UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

LUCIA ELAINE DE OLIVEIRA BRAGA

# ÓLEO ESSENCIAL DE *MENTHA AQUATICA* E ENANTIOMEROS DE CARVONA – EFEITOS SOBRE O TRATO GASTROINTESTINAL

# *MENTHA AQUATICA* ESSENTIAL OIL AND CARVONE ENANTIOMERS - EFFECTS ON THE GASTROINTESTINAL TRACT

Piracicaba
2020

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Odontologia, na Área de Farmacologia, Anestesiologia e Terapêutica.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Odontology, in Pharmacology, Anesthesiology and Therapeutics area.

Orientadora: Profa. Dra. Ana Lucia Tasca Gois Ruiz

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## RESUMO

Distúrbios do trato gastrointestinal, como as doenças ulcerativas e inflamatórias, estão relacionados com fatores como o uso contínuo de alguns medicamentos, fatores emocionais e físicos, consumo de álcool e tabaco, entre outros. Na busca por novas opções terapêuticas, as plantas representam uma importante fonte de novos fármacos. Destas, várias espécies da família Lamiaceae são usadas tradicionalmente para tratamento de distúrbios do TGI, com destaque para o gênero *Mentha.* A espécie *Mentha aquatica* é uma planta perene, cultivada no Brasil, cujo óleo essencial (OEMa) apresenta uma composição química variável, em parte, em função da região de cultivo. Estudos anteriores de nosso grupo de pesquisa indicaram que OEMa, constituído majoritariamente (≈ 56%) pelo monoterpeno carvona, apresentou efeito protetor contra úlceras gastrointestinais tanto em modelo de úlcera induzida por etanol quanto por doses repetidas de ácido acetilsalicílico (AAS), além de inibição da secreção ácida gástrica. O presente estudo propôs avaliar se os enantiômeros de carvona [*R*-(-)-carvona e *S*-(+)-carvona] estavam relacionados à atividade gastroprotetora apresentada por OEMa além de avaliar o potencial uso de OEMa e dos enantiômeros de carvona em modelo de colite induzida por dextran sulfato de sódio (DSS). Estudos *in vitro* (*scratch assay*) demonstraram que OEMa (25µg/mL) promoveu um aumento na migração de queratinócitos humanos HaCat para 45.6 ± 7.3% em relação ao controle negativo, enquanto ambos os enantiômeros de carvona não afetaram a migração celular. A avaliação da atividade antiproliferativa demonstrou que *S*-(+)-carvona inibiu em 50% a proliferação celular ($GI_{50}$) das linhagens PC-3 (próstata, $GI_{50}$ = 13.1µg/mL) e HaCat (queratinócitos não tumorais, $GI_{50}$ = 16.8 µg/mL) enquanto *R*-(-)-carvona e OEMa foram inativos. Em ratos, ambos os enantiômeros de carvona apresentaram atividade antiulcerogênica dependente da dose, sem diferença entre os isômeros, no modelo de úlcera induzida por etanol. Já a atividade antissecretória, avaliada por volume de secreção gástrica e acidez total no modelo de ligadura de piloro, foi independente da dose para ambos os isômeros, enquanto apenas a maior dose de ambos enantiômeros foi capaz de reduzir significativamente a acidez livre. Como observado anteriormente para OEMa, *R*-(-)-carvona e *S*-(+)-carvona não afetaram a motilidade intestinal, no modelo de deslocamento de carvão ativo. No modelo de úlcera induzida por doses repetidas de AAS, apenas *S*-(+)-carvona reverteu parcialmente a elevação nos níveis de glutationa (GSH) induzida pelo AAS, sem alterar significativamente os níveis de interleucina (IL)1β, *Macrophage inflammatory protein 2* (MIP-2), *Tumor necrosis fator* (TNF)-α, IL-10 e de atividade de mieloperoxidase (MPO). No modelo de colite induzida por DSS, OEMa foi capaz de retardar parcialmente o agravamento dos sinais clínicos. Ainda, OEMa e *S*-(+)-carvona reverteram parcialmente o aumento da atividade de MPO induzido por DSS sem alterarem significativamente as alterações histológicas induzidas pelo DSS. Concluindo, o estereoisomerismo pareceu afetar parcialmente os efeitos biológicos da carvona sendo a *S*-(+)-carvona mais ativa que a *R*-(-)-carvona em alguns modelos. Ainda, foi possível evidenciar a contribuição da carvona na atividade gastroprotetora de OEMa, sendo possível que *S*-(+)-carvona seja o isômero presente no óleo essencial.



**Palavras-chave:** *Mentha aquatica*, óleo essencial, atividade antiulcerogênica, esteroisômeros, enantiômeros, carvona, colite, trato gastrointestinal

## ABSTRACT

Disorders of the gastrointestinal tract (GIT), such as ulcerative and inflammatory diseases, are related to factors such as the continued use of some medications, emotional and physical factors, alcohol and tobacco consumption, among others. In the search for new therapeutic options, plants represent an important source of new drugs. Among them, several species of the Lamiaceae family are traditionally used to treat disorders of the GIT, with emphasis on the genus *Mentha*. The *Mentha aquatica* is a perennial species, cultivated in Brazil, whose essential oil (OEMa) has a variable chemical composition, partly depending on the region of cultivation. Previous studies of our research group indicated that EOMa, composed mainly by the monoterpene carvone (≈ 56%), demonstrated a gastroprotective effect in both ethanol and acetylsalicylic acid (ASA) repeated dose-induced ulcer models, besides gastric acid secretion inhibitory effect. The present study proposed to evaluate whether the carvone enantiomers [*R*-(-)-carvone and *S*-(+)-carvone] were related to the gastroprotective activity of EOMa besides the evaluation of EOMa and carvone enantiomers in the dextran sodium sulfate (DSS)-induced colitis model. *In vitro* studies (scratch assay) showed that EOMa at 25µg/mL increased the human keratinocytes HaCat migration to 45.6 ± 7.3% in comparison to the negative control, while both carvone enantiomers did not affect cell migration. The antiproliferative activity evaluation showed that the *S*-(+)-carvone inhibited by 50% the proliferation of PC-3 (human tumor prostate, $GI_{50}$ = 13.1µg/mL) and HaCat (human immortalized keratinocytes, $GI_{50}$ = 16.8 µg/mL) while *R*-(-)-carvone and EOMa were inactive. In rats, both the carvone enantiomers showed dose-dependent antiulcerogenic activity in ethanol-induced ulcer model of, with no difference between the isomers. Considering the anti-secretory activity, evaluated by gastric secretion volume and total acidity in the pylorus ligation model, both isomers were active dose independent while only the highest dose (100 mg/Kg) of both isomers were able to significantly reduce free acidity. As previously observed for EOMa, both carvone enantiomers did not affect intestinal motility in the active charcoal motility model. In the dose-repeated ASA-induced ulcer model, only the *S*-(+)-carvone partially reverted the ASA-induced glutathione (GSH) increased level without none significant changes in interleukin (IL)1β, Macrophage inflammatory protein 2 (MIP-2), Tumor necrosis factor (TNF) α, IL-10 levels and myeloperoxidase (MPO) activity. In the DSS-induced colitis model, EOMa was partially able to retard the worsening of the clinical signs. Also, EOMa and *S*-(+)-carvone partially reverted DSS-induced increase in MPO activity without significantly altering DSS-induced histological changes. In conclusion, chirality partially affected the biological effects of carvone been *S*-(+)-carvone more active in some models. More, carvone contributes in part to the gastroprotective effect of the essential oil of *M. aquatica* aerial parts (EOMa) and *S*-(+)-carvone may be the isomer present in the essential oil.



**Keywords:** *Mentha aquatica*, essential oil, antiulcerogenic activity, stereoisomers, enantiomers, carvone, colitis, gastrointestinal tract

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## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| µg - | micrograma |
| µL - | microlitro |
| 786-0 | Linhagem celular humana de adenocarcinoma de rim |
| AAS - | Ácido acetilsalicílico |
| AINES - | Anti-inflamatório não esteroidal |
| ASA - | acetylsalicylic acid |
| BHT - | Butil-hidroxi-tolueno |
| CMC - | carboximetilcelulose |
| CNPq - | Conselho Nacional de Pesquisa |
| CNS - | central nervous system |
| COX - | cicloxigenase |
| DAI - | disease activity index |
| DII - | Doenças inflamatórias intestinais |
| DMSO - | dimetilsulfóxico |
| DSS - | Dextran sodium sulfate |
| $ED_{50}$ - | Dose necessária para promover 50% do efeito farmacológico (dose efetiva 50) |
| EDTA - | Ácido etilenodiamino tetra-acético |
| EGF - | Epidermal Growth Factor |
| EOMa - | Essential oil of *M. aquatica* |
| FBS - | Fetal bovine serum (soro fetal bovino) |
| g - | gramas |
| $GI_{50}$ - | Concentração necessária para inibir em 50% a proliferação celular |
| GSH - | glutationa |
| h - | hour |
| $H_2O_2$ - | Peróxido de hidrogênio |
| HaCaT | Linhagem celular humana de queratinócitos imortalizados |
| HT29 | Linhagem celular humana de adenocarcinoma coloretal |
| i.p. - | intraperitoneal |
| IBD - | inflammatory bowel disease |
| IFN-γ - | Interferon gama |
| IL | interleucina |

| | |
|---|---|
| K562 | Linhagem celular humana de leucemia mielogênica crônica |
| kg - | quilograma |
| $LD_{50}$ - | Dose necessária para matar 50% dos animais |
| M - | molar |
| MCF-7 | Linhagem celular humana de adenocarcinoma de mama |
| mg - | miligrama |
| MIP-2 (ou CXCL-MIP-2)- | Macrophage inflammatory protein 2 |
| mL - | mililitro |
| mM - | Milimolar |
| MPO - | mieloperoxidase |
| MTD - | Máxima dose tolerada |
| N - | normal |
| NaCl - | Cloreto de sódio |
| NaOH - | Hidróxido de sódio |
| NCI-ADR/RES | Linhagem celular humana de adenocarcinoma de ovário com fenótipo de resistência a múltiplos fármacos. |
| NCI-H460 | Linhagem celular humana de carcinoma, tipo não pequenas células, de pulmão |
| OE - | Óleo essencial |
| OECD - | Organization for Economic Co-operation and Development |
| OEMa - | Óleo essencial de *Mentha aquatica* |
| PBS - | phosphate buffer saline solution |
| PC-3 | Linhagem celular humana de adenocarcinoma de prostata |
| pg - | picograma |
| $PGE_2$ - | Prostaglandinas $E_2$ |
| pH - | potencial hidrogeniônico |
| PNs - | Produtos naturais |
| R-C - | *R*-(-)-carvona |
| ROS - | Reactive oxygen species |
| rpm - | Rotação por minuto |
| RPMI 1640- | Meio de cultura desenvolvido no Roswell Park Memorial Institute |
| S-C - | *S*-(+)-carvona |

| | |
|---|---|
| SRB - | sulforhodamine B |
| TCA - | Ácido tricloroacético |
| TGF-β1 | Transforming growth factor, subtipo beta 1 |
| TNF-α - | Tumor necrosis fator |
| Tween 80 - | Polissorbato 80 |
| U - | unidades |
| U251 | Linhagem celular humana de glioblastoma |
| v.o. - | Via oral |
| VEGF - | vascular endothelial growth factor |
| w/v - | Relação peso/volume |
| WR - | wound reduction |

**SUMÁRIO**

# 1. INTRODUÇÃO

A úlcera péptica é caracterizada pela perda da continuidade da mucosa e da submucosa gastrointestinal e pode acometer o estômago, intestino, e em alguns casos, o esôfago. Essas lesões ocorrem quando há um desequilíbrio entre os fatores agressivos e protetivos do trato gastrointestinal (Zatorski, 2017).

A incidência e as complicações relacionadas a úlcera péptica na população no século XXI está sofrendo drásticas modificações. Dentre os principais fatores para o surgimento das úlceras gastrointestinais estão a presença da bactéria *Helicobacter pylori* e o uso contínuo de anti-inflamatórios não esteroidais (AINES). Além disso, fatores como alimentação, fumo, etilismo, tabagismo e estresse podem contribuir diretamente para o surgimento e/ou agravamento dessas lesões (Mustafa et al., 2015; Yegen, 2018; Azhari et al., 2019). Em algumas pessoas, as lesões ulcerativas podem ocorrer espontaneamente, de forma idiopática, ou seja, sem a presença de um agente danoso. Nestes casos, alterações genéticas que resultem em alterações moleculares na mucosa do trato gastrointestinal contribuem para o aparecimento das lesões ulcerativas (Chung et al., 2015).

O processo de envelhecimento contribui para o surgimento das lesões ulcerativas, pois há uma diminuição na produção da barreira muco-bicarbonato. Associado a isso, a população idosa apresenta uma incidência maior de doenças cardiovasculares e inflamações crônicas o que leva, muitas vezes, ao uso contínuo de outras classes de medicamentos tais como os anti-inflamatórios não esteroidais (AINES) (Scheiman, 2016).

Estima-se que cerca de 1,5 a 3% da população pode apresentar úlceras pépticas. Caso não seja tratada de modo eficaz, a lesão ulcerativa pode evoluir para sangramentos, perfurações, câncer gastrointestinal e morte (Chung e Shelat, 2017). No Brasil, estima-se que a incidência dos casos de úlcera pépticas duodenal seja de 3:1 em relação á gástrica, com maior prevalência na população do sexo masculino (Araújo et al., 2014).

Outro distúrbio do trato gastrointestinal (TGI) que merece destaque são as doenças inflamatórias intestinais (DII) que são distúrbios inflamatórios crônicos. As DII compreendem a colite ulcerativa, que corresponde a um processo inflamatório da mucosa do cólon e reto, e a doença de Crohn, inflamação que pode ocorrer em qualquer parte do trato gastrointestinal, desde a boca até o ânus (Pizzorno et al., 2016; Hedayat e Lapraz, 2019).

A incidência e a prevalência da colite ulcerativa vêm aumentando em todo o mundo (Kaplan, 2015), e a causa específica que favorece o aparecimento da doença ainda é desconhecida. Sabe-se que vários fatores podem contribuir para o surgimento da colite.

Alguns estudos sugerem uma relação direta entre o estilo de vida, como nível de estresse e tipo de alimentação, e o desenvolvimento da colite ulcerativa, visto que países com maior índice de industrialização apresentam uma maior incidência da doença (Ordás et al., 2012; Kaplan, 2015; GBD, 2017; Flies et al., 2019).

Ainda há divergências entre os pesquisadores quanto à etiologia da colite ulcerativa, mas a maioria concorda que alterações no sistema autoimune, na microbiota intestinal, além de idade e fatores ambientais, emocionais e genéticos sejam algumas das principais causas que desencadeiam o surgimento da doença (Ungaro et al., 2017; Kuhnen, 2019).

Na mucosa intestinal, a camada de muco constituída por vários tipos de mucinas corresponde a uma importante linha de defesa. Na colite ulcerativa, a produção de alguns subtipos de mucina encontra-se comprometida. Desta forma, antígenos presentes no lúmen intestinal conseguem permear a barreira epitelial, promovendo uma resposta inflamatória (Ordás et al., 2012). Fatores correlacionados com o aumento da incidência de casos da doença incluem o uso contínuo e/ou prolongado de AINES, de alguns tipos de antibióticos, de terapia de reposição hormonal e de anticoncepcionais orais (Feuerstein e Cheifetz, 2014)

Os tratamentos disponíveis para as úlceras pépticas, principalmente de esôfago e estomago, estão baseadas na antibioticoterapia contra *H. pylori* e no uso de inibidores de bomba de prótons, como omeprazol, e de receptores histamínicos, como ranitidina (Urs et al., 2014), enquanto o tratamento da colite é baseado principalmente em aminossalicilatos, imunomoduladores, glicocorticoides, antibióticos e biofármacos, como agentes anti-TNF-α (AlRuthia et al., 2019; Long et al, 2019; Parra et al., 2019). No entanto, a escolha do tratamento deve considerar a eficácia e resistência do fármaco, os possíveis efeitos adversos e principalmente o custo do medicamento (Moreau e Mas, 2015; Gomollón et al. 2017; AlRuthia et al., 2019; Long et al, 2019; Parra et al., 2019).

O desenvolvimento de novas opções de tratamento a partir de plantas medicinais constitui-se uma abordagem promissora, tendo em vista que todo o conhecimento adquirido a partir da sabedoria popular sobre plantas medicinais e seus metabólitos possibilitou o desenvolvimento da maior parte do arsenal terapêutico utilizado atualmente (Yuan et al., 2016). Produtos Naturais (PNs) são substâncias produzidas por organismos vivos, como plantas, fungos, bactérias e animais marinhos, cuja grande diversidade estrutural resulta em uma enorme variedade de efeitos biológicos (Katz e Baltz, 2016). Os PNs tanto podem ser usados como fármacos *per si* quanto servem de modelo para o desenvolvimento de novos

medicamentos. Cerca de 30% das drogas terapêuticas atualmente disponíveis são derivadas direta ou indiretamente de produtos naturais (Dutra et al., 2016).

Muitas plantas são utilizadas tradicionalmente para o tratamento de distúrbios do TGI, sendo que algumas delas tiveram seus efeitos comprovados cientificamente (Schmeda-Hirschmann e Yesilada, 2005). Como exemplo, pode-se citar *Cynara scolymus* (dispepsia e ação antioxidante), *Curcuma longa* (redução de citocinas pró-inflamatórias TNF-α, IL-1β, IL-6, no TGI), *Garcinia buchananii* (diarreia, desconforto e dor abdominal) e *Cannabis sativa* (dor abdominal, náusea, vômito e diarreia) entre outras (Brierley e Kelber, 2011; Fifi et al., 2018). Outro exemplo interessante é o fitoterápico Iberogast® (STW 5) comercializado na União Européia. Este fitoterápico é uma mistura de extratos de 9 plantas medicinais (*Iberis amara*, *Melissa officinalis, Matricaria chamomilla, Carum carvi, Mentha x piperita, Glycyrrhiza glabra, Angelicae archangelica, Silybum marianum, Chelidonium majus)* com uso aprovado para o tratamento de úlcera péptica e de DII (Brierley e Kelber, 2011; Ottillinger et al., 2013; Malfertheiner, 2017).

Dentre a grande variedade de plantas, pode-se destacar aquelas pertencentes à família Lamiaceae que são comumente encontradas em regiões temperadas. Esta família compreende aproximadamente 7.200 espécies distribuídas em 240 gêneros (Bräuchler et al., 2010). Dentre esses gêneros, destaca-se o gênero *Mentha* que compreende espécies com grande variabilidade biológica, e são utilizadas comumente na culinária e por suas atividades antiemética, antiespasmódicas, antiulcerogênica, anti-inflamatória, analgésica, entre outras (Agostini et al., 2009; Barros et al., 2015).

Uma das espécies mais conhecidas deste gênero é a *Mentha piperita* L. (hortelã pimenta) que é usada popularmente como relaxante da musculatura lisa intestinal e industrialmente como agente flavorizante (Grigoleit e Grigoleit, 2005). Os efeitos carminativo e antiespasmódicos do óleo essencial (OE) de *M. piperita* são em parte atribuidos ao mentol que é capaz de bloquear canais de cálcio modulando a contração muscular (McKay e Blumberg, 2006). Por via oral, a ação gastroprotetora do mentol está relacionada com aumento do teor de substâncias sulfidrílicas não proteicas, da produção de muco e de prostaglandina $PGE_2$, além de diminuição da concentração de íons hidrogênio no suco gástrico (atividade antissecretória) (Rozza et al., 2013).

Já *Mentha aquatica* L. (Figura 1) é uma planta perene, comum na Europa, Norte da África, e oeste da Ásia, introduzida na América e Austrália, e é encontrada em lugares úmidos e ao longo de cursos d'água. Esta planta tem uma fragrância marcante e sua

morfologia e composição são influenciados pela região em que elas se encontram (Bhat et al., 2002).

Figura 1- Coleta das partes aéreas (A) e detalhe das folhas de *Mentha aquatica*.

**A**  **B** 

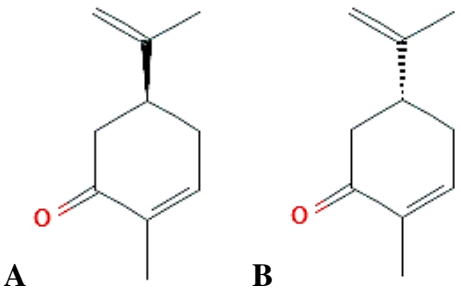
Fonte: A (Braga, 2015); B (worldofFloweringplants.com)

Uma das principais características das plantas da família Lamiacea é a produção de óleos essenciais (OE). Os OE's são misturas complexas de compostos voláteis, principalmente mono e sesquiterpenos, os quais são responsáveis por diversas interações das plantas com o meio ambiente. Esses óleos são amplamente utilizados em processos industriais, como flavorizantes, agentes bioativos ou ainda como matéria prima para síntese de fármacos (Aziz et al., 2018; Tetali, 2019).

Os compostos presentes no óleo essencial da *M. aquatica* (EOMa) podem variar significativamente de acordo com a região em que a planta é cultivada. Estudo recente de um exemplar cultivado em Paulínia, SP, Brasil, identificou a presença dos monoterpenos carvona, limoneno e 1,8-cienol como os principais constituintes do óleo, sendo a carvona o composto majoritário (≈ 56%) (Braga, 2016).

O monoterpeno carvona (Figura 2) possui um carbono quiral em sua estrutura, apresentando, desta forma, atividade óptica. Estudos de composição química de OEs indicaram que o isômero *R*-(-)-carvona é o principal constituinte do OE de *M. spicata,* enquanto o isômero *S*-(+)-carvona está presente no OE de *Carum cavi L.* (Apiaceae) (Buchbauer et al., 2005).

Figura 2- Estrutura química dos enantiômeros *S*-(+)-carvona (A) e *R*-(-)-carvona (B)




Fonte: PubChem

Isômeros são compostos que apresentam a mesma fórmula molecular, mas com diferente fórmula estrutural e/ou organização espacial. Na isomeria ótica, os pares de enantiômeros apresentam mesma fórmula molecular e mesma fórmula estrutural diferindo quanto à organização espacial da molécula. Essa diferença resulta em similaridade em quase todos os parâmetros físicos e químicos e possibilita que cada enantiômero apresente atividades biológicas diferentes. Isso porque os enantiômeros de uma determinada substância poderão interagir de maneira diversa com sítios específicos presentes em enzimas, proteínas e receptores gerando respostas diferentes (Nguyen et al., 2006; Smith, 2009).

Vários estudos demonstraram os efeitos promovidos pelos isômeros de carvona. Foi observado em modelos animais que o enantiômero *S*-(+)-carvona promoveu efeito depressor com diminuição da capacidade de resposta a estímulos, aumento na sedação e atividade anticonceptiva em ratos, maior do que o isômero *R*-(-)-carvona (de Sousa et al., 2007). Ambos os enantiômeros apresentaram atividade bloqueadora de canais de $Ca^{2+}$, sendo o isômero *S*-(+)-carvona mais potente (Souza et al., 2013).

## 1.1. Proposição:

Com base nos estudos realizados durante o mestrado (Braga, 2016) e no levantamento bibliográfico, este estudo consistiu na avaliação da participação da carvona, composto majoritário, na atividade gastroprotetora observada para o óleo essencial das partes aéreas de *Mentha aquatica*. Essa tese será apresentada no formato alternativo e encontra-se composta de um artigo científico que se encontra em fase de submissão em revista científica.

### 1.1.1. Proposição Geral

Avaliação da atividade dos enantiômeros *S*-(+)-carvona e *R*-(-)-carvona em distúrbios do Trato gastrointestinal visando ampliar os conhecimentos sobre os princípios ativos do óleo essencial de *Mentha aquatica*.

### 1.1.2. Proposições Específicas

- Avaliação *in vitro* dos enantiômeros *S*-(+)-carvona e *R*-(-)-carvona e do óleo essencial de *Mentha aquatica* em modelos de atividade antiproliferativa e de migração celular;
- Avaliação da atividade antiulcerogênica dos enantiômeros *S*-(+)-carvona e *R*-(-)-carvona em modelos de úlcera gástrica induzida por dose única de etanol;
- Avaliação do efeito antissecretório dos enantiômeros *S*-(+)-carvona e *R*-(-)-carvona no modelo de ligadura de piloro;
- Avaliação da atividade antiulcerogênica dos enantiômeros *S*-(+)-carvona e *R*-(-)-carvona em modelos de úlcera gástrica induzida por doses repetidas de ácido acetilsalicílico;
- Avaliação da atividade dos enantiômeros *S*-(+)-carvona e *R*-(-)-carvona sobre a motilidade gastrointestinal.
- Avaliação da atividade dos enantiômeros *S*-(+)-carvona e *R*-(-)-carvona e do óleo essencial de *Mentha aquatica* em modelo de colite induzida por DSS.

# 2. ARTIGO - *Mentha aquatica* essential oil and carvone enantiomers – Effects on the gastrointestinal tract

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**ABSTRACT**

Disorders of the gastrointestinal tract (GIT), such as ulcerative and inflammatory diseases, are related to factors such as the continued use of some medications, emotional and physical factors, alcohol and tobacco consumption, among others. In the search for new therapeutic options, plants represent an important source of new drugs. Among them, several species of the Lamiaceae family are traditionally used to treat disorders of the GIT, with emphasis on the genus *Mentha*. The *Mentha aquatica* is a perennial species, cultivated in Brazil, whose essential oil (OEMa) has a variable chemical composition, partly depending on the region of cultivation. Previous studies of our research group indicated that EOMa, composed mainly by the monoterpene carvone (≈ 56%), demonstrated a gastroprotective effect in both ethanol and acetylsalicylic acid (ASA) repeated dose-induced ulcer models, besides gastric acid secretion inhibitory effect. The present study proposed to evaluate whether the carvone enantiomers [*R*-(-)-carvona and *S*-(+)-carvone] were related to the gastroprotective activity of EOMa besides the evaluation of EOMa and carvone enantiomers in the dextran sodium sulfate (DSS)-induced colitis model. *In vitro* studies (scratch assay) showed that EOMa at 25µg/mL increased the human keratinocytes HaCat migration to 45.6 ± 7.3% in comparison to the negative control, while both carvone enantiomers did not affect cell migration. The antiproliferative activity evaluation showed that the *S*-(+)-carvone inhibited by 50% the proliferation of PC-3 (human tumor prostate, $GI_{50}$ = 13.1µg/mL) and HaCat (human immortalized keratinocytes, $GI_{50}$ = 16.8 µg/mL) while *R*-(-)-carvone and EOMa were inactive. In rats, both the carvone enantiomers showed dose-dependent antiulcerogenic activity in ethanol-induced ulcer model of, with no difference between the isomers. Considering the anti-secretory activity, evaluated by gastric secretion volume and total acidity in the pylorus ligation model, both isomers were active dose independent while only the highest dose (100 mg/Kg) of both isomers were able to significantly reduce free acidity. As previously observed for EOMa, both carvone enantiomers did not affect intestinal motility in the active charcoal motility model. In the dose-repeated ASA-induced ulcer model, only the *S*-(+)-carvone partially reverted the ASA-induced glutathione (GSH) increased level without none significant changes in interleukin (IL)1β, Macrophage inflammatory protein 2 (MIP-2), Tumor necrosis factor (TNF) α, IL-10 levels and myeloperoxidase (MPO) activity. In the DSS-induced colitis model, EOMa was partially able to retard the worsening the clinical signs. In addition, EOMa and *S*-(+)-carvone partially reverted DSS-induced increase in MPO activity without significantly altering DSS-induced histological changes. In conclusion, chirality partially affected the biological effects of carvone been *S*-(+)-carvone

more active in some models. More, carvone contributes in part to the gastroprotective effect of the essential oil of *M. aquatica* aerial parts (EOMa).

**Keywords:** *Mentha aquatica*, essential oil, antiulcerogenic, gastric motility, carvone isomers, colitis

## 1. Introduction

Gastric ulcer corresponds to lesions that appear in the mucosal and submucosal walls of the gastrointestinal tract by direct action of substances capable of promoting damage. Ulcers may occur in the stomach, proximal duodenum and gastroesophageal region. From the end of the 20$^{th}$ century to the beginning of the 21$^{st}$ century, a relative decrease in the incidence of peptic ulcers was associated to the improvement of diagnoses, treatments and overall quality of health conditions (Najm, 2011; Sharifi-Rad et al., 2018). However, factors such as the *Helicobacter pylori* presence, continuous use of some drugs, diet, alcohol, tobacco or cocaine consumption, aging and stress (physical and emotional) contribute to the appearance of lesions in the gastrointestinal tract and to the increasing incidence (Stewart and Ackroyd, 2011; Lanas and Chan, 2017).

In addition to peptic ulcer, other gastrointestinal concern are the chronic inflammatory disorders, such as inflammatory bowel disease (IBD), which encompasses ulcerative colitis and Crohn's disease. Like ulcers, IBD has become a global problem that affects all ages. Factors such as genetic predisposition, immune system dysfunction, together with environmental factors such as diet and microbiota, may trigger the onset of the IBDs (Molodecky et al., 2012; Büsch et al., 2014).

Since ancient times, natural products have been an important source of inspiration for new therapeutic options for various disorders, including the gastrointestinal disorders. (Xie et al., 2015; David et al., 2019). The genus *Mentha* comprises 42 species, 15 hybrids and numerous varieties and cultivars (Salehi et al., 2018), cultivated worldwide and used mainly in the food industry as flavorings. In traditional medicine, *Mentha* species are employed in the treatment of colds besides digestive and cardiovascular disorders. Many pharmacological activities have already been described for *Mentha* spp. such as antioxidant, antimicrobial, antitumoral, antiviral, antiallergic, anti-inflammatory and antihypertensive activities. The compounds present in its essential oil can vary according to the region in which they are cultivated, but are composed mainly by terpenoids and alcohols, among others (Rozza and Pellizzon, 2013; Anwar et al., 2019).

The aerial parts of *Mentha aquatica* L., found in humid regions and waterways, is popularly used due to its analgesic, anti-inflammatory, antioxidant, antispasmodic, antiemetic, digestive activities (Yarnell and Abascal, 2011; Do Ngoc Dai et al., 2015). Previous study demonstrated that *M. aquatica* essential oil (EOMa) obtained in Brazil, Paulínia, SP, presented the monoterpene carvone as the major component (≈ 56%). In addition, the EOMa showed significant antiulcerogenic activity in ethanol-induced ulcer models (preventive effect) and in dose-repeated acetylsalicylic acid-induce gastrointestinal lesions (curative effect) (Braga, 2016).

Present in several essential oils, the monoterpene carvone can be found in two enantiomeric forms. The *R*-(-)-carvone isomer was reported as the main constituent of the essential oils of some *Mentha* species, such as *M. spicata* and *M. longifolia* (Lamiaceae) while the *S*-(+)-carvone is present in the essential oils of *Carum cavi* L.and *Anethum graveolens L.* (Apiaceae) (Souza et al., 2013). Recently, the *S*-(+)-carvone was identified in *M. spicata* cultivated in India (Pragadheesh, Yadav, Chanotiya, 2015). Some differences in biological properties were already described for this enantiomeric pair. Besides different organoleptic characteristics (Souza et al., 2013), both carvone enantiomers showed antispasmodic effects probably attributed to the blockade of $Ca^{2+}$ channels in smooth muscle cells with different potencies depending on the used model (Gonçalves et al., 2013; Souza et al., 2013; Silva et al., 2015). More, *S*-(+)-carvone induced sedation and antinociceptive effects more potently than *R*-(-)-carvone in rats (de Sousa et al., 2007; Gonçalves et al., 2008; Nogoceke et al., 2016) while only *R*-(-)-carvone was already tested as anti-inflamatory agent (Sousa et al., 2010). Further, both carvone enantiomers were similarly able to improve gentamicin effect against methicillin-resistant *Staphylococcus aureus* (Mun et al., 2014).

In this context, we evaluated the participation of both carvone enantiomers in the gastroprotective effect of the EOMa using *in vitro* (scratch assay) and *in vivo* (ethanol-induced gastric ulcer, dose-repeated acetylsalicylic acid-induced gastric lesions and dextran sodium sulfate-induced acute colitis) models.

## 2. Material and Methods

### 2.1. General items:

Dextran Sulfate Sodium Salt (DSS) - Colitis Grade (36,000 - 50,000 MW) was acquired from MP Biomedical's, LLC. Doxorubicin chloridrate was acquired from Eurofarma. Pentobarbital 3% (Hypnol®) was acquired from Syntec. Absolute ethyl alcohol was acquired from J.T. Baker. Naphthylethylenediamine dihydrochloride was acquired from

Merck. Thiopental sodium (Thiopentax®) was acquired from Cristália. Ranitidine was acquired from Medley. Sulfanilamide was acquired from Ecibra. Naphthylethylenediamine dihydrochloride was acquired from Merck. Roswell Park Memorial Institute (RPMI) 1640 medium was acquired from GIBCO). Fetal bovine serum (FBS, origin: Brazil), 0.25% Trypsin-EDTA with phenol red, and penicillin:streptomycin (1000 U/ml:1000 g/ml) were acquired from Vitrocell. Ketamine chloridrate (Dopalen ®) and Xylasine (Anasedan ®) were acquired from Ceva. Anhydrous sodium sulfate and sodium hydroxide were acquired from Dinâmica.

Sulforhodamine B (SRB), trichloroacetic acid (TCA), Carbenoxolone disodium salt, N-Ethylmaleimide (NEM), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), Sucrose, Cimetidine, Ethylenediaminetetraacetic acid (EDTA), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), L-glutathione reduced, hexadecyltrimethylammonium bromide (HTAB), acetyl salicylic acid (ASA), *o*-dianisidine dihydrochloride, sodium nitrite, Tween 80, atropine sulfate salt, phosphoric acid, activated charcoal were acquired from Sigma-Aldrich.

Ethyl acetate, carboxymethylcellulose (CMC, medium viscosity, 400-800 cp), sodium chloride, magnesium chloride, phenolphthalein, potassium phosphate monobasic, potassium phosphate dibasic, hydrogen peroxide solution (30% w/w), dimethyl sulfoxide (DMSO), were acquired from Synth.

**2.2. Samples:**

The essential oil of *Mentha aquatica* aerial parts (EOMa) were obtained by hydrodestilation as previous described by Braga (2016). The standard compounds *S*-(+)-Carvone (>96% purity, catalog number 8.18410.0025, CAS: 2244-16-8), *R*-(-)-carvone (>99% purity, catalog number 8.18409.0100, CAS: 6485-40-1) were purchased from Merck.

**2.3 *In vitro Evaluations***

**2.3.1 *Cells lines:***

Human tumor and immortalized cell lines (Table 1S, supplementary information) were grown in complete medium [RPMI 1640 supplemented with 5% fetal bovine serum (FBS) and 1% (v/v) penicillin:streptomycin (1000 U/ml:1000 g/ml)] in a humidified atmosphere with 5% $CO_2$, at 37°C. For the experiments, cell lines were used between passages 4 to 12.

**2.3.2 Samples preparation:**

Aliquots of EOMa, *S*-(+)-Carvone and *R*-(-)-carvone were diluted (1:10 p/v) in DMSO before serial dilution in complete medium to afford the final concentrations described in each assay. For scratch assay, the complete medium was substituted by RPMI 1640 supplemented with 0.2% FBS and 1% (v/v) penicillin:streptomycin.

**2.3.3 Antiproliferative Activity Evaluation:**

Cells in 96-well plates (100 µL cells/well, Table 1S) were exposed to both carvone enantiomers (0.25, 2.5, 25 and 250 µg/mL, final concentrations) at 37 ºC, 5% of $CO_2$ for 48 h. Doxorubicin (0.025, 0.25, 2.5 and 25 µg/mL, final concentrations) was used as positive control. Final DMSO concentration (≤ 0.25%) did not affect cell viability. Before (T0 plate) and after sample addition (T1 plates), cells were fixed with 50% trichloroacetic acid and cell growth was determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B (SRB) assay. Using the concentration-cell growth curve for each cell line, $GI_{50}$ (concentration that inhibits 50% of cell growth) was determined through non-linear regression analysis using the software ORIGIN 8.0 (OriginLab Corporation, Northampton, MA, USA) (Monks et al., 1991).

**2.3.4 Scratch assay:**

Distributed into 12-well plates (3 x $10^5$ cel/ml in complete medium, 1 mL/well), the HaCaT keratinocytes were incubated for 24 h at 37 °C, 5% $CO_2$ humidified atmosphere. Then, using a sterile 200 µL-micropipette tip, one scratch was created in the central portion lengthwise of each well. After removal of complete medium with detached cells, the attached cells were washed with PBS (20 mM, pH 7.0, 2 x 1 mL/well). After, cells were treated with *R*-(-)-carvone, *S*-(+)-Carvone and EOMa (0.25; 2.5 and 25µg/mL) diluted in medium RPMI 1640 supplemented with 0.2% FBS. Complete medium was used as positive control and medium supplemented with 0.2% FBS as negative control. The plates were incubated for 24 h at 37 °C, in 5% $CO_2$ humidified atmosphere. Cell migration was assessed by observing the wound area at 0, 9, 18 and 24 h after treatment using Leika reversed-phase microscope equipped with digital camera Optikam B3 (Optika ®). The wound size was measured using ImageJ software and the wound reduction (WR, %) was calculated by WR = (100 x $T_x$ area/$T_0$ area) - 100, where $T_x$ = wound area at a given time (9, 18 or 24 h) after sample application and $T_0$ = wound area at time 0 (Liang et al., 2007).

## 2.4. *In vivo* Evaluation

### 2.4.1 Animals

Female Swiss mice (4 weeks old, 35 animals), male Wistar rats (4 weeks old, 175 animals) and female C57BL/6J mice (4 weeks old, 50 animals) were acquired from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB, UNICAMP). The animals were acclimatized to laboratory conditions, before handling, under temperature (20 °C ± 2 °C) and in light-dark cycle of 12 h controlled. The animals received commercial pelleted feed Nuvilab® and potable water *ad libitum*. The animals were kept (3 to 4 rats or 8 to 10 mice per cage) in autoclavable polypropylene cages (matte white) with disinfected softwood beddings. For the experiments, mice reached 8 weeks of life (body weight 20 – 30 g) and rats were 12 week old (body weight 250 – 300 g). In all experiments, euthanasia was performed by deepening anesthesia [Sodium Pentobarbital 3%, 80 mg/Kg, i.p., for rats; ketamine (Dopalen injetável® 300 mg/kg) and xylazine (Anasedan injetavel® 30mg/kg) for mice] followed by cervical displacement. The experiments were approved by the Animal Ethics Committee of UNICAMP (CEUA: 4472-1/2017; 4472-1(B)/2018) and conducted following the principles and guidelines adopted by Brazilian College of Animal Experimentation (COBEA) and The National Council for Animal Experiment Control (CONCEA).

### 2.4.2 Sample Preparation and Treatment Techniques

In all *in vivo* experiments, the vehicle consisted of 0.9% NaCl solution prepared in phosphate buffer solution 0.2 M and pH 7.0 (PBS). Both carvone enantiomers and EOMa were diluted in Tween 80 (0.5%, final concentration) before dispersion in the vehicle. For acetyl salicylic acid (ASA), carboxymethylcellulose (CMC, 0.2%, final concentration) was dispersed in hot vehicle under mechanical agitation followed by finely ground ASA resulting a homogeneous suspension. All oral treatments were done by gavage using a flexible orogastric catheter (Ø 2 mm, 5 cm length). All intraperitoneal and intraduodenal treatments were done using syringes (1 or 3 ml) and needles (13 x 0.45 mm or 20 x 0.55 mm).

### 2.4.3 Acute Oral Toxicity Study

After 12h fasting, thirty-five female Swiss mice were weighed, randomly distributed in seven groups (n = 5 animals/group) and orally treated with vehicle (10 ml/kg, G1), *R*-(-)-carvone (30, 100 e 300 mg/kg, G2-G4) or *S*-(+)-carvone (30, 100 e 300 mg/kg, G5-G7) in single dose (OECD 425, 2008). After administration, the animals were

continuously observed during 4h followed by daily observation during 14 days for clinical signs indicative of toxicity such as ambulation, convulsion, abdominal contractions, straub tail, and piloerection, among others. Body weight measurement was done at 0 (basal), $7^{th}$ and $14^{th}$ experimental days. At the $14^{th}$ day, all survivor animals were euthanized for macroscopic evaluation and internal organs (spleen, kidney and liver) weighing.

The higher dose (300 mg/kg) of both carvone enantiomers were estimated according to OECD Guide 129 (Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systematic Toxicity Tests), with adaptations. The $GI_{50}$ values for *R*-(-)-carvone and *S*-(+)-carvone against HaCaT (human keratinocytes) cells were used to calculate the estimated $LD_{50}$ following the Equation 1.

$$\log LD_{50}\ (mg/kg) = 0.372 \times \log GI_{50} + 2.024 \quad (Eq.\ 1)$$

### 2.4.4 Ethanol-induced gastric ulcer

After 12h fasting, fifty-six male Wistar rats were weighed, randomly separated into 8 groups (n = 7 animals/group) and orally treated (v.o.) with vehicle (10 ml/Kg, negative control), carbenoxolone (200 mg/kg, positive control), *R*-(-)-Carvone(10, 30 e 100 mg/kg) or *S*-(+)-Carvone (10, 30 e 100 mg/kg). After 1 h, all the animals were treated with ethanol P.A. (4 mL/Kg, v.o.) for the ulcerative lesion induction. Euthanasia was performed 1 h after the ethanol administration (Robert et al., 1979). The stomachs were removed, opened along the highest curvature and photographed with the aid of JVC – HD Everio (model GZ-HM860) camera with a minimum distance of 50 cm and 2x zoom. The images (1728 x 2304 pixels) were analyzed using the ImageJ® Software for determination of the total gastric and gastric ulcerated areas ($mm^2$). The relative ulcerative area (%) was calculated as [(ulcerated area)/(total gastric area)] x 100]. The gastroprotective effect (%) for each sample was calculated as [(ulcerated $area_{Vehicle}$) - (ulcerated $area_{Treatment}$)/(ulcerated $area_{Vehicle}$)] x 100.

### 2.4.5 Evaluation of gastric juice parameters

After 12h-fasting, fifty-six male Wistar rats were weighed, randomly distributed in 8 groups (n = 7 animals/Group) and anesthetized (sodium thiopental, 50 mg/kg, i.p.) to perform trichotomy and pylorus ligature. Immediately after ligation, the animals were treated by intraduodenal route with vehicle (2.5 mL/kg, negative control), cimetidine (100 mg/kg, positive control), *R*-(-)-Carvone(10, 30, 100 mg/kg) or *S*-(+)-Carvone (10, 30, 100 mg/kg). After surgical suture, the signs of pain and consciousness were monitored and the state of anesthesia was maintained throughout all the experiment. After 4 h of treatment, euthanasia

was performed, the stomachs were removed with the aid of the hemostatic clamp. The gastric contents were collected and the interior of each stomach was washed with deionized water (2 mL/stomach). The total volume of gastric secretion was measured with a graduated cylinder, adjusted to 10 mL with deionized water and centrifuged (2000 rpm, 10 min, room temperature). The free acidity was evaluated with pHmeter and the total acidity was evaluated by titration with NaOH (0.05 N) using phenolphthalein (1%) as a pH indicator (Monteiro et al., 2013).

### 2.4.6 Influence on intestinal motility

After 12h-fasting, twenty-eight male Wistar rats were weighed, randomly distributed in four groups (n = 7 animals/Group) and treated (v.o.) with vehicle (10mL/Kg, negative control, G1), atropine (3 mg/kg, positive control, G2), *R*-(-)-Carvone (15 mg/Kg, G3) and *S*-(+)-Carvone (15 mg/kg, G4). After 60 min, the animals received the activated carbon suspension (2%, 0.1 mL/animal, v.o.). Thirty minutes after active charcoal administration, all animals were euthanized and the whole gut was removed. The distance traveled by charcoal from the pyloric region to the ileocecal junction was measured. The intestinal transit was expressed, in percentage, as the distance traveled by charcoal related to the total length of the small intestine (Possenti et al., 2012).

### 2.4.7 Acetylsalicylic acid-induced gastric ulcer

Thirty-five male Wistar rats were weighed and randomly distributed in 5 groups (n = 7 animals/group). Animals from groups G2 – G5 were treated with ASA (200 mg/kg, 5 ml/Kg, v.o., in vehicle with CMC 0.2%) while animals from group G1 (Vehicle) were treated with CMC 0.2% in vehicle (5 ml/Kg, v.o.) every day during 14 days. From the 8$^{th}$ experimental day, one hour after ASA-treatment, animals were treated with vehicle (5 mL/kg, v.o., negative control, G2), ranitidine (2.5 mg/kg, 5 mL/kg, v.o., positive control, G3), *R*-(-)-Carvone(25 mg/kg, 5 mL/kg, v.o., G4) or *S*-(+)-Carvone (25 mg/kg, 5 mL/kg, v.o., G5) while animals at G1 received no additional treatment. On the 15$^{th}$ experimental day, the animals were euthanized, stomach and duodenum of each animal were removed, weighed and sliced into representative portions (50 to 100 mg) that were gathered by group and stored at -80 °C for subsequent biochemical (GSH, MPO, IL-1β, IL-10, TNF-α and MIP-2) analysis (Jin et al., 1999).

### 2.4.8 Dextran Sodium Sulphate (DSS)-induced Colitis Model in Mice

Fifty female C57BL-6 mice were randomly distributed in 2 groups, Satellite (n = 10 animals/group, 5 animals/cage, G1) and DSS (n = 40 animals/group, 5 animals/cage, G2-G5) groups. DSS-group was treated (v.o.) with sodium dextran sulfate solution (DSS, 3%) *ad libitum* while Satellite group received potable water *ad libitum,* for seven days. At 4$^{th}$ experimental day, the oral treatment with vehicle (5 mL/kg, v.o., negative control, G2), EOMa (25 mg/kg, 5 mL/kg, v.o., G3), *R*-(-)-Carvone(25 mg/kg, 5 mL/kg, v.o., G4) or *S*-(+)-Carvone (25 mg/kg, 5 mL/kg, v.o., G5) was initiated. In the DSS group, in each cage there was at least one animal receiving each proposed treatment. Animals from Satellite group (G1) received no treatment. During the experiment, body weight, bleed stool and stool consistence were evaluated every day. On the 8$^{th}$ experimental day, all animals were euthanized, the whole guts were removed and separated in small and large (colon) intestines. The colon length was measured using a ruler, weighed and sliced longitudinally in four segments. One segment was rolled (the Swiss rolling method), fixed in 4% paraformaldehyde (at least 24h) and then transferred for ethanol 70% for histological analysis (Bialkowska et al., 2016). The other segments were stored at -80 °C for MPO analysis while one fragment from each animal was weighed and stored at -80 °C in TCA (5%, 1 ml/fragment) for reduced glutathione (GSH) analysis. The disease activity index (DAI) was calculated by the daily combined scores attributed to body weight loss (0: without loss; 1: 5 a 10%; 2: 11 a 15%; 3: 16 a 20%; 4: >20%), bleeding presence (0: normal colored stools; 1: brown stools; 2: red colored stools; 3: bloody stools) and stool consistency (0: stool with normal consistency; 1: moderately pasty stools; 2: pasty stools; 3: diarrhea) (Melgar et al., 2005; Yao et al., 2010; Rose et al., 2012; Amaral et al., 2013; Hiratsuka et al., 2013).

#### 2.4.8.1 Histological analysis

Fixed colon segments were paraffin embedded, sectioned (7-µm thick) and stained with hematoxylin and eosin (HE). One slide with two sections were prepared for each animal, with blind examination by one of the authors (E.C.S. de Oliveira) using a Leika CME optic microscope. Representative microphotographs were acquired using one Axiovert25 optic microscope (Zeiss) at 20x magnification coupled to AxioCam ERc5s camera (Zeiss) and Zen Lite 2 (Blue edition) software.

### 2.4.9 Biochemical evaluations

#### 2.4.9.1 Determination of Glutathione levels (GSH)

Portions of stomach or duodenum, from each experimental group, were weighed and homogenized in trichloroacetic acid (5%, 1:20 w/v) by turbolization (IKA, Disperser T 10 Basic, Staufen, Germany). After centrifugation (2000 *g*, 10 min, 4 °C), aliquots of each supernatants (20 µL/well, in duplicate) were added in 96-well microplate and mixed with NADPH (0.248 mg/mL, 140 µL/well), potassium phosphate buffer (50 mM, pH 6.0, 5 µL/well) and 5.5 '-Ditiobis-2-nitrobenzoic acid (DTNB 0.01 M, 20µL/well). After 5 min, at room temperature and protected from light, the absorbance was readed at 412 nm (VersaMax spectrophotometer, Molecular Devices). Using a glutathione calibration curve, the results (average ± standard deviation) were expressed as glutathione equivalent (µg) *per gram* of tissue (Anderson, 1985).

#### 2.4.9.2 Myeloperoxidase activity (MPO)

After thawing and weighting, fragment of stomach or duodenum was homogenized by turbolization (IKA, Disperser T 10 Basic, Staufen, Germany) with HTAB solution (0.5% in PBS 50 mM pH 6.0, 1:20 w/v). After freezing and thawing three times, the homogenates were centrifuged (7000 *g*, 10 min, 4 ºC). Two aliquots (50 µL/well) of each supernatant were transferred to 96-well plate and mixed with reactive solution [150 µL/well; *o*-dianisidine dichloridrate (0.167 mg/mL) and $H_2O_2$ (0.0005%) in PBS 50 mM pH 6.0]. Absorbance at 450 nm was recorded using VersaMax (Molecular Devices) microplate reader adjusted to 37 ºC. Comparing to one horseradish peroxidase calibration curve (0.15 – 78 U), results were expressed as U/g of tissue (Krawisz et al., 1984).

#### 2.4.9.3 Cytokynes (TNF-α, IL-1β, IL-10) and Chemokynes (MIP-2) assays

After thawing, each fragment was homogenized by turbolization (IKA, Disperser T 10 Basic, Staufen, Germany) in extraction buffer [RIPA (1:3 w/v) plus protease inhibitor (1:0.03 w/v) and PBS (50 mM pH 6.0) q.s. 700 µL/sample] and centrifuged (2000 *g*, 10 min, 4 ºC). Protein level was determined by Bradford method and tissue levels of IL-1β, IL-6, IFN-γ, and TNF-α were determined using singleplex immunoassay with the Bio-Plex Pro cytokine assay kit (IL-1*β* cod.171L1008M, IL-10 cod.171L1014M, CXCL-MIP-2 cod.171L1022M, TNF-α cod.171L1025M, Bio-Rad) and the Bio-Plex 200 system (Bio-Rad), following the manufacturer's instructions. The results were expressed as pg/mg protein.

### 2.5 Statistical Analyses

The results were expressed as mean ± standard error. The difference between the averages was determined by analysis of variance (ANOVA) [one-way ANOVA followed by the Tukey, Kruskal-Wallis test followed by Dunn's test (for histological score) or two-way ANOVA followed by Bonferroni test] depending on the analysis. The analyses were performed using GraphPadPrism software version 5.0 (GraphPad Software). In all experiments $p < 0.05$ was considered significant.

## 3. Results:

### 3.1. *In vitro* Pharmacological evaluation

#### 3.1.1 Antiproliferative activity in cell tumoral panel

Antiproliferative effect of EOMa, *R*-(-)-Carvone and *S*-(+)-Carvone was evaluated against a panel (8 tumors and 1 non-tumor) of human cell lines and expressed as the concentration (µg/mL) required to inhibit cell proliferation by 50% (Table 1, Figure 1S).

Considering $GI_{50}$ values higher than 30 µg/mL as representative of inactivity (Fouche et al, 2008), the essential oil of *M. aquatica* aerial parts containing 54.82 ± 1.39% of carvone was inactive for all cell lines tested (Table 1, Figure 1S). Only *S*-(+)-Carvone inhibited selectively the proliferation of PC-3 cells (human adenocarcinoma of prostate, $GI_{50}$ = 13.1 µg/mL) and HaCaT (immortalized human keratinocytes, $GI_{50}$ = 16.8 µg/mL).

Table 1- The *in vitro* antiproliferative activity of *Mentha aquatica* essential oil, carvone enantiomers and doxorubicin expressed as $GI_{50}$ values ($GI_{50}$, µg/mL).

| | **Cell lines** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Samples** | **2** | **M** | **a** | **7** | **4** | **p** | **h** | **k** | **q** |
| **Doxorubicin#** | 0.030 | <0.025 | 0.49 | 0.044 | <0.025 | 0.063 | 0.13 | 0.14 | 0.025 |
| **EOMa** | >250 | 69.2 | >250 | >250 | >250 | >250 | >250 | >250 | 36.8 |
| ***R*-(-)-Carvone** | >250 | 70.6 | >250 | >250 | >250 | 94.6 | >250 | 64.1 | 30.8 |
| ***S*-(+)-Carvone** | 248.1 | 41.1 | 239.7 | >250 | >250 | 13.1 | 182.3 | 65.7 | 16.8 |

EOMa: essential oil of *Mentha aquatica* aerial parts; Human tumor cell lines: 2 = U251 (glioma), m = MCF-7 (breast); a = NCI-ADR/RES (ovary with multidrug resistance phenotype); 7= 786-0 (kidney); 4 = NCI-H460 (lung, non-small cell type); p = PC-3 (prostate); h = HT-29 (colon); k = K562 (leukemia);

Human non-tumor cell line: q= HaCat (immortalized keratinocytes)

#Doxorubicin = positive control.

$GI_{50}$: *Growth Inhibition 50* – concentration required to inhibit 50% of cell growth after 48h exposition. The values were determinate by nonlinear regression analysis using Origin ® 8.0 (OriginLab Corporation).

### 3.1.2 Scratch assay

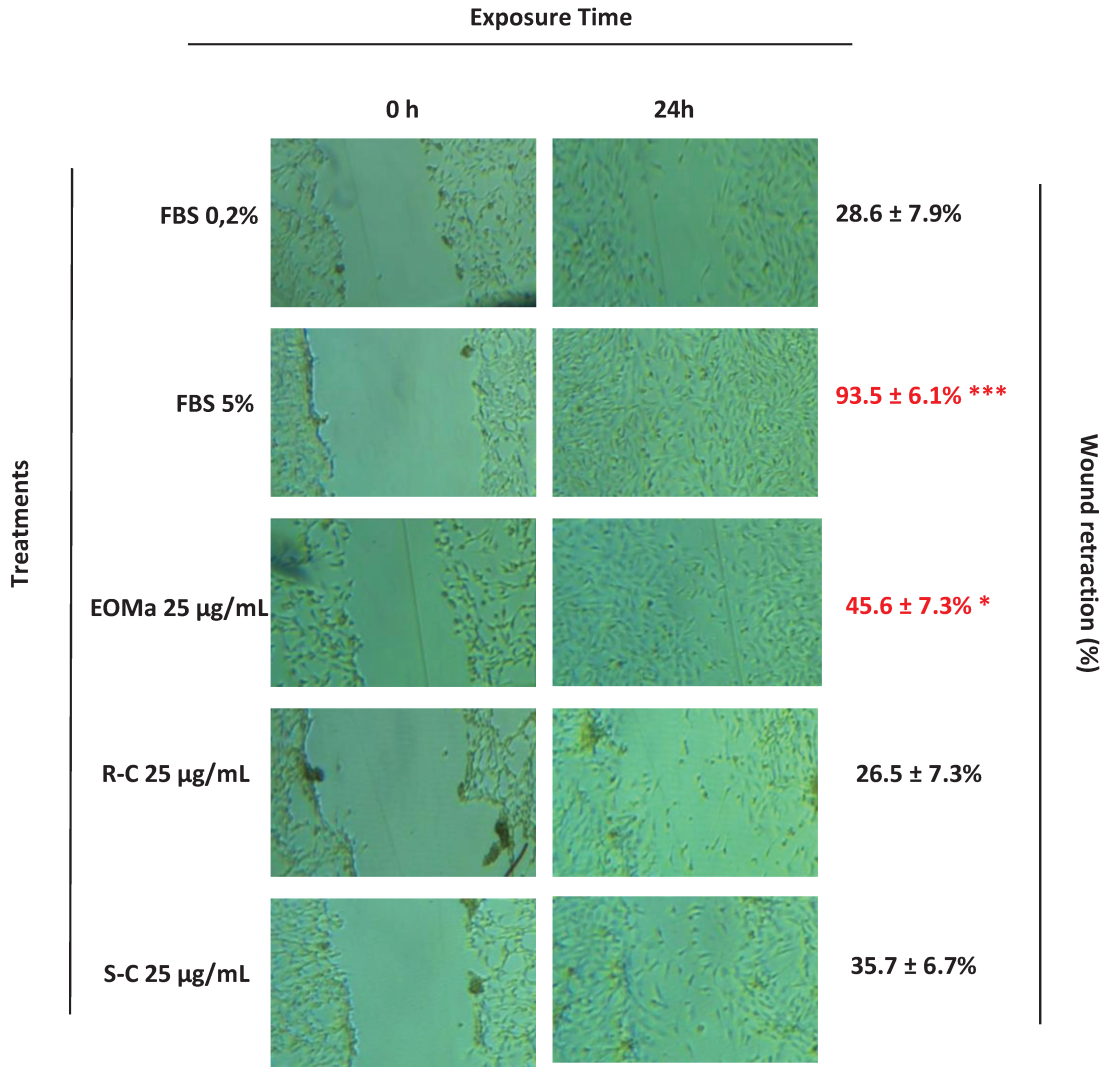
The positive control, medium RPMI 1640 supplemented with FBS 5%, induced HaCaT migration in a time dependent way reaching almost 94% of wound closure after 24 h (Table 2, Figure 1). Based on the antiproliferative activity assay, sample concentrations were established from 0.25 to 25 µg/mL. The EOMa promoted a significant wound closure at 25 µg/mL after 24h-exposure (45.6 ± 7.3%). More, both carvone enantiomers showed a similar profile to that observed for cells treated with medium RPMI 1640 supplemented with FBS 0.2% (Table 2, Figure 1).

Table 2- Analysis of HaCaT (immortalized human keratinocytes) migration (%) induced by *Mentha aquatica* essential oil and carvone enantiomers after 9, 18 and 24 h-exposure.

| **Treatment** | **Concentration (µg/mL)** | **Wound retraction (%)** | | |
|---|---|---|---|---|
| | | **9h** | **18h** | **24h** |
| **FBS** | 0.2# | 6.1 ± 4.0 | 20.6 ± 8.2 | 28.6 ± 7.9 |
| | 5.0# | 48.5 ± 8.3*** | 79.5 ± 16.2*** | 93.5 ± 6.1*** |
| **EOMa** | 0.25 | 9.1 ± 1.9 | 22.8 ± 4.1 | 31.0 ± 2.6 |
| | 2.5 | 8.8 ± 5.7 | 25.8 ± 8.3 | 39.3 ± 1.3 |
| | 25 | 9.2 ± 4.6 | 26.4 ± 7.9 | 45.6 ± 7.3* |
| ***R*-(-)-Carvone** | 0.25 | 14.46 ± 0.02 | 24. 5 ± 9.0 | 34.1 ± 6.8 |
| | 2.5 | 3.3 ± 3.6 | 15.8 ± 6.0 | 29.2 ± 8.2 |
| | 25 | 5.1 ± 4.3 | 15.5 ± 9.5 | 26.5 ± 7.3 |
| ***S*-(+)-Carvone** | 0.25 | 3.4 ± 2.5 | 12.5 ± 3.1 | 27.4 ± 1.7 |
| | 2.5 | 3.2 ± 2.7 | 22.5 ± 7.6 | 34.9 ± 2.36 |
| | 25 | 12.7 ± 5.1 | 22.9± 4.0 | 35.7 ± 6.7 |

FBS = fetal bovine serum, # concentration expressed in percentage; EOMa: essential oil of *Mentha aquatica* aerial parts. Results expressed as average ± SEM from triplicates of one experiment. Statistical analysis by two-way ANOVA followed by Bonferroni test (* p <0.05, *** p<0.001 related to FBS 0.2%-treated cells).

Figure 1- Representative photomicrographs of *Mentha aquatica* essential oil, *R*-(-)-Carvone and *S*-(+)-Carvone) effect on HaCaT migration (%) after 24-h exposure.




FBS = fetal bovine serum at 0.2% (negative control) and 5% (positive control) in RPMI 1640 medium; EOMa: essential oil of *Mentha aquatica* aerial parts at 25 µg/mL; R-C: *R*-(-)-Carvone at 25 µg/mL; S-C: *S*-(+)-Carvone at 25 µg/mL.

## 3.2 *In vivo* evaluation

### 3.2.1 Acute Oral Toxicity Study

During the first four-hour observation, one animal treated with *S*-(+)-Carvone (300 mg/kg) presented uncoordinated movements, looking drunk, while one animal treated with *R*-(-)-Carvone (300 mg/kg) presented straub tail. Twenty-four hours after, all these signal disappeared and following the clinical observation during 14 days, none toxic signal were evidenced for any treatment. Also, both treatments with *S*-(+)-Carvone or *R*-(-)-Carvone did not promote any significant changes in body weight evolution in comparison to

Vehicle group (Table 3). Considering the relative organs weight at the end of the experiment, animals treated with *R*-(-)-carvone (30mg/kg) showed a significant increase in spleen in comparison to vehicle group (Table 3) and non-significant difference was observed for kidneys and liver, independent of treatment (Table 2S).

Table 3- Body weight evolution and relative spleen weight in the oral acute toxicity evaluation of the carvone enantiomers.

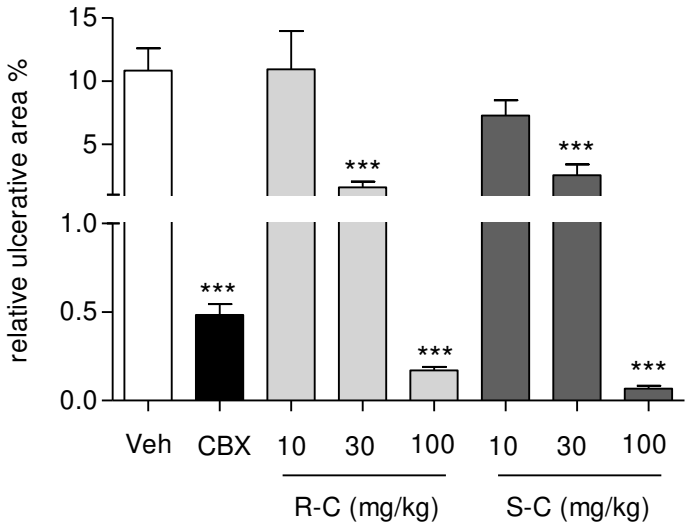
| Groups | Dose (mg/kg) | Body Weight | | | | | RSW (%)[b] |
|---|---|---|---|---|---|---|---|
| | | 1st day | 7th day | Δ[a] | 14th day | Δ[a] | |
| **Vehicle** | 10[#] | 24.6 ± 1.1 | 26.3 ± 1.6 | 1.7 ± 1.1 | 27.5 ± 1.7 | 2.9 ± 1.0 | 0.38 ± 0.06 |
| ***R*-(-)-Carvone** | 30 | 23.8 ± 1.5 | 24.9 ± 2.7 | 1.0 ± 1.4 | 26.1 ± 2.8 | 2.3 ± 1.5 | 0.58 ± 0.21* |
| | 100 | 24.1 ± 0.8 | 25.7 ± 0.5 | 1.6 ± 0.5 | 27.6 ± 0.7 | 3.52 ± 0.9 | 0.39 ± 0.03 |
| | 300 | 26.7 ± 1.2 | 27.1 ± 1.3 | 0.4 ± 1.0 | 30.1 ± 2.5 | 3.4 ± 1.8 | 0.41 ± 0.07 |
| ***S*-(+)-Carvone** | 30 | 25.3 ± 2.2 | 26.4 ± 2.2 | 1.1 ± 1.0 | 28.7 ± 2.1 | 3.3 ± 1.0 | 0.38 ± 0.09 |
| | 100 | 25.2 ± 2.7 | 27.1 ± 2.9 | 1.9 ± 1.0 | 28.6 ± 3.2 | 3.5 ± 1.4 | 0.45 ± 0.09 |
| | 300 | 24.8 ± 2.3 | 26.4 ± 1.9 | 1.6 ± 0.9 | 28.0 ± 2.5 | 3.2 ± 0.6 | 0.41 ± 0.03 |

Results expressed as average ± standard deviation; [#]: expressed in mL/kg; Body weight (g) of female mice Swiss (8 weeks old, n = 5 animals/group) evaluated at 1st, 7th and 14th experimental day; Oral treatment: Vehicle (10 mL/kg, positive control), *R*-(-)-Carvone (30, 100, 300 mg/kg), *S*-(+)-Carvone (30, 100, 300 mg/kg). (a) = body weight variation between the experimental day (7th or 14th day) and basal evaluation (at 1st day); (b) = RSW (%) = relative spleen weight [spleen weight (g)/spleen weight (g) x 100]. Statistical analysis of body weight variation by two-way ANOVA followed by Bonferroni test ($p>0.05$ related to the vehicle group); statistical analysis of RSW by one-way ANOVA followed by Tuckey test (* $p<0.05$ related to the vehicle group).

### 3.2.3 Gastroprotective effects of carvone enantiomers.

Based on the oral acute toxicity evaluation, 100 mg/Kg was considered as the maximum tolerable dose (MTD) for carvone enantiomers and two doses were proposed (1/3 and 1/10 of MTD). At this dose range, both *R*-(-) and *S*-(+)-Carvones inhibited the ethanol-induced gastric ulcer in a dose-dependent way and independent on the stereogenic configuration. At highest dose (100 mg/kg), both enantiomers protected gastric mucosa (ulcerative inhibition = 98.4 and 99.4 %, for *R*-(-)- and *S*-(+)-Carvone, respectively) similarly to carbenoxolone (200 mg/Kg, positive control, ulcerative inhibition = 95.5%) (Figure 2, Table 3S). The dose required to reduce in 50% the ethanol-induced ulcerative damage ($ED_{50}$) was calculated as 16.2 and 25.6 mg/Kg for *S*-(+)- and *R*-(-)-Carvones, respectively.

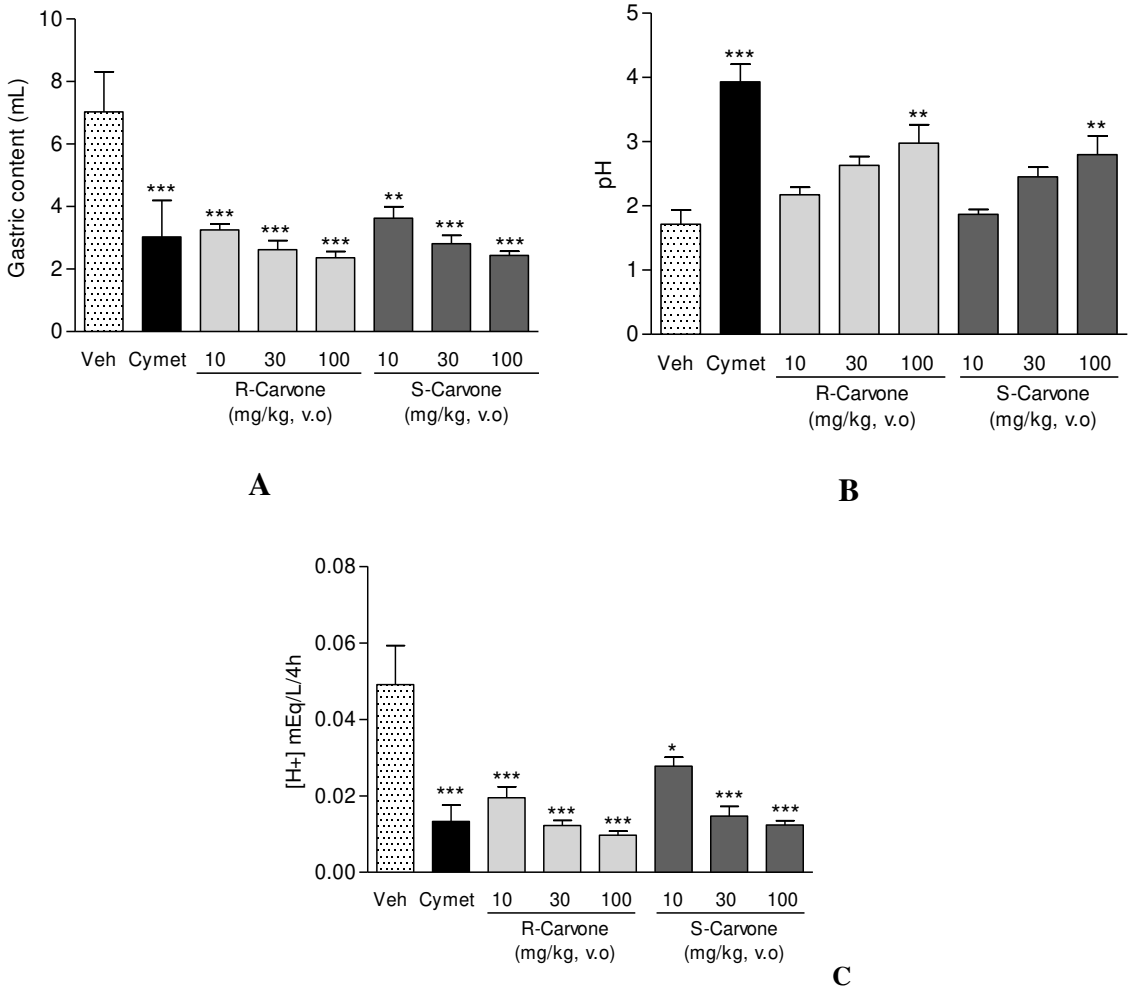
Figure 2- Antiulcerogenic effect of carvone enantiomers in the ethanol-induced gastric ulcer model in rats.




Results expressed as mean ± SEM (n = 7 male Wistar rats/group). Relative ulcerative area = [ulcerative gastric area ($mm^2$)/total gastric area ($mm^2$)] x 100, measured using ImageJ® software and calculated in Excel software. Oral treatments: Veh = Vehicle (PBS pH 7.0 with Tween 80 0.5%, 10 mL/kg); CBX = Carbenoxolone (200 mg/kg, positive control);R-C = *R*-(-)-Carvone (10, 30, 100 mg/kg), S-C = *S*-(+)-Carvone (10, 30, 100 mg/kg). Challenge with ethanol P.A. (0.4 mL/Kg, v.o.) 60 min after treatments. Statistical analysis by one-way ANOVA followed by Tukey's test (*** $p < 0.001$ compared to vehicle group).

Considering gastric secretion, both *R*-(-) and *S*-(+)-Carvones decreased the volume of gastric secretion, in a dose-independent way. Only at highest dose (100 mg/Kg), both *R*-(-) and *S*-(+)-Carvones reduced free acidity measured by pH values, despite both enantiomers were able to reduce total acidity, measured by hydrogenionic concentration, independent of the dose (Figure 3).

Figure 3- Effect of carvone enantiomers on gastric secretion, expressed as secretion volume (A), pH (B) and hydrogenionic concentration (C), in the pylorus ligature model.

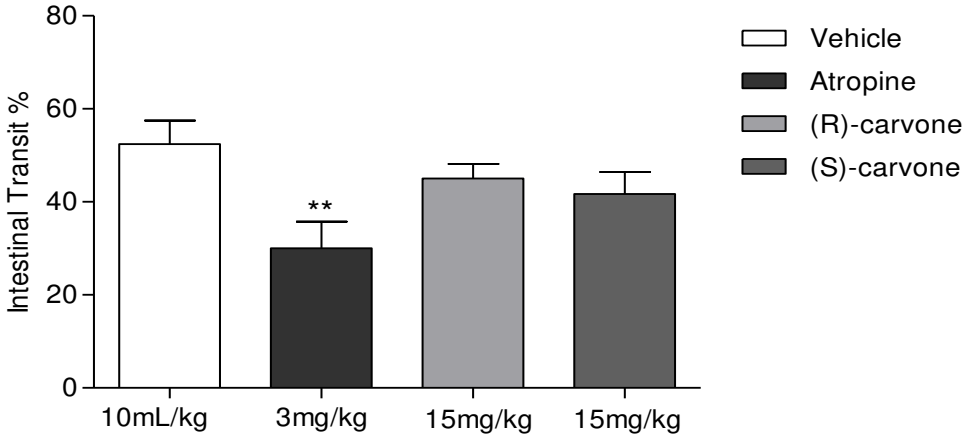



Results expressed as mean ± SEM (n = 7 male Wistar rats/group). A) Gastric juice volume; B) free acidity (pH); C) total acidity (hidrogenionic concentration). Intraduodenal treatments: Veh = Vehicle (PBS pH 7.0 with Tween 80 0.5%, 10 mL/kg); Cymet: Cimetidine (100 mg/Kg, positive control); *R*-(-)-Carvone (10, 30, 100 mg/kg), *S*-(+)-Carvone (10, 30, 100 mg/kg). Statistical analysis by one-way ANOVA followed by Tuckey's test (* $p < 0.05$, ** $p<0.01$, *** $p < 0.001$ compared to vehicle group).

### 3.2.4 Effect of carvone enantiomers on the activated charcoal gastrointestinal motility model

While atropine (3.0 mg/kg) promoted a significantly reduction on the intestinal motility both *R*-(-)- and *S*-(+)-carvone were inactive when tested in doses nearby the gastroprotective $ED_{50}$ of *S*-(+)-carvone (Table 4).

Figure 4- Effect of *R*-(-)-carvone, *S*-(+)-carvone and atropine in the activated charcoal gastrointestinal motility model.




Results expressed as mean ± SD (n = 10 male Wistar rats/group). #: mL/kg; ## Effect = inhibition (negative value) or stimulation (positive value). Oral treatments: Vehicle (PBS pH 7.0 with Tween 80 0.5%, 10 mL/kg); Atropine (3 mg/Kg, positive control); *R*-(-)-Carvone (15 mg/kg), *S*-(+)-Carvone (15 mg/kg). Challenge with activated charcoal (0.4 mL/Kg, 2 % in PBS, v.o.) 60 min after treatments. Statistical analysis by one-way ANOVA followed by Tukey's test (** $p<0.01$ compared to vehicle group).

### 3.2.4 Effect of carvone enantiomers on dose-repeated ASA-induced gastric ulcer.

As the MTD for acute treatment was determined as 100 mg/Kg for both carvone enantiomers, for the dose-repeated treatment the higher dose was estimated as the fourth part of MTD (25 mg/Kg) that corresponded to the gastroprotective $ED_{50}$ of *R*-(-)-carvone in the ethanol-induced gastric ulcer model.

The ASA challenge promoted significant reduction on body weight gain in comparison to CMC-treated animals (Satellite group). Treatment with *S*-(+)-carvone promoted a partial reversion of the ASA-loss weighting in rats. Ranitidine and *R*-(-)-carvone were not able to improve significantly the body weight gain (Table 5, Figure 2S).

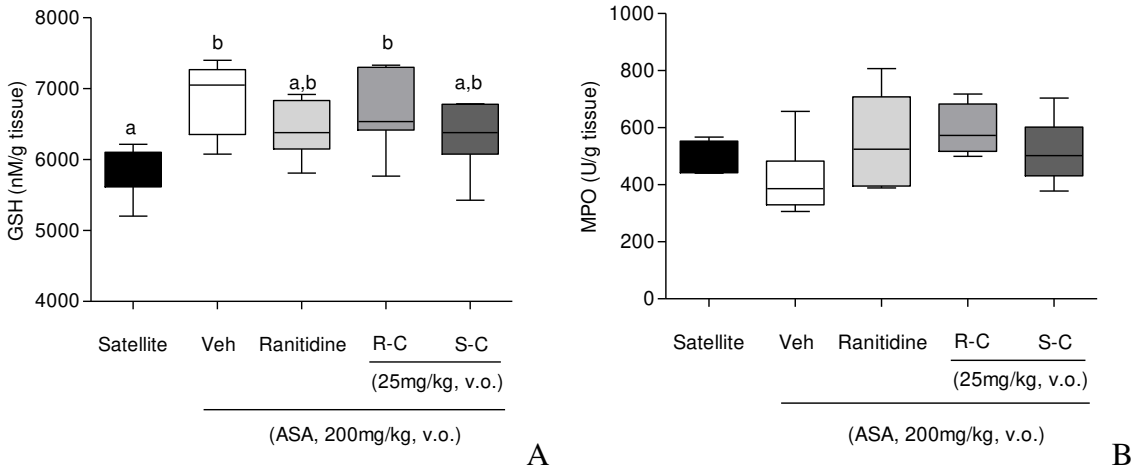
Table 4- Animal body weight variation in the dose-repeated acetylsalicylic acid ASA-induced ulcer model.

| | Day | Vehicle | Satellite | Ranitidine | *R*-(-)-Carvone | *S*-(+)-Carvone |
|---|---|---|---|---|---|---|
| Body weight variation (g) | 3rd | -15.1 ± 15.8[a] | 7.1 ± 1.5[b,c] | -10.4 ± 14.5[a,c] | -5.3 ± 8.6[a,c] | -8.0 ± 19.0[a,c] |
| | 5th | -17.1 ± 18.3[a] | 8.4 ± 3.6[b] | -12.7 ± 15.8[a,c] | -4.6 ± 13.2[a,b,c] | -6.3 ± 15.4[a,b,c] |
| | 7th | -14 ± 15.6[a] | 14.6 ± 5.1[b,c] | -5.3 ± 22.3[a] | -0.14 ± 11.8[a,c] | -2.3 ± 13.0[a,c] |
| | 8th | -8.4 ± 14.1[a] | 19.1 ± 5.3[b] | -5.3 ± 15.2[a] | 9.6 ± 12.2[a] | 5.0 ± 16.6[a] |
| | 12th | -2.3 ± 16.0 [a] | 28.6 ± 7.4 [b,c] | 2.8 ± 13.7 [a] | 7.4 ± 15.5 [a] | 11.6 ± 11.6 [a,c] |
| | 14th | -2.7 ± 14.2 [a] | 23.1 ± 6.4 [b,c] | -2.6 ± 10.1 [a] | 5.1 ± 14.0 [a,c] | 8.7 ± 13.8 [a,c] |
| | 15th | 7.1 ± 14.8 [a] | 36.9 ± 8.0 [b,c] | 6.7 ± 14.4 [a] | 10.0 ± 16.0 [a] | 19.8 ± 11.4 [a,c] |

Results expressed as mean ± SD (n = 7 male Wistar rats/group). Challenge: Acetylsalicylic acid [ASA. 200 mg/kg, diluted in Carboxymethyl cellulose (CMC) 0.2% in PBS, p.o., once a day, 15 days]. Treatments (starting on 8th experimental day, diluted in vehicle, p.o., once a day. 7 days). Treatment (v.o.): Vehicle = PBS pH 7.0 with Tween 80 0.5%, 10 mL/Kg; *R*-(-)-Carvone (25 mg/kg); *S*-(+)-Carvone (25 mg/kg); Ranitidine (2.5 mg/kg); Satellite (CMC 0.2% in PBS, 10 mL/Kg). Statistical analysis: two-way ANOVA followed by Bonferroni's test (different letters in the same row indicate significant difference).

Fourteen-day ASA-treatment (vehicle group) enhanced significantly the GSH tissue level in comparison to Satellite animals. Both ranitidine and *S*-(+)-carvone treatments promoted a partial reduction while treatment with *R*-(-)-carvone did not alter GSH levels in comparison to vehicle group (Figure 5). No significant difference in MPO activity was observed in any experimental group in comparison to satellite group (Figure 5). Corroborating the MPO analysis, there were no changes in the IL-1β, MIP-2, TNF-α and IL-10 levels (Table 4S).

Figure 5 -Effect of *R*-(-)-carvone, *S*-(+)-carvone and ranitidine on reduced glutathione level and myeloperoxidase activity in the dose-repeated ASA-induced ulcer model.

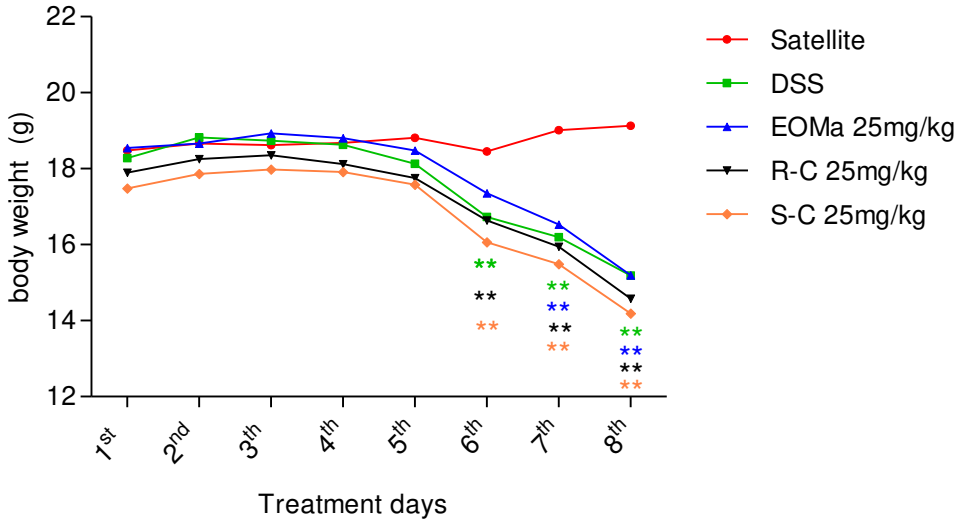



Results expressed as mean ± SEM (n = 7 male Wistar rats/group); A) GSH = glutathione reduced (µM/g tissue) stomach and B) MPO = mieloperoxidase activity (U/g tissue) stomach. Challenge: Acetylsalicylic acid [ASA. 200 mg/kg, diluted in Carboxymethyl cellulose (CMC) 0.2% in PBS, p.o., once a day, 15 days]. Treatments (starting on 8$^{th}$ experimental day, diluted in vehicle, p.o., once a day. 7 days). Treatment (v.o.): Vehicle = PBS pH 7.0 with Tween 80 0.5%, 10 mL/Kg; *R*-(-)-Carvone (25 mg/kg); *S*-(+)-Carvone (25 mg/kg); Ranitidine (2.5 mg/kg); Satellite (CMC 0.2% in PBS, 10 mL/Kg). Statistical analysis: one-way ANOVA followed by Tukey's test (different letters indicate a significant difference, $p < 0.05$ at least).

### 3.2.4 Effect of carvone enantiomers on Dextran Sodium Sulphate (DSS)-induced Colitis Model in Mice

The DSS-treatment promoted significant ($p < 0.001$) loss of body weight from the sixth experimental day. The EOMa treatment partially protected mice from DSS-induced loss weighting at sixth experimental day ($p > 0.05$ both in comparison to satellite and vehicle groups) while animals treated with both carvone enantiomers showed similar profile to DSS group (Figure 6).

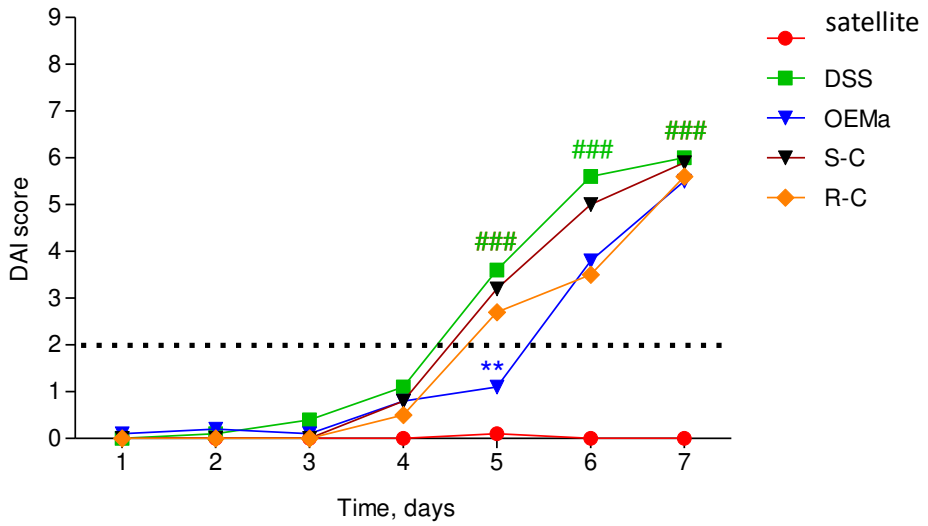
Figure 6- Effect of *Mentha aquatica* essential oil, *R*-(-)-carvone and *S*-(+)-carvone on mice body weight evolution during the DSS-induced colitis model.




Results expressed as mean ± SEM (n = 10 male C57BL/6J mice/group. DSS challenge = DSS 3% in potable water (*ad libitum*) during 8 days. Treatments (from 4$^{th}$ to 7$^{th}$ experimental day): Satellite (potable water *ad libitum* without DSS exposition); Vehicle = PBS (pH 7.0 with Tween 80 0.5%, 10 mL/Kg, v.o.); EOMA = essential oil of *Mentha aquatica* aerial parts (25 mg/Kg, v.o.); *R*-(-)-Carvone (25 mg/kg v.o.); *S*-(+)-Carvone (25 mg/kg v.o.). Statistical analysis: two-way ANOVA followed by Bonferroni's test (** $p < 0.01$ in comparison to Satellite group).

The disease activity index (DAI) was obtained from mice clinical observations during the experiment. Animals with DAI ≥ 2 were considered as representative of colitis presence. Only EOMa treatment significantly ($p < 0.05$) delayed the DAI improvement in one day, despite all DSS-treated animals reached DAI ≥ 2 at the end of DSS-exposition (Figure 7).

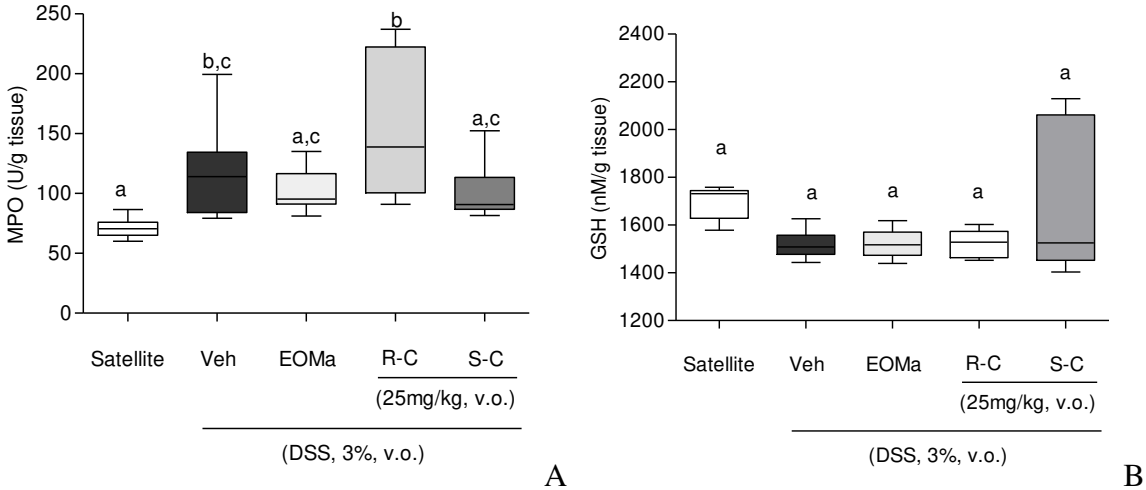
Figure 7. Effect of *Mentha aquatica* essential oil, *R*-(-)-carvone and *S*-(+)-carvone on disease activity index (DAI) during the DSS-induced colitis model.




Results expressed survival curve (n = 10 male C57BL/6J mice/group. DSS challenge = DSS 3% in potable water (*ad libitum*) during 8 days. Treatments (from 4th to 7th experimental day): Satellite (potable water *ad libitum* without DSS exposition); Vehicle = PBS (pH 7.0 with Tween 80 0.5%, 10 mL/Kg, v.o.); EOMA = essential oil of *Mentha aquatica* aerial parts (25 mg/Kg, v.o.); *R*-(-)-Carvone (25 mg/kg v.o.); *S*-(+)-Carvone (25 mg/kg v.o.). Statistical analysis: two-way ANOVA followed by Bonferroni's test (# $p < 0.05$, ## $p<0.01$, ### $p < 0.001$ compared to Satellite group and * $p < 0.05$, ** $p<0.01$, *** $p < 0.001$ compared to DSS group).

DSS-induced colitis promoted significant reduction on relative colon weight and length in comparison to animals in Satellite group. All treatments (EOMa, *R*-(-)- and *S*-(+)-carvones) were not able to revert the DSS effect (Figure 3S). Analysis of colon homogenates indicated that DSS exposure increased MPO activity in colonic tissue beside no significant influence on GSH level, comparing to animals in Satellite group. The EOMa and *S*-(+)-carvone treatments partially reduced MPO activity in colon while *R*-(-)-carvone did not affect this parameter in comparison to animals in DSS-group (Figure 8).

Figure 8- Effect of *Mentha aquatica* essential oil, *R*-(-)-carvone and *S*-(+)-carvone on GSH level and MPO activity in the DSS-induced colitis model.

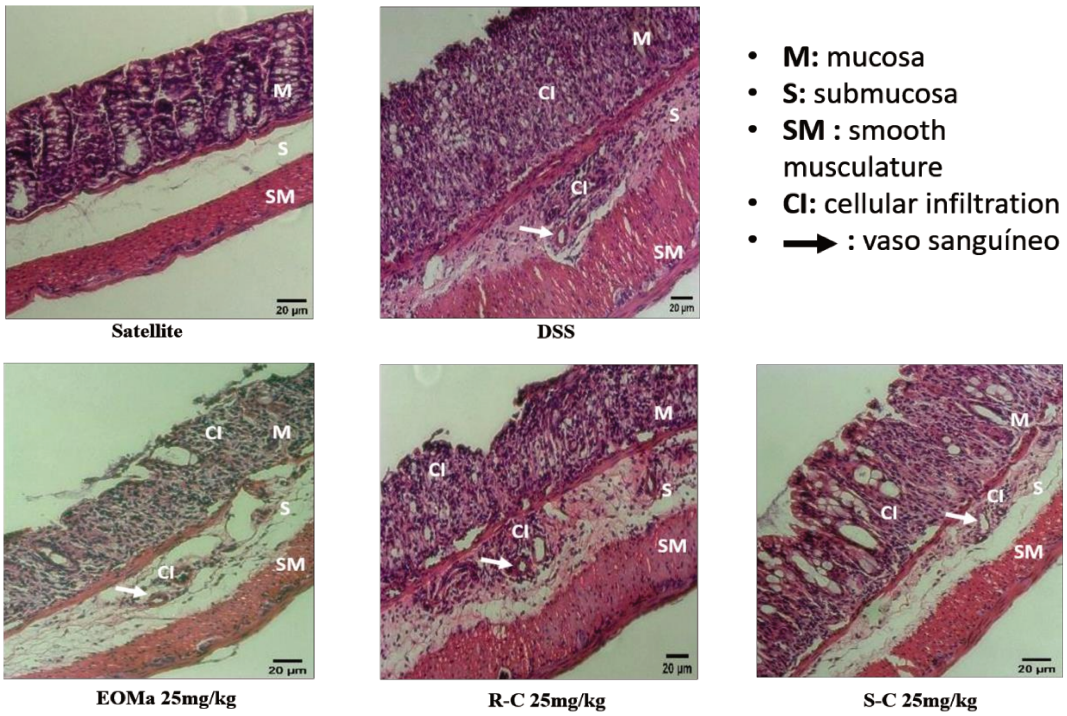


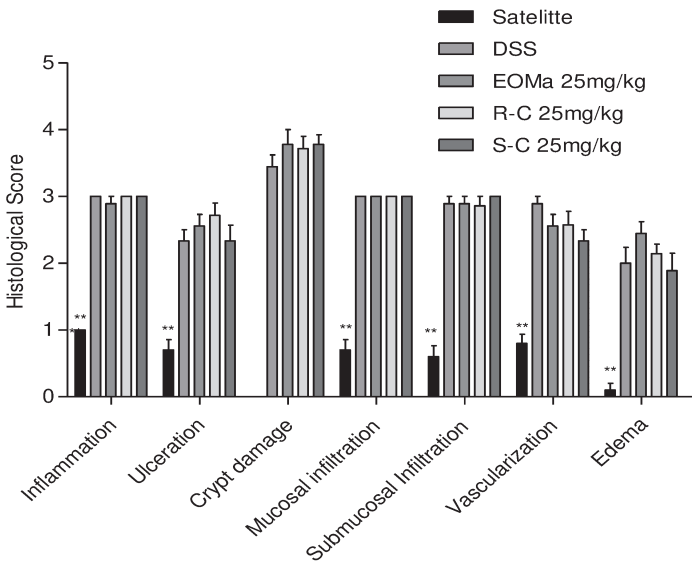

Results expressed as mean ± SEM (n = 10 male C57BL/6J mice/group. A) MPO (Mieloperoxidase) activity expressed as U/g tissue, B) GSH (reduced Glutathione) level expressed as nM/g tissue. DSS challenge = DSS 3% in potable water (*ad libitum*) during 8 days. Treatments (from 4$^{th}$ to 7$^{th}$ experimental day): Satellite (potable water *ad libitum* without DSS exposition); Vehicle = PBS (pH 7.0 with Tween 80 0.5%, 10 mL/Kg, v.o.); EOMA = essential oil of *Mentha aquatica* aerial parts (25 mg/Kg, v.o.); *R*-(-)-Carvone (25 mg/kg v.o.); *S*-(+)-Carvone (25 mg/kg v.o.). Statistical analysis: one-way ANOVA followed by Tukey's test (different letter indicate significant difference).

The histological evaluations showed that the animals in DSS-group presented a severe and intense inflammatory process, characterized by mucosal epithelium alteration, increased inflammatory cell infiltration, both in the mucosa and in the submucosa. It is also possible to observe the presence of edema together with loss of crypts in the mucosa (Figure 9). Despite the partial reversion on DSS-increased MPO activity promoted by EOMa and *S*-(+)-carvone treatments, none treatment has been able to reverse the DSS-induced damage.

Figure 9- Histopathological analysis of colon tissues in the DSS-induce colitis model after *Mentha aquatica* essential oil, *R*-(-)-carvone and *S*-(+)-carvone treatments.

**A) H & E Stain**




**B**

A) Representative photomicrographs (H&E stain, 20x) of each experimental group; B) Histopathological score (results expressed as average ± SEM, Statistical analysis: ANOVA by Kruskal-Wallis test followed by Dunn's test (*p < 0.05, **p<0.01, ***p < 0.001 compared to Satellite group). Experimental groups: Satellite group (potable water *ad libitum* without DSS exposition); DSS group (DSS challenge + PBS pH 7.0 with Tween 80 0.5%, 10 mL/Kg, v.o.); EOMa = essential oil of *Mentha aquatica* aerial parts group (DSS challenge + 25 mg/Kg, v.o.); R-C = *R*-(-)-Carvone group (DSS challenge + 25 mg/kg v.o.); S-C = *S*-(+)-Carvone group (DSS challenge + 25 mg/kg v.o.). DSS challenge = DSS 3% in potable water (*ad libitum*) during 8 days; Treatments (from 4$^{th}$ to 7$^{th}$ experimental day, PBS, EOMa, *R*-(-)-Carvone or *S*-(+)-Carvone).

## 4. Discussion and Conclusion

The present study showed the gastroprotective effect of the monoterpene carvone, with significant differences between *S*-(+)-carvone and *R*-(-)-carvone isomers, demonstrating that the stereoisomerism has an important influence on the pharmacological activities of carvone in different models of gastric lesions. More, these results pointed out to the contribution of carvone to the beneficial effects of the *Mentha aquatica* essential oil previously evidenced (Braga, 2016).

First, we evaluated the *in vitro* effects of EOMa and carvone enantiomers on cell proliferation and migration. The observed differences on antiproliferative profile (*R*-(-)-carvone inactive while *S*-(+)-carvone inhibited selectively the proliferation of PC-3 and HaCaT cell lines) could be explained by the changes in stereogenic center that can affect pharmacological properties (Wainer, 1992; Finefield et al., 2012). Despite been described as weak cytostatic agent against MCF-7 (breast adenocarcinoma, $GI_{50}$ = 24.96 µg/mL) (Bicas et al., 2011), in our study *R*-(-)-carvone required 70.6 µg/mL to inhibit in 50% the MCF-7 proliferation. These differences can be partially due to differences in cell sensibility during each experiment.

Following the *in vitro* analysis, the influence of EOMa and both carvone enantiomers on keratinocytes migration was evaluated using the "scratch" assay (Liang et al., 2007). Wound healing is a complex process involving many mechanisms and didactically separated in hemostasis, inflammation, proliferation and remodeling steps. Together with some growth factors (EGF and TGF-β1), cytokines (IL-1α, IL-1β, IL-4) and vascular endothelial growth factor (VEGF), keratinocytes are important for maintenance and restoration of epidermis integrity (Peplow and Chatterjee, 2013).

Although both carvone enantiomers did not promote cell migration, the EOMa induced *in vitro* wound retraction. Chemical analysis of EOMa using GC-MS showed the presence of limonene and 1,8-cineole together with carvone, as the main compounds (Braga, 2016). According to literature, both limonene and 1,8-cineole have been described as main component of essential oils with wound healing properties (Pérez-Recalde et al., 2018). The presence of these two monoterpenes could partially explain the EOMa effect on HaCaT migration.

The *in vitro* evaluation on HaCaT cells was also important to estimate the lethal oral dose for both carvone enantiomers allowing a rational way to select the higher dose to be evaluated in *in vivo* model. The acute systemic toxicity evaluation is important to evidence

the role of pharmacokinetic parameters such as absorption and metabolism in toxicological effects. Once established the maximum tolerable dose (MTD), pharmacological evaluations could be done using safe doses (Walum, 1998; Parasuraman, 2011).

During the initial phase of acute oral toxicity assay, 20% of animals in higher doses of both *S*-(+)-carvone and *R*-(-)-carvone presented a reversible alteration on locomotion activity suggesting action on the central nervous system (CNS) and/or on musculoskeletal system. Previous studies with animals pre-treated with analeptics or sedative drugs showed that both carvone enantiomers increased the sedative response and the isomer *R*-(-)-carvone showed more potent than *S*-(+)-carvone, maybe due to the blockade of voltage-gated sodium channels and anxiolytic-like effects (Buchbauer et al., 2005; de Sousa et al., 2007; Nogoceke et al., 2016).

Continued observation showed no alteration in the clinical parameters beside none significant variations on body weight during all the experiment. Relative organ weight evaluation is a parameter that might suggest histopathological alterations (Sellers et al., 2007). During necropsy, *S*-(+)-carvone or *R*-(-)-carvone-treated animals showed relative organ weights similar to those observed for vehicle-treated animals corroborating no significant alteration observed on internal organ aspects. The exception were the spleens; one animal treated with *R*-(-)-carvone, at 30 mg/Kg, showed increased relative spleen weight resulting in significant increase for the group average. Therefore, it was not possible to determine whether this animal already had a basal spleen disorder or was a hyper reactive animal.

Based on the oral acute toxicity assay, the maximum tolerated dose (MTD) was considered as 100 mg/Kg for both carvone enantiomers and was assumed as the highest dose to be evaluated in pharmacological studies.

Previously, we demonstrated that EOMa showed potent antiulcerogenic effect in the ethanol-induced ulcer model together with anti-secretory activity (Braga, 2016). Being the major component of EOMA, both *S*-(+)-carvone and *R*-(-)-carvone were evaluated in the same models. The ethanol-induced ulcer model is one of the most representative models of human gastric ulcers. After one single dose, ethanol is rapidly absorbed by the gastrointestinal mucosa promoting disturbances in the mucus-bicarbonate barrier and consequently increasing the epithelial permeability. Therefore, there is enhanced gastric acid effect on epithelial cells resulting in cellular exfoliation and rupture in the blood vessels wall. In addition, there are release of reactive oxygen species due oxidative stress together with

alteration on chemical mediators that contribute to the ulceration appearance (Ko and Cho, 1998; Siegmund et al., 2003; Simões et al., 2019).

Therefore, it was possible to demonstrate the carvone participation in the antiulcerogenic effect of EOMa. More, there was no stereoisomerism influence in this effect as both *S*-(+)-carvone and *R*-(-)-carvone showed similarly antiulcerogenic profile.

As EOMa also modulated gastric secretion, we investigated the ability of *S*-(+)-carvone and *R*-(-)-carvone to interfere on acid gastric production. Different pathways are responsible to regulation of gastric secretion, such as neural, hormonal, paracrine, and intracellular routs. The acid gastric secretion is important to correct functioning of digestive system and to control microorganism overgrowth. As gastric secretion and/or acidity increasing contributes to the occurrence of ulcerative lesions, gastric secretion modulators can contribute to gastroprotective effect (Schubert, 2014, 2015; Al Asmari et al., 2015).Thus, both *S*-(+)-carvone and *R*-(-)-carvone were able to reduce both the secretion volume and total acidity corroborating for the anti-secretory effect of EOMa. Again, the stereoisomerism did not affect the pharmacological effect.

Both carvone enantiomers showed antispasmodic effects probably attributed to the blockade of $Ca^{2+}$ channels in smooth muscle cells with different potencies depending on the used model (Gonçalves et al., 2013; Souza et al., 2013; Silva et al., 2015). Interestingly, all these studies were challenge evaluations as the animals were exposed to one spasmodic agent before or after carvone treatment. In our study, naïve animals were treated with *S*-(+)-carvone and *R*-(-)-carvone demonstrating no influence on gastrointestinal motility and corroborating the absence of EOMa activity on intestinal motility.

Following the analyses, we investigated the carvone enantiomers ability to reverse pre-established ulcerative lesions induced by dose-repeated acetylsalicylic acid (ASA). As a non-steroidal anti-inflammatory drug (NSAIDs), ASA can promote mucosal injury by two pathways. First, as weakly acid, ASA can be absorbed by gastric epithelial cells and converted in the cytoplasm to ionized form. This accumulation can result in local mucosal injury (Laine et al., 2008). Second, as nonspecific COX-inhibitor, ASA can reduce prostaglandin PGE2 synthesis resulting in decreased mucus and bicarbonate production and secretion followed by microvascular disturbances, neutrophil activation, increased ROS concentration and mucosal injury (Vonkeman and van de Laar, 2010; Patel et al., 2012; Drini, 2017; Wang et al., 2018).

In our experiment, ASA-treated animals presented a significant increase in the glutathione reduced (GSH) level comparing to animals of satellite group. Here, the

stereoisomerism affect the pharmacological action. While (*R*)-(-)-carvone-treated animals showed GSH levels similar to those from vehicle group (only treated with ASA), the *S*-(+)-carvone treatment was able to partially reverse the ASA-induced GSH increase. More, the ASA-challenge did not promote any change in MPO activity in gastric tissues.

The endogenous GSH plays an important role in maintenance of the gastric mucosa integrity by interacting with ROS. The ROS excess first results in increased GHS level followed by GSH depletion resulting in gastric ulcers generation whether the enhanced ROS production was continuous (Vendramini-Costa et al., 2014; da Silva et al., 2019).

The initial lesions of gastric mucosa result in release of inflammatory chemical mediators, such as IL-1β and TNF-α, which are responsible for initiating the inflammatory cascade (Dinarello, 2000; Umare et al., 2014; Elgorashi and McGaw, 2019). Mediated by TNF-α, some substances responsible for the leukocytes recruitment, such as MIP-2, are secreted in the injured tissue (McDonald and Kubes, 2010; Qin et al., 2017). Being the first line of defense against invading microorganisms, such as bacteria and fungi, the neutrophils are responsible to release myeloperoxidase (MPO) in the damaged tissue. Thus, the MPO activity is used as an important biomarker of the inflammatory process (Odashima et al., 2006; Khan et al., 2018). To reestablish the homeostasis, the cells also produce anti-inflammatory cytokines, such as IL-10, that play the suppression effect on the inflammatory cascade downregulating the production of inflammatory cytokines and chemokines (Dinarello, 2000; Umare et al., 2014; Elgorashi and McGaw, 2019).

Our results suggested that the ASA administration for fourteen days resulted in oxidative stress that induced increasing on GSH level without initiating the inflammatory cascade mediated by IL-1β and TNF-α. Therefore, there were not significant variation on IL-1β, TNF-α, MIP-2 and IL-10 levels and MPO activity between animals in satellite group and those from the ASA-challenge groups.

Finally, EOMa and both *S*-(+)-carvone and *R*-(-)-carvone were evaluated in the DSS-induced colitis model. Acting direct on epithelial cells, DSS promotes hyperemia, loss of mucosal integrity and ulcerations besides increased epithelial permeability and moderate to severe submucosal edema. At the cellular and molecular levels, DSS induces rupture of the *gap* junction followed by cellular apoptosis. These injuries result in inflammatory cells infiltration and increased cytokines levels in the gut mucosa. There are significant histopathological changes in mucosal structure characterizing the lesions in the intestinal mucosa (Rieder et al., 2012; Randhawa et al., 2014; Boal Carvalho and Cotter, 2017). Clinically, the DSS-induce colitis results in diarrhea, body weight loss and bloody stools that

can be observed and used to establish the disease activity index (DAI). Higher DAI values indicate the severity of colitis. More, the weight loss and the shortening of the colon's size are associated with severity level of colitis. (Sánchez-Fidalgo et al., 2013). As already explained, GSH level and MPO activity are good biochemical biomarkers of inflammation process in the intestinal mucosal (Krawisz et al., 1984; Khan et al., 2018).

Analyzing the clinical parameters, body weight evolution and DAI, EOMa treatment partially delayed both loss body weight and DAI aggravation while both carvone enantiomers showed none activity. None treatment reverted loss of relative colon weight and length. Despite none effect on clinical parameters, *S*-(+)-carvone promoted partial reduction on MPO activity such as EOMa treatment suggesting partial reduction on neutrophil infiltration. As none significant difference were observed in the histopathological parameters of infiltration, we hypothesized that EOMa and *S*-(+)-carvone might be affecting the production and/or the activity of MPO in a molecular level. Further studies are required to test this hypothesis. Further, none significant alteration in GSH level was evidenced among all experimental groups.

Some studies from medicinal plants claim that preparations, such as extract, are always more effective than isolated compounds due interaction of different constituents. However, in some cases, the better results can be reached by the isolated compounds (Caesar and Cech, 2019). Considering that each 100 g of EOMa contain 54.82 g of carvone, it is possible to say that the EOMa dose of 25 mg/Kg corresponded to the carvone dose of 13.7 mg/Kg. These results reinforce that carvone was important for the gastroprotective effects of EOMa. More, these effects could be related to the $Ca^{2+}$ channels inhibitory effect already described for both *R*-(-)-carvone and *S*-(+)-carvone. Mainly by the results of repeated-dose experiments (ASA-induced gastric lesions and DSS-induced colitis models) it was possible to suggest that the carvone enantiomer present in EOMa was the *S*-(+)-carvone. Recently, the *S*-(+)-carvone was identified in the essential oil of *Mentha* species cultivated in India (Pragadheesh, Yadav, Chanotiya, 2015).

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**Conflict of Interest**

All authors declare no conflict of interests

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**Supplementary Material:**

**Results:**

Table 1S – Cell lines used in activity assays antiproliferative properties and their inoculation densities (I.D.)

| Cell lines | Organ / Disease | I.D. (x $10^4$ cel/ml) |
|---|---|---|
| U251 | Glioma | 4,0 |
| MCF-7 | Breast; adecarcinoma | 6,0 |
| NCI-ADR/RES * | Ovary; adenocarinoma | 5,0 |
| 786-0 | Kidney; adenocarcinoma | 5,0 |
| NCI-H460 | Lung; non-small cells, carcinoma | 4,0 |
| PC-3 | Prostate; adenocarcinoma | 4,5 |
| HT29 | Colon; adenocarcinoma | 5,0 |
| K562 | Bone Marrow; chronic myelogenous leukemia | 6,0 |
| HaCaT | Skin; immortalized keratinocyte | 4,0 |

*cell line expressing multidrug resistance phenotype; Human tumor cell lines were kindly donated by National Cancer Institute/USA at Frederick; Human non-tumor cell line was donated by Prof. Dr. Ricardo Della Coletta, Piracicaba School of Dentistry, UNICAMP

Table 2S- Relative liver and kidney weights in the oral acute toxicity evaluation of the carvone enantiomers.

| | | | **Organs** | |
|---|---|---|---|---|
| | | | **Liver** | **Kidney** |
| Treatment (mg/kg) | | Vehicle[#] | 5.87 ± 0.55 | 1.22 ± 0.14 |
| | S-C | 30 | 5.67 ± 0.27 | 1.38 ± 0.09 |
| | | 100 | 6.14 ± 0.61 | 1.37 ± 0.13 |
| | | 300 | 5.60 ± 0.89 | 1.36 ± 0.05 |
| | R-C | 30 | 5.13 ± 1.33 | 1.29 ± 0.10 |
| | | 100 | 6.04 ± 0.49 | 1.39 ± 0.10 |
| | | 300 | 6.26 ± 0.68 | 1.35 ± 0.12 |

Results expressed as average ± standard deviation; [#]: expressed in mL/kg; Body weight (g) of female mice Swiss (8 weeks old, n = 5 animals/group) evaluated at 1st, 7th and 14th experimental day; Oral treatment: Vehicle (PBS pH 7.0 with Tween 80 0.5%, 10 mL/kg), R-C (*R*-(-)-Carvone 30, 100, 300 mg/kg), S-C (*S*-(+)-Carvone 30, 100, 300 mg/kg). Statistical analysis by one-way ANOVA followed by Tuckey test (related to the vehicle group).

Table 3S- Antiulcerative effect of carvone enantiomers in ethanol-induced gastric ulcer model.

| Treatment (mg/Kg) | | Relative ulcerative area (%) | Gastroprotective effect (%) |
|---|---|---|---|
| Vehicle | 10# | 10.82 ± 1.76 | - |
| Carbenoxolone | 200 | 0.48 ± 0.06*** | 95.5 |
| ©-carvone | 10 | 10.92 ± 3.03 | -0.9 |
| | 30 | 1.59 ± 0.47*** | 85.3 |
| | 100 | 0.17 ± 0.05*** | 98.4 |
| (S)-carvone | 10 | 7.26 ± 1.21 | 32.9 |
| | 30 | 2.56 ± 0.86*** | 76.4 |
| | 100 | 0.068 ± 0.02*** | 99.4 |

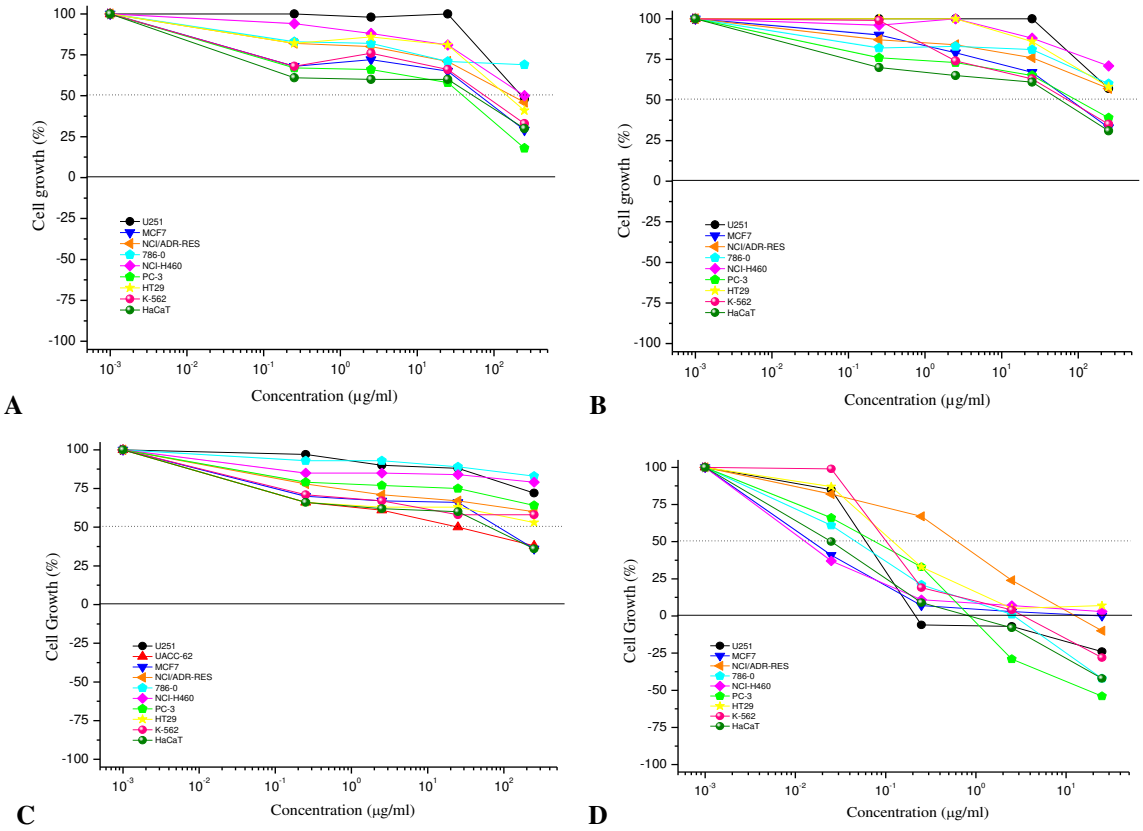
Results expressed as mean ± SEM (n = 7 male Wistar rats/group). # = mL/Kg; Relative ulcerative area (r.u.a.) = [ulcerative gastric area ($mm^2$)/total gastric area ($mm^2$)] x 100, measured using ImageJ® software and calculated in Excel software. Gastroprotective effect = $[(r.u.a._{treated\ group} - r.u.a._{vehicle\ group})/ r.u.a._{vehicle\ group}] \times 100$. Oral treatments= Vehicle (PBS pH 7.0 with Tween 80 0.5%, 10 mL/kg); Carbenoxolone (200 mg/kg, positive control); *R*-(-)-Carvone (10, 30, 100 mg/kg), *S*-(+)-Carvone (10, 30, 100 mg/kg). Statistical analysis by one-way ANOVA followed by Tuckey's test (*** $p < 0.001$ compared to vehicle group).

Table 4S- Effect of *R*-(-)-carvone, (*S*)-(+)-carvone and ranitidine on IL-1β, IL-10, TNF-α and MIP-2 levels in the dose-repeated acetylsalicylic acid ASA-induced ulcer model.

| Treatments | Cytokynes and Chemokynes | | | |
|---|---|---|---|---|
| | TNF-α | IL-1β | IL-10 | MIP-2 |
| **Vehicle** | 33.6 ± 8.8 | 47.3 ± 8.0 | 47.0 ± 3.6 | 37.8 ± 3.9 |
| **Satellite** | 25.1 ± 4.3 | 32.8 ± 3.2 | 43.2 ± 4.7 | 35.4 ± 3.5 |
| **Ranitidine** | 33.6 ± 5.2 | 44.9 ± 10.1 | 39.3 ± 3.0 | 32.7 ± 1.2 |
| **R-(-)-Carvone** | 38.0 ± 8.0 | 42.8 ± 4.4 | 50.5 ± 4.7 | 44.6 ± 4.6 |
| **S-(+)-Carvone** | 20.1 ± 3.5 | 35.1 ± 5.8 | 38.8 ± 4.4 | 32.2 ± 3.2 |

Results expressed as mean ± SEM (n = 7 male Wistar rats/group); TNF-α = alpha tumor necrosis factor (pg/µg total protein); IL-1β = Interleukin1-β (pg/µg total protein); IL-10 = Interleukin 10 (pg/µg total protein); MIP-2 = Macrophage Inflammatory Proteins (pg/µg total protein). Challenge: Acetylsalicylic acid [ASA. 200 mg/kg, diluted in Carboxymethyl cellulose (CMC) 0.2% in PBS, p.o., once a day, 15 days]. Treatments (starting on 8th experimental day, diluted in vehicle, p.o., once a day. 7 days). Treatment (v.o.): Vehicle = PBS pH 7.0 with Tween 80 0.5%, 10 mL/Kg; *R*-(-)-Carvone (25 mg/kg); *S*-(+)-Carvone (25 mg/kg); Ranitidine (2.5 mg/kg); Satellite (CMC 0.2% in PBS, 10 mL/Kg). Statistical analysis: one-way ANOVA followed by Tukey's test (* $p < 0.05$. ** $p<0.01$. *** $p < 0.001$ compared to vehicle group).

Figure 1S- Antiproliferative activity profile of *Mentha aquatica* essential oil, *R*-(-)-carvone, *S*-(+)-carvone and doxorubicin in human tumor and non-tumor cell lines.

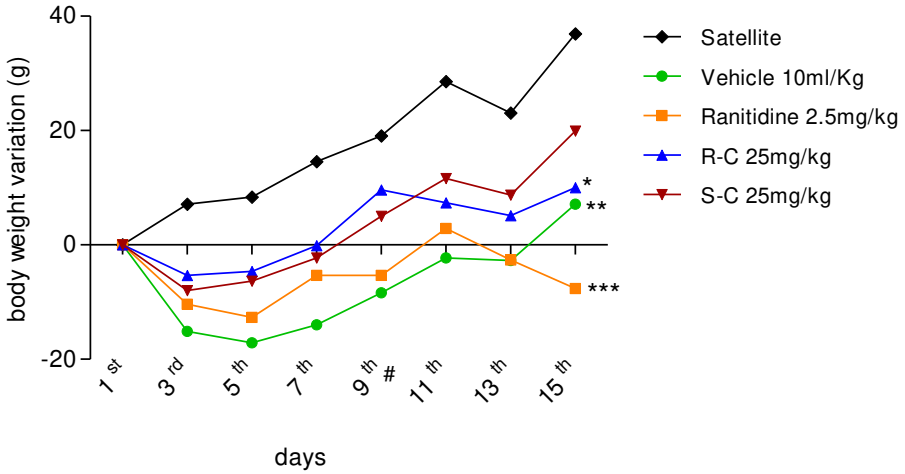



Cell growth profiles after 48 h-exposition to *S*-(+)-Carvone (A), *R*-(-)-Carvone (B), essential oil of *Mentha aquatica* aerial parts (C) and doxorubicin (D).

Tumor human cell lines: U251 (glioma); MCF-7 (breast); NCI/ ADR-RES (multidrug resistant ovarian); 786-0 (kidney); NCI-H460 (lung, non-small cells); PC-3 (prostate); HT29 (colon); K562 (leukemia)

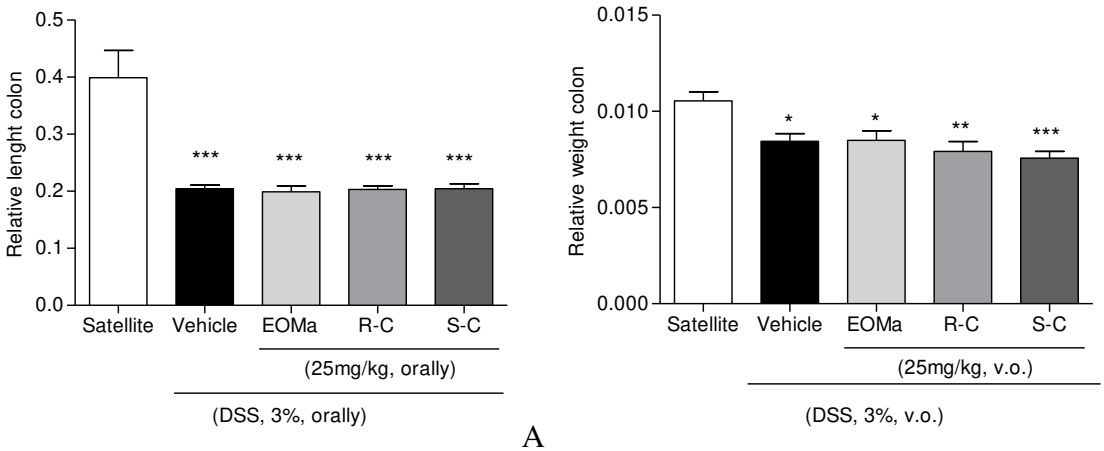
Non-tumor human line: HaCat (immortal keratinocyte).

Figure 2S- Animal body weight variation in the dose-repeated acetylsalicylic acid ASA-induced ulcer model.




Results expressed as mean ± SD (n = 7 male Wistar rats/group). Challenge: Acetylsalicylic acid (ASA. 200mg/kg. P.o., once a day, 15 days). Treatments (starting on 8th experimental day. P.o., once a day. 7 days). Treatment (v.o.): Vehicle = PBS pH 7.0 with Tween 80 0.5%; R-C: R-(-)-Carvone(25mg/kg); S-C: S-(+)-Carvone (25mg/kg); Ranitidine (2.5mg/kg); Satelitte (Carboxymethyl cellulose 0.2% in PBS). All samples were diluted in Vehicle. Statistical analysis: ANOVA followed by Tuckey's test (* $p < 0.05$. ** $p<0.01$. *** $p < 0.001$ compared to vehicle group). # (first weighing after starting treatments).

Figure 3S- Effect of *Mentha aquatica* essential oil, *R*-(-)-carvone and *S*-(+)-carvone on relative colon weight and length in the DSS-induced colitis model.




Results expressed as mean ± SEM (n = 10 male C57BL/6J mice/group. A) Relative Colon length (cm/g) = colon length (cm)/body weight (g) for each animal, B) Relative Colon weight = colon weight (g)/body weight (g) for each animal. DSS challenge = DSS 3% in potable water (*ad libitum*) for 8 days. Treatments (from 4th to 7th experimental day): Satellite (potable water *ad libitum* without DSS exposition); Vehicle = PBS (pH 7.0 with Tween 80 0.5%, 10 mL/Kg, v.o.); EOMA = essential oil of *Mentha aquatica* aerial parts (25 mg/Kg, v.o.); *R*-(-)-Carvone (25 mg/kg v.o.); *S*-(+)-Carvone (25 mg/kg v.o.). Statistical analysis: one-way ANOVA followed by Bonferroni's test (*$p<0.05$ **$p<0.01$ ***$p<0.001$ related to Satellite group).

# 3. CONCLUSÃO

Foi possível demonstrar que ambos os enantiômeros *S*-(+)-carvona e *R*-(-)-carvona apresentam efeito gastroprotetor em modelo de úlcera induzida por etanol muito provavelmente relacionado à atividade antisecretora sem afetarem a motilidade gastrointestinal. Ainda, o isômero *S*-(+)-carvona apresentou alguns efeitos benéficos em modelos nos quais tanto o agente deletério (ácido acetilsalicílico ou dextran sulfato de sódio) quanto o tratamento foi administrado em doses repetidas, enquanto a *R*-(-)-carvona mostrou-se inativa. Assim, foi possível demonstrar que a isomeria ótica exerce alguma influência sobre os efeitos biológicos dos enantiômeros de carvona. Os resultados obtidos ainda permitem inferir que a carvona, muito provavelmente o isômero *S*-(+)-carvona, tem participação no efeito farmacológico observado para o óleo essencial de *Mentha aquatica*, não sendo descartada a contribuição de outros monoterpenos identificados nesse óleo essencial.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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# ANEXOS

## Anexo 1 - Verificação de Originalidade e Prevenção de Plágio.

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# Anexo 2 - Aprovação CEUA / UNICAMP Nº 4472-1/201

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## CERTIFICADO

Certificamos que a proposta intitulada **Avaliação da atividade antiulcerogênica dos isômeros da carvona e sua ação em processos inflamatórios no Trato Gastrointestinal**, registrada com o nº **4472-1/2017**, sob a responsabilidade de **Dra. Ana Lúcia Tasca Gois Ruiz e Lucia Elaine De Oliveira Braga**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em **23 de março de 2017**.

| | |
|---|---|
| **Finalidade:** | **( ) Ensino ( X ) Pesquisa Científica** |
| **Vigência do projeto:** | **01/02/2017 -01/02/2020** |
| **Vigência da autorização para manipulação animal:** | **23/03/2017 -01/02/2020** |
| **Espécie / linhagem/ raça:** | **Camundongo heterogênico / Unib:SW (Swiss)** |
| **No. de animais:** | **35** |
| **Peso / Idade:** | **60 dias / 25g** |
| **Sexo:** | **fêmeas** |
| **Espécie / linhagem/ raça:** | **rato heterogênico / HanUnib: WH (Wistar)** |
| **No. de animais:** | **232** |
| **Peso / Idade:** | **02 meses / 180g** |
| **Sexo:** | **machos** |
| **Origem:** | **CEMIB/UNICAMP** |

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, 23 de março de 2017

Profa. Dra. Liana Maria Cardoso Verinaud
Presidente

Fátima Alonso
Secretária Executiva

IMPORTANTE: **Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos *sejam submetidos.***

# Anexo 3 - Aprovação CEUA / UNICAMP Nº 4472-1(B)/2017

UNICAMP

CEUA/UNICAMP

**CERTIFICADO**

Certificamos que a proposta intitulada **Avaliação da atividade antiulcerogênica dos isômeros da carvona e sua ação em processos inflamatórios no Trato Gastrointestinal**, registrada com o nº **4472-1(B)/2018**, sob a responsabilidade de **Profa. Dra. Ana Lúcia Tasca Gois Ruiz** e **Lucia Elaine De Oliveira Braga**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em reunião de **07 de fevereiro de 2018**.

| | |
|---|---|
| **Finalidade:** | **( ) Ensino ( X ) Pesquisa Científica** |
| **Vigência do projeto:** | **01/03/2018-01/02/2020** |
| **Vigência da autorização para manipulação animal:** | **01/03/2018-01/02/2020** |
| **Espécie / linhagem/ raça:** | **Camundongo isogênico / C57BL/6J** |
| **No. de animais:** | **50** |
| **Idade/Peso:** | **08 semanas / 20g** |
| **Sexo:** | **fêmeas** |
| **Origem:** | **CEMIB/UNICAMP** |
| **Biotério onde serão mantidos os animais:** | **Biotério da Experimentação Animal da Divisão de Farmacologia e Toxicologia, CPQBA/UNICAMP** |

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, 07 de fevereiro de 2018.

Prof. Dr. Wagner José Fávaro
Presidente

Fátima Alonso
Secretária Executiva

**IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.**

# Anexo 4 – Comprovante de submissão do artigo

Manuscript submitted to editorial office

Planta Medica Thieme

**Mentha aquatica essential oil and carvone enantiomers – Effects on the gastrointestinal tract**

| | |
|---|---|
| Journal: | *Planta Medica* |
| Manuscript ID | PLAMED-2020-04-0426-OP |
| Manuscript Type: | Original Papers |
| Date Submitted by the Author: | 23-Apr-2020 |
| Complete List of Authors: | Tasca Gois Ruiz, Ana Lúcia; Universidade Estadual de Campinas, Faculdade de Ciências Farmacêuticas<br>Oliveira Braga, Lucia; University of Campinas, Chemical, Biological, and Agricultural Pluridisciplinary Research Center (CPQBA), Division of Pharmacology and Toxicology<br>da Silva, Gisele; State University of Campinas<br>Oliveira, Ellen; Universidade Estadual Paulista Julio de Mesquita Filho Instituto de Biociencias Campus de Botucatu, Pharmacology; State University of Campinas<br>Candido, Tuany; State University of Campinas<br>Prado, Livia; State University of Campinas<br>de Carvalho, João; Universidade Estadual de Campinas |
| Keywords: | Mentha aquatica, Lamiaceae, essential oil, enantiomers, gastric ulcer, colitis |
| | |

SCHOLARONE™ Manuscripts