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PRISCILLA GUIMARÃES SILVA VASCONCELOS

ANÁLISE ANTIFÚNGICA DE COMPOSTOS BIOATIVOS, DA SUA AÇÃO EM FATORES DE VIRULÊNCIA DE *Candida albicans* E NA MODULAÇÃO DA INTERAÇÃO *Candida*- HOSPEDEIRO

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Tese apresentada ao programa de Pós-Graduação em odontologua da Universidade Estadual da Paraíba, como requisito parcial à obtenção do título de Doutorado em Clínica Odontológica

Área de concentração: Estudo dos Processos e Terapias Relacionadas aos Agravos à Saúde Bucal

.

Orientadora: Profª Drª Edja Maria Melo de Brito Costa
Universidade Estadual da Paraíba – UEPB

Coorientador: Prof. Dr. Ramiro Mendonça Murata
East Carolina University – ECU

CAMPINA GRANDE
2023

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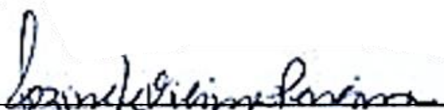
BANCA EXAMINADORA

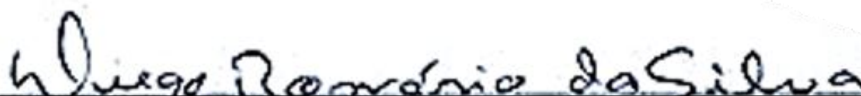
Profa. Dra. Edja Maria Melo de Brito Costa (Orientadora)
Universidade Estadual da Paraíba (UEPB)



Profa. Dra. Jozinete Vieira Pereira (Membro Titular Interno)
Universidade Estadual da Paraíba (UEPB)

Profa. Dra. Andréa Cristina Barbosa da Silva (Membro Titular Interno)
Universidade Estadual da Paraíba (UEPB)

Profa. Dra. Carolina Medeiros de Almeida Maia (Membro Titular Externo)
Faculdade Rebouças



Prof. Dr. Diego Romário da Silva (Membro Titular Externo)
Universidade de Cuiabá (UNIC)

Ao meu marido, Adbys Vasconcelos e aos meus pais Vanizia e Amarildo, pelo apoio incondicional.

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A vocês dedico este trabalho

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RESUMO

OBJETIVO: Analisar *in vitro* a atividade antifúngica sobre *Candida* spp do óleo essencial (OE) de *Syzygium aromaticum* e de compostos bioativos (eugenol, β-cariofileno, geraniol, citronelal e linalool), além da ação destes sobre fatores de virulência de *Candida albicans* e na modulação da interação *Candida*-hospedeiro, considerando também a toxicidade *in vivo*. **MATERIAL E MÉTODO**: A atividade antifúngica foi analisada por meio da microdiluição em caldo sobre espécies de *Candida* (*C. albicans*, *C. glabrata*, *C. tropicalis* e *C. dubliniensis*) e da atividade antibiofilme frente à *C. albicans*, para tal foi determinado respectivamente a Concentração Inibitória Mínima (CIM) e realizada a contagem de Unidades Formadoras de Colônia/mL (UFC/mL) padronizada pelo peso seco do biofilme (UFC/mL/g). A citotoxicidade foi analisada em células epiteliais orais humanas (TR146 - ECACC 10032305) e em monócitos humanos (THP-1 - ATCC TIB-202) pelo ensaio de viabilidade *Cell Titer Blue*. Com base nos resultados microbiológicos e citotóxicos, o geraniol (CIM) foi selecionado para análise da modulação da interação *Candida*-hospedeiro, por meio do método de co-cultura *dual chamber* com células THP-1 e células TR146 (infectadas com *C. albicans*), RT-PCR foi utilizado para avaliar expressão gênica de enzimas proteolíticas de *C. albicans* (ACT-1 e PLB-1) e citocinas inflamatórias do hospedeiro (IL-1β, IL-6, IL-17, IL-18, IL-10 e TNF), além disto, a co-cultura entre TR146 e *C. albicans* foi observada sob microscopia de fluorescência. Foi realizada análise da toxicidade sistêmica em modelo de *Galleria mellonella.* **RESULTADOS:** Todos os compostos, com excessão do β- cariofileno (CIM > 8000 μg/mL), apresentaram atividade antifúngica com CIM determinada em: 500-1000 μg/mL para o OE e eugenol, 1.25-5 mM/mL para o geraniol, 25-100 mM/mL para o linalol e 100-200 mM/mL para o citronelal. Todas as concentrações testadas para o OE, eugenol e geraniol reduziram a viabilidade da *C. albicans* durante a formação do biofilme e no biofilme maduro. O linalol inibiu a formação do biofilme, mas no biofilme maduro só 10xCIM foi eficaz. O citroenlal não apresentou atividade antibiofilme na concentração testada. Para citotoxidade foram definidas as LD_{50} , respectivamente, para TR146 e THP-1: OE 59.37 e 79.54 μg/mL; eugenol 55.35 e 84.16 μg/mL; geraniol 5.883 mM/mL e 8.027 mM/mL; linalol 1.432 mM/mL e 1.709 mM/mL; e citronelal 0.3006 mM/mL e 0.1825 mM/mL. Geraniol 5 mM/mL (CIM) foi capaz de modular a relação *Candida* hospedeiro por meio da infrarregulação da expressão das enzimas proteolíticas de *Candida* e das citocinas pró-inflamátorias do hospedeiro IL-1β, IL-6 e IL-18. A redução substancial do crescimento de *C. albicans* pela ação do geraniol também foi vista

microscopicamente. O geraniol (até 8000 mM/Kg) e o OE (até 100 mg/Kg) não induziram toxicidade. **Conclusão:** O óleo essencial de *S. aromaticum* e os compostos avaliados, com excessão do β-Cariofileno e do citronelal, apresentam atividade anti- *Candida*, capaz de inibir a formação de biofilme e reduzir a viabilidade celular de um biofilme maduro de *C. albicans.* O geraniol foi capaz de modular a interação *Candida*-hospedeiro, reduzindo a expressão de fatores de virulência e de citocinas pró-inflamatórias, sem evidências de toxicidade.

Palavras-chave: *Candida albicans;* biofilmes; fatores de virulência; candidíase oral; agentes antifúngicos; agentes antiinflamatórios.

ABSTRACT

OBJECTIVE: To analyze the *in vitro* antifungal activity against *Candida* spp of *S. aromaticum* essential oil (EO), and bioactive compounds (eugenol, β-caryophyllene, geraniol, citronellal and linalool), as well as its action on virulence factors of *Candida albicans*, and in the modulation of *Candida*-host interaction, considering the associated *in vivo* toxicity. **MATERIAL AND METHOD:** The antifungal activity was analyzed by Broth Microdilution Method on *Candida* species (*C. albicans, C. glabrata, C. tropicalis* and *C. dubliniensis*) and antibiofilm activity on *C. albicans*, for that it was established, respectively, the Minimal Inhibitory Concentration (MIC), and the counting of Colony Forming Units/mL (CFU/mL), normalized by the biofilm dry weight (CFU/mL/g). Cytotoxicity was analyzed in human oral epithelial cells (TR146 - ECACC 10032305) and in human monocytes (THP-1 - ATCC TIB-202) by the Cell Titer Blue viability assay. Based on the microbiological and cytotoxic results, geraniol (MIC) was selected for analysis of the capacity to modulate *Candida*-host interaction, through the dual chamber co-culture method. with THP-1 cells and TR146 cells (infected with *C. albicans*), RT-PCR were used to assess gene expression of *C. albicans* proteolytic enzymes (ACT-1 and PLB-1) and host inflammatory cytokines (IL-1β, IL-6, IL-17, IL-18, IL-10, and TNF). Fluorescence microscopy was performed with TR146 and *C. albicans* co- culture. An analysis of systemic toxicity was performed in a model of *Galleria mellonella*. **RESULTS:** All compounds, with the exception of β-caryophyllene (MIC > 8000 μg/mL), showed antifungal activity with MIC determined at: 500-1000 μg/mL for EO and eugenol, 1.25-5 mM/mL for geraniol, 25-100 mM/ml for linalool and 100-200 mM/ml for citronellal. All concentrations tested for EO, eugenol and geraniol reduced the viability of *C. albicans* during biofilm formation and in the mature biofilm. Linalool inhibited biofilm formation, but in the mature biofilm only 10xCIM was effective. Citronellal did not show antibiofilm activity at the tested concentration. For cell cytotoxicity, the LD_{50} were defined, respectively, for TR146 and THP-1: EO 59.37 and 79.54 μg/mL; eugenol 55.35 and 84.16 μg/mL; geraniol 5883 mM/ml and 8027 mM/ml; linalool 1432 mM/ml and 1709 mM/ml; and citronellal 0.3006 mM/mL and 0.1825 mM/mL. Geraniol 5 mM/mL (MIC) was able to modulate the *Candida*-host interaction by down-regulating the expression of *Candida* proteolytic enzymes and the host pro-inflammatory cytokines IL-1β, IL-6 and IL-18. The substantial reduction in growth of *C. albicans* by geraniol was also seen microscopically. Geraniol (up to 8000 mM/Kg) and EO (up to 100 mg/Kg) did not induce *in vivo* toxicity. **Conclusion:** The essential oil of *S. aromaticum* and the evaluated compounds, except for β-caryophyllene and citronellal, had anti-*Candida*

activity, capable of inhibiting biofilm formation and reducing the viability of a mature *C. albicans* biofilm. Geraniol modulates the *Candida*-host interaction, reducing the expression of virulence factors and pro-inflammatory cytokines, without evidence of toxicity**.**

Keywords: *Candida albicans;* biofilms; virulence factors; oral candidiasis; antifungal agents. anti-inflammatory agents.

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Artigo 1

- **Figure 1:** *S. aromaticum* essential oil (5xMIC - 2,500 µg/mL and 10xMIC 5,000 µg/mL) and eugenol (5xMIC – 5,000 µg/mL and 10xMIC - 10,000 µg/mL) action upon *C. albicans* ATCC MYA 2876 growth kinetics. DMSO 1%: Vehicle control; Fluconazole (10xMIC – 10 µg/mL): positive control.

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Artigo 2

- **Figure 1.:** Dual-chamber *in vitro* model for an oral epithelium upon fungal infection and treatment with geraniol (5mM/mL). The apical chamber represents the first barrier of the epithelial layer in which TR146 cells were subcultured until it reached confluency and subsequently infected with *C. albicans* ATCC MYA 2876. The inserts (apical chamber) were placed in a 6-well plate (basal chamber) containing THP-1 cells to evaluate the host inflammatory response.
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- **Table 1:** Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) for *S. aromaticum* essential oil, eugenol, ß-caryophyllene, and fluconazole according to *Candida* species.

Artigo 2

- **Table 1:** Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of geraniol, linalool, citronellal, and fluconazole according to the species of *Candida*. The ratio obtained from MFC/MIC is also shown.

LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de Variância
ATCC	American Type Culture Collection
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CFM	Concentração Fungicida Mínima
CFU	Counting forming unitys
CIM	Concentração Inibitória Mínima
CO_2	Dióxido de Carbono
DMSO	Dimethyl sulfoxide
ECU	East Carolina University
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
IL	Interleucina/Interleukin
LD_{50}	lethal dose 50
mg	Miligrama
mM	Milimolar
mL	Mililitro
MAPK	Mitogen-activated Protein Kinase
NIH	National Institutes of Health
NaOH	Hidróxido de Sódio
PAMP	Pathogen-associated Molecular Pattern
PB	Paraíba
PBS	Phosphate-buffered Saline
PPR	Pattern Recognition Receptors
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAP	Secreted Aspartyl Proteinase
TNF	Tumor necrosis factor
UEPB	Universidade Estadual da Paraíba
UFC	Unidades Formadoras de Colônias
YNB	Yeast Nitrogen Base
°C	Grau Celsius
µL	Microlitro
v/v	Volume/volume

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1 INTRODUÇÃO

A estomatite protética é uma das condições clínicas mais comuns na odontologia, com alta prevalência entre usuários de próteses dentárias removíveis (DIAS et al., 2018; REINHARDT et al., 2018), além de ser considerada a forma clinica mais prevalente da candidiase oral, constituindo cerca de 70-95% dos casos diagnosticados, sendo os idosos os mais acometidos (DIAS et al., 2018; REINHARDT et al., 2018; ROSA-GARCÍA et al., 2020)

Consiste na inflamação da mucosa oral subjacente a uma prótese dentária removível, ocorrendo, portanto, no palato e/ou na mucosa alveolar (GAD; FOUDA, 2020; HELLSTEIN; MAREK, 2019; LEWIS; WILLIAMS, 2017). Clinicamente, é caracterizada pela presença de eritema, hiperemia, edema e, em alguns casos, de petéquias hemorrágicas. Apesar de comumente ser assintomática, alguns pacientes relatam sensação de queimação, diminuição da salivação, halitose e alteração no paladar (GAD; FOUDA, 2020; GENDREAU; LOEWY, 2011; HELLSTEIN; MAREK, 2019; LEWIS; WILLIAMS, 2017).

Sua etiologia é multifatorial e está relacionada a diversos fatores como o uso prolongado de prótese dentária, trauma local, baixo fluxo salivar, hipersensibilidade ao material que constitui a prótese e/ou falta de higiene da cavidade oral e da prótese dentária (GAD; FOUDA, 2020; HELLSTEIN; MAREK, 2019; LEWIS; WILLIAMS, 2017). Adicionalmente, esta condição é comumente associada à infecção por espécies de *Candida*, em especial a *Candida albicans*, que encontra na superfície das próteses um ambiente favorável à sua colonização (MOUSA; LYNCH; KIELBASSA, 2020; REINHARDT et al., 2018).

A baixa qualidade de higiene favorece a adesão dos microrganismos à superfície da prótese e a formação de um biofilme que estará em contato direto com a mucosa adjacente, propiciando a invasão tecidual por *Candida*. Consequentemente, haverá quebra do equilíbrio entre os fatores de virulência do patógeno e a resposta imune do hospedeiro, alterando a microbiota normal, de um estado comensal, para uma forma patogênica (MOUSA; LYNCH; KIELBASSA, 2020; REINHARDT et al., 2018; VILA et al., 2020).

Durante a infecção por *C. albicans*, os Padrões Moleculares Associados a Patógenos (PAMPs) presentes em *Candida* induzem o seu reconhecimento pelo hospedeiro, por meio dos Receptores de Reconhecimento de Padrões (PRRs), estimulam a secreção de peptídeos antifúngicos com o intuito de controlar a proliferação do patógeno (D'ENFERT et al., 2021;

N AGLIK et al., 2017). Além disso, há liberação de citocinas pró-inflamatórias como IL-1α, IL-1β, IL-6, IL-8 e IL-17, que atuam no recrutamento de células do sistema imune para o local de infecção (GUPTA et al., 2021; NIKOU et al., 2022; ZHOU et al., 2021) instaurando assim o padrão inflamatório causado pela presença do patógeno e característico da estomatite protética. A inflamação pode também ser potencializada pelo trauma de uma prótese mal adaptada, criando um ambiente favorável a adesão, proliferação e invasão tecidual pela *Candida* (ABUHAJAR et al., 2023; BARS et al., 2001; D'ENFERT et al., 2021; DUTZAN et al., 2017).

O tratamento da estomatite protética inclui adaptação da prótese dentária, orientação de higiene oral e prescrição de antifúngicos (ABUHAJAR et al., 2023). No entanto, alguns dos antifúngicos utilizados na clínica já apresentam resistência microbiana (LUKASZUK; KRAJEWSKA-KULAK; KULAK, 2017; ROSA-GARCIA et al., 2020) e não atuam na modulação do fator inflamatório, que é presente na estomatite protética. Neste sentido, incentiva-se a busca por compostos bioativos que apresentem mecanismos moleculares capazes de modular os fatores de virulência associados à infecção causada por *C. albicans* e a resposta inflamatória do hospedeiro frente ao patógeno, podendo melhorar, assim, a resposta ao tratamento da estomatite protética.

Newman e Cragg (2020) destacam as plantas medicinais como uma fonte promissora de compostos bioativos, no entanto, ainda há muito o que ser explorado em especial no quesito antifúngicos. De acordo com os autores, apesar da crescente preocupação com a resistência de cepas fúngicas, nenhum antinfúngico derivado de produtos naturais foi registrado desde 2006, o que corrobora com a necessidade de novas pesquisas. Diante desta premissa, no presente estudo foram utilizados compostos originários de produtos naturais, como o óleo essencial de *Syzygium aromaticum* (*S. aromaticum*) e seus compostos majoritários eugenol e β-cariofileno, além dos monoterpenos geraniol, citronelal e linanol.

O óleo essencial de *Syzygium aromaticum* (popularmente conhecida como cravo ou cravo-da-índia) possui propriedades antioxidante (MARMOUZI et al., 2019), antinflamatória (BANERJEE et al., 2020; MARMOUZI et al., 2019), antineoplásica (NAJAR et al., 2020), analgésica (CORREIA et al., 2018), antifúngica (HEKMATPANAH et al., 2022; VASCONCELOS et al., 2021), antibacteriana (EL-DARIER et al., 2018; NIRMALA et al., 2019) e antibiofilme (VASCONCELOS et al., 2021), cujas atividades podem ser atribuídas aos seus compostos majoritários: eugenol e o β-cariofileno (HARO-GONZÁLEZ et al., 2021; VASCONCELOS et al., 2021).

O óleo essencial de *S. aromaticum* foi eficaz contra um biofilme multiespécie, constituído a partir da saliva de pacientes com candidíase oral (VASCONCELOS et al., 2021), no entanto, ainda não existe conhecimento sobre a sua aplicação clínica no gerenciamento da estomatite protética, seja como um agente auxiliar na limpeza da prótese ou no tratamento tópico, diretamente na mucosa oral.

Os monoterpenos: geraniol, linalol e citronelal estão presentes em diversos óleos essenciais de plantas ou frutos aromáticos, apresentam a mesma fórmula molecular (C10H18O), mas diferem na estrutura química (AMMAR, 2023; KAMATOU; VILJOEN, 2008; MĄCZKA et al., 2022; MACZKA; WINSKA; GRABARCZYK, 2020). Esses compostos apresentam atividade antioxidante (AMMAR, 2023; SABOGAL- GUÁQUETA et al., 2019; YIN et al., 2022), anticancer (AMMAR, 2023; FATIMA; LUQMAN, 2021; HOU et al., 2022), antibacteriana (LI et al., 2023; LIU et al., 2020; ZHANG et al., 2022), antifúngica (KAYPETCH et al., 2022; MANDRAS et al., 2021; MEDEIROS et al., 2022; SAIBABU et al., 2017; SINGH; FATIMA; HAMEED, 2016, 2016b; TRINDADE et al., 2015) e antinflamatória (AMMAR, 2023; LEE et al., 2018; RICCI et al., 2022).

Apesar da capacidade antifúngica e antinflamatória destes compostos já ser algo discutido na literatura, conhecer o potencial modulador na interação *Candida*-hospedeiro se torna um importante marco para determinar sua aplicação clínica no tratamento da estomatite protética, considerando sua ação nos componentes fúngico e inflamatório da condição. Portanto, este estudo aborda, simultâneamente, a ação dos compostos bioativos nos fatores de virulência da *Candida* e na modulação do componente inflamatório gerado pela presença da *C. albicans*, por meio de co-cultura celular em modelo *dual chamber*, com o intuito de aproximar o estudo da condição clínica em questão. Adicionalmente, não foram encontradas nas bases pesquisadas estudos que abordem a análise desses compostos na interação *Candida*-hospedeiro.

Este estudo teve como objetivo avaliar *in vitro* a atividade sobre *Candida* spp do óleo essencial de *S. aromaticum*, de seus compostos majoritários eugenol e β-cariofileno, e dos monoterpenos geraniol, citronelal e linalol, assim como, a sua ação sobre fatores de virulência de *Candida albicans* e na modulação da interação *Candida*-hospedeiro, além da sua toxicidade. em modelo de *Galleria mellonella.*

2 REFERENCIAL TEÓRICO

2.1 *Candida albicans*: fatores de virulência e relação com o hospedeiro

Quando ocorre quebra da relação comensal entre a *C. albicans* e o hospedeiro, os fatores de virulência associados ao microrganismo atuam proporcionando a instalação da infecção oral (D'ENFERT et al., 2021; LEMBERG et al., 2022; VILA et al., 2020). A adesão constitui o primeiro passo para proliferação e infecção pela *C. Albicans*, podendo ser estabelecida em superfícies bióticas, como o tecido epitelial, ou em superfície abióticas, como as próteses dentárias (TSUI; KONG; JABRA-RIZK, 2016). Dessa forma, a remoção de células não aderidas pelo fluxo salivar ou pela higienização consiste em um importante fator protetor contra a proliferação excessiva de *Candida* (LEWIS; WILLIAMS, 2017; TALAPKO et al., 2021).

Após a adesão, *C. albicans* pode alterar sua conformação, através do dimorfismo celular, de levedura para forma de hifa, a qual tem maior capacidade de adesão e invasão celular no hospedeiro (D'ENFERT et al., 2021; HO et al., 2021; TALAPKO et al., 2021). No processo de instalação da infecção outros fatores de virulência da *C. albicans* podem ser destacados como a secreção de enzimas hidrolíticas extracelulares (TALAPKO et al., 2021; VILA et al., 2020), como aspartil proteases e fosfolipases. As enzimas hidrolíticas atuam na degradação e destruição de membranas celulares, e também podem degradar moléculas inerentes ao sistema imune. Tais fatos, somado à resistência ao estresse oxidativo, são fatores que contribuem com a evasão da defesa imune do hospedeiro (D'ENFERT et al., 2021; KULSHRESTHA; GUPTA, 2023; TSUI; KONG; JABRA-RIZK, 2016; VILA et al., 2020).

Um dos principais fatores de virulência associado a *C. Albicans*, e com significante inferência clínica, é a capacidade de formar biofilmes (D'ENFERT et al., 2021; WALL et al., 2019). O biofilme, quando maduro, apresenta multicamadas heterogêneas de microrganismos envoltos por uma matriz de substâncias extracelulares poliméricas, rica em nutrientes e macromoléculas, como ácidos nucleicos e lipídios. Esta matriz promove a proteção do biofilme frente a toxinas, alterações osmóticas e de pH, resposta imune do hospedeiro e aos antimicrobianos (BOWEN et al., 2018; KUMAR et al., 2017; TSUI; KONG; JABRA-RIZK, 2016).

No processo de infecção por *Candida* há uma complexa interação *Candida*- hospedeiro,

na qual a quebra da relação de homeostase, pela prevalência de fatores de virulência leva a ativação e modulação do sistema imune. De forma geral, esta interação começa pelo reconhecimento do patógeno por meio de PAMPs presentes em sua parede celular, os quais são reconhecidas por receptpres (PRR) presentes em células do sistema imune inato do hospedeiro (macrofagos, neutrófilos, monócitos, dentre outras), sendo considerada a primeira linha de defesa do sistema imune (D'ENFERT et al., 2021). Este processo culmina na ativação de importantes vias de sinalização que desencadearão o processo inflamatório e o recrutamento do sistema imune para eliminação do patógeno (BECKER et al., 2015; ZHOU et al., 2021).

O reconhecimento da forma patogênica da *Candida* pelo hospedeiro geralmente está relacionado com a expressão dos fatores de virulência. (HO et al., 2021; NAGLIK et al., 2017). Células epiteliais, por exemplo, podem reconhecer o dimorfismo celular a partir da produção da candidalisina pelas hifas, uma toxina pepitídica citolítica que é importante na degradação celular e invasão tecidual. A produção desta toxina é capaz de ativar a cascata da MAPK (proteína quinase ativada por mitógeno), esta cascata é composta por três vias JNK, p38 e ERK1/2. Uma vez ativadas essas vias, serão liberadas citocinas inflamatórias como as interleucinas IL- 1β, IL-6, IL-8, IL-17 e TNF (GUPTA et al., 2021; HO et al., 2021; NIKOU et al., 2022; ZHOU et al., 2021). Apesar da importância relacionada à ativação da resposta imune, repercurssões clínicas podem ser causadas pela superexpressão destes fatores. A inflamação persistente causada por infecções, por exemplo, é um fator importante para carcinogênese sendo atribuída a cerca de 25% dos casos de câncer (GUPTA et al., 2021; HO et al., 2021; MURATA, 2018).

Candida spp faz parte da microbiota comensal e extiste a necessidade de manter a homeostase do meio, a partir do controle dos fatores de virulência, que podem levar a superexpressão de fatores pró-inflamatórios, por exemplo. Portanto, conhecer os fatores de virulência da *C. albicans* e estratégias para modulação da relação *Candida*-hospedeiro, durante a infecção são pontos importantes na bsuca por novos compostos que possam atuar nestes fatores.

2.2 *Syzygium aromaticum*

Dentre as plantas utilizadas na medicina popular destaca-se a *Syzygium aromaticum* (L.) Merr. & L.M. Perry, conhecida popularmente por cravo, cravo-da-índia ou cravo-do-reino.

Esta planta ainda possui diversos sinônimos taxonômicos para seu nome científico, dentre eles: *Caryophyllus aromaticum* L., *Eugenia aromática* (L.) *Baill, Eugenia caryophyllata Thunb., Jambosa caryophyllus* (Spreng.) Nied.. *S. aromaticum* pertence à família Myrtacea é conhecida pela riqueza em óleos essenciais (DE CERQUEIRA et al., 2009; GHEDIRA; GOETZ; LE JEUNE, 2010; HARO-GONZÁLEZ et al., 2021).

A medicina popular utiliza esta planta para o tratamento da halitose e dor de dente (SANTOS et al., 2009), como analgésico e anti-inflamatório (BACHIEGA et al., 2012; CHAIEB et al., 2007; HALDER et al., 2011, 2012), para o tratamento de ferimentos e infecções cutâneas, além de infecções orais e do trato geniturinário (KHAN; AHMAD, 2012; RODRIGUEZ et al., 2014).

O óleo essencial de *S. aromaticum* apresenta diferentes efeitos, como: ação antinflamatória e analgésica (BANERJEE et al., 2020; CORREIA et al., 2018; HAN; PARKER, 2017; MARMOUZI et al., 2019); antineoplásica (NAJAR et al., 2020); antifúngica, frente a diversas cepas de *Candida albicans* e não-*albicans* (HEKMATPANAH et al., 2022; VASCONCELOS et al., 2021); ação antibacteriana frente a espécies como o *Enterecoccus faecalis* e ao *Staphylococcus aureus* (BESRA; KUMAR, 2018; EL-DARIER et al., 2018; GUPTA et al., 2013; ISMAIL et al., 2017; NIRMALA et al., 2019) e também ação antibiofilme (BUDRI et al., 2015; BUDZYŃSKA et al., 2017; VASCONCELOS et al., 2021). Estas propriedades podem ser atribuídas aos compostos marjoritário constituíntes do óleo essencial, como o eugenol e o β-cariofileno (HARO- GONZÁLEZ et al., 2021; VASCONCELOS et al., 2021).

Em estudo prévio, constatou-se a capacidade antifúngica do óleo essencial de *S. aromaticum* frente a espécies de *Candida,* assim como, uma promissora atividade na inibição de biofilme multiespécie (VASCONCELOS et al., 2021). No entanto, outros estudos são necessários para caracterizar a sua aplicação no controle dos fatores inerentes a patogênese da estomatite protética, por exemplo. Estudos desta natureza são necessários para determinar e assegurar a ação do óleo essencial frente aos fatores relacionados à interação *Candida*-hospedeiro, assim como, análises de toxicidade *in vivo*, que poderão nortear ensaios clínicos.

2.3 Geraniol, citronelal e linalol

O geraniol, citronelal e linalol são monoterpenos de mesma fómula molecular (C10H18O), mas diferem em sua estrutura química. Estes compostos presentes em plantas

aromáticas de diversas famílias, como: Rutaceae, da qual se originam o limão, laranja e derivados cítricos; Lauraceae, família da qual se deriva a canela; e Lamiaceae, família que inclui a menta, hortelã e a lavanda (KAMATOU; VILJOEN, 2008; MĄCZKA et al., 2022; MACZKA; WINSKA; GRABARCZYK, 2020).

O geraniol apresenta diferentes atividades biológicas (AMMAR, 2023; MACZKA; WINSKA; GRABARCZYK, 2020), como: antinociceptiva (LA ROCCA et al., 2017), antiinflamatória, antioxidante (AMMAR, 2023; LEI et al., 2019; RICCI et al., 2022), anticâncer (AMMAR, 2023; DUAN et al., 2023), antibacteriana (KANNAPPAN et al., 2019; LI et al., 2023) e antifúngica (KAYPETCH et al., 2022; SINGH et al., 2019; SINGH; FATIMA; HAMEED, 2016a).

Há evidências que geraniol atua contra cepas de *C. albicans* e cepas não-albicans (SINGH et al., 2019; SINGH; FATIMA; HAMEED, 2016), com perfil fungicida (SINGH et al., 2019). Os resultados apontam para a ação do geraniol em importantes fatores de virulência da *C. albicans* como a inibição do dimorfismo celular, a interferência no processo de adesão às células epiteliais e na capacidade de formação do biofilme (SINGH; FATIMA; HAMEED, 2016). Adicionalmente, células sob o tratamento do geraniol apresentaram menor secreção da enzima fosfolipase, enzima hidrolítica importante no processo de adesão e evasão ao sistema imune (SINGH et al., 2019).

Estudos também apontam propriedades biológicas associadas ao linalol, como a atividade antinflamatória (LEE et al., 2018), antioxidante (SABOGAL-GUÁQUETA et al., 2019), antineoplásica (HOU et al., 2022; ZHAO et al., 2020), antifúngica (MANDRAS et al., 2021; MEDEIROS et al., 2022), antibacteriana (LIU et al., 2020; MĄCZKA et al., 2022) e antibiofilme (MANOHARAN et al., 2017). A atividade antifúngica foi observada frente às cepas de *C. albicans* e não-*albicans Candida.* (DIAS et al., 2018; MANDRAS et al., 2021). O modo de ação na *C. albicans* foi explorado no trabalho de Medeiros et al (2022), os quais constataram, com base em testes *in vitro* e *docking* molecular, que o linalol, possivelmente, interfere na integridade da parede celular e manutenção da membrana plamática da *C. albicans*, diminuindo a síntese de ergosterol e/ou aumentando a permeabilidade dos poros da membrana celular, levando a ruptura da mesma.

O citronelal possui atividade antioxidante (OUYANG et al., 2021; YIN et al., 2022), anticancer (FATIMA; LUQMAN, 2021), antibacteriana (ZHANG et al., 2022) e antifúngica contra cepas de *C. albicans* e não-*albicans Candida*. (SAIBABU et al., 2017; SINGH; FATIMA; HAMEED, 2016b; TRINDADE et al., 2022). O citronelal interfere na homeostase da membrana celular de *Candida,* aumentando sua permeabilidade e diminuindo níveis do

ergosterol, além de reduzir a capacidade de aderência das células e dimorfismo celular (SINGH; FATIMA; HAMEED, 2016b; OUYANG et al., 2021).

A literatura apresenta resultados da ação antifúngica do geraniol, citronelal e linalol. No entanto, não há evidências sobre a sua modulação na interação *Candida*-hospedeiro, durante a infecção por *Candida*. Isto se torna um fator importante, em especial, quando há o intuito de direcionar os compostos para o uso no tratamento da estomatite protética. Até o presente momento não há relatos na literatura da avaliação da capacidade destes compostos em modular o componente inflamatório gerado pela presença da *C. albicans*, aproximando assim a proposta deste trabalho com a finalidade de tratamento da condição em questão.

3 OBJETIVOS

3.1 Objetivo geral

Avaliar *in vitro* a atividade antifúngica de compostos bioativos, da sua ação em fatores de virulência de *Candida albicans* e na modulação da interação *Candida*-hospedeiro.

3.2 Objetivos específicos

3.2.1 Artigo 1:

- Determinar a atividade antifúngica do óleo essencial de *S. aromaticum*, do eugenol e do β-cariofileno sobre espécies de *Candida*;
- Analisar a ação do óleo essencial de *S. aromaticum* e do eugenol na cinética de crescimento de *C. albicans*;
- Verificar a capacidade do óleo essencial *S. aromaticum* e do eugenol em inibir a formação de biofilme e atuar sobre um biofilme de 72h de *C. albicans*;
- Determinar a citotoxidade do óleo essencial de *S. aromaticum* e do eugenol em linhagens humanas de células epiteliais TR146 e em monócitos – THP1;
- Observar *in vivo* a toxicidade sistêmica do óleo essencial de *S. aromaticum* em modelo de *Galleria mellonella*.

3.2.2 Artigo 2

- Determinar a atividade antifúngica do geraniol, citronelal e linalol sobre espécies de *Candida*;
- Verificar a capacidade do geraniol, citronelal e linalol em inibir a formação de biofilme e atuar sobre um biofilme de 72h de *C. albicans*;
- Determinar a citotoxidade do geraniol, citronelal e linalol em linhagens celulares TR146 e THP1;

- Avaliar o efeito do geraniol na expressão gênica de enzimas proteolíticas produzidas por *C. albicans* e na secreção de citocinas pró e anti-inflamatórias do hospedeiro utilizando modelo de co-cultura celular *dual-chamber*;
- Observar em modelo de co-cultura a distribuição qualitativa e a viabilidade da *C. albicans* e das células TR146 por meio de microscopia de fluorescência;
- Determinar *in vivo* a toxicidade sistêmica do geraniol em modelo de *G. mellonella.*

4 MATERIAIS E MÉTODOS

4.1 Materiais

As amostras utilizadas neste estudo foram: o óleo essencial de *S. aromaticum* (Lazslo, SP - Brasil), eugenol (Spectrum Chemical MFG, NJ – United States), β-cariofileno (Pfaltz & Bauer, CT – United States), geraniol (Alfa Aesar®, MA – United States), citronelal (MilliporeSigma®, MA – United States) e linalol (Alfa Aesar®, MA – United States). Todos as amostras foram preparadas utilizando dimetilsufóxido (DMSO, BDH Solvents®, GA - United States) como solvente.

4.2 Microorganismos

Foram utilizadas as seguintes linhagens de *Candida* ATCC (American Type Culture Collection): *C. albicans* ATCC 321182, *C. albicans* ATCC 90028, *C. albicans* ATCC MYA 2876, *C. albicans* ATCC MYA 274, *C. tropicalis* MYA 750, *C. dublinienses* ATCC MYA 646, and *C. glabrata* ATCC MYA 275.

4.3 Determinação da atividade antifúngica

4.3.1 Concentração inibitória mínima (CIM) e fungicida mínima (CFM)

Para determinação da CIM, o método de microdiluição em caldo foi utilizado (NCCLS/M27-A2, 2002). Inicialmente, o meio RPMI-1640 - *Roswell Park Memorial Institute Medium* (Corning®, AZ - United States) foi inserido em placas de 96 poços, seguido pela inserção das amostras a serem testadas, nas seguintes concentrações: *S. aromaticum:* 2000 – 15,6 µg/mL; eugenol: 2000 – 15,6 µg/mL; β-cariofileno: 8000 – 250 µg/mL; geraniol: 40 – 0,31 mM/ml; citronellal e linalool: 800 – 12,5 mM/ml. Como controle positivo foi usado o fluconazol (Alfa Aesar®, MA - United States) e como veículo DMSO à 1%. Por último foi adicionado o inóculo fúngico na concentração de $2.5x10^3$ unidades formadoras de colônia por mililitro (UFC/mL). Foram adicionados ao teste o controle negativo (inóculo e meio de cultura) e o controle do veículo (inóculo e DMSO à 1%). As placas foram incubadas à 37°C , 5% CO_2,,

por 24 h, e o crescimento fúngico foi observado por método visual. A menor concentração que inibiu o crescimento do microorganismo foi considerado como a CIM. Alíquotas referentes as concentrações iguais e maiores que a CIM foram subcultivadas em meio agar sabouraud dextrose (BD Difco®, NJ - United States) à 37°C, 5% CO_2, por 24 h, para determinação visual da CFM. A razão entre CIM e CFM foi utilizada para determinar o padrão de ação dos compostos entre fungicida (MFC/MIC<4) ou fungistático (MFC/MIC $\geq$ 4) (SIDIQUI et el., 2013).

4.3.2 Cinética de cresciemento

Com base nos resultados da CIM e CFM, o óleo essencial de *S. aromaticum* e o eugenol foram testados em relação a sua ação sobre a cinética de crescimento de *C. albicans* ATCC MYA 2876. O óleo essencial de *S. aromaticum* foi usado nas concentrações 2500 e 5000 µg/mL, enquanto o eugenol foi usado à 5000 e 10000 µg/mL, concentrações equivalentes, respectivamente, a 5xCIM e 10xCIM. Como controles foram utilizados: fluconazol 10 µg/mL (10xCIM) como o controle positivo; meio de cultura mais inóculo como controle negativo e DMSO 1% como controle do veículo. As amostras foram dispensadas em placa de 24 poços, em um volume correspondente a 10% do volume total do inóculo, que foi definido na concentração de 10^6 UFC/mL. As placas foram colocadas em um agitador de placas e incubadas à 37°C, 5% CO_2. Alíquotas de 10 µL das suspensões foram plaqueadas em àgar sabouraud dextrose, em tempos pré-determinados (0, 1, 5, 10 e 30 min, e 1, 2 e 4 horas). O número de UFC foi registrado após 48 h (SELEEM et al., 2016b; VASCONCELOS et al., 2021).

4.4 Atividade antibiofilme

Com base nos resultados obtidos na determinação da CIM e CFM o óleo essencial de *S. aromaticum*, eugenol, geraniol, citronelal e linalol foram avaliados quanto a sua capacidade de atuar sobre um biofilme inicial e quanto a sua ação sobre um biofilme de 72 h de *C. albicans*.

Para avaliar a ação sobre um biofilme inicial, um inóculo de $1x10^6$ UFC/mL *C. albicans* ATCC® MYA-2876 foi preparado em meio YNB - *Yeast Nitrogen Base* (Sigma Aldrich®, MO - United States), suplementado com 50 mM de glucose (VWR Life Science®, PA - United States). O inóculo foi distribuído em placas de 24 poços e incubados à 37°C, 5% CO_2, por 24

h, para estabelecer a formação inicial do biofilme. Em seguida, o sobrenadante foi removido e o biofilme lavado com uma solução PBS (*Phosphate Buffer Solution*). Por fim, um novo meio com os tratamentos (10% v/v) foi adicionado ao poço, sendo repetido até completar as 72 horas de tratamento. As concentrações testadas foram: *S. aromaticum* 2500 e 5000 µg/mL (5xCIM e 10xCIM), eugenol 5000 e 10000 µg/mL (5xCIM e 10xCIM), geraniol 5 e 50 mM/mL (CIM and 10xCIM), citronelal 200 mM/mL (CIM) e linalol 50 e 500 mM/mL (CIM and 10xCIM). O Fluconazol 10xCIM foi utilizado como controle positivo e DMSO 1% como controle negativo. As placas foram mantidas a 37°C, 5% CO_2 até completar as 72 h de tratamento.

O biofilme de 72 h foi formado seguindo o mesmo parâmetro descrito acima, no entanto, se manteve intacto pelo período determinado, incubado à 37°C, 5% CO_2. Após decorrido o tempo, o sobrenadante foi removido e o biofilme foi lavado duas vezes com PBS. Cada tratamento foi adicionado, seguindo as mesmas concentrações descritas acima. O biofilme foi incubado por 24 h à 37°C, 5% CO_2. Em seguida, o biofilme foi resuspendido em PBS e transferido para microtubos do tipo eppendorfs. Neste momento, alíquotas foram plaqueadas para determinar a contagem de UFC/mL. Os eppendorfs foram centrifugados à 10000 rpm por 5 min e a biomassa do biofilme (peso seco) foi obtida após a remoção do sobrenadante e secagem do pellet em concentrador à vácuo por 40 minutos. Os resultados foram normalizados com base na relação UFC/mL/peso seco (g) de biofilme (CHEN et al., 2018; SANTANA et al., 2013; SELEEM et al., 2016a, 2016b).

4.5 Ensaio de citotoxicidade

O efeito citotóxico dos compostos foi determinado através do método fluorométrico da resazurina (Cell Titer Blue Viability Assay, Promega Corp®, WI - United States). As concentrações de 2500 – 0,25 µg/mL foram utilizadas para o óleo essencial de *S. aromaticum* e para o eugenol. O geraniol, citronelal e linalol foram testados nas concentrações de 2500 – 0,25 mM/ml. O DMSO 0,1% foi utilizado como veículo.

Monócitos humanos - THP-1 (ATCC TIB-202) foram cultivadas em meio RPMI (FBS Gibco, Invitrogen, MA - United States) suplementado com 10% de soro fetal bovino (FBS) (Gibco, Invitrogen, Waltham, MA) e penicilina/streptomicina. As células incubadas à 37° C - 5% CO_2 por 24-72 h. O inóculo de $2.5x10^5$ células/mL foi adicionado em uma placa de 24 poços, seguido da adição dos compostos nas concentrações supracitadas respeitando uma proporção de 10% v/v. As células epiteliais orais humanas - TR146 (ECACC 10032305), por

sua vez, foram cultivadas em meio Ham`s F12 com L-glutamine (Lonza Bioscience®, MD - United States), também suplementado com 10% de FBS (Gibco, Invitrogen, Waltham, MA) e penicilina/streptomicina. As células ($1x10^6$ células/mL) foram subcultivadas em placas de 24 poços e incubadas à 37°C - 5% CO_2. As células foram observadas diariamente e o meio trocado a cada 2-3 dias até atingir confluência. Neste momento, as células foram lavadas com PBS e o tratamento foi adicionado de acordo com as concentrações citadas e na proporção de 10% v/v. Para ambas as células, após o tratamento, as placas foram mantidas em estufa a 37° C - 5% CO_2 por 24 h. Em seguida, foi adicionado o reagente *Cell titer blue*, seguindo a proporção de 20 µL do reagente para cada 100 µL de meio. A placa foi incubada por 3 h e a fluorescência do sobrenadante lida em leitor de microplacas (SpectraMax M3, Molecular Devices, Sunnyvale, CA) com excitação de 555 nm, emissão de 585 nm e *cut off* de 570 nm (O'BRIEN et al., 2000). Foi considerado como citotóxico a redução de mais de 30% da viabilidade celular quando comparada ao controle negativo (ISO 10993-5, 2009).

4.6 Modelo de co-cultura *in vitro dual-chamber*

O modelo *in vitro* de co-cultura *dual-chamber,* adaptado de Pasetto, Pardi e Murata (2014), foi utilizado para reproduzir o epitélio oral com infecção por *C. albicans*. Para este método, o geraniol (5mM/mL – MIC) foi escolhido como tratamento com base nos testes antifúngicos, antibiofilme e na citotoxicidade celular.

Células TR146 (ECACC 10032305) ($2x10^6$ células/mL) foram cultivadas utilizando meio de cultura Ham`s F12 com L-glutamine e 10% FBS, em insertos para cultura celular com membranas do tipo PET, poros de 1 µm e área de 452,4 mm² (Greiner Bio-One®, NC - United States). Os insertos foram posicionados em placas de 6 poços (Greiner Bio-One®, NC - United States), configurando a câmara apical do modelo *dual-chamber*. As placas foram incubadas a 37° C - 5% até que as células atingissem confluência, com troca do meio a cada 2-3 dias. Para determinar a confluência celular foi utilizado o *Trans Epithelial Electric Resistance* (TEER). Cada poço era medido utilizando um voltímetro *Millicell-ERS Volt-Ohm Meter* (Millipore, MA – United States) até atingir um valor para o TEER de 30 Ohm/cm^2, atingido no sexto dia de análise. Neste momento, os insertos foram lavados com PBS e transferidos para uma nova placa de 6 poços, na qual foi inserida o inóculo de células THP-1, preparado em meio RPMI ($2x10^5$ células/mL), configurando assim a câmara basal do modelo. Na câmara apical foi inserido o inóculo fúngico, preparado com *C. albicans* ATCC MYA 2876 (1×10^5 UFC/mL)

em RPMI sem FBS. O geraniol foi adicionado (10% v/v), utilizando DMSO 0.1% como veículo. O controle negativo constando só meio e DMSO 0,1% foi adicionado ao teste. A placa foi incubada a 37° C - 5% por 4 horas. Por fim, o RNA fúngico (câmara apical) e o RNA das células THP-1 (câmara basal) foram extraídos, como descrito abaixo, para determinar a ação do geraniol sobre a expressão gênica de fatores de virulência da *C. albicans* e sobre fatores inflamatórios do hospedeiro induzidos pela presença da *Candida*, respectivamente.

4.6.1 Expressão gênica através de RT-PCR

Passadas as 4 h de tratamento com geraniol, o RNA das células THP-1 e da *C. albicans* foi extraído utilizando, respectivamente, os kits Ilustra™ RNAspin Mini (GE Healthcare, IL – United States) e RiboPure™ Yeast (Invitrogen, VLN – Lithuania). As intruções dos fabricantes foram seguidas. A quantificação do RNA foi feita utilizando o reagente Qubit™ RNA High sensitivity (Invitrogen, MA - United States) e lida no Qubit 4 Fluorometer (Invitrogen, MA - United States).

Para avaliar as citocinas inflamatórias do hospedeiro foram selecionados os primers para os genes IL-1β, IL-6, IL-17, IL-18, IL-10, TNF (QuantTect® Primer Assay - Qiagen®) e o gene *housekeeping* GAPDH (QuantTect® Primer Assay - Qiagen®). Por sua vez, os primers utilizados para *C. albicans* foram aspartil protease (SAP-1), fosfolipase (PLB-1) e o gene *housekeeping* ACT-1. Todos os dados obtidos foram normalizados pelo respectivo gene *housekeeping*.

RT-PCR foi realizado utilizando o kit QuantiNova® SYBR® Green RT-PCR Kit (Qiagen®, Hilden, Germany), seguindo as instruções do fabricante. As reações foram realizadas em termociclador (QuantStudio 3 Real Time PCR System, ThermoFischer Scientific, Rockford, IL) a 50° C por 10 min (etapa de trasncrição reversa); 95°C por 2 min (ativação inicial da reação em cadeira da polimerase); seguido de 40 ciclos de 5 s a 95° C (etapa de desnaturação) e 10 s a 60° C (etapa de anelamento/extensão). O método $\Delta\Delta$Ct method foi utilizado para determinar a análise da expressão relativa de cada gene (CHEN et al., 2018; SELEEM et al., 2016a, 2016b).

4.7 Microscopia de fluorescência com modelo de co-cultura de TR146 e *C. albicans*

A ação do geraniol também foi analisada sob microscopia de fluorescência em um

modelo de co-cultura entre células TR146 (ECACC 10032305)e *C. albicans* ATCC MYA 2876. Inicialmente, células TR146 ($2x10^6$ células/mL) foram plaqueadas em uma placa de 24 poços com meio Ham`s F12 com L-glutamina e com 10% FBS. As placas foram mantidas a 37°C - 5% $CO_2$ até atingir a confluência. O meio foi substituído por um inóculo de *C. albicans* ATCC MYA 2876 ($5\times10^4$ UFC/mL) preparado em meio Ham`s F12 sem FBS. Foi adicionado também o tratamento com geraniol a 5mM/mL (10% v/v) e as placas foram incubadas por 24 h. O controle positivo (fluconazol 0,1 mM/mL) e o controle negativo (DMSO 0,1% e meio) foram adicionados ao teste. A distribuição e a viabilidade das células TR146 foram analisadas usando LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen, MA - United States). O calcofluor white (Sigma Aldrich, San Luis, MO) foi utilizado como fluorócromo para *C. albicans*. As imagens foram obtidas com microscópio invertido de fluorescência (Keyence All-in-One BZ-X810 Fluorescence Microscope, Itasca, IL).

4.8 Toxicidade *in vivo* em modelo de *Galleria mellonella*

O óleo essencial de *S. aromaticum* (5 – 100 mg/Kg) e o geraniol (8 – 80000 mM/Kg) foram avaliados quanto a sua toxicidade aguda em modelo de *Galleria mellonella.* No grupo teste foram introduzidos 5 µL dos compostos nas larvas, a injeção foi realizada na última pró-pata esquerda de cada larva. Foram utilizados dois grupos controle, em um dos grupos foi feita a injeção, sem que nenhuma substância fosse inoculada e outro inoculado com 5 µL do solvente (DMSO 1%). As larvas foram incubadas a 30°C e a sua sobrevivência avaliada até 96 horas. A morte foi determinada pela melanização e ausência de movimento das larvas frente ao estímulo (ROCHELLE et al., 2016).

4.9 Análises estatísticas

Todas as análises *in vitro* foram realizadas em triplicata e em três momentos distintos. Quando aplicável, os resultados foram expressos em média e desvio padrão. Os dados foram analisados estatisticamente com o software *GraphPad Prism* (versão 8.02), inicialmente foi avaliado a normalidade dos dados com teste de Shapiro-Wilk, em seguida, para os testes que foram considerados paramétricos foi usado análise de variância (ANOVA) de uma via e testes de comparação múltiplas de Dunnett em relação ao controle negativo ou controle do veículo.

Para análise de cinética de crescimento, a qual os dados foram considerados não paramétricos utilizou-se o teste de Friedman, seguido do teste de Kruskal-Wallis. A significância foi aceita para um valor de $p \leq 0,05$.

5 RESULTADOS

5.1 Artigo 1

Syzygium aromaticum essential oil and its major constituents: Assessment of activity against _Candida_ spp and toxicity

Priscilla Guimarães Silva Vasconcelos[a] (priscillags@hotmail.com.br), Gabriel Flores Abuna[b] (abunag20@ecu.edu), Edja Maria Melo de Brito Costa[a*], Ramiro Mendonça Murata[b] (muratar16@ecu.edu).

[a]Department of Dentistry, Postgraduate Program in Dentistry, State University of Paraiba, Campina Grande, Paraíba, Brazil

[b]Department of Foundational Sciences, School of Dental Medicine, East Carolina University, Greenville, North Carolina, United States of America

Corresponding Author:

Edja Maria Melo de Brito Costa https://orcid.org/0000-0002-3166-709X

Baraúnas St, 351 - Universitário, Campina Grande – PB. 58429-500, Brazil E-mail: edjacosta@gmail.com

Phone number: +55 (83) 996411201

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ABSTRACT

Syzigium aromaticum essential oil (EO), eugenol and β-caryophyllene, were evaluated regarding antifungal, antibiofilm, and *in vitro* toxicity. Additionally, *in vivo* toxicity of EO was observed. Anti-*Candida* activity was assessed through broth microdilution assay for all compounds. Time-kill assay (0, 1 ,10 30 min, 1, 2, and 4 h) was used to determine influence of EO and eugenol on *Candida* Growth kinetics. Thereafter, both compounds were evaluated regarding their capacity to act on a biofilm formation and on a mature biofilm, based on CFU/mL/g of dry weight. Cell Titer Blue Viability Assay was used for *in vitro* totoxicity, using oral epithelial cells (TR146) and human monocytes (THP-1). Lastly, *Galleria mellonella* model was used to define the EO *in vivo* systemic toxicity. All compounds, except B-cariofilene (MIC > 8000 µg/mL), presented antifungal activity against *Candida* strains (MIC 500-1000 µg/mL). Growth kinetics of *Candida* was affected by the EO (5xMIC 30 min onward; 10xMIC 10 min onward) and eugenol (5xMIC 10 min onward; 10xMIC 1 min onward). Fungal viability was also affected by 5xMIC and 10xMIC of both compounds during biofilm formation and upon mature biofilms. LD_{50} was defined for TR146 and THP1 cells at, respectively, 59.37 and 79.54 µg/mL for the EO and 55.35 and 84.16 µg/mL for eugenol. No sign of acute toxicity was seen *in vivo* up to 100 mg/Kg for the EO. *S. aromaticum* and eugenol presented antifungal and antibiofilm activity, acting on cell growth kinetics. *In vivo* test presented a safe parameter for the EO up to 100 mg/Kg.

KEY WORDS: Syzygium, Clove oil, Anti-Infective Agents, Biofilm

INTRODUCTION

Denture stomatitis can be characterized as the inflammation of the oral mucosa underlying a removable denture. Thus, it commonly affects the palate and alveolar mucosa sites[1,2]. Clinically, denture stomatitis is characterized by the presence of erythema, hyperemia, edema and, in some cases, hemorrhagic petechiae. Despite usually being asymptomatic, patients may report a burning sensation, low salivary flow, halitosis, and palate changes[3–6].

The etiology of this condition is multifactorial and may be associated with prolonged use of dental prosthesis, local trauma, low salivary flow, hypersensitivity to the material that constitutes the prosthesis and/or sub-optimal oral and denture hygiene[3,5,7]. Denture stomatitis is also considered the most prevalent clinical form of oral candidiasis, constituting 70-95% of the diagnosed cases, since local infection by *Candida* spp, especially *Candida albicans*, is frequently observed. The pathogen can find on denture surface a protected environment to adhere and colonize, forming a biofilm that would be in direct contact to the oral mucosa, leading to tissue invasion and pathogenesis development[2,8–10].

Adequate measures to solve most cases includes acting on risk factors such as adjustment of ill-fitting dentures, hygiene orientation, and the use of antifungal agents[6]. However, considering the limited number of antifungal agents available and the continuing rise of resistant *Candida* spp. strains, novel strategies towards identifying new bioactive compounds have been incentivized, prioritizing antifungal potential by controlling *Candida* virulence factors and the reestablishment of the homeostasis[10,11]. In the search for new bioactive compounds, natural products stand out due to its source of secondary metabolites[12].

Popularly known in Brazil as *cravo or cravo-da-índia* and as clove in English, *Syzygium aromaticum* stands out as one of the plants used in folk medicine in the treatment of skin, oral and genitourinary tract infections [13,14]. The literature has shown positive results for *S. aromaticum* regarding its antimicrobial action[13,15], anti-inflammatory and analgesic properties [16,17], and anticancer effect[18]. The biological activity of the essential oil may be attributed to its chemical composition, and among the major constituents of the oil we can highlight eugenol and β-Caryophyllene[19].

Preliminary results showed a good potential of *S. aromaticum* essential oil against a multispecies biofilm formed with saliva obtained from patients with oral candidiasis[19]. However, to the best of our knowledge no other study directed the use of *S. aromaticum* essential oil to denture stomatitis treatment, either as a auxiliar in denture disinfection or as a topical agent to be applied directly on oral mucosa. Therefore, the present study aims to evaluate the capacity of *S, aromaticum* essential oil and its major compounds eugenol and β-caryophyllene to act on *C. albicans* viability, growth kinetics, and biofilm formation, as well as to establish the essential oil toxicity *in vitro* and *in vivo*.

MATERIALS AND METHODS:

Materials

The present study used *S. aromaticum* flower bud's essential oil (Laszlo Aromaterapia Eireli, BH - Brazil), eugenol (Spectrum Chemical MFG, NJ – United States), and β-cariofileno (Pfaltz & Bauer, CT – United States). All samples were prepared with dimethyl sulfoxide (DMSO).

Microorganisms

The following standard ATCC (American Type Culture Collection) reference yeast of *Candida* were used: *C. albicans* ATCC 321182, *C. albicans* ATCC *90028, C. albicans* ATCC 2876, *C. albicans* ATCC MYA-274, *C. tropicalis* MYA-750*, C. dublinienses* ATCC MYA-646, and *C. glabrata* ATCC MYA-275.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

The microdilution method was used[20] to determine the MIC and MFC of the *Candida* strains. Roswell Park Memorial Institute Medium - RPMI-1640 (Corning®) was inserted into the wells, followed by different concentrations of *S. aromaticum* essential oil (2000 to 15.6 μg/ml), eugenol (2000 to 15.6 μg/ml), β-Caryophyllene (8000 to 62.5 μg/ml), and fluconazole (Sigma-Aldrich®) (256 to 0.12 μg/ml), diluted in 1% of dimethyl sulfoxide (DMSO). Lastly, fungal suspension (2.5×10^3 colony forming units -CFU/ml) was added to the wells. Wells containing medium with inoculum only served as the negative control and the ones with DMSO 1%, inoculum and medium were used as the vehicle control. Plates were incubated at 37°C – 5% CO_2 for 24 h and microbial growth was observed visually. Later, 10 μl of each well, with equal and/or higher concentrations of MIC were sub-cultured in sabouraud dextrose agar (Kasvi®) at 37°C – 5% CO_2 for 24 h, and the visual growth was analyzed to determine the MFC. The ratio between MFC and MIC was used to determine the compounds behavior as fungicidal (MFC/MIC<4) or fungistatic (MFC/MIC $\geq$ 4).

Time-kill assay

Based on MIC and MFC results, *S. aromaticum* essential oil and eugenol were evaluated on the growth kinetics of *C. albicans* ATCC MYA 2876. *S. aromaticum* essential oil were used at 2,500 and 5,000 µg/mL and eugenol at 5,000 and 10,000 µg/mL, concentrations respectively equivalent to 5xMIC and 10xMIC. The following controls were also added to the test: fluconazole 10 µg/ml (10xMIC) as the positive control, DMSO 1% and medium as negative control. Testing samples were added as 10% of inoculum final volume, which was defined as 10^6 CFU/mL. The solution was placed on a shaker and incubated at 37°C – 5% CO_2, Thereafter, samples of 10 µl was plated on sabouraud dextrose agar at predetermined time points (0, 1, 10, 30 min, 1, 2, and 4 h) and after 48 h visual growth was analyzed to establish

CFU/mL number[19,21].

Determination of antibiofilm potential

At this stage we used one minute treatment, simulating a mouthwash swish, to evaluate *S. aromaticum* essential oil and eugenol capacity to act upon an initial biofilm formation and to inhibit a 72h biofilm.

To evaluate the action upon an initial biofilm formation an inoculum of $1x10^6$ CFU/mL of *C. albicans* ATCC MYA-2876 was prepared using Yeast Nitrogen Base Medium (YNB) (Sigma Aldrich, Saint Luis, MO) supplemented with 50 mM of glucose (VWR Life Science, Radnor, PA) for 24 h at 37°C - 5% CO_2 to establish initial biofilm growth. After 24 hours of incubation, the biofilm was daily treated util it completed 72 hours, with 10% v/v of the samples prepared in 1% DMSO as the vehicle. *S. aromaticum* essential oil were used at 500, 2,500 and 5,000 µg/mL and eugenol at 1,000, 5,000 and 10,000 µg/mL, concentrations respectively equivalent to MIC, 5xMIC and 10xMIC. At each 24 hours' time the supernatant were removed, and samples were added for one- minute treatments, afterwards treatments were removed, the biofilm was washed twice with Phosphate Buffer Solution (PBS) (Lonza Bioscience, Walkersville, MD), and 1 mL of fresh YNB medium was added to the wells. The plates were incubated at 37°C - 5% CO_2 for 24 h, this process was repeated up until completing 72 hours of treatment. The vehicle control used was 1% DMSO, while positive control was Fluconazole 10 µg/mL (10xMIC).

The 72h biofilm was formed following the same concept described above, however, the biofilm remained untouched for the stablished time. Treatments were also applied as described. After treatment time of both methods, adhered biofilms were collected by scraping the bottom of each well plate and suspending in PBS, which was then centrifuged at 10,000 rpm for 5 minutes. Biomass (dry weight) of each biofilm sample was obtained by discarding the supernatant and placing the samples in a speed vacuum to dry for 40 minutes. CFU was determined by counting the colonies at Sabouraud Dextrose Agar plates, which were incubated at 37°C - 5% CO_2. Data was normalized based on the CFU/ml/dry weight of biofilm sample[21–24].

Cytotoxicity assay

The cytotoxic effect of *S. aromaticum* essential oil and eugenol were performed with concentrations ranging from 2,500 to 0.25 µg/mL. The resazurin fluorometric method (Cell Titer Blue Viability Assay, Promega Corp®, WI - United States) was employed using both

human monocytes - THP-1 (ATCC TIB-202) and human squamous cell carcinoma TR146 (ECACC 10032305) cells. DMSO with final concentration in the wells of 0.1% was used as the vehicle.

THP-1 cells were cultured in RPMI medium (FBS Gibco, Invitrogen, MA - United States) and kept at 37°C - 5% CO_2 for 48-96 h. Thereafter, an inoculum of $2.5x10^5$ cells/mL were seeded in a 24-well plate in fresh medium, followed by the compound's addition (10% v/v) in the predetermined concentrations. On the other hand, TR146 cells were cultured in Ham`s F12 medium with L-glutamine (Lonza Bioscience®, MD - United States), supplemented with 10% of FBS and Penicillin/Streptomcin. Cells ($1x10^6$ cells/mL) were initially seeded with fresh medium only in a 24-well plate until it reached confluency. Medium changes were made every 2-3 days. Then, cells were washed with PBS and the treatment was added (10% v/v) as mentioned above. The plates were incubated for 24 h at 37°C - 5% CO_2.

Afterwards, for both experiments, cell titer blue was added to each well, following a proportion of 20 µL of the reagent to each 100 µL of medium, cells were then incubated for 3 h. The fluorescence of the supernatant was read in a microplate reader with excitation of 555 nm, emission of 585 and 570 nm cut off[25]. A reduction in cell vitality of the sample(s) compared to an untreated control by more than 30% was considered cytotoxic[26].

In vivo systemic toxicity of _S. aromaticum_ essential oil in _G. mellonella_ larvae model

Different doses of *S. aromaticum* essential oil were tested at different concentrations (5 - 100 mg/Kg) to obtain the *in vivo* systemic toxicity in a *G. mellonella* model. A random selection of 10 healthy-looking larvae weighing between 0.2 and 0.3 g was made for each group. A volume of 5 µL of each treatment and control were injected into the left proleg of the larvae using a 25 µL Hamilton Syringe (Hamilton, Reno, NV). Two controls were added to the test, one in which there was only the injection and a second one with the vehicle control (1% DMSO). The larvae were incubated at 30°C and their survival was evaluated until the maximum time of 96 h. The larvae with a high degree of myelinization and lack of movement when touched were counted as dead[26].

Statistical analysis

All *in vitro* analysis were realized in triplicates in three distinct times. When applicable, the results were expressed as mean and standard deviation. Data were statistically analyzed using GraphPad Prism software (version 8.02). Initially, data normality was assessed by Shapiro-Wilk test. Parametric data were analyzed using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests in relation to the negative or vehicle control. In the time-kill assay, since the data was considered as non-parametric, Friedman, followed by the Kruskal-Wallis test were used. Significance was accepted for a value of $p \leq 0,05$.

RESULTS

Evaluation of antimicrobial activity

S. aromaticum essential oil and eugenol presented antifungal activity against *albicans* and non-*albicans Candida* strains (MIC 500-1000 µg/mL, MFC 1000-2000 µg/mL), however, such effect was not observed with β-Cariofillene (MIC and MFC > 8000 µg/mL). Values of MIC and MFC, as well as MFC/MIC ratio[27], for all *Candida* strains tested are presented in Table 1.

TABLE 1

Time-kill assay

S. aromaticum essential oil 5xMIC and 10xMIC, were able to interfere significantly in *C. albicans* ATCC MYA 2876 growth kinetics, respectively from 30 and 10 min onward when compared to the vehicle control (DMSO 1%). The points at which no visible growth was seen in the plates were respectively 2h and 1h. Regarding eugenol treatment, a significant difference in growth kinetics was seen for 5xMIC and 10xMIC, respectively, from 10 and 1 min onward. No visual growth was seen, respectively from 30 and 10 min onward (Figure 1)

FIGURE 1

Determination of antibiofilm potential

All tested concentrations of *S. aromaticum* essential oil and eugenol were capable of statistically ($p<0.05$) reduce fungal viability in the initial biofilm formation after the 1 min/day treatment. Regarding 72h biofilm, concentrations equivalents to 5xMIC and 10xMIC could statistically ($p<0.05$) reduce the yeast load in comparison to the vehicle control group (DMSO 1%) (Figure 2).

FIGURE 2

Cytotoxicity assay

S. aromaticum essential oil LD_{50} for TR146 and THP-1 cells were 59.37 and 79.54 µg/mL (Figure 3A). Whereas eugenol LD_{50} was establish at 55.35 µg/mL for TR146 and 84.16 µg/mL for THP-1 cells. Cell viability was kept above 70% for concentrations up to 2.5 (TR146- Figure 3A) and 0.25 (THP-1 - Figure 3B) µg/mL under *S. aromaticum* essential oil treatment. Regarding eugenol treatment, this pattern was seen for concentrations up to 25 (TR146- Figure 4A) and 2.5 (THP-1 - Figure 4B) µg/mL[26].

FIGURE 3 AND 4

***In vivo* systemic toxicity of *S. aromaticum* essential oil in *G. mellonella* larvae model**

No sign of toxicity was seen in the larvae under *S. aromaticum* treatment up to 100 mg/Kg of the larvae when compared to the control ($p > 0.05$).

DISCUSSION

The protocol of one-minute treatment with *S. aromaticum* essential was able to reduce *C. albicans* viability in an initial biofilm formation as well as to decrease CFU/mL/g of dry weight in the 72h biofilm. This result is important to support the possible use of *S. aromaticum* in a formulation with antifungal properties, guiding its appliance towards denture stomatitis treatment. Additionally, studies have shown that eugenol and β-caryophyllene are major compounds of *S. aromaticum* essential oil, thus it might be related to the main biological activities of the plant[19,28,29]. Using those isolated compounds under the same conditions of the essential oil allows us to better understand that hypothesis.

Based on the Holetz et al[30] criteria, our results showed a moderate antifungal activity (100µg/mL < MIC ≤ 500 µg/mL) against all *Candida* tested, except for *C. glabrata* upon which the essential oil presented a strong activity (MIC < 500µg/mL). Thus, we highlight the result obtained for *C. glabrata*, which has been increasingly isolated among patients with local and systemic fungal infections and is often associated with antifungal resistance[31]. Similar MIC values were found in other studies such as Hekmatpanah et al[32] and Vasconcelos et al[19] with MIC values ranging from 625-1250 µg/mL, and 500-1000 µg/mL, respectively. Mandras et al[33] have also found a good anticandidal activity against non-*albicans Candida* for the essential oil of *S. aromaticum.* Conversely, other studies found different MIC values such as 200

μg/mL[13].

Regarding the isolated compounds, only eugenol presented antifungal activity. MIC range of the compound was the same as the essential oil, 500 – 1000 μg/mL, however, most obtained MICs were stated at 1000 μg/mL, except for the *C. albicans* ATCC 321182. Thus, is possible to infer that a synergetic effect may happen among *S. aromaticum* essential oil compounds, since it took the same, or even higher, concentrations of the isolated compound (eugenol) to achieve similar results to *S. aromaticum* essential oil antifungal capacity. Other authors have compared MICs of *S. aromaticum* essential oil and eugenol, and similar values were also found, enhancing the possibility that antimicrobial properties are associated with eugenol[34,35].

To determine if we could use *S. aromaticum* essential oil and eugenol on a reduced treatment time (one-minute treatment) time-kill analysis were performed, since most of the published studies only used time points starting at 1 h[36,37]. Our results showed that the essential oil at 10xMIC were able to promote a significant reduction in the number of CFU/mL within the first 10 minutes of contact with the pathogen, whereas eugenol 10xMIC had the same effect within the first minute of contact. The period of inhibition of cell growth in the graphs shows that the strains failed to reach the Log phase, a phase of great cellular enzymatic activity[37,38]. Thus, the action of *S. aromaticum* may be associated with the inhibition of enzymatic activity and with an increase in cell permeability[13,39]. However, further studies should be conducted to evaluate its action upon *Candida* virulence factors to establish the essential oil effect on *Candida* pathogenesis.

A previous study evaluating *S. aromaticum* antifungal activity was conducted by our research group and the results showed that the *S. aromaticum* essential oil could inhibit the formation of a multispecies biofilm derived from the saliva of patients diagnosed with oral candidiasis, in which *C. albicans* and non-*albicans Candida* were presumptively identified alongside with different bacteria species[19]. However, in that study, as well as in others, 24 h treatment was used, which would not match real conditions of use if we considered, for instance, a mouthwash treatment. In the present study, antibiofilm activity was seen for both tested compounds under one-minute treatments, However, it is important to address that 1000 μg/mL of eugenol, the isolated compound, could not significantly reduce the yeast viability in an initial biofilm, whereas the essential oil was able to do that at 500 μg/mL, once more we can infer that a possible synergistic effect among the essential oil compounds may be responsible for that results.

Assessing *in vitro* and *in vivo* toxic parameters of the compound is an important step to future clinical studies. In our findings both *S. aromaticum* and eugenol had similar LD_{50} values for each tested cell type. LD_{50} obtained for TR146 cells were 59.37 and 55.35 µg/mL, whereas for THP-1 was 79.54 and 84.16 µg/mL, respectively for *S. aromaticum* essential oil and eugenol. Different values were found, for example, in Ribeiro et al[40] study, in which the *S. aromaticum* essential oil only showed cytotoxic for keratinocytes at the highest concentration of 250 µg/mL. Other studies approach S. *aromaticum* essential oil cytotoxicity on human fibroblasts and keratinocytes, however, the difference in the expression of concentrations makes it difficult to compare results[41,42]. According to ISO 10993-5[26], a sample is not considered cytotoxic if cell viability is sustained above 70% when compared to the control. Based on this criteria, *S. aromaticum* essential oil was considered non-cytotoxic in concentrations up to 2.5 (TR146) and 0.25 (THP-1) µg/mL, while eugenol treatment was considered non-cytotoxic in concentrations up to 25 (TR146) and 2.5 (THP-1) µg/mL.

The concentration obtained in the *in vitro* cytotoxicity test for both compounds were considered low when compared to MIC (500-1000 µg/mL) values, which would mean that using the compound in the MIC concentration would probably cause a distress in the cells. Moreover, we intended to use even higher doses (5xMIC and 10xMIC) in the biofilm test due to the stable environment formed by a structured biofilm associated to the reduced treatment time (one minute). However, *in vitro* tests with cell lines are considered sensible, in which a component can appear toxic, but further *in vivo* tests may show safety parameters, which is what we saw in the present study.

Following cytotoxicity tests, *S. aromaticum* was tested in the *G. mellonella in vivo* model to evaluate its sistemic toxicity. The innate immune response of *G. mellonella* shares several properties with mammalian immune system, also it is more advanced than other invertebrates' models, such as nematodes[43]. Thus, it qualifies as a well-accepted scientific method to be used as a preclinical stage. Our findings show that the *S. aromaticum* essential oil presented no toxic effect to the larvae up to 100 mg/Kg. To the best of our knowledge, no other study evaluated *in vivo* toxicity using the essential oil of *S. aromaticum* with *G. mellonella* model. However, *in vivo* toxicity of this compound was evaluated in mice, and value of 4500 mg/kg was found[44].

Our findings elucidate the safety *in vivo* parameters of using *S. aromaticum* essential oil up until 100 mg/Kg, as well as its antibiofilm capacity under one-minute treatments. These results can support the possible use of this compound in a formulation with antifungal potential since no clinical studies for this configuration have been published yet. The present study

showed *S. aromaticum* and eugenol antifungal activity against *C. albicans* and non-*albicans Candida* species. Both compounds also presented significant effects on cell growth kinetics of *C. albicans*, and antibiofilm properties, being able to inhibit biofilm formation and reduce viability in a mature biofilm. Finally, *in vivo* analysis showed a safe parameter up to 100 mg/Kg.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOLEDGMENTS

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REFERENCES

1. Mousa MA, Lynch E, Kielbassa AM. Denture-related stomatitis in new complete denture wearers and its association with Candida species colonization: a prospective case-series. *Quintessence Int (Berl)*. 2020;51(7):554-565. doi:10.3290/j.qi.a44630

2. Reinhardt LC, Nascente P da S, Ribeiro JS, Etges A, Lund RG. A single-center 18-year experience with oral candidiasis in Brazil: a retrospective study of 1,534 cases. *Braz Oral Res*. 2018;32:e92. doi:10.1590/1807-3107bor-2018.vol32.0092

3. Gad MM, Fouda SM. Current perspectives and the future of candida albicans- associated denture stomatitis treatment. *Dent Med Probl*. 2020;57(1):95-102. doi:10.17219/dmp/112861

4. Hellstein JW, Marek CL. Candidiasis: Red and White Manifestations in the Oral Cavity. *Head Neck Pathol*. 2019;13(1):25-32. doi:10.1007/s12105-019-01004-6

5. Lewis MAO, Williams DW. Diagnosis and management of oral candidosis. *Br Dent J*. 2017;223(9):675-681. doi:10.1038/sj.bdj.2017.886

6. Abuhajar E, Ali K, Zulfiqar G, et al. Management of Chronic Atrophic Candidiasis (Denture Stomatitis)—A Narrative Review. *Int J Environ Res Public Health*. 2023;20(4). doi:10.3390/ijerph20043029

7. Williams D, Lewis M. Pathogenesis and treatment of oral candidosis. *J Oral Microbiol*. 2011;3(2011):1-11. doi:10.3402/jom.v3i0.5771

8. Verhaeghe T V., Wyatt CC, Mostafa NZ. The effect of overnight storage conditions on complete denture colonization by Candida albicans and dimensional stability: A systematic review. *Journal of ProstheticDentistry*. 2020;124(2):176-182. doi:10.1016/j.prosdent.2019.07.014

9. Talapko J, Juzbašić M, Matijević T, et al. Candida albicans-the virulence factors and clinical manifestations of infection. *Journal of Fungi*. 2021;7(2):1-19. doi:10.3390/jof7020079

10. Vila T, Sultan AS, Montelongo-Jauregui D, Jabra-Rizk MA. Oral candidiasis: A disease of opportunity. *Journal of Fungi*. 2020;6(1):1-28. doi:10.3390/jof6010015

11. Lemberg C, de San Vicente KM, Fróis-Martins R, et al. Candida albicans commensalism in the oral mucosa is favoured by limited virulence and metabolic adaptation. *PLoS Pathog*. 2022;18(4). doi:10.1371/journal.ppat.1010012

12. Newman DJ, Cragg GM. Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *J Nat Prod*. 2020;83(3):770-803. doi:10.1021/acs.jnatprod.9b01285

13. Khan MSA, Ahmad I. Biofilm inhibition by Cymbopogon citratus and Syzygium aromaticum essential oils in the strains of Candida albicans. *J Ethnopharmacol*. 2012;140(2):416-423. doi:10.1016/j.jep.2012.01.045

14. Rodriguez O, Sánchez R, Verde M, Núñez M, Ríos R, Chávez A. Obtaining the essential oil of Syzygium aromaticum, identification of eugenol and its effect on Streptococcus mutans. *Journal of Oral Research*. 2014;3(4):218-224. doi:10.17126/joralres.2014.051

15. Besra M, Kumar V. In vitro investigation of antimicrobial activities of ethnomedicinal plants against dental caries pathogens. *3 Biotech*. 2018;8(257):1- 8. doi:10.1007/s13205-018-1283-2

16. Halder S, Mehta AK, Mediratta PK, Sharma KK. Acute effect of essential oil of Eugenia caryophyllata on cognition and pain in mice. *Naunyn Schmiedebergs Arch Pharmacol*. 2012;385(6):587-593. doi:10.1007/s00210-012-0742-2

17. Halder S, Mehta AK, Mediratta PK, Sharma KK. Essential oil of clove (Eugenia caryophyllata) augments the humoral immune response but decreases cell mediated immunity. *Phytotherapy Research*. 2011;25(8):1254-1256. doi:10.1002/ptr.3412

18. Najar B, Shortrede JE, Pistelli L, Buhagiar J. Chemical Composition and in Vitro Cytotoxic Screening of Sixteen Commercial Essential Oils on Five Cancer Cell Lines. *Chem Biodivers*. 2020;17(1). doi:10.1002/cbdv.201900478

19. Vasconcelos PGS, Maia CM de A, Vasconcelos VM, et al. In vitro inhibition of a multispecies oral cavity biofilm by Syzygium aromaticum essential oil. *Gerodontology*. Published online 2021. doi:10.1111/ger.12594

20. *Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Second Edition. NCCLS Document M27-A2 (ISBN 1-56238-469-4). Voluntary Consensus*. Vol 22.; 2002.

21. Seleem D, Benso B, Noguti J, Pardi V, Murata RM. In vitro and in vivo antifungal activity of lichochalcone-A against candida albicans biofilms. *PLoS One*. 2016;11(6). doi:10.1371/journal.pone.0157188

22. Santana IL, Gonçalves LM, Vasconcellos AA de, da Silva WJ, Cury JA, Cury AADB. Dietary Carbohydrates Modulate Candida albicans Biofilm Development on the Denture Surface. *PLoS One*. 2013;8(5). doi:10.1371/journal.pone.0064645

23. Seleem D, Chen E, Benso B, Pardi V, Murata RM. In vitro evaluation of antifungal activity of monolaurin against Candida albicans biofilms. *PeerJ*. 2016;2016(6). doi:10.7717/peerj.2148

24. Chen E, Benso B, Seleem D, et al. Fungal-Host Interaction: Curcumin Modulates Proteolytic Enzyme Activity of Candida albicans and Inflammatory Host Response in Vitro. *Int J Dent*. 2018;2018. doi:10.1155/2018/2393146

25. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem*. 2000;267(17):5421-5426. doi:10.1046/j.1432-1327.2000.01606.x

26. Rochelle SL de A, Sardi J de CO, Freires IA, et al. The anti-biofilm potential of commonly discarded agro-industrial residues against opportunistic pathogens. *Ind Crops Prod*. 2016;87:150-160. doi:10.1016/j.indcrop.2016.03.044

27. Siddiqui ZN, Farooq F, Musthafa TNM, Ahmad A, Khan AU. Synthesis, characterization and antimicrobial evaluation of novel halopyrazole derivatives. *Journal of Saudi Chemical Society*. 2013;17(2):237-243. doi:10.1016/j.jscs.2011.03.016

28. Budzyńska A, Różalska S, Sadowska B, Różalska B. Candida albicans/Staphylococcus aureus Dual-Species Biofilm as a Target for the Combination of Essential Oils and Fluconazole or Mupirocin. *Mycopathologia*. 2017;182(11-12):989-995. doi:10.1007/s11046-017-0192-y

29. Randünz M, Trindade MLM, Camargo TM, et al. Antimicrobial and antioxidant activity of unencapsulated and encapsulated clove (Syzygium aromaticum, L.) essential oil. *Food Chemestry*. 2018;276:180-186. doi:10.1016/j.foodchem.2018.09.173

30. Holetz FB, Pessini GL, Sanches NR, Cortez DAG, Nakamura CV, Filho BPD. Screening of Some Plants Used in the Brazilian Folk Medicine for the Treatment of Infectious Diseases. *Mem Inst Oswaldo Cruz, Rio de Janeiro*. 2002;97(7):1027-1031.

31. Kumar K, Askari F, Sahu MS, Kaur R. *Candida glabrata*: A Lot More Than Meets the Eye. *Microorganisms*. 2019;7(2):39. Published 2019 Jan 30. doi:10.3390/microorganisms7020039

32. Hekmatpanah A, Sharifzadeh A, Shokri H, Abbaszadeh S, Nikaein D. Efficacy of Syzygium aromaticum essential oil on the growth and enzymatic activity of pathogenic Candida albicans strains. *Curr Med Mycol*. 2022;8(1):12-19. doi:10.18502/cmm.8.1.9209

33. Mandras N, Roana J, Scalas D, et al. The inhibition of non-albicans candida species and uncommon yeast pathogens by selected essential oils and their major compounds. *Molecules*.

2021;26(16). doi:10.3390/molecules26164937

34. Moon SE, Kim HY, Cha JD. Synergistic effect between clove oil and its major compounds and antibiotics against oral bacteria. *Arch Oral Biol*. 2011;56(9):907- 916. doi:10.1016/j.archoralbio.2011.02.005

35. Xu JG, Liu T, Hu QP, Cao XM. Chemical composition, antibacterial properties and mechanism of action of essential oil from clove buds against staphylococcus aureus. *Molecules*. 2016;21(9). doi:10.3390/molecules21091194

36. Vasconcelos MAM, Alves SM, Filho APSS, Figueired FJC, Cunha RL.*Embrapa: Comunicado Técnico, 227 - Extração Do Óleo Essencial de Folhas e Galhos de Oriza (Pogostemon Heyneanus Benth.).*; 2011.

37. Latifah-Munirah B, Himratul-Aznita WH, Mohd Zain N. Eugenol, an essential oil of clove, causes disruption to the cell wall of Candida albicans (ATCC 14053). *Front Life Sci*. 2015;8(3):231-240. doi:10.1080/21553769.2015.1045628

38. Uppuluri P, Chaffin WLJ. Defining Candida albicans stationary phase by cellular and DNA replication, gene expression and regulation. *Mol Microbiol*. 2007;64(6):1572-1586. doi:10.1111/j.1365-2958.2007.05760.x

39. Ahmad A, Khan A, Khan LA, Manzoor N. In vitro synergy of eugenol and methyleugenol with fluconazole against clinical Candida isolates. *Journal of Medical Microbiology*. 2010;59(10):1178-1184. doi:10.1099/jmm.0.020693-0

40. Ribeiro SO, Fontaine V, Mathieu V, et al. Antibacterial and cytotoxic activities of ten commercially available essential oils. *Antibiotics*. 2020;9(10):1-17. doi:10.3390/antibiotics9100717

41. Han X, Parker TL. Anti-inflammatory activity of clove (Eugenia caryophyllata) essential oil in human dermal fibroblasts. *Pharm Biol*. 2017;55(1):1619-1622. doi:10.1080/13880209.2017.1314513

42. Kozics K, Bučková M, Puškárová A, Kalászová V, Cabicarová T, Pangallo D. The effect of ten essential oils on several cutaneous drug-resistant microorganisms and their cyto/genotoxic and antioxidant properties. *Molecules*. 2019;24(24). doi:10.3390/molecules24244570

43. Serrano I, Verdial C, Tavares L, Oliveira M. The Virtuous Galleria mellonella Model for Scientific Experimentation. *Antibiotics*. 2023;12(3):505. doi:10.3390/antibiotics12030505

44. Liu B Bin, Luo L, Liu XL, et al. Essential oil of syzygium aromaticum reverses the deficits of stress-induced behaviors and hippocampal p-erk/p-creb/brain- derived neurotrophic factor expression. *Planta Med*. 2015;81(3):185-192. doi:10.1055/s-0034-1396150

FIGURES

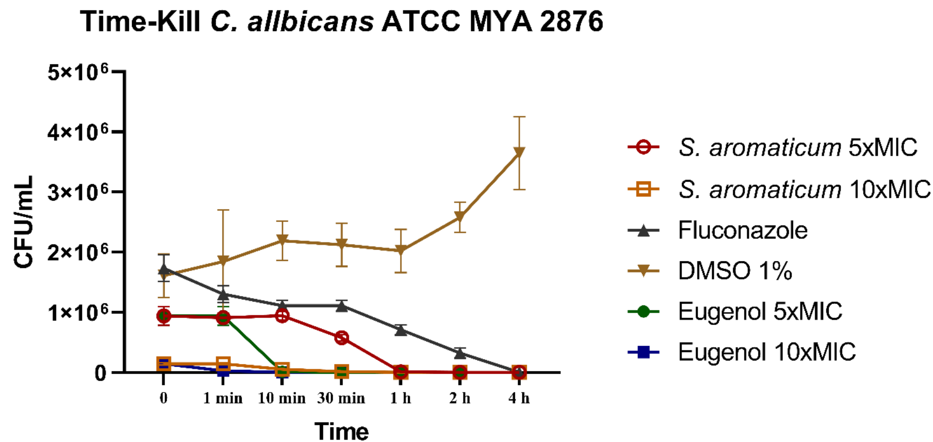

Figure 1: *S. aromaticum* essential oil (5xMIC - 2,500 µg/mL and 10xMIC 5,000 µg/mL) and eugenol (5xMIC – 5,000 µg/mL and 10xMIC - 10,000 µg/mL) action upon *C. albicans* ATCC MYA 2876 growth kinetics. DMSO 1%: Vehicle control; Fluconazole (10xMIC – 10 µg/mL): positive control.

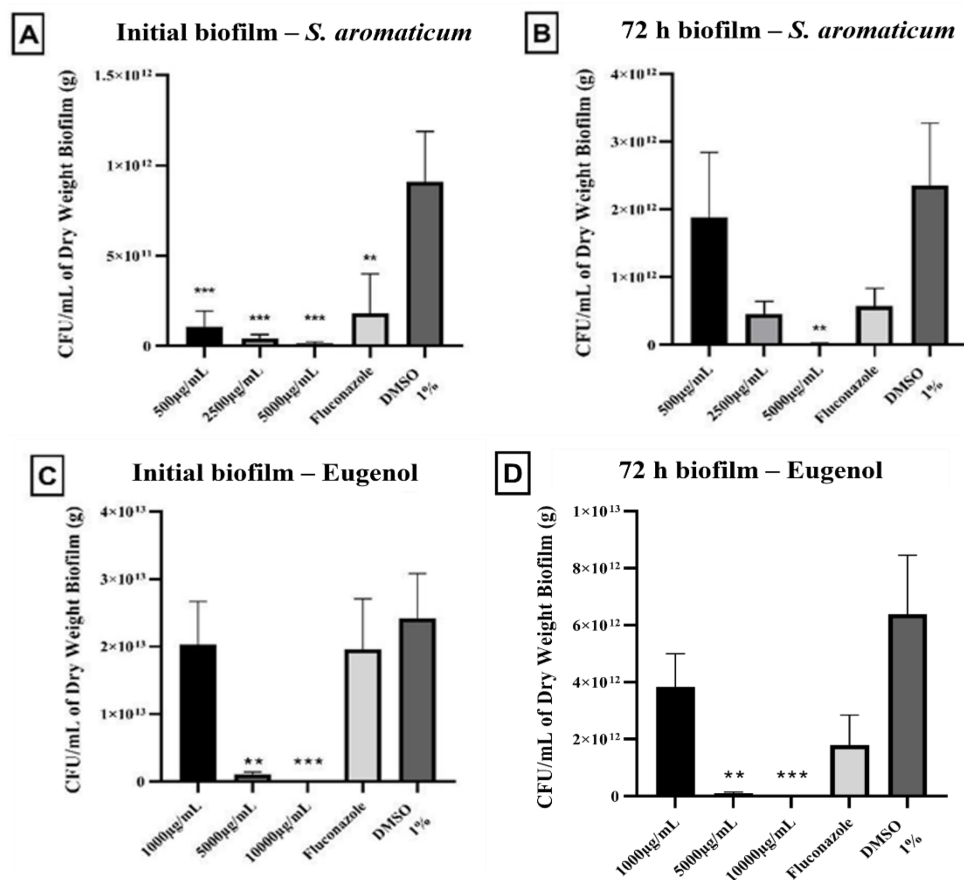

Figure 2: *S. aromaticum* essential oil (A and B) and eugenol (C and D) action upon an initial biofilm formation and against a 72h biofilm of *C. albicans.* (*$p < 0.05$; **$p<0.001$; ***$p<0.0001$).

Cell viability of TR146 and THP-1 cells after *S. Aromaticum* treatment

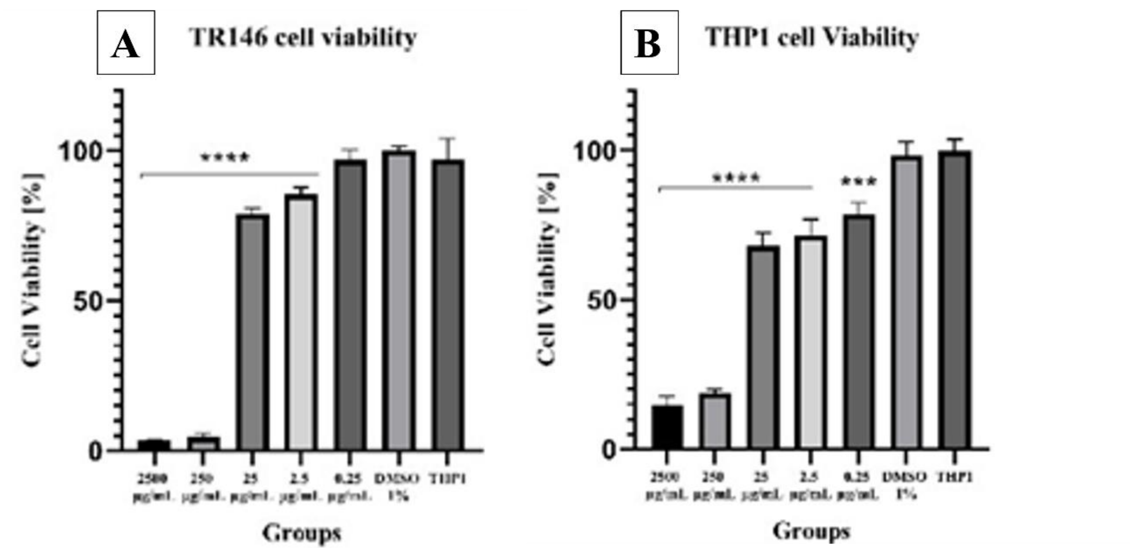

Figure 3: Cytotoxic effect of *S. aromaticum* essential oil (0.25 – 2500 µg/mL) on TR146 and THP-1 cells after 24 hours of treatment. Cell viability was kept above 70% for concentrations up to 2.5 (TR146- Figure 3A) and 0.25 (THP-1 - Figure 3B) µg/mL. TR146 and THP-1: Cells only; DMSO 0.1%: Vehicle control. significance values were considered as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$.

Cell viability of TR146 and THP-1 cells after eugenol treatment

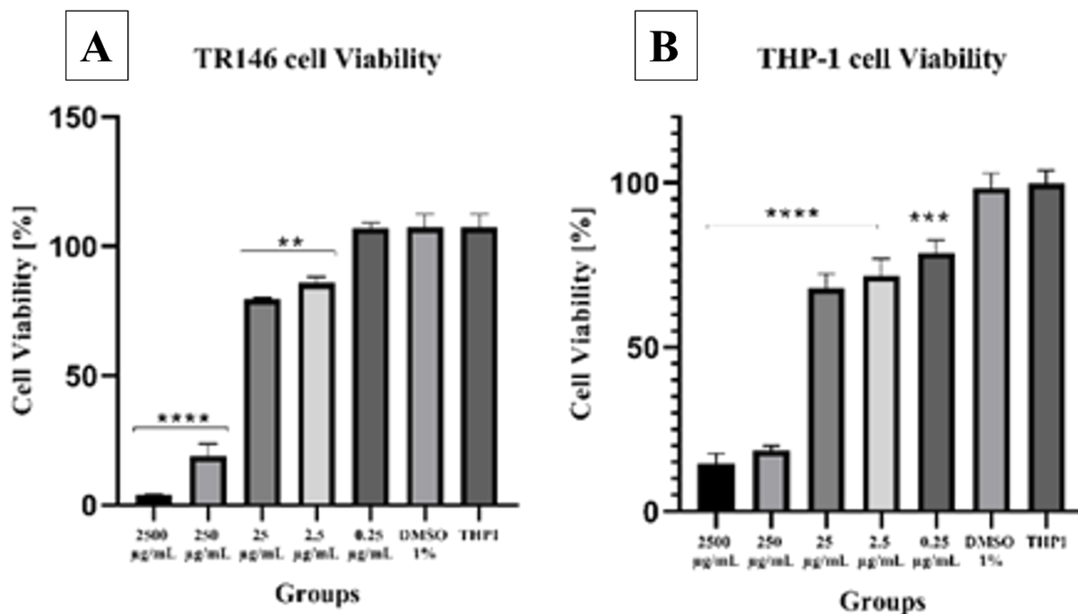

Figure 4: Cytotoxic effect of eugenol (0.25 – 2500 µg/mL) on TR146 and THP-1 cells after

24 hours of treatment. Cell viability was kept above 70% for concentrations up to 25 (TR146- Figure 4A) and 2.5 (THP-1 - Figure 4B) µg/mL. TR146 and THP-1: Cells only; DMSO 0.1%: Vehicle control. significance values were considered as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$.

TABLES

Table 1: Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) for *S. aromaticum* essential oil, eugenol, ß-caryophyllene, and fluconazole according to *Candida* species.

Microorganisms	*S. aromaticum* essential oil			Eugenol			ß-cariofillene			Fluconazole		
	MIC µg/mL	MFC µg/mL	MIC/ MFC	MIC µg/mL	MFC µg/mL	MIC/ MFC	MIC µg/mL	MFC µg/mL	MIC/ MFC	MIC µg/mL	MFC µg/mL	MIC/ MFC
C. albicans ATCC 321182	500	2000	4	500	2000	4	>8000	>8000	-	64	256	>4
C. albicans ATCC MYA 274	1000	2000	2	1000	1000	1	>8000	>8000	-	0.12	32-64	>4
C. albicans ATCC MYA 2876	500	2000	4	1000	2000	2	>8000	>8000	-	1	128	>4
C. albicans ATCC MYA 90028	500	2000	4	1000	2000	2	>8000	>8000	-	0.25	64	>4
*C. dubliniense*s ATCC MYA 646	500	2000	4	1000	1000	1	>8000	>8000	-	0.12	128	>4
C. tropicalis ATCC 750	1000	2000	2	1000	1000	1	>8000	>8000	-	1	>256	>4
C. glabrata ATCC MYA 275	250	1000	>4	1000	2000	2	>8000	>8000	-	0.5	256	>4

Fungicidal (MFC/MIC<4) and fungistatic (MFC/MIC $\geq$ 4)[27].

5.2 Artigo 2

Comparison of geraniol, citronellal, and linalool activity against *Candida* spp and assessment of the action on yeast-host interaction

Monoterpenes action upon *Candida* ssp and yeast-host interaction

Priscilla Guimarães Silva Vasconcelos[a] (priscillags@hotmail.com.br), Gabriel Flores Abuna[b] (abunag20@ecu.edu), Edja Maria Melo de Brito Costa[a*], Ramiro Mendonça Murata[b] (muratar16@ecu.edu).

[a]*Department of Dentistry, Postgraduate Program in Dentistry, State University of Paraiba, Campina Grande, Paraíba, Brazil*

[b]*Department of Foundational Sciences, School of Dental Medicine, East Carolina University, Greenville, North Carolina, United States of America*

Corresponding Author:

Edja Maria Melo de Brito Costa https://orcid.org/0000-0002-3166-709X

Baraúnas St, 351 - Universitário, Campina Grande – PB. 58429-500, Brazil E-mail: edjacosta@gmail.com

Phone number: +55 (83) 996411201

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ABSTRACT

Denture stomatitis consists in the inflammation of the mucosa subjacent of a removable denture, usually associated with *Candida* spp infection. Herein we evaluated monoterpenes geraniol, citronellal, and linalool capacity to act against *Candia* spp and used an *in vitro* co-culture model to assess geraniol capacity to modulate *Candida*-host interaction, furthermore geraniol *in vivo* toxicity was stablished. Initially, minimal inhibitory concentrations (MIC) was defined for all compounds, followed by antibiofilm and cytotoxic activity using human monocytes (THP-1) and oral squamous cell (TR146). A dual-chamber co-culture model was used to simulate *Candida* infection in oral mucosa. TR146 cells were infected with *C. albicans* in the apical chamber, and the basal chamber contained THP-1 cells. RNA was extracted from yeast and THP-1 cells, respectively, to evaluate the expression of *Candida* enzymes (SAP-1, PLB-1) and host inflammatory cytokines (IL-1β, IL-6, IL-17, IL-18, IL-10, and TNF) using RT-PCR. *In vivo* toxicity of geraniol was also assessed using *Galleria mellonella* model. The present study showed that, despite chemical similarities, geraniol presented better antifungal, antibiofilm and lower cytotoxicity when compared to the other monoterpenes. Geraniol was able to downregulate the expression of fungal enzymes and host pro-inflammatory cytokines IL- 1β. IL-6, and IL-18. Finally, safety parameters were observed *in vivo* up to 8000 mM/Kg. Our findings may sustain future clinical studies by elucidating geraniol as potential therapeutic option for denture stomatitis, with low *in vivo* toxicity and capacity to act on both fungal (reducing *Candida* virulence factors) and inflammatory (modulating host inflammatory reaction) aspects of the condition.

Keywords: Antifungal Agents; Anti-Inflammatory Agents; Oral Candidiasis, Denture Stomatitis

INTRODUCTION

Denture stomatitis is the most common oral condition amongst denture wearers. One in every three denture users are affected by this condition, which is also the most prevalent clinical form of oral candidiasis, constituting 70-95% of the diagnosed cases. Elderly people are the most affected as they are more likely to use removable dentures, especially due to the high rate of edentulism in this population[1–3].

The precise pathogenesis of denture stomatitis is not fully comprehended. However, several factors have been associated with this condition outcome including the overnight use of removable dentures, local trauma, low salivary flow, and/or sub-optimal oral and denture hygiene[4–7]. Additionally, this condition is commonly associated with *Candida* spp infection, especially *C. albicans*, which can find in the denture surface a protected environment to adhere and colonize[2,8]. The biofilm formed in this region would be in direct contact with the adjacent oral mucosa, allowing tissue invasion by *Candida.* The consequent unbalance between pathogen's virulence factors and host's immune response would change the normal microbiota to a pathogenic form[9,10].

The Pathogen Associated Molecular Patterns (PAMPs) present in *Candida* structure can be recognized by the host Pattern Recognition Receptors (PRRs), generating an inflammatory reaction aiming to control the pathogen proliferation[10–12]. Hence, pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, and IL-17[12–14] act recruiting cells from the immune system to the infection site, establishing the inflammatory pattern of the condition. On the other hand, the inflammatory reaction caused by local trauma, induced for instance by ill-fitting dentures, may be associated with a favorable environment for *Candida* adhesion, proliferation, and tissue invasion[10,15–17]. Thus, denture stomatitis pathogenesis duality can be approached as multifactorial with a fungal and inflammatory constituent.

Most denture stomatitis cases can be managed by acting on the risk factors, such as adjusting ill-fitting dentures and improving oral and denture hygiene, as well as prescribing topical antifungal agents[15]. However, available antifungal drugs are somewhat scarcer when compared to antibacterial, and the rise of *Candida* resistance must not be underestimated. Additionally, those agents do not act in inflammatory factors, which is present in denture stomatitis[18,19]. Therefore, novel therapeutic strategies toward identifying bioactive compounds that could act both in modulating the virulence factors of *C. albicans* and on the inflammatory response of the host against the pathogen would likely improve the clinical response to the treatment.

Geraniol, citronellal and linalool are monoterpenes with the same molecular formula ($C_{10}H_{18}O$), but different in chemical structure. These compounds cand be found as major components of essential oils extracted from aromatics plants and fruits[20–22]. Other studies have analyzed these compounds regarding different properties such as antioxidant[23–25], anticancer[23,26,27], antibacterial[28–30], antifungal[31–33], and anti- inflammatory [23,34,35].

Even though antifungal capacity of these compounds has already been discussed in the literature, there is little information on how they can regulate the host inflammation response under *Candida* infection. Thus, becoming an important milestone to determine its application in a possible formulation to treat denture stomatitis, acting on the fungal and inflammatory factor of the condition. Therefore, the present study evaluated geraniol, citronellal, and linalool antifungal, antibiofilm, and cytotoxicity activity. Furthermore, geraniol capacity to modulate *Candida*-host interaction, and its *in vivo* toxicity was assessed.

MATERIALS AND METHODS:

Material

The compounds tested in the present study were geraniol (Alfa Aesar®, MA – United States), citronellal (MilliporeSigma®, MA – United States) e linalool (Alfa Aesar®, MA – United States). All compounds were prepared using Dimethyl Sulfoxide (DMSO, BDH Solvents®, GA - United States) as the vehicle.

Microorganisms

The following standard ATCC (American Type Culture Collection) reference yeast of *Candida* were used: *C. albicans* ATCC 321182, *C. albicans* ATCC *90028, C. albicans* ATCC MYA 2876, *C. albicans* ATCC MYA 274, *C. tropicalis* MYA 750*, C. dublinienses* ATCC MYA 646, and *C. glabrata* ATCC MYA 275.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

Microdilution method[36] was used to determine the MIC and MFC of the *Candida* strains. Roswell Park Memorial Institute Medium - RPMI-1640 (Corning®, AZ - United States) was inserted into the wells, followed by different concentrations of geraniol (40 - 0.31 mM/ml), citronellal, linalool (800 – 12.5 mM/ml) and fluconazole (0.4 – 0.0004 mM/ml) (Alfa Aesar®, MA - United States), diluted in 1% of DMSO. Lastly, fungal suspension (2.5×10^3 CFU/ml) was added to the wells. Wells containing medium with inoculum only served as the negative control and the ones with DMSO 1%, inoculum and medium were used as the vehicle control. Plates were incubated at 37°C – 5% CO2 for 24 h and microbial growth was observed visually. Later, 10 µl of each well, with equal and/or higher concentrations of MIC were sub-cultured in Sabouraud Dextrose Agar medium (BD Difco®, NJ - United States) at 37°C – 5% CO2 for 24 h, and the visual growth was analyzed to determine the MFC. The ratio between MFC and MIC was used to determine the compounds behavior as fungicidal (MFC/MIC<4) or fungistatic (MFC/MIC $\geq$ 4).

Biofilm assay

Geraniol, citronellal and linalool were evaluated regarding both their capacity to act upon an initial biofilm formation and to inhibit a 72h biofilm.

To evaluate the action upon the initial biofilm of *C. albicans* ATCC® MYA-2876 inoculum (1×10^6 CFU/mL) was prepared using Yeast Nitrogen Base Medium (YNB) (Sigma Aldrich®, MO - United States) supplemented with 50 mM of glucose (VWR Life Science®, PA - United States). Initial biofilm growth was then established for 24 h at 37°C - 5% CO_2.

Thereafter, the biofilms were treated daily with 10% v/v of the samples prepared in 1% DMSO, until it reached 72 h. Geraniol was used at 5, and 50 mM/mL (MIC and 10xMIC), citronellal at 200 mM/mL (MIC), and linalool at 50 and 500 mM/mL (MIC and 10xMIC). At each 24 hours' time the supernatant were removed, biofilm was washed twice with Phosphate Buffer Solution (PBS) (Lonza Bioscience®, MD - United States), and 900 µL of fresh YNB medium with 100 µl of the test compounds was added to the wells. The vehicle control used was 1% DMSO, while positive control was Fluconazole 0.01 mM/mL (10xMIC). The 72h biofilm was formed following the same concept described above, however, the biofilm remained untouched for the described time, incubated at 37°C - 5% CO_2. Thereafter, supernatant medium was removed, the biofilm was washed with PBS, each treatment was applied (10% v/v) with fresh YNB medium and the plates were incubated overnight.

Afterwards, adhered biofilms of both methods were collected by scraping the bottom of each plate well and suspending the cells in PBS. The content of the wells was transferred to a microtube and centrifuged at 10,000 rpm for 5 minutes. Biomass (dry weight) of each biofilm sample was obtained by discarding the supernatant and placing the samples in a speed vacuum to dry for 40 minutes. Colony formation unit (CFU) was determined by counting the colonies at Sabouraud Dextrose Agar medium plates, which were incubated at 37°C - 5% CO_2. Data was normalized based on the CFU/ml/dry weight of biofilm sample[37–40].

Cytotoxicity assay

The resazurin fluorometric method (Cell Titer Blue Viability Assay, Promega Corp®, WI - United States) was used to assess the cytotoxic effect of geraniol (2,500–0.25 mM/ml), citronellal (2,500–0.0025 mM/ml) and linalool (2,500–0.25 mM/ml) using both THP-1 (ATCC TIB-202) human monocytes cells and oral squamous cell carcinoma cell line TR146 (ECACC 10032305). DMSO with final concentration in the wells of 0.1% was used as the vehicle.

THP-1 cells were cultured in RPMI medium supplemented with 10% of Fetal Bovine Serum (FBS, Gibco, Invitrogen, MA - United States) and Penicillin/Streptomcin (Lonza, MD – United states) and kept at 37°C - 5% CO_2 for 48-96 h. Thereafter, an inoculum of $2.5x10^5$ cells/mL were seeded in a 24-well plate in fresh medium, followed by the compound's addition (10% v/v) in the predetermined concentrations. On the other hand, TR146 cells were cultured in Ham`s F12 medium with L-glutamine (Lonza Bioscience®, MD - United States), supplemented with 10% of FBS and Penicillin/Streptomcin. Cells ($1x10^6$ cells/mL) were initially seeded with fresh medium only in a 24-well plate until it reached confluency. Medium changes were made every 2- 3 days. Then, cells were washed with PBS and the treatment was

added (10% v/v) as mentioned above. The plates were incubated for 24 h at 37°C - 5% CO_2.

Afterwards, for both experiments, cell titer blue was added to each well, following a proportion of 20 µL of the reagent to each 100 µL of medium, cells were then incubated for 3 h. The fluorescence of the supernatant was read in a microplate reader with excitation of 555 nm, emission of 585 and 570 nm cut off [41].

Dual-chamber *in vitro* model

The dual-chamber *in vitro* co-culture system, adapted from Pasetto, Pardi and Murata[42], (Figure 1) was used to mimic the oral epithelium upon a fungal infection. Geraniol (5mM/mL – MIC) was the chosen treatment based on the results obtained from the yeast susceptibility, biofilm, and cell cytotoxic assay. Thus, fungal and host inflammatory response were assessed after treatment.

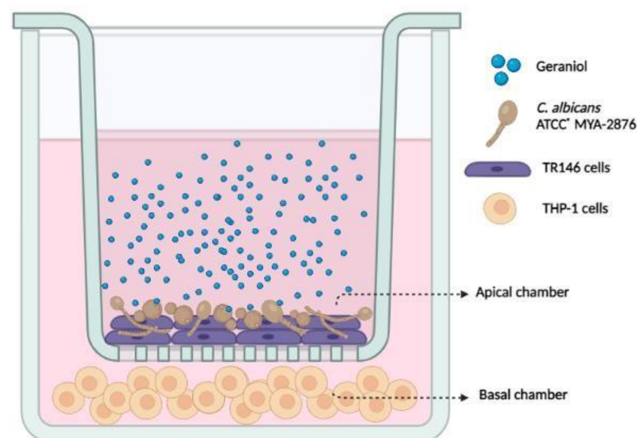

Figure 1.: Dual-chamber *in vitro* co-culture model to simulate an oral epithelium upon fungal infection and treatment with geraniol (5mM/mL). The apical chamber represents the first barrier of the epithelial layer in which TR146 cells were sub cultured until it reached confluency and subsequently infected with *C. albicans* ATCC MYA 2876. The inserts (apical chamber) were placed in a 6-well plate (basal chamber) containing THP-1 cells to evaluate the host inflammatory response.

Cell culture inserts for 6-well plates with a PET membrane of 1 µm pore size, and 452.4 mm² of culture surface (Greiner Bio-One®, NC - United States) were positioned in the wells of a 6-well plate (Greiner Bio-One®, NC - United States). Then, TR146 cells (2 x 10^6 cells/mL) were seeded into each transwell insert/apical chamber using Ham`s F12 with L-glutamine, and with 10% FBS. The plates were incubated at 37°C - 5% CO_2 until it reached confluency, the medium was changed every 2-3 days. To assess confluence, the Trans Epithelial Electric Resistance (TEER) of each insert well was measured daily with a Millicell-ERS Volt-Ohm Meter (Millipore, MA – United States) until the optimal TEER (30 Ohm/cm^2) was reached on

day 6. The insert was washed with PBS and transferred to a new 6-well plate containing THP-1 cells (2 x 10^5 cells/mL) in pre-warmed RPMI medium, constituting the basal chamber. The *C. albicans* inoculum (1 x 10^5 CFU/mL) was prepared from a 24 h *Candida* growth in RPMI without FBS and then transferred to the inserts on top of the TR146 cell layers (apical chamber). Then, geraniol treatment was added (10% v/v) using DMSO as the vehicle, with final concentration in the wells of 0.1%. A negative control (DMSO and medium only) was added to the experiment. The plate was incubated 37°C - 5% CO_2 for 4 hours. Then, yeast (apical chamber) and THP- 1 (basal chamber) RNAs were extracted, as described below, to evaluate the influence of geraniol respectively on fungal virulence factors and on host inflammatory modulation influenced by the yeast infection.

RNA extraction and Quantitative Real-Time PCR

Succeeding the 4 h of treatment with geraniol in the dual-chamber *in vitro* model, the RNA was isolated from THP-1 cells and from *C. albicans* using Ilustra™ RNAspin Mini (GE Healthcare, IL – United States) and RiboPure™ Yeast (Invitrogen, VLN – Lithuania) respectively, following each manufacture instruction. Qubit™ RNA High sensitivity (Invitrogen, MA - United States) was used to quantify the total RNA extracted using Qubit 4 Fluorometer (Invitrogen, MA - United States). RNA extracted from THP- 1 was used with the following primers for cytokines genes: IL-1β, IL-6, IL-17, IL-18, IL-10, TNF, and GAPDH (QuantTect® Primer Assay - Qiagen®) as the housekeeping gene. The selected *C. albicans* primers were: Secreted Aspartyl Proteinases-1 (SAP-1), Phospholipase (PLB-1), and ACT-1 as the housekeeping gene. All data from genes expression were normalized using the respective housekeeping gene. RT-PCR was conducted by using QuantiNova® SYBR® Green RT-PCR Kit (Qiagen®, Hilden, Germany). PCR amplification was performed by using 20 µl reaction mix per well in 0.2 ml 8-Strip PCR tubes. The reactions were conducted in thermocycler (QuantStudio 3 Real Time PCR System, ThermoFischer Scientific, Rockford, IL) at 50° C for 10 min (Reverse Transcription Step); 95°C for 2 min (PCR Initial Activation Step); followed by 40 cycles of 5 s at 95° C (Denaturation Step) and 10 s at 60° C (Annealing/Extension Step). Analysis of relative gene expression was achieved according to the ΔΔCt method[38–40].

Co-culture model for fluorescence microscopy

A co-culture model was conducted with TR146 cells and *C. albicans* ATCC MYA 2876 in a sterile 24-well plate. Initially, TR146 cells were seeded in Ham`s F12 with L- glutamine, supplemented with 10% FBS, and penicillin/streptomycin at 37°C - 5% CO2 until it reaches confluency. The medium was then replaced with a *C. albicans* inoculum of 5×10^4 CFU/mL

prepared in Ham`s F12 with L-glutamine only. Additionally, geraniol treatment (5mM/mL – MIC) was added to the wells (10% v/v). The plate was then incubated at 37° C - 5% CO_2 for 24 h. The vehicle control tested was 0.1% DMSO and the positive control was Fluconazole (0.01 mM/mL). The distribution of live and dead TR146 cells was examined using the LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells (Invitrogen, MA - United States), which contains a mixture of Calcein AM and EthDIII (Ethidium Homodimer III), and calcofluor white (Sigma Aldrich, San Luis, MO) was used to stain *C. albicans*. Fluorescent images of the double staining were captured using fluorescence microscopy (Keyence All-in-One BZ-X810 Fluorescence Microscope, Itasca, IL).

***In vivo* systemic toxicity of geraniol in *G. mellonella* larvae model**

Different doses of geraniol (8 – 80000mM/Kg) were tested in increasing order to obtain the *in vivo* systemic toxicity in a *G. mellonella* model. A random selection of 10 healthy-looking larvae weighing between 0.2 and 0.3 g was made for each group. A volume of 5 µL of each treatment and control were injected into the left proleg of the larvae using a 25 µL Hamilton Syringe (Hamilton, Reno, NV). Two controls were added to the test, one in which there was only the injection and a second one with the vehicle control (1% DMSO). The larvae were incubated at 30°C and their survival was evaluated until the maximum time of 96 h. The larvae with a high degree of melanization and lack of movement when touched were counted as dead[43].

Statistical analysis

All *in vitro* analysis were realized in triplicates in three distinct times. When applicable, the results were expressed as mean and standard deviation. Data were statistically analyzed using GraphPad Prism software (version 8.02), using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests in relation to the negative or vehicle control. Significance was accepted for a value of $p \leq 0,05$.

RESULTS

Evaluation of antifungal activity

Geraniol (MIC 1.25-5 mM/mL, MFC 10-20 mM/m) presented antifungal activity against all tested strains with lower MIC and MFC values when compared to linalool (MIC 25-100 mM/mL, MFC 25-100 mM/m) and citronellal (MIC 100-200 mM/mL, MFC 200

mM/mL). The MIC and MFC, as well as MFC/MIC[44] ratio, values for all *Candida* strains are presented in Table 1.

TABLE 1

Antibiofilm activity

Both tested concentrations of geraniol 5 and 50 Mm/mL (MIC and 10xMIC) showed a significant ($p<0.05$) activity against the biofilm of *C. albicans* – ATCC MYA 2876. Geraniol was able to reduce the fungal viability of the initial biofilm (Figure 2A) as well as to lower the 72h biofilm CFU/mL/g of dry weight count (Figure 2B). Linalool was able to inhibit initial biofilm formation at both tested concentrations 50 and 500 mM/mL (MIC and 10xMIC) (Figure 3A). However, only the 10xMIC concentration was able to reduce CFU/mL/g of dry weight in the 72h biofilm (Figure 3B). Conversely, citronellal (200 mM/mL – MIC) could neither inhibit the initial biofilm formation nor reduce the 72h biofilm viability when compared to the vehicle control (DMSO 1%) ($p> 0.05$) (Figure 4).

FIGURE 2-4

Cytotoxicity assay

Geraniol presented LD_{50} of 5.883 mM/mL and 8.027 mM/mL respectively for TR146 and THP-1 cells (Figure 5). LD_{50} values for linalool were 1.432 mM/mL for TR146 cells and 1.709 mM/mL for THP-1 cells (Figure 6). Lastly, citronellal presented LD_{50} of 0.3006 mM/mL for TR146 cells and 0.1825 mM/mL for THP-1 cells (Figure 7).

FIGURE 5-7

Inflammatory cytokines gene expression

A modulatory effect was seen on the expression of the host inflammatory genes after geraniol 5 mM/mL treatment in a dual chamber *in vitro* model. The gene expression of the pro-inflammatory genes IL-1β (Figure 8A), IL-6 (Figure 8B), and IL-18 (Figure 8C) were significantly ($p \leq 0.05$) down-regulated after the exposure to geraniol. Regarding IL-17 (Figure 8D) and TNF (Figure 8E), both known as pro-inflammatory genes, and the anti-inflammatory gene IL-10 (Figure 8F), no statistical difference ($p > 0.05$) was seen between each of these genes and the vehicle control group (DMSO 0.1%).

FIGURE 8

Proteolytic enzymes gene expression

Geraniol 5 mM/mL was able to significantly ($p \leq 0.05$) down-regulate the expression of SAP-1 and PLB-1 genes secreted by *C. albicans* MYA 2876 in the dual chamber *in vitro* model. The fold change was established as relative to the vehicle control group (DMSO 0.1%) (Figure 9).

FIGURE 9

Co-culture model for fluorescence microscopy

In the co-culture model with TR146 cells and *C. albicans*, geraniol showed a considerable decrease in *Candida* growth distribution (Figure 10B), as indicated by the reduction of the fluorescent blue color, less dense accumulation of cells clusters, and restrict hyphal presence in comparison to vehicle control (Figure 10A). TR146 cells remained viable (green color) with no significant increase in the fluorescent red color that would indicate a toxicity effect (Figure 10).

FIGURE 10

***In vivo* toxicity of geraniol in *G. mellonella* larvae model**

No sign of toxicity was seen in the larvae under geraniol treatment up to 8000 mM/Kg when compared to the control ($p > 0.05$). Only the concentration of 80000 mM/Kg affected larvae survival in which all of them died within the first 48 hours of test. (Figure 11).

FIGURE 11

DISCUSSION

The search for compounds with biological activities has gained attention over the years, and natural plants are considered a promising source of those compounds, as highlighted by Newman and Cragg[45]. The authors observed that 40% of FDA (Food and Drug Administration) approved compounds, between the years of 1940 and 2014, were obtained from natural products. Geraniol, linalool and citronellal are monoterpenes derived from different essential oils extracted from aromatic plants. Regarding biological activities, our study observed that geraniol showed better antifungal and cytotoxic results compared to citronellal and linalool. Thus, it was chosen to be used in the dual-chamber co-culture model and in the *in vivo* systemic toxicity test. The results of the present study highlighted geraniol as a promising bioactive compound, capable of interfering in *C. albicans* virulence factors and modulate host pro-inflammatory cytokines gene expression during fungal infection.

Previous studies have analyzed antifungal capacity of geraniol and found MIC values such as 225 µg/ml (equivalent to 1.45 mM/mL) for *C. albicans* and 300 µg/ml (equivalent to 1.94 mM/mL) for non-*albicans Candida* species (*C. krusei, C. tropicalis, C. parapsilosis and C. glabrata*)[46], similar values were found for some of the strains tested in the present study such as *C. albicans* ATCC 321182 (MIC 1.25 mM/mL), *C. albicans* ATCC 90028 (MIC 1.5 mM/mL), and *C. tropicallis* ATCC 750 (MIC 1.5 mM/mL). Opposing to Sigh et al[31], in which geraniol showed a fungicidal effect upon *C. albicans* strains, we found, based on MFC/MIC ratio[44], a fungistatic profile for all *Candida* strain tested. On the other hand, a fungicidal pattern was seen for citronellal and linalool.

The fungistatic profile of a compound, such as the one found for geraniol in the present study, might constitute a desirable effect rather than complete elimination of the pathogen[47,48]. *Candida* spp. are an important component of the oral microbiome, present in immunocompetent individuals as a commensal pathogen. Thus, controlling its virulence factors should prevent the rise of pathogenic strains and maintain microbiome homeostasis[10,48].

Even though the three monoterpenes have the same molecular formula ($C_{10}H_{18}O$), differences in the antifungal, antibiofilm, and cytotoxic effect could be seen. This difference in the biological properties might be related to the difference in chemical structure, changing the compounds' properties. Both linalool and citronellal antifungal activity showed higher MIC values (25-100 mM/mL and 100-200 mM/mL, respectively) when compared to geraniol (1.5-5 mM/mL). Regarding antibiofilm activity, geraniol also presented better performance, inhibiting biofilm formation and reducing the mature biofilm viability at MIC concentration (5 mM/mL), whereas linalool was effective against biofilm formation, but only the 10xMIC (50 mM/mL) had a significant effect on mature biofilm, and citronellal could neither inhibit biofilm formation nor reduce the mature biofilm viability when compared to the vehicle control (DMSO 1%).

The *C. albicans* ability to form biofilm is one of the major virulence factors related to candidiasis pathogenesis, mostly because of the extracellular polymeric matrix that enfolds the layers of microorganisms. The biofilm structure provides nutrients and protection against several factors such as aggression from toxins, pH changes, host immune response, and diffusion of antifungal agents[10,49,50]. Usually, higher doses of the antifungal agent, such as 10xMIC, are needed to have a potential effect against the stable environment created in mature biofilm structures, however we could see in our study that MIC concentration of geraniol was effective in reducing the viability of *C. albicans* mature biofilm.

The cytotoxic assay with TR146 and THP-1 cells was an important step of the present study, acting as a parameter to determine the compound therapeutic concentration that would be used in the dual-chamber co-culture model. In that context, citronellal and linalool presented higher cytotoxic activities compared to geraniol, respectively, the LD_{50} for TR146 cells were 0.3006 mM/mL and 1.432 mM/mL, whereas the LD_{50} for THP-1 was 0.1825 mM/mL and 1.709 mM/mL. The LD_{50} obtained for these compounds were lower than MIC values obtained. Conversely, geraniol had a LD_{50} of 5.883 mM/mL and 8.027 mM/mL respectively for TR146 and THP-1 cells, which indicates a minimal interference in cell viability during further tests when using MIC concentration, 5 mM/mL.

Semenova et al[51] obtained similar LD_{50} values for linalool (LD_{50} = 216.18 µg/mL; equivalent to 1.4 mM/mL), but lower values for geraniol LD_{50} = 157.54 µg/mL (equivalent to 1.02 mM/mL). The authors also observed a dose dependent pattern of toxicity for both compounds using mouse fibroblast cell line and bone-marrow-derived mesenchymal stromal cells. The dose dependent cytotoxic of geraniol was also observed for human lymphocytes by Gateva et al[52], still they found a low cytotoxic effect to those cells up to 25 µg/ml of geraniol.

Considering the purpose of analyzing geraniol capacity to modulate *Candida*-host interactions, we used a dual-chamber co-culture model. TR146 cells are commonly used to mimic the oral mucosa[53,54], thus, with *C. albicans* infection we would be able to simulate physiological conditions that happens in denture stomatitis, in which the *C. albicans* infection triggers important host responses. The use of this method allowed us, under the same conditions, to evaluate geraniol influence on *C. albicans* virulence factors and its modulatory effect in host inflammatory cytokines, an important initial step to support the production of an oral compound, such as a mouthwash, that could be used to help treat denture stomatitis.

The production of hydrolytic enzymes is an important virulence marker for *C. albicans*, thus, getting to know the influence of a compound in this context could be an important parameter to attest the antifungal efficiency against *C. albicans* infection[9,55]. In the present study it was possible to see the downregulation of the phospholipase (PLB) and aspartyl proteases (SAP) gene expression by RT-PCR. Those enzymes are secreted by *C. albicans* and are critical to important stages of fungal pathogeneses, such as yeast- hyphal transformation, adhesion, and penetration in the host tissue by the yeast[10,50,55]. Thus, our findings suggest that geraniol may have an important role by targeting those enzymes and potentially disrupt fungal virulence factors. However, further studies should be conducted to establish geraniol effect on

molecular and on signaling pathways during *Candida* infection.

Sigh et al[31] observed, in a proteomic level, that geraniol treatment resulted in a low phospholipase activity. Cell adherence is known to directly stimulate phospholipase activity, hence the authors also observed that adherence of *C. albicans* to human buccal epithelial cells was considerably reduced in geraniol presence, indicating the action of geraniol in potential *C. albicans* virulence factors. This finding corroborates the reduction of the phospholipase expression seen in our results.

Regarding the inflammatory component of the yeast-host interaction, *in vitro* studies have shown that innate immune response to *C. albicans* is related to the expression of the cytokines IL-6, IL-8, and IL-17, TNF. Those cytokines have also been identified in patients with *Candida* related lesions. Gupta et al[14], for instance, noticed elevated indices of the same pro-inflammatory cytokines in patients with *Candida* related oral leukoplakia. Whereas Ramírez-Amador et al[56], found elevated levels of IL-1β, IL-6 and TNF, while evaluating possible markers for oral candidiasis in leukemia patients. Modulating the overexpression or the continuous production of inflammatory cytokines is relevant to pathogenesis of chronic inflammatory diseases, tissue degradation and carcinogenesis[14,57–59].

In the present study, we could see the downregulation of IL-1β, IL-6 and IL-18 with statistical difference from vehicle control. The induction of IL-1β during oral candidiasis infection occurs in response to a biphasic MAPK response, which is related to hyphal formation, indicating an interesting mechanism by which the host can detect yeast switch from commensalism to pathogenicity[60]. IL-1β is related to neutrophil recruitment, Th1, and Th17 immunity[61,62]. Expression of IL-6 also seems to be related to hyphal recognition by the host[63]. Once it is secreted, IL-6 acts on adaptative immunity by activation of Th17, leading to production of antibodies and T cell help for fungal clearance[11,63]. IL-18 shares similar structure and signaling pathways with IL-1β, its signaling drives inflammation and is related to neutrophil recruitment and Th1 immunity during *Candida* infection[64–67].

Based on the discussed parameter, we can assume that IL-1β and IL-6 modulation may be related to reduction of hyphal formation, which was seen in the conducted fluorescent microscopy under calcofluor white stain (blue color). Hyphal structures were practically absent after geraniol treatment (Figure 10B) in comparison to the negative control (Figure 10A). This may demonstrate the action of geraniol in the yeast dimorphism, an important virulence factor of *C. albicans.*

Even though we couldn't see a statistical difference between geraniol treatment and vehicle control, a downregulation pattern was seen in the expression of the pro-inflammatory cytokines TNF and IL-17. Whereas an upregulation pattern was seen for the anti-inflammatory interleukin IL-10, which is an important component to reestablishment of immune homeostasis, being related to immune response control and inhibition of other cytokines such as IL-1β[68,69]. Further molecular studies should be conducted to confirm its modulation.

TNF signaling is important in the pro-inflammatory response of *C. albicans* via MAPK pathway, and has often elevated levels in patients with oral candidiasis[56,62], whereas IL-17 has been associated with *C. albicans* infection and the synergism of a wide range of inflammatory signals, for example, regulating neutrophil chemokines and other pro-inflammatory cytokines secretion such as IL-6[17,70,71]. IL-17 is also produced in Th17 cells, and studies have discussed the duality of Th17/IL-17 during fungal infection in the association with neutrophil response with direct antifungal activity, hence protecting barrier tissues. Conversely, it also has downsides regarding excessive neutrophil recruitment leading to tissue damage[17,70–72]. Thus, modulating IL-17 in denture stomatitis might have a positive repercussion in avoiding excess of neutrophil recruitment and possible related tissue damage, that could exacerbate local symptoms.

Geraniol immunomodulatory action upon cytokines, such as IL-1β, IL-6, TNF-α, IFN-γ, and IL-10 have already been discussed in some articles. However, to the best of our knowledge, no other study evaluated geraniol inflammatory modulation under *Candida* infection[23,73–78].

In the present study we also used *G. mellonella* model to assess *in vivo* the geraniol toxicity. The innate immune response of *G. mellonella* shares several properties with mammalian immune system, also it is more advanced than other invertebrates' models, such as nematodes[79]. Thus, it qualifies as a well-accepted scientific method to be used as a preclinical stage. Geraniol *in vivo* toxicity showed a non-toxic profile, up to 8000 mM per Kg of body weight of the larvae, with no appreciable defect to the larvae viability. *In vivo* tests with geraniol have also been performed by Sigh et al[31], using the *Caenorhabditis elegans* nematode model. The authors notice that geraniol treatment at 135 μg/mL (sub-MIC concentration) had no toxic effect in the worm. Additionally, the authors observed enhanced survival of the worm after infection with *C. albicans* and geraniol treatment (135 μg/mL) corroborating to an *in vivo* antifungal activity.

Our findings highlight the promising aspects of geraniol over citronellal and linalool.

Geraniol demonstrated better antifungal and antibiofilm activity, with lower cytotoxicity to host cells. Additionally, it was able to modulate *Candida*-host interaction by reducing virulence factors such as minimizing expression of proteolytic enzymes of *C. albicans*, and visually reducing hyphal formation as well as, under the same conditions, downregulate important pro-inflammatory cytokines such as IL-1β, IL-6 and IL-18. Collectively, those findings, along with the low *in vivo* toxicity, may sustain more studies to establish geraniol as a viable option for denture stomatitis, acting on both fungal and inflammatory components of the condition.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: RMM, EMMBC and PGSV. Investigation and Data Interpretation: PGSV. Formal analysis: RMM, EMMBC and PGSV. Writing – original draft: PGSV. Writing – review and editing: PGSV, EMMBC and RMM.

REFERENCES

1. Rosa-García E, Olalde-Hernández MJ, Irigoyen-Camacho ME, Mondragón-Padilla A, Mendoza-Juache A, Sánchez-Vargas LO. Antifungal susceptibility of oral isolates of Candida species from chronic kidney disease patients on chronic dialysis. *J Mycol Med*. 2020;30(3). doi:10.1016/j.mycmed.2020.101009

2. Reinhardt LC, Nascente P da S, Ribeiro JS, Etges A, Lund RG. A single-center 18-year experience with oral candidiasis in Brazil: a retrospective study of 1,534 cases. *Braz Oral Res*. 2018;32:e92. doi:10.1590/1807-3107bor-2018.vol32.0092

3. Verhaeghe T V., Wyatt CC, Mostafa NZ. The effect of overnight storage conditions on complete denture colonization by Candida albicans and dimensional stability: Asystematic review. *Journal of Prosthetic Dentistry*. 2020;124(2):176-182. doi:10.1016/j.prosdent.2019.07.014

4. Gad MM, Fouda SM. Current perspectives and the future of candida albicans-associated denture stomatitis treatment. *Dent Med Probl*. 2020;57(1):95-102. doi:10.17219/dmp/112861

5. Hellstein JW, Marek CL. Candidiasis: Red and White Manifestations in the Oral Cavity. *Head Neck Pathol*. 2019;13(1):25-32. doi:10.1007/s12105-019-01004-6

6. Williams D, Lewis M. Pathogenesis and treatment of oral candidosis. *J Oral Microbiol*. 2011;3(2011):1-11. doi:10.3402/jom.v3i0.5771

7. Lewis MAO, Williams DW. Diagnosis and management of oral candidosis. *Br Dent J*. 2017;223(9):675-681. doi:10.1038/sj.bdj.2017.886

8. Mousa MA, Lynch E, Kielbassa AM. Denture-related stomatitis in new complete denture wearers and its association with Candida species colonization: a prospective case- series. *Quintessence Int (Berl)*. 2020;51(7):554-565. doi:10.3290/j.qi.a44630

9. Vila T, Sultan AS, Montelongo-Jauregui D, Jabra-Rizk MA. Oral candidiasis: A disease of opportunity. *Journal of Fungi*. 2020;6(1):1-28. doi:10.3390/jof6010015

10. D'Enfert C, Kaune AK, Alaban LR, et al. The impact of the fungus-host-microbiota interplay upon Candida albicans infections: Current knowledge and new perspectives. *FEMS Microbiol Rev*. 2021;45(3). doi:10.1093/femsre/fuaa060

11. Naglik JR, König A, Hube B, Gaffen SL. Candida albicans–epithelial interactions and induction of mucosal innate immunity. *Curr Opin Microbiol*. 2017;40:104-112. doi:10.1016/j.mib.2017.10.030

12. Zhou Y, Cheng L, Lei YL, Ren B, Zhou X. The Interactions Between Candida albicans and Mucosal Immunity. *Front Microbiol*. 2021;12. doi:10.3389/fmicb.2021.652725

13. Nikou SA, Zhou C, Griffiths JS, et al. The Candida albicans toxin candidalysin mediates distinct epithelial inflammatory responses through p38 and EGFR-ERK pathways. *Sci Signal*. 2022;15(728). doi:10.1126/SCISIGNAL.ABJ6915

14. Gupta SR, Gupta N, Sharma A, Xess I, Singh G, Mani K. The association of Candida and antifungal therapy with pro-inflammatory cytokines in oral leukoplakia. *Clin Oral Investig*. 2021;25(11):6287-6296. doi:10.1007/s00784-021-03927-3

15. Abuhajar E, Ali K, Zulfiqar G, et al. Management of Chronic Atrophic Candidiasis (Denture Stomatitis)—A Narrative Review. *Int J Environ Res Public Health*. 2023;20(4). doi:10.3390/ijerph20043029

16. Bars P Le, Piloquet P, Daniel A, Giumelli B. Immunohistochemical localization of type IVcollagen and laminin (alpha1) in denture stomatitis. *Journal of Oral Pathology & Medicine* . 2001;30(2):98-103. doi:10.1034/j.1600-0714.2001.300206.x.

17. Dutzan N, Abusleme L, Bridgeman H, et al. On-going Mechanical Damage from Mastication Drives Homeostatic Th17 Cell Responses at the Oral Barrier. *Immunity*. 2017;46(1):133-147. doi:10.1016/j.immuni.2016.12.010

18. Costa-de-oliveira S, Rodrigues AG. Candida albicans antifungal resistance and tolerance in

bloodstream infections: The triad yeast-host-antifungal. *Microorganisms*. 2020;8(2). doi:10.3390/microorganisms8020154

19. Lukaszuk C, Krajewska-Kulak E, Kulak W. Retrospective observation of drug susceptibility of Candida strains in the years 1999, 2004, and 2015. *PeerJ*. 2017;2017(2):1-12. doi:10.7717/peerj.3038

20. Kamatou GPP, Viljoen AM. Linalool-A Review of a Biologically Active Compound of Commercial Importance. 2008;3(7):1183-1192.

21. Maczka W, Winska K, Grabarczyk M. One hundred faces of geraniol. *Molecules*. 2020;25(14). doi:10.3390/molecules25143303

22. Mączka W, Duda-Madej A, Grabarczyk M, Wińska K. Natural Compounds in the Battle against Microorganisms—Linalool. *Molecules*. 2022;27(20). doi:10.3390/molecules27206928

23. Ammar BR. Potential Effects of Geraniol on Cancer and Inflammation-Related Diseases: A Review of the Recent Research Findings. *Molecules*. 2023;28(9). doi:10.3390/molecules28093669

24. Sabogal-Guáqueta AM, Hobbie F, Keerthi A, et al. Linalool attenuates oxidative stress and mitochondrial dysfunction mediated by glutamate and NMDA toxicity. *Biomedicine and Pharmacotherapy*. 2019;118. doi:10.1016/j.biopha.2019.109295

25. Yin YL, Wang HH, Gui ZC, et al. Citronellal Attenuates Oxidative Stress–Induced Mitochondrial Damage through TRPM2/NHE1 Pathway and Effectively Inhibits Endothelial Dysfunction in Type 2 Diabetes Mellitus. *Antioxidants*. 2022;11(11). doi:10.3390/antiox11112241

26. Hou M, Zhang Y, Huang Q, et al. Linalool Impress Colorectal Cancer Deterioration by Mediating AKT/mTOR and JAK2/STAT3 Signaling Pathways. *Biomed Res Int*. 2022;2022. doi:10.1155/2022/4801703

27. Fatima K, Luqman S. Suppression of Molecular Targets and Antiproliferative Effect of Citronellal on Triple-Negative Breast Cancer Cells. *Curr Mol Pharmacol*. 2021;14(6):1156-1166. doi:10.2174/1874467214666210309120626

28. Li WR, Zeng TH, Zhang ZQ, Shi QS, Xie XB. Geraniol attenuates virulence factors by inhibiting quorum sensing of Pseudomonas aeruginosa. *Front Microbiol*. 2023;14. doi:10.3389/fmicb.2023.1190619

29. Liu X, Cai J, Chen H, et al. Antibacterial activity and mechanism of linaloolagainst Pseudomonas aeruginosa. *Microbial Pathogenesis* . Published online 2020. doi:10.1016/j.micpath.2020.103980.

30. Zhang M, Li H, Agyekumwaa AK, Yu Y, Xiao X. Effects of citronellal on growth and enterotoxinsproduction in Staphylococcus aureus ATCC 29213. *Toxicon*. Published online 2022:213-292. doi:10.1016/j.toxicon.2022.04.016.

31. Singh S, Fatima Z, Ahmad K, Hameed S. Fungicidal action of geraniol against Candida

albicans is potentiated by abrogated CaCdr1p drug efflux and fluconazole synergism. *PLoS One*. 2019;13(8). doi:10.1371/journal.pone.0203079

32. Mandras N, Roana J, Scalas D, et al. The inhibition of non-albicans candida species and uncommon yeast pathogens by selected essential oils and their major compounds. *Molecules*. 2021;26(16). doi:10.3390/molecules26164937

33. Trindade LA, Cordeiro LV, de Figuerêdo Silva D, et al. The antifungal and antibiofilm activity of Cymbopogon nardus essential oil and citronellal on clinical strains of Candida albicans. *Brazilian Journal of Microbiology*. Published online 2022. doi:10.1007/s42770- 022-00740-2

34. Ricci C, Rizzello F, Valerii MC, et al. Geraniol Treatment for Irritable Bowel Syndrome: A Double-Blind Randomized Clinical Trial. *Nutrients*. 2022;14(19). doi:10.3390/nu14194208

35. Lee SC, Wang SY, Li CC, Liu CT. Anti-inflammatory effect of cinnamaldehyde and linalool from the leaf essential oil of Cinnamomum osmophloeum Kanehira in endotoxin- induced mice. *J Food Drug Anal*. 2018;26(1):211-220. doi:10.1016/j.jfda.2017.03.006

36. *Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Second Edition. NCCLS Document M27-A2 (ISBN 1-56238-469-4). Voluntary Consensus*. Vol 22.; 2002.

37. Santana IL, Gonçalves LM, Vasconcellos AA de, da Silva WJ, Cury JA, Cury AADB. Dietary Carbohydrates Modulate Candida albicans Biofilm Development on the Denture Surface. *PLoS One*. 2013;8(5). doi:10.1371/journal.pone.0064645

38. Seleem D, Benso B, Noguti J, Pardi V, Murata RM. In vitro and in vivo antifungal activity of lichochalcone-A against candida albicans biofilms. *PLoS One*. 2016;11(6). doi:10.1371/journal.pone.0157188

39. Seleem D, Chen E, Benso B, Pardi V, Murata RM. In vitro evaluation of antifungal activity of monolaurin against Candida albicans biofilms. *PeerJ*. 2016;2016(6). doi:10.7717/peerj.2148

40. Chen E, Benso B, Seleem D, et al. Fungal-Host Interaction: Curcumin Modulates Proteolytic Enzyme Activity of Candida albicans and Inflammatory Host Response in Vitro. *Int J Dent*. 2018;2018. doi:10.1155/2018/2393146

41. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem*. 2000;267(17):5421-5426. doi:10.1046/j.1432-1327.2000.01606.x

42. Pasetto S, Pardi V, Murata RM. Anti-HIV-1 activity of flavonoid myricetin on HIV-1 infection in a dual-chamber in vitro model. *PLoS One*. 2014;9(12). doi:10.1371/journal.pone.0115323

43. Rochelle SL de A, Sardi J de CO, Freires IA, et al. The anti-biofilm potential of commonly discarded agro-industrial residues against opportunistic pathogens. *Ind Crops Prod*. 2016;87:150-160. doi:10.1016/j.indcrop.2016.03.044

44. Siddiqui ZN, Farooq F, Musthafa TNM, Ahmad A, Khan AU. Synthesis, characterization and antimicrobial evaluation of novel halopyrazole derivatives. *Journal of Saudi Chemical Society*. 2013;17(2):237-243. doi:10.1016/j.jscs.2011.03.016

45. Newman DJ, Cragg GM. Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *J Nat Prod*. 2020;83(3):770-803. doi:10.1021/acs.jnatprod.9b01285

46. Singh S, Fatima Z, Hameed S. Insights into the mode of action of anticandidal herbal monoterpenoid geraniol reveal disruption of multiple MDR mechanisms and virulence attributes in Candida albicans. *Arch Microbiol*. 2016;198(5):459-472. doi:10.1007/s00203-016-1205-9

47. Bhattacharya S, Sae-Tia S, Fries BC. Candidiasis and mechanisms of antifungal resistance. *Antibiotics*. 2020;9(6):1-19. doi:10.3390/antibiotics9060312

48. Lemberg C, de San Vicente KM, Fróis-Martins R, et al. Candida albicans commensalism in the oral mucosa is favoured by limited virulence and metabolic adaptation. *PLoS Pathog*. 2022;18(4). doi:10.1371/journal.ppat.1010012

49. Bowen WH, Robert AB, WU H, Koo H. Oral Biofilms: Pathogens, Matrix and Polymicrobial Interactions in Microenvironments. *Trends Microbiol*. 2018;26(3):229- 242. doi:10.1016/j.tim.2017.09.008

50. Kumar A, Alam A, Rani M, Ehtesham NZ, Hasnain SE. Biofilms: Survival and defense strategy for pathogens. *International Journal of Medical Microbiology*. 2017;307(8):481- 489. doi:10.1016/j.ijmm.2017.09.016

51. Semenova E, Presniakova V, Kozlovskaya V, et al. The In Vitro Cytotoxicity of Eremothecium oil and Its Components—Aromatic and Acyclic Monoterpene Alcohols. *Int J Mol Sci*. 2022;23(6). doi:10.3390/ijms23063364

52. Gateva S, Jovtchev G, Stankov A, Georgieva A, Dobreva A, Mileva M. The potential of geraniol to reduce cytotoxic and genotoxic effects of MNNG in plant and human lymphocyte test-systems. *South African Journal of Botany*. 2019;123:170-179. doi:10.1016/j.sajb.2019.03.005

53. Zwicker P, Zumpe M, Kramer A, Müller G. A 3D Model of Human Buccal Mucosa for Compatibility Testing of Mouth Rinsing Solutions. *Pharmaceutics*. 2023;15(3):721. doi:10.3390/pharmaceutics15030721

54. Lin GC, Leitgeb T, Vladetic A, et al. Optimization of an oral mucosa in vitro model based on cell line TR146. *Tissue Barriers*. Published online 2020. doi:10.1080/21688370.2020.1748459

55. Kulshrestha A, Gupta P. Secreted aspartyl proteases family: a perspective review on the regulation of fungal pathogenesis. *Future Microbiol*. 2023;18:295-309. doi:10.2217/fmb-2022-0143.

56. Ramírez-Amador V, Zambrano JG, Anaya-Saavedra G, et al. TNF as marker of oral candidiasis, HSV infection, and mucositis onset during chemotherapy in leukemia patients. *Oral Dis*. 2017;23(7):941-948. doi:10.1111/odi.12677

57. Tanaka T, Narazaki M, Kishimoto T. Interleukin (IL-6) immunotherapy. *Cold Spring Harb Perspect Biol*. 2018;10(8). doi:10.1101/cshperspect.a028456

58. Murata M. Inflammation and cancer. *Environ Health Prev Med*. 2018;23(1). doi:10.1186/s12199-018-0740-1

59. Ho J, Camilli G, Griffiths JS, Richardson JP, Kichik N, Naglik JR. Candida albicans and candidalysin in inflammatory disorders and cancer. *Immunology*. 2021;162(1):11-16. doi:10.1111/imm.13255

60. Moyes DL, Runglall M, Murciano C, et al. A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of candida albicans in epithelial cells. *Cell Host Microbe*. 2010;8(3):225-235. doi:10.1016/j.chom.2010.08.002

61. Griffiths JS, Camilli G, Kotowicz NK, Ho J, Richardson JP, Naglik JR. Role for IL-1 Family Cytokines in Fungal Infections. *Front Microbiol*. 2021;12. doi:10.3389/fmicb.2021.633047

62. Rai MN, Parsania C, Rai R. Mapping the mutual transcriptional responses duringCandida albicans and human macrophageinteractions by dual RNA-sequencing. *Microbial Pathogenesis* . Published online 2022:173. doi:10.1016/j.micpath.2022.

63. Nishikawa Y, Tomotake Y, Kawano H, et al. Effects of Candidalysin Derived from Candida albicans on the Expression of Pro-Inflammatory Mediators in Human Gingival Fibroblasts. *Int J Mol Sci*. 2023;24(4). doi:10.3390/ijms24043256

64. Stuyt RJL, Netea MG, Van Krieken JH, Van Der Meer JWM, Kullberg BJ. Recombinant Interleukin-18 Protects against Disseminated Candida albicans Infection in Mice. *J Infect Dis*. 2004;189(8):1524-1527. doi:10.1086/382955.

65. Reading PC, Smith GL. Vaccinia Virus Interleukin-18-Binding Protein Promotes Virulence by Reducing Gamma Interferon Production and Natural Killer and T-Cell Activity. *J Virol*. 2003;77(18):9960-9968. doi:10.1128/jvi.77.18.9960-9968.2003

66. Shen H, Yu Y, Chen SM, et al. Dectin-1 Facilitates IL-18 Production for the Generation of Protective Antibodies Against Candida albicans. *Front Microbiol*. 2020;11. doi:10.3389/fmicb.2020.01648

67. Tardif F, Goulet JP, Zakrzewski A, Chauvin P, Rouabhia M. Involvement of interleukin-18 in the infl ammatory response against oropharyngeal candidiasis. http://www.MedSciMonit.com/pub/vol_10/no_8/4477.pdf

68. Ouyang Q, Liu Y, Oketch OR, Zhang M, Shao X, Tao N. Citronellal exerts its antifungal activity by targeting ergosterol biosynthesis in penicillium digitatum. *Journal of Fungi*. 2021;7(6). doi:10.3390/jof7060432

69. Rutz S, Ouyang W. Regulation of interleukin-10 expression. *Adv Exp Med Biol*. 2016;941:89-116. doi:10.1007/978-94-024-0921-5_5

70. Li X, Bechara R, Zhao J, McGeachy MJ, Gaffen SL. IL-17 receptor–based signaling and implications for disease. *Nat Immunol*. 2019;20(12):1594-1602. doi:10.1038/s41590- 019-0514-y

71. Stockinger B, Omenetti S. The dichotomous nature of T helper 17 cells. *Nat Rev Immunol*. 2017;17(9):535-544. doi:10.1038/nri.2017.50

72. Gaffen SL, Moutsopoulos NM. Regulation of host-microbe interactions at oral mucosal barriers by type 17 immunity. *Sci Immunol*. 2020;5(43). doi:10.1126/sciimmunol.aau4594

73. Medicherla K, Sahu BD, Kuncha M, Kumar JM, Sudhakar G, Sistla R. Oral administration of geraniol ameliorates acute experimental murine colitis by inhibiting pro- inflammatory cytokines and NF-κB signaling. *Food Funct*. 2015;6(9):2984-2995. doi:10.1039/c5fo00405e

74. Huang Y, Yang XL, Ni YH, Xu ZM. Geraniol suppresses proinflammatory mediators in phorbol 12-myristate 13-acetate with A23187-induced HMC-1 cells. *Drug Des Devel Ther*. 2018;12:2897-2903. doi:10.2147/DDDT.S145702

75. Andrade BFMT, Conti BJ, Santiago KB, Fernandes AJ, Sforcin JM. Cymbopogon martinii essential oil and geraniol at noncytotoxic concentrations exerted immunomodulatory/anti-inflammatory effects in human monocytes. *Journal of Pharmacy and Pharmacology*. 2014;66(10):1491-1496. doi:10.1111/jphp.12278

76. Wu Y, Wang Z, Fu X, Lin Z, Yu K. Geraniol-mediated osteoarthritis improvement by down-regulating PI3K/Akt/NF-κB and MAPK signals: In vivo and in vitro studies. *Int Immunopharmacol*. 2020;86. doi:10.1016/j.intimp.2020.106713

77. Ye CJ, Li SA, Zhang Y, Lee WH. Geraniol targets KV1.3 ion channel and exhibits anti-inflammatory activity in vitro and in vivo. *Fitoterapia*. 2019;139. doi:10.1016/j.fitote.2019.104394

78. El Azab EF, Saleh AM, Yousif SO, et al. New insights into geraniol's antihemolytic, anti-inflammatory, antioxidant, and anticoagulant potentials using a combined biological and in silico screening strategy. *Inflammopharmacology*. 2022;30(5):1811-1833. doi:10.1007/s10787-022-01039-2

79. Serrano I, Verdial C, Tavares L, Oliveira M. The Virtuous Galleria mellonella Model for Scientific Experimentation. *Antibiotics*. 2023;12(3):505. doi:10.3390/antibiotics12030505

FIGURES

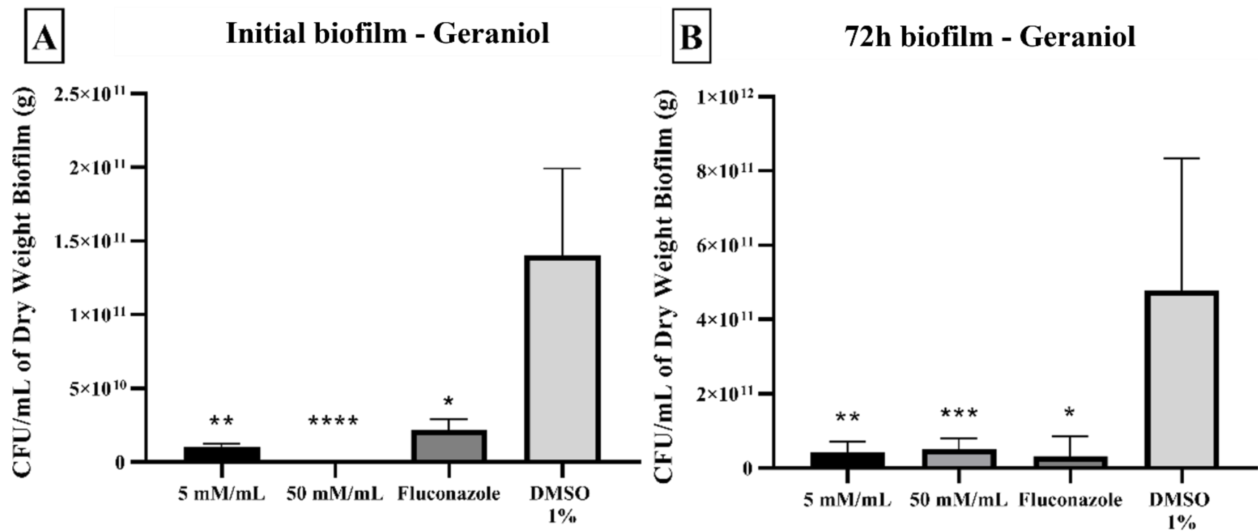


Figure 2: Geraniol (5 mM/mL - MIC and 50 mM/mL - 10xMIC) inhibited biofilm formation (A) and reduced fungal viability in a mature biofilm (B) when compared to the vehicle control (DMSO 1%). Results are expressed as CFU/mL/g of dry weight count and significance values were considered as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

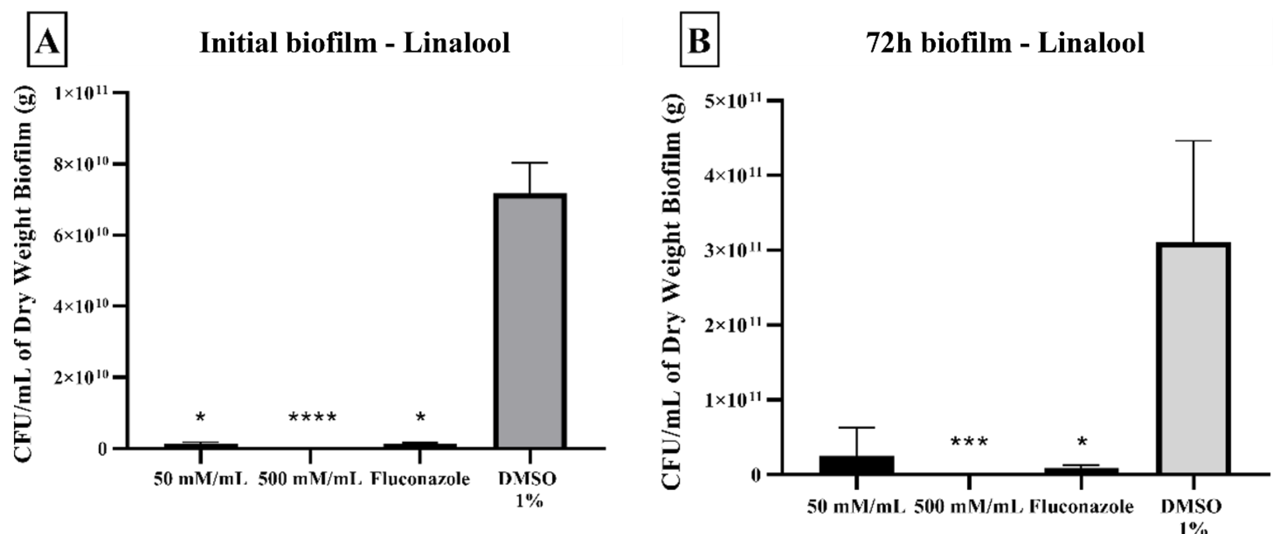


Figure 3: Linalool (50 mM/mL - MIC and 500 mM/mL - 10xMIC) inhibited biofilm formation (A). Regarding the mature biofilm, only 50 mM/mL concentration could reduce fungal viability (B) when compared to the vehicle control (DMSO 1%). Results are expressed as CFU/mL/g of dry weight count and significance values were considered as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

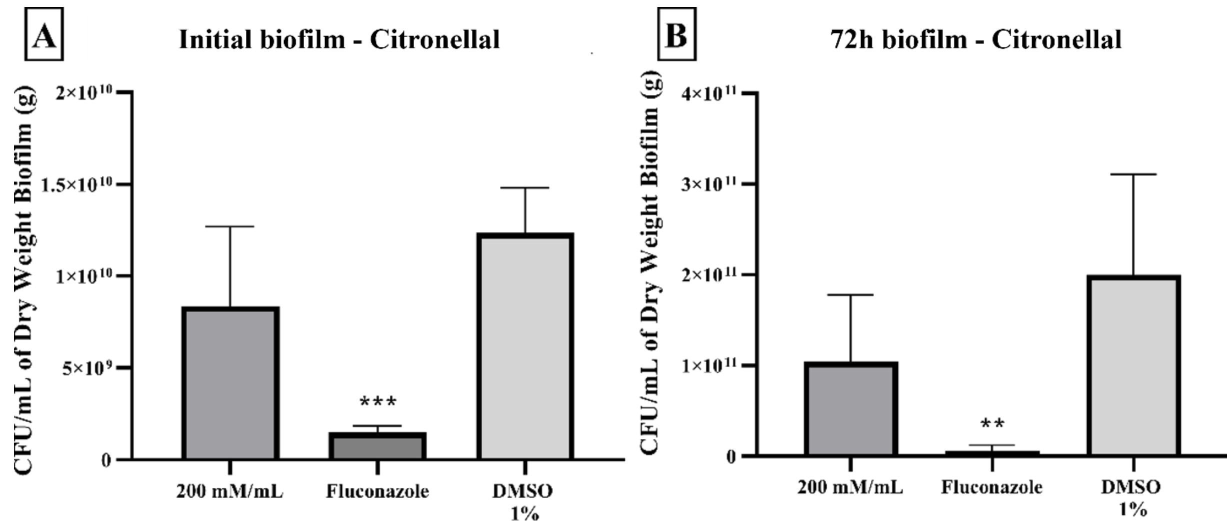
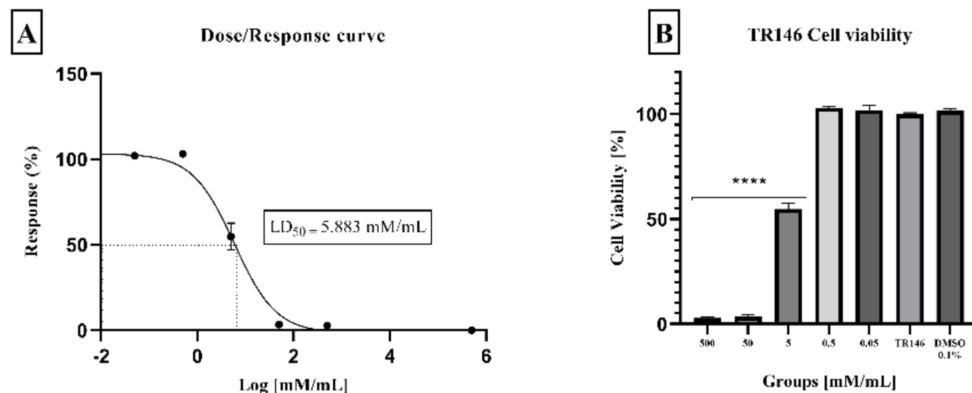


Figure 4: Citronellal (200 mM/mL - MIC) could not reduce biofilm viability when compared to the vehicle control (DMSO 1%). Results are expressed as CFU/mL/g of dry weight count and significance values were considered as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$.



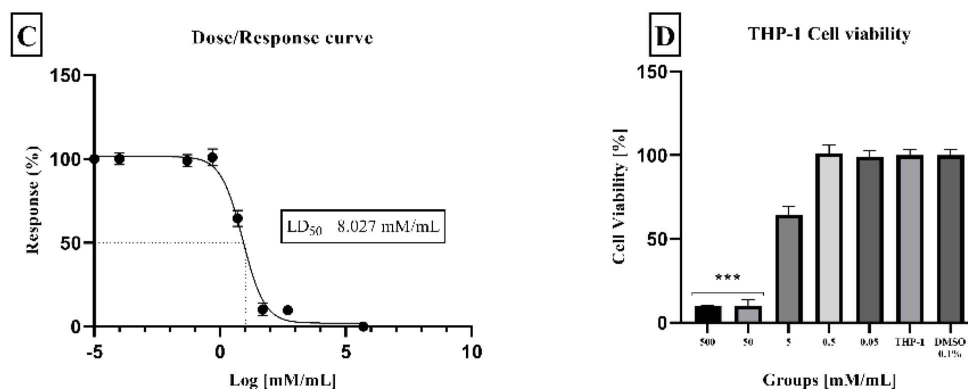


Figure 5: Cytotoxic effect of geraniol (500 – 0.05 mM/mL) on TR146 and THP-1 cells after 24 hours of treatment. LD_{50} of 5.883 mM/mL for TR146 cells (A) and LD_{50} of 8.027 mM/mL

for THP-1 cells (C). TR146 and THP-1: Cells only; DMSO 0.1%: Vehicle control. significance values were considered as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and
****$p \leq 0.0001$.

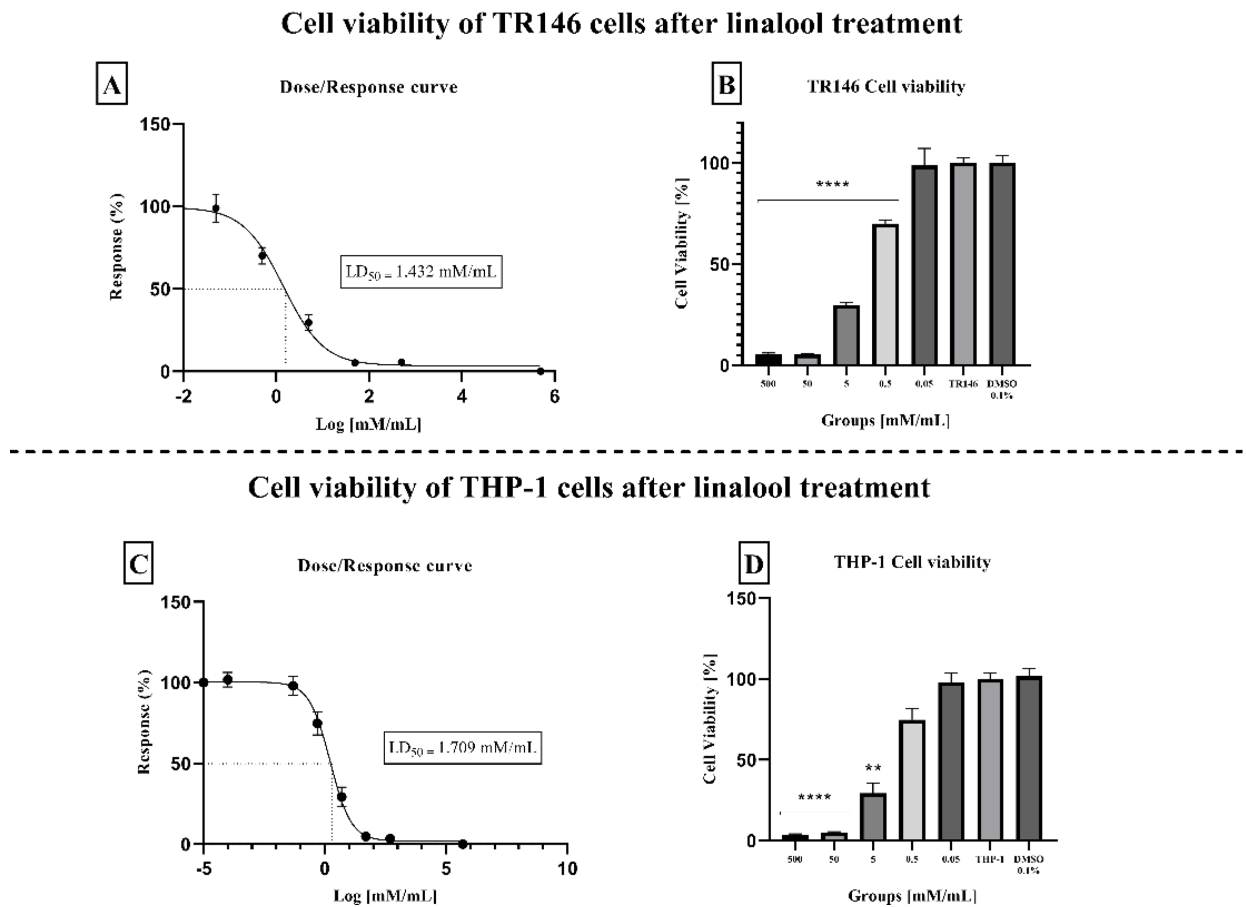
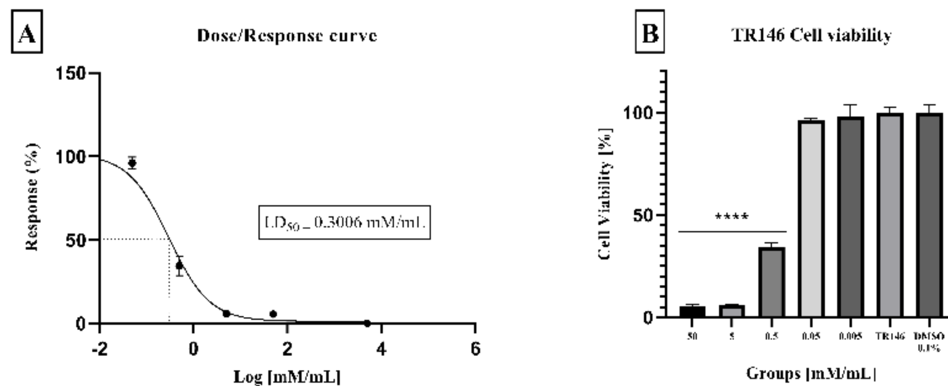


Figure 6: Cytotoxic effect of linalool (500 – 0.05 mM/mL) on TR146 and THP-1 cells after 24 hours of treatment. LD_{50} of 1.432 mM/mL for TR146 cells (A) and LD_{50} of 1.709 mM/mL for THP-1 cells (C). TR146 and THP-1: Cells only; DMSO 0.1%: Vehicle control. significance values were considered as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

Cell viability of TR146 cells after citronellal treatment



Cell viability of THP-1 cells after citronellal treatment

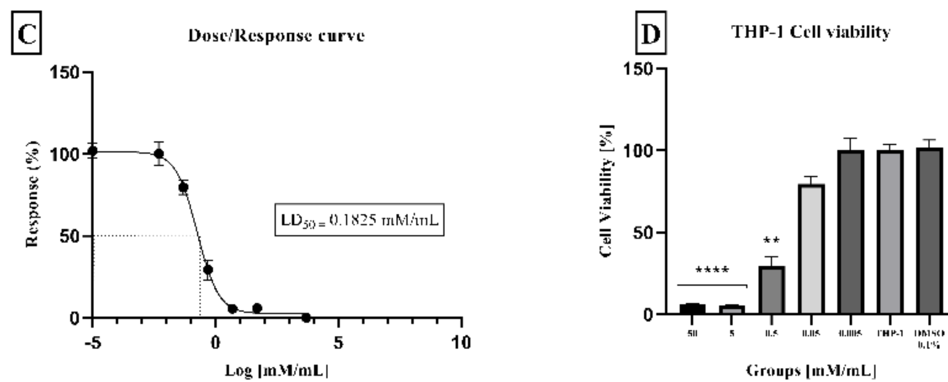


Figure 7: Cytotoxic effect of citronellal (500 – 0.05 mM/mL) on TR146 and THP-1 cells after 24 hours of treatment. LD_{50} of 0.3006 mM/mL for TR146 cells (A) and LD_{50} of 0.1825 mM/mL for THP-1 cells (C). TR146 and THP-1: Cells only; DMSO 0.1%: Vehicle control. significance values were considered as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$.

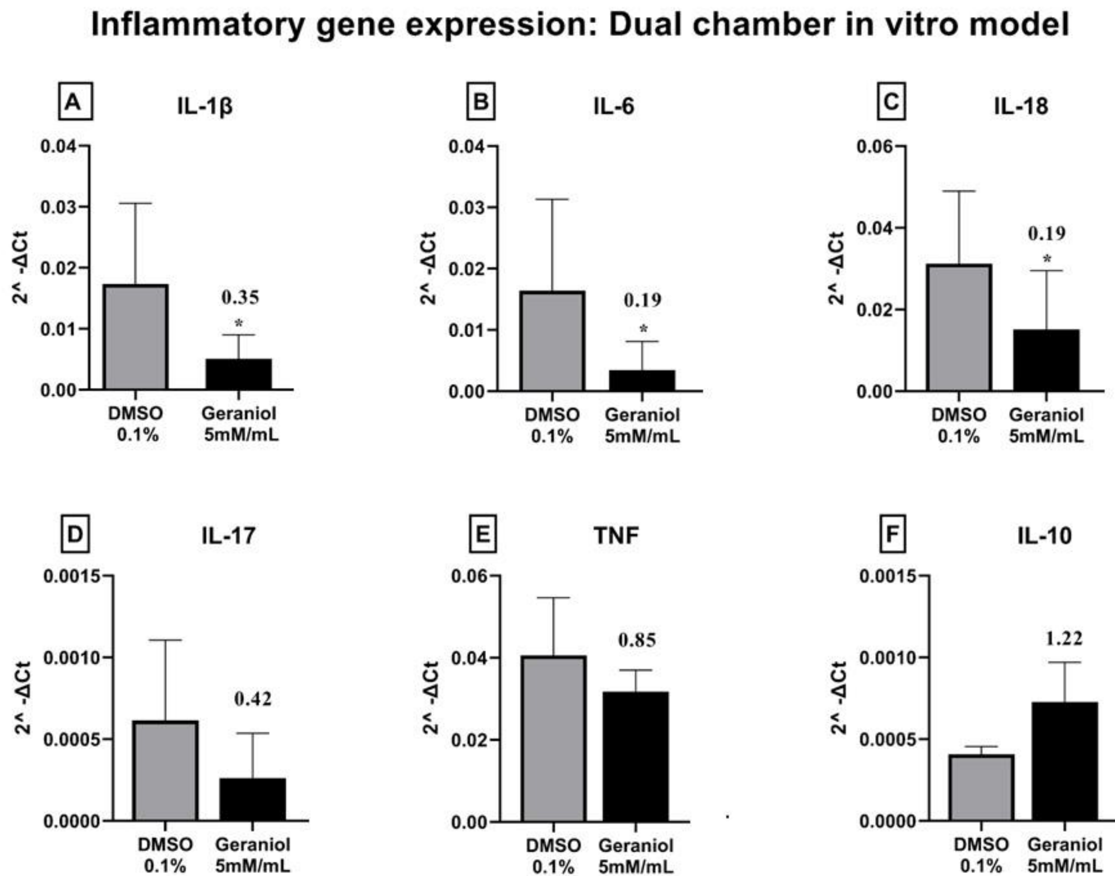


Figure 8: Relative gene expression of (A) IL-1β; (B) IL-6; (C) IL-18; (D) IL-17; (E) TNF; and (F) IL-10 of THP-1 cells after 4 h of *C. albicans* MYA 2876 infection in a dual- chamber *in vitro* model, and treatment with geraniol 5 mM/mL. The fold change was established as relative to the vehicle control group DMSO 0.1%. Significance values were considered as $*p \leq 0.05$.

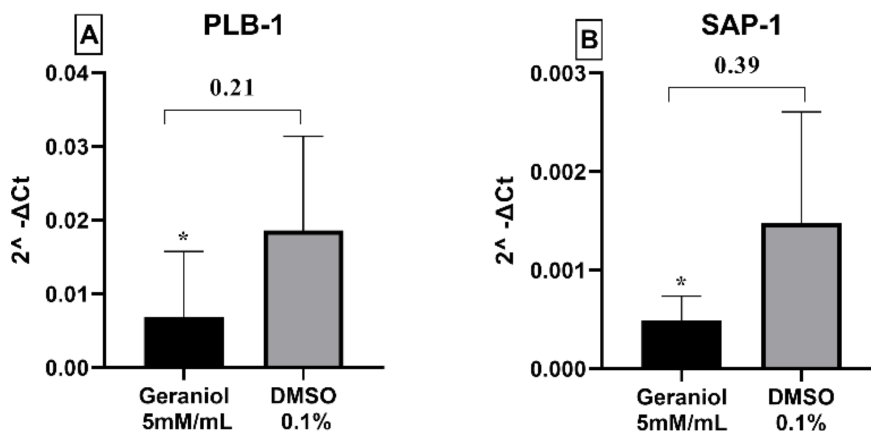


Figure 9: Relative gene expression of (A) PLB-1 and (B) SAP-1 secreted by *C. albicans* MYA 2876 after 4 h of infection in a dual-chamber *in vitro* model, and treatment with geraniol 5 mM/mL. The fold change was established as relative to the vehicle control group DMSO 0.1%. Significance values were considered as $*p \leq 0.05$.

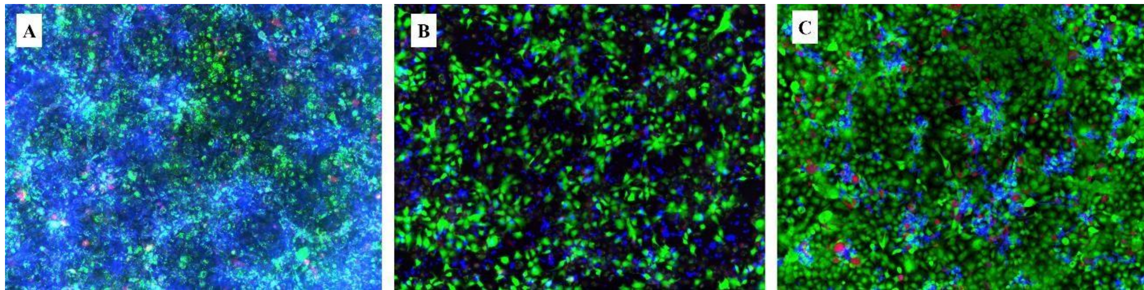


Figure 10: Fluorescence microscopy of 24 hours geraniol 5mM/mL treatment (B) in a co-culture of TR146 cells and *C. albicans*. DMSO 0.1% was used as control (A) and fluconazole 0.01 mM/mL as positive control (C). Magnification power of 20x.

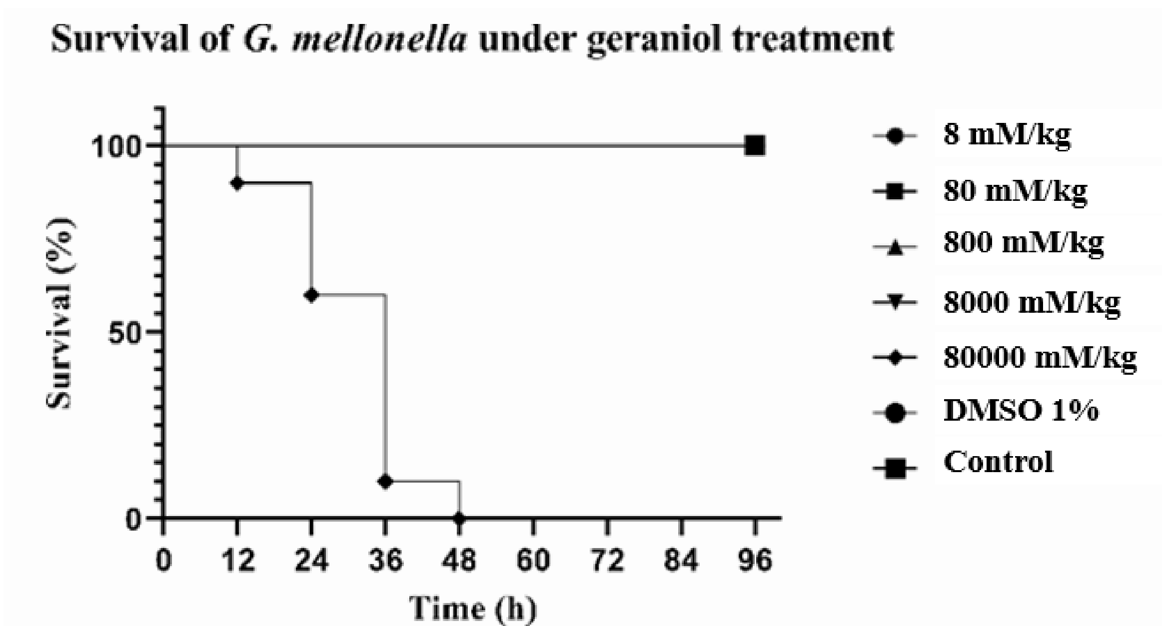


Figure 11: *In vivo* toxicity of geraniol (8 – 80000mM/Kg) in *G. mellonella* larvae model. Geraniol did not interfere with larvae survival up to 8000 mM/Kg. DMSO 1%: Vehicle control; Control: Injection only.

TABLES

Table 1: Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of geraniol, linalool, citronellal and fluconazole according to the species of *Candida*. The ratio obtained from MFC/MIC is also shown.

Microorganisms	**Geraniol**			**Linalool**			**Citronellal**			**Fluconazole**		
	MIC mM/mL	**MFC mM/mL**	**MIC/ MFC**	**MIC mM/mL**	**MFC mM/mL**	**MIC/ MFC**	**MIC mM/mL**	**MFC mM/mL**	**MIC/ MFC**	**MIC mM/mL**	**MFC mM/mL**	**MIC/ MFC**
C. albicans ATCC 321182	1.25	10	>4	25	50	<4	100	200	<4	0.1	0.4	>4
C. albicans ATCC MYA 274	2.5	10	4	100	100	<4	100	200	<4	0.0008	0.1	>4
C. albicans ATCC MYA 2876	5	20	4	50	100	<4	200	200	<4	0.001	0.1	>4
C. albicans ATCC MYA 90028	1.5	10	>4	50	50	<4	200	200	<4	0.0008	0.2	>4
C. dublinienses ATCC MYA 646	2.5	10	4	100	100	<4	200	200	<4	0.0008	0.1	>4
C. tropicalis ATCC 750	1.5	20	4	100	100	<4	200	200	<4	0.001	0.4	>4
C. glabrata ATCC MYA 275	5	20	4	100	100	<4	200	200	<4	0.0008	0.4	>4

Fungicidal (MFC/MIC<4) and fungistatic (MFC/MIC $\geq$ 4)[44]

6 CONSIDERAÇÕES FINAIS

Ao comparar os efeitos biológicos dos monoterpenos geraniol, citronelal e linalol, o presente trabalho concluiu que, apesar da comprovação do efeito antifúngico de todos os compostos, o geraniol apresentou melhores propriedas contra cepas de *C. albicans* e não-*albicans Candida*. Além de demonstar melhor efeito na inibição e ação frente a um biofilme maduro de *C. albicans* e menor citotoxidade avaliada *in vitro*, quando comparada aos demais monoterpenos. Portanto, o geraniol foi o composto escolhido para as demias análises. Este foi o primeiro estudo a avaliar a capacidade do geraniol em modular o fator inflamatório do hospedeiro quando há uma infecção por *Candida*. Para tal um modelo de infecção *in vitro* foi utilizado, simulando o epitélio oral. O geraniol foi capaz de reduzir a expressão de genes ligados à secreção enzimas proteolíticas de *C. albicans* e apresentou efeito modulador na expressão das citocinas pró-inflamatórias IL- 1β, IL-6 e IL-18. Além destes resultados, o geraniol apresentou baixa toxicidade *in vivo*, sem sinais de toxicidade às larvas de *G. mellonella* até 8000mM/Kg.

O presente estudo também avaliou a capacidade antifúngica e antibiofilme do óleo essencial de *S. aromaticum* e de seus compostos majoritários, eugenol e β-cariofileno . O óleo essencial e o eugenol apresentaram atividade contra cepas de espécies de *Candida* e foram capazes de interferir na cinética de crescimento de *C. albican*s. Adicionalmente, tratamentos de um minuto com o óleo essencial e com o eugenol foram capazes de interferir na formação de um biofilme e atuar sobre um biofilme maduro de *C. albicans.* Por fim, o óleo essencial foi avaliado quanto a sua toxicidade in vivo, metodologia mais robusta para atestar parâmetros seguros de uso de compostos em futuros ensaios clínicos, e para tal nenhum efeito tóxico foi observado até 100mg/Kg.

Os resultados obtidos no presente trabalho norteiam bases importantes para determinar o uso do geraniol e do óleo essencial de *S. aromaticum* no controle de fatores fúngicos que podem, por exemplo, estar relacionados a pacientes acometidos pela estomatite ptotética. No entanto, diante dos resultados, surge também o direcionamento para mais estudos que possam avaliar o efeito dos compostos em outrso fatores de virulência da *Candida* como, por exemplo, na expressão de adesinas de superfície e no mecanismo de destruição e invasão tecidual, cenhecendo melhor a ação dos compostos na patogenicidade da *Candida.* Além disto, tendo em vista a sensibilidade de modelos *in vitro* celulares, mais estudos *in vivo* podem ser realizados com o óleo essencial de *S. aromaticum*, para conhecer melhor sua ação na modulação de fatores de virulência da *Candida* e a resposta do hospedeiro frente ao tratamento.

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REFERÊNCIAS

ABUHAJAR, E. et al. Management of Chronic Atrophic Candidiasis (Denture Stomatitis)—A Narrative Review. **International Journal of Environmental Research and Public Health**, v. 20, n. 4, 1 fev. 2023.

AMMAR, B. R. Potential Effects of Geraniol on Cancer and Inflammation-Related Diseases: A Review of the Recent Research Findings. **Molecules**, v. 28, n. 9, 1 maio 2023.

ANDRADE, B. F. M. T. et al. Cymbopogon martinii essential oil and geraniol at noncytotoxic concentrations exerted immunomodulatory/anti-inflammatory effects in human monocytes. **Journal of Pharmacy and Pharmacology**, v. 66, n. 10, p. 1491– 1496, 1 out. 2014.

BACHIEGA, T. F. et al. Clove and eugenol in noncytotoxic concentrations exert immunomodulatory/ anti-inflammatory action on cytokine production by murine macrophages. **Journal of Pharmacy and Pharmacology**, v. 64, n. 4, p. 610–616, 2012.

BANERJEE, K. et al. Anti-inflammatory and wound healing potential of a clove oil emulsion. **Colloids and Surfaces B: Biointerfaces**, v. 193, 1 set. 2020.

BARS, P. LE et al. Immunohistochemical localization of type IVcollagen and laminin (alpha1) in denture stomatitis. **Journal of Oral Pathology & Medicine.** , v. 30, n. 2, p. 98–103, 2001.

BECKER, K. L. et al. Antifungal innate immunity: recognition and inflammatory networks. **Seminars in Immunopathology**, v. 37, n. 2, p. 107–116, 1 mar. 2015.

BESRA, M.; KUMAR, V. In vitro investigation of antimicrobial activities of ethnomedicinal plants against dental caries pathogens. **3 Biotech**, v. 8, n. 257, p. 1–8, 2018.

BHATTACHARYA, S.; SAE-TIA, S.; FRIES, B. C. Candidiasis and mechanisms of antifungal resistance. **Antibiotics**, v. 9, n. 6, p. 1–19, 1 jun. 2020.

BOWEN, W. H. et al. Oral Biofilms: Pathogens, Matrix and Polymicrobial Interactions in Microenvironments. **Trends in Microbiology**, v. 26, n. 3, p. 229–242, 2018.

BUDRI, P. E. et al. Effect of essential oils of Syzygium aromaticum and Cinnamomum zeylanicum and their major components on biofilm production in Staphylococcus aureus strains isolated from milk of cows with mastitis. **Journal of Dairy Science**, v. 98, n. 9, p. 5899–5904, 2015.

BUDZYŃSKA, A. et al. Candida albicans/Staphylococcus aureus Dual-Species Biofilm as a Target for the Combination of Essential Oils and Fluconazole or Mupirocin. **Mycopathologia**, v. 182, n. 11–12, p. 989–995, 2017.

CHAIEB, K. et al. The Chemical Composition and Biological Activity of Clove Essential Oil, Eugenia caryophyllata (Syzigium aromaticum L. Myrtaceae): A Short Review. **Phytotherapy Research**, v. 21, p. 501–506, 2007.

CHEN, E. et al. Fungal-Host Interaction: Curcumin Modulates Proteolytic Enzyme Activity of Candida albicans and Inflammatory Host Response in Vitro. **International Journal of Dentistry**, v. 2018, 2018.

CORREIA, A. M. et al. Basil, tea tree and clove essential oils as analgesics and anaesthetics in amphiprion clarkii (Bennett, 1830). **Brazilian Journal of Biology**, v. 78, n. 3, p. 436–442, 1 ago. 2018.

COSTA-DE-OLIVEIRA, S.; RODRIGUES, A. G. Candida albicans antifungal resistance and tolerance in bloodstream infections: The triad yeast-host-antifungal. **Microorganisms**, v. 8, n. 2, 1 fev. 2020.

DE CERQUEIRA, M. D. et al. Variação sazonal da composição do óleo essencial de myrcia salzmannii berg. (myrtaceae). **Quimica Nova**, v. 32, n. 6, p. 1544–1548, 2009.

D'ENFERT, C. et al. The impact of the fungus-host-microbiota interplay upon Candida albicans infections: Current knowledge and new perspectives. **FEMS Microbiology Reviews**, v. 45, n. 3, 2021.

DIAS, I. J. et al. Antifungal activity of linalool in cases of candida spp. isolated from individuals with oral candidiasis. **Brazilian Journal of Biology**, v. 78, n. 2, p. 368–374, 1 maio 2018.

DUAN, S. et al. A multi-bioresponsive self-assembled nano drug delivery system based on hyaluronic acid and geraniol against liver cancer. **Carbohydrate Polymers**, v. 310, 15 jun. 2023.

DUTZAN, N. et al. On-going Mechanical Damage from Mastication Drives Homeostatic Th17 Cell Responses at the Oral Barrier. **Immunity**, v. 46, n. 1, p. 133– 147, 17 jan. 2017.

EL AZAB, E. F. et al. New insights into geraniol's antihemolytic, anti-inflammatory, antioxidant, and anticoagulant potentials using a combined biological and in silico screening strategy. **Inflammopharmacology**, v. 30, n. 5, p. 1811–1833, 1 out. 2022.

EL-DARIER, S. M. et al. An in vitro study on antimicrobial and anticancer potentiality of thyme and clove oils. **Rendiconti Lincei**, v. 29, n. 1, p. 131–139, 1 mar. 2018.

FATIMA, K.; LUQMAN, S. Suppression of Molecular Targets and Antiproliferative Effect of Citronellal on Triple-Negative Breast Cancer Cells. **Current Molecular Pharmacology**, v. 14, n. 6, p. 1156–1166, 10 mar. 2021.

GAD, M. M.; FOUDA, S. M. Current perspectives and the future of candida albicans-associated denture stomatitis treatment. **Dental and Medical Problems**, v. 57, n. 1, p. 95–102, 2020.

GAFFEN, S. L.; MOUTSOPOULOS, N. M. Regulation of host-microbe interactions at oral mucosal barriers by type 17 immunity. **Science immunology**, v. 5, n. 43, 3 jan. 2020.

GATEVA, S. et al. The potential of geraniol to reduce cytotoxic and genotoxic effects of MNNG in plant and human lymphocyte test-systems. **South African Journal of Botany**, v. 123, p. 170–179, 1 jul. 2019.

GENDREAU, L.; LOEWY, Z. G. Epidemiology and Etiology of Denture Stomatitis. **Journal of Prosthodontics**, v. 20, n. 4, p. 251–260, 2011.

GHEDIRA, K.; GOETZ, P.; LE JEUNE, R. Syzygium aromaticum (L.) Merr. & Perry (Myrtaceae) Giroflier. **Phytotherapie**, v. 8, n. 1, p. 37–43, fev. 2010.

GRIFFITHS, J. S. et al. Role for IL-1 Family Cytokines in Fungal Infections. **Frontiers in Microbiology**, v. 12, 10 fev. 2021.

GUPTA, A. et al. Comparative evaluation of antimicrobial efficacy of Syzygium aromaticum, Ocimum sanctum and Cinnamomum zeylanicum plant extracts against Enterococcus faecalis: A preliminary study. **International Endodontic Journal**, v. 46, n. 8, p. 775–783, 2013.

GUPTA, S. R. et al. The association of Candida and antifungal therapy with pro-inflammatory cytokines in oral leukoplakia. **Clinical Oral Investigations**, v. 25, n. 11, p. 6287–6296, 1 nov. 2021.

HALDER, S. et al. Essential oil of clove (Eugenia caryophyllata) augments the humoral immune response but decreases cell mediated immunity. **Phytotherapy Research**, v. 25, n. 8, p. 1254–1256, ago. 2011.

HALDER, S. et al. Acute effect of essential oil of Eugenia caryophyllata on cognition and pain in mice. **Naunyn-Schmiedeberg's Archives of Pharmacology**, v. 385, n. 6, p. 587–593, jun. 2012.

HAN, X.; PARKER, T. L. Anti-inflammatory activity of clove (Eugenia caryophyllata) essential oil in human dermal fibroblasts. **Pharmaceutical Biology**, v. 55, n. 1, p. 1619–1622, 2017.

HARO-GONZÁLEZ, J. N. et al. Clove essential oil (Syzygium aromaticum l. myrtaceae): Extraction, chemical composition, food applications, and essential bioactivity for human health. **Molecules**, v. 26, n. 21, 1 nov. 2021.

HEKMATPANAH, A. et al. Efficacy of Syzygium aromaticum essential oil on the growth and enzymatic activity of pathogenic Candida albicans strains. **Current Medical Mycology**, v. 8, n. 1, p. 12–19, 1 mar. 2022.

HELLSTEIN, J. W.; MAREK, C. L. Candidiasis: Red and White Manifestations in the Oral Cavity. **Head and Neck Pathology**, v. 13, n. 1, p. 25–32, 2019.

HO, J. et al. Candida albicans and candidalysin in inflammatory disorders and cancer. **Immunology**, v. 162, n. 1, p. 11–16, 1 jan. 2021.

HOU, M. et al. Linalool Impress Colorectal Cancer Deterioration by Mediating AKT/mTOR and JAK2/STAT3 Signaling Pathways. **BioMed Research International**, v. 2022, 2022.

HUANG, Y. et al. Geraniol suppresses proinflammatory mediators in phorbol 12- myristate 13-acetate with A23187-induced HMC-1 cells. **Drug Design, Development and Therapy**, v. 12, p. 2897–2903, 2018.

ISMAIL, M. et al. Chemical composition, antibiotic promotion and in vivo toxicity of Piper nigrum and Syzygium aromaticum essential oil. **African Journal of Biochemistry Research**, v. 11, n. 10, p. 58–71, 2017.

ISO 10993-5:2009; Biological Evaluation of Medical Devices—Part 5: Tests for In Vitro Cytotoxicity. ICS 11.100.20. **International Organization for Standardization**: London, UK, 2017.

KAMATOU, G. P. P.; VILJOEN, A. M. Linalool-A Review of a Biologically Active Compound of Commercial Importance. v. 3, n. 7, p. 1183–1192, 2008.

KANNAPPAN, A. et al. In vitro and in vivo biofilm inhibitory efficacy of geraniol-cefotaxime combination against Staphylococcus spp. **Food and Chemical Toxicology**, v. 125, p. 322–332, 2019.

KAYPETCH, R. et al. Geraniol and thymoquinone inhibit Candida spp. biofilm formation on acrylic denturresin without affecting surface roughness or color. **Journal of Oral Science**, v. 64, n. 2, p. 161–166, 2022.

KHAN, M. S. A.; AHMAD, I. Biofilm inhibition by Cymbopogon citratus and Syzygium aromaticum essential oils in the strains of Candida albicans. **Journal of Ethnopharmacology**, v. 140, n. 2, p. 416–423, 2012.

KULSHRESTHA, A.; GUPTA, P. Secreted aspartyl proteases family: a perspective review on the regulation of fungal pathogenesis. **Future Microbiology**, v. 18, p. 295– 309, 2023.

KUMAR, A. et al. Biofilms: Survival and defense strategy for pathogens. **International Journal of Medical Microbiology**, v. 307, n. 8, p. 481–489, 1 dez. 2017.

LA ROCCA, V. et al. Geraniol Induces Antinociceptive Effect in Mice Evaluated in Behavioural and Electrophysiological Models. **Basic and Clinical Pharmacology and Toxicology**, v. 120, n. 1, p. 22–29, 1 jan. 2017.

LEE, S. C. et al. Anti-inflammatory effect of cinnamaldehyde and linalool from the leaf essential oil of Cinnamomum osmophloeum Kanehira in endotoxin-induced mice. **Journal of Food and Drug Analysis**, v. 26, n. 1, p. 211–220, 1 jan. 2018.

LEI, Y. et al. Pharmacological Properties of Geraniol-A Review. **Planta Medica**, v. 85, n. 1, p. 48–55, 2019.

LEMBERG, C. et al. Candida albicans commensalism in the oral mucosa is favoured by limited virulence and metabolic adaptation. **PLoS Pathogens**, v. 18, n. 4, 1 abr. 2022.

LEWIS, M. A. O.; WILLIAMS, D. W. Diagnosis and management of oral candidosis. **British Dental Journal**, v. 223, n. 9, p. 675–681, 2017.

LI, W. R. et al. Geraniol attenuates virulence factors by inhibiting quorum sensing of Pseudomonas aeruginosa. **Frontiers in Microbiology**, v. 14, 2023.

LI, X. et al. IL-17 receptor–based signaling and implications for disease. **Nature Immunology**, v. 20, n. 12, p. 1594–1602, 1 dez. 2019.

LIN, G. C. et al. Optimization of an oral mucosa in vitro model based on cell line TR146. **Tissue Barriers**, 2020.

LIU, X. et al. Antibacterial activity and mechanism of linaloolagainst Pseudomonas aeruginosa. **Microbial Pathogenesis** , 2020.

LUKASZUK, C.; KRAJEWSKA-KULAK, E.; KULAK, W. Retrospective observation of drug susceptibility of Candida strains in the years 1999, 2004, and 2015. **PeerJ**, v. 2017, n. 2, p. 1–12, 2017.

MĄCZKA, W. et al. Natural Compounds in the Battle against Microorganisms— Linalool. **Molecules**, v. 27, n. 20, 1 out. 2022.

MACZKA, W.; WINSKA, K.; GRABARCZYK, M. One hundred faces of geraniol. **Molecules**, v. 25, n. 14, 1 jul. 2020.

MANDRAS, N. et al. The inhibition of non-albicans candida species and uncommon yeast pathogens by selected essential oils and their major compounds. **Molecules**, v. 26, n. 16, 2 ago. 2021.

MANOHARAN, R. K. et al. Inhibitory effects of the essential oils α-longipinene and linalool on biofilm formation and hyphal growth of Candida albicans. **Biofouling**, v. 33, n. 2, p. 143–155, 7 fev. 2017.

MARMOUZI, I. et al. Modulatory effect of Syzygium aromaticum and Pelargonium graveolens on oxidative and sodium nitroprusside stress and inflammation. **Oriental Pharmacy and Experimental Medicine**, v. 19, n. 2, p. 201–210, 1 jun. 2019.

MEDEIROS, C. I. S. et al. Antifungal activity of linalool against fluconazoleresistant clinical strains of vulvovaginal Candida albicans and its predictive mechanism of action. **Brazilian Journal of Medical and Biological Research**, v. 55, 2022.

MEDICHERLA, K. et al. Oral administration of geraniol ameliorates acute experimental murine colitis by inhibiting pro-inflammatory cytokines and NF-κB signaling. **Food and Function**, v. 6, n. 9, p. 2984–2995, 1 set. 2015.

Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Second Edition. **NCCLS document M27-A2** (ISBN 1-56238-469-4). Voluntary Consensus.

MOUSA, M. A.; LYNCH, E.; KIELBASSA, A. M. Denture-related stomatitis in new complete denture wearers and its association with Candida species colonization: a prospective case-series. **Quintessence International**, v. 51, n. 7, p. 554–565, 2020.

MOYES, D. L. et al. A biphasic innate immune MAPK response discriminates between the yeast and hyphal forms of candida albicans in epithelial cells. **Cell Host and Microbe**, v. 8, n. 3, p. 225–235, 16 set. 2010.

MURATA, M. Inflammation and cancer. **Environmental Health and Preventive Medicine**, v. 23, n. 1, 20 out. 2018.

NAGLIK, J. R. et al. Candida albicans–epithelial interactions and induction of mucosal innate immunity. **Current Opinion in Microbiology**, v. 40, p. 104–112, 1 dez. 2017.

NAJAR, B. et al. Chemical Composition and in Vitro Cytotoxic Screening of Sixteen Commercial Essential Oils on Five Cancer Cell Lines. **Chemistry and Biodiversity**, v. 17, n. 1, 1 jan. 2020.

NEWMAN, D. J.; CRAGG, G. M. Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. **Journal of Natural Products**, v. 83, n. 3, p. 770-803, 2020. doi:10.1021/acs.jnatprod.9b01285

NIKOU, S. A. et al. The Candida albicans toxin candidalysin mediates distinct epithelial inflammatory responses through p38 and EGFR-ERK pathways. **Science Signaling**, v. 15, n. 728, 5 abr. 2022.

NIRMALA, J. M. et al. Anticancer and antibacterial effects of a clove bud essential oil-based nanoscale emulsion system. **International Journal of Nanomedicine**, v. 14, p. 6439–6450, 2019.

NISHIKAWA, Y. et al. Effects of Candidalysin Derived from Candida albicans on the Expression of Pro-Inflammatory Mediators in Human Gingival Fibroblasts. **International Journal of Molecular Sciences**, v. 24, n. 4, 1 fev. 2023.

O'BRIEN, J. et al. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. **European Journal of Biochemistry**, v. 267, n. 17, p. 5421–5426, 2000.

OUYANG, Q. et al. Citronellal exerts its antifungal activity by targeting ergosterol biosynthesis in penicillium digitatum. **Journal of Fungi**, v. 7, n. 6, 1 jun. 2021.

PASETTO, S.; PARDI, V.; MURATA, R. M. Anti-HIV-1 activity of flavonoid myricetin on HIV-1 infection in a dual-chamber in vitro model. **PLoS ONE**, v. 9, n. 12, 29 dez. 2014.

RAI, M. N.; PARSANIA, C.; RAI, R. Mapping the mutual transcriptional responses duringCandida albicans and human macrophageinteractions by dual RNA-sequencing. **Microbial Pathogenesis** , p. 173, 2022.

RAMÍREZ-AMADOR, V. et al. TNF as marker of oral candidiasis, HSV infection, and mucositis onset during chemotherapy in leukemia patients. **Oral Diseases**, v. 23, n. 7, p. 941–948, 1 out. 2017.

READING, P. C.; SMITH, G. L. Vaccinia Virus Interleukin-18-Binding Protein Promotes Virulence by Reducing Gamma Interferon Production and Natural Killer and T-Cell Activity.

Journal of Virology, v. 77, n. 18, p. 9960–9968, 15 set. 2003.

REINHARDT, L. C. et al. A single-center 18-year experience with oral candidiasis in Brazil: a retrospective study of 1,534 cases. **Brazilian oral research**, v. 32, p. e92, 2018.
RICCI, C. et al. Geraniol Treatment for Irritable Bowel Syndrome: A Double-Blind Randomized Clinical Trial. **Nutrients**, v. 14, n. 19, 1 out. 2022.

ROCHELLE, S. L. DE A. et al. The anti-biofilm potential of commonly discarded agro-industrial residues against opportunistic pathogens. **Industrial Crops and Products**, v. 87, p. 150–160, 2016.

RODRIGUEZ, O. et al. Obtaining the essential oil of Syzygium aromaticum, identification of eugenol and its effect on Streptococcus mutans. **Journal of Oral Research**, v. 3, n. 4, p. 218–224, 2014.

ROSA-GARCÍA, E. et al. Antifungal susceptibility of oral isolates of Candida species from chronic kidney disease patients on chronic dialysis. **Journal de Mycologie Medicale**, v. 30, n. 3, 1 set. 2020.

RUTZ, S.; OUYANG, W. Regulation of interleukin-10 expression. **Advances in Experimental Medicine and Biology**, v. 941, p. 89–116, 1 out. 2016.

SABOGAL-GUÁQUETA, A. M. et al. Linalool attenuates oxidative stress and mitochondrial dysfunction mediated by glutamate and NMDA toxicity. **Biomedicine and Pharmacotherapy**, v. 118, 1 out. 2019.

SAIBABU, V. et al. Insights into the intracellular mechanisms of citronellal in Candida Albicans: Implications for reactive oxygen species-mediated necrosis, mitochondrial dysfunction, and DNA damage. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 50, n. 4, p. 524–529, 1 jul. 2017.

SANTANA, I. L. et al. Dietary Carbohydrates Modulate Candida albicans Biofilm Development on the Denture Surface. **PLoS ONE**, v. 8, n. 5, 30 maio 2013.

SANTOS, E. B. et al. Estudo etnobotânico de plantas medicinais para problemas bucais no município de João Pessoa, Brasil. **Brazilian Journal of Pharmacognosy**, v. 19, n. 1 B, p. 321–324, jan. 2009.

SELEEM, D. et al. In vitro evaluation of antifungal activity of monolaurin against Candida albicans biofilms. **PeerJ**, v. 2016, n. 6, 2016.

SEMENOVA, E. et al. The In Vitro Cytotoxicity of Eremothecium oil and Its Components—Aromatic and Acyclic Monoterpene Alcohols. **International Journal of Molecular Sciences**, v. 23, n. 6, 1 mar. 2022.

SERRANO, I. et al. The Virtuous Galleria mellonella Model for Scientific Experimentation. **Antibiotics**, v. 12, n. 3, p. 505, 3 mar. 2023.

SHEN, H. et al. Dectin-1 Facilitates IL-18 Production for the Generation of Protective Antibodies Against Candida albicans. **Frontiers in Microbiology**, v. 11, 16 jul. 2020.

SIDDIQUI, Z. N. et al. Synthesis, characterization and antimicrobial evaluation of novel halopyrazole derivatives. **Journal of Saudi Chemical Society**, v. 17, n. 2, p. 237–243, 2013.

SINGH, S. et al. Fungicidal action of geraniol against Candida albicans is potentiated by abrogated CaCdr1p drug efflux and fluconazole synergism. **PLoS ONE**, v. 13, n. 8, 1 jul. 2019.

SINGH, S.; FATIMA, Z.; HAMEED, S. Insights into the mode of action of anticandidal herbal monoterpenoid geraniol reveal disruption of multiple MDR mechanisms and virulence attributes in Candida albicans. **Archives of Microbiology**, v. 198, n. 5, p. 459–472, 1 jul. 2016a.

SINGH, S.; FATIMA, Z.; HAMEED, S. Citronellal-induced disruption of membrane homeostasis in Candida albicans and attenuation of its virulence attributes. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 49, n. 4, p. 465–472, 1 jul. 2016b.

STOCKINGER, B.; OMENETTI, S. The dichotomous nature of T helper 17 cells. **Nature Reviews Immunology**, v. 17, n. 9, p. 535–544, 1 set. 2017.

STUYT, R. J. L. et al. Recombinant Interleukin-18 Protects against Disseminated Candida albicans Infection in Mice. **The Journal of Infectious Diseases**, v. 189, n. 8, p. 1524–1527, 2004.

TALAPKO, J. et al. Candida albicans-the virulence factors and clinical manifestations of infection. **Journal of Fungi**, v. 7, n. 2, p. 1–19, 2021.

TANAKA, T.; NARAZAKI, M.; KISHIMOTO, T. Interleukin (IL-6) immunotherapy. **Cold Spring Harbor Perspectives in Biology**, v. 10, n. 8, 1 ago. 2018.

TARDIF, F. et al. Involvement of interleukin-18 in the infl ammatory response against oropharyngeal candidiasis.

TRINDADE, L. A. et al. Inhibition of adherence of C. albicans to dental implants and cover screws by Cymbopogon nardus essential oil and citronellal. **Clinical Oral Investigations**, v. 19, n. 9, p. 2223–2231, 1 dez. 2015.

TRINDADE, L. A. et al. The antifungal and antibiofilm activity of Cymbopogon nardus essential oil and citronellal on clinical strains of Candida albicans. **Brazilian Journal of Microbiology**, 2022.

TSUI, C.; KONG, E. F.; JABRA-RIZK, M. A. Pathogenesis of Candida albicans biofilm. **Pathogens and disease**, v. 74, n. 4, p. ftw018, 1 jun. 2016.

VASCONCELOS, P. G. S. et al. In vitro inhibition of a multispecies oral cavity biofilm by Syzygium aromaticum essential oil. **Gerodontology**, 2021.

VERHAEGHE, T. V.; WYATT, C. C.; MOSTAFA, N. Z. The effect of overnight storage conditions on complete denture colonization by Candida albicans and dimensional stability: A systematic review. **Journal of Prosthetic Dentistry**, v. 124, n. 2, p. 176–182, 2020.

VILA, T. et al. Oral candidiasis: A disease of opportunity. **Journal of Fungi**, v. 6, n. 1, p. 1–28, 2020.

WALL, G. et al. Candida albicans biofilm growth and dispersal: contributions to pathogenesis. **Current Opinion in Microbiology**, v. 52, p. 1–6, 1 dez. 2019.

WILLIAMS, D.; LEWIS, M. Pathogenesis and treatment of oral candidosis. **Journal of Oral Microbiology**, v. 3, n. 2011, p. 1–11, 2011.

WU, Y. et al. Geraniol-mediated osteoarthritis improvement by down-regulating PI3K/Akt/NF-κB and MAPK signals: In vivo and in vitro studies. **International Immunopharmacology**, v. 86, 1 set. 2020.

YE, C. J. et al. Geraniol targets KV1.3 ion channel and exhibits anti-inflammatory activity in vitro and in vivo. **Fitoterapia**, v. 139, 1 nov. 2019.

YIN, Y. L. et al. Citronellal Attenuates Oxidative Stress–Induced Mitochondrial Damage through TRPM2/NHE1 Pathway and Effectively Inhibits Endothelial Dysfunction in Type 2 Diabetes Mellitus. **Antioxidants**, v. 11, n. 11, 1 nov. 2022.

ZHANG, M. et al. Effects of citronellal on growth and enterotoxinsproduction in Staphylococcus aureus ATCC 29213. **Toxicon**, p. 213–292, 2022.

ZHAO, Y. et al. Linalool inhibits 22Rv1 prostate cancer cell proliferation and induces apoptosis. **Oncology Letters**, v. 20, n. 6, 24 set. 2020.

ZHOU, Y. et al. The Interactions Between Candida albicans and Mucosal Immunity. **Frontiers in Microbiology**, v. 12, 21 jun. 2021.

ZWICKER, P. et al. A 3D Model of Human Buccal Mucosa for Compatibility Testing of Mouth Rinsing Solutions. **Pharmaceutics**, v. 15, n. 3, p. 721, 21 fev. 2023

ANEXO A – NORMAS DE PUBLICAÇÃO PARA O PERIÓDICO GERODONTOLOGY

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1. SUBMISSION

Authors should kindly note that submission implies that the content has not been published or submitted for publication elsewhere except as a brief abstract in the proceedings of a scientific meeting or symposium.

New submissions should be made via the Research Exchange submission portal **https://wiley.atyponrex.com/journal/GER**. Should your manuscript proceed to the revision stage, you will be directed to make your revisions via the same submission portal. You may check the status of your submission at any time by logging on to submission.wiley.com and clicking the "My Submissions" button. For technical help with the submission system, please review our FAQs or contact **submissionhelp@wiley.com**.

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Gerodontology accepts articles previously published on preprint servers.

Gerodontology will consider for review articles previously available as preprints. Authors are requested to update any pre-publication versions with a link to the final published article. Authors may also post the final published version of the article immediately after publication.

2. AIMS AND SCOPE

The ultimate aim of the subject area of gerodontology is to improve the quality of life and oral health of older people. *Gerodontology* fills the particular place of serving this subject area. The boundaries of most conventional dental specialties must be repeatedly crossed to provide optimal dental care for older people. Furthermore, management of other health problems impacts on their dental care and clinicians need knowledge in these numerous overlapping areas. Bringing together these diverse topics within one journal serves clinicians who have not time to scan many journals and it serves authors whose papers would therefore fail to access their target readership. The juxtaposition of papers from different specialties but sharing this patient-centered interest provides a synergy that serves progress in the subject of gerodontology.

3. MANUSCRIPT CATEGORIES AND REQUIREMENTS

Article Type	Description	Word Limit	Abstract / Structure	Other Requirements
Original Papers	reports of new research findings or conceptual analyses that make a significant contribution to knowledge	no	Yes, Structured	IRB Statement
Reviews	critical reviews of the literature, including systematic reviews and meta-analyses	no	Yes, Structured	PRISMA checklist
Invited Reviews	critical reviews of the literature, including systematic reviews and meta-analyses	no	Yes, Structured	PRISMA checklist
Case Reports	preliminary findings of research in progress or a case report of particular interest	1500 limit	Yes, structured, 75 words	IRB Statement 1-2 figures 5 references
Short Report	a succinct report of research findings	1500 limit	Yes, structured, 75 words	IRB Statement 1-2 figures 10 references No more than 5 subheadings
Letter to the Editor	Subject to approval by the Editor	no	no	no

Manuscripts should be submitted electronically via the online submission site **http://mc.manuscriptcentral.com/gerodontology**. Assistance can be obtained from the Editorial Office at **GERedoffice@wiley.com**.

3.1. Manuscript Categories

3.2. Blinded Review

All manuscripts accepted for publication in *Gerodontology* will be reviewed by at least two experts in the field. *Gerodontology* uses single-blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper, whereas the name(s) of the author(s) can be seen by the reviewers.

4. PREPARING THE SUBMISSION

Gerodontology now offers Free Format submission for a simplified and streamlined submission process.

Manuscripts can be uploaded either as a single document (containing the main text, tables and figures), or with figures and tables provided as separate files. Should your manuscript reach revision stage, figures and tables must be provided as separate files. The main manuscript file can be submitted in Microsoft Word (.doc or .docx) format.

Cover Letters

Cover letters are not mandatory; however, they may be supplied at the author's discretion.

Your main document file should include:

- A short informative title containing the major key words. The title should not contain abbreviations;
- The full names of the authors with institutional affiliations where the work was conducted, with a footnote for the author's present address if different from where the work was conducted;
- Acknowledgments;
- Abstract structured (intro/methods/results/conclusion) or unstructured;
- Up to seven keywords;
- Main body: formatted as introduction, materials & methods, results, discussion, conclusion
- References;
- Tables (each table complete with title and footnotes);
- Figures: Figure legends must be added beneath each individual image during upload AND as a complete list in the text.

Authorship

Please refer to the journal's Authorship policy in the Editorial Policies and Ethical Considerations section for details on author listing eligibility.

Acknowledgments

Contributions from anyone who does not meet the criteria for authorship should be listed, with permission from the contributor, in an Acknowledgments section. Financial and material support should also be mentioned. Thanks to anonymous reviewers are not appropriate.

Conflict of Interest Statement

Authors will be asked to provide a conflict of interest statement during the submission process. For details on what to include in this section, see the ‘Conflict of Interest’ section in the Editorial Policies and Ethical Considerations section below. Submitting authors should ensure they liaise with all co-authors to confirm agreement with the final statement.

Main Text

- **Authorship and Acknowledgements:**Gerodontology adheres to the definition of authorship set up by the International Committee of Medical Journal Editors (ICMJE). According to the ICMJE criteria, authorship should be based on (1) substantial contributions to conception and design of, or acquisition of data or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content and (3) final approval of the version to be published. Authors should meet conditions 1, 2 and 3. Contributors who have made substantive contributions but who do not qualify as authors should be mentioned under Acknowledgements. The letter of submission to the editor must identify the contributions made by each author to the three conditions, and state that each author approves of the submission.
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- **Font**: Manuscripts must be typed double-spaced.

References

References should be numbered consecutively in the text according to the AMA (American Medical Association) reference style. All references should be numbered consecutively in order of appearance and should be as complete as possible. In-text citations should cite references in consecutive order using Arabic superscript numerals. Only references cited in the Text, Tables and Figures may be included.). For abbreviations of journal names, consult **http://www.ncbi.nlm.nih.gov/nlmcatalog/journals**. Manuscripts accepted but not published may be cited in the reference list by placing 'in press" after the abbreviated title of the journal - all such references should be submitted to the Editor for approval. References must be verified by the author(s) against the original documents.

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Reference List Examples:

- **Journal article (1-6 authors):**
    - Hu P, Reuben DB. Effects of managed care on the length of time that elderly patients spend with physicians during ambulatory visits. Med Care. 2002;40(7):606-613.
- **Journal article with more than 6 authors:**

   - Geller AC, Venna S, Prout M, et al. Should the skin cancer examination be taught in medical school? Arch Dermatol. 2002;138(9):1201-1203.
- **Journal article with no named author or group name:**
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- **Electronic Journal article:**
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   - *If you do not have a doi:*
      - Aggleton JP. Understanding anterograde amnesia: disconnections and hidden lesions. Q J Exp Psychol. 2008;61(10):1441-1471. http://search.ebscohost.com/login.aspx?direct=true&db=pbh&AN=34168185&site=ehost-live. Accessed March 18, 2010.
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      - Chau NG, Haddad RI. Antiangiogenic agents in head and neck squamous cell carcinoma: tired of going solo [published online ahead of print September 20, 2016]. Cancer. doi: 10.1002/cncr.30352.
   - **Entire Book:**
      - McKenzie BC. Medicine and the Internet: Introducing Online Resources and Terminology. 2nd ed. New York, NY: Oxford University Press; 1997.
   - **Book Chapter:**
      - Guyton JL, Crockarell JR. Fractures of acetabulum and pelvis. In: Canale ST, ed. Campbell's Operative Orthopaedics. 10th ed. Philadelphia, PA: Mosby, Inc; 2003:2939-2984.
   - **Electronic Book:**
      - Rudolph CD, Rudolph AM. Rudolph's Pediatrics. 21st ed. New York, NY: McGraw-Hill Companies; 2002. http://online.statref.com/Document/Document.aspx?DocID=1&StartDoc=1&EndDoc=1882&FxID=13&offset=7&SessionId=A3F279FQVVFXFSXQ . Accessed August 22, 2007.
   - **Internet Document:**
      - American Cancer Society. Cancer Facts & Figures 2003. http://www.cancer.org/downloads/STT/CAFF2003PWSecured.pdf. Accessed March 3, 2003.
   - **Dissertation:**
      - Cloning and Characterization of Piccolo, a Novel Component of the Presynaptic Cytoskeletal Matrix [dissertation]. Birmingham: University of Alabama; 2000.
   - **Thesis:**
      - Undeman C. Fully Automatic Segmentation of MRI Brain Images Using Probabilistic Diffusion and a Watershed Scale-Space Approach [master’s thesis]. Stockholm, Sweden: NADA, Royal Institute of Technology; 2001.ase. Philadelphia: WB Saunders, 1974: 457-480.

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Additional Files

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- **Scientific Names:** Proper names of bacteria should be binomial and should be singly underlined in the typescript. The full proper name (e. g. Streptococcus sanguis) must

be given upon first mention. The generic name may be abbreviated thereafter with the first letter of the genus (e. g. S. sanguis). If abbreviation of the generic name could cause confusion, the full name should be used. If the vernacular form of a genus name (e. g. streptococci) is used, the first letter of the vernacular name is not capitalized and the name is not underlined. Use of two letters of the genus (e. g. Ps .for Peptostreptococcus) is incorrect, even though it might avoid ambiguity. With regard to drugs, generic names should be used instead of proprietary names. If a proprietary name is used, ® must be attached when the term is first used.

  - The term "elders" or "older people" should be used rather than "elderly' when using the word as a noun. The word "elderly" is an adjective used correctly as "elderly group," whereas "elders" is a noun used correctly as in a "group of elders." Refer to the "patients" or "subjects" who participated in the study - or who consented to the use of their health records in your research - as "participants." This more appropriately acknowledges the role they had in your research.

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- **Tables**: Tables should be numbered consecutively with Arabic numerals. Type each table on a separate sheet, self-explanatory titles. Due regard should be given to the proportions of the printed page.
- For instructions, see **Gerodontology Guide to Tables and Figures**
- **Figures**: At the Editor's discretion clinical photographs, photomicrographs, line drawings and graphs will be published as figures. All figures should clarify the text and their number should be kept to a minimum. Details must be large enough to retain their clarity after reduction in size. Illustrations should preferably fill a single column width (54 mm) after reduction, although in some cases 113 mm (double column) and 171 mm (full page) widths will be accepted. Micrographs should be designed to be reproduced without reduction, and they should be dressed directly on the micrograph with a linear size scale, arrows, and other designators as needed. The inclusion of color illustrations is at the discretion of the Editor. The author may pay for the cost of additional color illustrations.
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- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Human subject information in databases. The journal refers to the **World Health Medical Association Declaration of Taipei on Ethical Considerations Regarding Health Databases and Biobanks**.[RW12]

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9. EDITORIAL OFFICE CONTACT DETAILS

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Author Guidelines updated January 11, 2021

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ANEXO B – NORMAS DE PUBLICAÇÃO PARA O PERIÓDICO PHYTOTHERAPY RESEARCH

AUTHOR GUIDELINES

SECTIONS

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3. Manuscript Categories and Requirements
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1. SUBMISSION

Phytotherapy Research operates an online submission and peer review system that allows authors to submit articles online and track their progress via a web interface. Please read the remainder of these instructions to authors and then click **http://mc.manuscriptcentral.com/ptr** to navigate to the *Phytotherapy Research* online submission site.

Review process

The acceptance criteria for all papers are based on the quality and originality of the research and its significance to journal readership. Manuscripts are preliminarily evaluated by the Editorial Team (triage), which may return the manuscript to the authors (usually within 2-4 working days) if it is believed that the manuscript is out of scope, is more suited to a regional audience or to more specialized Journals or if it is believed that the content and the methods of the manuscript are below the usual scientific standard of the Journal. Otherwise, the manuscript will be sent for review (single-blind peer review).

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2. AIMS AND SCOPE

Phytotherapy Research is a monthly, pharmacologically-oriented international journal for the publication of original research papers (experimental and clinical), review articles (including systematic reviews and meta-analyses), letters on medicinal plant research. Key areas of interest are pharmacology, toxicology, and the clinical applications of herbs and natural products in medicine. Papers concerned with the effects of common food ingredients and standardised plant extracts, including commercial products, are particularly welcome, as are mechanistic studies on isolated natural products.

Phytotherapy Research does not publish agricultural, phytochemical, structure elucidation, quality control or botanical identification papers unless directly pertinent to the pharmacological effects or overall safety of plant based medicines currently in use.

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3. MANUSCRIPT CATEGORIES AND REQUIREMENTS

Submissions fall into the following categories:

a. Research papers (experimental and clinical)
b. Letters to the Editor
c. Review articles, including systematic reviews and meta-analyses
d. Editorials

a. Research papers (experimental and clinical). These should not exceed ten printed pages (where one page comprises 800 words or the equivalent in illustrative and tabular material). The text file should be presented in the following order: 1) title page, 2) abstract, 3) key words (up to six), main text (introduction, material and methods, results and discussion), references, figure legends. tables (with title and footnotes), acknowledgments, author contribution, conflict of interest. The abstract should be up to 250 words. The abstract must be structured into the following four sections: 1) background and aim, 2) experimental procedure, 3) key results and conclusions and implications. Abbreviations should be minimized and references not included. The introduction should be concise and depict the background to the subject, its significance and its relationship to earlier works. The introduction should end with the aim of the paper. Materials and methods should be presented with clarity and detail. Results illustrate original and important findings, with figures or tables where necessary. Do not repeat numerical values of any data presented in tables or figures. The discussion should emphasize the principal conclusions drawn from the results and their important implications. End the discussion with a clear conclusion and possibly clinical relevance. **Click here to download the Author checklist for experimental research papers.**

For clinical trials, manuscripts must comply with recognized guidelines for conducting and reporting clinical trials. Medical research involving human subjects must meet the current standards for clinical trials (Declaration of Helsinki, US Federal Policy for the Protection of Human Subjects or European Medicines Agency Guidelines for Good Clinical Practice). When reporting clinical trials, authors must follow the CONSORT and its extensions (for reporting randomised parallel or crossover trials), Strobe (for observational studies) or Care (for case reports) guidelines and provide their checklist and flowchart (documents can be downloaded from the respective website). To ensure transparent and unbiased reporting, clinical trials must be registered prospectively in a publicly accessible database. If they are not registered or have been registered retrospectively, the reasons should be explained. The methods section must state which ethics committee approved the study (including the full reference) and that the work described was conducted in accordance with recognized guidelines. The name of the trial register and the registration number of the clinical study should be stated in the methods section. PTR does not publish study protocols.

b. Letters to the Editor. These have no fixed format (no abstract), are intended for short constructive comments on published work or for putting forward new ideas and are published at the discretion of the Editor. Occasionally small research reports can be published as a letter to the Editor (max length: 3 double-spaced pages, max 2 figures two figures and/or two tables,

max 25 references, No subdivision into Introduction, Methods, Results and Discussion). **Click here to view example Letters to the Editor**.

c. Review articles. These will usually be written at the invitation of the Editors, but unsolicited reviews are welcome. Review articles should include a Table of Contents and will normally be limited to 20,000 words excluding references. No new and unpublished data should be submitted. The text file should be presented in the following order: title page, abstract, key words (up to six), introduction, relevant sections, discussion, conclusions, future directions, references, figure legends, tables (with title and footnotes), acknowledgments, author contribution and conflict of interest.

Systematic reviews should report on a detailed examination of previous research on a particular topic, using "*systematic and explicit methods to identify, select and critically appraise relevant primary research and to extract and analyse data from the studies included in the review*". **Systematic reviews and meta-analyses** must strictly adhere to PRISMA or MOOSE (for observational trials) guidelines. The PRISMA or MOOSE flowchart (as part of the main text) and checklist (as supplementary material) must be submitted. Both documents can be downloaded from the PRISMA or MOOSE website. We strongly recommend that authors register their detailed protocols in a public registry such as PROSPERO before starting data mining. A statement reporting compliance with PRISMA guidelines and registration information (if available) should be reported in the methods section. The abstract must be structured into the following sections: 1) background, 2) objective 3) Search methods, 4) selection criteria, 5) Data collection and analysis, 6) Main reslts, 7) Conclusions.

d. Editorials. These are written by the Editor-in-Chief or by editorial board members upon invitation. There is no fixed format.

Return to Guideline Sections

4. PREPARING THE SUBMISSION

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