. 2002; Gisbert and Pajares 2003).

Gastric ulcers are a common disorder of the gastrointestinal tract that affects millions of people around the world (O'Malley 2003). The development of gastric ulcers is a multifactorial process that occurs through an imbalance between aggressive (e.g., acid secretion and pepsin) and protective (e.g., mucus barrier, bicarbonate secretion, and antioxidant defenses) factors that are present in gastric mucosa (Choi et al. 2009). Additionally, chronic ethanol consumption can promote gastric ulceration by decreasing mucus production, cell proliferation, and mucosal blood circulation by increasing (ROS) production and causing an exaggerated inflammatory response (Issac et al. 2015; Takeuchi 2012).

The available strategies for the treatment of gastric ulcers include acid-suppressant drugs, such as type-2 histamine receptor antagonists (H2-RAs), proton pump inhibitors (PPIs), antibiotic drugs for the eradication of H. pylori,

and NSAID withdrawal (Kangwan et al. 2014). However, acid-suppressant drugs have failed to meet pharmacological expectations, and side effects have been reported, such as osteoporosis, hypergastrinemia, the hyperplasia of enterochromaffin-like cells, and the development of carcinoids in gastric mucosa (DeVault and Talley 2009; Eom et al. 2011; Penston and Wormsley 1987; Poynter et al. 1985; Sheen and Triadafilopoulos 2011). Moreover, this therapeutic approach is insufficient for complete ulcer healing and intimately associated with ulcer recurrence (Kangwan et al. 2014). In light of these considerations, there has been growing interest in the development of new pharmacological agents with protective effects against gastric ulcers that present good efficacy and fewer side effects.

Baccharis trimera (Less.) DC, popularly known as “carqueja” in Brazil, is a plant of the Asteraceae family that is often used in traditional medicine as a treatment for or prevention against gastrointestinal and hepatic diseases (Garcia et al. 2014). Its use was inherited from indigenous since centuries ago (Losqui et al. 2009). Several biological activities reported for B. trimera, including antihepatotoxic, antidiabetic, schistosomicidal, antioxidant, antinociceptive, and antiinflammatory effects are attribute to flavonoids, diterpenes, triterpenes, saponins, essential oils and caffeoyl quinic acids (Gené et al. 1996; Oliveira et al. 2005; Oliveira et al. 2012; Simões-Pires et al. 2005; Soicke and Leng-Peschlow 1987). Some studies also showed *B. trimera* antiulcerogenic activity. In a stress-induced ulcer model and pylorus ligature model, Biondo et al. (2011) reported the antiulcer and antisecretory activity of 1000 and 2000 mg·kg-1 dose of aqueous *B. trimera* extract. Mendonça et al. (2013) also described anti-ulcer activity of B. trimera in a stress-induced ulcer model and acute gastric ulcer induced by ethanol using 100, 200 and 400 mg·kg-1 dose of hydroethanolic extract. Finally, Dias et al. (2009) described protective effects of these doses of hydroethanolic *B. trimera* extract in a hydrochloric-induced ulcer model.

In despite of described antiulcerogenic activity of *B. trimera*, the reports in the literature are insufficient to elucidate the gastroprotective mechanisms that are promoted by this plant. Additionally, the studies described antiulcerogenic effects with high doses of extract, which makes unfeasible the proposition of *B. trimera* as a possible pharmacological agent for the treatment of gastric ulcer.

Beyond that, the researchers did not evaluate the gastric antioxidant system, which is strongly involved in the pathogenesis of gastric ulcer. Finally, there are no studies evaluating the effects of chronic ethanol consumption on the stomach neither the possible protective effects of *B. trimera* in this situation.

Considering these, the present study (1) evaluated the gastroprotective effects of lower doses of hydroethanolic extract of *B. trimera* (HEBT) against acute and chronic ethanol exposure, (2) the gastric ulcer healing activity of HEBT in acetic acid-induced chronic ulcers, (3) the possible action mechanisms of HEBT, (4) the effects of HEBT on gastric emptying and intestinal motility, and (5) investigated the toxicity of HEBT.

2. Material and methods

2.1 Chemicals

The following substances were used: Alcian blue, bovine serum albumin, 2',7'-dichlorofluorescein-diacetate, 2,2-diphenyl-1-picrylhydrazyl, 5,5'-dithiobis (2-nitrobenzoic acid), evans blue, reduced glutathione, omeprazole, pyrogallol, and xylenol orange (all from Sigma, St. Louis, USA); absolute ethanol (P.A), absolute methanol (P.A), acetic acid, ascorbic acid, diethyl ether, N,N-dimethylformamide, formaldehyde, hydrogen peroxide, magnesium chloride, sodium acetate, sodium carbonate, sucrose, and trichloroacetic acid (Vetec, Rio de Janeiro, Brazil). For chemical analyses, methanol (HPLC grade, Panreac), trifluoroacetic acid (analytical grade, Vetec), and ultra-pure water from a Milli-Q system were used for the eluent preparation. Deuterated methanol (CD3OD; Aldrich) was used for NMR analyses. 3,5-O-[E]-dicaffeoylquinic acid, previously isolated and identified by NMR (Strapasson et al. 2015), was used as authentic chemical reference.

2.2 Plant material and preparation of HEBT

Botanical material (aerial parts of *Baccharis trimera* [Less.] DC) was harvested in the Garden of Medicinal Plants of Paranaense University (UNIPAR), Umuarama, Paraná, Brazil, which is located at 430 m of altitude above sea level (coordinates 23°47′55'' S, 53°18′48'' W). A voucher specimen (no. 2220) was deposited in the Herbarium of UNIPAR. The material was dried in an oven with forced air circulation at 37°C for 5 days. After drying, it was

ground. Pulverization was performed in industrial crushers, and the material was placed in plastic bags until utilization. The hydroethanolic extract (ethanol: water, 9:1) was prepared by soaking at room temperature (Prista et al. 1975) until exhaustion, filtered, and concentrated at reduced pressure in a rotatory evaporator, with the temperature not exceeding 55°C. The final yield of dried *B. trimera* extract was 9.51%. After complete removal of the organic solvent, the residue was frozen in liquid nitrogen and freeze-dried. The final product (HEBT) was stored in a tightly sealed glass bottle and kept in a freezer until its utilization. The freeze-dried extract was dissolved in 2 ml of distilled water and 20 µl of 2% Tween immediately before the experiments.

2.3 Chemical analysis of HEBT

Nuclear magnetic resonance (NMR) spectra (^{1}H, HSQC and HMBC) of HEBT were recorded on a Bruker Avance 400 spectrometer, observing ^{1}H at 400 MHz and ^{13}C at 100 MHz, respectively. The solvent was CD_3OD, with TMS as the internal reference.

The high-performance liquid chromatography (HPLC) fingerprint analysis of HEBT was performed using a Waters high-performance liquid chromatograph equipped with a 2998 photodiode array detector and Waters X-Terra C18 column (250 × 4.6 mm, 5 µm particle size). The eluent consisted of MeOH-H_2O with 0.05% trifluoroacetic acid, applied in a linear gradient from 10:90 to 100:0 over 70 min. The flow rate was 1 ml·min^{-1}. The column effluent was monitored at 254 and 325 nm.

2.4 In vitro free radical scavenging activity of HEBT

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical that has been widely used as a tool to estimate the free radical scavenging activity of antioxidants. The reduction capacity of the DPPH radical was determined by the decrease in absorbance that was induced by antioxidants according to Blois (1958) and Chen et al. (2004), with modifications. To determine whether HEBT has free radical scavenging activity, different concentrations (1, 10, 100, and 1000 µg·ml^{-1}) were mixed with DPPH methanolic solution (10 µg·ml^{-1}). Ascorbic acid (50 µg·ml^{-1}) was used as a positive control, and distilled water was used as a negative control. The absorbance was measured at 517 nm using a

spectrophotometer and the values were interpolated into a standard curve (0.0 to 60.0 µg of DPPH) and are expressed as µM of DPPH.

2.2 Animals

The ethanol-induced gastric ulcer, acetic acid-induced chronic gastric ulcer, and pylorus ligature experiments were conducted using adult female Wistar rats, 8-10 weeks old and weighing 180-200 g. Swiss male or female mice, 8-10 weeks old and weighing 25-35 g, were used to evaluate the effects of chronic ethanol consumption on the stomach, the effects of HEBT on gastrointestinal motility, and the LD_{50} (median lethal dose) of HEBT. The rodents were housed at 22 ± 2°C under a 12 h/12 h light/dark cycle with *ad libitum* access to food and water. They were food-deprived for 12 h prior to the experiments. All of the experiments were approved by the Institutional Ethics Committee of the Federal University of Paraná (approval no. 810 and 619) and were performed in accordance with international standards and ethical guidelines on animal welfare.

2.3 Animal models used to evaluate HEBT gastroprotection

First of all the *in vivo* models used for investigating gastroprotection are described (section 2.6). The biological materials collected from these experiments were further processed in order to answer the aims of this study. The procedures realized with the collected material are sequentially described (sections 2.7 to 2.11).

2.3.1 Induction of acute gastric ulcers by ethanol in rats

Acute gastric ulcers were induced by the intragastric administration of ethanol P.A. as described by Robert et al. (1979). Omeprazole has been previously shown to inhibit ethanol-induced gastric lesion formation (Burci et al. 2013), so it was used as a positive control for lesion inhibition. Rats (n = 8) were pretreated with vehicle (control; water plus 2% Tween, 1 $ml \cdot kg^{-1}$, p.o.), omeprazole (40 $mg \cdot kg^{-1}$, p.o.), or HEBT (3, 10, and 30 $mg \cdot kg^{-1}$, p.o.) 1 h before the oral administration of ethanol P.A. (0.50 $ml \cdot 200\ g^{-1}$). One hour after ethanol administration, the rats were euthanized by cervical dislocation. The stomachs were removed and opened throughout the great curvature. The lesion area was

measured as length (mm) × width (mm) using Image Tool 3.0 software. The dose of 30 mg·kg^{-1} was chosen to evaluate all other parameters in this study.

2.3.2 Induction of chronic ulcers by acetic acid in rats

Chronic gastric ulcers were induced by acetic acid as described previously (Okabe et al. 1971), with modifications. The rats (*n* = 6) were anaesthetized with 7.50 mg·kg^{-1} xylazine and 60 mg·kg^{-1} ketamine (i.p.). After laparotomy, the stomach was exposed, and 80% acetic acid (v/v, 0.50 ml) instilled into a cylinder (6 mm diameter) was applied to the serosal surface of the stomach for 1 min. Acetic acid was removed by aspiration, the area of contact was washed with sterile saline, the stomach was replaced and the cavity sutured. Forty-eight hours after ulcer induction, the rats were orally treated with vehicle (water plus 2% Tween, 1 ml·kg^{-1}), omeprazole (40 mg·kg^{-1}), or HEBT (30 mg·kg^{-1}) twice daily for 7 days.

On the day following the last treatment, the animals were euthanized by cervical dislocation. The stomachs were removed and opened throughout the great curvature. The total ulcer area was measured as length (mm) × width (mm) using Image Tool 3.0 software.

2.3.3 Chronic ethanol consumption in mice

The effects of chronic ethanol consumption on the stomach were evaluated using the model proposed by Lívero et al. (2014). The mice were separated into individual cages, and food intake, fluid consumption, and body weight were controlled. During 6 experimental weeks, the mice received a low-protein diet (chow with 6% protein) and fluid that contained either 10% ethanol (*n* = 16) or water (*n* = 12). In the last 2 weeks, the animals were redistributed into four groups for the initiation of treatment with HEBT (30 mg·kg^{-1}, p.o.) or vehicle (water plus 2% Tween, 1 ml·kg^{-1}) once per day for 14 days. At the same time, we evaluate a naive group (normal-protein diet, chow with 23% protein). Thus, the final groups were the following: Naive (water + vehicle, *n* = 6), WV (negative control; water + vehicle, *n* = 6), EV (positive control; ethanol + vehicle, *n* = 8), and EHEBT (ethanol + 30 mg·kg^{-1} HEBT, *n* = 8). At the end of 6 experimental weeks, the animals were fasted for 12 h and then anesthetized with 80 mg·kg^{-1} ketamine and 10 mg·kg^{-1} xylazine intraperitoneally. Laparotomy

was performed to collect the stomach, and the animals were euthanized by puncture of the diaphragm, under anesthesia.

2.3.4 Induction of hypersecretion by pylorus ligature in rats

Pylorus ligature was carefully performed in fasted rats under anesthesia (Shay et al. 1945). The pylorus was located and ligated with a suture to maintain the gastric content in the stomach. The animals (n = 6) were treated with vehicle (water plus 2% Tween, 1 ml·kg^{-1}, intraduodenal [i.d.], p.o., or intraperitoneal [i.p.]), omeprazole (40 mg·kg^{-1}, p.o.), or HEBT (30 mg·kg^{-1}, i.d.; 30 mg·kg^{-1}, p.o.; or 30 mg·kg^{-1}, i.p.) immediately after (i.d. or i.p.) or 1 h before (p.o.) pylorus ligature. Four hours after pyloric ligation, the animals were euthanized by cervical dislocation. The stomach was opened, and gastric secretions were collected.

2.3.5 Determination of gastrointestinal motility

Fasted female Swiss mice (n = 6) were orally treated with HEBT (30 mg·kg^{-1}) or vehicle (water plus 2% Tween, 1 ml·kg^{-1}) or subcutaneously treated with atropine (3 mg·kg^{-1}) 60 min before oral administration of 0.50 ml of a semi-solid solution of 0.05% phenol red and 1.50% methylcellulose. After 15 min, the animals were euthanized by cervical dislocation, and the stomach and small intestine were quickly removed. Determinations were made using a modification of a previously described method (Suchitra et al. 2003). Gastric emptying was measured as the amount of marker that remained in the stomach at the end of the experiment. The stomachs were individually homogenized with 7 ml of distilled water and centrifuged at 1500 × g for 15 min. Equal amounts (1 ml) of supernatant and 0.025 M NaOH were mixed, and absorbance was read at 560 nm using a spectrophotometer. Gastric emptying (%GE) was calculated using the following equation: $\%GE = 100 - (X \times 100 \cdot Y^{-1})$, where X is the absorbance of phenol red recovered from the stomach in animals that were euthanized 15 min after marker administration, and Y is the mean (n = 8) absorbance of phenol red that was recovered from the stomach in control animals that were euthanized immediately after marker administration.

Intestinal transit was measured as the distance travelled by the marker in the small intestine. Briefly, the small intestine was dissected from the pylorus to

the ileocaecal junction. The total length of the small intestine and distance travelled by phenol red were then measured. Intestinal transit was calculated as the following: $\%IT = X \cdot Y^{-1} \times 100$, where X is the distance travelled by phenol red, and Y is the total length of the small intestine.

2.4 Preparation of stomach homogenate

Stomach samples from rats that were exposed to acute gastric lesions induced by ethanol P.A. and mice subjected to chronic consumption of 10% ethanol were homogenized with 200 mM potassium phosphate buffer, pH 6.5. The homogenate was used to measure reduced glutathione (GSH) and hydroperoxide (LOOH) levels. The material was then centrifuged at 9000 × *g* for 20 min, and the supernatant was used to determine superoxide dismutase (SOD), glutathione-*S*-transferase (GST) and protein levels.

2.5 Evaluation of antioxidant system

2.5.1 *Determination of reduced glutathione (GSH) levels*

Reduced glutathione levels were determined in gastric mucosa according to the method of Sedlak and Lindsay (1968). Aliquots of tissue homogenate were mixed with 12.50% trichloroacetic acid, vortexed, and centrifuged for 15 min at 6000 × *g.* The supernatant was reserved, and TRIS buffer (0.40 M, pH 8.9) and 5,5'-dithiobis 2-nitrobenzoic acid (DTNB; 0.01 M) were added. Absorbance was read at 415 nm using a spectrophotometer. The procedures were performed at 4°C, and the individual values were interpolated into a standard curve of GSH (0.37-3.0 $\mu g \cdot ml^{-1}$) and are expressed as $\mu g \cdot g$ of $tissue^{-1}$.

2.5.2 *Determination of lipid hydroperoxide (LOOH) content*

The levels of gastric LOOH were determined using the ferrous oxidation-xylenol orange (FOX2) method as described by Jiang et al. (1992). Briefly, 100 µl of methanol P.A. was added to 100 µl of supernatant, vortexed, and centrifuged at 9700 × *g* for 5 min at 4°C. The supernatant was mixed with FOX2 reagent (4 mM butylated hydroxytoluene [BHT], 250 mM $FeSO_4$, 25 mM H_2SO_4, and 100 mM xylenol orange) and incubated in the dark for 30 min at room

temperature. Absorbance was read at 560 nm in a microplate reader, and the concentration of LOOH is expressed as mmol hydroperoxide·mg of protein^{-1}.

2.5.3 *Determination of enzymatic activity of superoxide dismutase (SOD)*

Superoxide dismutase activity was measured in terms of the ability of SOD to inhibit pyrogallol autoxidation, according to Gao et al. (1998). Pyrogallol (1 mM) was added to buffer solution (200 mM Tris ethylenediaminetetraacetic acid, pH 8.5) and gastric glandular supernatant aliquots and then vortexed for 1 min. The reaction was incubated for 30 min at room temperature and stopped with the addition of 1N HCl. The absorbance of the resulting supernatant was measured at 405 nm using a spectrophotometer. The amount of SOD that inhibited the oxidation of pyrogallol by 50% (relative to the control) was defined as one unit of SOD activity.

2.5.4 Determination of glutathione S-transferase (GST) activity

Glutathione *S*-transferase activity was measured using the method of Habig et al. (1974). Reactions were performed in the presence of supernatant aliquots, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), 1 mM GSH, and 100 mM potassium phosphate buffer (pH 6.5) at room temperature. The conjugation of CDNB with GSH was monitored at 340 nm for 180 s. Specific activity was calculated using an extinction coefficient of 9.6·mM^{-1}·cm^{-1} for GSH, and the results are expressed as mmol·min^{-1}·mg of protein^{-1}.

2.8.5 Protein assay

The protein content in stomach tissue was measured to express the results of the oxidative stress parameters. Protein concentrations of the supernatants were determined by the Bradford method (Bio-Rad®, Hercules, CA, USA) using bovine serum albumin (125-1000 µg) as the standard and performed according to the manufacturer's instructions. 10 µL of supernatant was mixed with 250 µL of Bradford solution. Reading was performed at 595 nm and values are expressed as mg of protein.

2.6 Determination of gastric wall mucus

Gastric mucus was measured using glandular segments of the mucosa with or without acute lesions induced by ethanol P.A. The gastric tissues were weighed and immediately transferred to 0.10% Alcian blue solution prepared in 50 mM sucrose and 0.16 mM sodium acetate (pH 5) and stained for 2 h at room temperature. Excess dye was removed by two successive rinses with sucrose solution (0.25 mM), and the mucus-dye complex was extracted with 0.50 mM magnesium chloride solution, which was shaken for 1 min during 2 h at 30 min intervals. The resultant blue extract was then mixed with an equal volume of diethyl ether and centrifuged at 16100 × *g* for 10 min. The absorbance of the supernatant was read at 598 nm using a spectrophotometer. The amount of mucus was calculated using a standard curve for Alcian blue (6.25-100 $\mu g \cdot ml^{-1}$), and the results are expressed as µg of Alcian blue·g $tissue^{-1}$ (Corne et al. 1974).

2.7 Quantification of peptic activity

Measurements of volume and total gastric acidity were performed immediately after collecting gastric acid produced 4 h after pyloric ligation, as described previously (Baggio et al. 2005). To quantify pepsin activity, 100 µl of gastric acid secretions was collected, transferred to polypropylene tubes, and incubated with 500 µl of bovine albumin solution (5 $mg \cdot ml^{-1}$ in 0.06 N HCl) at 37°C for 10 min. The reaction was stopped by adding 500 µl of 10% trichloroacetic acid and centrifuged at 1500 × *g* for 20 min. The supernatant (1 ml) was then separated and alkalized with 5 ml of 0.55 M sodium carbonate. Afterward, 500 µl of 1N Folin reagent was added to the tubes and incubated for 30 min at room temperature. A volume of 300 µl from each tube was then transferred to a microplate, and absorbance was read at 660 nm using a spectrophotometer. Individual values were interpolated with a tyrosine standard curve (30-1000 $mmol \cdot ml^{-1}$), and the results are expressed as mmol of tyrosine·4 h^{-1} (Anson, 1938).

2.8 Histology

Stomach histology was performed to evaluate microscopic alterations that were induced by the aforementioned models (sections 2.6.1 to 2.6.3) and

recovery promoted by HEBT. Samples of gastric ulcers were quickly harvested, fixed in ALFAC solution (alcohol 80 ºGL, formaldehyde at 40% and glacial acetic acid), dehydrated with alcohol and xylene, embedded in paraffin wax, and sectioned at 6 µm for histological evaluation after hematoxylin/eosin (HE) staining. The gastric sections were observed and photographed with a slide scanner from MetaSystems (MetaViewer version 2.0.100) at 20 and 100× magnification.

2.9 In vivo toxicological effects of HEBT

The acute toxicity of HEBT was determined as the LD_{50}. Male Swiss mice (25-35 g) were separated into four groups (n = 6) that received one dose of HEBT (50, 500, 1000, and 5000 $mg \cdot kg^{-1}$) by oral gavage, and another group received one intraperitoneal dose of HEBT (1000 $mg \cdot kg^{-1}$). These animals were compared with the control group, which received 2% Tween solution (vehicle) orally.

The mice were monitored for the first 30 min post-administration and 1, 2, 3, and 4 h after treatment. Behavioral parameters and clinical symptoms (grooming, piloerection, dyspnea, ptosis, abdominal contraction, diarrhea, prostration, ataxia, anesthesia, coma and death) were recorded according to the methodology described by Almeida et al. (1999). After the first 4 h of observation, the animals received water and food and were observed daily for the next 14 days to record alterations or deaths. Water and food consumption were monitored during all the experiment. After this period, mice were anesthetized with isoflurane and underwent laparotomy to perform macroscopic observations of the liver, kidneys, adrenal glands, spleen, and lungs. The animals were then euthanized by puncture of the diaphragm.

2.10 Statistical analysis

The data were analyzed for homogeneity of variance and a normal distribution. Differences between means were determined by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The level of significance was set at 95% ($p < 0.05$). The data are expressed as mean ± standard error of the mean (SEM). GraphPad Prism 5.0 software was used to perform the statistical analysis and generate the graphs.

3 Results

3.1 Chemical characterization of HEBT

The main classes of compounds in HEBT were investigated by detailed analysis of selected regions of the NMR 1D and 2D spectra and HPLC-UV/PAD. The ^{1}H NMR spectrum of HEBT showed signals for aliphatic (δ 0.60-2.80), oxy-aliphatic (δ 3.20-5.30), olefinic (δ 5.30-5.50 ppm), and aromatic (δ 6.30-8.00) protons. An intense doublet at δ 5.40 (*J* = 3.90 Hz) showed a correlation with a carbon at δ 93.30 in the HSQC and cross-peaks in the HMBC with carbons at δ 74.50 and 104.40, suggesting the presence of free sucrose (Moccelini et al. 2009). Caffeoyl moieties were easily recognized in the ^{1}H NMR spectrum as doublets in δ 7.60 and 6.30 (*J* = 16 Hz), together with several multiplets around δ 7.00, 6.90, and 6.80. The protons at δ 7.60 showed correlations in the HSQC spectrum with carbons at δ 146.6 and cross-peaks in the HMBC with carbons at δ 115.30, 123.20, and 169.70, consistent with data for caffeoyl quinic acids (Lee et al. 2010), which were previously reported in *B. trimera* (Abboy et al. 2012; Simões-Pires 2005). The ^{1}H NMR spectrum also showed multiplets at δ 6.70, which presented a correlation in the HSQC with the carbon at δ 137.50, and cross-peaks in the HMBC with carbons at δ 47.20 and 172.40. These data are compatible with clerodane diterpenes that were previously isolated from *B. trimera* (Herz et al. 1977; Januário et al. 2004).

HEBT was analyzed by HPLC-UV/PAD using the method proposed by Simões-Pires et al. (2005). The chromatogram (Fig. 1) showed a different profile from the one previously reported under the same analytical conditions. The UV spectra at peaks **1**-**3** and **7**-**12** were characteristic of the caffeoyl group (Aboy et al. 2012; Simões-Pires 2005), whereas peaks **4**-**6** showed UV absorption that is typical of flavones (Rijke et al. 2006). Considering the elution sequence and comparisons with the literature (Aboy et al. 2012; Simões-Pires 2005), peaks **1**-**3** were identified as caffeoylquinic acid (CQA), peaks **7**-**9** were identified as dicaffeoylquinic acid (diCQA), and peaks **10**-**12** were identified as tricaffeoylquinic acid (triCQA). Peak **8** was identified as 3,5-*O*-[*E*]-dicaffeoylquinic acid by comparison with an authentic sample.

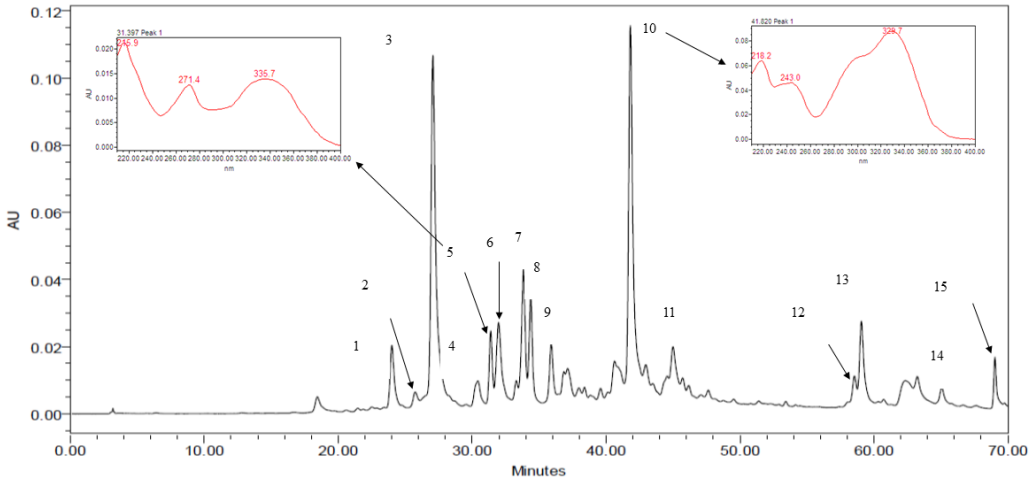


Figure 1 Chromatogram of the HEBT. The UV spectra of the peaks **1-3** identified monocaffeoylquinic acids, peaks **4-6** showed UV absorptions of flavones and peaks **7-9** and **10-12** were characteristic of dicaffeoylquinic and tricaffeoylquinic acids, respectively.

3.2 *Effect of HEBT on acute gastric lesions induced by ethanol*

Oral administration of HEBT (3, 10, and 30 mg·kg^{-1}) 1 h before the induction of gastric lesions with ethanol P.A. significantly reduced the lesion area by 68%, 63%, and 79%, respectively, compared with the control group (99.50 ± 14.50 mm^2). Omeprazole (the positive control) inhibited gastric lesions by 99% (Fig. 2A). The microscopic observations (data not shown) are in accordance with the macroscopic appearance of the acute gastric lesions that were induced by ethanol and treated with vehicle, omeprazole, or HEBT (Fig. 2B-D, respectively). Considering that all of the doses of HEBT effectively reduced the lesion area and because we sought to reduce the number of animals used in the experiments, we chose the 30 mg·kg¹ dose of HEBT for the subsequent analysis of the other parameters.

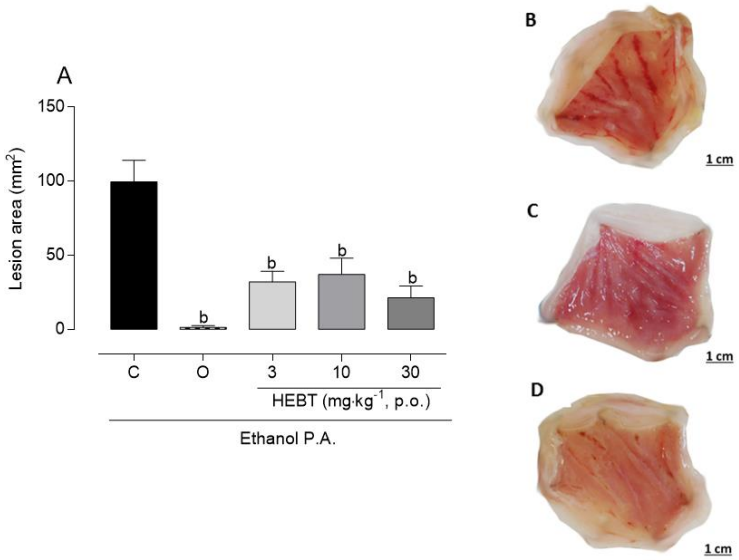


Figure 2 (A) Effect of HEBT on acute gastric lesions induced by ethanol P.A. in rats treated with vehicle (C: saline, 1 ml·kg^{-1}, p.o.), omeprazole (O: 40 mg·kg^{-1}, p.o.) or HEBT (3, 10 or 30 mg·kg^{-1}, p.o.) 1 hour before oral administration of ethanol P.A. (0.50 ml·200g^{-1}). Values are expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Tukey's test. Symbol: ^{b} $p<0.05$, when compared with control group. **(B-D)** Representative macroscopic photograph of stomachs of experimental acute gastric lesions induced by ethanol P.A. The animals were pretreated orally with vehicle (water plus 2% tween, 1 ml·kg^{-1}, panel **B**), omeprazole (40 mg·kg^{-1}, panel **C**) or HEBT (30 mg·kg^{-1}, panel **D**). Bars = 1 cm.

3.3 Effect of HEBT on gastric wall mucus

Ethanol P.A. administration decreased gastric mucus levels by approximately 49% compared with the non-lesioned group (naive: 1435 ± 174 µg of alcian blue·g of tissue^{-1}). However, treatment with HEBT and omeprazole did not restore mucus levels (Fig. 3).

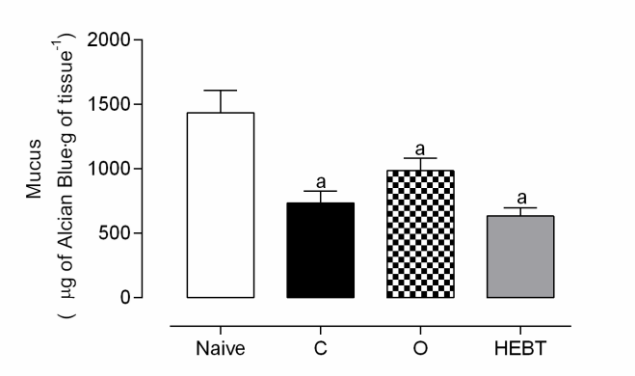


Figure 3 Effect of HEBT on gastric wall mucus of rats submitted to acute gastric lesions induced by ethanol and treated with vehicle (C: saline, 1ml·kg^{-1}, p.o.), omeprazole (O: 40 mg·kg^{-1}, p.o.) or HEBT (30 mg·kg^{-1}, p.o.). Values are expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Tukey's test. Symbol: ^{a} $p<0.05$, when compared with naive group.

3.4 *Effect of HEBT on markers of gastric oxidative stress in acute gastric lesions induced by ethanol*

The administration of ethanol P.A. decreased GSH levels and SOD activity by 19.50% and 69.81%, respectively, compared with the non-lesioned group (naive: 214.20 ± 6.20 µg GSH·g of tissue^{-1} and 47.91 ± 0.78 U SOD·mg of protein^{-1}; Fig. 4A and B, respectively) and increased GST activity by 165% compared with the non-lesioned group (naive: 2.00 ± 0.20 µmol of GST·min^{-1}·mg of protein^{-1}; Fig. 4C). Treatment with HEBT and omeprazole prevented the decrease in GSH but not the decrease in SOD activity or increase in GST activity. The administration of ethanol P.A. also increased LOOH levels by 409% compared with the non-lesioned group (naive: 27.50 ± 4.30 mmol LOOH·mg of protein^{-1}; Fig. 4D). HEBT and omeprazole reduced LOOH levels to 116.50 ± 2.60 and 111.60 ± 4.60 mmol·mg of protein^{-1}, respectively (Fig. 4D).

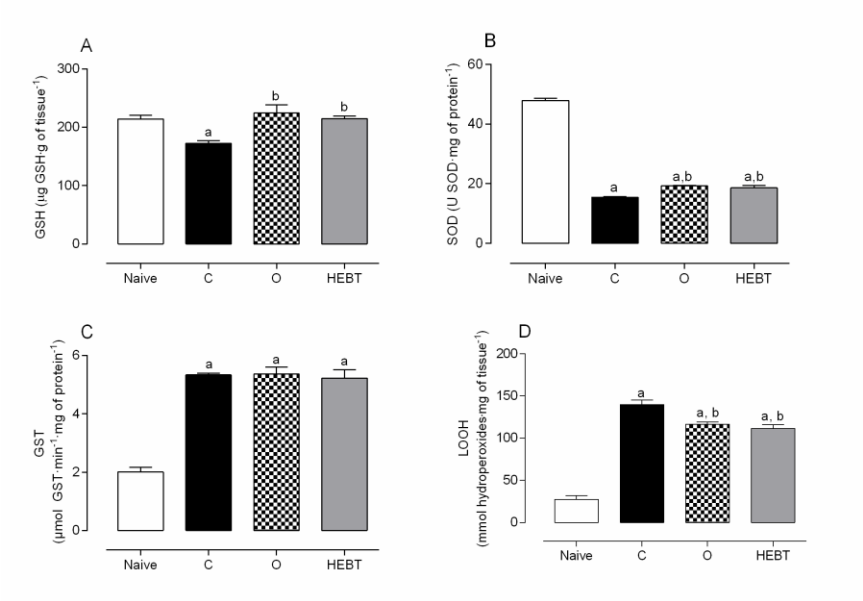


Figure 4 Gastric **(A)** reduced glutathione (µg GSH·g of tisse^{-1}), **(B)** superoxide dismutase (U SOD·mg protein^{-1}), **(C)** glutathione-*S*-Transferase (µmol GST·min^{-1}·mg protein^{-1}) and **(D)** LOOH levels (mmol hydroperoxides·mg of protein^{-1}) of rats submitted to acute gastric lesions induced by ethanol and treated with vehicle (C: saline, 1ml·kg^{-1}, p.o.), omeprazole (O: 40 mg·kg^{-1}, p.o.) or HEBT (30 mg·kg^{-1}, p.o.). Values are expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Tukey's test. Symbols: ^{a} $p<0.05$, when compared with naive group and ^{b} $p<0.05$, when compared with control group.

3.5 Effect of HEBT on gastric mucosa in mice exposed to *chronic ethanol consumption and the antioxidant activity of HEBT in vitro*

Although no macro- and microscopic lesions were observed in gastric mucosa in mice that were exposed to chronic 10% ethanol consumption and treated with vehicle or HEBT for 14 days (data not shown), several alterations were found in antioxidant system, which is closely related with the gastric ulcerations. When the animals received ethanol, the levels of GSH and GST decreased by 23.52% and 43.80%, respectively, compared with the naive group (0.51 ± 0.02 µg GSH·g of tissue^{-1} and 6.21 ± 0.29 µmol·min^{-1}·mg of protein^{-1}; Fig. 5A and C, respectively). Chronic ethanol consumption also increased LOOH levels and SOD activity by 60.71% and 248.67%, respectively, compared with the naive group (93.89 ± 7.35 mmol LOOH·mg of protein^{-1} and 8.69 ± 0.50 U SOD·mg of protein^{-1}; Fig. 5D and B, respectively). Interestingly, oral

treatment with HEBT restored GSH levels, GST activity, and LOOH content to basal levels and normalized SOD activity (Fig. 5).

Corroborating the *in vivo* antioxidant capacity of HEBT, the *in vitro* DPPH test showed that HEBT concentration-dependently scavenged DPPH radicals, with an IC_{50} of 1.707 $\mu g \cdot ml^{-1}$ (Fig. 6). Ascorbic acid was used as a positive control, which reduced DPPH levels by 82.50% compared with water (the negative control; 57.98 ± 2.28 $\mu g \cdot ml^{-1}$; Fig. 6).

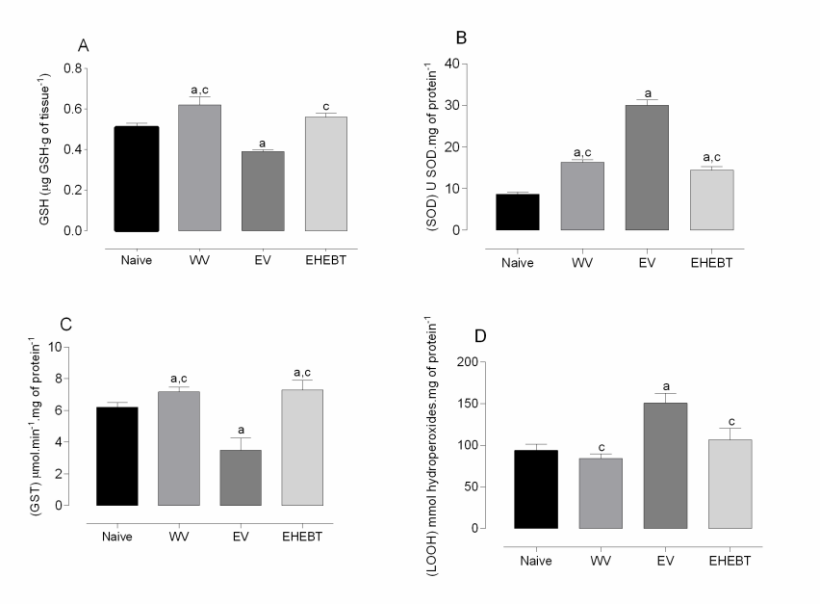


Figure 5 Gastric **(A)** reduced glutathione (µg GSH·g of tisse^{-1}), **(B)** superoxide dismutase (U SOD·mg protein^{-1}), **(C)** glutathione-*S*-Transferase (µmol GST·min^{-1}·mg protein^{-1}) and **(D)** LOOH levels (mmol hydroperoxides·mg of protein^{-1}) of mice submitted to chronic ethanol consumption. Groups: naive, WV (low-protein diet, water and vehicle, p.o.), EV (low-protein diet, ethanol and vehicle, p.o.) or EHEBT (low-protein diet, ethanol and 30 mg·kg^{-1} of HEBT, p.o.). Values are expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Tukey's test. Symbols: ^{a} $p<0.05$, when compared with naive group, ^{c} $p<0.05$, when compared with EV group.

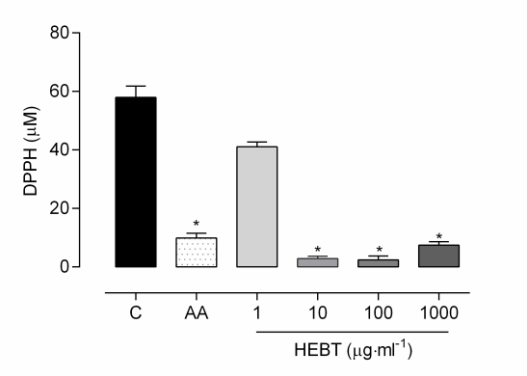


Figure 6 Effect of HEBT (1, 10, 100 and 1000 µg·ml^{-1}) on the ability to scavenge the stable free-radical DPPH. Ascorbic acid (AA, 50 µg·ml^{-1}) was used as positive control and distilled water (C) was used as negative control. Values are expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Tukey's test. Symbol: *p<0.05 negative control group.

3.6 *Effect of HEBT on chronic gastric ulcers induced by acid acetic*

The oral administration of HEBT or omeprazole twice daily for 7 days reduced gastric ulceration that was induced by acetic acid by 64% and 61%, respectively, compared with the control group (151.00 ± 12.80 mm^2; Fig. 7A). No mortality and no significant difference in body weight gain were found, with no signs of toxicity in any of the treatment groups during the 7 days of the study (data not shown).

Microscopically, extensive, deep damage was observed in the gastric mucosa in acetic acid-lesioned and vehicle-treated animals, consistent with the macroscopic appearance of this chronic gastric lesion (Fig. 7B and C). The histological examination indicated that oral treatment with omeprazole and HEBT promoted ulcer healing, with contraction of the base of the ulcer (Fig. 7D and E) and mucosal regeneration (Fig. 7F and G).

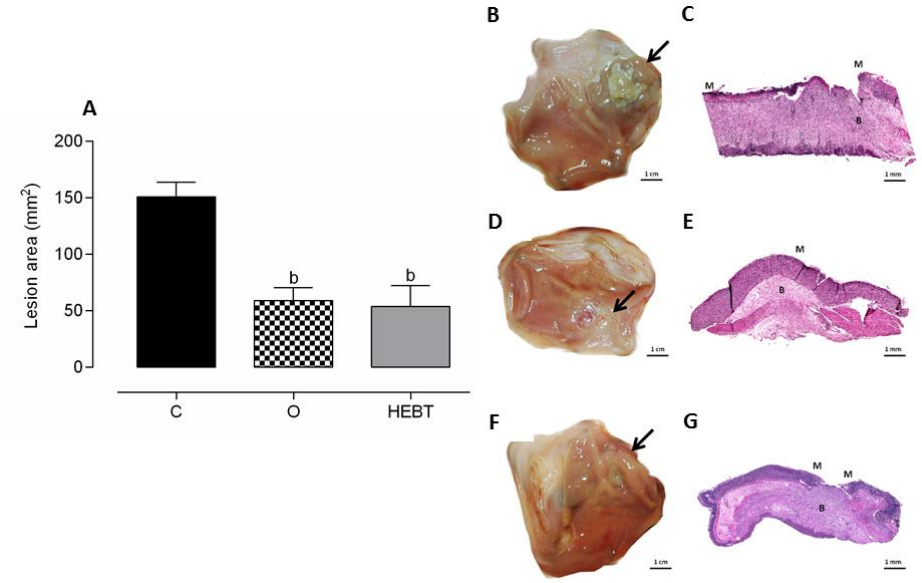


Figure 7 (A) Effects of HEBT on chronic gastric ulcer induced by 80% acid acetic in rats treated with vehicle (C: saline, 1ml·kg^{-1}, p.o.), omeprazole (O: 40 mg·kg^{-1}, p.o.) or HEBT (30 mg·kg^{-1}, p.o.) twice a day, for 7 days. Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Tukey's test. Symbol: ^{b} $p<0.05$, when compared with control group. **(B-G)** Representative macroscopic photograph of stomachs and histological hematoxylin/eosin (HE) sections (100x) of chronic gastric ulcer induced by 80% acetic acid in rats. Animals were orally treated with vehicle (water, 1 ml·kg^{-1}; Panel **B** and **C**), omeprazole (40 mg·kg^{-1}; Panel **D** and **E**) or HEBT (30 mg·kg^{-1}; Panel **F** and **G**) twice a day for seven days after the gastric ulcer induction. Bars = 1 cm (B, D, F) and 1 mm (C, E, G). M indicates margin and B indicates the base of the ulcer. Arrows indicated the ulcers area.

3.7 Effect of HEBT on gastric acid secretion

Intraduodenal, intraperitoneal, and oral administration of HEBT did not change the volume, total acidity, or peptic activity of gastric content in rats with hypersecretion that was induced by pylorus ligature for 4 h (Table 1). As expected, omeprazole reduced gastric volume, total acidity, and peptic activity by 52.31%, 67.92%, and 71.57%, respectively, compared with the control group (Table 1).

TABLE 1. Effects of HEBT on gastric acid secretion.

	Volume (ml)	**Total acidity** (mEq [H+]·ml^{-1})	**Peptic activity** (mmol of tirosine·4h^{-1})
Vehicle	7.34 ± 1.45	0.053 ± 0.011	2241.00 ± 133.80
Omeprazole	3.50 ± 0.31[a]	0.017 ± 0.001[a]	637.10 ± 75.89[a]
HEBT (p.o.)	5.80 ± 1.11	0.046 ± 0.008	1969.00 ± 110.70
HEBT (i.d.)	6.00 ± 0.88	0.041 ± 0.007	2195.00 ± 128.10
HEBT (i.p.)	4.96 ± 0.65	0.037 ± 0.007	1896.00 ± 232.10

Values are expressed as means ± S.E.M. (n=8). Statistical comparison was performed using ANOVA followed by Tukey's test. [a] $p<0.05$ when compared with vehicle.

3.8 Effect of HEBT on gastric emptying and intestinal transit

Treatment with atropine (the positive control) reduced gastric emptying by 42.30% compared with the vehicle group, but no effect of HEBT was found (Table 2). The oral administration of HEBT did not alter intestinal transit compared with the vehicle group. Atropine reduced intestinal transit by 48.30%.

TABLE 2. Effects of HEBT on gastric emptying and intestinal transit.

	Gastric emptying (%)	**Intestinal transit** (%)
Vehicle	73.58 ± 3.45	51.65 ± 3.94
Atropine	42.50 ± 11.19[a]	31.34 ± 5.08[a]
HEBT	56.42 ± 8.89	43.31 ± 3.74

Values are expressed as means ± S.E.M. (n=8). Statistical comparison was performed using ANOVA followed by Tukey's test. [a] $p<0.05$ when compared with vehicle.

3.9 Analysis of in vivo toxicity of HEBT

The oral or intraperitoneal administration of HEBT did not produce mortality or any behavioral disorders during the 14 days of observation. The oral LD_{50}

was > 5000 mg·kg^{-1}. Besides this, no mortality and no significant difference in body weight gain were found, with no signs of toxicity in mice orally treated with HEBT (30 mg·kg^{-1}, p.o.), once a day, during the 6 weeks (data not shown).

4. Discussion

The present study investigated the potential protective effects of *Baccharis trimera* hydroethanolic extract against acute and chronic stomach ulceration (induced by ethanol and acetic acid, respectively), the gastroprotective effects of HEBT on prolonged ethanol consumption, possible mechanism of gastroprotection of the extract, and acute toxicity of HEBT with several doses in different routes. The results are interesting because, unlike the previous reports, we found a potent anti-ulcerogenic action using lower doses of *B. trimera* extract. Also, this is the first study reporting gastric *B. trimera* antioxidant activity. In addition, using different doses and routes of administration we showed the safety of HEBT. This encourages the advancement of research indicating *B. trimera* as therapeutic agent for gastroprotection.

Biondo et al. (2011) reported the antiulcer activity of 1000 and 2000 mg·kg^{-1} dose of aqueous *B. trimera* extract in a stress-induced ulcer model, in mice submitted to 4ºC, during 2 hours. Mendonça et al. (2013) also described *(1)* anti-ulcer activity of *B. trimera* in a stress-induced ulcer model with rats submitted to -18ºC, during 45 minutes and *(2)* acute gastric ulcer induced by 90% ethanol, through gavage, using 100, 200 and 400 mg·kg^{-1} dose of hydroethanolic extract (70%). Finally, Dias et al. (2009) described protective effects of 100, 200 and 400 mg·kg^{-1} dose of hydroethanolic *B. trimera* extract (70%) in a hydrochloric-induced ulcer model (1 ml of 0.3 M of hydrochloric acid in 90% ethanol, through gavage). Our study observed pharmacological results with a lower dose of a more concentrate *B. trimera* extract (90%). The better antiulcerogenic activity was observed in rats treated with 30 mg·kg^{-1} dose, which motivated the choice of this dose for subsequent experiments. The oral administration of 30 mg·kg^{-1} dose of HEBT significantly reduced the lesion area and macroscopic appearance of acute and chronic ulcers models.

The model of acetic acid-induced chronic ulcers was used to investigate the lesion treatment efficacy of HEBT after identifying the gastroprotective effects of HEBT in the model of acute ethanol-induced ulcers. Lesions that are caused by

acetic acid are macroscopically and histologically similar to lesions in humans (Okabe and Amagase, 2005). The healing process is related to various cellular mechanisms, including migration, proliferation, re-epithelialization, angiogenesis, and extracellular matrix deposition, and these actions are mediated by cytokines, prostaglandins, nitric oxide, and growth factors (Tarnawski, 2000). The histological analysis in the present study revealed that HEBT treatment accelerated the healing of chronic gastric ulcers in rats, reflected by contraction of the ulcer base. These results in the acute ethanol and chronic acetic acid-induced gastric ulcer models indicate that HEBT has both gastroprotective and curative effects. Although HEBT presented these beneficial effects, it did not affect intestinal motility or gastric emptying, which differed from atropine (the positive control). Therefore, the active compounds that are present in 30 $mg \cdot kg^{-1}$ dose of HEBT likely did not interact with gastrointestinal muscarinic receptors.

Ethanol is commonly used as an ulcerogenic agent in rodents because it can produce severe gastric hemorrhagic lesions through the depletion of gastric mucus content, damage mucosal blood flow causing mucosal cell injury (Birdane et al. 2007). Our data corroborate these observations. For this reason, we evaluated whether the maintenance of gastric mucus barrier integrity was involved in the gastroprotective activity of *B. trimera*. HEBT did not protect against gastric wall mucus depletion that was caused by ethanol and did not alter gastric secretory volume, pH, or total acidity in pylorus-ligated animals. These results suggest that gastric mucus production or antisecretory activity may not be directly involved in the gastroprotective effect of 30 $mg \cdot kg^{-1}$ dose of HEBT. Biondo et al. (2011) proposed that *B. trimera* acts through inhibition of gastric acid secretion by acting on the cholinergic regulatory pathway. However, in this research, the authors used elevate doses (1000 and 2000 $mg \cdot kg^{-1}$) of an aqueous extract, which can explain our divergent results.

Besides gastric mucus depletion, ethanol also induces the overproduction of free radicals, leading to an increase in lipid peroxidation (Birdane et a. 2007). The generation of ROS is critically involved in the pathogenesis of ethanol-induced gastric damage. Experimental evidence indicates that compounds able to revert cell damage induced by ROS can be used to protect against stomach ulcers (Bonamin et al. 2014). Thus, the protection of gastric tissue against

oxidative stress by natural products may be a promising treatment against ulcer formation (Chen et al. 2003). In the present study, acute exposure and prolonged ethanol consumption induced oxidative stress in gastric mucosa. So, we evaluated the effects of HEBT on gastric redox status. In the experiment that administered ethanol acutely, HEBT prevented the decrease in GSH content and reduced LOOH. When mice received 10% ethanol chronically (during 6 weeks), the levels of GSH and GST decreased, and LOOH levels and SOD activity increased. Oral treatment with HEBT restored these parameters. Pádua et al. (2010) reported that a *B. trimera* extract significantly reduced ROS production and lipoperoxidation in neutrophils. The present results are consistent with these reports, in which the oral administration of HEBT reversed gastric ulceration by elevating the activity of antioxidant enzymes, reflected by a decrease in lipoperoxidation levels. These data indicate that the antiulcerogenic activity of HEBT may be at least partially attributable to the inhibition of free radical generation and subsequent prevention of lipid peroxidation.

The expressive antioxidant effect of HEBT may be linked to its high radical scavenging activity, which was observed in the DPPH test. This activity was also reported for others species of *Baccharis*, including *B. grisebachii*, *B. platypoda*, and *B. illinita* and is likely attributable to the presence of phenolic compounds (caffeoyl quinic acids and flavonoids) that are recognized as potent antioxidants (Tapia et al. 2004; Brighente et al. 2007).

In addition to caffeoylquinic acids, *B. trimera* contains essential oils (Brighente et al. 2007) and non-volatile compounds, such as diterpenes and flavonoids (Aboy et al. 2012; Simões-Pires et al. 2005; Verdi et al. 2005). In the present study, the NMR analysis identified sugars, caffeoylquinic acids, and clerodane diterpenes in HEBT. The HPLC-UV/PAD analysis confirmed the presence of nine caffeoylquinic acids (CQA, diCQA, and triCQA) and three flavones. One intense peak was identified as 3,5-*O*-[*E*]-dicaffeoylquinic acid, a compound that has been previously reported to be present in *B. trimera* (Aboy et al. 2012; Simões-Pires et al. 2005). Therefore, the actions of HEBT likely result from the concomitant action of several of its constituents.

Finally, we also investigated the toxic effects of HEBT *in vivo*. The acute toxicity test showed no signs of toxicity, such as alterations in water or food intake, behavioral changes, or organ weight (data not shown). Thus, suggesting

the absence of toxicity of different doses of HEBT administered orally (50-5000 $mg \cdot kg^{-1}$) or intraperitoneally (1000 $mg \cdot kg^{-1}$), and indicating the safety of HEBT, even at high doses. Our data corroborate those from Dias et al. (2009) and Nogueira et al. (2011), who did not find alteration in mice after 2 weeks of a single 5000 $mg \cdot kg^{-1}$ dose of *B. trimera*, p.o., and 4.20 or 42.00 $mg \cdot kg^{-1}$ of *B. trimera* isolated compounds. The last authors observed toxic effects of *B. trimera* compounds only on kidneys cells *in vitro*.

In conclusion, the present study provides evidence that the oral administration of HEBT has potent gastroprotective effects against acute and chronic ulcers, reflected by a reduction of the lesion area and suppression of gastric oxidative stress, without signs of systemic toxicity. Different from previous reports, we described the *Baccharis trimera* antiulcerogenic activity with lower doses. These results indicate that HEBT may have therapeutic efficacy against gastric lesions that are mainly caused by ethanol.

5. Conflict of interest

The authors declare no conflict of interest.

6. Author contributions

FARL was responsible for every step of the experiments, data analysis and manuscript preparation. LMS and DMF contributed with the *in vivo* experiments and manuscript writing. LFG and DGB contributed with the *in vivo* experiments. TBLP and ELBL contributed with *Baccharis trimera*'s production and extract preparation, while RLBS and MEAS performed the phytochemical analysis. MFPW, MEAS and AA were responsible for data discussion and manuscript correction. AA was the senior researcher responsible for the project.

7. Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The procedures performed in animals were in accordance with the ethical standards of the institution (UFPR).

8. References

Aboy AL, Apel MA, Debenedetti S et al (2010) Assay of caffeoylquinic acids in *Baccharis trimera* by reversed-phase liquid chromatography. J Chromatogr. doi: 10.1016/j.chroma.2011.11.042

Almeida RN, Falcão ACGM, Diniz RST, et al (1999) Metodologia para avaliação de plantas com atividade no sistema nervoso central e alguns dados experimentais. Rev Bras Farmacogn 80: 72-76

Anson ML (1938) The estimation of pepsin, trypsin, papain and catepsin with hemoglobin. J Gen Physiol 22: 78-89

Baggio CH De Martini Otofuji G, de Souza WM et al (2005) Gastroprotective mechanisms of indole alkaloids from *Himatanthus lancifolius*. Planta Med 71: 733–738

Birdane FM, Cemek M, Birdane YO et al (2007) Beneficial effects of *Foeniculum vulgare* on ethanol-induced acute gastric mucosal injury in rats. World J Gastroenterol 13: 607-611

Biondo TM, Tanae MM, Coletta ED et al (2011) Antisecretory actions of *Baccharis trimera* (Less.) DC aqueous extract and isolated compounds: analysis of underlying mechanisms. J Ethnopharmacol. doi: 10.1016/j.jep.2011.04.065

Blois MS (1958) Antioxidant determinations by use of a stable free radical. Nature 181: 1199–1200

Bonamin F, Moraes TM, Santos RC (2014) The effect of a minor constituent of essential oil from *Citrus aurantium*: The role of β-myrcene in preventing peptic ulcer disease. Chem Biol Interact. doi: 10.1016/j.cbi.2014.01.009

Brighente IMC, Dia M, Verdi LG et al (2007) Antioxidant Activity and Total Phenolic Content of Some Brazilian Species. Pharm Biol 45: 156-161

Burci LM, Pereira I, Silva LM, et al (2013) Antiulcer and gastric antisecretory effects of dichloromethane fraction and piplartine obtained from fruits of *Piper tuberculatum Jacq*. in rats. J Ethnopharmacol. doi: 10.1016/j.jep.2013.04.006

Chai J (2011). Peptic Ulcer Disease. Available from http://www.intechopen.com/books/peptic-ulcer-disease.

Chen W, Weng YM, Tseng CY (2003) Antioxidative and antimutagenic activities of healthy herbal drinks from Chinese medicinal herbs. Am J Chin Med 31: 523-532

Chen FA, Wu AB., Chen CY (2004) The influence of treatments on the free radical scavenging activity of burdock and variations of its activity. Food Chem 86: 479–484

Choi E, Hwang H, Kim I et al (2009) Protective effects of a polysaccharide from *Hizikia fusiformis* against ethanol toxicity in rats. Food Chem Toxicol. doi: 10.1016/j.tiv.2009.08.031

Corne SJ, Morrissey SM, Woods RJ (1974) Proceedings: A method for the quantitative estimation of gastric barrier mucus. J Physiol 242: 116–117

DeVault KR, Talley NJ (2009) Insights into the future of gastric acid suppression. Nat Rev Gastroenterol Hepatol. doi: 10.1038/nrgastro

Dias LFT, Melo ES, Hernandes LS et al (2009) Atividades antiúlcera e antioxidante *Baccharis trimera* (Less) DC (Asteraceae). Rev Bras Farmacogn. http://dx.doi.org/10.1590/S0102-695X2009000200022

Eom CS, Park SM, Myung SK, et al (2011) Use of acid-suppressive drugs and risk of fracture: a meta-analysis of observational studies. Ann Fam Med. doi: 10.1370/afm.1243

Franke A, Teyssen S, Singer MV (2005) Alcohol-related disease of the esophagus and stomach. Dig Dis 23: 204-213

Galuska B, Marazova K, Yankova T et al (2002) Effects of paracetamol and propacetamol on gastric mucosal damage and gastric lipid peroxidation caused acetylsalicylic acid (ASA) in rats. Pharmacol Res 46: 141–148

Gao R, Yuan Z, Zhao Z, et al (1998) Mechanism of pyrogallol autoxidation and determination of superoxide dimutase enzyme activity Bioelectrochem Bioenerg 45: 41–45

Garcia FAO, Tanae MM, Torres LMB et al (2014) A comparative study of two clerodane diterpenes from *Baccharis trimera* (Less.) DC on the influx and mobilization of intracellular calcium in rat cardiomyocytes. Phytomedicine. doi: 10.1016/j.phymed.2014.04.018

Gené RM, Cartana C, Adzet T et al (1996) Antiinflammatory and analgesic activity of *Baccharis trimera*: identification of its active constituents. Planta Med 62: 232–235

Gisbert J, Pajares M (2003) *Helicobacter pylori* infection and perforated peptic ulcer prevalence of infection and role of antimicrobial treatment. Helicobacter 8: 159-67

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249: 7130–7139

Herz W, Pilotti AM, Soderholm AC et al (1977) New ent-clerodane-type diterpernoids from *Baccharis trimera*. J Org Chem 42: 3913-1917

Issac A, Gopakumar G, Kuttan R et al (2015) Safety and anti-ulcerogenic activity of a novel polyphenol-rich extract of clove buds (*Syzygium aromaticum L*). Food Funct. doi: 10.1039/c4fo00711e

Januário AH, Santos SL, Marcussi S et al (2004) Neo-clerodane diterpenoid, a new metalloprotease snake venom inhibitor from *Baccharis trimera* (Asteraceae): anti-proteolytic and anti-hemorrhagic properties. Chem Biol Interact 150: 243-251

Jiang ZY, Hunt JV, Wolff SP (1992) Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. Anal Biochem 202: 384–389

Kangwan N, Park JM, Kim EH et al (2014) Quality of healing of gastric ulcers: Natural products beyond acid suppression. World J Gastrointest Pathophysiol. doi: 10.4291/wjgp.v5.i1.40

Lee BI, Nugroho A, Bachri MS et al (2010) Anti-ulcerogenic Effect and HPLC analysis of the caffeoylquinic acid-rich extract from *Ligularia stenocephala*. Biol Pharm Bull 33: 493-497

Lívero FAR, Stolf AM, Dreifuss AA, et al (2014) The FXR agonista 6ECDCA reduces hepatic steatosis and oxidative stress induced by ethanol and low protein diet in mice. Chem Biol Interact. doi: 10.1016/j.cbi.2014.03.014

Losqui YR, Rozete FSS, Almeida MB, et al (2009) Activity of *Baccharis trimera* (Less.) DC. Asteraceae on culture of retinal ganglion cells in vitro. Rev Bras Farmacogn. doi: http://dx.doi.org/10.1590/S0102-695X2009000600024

Mendonça NM, Silveira C, Oliveira JM, et al (2013) Avaliação da atividade antiulcerogênica da *Baccharis trimera* (Less) DC em ratos. Saud Pesq. doi: http://dx.doi.org/10.17765/1983-1870.2013v6n1p%25p

Moccelini SK, Silva VC, Ndiaye EA et al (2009) Estudo fitoquímico das cascas das raízes de *Zanthoxylum rigidum* Humb. & Bonpl. ex. Willd (Rutaceae). Quim Nova. doi: http://dx.doi.org/10.1590/S0100-40422009000100025

Nogueira NPA, Reis PA, Laranja GAT et al (2011) In vitro and in vivo toxicological evaluation of extract and fractions from *Baccharis trimera* with anti-inflammatory activity. J Ethnopharmacol. doi: 10.1016/j.jep.2011.09.051

O'Malley P (2003) Gastric ulcers and GERD: the new ''plagues'' of the 21st century update for the clinical nurse specialist. Clin Nurse Spec 17: 286–289

Okabe S, Roth JL, Pfeiffer CJ (1971) A method for experimental, penetrating gastric and duodenal ulcers in rats. Observations on normal healing. Am J Dig Dis 16: 277–284

Okabe S, Amagase K (2005) An overview of acetic acid ulcer models the history and state of the art of peptic ulcer research. Biol Pharm Bull 8: 1321–1341

Oliveira ACP, Endringer DC, Amorim LAS et al (2005) Effect of the extracts and fractions of *Baccharis trimera* and *Syzygium cumini* on glycaemia of diabetic and non-diabetic mice. J Ethnopharmacol 102: 465-469

Oliveira RN, Rehder VLG, Oliveira ASS et al (2012) *Schistosoma mansoni*: in vitro schistosomicidal activity of essential oil of *Baccharis trimera* (less) DC. Exp Parasitol. doi: 10.1016/j.exppara.2012.06.005

Pádua BC, Silva LD, Rossoni-Junior JV et al (2010) Antioxidant properties of *Baccharis trimera* in the neutrophils of Fischer rats. J Ethnopharmacol. doi: 10.1016/j.jep.2010.04.018

Penston J, Wormsley KG (1987) Achlorhydria: hypergastrinaemia: carcinoids-a flawed hypothesis? Gut 28: 488–505

Poynter D, Pick CR, Harcourt RA et al (1985) Association of long lasting unsurmountable histamine H2 blockade and gastric carcinoid tumours in the rat. Gut 26: 1284–1295

Prista LN, Correia AA, Morgado R (1975) Técnica Farmacêutica e Farmácia Galênica. 2 ed., Fundação Calouste Gulbenkian, Lisboa

Rijke E, Out P, Niessen WMA et al (2006) Analytical separation and detection methods for flavonoids. J Chromatogr 1112: 31-63

Robert A, Nezamis JE, Lancaster C et al (1979) Cytoprotection by prostaglandins in rats. Prevention of gastric necrosis produced by alcohol, HCl, NaOH, hypertonic NaCl, and thermal injury. Gastroenterol 77: 433–443

Rocco A, Compare D, Angrisani D et al (2014) Alcoholic disease: liver and beyond. World J Gastroenterol. doi: 10.3748/wjg.v20.i40.14652

Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 25: 192–205

Shay H, Komarov SA, Fels SS et al (1945) A simple method for the uniform production of gastric ulceration in the rat. Gastroenterol 5: 43–61

Sheen E, Triadafilopoulos G (2011) Adverse effects of long-term proton pump inhibitor therapy. Dig Dis Sci. doi: 10.1007/s10620-010-1560-3

Simões-Pires CA, Queiroz EF, Henriques AT et al (2005) Isolation and on-line identification of anti-oxidant compounds from three *Baccharis* species by HPLC-UV-MS/MS with post-column derivatization. Phytochem Anal 16: 307-314

Soicke H, Leng-Peschlow E (1987) Characterization of flavonoids from *Baccharis trimera* and their antihepatotoxic properties. Planta Med 53: 37–39

Strapasson RLB, Rüdiger AL, Burrow RA et al (2015) A new sesquiterpene lactone and other constituents of *Moquiniastrum polymorphum* subsp. *floccosum* (Asteraceae). Nat Prod Comm 9: 1541-1543.

Suchitra AD, Dkhar SA, Shewade DG et al (2003) Relative efficacy of some prokinetic drugs in morphine-induced gastrointestinal transit delay in mice. World J Gastroenterol 9: 779-783

Tapia A, Rodriguez J, Theoduloz C et al (2004) Free radical scavengers and antioxidants from *Baccharis grisebachii*, J Ethnopharmacol 95: 155-61

Tarnawski A (2000) Molecular mechanisms of ulcer healing. Drug News Perspect 13: 158–168

Takeuchi K (2012) Pathogenesis of NSAID-induced gastric damage: importance of cyclooxygenase inhibition and gastric hypermotility World J Gastroenterol. doi: 10.3748/wjg.v18.i18.2147

Verdi LG, Brighente MC, Pizzolatti MG (2005) Gênero *Baccharis* (Asteraceae): Aspectos químicos, econômicos e biológicos. Quim Nova. doi: http://dx.doi.org/10.1590/S0100-40422005000100017

WHO - World Health Organization (2008) Is harmful use of alcohol a public health problem? Available from http://www.who.int/features/qa/66/en/index.html

6. CONSIDERAÇÕES FINAIS

Diante dos resultados obtidos neste trabalho, conclui-se que a administração oral do extrato hidroetanólico de *Baccharis trimera*:

1. No modelo de estudo de esteatose hepática alcoólica, na dose oral de 30 mg.kg^{-1}:

- ✓ Diminuiu os níveis plasmáticos e hepáticos de colesterol, triglicerídeos, HDL e LDL;
- ✓ Aumentou a excreção fecal de triglicerídeos;
- ✓ Reverteu o estresse oxidativo hepático induzido pelo etanol em camundongos, refletido pela redução da lipoperoxidação e dos níveis de espécies reativas de oxigênio totais, bem como pela normalização dos níveis de GSH, e da atividade da Cat, SOD, GPx e GST;
- ✓ Reverteu as alterações histológicas hepáticas induzidas pelo etanol;
- ✓ Normalizou a expressão de genes envolvidos no metabolismo do etanol, no sistema antioxidante e na lipogênese, como o *Cyp2e1*, *Nrf2* e *Scd1*;
- ✓ Não induziu sinais de toxicidade nos animais.

2. Nos modelos de estudo de lesão gástrica, nas doses orais de 3 a 30 mg.kg^{-1}:

- ✓ Apresentou potente atividade gastroprotetora contra úlceras agudas e crônicas;
- ✓ Reduziu a área de lesão induzida por etanol e ácido acético;
- ✓ Diminuiu os níveis de GSH e LPO frente à administração aguda de etanol;

- ✓ Suprimiu o estresse oxidativo gástrico, restaurando os níveis de GSH, LPO e a atividade da GST e da SOD no modelo de administração prolongada de etanol;
- ✓ Não alterou a produção de muco e a secreção gástrica, bem como a motilidade intestinal;
- ✓ Doses mais baixas foram eficazes em promover ação gastroprotetora;
- ✓ Doses baixas ou elevadas do HEBT não induziram sinais de toxicidade nos animais.

Diante dos resultados supracitados o HEBT apresenta eficácia terapêutica contra lesões gástricas induzidas por etanol, bem como pode ser considerado um agente farmacológico promissor para o tratamento da esteatose hepática alcoólica.

7. REFERÊNCIAS ADICIONAIS

Albano E, Clot P, Morimoto M, Tomasi A, Ingelman- Sundberg M, French SW. Role of cytochrome P4502E1- dependent formation of hydroxyethyl free radicals in the development of liver damage in rats intragastrically fed with ethanol. *Hepatology* 1996; 23: 155–63.

Albano E. Free radical mechanisms in immune reactions associated with alcoholic liver disease. *Free Radic Biol Med* 2002; 32: 110–4.

Alonso JR. Tratado de Fitomedicina - bases clínicas y farmacológicas. ISIS Ediciones S. R. L., Buenos Aires, Argentina. 350-354, 1998.

Bagyánszki M, Torfs P, Krecsmarik M, et al. Chronic alcohol consumption induces an overproduction of NO by nNOS- and iNOS-expressing myenteric neurons in the murine small intestine. Neurogastroenterol Motil 2011; 23: 237-248.

Bailey SM, Mantena SK, Millender-Swain T, et al. Ethanol and tobacco smoke increase hepatic steatosis and hypoxia in the hypercholesterolemic ApoE$^{-/-}$ mouse: Implications for a “multihit” hypothesis of fatty liver disease. *Free Radic Biol Med* 2009; 46: 928–38.

Beckingham IJ. ABC of diseases of liver, pancreas, and biliary system: Investigation of liver and biliary disease. *BMJ* 2001; 322 :33-36.

Bellentani S, Saccoccio G, Costa G, et al. Drinking habits as cofactors of risk for alcohol induced liver damage. The Dionysos Study Group. *Gut* 1997; 41: 845-850.

Birdane FM, Cemek M, Birdane YO, et al. Beneficial effects of *Foeniculum vulgare* on ethanol-induced acute gastric mucosal injury in rats. *World J Gastroenterol* 2007; 13: 607-611.

Bode C, Bode C. Alcohol’s role in gastrointestinal tract disorders. *Alcohol Health & Research World* 1997; 21: 76-83.

Brown RS Jr. Transplantation for alcoholic hepatitis: time to rethink the 6-month “rule”. *N Engl J Med* 2011; 365: 1836–1838.

Bujanda L. The effects of alcohol consumption upon the gastrointestinal tract. Am J *Gastroenterol* 2000; 95: 3374-3382.

Cederbaum AI. CYP2E1–biochemical and toxicological aspects and role in alcohol-induced liver injury. *Mt Sinai J Med* 2006; 73: 657–72.

Ceni E, Mello T, Galli A. Pathogenesis of alcoholic liver disease: Role of oxidative metabolism. *World J Gastroenterol* 2014; 20: 17756–17772.

Day CP. Pathogenesis of steatohepatitis. *Best Pract Res Clin Gastroenterol* 2002; 16: 663-678.

de Groot H. Reactive oxygen species in tissue injury. *Hepatogastroenterology* 1994; 41: 328–32.

DeVault KR, Talley NJ, Insights into the future of gastric acid suppression. *Nat Rev Gastroenterol Hepatol* 2009; 6: 524-532.

Dixit N, Baboota S, Kohli K, Ahmad S, Ali J. Silymarin: A review of pharmacological aspects and bioavailability enhanced approaches. *Indian J Pharm* 2007; 39: 172–179.

Eom CS, Park SM, Myung SK, et al. Use of acid-suppressive drugs and risk of fracture: a meta-analysis of observational studies. *Ann Fam Med* 2011; 9: 257-267.

European Association for the Study of the liver. EASL Clinical practical guidelines: management of alcoholic liver disease. *J Hepatol* 2012; 57: 399–420.

Fendrick AM, Forsch RT, Harrison RV, Scheiman JM. University of Michigan Health System Peptic Ulcer Guideline, 2005.

Frazier TH, Stocker AM, Kershner NA, Marsano LS, McClain CJ. Treatment of alcoholic liver disease. *Therap Adv Gastroenterol* 2011; 4: 63–81.

Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000; 275: 2247-2250.

Gao B, Bataller R. Alcoholic fatty liver disease: Pathogenesis and new therapeutic targets. *Gastroenterology* 2011; 141: 1572–85.

Gené RM, Cartana C, Adzet T, et al. Antiinflammatory and analgesic activity of *Baccharis trimera*: identification of its active constituents. *Planta Med* 1996; 62: 232–235.

Ha HL, Shin HJ, Feitelson MA, Yu DY. Oxidative stress and antioxidants in hepatic pathogenesis. *World J Gastroenterol* 2010; 16: 6035–6043.

Jones DP. Redefining oxidative stress. *Antioxid Redox Signal* 2006; 8: 1865–1879.

Kangwan N, Park JM, Kim EH, et al. Quality of healing of gastric ulcers: Natural products beyond acid suppression. *World J Gastrointest Pathophysiol* 2014; 15: 40-47.

Koko V, Todorović V, Varagić J, et al. Gastrin producing G-cells after chronic ethanol and low protein nutrition. *Indian J Exp Biol* 1998; 36: 1093-1101.

Lazaratos S, Irukayama-Tomobe Y, Miyauchi T, et al. Oxygen radicals mediate the final exacerbation of endothelin-1-induced gastric ulcer in rat. *Eur J Pharmacol* 2001; 413: 121-129.

Levene AP, Goldin RD. The epidemiology, pathogenesis and histopathology of fatty liver disease. *Histopathology* 2012; 61: 141-152.

Liu, J. Ethanol and liver: Recent insights into the mechanisms of ethanol-induced fatty liver. *World J Gastroenterol* 2014; 20: 14672-14685.

Lorenzi H, Matos, FJA. Plantas Medicinais no Brasil: Nativas e Exóticas Cultivadas. Instituto Plantarum, Nova Odessa, SP. 2002; pp. 142–143.

Mashimo H, He XD, Huang PL, et al. Neuronal constitutive nitric oxide synthase is involved in murine enteric inhibitory neurotransmission. *J Clin Invest* 1996; 98: 8-13.

Massey VL, Arteel GE. Acute alcohol-induced liver injury. *Frontiers in physiology* 2012; 3: 1-8.

Massey VL, Beier JI, Ritzenthaler JD. Potential Role of the Gut/Liver/Lung Axis in Alcohol-Induced Tissue Pathology. *Biomolecules* 2015; 5: 2477-2503.

Menon KVN, Gores GJ, Shah VH. Pathogenesis, Diagnosis, and Treatment of Alcoholic Liver Disease. *Mayo Clin Proc* 2001; 76: 1021–1029.

Nagata K, Suzuki H, Sakaguchi S. Common pathogenic mechanism in development progression of liver injury caused by non-alcoholic or alcoholic steatohepatitis. *J Toxicol Sci* 2007; 32: 453–468.

Ning JW, Lin GB, Ji F, et al. Preventive effects of geranylgeranylacetone on rat ethanol-induced gastritis. *World J Gastroenterol* 2012; 18: 2262-2269.

O'Malley P. Gastric ulcers and GERD: the new ''plagues'' of the 21st century update for the clinical nurse specialist. *Clin Nurse Spec* 2003; 17: 286–289.

Oliveira ACP, Endringer DC, Amorim LAS, et al. Effect of the extracts and fractions of *Baccharis trimera* and *Syzygium cumini* on glycaemia of diabetic and non-diabetic mice. *J Ethnopharmacol* 2005; 102: 465-469.

Paiva FA, Bonomo LF, Boasquivis PF, et al. Carqueja (*Baccharis trimera*) protects against oxidative stress and β-amyloid-induced toxicity in *Caenorhabditis elegans*. *Oxidative medicine and cellular longevity* 2015; Article ID 740162.

Penston J, Wormsley KG. Achlorhydria: hypergastrinaemia: carcinoids-a flawed hypothesis? *Gut* 1987; 28: 488–505.

Poynter D, Pick CR, Harcourt RA, et al. Association of long lasting unsurmountable histamine H2 blockade and gastric carcinoid tumours in the rat. *Gut* 1985; 26: 1284–1295.

Rates SMK. Plants as source of drugs. *Toxicon* 2001; 39: 603-613.

Ratziu V, Goodman Z, Sanyal A. Current efforts and trends in the treatment of NASH. *J Hepatol* 2015; 62: 65–75.

Rehm J, Shield KD. Alcohol and mortality: global alcohol-attributable deaths from cancer, liver cirrhosis and injury in 2010. *Alcohol Research: Current Reviews* 2013; 35: 174–83.

Roberts DM. Chronic gastritis, alcohol, and non-ulcer dyspepsia. *Gut* 1972; 13: 768-774.

Rocco A, Compare D, Angrisani D, et al. Alcoholic disease: Liver and beyond. *World J Gastroenterol* 2014; 20: 14652-14659.

Ruiz ALTG, Taffarello D, Souza VHS, Carvalho, JE. Farmacologia e Toxicologia de *Pneumus boldus* e *Baccharis genistelloides*. *Brazilian Journal of Pharmacognosy* 2008; 18: 295-300.

Samuhasaneeto S, Thong-NgamD, Kulaputana O, et al. Effects of N-acetylcysteine on oxidative stress in rats with non-alcoholic steatohepatitis. *J Med Assoc Thai* 2007; 90: 788–97.

Seitz HK, Stickel F. Acetaldehyde as an underestimated risk factor for cancer development: role of genetics in ethanol metabolism. *Genes Nutr* 2010; 5: 121-128.

Sheen E, Triadafilopoulos G. Adverse effects of long-term proton pump inhibitor therapy. *Dig Dis Sci* 2011; 56: 931-50.

Singal AK, Guturu P, Hmoud B, et al. Evolving frequency and outcomes of liver transplantation based on etiology of liver disease. *Transplantation* 2013; 95: 755–760.

Simões-Pires CA, Queiroz EF, Henriques AT, et al. Isolation and on-line identification of anti-oxidant compounds from three *Baccharis* species by HPLC-UV-MS/MS with post-column derivatization. *Phytochem Anal* 2005; 16: 307-314.

Soden JS, Devereaux MW, Haas JE, et al. Subcutaneous vitamin E ameliorates liver injury in an in vivo model of steatocholestasis. *Hepatology* 2007; 46: 485–495.

Soicke H, Leng-Peschlow E. Characterization of flavonoids from *Baccharis trimera* and their antihepatotoxic properties. *Planta Med* 1987; 53: 37–39.

Sozio M, Crabb DW. Alcohol and lipid metabolism. *Am J Physiol Endocrinol Metab* 2008; 295: 10–16.

Szabo G, Mandrekar P. Focus on: alcohol and liver. *Alcohol Res Health* 2010; 33: 87–96.

Terelius Y, Norsten-Höög C, Cronholm T, Ingelman-Sundberg M. Acetaldehyde as a substrate for ethanolinducible cytochrome P450 (CYP2E1). *Biochem Biophys Res Commun* 1991; 179: 689–94.

Teyssen S, Singer MV. Alcohol-related diseases of the oesophagus and stomach. *Best Pract Res Clin Gastroenterol* 2003; 17: 557-573.

Todorović V, Koko V, Budec M, et al. G cells and gastrin in chronic alcohol-treated rats. *Alcohol* 2008; 42: 37-45.

Tsukamoto H, Machida K, Dynnyk A, Mkrtchyan H. “Second hit” models of alcoholic liver disease. *Semin Liver Dis* 2009; 29: 178–187.

U.S. Department of Health and Human Services. Results from the 2010 National Survey on Drug Use and Health; U.S. Department of Health and Human Services: Washington, DC, USA, 2010.

Verdi LG, Brighente IMC, Pizzolati MG. Gênero *Baccharis* (Asteraceae): Aspectos químicos, econômicos e biológicos. *Quim Nova* 2005; *28*: 85-94.

Viollet B, Guigas B, Leclerc J, et al. AMP-activated protein kinase in the regulation of hepatic energy metabolism: from physiology to therapeutic perspectives. *Acta Physiol* 2009; 196: 81–98.

Wilfred de Alwis NM, Day CP. Genetics of alcoholic liver disease and nonalcoholic fatty liver disease. *Semin Liver Dis* 2007; 27: 44–54.

World Health Organization. Global Status Report on Alcohol and Health. World Health Organization: Geneva, Switzerland, 2011.

Wu YS, Salmela KS, Lieber CS. Microsomal acetaldehyde oxidation is negligible in the presence of ethanol. *Alcohol Clin Exp Res* 1998; 22: 1165–1169.

Yamada H, Kimura K, Chen D. Effects of daily fat or ethanol ingestion on the cholecystokinin- pancreas and the gastrinenterochromaffin-like cell axes in rats. *Digestion* 1998; 59: 331-334

You M, Fischer M, Deeg MA, Crabb DW. Ethanol induces fatty acid synthesis pathways by activation of sterol regulatory element-binding protein (SREBP). *J Biol Chem* 2002; 277: 29342–29347.

8. PRODUÇÃO CIENTÍFICA (ARTIGOS PUBLICADOS) NO PERÍODO DO DOUTORADO

1. Molecular basis of alcoholic fatty liver disease: From incidence to treatment. **Lívero FA**, Acco A. Hepatol Res. 2015 Sep 29.

2. Sydnone 1: A Mesoionic Compound with Anti-tumoural and Hematological Effects In Vivo. Galuppo LF, **Lívero FA**, Martins GG, Cardoso CC, Beltrame OC, Klassen LM, Canuto AV, Echevarria A, Telles JE, Klassen G, Acco A. Basic Clin Pharmacol Toxicol. 2015 Dec 26.

3. Sesquiterpene lactones of *Moquiniastrum polymorphum* subsp. *floccosum* have antineoplastic effects in Walker-256 tumor-bearing rats. Martins GG, **Lívero FA**, Stolf AM, Kopruszinski CM, Cardoso CC, Beltrame OC, Queiroz-Telles JE, Strapasson RL, Stefanello MÉ, Oude-Elferink R, Acco A. Chem Biol Interact. 2015 Feb 25; 228:46-56.

4. The FXR agonist 6ECDCA reduces hepatic steatosis and oxidative stress induced by ethanol and low-protein diet in mice. **Lívero FA**, Stolf AM, Dreifuss AA, Bastos-Pereira AL, Chicorski R, de Oliveira LG, de Souza CE, Fabossi IA, Rabitto IS, Gremski LH, Henneberg R, Telles JE, Oude Elferink RP, Acco A. Chem Biol Interact. 2014 Jun 25; 217:19-27.

5. *Uncaria tomentosa* exerts extensive anti-neoplastic effects against the Walker-256 tumour by modulating oxidative stress and not by alkaloid activity. Dreifuss AA, Bastos-Pereira AL, Fabossi IA, **Lívero FA**, Stolf AM, Alves de Souza CE, Gomes Lde O, Constantin RP, Furman AE, Strapasson RL, Teixeira S, Zampronio AR, Muscará MN, Stefanello ME, Acco A. PLoS One. 2013;8(2):e54618.

6. Characterization of an Alcoholic Hepatic Steatosis Model Induced by Ethanol and High-Fat Diet in Rats. Alves-Souza CE, Stolf AM, Dreifuss AA, **Lívero FA**, Gomes LO, Petiz LL, Beltrame OC, Diettrich RL, Telles JEQ, Cadena SMSC, Acco A. Brazilian Archives of Biology and Technology (Online), v. 58, p. 367-378, 2015.