UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Propriedades antioxidantes da cerveja artesanal

Aluno: Lucas Brambilla Hilbig Feistauer

Orientador: Prof. Dr. José Cláudio Fonseca Moreira

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Resumo

Na cultura ocidental o hábito de consumir cerveja é bem disseminado, sendo essa, umas das bebidas mais consumidas no mundo, ocupando o primeiro lugar entre as alcoólicas. A cerveja é uma bebida complexa por incluir em seu preparo inflorescências de lúpulo, cevada e fermento, ingredientes que conferem atividades antioxidantes para essa bebida, provavelmente devido à presença de compostos fenólicos. Os polifenois são uma família de compostos, produzidos pelas plantas, muitos deles com capacidade antioxidante. Sabe-se que a ingestão de certos alimentos ricos nesses compostos tem se mostrado relevantes para a saúde humana. O consumo moderado de cerveja tem se mostrado benéfico como adjuvante no tratamento de doenças coronarianas, diabetes, osteoporose e alguns tipos de câncer. Existe muita variação entre as cervejas a respeito da quantidade de lúpulo utilizado em suas formulações, onde a maioria das cervejas comerciais possui pouca quantidade desse ingrediente em relação às cervejas artesanais. Além disso, cervejas artesanais possuem maior diversidade e quantidade de compostos fenólicos, entre eles o xantohumol e isoxantohumol. O objetivo do trabalho foi investigar o potencial antioxidante de cervejas, comparando algumas marcas artesanais com algumas marcas comercias, e também investigar se o consumo dessas bebidas confere algum tipo de hepatoproteção em um modelo de indução de dano hepático induzido por tetracloreto de carbono (CCl_4) em ratos Wistar. Trataram-se os animais durante 30 dias, na garrafa de beber, durante o período noturno de 12h, com: água, etanol 6,7%, cerveja comercial, cerveja artesanal ou silimarina, ministrada por gavagem (2 mL/kg do medicamento Legalon). Após o período de tratamento, o CCl_4 foi injetado (i.p. 2,5 mL/kg) e 12h após as injeções os animais foram sacrificados. Analisou-se o peso das gorduras abdnominais e peso do fígado, parâmetros redox do fígado e o perfil redox do soro. A cerveja artesanal demonstrou maior capacidade antioxidante, um aumento dos níveis de HDL, nenhum tipo de dano oxidativo, nem dano hepático. Entretanto não foi observado efeito de hepatoproteção em nenhum dos tratamentos testados.

Palavras-chave: Cerveja artesanal, estresse oxidativo, xantohumol.

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ABSTRACT:

In ocidental culture, beer consumption is a widespread habit. This is one of the most consumed beverages in the world, and the number one of alcoholic beverages. Beer is a complex beverage because includes in its brewing hops, barley malt and yeast, ingredients that endows antioxidant activity to this beverage, probably due to the presence of phenolic compounds. Polyphenols are a family of compounds produced by plants, many of them with antioxidant capacity. It is known that a high consumption of polyphenols is good for health. The moderate consumption of beer is beneficial as adjuvant in treatment of many dieseases as coronary diaseases, diabetes, osteoporosis, and some cancers. There are a lot of variations in beers formula, mainly in quantity of hops used. As craft beers have more quantities of this ingredient, they reveal more variety and quantity of phenolic compounds, among them xanthohumol and isoxanthohumol. The objective of this research was investigate the antioxidant potential of beers, comparing some commercial brands with some craft beers brands, and also investigates if a pré-treatment with beers could be hepatoprotective in a carbon tetrachloride (CCl_4) model in rats. They were trated for 30 days in drinking bottle, during night shift (12h), with: commercial beer, craft beer, ethanol 6,7 %, water or Silymarin, administered by gavage (2 mL/kg of Legalon medicine). After the treatment, CCl_4 was injected (i.p 2,5 mL/kg) and 12h after injection animals were sacrificed. It was analyzed abdominal fat weight, liver weight, liver redox parameters and serum redox profile. Craft beer presented higher antioxidant potential, raised HDL levels, no oxidative damage nor hepatic injury. However no hepatoprotection was observed with any of tested treatments.

Key words: Craft beer, oxidative stress, xanthohumol.

LISTA DE ABREVIATURAS:

ALT: Alanina-aminotransferase

AST: Aspartato-aminotrasferase

ATP: Adenosina-trifosfato

AUC: Área sob a curva

AVC: Acidente vascular encefálico

CAT: Catalase

CCl_4: Tetracloreto de Carbono

COX-1: Ciclo-oxigenase-1

ERN: Espécies reativas de nitrogênio

ERO: Espécies reativas de oxigênio

GPx: Glutationa-peroxidase

GST: Glutationa-S-Transferase

HDL: Lipoproteína de alta densidade

ICAM-1: Molécula de adesão intercelular-1

LDL: Lipoproteína de baixa densidade

NO: Óxido nítrico

SOD: Superóxido-dismutase

TAR: Reatividade antioxidate total

TBARS: Espécies reativas ao ácido tiobarbitúrico

TRAP: Potencial antioxidante total

I_0/I: Razão entre intensidade luminosa sem amostras/intensidade luminosa após adição da amostra

SUMÁRIO

1. INTRODUÇÃO

1.1 Cerveja no contexto histórico-cultural

Existem registros de bebidas fermentadas produzidas pelas civilizações Suméria e Assíria de 10.000 a.C. Nessa época, a mistura era nutritiva e considerada sagrada, oferecida aos deuses [Cruz et al., 2008]. A cerveja foi muito importante para os povos antigos, pois a primeira língua escrita tinha um símbolo para cevada muito utilizado [Cohen et al., 2004]. Também existem evidências na Mesopotâmia, de 6000 a.C, da utilização de cerveja exercendo funções medicinais como aplicando-a sobre ferimentos, como antisséptico bucal, utilizada para enema e lavagem vaginal, além da introdução na dieta alimentar [Darby, 1977]. Traços de um moderno antibiótico, a tetraciclina, foram encontrados em ossos de múmias egípcias com mais de 2000 anos de idade. Esse achado foi correlacionado com o consumo de cerveja, sugerindo um uso medicinal para bebida [Nelson et al., 2010]. A cerveja também era usada como uma bebida hidratante e, por não conter contaminantes, era dada para crianças beberem na idade média [Unger, 2004].

Os conceitos básicos de higiene e contaminação por microorganismos foi desenvolvido quando Pasteur olhou no microscópio uma cerveja estragada e associou a micro-organismos, que posteriormente foram associados a doenças. Ao longo dos anos, muitos nomes foram dados a bebida fermentada, mas somente no início do século XVI se definiu a palavra cerveja como: bebida fermentada por ação de leveduras; elaborada por cocção de cevada germinada (malte) e outros cereais; e aromatizada com lúpulo [Nogueira, 2006]. Sendo criada pelo império romano como denominação para a bebida fermentada de cevada (servisia) [Ferreira, 1986]. No século XVIII, foi criada na Bavária a Lei

da Pureza da Cerveja (*Reinheitsgebot*), que vigora até hoje em países como Alemanha e Grécia, restringindo a esta denominação somente a bebida fermentada obtida a partir de cevada malteada, água e lúpulo (cerveja conhecida como puro malte) [Garcia, 2012].

Na história do Brasil, a bebida demorou a tornar-se popular devido ao fato de Portugal incentivar o consumo de vinhos portugueses. A cerveja no Brasil era produzida de forma caseira e consumida somente pelas famílias de imigrantes. Somente após a vinda da corte Portuguesa para o Brasil (1808), a abertura dos portos possibilitou a entrada de cervejas importadas, principalmente inglesas, e com a popularização da bebida, iniciou-se um movimento de produção de cerveja brasileira [Coutinho, Quintella & Panzani, 2008].

A cerveja faz parte da construção da nossa sociedade e da nossa história. Hoje em dia, é uma das bebidas mais consumidas no mundo e dentre as alcoólicas é a mais consumida.

1.2 Comércio e indústria cervejeira

Em 2013, o consumo global de cerveja foi de 188.81 bilhões de litros e a produção globa foi de 192.94 bilhões de litros [Kirin Beer University]. O Brasil é o terceiro maior produtor de cerveja do mundo (13 bilhões de litros/ano), o terceiro maior consumidor (14 bilhões de litros/ano) e se encontra na 24ª posição do ranking de consumo per capita mundial, com 68 litros por ano [Kirin Beer University; Sindicerv, 2015]. Essa bebida é produzida a partir da fermentação do mosto (um líquido doce originado pela cocção da cevada, podendo ser outro cereal) por leveduras e adicionado o lúpulo, sendo um dos

alimentos da dieta humana que possui lúpulo como matéria prima. Cabe ressaltar, ainda, que atualmente já existem outros alimentos como: biscoitos, iogurtes etc. Porém, a cerveja é o alimento mais consumido dentre esses que contém lúpulo.

A cevada (*Hordeum sp.)* é uma gramínea cultivada em regiões de clima temperado [Hornsey, 1999], cultivada em vários estados brasileiros durante o inverno. O lúpulo (*Humulus lupulus)* é uma planta dioica da família das *Cannabinaceae* e é cultivado em regiões de baixas temperaturas [Hornsey, 1999]. As condições para o plantio de lúpulo no Brasil não são as mais favorávies, sendo essa matéria prima, praticamente toda importada [Hughes & Baxter, 2001]. O aroma e amargor característicos da cerveja são oriundos de diversos compostos presentes nas inflorescências femininas do lúpulo. Porém, cabe sinalizar que não é somente dessa parte, pois mais recentemente usam-se outras partes da planta.

O estilo de cerveja mais produzido e consumido no Brasil, e no mundo, é do tipo Pilsen (Standard American Lager), que possui grande percentual de adjuntos, pouco lúpulo, de cor clara, teor de extrato médio, baixa fermentação e baixo teor alcoólico [BJCP, 2008].

Diante desse fato, no Brasil, consumidores mais exigentes resgatam a história e a cultura de se fazer e beber boas cervejas [Rio, 2013], com a ideia de fabricar cerveja para consumo próprio, sem fins comerciais, de estilos europeus e norte-americanos, que apresentam propriedades organolépticas superiores às marcas encontradas no mercado brasileiro fornecido pelas grandes fábricas. Aumentando assim, o número de microcervejarias com produção própria e exclusiva [Rio, 2013].

O termo cerveja artesanal (craft beer) foi criado nos EUA na necessidade de distinguir as cervejas comerciais de larga escala, as tipo Pilsen. As cervejas artesanais, apesar de não representarem um estilo específico, seguem normas rígidas de qualidade. Entende-se por artesanal ou especial uma cerveja produzida com ingredientes diferenciados, com matérias primas de melhor qualidade, com nenhum ou poucos aditivos químicos e adjuntos. No Brasil, a matéria prima da cervejaria artesanal acaba sendo praticamente toda importada, já que as grandes cervejarias absorvem a cevada nacional. Em relação ao lúpulo, as cervejas artesanais possuem em suas receitas uma maior quantidade de lúpulo do que as cervejas comerciais [Wunderlich, Zürcher, Back, 2005]. A associação de cervejeiros dos EUA define a cervejaria artesanal como: pequena; independente e tradicional [Brewers Association, 2011]. Ao contrário da produção de cerveja em larga escala, que busca obter produtos padronizados e mercados cada vez mais homogêneos, as cervejas especiais representam uma forma genuína de inovação na produção desta bebida milenar.

1.3 Consumo saudável

A relação entre uma dieta equilibrada, com grandes quantidades de alimentos ricos em polifenois, está diretamente associada à redução de risco de doenças como acidente vascular encefálico (AVC); aterosclerose; diabetes; artrite; câncer [Patil et al., 2009, Joshipura et al., 1999]. Além de prevenir doenças, uma dieta rica em polifenois pode levar a melhores resultados em testes cognitivos e diminuir o risco de depressão [Akbaraly et al., 2009]. Alguns polifenois possuem potencial anti-inflamatório, com aplicações no tratamento de doenças como a hipertensão; alergia; hipercolesterolemia; cardiopatias e

problemas pulmonares [Ames et al., 1993; Witzum, 1994; Roy & Kulkarni, 1996; Stahl & Sies, 1997; Bravo 1998; Devi et al., 2014; Georgiev, Ananga & Tsolova, 2014].

O consumo moderado de bebidas alcoolicas diminui a mortalidade, pois o etanol é conhecido por reduzir doenças coronarianas, aumentando os níveis de HDL [Hendriks et al, 1998; Sierksma et al., 2002]; aumentando a atividade da paraoxonase [Van der Gaag et al., 1999] e o efluxo de colesterol [Van der Gaag et al., 2001]; estimulando fibrinólise [Hendriks et al., 1994] e diminuindo a coagulação [Dimmitt et al., 1998]; consequentemente diminuindo os processos inflamatórios [Imhof et al., 2001; Koenig et al., 1999]. Ainda, bebidas alcoólicas produzidas por fermentação, não por destilação, contêm estimulantes da secreção de ácido gástrico [Teyessen et al., 1997], além de estimulantes de gastrina [Yokoo et al., 1999; Kimura et al., 2000]. Apesar do alcoolismo aumentar as concentrações de homocisteína no soro, estudos demonstram que consumidores de cerveja apresentam níveis mais baixos, quando comparados com consumidores de vinho ou destilados [Cravo, 1996; van der Gaag et al., 2000]. Há ainda relatos que a ingestão de xantohumol, através do consumo de cerveja, reduz a inflamação, o estresse oxidativo e a angiogênese; melhorando o processo de cicatrização [Negrão et al., 2012]. A cerveja também pode melhorar a função endotelial mais do que outras bebidas e reduzir a onda de reflexões da pressão mais do que a cerveja sem álcool [Karatzi, 2013]. Alguns resultados mostram que o consumo, de cerveja, com e sem álcool, diminui o dano mitocondrial induzido por adriamicina [Valls-Belles et al., 2010]. Já na parte comportamental, estudos evidenciam que o consumo de cerveja é ansiolítico, porém atáxico [Gallate et al., 2003].

Alguns autores estudaram as chalconas e as flavonas das cervejas, observando uma inibição da produção de óxido nítrico (NO), influenciando uma variedade de proteínas pró-inflamatórias, inibindo a expressão de ciclo-oxigenase-1 (COX-1) e molécula de adesão intercelular-1 (ICAM-1) [Zhao et al., 2003; Motyl, 2013]. Além disso, certos compostos presentes no lúpulo foram descritos como citotóxicos contra algumas linhagens de cânceres [Gerhauser 2005; Ho et al., 2008; Dorn et al., 2010; Motyl, 2013] e capazes de efeitos sedativos e hipnóticos [Cooper, 1994]. Chen e colaboradores [2014] complementam a ação dos compostos presentes na cerveja como possuidores de vários efeitos bactericidas, anti-inflamatórios, antioxidativos, antiangiogênicos, antimelanogênicos, antiosteoporótico e anticarcinogênicos. Além disso, os efeitos na pele para tratamentos de diversas doenças também é descrito tanto no uso tópico, como na ingestão de cerveja, corroborando com o uso dos povos antigos, que o faziam como medicamento.

Já o consumo indiscriminado de bebidas alcoólicas é perigoso, prejudicial para saúde e nos deixa insociáveis, tendo efeitos como degeneração do sistema nervoso, hepatite, cirrose, aumentando a incidência de cânceres, cardiomiopatia e miopatia [Cooper, 1994]. Entretanto, baixas doses de álcool estimulam o apetite e melhoram a função intestinal na velhice [Dufour, 1992]. Além disso, o consumo excessivo de álcool é descrito por acarretar problemas na pele e no epitélio do trato digestório, aumentando a chance de desenvolvimento de cânceres [Reidy; McHugh; Stassen, 2011; Chen et al, 2014]. Ainda, a cerveja possui grandes quantidades de proteínas, além de conter peptídeos derivados das prolaminas, conhecidas por desencadear alergias em celíacos [Colgrave et al, 2013].

Embora o impacto do consumo de cerveja e a quantidade considerada moderada sejam muito discutidos na literatura, ainda não foi investigado um possível efeito hepatoprotetor. Porém, o impacto do consumo da cerveja artesanal brasileira é pouco descrito; principalmente o quanto dessa bebida pode ser consumido sem que haja um dano hepático.

1.4 Bioquímica da cerveja

A água é o principal constituinte da cerveja. A elevada quantidade de água associada à concentração de sais minerais similares a do suor tornam essa bebida hidratante e refrescante [Hughes & Baxter, 2001; Jiménez et al, 2009]. A dureza da água está associada à concentração total de íons cálcio e magnésio. O cálcio protege as enzimas alfa-amilases do calor da mostura, isso contribui para uma maior hidrólise do amido durante a mosturação. Além disso, sua presença estimula a ação enzimática de proteases e amilases, aumentando o teor de açúcares fermentáveis e compostos nitrogenados no mosto. O magnésio, proveniente principalmente do malte, atua como cofator enzimático durante a etapa de fermentação [Bernstein & Willox, 1977].

O pH influencia na mostura, na extração do lúpulo e na fermentação. A faixa ideal de pH para a produção cervejeira é entre 6,5 e 7, variando de acordo com o tipo de cerveja a ser produzido [Brigido, 2006]. Se o pH for alcalino, poderá dissolver materiais não desejados na mosturação que estão contidos na casca do malte, diminuindo os efeitos positivos do cálcio e do magnésio [Bernstein & Willox, 1977].

Hornsey [1999] destaca dentre os principais constituintes da cevada os polissacarídeos amiláceos, polissacarídeos não-amiláceos, as proteínas, lipídeos e outros constituintes como os monofenois, polifenois e alguns íons. O

malte de cevada torrado pode ser um ingrediente das cervejas artesanais que não é utilizado nas receitas das cervejas comerciais, no qual foi isolado um composto com atividade antioxidante considerada benéfica em biossistemas [Papetti et al., 2006].

A cevada é utilizada na produção cervejeira por suas características intrínsecas de teor de amido, riqueza proteica, uniformidade dos grãos e quantidade de cascas [Aquarone et al., 2001]. Após passar por um processo de germinação em condições controladas (malteação) a cevada recebe o nome de malte [Cruz et al., 2008]. O objetivo deste processo é: desenvolver enzimas (alfa-amilase e beta-amilase, amiloglucosidase, beta-glucanase, protease, beta-glucanasolubilase e pentosanase) que converterão o amido em açúcares solúveis; modificar o amido tornando-o mais solúvel e solubilizar proteínas [Nogueira, 2006]. A variedade da cevada, a temperatura, a aeração, o grau de torrefação, a umidade e a secagem influenciam no processo de malteação [Cruz et al., 2008]. Outros cereais podem ser adicionados como adjuntos cervejeiros, substituindo o malte de cevada por outras fontes de carboidrato [Morado, 2009].

González-Gross e colaboradores [2001] descreveram os principais constituintes do lúpulo, que são resinas, óleos essenciais e polifenois. As inflorescências secas de lúpulo contém 4-14 % de polifenois, principalmente ácidos fenólicos, chalconas preniladas, catequinas, proantocianidinas e flavonoides. Os flavonoides são responsáveis por uma série de propriedades bioativas, propriedades sedativas, estrogênicas e anticancerígenas. Os óleos essenciais, assim como as resinas, são provenientes da glândula lupulina, que contribuem para o aroma da cerveja. Eles apresentam sesquiterpenos,

monoterpenos, álcoois, ésteres, cetonas, aldeídos e ácidos carboxílicos [Hornsey, 1999]. As resinas são divididas em moles (alfa-ácidos: humulona, cohumulona, adhumulona; e beta-ácidos: lupulona, colupulona e adlupulona) e duras (alfa e beta-ácidos oxidados). Além disso, o lúpulo possui uma resina contento monoacilfloroglicídios, que são convertidos a ácidos amargos durante o processo cervejeiro [Gerhauser, 2005].

O lúpulo é o responsável por grande parte das propriedades antioxidantes conhecidas para a cerveja em virtude da presença de compostos como o xantohumol e isoxantohumol [Stevens et al., 1999; Ceh et al., 2007]. Esses compostos possuem atividade antioxidante comparável ao resveratrol do vinho [Vinson et al., 2003; Pinto et al., 2012]. Devido a presença de iso-alfa-ácidos o lúpulo possui ainda propriedades antibióticas e bacteriostáticas [Botelho, 2009].

O conteúdo total de antioxidantes depende do tipo de cerveja, das matérias-primas e do tipo de fabricação utilizado. Cerca de 70-80 % dos polifenois da cerveja provém do malte e 20-30 % do lúpulo [Humberstone & Briggs 2000]. Os polifenois do malte passam por alterações químicas durante a malteação e no processo cervejeiro, sendo menos caracterizados do que os fenólicos do lúpulo. Sem falar dos metabólitos das leveduras muito pouco conhecidos. O ácido ferúlico é o principal ácido fenólico encontrado na cerveja [Siqueira, Bolini, Macedo, 2008]. Ele provém das paredes celulares do malte de cevada, também conferindo propriedades antioxidantes para a cerveja, porém não se sabe o quanto é perdido ao longo do processo cervejeiro [Humberstone & Briggs 2000].

Estudos indicam que as concentrações de antioxidantes em cervejas especiais europeias estão diretamente relacionadas com as quantidades de lúpulo utilizadas para cada receita. Considerando a cerveja a única fonte alimentar significativa de lúpulo, os benefícios à saúde em relação a este ingrediente tornam-se, quase que exclusivos, desta bebida. Outros estudos indagam o possível efeito aditivo/sinérgico dos compostos presentes na matriz complexa dessa bebida [Monteiro et al., 2006].

A parte fermentativa da cerveja é realizada comumente por dois tipos de leveduras, as de alta fermentação (*Saccharomyces cerevisiae,* do tipo Ale), que fermentam mais rápido e em temperaturas mais altas entre 18 – 22 °C; e as de baixa fermentação (*Saccharomyces uvarum,* anteriormente denominada *carlsbergensis*, do tipo Lager) que fermentam mais devagar a temperaturas mais baixas entre 07 -15 °C [Stewart & Russel, 1996].

A diferença bioquímica entre tipos de leveduras (Lager e Ale) é a capacidade de fermentar o dissacarídeo melibiose (glicose-galactose). O transporte, a hidrólise e a fermentação da maltose (que corresponde a cerca de 50 a 60% dos açúcares fermentáveis no mosto) e da maltotriose são fundamentais na fabricação cervejeira [Nogueira, 2006]. Ao entrarem no interior da célula, os açúcares são convertidos pela via glicolítica a piruvato (Fig. I). Esta conversão irá gerar duas moléculas de adenosina trifosfato (ATP). A etapa de fermentação passa por uma etapa aeróbea seguida por uma etapa anaeróbea. Somente na segunda etapa ocorre a formação de etanol. Quando a

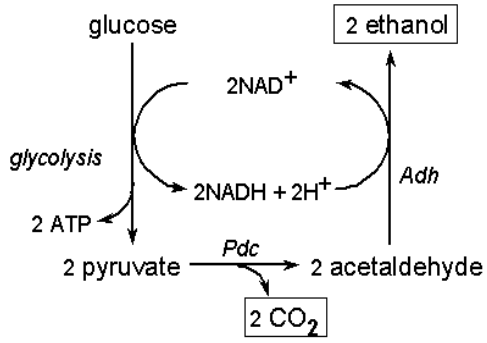

Fig. I: Via metabólica da fermentação alcoólica. [Flikweert 1999].

levedura está em condições anaeróbicas (estado fermentativo), o NAD^+ é regenerado, sendo utilizado o hidrogênio, tornando o meio mais ácido. O ácido pirúvico é convertido a dióxido de carbono e acetaldeído e este, posteriormente, a etanol. [Stewart & Russel, 1996]. Fatores como a cepa da levedura utilizada, a temperatura de fermentação, o tipo e a quantidade de adjunto adicionado, o pH do mosto e os compostos voláteis formados na fermentação contribuem para a variedade de compostos da bebida. Entre os compostos voláteis destacamos ácidos orgânicos, alcoois superiores, ésteres, carbonilas, compostos sulfúricos, aminas e fenois [Stewart & Russel, 1996; Hughes & Baxter, 2001].

A variedade de maltes utilizados; o processo de malteação; a(s) temperatura(s) e pH durante a mosturação; o método de aspersão dos grãos após a mostura; a variedade, a quantidade e o tempo de lúpulo adicionado durante a fervura; o método de resfriamento; a fermentação (tipo de fermento, temperatura, tempo); enfim, tudo isso influencia no tipo e na qualidade da cerveja [Gerhauser, 2005].

1.5 Espécies reativas e estresse oxidativo

Um radical livre é uma espécie química com um ou mais elétrons desemparelhados, sendo considerado uma espécie reativa. O alto potencial reativo deve-se a grande tendência de perder ou adquirir um segundo elétron (Halliwell 2007, Bittencourt Pasquali et al., 2013). Em condições celulares normais aeróbicas, existe um equilíbrio tênue entre a produção de espécies reativas de oxigênio (ERO) e as defesas antioxidantes [Negi et al., 2013]. O estresse oxidativo é considerado um desequilíbrio redox celular, causado pelo aumento da produção de ERO e/ou diminuição das defesas antioxidantes

[Schnorr et al., 2011]. Esse desequilíbrio é relacionado: à causa de doenças do envelhecimento [Valko et al., 2007]; à disfunção celular [Halliwell 2007,]; ao exercício físico [Pinho et al., 2006] e a diversas outras doenças [Ikawa et al., 2011, Uttara et al., 2009]. A superprodução de ERO resulta em um processo nocivo, embora o estresse oxidativo medeie danos às estruturas celulares, como lipídios de membrana, proteínas e DNA [Keisari et al., 1983]. As ERO ainda podem atuar na inativação de enzimas, como as de reparo ao DNA, devido a sua alta reatividade oxidante [Keisari et al., 1983; Valko et al., 2007; Saugstad 2001]. Os efeitos benéficos de ERO e espécies reativas de nitrogênio (ERN) ocorrem em concentrações moderadas, envolvendo funções fisiológicas para respostas celulares diversas, na defesa contra infecções, na modulação de vias de sinalização celular e na indução de resposta mutagênica [Valko et al., 2007]. Além disso, em condições fisiológicas, as ERO são conhecidas por estimularem a ativação de segundos mensageiros, aumentarem a produção de interleucina-2 em células T, ativarem fatores de transcrição como o NF-kB, controlarem a concentração de oxigêncio agindo como sensores e contribuir para a transdução de sinal de vários receptores de membrana. Já as ERN agem como moléculas regulatórias do relaxamento do músculo liso e na inibição da adesão de plaquetas [Droge, 2002].

Diante disso, compostos antioxidantes encontrados na dieta ou mesmo sintéticos são empregados nas indústrias de alimentos, cosméticos, bebidas e na medicina, atenuando assim os efeitos dos radicais livres [Valko et al., 2007]. Apesar da excelente capacidade do sistema antioxidante endógeno em sustentar a homeostase redox, nosso organismo requer outras fontes de antioxidantes, normalmente adquiridas pela dieta [Pietta, 2000]. Os

antioxidantes agem de forma a inibir, retardar ou diminuir o dano oxidativo, protegendo as células, reestabelecendo ou mantendo a homeostase redox [Valko et al., 2007].

Em termos gerais, os sistemas antioxidantes são divididos em dois principais grupos: enzimático e não-enzimático. Entretanto, Halliwell e Gutteridge [2000] apresentam o sistema antioxidante dividido em duas etapas: a) interceptação das espécies reativas e b) reparo dos danos causados pelas espécies reativas. A primeira etapa ocorreria através de substâncias enzimáticas e/ou não enzimáticas, como vitaminas e compostos antioxidantes; a segunda etapa aconteceria através de enzimas de reparo, as quais atuam em danos causados pelos radicais livres no DNA, esses danos desempenham um importante papel nos processos de mutagênese e carcinogênese [Poulsen et al. 1998].

Assim sendo, pesquisadores buscam nos alimentos efeitos protetores em órgãos alvos de estresse oxidativo (como fígado e sangue, por exemplo). No contexto do processo cervejeiro, considerando as cervejarias como laboratórios, a bioquímica foi fundada como ferramenta de estudo, controle e melhoramento do produto. Evidenciando o alto potencial de ensino de bioquímica no processo cervejeiro.

1.6 Defesas antioxidantes não enzimáticas

Muitos alimentos de origem vegetal são considerados ricos em defesas antioxidantes não enzimáticas que podem ser absorvidas, dependendo de sua biodisponibilidade, pelos animais que as consomem. Entre os antioxidantes mais conhecidos, estão os polifenois. Estes são um grupo de moléculas muito presente nos vegetais, sendo que suas características físico-químicas

conferem proteção contra patógenos e contra radiação ultravioleta [Kumazawa et al., 2006; Rudnicki et al., 2007]. Os flavonoides são os polifenois mais estudados, e apresentam uma estrutura comum de difenilpropano, com dois aneis aromáticos unidos por três carbonos (Fig. II). A ação bioquímica dos polifenois deve-se a sua capacidade de reação com radicais livres e/ou espécies reativas, eliminando-as ou reagindo com elas, impedindo que reajam com outras biomoléculas [Bravo 1998]. Os compostos fenólicos se apresentam como a principal classe de antioxidantes presentes nas plantas com funções redutoras e sequestradoras de radicais livres, estando associadas a capturar ERO [Craveiro, 2006].

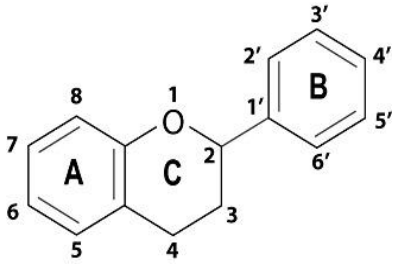

Fig. II: Estrutura química dos flavonoides. [Lillo, Lea & Ruoff, 2008].

As cervejas, por serem produzidas a partir de vegetais como o malte de cevada e o lúpulo, possuem uma matrix complexa rica em polifenois, nos quais já foram atribuídas diversas propriedades benéficas para a saúde humana [Gerhauser, 2005]. A estimativa do consumo de polifenois é incerta e varia na literatura, pois nos sistemas biológicos, os compostos não agem isolados ou como nos tubos de ensaio, havendo um sinergismo entre eles e o meio em que se encontram, além de uma possível interação com a microbiota particular de cada animal [Possemiers et al., 2005]. A cerveja artesanal, por não ser filtrada e conter células de leveduras, é considerada uma boa fonte alimentar desse tipo de moléculas, fornecendo diversos compostos tanto para a microbiota, quanto para o nosso organismo [Possemiers et al., 2005; Bamforth, 2002].

1.7 Modelo de indução de dano com tetracloreto de carbono

O CCl_4, quando administrado em um animal, é metabolizado no fígado e transformado no radical CCl_3^-, desencadenado uma série de reações e respostas nos biossistemas [Pinto et al., 2012; Van de Bovenkamp et al., 2006]. Uma substância potencialmente antioxidante é investigada através de um modelo com um dano prévio ou uma indução de dano. O modelo de indução oxidativa com tetracloreto de carbono é um dos mais utilizados na literatura em testes para moléculas com atividade hepatoprotetora [Al-Yahya et al., 2013; Rudinicki et al., 2007; Dani et al., 2009]. A disponibilidade de substâncias antioxidantes através da dieta rica em compostos fenólicos pode reduzir o dano causado pelo tetracloreto de carbono [Parise et al., 1992; Neves et al., 2003; Chehter et al., 1987]. Além disso, o Legalon®, um medicamento dito hepatoprotetor, é geralmente utilizado na literatura como controle interno do modelo de indução [Al-Yahya et al., 2013; Nasiri Bezenjani et al., 2012]. Esse é um fitoterápico obtido a partir da planta *Silybum marianum,* que contém grandes quantidades de silimarina (cerca de 80 % do extrato seco das folhas), seu componente ativo, que proporciona uma melhora nos sintomas clínicos de hepatite, cirrose e lesões do fígado. A silimarina é um flavonoide que age protegendo as membranas celulares dos hepatócitos, mantendo sua integridade [Vargas-Mendoza et al., 2014].

2. JUSTIFICATIVA

Por ser uma bebida amplamente consumida, pesquisas relevantes para a saúde humana contribuem para o conhecimento científico e popular. Além disso, pouco se conhece sobre os efeitos benéficos da cerveja. Wright e colaboradores [2008] mostraram em sua pesquisa que a cerveja é no mínimo equivalente ao vinho sob a ótica nutricional no combate a doenças cardiovasculares. Uma dieta rica em polifenois contribui para o sistema antioxidante do nosso organismo [Pietta, 2000]. Sabemos que as cervejas possuem uma grande quantidade de compostos como os polifenois [Gerhauser, 2005], porém não sabemos se esses são absorvidos pelo nosso organismo e nem se são capazes de prevenir algum tipo de estresse induzido.

Sabe-se que para observação de efeitos antioxidantes em biossistemas é necessário que haja um desequilíbrio redox, o estresse oxidativo. Para isso foi utilizado o modelo de indução de dano com CCl_4, por ser bem estabelecido e aceito na literatura [Van de Bovenkamp et al., 2006; Rudinicki et al., 2007; Dani et al., 2009; Pinto et al., 2012; Al-Yahya et al., 2013; Gasparotto et al., 2014]. Dentro desse modelo, como controle positivo, é utilizado um hepatoprotetor (silimarina do medicamento Legalon®) [Nasiri Bezenjani et al., 2012].

3. OBJETIVOS

O objetivo geral desse estudo foi investigar o impacto do consumo de cerveja artesanal e se o pré-tratamento crônico com essa bebida poderia prevenir ou atenuar o dano hepático induzido por CCl_4 em ratos Wistar machos.

Para atingir a este objetivo geral, desenvolveram-se os seguintes objetivos específicos:

1) Comparar o potencial antioxidante das cervejas artesanais, das cervejas comerciais e dos extratos de lúpulo *in vitro.*
2) Observar se o tratamento com cerveja altera parâmetros basais fisiológicos em animais.
3) Verificar o efeito do consumo de cerveja em modelo animal de dano hepático induzido por tetracloreto de carbono.

4. RESULTADOS

Nessa parte do trabalho, os resultados, bem como os materiais e métodos, serão apresentados em forma de artigo científico. Esse artigo será submetido ao periódico Journal of Food and Chemical Toxicology, intitulado:

Could craft beer really be efficient as hepatoprotector?

4.1 Could craft beer really be efficient as hepatoprotector?

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ABSTRACT

Polyphenols are molecules produced by plants known to have antioxidant potencial, pointing out that a diet rich in antioxidant compounds can be good for health. Beer is a complex beverage, rich in this compunds and is consumed all over the world. There are many different beer styles, although commercial beer is the most consumed style, it is very poor in bitterness and has only one variety of barley in its recipe. Hop is an ingredient of beers that contribute a lot to phenolic compounds and bitterness to this beverage. Craft beers usually have more hops in their recipies as well as a great variety of barley, suggesting that craft beers could contribute more for health. Our objective was to compare antioxidant potential of beers and investigate the effect of beer consumption in a carbon tetrachloride (CCl_4) model in rats. We treated them for 30 days in the drinking bottle containing a commercial beer, a craft beer, ethanol 6,7 %, water or silymarin. Then we injected intraperitoneally (i.p) CCl_4 or mineral oil, as control, 12 h before euthanasia. We analyzed abdominal fat, liver redox status, serum fat profile and redox status. Craft beer raised high-density-lipoprotein (HDL) levels and increased non-enzimatic antioxidant potential in serum. The consumption of beers did not affected the physiological basal oxidative status, but there was no protection against CCl_4.

INTRODUCTION

Beer is the most worldwide consumed alcoholic bevarage reaching 188.81 million kiloliters in 2013 [Kirin Beer University]. This beverage contains antioxidants, certain minerals, some vitamins, fibre and relatively low levels of ethanol [Bamforth, 2002]. The type and quality of beer are influenced by the variety of barley malt, the malting process, the temperature and pH during mashing, the way of sparging, the variety and the time-point of hops added

during wort-boiling, as well as the variety and quantity of yeast, and the addition of adjuncts and the equipaments used [Gerhauser, 2005]. For a cheaper brewing, big industries change barley malt for rice and/or corn (aproximatly 50 %) [BJCP], and use minimum quantities of hops [Wunderlich, 2005]. The term craft beer emerged in US from the need to distingue the large scale beers (Stantard American Lager Style). Craft breweries are defined as: small; independet and traditional [Breweres Association, 2011].

Hop (*Humulus lupulus*) is the raw material of beer that serves as an important source of polyphenols. Dried hop cones contain 4–14 % polyphenols, mainly phenolic acids, prenylated chalcones, flavonoids, catechins and proanthocyanidins [Stevens et al., 1998; De Keukeleire et al., 1999; Taylor et al., 2003]. Xanthohumol and isoxanthohumol are unique compounds found in beers, derivated from hops and isomerized in brewing process. They reveal several beneficial effects for cellular health, reducing inflammation, oxidative stress and angiogenesis; ameliorating the wound healing process [Negrao, 2012]. Isolated compounds of beer may have positive effects on health [Gerhauser 2005, Monteiro, 2006], however, the possible additive/synergistic effect of the compounds present in this beverage's complex matrix should be considered [Monteiro, 2006].

Moderate consumption of alchoolic beverages decrease mortality, because ethanol can reduce coronary diseases by: raising HDL levels [Hendriks et al., 1998; Sierksma et al., 2002]; increasing paraoxonase activity [Van der Gaag et al., 1999], cholesterol efflux [Van der Gaag et al., 2001]; stimulating fibrinolysis [Hendriks et al., 1994]; decreasing coagulation [Dimmitt et al., 1998]; and decreasing inflammatory processes [Imhof et al., 2001; Koenig et al., 1999].

However, abusive ethanol consumption induce cirrosis, cancers, and nervous system degeneration [Cooper, 1994]. Moderate consumption of beer and wine is known to bring beneficial effects for health, specially for cardioprotection [Chiva-Blanch, 2013]. Others data shows that chronic alcoholism leads to high concentrations of homocysteine in serum and that beer drinkers have lower serum concentrations in comparison with wine or spirits drinkers [Cravo, 1996]. Although beneficial effects for cardioprotection are observed just with ethanol consumption [Karatzi, 2013] alcoholic beverages produced by alcoholic fermentation, but not by distillation, contain powerful stimulants of gastric acid secretion [Teyssen et al., 1997]. In cardiovascular system, beer can improve endothelial function more than other drinks and reduce pressure wave reflections more than dealcoholized beer [Karatzi, 2013]. Nevertheless, beer consumption with and without alcohol decrease adriamycin-induced damage of mitochondrial chain components [Valls-Belles, 2013].

It is still argued what is the impact of drinking beer in biological systems and how much of it's consume can induce hepatic damage. Still, the possible hepatoprotective effect of craft beer consumption has not been investigated so far. The induction of hepatic damage by CCl_4 is a well establish model to investigate hepatoprotection. CCl_4 is metabolized in liver and transformed in CCl_3^- radical (Dani et al., 2008; Reckengel et al., 1989). For a better comparison, the concomitant use of silymarin, a hepatoprotector, is used as a positive control [Mourelle, 1989; Al-Yahya, 2013]. Therefore, the aim of this research was to compare the antioxidant potential of craft beer, commercial beer and hops extracts; also, investigate the effect of beer consumption against hepatic damage induced by carbon tetrachloride (CCl4) in male Wistar rats.

MATERIAL AND METHODS

Chemicals

Catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), thiobarbituric acid (TBA), hydrogen peroxide (H_2O_2), polyphenols standards (caffeic acid; epicatechin; catechin; isoxanthohumol and rutin) were from Sigma-Aldrich (St. Louis, USA). Carbon tetrachloride (CCl_4) were acquired from Dinâmica (São Paulo, Brazil). Ethanol from Zeppelin (Rio Grande do Sul, Brazil). β-actin primary antibodies and secondary-HRP linked antibodies were from Cell Signaling technology (Beverly, USA). Aspartate aminotransferase (AST), alanine aminotransferase (ALT) activities kits, lipid fraction kits and glucose kits were from Labtest (Minas Gerais, Brazil). Silymarin as Legalon® medicine (http://www.medicinanet.com.br/bula/3036/legalon.htm).

Beer samples

Commercial beers were from a common market and craft beers were made by Anner Brewery (Rio Grande do Sul, Brazil) (same lot and expiration date for all). Hops extracts were made exactly the same as breweing, but there was just water and hop (1,09; 1,83; 2,89; 2,05 g/L respectively for blond ale, tripel, bitter and red ale). Only hops extracts of craft beers were analyzed because there is no data indicating how much of hops are used in commercial beer. For biological experiment, only one of each group of beers was used. Red ale was used as craft beer. It was used 30 bottles with 600 mL of craft beer and 60 bottles with 350 mL of commercial beer in all experiment. Commercial beer had 5 % of ethanol and craf beer had 6,7 %.

Animals and experimental design

Male Wistar rats, 70 in total (60 days-old, weighting 200-250 g), were obtained from our breeding colony. Rats were maintained in a 12-hour light–dark cycle in a temperature-controlled colony room (21 °C). They were caged in 5 groups with free access to standard commercial food Chow Nuvilab CR1 (Paraná, Brazil) and supplemented for 30 days in the drinking bottle with: water, ethanol 6.7 %, commercial beer or craft beer every night (exception of Silymarin group, that received 2 mL/kg of Legalon drug by gavage). During the day, all groups had free acess to water. Experimental groups were further divided in 2 subgroups and received (intraperitoneally) CCl_4 or mineral oil (both 2,5 mL/kg) as a control. Biological samples (fats, liver and serum) were collected 12h after the injections; previously fasted for 12 h. All collected tissues were weighed (liver and abdominal fats) and liver was fixed in paraformol buffer 4 % for histological preparation. Serum biochemical profile and liver redox profile were analysed. All experimental procedures were performed in accordance with the guidelines of the National Institutes of Health [Research, US 2011]. Our research protocol (n° 27632) was approved by the Ethical Committee for Animal Experimentation of the Universidade Federal do Rio Grande do Sul. Also, we calculated the consumption of beer per cage and divided for the sum of rats weight. Converting the values, considering body surface área (BSA) for human equivalency [Reagan-Shaw, 2008].

Measurement of ALT, AST, blood glucose and lipid fraction in serum

Alanin-aminotransferase (ALT), aspartate-aminotransferase (AST), lipid fraction, and blood glucose in serum were determined using Labtest kits (Minas Gerais, Brazil) according to manufacturer instructions.

Antioxidant Enzymes (SOD, CAT and GPx) and GST

Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities were quantified in tissue homogenates (liver). SOD and GPx were also quantified in serum. CAT activity was evaluated by following the rate of hydrogen peroxide (H_2O_2) absorbance decrease at 240 nm [Aebi, 1984]. The activity of SOD was measured by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation to adrenochrome, which was monitored at 480 nm for 10 min (32 °C) in a spectrophotometer [Misra, 1972]. For quantification of SOD1 activity, cyanide (KCN) was used, and the difference of total SOD with SOD1 resulted in SOD2 activity. GPx activity was measured by following the decrease of NADPH at 340nm (37 °C) [Mannervik, 1985]. GST was measured by the synthesis of a thioeter dinitrofenilo monitorated at 340 nm [Habig, 1974]. The ratio SOD/peroxidases was calculated picking the values of total sod activity and divided by the sum of catalase and glutathione peroxidase activities.

Oxidative damage markers (Carbonyl, TBARS, and Sulfhydril content)

As an index of protein oxidative damage, the carbonyl groups were determined based on its reaction with 2,4-dinitrophenylhydrazine (DNPH) as previously described [Levine, 1990]. Lipoperoxidation was determined from the quantification of thiobarbituric acid reactive species (TBARS) originated from reaction of thiobarbituric acid with lipoperoxides in an acid-heating medium. After precipitation with trichloroacetic acid 10% (TCA), supernatant was mixed with 0.67 % of TBA and heated in a boiling water bath for 20 min. TBARS were determined by the absorbance in a spectrophotometer at 532 nm [Draper, 1990]. Oxidative status of thiol groups were assessed by quantification of total reduced sulfhydryl (SH) groups. Samples react with 5,5′-dithionitrobis-2-

nitrobenzoic acid (10 mM) for 60 minutes of incubation in room temperature and read in a spectrophotometer at 412 nm [Ellman, 1959]. These parameters were analysed in serum and liver.

Protein assay

Total protein was quantified by Lowry assay and used to normalize all data [Lowry et al., 1951].

Western blotting

To perform immunoblot experiments, the tissue was prepared using RIPA buffer protocol. The proteins content (20 µg/well) were fractionated by SDS-PAGE and electro-blotted onto nitrocellulose membranes with Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad (Hercules, CA, USA). Protein loading and electro-blotting efficiency were verified through Ponceau S staining, and the membrane was washed with Tween–Tris buffered saline (Tris 100 mM, pH 7.5, 0.9 % NaCl and 0.1 % Tween-20). Membranes were incubated 20 minutes at room temperature in SNAP i.d.® 2.0 Protein Detection System Merck Millipore (Billerica, MA, USA) with each primary antibody (anti-SOD1, anti-SOD2, anti-SOD-EC, anti-CAT, anti-GPx1, anti-HSP70 or anti β-actin; 1:500 dilution range) and subsequently washed with TTBS. Anti-rabbit or mouse IgG peroxidase-linked secondary antibody was incubated for additional 20 minutes in SNAP (1:5000 dilution), washed again and the immunoreactivity was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent kit (Thermo Scientific; Luminol/Enhancer and Stable Peroxide Buffer). Densitometric analysis of the films was performed using Image J. software. Blots were developed to be linear in the range used for densitometry. All results

were expressed as a relative ratio to β-actin (liver) or to ponceau image (serum).

Histological Analysis

Formalin-fixed samples of liver were dehydrated, diaphonized and embedded in paraffin according to protocols for routine histological procedures. Five-micrometer thick sections of the paraffin-embedded tissues were obtained and stained by means of haematoxylin eosin (H&E) histochemical method. The sections were examined microscopically for evaluation of histological changes [Frei, 1984].

Determination of total phenolic content in liver and serum

Total phenolic content in liver and serum was determined using the Folin-Ciocalteu method [Ainsworth, 2007]. One hundred µL of Folin-Ciocalteu reagent was mixed with 200 µL of sample and then 200 µL of Na_2CO_3 35% were added. The volume was completed to 1900 µL with ultra-pure H_2O and then homogenized. After 10 min, the absorbance was measured at 725 nm and compared to a gallic acid calibration curve. Total polyphenols in samples were expressed as gallic acid equivalents [Singleton, 1965].

Glycogen estimation

Gycogen was extracted from liver with hot KOH 30 % and precipiteded with ethanol. Further, glycogen reacted with iodine and glycogen content was measured at 460 nm [Krisman, 1962].

Non-enzymatic Antioxidant Potential

The total reactive antioxidant potential (TRAP) has been used as an index of the nonenzymatic antioxidant capacity, based on the peroxyl radical (generated by AAPH solution, 2,2′-azobis[2- amidinopropane], with luminol) quenching by

sample compounds. The system is an AAPH 10 mM solution prepared in glycine buffer (pH 8.4) with luminol (4 mM) and stabilized for 2h before the assay. The chemiluminescence emission is quantified. The results were transformed in percentual, and the area under curve (AUC) was calculated by GraphPad® software (version 5.00; GraphPad Software Inc., SanDiego, CA). The total antioxidant reactivity (TAR) was also analyzed, and it is based on the same technical principles of TRAP. The TAR results were calculated as the ratio of light in absence of samples (I_0) light intensity right after sample addition (I) (Lissi et al., 1992, 1995). For samples preparation, extracts were diluted 250x and added 10 µL in the system buffer (190 µL). For serum analysis, it was added 10 µL of fresh serum.

Mass Spectrometer LC-MS/MS for phenol quantification

An Agilent 1200 LC system (Germany) coupled to Applied Biosystems API 500 triple quadrupole mass spectrometer (Toronto, Canada) was employed. The chromatographic separation was carried out on a XTerra C18 column (3.5 µm, 100 mm × 2.1 mm; Waters, Milford, MA, USA) with guard column (Phenomenex, USA). The mobile phase gradient elution was water (A) and acetonitrile (B) both containing 0.1 % of formic acid. The flow rate was 300 $\mu L.min^{-1}$ and a 4 minutes equilibrate time was applied. The gradient started with 5 % of B increasing to 20 % in 2 min, 50 % in 6 min, 90 % in 10 min, kept for 2 min and decreasing to 5 % in 12 min and maintained until 13 min. The column temperature was maintained at 40 °C. The injection volume was 10 µL. Mass spectrometer resolution in multiple reactions monitoring (MRM) was unitary and dwell time applied was 50 ms for all transitions. The mass spectrometer was operated in negative electrospray ionization mode and precursor ions monitored

were [M-H] (Table 1). Nitrogen was used as nebulizer gas, curtain gas, heater gas and collision gas (psi units). Collision gas (CAD) was set at 4 psi, nebulizer gas (GS1) and dryer Gas (GS2) were set at 50 psi. Curtain gas and temperature was set at 20 psi and 600 ºC, respectively. Electrospray capillary voltage was set at -4.5 kV. Sample preparation: An aliquot of 500 µl of beer was placed in Eppendorf tube. Five hundred µL of acetonitrile was added in order to precipitate proteins and vortexed for 30s. The samples were centrifuged for 10 min at 12.000 g. A 200 µl aliquot of the supernatant was then diluted with 800 µl of water containing 0.1% formic acid and analyzed in LC-MS / MS system.

Table 1. Monitorated data for phenols quantification

Q1	Q3	Dwell Time (ms)	ID	DP (V)	EP (V)	CE(V)	CXP (V)
179,148	133,9	50	Caffeic acid 1	-60	-10	-34	-7
179,148	89,1	50	Caffeic acid 2	-60	-10	-44	-7
289,066	245	50	Epicatequin 1	-125	-10	-22	-13
289,066	108,9	50	Epicatequin 2	-125	-10	-36	-5
289,066	122,9	50	Epicatequin 3	-125	-10	-42	-7
353,084	212,9	50	Isoxanthohumol 1	-70	-10	-14	-11
353,084	119	50	Isoxanthohumol 2	-70	-10	-42	-9
609,237	299,9	50	Rutin 1	-240	-10	-52	-17
609,237	301	50	Rutin 2	-240	-10	-44	-17

Abbreviations: DP – Declustering Potential; EP – Entrance Potential; CE – Collision Energy; CXP – Exit Collision Cell Potential

Determination of heavy metals in the beers

The determination of Pb, Cd, and Cr was carried out using a Model 700 high-resolution continum source atomic absorption spectrometer (Analytic Jena AG, Germany), equipped with a transversely heated graphite tube atomizer. The contra 700 is equipped with a xenon short-arc lamp with a nominal power of 300 W operating in a hot-spot mode. The high-resolution double monochromator with a prism pre-monochromator, a high-resolution echelle monochromator and a linear charge coupled device (CCD) array detector with 588 pixels, 200 of

which are used for analytical purposes. The analytical lines at 283.306 nm for Pb, 357.869 nm for Cr, and 228.802 nm for Cd were used. The instrumental parameters were optimized and the method was adapted for Cd and Cr [Zmozinski et al., 2015] and for Pb [Zmozinski et al., 2013]. The standard solutions were prepared daily by appropriate dilution of the stock standard solutions of 1000 mg L^{-1} (Specsol, Brazil) for Pb, Cd and Cr. The chemical modifiers used were 0.05 % (m/v) Pd $(NO_3)_2$ + 0.03 % $Mg(NO_3)_2$ from (Merck, Germany). Nitric acid, purified in quartz sub-boiling still (Kürner Analysentechnik, Rosenheim, Germany) was used to prepare the aqueous calibration solution. All containers and glassware used for mineral determination were soaked in 1.4 mol L^{-1} nitric acid for at least 24 h and rinsed three times with deionized water before use. The following reagents were used for sample digestion: 65 % (v/v) HNO_3 and 30 % (v/v) H_2O_2 all from (Merck, Germany). A block wet-acid digestion in closed PTFE vessels was carried out. Initially, 3.0 mL of beer sample, 4 mL of HNO_3 (65 % v/v), 2 mL of H_2O_2 (30 % v/v) were added in the PTFE vessels. Then, the vessels were closed, placed inside de block and heated up to 150 °C for 2 hours. After cooling to environment temperature, the digested samples were diluted to a final volume of 10 mL with ultrapure water. All samples were digested in triplicate. The digested samples were analyzed injecting 30 µL of samples into the furnace with 10 µL of the chemical modifier solution for Pb. For Cr and Cd, it was not necessary modifier solution.

Statistical analysis

Statistical analysis was performed with IBM software SPSS statistics. The results were expressed as mean ± standard error (SEM). Data were evaluated

by univariate anova followed by Bonferroni's post hoc test. Differences were considered significant when $p < 0.05$ for all data. Some biological data have no variance homogeneity (Underwood, 1997) and some data were transformed using minimum ponderated squares method for homogeneity. Data with no differences are not shown.

RESULTS

The antioxidant potential of beers in TRAP assay revealed that craft beers presented better quenching potential for peroxyl radical than comecrial beers. For TRAP, lower AUC represent higher antioxidant potential. The respectives hops extracts did not show the same potential as craft beers, ethanol concentrantios had no antioxidant capacity. For TAR, values are directly proportional to antioxidant reativity, also craft beers had higher values than commercial beers (Fig. 1).

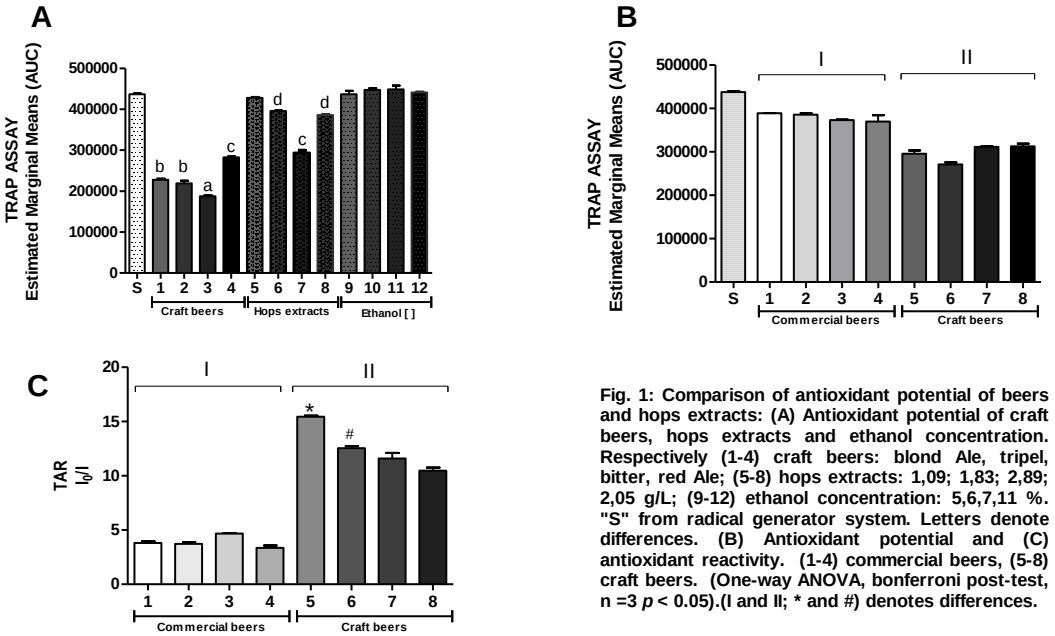


Fig. 1: Comparison of antioxidant potential of beers and hops extracts: (A) Antioxidant potential of craft beers, hops extracts and ethanol concentration. Respectively (1-4) craft beers: blond Ale, tripel, bitter, red Ale; (5-8) hops extracts: 1,09; 1,83; 2,89; 2,05 g/L; (9-12) ethanol concentration: 5,6,7,11 %. "S" from radical generator system. Letters denote differences. (B) Antioxidant potential and (C) antioxidant reactivity. (1-4) commercial beers, (5-8) craft beers. (One-way ANOVA, bonferroni post-test, n =3 $p < 0.05$).(I and II; * and #) denotes differences.

We analysed heavy metals (Pb, Cr, and Cd) in commercial beer choosen for biological experiment and in only three craft beers. The commercial beer is more watered down, however it showed the highests levels of Cr and Cd, craft

beers also differ in Cr and Cd levels. All beers were under harmful levels of heavy metals [Council 98] and no Pb was found (Table 2).

Table 2: Analytical results for Pb, Cd and Cr in beer samples by HR-CS GF AAS.

Beer	Pb (µg L^{-1})	Cd (µg L^{-1})	Cr (µg L^{-1})
Commercial	< LQ	0.60 ± 0.01 [a]	20.0 ± 2.1 [a]
Craft 1	< LQ	0.52 ± 0.01 [b]	15.0 ± 1.0 [b]
Craft 2	< LQ	0.49 ± 0.03 [b]	17.0 ± 1.7 [a]
Craft 3	< LQ	0.30 ± 0.02 [c]	18.0 ± 1.0 [a]

Commercial beer choosen for animal experiment. Craft 1: tripel; craft 2: red ale; craft 3: blond ale. (One-way ANOVA, bonferroni post-test, n =3 p < 0,05). Letters denote differences. Limit of quantification. Pb: 4.0 µg L^{-1} Cd: 0.22 µg L^{-1} Cr: 7.0 µg L^{-1}.

The polyphenol content was analysed only in the beers choosen for biological experiment. The craft beer tested has more compounds (sixteen), being a more complex matrix (Table 2). Six compounds were quantified and commercial beer tested has more catechin, caffeic acid, epicatechin and less isoxanthohumol and rutin than craft beer tested (Table 3).

Table 3: Polyphenol content in beers generated by LC-MS/MS.

	Compound	Mass Range	Craft	Commercial
1	Caffeic acid	179.0315-179.0375	x	x
2	Ferulic acid	193.0471-193.0531	x	x
3	Daidzein	253.0471-253.0531	x	
4	Apigenin	269.0420-269.0480	x	
5	Genistein	269.0420-269.0480	x	
6	(-)-Epicatechin	289.0682-289.0742	x	x
7	(+)-Catechin	289.0682-289.0742	x	x
8	Quercetin	301.0319-301.0379	x	
9	3,7-Dimethylquercetin	329.0632-369.0692	x	x
10	8-Prenylnaringenin	339.1203-339.1263	x	x
11	6-Prenylnaringenin	339.1203-339.1293	x	x
12	4-Caffeoylquinic acid	353.0843-353.0903	x	
13	5-Caffeoylquinic acid	353.0843-353.0903	x	
14	Isoxanthohumol	353.1359-353.1419	x	x
15	Xanthohumol	353.1359-353.1419	x	x
16	Procyanidin dimer B3	577.1316-577.1376		x
17	Prodelphinidin dimer B3	577.1316-577.1376		x
18	Quercetin 3-O-rutinoside (Rutina)	609.1426-609.1486	x	

Representative results from three samples.

Table 4: Quantification of polyphenols in beers generated by LC-MS/MS.

Beer	Caffeic acid ng/mL	Catechin ng/mL	Epicatequin ng/mL	Isoxanthohumol ng/mL	Rutin ng/mL
Craft	72 ± 0,7	596 ± 18,2	98 ± 10,4	1403 ± 15,6	658 ± 24,9
Commercial	163 ± 2,4	1423 ±28,9	253 ± 4,9	529 ± 6,9	41 ± 0,8

Representative results, as mean and standard error, from three samples.

Rats that received commercial beer had higher consumption and rats that received craft beer group ate less, but there were no changes in drinking water during the day nor in weight gain in all experimental groups. However, rats administred with silymarin decreased abdominal fat weight (Table 5).

Table 5: Consumption data, weight gain and abdominal fat weight.

Treatment	Drinking day shift (mL/kg)	Drinkining night shift (mL/kg)	Eating per day (g/kg)	Weight gain (g)	Abdominal fat weight/ total weigtht (g)
Commercial beer	24,69 ± 2,56	130,8 ± 12,43 *	75,14 ± 5,35	87,21 ± 4,85	2,91 ± 0,11
Craft beer	24,2 ± 3,81	100,4 ± 4,36	56,9 ± 5,88 *	94,23 ± 4,80	3,04 ± 0,28
Ethanol 6,7%	24,58 ± 2,95	88,13 ± 9,96	74,08 ± 5,26	82,42 ± 4,25	2,88 ± 0,11
Silymarin	17,91 ± 2,54	85,62 ± 2,50	78,65 ± 4,07	95,62 ± 2,56	2,72 ± 0,10 *
Water	20,27 ± 1,23	97,03 ± 8,35	87,3 ± 6,31	84,92 ± 4,95	3,14 ± 0,21

(Anova oneway, bonferroni post-test, n =14, $p < 0.05$). (*) denotes differences.

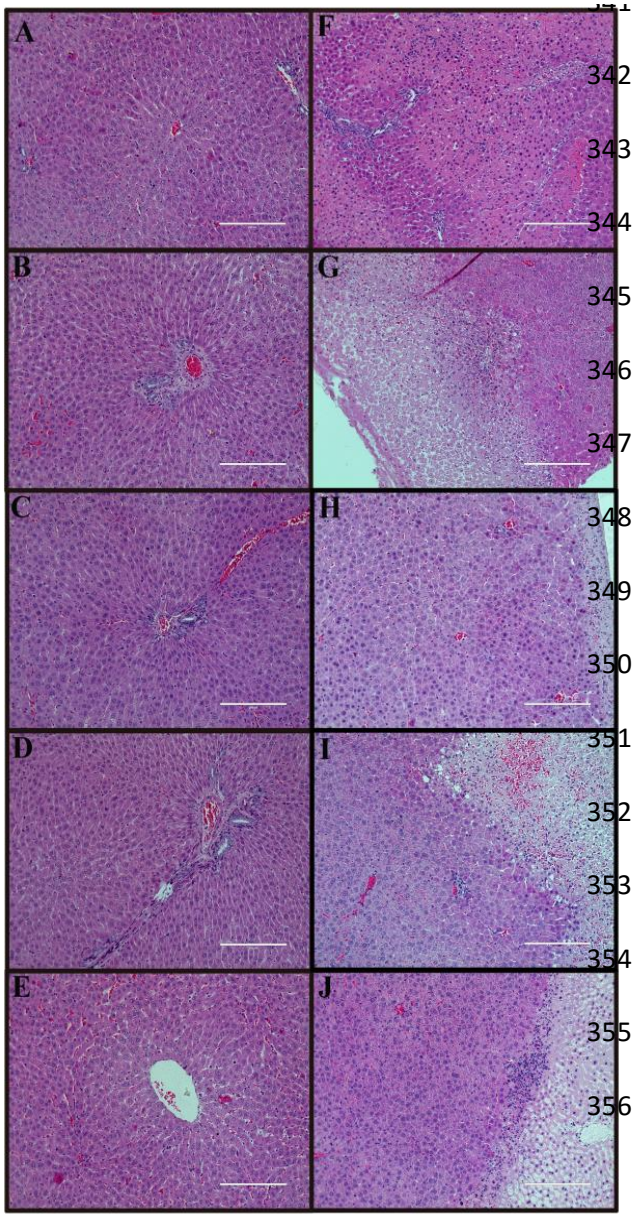


Fig. 2: Histological images of liver. (ABCDE) Control; (FGHIJ) CCl_4, respectively: water, silymarin, ethanol, commercial and craft beer. Representative results from one sample.

Beer consumption had no protective effect in oxidative parameters evaluated, such as: carbonils, TBARS, sulfhydril content, nitrotyrosine levels in serum and liver. Also, no differences in ALT and AST serum activity and liver weight were observed. These suggest that the cronic treatment with beer used in our experiment (aproximatly 40 mL per animal for 30 nigths) caused no increase: in oxidative base line, in fat weight or tissue

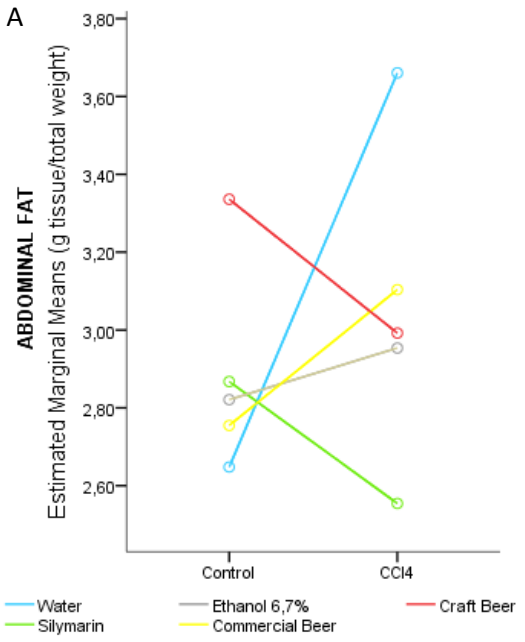
damage, as compared to control rats. Fig. 2 shows only the effect of CCl_4 injection, indicating no hepatic injury and no hepatoprotection caused by treatments. I.p. injection of CCl_4 was sucessfull as hepatic injury inducer evidenced by hydropic degeneration and nuclear polymorphism, which were characterized by presence of hepatocytes cloudy swelling with pale cytoplasm and poorly delineated and displaced nuclei (Fig. 2). CCl_4 increased liver weigth; ALT and AST activities and lipid fractions (LDL, total cholesterol and triglycerides) in serum. It decreased glucose levels, glycogen content in liver and antioxidant enzymes activity, GPX in serum and SOD 1 in liver (Table 6).

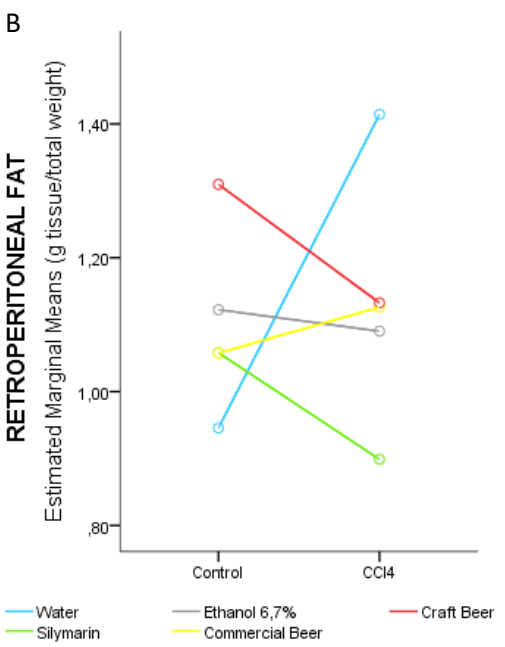
Table 6: Representation of CCl_4 effect in liver and serum.

	Analysis	Control	CCl_4
Serum	Cholesterol (mg/dL)	60,7 ± 1,6	↑ 72,8 ± 1,5
	Glucose (mg/dL)	96,2 ± 2,4	↓ 87,1 ± 2,4
	GPx activity	96,3 ± 3,6	↓ 52,5 ± 3,5
	HDL (mg/dL)	31,1 ± 0,6	↓ 28,5 ± 0,6
	LDL (mg/dL)	10,7 ± 0,9	↑ 19,7 ± 0,8
	TAR I_0 /I	8,1 ± 0,3	↑ 10,04 ± 0,3
	ALT (U/L)	253,9 ± 12,7	↑ 896,1 ± 73,45
	AST (U/L)	58,3 ± 2,0	↑ 216,04 ± 23,3
	TRAP (AUC)	274943 ± 2481	↓ 267231 ± 2446
	Triglicerides (mg/dL)	85,7 ± 6,6	↑ 112,3 ± 6,5
Liver	Abdominal fat + liver weight	5,8 ± 0,1	↑ 6,2 ± 0,1
	Glycogen (mg/mg tissue)	17,3 ± 1,4	↓ 10,2 ± 1,4
	Liver weight (g)	2,96 ± 0,03	↑ 3,16 ± 0,03
	SOD1 activity (U SOD/mg protein)	14,6 ± 0,5	↓ 9,41 ± 0,5

(Two-way ANOVA, bonferroni post-test, n =7, $p < 0.05$). Arrows denote differences.

We removed abdominal fats (gonadal fat, retroperitoneal fat, omental fat and visceral fat) and weighted after euthanasia. CCl_4 raised abdominal fats in water treated rats group, but silymarin could prevent this raise. Also, rats treated with silymarin decreased abdominal fat and retroperitoneal fat. Beer consumption did not affect fat weight (Fig. 3). In craft beer treated rats we may observe some adaptations in serum, such as a decrease in AUC of TRAP assay, an increase in HDL levels (Fig. 4) and an increase in imunocontent of SOD3 (Fig. 5).

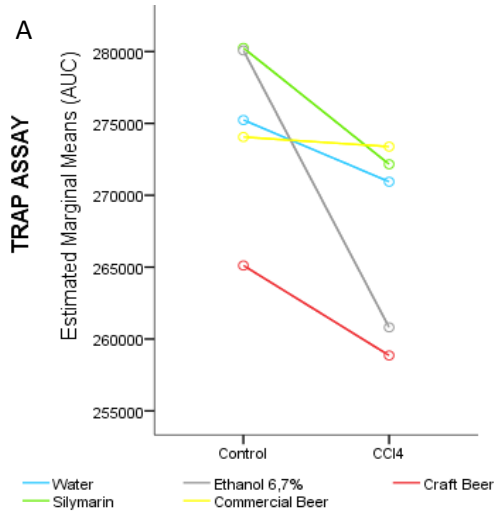


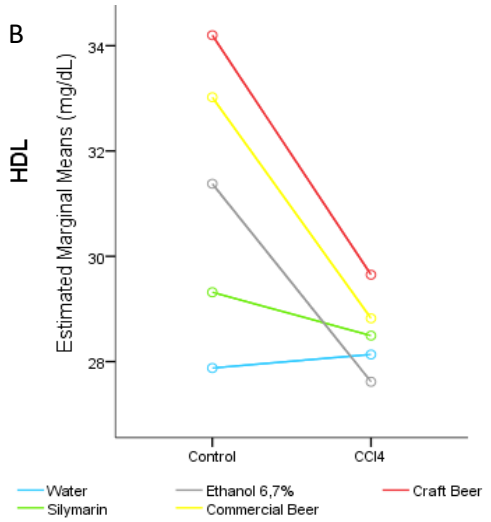


Treatments	Injection	Mean	Std. Error	Letters
Water	CCl_4	3,66	0,24	A
Commercial Beer	CCl_4	3,10	0,24	AB
Craft Beer	CCl_4	2,99	0,24	AB
Ethanol 6,7%	CCl_4	2,95	0,26	AB
Silymarin	CCl_4	2,55	0,24	B
Water	Control	2,65	0,24	
	CCl_4	3,66	0,24	↑

Treatments	Injection	Mean	Std. Error	Letters
Water	CCl_4	1,41	0,11	A
Craft Beer	CCl_4	1,13	0,11	AB
Commercial Beer	CCl_4	1,13	0,11	AB
Ethanol 6,7%	CCl_4	1,09	0,12	AB
Silymarin	CCl_4	0,90	0,11	B
Water	Control	0,95	0,11	
	CCl_4	1,41	0,11	↑

Fig. 3: Fats weight. Graphical representation of interaction in statystical analysis. (Two-way ANOVA, bonferroni post-test, n =7, $p < 0.05$). Tables indicate the differences with letters or arrows. (A) Total Abdominal fat weight and (B) retroperitoneal fat weight.

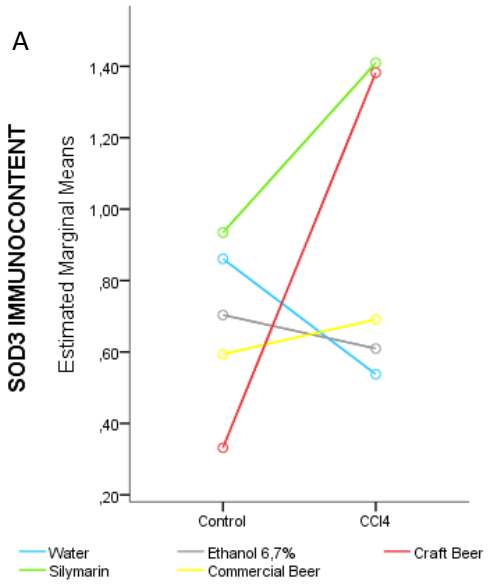


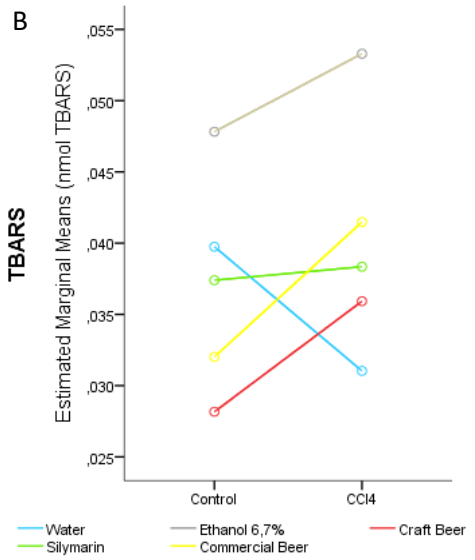


Treatments	Mean	Std. Error	Letters
Silymarin	276203	3950	A
Commercial Beer	273718	3801	A
Water	273089	3801	A
Ethanol 6,7 %	270441	3950	A
Craft Beer	261987	3950	B

Treatments	Mean	Std. Error	Letters
Craft Beer	31,9	1,0	A
Commercial Beer	30,9	0,9	AB
Ethanol 6,7 %	29,5	1,0	AB
Silymarin	28,9	1,0	AB
Water	28,0	0,9	B

Fig. 4: Trap assay and HDL levels in serum. Graphical representation of interaction in statystical analysis. (Two-way ANOVA, bonferroni post-test, n =7, $p < 0.05$). Tables indicate the differences with letters. (A) TRAP AUC of serum. (B) HDL levels in serum.





Treatments	Injection	Mean	Std. Error	Letters
Silymarin	CCl_4	1,41	0,16	A
Craft Beer	CCl_4	1,38	0,16	A
Commercial Beer	CCl_4	0,69	0,16	B
Ethanol 6,7 %	CCl_4	0,61	0,16	B
Water	CCl_4	0,54	0,16	B
Craft Beer	Control	0,33	0,16	
	CCl_4	1,38	0,16	↑

Treatments	Mean	Std. Error	Letters
Ethanol 6,7 %	0,051	0,003	A
Silymarin	0,038	0,003	B
Commercial Beer	0,037	0,002	B
Water	0,035	0,002	B
Craft Beer	0,032	0,003	B

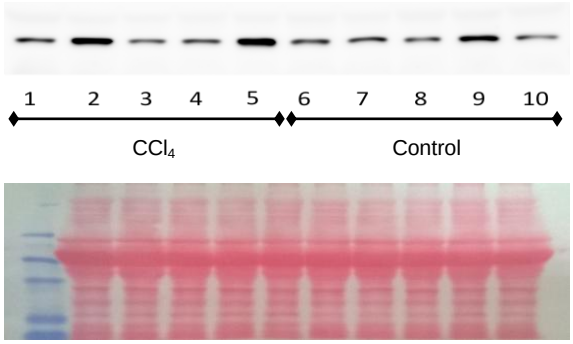
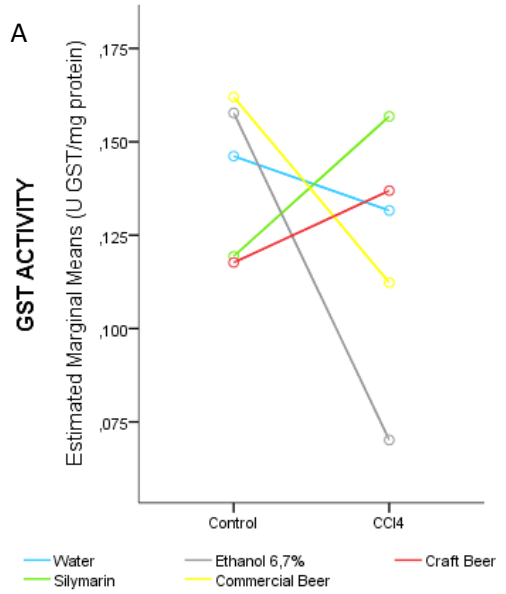
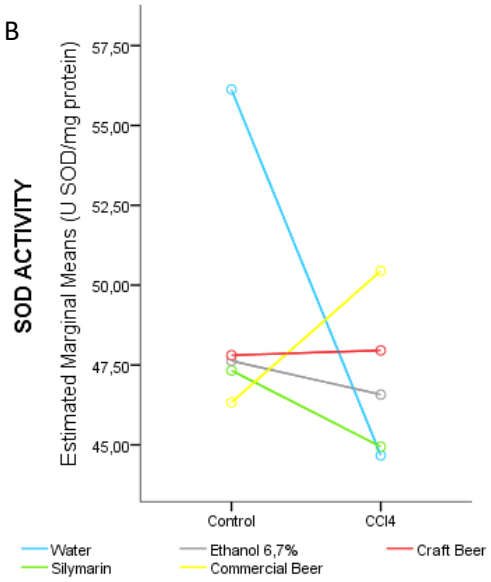


Fig. 5: Images of SOD3 imunocontent and TBARS levels in serum. Graphical representation of interaction in statystical analysis. (Two-way ANOVA, bonferroni post-test, n =7, $p < 0.05$). Tables indicate the differences with letters. (A) SOD3 immunocontent, results are expressed relatives to Ponceou; Western Blot image (1-5) CCl_4; (6-10) Control; respectively: water, silymarin, ethanol, commercial and craft beer; Ponceau image of serum. (B) TBARS levels in serum.

The result indicates that serum blood of craft beer drinkers had more non-enzimatic antioxidants as compared to other groups. Additionally, rats treated with ethanol increased TBARS levels, indicating a lipidperoxidation. Silymarin was also capable of increasing SOD3 immunocontent when chalenged with CCl_4 (Fig. 5).





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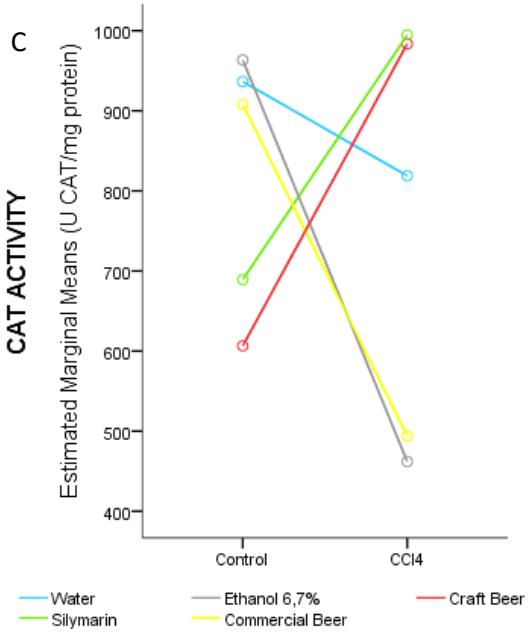
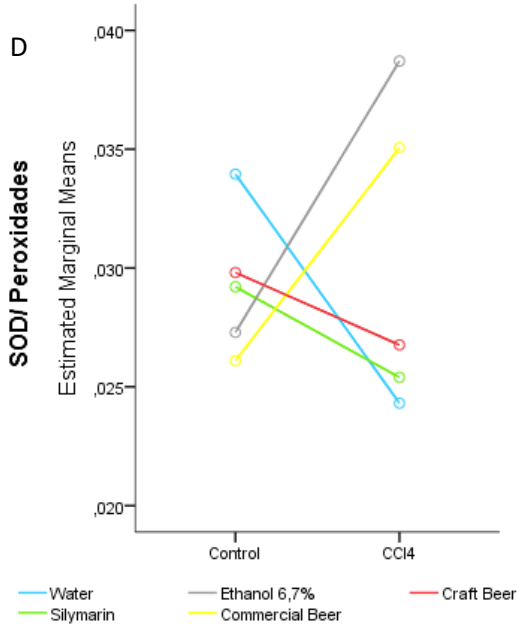
Treatments	Injection	Mean	Std. Error	Letters
Silymarin	CCl_4	0,16	0,02	A
Craft Beer	CCl_4	0,14	0,02	AB
Water	CCl_4	0,13	0,02	AB
Commercial Beer	CCl_4	0,11	0,02	AB
Ethanol 6,7 %	CCl_4	0,07	0,02	B
Ethanol 6,7 %	Control	0,16	0,02	
	CCl_4	0,07	0,02	↓

Treatments	Injection	Mean	Std. Error	Letters
Water	Control	56,13	2,22	A
Craft Beer	Control	47,81	2,39	AB
Ethanol 6,7 %	Control	47,63	2,22	AB
Silymarin	Control	47,33	2,39	AB
Commercial Beer	Control	46,33	2,22	B
Water	Control	56,13	2,22	
	CCl_4	44,67	2,22	↓





Treatament	Injection	Mean	Std. Error	Letters
Silymarin	CCl_4	994	135	A
Craft Beer	CCl_4	983	135	A
Water	CCl_4	818	135	AB
Commercial Beer	CCl_4	493	135	B
Ethanol 6,7%	CCl_4	461	146	B

Treatament	Injection	Mean	Std. Error	Letters
Ethanol 6,7%	CCl_4	0,039	0,021	A
Commercial Beer	CCl_4	0,035	0,021	AB
Craft Beer	CCl_4	0,027	0,021	AB
Silymarin	CCl_4	0,025	0,021	B
Water	CCl_4	0,024	0,023	B

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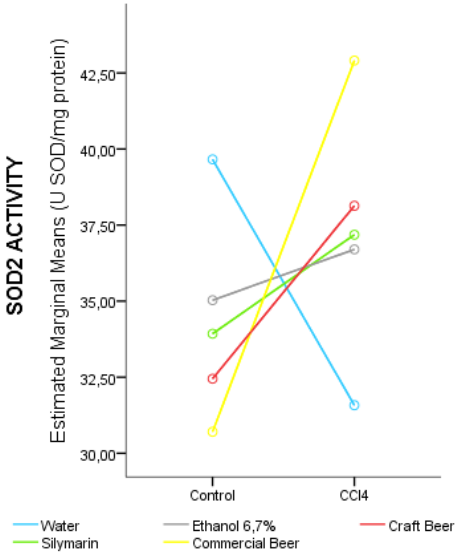
Fig. 6: Enzymes activities in liver. Graphical representation of interaction in statystical analysis. (Two-way ANOVA, bonferroni post-test, n =7, $p < 0.05$). Tables indicate the differences with letters. (A) GST activity. (B) Total SOD activity. (C) CAT activity. (D) Ratio SOD/ peroxidases.



Treatments	Injection	Mean	Std. Error	Letters
Commercial Beer	CCl_4	42,9	2,3	A
Craft Beer	CCl_4	38,1	2,4	AB
Silymarin	CCl_4	37,2	2,4	AB
Ethanol 6,7%	CCl_4	36,7	2,6	AB
Water	CCl_4	31,6	2,6	B
Commercial Beer	Control	30,7	2,4	
	CCl_4	42,9	2,4	↑

Fig. 7: SOD 2 activity in liver. Graphical representation of interaction in statystical analysis. (Two-way ANOVA, bonferroni post-test, n =7, $p < 0.05$). Tables indicate the differences with letters.

Analysing some liver enzymes activities we observe a decrease with CCl_4 in rats treated with water. Rats treated with ethanol decreased GST activity when chalenged with CCl_4 and only rats treated with silymarin could raise the activity of this enzyme (Fig. 6 A). Commercial beer consumption could also reveal some adaptations, decreasing the activity of SOD in liver. When chalenged with CCl_4 it increased only SOD2 activity (Fig. 6 B and Fig. 7). Catalase activity decreased with CCl_4 in rats treated with commercial beer and ethanol, but silymarin and craft beer increased the activity of this enzyme. Rats treated with ethanol, when chalenged with CCl_4, increased this ratio compared to silymarin and water treated animals (Fig. 6). The imunocontent of SOD1, SOD2, and CAT did not differ (data not shown).

DISCUSSION

There are many beneficial and antioxidant effects tested with isolated compunds from hops [Gerhauser, 2005; Monteiro et al., 2006; Negrão et al,, 2012]. So the antioxidant potential of beers could come only from hops extracts, however craft beers presented a higher antioxidant potential than their respectives extracts. This result indicates that craft beers have a complex matrix and their antioxidant potential does not come only from hops. Also, craft beers differ in antioxidant reactivity probably due to their especific recipes, where there are differences in malts, variety of hops and yeasts. Different from commercial beers that did not show any diference in TAR and presented less

antioxidant potential. Although TAR and TRAP evaluations are obtained in the same experiment, they represent different observations, because TAR is more related to the antioxidant quality (reactivity, the scavenging capacity in a shortterm period), and TRAP is more related to the antioxidants amount [Lissi et al., 1995].

It is known that heavy metals cause injury in biosystems and some them are present in beer [Eticha & Hymete, 2014]. This presence can be a problem for health due to a high consumption of this beverage [Mena et al., 1996]. As beers are made in INOX brewing tanks and craft beers are homebrewed, we searched for some heavy metals in beers (Cr, Cd, and Pb) and found higher levels of Cr and Cd in commercial beer, problably because of the water used in brewing process or from filtering accessories like diatomaceous earth. The levels were not criticals for human health [Council Directive 98], but this metals can accumulate in our body and are not metabolized.

All compounds found in our tested beers were found in beers as described [Hayes, 1987; Stevens, 1999; Cortacero-Ramírez 2004; Gerhauser 2005], with a slightly differcence of concentrations. The quantified compounds catechin, caffeic acid, epicatechin were higher in commercial beer tested probably due to the lager fermentation process. The isoxanthohumol comes from hops and this compound was higher in craft beer tested because it has more quantity of hops than commercial beer, rutin was also higher in craft beer tested probably because of the variety of malts used in red ales. The compounds found have many biological activities, for exemple, catechins are potent antioxidants that can help in cell survival, known as iron chelators [Kuwabara, 2008]. Some compounds can be metabolized by enteral microbiota,

activating its beneficial effect, as sterification make caffeic acid effective for protection agains hydrogen peroxide [Nakayama, 1992].

Beers presented powerful antioxidant effects *in vitro*, but in biological systems this effect could be otherwise prooxidant [Halliwell, 2007]. So, previous tests were performed with rats and no harmful results were found for craft beer consumption. Hops extracts did not have the same antioxidant potential, probably because there are potent antioxidants in yeast metabolites and/or in barley [Gerhauser, 2005].

In our biological experiment, the equivalent consumption for a human with 70 kg was 1134 mL per day for craft beer and 1474 mL per day for commercial beer. OMS suggest maximum 750 mL per day for prevention on cardiovascular diseases [OMS, 2015]. Drinking beer every night for 30 days, in our experimental model, did not cause any harmful effect. However, just ethanol raised TBARS levels in serum, indicating a possible lipid peroxidation. Craft Beer has the same percentage of ethanol, but did not affect any oxidative parameter, perhaps, beer ingredients can protect the harmful effect of ethanol itself, when in low levels (Gasbarrini, 1998).

The preference for commercial beer in rats ocurred probably because it is more watered down than craft beer and more caloric than water. As humans, rats preferred a softer drink that is not water. This corroborates with others [Hargreaves, 2011] that use beer to elevate the ethanol consumption in alcholism studies, because beer is easier to drink, a measure that brewers call “drinkability”. Rats have endogenous mecanisms for caloric intake regulation [Morton, Meek & Schwartz 2014], as craft beer is more caloric than others, the animals of this group ate less.

Besides what is described for ethanol intake and increase in HDL levels [Chiva-Blanch, 2013], only craft beer could raise HDL levels, corroborating with other results where beer show positive effect on plasma lipid profile and plasma antioxidant capacity [Gasowski, 2004]. This enhance the fact that craft beer is a more complex matrix and can have synergistic effects on biosystems.

It is known that CCl_4 is metabolized by the cytochrome P-450 system in liver, by microssomal enzymes, to form the true toxic compound [Mclean, 1966], the trichloromethyl radical. This radical reacts rapidly with oxygen to form a peroxyl radical [Recknagel, 1989], similar to peroxyl radical generated *in vitro* in TRAP assay, raising the possibility that craft beer could be efficient as hepatoprotective. CCl_4 can induce oxidative stress raising TBARS levels, carbonyls levels and others oxidative parameters [Dani et al., 2008, 2009; Gasparotto et al., 2014]. However, in our experiment, we observed a hepatic injury, a raise in ALT and AST levels but no raise in lipid peroxidation nor in carbonyl levels. Probably this occurred because the sequence of reaction is more often oxidative stress, cell damage, and secondary increase in lipid peroxidation of damaged cells. Lipid peroxidation is often a late event, accompanying rather than causing final cell death [Halliwell, 1984; Halliwell, 1993].

Also, CCl_4 inhibits calcium pumps, raising intracellular “free” calcium and activating a variety of hidrolytic enzymes [Schanne, 1979; Orrenius, 1989; Srivastava, 1990]. This leads to water influx, hidropic degeneration and displace nuclei as observed in our histological image. So, compounds calcium chelaters, as many polyphenols are [Radhakrishnan & Sivaprasad, 1980; Mcdonald, 1996] may protect against CCl_4 injury. The influx of water could be the responsable in

514 the raise of liver and fat weight observed. Silymarin slows the calcium
515 metabolism [Vargas-Mendoza, 2014] and it decreased total fat weight and
516 retroperitoneal fat weight, indicating that this could be the mechanism of
517 protection against the acute damage caused by CCl_4. The glycogen content in
518 liver, with animals fasted 12h, decreased with CCl_4 when compared to controls,
519 corroborating to depletion of energy stocks with this hepatic damage inducer
520 [Krahenbuhl, 1991].

521 As described (Halliwell, 1984) CCl_4 decreased activity of antioxidant
522 enzymes as SOD and GPX. Craft beer and silymarin could raise the expression
523 of SOD3 in serum and commercial beer raised the activity of SOD2, revealing
524 some protection. The raise in SOD2 activity corroborates with preventing results
525 with beer for mitochondrial chain [Valls-Belles, 2010]. CCl_3^- can affect cofactors,
526 oxidizing them, decreasing the activity of some enzymes [Halliwell, 84; Droge
527 2002]. Removing the excess of superoxide by SOD enzymes is an important
528 physiological antioxidant defense mechanism in aerobic organisms. Superoxide
529 can be reducer or oxidant, depending on medium, and reducing CCl_4 it will
530 displace Cl^- ion (Roberts & Sawyer, 1981). Beers and silymarin increased SOD
531 activity and expression in the CCl_4 treated animals helping the biosystem to
532 decrease superoxide in medium. However too much SOD may be harmful,
533 because the hydrogen peroxide is a reactive specie capable of triggering lipid
534 peroxidation and other biomolecules oxidations [Droge, 2002]. High
535 concentrations of hydrogen peroxide can inactivate the glycolytic enzyme
536 glyceraldehyde-3-phosphate dehydrogenase in mammalian cells [Hyslop,
537 1988]. Also, hydrogen peroxide play an important role in cell signalling [Droge,

2002 e Halliwel, 1993], ponting out that ratio SOD/peroxidases is extremely important and fundamental for CCl_4 protection.

CAT activity decreased with CCl_4, but craft beer and silymarin raised the activity of this enzyme, problaby to balance with SOD activity. The expression of CAT in our research did not differ in any group. The ratio between the activity of SOD/peroxidases show a possible explanation for biomolecules oxidation [Pinho, 2006]. Ethanol had higher ratios and this could explain the increase in TBARS levels in serum. As the depletion of antioxidant defenses and/or rises in ROS production can tip the ROS antioxidant balance and cause oxidative stress [Sies, 1997].

Nevertheless, TRAP and TAR assay revealed more antioxidant potential in serum with CCl_4 and GPx activity decreased in serum with CCl_4 too. This probably occurs due to glutathione mobilization from other organs to repair calcium pumps in liver [Sravstava, 1990]. The activity of GST in liver also decreased with CCl_4, but craft beer and silymarin raised the activity of GST indicating a possible protection for detoxification. Eventhough this raise could cause depletion of glutathione, disturbing physiological pathways where this molecule is essential. Altough some treatments revealed an intersting interaction with CCl_4, none of them could be hepatoprotective.

CONCLUSIONS:

Moderate consumption of beer, every day, is not harmful for health, however, beers are not hepatoprotective. The concept of moderate consumption for each organism is relative and should be studied and considered. Craft beer can enhance defenses in blood, being a healthier beverage than commercial beer, even commercial beer presenting more quantities of catechins and caffeic acid.

563 Both, commercial and craft beers, can influence the biosystems, changing
564 antioxidant enzymes activity.

565 CONFLICT OF INTEREST

566 The authors declare that there are no conflicts of interest

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5. DISCUSSÃO

A cerveja é uma fonte única de diversos compostos conhecidos por seus efeitos benéficos [Gerhauser, 2005]. Comparando as cervejas sob a ótica antioxidante, encontramos variação em seu potencial devido à forma de fabricação. Quando analisamos a resposta que as cervejas desencaderam no modelo animal, percebemos que houve uma diferença de resultados entre a cerveja artesanal e a comercial.

A produção de cerveja é realizada em tanques de aço inoxidável, sendo essa liga metálica passível de contaminação por metais pesados, decidiu-se analisar se havia presença de Pb, Cd e Cr, nas cervejas. Metais pesados podem causar danos a sistemas biológicos, acumulando-se no organismo, já que não são metabolizados. Apesar de todas as concentrações de metais estarem abaixo dos níveis críticos para a saúde humana [Directive, 1998], os maiores níveis foram encontrados na cerveja comercial. Esses metais são absorvidos e acumulados pelas plantas, podendo provir da cevada, do lúpulo, mas também da água, dos equipamentos ou dos adjuntos cervejeiros utilizados na fabricação da cerveja. As diferenças dos níveis de metais entre as cervejas artesanais ocorrem provavelmente pela variedade de maltes utilizados, já que os equipamentos e a água utilizada foram os mesmos.

Todos os compostos encontrados nas cervejas testadas já foram encontrados em outros estudos com cervejas [Gerhauser, 2005], mas com uma pequena diferença de concentrações. Os fenólicos da cevada passam por mudanças durante a malteação e fervura e são pouco conhecidos, o que acaba variando muito nas concentrações de cervejas. Já o xantohumol e isoxantohumol provindos do lúpulo normalmente são os compostos mais

presentes na cerveja, descritos nas concentrações de aproximadamente 0,04 mL/g e 0,73 mL/g, respectivamente [Walker et al., 2003; Wunderlich et al., 2003]. As catequinas são potentes antioxidantes capazes de ajudar na sobrevivência celular, conhecidas como quelantes de ferro [Kuwabara et al., 2008]. Alguns compostos podem ser metabolizados pela microbiota entérica, ativando os efeitos benéficos desses compostos, por exemplo, a esterificação do ácido cafeico torna-o eficiente contra o dano induzido por peróxido de hidrogênio [Nakayama et al., 1992].

As cervejas testadas apresentaram potentes efeitos antioxidantes *in vitro,* mas se tinha dúvidas se elas poderiam agir como próoxidantes em sistemas biológicos, uma vez que o teor de álcool das cervejas poderia ser um agravante ou atenuante em situações fisiológicas e/ou patológicas [Halliwell, 2007]. Por isso, fez-se alguns testes prévios e o consumo de cerveja artesanal não acarretou em nenhum tipo de dano ou prejuízo em ratos. Como existe uma vasta literatura sobre os efeitos benéficos de componentes presentes no lúpulo, testou-se *in vitro*, através do ensaio TRAP, os respectivos extratos de lúpulo das cervejas artesanais. Os extratos apresentaram um menor potencial antioxidante quando comparados às respectivas cervejas. Provavelmente isso ocorreu por que: existam potentes antioxidantes provenientes dos metabólitos da levedura e/ou os compostos fenólicos possam ser ativados pelo metabolismo fermentativo; os compostos fenólicos presentes na cevada podem ser importantes e não são totalmente eliminados no processo cervejeiro; o álcool auxiliaria os compostos fenólicos, solubilizando-os; ou talvez exista um sinergismo entre todos os componentes da cerveja.

Para uma interpretação dos resultados de consumo para humanos, calculou-se o quanto os animais beberam em média, levando em conta a área e o volume corporal [Reagan-Shaw, Nihal, Ahmad, 2008]. O consumo equivalente para um humano de 70 kg foi de 1134 mL para a cerveja artesanal e 1474 mL para cerveja comercial, por noite. A Organização Mundial da Saúde (OMS) sugere um máximo de 750 mL por dia [OMS, 2015].

Apesar do consumo dessa quantidade de cerveja por 30 noites não causar dano oxidativo nos animais, o consumo de álcool aumentou os níveis de TBARS no soro, indicando uma possível lipoperoxidação. A cerveja artesanal possui a mesma porcentagem de álcool (6,7 %), entretanto ela não afetou nenhum parâmetro oxidativo, e talvez, os ingredientes da cerveja possam proteger o efeito danoso do álcool, quando em baixas concentrações [Gasbarrini et al., 1998].

Assim como os humanos, os animais preferiram a cerveja comercial, provavelmente por ser mais diluída e suave ao paladar. Esse resultado corrobora com estudos de alcoolismo e alto consumo de etanol [Hargreaves et al., 2011] que utilizam cerveja para conseguirem um aumento no consumo de etanol, por essa bebida ser mais agradável ao paladar. O quão fácil uma bebida é consumida serve como um parâmetro de paladar que os cervejeiros chamam de “dringabilidade” (“drinkability”). Apesar de estar descrito que a ingestão de álcool aumenta os níveis de HDL [Chiva-Blanch et al., 2013], somente a cerveja artesanal aumentou esse parâmetro, corroborando com outros trabalhos em que a cerveja apresentou efeitos positivos no perfil lipídico plasmático e na capacidade antioxidante plasmática [Gasowski et al., 2004]. Isso reforça a ideia de que a cerveja artesanal possui uma matriz mais

complexa que excerça efeitos sinérgicos em sistemas biológicos. O álcool (6,7 %) por si foi capaz de aumentar os níveis de lipoperoxidação no soro. Embora a cerveja artesanal tenha a mesma concentração de álcool (6,7 %), essa apresentou um aumento dos níveis de HDL. Por outro lado, a cerveja é gaseificada, o que aumentaria a absorção dos nutrientes e de álcool e, além disso, a cerveja é descrita na literatura por aumentar a absorção de nutrientes [Gasowski et al., 2004]. Então, os componentes presentes na cerveja acabam agindo sinergisticamente e atenuando possíveis danos que componentes isolados poderiam ter.

O CCl_4 é descrito por ser metabolizado pelo sistema citocromo P-450 que compõe o sistema microssomal hepático, formando o verdadeiro composto tóxico [Mclean & Mclean, 1966], o radical triclorometil, que reage rapidamente com oxigênio formando um radical peroxil [Recknagel et al., 1989]. Essa molécula é semelhante ao radical gerado *in vitro* na técnica TRAP, reforçando a ideia de que a cerveja poderia ser eficiente como hepatoprotetora. O CCl_4 pode induzir estresse oxidativo, elevando níveis de TBARS, níveis de carbonilas e outros parâmetros oxidativos [Dani et al., 2008; 2009, Gasparotto et al., 2014]. Entretanto, no presente experimento, observou-se um dano hepático nas imagens histológicas, um aumento dos níveis de ALT e AST, mas nenhum aumento de parâmetros oxidativos no fígado. Provavelmente isso ocorreu porque a lipoperoxidação é muitas vezes um evento tardio, acompanhando ao invés de causando a morte celular [Halliwell & Gutteridge, 1984; Halliwell & Chirico, 1993]. Outros estudos também relatam para essa mesma dose de CCl_4 um aumento nos níveis de ALT e AST, caracterizando o dano hepático [Wang & Li et al., 2014; Rudnicki et al., 2007]

Além disso, o CCl_4 também é capaz de inibir as bombas de cálcio, o que acarreta em um aumento intracelular de cálcio livre, que consequentemente ativa uma grande variedade de enzimas hidrolíticas como nucleases e proteases [Schanne et al., 1979; Orrenius et al., 1989; Srivastava, Chen, Holtzman, 1990]. Isso leva a um influxo celular de água, ocasionando degeneração hidrópica e desaparecimento do núcleo, como observado nas imagens histológicas (Fig. 2 do artigo). Esse fato confirma a efetividade da injeção de CCl_4 como indutor de dano hepático. Tendo em vista o mecanismo de ação do CCl_4, aumentando o influxo de cálcio, compostos quelantes de cálcio, propriedade observada em muitos polifenois [Radhakrishnan & Sivaprasad, 1980; Mcdonald, Mila, Scalbert, 1996] sugeririam que estes poderiam exercer um efeito protetor contra o dano induzido por CCl_4.

O aumento de peso do fígado nos animais que receberam o CCl_4 pode ter ocorrido devido a um influxo celular de água. Um dos efeitos conhecidos da silimarina é a diminuição do metabolismo de cálcio [Vargas-Mendoza et al., 2014], o que poderia ser a explicação para a redução do peso das gorduras gonadal e retroperitoneal nos animais tratados com esse medicamento. Corroborando com o efeito de depleção dos estoques energéticos ocasionados pelo CCl_4 [Krahenbuhl, Weber, Brass 1991], observou-se uma diminuição do glicogênio presente no fígado, bem como uma diminuição nos níveis de glicose no soro dos animais que receberam a injeção com CCl_4.

Assim como descrito na literatura (Halliwell & Gutteridge, 1984), o CCl_4 diminuiu a atividade das enzimas antioxidantes, como a SOD e a GPX. Contudo, a cerveja artesanal e a silimarina, na presença de CCl_4, apresentaram um aumento no imunoconteúdo da enzima SOD3 presente no

soro, indicando uma resposta adaptativa ao insulto. A cerveja comercial aumentou a atividade da SOD2, aparentemente revelando certa proteção ao CCl_4. em um nível baixo, esses extratos podem ser considerados como hepatoprotetores pois eles ajudaram o organismo a diminuir o superóxido no meio. Isso concorda com resultados da literatura que descreveram proteção mitocondrial para a cerveja com o indutor de dano mitocondrial adriamicina [Valls-Belles et al., 2010]. Provavelmente, o CCl_4 afeta as coenzimas das enzimas antioxidantes como cobre, zinco e manganês [Halliwell & Gutteridge, 84; Dröge 2002]. Remover o excesso de superóxido pelas enzimas SOD é um importante mecanismo fisiológico de defesa antioxidante em organismos aeróbicos. Esse superóxido pode ser redutor ou oxidante, dependendo do meio que se encontra. No caso do CCl_4, o superóxido pode reduzí-lo, retirando um íon cloreto e transformando-o no radical triclorometil (Roberts & Sawyer, 1981).

Entretatanto, uma maior atividade da SOD pode ser danoso, pois o peróxido de hidrogênio é uma espécie reativa capaz de desencadear cascatas de oxidações lipídicas e de outras biomoléculas. Altas concentrações de peróxido de hidrogênio podem inativar enzimas da via glicolítica [Hyslop et al., 1988]. Apesar disso, o peróxido de hidrogênio desempenha um papel importante na sinalização celular [Halliwell, 2007], tornando a razão SOD/peroxidases fundamental para a homeostase celular e proteção contra o CCl_4.

A atividade da CAT diminuiu com CCl_4, mas a cerveja artesanal e a silimarina puderam aumentar a atividade dessa enzima, provavelmente para balancear a homeostase redox. Todavia a expressão da CAT não teve

diferenças entre os grupos. A razão entre a atividade das enzimas SOD/peroxidases exibe um resultado para uma possível explicação de oxidação à biomoléculas [Pinho et al., 2006]. O álcool apresentou a maior razão, indicando mais atividade da SOD do que das peroxidases e por isso gerando um excedente de peróxido de hidrogênio. Isso poderia explicar o aumento dos níveis de TBARS no soro desse grupo, mesmo sendo em outro órgão. É conhecido que a depleção de defesas antioxidantes e/ou aumento na produção de espécies reativas de oxigênio podem desequilibrar o balanço ERO/antioxidantes e causar estresse oxidativo [Sies, 1997].

O resultado obtido na técnica do TRAP/TAR revelou um potencial antioxidante maior no soro dos animais que receberam a injeção de CCl_4. Provavelmente esse resultado demonstra uma mobilização de glutationa de outros órgãos para reparar as bombas de cálcio danificadas no fígado [Srivastava et al., 1990]. Apesar da atividade da GST no fígado diminuir com o CCl_4, a cerveja artesanal e a silimarina aumentaram-na, indicando uma possível proteção para a detoxificação do CCl_4. Porém, um aumento da atividade dessa enzima pode depletar a glutationa disponível, prejudicando vias fisiológicas onde essa molécula é essencial.

A diferença dos teores de álcool entre as cervejas pode ser desconsiderada, pois existe, também, uma diferença de consumo. Apesar de alguns tratamentos revelarem uma interessante interação com o CCl_4, nenhum deles pode ser eficiente como hepatoprotetor

6. CONCLUSÕES

O tratamento com cerveja por 30 noites não apresentou elevação nos níveis de danos oxidativos do fígado e do soro analisados nessa pesquisa, bem

como se verificou que não houve aumento do peso das gorduras abdominais. O consumo moderado de cerveja artesanal (aproximadamente 40 mL por noite, por animal, com 6,7 % de álcool), apesar de conter álcool, aumentou os níveis de HDL, e da capacidade antioxidante do soro. A cerveja comercial foi mais consumida pelos animais e acredita-se que isso tenha se dado por esta ser menos calórica que a artesanal, já que os animais normais possuem um controle regulatório endógeno para ingesta de calorias. O grupo que consumiu etanol 6,7 % em água foi o único que apresentou algum tipo de dano oxidativo. Nenhum tratamento foi capaz de atenuar o dano hepático induzido por CCl_4.

7. PERSPECTIVAS

Estudar a biodisponibilidade dos compostos da cerveja, identificando possíveis metabólitos e relacionando com o efeito do consumo dessa bebida na microbiota intestinal.

Identificar qual o mecanismo de ação desses metabólitos nas nossas células e determinar modificações em vias de sinalização.

Fazer os estudos com humanos, analisando sua história e cultura com a cerveja e relacionando com a microbiota intestinal.

Investigar se o medicamento que continha a silimarina (Legalon) pode ser considerado termogênico, pois ele foi capaz de diminuir o peso da gordura abdominal sem alterar outros parâmetros fisiológicos do animal e nem mesmo aumentar os danos oxidativos.

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Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 27632
Título: Propriedades antioxidantes da cerveja artesanal

Vigência: 01/08/2014 à 28/02/2018

Pesquisadores:

Equipe UFRGS:

JOSE CLAUDIO FONSECA MOREIRA - coordenador desde 01/08/2014
Carlos Eduardo Schnorr - Aluno de Doutorado desde 01/08/2014
LUCAS BRAMBILLA HILBIG FEISTAUER - Aluno de Mestrado desde 01/08/2014

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 27/10/2014 - Sala I do Gabinete do Reitor - Prédio da Reitoria - Campus do Centro - Porto Alegre, em seus aspectos éticos e metodológicos, para a utilização de 145 ratos, Wistar, 60 dias, machos, provenientes do Biotério do Departamento de Bioquimica/ICBS-UFRGS, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Sexta-Feira, 27 de Novembro de 2015

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INTRODUCTION

Food and Chemical Toxicology (FCT), an internationally renowned journal, aspires to publish original research articles and reviews on **toxic effects**, in animals or humans, of natural or synthetic chemicals occurring in the human environment with particular emphasis on **food, drugs, and chemicals, including agricultural and industrial safety**, and **consumer product safety**. Areas such as safety evaluation of **novel foods and ingredients, biotechnologically-derived** products, and **nanomaterials** are included in the scope of the journal. FCT also encourages submission of papers on **inter-relationships between nutrition and toxicology** and on *in vitro* techniques, particularly those fostering the **3 Rs**. The principal aim of the journal is to publish high impact, scholarly work and to serve as a multidisciplinary forum for research in toxicology. Papers submitted will be judged on the basis of scientific originality and contribution to the field, quality and subject matter. Studies should address at least one of the following: Physiological, biochemical, or pathological changes induced by specific substances Techniques for assessing potential toxicity, including molecular biology Mechanisms underlying toxic phenomena Toxicological examinations of specific chemicals or consumer products, both those showing adverse effects and those demonstrating safety, that meet current standards of scientific acceptability Manuscripts concerning materials/substances of only local interest for which the chemical composition of the material/substance is **not clearly defined** will **not** be considered. manuscripts addressing only pharmacological properties, or only potentially beneficial effects using in *in vitro* or *in vivo* systems, are not within the scope of the journal. FCT is committed to the highest standards. Only papers that have not been previously published, that fit in the above mentioned scope, and that have been reviewed by experts in the field prior to publication will be accepted. Cover letters must state that the paper is new and original and not under consideration for publication elsewhere. Papers pending in other journals will not be considered. Coauthors should be individuals who have contributed substantially to the content of the papers.

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The Journal's main purpose is the publication of papers reporting and interpreting original unpublished toxicological research, particularly studies promoting an understanding of the mechanisms underlying toxic effects or improvements in methods for predicting adverse effects. Papers reporting the toxicological examination of specific foods, chemicals or consumer products will be published, irrespective of the positive or negative nature of the results, provided the tests and reporting meet current standards of acceptability. In addition, Short Communications will also be considered, as will concise interpretative Reviews of toxicological topics of contemporary significance. Letters to the Editor will be limited to comments on contributions already published in the journal; if a letter is accepted, a response (for simultaneous publication) will be invited from the authors of the original contribution. All Letters to the Editor should be submitted to the Editor in Chief, A. Wallace Hayes at the following address: awallacehayes@comcast.net.

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