UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE CIÊNCIAS FARMACÊUTICAS

DANIELE DAIANE AFFONSO

AVALIAÇÃO *IN VITRO* DO ÓLEO ESSENCIAL DE *MENTHA AQUATICA* E MONOTERPENOS CARVONA, LIMONENO E EUCALIPTOL SOBRE A MIGRAÇÃO CELULAR E A INFLUÊNCIA DA ESTEREOISOMERIA

IN VITRO EVALUATION OF THE ESSENCIAL OIL OF *MENTHA AQUATICA* AND THE MONOTERPENES CARVONE, LIMONENE AND EUCALYPTOL ON CELL MIGRATION AND THE INFLUENCE OF STEREOISOMERISM

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Orientadora: Profª Drª Ana Lucia Tasca Gois Ruiz

Coorientadora: Profª Drª Mary Ann Foglio

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RESUMO

A pele e seus anexos constituem o maior órgão do corpo humano. Para restaurar a integridade funcional, o processo de cicatrização, que envolve proliferação e migração celular, entre outras etapas, é desencadeado quando vários fatores externos ou internos causam danos à pele. Por outro lado, existem patologias que se caracterizam por hiperproliferação de queratinócitos com invasão de tecidos adjacentes, como a ceratose actínica. Diversas plantas medicinais têm sido utilizadas tradicionalmente para auxiliar no tratamento de enfermidades dérmicas. Muitas vezes esses efeitos estão relacionados à presença de óleos essenciais. Esses óleos são misturas de substâncias químicas voláteis, compreendendo álcoois e ésteres de cadeia curta, monoterpenos e sesquiterpenos entre as principais classes químicas. Estudos anteriores de nosso grupo de pesquisa indicaram que o óleo essencial de *Mentha aquatica* (OEMa), contendo cerca de 62% de carvona, inibiu *in vitro* a migração de queratinócitos humanos imortalizados (HaCaT). Assim, o presente projeto teve por objetivo avaliar o efeito sobre a migração e viabilidade celulares, em linhagem HaCaT, dos monoterpenos limoneno, carvona, eucaliptol e do OEMa. Por causa da possível interferência do estereo-isomerismo sobre as atividades biológicas, foram avaliados ambos os enantiômeros de limoneno e carvona. Em condições ideais de crescimento (5% de soro fetal bovino – SFB em meio RPMI 1640), a carvona inibiu a migração de queratinócitos, sem afetar a viabilidade, de forma dependente da concentração e independente do enantiômero. Já o limoneno não afetou a migração e a viabilidade das células HaCaT, independentemente da concentração, do tempo de exposição e do enantiomero avaliado. O eucaliptol demonstrou aumento parcial significativo na migração celular, sem alterar a proliferação celular. Nas menores concentrações avaliadas (≤ 250 μg/ml), OEMa estimulou a migração celular e apresentou efeito proliferativo. Na condição de restrição de nutrientes (0,2% SFB em meio de cultura), a carvona inibiu a migração de queratinócitos, de forma dependente da concentração e independente do enantiômero avaliado. O enântiômero *S*-(-)-limoneno inibiu de maneira mais potente a migração celular do que *R*-(+)-limoneno, na concentração de 125 μg/ml. Além disso, na maior concentração (500 μg/ml), ambos enantiômeros de limoneno reduziram significativamente a viabilidade celular, sendo o enantiomero *S* mais potente. Por sua vez, o eucaliptol estimulou a migração celular sem afetar a proliferação e o OEMa não afetou a migração e proliferação celular, apresentando um perfil semelhante ao controle negativo. Em modelo de esferoides (3D), tanto carvona quanto limoneno reduziram a viabilidade dos queratinócitos enquanto eucaliptol estimulou a proliferação. Além disso, o enantiômero *R*-(-)-carvona mostrou-se mais citotóxico do que o S-(+)-carvona, enquanto não houve diferença significativa entre os isômeros limoneno. Assim, foi observada influência significativa do estereoisomerismo sobre as atividades avaliadas. Com base nos resultados obtidos, carvona e limoneno parecem ser promissores candidatos para o tratamento de condições como queratose actínica e psoríase enquanto o eucaliptol parece ser um insumo interessante para formulações para tratamento de feridas. Essas descobertas abrem possibilidades para o desenvolvimento de novas opções terapêuticas para o tratamento de doenças de pele a partir de produtos naturais.

Palavras-chave: *Mentha aquatica*, óleo essencial, esteroisômeros, migração celular, ceratose actínica, cicatrização.

ABSTRACT

The skin and its annexes constitute the largest organ of the human body. To restore functional integrity, the healing process, which involves cell proliferation and migration, among other steps, is triggered when various external or internal factors cause damage to the skin. In addition, there are pathologies that are characterized by hyperproliferation of keratinocytes with invasion of adjacent tissues, such as actinic keratosis. Several medicinal plants have traditionally been used to aid in the treatment of dermal diseases. Often these effects are related to the presence of essential oils. These oils are mixtures of volatile chemicals, comprising short chain alcohols and esters, monoterpenes and sesquiterpenes among the major chemical classes. Previous studies by our research group indicated that *Mentha aquatica* essential oil (OEMa), containing about 62% carvone, inhibited in vitro migration of immortalized human keratinocytes (HaCaT). Thus, this project aimed to evaluate the effect of OEMa and the monoterpenes limonene, carvone, and eucalyptol on cell migration and viability, in HaCaT cell line. Considering the possible interference of stereoisomerism on biological activities, both limonene and carvone enantiomers were evaluated. Under ideal growth conditions (5% fetal bovine serum – FBS in RPMI 1640 medium), carvone inhibited keratinocyte migration, without affecting viability, in a concentration-dependent and enantiomer-independent manner. Limonene, on the other hand, did not affect the migration and viability of HaCaT cells, regardless of concentration, exposure time and the evaluated enantiomer. Eucalyptol showed a significant partial increase in cell migration, without altering cell proliferation. At the lowest evaluated concentrations (≤ 250 µg/ml), OEMa stimulated cell migration and showed a proliferative effect. In the starving condition (0.2% FBS in culture medium), carvone inhibited cell migration, in a concentration-dependent and enantiomer-independent manner. The *S*-(-)-limonene inhibited cell migration more potently than *R*-(+)-limonene at 125 µg/ml. Furthermore, at the highest concentration (500 µg/ml), both enantiomers of limonene significantly reduced cell viability, with the *S*-enantiomer being more potent. In turn, eucalyptol stimulated cell migration without affecting proliferation and OEMa did not affect cell migration and proliferation, presenting a profile similar to the negative control. In a spheroid (3D) model, both carvone and limonene reduced keratinocyte viability, while eucalyptol stimulated cell proliferation. Furthermore, the *R*-(-)-carvone enantiomer was more cytotoxic than the *S*-(+)-carvone, while there was no significant difference between the limonene isomers. Thus, a significant influence of stereoisomerism on the evaluated activities was observed. Based on the results obtained, carvone and limonene seem to be promising candidates for the treatment of conditions such as actinic keratosis and psoriasis, while eucalyptol seems to be an interesting ingredient for wound treatment formulations. These discoveries open possibilities for the development of new therapeutic options for the treatment of skin diseases with natural products.

Keywords: *Mentha aquatica*, essential oil, stereioisomers, cell migration, actinic keratosis, wound healing.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

786-0 – Linhagem celular humana de adenocarcinoma de rim

ANOVA – Análise de Variância

BVOCs – Compostos orgânicos voláteis biogênicos

CA – Ceratose actínica

CCD – Cromatografia em Camada Delgada

CNPq – Conselho Nacional de Pesquisa

CPNM – Câncer de pele não melanoma

CPQBA – Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas

D.I. – densidade de inoculação

DMBA – 7,12-dimetilbenz(a)antraceno

DMSO – dimetilsulfóxido

EDTA – Ácido etilenodiamino tetra-acético

EOMa – Óleo essencial de *Mentha aquatica*

Euc – Eucaliptol

HaCaT - Linhagem celular humana de queratinócitos imortalizados

HT29 - Linhagem celular humana de adenocarcinoma coloretal

IC_{50} – Concentração inibitória em 50%

INFABIC – Instituto Nacional de Ciência e Tecnologia de Fotônica Aplicada à Biologia Celular

Nrf2 – Fator nuclear eritroide 2 relacionado ao fator 2

OMS – Organização Mundial da Saúde

PBS – phosphate buffer saline solution

PNs – Produtos Naturais

R-C – *R*-(-)-carvona

R-L – *R*-(+)-limoneno

ROS – Espécies reativas ao oxigênio

S-C – *S*-(+)-carvona

S-L – *S*-(-)-limoneno

SFB – Soro fetal bovino

SNC – Sistema Nervoso Central

SRB – sulforrodamina B

TCA – ácido tricloroacético

WR – Would reduction

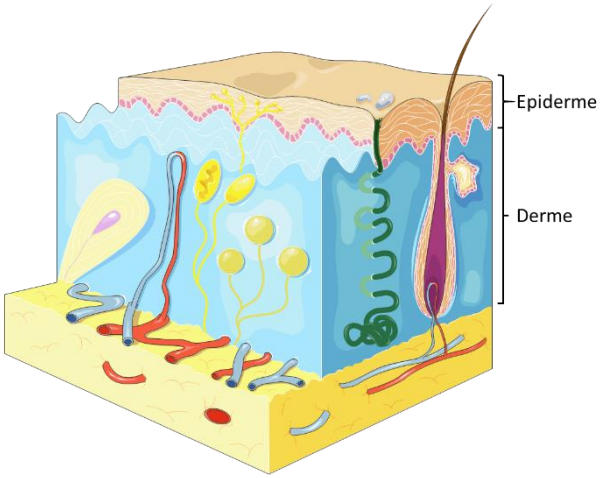
SUMÁRIO

1. INTRODUÇÃO

A pele é o órgão mais extenso do corpo humano, juntamente com seus anexos, que recobrem toda a superfície corpórea. A pele age como uma barreira protetora contra vários fatores ambientais, como a radiação UV prejudicial do sol, a invasão por patógenos e a perda excessiva de água por evaporação. Esses desafios ambientais podem comprometer a integridade da barreira da pele e levar a vários problemas e doenças de pele (Takeo; Lee; Ito, 2015).

Histologicamente, a pele é constituída por epiderme (porção epitelial de origem ectodérmica) e derme (porção conjuntiva de origem mesodérmica). A epiderme é formada principalmente por queratinócitos (95%, aproximadamente), responsáveis pela produção de queratina. Essas células passam por processo de renovação contínua e, à medida em que vão acumulando queratina, os queratinócitos migram para a superfície da pele. Os queratinócitos mortos, ricos em queratina, constituem o extrato córneo. Por sua vez, a derme é formada principalmente por fibroblastos que são responsáveis pela síntese das fibras elásticas e colágenas que compõem esse tecido. É também nessa camada que são encontrados os vasos sanguíneos, nervos, glândulas sebáceas e sudoríparas. Entre a pele e o tecido muscular subjacente, encontra-se uma camada de tecido adiposo, muitas vezes denominada como hipoderme, que ajuda a amortecer e proteger os órgãos internos e a manter a temperatura corporal (Junqueira e Carneiro, 2013) (Figura 1).

Figura 1: Estrutura da pele com órgãos acessórios (pelo e glandulas sudoríparas).



(Fonte: Figura modificada com texto após adaptação de “Skin” de Servier Medical Art by Servier, licenciada sob Creative Commons Attribution 3.0 Unported License)

Frequentemente exposta a traumatismos pela ação de diversos agentes, a perda de continuidade e interrupção da pele expõe o tecido conjuntivo subjacente ao meio externo e

desencadeia o processo de cicatrização. Assim, uma série de eventos bioquímicos e celulares são desencadeados a fim de restabelecer a integridade funcional da pele (Vitoriano et al., 2019; Yazarlu et al., 2021). A cicatrização de feridas é um processo complexo de substituição de estruturas celulares danificadas e/ou disfuncionais, além da substituição das camadas de tecido (Trinh et al., 2022).

Este processo pode ser dividido didaticamente em quatro fases denominadas fase hemostática, fase inflamatória, fase proliferativa e fase de remodelação do tecido. Durante a homostase, o processo de coagulação visa interromper a perda sanguínea. A ativação do sistema complemento e dos leucócitos inicia um processo inflamatório, com liberação de mediadores químicos (citocinas e quimiocinas), a fim de reduzir os riscos de contaminação microbiológica. Esses mediadores sinalizam a proliferação e migração de queratinócitos na epiderme e a proliferação de fibroblastos, com estímulo à sintese de colágeno. Nesta etapa de proliferação, o tecido lesionado é conhecido como tecido de granulação. Após a reepitelização, tem início a etapa de remodelação do tecido dérmico, caracterizada pela substituição dos tecidos temporários formados durante a fase proliferativa por tecidos mais permanentes, com reorganização das fibras colágenas e elásticas (Li et al., 2020; Masson-Meyers et al., 2020; (Trinh et al., 2022).

A cicatrização pode ser afetada por fatores como idade, doenças crônicas, tabagismo, entre outros. Além disso, as cicatrizes podem ser classificadas de acordo com sua aparência e textura, como cicatrizes hipertróficas, atróficas, queloides e por perda de pigmentação (González; Goldberg, 2019).

Em contrapartida, existem alterações patológicas da pele caracterizadas pela hiperproliferação de queratinócitos, podendo levar a invasão de tecidos adjacentes. A exposição excessiva e contínua a raios ultravioleta (UV) do sol pode induzir danos no DNA dos queratinócitos, tanto por ação direta sobre a molécula quanto pela indução de estresse oxidativo. Esses danos podem resultar em alterações mutagênicas e na proliferação anormal de queratinócitos. Outras causas de hiperproliferação incluem, exposição a certos reagentes químicos e substâncias tóxicas, imunossupressão, infecção viral, doenças genéticas e algumas condições médicas como obesidade e diabetes (Juráňová; Franková; Ulrichová, 2017). A hiperproliferação de queratinócitos, relacionada ou não à exposição aos raios UV, pode ocorrer em uma variedade de condições da pele, como verrugas, queloides, ceratose actínica e psoríase.

A psoríase é uma doença inflamatória crônica e autoimune que envolve a participação de citocinas estimuladoras e a presença de células T entre os queratinócitos, o que causa dano à membrana plasmática. Essa condição afeta cerca de 2% da população mundial e

é caracterizada por lesões avermelhadas e escamosas na pele, geralmente encontradas no couro cabeludo, joelhos, cotovelos e costas. Além disso, indivíduos com histórico familiar de psoríase têm maior probabilidade de desenvolver a doença. Outros fatores de risco para desencadear um quadro de psoríase incluem estresse, tabagismo, obesidade, uso contínuo de alguns medicamentos (como lítio e interferon) e infecções (como estreptocócicas) (Armstrong; Read, 2020).

Outra displasia queratinocítica é a ceratose actínica (CA) que, em cerca de 16% dos casos, possui potencial de transformação em carcionoma de células escamosas (CCE) cutâneo (Rigel; Stein Gold, 2013; Dirschka et al., 2017). CA é uma displasia intraepidérmica pré-maligna caracterizada pelo crescimento anormal de queratinócitos na camada basal da epiderme, com perda de polaridade e aparecimento de núcleos pleomórficos (Hammadi et al., 2021). As lesões ocorrem predominamente em superfícies expostas à radiação ultravioleta (UV) crônica, que pode levar a processos inflamatórios destrutivos e, finalmente, a danos estruturais substanciais ao DNA celular e aos lipídios da membrana (Berman; Cockerell, 2013; Zhao; He, 2010).

As anormalidades da pele são as doenças mais prevalentes dentre todas as patologias. Elas afetam indivíduos independentemente de sua localização geográfica, cultura ou idade, e são observadas em uma faixa entre 30% e 70% da população global. De acordo com a literatura, observa-se um aumento de 46,8% no número de casos de doenças de pele desde 1990, elevando-as para o quarto lugar dentre as doenças com maior prevalência (Maddheshiya et al., 2022). As doenças da pele representam um grande problema de saúde mundial sendo causadas tanto por fatores intrínsecos quanto extrínsecos. Atualmente existem várias limitações nas terapiais utilizadas como efeitos adversos e penetração limitada do fármaco (Majtan; Bucekova; Jesenak, 2021). Em relação à medicação convencional para problemas de pele, alguns medicamentos são usados para tratar alguns sintomas relacionados a doenças de pele, como os tratamentos tópicos com retinoides, agentes antimicrobianos e comedolíticos e tratamentos orais com agentes antimicrobianos, anticancerígenos e hormonais para formas mais graves (Ajjoun et al., 2022).

Na busca de novas opções terapêuticas, os Produtos Naturais (PNs) tem sido, ao longo dos anos, uma fonte rica de novas estruturas em função da grande diversidade química. Essas substâncias são metabólitos secundários de plantas, micro-organismos e animais e apresentam inúmeros efeitos biológicos. Até 2019, cerca de 25% dos medicamentos utilizados atualmente são derivados de compostos naturais, incluindo plantas, animais e micro-organismos, sendo as plantas uma das principais fontes. Além disso, 11% dos medicamentos

considerados essenciais pela Organização Mundial da Saúde (OMS) são de origem vegetal (Newman; Cragg, 2020; Katz; Baltz, 2016).

A crescente popularidade da medicina baseada em plantas medicinais pode ser atribuída à percepção de efetividade com baixo custo e mínimos efeitos colaterais, levando a maior aceitação pelo paciente. Por outro lado, há um aumento crescente de evidências científicas corroborando o importante papel dos produtos naturais no tratamento de muitas doenças, inclusive aquelas para as quais há poucas alternativas terapêuticas. Considerando-se apenas as condições dermatológicas, diversas plantas medicinais tem sido descritas como úteis no tratamento de câncer de pele, eczema, psoríase, ceratose actínica, além de infecções virais, bacterianas ou fúngicas, entre outras condições patológicas. Segundo a literatura, as plantas medicinais tem sido empregadas tanto em formas extemporâneas (preparações caseiras) quanto como insumo farmacêutico vegetal ativo para o desenvolvimento de medicamentos fitoterápicos e de produtos cosméticos (Tabassum; Hamdani, 2014; Ahuja; Gupta; Gupta, 2021; Čižmárová et al., 2023; Nurzyńska-Wierdak; Pietrasik; Walasek-Janusz, 2022; Castillo; González-Rosende; Martínez-Solís, 2023; Ma; Khachemoune, 2022; Costa; Magalhães; Di Stasi, 2022; Ng; Eh Suk; Gew, 2022).

Dentre as diferentes classes de produtos naturais, vários óleos essenciais tem sido descritos para tratamento de diferentes distúrbios dermatológicos. Por exemplo, o óleo essencial de erva-doce (*Foeniculum vulgare*) apresentou efeito antimicrobiano contra *Candida albicans*, podendo então ser utilizada no tratamento em casos de infecção fúngica. Já o óleo essencial de hortelã-verde (*Mentha spicata*) possui efeito antimicrobiano contra *Malassezia furfur*, podendo ser utilizado para o tratamento de eczema, dermatites e psoríase. Os óleos essenciais de diferentes espécies de *Citrus* inibem a proliferação de bactérias envolvidas no desenvolvimento da acne. Já os efeitos anti-inflamatórios e de inibição das enzimas elastase e colagenase descritos para os óleos de anis-estrelado japonês (alto teor de eucaliptol), de *Lavandula e* de *Cymbopogon citratus* (ricos em álcool perílico) indicam para o potencial uso desses óleos no tratamento de feridas e cânceres de pele (Maddheshiya et al., 2022; Asnaashari et al., 2023; Nurzyńska-Wierdak; Pietrasik; Walasek-Janusz, 2022).

Além de proporcionarem aromas e sabores agradáveis, as plantas aromáticas ou ervas-de-cheiro são valorizadas por seus benefícios para a saúde. Elas têm sido amplamente estudadas por sua capacidade de fornecer compostos ativos que são utilizados em diversos produtos, como medicamentos, cosméticos e alimentos (Riaz et al., 2021; Aziz et al., 2018). Obtidos a partir de plantas aromáticas, os óleos essenciais (OEs) são misturas de substâncias voláteis, também chamadas de compostos orgânicos voláteis biogênicos (BVOCs), que

pertencem principalmente à classe dos terpenos (monoterpenos e sesquiterpenos), dos fenilpropanoides e dos derivados de ácidos graxos. Em comum, essas substâncias apresentam baixo ponto de ebulição e caracteristicas lipofílicas, aliada à grande diversidade química. Além de serem amplamente utilizados em aromaterapia, cosméticos, produtos de limpeza e alimentos, essas substâncias diversas propriedades terapêuticas (Aziz et al., 2018; Sadgrove; Padilla-González; Phumthum, 2022; Bunse et al., 2022).

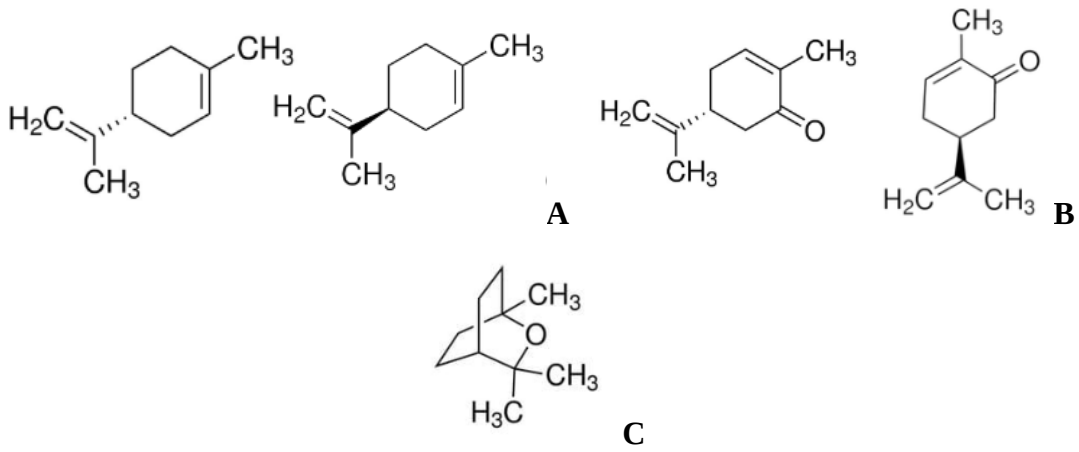
Uma das famílias de plantas aromáticas medicinais mais conhecidas é a família *Lamiaceae*. Dentro da família *Lamiaceae*, encontra-se o gênero *Mentha*. Com taxonomia complexa e nem sempre consensual entre os especialistas, acredita-se que o gênero *Mentha* compreenda 42 espécies, além de 15 híbridos e centenas de subespécies, variedades e cultivares. São plantas aromáticas perene, originária da Europa, sendo atualmente cultivada em praticamente todo o mundo. Com grande variação inter-espécies, os óleos essencias de espécies do gênero *Mentha* são armazenados em estruturas chamadas tricomas, sendo encontrados em maior quantidade nas flores e folhas do que nos caule. Ainda, em função da variação na composição química, esses OEs apresentam importantes propriedades farmacológicas, como atividades antialérgica, antioxidante, antimutagênica, antiproliferativa e quimioprotetora (Diniz Do Nascimento et al., 2020; De Sousa Barros et al., 2015; Tafrihi et al., 2021; Mimica-Dukic; Bozin, 2008; Anwar et al., 2019).

Dentre as espécies deste gênero, pode-se destacar a *Mentha aquatica*. Conhecida popularmente como hortelã-d'água, é uma planta herbácea perene que apresenta um crescimento rápido e é facilmente cultivada (Ferreira et al., 2014; Asadollah-Pour et al., 2021). Possui distribuição geográfica ampla, crescendo em ambientes úmidos da Europa, Norte da África e Oeste da Ásia, foi introduzida na América e na Austrália, ampliando ainda mais sua área de distribuição. Popularmente, a infusão das partes aéreas de *M. aquatica* é utilizada para tratamento de problemas gastrointestinais e pulmonares. No entanto, embora esta planta possua grande potencial como fonte de compostos bioativos, a maioria dos estudos disponíveis tem se concentrado na variabilidade química de seu óleo essencial (OE), que está relacionada com suas origens geográficas e com os tratamentos agronômicos aplicados no cultivo (Ferrati et al., 2023).

Estudos recentes de nosso grupo de pesquisa evidenciaram que o óleo essencial obtido a partir de *M. aquatica* cultivada no campo experimental do Centro Pluridisciplinar de Pesquisa Químicas, Biológicas e Agrícolas (CPQBA)/Unicamp, apresentou como componentes principais os monoterpenos carvona (62,34%), limoneno (19,5%) e eucaliptol (3,9%) (Figura 2). Esse óleo essencial demonstrou efeito antiulcerogênico em diferentes modelos *in vivo* de

úlcera gástrica induzida por etanol ou por fármacos anti-inflamatórios não esteroidais. Ainda, os efeitos protetores foram atribuidos à modulação de substâncias sulfidrilas não proteicas, óxido nítrico e da secreção gástrica, sem efeitos significativos sobre a motilidade intestinal (De Oliveira Braga et al., 2022).

Figura 2: Estrutura química dos monoterpenos *R*-(+)-limoneno, *S*-(-)-limoneno (A), *R*-(-)-carvona e *S*-(+)-carvona (B) e Eucaliptol (C)



Os monoterpenos, como carvona, limoneno e eucaliptol, são formados biossinteticamente na rota do ácido mevalônico e apresentam, como esqueleto químico básico, duas unidades de isopreno (Sadgrove; Padilla-González; Phumthum, 2022; Bunse et al., 2022).

A carvona é uma cetona monoterpênica com fórmula molecular $C_{10}H_{14}O$, muito encontrada nos óleos essenciais de plantas do gênero *Mentha* (67%). Por possuir um carbono assimétrico, a carvona ocorre em duas formas enantioméricas, *S*-(+)-carvona e *R*-(-)-carvona. Além da variação de concentração entre diferentes espécies, a concentração de carvona em uma mesma espécie (variação inter-espécie) pode variar em função das condições do ambiente de cultivo, da localização geográfica e parte ou estágio de desenvolvimento da planta (Scherer et al., 2013; Bouyahya et al., 2021; (Pina et al., 2022). Diversas propriedades farmacológicas foram descritas para a carvona, como por exemplo, atividades atividades antibacterianas, antifúngicas e antiparasitárias, assim como efeitos antioxidante, anti-inflamatória, anticancerígena, antiespamódico, antinoceptivo e anticonvulsionante (Bouyahya et al., 2021; Pina et al., 2022). Segundo revisões recentes (Bouyahya et al., 2021; Pina et al., 2022), nem sempre os estudos farmacológicos pré-clínicos indicaram qual o enantiômero de carvona testado. Alguns estudos foram realizados comparando os dois enantiômeros. Por exemplo, as atividades antimicrobiana, frente uma grande variedade de bactérias e fungos, e antioxidante

parece ser semelhante para *S*-(+)-carvona e *R*-(-)-carvona. Já o efeito antiparasitário, em espécies do gênero *Trypanosoma,* parece sugerir que cada enantiômero seria mais efetivo e seletivo para diferentes espécies. Diferenças relacionadas à isomeria também foram observadas para a ação sobre viabilidade celular e de inibição da enzima urease (Pina et al., 2022). Em *Staphylococcus aureus,* o mecanismo de ação antibacteriana da carvona parece envolver a desestabilização da estrutura dos fosfolipídeos e a interação com as proteínas de membrana. Além disso, atua como um trocador de prótons, reduzindo o pH através da membrana (Porfírio et al., 2017). Já os efeitos anti-inflamatórios, antidiabéticos e anticancerígenos envolvem ação em alvos celulares e moleculares, como indução de apoptose, autofagia e senescência (Bouyahya et al., 2021).

O limoneno é um terpeno monocíclico, constituído por duas unidades de isopreno, com fórmula molecular $C_{10}H_{16}$, que contém um carbono assimétrico (centro quiral) e ocorre em duas formas isoméricas ópticas: *R*-(+)-limoneno (ou d-limoneno) e *S*-(-)-limoneno (ou l-limoneno), ou ainda em uma mistura racêmica (Vieira et al., 2018). O isômero *R*-(+)-limoneno é o principal componente dos óleos essenciais presentes na casca de frutas cítricas e é produzido na natureza a partir de metabólitos secundários. Por sua vez, o isômero *S*-(-)-limoneno tem um odor de terebintina e é encontrado em plantas, como carvalhos e pinheiros, como o principal componente dos voláteis emitidos por essas espécies (Ciriminna et al., 2014). Isso confere ao limoneno uma grande variedade de propriedades e aplicações em diferentes áreas, como na indústria farmacêutica, alimentícia, e cosmética. Ademais, pesquisas pré-clínicas têm evidenciado o potencial terapêutico do limoneno em várias áreas, incluindo ação anti-inflamatória, antioxidante, anticancerígena, antinociceptiva, antidiabétics, além de efeitos no trato gastrointestinal e respiratório, bem como outras atividades (Eddin et al., 2021; Vieira et al., 2018). Estudos foram realizados comparando os dois enantiômeros. Um estudo conduzido para avaliar a atividade anti-inflamatória dos dois enantiômeros puros de limoneno, observou que o *R*-(+)-limoneno era aproximadamente 3x menos ativo do que o enantiômero *S*-(–)-limoneno na atividade inibitória da 5-lipoxigenase. A mistura racêmica exibiu uma atividade intermediária entre os valores dos dois isômeros testados separadamente. Essa observação é de relevância significativa, uma vez que a atividade farmacológica dos óleos essenciais pode depender de um enantiômero específico e/ou da proporção de enantiômeros presentes (Erasto; Viljoen, 2008). Outro estudo investigou os efeitos do *R*-(+)-limoneno e do S-(–)-limoneno no miométrio de ratas grávidas in vitro, bem como a modulação desses efeitos por outros medicamentos, como nifedipina, paxilina, tetraetilamônio e teofilina. Os resultados do estudo revelaram que o limoneno exibiu um efeito antioxidante, reduzindo o efeito inibitório do

metilglioxal (MGO), um indicador de dano oxidativo. Além disso, ambos os enantiômeros do limoneno causaram contração miometrial de forma dose-dependente. Esses achados destacam o potencial do limoneno como um composto com propriedades antioxidantes e efeitos no sistema reprodutivo, ressaltando a importância de considerar a estereoquímica e a interação com outros medicamentos ao avaliar seus efeitos farmacológicos (Vieira et al., 2018; Hajagos-Tóth et al., 2015).

O eucaliptol, também conhedido como 1,8-cineol, é um monoterpeno bicíclico oxigenado, com fórmula molecular $C_{10}H_{18}O$, presente em muitas plantas, como os óleos essenciais de eucalipto, cânfora, alecrim e sálvia. Apresenta diversas atividades farmacológicas, como anti-cancer e analgésico, sendo as atividades anti-inflamatória e antioxidante as propriedades farmacológicas dominantes. (Cai et al., 2021; De Cássia Da Silveira e Sá; Andrade; De Sousa, 2013; Guimarães; Quintans; Quintans-júnior, 2013). Por sua atividade anti-inflamatória, o eucaliptol é utilizado como opção de tratamento para sintomas de resfriado comum, asma e infecções respiratórias (Jiang et al., 2019; Kennedy-Feitosa et al., 2019). Além disso, tem sido estudado por seu potencial no tratamento de doenças cardiovasculares e digestivas, bem como a doença de Alzheimer (Cai et al., 2021).

Como apresentado anteriormente, os monoterpenos carvona e limoneno apresentam um centro quiral, sendo encontrado como enantiômeros. Enantiômeros são pares de moléculas que apresentam a mesma fórmula molecular e uma estrutura tridimensional diferente entre si, possuindo pelo menos um centro quiral ou assimétrico. Essas moléculas são semelhantes entre si em todas as propriedades fisico-químicas exceto pela capacidade de desviar o plano da luz polarizada. Essa propriedade permite separar os enantiômeros em dextrógiros (+) ou d, que desviam a luz polarizada para a direita, e levógiros (-) ou l, que desviam a luz polarizada (Lima, 1997). Levando-se em consideração o grau de complexidade dos ligantes do carbono assimétrico, a regra de nomenclatura estabelece que se a ordem da numeração arbitrária dos grupos (1 para o de maior prioridade e 4 para o de menor prioridade) seguir o sentido horário, a estrutura representada será denominada *R* (do latim *rectus*). Se a numeração seguir o sentido anti-horário, a estrutura representada será denominada *S* (do latim *sinister*) (Solomons e Fryhle, 2005).

Ao longo dos anos, tem sido dada considerável atenção especificamente à estereoquímica e ao mecanismo das reações de ciclização. A estereoquímica é um fator crucial no desenvolvimento de medicamentos, pois, apesar das semelhanças nas propriedades físico-químicas, os enantiômeros podem apresentar diferenças significativas em termos de eficácia terapêutica e toxicidade, devido às interações específicas que eles estabelecem com

macromoléculas, como proteínas e receptores. Essas diferenças resultam em respostas farmacocinéticas, farmacológicas e toxicológicas distintas (Finefield et al., 2012; Yu et al., 2022). Como resultado, é essencial que o enantiômero correto seja identificado e isolado para uso terapêutico, a fim de evitar efeitos indesejados e maximizar a eficácia (Eddin et al., 2021).

Por exemplo, o *S*-(-)-limoneno já foi identificado na *Mentha piperita* (hortelã-pimenta), enquanto o *R*-(+)-limoneno, na *M. spicata* (hortelã). Já a *S*-(+)-carvona foi identificada, por exemplo, em *Anethum graveolens* enquanto a *R*-(-)-carvona foi identificada em *M. cardiaca*. A presença dos diferentes pares de enantiômeros explica, em parte, a diferença de odor característico para cada uma dessas espécies (Finefield et al., 2012). Um estudo sobre os efeitos dos enantiomeros de carvona sobre o Sistema Nervoso Central (SNC) em camundongos demonstrou que, apesar de ambos apresentaram efeito depressor do SNC, apenas a *S*-(+)-carvona demonstrou um potencial efeito anticonvulsivante (De Sousa et al., 2007).

A busca de novas opções de tratamento envolve etapas pré-clínicas e clínicas. Antes que um novo fármaco ou fitoterápico possa ser avaliado em humanos, quanto à sua eficiência e segurança, é necessário reunir uma série de evidências em modelos experimentais *in silico*, *in vitro* e *in vivo*. O avanço do conhecimento sobre os aspectos moleculares e celulares da fisiopatologia das doenças, aliado ao desenvolvimento tecnológico, permitiram o desenvolvimento de uma ampla gama de modelos experimentais com diferentes linhagens celulares. Esse desenvolvimento permite a substituição e, consequentemente a redução do número, de animais de laboratório necessários para a comprovação de eficiência e segurança de novos fármacos. No entanto, é necessário conhecer as vantagens e limitações de cada modelo experimental *in vitro* a fim de aproveitar ao máximo as informações obtidas (Atanasov et al., 2015; Sneddon; Halsey; Bury, 2017; Jaroch; Jaroch; Bojko, 2018).

Nesse contexto, a busca por opções terapêuticas para tratamento de pele pode ser iniciada *in vitro* empregando-se linhagens celulares representativas como queratinócitos e fibroblastos. Aliado à avaliação de viabilidade e proliferação celulares, é importante avaliar como as amostras em estudo influenciam o processo de migração celular. Uma das técnicas mais utilizadas em função de sua simplicidade e economia é o teste de ranhura (do inglês *Scrath assay*). Este teste permite a medição de parâmetros básicos da migração celular, como a taxa de migração e a polaridade celular, por exemplo, através da análise da velocidade de fechamento da ferida em um determinado período de tempo. Através da avaliação desses parâmetros, é possível inferir informações sobre potenciais terapias para o tratamento de doenças relacionadas à migração celular. Isso porque o proceso de migração celular desempenha um papel essencial em uma série de processos biológicos, como embriogênese,

resposta imune, cicatrização de feridas, morfogênese e inflamação. Desta forma, o teste de ranhura, ou cicatrização, é uma ferramenta valiosa para a compreensão da atividade farmacológica de compostos e óleos essenciais, contribuindo para o desenvolvimento de novos fitoterápicos e medicamentos (Low et al., 2021; Bouchalova; Bouchal, 2022; Riss et al., 2016).

1.1. Proposição:

Com base nos estudos prévios realizados pelo grupo (De Oliveira Braga et al., 2022) e no levantamento bibliográfico, este trabalho buscou avaliar a influência do óleo essencial de *Mentha aquatica* e dos monoterpenos carvona, limoneno e eucaliptol sobre a proliferação, viabilidade e migração de queratinócitos, visando ampliar a utilização terapêutica dessas amostras. Essa dissertação será apresentada no formato alternativo, com um artigo científico descrevendo os resultados obtidos. Esse artigo se encontra em fase de submissão em revista científica.

1.1.1. Proposição Geral

Avaliação da influência de óleo essencial da *Mentha aquatica* e monoterpenos limoneno, carvona, eucaliptol sobre a migração de queratinócitos humanos.

1.1.2. Proposições Específicas

- Avaliação *in vitro* dos enantiômeros *S*-(+)-carvona, *R*-(-)-carvona, *S*-(-)-limoneno, *R*-(+)-limoneno, eucaliptol e do óleo essencial de *Mentha aquatica* em modelos de atividade antiproliferativa, de migração celular e de viabilidade celular em esferoides em queratinócitos humanos imortalizados HaCaT;
- Análise da influência da estereoisomeria sobre as atividades observadas para os pares de enantiômero *S*-(+)-Carvona/*R*-(-)-carvona e *R*-(+)-limoneno/*S*-(-)-limoneno.

2. ARTICLE - *IN VITRO* EVALUATION OF *MENTHA AQUATICA* ESSENTIAL OIL AND THE MONOTERPENES CARVONE, LIMONENE AND EUCALYPTOL ON CELL MIGRATION AND THE INFLUENCE OF STEREOISOMERISM.

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ABSTRACT

The skin and its annexes constitute the largest organ of the human body. To restore functional integrity, the healing process, which involves cell proliferation and migration, among other steps, is triggered when various external or internal factors cause damage to the skin. In addition, there are pathologies that are characterized by hyperproliferation of keratinocytes with invasion of adjacent tissues, such as actinic keratosis. Several medicinal plants have traditionally been used to aid in the treatment of dermal diseases. Often these effects are related to the presence of essential oils. These oils are mixtures of volatile chemicals, comprising short chain alcohols and esters, monoterpenes and sesquiterpenes among the major chemical classes. Previous studies by our research group indicated that *Mentha aquatica* essential oil (OEMa), containing about 62% carvone, inhibited in vitro migration of immortalized human keratinocytes (HaCaT). Thus, this project aimed to evaluate the effect of OEMa and the monoterpenes limonene, carvone, and eucalyptol on cell migration and viability, in HaCaT cell line. Considering the possible interference of stereoisomerism on biological activities, both limonene and carvone enantiomers were evaluated. Under ideal growth conditions (5% fetal bovine serum – FBS in RPMI 1640 medium), carvone inhibited keratinocyte migration, without affecting viability, in a concentration-dependent and enantiomer-independent manner. Limonene, on the other hand, did not affect the migration and viability of HaCaT cells, regardless of concentration, exposure time and the evaluated enantiomer. Eucalyptol showed a significant partial increase in cell migration, without altering cell proliferation. At the lowest evaluated concentrations (≤ 250 µg/ml), OEMa stimulated cell migration and showed a proliferative effect. In the starving condition (0.2% FBS in culture medium), carvone inhibited cell migration, in a concentration-dependent and enantiomer-independent manner. The *S*-(-)-limonene inhibited cell migration more potently than *R*-(+)-limonene at 125 µg/ml. Furthermore, at the highest concentration (500 µg/ml), both enantiomers of limonene significantly reduced cell viability, with the *S*-enantiomer being more potent. In turn, eucalyptol stimulated cell migration without affecting proliferation and OEMa did not affect cell migration and proliferation, presenting a profile similar to the negative control. In a spheroid (3D) model, both carvone and limonene reduced keratinocyte viability, while eucalyptol stimulated cell proliferation. Furthermore, the *R*-(-)-carvone enantiomer was more cytotoxic than the *S*-(+)-carvone, while there was no significant difference between the limonene isomers. Thus, a significant influence of stereoisomerism on the evaluated activities was observed. Based on the results obtained, carvone and limonene seem to be promising candidates for the treatment of conditions such as actinic keratosis and psoriasis, while eucalyptol seems to be an interesting ingredient for wound treatment formulations.

Keywords: *Mentha aquatica*, essential oil, stereioisomers, cell migration, actinic keratosis, wound healing.

1. Introduction

Natural Products (NPs) are a valuable source of new chemical structures for exploring therapeutic options. Originating from plants, microorganisms, and animals, these secondary metabolites exhibit various biological effects. Currently, approximately 25% of drugs in use are derived from natural compounds, with plants being the main source. Furthermore, 11% of essential drugs listed by the WHO are of plant origin (data up to 2019) (Newman; Cragg, 2020; Katz; Baltz, 2016).

Essential oils (EOs) are volatile compounds derived from aromatic plants and consist of mixtures of biogenic volatile organic substances (BVOCs). These substances, which include terpenes (monoterpenes and sesquiterpenes), phenylpropanoids, and fatty acid derivatives, are widely present in EOs.

Essential oils have been studied for the treatment of dermatological disorders. For example, fennel oil exhibits antimicrobial effects against Candida albicans, which is useful in the treatment of fungal infections. Peppermint oil has antimicrobial properties against Malassezia furfur, making it a potential treatment for eczema, dermatitis, and psoriasis. Citrus oils inhibit bacteria associated with acne. Moreover, Japanese star anise, Lavandula, and Cymbopogon citratus oils have anti-inflammatory effects and inhibit enzymes such as elastase and collagenase, suggesting potential use in wound healing and skin cancer. (Maddheshiya et al., 2022; Asnaashari et al., 2023; Nurzyńska-Wierdak; Pietrasik; Walasek-Janusz, 2022).

The Lamiaceae family is known for its medicinal aromatic plants, including the genus Mentha. With around 42 species, as well as hybrids and varieties, Mentha plants are perennial and cultivated globally. The essential oils of these plants, mainly found in the flowers and leaves, have a diverse chemical composition and important pharmacological properties such as anti-allergic, antioxidant, antimutagenic, antiproliferative, and chemoprotective activities.

Recent studies conducted by our research group have shown that the essential oil obtained from *M. aquatica* cultivated in the experimental field of the Center for Chemical, Biological, and Agricultural Research (CPQBA)/Unicamp, has carvone (62.34%), limonene (19.5%), and eucalyptol (3.9%) as its main components (Figure 2). This essential oil has demonstrated antiulcerogenic effects in different in vivo models of gastric ulcer induced by ethanol or nonsteroidal anti-inflammatory drugs. Furthermore, the protective effects were attributed to the modulation of non-protein sulfhydryl compounds, nitric oxide, and gastric secretion, with no significant effects on intestinal motility (De Oliveira Braga et al., 2022).

Monoterpenes, such as carvone, limonene, and eucalyptol, are biosynthetically

formed in the mevalonic acid pathway and have two isoprene units as their basic chemical skeleton (Sadgrove; Padilla-González; Phumthum, 2022; Bunse et al., 2022). Indeed, carvone and limonene monoterpenes possess a chiral center and exist as enantiomers. The stereochemistry and cyclization mechanisms of these compounds have been extensively studied. Stereochemistry plays a crucial role in drug development as enantiomers can exhibit significant differences in therapeutic efficacy and toxicity (Finefield et al., 2012; Yu et al., 2022; Eddin et al., 2021). Different enantiomers have been identified in *Mentha* species, such as *S*-(-)-limonene in *M. piperita* and *R*-(+)-limonene in *M. spicata*. Similarly, *S*-(+)-carvone was found in *Anethum graveolens* and *R*-(-)-carvone in *M. cardiaca*. These enantiomers contribute to the different biological effects. For example, a study in mice showed that *S*-(+)-carvone has anticonvulsant potential, while both enantiomers have a depressant effect on the Central Nervous System (CNS) (De Sousa et al., 2007). A study conducted to evaluate the anti-inflammatory activity of the two pure enantiomers of limonene observed that *R*-(+)-limonene was approximately 3 times less active than the *S*-(–)-limonene enantiomer in inhibiting 5-lipoxygenase activity (Erasmo, P; Viljoen, A, M, 2008).

The search for new treatments involves preclinical and clinical stages. *In silico*, *in vitro*, and *in vivo* experimental models are used to gather evidence on the efficacy and safety of new drugs. The advancement of knowledge and technological development has allowed the creation of in vitro models using different cell lines, reducing the need for laboratory animals. It is important to understand the advantages and limitations of each model to obtain relevant information. (Atanasov et al., 2015; Sneddon; Halsey; Bury, 2017; Jaroch; Jaroch; Bojko, 2018).

The search for therapeutic options for skin treatment can begin with *in vitro* evaluations using cell lines such as keratinocytes and fibroblasts. The scratch assay is a simple and cost-effective technique used to assess cell migration. It measures parameters such as migration rate and polarity, providing information about potential therapies for diseases related to cell migration. This test is valuable in understanding the pharmacological activity of compounds and essential oils, aiding in the development of new herbal medicines and skin treatment drugs. (Low et al., 2021; Bouchalova; Bouchal, 2022).

Based on previous studies conducted by the group (De Oliveira Braga et al., 2022) and the literature review, this work aimed to evaluate the influence of *Mentha aquatica* essential oil and the monoterpenes carvone, limonene, and eucalyptol on the proliferation, viability, and migration of keratinocytes, aiming to expand the therapeutic use of these samples.

2. Material and Methods

2.1. Samples

The essential oil of *Mentha aquatica* aerial parts (EOMa) used in this study was obtained by Braga et al. (2022), aliquoted and kept under refrigeration. The monoterpenes *S*-(+)-Carvone (96% purity, code 8.18410.0025, Merck, CAS: 2244-16-8), *R*-(-)-carvone (99% purity, code 8.18409.0100, Merck, CAS: 6485-40-1), *R*-(+)-limonene (97% purity, cat. no 183164, CAS: 5989-27-5), *S*-(-)-limonene (96% purity, cat. no 218367, CAS: 5989-54-8), and eucalyptol (99% purity, cat. no C80601, CAS: 470-82-6) were purchased from Merck/Sigma Aldrich.

This study was registered in the Brazilian National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the number AA0AAC4.

2.2. Qualitative analysis by Thin-Layer Chromatography (TLC)

The TLC analysis was performed following the protocol described by Wagner and Bladt (1996), with few adaptations. Using silicagel 60 F254 chromatographic alumni plates (Merck 1.05554), aliquots (30 mg/ml, 10 µL/application) of EOMa, *S*-(+)-carvone, *R*-(-)-carvone, *R*-(+)-limonene, *S*-(-)-limonene, and eucalyptol were eluted (90% of the plate) with hexane/ethyl acetate 70:30 (mobile phase). After solvent evaporation, TLC plate was observed under UV light (254 and 365 nm) and revealed with anisaldehyde solution followed by heating at 100°C for 5 min.

2.3. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis:

Following the protocol described by Braga et al. 2022, one aliquot of EOMa was diluted in ethyl acetate (20 mg/mL) and analyzed in a gas chromatograph (5977A model, Agilient Technologies, Little Falls, DE, USA) equipped with HP-5MS capillary column (30m x 0, 25mm x 0.25 µm), and coupled to a mass selective detector (Agilent, HP5975). The substance identification was attained by comparison of experimental data with those describe in the NIST 2.0 library fragmentation pattern.

2.4. *In vitro* Evaluations

2.4.1. Cells lines:

The seven human cancer cell lines (Table S1) were kindly donated by Frederick Cancer Research & Development Center, National Cancer Institute, Frederick, MA, USA and

one immortalized cell line (HaCat, human keratinocyte, Table S1) was kindly donated by Dr. Ricardo Della Coletta (University of Campinas). Stock cultures were grown in complete medium [RPMI-1640 (Nutricell, Brasil) supplemented with 5% fetal bovine serum (FBS, Vitrocell, Brasil) and 1% of penicillin:streptomycin mixture (1000 U/mL:1000 µg/mL, Vitrocell, Brasil)] at 37 °C and 5% of CO2. All experiments were done with cell at 5 to 12 passages after thawing. Subculture was done before convergence using tripsin/EDTA 0.25% solution.

2.4.2. Sample preparation:

For the anti-proliferative activity assay, aliquots of each sample were previously diluted in DMSO (1:10 p/v) followed to serial dilution in complete medium to afford the final concentrations (0.15, 1.5, 15, and 150 µg/ml).

For the scratch assay, aliquots of each sample were diluted in DMSO (2:5 p/v) followed to serial dilution in complete medium (normal condition) or in RPMI-1640 supplemented with 0.2% FBS (starving condition) to afford the final concentrations (62.5, 125, 250, and 500 µg/ml).

2.4.3. Anti-proliferative activity assay:

Each cell line (Table S1) was plated in 96-well plates (100 µL/well) for 24 h before samples addition (100 µL/well, *S*-(+)-carvone, *R*-(-)-carvone, *R*-(+)-limonene, *S*-(-)-limonene, and eucalyptol, 0.15 to 150 µg/mL) in triplicate and incubated for 48 h at 37 °C and 5% of CO_2. Doxorubicin (0.015 to 15 µg/mL) was used as positive control. Before (T0) and after (T1) sample addition, the cells were fixed with trichloroacetic acid (TCA, 50%, 50 µL/well) and cell viability was determined by sulforhodamine B (SRB) (50 µL/well) protocol at 540 nm using a microplate reader spectrophotometer (VersaMax, Molecular Devices). Considering the difference between T0 and T1 absorbance values as representing 100% of cell growth, the proliferation (%) of each cell line in the presence of each sample concentration was calculated. After plotting the cell growth versus sample concentration curves, an effective concentration representing the sample concentration required to promote 50% (GI_{50}) of growth inhibition for each cell line was calculated by sigmoidal regression using Origin 8.0 software (Monks et al., 1991).

2.4.4. 3D cell culture:

HaCaT cells (8×10^5 cell/ ml, 1.0 ml) in complete medium were mixed with

magnetic nanoparticles (1 μL/1 x 10^4 cell, NanoShuttleTM, Greiner Bio-One®). After magnetization, cell suspension was centrifuged (2500 rpm; 4 min) and the resulting pellet was washed with complete medium (3x, 1.5 ml followed by centrifugation). Magnetized keratinocytes were suspended in complete medium (1.2 × 10^5 cell/ml) and seeded in cell-repellent 96-well plate (100 μL/well, 1.2 × 10^4 cell/well, Greiner Bio-One®). The plate was kept on top of a magnetic drive during 12 h, under incubation, to allow cell aggregation and spheroid formation. After that, spheroids were exposed to *S*-(+)-carvone, *R*-(-)-carvone, *R*-(+)-limonene, *S*-(-)-limonene, and eucalyptol (62.5 to 500 μg/mL, in triplicate) and incubated for 48 h, without the magnetic drive. Before (T0) and after (T48) sample addition, spheroids images were captured, at 40x magnification, using digital camera OPTIKAM® B3 coupled to inverted microscopy Leica DM IL and OPTIKA® *software* version 1.2. All images (transversal area and the area of the central spheroid, disregarding radial growth when applicable.) were analyzed using ImageJ® *software*. After image capture, the medium was carefully replaced by MTT solution (0.5% in complete medium, 50 μL/well) and spheroids were incubated for 4 h before medium exchange for DMSO (100 μL/well) and spectrophotometric evaluation at 550 nm. Considering untreated spheroids as representative of 100% viable cells, relative cell viability was calculated by Equation 1 considering T_A = absorbance values of sample-treated spheroid and T_1 = absorbance values of untreated spheroid:

$$\text{Cell Viability (\%)} = \frac{TA}{T1} \times 100 \quad \text{(Eq. 1)}$$

Based on the cell viability versus sample concentration curves, the sample concentration required to reduce in 50% the cell viability in spheroids (IC_{50}) was calculated by sigmoidal regression using *software* Origin 8.0® (OriginLab *Corporation*).

2.4.5. Evaluation of cell migration - Scratch assay:

This evaluation followed the protocol described by (Liang; Park; Guan, 2007) with few adaptations. Briefly, after 24h-incubation, HaCaT cells (2 x 10^5 cel/ml, 1 ml/well) in 24-well plates were exposed to RPMI 1640 medium supplemented with 0.2% FBS for 24h. After that, a longitudinal scratch (1 per well) was done using a with a p200 pipet tip (2 well/tip). After careful medium removing and cell washing (PBS, 1x, 1 ml/well), the keratinocytes were exposed to *S*-(+)-carvone, *R*-(-)-carvone, *S*-(+)-limonene, *R*-(-)-limonene, eucalyptol, and EOMa (62.5 to 500 μg/ml, 2 ml/well, in duplicate). In the first set of experiments, all samples were diluted in complete medium (RPMI 1640 + 5% FBS) while in the second set, samples were diluted in RPMI 1640 + 0.2% FBS. As positive and negative controls, cells were kept in

RPMI 1640 medium supplemented with 5% or 0.2% FBS, respectively. Immediately after treatments, cells were observed in time lapse inverted microscope (Zeiss LSM780) for 24 h, with regular image capture (1 capture/h, 3 microphotographs/well). Wound area was analyzed at 0, 9, 12, 18, and 24 h using ImageJ® software. The wound retraction was calculated by Equation 2, considering T_x = wound area at a stablish time after sample addition, and T_0 = wound area at basal time (immediately after sample addition):

$$WR(\%) = \left(\frac{100 \times Tx}{T0}\right) - 100 \text{ (Eq. 2)}$$

After 24 h exposure, cell viability was accessed by SRB assay as described in item 2.4.3, considering HaCaT cells in negative control (RPMI 1640 + 0.2% FBS) as representative of 100% viable cells.

2.5. Statistical analysis:

Data were expressed as means ± SD of at least two independent experiments. Statistical analyses were done (software GraphPad Prism 8®) using One-way or Two-way ANOVA, followed by Tukey's or Bonferroni's test, depending on the evaluation, considering as significant p-values ≤ 0.05.

3. Results

Both TLC and GC-MS analysis confirmed the presence of carvone, limonene and eucalyptol in the *Mentha aquatica* aerial parts (EOMa) freeze-stored (Figures S1 and S2), as expected (Braga et al., 2022). As the analysis were performed in aquiral conditions, it was not possible to detect which enantiomer of carvone and limonene was produced by *M. aquatica*.

At the experimental conditions, none monoterpene showed anti-proliferative effect against human tumor and non-tumor cell lines (Figure S2). Assuming that 150 µg/ml was the highest concentration without anti-proliferative effect for 3 x 10^3 cell/well, the concentration range of 62.5 to 500 µg/ml was selected for scratch assay performed with 2 x 10^5 cell/well.

In the first set of experiments, samples were diluted in complete medium (RPMI 1640 + 5% FBS, ideal growing condition). Both *S*-(+)-carvone and *R*-(-)-carvone inhibited cell migration inhibition without affecting cell viability. Considering the results obtained at 18h-exposure, the inhibitory concentration (IC_{50}) was 206.0 ± 31.1 µg/ml for *R*-(-)-carvone and 177.2 ± 59.6 µg/ml for *S*-(+)-carvone (Table 1, Figures 1, 2, and S3). Independent on concentration or enantiomer evaluated, limonene did not affect both cell migration and viability (Table 2, Figures 1, 2, and S4). Interestingly, eucalyptol induced a slight and significant increase

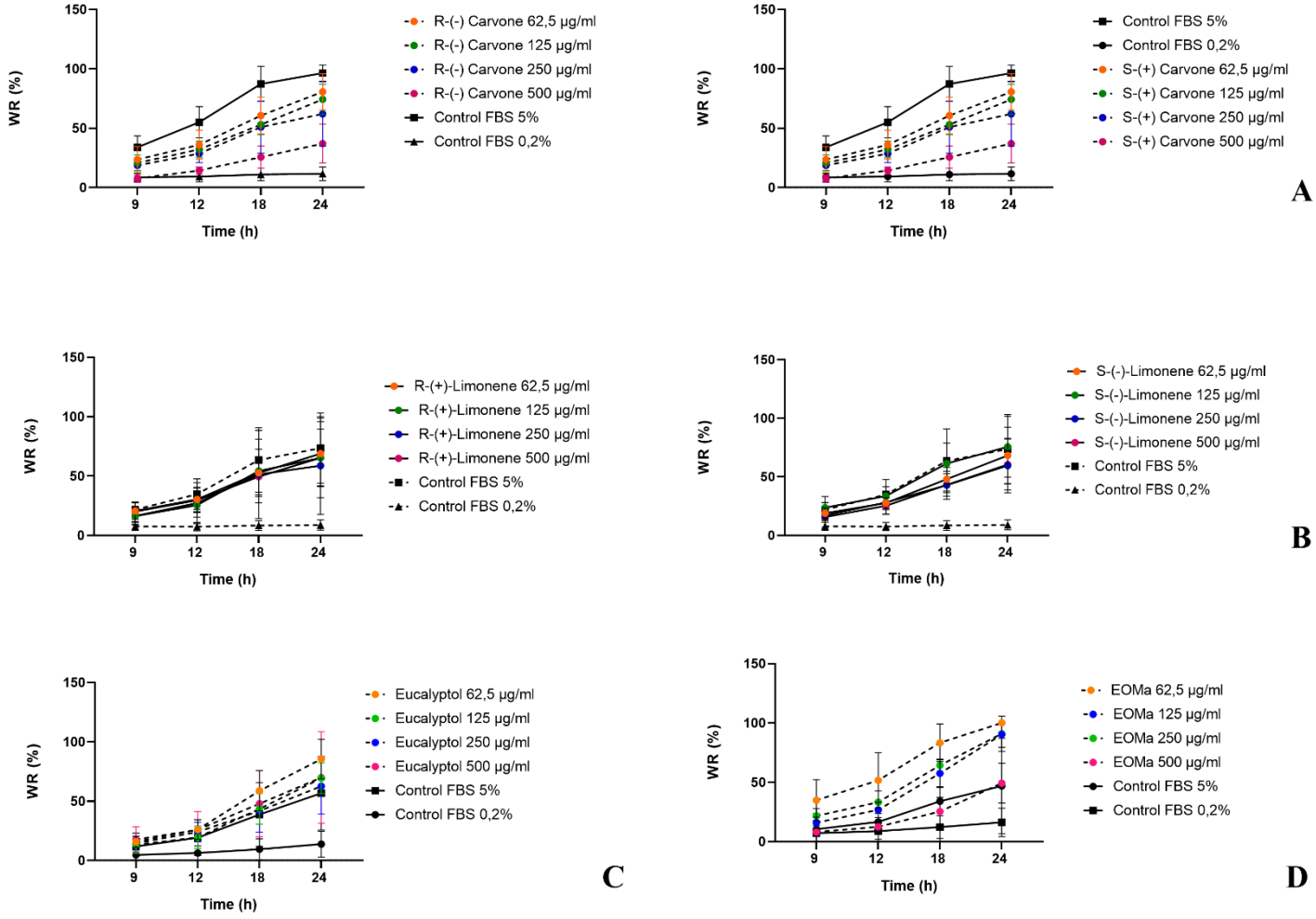
in cell migration compared to positive control (keratinocytes kept in RPMI 1640 + 5% FBS) (Table 3, Figures 1 and S5) without stimulated cell proliferation (Table 3, Figure 2). Moreover, the *Mentha aquatica* essential oil (EOMa), at 62.5 to 250 µg/ml, stimulated cell migration after 18 and 24h-exposure compared both to positive (5% FBS) and negative (0.2%) controls (Table 4, Figures 1 and S5). At same condition, EOMa showed proliferative effect (Table 4, Figure 2).

Table 1: Influence of *R*-(-)-carvone and *S*-(+)-carvone on the migration of HaCaT cells (human immortalized keratinocytes) under ideal growing condition[#].

Tratament	Concentration (µg/ml)	Wound Retraction (%)				Cell Viability(%)
		9h	12h	18h	24h	
FBS[##]	5.0	34.0 ± 9.6 [a]	55.1 ± 13.1 [a]	87.4 ± 14.8 [a]	96.5 ± 6.8 [a.c]	179.4 ± 15.9[a.c]
	0.2	8.7 ± 3.4 [b]	9.3 ± 4.5 [b]	11.1 ± 5.2 [b]	11.7 ± 5.8 [b]	101.9 ± 7.0[b]
	62.5	25.7 ± 9.2 [a.b.c]	45.4 ± 9.9 [c]	75.8 ± 13.2 [c]	90.5 ± 10.2 [a,c]	165.0 ± 13.1[a,c,d]
***R*-(-)-Carvone**	125	22.6 ± 7.7 [a.b.c]	39.9 ±14.5 [c]	65.5 ± 26.2 [c,d]	78.5 ± 10.2 [c,d,e]	147.8 ± 56.0[a]
	250	13.7 ± 6.0 [a.b.c]	23.3 ± 14.8 [c.e]	40.1 ± 28.5 [c]	56.2 ± 33.8 [d]	182.3 ± 21.3[d]
	500	11.3 ± 10.9 [b.c]	18.0 ± 15.0 [b.d.e]	31.0 ± 17.5 [b]	48.8 ± 26.0 [e]	188.3 ± 21.4[c]
	62.5	23.7 ± 9.2 [a.b.c]	36.1 ± 12.2 [c]	60.7 ± 15.4 [c]	80.7 ± 15.2 [c]	172.7 ± 29.7[a.c.d]
	125	20.8 ± 6.7 [a.b.c]	32.2 ± 7.0 [c]	53.1 ± 8.4 [c]	74.3 ±12.8 [c.f]	181.1 ± 23.4[a.c.d]
***S*-(+)-Carvone**	250	18.7 ± 4.3 [a.b.c]	28.7 ± 7.6 [c.e]	50.9 ± 21.9 [c]	62.1 ± 27.1 [d.f]	147.3 ± 28.9[a.d]
	500	7.7 ± 3.1 [b.c]	14.4 ± 3.1 [b.d.e]	25.7 ± 9.4 [b.d]	37.1 ± 16.4 [e]	167.3 ± 20.6[a.c.d]

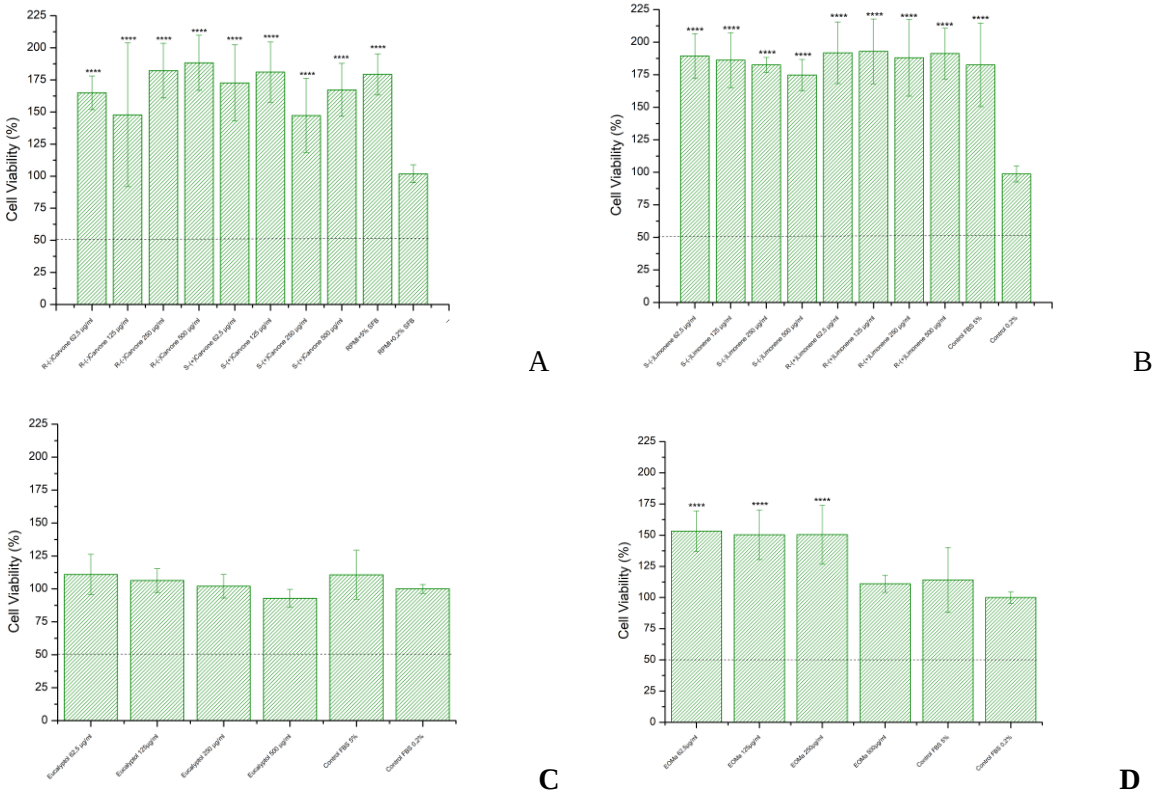
Ideal growing condition = samples diluted in complete medium (RPMI 1640 + 5% FBS); ##FBS = fetal bovine serum, concentration expressed in percentage (%). Results are expressed as mean ± SEM of technical duplicates from two independent experiments. Statistical analysis: two-way ANOVA followed by Bonferroni's test (wound retraction) and one-way ANOVA followed by Tukey's test (cell viability), different letters in the same column indicate significant difference with $p \leq 0.05$.

Figure 1: Migration of HaCaT cells exposed to carvone (A), limonene (B), eucalyptol (C), and EOMa (D) under ideal growing condition[#].



Ideal growing condition = samples (62.5 to 500 µg/ml) diluted in complete medium (RPMI 1640 + 5% FBS); WR (%): Wound retraction as a function of exposure time.

Figure 2: Effect of carvone (A), limonene (B), eucalyptol (C) and *Mentha aquatica* essential oil (EOMa, D) on the viability of human keratinocytes at the end of the scratch assay, under ideal growing condition#.



Ideal growing condition = samples (62.5 to 500 µg/ml) diluted in complete medium (RPMI 1640 + 5% FBS). Statistical analysis: one-way ANOVA followed by Tukey's test (asterisks indicate significant difference compared to the negative control (RPMI + 0.2% FBS), with *p<0.5 **p<0.1 *** p<0.01 **** p<0.0001).

Table 2: Influence of *R*-(+)-limonene and *S*-(-)-limonene on HaCaT (immortalized human keratinocytes) migration under ideal growing condition#.

Tratament	Concentration (µg/ml)	Wound Retraction (%)				Cell Viability (%)
		9h	12h	18h	24h	
FBS#	5.0	21.8 ± 6.2 [a]	34.8 ± 12.9 [a]	63.9 ± 27.2 [a]	73.6 ± 29.5 [a]	182.5 ± 32.1 [a.c]
	0.2	7.7 ± 3.4 [a]	7.5 ± 3.5 [b]	8.5 ± 4.1 [b]	8.8 ± 4.3 [b]	98.8 ± 6.2 [b]
***R*-(+)-limonene**	62,5	20.5 ± 7.0 [a]	30.4 ± 10.2 [a.b]	52.7 ± 20.0 [a]	69 ± 27.0 [a]	191.8 ± 23.6 [a.c]
	125	16.3 ± 6.9 [a]	25.5 ± 10.2 [a.b]	54.3 ± 26.7 [a]	65.3 ± 33.5 [a]	192.9 ± 24.8 [a.c]
	250	16.5 ± 7.6 [a]	27.3 ± 17.2 [a.b]	51.4 ± 37.2 [a]	58.9 ± 41.2 [a]	188.0 ± 29.4 [c]
	500	20.1 ± 8.1 [a]	29.7 ± 10.3 [a.b]	49.6 ± 15.1 [a]	65.4 ± 24.2 [a]	191.2 ± 19.5 [a.c]
***S*-(-)-limonene**	62,5	18.8 ± 4.5 [a]	27.5 ± 4.6 [a.b]	47.9 ± 10.0 [a]	68.1 ± 24.1 [a]	189.3 ± 17.3 [a.c]
	125	23.5 ± 9.5 [a]	33.6 ± 7.9 [a]	61.3 ± 17.5 [a]	75.5 ± 26.0 [a]	186.2 ± 21.1 [a.c]
	250	17.0 ± 4.3 [a]	28.0 ± 9.8 [a.b]	42.8 ± 11.9 [a]	59.6 ± 23.2 [a]	182.5 ± 5.7 [a.c]
	500	15.5 ± 8.8 [a]	25.1 ± 7.3 [a.b]	43.0 ± 9.6 [a]	60.4 ± 21.7 [a]	174.7 ± 12.0 [a]

Ideal growing condition = samples diluted in complete medium (RPMI 1640 + 5% FBS); ##FBS = fetal bovine serum, concentration expressed in percentage (%). Results are expressed as mean ± SEM of technical duplicates from two independent experiments. Statistical analysis: two-way ANOVA followed by Bonferroni's test (wound retraction) and one-way ANOVA followed by Tukey's test (cell viability), different letters in the same column indicate significant difference with $p \leq 0.05$.

Table 3: Influence of eucalyptol on HaCaT (immortalized human keratinocytes) migration under ideal growing condition[#].

Treatment	Concentration (µg/ml)	Wound Retraction (%)				Cell Viability (%)
		9h	12h	18h	24h	
FBS[##]	5.0	9.7 ± 11.2 [a]	19.2 ± 15.2 [a]	39.8 ± 26.8 [a]	66.0 ± 31.0 [a]	110.7 ± 18.5 [a]
	0.2	5.1 ± 5.9 [a]	5.7 ± 5.9 [a]	9.0 ± 8.7 [b]	14.3 ± 10.8 [b]	100.0 ± 3.4 [a.b]
Eucalyptol	62.5	15.5 ± 4.8 [a]	24.7 ± 8.2 [a]	58.1 ± 17.0 [a]	89.2 ± 16.7 [c]	110.9 ± 15.3 [a]
	125	13.6 ± 7.3 [a]	17.5 ± 9.5 [a]	43.5 ± 13.0 [a]	68.0 ± 12.9 [a.c]	106.4 ± 9.0 [a]
	250	12.3 ± 5.9 [a]	21.7 ± 8.2 [a]	43.9 ± 17.8 [a]	68.8 ± 23.5 [a]	102.1 ± 9.0 [a.b]
	500	16.2 ± 10.6 [a]	24.4 ± 15.4 [a]	57.6 ± 28.0 [a]	92.4 ± 38.6 [a.c]	92.9 ± 6.8 [b]

Ideal growing condition = samples diluted in complete medium (RPMI 1640 + 5% FBS); ##FBS = fetal bovine serum, concentration expressed in percentage (%). Results are expressed as mean ± SEM of technical duplicates from two independent experiments. Statistical analysis: two-way ANOVA followed by Bonferroni's test (wound retraction) and one-way ANOVA followed by Tukey's test (cell viability), different letters in the same column indicate significant difference with $p \leq 0.05$.

Table 4: Influence of *Mentha aquatica* oil essencial (EOMa) on HaCaT (immortalized human keratinocytes) migration under ideal growing condition[#].

Treatment	Concentration (µg/ml)	Would Reduction (%)				Cell Viability (%)
		9h	12h	18h	24h	
FBS[##]	5.0	10.5 ± 13.0 [a]	16.7 ± 17.5 [a.b]	34.1 ± 34.8 [a]	47.0 ± 40.2 [a]	114.1 ± 26.0 [a]
	0.2	7.2 ± 6.6 [a]	8.9 ± 6.7 [a]	12.4 ± 9.5 [b]	16.3 ± 11.9 [b]	100.0 ± 4.7 [a]
EOMa	62.5	25.3 ± 5.2 [b]	38.0 ± 2.9 [c]	79.5 ± 10.6 [d]	100.0 ± 0.0 [c]	153.2 ± 16.2 [b]
	125	12.9 ± 2.5 [a]	21.5 ± 3.3 [a.b]	48.1 ± 5.6 [c]	84.9 ± 8.6 [c]	150.3 ± 19.9 [b]
	250	25.4 ± 3.6 [a.b]	39.8 ± 5.9 [b.c]	74.4 ± 9.6 [c]	100.0 ± 0.0 [c]	150.4 ± 23.6 [b]
	500	5.6 ± 0.8 [a]	8.1 ± 0.7 [a]	15.2 ± 2.5 [a.b]	34.9 ± 1.5 [a]	111.0 ± 6.9 [a]

Ideal growing condition = samples diluted in complete medium (RPMI 1640 + 5% FBS); ##FBS = fetal bovine serum, concentration expressed in percentage (%). Results are expressed as mean ± SEM of technical duplicates from two independent experiments. Statistical analysis: two-way ANOVA followed by Bonferroni's test (wound retraction) and one-way ANOVA followed by Tukey's test (cell viability), different letters in the same column indicate significant difference with $p \leq 0.05$.

In the second set of experiments, all samples were diluted in RPMI 1640 medium supplemented with 0.2% FBS (starving condition). Once more, independent on time exposure

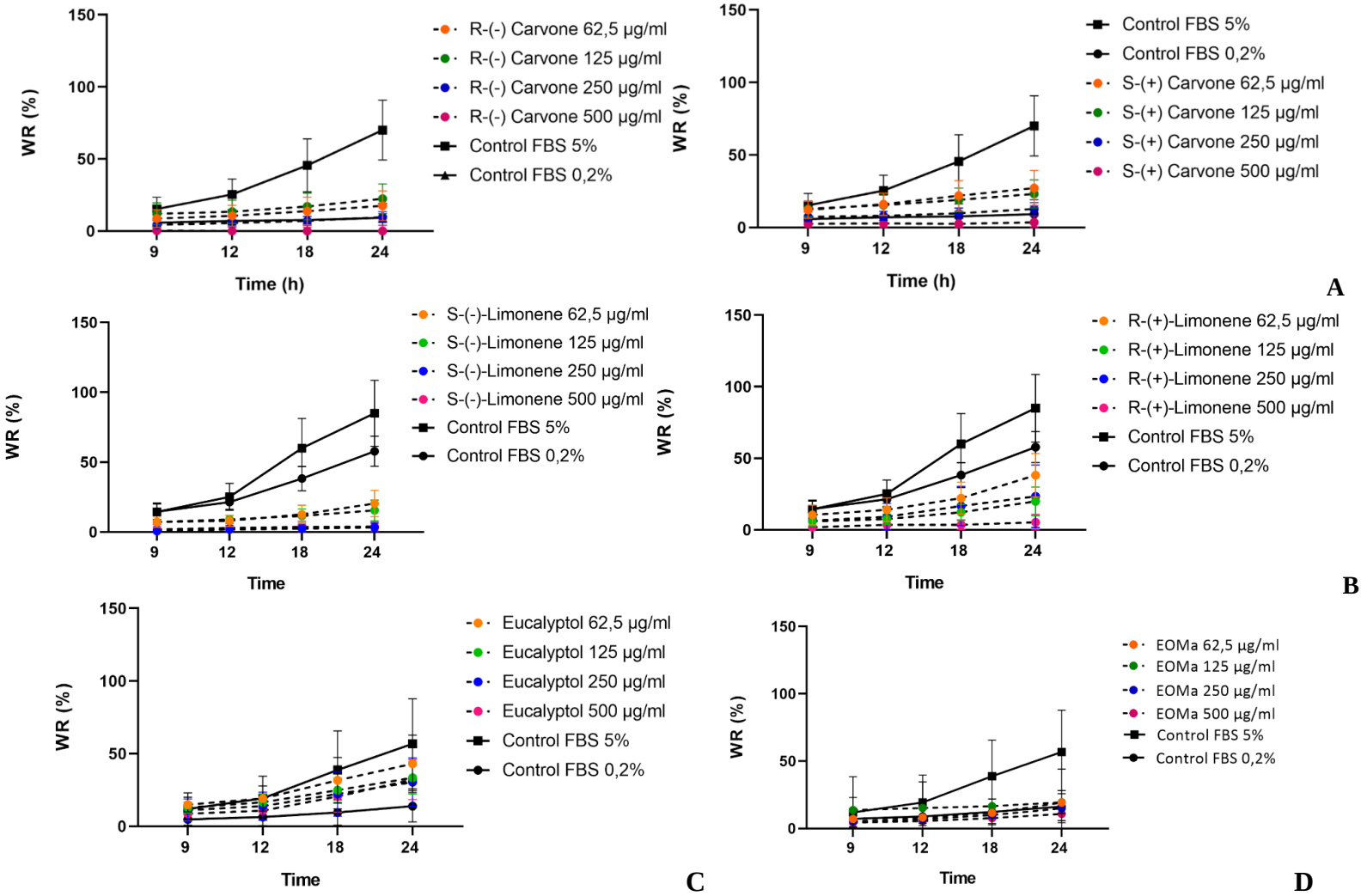
and sample concentration, both *R*-(-)-Carvona e *S*-(+)-Carvona inhibited cell migration with a slight proliferative effect (Table 5, Figures 3, 4, and S7). Interestingly, both *R*-(+)-limonene and *S*-(-)-limonene, at 62.5 μg/ml, inhibited cell migration without affecting cell proliferation in comparison to negative control (0.2% FBS). However, *S*-(-)-limonene showed a concentration dependent cytotoxic effect while *R*-(+)-limonene reduced cell viability to 40% at 500 μg/ml (Table 6, Figures 3, 4, and S8). Eucalyptol showed a significant increase on cell migration, without affecting cell proliferation in comparison to negative control (0.2% FBS). Moreover, this effect seemed to be inversely proportional to sample concentration (Table 7, Figures 3, 4, and S5). The EOMa did not affect cell migration and proliferation presenting similar profile to negative control (0.2% FBS) (Table 8, Figures 3, 4, and S5).

Table 5: Influence of *R*-(-)-carvone and *S*-(+)-carvone on the migration of HaCaT cells (human immortalized keratinocytes) under starving condition[#].

Treatment	Concentration (μg/ml)	Wound Retraction (%)				Cell Viability (%)
		9h	12h	18h	24h	
FBS[##]	5.0	15.4 ± 8.0 [a]	22.8 ± 10.6 [a]	41.0 ± 18.4 [a]	69.3 ± 20.3 [a]	203.0 ± 21.5 [a]
	0.2	3.4 ± 6.8 [a.b.c]	4.7 ± 7.6 [b.c]	5.7 ± 8.7 [b.e.f]	7.4 ± 10.0 [b.c.e.f]	100.0 ± 9.1 [b.c]
***R*-(-)-Carvone**	62.5	7.4 ± 6.1 [a.b.c]	9.0 ± 7.3 [c.d]	11.1 ± 9.6 [f]	16.7 ± 10.3 [f]	143.2 ± 10.2 [e]
	125	10.5 ± 7.5 [a.b]	12.2 ± 8.0 [c.d]	16.9 ± 9.2 [f]	23.5 ± 10.3 [d.f]	133.8 ± 9.2 [e]
	250	4.2 ± 2.2 [b.c]	5.7 ± 2.4 [b.c.d]	7.2 ± 3.1 [b.e.f]	9.5 ± 3.8 [e.f]	111.1 ± 8.2 [c]
	500	0.1 ± 1.7 [c]	0.6 ± 3.2 [b]	0.1 ± 4.0 [e]	1.9 ± 4.1 [e]	89.5 ± 10.5 [b]
***S*-(+)-Carvone**	62.5	10.9 ± 5.8 [a.b]	15.9 ± 7.7 [a.c.d]	21.9 ± 10.4 [d.f]	25.4 ± 12.1 [d.f]	125.2 ± 11.3 [e]
	125	11.8 ± 5.4 [a.b]	15.3 ± 6.8 [a.c.d]	19.2 ± 8.1 [c.d.f]	22.4 ± 9.7 [c.d.f]	134.2 ± 9.2 [d.e]
	250	8.0 ± 3.3 [a.b.c]	7.6 ± 3.2 [a.b.d]	9.6 ± 3.6 [b.c.f]	13.6 ± 4.3 [b.c.f]	117.1 ± 10.0 [c.e]
	500	1.5 ± 4.0 [b.c]	1.4 ± 3.7 [b.d]	1.0 ± 3.6 [b.e]	2.8 ± 4.3 [b.e]	105.2 ± 9.1 [c]

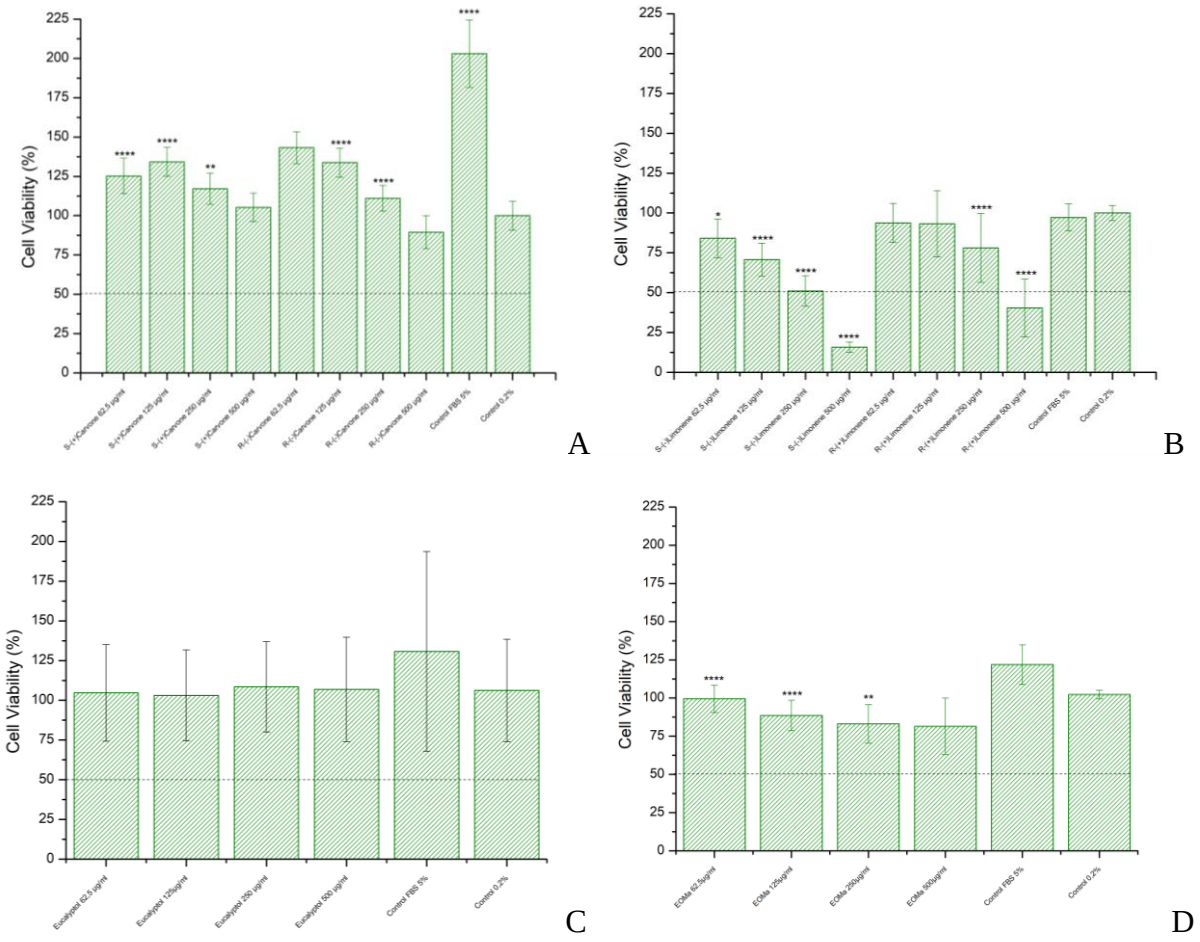
Starving condition = samples diluted in complete medium (RPMI 1640 + 0.2% FBS); ##FBS = fetal bovine serum, concentration expressed in percentage (%). Results are expressed as mean ± SEM of technical duplicates from two independent experiments. Statistical analysis: two-way ANOVA followed by Bonferroni's test (wound retraction) and one-way ANOVA followed by Tukey's test (cell viability), different letters in the same column indicate significant difference with $p \leq 0.05$.

Figure 3: Migration of HaCaT cells exposed to carvone (A), limonene (B), eucalyptol (C), and EOMa (D) under starving condition[#].



#Starving condition = samples (62.5 to 500 µg/ml) diluted in complete medium (RPMI 1640 + 0.2% FBS); WR (%): Wound retraction as a function of exposure time.

Figure 4: Effect of carvone (A), limonene (B), eucalyptol (C), and *Mentha aquatica* essential oil (EOMa, D) on the viability of human keratinocytes at the end of the scratch assay, under starving condition[#].



#Starving condition = samples (62.5 to 500 µg/ml) diluted in complete medium (RPMI 1640 + 0.2% FBS). Statistical analysis: one-way ANOVA followed by Tukey's test (asterisks indicate significant difference compared to the negative control (RPMI + 0.2% FBS), where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Table 6: Influence of *R*-(+)-limonene and *S*-(-)-limonene on HaCaT (immortalized human keratinocytes) migration under starving condition[#].

Treatment	Concentration (µg/ml)	Wound Retraction (%)				Cell Viability (%)
		9h	12h	18h	24h	
FBS[##]	5.0	13.6 ± 6.0 [a]	24.2 ± 9.3 [a.c]	58.8 ± 21.2 [a]	83.7 ± 24.2 [a]	97.2 ± 8.5 [a]
	0.2	14.6 ± 5.6 [a]	21.4 ± 5.2 [a.c]	38.3 ± 8.7 [b]	57.9 ± 10.8 [b]	100.0 ± 4.7 [a]
***R*-(+)-Limonene**	62.5	10.4 ± 6.9 [a.b]	14.2 ± 8.2 [a.b.c]	22.2 ± 11.2 [d]	38.2 ± 15.1 [e]	93.8 ± 12.2 [a.d]
	125	5.9 ± 3.6 [a.b]	7.5 ± 3.7 [b.c]	12.3 ± 5.7 [c.d]	20.0 ± 10.0 [d]	93.2 ± 20.8 [a.d]
	250	6.4 ± 4.4 [a.b]	9.1 ± 9.4 [b.c]	16.7 ± 13.7 [d]	23.5 ± 21.8 [d]	78.1 ± 21.6 [d]
	500	1.8 ± 4.8 [b]	3.6 ± 3.8 [b.c]	3.6 ± 3.8 [c]	5.3 ± 5.5 [c]	40.4 ± 18.1 [c]
***S*-(-)-Limonene**	62.5	7.2 ± 3.7 [a.b]	8.1 ± 4.0 [b.c]	12.7 ± 6.5 [c.d]	20.4 ± 9.4 [d]	84.1 ± 12.2 [a.d]
	125	7.2 ± 2.5 [a.b]	9.0 ± 3.0 [b.c]	11.6 ± 5.0 [c.d]	15.5 ± 7.5 [d]	70.7 ± 10.3 [d]
	250	0.7 ± 3.2 [b]	1.8 ± 2.9 [b]	2.4 ± 2.7 [c]	3.3 ± 3.1 [c]	51.0 ± 9.6 [c]
	500	1.9 ± 3.6 [b]	3.0 ± 3.6 [b.c]	3.6 ± 4.2 [c]	4.0 ± 3.3 [c.d]	15.8 ± 3.2 [b]

Starving condition = samples diluted in complete medium (RPMI 1640 + 0.2% FBS); ##FBS = fetal bovine serum, concentration expressed in percentage (%). Results are expressed as mean ± SEM of technical duplicates from two independent experiments. Statistical analysis: two-way ANOVA followed by Bonferroni's test (wound retraction) and one-way ANOVA followed by Tukey's test (cell viability), different letters in the same column indicate significant difference with $p \leq 0.05$.

Table 7: Influence of eucalyptol on HaCaT (immortalized human keratinocytes) migration under starving condition[#].

Treatment	Concentration (µg/ml)	Wound Reduction (%)				Cell Viability (%)
		9h	12h	18h	24h	
FBS*	5.0	9.1 ± 11.2 [a]	18.7 ± 15.2 [a]	39.3 ± 26.8 [a]	66.0 ± 31.0 [a]	130.8 ± 62.8 [a]
	0.2	5.1 ± 5.9 [a]	5.7 ± 5.9 [a]	9.0 ± 8.7 [b]	14.3 ± 10.8 [b]	106.2 ± 32.2 [a]
Eucalyptol	62.5	16.5 ± 5.1 [a]	19.7 ± 8.6 [a]	32.9 ± 15.8 [a]	50.0 ± 19.7 [a.c]	104.7 ± 30.4 [a]
	125	11.8 ± 4.0 [a]	13.3 ± 6.4 [a]	21.2 ± 8.9 [a.b]	26.9 ± 11.4 [c]	103.1 ± 28.7 [a]
	250	7.1 ± 5.6 [a]	8.3 ± 6.4 [a]	12.4 ± 10.0 [b]	19.4 ± 10.9 [c]	108.5 ± 28.5 [a]
	500	9.3 ± 5.3 [a]	10.3 ± 7.6 [a]	17.8 ± 12.5 [b]	31.0 ± 15.8 [c]	106.9 ± 32.8 [a]

Starving condition = samples diluted in complete medium (RPMI 1640 + 0.2% FBS); ##FBS = fetal bovine serum, concentration expressed in percentage (%). Results are expressed as mean ± SEM of technical duplicates from two independent experiments. Statistical analysis: two-way ANOVA followed by Bonferroni's test (wound retraction) and one-way ANOVA followed by Tukey's test (cell viability), different letters in the same column indicate significant difference with $p \leq 0.05$.

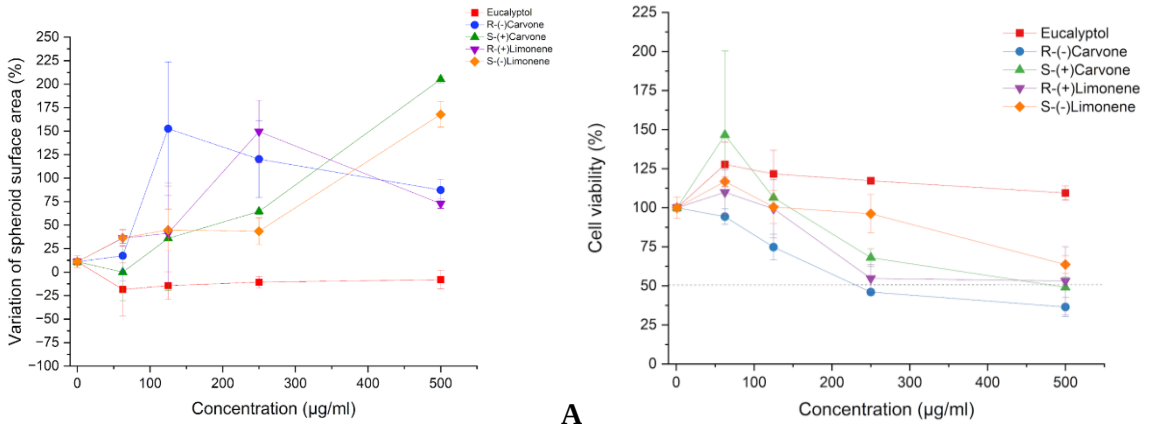
Table 8: Influence of *Mentha aquatica* essential oil (EOMa) on HaCaT (immortalized human keratinocytes) migration under starving condition[#].

Treatment	Concentration (µg/ml)	Wound Retraction (%)				Cell Viability (%)
		9h	12h	18h	24h	
FBS[##]	5.0	9.7 ± 11.2 [a]	19.2 ± 15.2 [a]	39.8 ± 26.8 [a]	66.0 ± 31.0 [a]	121.9 ± 13.0 [a]
EOMa	0.2	5.1 ± 5.9 [a]	5.9 ± 5.7 [a]	9.0 ± 8.7 [b]	14.3 ± 10.8 [b]	102.3 ± 2.8 [a]
	62,5	6.7 ± 3.9 [a]	7.9 ± 3.8 [a]	11.3 ± 4.4 [b]	17.5 ± 6.6 [b]	99.5 ± 9.0 [b]
	125	6.1 ± 24.8 [a]	6.7 ± 24.5 [a]	8.0 ± 24.5 [b]	12.4 ± 24.7 [b]	88.6 ± 9.9 [b]
	250	5.5 ± 2.6 [a]	6.6 ± 3.0 [a]	9.4 ± 2.2 [b]	14.4 ± 3.8 [b]	83.1 ± 12.5 [b]
	500	4.0 ± 3.0 [a]	4.7 ± 3.2 [a]	8.4 ± 4.0 [b]	10.1 ± 4.9 [b]	81.5 ± 18.4 [a]

Starving condition = samples diluted in complete medium (RPMI 1640 + 0.2% FBS); ##FBS = fetal bovine serum, concentration expressed in percentage (%). Results are expressed as mean ± SEM of technical duplicates from two independent experiments. Statistical analysis: two-way ANOVA followed by Bonferroni's test (wound retraction) and one-way ANOVA followed by Tukey's test (cell viability), different letters in the same column indicate significant difference with $p \leq 0.05$.

Using the same concentration range evaluated in the scratch assay, the effect of eucalyptol and the enantiomers of carvone and limonene on HaCaT spheroids was analyzed. Both carvone enantiomers reduced cell viability and promoted significant expansion of the spheroids' area. *R*-(-)-carvone (IC_{50} = 276.2 ± 41.5 µg/ml) was more effective than *S*-(-)-carvone (IC_{50} = 459.6 ± 134.3 µg/ml). Despite inducing reduction on cell viability and area expansion, the enantiomers of limonene were less active than carvone ones (IC_{50} > 500 µg/ml). Finally, eucalyptol induced a slight and significant increasing on cell viability together with a visual radial cell growth (Table 9, Figures 5 and S6).

Figure 5. Effect of carvone, limonene, and eucalyptol on the viability of human keratinocytes and area variation of spheroids on 3D assay.



A B

Results expressed as mean ± standard deviation of one experiment. Control (initial point): HaCaT cells in complete medium (RPMI 1640 + 5%FBS). Parameters: A) Relative area variation of the spheroid (%); B) Relative cell viability (%). Human non-tumoral cell line: HaCaT (immortalized human keratinocytes).

Table 9: Influence of *R*-(-)-carvone, *S*-(+)-carvone, *R*-(+)-limonene, *S*-(-)-limonene, and eucalyptol on cell viability (%) and spheroid area variation (%) in 3D model.

Samples	Parameters	Concentration (µg/ml)			
		62.5	**125**	**250**	**500**
***R*-(-)-Carvone**	**Viab. (%)**	94.3 ± 4.9	74.8 ± 8.2	46.0 ± 1.9**	36.5 ± 6.1**
	Δ area (%)	17.4 ± 27.1	152.6 ± 71.0****	120.2 ± 40.9***	87.3 ± 11.3*
***S*-(+)-Carvone**	**Viab. (%)**	146.6 ± 53.7*	106.4 ± 0.4	68.1 ± 5.7	49.1 ± 6.4**
	Δ area (%)	0.0 ± 30.5	35.9 ± 55.7	64.5 ± 3.1	205.1 ± 3.8****
***R*-(+)-Limonene**	**Viab. (%)**	109.9 ± 14.4	99.4 ± 18.6	54.8 ± 8.7***	53.2 ± 21.9***
	Δ area (%)	36.1 ± 8.4	41.4 ± 53.3	149.5 ± 33.1****	72.9 ± 4.9
***S*-(-)-Limonene**	**Viab. (%)**	116.8 ± 3.0	100.5 ± 10.6	96.2 ± 12.3	63.7 ± 5.7**
	Δ area (%)	36.5 ± 9.1	44.9 ± 22.2	43.5 ± 14.2	167.9 ± 13.7****
Eucalyptol	**Viab. (%)**	127.7 ± 14.3****	121.7 ± 15.1****	117.3 ± 1.1****	109.4 ± 4.5****
	Δ area (%)	-18.5 ± 28.2	-14.4 ± 14.8	-10.6 ± 6.2	-8.0 ± 9.9
Control	**Viab. (%)**	100.0 ± 6.9			
	Δ area (%)	11.1 ± 6.3			

Results expressed as mean ± standard deviation of one experiment. Control: HaCaT cells in complete medium (RPMI 1640 + 5%FBS). Parameters: Viab. (%) = relative cell viability (%); Δ area (%) = relative area variation of the spheroid. Statistical analysis: One-way ANOVA followed by Tukey's test (asterisks indicate significant difference compared to the control group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Human non-tumoral cell line: HaCaT (immortalized human keratinocytes).

4. Discussion

This study evaluated the influence of *Mentha aquatica* essential oil (EOMa) on cell viability and migration, finding evidence of the participation of *S*-(+)-carvone, *R*-(-)-carvone, *S*-(-)-limonene, *R*-(+)-limonene, and eucalyptol on these effects. Moreover, some differences on the biological effects could be explained by the stereoisomers.

Among the factors that can change the chemical composition of essential oils, the storage can be highlighted (Turek and Stintzing, 2013). In the present study, EOMa was stored in an amber glass bottle at -20 °C. This condition proved adequate to preserve the chemical composition as evidenced by the chromatographic analyzes (Figures S1 and S2). As previously reported (Braga et al 2022), EOMa did not affect HaCaT viability up to 250 µg/ml. All evaluated monoterpenes, up to 150 µg/ml, showed no anti-proliferative effect against almost all tumor and non-tumor human cell lines evaluated in the present study (Figure 3S).

Based in these results, the concentration of 150 µg/ml would be the highest

concentration that would not affect cell proliferation. However, considering the experimental conditions, the anti-proliferative (3×10^3 cell/well, 200 µl/well, 96-well plate) and the scratch (2×10^5 cell/well, 2000 µl/well, 24-well plate) assays were quite different. Thus, the simple transposition of the concentration could result in a negative result just because an insufficient amount of the sample was evaluated. To avoid this experimental error, it was important to stablish the correspondent dose. While the term "concentration" describes the amount of sample per unit volume, the term "dose" refers to the amount of sample per compartment used or per quantity of cells present in that compartment (Kisitu, 2019).

As the higher concentration without anti-proliferative effect (150 µg/ml) represented a dose of 30 µg/3×10^3 cell/well, the correspondent higher concentration in scratch assay was calculated as 1.000 µg/ml. At this concentration, all evaluated monoterpenes were cytotoxic (Table S2). Based on these results, the concentration range was established as 62.5 to 500 µg/ml.

Cell migration is a crucial step both in wound healing and cancer. Different assays have been developed to evaluate *in vitro* cell. One of the most used assays is the scratch assay. By a longitudinal fissure created in a confluent monolayer of cells, this protocol simulates a wound allowing the evaluation of both migration and cell viability (Martinotti; Ranzato, 2020). In this experimental model, the variation on fetal bovine serum (FBS) concentration in RPMI 1640 medium was used to establish positive (induction) and negative (inhibition) controls for cell migration. Together with minerals, lipids, and hormones, FBS also provides cells with growth factors that stimulate cell proliferation and factors that aid in cell adhesion (Subbiahanadar et al., 2021). Complete removal or reduction of serum concentration in the culture medium can result in cell cycle arrest at the G0/G1 phase, leading to cell cycle synchronization in a compartment (Wang, 2021). Thus, the positive control was defined as the culture medium supplemented with 5% FBS (ideal growing conditions), and the negative control was defined as the culture medium supplemented with 0.2% FBS (starving condition).

Representing the main cell found in epidermis, in vitro cultivated keratinocytes, such as HaCaT cell line, constitute an interesting experimental tool both in 2D and 3D models to evaluate several skin disorders. has been described previously (Gailit; Clark; Welch, 1994; Hamill et al., 2012; Larjava; Koivisto, 2013; Şenkal et al., 2022).

Despite stimulating both cell migration and viability in the ideal growing conditions, the EOMa showed none effect when evaluated in starving condition. Considering the isolated monoterpenes, the inhibitory effect of EOMa on keratinocytes' migration could be partially explained by the presence of high amount of carvone. In a concentration-dependent

way, carvone showed an inhibitory effect on HaCaT migration that was potentiated by the starving condition, without affecting cell viability. This potentiation induced by starving condition was also observed for limonene. However, in this condition and at higher concentration, limonene significantly reduced cell viability. Finally, eucalyptol partially enhanced cell migration and viability in both conditions. In this context, both carvone and eucalyptol could contribute to the absence of cytotoxic effect observed for EOMa in the experimental conditions.

According to literature, in triple negative breast tumor cells (MDA-MB- 231), *R*-(-)-carvone suppressed both cell migration and invasion at non-cytotoxic concentration (15 µg/ml). These effects were attributed to the *R*-(-)-carvone-induced reduction on both expression and gelatinolytic activity of MMP-2 and MMP-9, key enzymes in extracellular matrix degradation, together with down regulation of β1-integrin and FAK signaling (Lima et al., 2023). In DMBA-induced skin cancer mouse model, *S*-(+)-carvone (20 mg/kg, oral treatment, 3x week, 28 weeks) prevented tumor development. This effect was attributed to the restoration of hepatic redox balance, as evidenced by glutathione reductase and glutathione transferase levels, and metabolism, measured by cytochrome P540 activity. Furthermore, oral treatment with *S*-(+)-carvone reversed the DMBA-induced increase in p53 and Bcl-2 levels and the reduction on Bax level in mice skin (Gopalakrishnan et al., 2019).

On human tumor cell lines of lung (A549 and H1299), *R*-(+)-limonene at 0.75mM (≈ 100 µg/ml) showed a time-dependent effect on cell viability and proliferation, along with induction of phosphatidylserine residue exposure and loss of plasmatic membrane integrity (Yu et al., 2018). The potential chemopreventive effects of limonene on skin have been demonstrated on UV-induced damage model in HaCaT cells. At a non-cytotoxic level (up to 100 µM, ≈ 15 µg/ml), limonene prevented UV-induced reduction on keratinocytes viability. This effect was attributed to a concentration-dependent reduction on ROS generation after UV exposure and up-regulation of antioxidant activity via Nrf2 pathway. However, the authors did not report the enantiomeric form of limonene evaluated (Kumar; Vani; Wang, 2022).

In this context, the obtained results for *R*-(-)-carvone, *S*-(+)-carvone *R*-(+)-limonene, and *S*-(-)-limonene on the cell migration may be relevant in controlling the excessive proliferation of keratinocytes. Some skin conditions, such as psoriasis and actinic keratosis, may be characterized by an excessive proliferation of keratinocytes accompanied by local inflammation, with or without invasion of adjacent tissues (Griffiths et al., 2021). Affecting adults and children, psoriasis is a complex inflammatory disease, in which the interaction between T cells, dendritic cells and keratinocytes seems to play a crucial role in its

pathophysiology (Queiroz et al., 2021). Actinic keratosis are non-tumor skin lesions that could progress to squamous cell carcinoma, a high-incidence non-melanoma skin tumor, in around 30% of the cases. Cumulative exposure to UV light can cause genetic alterations in keratinocytes, disrupting or altering regulatory pathways involved in cell identification and differentiation. These alterations, together with the inflammatory micro-environment, have been related to the pathogenesis of actinic keratosis (Marques; Chen, 2023). Several natural products, such as the ethanolic extract of *Aloe vera* (immunomodulatory and inhibitory effects on the keratinocyte's proliferation) and the essential oils of *Zornia brasiliensis* and *Cymbopogon citratus*, have been described as promising candidates for the treatment of these pathologies (Krutmann et al. 2015; Steeb et al., 2021; Alesci et al. 2022; Maddheshiya et al. 2022).

Different from carvone and limonene, in the present study, eucalyptol stimulated both cell migration without affecting cell viability, at starving condition. Wound healing is a complex process that involves several steps, including inflammation, cell proliferation and tissue remodeling (Masson-Meyers et al., 2020) . In a skin burn model, limonene-treated rats (5% limonene-loaded ointment base, topic treatment, 7 and 14 days) showed an accelerated healing process compared to animals in negative group (without any treatment). This effect was correlated to modulation of anti-inflammatory response and redox balance induced by eucalyptol. However, the vehicle was not evaluated to exclude any effect induced by components of ointment base (Mohammed et al., 2022). Combined with black seed (*Nigella sativa*, 15%) oil, an 4% eucalyptol-loaded nanoemulsion was evaluated in a surgical wound model in rabbits (topic treatment, 15 days) accelerating wound healing in comparison to control group. Once more, empty nanoemulsion (without addition of black seed oil and eucalyptol) was not evaluated. (Anwar et al., 2019). Moreover, in chronic gastric ulcer model, eucalyptol (oral treatment, 100 mg/kg) reduced the gastric area lesion promoting restoration of mucus level and cell proliferation (Rocha Caldas et al. 2015). These results suggest that eucalyptol can modulate the inflammatory response during the healing process along with induction of cell proliferation, contributing to more efficient healing.

Continuing our evaluation of carvone, limonene and eucalytptol on imortalized keratinocytes, the effect on cell viability using a 3D model was accessed. Spheroids are an interesting 3D model to simulate a more representative environment in in vitro evaluations. Among the different techniques available for producing spheroids, magnetic levitation combines magnetic beads attached to cell membranes with an external magnetic field to induce cell aggregation. This technique allows for rapid and controlled aggregation, resulting in more

homogeneous spheroids from a wide variety of cell lines (Zanoni et al., 2016; Shen et al. 2021).

Using the same range concentration evaluated on scractch assay, *R*-(-)-carvone, *S*-(-)-carvone, *R*-(+)-limonene, and *S*-(-)-limonene increased transversal area of HaCaT spheroids, in a concentration-dependent way, associated to poorly defined edges. These morphological findings were suggestive of loss of cellular cohesion, indicating the permeation of evaluated compounds by the 3D structure. Moreover, the cell viability reduction corroborated these findings. Corroborating the cell migration induction and proliferation, eucalyptol-treated spheroids showed a more homogeneous aspect with increased radial cell growth, that was also evidenced by increased cell viability. This is the first evaluation of carvone, limonene and eucalyptol in HaCat spheroids.

Constituting and important aspect of cutaneous treatment, transdermal permeation is a complex process that depends on several factors, such as the characteristics of the substance, the formulation used and the properties of the skin (Todo 2017). Several monoterpenes, including *R*-(+)-limonene, eucalyptol and carvone, have been described as efficient penetration enhancers for topic formulation (Sapra, Jain, Tiwary, 2008). For example, in *ex vivo* model of permeation through third-degree burn eschar, both limonene and eucalyptol increased the permeation of silver sulphadiazine, an antimicrobial drug widely used in wound treatment (Moghimi, Makhmalzadeh, Manafi, 2009). Nanostructured formulations containing eucalyptol have been recently described as useful vehicle for transdermal drug delivery (Li et al. 2022; Kumar, Sahoo, Manchanda 2022). This permeation ability was evidenced in the HaCaT spheroids.

Finally, considering the enantiomers of carvone and limonene, the obtained results suggest that stereoisomerism small significantly influence the effects of carvone and limonene on the migration and viability of keratinocytes (HaCaT cell line). According to the literature, the main biological difference between these pairs of enantiomers is the distinct odor (Finefield et al., 2012).

5. Conclusion

This is the first report of the influence of cell migration and 3D cell viability of carvone, limonene and eucalyptol. Besides significant influence of stereoisomerism, carvone and limonene seem to be promisor candidates to further evaluations in pre-clinical models of actinic keratosis and non-melanoma tumor. Moreover, eucalyptol showed promisor biological effects to further evaluation on wound healing models.

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Supplementary Material:

Table S1: Cell lines used in anti-proliferative assay.

Cell lines	Organ / Disease	I.D. (x 10^4 cel/ml)
U251	Glioblastoma	4,0
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

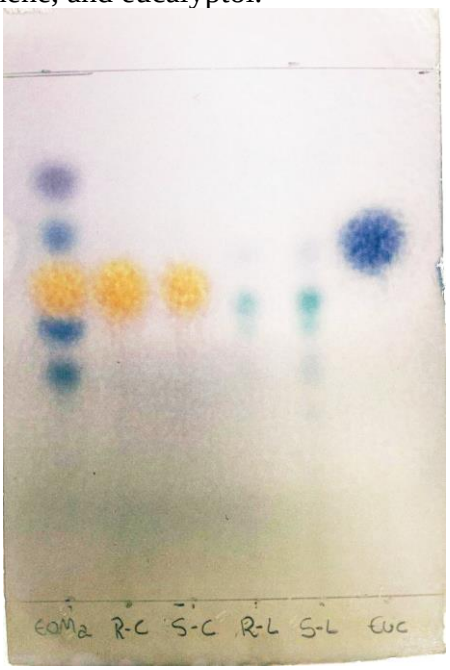
I.D.: inoculation density

Table S2: Influence of *R*-(-)-carvone, *S*-(+)-carvone, *R*-(+)-limonene, *S*-(-)-limonene, and eucalyptol on HaCaT (immortalized human keratinocytes) migration under starving condition[#].

Treatment[##]		Wound Retraction (%)[*] 9h	12h	24h	Cell Viability (%)
FBS	5.0	21.3 ± 4.7[a]	32.8 ± 3.7[a]	81.9 ± 16.3[a]	168,6 ± 6,8[a]
	0.2	8.8 ± 5.0[b]	10.5 ± 6.3[b]	12.4 ± 8.2[b]	100 ± 5,7[b]
***R*-(-)-Carvone**		2,3 ± 5,7[b]	4.1 ± 5.1[b]	4.9 ± 7.5[b]	12.6 ± 0.9[c.d]
S-(+)-Carvone		12,4 ± 3,1[b]	-0.2 ± 2.5[b]	-1.3 ± 3.7[b]	15.1 ± 0.7[c.e]
***R*-(+)-Limonene**		0,1 ± 4,7[b]	1.4 ± 2.5[b]	0.6 ± 2.2[b]	17.3 ± 2.7[d.e]
***S*-(-)- Limonene**		1,7 ± 0,9[b]	1.3 ± 1.0[b]	0.7 ± 0.4[b]	42.2 ± 2.1[f]
Eucalyptol		3,2 ± 3,4[b]	2.8 ± 3.0[b]	2.3 ± 2.1[b]	50.5 ± 0.9[f]

Starving condition = samples diluted in complete medium (RPMI 1640 + 0.2% FBS); ##FBS = fetal bovine serum, concentration expressed in percentage (%); *R*-(-)-carvone, *S*-(+)-carvone, *R*-(+)-limonene, *S*-(-)-limonene, and eucalyptol at 1,000 µg/ml. * technical problems in the microscope prevented the capture of images at 18h-exposure. Results are expressed as mean ± SEM of technical duplicates from one independent experiment. Statistical analysis – wound retraction: two-way ANOVA followed by Bonferroni's test (different letters in the same column indicate significant difference with $p \leq 0.05$). Statistical analysis – cell viability: one-way ANOVA followed by Tukey's test (different letters in the same column indicate significant difference with $p \leq 0.05$).

Figure S1: TLC analysis of *Mentha aquatica* essential oil and the monoterpenes carvone, limonene, and eucalyptol.



Sample	R_F
EOMa	0,79; 0,70;0,59; 0,51; 0,42
***R*-(-)-Carvone**	0,59
***S*-(+)-Carvone**	0,59
***R*-(+)-Limonene**	0065; 0,56; 0,51
***S*-(-)-Limonene**	0,65; 0,56; 0,52; 0,43
Eucalyptol	0,67

Samples: EOMa = essential oil of *Mentha aquatica* aerial parts*;* R-C = *R*-(-)-carvone; S-C = *S*-(+)-carvone, R-L = *R*-(+)-limonene, S-L = *S*-(-)-limonene, Euc = eucalyptol.

Stationary phase = silicagel 60 F254; Mobile phase = hexane/ethyl acetate 7:3; the plates were observed under UV (254 and 365 nm) and revealed by anisaldehyde solution

R_F = retention factor calculated as the ratio between the main spot observed after anisaldehyde revelation and the solvent front, both measured from the origin.

Figure S2: Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of *Mentha aquatica* essential oil.

Compound	Retention time (min)	Area (%)
Carvone	8,77	14,5
Limonene	8,67	25
Eucalyptol	17,2	47,3

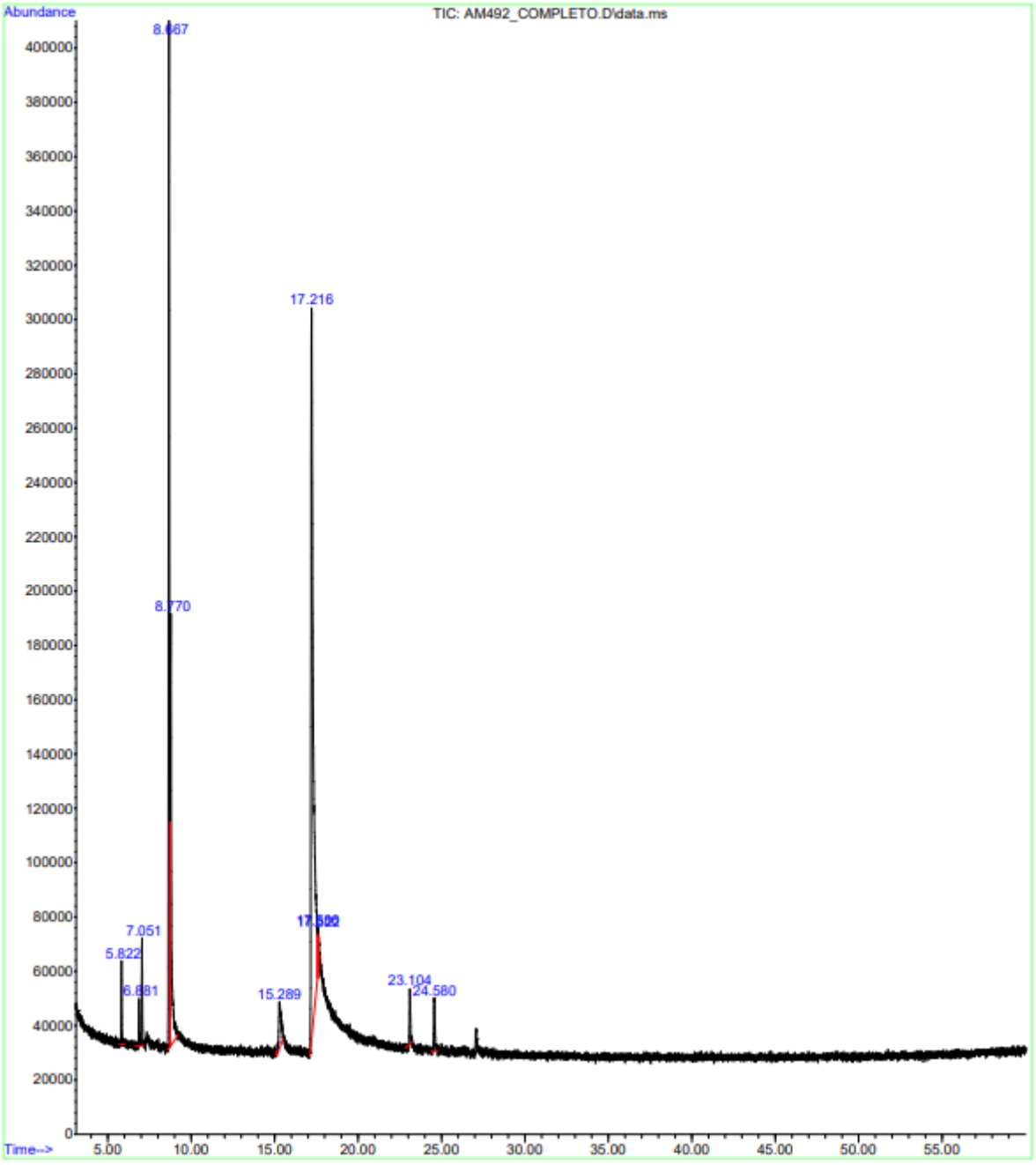
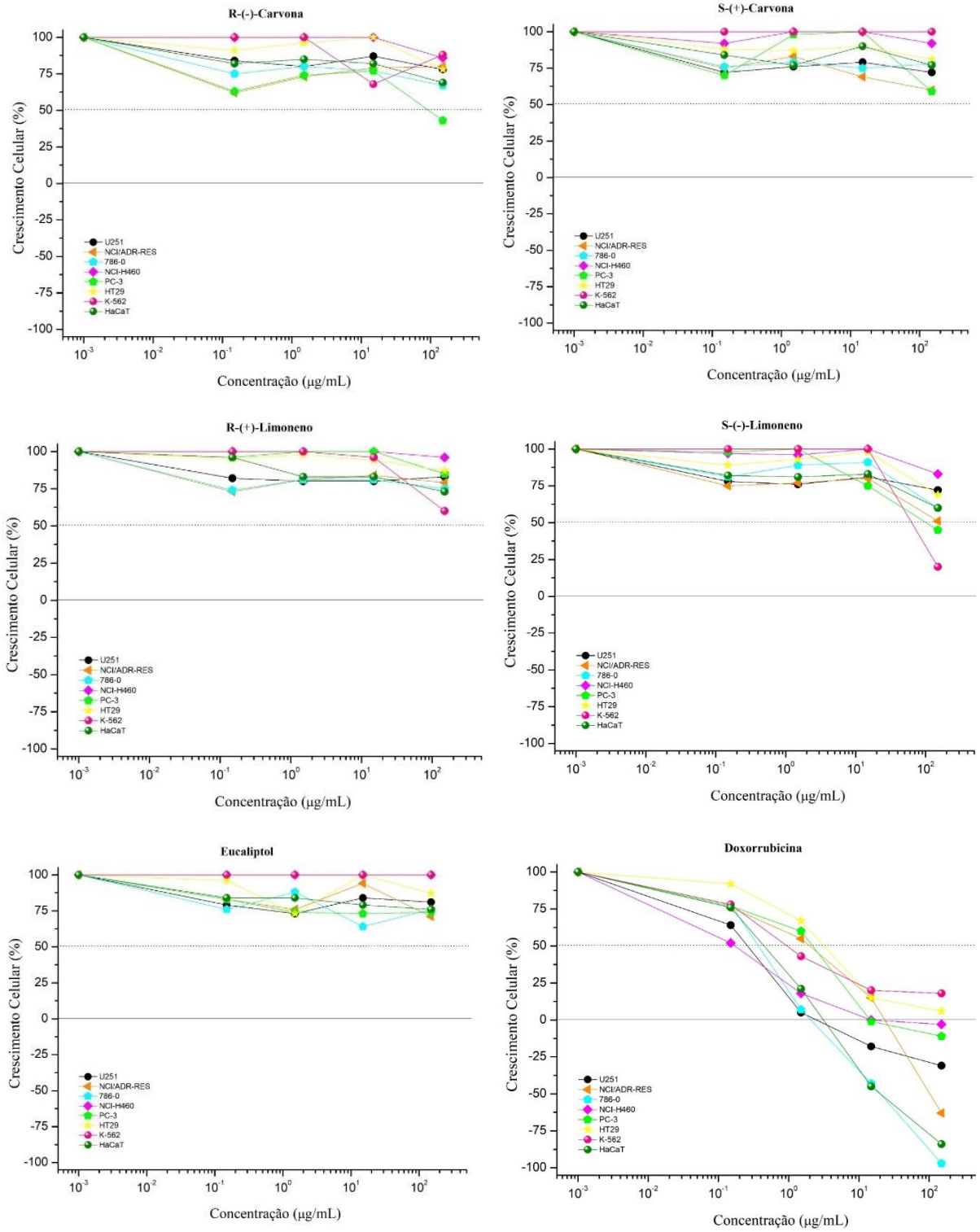


Figure S3: Anti-proliferative profile of *R*-(-)-carvona, *S*-(+)-carvona, *R*-(+)-limoneno, *S*-(-)-limoneno, eucalyptol, *Mentha aquatica* essential oil, and doxorubicin after 48h-exposure.



Human tumour cell lines: U251 (glioblastoma); NCI-ADR/RES (multidrug resistant ovarian adenocarcinoma); 786-0 (kidney, adenocarcinoma); NCI-H460 (lung, non-small cell carcinoma); PC-3 (prostate, adenocarcinoma); HT-29 (colorectal adenocarcinoma); K562 (Chronic Myelogenous Leukemia). Human non-tumour cell line: HaCaT, immortalized keratinocytes.

Figure S4: Representative photomicrographs, at 0 and 24 h, depicting the effect on the migration of HaCaT cells exposed to R-(-)-carvone, *S*-(+)-carvone (A), *R*-(+)-limonene, *S*-(-)-limonene (B), eucalyptol (C), and OEMa (D) (62.5 to 500 µg/ml, ideal growing condition: RPMI 1640 + 5% FBS) compared to positive (RPMI 1640 + 5% FBS) and negative (RPMI 1640 + 0.2% FBS) controls.

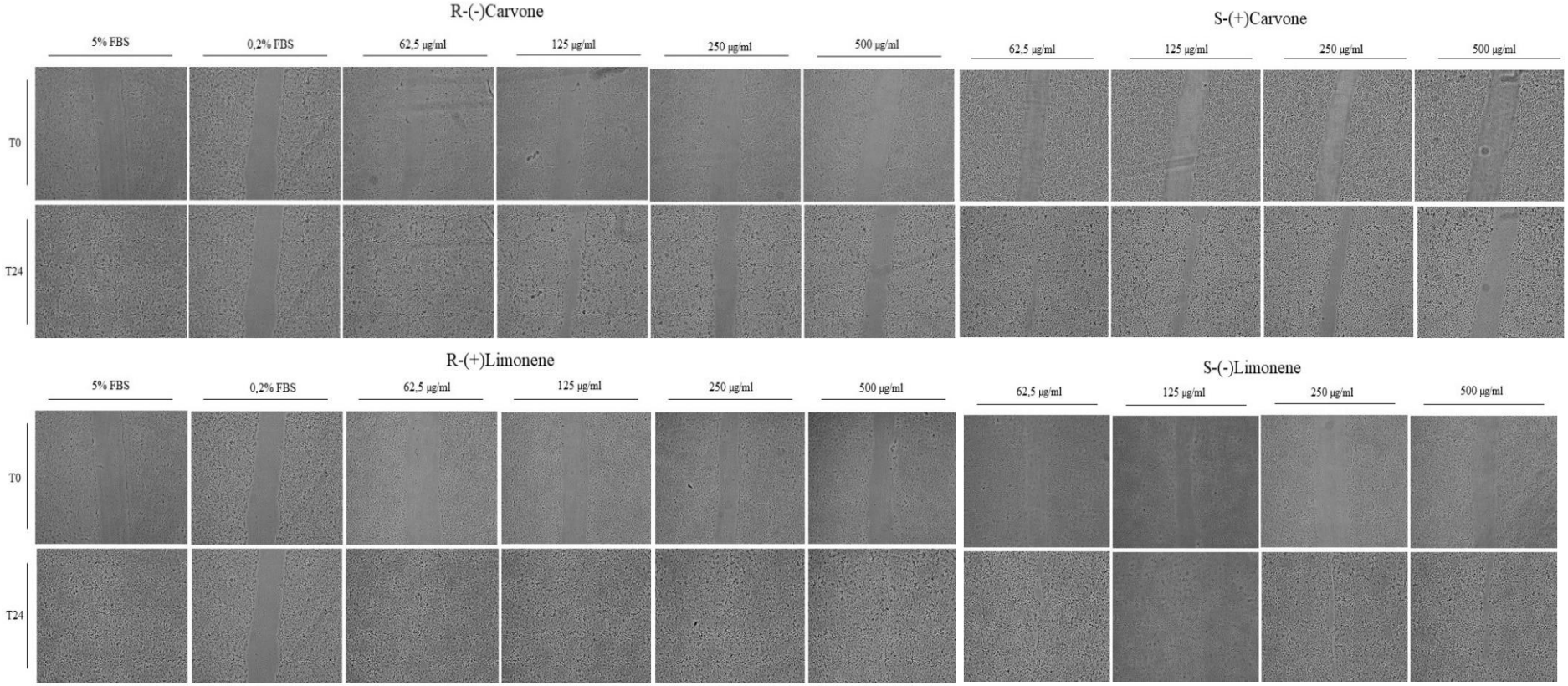


Fig. S4 (cont.):

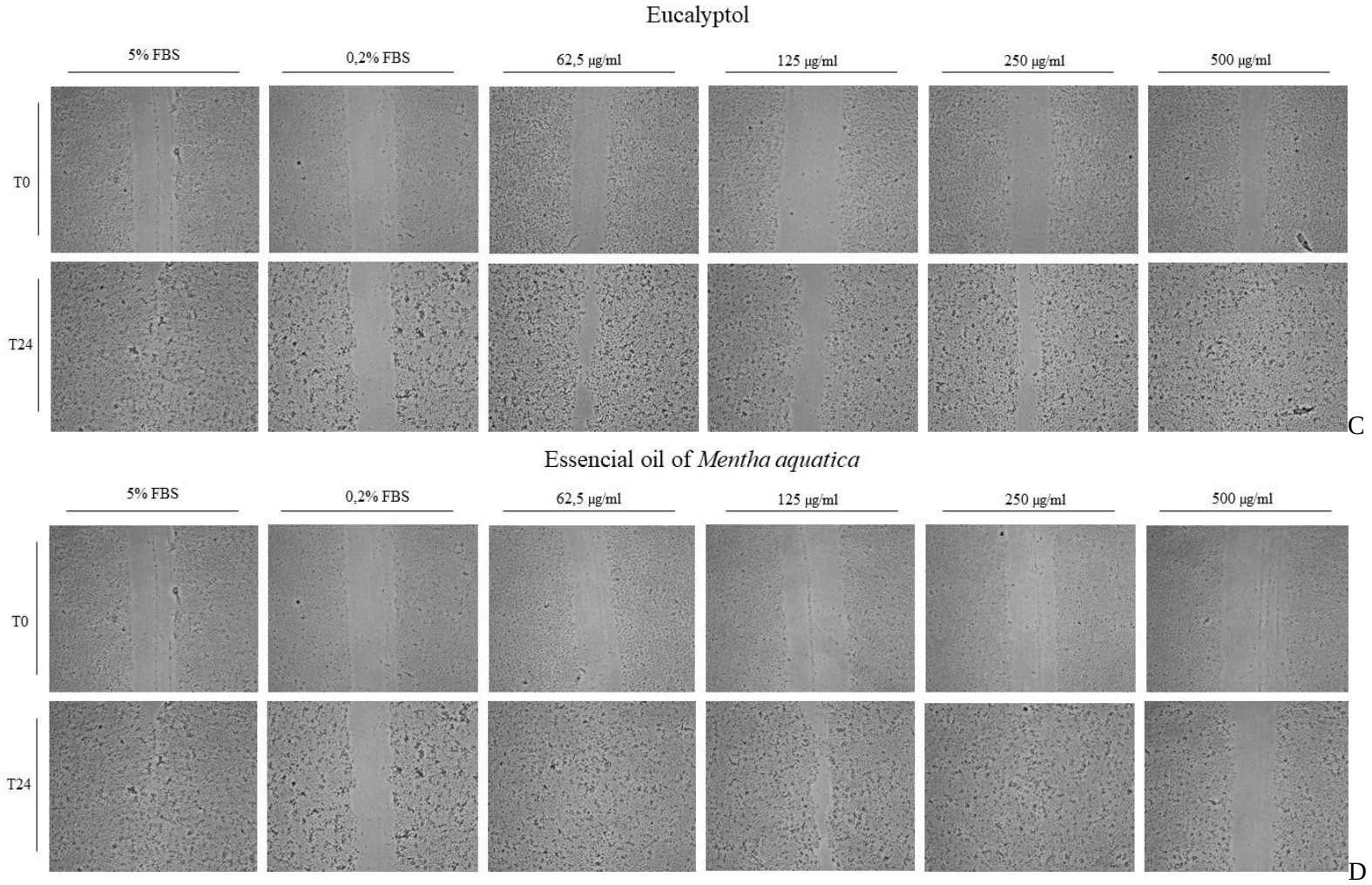

Eucalyptol
5% FBS
0,2% FBS
62,5 µg/ml
125 µg/ml
250 µg/ml
500 µg/ml
T0
T24
C
Essencial oil of *Mentha aquatica*
5% FBS
0,2% FBS
62,5 µg/ml
125 µg/ml
250 µg/ml
500 µg/ml
T0
T24
D

Figure S5: Representative photomicrographs at 0 and 24 h time points showing the effect on migration of HaCaT cells exposed to *R*-(-)-carvone, *S*-(+)-carvone (A), *R*-(+)-limonene, *S*-(-)-limonene (B), eucalyptol (C), and OEMa (D) (62.5 to 500 µg/ml, starving condition: RPMI 1640 + 0.2% FBS) compared to positive (RPMI 1640 + 5% FBS) and negative (RPMI 1640 + 0.2% FBS) controls.

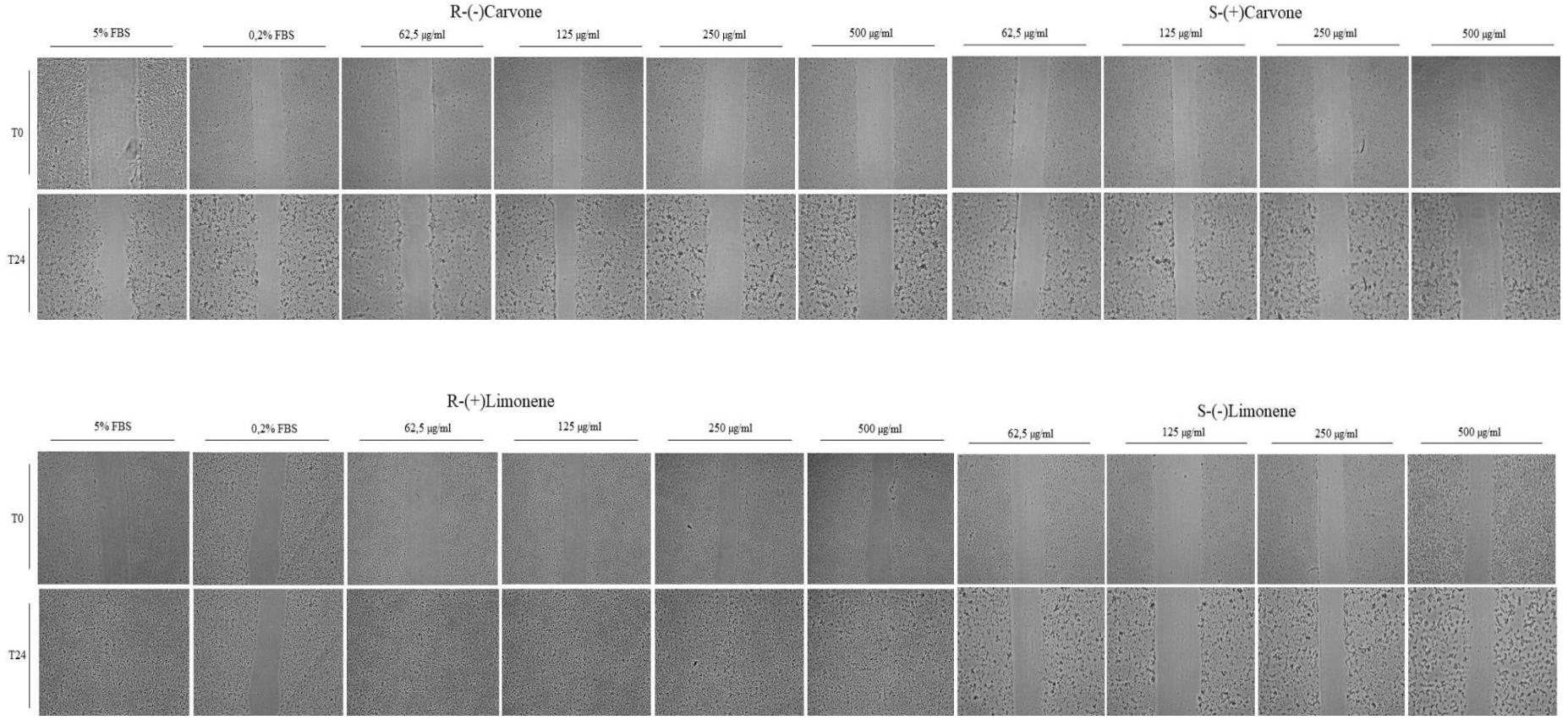


Fig. S5 (cont.):

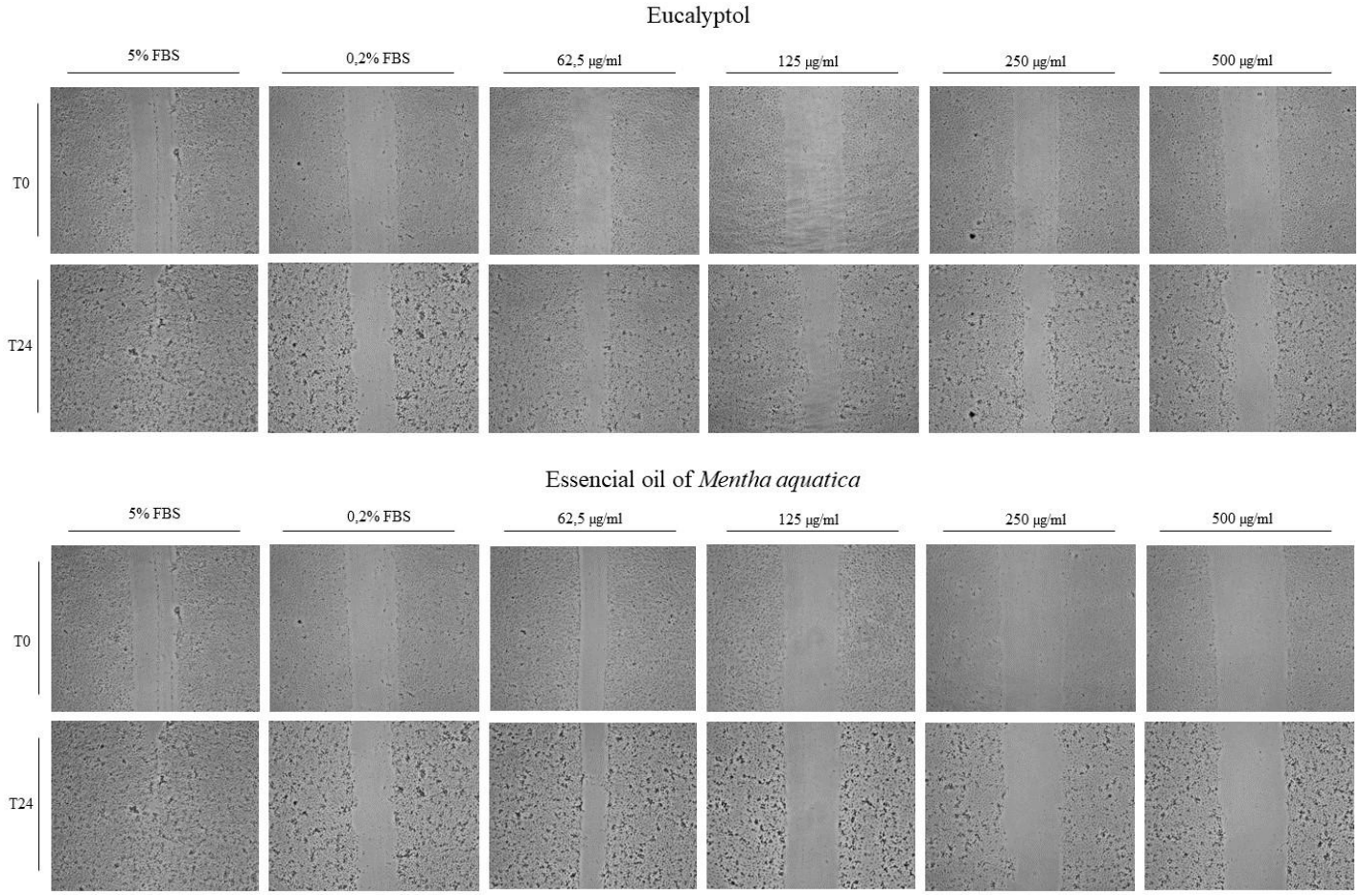

Eucalyptol
5% FBS
0,2% FBS
62,5 µg/ml
125 µg/ml
250 µg/ml
500 µg/ml
T0
T24
Essencial oil of *Mentha aquatica*
5% FBS
0,2% FBS
62,5 µg/ml
125 µg/ml
250 µg/ml
500 µg/ml
T0
T24

Figure S6: Representative photomicrographs of HaCaT cell spheroids exposed to *R*-(-)-carvone, *S*-(+)-carvone (A), *R*-(+)-limonene, *S*-(-)-limonene (B), and eucalyptol (C) (62.5 to 500 µg/ml, ideal growing condition: RPMI 1640 + 5% FBS) compared to untreated spheroids (control group) at T0 (sample addition) and T48 (after 48 h-exposure).

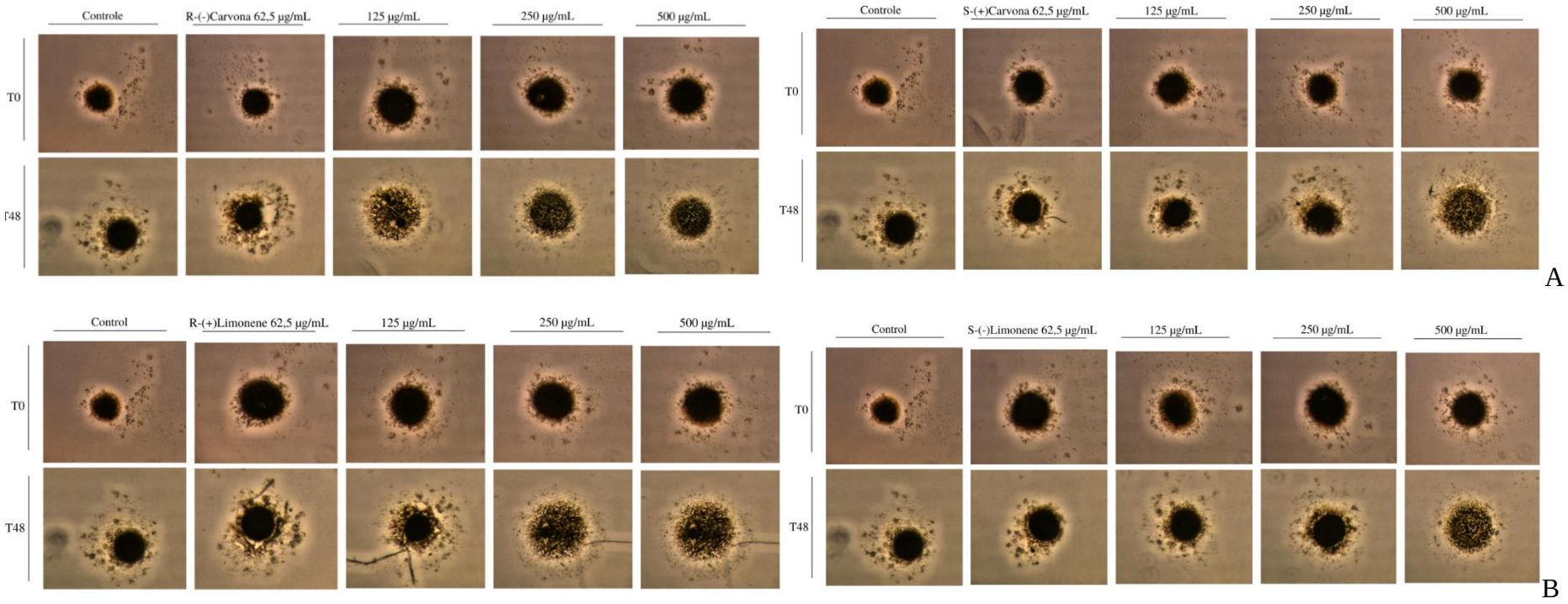
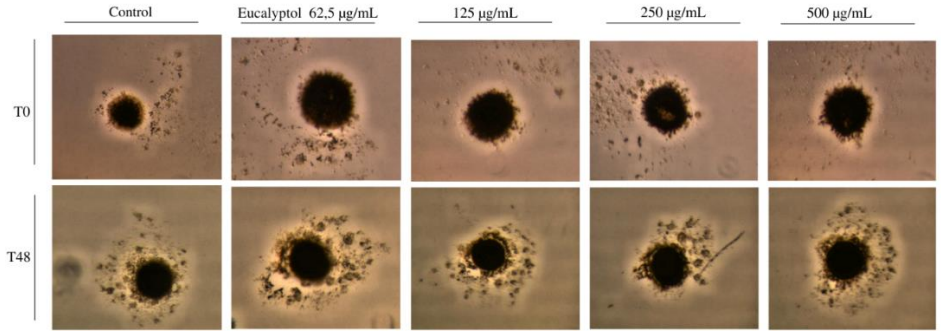


Figura S6 (cont.):



C

Spheroids were photographed using a digital camera for the OPTIKAM® B3 microscope attached to the Leica DM IL inverted microscope (40x magnification) using the OPTIKA® image capture software, version 1.2.

3. CONCLUSÃO

Através da utilização de modelos experimentais em 2D e 3D com linhagem de queratinócitos humanos imortalizados, foi possível evidenciar que o efeito do óleo essencial de *Mentha aquatica* sobre a migração celular dependeu da condição de cultivo empregada, com predominio do efeito inibidor. Esse efeito pode ser explicado pela presença de grande teor de carvona (cerca de 50%), uma vez que esse monoterpeno inibiu a migração de queratinócitos, de maneira concentração dependente e independente da condição de cultivo. Já o limoneno apenas reduziu a migração celular na ausência de estímulo (meio sem adição de soro fetal bovino). Em modelos 2D, não foram observados efeitos de redução de viabilidade celular nas condições empregadas para carvona e limoneno. No entanto, em modelo 3D, ambos afetaram negativamente a viabilidade celular. Esses achados indicam que ambos monoterpenos poderiam ser potenciais candidatos a fármacos adjuvantes no tratamento de problemas de pele caracterizados pela proliferação e migração desordenada de queratinócitos, como a psoríase e a queratose actínica.

Por outro lado, o eucaliptol estimulou a migração celular e, em algumas situações, aumentou a proliferação celular. Desta forma, este monoterpeno pode ser um insumo interessante no desenvolvimento de formulações para cicatrização dérmica.

Foram observadas pequenas diferenças significativas entre os enantiômeros avaliados sugerindo que, para as atividades biológicas estudadas, a estereoisomeria influência na ação farmacológica tanto da carvona quanto do limoneno.

Esses resultados contribuem para o melhor entendimento dos efeitos farmacológicos do óleo essencial de *M. aquatica* e dos monoterpenos em estudo para o desenvolvimento de novas opções terapêuticas para o tratamento de enfermidades de pele.

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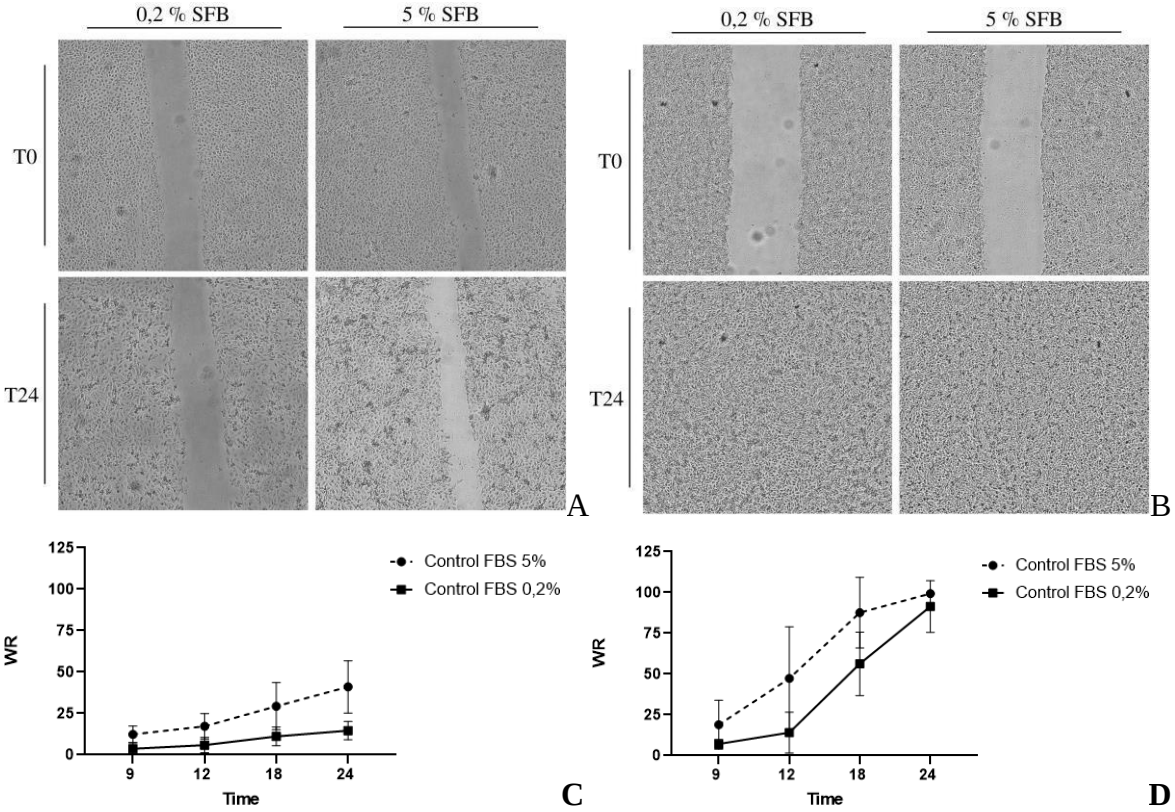
Appendix: Standardization of the cell migration experiment – scratch assay

For standardization of this experimental model, the first parameter evaluated was the media volume per well after the scratch. In the first experiment, 500 µL/well of culture media were used. Image analysis demonstrated that despite inducing a significant increase on cell migration compared to negative control (RPMI 1640 + 0.2% (v/v) FBS), the positive control RPMI 1640 + 5% (v/v) FBS promoted a wound closure effect lower than 50% (Table I, Figures IA and IC). When the volume was increased to 2 mL/well, the positive control resulted in wound retraction higher than 80% after 18 h-exposure (Table I, Figures IB and ID).

Figure I: Influence of compartment volume on cell migration.



Human HaCaT cell line (immortalized keratinocytes). **A** and **B**: Representative photomicrographs at 0 and 24 hours after treatments with RPMI 1640 media supplemented with 0.2% or 5% FBS (A = 500 µl/well; B = 2000 µl/well). **C** and **D**: Wound closure curve (WR, %) as a function of exposure time after treatments with RPMI 1640 media supplemented with 0.2% or 5% FBS (C = 500 µl/well; D = 2000 µl/well).

These results (Table I, Figure I) indicated the influence of the amount of active substances related to the number of cells per well. While the term "concentration" describes the amount of sample per unit of volume, the term "dose" refers to the amount of sample per compartment used or per quantity of cells present in that compartment (Kisitu, 2019). Based on these concepts, it was possible to explain the differences observed between the experiments

with different volumes. When HaCaT cells (3x105 cells/well) were treated with a smaller volume (500 µl/well) of RPMI 1640 supplemented with 5% and 0.2% FBS, the final dose of FBS was 25 µl and 1 µl per well, respectively. In the second experiment, the increased volume (2000 µl/well) resulted in higher final dose of FBS (100 and 4 µl/well, respectively) (Table I). This increase in dose enabled a wound area reduction higher than 50% at the 18h-exposure in positive control group (5% FSB).

Table I: Influence of fetal bovine serum (FBS) concentration (%) and dose (µl/3x10^5 cells) on the migration of HaCaT (immortalized human keratinocytes).

			Wound Retratction (%)			
Vol	**Conc**	**Dose**	**9h**	**12h**	**18h**	**24h**
500	5.0	250	12.4 ± 5.0	17.3 ± 7.6**	29.4 ± 14.2****	41.1 ± 15.8****
	0.2	1	3.7 ± 3.6	5.9 ± 4.6	11.1 ± 5.7	14.6 ± 5.5
2000	5.0	1,000	18.9 ± 15.0	47.4 ± 31.6**	87.7 ± 21.6*	99.3 ± 1.8
	0.2	4	7.2 ± 2.8	14.1 ± 12.5	56.3 ± 19.5	91.4 ± 15.8

Vol = volume (µl) of complete medium (RPMI 1640 + FBS) per well in 24-well plate; Conc = concentration (%) of fetal bovine serum (FBS) in RPMI 1640 medium; Dose = volume (µl) of FBS per 3x10^5 HaCaT cells. Statistical analysis: two-way ANOVA followed by Bonferroni's test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to cells cultivated in RPMI 1640 + 0.2% FBS).

The second parameter evaluated was the implementation of a serum starvation step in the culture medium 24 h before treatments, aiming to synchronize the cells in the G0/G1 phases. Regarding the positive (RPMI 1640 + 5% FBS) and negative (RPMI 1640 + 0.2% FBS) control groups, there was a significant difference in all time exposure evaluated (9, 12 and 24h). Furthermore, in addition to the wound closure, supplementation with 5% FBS promoted a significant increase in cell viability, suggesting a proliferative effect (Table II).

In the anti-proliferative activity assay conducted in a 96-well plate, it was determined that the concentration of 150 µg/ml would be the highest concentration without effect on cell proliferation for each monoterpene evaluated. Considering the cell amount per well, in 96-well plate there was 3x10^3 cells per well resulting, for each monoterpene, a dose of 30 µg/3x10^3 cell. As the cell migration assay was performed in a 24-well culture plate, the cell density was 3x10^5 cell/well. Considering the volume per well (2 ml) and the non-anti-proliferative dose (30 µg/3x10^3 cell), the calculated final concentration of each monoterpene was 1500 µg/ml. This way, the first experiment evaluating the effect of *R*-(-)-carvone, *S*-(+)-carvone, *R*-(+)-limonene, *S*-(-)-limonene, and eucalyptol on cell migration was performed at a

single concentration of 1000 µg/ml, in RPMI 1640 supplemented with 0.2% FBS (Table II).

Table II: Influence of the monoterpenes *R*-(-)-carvone, *S*-(+)-carvone, *R*-(+)-limonene, *S*-(-)-limonene, and eucalyptol, at a single concentration, on the migration of HaCaT (immortalized human keratinocytes) was investigated.

		Wound Retraction (%)*			Viability (%)
Tratament#		**9h**	**12h**	**24h**	**24h**
SFB	5.0	21.3 ± 4.7[a]	32.8 ± 3.7[a]	81.9 ± 16.3[a]	168.6 ± 6.8[a]
	0.2	8.8 ± 5.0[b]	10.5 ± 6.3[b]	12.4 ± 8.2[b]	100 ± 5.7[b]
***R*-(-)-Carvone**		2.3 ± 5.7[b]	4.1 ± 5.1[b]	4.9 ± 7.5 [b]	12.6 ± 0.9[c.d]
S-(+)-Carvone		12.4 ± 3.1[b]	-0.2 ± 2.5[b]	-1.3 ± 3.7 [b]	15.1 ± 0.7[c.e]
***R*-(+)-Limonene**		0.1 ± 4.7[b]	1.4 ± 2.5[b]	0.6 ± 2.2 [b]	17.3 ± 2.7[d.e]
***S*-(-)- Limonene**		1.7 ± 0.9[b]	1.3 ± 1.0[b]	0.7 ± 0.4 [b]	42.2 ± 2.1[f]
Eucalyptol		3.2 ± 3.4[b]	2.8 ± 3.0[b]	2.3 ± 2.1 [b]	50.5 ± 0.9[f]

#Treatments: SFB = RPMI 1640 medium supplemented with Fetal Bovine Serum at concentrations of 5% (positive control) or 0.2% (negative control); R-(-)-carvone, S-(+)-carvone, R-(+)-limonene, S-(-)-limonene, and eucalyptol = evaluated at a concentration of 1000 µg/ml. * Technical issue with the microscope prevented image capture at 18h-exposure. Statistical analysis: Area retraction by two-way ANOVA followed by Bonferroni's post hoc test; Cell viability by one-way ANOVA followed by Tukey's post hoc test. For both: different letters indicate significant difference within the same column, with $p \leq 0.01$.

In these conditions, all five monoterpenes inhibited the migration of human keratinocytes HaCaT. However, the evaluation of cell viability at the end of 24 h-exposure showed that the monoterpenes affected cell viability, suggesting that the observed inhibition of migration was due to a drastic reduction in the number of viable cells (Table II).

Based on these results, the concentration-effect relationship of each monoterpene was evaluated under the established experimental conditions (synchronization step 24 h before wound induction, monoterpenes solutions at concentration ranging from 62.5 to 500 µg/ml in both RPMI 1640 culture medium supplemented with 5% and 0.2% SFB and a final volume of 2 ml/well).

ANEXOS

Anexo 1 - SisGen

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº AA0AAC4

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**AA0AAC4**
Usuário:	**UNICAMP**
CPF/CNPJ:	**46.068.425/0001-33**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Mentha aquatica

Título da Atividade:	**Avaliação in vitro dos monoterpenos Carvona, Limoneno e Eucaliptol, e suas associações, sobre a migração celular**

Equipe

Ana Lucia Tasca Gois Ruiz	**UNICAMP**
DANIELE DAIANE AFFONSO	**Unicamp**
Mary Ann Foglio	**Unicamp**

Resultados Obtidos

Divulgação de resultados em meios científicos ou de comunicação

Identificação do meio onde foi divulgado:	**Dissertação de mestrado em andamento, partic**

Data do Cadastro:	**07/11/2022 08:12:41**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **8:13** de **07/11/2022**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN

Anexo 2 - Premiação em Congressos

Certificado de Honra ao Mérito

A Comissão Organizadora da XV Jornada Paulista de Plantas Medicinais tem a honra de conferia a **Daniele Daiane Affonso** o presente certificado de mérito pela apresentação do trabalho "EFEITO ANTIPROLIFERATIVO DE MONOTERPENOS IDENTIFICADOS NO ÓLEO ESSENCIAL DE *Mentha aquatica*." De autoria de Daniele Daiane Affonso, Kaio Eduardo Buglio, Ana Lucia Tasca Gois Ruiz.

Franca, 17 de setembro de 2021.

Profa. Dra. Patrícia Mendonça Pauletti
Presidente da Comissão Organizadora da XV JPPM

Certificado

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Certificamos que o trabalho
"AVALIAÇÃO IN VITRO DOS MONOTERPENOS LIMONENO, CARVONA E EUCALIPTOL SOBRE A MIGRAÇÃO CELULAR"
de Daniele D. Affonso, Ana Lucia. T. G. Ruiz, Mary Ann Foglio
recebeu Menção Honrosa na modalidade Melhores Painéis na categoria Mestrado,
no evento: III Simpósio Internacional em Investigações Químico-Farmacêuticas; I Encontro Ibero-Americano de Plantas Medicinais Dr. Mahabir Gupta; I Congresso Luso-Brasileiro de Ciências e Tecnologias em Saúde,
realizado(a) no dia 28 a 30 de setembro de 2022, em Itajaí-SC, Brasil.

Itajaí (SC), 13 de outubro de 2022.

Reitor
Prof. Dr. Valdir Cechinel Filho