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Laura Gizele Mascarin

FERMENTADO DE LARANJA COMPOSTO DE EXTRATO DE ERVAS AROMÁTICAS: DESENVOLVIMENTO, CARACTERIZAÇÃO E AVALIAÇÃO DO POTENCIAL BIOLÓGICO

Santa Maria, RS
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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de Doutora em Ciência e Tecnologia dos Alimentos.

Orientadora: Profª. Drª. Cláudia Kaehler Sautter

Santa Maria, RS
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_____

Cláudia Kaehler Sautter, Drª. (UFSM)
(Presidente/Orientadora)
(videoconferência)

_____

Amanda Leitão Gindri, Drª. (URI)
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_____

Liliane de Freitas Bauermann, Drª. (UFSM)
(videoconferência)

_____

Roberta de Oliveira Santos, Drª. (UNISC)
(videoconferência)

_____

Sabrina Somacal, Drª. (UFSM)
(videoconferência)

Santa Maria, RS, 03 de fevereiro de 2023

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Dedico este trabalho ao meu amado esposo Fernando, meu porto seguro e aos meus queridos pais Jorge e Zélia, meus grandes incentivadores nessa longa e desafiadora jornada. Por vocês!

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"O degrau de uma escada não serve simplesmente para que alguém permaneça em cima dele, destina-se a sustentar o pé de um homem pelo tempo suficiente para que ele coloque o outro um pouco mais alto".

Thomas Huxley

RESUMO

FERMENTADO DE LARANJA COMPOSTO DE EXTRATO DE ERVAS AROMÁTICAS: DESENVOLVIMENTO, CARACTERIZAÇÃO E AVALIAÇÃO DO POTENCIAL BIOLÓGICO

AUTORA: Laura Gizele Mascarin
ORIENTADORA: Prof. Cláudia Kaehler Sautter

Os consumidores têm se interessado cada vez mais por bebidas que além de diferenciadas no sabor, possam conferir benefícios à saúde. Para isso, a indústria é desafiada a desenvolver principalmente produtos funcionais, ricos em compostos bioativos. Nesse sentido, a fermentação é uma ferramenta importante na produção de bebidas funcionais a partir de frutas ricas em antioxidantes, plantas medicinais e aromáticas, uma vez que o meio etanólico contribui para a extração e biodisponibilidade de compostos bioativos que protegem contra a incidência de muitas doenças da sociedade moderna. As frutas cítricas são geralmente conhecidas por terem uma vida de prateleira baixa e enfrentam o problema de perdas pós-colheita, com isso, esforços têm sido feitos para o explorar seu potencial no desenvolvimento de bebidas alcoólicas. Este estudo desenvolveu uma bebida fermentada de laranja e avaliou a contribuição da adição de extratos de *Matricaria recutita* (camomila), *Cymbopogon citratus* (capim-limão) e *Mentha piperita* (hortelã) para o perfil fitoquímico e os potenciais efeitos biológicos *in vitro*, *in sílico* e *in vivo*. Por meio de análise sensorial foi determinado que as bebidas com adição de 2% dos extratos de ervas aromáticas obtiveram a melhor aceitabilidade, sendo assim essa concentração de extratos utilizada nos ensaios posteriores. As análises de caracterização *in vitro* mostraram que adição do extrato de ervas aromáticas contribuiu com um maior teor de fenólicos, flavonoides e potencial antioxidante em relação à bebida controle. Os principais compostos bioativos presentes nas formulações foram hesperidina (124-130 mg L^{-1}), narirutina (66-70 mg L^{-1}), ácido clorogênico (11-16 mg L^{-1}), ácido cafeico (5,3-5,5 mg L^{-1}) e ácido ferúlico (1-1,7 mg L^{-1}). A análise *in silico* apontou que esses compostos não apresentam risco de toxicidade (mutagenicidade, carcinogenicidade, hepatotoxicidade e capacidade de penetrar na barreira hematoencefálica). Além disso, os ensaios *in silico* apontaram que esses compostos podem contribuir com efeitos biológicos de importância terapêutica, como antioxidante, gastroprotetor e antiulcerativo, sendo o extrato de *M. piperita* com maior potencial entre as ervas avaliadas para uso em fermentados funcionais. A análise *in vivo* foi realizada através de um modelo de úlcera gástrica induzida por etanol (EtOH) em ratos. A avaliação histopatológica mostrou que a administração de EtOH resultou na formação de úlceras gástricas decorrentes da redução da camada de muco, presença de hemorragia e infiltração de neutrófilos no tecido estomacal de ratos, sendo que o tratamento com diferentes fermentados de laranja não foram capazes de reverter essas alterações. Além disso, a administração de EtOH alterou o volume do suco gástrico e induziu estresse oxidativo no tecido estomacal, observado através do aumento da peroxidação lipídica (TBARS), redução dos níveis de tióis não-proteicos (SHNP) e alteração da atividade da superóxido dismutase (SOD). A ingestão da bebida fermentada de laranja (controle) aumentou os níveis de SHNP e reduziu as alterações nos níveis de TBARS induzidos pelo EtOH. Portanto, esses achados sugerem que a bebida fermentada de laranja apresenta efeitos antioxidantes, conforme apontado por estudos *in silico*, mas não efeitos gastroreparadores e antiulcerativos. Estudos futuros podem ser conduzidos para investigar efeito gastroprotetor dos fermentados e também com bebidas desalcoolizadas.

Palavras-chave: Plantas medicinais. Bebidas funcionais. Antioxidantes. Úlcera gástrica.

ABSTRACT

FERMENTED ORANGE COMPOSED OF AROMATIC HERBS EXTRACT: DEVELOPMENT, CHARACTERIZATION AND EVALUATION OF THE ANTIOXIDANT AND BIOLOGICAL POTENTIAL

AUTHOR: Laura Gizele Mascarin
ADVISOR: Prof. Claudia Kaehler Sautter

Consumers have been increasingly interested in beverages that, in addition to being differentiated in flavor, can provide health benefits. To achieve this, the industry is challenged to develop mainly functional products, rich in bioactive compounds. In this sense, fermentation is an important tool in the production of functional beverages from antioxidant-rich fruits, medicinal and aromatic plants, since the ethanolic medium contributes to the extraction and bioavailability of bioactive compounds that protect against the incidence of many diseases of modern society. Citrus fruits are generally known to have a low shelf life and face the problem of post-harvest losses. Efforts have therefore been made to explore the potential of these fruits in the development of alcoholic beverages. Therefore, this study developed a fermented orange beverage and evaluated the contribution of the addition of extracts of *Matricaria recutita* (chamomile), *Cymbopogon citratus* (lemongrass) and *Mentha piperita* (peppermint) to the phytochemical profile and the potential *in vitro*, *in silico*, and *in vivo* biological effects. Sensory analysis determined that beverages with the addition of 2% of aromatic herb extracts obtained the best acceptability, so this concentration of extracts was used in subsequent assays. In vitro characterization analyses showed that the addition of aromatic herb extracts contributed to a higher content of phenolics, flavonoids, and antioxidant potential compared to the control beverage. The main bioactive compounds present in the formulations were hesperidin (124-130 mg L^{-1}), narirutin (66-70 mg L^{-1}), chlorogenic acid (11-16 mg L^{-1}), caffeic acid (5.3-5.5 mg L^{-1}), and ferulic acid (1-1.7 mg L^{-1}). *In silico* analysis indicated that these compounds do not present a risk of toxicity (mutagenicity, carcinogenicity, hepatotoxicity, and ability to penetrate the blood-brain barrier). In addition, in silico assays indicated that these compounds can contribute to biological effects of therapeutic importance, such as antioxidant, gastroprotective, and antiulcerative, with the extract of *M. piperita* showing greater potential among the herbs evaluated for use in functional fermented products. *In vivo* analysis was performed through a model of ethanol-induced gastric ulcer (EtOH) in rats. Histopathological evaluation showed that EtOH administration resulted in the formation of gastric ulcers due to the reduction of the mucous layer, presence of hemorrhage, and infiltration of neutrophils in the stomach tissue of rats, and the treatment with different orange fermented beverages was not able to reverse these changes. In addition, EtOH administration altered the volume of gastric juice and induced oxidative stress in stomach tissue, observed through the increase in lipid peroxidation (TBARS), reduction of non-protein thiols (SHNP) levels, and alteration of superoxide dismutase (SOD) activity. Consumption of the fermented orange beverage (control) increased SHNP levels and reduced changes in TBARS levels induced by EtOH. Therefore, these findings suggest that the fermented orange beverage has antioxidant effects, as indicated by in silico studies, but not gastro repair and antiulcerative effects. Future studies can be conducted to investigate the gastroprotective effect of fermented and also non-alcoholic beverages.

Keywords: Medicinal plants. Functional drinks. Antioxidants. Gastric ulcer.

LISTA DE FIGURAS

DESENVOLVIMENTO (REFERENCIAL TEÓRICO)

ARTIGO 2

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ARTIGO 1

LISTA DE SIGLAS

AAPH	Dicloridrato de 2,2'-azobis(2-metilpropionamidina)
AINES	Anti-inflamatórios não-esteroides
ANOVA	Análise de variância
AsA	Ascorbato
ATP	Adenosina trifosfato
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CAT	Catalase
CC	Capim-limão (*Cymbopogon citratus*)
DAD	Detector de arranjo de diodo, do inglês “diode array detector”
DPPH	Radical de nitrogênio orgânico, de nomenclatura química: 2,2-difenil-1-picrilidrazil
EtOH	Etanol
EtOH/H_2O	Etanol + água (solução hidroalcóolica)
EtOH-FOB	Etanol + Fermentado de laranja
EtOH-FOB-MP	Etanol + Fermentado de laranja com hortelã
EtOH-FOB-OMEP	Etanol + Fermentado de laranja + Omeprazol
FOB	Fermentado de laranja, do inglês “fermented orange beverage”
FOB-MP	Fermentado de laranja com hortelã
GPx	Glutationa peroxidase
GR	Glutationa redutase
GSH	Glutationa
GST	Glutationa S-tranferase
HPLC	Cromatografia líquida de alta performance, do inglês “high performance liquid chromatography”
IC_{50}	Concentração inibitória em 50%, do inglês “inhibitory concentration”
IST	Toxicologia *in silico*, do inglês “*in silico toxicology”*
LD_{50}	Dose letal para 50% das populações, do inglês “letal dose”
MeOH UV/HPLC	Metanol de grau ultravioleta para cromatografia líquida de alta eficiência
MP	Hortelã (*Mentha piperita*)
MR	Camomila (*Matricaria recutita*)
NIDAL	Núcleo integrado de análises laboratoriais

NPSH	Tióis não-proteicos, do inglês “non-protein thiols”
OMEP	Omeprazol
ON	Óxido nítrico
ORAC	Capacidade de absorção do radical de oxigênio, do inglês “oxygen radical absorbance capacity”
Pa	Provavelmente ativo
PGs	Prostaglandinas
Pi	Provavelmente inativo
QSAR	Quantitativo de relação estrutura-atividade, do inglês “quantitative structure-activity relationship”
QSPR	Quantitativo de relação estrutura-propriedade (QSPR), do inglês “quantitative structure-property relationship”
RL	Radical livre
ROS	Espécies reativas de oxigênio, do inglês “reactive oxygen species”
RT	Tempo de retenção, do inglês “retention time”
SOD	Superóxido dismutase
TBARS	Substâncias reativas do ácido tiobarbitúrico, do inglês “thiobarbituric acid reactive substances”
TFC	Conteúdo total de flavonoides, do inglês “total flavonoid content”
TPC	Conteúdo total de fenólicos, do inglês “total phenolic content”
TSS	Sólidos solúveis totais, do inglês “total soluble solids”
UV	Ultravioleta (espectro do comprimento de onda ultravioleta)

LISTA DE ABREVIATURAS

a.C.	Antes de Cristo
C. citratus	*Cymbopogon citratus* (capim-limão)
C. sinensis	*Citrus sinensis* (laranja)
M. piperita	*Mentha piperita* (hortelã)
M. recutita	*Matricaria recutita* (camomila)
O. europaea	*Olea europea* (oliveira)

LISTA DE SÍMBOLOS

BHT	Butil-hidroxi-tolueno
NaCl	Cloreto de sódio
CO_2	Gás carbônico
$O_2^{\bullet-}$	Radical superóxido
$^{\bullet}OH$	Radical hidroperóxido
$^{\bullet}NO$	Óxido nítrico
H_2O_2	Peróxido de hidrogênio
HCl	Ácido clorídrico
mg CAE L^{-1}	Miligrama por litro de equivalente de catequina
mg GAE L^{-1}	Miligrama por litro de equivalente de ácido gálico
µmol TE L^{-1}	Micromol por litro de equivalente de trolox
mEq L^{-1}	Miliequivalente por litro
Eq g^{-1}	Equivalente por grama
µmol	Micromol (= µM = micromolar)
Mm	Milimolar
nM	Nanomolar
M	Molar
N	Normal
µL	Microlitro
mL	Mililitro
g	Grama
kg	Quilograma
L	Litro
mg L^{-1}	Miligrama por litro
g L^{-1}	Grama por litro
g hL^{-1}	Grama por hecto litro (grama por 100 litros)
g%	Grama por cento
mg kg^{-1}	Miligrama por quilograma
k	Constante de reação
W	Watt (potência)
ppm	Parte por milhão
°C	Temperatura em graus Celsius
λ	Comprimento de onda (lâmbida)
b.w.	Peso corporal, do inglês “body weight”
h	Horas
min	Minutos
rpm	Rotação por minuto
v/v	Volume/volume

SUMÁRIO

1 INTRODUÇÃO

A produção de bebidas fermentadas é uma prática milenar que tem evoluído ao longo do tempo. O vinho é uma das bebidas alcoólicas mais consumidas e preferida devido às suas propriedades sensoriais e de aporte à saúde (SHIRADHONKAR *et al.*, 2014). Porém, além da uva, essa bebida pode ser produzida a partir de qualquer fruta açucarada, desde que designada como "fermentado de fruta" (BRASIL, 2009; UMEH; UDEMEZUE, 2015).

O crescente interesse dos consumidores em novas opções de bebidas fermentadas tem impulsionado a indústria a buscar alternativas aos substratos convencionais, como frutas que não somente a uva. Com isso, a indústria tem buscado atrair cada vez mais consumidores com produtos funcionais que façam parte da dieta e tragam benefícios à saúde (LV *et al.*, 2015; NAZIR *et al.*, 2019).

A laranja [*Citrus sinensis* (L.) Osbeck] é uma fonte abundante de vitamina C, apresentando consideráveis quantidades de açúcar e minerais e compostos bioativos como carotenoides, flavonoides, óleos essenciais e melatonina (LV *et al.*, 2015; SAE-TEAW *et al.*, 2013; TOUNSI *et al.*, 2011; ZACARÍAS-GARCÍA *et al.*, 2022). Além disso, possui boa aceitabilidade em pesquisas de consumo (GURAK; BORTOLINI, 2010) devido principalmente à variedade de produtos e novos sabores (VIEIRA, 2012).

Outros fatores que colaboram com a escolha da laranja é o fato do Brasil ser considerado o maior produtor mundial (FAO, 2021; KALAKI; NEVES, 2017; PASSOS *et al.*, 2018) e sua frutificação se dar em diferentes períodos do ano, permitindo diversas colheitas, evitando a concentração de safras, resultando em um preço de produção baixo durante o ano inteiro (PASSOS; CARVALHO; WIEST, 2010; EMBRAPA, 2010). Sua utilização como matéria-prima para produção de bebidas alcoólicas apresenta-se como uma opção atraente para agregar valor, diversificação do mercado, além de uma solução para minimizar as perdas pós-colheita destas frutas (TESSARO *et al.*, 2010).

Diversas pesquisas têm reportado que o consumo moderado de bebidas alcoólicas fermentadas é benéfico para a saúde humana. Dentre as principais propriedades reportadas estão a melhora do perfil lipídico, redução de biomarcadores de estresse oxidativo, com isso configurando proteção contra doenças neurológicas, cânceres, diabetes, doenças cardiovasculares e hipertensão (ARRANZ *et al.*, 2012; CERRILLO *et al.*, 2015; NAMBIAR *et al.*, 2016; NEMZER *et al.*, 2022; VASANTHI, PARAMESWARI, DELEIRIS, 2012).

Entretanto, quando se fala em bebidas alcoólicas, também é sabido que o consumo regular pode ser a causa de vários problemas de saúde, entre eles, lesões gástricas ulcerativas (KULSHRESHTHA; SRIVASTAVA; SINGH, 2017; NIETO, 2012).

Nesse contexto, os extratos de plantas têm mostrado resultados promissores no tratamento de úlceras, não somente proporcionando gastroproteção como também oferecendo gastroreparação às lesões, devido seus princípios farmacologicamente ativos, tais como, flavonoides, terpenos e taninos (BEZERRA *et al.*, 2009; FRANCISCO; FIGUEIRINHA; COSTA, 2014).

A aromatização de bebidas alcoólicas mediante a adição de extratos de plantas medicinais é uma prática milenar, muito utilizada em bebidas fermentadas e destiladas e que pode contribuir com a melhora do perfil sensorial, nutricional, fitoquímico e antioxidante do produto (ARORA; ANSARI; ARORA, 2019; JOSHI *et al.*, 2016; MASCARIN *et al.*, 2023; SHIRADHONKAR *et al.*, 2014).

Neste trabalho foi utilizado o extrato de três plantas comuns na medicina popular brasileira: camomila (*Matricaria recutita* L.), capim-limão [*Cymbopogon citratus* (DC.) Stapf] e hortelã (*Mentha piperita* L.) para serem incorporadas à bebida fermentada de laranja. Essas ervas apresentam capacidade anti-inflamatória, antioxidante (GOIS *et al.*, 2016; URIBE *et al.*, 2015) e atividade gastroreparadora bem elucidadas pela literatura (EL SOUDA *et al.*, 2015; GUL; ABBAS; QADIR, 2015; SAGRADAS *et al.*, 2015).

Sendo assim, esta pesquisa discorre sobre o desenvolvimento e a caracterização de uma bebida fermentada de laranja composta de extratos de ervas aromáticas a fim de, possivelmente, melhorar sua capacidade antioxidante e conferir efeito gastroreparador sobre úlcera gástrica induzida por etanol em modelo animal. De modo que, espera-se obter uma bebida funcional, inovadora e diferenciada.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Desenvolver e caracterizar *in vitro, in silico* e *in vivo* um fermentado de laranja composto de extrato de ervas aromáticas.

2.2 OBJETIVOS ESPECÍFICOS

• Desenvolver e caracterizar um fermentado de laranja composto;
• Desenvolver e caracterizar extratos hidro alcoólicos macerados a partir de flores de camomila (*M. recutita*), folhas de capim-limão (*C. citratus*) e folhas de hortelã (*M. piperita*);
• Eleger as concentrações de extratos a serem utilizadas no fermentado final, através diferentes testes sensoriais;
• Aplicar os extratos hidro alcoólicos macerados de camomila, capim-limão e hortelã no fermentado de laranja e avaliar *in vitro* a capacidade antioxidante dos tratamentos do fermentado de laranja composto;
• Avaliar *in silico* a predição de toxicidade dos compostos presentes na bebidas e a sua predição biológica para possíveis efeitos benéficos de aporte à saúde humana.
• Testar *in vivo* a possível capacidade gastroreparadora frente à úlcera gástrica induzida por etanol do fermentado alcoólico de laranja que apresentar a melhor aceitação no teste sensorial e nas análises antioxidantes.

3 DESENVOLVIMENTO

3.1 LARANJA E BIOCOMPOSTOS

O Brasil destaca-se como um dos maiores produtores mundial de laranjas (KALAKI; NEVES, 2017), sendo que as condições de solo, clima e período de frutificação permitem diversas colheitas, evitando a concentração de safras, resultando em um preço de produção baixo durante o ano inteiro (EMBRAPA, 2010).

As laranjas (*C. sinensis*) são divididas em dois grandes grupos, em função da sua coloração: as de coloração vermelha, ricas em antocianinas, são conhecidas como “sanguíneas” e as de coloração laranja, ricas em carotenoides, são conhecidas como “douradas”. No Brasil e em grande parte do mundo as laranjas mais cultivadas são da variedade dourada, também chamadas de “laranja doce”, usadas principalmente para extração de suco, dentre elas, a cultivar Valência (Figura 1), a qual apresenta poucas sementes e elevado rendimento em suco (SAUNT, 1991).

Figura 1 – Laranja Cultivar Valência

Fonte: Autor.

O suco de laranja é um dos sucos mais consumidos e apreciados em todo o mundo e constitui uma mistura aquosa complexa, formada por vários componentes orgânicos voláteis, responsáveis pelo seu sabor e aroma, além de açúcares, ácidos, sais minerais, vitaminas,

compostos bioativos como carotenoides, flavonoides, óleos essenciais e melatonina (SAE-TEAW *et al.*, 2013; TOUNSI *et al.*, 2011; ZACARÍAS-GARCÍA *et al.*, 2022).

É importante ressaltar que quando se trata de frutas cítricas, o conteúdo, a composição de nutrientes e metabólitos bioativos variam amplamente entre as diferentes espécies e variedades, estágios de maturação e forma de consumo, se *in natura* ou processada, o que influencia tanto na qualidade, valor nutricional e efeitos benéficos à saúde (ZACARÍAS-GARCÍA *et al.*, 2022).

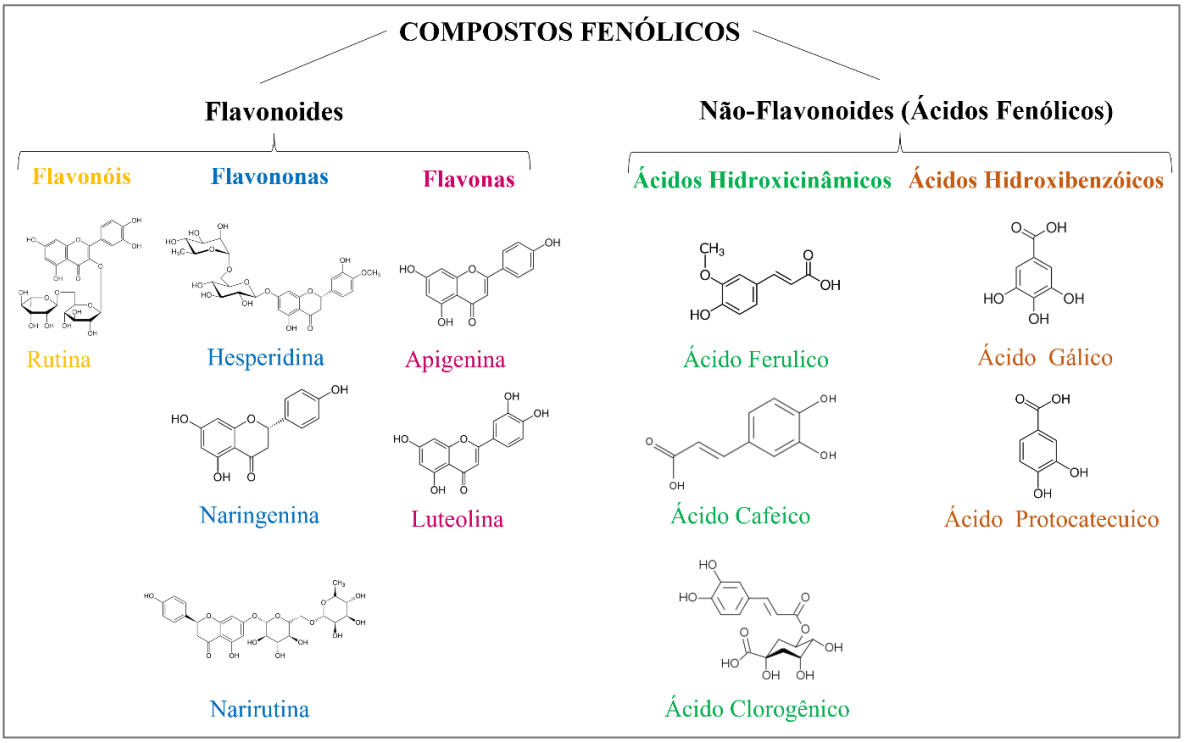
Na literatura estão descritos inúmeros benefícios de promoção à saúde associados ao consumo de frutas cítricas, dentre eles, principalmente fatores relacionados ao sistema imunológico, efeitos anti-inflamatórios, controle da obesidade, diminuição da incidência de doenças cardiovasculares, redução do risco de certos tipos de câncer prevenção de danos à pele, controle da pressão arterial, do nível de açúcar no sangue, entre outros (VAVOURA *et al.*, 2022; ZACARÍAS-GARCÍA *et al.*, 2022).

Sabe-se que o estresse oxidativo desempenha um papel central no desenvolvimento de doenças e as evidências indicam que os vários efeitos benéficos dos metabólitos de frutas cítricas na saúde humana estão associados à sua atividade antioxidante. A atividade antioxidante é a capacidade de um composto bioativo eliminar efetivamente radicais livres, inibindo as reações de peroxidação lipídica e prevenindo outros danos oxidativos (ZOU *et al.*, 2016).

Os compostos fenólicos estão entre os principais compostos bioativos responsáveis pela atividade antioxidante nas laranjas (COELHO; HERMSDORFF; BRESSAN, 2013). Dentre os compostos majoritários encontrados em laranjas, destacam-se a hesperidina, naringenina, rutina, narirutina, ácido ferulico, ácido cafeico e ácido protocatecúico (ZHAO *et al.*, 2020). Entretanto, esses compostos podem ser afetados pelo método de extração, além de sofrer biotransformação e biodegradação conforme sua maturação, de modo que uma mesma cultivar de laranja pode apresentar composição fenólica diferente a depender da época do ano em que foi colhida e analisada (HOU *et al.*, 2021).

A estrutura química que representa os compostos fenólicos é formada por um anel aromático com um grupamento carboxílico e um ou mais substituintes hidroxílicos e/ou metoxila na molécula, incluindo seus grupos funcionais. Encontram-se presentes nos vegetais na forma livre ou ligada a açúcares (heterosídeos), ácidos e /ou proteínas e são divididos em dois grupos: flavonoides e não-flavonoides (Figura 2) (MIRANDA, 2013).

Figura 2 – Principais compostos fenólicos presentes em *Citrus sinensis*



Fonte: Autor com adaptações (ZHAO *et al.*, 2020).

O metabolismo normal das células é capaz de gerar espécies reativas de oxigênio (em inglês “reactive oxygen species” - ROS) que desempenham um papel importante nas funções fisiológicas, incluindo expressão gênica, sinalização celular e respostas imunológicas. Os antioxidantes atuam neutralizando essas ROS em excesso, através da doação de um elétron para um radical livre (RL). Essa neutralização pode ser dar por diferentes mecanismos que vão desde a eliminação direta dos RL, inibição da peroxidação lipídica, entre outros, sendo que o sistema antioxidante pode ser enzimático ou não enzimático (CHAGAS *et al.*, 2022).

O sistema enzimático compreende enzimas antioxidantes, como superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa redutase (GR). Já no sistema antioxidante não enzimático, encontramos glutationa (GSH), ascorbato (AsA), ácidos fenólicos, e diferentes compostos flavonoides, entre outros (RANI; SINGH YADAV, 2015).

A busca por novos compostos antioxidantes como agentes terapêuticos para doenças associadas ao desequilíbrio na produção e liberação de ROS está ganhando atração dos pesquisadores. Antioxidantes naturais, como compostos fenólicos (flavonoides e ácidos

fenólicos), podem sequestrar as ROS e também atuar como agentes anti-inflamatórios (BOONDAENG *et al.*, 2022; CHAGAS *et al.*, 2022; YOO *et al.*, 2010).

Um estudo demonstrou que o consumo diário igual ou superior a 100 mL de sucos naturais (sem açúcar) é capaz de reduzir a expressão gênica de marcadores pró-inflamatórios (HERMSDORFF *et al.*, 2010). Além disso, diversos estudos têm demonstrado que o consumo de suco de laranja reduz a prevalência de fatores de risco cardiovasculares como estresse oxidativo ou inflamatório, dislipidemia, hiperglicemia, disfunção endotelial, hipertensão ou obesidade (APTEKMANN; CESAR, 2013; BUSCEMI *et al.*, 2012; O'NEIL *et al.*, 2012).

Segundo Nazir e colaboradores (2019), a busca por bebidas funcionais e medicinais é uma tendência das últimas décadas, uma vez que saúde e bem-estar estão entre os principais segmentos dos bens de consumo de alta rotatividade devido a consciência de saúde cada vez maior entre os consumidores (NAZIR *et al.*, 2019).

Os alimentos funcionais são definidos como "alimentos processados industrialmente ou naturais que, quando consumidos regularmente como parte de uma dieta diversificada em níveis eficazes, possuem efeitos positivos na saúde, além da nutrição básica". De fato, as frutas, por si só, podem ser consideradas alimentos funcionais, uma vez que contém quantidades significativas de compostos bioativos que podem prevenir de doenças metabólicas, cardiovasculares, entre outras (RODRÍGUEZ *et al.*, 2021).

Encontra-se então, uma nova oportunidade o desenvolvimento de bebidas inovadoras, com matérias primas diferentes das usuais (YANG *et al.*, 2021) e ricas em compostos bioativos que possam conferir benefícios de aporte à saúde humana, como é o caso da laranja (LIU *et al.*, 2021; LV *et al.*, 2015).

3.2 FERMENTADO ALCOÓLICO DE LARANJA

A produção de bebidas alcoólicas fermentadas é um dos processos mais antigos da humanidade (TARKO; KRANKOWSKI; DUDA-CHODAK, 2023), remontando seu preparo a uma antiguidade de pelo menos 5.000 a.C. (ZHANG; ROSENTRATER, 2019) e sendo o vinho, reconhecido como uma das bebidas alcoólicas mais consumidas e preferida devido às suas propriedades organolépticas e de aporte à saúde (SHIRADHONKAR *et al.*, 2014).

O termo "vinho" é exclusivo para a bebida fermentada a partir da uva, entretanto, qualquer fruta açucarada pode ser utilizada na produção de bebidas fermentadas. Nesse caso, essas bebidas precisam ser designadas como "fermentado de fruta", recendo assim, o nome da fruta utilizada como substrato (BRASIL, 2009; UMEH; UDEMEZUE, 2015).

A fermentação alcoólica é um processo bioquímico onde microrganismos, principalmente leveduras, convertem açúcares fermentescíveis (glicose, sacarose e frutose), gerando energia celular na forma de ATP (adenosina trifosfato) e produzindo como resíduos metabólicos etanol (álcool) e gás carbônico (CO_2) – Equação 1 (MEDEIROS, 2019).

$$C_6H_{12}O_{6\ (glicose)} + C_6H_{12}O_{6\ (frutose)} \rightarrow 2C_2H_5OH + 2CO_2 + 2ATP \qquad (1)$$

A literatura reúne uma extensa lista de frutas que são utilizadas para desenvolver bebidas alcoólicas fermentadas, dentre elas, uma das mais conhecidas, a cidra, feita da maçã (WAY *et al.*, 2022), e também outras frutas como abacaxi (BOONDAENG *et al.*, 2022), ameixa (LJEKOČEVIĆ *et al.*, 2019), cereja (YOO *et al.*, 2010), pêssego (LIANG *et al.*, 2022), mirtilo (SANTOS *et al.*, 2016), laranja (ESCUDERO-LÓPEZ *et al.*, 2016).

A laranja, por ser uma fruta açucarada apresenta as características necessárias para produzir uma bebida alcoólica fermentada (HU *et al.*, 2018). Um dos principais fatores relacionado a sua grande aceitação para uso em bebidas é o seu aroma, resultado da presença de aldeídos, cetonas, terpenos e álcoois (NISPEROS-CARRÍEDO; SHAW, 1990), sendo que, seu conteúdo volátil pode ser alterado durante a fermentação, devido à produção de compostos voláteis de levedura e do metabolismo dos compostos originais das frutas, apresentando no produto final a presença de álcoois, ácidos, aldeídos, cetonas e ésteres, particularmente importantes (SELLI *et al.*, 2008).

Estudo realizados também demonstraram que o fermentado alcoólico de laranja apresentou maior teor de flavononas, carotenoides e melatonina, aumentando sua capacidade antioxidante em relação ao suco (ESCUDERO-LO *et al.*, 2013; FERNÁNDEZ-PACHÕN *et al.*, 2014).

Dessa forma, além do fermentado de laranja ser uma bebida agradável sensorialmente (SHIRADHONKAR *et al.*, 2014; VÁZQUEZ-ARAÚJO *et al.*, 2013), a extração de seus compostos bioativos é favorecida pelo ambiente alcoólico (ARRANZ *et al.*, 2012; ESCUDERO-LÓPEZ *et al.*, 2015), podendo assim, exercer efeitos sinérgicos benéficos ao organismo (ARRANZ *et al.*, 2012) e sendo chamada de “bebida funcional” (BULMAN *et al.*, 2021; CONG; BREMER; MIROSA, 2020; GONÇALVES *et al.*, 2022). As “propriedades funcionais” podem ser representadas pelo alto teor de polifenóis, potencial antioxidante, probióticos, entre outras características (BULMAN *et al.*, 2021; CUVAS-LIMÓN *et al., 2022;* PINTO; VILELA, 2021). Inclusive, bebidas como vinho e cerveja já foram identificados como bebidas funcionais (RADONJIĆ *et al.*, 2020).

Além disso, estudos mostraram que o consumo moderado de bebidas alcoólicas mesmo com alto grau alcoólico, como licores e destilados, também têm efeito cardioprotetor (ARRANZ *et al.*, 2012), além de melhora principalmente no perfil lipídico plasmático, no sistema de coagulação e no processo de arteriosclerose (ARRANZ *et al.*, 2012; CERRILLO *et al.*, 2015; VASANTHI *et al.*, 2012).

No entanto, o consumo de bebidas alcoólicas também pode contribuir para vários problemas de saúde, entre eles, as úlceras gástricas (KULSHRESHTHA; SRIVASTAVA; SINGH, 2017; NIETO, 2012), uma vez que o álcool estimula o aumento de ânions superóxido, radicais hidroxila e peroxidação lipídica na mucosa gástrica, induzindo estresse oxidativo intracelular que leva ao desenvolvimento de lesões gástricas ulcerativas (MOUSAVI *et al.*, 2020).

3.3 LESÃO GÁSTRICA ULCERATIVA

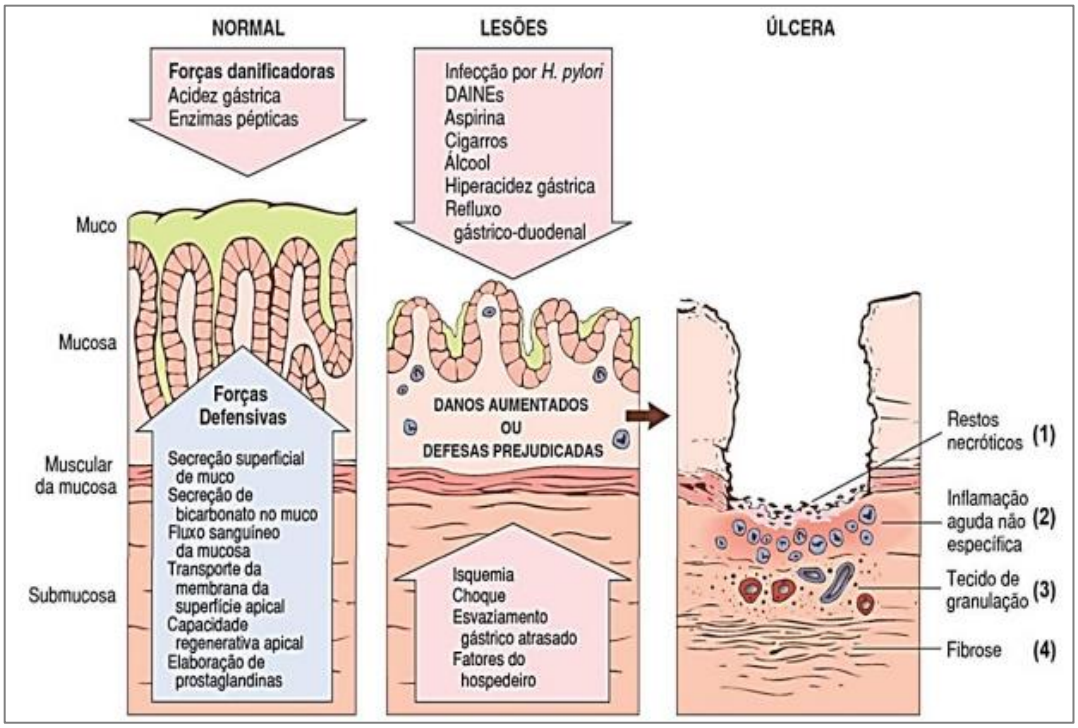
A úlcera é uma doença crônica, também conhecida como úlcera péptica, é caracterizada pelo desequilíbrio entre os fatores que danificam a mucosa do trato digestivo, como pepsina e ácido clorídrico e aqueles que a protegem, como muco, prostaglandinas, bicarbonato e óxido nítrico (BEIRANVAND, 2022; MOUSAVI *et al.*, 2020; NIETO, 2012).

É uma das doenças mais prevalentes no mundo, sendo considerada uma importante questão de saúde pública, pois, está associada a perda na qualidade de vida, perda de produtividade no trabalho e crescentes gastos no tratamento das complicações da doença (BARKUN; LEONTIADIS, 2010).

Quando a úlcera é encontrada no estômago, chama-se úlcera gástrica e quando está presente no duodeno, chama-se úlcera duodenal (BHOWMIK *et al.*, 2010).

As causas atribuídas ao desenvolvimento de úlcera péptica são multifatoriais. Os elementos ambientais, tais como consumo abusivo de álcool, estresse, tabagismo, infecções por *Helicobacter pilory*, uso indiscriminado de anti-inflamatórios não-esteroides (AINES), problemas com sono, hábitos alimentares, trabalho físico pesado e suscetibilidade genética são influenciadores de seu aparecimento e podem inibir ou reduzir a secreção de muco e bicarbonato, aumentando a secreção ácida (Figura 3) (SHAKER; MAHMOUD; MNAA, 2010; VOMERO; COLPO, 2014).

Figura 3 – Mecanismos de proteção e progressão das lesões gástricas.



A imagem ilustra a progressão da lesão ulcerativa, onde destacam-se 4 camadas: 1) tecido necrótico, geralmente do tipo fibrinóide, presente no fundo da lesão decorrente da digestão ácida; 2) exsudato inflamatório com predomínio de neutrófilos; 3) camada constituída principalmente por tecido de granulação; e 4) tecido fibroso cicatricial. Fonte: Adaptado (VALE, 2020).

A integridade da mucosa gástrica é comprometida mediante o desequilíbrio do sistema de defesa, uma vez que ocorre a redução na síntese de fatores chave de proteção como prostaglandinas (PGs), óxido nítrico (ON) e sistema antioxidante (LAINE; TAKEUCHI; TARNAWSKI, 2008). A inibição da síntese de PGs ocasiona a redução da produção de muco, redução do fluxo sanguíneo e lesões no endotélio vascular da mucosa, resultando na produção de ROS (PAN *et al.*, 2008).

O consumo de álcool tem sido relacionado a lesões da mucosa gástrica, incluindo gastrite, úlcera gástrica e até mesmo carcinoma gástrico (FRANKE; TEYSSEN; SINGER, 2006). No entanto, os mecanismos subjacentes à lesão gástrica induzida pelo etanol ainda não estão totalmente elucidados (ARAB *et al.*, 2015). Pesquisadores relatam que a ingesta excessiva de etanol causa hemorragia sub-epitelial, edema, esfoliação celular, degranulação de mastócitos e infiltração de neutrófilos na mucosa gástrica (ADINORTEY *et al.*, 2013; BEN BARKA *et al.*, 2017).

Dentre os maiores problemas no tratamento da úlcera péptica estão seu alto índice de reincidência, entre 60 e 80%, e também o fato da maioria das drogas prescritas produzir reações adversas como prejudicar a absorção de cálcio, ferro, magnésio e vitamina B12 (ABDELWAHAB *et al.*, 2013; DE BARROS *et al.*, 2007).

3.3.1 Úlcera gástrica induzida por etanol

Por se tratar de uma patologia comum, é necessário entender a patogênese das úlceras gástricas e, para isso, modelos experimentais em animais têm sido desenvolvidos e amplamente utilizados (DA SILVA, 2018).

O modelo mais utilizado é a indução de úlceras com etanol, que solubiliza a camada de muco proteção, expondo a mucosa gástrica às ações lesivas do ácido clorídrico e da pepsina, além de reduzir o fluxo sanguíneo e ativar enzimas que promovem a esfoliação e apoptose (BOLIGON *et al.*, 2014; KE *et al.*, 2020).

Os danos diretos e indiretos na mucosa gástrica induzem um quadro de estresse oxidativo, com produção excessiva de ânion radical superóxido ($O_2^{\bullet -}$), radicais hidroperóxidos ($^{\bullet}OH$), óxido nítrico ($^{\bullet}NO$), peróxido de hidrogênio (H_2O_2) e, como consequência, causando peroxidação lipídica (BEN BARKA *et al.*, 2017). Além disso, ocorre a redução das principais defesas antioxidantes, tais como superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GSH) (KE *et al.*, 2020) .

O modelo de úlcera gástrica induzida pelo etanol é semelhante às úlceras em humanos e, por isso é usado para avaliar compostos com potencial gastroprotetor e/ou antioxidante (DA SILVA *et al.*, 2020; FU *et al.*, 2022).

3.3.2 Toxicologia *in silico*: uma ferramenta complementar aos testes *in vitro* e *in vivo*

Mudanças legislativas, pressões comerciais e éticas tem motivado um movimento global para reduzir, refinar ou substituir o uso de animais em pesquisa (princípio dos 3Rs). Com isso, antes de iniciar os tradicionais ensaios *in vivo,* é possível utilizar de ferramentas computacionais para prever efeitos biológicos e de toxicidade no desenvolvimento de produtos e na avaliação de sua segurança (MADDEN *et al.*, 2020).

Os métodos de toxicologia *in silico* (IST) usam abordagens computacionais para prever a toxicidade e efeitos biológicos de produtos químicos. Essas abordagens são frequentemente usadas em combinação com outros testes para gerar informações de avaliação de toxicidade

com menos necessidade de realizar ensaios *in vitro* e *in vivo*, ou então, reduzir o número de animais utilizados nos testes, além de reduzir o custo analítico. Esses modelos são baseados em dados experimentais, relações estrutura-atividade e conhecimento científico reportado na literatura (MYATT *et al.*, 2018).

O princípio fundamental desses modelos é que as propriedades intrínsecas, as interações potenciais e os efeitos finais de um composto químico são codificados em sua estrutura molecular. Compreender isso permite desenvolver modelos quantitativos de relação estrutura-atividade (QSAR) ou quantitativos de relação estrutura-propriedade (QSPR) (MADDEN *et al.*, 2020).

Diversas organizações criam e disponibilizam softwares de computador que contêm um ou mais modelos, os quais predizem os possíveis efeitos toxicológicos ou mecanismos de ação de compostos químicos (AMBERG *et al.*, 2016). O Quadro 1 apresenta exemplos de softwares preditivos para uma variedade de relações estrutura-atividade.

Quadro 1 – Software de análise *in silico* e propriedades de predição de risco toxicológico e atividade biológica de compostos químicos.

Software	**Site para acesso e predições *in silico* disponíveis**
ADMET Predictor	http://lmmd.ecust.edu.cn/admetsar2 Previsão de propriedades: físico-químicas; ADMET (absorção, distribuição, metabolismo, excreção e toxicidade); pesquisa por similaridade.
pkCSM	https://biosig.lab.uq.edu.au/pkcsm/ Previsão de propriedades farmacocinéticas; ADMET.
Protox	https://tox-new.charite.de/protox_II/ Prever a toxicidade do composto; doses tóxicas e classes de toxicidade; alvos de toxicidade; vias toxicológicas.
Lazar	https://lazar.in-silico.ch/predict Prever a toxicidade do composto em espécies animais;
Way2Drug PASS	http://www.way2drug.com/passonline/ Atividade biológica; efeitos farmacológicos; mecanismos de ação; efeitos tóxicos e adversos; interação com enzimas metabólicas e transportadores; influência na expressão gênica; etc.

Fonte: Autor.

Umre et al. (2015) realizaram estudos *in silico* e *in vitro* e demonstraram o potencial antiulcerogênico do ácido ferúlico. Nas análises *in vivo* observaram uma redução na secreção gástrica, atrasando o deterioramento da mucosa gástrica em diferentes modelos de úlcera gástrica. Essas descobertas sobre o acoplamento *in silico* permitiram confirmar todas as interações dos efeitos observados no ensaio *in vivo*.

Em outro estudo, os pesquisadores avaliaram as propriedades hipocolesterolêmicas e antioxidantes de extrato de folhas de oliveira (*Olea europaea* L.). Seus achados *in vitro* e *in vivo* mostraram que os extratos de oliveira reduziram efetivamente os níveis de colesterol total, colesterol LDL, colesterol VLDL e triglicerídeos e aumentaram o nível de colesterol HDL. A ancoragem molecular *in silico* mostrou que existe interação de compostos fenólicos, rutina e luteolina, com a enzima HMG-CoA redutase, que limita a taxa de metabolismo do colesterol. Com a combinação de informações, os autores demonstraram o potencial para o uso terapêutico das folhas de *O. europaea* no tratamento da hipercolesterolemia e na inibição da aterosclerose (CHEURFA *et al.*, 2019).

Nesse contexto, o uso da IST para identificar os principais compostos biologicamente ativos com propriedades antioxidantes é de grande interesse para a indústria de alimentos e bebidas funcionais. Além disso, as novas tendências mundiais de preocupação com a biodiversidade e ideias de desenvolvimento sustentável trouxeram a importância do uso de métodos *in silico* para anteceder e corroborar com estudos experimentais *in vivo*, reduzindo tempo e custos de envolvidos na experimentação (JEAN-QUARTIER *et al.*, 2018).

3.4 APLICABILIDADE DE EXTRATOS DE PLANTAS EM BEBIDAS ALCOÓLICAS

A utilização de plantas medicinais na cura de inúmeras doenças é tão antiga quanto a própria humanidade e é conhecida como fitoterapia (KUNA *et al.*, 2019). Os extratos de plantas medicinais têm mostrado resultados promissores no tratamento de úlceras, não somente proporcionando gastroproteção como também oferecendo gastroreparação às lesões, devido seus princípios farmacologicamente ativos, tais como, flavonoides, terpenos e taninos (BEZERRA *et al.*, 2009; FRANCISCO; FIGUEIRINHA; COSTA, 2014).

Diversos estudos reportam o potencial gastroprotetivo das ervas Camomila (*M. recutita*), Capim-limão (*C. citratus*) e Hortelã (*M. piperita*) – Figura 4 (EL SOUDA *et al.*, 2015; GUL; ABBAS; QADIR, 2015; SAGRADAS *et al.*, 2015).

Figura 4 – Representação plantas medicinais com efeito gastroprotetivo

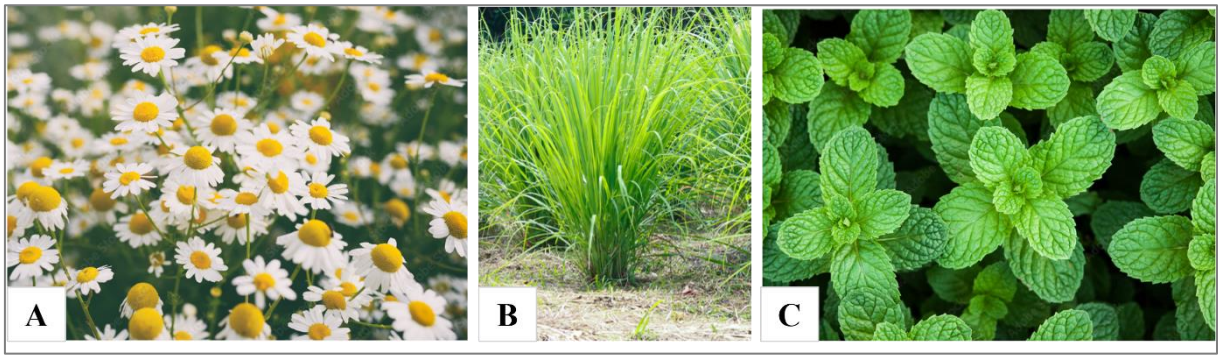


Figura A: Camomila (*Matricaria recutita*);
Figura B: Capim-limão [*Cymbopogon citratus*);
Figura C: Hortelã (*Mentha piperita*)
Fonte: Adaptado. Disponível em: https://stock.adobe.com/br/images/

O preparo de bebidas alcoólicas com uso de plantas aromáticas e especiarias data da história antiga do Mediterrâneo, quando sua maceração junto ao vinho era uma prática comum. Por outro lado, egípcios e gregos, ao descobrirem a tecnologia de destilação, aplicaram-na para produzir vários tipos de destilados de plantas aromáticas. Esses destilados foram então utilizados para aromatizar vinhos e outras bebidas (TONUTTI; LIDDLE, 2010).

A legislação brasileira permite a inclusão de extratos de ervas medicinais e especiarias às bebidas alcoólicas com a finalidade de conferir aroma, sabor e outras características (BRASIL, 2009; BRASIL, 2012).

Em um estudo realizado para avaliar os parâmetros que influenciam a qualidade do vermute utilizando diferentes ervas aromáticas (losna, coentro, anis, manjerona, camomila), os resultados indicaram que os macerados se encontravam dentro das exigências legais (europeias) e quanto às características organolépticas todos apresentaram certa turbidez, odor pungente de álcool e sabor agradável característico das ervas aromáticas (COLDEA; MUDURA, 2015).

Shiradhonkar et al (2014), desenvolveram uma bebida fermentada composta de laranja, realizando a adição de extratos de manjericão, capim-limão, hortelã e gengibre. Os atributos sensoriais mostraram o potencial de uso de extratos de ervas aromáticas e também ditam para um apelo aos possíveis benefícios à saúde humana.

Dessa forma, um merecido destaque pode ser atribuído aos extratos das plantas camomila, capim-limão e hortelã, uma vez que seus extratos não só contribuem com atividade anti-inflamatória e antioxidante (CHUKWUOCHA; FERNÁNDEZ-RIVERA; LEGORRETA-

HERRERA, 2016; MALE; DRAGOVI, 2022; ROZZA *et al.*, 2013; ROZZA; PELLIZZON, 2013; ZIELINSKI *et al.*, 2014), como também conferem o aroma característico das mesmas, devido a riqueza em compostos orgânicos voláteis (SOUALEH; SOULIMANI, 2016).

A Figura 5 representa a bebida desenvolvida na forma controle e contendo a adição dos extratos hidroalcóolicos das plantas estudadas.

Figura 5 – Fermentado de laranja composto de extrato de ervas aromáticas

Figura A: Fermentado de laranja,
Figura B: Fermentado de laranja composto de extrato de camomila,
Figura C: Fermentado de laranja composto de extrato de capim-limão,
Figura D: Fermentado de laranja composto de extrato de hortelã,
Fonte: Autor.

4 ARTIGOS CIENTÍFICOS INTEGRADOS

4.1 ARTIGO 1:

Effect of adding *Matricaria recutita* L., *Cymbopogon citratus*, or *Mentha piperita* L. extracts to fermented orange beverage: sensory evaluation, physicochemical characterization, and prediction of toxic risks and biological activity *in silico*[1*]

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Effect of Adding *Matricaria recutita* L., *Cymbopogon citratus,* or *Mentha piperita* L. Extracts to Fermented Orange Beverage: Sensory Evaluation, Physicochemical Characterization, and Prediction of Toxic Risks and Biological Activity *In Silico*

Laura Gizele Mascarin[1], Fernanda Wouters Franco[1], Rafaela Castro Dornelles[2], Kássia Caroline Figueredo[2], Roberta Oliveira Santos[3], Liliane de Freitas Bauermann[2], Tatiana Emanuelli[1], Sabrina Somacal[1*], and Cláudia Kaehler Sautter[1]

[1]Department of Food Technology and Science, Rural Sciences Center, Federal University of Santa Maria, Santa Maria, RS 97105-900, Brazil; lauragizele@gmail.com (L.G.M.); fernandafranco8@hotmail.com (F.W.F.); tatiana.emanuelli@ufsm.br (T.E.); claudia.sautter@ufsm.br (C.K.S.)

[2]Department of Physiology and Pharmacology, Health Sciences Center, Federal University of Santa Maria, Santa Maria, RS 97105-900, Brazil; rafaeladornelles@unipampa.edu.br (R.C.D.); kassia.tquimica@yahoo.com.br (K.C.F.); lgfbauermann@gmail.com (L.F.B.)

[3]Graduate Program in Industrial Systems and Processes, University of Santa Cruz, Santa Cruz do Sul, RS 96815-900, Brazil; eng.rosantos@gmail.com (R.O.S)

[*]Correspondence: sabrina.somacal@ufsm.br (S.S)

Abstract: Fermentation is an important tool in producing functional beverages through agro-industrial wastes, and medicinal and aromatic plants due to the specific content of bioactive molecules. Therefore, this study evaluated the contribution of *Matricaria recutita* (chamomile), *Cymbopogon citratus* (lemongrass), or *Mentha piperita* (peppermint) extracts to the phytochemical profile and potential biological effects of a functional fermented orange beverage in vitro and in silico. The concentrations of aromatic herbal extracts that yielded the best sensory performance for fermented beverages were selected for analyses that involved characterizing the fermented beverages. The beverages that received the extracts (2%) had the highest phenolic and flavonoid content and antioxidant potential compared to the control. Hesperidin (124-130 mg L^{-1}), narirutin (66-70 mg L^{-1}), chlorogenic (11-16 mg L^{-1}), caffeic (5.3-5.5 mg L^{-1}), and ferulic (1-1.7 mg L^{-1}) acids were found in the different formulations. The *in silico* analysis suggested that the evaluated compounds do not present a toxicity risk (mutagenicity, carcinogenicity, hepatotoxicity, and ability to penetrate the blood–brain barrier). Additionally, they can contribute to the biological effects of therapeutic importance, such as antioxidant, gastroprotective, and anti-ulcerative properties, and the *Mentha piperita* L. extract

presented the greatest potential among the evaluated herbs for use in functional fermented beverages.

Keywords: antioxidants; *Cymbopogon citratus*; fermented beverage; functional beverage; gastro-protective; *Matricaria recutita* L.; *Mentha piperita* L.; phenolic compounds

1 Introduction

Consumer dietary habits have drastically changed in recent decades and functional beverages now have a strong position in the market. Most of these beverages are produced using simple processes that use fruits, cereals, legumes, nuts, and food product waste, among other things [1,2]. Recently, wine, one of the most consumed and preferred alcoholic beverages, has been identified as a functional beverage [3]. In addition to the grape, wine can be produced from any sugary fruit as long as it is designated as fermented fruit [4]. In the last decade, various studies have reported the production of fruit wine and its possible therapeutic properties [4,5]. The main source of these beverages' beneficial potential is phenolic compounds [2,3]. After consuming foods rich in phenolic compounds, such as functional beverages, the colon is the main site of microbial fermentation. Phenolic compounds are transformed into phenolic acids or lactone structures by intestinal microbiota, which produces metabolites with biological and antioxidant activity, and evidence suggests those metabolites have health benefits for humans [2,6].

Oranges are an abundant source of vitamin C and have considerable amounts of sugar, minerals, and bioactive compounds such as carotenoids, phenolic compounds, and terpenoids [5,7]. Thus, their use as raw materials to produce functional beverages is an attractive option to add value to the product and diversify the market, and a solution to minimizing the losses of these fruits in the fields or during transportation [1]. In addition, Brazil is considered the largest producer of oranges [8], and its different fruiting periods enable various harvests, thus avoiding the concentration of crops and reducing production costs throughout the year [8].

The use of medicinal and aromatic plants in the production of functional beverages has become increasingly popular due to the specific content of structurally diverse bioactive molecules with numerous confirmed health benefits and specific sensory properties [9]. The aromatic features of *Matricaria recutita* L. (chamomile), *Cymbopogon citratus* (lemongrass), and *Mentha piperita* L. (peppermint) are mainly related to volatile compounds of essential oils. However, the presence of non-volatile compounds, including phenolics, also contribute to specific sensory and beneficial properties [10]. Phytochemical profiling of medicinal and

aromatic plants containing specific and complex mixtures of bioactive molecules provides numerous opportunities to develop new categories of functional beverages.

The herbs *Matricaria recutita* L., *Cymbopogon citratus*, and *Mentha piperita* L. have anti-inflammatory, antioxidant, antiseptic, gastroprotective, and many other properties attributed mainly to their polyphenols and essential oil components [11–13]. The beneficial effects of *Matricaria recutita* are related to the presence of phenolic compounds (e.g., apigenin or hydroxycinnamic acid derivatives) and the essential oil components (e.g., chamazulene, farnesene, α-bisabolol, and its oxides) [14]. Among the *Cymbopogon citratus* volatile compounds, the citral (mixture of terpenoids and geranial), myrcene, geraniol, citronellol, and α-oxobisabolene stand out [13]. In the case of *Mentha piperita*, the emphasis is on d-carvone, limonene, menthone, menthol, and pulegone, and among non-volatile compounds phenolic acids such as chlorogenic, caffeic and rosmarinic acids, and flavonoids, including luteolin, naringenin, and hesperetin derivatives [11]. This diverse profile of compounds allows them to be potentially included in beverages [9], generating a differentiated product.

In this context, identifying the main biologically active compounds with antioxidant properties is of great interest to the functional food and beverage industry. In addition, new global trends of concern with biodiversity and ideas of sustainable development have brought the importance of using in silico methods to precede experimental studies in vivo, reducing production time and costs [15]. *In silico* analysis, through the evaluation of the structural analysis of compounds, can be used to estimate their toxicity and/or predict their biological activity profile, among other effects [16].

Thus, this study aims to analyze the contribution of *Matricaria recutita* L., *Cymbopogon citratus*, and *Mentha piperita* L. extracts in the phytochemical profile and potential biological effects of a functional fermented orange beverage.

2 Materials and Methods

2.1 Chemicals

Milli-Q water (Millipore, Bedford, MA) was used in all experiments. 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH), catechin (98%), Folin–Ciocalteu reagent (2N), chlorogenic acid (95%), caffeic acid (98%), ferulic acid (99%), gallic acid (98%), protocatechuic acid (95%), synergic acid (95%), synaptic acid (95%), t -cinnamic acid (95%), and rutin (94%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hesperidin (98%), naringenin (98%),

narirutin (98%), apigenin (98%), and luteolin (98%) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). HPLC-grade methanol and acetic acid were acquired from Sigma-Aldrich (St. Louis, MO, USA) and used in the mobile phase. All other chemicals were of analytical grade.

2.2 Experiment 1. Production of fermented orange beverage and choice of aromatic herb concentrations

2.2.1 Raw Material: Oranges and Aromatic Herbs

In order to produce the fermented orange beverages, 200 kg of "Valencia" oranges (2018 harvest) were purchased in the city of Mata, southern Brazil. The aromatic herbs - *Matricaria recutita* L. (flowers), *Cymbopogon citratus* (leaves), and *Mentha piperita* L. (leaves) - were purchased locally and in dry form, which is suitable for making teas.

2.2.2 Fermented orange beverage production with the addition of aromatic herbal extracts

The oranges were selected and sanitized (200 ppm sodium hypochlorite). The juice was extracted by performing a latitudinal cut on the oranges, which were squeezed in an industrial extractor (JL Colombo® 650W, Itajobi, SP, Brazil). The juice was filtered to remove the solid residue, and a yield of 39% was obtained.

The total soluble solids (TSS) of the juice were determined by refractometry and the pH was measured with a digital pH meter (Digimed® model DM–22, São Paulo, SP, Brazil) [17].

The orange juice (must) was prepared for fermentation by adding 6% sodium metabisulfite (70 ppm) and left to rest for 60 min. Then, 230 g L^{-1} of sugar (26 °Brix) was added, homogenized, and the pectinolytic enzymes (Lafazin Extract®, 3 g hL^{-1}; NutriStart®, 40 g hL^{-1}) were added.

The yeast *Saccharomyces cerevisiae* (Blastosel Delta®, 40 g hL^{-1}) was inoculated and the alcoholic fermentation was carried out in 20 L polyethylene kegs at a controlled temperature (16 ± 2 °C), and with decreased and stabilized TSS to determine the endpoint of fermentation.

After fermentation, the temperature was maintained at 5 °C for 48 h to separate the yeasts and other solids. The must was racked and filtered, and 50 ppm of 6% sodium

metabisulfite was added and kept stabilized for three months with a controlled temperature of 16 ± 2 °C.

The hydro-alcoholic extracts of aromatic herbs (50% EtOH/H_2O) were prepared from equal volumes of 96 °GL cereal alcohol and distilled water. Then, 100 g of dry matter from each plant was weighed and placed in maceration in 1 L of 50% hydroalcoholic solution, which was the volume necessary to fully cover the dry matter, resulting in an extract concentration of 10 g% [9]. The herbs were kept in infusion in dark bottles at room temperature for 14 days to optimize the extraction of compounds [18]. Afterward, the extracts of *Matricaria recutita* L., *Cymbopogon citratus*, or *Mentha piperita* L. were added to the fermented beverage, thus forming 16 treatments: 1 control and 5 concentrations (0.5, 1.0, 2.0, 3.0, and 4.0%) (v/v) for every aromatic herb. The mixture remained stabilized for 2 months, in the dark, and at a controlled temperature of 16 ± 2 °C [9].

2.2.3 Sensory evaluation of functional fermented orange beverages

Sensory evaluation was carried out at the Federal University of Santa (Rio Grande do Sul State, southern Brazil) at the Integrated Center for Development and Laboratory Analysis (NIDAL), in a laboratory with partitioned booths under white light. Participants were fully informed of the experimental protocol and the possible risks and discomforts of the investigation before giving their written informed consent. The study was conducted according to the Declaration of Helsinki, and the study protocol was approved by the institutional ethics committee (CAAE number:10027519.3.0000.5346, no. 3.257.616, Supplementary material – Figure S5).

Twenty-five untrained panelists (men and women ages 18–60 years) were selected to participate based on their preference for wines, interest, and availability. The samples (20 mL) were randomly chosen and served chilled (7 ± 1 °C) in colorless vessels numbered with three random digits. Mineral water and water-and-salt biscuits were provided to clean the palate and powdered coffee for olfactory fatigue.

The evaluations were performed on three different days (day 1 = *Matricaria recutita* L., day 2 = *Cymbopogon citratus*, and day 3 = *Mentha piperita* L.). The samples were evaluated by an affective acceptance test using a 7-point hedonic scale (1 = disliked extremely, 2 = disliked a lot, 3 = disliked a little, 4 = disliked, 5 = liked, 6 = liked a lot, 7 = liked extremely), in which the color, aroma, and flavor attributes were judged. In the order preference test, the samples were ordered from the least preferred to the most preferred. The result is the sum of

the orders, where higher values for the samples represent greater preference. The purchase intention test was carried out using a 5-point scale (1 = would certainly buy, 2 = would likely buy, 3 = may or may not buy, 4 = would likely not buy, 5 = would certainly not buy) [19].

For each aromatic herbal extract tested, the concentration that yielded the best sensory performance for fermented beverages was selected for subsequent analyses.

2.3 Experiment 2. Physicochemical characterization and bioactive parameters of functional fermented orange beverages

The best concentration of the set of herbs in Experiment 1 led to the following treatments for Experiment 2: fermented control; fermented beverage with *Matricaria recutita* L. (MR); fermented beverage with *Cymbopogon citratus* (CC); and fermented beverage with *Mentha piperita* L. (MP).

2.3.1 Physicochemical characterization

All physicochemical characterization analyses were carried out in triplicate, with methodology indicated to meet the parameters of Brazilian legislation [17]. The alcohol content (% ethanol) was determined in a Gilbertini® enochemical distiller (Novate Milanese, MI, Italy). Total acidity (mEq L^{-1} of citric acid) was determined by titrimetry. Volatile acidity (mEq L^{-1} of acetic acid) was determined by steam dragging in a Gilbertini® enochemical distiller. The fixed acidity (mEq L^{-1} of citric acid) was determined by con-verting volatile acidity into fixed acidity, considering the gram-equivalent values (Eq g^{-1}) of citric acid per Eq g^{-1} of acetic acid. The pH of the samples was measured through digital reading, carried out in a pHgameter (Digimed® DM-22, São Paulo, SP, Brazil). Reducing sugar determination (g L^{-1}) was conducted according to Lane and Eynon's redox titration method. The reduced dry extract content (g L^{-1}) was determined as the dry residue's weight obtained after the volatile compounds' evaporation.

2.3.2 Total phenolic and total flavonoid content, and antioxidant potential

Total phenolic content (TPC) was determined by the colorimetric method with the Folin–Ciocalteau reagent [20] using a spectrophotometer at a wavelength of 765 nm. Quantification was performed using a calibration curve (0 to 80 mg L^{-1}; $y = 105.19x + 1.4331$;

R^2 = 0.989), and the results were expressed in milligrams of gallic acid equivalent per liter of the sample (mg GAE L^{-1}).

Total flavonoid content (TFC) was determined by spectrophotometry with absorbance at a wavelength of 510 nm [21]. Quantification was performed using a calibration curve (0 to 200 mg L^{-1}; y = 305.79x-3.0507; R^2 = 0.999), and the results were expressed in milligrams of catechin equivalents per liter of sample (mg CAE L^{-1}).

Antioxidant capacity was measured by eliminating DPPH radicals [22] in the samples of fermented orange beverages composed of aromatic herb extracts and samples of crude herbal extracts with a reading at 517 nm after 4 h of incubation in the dark. The effective sample concentration required to eliminate DPPH radicals by 50% (IC_{50} value) was obtained by linear regression analysis and represented the sample concentration in relation to the corresponding elimination effects. The lower the IC_{50} values, the greater the antioxidant activity.

The antioxidant potential of all beverages and crude extracts was also determined as their potential to protect against fluorescein oxidation by the peroxyl radical generated through the thermal degradation of AAPH using the oxygen radical absorbance capacity (ORAC) method [23]. Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4), where 25 μL of sample or Trolox and 150 μL of fluorescein (81 nM) were placed in the well of the black 96-well microplates. The mixture was pre-incubated for 10 min at 37 °C. The AAPH solution (25 μL; 152 mM) was rapidly added using a multichannel pipet, and the fluorescence was recorded every minute for 90 min. Fluorescence (λexc = 485 nm and λem = 528 nm) was measured using a Synergy H1 plate reader (Agilent, USA). The ORAC values were calculated by a regression equation obtained with the area under curve (AUC) of the fluorescein decay. This analysis was expressed as μmol of Trolox equivalents per liter of sample (μmol TE L^{-1}).

2.3.3 HPLC analysis of phenolic compounds

The samples' main phytochemicals with antioxidant potential were by high-performance liquid chromatography analysis combined with diode array detection analysis (HPLC-DAD), according to the adapted method [24]. The compounds were separated using liquid chromatography (SHIMADZU, Kyoto, Japan) composed of a pump (model LC-20AT), an automatic injector (SIL-20A), a diode array detector (DAD SPD-M20A), and a communicator (CBM 20A). The separation was controlled by the software LC SP1.

The Agilent Eclipse Plus C18 column (4.6 × 150 mm, 5 μm) was used with gradient elution (flow rate of 0.8 mL min^{-1}) using two mobile phases (A and B). Mobile phase A

consisted of 2% acetic acid in the water, while mobile phase B consisted of only UV/HPLC methanol. The injection volume of the samples was 40 µL, and the detection was monitored in a photodiode system at wavelengths between 230 - 400 nm for 55 min. Before injection, the samples and mobile phases were filtered through a 0.45 µm hydrophilic nylon membrane.

Compound identification was performed by comparing the retention time in the samples and visible UV absorption spectrum with solutions of authentic standards. The standards used were: gallic acid, protocatechuic acid, synergic acid, synaptic acid, t-cinnamic acid, chlorogenic acid, caffeic acid, ferulic acid, rutin, narirutin, hesperidin, naringenin, apigenin, and luteolin. The identified compounds were quantified by calibration curves obtained by preparing solutions at 1000 mg L^{-1} in MeOH UV/HPLC with the standards. The points used were: 5.0, 10.0, 25.0, 35.0, and 50.0 mg L^{-1}.

The contents of the quantified substances were calculated from the line equation and the results were expressed in milligrams equivalent to each standard per milliliter of each sample (mg L^{-1}).

2.3.4 Prediction of toxic risks *in silico*

A computer simulation study was carried out to estimate possible toxicity risks in the main compounds identified by HPLC-DAD using four online computer programs: admetSAR server (https://www.simulations-plus.com/software/admetpredictor - accessed on 15 December 2020), pkCSM platform (http://biosig.unimelb.edu.au/pkcsm - accessed on 16 December 2020), Protox (http://tox.charite.de/protox_II - accessed on 15 December 2020), and Lazar (http://lazar.in-silico.ch/predict - accessed on 15 December 2020). The toxicological safety comprised the predicted risk for the following toxic effects: mutagenicity, carcinogenicity, hepatotoxicity, blood-brain barrier permeability, and acute oral toxicity. Side effects were interpreted and expressed as: (+) at risk, (-) not at risk, and (nr) not reported.

Acute oral toxicity was classified based on the four categories of the United States Environmental Protection Agency, which divides the compounds according to their LD_{50} values (mean lethal dose). Category I contains compounds with LD_{50} values ≤ 50 mg kg^{-1}, Category II contains compounds with LD_{50} values > 50 mg kg^{-1} and < 500 mg kg^{-1}, Category III includes compounds with LD_{50} values ≥ 500 mg kg^{-1} and < 5000 mg kg^{-1}, and Category IV consists of compounds with LD_{50} values ≥ 5000 mg kg^{-1}.

2.3.5 Prediction of biological activity *in silico*

The possible biological effects of the main compounds identified by HPLC-DAD were predicted with computer simulations using the platform Way2Drug PASS online (http://www.way2drug.com - accessed on 17 December 2020).

The predicted results were expressed as a percentage of probably active (Pa) and probably inactive (Pi). Results with Pa greater than 70% indicate a high probability of pharmacological activity [25].

The criterion for selecting the main biological effects was the gastroprotective potential and/or healing potential of the identified compounds. The selected effects were anti-hemorrhagic, antioxidant, anti-free-radical, anti-inflammatory, and anti-ulcerative properties, gastritis treatment, mucous membrane protection, and vasoprotection.

2.4. Statistical analysis

The analyses were performed in triplicate, and the results were submitted to Pear-son's correlation analysis and analysis of variance (ANOVA), and the means compared by Tukey's test at 5% probability. All statistical analyses were performed using the Statistica 10.0 software. In the sensorial analysis, an experimental design of completely randomized blocks was used and analyzed by the Friedman method.

3 Results

3.1 Sensory evaluation

Different sensory tests were conducted to determine the most acceptable concentration of aromatic plant extract added to the fermented orange beverage.

The attributes "color" and "aroma" presented good evaluations (Table 1), which indicates that all extracts, in all concentrations, pleased the panelists. Nevertheless, they did not present significant differences ($p > 0.05$) between the concentrations of the samples; therefore, they cannot be considered criteria for selecting the best concentration.

Regarding "flavor," all extracts showed a significant difference ($p < 0.05$) between the lowest concentration (0.5%) and the highest concentration (4.0%). These data revealed that the panelists preferred the fermented orange beverage with lower concentrations of aromatic herbal

extracts (between 0.5 and 2.0%). Moreover, the panelists considered both beverages with CC and MP to be “very pleasant” and “very refreshing” (data not shown).

In the order preference test (Table 1), there was a significant difference ($p < 0.05$) in all extracts regarding the highest concentration (4.0%), which was indicated by the majority of the panelists as the least preferred. These data corroborate the assessments presented in the acceptance test.

For the consumption and purchase intention test (Table 1), the concentration of 4.0% showed a significant difference ($p < 0.05$) in all extracts, being indicated as “may or may not buy” and “would likely not buy.” The concentrations of 0.5, 1.0, and 2.0%, despite not showing significant differences ($p > 0.05$), received indications of “would certainly buy” and “would likely buy,” thus corroborating the other observations in the previous tests.

In our studies, the concentration of 2.0% was the highest in all extracts when there was no significant difference ($p > 0.05$) in any of the attributes (color, aroma, and flavor) (Table 1), with the highest concentration being accepted in the order preference test and purchase intention test, and, therefore, the concentration chosen for subsequent evaluations.

3.2. Physicochemical characterization

The physicochemical characterization of the beverages (Table 2) was in accordance with Brazilian legislation (BRASIL, 2012). The results showed no significant differences between the control and the other treatments ($p > 0.05$), suggesting that the added aromatic plant extracts did not alter the beverages’ physicochemical parameters.

3.3. Total phenolic and flavonoid content, and antioxidant capacity

The TPC, TFC, and antioxidant capacity of the extracts and beverages developed in this study can be seen in Table 2. The addition of the MP extracts increased TPC by 8%, indicating a possible increase in the reducing capacity of this beverage. The TPC of the beverages added with MP and CC extracts increased by 5 and 3%, respectively. Therefore, the results of our study show that the extracts contributed to the greater antioxidant potential in the beverages. Notably, despite being widely used to assess total phenolic content, this TPC assay measures the reducing capacity of samples [27].

The addition of aromatic herbal extracts contributed significantly ($p < 0.05$) to TFC. Hence, the MP extracts increased TFC by 101%, MR by 29%, and CC by 12%.

Antioxidant capacity evaluated by DPPH showed significant differences in all samples ($p < 0.05$), both in the crude herbal extracts and fermented orange beverages. The values found in the crude herbal extract had greater antioxidant capacity in the MP extract, as indicated by the lower IC_{50} values, followed by MR and CC. Nevertheless, the ORAC test showed that only the fermented orange beverage with the addition of MP extract had a greater capacity to neutralize the peroxyl radical when compared to the control beverage. These data suggest that the analyzed polyphenols are responsible for the antioxidant capacity of the extracts and corroborate the significant antioxidant capacity in vitro of these compounds, which can capture a wide range of reactive oxygen species. In the orange fermented samples with aromatic herbal extracts added, mainly MP, the antioxidant capacity was even more intense, indicating possible synergistic effects with bioactive compounds from the oranges, fermentation process, and extracts.

According to DPPH, adding the MP extract to the orange beverage helped increase antioxidant capacity by about 17%, followed by CC, which contributed to a 6% increase. The MR extract, however, resulted in a 6% reduction in antioxidant activity compared to the control, showing that the extract interacted with the matrix in the DPPH.

The addition of the extracts contributed to an increase in TPC ($r = 0.607$; $p < 0.05$) and flavonoids ($r = 0.775$; $p < 0.01$), where the antioxidant potential in the beverages followed the order MP > MR > CC. This same proportion was observed in the crude herbal extracts, where the evaluation of antioxidant activity using the DPPH method for them showed a positive correlation with the content of phenolics ($r = 0.932$; $p < 0.05$) and flavonoids ($r = 0.673$; $p < 0.01$).

3.4. Composition of phenolic compounds

Of the 14 analytes analyzed in this study, 3 phenolic acids and 5 flavonoids were identified in the samples of crude herbal extracts and fermented orange beverages (Table 3 and Figures S1-S4). The major compounds found in the crude herbal extracts were apigenin in the MR (138.4 mg L^{-1}) and MP extracts (132.4 mg L^{-1}). Other compounds identified in the MR and MP extracts were ferulic acid (95.2 and 98.7 mg L^{-1}, respectively), chlorogenic acid (49.1 and 47.4 mg L^{-1}, respectively), and luteolin (20.1 and 17.4 mg L^{-1}, respectively). In the CC extract, chlorogenic acid (8.4 mg L^{-1}), caffeic acid (3.5 mg L^{-1}), and luteolin (3.11 mg L^{-1}) were identified.

In the beverage control, the hydroxycinnamic acids found were chlorogenic acid (10.9 mg L^{-1}), caffeic acid (5.3 mg L^{-1}), and ferulic acid (1.6 mg L^{-1}). The flavonoids identified were narirutin (69.7 mg L^{-1}), rutin (6.1 mg L^{-1}), and hesperidin (130.3 mg L^{-1}).

The addition of the herbal extracts in the fermented beverages increased the concentration of chlorogenic acid compared to the control. Moreover, there was a 46% increase in the beverage with MR and 26% in the beverage with CC. The addition of the herbs did not change the concentration of caffeic acid. In the beverage with MP, the ferulic acid de-creased by 32% and CC by 39%.

Regarding the major flavonoids, hesperidin was reduced by 4% in the beverage with MP and 5% in the one with CC compared to the control. The additions of MR and MP did not alter the concentration of narirutin. However, it was reduced by 5% with CC compared to the control.

3.5. *In silico* toxicity prediction

The computational model suggested that the evaluated compounds did not present any risks of mutagenicity, carcinogenicity or hepatotoxicity, and could not penetrate the blood–brain barrier (Table 4).

Regarding the acute oral toxicity of the analytes, chlorogenic acid, narirutin, rutin, and hesperidin have median toxicity (LD_{50}) for concentrations between 500 and 5000 mg kg^{-1} and are identified as class III. Caffeic and ferulic acids have median toxicity (LD50) for concentrations ≥ 5000 mg kg^{-1} and are identified as class IV. Thus, the beverages with added plant extracts did not have the potential for acute toxicity, since the concentrations of these beverage analytes are lower than the LD_{50} (Table 3).

3.6 Prediction of biological activity *in silico*

To identify possible pharmacological effects, the major phytochemicals in the beverages were analyzed for their different types of predicted biological activity (Table 4).

The pharmacological analysis of chlorogenic acid showed that this compound likely has several biological properties of therapeutic importance (Pa > 70%), including antioxidant activity, free radical scavenging, and mucoprotective agents.

Caffeic acid has the potential to act as a mucoprotective and vasoprotective agent. Ferulic acid is a potential scavenger of free radicals and a mucoprotective and vasoprotective

agent. The flavonoids narirutin, rutin, and hesperidin have potential anti-hemorrhagic, antioxidant, and anti-inflammatory activity, free radical scavenging, and vasoprotective effects. Narirutin and hesperidin have potential antiulcerogenic effects.

4 Discussion

In recent years, our society began demanding healthier products, drastically changing dietary consumer habits. In this context, functional foods provide health benefits beyond basic nutritional functions, and beverages are by far the most important category [1]. The primary purposes of consuming these beverages are boosting energy, fighting aging, fatigue, and stress, weight management, and targeting specific diseases (e.g., hypercholesterolemia, helping decrease glucose levels, etc.) [2,3]. Nonetheless, producing attractive functional foods due to their sensory characteristics is a permanent challenge in the food industry. Gathering healthy and attractive items in a single food is an even greater obstacle. The extensive list of benefits associated with phenolic compounds, including antioxidant, anticancer, anti-inflammatory, and anti-aging properties, among others, fully justifies their use in the enrichment of various food products. Hence, this study sought to develop a functional fermented beverage composed of aromatic herbal extracts, with olfactory richness perceived by the panelists. The concentration of 2% (v/v) was the highest in which the "flavor" attribute stood out in the order preference, and consumption and purchase intention tests. Therefore, all the analyses reported below refer to the beverage developed and added with 2% (v/v) of chamomile, lemongrass, and mint extracts.

The physicochemical characterization of produced beverages is a legal requirement for the aspects that will make the product proper or not for commercialization and consumption. According to Brazil's identity and quality standards, the beverage developed should be designated as "Fermented Fruit Compound" [26]. Our findings showed that the beverage meets all the required legal parameters and has characteristics of being "dry" due to the low content of reducing sugars and "full-bodied," according to the high results obtained in the analysis of dry extract [26]. To meet legislative requirements and the purpose of the study with gastric ulcers (data not yet published), the developed beverage contains a higher alcohol content (15.9-16.2%) than the fermented ones reported in the literature.

In our study, the fermented beverages with CC and MP were well-accepted at the lowest concentrations. The concentration of 2.0% was the highest and most accepted in the preference ordering test, and the consumption and purchase intention tests. The consumption of fruit-based

beverages, fermented or not, rich in phenolic compounds has also been related to healthy diets, such as the Mediterranean diet, and the prevention of chronic diseases since they present antioxidant properties [1,2,5]. Although most phenolic compounds have low bioavailability after digestion, they attain remarkably high concentrations in the gastrointestinal tract [6], where they may exert direct radical scavenging and antioxidant properties. Phenolic content varies according to the cultivar due to the genetic potential of its biosynthesis. One study analyzed citrus juices and reported that the TPC was 784.7 mg L^{-1} for bitter oranges and 106.2 mg L^{-1} for mandarin oranges [7]. In another study with "Valencia" oranges, 571.0 mg L^{-1} of TPC was detected in the fruit [28]. This is the same cultivar utilized in our study in which a TPC of 459.4 mg L^{-1} was observed in the produced beverage. Furthermore, the TPC reported herein is relatively high, as a similar study with "Kozan" oranges reported a 48.7% loss in TPC from orange juice (317.36 mg L^{-1}) to orange wine (162.7 mg L^{-1}) [29].

The bioactive antioxidant properties of phenolic compounds are especially relevant for gastrointestinal disorders as this site attains the greatest concentrations. Phenolic compounds have demonstrated beneficial effects against gastrointestinal disorders associated with oxidative stress such as gastric ulcers or inflammatory bowel diseases [30]. Polyphenols are essential bioactive molecules with potential gastroprotection, which can prevent lesions of the gastric mucosa and reduce the number and intensity of lesions [12,30]. Among the total polyphenols, flavonoids are the major phenolic compounds in oranges, conferring a wide range of biological activities with potential beneficial effects against cardiovascular diseases, osteoporosis, and cancer [1,5,12].

In beverages with added aromatic herbal extracts, it was possible to verify the synergistic effects of bioactive compounds from the orange and extracts, for TPC and TFC. However, concerning the individually quantified analytes, there were slight differences. The extracts were added to the orange beverage that was already prepared, meaning they did not go through the fermentation process together with the beverage. Originally, the crude extract was in a concentration of 10 g% that, when added to the drinks, passed the concentration of 2% (v/v). Thus, it is expected that there will be a dilution of these bioactive compounds in the final product. This was observed with luteolin and apigenin which were present in the crude extracts but were not detected in the fermented ones. Rutin was not found in the extracts, although it was identified in the fermented control. However, adding the herbs was not detected, suggesting the interaction with the extracted matrix.

The chromatographic analysis identified chlorogenic acid, caffeic acid, ferulic acid, narirutin, rutin, and hesperidin. A different study on the composition of orange wine made with the

"Kozan" cultivar revealed the presence of chlorogenic acid (4.7 mg L^{-1}), caffeic acid (2.6 mg L^{-1}), narirutin (21.7 mg L^{-1}), and hesperidin (90.6 mg L^{-1}) in relatively lower values than those found here with the "Valencia" cultivar, except for ferulic acid (9.9 mg L^{-1}) [29]. The alcohol content of this study is high due to it being a fermented fruit compound beverage and is 25% higher than the "Kozan" fermented beverage [29]. This concentration may have favored the extractability of these analytes. The bioactive compound content in the extracts can be influenced by various factors, including the extraction method, climatic, geographic, and cultivar conditions, part of the plant material used, its origin, the processing, and even the particle size [31].

Another point of interest for future studies is predicting the bioactivity of the identified phytochemicals. The *in silico* methods precede *in vivo* experimental studies, reducing the time spent and laboratory costs [15]. Oral *in vivo* administration of the chamomile extract was effective in preventing gastric ulceration in mice and did not produce acute toxicity effects at doses up to 5000 mg kg^{-1} [32]. The beverage prepared here with chamomile extract and other herbs contains a proportion of 2 g% of the product, representing an in-take of 100 mg kg^{-1}.

Our results of the *in silico* analysis corroborate other data in the literature that attribute to the tested phytochemical groups a wide number of pharmacological activities such as anti-inflammatory, antioxidant, and gastroprotective activity [11–13]. Umre et al. (2015) conducted *in silico* and *in vitro* studies [33] and demonstrated the antiulcerogenic potential of ferulic acid, and their *in vivo* analysis reported reduced gastric secretion by delaying the deterioration of the gastric mucosa in different models of gastric ulcer. Moreover, their findings on *in silico* coupling allowed them to confirm all the interactions of effects observed in the *in vivo* assay.

In silico modeling for the aqueous extract of *Achyrocline satureioides* flowers revealed that isoquercitrin, quercetin, and caffeic acid had a low probability of toxic risk [34]. Toxicity tests aim to identify harmful effects caused by substances in humans, animals, plants, or the environment through acute or multiple exposures [35]. They also showed that *in silico* toxicology, by using computational resources, can organize, analyze, model, simulate, visualize, and predict the toxicity of chemical substances with possible beneficial or adverse effects for therapeutic purposes.

5 Conclusions

The addition of *Matricaria recutita, Cymbopogon citrates*, or *Mentha piperita* extracts at a 2% level in a fermented orange beverage had the best evaluation in sensory tests and

positively influenced the functional characteristics of the fermented orange beverage by increasing the total phenolic and flavonoid content, in addition to improving the antioxidant capacity without altering the physicochemical characteristics of the beverage. Among the evaluated extracts, the *Mentha piperita* proved to have the best characteristics to be added to the functional fermented beverage.

Fermented orange beverages have different bioactive compounds (flavonoids and phenolic acids) in their composition that demonstrate gastroprotective and anti-ulcerative potential through in silico evaluation, indicating potential beneficial properties related to the consumption of these beverages - the properties of which will be investigated in future *in vivo* complementary studies.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Representative chromatogram of fermented orange beverage acquired at 280 nm; Figure S2: Representative chromatogram of *Matricaria recutita* L. extract acquired at 280 nm; Figure S3: Representative chromatogram of *Cymbopogon citratus* extract acquired at 280 nm; Figure S4: Representative chromatogram of *Mentha piperita* extract acquired at 280 nm nm; Figure S5: Ethics committee approval document.

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Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

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Table 1. Sensory evaluation through acceptance, consumption intention and preference tests.

Sensory Test	Parameter	Herbal Extract	Concentration of Herbal Extract mL.100 mL^{-1}				
			0.5	**1.0**	**2.0**	**3.0**	**4.0**
Acceptance test [X]	Color	MR	5.72 [a]	5.63 [a]	5.64 [a]	5.63 [a]	5.64 [a]
		CC	5.76 [a]	5.72 [a]	5.48 [a]	5.52 [a]	5.36 [a]
		MP	5.72 [a]	5,68 [a]	5.24 [a]	5.08 [a]	5.04 [a]
	Aroma	MR	5.24 [a]	5.16 [ab]	5.32 [a]	4.72 [ab]	4.36 [b]
		CC	5.08 [a]	5.16 [a]	5.28 [a]	5.20 [a]	4.92 [a]
		MP	5.04 [a]	5.08 [a]	5.36 [a]	5.32 [a]	5.16 [a]
	Flavor	MR	5.28 [a]	4.44 [ab]	4.88 [a]	4.36 [ab]	3.48 [b]
		CC	5.12 [ab]	5.40 [a]	4.88 [ab]	4.32 [ab]	4.08 [b]
		MP	5.12 [a]	4.72 [ab]	4.72 [ab]	3.84 [b]	3.92 [b]
Preference test [Y]		MR	88 [a]	88 [a]	73 [a]	69 [a]	57 [b]
		CC	83 [a]	82 [a]	82 [a]	73 [a]	59 [b]
		MP	92 [a]	90 [a]	77 [a]	57 [b]	59 [b]
Purchase intention [Z]		MR	2.56 [ab]	2.88 [ab]	2.24 [b]	3.20 [ab]	3.48 [a]
		CC	2.44 [a]	2.52 [a]	2.64 [a]	3.28 [a]	3.32 [a]
		MP	2.28 [b]	2.56 [b]	3.00 [ab]	3.60 [a]	3.60 [a]

[X] Different letters in the same line indicate means with significant differences ($p < 0.05$) by the Tukey test (n = 25). [Y] Values represent the sum of the orders according to Friedman's method and Newell and MacFarlane's table. Different letters in the same line indicate values with significant differences ($p < 0.05$) by the Tukey test (n = 25). [Z] MR = *Matricaria recutita* L.; CC = *Cymbopogon citratus*; MP = *Mentha piperita* L. Acceptance tests were assessed using a 7-point hedonic scale where 1= strongly dislike, 2 = disliked a lot, 3 = disliked a little, 4 = disliked, 5 = liked, 6 = liked a lot, and 7 = strongly like. The preference test assessed sample order from the least preferred to the most preferred, yielding higher values for the most preferred samples. The purchase intention test was assessed using a 5-point hedonic scale where 1 = would certainly buy and 5 = would certainly not buy.

Table 2. Physicochemical characterization, total phenolic and flavonoid content, and antioxidant capacity of the functional fermented orange beverage containing aromatic herbal extracts.

Parameters	Crude Extract			Fermented Orange Beverage			
	MR	CC	MP	Control	Fermented + MR	Fermented + CC	Fermented + MP
Physicochemical characterization							
Alcohol (%)	na	na	na	16.2 [a]	16.1 [a]	15.9 [a]	16.2 [a]
Total acidity (mEq L^{-1})	na	na	na	122.0 [a]	119.3 [a]	118.9 [a]	122.7 [a]
Volatile acidity (mEq L^{-1})	na	na	na	10.1 [a]	9.1 [a]	10.4 [a]	10.8 [a]
Fixed acidity (mEq L^{-1})	na	na	na	111.2 [a]	109.6 [a]	105.8 [a]	111.1 [a]
pH	na	na	na	3.8 [a]	3.8 [a]	3.8 [a]	3.8 [a]
Reducing sugars (g L^{-1})	na	na	na	3.3 [a]	3.2 [a]	3.3 [a]	3.2 [a]
Reduced dry extract (g L^{-1})	na	na	na	27.1 [a]	27.1 [a]	27.3 [a]	27.4 [a]
Total phenolic and flavonoid content and antioxidant capacity							
TPC (mg L^{-1} GAE)	1965.0 [b]	1193.3 [c]	2843.3 [a]	459.4 [c]	481.9 [b]	474.3 [b]	495.2 [a]
TFC (mg L^{-1} CAE)	347.2 [b]	216.2 [c]	1247.0 [a]	32.1 [d]	41.4 [b]	35.9 [c]	64.4 [a]
DPPH (IC_{50})	119.7 [b]	223.6 [a]	44.3 [c]	26.9 [b]	28.0 [a]	25.2 [c]	22.4 [d]
ORAC (μmol L^{-1} TE)	2.5 [a]	2.6 [a]	2.6 [a]	1.6 [b]	1.8 [b]	1.9 [b]	2.2 [a]

MR = *Matricaria recutita* L. (chamomile); CC = *Cymbopogon citratus* (lemongrass); MP = *Mentha piperita* L. (peppermint); na = not analyzed; mEq = milliequivalent; TPC = total phenolic content; GAE = gallic acid equivalent; TFC = total flavonoid content; CAE = catechin equivalent; DPPH = 2,2-diphenyl-1-picril-hydrazine; IC_{50} = sample volume (μL) to remove 50% of DPPH; ORAC = oxygen radical absorbance capacity; TE = Trolox equivalent. Different lower-case letters, in extract or in fermented beverage, indicate means with significant differences ($p < 0.05$) by the Tukey test.

Table 3. Phenolic composition of the functional fermented orange beverage containing aromatic herbal extracts.

Parameters	RT (min)	Crude Extract			Fermented Orange Beverage			
		MR	CC	MP	Control	Fermented + MR	Fermented + CC	Fermented + MP
Phenolic acids								
Chlorogenic acid (mg L^{-1})	15.1	49.1 ^{a}	8.4 ^{c}	47.4 ^{b}	10.9 ^{c}	16.0 ^{a}	13.8 ^{b}	10.8 ^{c}
Caffeic acid (mg L^{-1})	16.5	nd	3.5	nd	5.3 ^{a}	5.5 ^{a}	5.3 ^{a}	5.3 ^{a}
Ferulic acid (mg L^{-1})	23.2	95.2 ^{b}	nd	98.7 ^{a}	1.6 ^{ab}	1.7 ^{a}	1.0 ^{c}	1.1 ^{bc}
Flavonoids								
Narirutin (mg L^{-1})	26.1	nd	nd	nd	69.7 ^{a}	70.6 ^{a}	66.5 ^{b}	69.6 ^{a}
Rutin (mg L^{-1})	27.1	nd	nd	nd	6.1	Nd	nd	nd
Hesperidin (mg L^{-1})	28.1	nd	nd	nd	130.3 ^{a}	129.9 ^{a}	124.3 ^{b}	125.1 ^{b}
Luteolin (mg L^{-1})	37.3	20.1 ^{a}	3.1 ^{c}	17.4 ^{b}	nd	Nd	nd	nd
Apigenin (mg L^{-1})	39.2	138.4 ^{a}	nd	132.4 ^{a}	nd	Nd	nd	nd

MR = *Matricaria recutita* L. (chamomile); CC = *Cymbopogon citratus* (lemongrass); MP = *Mentha piperita* L. (peppermint); nd = not detected. Different letters, in extract or in fermented beverage, indicate means with significant differences ($p < 0.05$) by the Tukey test.

Table 4. *In silico* prediction of toxicity and biological activity based on the major phenolic compounds present in fermented orange beverage.

	In Silico	**Chlorogenic Acid**	**Caffeic Acid**	**Ferulic Acid**	**Narirutin**	**Rutin**	**Hesperidin**
Prediction of toxicity	Mutagenic (AMES toxicity)	(-)pkCSM (-)admetSAR (-)Protox (-)Lazar	(-)pkCSM (-)admetSAR (-)Protox (-)Lazar	(-)pkCSM (-)admetSAR (-)Protox (-)Lazar	(-)pkCSM (-)admetSAR (-)Protox (-)Lazar	(-)pkCSM (-)admetSAR (-)Protox	(-)pkCSM (-)admetSAR (-)Protox
	Carcinogenic	(-)admetSAR (-)Protox (-)Lazar	(-)admetSAR (-)Lazar	(-)admetSAR (-)Protox (-)Lazar	(-)pkCSM (-)admetSAR (-)Protox	(-)pkCSM (-)admetSAR (-)Protox	(-) pkCSM (-)admetSAR (-)Protox
	Hepatotoxicity	(-)pkCSM	(-)pkCSM (-)Protox	(-)pkCSM (-) Protox	(-)pkCSM (-) Protox	(-)pkCSM (-) Protox	(-)pkCSM (-) Protox
	Blood–brain barrier penetration	(-)pkCSM (-)Lazar	(-)pkCSM (-)admetSAR (-)Lazar	(-)pkCSM (-)admetSAR (-)Lazar	(-)pkCSM (-)admetSAR (-)Lazar	(-)pkCSM (-)admetSAR (-)Lazar	(-)pkCSM (-)admetSAR (-)Lazar
	Acute oral toxicity	* III admetSAR	* IV admetSAR	* IV admetSAR	* III admetSAR	* III admetSAR	* III admetSAR
Prediction of biological activity	Antihemorrhagic	Pa PASS (16.4%)	n.r.	n.r.	Pa PASS (81.5%)	Pa PASS (90.4%)	Pa PASS (72.7%)
	Antioxidant	Pa PASS (80.9%)	Pa PASS (61.1%)	Pa PASS (54.7%)	Pa PASS (88.0%)	Pa PASS (92.7%)	Pa PASS (85.3%)
	Free radical scavenging	Pa PASS (85.6%)	Pa PASS (67.0%)	Pa PASS (74.1%)	Pa PASS (98.2%)	Pa PASS (99.0%)	Pa PASS (99.1%)
	Anti-inflammatory	Pa PASS (65.7%)	Pa PASS (64.8%)	Pa PASS (66.1%)	Pa PASS (71.6%)	Pa PASS (74.6%)	Pa PASS (70.4%)
	Antiulcerative	Pa PASS (54.2%)	Pa PASS (61.0%)	Pa PASS (60.4%)	Pa PASS (71.6%)	Pa PASS (58.5%)	Pa PASS (70,9%)
	Gastritis treatment	Pa PASS (27.1%)	Pa PASS (35.5%)	Pa PASS (38.4%)	Pa PASS (35.5%)	Pa PASS (49.6%)	Pa PASS (39,9%)
	Mucomembranous protection	Pa PASS (75.2%)	Pa PASS (94.5%)	Pa PASS (90.6%)	n.r.	n.r.	n.r.
	Vasoprotection	Pa PASS (44.2%)	Pa PASS (78.2%)	Pa PASS (75.3%)	PaPASS (97.0%)	Pa PASS (98.0%)	Pa PASS (97.4%)

* LD50 compounds are classified into four categories based on US EPA. (Category I: compounds with LD50 values ≤ 50 mg kg−1. Category II: compounds with LD50 values > 50mg kg−1 and < 500mg kg−1. Category III: compounds with LD50 values ≥ 500 mg kg−1 and < 5000 mg kg−1. Category IV: compounds with LD50 values ≥ 5000 mg kg−1 and < 5000 mg kg−1.) (+) = at risk; (-) = not at risk; (n.r.) = not reported; Pa = probably active; Pi = probably inactive.

SUPPLEMENTARY MATERIAL

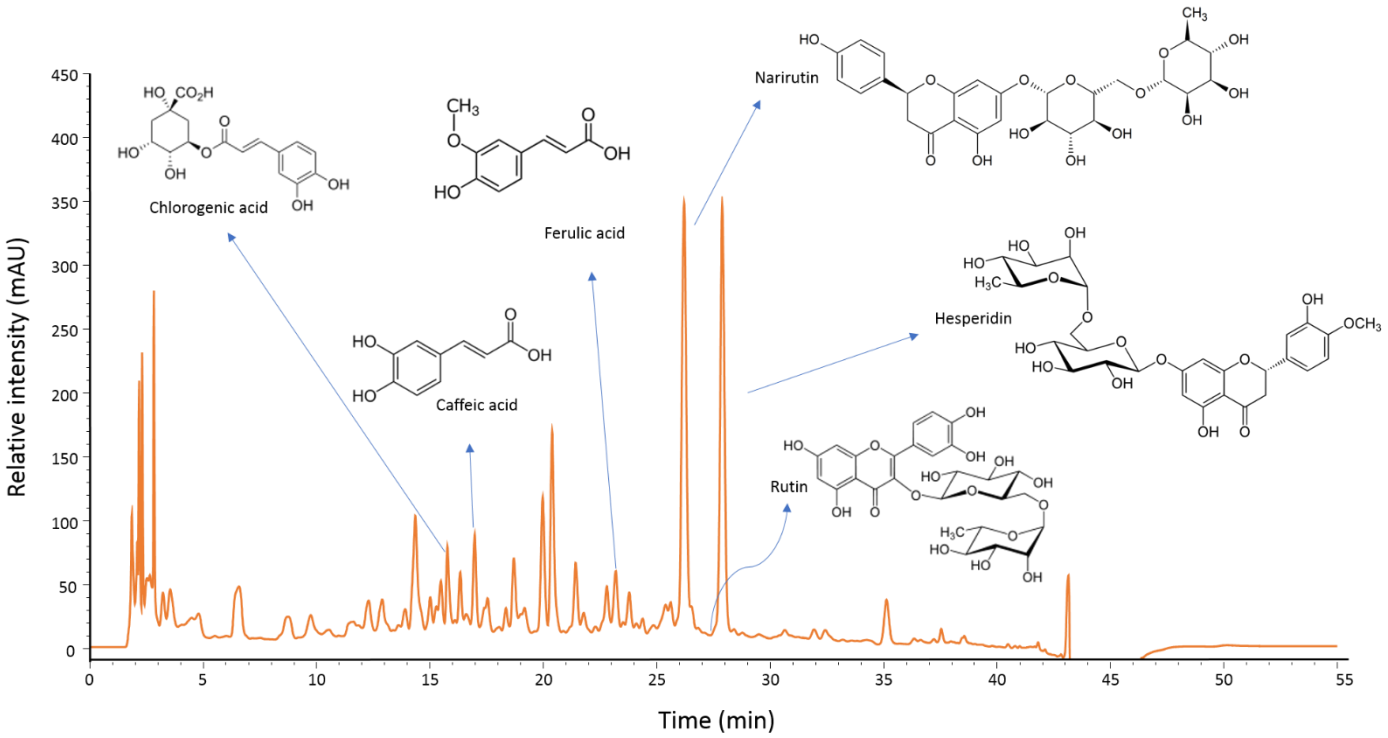


Figure S1. Representative chromatogram of fermented orange beverage acquired at 280 nm

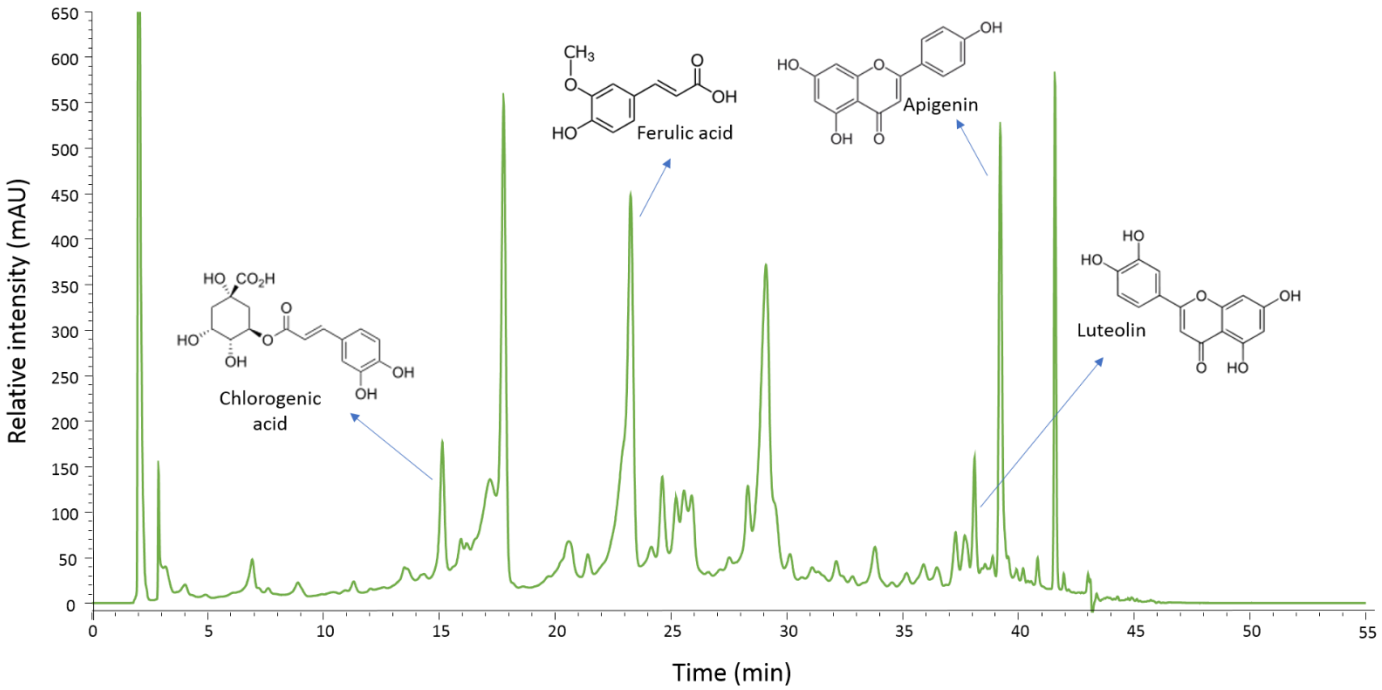


Figure S2. Representative chromatogram of *Matricaria recutita* L extract acquired at 280 nm

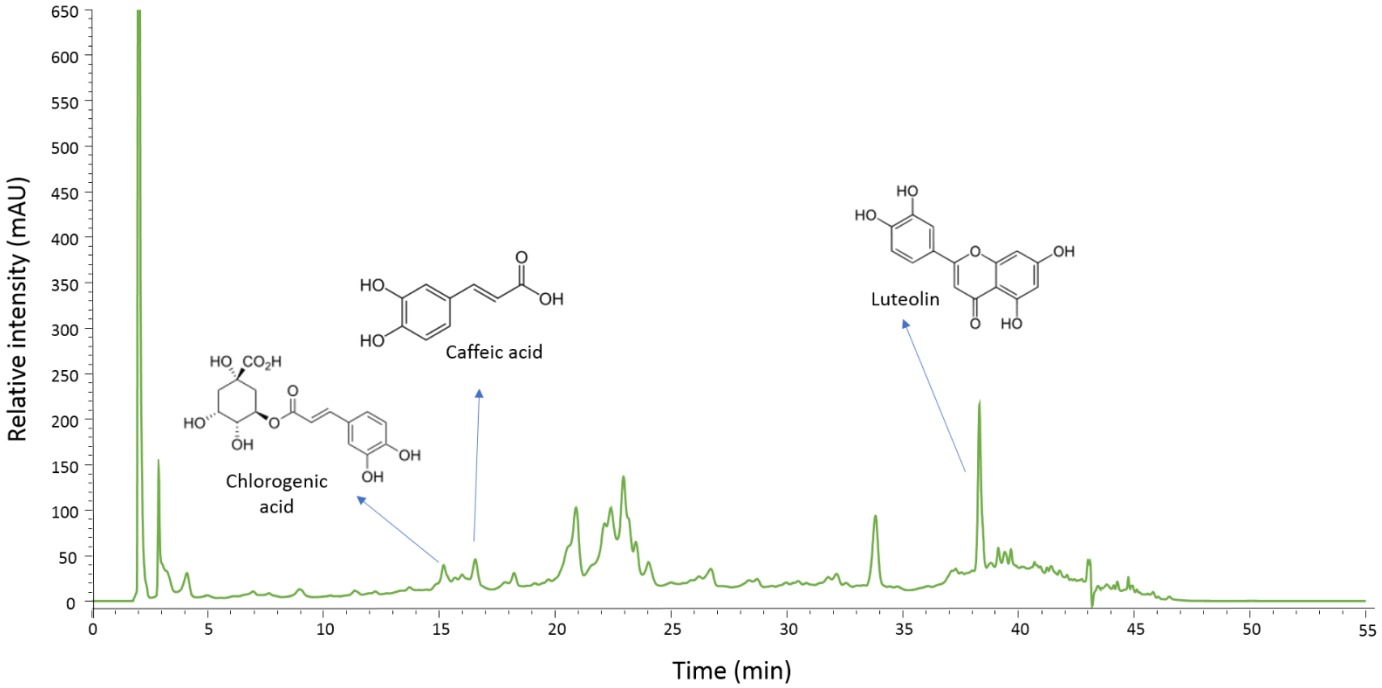


Figure S3. Representative chromatogram of *Cymbopogon citratus* extract acquired at 280 nm

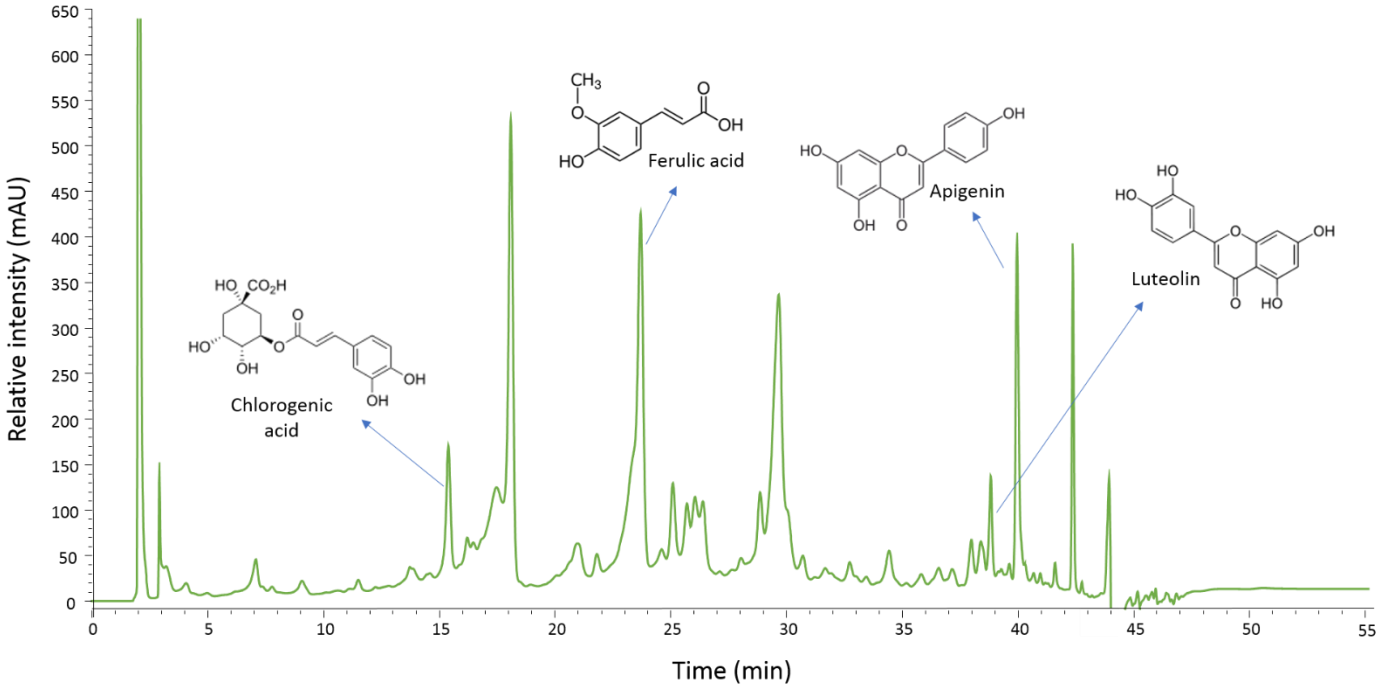
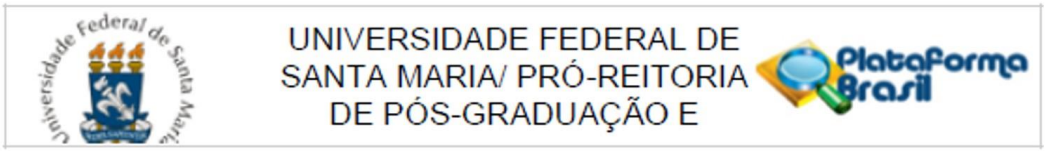


Figure S4. Representative chromatogram of *Mentha piperita* extract acquired at 280 nm



PARECER CONSUBSTANCIADO DO CEP

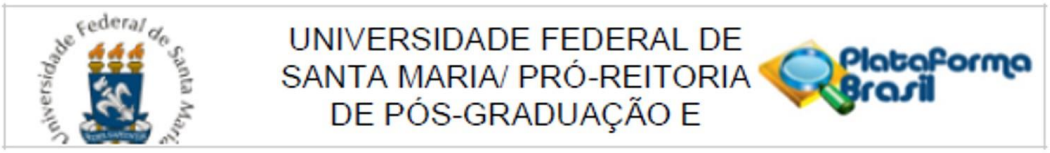
DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Fermentado de laranja composto de extrato de ervas aromáticas: desenvolvimento, caracterização e avaliação do potencial antioxidante e gastroprotetor.

Pesquisador: CLÁUDIA KAEHLER SAUTTER
Área Temática:
Versão: 1
CAAE: 10027519.3.0000.5346
Instituição Proponente: Universidade Federal de Santa Maria
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.257.616



Continuação do Parecer: 3.257.616

Investigador	Projeto_Sensorial.pdf	28/02/2019 11:33:09	CLÁUDIA KAEHLER SAUTTER	Aceito
Folha de Rosto	Folha_de_Rosto.pdf	28/02/2019 11:25:34	CLÁUDIA KAEHLER SAUTTER	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SANTA MARIA, 10 de Abril de 2019

Assinado por:
CLAUDEMIR DE QUADROS
(Coordenador(a))

Figure S5. Sensory evaluation approved by the Institutional Ethics Committee of the Federal University of Santa Maria (CEP/UFSM), CAAE number:10027519.3.0000.5346, nº 3.257.616.

4.2 ARTIGO 2:

Evaluation of the gastro repair potential of functional fermented orange beverage against ethanol-induced gastric ulcer in rats[2]

[2]Esse artigo foi submetido para a Revista Ciência Rural e está formatado conforme as normas da revista. Área de avaliação em Ciência de Alimentos A4.

Evaluation of the gastro repair potential of functional fermented orange beverage against ethanol-induced gastric ulcer in rats

Avaliação do potencial gastroreparador da bebida funcional fermentada de laranja contra úlcera gástrica induzida por etanol em ratos

Laura Gizele Mascarin[3], Fernanda Wouters Franco[1], Fernanda Ziegler Reginato[4], James Ramires Penteado Graiczik[2], William Nieckel Pereira[1], João Osmar Fruet da Silva[5], Jean Ramos Boldori[6], Silvio Teixeira da Costa[3], Cristiane Casagrande Denardin[4], Liliane de Freitas Bauermann[2], Sabrina Somacal[1], Jaime Sardá Aramburú Junior[5], Cláudia Kaehler Sautter[1*]

ABSTRACT

Previous investigations have revealed that a functional fermented orange beverage presented in its composition different phenolic compounds, which through *in silico* investigation demonstrated to have biological effects of therapeutic importance as antioxidant, gastro repair, and anti-ulcerative properties. Thus, the aim of this study was to confirm *in vivo*, through a model of ethanol (EtOH)-induced gastric ulcers in rats, the beneficial properties indicated by the *in silico* tests. Gastric ulcer was induced by EtOH (intragastric) and was treated after 1 h with fermented orange beverage with and without *Mentha piperita* extract (0.5 mL 100 g^{-1} $_{w.b}$).

[3] *Federal University of Santa Maria (UFSM), Department of Food Technology and Science, Center of Rural Sciences, Building 42, CEP 97105-900, Telephone +55 (55) 3220-8254, Santa Maria, RS, Brazil. e-mail address: claudia.sautter@ufsm.br (corresponding author).
[4] Federal University of Santa Maria (UFSM), Department of Physiology and Pharmacology, Center of Health Sciences, CEP 97105-900, Santa Maria, RS, Brazil.
[5] Federal University of Santa Maria (UFSM), Department of Morphology, Center of Health Sciences, CEP 97105-900, Santa Maria, RS, Brazil.
[6] Federal University of Pampa (UNIPAMPA), BR 472, Km 585, CEP 97501-970, Uruguaiana, RS, Brazil.
[5] Federal University of Santa Maria (UFSM), Pro-Rectory of Graduate Studies and Research, CEP 97105-900, Santa Maria, RS, Brazil.

Omeprazole was used as positive control. Histopathological evaluation revealed that EtOH administration resulted in the formation of gastric ulcers due to the reduction of the mucus layer, presence of hemorrhage, and infiltration of neutrophils in the stomach tissue of rats, and only treatment with omeprazole was able to reverse these changes. Additionally, EtOH administration altered the gastric juice volume and induced oxidative stress in the gastric tissue observed through the increase in lipid peroxidation (TBARS), reduction in the levels of non-protein thiols (NPSH), and alteration in the superoxide dismutase (SOD) activity. The ingestion of the fermented orange beverage increased NPSH levels and reduced changes in TBARS levels induced by ethanol. These findings suggest that the fermented orange beverage has antioxidant effects, as pointed out by *in silico* studies, but not gastro repair and antiulcerative effects.

Key words: gastric ulcer, oxidative stress, antioxidants defenses, polyphenols, functional beverages.

RESUMO

Investigações anteriores revelaram que uma bebida funcional fermentada de laranja apresentou em sua composição diferentes compostos fenólicos, que através de investigações *in silico* demonstraram ter efeitos biológicos de importância terapêutica como propriedades antioxidantes, gastro reparadoras e antiulcerativas. Assim, o objetivo deste estudo foi confirmar *in vivo,* através de um modelo de úlcera gástrica induzida por EtOH em ratos, as propriedades benéficas indicadas pelos ensaios *in silico*. A úlcera gástrica foi induzida por EtOH (intragástrico) e tratada após 1 h com bebida fermentada de laranja com e sem o extrato de *Mentha piperita* (0,5 mL 100 g^{-1} peso corporal). Omeprazol foi usado como controle positivo. A avaliação histopatológica revelou que a administração de EtOH resultou na formação de úlceras gástricas decorrentes da redução da camada de muco, presença de hemorragia e infiltração de neutrófilos no tecido estomacal de ratos, sendo apenas o tratamento com

omeprazol capaz de reverter essas alterações. Além disso, a administração de EtOH alterou o volume do suco gástrico e induziu estresse oxidativo no tecido estomacal observado através do aumento da peroxidação lipídica (TBARS), redução dos níveis de tióis não-proteicos (SHNP) e alteração da atividade da superóxido dismutase (SOD). A ingestão da bebida fermentada de laranja aumentou os níveis de SHNP e reduziu as alterações nos níveis de TBARS induzidos pelo EtOH. Esses achados sugerem que a bebida fermentada de laranja apresenta efeitos antioxidantes, conforme apontado por estudos *in sílico*, mas não efeitos gastroreparadores e antiulcerativos.

Palavras-chave: úlcera gástrica, estresse oxidativo, defesas antioxidantes, polifenóis, bebidas funcionais.

INTRODUCTION

Wine is one of the most consumed and preferred alcoholic beverages due to its organoleptic and health-promoting properties, and recently has been identified as a functional beverage (RADONJIĆ et al., 2020). In addition to the grape, this beverage can be produced from any sugary fruit as long as it is designated as fermented fruit (UMEH; UDEMEZUE, 2015). In the last decade, several studies have reported the production of fruit wine and the possible therapeutic properties (ESCUDERO-LÓPEZ et al., 2015; GONÇALVES et al., 2022; SHIRADHONKAR et al., 2014; UMEH; UDEMEZUE, 2015).

Brazil is considered the largest producer of oranges (FAO, 2021) and its different fruiting periods enable various harvests, thus avoiding the concentration of crops and reducing production costs throughout the year (PASSOS et al., 2018). Oranges are an abundant source of vitamin C and have considerable amounts of sugar, minerals, and bioactive compounds such as carotenoids, flavonoids, and essential oils (FERRARA; DE CANDIA; FERRANTI, 2023). Thus, their use as raw materials to produce alcoholic beverages is an attractive option to add

value to the product, diversify the market, and a solution to minimize the losses of these fruits in the fields or during transportation (FERRARA; DE CANDIA; FERRANTI, 2023).

Excessive consumption of alcoholic beverages contributes to increased superoxide anions, hydroxyl radicals, and lipid peroxidation in the gastric mucosa, inducing intracellular oxidative stress that leads to the development of ulcerative gastric lesions (MOUSAVI et al., 2020). Gastric ulcer is a chronic disease characterized by an imbalance between protective agents, such as mucus, prostaglandins, bicarbonate and nitric oxide, and aggressive agents, such as pepsin and hydrochloric acid (HCl), in the gastric mucosa (BEIRANVAND, 2022; SHAKER; MAHMOUD; MNAA, 2010).

On the other hand, several studies have shown that moderate consumption, especially of wines, may have positive effects on health (KANG et al., 2023). A study carried out with healthy mice showed that the ingestion of an alcoholic fermented orange beverage has greater protection against cardiovascular risk factors than orange juice (ESCUDERO-LÓPEZ et al., 2015). Another study reported that the regular consumption of low alcohol content orange beverages reversed metabolic parameters and modulated inflammatory response, lipid profile, and oxidative stress in rodents (ESCUDERO-LÓPEZ et al., 2016). These beneficial effects are related to the presence of phenolic compounds in these beverages and the mechanisms responsible for the potential health benefits of polyphenols are complex and generally attributed to their ability to directly eliminate free radicals and more recently to the upregulation of endogenous antioxidant defenses (MITHUL ARAVIND et al., 2021).

The synthetic drugs are used in the clinical treatment of gastric ulcers pose serious complications after prolonged use (FREEDBERG; KIM; YANG, 2017). Therefore, the discovery of natural compounds or nutritional strategies that can attenuate or prevent gastric ulcers are particularly relevant and of great interest to the food and pharmaceutical industry. In a recent study, we showed that functional fermented orange beverages have phenolic

compounds such as hesperidin, narirutin, and chlorogenic, caffeic, and ferulic acids in their composition (MASCARIN et al., 2023). These compounds through *in silico* studies have shown potential for biological effects of therapeutic importance, such as antioxidant, gastro repair, and antiulcerative properties, being the *Mentha piperita* (peppermint) extract presented the greatest potential among the evaluated herbs for use in functional fermented beverages (MASCARIN et al., 2023).

Plant extracts have shown promising results in the treatment of ulcers by not only providing gastric protection but also gastric healing due to their antioxidant pharmacological properties (BEZERRA et al., 2009; FRANCISCO; FIGUEIRINHA; COSTA, 2014). The herb *Mentha piperita* L. has anti-inflammatory, antioxidant, and gastroprotective activity that is widely reported in the literature (GOIS et al., 2016; GUL; ABBAS; QADIR, 2015; URIBE et al., 2015). Also, their volatile aromatic profile allows them to be potentially included in beverages (SHIRADHONKAR et al., 2014), generating a differentiated product.

In this context, this study aimed to evaluate the gastro repair, antiulcerative, and antioxidant effect of different functional fermented orange beverage (with or without addition of *M. piperita* extract) in a model of EtOH-induced gastric ulcer in rats, aiming to confirm *in vivo* the beneficial properties previously indicated by the *in silico* tests.

MATERIALS AND METHODS

2.1. Fermented orange beverage production with the addition of aromatic herbal extracts

The fermented orange beverage was produced according to Mascarin et al. (2023). Oranges of the cultivar ‘Valencia’ (2018 harvest) were purchased in the city of Mata, southern Brazil, and the juice was extracted, filtered to remove the solid residue, and a yield of 39% was obtained. The must was prepared for fermentation by adding 6% sodium metabisulfite (70 ppm) and left to rest for 60 min. Then, 230 g L^{-1} of sugar (26 $^{\circ}$Brix) was added, homogenized, and

the pectinolytic enzymes (Lafazin Extract®, 3 g hL^{-1}; NutriStart®, 40 g hL^{-1}) were added. The yeast Saccharomyces cerevisiae (Blastosel Delta®, 40 g hL^{-1}) was inoculated and the alcoholic fermentation was carried out in 20 L polyethylene kegs at a controlled temperature (16 ± 2 ºC) and with decreased and stabilized total soluble solids (TSS) to determine the endpoint of fermentation.

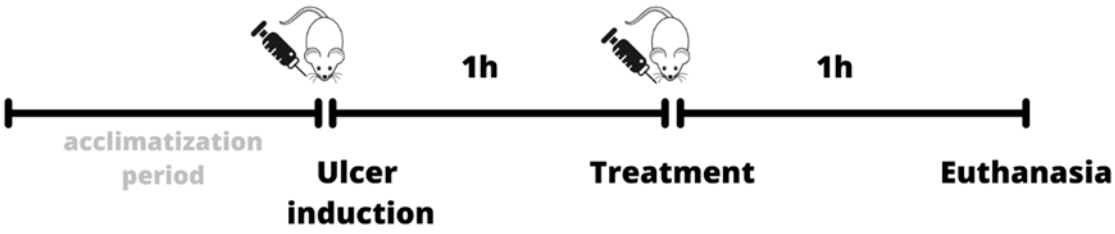
After fermentation, the temperature was maintained at 5 °C for 48 h to separate the yeasts and other solids. The must was racked, filtered, and 50 ppm of 6% sodium metabisulfite was added and kept stabilized for three months with a controlled temperature of 16 ± 2 °C.

The aromatic herb - *Mentha piperita* L. (leaves) - was purchased locally and in dry form, which is suitable for making teas. The hydro-alcoholic extract of aromatic herb (50% EtOH/H_2O) was prepared from equal volumes of 96 °GL cereal alcohol and distilled water. Then, 100 g of dry matter from each plant was weighed and placed in maceration in 1 L of 50% hydroalcoholic solution, which was the volume necessary to fully cover the dry matter, resulting in an extract concentration of 10 g% (SHIRADHONKAR et al., 2014). The herb was kept in infusion in dark bottles at room temperature for 14 days to optimize the extraction of compounds. Afterward, the aromatic herbal extract was added to the fermented beverage (2.0%, v/v). The mixture remained stabilized for 2 months, in the dark, and at a controlled temperature of 16 ± 2 °C (SHIRADHONKAR et al., 2014). The physical-chemical characterization of the beverages is available in Mascarin et al. (2023).

2.2. Animals and induction of gastric lesions by EtOH

Adult male Wistar rats were provided by the Central Animal House of the Federal University of Santa Maria and all procedures adopted were approved by the institutional Animal Ethics Committee (protocol nº 5178300819). Animals were housed in standard polypropylene cages (four rats/cage) and kept under controlled temperature (22 ± 2°C) and humidity (55 ± 5%)

at a 12/12 h light/dark cycle with access to food and water *ad libitum*. Animals were divided into seven experimental groups (Figure 1). The control groups contained 4 animals per group (reduced number according to the 3Rs practice) and the other groups contained 6 animals each.



Group	Ulcer induction	Treatment
Control	Received water (0.5 mL/100 g b.w.)	Received water (0.5 mL/100 g b.w.)
EtOH	Received ethanol (0.5 mL/100 g b.w.)	Received water (0.5 mL/100 g b.w.)
FOB	Received water (0.5 mL/100 g b.w.)	Received fermented orange beverage (FOB) (0.5 mL/100 g b.w.)
EtOH-FOB	Received ethanol (0.5 mL/100 g b.w.)	Received fermented orange beverage (FOB) (0.5 mL/100 g b.w.)
FOB-MP	Received water (0.5 mL/100 g b.w.)	Received FOB with *Mentha piperita* (MP) (0.5 mL/100 g b.w.)
EtOH-FOB-MP	Received ethanol (0.5 mL/100 g b.w.)	Received FOB with *Mentha piperita* (MP) (0.5 mL/100 g b.w.)
EtOH-OMEP	Received ethanol (0.5 mL/100 g b.w.)	Received omeprazole (30 mg/kg b.w.)

Figure 1 – Experimental design.

The dose of 0.5 mL 100 g^{-1} $_{b.w.}$ of fermented orange beverage was established based on the international recommendations which establishes the maximum volume administered by

gavage in rats as being 1% of body weight, that is, 1 mL for every 100 g^{-1} $_{b.w.}$ (TURNER et al., 2011). Thus, in the present investigations, the animals received 0.5 mL 100 g^{-1} $_{b.w.}$ of liquid (EtOH or water) in the gastric ulcer induction stage + 0.5 mL 100 g^{-1} $_{b.w.}$ in the treatment stage. Omeprazol (OMEP) was used as a positive control as it is a standard drug used for the clinical treatment of gastric ulcers.

After 7 days of acclimatization, rats were fasted for 12 h to ensure an empty stomach and efficient induction of gastric lesions. Gastric lesions were induced by an oral administration of 75% EtOH (0.5 mL 100 g^{-1} $_{b.w.}$) (BOLIGON et al., 2014). One hour after EtOH administration, the animals received the treatments [water, fermented orange beverage (FOB), fermented orange beverage with *Mentha piperita* (FOB-MP), or omeprazole (OMEP)] by gavage. After another 1 h, animals were anesthetized with isoflurane and euthanized by cardiac puncture (Figure 1). Blood samples were collected for determination of serum glucose and the stomach of each rat was quickly removed for determination of gastric juice/secretion volume. The weight of the stomach (without gastric juice) and of the liver were determined. After, each stomach was dichotomized, one part was immersed in 10% formalin solution for histopathological analyses, whereas the other part was homogenized in 0.01 M phosphate buffered saline with 0.136 M NaCl (pH 7.4) on ice using an Polytron mixer (Kinematica AG, Switzerland). One part of the homogenate was used to assess the levels of non-protein thiol groups (NPSH) and thiobarbituric acid-reactive substances (TBARS). Another part of the homogenate was centrifuged at 3500 rpm for 10 min to yield supernatant that was used to determine antioxidant enzyme activities and protein levels.

2.2.1. Histological analysis

Samples of stomach tissue fixed in 10% buffered formalin solution were processed and paraffin embedded. The histological sections were processed by a Thermo Scientific™ HM 325

rotary microtome with a thickness of 5 µm, in order to enable the production of two slides per section, which were later stained with Goldner-Masson trichrome. Each microscopic slide was evaluated in 10 histological fields using an Axio Scope A1 microscope coupled to an Axiocam 105 color camera (ZEISS®, Germany) and directly linked to software, which allows recording high-quality photos and recording them in sequence. Through the ImageJ program, random measurements were established throughout the entire sample in order to measure the thickness of the mucous, integrity of the gastric mucosa layer, hemorrhage, as well as neutrophil infiltration.

2.2.2. Markers of oxidative stress

In the homogenate of the stomach tissue, after the addition of 7.2 mM butylated hydroxytoluene (BHT) to prevent further oxidation, lipid peroxidation was estimated by the measurement of TBARS using a standard curve of 1,1,3,3-tetraethoxypropane (OHKAWA; OHISHI; YAGI, 1979). To the determination of NPSH levels, the homogenate fraction was deproteinized with 10% trichloroacetic acid (1:1 v/v), and NPSH content were determined as described by Ellman (ELLMAN, 1959) using a standard curve of cysteine.

2.2.3. Antioxidant defense system

Superoxide dismutase (SOD) activity was determined based on its ability to inhibit the auto-oxidation of epinephrine to adrenochrome at an alkaline pH (MISRA; FRIDOVICH, 1972) and catalase (CAT) activity was determined using hydrogen peroxide (H_2O_2) as substrate (AEBI, 1984). The pseudo-first order reaction constant (k) of the decrease in H_2O_2 absorption at 25°C was determined, and the activity was expressed as k ug^{-1} protein.

2.2.4. Protein determination

Protein was measured in homogenate and supernatant for the normalization of oxidative status analyses using bovine serum albumin as the standard (LOWRY et al., 1951).

2.3. Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Duncan's post hoc test when appropriate. Data that did not meet the ANOVA assumptions were analyzed by Kruskal Wallis analysis, followed by multiple comparison tests. Results were expressed as the mean ± SEM and differences were considered statistically significant when $p \leq 0.05$. Data were analyzed using the Statistica® V.7 software system (Statsoft Inc., 2004).

RESULTS AND DISCUSSION

In the present study, we investigated the gastro repair effects of a functional fermented orange beverages with or without addition of *M. piperita* extract in a model of gastric ulcers induced by EtOH in rats, aiming to confirm in vivo our previous findings that indicated beneficial properties through *in silico* tests (MASCARIN et al., 2023).

3.1. Body weight, organ weight, and serum and stomach parameters

No differences were found among the experimental groups with respect to body weight (Figure 2A; $p \leq 0.05$). Acute administration of ethanol or the different treatments did not change the weight of the liver or the stomach, nor did it alter the levels of serum glucose (Figure 2B – D, respectively; $p \leq 0.05$). Similarly, Da Luz and collaborators also did not observe changes in body weight and organ weight of animals that received ethanol and/or plant extracts rich in phenolic compounds (DA LUZ et al., 2021).

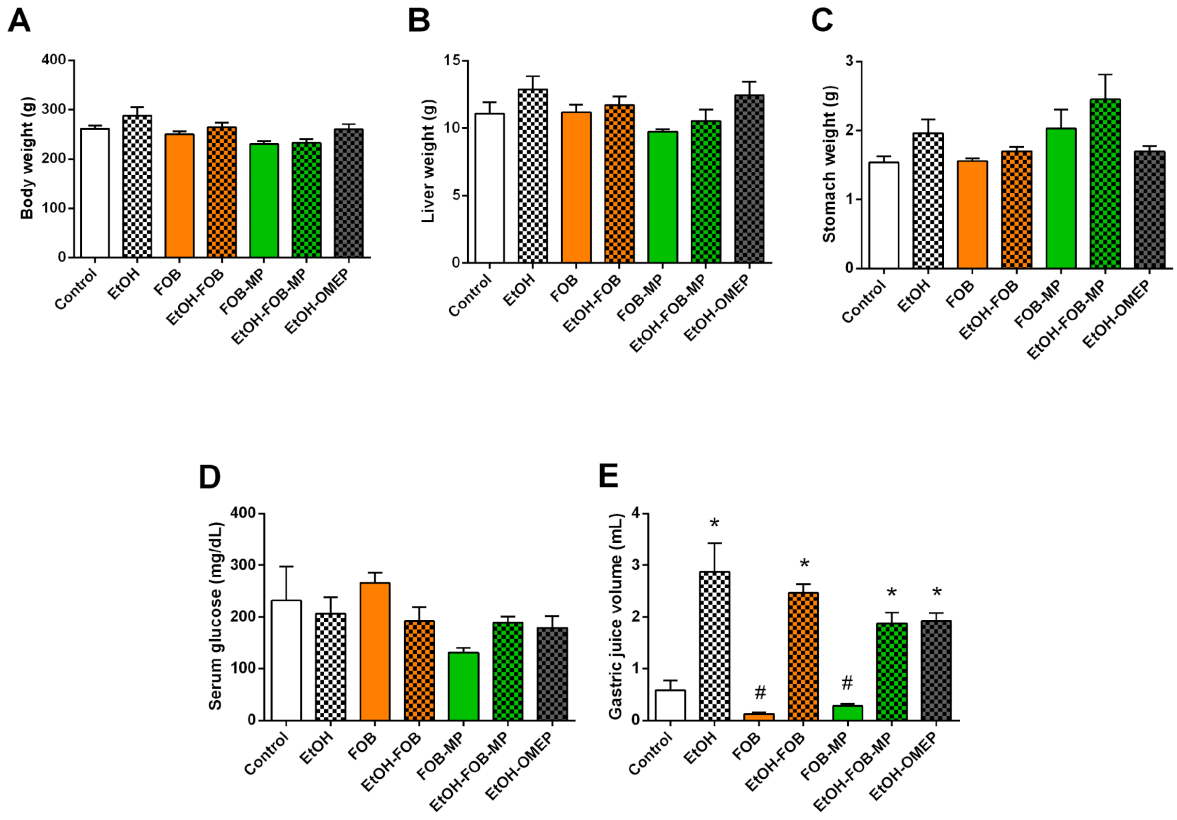


Figure 2 – Body, serum and gastric parameters of rats with gastric ulcer induced by ethanol and treated with water, FOB, FOB-MP, and omeprazole. Data are shown as the means ± S.E.M. Tested by ANOVA followed by post hoc Duncan's test. *Different from the control group ($p \leq 0.05$). #Different from EtOH group ($p \leq 0.05$). Control group: without gastric ulcer; EtOH group: with gastric ulcer; FOB group: without gastric ulcer and treated with fermented orange beverage; EtOH-FOB: with gastric ulcer and treated with fermented orange beverage; FOB-MP: without gastric ulcer and treated with fermented orange beverage with Mentha piperita extract; EtOH-FOB-MP: with gastric ulcer and treated with fermented orange beverage with Mentha piperita extract; EtOH-OMEP: with gastric ulcer and treated with omeprazole.

On the other hand, the induction of gastric ulcer through the administration of ethanol induced an increase in gastric juice volume (Figure 2E; $p \leq 0.05$). This increase in the volume of gastric juice was already expected, since it is already well known that in the stomach, alcohol can promote damage to the mucosa, influencing greater secretion of acid (BEIRANVAND, 2022; HUNT et al., 2015).The FOB and FOB-MP treatment in rats with gastric ulcer does not change the volume of gastric juice when compared to the EtOH group (Figure 2E; $p \leq 0.05$). It has already been reported that the beneficial activity of certain medicinal plants in the treatment

of gastric ulcer was not related to anti-secretory mechanisms (LI et al., 2008; NAGULSAMY; PONNUSAMY; THANGARAJ, 2015; SCHUBERT; PEURA, 2008). Although the current therapy for the treatment of gastric ulcers consists of the use of drugs with anti-secretory activity, this strategy can cause hypergastrinemia in chronic users, because once treatment is interrupted, hypergastrinemia is responsible for a rebound effect on gastric secretion. gastric acid (BEIRANVAND, 2022; HUNT et al., 2015). Thus, new drugs or therapeutic strategies with no anti-secretory action are desirable, as they prevent microbial growth and hypergastrinemia in patients who use it chronically.

3.2. Effect of fermented orange beverage on EtOH-induced gastric damage

EtOH can be used to induce experimental gastritis and gastric ulceration (BEIRANVAND, 2022; HUNT et al., 2015). EtOH is known to penetrate the gastric mucosa, causing membrane damage, erosion of gastric cells, impairment in proton (H^+) pumping into the gastric lumen, features most likely to precede cell death in gastric mucosal cells (BEIRANVAND, 2022). In this study, the intragastric administration of EtOH induced the formation of gastric ulcer evidenced by changes in the thickness of the mucus layer, highlighting that ulcerative lesions reduce the protective layer of mucus (Figure 3). Farther, histopathological evaluations revealed erosion of gastric mucosal epithelium, hemorrhage, and inflammatory infiltration of neutrophils (EtOH group, Table 1), which is in accordance with previous reports of this model (BOLIGON et al., 2014; DA SILVA et al., 2020). EtOH increases vascular permeability and exposes gastric mucosa to the proteolytic and hydrolytic actions of pepsin and HCl, besides it causes blood flow stasis and leading to vascular damage and necrosis (ADINORTEY et al., 2013).

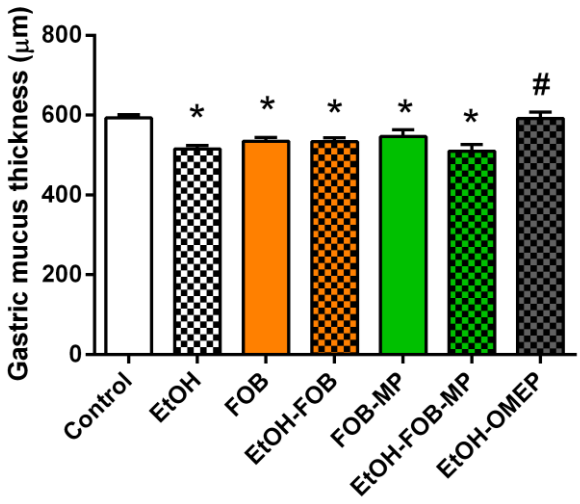


Figure 3 – Evaluation of the mucus layer in the stomach of rats with gastric ulcer induced by ethanol and treated with water, FOB, FOB-MP, or omeprazole. Data are shown as the means ± S.E.M. Tested by ANOVA followed by post hoc Duncan's test. *Different from the control group ($p \leq 0.05$). #Different from EtOH group ($p \leq 0.05$). Control group: without gastric ulcer; EtOH group: with gastric ulcer; FOB group: without gastric ulcer and treated with fermented orange beverage; EtOH-FOB: with gastric ulcer and treated with fermented orange beverage; FOB-MP: without gastric ulcer and treated with fermented orange beverage with Mentha piperita extract; EtOH-FOB-MP: with gastric ulcer and treated with fermented orange beverage with Mentha piperita extract; EtOH-OMEP: with gastric ulcer and treated with omeprazole.

Table 1 – Histopathological indexes of stomach from rats treated with ethanol and/or fermented orange beverages or omeprazole.

	Hemorrhage	Erosion of gastric mucosal epithelium	Neutrophil infiltrate
Control	-	†	-
EtOH	††	††	††
FOB	††	††	†
EtOH-FOB	††	††	††
FOB-MP	††	††	†
EtOH-FOB-MP	††	††	†
EtOH-OMEP	†	†	†

Gastric tissue with no negative features was given a score of 0 (-). Gastric tissue with mild histopathological damage was given a score of †. Those with moderate and severe negative features were given a score of †† and †††, respectively. Results were expressed as a histopathological score. Control group: without gastric ulcer; EtOH group: with gastric ulcer; FOB group: without gastric ulcer and treated with fermented orange beverage; EtOH-FOB:

with gastric ulcer and treated with fermented orange beverage; FOB-MP: without gastric ulcer and treated with fermented orange beverage with *Mentha piperita* extract; EtOH-FOB-MP: with gastric ulcer and treated with fermented orange beverage with *Mentha piperita* extract; EtOH-OMEP: with gastric ulcer and treated with omeprazole.

Treatment with FOB or FOB-MP did not reduce the ulcer induced by EtOH, as the ulcerative changes on the mucosal surface, hemorrhagic and neutrophil infiltrate are similar to the group that received only EtOH (gastric ulcer group, Table 1). The lack of effect may be related to the alcohol content of the fermented beverages used as a treatment in the present study – 16.2% (v/v) (MASCARIN et al., 2023). Since, surprisingly, the animals without gastric ulcer induction by EtOH and that received only FOB or FOB-MP also showed a reduction in the protective layer of mucus (Figure 3) and presented hemorrhage and erosion of the gastric epithelium, but do not infiltration of inflammatory cells (neutrophils) (Table 1). These results demonstrate that even much lower alcohol concentrations than those used for the induction of the model (75%) can have a negative impact on the gastric mucosa.

The gastric mucosa of animals that received OMEP showed little preservation of the histological aspects and were slightly more preserved than the EtOH group (Figure 3 and Table 1). OMEP is a proton pump inhibitor that has been widely used in the treatment and prevention of gastrointestinal disorders as gastric ulcers (BEIRANVAND, 2022) and was used as a positive control in this study. These results demonstrates that the induction of gastric ulcer was very aggressive in this study, since even a drug widely used in the clinic for this purpose was not able to completely treat the effects resulting from the administration of EtOH.

3.3. Effect of fermented orange beverage on EtOH-induced changes in gastric oxidative status and antioxidant defense

The histopathological damage by is related with overproduction of reactive oxygen species (ROS), lipid peroxidation, depletion of non-protein sulfhydryl compounds (NPSH) as

glutathione (GSH), as well as, impairment in the antioxidant defense system that also contribute to the EtOH-induced gastric damage and which plays a crucial role in the pathogenesis of gastric ulcer (MOUSAVI et al., 2020; DA SILVA et al., 2020). In this study, the EtOH administration caused an increase in ROS production leading to lipid peroxidation – evaluated through TBARS levels (Figure 4A; $p \leq 0.05$). In response to elevated ROS levels, the stomach tissue of rats with untreated gastric ulcer showed a decrease in its non-enzymatic antioxidant defenses, evaluated through NPSH content and compared to the control group (Figure 4B; $p \leq 0.05$), indicating a reduction in GSH levels, the major non-protein thiol in mammalian cells and tissues. GSH is a tripeptide that acts on the primary antioxidant defense system and its decrease has been used as a marker of oxidative stress (ZITKA et al., 2012).

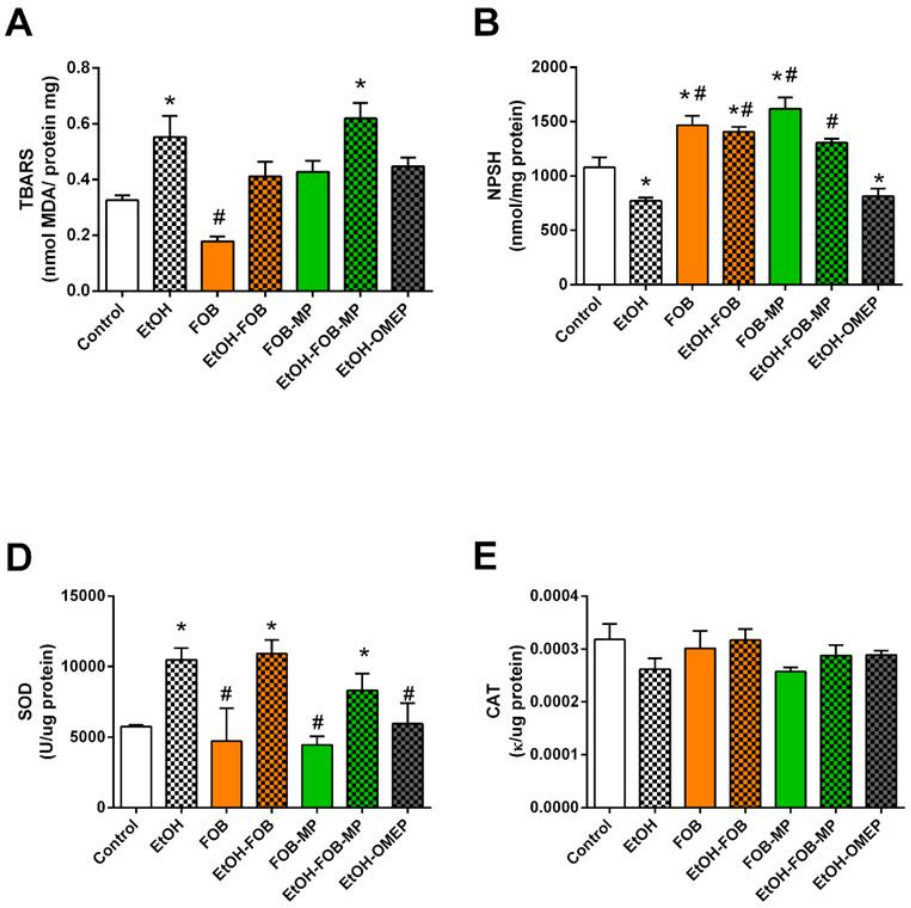


Figure 4 – Evaluation of the oxidative status of the stomach tissue through the levels of lipid peroxidation (A), non-protein thiols (B), and superoxide dismutase (C) and catalase (D) activities. Data are shown as the means ± S.E.M. Tested by ANOVA followed by post hoc Duncan's test. *Different from the control group ($p \leq 0.05$). #Different from EtOH group ($p \leq$

0.05). Control group: without gastric ulcer; EtOH group: with gastric ulcer; FOB group: without gastric ulcer and treated with fermented orange beverage; EtOH-FOB: with gastric ulcer and treated with fermented orange beverage; FOB-MP: without gastric ulcer and treated with fermented orange beverage with Mentha piperita extract; EtOH-FOB-MP: with gastric ulcer and treated with fermented orange beverage with Mentha piperita extract; EtOH-OMEP: with gastric ulcer and treated with omeprazole.

Additionally, EtOH administration significantly increased SOD activity in the stomach of the animals in the experimental conditions used in this protocol (Figure 4C, respectively; $p \leq 0.05$). This increase indicates a greater production of superoxide anion after EtOH administration. The release of superoxide anion in this type of injury may be related to the production of acetaldehyde, formed by the action of alcohol dehydrogenase on EtOH (BEIRANVAND, 2022). Acetaldehyde serves as a substrate for xanthine oxidase (a key enzyme in the metabolism of purines), which produces free radicals - among them superoxide (HALLIWELL; GUTTERIDGE, 1995).

Previous reports confirm that EtOH increases superoxide anion and hydroxyl radical production by neutrophils and these ROS cause lipid peroxidation in the gastric mucosa (DA LUZ et al., 2021; MOUSAVI et al., 2020; DA SILVA et al., 2020). These results show that EtOH administration caused overproduction of ROS that overloaded enzymatic and non-enzymatic antioxidant defense systems, leading to oxidative stress that contributed to gastric mucosal damage, as observed in this study.

The CAT activity, which is important for the decomposition of H_2O_2, was not altered by the administration of EtOH (or any treatment) (Figure 4D). We believe that the levels of H_2O_2 generated after the decomposition of the superoxide anion by SOD were low, so the most appropriate would be to evaluate the activity of glutathione peroxidase (GPx), which acts at low levels of H_2O_2 (HALLIWELL; GUTTERIDGE, 1995), but unfortunately it was not possible to evaluate this enzyme in the present study.

Considering that oxidative stress is a key factor in the development of gastric lesions resulting from EtOH administration, the use of antioxidants has been proposed as therapeutic agents to protect gastric damage (BEIRANVAND, 2022; DA SILVA et al., 2020; MOUSAVI et al., 2020). Here, the treatment with FOB was able to reduce oxidative stress in stomach tissue by partially reducing lipid peroxidation and increasing non-enzymatic antioxidant defenses (Figure 4A and 4B, respectively*; $p \leq 0.05$*). This protective effect of FOB may be related to the presence of phenolic compounds in this beverage, derived from the fermentation of orange juice. FOB had in its composition phenolic acids (caffeic, ferulic, and chlorogenic acid) and flavonoids (rutin, narirutin, and hesperidin), being the latter group the one with the highest concentration (MASCARIN et al., 2023).

The beneficial action observed in the present study can be related to the direct free radical scavenging capacity of flavonoid polyphenols, which has been mainly attributed to the presence of catechol groups in ring B, double bonds and hydroxyl substitutions in the aromatic ring (WANG et al., 2017; OGAWA et al., 2011).

The FOB direct antioxidant capacity had already been observed in our *in vitro* studies (MASCARIN et al., 2023). In these studies, we also observed that FOB showed greater antioxidant direct capacity (assessed by the DPPH method) than FOB-MP. Additionally, FOB showed higher levels of ferulic acid and hesperidin than FOB-MP, in addition to the presence of rutin that was not found in the beverage added with *Menta piperita* extract (MASCARIN et al., 2023). These differences in the antioxidant capacity and phytochemical composition between FOB and FOB-MP may be the reason why we observed a beneficial effect against oxidative stress in animals that were treated with FOB, but not in those that received FOB-MP.

In addition to the direct free radical scavenging capacity, the beneficial effect of FOB treatment observed in animals with gastric ulcer is also due to the improvement of endogenous non-enzymatic antioxidant defenses. We believe that the compounds present in FOB may also

have modulated enzymes related to the synthesis and/or recycling of GSH, strengthening non-enzymatic antioxidant defenses. This hypothesis can be confirmed by observing the increase in NPSH levels in all animals that received FOB (Figure 4B; $p \leq 0.05$), being a direct reflection of the increase in GSH levels. FOB-MP also seems to perform a similar action only in animals without gastric ulcer (Figure 4B; $p \leq 0.05$).

Recently, Da Silva and collaborators demonstrated that plant extracts rich in phenolic compounds were able to increase the reduced glutathione/oxidized glutathione (GSH/GSSG) ratio by modulating the activity of enzymes in the GSH synthesis/recycling cycle, such as GPx, glutathione reductase (GR), and glutathione-s-transferase (GST) (DA SILVA et al., 2020). Unfortunately, we did not determine the activity of these enzymes and this is a limitation of our study. Thus, the reduction in lipid peroxidation in the stomach tissue observed in the animals that received FOB may be, in addition to the improvement in GSH levels, due to a direct antioxidant effect of the phenolic compounds, which helped the antioxidant defenses in neutralizing the excess of ROS and, consequently, in reducing lipid peroxidation. Despite this, FOB treatment failed to reduce ethanol-induced tissue damage, and this lack of gastro repair effect may be related to the alcohol content of this beverage.

The FOB and FOB-MP treatments did not prevent changes in SOD activity when compared to the EtOH group (Figure 4C; $p \leq 0.05$). This non-modification of SOD activity may be due to EtOH administration in these groups. Despite having improved antioxidant status, by increasing NPSH levels, this was not enough to eliminate all the superoxide produced as a result of the decomposition of EtOH by alcohol dehydrogenase (BEIRANVAND, 2022).

The treatments with OMEP causes reduction in oxidative stress, evidenced by lower levels of TBARS and, consequently, SOD activity when compared to the EtOH group (Figure 4A and 4C, respectively; $p \leq 0.05$). This is possibly due to a lesser degree of damage to stomach tissue as pointed out by histological findings (Figure 2 and Table 1), with lower production of

ROS, which consequently requires less adaptive changes of the antioxidant defense system, and not to an antioxidant effect of OMEP, since this drug did not improve antioxidant status per se (did not increase NPSH levels when compared to the control; Figure 4D).

In this investigation, we refer to the fermented orange as functional since so-called "functional beverages" provide important bioactive compounds for maintaining health and/or contributing to the prevention and treatment of chronic diseases (BULMAN et al., 2021; CONG; BREMER; MIROSA, 2020; GONÇALVES et al., 2022). The "functional properties" can be characterized by high polyphenol content, antioxidant potential, probiotics, and other characteristics (BULMAN et al., 2021; CUVAS-LIMÓN et al., 2022; PINTO; VILELA, 2021), even alcoholic beverages such as wine and beer have already been identified as functional beverages (RADONJIĆ et al., 2020). FOB had already demonstrated good antioxidant capacity in different in vitro evaluations (MASCARIN et al., 2023) and now it has also demonstrated this property in vivo, which confirms that FOB is indeed a functional beverage.

CONCLUSION

In this investigation, treatment with FOB showed an antioxidant action *in vivo* in an EtOH-induced gastric ulcer model, confirming previous findings *in silico* studies. The phenolic composition, mainly flavonoids, is possibly the determining factor for FOB to have an antioxidant effect and FOB-MP not. The presence of high EtOH concentration in FOB and FOB-MP may have been the determining factor for the absence of gastro repair and antiulcerative effects. Gastroprotection trials may have more promising results than the gastro reparation trials we perform. The consumption of this functional beverage, preferably dealcoholized, can be further investigated as a nutritional adjuvant strategy in the prevention of diseases with the involvement of oxidative stress.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

L.G.M.: doctoral student, participated in the entire analyses, writing and revision of the work.

F.W.F.; F.Z.R.; J.R.P.G and W.N.P.: participated in the execution of the in vivo experiment.

J.O.F.S. and S.T.C: performed the histopathological analyzes and data interpretation.

J.R.B. and C.C.D.: performed the biochemical analyses.

J.A.S.J: veterinarian responsible for conducting the euthanasia of animals.

L.F.B.; S.S.; C.K.S.: participated in the entire analyses, writing and revision of the work.

All authors critically reviewed the manuscript and approved the final version.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The authors declare that the work was submitted for evaluation by the Ethics Committee of the Federal University of Santa Maria and was approved under number 5178300819 (ID 002808).

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5 CONCLUSÃO GERAL

De maneira geral, os resultados do presente estudo demonstraram a viabilidade da inclusão de extratos de *M. recutita, C. citratus* e *M. piperita* no fermentado alcoólico de laranja.

Entre as ervas adicionadas, o extrato de *M. piperita* teve a melhor avaliação nos testes sensoriais, contribuiu com o aumentando do teor de fenólicos totais e flavonoides e melhorou a capacidade antioxidante da bebida, sem alterar suas características físico-químicas.

Os compostos bioativos presentes na bebida desenvolvida foram principalmente flavonoides e ácidos fenólicos, que no estudo *in silico* indicaram potencial gastroprotetor e antiulcerativo da bebida desenvolvida.

O ensaio *in vivo* confirmou que a bebida fermentada de laranja (FOB) possui ação antioxidante em modelo de úlcera gástrica induzida por etanol (EtOH). Porém, o fermentado de laranja composto de hortelã (FOB-MP) não apresentou tal propriedade. Não houve efeito sinérgico na adição do extrato de hortelã à bebida, sendo possível que a presença de alta concentração de EtOH tenha sido o fator determinante para a ausência de efeitos gastroreparadores e antiulcerativos.

Com isso entendemos que o consumo dessa bebida funcional deva ser feito preferencialmente na sua forma desalcoolizada e sugerimos outras investigações como, por exemplo, a ensaios gastroprotetivos ao invés de gastroreparadores, como estratégia nutricional adjuvante na prevenção de doenças com envolvimento do estresse oxidativo. Outra abordagem futura sugerida é a investigação das adição de ervas aromáticas na vida de prateleira da bebida desenvolvida.

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